UNIVERSITY OF SOUTHAMPTON

Genetic analysis of aberrant histological structures in prophylactic oophorectomy specimens from women with germline BRCA1 and BRCA2 mutations

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ABSTRACT

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Ovarian cancer is the fifth most frequent cause of overall cancer related death for women in the Western World (Iwabuchi *et al*, 1995; Shelling *et al*, 1995). It has been estimated that inherited mutations of the breast and ovarian cancer susceptibility gene BRCA1 will be detected in 5% of common epithelial malignant ovarian tumours. The lifetime risk of ovarian or breast cancer development for women with BRCA1 mutations approaches 100% (Ford *et al*, 1994). As a result, some women with mutations elect to undergo prophylactic oophorectomy after completion of childbearing, in order to reduce the likelihood of tumour development. This study is based on the findings of Salazar *et al* (1996), who reported a significantly higher incidence of atypical features in the ovaries of BRCA1 mutation carriers when compared to a control population, raising the possibility of a histological pre-malignant phenotype. We examined prophylactic oophorectomy specimens from six women, five with BRCA1 mutations, and the other a BRCA2 mutant. DNA was extracted from microdissected areas of atypia within sections of paraffin-embedded ovarian tissue. Matched leucocyte DNA was obtained for comparison. Microsatellite PCR was used to examine for loss of heterozygosity (LOH) at candidate loci on all chromosome arms. Amongst BRCA1 carriers, we were able to identify LOH in both inclusion cyst material and in areas of microscopic malignancy incidentally discovered. Material from the BRCA2 mutant showed no LOH. The LOH detected was in keeping with that previously reported for ovarian tumours. The data we have obtained supports the concept of a histological pre-malignant phenotype in ovaries of women with BRCA1 mutations.

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PRESENTATIONS

1. **Low incidence of BRCA1 mutations in sporadic ovarian tumours: a consecutive series of 220 cases from a single centre**

   Poster presentation at 1999 Annual Meeting of the American Society of Clinical Oncology, Atlanta, Georgia, May 1999

   Poster presentation at 1999 CRC London and South East Grantees Meeting, St Georges Hospital, London, September 1999

2. **Genetic analysis of a potentially pre-malignant phenotype in ovaries of women with BRCA mutations**

   Poster presentation at 2000 Annual Meeting of the American Society of Clinical Oncology, New Orleans, LA, May 2000

   Poster presentation at 2000 British Cancer Research Meeting, University of Sussex, Falmer, Brighton, July 2000

ABBREVIATIONS

A	adenine
adm	'alleles don't match'
ALL	acute lymphoblastic leukaemia
ALP	alkaline phosphatase
APC	adenomatous polyposis coli
APS	ammonium persulphate
BCC	basal cell carcinoma
bcl-2	B cell lymphoma - 2
bp	base pair
BSA	bovine serum albumin
C	cytosine
Ca	carcinoma
CAT	catalase
CDKN2	cyclin dependent kinase N2
CI	confidence interval
CML	chronic myeloid leukaemia
dATP	deoxyadenosine triphosphate
DCC	deleted in colon cancer
DCIS	ductal carcinoma in situ
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dnw	did not work
DOB	date of birth
DOP-PCR	degenerate oligo-primed PCR
dTTP	deoxythymidine triphosphate
E.coli	*Escherichia coli*
EDTA	ethylenediamine-tetra-acetic acid
EGF	epidermal growth factor
EOC	epithelial ovarian cancer
FHIT	fragile histidine triad
FIGO	International Federation of Gynaecology and Obstetrics
G	guanine
HBOCS	hereditary breast and ovarian cancer syndrome
HET/het	heterozygous/retention of constitutional heterozygosity

H&E	haemotoxylin and eosin
HNPCC	hereditary non polyposis colorectal cancer
HOM/hom	homozygous
HRT	hormone replacement therapy
IC	inclusion cyst
ID	identification
IG	immunoglobulin
IGF	insulin-like growth factor
IL-6	interleukin 6
IRF	interferon regulatory factor
kV	kilovolts
leuc	leucocyte
LOH/loh	loss of constitutional heterozygosity
mg	milligram
$MgCl_2$	magnesium chloride
MI/mi	microsatellite instability
MIN/min	minute
ml	millilitre
mm	millimetre
MMR	mismatch repair
mRNA	messenger ribonucleic acid
n/no	number
na	not available
ND	not done
NF2	neurofibromatosis type II
ng	nanogram
ni	not informative/ non-informative due to constitutional homozygosity
P32	[α-^{32}P]dCTP
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEP	primer extension preamplification
PGH synthase	prostaglandin H synthase
pH	-log10[H+]
PLL	poly- L-lysine
PTT	protein truncation test
RB/Rb	retinoblastoma
RER	replication error
rpm	revolutions per minute

s.cerevisiae	*Saccharamyces cerevisiae*
SDS	sodium dodecyl sulphate
sec	second
SSCP	single stranded conformational polymorphism
STR	simple tandem repeats
T	thymidine
TAE	Tris-acetic acid-EDTA buffer
Taq	Thermus aquaticus DNA polymerase
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N, N, N, N-tetramethyl-ethylene-diamine
TGF-alpha	transforming growth factor alpha
TGF-beta	transforming growth factor beta
T_m	melting temperature
TNM	tumour, nodes, metastases
TSG	tumour suppressor gene
µg	microgram
µl	microlitre
µm	micrometre
UV	ultraviolet
VNTR	variable number of tandem repeats
WT1	Wilms tumour 1
WT2	Wilms tumour 2
^{0}c	degrees celsius

CHAPTER ONE
INTRODUCTION

1.1 OVARIAN CANCER

Amongst women in the Western World, ovarian cancer is the commonest fatal cancer of the reproductive tract (Banks *et al*, 1997), and is the 5th most frequent cause of overall cancer related death (the 4th most frequent for American women) (Iwabuchi *et al*, 1995; Shelling *et al*, 1995).

The lifetime risk of development of ovarian cancer is 1.4% (compared with 1.25% for cervix but 7.1% for breast) (Anderson *et al,* 1997). In the UK, there are more than 5000 new cases per year and 4275 deaths from the disease (Gore *et al*, 1994); the annual incidence in the USA is 24000 (Berchuck and Cirisano 1997).

The stage of disease at the time of diagnosis strongly predicts outcome, but the majority of cases present late, due to vague associated symptomatology, when the disease has already spread beyond the ovaries. Five year survival figures range from 89% for Stage I disease, through 57% and 24% respectively for Stage II and Stage III, to only 12% for Stage IV patients (Nguyen *et al*, 1993). Overall 5 year survival for all patients with ovarian carcinoma has remained fairly constant over several decades (at 35-40%), despite surgical and chemotherapeutic advances (Boente *et al*, 1996). It is, therefore, essential that we try to increase our understanding of the molecular genetic and biological factors involved in the disease, such that earlier diagnoses can be made, with a possible survival advantage to the patient.

1.2 AETIOLOGY OF OVARIAN CANCER

The incidence of ovarian cancer increases with age (Adami *et al*, 1990), with a substantial proportion diagnosed around the time of the menopause. A plateau in incidence occurs at approximately 55 years of age (Booth and Beral,1985). Marked alterations in hormonal balance seen at this time have been implicated in this observation (Berek *et al*, 1993).

The other established factors resulting in an increased risk are family history and the presence of BRCA1 and BRCA2 germline mutations. However, familial ovarian cancer is only responsible for a small minority of cases (Koch *et al*, 1989; Friedman *et al*, 1994a).

Factors which are known to be protective against tumour development are multiparity (Adami *et al*,1994; Risch *et al*, 1996) and the use of an oral contaceptive (Ory *et al*, 1983; Vessey *et al*, 1987; Parazzini *et al*, 1991a,b), whilst prolonged breastfeeding is also thought to reduce risk (Whittemore *et al*, 1992; Risch *et al*, 1996). Conversely, risk is increased for those who are nulliparous and have never lactated, and those who undergo regular menstruation (Parazzini *et al*, 1991b). There are various other suggested risk factors, although conflicting data exists. These include early menarche, late menopause, use of fertility stimulating drugs (Parazzini *et al*, 1991b; Whittemore *et al*, 1992), use of perineal talcuum powder or genital deodrants (Purdie *et al*, 1995; Cook *et al*, 1997), childhood viruses (Booth and Beral, 1985), and obesity (Casagrande *et al*, 1979; Purdie *et al*, 1995; Cook *et al*, 1997). In 1993, Parazzini and colleagues reported a greatly reduced risk of later development of ovarian cancer in those undergoing hysterectomy (Parazzini *et al*, 1993). There may be various reasons behind this, not least of which is the fact that ovaries will be examined at the time of abdominal hysterectomy. However, the reduced risk may also be linked to altered ovarian blood flow following hysterectomy. A reduced ovarian cancer risk amongst women who have undergone tubal ligation procedures has also been described (Miracle McMahill *et al*, 1997). Various possible reasons for this were suggested: a reduced blood supply to the ovaries following the procedure may result in inhibition of ovulation; reduced oestrogen and/or progesterone levels after ligation may lower the risk; tubal ligation blocks a potential pathway of exposure to carcinogens; the procedure may decrease or eliminate

uterine growth factors, which reach the ovaries via the utero-ovarian circulation and may be involved in ovarian carcinogenesis. In addition to these proposed mechanisms of risk reduction, the ovaries of women undergoing this operation will be screened at the time of surgery, with suspicious or malignant appearing ovaries being surgically removed.

1.3 MECHANISMS OF INDUCTION OF OVARIAN TUMORIGENESIS

Hypotheses regarding the mechanism of induction of cancer development in the ovary have been proposed on the basis of epidemiological data collected.

1.3.1 The 'Incessant Ovulation' Hypothesis (Fathalla 1971)

Ovulation traumatises the ovarian epithelium, which is repaired by proliferation of epithelial cells exposed to growth factors in follicular fluid (including TGF-alpha and IL-6). This repeated cell proliferation provides ample opportunity for neoplastic transformation to occur, as a result of mutations which stimulate somatic activation of oncogenes or inactivation of tumour suppressor genes (Berek *et al*, 1993; Jacobs and Lancaster 1996).

Conversely, reducing the number of ovulatory cycles, by pregnancy, breast feeding, or oral contraceptive use for example, results in less mitotic events within the ovary and, thus, less opportunity to acquire mutations (Casagrande *et al*, 1979).

1.3.2 The 'Gonadotrophin' Hypothesis

High levels of circulating gonadotrophins have a direct effect on transformation of epithelial cells of the ovary, or stimulate oestrogen production which, in turn, affects the epithelium (Cramer *et al*, 1994).

This hypothesis has proved less acceptable, some arguing that it does not fit with age specific ovarian cancer incidence, and also that hormone replacement therapy (HRT) does not appear protective despite reducing gonadotrophin levels (Mohle *et al*, 1985).

1.4 THE HISTOPATHOLOGY OF OVARIAN CANCER

Ovarian tumours are most commonly divided histopathologically into two main groups - epithelial and non-epithelial (Tables 1.1 and 1.2). Epithelial tumours account for approximately two thirds of all ovarian tumours, and are derived from surface epithelium (Godwin *et al*, 1993) or from inclusion cysts (Cramer and Welch 1983). Ninety percent of all ovarian cancers are epithelial. The ovarian surface epithelium is, in fact, mesothelium which, as it is reflected over the ovarian surface, comes to resemble epithelium. It migrates into the ovarian stroma to form inclusion cysts. These cysts are more prone to neoplastic transformation than the epithelium itself - immunohistochemical studies have shown ovarian carcinoma antigens (CA-125, CA19-9, placental-like ALP) are more frequently observed in inclusion cyst epithelium than surface epithelium ; metaplastic change is seen much more commonly in cyst epithelium than surface epithelium (Scully 1995).

Non-epithelial tumours, comprising mainly germ cell and sex cord stromal tumours, tend to affect a younger population, and are relatively rare.

Tumours can be further subdivided into benign, borderline (distinguished by absence of invasion of ovarian stroma), and frankly malignant (Link *et al*, 1996).

Table 1.1 Epithelial ovarian tumours

TYPE	% OF ALL EPITHELIAL LESIONS	FEATURES
SEROUS	45-50	**Histology**: similar to fallopian tube (*Scully 1977*); psammoma bodies present *Benign*: 50-70%; bilateral in up to 20% cases 1. unilocular cysts >1cm diameter, containing fluid 2. papillary lesions 3. adenofibromata - solid, rubbery lesions Borderline: 10-15%; bilateral in 26-34% cases Similar to benign, but finer and more exuberant papillary projections Malignant: 25-35%; bilateral in 2/3 1. well differentiated - cystic, multilocular, with soft, friable papillae; psammoma bodies in 60-70% 2. moderately differentiated - psammoma bodies in 20-30% 3. poorly differentiated - solid areas of crumbly grey/pink tissue; less papillae
MUCINOUS	35-40	Histology: similar to endocervix or large bowel (*Scully 1977*); goblet cells common; often see benign, borderline and malignant cell types within one specimen (*Gallion 1995*) Benign: 75-85% Cystic; lobules lined with mucin-secreting epithelium; may be huge Borderline: 10-15% Malignant: 5-10% Multilocular, thin walled cysts; may contain intestinal-like epithelium

ENDOMETRIOID	5-10	Histology: resemble endometrial tumours; may be solid or cystic (*Czernobilsky 1970*) Benign: rare Borderline: rare Malignant: most Often unilocular, containing turbid brown fluid; 15% associated with endometrial carcinoma in body of uterus
CLEAR CELL (MESONEPHROID)	5	**Histology**: may grow in solid masses or line glandular spaces; 10% bilateral Virtually all malignant Most thick walled unilocular cysts, containing turbid brown or blood-stained fluid
BRENNER TUMOURS	1-2	**Histology**: resemble transitional cells of urinary tract; may appear like ovarian fibroma Virtually all benign
UNDIFFERENTIATED CARCINOMA	1.5	
MIXED EPITHELIAL	<2	
SARCOMA	<1	
UNCLASSIFIED EPITHELIAL	0.5	
SQUAMOUS	rare	

From Russell 1987

Fox and Anderson, 1985

Anderson *et al*, 1997

Table 1.2 Non-epithelial ovarian tumours

I. SEX CORD STROMAL TUMOURS
A. Granulosa stromal cell tumour
B. Androblastoma - Sertoli-Leydig cell tumour
C. Gynandroblastoma
D. Unclassified
II. GERM CELL TUMOURS
A. Dysgerminoma
B. Endodermal sinus tumour - yolk sac tumour
C. Embryonal cell tumour
D. Polyembryoma
E. Choriocarcinoma
F. Teratoma
G. Mixed
III. LIPID CELL TUMOURS
IV. GONADOBLASTOMA
V. SOFT TISSUE TUMOURS NOT SPECIFIC TO OVARY
VI. UNCLASSIFIED TUMOURS
VII. METASTATIC TUMOURS

From Anderson *et al*, 1997

Scully 1977

1.5 GRADING AND STAGING OF OVARIAN TUMOURS

1.5.1 Tumour Grading

The cytological grading of ovarian tumours reflects the number of mitoses seen, the nuclear to cytoplasmic ratio, and the hyperchromicity of the nuclei (Broders, 1926). The effect of epithelial ovarian cancer grade on survival was shown by Decker *et al* - progressively higher grades of tumour were shown to have an increasingly poor prognosis, regardless of cell type (Decker *et al*, 1973, 1975). Malkasian *et al* (1975) also demonstrated that grade of tumour is important - grade for grade and stage for stage, the behaviour of different cell types was shown to be similar. Decker and Malkasian both used the grading system devised by Broders (1926), to ensure reproducibility of pathological evaluation. The grading system is dependent on the percentage of undifferentiated cells present (Table 1.3).

Table 1.3 Tumour grading

TUMOUR GRADE	CYTOLOGY	GROWTH PATTERN
I	<25% undifferentiated cells	Well differentiated
II	26-50% undifferentiated cells	Moderately differentiated
III	51-75% undifferentiated cells	Poorly differentiated
IV	>75% undifferentiated cells	Undifferentiated

From Malkasian *et al*, 1984

Other groups have modified the grading to include only three grades - I = well differentiated; II = moderately differentiated; III = poorly differentiated (Kosary 1994). Kosary (1994) has shown that, within FIGO stage, tumour grade has a large impact on survival. Within their study, survival declined from 94.6% for well differentiated stage I to 69.7% for poorly differentiated stage I lesions. For stage II, the figures were 78.3% and 48.2%; for stage III, 76.2% and 23.9%; and for stage IV, 50.2% and 13.8%. The

great prognostic significance of tumour grade was also demonstrated in a Swedish ovarian cancer series, in which the difference in 5 year survival between patients with well and poorly differentiated tumours, when subgrouped according to grade alone, was as large as the difference between stages I and IV in the complete series (Sorbe *et al*, 1982).

1.5.2 Tumour Staging

The most widely used methods for staging ovarian tumours are the FIGO (International Federation of Gynaecology and Obstetrics), and TNM systems (Table 1.5). The FIGO stage correlates well with patient outcome, but marked survival differences are seen within each stage (Table 1.4). More useful prognostic information can be gained by subcategorisation.

Table 1.4 Percentage five-year survival according to FIGO stage and sub-stage

FIGO stage	FIGO *(Pettersson et al 1988)*	*Nguyen et al 1993*	*Hogeberg et al 1993*
I		88.9	80
Ia	76.6	92	87.4
Ib	67.7	85	78.8
Ic	59.6	82	76.2
II		57	63
IIa	51.1	67	66.7
IIb	43.5	56	63.8
IIc	43.5	51	62.4
III	17.4	23.8	17.5
IIIa		39	50
IIIb		25	13.3
IIIc		17	15.5
IV	4.7	11.6	17.1
Total patients	**8082**	**5156**	**332**

From Friedlander 1998

Table 1.5 FIGO/TNM staging of ovarian tumours (Averette *et al*, 1995)

FIGO STAGE	TNM STAGE	DEFINITION
		PRIMARY TUMOUR (T)
IA	**TIa**	Tumour limited to 1 ovary Capsule intact No tumour on ovarian surface; no malignant cells in ascites or peritoneal washings
IB	**TIb**	Tumour in both ovaries Capsules intact No tumour on ovarian surface; no malignant cells in ascites or peritoneal washings
IC	**TIc**	Tumour in 1 or both ovaries With any of: ruptured capsule tumour on ovarian surface malignant cells in ascites or peritoneal washings
II	**T2**	Tumour in 1 or both ovaries With pelvic extension
IIA	**T2a**	Pelvic extension and/or implants on the uterus and/or tube(s) No malignant cells in ascites or peritoneal washings
IIB	**T2b**	Extension to other pelvic tissues No malignant cells in ascites or peritoneal washings
IIC	**T2c**	Pelvic extension (IIA or IIB) Malignant cells in ascites or peritoneal washings
III	**T3/N1**	Tumour in1 or both ovaries

		Microscopically confirmed peritoneal metastasis outside pelvis and/or regional lymph node metastasis
IIIA	**T3a**	Microscopic peritoneal metastasis outside pelvis
IIIB	**T3b**	</= 2cm macroscopic peritoneal metastasis outside pelvis
IIIC	**T3c/N1**	> 2cm peritoneal metastasis outside pelvis and/or regional lymph node metastasis
IV	**M1**	Distant metastasis (peritoneal metastasis excluded)
		REGIONAL LYMPH NODES (N)
	NX	Unable to assess regional nodes
	NO	No metastasis to regional nodes
	N1	Regional node metastasis
		DISTANT METASTASIS (M)
	MX	Unable to assess for distant metastasis
	MO	No distant metastasis
IV	**M1**	Distant metastasis (peritoneal metastasis excluded)

Liver capsule metastasis = Stage III/T3

Liver parenchymal metastasis = Stage IV/M1

Pleural effusion with positive cytology = Stage IV/M1

Presence of non-malignant ascites is not classified; ascites only affects staging if malignant cells seen

1.6 THE GENETICS OF CANCER

All cancer has a genetic origin, with a series of mutational events resulting in the development of a tumour. Cancers occur in both sporadic and familial forms, and are described as sporadic when all mutational events involved in their development have a somatic origin. For hereditary tumours, at least 1 mutation is inherited through the germline, and there is then a subsequent accumulation of additional somatic mutations.

1.6.1 Multistep Carcinogenesis

The main factors involved in controlling whether a cancer develops, and the rate of development and tumour progression, are sequential mutational events in cancer-related genes, and their selection and accumulation within a clonal cell population (Boyd and Barrett 1990; Vogelstein and Kinzler 1993).

For the majority of human epithelial tumours it has been suggested that 4-8 genetic mutational events are rate-limiting for tumour development (Renan 1993).

1.7 ONCOGENES, TUMOUR SUPPRESSOR GENES, AND THE MISMATCH REPAIR PATHWAY

Three major groups of genes responsible for regulating the proliferation and differentiation of cells are:

1. proto-oncogenes
2. tumour suppressor genes
3. mismatch repair genes

1.7.1 Proto-oncogenes and Oncogenes

Normal cells contain sequences (cellular protooncogenes) homologous to those of oncogenic viruses. They are responsible for appropriately driving cell proliferation, unless their cellular functions are activated or enhanced by mutation during carcinogenesis. Mutation of one allele results in oncogene activation, with the subsequent 'gain of function' effect of increased cell division and uncontrolled cell growth. Consequently, a neoplastic phenotype develops.

There are several mechanisms underlying proto-oncogene activation:

1. point mutation leads to activation of a mutant form of protein product
2. gene amplification leads to overexpression of a protein product which is otherwise normal.

These are the common mechanisms in solid tumours. However, in haematological malignancy, chromosomal translocation is more frequently seen (Table 1.6). This often results in the juxtaposition of an oncogene with the promoter region of an abnormally expressed gene, which in turn leads to overexpression of the oncogene-encoded protein.

Table 1.6 Chromosomal translocations in haematological malignancy

Chromosomal translocation	Haematological malignancy	Genes involved	Molecular effect
t(8;14)(q24;q32)	Burkitt's lymphoma B-cell ALL	myc(8q24) IG (14q32)	deregulated myc expression myc mutation
t(14;18)(q32;q21)	Follicular lymphoma	bcl-2(18q21) IG(14q32)	deregulated bcl-2 bcl-2 mutation
t(9;22)(q34;q11)	CML Some ALL	abl(9q34) bcr(22q11)	bcr-abl fusion protein
t(15;17)(q21;q22)	Acute promyelocytic leukaemia	PML(15q21) RARA(17q22)	PML-RARA fusion protein

IG - immunoglobulin

From Dyer 1996

Oncogenes and protooncogenes can be classified according to their function into several different categories (Ruddon 1995) (Table 1.7).

Table 1.7 Categories of oncogene

1. Class 1 - Growth factors

sis	PDGF B- chain growth factor
int-2 **hst(KS3)** **FGF-5**	FGF-related growth factor
int-1	? growth factor

Stimulate tumour cell proliferation by paracrine/autocrine mechanisms. May not, alone, be sufficient to sustain transformed phenotype

2. Class 2 - Receptor and non-receptor protein-tyrosine kinases

src **yes** **fgr** **lck**	Membrane-associated non receptor protein-tyrosine kinase
fps/fes **abl/bcr-abl**	Non receptor protein-tyrosine kinase
ros	Membrane-associated receptor-like protein-tyrosine kinase
erbB	Truncated EGF receptor protein-tyrosine kinase
neu	Receptor-like protein-tyrosine kinase
fms	Mutant CSF-1 receptor protein-tyrosine kinase
met **trk**	Soluble truncated receptor-like protein-tyrosine kinase
kit (W locus)	Truncated stem-cell receptor protein-tyrosine kinase
sea	Membrane-associated truncated receptor-like protein-tyrosine kinase
ret	Truncated receptor-like protein-tyrosine kinase

Code for altered growth factor receptors - many have associated tyrosine-kinase activity

3. Class 3 - Receptors lacking protein kinase activity

mas	Angiotensin receptor
α1β	Adrenergic receptor

4. Class 4 - Membrane-associated G (guanine nucleotide binding) proteins

H-ras **K-ras** **N-ras**	Membrane-associated GTP-binding/GTPase
gsp	Mutant activated form of $G_s\alpha$
gip	Mutant activated form of $G_i\alpha$

Proteins bind GTP, have associated GTPases, and act as signal transducers for cell surface growth factor receptors

5. Class 5 - Cytoplasmic protein-serine kinases

raf/mil **pim-1**	Cytoplasmic protein-serine kinase
mos	Cytoplasmic protein-serine kinase (cytostatic factor)
cot	? Cytoplasmic protein-serine kinase

Have serine/threonine protein kinase activity

6. Class 6 - Cytoplasmic regulators

crk	SH-2/3 protein that binds to (and ? regulates) phosphotyrosine-containing proteins

c-crk appears to act by stabilising tyrosine kinases associated with the src family of oncoproteins

7. Class 7 - Nuclear transcription factors

myc myb ets	Sequence-specific DNA-binding protein
N-myc L-myc lyl-1	? Sequence-specific DNA-binding protein
p53	Mutant form may sequester wild-type p53 growth suppressor
fos	Combines with c-jun product to form AP-1 transcription factor
jun	Sequence-specific DNA-binding protein; part of AP-1
erbA	Dominant negative mutant thyroxine (T_3) receptor
rel	Dominant negative mutant NF-αB-related protein
vav ski evi-1 gli-1 maf Hex2.4	? Transcription factor
pbx	Chimeric E2A-homeobox transcription factor

For a number of these, the oncogenic alteration making them transforming oncoproteins is a mutation resulting in loss of negative regulatory elements (eg. jun, fos, myb). For others, the activating mutations result in loss of active domains, leading to a mutant protein that prevents activity of the normal gene product (erbA, rel)-so-called "dominant-negative mutation"

8. Unclassified

dbl	? Cytoplasmic truncated cytoskeletal protein
bcl-2	? Plasma membrane signal transducer

From Ruddon 1995

1.7.2 Tumour Suppressor Genes

Cell proliferation is usually inhibited by the protein product of a tumour suppressor gene. Inactivation, through a loss of function mutation, is oncogenic. Studies of inherited predisposition for certain tumours provided evidence for existence of these genes.

1.7.2.1 *Knudson's '2 hit' Hypothesis*

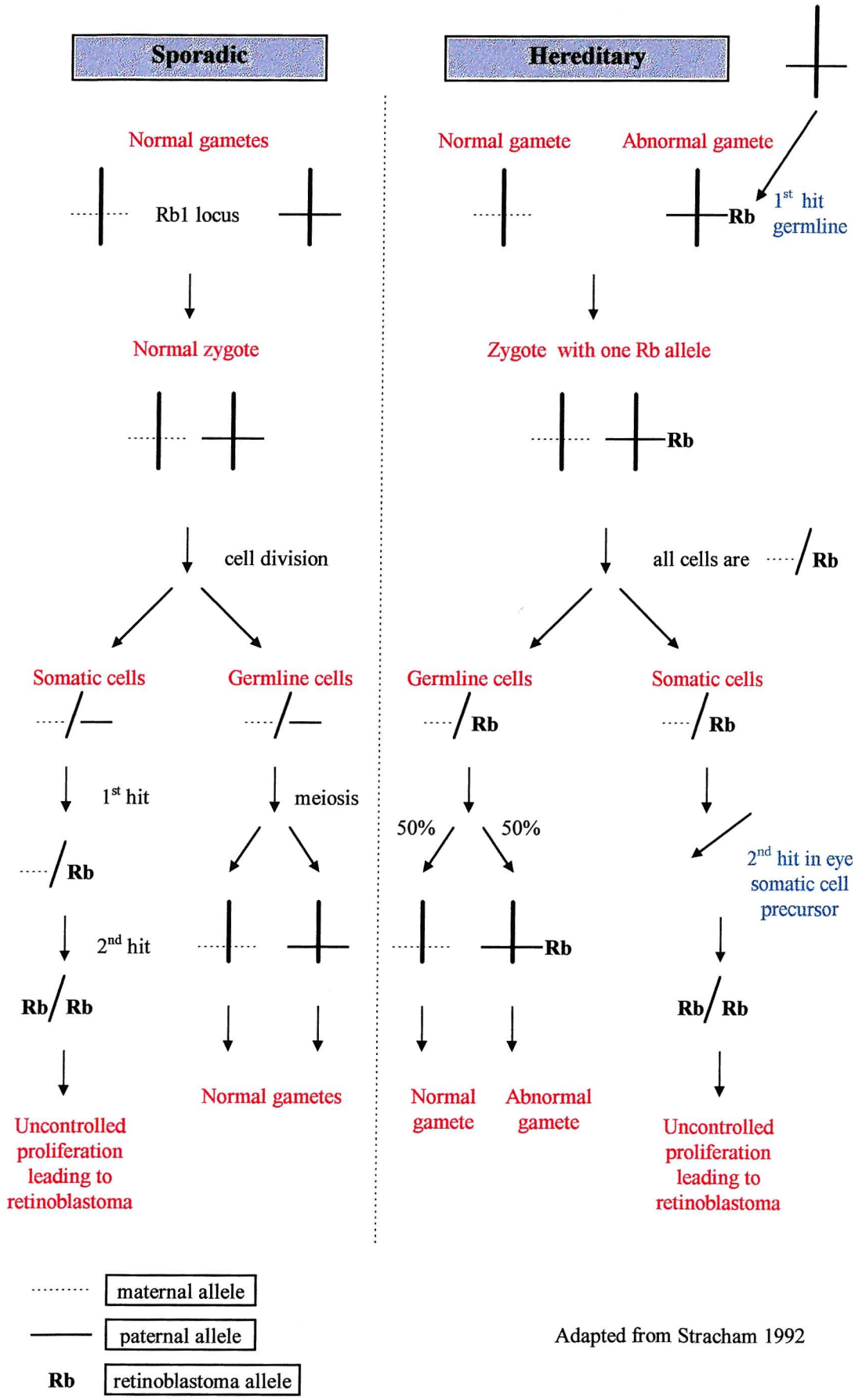
In 1971, Knudson formulated his '2 hit' hypothesis from the statistical analysis of a series of cases of hereditary retinoblastoma (Knudson 1971). He proposed the need for two mutational events or 'hits' in order for a tumour to develop.

For hereditary cancers, he suggested that the first mutation or 'hit' was inherited via germline cells, with the second occurring in somatic cells. As both events were required for tumour development, there was, therefore, a group of individuals who had inherited the germline (first) mutation but who did not have the disease, as the second, rate-limiting, somatic mutational event had not occurred. In other words, the predisposing inherited lesion was recessive, with the loss of function of one copy of a gene staying latent until the remaining normal allele was inactivated. He proposed that these individuals could, therefore, transmit the inherited trait to their offspring.

For non-hereditary cancers, he proposed that both mutational events occurred within somatic cells (Figure 1.1).

Subsequently, in 1989, Knudson proposed that heritable cancer genes are dominant in their ability to impart susceptibility to disease, but act in a recessive fashion with respect to carcinogenesis at the cellular level (Knudson 1989). Twenty five to thirty percent of cases of retinoblastoma are bilateral. Knudson proposed that these are hereditary, as the proportion of affected offspring closely approximates the 50% expected from dominant inheritance. Of the 70-75% with unilateral disease, only 15-20% are thought to be hereditary. Patients who inherit a mutation develop tumours earlier than those with the non-hereditary form of the disease, and in most hereditary cases more than one tumour develops. The probability of an individual with the non-hereditary form developing more than one lesion is vanishingly small.

Figure 1.1 Knudson's "2 hit" model in hereditary and sporadic retinoblastoma



Adapted from Stracham 1992

We now know that Knudson's '1st hit' results in inactivation of a single allele of a tumour suppressor gene, with the rate-limiting 2nd step being the loss of the remaining normal or 'wild type' allele, since mutation of both alleles is necessary for inactivation of a tumour suppressor gene.

The '1st hit' involves a tumour suppressor gene and its immediately adjacent chromosomal DNA. Several mechanisms of mutation may be responsible for allelic inactivation:

1. point mutation - either missense or nonsense
2. insertion/deletion of one or several nucleotides, resulting in a frameshift mutation
3. large deletions
4. translocation (rare)

The '2nd hit' may be similar to the 1st, but there is often loss of all or a large proportion of the chromosome which contains the 2nd 'wild type' allele of the tumour suppressor gene. Common mechanisms are:

1. chromosomal non-dysjunction at mitosis
2. deletion
3. mitotic recombination

The fact that the first mutation is inherited in familial cancer, such that only one mutational event is then required for tumour suppressor gene inactivation, perhaps accounts for the earlier age of onset seen in familial cancers.

A broader version of Knudson's model can be applied to tumours for which expression of a fully malignant phenotype requires more than two mutational events. In sporadic ovarian cancers, it has been suggested, from study of epidemiological data, that approximately six independent mutational events may be necessary (Armitage and Doll 1954).

1.7.3 Mismatch Repair Genes (MMR)

The mismatch repair pathway is responsible for stabilisation of the cellular genome. DNA replication errors are corrected, and recombination events between divergent DNA sequences are blocked. Defective mismatch repair genes result in a genetic instability phenotype. This, in turn, leads to a greater predisposition to tumour development, due to a huge increase in mutational events.

Defects in the MMR pathway were first described for the Hereditary Non Polyposis Colorectal Cancer (HNPCC or Lynch II) syndrome. In 1993, the first HNPCC locus was mapped to chromosome 2p, using microsatellite markers (Peltomaki *et al*, 1993). These DNA markers target tracts of $(A)_n$/ $(T)_n$ and $(CA)_n$/ $(GT)_n$ repeats, present in the human genome in approximately 100,000 copies. Microsatellite markers are useful in linkage studies, as the repeat lengths of the microsatellite loci vary between individuals, as well as between maternal and paternal alleles (Jiricny, 1996).

During the mapping process undertaken by Peltomaki *et al* (1993), a phenotype was discovered which was common to HNPCC and approximately 20% of sporadic colon tumours - a discrepancy in the repeat lengths between tumour and normal tissue from the same patient. It was suggested that this microsatellite instability (MI) resulted from abnormal function of a DNA metabolic pathway, eg. replication or repair, termed replication error (RER), and that this malfunction was the underlying cause of the tumour (Peltomaki *et al*, 1993; Thibodeau *et al*, 1993).

The best known such MMR pathway in bacteria was the *Escherichia coli* (*E.coli*) mut HLS system, promoting a 'long patch' methyl-directed repair of single base-pair mismatches and short mismatched loops in newly synthesised DNA. A similar pathway in *Saccharamyces cerevisiae* was known to require MSH (a homologue of bacterial mutS) and 2 MLH proteins (homologues of bacterial mutL proteins). If the bacterial mut HLS system fails, the consequence is that of genetic instability (Marra and Boland 1995).

Sequence homologies were identified between the *E.Coli* mutS gene, mouse Rep3 (MSH3) and human DUG1 (hMSH3), as well as between the pMS1 gene of *s.cerevisiae* and the *E.coli* mutL gene, implying that the MMR pathways of all organisms are closely related. These findings lead to the identification of human MMR genes (Jiricny, 1996). In 1993, Leach cloned the hMSH2 gene (a homologue of the prokaryotic DNA MMR gene mutS)(Leach *et al*, 1993; Marra and Boland 1995), and subsequently identified germline mutations within it. Also in 1993, Parsons was able to show that tumour cells which exhibited MI were deficient in mismatch repair (Parsons *et al*, 1993), an observation confirmed by Boland *et al* (1998). hMLH1, linked to chromosome 3p, and a homologue of the prokaryotic DNA MMR gene mutL, was also later cloned (Bronner *et al*, 1994; Marra and Boland 1995). In 1994, hPMS1 (2q) and

hPMS2 (7p), also homologues of mutL, were identified (Nicolaides *et al*, 1994; Papadopoulos *et al*, 1994).

hMSH2 comprises 2727bp, encoding a 909 amino acid protein with 41% identity to the yeast MSH2 protein, whilst hMLH1 contains 19 exons, encoding a transcript of more than 2525 nucleotides. hPMS1 comprises 2795bp, translating into a 932 amino acid protein which is 27% homologous to the yeast PMS1 protein, and hPMS2 has 2586bp, encoding an 862 amino acid protein with 32% identity to yeast PMS2 (Marra and Boland 1995).

1.7.3.1 *MMR enzymatic mechanism*

The MSH gene product is able to recognise a mismatch in newly synthesised DNA, and forms an MSH-DNA heteroduplex complex. MLH and pMS proteins are recruited, resulting in a complex which initiates excision of the incorrect nucleotide. Purified hMSH2 protein specifically binds DNA-containing single bp mismatched nucleotides or insertion-deletion loop-type mismatches (Marra and Boland 1995).

It is important for the MMR pathway that the template DNA strand is appropriately recognised and distinguished from the daughter strand containing the sequence error. In *E.coli*, the MMR system recognises the template by virtue of its degree of methylation, and preferentially removes nucleotides from the unmethylated daughter strand. A single-strand break is incised at varying distances proximal or distal to the mismatch (a bi-directional mechanism); DNA pieces of differing lengths, containing the mismatched nucleotides, can be removed. In mammalian cells, it has been suggested that gaps in the newly synthesised DNA strand indicate that it is the daughter strand, and thus signal its excision.

If any part of the MMR protein complex is defective, it becomes likely that a mismatch will not be detected or rectified. Thus, changes in the length of a microsatellite are seen in any tumour with defective MMR.

MMR genes are inactivated in a similar '2 hit' manner to tumour suppressor genes, and have been readily identified in numerous HNPCC kindreds. Mutations in hMSH2 appear to be responsible for more than 40% of HNPCC linked abnormalities, with hMLH1 mutations accounting for approximatley 30% (Jiricny, 1996). However, such mutations in sporadic tumours with an MI phenotype are rarely detected (Katabuchi *et*

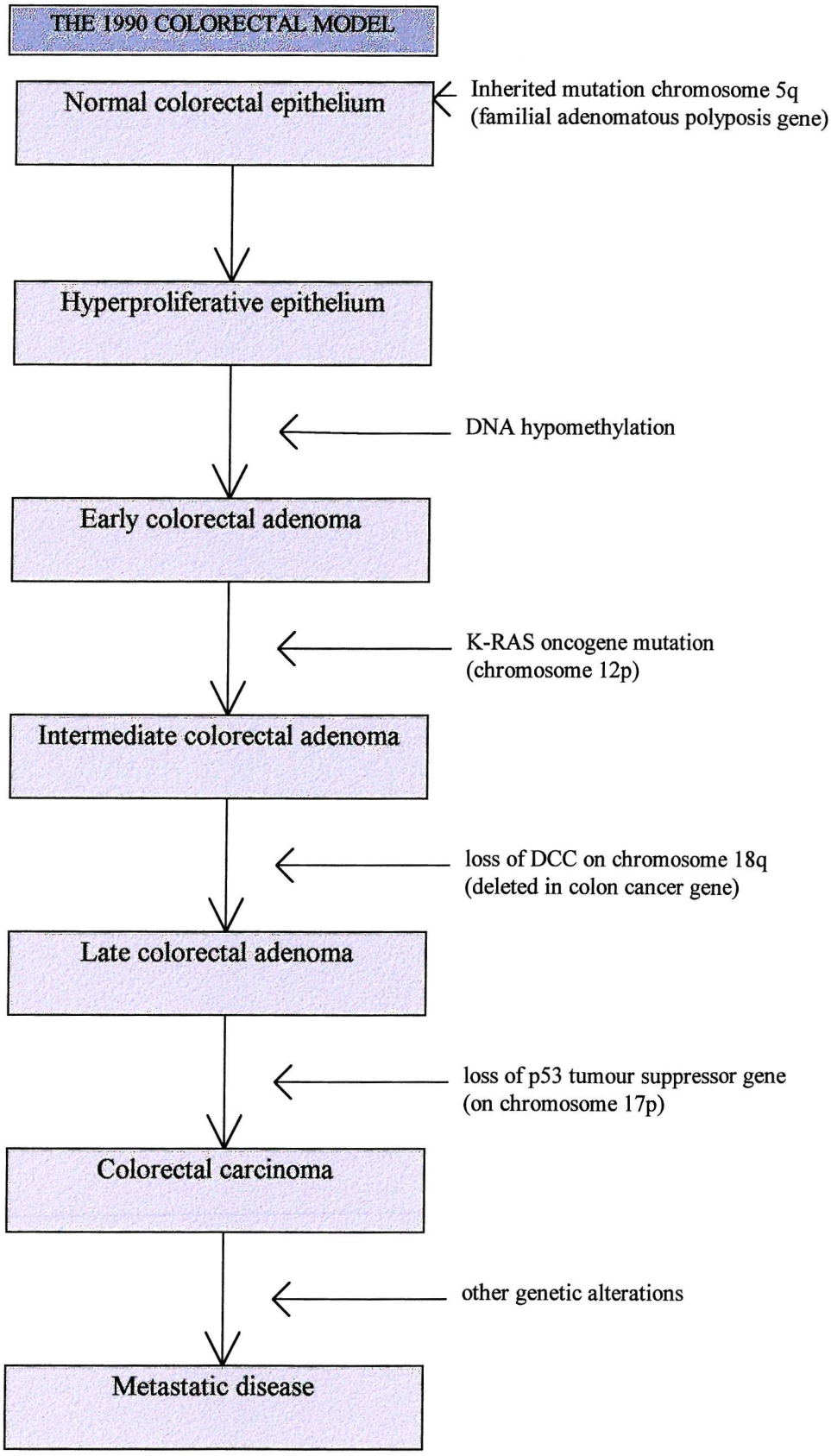
al, 1995; Liu *et al*, 1995). It is, therefore, possible that a different molecular genetic pathway from that in HNPCC-associated cancers is responsible for the genetic instability seen in sporadic lesions, or that there are other elements to the repair pathway which are yet to be identified. Other proteins, for example those responsible for replication fidelity or repair of different types of DNA lesion, may also play an important role in maintaining stability of microsatellites as well as coding sequences (Meuth, 1996) - MMR deficiency may be necessary for the mutator phenotype, but may not alone be sufficient.

Microsatellite instability is not limited to colorectal cancer, with reports published for various other tumour types, eg. gastric (Chong *et al*, 1993), endometrial (Risinger *et al*, 1993), bladder (Gonzales-Zulueta *et al*, 1993), non small cell lung (Shridhar *et al*, 1994), small cell lung (Merlo *et al*, 1994), breast (Yee *et al*, 1994), and ovary (Orth *et al*, 1994). Various studies have analysed sporadic ovarian cancers for microsatellite instability, concluding that mismatch repair defects and MI are quite uncommon in ovarian tumorigenesis (Osborne and Leech 1994, 8%; Schultz *et al*, 1995, 2%). Fujita *et al* (1995) found an overall frequency of 17% amongst 47 ovarian tumours, and observed an increasing incidence with advancing clinical stage for endometrioid lesions. When all histological types were combined, no association between stage and presence of MI was seen. The most commonly altered gene was hMSH2.

1.8 THE GENETIC MODEL OF COLORECTAL CARCINOGENESIS

In 1990 a model was proposed for colorectal neoplastic development and progression, which implied that specific histopathological stages in the disease process result from specific genetic events (Vogelstein *et al* 1988; Fearon and Vogelstein 1990) (Figure 1.2).

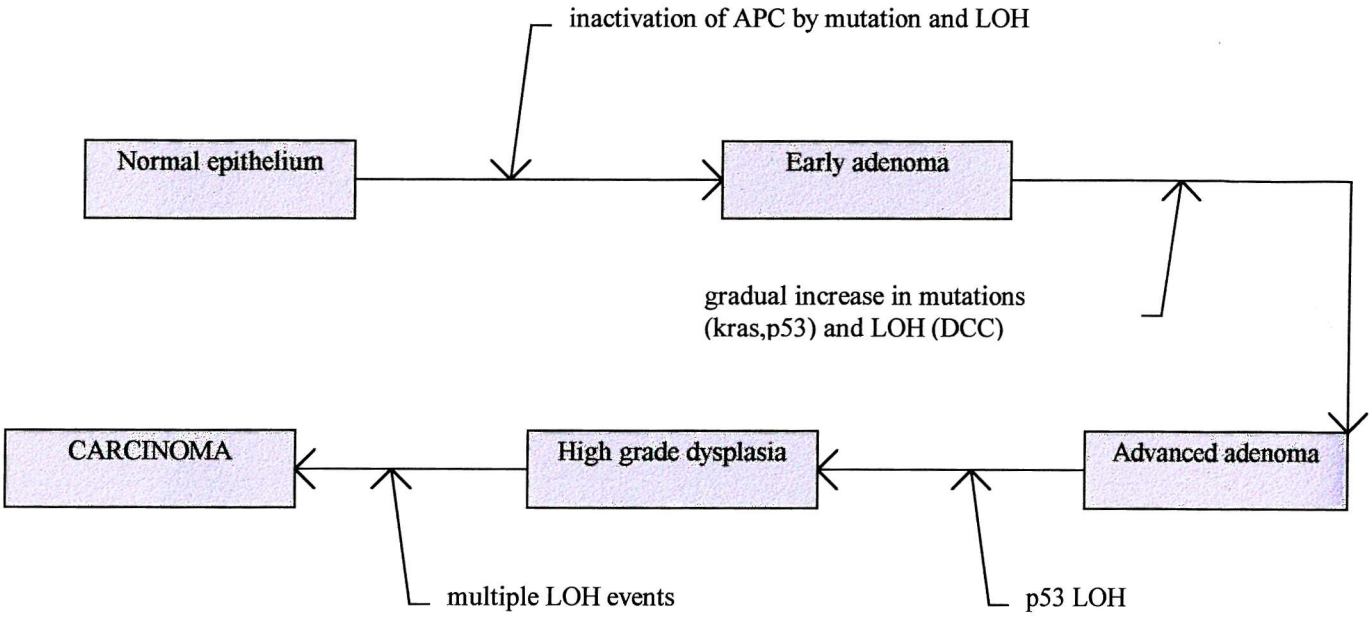
Figure 1.2



Adapted from Fearon and Vogelstein 1990

Studies by Boland and colleagues have confirmed parts of the model, and provided additional information allowing its modification (Boland *et al*, 1995, 1998) (Figure 1.3).

Figure 1.3 A subsequent model of colorectal carcinogenesis



Adapted from Boland *et al*, 1995

LOH - loss of constitutional heterozygosity

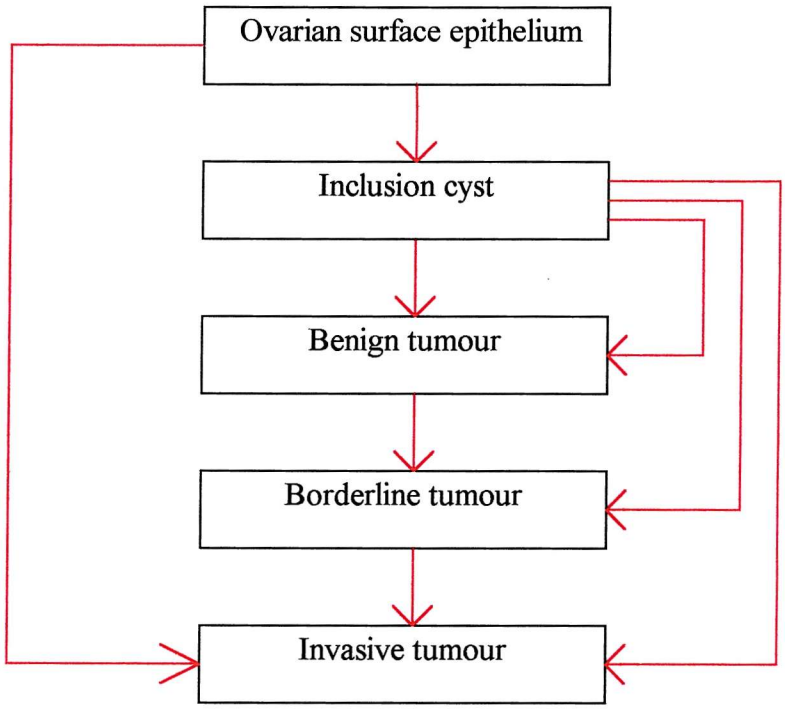
Neither Kras nor 18q (DCC) mutations appear to result in changes in histological appearance

p53 LOH is strongly associated with conversion of benign adenoma to carcinoma

The concept described, underlying the transition from normal colorectal epithelium to invasive cancer, is considered a likely model for other solid tumours, but despite attempts to devise a similar model for development and progression of ovarian cancer, the underlying molecular genetic mechanisms responsible are thus far not clear.

1.9 A PROPOSED MODEL FOR OVARIAN CARCINOGENESIS

Figure 1.4 The model proposed by Gallion and colleagues



From Gallion *et al*, 1995

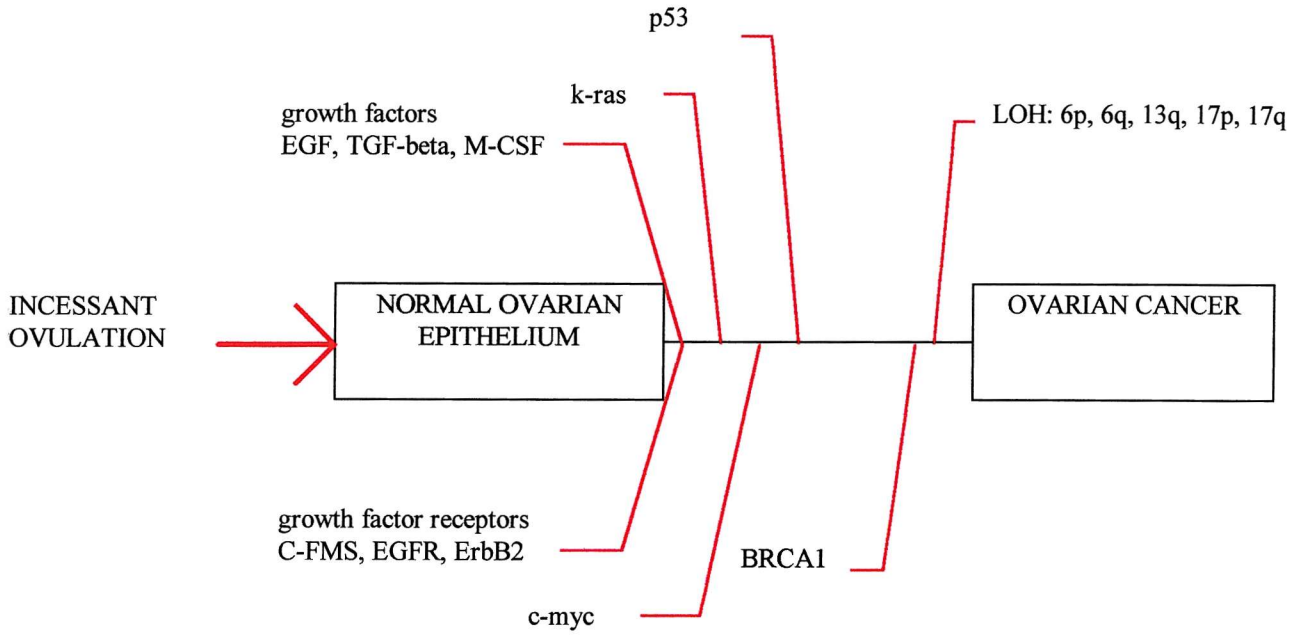
Epithelial cancers may arise directly from the surface epithelium or inclusion cysts (de novo), or from preexisting benign lesions

Gallion reported that benign epithelium is seen adjacent to borderline or malignant tissue in some serous and mucinous tumours (Gallion *et al*, 1995), an observation perhaps consistent with the hypothesis of other groups that certain benign and borderline lesions are precursors to invasive carcinoma (Stenback 1981; Zheng *et al*, 1995).

However, Bell and Scully reported 14 cases of apparently de novo microscopic malignancy within ovaries of grossly normal appearance, without intervening development of a benign tumour (Bell and Scully 1994).

Information relating to the temporal occurrence of genetic alterations and their possible associations with clinical stages of disease and prognosis will perhaps be gained from loss of heterozygosity studies with large numbers of ovarian tumours, and polymorphic markers spaced on each chromosome (Yang Feng *et al*, 1993). A further model of ovarian tumorigenesis, incorporating some such information already gained, has been put forward by Jacobs (Figure 1.5).

Figure 1.5 Jacobs' model for ovarian carcinogenesis



From Jacobs and Lancaster 1996

The order of events may vary from tumour to tumour, and the relationship of events to stage, grade, and histology is unknown

It is possible that some benign or borderline lesions represent early steps in carcinogenesis

1.10 LOSS OF HETEROZYGOSITY (LOH) ANALYSIS

Inactivation of a tumour suppressor gene is difficult to detect, as the effects are recessive in an individual cell; both gene copies require inactivation before loss of control occurs. The two step process of TSG inactivation provides a means of identification of TSG location - loss of the second allele by deletion can be detected by LOH analysis using polymorphic microsatellite repeats (simple tandem repeats - STR's). These are highly polymorphic, and present at short intervals throughout the genome. Polymerase chain reaction (PCR) amplification across the polymorphic repeat easily detects polymorphisms, the alleles being visualised by gel electrophoresis. Polymorphisms differ in size from one another, as they consist of varying numbers of tandemly repeated DNA sequences. Most individuals inherit a different sized region, or allele, from each parent (ie. are heterozygous for the allele). As a result, two different sized products will be seen on a polyacrylamide gel after PCR. If an individual is homozygous (inheriting alleles of the same size from each parent), only one band will be seen on the gel. For a heterozygous individual, if deletion of part or all of a chromosome occurs in a tumour, only one band will be seen following PCR and gel electrophoresis of tumour DNA, compared to the two bands seen for leucocyte (normal) DNA. This is referred to as loss of heterozygosity (LOH).

1.10.1 Loss of Heterozygosity (LOH) Studies in Ovarian Cancer

Loss of heterozygosity analyses have been widely used in attempts to identify chromosomal regions harbouring recessive genes involved in tumorigenesis (Shelling *et al*, 1995). There is a wealth of literature describing LOH in both ovarian cancer and many other tumour types (Table 1.8). Close chromosomal proximity of a putative TSG is implied by high levels of LOH (Marshall 1991). LOH can occur as a result of generalised genomic instability, and, therefore, a baseline level of 30% LOH is regarded as representing a potentially causative genetic event for most tumour types (Sato *et al*, 1991). However, Chenevix-Trench *et al* (1994) noted that LOH rates in ovarian tumours are seldom in excess of 40%, and suggested that moderate LOH may in fact be significant, as opposed to representing a high background level. The implication from this is that large deletions, detectable by LOH, are not commonly responsible for the inactivation of TSG's in ovarian cancer.

Widespread LOH has been reported for ovarian cancer, implying that many tumour suppressor genes may have a role in ovarian tumorigenesis (Tables 1.9 and 1.10).

Table 1.8 LOH reported in ovarian cancer and other tumour types

MARKER	CHROMOSOMAL LOCATION	LOH LITERATURE FOR OVARIAN TUMOURS	LOH LITERATURE FOR OTHER TUMOURS	ADDITIONAL COMMENTS
D1S243	**1p36.33**		**Tahara H, 1997** - 1p36 LOH parathyroid adenomata **Praml C, 1995** - 1p LOH 84% primary colorectal carcinoma; D1S243 LOH colorectal cancer	Failed to PCR with DNA from paraffin embedded material
D1S504	**1q22-23**		**Law MH, 1997** - 1q21 Wilm's tumours **Parker J, 1997** - 1q23-qter colorectal cancer	*1q23-qter - PGHsynthase II*
D2S391	**2p21-16**	**Leach FS, 1993** - 2p16 HNPCC (Lynch syndrome)		*Mut 5 homologue*
D2S93	**2q21-23**	**Saretzki G, 1997** - 2q21 LOH 8% benign, 11% borderline, 32.4% invasive		Failed to optimise
D2S102	**2q33-37**		**Otsuka T, 1996** - 2q33 deletion lung cancer	Failed to optimise *2q36.3 - site of DNA binding protein*
D3S1582	**3p21.2-21.1**	**Rimessi P, 1994** - 3p21.1-21.2 **Jones and Nakamura, 1992** - 3p21.1-22	**Pandis N, 1993** - 3p deletion breast cancer **Jones and Nakamura, 1992** - 3p13-21.1 endometrial; 3p13-14.3 cervix	
D3S1600	**3p14.2-14.1**	**Fullwood P, 1999** - 3p12-13, 3p14.2 (including FHIT locus), 3p24-25, 3p25-26	**Negrini M, 1996** - FHIT gene breast cancer **Larson AA, 1997** - 3p14.2 cervix cancer **Ogasawara S, 1995** - 3p squamous cell carcinoma oesophagus **Wistuba II, 1997** - 3p14.2, 3p21 early events cervix cancer	*3p14.2 - FHIT gene (fragile histidine triad)*
D3S196	**3q27-28**	**Osborne and Leech, 1994** - 26% LOH invasive with this marker		
D4S174	**4p11-15**	**Sato T, 1991** - 4p LOH 42% invasive **Osborne and Leech, 1994** - 15% LOH invasive with this marker		Failed to PCR with DNA from paraffin embedded material

D4S175	**4q21-qter**	**Osborne and Leech, 1994** - 4q25-q34 LOH 35% invasive		
D5S406	**5p15.3-14**		**Wieland and Bohm, 1994** - 5p13-q14 lung cancer **Murty VV, 1996** - 5p15.1-15.2 male germ cell tumours **Mitra AB, 1994** - 5p LOH cervix cancer	
D5S424 **D5S644**	**5q13.3** **5q13.3-21.1**	**Tavassoli M, 1996** - smallest common region of deletion flanked by D5S424 and D5S644 at 5q13.1-21 (proximal to APC locus)		
D5S346	**5q21-22**	**Osborne and Leech, 1994** - 40% LOH invasive **Chenevix-Trench G, 1997** - 40% LOH invasive with this marker **Saretzki G, 1997** - 30.4% LOH invasive at 5q21	**Mao EJ, 1998** - APC locus associated with squamous cell oral cancer	*5q21 - APC locus*
D5S399	**5q22.3-31**			*5q31 - Interferon Reg Factor I (IRF-1)*
D6S284	**6q13-15**	**Orphanos V, 1995a** - 50% LOH invasive **Roy WJ, 1997** - 15% LOH benign with this marker **Watson RH, 1998** - 0% LOH borderline with this marker	**Noviello C, 1996** - 6q13, 6q21, 6q24-25, 6q27 breast cancer **Cooney KA, 1996** - 6q14-21 prostate cancer	
D6S287	**6q21-23.3**	**Orphanos V, 1995a** - 40% LOH invasive; 57% LOH benign **Roy WJ, 1997** - 5% LOH benign with this marker **Watson RH, 1998** - 0% LOH borderline with this marker	**Ray ME, 1996** - 6q21 malignant melanoma **Bell DW, 1997** - 6q21-23.2, 6q14-21, 6q16.3-21, 6q25 malignant mesothelioma	
D6S1027	**6q25.3-27**	High frequency LOH at 6q24-27 - **Lee JH, 1990; Saito H, 1992; Foulkes W, 1993a** **Tibiletti M, 1996** - ? deletion of 6q27 an early lesion	**Orphanos V, 1995b** - 6q25.2-27 breast cancer	*6q24-27 - oestrogen receptor locus*

D7S654	**7p22.3-21.3**	**Roy WJ, 1997** - 10% LOH benign with this marker **Watson RH, 1998** - 60% LOH borderline with this marker		
D7S691	**7p14.2-12.2**	**Roy WJ, 1997** - 25% LOH benign with this marker **Watson RH, 1998** - 25% LOH borderline with this marker		
D7S670	**7p13**			
D7S672	**7q11.1-21.1**			
D7S491	**7q21-22**	**Chenevix-Trench G, 1997** - 7q21.3-22 LOH 40% invasive; 7q21-31 LOH 35% invasive **Roy WJ, 1997** - 13% LOH benign with this marker **Watson RH, 1998** - 20% LOH borderline with this marker	**Ishwad CS, 1997** - 7q22 benign lesions eg. uterine leiomyoma	
D7S633	**7q31**	**Zenklusen JC, 1995b** - 7q31.1 73% invasive **Koike M, 1997** - ? 7q31.1 deletion in advanced tumours	**Zenklusen JC, 1994a,b** - 7q31.1 LOH very common in breast (83%), and prostate (83%) cancers **Zenklusen JC, 1995a** - 7q31.1-7q31.2 80% colon, 53% squamous cell of head and neck **Lin JC, 1996** - 7q31 breast cancer	*7q31 - MET gene*
D8S258	**8p23-22**	**Emi M, 1992** - 8p23.1-21.3 deletion in some ovarian cancer **Wright K, 1998** - 8p22 and 8p23 **Brown MR, 1999** - 8p21 50% invasive; no LOH borderline	**Emi M, 1992** - 8p23.1-21.3 hepatocellular, colon, and non small cell lung cancer **Wood S, 1994** - 8p21 colon and lung **Yaremko ML, 1996** - 8p21-22 LOH ? late event in breast **Suzuki H, 1995** - 8p22-21.3 prostate cancer **Seitz S, 1997** - 8p12-22 breast cancer	*8p21 - SFTP2 locus (pulmonary surfactant SP5)*
D8S543	**8q12-13**			

D9S171 **D9S161**	**9p21** **9p21**	**Chenevix-Trench G, 1994** - candidate gene between D9S171 and IFNA **Weitzel J, 1994** - 53% LOH **Schultz DC, 1995** - putative TSG 9p21-24 **Rodabaugh K, 1995b** - 33% LOH invasive on 9p **Saretzki G, 1997** - 25% LOH invasive **Roy WJ, 1997** - 25% LOH with D9S171 benign; 22% LOH with D9S161 **Watson RH, 1998** - 29% LOH with D9S171 borderline; 67% with D9S161	**Orlow I, 1994** - 9p bladder cancer **An HX, 1996** - 9p21-22 sporadic breast cancer **Mead LJ, 1997** - region proximal to CDKN2 within 9p21 significant in non small cell lung cancer **Wiest JS, 1997** - 9p deletion proximal to p16/CDKN2 in squamous cell lung cancer	*9p21-22 - p16/CDKN2 gene*
D9S127	**9q31**	**Schultz DC, 1995** - putative TSG's at 9q31 and 9q32-34, spanning D9S127	**Cowan R, 1997** - 9q LOH in Gorlin syndrome (naevoid BCC)	
D10S89	**10p15.1-13**	**Osborne and Leech, 1994** - 0% LOH invasive	**Kimmelman AC, 1996** - 10p15 LOH glioma	
D10S1696 **D10S215**	**10q21.3-23.3** **10q22-23**		**Raskind WH, 1996** - 10q chondrosarcomas **Peiffer S, 1995**- 10q23-26 endometrial cancer **Rhei E, 1997** - 10q23 uncommon in sporadic breast cancer; present in Cowden's disease (hereditary breast cancer) **Wang SI, 1997**- 10q23 glioblastoma multiforme	*10q23 - PTEN/MMAC1*
D11S902	**11p15-13**	**Eccles DM, 1992** - 11p15.4 LOH 33%; 18% at 11p15.5 invasive; no LOH benign or borderline **Osborne and Leech, 1994** - 11p13-15 43% LOH invasive **Weitzel JW, 1994** - 11p15 LOH 50% invasive **Lu KH, 1997** - 11p15.5-15.3; 11p15.1 LOH in high grade ovarian tumours	**Gicquel C, 1997** - 11p15 malignant adrenocortical tumours **Shipman R, 1998** - 11p13 non small cell lung cancer	*11p13 - WT1* *11p13 - CAT* *(catalase gene)* *11p15 - IGF II* *(insulin-like growth factor II)* *11p15.5 - HRAS*

D11S912 **D11S1336** **D11S969**	**11q23** **11q23-24.2** **11q24.1-25**	**Foulkes W, 1993 b** - 49% LOH epithelial ovarian tumours **Davis M, 1996**- distal region of 11q LOH flanked by D11S912 and D11S439 **Foulkes W, 1993 b** - 66.7% LOH at 11q23.3-qter **Gabra H, 1995** - consensus region of deletion 11q23.3-24.1; 11q22-q23.3 and 11q23.3-24.3 LOH associated with poor survival **Davis M, 1996**- proximal region of 11q LOH flanked by D11S925 and D11S1336 **Roy WJ, 1997** - 12% LOH benign with this marker **Watson RH, 1998** - 43% LOH borderline with this marker	**Pabst T, 1996**- 11q23 LOH adult leukaemia	
D12S356 **D12S385**	**12p13** **12pter-qter**	**Hatta Y, 1997** - 12p12.2-13.1 26% LOH	**Cave H, 1997** - 12p12-13 childhood ALL **Takeuchi S, 1997** - 12p13 childhood ALL **Aihara, 1996** - 12q24.3-qter in hepatocellular carcinoma	*12p12.3-13.1 - TEL gene (ETS-family transcriptional factor); p27kip1 (cyclin dependent kinase inhibitor)*
D13S133 **D13S128**	**13q14.3** **13q32-34**	**Yang Feng TL, 1992 and 1993** - >40% LOH 13q **Kim TM, 1994** - chromosome 13 LOH only in high grade tumours	**Wooster R, 1994** - 13q12-13 breast (BRCA2) **Pabst T, 1996** - 13q14.3 CLL **Yamaguchi T, 1996** - 13q chondrosarcoma **Yokoyama J, 1996** - 13q14 head and neck tumours **Zeremski M, 1997** - 13q34 squamous tumours of head and neck	*13q12-13 - BRCA2* *13q14 - retinoblastoma (Rb) gene locus* *13q34 - ING1*

D14S80	**14q12-13**	**Osborne and Leech, 1994** - 46% 14q LOH invasive **Bandera CA, 1997** - LOH detected with this marker	**Young J, 1993** - distal 14q LOH colorectal cancer **Tse JYM, 1997** - 14q LOH meningiomas	
FES	**15q26.1**	**Osborne and Leech, 1994** - 40% LOH invasive with this marker		
D16S3072 **D16S3098**	**16pter-qter** **16pter-qter**	**Kawakami M, 1999** - high grade invasive 48% LOH at 16q23.1-23.2; 52% at 16q23.3-24.1; 57% at 16q24.2-24.3	**Godfrey TE, 1997** - 16q24.2-qter prostate cancer	*16q - H-cadherin (CDH13)* Failed to PCR with DNA from paraffin embedded material
D17S786	**17pter-qter**	**Leary J, 1993** - 82% LOH at 17p13.3 invasive **Wiper DW, 1998** - 17p13 LOH distal to TP53	**Pietsch T, 1997** - 17p13.3 medulloblastoma	*17p13.1 - TP53*
D17S855 **D17S1322** **D17S1323** **D17S1327**	**17q21.1** **17q21** **17q21** **17q21**	**Cliby W, 1993** - 76% invasive **Osborne and Leech, 1994** - 64% 17q LOH invasive **Weitzel J, 1994** - 50% invasive	**Caduff RF, 1999** - 17q21 16% LOH in sporadic infiltrating ductal carcinoma of breast	*17q21 - BRCA1*
D18S59	**18pter-p11.22**	**Dodson M, 1993** - 18p LOH 11% ovarian tumours		
D18S474	**18pter-qter**	**Enomoto T, 1995** - DCC LOH common **Chenevix-Trench G, 1997** - 18q21 LOH 54% invasive at DCC locus; 64% at BCL2 locus; 36% at PLANH2 locus **Takaknra S, 1999** - 36% LOH invasive with this marker (adjacent to SMAD4)	**Chenevix-Trench, 1992** - common DCC LOH in colorectal cancer **Ding SF, 1994** - 18q colorectal cancer **Latil A, 1995** - 18q prostate cancer	*18q21 - SMAD2* *18q21 - SMAD4* *18q21.3 - DCC* *18q21.3 - BCL2* *18q21.3 - PLANH2*

D19S886	**19pter-qter**	**Amfo K, 1995** - 19p **Wang ZJ, 1999** - 24% LOH sporadic adenocarcinomas with this marker	**Pabst T, 1996** - 19p13 adult ALL	*19p13.3 - AMH (Mullerian Inhibiting Substance gene)* *19p13.3 - Insulin receptor locus* *19p13.3 - LKB1 (Peutz-Jeghers locus)*
D19S412	**19q13.3**	**Bicher A, 1997** - 19q13.2-13.4	**Rubio MP, 1994** - 19q13.2-13.3 gliomas	Failed to optimise
D20S851	**20pter-qter**			
D20S17	**20q12**			
D21S1432	**21q21**	**Osborne and Leech, 1994** - 21q22.3 54% invasive; 21q22.3-qter 23%		
D22S274 **D22S276**	**22q13.3** **22q13.2**	**Dodson MK, 1993** - 52.8% invasive **Englefield P, 1994** - NF2 not involved in ovarian tumorigenesis **Bryan EJ, 1996** - 22q LOH 53% sporadic ovarian tumours	**Akagi K, 1995** - 22q meningiomas **Yana I, 1995** - 22q sporadic colorectal cancer **Gutmann DH, 1997** - 22q brain tumours **Urano T, 1997** - 22q11 rhabdoid tumours **Alllone F, 1998** - 66% 22q LOH sporadic breast cancer	Failed to optimise Failed to optimise *22q12 - NF2 gene (Neurofibromatosis type II)*
DXS538	**Xp11.21-21.1**	**Cliby W, 1993** - 28% Xp LOH invasive **Yang Feng TL, 1993** - 41% Xp LOH invasive **Osborne and Leech, 1994** - Xp11.21-21.1 40% LOH invasive **Cheng PC, 1996** - no Xp LOH benign; 4.8% LOH borderline	**Loupart ML, 1995** - Xp breast cancer	Failed to optimise
HPRT	**Xq26.1**	**Yang Feng TL, 1993** - Xq26 26% LOH invasive **Osborne and Leech, 1994** - 21% invasive		

* Markers illustrated are those chosen for LOH analysis in this study

Table 1.9 LOH and invasive ovarian cancer

	CHROMOSOME ARM																					
Investigators	**1p**	**1q**	**2p**	**2q**	**3p**	**3q**	**4p**	**4q**	**5p**	**5q**	**6p**	**6q**	**7p**	**7q**	**8p**	**8q**	**9p**	**9q**	**10p**	**10q**	**11p**	**11q**
Ehlen /Dubeau 1990					57.1				0		60	28.6									44.4	
Lee et al 1990		0		20						0	0	64		20							46	0
Russell et al 1990																						
Okamoto et al 1991																						
Sato et al 1991	7	29	0	14	18	6	42	6	-	0	50	17	43	21	-	31	-	10	11	21	24	5
Eccles et al 1992																						
Gallion et al 1992																						
Saito et al 1992												33.3										
Vandamme et al 1992																					50	
Viel et al 1992																					41	
Yang Feng et al 1992																						
Cliby et al 1993	24	28	17	33	0	17	34	19	21	43	62	57	36	13	40	29	33	54	12	11	35	33
Foulkes et al 1993a											28	55										
Foulkes et al 1993b																					67	
Foulkes et al 1993c																						
Jacobs et al 1993																						
Leary et al 1993					38							23									33	
Saito et al 1993																						
Allan et al 1994									38	50												
C-Trench et al 1994																	40.8					
Englefield et al 1994																						
Kim et al 1994																						
Osborne /Leech 1994	19	23	17	38	26	32	18	33	19	40	27	35	17	19	26	26	30	48	22	19	43	30
Weitzel et al 1994	24									42	28	33					48				50	29
Campbell et al 1995																	50					

Investigators	1p	1q	2p	2q	3p	3q	4p	4q	5p	5q	6p	6q	7p	7q	8p	8q	9p	9q	10p	10q	11p	11q
Enomoto et al 1995																						
Gabra et al 1995																					72	66
Orphanos et al 1995a												44										
Pieretti 1995		9.5	5.7				14.3			10.8		2.5		7.7		16.7		17.4		17.5		
Rodabaugh et al 1995a												29										
Rodabaugh et al 1995b																	38					
Schultz et al 1995																	9	61.8				
Arnold et al 1996								23														
Davis et al 1996																						64.5
Kerr et al 1996														38								
Tavassoli et al 1996									5	53.5												
Bandera et al 1997																						
Bicher et al 1997																						
C-Trench et al 1997	56		27		-			-		62		52		40			64	50			0	29
Choi et al 1997																						
Hatta et al 1997																						
Koike et al 1997														50								
Lu et al 1997																					48	
Saretzki 1997				32.4						30.4							25					
Colitti et al 1998												44										
Edelson et al 1998																						
Launonen et al 1998																						61
Suzuki et al 1998												54										
MEAN LOH	**26**	**17.9**	**13.3**	**27.5**	**27.8**	**18.3**	**27.1**	**20.3**	**16.6**	**33.2**	**36.4**	**38.1**	**32**	**26.1**	**33**	**25.7**	**37.5**	**40.2**	**15**	**17.1**	**42.6**	**35.3**

* If more than one marker used for a particular chromosome arm, and no total LOH calculated in literature, highest rate recorded here

CHROMOSOME ARM

Investigators	12p	12q	13q	14q	15q	16p	16q	17p	17q	18p	18q	19p	19q	20p	20q	21q	22q	Xp	Xq
Ehlen / Dubeau 1990																0			
Lee et al 1990	0							75	37.5				14				20		
Russell et al 1990								40	80										
Okamoto et al 1991								79											
Sato et al 1991	38	33	22	18	11	33	37	46	39	0	0	33	25	13					
Eccles et al 1992								54	70										
Gallion et al 1992			68.2																
Saito et al 1992																			
Vandamme et al 1992																			
Viel et al 1992																			
Yang Feng et al 1992			46																
Cliby et al 1993	9	24	56	47	36	21	35	81	76	10	43	15	17	0	16	36	71	28	-
Foulkes et al 1993a																			
Foulkes et al 1993b																			
Foulkes et al 1993c								78	75										
Jacobs et al 1993									55.4										
Leary et al 1993								82	62										
Saito et al 1993									39.3										
Allan et al 1994																			
C-Trench et al 1994																			
Englefield et al 1994																	72		
Kim et al 1994			37.9																
Osborne /Leech 1994	6	13	12	46	40	0	16	61	64	6	27	45	23	21	21	33	28	40	21
Weitzel et al 1994			14					64	50										
Campbell et al 1995																			

Investigators	12p	12q	13q	14q	15q	16p	16q	17p	17q	18p	18q	19p	19q	20p	20q	21q	22q	Xp	Xq
Enomoto et al 1995											33								
Gabra et al 1995																			
Orphanos et al 1995a																			
Pieretti 1995			16.7	15.6	0		10.2		48							3.9	14.9		
Rodabaugh et al 1995a																			
Rodabaugh et al 1995b																			
Schultz et al 1995																			
Arnold et al 1996			17				17				23								
Davis et al 1996																			
Kerr et al 1996																			
Tavassoli et al 1996																			
Bandera et al 1997				51.4															
Bicher et al 1997													33.3						
C-Trench et al 1997	7		47					82	80		72						29	20	37
Choi et al 1997																			31.1
Hatta et al 1997	28	38																	
Koike et al 1997																			
Lu et al 1997																			
Saretzki 1997								56	40.5		21.4								
Colitti et al 1998																			
Edelson et al 1998																			38
Launonen et al 1998																			
Suzuki et al 1998																			
MEAN LOH	**14.7**	**27**	**33.7**	**35.6**	**21.8**	**18**	**23**	**66.5**	**58.3**	**5.3**	**31.1**	**31**	**22.5**	**11.3**	**18.5**	**18.2**	**39.2**	**29.3**	**31.8**

* If more than one marker used for a particular chromosome arm, and no total LOH calculated in literature, highest rate recorded here

Table 1.10 LOH in benign and borderline ovarian tumours

	CHROMOSOME ARM																					
Investigators	**1p**	**1q**	**2p**	**2q**	**3p**	**3q**	**4p**	**4q**	**5p**	**5q**	**6p**	**6q**	**7p**	**7q**	**8p**	**8q**	**9p**	**9q**	**10p**	**10q**	**11p**	**11q**
Russell et al 1990																						
Eccles et al 1992																						
Gallion et al 1992																						
Vandamme et al 1992																					- 0	
Foulkes et al 1993a											0 0	0 0										
Jacobs et al 1993																						
Leary et al 1993												0 -									0 -	
C-Trench et al 1994																	50 15.4					
Kim et al 1994																						
Campbell et al 1995																	- 0					
Orphanos et al 1995a												57 66.7										
Pieretti 1995		- 0	- 0				- 0			- 0		- 6.3		- 6.7		- 0		- 0		- 0		
Rodabaugh et al 1995a												- 11.5										
Rodabaugh et al 1995b																	- 20					
Schultz et al 1995																	50 -	0 -				
Cheng et al 1996																						
Davis et al 1996																						0 66.7
Kerr et al 1996														0 3.7								
Tavassoli et al 1996									0 0	0 0												

Investigators	1p	1q	2p	2q	3p	3q	4p	4q	5p	5q	6p	6q	7p	7q	8p	8q	9p	9q	10p	10q	11p	11q
Bandera et al 1997																						
Bicher et al 1997																						
C-Trench et al 1997	0 0		0 0		0 0			0 0		0 0		16.7 7.4		0 4.5	0 0		6.3 16.7	0 0				
Roy et al 1997																						9 -
Saretzki 1997				7.7 7.1						0 10	0 9.1	0 0					0 0					0 0
Edelson et al 1998																						
Watson et al 1998												- 0					- 42.9					
MEAN LOH	**0** **0**	**-** **0**	**0** **0**	**7.7** **7.1**	**0** **0**	**-** **-**	**-** **0**	**0** **0**	**0** **0**	**0** **2.5**	**0** **4.6**	**14.7** **13.1**	**-** **-**	**0** **5**	**0** **0**	**-** **0**	**26.6** **15.8**	**0** **0**	**-** **-**	**-** **0**	**0** **0**	**3** **33.4**

* If more than one marker used for a particular chromosome arm, and no total LOH calculated in literature, highest rate recorded here

Data recorded benign (%) - = not done

borderline

CHROMOSOME ARM

Investigators	12p	12q	13q	14q	15q	16p	16q	17p	17q	18p	18q	19p	19q	20p	20q	21q	22q	Xp	Xq
Russell et al 1990								- 0	- 50										
Eccles et al 1992								7.7 14.3	13 12.5										
Gallion et al 1992			25 40																
Vandamme et al 1992																			
Foulkes et al 1993a																			
Jacobs et al 1993									50 16.7										
Leary et al 1993								0 -	0 0										
C-Trench et al 1994																			
Kim et al 1994			0 0																
Campbell et al 1995																			
Orphanos et al 1995a																			
Pieretti 1995			- 0	- 6.3	- 6.7		- 0									- 7.1	- 0		
Rodabaugh et al 1995a																			
Rodabaugh et al 1995b																			
Schultz et al 1995																			
Cheng et al 1996																			- 50
Davis et al 1996																			
Kerr et al 1996																			
Tavassoli et al 1996																			

Investigators	12p	12q	13q	14q	15q	16p	16q	17p	17q	18p	18q	19p	19q	20p	20q	21q	22q	Xp	Xq
Bandera et al 1997				- 0															
Bicher et al 1997													- 0						
C-Trench et al 1997			0 0									0 0					- 0		40 12.5
Roy et al 1997																			
Saretzki 1997								6.7 4.8	0 4.5		0 0						0 0		
Edelson et al 1998																			- 25
Watson et al 1998																			
MEAN LOH	- -	- -	**8.3** **10**	- **3.2**	- **6.7**	- -	- **0**	**4.8** **6.4**	**15.8** **16.7**	- -	**0** **0**	**0** **0**	- **0**	- -	- -	- **7.1**	**0** **0**	- -	**40** **29.2**

* If more than one marker used for a particular chromosome arm, and no total LOH calculated in literature, highest rate recorded here

Data recorded benign (%) - = not done

borderline

1.11 FAMILIAL OVARIAN CANCER

Between 5 and 10% of ovarian cancers result from an inherited predisposition to the disease (Lynch *et al,* 1990; Narod *et al,* 1994). Apart from age, family history of ovarian cancer confers the greatest risk of all known factors (Schildkraut and Thompson 1988; Amos *et al*, 1992; Amos 1993). Analysis of the majority of case-control studies examining this association showed a 3.6 fold increase in risk (Amos 1993).

There are three main predisposing syndromes for ovarian cancer (Liede *et al*, 1998), all of which are rare and have an autosomal dominant mode of transmission of susceptibility:

1. Hereditary Breast Ovarian Cancer Syndrome (HBOCS) - the most common
2. Hereditary Non-Polyposis Colorectal Carcinoma Syndrome (HNPCC/ Lynch II Syndrome/ Cancer Family Syndrome) (Lynch and Lynch 1979)
3. Site-specific Ovarian Cancer Syndrome - the least common (Gallion *et al*, 1995)

1.11.1 The Hereditary Breast Ovarian Cancer Syndrome

65-75% of hereditary cases of ovarian cancer are accounted for by this syndrome (Bewtra *et al*, 1992). The syndrome encompasses families in which there are at least 5 cases of breast or ovary cancer in first and second degree relatives (Boyd 1998), and also those where there are at least 3 cases of early onset breast or ovarian cancer (Narod *et al*, 1995a,b).

76-100% of HBOCS families are linked to BRCA1 (Easton *et al*, 1993; Narod *et al*, 1995a,b; Szabo and King 1995). The majority of families not linked to BRCA1 are linked to BRCA2, particularly those families which feature cases of male breast cancer. The incidence of ovarian cancers, compared with breast, seems to be much lower in BRCA2-linked families.

1.11.2 The Hereditary Non-Polyposis Colorectal Cancer Syndrome

This syndrome was previously known as Lynch Syndrome II or The Cancer Family Syndrome. It accounts for 10-15% of all hereditary ovarian cancer. Inherited in an autosomal dominant fashion, HNPCCS encompasses families in which at least 3 first

degree relatives have carcinomas of the colon or endometrium, with at least 2 of the cases of colonic carcinoma diagnosed at less than 50 years of age (Lynch *et al*, 1993; Watson and Lynch 1993). Within these families there is also a substantially increased risk of development of tumours elsewhere in the gastrointestinal tract eg. stomach and small bowel, in the upper urological tract eg. kidney and ureter, and in the ovary (Watson and Lynch 1993). The risk of development of ovarian cancer is increasd by 4 fold compared with that of the general population (Watson and Lynch 1993).
There are four known genes contributing to the HNPCC phenotype, all of which are MMR genes - hMSH2 (on chromosome 2p), hMLH1 (on chromosome 3p), hPMS1 (on chromosome 2q), and hPMS2 (on chromosome 7p) (Fishel and Kolodner 1995; Marra and Boland 1995). More than 90% of mutations affect either hMSH2 or hMLH1.

1.11.3 Site-specific Ovarian Cancer Syndrome

Whether Hereditary Site-specific Ovarian Cancer represents a genetic entity separate from HBOCS is not clear (Liede *et al*, 1998). It seems likely that it simply represents a variant of HBOCS, attributable to either BRCA1 or BRCA2 (Steichen-Gersdorf *et al* , 1994; Shattuck-Eidens *et al*, 1995). A Site-specific Ovarian Cancer family is defined as one in which there are at least 3 cases of epithelial ovarian cancer (diagnosed at any age) and no cases of breast cancer diagnosed before 50 years old (Steichen-Gersdorf *et al*, 1994). A series of nine families with multiple ovarian cancer cases, seven of which had positive linkage to BRCA1, was described by Steichen-Gersdorf.

1.12 BRCA1 AND BRCA2

1.12.1 BRCA1

Broca, in 1886, suggested that familial clustering of breast and ovarian cancer was caused by a genetic or heritable factor (Broca 1886).

Hall, in 1990, demonstrated, by linkage analysis, the existence of a breast and ovarian cancer susceptibility gene, and mapped this putative gene to chromosome 17q21 for breast cancer (Hall *et al*, 1990). In 1993, a series of 214 families was studied (Easton *et al*, 1993). For nearly all HBOCS families, and 45% of breast cancer only families, linkage to 17q was demonstrated. Using positional cloning methods, the gene was subsequently localised to 17q12-22: following examination of 65 transcription sequences, 3 emerged into a single sequence highly suggestive of the susceptibility gene (Miki *et al*, 1994).

BRCA1 was finally cloned in 1994 (Miki *et al*, 1994). It comprises 22 coding exons, and 2 non-coding exons, covers 100kb of genomic DNA, and encodes a protein of 1863 amino acids.

1.12.1.1 *Structure and functional regions of BRCA1*

Subsequently, domains of potential functional significance have been identified in BRCA1, which are conserved between mammalian species (Figure 1.6). These include:

1. **an N terminal 'zinc finger' motif (RING finger)**, containing cystine and histidine residues which are present in the consensus Cys3-His-Cys4 (C3HC4) zinc finger motif, and sharing many other residues with zinc finger proteins in the databases (Miki *et al*, 1994). The presence of this motif suggests that BRCA1 encodes a transcription factor, and it would, therefore, be expected that inactivation of BRCA1 would affect expression of other genes involved in the regulation of growth and differentiation of breast and ovarian epithelium (Boyd and Rubin 1997).

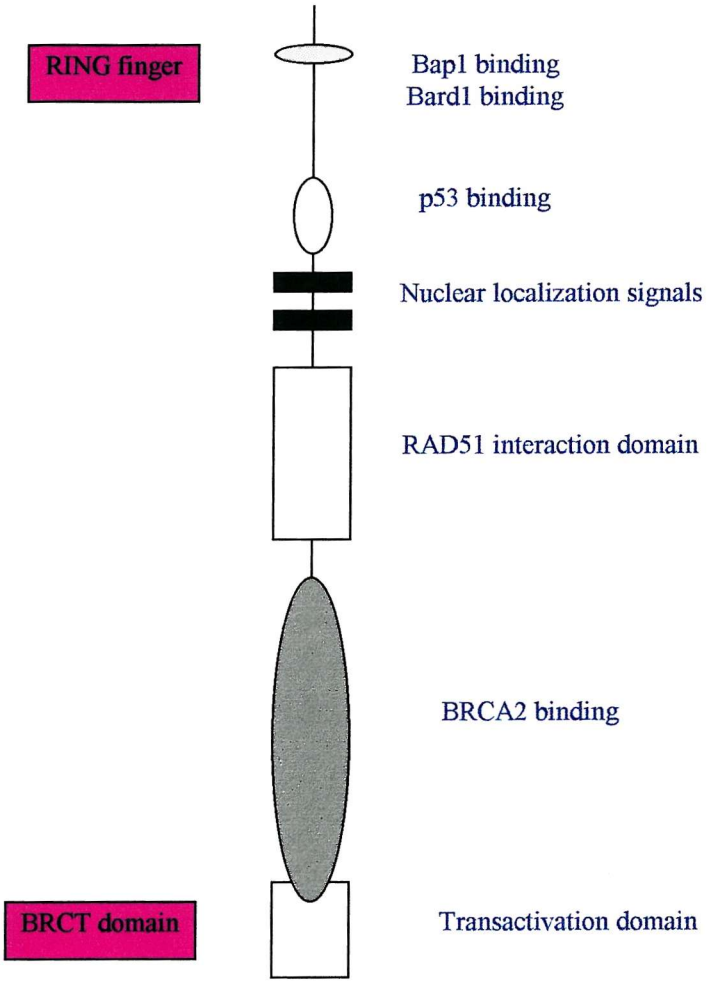
Several RING finger proteins facilitate ubiquitination. In vitro, the BRCA1 RING finger and its adjacent domains are ubiquitinated in the presence of ubiquitin-conjugating enzymes. Thus, it may function with, or as, a ubiquitin-protein ligase targeting proteins for proteosomal degradation. If this is the case, loss of the RING finger of BRCA1 would lead to an increase in steady state levels of proteins which stimulate proliferation.

The BRCA1 RING finger region is also the site of heterodimerization of BRCA1 and BARD1 (BRCA1-associated RING domain 1), which is another N-terminal RING finger protein. In addition the domain binds the ubiquitin hydrolase BAP1 (BRCA1-associated protein 1). BAP1 may modulate the BRCA1 ubiqutination of other proteins and/or ubiquitin-dependent degradation of BRCA1 itself (Ingvarsson 1999;TIG 2000).
2. **the 'BRCT' domain** (BRCA1 C terminal domain), containing an amino acid sequence common to other polypeptides with a role in cell cycle control (the p53 binding protein 53BP1, and the yeast RAD9 protein which mediates cell cycle arrest in response to DNA damage) (Miki *et al*, 1994; Koonin *et al*, 1996; Wu *et al*, 1996).
3. **a 'granin' motif** at amino acids 1214-1223 (Jensen *et al*, 1996). The function of granins is not clear, but they may be involved in packaging and processing of peptide hormones. Presence of a granin motif is characteristic of a family of secreted proteins, but there has been controversy over the subcellular localisation of BRCA1. In various studies, it has been characterised as nuclear, cytoplasmic, and secreted (Chen *et al*, 1995; Jensen *et al*, 1996; Scully *et al*, 1996).

1.12.1.2 *Cellular expression of BRCA1*

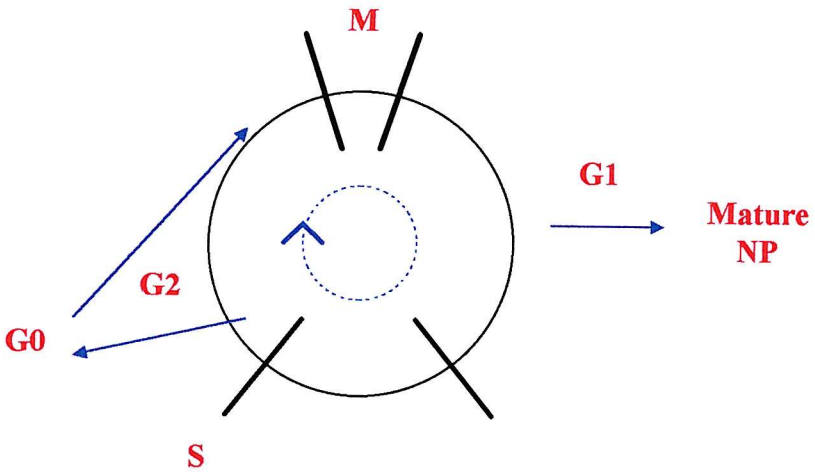
BRCA1 mRNA and protein are preferentially expressed in the late G1-early S phase of the cell cycle (Figure 1.7), when hyperphosphorylation occurs. Dephosphorylation occurs in the M phase. A function of BRCA1 during the G1-S transition may be to arrest cell cycle progression by binding hyperphosphorylated retinoblastoma protein. BRCA1 may also have a role in regulation of the G2-M checkpoint, by controlling the assembly of mitotic spindles and ensuring appropriate segregation of chromosomes to daughter cells (TIG 2000).

Figure 1.6 **The BRCA1 protein (1863 amino acids)**



Adapted from Ingvarsson 1999

Figure 1.7 **The normal cell cycle**



M	Mitosis (prophase/ metaphase/ anaphase/ telophase)	**G1**	Early growth phase
G	Gap	**G2**	Later growth phase
S	DNA synthesis	**NP**	Non-proliferating
G0	Resting phase		

1.12.1.3 *Expression of BRCA1 in murine development*

Marquis *et al* (1995) investigated the developmental expression of BRCA1, using the murine homologue, by in situ hybridisation of BRCA1 mRNA in embryonic tissues. BRCA1 expression was ubiquitous up to day 20 post conception. Subsequently, localisation to specific organs was observed - thymus, liver, lung, brown fat, renal cortex, and salivary gland - overlapping with the pattern described for p53. During mammary gland development, BRCA1 was expressed in ductal epithelium of both females and males. At 2-5 weeks (pubertal period), expression was higher in females, and higher in terminal buds than ducts, but the high pubertal levels were declining by 10-16 weeks of age. In early pregnancy, mammary BRCA1 mRNA levels showed a sharp early rise, then remaining elevated throughout pregnancy and lactation. Even when breast tissue regressed after lactation, BRCA1 levels remained higher than those of virgin mice. Adult mouse tissues showed significant BRCA1 levels in ovary, breast, uterus, spleen, lymph nodes and liver, with the highest levels in thymus and testis. Tissues undergoing proliferation and differentiation also expressed BRCA1.
Wu *et al* (1996), also studying mouse embryos, was able to identify the murine homologue of BRCA1 in many growing and differentiating tissues.
When compared with the human BRCA1 sequence, that of the mouse shows 60% identity and 72% similarity at the nucleic acid level (Marquis *et al*, 1995, TIG 2000). The N-terminal 100 residues and BRCT domain are conserved (Wu *et al*, 1996).

The patterns of expression of BRCA1 during mouse development, together with the cell-cycle and hormone-mediated expression of the gene, suggest its relationship with cell proliferation and differentiation. BRCA1 expression has been shown to increase during cell cycle progression, suggesting it may act to regulate transcription of genes involved in cellular proliferation (Gudas *et al*, 1995).

1.12.1.4 *The role of BRCA1 in DNA repair*

BRCA1 (together with BRCA2) may also have a role in the maintenance of genomic stability - they may, together with RAD51, be involved in repair of double-stranded DNA breaks (Scully *et al*, 1997; Ingvarsson, 1999). BRCA2 and RAD51 interact, and co-localise in a BRCA1-BRCA2-RAD51 complex. BRCA1 also associates with

RAD50, which is also involved in the response to DNA damage (TIG 2000). A possible model for the involvement of BRCA1 (and BRCA2) in repair of DNA damage is shown in Figure 1.8.

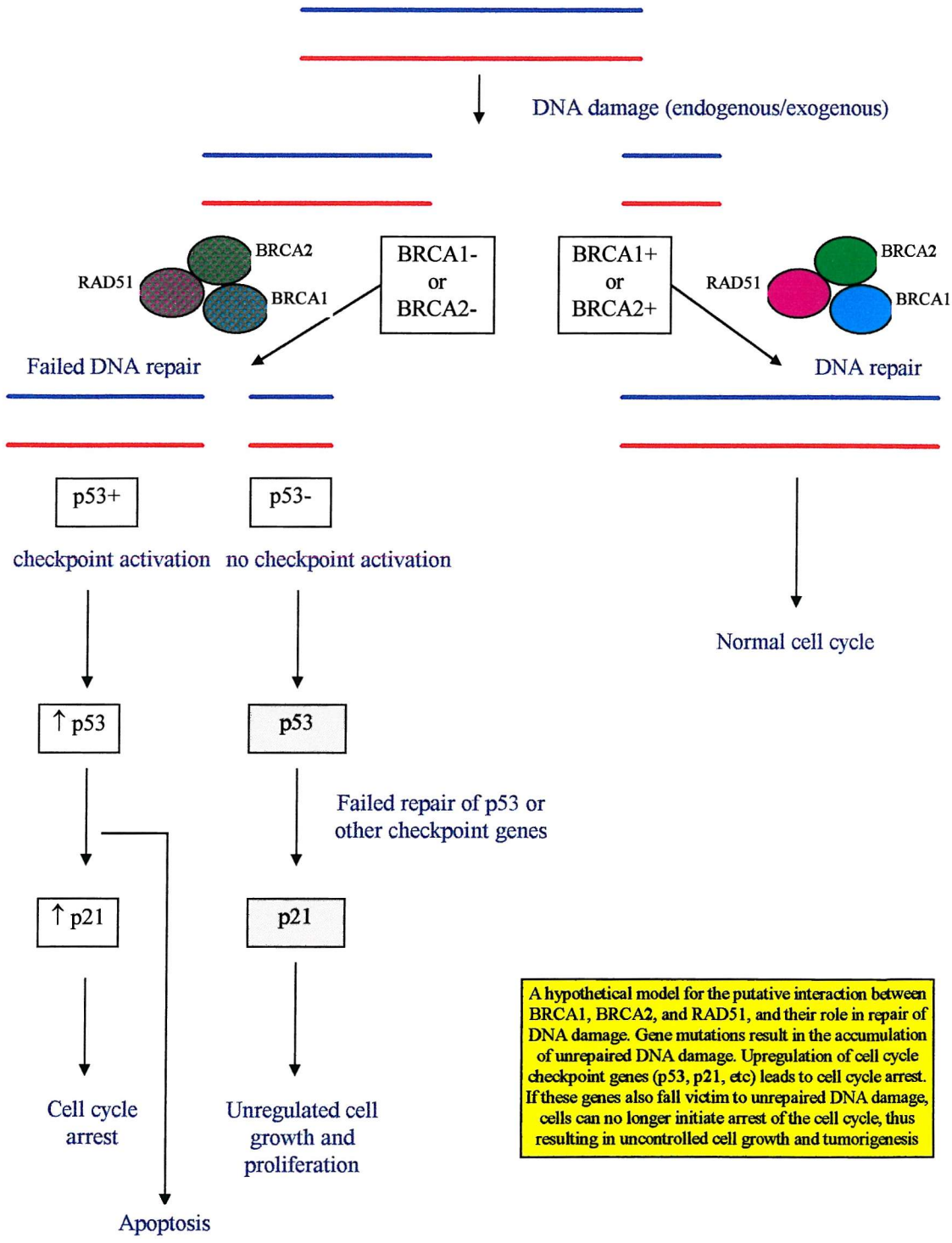
1.12.1.5 *The role of BRCA1 in transcriptional regulation*

A potential role has been suggested by the observed interactions of BRCA1 with RNA helicase A and with ctlp (a protein of unknown function that associates with the transcriptional repressor ctBP), together with the behaviour of BRCA1 in transcriptional activation assays. Clinically relevant mutations at the 3' end of BRCA1 have been seen to reduce its association with RNA helicase A. Protein-truncating mutations of BRCA1, which lack the extreme C terminal part of the transactivation domain, fail to induce transcription of reporter plasmids, which suggests a link between tumour suppression and transcriptional regulation (TIG 2000).

1.12.1.6 *Evidence for BRCA1 as a tumour suppressor gene*

Germline BRCA1 mutations found in various breast and ovarian cancer families support its function as a TSG (Struewing *et al*, 1995; Serova *et al,* 1996; Hamann *et al*, 1997). Approximately 80% of mutations are frameshift or nonsense 'loss of function' mutations (Shattuck-Eidens *et al*, 1995), and LOH detected allelic deletions in the 17q21 region of BRCA1-linked ovarian or breast cancer always involve the wild-type allele (Smith *et al*, 1992; Merajver *et al,* 1995). Functional studies also support the TSG classification - retroviral transfer of wild-type BRCA1 suppresses in vitro growth of breast and ovarian carcinoma cells, whilst mutants of BRCA1 fail to inhibit growth (Holt *et al*, 1996).

Figure 1.8 **BRCA1 and BRCA2 in DNA repair**



A hypothetical model for the putative interaction between BRCA1, BRCA2, and RAD51, and their role in repair of DNA damage. Gene mutations result in the accumulation of unrepaired DNA damage. Upregulation of cell cycle checkpoint genes (p53, p21, etc) leads to cell cycle arrest. If these genes also fall victim to unrepaired DNA damage, cells can no longer initiate arrest of the cell cycle, thus resulting in uncontrolled cell growth and tumorigenesis


Mutant BRCA1

p53 Mutant p53


Mutant BRCA2

p21 Mutant p21


Mutant RAD51

Adapted from Gayther and Ponder 1998
TIG 2000

1.12.1.7 *Risks of breast and ovarian cancer conferred by BRCA1 mutations*

The estimated gene frequency for aberrant BRCA1 is 0.0006. This means that one would expect 1 in 800 women to carry a mutation (Ford *et al*, 1995a).

It has been suggested that mutations in BRCA1 confer a risk, by 70 years of age, of 87% for breast cancer (Easton *et al*, 1993) and 44% for ovarian cancer (Ford *et al*, 1994). The lifetime risk for either breast or ovarian cancer in individuals carrying these mutations approaches 100% (Ford *et al*, 1994). The risk appears variable, depending on the site of mutation within the gene (Gayther *et al*, 1995; Holt *et al*, 1996). Mutations near the 3' end of the gene seem to be associated predominantly with breast cancer, whilst those closer to the 5' end confer a greater risk of ovarian cancer. The risk of ovarian cancer in BRCA1 mutation carriers is higher when rare alleles of the HRAS1 variable number of tandem repeats (VNTR) polymorphism - located 1 kilobase downstream of the HRAS1 protooncogene (chromosome 11p15.5) - are present. This is consistent with the idea that penetrance of hereditary ovarian cancer may be affected by modifying genetic loci (Phelan *et al*, 1996).

However, the risks quoted may be overestimates, due to biased ascertainment - families in which mutations are detected may have been selected on the basis of a history of early onset breast or ovarian cancer. In a study of an unselected population of Ashkenazi Jews in Washington, USA (Struewing *et al*, 1997), the risk of breast cancer for BRCA1 carriers was calculated to be 56%, and that for ovarian cancer 16%, by 70 years of age. The French National Ad Hoc Committee therefore suggests that the following ranges of risk should be used: 56-87% for breast cancer and 16-63% for ovarian cancer (Eisinger *et al*, 1998). It has been estimated that a study of 100,000 individuals would be necessary to reliably assess the penetrance of mutations in BRCA1 and BRCA2 in the general population, since the prevalence of individual mutations is so low (Easton 1997).

1.12.1.8 *BRCA1 mutations detected*

The Human Gene Mutation Database in Cardiff, UK currently contains 277 novel BRCA1 mutations (to April 2000) detected in families with breast and ovarian cancer (Tables 1.11 and 1.12) , and these show little evidence of clustering. Of those identified, some are seen much more frequently than others. The two commonest mutations are 185delAG on exon 2 and 5382insC on exon 20, which together account for about 20% of all mutations.

Most mutations (more than 90%) are nonsense, frameshift, or splice site mutations (Szabo and King 1995), and result in premature termination of the protein product. Mutations usually only affect one or two nucleotides. The wild-type protein is lost, and a mutant, truncated protein synthesised, varying from 5-99% of the length of the wild-type protein (Holt *et al*, 1996).

Table 1.11 BRCA1 and BRCA2 mutations identified

Mutation type	Total number mutations BRCA1	Total number mutations BRCA2
Missense/nonsense	94	28
Substitutions (splicing)	21	5
Substitutions (regulatory)	0	0
Small deletions	110	66
Small insertions	36	21
Small indels	3	1
Gross deletions	12	0
Gross insertions/duplications	1	0
Complex rearrangements	0	2
Repeat variations	0	0
TOTAL	277	123

Table 1.12 BRCA1 and BRCA2 mutations according to phenotype

Phenotype	Nucleotide substitutions	Microlesions	Gross lesions
BRCA1			
Breast	112	136	13
Ovary	3	13	0
BRCA2			
Breast	31	84	2
Male breast	2	4	0

From The Human Gene Mutation Database, Cardiff
(http://www.uwcm.ac.uk)

1.12.2 BRCA2

The methods used to localise and clone BRCA2 were similar to those for BRCA1. High risk breast cancer families, known not to be linked to BRCA1, were used in linkage analysis, and a locus at 13q12-13 was identified (Wooster *et al*, 1994). BRCA2 comprises 26 coding exons over 70kb of genomic DNA, and encodes an 11-12kb transcript (Tavtigian *et al*, 1996). Mutations occur at sites throughout the gene, and most are frameshift in nature (Wooster *et al*, 1995; Tavtigian *et al*, 1996).

BRCA2 is predicted to account for about a third of families with multiple cases of early onset breast cancer, and is also involved in the genetic predisposition to male breast cancer (Stratton *et al*, 1994). Tumour incidence rates in BRCA2 linked families suggest that it results in a substantially lower ovarian cancer risk than that for breast cancer (Wooster *et al*, 1995). Indeed, the analysis of unselected ovarian cancer cases shows that germline BRCA2 mutations are found in patients with no remarkable family history for breast or ovarian cancer and with late onset disease (Foster *et al*, 1996; Takahashi *et al*, 1996). This is consistent with the suggestion that mutations in BRCA1 contribute to ovarian cancer with a much higher penetrance than BRCA2. Mutations in exon 11, near the centre of the BRCA2 gene, appear to result in a higher ratio of ovarian tumours to breast tumours when compared with other BRCA2 mutations (Gayther *et al*, 1997). This is known as the ovarian cancer cluster region (OCCR).

1.12.2.1 *Structure and functional regions of BRCA2* (Figure 1.9)

A potentially important functional domain is located in the central part of the gene (exon 11), and consists of eight copies of a 30-80 amino acid sequence (known as the BRC repeats). These are conserved across mammalian BRCA2 proteins, and it has been shown, using yeast 2-hybrid screens, that six of the eight BRC repeats bind specifically to the RAD51 protein implicated in repair of double-strand DNA breaks (Gayther and Ponder, 1998; TIG 2000).

Another BRCA2 region which is highly conserved between humans and mice is located at the amino terminus, includes exon 3, and has been shown in vitro to activate transcription. It is homologous to the activation domain of c-Jun, which is a known transcription factor (Gayther and Ponder, 1998). It has been shown that introduction of

a naturally occurring missense mutation into the sequence results in substantially reduced ability to activate transcription.

1.12.2.2 *Cellular expression of BRCA2*

As with BRCA1, expression of BRCA2 mRNA is cell-cycle dependent, and is induced in the late G1-early S phase. Expression seems greatest in cells which are proliferating rapidly, and is reduced in response to deprivation of growth factors (Gayther and Ponder, 1998).

BRCA2 may also have a role in regulation of mitotic checkpoints - tumours from mice with BRCA2 mutations have been shown to be defective in the spindle assembly checkpoint (TIG 2000).

1.12.2.3 *BRCA2 as a tumour suppressor gene*

As with BRCA1, LOH at the BRCA2 locus in tumours from BRCA2-linked individuals invariably affects the wild-type allele, consistent with a TSG classification (Gudmundsson *et al*, 1995).

For BRCA2, the Human Gene Mutation Database in Cardiff, UK currently contains 123 novel mutations (to April 2000), details of which are shown in Tables 1.11 and 1.12.

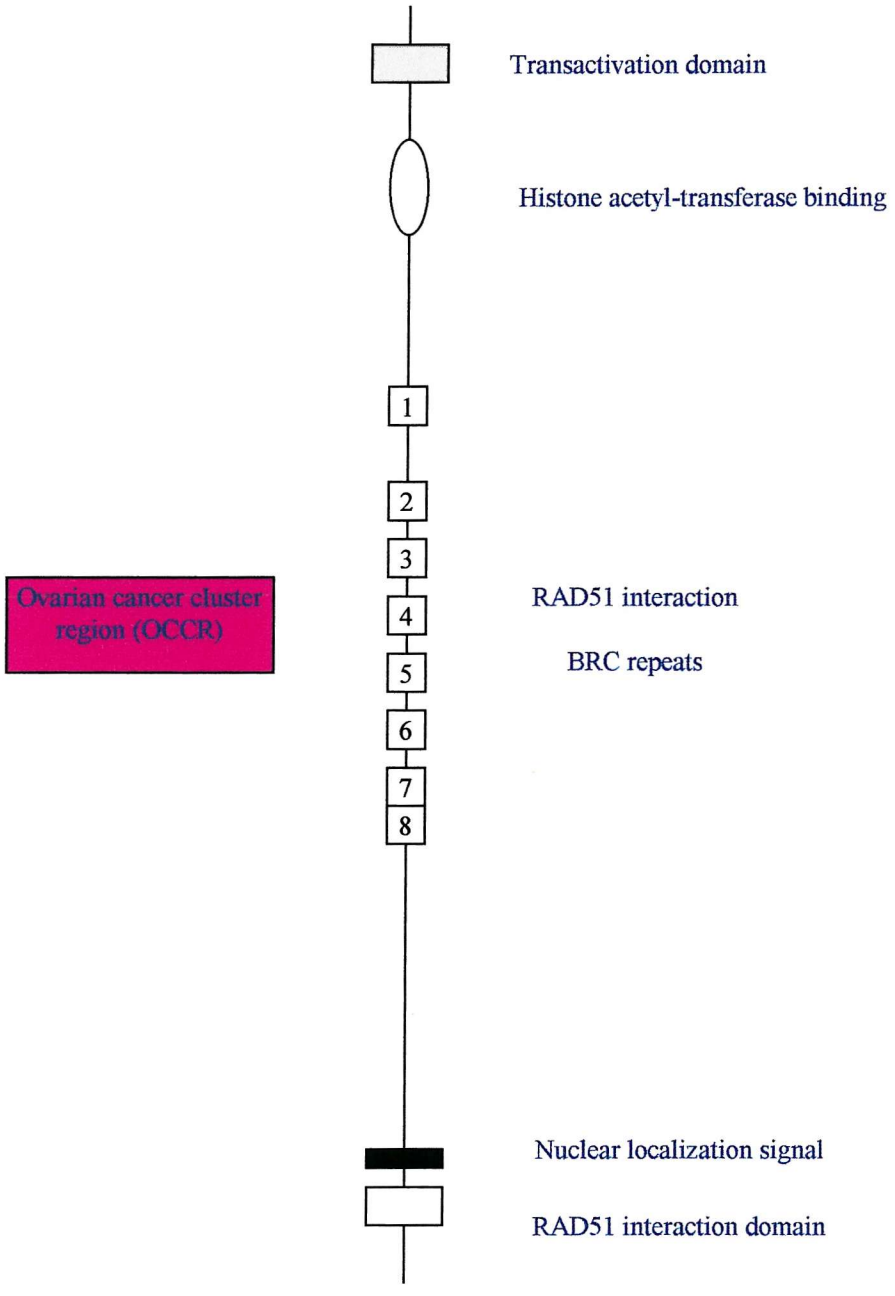
1.12.3 Similarities between BRCA1 and BRCA2

Both are very large genes, in terms of the number of exons and the size of their encoded message.

In both cases, exon 11 is large (3,426bp for BRCA1, 4,932bp for BRCA2), and contains approximately half the coding region (Tavtigian *et al*, 1996). Both are A/T-rich, and both have translation start sites in exon 2.

It has proved difficult to define the normal cellular functions of these genes, but both contribute to DNA repair, to embryonic proliferation, and to regulation of transcription.

Figure 1.9 **The BRCA2 protein (3418 amino acids)**



Adapted from Ingvarsson 1999

1.12.4 Founder Effects and Inherited Mutations

Evidence suggests that many of the known BRCA1 and BRCA2 mutations recur. A third of BRCA1 mutations have been identified more than once in unrelated families, and account for 57% of the mutations identified (Shattuck-Eidens *et al*, 1995). It is possible to show whether frequently occurring mutations result from spontaneous mutational events which occur commonly or as a result of a founder effect (mutations arising in a single ancestor), by comparing haplotypes.

Thorlacius *et al* (1997) gave very strong evidence for founder effects in a series of individuals from Iceland. One particular mutation in BRCA2 - 999del5 - was shown to be common in the Icelandic population, all mutation positive individuals tested showing the same BRCA2 haplotype (suggesting a common origin). In this particular study, 7.7% overall of female breast cancer patients in Iceland had the 999del5 mutation (16% for cases diagnosed at <50 years old). Irrespective of age, the same mutation was shown to be involved in 40% of male breast cancer. However, the presence of the mutation was not limited to those with a strong family history of breast cancer. This may reflect small family size, young relatives, male transmission, or low penetrance. Although the mutation was seen more commonly in younger women, the age of onset spanned 50 years. The variable age of onset and family histories may reflect varying penetrance of the mutation, whilst an observed increased incidence in male mutation carriers and decreasing age of onset for female mutation carriers indicate that there may be new environmental factors affecting the phenotypic expression of the mutation.

Friedman *et al* (1995) described the BRCA1 185delAG mutation in exon 2 in five Ashkenazi Jewish families, all sharing the same haplotype. Within these families, a range of expressivity of the mutation, from early onset ovarian cancer and bilateral breast cancer to late onset breast cancer with no ovarian cancer, was observed. The penetrance of the mutation for known carriers within these five families combined was 38% by 40 years of age, 69% by 50 years of age, and 85% by 65 years of age, which is comparable with cumulative risk estimates from Easton's evaluation of high risk families (Easton *et al*, 1993).

Berman and colleagues (1996) similarly found 13 Ashkenazi women, in a series of 64, with the 185delAG mutation, all of whom shared the same haplotype. However, they

also describe two non-Jewish individuals carrying the mutation, with a different haplotype, suggesting a distinct ancestry separate from the other group.
Founder effects have also been widely described by other groups (Simard *et al*, 1994; Johannsson *et al* , 1996; Neuhausen *et al*, 1996).

1.12.5 BRCA1 and BRCA2 in Sporadic Ovarian Cancer

Loss of heterozygosity on chromosome 17q is seen in up to 80% of sporadic ovarian tumours, and it was, therefore, felt that BRCA1 would be found to play a significant role in their development (Sato *et al*, 1991; Eccles *et al*, 1992; Cliby *et al*, 1993; Yang-Feng *et al*, 1993). However, an analysis of unselected series of ovarian tumours, in which most cases would be expected to be sporadic, has suggested that somatic BRCA1 mutations are, in fact, rare occurrences (Futreal *et al*, 1994; Hosking *et al*, 1995; Matsushima *et al*, 1995; Merajver *et al*, 1995; Takahashi *et al*, 1995; Stratton *et al*, 1997).
Various studies employing fine deletion mapping have identified at least one region distal to BRCA1 which may contain further TSG's which may be of greater relevance to sporadic tumours (Jacobs *et al*, 1993; Saito *et al*, 1993; Godwin *et al*, 1994).
As with BRCA1, somatic mutations in BRCA2 have also been shown to be rare (Foster *et al*, 1996; Takahashi *et al*, 1996).

1.12.6 BRCA1 and BRCA2 Mutation Studies

The rates of identification of mutations within these genes is dependent on both the criteria for selection of those individuals to be screened, and the methods of analysis of DNA used.
The probability of finding a mutation in BRCA1 in a woman with breast cancer over the age of 50 who is the only affected individual in the family is <3% (Shattuck-Eidens *et al*, 1995). In families with two cases of ovarian cancer or two cases of breast cancer, the probability is 80-90% (Berchuck *et al*, 1999). In practical terms, the testing of 'high risk' individuals means those with a family history of two individuals with ovarian cancer at any age or breast cancer at less than 50 years of age who are first or second degree relatives (Berchuck *et al*, 1999). However, it has been suggested by some investigators that patients with ovarian cancer who have germline BRCA1 mutations

may have a more indolent disease than those with sporadic cancer (Buller *et al*, 1993; Rubin *et al*, 1996), making it essential to identify all mutation carriers. Unfortunately, with methodology currently available, this would not be practical, as it would require testing of all individuals with ovarian cancer for BRCA1, BRCA2, and HNPCC gene mutations (Rubin *et al*, 1998).

Currently, the most widely used technique is that of single strand conformational polymorphism (SSCP) and heteroduplex analysis, which, when used in conjunction with automated sequencing has a sensitivity of 70-80%. However, mutations affecting splicing, or expression or stability of the BRCA1 transcript, are particularly difficult to identify, although they account for 15-20% of disease-causing mutations (Swensen *et al*, 1997).

An alternative method is the protein truncation test (PTT), which detects mutations resulting in premature termination of protein synthesis (Hogervorst *et al*, 1995). It involves the translation of BRCA1 RNA into protein, with subsequent electrophoresis to detect alterations in its size. With this method, mutations which only alter single amino acids will not be seen (accounts for ~10% of BRCA1 mutations). Sensitivity is also reduced for very large protein products with mobility similar to the wild-type protein, and for small products which migrate off the electrophoretic gel. However, splice site mutations not detected by SSCP and direct sequencing should be seen using this method (Berchuck *et al*, 1996).

1.13 OTHER TUMOUR SUPPRESSOR GENES ASSOCIATED WITH OVARIAN CANCER

1.13.1. The Familial Adenomatous Polyposis Gene, APC - on 5q21.

Cliby et al reported high frequencies of LOH in the APC region in sporadic ovarian tumours (Cliby *et al*, 1993), a finding confirmed by Weitzel et al, who described 50% LOH (9/18 informative cases) (Weitzel *et al*, 1994).

1.13.2. The Deleted in Colon Cancer Gene, DCC - on 18q21.

Yamashita et al described altered expression of the DCC gene in 67% of ovarian tumours (6/9) and 100% of ovarian epithelial cell lines (6/6). LOH was confined to serous and endometrioid tumour types (Yamashita *et al*, 1996).

Chenevix-Trench reported 60% LOH on 18q (31/52 informative tumours), but found that the smallest region of overlap infact did not include the DCC locus, raising the possibility of another 18q gene involved in ovarian tumorigenesis (Chenevix-Trench *et al*, 1992).

1.13.3. The Wilm's Tumour Genes, WT1 and WT2 - on 11p13 and 11p15 respectively.

Chromosomal deletions in the region of WT1 and WT2 have frequently been reported in ovarian cancer (Vandamme *et al*, 1992; Viel *et al*, 1992), but subsequent direct analysis of the WT1 locus infers that the gene is in fact not involved. Foulkes has described a minimal region of loss at 11q23.3-qter which may mean there is another chromosome 11 TSG involved in ovarian carcinogenesis (Foulkes *et al*, 1993b).

1.13.4. The Retinoblastoma Gene, RB - on 13q14.

This was the first TSG to be identified (Knudson 1971). Its mutation in ovarian cancer is rare (Dodson *et al*, 1994).

1.14 PARTICULAR CHARACTERISTICS OF FAMILIAL OVARIAN TUMOURS

Hereditary ovarian cancer syndromes have an autosomal dominant mode of inheritance, but it is often difficult to demonstrate this from family pedigrees. There are various reasons for this , including small family size, families with predominantly male offspring, difficulties accessing medical records, and incorrect classification of ovarian cancers (Piver *et al*, 1993a; Rubin *et al*, 1998). All these factors make the distinction between hereditary and non-hereditary ovarian cancer difficult.

Most women with a family history of ovarian cancer do not come from families with hereditary syndromes. 7% of all women with ovarian cancer describe a positive family history; in the majority (>90%) only one relative is affected (Schildkraut and Thompson 1988).

Women from families with ovarian cancer syndromes who develop ovarian cancer tend to be diagnosed 7-14 years earlier than the mean population age at diagnosis of 59 years (Lynch *et al*, 1991) - (7 years for HBOCS, 10 years for Site-specific Ovarian Cancer, 14 years for HNPCCS). In a study of 53 women with known BRCA1 mutations, Rubin et al confirmed this - the average age at diagnosis of ovarian cancer in his group was 48 years (Rubin *et al*, 1996). Bewtra *et al* (1992) reported ages at diagnosis of 50.2 years for HBOCS and 41 years for HNPCCS.

The age at diagnosis for women who simply have a family history of ovarian cancer is no different from the population mean age at diagnosis.

Most tumours occurring within ovarian cancer syndromes are serous cystadenocarcinomas (Lynch *et al*, 1991), whilst only 1/3-1/2 of all cases of ovarian carcinoma are of this histopathological type. This finding was also confirmed by Rubin's group (1996) - in his group of 53 women, 43 had serous tumours, 3 mucinous, 3 endometrioid, and 4 undifferentiated.

Rubin and colleagues also noted other characteristics amongst their population of patients with familial ovarian cancer. Grade and stage distribution was similar to that for a matched group of sporadic ovarian tumours. Tumours of low malignant potential were seen amongst the group of BRCA1 mutation carriers. Most importantly, survival for those with known BRCA1 mutations and advanced stage disease was significantly

longer than for sporadic matched cases (actuarial mean survival 77 months compared with 29 months, $p<0.001$).

Another study comparing 34 cases of familial ovarian cancer with a non-familial group matched for age at diagnosis, tumour histology, and preponderance of advanced grade and stage, has also shown this apparent survival advantage (Buller *et al*, 1993), revealing a 5 year survival for the familial group of 67% compared with 17% in the non-familial cases.

However, the data is conflicting, and indeed a more recent study by Pharoah *et al* (1999) has shown inferior survival in familial ovarian cancer (whether or not it was possible to identify a BRCA1 or BRCA2 mutation) compared with sporadic cases.

As mutational data continues to accumulate, it should become easier to correlate the clinical and histopathological features of these hereditary cancers (Boyd and Rubin 1997).

1.15 MANAGEMENT STRATEGIES IN FAMILIAL OVARIAN CANCER

1.15.1 The Role of Prophylactic Oophorectomy

Kerlikowske *et al* (1992) have estimated the lifetime probabilities of ovarian cancer for women with a family history of the disease, and also for women from families with known hereditary ovarian cancer syndromes. They estimate the lifetime risk for a 35 year old without a family history to be 1.6%, compared with 5% for a woman with one affected relative, and 7% for someone with two affected relatives. For women from families with known syndromes, the risk is probably of the order of 50%.

On the basis of these risks, the Gilda Radner Familial Ovarian Cancer Registry recommends prophylactic oophorectomy, by age 35, in all women from families with hereditary syndromes, and those with two or more first degree relatives with the disease (Kerlikowske *et al* , 1992). However, for women who simply have a family history of disease, surgery can probably be safely delayed until later, since ovarian cancer is rare under the age of 45 years (~11 per 100,000), even in women with a positive family history (~33 per 100,000).

Whether a lifetime probability of development of ovarian cancer of 5%, for women with one affected relative, is great enough to justify prophylactic surgery is more difficult to gauge, and needs to be weighed against issues such as potential post operative complications, risks resulting from the consequent early menopause induced by surgery, particularly cardiovascular and osteoporotic events, and, of course, the psychological impact associated with oophorectomy (Kerlikowske *et al*, 1992; Nguyen *et al*, 1994).

Other groups have attempted to quantify the benefits of prophylactic surgery in women carrying BRCA1 or BRCA2 mutations. Schrag *et al* (1997) describe significant gains in life expectancy in young women (30 years old) from both prophylactic mastectomy and oophorectomy, the effects of mastectomy being by far the greater. The group estimated an increased life expectancy of 2.9-5.3 years for mastectomy and 0.3-1.7 years for oophorectomy. Life expectancy was not affected much by delaying oophorectomy until 40 years of age. Similar findings were reported by Grann *et al* (1998). However, these figures need to be regarded with some caution, as thay do not take into account the myriad of other factors which may affect a patient's life expectancy.

It must also be remembered that undergoing prophylactic surgery does not completely eliminate the risk of subsequent ovarian cancer development. Cases of primary peritoneal carcinomatosis after prophylactic oophorectomy are well described (Tobacman *et al*, 1982; Piver *et al*, 1993b), and it may be that other tissues derived from coelomic epithelium are also predisposed to tumour development (Lynch *et al*, 1986). In a few cases, it is possible to show that microscopic foci of cancer were in fact present in the prophylactic oophorectomy specimen, but not appreciated in the initial pathological examination. It is, therefore, essential to examine multiple sections from each ovary to ensure the absence of occult disease (Berchuck *et al*, 1996).

1.15.2 The Role of Oral Contraceptive Therapy

It has been shown that women with a family history of one relative with ovarian cancer may be able to reduce their lifetime probability of disease development to 3% by taking an oral contraceptive for 5-9 years, and to 2.5% by ten years' usage (Kerlikowske *et al*, 1992).

Women with hereditary ovarian cancer syndromes may also benefit from use prior to prophylactic oophorectomy, although studies to assess this have not been performed.

1.15.3 The Role of Surveillance

For women with hereditary cancer syndromes, some groups have recommended once or twice yearly screening, with pelvic and abdominal clinical examination, serum CA125, abdomino-vaginal ultrasound, and colour flow Doppler imaging of the ovarian vascular tree, from the age of 20-25 years until prophylactic oophorectomy has been carried out (Lynch *et al*, 1991). The French Ad Hoc Committee recommends two pelvic examinations per year, and a yearly transvaginal ultrasound with colour flow Doppler in women, from the age of 35 years, who have a greater than 20% chance of harbouring a mutation (Eisinger *et al*, 1998). However, clinical trials have yet to show that screening is effective.

1.16 HISTOLOGICAL FEATURES OF PROPHYLACTIC OOPHORECTOMY SPECIMENS

There have been suggestions that prophylactic oophorectomy specimens from women with strong family histories of ovarian cancer show high frequencies of epithelial abnormalities compared with ovaries removed from women for other reasons (Berchuck *et al*, 1999). This was first reported in a study of identical twins, in which one twin underwent prophylactic surgery following development of ovarian cancer in the other (Gusberg and Deligdisch 1984).

Various epithelial abnormalities have been described (Salazar *et al*, 1996):

1. surface epithelial pseudostratification and papillomatosis
2. deep cortical invaginations, often with multiple papillary projections within small cystic spaces (microscopic papillary cystadenomas)
3. epithelial inclusion cysts, frequently accompanied by epithelial hyperplasia and papillary formations
4. cortical stromal hyperplasia and hyperthecosis
5. increased follicular activity
6. corpus luteum hyperplasia
7. hilar cell hyperplasia

Similar histological features have been described in normal ovarian epithelium adjacent to early ovarian cancer, and in the contralateral normal ovary of Stage I ovarian cancer (Plaxe *et al*, 1990; Mittal *et al*, 1993; Resta *et al*, 1993).

These studies suggest that prophylactic surgery in high risk individuals may serve to remove premalignant lesions. If precursor lesions do exist, they would be expected to show specific genetic alterations seen in early invasive disease. Also, areas of invasive disease adjacent to a putative precursor lesion would be expected to have the same clonal origin (Jacobs and Lancaster 1996). Therefore, identification of a precursor lesion would have a huge impact on our understanding of ovarian tumorigenesis, and would also have implications for patient management.

1.17 AIMS OF STUDY

The primary aim of this study was the genetic analysis of aberrant histological structures in prophylactic oophorectomy specimens from women with germline BRCA1 and BRCA2 mutations. The concept that ovarian tumours develop via an adenoma/adenocarcinoma route, similar to that described for colorectal tumours, would be supported if the atypical features documented by Salazar *et al* (1996) could be shown to exhibit DNA deletions. This could have a significant impact on the diagnosis and management of ovarian tumours, by facilitating identification of genetic events which initiate tumour development.

A secondary aim was the BRCA1 mutation screening of a consecutive series of over 200 cases of sporadic ovarian tumours from our centre. The reasons behind this analysis were twofold: firstly to assess the incidence of such mutations in unselected sporadic tumours, since few studies have investigated this and most of those published have been with small numbers of patients (Futreal *et al*, 1994; Hosking *et al*, 1995; Matsushima *et al*, 1995; Merajver *et al*, 1995; Takahashi *et al*, 1995; Stratton *et al*, 1997; Rubin *et al*, 1998); and secondly in an attempt to identify additional BRCA1 mutation carriers, with benign or borderline ovarian lesions, for possible inclusion in the loss of heterozygosity (LOH) analysis described for prophylactic oophorectomy specimens.

CHAPTER TWO
MATERIALS AND METHODS

2.1 BRCA1 MUTATION SCREENING IN SPORADIC EPITHELIAL OVARIAN TUMOURS

2.1.1 Collection of DNA Specimens

This was a retrospective review of leucocyte DNA extracted from venous blood samples at the time of clinical presentation with an ovarian tumour.
An initial consecutive series of 300 samples was used, from patients presenting to the Princess Anne Hospital, Southampton, between 1992 and 1998. Local ethical committee approval had been obtained for sample collection for mutation analysis. Those within the series with histological diagnoses of non-epithelial tumours were later excluded, together with those for which mutation screening experiments failed. A final series of 223 was therefore analysed.
Patient information and histological data is documented in Table 3.1.

2.1.2 DNA Extraction Technique

Leucocyte DNA samples for inclusion in the study had already been prepared in the laboratory, using the following 'salt chloroform DNA extraction technique'.
Each venous sample was spun for 10 minutes at 10,000rpm in a Hermle centrifuge (Z382K, Germany) within a 30ml 'Oak Ridge' tube. Careful aspiration of the serum was then performed, using a Pasteur pipette. An equal volume of ice cold 2x sucrose/triton solution was then added to the remaining volume and left for 10 minutes on ice. This was in order to achieve lysis of red blood cells and leucocytes. Following this, the nuclear fraction of DNA was pelleted by centrifugation at 10,000rpm for 10 minutes at 4°C. After removal of the supernatant, again using a Pasteur pipette, the remaining pellet was resuspended in 5ml of 1x sucrose/triton lysis buffer, to remove heme traces. The mixture was subjected to 5 minutes of centrifugation at 10,000rpm and 4°C, prior to aspiration of supernatant.

2.1.2.1 *SDS/proteinase K extraction*

The DNA pellet was suspended in 5mls of SDS lysis buffer, to which was added 50µl of a 40mg/ml proteinase K solution. This mixture was incubated at 55°C overnight. 1.65mls of pre-warmed sodium chloride solution and 7mls of chloroform were added, and the tube put on a rotator for 1 hour. The suspension produced was centrifuged for 10 minutes at 10,000rpm at 4°C. The aqueous phase was then transferred to a clean 30ml Oak Ridge tube, following aspiration and disposal of the top layer. 7mls of 2-propanol was added, a white precipitate forming within a few minutes at room temperature. Further centrifugation for 20 minutes at 15,000rpm and 4°C was performed, prior to removal of the supernatant. The remainder was washed with 75% ethanol, and again centrifuged for 5 minutes at 10,000rpm and 4°C. Visible ethanol was aspirated with a pipette and the tube left open to allow evaporation of any further traces. 500µl of TE buffer was used to resuspend the DNA, which was left at 4°C overnight to dissolve completely. The DNA solution was transferred to a 500µl Eppendorf tube. A further 200µl of TE was used to rinse the Oak Ridge tube for recovery of DNA traces. Stock solution was stored at 4°C. A 1:5 dilution was also prepared, and stored under the same conditions.

2.1.3 Polymerase Chain Reaction(PCR)

PCR is a rapid, versatile, in-vitro method, using enzymatic synthesis, to exponentially amplify defined target sequences of DNA. The technique described by Saiki *et al* (1985) has proved of great importance in the field of molecular genetics, and is powerful enough to amplify a single copy of a DNA sequence millions of times.

A standard PCR reaction utilises two oligonucleotide primer sequences (usually 15-30 nucleotides long) which specifically bind to complementary DNA sequences flanking the region of interest in the target DNA.

The reaction then comprises three basic steps:

1. **Template denaturation** - a temperature of 90-94°C for up to 2 minutes ensures separation of the 2 strands of the DNA helix.
2. **Primer annealing** - the reaction is cooled in the presence of a large excess of the two oligonucleotide primers. At the appropriate temperature (dependent on the nucleotide composition of the primers), specific annealing of the primers to their complementary target DNA sequences occurs. Typically, the conditions required vary from 50-60°C for 1/2-1 minute.
3. **Extension** - this step takes place at 72°C. DNA polymerase (thermostable Taq polymerase from the algae Thermus aquaticus) catalyses synthesis of new DNA strands from the primers, complementary to the single-strand DNA template to which the primers were annealed (Figure 2.1).

PCR is a chain reaction, so newly synthesised DNA becomes a template for further synthesis in subsequent cycles. DNA is amplified in an exponential fashion - in theory 2^n times, where n is the number of cycles; in practice less than this, as the system is not 100% efficient, and product generation eventually plateaus. The optimum number of cycles for a PCR reaction depends on the starting concentration of target DNA - for lymphocyte DNA, 30-35 cycles is usually sufficient, whilst higher numbers of cycles may be needed for DNA from small biopsies or paraffin-embedded material. However, too many cycles may result in generation of non-specific products, so care must be taken to keep cycle numbers to a minimum.

2.1.3.1 *Specificity of PCR*

Specificity of the reaction is dependent on primer design, such that primers used only recognise the DNA sequence intended to be amplified. For the complex genomic DNA of a human cell, the design of primers of ~20 nucleotides in length is usually adequate, as the probability of an accidental match for either primer elsewhere in the genome is extremely low. PCR conditions are usually chosen such that only strongly matched primer-target duplexes are stable.

2.1.3.2 *Sensitivity of PCR*

PCR is extremely sensitive, so care to prevent contamination of reagents is very important, especially when using archival tissue with low concentrations of DNA which could easily be masked by DNA contamination.

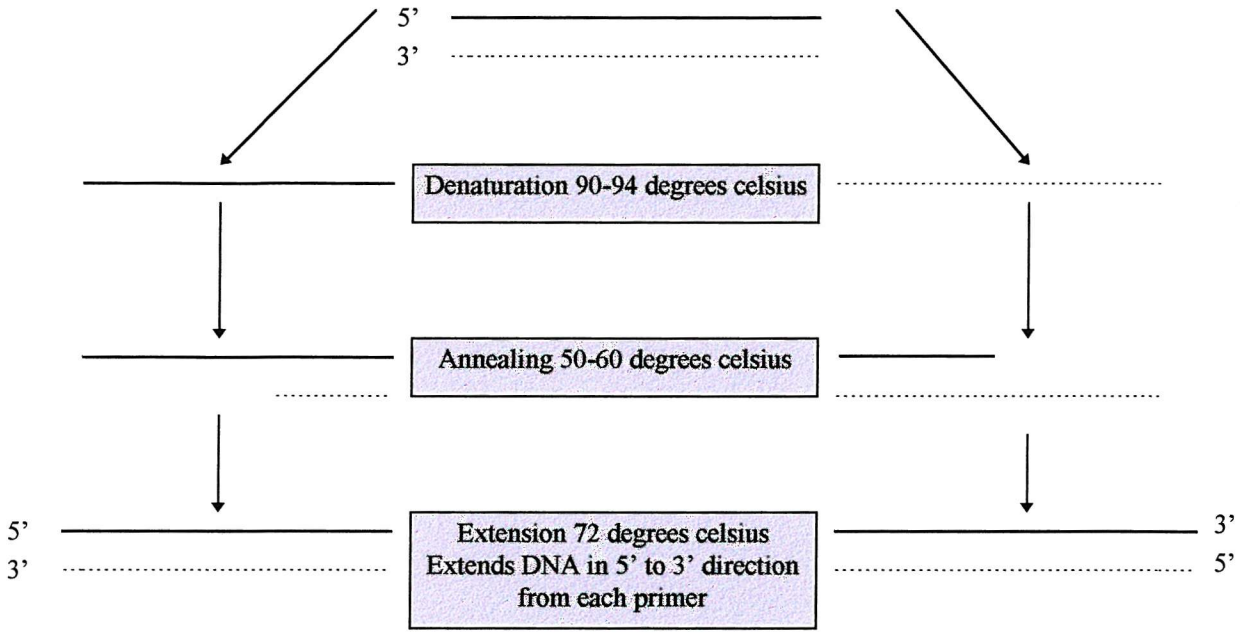
Therefore, clean gloves are always worn and regularly changed, and pipettes containing filter tips (Advanced Biotechnologies Ltd, UK) used - the tips prevent cross-contamination via the pipette.

2.1.3.3 *Contamination control*

It is always necessary to include negative controls (all the reagents except DNA) in each PCR reaction - contamination is indicated by the presence of PCR products within the controls. If contamination occurs, all reagents should be discarded, and new ones used to repeat the reaction.

UV irradiation can also be used to convert contaminating DNA into a non-amplifiable form. This is achieved by exposing PCR reaction mixtures (without DNA and P32) to 5 minutes of 260nm UV irradiation in a UV crosslinker (CL-1000, Genetic Research Instruments Ltd, UK).

Figure 2.1 A single cycle of a PCR reaction



2.1.4 Single Strand Conformational Polymorphism (SSCP)

This is a gel electrophoresis method for detecting mutations in defined regions of DNA, which relies on electrophoretic mobility of a PCR product being a function of the size, shape, and charge of the molecule.

A region of DNA is amplified by PCR. The product is then heated in formamide to denature it (make it single stranded) and electrophoresed on a non-denaturing polyacrylamide gel. When using these conditions, formation of intrastrand hydrogen bonds can occur, thus producing secondary structure. A PCR product obtained from genomic DNA which contains a mutation will not adopt the same conformation as a wild-type product, and will, therefore, have different electrophoretic mobility. Mutants can, therefore, be identified by comparison with known normal controls.

This method is useful for products up to approximately 500bp, so multiple pairs of PCR primers are needed in order to analyse a whole gene for the presence of mutations.

SSCP will detect about 80% of mutations (Rocques, 1996).

2.1.5 PCR Amplification of BRCA1 Exons in DNA Samples

Primers exist for BRCA1 for exons 2 to 24, omitting exon 4. Due to their size, exons 16 and 11 are amplified in 2 and 18 segments, respectively. For this study, only the 'priority' primers were studied. These were those for which the majority of mutations in BRCA1 have been found in the Wessex region (Eccles *et al*, 1998), and comprise 11B, 11J, 11L, 11O, 11P, 11Q, 11R, 2, 12, and 20. Exons were multiplexed to save time and reagents; the combinations used are shown in Table 2.1.

Table 2.1 Primers studied and PCR amplicon sizes

PRIMER PAIR	PCR PRODUCT SIZE (bp)
11B+11Q	248 / 302
11J+11O	265 / 288
11R+12	350 / 265
11P+2	298 / 264
11L+20	281 / 399

The primers used were approximately 15-20 nucleotides long; mixes contained both 'forward' and 'reverse' exon primers (see Table 2.2).

Table 2.2 BRCA1 primers

EXON	FORWARD/REVERSE PRIMER	SEQUENCE
2	F	GAAGTTGTCSTTTTATAAACCTTT
	R	TGTCTTTTCTTCCCTAGTATGT
11B	F	ACAGCATGAGAACAGCAGTTT
	R	CTCTAGGATTCTCTGAGCATGG
11J	F	GGGTTTTGCAAACTGAAAGA
	R	CTTGTTTCCCGACTGTGGTT
11L	F	TTTGCTCCGTTTTCAAATCC
	R	TCGTTGCTCTGAACTGAGA
11O	F	CCAGTGATGAAAACATTCAAGC
	R	TTCACCATCATCTAACAGGTCA
11P	F	AACTTAGAACAGCCTATGGGAA
	R	AACAAGTGTTGGAAGCAGGG
11Q	F	AGGGGCCAAGAAATTAGAGTC
	R	CTTCCAATTCACTGCACTGTG
11R	F	AAAGGCATCTCAGGAACATCA
	R	GTGCTCCCAAAAGCATAAA
12	F	GTCCTGCCAATGAGAAGAAA
	R	TGTCAGCAAACCTAAGAATGT
20	F	ATATGACGTGTCTGCTCCAC
	R	GGGAATCCAAATTACACAGC

2.1.5.1 *Standard PCR protocol*

Stock solutions of DNA were diluted to 1:20 with a 1xTE buffer (resulting in a DNA concentration of 10-200ng/μl). For each PCR reaction, 1μl of DNA was utilised, overlaid with approximately 30μl of mineral oil (Sigma, UK), to prevent evaporation during the PCR reaction. A 10μl PCR volume was used (Table 2.3). Preparation of PCR mastermixes, containing all the reagents needed to generate the total number of PCR reactions, was carried out on ice in 1.5ml Eppendorf tubes. Thorough mixing and dispensing from a mastermix ensured all PCR tubes contained equal concentrations of reagents, and resulted in less risk of cross-contamination between samples.

Table 2.3 Composition of standard PCR mixture (all volumes in μl)

REAGENT	REACTION QUANTITY PER 10μl PCR
Deionised water	7.025
Low dCTP nucleotide mix	0.25
10x buffer solution	1.0
Primer 1	0.25
Primer 2	0.25
1:5 'Red hot' Taq polymerase solution	0.2
P32	0.025

Details of reagents are outlined in Appendix 1

'Red hot' Taq polymerase (Advanced Biotechnologies, UK) and P32 (Amersham International) were added immediately prior to aliquoting 9μl of the mix to each tube containing DNA. The PCR mixtures were then subjected to thermal cycling (Table 2.4).

Table 2.4 Thermal cycling conditions for PCR reactions for BRCA1 exons

PCR STEP	FUNCTION	TEMPERATURE (degrees celsius)	TIME (seconds)
Initial step	Template melting	94	300
Cycling (35-40 cycles)	Denaturing	94	30
	Annealing	53-57	45
	Extending	72	45
Final step	Extending	72	300

2.1.6 Agarose Gel Electrophoresis of PCR Products

Agarose gel electrophoresis was used to confirm the presence of amplified DNA following the PCR reaction (BRL Life Technologies, Scotland, UK).

A 1.5% agarose gel, containing 0.5μg/ml ethidium bromide, was used. Gels were prepared in a tank (Hybaid, UK), with a well-forming comb which was removed once the gel was set. A 1xTAE buffer was used for electrophoresis.

3μl of PCR product was mixed with tracking dye and loaded into each well of the submerged gel. A 100bp ladder was run in the first lane of the gel, to enable estimation of product size (BRL Life Technologies, UK). Gels were run at room temperature and 150 volts.

Following electrophoresis, DNA products were visualised using a TM-20 dual-intensity ultraviolet transilluminator (Genetic Research Instruments Ltd, UK).

If PCR proved successful, the products were then subjected to SSCP analysis.

All PCR reactions were repeated at least twice, to ensure reliability of results.

2.1.7 SSCP Analysis of PCR Products

2.1.7.1 *Preparation of polyacrylamide gel*

Polyacrylamide gel electrophoresis (PAGE) was used. Two sizes of electrophoresis tank were available for use:

0.4mmx200nmx430mm (Hybaid, UK)

0.4mmx400mmx430mm (BRL, Life Technologies, UK)

Glass plates were thoroughly cleaned, to remove all residues, using detergent, followed by acetone and 100% ethanol. One of the pair of plates was then siliconised using 'Repelcote' (BDH Laboratory Supplies, Poole, UK), to facilitate later separation from the gel after electrophoresis. 0.4mm spacers were placed between the plates, which were clipped together and assembled at approximately 45 degrees to the horizontal. Reagents were mixed by gentle swirling (to avoid air bubbles) in a clean glass beaker (Table 2.5).

Table 2.5 Composition of 6% Polyacrylamide gel with glycerol

REAGENT	QUANTITY FOR 40ml HYBAID GEL	QUANTITY FOR 60ml BRL GEL
30% polyacrylamide solution	8ml	12ml
5xTBE buffer	4ml	6ml
Distilled water	26ml	39ml
Glycerol	2ml	3ml
20% APS	90µl	135µl
TEMED	70µl	105µl

Polyacrylamide solution - 30% acrylamide / Bis-acrylamide, 29:1 (Anachem, UK)

APS - ammonium persulphate (Sigma, USA)

TEMED - N,N,N,N' - Tetramethyl ethylenediamine (Sigma, USA)

The mixture was drawn into a 60ml plastic syringe, and injected between the glass plates to produce a 0.4mm thick gel. Any bubbles which formed in the gel during pouring were removed to ensure even electrophoresis. The gel was laid horizontally, and the flat side of a lane-forming shark's tooth comb applied to the top of the gel. The gel required 45-60 minutes at room temperature for polymerisation prior to use.
The plates were set up vertically in a gel tank which was filled with 0.5xTBE buffer. The shark's tooth comb was gently removed, the top surface of the gel rinsed with buffer, to remove any loose gel, and the lane-forming comb gently reapplied, such that the teeth formed loading lanes.

2.1.7.2 *Electrophoresis of PCR products*

PCR products were denatured by heating to 95°C for 5 minutes with an equal volume of formamide. Samples were then cooled on ice prior to loading onto the electrophoretic gel. 1.7µl of each product was loaded, using one lane per sample. Electrophoresis was carried out over 18-22 hours, at a voltage of 0.26kV, depending on the PCR product size.

2.1.7.3 *Gel drying and autoradiography*

On completion of electrophoresis, the glass gel plates were carefully separated, and the gel transferred to Whatman 3mm filter paper. The gel was covered with film (Saran film, UK) and dried using a vacuum dryer (AC-3750, Genetic Research Instrumentation Ltd, UK). Dry gel was then exposed to GRI X-ray film, either at room temperature or at -80°C, to produce an image of single and double strands of DNA. Exposure at -80°C is faster than at room temperature, but results in inferior images. Films were developed in an automatic processor (Fuji, Japan).

Where shifts in the DNA electrophoretic pattern were shown, the sample DNA was subjected to direct sequencing for that exon or part exon.

2.1.8 Direct Sequencing of DNA

2.1.8.1 *'Cold' PCR*

For efficient primer-template annealing to be achieved, DNA sequencing requires a clean DNA template. This was produced by performing a repeat PCR for the BRCA1 exon or part exon involved, in a 20µl reaction mix without P32 (Table 2.6). The PCR thermal cycling conditions were 35 cycles at 55°C. Following PCR, the presence of a product was again confirmed on an agarose gel.

Table 2.6 Composition of 'cold' PCR mixture

REAGENT	QUANTITY PER 20µl REACTION
Deionised water	15.4µl
Nucleotide mix	0.5µl
10x buffer solution	2.0µl
Primer	0.5µl
'Red hot' Taq polymerase solution	0.6µl

2.1.8.2 *Purification of DNA product from 'cold' PCR*

The Wizard PCR Preps DNA Purification System (Promega, UK) was used to remove contaminants such as primer-dimers and amplification primers. The sample was then resuspended in 50µl of water, and either subjected to the sequencing protocol straight away or stored at -20°C.

2.1.8.3 *Thermal cycling*

A 'Thermo-Sequenase' cycle sequencing kit (Amersham Life Sciences, UK) was utilised, involving two stages of thermal cycling:

1. a primer labelling reaction, using P32
2. an end extension/termination reaction

1. Primer labelling

Purified DNA was subjected to further polymerase chain reaction with a selected 'forward' or 'reverse' primer (Tables 2.7 and 2.8).

Table 2.7 Mixture for primer labelling reaction

REAGENT	QUANTITY (µl)
Purified DNA	1.0
Deionised water	9.0
Reaction buffer	2.0
Primer	1.0
dATP	1.0 / 0.0
dGTP	1.0 / 0.0
dTTP	1.0 / 0.0
P32 dCTP	1.0
Diluted sequencing polymerase	2.0

The mixture was overlaid with mineral oil to avoid evaporation during PCR.

Table 2.8 Thermal cycling conditions for primer labelling reaction (50 cycles)

Denaturing step	95 degrees for 15 seconds
Annealing step	50 degrees for 25 seconds
Extension step	72 degrees for 45 seconds

2. End extension/termination

3.5µl of PCR product was aliquoted to each of four tubes labelled G, A, T, and C. 4µl of the appropriate di-deoxynucleotide was added to each tube. The mixtures were overlaid with mineral oil, and then subjected to thermal cycling of the end extension/ termination reaction (Table 2.9).

Table 2.9 Thermal cycling conditions for end extension/termination reaction (40 cycles)

Denaturing step	95 degrees for 15 seconds
Annealing step	55 degrees for 30 seconds
Extension step	72 degrees for 60 seconds

At the end of this reaction, 4µl of 'stop solution' was added to each tube (Amersham Life Sciences, UK).

This solution contained: 95% formamide buffer
20mM EDTA
0.05% bromophenol blue
0.05% xylene cyanol

Samples were then heated to 70°C for two minutes and cooled rapidly on ice prior to loading on a sequencing gel.

2.1.8.4 *Sequencing gel electrophoresis*

The technique for gel preparation was as for SSCP electrophoresis, using a 0.4mm x 200mm x 430mm gel (Hybaid, UK). The gel was left for 60 minutes at room temperature to polymerise. The composition of the gel used is shown in Table 2.10.

Table 2.10 Polyacrylamide gel for sequencing

Gene Page 6% (6% Acrylamide: Bis- acrylamide 19:1) (Anachem International).

REAGENT	CONCENTRATION
Acrylamide	5.7% (w/v)
Bis-acrylamide	0.3% (w/v)
Urea	7M
Tris	0.089M
Boric acid	0.089M
EDTA	0.002M

The running buffer was 1xTBE. Gels were warmed prior to loading by running a current of 50 watts for 10 minutes. Following gel warming, wells were rinsed with buffer, to remove extra urea. 1.5-2µl of each denatured sample was loaded. Gels were run at 50 watts for approximately an hour. Samples were then reloaded in further lanes of the gel, and the gel rerun at 50 watts until the bromophenol blue dye reached the bottom of the gel.

When electrophoresis was complete, gels were fixed by soaking in a solution containing 10% acetic acid and 10% methanol for 10 minutes.
Gels were dried as previously described, and exposed to x-ray film overnight at room temperature.

2.2 LOH ANALYSIS OF MICRODISSECTED TISSUE FROM PROPHYLACTIC OOPHORECTOMY SPECIMENS

2.2.1 Tissue Microdissection Technique

Detection of genetic alterations in histologically abnormal tissue areas relies on removal of the majority of 'normal' cells from a tissue specimen prior to DNA extraction; genetic abnormalities such as point mutations and allelic deletions only occur in 'tumour' cells, not in normal cells, and the presence of a high proportion of normal will thus obscure such change.

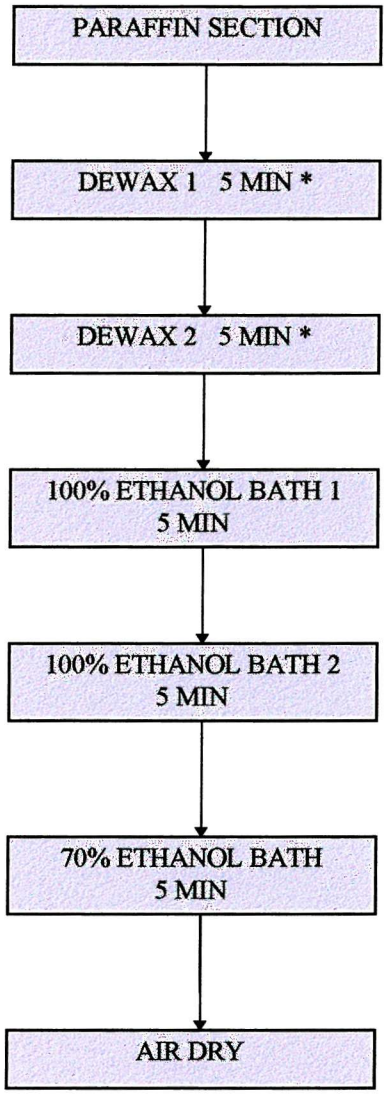
For the purposes of my study, this was of paramount importance, since an ovarian inclusion cyst may comprise only a single layer of cells, and thus stromal contamination of a sample may completely mask any genetic abnormalities present within its DNA. Therefore, a tissue microdissection technique was used to obtain the appropriate material. The technique is suitable for both paraffin-embedded and fresh tissue (only paraffin-embedded material was available for this study). Its main disadvantage is that of possible contamination with tissue flakes, but with care this can be avoided (Bohm and Wieland 1997).

2.2.1.1 *Preparation of tissue sections*

10μm sections were prepared using a microtome (Model 5030, Bright Instrument Company Ltd, England), and fixed onto PLL coated glass slides (Poly-L-Lysine, Sigma, USA). Six sections were prepared from each paraffin-embedded block. Sections were dewaxed, and rehydrated in alcohol (Figure 2.2). One was then stained with H and E and mounted with a coverslip for use as a template to guide microdissection. The other serial sections were thoroughly dried. Preparation was performed away from possible sources of contaminating DNA, and clean gloves were worn throughout.

Paraffin sections were stored at room temperature.

Figure 2.2 Protocol for 'dewaxing' of paraffin sections



* Low toxicity processing solvent (Metaclean Solvo 8000) (BDH Laboratory Supplies, Poole, UK)

H and E stained template slides were reviewed by a gynaecological pathologist (Dr N Singh, Southampton General Hospital) to identify areas of abnormality for microdissection.

2.2.1.2 *Microdissection*

A Stemi 2000 Stereomicroscope (Zeiss, Germany) was used. The H and E stained template slide was used to allow identification of the area to be dissected on the non-stained slides.

A 21 gauge needle was used to scrape the required cells from the sections. The needle tip was positioned approximately 1mm above the area to be dissected, using the microscope to view. Commencing at the edge of the identified inclusion cyst/area of histological abnormality, cells were separated from adjacent tissue and moved to the centre of the cyst/ abnormal region, to form a small heap. The dissected tissue was affixed to the needle tip, and transferred to an Eppendorf tube containing 50µl Tween lysis buffer (pretreated with UV irradiation for 5 minutes). Tissue from paraffin sections was brittle, and transfer to the needle tip often difficult. This was facilitated by careful moistening of the tip with lysis buffer. The process was repeated for each of the five unstained serial sections from each block.

2.2.1.3 *DNA extraction from microdissected paraffin-embedded tissue*

Following microdissection, 0.5µl of UV treated proteinase K was added to the Eppendorf tubes, and the mixtures overlaid with 20-40µl of mineral oil to avoid evaporation. The lids of the closed tubes were covered with parafilm, and the tubes incubated at 55°C for approximately 3 hours.

Samples were then heated to 95°C for 10 minutes, in order to destroy proteinase K activity. DNA was stored at 4°C, and used undiluted for PCR.

On completion of microdissection, paraffin sections were H and E stained and mounted with coverslips, to enable a permanent photographic record of dissected areas to be obtained.

2.2.2 Primer Extension Pre-amplification (PEP) Procedure

The quantity and quality of DNA which can be obtained from formalin-fixed, paraffin-embedded tissue is limited. When pathological material is fixed in formalin, DNA is rendered brittle, and is then susceptible to shearing when subjected to paraffin-embedding and sectioning. As a result, the average size of DNA fragments which can subsequently be recovered is in the region of only 100-200 bp (Bianchi *et al*, 1991).
A second limiting factor to DNA availability is the size of lesion microdissected. Ovarian inclusion cysts are usually very small, microdissection perhaps providing of the order of 100 cells. Dissection of the same lesion from a number of serial sections can then be performed, in order to increase the number of cell genomes obtained.
In order to amplify the entire genome, prior to performing locus-specific PCR, the primer extension pre-amplification procedure (PEP) can be utilised. The technique was initially developed by Zhang *et al (*1992*)*, using totally degenerate, short, 15 mer oligonucleotide primers containing all possible nucleotide sequences. At an annealing temperature of 37°C, Zhang estimated that no less than 78% of genomic DNA sequences from a single cell were amplified at least thirty times. An alternative method of amplification, using partially degenerate primers, is that of DOP-PCR (degenerate oligonucleotide-primed PCR), which was first developed for use in genome mapping studies (Telenius *et al*, 1992a).
DNA pre-amplification thus allows amplification of minute quantities of DNA, such as that obtained during fine needle aspiration or from tissue microdissection, and also enables amplification of unknown DNA sequences, thus facilitating construction of DNA libraries (Von Eggeling and Spielvogel 1995).

2.2.2.1 *Method*

The protocol used for this study was based on that of Zhang *et al*, 1992.

The reaction mixture used comprised:
5µl of a 400µM solution of 15N random primers (MWG Biotech GmbH, Germany)
5µl of a mixture of 4 dNTPs, each at 4mM (Promega, UK)
5µl of 10x reaction buffer, containing 15mM $MgCl_2$ (Genpak, UK)
5µl of Taq express DNA polymerase (Genpak, UK)

15µl of DNA

Ultrapure water to make a total volume of 50µl

Cycling conditions, using a PTC 100 thermal cycler (MJ Research Inc, USA) were as shown in Table 2.11.

Table 2.11 Cycling conditions for PEP procedure

96°C 12 min

Then 50 cycles of:

94 degrees celsius	1 min
37 degrees celsius	3 min
37-55 degrees celsius	a ramp of 10 secs/degree
55 degrees celsius	4 min
72 degrees celsius	5 min

Each PEP-PCR procedure lasted 14 hours.

A successful PEP experiment was indicated by the appearance of a DNA smear when the sample was run on an agarose electrophoretic gel (Figure 2.3). The intensity of the smear seen reflects the efficiency of the PCR reaction, and/or the quantity of DNA initially added to the PEP reaction.

2.2.3 Loss of Heterozygosity Analysis (LOH)

50 microsatellite markers were used in the study. Details of primer locations and sequences can be found in Table 2.12 and PCR conditions in Table 2.13. Microsatellite markers were chosen on the basis of the existing literature for both ovarian cancer and other tumour types (Tables 1.8, 1.9, and 1.10). Markers were selected to enable analysis of all chromosome arms.

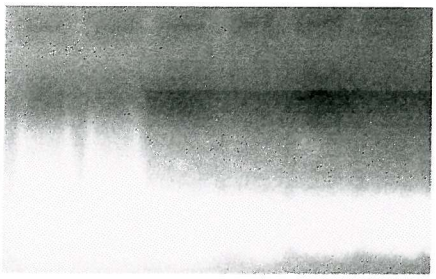
Figure 2.3 Agarose electrophoretic gel of PCR products from PEP-PCR procedure

Lanes 1 and 2 PEP-PCR product with DNA extracted from fresh tissue

Lanes 3 to 6 PEP-PCR product with DNA extracted from paraffin-embedded archival material

The intensity of the smear reflects the efficiency of the PCR reaction, and/or the quantity of DNA initially added to the PEP reaction.
In this example, equal quantities (15µl) of DNA were added to each reaction volume.

Figure 2.3 Agarose electrophoretic gel of PCR products from PEP-PCR procedure



1 2 3 4 5 6

Table 2.12 Microsatellite markers for LOH analysis

MARKER	CHROMOSOME LOCATION	PCR PRODUCT SIZE (bp)	HET.	PRIMER SEQUENCE (5'-3') CA STRAND	PRIMER SEQUENCE (5'-3') GT STRAND	REFERENCE
D1S504	1q22-23	124-138	0.51	TGTGGAAAGACAAGATGC	GCAAGGTTACATGGAGC	Gyapay, G '94, Dib, C '96
D2S391	2p21-16	142-152	0.79	ATGGAGCCAGTAGGTTACAGC	GGTGAGAGGGTATGATGGAA	Dib, C '96, Gyapay, G '96
D3S1582	3p21.2-21.1	154-178	0.80	CAGCAGGTACTATGAAAGCCTGT	GGAACAGCCCTATGGTTCAC	Gyapay, G '94, Dib, C '96
D3S1600	3p14.2-14.1	182-198	0.72	ATCACCATCATCTGCCTGTC	TGCTTGCCTTGGGATTTA	Gyapay, G '94, Dib, C '96
D3S196	3q21.3-25.2	86-98	0.728*	ACTCTTTGTTGAATTCCCAT	TTTCCACTGGGGAACATGGT	Weber, JL '90, Naylor, SL '94
D4S175	4q21-qter	112-134	0.842*	ATCTCTGTTCCCTCCCTGTT	CTTATTGGCCTTGAAGGTAG	Goold, RD '93
D5S406	5p15.3-14	160-186	0.78	CCTGCCAATACTTCAAGAAA	GGGATGCTAACTGCTGACTA	Weissenbach, J '92, Gyapay, G '96
D5S424	5q13.3	116-146	0.668	GGGTACATGGGAGTTCATTAGG	TCTCATGCTGGCAGGGATA	Weissenbach, J '92, Dib, C '96
D5S644	5q13.3-21.1	81-101	0.85	ACTAACTGGTAGATCAATGTGC	TTGGATTTGCTAAGACTGTG	Gyapay, G '94, Dib, C '96
D5S346	5q21-22	96-122	0.82*	ACTCACTCTAGTGATAAATCGGG	AGCAGATAAGACAGTATTACTAGTT	Spirio, L '91
D5S399	5q22.3-31	116-132	0.79	GAGTGTATCAGTCAGGGTGC	GGCCTCAACTTCATAATCAA	Weissenbach, J '92, Dib, C '96
D6S284	6q13-15	233-251	0.72	CATGGCTGTCTATCAAACCC	AAGCATTTGTGTGGCTCTTG	Weissenbach, J '92, Dib, C '96
D6S287	6q21-23.3	143-171	0.85	ATATTAGTGCCTTATGCTTCTG	AAATTGGATATTCATGCTTG	Weissenbach, J '92, Dib, C '96
D6S1027	6q25.3-27	117-138	na	CGTTCTGCACATGTATCCTG	TGCTCTGTCTATGGAGTAGCC	Murray, J pers comm '95
D7S654	7p22.3-21.3	207-227	0.73	TTGCTGGTGATTTTCCAGGT	CCACTCACTCTGTGGCATTT	Gyapay, G '94, Dib, C '96
D7S691	7p14.2-12.2	128-146	0.74	GGGTGATTAATGCTTGCTTA	GCTTGATTTTCCAACAGG	Gyapay, G '96, Dib, C '96
D7S670	7p13	100-110	0.76	GTCCATTTTNATTTAGCATTTCA	AACCGAAGCAGGATTTTATT	Gyapay, G '96, Dib, C '96
D7S672	7q11.1-21.1	132-160	0.83	ACATGAAGGTCTACCAGTAGCC	CACTTTGGTTGGAGCAAGG	Gyapay, G '94, Dib, C '96

D7S491	7q21-22	115-131	0.74	AGCTCCAAAACCTAACTCCA	TCAAAATTATTTGACTTCTTGATTT	Weissenbach, J '92, Dib, C '96
D7S633	7q31	168-182	0.62	TGAGCCTCGCATCACTG	TCTGGGGAGTCCTTTAACAGTA	Gyapay, G '94, Dib, C '96
D8S258	8p23-22	144-154	0.70	CTGCCAGGAATCAACTGAG	TTGACAGGGACCCACG	Weissenbach, J '92, Dib, C '96
D8S543	8q12-13	116-140	0.75	TGGTGTCATTGCTTTCTAGTCT	TGCACAGGTGAGTAAATTTGTAA	Gyapay, G '96, Dib, C '96
D9S171	9p21	159-177	0.79	AGCTAAGTGAACCTCATCTCTGTCT	ACCCTAGCACTGATGGTATAGTCT	Weissenbach, J '92, Dib, C '96
D9S161	9p21	119-135	0.77	TGCTGCATAACAAATTACCAC	CATGCCTAGACTCCTGATCC	Weissenbach, J '92, Dib, C '96
D9S127	9q31	149-159	0.72	CCCTCAAAATTGCTGTCTAT	AGATTGATTGATACAAGGATTTG	Lyall, JE '92
D10S89	10p15.1-13	142-156	0.745*	AACACTAGTGACATTATTTTCA	AGCTAGGCCTGAAGGCTTCT	Weber, JL '90
D10S1696	10q21.3-23.3	124-136	0.65	TCCTGGGTGACAGAGTGA	GAGACAGCATTTCCATTATGA	Dib, C '96
D10S215	10q22-23	152-208	0.81	TGGCATCATTCTGGGGA	GCTTTACGTTTCTTCACATGGT	Weissenbach, J '92, Gyapay, G '94
D11S902	11p15-13	145-163	0.80	CCCGGCTGTGAATATACTTAATGC	CCCAACAGCAATGGGAAGTT	Weissenbach, J '92
D11S912	11q23	101-123	0.80	TCGTGAGANTACTGCTTTGG	TTTTGTCTAGCCATGATTGC	Weissenbach, J '92, Gabra, H '96
D11S1336	11q23-24.2	232-252	0.68	TGCAGCAAGCCGAGATT	CTGGATGGCATGAGGTTTC	Gyapay, G '94, Dib, C '96, Gabra, H '96
D11S969	11q24.1-25	141-149	0.75	TTGATTTGGAAGATTTTCAC	GGGGCAGAATGGGTAT	Gyapay, G '94, Dib, C '96
D12S356	12p13	205-225	0.80	CTGGGTGAAACTGCTAGGAAATACT	GAGGCTTGTCAAAAAATCCTGC	Gyapay, G '94, Dib, C '96
D12S385	12pter-qter	125-151	0.82	GGGAACAGTGAGTTACAAAC	GGATTGTCTAGCCAGTGGG	Weber, JL pers comm '93
D13S133	13q14.3	130-187	0.82	GCTAGGACTACAGGTGCAAACC	GGCAACATAGGGAAACCCTAGC	Gilliam, CT pers comm '92
D13S128	13q32-34	144-178	0.91	GGACACAACTGACTTCATAATGAAT	TTCACCGAAGTGGAAGAGAGAT	Gilliam, CT pers comm '92
D14S80	14q12-13	132-160	0.83	CATCTACCTGCCGCAA	TAGCCAATTTATGGATACAACTT	Weissenbach, J '92, Dib, C '96
FES	15q26.1	143-163	0.75	GGAAGATGGAGTGGCTGTTA	CTCCAGCCTGGCGAAAGAAT	Polymeropoulos, MH '91
D16S3072	16pter-qter	148-168	0.80	ACCACTGCACTCCAGCCT	GAGACCCAGATGTAGCATCCT	Dib, C '96

D17S786	17pter-qter	135-157	0.76	TACAGGGATAGGTAGCCGAG	GGATTTGGGCTCTTTTGTAA	Weissenbach, J '92, Gyapay, G '96
D17S855	17q21.1	143-155	0.822*	GGATGGCCTTTTAGAAAGTGG	ACACAGACTTGTCCTACTGCC	Anderson, LA '93
D17S1322	17q21	121-139	0.67	CTAGCCTGGGCAACAAACGA	GCAGGAAGCAGGAATGGAAC	Goldgar, D pers comm '94
D17S1323	17q21	151-161	0.44	TAGGAGATGGATTATTGGTG	AAGCAACTTTGCAATGAGTG	Goldgar, D pers comm '94
D17S1327	17q21	128-152	0.55	CTAAGGAGGTTTCTCTGGAC	TTCACAACTCAAGGTAAGATAGG	Goldgar, D pers comm '94
D18S59	18pter-18p11.22	148-164	0.81	AGCTTCTATCCAACAGGGGC	ACCAGAATGTGAACGACCCT	Weissenbach, J '92, Gyapay, G '96
D18S474	18pter-qter	119-139	0.82	TGGGGTGTTTACCAGCATC	TGGCTTTCAATGTCAGAAGG	Dib, C '96, Gyapay, G '96
D19S886	19pter-qter	142-158	0.64	TGGATCTACACTCCGGC	ATTTTACTGGCTGGCACTTG	Dib, C '96, Gyapay G '96
D20S851	20pter-qter	128-150	0.74	ACTTCAAGTTATGTGTGGCACAA	GCCCAGACTCTGACACCTTT	Dib, C '96
D21S1432	21q21	139-151	na	CTTAGAGGGACAGAACTAATAGGC	AGCCTATTGTGGGTTTGTGA	Murray, J pers comm '95
HPRT	Xq26.1	151-167	0.715*	TCTCTATTTCCATCTCTGTCTCC	TCACCCCTGTCTATGGTCTCG	Hearne, CM '91

HET= heterozygosity * heterozygosity calculated, all others observed na = not available

Table 2.13 Optimised PCR conditions for microsatellite markers

MARKER	ANNEALING		
	TEMPERATURE (degrees celsius)	TIME (seconds)	NO. OF CYCLES
D1S504	55	30	35
D2S391	55	30	35
D3S1582	53	45	38
D3S1600	55	30	35
D3S196	52	45	40
D4S175	52	45	40
D5S406	53	45	40
D5S424	53	45	38
D5S644	55	45	40
D5S346	50	30	35
D5S399	52	45	40
D6S284	55	45	40
D6S287	52	45	40
D6S1027	55	30	35
D7S654	50	45	35
D7S691	50	45	35
D7S670	53	45	35
D7S672	53	45	35
D7S491	53	45	35
D7S633	53	30	35
D8S258	55	30	35
D8S543	53	30	35
D9S171	52	30	38

D9S161	52	30	38
D9S127	53	30	35
D10S89	55	30	35
D10S1696	55	30	35
D10S215	52	45	40
D11S902	55	45	40
D11S912	55	30	35
D11S1336	55	45	40
D11S969	55	30	35
D12S356	55	45	40
D12S385	55	30	35
D13S133	53	30	35
D13S128	53	30	35
D14S80	50	30	35
FES	55	30	35
D16S3072	53	30	35
D17S786	50	30	35
D17S855	55	20	38
D17S1322	55	20	38
D17S1323	55	20	38
D17S1327	55	20	38
D18S59	55	30	35
D18S474	53	30	35
D19S886	53	45	40
D20S851	55	30	35
D21S1432	55	30	35
HPRT	55	30	35

2.2.4 Optimisation of Primer Annealing Temperature

PCR specificity depends on the primer annealing step. The amount of PCR product will be reduced if the annealing temperature is too high, whilst setting the temperature too low allows non-specific binding with consequent generation of multiple products. The base composition, length, and concentration of primers determine the optimal temperature and time for annealing.

A widely used starting point for PCR optimisation is an annealing temperature 3-5°C below the melting temperature (T_m) at which DNA is 50% melted. The formula used to calculate this (only accurate for primers up to 20 nucleotides long) is as follows:

T_m= [(number of A+T) x 2°C + (number of G+C) x 4°C]

Optimal annealing temperatures may be 3-12°C higher than the calculated T_m. For the majority of primers, annealing temperatures range from 55°C to 72°C.

Within this study, 55°C was initially tried for PCR amplification. According to the PCR results, this was adjusted until the optimal temperature was found.

2.2.5 Polymerase Chain Reaction

PCR was performed as described in section 2.1.3

2.2.5.1 *Preparation of PCR mixture*

'PEPed' DNA was used. Preparation of the mixture was carried out on ice in a 1.5ml Eppendorf tube. A 10µl PCR reaction, containing 1µl DNA was used (Table 2.14), the DNA being aliquoted to a 50µl Eppendorf tube, and overlaid with mineral oil as previously described.

Table 2.1.4 Composition of PCR mixture (all volumes in μl)

REAGENT	QUANTITY PER 10μl PCR REACTION
Deionised water	7.25
Low dCTP nucleotide mix	0.25
Taq express buffer	1.0
Primer mix	0.5
Taq express	0.1
P32	0.05

Taq express (Genpak Ltd, UK) and P32 were added immediately prior to aliquoting 9μl of mix to each tube containing DNA. PCR mixtures were subjected to thermal cycling (see Table 2.13 for cycling conditions for individual markers used).

After confirmation of the presence of a PCR product by agarose gel electrophoresis, products were analysed on 6% non-denaturing gels (see Table 2.10). The running buffer used was 1xTBE.

Approximately 30μl of formamide was added to each sample, prior to heating to 95°C for 5 minutes to denature. 2μl of each sample was loaded on the gels. Gels were run at 50 watts (Hybaid) or 70 watts (BRL) for 4-5 hours, depending on the expected PCR product size, and processed as previously described.
LOH was determined by comparison of allele intensity between normal DNA (from leucocytes) and DNA extracted from dissected areas of abnormality. Samples were scored as showing LOH when relative allele intensity differed markedly between blood and 'abnormal' tissue areas. All reactions were repeated at least twice, to ensure consistency of PCR results.

CHAPTER THREE

BRCA1 MUTATION SCREENING IN SPORADIC OVARIAN TUMOURS

3.1 INTRODUCTION

Few studies have investigated the incidence of BRCA1 mutations in unselected sporadic ovarian tumours. The frequency of mutations reported by other groups ranges from 0 to 8.6% (Futreal *et al*, 1994; Hosking *et al*, 1995; Matsushima *et al*, 1995; Merajver *et al*, 1995; Takahashi *et al*, 1995; Stratton *et al*, 1997; Rubin *et al*, 1998).

Within this study, 223 consecutive cases of epithelial ovarian tumours presenting to the Princess Anne Hospital, Southampton, were analysed for germline BRCA1 mutations. Details of cases included are documented in Table 3.1 (case numbers recorded are Princess Anne Hospital identification numbers), with a breakdown by histology presented in Table 3.2. As the majority of previously identified mutations in BRCA1 have occurred in exons 2, 11, 12, and 20 in the Wessex region (Eccles *et al*, 1998) (Figure 3.1), mutation analysis was carried out for these exons, using Single Stranded Conformational Polymorphism (SSCP) and sequencing.

3.2 AIMS OF THE STUDY

The study was carried out for two reasons:

1. To assess the incidence of BRCA1 mutations in a large series of sporadic ovarian tumours, since previous studies, with the exception of that by Stratton *et al* 1997 (who studied 374 patients), have been with small numbers of patients.
2. In an attempt to identify additional BRCA1 mutation carriers, with benign or borderline ovarian lesions, for possible inclusion in the LOH analysis described for prophylactic oophorectomy specimens.

Table 3.1 Clinical and histological details for patients included in the BRCA1 mutation screening study

CASE NO.	DOB.	AGE AT DIAGNOSIS (YEARS)	TUMOUR HISTOLOGY	TUMOUR STAGE (FIGO)	TUMOUR GRADE
1	15/03/31	62	Benign serous cystadenofibroma	-	-
2	11/01/33	60	Borderline serous tumour	IC	-
5	7/11/11	81	Benign serous cyst	-	-
7	26/05/14	78	Benign serous cystadenoma	-	-
10	13/12/34	58	Benign serous cystadenofibroma	-	-
11	17/03/57	36	Benign mucinous cystadenoma	-	-
13	19/07/15	77	Benign mucinous cystadenoma	-	-
14	27/10/36	56	Serous cystadenocarcinoma	II	1
15	15/01/09	84	Benign mucinous cystadenoma	-	-
16	5/11/35	57	Serous adenocarcinoma	II	3
18	17/07/20	72	Serous adenocarcinoma	IA	3
19	21/10/07	85	Serous adenocarcinoma	III	3
20	22/09/51	41	Borderline serous tumour	IIIA	-
22	28/05/28	65	Serous adenocarcinoma	III	3
23	18/10/46	46	Serous cystadenocarcinoma	IA	1
24	7/10/16	76	Benign serous cystadenoma	-	-
25	11/09/28		Adenocarcinoma	III	-
26	30/05/42	51	Serous adenocarcinoma	III	2
27	10/02/46	46	Serous cystadenocarcinoma	IB	2
28	9/11/46		Serous adenocarcinoma	IB	-
29	5/11/61		Mucinous cystadenocarcinoma	IA	-
30	7/03/34	59	Endometrioid and mucinous adenocarcinoma	III	3

31	1/01/31		Serous cystadenocarcinoma	III	-
32	14/02/22	71	Serous cystadenocarcinoma	II	1
34	16/02/47	46	Serous cystadenocarcinoma Benign serous cystadenoma	IC	-
35	15/12/31		Serous cystadenocarcinoma	IIA	-
36	15/12/39	53	Endometrioid adenocarcinoma	IA	1
37	3/07/24	68	Endometrioid adenocarcinoma	I	2
38	4/02/46		Endometrioid and clear cell carcinoma	IA	-
39	3/06/12	81	Benign serous cystadenofibroma	-	-
40	6/01/20	73	Mucinous cystadenocarcinoma	II	3
41	11/11/39		Mucinous cystadenocarcinoma	IB	-
42	5/07/28		Serous cystadenocarcinoma	IA	-
43	18/11/42	50	Serous cystadenocarcinoma	II	2
44	4/08/16	74	Mucinous cystadenocarcinoma	I	1
45	20/10/43	49	Serous cystadenocarcinoma	III	3
47	10/02/34	59	Adenocarcinoma	III	4
49	3/12/44	48	Papillary high grade carcinoma of Mullerian origin	III	3
51	30/09/11	81	Mucinous cystadenocarcinoma	IA	1
52	9/01/22	71	Serous cystadenocarcinoma	III	-
53	13/12/33	59	Adenocarcinoma	III	-
54	4/05/29	64	Endometrioid carcinoma	IB	-
55	22/03/33	60	Endometrioid carcinoma	IIC	-
56	26/10/33	59	Adenocarcinoma	III	-
57	11/04/36	57	Serous adenocarcinoma	III	-
58	22/03/38	55	Cystadenocarcinoma	IIC	1
59	31/08/36	56	Endometrioid carcinoma		-
61	30/03/17	76	Mucinous cystadenocarcinoma	IA	1

63	16/06/26	66	Serous adenocarcinoma	I	3
64	21/10/30	62	Endometrioid carcinoma	III	-
66	17/08/33		Serous adenocarcinoma	IIIC	-
67	3/04/35		Serous cystadenocarcinoma	IA	2
70	17/05/36	57	Endometrioid and clear cell carcinoma	IA	3
72	6/04/33	60	Adenocarcinoma	IIB	-
73	7/12/23	69	Endometrioid carcinoma	IIC	-
74	1/02/31	62	Cystadenocarcinoma	IIB	-
75	20/05/25	68	Benign mucinous cystadenoma	-	-
78	29/09/17	75	Endometrioid carcinoma	IA	2
80	19/06/22	71	Mucinous cystadenocarcinoma	IA	1
81	16/10/31	61	Endometrioid carcinoma	IC	-
85	25/01/24	69	Benign mucinous cystadenoma + Brenner tumour	-	-
86	7/08/19	74	Serous adenocarcinoma	IIIA	3
87	5/12/26	66	Adenocarcinoma	IA	-
89	15/08/26	67	Adenocarcinoma	II	-
90	26/10/31		Mucinous adenocarcinoma	IC	1
91	15/10/44	49	Serous adenocarcinoma	III	-
92	11/12/44	49	Borderline mucinous tumour	IA	-
93	4/03/53		Adenocarcinoma	III	-
94	19/11/45	47	Benign mucinous cystadenoma	-	-
95	31/08/27	66	Endometrioid carcinoma	II	3
96			Benign mucinous cystadenoma with borderline changes	-	-
97	24/09/19	74	Serous cystadenocarcinoma	III	2-3
99	13/09/23	70	Adenocarcinoma	III	-
100	3/12/40	52	Endometrioid carcinoma	III	-

101	26/08/46	47	Cystadenocarcinoma	IA	-
103	19/01/37	56	Benign serous cystadenofibroma	-	-
105	1/08/29		Endometrioid carcinoma	III	-
106	24/07/21	72	Clear cell carcinoma	IB	-
107	30/03/35	48	Mucinous cystadenocarcinoma	IC	1
108	6/10/25	68	Mucinous and endometrioid carcinoma	IA	-
109	4/04/40	53	Endometrioid carcinoma	III	-
110	25/03/46	47	Serous cystadenocarcinoma	IIC	-
111	29/10/19	73	Borderline adenocarcinoma	-	-
112	15/08/31	62	Borderline mucinous cystadenocarcinoma	IC	-
113	4/01/57	36	Benign mucinous cystadenoma	-	-
114	3/04/23	70	Endometrioid and clear cell carcinoma	IA	2
117	6/11/30		Mucinous and endometrioid carcinoma	I	2
118	3/10/16	77	Endometrioid carcinoma	IC	-
119	13/09/34	59	Serous cystadenocarcinoma	IC	2
120	1/02/19	74	Benign cystadenofibroma	-	-
121	16/05/16	77	Mucinous cystadenocarcinoma	III	1
122	1/12/45	48	Serous cystadenocarcinoma	III	3
123	4/06/11	82	Endometrioid carcinoma	IB	3
125	6/10/37	56	Benign mucinous cystadenoma	-	-
127	14/01/26	67	Serous cystadenocarcinoma	-	2
128	11/11/38	55	Endometrioid carcinoma	IC	2
129	24/12/25	67	Malignant mixed Mullerian tumour	IV	-
131	22/12/20	73	Serous cystadenocarcinoma	III	2-3
133	26/09/46	47	Mucinous cystadenocarcinoma	-	1
134	11/12/30	63	Serous cystadenocarcinoma	IIIC	3
136	25/03/16	77	Endometrioid carcinoma	I	3

138	13/04/47	46	Mucinous cystadenocarcinoma	IA	1
139	12/06/29	64	Serous cystadenocarcinoma	III	3
140	31/12/48	45	Benign mucinous cystadenoma	-	-
141	16/03/38	55	Serous adenocarcinoma	IIIC	2
144	18/04/31	62	Mucinous cystadenocarcinoma	IC	1
147	24/11/35	58	Benign serous cystadenoma	-	-
148	15/08/27	66	Benign serous cystadenoma	-	-
149	11/04/28	65	Benign serous cystadenofibroma	-	-
150	10/03/27	67	Endometrioid carcinoma	IA	-
151	3/09/38	55	Endometrioid carcinoma	IIC	2
155	17/02/02	92	Endometrioid carcinoma	IA	2
156	3/05/47	47	Benign mucinous cystadenoma	-	-
158	9/09/12	81	Benign cystadenofibroma	-	-
159	10/12/46	47	Benign mucinous cystadenoma	-	-
160	14/05/46	48	Benign cystadenofibroma	-	-
161	28/07/28	65	Cystadenocarcinoma	-	-
162	19/02/22	72	Adenocarcinoma	-	3
163	2/09/43	50	Serous adenocarcinoma	III	3
164	8/12/22	71	Benign serous cystadenofibroma	-	-
165	15/01/21	73	Adenocarcinoma	IVC	-
166	28/01/29	65	Serous cystadenocarcinoma	IVC	-
167	11/01/28	66	Benign serous cystadenofibroma	-	-
170	9/03/34	60	Endometrioid carcinoma	IA	2
172	22/08/22	71	Benign cystadenofibroma	-	-
176	13/11/32	61	Mucinous cystadenocarcinoma	IA	1
177	19/09/41	52	Clear cell adenocarcinoma	IA	3
179	8/11/32	61	Endometrioid carcinoma	III	3
182	5/12/30	59	Adenocarcinoma	III	-

184	13/04/47	47	Adenocarcinoma	II	-
185	11/04/33	61	Serous cystadenocarcinoma	III	2
186	3/08/16	77	Borderline mucinous tumour	IC	-
187	2/08/22	72	Adenocarcinoma	IV	4
188	7/06/47	47	Serous cystadenocarcinoma	IIIC	3
189			Endometrioid carcinoma	II	-
190	12/11/43	50	Serous cystadenocarcinoma	II	1
195	1/06/29	65	Serous and endometrioid carcinoma + Brenner tumour	II	2-3
196	13/10/48	45	Benign serous cystadenoma	-	-
198	4/03/37	57	Adenocarcinoma	IIC	-
199	18/05/18	76	Adenocarcinoma	IIIC	-
201	5/10/14	79	Serous adenocarcinoma	IIIB	2
202	28/05/29	65	Endometrioid carcinoma	II	1
203	28/06/31	63	Mucinous adenocarcinoma	II	-
204	9/12/21	72	Serous cystadenocarcinoma	IIIC	3
205	27/06/09		Endometrioid carcinoma	II	2
207	28/02/20	74	Adenocarcinoma	III	3
208	29/10/41	52	Adenocarcinoma	IA	-
209	15/12/21		Adenocarcinoma	III	-
210	17/02/32	62	Adenocarcinoma	IIIC	3
211	30/04/39	55	Endometrioid carcinoma	IIC	-
212	2/05/18	76	Mucinous cystadenocarcinoma	III	-
213	30/01/25	69	Serous cystadenocarcinoma	IIC	-
214	2/04/19	75	Benign mucinous cystadenoma	-	-
215	4/02/39	55	Serous adenocarcinoma	IC	3
216	15/06/20	74	Serous adenocarcinoma	III	-
217	6/04/40	54	Endometrioid carcinoma	IA	-

219	21/01/31	63	Mucinous cystadenocarcinoma	IC	1
220	9/02/32	62	Malignant mixed Mullerian tumour	IIIC	2
221	23/12/40		Adenocarcinoma	IV	-
222	10/02/26	68	Serous adenocarcinoma	IIIC	-
223	10/05/34	60	Endometrioid carcinoma	IA	-
224	12/03/38	56	Clear cell carcinoma	IC	-
225	10/10/36	58	Mucinous cystadenocarcinoma	IIIA	2
228	2/07/21	73	Serous cystadenocarcinoma	IIC	-
229	27/05/30	64	Borderline serous fibrocystadenoma		-
230	3/05/45	49	Serous cystadenocarcinoma	IV	2-3
231	2/12/28	65	Serous cystadenocarcinoma	IIC	-
232	5/07/31	63	Cystadenocarcinoma	IC	-
233	28/01/26	68	Endometrioid carcinoma	IC	2
234	23/03/25	69	Cystadenocarcinoma	IIC	-
235	24/10/46	48	Endometrioid carcinoma	IC	-
236	30/10/42		Prophylactic oophorectomy	-	-
237	8/02/28	66	Endometrioid carcinoma	IC	2
238	9/10/36	58	Adenocarcinoma	IC	-
239	10/08/34	60	Adenocarcinoma	IA	3
240	28/06/47	47	Endometrioid carcinoma	IIIC	-
241	11/10/18	76	Adenocarcinoma	IIIB	3
242	26/03/50	44	Endometrioid carcinoma	IC	1
243	10/02/28	66	Benign cystadenofibroma	-	-
246	16/04/47	47	Benign mucinous cystadenoma	-	-
247	24/09/39	55	Cystadenocarcinoma	III	-
248	4/06/19	75	Adenocarcinoma	IIIC	-
249	28/07/29	65	Adenocarcinoma	IIIC	-
250	25/11/56	38	Serous adenocarcinoma	III	1

251	21/12/21	72	Adenocarcinoma	IC	1
253	13/07/24	70	Endometrioid carcinoma	IIIA	2
254	6/04/49	45	Adenocarcinoma	IIIC	2
255	28/08/44	50	Endometrioid carcinoma	IIIC	1
256			Cystadenocarcinoma	IIIC	2
257	7/04/34	60	Mucinous cystadenocarcinoma	IA	1
258	13/12/25	69	Endometrioid carcinoma	IIIC	3
262	29/12/20	73	Benign serous cystadenoma	-	-
263	26/10/32	62	Borderline mucinous tumour	IA	-
264	23/02/25		Serous cystadenocarcinoma	IV	-
267	13/06/61	33	Endometrioid carcinoma	IA	2
271	17/04/04	90	Mucinous cystadenocarcinoma	IA	1
272	15/03/36	58	Clear cell carcinoma	IIIA	1
274	20/02/42	52	Clear cell carcinoma	-	-
280	25/03/34	60	Clear cell carcinoma	IC	1
282	13/09/22	72	Serous cystadenocarcinoma	III	-
286	15/08/24	70	Endometrioid carcinoma	IIA	3
289	11/09/42	52	Borderline mucinous tumour	I	-
292	2/03/15	80	Mucinous adenocarcinoma	III	2
293	10/01/36	59	Adenocarcinoma	I	3
294	5/12/35	59	Benign mucinous cystadenoma	-	-
299	1/11/19	75	Clear cell carcinoma	IC	-
306	24/10/32	62	Prophylactic oophorectomy	-	-
308	9/05/70	24	Benign serous cystadenofibroma	-	-
360	29/08/27	68	Benign serous cystadenoma	-	-
362	10/12/19	76	Borderline serous tumour	-	-
367	8/09/17	78	Benign serous cystadenoma	-	-
412	6/11/32	63	Benign serous cystadenofibroma	-	-

415	25/10/33	63	Benign serous cystadenoma	-	-
426	5/05/33	63	Benign serous cystadenofibroma	-	-
438	18/03/40	56	Benign serous cystadenoma	-	-
447	2/04/16	80	Borderline serous cystadenofibroma	I	-
449	11/03/24	73	Benign serous cystadenofibroma	-	-
467	4/02/45	52	Benign serous cystadenofibroma	-	-
479	31/05/26	71	Benign serous cystadenofibroma	-	-
480	20/03/20	77	Benign serous cystadenofibroma	-	-
486	1/10/40		Borderline serous tumour	-	-
527	20/08/24	73	Benign serous cystadenofibroma	-	-
530	23/01/51	47	Benign serous cystadenoma	-	-

* Case numbers used are Princess Anne Hospital identification numbers

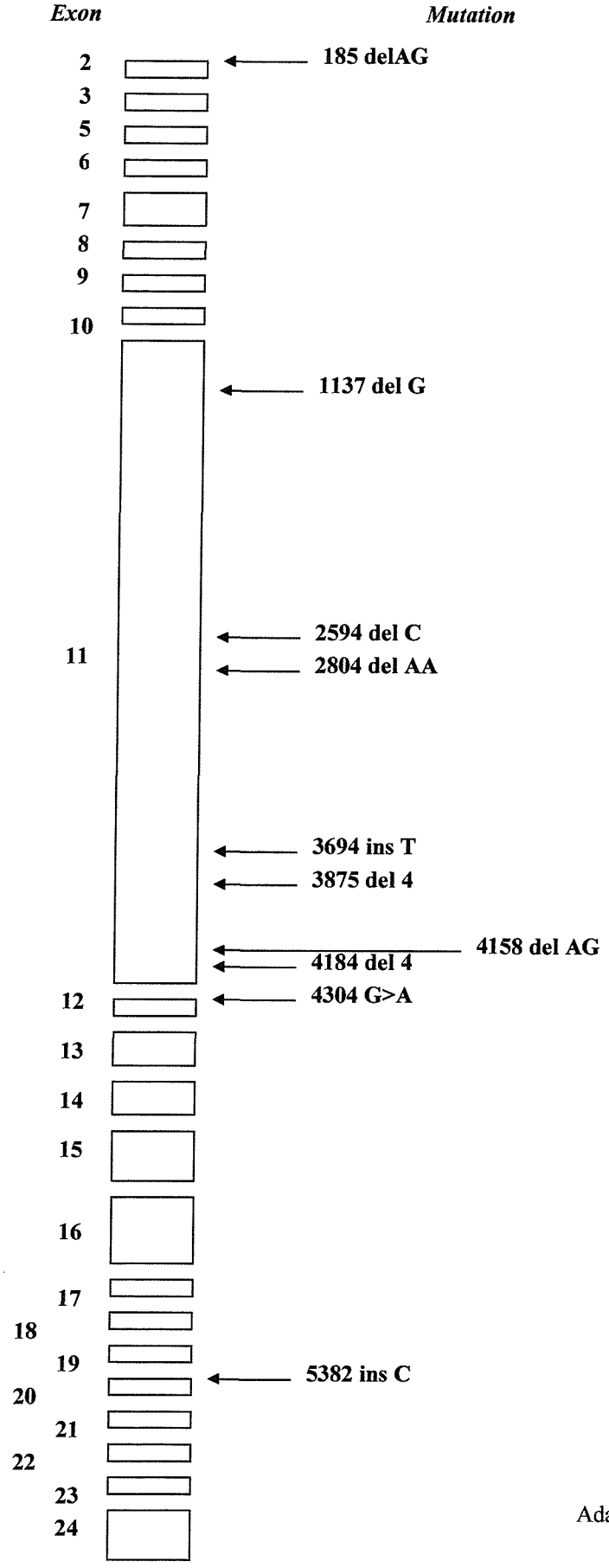
Table 3.2 Tumour details

Histology	Malignant n=162	Borderline n=12	Benign n=47
	Number (%)	Number (%)	Number (%)
Serous	49 (30.3)	6 (50)	27 (57.5)
Mucinous	21 (13)	5 (42)	15 (31.9)
Endometrioid	41 (25.3)	-	-
Clear cell	7 (4.3)	-	-
Mixed Mullerian	7 (4.3)	-	-
Adenocarcinoma (unspecified histology)	29 (17.9)	1 (8)	-
Undifferentiated	8 (4.9)	-	5 (10.6)

Two prophylactic oophorectomy samples were also included in the total of 223 cases, as they were amongst the consecutive series of oophorectomies performed

Histological diagnoses were taken from written pathology reports, issued by a number of pathologists over the time period studied

Figure 3.1 BRCA1 mutations in Wessex families



Adapted fron Eccles *et al*, 1998

3.3 RESULTS

Three unambiguous inactivating mutations were detected (patients 27, 134, and 179), all in malignant lesions (two serous and one endometrioid). One patient had a clear family history of Breast-Ovarian cancer, whilst in the other two cases there was no known relevant family history (see Table 3.3).

All three mutations were in exon 11, with the endometrioid tumour (patient 179) exhibiting an identical mutation to that observed in one of the serous tumours (patient 27). The frequency of mutations detected in serous malignant lesions was 2 of 49 (4.1%), and that of endometrioid 1 of 41 (2.4%).

Figure 3.2 shows SSCP gel electrophoresis for all three cases. Figure 3.3 shows the 11p mutation (11p3695insT) shown by direct sequencing, whilst Figure 3.4 shows direct sequencing for the 11q mutation (11qdel4 3875).

3.4 LITERATURE REVIEW

Few studies have reported the incidence of BRCA1 mutations in unselected sporadic ovarian tumours, and, in most of those published, patient numbers have been small (see Table 3.4). The first such study, by Futreal *et al (*1994*)*, described a single germline BRCA1 mutation (pro1637leu) amongst a series of 12 ovarian carcinomas showing LOH with polymorphic markers in the BRCA1 region (D17S1323, D17S855, D17S1327). The patient had no relevant family history. Hosking *et al (*1995*)* again demonstrated LOH on chromosome 17q in a series of 17 cases, and, amongst these, demonstrated 1 germline mutation (exon 4) and 1 somatic mutation (exon 23). Again, the individuals involved had no family history of breast or ovarian cancer. The study carried out by Matsushima *et al (*1995*)* involved greater patient numbers, revealing 4 germline BRCA1 mutations (1 exon 2, 2 exon 11, 1 intronic substitution distal to exon 22) from a series of 76 cases. Merajver's group (Merajver *et al*, 1995) again only detected 1 germline mutation (exon 11) in their 47 patient series, but were also able to demonstrate 4 somatic mutations (exons 2, 5, 19, 22). No data regarding family history is available within this report. There have, to date, been 3 larger studies reported, all involving in excess of 100 patients. The largest is that by Stratton *et al (*1997*)*,

describing a series of 374 cases, amongst which 13 germline mutations (exons 3, 5, 11, 13, 17, 20, 24) were detected, 6 of them novel. Twelve of the mutations resulted in a truncated protein product, whilst the other caused an in-frame deletion just outside the putative zinc-finger domain. The patients included in this study were not selected on the basis of family history, but on subsequent enquiry 9 of the 12 with truncating mutations described a relevant family history of breast or ovarian cancer; 6 had affected first degree relatives. Takahashi *et al (*1995*)* reported a series of 115 patients, from which 6 germline BRCA1 mutations were detected (exons 2, 5, 11), all having shown LOH at polymorphic markers in the BRCA1 region. Five of the patients exhibited relevant family histories - 4 gave a history of maternal breast cancer, and 1 had multiple relatives with breast, ovary, prostate, and colon cancer. Rubin's group (Rubin *et al*, 1998) found a somewhat higher mutation incidence in a population of the same size (10/116). Of these, 3 had no relevant family history of breast or ovarian cancer, whilst another 4 had only a single relative affected by either tumour type. Only 3 patients had 2 or more affected relatives. Again, all mutations detected were germline.

Table 3.3 Patients with mutations

Patient	Age at diagnosis	Family history	Tumour histology	Stage	Grade	Exon affected	Mutation
27	46	Yes	Serous	IB	2	11	11p3695insT
134	63	None relevant	Serous	IIIC	3	11	11qdel4 3875
179	61	None relevant	Endometrioid	III	3	11	11p3695insT

Figure 3.2 SSCP gel electrophoresis

11p Lane 1. Patient 27

Lane 2. Patient 45 (normal control)

Lane 3. Patient 46 (normal control)

Lane 4. Patient 179

Lanes 1 and 4 show band shifts when compared with the normal controls

11q Lane 1. Patient 134

Lane 2. Patient 28 (normal control)

Lane 3. Patient 29 (normal control)

Lane 1 shows a band shift when compared with the normal controls

Figure 3.2 SSCP gel electrophoresis

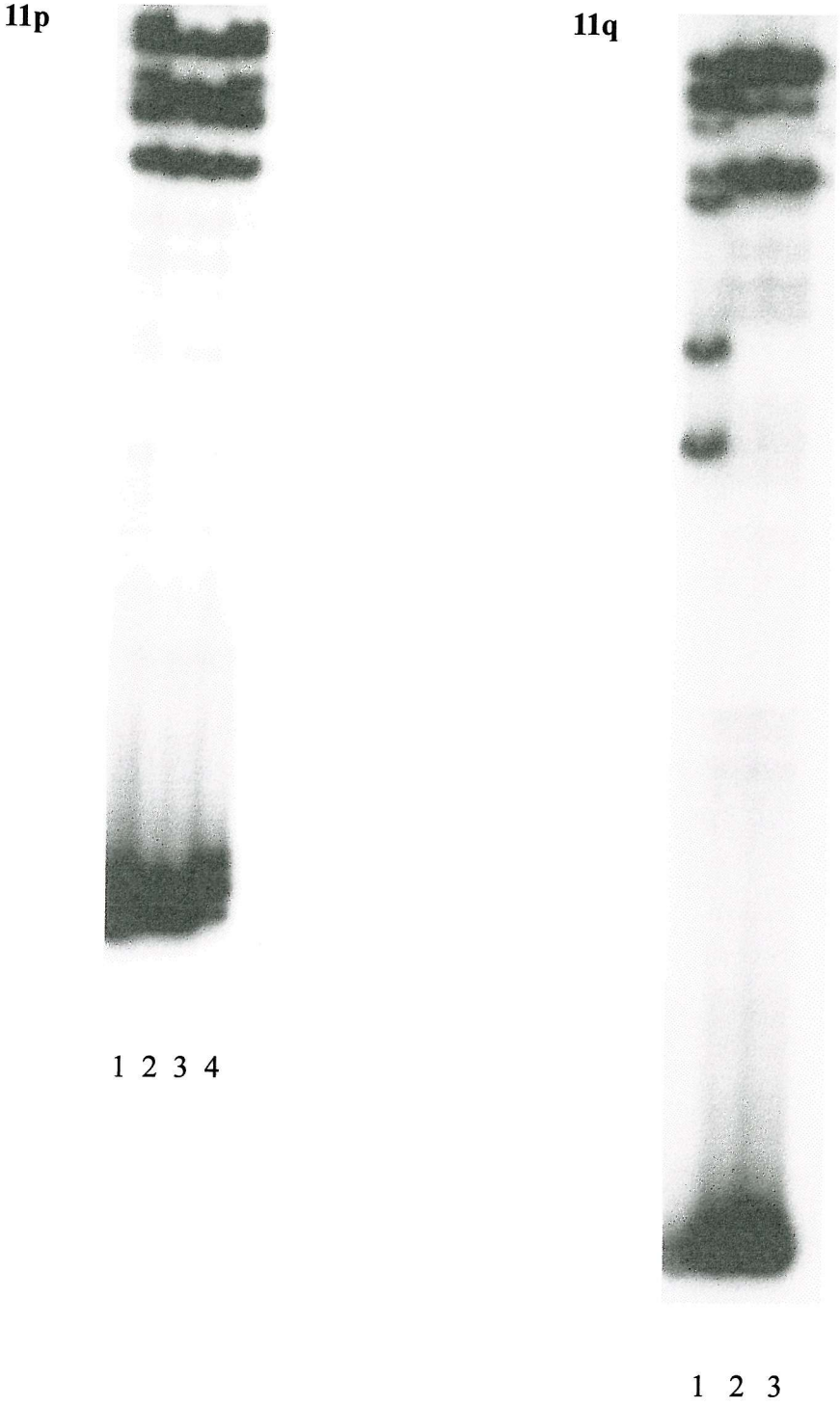


Figure 3.3 11p mutation

Direct sequencing of leucocyte DNA from patients 27 and 179 revealed identical mutations in these two individuals (11p3695insT). One sequencing autoradiograph is shown; the mutation is marked by an arrow.

Figure 3.3 11p mutation shown by direct sequencing

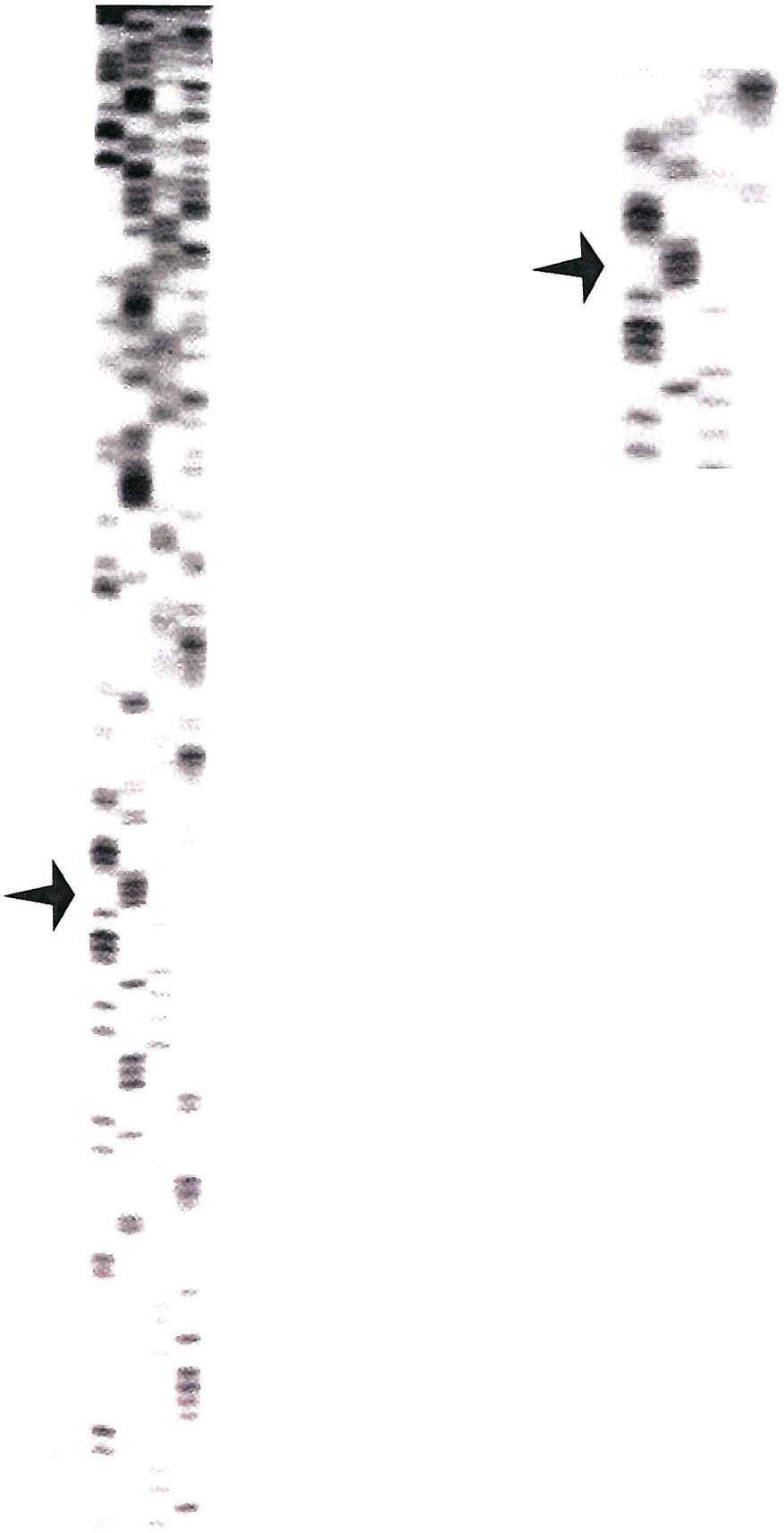


Figure 3.4 11q mutation

Direct sequencing of leucocyte DNA from patient 134 revealed an 11qdel4 3875 mutation as marked by the arrow.

Figure 3.4 11q mutation shown by direct sequencing

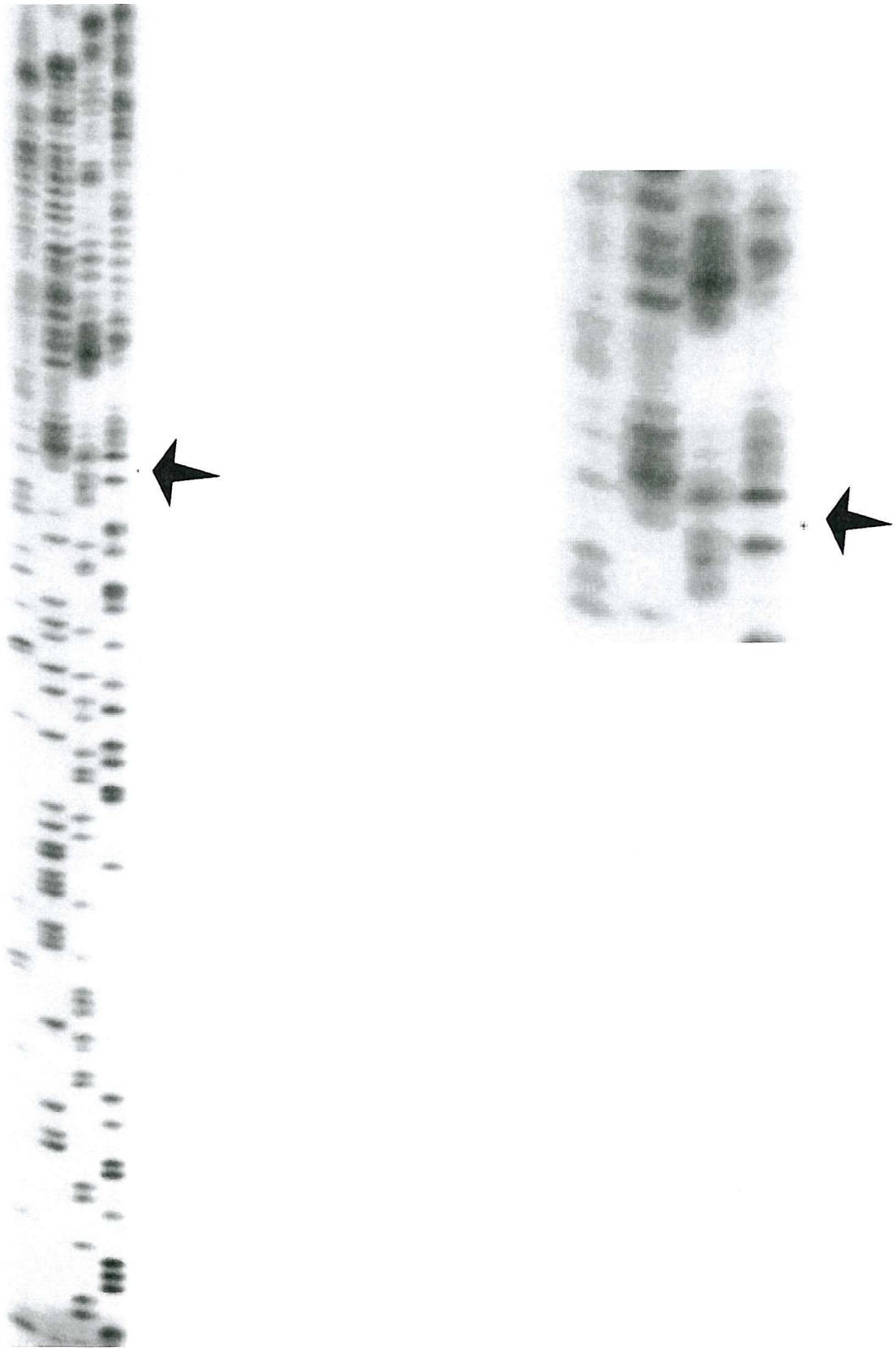


Table 3.4 Previous studies

Investigators	No. mutations/No. patients	% mutations	Information re mutations	Reference
Futreal et al	1/12	8.3	Mutation seen in patient with early onset disease	Science 266: 120-122, 1994
Hosking et al	1/17	5.9	Single base substitution in exon 4	Nature Genet 9:343-344, 1995
Matsushima et al	4/76	5.3	Two patients with relevant family history	Hum Mol Genet 4:1953, 1995
Merajver et al	1/47	2.0	Missense mutation	Nature Genet 9:439-443, 1995
Takahashi et al	6/115	5.2	Five patients with relevant family history -4 frameshift, 1 missense mutation Other mutation frameshift	Cancer Res 55:2998-3002, 1995
Stratton et al	13/374	3.5	Nine patients with relevant family history Six novel mutations Twelve mutations resulting in truncated protein product; one in-frame deletion just outside the putative zinc-finger domain	NEJM 336:1125-1130, 1997
Rubin et al	10/116	8.6	One missense mutation; all others frameshift	Am J Obstet Gynecol 178:670-677, 1998

3.5 DISCUSSION

This study is the second largest reported, and has, as described, revealed 3 germline exon 11 BRCA1 mutations. Two of our cases were serous ovarian tumours and the other an endometrioid lesion. None of the other studies quoted has demonstrated mutations in endometrioid ovarian cancer. All the cases described by Takahashi were serous, as were 12 of the 13 reported by Stratton. The other patient with a mutation from Stratton's study had a borderline mucinous tumour (the fifth recorded case of a borderline tumour in a BRCA1 mutation carrier, and the second reported case of a mucinous tumour). The individual in question had a family history of breast cancer in a paternal aunt, and had an exon 11 mutation. Several epidemiological and linkage studies describe extreme underrepresentation of mucinous tumours in all types of familial ovarian cancer (Greggi *et al*, 1990; Bewtra *et al*, 1992; Piver *et al*, 1993a; Narod *et al*, 1994; Narod *et al*, 1995a; Narod *et al*, 1995b). The individual in our study with an endometrioid tumour and demonstrable BRCA1 mutation had no known relevant family history.

Within our study, the frequency of mutations detected was 4.1% for serous malignant and 2.4% for endometrioid malignant lesions. However, this is likely to be an underestimate for several reasons:

1. The techniques used for mutation analysis are not 100% sensitive. Stratton *et al* estimated the overall sensitivity of heteroduplex analysis for the detection of coding alterations to be 70%, and calculated that the true proportion of ovarian cancers attributable to BRCA1 mutations would be 5% (95% CI 3-8%). This is consistent with the prevalence estimated by Ford *et al (*1995b*)*.
2. Even amongst women with ovarian or breast cancer clearly linked to BRCA1 mutations, approximately 20% will have no detectable coding sequence alterations (Castilla *et al*, 1994; Friedman *et al*, 1995; Miki *et al*, 1994; Simard *et al*, 1994).
3. Mutations outside the region screened will not have been detected.

This study has subsequently been extended using primers to examine the whole of exon 11 of BRCA1, in addition to the 'priority' BRCA1 primers described previously. However, other BRCA1 exons have not been examined. This work has been carried out by Mrs P Englefield, University Department Obstetrics and Gynaecology, Princess

Anne Hospital, Southampton. No additional mutations have been detected amongst those individuals for whom sufficient DNA was available to carry out further analysis - a subgroup of 123 patients (Table 3.5). The number of patients included in the study has also been extended by a further 61 (Table 3.6). These are, however, not consecutive cases, and comprise predominantly patients with serous malignant tumours. Amongst these additional individuals, two have been shown to carry mutations. Patient 560 exhibited a 4bp deletion of exon 11q, whilst patient 565 was found to have an 11p/11q mutation. Two further patients (22 and 67) exhibited unclassified variants (it is unclear whether these represent disease causing mutations or polymorphisms (non-pathological sequence variants)). For patient 22, the variant was in exon 11a (855 TTG to GTG at codon 246). For patient 67, an exon 11e variant was detected (1606 CGT to CAT at codon 496).

Of note is that, in the studies described, few somatic mutations of BRCA1 were detected (Stratton's study and our own were only analysing for germline mutations). This is surprising given the high frequency of 17q LOH seen in ovarian cancer. It is probable that 17q LOH does not reflect somatic inactivation of BRCA1, and that the presence of other TSG's on 17q may be contributory. Fine deletion mapping on chromosome 17q in ovarian cancer is not straightforward, as the whole chromosome arm is usually lost in tumours showing LOH. However, there have been reported ovarian cancer cases with partial deletions, implicating distinct regions distal to BRCA1 as harbouring presumptive TSG's (Jacobs *et al*, 1993; Saito *et al*, 1993; Godwin *et al*, 1994).

We thus conclude that BRCA1 mutations are unlikely to play a major role in the pathogenesis of 'sporadic' ovarian tumours, although fuller analysis of other exons is required to confirm this.

Table 3.5 Patients screened for priority primers + rest of exon 11

CASE NO.	HISTOLOGY	CASE NO.	HISTOLOGY
1	Benign serous cystadenofibroma	34	Serous cystadenocarcinoma + benign serous cystadenoma
2	Borderline serous tumour	35	Serous cystadenocarcinoma
5	Benign serous cyst	36	Endometrioid adenocarcinoma
7	Benign serous cystadenoma	37	Endometrioid adenocarcinoma
10	Benign serous cystadenofibroma	38	Endometrioid + clear cell carcinoma
11	Benign mucinous cystadenoma	42	Serous cystadenocarcinoma
13	Benign mucinous cystadenoma	43	Serous cystadenocarcinoma
14	Serous cystadenocarcinoma	45	Serous cystadenocarcinoma
15	Benign mucinous cystadenoma	52	Serous cystadenocarcinoma
16	Serous adenocarcinoma	53	Adenocarcinoma
18	Serous adenocarcinoma	54	Endometrioid carcinoma
19	Serous adenocarcinoma	55	Endometrioid carcinoma
20	Borderline serous tumour	56	Adenocarcinoma
22	Serous adenocarcinoma	57	Serous adenocarcinoma
23	Serous cystadenocarcinoma	58	Cystadenocarcinoma
24	Benign serous cystadenoma	59	Endometrioid carcinoma
25	Adenocarcinoma	63	Serous adenocarcinoma
26	Serous adenocarcinoma	64	Endometrioid carcinoma
27	Serous cystadenocarcinoma	66	Serous adenocarcinoma
28	Serous adenocarcinoma	67	Serous cystadenocarcinoma
29	Mucinous cystadenocarcinoma	70	Endometrioid + clear cell carcinoma
30	Endometrioid + mucinous carcinoma	72	Adenocarcinoma
31	Serous cystadenocarcinoma	73	Endometrioid carcinoma

32	Serous cystadenocarcinoma		**74**	Cystadenocarcinoma
81	Endometrioid carcinoma		**165**	Adenocarcinoma
86	Serous adenocarcinoma		**166**	Serous cystadenocarcinoma
87	Adenocarcinoma		**182**	Adenocarcinoma
89	Adenocarcinoma		**184**	Adenocarcinoma
91	Serous adenocarcinoma		**185**	Serous cystadenocarcinoma
93	Adenocarcinoma		**188**	Serous cystadenocarcinoma
97	Serous cystadenocarcinoma		**189**	Endometrioid carcinoma
99	Adenocarcinoma		**190**	Serous cystadenocarcinoma
100	Endometrioid carcinoma		**198**	Adenocarcinoma
101	Cystadenocarcinoma		**199**	Adenocarcinoma
105	Endometrioid carcinoma		**201**	Serous adenocarcinoma
106	Clear cell carcinoma		**204**	Serous cystadenocarcinoma
108	Endometrioid + mucinous carcinoma		**207**	Adenocarcinoma
109	Endometrioid carcinoma		**210**	Adenocarcinoma
110	Serous cystadenocarcinoma		**211**	Endometrioid carcinoma
118	Endometrioid carcinoma		**213**	Serous cystadenocarcinoma
119	Serous cystadenocarcinoma		**215**	Serous adenocarcinoma
122	Serous cystadenocarcinoma		**216**	Serous adenocarcinoma
123	Endometrioid carcinoma		**217**	Endometrioid carcinoma
127	Serous cystadenocarcinoma		**221**	Adenocarcinoma
131	Serous cystadenocarcinoma		**222**	Serous adenocarcinoma
134	Serous cystadenocarcinoma		**223**	Endometrioid carcinoma
139	Serous cystadenocarcinoma		**224**	Clear cell carcinoma
141	Serous adenocarcinoma		**228**	Serous cystadenocarcinoma
150	Endometrioid carcinoma		**230**	Serous cystadenocarcinoma
161	Cystadenocarcinoma		**231**	Serous cystadenocarcinoma
162	Adenocarcinoma		**232**	Cystadenocarcinoma

163	Serous adenocarcinoma		**233**	Endometrioid carcinoma
234	Cystadenocarcinoma		**250**	Serous adenocarcinoma
235	Endometrioid carcinoma		**251**	Adenocarcinoma
237	Endometrioid carcinoma		**255**	Endometrioid carcinoma
238	Adenocarcinoma		**256**	Cystadenocarcinoma
239	Adenocarcinoma		**264**	Serous cystadenocarcinoma
240	Endometrioid carcinoma		**267**	Endometrioid carcinoma
241	Adenocarcinoma		**272**	Clear cell carcinoma
242	Endometrioid carcinoma		**274**	Clear cell carcinoma
247	Cystadenocarcinoma		**282**	Serous cystadenocarcinoma
248	Adenocarcinoma			

* Case numbers used are Princess Anne Hospital identification numbers

Table 3.6 Additional patients subsequently studied for the BRCA1 mutation screening study

CASE NO.	DOB.	AGE AT DIAGNOSIS (YEARS)	TUMOUR HISTOLOGY	TUMOUR STAGE (FIGO)	TUMOUR GRADE
302	22/05/31	63	Serous adenocarcinoma	-	2
313	25/07/20	74	Serous cystadenocarcinoma	IC	2-3
314	2/03/21	74	Papillary adenocarcinoma	-	-
315	20/03/13	82	Serous cystadenocarcinoma	II	3
316	13/10/51	43	Endometrioid carcinoma	III	3
318	28/04/25	70	Serous cystadenocarcinoma	III	3
319	15/01/42	53	Serous cystadenocarcinoma	I	2
323	23/04/41	54	Serous adenocarcinoma	II	2
325	5/10/31	63	Serous cystadenocarcinoma	-	-
326	21/02/17	78	Serous cystadenocarcinoma	IIIA	2
328	31/03/29	66	Serous cystadenocarcinoma	-	2
329	25/09/28	67	Serous adenocarcinoma	IV	3
331	2/08/29	66	Serous adenocarcinoma	III	3
332	30/09/41	54	Serous adenocarcinoma	I	2
338	10/11/26	69	Serous cystadenocarcinoma	I	2
341	21/07/33	62	Serous adenocarcinoma	IC	2
349	3/09/57	38	Clear cell adenocarcinoma	IA	2
365	19/04/38	57	Serous adenocarcinoma	IV	2
376	7/11/34	61	Serous cystadenocarcinoma	II	2-3
378	19/02/27	69	Serous adenocarcinoma	I	2
382	6/09/37	58	Serous cystadenocarcinoma	III	3
407	18/01/13	83	Serous cystadenocarcinoma	III	2

413	12/04/60	36	Serous cystadenocarcinoma	III	3
427	25/01/30	66	Serous cystadenocarcinoma	III	2
436	21/08/47	49	Serous adenocarcinoma	III	2
454	21/12/15	81	Serous cystadenocarcinoma	IIIC	3
456	9/04/39	58	Serous adenocarcinoma	III	3
462	27/10/74	22	Serous adenocarcinoma	III	1
469	18/06/26	71	Serous cystadenocarcinoma	III	3
471	10/07/14	83	Serous cystadenocarcinoma	-	2
472	15/01/33	64	Serous cystadenocarcinoma	IC	2
474	11/05/41	56	Serous adenocarcinoma	IC	2
482	5/07/24	73	Serous cystadenocarcinoma	II	1
484	27/03/38	59	Serous cystadenocarcinoma	III	3
487	12/05/56	41	Serous cystadenocarcinoma	III	1
489	22/12/55	41	Serous adenocarcinoma	III	3
491	30/04/45	52	Serous cystadenocarcinoma	IV	3
492	1/04/44	53	Serous cystadenocarcinoma	III	2
493	12/10/36	61	Serous cystadenocarcinoma	III	2
498	13/01/17	80	Serous adenocarcinoma	III	2
499	19/04/48	49	Serous adenocarcinoma	III	-
501	1/04/35	62	Papillary adenocarcinoma		
509	11/08/32	65	Serous cystadenocarcinoma	III	2
515	15/12/31	66	Serous cystadenocarcinoma	IV	1
517	26/09/32	65	Serous cystadenocarcinoma	IIIC	2
519	8/11/34	63	Serous adenocarcinoma	IC	1-2
520	20/04/42	56	Serous adenocarcinoma	III	3
522	11/09/62	35	Endometrioid carcinoma	III	3-4
524	16/02/33	65	Serous adenocarcinoma	IIIC	3
542	13/11/30	67	Serous adenocarcinoma	III	2

546	8/11/57	40	Carcinoma	I	3
548	19/05/37	61	Serous cystadenocarcinoma	III	1
549	23/04/56	42	Clear cell carcinoma	-	3
551	4/10/43	54	Serous adenocarcinoma	III	3
560	8/10/46	52	Serous adenocarcinoma	IV	2-3
565	8/01/35	63	Serous cystadenocarcinoma	II	2-3
571	17/06/46	52	Serous cystadenocarcinoma	-	2-3
574	20/06/23	75	Serous adenocarcinoma	-	2
575	21/04/36	62	Serous adenocarcinoma	IV	2-3
576	31/10/68	30	Endometrioid carcinoma	I	1
583	30/08/52	46	Serous adenocarcinoma	IIIC	-

*The additional series shown does not include any individuals with mucinous histology and is, therefore, not consecutive

* Case numbers used are Princess Anne Hospital identificaton numbers

CHAPTER FOUR

GENETIC ANALYSIS OF ABERRANT HISTOLOGICAL STRUCTURES IN PROPHYLACTIC OOPHORECTOMY SPECIMENS FROM WOMEN WITH GERMLINE BRCA1 AND BRCA2 MUTATIONS

4.1 INTRODUCTION

This part of my study was prompted by the findings of Salazar *et al (*1996*)* who reported a significantly higher incidence of atypical features in the prophylactically removed ovaries of BRCA1 mutation carriers when compared with a control population. In 85% of the 20 cases examined by Salazar et al, two or more atypical features were seen; 75% of cases revealed three or more such features. These included epithelial inclusion cysts, microscopic papillary cystadenomas, cortical stromal hyperplasia, surface epithelial pseudostratification, surface papillomatosis, and corpus luteum hyperplasia. In two of his cases, microscopic malignancies were described. Cortical superficial inclusion cysts were seen in 70%, compared with 25% of control subjects. These were of variable shape and size, and were lined with pseudostratified epithelium of either a serous or tubal type. Most had no contents within the space, and in some cases groups of cysts were seen together, giving the appearance of microscopic cystadenomas or adenofibromas.

This study raised the possibility of a histological pre-malignant phenotype in the ovaries of BRCA1 mutation carriers. If the atypical features documented by Salazar could be shown to exhibit DNA deletions, the concept that ovarian cancers develop via an adenoma/adenocarcinoma route, similar to that described for colorectal tumours, would be supported. This could have a significant impact on the diagnosis and management of ovarian tumours, by facilitating the identification of genetic events initiating tumour development. If a series of morphological precursors of clinical ovarian cancer could be defined, studies with the currently available molecular biologic strategies could help clarify which of the many genetic alterations seen in late stage disease are causal.

4.2 AIMS OF THE STUDY

The aim of this part of my study was to identify BRCA1 and BRCA2 carriers who had undergone prophylactic oophorectomy, to review their ovarian tissue for atypical features, to extract DNA from atypical areas, and, using microsatellite PCR, to analyse for loss of heterozygosity (LOH) at candidate loci on all chromosome arms. We hoped to be able to show loss of heterozygosity consistent with that seen in later lesions (ie. benign, borderline, and malignant ovarian tumours).

4.3 STUDY METHODOLOGY

The BRCA1/BRCA2 Clinical Genetics Service in Southampton has resulted in identification of mutations in 17 unrelated families between 1995 and 1997. Family members are offered predictive testing, and, as a result of this, 8 women have elected to undergo prophylactic oophorectomy.

For my study, paraffin-embedded ovarian tissue was available from prophylactic oophorectomy specimens from six women, five of whom had BRCA1 and one a BRCA2 mutation. Patient details are documented in Table 4.1, with family pedigrees shown in Appendices 2-6 (no pedigree was available for patient JL, whose surgery was performed in the private sector, but her mother and sister are both known to have died from ovarian cancer).

Atypical features were identified by a specialist gynaecological pathologist (Dr N Singh, Southampton General Hospital). Most sections revealed inclusion cysts, whilst, for one individual, there were also multiple areas of microscopic malignancy. In order to use LOH analysis for detection of genetic alterations, it was essential to remove as much normal tissue from the specimens as possible prior to DNA extraction. Therefore, a tissue microdissection technique was used to dissect areas of interest from surrounding tissue, as described in section 2.2.1. Cells were microdissected from five consecutive 10μm sections in each case; matching normal cells were obtained from other areas of the same section. Examples of lesions dissected are shown in Figures 4.1-4.4. DNA was extracted from dissected tissue and leucocytes for each individual (for one individual no leucocyte DNA was available). Following PEP pre-amplification of the extracted DNA,

LOH analysis using microsatellite PCR was carried out with 50 markers, as shown in Table 2.12. The reliability of PCR results was tested by always repeating the reactions a second time.

Table 4.1 Patient details

PATIENT ID	DOB	DETAILS OF MUTATION	AGE AT PROPHYLACTIC OOPHORECTOMY	HISTOLOGY FROM OOPHORECTOMY	PREVIOUS HISTORY OF BREAST SURGERY
RF	17/08/44	BRCA1 Exon 11q 3875 del 4 1111 stop @ 1262	49 years	Bilateral microscopic serous malignancies	Benign breast disease at 34yr
HJ	9/06/47	BRCA1 Exon 11q 3875 del 4 1111 stop@ 1262	48 years	Atrophic ovaries	Bilateral mastectomies at 38yr and 43yr Invasive carcinoma + DCIS age 38yr (R) Invasive ductal carcinoma age 43yr (L)
JL	9/12/45	BRCA1	51 years	Serous inclusion cysts	-
MR	13/07/49	BRCA1 Exon 11r 4185 del AG	48 years	Surface inclusion cysts No malignancy	Medullary carcinoma age 35yr
CB	25/08/49	BRCA1 Exon 12 G to A transition at 4304	44 years		Bilateral mastectomies age 47yr Invasive ductal carcinoma (R); prophylactic (L)
SS	5/02/50	BRCA2 Exon 11f ins A 2950	46 years	Bilateral leiomyomata	Bilateral mastectomies at 30yr and 41yr Invasive ductal carcinoma age 30yr (L) Medullary carcinoma age 41yr (R)

Figure 4.1 Examples of inclusion cysts microdissected

Haematoxylin-eosin (H&E) stained formalin fixed paraffin embedded tissue sections

RFIC1/RFIC3 - inclusion cysts 1 and 3 from patient RF

JLIC - inclusion cyst from patient JL

CBIC1/CBIC2 - inclusion cysts 1 and 2 from patient CB

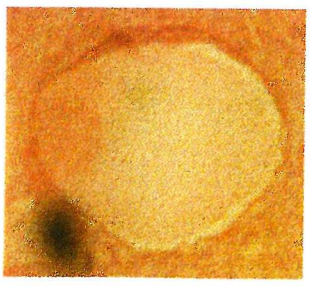
Figure 4.1 Examples of inclusion cysts microdissected

RFIC1

RFIC3

JLIC

CBIC1



CBIC2

Figure 4.2 Areas of microscopic malignancy microdissected

H&E stained formalin fixed paraffin embedded tissue sections

RFT1-T9 - microscopic malignancy samples 1 to 9

Figure 4.2 Areas of microscopic malignancy microdissected

RFT1

RFT2

RFT3

RFT4

RFT5

RFT6

RFT7

RFT8

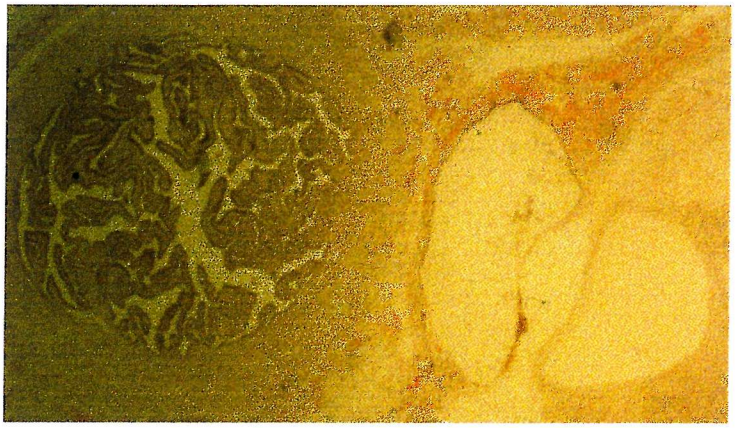
RFT9

Figure 4.3 Inclusion cyst before and after microdissection

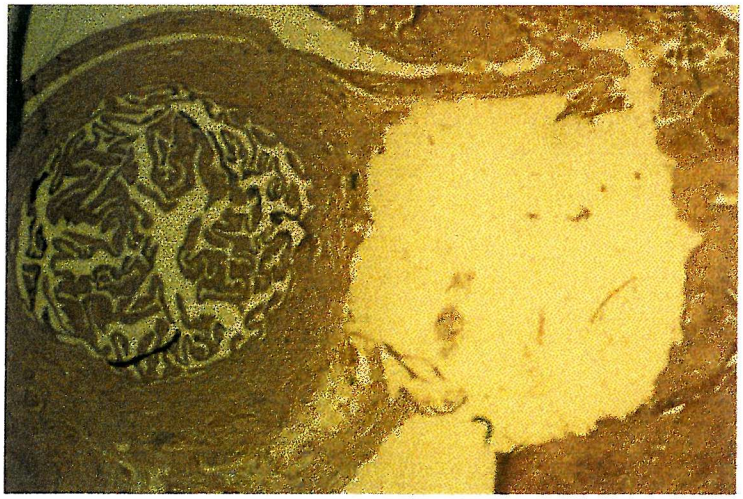
H&E stained formalin fixed paraffin embedded tissue sections from patient SS (BRCA2 mutation carrier)

SSIC1 - inclusion cyst 1 from patient SS

Figure 4.3 Inclusion cyst before and after microdissection



SSIC1 before



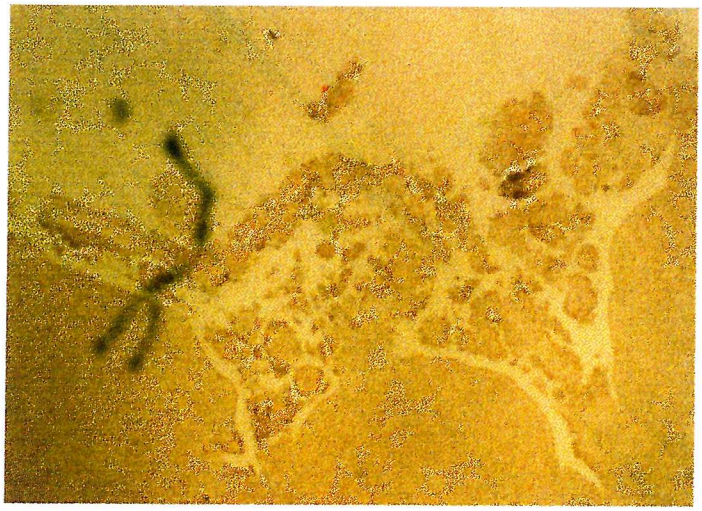
SSIC1 after

Figure 4.4 Area of microscopic malignancy before and after microdissection

H&E stained formalin fixed paraffin embedded tissue sections

RFT4 - microscopic malignancy sample 4 from patient RF

Figure 4.4 Area of microscopic malignancy before and after microdissection



RFT4 before

RFT4 after

4.4 RESULTS

Data obtained from a loss of heterozygosity analysis using 50 microsatellite markers spanning all chromosome arms is recorded in Table 4.2.

Loss of heterozygosity in inclusion cyst DNA was seen with at least one microsatellite marker for three of the five BRCA1 mutation carriers (patients RF, MR, and CB) (see Tables 4.2 and 4.3). Inclusion cyst DNA from the BRCA2 mutation carrier (patient SS) revealed no LOH with any of the 50 microsatellite markers studied. This was true for DNA from two separate inclusion cysts from this individual. Amongst those samples showing LOH, loci on chromosomes 2p, 5p, 5q, 6q, 8q, 9p, 11q, 13q, 15q, 17q, and Xq were affected (see Table 4.4), with the highest frequencies being on 5p, 6q, 11q, 13q, 15q, and 17q (all>25%). Nine sample areas of microscopic malignancy, in addition to four inclusion cysts, were examined for patient RF. LOH was seen with at least one marker in all except one of the malignant samples (see Table 4.5). For these areas of microscopic malignancy, LOH was again seen at various chromosomal loci on 2p, 5q, 6q, 7q, 8q, 9p, 9q, 13q, 15q, and 17q. Frequencies of >25% were seen for markers on 9p, 9q, 15q, and 17q. Allelic losses observed can be seen in Figures 4.5-4.9.

Areas of tissue with 'normal' microscopic appearance were also dissected for each individual included in the study, for comparison with PCR results from their leucocyte DNA. One would not expect to see LOH amongst these samples. All samples except three matched the results seen with leucocyte DNA. However, apparent LOH was seen in the following cases: HJ 'normal' DNA with marker D13S128 (chromosome 13q), CB 'normal' DNA with D13S128, and MR 'normal' DNA with D21S1432 (chromosome 21q) (see Figure 4.10 for examples). MR 'normal' DNA also showed apparent microsatellite instability (MI) with three markers [D6S287 (6q), D7S691 (7p), and D9S171 (9p)] (see Table 4.6 and Figure 4.11).

Table 4.2 Loss of heterozygosity analysis (1)

MARKER

	D1S504 (1q22-23) **124-138 bp**	**D2S391** (2p21-16) **142-152 bp**	**D3S1582** (3p21.2-21.1) **154-178 bp**	**D3S1600** (3p14.2-14.1) **182-198 bp**	**D3S196** (3q21.3-25.2) **86-98 bp**	**D4S175** (4q21-qter) **112-134 bp**	**D5S406** (5p15.3-14) **160-186 bp**	**D5S424** (5q13.3) **116-146 bp**	**D5S644** (5q13.3-21.1) **81-101 bp**	**D5S346** (5q21-22) **96-122 bp**
PATIENT ID										
RF T1	het	het	ni	het	het	dnw	adm	het	het	het
RF T2	het	het	ni	het	het	het	adm	het	het	het
RF T3	het	dnw	ni	het	het	dnw	adm	het	het	het
RF T4	het	dnw	ni	het	het	het	adm	het	het	het
RF T5	het	het	ni	dnw	het	dnw	adm	het	het	loh(2)
RF T6	het	het	ni	het	het	het	adm	het	het	het
RF T7	het	het	ni	het	het	het	adm	dnw	het	het
RF T8	het	loh(1)	ni	dnw	het	het	adm	dnw	het	het
RF T9	het	het	ni	het	het	het	adm	het	het	het
RF IC1	het	het	ni	het	het	dnw	adm	het	het	het
RF IC2	het	het	ni	het	het	het	adm	het	het	het
RF IC3	dnw	dnw	ni	het	dnw	het	adm	dnw	het	dnw
RF IC4	het	loh(1)	mi	het	het	het	adm	het	het	het
HJ IC	het	het	het	het	het	dnw	ni	het	het	het
JL IC	het	het	ni	het	ni	dnw	ni	het	het	het
MR IC	dnw	ni	dnw	dnw	het	dnw	ni	dnw	dnw	dnw
CB IC1	het	dnw	dnw	dnw	ni	dnw	het	dnw	dnw	dnw
CB IC2	het	dnw	het	het	ni	het	loh(1)	het	dnw	het
SS IC1	het	het	het	het	het	dnw	het	het	het	het
SS IC2	het	het	het	het	het	het	het	het	het	het

het = retention of constitutional heterozygosity
loh(1) = loss of larger allele
loh(2) = loss of smaller allele
ni = non-informative due to constitutional homozygosity

adm = alleles don't match (when different samples from an individual compared)
mi = microsatellite instability
dnw = PCR did not work

RF; HJ; JL; MR; CB = BRCA1 carriers
SS = BRCA2 carrier

MARKER

	D5S399 (5q22.3-31) **116-132 bp**	**D6S284** (6q13-15) **233-251 bp**	**D6S287** (6q21-23.3) **143-171 bp**	**D6S1027** (6q25.3-27) **117-138 bp**	**D7S654** (7p22.3-21.3) **207-227 bp**	**D7S691** (7p14.2-12.2) **128-146 bp**	**D7S670** (7p13) **100-110 bp**	**D7S672** (7q11.1-21.1) **132-160 bp**	**D7S491** (7q21-22) **115-131 bp**	**D7S633** (7q31) **168-182 bp**
PATIENT ID										
RF T1	het	ni	het	het	adm	ni	het	dnw	het	dnw
RF T2	het	ni	het	het	adm	ni	het	dnw	het	het
RF T3	het	ni	loh(2)	het	adm	ni	het	dnw	het	het
RF T4	het	ni	het	loh(2)	adm	ni	het	dnw	het	het
RF T5	het	ni	dnw	loh(1)	adm	ni	het	dnw	het	dnw
RF T6	het	ni	dnw	het	adm	ni	het	dnw	loh(1)	dnw
RF T7	het	ni	dnw	het	adm	ni	het	dnw	het	het
RF T8	het	ni	het	het	adm	ni	het	dnw	het	dnw
RF T9	het	ni	het	het	adm	ni	het	dnw	het	het
RF IC1	het	ni	het	het	adm	ni	het	dnw	het	dnw
RF IC2	het	ni	het	het	adm	ni	het	dnw	het	het
RF IC3	het	ni	dnw	loh(2)	adm	ni	het	dnw	het	dnw
RF IC4	het	ni	mi	loh(2)	adm	ni	het	dnw	het	het
HJ IC	het	ni	ni	het	het	het	het	het	het	het
JL IC	het	het	ni	het	ni	het	het	ni	het	het
MR IC	loh(1)	ni	mi	dnw	ni	mi	het	ni	ni	dnw
CB IC1	het	dnw	ni	dnw	dnw	ni	het	dnw	dnw	dnw
CB IC2	het	het	ni	dnw	dnw	ni	het	het	het	het
SS IC1	het	ni	ni	het	het	ni	het	het	ni	het
SS IC2	het	ni	ni	het	het	ni	het	het	ni	het

het = retention of constitutional heterozygosity
loh(1) = loss of larger allele
loh(2) = loss of smaller allele
ni = non-informative due to constitutional homozygosity

adm = alleles don't match (when different samples from an individual compared)
mi = microsatellite instability
dnw = PCR did not work

RF; HJ; JL; MR; CB = BRCA1 carriers
SS = BRCA2 carrier

MARKER

	D8S258 (8p23-22) **144-154 bp**	**D8S543** (8q12-13) **116-140 bp**	**D9S171** (9p21) **159-177 bp**	**D9S161** (9p21) **119-135 bp**	**D9S127** (9q31) **149-159 bp**	**D10S89** (10p15.1-13) **142-156 bp**	**D10S1696** (10q21.3-23.3) **124-136 bp**	**D10S215** (10q22-23) **152-208 bp**	**D11S902** (11p15-13) **145-163 bp**	**D11S912** (11q23) **101-123 bp**
PATIENT ID										
RF T1	het	het	het	het	het	dnw	het	ni	het	dnw
RF T2	het	het	het	het	het	dnw	het	ni	het	dnw
RF T3	dnw	het	loh(1)	het	loh(1)	dnw	het	ni	dnw	dnw
RF T4	het	het	het	het	het	het	het	ni	het	dnw
RF T5	dnw	het	dnw	het	het	dnw	dnw	ni	het	dnw
RF T6	het	het	loh(2)	het	loh(1)	dnw	dnw	ni	het	dnw
RF T7	het	het	mi	het	dnw	dnw	dnw	ni	het	dnw
RF T8	het	loh(1)	het	dnw	mi	dnw	dnw	ni	het	dnw
RF T9	het	het	het	het	het	dnw	dnw	ni	het	dnw
RF IC1	het	het	het	het	dnw	dnw	het	ni	het	dnw
RF IC2	het	het	het	het	het	dnw	het	ni	het	dnw
RF IC3	dnw	dnw	dnw	het	dnw	dnw	dnw	ni	dnw	dnw
RF IC4	het	loh(1)	loh(1)	het	het	dnw	dnw	ni	het	dnw
HJ IC	het	het	ni	het	ni	het	het	ni	het	ni
JL IC	het	het	het	het	ni	het	het	het	ni	het
MR IC	dnw	dnw	mi	dnw	dnw	dnw	dnw	ni	dnw	dnw
CB IC1	dnw	dnw	ni	het	dnw	dnw	ni	dnw	het	dnw
CB IC2	het	het	ni	loh(1)	het	dnw	ni	het	het	dnw
SS IC1	het	het	ni	ni	ni	het	het	ni	het	het
SS IC2	het	het	ni	ni	ni	het	het	ni	het	het

het = retention of constitutional heterozygosity
loh(1) = loss of larger allele
loh(2) = loss of smaller allele
ni = non-informative due to constitutional homozygosity

adm = alleles don't match (when different samples from an individual compared)
mi = microsatellite instability
dnw = PCR did not work

RF; HJ; JL; MR; CB = BRCA1 carriers
SS = BRCA2 carrier

MARKER

	D11S1336 (11q23-24.2) **232-252 bp**	**D11S969** (11q24.1-25) **141-149 bp**	**D12S356** (12p13) **205-225 bp**	**D12S385** (12pter-qter) **125-151 bp**	**D13S133** (13q14.3) **130-187 bp**	**D13S128** (13q32-34) **144-178 bp**	**D14S80** (14q12-13) **132-160 bp**	**FES** (15q26.1) **143-163 bp**	**D16S3072** (16pter-qter) **148-168 bp**	**D17S786** (17p13.1) **135-157 bp**
PATIENT ID										
RF T1	adm	het	adm	ni	het	dnw	dnw	loh(1)	het	adm
RF T2	adm	het	adm	ni	het	het	dnw	loh(1)	het	adm
RF T3	adm	het	adm	ni	dnw	dnw	dnw	loh(2)	dnw	adm
RF T4	adm	het	adm	ni	dnw	het	dnw	loh(1)	het	adm
RF T5	adm	het	adm	ni	dnw	dnw	dnw	loh(1)	het	adm
RF T6	adm	dnw	adm	ni	het	dnw	dnw	het	dnw	adm
RF T7	adm	dnw	adm	ni	het	dnw	dnw	het	het	adm
RF T8	adm	dnw	adm	ni	loh(1)	dnw	dnw	het	dnw	adm
RF T9	adm	het	adm	ni	het	het	dnw	het	het	mi
RF IC1	adm	het	adm	ni	het	dnw	dnw	loh(1)	het	adm
RF IC2	adm	het	adm	ni	het	loh(1)	dnw	loh(1)	het	adm
RF IC3	adm	het	adm	ni	dnw	dnw	dnw	loh(1)	dnw	adm
RF IC4	adm	het	adm	ni	het	loh(2)	dnw	loh(2)	het	adm
HJ IC	het	ni	ni	het	het	het	ni	het	het	het
JL IC	het	ni	ni	ni	het	ni	het	het	het	het
MR IC	loh(1)	ni	mi	dnw	dnw	dnw	dnw	het	het	het
CB IC1	loh(1)	ni	dnw	ni	het	dnw	ni	dnw	dnw	het
CB IC2	het	ni	het	ni	het	loh(1)	ni	het	het	het
SS IC1	ni	het	ni	ni	het	ni	het	ni	het	ni
SS IC2	ni	het	ni	ni	het	ni	dnw	ni	het	ni

het = retention of constitutional heterozygosity
loh(1) = loss of larger allele
loh(2) = loss of smaller allele
ni = non-informative due to constitutional homozygosity

adm = alleles don't match (when different samples from an individual compared)
mi = microsatellite instability
dnw = PCR did not work

RF; HJ; JL; MR; CB = BRCA1 carriers
SS = BRCA2 carrier

MARKER

	D17S855 (17q21.1) 143-155 bp	D17S1322 (17q21) 121-139 bp	D17S1323 (17q21) 151-161 bp	D17S1327 (17q21) 128-152 bp	D18S59 (18pter-11.22) 148-164 bp	D18S474 (18pter-qter) 119-139 bp	D19S886 (19pter-qter) 142-158 bp	D20S851 (20pter-qter) 128-150 bp	D21S1432 (21q21) 139-151 bp	HPRT (Xq26.1) 151-167 bp
PATIENT ID										
RF T1	het	het	ni	loh(1)	het	het	het	dnw	het	het
RF T2	het	het	ni	het	het	het	het	het	het	het
RF T3	het	loh(1)	ni	loh(1)	dnw	het	het	het	het	het
RF T4	loh(1)	het	ni	het	het	het	het	het	het	het
RF T5	dnw	het	ni	het	dnw	het	dnw	het	het	het
RF T6	het	loh(1)	ni	mi	het	het	dnw	het	het	het
RF T7	het	loh(1)	ni	loh(1)	dnw	het	dnw	het	het	het
RF T8	het	het	ni	dnw	dnw	het	dnw	het	het	het
RF T9	het	het	ni	het	het	het	dnw	het	het	het
RF IC1	het	loh(1)	ni	loh(1)	het	het	het	dnw	het	het
RF IC2	het	loh(1)	ni	het	het	het	het	het	het	het
RF IC3	het	het	ni	loh(1)	dnw	het	dnw	het	dnw	dnw
RF IC4	loh(1)	loh(1)	ni	het	dnw	het	het	het	het	dnw
HJ IC	het	het	het	het	het	het	het	het	mi	het
JL IC	het	het	het	het	het	het	het	ni	het	het
MR IC	het	het	ni	ni	dnw	dnw	dnw	het	dnw	loh(1)
CB IC1	het	dnw	het	ni	dnw	dnw	dnw	dnw	dnw	ni
CB IC2	het	het	het	ni	dnw	het	het	het	het	ni
SS IC1	het	het	ni	ni	het	het	het	het	het	ni
SS IC2	dnw	het	ni	ni	het	het	het	het	het	ni

het = retention of constitutional heterozygosity
loh(1) = loss of larger allele
loh(2) = loss of smaller allele
ni = non-informative due to constitutional homozygosity

adm = alleles don't match (when different samples from an individual compared)
mi = microsatellite instability
dnw = PCR did not work

RF; HJ; JL; MR; CB = BRCA1 carriers
SS = BRCA2 carrier

Table 4.3 Loss of heterozygosity analysis (2) - Number of markers showing LOH for each lesion analysed

PATIENT ID	NUMBER OF MARKERS SHOWING LOH
RF T1	2
RF T2	1
RF T3	6
RF T4	3
RF T5	3
RF T6	4
RF T7	2
RF T8	3
RF T9	0
RF IC1	3
RF IC2	3
RF IC3	3
RF IC4	8
HJ IC	0
JL IC	0
MR IC	3
CB IC1	1
CB IC2	3
SS IC1	0
SS IC2	0

T = area of microscopic malignancy

IC = inclusion cyst

Table 4.4 Loss of heterozygosity analysis (3) - Markers for which LOH detected

MARKER	CHROMOSOME LOCATION	INCLUSION CYSTS WITH LOH (n=11) [no/no informative]	% LOH	MALIGNANCY WITH LOH (n=9) [no/no informative]	% LOH	TOTAL SAMPLES WITH LOH (n=20)
D2S391	2p21-16	1/7	14.3	1/6	16.7	2
D5S406	5p15.3-14	1/4	25.0	0/0	0	1
D5S346	5q21-22	0/8	0	1/9	11.1	1
D5S399	5q22.3-31	1/11	9.1	0/9	0	1
D6S287	6q21-23.3	0/4	0	1/6	16.7	1
D6S1027	6q25.3-27	2/8	25.0	2/9	22.2	4
D7S491	7q21-22	0/7	0	1/9	11.1	1
D8S543	8q12-13	1/8	12.5	1/9	11.1	2
D9S171	9p21	1/5	20.0	2/8	25.0	3
D9S161	9p21	1/8	12.5	0/8	0	1
D9S127	9q31	0/3	0	2/8	25.0	2
D11S1336	11q23-24.2	2/5	40.0	0/0	0	2
D13S133	13q14.3	0/9	0	1/6	16.7	1
D13S128	13q32-34	3/4	75.0	0/3	0	3
FES	15q26.1	4/8	50.0	5/9	55.6	9
D17S855	17q21.1	1/10	10.0	1/8	12.5	2
D17S1322	17q21	3/10	30.0	3/9	33.3	6
D17S1327	17q21	2/6	33.3	3/8	37.5	5
HPRT	Xq26.1	1/5	20.0	0/9	0	1

Table 4.5 Loss of heterozygosity analysis (4) - Patient RF

	D2S391 2p21-16	D5S346 5q21-22	D6S287 6q21-23.3	D6S1027 6q25.3-27	D7S491 7q21-22	D8S543 8q12-13	D9S171 9p21	D9S127 9q31	D13S133 13q14.3	D13S128 13q32-34	FES 15q26.1	D17S855 17q21.1	D17S1322 17q21	D17S1327 17q21	MARKERS WITH LOH
LESION															
IC1											LOH		LOH	LOH	**2**
IC2										LOH	LOH		LOH		**3**
IC3				LOH							LOH			LOH	**3**
IC4	LOH			LOH		LOH	LOH			LOH	LOH	LOH	LOH		**7**
T1											LOH			LOH	**2**
T2											LOH				**1**
T3			LOH				LOH	LOH			LOH		LOH	LOH	**5**
T4				LOH							LOH	LOH			**3**
T5		LOH		LOH							LOH				**3**
T6					LOH		LOH	LOH					LOH		**4**
T7													LOH	LOH	**1**
T8	LOH					LOH			LOH						**3**
T9															**0**
n LOH/ n inform	2/9	1/12	1/9	4/13	1/13	2/12	3/11	2/10	1/9	2/5	9/13	2/12	6/13	5/12	
% LOH	22.2	8.3	11.1	30.8	7.7	16.7	27.3	20.0	11.1	40.0	69.2	16.7	46.2	41.7	

IC = inclusion cyst

T = area of microscopic malignancy

* Only samples showing LOH are documented; unfilled boxes represent a number of different PCR results (see Table 4.2)

Figure 4.5 Allelic losses observed (excluding patient RF)

Chromosome 5 D5S406 *(5p15.3-14)*

1. CBIC1(het); 2. CBIC2 (loh1); 3. CBleuc (het)

D5S399 *(5q22.3-31)*

1. MRnorm (het); 2. MRIC (loh1); 3. MRleuc (het)

Chromosome 9 D9S161 *(9p21)*

1. CBIC1 (het); 2. CBIC2 (loh1)

Chromosome 11 D11S1336 *(11q23-24.2)*

a. 1. MRIC (loh1); 2. MRnorm (het)

b. 1. CBIC1 (loh1); 4. CBleuc (het)

Chromosome 13 D13S128 *(13q32-34)*

1. CBIC2 (loh1); 2. CBleuc (het)

Chromosome X HPRT *(Xq26.1)*

1. MRIC (loh1); 3. MRleuc (het)

IC = inclusion cyst
norm = microscopically normal tissue
leuc = leucocyte DNA
het = retention of constitutional heterozygosity
loh1 = loss of larger allele
loh2 = loss of smaller allele

Figure 4.5 Allelic losses observed

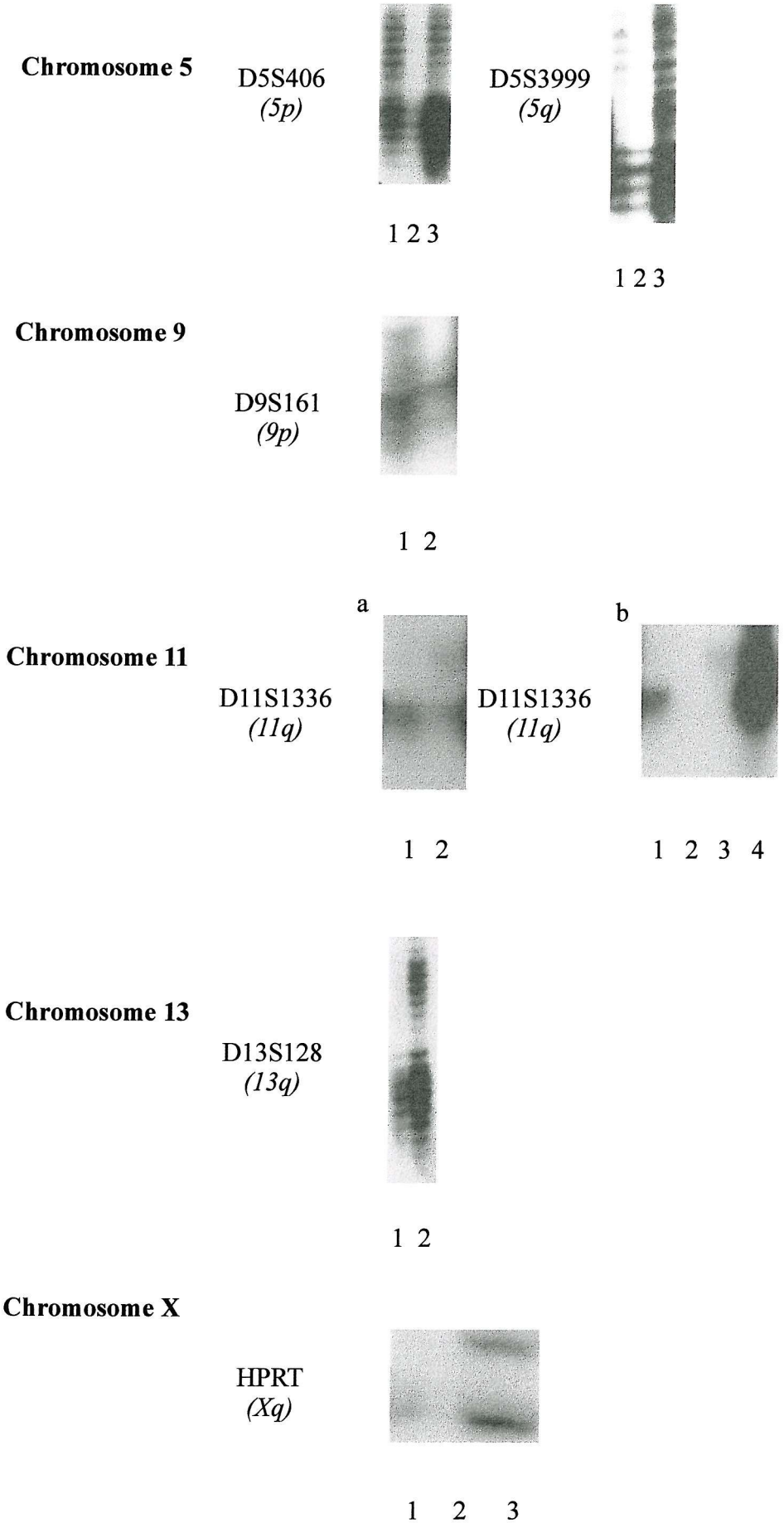


Figure 4.6 LOH in inclusion cysts (patient RF)

D2S391 *(2p21-16)*

1. het (RFT6); 2. het (RFT7); 3. loh1 (RFT8); 4. RFIC4 (loh1)

D6S287 *(6q21-23.3)*

1. het (RFT9); 2. RFIC4 (MI); RFleuc (het)

D6S1027 *(6q25.3-27)*

1. RFIC2 (het); 2. het (RFT3); 3. loh2 (RFT4); 4. loh1 (RFT5);
5. RFIC3 (loh2); 6. het (RFT6)

No image available for RFIC4 (loh2)

D8S543 *(8q12-13)*

1. het (RFT9); 2. RFIC4 (loh1)

D9S171 *(9p21)*

1. het (RFT8); 3. RFIC4 (loh1)

D13S128 *(13q32-34)*

a. 1. het (RFT2); 2. RFIC2 (loh1)

b. 1. het (RFT9); 2. RFIC4 (loh2)

IC = inclusion cyst

T = area of microscopic malignancy

leuc = leucocyte DNA

het = retention of constitutional heterozygosity

loh1 = loss of larger allele

loh2 = loss of smaller allele

MI = microsatellite instability

Figure 4.6 LOH in inclusion cysts (patient RF)

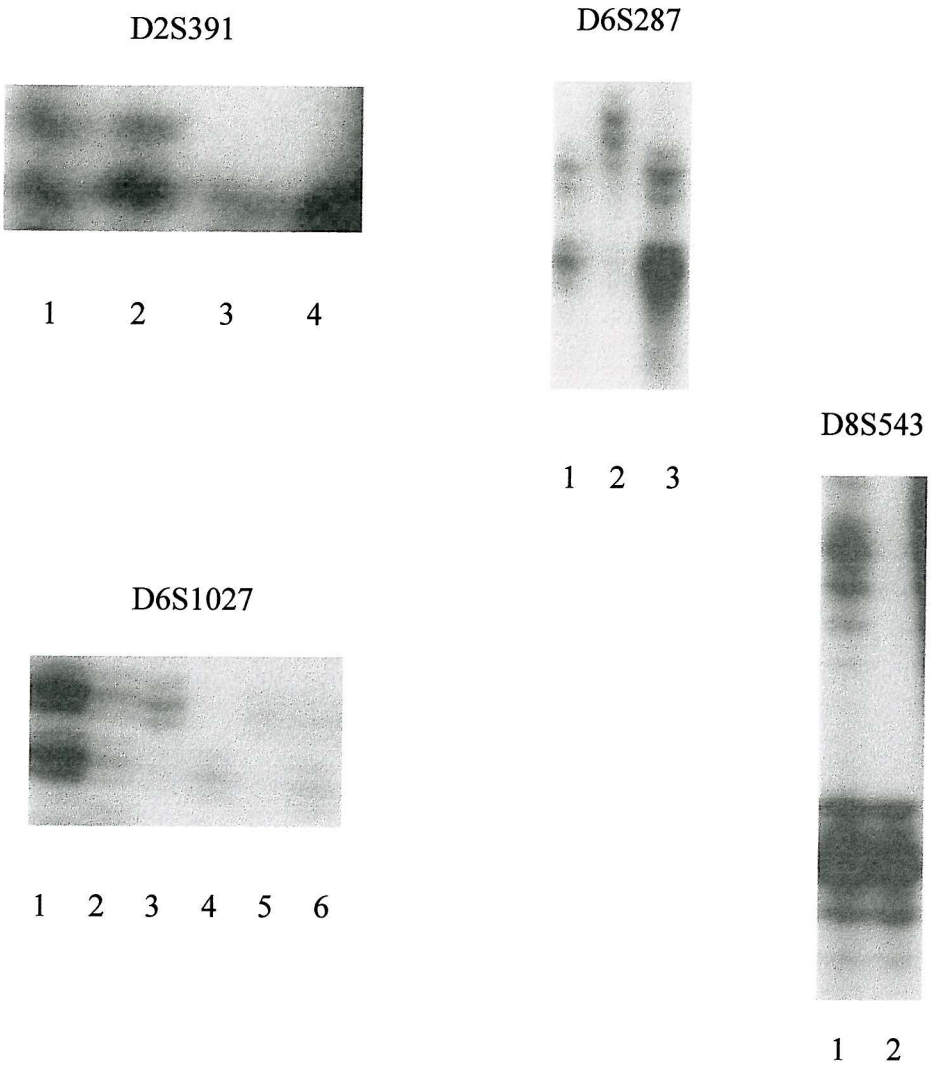


Figure 4.7 LOH in inclusion cysts (patient RF)

FES *(15q26.1)*

a. 1. RFIC1 (loh1); 2. RFT1; 3. RFT2; 4. het

b. 1. het (RFT9); 2. RFIC4 (loh2)

No images available for RFIC2 and RFIC3 (both loh1)

D17S855 *(17q21.1)*

No image available for RFIC4 (loh1)

D17S1322 *(17q21)*

a. 1. RFIC1 (loh1); 3. het (RFT2); 4. RFIC2 (loh1)

b. 1. RFIC4 (loh1)

D17S1327 *(17q21)*

1. RFIC1 (loh1); 2. het (RFT2); 3. RFIC3 (loh1)

IC = inclusion cyst

T = area of microscopic malignancy

het = retention of constitutional heterozygosity

loh1 = loss of larger allele

loh2 = loss of smaller allele

Figure 4.7 LOH in inclusion cysts (patient RF)

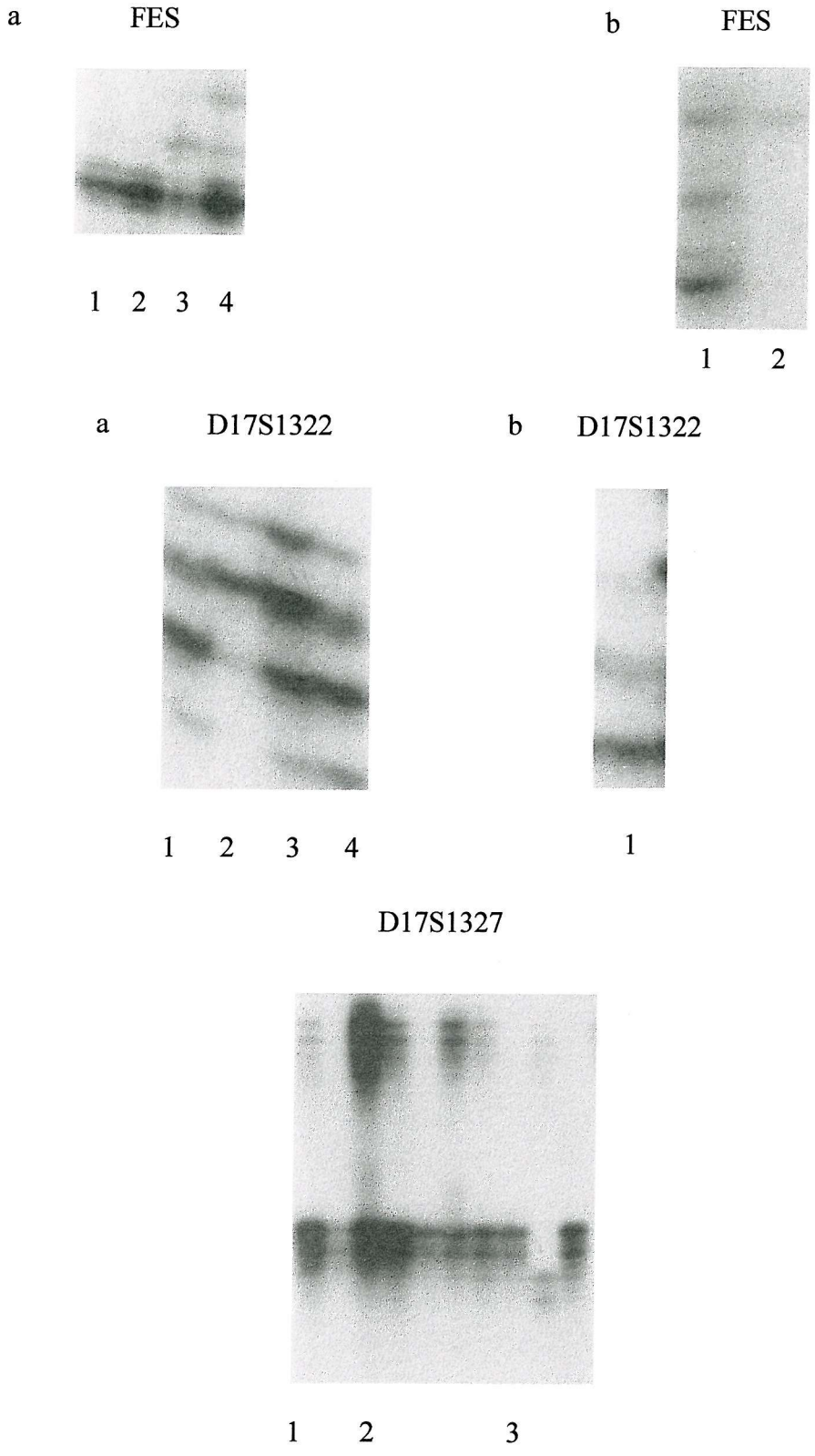


Figure 4.8 LOH in areas of microscopic malignancy (patient RF)

D2S391 *(2p21-16)*

1. RFT7 (het); 2. RFT8 (loh1)

D5S346 *(5q21-22)*

No image available for RFT5 (loh2)

D6S287 *(6q21-23.3)*

1. RFT2 (het); 2. RFIC2; 3. RFT3 (loh2)

D6S1027 *(6q25.3-27)*

1. RFIC2; 2. RFT3 (het); RFT4 (loh2); 4. RFT5 (loh1)

D7S491 *(7q21-22)*

1. RFT3 (het); 2. RFT6 (loh1)

D8S543 *(8q12-13)*

1. RFT8 (loh1); 2. RFT9 (het)

D9S127 *(9q31)*

a. 1. RFIC2; 2. RFT3 (loh1); 4. RFT5 (het); 6. RFT6 (loh1)

b. 1. RFT8 (MI); RFleuc (het)

D9S171 *(9p21)*

a. 1. RFIC2; 2. RFT3 (loh1)

b. 1. RFT6 (loh2); 3. RFT8 (het)

c. 1. RFT7 (MI); 2. RFT9 (het)

T = area of microscopic malignancy
IC = inclusion cyst
leuc = leucocyte DNA
het = retention of constitutional heterozygosity
loh1 = loss of larger allele
loh2 = loss of smaller allele

Figure 4.8 LOH in areas of microscopic malignancy (patient RF)

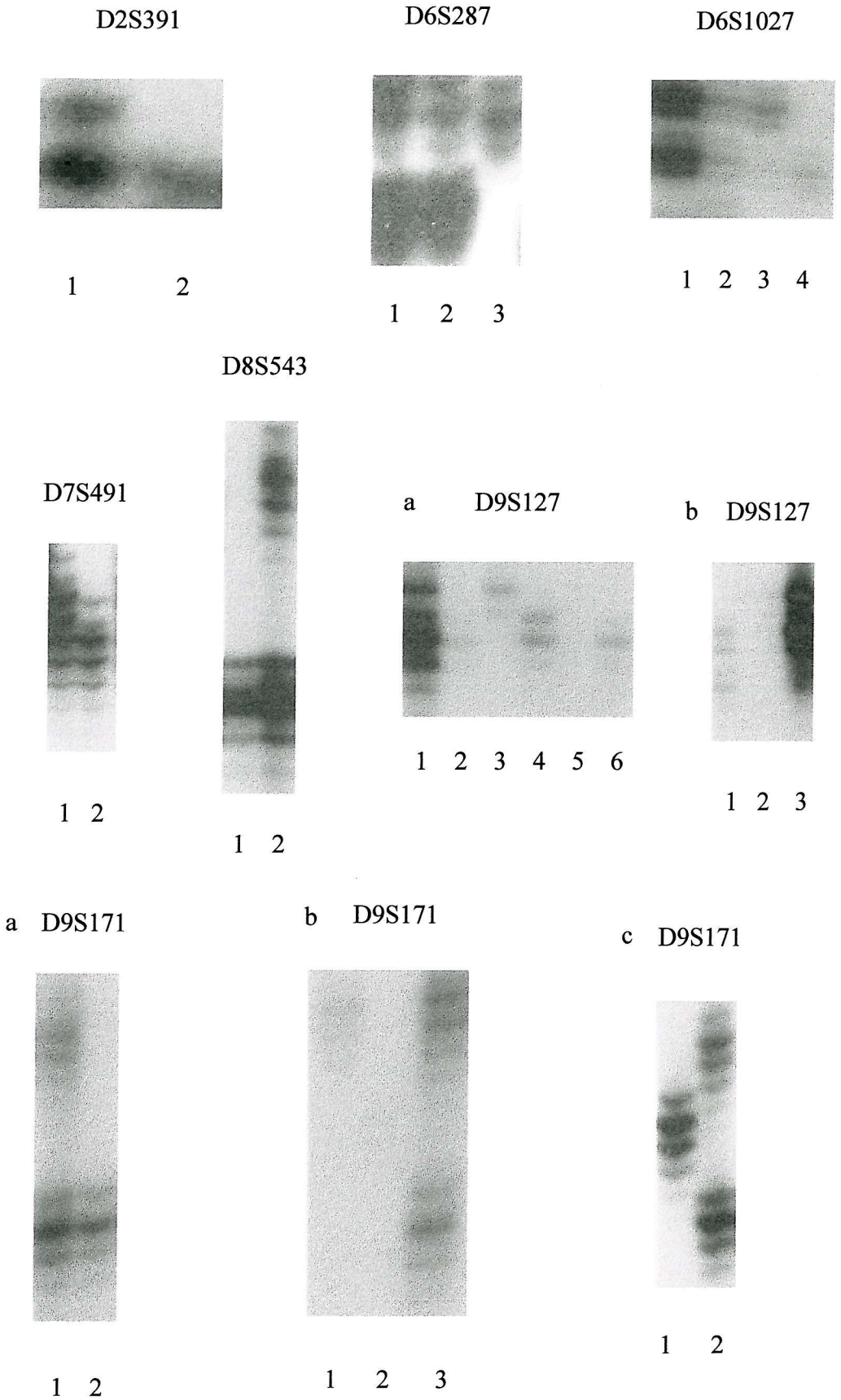


Figure 4.9 LOH in areas of microscopic malignancy (patient RF)

D13S133 *(13q14.3)*

1. RFT7 (het); 2. RFT8 (loh1)

FES *(15q26.1)*

1. het; 2. RFT1 (loh1); 3. RFT2 (loh1); 4. RFT3 (loh2); 5. RFT4 (loh1);
6. RFT5 (loh1)

D17S855 *(17q21.1)*

1. RFnorm (het); 2. RFT4 (loh1)

D17S1322 *(17q21)*

a. 1. RFT1 (het); 2. RFT3 (loh1)

b. 1. RFT4 (het); 2. RFT5 (het); 3. RFIC3; 4. RFT6 (loh1); 5. RFT7 (loh1)

D17S1327 *(17q21)*

1. RFIC1; 2. RFT1 (loh1); 3. RFT2 (het); 4. RFIC2; 5. RFT3 (loh1);
6. RFT4 (het); 7. RFT5 (het); 8. RFIC3; 9. RFT6 (mi); 10. RFT7 (loh1)

T = area of microscopic malignancy
IC = inclusion cyst
norm = microscopically normal tissue
het = retention of constitutional heterozygosity
loh1 = loss of larger allele
loh2 = loss of smaller allele
mi = microsatellite instability

Figure 4.9 LOH in areas of microscopic malignancy (patient RF)

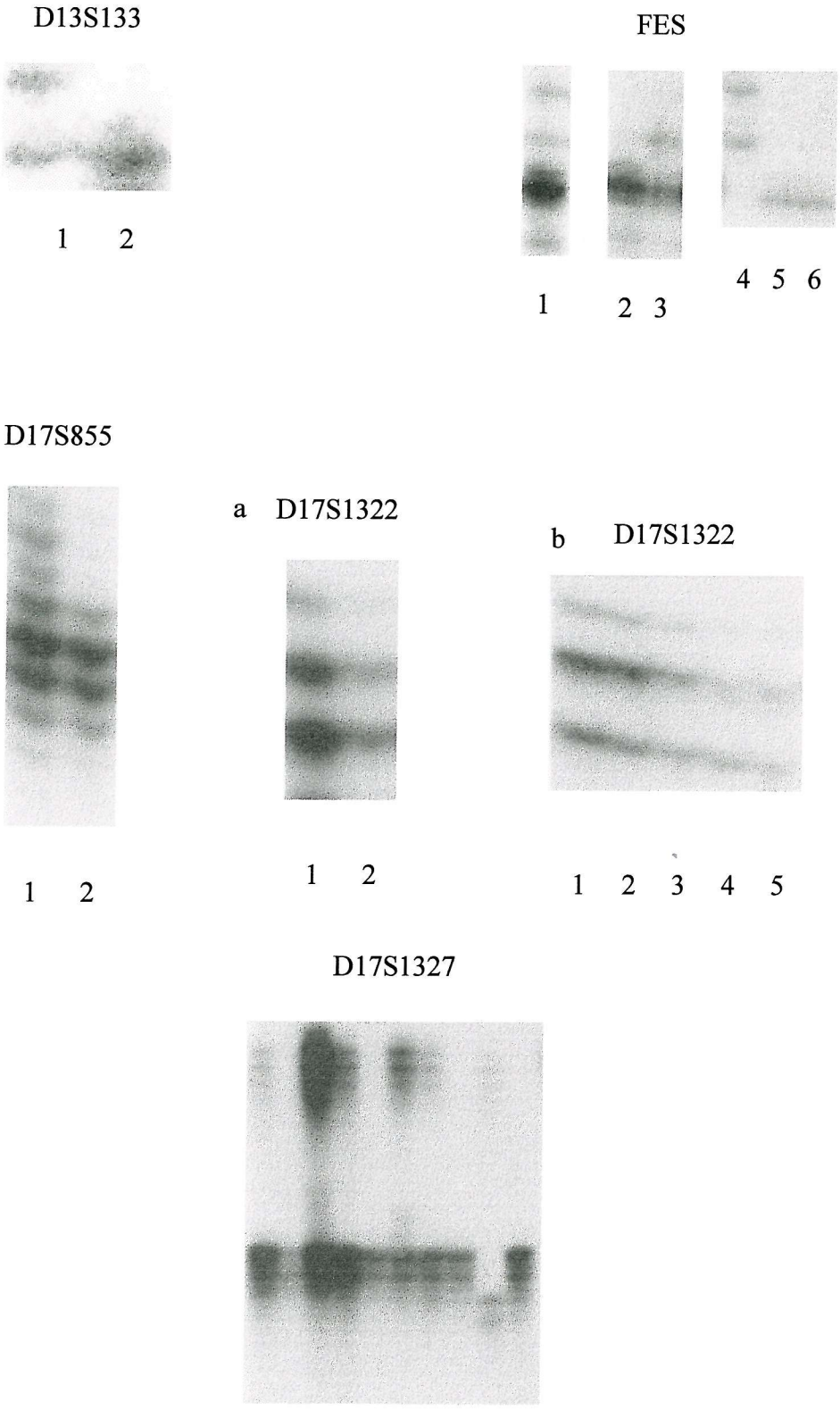


Figure 4.10 Examples of apparent allelic loss and microsatellite instability in areas of microscopically normal tissue

Apparent LOH

D13S128 1. CBnorm (loh1); 3. CBIC2 (loh1); 4. CBleuc (het)

D21S1432 1. MRnorm (loh1); 3. MRleuc (het)

Apparent MI

D6S287 1. MRnorm (mi); 3. MRleuc (het)

D9S171 1. MRnorm (mi); 3. MRleuc (het)

norm = microscopically normal tissue
IC = inclusion cyst
leuc = leucocyte DNA
het = retention of constitutional heterozygosity
loh1 = loss of larger allele
mi = microsatellite instability

Figure 4.10 Apparent LOH and MI in areas of microscopically normal tissue

Apparent LOH

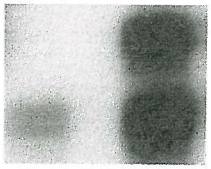
D13S128

(13q32-34)

1 2 3 4

D21S1432

(21q21)

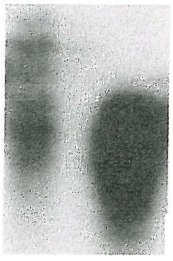


1 2 3

Apparent MI

D6S287

(6q21-23.3)



1 2 3

D9S171

(9p21)

1 2 3

Table 4.6 Loss of heterozygosity analysis (5) - Microdissected normal areas

	RFN1	RFN2	RFN3	RFN4	RFN5	RFB	HJN	HJB	JLN	JLB	SSN1	SSN2	SSB	MRN	MRB	CBN	CBB
Marker																	
D1S504	het	het	het	het	het	het	het	het	het	n/a	het	het	het	het	het	dnw	het
D2S391	het	dnw	het	het	het	het	het	het	het	n/a	het	het	het	ni	ni	het	het
D3S1582	ni	ni	ni	ni	ni	ni	het	het	ni	n/a	het	het	het	het	het	het	het
D3S1600	het	het	het	het	het	het	het	het	het	n/a	het	het	het	het	het	het	het
D3S196	het	dnw	dnw	het	het	het	het	het	ni	n/a	het	het	het	het	het	ni	ni
D4S175	het	het	het	het	het	dnw	het	het	dnw	n/a	het	het	het	het	het	het	het
D5S406	adm	adm	adm	adm	adm	adm	ni	ni	ni	n/a	het	het	het	ni	ni	dnw	het
D5S424	dnw	het	het	het	het	het	het	het	het	n/a	het	het	het	dnw	het	het	het
D5S644	het	dnw	dnw	dnw	dnw	het	het	het	het	n/a	het	het	het	het	het	dnw	het
D5S346	dnw	dnw	het	dnw	dnw	het	het	het	het	n/a	het	het	het	het	het	het	het
D5S399	het	het	het	het	het	het	het	het	het	n/a	het	het	het	het	het	het	het
D6S284	ni	ni	ni	ni	ni	ni	ni	ni	het	n/a	ni	ni	ni	ni	ni	het	het
D6S287	het	het	het	het	het	het	ni	ni	ni	n/a	ni	ni	ni	mi	het	ni	ni
D6S1027	het	dnw	dnw	dnw	dnw	het	het	het	het	n/a	het	het	het	het	het	dnw	het
D7S654	adm	adm	adm	adm	adm	adm	het	het	ni	n/a	het	het	het	ni	ni	dnw	het
D7S691	ni	ni	ni	ni	ni	ni	het	het	het	n/a	ni	ni	ni	mi	het	ni	ni
D7S670	het	het	het	het	het	het	het	het	het	n/a	het	het	het	het	het	het	het
D7S672	dnw	dnw	dnw	dnw	dnw	het	dnw	het	ni	n/a	het	het	het	ni	ni	dnw	het
D7S491	het	het	het	het	het	het	dnw	het	het	n/a	ni	ni	ni	ni	ni	het	het
D7S633	het	het	het	het	het	het	het	het	het	n/a	het	het	het	het	het	het	het
D8S258	dnw	het	het	het	het	het	het	het	het	n/a	het	het	het	dnw	het	het	dnw
D8S543	dnw	dnw	het	dnw	dnw	het	het	het	het	n/a	het	het	het	dnw	het	dnw	het
D9S171	het	het	het	het	het	het	ni	ni	het	n/a	ni	ni	ni	mi	het	ni	ni
D9S161	het	het	het	het	het	het	het	het	het	n/a	ni	ni	ni	het	het	het	het
D9S127	het	het	het	het	het	het	ni	ni	ni	n/a	ni	ni	ni	dnw	het	het	het
D10S89	dnw	dnw	dnw	dnw	dnw	het	dnw	het	het	n/a	het	het	het	dnw	het	dnw	het
D10S1696	het	het	het	het	dnw	het	het	het	het	n/a	het	het	dnw	dnw	het	ni	ni
D10S215	ni	ni	ni	ni	ni	ni	ni	ni	het	n/a	ni	ni	ni	ni	ni	het	het
D11S902	het	het	het	het	het	het	dnw	het	ni	n/a	het	het	het	het	dnw	het	het
D11S912	dnw	dnw	dnw	dnw	dnw	het	ni	ni	het	n/a	het	het	het	dnw	het	dnw	het
D11S1336	adm	adm	adm	adm	adm	adm	het	het	het	n/a	ni	ni	ni	het	het	dnw	het
D11S969	het	het	het	het	dnw	het	ni	ni	ni	n/a	het	het	het	ni	ni	ni	ni
D12S356	adm	adm	adm	adm	adm	adm	ni	ni	ni	n/a	ni	ni	ni	het	het	het	het
D12S385	ni	ni	ni	ni	ni	ni	dnw	het	ni	n/a	ni	ni	ni	het	het	ni	ni
D13S133	dnw	dnw	dnw	dnw	dnw	het	het	het	het	n/a	het	het	het	het	het	het	het
D13S128	het	het	het	het	het	het	loh (1)	het	ni	n/a	ni	ni	ni	ni	ni	loh (1)	het
D14S80	dnw	dnw	dnw	dnw	dnw	dnw	ni	ni	het	n/a	het	dnw	het	dnw	het	ni	ni
FES	dnw	dnw	dnw	dnw	dnw	het	het	het	het	n/a	ni	ni	ni	het	het	dnw	het
D16S3072	het	het	het	het	het	hett	het	het	het	n/a	het	het	het	het	het	het	het
D17S786	adm	adm	adm	adm	adm	adm	het	het	het	n/a	ni	ni	ni	het	het	het	het
D17S855	het	het	het	het	het	het	het	dnw	het	n/a	het	het	dnw	het	dnw	het	dnw
D17S1322	het	het	het	het	het	het	het	het	het	n/a	het	het	het	het	het	het	het
D17S1323	ni	ni	ni	ni	ni	ni	het	het	het	n/a	ni	ni	ni	ni	ni	het	het
D17S1327	het	dnw	het	het	het	het	het	het	het	n/a	ni	ni	ni	ni	ni	ni	ni
D18S59	het	het	het	het	het	het	het	het	het	n/a	het	het	het	het	het	dnw	dnw
D18S474	het	het	het	het	het	het	het	het	het	n/a	het	het	het	het	het	het	het
D19S886	dnw	dnw	dnw	dnw	het	het	het	dnw	het	n/a	het	het	dnw	dnw	dnw	dnw	het
D20S851	het	het	het	het	het	het	het	het	ni	n/a	het	het	het	het	het	het	het
D21S1432	het	het	het	het	dnw	het	ni	ni	het	n/a	het	het	het	loh (1)	het	het	het
HPRT	het	het	het	het	het	het	het	het	het	n/a	ni	ni	ni	het	het	ni	ni

N = microscopically normal area
het = retention of constitutional heterozygosity
loh(1) = loss of larger allele
ni = non-informative due to constitutional homozygosity
dnw = PCR did not work
mi = microsatellite instability
adm = alleles don't match

Figure 4.11 Examples of microsatellite instability

Chromosome 6 1. MRnorm (mi); 3. MRleuc (het)

Chromosome 9 1. MRnorm (mi); 3. MRleuc (het)

norm = microscopically normal tissue

leuc = leucocyte DNA

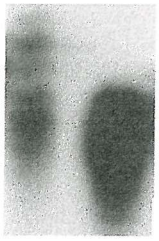
het = retention of constitutional heterozygosity

mi = microsatellite instability

Figure 4.11 Microsatellite instability

Chromosome 6

D6S287
(6q21-23.3)



1 2 3

Chromosome 9

D9S171
(9p21)

1 2 3

4.5 DISCUSSION

The prophylactic oophorectomy specimens examined for the purposes of this study provide tissue with a high potential for malignant transformation, by virtue of the individuals' BRCA1 or BRCA2 mutation carrier status. This is well evidenced by the unexpected finding of several areas of microscopic malignancy in the ovarian tissue taken from one of the BRCA1 mutation carriers included.

The study was based around the findings of Salazar *et al* in 1996, suggesting that prophylactically removed ovaries from women at risk of development of ovarian cancer - due to positive family history, positive linkage, or mutational analysis of BRCA1 - revealed greater numbers of inclusion cysts than a matched control group of ovaries removed for reasons unrelated to cancer, thus raising the possibility of a histological pre-malignant phenotype in these ovaries. The observations made by Salazar were initially reported in women who underwent prophylactic surgery because an identical twin developed ovarian cancer (Gusberg and Deligdisch 1984). Similar atypical features have also been reported in normal ovarian epithelium adjacent to early ovarian cancer, and in the contralateral normal ovary in cases of Stage I ovarian cancer (Plaxe *et al*, 1990; Resta *et al*, 1993). However, a subsequent study by Stratton *et al*, 1999, has been unable to confirm Salazar's data. Ovarian tissue, prophylactically removed from 37 women from families with BRCA1 or BRCA2 mutations, was reviewed, looking for inclusion cysts, clefts and fissures, ovarian epithelial metaplasia, and the presence of papillae on the ovarian surface epithelium. Samples were also examined for evidence of dysplasia or occult malignancy. The women were tested for BRCA1 and BRCA2 mutations, revealing that 11 had inherited a mutation. The investigators were unable to demonstrate any difference between the groups of mutation carriers and non-carriers for the features described.

The data of Salazar, Gusberg, Plaxe, and Resta is all suggestive of a histological pre-malignant phenotype. However, if the data of Stratton is accurate, with approximately equal frequencies of atypical lesions amongst carriers and non-carriers of BRCA1 and BRCA2 mutations, the implication is that mutation carriers may have higher frequencies of genetic alterations than non-carriers, thus resulting in a higher incidence of progression to ovarian carcinoma in this population.

4.5.1 Detection of LOH in Inclusion Cyst DNA

This study shows that it is possible to detect LOH in DNA extracted from microdissected inclusion cysts from the ovaries of women who carry BRCA1 mutations and have undergone prophylactic oophorectomy. The pathological material of all the individuals studied revealed inclusion cysts, and, for one individual, multiple areas of microscopic malignancy were also present (patient RF). This finding is in keeping with the data of Salazar, who described microscopic malignancies in two of his samples. It is, therefore, essential that careful histological examination of these ovaries is performed, to ensure that such lesions are not missed. It is possible that microscopic neoplasia may have early metastatic potential, and may thus be responsible for the occurrence of peritoneal carcinomatosis after prophylactic surgery (Tobacman *et al*, 1982).

4.5.2 Primer Extension Pre-amplification (PEP)

DNA availability is a major problem of studies involving small lesions. Epithelial germline inclusion cysts and benign cysts, thought to be the origin of ovarian cancer, comprise only a single layer of epithelial cells. This makes it difficult to obtain pure epithelium, uncontaminated by stromal cells, for analysis. An additional confounding factor is the poor quality of the DNA obtainable from paraffin-embedded material. DNA pre-amplification, prior to locus specific PCR, seems a reasonable way to attempt to overcome some of these difficulties.

The procedure has been validated by other investigators. Barrett *et al* (1995) reported the reliability of PEP amplified DNA in microsatellite PCR analysis, provided at least 50 cell genomes were present in each PEP reaction. The number of cells is critical for detection of LOH and MI, as too few cells or too little DNA may result in the phenomenon of preferential allelic amplification, giving a false impression of LOH. When microsatellite alleles are amplified, one (usually the smaller) often amplifies more efficiently. This bias may be greater in the presence of low DNA concentrations, resulting in disappearance of the larger allele, even if it was initially equimolar to the smaller (Barrett *et al*, 1995). Barrett's group also showed that LOH could still be detected in the presence of up to 33% of 'normal' cells within the biopsied tissue.

Dietmaier *et al* (1999) reported a lower limit of 10 cells per PCR reaction to enable amplification from frozen tissue, but suggested that, for paraffin-embedded, formalin-fixed material, at least 1000 cell genomes are required for good PEP amplification. The technique is simple, inexpensive, and reliable, and is well suited to studies where large numbers of samples are required for microsatellite PCR and mutation analysis. It was, therefore, felt to be suitable for use within this study.

4.5.3 The Choice of Microsatellite Markers Used

Within this study, 50 microsatellite markers were PCR'ed with each DNA sample. Markers were chosen following review of the existing literature documenting LOH studies in ovarian cancer and other tumour types (Table 1.8). Markers were selected to enable analysis of all chromosome arms. However, satisfactory PCR optimisation was not possible for markers on chromosome 2q (D2S93, D2S102), 19q (D19S412), 20q (D20S17), 22q (D22S274, D22S276), or Xp (DXS538). In addition, several markers failed to PCR with any of the DNA extracted from paraffin-embedded material, despite satisfactory optimisation with leucocyte DNA, and with control DNA extracted from fresh ovarian tumour samples. The affected markers were D1S243 (1p), D4S174 (4p), and D16S3098 (16q). Consequently, several chromosome arms are not represented - 1p, 2q, 4p, 6p, 16q, 19q, 20q, 22q, and Xp.

4.5.4 Loss of Heterozygosity Detected

Loss of heterozygosity observed for microsatellite markers on 17q fits with the BRCA1 mutation background of the individuals studied. Findings of LOH on 2p (14.3%), 5p (25%), 5q (9.1%), 6q (25%), 8q (12.5%), 9p (20%), 11q (40%), 13q (75%), 15q (50%), 17q (33%), and Xq (20%) in inclusion cysts, and 2p (16.7%), 5q (11.1%), 6q (22.2%), 7q (11.1%), 8q (11.1%), 9p (25%), 9q (25%), 13q (16.7%), 15q (55.6%), and 17q (37.5%) in areas of microscopic malignancy (seen in Figures 4.5-4.9) can be compared with the existing literature. It must be noted that all the literature relates to sporadic ovarian lesions. For malignant ovarian tumours, loss of heterozygosity has been described in numerous studies, on virtually all chromosome arms. The finding of significant LOH (>20%) on chromosomes 6q, 9p, 11q, and 17q in inclusion cyst DNA is in keeping with previously published studies of benign and borderline ovarian lesions.

Similar rates of LOH were also observed for 5p, 13q, 15q, and Xq. The detection of LOH in inclusion cysts fits with the hypothesis of these being very early lesions in the model of progression to ovarian cancer. Within this study, >20% LOH for areas of microscopic malignancy was detected on 6q, 9p, 9q, 15q, and 17q.

4.5.5 Literature Review for Chromosome Arms Showing LOH in This Study

4.5.5.1 *Studies of LOH on 2p*

Pieretti *et al* (1995) studied 74 sporadic ovarian tumours, including 18 borderline lesions, for LOH on 2p. None of 13 informative borderline tumours showed allelic loss, whilst the rate of LOH for invasive cancers was 5.7% (2/35).
A further study by Chenevix-Trench *et al* (1997) examined 25 benign, 31 borderline, and 93 invasive tumours. LOH was observed in 26.9% (7/26) of invasive lesions, but was not seen in either benign or borderline tumours (0/6 and 0/8 respectively).
Allelic loss on 2p has also been reported within the allelotyping studies for invasive ovarian cancer of Cliby *et al* (1993) (17%) and Osborne and Leech (1994) (17%).

Within our study, LOH on 2p was observed in 14.3% of inclusion cysts and 16.7% of samples of microscopic malignancy.

4.5.5.2 *Studies of LOH on chromosome 5*

Allan *et al* (1994) studied 27 malignant ovarian lesions for allelic loss on chromosome 5, using 2 markers on 5p and 8 on 5q. 50% LOH was detected on 5q (12/24 informative) and 38% on 5p (8/21). Of the 12 tumours showing 5q LOH, 7 showed loss at all informative markers.
Tavassoli *et al* (1996) subsequently studied a series of 49 ovarian tumours for LOH on chromosome 5. The series included 2 borderline serous tumours, 1 mucinous adenoma, 1 serous adenoma, and 2 granulosa cell tumours. For malignant epithelial lesions, LOH on 5q was detected in 23/43 informative samples (53.5%). The rate for 5p LOH was only 5% (1/22). 5q LOH was seen in 33% of stage 1 lesions (6/18). None of the benign or borderline tumours demonstrated LOH on chromosome 5.

Saretzki *et al* (1997) examined 25 benign, 35 borderline, and 72 invasive ovarian tumours for LOH on 5q. No allelic loss was detected amongst benign lesions, but rates of 10% (1/10) and 30.4% (7/23) were detected for borderline and invasive tumours respectively.

Amongst our samples, LOH on 5p was observed in 25% of inclusion cysts, but was not detected amongst microscopic malignancy specimens. For 5q, rates were 9.1% and 11.1% respectively.

4.5.5.3 *Studies of LOH on 6q*

Foulkes *et al* (1993c) showed LOH on chromosome 6q in 16 of 29 malignant ovarian tumours studied (55%). Of these 16, 63% showed loss of all informative markers on that chromosome arm. Frequencies of LOH for five loci on 6q27 were 59-73%. No LOH was found amongst benign or borderline lesions.

A further study by Leary *et al* (1993) also showed 6q LOH in malignant ovarian lesions (3 of 13 samples, ie 23%), using a marker for 6q24-27; again no allelic loss was detected in benign lesions (3 informative samples). No borderline lesions were examined.

In 1995, Orphanos *et al* (1995a) presented evidence for involvement of different regions of chromosome 6q in ovarian tumours of differing histologies. The findings of Foulkes, of high frequencies of distal 6q LOH in malignant tumours, were confirmed. In addition, a locus at 6q21-23.3 was identified which was commonly involved in benign and endometrioid tumours (4/7 benign samples = 57%; 5/8 endometrioid samples = 62%). A further locus at 6q14-15 was found to be involved in endometrioid lesions (5/7 samples = 71%), and a third, at 6q16.3-21, apparently involved in early-stage malignant lesions.

Rodabaugh *et al* (1995) studied patterns of allelic loss at 6q in borderline ovarian lesions compared with invasive cancers. In a study of 46 borderline and 20 invasive lesions, LOH at 6q25-27 was seen in 29% of invasive (13/45) and 11% of borderline (3/26) cases.

The first report of distal 6q LOH (6q27) as an early lesion in the pathogenesis of ovarian carcinoma came from Tibiletti *et al* (1996). Forty cases of surface epithelial

ovarian tumours were analysed using conventional cytogenetics and FISH (fluorescence in-situ hybridization). Thirty two cases were cytogenetically analyzable and, of these, chromosome 6q deletion was demonstrated in 25 (78%). All cases with 6q deletion shared the 6q27-qter segment as a minimal common deleted region. Using FISH analysis, loss of 6q27 sequences in 3 karyotypically normal malignant serous tumours (with no cytogenetically detectable chromosome 6 abnormalities) was shown. This suggests that deletions not detected with conventional cytogenetic techniques may be early events in ovarian carcinogenesis. This is supported by the finding of distal 6q deletion in borderline lesions and low-grade ovarian carcinomas.

In contrast to the work of Tibiletti et al, Watson *et al* (1998) found 6q LOH was relatively uncommon in early ovarian cancer (0/7 borderline lesions; 4/34 early malignant tumours = 12%). The cases examined by Watson and colleagues were mostly of mucinous and endometrioid histologies, whilst those of Tibiletti were serous. This perhaps suggests that distal 6q LOH is specific to serous ovarian lesions, in keeping with studies of advanced ovarian carcinomas (Saito *et al*, 1992; Orphanos *et al*, 1995a). Other studies have also confirmed high rates of distal 6q LOH in invasive malignancies. Amongst 54 invasive lesions, Colitti *et al* (1998) observed rates of 44% (14/32) and 43% (17/40) for 2 markers between 6q25.1 and 6q25.2 (D6S473 and D6S448 respectively), whilst Suzuki *et al* (1998) reported 54% LOH (26/48) at 6q27.

For our series of samples, 6q LOH was seen in 25% of inclusion cysts and 22.2% of microscopic malignancy specimens. If the hypothesis is correct, that inclusion cysts represent very early lesions in the pathway of ovarian carcinogenesis, our finding of 6q LOH amongst these lesions is in keeping with the data for allelic loss in benign and borderline tumours.

4.5.5.4 *Studies of LOH on 7q*

Within allelotyping studies for invasive ovarian cancer, the following rates of 7q LOH have been reported - Lee *et al* (1990) 20%, Sato *et al* (1991) 21%, Cliby *et al* (1993) 13%, Osborne and Leech (1994) 19%.

Pieretti *et al* (1995) observed LOH in 3 of 39 invasive lesions (7.7%) and 1/15 borderline tumours (6.7%).

Kerr *et al* (1996) analysed 139 epithelial ovarian tumours for LOH at 4 loci on 7q21-q31. The highest rates of LOH were seen in malignant lesions (38% (20/52) at D7S471; 34% (15/44) at D7S522). Only 1 of 27 tumours of low malignant potential (borderline tumours) showed LOH (4%), whilst no allelic loss was seen amongst benign samples. Chenevix-Trench *et al* (1997) examined 25 benign and 31 borderline tumours, and found 7q LOH in 0/18 benign and 1/22 borderline lesions (4.5%). For invasive cancers, the rate of LOH observed was 40%.

We did not detect 7q LOH amongst inclusion cysts, which is consistent with the findings of Kerr for benign and borderline tumours. Our LOH rate for malignant samples was 11.1%.

4.5.5.5 *Studies of LOH on 8q*

Various studies have detected chromosome 8q LOH amongst invasive ovarian carcinomas - Sato *et al* (1991) 31%, Cliby *et al* (1993) 29%, Osborne and Leech (1994) 26%, Pieretti *et al* (1995) 16.7%.
Data regarding LOH in benign or borderline lesions is lacking - Pieretti *et al* (1995) detected no allelic loss amongst 17 informative borderline lesions.

We detected 8q LOH in 12.5% of inclusion cysts and 11.1% of malignant samples examined.

4.5.5.6 *Studies of LOH on 9p*

Chenevix-Trench *et al* (1994) were able to show loss of heterozygosity on the short arm of chromosome 9 in 34 of 91 informative sporadic ovarian tumours (37%). The tumours studied included some benign, low malignant potential, and early-stage malignant lesions, suggesting 9p LOH as an early event in ovarian carcinogenesis. Rodabaugh *et al* (1995) studied the pattern of chromosome 9p LOH in borderline (n=25) and invasive (n=17) ovarian tumours. For invasive carcinomas, 3 regions showed high frequencies of allelic loss - 9p23-22 (38% with D9S144, 31% with D9S156); 9p21 (33%); 9p13 (31%). For borderline lesions, LOH was much less

common. The highest frequency was seen at 9p23 (20%). For markers at 9p21, rates were 7-8%, with 8% LOH at 9p23-22 and 9p13.
Other studies have also investigated allelic loss on chromosome 9p. Campbell *et al* (1995) studied a series of 67 primary ovarian tumours (including 2 granulosa cell tumours and 3 borderline lesions) for LOH at 9p21. Amongst malignant tumours, LOH was detected in 24/48 informative samples, ie. 50%, and was a common finding in early lesions (50% for stage I lesions). No LOH was observed in borderline tumours.
Roy *et al* (1997) reported a study of 31 microdissected benign ovarian tumours, in which 9p LOH was investigated using 2 microsatellite markers at 9p21.3 (D9S161 and D9S171). The total LOH observed was 26% (7/27).
Subsequently, in 1998, Watson *et al* reported a loss of heterozygosity analysis for chromosome 9 involving 40 early-stage malignant and 7 borderline ovarian lesions. In the early-stage tumours 9p LOH was detected in 42% (14/33), and in borderline lesions in 43% (3/7).

Amongst our samples, 9p LOH was observed in 20% of inclusion cysts and 25% of microscopic malignancy specimens. Our data is in keeping with that of Chenevix-Trench *et al* (1994), Roy *et al* (1997), and Watson *et al* (1998), suggesting 9p LOH as an early event in ovarian carcinogenesis.

4.5.5.7 *Studies of LOH on 9q*

Schultz *et al* (1995) examined 41 ovarian tumour specimens (29 epithelial, 2 recurrent adenocarcinoma, 2 mixed Mullerian, 1 clear cell, 1 teratoma, 1 granulosa cell, 1 Brenner, 2 mucinous cystadenomata, 2 thecoma) for genetic alterations on chromosome 9. Allelic loss on 9q was commonly found in late- stage malignant tumours (63%, 17/27) and poorly differentiated lesions (75%, 15/20). In benign and early- stage malignant tumours, the rate of LOH was 30% (3 of 10).

25% of our microscopic malignancy specimens showed 9q LOH, whilst allelic loss on this chromosome arm was not detected amongst inclusion cysts. Higher LOH rates amongst 'later' lesions fits with the data of Schultz.

4.5.5.8 *Studies of LOH on 11q*

Foulkes *et al* (1993b) examined 28 epithelial ovarian tumours for 11q LOH. All were malignant. High frequency LOH (8/12) was detected at 11q23.3-qter, suggesting the presence of a tumour suppressor gene in this region.

Subsequently, Gabra and colleagues (1995) reported a study of 60 ovarian tumours (47 epithelial ovarian cancers, 5 borderline lesions, 3 adenofibromas, 2 mixed mesodermal tumours, 2 granulosa cell tumours, and 1 teratoma) and reported 11q LOH for at least 1 locus in 65% of all informative tumours (39/60). The rate for epithelial ovarian cancers (EOC) was 66% (31/47). High frequencies of LOH were detected at several loci: 11q14.3 in 11/25 of all informative tumours (44%) and 9/22 EOC (41%); 11q23.3 in 24/45 of all informative lesions (53%) and 18/33 EOC (54%); 11q24.1 in 23/49 of all informative tumours (47%) and 18/37 EOC (49%). For the benign and borderline lesions only 1/7 had allelic loss at 11q24.1.

Davis *et al* (1996) set out to further refine a minimum region of 11q loss for ovarian cancer. Of 67 tumours analysed for allelic losses with 8 microsatellite markers spanning 11q23.3-qter, 40 (61%) showed LOH. Two of three borderline tumours, but neither of 2 benign lesions, also showed allelic loss. Higher frequency LOH was seen in both later stage and higher grade tumours. However, the observation of LOH in 50% of grade 1 (5/10) and 47% of stage 1 lesions (7/15) suggests that 11q LOH is a relatively early feature.

In 1997, Roy *et al* examined 31 benign ovarian tumours for LOH on 11q, using 2 microsatellite markers (D11S1336 for 11q23.3 and D11S1328 for 11q24.1). No LOH was detected at the latter locus. The total LOH for 11q was 9% (2/22).

A further study by Watson *et al* (1998) showed 34% LOH in early- stage malignant tumours (11/32) and 57% in borderline lesions (4/7).

Launonen *et al* (1998) have shown rates of LOH in agreement with other studies. 61% LOH was observed amongst 49 epithelial ovarian tumours, using 9 markers covering 11q22.3-q25.

We were unable to detect LOH on this chromosome arm amongst our malignant specimens (or inclusion cysts from patient RF), but 40% of inclusion cysts did show 11q allelic loss. This is more frequent than that described by Roy *et al* (1997) for benign

tumours (9%), but in keeping with data for borderline lesions of 57% LOH (Watson *et al,* 1998).

4.5.5.9 *Studies of LOH on 13q*

Yang Feng *et al* (1992) reported LOH on 13q in a series of 42 tumours. Frequencies of 46% (12/26) and 42% (5/12) were observed with markers D13S32 and D13S34. A somewhat higher rate was observed by Gallion *et al* (1992) - 58% (18/31). In Gallion's series, 1 of 4 benign, 2 of 5 borderline, and 15 of 22 invasive tumours showed LOH. Kim *et al* (1994) also investigated 77 primary ovarian epithelial tumours for 13q loss of heterozygosity. Tumours were divided into benign cystadenomas (n=12), tumours of low malignant potential (n=21), low grade carcinomas (n=15) and high grade carcinomas (n=29). LOH was only detected in high grade carcinomas, at a rate of approximately 50%.

75% of our inclusion cysts showed 13q LOH, together with 16.7% of malignant lesions. High rates of 13q allelic loss in 'early' (benign and borderline) lesions has not been previously reported. This raises the question of whether this finding is particular to our patient population with their BRCA1 mutation background.

4.5.5.10 *Studies of LOH on 15q*

Pieretti *et al* (1995) observed 15q allelic loss in 1/15 borderline lesions (6.7%) but did not find any LOH amongst 44 informative invasive tumours.
However, other studies have reported LOH in invasive ovarian tumours - Sato *et al* (1991) 11%, Cliby *et al* (1993) 36%, Osborne and Leech (1994) 40%.

Within our study, the rate of 15q LOH amongst inclusion cysts was 50%, with that for malignant lesions being slightly higher (55.6%).

4.5.5.11 *Studies of LOH on 17q*

Russell *et al* (1990) examined 19 ovarian tumours for LOH on chromosome 17, using one probe for each chromosome arm. 77% (10/13) showed 17q LOH, and 31% (4/13) demonstrated 17p LOH. All those showing 17p LOH were invasive lesions, whilst

amongst those with 17q loss were 1 mucinous cystadenoma, 1 borderline mucinous lesion, and 1 teratoma.

Eccles *et al* (1992) examined a large series of 146 ovarian tumours for chromosome 17q allelic loss (94 carcinomas, 22 tumours of low malignant potential, 30 benign lesions). The locus examined was 17q23-qter. For malignant lesions, 70% showed LOH (45/64). Amongst benign tumours, the rate was 13% (3/23), and for borderline lesions 2/16 (13%).

Foulkes *et al* (1993) confirmed the finding of high rates of 17q LOH in malignant tumours. Within his study, using 6 markers flanking the BRCA1 locus, 75% LOH was observed.

Jacobs *et al* (1993) studied 120 ovarian tumours for 17q LOH. Rates observed were 50% for benign lesions (1/2), 16.7% for borderline tumours (1/6), and 55.4% (62/112) for invasive malignancies.

In a study by Leary *et al* (1993), 17q LOH was identified in 62% of informative malignant tumours (13/21). Three benign and one borderline lesion were informative, but no allelic loss was identified.

Saretzki *et al* (1997) examined 72 invasive carcinomas, 35 tumours of borderline malignancy, and 25 benign lesions for 17q allelic loss. No LOH was detected amongst the benign tumours (0/4), but the rates for borderline and invasive lesions were 4.5% (1/22) and 40.5% (15/37) respectively.

33% of inclusion cyst samples and 37.5 % of malignant specimens showed 17q LOH in our study. 17q allelic loss was an expected finding, due to the BRCA1 mutation background of our patient series.

4.5.5.12 *Studies of LOH on Xq*

Cheng *et al* (1996) found that 8 of a series of 16 tumours of low malignant potential informative for a locus in the proximal portion of Xq showed LOH. The inactive copy of the X chromosome was exclusively involved. Similar allelic losses in Xq were not seen in either cystadenomas or low grade carcinomas.

Chenevix-Trench *et al* (1997) were able to detect LOH in benign tumours (33%) and in a small number of borderline lesions.

A subsequent study by Choi *et al* (1997) investigated for chromosome X LOH in 123 epithelial ovarian tumours. Frequent LOH was observed in the Xq 25-26.1 region (34.5% with DXS1206; 27.7% with DXS1047; 24.1% with HPRT; 33.3% with DXS1062). The series included 9 borderline and 2 benign tumours, none of which showed LOH at any of the loci documented. However, 2 of the borderline lesions showed LOH at the more proximal AR (androgen receptor) locus (Xq12), suggesting that there might be two different TSG's on the X chromosome, one responsible for genesis of borderline lesions and the other for tumour progression.
Edelson *et al* (1998) also examined Xq LOH in 41 borderline and 65 invasive tumours. Eight microsatellite markers were used, spanning Xq11.2-q12. Within this study, the highest frequency of LOH for borderline lesions was 25%, and for invasive tumours was 38% (at the AR locus).

Our finding of 20% LOH amongst inclusion cysts is in keeping with the data for benign lesions of Chenevix-Trench *et al* (1997). Our malignant samples did not show LOH.

4.5.5.13 *Allelotyping studies*

Numerous allelotyping studies in ovarian cancer have been carried out. Lee *et al* (1990) analysed DNA from 19 ovarian cancer patients for LOH at 18 chromosomal loci. Sato *et al* (1991) examined 37 tumours, as did Cliby *et al* (1993). A similar study by Yang Feng *et al* (1993) investigated 50 tumours and 86 chromosomal loci, whilst that of Osborne and Leech (1994) used a panel of 25 samples. Data from these studies is shown in Table 4.7.
Saretzki *et al* (1997) analysed 35 borderline and 25 benign lesions, in addition to 72 invasive cancers, for LOH on 2q, 5q, 6p, 6q, 9p, 11q, 17p, 17q, 18q, and 22q. Amongst benign tumours, LOH was only detected in 8% (at 2q21 and 17p13). 11% of borderline lesions showed allelic loss (at 2q21, 5q21, 6p21, 17p13, and 17q21), whilst the rate for invasive cancers was 77.7%. Amongst invasive lesions, the highest frequencies were 56% at 17p13 (TP53) and 40.5% at 17q21 (BRCA1). 30.4% of informative cases also showed 5q21 loss, 21.4% at 18q21, 32.4% at 2q21-22, and 25% at 9p21.

Table 4.7 Allelotyping studies in ovarian cancer

	Lee 1990	Sato 1991	Cliby 1993	Yang Feng 1993	Osborne and Leech 1994
Chromosome location	**% LOH detected**				
1p		7	24	11	19
1q	0	29	28	11	23
2p		0	17	23	17
2q	20/0	14	33	11	38
3p		18	0	25	26
3q		6	17	11	32
4p		42	34	21	18
4q		6	19	10	33
5p		-	21	5	19
5q	0	0	43	7	40
6p	0	50	62	21/25	27
6q	64/13	17(42*)	57	22	35
7p		43	36	15	17
7q	20/0	21	13	13	19
8p		-	40	26	26
8q		31	29	11	26
9p		-	33	31	30
9q		10	54	19	48
10p		11	12	14	22
10q		21	11	12	19
11p	46	24	35	8	43
11q	0	5	33	0	30

	Lee 1990	Sato 1991	Cliby 1993	Yang Feng 1993	Osborne and Leech 1994
Chromosome location	**% LOH detected**				
12p	0	38	9	10	6
12q		33	24	8	13
13q		22(56*)	56	42	12
14q		18	47	8	46
15q		11	36	8	40
16p		33	21	15	0
16q		37	35	23	16
17p	75/64	46	81	42	61
17q	37.5	39	76	45	64
18p		0	10	18	6
18q		0	43	29	27
19p		34	15	26	45
19q	14	25(67*)	17	22	23
20p		13	0	15	21
20q		-	16	0	21
21q		-	36	8/23	33
22q	20	-	71	-	28
Xp		-	28	41	40
Xq		-	-	26	21

* serous tumours only

For the majority of chromosome arms, in most studies presented, total LOH was calculated if more than one marker was studied

x/y = LOH values recorded for two markers; no total LOH calculated

= chromosome arms for which LOH detected in microscopic malignancy in our study

= chromosome arms for which LOH detected in inclusion cysts in our study

= chromosome arms for which LOH detected in both inclusion cysts and areas of malignancy

4.5.6 Comparison of Patterns of LOH for Individuals with More Than One Inclusion Cyst Examined

For three of the six individuals included in this study, it was possible to identify and microdissect more than one inclusion cyst from the paraffin-embedded material available, thus enabling comparison of LOH patterns between DNA samples from more than one lesion for an individual.

Such comparison suggests that the inclusion cysts for a particular individual are unrelated lesions, as patterns of LOH differ between them. This is evident when looking at the data for inclusion cyst DNA from patient RF. For the microsatellite marker D13S128, sample RF IC2 showed loss of the larger allele, whilst RF IC4 showed loss of the smaller. Again, with marker FES, samples RF IC1, RF IC2, and RF IC3 all showed loss of the larger allele, whilst RF IC4 showed loss of the smaller.

4.5.7 Areas of 'Normal' Tissue Studied

As previously stated, for all individuals included in the study, areas of tissue of 'normal' microscopic appearance, dissected some distance from any inclusion cyst or malignant material, were analysed. Three spurious results were seen, when compared with matched leucocyte DNA.

Apparent loss of heterozygosity was seen with HJ 'normal' DNA following PCR with marker D13S128 (Figure 4.10), whilst no allelic loss was detected in the associated inclusion cyst DNA. This suggests that the PCR result with 'normal' DNA reflects PCR artefact rather than true LOH. The results described with MR 'normal', of apparent LOH with D21S1432 and MI with 3 markers (D6S287, D7S691, and D9S171) (Figure 4.10), probably reflect poor quality DNA - it is apparent (from Table 4.2) that the associated inclusion cyst DNA failed to PCR with many of the microsatellite markers used (25/50). However, in view of the MI detected, this individual has subsequently been screened for mismatch repair gene mutations, and has no detectable mutation in either hMLH1 or hMSH2. The spurious result with CB 'normal' DNA and D13S128 is difficult to interpret, since the associated inclusion cyst DNA failed to PCR with this particular marker.

4.5.8 The Effect of Product Size on PCR Results

It became apparent whilst carrying out PCR reactions with our series of microsatellite markers that PCR results were less reliable for those with large PCR products (>200 base pairs). Figure 4.12 shows the mean % of samples for which PCR reactions were successful and consistent at varying product sizes. PCR product size was less than 100 base pairs (bp) for only 2 of the markers used (D3S196 and D5S644). With the marker on chromosome 3, PCR was successful with 95% of samples (19/20); for D5S644 the figure was 85% (17/20). The majority of the microsatellite markers studied gave product sizes between 100 and 200bp (Table 4.8). As can be seen, no significant differences in success rates were seen across this range of product size. Markers with product sizes in the range 101-125bp (n=6) gave a mean PCR success rate of 76%. For products of 126-150bp (n=22) the rate was 74%, and for those of 151-175bp (n=14) and 176-200bp (n=2) the rates were 75.4% and 87.5% respectively. However, markers giving PCR products in excess of 200bp were much less reliable, with the exception of the chromosome 6 marker D6S284 which resulted in a product of 233-251bp and was successful with 95% of samples. Although a PCR product was generated for most samples with each of the other 3 markers (D7S654, D11S1336, and D12S356), the alleles often did not match when samples from an individual were compared (as seen in Figure 4.13 for D11S1336). The mean PCR success rate for the 4 markers in this category was 43.7%.

We therefore suggest that careful selection of microsatellite markers is required when studying DNA extracted from small paraffin-embedded lesions, to increase reliability of PCR results. Markers which give products in excess of 200bp are unlikely to be useful in this setting, as PCR results appear to become significantly less reliable once this threshold of product size is reached.

Figure 4.12 Effect of PCR product size on PCR reliability

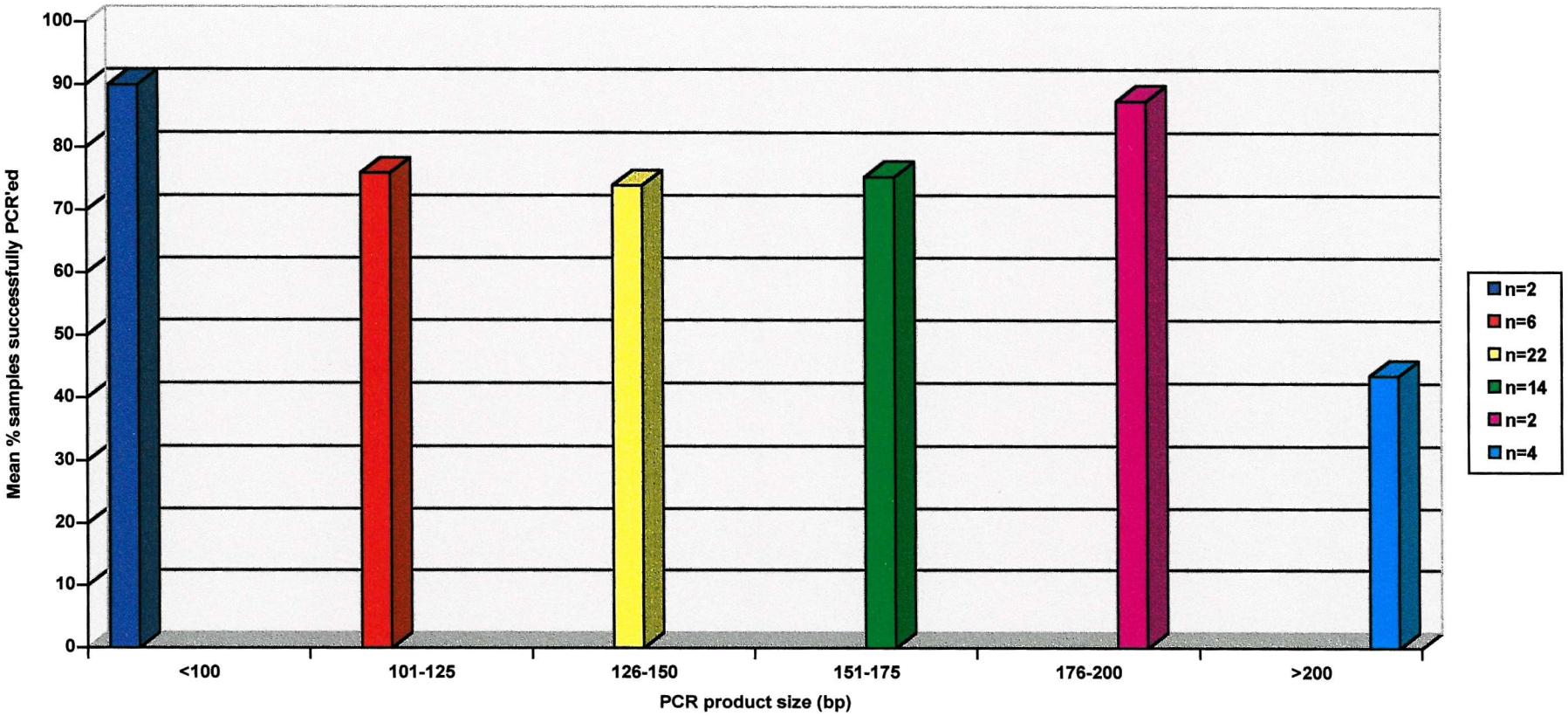


Table 4.8 Effect of PCR product size on PCR reliability

	PRODUCT SIZE					
	<100	101-125	126-150	151-175	176-200	>200
MARKER (% samples PCR'ed successfully)	D3S196 (95)	D4S175 (55)	D1S504 (90)	D3S1582 (90)	D3S1600 (80)	D6S284 (95)
	D5S644 (85)	D5S346 (85)	D2S391 (70)	D5S406 (35)	D10S215 (95)	D7S654 (15)
		D5S399 (100)	D5S424 (75)	D6S287 (80)		D11S1336 (35)
		D7S670 (100)	D6S1027 (85)	D7S633 (60)		D12S356 (30)
		D7S491 (95)	D7S691 (100)	D9S171 (90)		
		D11S912 (20)	D7S672 (30)	D9S127 (75)		
			D8S258 (75)	D11S902 (85)		
			D8S543 (85)	D13S133 (75)		
			D9S161 (90)	D13S128 (50)		
			D10S89 (25)	FES (95)		
			D10S1696 (60)	D16S3072 (75)		
			D11S969 (85)	D17S1323 (100)		
			D12S385 (95)	D18S59 (55)		
			D14S80 (25)	HPRT (90)		
			D17S786 (40)			
			D17S855 (90)			
			D17S1322 (95)			
			D17S1327 (95)			
			D18S474 (90)			
			D19S886 (60)			
			D20S851 (85)			
			D21S1432 (85)			
MEAN % PCR SUCCESS	**90**	**76**	**74**	**75.4**	**87.5**	**43.7**

Figure 4.13 Effect of PCR product size >200bp on PCR reliability

Chromosome 11 D11S1336

1. & 6. - areas of microscopically normal tissue from patient RF

4. RFT5

7. RFT6

8. RFT7

11. RFT9

14. - leucocyte DNA from patient RF

The figure shows inconsistency of PCR product for samples from patient RF with this marker

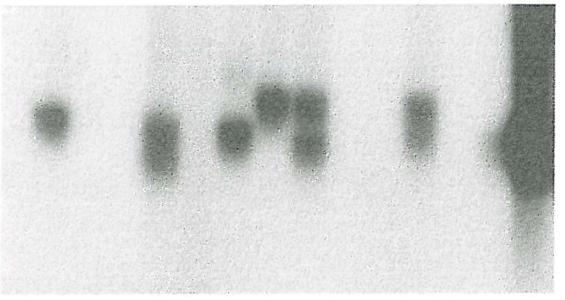
RFT = sample of microscopic malignancy from patient RF

Figure 4.13 Effect of product size >200bp

Chromosome 11

D11S1336
(11q23-24.2)

Product 232-252bp



1 2 3 4 5 6 7 8 9 10 11 12 13 14

4.5.9 Data Collected for Patient RF

The availability of multiple DNA samples for this individual, extracted from both inclusion cysts and areas of microscopic malignancy, provides an excellent opportunity to study patterns of LOH in the proposed lesions in the pathway of progression to ovarian cancer in an individual at increased genetic risk (Figures 4.6-4.9).

Following PCR with markers in the BRCA1 region of 17q, multiple DNA samples, from both inclusion cysts and areas of microscopic malignancy, showed allelic loss (Figure 4.14). All affected samples demonstrated loss of the same allele. Loss of heterozygosity with several 17q markers in DNA from a number of inclusion cysts of BRCA1 mutation carriers, fits with the hypothesis that thay are early lesions in the pathway of ovarian carcinogenesis for these individuals.

Multiple allelic losses were observed within areas of microscopic malignancy studied. This is in keeping with the known role of BRCA1 in the maintenance of genomic stability - loss of this function, as a result of BRCA1 mutation, allows the occurrence of multiple mutational events, and, potentially, rapid progression from inclusion cyst to malignant tumour.

Of particular note for this individual is the pattern of allelic loss seen with the chromosome 15q marker FES. All 4 inclusion cyst samples showed LOH, 3 of the 4 showing loss of the same allele (Figure 4.15). Of the 9 sample areas of microscopic malignancy, allelic loss was seen in 5 (4 samples losing the same allele). Chromosome 15q LOH in ovarian cancer is well documented: the frequency reported by Cliby *et al* (1993) for invasive ovarian lesions was 36%, and by Osborne and Leech (1994) was 40%.

Of interest is the question of whether the areas of microscopic malignancy studied are related or independent lesions. For several of the microsatellite markers used (D6S1027, D9S171, FES) individual samples lost differing alleles. This implies that the lesions studied are independent of each other, and the tissue very heterogeneous. The same observation applies for inclusion cyst DNA samples (with D13S128, and FES). We hypothesise that BRCA1 mutation, and subsequent loss of genomic stability, allows occurrence of multiple unrelated mutational events in different areas of ovarian tissue. Our findings raise questions regarding the existence of cases of apparently de novo ovarian cancer, as described by Bell and Scully (1994). They described a series of 14

patients with early ovarian cancer detected as microscopic abnormalities in grossly normal ovaries. The patients studied were aged between 27 and 65 years. Three women had first degree relatives with ovarian cancer, and the mother of a further individual had been diagnosed with breast cancer at 40 years of age. The tumours were of varying size (from microscopic to 7mm maximum dimension). Ten were serous lesions. The noncarcinomatous epithelium and inclusion cysts were described as severely atypical in 3 cases, and moderately abnormal in a further 8. On the basis of these findings, the authors suggested that at least a subset of epithelial ovarian cancers develop de novo, without intervening development of a benign tumour. However, we would suggest that the cases described were atypical. Four of the patients had an identifiable relevant family history, 5 had no obvious family history, and information was not available for the remainder; we would hypothesise that the malignancies reported in this series may have arisen on the background of BRCA1 mutation, with loss of genomic stability and multiple mutational events resulting in accelerated transformation from inclusion cysts to malignancy (rather than transformation from surface epithelium or inclusion cyst to tumour without an intervening benign stage).

The implications for individuals carrying BRCA1 mutations are those of development of malignancy at a young age, and potentially occurrence of multiple independent malignancies within one or both ovaries.

Figure 4.14 Chromosome 17q LOH for patient RF

D17S855 *(17q21.1)*

1. RFnorm (het); 2. RFT4 (loh1)

No image available for RFIC4 (loh1)

D17S1322 *(17q21)*

a. 1. RFT1 (het); 2. RFT3 (loh1)

b. 1. RFIC1 (loh1); 2. RFT2 (het); 3. RFIC2 (loh1); 4. RFT4 (het);

5. RFT6 (loh1); 6. RFT7 (loh1); 7. RFIC4 (loh1)

D17S1327 *(17q21)*

1. RFIC1 (loh1); 2. RFT1 (loh1); 3. RFT2 (het); 4. RFIC2 (het);

5. RFT3 (loh1); 8. RFIC3 (loh1); 9. RFT6 (mi); 10. RFT7 (loh1)

norm = area of microscopically normal tissue
IC = inclusion cyst
T = area of microscopic malignancy
het = retention of constitutional heterozygosity
loh1 = loss of larger allele
mi = microsatellite instability

Figure 4.14 Chromosome 17q LOH for patient RF

D17S855

1 2

D17S1322

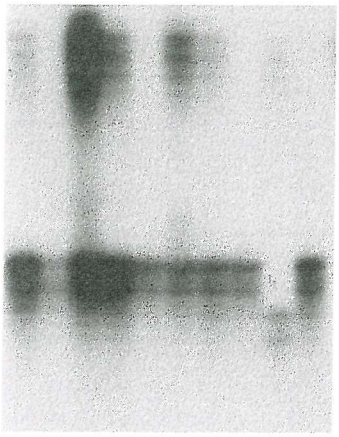
a

1 2

b

1 2 3 4 5 6 7

D17S1327



1 2 3 4 5 6 7 8 9 10

Figure 4.15 Chromosome 15q LOH for patient RF

FES *(15q26.1)*

1. het; 2. RFIC1 (loh1); 3. RFT1 (loh1); 4. RFT2 (loh1); 5. RFT3 (loh2);

6. RFT4 (loh1); 7. RFT5 (loh1); 8. RFT6 (het); 9. RFT7 (het); 10. RFT8 (het);

11. RFT9 (het); 12. RFIC4 (loh2)

No images available for RFIC2 and RFIC3 (both loh1)

IC = inclusion cyst

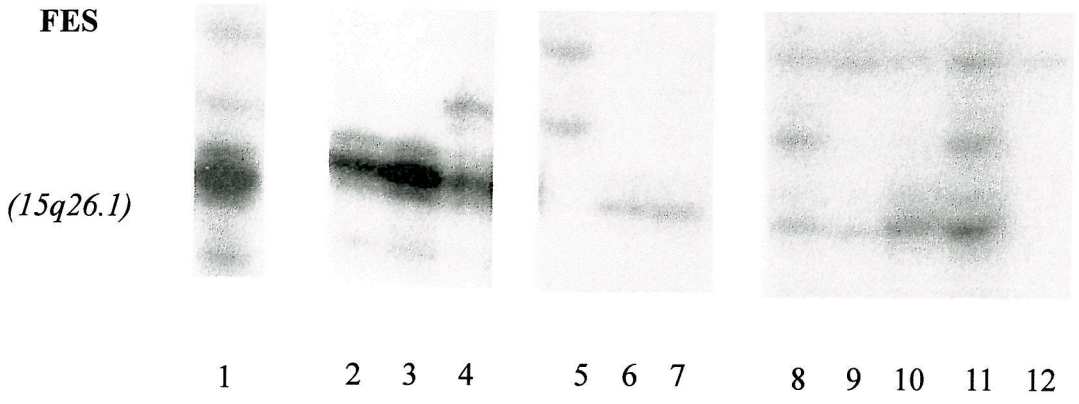
T = area of microscopic malignancy

het = retention of constitutional heterozygosity

loh1 = loss of larger allele

loh2 = loss of smaller allele

Figure 4.15 Chromosome 15q LOH for patient RF



CHAPTER FIVE
SUMMARY AND FUTURE DIRECTIONS

5.1 BRCA1 MUTATION SCREENING IN SPORADIC OVARIAN TUMOURS

The incidence of BRCA1 mutations in unselected sporadic ovarian tumours has only been investigated in a few studies, the majority of which have been with small patient numbers. Of those published, only three examined more than 100 cases (Takahashi *et al*, 1995; Stratton *et al*, 1997; Rubin *et al*, 1998). Our aim was to study a large series of cases to both assess mutation incidence and attempt to identify additional BRCA1 mutation carriers, presenting with benign or borderline ovarian lesions, who might be suitable for inclusion in our loss of heterozygosity analysis of inclusion cyst DNA.

Amongst our initial series of 223 patients, screened for BRCA1 mutations using the Wessex 'priority' primers (exons 11B, 11J, 11L, 11O, 11P, 11Q, 11R, 2, 12, and 20), three unambiguous mutations were detected. Two patients carried exon 11p mutations (one presenting with a serous tumour and the other an endometrioid lesion), whilst the third patient exhibited an 11q mutation (presenting with a serous malignancy). Only one of these individuals had a relevant family history of malignancy. All presented with invasive lesions.

Subsequently, for a subset of 123 of our initial 223 cases, the whole of exon 11 was examined, with no additional mutations detected. Unfortunately, sufficient DNA was not available to analyse the remainder of our cases in this way. When the number of patients included in our study was extended by a further 61, all of whom were analysed using the 'priority' primers and those covering the whole of exon 11, only two additional mutations were found, again on 11p and 11q, and again in patients presenting with invasive malignancy. Two unclassified variants were also detected (one in exon 11a and one in exon 11e).

Thus, this part of our study failed to identify any additional BRCA1 mutation carriers for inclusion in our LOH study - all those found to have mutations presented with invasive ovarian lesions.

Of interest, however, is the finding of a BRCA1 mutation in an individual presenting with an endometrioid malignancy, as this has not previously been described. In the other published studies described in Chapter 3 (Table 3.4), all except one of the mutations observed have been in individuals with serous ovarian malignancies. Stratton *et al* (1997) reports one mutation in a borderline mucinous tumour, in an individual with a family history of breast cancer.

5.2 GENETIC ANALYSIS OF ABERRANT HISTOLOGICAL STRUCTURES IN PROPHYLACTIC OOPHORECTOMY SPECIMENS FROM WOMEN WITH GERMLINE BRCA1 AND BRCA2 MUTATIONS

Extensive molecular genetic research aimed at identifying genes involved in the development of ovarian cancer has been carried out. Studies in various tumour types have shown that alterations in oncogenes and tumour suppressor genes are vital to the development of malignancy, as well as the formation of precursor lesions.

We hypothesised that the ovaries of women carrying BRCA1 and BRCA2 mutations might contain increased numbers of precursor lesions when compared with ovaries of women not carrying these mutations (as per Salazar *et al*, 1996), and that genetic change within these precursor lesions might be responsible for the high incidence of early malignant transformation seen in these individuals.

We had available to us prophylactically removed ovaries from six women, five of whom were BRCA1 mutation carriers. The other carried a BRCA2 mutation. We were able to identify inclusion cysts in all these individuals (one of the structures identified as an atypical, potential precursor lesion by Salazar *et al*, 1996). For one of our BRCA1 carriers we also identified multiple areas of microscopic malignancy. Our aim was to analyse for genetic alterations within these lesions, in order to provide data to support the hypothesis of their role as malignant precursors.

The lesions described are very small, and until the advent of PCR (polymerase chain reaction) and tissue microdissection it was not possible to adequately study their molecular genetics. However, the tissue microdissection technique described in Chapter 2 (section 2.2.1) is simple, reliable, and inexpensive, allowing isolation of selected populations of cells from archival tissue.

The LOH (loss of heterozygosity) analysis described in this study has been widely used to investigate the molecular genetics of many tumour types. However, there are difficulties inherent to this technique. Tumour samples can only be analysed for LOH if matched normal DNA is available for comparison. Hence, we had to ensure that either leucocyte DNA was available for the individuals included in our study, or that areas of microscopically normal tissue were dissected from our archival ovarian samples. Secondly, results are often ambiguous, as a result of contaminating stromal tissue - presence of 'normal' cells within a 'tumour' sample results in loss of allelic intensity at a microsatellite marker for which the 'tumour' sample shows LOH. This may result in LOH being 'missed'.
Inconsistencies were sometimes observed in our PCR results, but all reactions were repeated at least twice, and we were conservative in our interpretation of LOH. The data presented may, therefore, represent an underestimate of the frequency of genetic alterations present.

We were able to identify allelic losses in inclusion cyst material in three of the five BRCA1 mutation carriers included in our study. No LOH was detected, with any of the 50 microsatellite markers used, in the inclusion cyst DNA from the BRCA2 mutation carrier. Allelic losses were also observed in the samples of microscopic malignancy dissected for one of the BRCA1 mutation carriers. The LOH observed was in keeping with previously published literature for LOH in ovarian tumours. The finding of significant LOH (>20%) on chromosome arms 6q, 9p, 11q, and 17q in inclusion cyst DNA compares well with previously published studies of benign and borderline ovarian lesions. This suggests that the inclusion cysts detected may represent even earlier ovarian lesions in the pathway to malignant transformation.

It would be useful to perform a study with larger numbers of patients, in order to assess whether the lack of LOH in inclusion cyst DNA from a BRCA2 mutation carrier is a phenomenon true of all BRCA2 associated lesions. Larger numbers of BRCA1 mutation carriers would also permit greater comparison of LOH patterns between inclusion cysts from different individuals.

A future study using a greater number of microsatellite markers would also be worthwhile. Whilst our study included 50 markers, chosen following review of existing LOH data for ovarian cancer and other tumour types, and covering the majority of chromosome arms, there were several chromosome arms for which PCR optimisation was not possible, and there were also a number of markers which, despite satisfactory optimisation with leucocyte DNA, failed to PCR with DNA from archival ovarian material.
Our study highlighted the importance of using microsatellite markers with PCR products <200bp, in order to ensure PCR reliability, and this should be borne in mind when choosing markers for use in future similar studies.

A comparison of the prevalence of inclusion cysts in carriers and non-carriers of BRCA1 and BRCA2 mutations would allow clarification of the conflicting findings of data published to date. Whilst Salazar *et al* (1996) reported an increased incidence of such lesions in mutation carriers, when compared with non-carriers, Stratton *et al* (1999) was unable to confirm this finding, reporting approximately equal frequencies.

A study similar in design to ours, but using ovarian tissue from non-carriers of BRCA1 or BRCA2, would also be useful. If the data of Stratton *et al* (1999) is accurate, one might expect to observe lower frequencies of LOH in inclusion cyst DNA from non-carriers, which would in turn help to explain the pattern of progression to ovarian cancer at a young age in BRCA1 and BRCA2 mutation carriers.

Technological advances, with the advent of high-density DNA microarray techniques, mean that it is now possible to screen large numbers of genes and assess their activity under various conditions (called gene-expression profiling). Tumour behaviour is

believed to reflect expression of thousands of genes and, thus, microarray technology should enable predictions to be made about tumour behaviour and the clinical consequences thereof (Berns, 2000).

Such technology could be useful in the setting of our study, allowing comparisons of gene expression to be made between 'normal' DNA, DNA from potential precursor lesions observed in prophylactic oophorectomy specimens, and DNA from malignant ovarian lesions in BRCA1 and BRCA2 mutation carriers. In this way, genes and pathways of importance for the tumorigenic process might be identified.

Appendix 1.

REAGENTS USED IN POLYMERASE CHAIN REACTION

1. DNA polymerase

a. **'Red Hot' Taq polymerase** (Advanced Biotechnologies, UK) was used at a concentration of 1U/µl.

b. **'Taq Express'** (Genpak Ltd, UK)

2. Reaction buffer

a. **10x reaction buffer** was supplied with the 'Red Hot' Taq polymerase, and contained: 200mM $(NH_4)_2SO_4$
750mM Tris Hcl
Tween 0.1% (w/v) $MgCl_2$ 15mM

b. **'Taq Express' reaction buffer** contained: 50mM Tris Hcl
16mM ammonium sulphate
3.5mM $MgCl_2$
150µg/ml BSA

3. Low dCTP nucleotide solution

A 250µl solution containing 1µl dCTP (0.4mM)
10µl dATP (4mM)
10µl dGTP (4mM)
10µl dTTP (4mM) in 219µl ultrapure water

Stock solutions of dCTP, dATP, dGTP, dTTP from Promega, UK

4. Nucleotide solution (for 'cold' PCR reaction prior to sequencing)

A 250µl solution containing	10µl dCTP	
	10µl dATP	
	10µl dGTP	
	10µl dTTP	in 210µl ultrapure water

Stock solutions from Promega, UK

All the above solutions were stored at -20°C when not in use.

5. P32 solution

A commercial preparation containing radio-labelled deoxycytidine-5'-triphosphate, [α-^{32}P] dCTP was used (Amersham International). The solution was stored at 4°C.

6. Buffers

A. Water

i. Standard purified water was used for preparation of electrophoresis buffers and gels (Elga UHQ, PS, Elga, Bucks, UK).

ii. Ultrapurified UV treated water was used for all other buffers, and for dilution of DNA, dNTP solutions, primers, and PCR reactions (Elga UHQ11, Elga, Bucks, UK).

B. Lysis buffers

i. 2x Sucrose/triton lysis buffer	634mM sucrose, 10mM $MgCl_2$, 2% (v/v) Triton x 100, 10mM Tris, pH 7.5
ii. SDS lysis buffer	75mM NaCl, 25mM EDTA, 1% SDS (v/v), pH 8

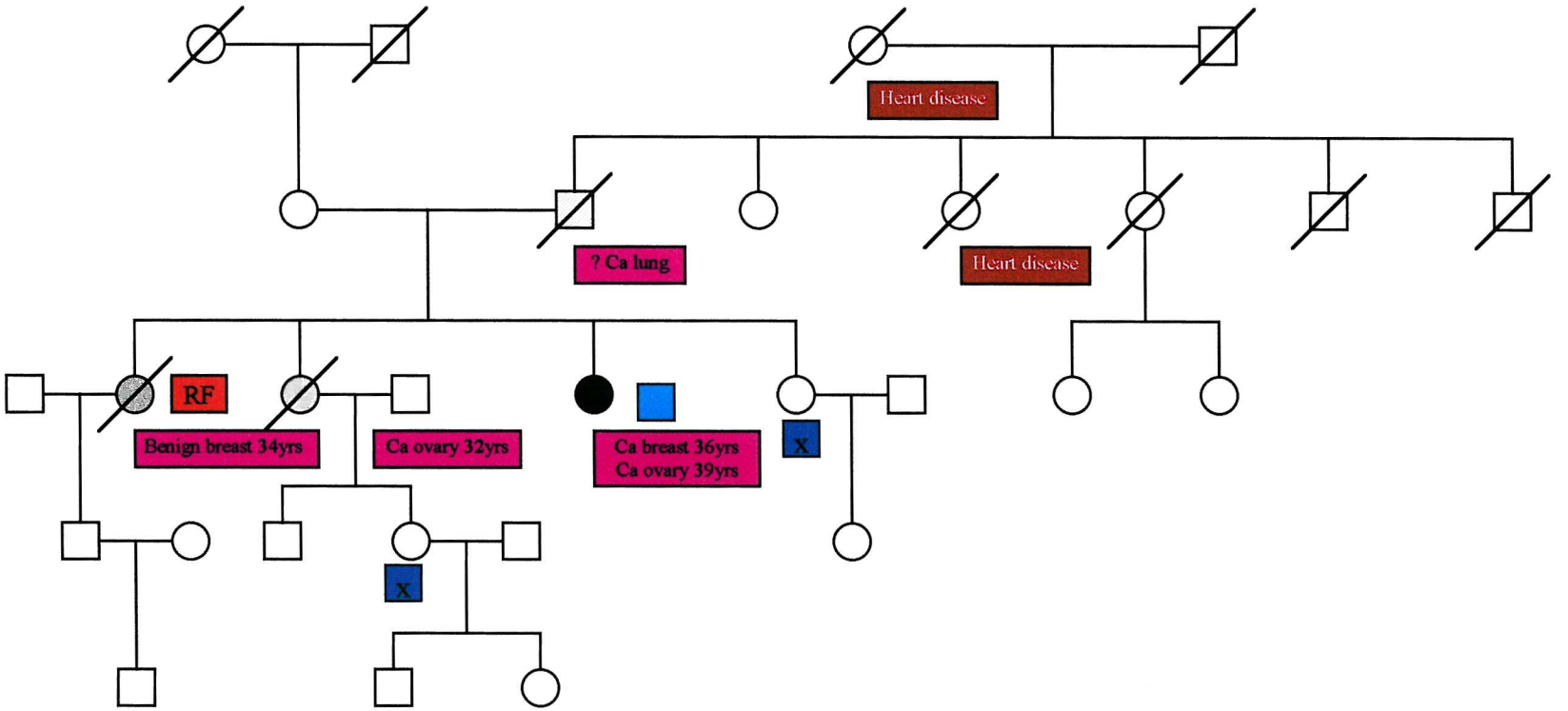
iii. Tween lysis buffer	50mM Tris, 1mM EDTA, 0.5% Tween 20 (v/v)
C. TE buffer	10mM EDTA, Tris base 100mM, pH8
D. TAE buffer (x50)	50mM EDTA, Tris base 2M, 57.1% Glacial acetic acid (v/v), pH 8
E. TBE buffer (x5)	20mM EDTA, Tris base 0.44M, Boric acid 0.44M, pH 8
F. Tracking dye (for agarose gel electrophoresis)	30% glycerol (v/v), 0.25% xylene cyanol (w/v)
G. 100bp ladder	50µl 100 bp ladder, 350µl 1xTE, 100µl tracking dye
H. Formamide buffer	90% formamide (v/v), 0.25% bromophenol blue (w/v), 0.25% xylene cyanol (w/v)

THERMAL CYCLING MACHINES USED FOR PCR

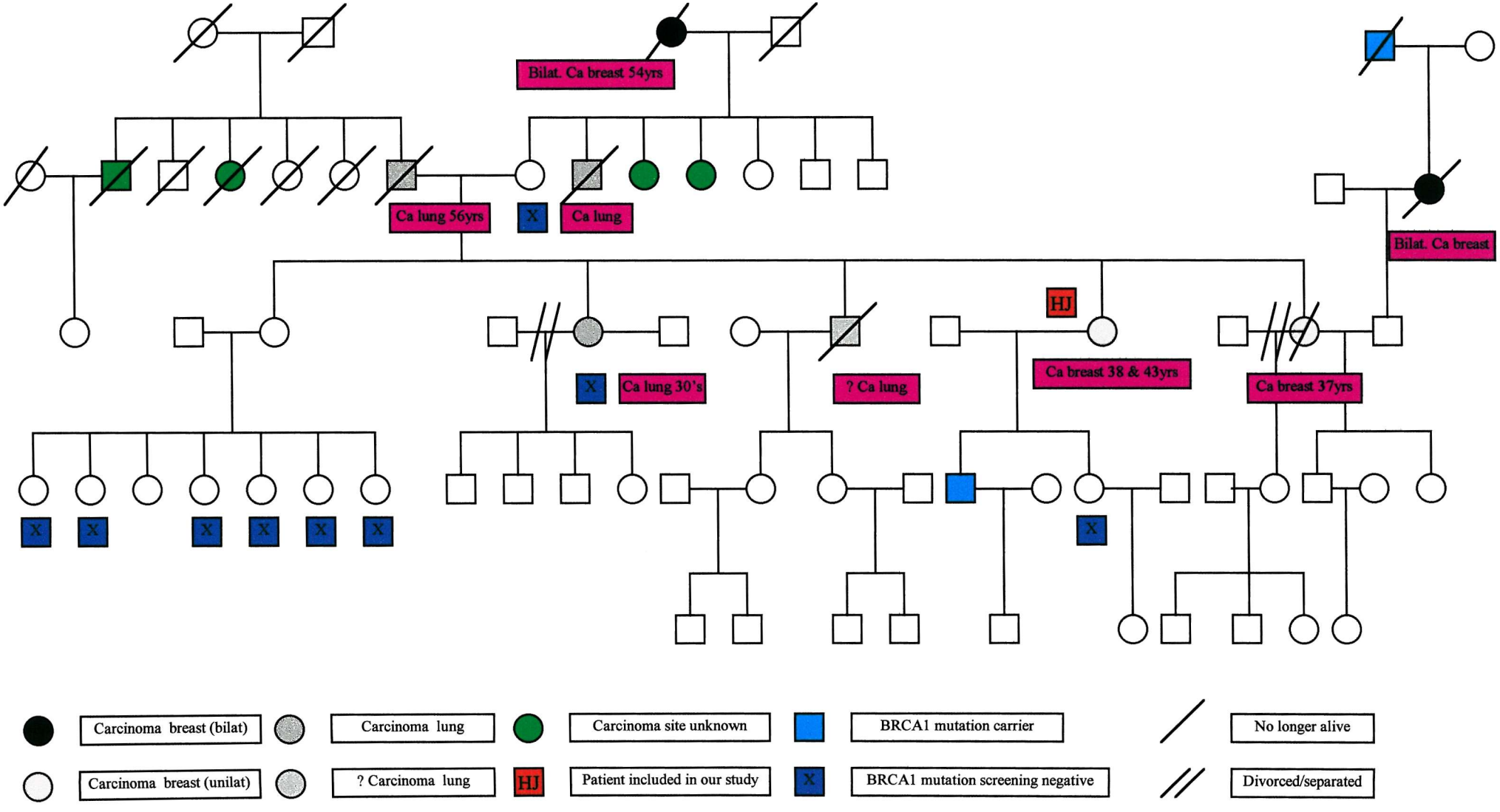
1. PTC 100 (MJ Research Inc, USA)

2. Progene (Techni, UK)

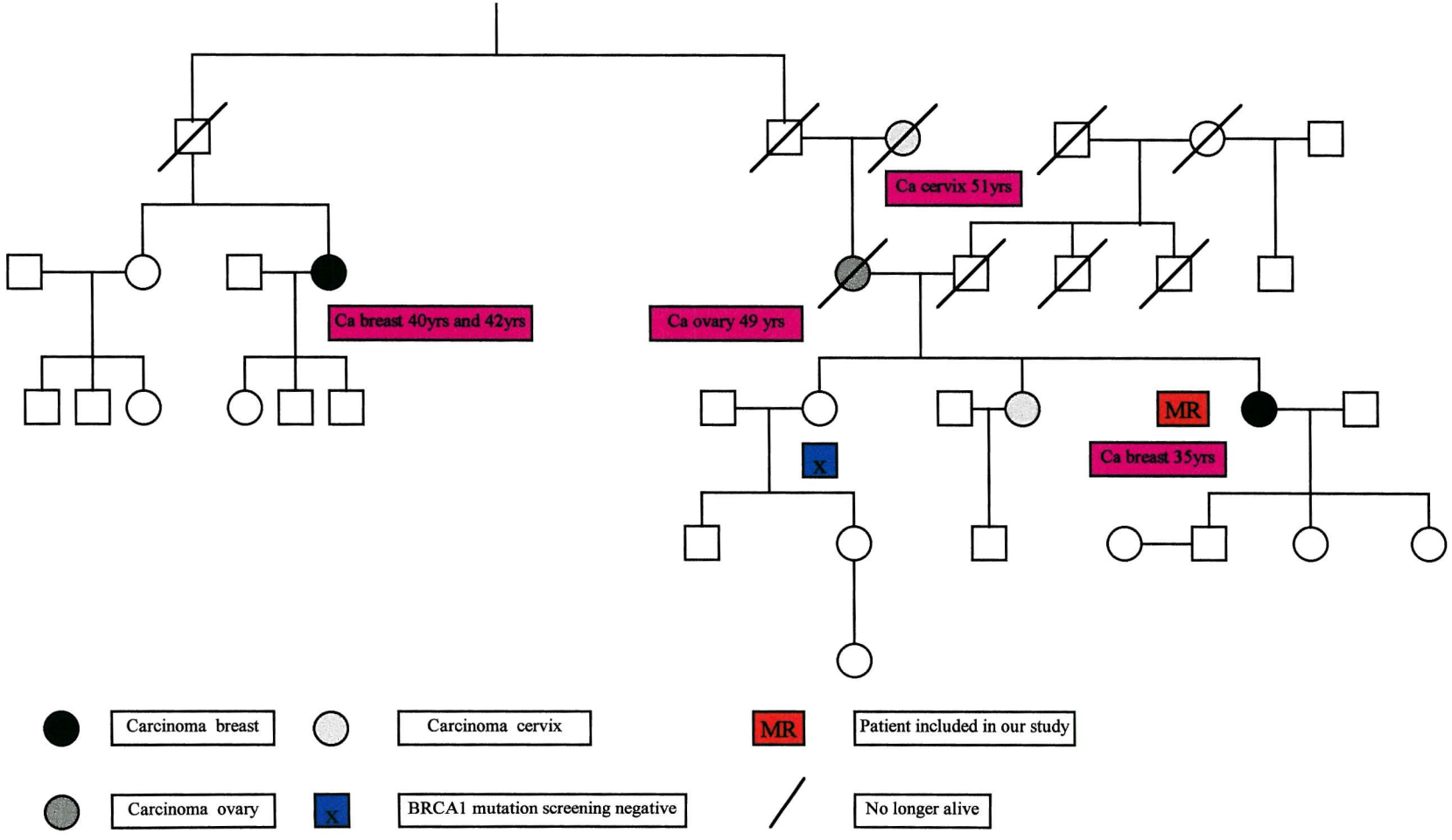
Appendix 2. Family pedigree for patient RF (BRCA1 exon 11q 3875 del 4 mutation - 1111 stop @ 1262)



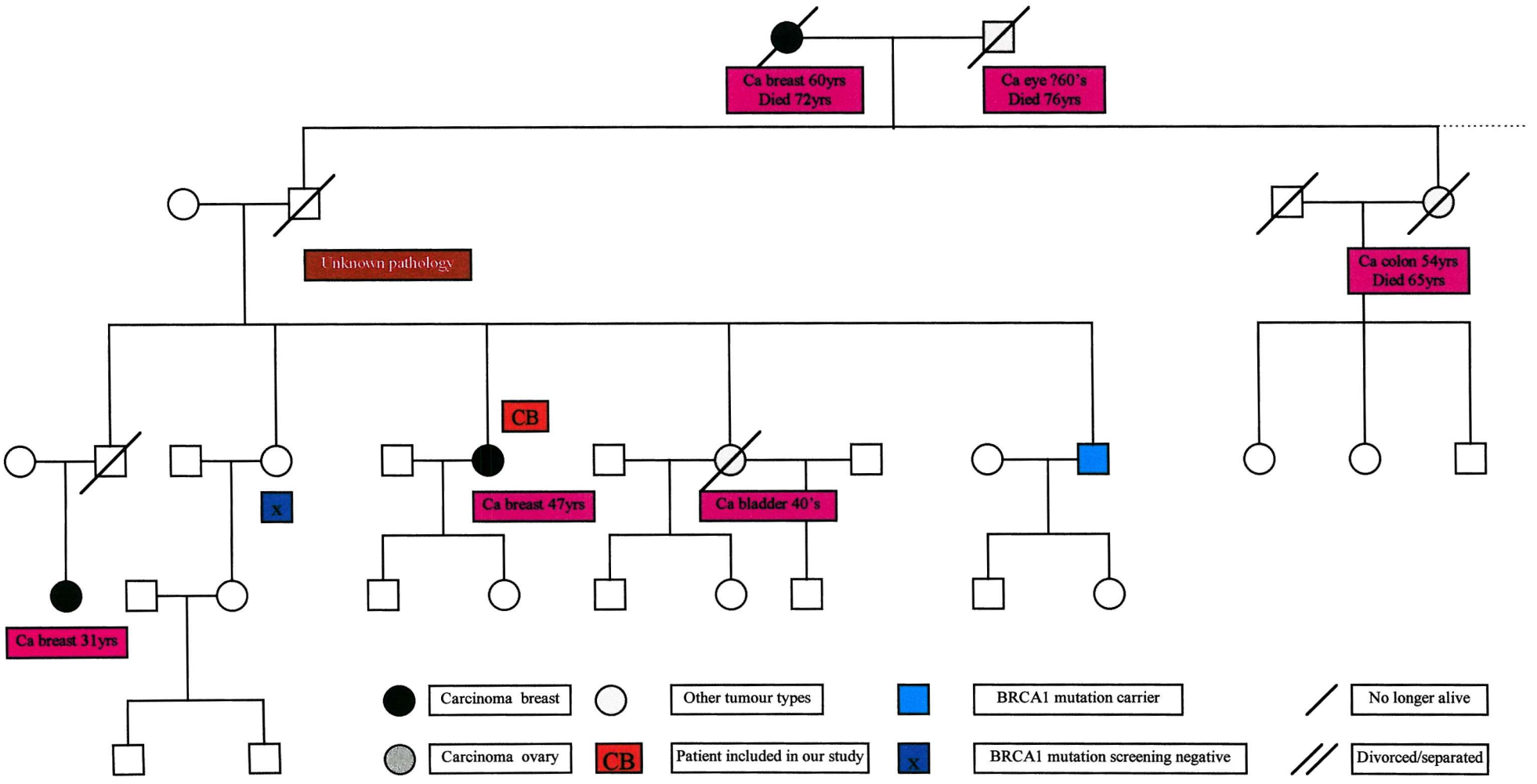
Appendix 3. Family pedigree for patient HJ (BRCA1 exon 11q 3875 del 4 mutation - 1111 stop @ 1262)



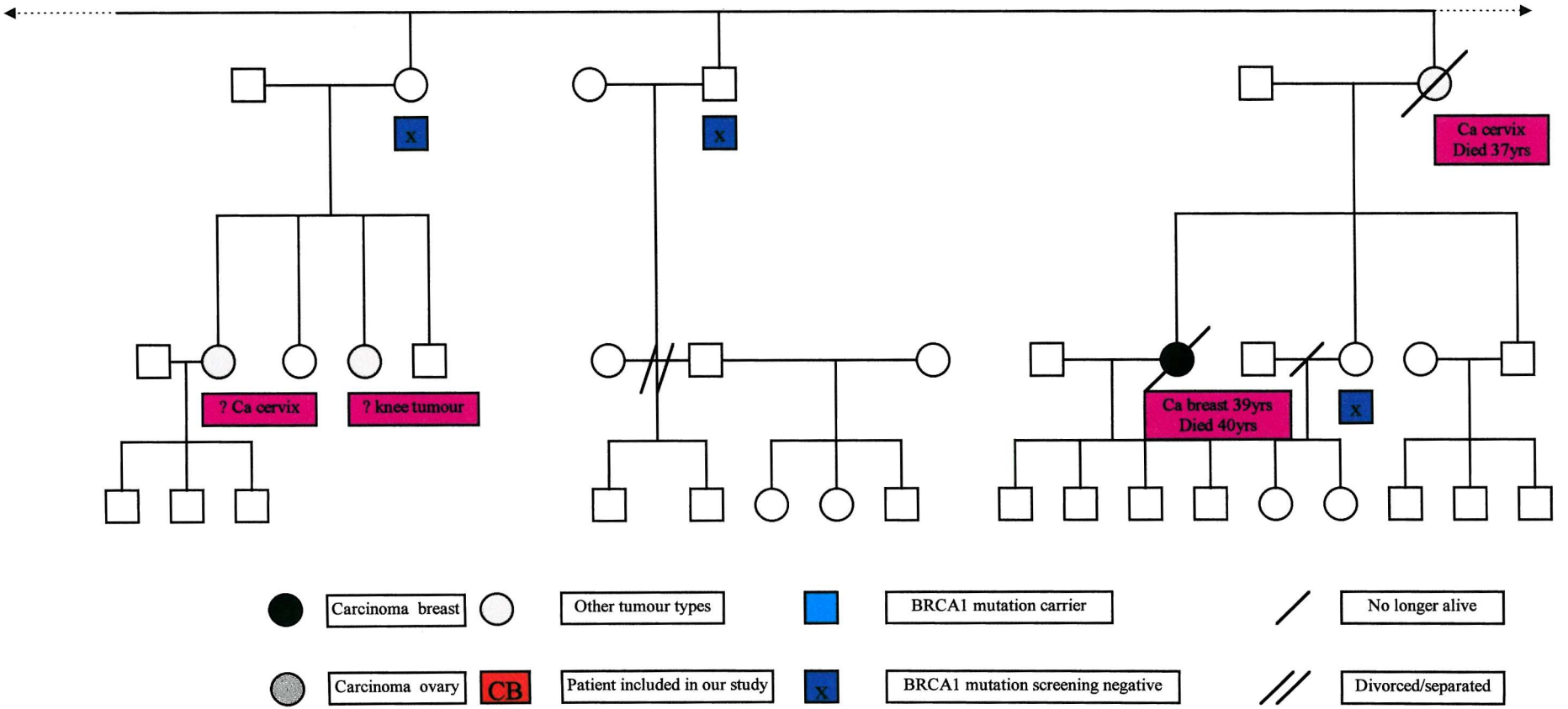
Appendix 4. Family pedigree for patient MR (BRCA1 exon 11r 4185 del AG mutation)



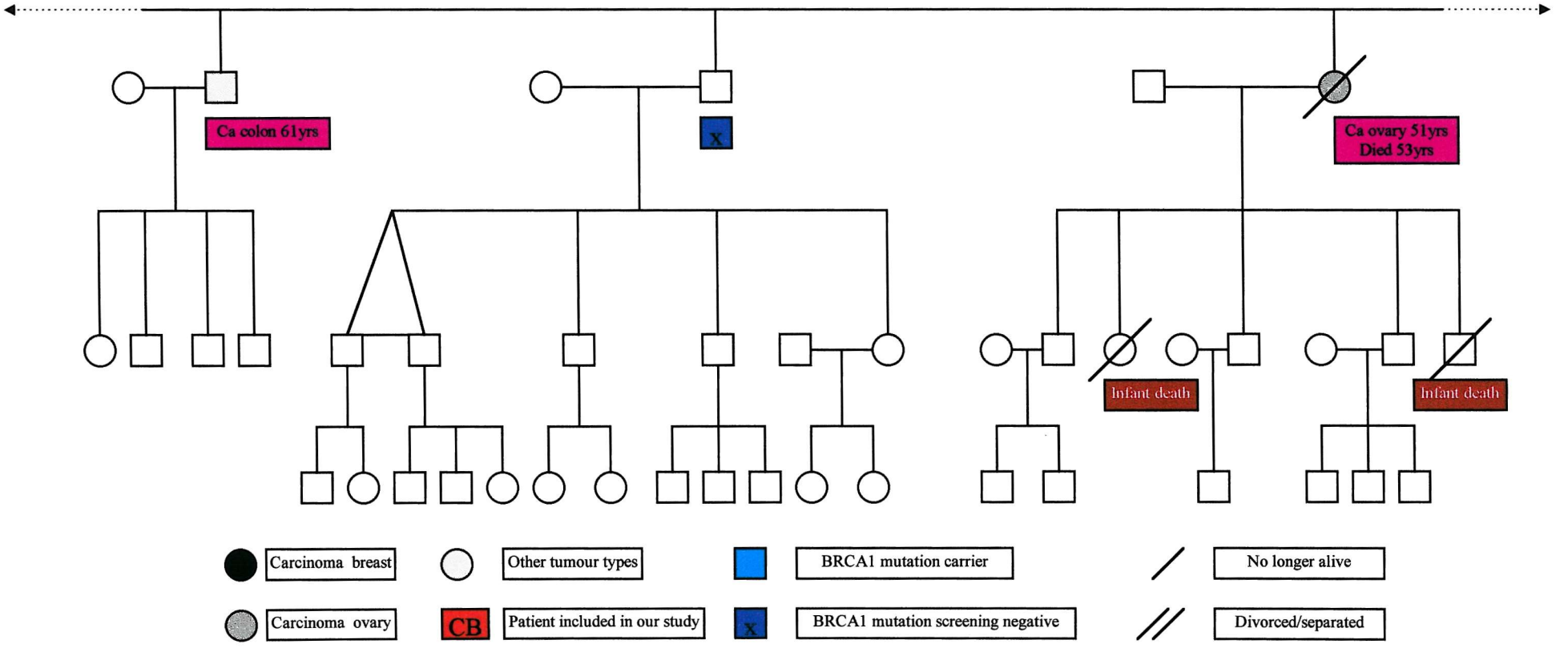
Appendix 5. Family pedigree for patient CB (BRCA1 exon 12 mutation - G to A transition at 4304)



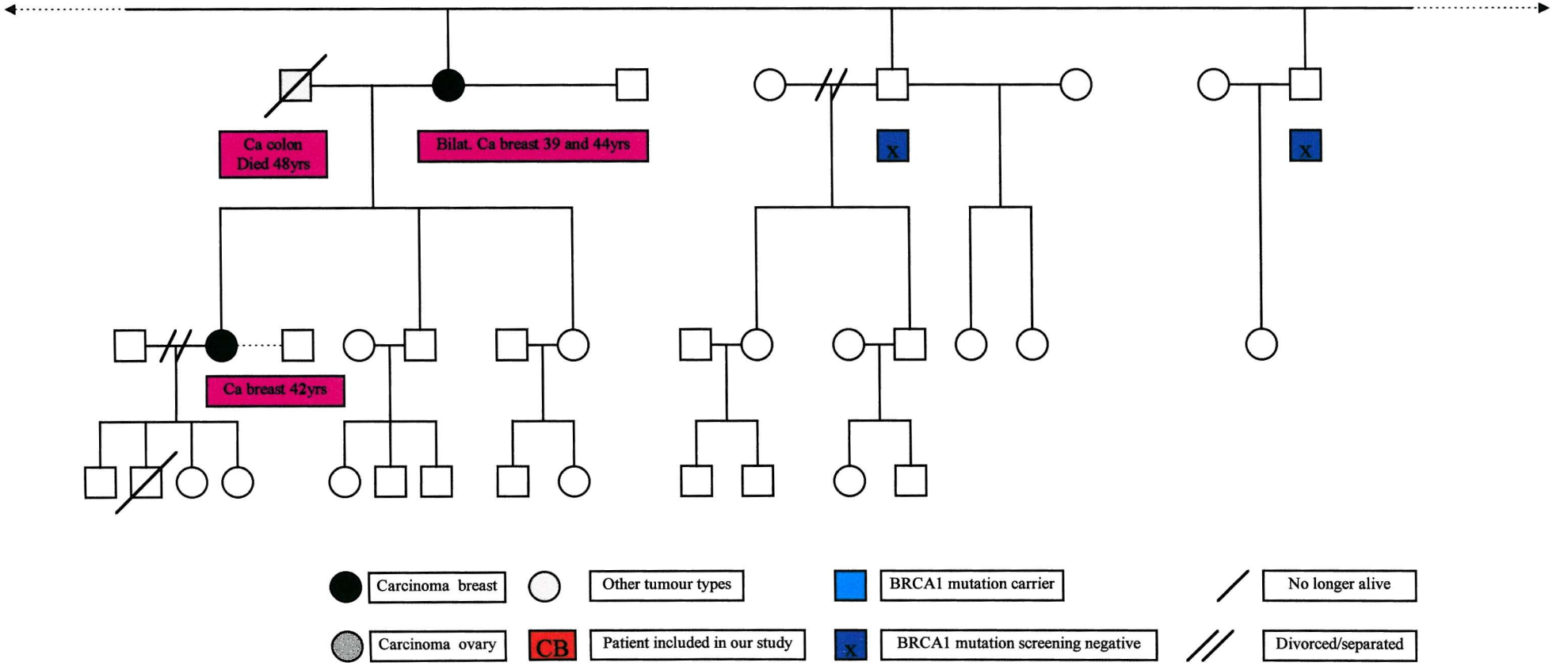
Family pedigree for patient CB (continued)



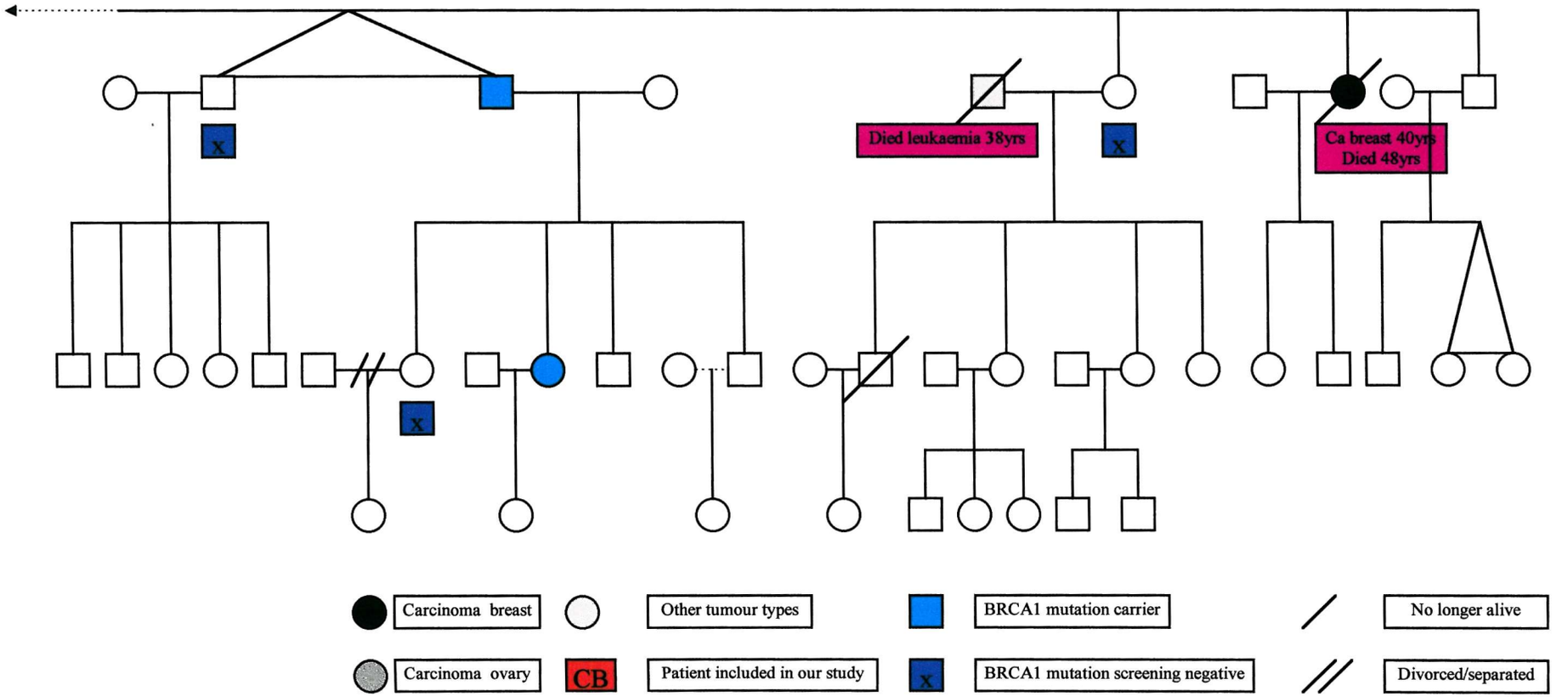
Family pedigree for patient CB (continued)



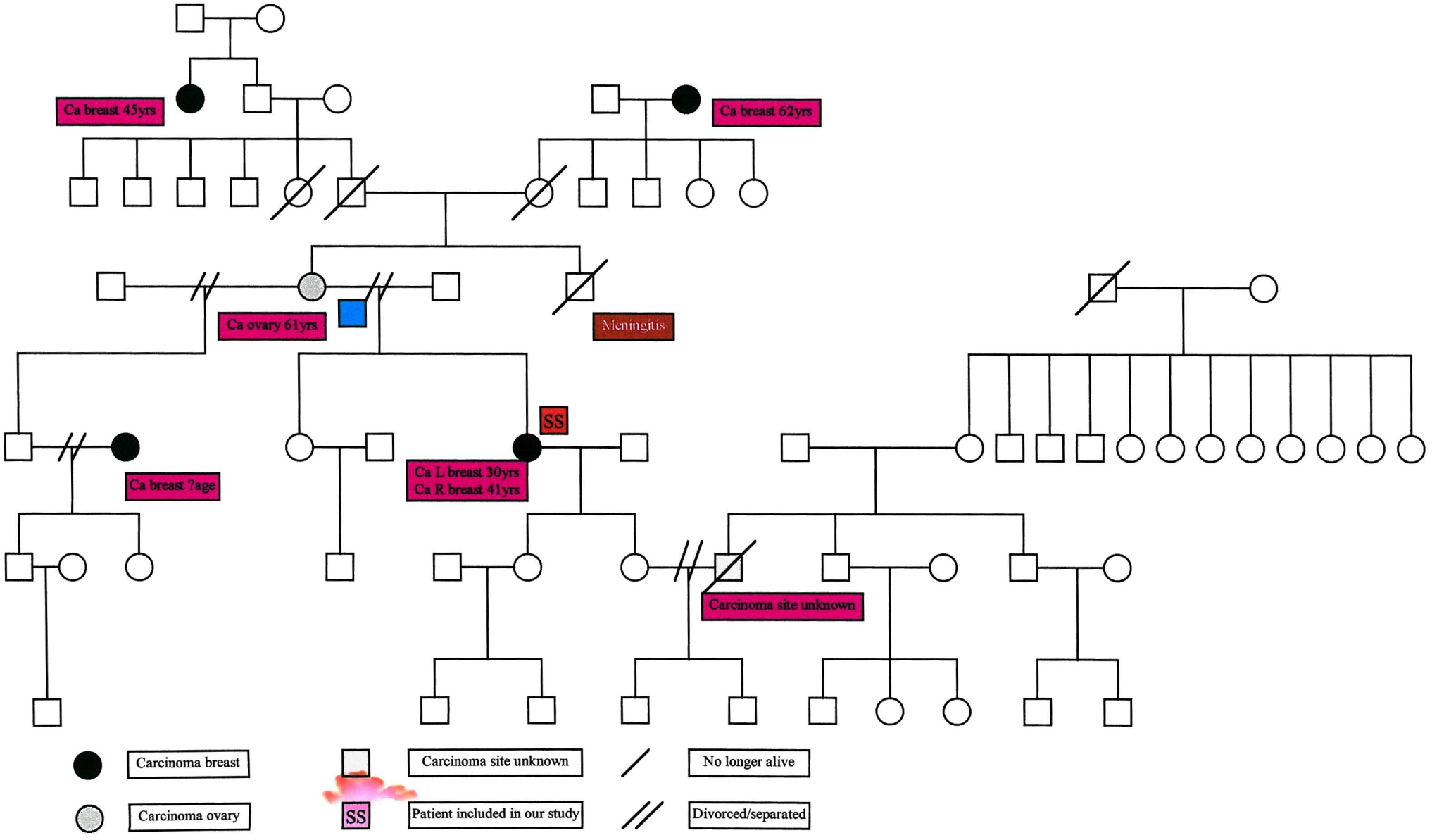
Family pedigree for patient CB (continued)



Family pedigree for patient CB (continued)



Appendix 6. Family pedigree for patient SS (BRCA2 exon 11f 2950 ins A)



REFERENCES

1. Insights into the functions of BRCA1 and BRCA2. *TIG* 2000;16:69-74.

2. Adami H-O, Bergstrom R, Persson I, Sparen P. The incidence of ovarian cancer in Sweden, 1960-1984. *Am. J. Epidemiol* 1990;132:446-452.

3. Adami H-O, Hsieh C-C, Lambe M, Trichopoulos D, Leon D, Persson I, et al. Parity, age at first childbirth, and risk of ovarian cancer. *Lancet* 1994;344:1250-1254.

4. Aihara T, Noguchi S, Sasaki Y, Nakano H, Monden M, Imaoka S. Clonal analysis of precancerous lesion of hepatocellular carcinoma. *Gastroenterology* 1996;111:455-461.

5. Akagi K, Kurahashi H, Arita N, Hayakawa T, Monden M, Mori T, et al. Deletion mapping of the long arm of chromosome 22 in human meningiomas. *Int. J. Cancer* 1995;60:178-182.

6. Allan G, Cottrell S, Trowsdale J, Foulkes W. Loss of heterozygosity on chromosome 5 in sporadic ovarian carcinoma is a late event and is not associated with mutations in APC at 5q21-22. *Human Mutation* 1994;3:283-291.

7. Allione F, Eisenger F, Parc P, Noguchi T, Sobol H, Birnbaum D. Loss of heterozygosity at loci from chromosome arm 22Q in human sporadic breast carcinomas. *Int. J. Cancer* 1998;75:181-186.

8. Amfo K, Neyns B, Teugels E, Lissens W, Bourgain C, Sutter PD, et al. Frequent deletion of chromosome 19 and a rare rearrangement of 19p13.3 involving the insulin receptor gene in human ovarian cancer. *Oncogene* 1995;11:351-358.

9. Amos C, Shaw G, Tucker M, Hartge P. Age at onset for familial epithelial ovarian cancer. *JAMA* 1992;268:1896-1899.

10. Amos C, Struewing J. Genetic epidemiology of epithelial ovarian cancer. *Cancer* 1993;71:566-572.

11. An H, Niederacher D, Picard F, Roeyen Cv, Bender H, Beckmann M. Frequent allelic loss on 9p21-22 defines a smallest common region in the vicinity of the CDKN2 gene in sporadic breast cancer. *Genes Chromosomes Cancer* 1996;17:14-20.

12. Anderson L, Friedman L, Osborne-Lawrence S, Lynch E, Weissenbach J, Bowcock A, et al. High-density genetic map of the BRCA1 region of chromosome 17q21-q21. *Genomics* 1993;17:618-623.

13. Anderson M, Lambert H, Rustin G, Soutter W. Carcinoma of the Ovary and Fallopian Tube. In: Shaw R, Soutter W, Stanton S, editors. *Gynaecology.* 2nd ed: Churchill Livingstone, 1997:627-648.

14. Armitage P, Doll R. The age distribution of cancer and a multi-stage theory of carcinogenesis. *Br. J. Cancer* 1954;8:1-10.

15. Arnold N, Hagele L, Walz L, Schempp W, Pfisterer J, Bauknecht T, et al. Overrepresentation of 3q and 8q material and loss of 18q material are recurrent findings in advanced human ovarian cancer. *Genes Chromosomes Cancer* 1996;16:46-54.

16. Averette H, Janicek M, Menck H. The National Cancer Data Base report on ovarian cancer. *Cancer* 1995;76(6):1096-1103.

17. Bandera C, Takahashi H, Behbakht K, Liu P, LiVolsi V, Benjamin I, et al. Deletion mapping of two potential chromosome 14 tumor suppressor gene loci in ovarian carcinoma. *Cancer Res* 1997;57:513-515.

18. Banks E, Beral V, Reeves G. The epidemiology of epithelial ovarian cancer: a review. *Int. J. Gynecol. Cancer* 1997;7:425-438.

19. Barrett M, Reid B, Joslyn G. Genotypic analysis of multiple loci in somatic cells by whole genome amplification. *Nucl. Acids Res* 1995;23:3488-3492.

20. Bell D, Scully R. Early de novo ovarian carcinoma. *Cancer* 1994;73:1859-1864.

21. Bell D, Jhanwar S, Testa J. Multiple regions of allelic loss from chromosome arm 6q in malignant mesothelioma. *Cancer Res* 1997;57:4057-4062.

22. Berchuck A, Cirisano F, Lancaster J, Schildkraut J, Wiseman R, Futreal A, et al. Role of BRCA1 mutation screening in the management of familial ovarian cancer. *Am. J. Obstet. Gynecol* 1996;175:738-746.

23. Berchuck A, Cirisano F. Genetics of ovarian cancer. *The Endocrinologist* 1997;7:103-106.

24. Berchuck A, Carney M, Futreal P. Genetic susceptibility testing and prophylactic oophorectomy. *Eur. J. Obstet. Gynecol. Reprod. Biol* 1999;82:159-164.

25. Berek J, Martinez-Maza O, Hamilton T, Trope C, Kaern J, Baak J, et al. Molecular and biological factors in the pathogenesis of ovarian cancer. *Ann. Oncol.* 1993;4(Suppl. 4):S3-S16.

26. Berman D, Wagner-Costalas J, Schultz D, Lynch H, Daly M, Godwin A. Two distinct origins of a common BRCA1 mutation in breast-ovarian cancer families: a genetic study of 15 185delAG-mutation kindreds. *Am. J. Hum. Genet* 1996;58:1166-1176.

27. Berns A. Gene expression in diagnosis. *Nature* 2000;403:491-492.

28. Bewtra C, Watson P, Conway T, Read-Hippee C, Lynch H. Hereditary ovarian cancer: a clinicopathological study. *Int. J. Gynecol. Pathol* 1992;11(3):180-187.

29. Bianchi A, Navone N, Conti C. Detection of loss of heterozygosity in formalin-fixed paraffin-embedded tumor specimens by the polymerase chain reaction. *Am. J. Pathol* 1991;138:279-284.

30. Bicher A, Ault K, Kimmelman A, Gershenson D, Reed E, Liang B. Loss of heterozygosity in human ovarian cancer on chromosome 19q. *Gynecol. Oncol* 1997;66:36-40.

31. Boente M, Hamilton T, Godwin A, Buetow K, Kohler M, Hogan W, et al. Early ovarian cancer: a review of its genetic and biologic factors, detection, and treatment. *Curr. Probl. Cancer* 1996;20:83-137.

32. Bohm M, Wieland I. Analysis of tumour-specific alterations in native specimens by PCR: how to procure the tumour cells! *Int. J. Oncology* 1997;10:131-139.

33. Boland C, Sato J, Appelman H, Bresalier R, Feinberg A. Microallelotyping defines the sequence and tempo of allelic losses at tumour suppressor gene loci during colorectal cancer progression. *Nature Med* 1995;1(9):902-909.

34. Boland C, Sato J, Saito K, Carethers J, Marra G, Laghi L, et al. Genetic instability and chromosomal aberrations in colorectal cancer: a review of the current models. *Cancer Detection and Prevention* 1998;22(5):377-382.

35. Booth M, Beral V. The Epidemiology of Ovarian Cancer. In: Hudson C, editor. *Ovarian cancer*: Oxford University Press, 1985:22-44.

36. Boyd J, Barrett J. Genetic and cellular basis of multistep carcinogenesis. *Pharmac. Ther* 1990;46:469-486.

37. Boyd J, Rubin S. Hereditary ovarian cancer: molecular genetics and clinical implications. *Gynecol. Oncol* 1997;64:196-206.

38. Boyd J. Overview - The Genetics of Ovarian Cancer. In: Sharp F, Blackett T, Berek J, Bast R, editors. *Ovarian cancer*. 5th ed: ISIS Medical Media Ltd, 1998:3-16.

39. Broca P. *Traite des tumeurs*. Paris, France, 1886.

40. Broders A. Carcinoma. Grading and practical application. *Arch. Pathol* 1926;2:376-381.

41. Bronner C, Baker S, Morrison P, Warren G, Smith L, Lescoe M, et al. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. *Nature* 1994;368:258-261.

42. Brown M, Chuaqui R, Vocke C, Berchuck A, Middleton L, Emmert-Buck M, et al. Allelic loss on chromosome arm 8p: analysis of sporadic epithelial ovarian tumors. *Gynecol. Oncol* 1999;74:98-102.

43. Bryan E, Watson R, Davis M, Hitchcock A, Foulkes W, Campbell I. Localization of an ovarian cancer tumor suppressor gene to a 0.5-cM region between D22S284 and CYP2D, on chromosome 22q. *Cancer Res* 1996;56:719-721.

44. Buller R, Anderson B, Connor J, Robinson R. Familial ovarian cancer. *Gynecol. Oncol* 1993;51:160-166.

45. Caduff R, Svoboda-Newman S, Ferguson A, Frank T. Comparison of alterations of chromosome 17 in carcinoma of the ovary and of the breast. *Virchows Arch* 1999;434:517-522.

46. Campbell I, Beynon G, Davis M, Englefield P. LOH and mutation analysis of CDKN2 in primary human ovarian cancers. *Int. J. Cancer* 1995;63:222-225.

47. Casagrande J, Pike M, Ross R, Louie E, Roy S, Henderson B. 'Incessant ovulation' and ovarian cancer. *Lancet* 1979(July 28):170-172.

48. Castilla LH, Couch FJ, Erdos MR, Hoskins KF, Calzone K, Garber JE, et al. Mutations in the BRCA1 gene in families with early-onset breast and ovarian cancer. *Nature Genet* 1994;8:387-391.

49. Cave H, Cacheux V, Raynaud S, Brunie G, Bakkus M, Cochaux P, et al. ETV6 is the target of chromosome 12p deletions in t(12;21) childhood acute lymphocytic leukaemia. *Leukaemia* 1997;11:1459-1464.

50. Chen Y, Chen C-F, Riley D, Allred D, Chen P-L, Hoff DV, et al. Aberrant subcellular localisation of BRCA1 in breast cancer. *Science* 1995;270:789-791.

51. Chenevix-Trench G, Leary J, Kerr J, Michael J, Kefford R, Hurst T, et al. Frequent loss of heterozygosity on chromosome 18 in ovarian adenocarcinoma which does not always include the DCC locus. *Oncogene* 1992;7:1059-1065.

52. Chenevix-Trench G, Kerr J, Friedlander M, Hurst T, Sanderson B, Coglan M, et al. Homozygous deletions on the short arm of chromosome 9 in ovarian adenocarcinoma cell lines and loss of heterozygosity in sporadic tumors. *Am. J. Hum. Genet* 1994;55:143-149.

53. Chenevix-Trench G, Kerr J, Hurst T, Shih Y-C, Purdie D, Bergman L, et al. Analysis of loss of heterozygosity and KRAS2 mutations in ovarian neoplasms: clinicopathological correlations. *Genes Chromosomes Cancer* 1997;18:75-83.

54. Cheng P, Gosewehr J, Kim T, Velicescu M, Wan M, Zheng J, et al. Potential role of the inactivated X chromosome in ovarian epithelial tumor development. *J. Natl. Cancer. Inst* 1996;88:510-518.

55. Choi C, Cho S, Horikawa I, Berchuck A, Wang N, Cedrone E, et al. Loss of heterozygosity at chromosome segment Xq25-26.1 in advanced human ovarian carcinomas. *Genes Chromosomes Cancer* 1997;20:234-242.

56. Chong J-M, Fukayama M, Hayashi Y, Takizawa T, Koike M, Konishi M, et al. Microsatellite instability in the progression of gastric carcinoma. *Cancer Res* 1994;54:4595-4597.

57. Cliby W, Ritland S, Hartmann L, Dodson M, Halling K, Keeney G, et al. Human epithelial ovarian cancer allelotype. *Cancer Res* 1993;53:2393-2398.

58. Colitti C, Rodabaugh K, Welch W, Berkowitz R, Mok S. A novel 4cM minimal deletion unit on chromosome 6q25.1-q25.2 associated with high grade invasive epithelial ovarian carcinomas. *Oncogene* 1998;16:555-559.

59. Cook L, Kamb M, Weiss N. Perineal powder exposure and the risk of ovarian cancer. *Am. J. Epidemiol* 1997;145:459-465.

60. Cooney K, Wetzel J, Consolino C, Wojno K. Identification and characterization of proximal 6q deletions in prostate cancer. *Cancer Res* 1996;56:4150-4153.

61. Cowan R, Hoban P, Kelsey A, Birch J, Gattamaneni R, Evans D. The gene for the naevoid basal cell carcinoma syndrome acts as a tumour-suppressor gene in medulloblastoma. *Br. J. Cancer* 1997;76:141-145.

62. Cramer D, Welch W. Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. *JNCI* 1983;71:717-721.

63. Cramer D, Barbieri R, Muto M, Kelly A, Brucks J, Harlow B. Characteristics of women with a family history of ovarian cancer. *Cancer* 1994;74:1318-1322.

64. Czernobilsky B, Silverman B, Mikuta J. Endometrioid carcinoma of the ovary. *Cancer* 1970;26:1141-1152.

65. Davis M, Hitchcock A, Foulkes W, Campbell I. Refinement of two 11q regions of loss of heterozygosity in ovarian cancer. *Cancer Res* 1996;56:741-744.

66. Decker D. Philadelphia, editor. Grading of gynecologic malignancy: epithelial ovarian cancer. Epithelial ovarian cancer, 7th national cancer conference; 1973.

67. Decker D, Malkasian G, Taylor W. Prognostic importance of histologic grading in ovarian carcinoma. *Natl. Cancer Inst. Monogr* 1975;42:9-11.

68. Dib C, Faure S, Fizames C, Sampson D, Drouot N, Vignal A, et al. A comprehensive genetic map of the human genome based on 5264 microsatellites. *Nature* 1996;380:152-154.

69. Dietmaier W, Hartmann A, Wallinger S, Heinmoller E, Kerner T, Endi E, et al. Multiple mutation analyses in single tumor cells with improved whole genome amplification. *Am. J. Pathol* 1999;154:83-95.

70. Ding S, Delhanty J, Zografos G, Michail N, Dooley J, Habib N. Chromosome allele loss in colorectal liver metastases and its association with clinical features. *Br. J. Surg* 1994;81:875-878.

71. Dodson M. Comparison of loss of heterozygosity patterns in invasive low-grade and high-grade epithelial ovarian carcinomas. *Cancer Res* 1993;53:4456-4460.

72. Dodson M, Cliby W, Xu H-J, DeLacey K, Hu S-X, Keeney G, et al. Evidence of functional RB protein in epithelial ovarian carcinomas despite loss of heterozygosity at the RB locus. *Cancer Res* 1994;54:610-613.

73. Dyer M. Chromosomal Translocations in Cancer. In: Yarnold J, Stratton M, McMillan T, editors. *Molecular Biology for Oncologists*. 2nd ed: Chapman and Hall, 1996:38-44.

74. Easton D, bishop D, Ford D, Crockford G. Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. *Am. J. Hum. Genet* 1993;52:678-701.

75. Easton D. Breast cancer genes - what are the real risks? *Nature Genet* 1997;16:210-211.

76. Eccles D, Russell S, Haites N. Early loss of heterozygosity on 17q in ovarian cancer. *Oncogene* 1992;7:2069-2072.

77. Eccles D, Gruber L, Stewart M, Steel C, Leonard R. Allele loss on chromosome 11p is associated with poor survival in ovarian cancer. *Dis. Markers* 1992;10:95-99.

78. Eccles D, Englefield P, Soulby M, Campbell I. BRCA1 mutations in southern England. *B. J. Cancer* 1998;77:2199-2203.

79. Edelson M, Lau C, Colitti C, Welch W, Bell D, Berkowitz R, et al. A one centimorgan deletion unit on chromosome Xq12 is commonly lost in borderline and invasive epithelial ovarian tumors. *Oncogene* 1998;16:197-202.

80. Ehlen T, Dubeau L. Loss of heterozygosity on chromosomal segments 3p, 6q, and 11p in human ovarian carcinomas. *Oncogene* 1990;5:219-223.

81. Eisinger F, Alby N, Bremond A, Dauplat J, Espie M, Janiaud P, et al. Recommendations for medical management of hereditary breast and ovarian cancer: The French National Ad Hoc Committee. *Ann. Oncol* 1998;9:939-950.

82. Emi M, Fujiwara Y, Nakajima T, Tsuchiya E, Tsuda H, Hirohashi S, et al. Frequent loss of heterozygosity for loci on chromosome 8p in hepatocellular carcinoma, colorectal cancer, and lung cancer. *Cancer Res* 1992;52:5368-5372.

83. Englefield P, Foulkes W, Campbell I. Loss of heterozygosity on chromosome 22 in ovarian carcinoma is distal to and is not accompanied by mutations in NF2 at 22q12. *B. J. Cancer* 1994;70:905-907.

84. Enomoto T, Fujita M, Cheng C, Nakashima R, Ozaki M, Inoue M, et al. Loss of expression and loss of heterozygosity in the DCC gene in neoplasms of the human female reproductive tract. *B. J. Cancer* 1995;71:462-467.

85. Fathalla M. Incessant ovulation - a factor in ovarian neoplasia? *Lancet* 1971;2:163.

86. Fearon E, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759-767.

87. Fishel R, Kolodner R. Identification of mismatch repair genes and their role in the development of cancer. *Curr. Opin. Genet. Dev* 1995;5:382-395.

88. Ford D, Easton D, Bishop D, Narod S, Goldgar D. Risks of cancer in BRCA1-mutation carriers. *Lancet* 1994;343:692-695.

89. Ford D, Easton D, Peto J. Estimates of the gene frequency of BRCA1 and its contribution to breast and ovarian cancer incidence. *Am. J. Hum. Genet* 1995;57:1457-1462. (a)

90. Ford D. The genetics of breast and ovarian cancer. *B. J. Cancer* 1995;72:805-812. (b)

91. Foster K, Harrington P, Kerr J, Russell P, DiCioccio R, Scott I, et al. Somatic and germline mutations of the BRCA2 gene in sporadic ovarian cancer. *Cancer Res* 1996;56:3622-2625.

92. Foulkes W, Ragoussis J, Stamp G, Allan G, Trowsdale J. Frequent loss of heterozygosity on chromosome 6 in human ovarian carcinoma. *B. J. Cancer* 1993;67:551-559. (a)

93. Foulkes W, Campbell I, Stamp G, Trowsdale J. Loss of heterozygosity and amplification on chromosome 11q in human ovarian cancer. *B. J. Cancer* 1993;67:268-273. (b)

94. Foulkes W, Black D, Stamp G, Solomon E, Trowsdale J. Very frequent loss of heterozygosity throughout chromosome 17 in sporadic ovarian carcinoma. *Int. J. Cancer* 1993;54:220-225. (c)

95. Fox H, Anderson J. The Female Reproductive Tract and the Breast. In: Anderson J, editor. *Muir's Textbook of Pathology*. 12th ed: Edward Arnold, 1985.

96. Friedlander M. Prognostic Factors. In: Sharp F, Blackett T, Berek J, Bast R, editors. *Ovarian Cancer*. 5th ed: ISIS Medical Media Ltd, 1998:199-215.

97. Friedman L, Ostermeyer E, Lynch E, Szabo C, Anderson L, Dowd P, et al. The search for BRCA1. *Cancer Res* 1994;54:6374-6382. (a)

98. Friedman L. Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families. *Nature Genet* 1994;8:399-404. (b)

99. Friedman L, Szabo C, Ostermeyer E, Dowd P, Butler L, Park T, et al. Novel inherited mutations and variable expressivity of BRCA1 alleles, including the founder mutation 185delAG in Ashkenazi Jewish families. *Am. J. Hum. Genet* 1995;57:1284-1297.

100. Fujita M, Enomoto T, Yoshino K, Nomura T, Buzard G, Inque M, et al. Microsatellite instability and alterations in the hMSH2 gene in human ovarian cancer. *Int. J. Cancer* 1995;64:361-366.

101. Fullwood P, Marchini S, Rader J, Martinez A, Macartney D, Broggini M, et al. Detailed genetic and physical mapping of tumor suppressor loci on chromosome 3p in ovarian cancer. *Cancer Res* 1999;59:4662-4667.

102 Futreal P, Liu Q, Shattuck-Eidens D, Cochran C, Harshman K, Tavtigian S, et al. BRCA1 mutations in primary breast and ovarian carcinomas. *Science* 1994;266:120-122.

103. Gabra H, Taylor L, Cohen B, Lessels A, Eccles D, Leonard R, et al. Chromosome 11 allele imbalance and clinicopathological correlates in ovarian tumours. *B. J. Cancer* 1995;72:367-375.

104. Gabra H, Watson J, Taylor K, Mackay J, Leonard R, Steel C, et al. Definition and refinement of a region of loss of heterozygosity at 11q23.3-q24.3 in epithelial ovarian cancer associated with poor prognosis. *Cancer Res* 1996;56:950-954.

105. Gallion H, Powell D, Morrow J, Pieretti M, Case E, Turker M, et al. Molecular genetic changes in human epithelial ovarian malignancies. *Gynecol. Oncol* 1992;47:137-142.

106. Gallion H, Pieretti M, DePriest P, Jr JvN. The molecular basis of ovarian cancer. *Cancer* 1995;76:1992-1997.

107. Gayther S. Germline mutations of the BRCA1 gene in breast and ovarian cancer families provide evidence for a genotype-phenotype correlation. *Nature Genet* 1995;11:428-433.

108. Gayther S, Mangion J, Russell P, Seal S, Barfoot R, Ponder B, et al. Variation of risks of breast and ovarian cancer associated with different germline mutations of the BRCA2 gene. *Nature Genet* 1997;15:103-105.

109. Gayther S, Ponder B. Clues to the function of the tumour susceptibility gene BRCA2. *Dis. Markers* 1998;14:1-8.

110. Gicquel C, Raffin-Sanson M, Gaston V, Bertagna X, Plouin P, Schlumberger M, et al. Structural and functional abnormalities at 11p15 are associated with the malignant phenotype in sporadic adrenocortical tumors: study on a series of 82 tumors. *J. Clin. Endocrinol. Metab* 1997;82:2559-2565.

111. Godfrey T, Cher M, Chhabra V, Jensen R. Allelic imbalance mapping of chromosome 16 shows two regions of common deletion in prostate adenocarcinoma. *Cancer Genet. Cytogenet* 1997;98:36-42.

112. Godwin A, Testa J, Hamilton T. The biology of ovarian cancer development. *Cancer* 1993;71:530-536.

113. Godwin A, Vanderveer L, Schultz D, Lynch H, Altomare D, Buetow K, et al. A common region of deletion on chromosome 17q in both sporadic and familial epithelial ovarian tumors distal to BRCA1. *Am. J. Hum. Genet* 1994;55:666-677.

114. Gonzalez-Zulueta M, Ruppert J, Tokino K, Tsai Y, III CS, Miyao N, et al. Microsatellite instability in bladder cancer. *Cancer Res* 1993;53:5620-5623.

115. Goold R, diSibio G, Xu H, Lang D, Dadgar J, Magrane G, et al. The development of sequence-tagged sites for human chromosome 4. *Hum. Mol. Genet* 1993;2:1271-1288.

116. Gore M, Chang J, Bridgewater J, Fisher C, Schofield J, Ponder B, et al. Non-surgical aspects of ovarian cancer. *Lancet* 1994;343:335-340.

117. Grann V, Panageas K, Whang W, Antman K, Neugut A. Decision analysis of prophylactic mastectomy and oophorectomy in BRCA1-positive or BRCA2-positive patients. *J. Clin. Oncol* 1998;16:979-985.

118. Greggi S, Genuardi M, Benedetti-Panici P, Cento R, Scambia G, Neri G, et al. Analysis of 138 consecutive ovarian cancer patients: incidence and characteristics of familial cases. *Gynecol. Oncol* 1990;39:300-304.

119. Gudas J, Nguyen H, Li T, Cowan K. Hormone-dependent regulation of BRCA1 in human breast cancer cells. *Cancer Res* 1995;55:4561-4565.

120. Gudmundsson J, Johannesdottir G, Bergthorsson J, Arason A, Ingvarsson S, Egilsson V, et al. Different tumor types from BRCA2 carriers show wild-type chromosome deletions on 13q12-q13. *Cancer Res* 1995;55:4830-4832.

121. Gusberg S, Deligdisch L. Ovarian dysplasia. *Cancer* 1984;54:1-4.

122. Gutmann D, Giordano M, Fishback A, Guha A. Loss of merlin expression in sporadic meningiomas, ependymomas and schwannomas. *Neurology* 1997;49:267-270.

123. Gyapay G, Morissette J, Vignal A, Dib C, Fizames C, Millasseau P, et al. The 1993-94 Genethon human genetic linkage map. *Nature Genet* 1994;7:246-339.

124. Gyapay G, Schmitt K, Fizames C, Jones H, Vega-Czarny N, Spillett D, et al. A radiation hybrid map of the human genome. *Hum. Mol. Genet* 1996;5:339-346.

125. Hall J, Lee M, Newman B, Morrow J, Anderson L, Huey B, et al. Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* 1990;250:1684-1689.

126. Hamann U. Low frequency of BRCA1 germline mutations in 45 German breast/ovarian cancer families. *J. Med. Genet* 1997;34:884-888.

127. Hatta Y, Takeuchi S, Yokota J, Koeffler H. Ovarian cancer has frequent loss of heterozygosity at chromosome 12p12.3-13.1 (region of TEL and Kip 1 loci) and chromosome 12q23-ter:evidence for two new tumour-suppressor genes. *B. J. Cancer* 1997;75:1256-1262.

128. Hearne C, Todd J. Tetranucleotide repeat polymorphism at the HPRT locus. *Nucleic Acids Res* 1991;19:5450.

129. Hogberg T, Carstensen J, Simonsen E. Treatment results and prognostic factors in a population-based study of epithelial ovarian cancer. *Gynecol. Oncol* 1993;48:38-49.

130. Hogervorst F, Cornelis R, Bout M, Vliet Mv, Oosterwijk J, Olmer R, et al. Rapid detection of BRCA1 mutations by the protein truncation test. *Nature Genet* 1995;10:208-212.

131. Holt J, Thompson M, Szabo C, Robinson-Benion C, Arteaga C, King M-C, et al. Growth retardation and tumour inhibition by BRCA1. *Nature Genet* 1996;12:298-302.

132. Hosking L, Trowsdale J, Nicolai H, Solomon E, Foulkes W, Stamp G, et al. A somatic BRCA1 mutation in an ovarian tumour. *Nature Genet* 1995;9:343-344.

133. Ingvarsson S. The BRCA1 and BRCA2 proteins and tumor pathogenesis. *Anticancer Res* 1999;19:2853-2862.

134. Ishwad C, Ferrell R, Hanley K, Davare J, Meloni A, Sandberg A, et al. Two discrete regions of deletion at 7q in uterine leiomyomas. *Genes Chromosomes Cancer* 1997;19:156-160.

135. Iwabuchi H, Sakamoto M, Sakunaga H, Ma Y-Y, Carcangiu M, Pinkel D, et al. Genetic analysis of benign, low-grade, and high-grade ovarian tumours. *Cancer Res* 1995:6172-6180.

136. Jacobs I, Smith S, Wiseman R, Futreal P, Harrington T, Osborne R, et al. A deletion unit on chromosome 17q in epithelial ovarian tumors distal to the familial breast/ovarian cancer locus. *Cancer Res* 1993;53:1218-1221.

137. Jacobs I, Lancaster J. The molecular genetics of sporadic and familial epithelial ovarian cancer. *Int. J. Gynecol. Cancer* 1996;6:337-355.

138. Jensen R, Thompson M, Jetton T, Szabo C, Meer Rvd, Helou B, et al. BRCA1 is secreted and exhibits properties of a granin. *Nature Genet* 1996;12:303-308.

139. Jiricny J. Mismatch repair and cancer. *Cancer Surveys* 1996;28:47-68.

140. Johannsson O, Ostermeyer E, Hakansson S, Friedman L, Johansson U, Sellberg G, et al. Founding BRCA1 mutations in hereditary breast and ovarian cancer in Southern Sweden. *Am. J. Hum. Genet* 1996;58:441-450.

141. Jones M, Nakamura Y. Deletion mapping of chromosome 3p in female genital tract malignancies using microsatellite polymorphisms. *Oncogene* 1992;7:1631-1634.

142. Katabuchi H, Rees Bv, Lambers A, Ronnett B, Blazes M, Leach F, et al. Mutations in DNA mismatch repair genes are not responsible for microsatellite instability in most sporadic endometrial carcinomas. *Cancer Res* 1995;55:5556-5560.

143. Kawakami M, Staub J, Cliby W, Hartmann L, Smith D, Shridhar V. Involvement of H-cadherin (CDH13) on 16q in the region of frequent deletion in ovarian cancer. *Int. J. Oncol* 1999;15:715-720.

144. Kerlikowske K, Brown J, Grady D. Should women with familial ovarian cancer undergo prophylactic oophorectomy? *Obstet. Gynecol* 1992;80:700-707.

145. Kerr J, Leary J, Hurst T, Shih Y-C, Antalis T, Friedlander M, et al. Allelic loss on chromosome 7q in ovarian adenocarcinomas: two critical regions and a rearrangement of the PLANH1 locus. *Oncogene* 1996;13:1815-1818.

146. Kim T, Benedict W, Xu H-J, Hu S-X, Gosewehr J, Velicescu M, et al. Loss of heterozygosity on chromosome 13 is common only in the biologically more aggressive subtypes of ovarian epithelial tumors and is associated with normal retinoblastoma gene expression. *Cancer Res* 1994;54:605-609.

147. Kimmelman A, Ross D, Liang B. Loss of heterozygosity of chromosome 10p in human gliomas. *Genomics* 1996;34:250-254.

148. Knudson A. Mutation and cancer: statistical study of retinoblastoma. *Proc. Nat. Acad. Sci. USA* 1971;68(4):820-823.

149. Knudson A. Hereditary cancers disclose a class of cancer genes. *Cancer* 1989;63:1888-1891.

150. Koch M, Gaedke H, Jenkins H. Family history of ovarian cancer patients: a case-control study. *Int. J. Epidemiol* 1989;18:782-785.

151. Koike M, Takeuchi S, Yokota J, Park S, Hatta Y, Miller C, et al. Frequent loss of heterozygosity in the region of the D7S523 locus in advanced ovarian cancer. *Genes Chromosomes Cancer* 1997;19:1-5.

152. Koonin E, Altschul S. Functional motifs. *Nature Genet* 1996;13:266-267.

153. Kosary C. FIGO stage, histology, histologic grade, age, and race as prognostic factors determining survival for cancers of the female gynecological system: an analysis of 1973-87 SEER cases of cancers of the endometrium, cervix, ovary, vulva, and vagina. *Semin. Surg. Oncol* 1994;10:31-46.

154. Larson A, Kern S, Curtiss S, Gordon R, Cavenee W, Hampton G. High resolution analysis of chromosome 3p alterations in cervical carcinoma. *Cancer Res* 1997;57:4082-4090.

155. Latil A, Baron J, Cussenot O, Fournier G, Soussi T, Boccon-Gibod L, et al. Genetic alterations in localised cancers of the prostate: identification of a common region of deletion on the chromosome 18q. *Bull. Cancer* 1995;82:589-597.

156. Launonen V, Stenback F, Puistola U, Bloigu R, Huusko P, Kytola S, et al. Chromosome 11q22.3-q25 LOH in ovarian cancer: association with a more aggressive disease course and involved subregions. *Gynecol. Oncol* 1998;71:299-304.

157. Law M, Algar E, Little M. Allelic imbalance at chromosome 1q21 in Wilms tumor. *Cancer Genet. Cytogenet* 1997;97:54-59.

158. Leach F, Nicolaides N, Papadopoulos N, Liu B, Jen J, Parsons R, et al. Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell* 1993;75:1215-1225.

159. Leary J, Doris C, Boltz E, Houghton C, Kefford R, Friedlander M. Investigation of loss of heterozygosity at specific loci on chromosomes 3p, 6q, 11p, 17p and 17q in ovarian cancer. *Int. J. Gynecol. Cancer* 1993;3:293-298.

160. Lee J, Kavanagh J, Wildrick D, Wharton J, Blick M. Frequent loss of heterozygosity on chromosomes 6q, 11, and 17 in human ovarian carcinomas. *Cancer Res* 1990;50:2724-2728.

161. Liede A, Tonin P, Sun C, Serruya C, Daly M, Narod S, et al. Is hereditary site-specific ovarian cancer a distinct genetic condition? *Am. J. Med. Genet* 1998;75:55-58.

162. Lin J, Scherer S, Tougas L, Traverso G, Tsui L, Andrulis I, et al. Detailed deletion mapping with a refined physical map of 7q31 localizes a putative tumor suppressor gene for breast cancer in the region of MET. *Oncogene* 1996;13:2001-2008.

163. Link C. The relationship between borderline ovarian tumors and epithelial ovarian carcinoma: epidemiologic, pathologic, and molecular aspects. *Gynecol. Oncol* 1996;60:347-354.

164. Liu B, Nicolaides N, Markowitz S, Willson J, Parsons R, Jen J, et al. Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nature Genet* 1995;9:48-55.

165. Loupart M, Adams S, Armour J, Walker R, Brammar W, Varley J. Loss of heterozygosity on the X chromosome in human breast cancer. *Genes Chromosomes Cancer* 1995;13:229-238.

166. Lu K, Weitzel J, Kodali S, Welch W, Berkowitz R, Mok S. A novel 4-cM minimally deleted region on chromosome 11p15.1 associated with high grade nonmucinous epithelial ovarian carcinomas. *Cancer Res* 1997;57:387-390.

167. Lyall J, Furlong R, Yuille M, Goudie D, Leversha M, Affara N, et al. A dinucleotide repeat polymorphism at the D9S127 locus. *Nucleic Acids Res* 1992;20:925.

168. Lynch H, Lynch P. Tumor variation in the Cancer Family Syndrome: ovarian cancer. *Am. J. Surg* 1979;138:439-442.

169. Lynch H, Bewtra C, Lynch J. Familial peritoneal ovarian carcinomatosis: a new clinical entity? *Med. Hypothesis* 1986;21:171-177.

170. Lynch H, Conway T, Lynch J. Hereditary Ovarian Cancer. In: Sharp F, Mason W, Leake R, editors. *Ovarian Cancer: Biological and Therapeutic Challenges*: Chapman and Hall Medical, 1990:7-19.

171. Lynch H, Watson P, Bewtra C, Conway T, Hippee C, Kaur P, et al. Hereditary ovarian cancer. *Cancer* 1991;67:1460-1466.

172. Lynch H, Smyrk T, Watson P, Lanspa S, Lynch J, Lynch P, et al. Genetics, natural history, tumor spectrum, and pathology of hereditary nonpolyposis colorectal cancer: an updated review. *Gastroenterology* 1993;104:1535-1549.

173. Malkasian G, Decker D, Webb M. Histology of epithelial tumors of the ovary: clinical usefulness and prognostic significance of the histologic classification and grading. *Seminars Oncol* 1975;2:191-201.

174. Malkasian G, III LM, O'Brien P, Greene M. Prognostic significance of histologic classification and grading of epithelial malignancies of the ovary. *Am. J. Obstet. Gynecol* 1984;149:274-284.

175. Mao E, Schwartz S, Daling J, Beckmann A. Loss of heterozygosity at 5q21-22 (adenomatous polyposis coli gene region) in oral squamous cell carcinoma is common and correlated with advanced disease. *J. Oral Pathol. Med* 1998;27:297-302.

176. Marquis S, Rajan J, Wynshaw-Boris A, Xu J, Yin G-Y, Abel K, et al. The developmental pattern of BRCA1 expression implies a role in differentiation of the breast and other tissues. *Nature Genet* 1995;11:17-26.

177. Marra G, Boland C. Hereditary Nonpolyposis Colorectal Cancer: the syndrome, the genes, and historical perspectives. *J. Natl. Cancer Inst* 1995;87:1114-1125.

178. Marshall C. Tumor suppressor genes. *Cell* 1991;64:313-326.

179. Matsushima M, Kobayashi K, Emi M, Saito H, Saito J, Suzumori K, et al. Mutation analysis of the BRCA1 gene in 76 Japanese ovarian cancer patients: four germline mutations, but no evidence of somatic mutation. *Hum. Mol. Genet* 1995;4:1953-1956.

180. Mead L, Gillespie M, Hung J, Rane U, Rayeroux K, Irving L, et al. Frequent loss of heterozygosity in early non-small cell lung cancers at chromosome 9p21 proximal to the CDKN2a gene. *Int. J. Cancer* 1997;71:213-217.

181. Merajver S, Pham T, Caduff R, Chen M, Poy E, Cooney K, et al. Somatic mutations in the BRCA1 gene in sporadic ovarian tumours. *Nature Genet* 1995;9:439-443.

182. Merlo A, Mabry M, Gabrielson E, Vollmer R, Baylin S, Sidransky D. Frequent microsatellite instability in primary small cell lung cancer. *Cancer Res* 1994;54:2098-2101.

183. Meuth M. Patterns of mutation in cancer cells. *Cancer Surveys* 1996;28:33-45.

184. Miki Y, Swensen J, Shattuck-Eidens D, Futreal P, Harshman K, Tavtigian S, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994;266:66-71.

185. Miracle-McMahill H, Calle E, Kosinski A, Rodriguez C, Wingo P, Thun M, et al. Tubal ligation and fatal ovarian cancer in a large prospective cohort study. *Am. J. Epidemiol* 1997;145:349-357.

186. Mitra A, Murty V, Li R, Pratap M, Luthra U, Chaganti R. Allelotype analysis of cervical carcinoma. *Cancer Res* 1994;54:4481-4487.

187. Mittal K, Zeleniuch-Jacquotte A, Cooper J, Demopoulos R. Contralateral ovary in unilateral ovarian carcinoma: a search for preneoplastic lesions. *Int. J. Gynecol. Pathol* 1993;12(1):59-63.

188. Mohle J, Whittemore A, Pike M, Darby S. Gonadotrophins and ovarian cancer risk. *J. Natl. Cancer Inst* 1985;75:178-179.

189. Murty V, Reuter V, Bosl G, Chaganti R. Deletion mapping identifies loss of heterozygosity at 5p15.1-15.2, 5q11 and 5q34-35 in human male germ cell tumors. *Oncogene* 1996;12:2719-2723.

190. Narod S, Madlensky L, Bradley L, Cole D, Tonin P, Rosen B, et al. Hereditary and familial ovarian cancer in Southern Ontario. *Cancer* 1994;74:2341-2346.

191. Narod S, Ford D, Devilee P, Barkardottir R, Lynch H, Smith S, et al. An evaluation of genetic heterogeneity in 145 breast-ovarian cancer families. *Am. J. Hum. Genet* 1995;56:254-264. (a)

192. Narod S, Ford D, Devilee P, Barkardottir R, Eyfjord J, Lenoir G, et al. Genetic heterogeneity of breast-ovarian cancer revisited. *Am. J. Hum. Genet* 1995;57:957-958. (b)

193. Naylor S, Buys C, Carritt B. Report of the Fourth International Workshop on human chromosome 3 mapping. *Cytogenet. Cell. Genet* 1994;65:2-50.

194. Negrini M, Monaco C, Vorechovsky I, Ohta M, Druck T, Baffa R, et al. The FHIT gene at 3p14.2 is abnormal in breast carcinomas. *Cancer Res* 1996;56:3173-3179.

195. Neuhausen S, Mazoyer S, Friedman L, Stratton M, Offit K, Caligo A, et al. Haplotype and phenotype analysis of six recurrent BRCA1 mutations in 61 families: results of an International study. *Am. J. Hum. Genet* 1996;58:271-280.

196. Nguyen H, Averette H, Hoskins W, Sevin B-U, Penalver M, Steren A. National survey of ovarian cancer VI. *Cancer* 1993;72:3007-3011.

197. Nguyen H, Averette H, Janicek M. Ovarian carcinoma. *Cancer* 1994;74:545-555.

198. Nicolaides N, Papadopoulos N, Liu B, Wei Y-F, Carter K, Ruben S, et al. Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. *Nature* 1994;371:75-80.

199. Noviello C, Courjal F, Theillet C. Loss of heterozygosity on the long arm of chromosome 6 in breast cancer: possibly four regions of deletion. *Clin. Cancer. Res* 1996;2:1601-1606.

200. Ogasawara S, Maesawa C, Tamura G, Satodate R. Frequent microsatellite alterations on chromosome 3p in esophageal squamous cell carcinoma. *Cancer Res* 1995;55:891-894.

201. Okamoto A, Sameshima Y, Yokoyama S, Terashima Y, Sugimura T, Terada M, et al. Frequent allelic losses and mutations of the p53 gene in human ovarian cancer. *Cancer Res* 1991;51:5171-5176.

202. Orlow I, Lianes P, Lacombe L, Dalbagni G, Reuter V, Cordon-Cardo C. Chromosome 9 allelic losses and microsatellite alterations in human bladder tumors. *Cancer Res* 1994;54:2848-2851.

203. Orphanos V, McGown G, Hey Y, Thorncroft M, Santibanez-Koref M, Russell S, et al. Allelic imbalance of chromosome 6q in ovarian tumours. *B. J. Cancer* 1995;71:666-669. (a)

204. Orphanos V, McGown G, Hey Y, Boyle J, Santbanez-Koref M. Proximal 6q, a region showing allele loss in primary breast cancer. *B. J. Cancer* 1995;71:290-293. (b)

205. Orth K, Hung J, Gazdar A, Bowcock A, Mathis J, Sambrook J. Genetic instability in human ovarian cancer cell lines. *Proc. Natl. Acad. Sci. USA* 1994;91:9495-9499.

206. Ory H, The Centers for Disease Control Cancer and Steroid Hormone Study. Oral contraceptive use and the risk of ovarian cancer. *JAMA* 1983;249:1596-1599.

207. Osborne R, Leech V. Polymerase chain reaction allelotyping of human ovarian cancer. *B. J. Cancer* 1994;69:429-438.

208. Otsuka T, Kohno T, Mori M, M, Noguchi, Hirohashi S, et al. Deletion mapping of chromosome 2 in human lung carcinoma. *Genes Chromosomes Cancer* 1996;16:113-119.

209. Pabst T, Schwaller J, Bellomo M, Oesteicher M, Muhlematter D, Tichelli A, et al. Frequent clonal loss of heterozygosity but scarcity of microsatellite instability at chromosomal breakpoint cluster regions in adult leukaemias. *Blood* 1996;88:1026-1034.

210. Pandis N, Jin Y, Limon J, Bardi G, Idvall I, Mandahl N, et al. Interstitial deletion of the short arm of chromosome 3 as a primary chromosome abnormality in carcinomas of the breast. *Genes Chromosomes Cancer* 1993;6:151-155.

211. Papadopoulos N, Nicolaides N, Wei Y-F, Ruben S, Carter K, Rosen C, et al. Mutation of a mutL homolog in hereditary colon cancer. *Science* 1994;263:1625-1629.

212. Parazzini F, Vecchia CL, Negri E, Bocciolone L, Fedele L, Franceschi S. Oral contraceptive use and the risk of ovarian cancer: an Italian case-control study. *Eur. J. Cancer* 1991;27:594-598. (a)

213. Parazzini F, Franceschi S, Vecchia CL, Fasoli M. The epidemiology of ovarian cancer. *Gynecol. Oncol* 1991;43:9-23. (b)

214. Parazzini F, Negri E, Vecchia CL, Luchini L, Mezzopane R. Hysterectomy, oophorectomy, and subsequent ovarian cancer risk. *Obstet. Gynecol* 1993;81:363-366.

215. Parker J, Kaplon M, Alvarez C, Krishnaswamy G. Prostaglandin H synthase expression is variable in human colorectal adenocarcinoma cell lines. *Exp. Cell. Res* 1997;236:321-329.

216. Parsons R, Li G-M, Longley M, Fang W, Papadopoulos N, Jen J, et al. Hypermutability and mismatch repair deficiency in RER+ tumor cells. *Cell* 1993;75:1227-1236.

217. Peiffer S, Herzog T, Tribune D, Mutch D, Gersell D, Goodfellow P. Allelic loss of sequences from the long arm of chromosome 10 and replication errors in endometrial cancers. *Cancer Res* 1995;55:1922-1926.

218. Peltomaki P, Aaltonen L, Sistonen P, Pylkkanen L, Mecklin J-P, Jarvinen H, et al. Genetic mapping of a locus predisposing to human colorectal cancer. *Science* 1993;260:810-812.

219. Pettersson F. *Annual report on the results of treatment in gynecological cancer*: International Federation of Gynecology and Obstetrics, 1988.

220. Pharoah P, Easton D, Stockton D, Gayther S, Ponder B. Survival in familial, BRCA1-associated, and BRCA2-associated epithelial ovarian cancer. *Cancer Res* 1999;59:868-871.

221. Phelan C, Rebbeck T, Weber B, Devilee P, Ruttledge M, Lynch H, et al. Ovarian cancer risk in BRCA1 carriers is modified by the HRAS1 variable number of tandem repeat (VNTR) locus. *Nature Genet* 1996;12:309-311.

222. Pieretti M. Genetic alterations distinguish different types of ovarian tumours. *Int. J. Cancer* 1995;64:434-440.

223. Pietsch T, Koch A, Wiestler O. Molecular genetic studies in medulloblastomas: evidence for tumor suppressor genes at the chromosomal regions 1q31-32 and 17p13. *Klin. Padiatr* 1997;209:150-155.

224. Piver M, Baker T, Jishi M, Sandecki A, Tsukada Y, Natarajan N, et al. Familial ovarian cancer. *Cancer* 1993;71:582-588. (a)

225. Piver M, Jishi M, Tsukada Y, Nava G. Primary peritoneal carcinoma after prophylactic oophorectomy in women with a family history of ovarian cancer. *Cancer* 1993;71:2751-2755. (b)

226. Plaxe S, Deligdisch L, Dottino P, Cohen C. Ovarian intraepithelial neoplasia demonstrated in patients with stage I ovarian carcinoma. *Gynecol. Oncol* 1990;38:367-372.

227. Polymeropoulos M, Rath D, Xiao H, Merril C. Tetranucleotide repeat polymorphism at the human c-fes/fps proto-oncogene (FES). *Nucleic Acids Res* 1991;19:4018.

228. Praml C, Finke L, Herfarth C, Schlag P, Schwab M, Amler L. Deletion mapping defines different regions in 1p34.2-pter that may harbor genetic information related to human colorectal cancer. *Oncogene* 1995;11:1357-1362.

229. Purdie D, Green A, Bain C, Siskind V, Ward B, Hacker N, et al. Reproductive and other factors and risk of epithelial ovarian cancer: an Australian case-control study. *Int. J. Cancer* 1995;62:678-684.

230. Raskind W, Conrad E, Matsushita M. Frequent loss of heterozygosity for markers on chromosome arm 10q in chondrosarcomas. *Genes Chromosomes Cancer* 1996;16:138-143.

231. Ray M, Su Y, Meltzer P, Trent J. Isolation and characterization of genes associated with chromosome-6 mediated tumor suppression in human malignant melanoma. *Oncogene* 1996;12:2527-2533.

232. Renan M. How many mutations are required for tumorigenesis? Implications from human cancer data. *Mol. Carcinog* 1993;7:139-146.

233. Resta L, Russo S, Colucci G, Prat J. Morphologic precursors of ovarian epithelial tumors. *Obstet. Gynecol* 1993;82:181-186.

234. Rhei E, Kang L, Bogomolniy F, Federici M, Borgen P, Boyd J. Mutation analysis of the putative tumor suppressor gene PTEN/MMAC1 in primary breast carcinomas. *Cancer Res* 1997;57:3657-3659.

235. Rimessi P, Gualandi F, Morelli C, Trabanelli C, Wu Q, Possati L, et al. Transfer of human chromosome 3 to an ovarian carcinoma cell line identifies three regions on 3p involved in ovarian cancer. *Oncogene* 1994;9:3467-3474.

236. Risch H, Marrett L, Jain M, Howe G. Differences in risk factors for epithelial ovarian cancer by histologic type. *Am. J. Epidemiol* 1996;144:363-372.

237. Risinger J, Berchuck A, Kohler M, Watson P, Lynch H, Boyd J. Genetic instability of microsatellites in endometrial carcinoma. *Cancer Res* 1993;53:5100-5103.

238. Rocques P. Techniques of Molecular Biology. In: Yarnold J, Stratton M, McMillan T, editors. *Molecular Biology for Oncologists*. 2nd ed: Chapman and Hall, 1996:322-331.

239. Rodabaugh K, Blanchard G, Welch W, Bell D, Berkowitz R, Mok S. Detailed deletion mapping of chromosome 6q in borderline epithelial ovarian tumors. *Cancer Res* 1995;55:2169-2172. (a)

240. Rodabaugh K, Biggs R, Qureshi J, Barrett A, Welch W, Bell D, et al. Detailed deletion mapping of chromosome 9p and p16 gene alterations in human borderline and invasive epithelial ovarian tumors. *Oncogene* 1995;11:1249-1254. (b)

241. Roy W, Watson R, Hitchcock A, Campbell I. Frequent loss of heterozygosity on chromosome 7 and 9 in benign epithelial ovarian tumours. *Oncogene* 1997;15:2031-2035.

242. Rubin S, Benjamin I, Behbakht K, Takahashi H, Morgan M, LiVolsi V, et al. Clinical and pathological features of ovarian cancer in women with germ-line mutations of BRCA1. *N. Engl. J. Med* 1996;335:1413-1416.

243. Rubin S, Blackwood M, Bandera C, Behbakht K, Benjamin I, Rebbeck T, et al. BRCA1, BRCA2, and hereditary nonpolyposis colorectal cancer gene mutations in an unselected ovarian cancer population: relationship to family history and implications for genetic testing. *Am. J. Obstet. Gynecol* 1998;178:670-677.

244. Rubio M, Correa K, Ueki K, Mohrenweiser H, Gusella J, Deimling Av, et al. The putative glioma tumor suppressor gene on chromosome 19q maps between APOC2 and HRC. *Cancer Res* 1994;54:4760-4763.

245. Ruddon R. Oncogenes. In: Ruddon R, editor. *Cancer Biology*. 3rd ed: Oxford University Press, 1995:277-317.

246. Russell P. Common Epithelial Tumours of the Ovary. In: Fox H, editor. *Obstetrical and Gynaecological Pathology*. 3rd ed: Churchill Livingstone, 1987:556-572.

247. Russell S, Hickey G, Lowry W, White P, Atkinson R. Allele loss from chromosome 17 in ovarian cancer. *Oncogene* 1990;5:1581-1583.

248. Saiki R, Scharf S, Faloona F, Mullis K, Horn G, Erlich H, et al. Enzymatic amplification of B-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 1985;230:1350-1354.

249. Saito S, Saito H, Koi S, Sagae S, Kudo R, Saito J, et al. Fine-scale deletion mapping of the distal long arm of chromosome 6 in 70 human ovarian cancers. *Cancer Res* 1992;52:5815-5817.

250. Saito H, Inazawa J, Saito S, Kasumi F, Koi S, Sagae S, et al. Detailed deletion mapping of chromosome 17q in ovarian and breast cancers: 2-cM region on 17q21.3 often and commonly deleted in tumors. *Cancer Res* 1993;53:3382-3385.

251. Salazar H, Godwin A, Daly M, Laub P, Hogan W, Rosenblum N, et al. Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies. *J. Natl. Cancer Inst* 1996;88(24):1810-1820.

252. Saretzki G. Identification of allelic losses in benign, borderline, and invasive epithelial ovarian tumours and correlation with clinical outcome. *Cancer* 1997;80:1241-1249.

253. Sato T, Saito H, Morita R, Koi S, Lee J, Nakamura Y. Allelotype in human ovarian cancer. *Cancer Res* 1991;51:5118-5122.

254. Schildkraut J, Thompson W. Familial ovarian cancer: a population-based case-control study. *Am. J. Epidemiol* 1988;128:456-466.

255. Schrag D, Kuntz K, Garber J, Weeks J. Decision analysis - effects of prophylactic mastectomy and oophorectomy on life expectancy among women with BRCA1 or BRCA2 mutations. *N. Engl. J. Med* 1997;336:1465-1471.

256. Schultz D, Vanderveer L, Buetow K, Boente M, Ozols R, Hamilton T, et al. Characterization of chromosome 9 in human ovarian neoplasia identifies frequent genetic imbalance on 9q and rare alterations involving 9p, including CDKN2. *Cancer Res* 1995;55:2150-2157.

257. Scully R. Ovarian tumors. *Am. J. Pathol* 1977;87:686-720.

258. Scully R. Pathology of ovarian cancer precursors. *J. Cell. Biochem* 1995;23:208-218.

259. Scully R, Ganesan S, Brown M, Caprio JD, Cannistra S, Feunteun J. Location of BRCA1 in human breast and ovarian cancer cells. *Science* 1996;272:123-126.

260. Scully R, Chen J, Plug A, Xiao Y, Weaver D, Feunteun J, et al. Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell* 1997;88:265-275.

261. Seitz S, Rohde K, Bender E, Nothnagel A, Pidde H, Ullrich O, et al. Deletion mapping and linkage analysis provide strong indication for the involvement of the human chromosome region 8p12-p22 in breast carcinogenesis. *B. J. Cancer* 1997;76:983-991.

262. Serova O, Montagna M, Torchard D, Narod S, Tonin P, Sylla B, et al. A high incidence of BRCA1 mutations in 20 breast-ovarian cancer families. *Am. J. Hum. Genet* 1996;58:42-51.

263. Shattuck-Eidens D, McClure M, Simard J, Labrie F, Narod S, Couch F, et al. A collaborative survey of 80 mutations in the BRCA1 breast and ovarian cancer susceptibility gene. *JAMA* 1995;273:535-541.

264. Shelling A, Cooke I, Ganesan T. The genetic analysis of ovarian cancer. *B. J. Cancer* 1995;72:521-527.

265. Shipman R, Schraml P, Colombi M, Ludwig C. Allelic deletion at chromosome 11p13 defines a tumour suppressor region between the catalase gene and D11S935 in human non-small cell lung carcinoma. *Int. J. Oncol* 1998;12:107-111.

266. Shridhar V, Siegfried J, Hunt J, Alonso MdM, Smith D. Genetic instability of microsatellite sequences in many non-small cell lung carcinomas. *Cancer Res* 1994;54:2084-2087.

267. Simard J, Tonin P, Durocher F, Morgan K, Rommens J, Gingras S, et al. Common origins of BRCA1 mutations in Canadian breast and ovarian cancer families. *Nature Genet* 1994;8:392-398.

268. Smith S. Allele losses in the region 17q12-21 in familial breast and ovarian cancer involve the wild-type chromosome. *Nature Genet* 1992;2:128-131.

269. Sorbe B, Frankendal B, Veress B. Importance of histologic grading in the prognosis of epithelial ovarian carcinoma. *Obstet. Gynecol* 1982;59:576-582.

270. Spirio L, Joslyn G, Nelson L, Leppert M, White R. A CA repeat 30-70 KB downstream from the adenomatous polyposis coli (APC) gene. *Nucleic Acids Res* 1991;19:6348.

271. Steichen-Gersdorf E, Gallion H, Ford D, Girodet C, Easton D, DiCioccio R, et al. Familial site-specific ovarian cancer is linked to BRCA1 on 17q12-21. *Am. J. Hum. Genet* 1994;55:870-875.

272. Stenback F. Benign, borderline, and malignant serous cystadenomas of the ovary. *Path. Res. Pract* 1981;172:58-72.

273. Stracham T. *The Human Genome*: BIOS Scientific Publishers Ltd, 1992.

274. Stratton M, Ford D, Neuhasen S, Seal S, Wooster R, Friedman L, et al. Familial male breast cancer is not linked to the BRCA1 locus on chromosome 17q. *Nature Genet* 1994;7:103-107.

275. Stratton J, Gayther S, Russell P, Dearden J, Gore M, Blake P, et al. Contribution of BRCA1 mutations to ovarian cancer. *N. Eng. J. Med* 1997;336:1125-1130.

276. Stratton J. Comparison of prophylactic oophorectomy specimens from carriers and noncarriers of a BRCA1 or BRCA2 gene mutation. *J. Natl. Cancer. Inst* 1999;91:626-628.

277. Struewing J, Brody L, Erdos M, Kase R, Giambarresi T, Smith S, et al. Detection of eight BRCA1 mutations in 10 breast/ovarian cancer families, including 1 family with male breast cancer. *Am. J. Hum. Genet* 1995;57:1-7.

278. Struewing J. The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. *N. Engl. J. Med* 1997;336(20):1401-1408.

279. Suzuki H, Emi M, Komiya A, Fujiwara Y, Yatani R, Nakamura Y, et al. Localization of a tumor suppressor gene associated with progression of human prostate cancer within a 1.2Mb region of 8p22-p21.3. *Genes Chromosomes Cancer* 1995;13:168-174.

280. Suzuki M, Saito S, Saga Y, Ohwada M, Sato I. Loss of heterozygosity on chromosome 6q27 and p53 mutations in epithelial ovarian cancer. *Medical Oncology* 1998;15:119-123.

281. Swensen J, Hoffman M, Skolnock M, Neuhausen S. Identification of a 14kb deletion involving the promoter region of BRCA1 in a breast cancer family. *Hum. Mol. Genet* 1997;6:1513-1517.

282. Szabo C, King M-C. Inherited breast and ovarian cancer. *Hum. Mol. Genet* 1995;4:1811-1817.

283. Tahara H, Smith A, Gaz R, Zariwala M, Xiong Y, Arnold A. Parathyroid tumor suppressor on 1p: analysis of the p18 cyclin-dependent kinase inhibitor gene as a candidate. *J. Bone Miner. Res* 1997;12:1330-1334.

284. Takahashi H, Behbakht K, Mc Govern PE, Chiu H-C, Couch FJ, Weber BL, et al. Mutation analysis of the BRCA1 gene in ovarian cancers. *Cancer Res* 1995;55:2998-3002.

285. Takahashi H, Chiu H-C, Bandera C, Behbakht K, Liu P, Couch F, et al. Mutations of the BRCA2 gene in ovarian carcinomas. *Cancer Res* 1996;56:2738-2741.

286. Takakura S, Okamoto A, Saito M, Yasuhara T, Shinozaki H, Isonishi S, et al. Allelic imbalance in chromosome band 18q21 and SMAD4 mutations in ovarian cancers. *Genes Chromosomes Cancer* 1999;24:264-271.

287. Takeuchi S, Seriu T, Bartram C, Golub T, Reiter A, Miyoshi I, et al. TEL is one of the targets for deletion on 12p in many cases of childhood B-lineage acute lymphoblastic leukaemia. *Leukaemia* 1997;11:1220-1223.

288. Tavassoli M, Steingrimsdottir H, Pierce E, Jiang X, Alagoz M, Farzaneh F, et al. Loss of heterozygosity on chromosome 5q in ovarian cancer is frequently accompanied by TP53 mutation and identifies a tumour suppressor gene locus at 5q13.1-21. *B. J. Cancer* 1996;74:115-119.

289. Tavtigian S, Simard J, Rommens J, Couch F, Shattuck-Eidens D, Neuhausen S, et al. The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds. *Nature Genet* 1996;12:333-337.

290. Telenius H, Carter N, Bebb C, Nordenskjold M, Ponder B, Tunnacliffe A. Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. *Genomics* 1992;13:718-725.

291. Thibodeau S, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 1993;260:816-819.

292. Thorlacius S, Sigurdsson S, Bjarnadottir H, Olafsdottir G, Jonasson J, Tryggvadottir L, et al. Study of a single BRCA2 mutation with high carrier frequency in a small population. *Am. J. Hum. Genet* 1997;60:1079-1084.

293. Tibiletti M, Bernasconi B, Furlan D, Riva C, Trubia M, Buraggi G, et al. Early involvement of 6q in surface epithelial ovarian tumors. *Cancer Res* 1996;56:4493-4498.

294. Tobacman J, Tucker M, Kase R, Greene M, Costa J, Fraumeni J. Intra-abdominal carcinomatosis after prophylactic oophorectomy in ovarian-cancer-prone families. *Lancet* 1982: 795-797.

295. Tse J, Ng H, Lau K, Lo K, Poon W, Huang D. Loss of heterozygosity of chromosome 14q in low- and high-grade meningiomas. *Hum. Pathol* 1997;28:779-785.

296. Urano T, Nishimori H, Han H, Furuhata T, Kimura Y, Nakamura Y, et al. Cloning of P2XM, a novel human P2X receptor gene regulated by p53. *Cancer Res* 1997;57:3281-3287.

297. Vandamme B, Lissens W, Amfo K, Sutter PD, Bourgain C, Vamos E, et al. Deletion of chromosome 11p13-1p15.5 sequences in invasive human ovarian cancer is a subclonal progression factor. *Cancer Res* 1992;52:6646-6652.

298. Vessey M, Metcalfe A, Wells C, McPherson K, Westhoff C, Yeates D. Ovarian neoplasms, functional cysts, and oral contraceptives. *BMJ* 1987;294:1518-1520.

299. Viel A, Tumiotto G, Sopracordevole F, Visentin M, Boiocchi M. Chromosomal localisation of two putative 11p oncosuppressor genes involved in human ovarian tumours. *Br. J. Cancer* 1992;66:1030-1036.

300. Vogelstein B, Fearon E, Hamilton S, Kern S, Preisinger A, Leppert M, et al. Genetic alterations during colorectal-tumor development. *N. Engl. J. Med* 1988;319:525-532.

301. Vogelstein B, Kinzler K. The multistep nature of cancer. *TIG* 1993;9:138-141.

302. VonEggeling F, Spielvogel H. Applications of random PCR. *Cell Molec. Biol* 1995;41:653-670.

303. Wang S, Puc J, Li J, Bruce J, Cairns P, Sidransky D, et al. Somatic mutations of PTEN in glioblastoma multiforme. *Cancer Res* 1997;57:4183-4186.

304. Wang Z, Churchman M, Campbell I, Xu W, Yan Z, McCluggage W, et al. Allele loss and mutation screen at the Peutz-Jeghers (LKB1) locus (19p13.3) in sporadic ovarian tumours. *B. J. Cancer* 1999;80:70-72.

305. Watson P, Lynch H. Extracolonic cancer in Hereditary Nonpolyposis Colorectal Cancer. *Cancer* 1993;71:677-685.

306. Watson R, Neville P, Roy W, Hitchcock A, Campbell I. Loss of heterozygosity on chromosomes 7p, 7q, 9p, and 11q is an early event in ovarian tumorigenesis. *Oncogene* 1998;17:207-212.

307. Weber J, May P, Patterson D, Drabkin H, Killary A. Dinucleotide repeat polymorphism at the D3S196 locus. *Nucleic Acids Res* 1990;18:4635-4637.

308. Weissenbach J, Gyapay G, Dib C, Vignal A, Morissette J, Millasseau P, et al. A second-generation linkage map of the human genome. *Nature* 1992;359:794-801.

309. Weitzel J, Patal J, Smith D, Goodman A, Safaii H, Ball H. Molecular genetic changes associated with ovarian cancer. *Gynecol. Oncol* 1994;55:245-252.

310. Whittemore A, Harris R, Itnyre J. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. *Am. J. Epidemiol* 1992;136:1184-1203.

311. Wieland I, Bohm M. Frequent allelic deletion at a novel locus on chromosome 5 in human lung cancer. *Cancer Res* 1994;54:1772-1774.

312. Wiest J, Franklin W, Otstot J, Forbey K, Varella-Garcia M, Rao K, et al. Identification of a novel region of homozygous deletion on chromosome 9p in squamous cell carcinoma of the lung: the location of a putative tumor suppressor gene. *Cancer Res* 1997;57:1-6.

313. Wiper D, Zanoti K, Kennedy A, Belinson J, Casey G. Analysis of allelic imbalance on chromosome 17p13 in stage I and stage II epithelial ovarian cancers. *Gynecol. Oncol* 1998;71:77-82.

314. Wistuba I, Montellano F, Milchgrub S, Virmani A, Behrens C, Chen H, et al. Deletions of chromosome 3p are frequent and early events in the pathogenesis of uterine cervical carcinoma. *Cancer Res* 1997;57:3154-3158.

315. Wood S, Yaremko M, Schertzer M, Kelemen P, Minna J, Westbrook C. Mapping of the pulmonary surfactant SP5 (SFTP2) locus to 8p21 and characterization of a microsatellite repeat marker that shows frequent loss of heterozygosity in human carcinomas. *Genomics* 1994;24:597-600.

316. Wooster R, Neuhausen S, Mangion J, Quirk Y, Ford D, Collins N, et al. Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. *Science* 1994;265:2088-2090.

317. Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, et al. Identification of the breast cancer susceptibility gene BRCA2. *Nature* 1995;378:789-792.

318. Wright K, Wilson P, Kerr J, Do K, Hurst T, Khoo S, et al. Frequent loss of heterozygosity and three critical regions on the short arm of chromosome 8 in ovarian adenocarcinomas. *Oncogene* 1998;17(9):1185-1188.

319. Wu L, Wang Z, Tsan J, Spillman M, Phung A, Xu X, et al. Identification of a RING protein that can interact in vivo with the BRCA1 gene product. *Nature Genet* 1996;14:430-440.

320. Yamaguchi T, Toguchida J, Wadayama B, Kanoe H, Nakayama T, Ishizaki K, et al. Loss of heterozygosity and tumor suppressor gene mutations in chondrosarcomas. *Anticancer Res* 1996;16:2009-2015.

321. Yamashita T, Yaginuma Y, Yamashita K, Fujita M, Ishikawa M. DCC gene alterations in histological types and clinical stages of epithelial ovarian cancer. *Int. J. Oncol* 1996;9:291-296.

322. Yana I, Kurahashi H, Nakamori S, Kameyama M, Nakamura T, Takami M, et al. Frequent loss of heterozygosity at telomeric loci on 22q in sporadic colorectal cancers. *Int. J. Cancer* 1995;60:174-177.

323. Yang-Feng T, Li S, Han H, Schwartz P. Frequent loss of heterozygosity on chromosomes Xp and 13q in human ovarian cancer. *Int. J. Cancer* 1992;52:575-580.

324. Yang-Feng T, Han H, Chen K-C, Li S, Claus E, Carcangiu M, et al. Allelic loss in ovarian cancer. *Int. J. Cancer* 1993;54:546-551.

325. Yaremko M, Kutza C, Lyzak J, Mick R, Recant W, Westbrook C. Loss of heterozygosity from the short arm of chromosome 8 is associated with invasive behavior in breast cancer. *Genes Chromosomes Cancer* 1996;16:189-195.

326. Yee C, Roodi N, Verrier C, Parl F. Microsatellite instability and loss of heterozygosity in breast cancer. *Cancer Res* 1994;54:1641-1644.

327. Yokoyama J, Shiga K, Sasano H, Suzuki M, Takasaka T. Abnormalities and the implication of retinoblastoma locus and its protein product in head and neck cancers. *Anticancer Res* 1996;16:641-644.

328. Young J, Leggett B, Ward M, Thomas L, Buttenshaw R, Searle J, et al. Frequent loss of heterozygosity on chromosome 14 occurs in advanced colorectal carcinomas. *Oncogene* 1993;8:671-675.

329. Zenklusen J, Bieche I, Lidereau R, Conti C. (C-A)n microsatellite repeat D7S522 is the most commonly deleted region in human primary breast cancer. *Proc. Natl. Acad. Sci. USA* 1994;91:12155-12158. (a)

330. Zenklusen J, Thompson J, Troncoso P, Kagan J, Conti C. Loss of heterozygosity in human primary prostate carcinomas: a possible tumor suppressor gene at 7q31.1. *Cancer Res* 1994;54:6370-6373. (b)

331. Zenklusen J, Thompson J, Klein-Szanto A, Conti C. Frequent loss of heterozygosity in human primary squamous cell and colon carcinomas at 7q31.1: evidence for a broad range tumor suppressor gene. *Cancer Res* 1995;55:1347-1350. (a)

332. Zenklusen J, Weitzel J, Ball H, Conti C. Allelic loss at 7q31.1 in human primary ovarian carcinomas suggests the existence of a tumor suppressor gene. *Oncogene* 1995;11:359-363. (b)

333. Zeremski M, Horrigan S, Grigorian I, Westbrook C, Gudkov A. Localization of the candidate tumor suppressor gene ING1 to human chromosome 13q34. *Somat. Cell. Mol. Genet* 1997;23:233-236.

334. Zhang L, Cui X, Schmitt K, Hubert R, Navidi W, Arnheim N. Whole genome amplification from a single cell: implications for genetic analysis. *Proc. Natl. Acad. Sci. USA* 1992;89:5847-5851.

335. Zheng J, Benedict W, Xu H-J, Hu S-X, Kim T, Velicescu M, et al. Genetic disparity between morphologically benign cysts contiguous to ovarian carcinomas and solitary cystadenomas. *J. Natl. Cancer Inst* 1995;87:1146-1153.