UNIVERSITY OF SOUTHAMPTON

MOLECULAR GENETIC ANALYSIS OF CHROMOSOME 7 AND ITS ROLE IN HUMAN OVARIAN CANCER

Phillippa Joanne Neville

Doctor of Philosophy

Department of Obstetrics and Gynaecology

Faculty of Medicine, Health & Biological Sciences

September 2000

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH & BIOLOGICAL SCIENCES

OBSTETRICS AND GYNAECOLOGY

Doctor of Philosophy

MOLECULAR GENETIC ANALYSIS OF CHROMOSOME 7 AND ITS ROLE IN HUMAN OVARIAN CANCER

by Phillippa Joanne Neville

Loss of heterozygosity (LOH) of chromosome 7q has been shown to occur frequently in many tumour types including that of the ovary, suggesting the presence of a tumour suppressor gene (TSG). This investigation used thirty-four microsatellite markers from across chromosome 7 to analyse LOH in a large bank of over 200 primary epithelial ovarian tumours. Several distinct regions of LOH were identified indicating that chromosome 7 may harbour more than one tumour suppressor gene associated with ovarian cancer. The data presented here suggests that chromosome 7 LOH is an early event in ovarian tumorigenesis, and potentially involved in the switch from benign or borderline disease to malignancy.

Three genes from the most commonly deleted regions at 7q22 and 7q31 were analysed for mutations. The proto-oncogene c-*MET* at 7q31, did not exhibit any mutations and does not appear to be the gene being targeted for deletion in this region. *PAI-1*, a member of the plasminogen/plasmin proteolytic pathway previously implicated in tumor growth and metastasis, did not appear to harbour any mutations within its coding region, strongly suggesting that it is not involved in the LOH observed at 7q22 in ovarian cancer. Another 7q22 candidate, *CUTL1*, a putative repressor of gene expression, revealed a polymorphism within a 'cut repeat' domain, and several silent, intronic, germline alterations. The lack of functional mutations implies that *CUTL1* is not the 7q22 TSG candidate. Further refinement of the candidate TSG loci identified here will facilitate the isolation of further putative genes from these regions. Using this approach a model will hopefully be established demonstrating the role of chromosome 7 and the genes harboured within it, in the progression of ovarian carcinogenesis.

This study also demonstrates the ability to identify LOH and genetic mutations in DNA isolated from the blood plasma of ovarian cancer patients. In many cases the pattern of genetic aberration in plasma DNA is identical to that seen in the patients tumour DNA, however one case showed distinct LOH in plasma DNA but not in the corresponding tumour DNA, possibly indicating the presence of metastases or a second primary tumour. This feasibility study highlights the potential for using such a non-invasive technique in the early detection, therapy and follow-up of ovarian cancer in a clinical setting.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER ONE

Figures

Tables

CHAPTER TWO

Figures

Tables

CHAPTER THREE

Figures

Tables

CHAPTER FOUR

Figures

Tables

CHAPTER FIVE

Figures

CHAPTER SIX

Figures

CHAPTER SEVEN

Figures

Tables

ACKNOWLEDGMENTS

I would like to take this opportunity to thank the following people for providing me with the very much appreciated help, advice and support during my studies and completion of this thesis.

Thanks go to all members of the Department of Obstetrics and Gynaecology, University of Southampton. In particular, Associate Professor Ian Campbell, whose supervision has proved invaluable. I am also grateful to Ms. Karen Creed and Mrs. Hazel Hills for their assistance in all things clerical.

Special thanks go to my colleagues, especially Dr. Kirsty Ratcliffe, Ms Angeliki Koumentaki, Dr. Joanna Gale and Dr. Emma Bryan for helpful brainstorming and light entertainment, Mrs. Pat Englefield for technical advice and DNA preparation and Sister Maggie Barron for tumour collection. Also Dr. Sarah Morland, who 'crossed the pond' and embarked on the great adventure to the USA with me.

Finally I would like to thank my mother Susan Neville for her support, encouragement and occasional loan, my brother Stewart Neville for belief in his sister and Anthony Curran for his advice, understanding and unerring faith in me throughout my PhD studies.

I would like to dedicate this thesis to the memory of my late father Richard Neville, whose encouragement will never be forgotten.

PRESENTATIONS AND PUBLICATIONS

PJ Neville, N Thomas and IG Campbell (2000). Loss of heterozygosity at 7q22 and mutation analysis of the CDP gene in human epithelial ovarian tumors. (In Press) International Journal of Cancer.

Campbell, I.G, Morland, S.J., Obata, K., Neville, P.J., Thomas, N. Genetic analysis of putative precursors of ovarian cancer. *Ovarian Cancer.* Volume 6, Isis Medical Media Limited, 1999.

RH Watson, PJ Neville, WJ Roy Jr, A Hitchcock and IG Campbell (1998). Loss of heterozygosity on chromosomes 7p, 7q, 9p and 11q is an early event in ovarian tumorigenesis. *Oncogene* **17**, 207-212.

Phillippa J Neville, Richard H Watson, Andrew Hitchcock and Ian G Campbell (1997). LOH evidence for a putative tumour suppressor gene on chromosome 7 in human ovarian cancer. Presented at the Second Joint Conference of the American Association for Cancer Research and the European Association for Cancer Research, 9-13 September1997, Oxford UK.

RH Watson, PJ Neville, WJ Roy, A Hitchcock and IG Campbell (1997). Loss of heterozygosity on chromosomes 6q, 7p, 7q, 9p, 11q and 17p in benign, borderline and early stage malignant ovarian cancer: a preliminary model of ovarian tumorigenesis. Presented at the Blair Bell Jubilee Competition, 11-12 December 1997, London UK.

ABBREVIATIONS

ABL	Abelson murine leukaemia virus transforming-human homologue
bp	base pair
BRCA1/2	breast cancer gene 1/2
c-erbB-2	*HER2/neu*
cM	Centimorgan
c-*MET*	Met tyrosine kinase gene
CUTL1	cut-like-1 gene
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanine triphosphate
dnw	did not work
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine-tetra-acetic acid
EGF	epithelial growth factor
FIGO	International Federation of Gynaecology & Obstetrics
FISH	fluorescence *in situ* hybridisation
HET/het	heterozygosity
HGF	hepatocyte growth factor
HNPCC	hereditary non-polyposis colorectal cancer
IGF1/2	insulin-like growth factor 1/2
JPS	juvenile polyposis syndrome
K-*Ras*	Kirsten murine sarcoma virus oncogene-human homologue
LOH	loss of heterozygosity
M	molar
Mb	megabase
M-CSF	macrophage-colony stimulating factor
MDE	mutation detection enhancement
mi	microsatellite instability
ml	millilitre
mM	millimolar

MMR	**mismatch repair**
myc	**avian myelocytomatosis virus oncogene-human homologue**
N	**normal tissue**
na	**not available**
nd	**not done**
ni	**not informative**
ng	**nanogram**
nM	**nanomolar**
P	**plasma**
PAI-1	**plasminogen activator inhibitor-1 gene**
PCR	**polymerase chain reaction**
PDGF	**platelet-derived growth factor**
PEP	**primer extension protocol**
PTEN	**phosphatase and tensin homologue**
RB1	**retinoblastoma 1**
RER	**replication error**
SDS	**sodium dodecyl sulphate**
SF	**scatter factor**
SSCP	**single-strand conformational polymorphism**
T	**tumour**
Taq	**Thermus aquaticus polymerase**
TGFα	**transforming growth factor-α**
TNF	**tumour necrosis factor**
tPA	**tissue-type plasminogen activator**
TPR	**translocated promoter region (to activated *MET* oncogene)**
TP53	**tumour protein P53**
TSG	**tumour suppressor gene**
μg	**microgram**
μl	**microlitre**
μM	**micromolar**
uPA	**urinary-type plasminogen activator**
uPAR	**urinary-type plasminogen activator receptor**
UV	**Ultraviolet**
WHO	**World Health Organisation**

CHAPTER ONE

INTRODUCTION

1.1 THE OVARY

The ovaries, situated in the pelvis and each weighing approximately 15 g, consist of three zones: the cortex; medulla; and hilum (Berne & Levy, 1990). The cortex is the dominant region, responsible for the production of steroid hormones and the maturation and cyclical release of ova. The surface of the ovary is protected by a layer of epithelial cells. It is from this germinal epithelium that the majority of ovarian neoplasms arise. Figure 1.1 shows the ovary, its major structures and anatomy.

1.2 OVARIAN CANCER

Ovarian cancer is the fifth most common neoplasm amongst women in the western world (Parazzini *et al.*, 1997), with 5800 cases diagnosed in the United Kingdom in 1989 (Anderson *et al.*, 1997). Worldwide, women are estimated to have a 1.4% lifetime risk of developing this disease.

With an overall survival rate of between 35 and 40% (Boente, 1996) ovarian cancer is the most common cause of death amongst gynaecological tumours. The poor survival rate can be attributed to the fact that approximately 70% of newly diagnosed patients present with late stage (III-IV) disease (Iwabuchi *et al.*, 1995). This is mainly due to the absence of early symptoms and the lack of efficient early screening techniques. However, studies of women with early stage disease reveal a more optimistic 5 year survival rate of approximately 80%. Therefore any knowledge of the mechanisms involved in tumour initiation and progression could be an invaluable help in the diagnosis and treatment of this disease.

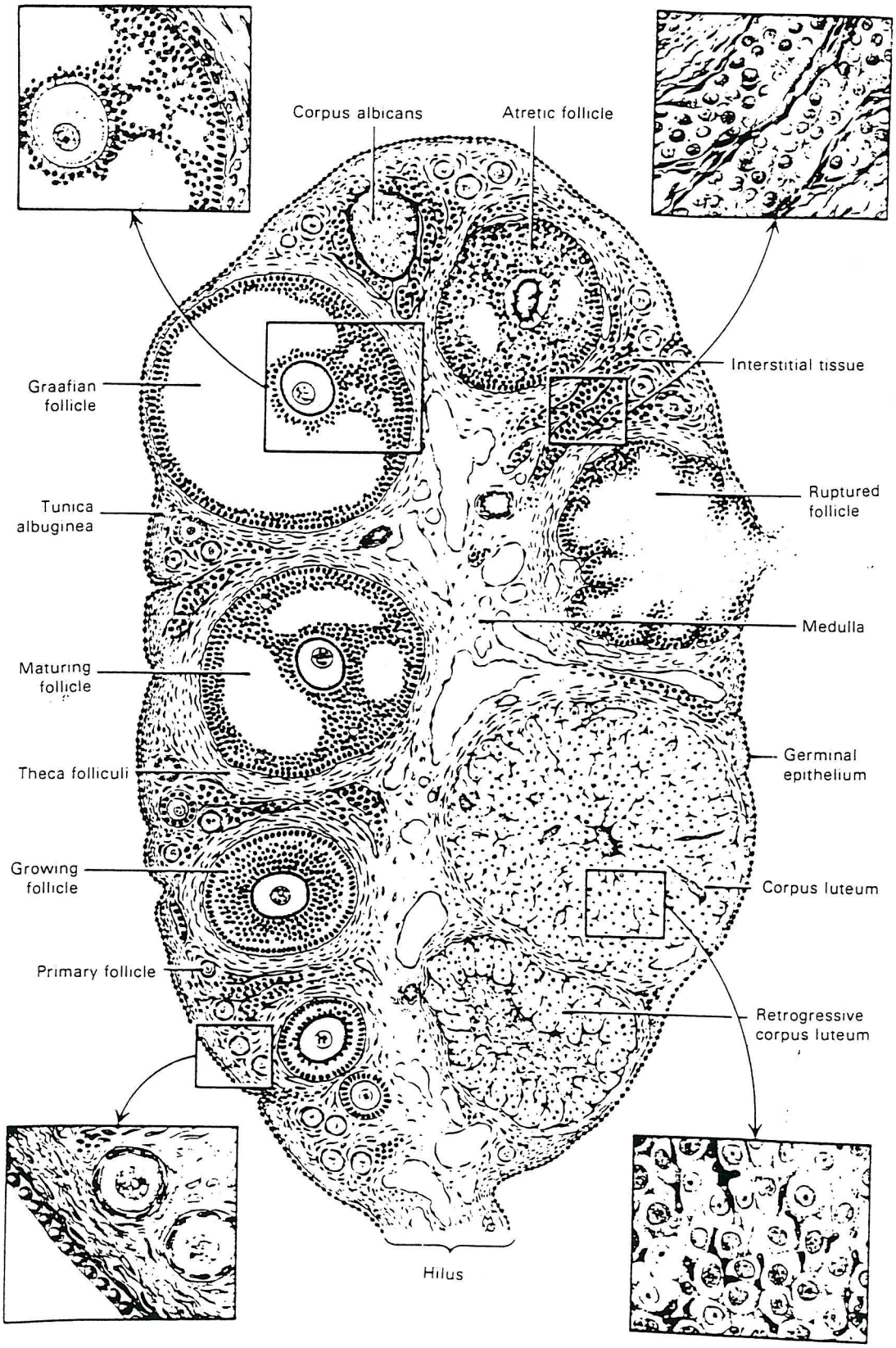
1.3 AETIOLOGY

Two major theories have been put forward to explain the mechanism of ovarian tumour initiation. The first, proposed by Fathalla in 1971 (Fathalla, 1971) is known as the 'incessant

Figure 1.1. Anatomy of the human ovary.

Schematic diagram showing the follicular and ovulatory cycle.

Taken from (Turner, 1971)


Corpus albicans
Atretic follicle
Interstitial tissue
Graafian follicle
Ruptured follicle
Tunica albuginea
Medulla
Maturing follicle
Germinal epithelium
Theca folliculi
Corpus luteum
Growing follicle
Primary follicle
Retrogressive corpus luteum
Hilus

ovulation' hypothesis and links a women's lifetime frequency of ovulation to her risk of marked effect on the risk of ovarian cancer development. The second proposes that the levels of circulating gonadotrophins have a marked effect on the risk of ovarian cancer development.

1.3.1 Incessant Ovulation theory - reproductive effects

A decreased risk in ovarian cancer has been shown to be associated with multiple pregnancies (Risch *et al.*, 1994), the use of oral contraceptives, breast-feeding, hysterectomy (with and without oophorectomy) and tubal ligation (Green *et al.*, 1997). Observations of the protective effects against ovarian cancer arising from conditions which prevent ovulation have lead to the proposal that the increase in damage to the ovarian epithelium, due to raised frequency of ovulation and subsequent mitosis at the site of trauma following exposure to high levels of oestrogen in the follicular fluid, may induce neoplastic transformation (Parazzini *et al.*, 1997). Studies carried out on chickens have shown that artificially increased egg production can induce epithelial ovarian cancer. In addition, the observations that nulliparity, older age at first birth, early menarche, late menopause and infertility show increased rates of ovarian cancer support this theory.

Whilst the majority of the protective practices detailed above can be explained by their ability to reduce the number of ovulations, others such as hysterectomy without oophorectomy and tubal ligation cannot be understood so easily. One possibility is that these surgical procedures lead to the alteration of ovarian blood flow and therefore interfere with normal ovarian function and ovulation.

Although this hypothesis seems to be supported by a large amount of data, it does not fully explain the degree of protection afforded by several factors. For example Wu et al. (1988) showed an increased ovarian cancer risk of 56% for each additional 5 years of ovulation and Persson (1996) reported that the reduction in ovulations of 10-20 (2-4% of total lifetime ovulations) due to a single pregnancy lead to a reduction in risk of cancer development of 20-40%. This data suggests that there must be alternative or additional mechanisms at work which lead to the induction and promotion of ovarian carcinogenesis.

1.3.2 Gonadotrophin Theory of ovarian tumorigenesis

The observation that the incidence of ovarian cancer greatly increases in women of menopausal age, as do the circulating levels of gonadotrophins, lead to the second hypothesis for human ovarian tumorigenesis. The gonadotrophin theory suggests that high circulating levels of gonadotrophins play a role in the initiation and progression of ovarian tumours by stimulating the ovarian surface epithelium (Riman *et al.*, 1998).
Ovarian tumours are associated with high levels of gonadotrophins in animal models. In addition, the suppression of gonadotrophins, in mice genetically predisposed to develop ovarian tumours, was shown to prevent tumorigenesis. The growth of cell lines, derived from human ovarian carcinomas, is stimulated by gonadotrophins, LH (luteinising hormone) and FSH (follicle stimulating hormone), suggesting that they may indeed play a role in ovarian carcinogenesis. Further evidence to support this theory comes from a study in women with Polycystic Ovary Syndrome (PCO) (Schildkraut *et al.*, 1996). These women exhibit an increased LH/FSH ratio and a higher risk of developing epithelial ovarian cancer.

The protective effects of pregnancy, parity and lactation (Adami *et al.*, 1994; Parazzini *et al.*, 1997), against ovarian cancer, fit this hypothesis, as each down-regulates the production of gonadotrophins by inducing anovulation. Use of the combined oral contraceptive has also been shown to decrease ovarian cancer risk significantly (Purdie *et al.*, 1995). This may be due to its ability to suppress the mid-cycle surge of gonadotrophins. Post-menopausal HRT is also thought to decrease the secretion of pituitary gonadotrophins, via a negative feedback loop, in a similar manner to that of the oral contraceptive. However, HRT has not been consistently shown to reduce ovarian cancer risk (Purdie *et al.*, 1999).
As previously mentioned, tubal ligation, and to a lesser extent hysterectomy, have been shown to decrease the likelihood of developing ovarian cancer (Green *et al.*, 1997; Hankinson *et al.*, 1993; Purdie *et al.*, 1995). However, the gonadotrophin hypothesis would predict that these procedures might increase the risk, as they decrease production of oestrogens and progestins and so remove any regulation of gonadotrophin production via the negative feedback loop. Alternatively, surgery may disrupt the ovarian blood supply, decreasing the amount of gonadotrophins that reach and stimulate the ovary.

1.3.3 Pelvic contamination theory

A third hypothesis rests on the supposition that exogenous chemicals, such as talc, may infiltrate the pelvic cavity via the vagina and uterus, and inflict direct damage on the ovary or play a role in initiating ovarian tumorigenesis (Cook *et al.*, 1997). Early studies showed that inert particles of carbon, deposited into the vagina, could be identified 30 minutes later in the fallopian tubes (Egli & Newton, 1961). Another group (Cook *et al.*, 1997) showed the migration of a radioactive tracer from the vagina to the peritoneal cavity and ovaries. This demonstrated the ease at which chemicals may be transported across the female reproductive tract.

Perineal talc application has been proposed as a risk factor for epithelial ovarian cancer following the detection of talc particles in the ovaries of patients with both benign (Heller *et al.*, 1996) and invasive ovarian cancer (Henderson *et al.*, 1971). In addition, the chemical similarity of talc to asbestos and the observation that the introduction of asbestos into the peritoneal cavity of guinea pigs and rabbits results in ovarian hyperplasia (Wong *et al.*, 1999), suggests a possible link between talcum powder and ovarian cancer. Further evidence supporting this theory comes from the decreased risk of ovarian cancer afforded by tubal ligation (Hankinson *et al.*, 1993). It is possible that this procedure may prevent the retrograde transfer of talc particles to the ovaries and therefore lead to a decrease in cancer risk.

Several case-control studies have found an association between talc use and ovarian cancer (Chang & Risch, 1997; Cook *et al.*, 1997), however, a similar number have also failed to show any association, especially when the results were adjusted for other risk factors and gynaecological procedures (Wong *et al.*, 1999). The most recent prospective study to date (Gertig *et al.*, 2000) reports a lack of evidence to support for a significant association between perineal talc use and ovarian cancer in general, although a slight increase in risk of invasive serous ovarian cancer was reported.

1.3.4 Inflammation of the ovarian epithelium

To date, none of the proposed theories for ovarian carcinogenesis are entirely consistent with the biological and epidemiological data available. In a recent review, Ness and Cottreau (1999) suggest that inflammation of the ovarian epithelium, due to a number of factors, may

play a role, alongside that of steroid hormones and ovulation, in mediating ovarian tumorigenesis. Ovulation itself is associated with inflammation due to wound generation at the surface epithelium of the ovary in the vicinity of the ovulatory follicles. During ovulation, many inflammatory (and anti-inflammatory) compounds are seen to be elevated around the rupture site. Of these inflammation-associated substances, cytokines and prostaglandins have also been shown to be produced by ovarian cancer cells. Prostaglandins in particular are more common in tumour cells than in normal cells, and their overexpression is seen to increase the invasive potential of tumour cells. In addition, inhibitors of the prostaglandin synthetic pathway protect against several tumour types in animal studies (Ness & Cottreau, 1999). Inflammation also leads to the production of oxidants, capable of killing pathogens. These toxins can directly damage DNA, which in a rapidly proliferating cell population, also a consequence of the inflammatory and wound repair responses, will lead to repair errors and the introduction of mutations. Increased levels of gonadotrophins and therefore steroid hormones, may also be involved in the inflammatory pathway.

1.4 CLASSIFICATION OF OVARIAN TUMOURS

The ovary has the greatest diversity of tumour types than any other organ in the body. The recognition and appropriate classification of these tumours is therefore very important both in terms of the mechanisms of tumour function and growth, and the selection of appropriate therapy. The World Health Organisation's (WHO) classification of ovarian neoplasms (Talerman, 1992) is the most commonly used system for determining the histological sub-types of ovarian tumour. A simplified version of the WHO classification is shown in table 1.1.

Broadly, ovarian tumours can be classified into epithelial and non-epithelial. In addition, tumours may be defined as benign, of low malignant potential (borderline), or malignant.

1.4.1 Non-epithelial ovarian tumours

Approximately 10% of all ovarian neoplasms are non-epithelial. As can be seen from table 1.1, there are many sub-classifications of non-epithelial ovarian tumours, the most common of which are the stromal and germ cell tumours (Merino & Jaffe, 1993). Germ cell tumours predominate in children and young women, accounting for 90% of ovarian tumours in pre-pubescents and 60% in women under twenty years of age.

I Surface epithelial -stromal tumours (benign, borderline or malignant)

- Serous tumours
- Mucinous tumours
- Endometioid tumours
- Clear cell tumours
- Transitional cell tumours (Brenner tumours)
- Squamous cell tumours
- Mixed epithelial tumours
- Undifferentiated carcinomas
- Unclassified

II Sex-cord stromal cell tumours

- Granulosa-stromal cell tumours
- Sertoli-stromal cell tumours; Androblastomas
- Sex-cord tumour with annular tubes
- Gynandroblastoma
- Steroid (lipid) cell tumours

III Germ cell tumours

- Dysgerminoma
- Yolk sac tumour (endodermal sinus tumour)
- Embryonal carcinoma
- Polyembryoma
- Choriocarcinoma
- Teratoma
- Mixed tumours

IV Gonadoblastoma

V Germ cell--sex-cord stromal tumour

VI Tumours of rete ovarii

VII Mesothelial tumours

VIII Tumours of uncertain origin

IX Gestational trophoblastic diseases

X Soft tissue tumours not specific to ovary

XI Malignant lymphomas

XII Unclassified tumours

XIII Secondary (metastatic) tumours

XIV Tumour-like lesions

Table 1.1: WHO Histologic classification of ovarian tumours.

1.4.2 Epithelial ovarian tumours

Epithelial tumours account for the majority, ~90%, of ovarian neoplasms and are thought to arise from the surface epithelium of the ovary or from the lining of ovarian inclusion cysts, believed to represent invaginations of the surface epithelium (Deligdisch, 1997). These tumours are uncommon in young women, who tend to develop benign or borderline lesions, and extremely rare prior to menarche (Merino & Jaffe, 1993). However, around menopausal age, the incidence of epithelial tumours increases significantly.
The most common sub-types of ovarian tumour are listed in table 1.2. Along with their characteristic features and incidence (Berek & Hacker, 1994).

Histological Subtype	Characteristics	Frequency
Serous	Composed of epithelial cells resembling those of the fallopian tubes which secrete serous fluid. Derived from the surface epithelium.	80%
Mucinous	Characterised by tall columnar epithelial cells which form mucin-secreting cysts.	10%
Endometrioid	Comprised of epithelial cells resembling those of the endometrium.	8%

Table 1.2. Characteristics and incidence of common histological types of ovarian tumour.

1.5 STAGING OF OVARIAN TUMOURS

The staging of ovarian tumours is obviously of extreme importance for the management and prognosis of individual cases. FIGO, International Federation of Gynaecology & Obstetrics, is the body responsible for standardising the pathological staging of ovarian tumours. Stage indicates the extent to which the tumour has progressed and metastasised. The criteria by which the surgeon assigns stage to an ovarian tumour is as follows:

- Stage I Growth limited to ovaries.
- Stage II Pelvic extension - involvement of other pelvic organs or tissues.
- Stage III Peritoneal implants outside pelvis or positive retroperitoneal nodes.
- Stage IV Parenchymal liver metastasis, pleural effusions, distant metastasis.

1.6 GRADING OF OVARIAN TUMOURS

Grade refers to the level of cell differentiation and morphology. The higher the grade the less differentiated the tumour, indicating that the tumour cells have lost many or all of the characteristic features of the original parental cell. Higher grade is usually associated with poor prognosis since the tumour cells are undifferentiated and unresponsive to the normal controls on cell growth

1.7 GENETIC BASIS OF CANCER

Approximately 5% of ovarian tumours are believed to arise due to inherited factors, the remaining 95% of ovarian tumours are sporadic (Boente, 1996). It is generally accepted that carcinogenesis occurs due to a gradual accumulation of mutations within a population of cells which lead to a breakdown of cell growth control. These mutations occur in oncogenes, causing their activation, tumour suppressor genes leading to their inactivation, or DNA repair genes, disrupting DNA repair mechanisms.

1.7.1 ONCOGENES

Proto-oncogenes encode proteins with normal physiological roles in cellular growth and differentiation. The alteration of these genes either through activating mutations or over-expression, following stimulation from other signalling pathways or loss of inhibitory activities, results in a loss of control over growth and proliferation, and subsequent tumorigenesis. Oncogenes are dominant-transforming genes, they can be activated following an alteration in one copy of the gene. These activating alterations include:

- point mutation
- mitotic-recombination
- over-expression
- hypermethylation

Many oncogenes have now been recognised, the most extensively studied of these are discussed here.

Generally oncogenes can be grouped according to the type of protein that they encode.

- **Receptor Tyrosine Kinases**: *c-erbB*-2; *fms*
- **Non-Receptor Tyrosine Kinases**: *abl*; *src*
- **Signal transduction membrane proteins/ G proteins**: *ras*
- **Nuclear transcriptional regulatory proteins**: *myc*; *myb*; *jun*
- **Peptide Growth Factors**: *EGF*; *PDGF*; *M-CSF*; *TGFα*; *c-sis*; *IGF1*; *IGF2*

Many of these have shown alterations of expression in several cancers, including ovarian.

1.7.1.1 *c-erbB*-2

c-erbB-2 (HER2/*neu)* maps to chromosome 17q, encodes an epidermal growth factor-like receptor tyrosine kinase and is involved in signalling pathways including that of Ras and c-Src (Engelman *et al.*, 1998).

Mutations within the rat *neu* gene have been shown to be very oncogenic, inducing mammary tumours. It is also of interest that over-expression of the wildtype gene also leads to mammary tumour formation.

There are 3 major mechanisms by which *neu* activation can occur:

- Over-expression.
- A point mutation within the transmembrane domain.
- Small deletions in the extracellular domain close to the membrane.

The activation of *c-erbB*-2 may then confer the ability of cells to resist cytotoxicity (Meden *et al.*, 1998). This was suggested following the observation that ovarian tumour-derived cell lines, over-expressing *c-erbB*-2, were more resistant to TNF cell lysis than cell lines expressing lower *c-erbB*-2 levels. Indeed, *c-erbB*-2 has been shown to be overexpressed in approximately 30%, and amplified in 8% of ovarian malignancies (Berek *et al.*, 1993); (Katsaros *et al.*, 1995). This overexpression is associated with a poor prognosis in both ovarian and breast tumours (Katsaros *et al.*, 1995).

1.7.1.2 *Ras*

Ras proteins generally function in a similar way to regulatory G-proteins, controlling cell growth by regulating signal transduction at the cell membrane. Thirty percent of all human cancers display a mutated version of one of the 3 human *Ras* genes, suggesting that they have an important role in the process of carcinogenesis (Cox & Der, 1997). Mutations of K*ras* and N*ras* are more common than that of H*ras*, and are most frequently seen in pancreatic (90%), lung (40%) and colorectal (50%) cancers. They are relatively rare events in ovarian tumours (5-15%), suggesting that these genes do not play a pivotal role in the development of ovarian neoplasms. However, Chenevix-Trench and colleagues (1997) showed that mutations in K*ras* were more common in borderline epithelial ovarian tumours than their malignant counterparts, and that mucinous tumours showed a significantly higher mutation rate than other histological types.

Although, ovarian tumours do not show a high rate of ras mutation, it is possible, considering the range of signalling pathways that ras proteins are involved in, that altered regulatory function of other members of these cascades can cause changes in ras function within these tumours. For example, the loss of function of negative ras regulators, such as NF1-GAP (NeuroFibromatosis type-1-GTPase Activating Protein), leads to the up-regulation of ras activity (Cox & Der, 1997). In addition, other over-expressed oncogenes, such as *c-erbB*-2 and *EGF*, exert their transforming capabilities through ras-dependent pathways, indicating that aberrant ras function may be more wide-ranging than simply those tumours harbouring *ras* mutations.

1.7.1.3 c-*myc*

c-*myc*, located on human chromosome 8, is a transcription factor belonging to the helix-loop-helix/leucine zipper family. Transcription factors play an important role in promoting the switch from resting to proliferating cells. Changes in the regulation or function of these factors can therefore lead to uncontrolled cell growth and proliferation and the promotion of tumorigenesis. Mutations of c-*myc* or in its regulatory pathways, results in growth factor-independent expression of c-*myc* and potentially cell transformation. Conversely, the inhibition of c-*myc* impairs cell proliferation even in the presence of growth factors. Further studies have shown that fibroblasts, in which both *myc* alleles have been inactivated, are viable but exhibit severely diminished proliferative activity. In addition, *both* c-*myc* and *ras*

individually, can transform cell lines but not primary cells. This evidence would seem to suggest that whilst c-*myc* is important in cell proliferation, it is not essential, nor does the activation of c-*myc* alone, confer transformation. c-myc is seen to be over-expressed in approximately 26% of ovarian tumours (Katsaros *et al.*, 1995), with the more aggressive tumours showing the highest levels of c-myc (Tanner *et al.*, 1998). In addition, the amplification of the c-*myc* gene has also been detected in ovarian cancer cell lines in a number of studies (Abeysinghe *et al.*, 1999), providing further evidence for the activation of this oncogene in ovarian neoplasia.

1.7.2 TUMOUR SUPPRESSOR GENES

Tumour suppressor genes (TSG) normally act as the 'brakes' on cell growth. Therefore, if they are mutated, control of cell proliferation may be lost and tumorigenesis result. Knudson (1971) explained this inactivation of TSGs using the 'Two-Hit' hypothesis. which states that both alleles of a gene must be mutated or deleted to remove the function of the TSG.

1.7.2.1 The Two Hit Hypothesis

Retinoblastoma is a childhood disease in which tumours develop in the eyes, either unilaterally or bilaterally. Prior to the discovery of the Retinoblastoma gene (*RB1*), it was noted that the disease presented at an earlier age and with many more tumours, in children with a family history of the disease. In addition, these children often exhibited bilateral tumours, in contrast to patients with no family history, who only display in one eye. It therefore appeared that Retinoblastoma could arise either from a germinal or somatic mutation. Knudson (1971) studied 48 cases, both hereditary and sporadic, in an attempt to determine the number of genetic events required to produce these two forms of the disease. He predicted that two mutations, occurring at the same rate, must be experienced to cause Retinoblastoma. One of these may be inherited as a germline mutation occurring at a similar rate (figure 1.2). Further work has shown that the *RB1* gene is indeed mutated and 'knocked out' in Retinoblastoma patients.

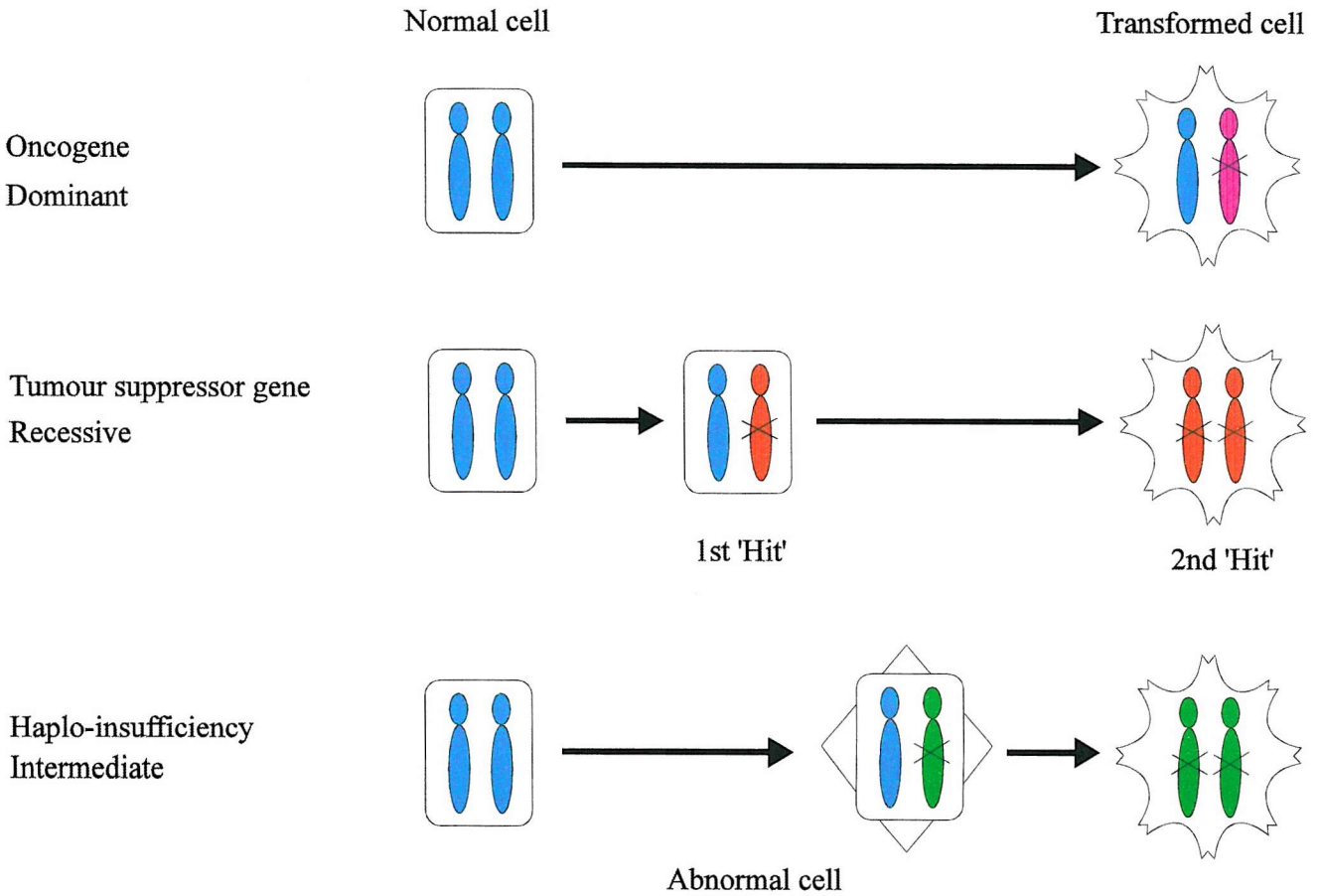


Figure 1.2. Different types of mutation in tumorigenesis.

Oncogenes are dominantly mutated and are activated following a change in one gene copy.
Tumour suppressor genes are recessively inactivated, requiring mutations of both copies for activity to be lost. This is known as the 'Two Hit' hypothsis
The mutation at one allele of a tumour suppressor gene is sometimes sufficient to produce an altered phenotype (p53, PTEN). This intermediate mutation is often referred to as haplo-insufficiency.

By whichever mechanism the 'first hit' occurs, the 'second hit' often results in a phenomenon known as loss of heterozygosity (LOH), generally accepted as being indicative of the presence of a TSG. The most common way for this to occur, though by no means the only mechanism, is for the first allele to be inactivated due to a genetic mutation, either germline or somatic, involving just one or a few nucleotides and the second by the deletion of the entire gene along with part or all of the chromosome. This phenomenon is discussed in more detail in section 1.3.1.

Many chromosomes exhibit deletions in ovarian cancer, including chromosome 7, suggesting that multiple TSGs are involved in the aetiology of ovarian malignancies. Since the *RB1* (Retinoblastoma) gene was identified as a TSG, many others have been discovered and extensively studied. Some of the TSGs known to date to be involved in the aetiology of ovarian cancer are discussed here.

1.7.2.2 *RB1* and $p16^{INK4A}$

The Retinoblastoma gene (*RB1*) maps to 13q14, a region exhibiting high rates of LOH (30-60%) in ovarian tumours. This LOH is associated with more aggressive and higher grade tumours (Dodson *et al.*, 1994; Kim *et al.*, 1994; Li *et al.*, 1991; Liu *et al.*, 1994). It has been suggested that *RB1* is the target for LOH at 13q, since truncation of the remaining allele has been observed. However, the low frequency of mutations in *RB1* observed in ovarian tumours (Liu *et al.*, 1994), even those exhibiting LOH within this region, express wildtype *RB1* mRNA and protein (Dodson *et al.*, 1994; Kim *et al.*, 1994), suggest that the *RB1* gene is not the major target in ovarian cancer. In contrast, in a study using immunohistochemistry (Taylor *et al.*, 1995), benign and borderline tumours are seen to express Rb protein, whereas a small proportion of malignant tumours are pRb negative, implying that alterations in *RB1* occur in the late stages of a particular subset of malignant ovarian tumours.

The RB protein (pRb) is involved in cell cycle regulation, where it acts to inhibit the switch between the G1 (Gap 1) and S (Synthesis) phases. Thus, absence of pRb function removes this block and leads to uncontrolled cellular proliferation. It has recently been proposed (Todd *et al.*, 2000) that it is a defect of the Rb/cyclin D1/p16 pathway, other than the loss of the individual *RB1* or $p16^{INK4A}$ genes, that may play a major role in the development of ovarian cancer. The phosphorylation of Rb by molecules such as cyclin D1 and cyclin-dependent kinases (CDK4/CDK6), results in the transition of the cell from G1 to S phase. Cyclin-dependent kinase inhibitors such as p16, encoded by the $p16^{INK4A}$ (*CDKN2A*) gene,

prevent the phosphorylation of pRb and therefore cellular progression through the cell cycle. In the majority of human cancers, a significant change in expression of at least one of the components of the Rb/cyclin D1/p16 pathway is observed, with the reduction in pRb or p16 expression being the most common finding. However, in a study of ovarian cell lines and primary tumour tissue (Todd *et al.*, 2000), the majority of samples showed co-expression of pRb, p16 and cyclin D1 proteins. In addition, the introduction of high levels of adenoviral-mediated p16 protein, produced growth arrest in an ovarian cell line expressing pRb but not p16, but did not have any effect on the growth of cell lines coexpressing pRb and p16, implying that these cells harbour a defect downstream of p16 in the Rb/cyclin D1/p16 pathway.

1.7.2.3 *TP53*

The *p53* tumour suppressor gene is located at 17p13.3, a chromosomal region exhibiting high rates of genomic instability in the majority of human cancers (Mertens *et al.*, 1997). Indeed, studies indicate that LOH at 17p occurs in approximately 60-70% of epithelial ovarian tumours (Gallion *et al.*, 1992; Lee *et al.*, 1990; Eccles *et al.*, 1992a; Osborne & Leech, 1994). This loss is observed in all grades (Dodson *et al.*, 1993) and stages of ovarian cancer including benign lesions indicating that it is an early event in ovarian tumorigenesis. In addition, comparative genomic hybridisation (CGH) studies (Iwabuchi *et al.*, 1995) have shown reduced copy number of the 17pter-q21 region in invasive ovarian carcinomas, which may well represent loss of the *TP53* locus.

p53 is a 53 kDa nuclear phosphoprotein expressed in normal cells and involved in the control of cell growth and development via its action as a transcription factor. It is activated following cellular stresses such as DNA damage or the activation of oncogenes (Meek, 1999; Oren, 1999). Functional p53 acts to either induce growth arrest, allowing the DNA to be repaired prior to replication, or apoptosis, leading to the death of the damaged cell (Soussi, 1996). In this capacity p53 acts as a cell-cycle checkpoint and is often referred to as the 'guardian of the genome'. The absence of normal p53 activity results in the inefficient repair of DNA and the appearance of genetically unstable cell populations with the potential to become immortalised and tumorigenic (Carson & Lois, 1995). Loss of p53 function results from either a mutation followed by overexpression of the mutated copy of the gene, or by a mutation followed by the deletion of the remaining wild-type copy. This inactivation is seen to be associated with the production of an aggressive, malignant phenotype, often resistant to

therapy. One of the most frequent mechanisms of *p53* inactivation occurs via somatic point mutation and is believed to be the most common genetic aberration in human cancer. Indeed, approximately 30-50% of ovarian tumours harbour *p53* mutations (Suzuki *et al.*, 1998) and in addition show overexpression of the gene (Eccles *et al.*, 1992a). The majority of mutations of p53 observed in ovarian tumours occur within the mutational 'hot spot' of the gene, which encompasses exons 5-8. Point mutations, particularly missense, are the most common alterations seen, although deletions and insertions are also detected within ovarian tumours (Milner *et al.*, 1993).

1.7.2.4 *BRCA1 & BRCA2*

The BRCA1 and BRCA2 nuclear phosphoproteins were first identified as tumour suppressors in familial breast and ovarian cancer. Mutations of *BRCA1/BRCA2* account for approximately 80% of hereditary site-specific ovarian cancers (Gallion *et al.*, 1995), and almost all cases of the disease in families with a history of breast-ovarian cancer. Indeed, a women's lifetime risk for developing ovarian cancer if she carries a mutation in *BRCA1* is 60% and 30% if she harbours a *BRCA2* mutation .

Regions of LOH on 17q21 and 13q12-13, that include the *BRCA1* and *BRCA2* loci, have been frequently observed in both hereditary and sporadic ovarian cancer, suggesting that the loss of *BRCA1/BRCA2* activity is associated with both forms of the disease. In addition, decreased levels of *BRCA1* mRNA have been reported in sporadic ovarian cancers, as would be expected for a classical TSG. However, the BRCA1 protein is observed in the cytoplasm of many ovarian cancers, although often at reduced levels (Zheng *et al.*, 2000), methylation of the promoter of *BRCA1* occurs in only 5% of tumours (Cattreau *et al.*, 1999) and mutations of *BRCA1* are seen only at very low frequency in sporadic ovarian cancer (Hosking *et al.*, 1995; Merajver *et al.*, 1995) compared to the hereditary variants, suggesting that the alteration of *BRCA1* is not a critical step in these tumours. Somatic mutations of BRCA2, accompanied by loss of the second BRCA2 allele, have also been reported in cases of sporadic ovarian cancer, although at low frequency (Foster *et al.*, 1996).

1.7.2.5 *PTEN*

The *PTEN* (phosphatase and tensin homologue deleted on chromosome ten) or *MMAC1* (mutated in multiple advanced cancers) tumour suppressor gene is located on 10q23, a region frequently targeted by LOH in many tumour types (Saito *et al.*, 2000). *PTEN* encodes a phosphatidylinositol phosphatase, which acts to antagonise and inactivate the second messengers of the P13Kinase growth-control pathway, in particular phosphatidylinositol (3,4,5)-triphosphate (PIP3). Mutations of the gene have been observed in several cancers including those of prostate and endometrium (Lin *et al.*, 1998). Ovarian cancer is no exception, with frequent mutations occurring in early stage endometrioid tumours (21%), suggesting that *PTEN* plays a significant role in the aetiology of this histological sub-type (Obata *et al.*, 1998). In addition, *PTEN* has recently been shown to exhibit growth-suppressing properties when transfected into ovarian carcinoma-derived cell lines (Minaguchi *et al.*, 1999), providing further evidence to support *PTEN*'s classification as an ovarian tumour suppressor gene. Also of interest is the observation that the function of *PTEN* can become diminished through a phenomenon known as haplo-insufficiency (Macleod, 2000). In this scenario the activity of *PTEN* is impaired following the loss of just one gene copy, unlike a classical TSG which loses its activity following the mutation and/or deletion of both copies of the gene (figure 1.2). It has been suggested that the function of many more TSGs may be impaired in this way.

1.7.3 DNA MISMATCH REPAIR GENES

The ability of a cell to repair any damage to its complement of DNA is imperative to its survival and subsequent proliferation. The human genome contains many stretches of nucleotide repeats. During replication, there is the potential for the replicative polymerases to alter these repeats by allowing the primer strand to 'slip' along the template strand. This 'frameshift' results in a deletion, if the new primer strand slips forward, or an insertion if the primer slides backwards. These mutations are usually repaired by a group of Mismatch Repair (MMR) genes. However, during microsatellite analysis studies of Hereditary Non-Polyposis Colorectal Cancer (HNPCC), it was noticed that there was a discrepancy in allelic pattern between the normal and tumour DNA (Jicriny, 1996), the repeat lengths of the microsatellites were different. This microsatellite instability (MI) is now generally thought to occur following a malfunction of DNA replication or repair, known as replication error

(RER) and can be used as a surrogate marker to indicate the presence of a possible mutation within an MMR gene.

Further studies into the HNPCC syndrome have identified mutations within the known human mismatch repair genes, *hMSH2*, *hPMS1*, *hPMS2*, *hMLH1* and *GTBP* (GT binding protein), suggesting that it is these genetic alterations that lead to this particular disease. Microsatellite instability has been observed in many different cancers, including that of the ovary (King *et al.*, 1995), implying that changes in the DNA repair mechanism may play a role in multiple tumour types. However, although mismatches may occur fairly frequently, it is only when these affect a functionally important gene that cancer initiation may result.

1.8 GATEKEEPERS, CARETAKERS AND LANDSCAPERS

Recently, Kinzler and Vogelstein (1997) have suggested that considering cancer as merely a balance between the activation of oncogenes and the inactivation of TSGs over-simplifies the carcinogenic mechanism. Instead they have hypothesised that genes may act as 'gatekeepers', 'caretakers' and/or 'landscapers' and should, therefore, also be assigned to these alternative cancer susceptibility categories.

1.8.1 Gatekeepers

As the term suggests, the inactivation of a gatekeeper gene may be necessary for cells to pass the genetic 'boundary' of tumour initiation (Sidransky, 1996). Normal copies of these genes act to directly control cell proliferation, achieving this by inhibiting tumour growth or by promoting cell death. Most TSGs can be assumed to act as gatekeepers for a specific cell or tissue type, and in keeping with the 'two-hit' hypothesis (Knudson, 1971), both copies of the TSG must be inactivated to remove its gatekeeping effects (figure 1.3). In addition, proto-oncogenes can also act as gatekeepers, but unlike TSGs only require a mutation in one allele in order to exert their influence.

1.8.2 Caretakers

Caretaker genes are involved in maintaining the stability and integrity of the genome and as such do not have a direct role in the control of cell growth. Instead, the inactivation of a

Figure 1.3. Schematic representation of the different pathways to tumour initiation.

When a proto-oncogene acts as a gatekeeper, only one mutation is needed to initiate tumorigenesis.
In the case of a tumour suppressor gene acting as the gatekeeper, mutations of both alleles are required.
In the caretaker pathway, mutations in the two caretaker alleles and in both gatekeeper alleles are required in order for oncogenesis to occur.

GATEKEEPER PATHWAY
ONCOGENES
TSGs
GK
Precursor lesion
GK
GK
GK
Precursor lesion
CARETAKER PATHWAY
CT
CT
CT
GK
CT
CT
GK
GK
CT
CT
Precursor lesion
MALIGNANT TUMOUR
1ST MUTATION
2ND MUTATION
3RD MUTATION
4TH MUTATION
ADDITIONAL MUTATIONS
GENETIC INSTABILITY

caretaker gene results in genetic instability, which in turn leads to increased mutation rates of all genes, including the gatekeepers (figure 1.3). However, following the inactivation of the caretaker gene, even if other genetic hits occur, a precursor lesion will not arise until the gatekeeper is also knocked out. DNA repair genes are believed to be caretaker genes and recent evidence seems to suggest that *BRCA1*, *BRCA2* and even *P53* may act as caretakers as well as gatekeepers (Pearson & Van Der Luijt, 1998).

Both *BRCA1* and *BRCA2* have regions that can act as transcription activators when bound to the DNA-binding domain of another gene. The protein products of the *BRCA* genes have been shown to bind to the product of the DNA repair gene *Rad51,* implying that the BRCA genes may have a role in activating *Rad51*. A mutation in *BRCA1* or *BRCA2* may then impede the production of Rad51, and the effective prevention of genetic instability. In addition the embryonal cells from *BRCA1*, *BRCA2* and *Rad51* knockout mice show increased sensitivity to radiation. This may result from the inability to detect and repair any induced genomic damage, due to the absence of the caretaker gene usually involved.
Following DNA damage, *P53* has been shown to act as a controller of cell fate. It is believed that *P53*-mediated cell cycle arrest occurs to allow more time for DNA repair. Failure to repair this damage results in apoptosis, preventing the survival and proliferation of cells with genomic damage. This could be interpreted as a caretaker role for *P53*.

1.8.3 Landscapers

These 'landscaper' tumour suppressor genes are proposed to exert their effect by modifying the environment in which tumour cells grow (Macleod, 2000). This modulation may occur in the stromal tissue, extracellular matrix or regulatory proteins such as growth factors, and lead to an unfavourable environment for neoplastic change. Loss of the 'landscapers' function would change the environment and may promote transformation of nearby epithelia. This hypothesis was suggested following the observation that what appeared to be the initiating lesions of juvenile polyposis syndrome (JPS), occurred in the stroma that surrounded the tumour and not in the tumour cells *per se*.

1.9 EPIGENETIC MECHANISMS OF TSG INACTIVATION - METHYLATION

As epigenetic events occur at a higher frequency than genetic events, and as these changes are passed onto daughter cells during cell division, alterations in methylation patterns may well lead to the growth and proliferation of clonally selected populations, a characteristic of cancer.

In general, the more heavily methylated the gene (usually at the 5' end), the less transcriptionally active it is (Laird, 1997). Therefore in the case of proto-oncogenes and tumour suppressor genes, the reduction or increase of DNA methylation could lead to the respective increase or decrease in expression of these genes with oncogenic implications. The hypermethylation of TSGs is often targeted at CpG islands. $p16^{INK4A}$ (*CDKN2A*) is one such gene that is not commonly mutated in sporadic cancers but is transcriptionally silenced via methylation of its promoter region (Toyota & Issa, 1999; Merlo *et al.*, 1995).

1.10 IDENTIFICATION OF ALTERED GENES

1.10.1 LOH analysis

In this study LOH was assessed by utilising polymorphic microsatellite markers (known regions of nucleotide repeats) which are present throughout the genome. These markers are amplified using PCR (section 2.4) and analysed by autoradiography to determine if the patient is heterozygous (has retained both alleles from the respective genomic fragment, one from each parent) or has lost one allele in the tumour DNA (figure 1.4). By mapping the pattern of LOH in tumours, across a spread of microsatellite markers, it is possible to identify common regions of loss and therefore putative TSG loci. The identification of several genes has been aided by the use of LOH, including that of *WT1* (Wilm's tumour) and the *BRCA1* and *BRCA2* genes.

1.10.2 Alternative methods of 'gene hunting'

As technology progresses, the methods by which TSGs are isolated and identified are becoming more sophisticated. Prior to the use of LOH analysis, familial linkage studies were, and still are, used to elucidate the presence of TSGs that may have a role in inherited or familial disease including cancer. This linkage can be undertaken by studying whole

Figure 1. 4. Diagrammatic representation of LOH analysis using microsatellite markers.

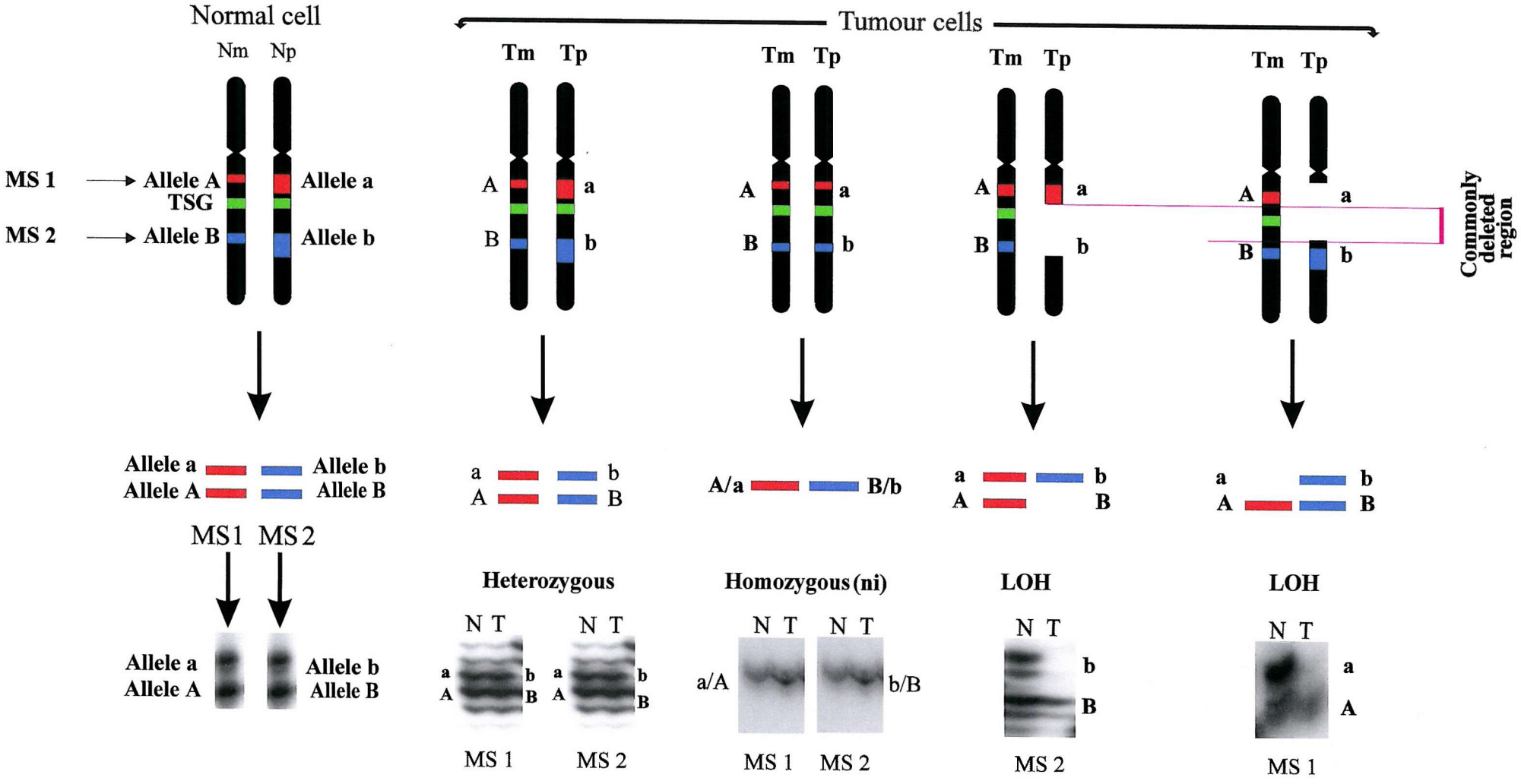
N(m/p) = Normal (maternal/paternal) DNA

T(m/p) = Tumour (maternal/paternal) DNA

MS = Microsatellite marker loci

TSG = Tumour suppressor gene

LOH = Loss of heterozygosity


Normal cell
Nm Np
MS 1
Allele A
TSG
MS 2
Allele B
Allele a
Allele b
Allele a
Allele A
Allele b
Allele B
MS1 MS2
Allele a
Allele A
Allele b
Allele B
Tumour cells
Tm Tp
A
a
B
b
a
A
b
B
Heterozygous
N T
N T
a
A
b
B
MS 1
MS 2
A/a
B/b
Homozygous (ni)
N T
N T
a/A
b/B
MS 1
MS 2
a
A
b
B
LOH
N T
b
B
MS 2
LOH
N T
a
A
MS 1
Commonly deleted region

pedigrees, twins or even siblings to indicate the relationship between both phenotypic and genotypic markers, and the disease in question. Techniques such as LOH and representational difference analysis (RDA) have been utilised to identify changes in overall genomic structure, ranging from point mutations and deletions to rearrangements and amplifications, which can then be used to indicate potential TSG or oncogene loci. Differential display and now micro-arraying and gene-chip technology are allowing researchers to rapidly collect gene expression data from many hundreds and even thousands of genes, in a wide range of cellular environments including that of the tumour cell. Analysis of the up and down-regulation of a range of genes, within a particular tumour type, stage or grade should provide invaluable information about the tumorigenic pathway and the roles that genes may play.

As the human genome project nears completion, the interest in gene discovery has shifted towards the new field of proteomics, the study of the entire protein complement of an organism. By studying the range of proteins expressed in tumours compared to normal tissue, and vice versa, it is hoped that a new insight into carcinogenesis can be achieved, by using the central dogma - in reverse.

1.11 DISTINCT GENETIC ALTERATIONS OCCUR THROUGHOUT TUMORIGENESIS

The model for colorectal carcinoma progression proposed by Fearon and Vogelstein (1990) hypothesises that cells accumulate mutations, in proto-oncogenes and TSGs, that offer selective advantage (Bodmer *et al.*, 1994), leading to expanded populations of cells within which further mutations take place and tumorigenesis progresses (Weinberg, 1996). Microsatellite analysis of colorectal adenomas and adjacent carcinomas has revealed a sequence of LOH events that occur during this particular neoplastic progression (Boland *et al.*, 1995). No LOH was observed in normal tissue surrounding the tumours, but LOH at 5q was detected at the transition between normal tissue and colorectal adenoma, and 17p LOH at the transition from benign tissue to carcinoma. Several other genetic events have been associated with specific steps along the colorectal tumorigenic pathway, providing the basis for a molecular model of this disease (figure 1.5). Although in many colorectal tumours this pathway holds true, in others it does not, implying that although the accumulation of these alterations is critical the order in which they occur is not.

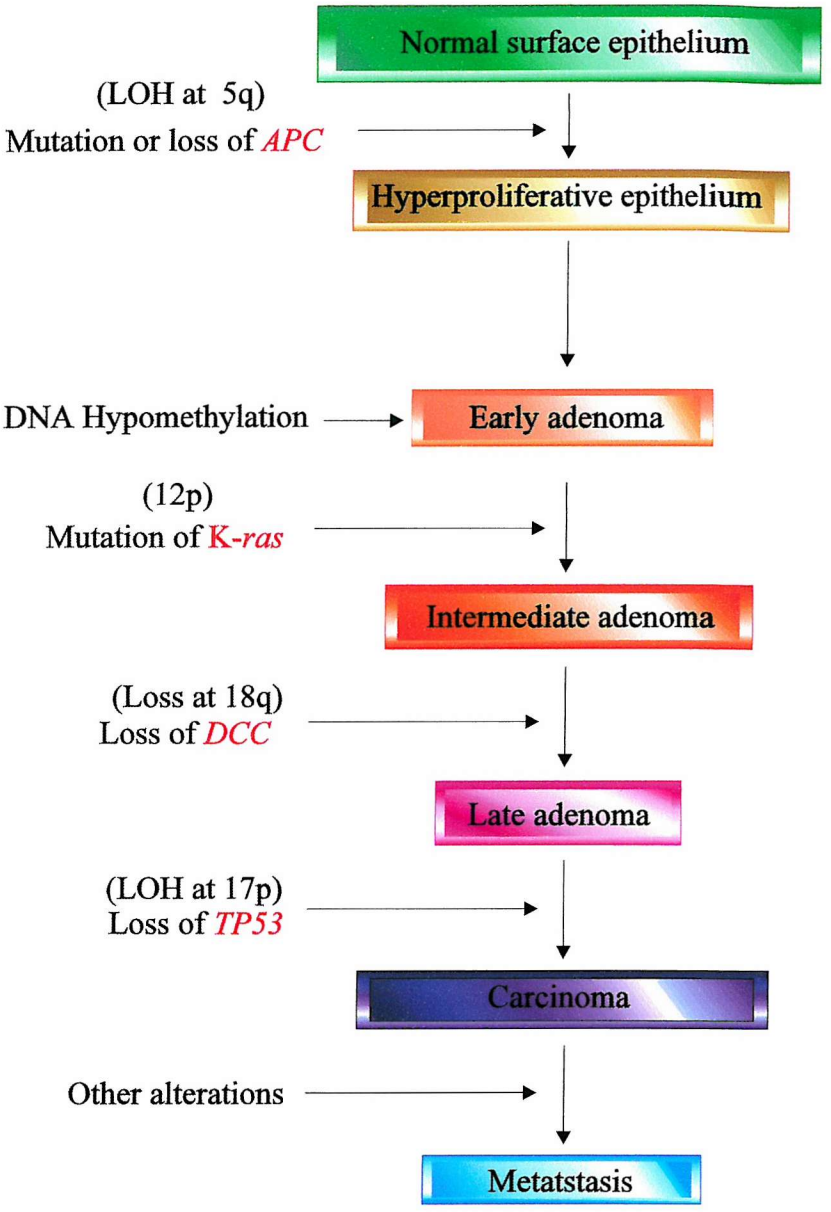


Figure 1.5. Proposed model of colorectal tumorigenesis.

Adapted from Fearon & Vogelstein, 1990.

Unlike colorectal cancer, the molecular genetic events that occur during the initiation, promotion and progression of ovarian cancer have not yet been well characterised, however many studies have attempted to elucidate these steps. Figure 1.6 summarises the reported LOH frequencies for each chromosome arm in ovarian cancer. By combining data collected using many different methods of study, including LOH, CGH (comparative genome hybridisation) and mutation screens, from ovarian tumours spanning a range of stages, grades and histologies, it may be possible to suggest a genetic model for this disease. The ability to define the genetic alterations occurring before and during tumour initiation, promotion, progression and finally invasion and metastasis, would provide a good base from which to develop, and target, novel diagnostic and therapeutic approaches. Figure 1.7 shows a tentative model for ovarian tumorigenesis based on a consensus of the literature to date. This is by no means the whole story, but provides a 'time-line' for some of the more intensely studied alterations.

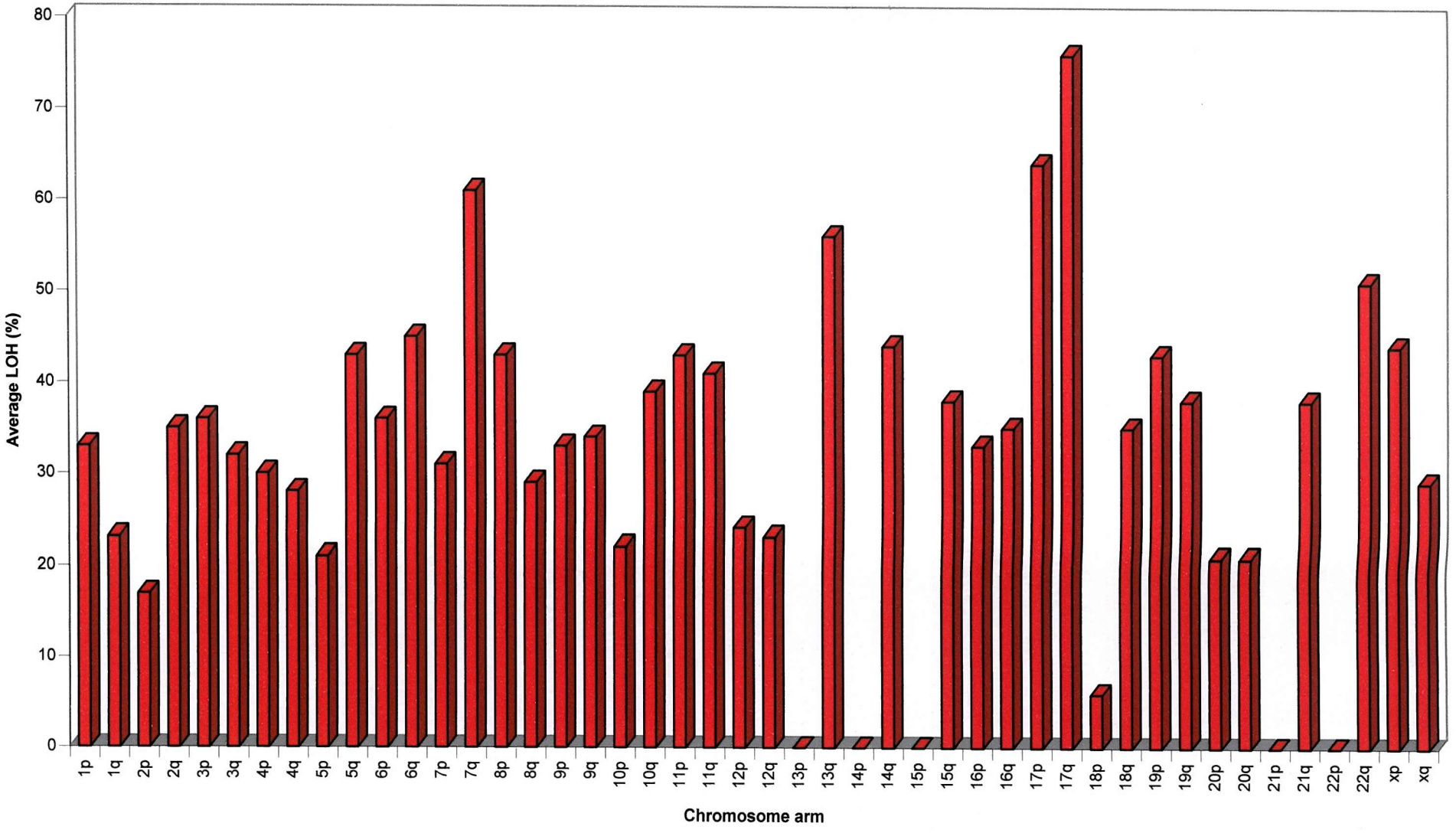
1.12 CHROMOSOME 7 AND CANCER

Approximately 5% of the human genome is encoded by chromosome 7, which is believed to harbour more than 4000 genes across its 170 Mb of DNA (Glockner *et al.*, 1998). Many studies have highlighted genetic changes across chromosome 7 in a wide range of solid tumour types and leukaemias, suggesting that a gene or genes located on this chromosome may play an important role in the tumorigenic pathway. As can be seen from figure 1.6, studies of ovarian cancer indicate that LOH appears to be much more frequent on the long arm of chromosome 7 (61%) than the p arm (31%). Indeed LOH on chromosome 7q seems to be among the most common alterations in ovarian tumours. Studies in a wide variety of malignancies have also demonstrated frequent LOH on 7q suggesting the presence of a TSG involved in many different neoplasms (figure 3.1). In addition, some tumour types, for example renal and ovarian, exhibit distinct LOH at both 7q22 and 7q31. This frequent pattern of allelic loss and the presence of multiple deleted regions, warrants the search for a TSG or TSGs on this chromosome arm. Evidence for the presence of a TSG on chromosome 7 is discussed further in chapter 3.

Figure 1.6. Histogram illustrating the reported rates of LOH in ovarian cancer with respect to chromosome arm.

Abeln *et al.*, 1994; Allan *et al.*, 1994; Arnold *et al.*, 1996; Bandera *et al.*, 1997; Bicher *et al.*, 1997; Brown *et al.*, 1999; Bryan *et al.*, 1996; Chenevix-Trench *et al.*, 1992; ChenevixTrench *et al.*, 1997; Choi *et al.*, 1997; Cliby *et al.*, 1993; Colitti *et al.*, 1998; Cornelis *et al.*, 1995; Davis *et al.*, 1996; Devlin *et al.*, 1996; Dodson *et al.*, 1994; Eccles *et al.*, 1992b; Edelson *et al.*, 1997; Foulkes *et al.*, 1993a; Foulkes *et al.*, 1993b; Foulkes *et al.*, 1993c; Fullwood *et al.*, 1999; Gabra *et al.*, 1995; Gallion *et al.*, 1992; Gallion *et al.*, 1995; Hatta *et al.*, 1997; Huang *et al.*, 1999; Imyanitov, 1999; Kawamaki, 1999; Kerr *et al.*, 1996; Kiechle-Schwarz M *et al.*, 1993; Koike *et al.*, 1997; Koike, 1999; Lancaster *et al.*, 1996; Launonen *et al.*, 1998; Lin *et al.*, 1998; Liu *et al.*, 1994; Lounis *et al.*, 1998; Lu *et al.*, 1997; Niederacher *et al.*, 1999; Obata *et al.*, 1998; Osborne & Leech, 1994; Otis, 2000; Papp *et al.*, 1996; Rodabaugh *et al.*, 1995; Roy *et al.*, 1997; Sakamoto *et al.*, 1996; Saretzki *et al.*, 1997; Sato *et al.*, 1991; Suzuki *et al.*, 1998; Tavassoli *et al.*, 1996; Viel *et al.*, 1992; Villeneuve *et al.*, 1999; Wang *et al.*, 1999; Watson *et al.*, 1998; Weitzel *et al.*, 1994; Wright *et al.*, 1998; Yang-Feng *et al.*, 1992; Zborovskaya *et al.*, 1999; Zenklusen *et al.*, 1995b.

Reported LOH in ovarian cancer



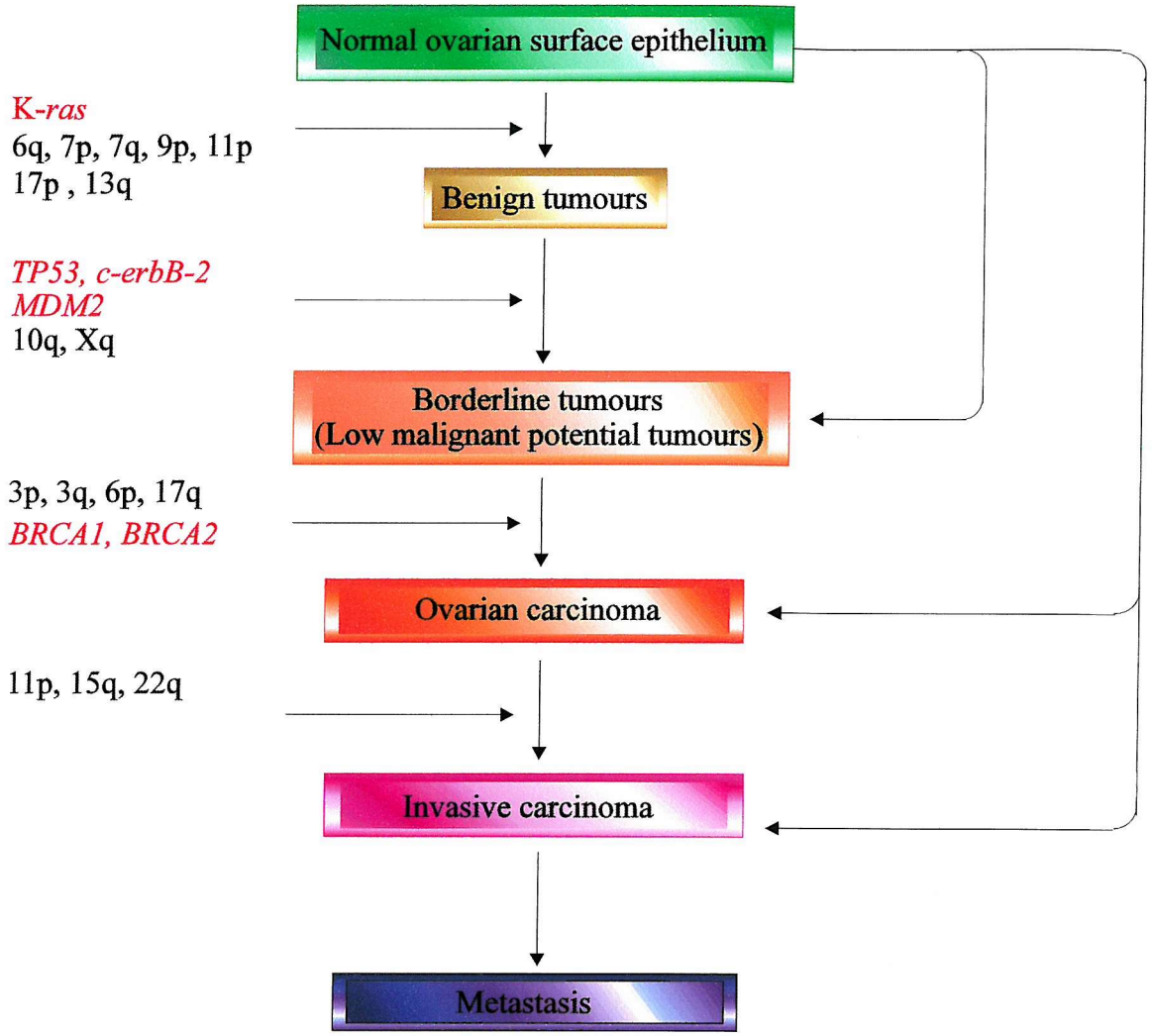


Figure 1.7: Proposed model of ovarian tumorigenesis.
Reported LOH shown in black text.
Reported genetic alterations are highlighted in red text.
Adapted from Chuaqui et al., 1997.

1.13 AIMS OF THIS STUDY

The first goal of this study was to determine the overall frequency of LOH on chromosome 7 in human epithelial ovarian cancer, utilising a large cohort of ovarian tumours and a relatively high number of microsatellite markers.

From this data I also hoped to establish if any specific regional deletion on chromosome 7 was associated with any particular histological sub-type, stage or grade of ovarian tumour.

Further, it was hoped that by using a dense array of microsatellite markers some tumours would be identified with relatively small deletions which could aid in the identification of candidate TSGs.

In addition to the identification of candidate ovarian TSGs, this study aimed to determine whether chromosomal aberrations, such as LOH or somatic mutations, could be detected in the plasma of ovarian cancer patients, with the view to a future diagnostic or prognostic use.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 CLINICAL SAMPLES

2.1.1 Ovarian tumour specimens

A total of 219 specimens consisting of 130 malignant, 23 borderline and 66 benign fresh ovarian tumours were acquired from hospitals throughout the Wessex region. Fresh tumours were snap-frozen and stored in liquid nitrogen until required. Histological information where available is shown in table 2.1.

2.1.2 Normal DNA samples

For each tumour, matching normal DNA was obtained from blood lymphocytes. In very few cases however, lymphocyte DNA was unavailable and DNA was extracted from cells that had been microdissected from normal areas of tissue sections (section 2.3).

2.1.3 Plasma Samples

Fifteen blood samples were obtained from patients prior to them undergoing de-bulking surgery in the Princess Anne Hospital, Southampton. Plasma was removed from whole blood as explained in section 2.2.5, and stored at -80°C until processed further.

2.2 DNA EXTRACTION

2.2.1 Buffers and reagents

10X TE

100mM Tris (pH 8.0)

10 mM EDTA (pH 8.0)

Table 2.1: Clinical and histological information for ovarian tumours.

Case number	Age[1]	Histology[2]	Grade[3]	Stage	Nature[4]
1	62	serous fibroma	na	na	b
2	60	serous	na	1c	bl
5	82	serous	na	na	b
7	79	serous	na	na	b
10	59	serous	na	na	b
10.1	na	serous	3	na	m
11.1	na	serous	3	na	m
12	57	serous	1	1b	m
13	78	mucinous	na	na	b
13.1	na	mucinous	1	na	m
14	57	serous	2	3b	m
15	84	mucinous thecoma	na	na	b
17.1	na	serous	3	na	m
19	86	serous	3	3	m
20	42	serous	na	3a	bl
20.1	na	serous	2	na	m
22	65	serous	3	3	m
23	47	serous	1	1	m
24	77	serous	na	na	b
24.1	na	undifferentiated	3	3	m
26	51	serous	2	3	m
27	na	serous	2	1b	m
28	na	serous	na	1b	m
28.1	na	serous	3	na	m
29	na	mucinous	na	1a	m
29.1	na	serous	3	3	m
30	59	endometrioid/mucinous	3	3	m
30.1	na	mucinous	1	3	m
32	71	serous	1	2	m
32.1	na	serous	3	3	m
36	54	endometrioid	1	1a	m
37	69	endometrioid	2	1	m
38	na	clear/endometrioid	na	1a	m
39	81	serous fibroma	na	na	b
40	73	mucinous	3	2	m
41.1	na	serous	3	3	m
43	51	serous/endometrioid	2	2	m
44	75	mucinous	1	1	m
45	50	serous	3	3	m
48	50	serous	2	3	m
48.1	na	serous	3	2	m
49	49	Müllerian origin	3	3	m
50	81	mucinous	2	1	m
50.1	na	mixed Müllerian tumour	3	3	m
51	82	mucinous	1	1a	m
60	47	granulosa cell tumour	1	1a	m
61	76	mucinous	1	1a	m
63	66	serous	3	1	m
67	na	serous	2	1a	m
67.1	na	serous	3	3	m
69	55	serous	2	1b	m
70	57	endometrioid	1	1a	m
71	68	serous	2	1a	m
72.1	na	serous	3	4	m
75	68	mucinous	na	na	b
76	66	serous	1	1	m
77	74	thecoma	na	na	b

Table 2.1: Clinical and histological information for ovarian tumours.

Case number	Age[1]	Histology[2]	Grade[3]	Stage	Nature[4]
78	76	endometrioid	2	1a	m
80	71	mucinous	1	1a	m
80.1	na	serous	3	3c	m
83.1	na	serous	3	3c	m
85	69	mucinous/brenner	na	na	b
86	84	serous	3	3a	m
92	50	mucinous	na	1a	bl
94	48	mucinous	na	na	b
95	66	endometrioid	3	2	m
97	74	serous	3	3	m
103	56	serous cystadenofibroma	na	na	b
107	48	mucinous	1	1c	m
113	36	mucinous	na	na	b
114	70	endometrioid	2	1a	m
117	na	mucinous/endometrioid	2	1	m
119	59	serous	2	1c	m
121	77	mucinous	1	3	m
122	na	serous	3	3	m
123	82	endometrioid	3	1b	m
124	53	granulosa cell tumour	na	3	m
125	57	mucinous	na	na	b
128	56	endometrioid	2	1c	m
131	74	serous	3	3	m
134	64	serous	3	3c	m
135	66	serous	2	2	m
136	78	endometrioid	3	1	m
138	47	mucinous	1	1a	m
139	65	serous	3	3	m
140	46	mucinous	na	na	b
142	66	serous	3	na	m
143	71	serous	na	na	b
144	63	mucinous/endometrioid	1	1c	m
146	67	endometrioid	2	1c	m
147	59	serous	na	na	b
148	67	serous	na	na	b
149	66	serous	na	na	b
151	56	endometrioid	2	2c	m
154	52	serous	2	3a	m
155	92	endometrioid	2	1a	m
156	47	mucinous	na	na	b
158	82	serous cystadenofibroma	na	na	b
164	72	serous	na	na	b
167	66	serous fibroma	na	na	b
170	60	endometrioid	2	1a	m
176	62	mucinous	1	1a	m
177	53	endometrioid	3	1a	m
179	62	endometrioid	3	3	m
183	51	serous/mucinous/brenner	na	na	b
184	47	undifferentiated	na	2	m
186	78	mucinous	na	1c	bl
187	72	undifferentiated	3	4	m
188	47	serous	3	3c	m
190	51	serous	2	1	m
192	41	serous	3	3c	m
194	63	papillary	3	2	m
195	65	endometrioid/serous	3	2	m
196	46	serous	na	na	b

Table 2.1: Clinical and histological information for ovarian tumours.

Case number	Age[1]	Histology[2]	Grade[3]	Stage	Nature[4]
201	80	serous	2	3b	m
202	65	endometrioid	1	2	m
204	73	serous	3	3c	m
205	na	endometrioid	na	2	m
206	73	mucinous	na	na	b
211	55	endometrioid	na	2c	m
214	75	mucinous	na	na	b
215	55	serous	3	1c	m
219	63	mucinous	1	1c	m
220	62	endometrioid	2	3c	m
225	58	mucinous	2	3a	m
229	64	serous	na	na	bl
230	49	serous	3	4	m
245	53	teratoma	na	na	b
246	47	mucinous	na	na	b
253	70	endometrioid	2	3a	m
257	60	mucinous	1	1a	m
258	69	endometrioid	3	3c	m
259	67	serous	2	1c	m
262	74	serous	na	na	b
263	62	mucinous	na	1a	bl
267	34	endometrioid	2	1a	m
269	59	fibroma	na	na	b
271	91	mucinous	1	1a	m
276	49	mucinous	na	na	b
279	70	undifferentiated	2	1c	m
282	73	serous	na	3	m
285	61	serous	2	2c	m
286	71	endometrioid	3	3b	m
287	57	endometrioid	1	1a	m
289	53	mucinous	na	1	bl
291	87	serous	3	3	m
293	59	undifferentiated	3	1	m
294	60	mucinous	na	na	b
295	63	adenofibroma	na	na	b
297	53	serous	3	3	m
298	82	serous	3	1c	m
299	na	clear cell	na	1c	m
300	68	endometrioid	3	3	m
301	71	serous	na	na	b
304	53	mucinous	na	na	bl
308	25	serous	na	na	b
311	52	endometrioid	2	1	m
322	22	mucinous	na	1	bl
324	32	mucinous	2	1	m
335	50	mucinous	na	na	b
336	85	mucinous	na	na	b
338	69	serous	2	1	m
339	75	serous	na	na	b
341	62	serous	2	1	m
351	77	serous	na	na	b
357	72	clear cell	3	1	m
360	69	serous	na	na	b
362	77	serous	na	na	bl
372	72	mucinous	na	na	b
376	62	serous	3	1c	m
377	77	serous/endometrioid	2	1c	m

Table 2.1: Clinical and histological information for ovarian tumours.

Case number	Age[1]	Histology[2]	Grade[3]	Stage	Nature[4]
378	69	serous	2	1	m
379	36	mucinous	na	na	bl
388	81	mucinous	na	1a	bl
389	76	mucinous	na	1a	bl
397	70	mucinous	na	na	bl
408	39	teratoma	na	na	b
411	84	mucinous	na	na	b
412	64	serous	na	na	b
416	45	thecoma	na	na	b
418	61	mucinous	na	na	bl
420	69	mucinous	na	na	bl
426	63	serous adenofibroma	na	na	b
429	73	serous cystadenofibroma	na	na	b
433	34	mucinous	na	na	b
438	57	serous	na	na	b
446	77	mucinous	na	na	b
447	81	serous	na	1	bl
449	73	serous	na	na	b
450	68	serous	na	na	b
451	84	adenofibroma	na	na	b
452	73	mucinous	na	1	bl
463	61	mucinous	na	na	b
466	79	serous	na	na	b
467	52	serous	na	na	b
486	57	serous	na	na	bl
494	53	fibroma	na	na	b
508	53	serous	1	1	bl
512	61	mucinous	na	na	b
516	40	mucinous	na	na	b
527	74	serous	na	na	b
531	59	mucinous	1	1	bl
537	61	mucinous	1	1	bl
545	67	mucinous/serous	na	1	bl
548	61	serous	na	3	m
549	42	clear cell	na	na	m
550	72	endometrioid	na	na	m
551	na	serous	na	3	m
557	35	mucinous	na	na	m
560	52	serous	na	4	m
568	60	serous	na	3c	m
569	na	serous/endometrioid	na	3c	m
574	75	undifferentiated	na	na	m
575	62	serous	na	4	m
577	79	serous/endometrioid	na	4	m
580	69	endometrioid	na	na	m
588	na	mucinous	na	na	m
592	na	fibrothecoma	na	na	b
594	na	endometrioid	na	1	m

[1]na = not available

[2]The collection includes 98 serous, 57 mucinous, 28 endometrioid and 6 undifferentiated tumours.

[3]na = not available

[4]b = benign, bl = borderline and m = malignant tumours.

2X Sucrose/Triton

634mM Sucrose

10mM $MgCl_2$

2% (v/v) TritonX100

10mM Tris (pH 7.5)

SDS-Lysis Buffer

75 mM NaCl

25mM EDTA (pH 8.0)

1% (v/v) SDS

TWEEN Lysis Buffer

50mM Tris

1mM EDTA

0.5% (v/v) TWEEN 20

5X TBE (1 litre)

0.45M Tris

0.4M Boric Acid

20mM EDTA (pH 8.0)

50X TAE (1 litre)

2 M Tris Base

50mM EDTA (pH 8.0)

57.1% (v/v) Glacial Acetic Acid

Loading dye

30% (v/v) Glycerol

0.25% (w/v) Xylene Cyanol

90% Formamide buffer

90% (v/v) Formamide

10mM EDTA

0.25% (w/v) Xylene cyanol

0.25% (w/v) Bromophenol blue

2.2.2 DNA extraction from blood lymphocytes

DNA was extracted by the standard salt chloroform method, as described previously (Mullenbach *et al.*, 1989).

Preparation of nuclear fraction

Blood samples were spun in a 30 ml Oak Ridge tube at 10,000rpm for 10 minutes at 4°C in a Hermle centrifuge (Z382K, Germany). The plasma was removed using a disposable Pasteur pipette and stored in 25 ml Falcon tubes at -80°C, and an equal volume of ice-cold 2X sucrose/triton solution was added to the remaining cellular fraction [consisting of some plasma, red blood cells (RBC) and white blood cells (WBC)]. To ensure efficient lysis of cell membranes the solution was mixed and left on ice for 10 minutes and then centrifuged at 10,000rpm for 10 minutes at 4°C to pellet the nuclear fraction of the lymphocytes. The supernatant was removed and 5 ml of 1X sucrose/triton solution was added to remove traces of heme, a potent inhibitor of *Taq* DNA polymerase. This was centrifuged at 10,000rpm and 4°C for 5 minutes and the supernatant removed.

DNA extraction from nuclear fraction

The pellet was resuspended in 5 ml of SDS lysis buffer by drawing the pellet up and down several times into a 10 ml pipette. Fifty µl of 40mg/ml proteinase K was added and the samples incubated overnight at 55°C. Following the incubation, 1.65 ml of pre-warmed saturated NaCl solution and 7 ml of chloroform were added and the samples rotated for 1 hour. The samples were then spun at 10,000rpm for 10 minutes. The top layer was transferred to a new tube and the DNA precipitated by adding 7 ml of 2-propanol. The DNA was pelleted by centrifugation at 15,000rpm for 20 minutes. The pellet was washed with 75% ethanol, resuspended in 500 µl TE and stored at 4°C to ensure that the DNA had completely dissolved. The DNA solution was then transferred to a 1.5 ml Eppendorf tube. Aliquots of stock were diluted 1/20 with 1 X TE buffer for use in PCR.

2.2.3 DNA extraction from fresh tumour tissue

The tumour tissue (200 - 1000 mg) was prepared for tissue extraction by homogenising the fresh frozen samples using a Mikro-dismembrator (B.Braun Biotech International, Germany) and then suspending this powdered tissue in 10 ml SDS lysis buffer. DNA was extracted from the fresh tissue samples using the SDS/Proteinase-K DNA extraction protocol described above in section 2.2.2, except that all following volumes were doubled.

2.2.4 DNA extraction from microdissected tissue

DNA was extracted by incubating the harvested material at 55°C overnight in a solution of 200 µl TWEEN lysis buffer (section 2.2.1) and 1 µl Proteinase K (200µg/µl). The solution was heated to 94°C for 8 minutes before use, to denature the proteinase K.

2.2.5 DNA extraction from plasma

The very small quantity of DNA present in plasma samples necessitated the use of the QIAmp tissue kit (QIAGEN Ltd., UK) in order to purify the DNA available. Purification was carried out according to the manufacturers instructions, from 600µl of harvested plasma sample. The extracted DNA was resuspended in 50 µl of and stored at 4°C.

2.3 MICRODISSECTION

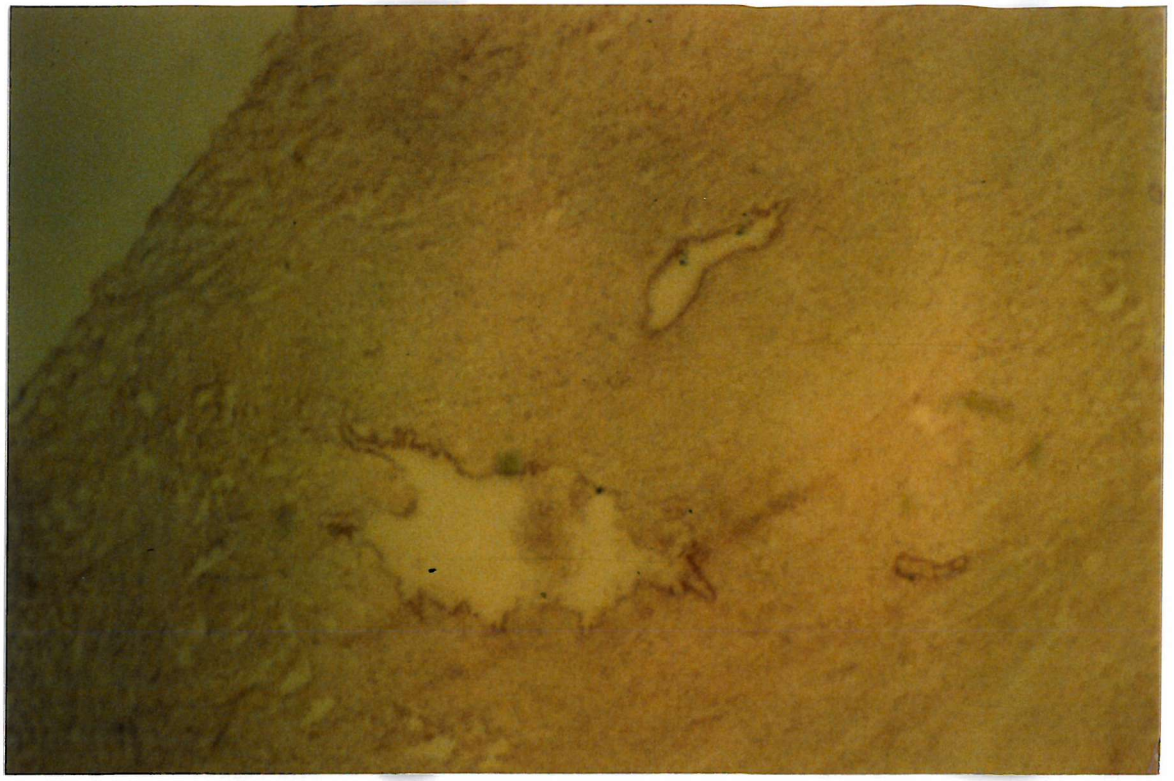
Six sequential five micron sections were cut from fresh, snap-frozen, tumour samples. One section was then stained with haematoxylin and eosin, using standard techniques, to facilitate identification of the desired regions of tumour tissue. Using this slide as a template the required areas of tumour material were carefully scraped from the remaining unstained sections with a 25-gauge needle (figure 2.1). The tissue was then transferred to a 1.5 ml Eppendorf tube containing 200 µl TWEEN lysis buffer (section 2.2.4).

2.4 PCR AND MICROSATELLITE ANALYSIS

2.4.1 Primer Extension Pre-amplification (PEP) Protocol.

This technique uses whole genome amplification, as described previously (Zhang *et al.*, 1992; Beltinger *et al.*, 1997), to produce an amplified representation of the DNA from microdissected tissue. The microdissected tissue in lysis buffer was incubated at 94°C for 10 minutes. PCR was carried out in reaction volumes of 50 µl containing 15 µl of microdissected tissue DNA (genomic DNA) in lysis buffer, 5 µl of a 400 µM solution of a random 15mer primer (MWG Biotech GmbH, Germany), 5 µl 10X reaction buffer (Applied Biotechnologies, UK), 5 µl of a nucleotide mix containing 4mM each of dATP, dCTP, dGTP and dTTP (Promega, UK), 1.25u Taq polymerase (Red Hot Taq, Applied Biotechnologies, UK) and 17.5 µl distilled dH_2O. The thermal cycling conditions for the PEP protocol were as described by Zhang *et al.* (1992):

A.



B.

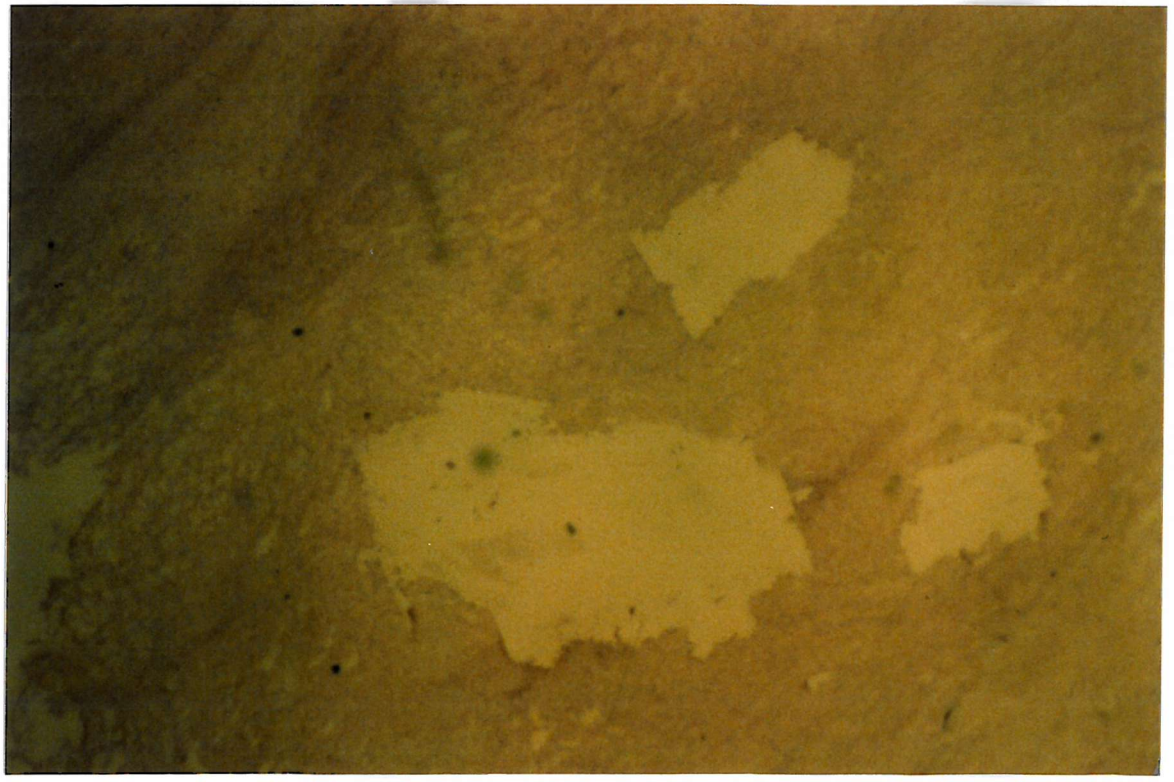


Figure 2.1. Photographic illustration of microdissection.

A. Slide used as template for microdissection.

B. Slide following microdissection of tumour foci.

96°C	12 min
then 50 cycles of:	
94°C	1 min
37°C	3 min
37°C - 55°C	A ramp of 10sec/°C
55°C	4 min
72°C	5 min

Negative controls in which the DNA was replaced with equal volumes of lysis buffer were used to detect contaminating DNA.

2.4.2 PCR amplification of microsatellite markers.

All PCRs were carried out in 0.5 µl Eppendorf tubes in reaction volumes of 10 µl. Each PCR reaction contained 1 µl of DNA (containing 10 - 200ng of DNA); 0.5 µl of a primer mix (containing 50ng of each of the forward and reverse primers); 1 µl of a 10X PCR reaction buffer (Applied Biotechnologies, UK); 0.5 µl of a nucleotide mix containing dATP, dGTP and dTTP at a concentration of 4mM and dCTP at a concentration of 0.4 mM (Promega, UK); 0.1 µl of a 10 µCi/µl solution of [α-^{32}P]dCTP and 0.5 µl of 1 unit/µl Taq DNA polymerase solution (Red Hot Taq, Applied Biotechnologies, UK). To prevent evaporation during the reaction, one drop of mineral oil (Sigma, UK) was added to each tube. Thirty-four microsatellite markers were used to study LOH, the sequences and PCR conditions for which are given in table 2.2. In addition, three chromosome 17 microsatellites were used for the plasma DNA detection study, the sequences and PCR conditions for which are also given in table 2.2.

The standard programme used for PCR amplification was as follows: one initial denaturation cycle of 94°C for 5 minutes, then either 35 or 40 cycles of;

annealing step:	X°C for 45 seconds, (for X see table 2.2).
elongation step:	72°C for 45 seconds
denaturation step:	94°C for 30 seconds

With a final elongation cycle of 72°C for 5 minutes. A hot start procedure was utilised to reduce mis-priming. Negative controls in which the DNA was replaced with equal volumes of water were used to detect contaminating DNA. PCR-amplified products were separated on non-denaturing or denaturing 5-8% polyacrylamide gels (section 2.8.3) which were dried

Table 2.2: Microsatellite markers, their sequences and conditions for use in LOH analysis.

Marker	[a]Cytogenic location	[b]Primer sequence	Annealing temperature
D7S481	7p	TGATTCTCATTCTCACCCCC ATCCCCCACTGTCTCCAAAA	52°C
D7S654	7p	TTGCTGGTGATTTTCCAGGT CCACTCACTCTGTGGCATTT	50°C
D7S691	7p	GGGTGATTAATGCTTGCTTA GCTTGATTTTCCAACAGG	50°C
D7S678	7p11	AGCCCATTTGAGTGGTCTT TGCCATCCTCAGCACTTAG	54°C
D7S670	7p11	AACCGAAGCAGGATTTTATT GTCCATTTTNATTTAGCATTTCA	54°C
D7S506	7p11.1	CCCTTCAAATGCACAGATA GCGTCAGTTACTGGAACTT	52°C
D7S672	7q11.21	ACATGAAGGTCTACCAGTAGCC CACTTTGGTTGGAGCAAGG	53°C
D7S634	7q11.22	AATCCTCCAAATGAAACAGA CTCAGTACACGTTGCTGGTA	53°C
D7S524	7q11.23	AAGTAATGCAAAACAGCCTTGA ACCCACTGAAAAGATTTGTGTC	53°C
D7S630	7q21.11	TCCATTCTGAGGTTTGATGT CCATGGTCTTTTCAATGAAC	55°C
D7S492	7q21.11	ATCTTGGATTTAGGGTTGGC GGCTCTGCTCCATCTTCATA	45°C
D7S2410	7q21.12	CGGTGCCAAGACATTCAG TATAGAAGCAAAGCCAAAAGATCCT	55°C
D7S689	7q21.13	CCTCAACCTGAATCTCACATC CAATGGAGCCAGACTCTGT	54°C
D7S2431	7q21.2	TTAGAAATCCGAGCCAATAGAA TCCACATTGCATGAGCC	53°C
D7S491	7q22.1	AGCTCCAAAACCTAACTCCA TCAAAATTATTTGACTTCTTGATTT	53°C
D7S2480	7q22.1	ACCTTGACTGTGGTAGTTGG GTTCACCTCATAGGAAAATCTTG	54°C
PAI-1	7q22.1	GGGGACAGAGCAAGAATCT GATAGCAGCAAGAGGCTG	53°C
D7S518	7q22.1	GGGTGTGTCTGTGTGACAAC CAGTAGGCAGGGGTGG	55°C
D7S658	7q22.2	CATCACACACCAGGGC AACAGAAGGACTGAACTTCATC	50°C
D7S692	7q31.31	CTGATGATTGCTATAGATATTCATC TGTAAACACTTTTGTAGAAGAACCT	55°C
D7S2502	7q31.31	CCAGTGGTTTTAGTCATTTGTTTTG CATGTATGCTCATGGTTGGA	52°C
D7S486	7q31.31	GCCCAGGTGATTGATAGTGC AAAGGCCAATGGTATATCCC	55°C
7G14	7q31.31	CATCCCAGATACAATTGAG CCCAATAAACCAACCAGA	45°C
D7S522	7q31.31	GCCAAACTGCCACTTCTC ACGTGTTATGCCACTCCC	55°C
D7S633	7q31.31	TGAGCCTCGCATCACTG TCTGGGGAGTCCTTTAACAGTA	53°C

Table 2.2: Microsatellite markers, their sequences and conditions for use in LOH analysis.

Marker	[a]Cytogenic location	[b]Primer sequence	Annealing temperature
D7S655	7q31.31	CAAAATAGTGGGGTATTGGTAAA CCAAGTTAATCTNTGTGAAAGTGTA	50°C
D7S643	7q31.31	AGCTAATATTGCTGCCTTTT CAATCTCTTGCTAGATGCCA	50°C
D7S530	7q32.1	TGCATTTTAGTGGAGCACAG CAGGCATTGGGAACTTTG	53°C
D7S684	7q34	GCTTGCAGTGAGCCGAC GATGTTGATGTAAGACTTTCCAGCC	55°C
D7S676	7q35	TGANTCTAAGCAGCCACCT GCAACATGATCCAGAAAACA	53°C
D7S688	7q36.1	ACAGGAAACGATTGCCATCCTT TGCAGAAATGCCTCCCTAAATATAA	53°C
D7S636	7q36.1	GGAGTGACTGGGCAGGAA AGCTTGTGTGGGGTTTCA	56°C
D7S2546	7q36.2	CACGCCAGGGTCTATCTT GGAGGTTGAACAACTCTGAATAC	57°C
D7S2465	7q36.2	CTTCAAAGAGTTTATGCTTATGTGG ACCTGGGCAACAGAGTGAG	55°C
D17S786	17p	TACAGGGATAGGTAGCCGAG GGATTTGGGCTCTTTTGTAA	55°C
D17S855	17q21.1	GGATGGCCTTTTAGAAAGTGG ACACAGACTTGTCCTACTGCC	55°C
TP53 CA	17q13.1	ACTGCCACTCCTTGCCCCATTC AGGGATACTATTCAGCCCGAGGTG	55°C

[a]The cytogenic location and sequences of the primers were obtained from various databses accessed through the National Center for Human Genome Research (http://www.nih.gov).

[b]The primer sequences are shown in the 5' to 3' orientation.

under vacuum and examined after autoradiographic exposure. The intensities of the alleles obtained from the normal and tumour DNA samples were compared and used to assess for LOH.

2.5 PCR AND GENETIC MUTATION ANALYSIS

2.5.1 PCR of *CUTL1* gene exons.

PCR was carried out as described in section 2.4.3. The primers, the exon they amplify, their sequences and conditions used are shown in table 2.3. In general, primers with sequences complementary to intronic sequences were used with the exception of large exons in which case additional primers complementary to exon sequences were also employed.

2.5.2 PCR of *PAI-1, TP53* and *K-RAS* gene exons.

PCR was carried out as described in Section 2.4.3. Exon primers, their sequences and conditions used are shown in tables 2.4, 2.5 and 2.6 respectively. Again, primers complementary to intronic sequences were utilised.

2.5.3 PCR of *c-MET* gene exons.

Multiplex PCR was carried out as described in Section 2.4.3 with the exception that 50ng of all three exon primers was included in each reaction. Primers complementary to intronic sequences were utilised for amplifying each exon (table 2.7).

2.6 PREPARATION OF PCR PRODUCTS FOR DNA SEQUENCING

PCR amplification was carried out in 30 µl volumes as describe in section 2.4.2 but without the addition of [α-^{32}P]dCTP. The PCR products were purified using the Wizard PCR Preparation System (Promega, UK) according to the manufacturers instructions and resuspended in 50 µl of water. To assess the quality and quantity of DNA, a 2 µl sample was taken before and after purification and analysed on an agarose gel.

2.7 SEQUENCING: 3-dNTP INTERNAL LABEL PROTOCOL

The Thermo Sequenase cycle sequencing kit (Amersham Life Science, UK) was used and sequencing was performed using a 3-dNTP internal labelled primer with [α-^{32}P]dCTP. The

Table 2.3: *CUTL1* Primers, their sequences and conditions for use in SSCP-HD analysis.

Protein Domain Encoded	Primer	[a]Primer Sequence	Annealing temperature
---	Exon 3	TTCTCAAATGGCTGCTTCCT GCCCGGAGCATTTCTTTAT	60°C
---	Exon 4	TCGACAAATGTTGAGCTCTTTG CACCGACTCACCAGACACAA	55°C
---	Exon 5	AGTGGGGTGATGGCTGTTT GAAGAAAAGAAAAAGCAAAGAACG	56°C
---	Exon 6	GCCATATGGAATTGTGTGCTT GGGGCAAATGCTATGTCAGT	55°C
---	Exon 7	ATGACCAATTTGGCTTCGTC CTCCCCACATCCATGGTACT	53°C
---	Exon 8	TGGGAATAGATTACCTGTGTATGC TGTATGGATAAGGGAGGCCA	60°C
---	Exon 9	GCTAGGAAGCTGTCCCATCA ATCTTCCGAGATGCCAGAAA	55°C
---	Exon 10	TTTCTCTTTCACCCCTTTTCA CAAGCGGCTGTAACTCGAAG	55°C
Coiled coil	Exon 11	CAGCGCAGTTTTGTCATCTC GAAAGGCAGAGGGTGTGTGT	60°C
Coiled coil	Exon 12	GTGAAGTCCGTCGACCTGTT AGCCTGGCATTGTTAGCAGA	60°C
---	Exon 13	CTAGCCTGGGTGACAGAACG GCTGATGCTAGTGCTGACCA	60°C
---	Exon 14	CAGTGAGACCCCCGCTCT CTGGAAGCGAACCAACCAC	60°C
Cut repeat 1	Exon 15a	ATGCATTCTGTTTGCCCTTC GTAGAAGCCAGGGGTAGGCT	60°C
Cut repeat 1	Exon 15b	CTTTTTCAGCTCATCCCTGG CGATTTCTGCAGTGTCCATC	60°C
Cut repeat 1	Exon 15c	AGTCAGAAAGTGCTGGGAGC CATGTGCAACTCTGTGACGG	60°C
---	Exon 16	TGTCACACAGCCCATATCGT CCCTAGGTGACTGACTGCAA	60°C
---	Exon 17	ATGTCATTGGCGCAACTTCT ACTGCTGACCAGAGCACTCA	60°C
Cut repeat 2	Exon 18a	CCACACTCTCACCCCTGTTT GTGCACAATCGGCTGCTC	60°C
Cut repeat 2	Exon 18b	CAAAAAGGAGGCCCAGGAC GGGCTGTTCTGTGGTGTCTC	57°C
Cut repeat 2	Exon 18c	AGAGCTGAGTCTGACCGGG CAGCAAGGAGAAGAGATGGG	60°C
Cut repeat 2	Exon 19	CACTGATGGCCTGTGTGTTC TGACCAGGTCCTTGCTATGG	60°C
Cut repeat 2	Exon 20	GGCCTTGCCACATTCAGTTA AGCACGGGACAACAGAAAAG	60°C
Cut repeat 3	Exon 21	ACAGCTATTTTCAGGCACGG CTGACTCAGCAGCCGAGAG	60°C
Cut repeat 3	Exon 22	GGCTCTCGGTGACAATACCT GCCTGTCTATGAATGCGGAC	60°C
Homeobox	Exon 23	CTGGGCCTGACCTTAGTCTG ACAGATCAGCCCCTTCCC	55°C
---	Exon 24a	CGCCGCTTGTTGTCTTGTAG CTCCCTCGTGGTCGTCGT	-
---	Exon 24b	GAGGCCGAGCGGGAGGAG CAGAGCCTTTTCGGCCTC	-
---	Exon 24c	CCAGCAACAGCAGCAGCA CTGGACGGGGTCGGACGG	55°C

[a]The primer sequences are shown in the 5' to 3' orientation.

Table 2.4: *PAI-1* Primers, their sequences and conditions for use in SSCP-HD analysis.

Primer	[a]Primer Sequence	Annealing temperature
Exon 2	ATTCCTTCACCAGCCCTCTT TCCTTGGCTTCTGGTAGGTC	55°C
Exon 3	AAGCCTCACATGTCCTCTCC TCTCTTGAGGCCCAGGAAAT	60°C
Exon 4	TTCCTGATCTTCCTGATCATTG ATGAGATGCAGTTGCTGTGG	60°C
Exon 5	CCATTTCCAACGAACCATCT GTTGGTAGGTCGGGGAGATA	60°C
Exon 6	GTAGGGGATGGGGAAAGGT GGGTGAGAAAATGCAAAGGA	55°C
Exon 7	AGAGCGGCAGCGATCTAAT TCTGGGGACCAGTTATCCTG	55°C
Exon 8	GCCAGCATCCCTCTGTTCTA GAAAGAATGGGTGGGAGGAT	60°C

[a]The primer sequences are given in the 5' to 3' orientation.

Table 2.5: *TP53* Primers, their sequences and conditions for use in SSCP-HD analysis.

Primer	[a]Primer sequence	Annealing temperature
Exon 5	CACTTGTGCCCTGACTTTCA AACCAGCCCTGTCGTCTCT	55°C
Exon 6	CAGGCCTCTGATTCCTCACT CTTAACCCCTCCTCCCAGAG	55°C
Exon 7	CCTGCTTGCCACAGGTCT GTGTGCAGGGTGGCAAGT	55°C
Exon 8	TTTCCTTACTGCCTCTTGCTTC TAACTGCACCCTTGGTCTCC	55°C

[a]The primer sequences are shown in the 5' to 3' orientation.

Table 2.6: *K-RAS* Primers, their sequences and conditions for use in SSCP-HD analysis.

Primer	[a]Primer sequence	Annealing temperature
Codon 1-36	GGCCTGCTGAAAATGACTGA GTCCTGCACCAGTAATATGC	55°C
Codon 38-80	TTCCTACAGGAAGCAAGTAG CACAAAGAAAGCCCTCCCCA	55°C

[a]The primer sequences are shown in the 5' to 3' orientation.

Table2.7: *c-MET* Primers, their sequences and conditions for use in SSCP-HD analysis.

Primer	[a]Primer Sequence	Annealing temperature
Exon 17	TGTGGTTTACCATTTCATTGC GCTTGGCAGTCAACTTACATG	55°C
Exon 18	AAGGTCAAAATTAGAACAGTAGATGC TTTGGATTGTGGCACAGAGA	55°C
Exon 19	TTCTATTTCAGCCACGGGTAA GAGGAGAAACTCAGAGATAACCAA	55°C

[a]The primer sequences are shown in the 5' to 3' orientation.

sequencing procedure is carried out in two steps: The first is the primer labelling step and the second is the chain extension/termination reaction using dideoxynucleotides. The PCR for the labelling step was carried out in a reaction volumes of 18 µl containing 1 µl of purified DNA, 1 µl of primer (5ng/µl), 2 µl reaction buffer (Amersham Life Science, UK), 1µl 7-deaza-dGTP Cycle mix (Amersham Life Science, UK), 1µl dATP Cycle mix (Amersham Life Science, UK), 1µCi of [α-^{32}P]dCTP, 9µl dH_2O and 2µl Thermo Sequenase (Amersham Life Science, UK).

PCR conditions were as follows: 50 cycles of

95°C	15 seconds
55°C	45 seconds
72°C	30 seconds

Aliquots of 3.5 µl of the reaction were then transferred to four labelled microtubes containing 4 µl of the ddGTP, ddATP, ddTTP and ddCTP termination mixes respectively for the extension/termination step.

PCR conditions were as follows: 50 cycles of

95°C	30 seconds
72°C	60 seconds

After the thermocycling programme had been completed, 4 µl of stop solution (Amersham Life Science, UK) was added to each tube to terminate the reaction. The reaction samples were heated to 72°C for 5 minutes and cooled rapidly on ice. The products were separated on a 6% polyacrylamide-urea denaturing gel, dried under vacuum and examined after autoradiographic exposure.

2.8 GEL ELECTROPHORESIS

2.8.1 Gels

1.5% Agarose gel

1 X TAE	400 ml
Electrophoresis grade agarose	6g
5 mg/ml Ethidium bromide	5 µl

8% Polyacrylamide gel (PAGE) - 60 ml

Acrylamide:bis-acrylamide (29:1)	21.2 ml
5 X TBE	16.0 ml

dH_2O	42.8 ml
20% APS	180 µl
TEMED (Sigma, UK)	140 µl

Gels were run in 1 X TBE

6% Polyacrylamide gel - 60 ml

Acrylamide:bis-acrylamide (29:1)	12.0 ml
5X TBE	12.0 ml
dH_2O	36.0 ml
20% APS	135 µl
TEMED	105 µl

Gels were run in 1 X TBE

6% PAG + 5% Glycerol (SSCP) - 60 ml

Acrylamide:bis-acrylamide (29:1)	12 ml
5X TBE	6 ml
dH_2O	39 ml
Glycerol	3 ml
20% APS	135 µl
TEMED	105 µl

Gels were run in 0.5 X TBE

0.5X MDE gel - 60 ml

MDE (FMC Bioproducts, USA)	15 ml
5X TBE	7.2 ml
dH_2O	37.8 ml
20% APS	135 µl
TEMED	105 µl

Gels were run in 0.6 X TBE.

6% Denaturing polyacrylamide-urea gel - 60 ml

Gene-PAGE Acrylamide/bis-acrylamide (Amresco, USA)	60 ml
20% APS	135 µl

TEMED	105 µl

Gels were run in 1 X TBE.

2.8.2 Visualising PCR products using agarose gels

PCR products were visualised on 1.5% agarose gels prepared and run using an Easi-cast system (Hybaid, UK). Two µl drops of loading dye were placed on a sheet of parafilm (Sigma, USA), to which 2 µl of PCR sample was added and mixed by drawing the PCR/dye mix up and down into the pipette several times. Three µl of this mix was loaded onto the gel which was then run in 1X TAE buffer at 150V for approximately 15 minutes. A 100bp ladder (Gibco, BRL, UK) was also run on each gel in order to verify the PCR product sizes. The DNA was visualised by studying the gel on an ultraviolet transilluminator (Chromato-vue Transilluminator, UVP, USA). DNA bands are seen to fluoresce under UV light due to the intercalation of ethidium bromide within DNA molecules.

2.8.3 Microsatellite analysis via polyacrylamide gels

Both non-denaturing and denaturing gels were used in this study.

Non-denaturing 6-8% polyacrylamide gels

The percentage of acrylamide used in each gel was determined by the size of the PCR products to be separated (table 2.8). The preparation of gels was carried out in 40cm X 45cm X0.4mm gel apparatus (S2 sequencing gel system, BRL Life Technologies, UK). Five µl of loading dye was added to each PCR reaction and 2 µl of this mixture was loaded onto the gel. Electrophoresis, using 1X TBE as the running buffer, was then carried out either overnight at 300V or at 1kV for approximately 5 hours. The gel was then dried on a vacuum gel dryer (AE-3750, Genetic Research Instruments Ltd., UK) placed in a film cassette and exposed for an appropriate length of time to X-ray film (Kodak X-AR).

6% polyacrylamide-urea denaturing gels

Gels were prepared as for non-denaturing gels with the exception that loading dye was not added. Thirty-five µl of 90% formamide was added to each PCR reaction which were then heated to 94°C for 5 minutes in order to denature the DNA. Following heating, the samples were rapidly cooled by placing them on wet ice, and then 2 µl of each was loaded onto the gel. The gels were electrophoresed in 1X TBE at 70W for approximately 3 hours (table 2.9) and dried as explained above.

Table 2.8: Separation range of DNAs in non-denturing polyacrylamide gels and corresponding migration rates of marker dyes .

Acrylamide % (w/v)	Range of separation (bp)	[a]Bromophenol blue	[a]Xylene cyanol
3.5	1000-2000	100	460
5.0	80-500	65	260
8.0	60-400	45	160
12.0	40-200	20	70

[a]Numbers represent the approximate sizes of double-stranded DNA (bp) with which the dyes comigrate.

Table 2.9 : Migration rates of marker dyes through denaturing polyacrylamide gels.

Acrylamide % (w/v)	[a]Bromophenol blue (bp)	[a]Xylene cyanol (bp)
5	35	130
6.0	26	106
8.0	19	76
10.0	12	55

[a]Numbers represent the approximate sizes (bp) of DNA fragments with which the dyes comigrate.

Adapted from Sambrook J,Fritsch EF and Maniatis T. *Molecular Cloning* 2nd Ed (1989).

2.8.4 Single-stranded Conformational Polymorphism and Heteroduplex (SSCP-HD) analysis.

PCR products were denatured by adding 15 µl of 90% formamide buffer. The samples were incubated at 95°C for 5 minutes and immediately put on ice. Two µl of the PCR/formamide mix was loaded into each well on the gel. The products were separated on 6% non-denaturing polyacrylamide gels with 5% glycerol and on 0.5X MDE (FMC Bioproducts, UK) matrix gels run in 0.5X and 0.6X TBE respectively at 350V for approximately 18 hours. The use of two different gel types increased the likelihood of detecting mutations.

The gels were dried under vacuum as in section 2.8.3 and examined after suitable autoradiographic exposure for aberrant SSCP-HD band shifts.

CHAPTER THREE

ANALYSIS OF LOSS OF HETEROZYGOSITY ON HUMAN CHROMOSOME 7 AND MAPPING OF CANDIDATE OVARIAN CANCER TUMOUR SUPPRESSOR LOCI

3.1 INTRODUCTION

The inactivation of TSGs, commonly via somatic mutations, is a critical step in the development of tumours (Fearon & Vogelstein, 1990). Evidence for the location of such genes can be revealed by the analysis of loss of heterozygosity (LOH), a phenomenon indicative of the presence of a TSG (section 1.9). Indeed, LOH studies have previously identified common deletions on 7q, characteristic of a TSG which may be involved in the development of tumours in many tissue types (figure 3.1) including breast (Lin *et al.*, 1996), colon (Zenklusen *et al.*, 1995a), head and neck (Nawroz *et al.*, 1994), kidney (Shridhar *et al.*, 1997), pancreas (Achille *et al.*, 1996), prostate (Latil *et al.*, 1995; Takahashi *et al.*, 1995), stomach (Nishizuka *et al.*, 1997), thyroid (Zhang *et al.*, 1998) and ovary (Zenklusen *et al.*, 1995b). Overall frequencies of LOH on the long arm of chromosome 7 in ovarian cancer range from 50% (Koike *et al.*, 1997) to 71% (Huang et al., 1999) with the most common deletions occurring at 7q22 and 7q31. LOH on chromosome 7 has also been shown to be an early event in ovarian tumorigenesis (Watson *et al.*, 1998) and occurs frequently in benign ovarian tumours (Martin *et al.*, 1998; Roy *et al.*, 1997). Indeed, benign ovarian tumours are often seen adjacent to areas of carcinoma suggesting that benign lesions may undergo transition to malignancy, in a similar manner to those observed in colorectal tissue (Puls *et al.*, 1992).

The aims of this study were to quantitate the frequency of LOH on chromosome 7, identify candidate TSG loci and delineate the temporal sequence of deletions occurring within this region in benign, borderline and malignant ovarian tumours. To ensure a comprehensive analysis of chromosome 7, thirty-four microsatellite markers from across the entire chromosome were used to assess for LOH, in a large bank of ovarian tumours comprising a range of histologies, stages and grades. Previous studies have generally focused on either large sample numbers and relatively large patient numbers and a sizeable panel of markers,

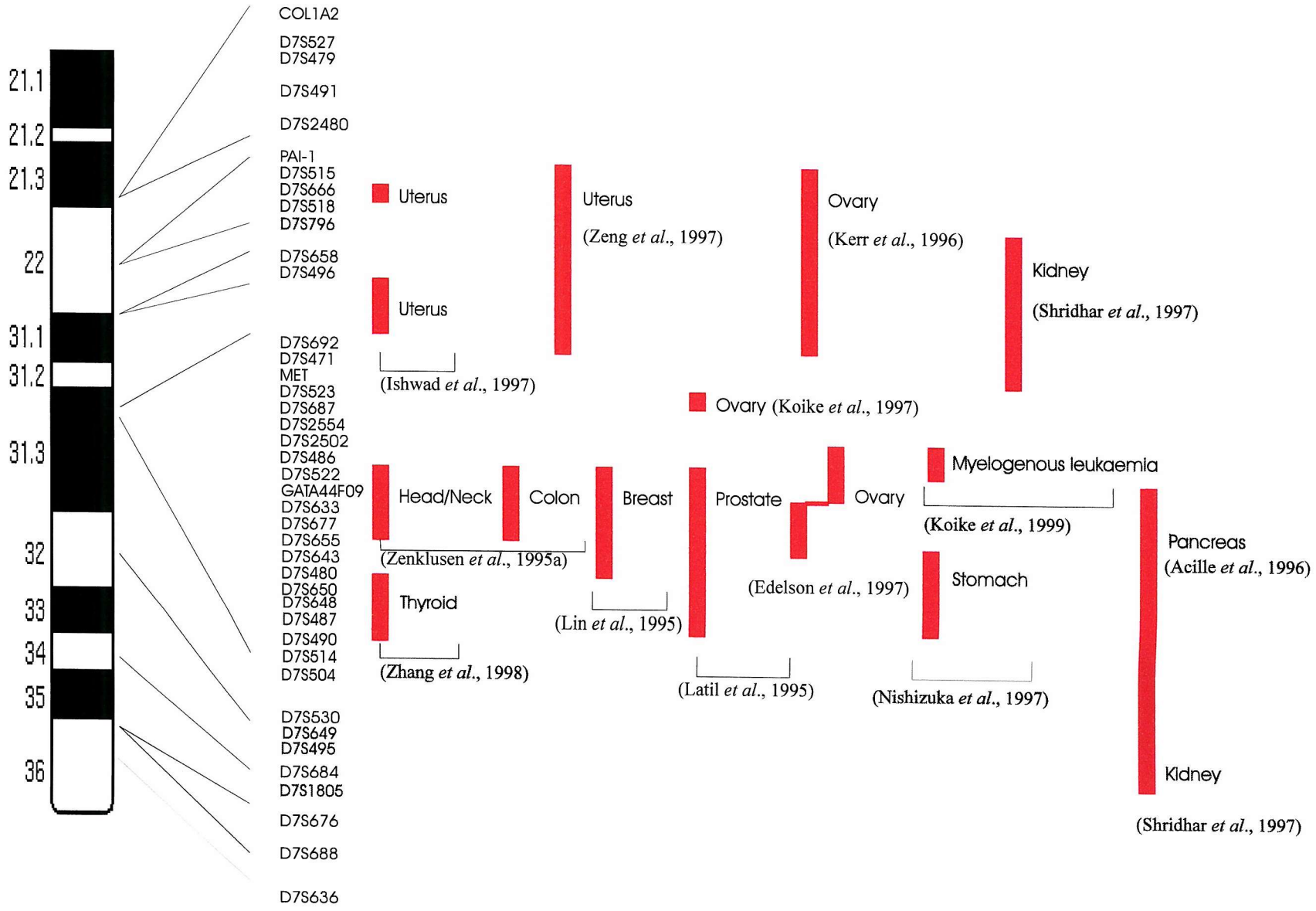


Figure 3.1. Regions of Chromosome 7q previously implicated in cancer.

this study would provide a more representative overview of the deletions occurring in ovarian cancer.

3.2 RESULTS

3.2.1 Clinical specimens

Two-hundred and ten fresh frozen epithelial and 9 non-epithelial ovarian tumour samples and their corresponding lymphocyte DNA were analysed for LOH on chromosome 7. Specimens were collected and DNA prepared as described in sections 2.1 and 2.2. These samples comprised of 98 serous, 57 mucinous, 28 endometrioid, 6 undifferentiated and 30 other tumours, the comprehensive histological information for which is shown in table 2.1. The samples could also be sub-divided according to their nature as follows: 130 malignant; 66 benign and 23 borderline tumours. Tables 3.4 and 3.5 indicate the breakdown of the samples with respect to grade and stage.

3.2.2 Microsatellite markers

Thirty-four microsatellite markers were used for the LOH study, 6 on the p arm and 28 on the q arm of chromosome 7. Figure 3.2 shows a schematic representation of the relative positions of these markers, in relation to cytogenetic banding and to each other. The primer sequences and PCR conditions for these markers are listed in table 2.2. Sequence and cytogenetic location data for these markers can be accessed through the National Center for Human Genome Research database (http://www.nih.gov).

3.2.3 Overall LOH on chromosome 7

LOH on chromosome 7 was a frequent event, with 113 (52%) of the 219 tumours studied, exhibiting LOH at one or more microsatellite markers. Table 3.1 shows the LOH data for all cases and all microsatellite markers. LOH with at least 1 marker on chromosome 7q was observed in 49% (107/219) of tumours versus 27% (58/219) with LOH of at least 1 marker on chromosome 7p.

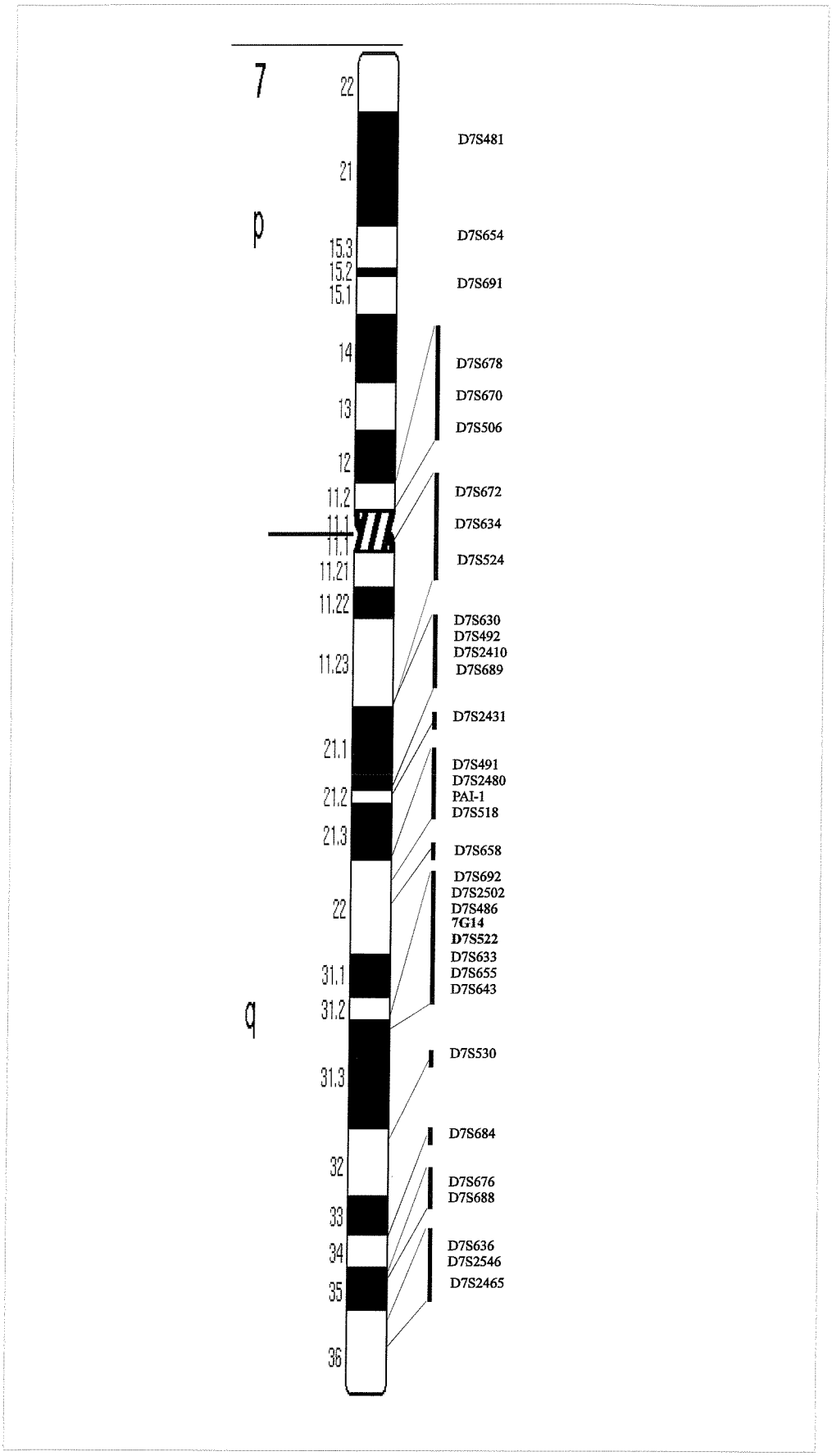


Figure 3.2: Schematic representation of chromosome 7 and the positions of microsatellite markers used in LOH analysis.

Table 3.1. LOH results for all loci in ovarian tumours.

LOH is indicated by red shading and the complete extent of the deletion within each tumour is indicated by pink shading, with the bordering retention of heterozygosity indicated in white.

The number of tumours showing LOH, and the percentage (%) LOH for each marker is indicated at the end of the table.

[1]Numbers with the suffix ".1" correspond to tumour samples donated by the ICRF, London.
[2]LOH present: y = LOH present; n = LOH absent.
[3]Nature: b = benign; bl = borderline; m = malignant.
[4]Histology: S = serous; M = mucinous; E = endometrioid; U = undifferentiated;
P = papillary; T = teratoma; th = thecoma; F = fibroma; Mn = Mullerian origin;
A = adenocarcinoma; Br = Brenner tumour; CC = clear cell carcinoma.
[5]Complete pathological data was not available for all tumours studied.
[6]Microsatellite markers pre-fixed by "D7S", correspond to STSs.
7G14 corresponds to a microsatellite within the FRA7G fragile site.
PAI-1 corresponds to a microsatellite marker within the respective gene loci.
het = constitutional heterozygosity
loh = loss of constitutional heterozygosity
na = non-informative
dnw = did not work
LOH = total number of samples showing LOH for particular marker.
INF = total number of informative samples for that marker.
%LOH = tumours showing LOH/informative tumours (%).

Table 3.1. LOH results for all loci in ovarian tumours

Case number[1]	LOH?[2]	Nature[3]	Histology[4]	Stage[5]	Grade	D7S481[6]	D7S654	D7S691	D7S678	D7S670	D7S506	D7S672	D7S634	D7S524	D7S630	D7S492	D7S2410	D7S689	D7S2431	D7S491	D7S2480	PAI-1	D7S518	D7S658	D7S692	D7S2502	D7S486	7G14	D7S522	D7S633	D7S655	D7S643	D7S530	D7S684	D7S676	D7S688	D7S636	D7S2546	D7S2465
10	y	b	S			het	loh	ni	ni	loh	loh	het	het	het	ni		het	het	dnw	het	het		het	het	het	ni	het		het	het	het	dnw		het	het	het	het	het	het
24	y	b	S			het	loh	het	ni	ni	ni	het	ni	dnw	dnw			dnw	dnw	het	het		het	ni	ni	ni	dnw		het	loh	ni	dnw		dnw	dnw	dnw	het	dnw	
113	y	b	M			dnw	het	het	het	loh	dnw	loh	ni	dnw	loh		het	ni	dnw	het	het		het	ni	het	het	ni		ni	het	het	loh		mi	het	het	het	het	het
140	y	b	M			ni	loh	loh				dnw								loh									loh								het		
143	y	b	S			het	het	het					loh							ni									ni								het		
147	y	b	S			het	het	het	het	ni	het	het	het	ni	ni	dnw	het	het	het	loh	het	dnw	het	het	ni	ni			ni	het	ni	dnw	loh	het	ni	dnw	het	het	loh
156	y	b	M			loh	het	het	het	het	het	het	dnw	ni	het		dnw	ni	dnw	het	het		het	dnw	ni	het	het		het	dnw	ni	het		het	dnw	mi	het	dnw	het
206	y	b	M			loh	loh	het	het	ni	het	het	het	ni	ni	dnw	het	het	het	het	ni	dnw	het	dnw	ni	het			ni	het	ni	dnw	het	het	het	dnw	ni	ni	het
245	y	b	T			het	ni	het	het	ni	loh	loh	loh	dnw	loh		dnw	ni	het	het	loh		loh	ni	het	het	dnw			het	ni	het		het	ni	het	het	dnw	het
269	y	b	F			het	dnw	het	ni	het	het	het	het	dnw	het		ni	het	dnw	ni	loh		loh	dnw	het	het				dnw	dnw			dnw	het	dnw		dnw	loh
408	y	b	T			het	ni	het	ni	het	het	het	dnw	het	loh		dnw	ni	loh	ni	loh		ni	ni	dnw	het	het		ni	dnw	ni			ni	ni	dnw	dnw	ni	het
416	y	b	th			mi	ni	het	loh	dnw	mi	mi	dnw	dnw	het		dnw	ni	dnw		dnw		ni	dnw	dnw	dnw	mi			dnw	ni			dnw	dnw	dnw	dnw	ni	het
426	y	b	S			het	het	het	het	het	het	het	het	ni	ni	dnw	het	het	ni	het	het	dnw	ni	het	ni	ni			ni	het	het	het	het	het	loh	het	het	het	het
433	y	b	M			het	het	ni	loh	het	het	loh	ni	ni	het	dnw	het	ni	het	het	het	dnw	het	het	het	ni			ni	het	ni	het	het	ni	het	het	het	het	ni
446	y	b	M			het	het	het	ni	loh	het	het	ni	ni	ni	dnw	het	ni	het	het	het	dnw	ni	het	het	het			het	het	het	ni	het	het	het	het	het	ni	het
450	y	b	S			ni	ni	het	het	het	het	het	ni	ni	het	dnw	het	ni	het	het	het	dnw	het	het	het	ni			ni	het	het	ni	het	het	het	het	het	ni	loh
467	y	b	S			ni	loh	het	het	het	ni	het	dnw	dnw	het		dnw	ni	dnw	het	het		dnw	het	dnw	dnw	ni		ni	dnw	dnw			dnw	dnw	dnw	dnw	ni	het
516	y	b	M				dnw					het								loh									ni										
322	y	bl	M	1		het		ni	dnw	het	het	ni	loh		het		ni	dnw					dnw	ni	ni	dnw	ni		dnw	dnw	dnw	dnw		het	het	het	dnw		dnw
537	y	bl	M	1	1	dnw		dnw	het	dnw	ni	het	het		het		dnw						het	het	het	het	het		dnw	het	loh	dnw		het	het	het	dnw		het
263	y	bl	M	1a		ni	ni	het	het	het	het	het	het		het		het	het	het	het	ni		loh	ni	het	het	het		ni	loh	ni	loh		loh	ni	loh	loh	het	dnw
2	y	bl	S	1c		het	het	ni	het	het	het	het	ni	het	loh		ni	ni		het	ni		het	het	ni	ni	het		het	het	het	het		het	het	het	het	dnw	dnw
20	y	bl	S	3a		het	het	het	het	loh	het	het	het	ni	het	dnw	het	het	loh	ni	loh	dnw	loh	ni	ni	loh	loh		loh	loh	loh	ni	dnw	loh	loh	loh	loh	loh	het
23	y	m	S	1	1	het		loh							loh		dnw			ni			het		het		het	het	het		ni		het	het		dnw	het		het
37	y	m	E	1	2	het	het	ni							het		dnw			het			dnw		ni		dnw	dnw	het	loh	dnw		het	dnw		het	het		het
44	y	m	M	1	1										het		dnw						dnw		dnw		dnw		dnw		ni		het	loh		het	dnw		
63	y	m	S	1	3			loh							het		het			het			ni		loh		loh	loh	loh		loh		ni	ni	dnw	loh	dnw		loh
76	y	m	S	1	1	ni		ni							dnw		dnw			het		het			loh				loh				dnw			dnw	loh		
117	y	m	M/E	1	2			loh							dnw		dnw			loh					dnw			dnw	loh				dnw		ni		dnw	dnw	ni	dnw	ni		het
324	y	m	M	1	2			loh																														ni		
341	y	m	S	1	2			ni												het																		loh		
32	y	m	S	2	1	het	het	het	het	het	het	ni	loh	dnw	loh	dnw	ni	het	het	het	het	ni	het	het	het	het	ni	ni	het	ni	loh	ni	loh	ni	het	het	het	ni	ni
40	y	m	M	2	3	het	ni	ni	het	ni	het	het	loh	ni	loh	ni	het	het	het	dnw	het	het	ni	ni	het	het	het	dnw	het	loh	ni	ni	het	ni	ni	het	ni	het	het
43	y	m	S/E	2	2	het	het	het	ni	loh	ni	ni	ni	ni	loh	dnw	ni	ni	loh	loh	loh	ni	ni	het	ni	ni	het	loh	loh	loh	ni	loh	loh	loh	ni	loh	loh	loh	loh
95	y	m	E	2	3	dnw	loh	het	ni	het	ni	dnw	ni	dnw	het	ni	het	ni	het	ni	ni	het	ni	ni	ni	dnw	dnw	dnw	ni	het	het	ni	het	ni	ni	het	het	het	het
135	y	m	S	2	2	het	dnw	het	het	loh	ni	loh	loh	loh	loh	loh	loh	ni	dnw	het	het	ni	ni	ni	ni		dnw	dnw	ni	het	ni		dnw	het	mi	dnw	het	dnw	dnw
194	y	m	P	2	3							het	ni		het		het	het				ni	ni	ni	het		ni	dnw	dnw		het	loh	dnw	dnw	het	het	ni		dnw
22	y	m	S	3	3	loh	loh	ni	het	het	het	loh	ni	ni	ni	ni	loh	ni	dnw	dnw	het	ni	het	het	het	loh	loh	ni	loh	loh	loh	ni	het	het	ni	het	loh	ni	loh

Table 3.1. LOH results for all loci in ovarian tumours

Case number[1]	LOH?[2]	Nature[3]	Histology[4]	Stage[5]	Grade	D7S481[6]	D7S654	D7S691	D7S678	D7S670	D7S506	D7S672	D7S634	D7S524	D7S630	D7S492	D7S2410	D7S689	D7S2431	D7S491	D7S2480	PAI-1	D7S518	D7S658	D7S692	D7S2502	D7S486	7G14	D7S522	D7S633	D7S655	D7S643	D7S530	D7S684	D7S676	D7S688	D7S636	D7S2546	D7S2465
24	y	m	U	3	3							loh	ni		ni									het						het					ni				
26	y	m	S	3	2	loh	dnw	dnw	het	ni	het	ni	het	loh	loh	ni	dnw	ni	loh	dnw	loh	dnw	loh	ni	ni	ni	dnw	loh	ni	loh	loh	ni	loh	loh	loh	het	het	het	loh
29	y	m	S	3	3							loh	ni		loh	loh					ni			ni						het					ni	het			
30	y	m	E/M	3	3	loh	loh	loh	ni	loh	ni	loh	loh	het	het	ni	loh	ni	loh	ni	loh	loh	loh	loh	ni	ni	het	ni	ni	het	loh	loh	het	het	het	het	het	het	het
41	y	m	S	3	3							loh	loh		loh	loh								loh						loh	dnw	dnw			loh				
45	y	m	S	3	3	loh	dnw	dnw	loh	loh	loh	dnw	ni	loh	het	het	ni	ni	het	ni	het	het	loh	loh	loh	ni	loh	loh	ni	het	het	ni	het	het	ni	loh	dnw	loh	loh
48	y	m	S	3	2	dnw	ni	dnw	mi	mi	het	het	het	het	het	het	ni	het	ni	ni	ni	loh	ni	loh	loh	ni	ni		dnw	het	dnw	dnw	ni	het	ni	ni	dnw	het	het
49	y	m	Mn	3	3	het	het	ni	ni	ni	het	het	ni	ni	het	dnw	ni	ni	dnw	dnw	ni	dnw	dnw	ni	dnw	dnw			dnw	het	dnw	ni	dnw	loh	ni	ni	ni	loh	ni
50	y	m	Mn	3	3							ni	ni		loh	ni							loh	ni					ni		loh			loh					
97	y	m	S	3	3	dnw	ni	dnw	het	ni	ni	het	ni	het	het	ni	het	ni	dnw	dnw	het	dnw	het	het	het	ni	het	ni	ni	ni	ni	ni	het	loh	loh	loh	loh	loh	loh
121	y	m	M	3	1		ni	dnw	ni	het		loh	het	het	ni	het	dnw	ni	dnw		het		dnw	het	het		dnw	dnw	het	ni	het	loh	dnw	het	mi	ni	dnw	het	dnw
122	y	m	S	3	3		ni	ni	ni	ni		loh	loh	ni	loh	loh	loh	ni	loh	ni	loh	ni	loh	loh	ni	loh	ni	ni	ni	loh	loh	ni	loh	ni	loh	loh	loh	loh	loh
131	y	m	S	3	3	ni	ni	het	ni	loh	ni	loh	loh	loh	ni	ni	loh	ni	loh	ni	loh	loh	loh	loh	loh	ni	ni	ni	ni	loh	ni	loh	dnw	het	ni	het	dnw	het	het
134	y	m	S	3	3	dnw	ni	het	ni	het	ni	loh	dnw	loh	loh		loh	ni	loh		loh		loh	ni	loh		ni	dnw	loh	loh	dnw		dnw	ni	loh	loh	mi	loh	loh
179	y	m	E	3	3	dnw	ni	ni	ni	het	dnw	loh	ni	loh	loh	loh	ni	ni	dnw	dnw	loh		ni	loh	loh	loh	het	het	dnw	loh	het	ni	het	loh	ni	het	het	het	het
282	y	m	S	3		ni	het	het	het	het	het	het	ni	loh	loh	loh	het	het	het	het	ni	dnw	het	ni	het	ni	het	dnw	ni	het	loh	loh	het	ni	het	ni	het	dnw	
291	y	m	S	3	3	ni	het	het	ni	het		het	het	het	het	ni	het	ni	loh	het	ni		het	het	het	loh	loh	dnw	loh	het	ni	het	het	het	het	het	het	het	
297	y	m	S	3	3	loh	loh	loh	ni	loh	ni	loh	loh	ni	loh	loh	loh	ni	loh	ni	ni	ni	loh	ni	loh	ni	ni	dnw	loh	loh	loh	ni	loh	loh	ni	loh	het	het	
300	y	m	E	3	3	het	loh	loh	het	het	het	ni		het	het	het	het	het	het	ni	ni		het	het	het	het	het		ni	het	loh	ni	het	loh	ni	loh	het	het	
548	y	m	S	3																			het	ni			het		loh										
551	y	m	S	3																			loh	ni			loh		loh										
72	y	m	S	4	3							het	loh		loh	loh							ni											ni				
187	y	m	U	4	3										het						ni				loh								het		het			
230	y	m	S	4	3	loh	het	het	het	ni	het	het	dnw		loh	het	het	het	ni	het	loh	loh	ni	het	het	het	ni	het	het	het	ni	ni	het	ni	het	het	het	het	
560	y	m	S	4																			dnw	loh			loh		het										
29	y	m	M	1a		het	loh	ni							loh					loh				loh	het				het	loh					ni			
36	y	m	E	1a	1	loh	loh	ni	loh	ni	het	het	het	ni	loh	ni	ni	ni	loh	loh	ni	loh	loh	loh	loh	ni	het	het	het	het	het	dnw	dnw	het	dnw	het	het	het	ni
38	y	m	S/E	1a		ni	het	het							loh					loh					loh				ni	loh						ni			
51	y	m	M	1a	1	ni		loh							dnw		dnw			loh			dnw		loh		dnw	dnw	loh	dnw	dnw		dnw	ni	dnw	dnw	loh		ni
60	y	m	G	1a	1	ni	loh	loh							het					ni					ni					ni	het					ni			
61	y	m	M	1a	1	het	het	ni	ni	het	loh	loh	ni	dnw	ni	dnw	dnw	ni	het	ni	het	het	dnw	ni	ni	ni	dnw	dnw	ni	ni	ni	het	ni	het	het	het	het	dnw	dnw
67	y	m	S	1a	2			het												het									loh										
70	y	m	E	1a	1	het	het	het							dnw		het			loh	loh	het	het		het		ni	loh	ni	het	het		dnw	het	dnw	dnw	het		het
71	y	m	S	1a	2	het	het	het	het	het	het	loh	ni	loh	loh	ni	ni	loh	het	ni	het		het	het	loh	loh	ni	ni	ni	loh	loh	loh	ni	het	het	het	het	het	het
78	y	m	E	1a	2	ni	het	het	het	het	het	loh	loh	ni	loh	ni	ni	ni	loh	loh	loh	loh	loh	het	het	loh	loh	dnw	loh	loh	loh	dnw	dnw	het	dnw	het	het	het	het
80	y	m	M	1a	1	ni	het	het	het	het	het	het	het	het	het	het	ni	het	dnw	ni	het	het	ni	het	het	ni	loh	loh	loh	het	het	ni	dnw	het	het	het	het	het	het
114	y	m	E	1a	2	ni	ni	ni	ni	ni					ni		ni	ni		loh	loh	loh	ni		loh		dnw	ni	loh	loh	ni		dnw	mi	loh	loh	het		dnw
170	y	m	E	1a	2			het							het		dnw			ni		ni	ni		dnw		het	dnw	het		dnw		dnw	ni	het	dnw	loh		het

Table 3.1. LOH results for all loci in ovarian tumours

Case number[1]	LOH?[2]	Nature[3]	Histology[4]	Stage[5]	Grade	D7S481[6]	D7S654	D7S691	D7S678	D7S670	D7S506	D7S672	D7S634	D7S524	D7S630	D7S492	D7S2410	D7S689	D7S2431	D7S491	D7S2480	PAI-1	D7S518	D7S658	D7S692	D7S2502	D7S486	7G14	D7S522	D7S633	D7S655	D7S643	D7S530	D7S684	D7S676	D7S688	D7S636	D7S2546	D7S2465
177	y	m	E	1a	3	het	het	ni				ni			het		het			het			het		mi		ni	dnw	ni	het	ni		het	het	loh	dnw	ni		het
257	y	m	M	1a	1			het		het		het	het	ni	ni	het	het	ni		het	het	het	het	ni	het	het	loh	dnw	ni	het	ni	het	het	ni	het	ni	het		
267	y	m	E	1a	2			ni									het			ni					ni				loh							loh		
271	y	m	M	1a	1			ni									ni			het					het				het							loh		
287	y	m	E	1a	1			het							het		loh			loh					het				het							ni		
12	y	m	S	1b	1	het		het							ni		dnw			loh			dnw		dnw		dnw	dnw	loh		ni		het	dnw		het	het		dnw
27	y	m	S	1b	2			ni							het		dnw			ni	ni	loh	loh		dnw		dnw	dnw	ni		dnw		dnw	het		het	het		dnw
28	y	m	S	1b		ni	ni	ni							loh		dnw			ni		het	dnw		dnw		dnw		het	loh	dnw		dnw	dnw		het	het		het
69	y	m	S	1b	2			het												het									ni							ni	loh	
123	y	m	E	1b	3	loh	ni	ni							ni		dnw			loh					ni				het	het						loh		
107	y	m	M	1c	1	loh		het							ni		dnw			ni			ni		dnw		het		loh		dnw		dnw	mi	ni	dnw	het		dnw
119	y	m	S	1c	2	ni	ni	ni	het	het	dnw	het	het	het	ni	ni	ni	het	loh	het	het		dnw	het	het	het	het	dnw	loh	het	dnw	ni	het	het	het	het	het	ni
128	y	m	E	1c	2	het	het	het							het		dnw	ni		het		het	loh		het		het	dnw	loh	het	dnw		dnw	ni	ni	dnw	het		dnw
144	y	m	M/E	1c	1	ni	het	ni	het	het	dnw	mi	het	loh	loh	het	het	loh	het	ni	het		ni	het	het	dnw			het	ni	ni	loh	dnw	ni	ni	loh	loh	het	ni
146	y	m	E	1c	2	het	ni	het	ni	het	het	dnw	het	mi	mi	dnw	mi	ni	dnw	ni	mi	ni	ni	ni	mi	dnw	het	dnw	het	het	het	dnw	loh	mi	het	het	het	mi	mi
215	y	m	S	1c	3	het	loh	ni	dnw	het	dnw	het	ni	het	het	ni	het	het	het	het	het	ni	ni	ni	ni	loh	loh	ni	loh	ni	ni	loh	mi	loh	het	ni	het	het	het
219	y	m	M	1c	1	het	het	het	ni	het	dnw	het	het	ni	loh	het	het	het	het	het	ni	het	het	het	ni	ni	ni	dnw	het	het	het	het	het	het	het	het	het	het	het
376	y	m	S	1c	3			het												loh																	het		
211	y	m	E	2c											ni		het						dnw		ni		ni	dnw	het		dnw				ni	loh			ni
285	y	m	S	2c	2	het	ni	het	dnw	ni	ni	het		ni	ni	het	het	het	ni	dnw	het		dnw	ni	loh		dnw	dnw	loh	ni	dnw	loh		het	het		het	dnw	
86	y	m	S	3a	3	ni	ni	het	het	ni	het	het	het	ni	ni	het	het	ni	ni	dnw	het	ni	het	ni	het	het	het	dnw	ni	het	het	het	ni	ni	het	loh	loh	het	ni
253	y	m	E	3a	2	het	het	het	ni	het	het	het	ni		ni	het	het	het	het	het	ni		het	het	loh	het	het	het	het	het	het	het	het	het	dnw	het	het	het	
14	y	m	S	3b	2	het	het	het	ni	dnw	het	het	het	het	het	ni	het	ni	ni	dnw	het	dnw	het	het	ni	het	ni	het	dnw	het	ni	het	het	het	het	het	loh	ni	het
201	y	m	S	3b	2	dnw	dnw	het	dnw	loh	dnw	loh	loh	dnw	ni	loh	loh	ni	dnw	dnw	het	dnw	ni	ni	het	dnw	dnw	dnw	ni	het	ni	dnw	dnw	ni	dnw	het	dnw	het	dnw
286	y	m	E	3b	3	loh	loh	het	ni	ni	het	het	ni	het	ni	het	het	het	loh	loh	loh	loh	loh	loh	het	ni	ni	het	ni	het	het	het	het	het	het	ni	het	het	
80	y	m	S	3c	3							loh	ni		loh	loh							loh							ni		loh			ni				
83	y	m	S	3c	3										het	loh					ni									loh									
188	y	m	S	3c	3	dnw	ni	het	het	het	dnw	ni	het	loh	dnw	loh	het	ni	ni	dnw	het	het	ni	het	het	ni	loh	dnw	loh	ni	ni	loh	dnw	loh	het	het		dnw	loh
192	y	m	S	3c	3	dnw	het	het	ni	het	dnw	het	het	het	het	het	loh	loh	het	ni	ni	het	het	het	het		het	dnw	dnw	ni	dnw		het	loh	loh	loh		loh	loh
204	y	m	S	3c	3	ni	het	ni	het	dnw	dnw	het	dnw	het	het	het	dnw	ni	dnw	het	ni	dnw	het	ni	loh	dnw	het	dnw	ni	het	ni	ni	het	het	het	het	het	het	het
220	y	m	E	3c	2	dnw	ni	het	het	het	dnw	het	het	ni	het	ni	het	het	het	het	het		het	ni	loh	het	het	ni	ni	het	het	het		het	ni	loh	ni	het	het
11	y	m	S		3							loh											dnw				loh		loh	loh	dnw	dnw		ni	loh		loh		
17	y	m	S		3							ni	ni										dnw				dnw		het	loh	dnw	dnw	het		het		het		
20	y	m	S		2																		dnw				dnw		het	het	dnw	dnw			dnw		loh		
142	y	m	S		3	ni	loh	het	het	ni	het	loh	het	dnw	ni	ni	het	ni	dnw	ni	het	het	het	het	ni	dnw	dnw	het	ni	het	loh	loh	dnw	ni	mi	het	het	het	het
550	y	m	E																				loh	dnw			het		dnw										
557	y	m	M																				dnw	dnw			loh		dnw										
1	n	b	SF			het	ni	het												ni									het							het		

Table 3.1. LOH results for all loci in ovarian tumours

Case number[1]	LOH?[2]	Nature[3]	Histology[4]	Stage[5]	Grade	D7S481[6]	D7S654	D7S691	D7S678	D7S670	D7S506	D7S672	D7S634	D7S524	D7S630	D7S492	D7S2410	D7S689	D7S2431	D7S491	D7S2480	PAI-1	D7S518	D7S658	D7S692	D7S2502	D7S486	7G14	D7S522	D7S633	D7S655	D7S643	D7S530	D7S684	D7S676	D7S688	D7S636	D7S2546	D7S2465
5	n	b	S			het	het	het	dnw	ni	het	het	dnw		het				ni	het	het		ni	het	het	het			het	ni	het	het		het	het	dnw	het	het	het
7	n	b	S			het	het	het	ni	ni	het	het	dnw	het	het		dnw	ni	dnw	het	ni		het	het	ni	het	ni		het	het	ni	het		dnw	ni	dnw	het	dnw	ni
13	n	b	M			het	het	het	het	het	ni	het	ni	het	het		dnw	ni	dnw	het	het		het	ni	het	ni	het		ni	het	ni	het		het	ni	dnw	het	het	het
15	n	b	Mth			het	het	ni	ni	het	dnw	het	dnw	ni	ni		dnw	dnw	dnw	het	het		het	ni	het	het	het		het	ni	ni	het		het	het	dnw	het	het	het
39	n	b	SF			het	het	het	het	het	het	het	het	ni	ni	dnw	het	het	het	het	het	dnw	het	het	het	het			ni	het	het	dnw	het	ni	ni	het	ni	het	het
75	n	b	M			het	ni	het	het	ni	het	het	het	het	ni	ni	dnw	ni	dnw	het	ni	dnw	het	het	ni	het	het		ni	het	ni	ni	ni	ni	het	het	het	ni	ni
77	n	b	th					het												ni									het										
85	n	b	M/Br			het	ni	ni												het									ni								het		
94	n	b	M			het	het	ni	het	het	ni	ni	ni	dnw	het		dnw	het	dnw	het	het		het	het	het	het	het		ni	het	het	het		ni	het	het	het	het	het
103	n	b	S			het	het	ni	het	het	het	het	het	het	het		het	ni	ni	het	het		het	het	ni	het	ni		het	het	ni	het		het	ni	het	het	ni	het
125	n	b	M			ni	ni	ni	ni	het	ni	het	het	ni	het		het	dnw	dnw	het	het		het	ni	ni	het	het		het	ni	ni	het		het	ni	het	het	ni	het
148	n	b	S			dnw	het	het	ni	het	het	ni	ni	het	ni		dnw	het	dnw	het	het		het	dnw	het	dnw	het		ni	het	het	het		het	ni	ni	ni	het	het
149	n	b	S			het	het	het												ni									ni								het		
158	n	b	S			ni	het	het	ni	het	het	het	ni	ni	het		dnw	het	dnw	het	het		het	ni	het	het	het		het	dnw	het	het		het	het	dnw	het	ni	het
164	n	b	S			het	het	het	ni	ni	ni	het	dnw	dnw	ni		dnw	het	dnw	het	het		ni	het	het	dnw	dnw		het	het	het	het		het	het	dnw	het	dnw	het
167	n	b	SF			het	ni	ni	het	ni	het	het	dnw	dnw	het		dnw	het	dnw	het	het		het	ni	het	dnw	het		ni	het	het	het		het	dnw	het	het	dnw	
183	n	b	S/M			het	ni	ni	dnw	dnw	ni	mi	dnw	dnw	dnw		dnw	dnw	dnw	ni	het		dnw	dnw	het	dnw	dnw		ni	dnw	dnw	dnw		dnw	dnw	dnw	ni	dnw	het
196	n	b	S			ni	ni	het	het	het	dnw	het	dnw	dnw	het		dnw	dnw	dnw	het	dnw		het	ni	ni	dnw	het		ni	het	dnw	het		het	ni	dnw	ni	dnw	ni
214	n	b	M			ni	het	het				dnw								het									ni	ni							ni		
246	n	b	M			het	het	ni	ni	ni	het	het	het	dnw	het		ni	ni	het	het	het		dnw	ni	ni	ni	het		ni	het	dnw	het		ni	het	dnw	het	dnw	het
262	n	b	S			ni	ni	het	het	het	het	het	ni	het	het		het	ni	het	het	het		het	het	het	het	het		ni	het	het	het		ni	ni	dnw	het	dnw	het
276	n	b	M			het	het	het	het	het	het	het	het	het	het		ni	het	het	het	het		het	het	ni	het	het		ni	het	het	het		het	het	het	ni	het	het
294	n	b	M			het	het	het	het	het	ni	het	het	dnw	ni		het	het	het	het	het		het	het	het	ni	het		ni	het	het	het		het	het	ni	het	het	het
295	n	b	A			het	het	het				dnw								het									ni								*ni*		
301	n	b	S			ni	het	het	ni	het	het	het	dnw	dnw	het		het	het	het	het	het		het	het	dnw	het	ni		ni	het	ni	het		dnw	ni	dnw	ni	het	het
308	n	b	S			ni	het	het	het	het	het	het	ni	dnw	het		het	het	het	het	het		het	ni	het	het	het		het	dnw	het	het		het	het	dnw	het	het	het
335	n	b	M			het	het	ni	het	ni	het	het	ni	ni	ni	ni	ni	het	het	het	het	dnw	het	het	ni	het			het	het	ni	het	het	het	het	het	het	het	het
336	n	b	M			ni	het	het	ni	het	het	het	het	het	het	dnw	dnw	ni	het	het	ni	dnw	het	het	het	het			het	het	het	het	het	het	ni	het	het	ni	het
339	n	b	S			het	het	het	het	het	het	het	ni	ni	het	het	het	het	dnw	het	het	dnw	ni	het	het	ni			ni	het	het	dnw	het	het	het	dnw	het	ni	het
351	n	b	S			het	het	het	het	het	het	het	het	ni	het	het	ni	ni	het	het	het	dnw	ni	het	het	het			ni	het	het	dnw	het	het	het	het	het	ni	het
360	n	b	S			het	ni	het	het	het	het	het	ni	ni	het	dnw	het	ni	het	het	het	dnw	ni	het	het	het			ni	het	het	het	het	het	het	het	het	ni	het
372	n	b	M			ni	het	het	het	het	het	het	dnw	ni	het		ni	ni	dnw	ni	het		het	ni	dnw	ni	het		ni	het	ni			het	ni	dnw	het	ni	het
411	n	b	M			het	ni	het	ni	het	het	het	het	ni	ni	dnw	het	ni	het	het	het	dnw	het	het	het	ni			ni	het	ni	het	het	het	het	het	het	het	het
412	n	b	S			ni	het	het	het	ni	het	ni	het	ni	het	dnw	het	het	het	het	het	dnw	het	het	ni	het			ni	ni	ni	ni	het	het	ni	het	ni	het	het
429	n	b	S			het	het	het	ni	het	het	het	het	het	ni	dnw	ni	ni	het	het	ni	dnw	het	het	dnw	ni			het	het	ni	ni	ni	het	het	het	het	ni	ni
438	n	b	S			ni	het	het	het	ni	ni	het	het	het	het	dnw	het	ni	het	het	het	dnw	het	het	het	het			ni	het	het	het	het	het	ni	het	het	het	het
449	n	b	S			het	het	het	het	dnw	het	het	het	ni	ni	dnw	het	ni	het	het	het	dnw	het	het	het	het			het	ni	het	het	ni	het	het	het	het	het	ni
451	n	b	A			het	het	ni	het	ni	het	het	het	het	het	dnw	het	het	het	het	het	dnw	het	het	ni	ni			het	het	het	het	het	het	het	het	het	ni	ni

Table 3.1. LOH results for all loci in ovarian tumours

Case number[1]	LOH?[2]	Nature[3]	Histology[4]	Stage[5]	Grade	D7S481[6]	D7S654	D7S691	D7S678	D7S670	D7S506	D7S672	D7S634	D7S524	D7S630	D7S492	D7S2410	D7S689	D7S2431	D7S491	D7S2480	PAI-1	D7S518	D7S658	D7S692	D7S2502	D7S486	7G14	D7S522	D7S633	D7S655	D7S643	D7S530	D7S684	D7S676	D7S688	D7S636	D7S2546	D7S2465
463	n	b	M				het													dnw									ni									
466	n	b	S				dnw					het								het									dnw									
494	n	b	F			het	ni	het	het	ni	het	het	het	het	het		ni	het	het	het	het		dnw	het	dnw	dnw	het		dnw	dnw	dnw			dnw		dnw	dnw	ni	ni
512	n	b	M				het																						ni									
527	n	b	S				het																						het									
592	n	b	F						dnw														dnw				dnw		dnw									
289	n	bl	M	1		het	het	het	het	het	het	het	ni		ni		ni	het	het	het	het		het	ni	het	het	het		ni	ni	het	dnw		het	het	het	het	dnw	het
447	n	bl	S	1		dnw		ni	dnw	dnw	dnw	dnw	dnw		dnw		dnw						dnw	dnw	ni	ni	dnw		dnw	dnw	dnw	dnw		ni	dnw	dnw	dnw		dnw
452	n	bl	M	1		dnw		dnw	dnw	dnw	dnw	dnw	dnw		dnw		ni						dnw	ni	dnw	dnw	dnw		dnw	dnw	ni	dnw		dnw	dnw	dnw	dnw		dnw
508	n	bl	S	1		het		het	dnw	dnw	het	dnw	het		het		het						dnw	dnw	dnw	het	dnw		dnw	dnw	het	dnw		het	ni	het	het		het
531	n	bl	M	1		het		het	het	het	het	het	het		ni		het						het	dnw	het	het	het		dnw	het	het	dnw		het	het	het	het		het
545	n	bl	M/S	1		dnw		het	dnw	dnw	dnw	dnw	dnw		dnw		dnw			dnw			dnw	dnw	dnw	dnw	dnw		dnw	dnw	ni	dnw		dnw	dnw	dnw	dnw		dnw
92	n	bl	M	1a		het	het	ni	het	het	het	ni	het	dnw	het	dnw	het	het	het	het	het	dnw	dnw	het	het	ni	mi		het	het	het	het	dnw	het	ni	het	het	ni	het
388	n	bl	M	1a		dnw		dnw	dnw	dnw	dnw	dnw	dnw		dnw		dnw								dnw	dnw	dnw		dnw	dnw	dnw	dnw		dnw	dnw	dnw	dnw		dnw
389	n	bl	M	1a		dnw		dnw	dnw	dnw	dnw	dnw	dnw		dnw		ni						dnw	ni	ni	dnw	dnw		dnw	dnw	dnw	dnw		dnw	ni	dnw	dnw		dnw
186	n	bl	M	1c		het	het	het	mi	ni	het	het	ni		dnw		dnw	dnw		het			dnw	ni	dnw	dnw	dnw		het	het	het	het		het	dnw	dnw	het		dnw
229	n	bl	S			het		het	het	het	ni	het	het		het		het	het					het	het	ni	het	het		ni	het	ni	het		het	het	het	het		het
304	n	bl	M			het	ni	het	ni	ni	het	het	het		het		het	het		ni			het	dnw	ni	het	het		ni	het	het	het		ni	het	het	het		dnw
362	n	bl	S			het		het	dnw	het	het	het	het		het		ni	dnw					dnw	dnw	het	het	het		het	het	ni	dnw		dnw	ni	het	het		dnw
379	n	bl	M			dnw		dnw	dnw	dnw	dnw	dnw	ni		dnw		dnw	dnw					dnw	dnw	dnw	dnw	dnw		dnw	dnw	dnw	dnw		dnw	dnw	dnw	dnw		dnw
397	n	bl	M			het		het	dnw	het	dnw	ni	ni		ni		dnw						dnw	dnw	ni	ni	het		dnw	dnw	het	dnw		dnw	ni	ni	dnw		dnw
418	n	bl	M			dnw		dnw	dnw	dnw	ni	dnw	dnw		dnw		dnw						dnw	ni	dnw	dnw	dnw		dnw	dnw	dnw	dnw		dnw	dnw	dnw	dnw		dnw
420	n	bl	M			ni		het	dnw	het	het	het	ni		het		dnw						dnw	ni	het	ni	het		dnw	dnw	het	dnw		het	het	het	dnw		dnw
486	n	bl	S			dnw		dnw	dnw	dnw	dnw	dnw	ni		het		dnw						dnw	dnw	dnw	dnw	dnw		dnw	dnw	ni	dnw		ni	ni	dnw	dnw		dnw
50	n	m	M	1	2	het	het	ni	ni	ni	ni	het	ni	het	het	het	het	het	dnw	ni	het	het	het	het	ni	dnw	het	ni	ni	ni	ni	dnw	ni	het	ni	dnw	het	het	het
136	n	m	E	1	3			ni												het									ni									
190	n	m	S	1	2										het		ni						het		het		het	dnw	het		dnw		het	het	het	dnw			ni
293	n	m	U	1	3			ni							het																								
311	n	m	E	1	2			ni												het																het			
338	n	m	S	1	2			het				het																								ni			
357	n	m	CC	1	3			het												ni																het			
378	n	m	S	1	2			het												het																het			
594	n	m	E	1					dnw														het				dnw		dnw							het			
48	n	m	S	2	3							ni	het		het	het								ni						ni				het	het				
184	n	m	U	2											het																				het				
195	n	m	E/S	2	3			ni	het			het	ni		het	het	het	ni		het			het	ni	het	ni	het	het	ni	ni	ni	ni	het	het	het	het	het		het
202	n	m	E	2	1	het	het	het	ni	het	dnw	dnw	ni	het	ni	ni	het	ni	dnw	ni	het	ni	het	ni	het	dnw	het	dnw	ni	het	ni	het	het	het	ni	ni	ni	het	ni
205	n	m	E	2													mi						dnw		ni		mi	dnw	ni		dnw				dnw	het			dnw

Table 3.1. LOH results for all loci in ovarian tumours

Case number[1]	LOH?[2]	Nature[3]	Histology[4]	Stage[5]	Grade	D7S481[6]	D7S654	D7S691	D7S678	D7S670	D7S506	D7S672	D7S634	D7S524	D7S630	D7S492	D7S2410	D7S689	D7S2431	D7S491	D7S2480	PAI-1	D7S518	D7S658	D7S692	D7S2502	D7S486	7G14	D7S522	D7S633	D7S655	D7S643	D7S530	D7S684	D7S676	D7S688	D7S636	D7S2546	D7S2465
19	n	m	S	3	3	het	het	het	het	dnw	ni	het	het	ni	het	het	ni	het	het	dnw	ni	ni	het	het	het	ni	het	het	dnw	het	het	ni	het	het	ni	het	het	het
30	n	m	M	3	1																		dnw				dnw		dnw		dnw	dnw			dnw			
32	n	m	S	3	3							het	ni			ni							dnw				dnw			het	dnw	dnw			het			
67	n	m	S	3	3							het	ni		ni	ni								ni						ni		ni			het	het		
139	n	m	S	3	3										dnw								ni				mi	dnw					dnw	mi	het	ni	dnw		dnw
575	n	m	S	4					dnw														dnw	dnw			dnw		dnw										
577	n	m	S/E	4					ni														het	dnw			ni		dnw										
138	n	m	M	1a	1			het							het					het									ni										
155	n	m	E	1a	2	het	het	het	het	ni	het	het	het	ni	ni	dnw	het	ni	het	het	het	dnw	het	het	het	ni			ni	het	ni	dnw	het	het	het	het	het	ni	ni
176	n	m	M	1a	1	ni		ni				het		het	het	het	het	het		ni		ni	het	ni	het	het	het	dnw	het	het	dnw	ni	ni	het	het	het	het		het
259	n	m	S	1c	2												het						dnw		het		het	dnw	het		dnw	dnw			het		het		
279	n	m	U	1c	2			ni												het																			
298	n	m	S	1c	3			het																															
377	n	m	S/E	1c	2																								het										
151	n	m	E	2c	2			het				het	ni	het	het		ni	ni		ni			het	het	het		ni	dnw	ni	ni	het	ni	het	het	het	het	het		het
154	n	m	S	3a	2	dnw	ni	het	ni	ni	dnw		ni	dnw	dnw	het	dnw	ni	dnw		het	het	dnw	het	ni		het	dnw	het	dnw	dnw		dnw	het	het	het	het	dnw	het
225	n	m	M	3a	2	het	het	ni	het	het	het	het	het	het	ni	ni	ni	het	het	het	het		het	ni	ni	het	het	dnw	het	het	ni	ni		het	het	ni	het	het	dnw
258	n	m	E	3c	3	het	ni	het	ni	het	het	het	dnw	het	het	het	het	ni	het	het	het		het	ni	het	het	het	het	ni	ni	het	het	ni	ni	het	het	het	het	
568	n	m	S	3c																			het	ni			het		het										
569	n	m	S/E	3c																			dnw	dnw			dnw		dnw										
10	n	m	S		3																		dnw				dnw		dnw		dnw	dnw			ni			
13	n	m	M		1																		dnw				dnw		dnw		dnw	dnw			dnw		ni	
28	n	m	S		3							het	ni		ni	het							dnw	ni			dnw		dnw		dnw	dnw			ni			
124	n	m	G		3										ni			ni							ni									het					
299	n	m	CC					het												het			het													ni		
549	n	m	CC																				het	dnw			ni		dnw										
574	n	m	U						dnw														dnw	dnw			dnw		dnw										
580	n	m	E						dnw														dnw				dnw		dnw										
588	n	m	M						dnw														het				dnw		het										
LOH						12	16	10	4	10	4	22	14	10	29	12	11	4	14	17	16	9	20	11	18	8	14	6	26	24	15	16	7	13	12	16	19	9	13
Informative						88	85	113	64	75	72	111	65	44	106	37	71	48	59	100	84	25	96	70	87	56	75	17	77	103	62	62	53	94	80	92	120	64	84
% LOH						14	19	9	6	13	6	20	22	23	27	32	16	8	24	17	19	36	21	16	21	14	19	35	33	23	24	26	13	14	15	17	16	14	16

3.2.4 LOH versus tumour nature

The LOH frequencies with respect to tumour nature are shown in table 3.2. No significant difference (p=0.6) in frequency was observed between benign (18/66; 24%) and borderline (5/23; 22%) tumours, but a significant increase in LOH rate was seen in the malignant tumours (90/130; 69%) compared to the benign and borderline lesions (p=<0.0001 & p=<0.0001 respectively).

3.2.5 Frequent LOH irrespective of tumour histology, stage or grade

No significant difference in LOH rate could be determined between histological sub-types (table 3.3) of epithelial ovarian tumours, implying that deletion of chromosome 7 is a common event in all variations of ovarian neoplasia. In addition, the rate of LOH did not differ between tumour stage or grade (tables 3.5 and 3.4), ranging between 57-76% and 65-74% respectively.

3.2.6 Identification of candidate TSG loci

Figure 3.3 shows the percentage of LOH observed for each marker studied. The highest rate of LOH was with the marker PAI-1 (36%) located at 7q22 followed by D7S522 (33%) and 7G14 (35%) which are both located at 7q31. One other peak was observed at D7S492 (32%) on 7q21. The complexity of the LOH observed on chromosome 7 and in particular the multiple independent regions of LOH seen in individual tumours made interpretation of the data difficult. Indeed, from figure 3.4 it is apparent that there are several regions of chromosome 7 that are commonly deleted in benign, borderline and malignant tumours. Three of these regions correspond to those identified by studying the percentage of LOH observed for each microsatellite marker used (figure 3.3). Each region is best described by a small number of tumours with relatively well defined deletions and are also consistent with the broader deletions observed in several other tumours. The first region of loss, is defined by tumours 416, 433, 144, 188 and 219 is located at 7q11.2 between the markers D7S634 and D7S630. The second is at 7q22 and is best described by tumours 128 and 230 and is bounded by PAI-1 and D7S658, centring around D7S518. The third region is situated at 7q31, bounded by the markers D7S522 and D7S633 and identified by several tumours including 37, 40, 70, 80, 119 and 128. Representative autoradiographs from these three most well defined regions are shown in figures 3.5-3.7. Other regions of deletion can also be identified from the

Table 3.2: LOH on chromosome 7 with respect to tumour nature[1].

Benign	Borderline	Malignant
18/66 (24%)	5/23 (22%)	90/130 (69%)

Numerator is the number of tumours showing LOH on chromosome 7.

Denominator is the total number of tumours studied.

[1]Complete pathological data was not available for all tumours studied.

Table 3.3: LOH on chromosome 7 with respect to histological subtype of epithelial ovarian tumours[1].

Serous	Mucinous	Endometrioid	Undifferentiated
55/98 (56%)	23/57 (40%)	20/28 (71%)	2/6 (33%)

Numerator is the number of tumours showing LOH on chromosome 7.

Denominator is the total number of tumours studied.

[1]Complete pathological data was not available for all tumours studied.

Table 3.4: LOH on chromosome 7 with respect to grade of tumour[1].

Grade 1	Grade 2	Grade 3
19/26 (73%)	24/37 (65%)	37/50 (74%)

Numerator is the number of tumours showing LOH on chromosome 7.

Denominator is the total number of informative tumours of that grade.

106 tumours were ungraded.

[1]Complete pathological data was not available for all tumours studied.

Table 3.5: LOH on chromosome 7 with respect to stage of tumour[1].

Stage I	Stage II	Stage III	Stage IV
43/70 (61%)	8/14 (57%)	34/45 (76%)	4/6 (67%)

Numerator is the number of tumours showing LOH on chromosome 7.

Denominator is the total number of informative tumours of that stage.

84 tumours were not assigned stages.

[1]Complete pathological data was not available for all tumours studied.

Figure 3.3: Percentage of LOH shown by microsatellite markers on chromosome 7.

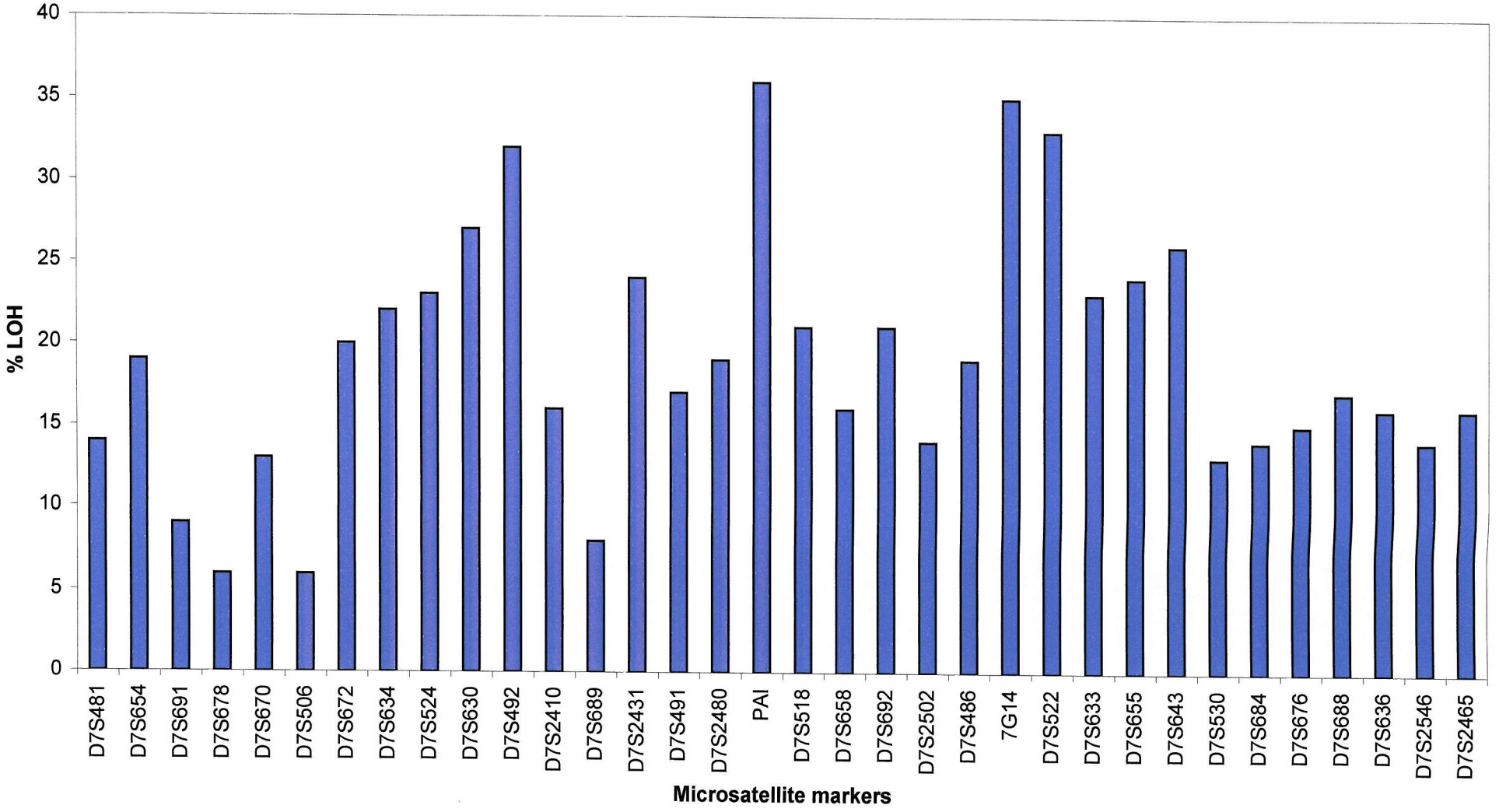
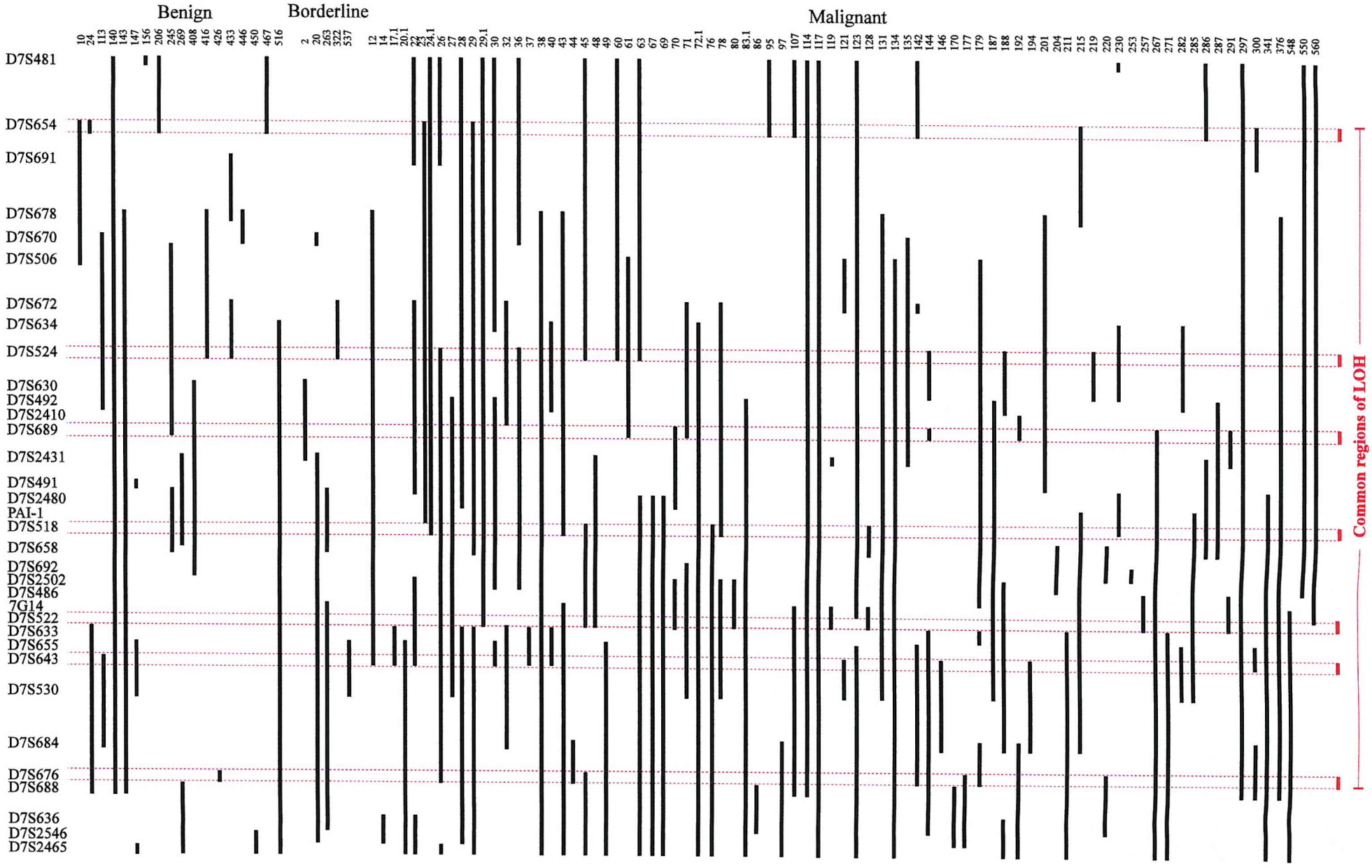


Figure 3.4. Diagrammatic representation of 104 benign, borderline and malignant ovarian tumours showing partial loss of chromosome 7.

Black bars indicate the extent of the deletion observed in each tumour.

Red bars indicate the common regions of LOH.

Microsatellite markers are represented in their relative positions and distances from each other.


Benign
Borderline
Malignant
D7S481
D7S654
D7S691
D7S678
D7S670
D7S506
D7S672
D7S634
D7S524
D7S630
D7S492
D7S2410
D7S689
D7S2431
D7S491
D7S2480
PAI-1
D7S518
D7S658
D7S692
D7S2502
D7S486
7G14
D7S522
D7S633
D7S655
D7S643
D7S530
D7S684
D7S676
D7S688
D7S636
D7S2546
D7S2465
Common regions of LOH

interstitial deletions shown in figure 3.4 and span the markers D7S654, D7S689, D7S643 and D7S676. Whilst these do not correspond as well with the overall LOH frequency distribution (figure 3.3) they are still valid.

3.3 DISCUSSION

Ovarian cancers are characterised by numerous regions of LOH across the genome. This suggests either that there are dozens of TSGs involved in the initiation, promotion and progression of ovarian cancer or that all or most of these losses are non-pathological random events due to generalised genomic instability. Chromosome 7 in particular appears to be especially prone to genomic instability in ovarian cancer. However if this is due to random events it is not restricted to ovarian cancers as LOH analysis has identified many putative TSGs on this chromosome in many tumour types (figure 3.1). Further evidence for the existence of at least one chromosome 7 TSG comes from microcell studies which have shown that the transfer of human chromosome 7 into both transformed human fibroblasts (Ogata *et al.*, 1993) and murine squamous cell carcinoma cell lines (Zenklusen *et al.*, 1994a) suppresses tumorigenic potential. Recently Zenklusen et al., (2000b) published a refined study of the 7q31 region which provided functional evidence for the presence of a TSG on 7q31. Single copies of human chromosome 7 were introduced into the highly aggressive prostate cancer cell-line, PC3. When these hybrids were introduced into nude mice they were seen to increase tumour latency compared to the parental PC3 cell-line, implying that a TSG is indeed present on chromosome 7. Also of interest was the observation that when grown *in vitro*, the hybrid cells grew at about the same rate as the PC3 cells. This suggests that the chromosome 7 TSG probably does not act to regulate the cell cycle but instead exerts its effect via a cell-cell or cell-matrix interaction and potentially plays a role within a progression pathway such as extracellular matrix remodelling (Zenklusen *et al.*, 2000b). Further, due to the tendency for hybrids of this nature to revert to the tumorigenic phenotype it was possible to use microsatellite analysis to identify deletions within the introduced chromosome. These deletions can occur following mitotic recombination and lead to the inactivation of TSG within the region of interest. By mapping the deletions observed in nine hybrids, Zenklusen et al. (2000b) was able to refine the candidate TSG loci to a 1.5 Mb region between the microsatellite markers D7S486 and D7S655. In this study we have shown that chromosome 7 is a site of frequent LOH (52%) in ovarian tumours. The observation that LOH occurs at similar frequencies in both epithelial (51%) and non-epithelial (56%) tumours implies that this chromosome harbours gene(s) involved in a wide range of tumour types which is

Figure 3.5. Representative autoradiographs for LOH results around 7q11-21.

Locus names are shown to the left, tumour case numbers are indicated at the top.

■ = LOH

□ = retention of heterozygosity

▒ = non-informative

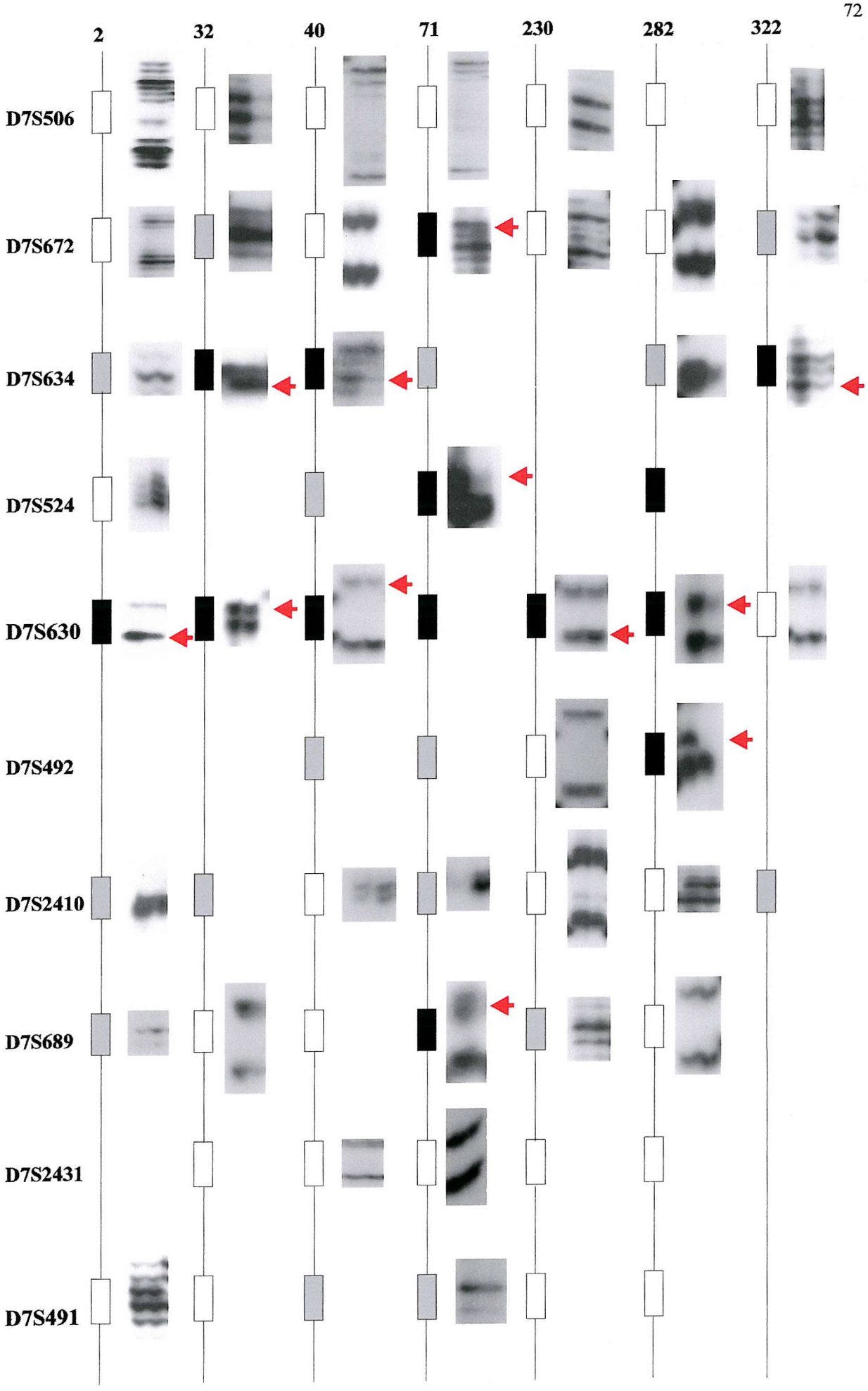

2
32
40
71
230
282
322
D7S506
D7S672
D7S634
D7S524
D7S630
D7S492
D7S2410
D7S689
D7S2431
D7S491

Figure 3.6. Representative autoradiographs for LOH results around 7q21-31.

Locus names are shown to the left, tumour case numbers are indicated at the top.

■ = LOH

□ = retention of heterozygosity

▒ = non-informative

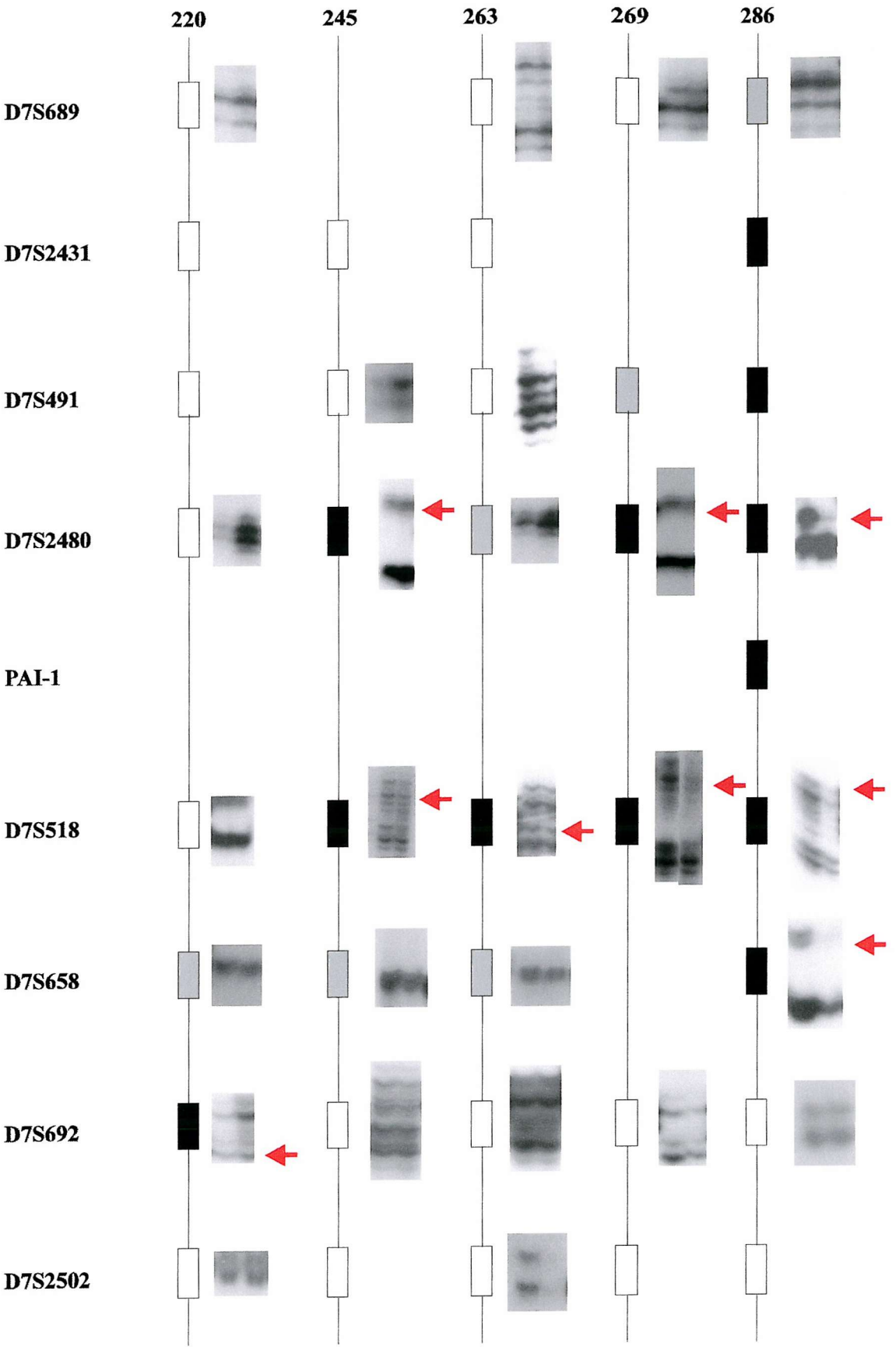

220
245
263
269
286
D7S689
D7S2431
D7S491
D7S2480
PAI-1
D7S518
D7S658
D7S692
D7S2502

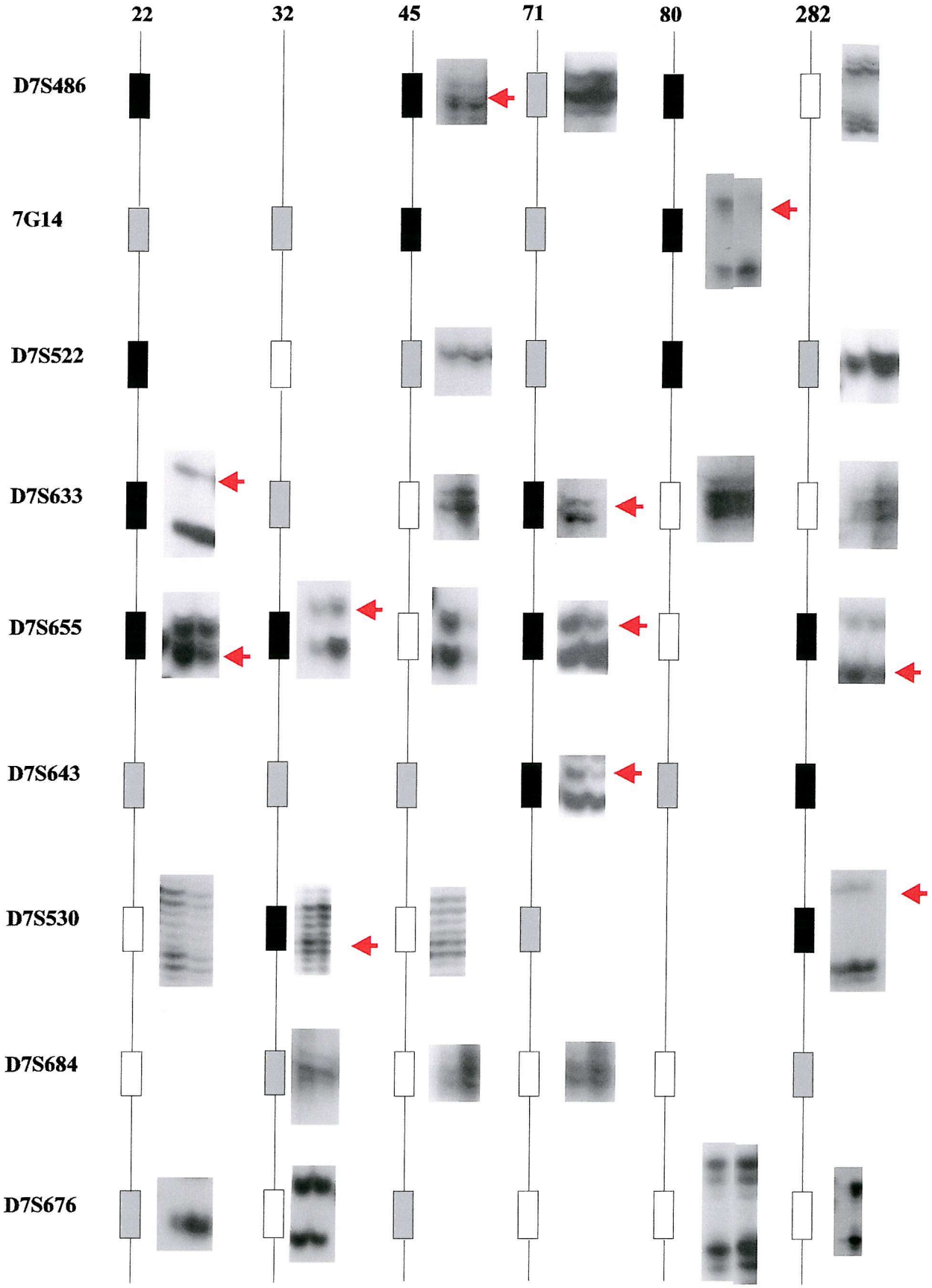
Figure 3.7. Representative autoradiographs for LOH results around 7q31-34.

Locus names are shown to the left, tumour case numbers are indicated at the top.

■ = LOH

□ = retention of heterozygosity

▒ = non-informative


22
32
45
71
80
282
D7S486
7G14
D7S522
D7S633
D7S655
D7S643
D7S530
D7S684
D7S676

consistent with previous studies of this chromosome suggesting that it harbours at least one broad range tumour suppressor. From the data shown (table 3.1) it appears that chromosome 7 LOH is a relatively early event in ovarian cancer, occurring at similar frequencies in benign and borderline tumours. The frequency of LOH was higher in malignant tumours, as might be expected due to the trend toward increased genetic instability in malignancies. However, the frequency of LOH did not differ between stage or grade of malignant tumour (tables 3.3 & 3.4) implying that the acquisition of this alteration may be necessary to allow a tumour to progress from either benign or borderline status to malignancy. In addition, there are overlaps in the patterns of chromosome 7 LOH seen in benign, borderline and malignant tumours, which may be evidence of progression from benign lesions to borderline and malignant lesions. This is a contentious point since there is still no well-defined precursor lesion for ovarian cancer and many reports suggest that these lesions are aetiologically distinct and do not progress to malignancy. There is support for this view as some studies have reported that borderline tumours harbour a range of genetic alterations that are distinct from those found in their malignant counterparts (Berchuck *et al.*, 1994; Saretzki *et al.*, 1997; Wertheim *et al.*, 1996). Conversely, inclusion cysts and borderline lesions have been shown to occur synchronously with frankly malignant ovarian tumours (Puls *et al.*, 1992), providing evidence that malignant progression may indeed occur.

The long arm of chromosome 7 exhibits a higher rate of LOH than 7p and from the data presented here it is possible to define several common regions of loss on chromosome 7q (figure 3.4). However, due to the complexity of the LOH observed in this study and the possibility that a proportion of the deletions seen are in fact regions of amplification, it is important to note that the interpretation of the results given here is one of many possible scenarios. Indeed the identification of commonly deleted regions often relies on a handful of tumours with small but unambiguous deletions. Comparing the overall patterns of LOH with the rates of LOH for each marker (figure 3.3), it is possible to describe three regions that are the most consistent between the two. These are situated at 7q11.2 centred around D7S524, 7q22 between the PAI-1 locus and D7S658, and at 7q31 between markers D7S522 and D7S633 (~1100kb). Additional common regions of LOH can be identified from the tumours showing interstitial deletion of chromosome 7q (figure 3.4), these span markers D7S654, D7S689, D7S643 and D7S676 respectively. Two of these deleted regions, 7q22 and 7q31, have been alluded to in previous studies (figure 3.1) of ovarian tumours (Edelson *et al.*, 1997; Kerr *et al.*, 1996; Koike *et al.*, 1997; Zenklusen *et al.*, 1995b) and several other tumour types (Nishizuka et al., 1997; Zenklusen *et al.*, 1994b), as potentially harbouring a TSG. For this

reason, candidate gene analysis was confined to these two regions, as discussed in chapters 4, 5 and 6. Of interest is the observation that although LOH is seen in benign, borderline and malignant tumours, within each commonly deleted region, the patterns of LOH appears to differ with tumour nature. Figure 3.4 shows that the defined deletion identified at 7q31 between markers D7S522 and D7S633 seen in malignant tumours, does not correspond to the smallest defined region in the non-malignant lesions. This could indicate that the loss seen between D7S522 and D7S633 occurs at the transition between benign or borderline lesions and malignancy, or that these tumours develop as completely separate entities. However, sample numbers are smaller for both benign and borderline cases and any difference in LOH pattern between these and the malignant tumours may therefore be ambiguous.

The commonly deleted region situated at 7q22, has been intensively studied in uterine leiomyomas (Ishwad *et al.*, 1995; vanderHeijden *et al.*, 1998; Zeng *et al.*, 1999) and myeloid disorders (Kere *et al.*, 1989; Tosi *et al.*, 1999; Zeng *et al.*, 1997). These diseases have both demonstrated distinct LOH around 7q22 and more recently a similar pattern of deletion has been identified in primary breast carcinomas (Zeng *et al.*, 1999). Figure 3.1 illustrates the overlapping deletions from several studies (Ishwad *et al.*, 1997; Kerr *et al.*, 1996; Shridhar *et al.*, 1997; Zeng *et al.*, 1997) which indicate a frequently deleted region lying between D7S666 and D7S658 that includes the *CUTL1* (cut-like 1) locus (Zeng *et al.*, 1997). Chromosome 7q22 is approximately 20 Mb in length and is thought to be one of the most gene-rich regions of the human genome (Glockner *et al.*, 1998), making the identification of TSGs difficult in the absence of a reasonably small candidate region from which to begin the hunt for known and novel genes. In the absence of adequate sequencing coverage of this region, which until recently was not represented in a YAC contig, the analysis of 7q22 was difficult. Glockner et al,. (Glockner *et al.*, 1998) therefore undertook sequencing of areas surrounding two known genes from within this region, *EPO* (erythropoietin) and *CUTL1*. Analysis of the EPO and CUTL1 contigs, 228 kb and 416 kb respectively, identified 17 genes, 11 known and 6 novel. The CUTL1 contig is of particular interest since LOH analysis has implicated the region around this gene as harbouring a TSG (Zeng *et al.*, 1999). Previously known genes that map within this contig, in addition to *CUTL1*, are *PMSL12* (postmeiotic segregation), a mismatch repair gene, and the adaptor protein *APS* (adaptor protein with PH and SH2 domain). *CD55* is the only novel gene identified, the function of which is still to be determined, although the protein it encodes is believed to reside in the nucleus (Glockner *et al.*, 1998). Considering its position in relation to the commonly deleted region defined by LOH studies and its potential gene repressing properties (Dufort & Nepveu,

1994; Mailly *et al.*, 1996), *CUTL1* has been put forward as a candidate TSG. Mutation analysis of this gene was carried out as part of this project and is discussed further in chapter 4.

In our studies, the commonly deleted region at 7q22 is seen to lie between the *PAI-1* (*p*lasminogen activator inhibitor-1) locus and D7S658, centred around D7S518, a marker that has been mapped within intron 20 of *CUTL1*. However, also of interest is the locality of the *PAI-1* gene (Kerr *et al.*, 1996) which has been previously indicated to have potential tumour suppressing properties, since it acts to inhibit plasminogen activator and therefore proteolysis of the extracellular matrix and subsequent tumour invasion (Stack *et al.*, 1998). A tumour suppressor function of PAI-1 has been challenged by the observation that PAI-1 is often elevated, along with its target uPA, in ovarian cancer (Schmalfeldt *et al.*, 1995; Stack *et al.*, 1998). It appears, therefore, that PAI-1 may in fact facilitate metastasis, presenting a paradox surrounding its role in tumour progression. This complex relationship is further discussed in chapter 5, along with the results of mutational analysis of this gene in ovarian tumours.
The close proximity of the postmeiotic segregation gene (*PMSL12*), to the deleted region described by LOH studies, is also a potentially interesting observation. *PMSL12* is one of several mismatch repair genes (section 1.10) that have been mapped to chromosome 7 and would have been another candidate for further analysis, time permitting. However, as yet the structure of the gene, although predicted by alignment with *PMS2* (another mismatch repair gene previously mapped to 7q22), has not been confirmed (Glockner *et al.*, 1998).
APS is located proximal of both *CUTL1* and *PMSL12*, but may also prove to be of importance due to evidence suggesting that it may function as a mediator between immune receptors and tyrosine phosphorylation signalling pathways (Yokouchi *et al.*, 1997). Such an activity may also have the potential for a role in controlling tumour progression.
Also of interest is a recent report indicating that an aphidicolin-inducible fragile site, FRA7B, is present within 7q22 (Guven *et al.*, 1999). However, this site does not appear to be very common and has yet only been identified in normal healthy individuals (Guven *et al.*, 1999), leaving its relevance to human cancer open for investigation.

The commonly deleted region within 7q31, identified in the present study, is the most frequently reported region of loss on chromosome 7 throughout a wide range of tumour types, including that of the ovary, suggesting the presence of a multi-tissue tumour TSG. The reported frequency of LOH occurring at one or more loci within 7q31 show a wide variation from 12.3%-100 % (Lin *et al.*, 1996; Zenklusen *et al.*, 1995b) and is likely to be due to biases

introduced by low sample numbers or the use of markers previously shown to exhibit high rates of LOH. However, the reports of frequent LOH are consistent and the patterns of deletion appear to overlap (figure 3.1), implying that this region is of importance. The results from the present study further support and refine this region to approximately 1.1 Mb between the markers D7S522 and D7S633.

To date there are few genes known to reside within 7q31. The c-*MET* oncogene, discussed further in chapter 6, was of interest due to its location within this the 7q31 candidate region. Caveolin-1 has also been mapped to this region, however it has recently been dismissed as the 7q31 TSG since no mutations or aberrant methylation of the gene have been identified (Hurlstone *et al.*, 1999). More recently, Zenklusen et al., (2000a) presented data indicating that they had identified a broad range TSG from 7q31 using a high-resolution physical map of the region between D7S522 and D7S677 (which lies distal to D7S633). Six known genes, including c-MET and WNT2 (wingless-type MMTV integration site), and 3 unknown genes were identified from the area of interest. The three unknown genes were then analysed for aberrations in prostate and breast carcinoma cell lines and primary colon carcinomas. One of the genes, now referred to as *PITS* (Proliferation Independent Tumour Suppressor), exhibited mutations, resulting in truncated protein products, in 50% of the cell lines and 40% of the primary tumours studied (Zenklusen *et al.*, 2000a). This gene comprises 16 exons, spans 130 kb of genomic sequence and translates to a 525 aa globular protein which has no sequence homology with any other known gene, or any known protein motifs. In addition *PITS* is expressed at substantial levels in most human tissues and is highly conserved between species, suggesting that it may have housekeeping activity, all of which is consistent with the association with many tumour types. This data is compelling but further functional work is required before *PITS* is regarded as the TSG targeted by LOH on 7q31.

Interestingly, recent data (Huang *et al.*, 1998a) confirms the presence of an aphidicolin-inducible fragile site, FRA7G, the second most common in the human genome (Zenklusen *et al.*, 2000b), which spans this region and may be responsible for a proportion of the LOH observed in malignant lesions, rather than the inactivation of a TSG. Indeed, some of the highest frequencies of LOH on chromosome 7q in ovarian cancer have been reported with microsatellite markers that map within FRA7G, for example 7G14 which showed 37% (15/41) LOH in a study by Huang et al., (Huang *et al.*, 1999) and 35% (6/17) LOH in the present study, implying that this fragile site may well be a 'hot-spot' for LOH. Fragile sites are regions of the genome prone to breakage when cells are exposed to chemicals such as caffeine, aphidicolin or inhibitors of the folic acid pathway (Huang *et al.*, 1999). FRA7G is

an aphidicolin-inducible fragile site spanning at least 300kb of genomic DNA. Of particular interest is the observation that this fragile site contains both small polydispersed circular DNA (spcDNA) and evidence of human retroviral sequence (Huang *et al.*, 1998b), a phenomenon also described within the FRA3B common fragile site on chromosome 3p14.2. The presence of human endogenous retroviral sequence, homologous to HERV-H would seem to suggest that fragile sites provide integration sites for viruses, although it is still unknown if this sequence, or the presence of spcDNA, is a cause of fragility within this site (Huang *et al.*, 1998b). The potential viral association is of further interest due to the link between specific types of neoplasia, such as cervical cancer, and viral infection. The presence of a fragile site at 7q31 does not however, preclude the possibility that a TSG is also located within this region. Indeed, a similar situation is observed between the Fragile Histidine Triad gene (FHIT) and the common fragile site FRA3B (Huebner *et al.*, 1997). This is the most common fragile site in the human genome and like FRA7G, is aphidicolin-inducible. FRA3B spans over 300 kb of sequence, encompasses a region that is frequently deleted in several tumour types (Huang *et al.*, 1998b) and lies close to the t[3;8](p14.2;q24.1) translocation breakpoint often found in hereditary renal cell carcinoma (Huebner *et al.*, 1997). *FHIT* is now known to cross this FRA3B region and has been shown to undergo homozygous deletions in many types of cancer cell-lines, earning it candidate TSG status (Huebner *et al.*, 1997; Zenklusen *et al.*, 2000b). The majority of the breakpoints reported within FRA3B occur within introns either side of the first coding exon of the *FHIT* gene which may affect splicing (Huebner *et al.*, 1997). It is believed that FRA7G may provide a choice location for a TSG just as FRA3B does for *FHIT*, since the instability of the fragile site extends the opportunity for alterations to occur, leading to the inactivation of the gene (Zenklusen *et al.*, 2000b). Indeed Zenklusen further suggests that by mapping the positions of other fragile sites, additional TSGs may be identified.

The final region of common LOH identified in this present study was centred around D7S524, located at 7q11.2. This novel region did not show an LOH frequency as high as that for either of the other 2 regions observed, but did appear to be a distinct deletion, separate from the others.

Whilst other regions of LOH could be seen amongst the malignant tumours, these were not as consistent as the three discussed above and did not seem to correspond with the peaks in percentage LOH shown in figure 3.3. It is impossible to say at this stage whether these other regions are bona fide TSG loci or if they are the result of the generalised genomic instability

and not associated with the inactivation of any TSG. Indeed, until a gene from each or any of the candidate regions is shown to harbour somatic alterations and/or shown to possess tumour suppressor function, the exact number and nature of ovarian TSGs on chromosome 7 will remain unclear.

3.4 SUMMARY

We have provided additional evidence in support of previous studies showing that LOH on chromosome 7 is a frequent event in ovarian cancers. In addition, the data presented here suggests that chromosome 7 LOH may trigger a switch from benign or borderline status to malignancy. The deletions identified on chromosome 7 also appear to be involved in a range of histological sub-types of both epithelial and non-epithelial tumours, implying that any TSG or TSGs found are likely to be multi-tissue type tumour suppressors. We have also refined these common regions of loss with respect to ovarian cancer. In addition to two regions that have previously been indicated at 7q22 and 7q31, we have identified a novel deletion at 7q11.2, centred around D7S524. The presence of the common aphidicolin-inducible fragile site within the common region of loss at 7q31 is also of interest, as is a second fragile site, FRA7B, situated at 7q22..

CHAPTER FOUR

MUTATION ANALYSIS OF *CUTL1* IN HUMAN EPITHELIAL OVARIAN TUMOURS

4.1 INTRODUCTION

Chromosome 7q22 has been shown to contain defined regions of LOH in ovarian tumours (discussed further in section 3), malignant myeloid tumours (Fischer *et al.*, 1997; Johnson *et al.*, 1996; Kere *et al.*, 1989), uterine leiomyomas (Ishwad *et al.*, 1997; Zeng *et al.*, 1997) and recently, breast tumours (Zeng *et al.*, 1999). This frequent deletion suggests the presence of one or more TSGs within this chromosomal band. Several genes have now been mapped to this area (Zeng *et al.*, 1997) including *PAI-1* (Plasminogen Activator Inhibitor-*1)* (Chapter 5), *EPO* (erythropoietin), *MDR* 1 and 2 (multidrug resistance 1 and 2) (Kere *et al.*, 1989), *TRIP6* (thyroid receptor interacting protein-6) (Yi & Beckerle, 1998), *MSSI* (mammalian suppressor of sgv1) (Tanahashi *et al.*, 1998) and *CUTL1* (cut-like-1) (Scherer *et al.*, 1993).

4.1.1 Deletion of 7q22 in tumours

The deletion of all or part of the long arm of chromosome 7 is a frequent occurrence in many tumour types. Several cytogenetic and LOH studies have shown 7q22 to be one of the most common breakpoint regions on chromosome 7q. In 1989, Kere *et al.*, (1989) identified a narrow breakpoint of 7q22 in myeloid disorders, using restriction fragment length polymorphism analysis. This deletion was located between the *PAI-1* and *EPO* gene loci, a distance of approximately 3cM. Further studies on myelodysplasia and myeloid leukaemias (Johnson *et al.*, 1996; Fischer *et al.*, 1997) using Fluorescence *In Situ* Hybridisation (FISH) with cosmid and Yeast Artificial Chromosome(YAC) clones, have identified both translocations and deletions of 7q, the breakpoints of which map to 7q22. Cytogenetic studies have shown that many uterine leiomyomas exhibit 7q deletion as the only chromosomal abnormality (Ishwad *et al.*, 1995).

In an attempt to further define this deletion, LOH analyses have been carried out by several groups (Ishwad *et al.*, 1995; Ishwad *et al.*, 1997; Zeng *et al.*, 1997; van der Heijden *et al.*,

1998). Three of these studies have localised the deletion to a 340 kb region located between the markers D7S518 and D7S515 (see figure 3.2 for marker positions on chromosome 7). However, Van der Heijden *et al.*, (1998) placed the breakpoint at a more distal position, in a 4cM interval bounded by the markers D7S2453 and D7S496. It is possible that each of these two discrete regions may contain a potential TSG in leiomyomas.

More recently, Zeng *et al.*, (1999) has shown that a region of LOH at 7q22 is also observed in all stages and grades of breast tumours. This deletion appears to occur between D7S666 and D7S658, which overlaps one of the putative leiomyoma TSG loci. Finally, from the data in chapter 3 and that of Watson *et al.* (1998), it is clear that LOH at 7q22 is also very common in epithelial ovarian tumours with little or no difference in frequency between early and advanced tumours. Consideration of all the data for 7q22 suggests the region harbours a TSG(s) involved at an early stage in the transformation of a wide range of tumour types.

It is of interest that the frequently deleted markers D7S518, D7S515 and D7S666 are intergenic to the *CUTL1* gene (Zeng *et al.*, 2000) mapping to introns 3, 6 and 20 respectively. The *CUTL1* (cut-like 1) gene (figure 4.1) encodes the 1505aa CCAAT displacement protein (CDP), a putative repressor of gene expression (Neufeld *et al.*, 1992); (Scherer *et al.*, 1993). *CUTL1* therefore appeared to be a promising candidate TSG.

4.1.2 Aim of the study

This study aimed to determine if *CUTL1* was the target of the LOH observed at 7q22 in ovarian tumours, and therefore a potential tumour suppressor. In order for *CUTL1* to be considered a classical TSG, one would expect to find a mutation in the retained copy of the gene. In an attempt to elucidate this, the majority of the known coding exons of *CUTL1* were analysed for mutations in samples with and without LOH at 7q22.

4.2 RESULTS

Forty-seven tumour samples, comprising 42 malignant, 3 benign and 2 borderline tumours were used in this study. Within this panel of tumours, 29 were serous, 5 were mucinous, 9 endometrioid and the remainder were of mixed histology. Histological data is shown in table 4.1. Forty-two of the tumours exhibited LOH at 7q, of which, 35 included LOH of 7q22 (chapter 3).

Figure 4.1 : *CUTL1* Amino acid sequence.

Identified protein domains are shown in pink.

Exon 20 polymorphism is located at aa 1028, indicated in red.

Table 4.1. Mutation analysis of *CUTL1*, *PAI-1* and c-*MET*.

[1]Suffix '.1' indicates samples provided by the ICRF, London.

[2]Age at diagnosis.

[3]Histological subtype of ovarian tumour.

[4]m = malignant; b = benign; bl = borderline.

[5]LOH: y = LOH observed; n = LOH absent

[6]Mutation analysis: y = tumour has been analysed for the gene specified;

n = tumour has not been analysed for the specific gene.

Table 4.1. Mutation analysis of *CUTL1*, *PAI-1* and c-*MET*

Case Number[1]	Age[2]	Histology[3]	Grade	Stage	Nature[4]	7q loh[5]	7q22 LOH[5]	7q31 LOH[5]	Mutation analysis[6]		
									CUTL1	*PAI-1*	c-*MET*
2	60	serous	na	1c	bl	y	n	n	n	y	y
5	82	serous	na	na	b	n	n	n	n	n	y
7	79	serous	na	na	b	n	n	n	n	y	n
10	59	serous	na	na	b	n	n	n	y	y	n
10.1	na	serous	3	na	m	n	n	n	n	y	n
12	57	serous	1	1b	m	y	y	y	y	n	n
13	na	mucinous	1	na	m	n	n	n	n	y	y
14	57	serous	2	3b	m	y	n	n	y	y	y
15	84	mucinous thecoma	na	na	b	n	n	n	n	n	y
19	86	serous	3	3	m	n	n	n	y	y	y
20	42	serous	na	3a	bl	y	y	y	y	y	y
20.1	na	serous	2	na	m	y	n	n	n	y	n
22	65	serous	3	3	m	y	y	y	y	y	y
23	47	serous	1	1	m	y	y	n	n	y	y
24	77	serous	na	na	b	y	n	y	y	y	n
26	51	serous	2	3	m	y	y	y	y	y	y
27	na	serous	2	1b	m	y	y	y	y	y	y
29.1	na	serous	3	3	m	y	y	y	y	n	n
30	59	endometrioid/mucinous	3	3	m	y	y	y	y	y	y
30.1	na	mucinous	1	3	m	n	n	n	n	y	n
32	71	serous	1	2	m	y	n	n	n	y	y
36	54	endometrioid	1	1a	m	y	y	y	intron 7 A>G	y	y
40	73	mucinous	3	2	m	y	n	y	n	y	y
41	na	serous	3	3	m	y	y	y	n	y	n
41.1	na	serous	3	3	m	y	y	y	y	n	n
43	51	serous/endometrioid	2	2	m	y	y	y	y	y	y
44	75	mucinous	1	1	m	y	n	n	n	y	n
45	50	serous	3	3	m	y	y	y	y	y	y
48	50	serous	2	3	m	y	y	y	y	y	y
48.1	na	serous	3	2	m	n	n	n	n	y	n
49	49	Mullerian origin	3	3	m	y	n	y	n	y	y
50	81	mucinous	2	1	m	n	n	n	n	y	y
50.1	na	mixed Müllerian tumour	3	3	m	y	y	y	y	y	n
51	82	mucinous	1	1a	m	y	y	y	y	n	n
60	47	granulosa cell tumour	1	1a	m	y	n	n	n	n	y
61	76	mucinous	1	1a	m	y	n	n	n	y	y
63	66	serous	3	1	m	y	y	y	n	y	y
70	57	endometrioid	1	1a	m	y	y	y	y	y	y
71	68	serous	2	1a	m	y	y	y	y	y	y
72.1	na	serous	3	4	m	y	y	y	y	n	n
75	68	mucinous	na	na	b	n	n	n	n	y	y
78	76	endometrioid	2	1a	m	y	y	y	codon 1028 TCT>TCC	y	n
80	71	mucinous	1	1a	m	y	n	y	y	y	y
80.1	na	serous	3	3c	m	y	y	y	y	n	n
86	84	serous	3	3a	m	y	n	n	n	y	y
92	50	mucinous	na	1a	bl	n	n	n	n	y	n
94	48	mucinous	na	na	b	n	n	n	n	y	n
95	66	endometrioid	3	2	m	n	n	n	n	y	y

Table 4.1. Mutation analysis of *CUTL1*, *PAI-1* and c-*MET*

Case Number[1]	Age[2]	Histology[3]	Grade	Stage	Nature[4]	7q loh[5]	7q22 LOH[5]	7q31 LOH[5]	Mutation analysis[6]		
									CUTL1	*PAI-1*	c-*MET*
97	74	serous	3	3	m	y	n	n	n	y	y
103	56	serous cystadenofibroma	na	na	b	n	n	n	n	y	n
113	36	mucinous	na	na	b	y	n	n	n	y	n
114	70	endometrioid	2	1a	m	y	y	y	y	y	n
119	59	serous	2	1c	m	y	n	y	n	y	y
121	77	mucinous	1	3	m	y	n	n	n	y	y
122	na	serous	3	3	m	y	y	y	y	y	n
124	53	granulosa cell tumour	na	3	m	n	n	n	n	n	y
128	56	endometrioid	2	1c	m	y	y	y	n	n	y
131	74	serous	3	3	m	y	y	y	y	n	y
134	64	serous	3	3c	m	y	y	y	y	n	y
135	66	serous	2	2	m	y	n	n	y	y	y
139	65	serous	3	3	m	n	n	n	n	y	y
140	46	mucinous	na	na	b	y	y	y	n	y	n
142	66	serous	3	3	m	y	n	y	y	y	y
144	63	mucinous/endometrioid	1	1c	m	y	y	y	y	y	y
146	67	endometrioid	2	1c	m	y	n	n	intron 11 G>A	y	y
148	67	serous	na	na	b	n	n	n	n	n	y
151	56	endometrioid	2	2c	m	n	n	n	n	y	y
154	52	serous	2	3a	m	n	n	n	n	y	n
156	47	mucinous	na	na	b	n	n	n	n	y	n
158	82	serous cystadenofibroma	na	na	b	n	n	n	n	y	n
164	72	serous	na	na	b	n	n	n	n	y	n
167	66	serous fibroma	na	na	b	n	n	n	n	y	n
170	60	endometrioid	2	1a	m	y	n	n	n	y	y
176	62	mucinous	1	1a	m	n	n	n	n	y	n
177	53	endometrioid	3	1a	m	y	n	n	n	y	n
179	62	endometrioid	3	3	m	y	y	y	y	y	n
183	51	serous/mucinous/brenner	na	na	b	n	n	n	n	n	y
186	78	mucinous	na	1c	bl	n	n	n	n	n	y
188	47	serous	3	3c	m	y	n	y	y	y	n
190	51	serous	2	1	m	n	n	n	n	y	n
192	41	serous	3	3c	m	y	y	n	y	y	n
194	63	papillary	3	2	m	y	n	n	n	y	n
195	65	endometrioid/serous	3	2	m	n	n	n	n	y	n
196	46	serous	na	na	b	n	n	n	n	n	y
201	80	serous	2	3b	m	y	y	n	y	y	n
202	65	endometrioid	1	2	m	n	n	n	n	y	n
204	73	serous	3	3c	m	y	y	y	n	y	n
205	na	endometrioid	na	2	m	n	n	n	n	y	n
211	55	endometrioid	na	2c	m	y	n	y	n	y	n
214	75	mucinous	na	na	b	n	n	n	n	n	y
215	55	serous	3	1c	m	y	y	y	n	y	n
219	63	mucinous	1	1c	m	y	y	n	y	y	n
220	62	endometrioid	2	3c	m	y	y	n	y	y	n
225	58	mucinous	2	3a	m	y	y	n	y	y	n
230	49	serous	3	4	m	y	y	n	y	y	n
246	47	mucinous	na	na	b	n	n	n	n	n	y
253	70	endometrioid	2	3a	m	y	y	n	n	y	n

Table 4.1. Mutation analysis of *CUTL1*, *PAI-1* and c-*MET*

									Mutation analysis[6]		
Case Number[1]	Age[2]	Histology[3]	Grade	Stage	Nature[4]	7q loh[5]	7q22 LOH[5]	7q31 LOH[5]	*CUTL1*	*PAI-1*	c-*MET*
257	60	mucinous	1	1a	m	y	n	y	n	y	n
258	69	endometrioid	3	3c	m	n	n	n	y	y	n
259	67	serous	2	1c	m	n	n	n	n	y	n
262	74	serous	na	na	b	n	n	n	y	y	y
263	62	mucinous	na	1a	bl	y	y	y	n	y	y
276	49	mucinous	na	na	b	n	n	n	n	y	y
282	73	serous	2	3	m	y	n	y	y	y	y
285	61	serous	2	2c	m	y	y	y	n	y	n
286	71	endometrioid	3	3b	m	y	y	n	n	y	y
289	53	mucinous	na	1	bl	n	n	n	y	y	y
291	87	serous	3	3	m	y	n	y	y	y	y
294	60	mucinous	na	na	b	n	n	n	n	n	y
295	63	adenofibroma	na	na	b	n	n	n	n	n	y
297	53	serous	3	3	m	y	y	y	y	y	y
300	68	endometrioid	3	3	m	y	n	n	y	y	y
301	71	serous	na	na	b	n	n	n	n	n	y
304	53	mucinous	na	na	bl	n	n	n	n	n	y
308	25	serous	na	na	b	n	n	n	n	n	y
338	69	serous	2	1	m	n	n	n	n	n	y
339	75	serous	na	na	b	n	n	n	n	n	y
341	62	serous	2	1	m	y	y	y	n	n	y
357	72	clear cell	3	1	m	n	n	n	n	n	y
360	69	serous	na	na	b	n	n	n	n	n	y
362	77	serous	na	na	bl	n	n	n	n	n	y
376	62	serous	3	1c	m	y	y	y	n	n	y

The coding exons 3-24 of the *CUTL1* gene, were examined for mutations using SSCP-HD analysis (table 4.1). The intron/exon boundaries of CUTL1 were determined from public sequence databases and intronic primers were then designed using the primer3 program (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Primer sequences and PCR conditions are given in table 2.3.

No somatic mutations were identified in any exon. However, a number of exons appeared to harbour common germline alterations. Representative tumours showing alterations in these exons were sequenced (table 4.2). Exon 6 was found to be highly polymorphic and as such it was decided that these alterations were unlikely to be pathological and so were not investigated further.

Tumour 36 displayed an aberrant band pattern in exon 7 (figure 4.2A). Sequencing revealed this to be due to an A>G base change (figure 4.2B) 6 bases downstream from the exon 7/intron 7 splice junction. It can also be seen from figure 4.2A, that 36N is homozygous and 45T heterozygous for this variant, indicating that this is likely to prove a fairly common variant and unlikely to be of pathological significance. However, the alteration does fall within the splice consensus sequence, although it is a reasonably common variant of the consensus sequence occurring in approximately 16% of the population, further dismissing a pathological role for this variant (1998). Tumour 146 was shown to harbour a G>A transversion within intron 11 (figure 4.3C) 21 bases downstream of exon 11, this places it outside the splice consensus sequence and is therefore likely to represent a rare neutral polymorphism. Exon 20 was shown to harbour a C>T transition mutation of codon 1028 (figure 4.4C). This alteration did not change the amino acid, suggesting that it is a rare, non-pathological sequence variant.

4.3 DISCUSSION

CUTL1, also known as CDP (CCAAT displacement protein) was first identified in humans as a repressor of the gp91-pho gene in immature myeloid cells (Skalnik *et al.*, 1991). CUTL1 was named CDP when it was shown to compete with the positively acting CCAAT factor CP1, to bind to a region containing a duplicated CCAAT box within the promoter of gp91-phox (Mailly *et al.*, 1996). However, CUTL1 has been shown to recognise many DNA sequences that do not exhibit the CCAAT region (Harada *et al.*, 1994). It is therefore conceivable that CUTL1 may also act via means that are independent of CCAAT binding.

Case Number	Age	Histology	Stage	Grade	7q LOH	7q22 LOH	Band shift	Base Change
36	54	Endometrioid	1a	1	y	y	intron 7 (+ 6 bases)	A>**G**
78	76	Endometrioid	1a	2	y	y	exon 20: codon 1028	TC**T**>TC**C**
146	67	Endometrioid	1c	2	y	n	intron 11 (+ 21 bases)	G>**A**

Table 4.2 Polymorphisms detected in the *CUTL1* gene in epithelial ovarian tumours.

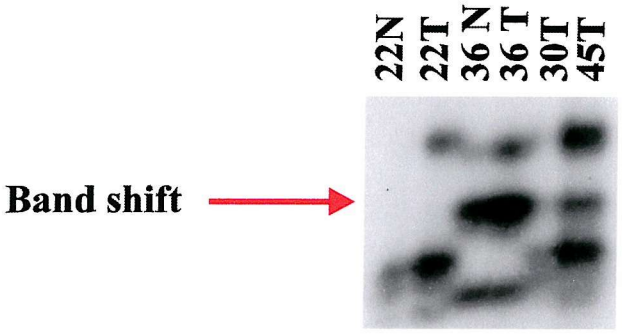
Figure 4.2. Autoradiographs showing the mutation observed in *CUTL1* intron 7.

A. Tumour number 36 shows a band shift in intron 7 of *CUTL1*. The band shift can be seen in both normal (N) and tumour (T) DNA, indicating a germline polymorphism

.

B. An intronic, transversion mutation A>G is shown.

A.


22N
22T
36 N
36 T
30T
45T
Band shift

EXON 7

B.

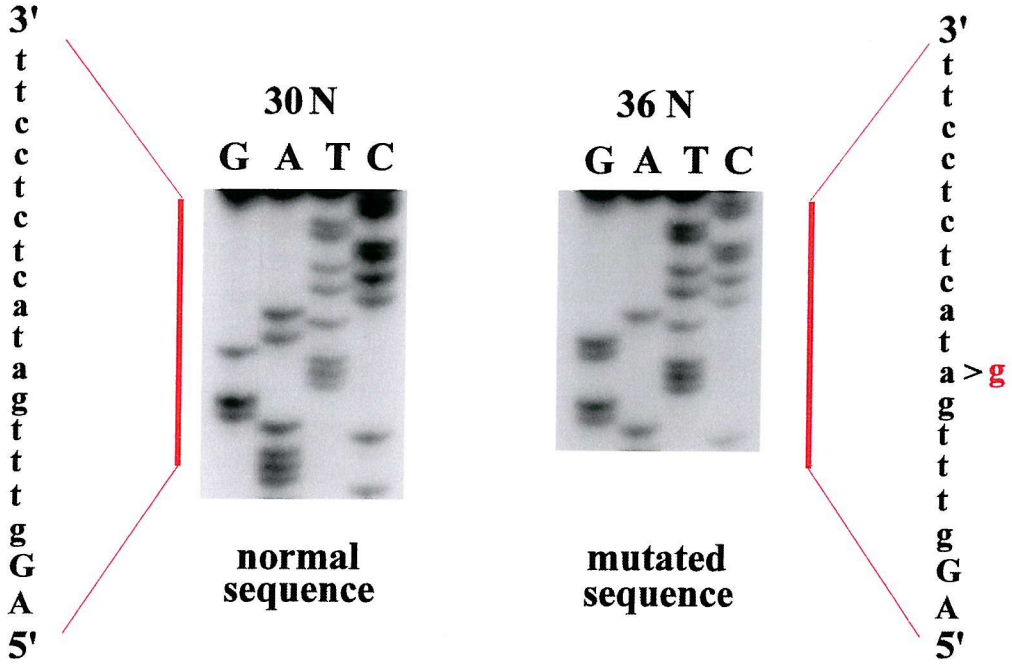

3'
t t c c t c t c a t a g t t t g G A
5'
30 N
G A T C
36 N
G A T C
normal sequence
mutated sequence
3'
t t c c t c t c a t a > g t t t g G A
5'

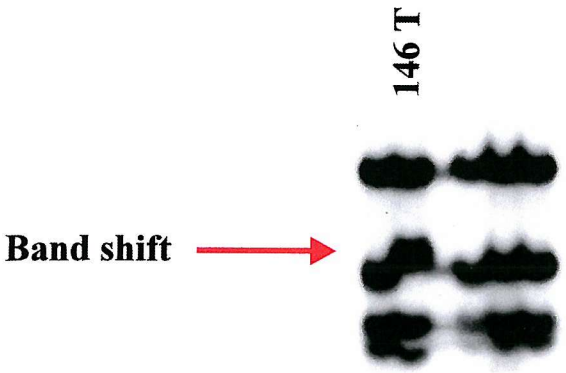
Figure 4.3. Autoradiographs showing the mutation observed in *CUTL1* intron 11.

A. Tumour number 146 shows a band shift in intron 11 of *CUTL1*.

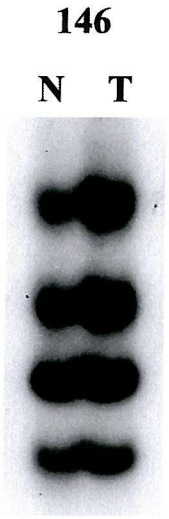
B. The band shift can be seen in both normal (N) and tumour (T) DNA, indicating a germline polymorphism.

C. An intronic, transversion mutation G>A is shown.

A.


146 T
Band shift

B.


146
N T

C.

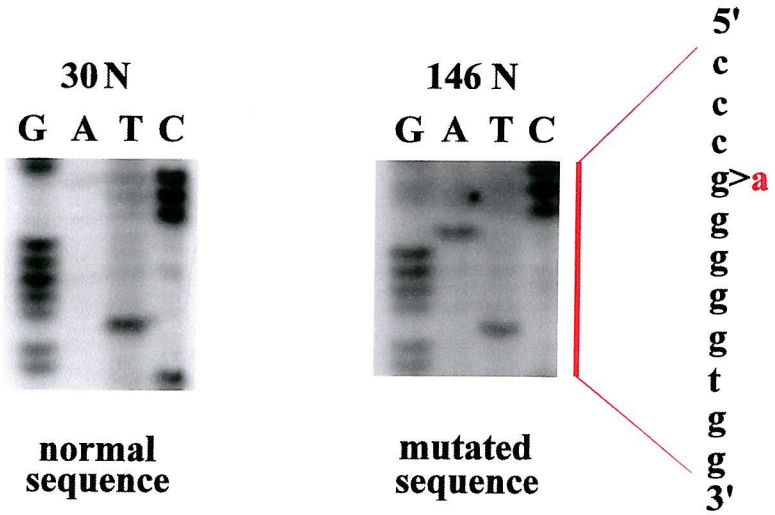

30 N
G A T C
146 N
G A T C
5'
c
c
c
g>a
g
g
g
g
t
g
g
3'
normal sequence
mutated sequence

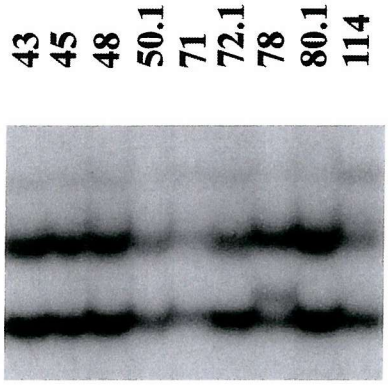
Figure 4.4. Autoradiographs showing the mutation observed in *CUTL1* exon 20.

A. Tumour number 78 shows a band shift in exon 20 of *CUTL1*.

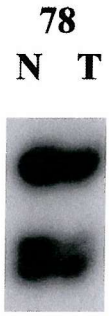
B. The band shift can be seen in both normal (N) and tumour (T) DNA, indicating a germline polymorphism.

C. A silent transversion mutation in codon 1028 (TCT>TCC) is shown.
Ser>Ser

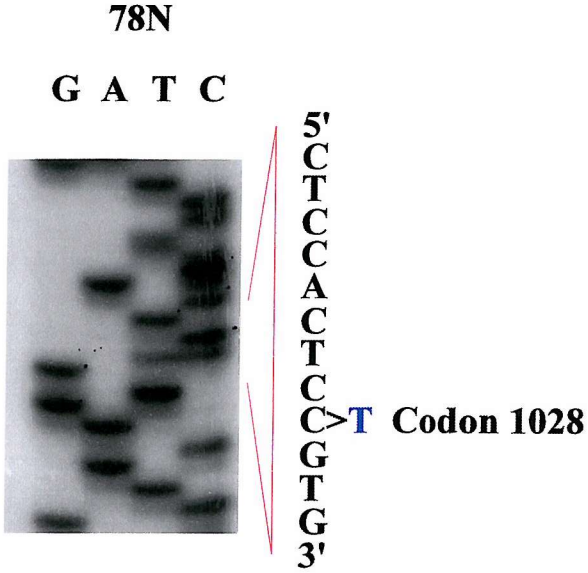
A


43
45
48
50.1
71
72.1
78
80.1
114
Band shift

B


78
N T

C


78N
G A T C
5'
C
T
C
C
A
C
T
C
C>T Codon 1028
G
T
G
3'

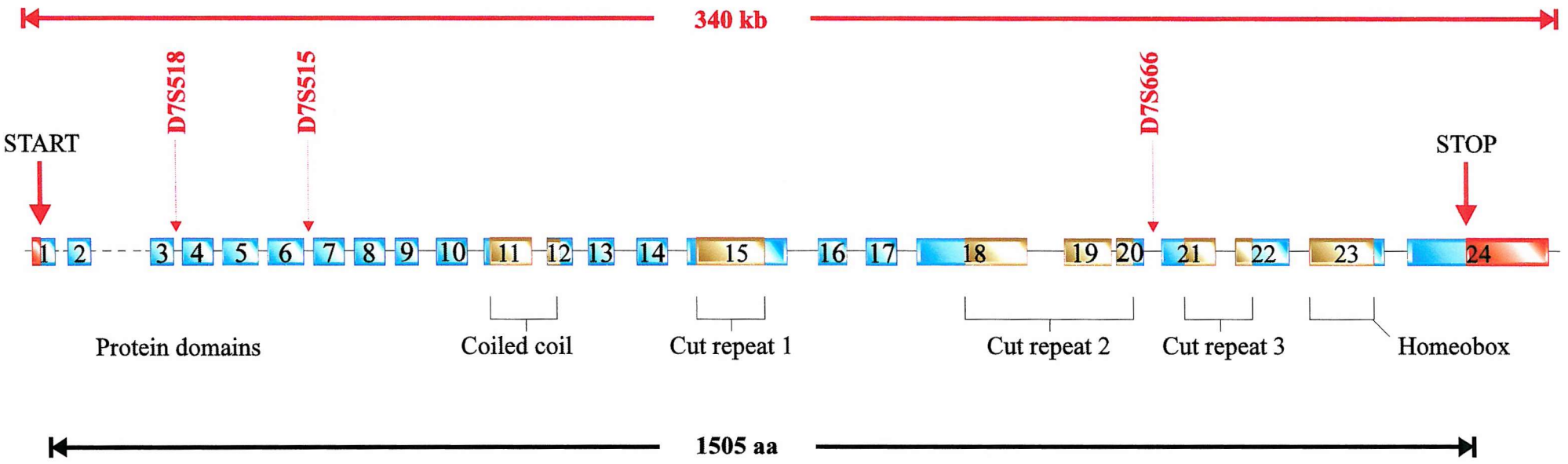
CUTL1 contains a homeobox domain, thought to be important in regulating DNA-binding activity, and three 'cut repeats' (figure 4.5) (Harada *et al.*, 1994). The role of these cut repeats is unknown but are prominent features of both CUTL1 and Cut, a homeoprotein found in Drosophila, suggesting a high degree of evolutionary conservation and therefore important functionality (Neufeld *et al.*, 1992). Other families of homeoproteins exhibit a second DNA-binding region in addition to the homeobox and it is possible that cut repeats fulfil this role (Harada *et al.*, 1994). Alternatively, they may act as interfaces for protein-protein interaction.

Mutations that lead to the loss of function of Cut in Drosophila often cause a change in cell fate decisions e.g. the conversion of one type of peripheral nervous system organ into another. By analogy, CUTL1 may act in a similar fashion and act to determine cell fate in several tissues. The proposed action of CUTL1 along with its expression in a broad range of mammary cell lines, implies that it may prove to be a general repressor of developmentally regulated genes.

Zeng et al. (Zeng *et al.*, 1997) carried out *CUTL1* expression studies on uterine leiomyomas and demonstrated that not only was *CUTL1* expressed in the uterus but at much reduced levels in the tumours, as would be expected if it was acting as a TSG. Homeobox genes act as controllers on cell fate and therefore have the potential to behave as tumour suppressors. Indeed, CUTL1 has been shown to bind to the promoter of the *c-Myc* proto-oncogene repressing its expression (Dufort & Nepveu, 1994). However, the decrease in *CUTL1* expression in leiomyomas did not lead to a proportional increase in *c-Myc* expression suggesting that additional factors are involved in the regulation of the proto-oncogene, such as hormone levels. Further evidence for a possible role for CUTL1 in tumour promotion comes from a study (Webster *et al.*, 1998) in which murine *CUTL1* protein is shown to bind to and form complexes with the large T antigen (LT) of the SV40 (Simian virus 40) polyomavirus (PyV). PyV LT is one of the principle viral oncoproteins involved in cellular transformation and has also been shown to complex with the products of both Rb and P53 tumour suppressor genes. In addition, transgenic mice which express PyV LT in their mammary epithelium, readily develop mammary tumours and uterine leiomyomas. Although the effect of PyV LT on murine or human CUTL1 is unknown, by analogy to the probable action of the oncoprotein on the Rb and p53 growth regulatory proteins, and the observations described above, it is possible that CUTL1 may play a part in cellular proliferation.

Figure 4.5. Diagrammatic representation of the intron/exon boundaries and domains of the *CUTL1* gene.

Transcribed regions are shown in blue and gold. Gold represents known translated domains. *CUTL1* spans at least 340 kb of genomic sequence and translates to a 1505 amino acid protein.
Microsatellite markers that have been mapped within the *CUTL1* locus are indicated in red.


340 kb
D7S518
D7S515
D7S666
START
STOP
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
Protein domains
Coiled coil
Cut repeat 1
Cut repeat 2
Cut repeat 3
Homeobox
1505 aa

Above and beyond all of the functional data, the *CUTL1* gene has been mapped to the distal boundary of 7q22 (Scherer *et al.*, 1993), a site of frequent LOH in many tumour types and very specific cytogenetic aberrations in uterine leiomyomas. Although both functional and genetic evidence has been produced to support the potential involvement of CUTL1 and 7q22, in the development of tumours, particularly leiomyomas, there have, to date, been no studies that have specifically searched for somatic alterations within the gene. The present study is the first to attempt to identify such variations. No somatic alterations of *CUTL1* were detected during this study, despite analysing over 90% of the coding region in a relatively large panel of tumours displaying LOH at 7q22. Even allowing for the limitations of SSCP-HD analysis, it would seem unlikely that alterations of CUTL1 are common in ovarian cancers.

The *CUTL1* locus also contains an alternative splice variant called *CASP* (cut alternatively spliced product) (Glockner *et al.*, 1998). *CASP* comprises of 22 exons but lacks the cut-like repeats and homeobox domains found in *CUTL1* (Lievens *et al.*, 1997). The absence of these functional domains may well remove the ability of CASP to bind DNA and any of the transcription repressing properties seen in CUTL1. The splice junction, at which the *CUTL1* and *CASP* sequences diverge, lies between exons14 and 15, and just before the region encoding the first cut repeat. A mutation within this splice junction could cause preferential transcription of one of these two variants. In the case of *CASP* being the favoured transcript, DNA-binding activity and therefore the ability of CUTL1 to suppress gene expression would be removed, with potential tumourigenic consequences. This could also explain the decrease in expression of *CUTL1* in uterine leiomyomas (Zeng *et al.*, 1997). Although several germline variants were detected in *CUTL1* in my study, none were found in the exon 14 and 15 splice junctions, suggesting that this potential mechanism of down-regulating *CUTL1* does not occur in ovarian tumours.
Of course, the results presented here do not preclude the hypothesised TSG role for *CUTL1* in other tumour types. In addition, it is conceivable that an alternative pathway, such as epigenetics, down regulation, or simply haplo insufficiency, may be the principle mechanism of *CUTL1* inactivation in ovarian and other cancers.

In a very recent article, published following the completion of my laboratory work, CUTL1 has been shown to span a region of at least 340 kb (Zeng *et al.*, 2000). This group has also described the identification of a further 9 exons in the *CUTL1* gene, in addition to the 24 already known, and five alternatively spliced mRNAs. The intron/exon boundaries for exons

2-24 were identical to those predicted by previously published sequence data, and therefore the structure used in my study is correct. It is possible that these additional exons may contain mutations, however it was also shown that the most abundant transcription product was the full length, 24 exon, isoform analysed during the present study and therefore it is unlikely that any functionally significant alterations were missed. All possible mRNA isoforms were shown to be expressed in most adult human tissues, including ovary, without significant variation in relative abundance between tissues.

4.4 SUMMARY

Although *CUTL1* does not appear to be the TSG, at 7q22, involved in human ovarian cancer, it is feasible, considering the large genetic distance covered by this gene, >340 kb, that another reading frame is contained within the *CUTL1* locus (Webster *et al.*, 1998). Indeed, analysis of the intronic sequences has identified the presence of several open reading frames (Glockner *et al.*, 1998). Further work in isolating alternative transcription units is needed to determine the possibility of such a scenario. Although the newly characterised *CUTL1* exons do not appear to be the major transcripts, they are expressed in most human tissues and therefore, for completeness, should be analysed for possible mutations.

CHAPTER FIVE

MUTATION ANALYSIS OF *PAI-1* IN HUMAN EPITHELIAL OVARIAN TUMOURS

5.1 INTRODUCTION

The plasminogen activator inhibitor-1 gene (*PAI-1*), shown to reside in 7q22, has previously been associated with the LOH observed in this region (Kerr *et al.*, 1996). Its role in regulating proteolytic processes would suggest that the down regulation or absence of this inhibitory protein would lead to increased proteolysis and therefore tumour invasion. Further, this could suggest a potential tumour suppressing role for *PAI-1*.

The growth of ovarian tumours is thought to involve the initial formation of a provisional fibrin/fibronectin matrix surrounding the tumour which is then replaced by mature stroma. Ascites, collected from patients with advanced ovarian carcinoma, are shown to contain the degradation products of fibrin and fibronectin from the proteolysis (Stack *et al.*, 1998) of the provisional stroma, suggesting that the plasminogen activator (PA)/plasmin pathway may well play a role in ovarian cancer pathology.

It has been shown by several studies that both urinary-type plasminogen activator (uPA) and PAI-1 levels are elevated in ovarian cancer patients (Schmalfeldt *et al.*, 1995; Stack *et al.*, 1998). In addition, immunohistochemistry has shown that both proteins are expressed to a greater extent in metastatic rather than primary tumours (Schmalfeldt *et al.*, 1995), further suggesting that this system of proteases and their inhibitors, plays a role in metastasis. However, these findings are not consistent with the suggestion that *PAI-1* could function as a classical TSG.

Despite the fact that some aspects of PAI-1 biology such as elevated rather than reduced levels in OC are not consistent with a TSG function the observation of LOH around 7q22, including the *PAI-1* tetranucleotide microsatellite marker, prompted us to investigate this gene for somatic genetic alterations.

5.2 RESULTS AND DISCUSSION

Ninety-four tumour samples were included in this study, comprising 74 malignant, 15 borderline and 5 benign tumours. Of these 94 tumours, 44 were of serous histology, 22 mucinous, 18 endometrioid and 10 mixed. Sixty of the tumours studied exhibited LOH of chromosome 7q and of these 30 showed LOH that included 7q22 (table 3.1). 7q22 LOH was observed in all stages, grades and histologies.

The PAI-1 gene comprises nine exons (figure 5.1A). Intronic primers for each of the seven coding exons (table 2.4) were designed according to the published sequence available on public genome sequence databases. All seven coding exons of the coding region of *PAI-1* were amplified using PCR (section 2.5.2) and examined using SSCP-HD analysis (section 2.8.4). Figure 5.1A shows the intron/exon boundaries of the *PAI-1* gene. No aberrant band shifts were identified in any of the 94 tumour samples analysed, implying that no mutations were present in the exons tested (table 4.1).

5.2.1 Plasminogen activator inhibitor 1

The 50 kDa PAI-1 gene comprised of 9 exons (Falk *et al.*, 1995) (figure 5.1A), distributed over 12.3 kb of DNA at 7q22. PAI-1, a glycoprotein, composed of 376 amino acids (figure 5.1B), (Bjorquist *et al.*, 1994) is a member of the serine proteinase inhibitor superfamily (Serpins). This is a group of over 40 proteins with approximately 35% sequence homology, that includes $\alpha_{1,2}$-antitrypsin and anti-thrombin III. PAI-1 is synthesised by many cell types including, hepatocytes, muscle cells and endothelial cells (Aertgeerts *et al.*, 1994).
There are 3 forms of PAI-1; a reactive inhibitory form, which is heat labile and forms a stable covalent bond with Urinary-type Plasminogen Activator (u-PA, or Urokinase); a reactive non-inhibitory substrate form, which is cleaved by serine proteases (such as u-PA) without the formation of a stable complex, allowing the resumption of proteinase activity; and a latent form, which is a heat stable molecule that is spontaneously generated from the reactive inhibitory PAI-1 following conformational change. This labile form is not cleaved by plasminogen activators (PAs), but it can be returned to its former reactive self by protein denaturing agents such as SDS and urea (Aertgeerts *et al.*, 1995).

PAI-1 is the primary plasminogen activator inhibitor in plasma (the other being PAI-2), where it is found in platelets, but it is also found in extracellular matrix bound to vitronectin

Figure 5.1. The structure of Plasminogen Activator Inhibitor-1.

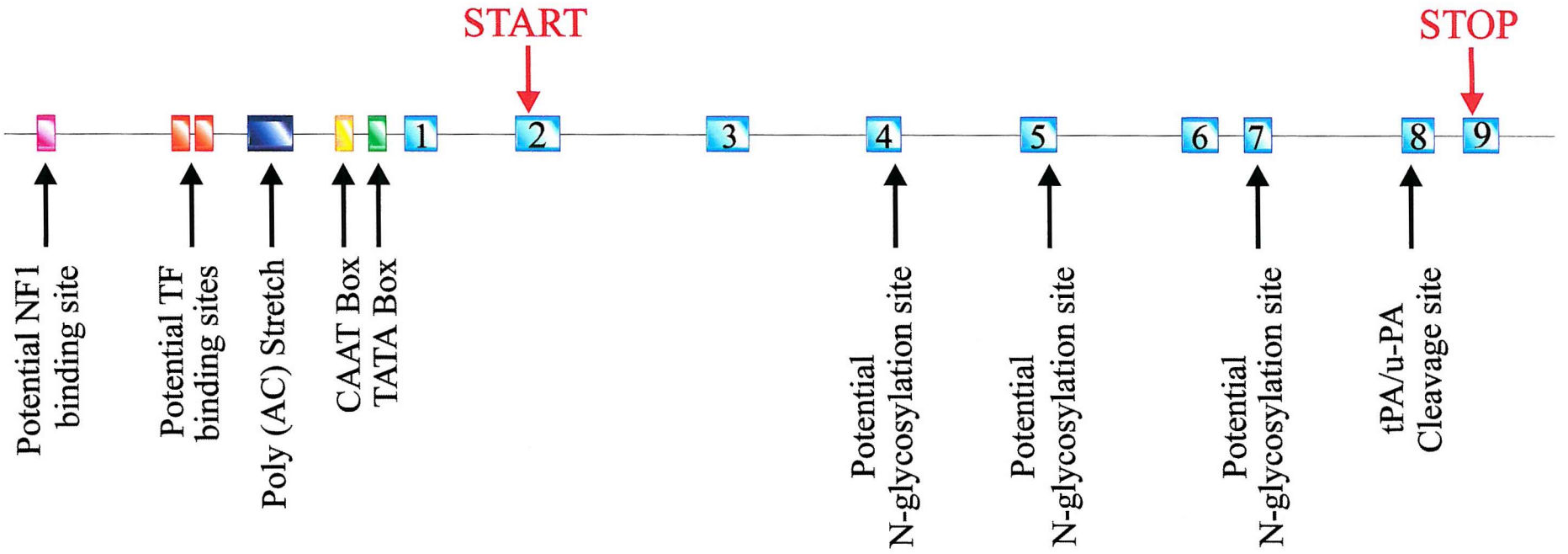
A. Intron/exon boundaries and reactive sites of the *PAI-1* gene.

B. PAI-1 amino acid sequence.

The cleavage site for both tPA and uPA is highlighted in pink.

Proposed N-glycosylation sites are shown in red.

A



B

MQMSPALTCLVLGLALVFGEGSAVHHPPSYVAHLASDFGVRVFQQVAQASK
DRNVVFSPYGVASVLAMLQLTTGGETQQQIQAAMGFKIDDKGMAPALRHLY
KELMGPWNKDEISTTDAIFVQRDLKLVQGFMPHFFRLFRSTVKQVDFSEVERA
RFIINDWVKTHTKGMISNLLGKGAVDQLTRLVLVNALYFNGQWKTPFPDSST
HRRLFHKSDGSTVSVPMMAQTNKFNYTEFTTPDGHYYDILELPYHGDTLSMFI
AAPYEKEVPLSALTNILSAQLISHWKGNMTRLPRLLVLPKFSLETEVDLRKPLE
NLGMTDMFRQFQADFTSLSDQEPLHVAQALQKVKIEVNESGTVASSSTAVIVS
ARMAPEEIIMDRPFLFVVRHNPTGTVLFMGQVMEP

(Manchanda & Schwartz, 1995). This complex is believed to protect the mature PAI-1 from oxidation and degradation (Dawson *et al.*, 1993).

5.2.2 PAI-1 and its role in the regulation of proteolysis.

PAI-1 plays an important role in the regulation of the fibrinolytic pathway (figure 5.2), itself a central player in vascular thrombolysis, neovascularisation and tumour invasion. Plasminogen activator inhibitors have a high affinity for both urinary-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), therefore effectively regulating their enzymatic activity. These two serine proteases cleave plasminogen to produce another widely acting protease, plasmin, which has been shown to digest both fibrin clots and extracellular matrix proteins and to activate other members of the proteolytic cascade such as collagenases (Sourla *et al.*, 1996). This activation of plasminogen to plasmin is made all the more efficient due to the co-localisation of the uPA and plasminogen receptors on the cell surface. Plasmin also acts in a positive feedback loop, cleaving the low activity single-chain uPA (scuPA) into the double-chain high activity uPA (figure 5.3). In order for this cleavage to take place, scuPA must first bind to the glycosyl phosphatidyl-inositol (GPI)-anchored uPA receptor (uPAR) on the cell surface, via the amino terminal fragment (ATF) of uPA (Stack *et al.*, 1998). It has been suggested that the GPI anchor prevents degradation of the uPA receptor by proteinases and phospholipases.

The reactive inhibitory form of PAI-1 can bind both scuPA and double-chain uPA bound to the uPAR. The binding of PAI-1 to uPA involves an initial reversible interaction followed by the formation of a covalent bond between the hydroxyl group of the active-site serine of uPA, and a carboxyl group of the reactive-site loop of PAI-1. While the uPA/PAI-1 interaction forms a 'locked' inactive complex, only a fraction of the corresponding scuPA/PAI-1 complexes remain stable (Manchanda & Schwartz, 1995), freeing PAI-1 to scavenge the more active uPA. The stable uPAR/uPA/PAI-1 complex is then internalised by the cell (Schmalfeldt *et al.*, 1995), removing the serine protease activity and regulating plasmin production.

5.2.3 PAI-1 and metastasis

In order to be able to migrate and infiltrate, cells require a surface of extracellular matrix (ECM) proteins. Contacts are formed between the cells and these ECM proteins, such as

Figure 5.2. Plasminogen activation and the fibrinolytic pathway.

Activation can occur via three pathways:

a) Intrinsic.

b) Extrinsic

c) Exogenous

PAI-1 is involved in regulating the extrinsic pathway of plasminogen activation.

Once plasmin is produced it can act to degrade fibrinogen and fibrin.

XI; XIa; XII; XIIa = Contact system factors
Hka = High molecular weight kininogen
tPA = Tissue-type plasminogen activator
scuPA = Single chain plasminogen activator
SK-PGN = Streptokinase-plasminogen complex

Adapted from Ouimet & Loscalzo, 1994.

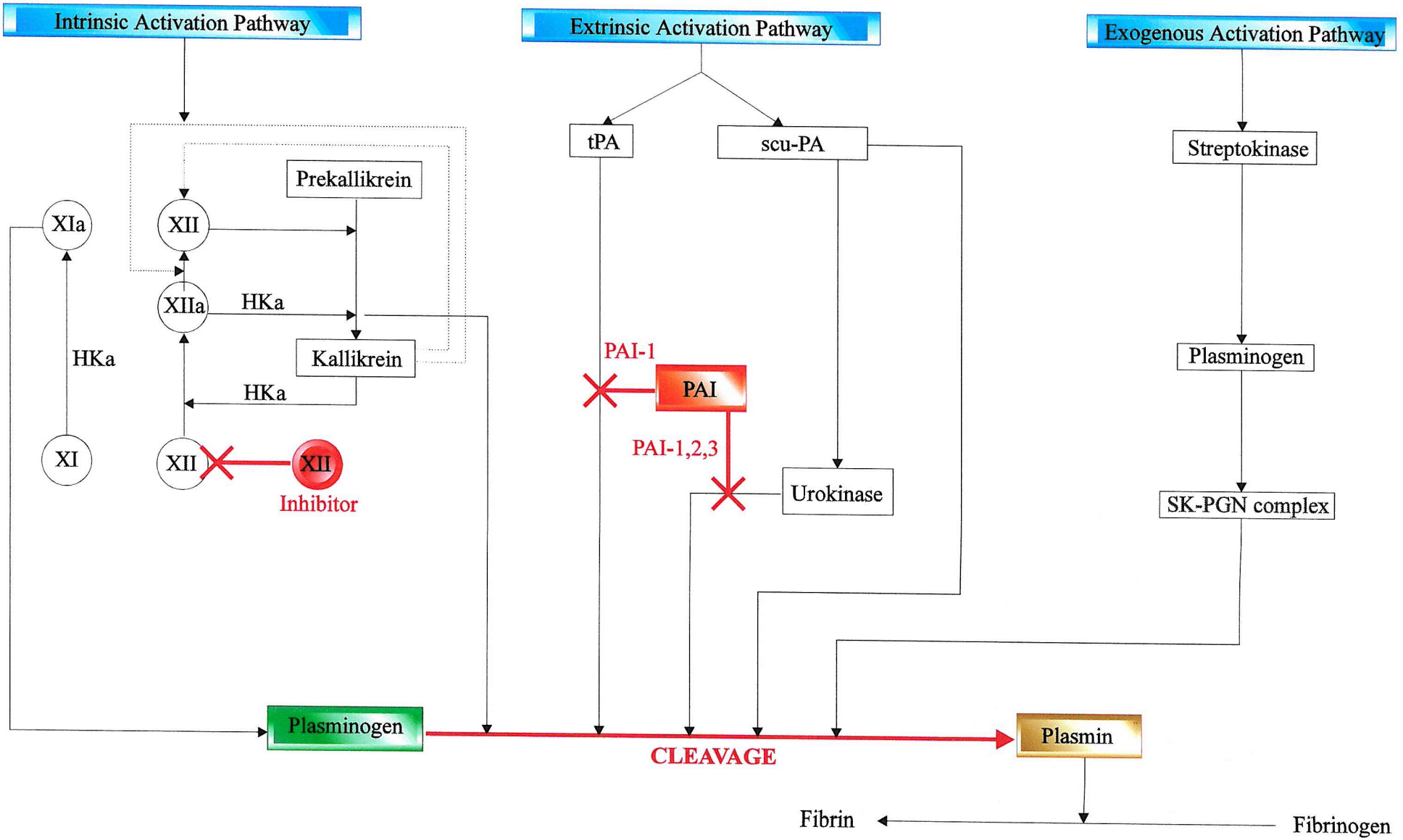
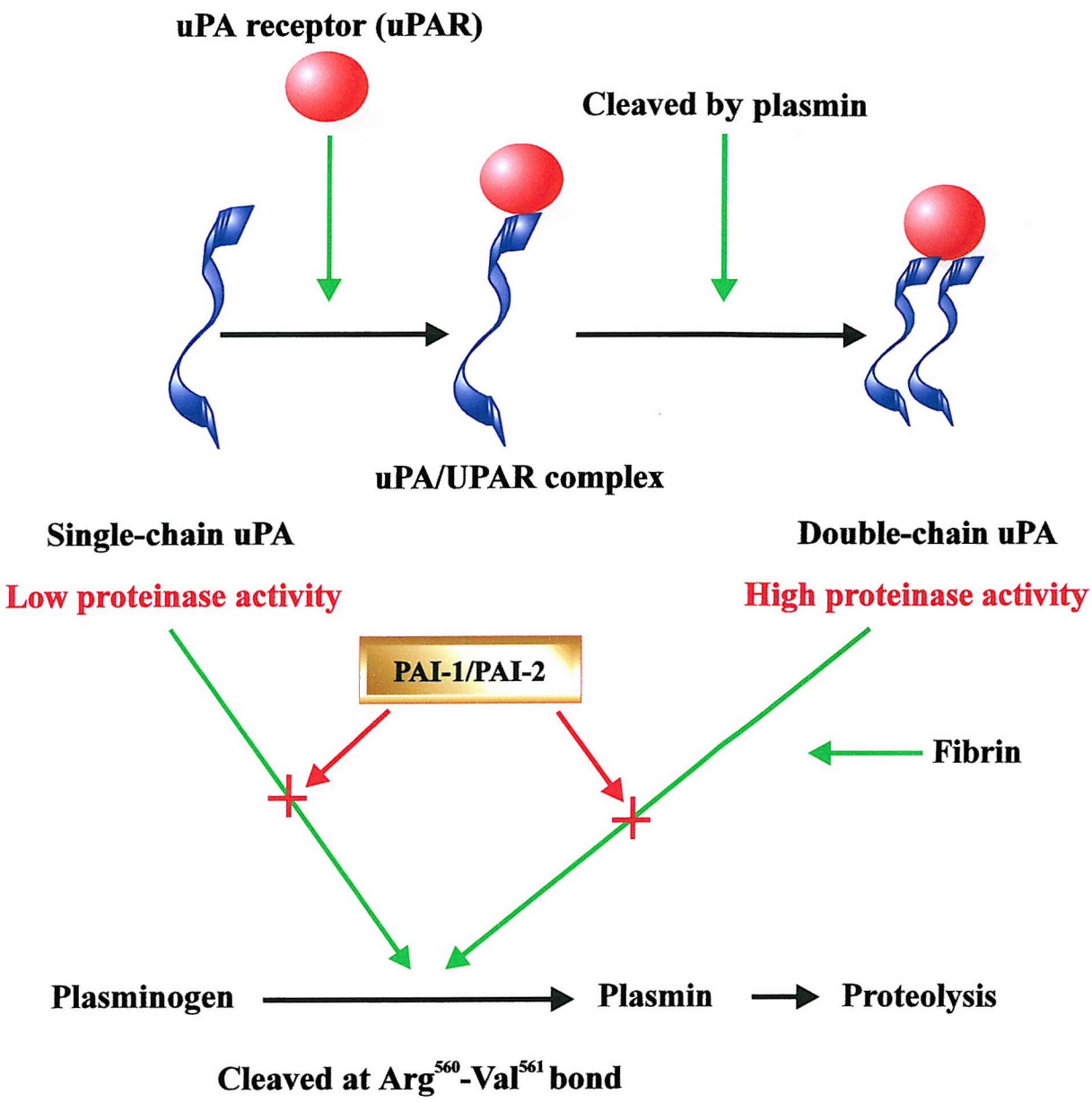

Intrinsic Activation Pathway
Extrinsic Activation Pathway
Exogenous Activation Pathway
Prekallikrein
XIa
XII
XIIa
HKa
HKa
Kallikrein
HKa
XI
XII
XII
Inhibitor
tPA
scu-PA
PAI-1
PAI
PAI-1,2,3
Urokinase
Streptokinase
Plasminogen
SK-PGN complex
Plasminogen
CLEAVAGE
Plasmin
Fibrin
Fibrinogen

Figure 5.3. The role of Plasminogen Activator Inhibitor-1 (PAI-1) in the plasminogen activator (PA)/plasmin pathway.

uPA = urinary-type plasminogen activator.

uPAR = urinary-type plasminogen activator receptor.



vitronectin and fibronectin, via an association with cell surface receptors known as integrins. Highly motile metastatic tumour cells are known to express integrins including $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Stefansson *et al.*, 1998). Conversely, many transformed cells are unable to express integrins, yet can still become adhesive, implying that an integrin independent pathway is at work. The introduction of TGF-β1 and vitamin D, known to stimulate uPA, uPAR and PAI-1 expression, restores the ability of these cells to adhere to vitronectin (Deng *et al.*, 1996).

The plasminogen activator (uPA), its receptor (uPAR) and paradoxically, its inhibitor (PAI-1) have all been shown to be expressed at high levels in tumour cells compared to normal tissue. uPA has been shown to promote vitronectin-cell adhesion. This is achieved when uPA binds to uPAR, changing the receptors conformation and increasing its affinity for the somatomedin B domain (SMB) of vitronectin. Studies indicate that PAI-1 accumulates at points of adhesion (Deng *et al.*, 1996) and that its active conformation binds to this SMB domain with high affinity (Carrell, 1999), producing a stable complex. In fact, uPAR and PAI-1 appear to compete for this binding site. However when PAI-1 binds to vitronectin it also blocks an arginine-glycine-aspartic acid site (RGD), which is required for vitronectin-integrin interaction (Stefansson *et al.*, 1998). The inability of the cell surface integrin to bind to the ECM protein prevents cell adhesion. PAI-1 is still capable of binding uPA whilst in complex with vitronectin. Following this, the uPA/PAI-1 unit dissociates from vitronectin, freeing both the RGD and SMB domains and cell adhesion takes place via integrins and uPAR.

The production of PAI-1 can be rapidly stimulated by various growth factors and hormones. In addition, this protein has a half-life of only 10 minutes, suggesting that it may act as a molecular switch, regulating the adhesion and movement of cells. Many recent reports have indicated that increased levels of PAI-1 in many tumour types including that of breast (Foekens *et al.*, 2000), colon (Herszenyl *et al.*, 1999), endometrium (Kohler *et al.*, 1997), stomach (Plebani *et al.*, 1997), head and neck (Yasuda *et al.*, 1997), lung (Robert *et al.*, 1999) and ovary (Ho *et al.*, 1999), does indeed correlate with poor prognosis. Indeed, in a study concerned with gastric cancer, increased PAI-1 was correlated with other indicators of poor prognosis, such as lymph node and liver metastasis and undifferentiated tumours. Studies of ovarian tumours have also shown elevated PAI-1 levels to be independent poor prognostic factors in advanced epithelial ovarian cancers, and have also suggested that, in addition, PAI-1 may play a role in tumour protection (Chambers *et al.*, 1998).

5.2.4 PAI-1 and cancer

The malignant potential of tumours is related to their ability to degrade extracellular matrix and basement membrane components. In order to spread further into adjacent tissue, lay down new blood vessels and eventually metastasise, cancer cells depend on several groups of proteolytic enzymes: matrix metalloproteinases (MMPs); cysteine-aspartyl proteinases; and serine proteases (Heiss *et al.*, 1995). These lytic enzymes are shown to be expressed at higher levels in malignant tumours compared to normal or benign tissue (Schmalfeldt *et al.*, 1995; Sourla *et al.*, 1996). In addition, the observation that transformed cells often show a significant increase in the production of uPA, and an altered expression of PAI-1 (Kunz *et al.*, 1995), would seem to suggest that the balance between these two molecules is critical for the invasive phenotype of cancer cells. Should this balance tip in favour of uPA production, either due to increased expression of uPA or suppressed expression of PAI-1, then matrix degradation may be increased and the promotion of a malignant phenotype may result. In this scenario it could be postulated that PAI-1 could function as a tumour suppressor. However, reduced levels of PAI-1 are not observed in ovarian cancer, indeed they are significantly elevated (Schmalfeldt *et al.*, 1995; Stack *et al.*, 1998). This could be explained, in terms of the putative tumour suppressing capabilities of PAI-1, if the increase lead to an accumulation of a mutated, non-functional PAI-1, in a similar mechanism to the inactivation of p53. Yet, this would seem unlikely given the absence of somatic alterations in ovarian tumours in this study. Indeed, these results and previous studies would argue against *PAI-1* behaving as a classical TSG.

Although other studies have not looked for mutations within the *PAI-1* gene in many ovarian tumour samples, Turkmen and colleagues (1997) did analyse PAI-1 for mutations in ovarian cancer cell lines and then in 22 ovarian cancer carcinomas. Two polymorphisms were identified, but similar frequencies of the variants observed in tumour tissue were also seen in the leucocyte DNA of healthy donors, implying that these polymorphisms are unlikely to be linked to ovarian cancer.

Studies using PAI-1 deficient mice, also support a role for PAI-1 in the promotion of metastasis (Bajou *et al.*, 1998). Wild-type and PAI-1 -/- mice were implanted with malignant keratinocytes. Local invasion and tumour vascularisation were observed in the wild-type mice but not in the PAI-1 null mice. The introduction of an adenoviral vector, expressing PAI-1, restored these invasive properties. All of the evidence presented here would suggest that PAI-1 is required for cancer invasion and metastasis.

5.3 SUMMARY

Based on the data presented here and in previous studies, it seems highly unlikely that *PAI-1* is the TSG target of the LOH seen at 7q22 and instead appears to promote tumour spread and metastasis.

CHAPTER SIX

MUTATION ANALYSIS OF *c-MET* IN BENIGN, BORDERLINE AND MALIGNANT OVARIAN TUMOURS

6.1 INTRODUCTION

The region 7q31 is frequently deleted in many tumour types (figure 3.1) including that of the ovary, breast, kidney, stomach, colon and prostate (Edelson *et al.*, 1997; Watson *et al.*, 1998; Lin *et al.*, 1996; Shridhar *et al.*, 1997; Nishizuka *et al.*, 1997; Zenklusen *et al.*, 1995; Latil *et al.*, 1995), suggesting that it may harbour a tumour suppressor gene. A few genes have been identified within this location, these include the oncogene c-*MET* and the putative tumour suppressor caveolin1 (Engelman *et al.*, 1998; Genemap'98:http://www.ncbi.nlm.nih.gov/genemap98).

The caveolin gene has been implicated as a potential tumour suppressor since the expression of caveolin1 is reduced in cells transformed by oncoproteins including H-ras, v-abl and bcr-abl (Engelman *et al.*, 1997). This reduction is correlated with the capability of the transformed cells to proliferate in soft agar. In addition, caveolin1 exhibits the ability to suppress the anchorage-independent growth and induce apoptosis in human breast carcinoma cell lines and transformed fibroblasts, further suggesting a putative tumour suppressor role. However, a more recent study (Hurlstone *et al.*, 1999) failed to identify mutations or aberrant methylation of caveolin1 in human cancers (both primary tumours and cell lines) implying that caveolin1 is unlikely to be a TSG and therefore not the target of the 7q31 LOH.

Although not a tumour suppressor, the presence of the c-*MET* oncogene within this region is of interest. In order to determine whether or not c-met was involved in the LOH observed at 7q31.1, mutation analysis was carried out on the three exons that commonly exhibit mutations in other human tumours (Schmidt *et al.*, 1997; Schmidt *et al.*, 1998).

6.2 RESULTS

6.2.1 Clinical specimens

Forty-six malignant, 17 benign and 7 borderline fresh epithelial ovarian tumours that had shown some degree of chromosomal deletion around 7q31 and 5 normal tissue samples were analysed for mutations within exons 17, 18 and 19 of the *c-Met* proto-oncogene.

6.2.2 SSCP-HD analysis of *c-MET* in ovarian neoplasia

The three most commonly mutated exons of *c-MET* were examined by SSCP-HD analysis (section 2.8.4) using intronic primers (table 2.7). Exons 17, 18 and 19 represent approximately 10% of the cDNA, and encode the majority of the tyrosine kinase domain of the Met protein (figure 6.1). Of the 70 tumours studied, 28 had no 7q LOH, 42 showed LOH of at least one marker on 7q and 28 exhibited LOH at 7q31. A representative autoradiograph for the SSCP-HD analysis of *MET* is shown in figure 6.2. No band shifts were detected in the 70 tumours studied (table 4.1) suggesting that *MET* is not involved in ovarian carcinogenesis.

6.3 DISCUSSION

The *MET* proto-oncogene has previously been shown to be overexpressed in ovarian carcinomas. It has been mapped to a position within 7q31, a region frequently deleted in both ovarian and other tumour types, and several activating mutations of the tyrosine kinase region of the translated protein have been identified in papillary renal carcinomas.

6.3.1 Met tyrosine kinase

The *c-met* proto-oncogene, comprising 20 coding exons and one non-coding exon (Duh *et al.*, 1997), encodes the 190kDa protein Met tyrosine kinase (Met) (figure 6.1), a high-affinity receptor for Hepatocyte Growth Factor/Scatter Factor (HGF/SF). It is a member of the receptor tyrosine kinase family which also includes the RET proto-oncogene, PDGF and EGF-R. The wild-type *MET* produces a 1408 amino acid product (figure 6.3) which is cleaved post-transationally into a 45 kDa α chain (307 aa) and 145 kDa β chain (1101 aa) held together by a disulphide bond to form the mature transmembrane heterodimer (Lin *et al.*,

MKAPAVLAPGILVLLFTLVQRSNGECKEALAKSEMNVNMKYQLPNFTAETPI
QNVILHEHHIFLGATNYIYVLNEEDLQKVAEYKTGPVLEHPDCFPCQDCSSKA
NLSGGVWKDNINMALVVDTYYDDQLISCGSVNRGTCQRHVFPHNHTADIQS
EVHCIFSPQIEEPSQCPDCVVSALGAKVLSSVKDRFINFFVGNTINSSYFPDHPL
HSISVRRLKETKDGFMFLTDQSYIDVLPEFRDSYPIKYVHAFESNNFIYFLTVQ
RETLDAQTFHTRIIRFCSINSGLHSYMEMPLECILTEKRKKRSTKKEVFNILQAA
YVSKPGAQLARQIGASLNDDILFGVFAQSKPDSAEPMDRSAMCAFPIKYVNDF
FNKIVNKNNVRCLQHFYGPNHEHCFNRTLLRNSSGCEARRDEYRTEFTTALQ
RVDLFMGQFSEVLLTSISTFIKGDLTIANLGTSEGRFMQVVVSRSGPSTPHVNF
LLDSHPVSPEVIVEHTLNQNGYTLVITGKKITKIPLNGLGCRHFQSCSQCLSAPP
FVQCGWCHDKCVRSEECLSGTWTQQICLPAIYKVFPNSAPLEGGTRLTICGWD
FGFRRNNKFDLKKTRVLLGNESCTLTLSESTMNTLKCTVGPAMNKHFNMSIIIS
NGHGTTQYSTFSYVDPVITSISPKYGPMAGGTLLTLTGNYLNSGNSRHISIGGK
TCTLKSVSNSILECYTPAQTISTEFAVKLKIDLANRETSIFSYREDPIVYEIHPTK
SFISTWWKEPLNIVSFLFCFASGGSTITGVGKNLNSVSVPRMVINVHEAGRNFT
VACQHRSNSEIICCTTPSLQQLNLQLPLKTKAFFMLDGILSKYFDLIYVHNPVF
KPFEKPVMISMGNENVLEIKGNDIDPEAVKGEVLKVGNKSCENIHLHSEAVLC
TVPNDLLKLNSELNIEWKQAISSTVLGKVIVQPDQNFTGLIAGVVSISTALLLLL
GFFLWLKKRKQIKDLGSELVRYDARVHTPHLDRLVSARSVSPTTEMVSNESV
DYRATFPEDQFPNSSQNGSCRQVQYPLTDMSPILTSGDSDISSPLLQNTVHIDL
SALNPELVQAVQHVVIGPSSLIVHFNEVIGRGHFGCVYHGTLLDNDGKKIHCA
VKSLNRITDIGEVSQFLTEGIIMKDFSHPNVLSLLGICLRSEGSPLVVLPYMKHG
DLRNFIRNETHNPTVKDLIGFGLQVAKAMKYLASKKFVHRDLAARNCMLDEK
FTVKVADFGLARDMYDKEYYSVHNKTGAKLPVKWMALESLQTQKFTTKSDV
WSFGVVLWELMTRGAPPYPDVNTFDITVYLLQGRRLLQPEYCPDPLYEVMLK
CWHPKAEMRPSFSELVSRISAIFSTFIGEHYVHVNATYVNVKCVAPYPSLLSSE
DNADDEVDTRPASFWETS

Figure 6.1 : Amino acid sequence of the *MET* proto-oncogene.

Exon 17 is shown in green.

Exon 18 is shown in pink.

Exon 19 is shown in blue.

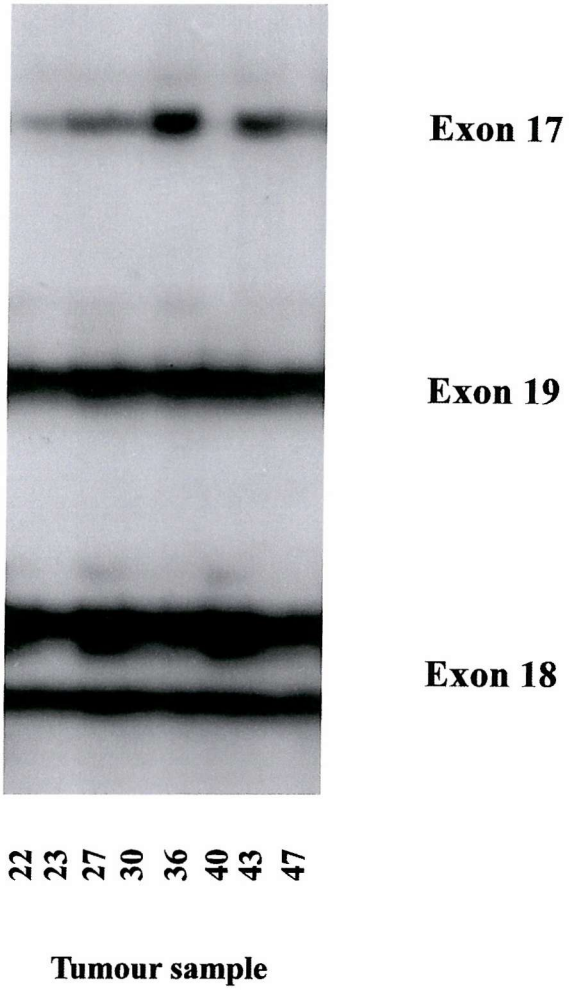


Figure 6.2. Representative autoradiograph of c-MET SSCP-HD analysis. Multiplex of exons 17, 18 and 19 is shown.

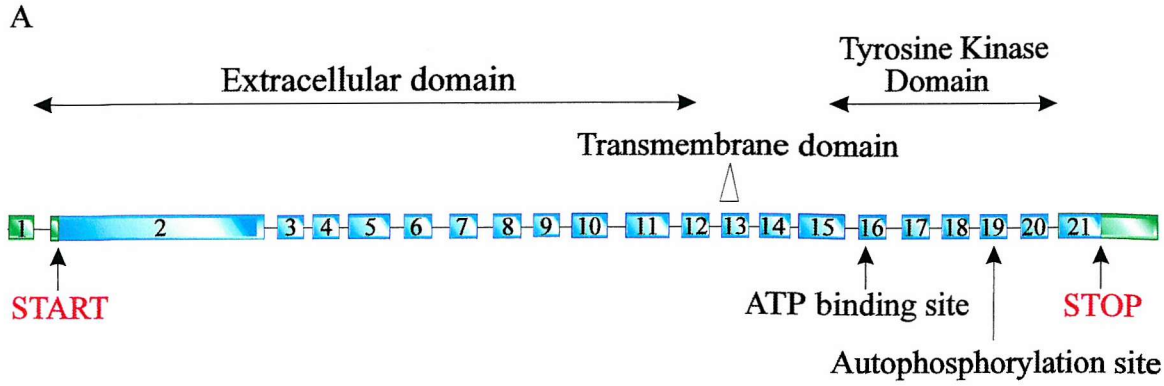
Figure 6.3. Schematic representation of the *MET* proto-oncogene.

A. Exon/Intron boundaries and domains of the MET proto-oncogene.

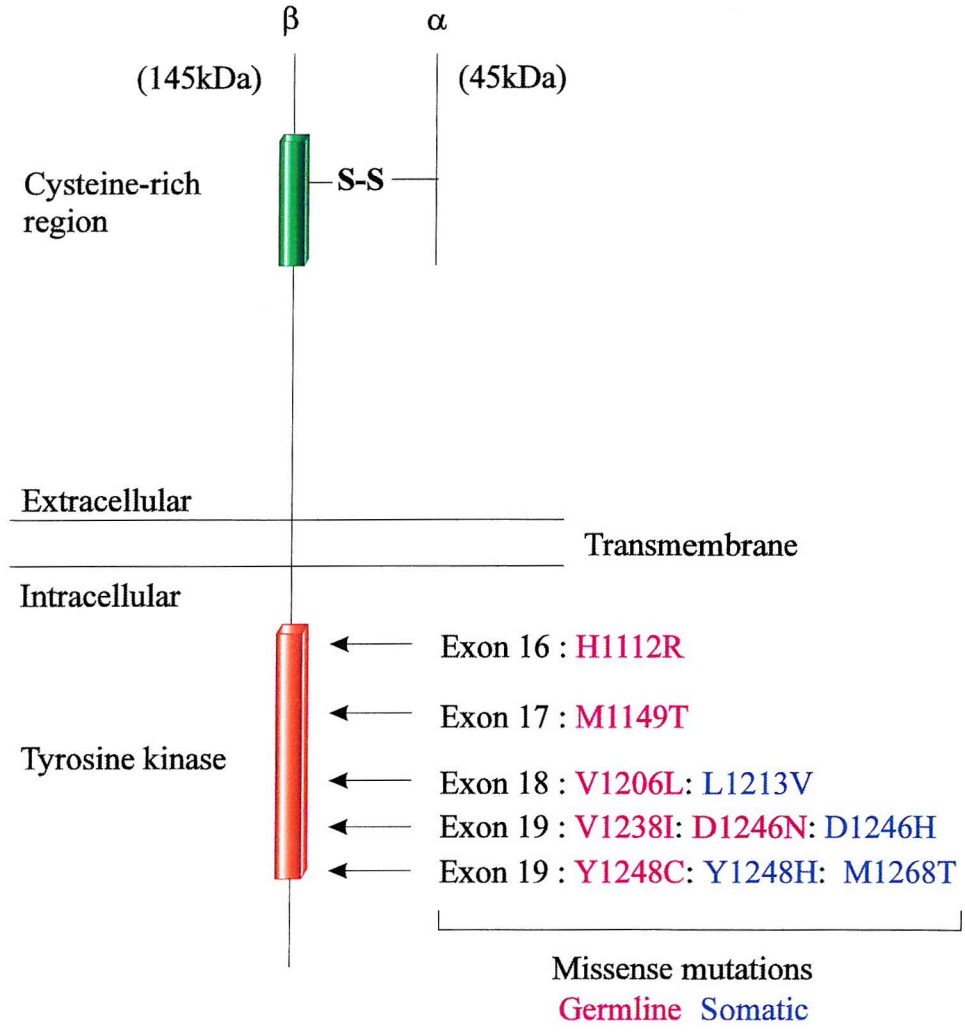
B. Arrows indicate the positions of previously identified missense mutations.

Adapted from Schmidt *et al.*, 1998 and Duh *et al.*, 1997.

A


Extracellular domain
Tyrosine Kinase Domain
Transmembrane domain
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
START
ATP binding site
STOP
Autophosphorylation site

B


β
α
(145kDa)
(45kDa)
Cysteine-rich region
S-S
Extracellular
Transmembrane
Intracellular
Tyrosine kinase
Exon 16 : H1112R
Exon 17 : M1149T
Exon 18 : V1206L: L1213V
Exon 19 : V1238I: D1246N: D1246H
Exon 19 : Y1248C: Y1248H: M1268T
Missense mutations
Germline Somatic

1998). Met was originally isolated as an oncogenic variant produced by a genetic rearrangement of *TPR* (1q25) and *MET* (7q31) in a chemically transformed tumour cell line. This *TPR* (Translocated Promoter Region [to activated *MET* oncogene])-*MET* gene translated to an altered Met protein that possessed both kinase activity and transforming capabilities (Park *et al.*, 1987). Indeed, the expression of tpr-met in transgenic mice has been shown to lead to the development of mammary tumours (Liang *et al.*, 1996). However, such genetic rearrangements are rare in primary human tumours and it has recently been shown that missense mutations of the *MET* gene are associated with papillary renal carcinomas (Schmidt *et al.*, 1997).

The *MET* proto-oncogene is distributed over approximately 130 kb of genomic DNA and produces two common mRNA transcripts, one of 8kb encoding the 190 kDa wildtype Met protein and the other, alternatively spliced 7kb mRNA, which does not appear to be translated *in vivo* although it is ubiquitously expressed (Lin *et al.*, 1998). This splice variant does not contain exon 2, which codes for the α subunit, and is expressed in both normal tissue and tumour cell lines. The retention of exon 2 in the 8kb mRNA which encodes the mature Met protein, suggests that it is selected for *in vivo* and that the highly expressed but non-translated 7kb transcript may act to regulate the amount of functional Met produced.

6.3.2 The Met/HGF complex

HGF, a 97 kDa peptide growth factor, has been shown to act as a potent mitogen and also to stimulate cell motility, invasion and morphological change (Corps *et al.*, 1997). The binding of the 97 kDa HGF (Clifford *et al.*, 1998) to the extracellular domain of the c-Met leads to phosphorylation of tyrosine residues within the intracellular kinase domain of the β subunit and the activation of Met. This tyrosine phosphorylation produces 'docking sites' for Src Homology 2 (SH2) domain-containing signal-transduction proteins such as the p85 subunit of PI 3-kinase and the *Grb*-2 adaptor protein (section 6.3.6) (Bardelli *et al.*, 1997).

Both HGF and Met are primarily expressed in epithelial and mesenchymal tissues (Jeffers *et al.*, 1997), and together are considered to be major paracrine mediators of mesenchymal-epithelial signalling i.e. in the ovarian follicle HGF is produced by the thecal cells and acts on granulosa cells which express Met. Co-expression of both wild-type Met and HGF within the same cell has also been shown to be oncogenic (Rong *et al.*, 1992), questioning a possible role for the HGF/c-Met system in the initiation and growth of these malignancies. In fact,

Ebert and co-workers (Ebert *et al.*, 1994) showed a 7-fold increase in Met mRNA and a 10-fold increase in HGF mRNA in pancreatic carcinomas compared to that of the normal pancreatic tissue, although Southern blot analysis did not reveal any alteration or amplification of either gene. Another point of interest is the observation that the *HGF* gene maps to 7q21, another region of chromosome 7 frequently deleted in many tumour types (chapter 3).

6.3.3 Mutations of c-*MET* and its oncogenic properties

Increased expression of the *MET* gene has been detected in papillary carcinomas of the kidney and thyroid (Di Renzo *et al.*, 1991; Ivan *et al.*, 1997; Schmidt *et al.*, 1999; Fischer *et al.*, 1998) and carcinomas of the breast (Bieche *et al.*, 1999), liver (Boix *et al.*, 1994), stomach (Nakajima *et al.*, 1999), colon, pancreas and ovary (Schmidt *et al.*, 1997; Corps *et al.*, 1997). It has also been suggested that this overexpression of *MET* is associated with poor prognosis in hepatocellular (Ueki *et al.*, 1997), gastric (Nakajima *et al.*, 1999) and breast carcinomas (Yamashita *et al.*, 1994).

c-Met has been shown to harbour activating missense mutations in both familial and sporadic papillary renal carcinomas (Schmidt *et al.*, 1997; Schmidt *et al.*, 1998; Duh *et al.*, 1997). These mutations appear to increase the expression of the Met protein, even in the absence of HGF, and perhaps more interesting, somatic mutations are more strongly activating than germ-line mutations (Jeffers *et al.*, 1997), possibly because highly-activating *c-met* mutations in the germ-line would be incompatible with life. Biochemical assays have also shown that the Met mutants exhibit higher levels of tyrosine phosphorylation due to increased sensitivity of the kinase to its substrate, HGF, rather than due to their increased expression over that of the wildtype (Jeffers *et al.*, 1997). Met mutants may also be less specific in terms of their ligand specificity. Mutations seen in the receptor tyrosine kinases *Ret* and *Kit,* that correspond to positions of known Met mutations, lead to a change of residue from one that is conserved in receptor tyrosine kinases to one that is observed in non-receptor tyrosine kinases. This may alter the conformation of the receptor so that it will recognise SH2 - domain-containing proteins which would normally interact with non-receptor tyrosine kinases.

The majority of somatic mutations observed in the *c-met* gene occur in exons 17, 18 and 19, which represent a large proportion of the tyrosine kinase domain of the encoded protein

(figure 6.3). The mechanisms by which these mutations activate the Met receptor are not yet understood. However, it has been noted that the majority of these mutations occur in the COOH-terminal lobe of the tyrosine kinase domain (Jeffers *et al.*, 1997), a region with considerable structural homology with other receptor tyrosine kinases. This region is thought to act as an intermolecular substrate which in the absence of a ligand blocks the active site and inhibits enzymatic activity. It is possible that mutations within this region alter the structure of this substrate so that it can no longer block the active site, allowing the kinase to be stimulated inappropriately. Both *Ret* and *Kit* have shown activating mutations that correspond to amino acid changes at methionine and aspartic acid residues seen in *Met* within this COOH-terminal region. Implications of this observation and that of the structural homology between the receptor tyrosine kinase supergene family, are that some of the mutations observed in *Met* may well be activating when introduced into other members of this group of genes. In addition, the introduction of cells expressing the mutant Met protein into nude mice lead to tumour growth, with the more strongly activating mutations proving to be the most tumorigenic (Jeffers *et al.*, 1997).

6.3.4 No evidence of mutations within the tyrosine kinase domain of Met in many tumour types

The failure to find any mutations amongst the tumours analysed in this study would seem to suggest that the tyrosine kinase domain of Met is not altered in ovarian cancer. The inability to detect mutations is unlikely to be due to either technique or sample numbers. The combination of SSCP and heteroduplex analysis should allow the identification of at least 70% of mutations (Sheffield *et al.*, 1993), and since 70 tumours were analysed, a proportion of any mutations present should have been detected. A recent study in breast tumours (Bieche *et al.*, 1999) appears to provide further evidence to support our findings. Bieche and colleagues studied 6 primary breast tumours showing 7q31 LOH and 5 without LOH. cDNA was prepared from the tumours, PCR was then utilised to amplify exons 16, 17, 18 and 19 of the *MET* proto-oncogene. Sequencing was then employed in an attempt to detect any mutations present. No mutations were identified, suggesting that the tyrosine kinase domain of Met is not involved in sporadic breast cancer. In addition, a group that had previously detected mutations in papillary renal cell carcinomas, failed to find any mutations in a panel of 199 solid tumours of varying type (Schmidt *et al.*, 1999). It is possible that there are mutations present within this gene, in both ovarian and breast tumours, as suggested by the over-expression of Met in many tumour types, but they occur outside the kinase domain.

Another explanation for these observations could be the presence of epigenetic alterations, such as methylation of the promoter.

6.3.5 Duplication of *MET* - Gene Dosage

Proto-oncogenes are believed to exert their tumorigenic effect following their mutation and/or amplification. Amplification can be thought of as increasing the dosage of a particular gene within a cell. Trisomy of chromosome 7 has been identified in papillary renal cell carcinomas along with a mutation of *MET* (Zhuang *et al.*, 1998). It has also been shown that it is the mutant copy of MET that is duplicated in these tumours, giving a 'double dose' of the mutated gene. Amplification of MET has also been observed in gastric carcinomas, where it is associated with tumour invasion and poor prognosis (Nakajima *et al.*, 1999). The amplification of the mutated allele of a proto-oncogene results in hyper-activation of the gene. However, in two cases of hereditary renal carcinoma from the same family, the duplication of the same parental allele was seen in the absence of a germline mutation of *MET* (Fischer *et al.*, 1998). This would seem to suggest that there may an alternative genetic alteration occurring in these two family members. In addition, sporadic tumours, without apparent *MET* mutations, also showed duplication of the same alleles at various loci on chromosome 7. All of this evidence suggests that *MET* may be amplified and activated through a mechanism other than genetic mutation.

6.3.6 Induction of *MET* by Oncogenes

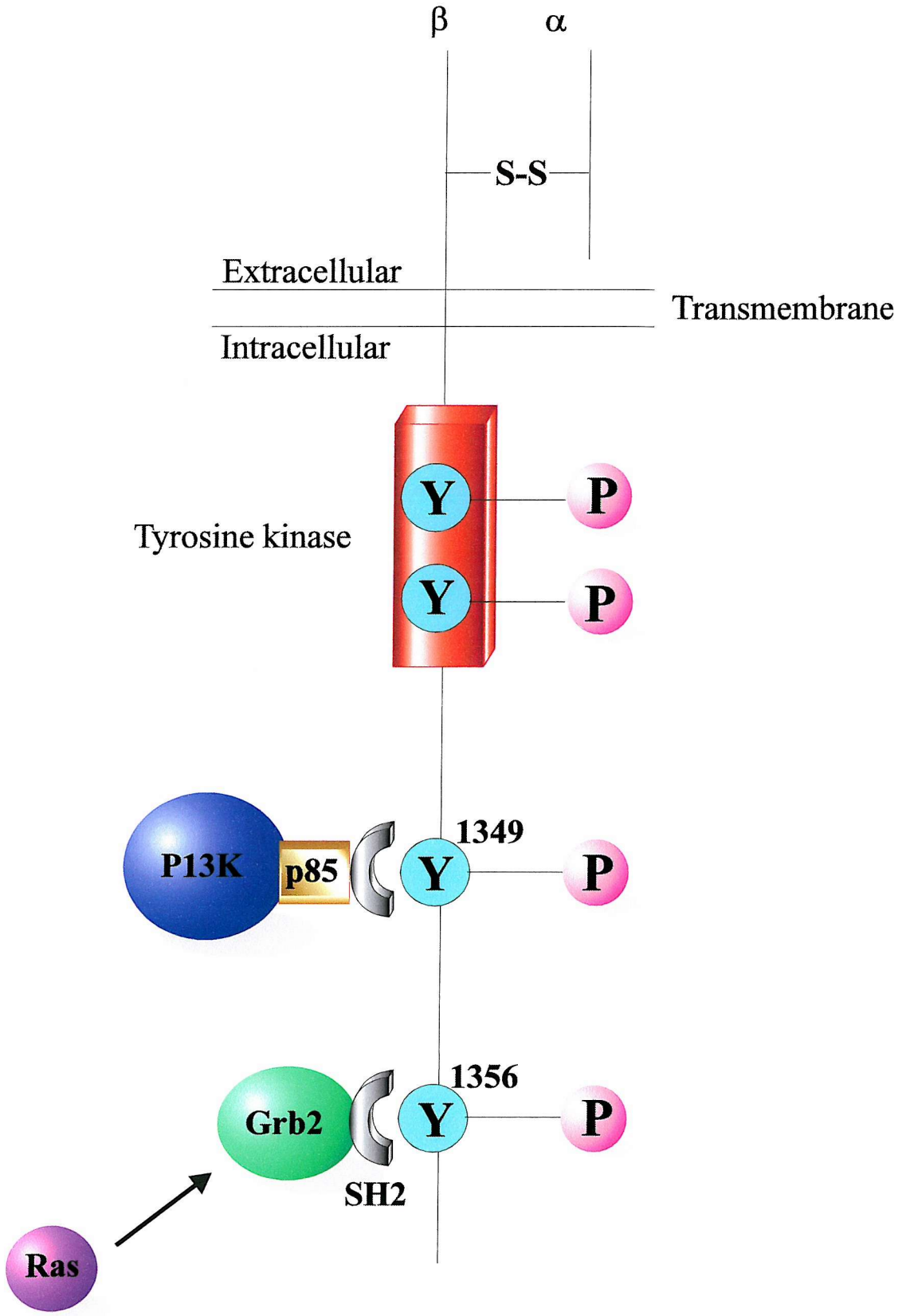
The absence of any underlying mutation in the *MET* proto-oncogene, in many tumour types exhibiting over-expression of the Met protein, would seem to suggest that this response is modulated by the mutation of another gene(s). One of the most likely mechanisms by which this induction of MET could occur is that of the promotion of a signalling pathway following the mutation and activation of another oncogene (Ivan *et al.*, 1997). This increased expression of Met may then lead to the increased sensitivity of tumour cells to HGF, and their proliferation. Indeed, Ivan *et al.*, (1997) demonstrated that the activated oncogenes H-*Ras* and *Ret* could induce expression of c-*Met* in human thyroid cells. In addition, HGF has also been shown to stimulate the growth of thyrocyte cells that have been induced by *Ret*. Structural analysis of Met, has revealed that the carboxyl-terminal tail of the tyrosine kinase domain harbours a multifunctional docking site, comprising two tyrosine residues, Y^{1349} and Y^{1356} (Giordano *et al.*, 1997). The phosphorylation of these two tyrosines mediates the signal

Figure 6.4. Schematic representation of the multifunctional docking site of Met Tyrosine Kinase.

Phosphate group

Tyrosine residue

S-S Disulphide bond


β
α
S-S
Extracellular
Transmembrane
Intracellular
Tyrosine kinase
Y
P
Y
P
P13K
p85
1349
Y
P
Grb2
1356
Y
P
SH2
Ras

transduction properties of Met. The adaptor protein, Grb2, which links the *Ras* pathway to Met, selectively binds to Y^{1356} via its Src homology 2 (SH2) domain (figure 6.4). Blocking this interaction of Grb2 with Y^{1356} greatly diminishes the ability of cells to become transformed, but does not disrupt their motility or 'scattering' response. This would suggest that non-Grb2-mediated pathways are sufficient for inducing motility but does not confer transforming ability. Indeed, of these two Met-mediated responses, only transformation requires a direct link to Ras. Substituting the two tyrosine residues for phenylalanine, results in the prevention of both transformation and metastasis. In addition, the introduction of a second Grb2 binding site at Y^{1349}, produces a highly transforming phenotype with no metastatic activity. These studies indicate that although the Ras-Grb2 pathway is required for the transformation and metastasis of tumour cells, it must act in conjunction with other signalling pathways, such as PI3 Kinase via its p85 subunit, to produce such an effect. It therefore appears that the oncogene *Ras* can play a role in inducing the expression of *MET*.

In addition, p53 has also been implicated in the induction of *MET*. The *MET* promoter contains a wild-type p53 binding site, or p53 response element (Seol *et al.*, 1999), between nucleotide 278 and 216. The *MET* promoter also has two binding sites for the transcription factor Spl, which is known to be involved in the regulation of *MET*. p53 has been shown to interact with Spl, raising the possibility that p53 may act to regulate the expression of MET by also interacting with Spl.

Finally, two other potential mechanisms of MET activation should be mentioned for completeness. The first is that of the overexpression of the Met ligand, HGF/SF. The observation that HGF is expressed simultaneously with Met, would indicate that any increase in HGF expression could lead to the overexpression of Met. Secondly, inflammatory cytokines, such as Tumour Necrosis Factor (TNF)-α, Transforming Growth Factor (TGF)-β1 and Interleukins 1 and 6 (IL-1 and IL-6), that are often seen to be increased within tumours, can themselves upregulate MET expression (Fischer *et al.*, 1998). It seems highly likely that a combination of all of these mechanisms induces Met-mediated tumorigenesis.

6.4 SUMMARY

Exons 17, 18 and 19, encoding the majority of the tyrosine kinase domain of the *MET* proto-oncogene, were analysed in an attempt to identify somatic mutations. The lack of observed mutations within the 70 tumours exhibiting 7q31 LOH strongly implies that *c-MET* is not the

ovarian TSG present at 7q31. Although no functional mutations in any regions outside the tyrosine kinase domain have been reported, providing further evidence against a TSG role for *MET* in ovarian carcinogenesis, it is not possible to exclude *MET* as the target of 7q31 LOH until the possibility of alterations in the remaining exons and promoter region, not analysed here, have been studied. In addition, the action of other signalling pathways must be studied in further detail to elucidate other activating mechanisms.

CHAPTER SEVEN

DETECTION OF TUMOUR DNA IN PLASMA - MUTATION ANALYSIS OF *TP53* AND *K-RAS* AND STUDY OF LOSS OF HETEROZYGOSITY

7.1 INTRODUCTION

DNA can be detected in many clinical samples including blood (Leon *et al.*, 1977), sputum (Mao *et al.*, 1994), urine and stool (Sidransky, 1997). In contrast to the DNA concentration observed in blood form healthy individuals of approximately 10ng/ml (equivalent to genomic content of about 1600 diploid cells), the levels detected in serum of cancer patients is considerable increased (Leon *et al.*, 1977). Several studies have reported levels of free DNA in the serum of cancer patients of up to 5000 ng/ml with mean values approximately 180 ng/ml (Leon *et al.*, 1977; Shapiro *et al.*, 1983). However, DNA can be released during the clotting of blood (Sorenson *et al.*, 1994) leading to falsely high yields of DNA from serum and an biased reflection of conditions *in vivo*. In an attempt to negate this effect, plasma is frequently assessed for free DNA instead of serum.

The observation that DNA levels are raised in the plasma of cancer patients suggests that this additional circulating DNA originates from the cancer. This is supported by Leon *et al.*, (1977) who showed that following radiotherapy, DNA levels measured in plasma were markedly decreased in 40% of individuals with a variety of malignancies. Further evidence for a tumour origin of excess plasma DNA comes from studies of nude mice bearing human lung adenocarcinomas (Fournie *et al.*, 1995). Plasma DNA from these mice was shown to hybridise to both human and mouse plasma DNA, whereas, plasma DNA from mice injected with endotoxin only hybridised to mouse plasma DNA, suggesting that at least some of the plasma DNA must originate from the human cancerous cells. A tumour origin of elevated plasma DNA has also been demonstrated directly in studies of K-*ras* mutations in pancreatic and colorectal carcinomas (Sorenson *et al.*, 1994; Mulcahy *et al.*, 1998; Anker *et al.*, 1997). These groups were able to identify identical mutations in corresponding plasma and tumour DNA using both PCR-based restriction-enzyme assays and direct sequencing. Mutations were not detected in the plasma DNA of normal controls or in that of cancer patients whose

tumours had failed to show K-*ras* alterations, again supporting the cancer origin of plasma DNA. Indeed, a more recent study of pancreatic tumours (Yamada *et al.*, 1998) has shown that following treatment, K-*ras* mutations identified in plasma DNA prior to therapy could no longer be detected in 6 of 9 patients (67%), whereas 3 patients with retained high levels of plasma DNA had either early relapses or progressive disease.

The mechanisms by which tumour DNA is released into the circulation has not yet been elucidated but several hypotheses have been put forward (Anker *et al.*, 1999). Figure 7.1. gives an overview of these potential mechanisms.

a) Lysis of circulating cancer cells or micrometastases.
b) Necrosis.
c) Apoptosis.
d) Active release of DNA from tumours.

7.1.1 Lysis of cancer cells

The release of DNA following the lysis of cancerous cells, shed into the circulation, is unlikely on the basis of cell number (Anker *et al.*, 1999). The quantity of cells required to produce the amounts of plasma DNA observed when using efficient methods of DNA extraction, have been calculated to be between 1 and 10 thousand cells per ml. This is far more than has ever been observed in the circulation, implying that the appearance of plasma DNA is a result of leakage from tumours rather than lysis of sheared cancer cells.

7.1.2 DNA release via necrosis

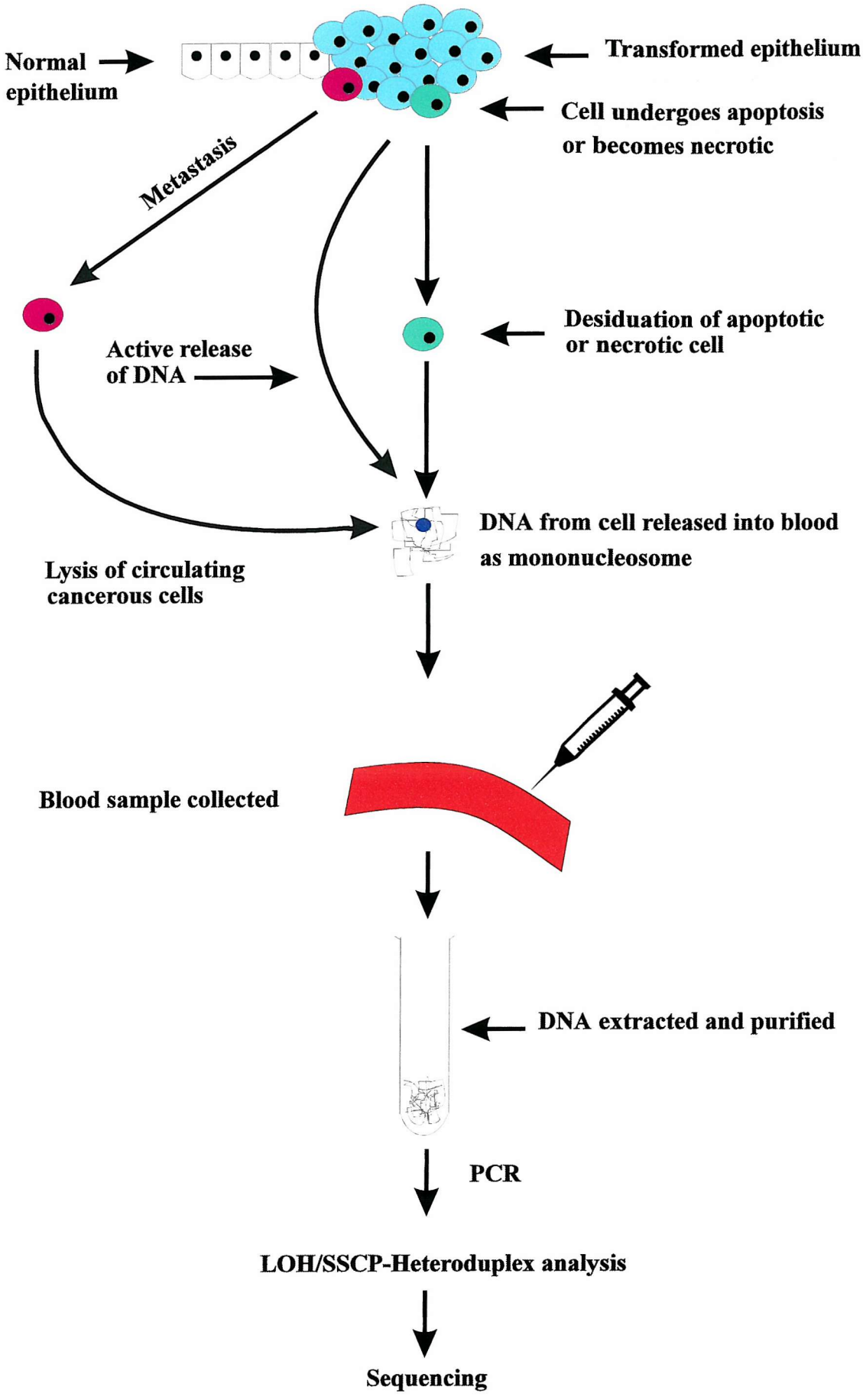
Necrosis of tumours has also been suggested as a route for the release of DNA into the blood. The finding that plasma DNA is increased in individuals with later stage disease and metastatic spread (Leon *et al.*, 1977) or larger tumours is the basis for this argument. However, as mentioned previously, radiation treatment produces a decrease in plasma DNA concentrations without an initial surge which would be predicted by the necrosis hypothesis. One explanation for this drop in DNA level is that radiotherapy leads to an arrest in cell proliferation and therefore DNA release. Another possibility is that there is a reduction in the

Figure 7.1. Diagrammatic representation of the possible mechanisms of tumour DNA release into the bloodstream.

Blue cells = transformed

Green cells = apoptotic/necrotic

Pink cells = Metastatic


Normal epithelium
Transformed epithelium
Cell undergoes apoptosis or becomes necrotic
Metastasis
Active release of DNA
Desiduation of apoptotic or necrotic cell
DNA from cell released into blood as mononucleosome
Lysis of circulating cancerous cells
Blood sample collected
DNA extracted and purified
PCR
LOH/SSCP-Heteroduplex analysis
Sequencing

release of DNAse inhibitor from the tumour following treatment, allowing increased degradation of circulating DNA.

7.1.3 DNA release via apoptosis

DNA found in plasma of cancer patients exhibits a 'ladder' pattern similar to that derived from apoptotic cells after electrophoresis, but different from the banding pattern of DNA from that of normal plasma DNA (Anker *et al.*, 1999). This observation and that of the presence of mononucleosomes in the blood (Fournie *et al.*, 1995), has lead to the argument that apoptosis may play a significant role in the release of DNA into the circulation. This nucleo-protein structure is believed to arise following the inter-nucleosomal cleavage of chromatin, a characteristic of apoptosis. Indications of this mononucleosomal release come from both *in vitro* and *in vivo* studies. In cell culture, the supernatant from apoptotic cells is seen to contain mononucleosomes (Fournie *et al.*, 1995). In mouse models, anti-Fas antibody induced apoptosis of hepatocytes is responsible for nucleosomal DNA release into the plasma within 2 hours of antibody injection (Fournie *et al.*, 1995). However, although the release of mononucleosomes is an indication of apoptosis, programmed cell death is arrested in cancer cells and so it seems unlikely that this theory could account for the excessive levels of tumour DNA observed in many patients.

7.1.4 Active release of DNA

When grown in culture, cells release DNA-protein complexes, preferentially those comprising newly synthesised DNA, into their surrounding medium (Fournie *et al.*, 1995). In addition, phytohaemagglutinin-activated lymphocytes replicate specific regions of their genome, spontaneously releasing the copies into the culture supernatant (Anker *et al.*, 1975; Rogers, 1976). Portions of this DNA are actively capped and attached to the plasma membrane of the cell (Sidransky, 1997). These observations point towards an active mechanism for DNA release into the circulation.

Whichever mechanism(s) is involved it seems likely that DNA is released into the blood stream as mononucleosomes (Fournie *et al.*, 1995). This structure (figure 7.2) is likely to protect DNA from nuclease attack and minimises the possibility of it being degraded.

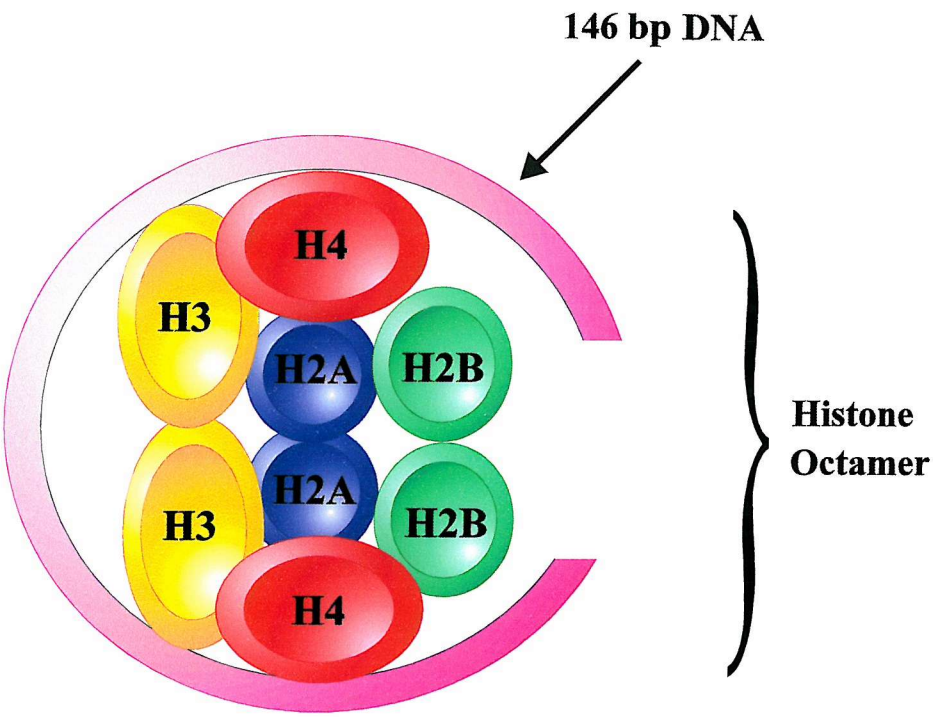


Figure 7.2 : Schematic representation of the core particle of a mononucleosome. DNA lies around the outside of a histone octamer, arranged in a H32.H42 model.

7.1.5 Detection of genetic abnormalities in plasma DNA

The first genetic mutations to be identified within plasma DNA were that of K-*ras* and N-*ras* (Anker *et al.*, 1999). These two genes were known to be mutated in many tumour types at relatively high frequencies. The mutations exhibited are also reliably detected by PCR-based assays and therefore seemed the ideal candidates for analysis. K-*ras* has been shown to be mutated in 90% of pancreatic tumours and 50% of colorectal tumours. This gene also has well-defined, clustered mutations occurring in codons 12 and 13, making it ideal for use in mutation scanning as opposed to larger genes such as *APC* (8.5kb), which have diverse mutations spread across a very large coding region (Sidransky, 1997). Sorenson *et al.*, (1994) used allele-specific amplification (ASA) to detect K-*ras* mutations in plasma DNA of 3 pancreatic carcinoma patients. They were also able to determine the presence of identical alterations in the corresponding tumour DNA. In a more recent study, 17 of 21 (81%) patients with pancreatic carcinoma exhibited K-*ras* mutations within codon 12, in their plasma DNA (Mulcahy *et al.*, 1998). In all ten patients for whom biopsy samples were available, similar mutations could be detected in the matching tumour DNA.

Observations of mutation analyses in colorectal carcinoma are in agreement with those for pancreatic neoplasms, with 6 of 7 (86%) patients harbouring tumour DNA K-*ras* mutations, also exhibiting identical mutations in their plasma DNA (Anker *et al.*, 1997). No correlation has been observed between the presence of mutated plasma DNA and histology, patient age, sex, location of primary and secondary tumours or recent therapy. However, Yamada et al., (1998), showed that patients with larger tumours tended to show plasma DNA mutations.

Mulcahy (1998) and colleagues also noted with interest, that K-*ras* alterations were detected in the plasma DNA of four patients before diagnosis of disease. Biopsies indicated one case of pancreatitis, two of fibrosis and one of normal tissue. Each patient had a detectable K-*ras* point mutation in their plasma DNA and all but one had wild-type K-*ras* in their biopsy sample. Clinical diagnosis of pancreatic adenocarcinoma in all cases was made 5-14 months after initial mutation screening, with identical alterations observed in both plasma and tumour DNA.

The persistence of mutated plasma DNA following treatment has been identified as a marker for early relapse or progression of disease (Anker *et al.*, 1999). N-*ras* mutations have been detected by ASA in the plasma of patients with the myeloid disorders myelodysplastic

syndrome (MDS) and acute myelogenous leukaemia (AML) (Vasioukhin *et al.*, 1994). These mutations were sometimes absent in the corresponding blood cells and bone marrow, suggesting that bone marrow biopsies did not represent the complete spectra of malignant clones associated with the disease, whereas plasma DNA appeared to exhibit characteristic alterations associated with each of these sub-clones. In addition, two or more different N-*ras* mutations were identified in several MDS cases but not in patients with AML. This may indicate that several somatic alterations may occur in the pre-cancerous state but that only one sub-clone, carrying a particular N-*ras* mutation, progresses to acute leukaemia.

The observations outlined here indicate the possible use of plasma DNA mutation screens as tools for staging, monitoring of treatment and follow-up of cancer patients. However, as mentioned earlier, not many known oncogenes or TSGs lend themselves to easy screening, either due to their size or wide range of mutations, and so alternative DNA-based assays are being assessed.

7.1.6 Hypermethylation of tumour suppressor genes

Epigenetic mechanisms are now widely thought to play a role in oncogenesis alongside genetic mutations. One of the best understood of these mechanisms is that of DNA methylation (section 1.12). Several publications have shown hypermethylation of TSGs including *TP53* and *P16* (Laird, 1997), in tumours and cell lines and more recently (Anker *et al.*, 1999), aberrant DNA methylation patterns have been detected in both the plasma and serum of cancer patients.

7.1.7 LOH and microsatellite instability

The large number of microsatellite markers across the human genome and their highly polymorphic characteristics make them ideal markers for genetic alterations. The common changes seen are LOH, which identifies deleted chromosomal regions, and microsatellite instability (MI), described as the presence of novel alleles at a given microsatellite locus. Two studies in 1996, identified microsatellite alterations in the serum DNA of head and neck cancer patients (Nawroz *et al.*, 1996), and in the plasma of small cell lung cancer patients (Chen *et al.*, 1996). Hickey *et al* (1999) demonstrated the ability to detect both LOH and MI in the serum DNA of ovarian cancer patients.

LOH of chromosome 7q has been shown to occur frequently in many tumour types including that of the ovary (chapter 3), suggesting the existence of an important TSG. This high frequency of loss, even at the earliest stages of ovarian tumourigenesis (section 3.2), could be utilised in the development of a novel, and relatively non-invasive, assay for the detection of ovarian tumours. In addition, Hickey and co-workers (1999) identified LOH and MI on the long arm of chromosome7 in matching tumour and serum pairs, further suggesting that screening serum, and indeed, plasma DNA for the presence of chromosome 7 aberrations, may provide a reasonably simple and cost-effective diagnostic tool.

7.2 RESULTS

7.2.1 Clinical samples

Fifteen matching sets of normal, tumour and plasma DNA from ovarian cancer patients were used in this study. Histological data can be seen in table 7.1. The DNA samples were prepared as in sections 2.2.2, 2.2.3. and 2.2.5 respectively.

7.2.2. LOH and mutation analysis

LOH analysis was carried out on matching lymphocyte DNA , plasma DNA and tumour DNA from 15 cases using 2 microsatellite markers from 7q22, 2 from 7q31, 2 from within the *TP53* locus and 1 from the *BRCA1* locus (Table 7.1). The analysis was performed as shown in section 2.4.3. LOH analysis was repeated for any cases showing LOH on the first round to confirm the result.

Among the tumour components 7/15 (47%) showed LOH with one or more markers (Table 7.1). Among the plasma DNA components there were a total of 15 LOH events distributed among 9 cases. In four of these cases some of the LOH events were identical to those observed in the matching tumour DNA. In total there were 7 common losses; 2 in case 550, 3 in case 551, 1 in case 560 and 1 in case 568. Examples of some of these common losses are shown in Figure 7.3. In case 548 the DNA from the plasma showed LOH for markers D7S522 and D17S855 but was heterozygous for both markers in the corresponding tumour DNA. In several cases LOH was detected in the tumour DNA (cases 551, 560, 569, 577) or plasma DNA (cases 569, 588, 592) but PCR amplification of the matching plasma or tumour DNA was not successful and so it was not possible to determine the identical mutation was present.

Table 7.1. Comparison of genetic alterations between tumour and plasma DNA samples.

[1]Age at diagnosis
na = not available
S = serous; CC = clear cell; E = endometrioid; Mu = mucinous; UD = undifferentiated; FT = fibrothecoma
het = retention of constitutional heterozygosity
loh = loss of constitutional heterozygosity
ni = non-informative
dnw = did not work
no = no mutation observed
R = arginine; M = methionine; C =cysteine; G = glycine

Tumour	Age	Stage	Grade	Histology	CA125	Component	D7S518	D7S658	D7S486	D7S522	p53 CA	D175786	D175855	TP53	Kras
548	61	3	1	S	45	T	het	ni	het	het	het	het	het	no	no
						P	dnw	ni	het	loh	dnw	dnw	loh	no	no
549	42	na	3	CC	92	T	het	dnw	ni	ni	het	ni	het	no	no
						P	dnw	dnw	ni	ni	dnw	ni	dnw	no	no
550	72	3	2	E	555	T	het	dnw	het	dnw	loh	loh	ni	no	no
						P	het	het	het	het	loh	loh	ni	no	no
551	56	3	3	S	1032	T	het	ni	loh	loh	loh	ni	loh	no	no
						P	het	ni	loh	dnw	loh	ni	loh	no	no
557	35	1	1	Mu	51	T	het	ni	loh/mi	dnw	het	het	het	no	12 C>G
						P	dnw	ni	dnw	dnw	dnw	dnw	dnw	no	dnw
560	52	4	3	S	2896	T	ni	loh	loh	loh	loh	loh	loh	249 R>M	no
						P	ni	het	het	dnw	dnw	loh	het	no	no
568	60	3c	3	S	124	T	het	ni	ni	het	het	loh	het	no	no
						P	het	dnw	ni	dnw	het	loh	het	no	no
569	72	3c	3	S/E	na	T	dnw	dnw	tdnw	dnw	dnw	loh	dnw	no	no
						P	dnw	dnw	loh	dnw	het	dnw	loh	no	no
574	75	3	2	UD	1717	T	dnw	dnw	dnw	dnw	dnw	ni	ni	no	no
						P	dnw	dnw	loh	dnw	het	ni	ni	no	no
575	62	4	3	S	7825	T	dnw	dnw	dnw	dnw	dnw	ni	ni	no	no
						P	dnw	dnw	ni	dnw	het	ni	ni	no	no
577	79	4	3	S/E	na	P	het	dnw	ni	dnw	dnw	dnw	loh	no	no
						P	mi	dnw	ni	dnw	dnw	dnw	dnw	no	no
580	69	1c	2	E	59.5	T	dnw	nd	dnw	ni	nd	het	dnw	no	no
						P	dnw	nd	dnw	ni	nd	dnw	het	no	no
588	60	1c	1	Mu	20.4	T	het	nd	dnw	ni	dnw	dnw	dnw	no	no
						P	het	nd	loh	mi	het	loh	dnw	no	no
592	57	na	na	FT	33.4	T	dnw	nd	dnw	dnw	nd	ni	dnw	no	no
						P	dnw	nd	het	loh	nd	ni	het	no	no
594	47	1	3	E	91.9	T	ni	nd	dnw	dnw	nd	dnw	dnw	no	no
						P	dnw	nd	het	dnw	nd	het	het	no	no

Figure 7.3. Representative autoradiographs showing LOH detected in both tumour and plasma DNA.

Uppermost number indicates marker used.

Lower number indicates sample analysed.

N = normal DNA; T = tumour DNA; P = plasma DNA

D17S786

550

N T P

LOH LOH

D17S855

551

N T P

LOH LOH

P53 CA repeat

550

N T P

LOH LOH

P53 CA repeat

551

N T P

LOH LOH

No correlation was observed between LOH and tumour stage, grade, histological sub-type or CA125 level.

Mutation analysis of *TP53* and K-*ras* was carried out on all 15 DNA sets, using SSCP-HD analysis (section 2.8.4). Exons 5, 6, 7 and 8 of *TP53* were screened for mutations as they have been shown to harbour the majority of alterations in ovarian cancers. Likewise, K-*ras* was analysed for mutations around codon 12 and 61, common sites for sequence changes. SSCP-HD analysis was repeated for any sample showing a band shift and the sample was then sequenced.

Analysis of *TP53* revealed only one consistent SSCP band shift, in exon 7 of tumour sample 560 (figure 7.4A). This shift was not detected in the respective normal DNA or corresponding plasma DNA. Sample 560 was sequenced, resulting in the identification of a base substitution within codon 249 of tumour sample 560 (figure 7.4B). This G>T transversion results in an amino acid change, arginine to methionine. No aberrant bands were observed in any sample for the remaining *TP53* exons studied.

The analysis of K-*ras* identified a mutation within codons 1-36 in tumour sample 557 (figure 7.5A). Sequencing revealed that tumour sample 557 harboured a T>G transversion mutation within codon 12 of K-*ras* (figure 7.5B). This translated to an amino acid change from cysteine to glycine. Unfortunately, the corresponding plasma sample could not be amplified efficiently enough to determine whether an identical shift was present. The normal DNA did not exhibit the mutation observed in the tumour sample.

7.3 DISCUSSION

Cancer has long been recognised as a genetic disease, arising through the accumulation of somatic mutations within populations of cells. These mutations generally involve the activation of proto-oncogenes and inactivation of TSGs, although exactly when these changes occur in the tumourigenic pathway of the many tumour types remains largely unknown. The ability to identify these alterations and correctly assign them to specific stages of disease may provide accurate molecular diagnosis of neoplasms and therefore greatly improve the management and therapy of this disease.

Figure. 7.4. Mutation analysis of *TP53*.

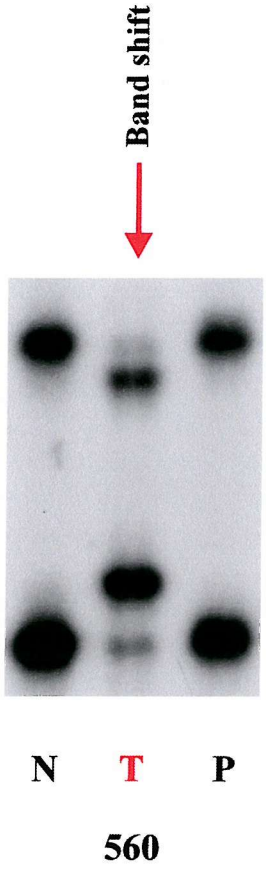
A. Band shifts detected by SSCP-HD analysis of *TP53* exon 7.

B. Sequence of normal and mutated regions of TP53.

Tumour sample shows a G>T transversion mutation.
Arg>Met

N = normal DNA; T = tumour DNA; P = plasma DNA.

A.


Band shift
N
T
P
560

B.

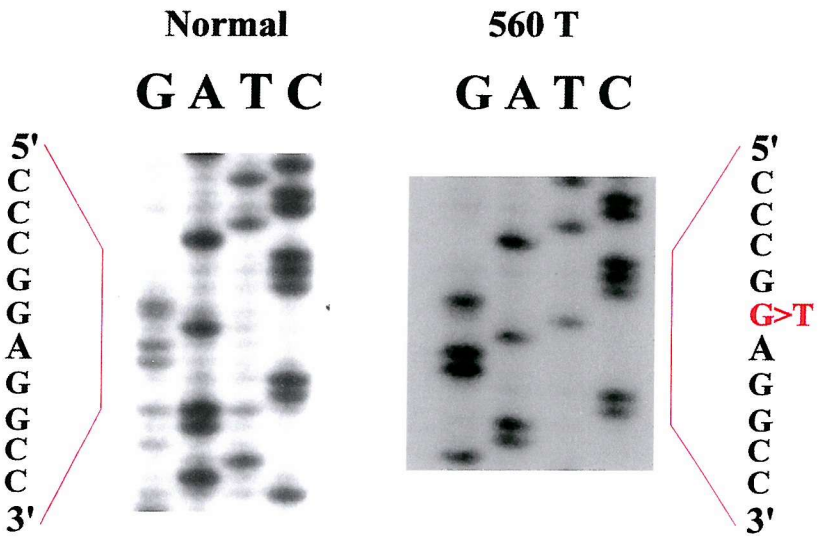

Normal
560 T
G A T C
G A T C
5'
C
C
C
G
G
A
G
G
C
C
3'
5'
C
C
C
G
G>T
A
G
G
C
C
3'

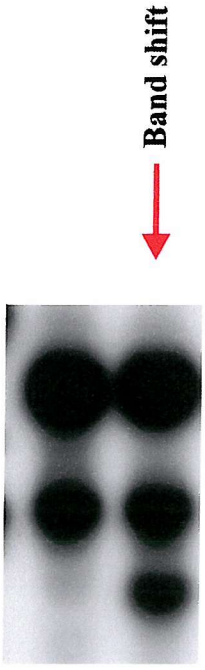
Figure. 7.5. Mutation analysis of K-*ras*.

A. Band shift detected by SSCP-HD analysis of K-*ras*, codons 1-36.

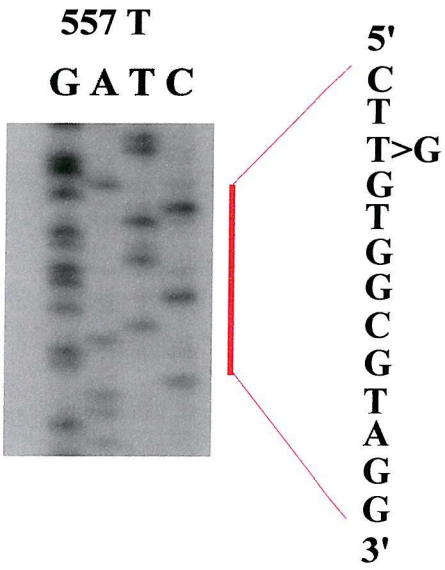
B. Sequence analysis reveals a T>G transversion mutation
Cys>Gly

N = normal DNA; T = tumour DNA; P = plasma DNA.

A.



B.



The finding that tumour DNA can be readily detected in plasma may well prove a reliable and non-invasive method for early diagnosis of tumours. However, care must be exercised when designing molecular tests for use in diagnosis, as the DNA present in clinical samples including plasma may be predominantly from normal and not tumour cells and the ratio may vary considerably according to the tumour. For example, over 50% of DNA detected in the urine of bladder cancer patients is believed to originate from tumour cells, making a molecular test for tumour DNA in urine plausible (Sidransky, 1997). In the case of lung cancer, only 0.2% of DNA detected in sputum is likely to arise from tumour cells, suggesting that a similar molecular test for tumour DNA in sputum would be highly inadequate (Sidransky, 1997). There are also disadvantages associated with using highly sensitive PCR-based assays. These may detect alterations in as little as one cell, identifying the patient as at risk of developing cancer even though that cell has not begun to proliferate, leading to a false positive diagnosis of cancer. Clearly, any screening assay must be both highly specific and highly sensitive. At this stage the use of plasma DNA as a screening test is in its infancy and there is a need to assess the potential of this mode of screening in a wide variety of situations. At the time this work commenced there had been no reports of plasma DNA screening in ovarian cancer patients. The aim of this study was to provide some preliminary data about the feasibility of detecting both point mutations and LOH in plasma DNA from ovarian cancer patients.

Since this study was undertaken towards the end of my research scholarship, only the most recently obtained tumours had corresponding plasma DNA available for analysis. Prior to this work, the diagnostic potential of tumour DNA detected in plasma samples had not been realised and plasma had not been stored. With only a few cases available for analysis, the priority of the study was to maximise the chances of detecting genetic abnormalities. To achieve this, LOH analysis was performed using markers known to be lost at a high frequency in the majority of ovarian tumours. These included markers at the TP53 and BRCA1 loci on chromosome 17 which show LOH in more than 50% of ovarian cancers. In addition, four markers were chosen from the long arm of chromosome 7, which as demonstrated in chapter 3 is the site of particularly frequent LOH in ovarian tumours which significantly, is equally common among early and late stage ovarian tumours.

It is of particular interest that four out of the 15 patients studied, exhibited common losses between their tumour and plasma DNA samples, with cases 550 and 551 showing 2 and 3 common losses respectively. If the additional cases, which either did not show LOH in the tumour sample, or in which the tumour or plasma sample could not be amplified, are

disregarded, it would appear that this technique allows the detection of 4 out of 4 common losses. A previously published study (Hickey *et al.*, 1999) on ovarian cancer showed that of 27 LOH events observed in serum samples, 19 of these (73%) also occurred in the corresponding tumour DNA. The overall detection rate reported by Hickey (Hickey *et al.*, 1999) appears higher than that found in the present study, however sample number was slightly increased. In the present study, the number of matching tumour/plasma pairs showing identical alterations may have been higher than documented, however several samples could not be sufficiently amplified by PCR to enable analysis to be carried out. It is conceivable that if the PCR protocol could be designed to work more reliably, a proportion of these samples would amplify and also show common losses. Further work is required to optimise the technique for preparing and amplifying plasma DNA for the detection of genetic abnormalities.

In one case, 548, LOH of two distinct markers was detected in the plasma DNA but not in the matching tumour DNA. Hickey and co-workers also demonstrated LOH within serum samples which did not occur in the respective tumour sample. This may show that the primary tumour is not monoclonal and instead contains sub-clonal populations arising from several individual progenitor cells, which have undergone different genetic alterations. Only the progeny of one of these clones has undergone this particular mutation, allowing them to become more aggressive and able to release DNA into the plasma. It is therefore possible, that the area of microdissected tissue used in LOH analysis does not correspond to the region of tumour responsible for the circulating DNA in plasma. Alternatively, there may be other tumours present, distinct from the primary tumour. These may prove to be secondary sites, arising from primary tumour cells that have accumulated additional genetic alterations, including those identified only in the plasma DNA, and metastasised, or they could prove to be another distant and unrelated primary tumour.

Mutation studies, in addition to clonality analysis, of further matched samples is required in order to determine whether similar patterns emerge and if these are related to different sub-populations within one tumour or if the technique is identifying the presence of additional tumours.

The ability to detect LOH within the plasma samples of patients with stage I disease further suggests that this technique could potentially be used as an diagnostic tool for detecting early stage disease, as well as for monitoring the progress of advanced disease during and after therapy.

The oncogene K-*ras* and tumour suppressor gene *p53* were analysed for mutations in this study because of the well-defined alterations that have been previously reported in ovarian (Pieretti *et al.*, 1995; Chenevix-Trench *et al.*, 1997; Milner *et al.*, 1993) and other tumours. However, these two genes would not be the ideal candidates for use in the further development of this technique. Although K-*ras* mutations are more definitive than LOH, they are not very common in ovarian cancer (Pieretti *et al.*, 1995; Chenevix-Trench *et al.*, 1997) and so are inappropriate for ovarian tumour screening. Similarly *p53* would not be an ideal choice since mutations of this gene are a relatively late event in the development of this disease and would not be a useful marker for the early detection of ovarian cancer. However, in the preliminary phase of this technique, these genes and others, such as *PTEN*, some of the few known to be mutated in ovarian cancer, should still be utilised to assess the feasibility of such an approach to screening, since the presence a point mutation in both tumour and plasma samples is unlikely to have occurred independently or as an artefact.. The use of LOH, on the other hand, is not very precise due to the possibility that any common LOH observed may well have arisen independently or as the result of a PCR artefact.

Despite this, LOH analysis is useful for this stage of the study as it is often seen as an early event, but it is dependent on the comparison of tumour and plasma DNA with normal DNA, the presence of which can mask any LOH in the plasma. These points indicate that any future test would need to be performed only on plasma DNA, with the aim of detecting a specific TSG or oncogene mutation. Since LOH studies indicate that deletions of chromosome 7 are an early event that is common in all histological sub-types of ovarian tumour, the TSG harboured within this region, once it is cloned, may well provide the perfect marker for this technique, as long as the mutations are not distributed all across the gene. In order to maximise the potential for this technique to detect a particular tumour, a bank of genetic markers would be required. This plasma screening method could also be used to complement existing diagnostic tools such as the CA125 tumour marker. Indeed, it can be seen from table 7.1, that LOH was detected in the plasma of patients whose CA125 level was low.

Given the time and resources I would have liked to investigate the optimal method of extracting and purifying tumour DNA from plasma samples, since the ability to amplify DNA from the15 cases I used was variable, suggesting that the quality of DNA may not have been adequate. In addition, the region of deletion on chromosome 7 has recently been further defined by Zenklusen *et al.*, (2000b) who also believe they have identified the candidate TSG located within this region (Zenklusen *et al.*, 2000a). The next step would therefore be to screen this gene for mutations in primary tumours and if any are found, to screen plasma DNA for the corresponding alteration.

7.4 SUMMARY

Both LOH and gene mutations were detected in the plasma DNA samples, and in several cases, similar alterations were detected in the corresponding tumour DNA. This suggests that tumour DNA can be detected in the blood plasma of ovarian cancer patients, and that genetic alterations can also be observed in these clinical samples.

CHAPTER EIGHT

SUMMARY AND CONCLUSIONS

8.1 MOLECULAR GENETIC ANALYSIS OF OVARIAN CANCER

A woman's lifetime risk for developing ovarian cancer is approximately 2% (Risch, 1998), making it the most common, and most fatal, of all gynaecological cancers (Auersperg *et al.*, 1997). The construction of a genetic model for this disease would provide a great step forward in the understanding and treatment of ovarian neoplasia. The identification of the breast and ovarian cancer predisposing genes *BRCA1* and *BRCA2* has resulted in some early screening and prophylactic intervention programs to be set up for individuals with a family history of either cancer. Knowledge of the genetic alterations that occur throughout ovarian tumourigenesis, from initiation, to promotion, to progression and finally metastasis, may provide the basis for other early detection regimes.

In order to develop this model many groups have studied ovarian tumour DNA for regions of amplification, that may contain oncogenes, and deletions, that may harbour TSGs with the ultimate goal of identifying the actual gene involved. There any many ways in which candidate oncogenes and TSGs can be identified. Positional cloning approaches such as LOH, comparative genomic hybridisation and linkage analysis in the case of familial syndromes are often used as starting points to identify genomic regions which may be of interest. Other methods such as candidate gene analysis can be used when functional evidence implicates the involvement of a particular gene. With the advances in technology, high throughput methods such as cDNA arrays can be utilised to identify many genes that are differentially expressed throughout tumourigenesis. All of these approaches have advantages and disadvantages and it is recognised that no single approach will be able to identify every gene with a role in ovarian cancer. In this study LOH was used as we had access to a large bank of ovarian tumours and the technique is relatively easy and inexpensive to perform and, in principle at least, has the potential to provide precise localisation of TSG loci. Interestingly the relative ease with which LOH studies can be carried out has lead to some bad press concerning this approach. In the last 10 years there have been thousands of LOH studies reported but few, if any, have led to the identification of a TSG. However only a minority of the laboratories conducting these studies had the facilities or even an intention to

proceed further than a low density LOH analysis. With the announcement of the first draft sequence from the Human Genome Project the translation of a region of common LOH to the identification of an actual gene will be greatly simplified and will not be restricted to the major genome centres.

8.2 LOH ANALYSIS OF CHROMOSOME 7 IN OVARIAN CANCER

Over the last decade chromosome 7 has been extensively studied in many types of tumour in attempt to identify any TSGs that may reside within it. LOH studies carried out in the early 1990s identified frequent deletions of chromosome 7p in ovarian cancers (Cliby *et al.*, 1993; Sato *et al.*, 1991), but as very few markers were used these regions of deletion remained very large. In subsequent studies of ovarian and other cancer types, the focus began to shift to the long arm of chromosome 7, in particular 7q31 and 7q22 (figure 3.1). Since these were the first regions identified they were extensively analysed whilst the remainder of the chromosome was largely ignored. To my knowledge this work represents the largest single study of the role of chromosome 7 in ovarian cancer. I have shown that not only is LOH on chromosome 7 a frequent event in the majority of ovarian tumours, but that it appears to be associated with the transition from a benign or borderline state to malignancy. In addition, this study has demonstrated the presence of multiple deletions across chromosome 7 suggesting the presence of multiple TSGs with a role in various stages of ovarian tumourigenesis. Indeed many of the tumours studied displayed multiple, overlapping regions of deletion. It is likely that some of the LOH is due to an underlying degree of random genetic instability, especially amongst the later stage tumours, which has made interpretation of the LOH difficult. Support for the results obtained here comes from the observation that two of the commonly deleted regions identified in this study, at 7q31 and 7q22, correspond to those which have previously been implicated in several tumour types, including ovarian (Devilee *et al.*, 1997; Edelson *et al.*, 1997; Huang *et al.*, 1999; vanderHeijden *et al.*, 1998; Zeng *et al.*, 1999; Zenklusen *et al.*, 1994).

Although the LOH evidence is compelling, the common fragile site FRA7G (Huang *et al.*, 1999) is located within the 7q31 locus. Since fragile sites, as their name suggests, are prone to breakage it is possible that the LOH observed within this region is simply an artefact of this process. If this were the case one might expect this breakage to occur more often in late stage or high grade tumours than in early disease, since the tumour is more aggressive, proliferating at a higher rate and therefore providing greater opportunity for genetic damage.

However, this study indicates that although the rate of LOH increases following the progression of a lesion to malignancy, no significant change is observed between stages of malignant disease. Whilst this data appears to contradict a role for FRA7G in the LOH seen at 7q31, until a tumour suppressor gene is located within this region such a possibility must still be considered. A similar situation is seen for 7q22 which also encompasses a fragile site, FRA7B (Guven *et al.*, 1999). However to date this site has not been extensively mapped and the role of this aphidicolin-inducible site has not been investigated.

The 7q31 TSG has long been sought, but as yet remains elusive. A handful of candidates have been screened including the caveolin 1 gene (Engelman *et al.*, 1998; Hurlstone *et al.*, 1999), but no alterations have been identified. Whilst carrying out my laboratory study, the mapping and sequencing of 7q was not sufficiently advanced to permit the identification of novel transcripts from within 7q31. One gene that was known to reside within the candidate region was the gene *MET*. MET protein has been shown to be present in primary ovarian tumours at high levels (DiRenzo *et al.*, 1994), implying that it is up-regulated in ovarian cancer. However, this overexpression is highly variable between ovarian tumours, with one study indicating high levels of MET in clear cell carcinomas, but relatively low levels in the serous sub-type (Moghul *et al.*, 1994). Although MET was traditionally thought of as a proto-oncogene we felt it warranted further investigation for inactivating somatic mutations because it was located within a region frequently showing LOH. However, my studies failed to find any mutations within the 3 exons of MET commonly altered in other tumour types, including renal carcinomas (Schmidt *et al.*, 1997; Schmidt *et al.*, 1999; Schmidt *et al.*, 1998), suggesting that MET is not involved in ovarian tumourigenesis or that alterations occur elsewhere in the gene or via post-transcriptional mechanisms.

Whilst this thesis was in preparation Zenklusen et al., (2000a) presented a meeting abstract alleging they had identified somatic alterations in a gene, PITS, from within the most commonly deleted region of chromosome 7q. If substantiated this is likely to be the long sought after 7q31 TSG. Assuming the data is correct and PITS is indeed the 7q31 multi tissue TSG, then studies regarding its role in the tumourigenic pathway will be required to determine the impact of this gene with respect to future diagnostics and therapy. Indeed, considering the already promising evidence surrounding this gene and its potential as a tumour suppressor, it would have proved to be the next gene to be analysed within this study, for mutations in primary ovarian tumours, since work on PITS to date has involved primary colon carcinomas and breast cancer cell-lines (Zenklusen *et al.*, 2000a).

Chromosome band 7q22 has previously been implicated as playing a role in uterine leiomyomas (Ishwad *et al.*, 1995; vanderHeijden *et al.*, 1998; Zeng *et al.*, 1999) and a variety of myeloid disorders (Fischer *et al.*, 1997; Tosi *et al.*, 1999). In addition, this gene-rich location has also been implicated in ovarian cancer (Kerr *et al.*, 1996) although it has received much less attention than 7q31. Two candidate genes from this region were chosen for mutation screening in our panel of ovarian tumours, *PAI-1* due to its potential negative effects on tumour invasion and close proximity to the most frequently deleted markers (Kerr *et al.*, 1996) and *CUTL1* (Zeng *et al.*, 1997), a potential suppressor of gene expression, because it straddles the 7q22 breakpoint. No somatic mutations were identified implying that neither is the TSG residing within this region of chromosome 7.

8.3 THE DETECTION OF OVARIAN TUMOUR DNA IN PLASMA SAMPLES

Women with ovarian cancer usually present at late stage, as the earlier stages of the disease are largely asymptomatic. The ability to diagnose this disease in the early stages would greatly improve an individuals prognosis as therapy has been shown to be much more successful in patients with early lesions compared to those with stage III or IV disease. However, few specific markers for ovarian cancer exist, and other tumour antigens, such as CA125, do not always accurately predict the presence of tumours, particularly those of early stage or grade. Furthermore, the ability to detect tumour DNA in the plasma of cancer patients had previously been reported and proposed as an alternative method of screening and diagnosis. This technique offers the promise of providing a rapid, highly sensitive, non invasive and cost effective approach to early diagnosis of ovarian cancer. As only one study of tumour DNA in the serum of ovarian cancer patients has been reported (Hickey *et al.*, 1999), my study was designed to determine the feasibility of using such a technique to detect ovarian tumour DNA in the blood plasma of patients. Since the results from my LOH study indicated that the deletions of chromosome 7q were very common early events in the progression of ovarian tumours, the identification of such aberrations appeared to provide an ideal tumour marker.

The results obtained from this study have shown that it is indeed feasible to use plasma DNA for the detection of ovarian tumour DNA and the genetic aberrations within it. It could be argued that the LOH detected was simply the result of a PCR artefact possibly due to the poor quality of DNA obtained, however several cases exhibited deletions in more than one marker and in matched tumour and plasma samples implying that the data is real. One case was of particular interest since it displayed LOH of two microsatellite markers in the plasma DNA

but not in the matching tumour DNA sample, a phenomenon also seen by another group in serum samples of ovarian cancer patients (Hickey *et al.*, 1999). Whilst it is a possibility that this is indeed the result of a PCR artefact, other explanations cannot be disregarded. Firstly the tumour sample used in this study may not be monoclonal and instead may comprise of discrete cellular populations that have arisen via distinct genetic changes. One such sub-population may have acquired a more aggressive phenotype following these alterations and therefore gained the ability to release DNA into the blood. If the microdissected tissue used in the study did not contain this aggressive region of tumour the corresponding LOH would not be detected. However, by definition these more aggressive cells would be expected to proliferate more rapidly and therefore account for the majority of the tumour tissue present. If this were the case any genetic abnormality identified in the plasma DNA should also be observed in the corresponding tumour sample. An alternative explanation may be the presence of multiple tumours, either primary or metastatic, which may have acquired different or additional mutations enabling the release of DNA into the bloodstream. This could prove clinically valuable if shown to be true, providing a method to potentially detect microscopic metastatic lesions in addition to the early detection of primary ovarian cancer.

Whilst this study proved successful extracting DNA from plasma samples and successfully amplifying it for use in analysis occasionally proved difficult. This may have been due to varying lengths of time between blood sample collection and DNA extraction, which could allow the DNA to deteriorate. This was reduced to a minimum by immediately processing and extracting DNA from the samples once received, or if not possible, by storing the plasma sample in a -80(C freezer until it could be processed further. Subsequent freeze/thaw cycles were also kept to a minimum to avoid additional DNA deterioration. Another major obstacle is the presence of normal DNA within the plasma DNA sample. If this normal component is greater than the tumour DNA component, any LOH will likely be masked. Unless the contaminating normal DNA could be tagged, it would be impossible to distinguish it from the tumour DNA and remove it. In order to reduce this contamination, blood samples were collected prior to any surgery or chemotherapy, which could otherwise introduce more normal DNA, or falsely elevate circulating tumour DNA. In addition, since clotting is known to result in the release of DNA from lymphocytes (Sorenson *et al.*, 1994), we opted to use blood plasma for these studies, which by definition is the non-cellular component of un-clotted blood, instead of serum, which is the non-cellular component of whole blood collected following clotting.

Finally, as PCR relies on the quality of DNA used and the ability of primers to anneal the template provided, this technique may be improved by utilising microsatellite markers that are much shorter in length, since the tumour DNA found in plasma samples may well have been degraded, producing relatively small fragments. However, other changes to PCR conditions may not improve the success of this technique. Indeed, although lowering the initial annealing temperature used in PCR reactions, allows primers to bind to the DNA template and amplification to proceed, potentially improving the yield of the reaction, the primer is more likely to anneal randomly, resulting in non-specific binding and multiple products or artefacts. Once these technical obstacles have been overcome this tumour DNA detection method could provide the basis for many novel screening and diagnostic protocols.

Since this study has proved that tumour DNA, derived from the plasma of ovarian cancer patients, can be utilised to identify genetic mutations and regions of LOH in a relatively small sample set it would seem appropriate to extend the analysis to a larger group of cases. Initially total plasma DNA should be measured and a larger bank of microsatellite markers used to identify the distinct regions of LOH involved in each case. In addition the study should include the full clinical follow-up of patients to determine the prognostic characteristics associated with both total plasma DNA and the patterns of LOH identified. Ultimately however, the test will need to incorporate specific gene mutations, as contaminating normal DNA will limit the ability to detect LOH while specific somatic mutations can be identified even with gross normal DNA contamination. Such a regime has recently been utilised by Kopreski *et al.*, (2000) to identify K-ras mutations in colorectal cancer patients by specifically amplifying the K-ras gene from both plasma and tissue samples.

8.4 SUMMARY

The aim of this study has been to gain a detailed picture of the frequency and site of LOH on chromosome 7 in ovarian cancer and thereby extend the genetic model of ovarian carcinogenesis.

I have shown that deletions of defined regions of chromosome 7 are extremely common in all stages, grades and histological sub-types of epithelial ovarian cancer. These deletions are therefore likely to represent important, early alterations across the broad spectrum of ovarian neoplasia and further, may play a role in the switch from benign or borderline lesions to malignancy. Our LOH analysis has allowed the further refinement of a number of regions,

including one novel deletion, which may prove to harbour TSGs involved in this transformation. In this respect our study may provide further evidence to support the theory that a subset of benign and borderline ovarian tumours can progress to malignancy.

In addition, we have shown that circulating tumour DNA can be detected in and purified from plasma samples, and utilised in LOH and mutation studies. Since the collection of biopsy tissue is invasive and time consuming, this technique once optimised, could potentially be used as an early tumour marker.

BIBLIOGRAPHY

Abeln, E.C.A., Corver, W.E., Kuipersdijkshoorn, N.J., Fleuren, G.J. & Cornelisse, C.J. (1994). Molecular-genetic analysis of flow-sorted ovarian tumor-cells - improved detection of loss of heterozygosity. *British Journal Of Cancer*, **70,** 255-262.

Abeysinghe, H.R., Cedrone, E., Tyan, T., Xu, J. & Wang, N. (1999). Amplification of C-*MYC* as the origin of the homogeneous staining region in ovarian carcinoma detected by micro-FISH. *Cancer Genetics and Cytogenetics*, **114,** 136-143.

Achille, A., Biasi, M.O., Zamboni, G., Bogina, G., Magalini, A.R., Pederzoli, P., Perucho, M. & Scarpa, A. (1996). Chromosome 7q allelic losses in pancreatic-carcinoma. *Cancer Research*, **56,** 3808-3813.

Adami, H.O., Hsieh, C.-C., Lambe, M., Trichopoulos, D., Leon, D., Persson, I., Ekbom, A. & Janson, P.O. (1994). Parity, age at first childbirth, and risk of ovarian cancer. *The Lancet*, **344,** 1250-1254.

Aertgeerts, K., Debondt, H.L., Deranter, C. & Declerck, P.J. (1994). A model of the reactive form of plasminogen-activator inhibitor-1. *Journal Of Structural Biology*, **113,** 239-245.

Aertgeerts, K., Debondt, H.L., Deranter, C.J. & Declerck, P.J. (1995). Mechanisms contributing to the conformational and functional flexibility of plasminogen-activator inhibitor-1. *Nature Structural Biology*, **2,** 891-897.

Allan, G.J., Cottrell, S., Trowsdale, J. & Foulkes, W.D. (1994). 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*, **3,** 283-291.

Anderson, M.C., Lambert, H.E., Rustin, G.J.S. & Soutter, W.P. (1997). Carcinoma of the ovary and fallopian tube. In *Gynaecology*, Shaw, R.W., Soutter, W.P. & Stanton, S.L. (eds) pp. 627-649. Churchill Livingstone.

Anker, P., Lefort, F., Vasioukhin, V., Lyautey, J., Lederrey, C., Chen, X.Q., Stroun, M., Mulcahy, H.E. & Farthing, M.J.G. (1997). K-ras mutations are found in DNA extracted from the plasma of patients with colorectal cancer. *Gastroenterology*, **112,** 1114-1120.

Anker, P., Stroun, M. & Maurice, P. (1975). Spontaneous release of DNA by human blood lymphocytes as shown in an *in vitro* system. *Cancer Research*, **35,** 2375-2382.

Anker, P., Mulcahy, H., Chen, X.Q. & Stroun, M. (1999). Detection of circulating tumour DNA in the blood (plasma/serum) of cancer patients. *Cancer and Metastasis Reviews*, **18,** 65-73.

Arnold, N., Hagele, L., Walz, L., Schempp, W., Pfisterer, J., Bauknecht, T. & Kiechle, M. (1996). Overrepresentation of 3q and 8q material and loss of 18q material are recurrent findings in advanced human ovarian cancer. *Genes Chromosomes & Cancer*, **16,** 46-54.

Auersperg, N., MainesBandiera, S.L. & Dyck, H.G. (1997). Ovarian carcinogenesis and the biology of ovarian surface epithelium. *Journal Of Cellular Physiology*, **173,** 261-265.

Bajou, K., Noel, A., Gerard, R.D., Masson, V., Brunner, N., Holst-Hansen, C., Skobe, M., Fusenig, N.E., Carmeliet, P., Collen, D. & Foidart, J.M. (1998). Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularisation. *Nature Medicine*, **4,** 923-928.

Bandera, C.A., Takahashi, H., Behbakht, K., Liu, P.C., LiVolsi, V.A., Benjamin, I., Morgan, M.A., King, S.A., Rubin, S.C. & Boyd, J. (1997). Deletion mapping of two potential chromosome 14 tumor suppressor gene loci in ovarian carcinoma. *Cancer Research*, **57,** 513-515.

Bardelli, A., Pugliese, L. & Comoglio, P.M. (1997). "Invasive-growth" signalling by the Met/HGF receptor. The hereditary renal carcinoma connection. *Biochimica et Biophysica Acta*, **1333,** M41-M51.

Beltinger, C.P., Klimek, F. & Debatin, K.M. (1997). Whole genome amplification of single cells from clinical peripheral blood smears. *Journal of Clinical Pathology: Molecular Pathology*, **50,** 272-278.

Berchuck, A., Kohler, M.F., Hopkins, M.P., Humphrey, P.A., Robboy, S.J., Rodriguez, G.C., Soper, J.T., Clarkepearson, D.L. & Bast, R.C. (1994). Overexpression of p53 is not a feature of benign and early stage borderline epithelial ovarian tumors. *Gynecologic Oncology*, **52,** 232-236.

Berek, J.S. & Hacker, N.F. (1994). *Practical Gynecologic Oncology*. Williams and Wilkins.

Berek, J.S., Martinezmaza, O., Hamilton, T., Trope, C., Kaern, J., Baak, J. & Rustin, G.J.S. (1993). Molecular and biological factors in the pathogenesis of ovarian- cancer. *Annals Of Oncology*, **4,** 3-16.

Berne, R.M. & Levy, M.N. (1990). *Principles of physiology*. Wolfe Publications Limited.

Bicher, A., Ault, K., Kimmelman, A., Gershenson, D., Reed, E. & Liang, B. (1997). Loss of heterozygosity in human ovarian cancer on chromosome 19q. *Gynecologic Oncology*, **66,** 36-40.

Bieche, I., Champeme, M.H. & Lidereau, R. (1999). Infrequent mutations of the *MET* gene in sporadic breast tumours. *International Journal of Cancer*, **82,** 908-910.

Bjorquist, P., Brohlin, M., Ehnebom, J., Ericsson, M., Kristiansen, C., Pohl, G. & Deinum, J. (1994). Plasminogen-activator inhibitor type-1 interacts exclusively with the proteinase domain of tissue-plasminogen activator. *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology*, **1209,** 191-202.

Bodmer, W., Bishop, T. & Karran, P. (1994). Genetic steps in colorectal-cancer. *Nature Genetics*, **6,** 217-219.

Boente, M.P. (1996). Early ovarian cancer: A review of its genetic and biologic factors, detection, and treatment. *Current Problems in Cancer*, **10,** 83-137.

Boix, L., Rosa, J.L., Ventura, F., Castells, A., Bruix, J., Rodes, J. & Bartrons, R. (1994). c-*met* mRNA overexpression in human hepatocellular carcinoma. *Hepatology*, **19,** 88-91.

Boland, C.R., Sato, J., Appelman, H.D., Bresalier, R.S. & Feinberg, A.P. (1995). Microallelotyping defines the sequence and tempo of allelic losses at tumor-suppressor gene loci during colorectal-cancer progression. *Nature Medicine*, **1,** 902-909.

Brown, M.R., Chuaqui, R., Vocke, C.D., Berchuck, A., Middleton, L.P., Emmert-Buck, M.R. & Kohn, E.C. (1999). Allelic loss on chromosome arm 8p: Analysis of sporadic epithelial ovarian tumors. *Gynecologic Oncology*, **74,** 98-102.

Bryan, E.J., Watson, R.H., Davis, M., Hitchcock, A., Foulkes, W.D. & Campbell, I.G. (1996). Localization of an ovarian-cancer tumor-suppressor gene to a 0.5-cm region between d22s284 and cyp2d, on chromosome 22q. *Cancer Research*, **56,** 719-721.

Carrell, R.W. (1999). How serpins are shaping up. *Science*, **285,** 1861-1862.

Carson, D.A. & Lois, A. (1995). Cancer progression and p53. *The Lancet*, **346,** 1009-1011.

Cattreau, A., Harris, W.H., Xu, C.-F. & Solomon, E. (1999). Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. *Oncogene*, **18,** 1957-1965.

Chambers, S.K., Ivins, C.M. & Carcangiu, M.L. (1998). Plasminogen activator inhibitor-1 is an independent poor prognostic factor for survival in advanced stage epithelial ovarian cancer patients. *International Journal of Cancer*, **79,** 449-454.

Chang, S. & Risch, H.A. (1997). Perineal talc exposure and risk of ovarian carcinoma. *Cancer*, **79,** 2396-2401.

Chen, X.Q., Stroun, M., Magnenat, J.L., Nicod, L.P., Kurt, A.M., Lyautey, J., Lederrey, C. & Anker, P. (1996). Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nature Medicine*, **2,** 1033-1035.

Chenevix-Trench, G., Kerr, J., Hurst, T., Shih, Y.C., Purdie, D., Bergman, L., Friedlander, M., Sanderson, B., Zournazi, A., Coombs, T., Leary, J.A., Crawford, E., Shelling, A.N., Cooke, I., Ganesan, T.S., Searle, J., Choi, C., Barrett, J.C., Khoo, S.K. & Ward, B. (1997). Analysis of loss of heterozygosity and KRAS2 mutations in ovarian neoplasms: Clinicopathological correlations. *Genes Chromosomes & Cancer*, **18,** 75-83.

Chenevix-Trench, G., Leary J., Kerr J., Michel J., Kefford R., Hurst T., Parsons P.G., Friedlander M. & Khoo S.K. (1992). Frequent loss of heterozygosity on chromosome 18 in ovarian adenocarcinoma which does not always include the DCC locus. *Oncogene*, **7,** 1059-1065.

Choi, C., Cho, S.H., Horikawa, I., Berchuck, A., Wang, N., Cedrone, E., Jhung, S.W., Lee, J.B., Kerr, J., Chenevix-Trench, G., Kim, S.Y., Barrett, J.C. & Koi, M. (1997). Loss of heterozygosity at chromosome segment Xq25-26.1 in advanced human ovarian carcinomas. *Genes Chromosomes & Cancer*, **20,** 234-242.

Chuaqui, R.F., Zhuang, Z. & Merino, M.J. (1997). Molecular genetic events in the development and progression of ovarian cancer in humans. *Molecular Medicine Today*, **3,** 207-213.

Cliby, W., Ritland, S., Hartmann, L., Dodson, M., Halling, K.C., Keeney, G., Podratz, K.C. & Jenkins, R.B. (1993). Human epithelial ovarian cancer allelotype. *Cancer Research*, **53,** 2393-2398.

Clifford, S.C., Czapla, K., Richards, F.M., Odonoghue, D.J. & Maher, E.R. (1998). Hepatocyte growth factor stimulated renal tubular mitogenesis: effects on expression of c-myc, c-fos, c-met, VEGF and the VHL tumour-suppressor and related genes. *British Journal of Cancer*, **77,** 1420-1428.

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

Cook, L.S., Kamb, M.L. & Weiss, N.S. (1997). Perineal powder exposure and the risk of ovarian cancer. *American Journal Of Epidemiology*, **145,** 459-465.

Cornelis, R.S., Neuhausen, S.L., Johansson, O., Arason, A., Kelsell, D., Ponder, B.A.J., Tonin, P., Hamann, U., Lindblom, A., Lalle, P., Longy, M., Olah, E., Scherneck, S., Bignon, Y.J., Sobol, H., Changclaude, J., Larsson, C., Spurr, N., Borg, A., Barkardottir, R.B., Narod, S. & Devilee, P. (1995). High allele loss rates at 17q12-q21 in breast and ovarian-tumors from brca1-linked families. *Genes Chromosomes & Cancer*, **13,** 203-210.

Corps, A.N., Sowter, H.M. & Smith, S.K. (1997). Hepatocyte growth factor stimulates motility, chemotaxis and mitogenesis in ovarian carcinoma cells expressing high levels of c-MET. *International Journal of Cancer*, **73,** 151-155.

Cox, A.D. & Der, C.J. (1997). Farnesyltransferase inhibitors and cancer treatment: targeting simply Ras? *Biochimica et Biophysica Acta*, **1333,** F51-F71.

Davis, M., Hitchcock, A., Foulkes, W.D. & Campbell, I.G. (1996). Refinement of two chromosome 11q regions of loss of heterozygosity in ovarian cancer. *Cancer Research*, **56,** 741-744.

Dawson, S.J., Wiman, B., Hamsten, A., Green, F., Humphries, S. & Henney, A.M. (1993). The two allele sequences of a common polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene respond differently to interleukin-1 in HepG2 cells. *Journal of Biological Chemistry*, **268,** 10739-10745.

Deligdisch, L. (1997). Ovarian dysplasia: A review. *International Journal Of Gynecological Cancer*, **7,** 89-94.

Deng, G., Curriden, S.A., Wang, S., Rosenberg, S. & Loskutoff, D.J. (1996). Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release. *The Journal of Cell Biology*, **134,** 1563-1571.

Devilee, P., Hermans, J., Eyfjord, J., Borresen, A.L., Lidereau, R., Sobol, H., Borg, A., CletonJansen, A.M., Olah, E., Cohen, B., Scherneck, S., Hamann, U., Peterlin, B., Caligo, M., Bignon, Y.L., Maugard, C., Andersen, T.I., Odegand, A.E.M., Bieche, I., Birnbaum, D., Cornelisse, C.J., Sztan, M., Steel, M.R., Seitz, S., Logar, N., Golouh, R., Ghimenti, C., Bruelle, S. & Joalland, M.P. (1997). Loss of heterozygosity at 7q31 in breast cancer: Results from an international collaborative study group. *Genes Chromosomes & Cancer*, **18,** 193-199.

Devlin, J., Elder, P.A., Gabra, H., Steel, C.M. & Knowles, M.A. (1996). High-frequency of chromosome-9 deletion in ovarian-cancer - evidence for 3 tumor-suppressor loci. *British Journal Of Cancer*, **73,** 420-423.

DiRenzo, M.F., Narsimhan, R.P., Olivaro, M., Bretti, S., Giordano, S., Medico, E., Gaglia, P., Zara, P. & Comoglio, P.M. (1991). Expression of the Met/HGF receptor in normal and neoplastic human tissues. *Oncogene*, **6,** 1997-2003.

DiRenzo, M.F., Olivero, M., Katsaros, D., Crepaldi, T., Gaglia, P., Zola, P., Sismondi, P. & Comoglio, P.M. (1994). Overexpression of the *Met*/HGF receptor in ovarian cancer. *International Journal of Cancer*, **58,** 658-662.

Dodson, M.K., Cliby, W.A., Xu, H.-J., DeLacey, K.A., Hu, S.-X., Keeney, G.L., Li, J., Podratz, K.C., Jenkins, R.B. & Benedict, W.F. (1994). Evidence of functional RB protein in epithelial ovarian carcinomas despite loss of heterozygosity at the *RB* locus. *Cancer Research*, **54,** 610-613.

Dodson, M.K., Hartmann, L.C., Cliby, W.A., DeLacey, K.A., Keeney, G.L., Ritland, S.R., Su, J.Q., Podratz, K.C. & Jenkins, R.B. (1993). Comparison of loss of heterozygosity patterns in invasive low-grade and high-grade epithelial ovarian carcinomas. *Cancer Research*, **53,** 4456-4460.

Dufort, D. & Nepveu, A. (1994). The human cut homeodomain protein represses transcription from the c-myc promoter. *Molecular and Cellular Biology*, **14,** 4251-4257.

Duh, F.M., Scherer, S.W., Tsui, L.C., Lerman, M.I., Zbar, B. & Schmidt, L. (1997). Gene structure of the human MET proto-oncogene. *Oncogene*, **15,** 1583-1586.

Ebert, M., Yokoyama, M., Friess, H., Buchler, M.W. & Kore, M. (1994). Co-expression of the c-*met* proto-oncogene and hepatocyte growth factor in human pancreatic cancer. *Cancer Research*, **54,** 5775-5778.

Eccles, D.M., Brett, L., Lessels, A., Gruber, L., Lane, D., Steel, C.M. & Leonard, R.C.F. (1992a). Overexpression of the p53 protein and allele loss at 17p13 in ovarian carcinoma. *British Journal Of Cancer*, **65,** 40-44.

Eccles, D.M., Gruber, L., Stewart , M., Steel, C.M. & Leonard, R.C. (1992b). Allele loss on chromosome 11p is associated with poor survival in ovarian cancer. *Disease Markers*, **10,** 95-99.

Edelson, M.I., Scherer, S.W., Tsui, L.C., Welch, W.R., Bell, D.A., Berkowitz, R.S. & Mok, S.C. (1997). Identification of a 1300 kilobase deletion unit on chromosome 7q31.3 in invasive epithelial ovarian carcinomas. *Oncogene*, **14,** 2979-2984.

Egli, G.E. & Newton, M. (1961). The transport of carbon particles in the human female reproductive tract. *Fertility and Sterility*, **12,** 151-155.

Engelman, J.A., Wykoff, C.c., Yasuhara, S., Song, K.S., Okamoto, T. & Lisanti, M.P. (1997). Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth. *Journal of Biological Chemistry*, **272,** 16374-16381.

Engelman, J.A., Zhang, X.L. & Lisanti, M.P. (1998). Genes encoding human caveolin-1 and -2 are co-localized to the D7S522 locus (7q31.1), a known fragile site (FRA7G) that is frequently deleted in human cancers. *Febs Letters*, **436,** 403-410.

Falk, G., Almqvist, A., Nordenhem, A., Svensson, H. & Wiman, B. (1995). Allele-specific pcr for detection of a sequence polymorphism in the promoter region of the plasminogen-activator inhibitor-1 (pai-1) gene. *Fibrinolysis*, **9,** 170-174.

Fathalla, M.F. (1971). Incessant ovulation - a factor in ovarian neoplasia? *Lancet*, **2,** 163.

Fearon, E.R. & Vogelstein, B.A. (1990). A genetic model for colorectal tumorigenesis. *Cell*, **61,** 759-767.

Fischer, J., Palmedo, G., Von Knochbloch, R., Bugert, P., Prayer-Galetti, T., Pagano, F. & Kovacs, G. (1998). Duplication and overexpression of the mutant allele of the MET proto-oncogene in multiple hereditary papillary renal cell tumours. *Oncogene*, **17,** 733-739.

Fischer, K., Frohling, S., Scherer, S.W., Brown, J.M., Scholl, C., Stilgenbauer, S., Tsui, L.C., Lichter, P. & Dohner, H. (1997). Molecular cytogenetic delineation of deletions and translocations involving chromosome band 7q22 in myeloid leukemias. *Blood*, **89,** 2036-2041.

Foekens, J.A., Peters, H.A., Look, M.P., Portengen, H., Schmitt, M., Kramer, M.D., Brunner, N., Janicke, F., Meijer-van Gelder, M.E., Henzen-Logmans, S.C., van Putten, W.L.J. & Klijn, J.G.M. (2000). The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients. *Cancer Research*, **60,** 636-643.

Foster, K.A., Harrington, P., Kerr, J., Russell, P., DiCioccio, R.A., Scott, I.V., Jacobs, I., Chenevix-Trench, G., Ponder, B.A.J. & Gayther, S.A. (1996). Somatic and germline mutations of the BRCA2 gene in sporadic ovarian cancer. *Cancer Research*, **56,** 3622-3625.

Foulkes, W.D., Black, D.M., Stamp, G.W., Solomon, E. & Trowsdale, J. (1993b). Very frequent loss of heterozygosity throughout chromosome 17 in sporadic ovarian carcinoma. *International Journal of Cancer*, **54,** 220-225.

Foulkes, W.D., Campbell, I.G., Stamp, G.W. & Trowsdale, J. (1993c). Loss of heterozygosity and amplification on chromosome 11q in human ovarian cancer. *British Journal of Cancer*, **67,** 268-273.

Foulkes, W.D., Ragoussis, J., Stamp, G.W., Allan, G.J. & Trowsdale, J. (1993a). Frequent loss of heterozygosity on chromosome 6 in human ovarian carcinoma. *British Journal of Cancer*, **67,** 551-559.

Fournie, G.J., Courtin, J.P., Laval, F., Chale, J.J., Pourrat, J.P., Pujazon, M.C., Lauque, D. & Carles, P. (1995). Plasma DNA as a marker of cancerous cell-death - investigations in patients suffering from lung-cancer and in nude-mice bearing human tumors. *Cancer Letters*, **91,** 221-227.

Fullwood, P., Marchini, S., Rader, J.S., Martinez, A., Macartney, D., Broggini, M., Morelli, C., Barbanti-Brodano, G., Maher, E.R. & Latif, F. (1999). Detailed genetic and physical mapping of tumor suppressor loci on chromosome 3p in ovarian cancer. *Cancer Research*, **59,** 4662-4667.

Gabra, H., Langdon, S.P., Watson, J.E.V., Hawkins, R.A., Cohen, B.B., Taylor, L., Mackay, J., Steel, C.M., Leonard, R.C.F. & Smyth, J.F. (1995). Loss of heterozygosity at 11q22 correlates with low progesterone- receptor content in epithelial ovarian-cancer. *Clinical Cancer Research*, **1,** 945-953.

Gallion, H.H., Pieretti, M., Depriest, P.D. & Vannagell, J.R. (1995). The molecular-basis of ovarian-cancer. *Cancer*, **76,** 1992-1997.

Gallion, H.H., Powell, D.E., Morrow, J.K., Pieretti, M., Case, E., Turker, M.S., DePriest, P.D., Hunter, J.E. & van Nagell, J.J., Jr. (1992). Molecular genetic changes in human epithelial ovarian malignancies. *Gynecologic Oncology*, **47,** 137-142.

Gertig, D.M., Hunter, D.J., Cramer, D.W., Colditz, G.A., Speizer, F.E., Willett, W.C. & Hankinson, S.E. (2000). Prospective study of talc use and ovarian cancer. *Journal of the National Cancer Institute*, **92,** 249-252.

Giordano, S., Bardelli, A., Zhen, Z., Menard, S., Ponzetto, C. & Comoglio, P.M. (1997). A point mutation in the *MET* oncogene abrogates metastasis without affecting transformation. *Proceedings of the National Academy of Sciences of the USA*, **94,** 13868-13872.

Glockner, G., Scherer, S., Schattevoy, R., Boright, A., Weber, J., Tsui, L.C. & Rosenthal, A. (1998). Large-scale sequencing of two regions in human chromosome 7q22: analysis of 650 kb of genomic sequence around the *EPO* and *CUTL1* loci reveals 17 genes. *Genome Research*, **8,** 1060-1073.

Green, A., Purdie, D., Bain, C., Siskind, V., Russell, P., Quinn, M., Ward, B., Campbell, S., Dalrymple, C., Day, A., Ferrier, A., Free, K., Grant, P., Hacker, N., Harnett, P., Harvey, P., Houghton, R., Jobling, T., Planner, R., Proietto, T., Rome, R., Solomon, J., Susil, B., Wain, G., Wright, G., Birks, C., Brisley, P., Candlish, P., Clarke, S., Lockwood, K.A., Luck, L., Patterson, R., Potter, L., Ward, A., Watson, M. & Yarker, M.E. (1997). Tubal sterilisation, hysterectomy and decreased risk of ovarian cancer. *International Journal Of Cancer*, **71,** 948-951.

Guven, G.S., Hacihanefioglu, S. & Cenami, A. (1999). Expression of aphidicolin, FUdR and caffeine-induced fragile sites in lymphocytes of healthy Turkish individuals. *Genetica*, **105,** 109-116.

Hankinson, S.E., Hunter, D.J., Colditz, G.A., Willett, W.C., Stampfer, M.J., Rosner, B., Hennekens, C.H. & Speizer, E. (1993). Tubal ligation, hysterectomy, and risk of ovarian cancer: A prospective study. *The Journal of the American Medical Association*, **270,** 2813-2818.

Harada, R., Dufort, D., Denis-Larose, C. & Nepveu, A. (1994). Conserved cut repeats in the human cut homeodomain protein function as DNA binding domains. *Journal of Biological Chemistry*, **269,** 2062-2067.

Hatta, Y., Takeuchi, S., Yokota, J. & Koeffler, H.P. (1997). Ovarian cancer has frequent loss of heterozygosity at chromosome 12p12.3-13.1 (region of TEL and Kip1 loci) and chromosome 12q23-ter: evidence for two new tumour-suppressor genes. *British Journal of Cancer*, **75,** 1256-1262.

Heiss, M.M., Babic, R., Allgayer, H., Gruetzner, K.U., Jauch, K.W., Loehrs, U. & Schildberg, F.W. (1995). Tumor-associated proteolysis and prognosis - new functional risk-factors in gastric-cancer defined by the urokinase-type plasminogen- activator system. *Journal Of Clinical Oncology*, **13,** 2084-2093.

Heller, D.S., Westhoff, C., Gordon, R.E. & Katz, N. (1996). The relationship between perineal cosmetic talc usage and ovarian talc particle burden. *American Journal of Obstetrics and Gynecology*, **174,** 1507-1510.

Henderson, W.J., Joslin, C.A., Turnbull, A.C. & Griffiths, K. (1971). Talc and carcinoma of the ovary and cervix. *Journal of Obstetrics and Gynaecology in Britain and the Commonwealth*, **78,** 266-272.

Herszenyl, L., Plebani, M., Carraro, P., De Paoli, M., Roveroni, G., Cardin, R., Tulassay, Z., Naccarato, R. & Farinati, F. (1999). The role of cysteine and serine proteases in colorectal carcinoma. *Cancer*, **86,** 1135-1142.

Hickey, K.P., Boyle, K.P., Jepps, H.M., Andrew, A.C., Buxton, E.J. & Burns, P.A. (1999). Molecular detection of tumour DNA in serum and peritoneal fluid from ovarian cancer patients. *British Journal of Cancer*, **80,** 1803-1808.

Ho, C.H., Yuan, C.C. & Liu, S.-M. (1999). Diagnostic and prognostic values of plasma levels of fibrinolytic markers in ovarian cancer. *Gynecologic Oncology*, **75,** 397-400.

Hosking, L., Trowsdale, J., Nicolai, H., Solomon, E., Foulkes, W., Stamp, G., Signer, E. & Jeffreys, A. (1995). A somatic BRCA1 mutation In an ovarian tumor. *Nature Genetics*, **9,** 343-344.

Huang, H.J., Qian, C.P., Jenkins, R.B. & Smith, D.I. (1998a). Fish mapping of YAC clones at human chromosomal band 7q31.2: Identification of YACS spanning FRA7G within the common region of LOH in breast and prostate cancer. *Genes Chromosomes & Cancer*, **21,** 152-159.

Huang, H.J., Qian, J.Q., Proffit, J., Wilber, K., Jenkins, R. & Smith, D.I. (1998b). FRA7G extends over a broad region: coincidence of human endogenous retroviral sequences (HERV-H) and small polydispersed circular DNAs (spcDNA) and fragile sites. *Oncogene*, **16,** 2311-2319.

Huang, H.J., Reed, C.P., Mordi, A., Lomberk, G., Wang, L.A., Shridhar, V., Hartmann, L., Jenkins, R. & Smith, D.I. (1999). Frequent deletions within FRA7G at 7q31.2 in invasive epithelial ovarian cancer. *Genes Chromosomes & Cancer*, **24,** 48-55.

Huebner, K., Hadaczek, P., Siprashvili, Z., Druck, T. & Croce, C.M. (1997). The *FHIT* gene, a multiple tumor suppressor gene encompassing the carcinogen sensitive chromosome fragile site, *FRA3B*. *Biochimica et Biophysica Acta*, **1332,** M65-M70.

Hurlstone, A.F.L., Reid, G., Reeves, J.R., Fraser, J., Strathdee, G., Rahilly, M., Parkinson, E.K. & Black, D.M. (1999). Analysis of the CAVEOLIN-1 gene at human chromosome 7q31.1 in primary tumours and tumour-derived cell lines. *Oncogene*, **18,** 1881-1890.

Imyanitov, E.N. (1999). Frequent loss of heterozygosity at 1p36 in ovarian adenocarcinomas but the gene encoding p73 is unlikely to be the target. *Oncogene*, **18,** 4640-4642.

Ishwad, C.S., Ferrell, R.E., Davare, J., Meloni, A.M., Sandberg, A.A. & Surti, U. (1995). Molecular and cytogenetic analysis of chromosome-7 in uterine leiomyomas. *Genes Chromosomes & Cancer*, **14,** 51-55.

Ishwad, C.S., Ferrell, R.E., Hanley, K., Davare, J., Meloni, A.M., Sandberg, A.A. & Surti, U. (1997). Two discrete regions of deletion at 7q in uterine leiomyomas. *Genes, Chromosomes & Cancer*, **19,** 156-160.

Ivan, M., Bond, J.A., Prat, M., Comoglio, P.M. & Wynford Thomas, D. (1997). Activated ras and ret oncogenes induce over-expression of c-met (hepatocyte growth factor receptor) in human thyroid epithelial cells. *Oncogene*, **14,** 2417-2423.

Iwabuchi, H., Sakamoto, M., Sakunaga, H., Ma, Y.Y., Carcangiu, M.L., Pinkel, D., YangFeng, T.L. & Gray, J.W. (1995). Genetic analysis of benign, low-grade, and high-grade ovarian tumors. *Cancer Research*, **55,** 6172-6180.

Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C.P., Weirich, G., Kishida, T., Zbar, B. & Vande Woude, G.F. (1997). Activating mutations for the Met tyrosine kinase receptor in human cancer. *Proceedings for the National Academy of Sciences of the USA*, **94,** 11445-11450.

Jicriny, J. (1996). Mismatch repair and cancer. *Cancer Surveys*, **28,** 47-68.

Johnson, E.J., Scherer, S.W., Osborne, L., Tsui, L.C., Oscier, D., Mould, S. & Cotter, F.E. (1996). Molecular definition of a narrow interval at 7q22.1 associated with myelodysplasia. *Blood*, **87,** 3579-3586.

Katsaros, D., Theillet, C., Zola, P., Louason, G., Sanfilippo, B., Isaia, E., Arisio, R., Giardina, G. & Sismondi, P. (1995). Concurrent abnormal expression of erbB-2, myc and ras genes is associated with poor outcome of ovarian cancer patients. *Anticancer Research*, **15,** 1501-1510.

Kawamaki, M. (1999). Involvement of H-cadherin (CDH13) on 16q in the region of frequent deletion in ovarian cancer. *International Journal of Oncology*, **15,** 715-720.

Kere, J., Ruutu, T., Davies, K.A., Roninson, I.B., Watkins, P.C., Winqvist, R. & De la Chapelle, A. (1989). Chromosome 7 long arm deletion in myeloid disorders: A narrow breakpoint region in 7q22 defined by molecular mapping. *Blood*, **73,** 230-234.

Kerr, J., Leary, J.A., Hurst, T., Shih, Y.C., Antalis, T.M., Friedlander, M., Crawford, E., Khoo, S.K., Ward, B. & Chenevix-Trench, G. (1996). Allelic loss on chromosome 7q in ovarian adenocarcinomas - 2 critical regions and a rearrangement of the planh1 locus. *Oncogene*, **13,** 1815-1818.

Kiechle-Schwarz M, Bauknecht T, Wienker T, Walz L & Pfleiderer A. (1993). Loss of constitutional heterozygosity on chromosome 11p in human ovarian cancer. Positive correlation with grade of differentiation. *Cancer*, **72,** 2423-2432.

Kim, T.M., Benedict, W.F., Xu, H.J., Hu, S.X., Gosewehr, J., Velicescu, M., Yin, E., Zheng, J.P., Dablaing, G. & Dubeau, L. (1994). 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 Research*, **54,** 605-609.

King, B.L., Carcangiu, M.L., Carter, D., Kiechle, M., Pfisterer, J. & Kacinski, B.M. (1995). Microsatellite instability in ovarian neoplasms. *British Journal Of Cancer*, **72,** 376-382.

Kinzler, K.W. & Vogelstein, B. (1997). Cancer susceptibility genes. Gatekeepers and caretakers. *Nature*, **386,** 761-763.

Kopreski, M.S., Benko, F.A., Borys, D.J., Khan, A., McGarrity, T.J. & Gocke, C.D. (2000). Somatic mutation screening: identification of individuals harboring K-ras mutations with the use of plasma DNA. *Journal of the National Cancer Institute*, **92,** 918-923.

Knudson, A.G. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proceedings of the National Academy of Sciences of the USA*, **68,** 820-823.

Kohler, U., Hiller, K., Martin, R., Langanke, G., Naumann, G., Bilek, K., Janicke, F. & Schmitt, M. (1997). Tumor-associated proteolytic factors uPA and PAI-1 in endometrial carcinoma. *Gynecologic Oncology*, **66,** 268-274.

Koike, M., Takeuchi, S., Yokota, J., Park, S., Hatta, Y., Miller, C.W., Tsuruoka, N. & Koeffler, H.P. (1997). Frequent loss of heterozygosity in the region of the D7S523 locus in advanced ovarian cancer. *Genes Chromosomes & Cancer*, **19,** 1-5.

Koike, M., Tasaka, T., Spira, S., Tsuruoka, N. & Koeffler, H.P. (1999). Allelotyping of acute myelogenous leukemia: loss of heterozygosity at 7q31.1 (D7S486) and q33-34 (D7S498, D7S505). *Leukemia Research*, **23,** 307-310.

Kopreski, M.S., Benko, F.A., Borys, D.J., Khan, A., McGarrity, T.J. & Gocke, C.D. (2000). Somatic mutation screening: identification of individuals harboring K-ras mutations with the use of plasma DNA. *Journal of the National Cancer Institute*, **92,** 918-923.

Kunz, C., Pebler, S., Otte, J. & Vonderahe, D. (1995). Differential regulation of plasminogen-activator and inhibitor gene- transcription by the tumor-suppressor p53. *Nucleic Acids Research*, **23,** 3710-3717.

Laird, P.W. (1997). Oncogenic mechanisms mediated by DNA methylation. *Molecular Medicine Today*, **5,** 223-229.

Lancaster, J.M., Wooster, R., Mangion, J., Phelan, C.M., Cochran, C., Gumbs, C., Seal, S., Barfoot, R., Collins, N., Bignell, G., Patel, S., Hamoudi, R., Larsson, C., Wiseman, R.W., Berchuck, A., Iglehart, J.D., Marks, J.R., Ashworth, A., Stratton, M.R. & Futreal, P.A. (1996). BRCA2 mutations in primary breast and ovarian cancers. *Nature Genetics*, **13,** 238-240.

Latil, A., Cussenot, O., G. Fournier, Baron, J.C. & Lidereau, R. (1995). Loss of heterozygosity at 7q31 is a frequent and early event in prostate cancer. *Clinical Cancer Research*, 1385-1389.

Launonen, V., Stenback, F., Puistola, U., Bloigu, R., Huusko, P., Kytola, S., Kauppila, A. & Winqvist, R. (1998). Chromosome 11q22.3-q25 LOH in ovarian cancer: Association with a more aggressive disease course and involved subregions. *Gynecologic Oncology*, **71,** 299-304.

Lee, J.H., Kavanagh, J.J., Wildrick, D.M., Wharton, J.T. & Blick, M. (1990). Frequent loss of heterozygosity on chromosomes 6q, 11, and 17 in human ovarian carcinomas. *Cancer Research*, **50,** 2724-2728.

Leon, S.A., Shapiro, B., Sklaroff, D.M. & Yaros, M.J. (1977). Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Research*, **37,** 646-650.

Li, S.B., Schwartz, P.E., Lee, W.H. & Yang-Feng, T.L. (1991). Allele loss at the retinoblastoma locus in human ovarian cancer. *Journal of the National Cancer Institute*, **83,** 637-640.

Liang, T.J., Reid, A.E., Xavier, R., Cardiff, R.D. & Wang, T.C. (1996). Transgenic expression of tpr-met oncogene leads to development of mammary hyperplasia and tumors. *Journal of Clinical Investigation*, **97,** 2872-2877.

Lievens, P.M., Tufaralli, C., Donady, J.J., Stagg, A. & Neufeld, E.J. (1997). CASP, a novel, highly conserved alternative-splicing product of the CDP/cut/cux gene, lacks cut-repeat and homeo DNA-binding domains and interacts with full-length CDP in vitro. *Gene*, **197,** 73-81.

Lin, J.C., Naujokas, M., Zhu, H., Nolet, S. & Park, M. (1998). Intron-exon structure of the MET gene and cloning of an alternatively-spliced Met isoform reveals frequent exon-skipping of a single large internal exon. *Oncogene*, **16,** 833-842.

Lin, J.C., Scherer, S.W., Tougas, L., Traverso, G., Tsui, L.C., Andrulis, I.L., Jothy, S. & Park, M. (1996). 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*, **13,** 2001-2008.

Lin, W.M., Forgacs, E., Warshal, D.P., Yeh, I.T., Martin, J.S., Ashfaq, R. & Muller, C.Y. (1998). Loss of heterozygosity and mutational analysis of the PTEN/MMAC1 gene in synchronous endometrial and ovarian carcinomas. *Clinical Cancer Research*, **4,** 2577-2583.

Liu, Y., Heyman, M., Wang, Y., Falkmer, U., Hising, C., Szekely, L. & Einhorn, S. (1994). Molecular analysis of the retinoblastoma gene in primary ovarian cancer cells. *International Journal of Cancer*, **58,** 663-667.

Lounis, H., MesMasson, A.M., Dion, F., Bradley, W.E.C., Seymour, R.J., Provencher, D. & Tonin, P.N. (1998). Mapping of chromosome 3p deletions in human epithelial ovarian tumors. *Oncogene*, **17,** 2359-2365.

Lu, K.H., Weitzel, J.N., Kodali, S., Welch, W.R., Berkowitz, R.S. & Mok, S.C. (1997). A novel 4-cM minimally deleted region on chromosome 11p15.1 associated with high grade non-mucinous epithelial ovarian carcinomas. *Cancer Research*, **57,** 387-390.

Macleod, K. (2000). Tumor suppressor genes. *Current Opinion in Genetics & Development*, **10,** 81-93.

Mailly, F., Berube, G., Harada, R., Mao, P.L., Phillips, S. & Nepveu, A. (1996). The human cut homeodomain protein can repress gene expression by two distinct mechanisms: active repression and competition for binding site occupancy. *Molecular and Cellular Biology*, **16,** 5346-5357.

Manchanda, N. & Schwartz, B.S. (1995). Interaction of single-chain urokinase and plasminogen-activator inhibitor type-1. *Journal Of Biological Chemistry*, **270,** 20032-20035.

Mao, L., Hruban, R.H., Boyle, J.O., Tockman, M. & D., S. (1994). Detection of oncogene mutations in sputum precedes diagnosis of lung cancer. *Cancer Research*, **54,** 1634-1637.

Martin, R.J.L., Hurlstone, A., Black, D.H., Hickey, I. & Russell, S.E.H. (1998). Microsatellite analysis of chromosome 7 in ovarian cancer identifies 7q31 as a region frequently deleted in benign disease. *British Journal Of Cancer*, **78,** 181.

Meden, H., Marx, D., Roegglen, T., Schauer, A. & Kuhn, W. (1998). Overexpression of the oncogene c-erbB-2 (HER2/neu) and response to chemotherapy in patients with ovarian cancer. *International Journal of Gynecological Pathology*, **17,** 61-65.

Meek, D.W. (1999). Mechanisms of switching on p53: a role for covalent modification. *Oncogene*, **18,** 7666-7675.

Merajver, S.D., Pham, T.M., Caduff, R.F., Chen, M., Poy, E.L., Cooney, K.A., Weber, B.L., Collins, F.S., Johnston, C. & Frank, T.S. (1995). Somatic mutations in the BRCA1 gene in sporadic ovarian-tumors. *Nature Genetics*, 439-443.

Merino, M.J. & Jaffe, G. (1993). Age contrast in ovarian pathology. *Cancer*, **71,** 537-544.

Merlo, A., Herman, J.G., Mao, L., Lee, D.J., Gabrielso, E., Burger, P.C., Baylin, S.B. & Sidransky, D. (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nature Medicine*, **1,** 686-692.

Ness, R.B. & Cottreau, C. (1999). Possible role of ovarian epithelial inflammation in ovarian cancer. *Journal of the National Cancer Institute*, **91,** 1459-1467.

Mertens, F., Johansson, B., Hoglund, M. & Mitelman, F. (1997). Chromosomal imbalance maps of malignant solid tumours: A cytogenetic survey of 3185 neoplasms. *Cancer Research*, **57,** 2765-2780.

Milner, B.J., Allan, L.A., Eccles, D.M., Kitchener, H.C., Leonard, R.C.F., Kelly, K.F., Parkin, D.E. & Haites, N.E. (1993). p53 mutation is a common genetic event in ovarian carcinoma. *Cancer Research*, **53,** 2128-2132.

Minaguchi, T., Mori, T., Kanamori, Y., Matsushima, M., Yoshikawa, H., Taketani, Y. & Nakamura, Y. (1999). Growth suppression of human ovarian cancer cells by adenovirus-mediated transfer of the *PTEN* gene. *Cancer Research*, **59,** 6063-6067.

Moghul, A., Lin, L., Beedle, A., Kanbour-Shakir, A., DeFrances, M.C., Liu, Y. & Zarnegar, R. (1994). Modulation of c-MET proto-oncogene (HGF receptor) mRNA abundance by cytokines and hormones: evidence for rapid decay of the 8 kb c-MET transcript. *Oncogene*, **9,** 2045-2052.

Mulcahy, H.E., Lyautey, J., Lederrey, C., Chen, X.Q., Anker, P., Alstead, E.M., Ballinger, A., Farthing, M.J.G. & Stroun, M. (1998). A prospective study of K-ras mutations in the plasma of pancreatic cancer patients. *Clinical Cancer Research*, **4,** 271-275.

Mullenbach, R., Lagoda, P.J.L. & Welter, C. (1989). An efficient salt chloroform extraction of DNA from blood and tissues. *Trends in Genetics.*, **5,** 391.

Nakajima, M., Sawada, H., Yamada, Y., Watanabe, A., Tatsumi, M., Yamashita, J., Matsuda, M., Sakaguchi, T., Hirao, T. & Nakano, H. (1999). The prognostic significance of amplification and overexpression of c-*met* and c-*erb* B-2 in human gastric carcinomas. *Cancer*, **85,** 1894-1902.

Nawroz, H., Koch, W., Anker, P., Stroun, M. & Sidransky, D. (1996). Microsatellite alterations in serum DNA of head and neck cancer patients. *Nature Medicine*, **2,** 1035-1037.

Nawroz, H., van der Riet, P., Hruban, R.H., Koch, W., Ruppert, J.M. & Sidransky, D. (1994). Allelotype of head and neck squamous cell carcinoma. *Cancer Research*, **54,** 1152-1154.

Ness, R.B. & Cottreau, C. (1999). Possible role of ovarian epithelial inflammation in ovarian cancer. *Journal of the National Cancer Institute*, **91,** 1459-1467.

Neufeld, E.J., Skalnik, D.G., Lievens, P.M.-J. & Orkin, S.H. (1992). Human CCAAT displacement protein is homologous to the *Drosophila* homeoprotein, *cut. Nature Genetics*, **1,** 50-55.

Niederacher, D., Yan, H.Y., An, H.X., Bender, H.G. & Beckmann, M.W. (1999). CDKN2A gene inactivation in epithelial ovarian cancer. *British Journal of Cancer*, **80,** 1920-1926.

Nishizuka, S., Tamura, G., Terashima, M. & Satodate, R. (1997). Commonly deleted region on the long arm of chromosome 7 in differentiated adenocarcinoma of the stomach. *British Journal of Cancer*, **76,** 1567-1571.

Obata, K., Morland, S.J., Watson, R.H., Hitchcock, A., Chenevix-Trench, G., Thomas, E.J. & Campbell, I.G. (1998). Frequent PTEN/MMAC mutations in endometrioid but not serous or mucinous epithelial ovarian tumors. *Cancer Research*, **58,** 2095-2097.

Ogata, T., Ayusawa, D., Namba, M., Takahashi, E., Oshimura, M. & Oishi, M. (1993). Chromosome 7 suppresses indefinite division of non-tumorigenic immortalised human fibroblast cell lines KMST-6 and SUSM-1. *Molecular Cell Biology*, **13,** 6036-6043.

Oiumet, H. & Loscalzo, J. (1994). Fibrinolysis. In *Thrombosis and Haematosis* pp. 127-143.
Oren, M. (1999). Regulation of the p53 tumor suppressor protein. *Journal of Biological Chemistry*, **274,** 36031-36034.

Osborne, R.J. & Leech, V. (1994). Polymerase chain reaction allelotyping of human ovarian cancer. *British Journal of Cancer*, **69,** 429-38.

Otis, C.N. (2000). Loss of heterozygosity in P53, BRCA1, and estrogen receptor genes and correlation to expression of P53 protein in ovarian epithelial tumors of different cell types and biological behaviour. *Human Pathology*, **31,** 233-238.

Papp, J., Csokay, B., Bosze, P., Zalay, Z., Toth, J., Ponder, B. & Olah, E. (1996). Allele loss from large regions of chromosome-17 is common only in certain histological subtypes of ovarian carcinomas. *British Journal of Cancer*, **74,** 1592-1597.

Parazzini, F., Franceschi, S., LaVecchia, C., Chatenoud, L. & DiCintio, E. (1997). The epidemiology of female genital tract cancers. *International Journal of Gynecological Cancer*, **7**, 169-181.

Park, M., Dean, M., Kaul, K., Braun, M.J., Gonda, M.A. & Vande Woude, G.F. (1987). Sequence of MET proto-oncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. *Proceedings of the National Academy of Sciences of the USA*, **84**, 6379-6383.

Pearson, P.L. & Van Der Luijt, R.B. (1998). The genetic analysis of cancer. *Journal of Internal Medicine*, **243**, 413-417.

Persson, I. (1996). Cancer risk in women receiving estrogen-progestin replacement therapy. *Maturitas*, **23**, S37-S45.

Pieretti, M., Cavalieri, C., Conway, P.S., Gallion, H.H., Powell, D.E. & Turker, M.S. (1995). Genetic alterations distinguish different types of ovarian tumors. *International Journal of Cancer*, **64**, 434-440.

Plebani, M., Herszenyi, L., Carraro, P., De Paoli, M., Roveroni, G., Cardin, R., Tulassay, Z., Naccarato, R. & Farinati, F. (1997). Urokinase-type plasminogen activator receptor in gastric cancer: tissue expression and prognostic role. *Clinical and Experimental Metastasis*, **15**, 418-425.

Puls, L.E., Powell, D.E., Depriest, P.D., Gallion, H.H., Hunter, J.E., Kryscio, R.J. & Vannagell, J.R. (1992). Transition from benign to malignant epithelium in mucinous and serous ovarian cystadenocarcinoma. *Gynecologic Oncology*, **47**, 53-57.

Purdie, D., Green, A., Bain, C., Siskind, V., Ward, B., Hacker, N., Quinn, M., Wright, G., Russell, P. & Susil, B. (1995). Reproductive and other factors and risk of epithelial ovarian-cancer - an Australian case-control study. *International Journal Of Cancer*, **62**, 678-684.

Purdie, D.M., Bain, C.J., Siskind, V., Russell, P., Hacker, N.F., Ward, B.G., Quinn, M.A. & Green, A.C. (1999). Hormone replacement therapy and risk of epithelial ovarian cancer. *British Journal of Cancer*, **81**, 559-563.

Riman, T., Persson, I. & Nilsson, S. (1998). Hormonal aspects of epithelial ovarian cancer: review of epidemiological evidence. *Clinical Endocrinology*, **49,** 695-707.

Risch, H.A., Jain, M., Marrett, L.D. & Howe, G.R. (1994). Dietary lactose intake, lactose-intolerance, and the risk of epithelial ovarian-cancer in southern Ontario (Canada). *Cancer Causes & Control*, **5,** 540-548.

Risch, H.A. (1998). Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. *Journal of the National Cancer Institute*, **90,** 1774-1786.

Robert, C., Bolon, I., Gazzeri, S., Veyrenc, S., Brambilla, C. & Brambilla, E. (1999). Expression of plasminogen activator inhibitors 1 and 2 in lung cancer and their role in tumor progression. *Clinical Cancer Research*, **5,** 2094-2102.

Rodabaugh, K.J., Biggs, R.B., Qureshi, J.A., Barrett, A.J., Welch, W.R., Bell, D.A., Berkowitz, R.S. & Mok, S.C. (1995). Detailed deletion mapping of chromosome 9p and p16 gene alterations in human borderline and invasive epithelial ovarian tumors. *Oncogene*, **11,** 1249-1254.

Rogers, J.C. (1976). Characterization of DNA excreted from phytohaemagglutinin stimulated lymphocytes. *Journal of Experimental Medicine*, **143,** 1249-1264.

Rong, S., Bodescot, M., Blair, D., Dunn, J., Nakamura, T., Mizuno, K., Park, M., Chan, A., Aaronson, S. & Vande Woude, G.F. (1992). Tumorigenicity of the met proto-oncogene and the gene for hepatocyte growth factor. *Molecular Cellular Biology*, **12,** 5152-5158.

Roy, W.J., Watson, R.H., Hitchcock, A. & Campbell, I.G. (1997). Frequent loss of heterozygosity on chromosomes 7 and 9 in benign epithelial ovarian tumours. *Oncogene*, **15,** 2031-2035.

Saito, M., Okamoto, A., Kohno, T., Takakura, S., Shinozaki, H., Isonishi, S., Yasuhara, T., Yoshimura, T., Ohtake, Y., Ochiai, K., Yokota, J. & Tanaka, T. (2000). Allelic imbalance and mutations of the *PTEN* gene in ovarian cancer. *International Journal of Cancer*, **85,** 160-165.

Sakamoto, T., Nomura, N., Mori, H. & Wake, N. (1996). Poor correlation with loss of heterozygosity on chromosome 17p and p53 mutations in ovarian cancers. *Gynecologic Oncology*, **63,** 173-179.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular cloning: a laboratory manual, Vol 1, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.*

Saretzki, G., Hoffmann, U., Rohlke, P., Psille, R., Gaigal, T., Keller, G., Hofler, H., Loning, T., Petersen, I. & Dietel, M. (1997). Identification of allelic losses in benign, borderline, and invasive epithelial ovarian tumors and correlation with clinical outcome. *Cancer*, **80,** 1241-1249.

Sato, T., Saito, H., Morita, R., Koi, S., Lee, J.H. & Nakamura, Y. (1991). Allelotype of human ovarian cancer. *Cancer Research*, **51,** 5118-5122.

Scherer, S.W., Neufeld, E.J., Lievens, J., Orkin, S.H., Kim, J. & Tsui, L.C. (1993). Regional localisation of the CCAAT displacement protein gene (*CUTL1*) to 7q22 by analysis of somatic cell hybrids. *Genomics*, **15,** 695-696.

Schildkraut, J.M., Schwingl, P.J., Bastos, E., Evanoff, A. & Hughes, C. (1996). Epithelial ovarian cancer risk among women with polycystic ovary syndrome. *Obstetrics and Gynecology*, **88,** 554-559.

Schmalfeldt, B., Kuhn, W., Reuning, U., Pache, L., Dettmar, P., Schmitt, M., Janicke, F., Hofler, H. & Graeff, H. (1995). Primary tumor and metastasis in ovarian-cancer differ in their content of urokinase-type plasminogen-activator, its receptor, and inhibitors type-1 and type-2. *Cancer Research*, **55,** 3958-3963.

Schmidt, L., Duh, F.M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S.W., Zhuang, Z.P., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U.R., Feltis, J.T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C.J.M., Walther, M.M., Tsui, L.C., Geil, L., Orcutt, M.L., Stackhouse, T., Lipan, J., Slife, L., Brauch, H., Decker, J., Niehans, G., Hughson, M.D., Moch, H., Storkel, S., Lerman, M.I., Linehan, W.M. & Zbar, B. (1997). Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nature Genetics*, **16,** 68-73.

Schmidt, L., Junker, K., Nakaigawa, N., Kinjerski, T., Weirich, G., Miller, M., Lubensky, I., Neumann, H.P.H., Brauch, H., Decker, J., Vocke, C., Brown, J.A., Jenkins, R., Richard, S., Bergerheim, U., Gerrard, B., Dean, M., Linehan, W.M. & Zbar, B. (1999). Novel mutations of the MET proto-oncogene in papillary renal carcinomas. *Oncogene*, **18,** 2343-2350.

Schmidt, L., Junker, K., Weirich, G., Glenn, G., Choyke, P., Lubensky, I., Zhuang, Z.P., Jeffers, M., Woude, G.V., Neumann, H., McClellan, W., Linehan, W.M. & Zbar, B. (1998). Two North American families with hereditary papillary renal carcinoma and identical novel mutations in the MET proto-oncogene. *Cancer Research*, **58,** 1719-1722.

Seol, D.-W., Chen, Q., Smith, M.L. & Zarnegar, R. (1999). Regulation of the c-MET proto-oncogene promoter by p53. *Journal of Biological Chemistry*, **274,** 3565-3572.

Shapiro, B., Chakrabarty, M., Cohn, E.M. & Leon, S.A. (1983). Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. *Cancer*, **51,** 2116-2120.

Sheffield, V.C., Beck, J.S., Kwitek, A.E., Sandstorm, D.W. & Stone, E.M. (1993). The sensitivity of single-stranded conformation polymorphism analysis for the detection of single base substitutions. *Genomics*, **16,** 325-332.

Shridhar, V., Sun, Q.C., Miller, O.J., Kalemkerian, G.P., Petros, J. & Smith, D.I. (1997). Loss of heterozygosity on the long arm of human chromosome 7 in sporadic renal cell carcinomas. *Oncogene*, **15,** 2727-2733.

Sidransky, D. (1996). Is human *patched* the gatekeeper of common skin cancers? *Nature Genetics*, **14,** 7-8.

Sidransky, D. (1997). Nucleic acid-based methods for the detection of cancer. *Science*, **278,** 1054-1058.

Skalnik, D.G., Strauss, E.C. & Orkin, S.H. (1991). CCAAT displacement protein as a repressor of the myelomonocytic-specific gp91-phox gene promoter. *Journal of Biological Chemistry*, **266,** 16736-16744.

Sorenson, G.D., Pribish, D.M., Valone, F.H., Memoli, V.A., Bzik, D.J. & Yao, S.L. (1994). Soluble normal and mutated DNA sequences from single-copy genes in human blood. *Cancer Epidemiology, Biomarkers and Prevention*, **3,** 67-71.

Sourla, A., Polychronakos, C., Zeng, W.R., Nepveu, A., Kukuvitis, A., Naud, F. & Koutsilieris, M. (1996). Plasminogen activator inhibitor 1 messenger RNA expression and molecular evidence for del(7)(q22) in uterine leiomyomas. *Cancer Research*, **56,** 3123-3128.

Soussi, T. (1996). The p53 tumour suppressor gene: a model for molecular epidemiology of human cancer. *Molecular Medicine Today*, **2,** 32-37.

Stack, M.S., Ellerbroek, S.M. & Fishman, D.A. (1998). The role of proteolytic enzymes in the pathology of epithelial ovarian carcinoma (Review). *International Journal Of Oncology*, **12,** 569-576.

Stefansson, S., Haudenschild, C.C. & Lawrence, D.A. (1998). Beyond fibrinolysis: the role of plasminogen activator inhibitor-1 and vitronectin in vascular wound healing. *Trends in Cardiovascular Medicine*, **8,** 175-180.

Suzuki, M., Saito, S., Saga, Y., Ohwada, M. & Sato, I. (1998). Loss of heterozygosity on chromosome 6q27 and p53 mutations in epithelial ovarian cancer. *Medical Oncology*, **15,** 119-123.

Takahashi, S., Shan, A.L., Ritland, S.R., Delacey, K.A., Bostwick, D.G., Lieber, M.M., Thibodeau, S.N. & Jenkins, R.B. (1995). Frequent loss of heterozygosity at 7q31.1 in primary prostate-cancer is associated with tumor aggressiveness and progression. *Cancer Research*, **55,** 4114-4119.

Talerman, A. (1992). Ovarian pathology. *Current Opinion in Obstetrics and Gynecology*, **4,** 608-615.

Tanahashi, N., Suzuki, M., Fujiwara, T., Takahashi, E., Shimbara, N., Chung, C.H. & Tanaka, K. (1998). Chromosomal localization and immunological analysis of a family of human 26S proteosomal ATPases. *Biochemical and Biophysical Research Communications*, **243,** 229-232.

Tanner, B., Hengstler, J.G., Luch, A., Meinert, R., Kreutz, E., Arand, M., Wilkens, C., Hofmann, M., Oesch, F., Knapstein, P.G. & Becker, R. (1998). C-myc mRNA expression in epithelial ovarian carcinomas in relation to estrogen receptor status, metastatic spread, survival time, FIGO stage, and histologic grade and type. *International Journal of Gynecological Pathology*, **17,** 66-74.

Tavassoli, M., Steingrimsdottir, H., Pierce, E., Jiang, X., Alagoz, M., Farzaneh, F. & Campbell, I.G. (1996). Loss of heterozygosity on chromosome 5q in ovarian-cancer is frequently accompanied by tp53 mutation and identifies a tumor- suppressor gene locus at 5q13.1-21. *British Journal Of Cancer*, **74,** 115-119.

Taylor, R.R., Linnoila, R.I., Gerardts, J., Teneriello, M.G., Nash, J.D., Park, R.C. & Birrer, M.J. (1995). Abnormal expression of the retinoblastoma gene in ovarian neoplasms and correlation to p53 and k-ras mutations. *Gynecologic Oncology*, **58,** 307-311.

Todd, M.C., Sclafani, R.A. & Langan, T.A. (2000). Ovarian cancer cells that co-express endogenous Rb and p16 are insensitive to overexpression of functional p16 protein. *Oncogene*, **19,** 258-264.

Tosi, S., Scherer, S.W., Giudici, G., Czepulkowski, B., Biondi, A. & Kearney, L. (1999). Delineation of multiple deleted regions in 7q in myeloid disorders. *Genes Chromosomes & Cancer*, **25,** 384-392.

Toyota, M. & Issa, J-P. (1999). CpG island methylator phenotypes in ageing and cancer. *Seminars in Cancer Biology*, **9,** 349-357.

Turkmen, B., Schmitt, M., Schmalfeldt, B., Trommler, P., Hell, W., Creutzburg, S., Graeff, H. & Magdolen, V. (1997). Mutational analysis of the genes encoding urokinase-type plasminogen activator (uPA) and its inhibitor PAI-1 in advanced ovarian cancer. *Electrophoresis*, **18,** 686-689.

Turner, C. (1971). *General Endocrinology*. WB Saunders: Philadelphia.

Ueki, T., Fujimoto, J., Suzuki, T., Yamamoto, H. & Okamoto, E. (1997). Expression of hepatocyte growth factor and its receptor *c-met* proto-oncogene in hepatocellular carcinoma. *Hepatology*, **25,** 862-866.

VanderHeijden, O., Chiu, H.C., Park, T.C., Takahashi, H., LiVolsi, V.A., Risinger, J.I., Barrett, J.C., Berchuck, A., Evans, A.C., Behbakht, K., Menzin, A.W., Liu, P.C., Benjamin, I., Morgan, M.A., King, S.A., Rubin, S.C. & Boyd, J. (1998). Allelotype analysis of uterine leiomyoma: Localization of a potential tumor suppressor gene to a 4-cM region of chromosome 7q. *Molecular Carcinogenesis*, **23,** 243-247.

Vasioukhin, V., Anker, P., Maurice, P., Lyautey, J., Lederrey, C. & Stroun, M. (1994). Point mutations of the N-*ras* gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. *British Journal of Haematology*, **86,** 774-779.

Viel, A., Giannini, F., Tumiotto, L., Sopracordevole, F., Visentin, M.C. & Boiocchi, M. (1992). Chromosomal localisation of two putative 11p oncosuppressor genes involved in human ovarian tumours. *British Journal of Cancer*, **66,** 1030-1036.

Villeneuve, J.B., Silverman, M.B., Alderete, B., Cliby, W.A., Li, H.Z., Croghan, G.A., Podratz, K.C. & Jenkins, R.B. (1999). Loss of markers linked to BRCA1 precedes loss at important cell cycle regulatory genes in epithelial ovarian cancer. *Genes Chromosomes & Cancer*, **25,** 65-69.

Wang, Z.J., Churchman, M., Campbell, I.G., Xu, W.H., Yan, Z.Y., McCluggage, W.G., Foulkes, W.D. & Tomlinson, I.P.M. (1999). Allele loss and mutation screen at the Peutz-Jeghers (LKB1) locus (19p13.3) in sporadic ovarian tumours. *British Journal Of Cancer*, **80,** 70-72.

Watson, R.H., Neville, P.J., Roy, W.J., Hitchcock, A. & Campbell, I.G. (1998). Loss of heterozygosity on chromosomes 7p, 7q, 9p and 11q is an early event in ovarian tumorigenesis. *Oncogene*, **17,** 207-212.

Webster, M.A., MartinSoudant, N., Nepveu, A., Cardiff, R.D. & Muller, W.J. (1998). The induction of uterine leiomyomas and mammary tumors in transgenic mice expressing polyomavirus (PyV) large T (LT) antigen is associated with the ability of PyV LT antigen to form specific complexes with retinoblastoma and CUTL1 family members. *Oncogene*, **16,** 1963-1972.

Weinberg, R.A. (1996). How cancer arises. *Scientific American*, **275,** 62-70.

Weitzel, J.N., Patel, J., Smith, D.M., Goodman, A., Safaii, H. & Ball, H.G. (1994). Molecular genetic changes associated with ovarian cancer. *Gynecologic Oncology*, **55,** 245-252.

Wertheim, I., Tangir, J., Muto, M.G., Welch, W.R., Berkowitz, R.S., Chen, W.Y. & Mok, S.C. (1996). Loss of heterozygosity of chromosome 17 in human borderline and invasive epithelial ovarian tumors. *Oncogene*, **12,** 2147-2153.

Wong, C., Hempling, R.E., Piver, M.S., Natarajan, N. & Mettlin, C.J. (1999). Perineal talc exposure and subsequent epithelial ovarian cancer: A case control study. *Obstetrics and Gynecology*, **93,** 372-376.

Wright, K., Wilson, P.J., Kerr, J., Do, K., Hurst, T., Khoo, S.K., Ward, B. & Chenevix-Trench, G. (1998). Frequent loss of heterozygosity and three critical regions on the short arm of chromosome 8 in ovarian adenocarcinomas. *Oncogene*, **17,** 1185-1188.

Wu, M.L., Whittemore, A.S., Paffenberger Jr, R.S., Sarles, D.L., Kampert, J.B., Grosser, S., Jung, D.L., Ballon, S., Hendrickson, M. & Mohle-Boetani, J. (1988). Personal and environmental characteristics related to epithelial ovarian cancer. I. Reproductive and menstrual events and oral contraceptive use. *American Journal of Epidemiology*, **128,** 1216-1227.

Yamada, T., Nakamori, S., Ohzato, H., Oshima, S., Aoki, T., Higaki, N., Sugimoto, K., Agaki, K., Fujiwara, Y., Nishisho, I., Sakon, M., Gotoh, M. & Monder, M. (1998). Circulating DNA K-ras mutation in pancreatic adenocarcinoma. *Clinical Cancer Research*, **4,** 1527-1532.

Yamashita, J., Ogawa, M., Yamashita, S., Nomura, K., Kuramoto, M., Saisho, T. & Shin, S. (1994). Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. *Cancer Research*, **54,** 1630-1633.

Yang-Feng, T.L., Li, S., Han, H. & Schwartz, P.E. (1992). Frequent loss of heterozygosity on chromosomes Xp and 13q in human ovarian cancer. *International Journal of Cancer*, **52,** 575-580.

Yasuda, T., Sakata, Y., Kitamura, K., Morita, M. & Ishida, T. (1997). Localization of plasminogen activators and their inhibitors in squamous cell carcinomas of the head and neck. *Head & Neck*, **19,** 611-616.

Yi, J. & Beckerle, M.C. (1998). The human *TRIP6* gene encodes a LIM domain protein and maps to chromosome 7q22, a region associated with tumorigenesis. *Genomics*, **49,** 314-316.

Yokouchi, M., Suzuki, R., Masuhara, M., Komiya, S., Inoue, A. & Yoshimura, A. (1997). Cloning and characterization of APS, an adaptor molecule containing PH and SH2 domains that is tyrosine phosphorylated upon B-cell receptor stimulation. *Oncogene*, **14,** 7-15.

Zborovskaya, I., Gasparian, A., Karseladze, A., Elcheva, I., Trofimova, E., Driouch, K., Trassard, M., Tatosyan, A. & Lidereau, R. (1999). Somatic genetic alterations (LOH) in benign, borderline and invasive ovarian tumours: intratumoral molecular heterogeneity. *International Journal of Cancer*, **82,** 822-826.

Zeng, W.R., Scherer, S.W., Koutsilieris, M., Huizenga, J.J., Filteau, F., Tsui, L.C. & Nepveu, A. (1997). Loss of heterozygosity and reduced expression of the CUTL1 gene in uterine leiomyomas. *Oncogene*, **14,** 2355-2365.

Zeng, W.R., Soucie, E., Moon, N.S., Martin-Soudant, N., Berube, G., Leduy, L. & Nepveu, A. (2000). Exon/intron structure and alternative transcripts of the CUTL1 gene. *Gene*, **241,** 75-85.

Zeng, W.R., Watson, P., Lin, J., Jothy, S., Lidereau, R., Park, M. & Nepveu, A. (1999). Refined mapping of the region of loss of heterozygosity on the long arm of chromosome 7 in human breast cancer defines the location of a second tumor suppressor gene at 7q22 in the region of the CUTL1 gene. *Oncogene*, **18,** 2015-2021.

Zenklusen, J.C., Conti, C.J. & Green, E. (2000a). Cloning and characterization of a broad range tumor suppressor gene on chromosome 7q31.1. In *91st Annual meeting of the American Association for Cancer Research*, Vol. 41. pp. 777-778: San Francisco, California.

Zenklusen, J.C., Hodges, L.C., LaCava, M., Green, E.D. & Conti, C.J. (2000b). Definitive functional evidence for a tumor suppressor gene on human chromosome 7q31.1 neighbouring the Fra7G site. *Oncogene*, **19,** 1729-1733.

Zenklusen, J.C., Oshimura, M., Barrett, J.C. & Conti, C.J. (1994a). Inhibition of tumorigenicity of a murine squamous cell carcinoma (SCC) cell line by a putative tumor suppressor gene on human chromosome 7. *Oncogene*, **9,** 2817-2825.

Zenklusen, J.C., Thompson, J.C., Kleinszanto, A.J.P. & Conti, C.J. (1995a). Frequent loss of heterozygosity in human primary squamous-cell and colon carcinomas at 7q32.1 - evidence for a broad range tumor- suppressor gene. *Cancer Research*, **55,** 1347-1350.

Zenklusen, J.C., Thompson, J.C., Troncoso, P., Kagan, J. & Conti, C.J. (1994b). Loss of heterozygosity in human primary prostate carcinomas - a possible tumor-suppressor gene at 7q31.1. *Cancer Research*, **54,** 6370-6373.

Zenklusen, J.C., Weitzel, J.N., Ball, H.G. & Conti, C.J. (1995b). Allelic loss at 7q31.1 in human primary ovarian carcinomas suggests the existence of a tumor-suppressor gene. *Oncogene*, **11,** 359-363.

Zhang, J.S., Nelson, M., McIver, B., Hay, I.D., Goellner, J.R., Grant, C.S., Eberhardt, N.L. & Smith, D.I. (1998). Differential loss of heterozygosity at 7q31.2 in follicular and papillary thyroid tumors. *Oncogene*, **17,** 789-793.

Zhang, L., Cui, X., Schmitt, K., Hubert, R. & Naridie, W. (1992). Whole genome amplification from a single cell: implications for genetic analysis. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, **89,** 5847-5851.

Zheng, W., Luo, F., Lu, J.J., Baltayan, A., Press, M.F., Zhang, Z.F. & Pike, M.C. (2000). Reduction of BRCA1 expression in sporadic ovarian cancer. *Gynecologic Oncology*, **76,** 294-300.

Zhuang, Z.P., Park, W.S., Pack, S., Schmidt, L., Vortmeyer, A.O., Pak, E., Pham, T., Weil, R.J., Candidus, S., Lubensky, I.A., Linehan, W.M., Zbar, B. & Weirich, G. (1998). Trisomy 7-harbouring non-random duplication of the mutant MET allele in hereditary papillary renal carcinomas. *Nature Genetics*, **20,** 66-69.