**Folic acid supplementation *in vitro* induces cell type-specific changes in BRCA1 and BRCA 2 mRNA Expression, but does not alter DNA methylation of their promoters or DNA repair§**

*R. Jordan Pricea, Karen A. Lillycropb, Graham C. Burdgea\**

*aAcademic Unit of Human Health and Development, Faculty of Medicine University of Southampton, Southampton, UK.*

*bCentre for Biological Sciences, Faculty of Natural and Environmental Sciences, University of Southampton, Southampton, UK*.

\*Corresponding author at:- Institute of Developmental Sciences Building (MP887), Academic Unit of Human Health and Development, Faculty of Medicine, University of Southampton, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, UK. Tel: +44(0)2380795259; Fax: +44(0)23804221; E-mail address:g.c.burdge@soton.ac.uk

**§**This work as supported byan award (2011/42) by the World cancer Research Fund UK (WCRF UK) to GCB and KAL.

**ABSTRACT**

Dietary supplementation with folic acid (FA) has been shown to induce opposing effects on cancer-related outcomes. The mechanism underlying such heterogeneity is unclear. We hypothesized that FA supplementation induces changes in breast cancer-associated (BRCA) genes 1 and 2 expression and function through altered epigenetic regulation in a cell-type dependent manner. We investigated the effect of treating normal and cancer cells with physiologically-relevant FA concentrations on the mRNA and protein expression, capacity for DNA repair and DNA methylation of BRCA1 and 2. FA treatment induced dose-related increases in BRCA1 mRNA expression in HepG2, Huh-7D12, Hs578T, and JURKAT and in BRCA2 in HepG2, Hs578T, MCF7 and MDA-MB-157 cells. FA did not affect the corresponding normal cells or on any of the ovarian cell lines. FA induced increased BRCA1 protein expression in Hs578T, but not HepG2 cells, while BRCA2 protein levels were undetectable. FA treatment did not alter DNA repair in liver-derived cells, while there were transient effects on breast-derived cells. There was no effect of FA treatment on BRCA1 or BRCA2 DNA methylation, although there was some variation in the methylation of specific CpG loci between some cell lines. Overall, these findings show that the effects of FA on BRCA-related outcomes differ between cells lines, but the biological consequences of induced changes in BRCA expression appear to be at most limited.

*Abbreviations: ECCAC, European collection of animal cell cultures; EGF,* epidermal growth factor; *BRCA, breast cancer-associated gene;* FA, folic acid; FBS, fetal bovine serum; PBMC, peripheral blood mononuclear cells;

*Key words*: BRCA; folic acid; cancer; gene expression; DNA methylation; DNA repair

**1. INTRODUCTION**

Folic acid (FA) is the synthetic form of folate that is used widely as a nutritional supplement or in dietary fortification. The effect of FA on cancer risk is unclear and there are conflicting reports that suggest that FA intake is either associated with increased or decreased risk of cancer, in particular colorectal cancer [1]. FA fortification has been associated with a lower incidence of neuroblastoma, but had no effect on lymphoblastic leukaemia or hepatoblastoma [2]. Maternal FA intake has been associated negatively with risk of childhood neuroectodermal tumours [3] and neuroblastoma [4]. In adults, supplementation with 5mg FA/day for reduced reoccurrence of adenomas by 56% [5] compared to placebo, while co-supplementation of FA and aspirin had no significant effect on reoccurrence [6, 7]. The extent to which such effects are associative rather than causal is unclear [8]. Furthermore, the incidence of colorectal cancer in the United States of America and Canada appeared to increase transiently following the introduction of mandatory FA fortification [9]. This positive association between FA and risk of colon cancer is supported by an increase in incidence by 2.6 to 2.9 between pre and post introduction of FA fortification in Chile [10]. In contrast, FA intake was negatively associated with colorectal cancer risk in a case-cohort study of 5,629 women [11]. A meta-analysis of randomised controlled trials of FA supplementation based on thirteen studies failed to show a significant effect on total cancer incidence, or the incidence of specific cancers [12]. Women who received a supplement containing FA and vitamins B12 and B6 showed reduction in risk of total invasive cancer and of breast cancer, although these effects were not statistically significant [13]. While such heterogeneity may reflect differences between study cohorts and between the design of the intervention, and level of FA given is also possible that different tissues or cancer subtypes may differ in their response to FA.

Tetrahydrofolate is the biologically active metabolite of FA and is a co-factor for the rate limiting reaction in the supply of methyl groups to the homocysteine/ methionine remethylation cycle in which DNA is a terminal acceptor. Epigenetic regulation of transcription by DNA methylation involves differential methylation of CpG dinucleotides in gene promoters as well as covalent modifications of histones and non-coding RNAs [14]. Methylation of gene promoters is a relatively stable epigenetic mark that is induced during development. However, some genes retain epigenetic plasticity beyond early development and are susceptible to interventions in later life, including folic acid intake [15]. Furthermore, aging is associated with carcinogenesis with both global hypomethylation and hypermethylation of tumour suppressor genes [16]. Diets low or enriched in folic acid have been shown to induce altered DNA methylation in experimental models [17-20] and I humans [21]. Thus variations in folate status or FA intake may modify cancer risk by altering the epigenetic regulation of genes.

The breast cancer associated genes (BRCA) 1 and 2 are tumour suppressor genes with several key functions related to maintaining DNA integrity [22]. The proteins encoded by these genes are expressed in all cells and are critical for repair of single and double stranded DNA breaks. Mutations in the BRCA1 and 2 genes have been implicated in primarily in the development of breast and ovarian cancers, but germline mutation carriers of BRCA1 and BRCA2 also have a small increased risk of stomach, pancreas, prostate and colon cancer [23]. Impaired BRCA 1 and 2 activities lead to gross chromosomal rearrangements and gene dysregulation [22]. Approximately 90% of cases of breast and ovarian cancer are sporadic and are not associated with mutations in the BRCA genes [24]. In these cases, reduced BRCA1 activity involves hypermethylation of its promoter leading to transcriptional repression [25-29]. In contrast, the BRCA2 promoter has been shown to be hypomethylated and over-expressed in ovarian cancers compared to normal tissue [29]. Thus one possible additional source of heterogeneity in the effects of FA on cancer risk is the differential effects on the epigenetic regulation of BRCA 1 and 2 leading to genomic instability [30-32]. In order to inform nutritional guidelines about FA intake and cancer risk, it is important to know if FA supplementation induces differential effects on the epigenetic regulation of BRCA 1 and 2, and whether such effects are specific to individual tissues or cancer subtypes and if such effects differ between cancer and normal cells.

We tested the hypothesis that treatment with FA induces differential effects of the epigenetic regulation of BRCA 1 and 2 transcription leading to variation between cell types in capacity for DNA repair. To address this, normal and cancer cells were treated *in vitro* with concentrations of FA that were within the range of unmetabolised FA in plasma (0 – 100nmoles/l) [33-37] reported in humans taking ≥ 200μg/day FA per day on the mRNA expression of BRCA 1 and 2. Cells arising from different tissues were tested in order to determine whether any effects of FA on BRCA 1 and 2 were specific to a specific cancer type or subtype. In order to determine whether any changes in BRCA 1 or 2 mRNA expression were associated altered function of these genes, we investigated the effect of FA treatment on BRCA 1 and 2 protein expression and on the DNA methylation of their promoters, and on capacity of cells to repair radiation-induced DNA damage.

**2. Methods and materials**

*2.1 Cell lines*

SK-HEP-1 human liver adenocarcinoma, PLC/PRF/5 human liver hepatoma, Huh-7D12 human hepatocellular carcinoma, HMT-3522 S1 human breast epithelia, Hs578T human breast adenocarcinoma, MDA-MB-157 human breast medulla carcinoma, MDA-MB-231 human breast adenocarcinoma, A2780 human ovarian carcinoma, COV434 human ovarian granulosa tumour and PEA1 human ovarian carcinoma were obtained from the European Collection of Cell Cultures (ECACC). MCF10a human non-tumourigenic breast epithelia were obtained from American Type Culture Collection, peripheral blood mononuclear cells (PBMC) were obtained from Stem Cell Technologies, and primary hepatocytes were obtained from Life Technologies. HepG2 human hepatocellular carcinoma, MCF7 human breast adenocarcinoma, THP1 human acute monocytic leukaemia and JURKAT human acute T cell leukaemia cells were from our archive which was derived originally from cells purchased from ECACC.

*2.2 Cell culture procedures*

All cell lines were cultured at 37oC in an atmosphere containing 5% (v/v) CO2, in Dulbecco’s Modified Eagle Medium without folic acid (Sigma), supplemented with 10% (v/v) fetal bovine serum (FBS), 2mM glutamine, 10U/ml penicillin and 100µg/ml streptomycin. The media for the MCF10a cell line was further supplemented with 20ng/ml epidermal growth factor (EGF) and 100µg/ml hydrocortisone. The media for the HMT-3522 S1 cell line was also supplemented with 10ng/ml EGF and 500ng/ml hydrocortisone.

*2.3 Measurement of BRCA 1 and 2 mRNA expression by real time RTPCR*

To determine the effect of FA supplementation on BRCA1 and 2 mRNA expression, all cell lines were treated with 0, 25, 50, 75 or 100 nmoles/l FA for 72 hours before harvesting in TRI Reagent (Sigma) according to the manufacturer’s instructions. Background folate concentration derived from FBS was 1.5 nmoles/l. Measurement of mRNA expression was carried out essentially as described previously [38]. Briefly, complementary DNA was prepared using Moloney-murine leukaemia virus reverse transcriptase (Promega). Real time RTPCR was performed with SYBR Green JumpStart Taq ReadyMix (Sigma) to amplify BRCA1 and BRCA2 mRNA using QuantiTect Primer assays (Qiagen) QT00039305 and QT00008449, respectively. mRNA levels were determined by the standard curve method [39] and normalised to cyclophilin expression (QuantiTect assay QT01866137) [38]. All samples were analysed in duplicate.

*2.4 Measurement of BRCA 1 and 2 protein expression by western blotting*

BRCA1 and BRCA2 protein levels were assessed in cell lines in which FA treatment induced significant changes in BRCA1 and/or BRCA2 mRNA expression. Cells were treated with either 0 or 100 nmoles/l FA. Protein extracts were prepared in 50mM Tris pH8, 150mM NaCl, 0.5% sodium deoxycholate and 1% nonidet-P40 containing 10% (v/v) Protease Inhibitor Cocktail (Sigma). Protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific). Western blot analysis of protein expression was performed as described previously [40]. 25µg of cell extract was resolved by SDS PAGE using a 4-15% polyacrylamide gradient gel (Bio-Rad) and transferred to PVDF membrane (Amersham) in 25mM Tris pH8, 192mM glycine, 20% (v/v) methanol and 0.1% (w/v) SDS for 3hr at 4oC. The membrane was blocked with 5% (w/v) skimmed milk powder/tris-buffered saline (TBS; 10mM Tris pH8.0, 150mM NaCl) containing 0.1% (v/v) Tween-20 for 1hr at room temperature and then incubated overnight at 4oC with anti-BRCA1 antibody (1µg/ml; Abcam) or anti-BRCA2 antibody (2 µg/ml; Abcam) in 2% (w/v) skimmed milk powder/TBS/0.1% Tween-20. The membrane was then washed four times for 10min each in TBS/0.1% Tween-20 before being incubated with a horseradish peroxidase- conjugated anti-mouse secondary antibody (1:50 000; Sigma) in 2% (w/v) skimmed milk powder/TBS/0.1% Tween-20 for 1hr at room temperature. After washing in TBS/0.1% Tween-20, the protein bands were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and were visualised on a VersaDoc 4000MP imaging system (Bio-Rad). Protein molecular weights were determined using a Fermentas Spectra Multicolor Broad Range protein ladder (Fisher Scientific) and protein band intensities were analysed using ImageJ software (NIH). Anti-β-actin (1:2000; Sigma) was used as the primary antibody to normalise for differences in protein loading.

*2.5 Measurement of BRCA 1 and 2 promoter methylation by sodium bisulphite pyrosequencing*

The regions of the BRCA1 and 2 genes that were analysed for DNA methylation by pyrosequencing are indicated in Fig. 1. The region of the BRCA1 promoter that was analysed has been shown to be unmethylated in normal cells and hypermethylated in cancer [41], to be involved in the regulation of transcription [42] and to contain the minimal promoter [43] and several transcription factor binding sites [44-46]. The region of the BRCA 2 gene that was analysed has previously been shown to be hypermethylated in sporadic breast cancers [47]. This region encompasses the BRCA2 minimal promoter region [48] and contains a number of transcription factor binding sites that regulate BRCA2 expression [48-50].

The level of methylation of individual CpG dinucleotides in the BRCA1 (Fig. 1A) and BRCA2 (Fig. 1B) promoters was measured using bisulphite pyrosequencing essentially as described previously [38]. Genomic DNA was isolated and bisulphite conversion was performed using the EZ DNA Methylation-Gold kit (ZymoResearch). The bisulphite-modified DNA was then amplified using the primers listed in Table 1 with KAPA2G Robust HotStart ReadyMix (Labtech). Biotinylation of the PCR products allowed them to be immobilised on streptavidin-sepharose beads (GE Healthcare), washed and denatured, and then released into annealing buffer containing the sequencing primers in Table 1. Pyrosequencing was performed using PyroMark Gold Q96 CDT reagents (Qiagen) on a PSQ 96MA machine (Biotage) and the percentage methylation for each CpG loci was calculated using the PyroQ CpG software (Biotage). Internal controls were included within each pyrosequencing assay to verify bisulphite-conversion efficiency. Human genomic DNA methylated at 100% of CpG loci (Millipore) or at 0% CpG loci (Promega) were included for each assay.

*2.6 Measurement of DNA repair*

Cells were treated with either 0 or 100 nmoles/l FA for 72 hours prior to irradiation with UVC (λ = 254nm) at a dose of 0.1J/m2/s for 18 seconds (1.8J/m2) using a CL-1000 UV X-linker (UVP). Cells were cultured for a further 0, 1 or 4 hours and then collected in Ca2+ and Mg2+-free PBS at approximately 105cells/ml. Cell viability was determined using trypan blue exclusion (in all experiments viability was ≥90%). The single cell gel electrophoresis assay [51] was performed under alkaline conditions using a Comet Assay Kit (Trevigen). All steps were performed in low light level conditions and at 4oC, unless otherwise stated. Cells were combined with molten low melting point agarose at 37oC at a ratio of 1:10 (v/v), 50µl was spread onto a CometSlide and the agarose was left to adhere for 30min. The slides were immersed in cold Lysis Solution overnight and then in freshly prepared, cold Alkaline Solution (300nM NaOH, 1mM EDTA, pH13) for 1 hour. Slides were then placed in a horizontal electrophoresis tank on ice in Alkaline Solution and electrophoresis was performed at 15V (1V/cm), 300mA for 1 hour. The slides were washed twice in distilled water and then in 70% ethanol, before being dried for 20min at 37oC. SYBR Gold (Life Technologies) was used to stain the DNA for 30min at RT and the slides were then rinsed with distilled water before being completely dried at 37oC. Comets were imaged using a Nikon D3100 DSLR camera attached to an Axiovert 25CFL microscope (Zeiss). For each treatment, at least 50 cells were analysed using CASP software (CaspLab) and the amount of DNA damage was expressed as the percentage of total DNA in the comet tail.

*2.7 Statistical analysis*

Data are expressed as mean ± SE. Statistical analyses were carried out using SPSS (v21, IBM Corporation, Armonk, NY). FA dose-response groups for each cell line were compared by 1-way ANOVA with Dunnett’s *post hoc* test. Pairwise comparisons of protein expression and DNA methylation were by Student’s unpaired t-test. DNA repair capacity was compared by 2-way ANOVA with Bonferroni’s *post hoc* test. Differences were considered to be statistically significant at P <0.05. For the primary outcome measure, mRNA expression, a sample size of 10 cultures provided statistical power of at least 85% for detecting a 10% difference with a two-tailed probability of < 0.05. This sample size provided at least this level of statistical power for the other outcomes.

**3. Results**

*3.1 Effect of FA supplementation on BRCA 1 and 2 mRNA expression*

FA treatment of liver cancer cell lines induced cell type and cell line specific effects on BRCA1 and BRCA2 expression. FA treatment induced a significant increase in BRCA1 and BRCA2 mRNA expression in the hepatocellular carcinoma cell line HepG2 (Tables 2 and 3). FA treatment induced a dose-related increase in BRCA2 expression in hepatocellular PLC/PRF/5 cells, but did not alter BRCA1 mRNA expression significantly. In contrast in FA treated hepatocellular carcinoma Huh-7D12 cells, BRCA1 mRNA expression was lower and BRCA2 expression was unchanged. There was no significant effect of FA treatment on BRCA1 or 2 mRNA levels in the liver adenocarcinoma SK-HEP-1 cells or primary hepatocytes (Tables 2 and 3).

There was no significant effect of FA treatment on BRCA1 or 2 mRNA expression in transformed mammary epithelial HMT-3522 cells nor on BRCA1 mRNA expression in the immortalised but non transformed mammary epithelial MCF10a cells. BRCA2 expression in MCF10a cells was consistently below the detection limit of the assay (Tables 2 and 3). FA treatment increased BRCA1 and 2 mRNA expression in breast adenocarcinoma MCF7 cells. Treatment with FA did not alter BRCA1 mRNA expression significantly in breast medullary MDA-MB-157 cells, but decreased the expression of BRCA2 in a dose-related manner. In contrast, FA treatment induced increased BRCA1 and 2 expression at 25 nmoles/l, but the expression of these genes was reduced at higher FA concentrations.

There was no significant effect of FA treatment on BRCA1 expression in any of the ovarian cancer cell lines tested (Table 2), while BRCA2 expression was below the assay detection limit (Table 3). Treatment with FA decreased BRCA1 mRNA expression in JURKAT cells, but did not significantly alter its expression in primary peripheral blood mononuclear cells (PBMC) or THP1 cells (Table 2). There was no significant effect of FA treatment on BRCA2 mRNA expression in PBMC, while the level of BRCA2 in THP1 and JURKAT cells was below the detection limit of the assay (Table 3).

*3.2 Effect of FA supplementation on BRCA 1 and 2 protein expression*

BRCA1 protein expression was not significantly altered in HepG2 cells exposed to 100 nmoles/l FA (Fig. 2A). In contrast, FA treatment of Hs578T cells induced a significant increase in BRCA1 protein (Fig. 2B). The level of BRCA2 protein was below the level of detection in all cells tested (data not shown).

*3.3 Effect of FA supplementation on DNA repair*

Significant DNA damage was induced in all of the liver cell lines which were tested (all P < 0.0001). Treatment with 100 nmoles/l FA had no effect on DNA damage in any of the cell lines at any of the time points that were measured (Fig. 3). DNA damage increased significantly in primary hepatocytes one hour after being irradiated and the amount of damage returned to similar levels prior to irradiation (Fig. 3A). HepG2 cells had much lower levels of DNA damage, which were highest immediately after irradiation and then decreased to baseline damage levels after four hours (Fig. 3B). Conversely, the damage observed in the PLC/PRF/5 cell line significantly increased with every time point (Fig. 3C).

Significant DNA damage was also induced in all of the breast lines that were tested (all P < 0.0001) (Fig. 4). There was a significant time\*treatment interaction effect on DNA damage in MCF10a cells (F= (3,907) 12.0, P <0.0001) (Fig. 4A). Treatment with 100nmoles/l FA decreased the amount of damage observed in MCF10a cells after one hour, however, the damage in both treatment groups had returned to baseline levels after 4 hours (Fig. 4A). There was also a significant time\*treatment interaction effect on DNA damage in Hs578T cells (F=(3,911) 7.2, P < 0.0001) (Fig. 4B). DNA damage immediately after irradiation was significantly higher in cells treated with 100 nmoles/l FA compared to untreated cells. However, DNA damage levels were significantly lower in the FA treated cells than the control group after 1 hour recovery (Fig. 4B). After 4 hours, DNA damage levels for both groups had increased to similar levels. There was no significant effect of FA treatment on the induction of DNA damage or recovery in either MCF7 or MDA-MB-157 cells (Fig. 4 C, D).

*3.4 BRCA 1 and 2 DNA methylation*

We compared baseline methylation levels at 0 nmoles/l FA for all of the cell lines (Fig. s 5 and 6). Because of the detection limit of pyrosequencing assays [52], CpG loci that had methylation levels of 5% or less were regarded as essentially unmethylated. Statistical analysis was only carried out for loci at which the level of methylation was at least 5% in all the cell lines tested for a specific tissue.

BRCA1 promoter methylation was below 15% at the majority of CpGs investigated in all liver cell lines, with small significant differences (≤5%) between cell lines at specific CpG loci (Fig. 5A). Methylation of BRCA1 in the breast cancer cells was more variable than in liver or ovarian cells, or leukocytes (Fig. 5). HMT-3522 and Hs578T cells significantly higher methylation (≥ 20%) at CpG loci -567, -565 and in HMT-3522 cells alone at CpGs -533 and -518 compared to the other breast cell lines for which methylated was approximately 5% for all CpG loci (Fig. 5B). There were also small, significant differences (≤5%) between ovarian cells lines in the level of methylation at CpGs -533 and -518. Methylation of CpGs -567 and -565 in PBMCs and THP1 cells was significantly higher (20 - 30 %) at CpGs -567, -565 and at CpGs -533 and -518 (≥10%) compared to the JURKAT cells (Fig. 5D). However, the level of methylation for all other CPG loci was close to or less than 5% for all three leukocyte cell lines which were tested. DNA methylation across the BRCA2 promoter region was close to or below 5% in all of the cell lines investigated (Fig. 6). There was no significant effect of FA treatment on the methylation status of either BRCA1 or 2 in any of the cell lines tested (data not shown).

**4. Discussion**

The findings of previous studies have suggested that the effect of dietary supplementation with FA on cancer risk is variable and may depend, in part, upon the nature of the cancer [2-5, 7, 8, 10-12, 53, 54]. Our findings are consistent with these observations. Treatment of cell cultures with FA at concentrations that were within the range which can be achieved in human subjects *in vivo* [34, 55-57] induced differential changes in the mRNA and protein expression of BRCA 1 and 2 between primary and cancer cells derived from the same tissue, and between cell lines derived from the same cell type. These findings show for the first time that physiological concentrations of FA are able to modulate the level of mRNA of two genes that encode proteins that are critical for maintenance of DNA integrity. None of the primary or non-transformed cells showed significant FA-induced changes in BRCA1 or BRCA 2 mRNA expression. In contrast, 2/4 of the liver cancer cells lines, 3/5 breast cancer cells lines and 1/2 leukaemia cells lines, but none of the ovarian cancer cell lines, showed altered BRCA1 mRNA expression. 2/4 liver and 2/5 breast, but not ovarian or leukaemia, cancer cell lines showed altered BRCA 2 mRNA expression. Although these findings do not represent a comprehensive analysis of all possible cancer cell types that may be derived from these tissues, these findings support the suggestion that any effect of FA supplementation on the mRNA expression of BRCA 1 or BRCA 2 may reflect the particular type of cancer. Thus these findings are consistent with and suggest an explanation for the inconsistent reports in the literature regarding the effect of FA on cancer risk

*4.1 mRNA expression*

Treatment with the highest concentration of FA (100 nmol/l)induced changes in the level of BRCA1 protein in the same direction as the mRNA transcript in HepG2 and Hs578T cells, although this was only significant for the Hs578T cell line. The effect of varying FA concentration on protein expression was not tested for practical reasons. Although MCF7 and MDA-MB-231 cells showed an overall significant effect of FA treatment on BRCA 1 mRNA expression, pairwise testing did not detect a significant difference between treated cells and controls, and so the effect of FA on the levels of BRCA 1 protein was not determined in these cells. Although the BRCA2 transcript was detected in some cell lines, the level of BRCA2 protein expression was below the detection limit of the western blot assay. Nevertheless, these findings suggested that, at least in some cell types, FA treatment modified the level of both BRCA 1 mRNA and protein. These findings are in contrast to the effect of supra-physiological folic acid concentrations on normal cells [58]. This highlights the importance of using physiological concentrations in studies of the effects of nutrients on cancer-related outcomes *in vitro*.

*4.2 DNA repair*

Capacity to repair radiation-induced DNA damage was used to test whether the changes induced in BRCA 1 and/ or 2 mRNA or protein expression might be biologically significant. All cell types showed significant DNA damage as a result of exposure to non-ionising radiation. However, there were differences between cells types in their ability to repair DNA damage. Primary hepatocytes, HepG2, MCF10a, MCF7 and MDAMB157 cells exhibited DNA repair by 4 hours after irradiation, the extent of which was greater for the non-cancer cells hepatocytes and MCF10a cells. However, the other cancer cell lines, PLCPRF5 and Hs578T cells, showed significantly greater DNA damage at 4 hours after irradiation than at earlier time points. Such differences in DNA repair capacity between cell lines may reflect variation in the expression and functional activity of other genes involved in DNA repair. For example, p53 is mutated in Hs578T cells [59] and CDKN2A in PLC/PRF/5 cells [60]. There was no effect of FA treatment on DNA repair in liver-derived cells while there were transient effects of FA treatment on breast tissue-derived cells. One possible explanation is that although FA treatment altered BRCA 1 or 2 mRNA expression, the magnitude of this effect maybe too small to result in a significance change in DNA repair capacity . In cancer cells this may have been due to impaired expression of other genes involved in DNA repair. One implication of these findings is that dietary FA may have a limited effect on the susceptibility of liver or breast tumour cells to radiation and hence may not be a consideration in patients undergoing radiotherapy.

*4.3 DNA methylation*

Variations in folate status have been associated with changes in the DNA methylation status of specific genes [17-20]. Furthermore, DNA hypermethylation of the BRCA1 promoter has been associated with decreased mRNA expression [61-63] and with sporadic breast cancer [30, 64-66]. We investigated whether the changes in BRCA1 or 2 mRNA expression induced by FA treatment were associated with altered DNA methylation of these genes. The region of BRCA 1 that was analysed has been shown previously to be hypermethylated in some sporadic breast cancer cells, but essentially unmethylated in others including MCF7 cells, and in peripheral blood mononuclear cells, fibroblasts and normal mammary epithelium [63]. To our knowledge, there have not been any study that have reported in detail the methylation status of individual CpG loci in BRCA 2 using sequencing techniques. One study reported average methylation (60%) at CpGs -176 and -148 bp relative to the transcription start site (TSS) [67], but no information is available about the level of methylation of CpG loci more proximal to the TSS. We found that the proximal promoter region of BRCA 1 was essentially unmethylated in all cells tested in the absence of FA treatment. However, specific CpG loci were more highly methylated in some, but not all, breast, ovary and leukocyte-derived cells. In contrast, the region of BRCA 2 that was analysed was essentially unmethylated in all cells tested. One possible implication of these findings is that the background level of DNA methylation, particularly of BRCA 1, may influence the choice of cell type for studies on epigenetic processes in cancer.

There was no significant effect of FA treatment on the methylation of the regions of sequenced within the BRCA 1 or 2 promoters. Thus any effect of FA treatment on the levels of the transcripts of these genes is unlikely to be mediated through changes in DNA methylation of these sequences, although it is possible that other regions could be involved. However, since the duration of FA treatment was relatively short, other mechanisms such as changes in histone methylation could be involved which may subsequently lead to altered DNA methylation over a longer period [68].

**5. Conclusions**

These findings are consistent with the uncertainty in the literature regarding the effects of FA on cancer risk, but indicate that any effect of FA on BRCA 1 or 2 expression may be specific to a particular cell type. Furthermore, the functional consequences of FA appear to be modest at least in terms of DNA repair. Extrapolation of the findings of *in vitro* studies to patients must be cautious and limited. However, one possible implication is that, even if replicated in primary tumour cells, it may not be possible to make general recommendations for FA intake in cancer.

**Competing interests:** The authors have declared that no competing interests exist.

**Author contributions**

Conceived and designed the experiments: GCB, KAL. Performed the experiments: RJP. Analysed the data: GCB RJP. Wrote the paper: GCB, RJP, KAL.

**REFERENCES**

[1] Mathers JC. Folate intake and bowel cancer risk. Genes Nutr. 2009;4:173-8.

[2] French AE, Grant R, Weitzman S, Ray JG, Vermeulen MJ, Sung L, et al. Folic acid food fortification is associated with a decline in neuroblastoma. Clin Pharmacol Therap. 2003;74:288-94.

[3] Bunin GR, Kuijten RR, Buckley JD, Rorke LB, Meadows AT. Relation between maternal diet and subsequent primitive neuroectodermal brain tumors in young children. N Eng J Med. 1993;329:536-41.

[4] Olshan AF, Smith JC, Bondy ML, Neglia JP, Pollock BH. Maternal vitamin use and reduced risk of neuroblastoma. Epidemiol. 2002;13:575-80.

[5] Jaszewski R, Misra S, Tobi M, Ullah N, Naumoff JA, Kucuk O, et al. Folic acid supplementation inhibits recurrence of colorectal adenomas: a randomized chemoprevention trial. World JGastroenterol. 2008;14:4492-8.

[6] Logan RF, Grainge MJ, Shepherd VC, Armitage NC, Muir KR. Aspirin and folic acid for the prevention of recurrent colorectal adenomas. Gastroentero. 2008;134:29-38.

[7] Cole BF, Baron JA, Sandler RS, Haile RW, Ahnen DJ, Bresalier RS, et al. Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. JAMA. 2007;297:2351-9.

[8] Kim YI. Folic acid supplementation and cancer risk: point. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the Am Soc Prev Oncol. 2008;17:2220-5.

[9] Mason JB, Dickstein A, Jacques PF, Haggarty P, Selhub J, Dallal G, et al. A temporal association between folic acid fortification and an increase in colorectal cancer rates may be illuminating important biological principles: a hypothesis. Cancer Epidemiol Biomarkers Prev. 2007;16:1325-9.

[10] Hirsch S, Sanchez H, Albala C, de la Maza MP, Barrera G, Leiva L, et al. Colon cancer in Chile before and after the start of the flour fortification program with folic acid. Eur J Gastroenterol Hepatol. 2009;21:436-9.

[11] Terry P, Jain M, Miller AB, Howe GR, Rohan TE. Dietary intake of folic acid and colorectal cancer risk in a cohort of women. Int J Cancer. 2002;97:864-7.

[12] Qin X, Cui Y, Shen L, Sun N, Zhang Y, Li J, et al. Folic acid supplementation and cancer risk: a meta-analysis of randomized controlled trials. Int J Cancer. 2013;133:1033-41.

[13] Zhang SM, Cook NR, Albert CM, Gaziano JM, Buring JE, Manson JE. Effect of combined folic acid, vitamin B6, and vitamin B12 on cancer risk in women: a randomized trial. JAMA. 2008;300:2012-21.

[14] Bird A. DNA methylation patterns and epigenetic memory. Genes Dev. 2002;16:6-21.

[15] Anderson OS, Sant KE, Dolinoy DC. Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. J Nutr Biochem. 2012;23:853-9.

[16] Burdge GC, Lillycrop KA, Jackson AA. Nutrition in early life, and risk of cancer and metabolic disease: alternative endings in an epigenetic tale? Br J Nutr. 2009;101:619-30.

[17] Kotsopoulos J, Sohn KJ, Kim YI. Postweaning dietary folate deficiency provided through childhood to puberty permanently increases genomic DNA methylation in adult rat liver. J Nutr. 2008;138:703-9.

[18] Wolff GL, Kodell RL, Moore SR, Cooney CA. Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. FASEB J. 1998;12:949-57.

[19] McKay JA, Williams EA, Mathers JC. Folate and DNA methylation during in utero development and aging. Biochem Soc Trans. 2004;32:1006-7.

[20] McKay JA, Williams EA, Mathers JC. Gender-specific modulation of tumorigenesis by folic acid supply in the Apc mouse during early neonatal life. Br J Nutr. 2008;99:550-8.

[21] Ingrosso D, Cimmino A, Perna AF, Masella L, De Santo NG, De Bonis ML, et al. Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocysteinaemia in patients with uraemia. Lancet. 2003;361:1693-9.

[22] Venkitaraman AR. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell. 2002;108:171-82.

[23] Friedenson B. BRCA1 and BRCA2 pathways and the risk of cancers other than breast or ovarian. Med Gen Med. 2005;7:60.

[24] Wilson CA, Ramos L, Villasenor MR, Anders KH, Press MF, Clarke K, et al. Localization of human BRCA1 and its loss in high-grade, non-inherited breast carcinomas. Nat Genet. 1999;21:236-40.

[25] Jones PA. DNA methylation and cancer. Oncogene. 2002;21:5358-60.

[26] Rice JC, Ozcelik H, Maxeiner P, Andrulis I, Futscher BW. Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. Carcinogenesis. 2000;21:1761-5.

[27] Catteau A, Harris WH, Xu CF, Solomon E. Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. Oncogene. 1999;18:1957-65.

[28] Birgisdottir V, Stefansson OA, Bodvarsdottir SK, Hilmarsdottir H, Jonasson JG, Eyfjord JE. Epigenetic silencing and deletion of the BRCA1 gene in sporadic breast cancer. Breast Cancer Res. 2006;8:R38.

[29] Chan KY, Ozcelik H, Cheung AN, Ngan HY, Khoo US. Epigenetic factors controlling the BRCA1 and BRCA2 genes in sporadic ovarian cancer. Cancer Res. 2002;62:4151-6.

[30] Esteller M. Epigenetic lesions causing genetic lesions in human cancer: promoter hypermethylation of DNA repair genes. Eur J Cancer. 2000;36:2294-300.

[31] Vineis P, Chuang SC, Vaissiere T, Cuenin C, Ricceri F, Genair EC, et al. DNA methylation changes associated with cancer risk factors and blood levels of vitamin metabolites in a prospective study. Epigenetics. 2011;6:195-201.

[32] Stidley CA, Picchi MA, Leng S, Willink R, Crowell RE, Flores KG, et al. Multivitamins, folate, and green vegetables protect against gene promoter methylation in the aerodigestive tract of smokers. Cancer Res. 2010;70:568-74.

[33] Sweeney MR, McPartlin J, Scott J. Folic acid fortification and public health: report on threshold doses above which unmetabolised folic acid appear in serum. BMC Public Health. 2007;7:41.

[34] Tam C, O'Connor D, Koren G. Circulating unmetabolized folic Acid: relationship to folate status and effect of supplementation. Obstet Gynecol Int. 2012;2012:485179.

[35] Boilson A, Staines A, Kelleher CC, Daly L, Shirley I, Shrivastava A, et al. Unmetabolized folic acid prevalence is widespread in the older Irish population despite the lack of a mandatory fortification program. Am J Clin Nutr. 2012;96:613-21.

[36] Kelly P, McPartlin J, Scott J. A combined high-performance liquid chromatographic-microbiological assay for serum folic acid. Anal Biochem. 1996;238:179-83.

[37] Kelly P, McPartlin J, Goggins M, Weir DG, Scott JM. Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. Am J Clin Nutr. 1997;65:1790-5.

[38] Hoile SP, Irvine NA, Kelsall CJ, Sibbons C, Feunteun A, Collister A, et al. Maternal fat intake in rats alters 20:4n-6 and 22:6n-3 status and the epigenetic regulation of Fads2 in offspring liver. J Nutr Biochem. 2013; 24:1213-20.

[39] Cikos S, Bukovska A, Koppel J. Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. BMC Mol Biol. 2007;8:113.

[40] Rodway HA, Hunt AN, Kohler JA, Postle AD, Lillycrop KA. Lysophosphatidic acid attenuates the cytotoxic effects and degree of peroxisome proliferator-activated receptor gamma activation induced by 15-deoxyDelta12,14-prostaglandin J2 in neuroblastoma cells. BiochemJ. 2004;382:83-91.

[41] Catteau A, Morris JR. BRCA1 methylation: a significant role in tumour development? Sem Cancer Biol. 2002;12:359-71.

[42] Wei M, Grushko TA, Dignam J, Hagos F, Nanda R, Sveen L, et al. BRCA1 promoter methylation in sporadic breast cancer is associated with reduced BRCA1 copy number and chromosome 17 aneusomy. Cancer Res. 2005;65:10692-9.

[43] Xu CF, Chambers JA, Solomon E. Complex regulation of the BRCA1 gene. The J Biol Chem. 1997;272:20994-7.

[44] Atlas E, Stramwasser M, Whiskin K, Mueller CR. GA-binding protein alpha/beta is a critical regulator of the BRCA1 promoter. Oncogene. 2000;19:1933-40.

[45] Atlas E, Stramwasser M, Mueller CR. A CREB site in the BRCA1 proximal promoter acts as a constitutive transcriptional element. Oncogene. 2001;20:7110-4.

[46] Bindra RS, Gibson SL, Meng A, Westermark U, Jasin M, Pierce AJ, et al. Hypoxia-induced down-regulation of BRCA1 expression by E2Fs. Cancer Res. 2005;65:11597-604.

[47] Cucer N, Taheri S, Ok E, Ozkul Y. Methylation status of CpG islands at sites -59 to +96 in exon 1 of the BRCA2 gene varies in mammary tissue among women with sporadic breast cancer. J Genetics. 2008;87:155-8.

[48] Wu K, Jiang SW, Thangaraju M, Wu G, Couch FJ. Induction of the BRCA2 promoter by nuclear factor-kappa B. J Biol Chem. 2000;275:35548-56.

[49] Jin W, Chen Y, Di GH, Miron P, Hou YF, Gao H, et al. Estrogen receptor (ER) beta or p53 attenuates ERalpha-mediated transcriptional activation on the BRCA2 promoter. J Biol Chem. 2008;283:29671-80.

[50] Tripathi MK, Misra S, Khedkar SV, Hamilton N, Irvin-Wilson C, Sharan C, et al. Regulation of BRCA2 gene expression by the SLUG repressor protein in human breast cells. J Biol Chem. 2005;280:17163-71.

[51] Lemay M, Wood KA. Detection of DNA damage and identification of UV-induced photoproducts using the CometAssay kit. Bio Techniques. 1999;27:846-51.

[52] Tsiatis AC, Norris-Kirby A, Rich RG, Hafez MJ, Gocke CD, Eshleman JR, et al. Comparison of Sanger sequencing, pyrosequencing, and melting curve analysis for the detection of KRAS mutations: diagnostic and clinical implications. J Mol Diag. 2010;12:425-32.

[53] Gao QY, Chen HM, Chen YX, Wang YC, Wang ZH, Tang JT, et al. Folic acid prevents the initial occurrence of sporadic colorectal adenoma in Chinese older than 50 years of age: a randomized clinical trial. Cancer Prev Res. 2013;6:744-52.

[54] Mungall AJ, Palmer SA, Sims SK, Edwards CA, Ashurst JL, Wilming L, et al. The DNA sequence and analysis of human chromosome 6. Nature. 2003;425:805-11.

[55] Bradbury KE, Williams SM, Mann JI, Brown RC, Parnell W, Skeaff CM. Estimation of serum and erythrocyte folate concentrations in the New Zealand adult population within a background of voluntary folic acid fortification. J Nutr. 2014;144:68-74.

[56] von Arnim CA, Dismar S, Ott-Renzer CS, Noeth N, Ludolph AC, Biesalski HK. Micronutrients supplementation and nutritional status in cognitively impaired elderly persons: a two-month open label pilot study. Nutr J. 2013;12:148.

[57] Wald DS, Bishop L, Wald NJ, Law M, Hennessy E, Weir D, et al. Randomized trial of folic acid supplementation and serum homocysteine levels. Arch Internal Med. 2001;161:695-700.

[58] Charles MA, Johnson IT, Belshaw NJ. Supra-physiological folic acid concentrations induce aberrant DNA methylation in normal human cells in vitro. Epigenetics. 2012;7:689-94.

[59] Sjoblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, et al. The consensus coding sequences of human breast and colorectal cancers. Science. 2006;314:268-74.

[60] Kaneko Y, Tsukamoto A. Apoptosis and nuclear levels of p53 protein and proliferating cell nuclear antigen in human hepatoma cells cultured with tumor promoters. Cancer Lett. 1995;91:11-7.

[61] Rice JC, Massey-Brown KS, Futscher BW. Aberrant methylation of the BRCA1 CpG island promoter is associated with decreased BRCA1 mRNA in sporadic breast cancer cells. Oncogene. 1998;17:1807-12.

[62] Rice JC, Ozcelik H, Maxeiner P, Andrulis I, Futscher BW. Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. Carcinogenesis. 2000;21:1761-5.

[63] Rice JC, Futscher BW. Transcriptional repression of BRCA1 by aberrant cytosine methylation, histone hypoacetylation and chromatin condensation of the BRCA1 promoter. Nucleic Acids Res. 2000;28:3233-9.

[64] Birgisdottir V, Stefansson OA, Bodvarsdottir SK, Hilmarsdottir H, Jonasson JG, Eyfjord JE. Epigenetic silencing and deletion of the BRCA1 gene in sporadic breast cancer. Breast Cancer Res. 2006;8:R38.

[65] Dobrovic A, Simpfendorfer D. Methylation of the BRCA1 gene in sporadic breast cancer. Cancer Res. 1997;57:3347-50.

[66] Xu X, Gammon MD, Zhang Y, Bestor TH, Zeisel SH, Wetmur JG, et al. BRCA1 promoter methylation is associated with increased mortality among women with breast cancer. Breast Cancer Res Treat. 2009;115:397-404.

[67] Szaumkessel M, Richter J, Giefing M, Jarmuz M, Kiwerska K, Tonnies H, et al. Pyrosequencing-based DNA methylation profiling of Fanconi anemia/BRCA pathway genes in laryngeal squamous cell carcinoma. Int J Oncol. 2011;39:505-14.

[68] Rothbart SB, Krajewski K, Nady N, Tempel W, Xue S, Badeaux AI, et al. Association of UHRF1 with methylated H3K9 directs the maintenance of DNA methylation. Nature structural & molecular biology. 2012;19:1155-60.

**Table 1 -** PCR and pyrosequencing primers

|  |  |  |  |
| --- | --- | --- | --- |
| **CpGs covered** | **Forward Primer** | **Reverse Primer**  **(Biotin Labelled)** | **Sequencing Primer** |
|  |  | BRCA1 |  |
| -567 to -518 | ATGGGAATTGTAGTTTTTTTAAAGAGTT | AAAAATCCCAATCCCCCACT | AGTTTATAATTGTTGATAAGTA |
| -355 to -300 | AGATTATAGTTTTTAAGGAATATTGTGG | TAAAATACCTACCCTCTAACCTCTACT | ATTGGAGATTTTTATTAGGG |
| -189 to -166 | AGGTTAGAGGGTAGGTATTTTAT | ACTCTAAATTAACCACCCAATCTAC | ATGGTAAATTTAGGTAGAATTTTT |
| -80 to -19 | GGGGTAGATTGGGTGGTTAATTTAGAG | CCAATTATCTAAAAAACCCCACAACC | TTATTTTTTGATTGTATTTTGATTT |
| +8 to +44 | GGGGTAGATTGGGTGGTTAATTTAGAG | CCAATTATCTAAAAAACCCCACAAC | GGGAATTATAGATAAATTAAAATTG |
|  |  | BRCA2 |  |
| -56 to +7 | GTTGGGATGTTTGATAAGGAATTTT | CACAAATCTATCCCCTCAC | GGT TTA TTTAGG TTTGATTT |
| +25 to +102 | GTTGGGATGTTTGATAAGGAATTTT | CACAAATCTATCCCCTCAC | GAGTTT TTG AAATTAGG |

**Table 2 -** Effect of FA treatment on BRCA 1 mRNA expression

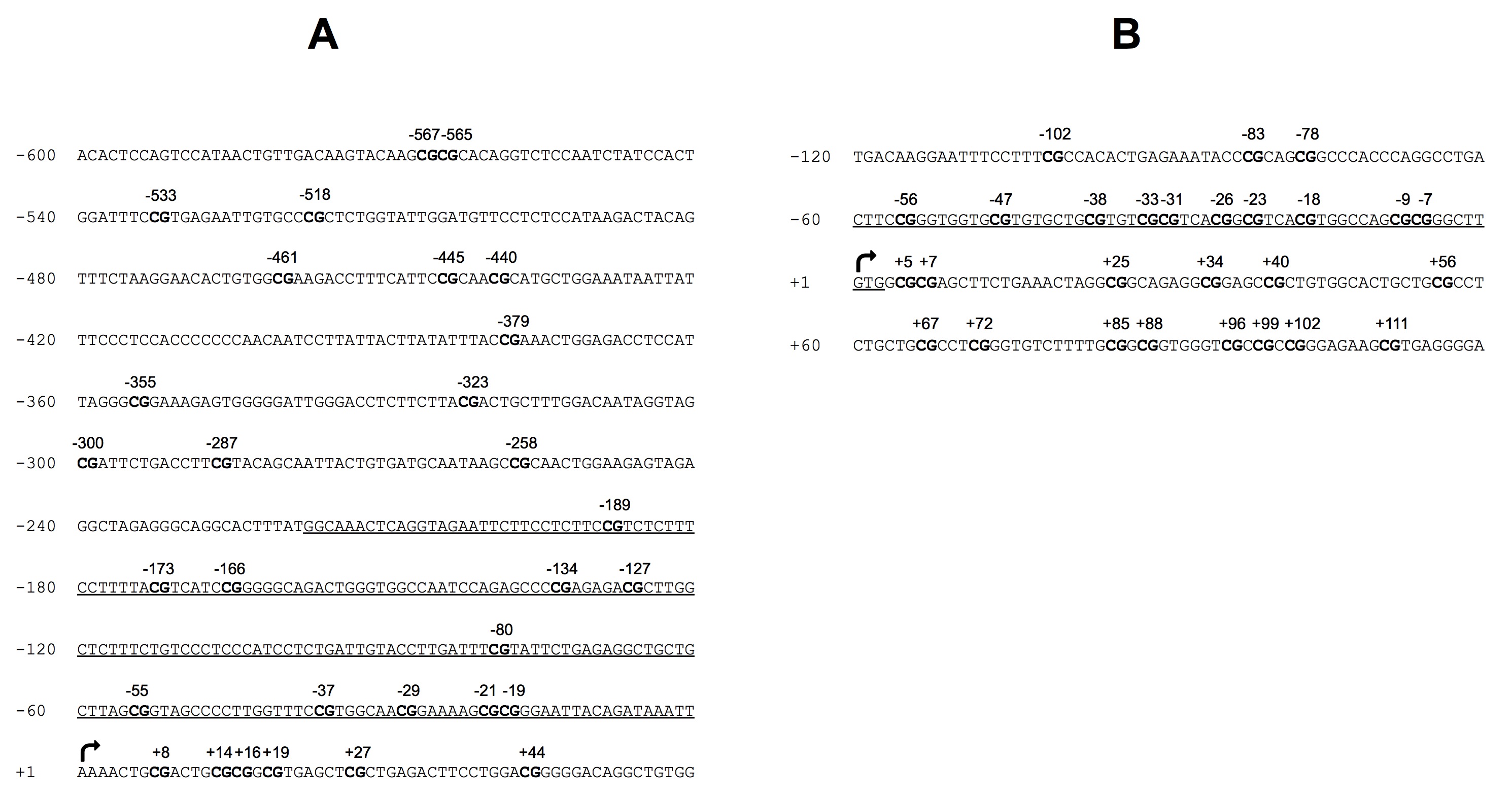
|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Cell  line | Cell  type | Relative mRNA expression | | | | | ANOVA  (P) |
| Folic acid concentration (nmoles/l) | | | | |
| 02 | 25 | 50 | 75 | 100 |
| Liver | | | | | | | |
| Hepatocytes | Primary | 100±22.4 | ND | ND | ND | 98.1±18.9 | 0.951 |
| SK-HEP-1 | Adenocarcinoma | 100±6.2 | 91.6±6.3 | 90.3±5.3 | 93.9±6.4 | 108.1±8.3 | 0.32 |
| PLC/PRF/5 | Hepatoma | 100±4.9 | 99.6±3.1 | 96.9±5.1 | 105.6±2.6 | 107.5±5.9 | 0.45 |
| HepG2 | Hepatocellular  carcinoma | 100±2.4 | 114.6±2.6\*\* | 116.8±3.1\*\* | 113.5±1.7\* | 115.8±4.3\*\* | 0.003 |
| Huh-7D12 | Hepatocellular  carcinoma | 100±4.1 | 95.5±4.5 | 79.0±5.4\* | 82.0±7.2 | 98.9±5.0 | 0.024 |
| Breast | | | | | | | |
| HMT-3522 | Transformed  epithelial | 100±35.8 | 139.6±36.0 | 143.9±24.0 | 174.5±32.1 | 62.9±58.5 | 0.30 |
| Hs578T | Ductal  carcinoma | 100±17.0 | 146.1±11.8 | 218.1±21.6\*\*\* | 181.5±14.3\* | 180.5±22.2\* | 0.001 |
| MCF7 | Adenocarcinoma (ER+) | 100±7.9 | 92.3±10.0 | 99.6±13.4 | 66.8±13.3 | 119.7±10.5 | 0.028 |
| MDA-MB-157 | Medulla  carcinoma | 100±1.4 | 121.9±8.0 | 112.2±6.6 | 108.5±6.2 | 98.0±6.4 | 0.080 |
| MDA-MB-231 | Adenocarcinoma | 100±7.5 | 124.3±12.8 | 84.0±4.6 | 72.4±9.0 | 77.0±5.0 | 0.0004 |
| MCF10a | Non-tumourigenic  epithelial | 100±20.8 | 88.7±24.7 | ND | 64.1±41.1 | 61.8±27.5 | 0.55 |
| Ovarian | | | | | | | |
| A2780 | Serous  carcinoma | 100±31.5 | 163.8±66.0 | 103.1±32.2 | 171.4±29.7 | 131.0±26.4 | 0.52 |
| COV434 | Granulosa  carcinoma | 100±72.8 | 89.8±62.3 | 135.7±52.7 | 141.1±28.6 | 139.8±33.5 | 0.91 |
| PEA1 | Serous carcinoma (ER+) | 100±31.6 | 61.2±21.6 | 27.8±8.4 | 43.1±26.0 | 95.0±29.2 | 0.19 |
| Leukocyte | | | | | | | |
| PBMC | Primary | 100±19.7 | 97.0±16.3 | 153.2±23.6 | 128.7±13.4 | 119.7±14.2 | 0.197 |
| THP1 | Acute monocytic leukaemia | 100±31.4 | 150.5±52.8 | 22.2±7.7 | 103.6±43.3 | 169.7±89.7 | 0.0827 |
| JURKAT | Acute T cell leukaemia | 100±18.2 | 14.3±12.2\* | 79.9±36.5 | 26.8±6.1\* | 24.8±5.4 | 0.0167 |

Values are mean ± SE (n = 10 replicate cultures). Expression levels were normalised to reference gene and are relative to untreated cells. Data were analysed by 1-way ANOVA using Dunnett’s post hoc correction except 1where data were analysed using Student’s t test. Values significantly different from baseline untreated cells are indicated by \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

**Table 3 -** Effect of FA treatment on BRCA 2 mRNA expression

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Cell  line | Cell  type | Relative mRNA expression | | | | | ANOVA  (P) |
| Folic acid concentration (nmoles/l) | | | | |
| 02 | 25 | 50 | 75 | 100 |
| Liver | | | | | | | |
| Hepatocytes | Primary | 100±11.9 | ND | ND | ND | 89.9±17.5 | 0.641 |
| SK-HEP-1 | Adenocarcinoma | 100±3.5 | 109.1±13.3 | 92.8±5.9 | 113.3±3.0 | 126.3±13.8 | 0.20 |
| PLC/PRF/5 | Hepatocellular carcinoma | 100±7.0 | 100.6±5.4 | 105.7±5.7 | 119.2±1.8\* | 129.2±3.2\*\* | 0.0009 |
| HepG2 | Hepatocellular  carcinoma | 100±4.1 | 114.2±2.3 | 112.0±3.7 | 111.7±3.8 | 110.2±5.2 | 0.003 |
| Huh-7D12 | Hepatocellular  carcinoma | 100±6.4 | 101.1±5.6 | 88.2±3.2 | 87.2±3.4 | 94.6±2.3 | 0.14 |
| Breast | | | | | | | |
| HMT-3522 | Transformed breast  epithelial | 100±40.7 | 148.9±55.1 | 221.7±85.4 | 278.1±111.9 | 120.4±120.4 | 0.51 |
| Hs578T | Ductal breast  carcinoma | 100±18.1 | 182.9±21.9 | 334.5±24.9\*\*\* | 228.5±19.1 | 246.4±26.3\* | 0.0001 |
| MCF7 | Breast Adenocarcinoma (ER+) | 100±10.8 | 106.0±7.8 | 123.2±12.6 | 108.2±8.4 | 158.0±11.3\*\*\* | 0.0024 |
| MDA-MB-157 | Medulla  Breast carcinoma | 100±4.4 | 86.6±4.4 | 72.7±4.6 | 71.4±4.9 | 62.2±2.0\*\* | 0.0029 |
| MDA-MB-231 | Breast Adenocarcinoma | 100±9.6 | 127.9±14.0 | 110.7±9.8 | 74.6±10.9 | 105.6±8.8 | 0.023 |
| MCF10a | Immortalised breast  epithelial | ND | ND | ND | ND | ND | ND |
| Ovarian | | | | | | | |
| A2780 | Serous  carcinoma | Undetectable | | | | | ND |
| COV434 | Granulosa  carcinoma | Undetectable | | | | | ND |
| PEA1 | Serous carcinoma (ER+) | Undetectable | | | | | ND |
| Leukocyte | | | | | | | |
| PBMC | Primary | 100±23.4 | 142.4±15.8 | 214.2±41.8 | 235.3±25.6 | 179.5±18.9 | 0.0672 |
| THP1 | Acute monocytic leukaemia | Undetectable | | | | | ND |
| JURKAT | Acute T cell leukaemia | Undetectable | | | | | ND |

Values are mean ± SE (n = 10 replicate cultures). Expression levels were normalised to reference gene and are relative to untreated cells. Data were analysed by 1-way ANOVA using Dunnett’s post hoc correction except 1where data were analysed using Student’s t test. Values significantly different from baseline 2folate concentration (15 nmoles/l) are indicated by \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

****

**Fig. 1.** Regions of the (A) BRCA1 and (B) BRCA2 genes that were analysed by pyrosequencing. The minimal promoter regions of BRCA1 [43] and BRCA2 [48] are indicated by the underlined sequences. CpG loci are indicated in bold font and numbered relative to the transcription start site. Arrows indicates the transcription start sites.



**Fig. 2.** Effect of FA treatment on BRCA1 protein expression in HepG2 and Hs578T cells. Cell extracts from (A) HepG2 and (B) Hs578T cells treated with 0 nmoles/l FA or 100 nmoles/l FA for 72h and analysed by Western blotting with anti-BRCA1 and anti-β-Actin antibodies. Values are mean ± SE (n = 5 replicate cultures). Data were analysed using Student’s t-test. Values significantly different from untreated cells are indicated by \*P < 0.05.



**Fig. 3.** Effect of FA supplementation on DNA repair capacity in liver cells. Primary hepatocyte (A), HepG2 (B) and PLC/PRF/5 (C) cells were treated with 0 nmoles/l FA or 100 nmoles/l FA for 72h, irradiated with 1.8J/m2 UVC and DNA damage was analysed by Comet assay. Values are mean ± SE (n = >50 comets). Data were analysed by 1-way ANOVA using Bonferroni’s post hoc correction. Means without a common letter differ significantly (P < 0.05).



**Fig. 4.** Effect of FA supplementation on DNA repair capacity in breast cells. MCF10a (A), Hs578T (B) MCF7 (C) and MDA-MB-157 cells were treated with 0 nmoles/l FA or 100 nmoles/l FA for 72h, irradiated with 1.8J/m2 UVC and DNA damage was analysed by Comet assay. Values are mean ± SE (n = >50 comets). Data were analysed by 1-way ANOVA using Bonferroni’s post hoc correction. Means without a common letter differ significantly (P < 0.05).



**Fig. 5.** BRCA1 DNA methylation. The methylation status of individual CpG loci was measured in liver (A), breast (B), ovarian (C) and leukocyte (D) cell lines without the addition of FA by bisulphite pyrosequencing. Values are mean ± SE (n = 10 replicate cultures). Data were analysed by 1-way ANOVA using Bonferroni’s *post hoc* correction. For each CpG loci, means without a common letter differ significantly (P < 0.05). (only differences that were ≥ 5% methylation are marked). Dotted line indicates the limit of detection of the analysis.



**Fig. 6.** BRCA2 DNA methylation. The methylation status of individual CpG loci was measured in liver (A), breast (B), ovarian (C) and leukocyte (D) cell lines without the addition of FA by bisulphite pyrosequencing. Values are mean ± SE (n = 10 replicate cultures). Data were analysed by 1-way ANOVA using Bonferroni’s *post hoc* correction. For each CpG loci, means without a common letter differ significantly (P < 0.05) (only differences that were ≥ 5% methylation are marked). Dotted line indicates the limit of detection of the analysis.