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Lymphocytes of *BRCA1* and *BRCA2* germ-line mutation carriers, with or without breast cancer, are not abnormally sensitive to the chromosome damaging effect of moderate folate deficiency

Sasja Beetstra[1,2], Carolyn Salisbury[1], Julie Turner[1], Meryl Altree[3], Ross McKinnon[2], Graeme Suthers[3,4] and Michael Fenech[1,*]

[1]CSIRO Human Nutrition, PO Box 10041, Gouger Street, Adelaide BC 5000, SA, Australia, [2]Sansom Research Institute, The University of South Australia, Adelaide 5000, SA, Australia, [3]Familial Cancer Unit, SA Clinical Genetics Service, Children's, Youth and Women's Health Service, North Adelaide 5006, SA, Australia [4]Department of Pediatrics, University of Adelaide, Adelaide 5003, SA, Australia

[*]To whom correspondence should be addressed. Tel: +61 0 8 8303 8880; Fax: +61 0 8 8303 8899;
Email: michael.fenech@csiro.au

Mutations in *BRCA1* and *BRCA2* genes may cause defective DNA repair and increase the risk for breast cancer. Folate deficiency is associated with increased breast cancer risk and induces chromosome abnormalities. We hypothesized that *BRCA1* and *BRCA2* germline mutation carriers are more sensitive to the genome damaging effect of folate deficiency compared with healthy non-carrier controls and that this sensitivity is further increased in those carriers who develop breast cancer. We tested these hypotheses in lymphocytes cultured in a medium containing 12 or 120 nM folic acid (FA) for 9 days and measured proliferative capacity and chromosomal instability using the cytokinesis-block micronucleus assay. *BRCA1* and *BRCA2* mutation carriers with or without breast cancer were not abnormally sensitive to FA deficiency-induced chromosome instability; however, *BRCA2* mutation carriers had significantly reduced cell proliferation. FA deficiency reduced cell proliferation and increased micronucleus formation significantly, accounting for 45–59% and 70–75% of the variance in these parameters compared with 0.3–8.5% and 0.2–0.3% contributed by *BRCA1* or *BRCA2* mutation carrier status, respectively. The results of this study suggest that moderate folate deficiency has a stronger effect on chromosomal instability than *BRCA1* or *BRCA2* mutations found in breast cancer families.

Introduction

Folate, a B-vitamin found in a wide variety of plant foods, is required for the synthesis of dTMP from dUMP, methionine and ultimately *S*-adenosylmethionine, the primary methyl donor required for methylation at CpG sequences in DNA. Folate deficiency causes DNA hypomethylation and alters both gene expression and chromatin structure, all of which are considered initiating events in several malignancies (1–4). In addition, folate deficiency reduces dTMP synthesis and consequently increases uracil incorporation into DNA, which may result in the generation of single- and double-strand breaks, chromosome aberrations and micronuclei (5–9). Reduced dTMP synthesis is also associated with folate-sensitive fragile sites expression (1), which has been suggested to be involved in carcinogenesis (10).

Breast cancer is the most common malignancy affecting women in developed countries (11,12) (www.dep-iarc.fr). Besides a history of familial breast cancer, age at menarche, age of first full-time pregnancy and lifestyle factors such as socioeconomic status and diet are important factors in the development of breast cancer. Excessive alcohol consumption (13) has been reported to be a breast cancer risk factor, whereas a diet rich in vitamins A, C, E, B_{12} and folate may have a protective effect (14–22).

Approximately 5–10% of all breast cancer cases are considered to be hereditary. Hereditary breast cancer, usually characterized by its early onset, has been linked to the *BRCA1* and *BRCA2* germline mutation (23). *BRCA1*, mapped to chromosome 17q21 (24–26), and *BRCA2*, mapped to 13q12–q13, (27) code for important proteins required for genome stability maintenance because of their functions in cell cycle checkpoint control, ubiquitylation, mitotic spindle formation, transcriptional regulation and DNA repair. *BRCA1* and *BRCA2* are involved in homologous recombination repair, whereas *BRCA1* is also involved in non-homologous end joining and nucleotide excision repair (especially transcription-coupled repair of oxidative damage) (28–31).

Peripheral blood lymphocytes from *BRCA1* and *BRCA2* carriers (with or without breast cancer) show enhanced sensitivity to micronucleus induction by a wide variety of clastogens compared with controls (32,33). Folate deficiency has been shown to mimic ionizing radiation in damaging DNA by inducing single- and double-strand breaks and micronuclei (34–37). It is therefore plausible that cells exhibiting an impaired single- and/or double-strand break repair system may be more sensitive to the chromosome damaging effects of folate deficiency. Therefore, we hypothesized that cells with an inactivating mutation in *BRCA1* or *BRCA2* are more susceptible than non-mutant controls to the genome damaging effect of folic acid (FA) deficiency, and that this effect is greater in cells of *BRCA1* or *BRCA2* carriers who developed breast cancer compared with those carriers who are breast cancer free.

The model used to test these hypotheses consisted of 9-day cultures, in folate replete or deficient medium, of human peripheral lymphocytes from *BRCA1* or *BRCA2* germline mutation carriers, with or without breast cancer, and non-carrier relatives, and the cytokinesis-block micronucleus (CBMN) assay. In its comprehensive mode the CBMN-assay can be used to measure micronuclei (biomarkers of chromosome breakage and loss), nucleoplasmic bridges (biomarker of asymmetrical chromosome rearrangements and DNA mis-repair), nuclear buds (biomarker of gene amplification),

Abbreviations: BMI, body mass index; FA, folic acid; MNed BNC, binucleated cell containing micronuclei; NBud BNC, binucleated cell containing nuclear buds; NDI, nuclear division index; Npb BNC, binucleated cell containing nucleoplasmic bridges.

necrosis and apoptosis (biomarkers of cell death), and nuclear division index (NDI) (biomarker of mitogen responsiveness in lymphocytes and/or cytostatic effect) (7). Additionally, in combination with the long-term cultures of lymphocytes, viable cell growth was also measured over 9 days. This model has previously been used to demonstrate the genome damaging effect of moderate folate deficiency and the modulating effect of the *MTHFR* C677T polymorphism and riboflavin concentration on sensitivity to folate deficiency-induced genome damage (34).

Materials and methods

Approval for this study was obtained from the Human Experimental Ethics committees of CSIRO Health Sciences and Nutrition, University of South Australia and Women's and Children's Hospital in Adelaide, South Australia. A total of 66 female volunteers were recruited from a database of breast cancer families who had previously undergone *BRCA1* or *BRCA2* gene mutation testing at the South Australian Clinical Genetics Service. We recruited 8–15 participants for each of the following groups amongst BRCA1 or BRCA2 breast cancer families: (i) controls, (ii) mutation carriers without breast cancer, (iii) mutation carriers with breast cancer. Controls were non-carrier relatives of *BRCA1* or *BRCA2* mutation carriers and had no history of breast cancer. All participants with breast cancer completed chemotherapy and/or radiotherapy 6 months prior to blood sample collection with the exception of one participant who completed treatment 1 month before blood collection. Volunteer age and body mass index (BMI) and the specific mutations in *BRCA1* and *BRCA2* mutation carriers are listed in Tables I and II, respectively. With one exception, the *BRCA1* and *BRCA2* mutations in the carriers were pathogenic and place a woman at high risk of developing breast cancer. There is uncertainty about the pathogenicity of the 4486G>T variant in *BRCA2* (38); two women in the study carried this variant, one having had breast cancer and the other being unaffected. The controls were at 25–50% risk of having the pathogenic mutation which had been documented in another relative. They were only tested for that mutation and were not screened for mutations elsewhere in the *BRCA1* or *BRCA2* genes. The frequency of BRCA mutations in the general population is low (~1:1000) and mutation screening was not pursued in non-carriers of the family's mutation. Lymphocytes were isolated from heparinized blood samples using Ficoll-Paque gradients (Amersham Biosciences, Adelaide, Australia) and cultured, in duplicate, in 1 ml of RPMI-1640 without FA containing 10% dialysed foetal bovine serum (Trace Scientific, Melbourne, Australia), 2 mM L-glutamine (Sigma, Sydney, Australia), 1% penicillin (5000 IU/ml)/streptomycin (5 mg/ml) solution (Trace Scientific) and either 12 or 120 nM FA (Sigma). The FA deficiency concentration chosen (12 nM) is the lowest possible that allows cell growth, maximizes induced chromosomal damage and is within the physiological range normally seen in blood of folate deficient individuals. The long-term culture procedure, summarized in Figure 1, was performed as described by Crott *et al*. (39) with minor modifications. In brief, cultures were set up at 1.0×10^6 cells/ml in 10 ml sterile conical tubes (Technoplas, Adelaide, Australia), stimulated to divide with phytohaemagglutinin (PHA; 30 μg/ml; Murex Biotech, Kent, England), and then incubated at 37°C and 5% CO_2 in a humidified atmosphere for 9 days. Culture medium was replaced with fresh medium 3 and 6 days after mitogen stimulation. Before changing the culture medium, cell number and viability were determined using a Coulter counter (Coulter Electronics, Hertfordshire UK) and Trypan Blue exclusion (Sigma), respectively. After gentle spinning (125 *g* for 10 min), a 50 μl aliquot of the supernatant was removed from each culture and transferred to a new sterile culture tube. The remaining supernatant was discarded and the cell pellet resuspended in 500 μl of the appropriate fresh culture medium. A second cell count was performed to calculate the volume of cell solution needed to set up the fresh cultures at 0.5×10^6 viable cells/ml. Fresh medium and 10 units of interleukin-2 (Roche Diagnostics, Basel, Switzerland) were added to the culture tubes with the supernatant aliquot. On day 9, cell number and viability were also determined. The extent of viable cell growth was determined using the starting concentrations on day 0, 3 and 6, and the concentration of viable cells on days 3, 6 and 9. Only cell counts for day 9 are shown because this figure is based on the estimated results from growth curves generated from the viable cell counts on days 3, 6 and 9.

Table I. Volunteer age and BMI

	BRCA1			BRCA2		
	Controls	Mutation carriers without breast cancer	Mutation carriers with breast cancer	Controls	Mutation carriers without breast cancer	Mutation carriers with breast cancer
N	15	8	12	9	12	10
Age (years)*	57.5 ± 3.5[c]	41.8 ± 3.5[a]	51.4 ± 3.2[b]	54.6 ± 3.0	48.6 ± 4.4	50.5 ± 3.7
BMI (kg/m^2)#	27.3 ± 1.1[d]	25.3 ± 1.3[d]	30.1 ± 1.9[e]	28.0 ± 1.4	27.9 ± 1.3	27.7 ± 1.7

Groups that do not share the same superscript letter within a row are significantly different from each other ($P < 0.05$).
*One-way ANOVA $P < 0.001$.
#One-way ANOVA $P = 0.002$.

Table II. Description and distribution of *BRCA1* and *BRCA2* mutations amongst carriers with or without cancer

BRCA1				BRCA2			
n	Without breast cancer	*n*	With breast cancer	*n*	Without breast cancer	*n*	With breast cancer
3	300 T>G (C61G)[a]	1	188del11 (39X)	1	IVS4-12del5	3	2988delC (959X)[e]
2	3717 C>T (Q1200X)[b]	1	300 T>G (C61G)	1	2988delC (959X)[e]	1	3031 G>A (D935N)[f]
1	IVS18+1G>T	1	1294del40 (397X)	1	3031 G>A (D935N)[f]	1	4486 G>T (D1420Y)[g]
1	exon13dup (1460X)[c]	1	3717 C>T (Q1200X)[b]	1	4075delGT (1284X)	1	6024delTA (1943X)
1	IVS4-1 G>T [d]	1	3988delAA (1293X)	1	4486 G>T (D1420Y)[g]	1	6468insA (STOP2084)
		2	4184-4187delTCAA (N1364X)	1	4706delAAAG (1502X)	1	7180 C>T (R2318X)
		2	5385insC (1829X)	1	5910 C>G (Y1894X)	1	9161 C>A (S2978X)
		1	exon13dup (1460X)[c]	1	8034insAG (2648X)	1	IVS7-1 G>A
		2	IVS4-1 G>T[d]	1	8205-1 G>C		
				1	8714 A>G (del exon 19)		
				1	9132delC (2975X)		
				1	IVS7-1 G>A		

Individuals or groups sharing the same superscript letter are members of the same family.

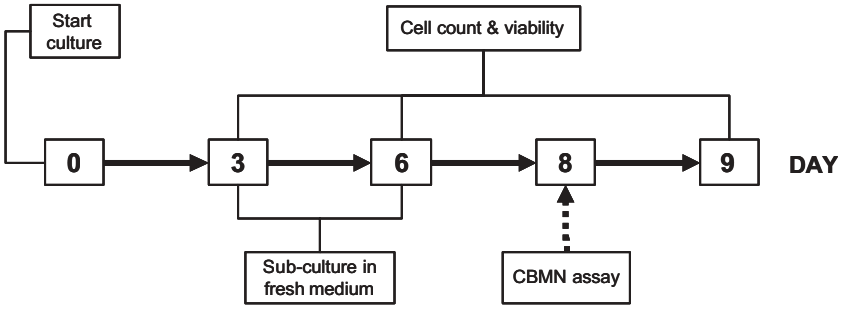

Fig. 1. Experimental design showing the days when cell counts and viabilities, subcultures and CBMN-assay were performed. Cells were initiated at 1.0×10^6 cells/ml and subcultured on day 3 and day 6 to a concentration of 0.5×10^6 cells/ml. In the CBMN-assay, cytochalasin-B was added on day 8 and cells were harvested on day 9.

Table III. Pearson correlation matrix

	[FA] in medium (nM)		Viable cell growth (day 9)	NDI	% Apoptotic	% Necrotic	MNed BNCs	Npb BNCs	NBud BNCs
Age	12	*r*	−0.184**	0.131*	0.048	0.162**	−0.028	0.027	−0.274**
	120	*r*	−0.257**	0.258*	0.112	0.015	0.042	0.050	−0.124
BMI	12	*r*	−0.153*	−0.047	0.011	0.200**	−0.032	0.080	0.024
	120	*r*	−0.056	−0.005	0.046	0.006	0.031	0.035	0.045

*Correlation is significant at the 0.05 level (two-tailed).
**Correlation is significant at the 0.01 level (two-tailed).

To perform the CBMN-assay cytochalasin-B (4.5 μg/ml; Sigma) was added to the cultures 8 days after PHA stimulation, and 24 h later the cells were transferred onto microscope slides using a cyto-centrifuge (Shandon Southern Products, Cheshire, UK). Slides were then air dried, fixed and stained using Diff-Quik (LabAids, Narrabeen Australia). Coded slides were examined by one person at 1000× magnification using a light microscope. A total of 250 cells were scored for each duplicate culture (total of 500 cells) to determine the ratios of mononucleated-, binucleated-, multinucleated-, apoptotic and necrotic cells and to calculate the NDI (40). Binucleated cells (BNCs) containing micronuclei (MNed BNCs), nucleoplasmic bridges (Npb BNCs) and nuclear buds (NBud BNCs) were scored in at least 500 BNCs per duplicate slide (total 1000 BNCs). The slides were scored using the scoring criteria as described by Fenech (40) and Fenech *et al*. (41).

Statistics

The study was designed to detect an increase of at least 6 MNed BNCs per 1000 BNCs, which is equivalent to the amount induced by 0.10 Gy of X-rays, a biologically relevant dose that is ∼100 times the annual radiation exposure limit for the general public (6). Based on an observed mean value of 11.4 MNed BNCs per 1000 BNCs and a SD of 4.1 in our previous study (34), it was estimated that it should be possible to detect an increase of 6 MNed BNCs per 1000 BNCs with 9 subjects per group with 80% power and a *P* value of 0.05.

Pearson correlation and regression analysis were used to determine the relationships between biomarkers and age or BMI. When a significant correlation was observed the relevant biomarker was adjusted for age and/or BMI. For adjustment the following formula were used: (i) for age: $Y_{51.7y} = (51.7 - X)S + M$ and (ii) for BMI: $Y_{27.9} = (27.9 - X)S + M$, where X = actual age in years, S = slope of the regression line for the relationship between age and the biomarker, M = actual value measured, $Y_{51.7y}$ = biomarker adjusted to the value expected at age 51.7 years (average age of all study subjects) and $Y_{27.9}$ = biomarker adjusted to the value expected for a BMI of 27.9 (average BMI of all study subjects). One-way ANOVA, followed by Tukey's *post hoc* multiple comparison test, was used to compare controls, mutation carriers without cancer and mutation carriers with cancer. Paired *t*-test (two-tailed) was used to compare the effect of low and high FA on various biomarkers. To estimate the relative sensitivity to the effects of FA deficiency we subtracted the values for 120 nM FA cultures from those for corresponding 12 nM FA cultures and compared results across groups using one-way ANOVA. Two-way ANOVA was used to determine the percentage of the variance in the biomarkers measured and any interactive effects attributable to FA concentration and mutation carrier status or FA and breast cancer status in the *BRCA1* and *BRCA2* groups. All data are expressed as mean ± SEM. Significance was accepted at $P < 0.05$. Two-way ANOVA statistical analysis was performed using GraphPad Prism 4.00 (GraphPad, San Diego, CA). All other statistical analyses were performed using SPSS 11.5 (SPSS, Chicago, IL).

Results

Volunteer age and BMI

Age and BMI of participants are displayed in Table I, and BRCA1 and BRCA2 mutations in carriers are presented in Table II. The BRCA1 subgroups differed significantly in age ($P < 0.01$). Additionally, the BMI of *BRCA1* carriers with breast cancer was significantly higher compared with *BRCA1* controls and carriers without breast cancer ($P = 0.002$). Age and BMI did not differ significantly among the BRCA2 subgroups. The average age and BMI in the BRCA1 study group was not significantly different compared with the BRCA2 study group. Age was significantly correlated with viable cell growth on day 9, percentage of necrotic cells, NDI and NBud BNCs, while BMI was significantly correlated with viable cell growth on day 9 and percentage of necrotic cells only (Table III).

Effect of FA concentration and BRCA1 or *BRCA2* carrier- and breast-cancer status on viable cell growth and CBMN-assay biomarkers

Viable cell growth data are shown in Figure 2. Results for CBMN-assay biomarkers and one-way ANOVA analysis are shown in Table IV. Two-way ANOVA analysis results for all data are shown in Table V.

Viable cell growth

Cell growth did not significantly differ between *BRCA1* mutation carriers (with or without breast cancer) and *BRCA1* controls under both low and high FA conditions. However, *BRCA2* mutation carriers with breast cancer had significantly lower viable cell growth compared with *BRCA2* controls in both 12 and 120 nM FA cultures ($P = 0.001$ and 0.021, respectively); the percentage by which viable cell growth was induced in 120 nM relative to 12 nM FA was not significantly different in mutation carriers compared with controls (data not shown). Two-way ANOVA revealed a significant

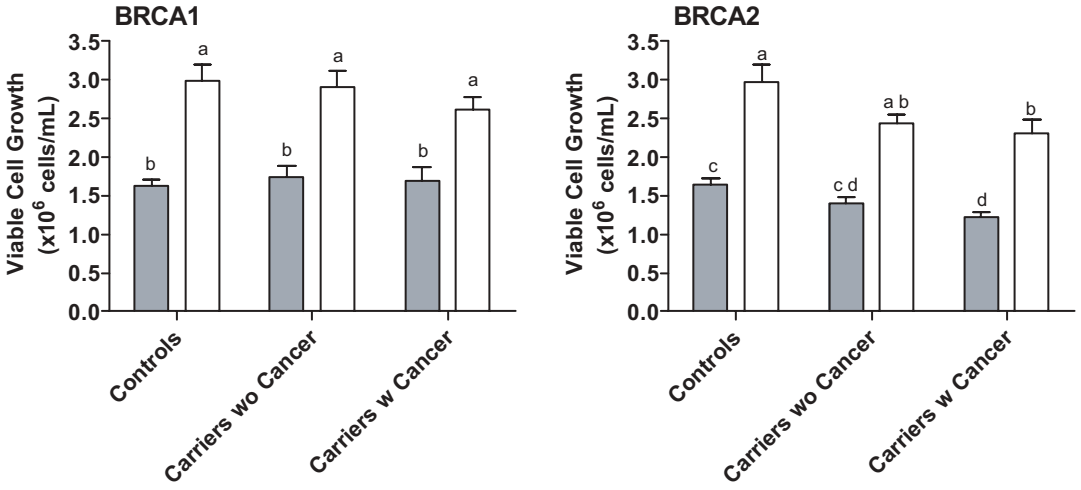

Fig. 2. Viable cell growth of peripheral lymphocytes from *BRCA1* and *BRCA2* carriers with or without breast cancer and non-carrier controls, on day 9 in media containing 12 or 120 nM FA. Cultures were initiated at 1.0×10^6 cells/ml and subcultured at 0.5×10^6 cells/ml on day 3 and day 6. Grey bars represent the viable cell growth in 12 nM FA, black bars the viable cell growth in 120 nM FA. Bars that do not share the same letter are significantly different from each other ($P < 0.05$). Data shown were adjusted for the effect of age and BMI.

Table IV. Experimental data for BRCA1 and BRCA2

	[FA] in medium	Controls	Mutation carriers without breast cancer	Mutation carriers with breast cancer	One-way ANOVA *P*
BRCA1					
NDI	12 nM*	1.29 ± 0.01^{a}	$1.27 \pm 0.02^{a,b}$	1.24 ± 0.02^{b}	0.039
	120 nM*	1.40 ± 0.02^{a}	1.31 ± 0.03^{b}	$1.37 \pm 0.03^{a,b}$	0.047
	t-test *P*	<0.001	NS	<0.001	
% Apoptotic	12 nM	14.6 ± 0.8^{a}	14.3 ± 0.9^{a}	14.0 ± 0.6^{a}	NS
	120 nM	14.7 ± 0.7^{a}	14.8 ± 0.8^{a}	15.7 ± 1.0^{a}	NS
	t-test *P*	NS	NS	NS	
% Necrotic	12 nM**	12.7 ± 0.8^{a}	13.3 ± 0.9^{a}	11.2 ± 0.9^{a}	NS
	120 nM	11.2 ± 0.8^{a}	11.4 ± 1.0^{a}	9.5 ± 0.7^{a}	NS
	t-test *P*	NS	0.050	NS	
MNed BNCs	12 nM	17.4 ± 0.8^{a}	17.2 ± 1.3^{a}	17.9 ± 1.0^{a}	NS
	120 nM	7.8 ± 0.4^{a}	8.3 ± 1.0^{a}	8.6 ± 0.5^{a}	NS
	t-test *P*	<0.001	<0.001	<0.001	
Npb BNCs	12 nM	11.5 ± 1.0^{a}	12.4 ± 1.7^{a}	14.3 ± 1.5^{a}	NS
	120 nM	7.0 ± 0.8^{a}	6.0 ± 0.6^{a}	9.7 ± 1.8^{a}	NS
	t-test *P*	<0.001	0.004	0.033	
NBud BNCs	12 nM*	31.4 ± 2.0^{a}	35.4 ± 4.1^{a}	36.7 ± 3.1^{a}	NS
	120 nM	16.5 ± 1.8^{a}	25.7 ± 2.6^{b}	$20.3 \pm 2.2^{a,b}$	0.021
	t-test *P*	<0.001	0.018	<0.001	
BRCA2					
NDI	12 nM*	1.24 ± 0.02^{a}	1.31 ± 0.02^{b}	1.30 ± 0.02^{b}	0.009
	120 nM*	1.31 ± 0.02^{a}	$1.38 \pm 0.02^{a,b}$	1.43 ± 0.02^{b}	0.001
	t-test *P*	0.003	0.004	<0.001	
% Apoptotic	12 nM	12.1 ± 0.8^{a}	$14.6 \pm 0.7^{a,b}$	15.4 ± 1.0^{b}	0.018
	120 nM	14.6 ± 1.0^{a}	14.8 ± 0.8^{a}	16.2 ± 0.9^{a}	NS
	t-test *P*	0.011	NS	NS	
% Necrotic	12 nM**	10.5 ± 1.0^{a}	11.3 ± 0.9^{a}	12.4 ± 0.9^{a}	NS
	120 nM	8.9 ± 0.7^{a}	$9.6 \pm 0.5^{a,b}$	11.7 ± 0.8^{b}	0.019
	t-test *P*	0.051	0.068	NS	
MNed BNCs	12 nM	19.3 ± 1.1^{a}	19.3 ± 1.3^{a}	20.1 ± 1.3^{a}	NS
	120 nM	8.2 ± 0.7^{a}	10.3 ± 1.0^{b}	7.7 ± 0.5^{a}	0.045
	t-test *P*	<0.001	<0.001	<0.001	
Npb BNCs	12 nM	18.0 ± 2.6^{a}	12.1 ± 1.2^{b}	11.7 ± 1.3^{b}	0.019
	120 nM	$7.9 \pm 1.0^{a,b}$	9.7 ± 1.6^{a}	5.7 ± 0.5^{b}	0.054
	t-test *P*	0.001	NS	<0.001	
NBud BNCs	12 nM*	32.9 ± 2.6^{a}	32.5 ± 3.2^{a}	32.0 ± 3.3^{a}	NS
	120 nM	20.6 ± 2.4^{a}	18.9 ± 2.1^{a}	17.0 ± 2.9^{a}	NS
	t-test *P*	<0.001	<0.001	<0.001	

Groups in one row that do not share the same superscript letter are significantly different from each other ($P < 0.05$). *t*-Test *P* values refer to companson of 12 nM and 120 nM FA cultures.
NS, not significant;
*Data adjusted for age;
**Data adjusted for age and BMI.

Table V. Percentage variation attributable to FA concentration in the medium and *BRCA* mutation carrier status or cancer status

	BRCA1				BRCA2			
	[FA] in medium	Mutation carrier status	[FA] in medium	Cancer status	[FA] in medium	Mutation carrier status	[FA] in medium	Cancer status
Viable cell growth (day 9)	50.2****	0.3	44.8****	1.0	58.5****	8.5***	58.7****	4.9**
NDI	33.4****	7.2**	34.6****	1.2	25.0****	19.8*****	34.6*****	7.8**
% Apoptotic	1.5	0.0	2.8	0.1	5.5	8.4*	2.7	8.0*
% Necrotic	7.4*	1.9	6.6*	7.4*	5.6	5.9	3.9	10.3*
MNed BNCs	74.8****	0.2	72.9****	0.3	69.6****	0.3	71.7****	0.2
Npb BNCs	22.6****	2.7	20.9****	6.8*	30.1^{a,****}	5.6^{a,****}	24.4****	6.7*
NBud BNCs	32.0****	6.2*	33.7****	2.2	25.4****	0.9	28.4****	0.1

Result of two-way ANOVA analysis.
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.
[a]Interaction between these two factors explained 5.3% of the variance ($P < 0.05$).

impact of FA concentration ($P < 0.0001$), *BRCA2* carrier status ($P = 0.0002$) and *BRCA2* breast cancer status ($P = 0.0069$) on adjusted viable cell growth, but no interaction between these variables was observed. The percentage of variation in cell growth that could be explained by the concentration of FA ranged between 45 and 59%, whereas *BRCA2* breast cancer status and *BRCA2* carrier status explained 4.9 and 8.5% of variation, respectively (Table V).

NDI

NDI tended to be decreased in *BRCA1* mutation carriers relative to controls ($P < 0.05$). In contrast, NDI of *BRCA2* mutation carriers tended to be increased compared with controls ($P < 0.01$). NDI was significantly increased in 120 nM FA relative to 12 nM FA in all groups (paired *t*-test $P < 0.05$) except for *BRCA1* mutation carriers without breast cancer. The effect of FA concentration accounted for 25–35% of the total variation (two-way ANOVA $P < 0.0001$) compared with *BRCA1* and *BRCA2* germline mutation and breast cancer status in the BRCA2 group, which accounted for 7, 20 and 8% of the variance (two-way ANOVA $P < 0.01$), respectively. No interaction between FA and these parameters was observed.

Apoptosis and necrosis

There was no difference in BRCA1 groups with respect to apoptotic and necrotic cell frequency, but FA deficiency tended to marginally increase necrotic cell frequency in *BRCA1* carriers without cancer ($P = 0.05$). In contrast, *BRCA2* mutation carriers with breast cancer tended to have a higher apoptotic and necrotic cell frequency relative to *BRCA2* carriers without breast cancer and non-carrier controls, with significant differences between *BRCA2* carriers with breast cancer and *BRCA2* controls for apoptosis at 12 nM FA ($P = 0.016$) and necrosis at 120 nM FA ($P = 0.021$). Apoptosis was not affected by FA concentration in the BRCA1 group. *BRCA2* carrier and breast cancer status affected apoptosis frequency significantly, explaining 8.4% (two-way ANOVA $P = 0.0192$) and 8.0% (two-way ANOVA $P = 0.0257$) of the variance, respectively. Cancer status explained 7.4 and 10.3% of the necrosis variance in *BRCA1* and *BRCA2* mutation carriers, respectively ($P < 0.05$). FA contributed between 6.6 and 7.4% of the necrosis variance in the BRCA1 group but had no significant impact in the BRCA2 group.

MNed BNCs

MNed BNC frequency did not differ significantly between controls, mutation carriers without breast cancer and those with breast cancer in the BRCA1 group (in low as well as high FA culture medium). The same observation was made in the BRCA2 group for low FA culture medium but in the 120 nM culture medium MNed BNC frequency was significantly elevated in the mutation carriers without cancer. There was no significant difference in the percentage of reduction in MNed BNCs in 120 nM cultures with respect to 12 nM in both BRCA1 and BRCA2 mutation carriers (with or without breast cancer) relative to their respective controls (data not shown). Two-way ANOVA indicated that only FA concentration influenced MNed BNC frequency significantly, accounting for 70–75% of the variance ($P < 0.0001$) (Table V).

Npb BNCs

The frequency of Npb BNCs tended to be increased in *BRCA1* mutation carriers with cancer but the differences relative to the other groups were not statistically significant. In contrast, *BRCA2* mutation carriers (with or without cancer) had a significant decreased frequency of Npb BNCs in 12 nM FA cultures compared with controls ($P < 0.05$); however, this difference was less evident in 120 nM FA. FA deficiency increased Npb BNC frequency in both *BRCA1* and *BRCA2* groups ($P < 0.05$). However, the percentage decrease in Npb BNC frequency in 120 nM relative to 12 nM FA cultures was not significantly different for *BRCA1* and *BRCA2* mutation carriers (with and without cancer) relative to controls (data not shown). FA concentration was the most significant factor affecting Npb BNC frequency, explaining 21–30% of the variance ($P < 0.0001$). The effect of *BRCA2* carrier status and breast cancer status (in both *BRCA1* and *BRCA2* mutation carriers) on Npb BNC frequency was smaller, contributing 5.6% ($P = 0.022$), 6.8% ($P = 0.015$) and 6.7% ($P = 0.021$) of the variance, respectively. A significant interaction was observed between FA and *BRCA2* carrier status ($P = 0.026$), accounting for 5.3% of the variance.

NBud BNCs

The frequency of NBud BNCs tended to be increased in *BRCA1* mutation carriers relative to controls, with significance for this effect observed in 120 nM FA cultures ($P = 0.015$). In contrast, *BRCA2* mutation did not have a significant impact on frequency of NBud BNCs compared with controls.

The decrease in NBud BNC frequency observed after increasing the FA concentration in the cultures from 12 to 120 nM did not significantly differ between *BRCA2* mutation carriers (with and without breast cancer) relative to controls (data not shown). However, the percentage by which NBud BNC frequency was reduced in unaffected *BRCA1* carriers was significantly less than the decrease observed in controls (data not shown; $P = 0.027$). FA concentration was the main determinant of NBud BNC frequency, explaining between 25 and 34% of the variance compared with 6.2 and 0.9% contributed by *BRCA1* or *BRCA2* mutation carrier status, respectively (Table V).

Discussion

The results of this study indicate that *BRCA1* and *BRCA2* germline mutation carriers, with or without breast cancer, neither exhibit a marked difference in chromosome instability relative to controls nor appear to be abnormally sensitive towards chromosome damage induced by FA deficiency. It is evident from these results that moderate FA deficiency has a much stronger impact on cell growth and chromosomal stability than *BRCA1* or *BRCA2* mutation and/or breast cancer status. The observed incremental effect of 12 nM FA on MNed BNCs, Npb BNCs and NBud BNCs is in agreement with those of our previous studies (33,34,42).

The apparent lack of an aggravating effect of *BRCA1* or *BRCA2* mutation on base-line genome damage seems surprising because *BRCA1* is required for homologous recombination repair (28), transcription-coupled repair (29) and possibly non-homologous end joining (30), and BRCA2 plays an important role in homologous recombination repair (31). Upon DNA damage BRCA1 activates p21 expression which results in the arrest of the cell cycle to allow DNA damage to be repaired (43,44). Mutations in BRCA1 affect the G_2/M cell cycle checkpoint and mitotic spindle formation and induce centrosome amplification, and reduced BRCA1 expression prevents DNA damage-induced mitotic exit delay, which does not appear to explain the reduced NDI in BRCA1 mutation carriers in our study unless these events cause fewer cells to undergo further mitoses after the initial mitotic mishap (45). BRCA2 deficient cells are reported to show decreased levels of DNA repair, an increased frequency of aneuploidy, chromosome aberrations, micronuclei and centrosomes together with decreased cell proliferation, the latter being in agreement with our observation of reduced cell growth in *BRCA2* mutation carriers (46–48).

Cell growth of *BRCA2*, but not of *BRCA1*, mutation carrier cells was reduced in our study; however, the percentage reduction in growth caused by FA deficiency did not differ between *BRCA1* or *BRCA2* mutation carriers and non-carrier controls. This indicates that although *BRCA2* mutation carriers exhibit reduced cell growth relative to controls they were not more sensitive to the effect of folate depletion on cell growth. However, *BRCA2* carriers showed increased NDI with decreased cell growth, which seems counterintuitive. Increased NDI in *BRCA2* germline mutation carriers could be due to a defect in cytokinesis that may have increased sensitivity to the cytokinesis-blocking action of cytochalasin-B. Daniels *et al.* (49) recently reported that targeted gene disruption of BRCA2 or reduced transcription of BRCA2 by RNA interference delays or prevents cytokinesis in mammalian cells causing an accumulation of binucleated cells in culture. It is therefore possible that, in our study, the number of binucleated *BRCA2* mutant cells increased during passage of the cells in culture and that the induced block in cytokinesis by cytochalasin-B further enhanced this effect resulting in an unexpectedly high NDI relative to controls. An extended delay in cytokinesis may also explain why nucleoplasmic bridges tended to be reduced in *BRCA2* mutation carriers because this may have allowed more time for the bridges to break prior to cell harvesting. Whether results of BRCA gene disruption or gene expression knockdown studies in cell lines are relevant to normal cells of *BRCA* mutation carriers with only a single defective copy remains uncertain; however, they may reflect the situation that may emerge if a second mutation in the residual normal gene is acquired due to a genotoxic event. Further research is required to verify these possible explanations.

In contrast to previous mutagen and/or clastogen-sensitivity studies in G_o-treated peripheral blood lymphocytes (cultured for 3 days) from *BRCA1* and *BRCA2* mutation carriers using the micronucleus assay (32,33,50,51), no significant effect was observed in our study with regard to FA-deficiency induction of micronuclei following 9-day culture. Baeyens *et al.* (52) reported a lack of radiation-sensitivity, measured by the micronucleus assay, after long-term culture (interleukin-2 cultures) of lymphocytes from healthy individuals and breast cancer patients. This could be explained by either a lack of sensitivity to DNA damage induced in the G_1-, S- or G_2-phases of the cell cycle or improved DNA repair in long-term *in vitro* cultures due to optimal supply of other micronutrients required for DNA repair (e.g. magnesium, niacin) which may have been suboptimal *in vivo*. Long-term cultures are necessary to observe the effect of folate deficiency on genome damage (35); however, it is possible that the otherwise optimal nutritional status in long-term cultures may have altered the impact of *BRCA1* and *BRCA2* germline mutation causing the anticipated increased genome instability in *BRCA1* and *BRCA2* mutation carriers to become undetectable. It may also be that the effect of a mutant copy of the *BRCA1* or *BRCA2* gene only becomes evident in G_o because at this stage of the cell cycle fewer DNA repair genes are expressed and repair capacity is already somewhat compromised; however, the effect of folate deficiency is likely to manifest itself during the cell cycle because it is during S-phase that uracil is incorporated into DNA. An alternative explanation is that the *BRCA1* and *BRCA2* genes may not be involved in the repair of DNA lesions induced by folate deficiency possibly because the type of double-strand breaks in DNA that might result from simultaneous excision of uracil (in close proximity) on opposite strands of DNA may not be a substrate of homologous recombinational repair. In fact, it was recently shown by gene expression array analysis that folate deficiency did not induce expression of genes involved in DNA double-strand break repair (53). Therefore, although experiments by Dianov *et al.* (8) indicated that simultaneous excision of uracil on opposite strands of DNA within 12 bases of each other leads to formation of a double-strand break in plasmid DNA, it remains unclear whether folate deficiency-induced uracil incorporation leads to double-strand DNA breaks in human cells.

It was hypothesized that those *BRCA1* and *BRCA2* mutation carriers who develop breast cancer may exhibit a higher level of chromosomal instability compared with carriers without

cancer. Our results did not support this hypothesis and furthermore show that those who develop breast cancer are not more susceptible to the genome damaging effects of folate deficiency either. This may imply that other dietary/lifestyle factors, such as excessive alcohol intake, may be more important in breast cancer aetiology. Alternatively, micronutrient deficiencies that may have been extant *in vivo* may have been corrected or masked by the micronutrient composition of the culture medium, which is supra-physiological for certain micronutrients, such as riboflavin and methionine, and deficient for other micronutrients, such as natural antioxidant phenolic compounds and selenium (34). The possibility that altered nutrient status could modify DNA repair deficiency phenotype is supported by a recent study showing that dietary supplementation with selenium corrected the mutagen-sensitivity phenotype of *BRCA1* mutation carriers (54).

It is reasonable to consider the possibility that experimental outcomes might have been affected by certain weaknesses in the study design. We cannot entirely exclude the possibility that previous exposure to chemotherapy and/or radiotherapy in the breast cancer patients may have up-regulated DNA repair response mechanisms or altered chromosome instability in lymphocytes; however, this seems unlikely because virtually all participants with cancer completed chemotherapy and/or radiotherapy at least 6 months prior to sample collection and there was no evidence of altered genome instability in the cancer cases relative to controls. Those with cancer may have modified their diet post-diagnosis, which may have impacted on the *in vivo* nutrient status of the cells; however, our analysis of plasma micronutrients (folate, vitamin B_{12} and selenium) does not suggest marked dietary differences between groups (data not shown). Perhaps, the use of culture medium with physiological levels of micronutrients reflecting *in vivo* status should be considered because this strategy may prevent the possibility of masking gene–nutrient interaction that may be operational *in vivo*. Another point to consider was that the *BRCA1* and *BRCA2* mutation profiles of the carriers, with or without breast cancer, were not perfectly matched; however, the mutations in the groups studied are known pathological mutations, with the exception of the 4486G>T variant in *BRCA2*, for which pathological effects remain uncertain. However, there are several other DNA repair and folate metabolism genes that could impact on genome stability and it is impossible to control for differences in genotype across a host of genes. Nevertheless, the study could be improved by substantially increasing the size of the cohort so that better matching of genotypes becomes increasingly possible.

It cannot be excluded that the response of mammary epithelial cells to FA deficiency may be different to the response of lymphocytes as there are no published studies on folate metabolism in mammary cells. In addition, it is possible that folate metabolism in mammary cells may be different to lymphocytes. Clearly it would have been ideal to use primary mammary cell cultures from donors with *BRCA1* and *BRCA2* mutations but these cells are not readily available other than from mastectomies. This could be a possible investigation for the future. Therefore until comparative studies are performed between lymphocytes and mammary epithelial cells the relevance of our results in lymphocytes to mammary cells *in vivo* or *ex vivo* remains questionable.

In conclusion, the results of our study on *BRCA1* and *BRCA2* mutations in breast cancer families show no marked effect of these genetic defects on chromosomal instability under folate replete or deficient conditions. It was also demonstrated that folate deficiency is a more important determinant of chromosomal instability than defects in the *BRCA1* and *BRCA2* genes in the breast cancer families studied. More research is needed to determine whether these observations can be reproduced under cell culture conditions with physiological concentrations of other micronutrients involved in DNA metabolism and strand break repair.

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