BRCA1 mRNA levels following a 4–6-week intervention with oral 3,3′-diindolylmethane

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Background: Haploinsufficiency may contribute to the development of breast cancer among women with a BRCA1 mutation. Thus, interventions that enhance BRCA1 expression may represent avenues for prevention. Studies have shown that 3,3′-diindolylmethane (DIM) can upregulate BRCA1 expression in breast cancer cells. This has yet to be demonstrated in vivo.

Methods: We conducted a study to evaluate the ability of oral DIM to upregulate BRCA1 mRNA expression in white blood cells. A total of 18 women were enrolled in the study, including 13 BRCA1 mutation carriers who received 300 mg per day of Rx Balance BioResponse DIM for 4–6 weeks (intervention group) and 5 BRCA1 mutation carriers who did not take DIM (control group). BRCA1 mRNA expression was assessed at baseline and at 4–6 weeks by real-time, quantitative PCR and the relative change in BRCA1 mRNA expression (that is, $2^{-\Delta \Delta C_T}$) was calculated.

Results: The relative change in BRCA1 mRNA expression among women in the intervention group achieved borderline significance ($P$ paired $t$-test $= 0.05$). In the intervention group, BRCA1 mRNA expression increased in 10 of the participants, decreased in 2 and remained unchanged in 1 of the participants following DIM intervention ($P$ sign test $= 0.02$). On average, women in the intervention group experienced a 34% increase in BRCA1 mRNA expression (range 24 to 194%). There was no significant difference in the relative change in BRCA1 mRNA expression among women in the control group ($P$ paired $t$-test $= 0.45$).

Conclusions: Under the tested conditions, oral DIM was associated with an increase in BRCA1 mRNA expression in women with a BRCA1 mutation. The possibility of mitigating the effect of an inherited deleterious BRCA1 mutation by increasing the physiologic expression of the gene and normalising protein levels represents a clinically important paradigm shift in the prevention strategies available to these high-risk women. Future studies with a larger sample size and higher doses of DIM are warranted.
caffeine (Nkondjock et al, 2006), selenium (Kowalska et al, 2005; Kotsopoulos et al, 2010) and weight loss in early adult life (Kotsopoulos et al, 2008b) protect against BRCA1-related breast cancer. In addition, we have recently reported possible relationships between plasma iron and antimyel levels and BRCA1-associated breast cancer (Kotsopoulos et al, 2012). Cancer risk reduction options that are nonsurgical and modifiable are needed.

When identifying possible lifestyle interventions that may help in the prevention of breast cancer, it is important to consider that the mechanism underlying the predisposition is likely to be different in BRCA1 mutation carriers from that of women in the general population (Salmena and Narod, 2012). BRCA1 helps maintain genomic integrity through participating in the cellular response to DNA damage, specifically in the repair of double-stranded DNA breaks (Scott, 2004). Haploinsufficiency refers to a state in which an individual has only one functional copy of a gene due to mutation or gene loss, and thus may produce an insufficient amount of protein (Berger and Pandolfi, 2011; Berger et al, 2011; Konishi et al, 2011). Although limited, there is evidence to support that the predisposition to breast cancer among BRCA1 mutation carriers is due to haploinsufficiency associated with heterozygosity, which increases genomic instability and accelerates the mutation rate of other critical genes, including the second copy of BRCA1 (Konishi et al, 2011; Salmena and Narod, 2012). Thus, factors that might increase the cellular expression of the normal copy of the gene and thereby help normalise protein levels may mitigate against the effect of the mutation.

One dietary supplement that is thought to have potential for preventing cancer in BRCA1 mutation carriers is 3,3'-diindolylmethane (DIM), a phytochemical derived from cruciferous vegetables (Higdon et al, 2007). DIM has been found to have various cancer-protective effects, particularly for hormone-dependent cancers such as that of the breast (Higdon et al, 2007). In vitro administration of a relatively low dose of the phytochemical DIM (and its precursor indole-3-carbinol (I3C)) can significantly upregulate both the BRCA1 and BRCA2 mRNA and the protein expression in breast and prostate cancer cells (Meng et al, 2000a, b, c; Fan et al, 2006, 2009).

If haploinsufficiency predisposes mutation carriers to cancer development, ultimately, the ability to upregulate BRCA1 expression in female BRCA1 heterozygotes may translate into a reduced cancer risk. To date, no studies have evaluated whether or not oral administration of I3C or DIM enhances the expression of normal BRCA1 mRNA or protein in vivo. We conducted a 4–6-week dietary intervention trial to evaluate the ability of DIM, a stable metabolite of I3C, to upregulate BRCA1 mRNA expression in women with a BRCA1 mutation.

MATERIALS AND METHODS

Study population. Potential study participants were identified from the Familial Breast Cancer Research Unit, Women’s College Hospital (Toronto, Ontario, Canada) and included women who were enrolled in previous and ongoing clinical research protocols. Eligible subjects were healthy women with no personal history of cancer, who were not pregnant or breastfeeding and were between the ages of 25 and 65 years. Women who had taken DIM were eligible to participate if they discontinued DIM use for at least 1 month before study enrolment. We included 21 healthy BRCA1 mutation carriers (BRCA1*+/*). All study subjects provided their written informed consent. This study was approved by the Women’s College Hospital research ethics board and an independent Data Safety Monitoring Board was in place during the study.

Data collection. Women were invited to participate in the study by letter. After a woman expressed interest in the study, a package consisting of a consent form and a short questionnaire about diet and lifestyle was sent to her. The research coordinator contacted the participants to arrange a preliminary clinic visit.

DIM intervention. The intervention group included 15 BRCA1 mutation carriers who were assigned to receive 300 mg per day (150 mg twice daily) of Rx Balance BioResponse DIM for 4–6 weeks provided kindly by Michael Zeligs, BioResponse, LLC, Boulder, CO, Canada. The control group consisted of six BRCA1 mutation carriers who did not take DIM. At the first clinic visit, the research coordinator assigned the BRCA1 mutation carriers to either the intervention group or control group. DIM was not randomly assigned, given that women who did not want to take the intervention were assigned to the control group. A study diary was provided to the participants in the intervention group to record information on compliance and any side effects. The research coordinator also completed a reporting form for all participants to log clinic visits, compliance with the medication and any side effects. Adverse events were reported to the Data Safety Monitoring Board at the Women’s College Hospital.

Two women in the intervention group reported adverse side effects with DIM use and withdrew from the study, thus only providing baseline blood and urine samples and one woman in the intervention group did not return for a second clinic visit. One of these subjects experienced nausea and painful menstrual cramps, whereas the other woman had an adverse reaction to the gadolinium received during MRI appointment and not due to the study intervention. One of the subjects in the control group did not complete the study. Eight of the 16 women in the intervention group reported side effects on initiation of DIM supplementation, which were of mild severity and self-limited, resolving with continued use. Side effects included loose stools, change in urine colour, headaches, decreased appetite, feeling thirsty, joint pain and abdominal pain. All adverse events were reviewed by the Data Safety Monitoring Board at the Women’s College Research Institute and deemed to be of minimal severity.

Biological sample collection. Participants were invited to come to the Familial Breast Cancer Research Unit for a 15-min visit for biological sample collection and dietary intervention assignment. All participants had a blood sample collected at baseline during the first clinic visit and an additional sample collected 4–6 weeks after the first appointment. Subjects arrived to the clinic between 9 am and 10 am after an overnight fast. Approximately 20 ml of blood was collected from the participants by venipuncture into two 10 ml EDTA-containing vials. Women were also asked to bring their first morning urine sample on ice that was collected at home. Samples were labelled, placed on ice and delivered immediately (within half an hour) to the laboratory of Dr Steven Narod (Women’s College Hospital). The blood was immediately processed for RNA extraction, and 5 ml of urine was aliquotted into two 15 ml tubes containing 10 mg of ascorbic acid and stored for future use at –80 °C in freezers, which are alarmed and continuously monitored.

BRCA1 mRNA expression. BRCA1 mRNA expression was quantified in the white blood cells of the study subjects using real-time, quantitative PCR in the laboratory of Dr Steven Narod (Women’s College Hospital) at baseline and following DIM supplementation. The LeukoLOCK Total RNA Isolation System (Ambion, Austin, TX, USA) was used for total RNA extraction from the leucocyte population according to manufacturer’s instructions. The Taqman PCR system (Applied Biosystems, Foster City, CA, USA) was used for reverse-transcription real-time PCR and the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) was used to quantify mRNA levels. The glyceraldehyde phosphate dehydrogenase (GAPDH) gene was used as a control measuring changes in BRCA1 mRNA by the comparative C_\text{t} method (\Delta\DeltaC_\text{t} method). The Taqman

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BRCA1 mRNA levels following a 4–6-week intervention

1270 www.bjcancer.com | DOI:10.1038/bjc.2014.391
assay IDs for BRCA1 and GADPH were Hs01556194_m1 and Hs99999905_ml, respectively (Applied Biosystems). All the samples were run in triplicate to evaluate accuracy and reproducibility. The largest coefficient of variation was <2% and the mean s.e. for the \( C_T \) was 0.09 and 0.10 for BRCA1 and GADPH, respectively. Samples from the same study subject (both baseline and second visit samples) were assayed in the same batch. Data were collected by the Applied Biosystems 7500 software v2.0.6 in the form of threshold cycle number (\( C_T \)) for the target gene and the endogenous control gene. The mean \( C_T \) values of the three replicate runs for each sample were used for calculations. The \( C_T \) values were normalised to the endogenous control gene and the corresponding \( \Delta C_T \) values were obtained by subtracting the mean GAPDH value from the mean BRCA1 \( C_T \) value. The corresponding \( \Delta C_T \) values were obtained by subtracting the \( \Delta C_T \) of the mRNA sample at baseline from the \( \Delta C_T \) of the mRNA at the second clinic visit. The relative change in BRCA1 mRNA expression is given by \( 2^{-\Delta C_T} \), which represents the change in BRCA1 mRNA expression at the second visit compared with the baseline levels (Livak and Schmittgen, 2001).

### Statistical analyses.

For descriptive statistics, the \( t \)-test and \( \chi^2 \) test were used to test for differences in continuous (including \( 2^{-\Delta C_T} \)) and categorical variables, respectively. A paired \( t \)-test was used to compare the mean ratio of the change in BRCA1 mRNA levels at baseline versus the second clinic visit (that is, specifically the \( 2^{-\Delta C_T} \)) compared with one (which represents the ratio for no change in BRCA1 mRNA expression) in the intervention group and control group separately. A sign test was used for differences in the distribution in the number of women in the intervention group that experienced an increase versus decrease in the \( 2^{-\Delta C_T} \). A \( P \)-value of \( \leq 0.05 \) was considered statistically significant and all analyses were carried out using SAS Version 9.1 (SAS Institute, Cary, NC, USA).

### RESULTS

Table 1 shows the baseline characteristics of the 18 participants in the intervention (\( n = 13 \)) and control (\( n = 5 \)) groups who provided two blood samples. Participants in the intervention group were on average significantly younger than those in the control group (\( P = 0.02 \)), and thus were more likely to be premenopausal (\( P = 0.0004 \)). There was no significant difference with respect to the other baseline characteristics between the two groups, including current oral contraceptive use, alcohol consumption and multivitamin use.

Figure 1 shows the distribution of the \( 2^{-\Delta C_T} \) values among all the study participants. The relative change in BRCA1 mRNA expression among women in the intervention group achieved borderline significance (\( P \) paired \( t \)-test = 0.05). In the intervention group (\( n = 13 \)), BRCA1 mRNA expression increased in 10 of the participants, decreased in 2 and remained unchanged in 1 of the participants following DIM intervention (\( P \) sign test = 0.02). On average, these women experienced a 34% increase in BRCA1 mRNA expression (range –24 to 194%). There was no significant difference in the relative change in BRCA1 mRNA expression among women in the control group (\( P \) paired \( t \)-test = 0.45). Among women in control group (\( n = 5 \)), BRCA1 mRNA expression decreased in two of the participants, increased in two of the participants and remained unchanged in one of the participants when comparing baseline and second visit levels with a mean increase of 15% (range –18 to 81%). There was no significant difference in the relative change in BRCA1 mRNA expression between women in the intervention group and those in the control group (\( P = 0.43 \)).

### DISCUSSION

There were three common mutations among the women in this study: (1) 2190delA (exon 11), (2) 185delAG (exon 2) and (3) 5382insC (exon 2). We found no significant difference in BRCA1 mRNA levels among the three (\( P \) from ANOVA = 0.32; data not shown).

The objective of this study was to evaluate the effect of a 4–6-week intervention with oral DIM on BRCA1 mRNA levels in women with an inherited BRCA1 mutation. We found that supplementation with 300 mg per day of Rx Balance BioResponse DIM for 4–6 weeks resulted in a borderline significant 34%
shown that endoplasmic reticulum stress-like pathway. This group has also observed that the induction of BRCA expression is oestrogen independent and may be via a time-dependent manner. The authors proposed that the induction of BRCA expression in human breast and prostate cancer cells in a dose- and time-dependent manner is significantly upregulated by DIM (Bradlow and Zeligs, 2010). In a series of cell culture experiments, Fan et al. (2006) demonstrated that incubation of noncancerous human breast epithelial cells with DIM results in the formation of sulphur-containing isothiocyanates and indole-based I3C and DIM (Higdon et al., 2007; Ciska et al., 2009). Under the acidic environment of the stomach, I3C further dimerises to form DIM within 24 h of incubation (Higdon et al., 2002). Numerous cancer-preventive properties have been associated with I3C and its derivatives, including cell cycle arrest, induction of apoptosis and modulation of oestrogen metabolism (Aggarwal and Ichikawa, 2005). Of particular relevance for BRCA1 mutation carriers is the ability of DIM to enhance BRCA1 expression (Fan et al., 2006).

In cell culture media, I3C is known to spontaneously auto-react, yielding DIM (Bradlow and Zeligs, 2010). In a series of cell culture experiments, Fan et al. (2006) demonstrated that incubation of human breast cancer cells (that is, MCF-7 and T47D) with I3C significantly upregulated BRCA1 and BRCA2 mRNA and protein expression in human breast and prostate cancer cells in a dose- and time-dependent manner. The authors proposed that the induction of BRCA expression is oestrogen independent and may be via an endoplasmic reticulum stress-like pathway. This group has also shown that in vitro exposure to low doses of DIM (<0.5–1 μM) for 72 h (rather than I3C) significantly increased BRCA1 and BRCA2 protein expressions and protects against oxidative stress (Fan et al., 2009). This dose of DIM was ~40 times lower than that of I3C required to produce similar effects. Collectively, the findings of Fan et al. provide preliminary evidence that expression of the normal BRCA1 protein may also be enhanced in BRCA1-deficient cells by exposing them to low doses of DIM. Zhang and colleagues have shown that intraperitoneal administration of DIM (10–50 mg kg⁻¹ body weight) is able to significantly increase expression of BRCA1 in colon and cardiac tissue from mice with experimental colitis and adriamycin-induced cardiac fibrosis, respectively (Huang et al., 2011; Yao et al., 2013). This group also demonstrated a concomitant reduction in levels of oxidative stress.

Various groups (including ours) have evaluated mutagen sensitivity (as a marker of impaired cellular response to DNA damage) in fibroblasts, lymphoblastoid cell lines and peripheral blood lymphocytes of heterozygous BRCA1 (and BRCA2) mutation carriers; however, the results have been conflicting (Speit and Trenz, 2004; Kotsopoulos et al., 2007). Although limited, there is evidence to support the idea that the predisposition to breast cancer among BRCA1 mutation carriers is the haploinsufficiency associated with a heterozygosity, which increases genomic instability and accelerates the mutation rate of other critical genes, including the second copy of BRCA1 (Foray et al., 1999; Rothfuss et al., 2000; Baldeyron et al., 2002; Coupier et al., 2004; Kowalska et al., 2005; Konishi et al., 2011; Salmena and Narod, 2012). Baldeyron et al. (2002) have shown that BRCA1 protein levels in lymphoblastoid cell lines established from peripheral blood lymphocytes of heterozygous BRCA1 mutation carriers were 50% lower in comparison with the normal control cell lines. Further, these heterozygous cells displayed impaired DNA end joining, a major double-strand break repair pathway in mammals. Similarly, Konishi et al. (2011) have reported reduced capacity for homologous recombination-mediated DNA damage repair in human cell lines with a single mutant copy of BRCA1 derived from noncancerous human breast epithelial cells. In contrast, we and others have failed to demonstrate differences in DNA repair BRCA1 mutagen sensitivity using fibroblasts or lymphocytes with heterozygous mutations (Baria et al., 2001; Nieuwenhuis et al., 2002; Kotsopoulos et al., 2007; Lovelock et al., 2007).

Although some of the initial in vitro studies of I3C and BRCA1 expressions were conducted using I3C, the current study utilised DIM supplementation for several reasons. I3C is highly reactive and poorly absorbed as it does not leave the stomach or circulates in the bloodstream after oral administration to humans (Reed et al., 2006). In vitro studies have shown that at least 50% of I3C spontaneously dimersises to form DIM within 24 h of incubation (Bradlow and Zeligs, 2010), whereas in vivo studies have shown that following administration of I3C to humans, only DIM and not I3C is detectable in the bloodstream (Reed et al., 2006). Further, following oral administration of I3C to humans, only DIM and not I3C is detectable in the bloodstream (Reed et al., 2006). Further, following oral administration of I3C to humans, only DIM and not I3C is detectable in the bloodstream (Reed et al., 2006). This is in agreement with the observation that DIM is a pro-drug that requires bioactivation in the acidic environment of the stomach (De Kruijf et al., 1991). A major limitation of our study was the quantification of BRCA1 mRNA in the genetic material derived from peripheral blood lymphocytes, which might not be representative of the breast tissue. Studies that directly quantify BRCA1 mRNA (or protein) levels in the breast before and following DIM administration are warranted. Despite this, the goal of this study was to elucidate the role of DIM in BRCA1 expression using a convenient, minimally invasive approach. Our small sample size may have precluded us from detecting a greater effect of DIM on BRCA1 expression; however, this was due to the need for immediate processing of the blood samples for RNA extraction. Nonetheless, we anticipated a substantial increase in BRCA1 mRNA with the intervention. Perhaps, the dose of DIM was not high enough or the duration of intervention was not long enough to induce a substantial change in gene expression, although this is unlikely given that this duration and dose were based on prior studies reporting plasma and tissue accumulation of DIM as well as shifts in oestrogen metabolism (Dalessandri et al., 2004; Reed et al., 2008; Heath et al., 2010; Rajoria et al., 2011), changes in gene expression (Kong et al., 2012) and activation of DNA repair (Fan et al., 2013). Pertinent to our study is that Fan et al. (2009) showed increases in both BRCA1 mRNA and protein expression in cell lines exposed to ≤0.5 μM of DIM.

Figure 1. Relative change in BRCA1 expression normalized to GAPDH in all participants. The paired t-test used to test for the relative change in BRCA1 mRNA expression for women in the intervention group and in control group (P = 0.05 and 0.45, respectively). The student’s t-test was used to test for differences in the relative change in BRCA1 mRNA expression between women in the intervention group and those in the control group (P = 0.43).
This concentration of DIM corresponds to ≤125 ng ml⁻¹ of DIM in the human plasma, which has been achieved at lower doses of DIM (that is, ~200 mg per day; Reed et al. 2008). Thus, the literature suggests that the dose and duration of intervention we used were adequate to induce changes in gene expression. Further, we did not have information on intake of cruciferous vegetables; however, we asked women to discontinue DIM use for at least 1 month before study enrolment. Finally, we did not evaluate the effect of DIM on a non-carrier population. Despite these limitations, strengths of the current study include the inclusion of unaffacted participants with a known BRCA1 mutation status and the use of a single technician (who was blinded to the intervention group) who analysed all of the samples from the same participant in a single batch under the same conditions.

In summary, the findings from this study suggest that an intervention of 300 mg per day of Rx Balance BioResponse DIM significantly modulates BRCA1 mRNA expression in BRCA1 mutation carriers. The resultant effect on BRCA1 protein expression, and more importantly cancer risk, requires further exploration. Future studies that evaluate dietary and/or lifestyle determinants of BRCA1 expression at the gene or protein level in a large sample of BRCA1 mutation carriers may help delineate a role of non-genetic factors in the aetiology of this disease. The possibility of mitigating the effect of an inherited deleterious BRCA1 mutation by increasing the physiologic expression of the gene and normalising protein levels represents a clinically important paradigm shift in the prevention strategies available to these high-risk women.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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