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The DNMT3B C→T promoter polymorphism and risk of breast cancer in a British population: a case-control study

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Abstract

Background Gene promoter methylation is an important regulator of expression and is a key epigenetic factor in tumorigenesis. DNA methylation is mediated by DNA methyltransferases (DNMTs), of which three active forms have been identified: DNMT1, DNM3A and DNMT3B. The C→T transition polymorphism (C46359T) in the promoter of the *DNMT3B* gene, which significantly increases transcriptional activity, has been postulated to increase the propensity for promoter-hypermethylation-mediated silencing of tumour suppressor genes.

Methods To determine the role of this polymorphism in breast cancer, we genotyped 352 cases and 258 controls from a British population. The breast cancer cases were selected on the basis of either an age at onset of less than 40 years, a family history of breast cancer irrespective of age at onset, or bilateral breast cancer diagnosed after 39 years of age irrespective of family history.

Results The C allele was found to be more common in case subjects than in control subjects (cases, 0.59; controls, 0.54) corresponding to a nominally significant increase in breast cancer risk to heterozygotes and CC homozygotes (odds ratio 1.51, 95% confidence interval 1.01–2.25) in the dominant inheritance model.

Conclusions Our findings contrast with those of a previous study, which showed that individuals carrying at least one T allele have a significantly increased risk of developing lung cancer. This discrepancy might be an artefact resulting from a chance variation, or it might point to differing influences of promoter hypermethylation in these cancer types.

Keywords: breast cancer, *DNMT3B*, methylation, polymorphism

Introduction

Genetic factors are increasingly being recognised as a major contributor to cancer risk [1,2]. Although genes with highly penetrant mutations, exemplified by *BRCA1* and *BRCA2*, confer a high relative risk, they are rare in the general population and therefore the population attributable risk is low. It is now suspected that most of the population attributable genetic risk is due to relatively common 'low-penetrance' disease-associated allelic variants. Although these variants might confer a small absolute cancer risk, this is outweighed by the fact that they are very common in the general population [2]. Recognition of this fact has led to an explosion of epidemiological studies searching for common polymorphisms in genes that might represent susceptibility alleles [3,4]. Among the most promising candidates are genes involved in the maintenance of genomic integrity and DNA repair, which include genes such as *BRCA1*, *BRCA2* and *TP53* [5-8]. These and other functionally related genes are currently under intense scrutiny for common low-penetrance cancer-predisposing alleles. However, another group of genes, which have not received much attention, are those responsible for epigenetic modification of DNA through CpG methylation.

DNA methylation is a major epigenetic modification involving the addition of a methyl group to the 5' position of a cytosine in a CpG dinucleotide. These dinucleotides are not equally distributed throughout the genome: most are clustered in so-called 'CpG islands', which span the promoter region and the first few exons of most housekeeping and tumour suppressor genes [9]. Most CpG dinucleotides present in islands are unmethylated in normal tissues, whereas those present in other regions of the genome are usually methylated. However, during the process of

CI = confidence interval; DNMT = DNA methyltransferase; OR = odds ratio; PCR = polymerase chain reaction.

carcinogenesis this balance is reversed. Consequently, even though many gene promoters become hypermethylated, overall cancers are hypomethylated compared with matching normal tissues. Hypermethylation of CpG islands located in the promoter regions of tumour suppressor genes is now firmly established as an important, if not the most important, mechanism of tumour suppressor gene inactivation [10] and might also influence genomic stability.

DNA methylation is mediated by a family of DNA methyltransferases (DNMTs), of which three active forms (DNMT1, DNMT3A and DNMT3B) have been identified in mammalian cells [11]. An overall increase in the enzymatic DNA methyltransferase activity of the two maintenance DNA methyltransferases, DNMT1 and DNMT3B, has been shown to occur in tumours [12-17].

The *DNMT3B* gene, located on chromosome 20q11.2, contains a C→T transition polymorphism (C46359T) at a novel promoter region, -149 base pairs from the transcription start site, which in assays *in vitro* confers a 30% increase in promoter activity [11,18]. The first evidence that this polymorphism might be associated with cancer was reported in lung cancer in which carriers of the T allele, particularly heterozygotes, had a significant increase in risk (odds ratio [OR] 2.13, 95% confidence interval [CI] 1.47–3.08) [11]. Although the mechanism for this association was not clear, it was postulated that the T variant might upregulate DNMT3B expression and thereby increase the propensity for the epigenetic silencing of some tumour suppressor genes. Such an association is supported by the observation that DNMT3b is upregulated in lung cancer cell lines [19], and comparative genomic hybridization studies have shown that the 20q region is often amplified in primary lung cancers [20].

In the present study we evaluated the association between the DNMT3B C46359T polymorphism and breast cancer risk in a hospital-based case-control study in a British population.

Materials and methods

Subjects

Subjects were recruited in a hospital-based case-control study of genes predisposing to breast cancer, performed at breast clinics in the South of England. The breast cancer cases were selected on the basis of either an age at onset of less than 40 years (203 cases), a family history of breast cancer (defined as two or more cases of breast cancer in a first-degree or second-degree female relative) irrespective of age at onset (105 cases), or bilateral breast cancer diagnosed after 39 years of age irrespective of family history (44 cases). All breast cancer cases were systematically ascertained through breast clinics in the South of England as described previously [21,22]. In brief, women were invited to take part in a research study, the primary goal of which was to ascertain and verify family histories for segregation analysis. The breast cancer cases diagnosed before 40 years of age were consecutively ascertained without regard to family history. The group of women with bilateral breast cancer were ascertained in the same clinics but the selection criterion was the presence of bilateral breast cancer diagnosed after 39 years of age. The familial breast cancer cases consisted of women presenting to the same clinics with a strong family history of breast or ovarian cancer or both. Family histories were verified as far as possible from medical records and death certificates. Blood was taken from all recruits who consented to molecular analysis for breast cancer predisposition genes. The age range of case participants was 19–76 years, with a mean of 40.

The control subjects ($n = 258$) were all white female volunteers who were either staff at the Princess Anne Hospital, Southampton ($n = 117$) or outpatients ($n = 141$) attending for obstetric-related, non-neoplastic disease conditions. The age of the controls ranged from 18–84 years, with a mean of 39.

For all groups, normal genomic DNA was prepared from blood lymphocytes. Epidemiological data such as reproductive factors, oral contraceptive use, smoking and obesity were not available for either the cases or controls. However, both control and cancer groups were residents of the greater Southampton area, which is a predominantly Anglo-Saxon population.

Molecular analysis

A total of 352 case and 258 control subjects were genotyped for the DNMT3B C46359T polymorphism by using a dual-colour allele-specific polymerase chain reaction (PCR) assay. PCR amplifications were performed with a common forward primer (5'-TGCTGTGACAGGCAGAGCAG-3') and HEX and FAM labelled reverse primers (5'-GCCTTAGGTGACTGGAGGCCTG-3' and 5'-GGCTTAGGTGACTGGAGGCCTA-3', respectively). All PCRs were performed in 10 μl volumes containing 10–200 ng of genomic DNA, 200 nM dNTPs (Promega, Annandale, NSW, Australia), 25 ng of each primer, 1× ReddyMix buffer (Abgene, Epsom, Surrey, UK) and 0.2 units of Thermoprime Plus DNA Polymerase (Abgene). PCR amplification cycle conditions involved an initial denaturation step at 94°C for 5 min, 40 cycles of denaturation at 94°C for 20 s, annealing at 68°C for 20 s, and extension at 72°C for 45 s. This was followed by a further extension step at 72°C for 5 min. The alleles were then separated through an unstained 3% agarose gel and analysed with a scanning laser fluorescence imager (Molecular Imager FX; Bio-Rad); 10% of the genotyping was confirmed with a PCR and restriction fragment length polymorphism assay described previously [3].

Statistical methods

The Hardy–Weinberg equilibrium assumption was assessed by the standard methods.

The data were considered by using models assuming either dominant inheritance (that is, women with one or two C alleles had the same relative hazard), co-dominant inheritance (that is, the relative hazard differed between women with one C allele and those with two C alleles) or recessive inheritance (that is, only women with two C alleles were at increased risk).

Case subjects were also stratified by family history, bilateral breast cancer status and age of breast cancer onset. For all analyses, the control subjects were treated as a single group without stratification.

Comparisons of frequencies were analysed with Fisher's exact test. ORs and 95% CIs were calculated by using the relevant 2 × 2 contingency tables. All statistical calculations were two-sided and performed with InStat version 3.01 (GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered statistically significant. Power calculations were performed with the online Binomial Distribution-Case Control Power Calculator available from the UCLA Department of Statistics http://calculators.stat.ucla.edu/.

Results

There was no evidence of a deviation from Hardy–Weinberg equilibrium among the case or control subjects. The frequency of the C allele in control subjects (0.54) was similar to that found in the previous study among Caucasians (0.56); however, the frequency in case subjects was substantially lower (0.59 versus 0.51) [3]. All the breast cancer cases have been screened for germline *BRCA1* and *BRCA2* mutations [22]. A total of 18 *BRCA1* and 9 *BRCA2* mutations were detected and the frequencies of the *DNMT3b* genotypes were 25.9% TT, 44.5% TC and 29.6% CC, which was not significantly different from the breast cancer group as a whole ($P = 0.22$).

Table 1 shows the breast cancer risk (given by OR, 95% CI and *P* value) for the different DNMT3B C46359T polymorphism genotypes according to co-dominant and dominant models of inheritance. Under a dominant model of inheritance, there was a nominally significant increase in breast cancer risk (OR 1.51, 95% CI 1.01–2.25) when grouping CC and CT genotypes and comparing them with the TT genotype. A similar excess of C allele genotypes was also observed in cases under co-dominant and recessive models, but these did not reach statistical significance. The frequency of the C allele was higher among the breast cancers than the controls (0.59 versus 0.54) but this difference was not statistically significant ($P = 0.13$).

The distribution of genotypes among subjects diagnosed with breast cancer at the age of less than 40 years and among subjects with a family history of breast cancer (defined as an individual with two or more cases of breast cancer in a first-degree or second-degree female relative) and/or with bilateral breast cancer, was similar to that observed for the entire cohort. Under each model of inheritance, a higher frequency of genotypes containing a C allele was observed than in the controls, although these differences were not statistically significant. Analysis of the data according to the criteria on which the cases were recruited revealed that the main contribution to the significant result came from the under-40 and family history groups (data not shown). The cases selected on the basis of bilateral breast cancer showed a genotype distribution very similar to the control group (TT, 25.0%; CT, 43.2%; CC, 31.8%). The study had 80% power to detect an OR of 1.6 or more for carriers heterozygous for the C allele and an OR of 1.7 or more for carriers homozygous for the C allele.

Table 1

DNMT3B genotype frequencies of breast cancer cases and controls

Group	No. of subjects	TT No. (%)	TC No. (%)	*P*[a]	OR (95% CI)	CC No. (%)	*P*[a]	OR (95% CI)	TC+CC No. (%)	*P*[a]	(OR, 95% CI)
Control	258	60 (23.3)	116 (45.0)			82 (31.8)			198 (76.8)		
All breast cancer	352	59 (16.8)	173 (49.1)	0.06	1.52 (0.99–2.33)	120 (34.1)	0.10	1.49 (0.94–2.35)	293 (83.2)	0.05	1.51 (1.01–2.25)
Under 40[b]	212	34 (16.0)	103 (48.6)	0.08	1.57 (0.95–2.58)	75 (35.4)	0.09	1.61 (0.96–2.73)	178 (84.0)	0.06	1.21 (1.01–1.45)
Family history bilateral cancer[c]	245	46 (18.5)	119 (47.8)	0.24	1.34 (0.84–2.12)	84 (33.7)	0.26	1.34 (0.82–2.18)	203 (81.5)	0.19	1.34 (0.87–2.06)

OR, odds ratio; CI, 95% confidence interval. [a]*P* was determined by Fisher's exact test (two-sided) for the relevant genotype, with the TT homozygotes as reference. [b]This group includes cases that were originally selected on the basis of family history but where the diagnosis was made earlier than 40 years of age. [c]This group includes cases that were originally selected on the basis of diagnosis earlier than 40 years of age but where there was also bilateral disease or a family history of cancer.

Discussion

DNMT3B has been postulated to be important in cancer because of its ability to mediate *de novo* DNA methylation, which in turn might silence tumour suppressor gene expression through promoter hypermethylation. Overexpression of DNMT3B has been observed in some cancer types [13-17], providing circumstantial evidence for a role in cancer development. In this context, the C46359T promoter polymorphism, which is reported to increase promoter activity, represents a highly plausible cancer-predisposing allele [11].

Our study of British women diagnosed with early-onset breast cancer, bilateral breast cancer and/or with a family history of breast cancer has suggested that individuals carrying one or more C alleles for the *DNMT3B* C46359T promoter polymorphism have a nominally significant increase in breast cancer risk in comparison with TT homozygotes. If we assume that the T allele is associated with increased promoter activity, our findings are counter to the underlying hypothesis that higher DNMT3B activity would increase *de novo* methylation of tumour suppressor gene promoters, and would thereby confer increased cancer risk. Because the association with the C allele was of marginal significance ($P = 0.05$), and because we could not adjust for known breast cancer risk factors, we cannot exclude the possibility that such confounding factors might have led to a type I error. However, confounding due to differences in ethnicity is unlikely because both the cases and the controls were residents of Southampton or nearby towns, which is a predominantly Anglo-Saxon population.

This is the first study of any DNMT3b polymorphism in breast cancer. The only other cancer association study reported an increased risk of lung cancer in carriers of the allele with putatively higher activity (the T allele) [11]. Intriguingly, the lung cancer study found the most pronounced effect with the CT heterozygotes under a co-dominant model, which is similar to our observation. This raises the possibility that intermediate DNMT3B activity might be more biologically conducive to cancer development than either extreme in activity. Alternatively, it is possible that both associations are real and that the discrepancy is a reflection of differing roles of DNMT3B in different cell types. It has certainly been noted that different splice variants of DNMT3B, with potentially altered catalytic activity, are expressed in a tissue-specific manner [17]. In addition, it has been shown that repression of DNMT3B activity does not result in the re-expression of all hypermethylated tumour suppressor genes in some cell systems [23], suggesting that there is a complex interplay of the different DNMTs that might operate in a tissue-specific manner. Finally, it is possible that linkage disequilibrium of the C46359T promoter polymorphism alleles to other, as yet unidentified, functional polymorphisms could explain the lack of consensus with regard to disease association. This possibility seems very likely given the very recent finding that a T/C polymorphism (T46222C) located 138 base pairs upstream of the C46359T polymorphism decreases promoter activity by up to 90% [18]. In addition, an inverse allele-dose response between DNMT3b mRNA expression and the number of T alleles of the C46359T polymorphism has been reported in bladder cancer, suggesting that it might be the C allele that is associated with higher DNMT3b expression [24]. Clearly, our understanding of the genetic factors influencing the expression of DNMT3b is incomplete, and definitive evidence defining how each allelic variant influences expression is required. Nevertheless, the existing data suggest that genetic variants located in the DNMT3b promoter can profoundly influence mRNA expression.

Conclusions

Our study suggests that there may be an association between the C allele of the DNMT3B promoter polymorphism and women with early-onset breast cancer, bilateral breast cancer or with a family history of the disease. As this is the first report of this polymorphism in breast cancer, independent studies are needed to verify this association.

Competing interests

None declared.

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