RESEARCH NOTE

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

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Introduction

Germline mutations of the *BRCA2* gene on chromosome 13q12-13 predispose humans to the development of early-onset breast cancer and ovary cancer (Wooster *et al*. 1995). Most germline mutations in *BRCA2* are predicted to result in truncation (Wooster *et al*. 1995; Tavtigian *et al*. 1996) and hence, inactivates the critical functions of the encoded protein (Shamoo 2003). Tumours arising in carriers of *BRCA2* germline mutations usually exhibit loss of heterozygosity (LOH) of chromosome 13q polymorphic markers flanking *BRCA2*. The allele lost is the wild-type allele inherited from the nonmutation-carrying parent (Collins *et al*. 1995), a pattern that is characteristic of a tumour-suppressor gene and that is predicted to result in the absence of the functional protein in the tumour cell.

In addition to germline mutations, many cancer predisposition genes including *RB1*, *p53*, *NF-2*, *APC*, *WT1* (Fearon 1997), *VHL* (Shuin *et al*. 1994) *NF-1* (Li *et al*. 1992) and *MTS1* (Caldas *et al*. 1995) are somatically mutated in sporadic cancers. These somatic mutations are usually associated with a high frequency of LOH near the susceptibility gene, relevant to the sporadic cancers. Loss of heterozygosity at the *BRCA2* locus has been observed in 30%–40% of sporadic primary breast cancers (Callens *et al*. 2003) and approximately 50% of sporadic primary ovarian cancers (Takahashi *et al*. 1996). However, exhaustive analyses of many sporadic breast, ovarian and other cancers have indicated that somatic mutations in *BRCA2* gene are very rare (Collins *et al*. 1997).

For certain tumour-suppressor genes, an alternative epigenetic mechanism of inactivation within tumour cells has been proposed. In some cancers, normally unmethylated cytosine residues within or near the promoter region of genes such as *MTS1* (*p161NK4a*) (Gonzales-Zulueta *et al*. 1995), *RB1* (Ohtani *et al*. 1993), *E-cadherin* (Yoshiura *et al*. 1995), *VHL* (Herman *et al*. 1994, 1995); *FHIT*, *FANCF*, *cyclin-D2*, *RUNX3* (Dhillon *et al*. 2004) and *RASSF1A* (Lehmann *et al*. 2002) become methylated. There is a shift in methylation patterns and some promoter region CpG islands become methylated leading to silencing of the adjacent genes, and this process is considered to be a critical step in cancer development (Herman and Baylin 2003).

Although preliminary studies on breast (Collins *et al*. 1997) and ovarian (Gras *et al*. 2001) tumours, reported the absence of cytosine methylation in *BRCA2* gene promoter, a later study showed methylation of *BRCA2* gene promoter in 4% of the patients with ovarian cancer (Dhillon *et al*. 2004). The structure of *BRCA2* gene, and the high density of CpG dinucleotides (more than 50) within a 1.1-kb region, prompted us to study the cytosine methylation status on two unstudied sites of the gene in women with sporadic breast cancer.

Materials and methods

Archival tumour tissue samples of six, and both tumour and normal tissue samples of 12 women with the diagnosis of sporadic breast cancer were the subjects of this study. The normal breast tissue samples were used as controls in order to show the differences between *BRCA2* gene methylation patterns of normal and cancerous cells of the same individuals. All patients were untreated at the time of surgery, none were smokers, and their age ranged between 40 and 58 years, and

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they did not exhibit LOH of *BRCA2* gene during routine analysis. We obtained the genomic DNA sequence (from −79 to +420) at the 5′ end of the *BRCA2* gene from the literature (Collins *et al*. 1997). Site 4 (from +97 to +282) and site 5 (−59 to +96) were determined according to their high CpG contents (figure 1).

DNA isolation

The DNA isolation was performed by following QI-Amp DNA mini Kit (Qiagen, Valencia) protocol for fresh tumour and normal tissue samples, and by applying the cell lysing technique (Williams *et al*. 1988) for archival breast tissue samples. We made slight modifications to the homogenization step of both the methods, prolonging it to overnight, and adding proteinase K (20 μl) from time to time, as needed, due to the loss of enzymatic activity. DNA samples from tumour and normal tissue samples were exposed to bisulphite treatment, following Herman *et al*. (1994).

Designing of primers

The unmethylated primer pairs designed originally for methylation specific amplification of sites 4 and 5 were:

(Site 4) *BRCA* 211 U → 5′ - TTCCGGCTGGTGCGTGTG - 3′ sense
212 U → 5′ - CGGCGACCACCGCCGAA - 3′ antisense

(Site 5) *BRCA* 221 U → 5′ - CCGGGAGAAGCGTGAGG – 3′ sense
222 U → 5′ - CCGCGGCAGGCGACCG - 3′ antisense,

and the methylated primers were:

(Site 4) *BRCA* 211 M → 5′ - TTTTGGTTGGTGTGTGTG - 3′ sense
212 M → 5′ - CAACAACCACCACCAA - 3′ antisense

(Site 5) *BRCA* 211 M → 5′ - TTGGGAGAAGTGTGAGG - 3′ sense
212 M → 5′ - CCACAACAAACAACCA - 3′ antisense.

All cytosines in sense-methylated primers were converted into thymine. All guanines of the antisense-methylated primers were converted into adenine, because of the conversion of methyl-cytosines of the template into uracil by bisulphite treatment. The primers were synthesized by Proligo-Sigma, Paris.

PCR mixture and cycles

Amplification by the methylation specific PCR (MS-PCR) and detection of the CpG methylation of sites 4 and 5 was performed under the following conditions. MS-PCR mixtures were prepared for methylated and unmethylated primers separately. Contents of the PCR mixture includes 5 μl of bisulphite treated template DNA, 5 μl of PCR buffer, 4 μl of $MgCl_2$ (25 mM), 4 μl of dNTPs (2.5 mM), 5 μl of primer 1-1 (10 ppm), 5 μl of primer 1-2 (10 ppm), 5 μl of DMSO and 0.5 μl of *Taq* DNA polymerase (Sigma, St Louis), made up to a total of 50 μl by adding distilled water.

The PCR programme was as follow: after the denaturation step at 95°C for 5 min, 95°C (30 sec), followed by 57°C (30 s) and 72°C (30 s), for a total of 40 cycles, followed by a final extension step at 72°C for 10 min. MS-PCR products were loaded on 2% agarose gel and electrophoresed at 75 V for 20–30 min, and then the bands were evaluated via photographs of the gels.

Results and discussion

We examined both site 4, extending from +97 to +282 positions, and site 5 extending from −59 to +96 positions, in the exon 1 of the *BRCA2* gene, using sodium bisulphite treatment, and MS-PCR amplification of the genomic DNA. Site 4 overlapped with three sites (sites1 and 3 partially and site 2 completely) that have been studied previously (Collins *et al*. 1997). The extra part from +96 to +103 at 5′ upstream of site 1, and the excluded part of site 3 from +282 to +334 positions were nonoverlapping regions of site 4, and we found cytosine methylation at site 4 in neither normal nor tumour samples of 12 patients, nor in the six archival tumour samples (figure 2).

However, we found CpG methylation at site 5 in the promoter of *BRCA2* gene. Interestingly, five out of twelve patients (41.67%) had cytosine methylation at site 5 in both the tumours and normal DNA, and the other seven (58.33%) cases did not show methylated CpGs in the same site of either normal or tumour tissue DNA (figure 3). In addition, four of six archival tumour samples (66.67%) also showed cytosine methylation at site 5 (figure 2).

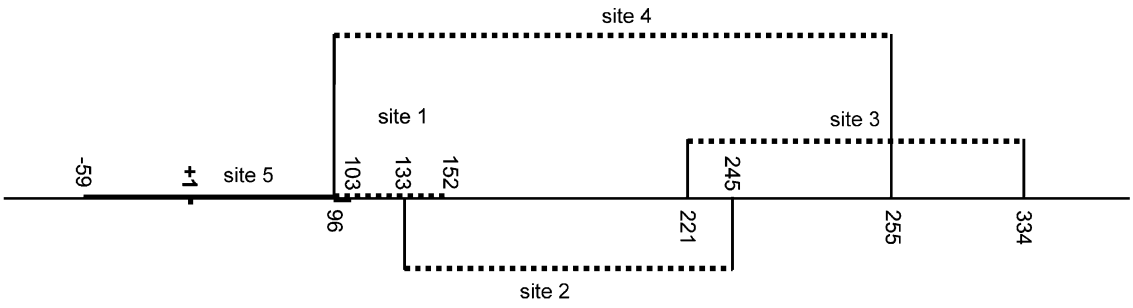

Figure 1. The illustration of the sites 1–5 on the promoter region of *BRCA2* gene. Methylation status of the sites 1–3 was examined by Collins *et al*. (1997) previously, and the sites 4 and 5 have been examined in the present study.

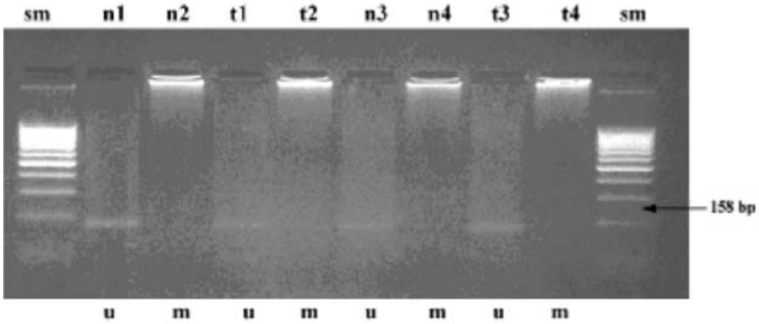

Figure 2. Methylation specific PCR amplified products of site 5 DNA after gel electrophoresis. sm, size marker; n, normal tissue DNAs of patients 1–4; t, tumour tissue DNAs of the same patients; u, unmethylated; m, methylated.

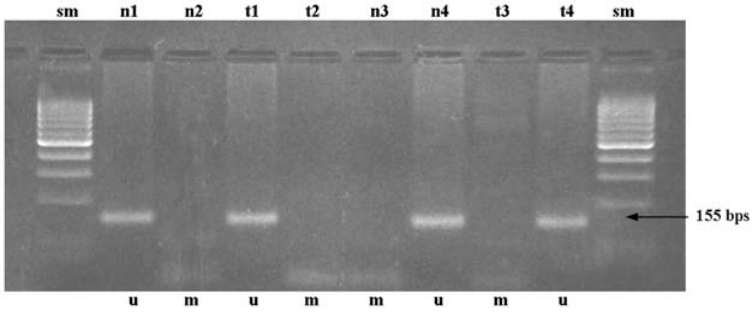

Figure 3. Methylation specific PCR amplified products of site 4 DNA after gel electrophoresis. sm, size marker; n, normal tissue DNAs of patients 1–4; t, tumour.

BRCA2 is a large gene containing 10,274 nucleotides encoded by 26 exons (Wooster *et al*. 1995; Tavtigian *et al*. 1996). As mentioned in the introduction, within the 1.1-kb region of *BRCA2* extending from −380 to +700, with 0 indicating the transcription start site, the G+C content exceeds 60% and there is an elevated CpG/GpC ratio. The high density of CpG dinucleotides and the location of this region are characteristic of 'CpG islands' that are found in the vicinity of the transcriptional start site of approximately 60% of most known housekeeping and tissue specific genes (Bird 1986). This 1.1-kb region also contains short sequences corresponding to the recognition sites of the transcription factors SP1, USF, AP2 and CP2, that are often localized within the promoters of the genes. However, this region presents no TATA or CAAT box (Collins *et al*. 1997). Previous research to identify the methylation status of the CpG rich promoter region of the *BRCA2* gene has been limited to one study on breast cancer (Collins *et al*. 1997), and the two others performed with ovarian tumours (Gras *et al*. 2001; Dhillon *et al*. 2004).

Among the two sites studied by us, site 4 did not show any methylated cytosine residues. Although the methods used are different, our data related to overlapping regions of site 4 and earlier studied sites is consistent with previous observations of Collins *et al*. (1997). As far as we know, the methylation status of the nonoverlapping part from +96 to +103 at 5′ upstream of the site 1, which includes a CpG island, has not been studied before.

In contrast to site 4, we observed methylation in site 5, extending from −59 to +96 positions in the promoter region of the *BRCA2* gene. To our knowledge, this is the first study to examine the methylation status of a site which extends to the 5′ upstream of the start site of exon 1 of the *BRCA2* gene. This site was found methylated in 50% of the studied women with breast cancer. Site 5 was methylated both in normal and tumour cells in same patients, but surprisingly it was unmethylated in both normal and tumour cells in 58.33% of sporadic breast cancer women, and 66.67% of archival breast tumours with missed normal cell samples, because of the retrospective character. The importance of this data, in the context of the epigenetic role of DNA methylation on *BRCA2* gene transcription activity, is not supported by some previous reports (Ohtani *et al*. 1993; Herman *et al*. 1994, 1995; Yoshiura *et al*. 1995; Collins *et al*. 1997). In these previous studies, the increases in methylation on promoter regions in cancers were associated with increases in methylation at all the CpG dinucleotides studied. However, one cannot predict how many CpG methylations for a given gene are required to trigger tumourigenesis (Kang *et al*. 2001). For example, it has been shown that a single-site methylation that occurs 216 nucleotides upstream from 85-bp reduced promoter ac-

tivity by 85% and was associated with transcriptional inactivation of *p53* gene in mouse hepatocarcinogenesis (Pogribny *et al*. 2000). Similar data, related to the *p53* gene methylation has also been reported for human breast cancers (Kang *et al*. 2001). Therefore, it is hard to draw any definitive conclusions about the possible role of methylation as an epigenetic factor in tumourigenesis, without expression studies of the *BRCA2* gene, with a comparison of methylated and unmethylated site 5 in women with sporadic breast cancer.

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