Research article

Epigenetic silencing and deletion of the \textit{BRCA1} gene in sporadic breast cancer

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Abstract

\textbf{Introduction} \textit{BRCA1} or \textit{BRCA2} germline mutations increase the risk of developing breast cancer. Tumour cells from germline mutation carriers have frequently lost the wild-type allele. This is predicted to result in genomic instability where cell survival depends upon dysfunctional checkpoint mechanisms. Tumorigenic potential could then be acquired through further genomic alterations. Surprisingly, somatic \textit{BRCA} mutations are not found in sporadic breast tumours. \textit{BRCA1} methylation has been shown to occur in sporadic breast tumours and to be associated with reduced gene expression. We examined the frequency of \textit{BRCA1} methylation in 143 primary sporadic breast tumours along with \textit{BRCA1} copy number alterations and tumour phenotype.

\textbf{Methods} Primary sporadic breast tumours were analysed for \textit{BRCA1}\textsubscript{\alpha} promoter methylation by methylation specific PCR and for allelic imbalance (AI) at \textit{BRCA1} and \textit{BRCA2} loci by microsatellite analysis and \textit{TP53} (also known as \textit{p53}) mutations by constant denaturing gel electrophoresis. The \textit{BRCA1} methylated tumours were analysed for \textit{BRCA1} copy alterations by fluorescence \textit{in situ} hybridisation and \textit{BRCA1} expression by immunostaining.

\textbf{Results} \textit{BRCA1} methylation was found in 13/143 (9.1\%) sporadic breast tumours. The \textit{BRCA1} methylated tumours were significantly associated with estrogen receptor (ER) negativity (\(P = 0.0475\)) and displayed a trend for \textit{BRCA1} AI (\(P = 0.0731\)) as well as young-age at diagnosis (\(\leq 55; P = 0.0898\)). \textit{BRCA1} methylation was not associated with \textit{BRCA2} AI (\(P = 0.5420\)), although a significant association was found between \textit{BRCA1} AI and \textit{BRCA2} AI (\(P < 0.0001\)).

Absent/markedly reduced \textit{BRCA1} expression was observed in 9/13 \textit{BRCA1} methylated tumours, most of which had \textit{BRCA1} deletion. An elevated \textit{TP53} mutation frequency was found among \textit{BRCA1} methylated tumours (38.5\%) compared with non-methylated tumours (17.2\%). The \textit{BRCA1} methylated tumours were mainly of tumour grade 3 (7/13) and infiltrating ductal type (12/13). Only one methylated tumour was of grade 1.

\textbf{Conclusion} \textit{BRCA1} methylation is frequent in primary sporadic breast tumours. We found an indication for \textit{BRCA1} methylation to be associated with AI at the \textit{BRCA1} locus. Almost all \textit{BRCA1} methylated tumours with absent/markedly reduced \textit{BRCA1} expression (8/9) displayed \textit{BRCA1} deletion. Thus, epigenetic silencing and deletion of the \textit{BRCA1} gene might serve as Knudson’s two ‘hits’ in sporadic breast tumorigenesis. We observed phenotypic similarities between \textit{BRCA1} methylated and familial \textit{BRCA1} tumours, based on \textit{BRCA1} deletion, \textit{TP53} mutations, ER status, young age at diagnosis and tumour grade.

\textbf{Introduction} Germline mutations in one allele of the \textit{BRCA1} or \textit{BRCA2} genes significantly increase the risk of developing early-onset breast cancer [1]. Tumour cells from predisposed individuals have consistently lost the wild-type \textit{BRCA} allele [2,3]. The most prominent feature of \textit{BRCA} deficient cells is the inability...
to repair DNA cross-links and DNA double-strand breaks by
error-free homologous recombination, which probably under-
lies genomic instability and cancer predisposition [4]. Survival
of BRCA deficient cells is generally thought to be dependent
upon dysfunctional checkpoint mechanisms, in which case
tumorigenic potential could be acquired through additional
genomic rearrangements and gene mutations. Indeed, familial
BRCA1 tumours are associated with mutations in the TP53
checkpoint gene, supporting the notion that genomic instabil-
ity is an important driving force in early-onset familial BRCA1
tumorigenesis [5].

Although inherited cancer syndromes are rare, the genes
accounting for them are generally believed to play an important
role in sporadic cancer. It was anticipated, therefore, that
somatic BRCA mutations would be found to contribute to spo-
radic breast carcinogenesis. Surprisingly, somatic BRCA
gene mutations have not been found in sporadic breast
tumours [6,7]. On the other hand, allelic imbalance (AI) at the
BRCA loci, an indicator for loss of heterozygosity, is know to
be a fairly common event in breast cancer [8]. The implications
of AI at the BRCA loci are unknown since Knudson’s hypoth-
esis predicts an additional inactivating event on top of AI to be
required for tumorigenesis to occur [9]. For these reasons, the
involvement of the BRCA genes in sporadic breast tumours
has been questioned. An alternative mechanism for BRCA1
inactivation has been suggested to be gene silencing by epi-
genetic mechanisms. Hypermethylation of CpG-island pro-
moters is known to be strongly associated with gene silencing.
Once established, methylation is passed on to daughter cells
during DNA replication by the activity of DNA methyltrans-
fersases, thereby conserving the overall pattern of methylated
CpG-islands [10]. The methylation patterns of virtually all
types of cancer, including breast carcinoma, have been found
to differ extensively from that of the corresponding normal tis-
ue. These alterations are cancer-type specific and include
global genomic hypomethylation as well as non-random hyper-
methylation of normally unmethylated CpG-island promoters
[11,12]. These observations, and others, indicate that epige-
netic modifications could be important in cancer etiology [13].

Several studies have reported hypermethylation of the BRCA1
promoter in sporadic breast and ovarian tumours. Further-
more, BRCA1 methylation has only been found in breast and
ovarian tumours and has been associated with AI at the
BRCA1 locus and reduced BRCA1 gene expression [12,14].
BRCA2 promoter hypermethylation has not been found in
breast tumours, although it has been reported in ovarian
tumours [15,16].

Familial BRCA1 and BRCA2 tumours are associated with
young age of onset and are phenotypically distinct from each
other as well as from sporadic breast tumours [1,17-19]. Con-
ventional histopathological and molecular analyses have dem-
onstrated familial BRCA1 tumours to have a basal-like
phenotype and to be significantly associated with certain fea-
tures, such as AI at the BRCA1 locus, a negative estrogen
receptor (ER) and progesterone receptor (PR) status, a med-
ullary tumour histological type, TP53 mutations and, depend-
ing on the mutation involved, a high tumour grade
[3,5,18,20,21]. Interestingly, gene expression profiling has
revealed similarities between BRCA1 methylated and familial
BRCA1 tumours [17,22]. Similarly, a comparative genomic
hybridisation study has reported a specific pattern of genetic
alterations to be predictive of familial BRCA1 tumours and
BRCA1 methylated tumours [23]. This lends support to the
idea that epigenetic silencing of the BRCA1 gene might chan-
nel tumour progression, akin to an underlying BRCA1 germ-
line mutation resulting in a BRCA-like phenotype. However, a
recent report showing high levels of BRCA1 expression and a
low frequency of BRCA1 promoter methylation in basal-like
sporadic tumours suggests that this might be more complex
[24].

In the present study, we examined the frequency of BRCA1α
promoter hypermethylation in 143 unselected primary spo-
radic breast tumours. All tumours were analysed for AI at the
BRCA1 and BRCA2 loci, TP53 mutations, hormonal receptor
status and age at diagnosis. Copy number alterations at the
BRCA1 locus were further examined by fluorescence in situ
hybridisation (FISH) in the BRCA1 methylated tumours which
were also analysed for BRCA1 protein expression, histological
type and tumour grade. The purpose of the study was to exam-
ine whether the BRCA1 gene could be implicated in sporadic
breast tumorigenesis through epigenetic modifications.

Materials and methods

Study group

The study group consisted of 143 female breast cancer
patients that carried neither the Icelandic BRCA1 5193G→A
nor the BRCA2 999del5 germine mutations [25,26]. DNA
samples from these patients were obtained from the Biological
Specimen Bank of the Icelandic Cancer Society. Tumour DNA
(obtained from fresh/frozen primary breast cancer tissue) and
normal DNA (obtained from blood or from fresh/frozen breast
tissue adjacent to the breast cancer tissue) were available
from each of the patients. Data on tumour grade (Nottingham
tumour grade), histological type, ER and PR status, flow-cyto-
metric DNA index and aneuploidy of the tumours were
obtained from the Department of Pathology, Landspitali Uni-
versity Hospital (Reykjavik, Iceland). This work was carried out
according to permits from the Icelandic Data Protection Com-
mission (2004040264; 200403147) and Bioethics Commit-
tee (99041V2S1; 99111V1S1).

Methylation specific PCR

DNA methylation of the BRCA1 promoter region was assessed
by methylation specific PCR of sodium bisulphite
treated DNA [27]. Tumour DNA and controls (1 µg) were
treated with sodium bisulphite and purified using the Wizard
DNA Clean-Up System (catalogue no. A7280, Promega, Madison, WI) following the manufacturer’s recommendations. Modified DNA was amplified with published PCR primers that distinguish unmethylated and methylated DNA. Primer sequences for unmethylated and methylated DNA were as follows: unmethylated forward, gtt taat gtta gtt tag aga gat g; unmethylated reverse, tca caa act cac acc caa tca; methylated forward, gtt taat gtta gtt tcg aga gag g; and methylated reverse, tca acg aac tca cgc cgc gca atc g [28]. The primers amplified a 182 base-pair (bp) product corresponding to nucleotides -150 to +32 relative to the main transcription start site of BRCA1. DNA extracted from blood was used as a negative control for methylated BRCA1 alleles. DNA extracted from blood and methylated in vitro was used as a positive control. The PCR solution (15 µl) contained 1 µl of modified DNA in 1X Thermo-Start PCR Master Mix (ABgene, Epsom, UK) and 5 pmol of each primer. The PCR was carried out in a thermocycler with the following conditions: one cycle of 95°C for 15 minutes followed by 35 cycles of 94°C for 30s, 65°C for 30s and 72°C for 60s, ending with one cycle of 72°C for 5 minutes. Then, 6 µl of the PCR product were mixed with 6 µl of 1X loading buffer (98% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue and 10 mM EDTA) and electrophoresed on 7.5% polyacrylamide gels.

**Allergic imbalance by microsatellite analysis**

AI at polymorphic microsatellite markers was analysed by laser quantification of PCR products. We analysed two intragenic BRCA1 markers (D17S855 and D17S1323), located within introns 12 and 20, respectively, and one marker centromeric to the BRCA1 gene (D17S846) located in region 17q12. Two BRCA2 markers were analysed, located in region 13q12, centromeric (D13S260) and telomeric (D13S171) to the gene. The marker primers were of published sequences available from The GDB Human Genome Data Base [29]. The primers were purchased HPLC purified from Eurogentec (Seraing, Belgium) with the forward primers Cy5 indocarbocyanin labelled.

The PCR solution (15 µl) contained 50 ng of DNA, 5 pmol of each primer, 0.2 mM Ultrapure dNTPs (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK) and 0.36U Dynazyme enzyme mix (Finnzymes, Espoo, Finland) with supplied 1X reaction buffer. A Hot Start was performed by heating the PCR solution in a thermocycler at 94°C for 2 minutes and cooling to 85°C before the enzyme was added to the solution. This was followed by 30 cycles at 94°C for 30s, 64°C to 69°C (annealing temperature varied depending on which primers were used) for 30s and 72°C for 60s, ending with 1 cycle of 72°C for 5 minutes.

The PCR products were mixed in a stop solution (100% deionized formamide and Dextran Blue 2000 (5 mg/ml; Amersham Pharmacia) in ratios varying from 0.13 to 1, denatured at 95°C for 5 minutes and resolved on a 3 mm thick HighResolution Repropel (Amersham Pharmacia) using an automated laser fluorescent sequencer (ALF Express DNA Sequencer, Amersham Pharmacia). Aliquots of 3 to 5 µl of each sample were loaded onto the gel. The following running parameters were used: 1,500 V, 60 mA, 25 W, 55°C. The sample interval was 2s, the running time 300 minutes and the running buffer 1X TBE (Tris-Boric Acid-EDTA). ALFwin Fragment analyser 1.0 software (Amersham Pharmacia) was used to compare the relative quantity of the PCR products. AI was defined if the relative difference of peaks representing alleles in the tumour and the corresponding normal DNA reactions was more than 25%.

**Fluorescence in situ hybridization**

FISH analysis was performed on paraffin embedded and formalin fixed breast tumour tissue sections (sliced in 4 µm sections) using DNA probes for the BRCA1 region and the centromere region of chromosome 17, simultaneously. The probe for the BRCA1 region (PAC103014; the Human BAC Clone Library, Sanger Centre, Hinxton, Cambridge, UK) which spans the entire BRCA1 gene was labelled with SpectrumOrange-dUTP (Vysis, Des Plaines, IL, USA) by nick-translation. Hybridisation efficiency of the BRCA1 probe has previously been tested in non-malignant breast samples [30]. The probe for the centromere region of chromosome 17 (clone D17Z1 in pUC 19: American Type Culture Collection, USA), was used as a copy number reference for BRCA1 and labelled with green fluorescein-11-dUTP (Amersham Pharmacia) by nick-translation.

**Figure 1**

Methylation-specific PCR analysis of the BRCA1 promoter region. Positive (+) and negative (-) controls are seen on the far right. Each lane contains products generated from separate PCR reactions using primers specific for (a) non-methylated and (b) methylated BRCA1 alleles. The tumour sample in lane 1 (L1) shows the presence of a band in both PCR reactions, indicating methylation of the BRCA1 promoter region.
Tissue sections were deparaffinized, placed in 0.01 M citric acid solution (pH 6) and heated for 2 × 10 minutes in a microwave oven at maximum power. After cooling, tissue sections were incubated with pepsin at 37°C for 20 minutes followed by dehydration. Probes were diluted in t-DenHyb-2 hybridization buffer (InSitus Biotechnologies, Albuquerque, NM, USA) as described by the manufacturer. Tissue section chromosomes and probes were simultaneously denatured at 95°C for 10 minutes. This was followed by overnight hybridisation at 37°C in a humid chamber and washing of tissue sections for 3 × 5 min in 0.1X SSC (Saline-Sodium-Citrate) at 60°C and mounting with 4′-6-Diamidino-2-phenylindole (DAPI) counterstaining. Fluorescence signals were scored in each sample by counting the number of single-copy gene and centromeric signals in at least 100 well-defined nuclei. Deletion of BRCA1 was defined if the copy number ratio was 0.8 or less, which has previously been used to detect deletion [30]. Deletion of chromosome 17 was defined if both BRCA1 and centromere mean copy numbers were 1.5 or less.

**Immunohistochemistry**

BRCA1 protein expression analysis was performed on formalin fixed and paraffin embedded malignant breast tissue and adjacent normal tissue (sliced in 4 µm sections), with BRCA1 MS110 antibody (Oncogene Research Products, San Diego, CA, USA). Tissue sections were deparaffinized, placed in 0.01 M citric acid solution (pH 6) and heated for 2 × 10 minutes in a microwave oven at maximum power. The sections were then incubated in 3% H2O2 in order to block endogenous peroxidase activity. The BRCA1 MS110 antibody (100 µg/ml) was used in 1:50 dilution in 1X Tris buffer and incubated in a humid chamber at room temperature overnight. For antibody detection all slides were incubated with StreptABComplex/HRP Duet, Mouse/Rabbit Kit (Code No. K0492: Dako, Glostrup, Denmark) reagents following the manufacturer’s recommendations. Counterstaining was performed with haemotoxylin.

Positive staining of normal breast epithelial cells that either co-existed on the tumour sections and/or normal breast tissue sections from the same breast was used as a control. The protein expression levels in tumour sections were measured by eye in three discontinuous classes, as previously described [31]. When the immunoreactivity was comparable to that of the normal breast epithelium or nuclear staining was observed in >50% of tumour cells, it was classified as level 3, that is, wild-type expression. When the staining was clearly weaker than normal surrounding cells or nuclear staining occurred in 20% to 50% of tumour cells, it was classified as level 2, that is, reduced expression. When there was no staining or nuclear staining occurred in <20% of tumour cells, it was classified as level 1, that is, absent/markedly reduced expression.

**TP53 mutation analysis**

TP53 mutation analysis was carried out by PCR amplification and constant denaturing gel electrophoresis on exons 5–8. Mutations were confirmed by direct DNA sequencing in an
ALF Express DNA Sequencer (Amersham Pharmacia) as previously described [32].

Statistical analysis
Proportions were compared by two-tailed Fisher’s exact test using GraphPad InStat3 (GraphPad Software Inc., San Diego, CA, USA). Associations with P values of <0.05 were considered to be significant and P values within the range of 0.05 to 0.10 as an indication of an association.

Results
Methylation of the BRCA1 promoter
Hypermethylation of the BRCA1α promoter was assayed in 143 primary sporadic breast tumours. Methylation was detected in 9.1% (13/143) of the tumours (Figure 1).

Allelic imbalance at the BRCA1 and BRCA2 loci
AI in the BRCA1 region was assessed in all 143 samples (Figure 2a,b). The frequencies of informative cases for polymorphism at the BRCA1 microsatellite marker regions were 72.7% (D17S846), 89.5% (D17S855) and 40.6%
The frequencies of informative cases for polymorphism at the BRCA2 microsatellite marker regions D13S260 and D13S171 were 74.1% and 71.3%, respectively. The D13S260 marker showed AI in 32.1% of informative cases, and the D13S171 marker in 35.3% of informative cases (Additional file 1). AI at the BRCA2 locus was defined if one or both markers displayed AI. According to this definition, 31.1% (42/135) of informative tumours had AI at the BRCA2 locus. Of these 42 tumours, 29 were informative for both markers, of which all but one displayed AI at both regions (96.6%).

AI at the BRCA1 locus was found to be strongly associated with AI at the BRCA2 locus (P < 0.0001, OR = 7.0, 95%CI = 3.0–16.4) with 26 of 124 (21.0%) informative tumours having AI at both loci (Table 1). However, AI at the BRCA2 locus was not found to be associated with BRCA1 methylation (P = 0.5420, OR = 1.4, 95%CI = 0.3–5.3).

**FISH analysis at the BRCA1 locus**

BRCA1 gene copy number was determined in the BRCA1 methylated tumours by FISH analysis (Additional file 1). Considerable heterogeneity was evident in the nuclei of these tumour cells (Figure 3a).

A physical deletion was detected at the BRCA1 locus in six tumours, including four with deletion of chromosome 17 (Table 2). Of these six tumours, all but one (sample 6 in Table 2) showed AI at the BRCA1 locus.

**BRCA1 protein expression**

BRCA1 protein expression was estimated in all BRCA1 methylated tumours by immunostaining. Nine of the methylated tumours were estimated to have class 1 BRCA1 protein expression, indicating absent or markedly reduced BRCA1 expression (Figure 3b,c; Table 2). Of these nine tumours, all but two had AI at the BRCA1 locus (Table 2). Four tumours were estimated to have class 2 or 3 BRCA1 protein expression (Table 2). Of these four tumours, AI at the BRCA1 locus was detected in one case (Table 2).

**TP53 mutation analysis**

Of the 143 primary sporadic breast tumours in this study, 141 were available for TP53 mutation analysis. Mutation was found in 19.1% (27/141) of the tumours. Although not statistically significant, we found the frequency of TP53 mutations to be much higher within the subset of BRCA1 methylated tumours compared with the non-methylated BRCA1 tumours or 38.5% (5/13) compared to 17.2% (22/128), respectively (P = 0.1299, OR = 3.0, 95%CI = 0.9–10.1; Table 1). However, TP53 mutations were only found in those BRCA1 methylated tumours that exhibited absent or markedly reduced BRCA1 expression, in which case the TP53 mutation frequency becomes 55.5% (5 of 9) and the association statistically significant (P = 0.01317, OR = 6.13, 95%CI = 1.21–33.51; Table 2). All the five tumours with BRCA1 methylation and...
TP53 mutation were found to have AI at the BRCA1 locus (Table 2). Furthermore, all five tumours showed absent or markedly reduced BRCA1 protein expression (Table 2).

**Hormonal receptor status and age at diagnosis**

Association was found between a negative ER status and BRCA1 methylation ($P = 0.0475$, OR = 3.4, 95%CI = 1.1–10.9; Table 1). No associations were found between BRCA1 methylation and a negative/positive PR status.

An indication for an association between young age (= 55) at diagnosis and BRCA1 methylation was found ($P = 0.0898$, OR = 2.9, 95%CI = 0.8–13.4).

**Tumour grade and histological type**

Of all the 13 BRCA1 methylated tumours, seven were of grade 3, five of grade 2 and one of grade 1. All BRCA1 methylated tumours were of infiltrating ductal type except for one that was of a lobular type.

**Discussion**

We report here that hypermethylation of the BRCA1 gene promoter is found in a considerable proportion of primary sporadic breast carcinomas, that is, 13 of 143 (9.1%), which is in the lower end of previously reported frequencies for this alteration in sporadic breast tumours [14,33,34].

Absent or markedly reduced BRCA1 protein expression was evident in the majority of the BRCA1 methylated tumours (9 of 13), suggesting transcriptional silencing in these tumours by epigenetic modifications. A trend for AI at the BRCA1 locus was observed in the subset of BRCA1 methylated tumours ($P = 0.0731$). All the BRCA1 methylated tumours that had AI at the BRCA1 locus and were informative for AI at the exogenic and an intragenic marker displayed AI at both regions, indicating a rather large deletion at chromosome 17. This is supported by the FISH analysis, which revealed deletion of chromosome 17 in most of the BRCA1 methylated tumours that had a detectable BRCA1 deletion. Importantly, the FISH analysis revealed substantial heterogeneity in BRCA1 gene copy numbers between individual cells in the BRCA1 methylated tumours, demonstrating that AI as detected by polymorphic microsatellite PCR analysis does not infer a simple loss of one BRCA1 allele but, rather, it appears to reflect complex genetic rearrangements.

AI at the BRCA1 and BRCA2 loci are known to be relatively common in breast tumours [8]. The implications of AI at the BRCA1 and/or BRCA2 loci for sporadic breast tumorigenesis remain unknown since Knudson’s hypothesis predicts that two ‘hits’ are required for tumorigenesis to occur [9]. Our results confirm that AI at the BRCA1 and BRCA2 loci are common events in sporadic breast tumours, present in 37.4% (49/131) and 31.1% (42/135) of primary sporadic breast tumours, respectively. A significant association was found between AI at the BRCA1 and BRCA2 loci ($P < 0.0001$). Importantly, we found an indication for AI at the BRCA1 locus to be associated with BRCA1 methylation ($P = 0.0731$) whereas AI at the BRCA2 locus was not found to be associated with BRCA1 methylation ($P = 0.5420$). This has not been shown previously and suggests that AI at the BRCA1 locus is specifically associated with BRCA1 methylation. Thus, copy number alter-
tions and epigenetic silencing of the \textit{BRCA1} gene in sporadic breast cancer could serve as Knudson's 'hits', which has previously been proposed by Esteller and colleagues [35]. Indeed, all but one of the \textit{BRCA1} methylated tumours that had absent/markedly reduced \textit{BRCA1} protein expression (8 of 9) also had a detectable deletion of the \textit{BRCA1} gene. Collectively, these results suggest that the \textit{BRCA1} gene is implicated in sporadic breast tumorigenesis through epigenetic silencing and deletion of the \textit{BRCA1} gene. Indications that \textit{BRCA1} methylation is important in hereditary breast cancer have been reported [35].

The failure to detect a \textit{BRCA1} deletion in one of the tumours that exhibited absent or markedly reduced \textit{BRCA1} expression could mean that promoter hypermethylation is present on both alleles, thereby alleviating any selection pressure for deletion at the \textit{BRCA1} locus. Alternatively, the level of detection in the FISH analysis could be limited by the small proportion of tumour cells present in each tumour section analysed. This might also apply for those tumours in which Al was present without a detectable deletion by FISH. Conversely, the detection level of the Al analysis was limited by the fact that none of the tumours were micro/macrodissected prior to DNA isolation, which also means that unmethylated \textit{BRCA1} alleles are always detected in the tumour samples due to the presence of normal DNA.

The four \textit{BRCA1} methylated tumours that did not exhibit significantly reduced \textit{BRCA1} expression could possibly be heterogeneous with respect to this alteration. None of the four tumours exhibited \textit{BRCA1} deletion by FISH and only one displayed Al at the \textit{BRCA1} locus. Alternatively, DNA methylation might not bring about transcriptional silencing in all instances.

Although the etiology of cancer predisposition in individuals carrying a germline \textit{BRCA1} mutation is not clear, increased genomic instability in \textit{BRCA1} deficient cells is undoubtedly of importance since it is predicted to result in increased probability of further genetic alterations and gene mutations, which might result in functional consequences by which tumorigenic potential could be acquired. Genomic instability, however, is a potent inducer of apoptosis where cell survival is dependent upon dysfunctional checkpoint mechanisms [4]. Indeed, familial \textit{BRCA1} tumours are associated with mutations in the \textit{TP53} checkpoint gene, supporting the notion that genomic instability is an important driving force in early-onset familial \textit{BRCA1} tumorigenesis [5]. Association of \textit{BRCA1} methylation with \textit{TP53} mutations has not been shown previously. Our results show a higher frequency of \textit{TP53} mutations among the \textit{BRCA1} methylated tumours compared with the non-methylated tumours or 38.5% (5 of 13) and 17.2% (22 of 128), respectively (P = 0.1299, OR = 3.0, 95%CI = 0.9–10.1). This association was not statistically significant, although the \textit{TP53} mutations were found to be entirely limited to those \textit{BRCA1} methylated tumours that exhibited absent or markedly reduced \textit{BRCA1} expression, in which case the frequency of \textit{TP53} mutations becomes 55.5% (5 of 9) and the association statistically significant (P = 0.01317, OR = 6.13, 95%CI = 1.21–33.51). Reinforcing this idea is the observation that all the five \textit{BRCA1} methylated tumours with a \textit{TP53} mutation had a detectable \textit{BRCA1} copy number reduction and the majority of these tumours had a relatively high DNA index, suggesting genomic instability (Table 2).

It has previously been suggested that \textit{BRCA1} methylated tumours might phenocopy familial \textit{BRCA1} tumours [36]. In support of this notion, we observed ER negativity to be significantly associated with \textit{BRCA1} methylation (P = 0.0475), a well established characteristic of familial \textit{BRCA1} tumours previously reported by Catteau and colleagues [37] and others. However, Matros and colleagues [24], looking at gene expression profiles, found a high frequency of \textit{BRCA1} promoter methylation among high-grade ER positive tumours, suggesting a more complex phenotype association. We found an indication for \textit{BRCA1} methylation to be specifically associated with Al at the \textit{BRCA1} locus and an elevated frequency of \textit{TP53} mutations, which has not been reported previously. In addition, we found a considerable proportion of the \textit{BRCA1} methylated tumours (7 of 13) to be of grade 3, with only one tumour of grade 1, as well as an indication of an association between \textit{BRCA1} methylation and an early age of onset (P = 0.0898) as previously reported by Wei and colleagues [34].

It has been suggested that breast cancers arising in individuals carrying a germline mutation in the \textit{BRCA} genes could benefit from therapeutic agents that lead to DNA cross-links or double-strand breaks at replication forks, for example, mitomycin C, cisplatin, diepoxybutane and, more recently, poly(ADP-ribose) polymerase (PARP) inhibitors [38]. These therapeutic agents could also be effective for sporadic breast cancers with abnormalities in the \textit{BRCA} genes, which is, as shown here, a considerably larger proportion of all breast cancer patients than germline \textit{BRCA1} or \textit{BRCA2} mutation carriers. In addition, abnormalities in other genes regulating homologous recombination could also be of relevance. This emphasizes the importance of developing methods for identifying \textit{BRCA}-like cancers, regardless of the underlying alterations [36].

**Conclusion**

Our results show promoter hypermethylation of the \textit{BRCA1} gene in a considerable proportion of all primary sporadic breast tumours. The majority of the \textit{BRCA1} methylated tumours were found to have absent or markedly reduced \textit{BRCA1} expression, suggesting transcriptional silencing by epigenetic modifications. In addition, we found an indication for Al at the \textit{BRCA1} locus to be associated with \textit{BRCA1} methylation whereas Al at the \textit{BRCA2} locus was not associated with \textit{BRCA1} methylation. This indicates that Al at the \textit{BRCA1} locus is specifically associated with \textit{BRCA1} methylation. The genetic alterations at the \textit{BRCA1} locus were further
examined by FISH, which revealed chromosome 17 deletions and heterogeneity with respect to chromosomal abnormalities. These results imply that methylation of the BRCA1 gene is accompanied by genomic rearrangements at the BRCA1 locus, resulting in loss of genetic material containing non-methylated BRCA1 alleles and retention of methylated BRCA1 alleles. We also found a substantially elevated frequency of TP53 mutations in the subset of BRCA1 methylated tumours, which has not been reported previously, suggesting that BRCA1 methylation might lead to alterations in the same molecular pathways as those known to be commonly altered in familial BRCA1 tumours. Collectively, these results implicate epigenetic silencing of the BRCA1 gene in sporadic breast tumorigenesis.

Medullary histological type was not found in the BRCA1 methylated tumours. However, we observed ER negativity to be significantly associated with BRCA1 methylation. We also found a substantial proportion of the BRCA1 methylated tumours to be of grade 3 and an indication for an association between BRCA1 methylation and early age of onset. Thus, our results indicate phenotypic similarities between BRCA1 methylated and familial BRCA1 breast tumours.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
VB and OAS contributed equally to this work, performing a substantial part of the analysis, participation in design and contribution to the writing of the manuscript. SKB contributed to FISH analysis, HH to TP53 analysis and JGJ to supervision and analysis of pathological data and immunostaining. JEE conceived of the study, was in charge of its design and coordination and the writing of the manuscript. All authors read and approved the final manuscript.

Additional files

The following Additional files are available online:

**Additional file 1**
An Excel file containing results of the FISH analysis along with DNA index and proportion of aneuploid cells. See http://www.biomedcentral.com/content/supplementary/bcr1522-S1.xls

**Additional file 2**
An Excel file containing results for each of the microsatellite markers analysed for AI. See http://www.biomedcentral.com/content/supplementary/bcr1522-S2.xls

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