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Decreased *BRCA1* Expression Levels May Arrest the Cell Cycle through Activation of *p53* Checkpoint in Human Sporadic Breast Tumors

George Sourvinos and Demetrios A. Spandidos
Laboratory of Virology, Medical School, University of Crete, Heraklion 71409, Crete, Greece

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Predisposition to breast cancer has been attributed to mutant *BRCA1* alleles whereas no *BRCA1* mutation has been described yet in sporadic breast tumours. As an initial characterization of the regulation and function of the *BRCA1* gene in sporadic breast cancer, we have compared the expression of *BRCA1* in thirty-five paired tumour specimens versus their corresponding adjacent normal tissue. We found two- to five-fold reduced *BRCA1* expression levels in tumour specimens as compared to normal tissue. Decreased *BRCA1* expression was significantly associated with loss of heterozygosity (LOH) at the *BRCA1* region, as well as with negative estrogen receptor (ER) status. Our results offer an alternative explanation of how *BRCA1* could play an important role in sporadic breast cancer, not via mutations in coding sequences but due to transcriptional disregulation. Decreased *BRCA1* mRNA may be caused due to loss of gene copies, deletions of regulatory elements in the *BRCA1* promoter or failure of transcriptional regulation by estrogen receptors. We also investigated possible relationships between *BRCA1, p53, mdm-2* and $p21^{WAF1/CIP1}$ at the expression level. *p53* expression was unaffected in almost all the specimens, *mdm-2* was overexpressed in 18/35 specimens while 21/35 overexpressed *p21.* Samples exhibiting reduced *BRCA1* levels simultaneously overexpressed both *p21* and *mdm-2,* showing that *BRCA1,* at certain levels, even reduced up to 2.7-fold, is functional and sufficient to upregulate *p21,* when p53 activity is inhibited by its negative regulator, the *mdm-2.* On the contrary, specimens exhibiting more than 2.7-fold reduced *BRCA1* levels, overexpressed *p21* while *mdm-2* expression was normal, allowing us to speculate that *p21* transcriptional activation is due to p53 activity, in cases with dramatically decreased *BRCA1* expression. Our findings provide evidence, indicating that BRCA1 might affect cell cycle regulation and loss of BRCA1 function due to decreased expression leads to cell cycle arrest, through *p53* and *p21* genes.

***Key Words:* *BRCA1* gene; cell cycle; *p53; mdm-2;* $p21^{WAF1/CIP1}$ Breast Tumors.**

Breast cancer is the most frequent malignancy in women and may occur either in hereditary or sporadic form. The *BRCA1* gene mutations in humans are associated with predisposition to breast and ovarian cancer (1-3). Many tumours arising in patients with germline *BRCA1* mutations display loss of heterozygosity at this locus which uniformly involves loss of the wild-type *BRCA1* allele, suggesting that *BRCA1* is a tumour suppressor gene (4). Furthermore, *BRCA1* antisense strategies using cultured cells resulted either in acceleration of proliferation (5) or cell transformation (6), supporting the tumour-suppressive function of *BRCA1.*

The *BRCA1* gene contains 22 coding exons and encodes a nuclear phosphoprotein of 1863 amino acid residues (7). The N-terminus of the BRCA1 protein contains a zing finger domain, indicating that it is a putative DNA binding protein and may be implicated in transcriptional regulation, DNA repair and site specific recombination (8). A distinct domain of BRCA1 protein interacts with the Rad51 protein, shown to be involved in the regulation of recombination and double-stranded DNA repair in mammalian systems, consistent with a putative DNA repair function of *BRCA1* (9). The carboxy-terminal region of BRCA1, which acts as a transcriptional transactivator when fused with GAL4 DNA-binding domain (10, 11), contains two tandem regions termed BRCT (12), exhibiting similarity with the mammalian p53-binding protein 53BP1 and with the yeast RAD9 protein that is involved in cell cycle arrest at checkpoints on DNA damage and with other proteins (12, 13), including BARD1 (14) that interacts with BRCA1 *in vivo.* It has also been reported that *BRCA1* negative regulation of the mammalian cell cycle is due to the ability of *BRCA1* to induce p21 (15).

Despite the great number of germline mutations which have already been detected in familial breast tumours, the gene has not been shown to be mutated in any truly sporadic breast cancer although these tumours show frequent LOH in the *BRCA1* region. We searched for an alternative role of *BRCA1* in sporadic

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breast cancer, not via alterations in the coding sequences of *BRCA1*, but due to aberrant expression of the gene. As an activation of the *p53* checkpoint in response to loss of *BRCA1* function takes place in mouse embryos during embryogenesis (16, 17), we investigated potential relationships between the *BRCA1* and *p53, mdm-2* and $p21^{WAF1/CIP1}$ genes responsible for the genome stability and proliferation status of the cell. Our results show decreased expression levels of *BRCA1* in tumour tissue in comparison to normal breast, suggesting that abrogation of *BRCA1* function by decreased expression is possibly a critical step in the development of sporadic breast cancer. Combined alterations in the expression of *BRCA1 p53, mdm-2* and $p21^{WAF1/CIP1}$ were observed, providing clues for the contribution of *BRCA1* to cell cycle arrest and growth suppression through these genes.

MATERIALS AND METHODS

Tissue specimens. Thirty-five tumour specimens from sporadic breast cancer paired with their corresponding adjacent normal tissue were surgically obtained and frozen at −70°C (18). All the specimens corresponded to primary tumours. Clinical data (stage, grade, lymph node metastasis, estrogen and progesterone receptors, age and family history) were available for all the specimens examined.

RNA and DNA extraction. Total RNA was isolated from fresh tissues using Trizol (Life Technologies) following the manufacturer's instructions. RNA samples were digested with DNaseI (Gibco BRL) in order to discard genomic DNA. DNA from fresh tissues was extracted as previously described (19) and stored at 4°C until PCR amplification.

cDNA synthesis and PCR. For first strand cDNA synthesis, 1-5 μg of total RNA was reverse transcribed in a 20 μl reaction volume containing 2 μl of 10× PCR buffer, 50 ng random hexamers, 50 mM $MgCl_2$, 200 ng dNTPs, 0.1M DTT and 200U Reverse Transcriptase, (SuperScript II RT, Life Technologies) for 50 min at 42°C. PCR amplification of cDNA was performed in a 50 μl reaction volume containing 1 μg cDNA, 1 μM of each primer, 200 ng dNTPs, 5 μl of 10× buffer (670 mM Tris.HCl, pH 8.5; 166 mM ammonium sulphate; 67 mM magnesium chloride; 1.7 mg/ml BSA; 100 μM β-mercaptoethanol and 1% (w/v) Triton X-100) and 1 U of *Taq* DNA polymerase. The oligonucleotide primers and the annealing temperatures used for each gene, were as follows: *BRCA1* (sense) 5′ GAT TTG ACG GAA ACA TCT TAC 3′, (antisense) 5′ CCA GCA GTA TCA GTA GTA TGA 3′, annealing temperature 57°C. PCR primers for *BRCA1,* which amplify a 236 bp region from exons 14-15, were derived from the *BRCA1* sequence in GenBank (# U14680). This region was selected because these exons have not been reported to be differentially spliced unlike 5′ exons. Primers for *p21,* (sense) 5′ AGG CGC CAT GTC AGA ACC GGC TGG 3′, (antisense) 5′ GGA AGG TAG AGC TTG GGC AGG C 3′, with annealing tepmerature 62°C, amplify 243 bp from a part of exon 2 and were derived from the *p21* sequence in GenBank (# HS03106), Primers for *p53,* (sense) 5′ CAT GAC GGA GGT TGT GAG GC 3′, (antisense) 5′ CGC AAA TTT CCT TCC ACT CG 3′ 102 bp, with annealing temperature 60°C, were designed in order to amplify 102 bp from exons 5-6 of the gene while primers for *mdm-2,* (sense) 5′ TTA TTA AAG TCT GTT GGT GCA 3′, (antisense) 5′ TGA AGG TTT CTC TTC CTG AAG 3′, with annealing temperature 57°C, amplifying 335 bp, were previously described (20). The PCR program consisted at 94°C for 40 sec, annealing temperature depending on each set of primers for 40 sec and 72°C for 35 sec. Cycles were preceded by incubation for 5 min at 94°C to ensure full denaturation and 72°C after the final cycle to ensure full extension of the product.

Samples were amplified through 27 consecutive cycles. The PCR reactions were performed on a DNA thermal cycler (Perkin Elmer-Cetus Instruments, Norwalk, CT). Preliminary experiments had revealed the conditions in which the amplification reaction remained in the exponential phase (data not shown) and thus the results could be used for quantitation of the template. 10 μl of the PCR product was electrophosed through a 10% polyacrylamide gel, silver stained and the intensity of the bands was analysed by a UVP image analysis system.

The quantity and the quality of mRNA samples was normalised after the amplification 120 bp of β2 microglobulin ($\beta 2m$) mRNA using the primers (sense) 5′ AAAGATGAGTATGCCTGCCG 3′ and (antisense) 5′ ACTCAATCCAAATGCGGC 3′ with annealing temperature 57°C. The mRNA levels for each gene were expressed as the ratio of the intensity of the bands in tumour tissues versus the corresponding levels of normal tissues. We arbitrarily considered as decreased expression, levels lower than 1.5-fold and as overexpression levels higher than 1.5-fold in malignant specimens compared to corresponding normal tissues.

Microsatellite and LOH analysis. The DNA samples were examined for genetic alterations at 17q12-q21, using a bank of four highly polymorphic microsatellite markers (18). Two of the microsatellite markers lie proximal to *BRCA1* (D17S250, THRA1), D17S855 lies within the *BRCA1,* and D17S579 lies distal to *BRCA1.* PCR analysis was performed in a 50 μl reaction volume as described above. The reactions were denatured for 5 min at 94°C and the DNA was subsequently amplified for 30 cycles at 94°C, 57°C and 72°C each step. Ten μl of the PCR product was analysed in a 10% polyacrylamide gel and stained with silver staining.

Microsatellite instability (MI) was scored by comparing the electrophoretic pattern of the microsatellite markers amplified from the paired DNA preparations that corresponded to the tumour with adjacent normal tissue, demonstrating a shift of one or both of the alleles in the tumour DNA specimen. The shift was indicated by either an addition or deletion of one or more repeat units resulting in the generation of novel microsatellite alleles. The analysis in the MI positive cases was repeated at least twice and the results were highly reproducible. Gels were scanned and the intensity of the bands corresponding to the microsatellite alleles was quantitated by a UVP image analysis system. Allelic loss was scored as a decrease in intensity of one allele relative to the other as determined from comparison of tumour and normal DNAs.

Statistical analyses. All statistical analyses were performed using the X^2-test or Fisher's exact test. One-tailed P-values ≥ 0.05 were considered statistically significant.

RESULTS

Thirty-five paired tumour specimens from sporadic breast cancer with their corresponding adjacent normal tissue were used for evaluating the expression of *BRCA1.* Twenty-six out of 35 tumour specimens (74%) exhibited reduced expression as compared with paired normal samples (Table I). *BRCA1* mRNA was expressed at two- to five-fold higher levels in normal mammary tissue than in breast cancer samples (Fig. 1).

We investigated the incidence of genetic alterations at the microsatellite level, either LOH or MI, using highly polymorphic markers flanking the *BRCA1* or located within the gene. Twenty of the 26 specimens representing decreased expression of *BRCA1* exhibited LOH, mainly for the intragenic marker D17S855 and

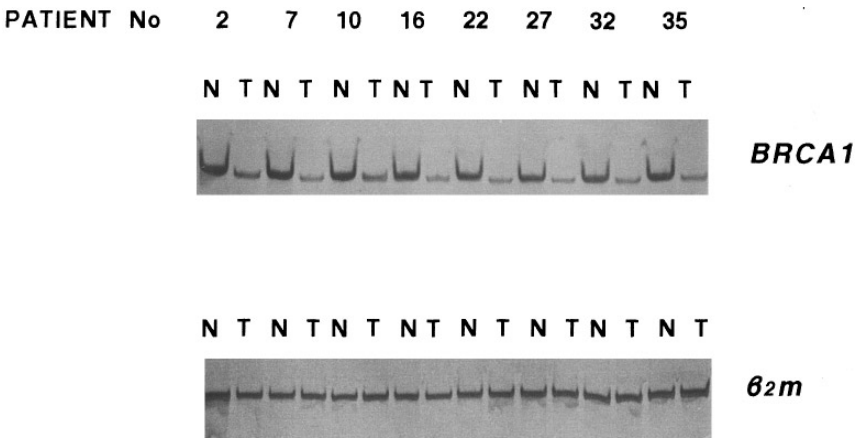

FIG. 1. Expression of *BRCA1* mRNA in sporadic breast cancer. N, normal and T, tumour specimens.

the THRA1 marker, which is located in the 5′ end of *BRCA1* (Table I) (Fig. 2). On the contrary, only two of the remaining nine cases (cases 3, 32) with normal expression of the gene displayed LOH. This finding supports a significant correlation between the decreased levels of *BRCA1* mRNA and allelic imbalances in the *BRCA1* region (p=0.006). Eight specimens exhibited MI, but no statistically significant association was found between the expression levels of *BRCA1* and MI. Furthermore, 21 of the 26 samples with diminished expression of *BRCA1* gene were negative for estrogen receptors (ER), whereas almost all of the specimens (8/9) expressing *BRCA1* in normal levels were ER positive (Table I), suggesting a significant relationship between aberrant expression of *BRCA1* and ER negative status (p=0.0004). We did not detect an association between decreased *BRCA1* expression and progesterone receptor status, neither any significant statistical correlation between *BRCA1* expression and other clinical parameters (stage, grade, age).

As *BRCA1* has been proposed to participate in the cell cycle regulation and DNA repair in mouse embryos (21), we investigated the expression levels of *p53, mdm-2* and $p21^{WAF1/CIP1}$, genes implicated in the same cellular functions, in human breast tumours. The expression of *p53* remained unaffected in 32 out of 35 specimens (91%). In contrast, half of them (18/35) exhibited overexpression of *mdm-2* gene while in 21 of 35 samples (60%) an overexpression of $p21^{WAF1/CIP1}$ was observed (Table I) (Fig. 3). Interestingly, 20/26 specimens displaying reduced levels of *BRCA1* expression, overexpressed $p21^{WAF1/CIP}$, while 1/8 samples with normal *BRCA1* levels overexpressed p21 (p=0.0009) (Table I).

The latter indicates a significant association of reduced *BRCA1* expression with *p21.* Nine of 26 specimens expressing *BRCA1* up to 2.7-fold lower than their paired normal tissues, overexpressed *p21* and *mdm-2* at the same time, whereas eleven specimens expressing more than 2.7-fold reduced *BRCA1* mRNA levels, expressed *p21* at high levels and *mdm-2* was constant (p=0.05) (Table I). Thus, we may assume that certain levels of *BRCA1,* even reduced (up to 2.7-fold), may be functional and sufficient to upregulate *p21,* when *p53* activity is inhibited by its negative regulator, the *mdm-2.* Indeed, statistically significant association was observed between the normal expression of *mdm-2* and *p21* overexpression, in specimens with more than 2.7-fold decreased *BRCA1* levels (p=0.03), indicating that *p21* is upregulated by p53 in cases with dramatic reduction of BRCA1 protein (Table I).

DISCUSSION

The involvement of *BRCA1* in familial breast and ovarian cancer is well established. However, the contribution of *BRCA1* in the genesis of the far more common sporadic form of these tumours remains obscure. Thus, as mutations in the genomic sequences of *BRCA1* has not been described yet in sporadic breast cancer, we investigated alterations at the expression level of the gene. Because previous studies suggested that invasive breast cancers exhibit lower mRNA levels than normal breast epithelial cells (5, 22), we compared the expression of *BRCA1* in sets of paired samples from cases that had both normal breast and invasive tumour material available. Our results demonstrate that *BRCA1* is expressed at higher levels in normal adjacent tissues than in malignancies. These results support a possible critical role of *BRCA1* gene in sporadic breast cancer. *BRCA1*-related carcinogenesis in the breast occurs primarily when expression of this gene is absent during critical phases of development (23).

Decreased expression of *BRCA1* supports the tumour-suppressive function of the gene, suggesting that a certain level of normally functioning *BRCA1* is needed for the maintenance of negative growth regulatory mechanisms and that cancer may arise due to the reduction in its relative level of expression.

TABLE I

Cumulative Results of Gene Expression, Microsatellite Analysis and Estrogen Receptor Status

Pt No	BRCA1	p53	mdm-2	$p21^{WAF1/CIP1}$	LOH	MI	ER
1	↓ (>2.7-fold)	N	N	↑	D17S855	THRA1, D17S855	−
2	↓ (≤2.7-fold)	N	↑	↑	THRA1, D17S855	−	−
3	N (1.15-fold)	N	N	N	THRA1	THRA1, D17S579	+
4	N (1.22-fold)	N	↑	↑	−	D17S855	+
5	N (1.31-fold)	N	↑	N	−	−	+
6	N (1.27-fold)	N	N	N	−	−	+
7	↓ (>2.7-fold)	↑	N	↑	D17S855	THRA1	−
8	↓ (>2.7-fold)	N	N	N	D17S855	−	−
9	↓ (>2.7-fold)	N	N	↑	THRA1	−	−
10	N (1.36-fold)	N	↑	N	−	−	+
11	↓ (>2.7-fold)	N	N	↑	THRA1, D17S855	−	−
12	↓ (>2.7-fold)	N	↑	N	−	−	−
13	↓ (≤2.7-fold)	N	↑	↑	D17S855	−	−
14	↓ (>2.7-fold)	↑	↑	N	−	−	−
15	↓ (≤2.7-fold)	N	↑	↑	THRA1	−	−
16	↓ (≤2.7-fold)	N	↑	↑	D17S250	−	−
17	N (1.28-fold)	N	↑	N	−	D17S250	+
18	↓ (>2.7-fold)	N	N	↑	D17S855	−	−
19	↓ (>2.7-fold)	N	N	↑	THRA1	−	−
20	↓ (>2.7-fold)	N	N	↑	D17S250	D17S579	−
21	↓ (≤2.7-fold)	↑	↑	↑	−	D17S855	−
22	↓ (≤2.7-fold)	N	↑	↑	THRA1	−	+
23	↓ (≤-fold)	N	↑	↑	THRA1, D17S855	−	−
24	↓ (>2.7-fold)	N	N	↑	D17S250, THRA1	−	+
25	↓ (>2.7-fold)	N	N	N	−	−	−
26	↓ (>2.7-fold)	N	↑	N	−	−	−
27	↓ (≤2.7-fold)	N	↑	↑	D17S855	−	+
28	N (1.21-fold)	N	↑	N	−	−	+
29	N (1.36-fold)	N	N	N	−	−	−
30	↓ (>2.7-fold)	N	N	↑	D17S855	THRA1	−
31	↓ (>2.7-fold)	N	N	N	−	−	+
32	N (1.18-fold)	N	↑	N	THRA1, D17S855	−	+
33	↓ (>2.7-fold)	N	N	↑	D17S855, D17S579	−	−
34	↓ (>2.7-fold)	N	N	↑	D17S579	−	−
35	↓ (≤2.7-fold)	N	↑	↑	D17S855	−	+

↓, Decreased expression; ↑, Overexpression; N, normal expression; LOH, loss of heterozygosity; MI, microsatellite instability; ER, estrogen receptor; +, positive ER; −, negative ER

There are several explanations for the low *BRCA1* activity in breast tumour cells and we may consider that more than one mechanism lead to decreased expression. Our results indicate significant correlation between decreased *BRCA1* expression and allelic deletions within the gene, suggesting that gene dosage is a mechanism that frequently accounts for decreased levels of expression. Furthermore, LOH in a region at the 5′ end of *BRCA1* indicates a possible deletion of regulatory elements of the *BRCA1* promoter, following failure of potential transcription factors to regulate *BRCA1* expression. A further explanation is derived from our observation of negative estrogen receptor status in specimens displaying low levels of *BRCA1*. Estrogen is known to modulate the growth and differentiation of human breast epithelium (24) and several studies have shown that *BRCA1* levels are elevated upon estrogen stimulation (25-28). These observations suggest that the expression of *BRCA1* may be modified, either directly or indirectly by estrogen. An alternative mechanism may lead to decreased *BRCA1* expression and could be the regulatory control of *BRCA1* expression by a differential splicing mechanism, although our data cannot provide evidence for this hypothesis, because *BRCA1* expression was quantitated with a pair of primers amplifying mRNA from exons 14-15 which is not a region where differential splicing is reported to occur, hypermethylation of the *BRCA1* promoter region as an epigenetic mechanism (29) or the occurrence of mutations in sporadic cases distinct from that of the hereditary cases.

Because several known tumour-suppressor genes interact with or negatively regulate the cell-cycle machinery (30), we investigated the *BRCA1* expression levels in relevance with the expression levels of *p53*, *mdm-2* and $p21^{WAF1/CIP1}$, genes implicated in cell cycle progression. We found combined alterations in the expression levels of these genes. Specimens expressing

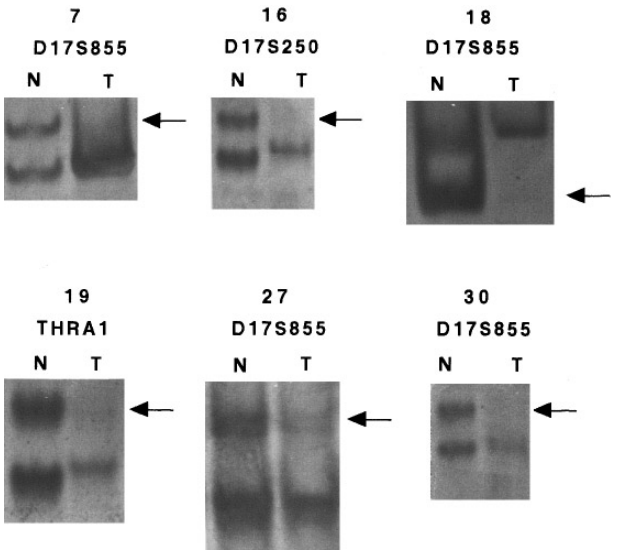

FIG. 2. Representative examples of LOH detected in breast cancers. N, normal DNA; T, tumour DNA. Arrows indicate the position of a deleted allele. The faint bands in the position of the deleted alleles are interpreted as contamination by the adjacent normal tissue.

BRCA1 up to 2.7-fold lower than their paired normal tissues, overexpressed *p21* and *mdm-2* at the same time, whereas specimens expressing more than 2.7-fold reduced *BRCA1* mRNA levels expressed *p21* at high levels and *mdm-2* was unchanged. *p21* is a universal cyclin dependent kinase inhibitor (31, 32) and its expression is directly regulated by p53 protein (33). In addition, BRCA1 has been shown to be efficient in activating *p21* expression (15). Our results allow us to speculate that certain levels of *BRCA1*, even reduced (threshold 2.7-fold in our study), are sufficient to upregulate *p21*, when p53 activity is inhibited by its negative regulator, the *mdm-2*. *p53* expression levels were unaffected, although expression of *mdm-2*, a gene coding for a negative regulator of p53 activity, was elevated in some cases. The latter indicates a critical role for *p53*, not at the expression level but in the activity of the gene. Furthermore, specimens exhibiting more than 2.7-fold reduced *BRCA1* levels, overexpressed *p21* while *mdm-2* expression was normal, proposing that *p21* transcriptional activation is due to p53 activity, in cases with dramatically decreased *BRCA1* expression.

BRCA1 can inhibit S-phase progression and thus negatively regulate the cell cycle in human cancer cells (15). This inhibition may occur through transcriptional transactivation by the C-terminal portion of *BRCA1* or by its ability to act as an RNA polymerase II holoenzyme-associated protein (34). The inhibition of S-phase progression has been proposed to take place during absence of BRCA1 function, leading to activation of the *p53* checkpoint (35). BRCA1 can also induce *p21* directly (15). *p21* expression may be essential for *BRCA1* to inhibit new DNA synthesis. Our results show that decreased levels of *BRCA1* in sporadic breast cancer are related to overexpression of *p21*, leading to the par-

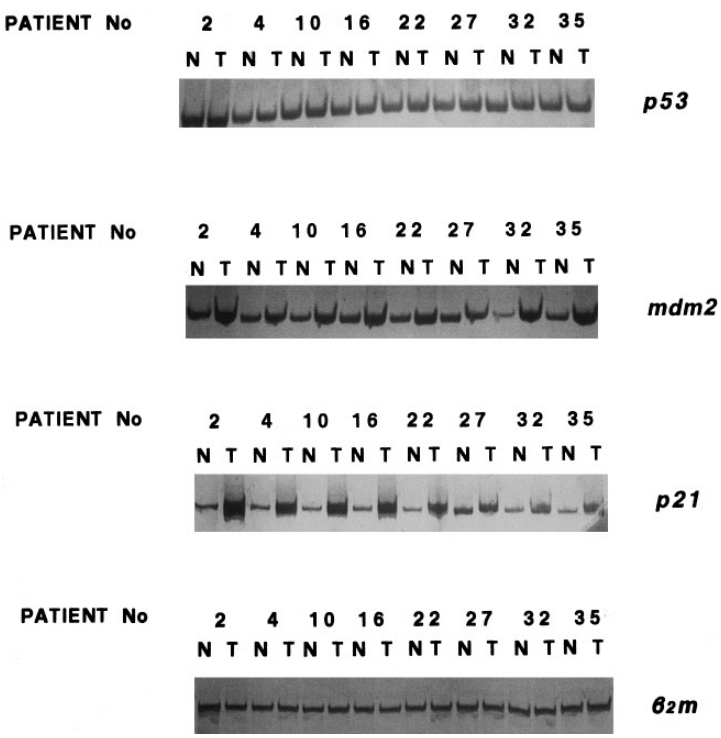

FIG. 3. Expression of *p53*, *mdm-2* and $p21^{WAF1/CIP1}$ in sporadic breast cancer. N, normal and T, tumour specimens.

adox suggestion of an inhibitory effect of *BRCA1* on *p21* expression. However, it has been proved that mutants of *BRCA1* lacking a functional nuclear localization signal, the C-terminal transactivation domain, the RAD51 interacting domain or all three domains are deficient in activating *p21* expression (15). As no BRCA1 mutation has been described in sporadic breast cancer and obviously no mutant BRCA1 protein has been found, BRCA1 protein produced by tumour cells from sporadic cases, although reduced, is complete. Thus, we may propose that certain levels of complete BRCA1 protein are required for the maintenance of negative growth regulatory mechanisms in mammary epithelial cells and sufficient to activate *p21* leading to cell cycle arrest.

Our findings provide several lines of evidence, suggesting that *BRCA1* may affect cell cycle regulation. Decreased *BRCA1* expression levels in sporadic breast cancer may result in activation of the checkpoint mechanisms implying *p53* and *p21* genes responsible for cell cycle arrest. Further studies are needed to determine the precise mechanisms through which the genes interact, leading to cell cycle arrest and repair of the genome.

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