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Article *in* Oncogene · November 1998

DOI: 10.1038/sj.onc.1202106 · Source: PubMed

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p53 mutation with frequent novel codons but not a mutator phenotype in BRCA1- and BRCA2-associated breast tumours

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The status of p53 was investigated in breast tumours arising in germ-line carriers of mutant alleles of BRCA1 and BRCA2 and in a control series of sporadic breast tumours. p53 expression was detected in 20/26 (77%) BRCA1-, 10/22 (45%) BRCA2-associated and 25/72 (35%) grade-matched sporadic tumours. Analysis of p53 sequence revealed that the gene was mutant in 33/50 (66%) BRCA-associated tumours, whereas 7/20 (35%) sporadic grade-matched tumours contained p53 mutation ($P<0.05$). A number of the mutations detected in the BRCA-associated tumours have not been previously described in human cancer databases, whilst others occur extremely rarely. Analysis of additional genes, $p16^{INK4}$, Ki-*ras* and β-globin revealed absence or very low incidence of mutations, suggesting that the higher frequency of p53 mutation in the BRCA-associated tumours does not reflect a generalized increase in susceptibility to the acquisition of somatic mutation. Furthermore, absence of frameshift mutations in the polypurine tracts present in the coding sequence of the TGF β type II receptor (TGF β IIR) and *Bax* implies that loss of function of BRCA1 or BRCA2 does not confer a mutator phenotype such as that found in tumours with microsatellite instability (MSI). $p21^{Waf1}$ was expressed in BRCA-associated tumours regardless of p53 status and, furthermore, some tumours expressing wild-type p53 did not express detectable $p21^{Waf1}$. These data do not support, therefore, the simple model based on studies of BRCA−/− embryos, in which mutation of p53 in BRCA-associated tumours results in loss of $p21^{Waf1}$ expression and deregulated proliferation. Rather, they imply that proliferation of such tumours will be subject to multiple mechanisms of growth regulation.

Keywords: BRCA1; BRCA2; p53; mutation(s); breast; tumour; familial

Introduction

Inheritance of mutant alleles of the breast cancer susceptibility genes BRCA1 and BRCA2 confers a substantial risk of developing breast, ovarian and some other cancers (Mikki *et al*., 1994; Wooster *et al*., 1995). BRCA1 and BRCA2 have numerous similarities (Zhang *et al*., 1998). Tumorigenesis in carriers of germ-line mutations in BRCA1 and BRCA2 is invariably accompanied by loss of the wild-type allele (Smith *et al*., 1992; Neuhausen and Marshall, 1994; Collins *et al*., 1995; Gudmundsson *et al*., 1995; Kelsell *et al*., 1996) suggesting that the proteins encoded by the two genes operate as tumour suppressors. An increasing-body of evidence favours a role for both BRCA1 and BRCA2 in cellular response to DNA damage (Connor *et al*., 1997; Scully *et al*., 1997; Patel *et al*., 1998). It is postulated that the BRCA genes act as caretakers, functioning to maintain genomic stability, rather than as gatekeepers which regulate cellular proliferation (Brugarolas and Jacks, 1997; Kinzler and Vogelstein, 1997). Embryos homozygously deleted for BRCA1 or 2 are inviable, cells undergoing a $p21^{Waf1}$-mediated growth arrest at around day 5–6 (Hakem *et al*., 1996; Suzuki *et al*., 1997). This lethality is partially reversed by breeding into a p53 or $p21^{Waf1}$ null background (Hakem *et al*., 1997; Ludwig *et al*., 1997). Paradoxically, tumours arising in carriers of BRCA1 mutations are frequently highly proliferative, despite being functionally null for BRCA1 (Eisenger *et al*., 1996; Marcus *et al*., 1996; Johannsson *et al*., 1997). The molecular basis for this is not known. Analysis of fibroblasts derived from mice homozygous for a truncating mutation in BRCA2 has revealed a proliferation defect associated with p53-dependent induction of $p21^{Waf1}$ expression, and a DNA repair defect (Connor *et al*., 1997). In another study, mouse embryo fibroblasts carrying BRCA2 truncations, were shown to be significantly more sensitive to UV irradiation and the alkylating agent methylmethanesulphonate than wild-type controls, leading to the suggestion that BRCA2 may be associated with a nucleotide excision repair pathway (Patel *et al*., 1998). However, it is not known whether these defects occur in tumours arising in carriers of germline mutant alleles of BRCA1 and BRCA 2.

In sporadic breast cancer the frequency of p53 mutation is 20–40%, the proportion of mutant tumours increasing with higher grade. In a previous study using DNA extracted from paraffin sections, p53 mutations were reported to occur at high frequency in familial tumours (of unknown BRCA status) but not sporadic breast tumours (Glebov *et al*., 1994). Furthermore, analysis of a small series of breast and ovarian tumours arising in carriers of mutant BRCA1 alleles revealed a high incidence of p53 mutation (Crook *et al*., 1997). In the present study we have

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Received 2 January 1998; revised 1 May 1998; accepted 5 May 1998

investigated the status of p53 in a larger series of BRCA1- and BRCA2-associated breast carcinomas and in a group of sporadic breast tumours matched for grade, and have compared both the frequency and nature of the p53 mutations detected in the two groups.

Results

The expression and structure of p53 was examined in 50 tumours arising in carriers of germ-line mutations in BRCA1 (28 tumours) and BRCA2 (22 tumours). The germ-line mutations in these families have been described previously (Gayther *et al*., 1995, 1997). To control for a potentially biasing effect of grade, a series of sporadic grade 3 breast tumours, previously characterized by the Breast Cancer Linkage Consortium (1997), was analysed in parallel.

Expression of p53 in BRCA1- and BRCA2-associated breast tumours

Expression of p53 in sporadic and familial tumours was analysed by immunocytochemistry (IC) in paraffin sections of formalin-fixed tissues, using the DO-1 antibody. 20/26 (77%) of BRCA1-, 10/22 (45%) of BRCA2-associated tumours and 25/72 (35%) of grade-matched sporadic tumours were positive (Figure 1 and Table 1). In all cases of BRCA1- and BRCA2-associated tumours where ductal carcinoma *in situ* (DCIS) was also present in the tissue sections examined, the p53 immunostaining detected in invasive tumour tissue was also present in the DCIS. In no section examined was p53 staining observed in normal tissue. Detection of p53 by IC in breast tumours frequently reflects stabilization due to mutation (Visscher *et al*., 1996). The absence of immunocytochemically detectable p53 in normal breast tissue in our study therefore implies somatic acquisition, rather than germline inheritance, of p53 mutation. It is estimated, however, that 10–15% of mutants do not give rise to stabilized protein and that p53 becomes stabilized (and thereby detectable) in some breast tumours despite being wild-type (Visscher *et al*., 1996). We therefore analysed the sequence of p53 in the sporadic and BRCA-associated tumours.

p53 is mutant at high frequency in BRCA-associated tumours

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tumour sections from 28 BRCA1-, 22 BRCA2-associated and 20 grade 3 sporadic tumours and used for analysis of the sequence of various genes. For p53 analysis, DNA was subjected to single strand conformation polymorphism analysis (SSCP), cloning and sequencing of each p53 exon (Visscher *et al*., 1996). However, when it was observed in preliminary studies that p53 mutation was common in the BRCA1- and BRCA2-associated tumours, the coding sequence of p53 was then analysed in its entirety in each BRCA-associated and sporadic tumour by sequencing at least 12 independent plasmid clones for each exon for each tumour, irrespective of the presence or not of SSCP mobility shifting. In the BRCA1-associated tumours, p53 mutations were detected in 19/28 tumours analysed, two of which were negative by IC (Table 1). In the case of the BRCA2-associated tumours, 14/22 tumours were mutant for p53 these being all nine tumours positive by IC and five negative by IC (Table 1, Figure 2). Similar sequencing analysis of DNA extracted from paraffin sections of 20 sporadic grade 3 tumours revealed single missense mutations in 7/20 tumours (Table 1). The difference in p53 mutation frequency between the sporadic and BRCA-associated tumours was statistically significant ($P<0.05$).

Multiple p53 mutations occur in some BRCA-associated but not sporadic breast tumours

Sequencing analysis revealed that five BRCA1-associated and three BRCA2-associated tumours contained two p53 mutations. One BRCA1-associated tumour and one BRCA2-associated tumour contained three independent mutations (Table 1). In four BRCA1- and three BRCA2-associated tumours with two mutations, a different coding change occurred with the identical silent, second mutation (codon 211 ACT>ACC). In the informative tumours, sequencing of multiple plasmid clones revealed that the mutations usually occurred on the same allele (Figure 2a). Thus in the BRCA2 tumour with three mutations (202R>S, 214 H>R and 219 P>H), all three occurred on the same allele. Similarly, in the two informative BRCA2

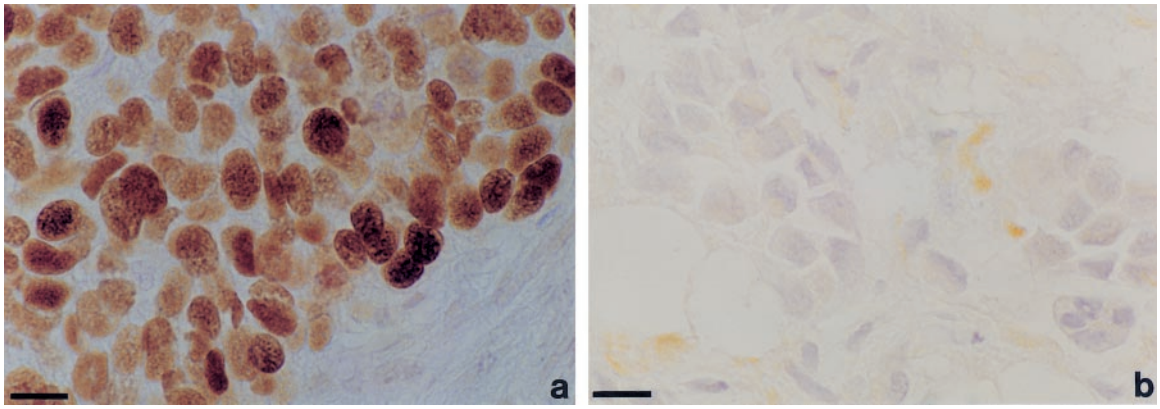

Figure 1 Immunocytochemical analysis of p53 expression in BRCA1-associated breast cancer. (**a**) positive tumour: (**b**) negative tumour. The scale bars are equivalent to 10 μm

tumours with two mutations (202R > S, 211 Sil.) and (220Y > H, 211 Sil.), both mutations were on the same allele. Further sequence analysis of multiple independent plasmid clones from these two tumours identified numerous cases in which only the mutation resulting in the coding change (202R > S or 220Y > H) was present. However, no clone was observed in which the silent mutation at codon 211 alone was detected, implying that it arose subsequent to the coding mutation (Table 1). Previous studies using DNA extracted from paraffin sections have reported multiple p53 mutations in familial tumours (of unknown BRCA status) but not sporadic breast tumours. To further verify that the mutations in the present study were not artefacts attributable to tissue fixation or contamination, and to confirm that they were somatically acquired and not germ-line or polymorphisms, microdissected tumour, DCIS and normal from selected tissue sections were analysed for the presence of mutations. In each of the analysed cases, the coding mutations originally detected in DNA from undissected tissue sections were also identified in the microdissected tumour and the single case of DCIS (Table 2). In the two cases analysed where the tumour contained a non-coding change at codon 211, in addition to coding changes, sequencing analysis did not detect the codon 211 change in the microdissected DCIS. These observations are consistent with (i) absence of p53 expression in normal tissue present in any of the tissue sections analysed (ii) the presence of immunocytochemically detectable p53 protein in cases of DCIS where the associated tumours were also positive for p53 expression (iii) somatic acquisition rather than germline inheritance of the p53 sequence changes.

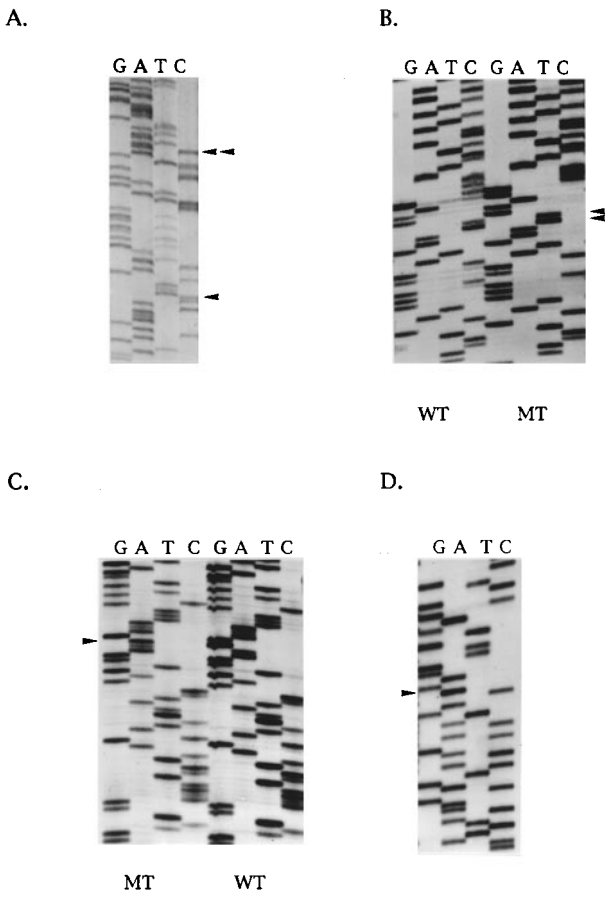

Figure 2 Sequence analysis of p53 in BRCA-associated breast tumours. (**a**) Presence of two p53 mutations in the same allele of p53 in BRCA1-associated tumour. The sequence changes at codon 224 gAg > gAC and codon 211 ACT > ACC are arrowed; (**b**) Tandem CC > TT transition at codons 247/248 (arrowed in lane MT) in BRCA2-associated tumour. The wild-type sequence (WT) is also shown; (**c**) Codon 199 mutation (ggA > AgA) arrowed in BRCA1-associated tumour; (**d**) Silent sequence change at codon 170 ACg > ACA in BRCA1-associated tumour

Absence of mutator phenotype in BRCA-associated tumours

The frequency and occasional multiplicity of p53 mutation suggested that loss of BRCA1 and BRCA2 function might confer a generalized increase in sensitivity to somatic mutation of cellular genes. To investigate this possibility, we performed analysis of the structure of additional genes which are rarely mutant (Ki-*ras*) or almost never mutant ($p16^{INK4}$) in sporadic breast cancer. We also analysed a gene in which mutation could not provide a selective advantage (β-globin). Consistent with previous studies, no mutations were detected in $p16^{INK4}$ in paraffin-extracted DNA from the sporadic grade 3 tumours, nor in any of the BRCA-associated tumours. The proposed absence of $p16^{INK4}$ mutations in BRCA tumours was further confirmed by direct sequencing in 12 of the BRCA1-associated and 12 of the BRCA2-associated tumours. In the case of the Ki-*ras* oncogene which is often mutant in pancreatic carcinomas (Bos, 1987), but only rarely in sporadic breast tumours, a codon 12 mutation (GGT > GAT, Gly > Asp) was detected in one BRCA2-associated tumour, whereas 13 further tumours analysed (eight BRCA1 and five BRCA2) did not contain activating mutations at these sites. Mutation at this codon has been previously detected in sporadic breast cancer and in a breast carcinoma cell line (Prosperi *et al*., 1990). Furthermore, this frequency of Ki-*ras* mutation is comparable to that reported in sporadic breast tumours (Rochlitz *et al*., 1989; Clark *et al*., 1995). Finally a 268 bp fragment of the β-globin gene was analysed by SSCP in each of the BRCA1 and BRCA2 tumours and the sporadic grade 3 breast tumours. No mobility shifts indicative of mutation were detected. Because the incidence of p53 mutations suggested the possibility of a mutator phenotype, we analysed the $poly(A)_{10}$ tract of TGF-βIIR and the $poly(G)_8$ tract of *Bax* in the BRCA-associated tumours, since studies of human tumours with microsatellite instability (MSI) arising in a classical mutator background have revealed frameshift mutations within these regions of each gene. No aberrant mobility bands suggestive of frameshift mutation were observed in any of 28 tumours analysed (16 BRCA1, 12 BRCA2).

Expression of $p21^{WAF1}$ occurs in BRCA1- and BRCA2-associated tumours irrespective of p53 status

Studies of embryos deleted for both BRCA2 and p53, or BRCA2 and $p21^{Waf1}$, have revealed that development progresses to a significantly later stage (in such double knockouts) than in BRCA2−/− embryos which undergo cell cycle arrest at day 5–6. This suggests

Table 1 *p53* mutations in tumours arising in carriers of mutant BRCA1/2 alleles and grade-matched sporadic breast tumours

Family	*p53 expression*	*p53 mutation*	*p21*^{Waf1} expression	*Mitoses/10 hpf*
BRCA1				
B117	12	144CAG > CCG (Q > P);[d] 211 ACT > ACC (Sil.)[a] 224 GAG > GAC (E > D)[a]	4	49
B35	12	212 TTT > TCT (F > S)	4	36
B35	0	Wild-type	4	6
B35	12	197 GTG > TTG (V > L)[a] 211 ACT > ACC (Sil)[a]	0	29
B43	15	169 ATG > ATA (M > I);[d] 211 ACT > ACC (Sil.)[d]	0	122
B64	10[b]	Wild-type	0	N/D
B64	15	220 TAT > CAT (Y > H);[d] 234 TAC > TGC (Y > C)[d]	0	56
B138	0	Wild-type	2	42
B138	0	Wild-type	4	46
B138	4	Wild type	0	26
B146	0	150 ACA > ATA (T > I)	0	51
b146	N/D	163 TAC > AAC (Y > N)	N/D	110
B155	12	Wild-type	0	53
B176	15	Wild-type	0	35
B176	15	248 CGG > TGG (R > W)	0	100
B176	18	247, 248 AACCGG[d] AATTGG (NR > NW) 211 ACT > ACC (Sil.)[d]	0	56
B176	10	212 TTT > TCT (F > S)	0	64
B178	N/D	Wild-type	N/D	13
B202[e]	18	163 TAC > AAC (Y > N)	6	90
B202[e]	18	163 TAC > AAC (Y > N)	2	89
B202[e]	12	158 CGC > CAC (R > H)	2	32
B217[e]	12	168 CAC > TAC (H > Y)	0	87
B217[e]	12	133 ATG > ATA (M > I)	0	12
B218[e]	18	163 TAC > AAC (Y > N)	0	79
B227[e]	15	251 1 bp deletion;[c] 249 AGG > AGA (Sil.)[c]	0	70
B227	0	Wild-type	1	30
B229	0	199 GGA > AGA (G > R)[a] 211 ACT > ACC (Sil.)[a]	0	28
B229	6	170 ACG > ACA (Sil.)[c] 181 CGC > TGC (R > C)[c]	0	N/D
BRCA2				
B97	2	Wild-type	0	9
B105	6	247/248 AACCGG > AATTGG(NR > NW)	0	110
B105	0	202 CGT > AGT(R > R)[a] 214 CAT > CGT (R > S) [a] 219 CCC > CAC (P > H)[a]	0	65
B124	0	Wild-type[b]	0	N/D
B135	0	Wild-type	0	1
B135	0	Wild-type	0	0
B135	0	240 AGT > AGG(R > S)	0	0
B150	0	155 ACC > ATC(T > I)	0	76
B150	0	Wild type	10	36
B164	0	228 GAC > CAC (D > H)	0	10
B169	15	164 AAG > AAT (K > N)	0	56

Table 1 continued

Family	*p53 expression*	*p53 mutation*	*p21*^{Waf1} expression	*Mitoses/10 hpf*
B186	18	220 TAT > CAT (Y > H) [a] 211 ACT > ACC (Sil.)[a]	0	36
B186	8	207 GAT > GGT (D > G)	1	30
B186	0	202 CGT > AGT (R > S)[a] 211 ACT > ACC (Sil.)[a]	6	56
B186	0	Wild-type	0	12
B186	2	Wild-type	9	23
B186	0	Wild-type	0	1
B186	15	163 TAC > AAC (Y > N)	0	98
B186	15	156 CGC > CCC (R > P)	0	72
B196	6	202 CGT > AGT (R > S)	0	36
B196	0	181 CGC > TGC (R > C)	6	36
B211	18	163 TAC > AAC (Y > N)[c] 211 ACT > ACC (Sil.)[c]	0	136
Sporadic	15	175 CGC > CAC (R > H)	0	120
Sporadic	15	245 GGC > GTC (G > V)	0	150
Sporadic	0	Wild-type	2	61
Sporadic	0	Wild-type	0	33
Sporadic	0	Wild-type	1	32
Sporadic	0	Wild-type	0	7
Sporadic	18	248 CGG > CAG (R > Q)	0	100
Sporadic	0	Wild-type	0	110
Sporadic	0	Wild-type	0	160
Sporadic	0	Wild-type	0	45
Sporadic	0	Wild-type	0	19
Sporadic	0	Wild-type	0	4
Sporadic	12	258 GAA > AAA (E > K)	0	66
Sporadic	0	Wild-type	0	46
Sporadic	10	171 GAG > AAG (E > K)	0	N/D
Sporadic	10	Wild-type	0	91
Sporadic	8	Wild type	0	44
Sporadic	10	Wild type	0	28
Sporadic	10	163 TAC > TCC(Y > S)	0	16
Sporadic	15	272 GTG > GCG (V > A)	0	46

[a]Mutations occurring on same allele; [b]Ductal Carcinoma *in situ*; [c]Mutations occurring on different alleles; [d]Cannot be determined whether mutations are on the same allele; [e]Mutation previously reported (Crook *et al*., 1997); N/D: not determined

Table 2 p53 status of microdissected BRCA1-associated tissues

Family	*Mutation*	*Tumour*	*DCIS*	*Normal*
B35	197 GTG > TTG	5/12 [a]	5/12[a]	0/12
	211 ACT > ACC	3/12[b]	3/12[b]	0/12
B176	247,248 AACCGG > AATTGG	8/12[a,c]	N/D	0/12
	211 ACT > ACC	3/12[c]	N/D	0/12
B227	251 1 bp deletion	7/12[a]	N/D	0/6
	249 AGG > AGA	2/12[d]	N/D	0/6
B229	199 GGA > AGA	3/12[a]	N/D	0/6
	211 ACT > ACC	3/12[b]	N/D	0/6

[a]Total number of clones in which coding change was detected. [b]Number of clones in which non-coding change was detected with coding change. [c]Cannot be determined whether these changes occur together on same allele. [d]Number of clones in which silent mutation was detected. N/D: Not done (no DCIS in section)

that loss of the p53-dependent, p21^{Waf1} mediated G_1-S checkpoint confers partial rescue of the cell cycle arrest consequent to abrogation of BRCA1 or BRCA2 function. To determine whether expression of p21^{Waf1} is affected by p53 status in BRCA1- and BRCA2-associated tumorigenesis, expression was analysed in tumours by IC. In the 20 sporadic grade 3 tumours, expression was absent or at extremely low levels irrespective of p53 status or expression, even in the three tumours which expressed significant wild-type p53 (Table 1). In the case of the BRCA1-associated tumours, expression of p21^{Waf1} was detected in 9/26 tumours analysed, these comprising four wild-type and five mutant for p53. Three tumours wild-type for p53 did not express p21^{Waf1} despite having high levels of p53 protein (Table 1). Analysis of BRCA2-associated tumours revealed expression of p21^{Waf1} in 5/22 tumours including three tumours mutant for p53 (Table 1). The highest expression of p21^{Waf1} was observed in two of the eight BRCA2-associated tumours which retained wild-type p53 (Table 1). However, in these tumours p21^{Waf1} expression was not detected in every tumour cell. Rather, intense expression was observed in some cells, whereas expression was either much reduced or absent in other cells. Of the remaining six BRCA2 tumours wild-type

for p53, only a single tumour expressed detectable p53 protein and none expressed $p21^{Waf1}$ (Table 1). Taken together, these data imply that, as in sporadic breast and other tumours, $p21^{Waf1}$ expression is induced in some cases by p53-independent mechanisms.

Effect of p53 mutation on mitosis in BRCA1- and BRCA2-associated tumours

The higher frequency of p53 mutation in the BRCA1 and BRCA2 tumours than in sporadics implies some form of selective pressure. Although abrogation of $p21^{Waf1}$ expression does not appear to be the basis for this, we sought to establish whether proliferation was affected by p53 status by determining mitotic fraction in BRCA-associated tumours mutant or wild-type for p53. These analyses suggested that no clear relationship exists between p53 mutation and proliferation (Table 1). For example, although mitosis is higher in some tumours with mutant p53 in certain families (see for example family B186), in other families mitotic fraction was not elevated despite the presence of mutation. For example in family B135, two of the three analysed tumours retained wild-type p53 and exhibited an extremely low mitotic fraction. In the third tumour this extremely low proliferation was unaffected by p53 mutation (codon 240R > S). Taken together, with the analysis of $p21^{Waf1}$ expression, these results are not consistent with a model in which p53 mutation simply reflects a requirement to abrogate a $p21^{Waf1}$-dependent proliferation block.

Discussion

In this study we demonstrate that p53 is mutant in a higher proportion of BRCA1- and BRCA2-associated breast tumours than in a series of sporadic breast tumours matched for grade. The higher frequency implies that mutation in p53 may have a role in tumorigenesis in a proportion of the BRCA1- and BRCA2-associated tumours in the present series. However, the presence of a significant number of tumours which retain wild-type p53 and of a number of tumours which express $p21^{Waf1}$, suggests that neither mutation of p53 nor loss of $p21^{Waf1}$ expression is required for BRCA-associated tumorigenesis.

The identity of the p53 mutations detected in both BRCA1- and BRCA2-associated breast tumours is most unusual and of interest in comparison with numerous previous analyses of p53 in human cancer in general, and in breast cancer specifically. Although some of the mutants, such as 163Y > N, have been previously documented in human breast cancer, a significant number have not been described. For example, analysis of the IARC human cancer p53 mutation database revealed codon 211 mutations in occasional tumours, but the ACT > ACC mutation detected in the BRCA-associated tumours herein is not present. Similarly, mutation at codon 150 is extremely rare, being reported in only three cases, two of which involve deletion. The ACA > ATA mutation we have observed is the first report of this sequence change, and only the second observation of any missense mutation at this codon in human cancer. A third example of an apparently previously unobserved mutation is the codon 202 CGT > AGT change we have detected in three BRCA2-associated tumours. Although mutations at codon 202 have been occasionally described, the CGT > AGT mutation has not. Yet another example is at codon 155. Ten point mutations at this codon are in the IARC database, of which five are ACC > CCC, two are ACC > GCC, two ACC > AAC and one ACC > ACT. The mutation detected in the BRCA2-associated tumour in our study, ACC > ATC, has not been previously described. Similarly, 11 point mutations at codon 197 are present in the database, yet the GTG > TTG mutation observed in a BRCA1-associated tumour in the present study has not been described. Additionally, in some tumours, mutations were detected which are exceedingly rare in human cancer. One such is the codon 199 change GGA > AGA. This mutation occurs only once, this being in a Wilm's tumour, in the IARC database. A second, and related, observation of interest from our work is the occurrence of p53 mutations in BRCA-associated breast tumours which have previously only been reported in cutaneous tumours. The most striking example of this is the presence of a CC-TT tandem transition at codons 247, 248 in one BRCA1- and one BRCA2-associated tumour. Previously, such tandem mutations at pyrimidine dimers have been described exclusively, with a single exception, in cutaneous neoplasia, their presence being attributed to production of photolesions between adjacent pyrimidine bases by UVB and subsequent impaired repair of the lesion (Hutchinson, 1994). The observation of such sequence changes in familial breast cancer, although most unexpected, may result from exposure to oxidising or other mutagenic agents which act with increased efficiency in cells with impaired DNA repair function (Reid and Loeb, 1993). This hypothesis is strengthened by the observation of the mutation TTT > TCT at codon 212 in 2 BRCA1-associated tumours. Point mutation at this codon is rare in human tumours, the change TTT > TTA being reported in just three tumours in the IARC database. Only a single other codon 212 mutation is represented on the IARC database, this being TTT > TCT in a skin tumour. The recent demonstration that mouse fibroblasts with truncated BRCA2 are more sensitive to UV irradiation than controls (perhaps due to defective nucleotide or base excision repair) is clearly of interest in the context of this observation (Patel *et al*., 1998). Previous studies of sporadic breast cancer have revealed three codons at which mutation most commonly occurs: 175, 248 and 273 (Levine *et al*., 1994; Hollstein *et al*., 1994). Two BRCA1-associated and one BRCA2-associated tumour contained mutation at these 'hot spot' codons, these being the tandem 247, 248 CC > TT mutation alluded to above in two cases and a single CGG > TGG transition at codon 248 in one BRCA1 tumour. The biological significance of the unusual spectrum of p53 changes, and the mechanism underlying their presence in BRCA-associated but not sporadic breast cancer requires additional study. The presence of multiple p53 mutations, some of which were non-coding, was described in by Glebov *et al*. (1994) in a study of familial breast tumours of unknown BRCA status and we now demonstrate multiple mutations in eight of the BRCA1 and four of the BRCA2-associated tumours.

The explanation for the multiple mutations is not clear. However, it is noteworthy that in tumours with both coding and silent mutations, the coding mutation is clearly acquired before the silent change. It is not possible from the present data to determine whether loss of the wild-type BRCA allele precedes somatic acquisition of p53 mutation or *vice versa*. The relatively high frequency of mutation and presence of multiple p53 mutations in some tumours is consistent with a generalised increase in sensitivity to acquisition of somatic gene mutations in BRCA-associated tumourigenesis. To address this possibility, we performed sequence analysis of five additional genes. We analysed two genes in which mutation confers growth advantage (Ki-*ras* by gain of function, $p16^{INK4}$ by loss of function), but which are infrequently mutant in sporadic breast cancer, and it was reasoned that mutation in either would be likely to confer a selective advantage to cells in which they occur and thereby favour their retention in tumour progression. However, our analysis did not reveal a frequency of mutation in either BRCA1- or BRCA2-associated tumours above that observed in sporadic breast tumours of similar grade. Thus, no mutations were detected in $p16^{INK4}$ in any of the familial tumours analysed, as has previously been demonstrated in sporadic breast tumours (Xu *et al*., 1994; Quesnel *et al*., 1995) and verified in this study. Similarly, analysis of Ki-*ras* revealed only a single codon 12 mutation in 14 analysed tumours. Both the identity and frequency of this change is consistent with previous studies of *ras* genes in breast cancer (Clark and Der, 1995). The absence of mutation in globin further argues against a global increase in mutation frequency in BRCA1- and BRCA2-associated tumours. The presence of a mutator phenotype, similar to that observed in Hereditary Non-Polyposis Colon Cancer (HNPCC) and gastric carcinomas was investigated by searching for frameshift mutations in TGF-β type IIR and *Bax*. Mutations in polypurine tracts within these genes have been reported in human tumours, such as gastric carcinoma, with MSI (Markowitz *et al*., 1995; Parsons *et al*., 1995; Chung *et al*., 1997). However, the absence of any such changes in the BRCA-associated tumours in the present series implies that a classical mutator phenotype is not present in BRCA-associated tumours. Consistent with this observation, we failed to observe instability in several microsatellites in the same tumours (data not shown). Taken together, these results suggest that the high frequency of p53 mutation is not the result of a mutator phenotype.

Given the apparent absence of a global mutator phenotype in BRCA1- and BRCA2-associated tumours, the implication of our data is, therefore, that selective pressure to abrogate one or more of the wild-type functions of p53, or to acquire some gain of function phenotype, (at least in some tumours) underlies the increased mutation frequency. Neither the expression of $p21^{Waf1}$ in a significant number of tumours mutant for p53 nor the absence of a clear relationship between p53 status and proliferation is consistent with the hypothesis that selection for p53 mutation in such tumours simply reflects a requirement to abrogate a $p21^{Waf1}$-dependent G1 block. The presence of a significant proportion of tumours which retain (and in some cases express) wild-type p53 further refutes this hypothesis. Taken together, therefore, our data imply that proliferation of BRCA tumours, like sporadic breast cancers, is subject to regulation by multiple factors, genetic, epigenetic and hormonal, rather than by activation of a single cell cycle checkpoint. The role of the p53 mutations will require further study.

Materials and methods

Tumours

The tumours analysed in this study arose in confirmed carriers of germ-line mutant alleles of BRCA1 and BRCA2 and were identified from databases compiled by Professor M Stratton. The cases were unselected and were all of those available to us where paraffin blocks of the tumours had been collected into the department and sections had been taken for immunohistochemistry and thick sections taken for DNA isolation. On pathology review by BG and PO, there were 27 grade 3 BRCA1 tumours and one case of pure DCIS. The BRCA2 tumours comprised 16 cases that were grade 3 and six cases that were grade 2. Although this reflects the previous high percentage of BRCA1 and BRCA2 tumours of high grade, it was clearly important to use as controls a group of tumours matched for grade. We therefore took 72 grade-matched tumours from the control group used by the Breast Cancer Linkage Consortium (1997) for immunocytochemical analysis of p53. These comprised seven grade 2, 64 grade 3 and one pure DCIS of comedo type. In addition to the selection of these tumours on the basis of grade, it was possible to assess whether they were biologically representative of sporadic tumours of the designated grade on the basis of published data for expression of $p27^{Kip1}$ (Fredersdorf *et al*., 1997) and the oestrogen receptor. These analyses confirmed that the control sporadic tumours were representative of their allocated grade. All tumour grading was carried out using the recommendations of the National Co-ordinating Group for Breast Cancer Screening Pathology. For sequencing, 20 of the control grade 3 tumours were selected for DNA isolation and subsequent SSCP and sequencing analysis. Proliferation was assessed by counting the number of mitotic figures per 10 high-powered fields, using the methodology advised by the National Co-ordinating Group for Breast Screening Pathology.

Immunocytochemistry

Formalin-fixed, paraffin-embedded 5 μm sections were mounted on glass slides then deparaffinized by passage through graded alcohols. Following microwave antigen retrieval, immunocytochemisty was performed as follows: For p53: antibody DO-1 (purchased from Oncogene Science) was diluted 1 : 1000 then applied to sections. For $P21^{Waf1}$: Tissue culture supernatant containing antibody SX21 (Fredersdorf *et al*., 1996) was applied directly to sections. The detection system used a biotinylated rabbit anti-mouse polyclonal serum (Dako Cat. No. E0354) at 1 : 200 dilution, followed by a streptAB complex/HRP (Dako. Cat. No. K0377) according to the manufacturer's instructions.

p53 and $p21^{Waf1}$ scoring

Each tumour was evaluated by two breast pathologists (PO and BAG) without prior knowledge of the patient group. Sections were scored for immunocytochemical positivity using the 'quick score' method (Detre *et al*., 1995). This combines both intensity of staining (scored 0 – 3), multiplied by the percentage of tumour cells positive (on a scale

1–6) giving a range of 1–18 (within which range tumours were designated positive).

Analysis of gene sequence

DNA was isolated from paraffin-embedded tissue following xylene dewaxing by incubation for 5 days in SDS/proteinase K at 55°C. DNA was recovered by phenol extraction and ethanol precipitation. For analysis of mutation in exons 2–11 of p53, SSCP was performed as described (Visscher *et al*., 1996). Briefly, 50 ng of genomic DNA was subjected to PCR in a total volume of 50 μl containing $\times$1 PCR buffer (10 mM Tris.Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin), 200 μM each dNTP, 0.4 μM primers, 4 μCi α-^{32}P dCTP and 2 units AmpliTaq DNA polymerase (Cetus). Amplified products were denatured, then resolved on 5 or 6% native polyacrylamide gels with and without 10% glycerol. DNA samples with apparently abnormally migrating conformers were reamplified, then ligated into pGEM-T. Multiple plasmid clones were sequenced using T7 DNA polymerase. Proposed mutations were sequenced on both strands and from at least two paraffin sections. In view of the high frequency of p53 mutations in the BRCA-associated tumours suggested by initial SSCP analysis, the coding sequence of p53 was determined in its entirety for each tumour (irrespective of the presence of SSCP shifts). At least 12 clones from each exon for each tumour were sequenced to exclude the possible presence of p53 mutations which were not detected by SSCP. For analysis of $p16^{INK4}$, SSCP was performed using the PCR primers and conditions described by Zhang *et al*. (1994). Following PCR, amplified products were resolved on 6% native polyacrylamide gels. All reactions were run on gels with 5% glycerol (in 0.5$\times$ TBE) and 10% glycerol (in 1$\times$ TBE). Each gel run included positive control DNA samples known to contain point mutations in the region of p16 under analysis. To verify absence of mutations, the sequence of exons 1 and 2 of p16 was determined in 24 tumours (12 BRCA1 and 12 BRCA2). Sequencing templates were generated by asymmetric PCR and sequenced in both directions. For analysis of Ki-*ras*, a 106 bp region of the gene spanning codons 12 and 13 was amplified with the following primers: 5′-GACTGAATA-TAAACTTGTGG-3′ (sense) and 5′-CTATTGTTGGAT-CATATTCG-3′ (anti-sense). Reactions were performed in $\times$1 reaction buffer (60 mM KCl, 15 mM Tris pH 8.8, 2.2 mM $MgCl_2$ 200 μM of each dNTP, 20 pmol each primer and 1 U Taq DNA polymerase. Reactions were cycled 40 times at 94°C (1 min), 55°C (2 min) and 72°C (1 min). Amplified products were resolved on 1.5% agarose TAE gels, excised and purified using a Qiaex II gel extraction system, then ligated into pGEM T-easy and sequenced. The presence of sequence changes in a 268 bp fragment of β-globin was tested by SSCP using PCR conditions previously described (Bauer *et al*., 1991). Analysis for the presence of MSI-associated frameshift mutations in the coding regions of TGF βRII [poly$(A)_{10}$] tract and *Bax* [poly$(G)_8$] tract was performed as described previously by Chung *et al*. (1997).

Acknowledgements
T Crook is a Leopold Muller Fellow. J Waller is funded by US Army grant number DAMD 17-94-4066, L Brooks by the Medical Research Council, P Osin by the Gilbert Fellowship and G Parker by the Wellcome Trust. IG Yulug received an award from the British Council in Turkey. Various parts of the study were supported by the Cancer Research Campaign and Breakthrough Breast Cancer. We thank X Lu for supplying the anti-$p21^{Waf1}$ antibody. Professors Mike Stratton, Doug Easton and Bruce Ponder generously facilitated access to clinical material used in the study.

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