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High incidence of loss of heterozygosity at chromosome 17p13 in breast tumours from *BRCA2* mutation carriers

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Breast tumours from *BRCA1* and *BRCA2* mutation carriers are genetically instable and display specific patterns of chromosomal aberrations, suggestive of distinct genetic pathways in tumour progression. The frequency of abnormalities affecting chromosome 17p and the *TP53* gene was determined in 27 breast tumours from 26 female patients carrying the Icelandic *BRCA2* founder mutation (999del5). Loss of heterozygosity (LOH) was detected in 23 of the 27 tumours (85%). The majority of tumours manifesting LOH had lost a large region on 17p, although a more restricted loss, including the *TP53* locus was seen in a few tumours. Positive p53 immunostaining was observed in 18 of 26 tumours (69%). However, mutations in the *TP53* gene were detected in only three tumours (11%), including a missense (codon 139) and a nonsense mutation (codon 306) in two tumours with moderate p53 expression and a frameshift deletion (codon 182) in a tumour with no detectable p53 expression. Positive p53 immunostaining, mainly weak, was observed in 16 of the 24 tumours (66%) without *TP53* mutation. The high frequency of LOH at chromosome 17p13 suggests that one or more genes from this region are involved in the development of *BRCA2*-induced breast cancer. The frequent finding of weak overexpression of, presumably wild type p53 protein, suggests an alternative mechanism of *TP53* involvement specific to these tumours.

Keywords: breast cancer; chromosome 17p13; *TP53*, *BRCA2* carriers; LOH

Introduction

Breast cancer is one of the most common malignancies in women living in the Western countries. About 5–10% of the cases are believed to be due to germline mutations in breast cancer susceptibility genes (Newman *et al*., 1988; Lynch *et al*., 1990). In the majority of the families with multiple cases of breast cancer the disease has been shown to be due to mutations in either of the two tumour suppressor genes *BRCA1* and *BRCA2* (Easton *et al*., 1993; Wooster *et al*., 1994). Since mutations in the *BRCA1* and *BRCA2* genes are rarely found in sporadic tumours (Futreal *et al*., 1994; Teng *et al*., 1996) the question arises whether the progression pathway of carcinogenesis may be different in breast tumours in genetically predisposed individuals and sporadic cases. A recent study using comparative genomic hybridization (CGH) to evaluate genomic changes in tumours from *BRCA1* and *BRCA2* carriers showed a high incidence of genetic abnormalities in these tumours (Tirkkonen *et al*., 1997). The tumours from *BRCA2* carriers showed a high frequency of aberrations at many of the same chromosome regions shown to be involved during sporadic tumour development such as 13q, 1p, 3p, 6q, 8p, 11q, 17q22-q24, 17p, 18q and 20q13 but lower frequency at chromosomes such as 7q and 16q. Chromosome 17p harbours the p53 gene. *TP53* is considered to be a tumour suppressor gene exerting growth-regulatory functions in response to DNA damage by directly inhibiting DNA replication (Soddu *et al*., 1996; Cox *et al*., 1995) or inducing apoptosis (White, 1996; Levine 1997). Mutations in the *TP53* gene are among the most common genetic alterations observed in human neoplasia (Hollstein *et al*., 1991) and often result in the accumulation of a non-functional protein (Iggo *et al*., 1990; Kastan *et al*., 1991; Levine *et al*., 1991). *TP53* mutations have been reported in sporadic breast cancer and are in general associated with adverse prognosis (Saitoh *et al*., 1994; Bergh *et al*., 1995). In this paper genomic changes occurring at chromosome 17p in tumours from *BRCA2* carriers were studied. We used microsatellite markers to map loss of heterozygosity, and we evaluated the involvement of the *TP53* gene in the tumour progression by mutational analysis of the gene and immunohistochemical staining of the p53 protein. We also analysed the samples for LOH at chromosome 13q12-q14, harbouring the *BRCA2* gene, and at chromosome 7q21-q35 which according to the CGH results is rarely deleted in *BRCA2* tumours (Tirkkonen *et al*., 1997).

Results

Twenty-seven tumours from female breast cancer cases with the 999del5 *BRCA2* mutation were analysed. Summarised in Table 1 are the results of the LOH mapping with ten markers at 17p, the p53 immunohistochemical staining and the mutational analysis of the *TP53* gene. Clinico-pathological information is also included, and data from the LOH analysis at chromosomes 13q12-q14 and 7q21-q35 based on six and nine markers, respectively. The two metastatic tumours analysed (121ii and 123ii) showed the same LOH and mutation pattern as the primary tumour (see Table 1).

LOH analysis

We detected LOH at 17p13 with at least one marker in 23 of the 27 tumours analysed (85%). Twenty-one

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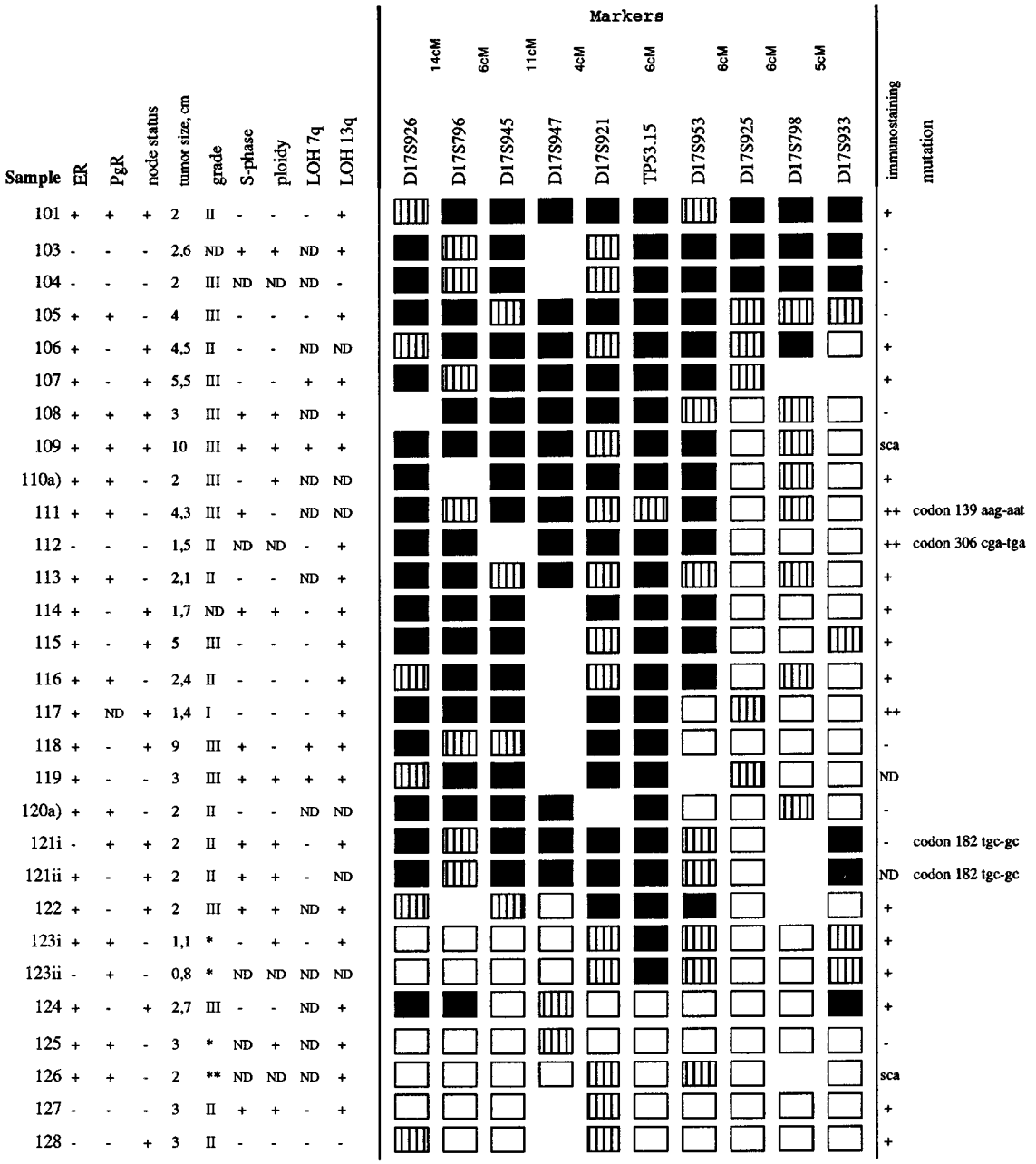
Table 1 Patterns of allelic imbalance at chromosome 17p and the results of the immunohistochemical staining of p53 protein and mutation analysis of the *TP53* gene. Also shown are the clinico-pathological factors determined for the tumours and information on LOH at 13q12–q14 and 7q21–q35.[a]



[a]Key to the information in the Table: Solid boxes show LOH, open boxes show retention of heterozygosity and cross-hatched boxes are homozygous for the given marker. Distances between markers according to Gyapay *et al.* (1994) are shown in centimorgans (cM). a: bilateral case; ii: metastatic tumour from i; ND: not determined; ER: estrogen receptor content: + = ⩾10 fmol/mg protein, − = <10 fmol/mg protein; PgR: progesterone receptor content: + = ⩾25 fmol/mg protein, − = <25 fmol/mg protein; Node status: + = positive, − = negative; grade: *lobular cancer; ***in situ* cancer; S-phase: + = ⩾7%, − = <7%; Ploidy: + = aneuploid, − = diploid; immunostaining: − = negative, sca = scattered, + = weak, + + = moderate; LOH at 7q or 13q: + = loss of heterozygosity at one or more markers tested, − = retention of heterozygosity at all informative markers

tumours showed LOH at the *TP53* locus. In one tumour (no. 124) LOH was detected at a region telomeric to *TP53*, but not at the *TP53* locus itself (Figure 1a). In sample 123i LOH was detected at the *TP53* locus and not at other informative markers at 17p (Figure 1b).

Fifteen samples were analysed with markers at 7q21-q35 and LOH at one or more markers was detected in four tumours (27%). All but two of 23 samples analysed showed LOH at the *BRCA2* locus (91%).

Staining and mutational analysis of p53

Positive staining of the p53 protein was seen in 18 of the 26 tumour samples (69%) available for analysis, including one case (no. 126) diagnosed with *in situ*

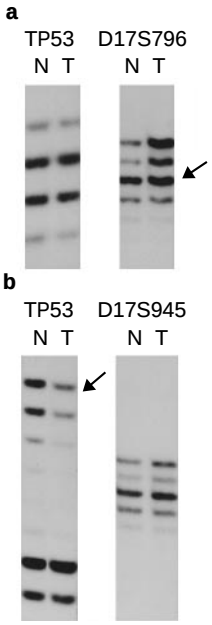


Figure 1 Autoradiograms of PCR products with mocrosatellite markers at 17p in matched normal (N) and tumour (T) breast tissue of 999del5 *BRCA2* carriers. (**a**) Sample 124 amplified with markers TP53.15 and D17S796. Loss of heterozygosity is seen with marker D17S796 and retention of heterozygosity with marker TP53.15. (**b**) Sample 123i amplified with markers TP53.15 and D17S945. Loss of heterozygosity is seen with marker TP53.15 and retention of heterozygosity with marker D17S945

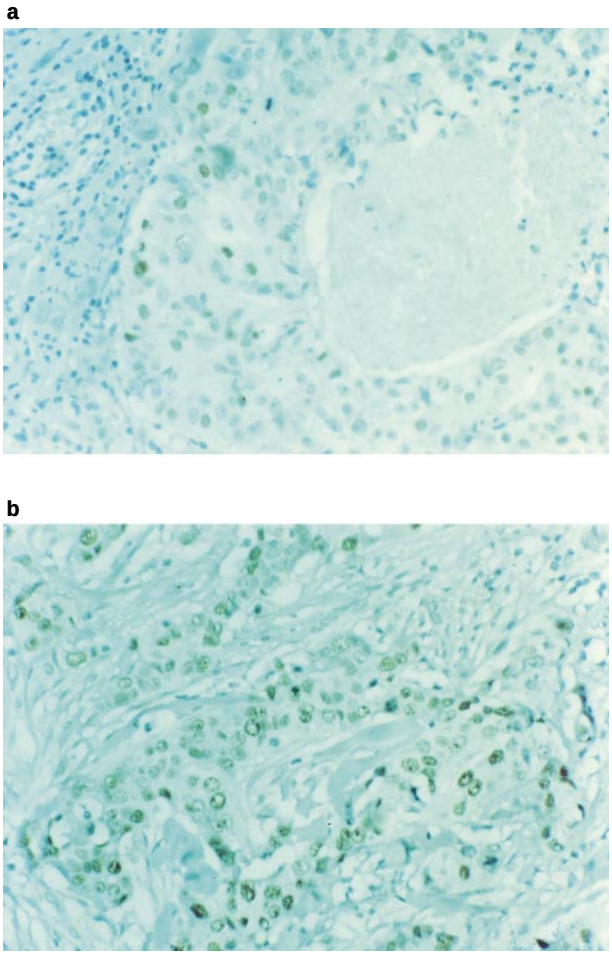


Figure 2 Immunohistochemical staining of p53 in breast tumour tissue of a 999del5 *BRCA2* carrier. Representative staining of tumour sections in which *in situ* (**a**) and invasive tumour growth is seen (**b**)

cancer. In 12 cases with positive staining where both *in situ* and invasive tumour growth was seen, staining was detected in cells of both components (Figure 2). Four of the five tumours that did not show LOH at the *TP53* locus stained positively for the p53 protein (Table 1). We used SSCP to look for *TP53* mutations and were able to identify band shifts in only four of the tumours. A nucleotide deletion at codon 182 (TGC to GC), which leads to a frameshift mutation and a premature translation termination (246ter), was identified in one of the negatively staining tumours (no. 121i), and the same mutation was detected in the metastatic growth from this tumour (121ii). A missense mutation at codon 139 (Lys to Asn) and a nonsense mutation at codon 306 (Arg to Stop) were observed in two tumours (nos. 111 and 112) with positive immunostaining. The remaining SSCP-band shift, observed in a tumour without detectable staining, was found to be due to a known polymorphism at codon 213 (CGA to CGG). The same mutations have previously been detected in studies of tumours from sporadic breast cancer cases and are listed in the Human Gene Mutation Database (Cardiff, UWCM; http://www.cf.ac.uk/UWCM/mg/hgmdO.html).

Discussion

We have recently reported a high frequency of genomic alterations, as seen by CGH analysis, in breast tumours from both *BRCA1* and *BRCA2* mutation carriers (Tirkkonen *et al*., 1997). The CGH results clearly suggested that the accumulation of somatic genetic changes is not random and some chromosome regions are affected more frequently than others. The LOH results presented in this paper support the CGH data, where the involvement of 7q is seen in 27% of the tumours analysed *versus* 85% and 91% at 17p and 13q respectively. The LOH mapping results indicated the *TP53* gene as a possible target of the allelic deletions. This is supported by the high number of tumours positively staining for the p53 protein, taking into consideration the known relationship between gene mutation and protein accumulation (Iggo *et al*., 1990; Kastan *et al*., 1991; Levine *et al*., 1991). However, *TP53* mutations were detected in only 11% of the tumours, suggesting the involvement of alternative mechanisms that give rise to (mainly weak), overexpression of wildtype p53. For instance, the p53 protein is known to be modified and stabilised by phosphorylation in response to DNA damage and the action of DNA damage sensor proteins (Kastan *et al*., 1992; Levine, 1997). One cannot exclude the possibility that the deletions are targeted to another gene on 17p as the majority of tumours manifesting LOH had lost a large region including the *TP53* gene as well as markers centromeric and, most often, telomeric to *TP53*. In two cases (see 122, 123i and 123ii in Table 1) the deletion pattern was in accordance with the specific loss of *TP53* or a closely located gene, while in one tumour (no. 124) LOH was detected both telomeric and centromeric to *TP53* but not at the *TP53* locus itself. The existence of a tumour suppressor gene telomeric to *TP53* has been suggested previously

(Merlo *et al*., 1994; Cornelis *et al*., 1994) and cannot be excluded as a target gene in these tumours.

It should be emphasised that in this study any positive staining was judged to be significant and the staining in all but one of these tumours was weak. According to previously published studies disparity is frequently encountered when correlating immunohistochemical findings with DNA mutations of the *TP53* gene (Jacquemier *et al*., 1994; Yandell and Thor, 1993), especially regarding samples with weak staining (Gretarsdottir *et al*., 1996; Hall and Lane, 1994). Possible explanations for the positive staining of p53 in the 16 cases where no *TP53* mutations were detected are that the protein expression was caused by mutations in another gene that prolongs the half-life of the p53 protein, by mutations that were not detected by SSCP due to co-migration of wild-type and mutant alleles, by mutations in exons other than those screened in this study by SSCP or by mutations that were not detected due to a dilution factor of normal cells. The two last explanations are not very likely since relatively few mutations of the *TP53* gene have been found to occur outside the exons tested in this study (Saitoh *et al*., 1994; Sjögren *et al*., 1996) and contamination of the tumours by normal cells is not large as LOH was detected in 91% of the samples.

The high frequency of positive staining of p53 in the tumours of *BRCA2* carriers determined here is comparable to what has been reported earlier for *BRCA1* tumours (Johannsson *et al*., 1997; Sobol *et al*., 1997) and higher than reported in similar studies on sporadic breast cancer (Gretarsdottir *et al*., 1996; Cornelis *et al*., 1994; Runnebaum *et al*., 1991). In agreement with results of histopathological studies showing *BRCA2* tumours to be of significantly higher grade than sporadic tumours (Marcus *et al*., 1996; Lakhani *et al*., 1997), most of the tumours in this study are grade II and III. A significant association between p53 abnormalities and higher histological grade has been shown in sporadic tumours (Gretarsdottir *et al*., 1996; Umekita *et al*., 1994; Bhargava *et al*., 1994). In this study no association was seen between p53 aberrations and histological grade or the other recognised prognostic factors listed in Table 1. In 12 cases where both *in situ* and invasive tumour growth was seen, staining was detected in cells of both components. Staining was also seen in the tumour cells of one case (no. 126) diagnosed with *in situ* cancer.

The interaction recently suggested between the BRCA proteins and Rad51 protein in meiotic and mitotic recombination and DNA repair (Scully *et al*., 1997; Sharan *et al*., 1997), is in accordance with a role of *BRCA2* and *BRCA1* in preservation of genome integrity. Thus, cells without *BRCA1* or *BRCA2* activity might be defective in the repair of lesions such as DNA double-strand breaks (Scully *et al*., 1997; Sharan *et al*., 1997). In order to tolerate and survive with such DNA damage, cells must also acquire a deregulation in growth arrest and apoptosis. The results of the present study indicate that at an early stage in the development of *BRCA2* predisposed tumours the p53 pathway is activated, and at some point either bypassed or inactivated by an unknown mechanism.

Materials and methods

Patients and tumour material

Screening of 459 randomly chosen breast cancer cases diagnosed in the years 1989–1994 revealed 39 female patients with the 999del5 *BRCA2* mutation (Johannesdottir *et al*., 1996). Paired blood and tumour DNA were available from 27 primary breast tumours from 26 females (one bilateral case). These tumours were removed from the patients prior to any treatment. Two metastatic tumours were also available from 999del5 *BRCA2* mutation carriers. Conventional methods were used for DNA extraction from blood and tumour material. The samples were subjected to PCR analysis using DynaZyme™ polymerase (Finnzymes Oy, Espoo, Finland) in the buffer solution provided by the manufacturer. The TP53.15 marker, described by Jones and Nakamura (1992) and mapping within the *TP53* gene, was obtained from Pharmacia Biotech (Denmark). All the other markers were obtained from Research Genetics (Huntsville, AL); D17S926, D17S796, D17S945, D17S947, D17S921, D17S953, D17S925, D17S798, D17S933, D13S283, D13S1246, D13S260, D13S171, D13S267, D13S263, D7S492, D7S518, D7S515, D7S501, D7S523, D7S471, D7S522, D7S480, D7S500. PCR products were subjected to 6.5% acrylamide denaturing gels and detected by the method of Vignal *et al*. (1993). Autoradiograms were inspected visually by three independent viewers, comparing the intensity of alleles from normal and tumour DNA. Any absence or significant decrease of one allele relative to the other was considered loss of heterozygosity (LOH). Relevant information regarding pathological factors of the tumours was recorded by pathologists at our department. Tumour grade was assessed by a modified Bloom-Richardson system (Elston, 1987). Estrogen- and progesterone-receptors (ER and PgR) were analysed using a ligand binding assay. S-phase fraction and DNA-ploidy were analysed using a FACScan flow cytometer (Becton-Dickinson) utilising paraffin embedded tissue. The Fisher's exact test (Abacus Concepts, Survival Tools for StatView, Berkeley, CA) was used to assess the relationship between p53 staining and/or *TP53* mutations and clinico-pathological factors.

Mutation and sequencing analysis

The PCR amplification and SSCP conditions for exons 5–9 are described by El-Naggar *et al*. (1995), and for exon 4 by Deng *et al*. (1994). PCR products were diluted 1:4 in 95% formamide, 10 mM NaOH, 0.025% xylene cyanol and 0.025% bromophenol blue and denatured at 95°C for 8–10 min. The mixture was applied to 0.5 × MDE (Mutation Detection Enhancement, AT Biochem, no 1-500-01), 0.6 × TBE gels with or without 5 or 10% glycerol. The gels without glycerol and the 5% glycerol gels were run at 30 W for 6–10 h at room temperature; the 10% glycerol gels were run at 4°C for 4 h. The PCR products were detected with the same non-radioactive method as used for the microsatellite markers mentioned above. Samples positive in the SSCP analysis were subjected to a new PCR reaction with a biotinylated reverse primer and solid phase sequencing was carried out. The PCR products were immobilized on streptavadin beads (M-280; Dynal) and denatured with alkaline. The single-stranded biotinylated DNA template was sequenced using Sequenase 2.0 and [α-^{33}P]dATP (Amersham, Buckinghamshire, England).

Immunohistochemistry

Immunohistochemical staining of the p53 protein was performed with a commercially available antibody (clone

D07, Novocastra Laboratories, Newcastle upon Tyne, UK) utilizing formalin fixed 4μ paraffin sections. This antibody recognizes both mutant and wild type p53. Sections were pretreated in an 850 watt microwave oven in citrate buffer at pH 6.0 for 2×5 min at full power. The sections were incubated with the primary antibody for 30 min at a 1 : 50 dilution and subsequently incubated with a second goat anti-mouse biotinylated antibody (Dako, Glostrup, Denmark) for 30 min before being reacted with a streptavadin-biotin-peroxidase complex (Dako) for 30 min (DAKO, Glostrup, Denmark). Diaminobenzidine tetrahydrochloride was used as chromogen to detect the peroxidase activity. Results were scored according to the method of Fisher *et al*. (1994). In brief, strong staining is classified as dark nuclear staining in $>50\%$ of cells; moderate staining is dark staining in $<50\%$ of cells or moderate staining in $>50\%$ of cells; and weak staining is moderate staining in $<50\%$ of cells or pale staining in any proportion of cells. Scattered staining is dark nuclear staining in widely scattered cells.

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