Protein Accumulation and Gene Mutation of p53 in Bilateral Breast Cancer

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Acta Oncologica Vol. 40, No. 1, pp. 56–62, 2001

The aim of this study was to investigate the frequency of p53 alterations in bilateral breast cancer and to evaluate a possible clonal relationship between the paired tumours regarding p53 alteration and other pathobiological variables. Tumours from 34 patients were investigated with immunohistochemistry, single strand conformation polymorphism analysis and DNA-sequence analysis applied to exons 5–8. Fifteen percent of the 68 tumours showed positive immunoreaction and/or presence of mutation. The occurrence of p53 accumulation was 9% and the prevalence of gene mutation 10%. No significant concordance was found between the tumours in the same patient for p53 alterations, progesterone receptor status or DNA ploidy. S-phase fraction showed a weak correlation, not statistically significant. Oestrogen receptor status was the only variable that exhibited a significant concordance. No convincing evidence was found for other associations between the paired tumours or for a high prevalence of p53 alterations in bilateral breast cancer.

Received 5 June 2000
Accepted 9 November 2000

Women already diagnosed with breast cancer have an increased risk of new primary tumours of the contralateral breast compared with women without previous breast neoplasms. Adami et al. reported the relative risk to be 10 times higher for women younger than 50 years and 2 times higher for women older than 50 (1). It has been questioned whether there is a clonal relationship between the tumours in bilateral breast cancer and whether p53 alterations are more frequent in bilateral breast cancer, which could indicate an aggressive disease with poor outcome. Only a small number of breast cancer cases are associated with the rare disorder Li-Fraumeni syndrome, which is linked to a germline mutation of the tumour suppressor gene p53. Li-Fraumeni syndrome has been reported in association with bilateral breast cancer and poor prognosis (2, 3). However, most cases of bilateral breast cancer do not involve this rare syndrome (3) and lack of concordance concerning other tumour characteristics of the paired tumours has also been reported (4). With regard to prognosis, an increased and a similar risk for distant metastasis to that in unilateral disease has been found (5, 6). The prognosis for these women is also influenced by age and the interval between diagnosis of the tumours, higher age and longer interval being the more favourable (7, 8).

Mutation of the p53 gene and/or inactivation of its protein are common events in several human cancers and altered p53 was shown to be a strong prognostic factor in unilateral breast cancer, with a significantly increased risk of distant relapses (9, 10). The p53 gene, located on chromosome 17, encodes a protein that binds specific stretches of DNA and transactivates genes regulating the cell cycle as well as apoptosis, and in this way suppresses tumour progression. Mutations of the p53 gene in breast cancer have been found in most of its 11 exons, but are concentrated within exons 5–8 (11, 12). Mutations altering the protein structure are believed to cause accumulation of the protein in the nucleus thus enabling detection by immunohistochemistry (IHC) (13). Much work has been done to describe the spectrum of p53 mutations in breast cancer. So far, these studies have been focused on unilateral breast cancer. Only a few studies have reported on bilateral breast cancer and p53 status. One study has confirmed the mutations detected by screening methods with DNA sequence analysis while others have not (14–16).

 ISSN 0284-186X

The purpose of our study was to investigate the frequency of p53 alterations at both the protein and the gene level in the paired tumours of patients registered for early bilateral breast cancer. The aim was also to evaluate whether p53 status and other tumour characteristics could indicate a clonal relationship in the paired tumours of bilateral breast cancer.

MATERIAL AND METHODS

Patients and tumours

The study included 34 patients registered in the South-East Sweden Healthcare Region with bilateral breast cancer during the years 1982–1995. Twenty patients had synchronous tumours diagnosed within 6 months and 14 patients had asynchronous tumours, with the second diagnosis after more than 6 months. The tumours included in the study did not exceed 50 min in size. There was no clinical evidence of lymph node involvement of the contralateral axilla or tumour extension to chest wall. In one patient the first tumour and in another patient both tumours were diagnosed as cancer in situ. None of the patients had received any preoperative treatment. Patients were treated with breast-conserving surgery plus 54 Gy breast irradiation or modified radical mastectomy. Distant recurrence or death in breast cancer was registered in 9 cases. The median follow-up period was 40 months.

For comparison, an additional series of 7 patients with advanced breast cancer with involvement of both breasts and distant metastasis before presentation of the contralateral tumour was also studied. Tumour data are presented in Table 1.

Immunohistochemistry (IHC)

Cell imprints from frozen tumour tissue were investigated with the monoclonal antibody PAb 1801 (Oncogene Science, Manhasset, NY, USA). As negative control, IgG antibodies (Sigma, St Louis, MI, USA) were used. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 5 min. Normal rabbit serum (10%) was used for 10 min in order to block non-specific immunostaining. The slides were incubated with the primary antibody (1 μg/ml) at room temperature for 2 h. The sections were subsequently incubated with biotinylated rabbit-anti-mouse immunoglobulin (1 : 200) and streptavidin horseradish peroxidase (1 : 300), 30 min for each step, staining with 3.3-diaminobenzidien tetrahydrochloride (DAB) in phosphate-buffered saline (pH 7.5) with 0.036% (v/v) hydrogen peroxide for 8 min. After each step, with the exception of the incubation with normal rabbit serum, the sections were rinsed in phosphate-buffered saline containing 0.1% bovine serum albumin and after staining with DAB rinsed in water. Thereafter the sections were counterstained with haematoxylin, dehydrated in a series of ethanols and mounted.

Extraction of DNA from fresh tissue

Tumour tissue was first disintegrated with scissors and then digested with proteinase-K (2 mg/ml, Boeringer Mannheim) in a digestion buffer containing 8 mM TRIS-HCI (pH 8.0), 0.8 mM EDTA, 80 mM NaCl and 2% SDS and incubated at 55°C. DNA was separated from proteins by repeated extractions with phenol, phenol/chloroform and chloroform. Nucleic acids were precipitated in 65% (v/v) ethanol with 0.1 M sodium acetate incubated at −20°C for 1h. DNA was pelleted by centrifugation at 12000 × g for 1 h, after which salt residues and RNA were removed by washing with 70% (v/v) ethanol. Nucleic acids were re-pelleted as above for 5 min and then vacuum-dried and suspended in sterile double-distilled water.

Polymerase chain reaction (PCR)

The DNA was examined for mutations in exon 5–8 of the p53 gene. Amplification of DNA was carried out using 30–35 cycles of PCR with denaturation and extension steps performed at 94°C for 45 s and 72°C for 40 s, respectively. Annealing temperatures were optimized for

Table 1

Patient and tumour data of early and advanced bilateral breast cancer cases

	Early no. of patients/tumours	Advanced no. of patients/tumours
Age at the first diagnosis		
<50 years	5	3
≥ 50 years	29	4
Tumour size		
≤ 20 mm	42	5
>20 mm	23	8
In situ	3	1
Number of positive nodes [1]		
N0	36	3
N1	21	7
Oestrogen receptor status		
Negative	17	6
Positive	51	8
Progesterone receptor status		
Negative	39	8
Positive	29	6
DNA ploidy [2]		
Diploid	28	0
Aneuploid	32	12
S-phase fraction [3]		
$<10\%$	35	3
$\geq 10\%$	15	6

[1] Data were missing in 11 of the early cases and in 4 of the advanced cases.
[2] Data were missing in 8 of the early cases and in 2 of the advanced cases.
[3] Data were missing in 18 of the early cases and in 5 of the advanced cases.

Table 2

Primers and annealing temperatures used for PCR amplification of DNA

Location	Forward (5′→3′)	Reverse (5′→3′)	Annealing temperature
Exon 5	GCCCTGACTTTCAACTCT	CAGCCCTGTCGTCTCTCC	57°C
Exon 6	GTCCCCAGGCCTCTGATTC	AACCCCTCCTCCCAGAGAC	58°C
Exon 7	TCTTGGGCCTGTGTTATCTC	GGTGGATGGGTAGTAGTATG	56°C
Exon 8	CTGCCTCTTGCTTCTCTTTT	CTCCTCCACCGCTTCTTGTC	55°C

each set of primers (Table 2). PCR was performed in a total reaction volume of 22 μl containing 25 ng DNA, 2 mM $MgCl_2$, 1 × Taq Polymerase Buffer solution (20 mM $(NH_4)_2SO_4$, 75 mM TRIS-HCI (pH 8.5), 0.1% Tween 20 (v/v), 1 μM of each primer, 0.2 mM of each dNTP and 0.5 u Taq Polymerase (SDS/Promega). An identical reaction mixture, but excluding DNA, was used as a negative control. PCR products and control were confirmed using agarose (2%) gel separation and ethidium bromide staining.

Single strand conformational polymorphism (SSCP) analysis

Amplified products were subjected to radioactive labelling using PCR by incorporation of α-dATP^{32} (Amersham). The labelling was done with 15 cycles of PCR using the same conditions as those for the primary reaction. Five μl labelled PCR product was diluted in 15 μl loading buffer (0.05% SDS, 5 mM EDTA) denatured at 94°C for 5 min and thereafter put on ice before being loaded on a non-denaturing 0.5 × MDE gel (FMC BioProducts). Electrophoresis was carried out in a buffer consisting of 50 mM TRIS-HCL (pH 8.0), 50 mM boric acid, 1 mM EDTA and run overnight at 3W at room temperature. The gel was dried and exposed on x-ray film (Cronex 4, DU PONT) using an intensifying screen, for 6–48 h. The film was viewed independently by at least two investigators. Bands showing mobility shifts were selected for DNA sequencing.

DNA sequencing

DNA excised from SSCP-gel was amplified by 30 cycles of PCR and purified from primers using Wizard™ PCR Preps DNA Purification System (Promega/SDS). The sequencing was performed with a thermo sequenase radiolabelled terminator cycle sequencing kit (Amersham), where γ-ddNTP^{33} (G, A, T and C) were incorporated into the amplified DNA fragments. For each exon the forward primer was used for sequencing. Exon 5 was cut in two parts, using forward TGCCCTGACTTTCAACTC and forward GCAGCTGTGGGTTGATTC, creating one upstream and one downstream product. The samples were run on a denaturing polyacrylamide (6%) gel containing 8 M urea at 45 W in a buffer solution containing 50 mM TRIS-HCI (pH 8.0), 50 mM boric acid, 1 mM EDTA. The running time was optimized for each exon. Finally, the gel was dried and exposed on x-ray film (Kodak, BioMax MR).

RESULTS

Immunohistochemistry

Table 3 summarizes the IHC staining pattern, mutations found in exon 5–8 and the presence of distant recurrence.

Nuclear staining was scored as positive when more than 5% of the tumour cells were stained. An example of positive staining is shown in Fig. 1. Cytoplasmic accumulation was also registered. Six tumours from 5 patients showed nuclear staining, with five of the tumours also exhibiting cytoplasmic staining. None of the tumours expressed cytoplasmic accumulation alone. One patient had nuclear staining in both tumours, whereas 4 patients showed nuclear accumulation in only one tumour.

Mutational analysis

The DNA showing mobility shifts in SSCP analyses were sequenced. Seven tumours from 6 patients exhibited gene mutation. Altogether, 10 tumours from 8 patients were positive for either gene mutation and/or IHC. One patient exhibited the same missense mutation at codon 193 in both tumours (lab). Five patients showed mutations in only one tumour, deletion at codon 294 (5a), missense mutation at codon 239 (4b), 264 (6b), 163 (7a) and insertion at codon 242 (2a) respectively. Tumours leading to frameshift mutations were negative for IHC (2a, 5a) (Table 3). Two patients showed polymorphism at codon 213 in both tumours. This polymorphism was not classified as p53 alteration. Examples of autoradiographs showing mobility shifts from SSCP along with the confirmative sequence analysis are shown in Fig. 2a and b.

p53 status, hormone receptor status, S-phase fraction and DNA ploidy

Comparisons of p53 status and different pathobiological variables between the first versus the second tumour are shown in Table 4. A significant correlation was found for oestrogen receptor (ER) status, whereas progesterone receptor (PgR) status, DNA ploidy and p53 alterations showed no significant correlation. S-phase fraction showed a trend toward concordance.

Advanced breast tumours

Two patients exhibited p53 alterations. The paired tumours were positive for both p53 accumulation and gene mutation. The mutations affected exon 8, codon 281 (Asp

Table 3

Mutations in exons 5-8 in the p53 gene and immunohistochemical staining of p53 protein in patients with bilateral breast cancer

Case [1]	Synchronic (S) Asynchronic (AS)	Mutation	Gene analysis of exons 5–8			Type	IHC [2]	Distant recurrence
			Exon	Codon	Effect on aa sequence			
1a, 1b	AS	CAT → CTT	6	193	His → Leu	Missense	Negative	Yes
2a	S	Insertion 8-bp	7	242	Stop at codon 249	Frameshift	Negative	
2b							Positive	Yes
3a, 3b	AS						Positive	Yes
4b	S	AAC → GAC	7	239	Asn → Asp	Missense	Positive	No
5a	S	GAG → GA and CCT → ACC	8	294 and 295	Stop at codon 305	Frameshift	Negative	No
6b	AS	CTA → ATA	8	264	Leu → Ile	Missense	Positive	No
7a	AS	TAC → TGC	5	163	Tyr → Cys	Missense	Positive	No

[1] The first and the second tumours diagnosed are referred to as 'a' and 'b', respectively.
[2] Immunohistochemistry.

to Glu) and exon 5, codon 132 (Lys to Arg), respectively. For all seven patients investigated, hormone receptor status, DNA ploidy and S-phase fraction showed concordant data for the paired tumours in cases with available data (Table 1).

DISCUSSION

p53 alterations are found in early as well as in advanced breast cancer (17–19). The occurrence of p53 alterations reported in the literature varies depending on the methods applied and the investigated material. Studies of node-negative breast cancer have reported frequencies ranging between 20 and 52% with immunohistochemistry (9, 20, 21). Our results do not indicate that altered protein expression of p53 is more frequent in bilateral breast cancer compared with unilateral disease. Few studies have investigated p53 alterations in relation to bilateral disease. Ackerman et al. (16) analysed p53 expression with IHC and reported a prevalence of 22% being p53 positive. Kinoshita et al. (15) used SSCP in order to detect p53 mutations in bilateral breast cancer cases and found mobility shifts in 50% of these cases. Compared with these studies, the prevalence of p53 alteration of 15% in our study, 9% being positive with IHC and 10% having gene alterations, is a small number. One explanation for this result could be that the material was confined to an early stage. Approximately two-thirds of the tumours measured 20 mm or less. Another reason could be the cut-off level for positive staining of 5%. A higher percentage might have been achieved if a lower cut-off level had been applied. However, the 5% cut-off level was also used by Ackerman et al. (16). The high percentage of mutation reported by Kinoshita et al. (15) can perhaps be explained by the use of SSCP only. If SSCP alone is used as a means of detecting mutations, there is a risk of both false-positive and negative samples (22). We performed sequence analysis to verify the presence of mutation. We found polymorphisms in four tumours that with SSCP only would have been falsely regarded as mutations. Kinoshita et al. (15) defined patients having bilateral breast cancer on the basis of clinical parameters and the presence of an additional intraductal component in both tumours. In their report they also studied unilateral tumours, but to our understanding these tumours were not selected with regard to a concomitant in situ component. Done et al. (23) reported the simultaneous occurrence of p53 alterations of in situ lesions and the invasive component. To our knowledge, no study has been performed to investigate whether invasive tumours with an in situ component are more likely to be p53 positive than invasive tumours lacking an intraductal component. If such an association exists, it might in part explain the high

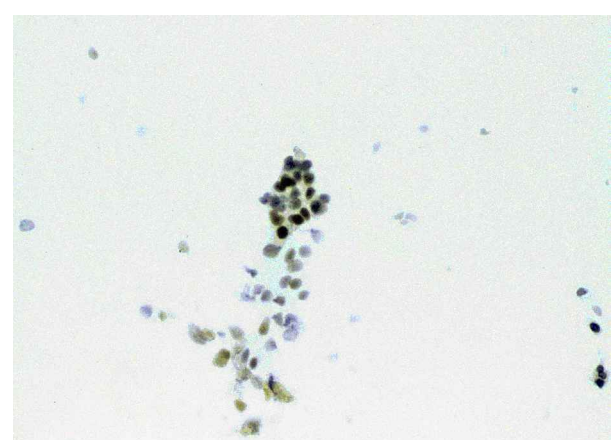

Fig. 1. p53 immunoreactivity in breast cancer with PAb1801. The imprints was counterstained with haematoxylin.

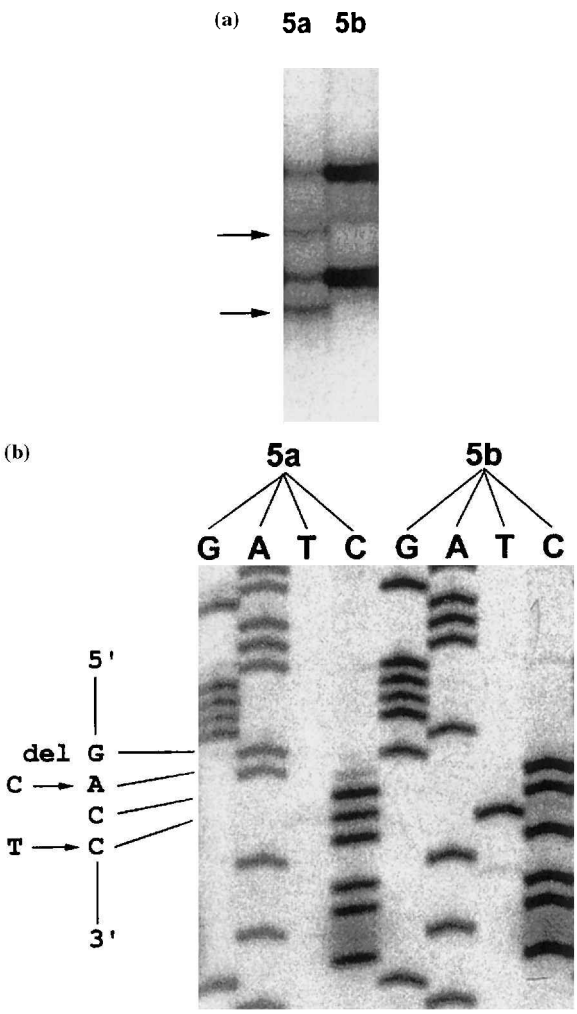

Fig. 2. (a) Single strand conformational polymorphism (SSCP) analysis of exon 8 from a patient with bilateral breast cancer. Sample 5a is from the first tumour where arrows indicate shifted bands. 5b is the second tumour of the same patient without mobility shift. (b) The confirmative DNA-sequence analysis from the patient exhibiting SSCP mobility shifts in Fig. 1. The analysis showed both deletion (del) in codon 294, GAG→GA, and substitution in codon 295, CCT→ACC, giving rise to a frameshift mutation and stop at codon 305.

number of p53-positive tumours in the bilateral group compared with the unilateral one described by Kinoshita et al. (15). Allred et al. (24) reported a twice as high prevalence of HER-2/neu over-expression in breast carcinomas having a coexisting in situ component compared with tumours lacking this lesion.

By using sequence analysis, we also had the possibility to investigate a clonal relationship in the paired tumours. Only in one patient was it suspected that the tumours had the same clonal origin, as they exhibited the same missense mutation in both tumours. Shibata et al. have also addressed the question of whether bilateral breast cancer is a monoclonal or a polyclonal disease (14). The prevalence of p53 mutations in this study was 13%. Their results also indicated that the tumours in bilateral disease have different clonal origins.

To minimize the risk of the second tumour simply having metastasized from the first one, we only included tumours less or equal to 50 mm without clinical evidence of other engagement than the axillary lymph nodes on the ipsilateral side. Ackerman et al. (16) and Shibata et al. (14) did not account for stage and Kinoshita et al. (15) included stage III tumours as well. We believe that the additional cases of advanced breast cancer in our study provide further information. These patients with involvement of both breasts and distant metastasis before presentation of the contralateral tumour exhibited quite similar characteristics in the paired samples, which suggests that the second tumour had metastasized from the first tumour.

To investigate whether there is a discrepancy between protein alterations and gene mutations of p53, we performed both IHC and sequence analysis. In our material one patient showed negative immunostaining despite having the same missense mutation in both tumours. One explanation to this could be that IHC is false negative because of not being representative material or that the p53 protein has degraded. Another explanation could be associated with the mutation being positioned at codon 193 in the zinc-binding domain and the amino acid shift from histidine to leucine. Such a mutation could disrupt the three-dimensional structure of the protein, thus making detection by IHC impossible.

Two patients exhibited the same polymorphism in both tumour pairs at codon 213, a polymorphism also reported by Carbone et al. (25). Being a silent mutation, it was surprising to find that one of the patients showed no

Table 4

Comparison of p53-status and different pathobiological variables between the paired bilateral tumours

1st tumour	2nd tumour		p-value [1]
ER	Negative	Positive	
Negative	5	4	0.016
Positive	3	22	
PgR	Negative	Positive	
Negative	12	8	0.23
Positive	7	7	
S-phase	Low	High	
Low	10	2	0.053
High	2	4	
DNA ploidy	Diploid	Aneuploid	
Diploid	6	4	0.31
Aneuploid	9	7	
p53 IHC	Negative	Positive	
Negative	29	3	0.20
Positive	1	1	
p53 mutation	Negative	Missense	
Negative	28	2	
Missense	3	1	0.29

[1] Fisher's exact test.

immunostaining while the other patient was positive for protein accumulation in one tumour. A point mutation in a non-investigated part of the sequence could explain this finding or the protein could have been bound to other cellular proteins, which could also be true in the case of normal SSCP and positive immunostaining. Tumours having a frameshift mutation were negative with IHC, presumably because these types of mutation lead to truncated transcripts not readily detectable by IHC. Casey et al. (12) and Sjögren et al. (26) have also reported that these mutations have been negative for protein accumulation with IHC.

We chose to examine only exons 5–8. These comprise the sequence-specific, DNA-binding domain and the two zinc-binding regions. Both regions are vital for protein function (27, 28). Some studies have reported that approximately 80% of the mutations are situated within these exons (11, 12, 26). We are aware that additional information could have been gained if we had performed analyses of all exons, since the mutations outside exon 5–8 very often are mutations that do not give rise to positive staining with IHC (26). As we did not examine any normal cells, we could not assess whether the observed mutations were inherited or not. However, germline mutations of the p53 gene are quite rare.

Oestrogen receptor (ER) status showed a significant concordance between the two primary tumours. Dawson et al. (4) have reported the same correlation. This might possibly reflect the existence of an oestrogen-mediated pathway to tumour progression, which could be related to polymorphisms concerning oestrogen metabolism that cause different levels of oestrogen. Interestingly, we did not find any concordance for progesterone status. Oestrogen and its receptor regulate the synthesis of the PgR. PgR is rarely expressed in tumours lacking ER and not all ER-positive tumours express PgR. The lack of concordance for PgR status, p53, S-phase fraction and DNA ploidy in the present study might reflect the absence of an association between the tumours in bilateral breast cancer at the clinical stage.

In summary, we did not find any high prevalence of p53 alterations in bilateral breast cancer. The p53 status, S-phase fraction, DNA index or PR status did not show any common evidence of a clonal relationship between the paired tumours. Only oestrogen receptor status exhibited a significant concordance.

ACKNOWLEDGEMENTS

This work was supported by grants from the Linköping Läkaresällskaps Research Fund, the Lion's Cancer Research Fund, Landstinget i Östergötland, Östgötaregionens Cancerfond and the Swedish Cancer Society. We thank Dr T Jahnberg and Dr G Westman for kindly providing some of the patient and tumour data.

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