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Original Paper

p53 and Survival in Early Onset Breast Cancer: Analysis of Gene Mutations, Loss of Heterozygosity and Protein Accumulation

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The p53 protein has proven to be central in tumorigenesis by its cell cycle regulatory properties and both gene mutations and protein accumulation have been associated with poor prognosis in breast cancer. The present study was undertaken to investigate the prognostic significance of gene mutations, p53 protein accumulation and of loss of heterozygosity (LOH) at the *TP53* locus in young (age <37 years) breast cancer patients. In total, gene mutations were found in 21 of the 123 patients (17%), LOH in 20 of the 47 informative cases (43%) and protein accumulation in 47 of the 102 available cases (46%). Log rank analysis revealed no significant association between survival and *TP53* mutations (in general), p53 protein accumulation or LOH. However, missense mutations localised to the zinc binding domain were significantly ($P = 0.0007$) associated with poorer prognosis. As indicated in this as well as other studies, p53 protein accumulation is frequently found in young breast cancer patients, but this protein overexpression appears to be of minor significance for survival. Nevertheless, the present report also suggests that specific mutations contribute substantially to tumour aggressiveness.

Key words: breast cancer, young age, early onset, prognosis, p53 protein, TP53 gene, zinc binding domain
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INTRODUCTION

COMPARED WITH older women, young women with breast cancer are more likely to have adverse pathological features, such as a high proliferation rate, a lack of oestrogen receptors, positive lymph nodes and high nuclear grade [1, 2]. In addition, several studies have demonstrated a poorer prognosis than older women and that young age at the time of diagnosis independently predicts an unfavourable outcome [3–7], although conflicting results have been reported [8]. These findings have raised the hypothesis that breast cancer affecting younger and older women may have a biologically different origin [1, 3, 7], possibly caused by diverse pathways of genetic transformation.

Among the growing number of oncogenes and tumour suppressor genes reported to be involved in human cancer, the *TP53* gene product has proven to be central in tumorigenesis by its cell cycle regulatory properties. The *TP53* tumour suppressor gene, which is located on chromosome 17p13.1 and encodes for a 53 kD protein, contains a motif with sequence-specific DNA binding properties and a zinc binding domain necessary for providing the DNA contact [9, 10]. The protein has the ability to trigger cell cycle arrest or apoptosis in response to DNA damage and cancer cells with mutation in the *TP53* gene appear to have a selective growth advantage, presumably as a result of down regulation or loss of the suppressive function [11–13]. Inactivation of the protein may occur as a result of inhibitory interaction with viral-transforming proteins, oncogene or heat shock proteins or through alteration of the gene, such as allelic losses or mutations in the gene sequence.

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Mutations in the *TP53* gene and immunohistochemically detectable p53 protein are commonly observed in human breast tumours, although the reported frequency of these p53 abnormalities varies considerably. Both gene mutations and protein accumulation have been associated with poor prognosis, reviewed in [14, 15]. Several studies have also shown that p53 alterations are an independent prognostic marker in breast cancer [12, 16, 17]. The majority of the gene alterations are missense mutations resulting in amino acid substitutions [18, 19]. These missense mutations are predominantly found in the DNA-binding domain, and it has been suggested that these mutations are associated with a more adverse prognosis than mutations in other domains [19–21].

Few studies have focused on p53 status in sporadic breast cancer with early onset. Immunohistological studies have revealed a higher incidence of p53 protein accumulations in younger patients (aged <35 years) compared with older patients [2, 4]. These results are in agreement with findings of Caleffi and colleagues [22], showing a significantly higher incidence of *TP53* gene mutations in the youngest age category (aged <45 years). The impact of the *TP53* gene in breast cancer tumorigenesis is further illustrated by the frequent allelic imbalance found at the chromosomal locus for the *TP53* gene [23]. However, due to the limited number of studies and the few patients under investigation, the knowledge about the influence of p53 alterations on tumour behaviour is still relatively scarce in this youngest age category. Thus, the present study was undertaken with the intent to investigate the association between p53 alterations and the prognosis in a breast cancer population diagnosed at a young age. Alterations were assessed by means of analysing p53 for loss of heterozygosity, protein accumulation and gene mutations.

MATERIAL AND METHODS

Patients

The study included women diagnosed with primary breast cancer in the South-east Sweden Health Care Region, between 1980 and 1993. All patients under the age of 37 years at the time of diagnosis, for whom archival material was available, were included in the investigation. In all, specimens were obtained in 123 cases, ranging from 24 to 36 years of age, with a median age of 34 years. Survival data were obtained from the Cause of Death Register, provided by the National Board of Health and Welfare. At the time of follow-up, i.e. 31 December 1996, 50 of the patients were reported to be deceased due to breast cancer and additionally 2 patients were dead due to malignancies of unspecified origin. The median follow-up was 67 months. The formalin-fixed and paraffin-embedded tumours were obtained from the departments of pathology in Linköping, Kalmar, Jönköping and Norrköping.

Immunohistochemistry

Tumour sections, used for immunohistochemistry and genetical analysis, were selected by a pathologist from routinely stained formalin-fixed and paraffin-embedded material. In a minority of cases the tumour sections also contained parts of normal breast parenchyma, which was removed before extracting the DNA. Immunohistochemistry analysis was carried out using standard procedures. Briefly, 5 μm sections were deparaffinised in xylene, rehydrated in ethanol and water before treatment in a microwave oven in a 0.01 M citrate-buffer (pH 6.0) during 30 min at 750 W. The tissue sections were then incubated with a mouse monoclonal antibody (MAb) (DAKO, Denmark, clone DO-7, diluted 1:100) against the p53 protein. The primary antibody was coupled with the avidin-biotin-peroxidase complex and stained by adding 3,3′-diaminobenzidine. Cells were counterstained with Mayer's Haematoxylin. The immunostaining was graded semi-quantitatively in three groups by estimating the content of p53 protein accumulation. The different grading groups were scored as follows: *weak* when only a few cells were stained, *medium* if significant but not extensive staining was observed, and finally *strong* if the staining was extensive, i.e. including most of the tumour cells.

Isolation of DNA

DNA from 30 μm sections of the selected paraffin-embedded tumours and matched uninvolved lymph nodes were prepared using a standard protocol [24]. Briefly, the paraffin was removed by repeated extractions with xylene after which the specimens were washed with decreasing concentrations of ethanol. The tissue was then digested in a proteinase-K solution at 55°C during 36 h, followed by extraction of the DNA with phenol, phenol–chloroform (1:1) and chloroform. The nucleic acids were precipitated in 95% ethanol, containing sodium acetate (0.1 M), during approximately 1 h at −70°C and then pelleted by centrifugation at 12 000×*g*. The DNA was washed with 70% ethanol, dried in vacuum, and finally dissolved in sterile water. The concentration of DNA was determined by spectrophotometry.

Polymerase chain reaction

The tumours were examined for mutations in exons 5–8 of the *TP53* gene and loss of heterozygosity (LOH) at the HP53 locus intragen to *TP53*. Polymerase chain reaction (PCR) was performed in a total reaction volume of 22 μl, containing 25–50 ng of genomic DNA, 2 mM $MgCl_2$, 1 Taq polymerase buffer solution (20 mM$(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 8.5), 0.1% Tween 20), 1 μM of each primer, 0.2 mM of each dNTP and 0.5 U Taq polymerase (SDS/Promega, Madison, Wisconsin, U.S.A.). Amplification was carried out by 30–40 cycles of PCR with the following conditions: denaturation steps were performed for 1 min at 94°C, except in the first cycle where the time was extended to 3 min, annealing temperatures ranged from 53 to 59°C for 45 sec, and the extension steps were performed at 72°C for 45 sec. The primers and corresponding annealing temperature are shown in Table 1.

Mutation analysis

The PCR products of exons 5–8 were labelled by inclusion of [α-^{32}P] dATP through 10–15 cycles of PCR. Electrophoresis was performed on a non-denaturating 6% polyacrylamide gel at 4 W for 14–18 h. The dried gel was exposed on an X-ray film (Cronex 4, Du Pont, Hamburg, Germany). The DNA showing mobility shift were collected from the gel, amplified with 5–15 PCR cycles, purified by Wizard™ purification system (SDS/Promega) and then used in the DNA-sequencing protocol. The DNA samples were labelled with the Thermo Sequenase Radio Labelled Terminator Cycle Sequencing kit (Amersham, Little Chalfont, Buckinghamshire, U.K.) with termination at γ-ddNTP^{33}, followed by electrophoresis on a denaturating polyacrylamide gel (6%) containing 8 M urea at 70 W. Gels were run for approximately 2 h, and dried and exposed on Kodax Biomax MR or Biomax MS film using a TransScreen LE intensifying screen, for 5–40 h at −70°C.

Table 1. *Annealing temperatures and DNA sequences of primers used for PCR amplification of exons* 5–8 *and the intragenic microsatellite marker of the* TP***53*** *gene*

		Forward (5′→3′)	Reverse (5′→3′)	Annealing temperature °C
TP53 gene	Exon 5	GCCCTGACTTTCAACTCT	CACCCCTGTCGTCTCTCC	53
	Exon 6	GTCCCCAGGCCTCTGATTC	AACCCCTCCTCCCAGAGAC	59
	Exon 7	TCTTGGGCCTGTGTTATCTC	GGTGGATGGGTAGTAGTATG	55
	Exon 8	CTGCCTCTTGCTTCTCTTTT	CTCCTCCACCGCTTCTTGTC	55
Microsatellite	HP53	GCACTTTCCTCAACTCTACA	ACCAGCTCCTTTAATGGCAG	68*

*Annealing and extension steps were combined at 68°C for 75 sec.

LOH analysis

The PCR was carried out using a two-step technique consisting of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec and combined annealing and extension steps at 68°C for 75 sec. PCR products were subjected to radioactive labelling with PCR by incorporation of α-dATP^{32}. Labelling conditions were identical to those used for the primary PCR, except that the number of cycles was decreased to 15. The different alleles were then separated on a denaturing polyacrylamide (6%) gel containing 8 M urea, at 45 W for 2–3 h. Gels were dried and exposed as earlier described. The evaluation of LOH was made by visual inspection by at least two independent investigators. LOH was considered to have occurred if the signal intensity of one allele in the tumour DNA was significantly reduced, in relation to the other allele, when compared to the signal intensity observed for the alleles in the corresponding normal DNA.

Statistical method

The log rank test was used to assess differences in survival between patients with and without p53 alterations.

RESULTS

In total, mutations by sequence analysis in the *TP53* gene were found in 21 of 123 patients (17%) (Table 2). In addition, two silent mutations were also found. 2 of the patients had multiple mutations (Patients 46 and 85). As shown in Table 2, 14 of 26 mutations were situated in the zinc binding domain (an illustration of the protein structure including the functional domains is given in Figure 1). The distribution of mutations according to type was: 18 point mutations leading to amino acid substitution, 1 nonsense mutation, 3 deletions, 2 silent mutations and 2 splicing site mutations. Furthermore, loss of heterozygosity was found in 20 of the 47 cases (43%) informative at the intragenic marker HP53.

Table 2. *Type and localisation of* TP53 *mutations, patients age and survival*

Patient	Codon	Nucleotide change	AA change	Domain	Age (years)	D/A
1	248	CGG→CAG	arg→gln	Z, L3	36	D
2	247	GGG→GAG	arg→gln	Z, L3	34	D
6	233	CAC→CTC	his→leu		31	D
12	183	TCA→TTA	ser→leu	Z, L2	29	A
17	229	TGT→CGT	cys→arg		31	A
30	228	CAC→AAC	asp→asn		36	A
46	228	GAC→GGC	asp→gly		29	D
	248	CGG→CAG	arg→gln	Z, L3	29	D
	251	ATC→GTC	ile→val	Z, L3	29	D
49	253	ACC→GCC	thr→ala		34	A
54	248	CGG→CAG	arg→gln	Z, L3	31	D
57	260	TCC→TCT	ser→ser		35	A
64	Exon 7/8	GT→CT	Splicing		34	D
65	186	GAT→GAC	asp→asp	Z, L2	34	A
67	174	AGG→TGG	arg→trp	Z, L2	24	A
68	186–191	Deletion 18 bases	In frame	Z, L2	36	D
75	243	ATG→ACA	met→thr	Z, L3	33	D
85	142	CCT→ACT	pro→thr		33	A
	144	CAG→CAT	gln→his		33	A
90	139–141	Deletion, 9 bases	In frame		35	A
91	174	AGG→TGG	arg→trp	Z, L2	32	D
99	176–177	Deletion 6 bases	In frame	Z, L2	32	D
104	139	AAG→AAT	lys→asn		36	A
107	158	CGC→CTC	arg→leu		36	D
114	Exon 5/6	GT→AT	Splicing	Z, L2	24	D
123	192	CAG→TAG	gln→stop	Z, L2	31	D

AA, amino acid; A, alive; D, deceased in breast cancer; Z, zinc binding domain; L2, DNA residues 163–195; L3, DNA residues 236–251.

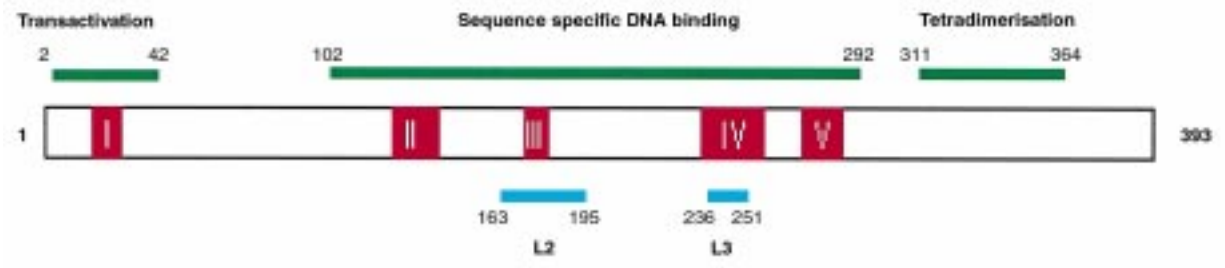

Figure 1. Schematic representation of the functional domains of the p53 proteins. Red boxes represent the five different evolutionary conserved regions, green lines show the different functional domains and blue lines show the extension of the two loops that provide direct DNA-contact and protein stabilisation. Numbers refer to positions along the 393 amino acid chain of the protein.

Results of the immunohistochemical staining of the p53 protein were available in 102 patients. Forty-seven tumours (46%) were judged as containing p53 protein accumulation. 14, 19 and 14 of the tumours were scored as having weak, medium and strong immunostaining, respectively.

Of the 123 breast cancer patients included in the study, 50 cases (41%) died due to breast cancer. As illustrated in Table 3, the proportion of deaths among patients with a gene mutation, loss of heterozygosity or protein accumulation was 62, 35 and 43%, respectively. Furthermore, 45% of the patients with medium or strong p53 protein accumulation died due to breast cancer. The statistical evaluation revealed no significant association between survival and *TP53* gene mutation (overall), LOH in the intragenic marker HP53, or protein accumulation (Table 3). However, in relation to wild-type *TP53*, missense mutations in the L2 and L3 domain were significantly ($P = 0.0009$) associated with a reduced survival rate. Moreover, missense mutations localised to the zinc binding domain were associated with poorer survival ($P = 0.0007$) also when compared with wild-type *TP53* and other mutations (Figure 2). Among the 12 patients having mutations leading to amino acid aberrations in this domain, 10 (83%) died due to breast cancer (Table 3).

Table 3. Gene mutations, accumulation of p53 protein and loss of heterozygosity at marker HP*53 in relation to survival*

	Number (%)	Number of deaths (%)	*P*-value*
Gene mutations			
WT	102 (83)	37 (38)	
All mutations	21 (17)	13 (62)	0.10
WT	102 (89)	37 (36)	
L2 and L3†	12 (11)	10 (83)	0.0009
WT and outside L2 and L3	111 (90)	40 (36)	
L2 and L3	12 (10)	10 (83)	0.0007
Loss of heterozygosity			
ROH	27 (57)	8 (30)	
LOH	20 (43)	7 (35)	0.24
P53 protein accumulation			
No accumulation	55 (54)	23 (42)	
Accumulation‡	47 (46)	20 (43)	0.8
None or weak	69 (68)	28 (41)	
Medium or strong§	33 (32)	15 (45)	0.37

*The association with survival was calculated using log rank analysis. †Mutations in the L2 and L3 domain leading to amino acid substitution. ‡Comparison of patients without versus with presence (i.e. weak, medium and strong) immunohistochemical p53 staining. §Comparison of patients with none or weak versus patients with medium or strong p53 staining of the protein. WT, wild type *TP53*, L2 and L3 loops comprise residues 163–195 and 236–251 of the protein. ROH, retention of heterozygosity and LOH, loss of heterozygosity.

DISCUSSION

In the large number of studies performed on breast cancer populations not selected for age, p53 protein accumulation and *TP53* gene mutations are usually associated with worse prognosis [12, 14–17]. However, as indicated both in the present study and in the few earlier reports on breast cancer in young women, p53 status may be of less prognostic significance in early onset breast carcinomas [25–27]. Clahsen and colleagues [27] used immunohistochemical analysis to determine p53 expression in premenopausal (median age 44 years) breast cancer patients without lymph node metastasis. Disease-free survival did not differ with respect to p53 status, among the 210 cases not receiving peri-operative chemotherapy. Furthermore, Bertheau and colleagues [26] investigated the prognostic impact of p53 accumulation, determined by immunohistochemical staining, in a cohort of 112 patients, consisting of 50 subjects 35 years or younger and 62 cases who were aged 36 to 50 years. In the total population, p53 was significantly correlated to reduced overall survival. However, when survival analysis was performed in the different age groups, the significance remained only in the oldest population ($P = 0.0059$). Moreover, Porter and colleagues

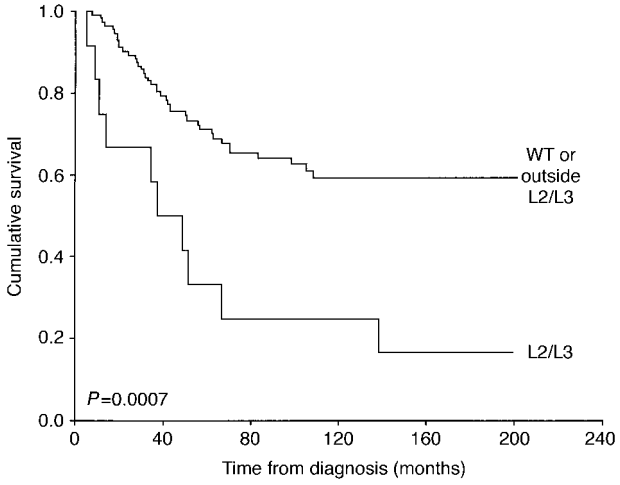

Figure 2. Patient survival (Kaplan–Meier) in relation to type of *TP53* mutation. L2/L3 represents mutations in the zinc-binding domains (amino acids 163–195 and 236–251), whereas WT represents the unaltered wild-type gene. Log rank analysis was used to evaluate the difference in survival ($P = 0.0007$). Deaths due to causes other than breast cancer were censored.

[25] investigated prognostic factors in a cohort of 246 patients (younger than 44 years) and found no association between overall survival and p53 protein accumulation. The observed difference in prognostic value of the p53 status between younger and older women with breast cancer may be a consequence of differences in adjuvant treatment, since p53 status has been associated with the response to systemic therapy [21, 28, 29]. Another plausible explanation might be influence from the adverse prognostic features, such as lymph node metastasis, high S-phase fraction or absence of hormonal receptors, frequently found in early onset breast cancer [2, 4–6]. It is also possible that breast cancer in young women is biologically different and thus follows a different pathway of genetical events. The lack of association between LOH at the intragenic HP53 locus and adverse prognosis is consistent with a previous report by Andersen and colleagues [30]. Possibly, the high frequency of LOH observed in the present study is not only a consequence of alterations at the *TP53* gene, but also due to other genes residing in this chromosomal region.

The frequency of gene mutations and p53 protein accumulation in the present study fall within the range previously reviewed by Hartman and colleagues [15] and Elledge and colleagues [14]. Moreover, as found in the present work and by others, reviewed in [14], the proportion of tumours with p53 protein accumulation is usually higher than the frequency of gene mutations. The strikingly high discrepancy observed in the present investigation may be explained not only by methodological differences but could also be due to biological reasons. Tumours affecting young women often have high S-phase fraction which in turn is associated with p53 protein accumulation [12, 13]. The elevated cell turnover may result in an increased risk for DNA damage, thereby causing wild-type p53 protein response [30]. Detectable levels of wild-type p53 protein may also result from interactions with cellular proteins [31–33] or failures in the degradation pathways, reviewed in [34]. However, the proportion of tumours with p53 accumulation found in the present report are consistent with the results of Albain and colleagues [4] and Walker and colleagues [2], who both found increased expression of p53 protein in women less than 35 years of age.

Of the different types of mutations, missense mutations were the most frequently found in the present study, which is similar to that reported by others [19, 20]. Also, the majority of the mutations were localised to the L2 and L3 loops (residues 163–195 and 236–251, respectively) of the protein, a domain involved in DNA contact and stabilisation of the protein structure. Moreover, in agreement with previous reports [19–21], mutations in the L2 and L3 domain were significantly associated with poor survival. Among the 12 cases having mutations leading to changes in the amino acid sequences in the L2 and L3 domains, 10 died of breast cancer. Presumably, these mutations have a direct impact on the DNA-binding properties of p53, possibly abolishing or diminishing the ability to specifically regulate cell-cycle regulatory proteins [35]. Aggressive tumour behaviour could arise as a result of reduced growth suppression or by compromised cell-cycle checkpoint activity [36]. A less likely, but none the less intriguing mechanism whereby such tumours might develop aggressive features, could involve the activation of new genes and their protein products, caused by mutations which might alter the DNA-binding specificity of wild-type p53 [36]. Another plausible explanation becomes evident when considering the matter from the opposite point of view. As suggested in the present study, as well as by others [19–21], mutations outside the L2/L3 domains, or outside the conserved regions, are associated with less aggressive tumour behaviour when compared with mutations in the L2/L3 domains. Furthermore, our results indicate that this type of mutation does not result in any significant difference in survival when compared with wild-type p53. These observations suggest that such mutations might not alter the structure or function of the protein, leading to a mutant p53 with, at least to some extent, retained wild-type properties.

In conclusion, the results from the present and other investigations of young women with breast cancer demonstrate a high frequency of p53 protein accumulations, although this protein overexpression appears to be of minor significance for survival. Nevertheless, the present report also suggests that missense mutations located in the zinc binding domain of the protein contribute substantially to tumour aggressiveness and adverse prognosis.

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