Pergamon

European Journal of Cancer, Vol. 35, No. 3, pp. 398–405, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(98)00400-6

Original Paper

Prognostic Significance of Mutations in the *p53* Gene, Particularly in the Zinc-binding Domains, in Lymph Node- and Steroid Receptor Positive Breast Cancer Patients

E. Kucera,[1] P. Speiser,[1] M. Gnant,[2] L. Szabo,[1] H. Samonigg,[2] H. Hausmaninger,[2] M. Mittlböck,[3] M. Fridrik,[2] M. Seifert,[1] E. Kubista,[1] A. Reiner,[4] R. Zeillinger,[1] R. Jakesz[3] and the Austrian Breast Cancer Study Group

[1]Department of Gynaecology and Obstetrics; [2]Department of Surgery; [3]Department of Medical Computer Sciences; and [4]Department of Pathology, University of Vienna Medical School, Waehringer Guertel, 1090 Vienna, Austria

The aim of our study was to evaluate if *p53* mutations, especially those in the L2/L3 domains of the *p53* gene, add prognostic information for node-positive and steroid receptor positive breast cancer patients. Two hundred and five tumour samples from a randomised clinical trial of 596 lymph node- and steroid receptor positive breast cancer patients were included. All patients had been randomly allocated to receive 20 mg of adjuvant tamoxifen (TAM) daily for 2 years or TAM plus one cycle of low-dose, short-term chemotherapy. For detection of *p53* mutations we used *in vitro* amplification by polymerase chain reaction and consecutively performed temperature gradient gel electrophoresis (PCR-TGGE) and direct sequencing. We found *p53* mutations in 42/205 (20%) cases: 16/42 (38%) *p53* mutations occurred within the L2/L3 domains of the *p53* gene, and 26/42 (62%) outside the L2/L3 domains. *p53* mutation served as a statistically significant parameter in predicting disease-free survival in univariate ($P = 0.02$) and multivariate ($P = 0.009$) analysis. For overall survival, no significant differences were observed. Patients with tumours that had *p53* mutations within the L2/L3 domains of the gene showed no significant difference to those with mutations outside the L2/L3 domains for disease-free survival. For overall survival, mutations in the L2/L3 domains showed a marginally significant difference ($P = 0.05$) in multivariate analysis, but not in univariate analysis ($P = 0.13$). We conclude that mutation in the L2/L3 domains of the *p53* gene is not an independent prognostic indicator of disease outcome for patients suffering from breast cancer with lymph node metastases and positive steroid receptors.

Key words: breast cancer, lymph node-positive, steroid receptor positive, prognosis, *p53* mutations, L2/L3 domains, PCR, temperature gradient gel electrophoresis, low-dose chemotherapy
Eur J Cancer, Vol. 35, No. 3, pp. 398–405, 1999

INTRODUCTION

THE MOST common genetic changes identified in cancer cells are mutations in the *p53* tumour suppressor gene. Mutations in the *p53* gene occur in different human malignancies [1, 2]. Breast cancer has been shown to have an especially high rate of *p53* mutations [3–5]. Several studies have shown *p53* mutation to be associated with increased metastatic potential, to serve as a prognostic marker of shortened survival and as an indicator of poor prognosis in breast cancer [6–13].

The majority of the mutations occur in the core domain of the *p53* gene (residues 102–292), which contains the sequence-specific DNA binding activity. Mutations in the core domain lead to loss of DNA binding, which is estimated to be critical for the biological activity of *p53* [14]. The L2/L3 domains are parts of the core domain of the *p53* gene and contain a zinc atom necessary for DNA binding. Exons 5 and 6 include the L2 domain (residues 163–195), exon 7 includes

Correspondence to E. Kucera, e-mail: elisabeth.kucera@akh-wien.ac.at
Received 31 Jul. 1998; revised 20 Oct. 1998; accepted 19 Nov. 1998.

the L3 domain (residues 236–251). Mutations in the L2/L3 domains have been described to account for almost 50% of *p53* mutations and have been reported to correlate with shortened disease-free and overall survival [7].

Mutations of the *p53* gene lead to protein stabilisation, therefore, immunohistochemistry (IHC) has frequently been used for detecting altered p53 protein [11, 12, 15]. However, Sjögren and colleagues showed that complementary DNA-sequencing is superior in determining *p53* alterations [16].

In the current study, 205 steroid receptor positive breast cancer samples from women with axillary lymph node metastases were analysed for *p53* mutations using PCR and subsequent temperature gradient gel electrophoresis as a screening method (PCR-TGGE). We further characterised possible mutations, indicated by altered band mobility in TGGE, by direct sequencing. The objective was to determine if *p53* gene mutations, especially those in the L2/L3 domains, could identify a high risk group of node-positive breast cancer patients. Therefore, we primarily investigated the correlation between *p53* mutation and disease-free and overall survival. Furthermore we focused on *p53* mutations in the L2/L3 domains versus outside the L2/L3 domains and disease-free and overall survival in the high risk group of patients with positive lymph nodes.

PATIENTS AND METHODS

Study population

The Austrian Breast Cancer Study Group conducted a randomised trial from 1984 to 1990 (dates of enrolment) investigating additional chemotherapy to tamoxifen treatment at a dose of 20 mg per day for 2 years. The randomised trial included 596 lymph node-positive and steroid receptor positive patients. Patients who had low oestrogen receptors (< 10 fmol/mg) only were included in the randomised trial if they had positive progesterone receptors. Patients were eligible for the randomised trial if they were younger than 70 years and above 18 years of age and showed histologically confirmed complete removal of a unilateral carcinoma of the breast, level I and level II axillary nodal dissection, histological examination of at least six axillary nodes with at least one being involved, and oestrogen- and/or progesterone-receptor level $\geq$ 10 fmol/mg cytosol protein. Distant metastases had to be excluded by lung X-ray, liver ultrasound and bone scan or, if indicated, CT-scan. All patients were treated with either quadrantectomy or modified radical mastectomy and axillary lymphadenectomy. Breast conservation surgery was combined with adjuvant irradiation of the breast. Patients were randomly allocated to receive 20 mg of adjuvant tamoxifen (TAM) daily for 2 years or TAM plus one cycle of adjuvant low-dose, short-term chemotherapy. The regimen of adjuvant chemotherapy consisted of one cycle of doxorubicin (20 mg/m^2) and vincristine (1 mg/m^2) (AV) and one cycle of cyclophosphamide (300 mg/m^2), methotrexate (25 mg/m^2) and 5-fluorouracil (600 mg/m^2) (CMF). This treatment was chosen in order to test the hypothesis that this regimen of adjuvant low-dose chemotherapy could improve the prognosis of patients treated with TAM alone. The regimens were the same between premenopausal and postmenopausal patients. Out of the tumour samples of 596 node-positive and steroid receptor positive (oestrogen or progesterone receptor positive) breast cancer patients, we screened 205 paraffin sections for mutations of the *p53* gene. Our correlative study included 34.4% (205/596) of the lymph node-positive and steroid receptor positive patients. Half of our 205 patients received adjuvant low-dose chemotherapy and the other did not. All patients underwent a close follow-up scheme consisting of regular visits with a complete physical examination. Ultrasound examination of the abdomen and chest X-ray were performed every 6 months. Mammography and bone scan were performed every 12 months or in cases of suspect clinical findings.

p53 status was evaluated retrospectively, and the patient cohort was divided into two groups (*p53* positive and *p53* negative breast cancer patients) for further analysis. The mean age at diagnosis of the 163 *p53* negative patients was 55 ± 10 years, with a median of 57 years (range 26–71 years). The mean age of the 42 *p53* positive patients was 50 ± 9 years, with a median of 48 years (range 35–71 years). Survival data were available for all patients and the median observation time was 107 months. All DNA preparations were made from paraffin-embedded tissue sections by standard techniques.

DNA extraction

Two paraffin sections (30 μm thickness) of each tumour were deparaffinised with 1 ml xylene. Extraction was done at room temperature by vortexing followed by slight agitation for 2 min. Then the samples were centrifuged at 10 000 ***g*** for 2 min. This extraction procedure was repeated twice with xylene and three times with ethanol to remove traces of xylene. The samples were then dried in a vacuum for approximately 1 h. Next 200 μl of PCR-buffer (10 mM Tris–HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatine, 0.45% Nonidet P40, 0.45% Tween 20) containing 10 mg of glass beads (GG-500-200 Glycerol Glass Controlled Pore, 120-200 mesh, Sigma, St Louis, Missouri, U.S.A.) and proteinase-K at a final concentration of 0.6 mg/ml were added. The sample tubes were sonicated for 30 min at 55°C in a sonicating water bath (Branson, U.S.A.). After sonication the samples were boiled for 10 min in a water bath. The tubes were spun at 10 000 ***g*** and the supernatant containing the DNA was collected.

Polymerase chain reaction and temperature-gradient gel electrophoresis (PCR-TGGE)

Four regions of the *p53* gene corresponding to exons 5–8 were amplified by PCR. Fragment I comprises exon 5, intron 5 (81 bp) and exon 6; fragment II comprises exon 5, fragment III exon 7, and fragment IV exon 8, respectively. Primer sequences are shown in Table 1. Primer sequences were chosen from the *p53* cDNA sequence [17]. The sense primers contained the sequence for a previously described 40 bp 'GC-clamp' [18]. Approximately 5 μl of the DNA solution extracted as described above were used as template for PCR. Reactions were performed in a total volume of 50 μl in PCR-buffer containing 50 pmol of each primer, 250 μM each dNTP and 0.25 unit ''HiTaq'' polymerase (ViennaLab, Vienna, Austria) for 30 cycles at 94°C (30 sec), 62°C (30 sec) and 72°C (45 sec) and a final extension time of 5 min at 72°C in a Perkin Elmer Cetus 9600 DNA Thermal Cycler (Perkin Elmer, Norwalk, Connecticut, U.S.A.). PCR-products were analysed in a 4% (3/1 NuSieve/GTG-Agarose) agarose gel (FMC BioProducts, Rockland, Maryland, U.S.A.). Experimental parameters for system optimisation were obtained by performing perpendicular TGGE using the Diagen-TGGE system (Diagen, Hilden, Germany).

Gels (19×19×0.1 cm) for TGGE contained 8% acrylamide and 8 M urea in a MOPS/EDTA buffer (20 mM 4-morpholinepropane sulphonic acid (MOPS), 1 mM EDTA, pH 8.0) and were polymerised with 0.015% (w/v) ammonium persulphate and 0.17% (v/v) N,N,N',N'-tetramethylethylenediamine. Ten microlitres of 1:10 diluted PCR-products were loaded on to the gel at starting positions at the following temperatures: 43°C for fragments I and III, 48°C for fragment II, and 46°C for fragment IV. Gels were run at 6 W (10 V/cm) at 20°C for 15 min to allow entering of the samples into the gel at native conditions. Then, a temperature-gradient ($T_1 = 30°C$ and $T_2 = 70°C$) was superimposed on the gel parallel to the electric field. Running times were 30 min at 7.5 W (8 V/cm). After electrophoresis, the gels were silver-stained (incubation of 0.1% silver nitrate for 10 min followed by a 20 min incubation in 1.5% NaOH, 0.15% formaldehyde, and 0.01% natrium borhydride and fixation in 0.75% natrium carbonate).

DNA sequencing

Sequence-analysis of PCR-products was performed with the ABI 373A automatic sequencer (Perkin Elmer, Foster City, California, U.S.A.). PCR products were either cloned into the PCR™2.1 vector (TA cloning kit, Invitrogen, Carlsbad, California, U.S.A.) or directly sequenced according to the manufacturer's instructions (ABI Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer). Primers for sequencing were the same as used for PCR, but without 'GC-clamp'.

Statistical analysis

The association between all *p53* mutations or mutations in versus outside the L2/L3 domains and clinicohistopathological factors were assessed by χ^2-test and the Fisher's exact test (two-tailed) where appropriate. A trend test was used if applicable. A P value < 0.05 was considered to indicate statistically significant differences. Survival curves for overall and disease-free survival were estimated by the method of Kaplan–Meier for each level of the prognostic factors and corresponding differences were tested in an univariate manner by Mantel's log-rank test. Multiple analysis of overall and disease free survival was made by a stepwise Cox's proportional hazards regression model to test and quantify the role of independent prognostic factors. The following parameters were included in the multivariate analysis: *p53* status, age, menopausal status, lymph node status, differentiation grade, oestrogen receptor and progesterone receptor status, and histological type. Chemotherapy was not included as a factor in the multivariate survival analysis. All P values reported are two-sided. The statistical software SAS and BMDP were used for all statistical analysis.

RESULTS

Clinical results

There were 88 cases of recurrences in our series. Whenever possible, recurrences were proven histologically, or otherwise indicated by X-ray, computer tomography (CT) or bone scan as measurable disease. 26/88 recurrences were local failures, 57/88 recurrences were metastases, and 5/88 were considered as both. There were 96 cases of deaths within a median observation time of 107 months. Time of enrolment was from 1984 to 1990, and all patients were followed up until November 1997. 3 out of 205 patients were lost to follow-up up to 1990, another 64 patients were lost to follow-up by the end of 1996, whilst in the last year another 42 patients were lost to follow-up. Those patients who were lost from follow-up were regarded as censored.

Mutations in the p53 *gene*

In 62/205 cases (30%) we found an altered band mobility in TGGE. In 5 of the 62 cases sequencing was not possible because of insufficient tumour material. 48 out of the remaining 57 cases showed nucleotide substitutions (Table 2). Ten of these mutations did not result in an amino acid change and were considered silent. 4 mutations resulted in stop codons leading to protein truncation and 34 mutations resulted in an amino acid change. Frameshift mutations (deletions) were detected in 9 cases. Clinicopathological characteristics did not differ between patients with silent mutations and functional mutations. Three tumour samples contained two, and one sample contained three independent mutations (Table 2). 20 mutations leading to functional protein alterations were found in exon 5, one in exon 6, 10 in exon 7, and 16 in exon 8. The majority of point mutations appeared at G:C base pairs (29/40 cases, i.e. 74%): G:C→A:T transitions were detected 11 times, C:G→T:A transversions 10 times, G:C→T:A and C:G→G:C transversions 3 times each, and G:C→C:G twice. Further transitions were A:T→G:C ($n = 6$), and T:A→C:G ($n = 1$). Further transversions were A:T→T:A ($n = 3$), A:T→C:G ($n = 1$), and T:A→A:T ($n = 1$). Out of 205 samples, 42 (20%) were considered to have mutations for the final analysis. Thirty-eight per cent ($n = 16$) of the mutations were located within the L2/L3 domains, and 62% ($n = 26$) were located outside the L2/L3 domains.

Table 1. *Primer sequences*

Sense primer, fragment I
5′-CGCCCGCCGCGC-CCCGCGCCCGCCCCGCCGCCCCCGCCCCTT-CCTCTTCCTGCAGTACTCC-3′
Antisense primer, fragment I
5′-AGTTGCAAACCAGA-CCTCAGG-3′
Antisense primer, fragment II, sense primer see fragment I
5′-GCCCCAGCTGCTCACCATCGCT-3′
Sense primer, fragment III
5′-CGCCCGCCGCGCCCCGCGCCCGCCCCGCCGCCCCCGCCCC-GTGTTGTCTCCTAGGTTGGC-3′
Antisense primer, fragment III
5′-CAAGTGGCTCCTGACCTGGAG-3′
Sense primer, fragment IV
5′-TGGTAATCTACTGGGACGGAACAGC-3′
Antisense primer, fragment IV
5′-CGCCCGCCGCGCCCCGCGCCCGCCCCGCCGCCCCCGCCCCTTACCTCGCTTAGT-GCTCC-3′

Association between p53 *mutations and patient and tumour characteristics*

Associations between *p53* mutation and clinicohistopathological parameters are shown in Table 3. There were no significant correlations, except that patients with *p53* mutated tumours were found to be significantly younger ($P = 0.04$) compared with patients whose tumours showed no *p53* mutations. Similarly, none of the same clinicohistopathological

Table 2. p53 *mutations and specific alterations in the amino acid sequence*

Patient No.	Base change	Substitution	Codon	Exon	Amino acid change
1	TGG > TGA	G > A	146	5	Trp > stop
2	CCC > TCC	C > T	151	5	Pro > Ser
3	CCC > TCC	C > T	151	5	Pro > Ser
4	Del. CCG > C_G		152	5	Frameshift
	CGC > TGC	C > T	158	5	Arg > cys
5	Del. TAC > TA_		163	5	Frameshift
6	CAG > TAG	C > T	167	5	Gin > stop
7	GTT > GTG	T > G	172	5	Silent
8	CGC > CAC	G > C	175	5	Arg > His
9	CGC > CAC	G > A	175	5	Arg > His
10	TTG > TTA	G > A	201	6	Silent
11	CGA > CGG	A > G	213	6	Silent
12	CAT > CAC	T > C	214	6	His > His silent
13	Del. TCC > TC_		241	7	Frameshift
14	GGC > GTC	G > T	244	7	Gly > Val
15	CGG > CAG	G > A	248	7	Arg > Gin
16	CGG > TGG	C > T	248	7	Arg > Trp
17	CGG > TGG	C > T	248	7	Arg > Trp
18	CGG > CAG	G > A	248	7	Arg > Gln
19	CGG > CAG	G > A	248	7	Arg > Gln
20	ATC > TTC	A > T	248	7	Ile > Phe
21	CGT > TGT	C > T	273	8	Arg > Cys
22	CCT > TCT	C > T	278	8	Pro > Ser
23	AGA > ACA	G > C	280	8	Arg > Thr
24	GAC > AAC	G > A	281	8	Asp > Asn
25	CGG > GGG	C > G	282	8	Arg > Gly
26	Del. CG > _GG		282	8	Frameshift
27	CGG > GGG	C > G	282	8	Arg > Gly
28	Del. GAG > G_		285	8	Frameshift
29	GAG > AAG	G > A	285	8	Glu > Lys
30	GAG > AAG	G > A	285	8	Glu > Lys
31	AAA > AGA	A > G	292	8	Lys > Arg
32	GAG > TAG	G > T	294	8	Glu > stop
33	GAG > GAA	G > A	294	8	Silent
34	Del. CCT > C_T		295	8	Frameshift
35	CAC > CTC	A > T	296	8	Leu > His
36	CAC > CCC	A > C	297	8	His > Pro
37	CCT > CCC	T > C	278	8	Silent
38	ACC > ACT	C > T	155	5	Silent
	GCC > GTC	C > T	159	5	Ala > Val
39	GCC > ACC	G > A	159	5	Ala > Thr
	CGA > CGG	A > G	213	6	Silent
40	ATG > GTG	A > G	169	5	Met > Val
	CGC > GGC	C > G	181	5	Arg > Gly
41	GCC > ACC	G > A	129	5	Ala > Thr
	CAG > TAG	C > T	165	5	Gln > stop
42	GAA > GAG	A > G	268	8	Silent
	CTG > CCG	T > C	299	8	Leu > Pro
43	TGC > AGC	T > A	135	5	Cys > Ser
	ATC > TTC	A > T	162	5	Ile > Phe
	TGC > TTC	G > T	176	5	Cys > Phe
44	GGC > GAC	G > A	244	7	Gly > Asp
	CGG > AGG	C > A	248	7	Silent
	ACA > GCA	A > G	256	7	Thr > Ala
45	Del. TGC____CGC		176___181	5	Frameshift
46	Del. GTG____CCC		216___219	6	Frameshift
47	Del. TC____G		149___160	5	Frameshift
48	TGA > TGC	A > C	Intron 5		Splicing ?

Del., deletion.

parameters, including age, was statistically significantly associated with mutations of the *p53* gene within the L2/L3 domains or outside the L2/L3 domains (data not shown).

Association between p53 *mutations and survival*

For disease-free survival, univariate analysis revealed *p53* mutated tumours ($P = 0.02$) (Figure 1a) and the number of involved lymph nodes ($P = 0.0001$) as prognostic parameters. Multivariate analysis confirmed the prognostic significance of *p53* mutations ($P = 0.009$) and the number of involved lymph nodes ($P = 0.0001$) for predicting relapse-free survival. For overall survival, univariate analysis revealed the number of involved lymph nodes ($P = 0.0001$) and tumour size ($P = 0.04$) as independent prognostic parameters, whereas in multivariate analysis only the number of involved lymph nodes remained an independent prognostic parameter ($P = 0.0001$). For overall survival no significant differences were found for *p53* mutated tumours in univariate ($P = 0.29$) (Figure 1b) or multivariate ($P = 0.14$) analysis.

A difference in the 5-year survival of 72% in *p53* negative ($n = 163$) patients and 60% in *p53* positive ($n = 42$) patients could be detected with a power of 44% with a two-sided Mantel test, given a significance level of 5% and the observed follow-up times. A difference in the 5-year disease-free survival of 65% in *p53* negative and 45% in *p53* positive patients could be detected with a power of 77% with the same test and assumptions.

Association between p53 *mutations within and outside the zinc-binding domains and survival*

16/42 (38%) *p53* mutations occurred in the L2/L3 domains, and 26/42 (62%) *p53* mutations occurred outside the L2/L3 domains. We found 8/16 (50%) mutations in the L2 domain, and 8/16 (50%) in the L3 domain. For mutations in the L2/L3 domains compared with mutations outside the L2/L3 domains, univariate ($P = 0.17$) (Figure 1c) and multivariate ($P = 0.10$) analysis demonstrated no statistically significant difference for disease-free survival. Only lymph node involvement was statistically significant for predicting disease-free survival in univariate ($P = 0.001$) and multivariate ($P = 0{\cdot}003$) analysis.

For overall survival, we found that patients whose tumours had mutations within the L2/L3 domains showed no significant differences in univariate analysis ($P = 0.13$) (Figure 1d), to those with mutations outside the L2/L3 domains of the *p53* gene, whereas multivariate analysis revealed marginally significant differences ($P = 0{\cdot}05$). The P values for lymph node involvement were $P = 0.001$ in univariate analysis and $P = 0.003$ in multivariate analysis, respectively.

Association between p53 *mutations, chemotherapy and survival*

No significant association between p53 status, chemotherapy and survival was shown in the univariate analysis. Although chemotherapy was not included in the multivariate survival analysis, chemotherapy neither influenced disease-free nor overall survival in the whole study population of 596 patients (data not shown).

DISCUSSION

Patients suffering from lymph node-positive breast cancer are usually treated with adjuvant therapy after surgical treatment. Nowadays certain gene alterations are estimated to support or even replace traditional prognostic factors (e.g.

Table 3. Association of patient and tumour characteristics with p53 *status*

Characteristics	*n* (%)	*p53* mutation present (%)	Statistical analysis *P*
Age			
≤ 50 years	83 (40)	23 (28)	
> 50 years	122 (60)	19 (16)	0.04 (χ^2)
Menopause			
Premenopausal	91 (44)	24 (26)	
Peri-, postmenopausal	114 (56)	18 (16)	0.06 (χ^2)
Tumour size			
T1	67 (33)	7 (10)	
T2	115 (56)	32 (28)	
T3/T4	23 (11)	3 (13)	0.17 (χ^2 trend test)
Nodal status			
1–3 positive nodes	125 (61)	26 (21)	
4-10 positive nodes	57 (28)	12 (21)	
> 10 positive nodes	23 (11)	4 (17)	0.93 (χ^2 trend test)
Grade			
G1 + G2	149 (73)	26 (17)	
G3	56 (27)	16 (29)	0.09 (χ^2)
Oestrogen receptor status			
< 10 fmol/mg	16 (8)	5 (31)	
10–100 fmol/mg	127 (62)	27 (21)	
> 100 fmol/mg	62 (30)	10 (16)	0.18 (χ^2 trend test)
Progesterone receptor status			
< 10 fmol/mg	46 (22)	11 (24)	
10–100 fmol/mg	67 (33)	14 (21)	
> 100 fmol/mg	92 (45)	17 (18)	0.45 (χ^2 trend test)
Histological type			
Invasive ductal	187 (91)	41 (22)	
Invasive lobular	18 (9)	1 (6)	0.13 (Fisher's exact)

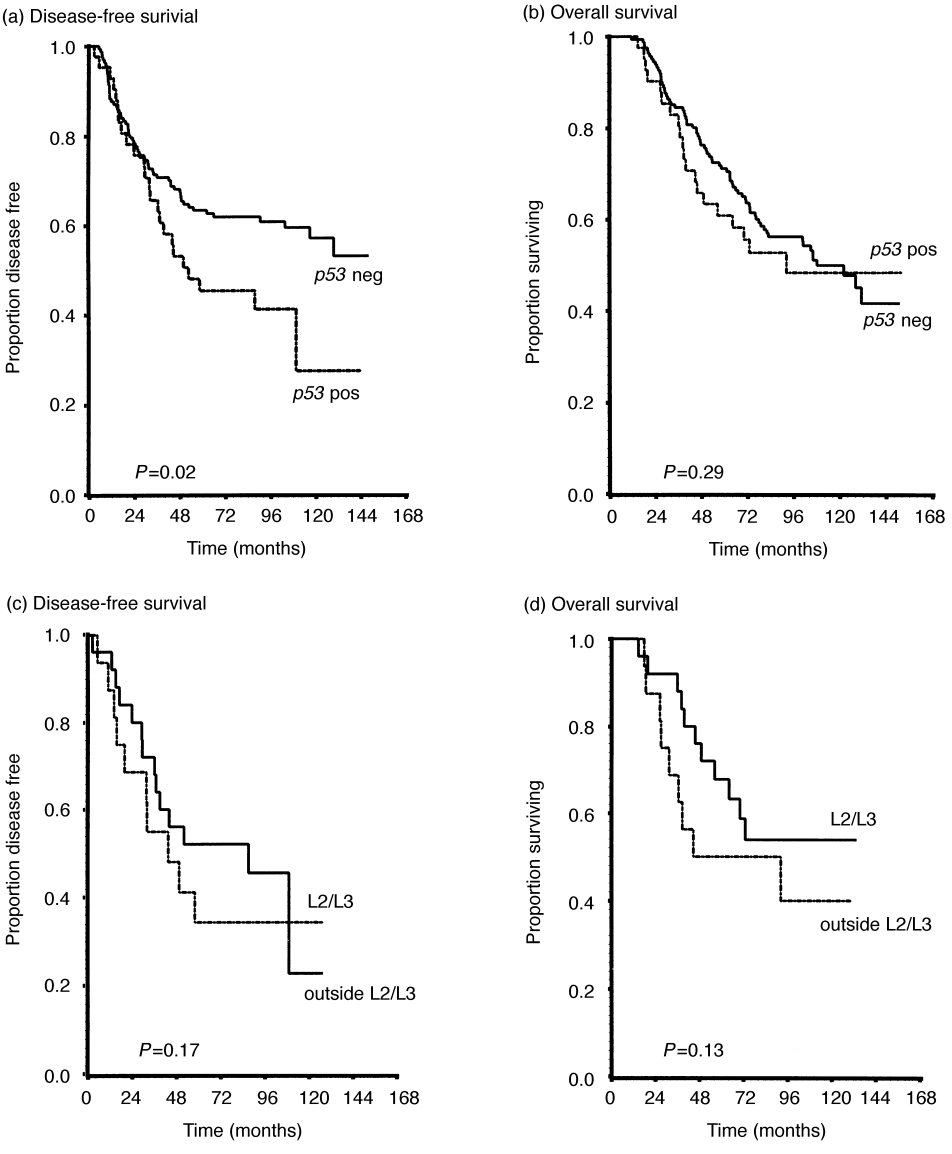

Figure 1. (a) Disease-free and (b) overall survival curves for *p53* mutated tumours in comparison with tumours without *p53* mutations. (c) Disease-free, and (d) overall survival curves for tumours with mutations within and outside the L2/L3 domains.

lymph node status, tumour size, histological and nuclear grades of atypia and mitotic indices of cancer cells) for the prediction of clinical outcomes in human cancer [19–22].

Recent evidence [5] has shown that the prognosis of breast cancer patients with lymph node metastases and p53 immunoreactivity is poorer under identical adjuvant systemic therapies in comparison with patients without p53 immunoreactivity. The Austrian Breast Cancer Study Group initiated a randomised trial in 1984 to test the hypothesis that adjuvant low-dose chemotherapy could improve the prognosis of patients treated with tamoxifen alone. At that time adjuvant chemotherapy after operation for breast cancer had been shown to be effective in improving disease-free and overall survival in premenopausal patients. Adjuvant tamoxifen had shown a significant reduction in recurrence rates, predominantly in postmenopausal patients [25]. In fact, there were conflicting results with regard to age dependency of tamoxifen [26]. For the efficacy of tamoxifen, steroid receptors had been shown to predict response in the adjuvant and metastatic status. Bergh and associates already supposed that adjuvant therapy with tamoxifen might be of less value to patients with *p53* mutated tumours [10]. Our main interest was to evaluate the prognostic significance of *p53* mutations, particularly of mutations in the zinc-binding domains of the *p53* gene. Additionally, we evaluated if *p53* mutated tumours, especially tumours with mutations in the L2/L3 domains of the gene had a worse prognosis despite tamoxifen or tamoxifen plus low-dose chemotherapy.

Verified mutations (by complementary DNA sequencing) of the *p53* gene have been reported to be an indicator of poorer prognosis, although with less impact than p53 immunoreactivity [16]. In contrast, Sjögren and associates suggested that using complementary DNA sequencing to determine the status of the *p53* gene in primary breast cancers provides better prognostic information than immunohistochemistry [16]. They performed an immunohistochemical analysis of the p53 status with a mouse monoclonal antibody, which recognises wild-type as well as mutant forms of *p53* [16]. Because one third of verified *p53* mutations (by complementary DNA sequencing) failed to be detected by

immunohistochemistry (IHC), and a 30% false-positive frequency for IHC-positive tumours was reported [16, 23], we decided to perform PCR-TGGE as a screening method for *p53* mutations and further characterised possible mutations by direct sequencing.

The percentage of *p53* gene mutations (20%) in our patient series was similar to that reported by Tsuda and colleagues (25%). The higher frequency of *p53* gene mutations in younger patients seems to correlate with more aggressive tumours in these patients. Mutations in the *p53* gene lead to loss of the usual negative growth regulation and therefore more rapid cell proliferation. In accordance with other authors [5, 23] statistical analysis of our data revealed that lymph node status and mutation of the *p53* gene were statistically significant parameters for predicting disease-free survival, but we could not confirm histological grade, nor tumour size as significant indicators of shortened disease-free survival. For overall survival only lymph node status served as a prognostic indicator in our patient series, whereas Tsuda and colleagues [5] additionally found *p53* gene mutations, steroid receptor status and histological grade as significant prognostic indicators of poorer overall survival. These different results might be due to different patient series, as the number of patients with grade 3 tumours was 2-fold higher in their study [5]. Whilst Tsuda and colleagues [5] suggested that the combination of p53 immunoreactivity and c-erbB-2 amplification (both strongly correlated with the histological grade) with conventional factors might help to identify highly aggressive node-positive breast cancers, we investigated whether *p53* gene mutations in the core domain, especially in the L2/L3 domains, could identify a high risk group of node-positive breast cancer patients with poor prognosis. We found a marginally significant difference towards shortened overall survival ($P = 0.05$) in multivariate statistical analysis for patients whose tumours showed mutations in the L2/L3 domains of the *p53* gene.

Borresen and associates [7, 24] indicated that *p53* mutations in the zinc-binding domains (L2/L3 domains) predict poorer survival rates for patients suffering from breast cancer and colorectal cancer. In accordance with other authors [7, 9], the majority of the mutations in the *p53* gene in our tumour samples were detected in exons 5–7. Most were point mutations and occurred at G:C base pairs. Transversions and transitions occurred in nearly equal numbers. Thirty-eight per cent of the *p53* mutations were located in the L2/L3 domains. Borresen and associates [7] found 34% of all *p53* mutations located in the L2 or L3 domain and showed a significantly shorter relapse-free and overall survival in univariate analysis for patients with *p53* mutations in these domains, whereas multivariate survival analysis confirmed tumour size and nodal status as the only significant prognostic parameters. The marginally significant difference towards shortened overall survival ($P = 0.05$) in our series might be explained by the median observation time of 107 months versus 40 months in Borresen's study [7].

Our results confirmed that nodal status is still the most reliable prognostic parameter in breast cancer patients. We also confirmed that mutation of the *p53* gene adds significant prognostic information regarding disease-free survival, whilst screening for mutations in the L2/L3 domains of the *p53* gene in our lymph node-positive and steroid receptor positive tumour series did not provide additional prognostic information.

1. Lane DP. p53 and human cancers. *Br Med Bull* 1994, **50**, 582–599.
2. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991, **253**, 49–53.
3. Osborne RJ, Merlo GR, Mitsudomi T, *et al.* Mutations in the *p53* gene in primary human breast cancers. *Cancer Res* 1991, **51**, 6194–6198.
4. Elledge RM, Allred DC. The *p53* tumor suppressor gene in breast cancer. *Breast Cancer Res Treat* 1994, **32**, 39–47.
5. Tsuda H, Sakamaki C, Tsugane S, Fukutomi T, Hirohashi S. A prospective study of the significance of gene and chromosome alterations as prognostic indicators of breast cancer patients with lymph node metastases. *Breast Cancer Res Treat* 1998, **48**, 21–32.
6. Mazars R, Spinardi L, BenCheikh M, Simony Lafontaine J, Jeanteur P, Theillet C. p53 mutations occur in aggressive breast cancer. *Cancer Res* 1992, **52**, 3918–3923.
7. Borresen AL, Andersen TI, Eyfjord JE, *et al.* TP53 mutations and breast cancer prognosis: particularly poor survival rates for cases with mutations in the zinc-binding domains. *Genes Chromosomes Cancer* 1995, **14**, 71–75.
8. Thorlacius S, Thorgilsson B, Bjornsson J, *et al.* TP53 mutations and abnormal p53 protein staining in breast carcinomas related to prognosis. *Eur J Cancer* 1995, **31A**, 1856–1861.
9. Andersen TI, Holm R, Nesland JM, *et al.* Prognostic significance of TP53 alterations in breast carcinoma. *Br J Cancer* 1993, **68**, 540–548.
10. Bergh J, Norberg T, Sjogren S, Lindgren A, Holmberg L. Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. *Nat Med* 1995, **1**, 1029–1034.
11. Silvestrini R, Benini E, Daidone MG, *et al.* p53 as an independent prognostic marker in lymph node-negative breast cancer patients. *J Natl Cancer Inst* 1993, **85**, 965–970.
12. Thor AD, Moore DH, II, Edgerton, S. M., *et al.* Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. *J Natl Cancer Inst* 1992, **84**, 845–855.
13. Tsuda H, Iwaya K, Fukutomi T, Hirohashi S. p53 mutations and c-erbB-2 amplification in intraductal and invasive breast carcinomas of high histologic grade. *Jpn J Cancer Res* 1993, **84**, 394–401.
14. Cho Y, Gorina S, Jeffrey PD, Pavletich NP. Crystal structure of a p53 tumor suppressor–DNA complex: understanding tumorigenic mutations (see comments). *Science* 1994, **265**, 346–355.
15. Allred DC, Clark GM, Elledge R, *et al.* Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer (see comments). *J Natl Cancer Inst* 1993, **85**, 200–206.
16. Sjögren S, Inganäs M, Norberg T, *et al.* The p53 gene in breast cancer: prognostic value of complementary DNA sequencing versus immunohistochemistry. *J Natl Cancer Inst* 1996, **88**, 173–182.
17. Zakut Houri R, Bienz Tadmor B, Givol D, Oren M. Human p53 cellular tumor antigen: cDNA sequence and expression in COS cells. *EMBO J* 1985, **4**, 1251–1255.
18. Sheffield VC, Cox DR, Lerman LS, Myers RM. Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc Natl Acad Sci USA* 1989, **86**, 232–236.
19. Seeger RC, Brodeur GM, Sather H, *et al.* Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. *N Engl J Med* 1985, **313**, 1111–1116.
20. Wong AJ, Ruppert JM, Eggleston J, Hamilton SR, Baylin SB, Vogelstein B. Gene amplification of c-myc and N-myc in small cell carcinoma of the lung. *Science* 1986, **233**, 461–464.
21. Yokota J, Yamamoto T, Toyoshima K, *et al.* Amplification of c-erbB-2 oncogene in human adenocarcinomas *in vivo*. *Lancet* 1986, **1**, 765–767.
22. Kitagawa Y, Ueda M, Ando N, Shinozawa Y, Shimizu N, Abe O. Significance of int-2/hst-1 coamplification as a prognostic factor in patients with esophageal squamous carcinoma. *Cancer Res* 1991, **51**, 1504–1508.
23. Tsuda H, Hirohashi S. Association among p53 gene mutation, nuclear accumulation of the p53 protein and aggressive phenotypes in breast cancer. *Int J Cancer* 1994, **57**, 498–503.
24. Borresen DA, Lothe RA, Meling GI, Hainaut P, Rognum TO, Skovlund E. TP53 and long-term prognosis in colorectal cancer: mutations in the L3 zinc-binding domain predict poor survival. *Clin Cancer Res* 1998, **4**, 203–210.

25. Controlled trial of tamoxifen as adjuvant agent in management of early breast cancer: interim analysis at four years by Nolvadex Adjuvant Trial Organisation. *Lancet* 1983, **1**, 257–261.
26. Ribeiro G, Swindell R. The Christie Hospital adjuvant tamoxifen trial: status at 10 years. *Br J Cancer* 1988, **57**, 601–603.

APPENDIX

Austrian Breast Cancer Study Group: G. Reiner, P. Götzinger, T Grünberger, S. Taucher, B. Gebhard, M. Rudas, G.Steger, Ch. Wiltschke (University of Vienna, Department of Surgery, Department of Pathology, Department of Internal Medicine I); M. Smola, G Rosanelli, H. Hauser, L. Kronberger, M. Hoff, H. Stöger, A. Kasparek, M. Schmid, Th. Bauernhofer, R. Moser (University of Graz, Department of Surgery, Department of Medicine); R. Obwegeser, L. Auerbach, C. Dadak, Ch. Kainz, G. Sliutz (University of Vienna, Department of Gynaecology and Obstetrics); W. Horvath, O. Langer (Oberwart); M. Stierer, H. Spoula, M. Hanak (Hanusch Krankenhaus Wien); V. Wette, G. Jatzko (St. Veit an der Glan); G. Michlmayr, D. Nitsche, G. Wahl (Linz); F. Hofbauer, M. Lang (Oberpullendorf).