British Journal of Cancer (1998) 77(7), 1130–1136

Mutations in residues of *TP53* that directly contact DNA predict poor outcome in human primary breast cancer

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Summary The tumour-suppressor gene *TP53* is frequently mutated in breast tumours, and the majority of the mutations are clustered within the core domain, the region involved in DNA binding. We searched for alterations in this central domain of the *TP53* gene in 222 human breast cancer specimens using polymerase chain reaction–single-strand conformation analysis (PCR–SSCA) followed by sequencing. *TP53* gene mutations were observed in 66 tumours (31%), including three tumours that contain two mutations. Fifty-four (78%) of these mutations were missense point mutations, one was a nonsense mutation and four were deletions and/or insertions causing disruption of the protein reading frame, whereas four mutations were either silent or a polymorphism (at codon 213; $n = 6$). Interestingly, the majority of missense mutations were observed at codon 248. The outcome has been related with patient and tumour characteristics, and with prognosis in 177 patients who were eligible for analysis of both relapse-free and overall survival (median survival for patients alive was 115 months). There was no significant association between the frequency of *TP53* mutations and menopausal or nodal status, or tumour size. In a Cox univariate analysis, *TP53* gene mutation was significantly associated with poor relapse-free survival (RFS: $P = 0.02$) but not with overall survival (OS: $P = 0.07$). In a Cox multivariate analysis, including classical prognostic factors, *TP53* gene mutation independently predicted poor RFS and OS (RHR = 1.8 and 1.6 respectively). Unexpectedly, the median relapse-free survival of patients with a polymorphism at codon 213 or with a silent mutation was shorter (median 11 months) than the median relapse-free survival of patients with or without a *TP53* gene mutation (median 34 or 48 months respectively). In an exploratory subset analysis, mutations in codons that directly contact DNA were related with the poorest relapse-free ($P < 0.05$) and overall survival ($P < 0.02$). These data imply that in the analysis of the prognostic value of *TP53*, the type of mutation and its biological function should be considered.

Keywords: *TP53*; mutation; breast cancer prognosis; DNA contact residue

The tumour-suppressor gene *TP53* (also known as p53) plays a key role in cell cycle regulation, gene transcription, genomic stability, DNA repair, senescence and apoptosis (reviewed in Haffner and Oren, 1995; Kinzler and Vogelstein, 1996; Velculescu and El-Deiry, 1996; Harris, 1996*a*). Inactivation of wild-type functions of *TP53* by either mutation of the gene, nuclear exclusion, interaction of its protein product with either cellular proteins (for example MDM2) or oncogene products of DNA tumour viruses can lead to cancer (Levine et al, 1991). Point mutation is the most common event and as this is often accompanied by deletion of the second allele, all wild-type *TP53* activity will be eliminated.

The structure–function relationship of the TP53 protein provided a basis for understanding how *TP53* mutations might inactivate its normal cell function. The central portion of TP53 consists of three loops involved in DNA binding (Cho et al, 1994). Most mutations are clustered within the core domain (residues 102–292) and mutations are particularly common in the four conserved regions located in this core domain (Cariello et al, 1994; Greenblatt et al, 1994; Harris, 1996*b*). In general, these mutations are missense (Cariello et al, 1994), resulting in a defective or conformationally altered non-functional protein (Harris, 1996*b*). Up to 20% of the mutations, however, have been reported outside exons 5–8, and these are predominantly of the 'null type' (Bergh et al, 1995; Hartmann et al, 1995). There are two functional classes of mutations, of which class I affects residues that directly contact DNA (for example hotspots Arg-248 and Arg-273) and of which class II affects residues that have a critical role in stabilizing the structural integrity of the domain (for example hotspot Arg-175) (Prives, 1994).

TP53 gene mutation is the most common single gene alteration in breast cancer and, depending on the method of detection, the frequencies of *TP53* mutations reported in invasive breast cancer range from 12% to 46% (Andersen and Børresen, 1995). The transitions of the major CpG dinucleotide hotspots at codons 175, 248 and 273 are the most prevalent (Cariello et al, 1994; Greenblatt et al, 1994; Hartmann et al, 1997). In general, *TP53* gene alterations have been associated with worse prognosis of breast cancer patients. However, various mutations can alter the TP53 protein distinctly, which may lead to different biological characteristics and tumorigenic potential (Cho et al, 1994; Friend, 1994). Analysis of these various mutations allows us to focus attention on the biological significance of particular mutations, which may abet selection of those residues that could be of predictive value in breast cancer.

In the present study on breast carcinoma samples, both type and location of *TP53* gene alterations were studied. The outcome, by function of affected codons and regions, was related with patient and tumour characteristics and with (relapse-free) survival.

Received 18 April 1997
Revised 23 September 1997
Accepted 1 October 1997

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PATIENTS AND METHODS

Patients and tumour samples

TP53 gene alterations were studied in a series of 222 female breast tumours. Of the patients, 177 were eligible for evaluation of relapse-free and overall survival according to the strict criteria described previously (Putten et al, 1996). The 45 patients who were excluded from the analyses of (relapse-free) survival involved 37 for whom tumour characteristics were unknown and no records were available and eight for whom follow-up was not detailed enough. Patients underwent surgical tumour removal (124 mastectomy, 53 lumpectomy) between 1978 and 1990. Radiotherapy on the breast or thoracic wall was given to 89 patients, of the axilla to 81 patients and of other lymph node areas (supraclavicular and/or parasternal) to 143 patients. The median age of these patients was 59 years (range, 28–82 years), 33% were premenopausal, 36% had no involved lymph nodes, and the majority of the patients had tumours ≤ 5 cm.

Of the node-positive patients, 39 patients (35%) received systemic adjuvant therapy (30 patients received CMF; seven patients tamoxifen and two patients a combination of hormonal therapy and chemotherapy). One node-negative patient received systemic adjuvant therapy. These and further details of the patients, with a medium follow-up of patients alive of 115 months (range, 47–183 months), are given in Table 1. One-hundred and eight patients (61%) experienced a relapse and 106 patients (60%) died during follow-up of this study.

Table 1 Patient characteristics and *TP53* gene alterations

Characteristics	Number of tumours with TP53 alterations	%
Patients with complete sequence (n = 214)	66 with mutations[a]	31
Patients eligible for the analysis of RFS and OS (n = 177)	53 with mutations	30
Nodal status[b]		
Node-negative (n = 64)	20 with mutations	31
Node-positive (n = 111)	32 with mutations	29
Tumour size[c]		
≤ 2 cm (n = 46)	15 with mutations	33
2–5 cm (n = 95)	25 with mutations	26
> 5 cm (n = 33)	12 with mutations	36
Adjuvant therapy		
Yes (n = 40)[d]	11 with mutations	31
No (n = 137)	42 with mutations	28
Relapse		
No (n = 69)	16 with mutations	23
Yes (n = 108)	37 with mutations	34
Survival		
Alive (n = 71)	18 with mutations	25
Dead (106)	35 with mutations	33

[a] In 66 tumour samples, 69 mutations were observed (see text). [b]Nodal status missing for two patients, one with a mutation. [c] Tumour size missing for three patients, one with a mutation. [d] Of the node-positive patients, 39 patients (35%) received systemic adjuvant therapy (see text). One node-negative patient received systemic adjuvant therapy.

DNA isolation and sequence analysis

Breast tumour specimens, stored in liquid nitrogen, were pulverized and homogenized in phosphate buffer according to the procedures recommended by the EORTC (EORTC Breast Cancer Cooperative Group, 1973). High-molecular-weight chromosomal DNA was isolated from an aliquot of the total tissue homogenate as described previously (Berns et al, 1992). Exons 5 through 8 of the *TP53* gene were analysed by polymerase chain reaction (PCR) driven single-strand conformation analysis (PCR-SSCA), as described previously (Berns et al, 1996). The mammary tumour cell line EVSA-T was included in the assay as a positive control for the neutral polymorphism at codon 213 (exon 6), which occurs in 3–10% of the normal population (Carbone et al, 1991). Because of possible errors that may accumulate in the initial PCR step, samples showing an altered electrophoretic mobility of single-stranded nucleic acids were analysed again with an independent PCR product. A third PCR product was sequenced (Ampli-Cycle sequencing kit, Perklin Elmer, Branchbury, NJ, USA) with 5-prime ^{33}P end-labelled primers. The DNA sequence was determined by separation of the terminated products on a 6% polyacrylamide gel containing 8 M urea, followed by autoradiography. The naturally occurring restriction sites of *HaeII*, *TaqI* and *MspI* were used to verify mutations in codons 175 (exon 5), 213 (A→G polymorphism in exon 6) and 248 (exon 7) respectively.

Luminometric immunoassay

The TP53 protein levels were measured in 151 breast tumour cytosols, which were available from the cytosol bank, using a quantitative luminometric immunoassay (LIA; AB Sangtec Medical, Bromma, Sweden), described previously by us (De Witte et al, 1996). In brief, the luminometric immunoassay (LIA) which detects both wild-type and mutant TP53 protein in a sandwich-type assay, is based on a combination of two monoclonal antibodies: 1801, as catching antibody, and DO1, as detecting antibody labelled with the chemiluminescent compound amino-butyl-ethyl-isoluminol. The immunoassay was performed by incubating either 100 µl of TP53 standard (range: 0–80 ng ml^{-1}), controls or tumour cytosols, as recommended by the supplier.

Statistical analysis

The associations between *TP53* mutations and other prognostic variables were examined using non-parametric tests: the Kruskall–Wallis test for ordered variables (menopausal status, grade) and the Spearman's rank correlation (r_s) for continuous variables (age, tumour size, nodal status). Two-sided P-values below 0.05 were considered significant. The likelihood ratio test in the univariate Cox regression model was used to test for differences and trend. The relapse-free and overall survival probabilities were calculated by the actuarial method of Kaplan and Meier (1958).

RESULTS

Analysis of *TP53* gene mutations in 222 breast tumours

Two-hundred and twenty-two female breast tumour specimens were studied. Altered migration patterns on SSCA, indicative of *TP53* gene mutations, were observed in 77 (35%) samples. The sequence data were successfully obtained on 214 tumours

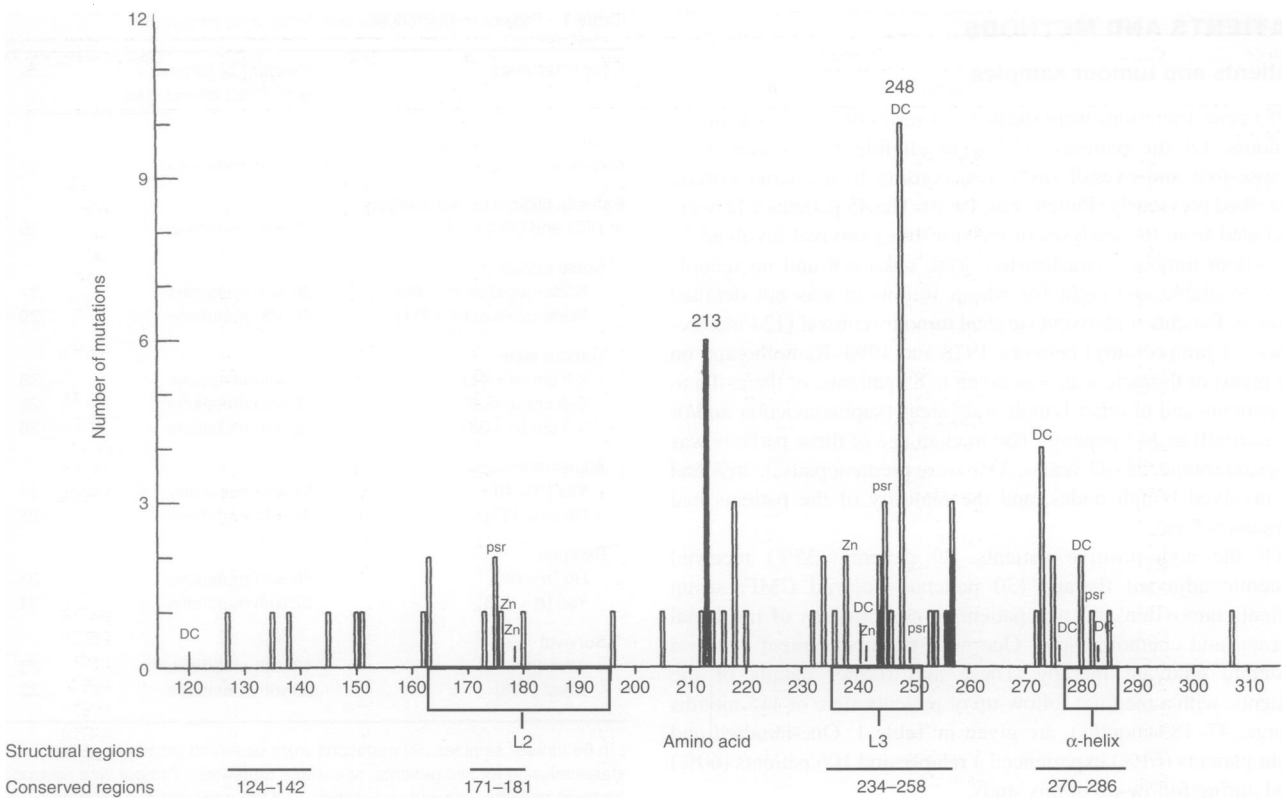

Figure 1 Mutation spectrum of *TP53* gene alterations in 222 breast tumours. The four *silent* mutations [at codons 244, 256, 257 ($n = 2$)] and the six cases with neutral *polymorphism* at codon 213 (exon 6) are shaded. Psr, protein stabilizing region; Zn, Zinc contact; DC, DNA contact. Exon 5, codon 126–186; exon 6, codon 187–224; exon 7, codon 225–261 and exon 8, codon 262–306

(see Table 1). Sixty-six tumour samples were successfully sequenced and 69 *TP53* gene mutations were found in these 66 tumour samples (see Table 1 and Figure 1). We observed 54 missense point mutations, comprising 78% of all mutations, and five mutations leading to a premature termination of the protein [one nonsense mutation (codon 196) and four deletions and/or insertions (codons 150, 216 and two times at codon 218, in exons 5 and 6)]. As expected, the majority (81%) of the mutations are transitions, and mutations of the amino acid arginine (Arg, $n = 20$) are the most prevalent (34%). These mutations were distributed over 37 distinct codons and resided especially in exon 7 (41%). In addition, we observed six cases of polymorphism of codon 213 (exon 6) and four silent mutations (codons 244, 256 and two times codon 257; all within exon 7).

Forty-one out of 59 mutations (69%) were restrained to the conserved regions, and 25 mutations (42%) were within the zinc-binding domains (regions L2 and L3; Figure 1). Moreover, our analysis identified mutations in three of the seven amino acids important in direct DNA binding (i.e. codons 248, 273 and 280), in total 16 mutations were observed (Figure 1).

Recently, a LIA became available for the measurement of TP53 protein levels in cytosolic extracts. This assay, which detects both wild-type and mutant TP53 protein, is based on the principle that mutated TP53 has a prolonged half-life and is thus accumulated in the cell. In a subset of 151 primary breast tumour samples, the LIA values were related with the outcome of mutation analysis. The median TP53 level of 4.2 ng mg^{-1} protein (range 0.0–176.0) in 46 tumours with missense mutations was higher (eightfold) than those levels measured in ten tumours with a silent mutation or in 91 tumours without a *TP53* gene alteration [median levels of 0.5 (range 0.0–11.9) or 0.4 (range 0.0–70.8 ng mg^{-1} protein) respectively]. The level in tumours with deletions/insertions, however, was also low, i.e. 0.1 ng mg^{-1} protein (range 0.0–0.16).

TP53 gene mutations related with tumour and patient characteristics and (relapse-free) survival of 177 patients

Of the 177 patients who were evaluable for analysis of relapse-free survival (RFS) and overall survival (OS), we observed 43 missense point mutations, four mutations leading to premature termination of the protein (one nonsense and three deletions and/or insertions) and six silent alterations in the primary tumours (Table 2). The frequency of *TP53* mutations in the primary tumours was not significantly related with tumour size or nodal status (Table 1) nor was there a relation with failure type (Table 2). The median RFS in the six patients with a mutation at codon 213 (neutral polymorphism) or a silent mutation (in exon 7) was shorter (median RFS = 11; range 7–107 months) than the RFS of the 47 patients with a mutation (median RFS = 34; range 3–160 months) and with the median RFS of 124 patients with wild-type *TP53* (median RFS = 48; range 2–183 months).

In an exploratory analysis the *TP53* gene mutations were stratified according to type of mutation, for example evolutionarily conserved regions, zinc-binding domains L2 (residues 163–195) and L3 (residues 236–251) of the protein, by residues that directly

Table 2 Patient and tumour characteristics and *TP53* gene mutations of patients with follow-up

Number	Codon[a]	Change[b]		Lia[c] (ng mg^{-1} P)	Age (years)	T/N[d]	DFS[e] (months)	Site of metastasis[f]
		Effect	Site					
Missense								
1	127	Ser→Pro		2.0	49	1/1	> 118	
2	135	Cys→Arg		5.8	70	4/1	4	SCCI
3	138	Ala→Val		4.2	67	2/12	8	META
4	145	Leu→Arg		n.d.	61	3/0	42	LRR
5	151	Pro→Ala		41.9	49	2/1	3	META
6	162	Ile→Thr		0.2	64	1/1	122	LRR
7	163	Tyr→Cys	L2	0.1	73	2/1	17	SCCI
8	173	Val→Ala	L2	1.2	62	1/1	> 100	
9	175	Arg→His	L2/psr	6.1	40	2/1	13	META
10	175	Arg→His	L2/psr	1.9	68	2/0	> 72	
11	176	Cys→Tyr	L2/Zn	4.8	50	1/1	60	META
12	180	Glu→Gly	L2	2.8	55	1/1	17	META
13	205	Tyr→Phe		11.7	57	2/1	34	SCCI
14	212	Phe→Leu		n.d.	42	1/0	36	META
15	218	Val→Glu		13.0	75	2/2	34	META
16	220	Tyr→Ser		4.1	54	2/0	> 122	
17	232	Ile→Ser		9.8	52	2/1	> 116	
18	234	Tyr→Cys		0.6	46	1/1	> 54	
19	234	Tyr→Cys		5.8	82	2/0	> 108	
20	236	Tyr→Cys	L3	0.1	72	2/x	26	META
21	238	Cys→Ser	L3/Zn	nd	62	2/0	30	META
22	238	Cys→Tyr	L3/Zn	0.4	44	1/1	111	LRR
23	244	Gly→Ser	L3	1.7	74	2/1	32	META
24	245	Gly→Ser	L3/psr	4.8	50	3/1	14	META
25	245	Gly→Ser	L3/psr	2.0	77	4/1	15	META
26	245	Gly→Ser	L3/psr	n.d.	47	3/1	18	META
27	246	Met→Val	L3	n.d.	52	1/0	> 113	
28	248	Arg→Gln	L3/DNA	176.0	72	3/1	4	META
29	248	Arg→Gln	L3/DNA	49.0	58	2/1	6	META
30	248	Arg→Gln	L3/DNA	26.3	70	3/0	12	META
31	248	Arg→Gln	L3/DNA	87.3	75	1/1	3	D-BRCA
32	248	Arg→Trp	L3/DNA	n.d.	51	2/0	46	META
33	248	Arg→Trp	L3/DNA	0.3	57	3/0	> 141	
34	254	Ile→Ser		n.d.	52	1/0	> 160	
35	257	Leu→Pro		1.1	61	1/1	50	D-BRCA
36	272	Val→Met		9.0	34	2/1	10	META
37	273	Arg→His	DNA	20.6	57	x/1	6	META
38	273	Arg→His	DNA	6.0	39	2/0	34	META
39	275	Cys→Tyr		12.7	53	2.0	> 100	
40	280	Arg→Thr	DNA	0.2	46	3/0	25	META
41	280	Arg→Thr	DNA	3.3	73	2/1	23	D-BRCA
42	282	Arg→Gly	psr	5.7	59	2/1	44	META
43	307	Gly→Asp		0.3	54	2/0	7	META
Deletion/insertion/nonsense								
44	150	Frameshift; 449–467*, 19bp del		0.2	61	2/0	45	META
45	196	Arg→stop	n	n.d.	38	1/0	26	MAM2
46	218	Frameshift; 653–657, 5bp d/i 4bp		0.0	54	4/1	21	META
47	218	Frameshift; 654 ins 5bp		n.d.	75	1/0	42	D-BRCA
Silent								
48	213	Arg→Arg		11.9	42	4/1	7	SCCI
49	213	Arg→Arg		0.3	77	3/1	9	META
50	213	Arg→Arg		0.7	44	1/1	11	META
51	213	Arg→Arg		0.4	73	2/2	17	META
52	213	Arg→Arg		0.7	68	2/0	> 107	
53	244	Gly→Gly	L3	0.0	53	2/0	11	SCCI

[a]Double mutants, number 8 has an additional frameshift at codon 216 (d/i) and number 25 has an additional mutation at codon 253 (Thr→Ala). [b]Site, L2/L3, loop 2 or loop 3; psr, protein stabilizing region; Zn, zinc-binding domain; DNA, direct contact with DNA. Del/d, deletion; ins/i, insertion; n, nonsense; *position of base. [c]LIA, luminometric immunoassay (see Patients and methods). [d]T tumour size (1, ≤ 2 cm; 2, 2–5 cm; 3/4, > 5 cm); N, nodal status (0, node negative; 1, node positive; x, unknown). [e]DFS, disease-free survival in months. [f]Site of relapse: SCCI, distant nodes; META, distant metastasis; LRR, local regional relapse; D-BRCA, dead without evidence of recurrent disease. Only patients with complete tumour characteristics available were included in this table.

Table 3 Cox univariate (relapse-free) survival analysis of 177 breast cancer patients, stratified by TP53 mutation type

Patient group	n	Five-year RFS			Five-year OS		
		RHR	95% CL	P-value	RHR	95% CL	P-value
WT	124	1			1		
All mutations	53	1.7	1.1–2.6	0.01	1.8	1.1–2.8	0.02
WT	124	1			1		
Non-conserved	19	1.6	0.96–2.6	n.s.	1.8	0.95–3.6	n.s.
Conserved region	34	1.9	1.1–3.5	0.03	1.7	1.0–3.0	0.05
WT	124	1			1		
Outside loops	33	1.6	0.95–2.6	n.s.	1.7	0.97–2.9	n.s.
Inside loops, L2 and L3	20	2.0	1.1–3.6	0.02	1.9	1.0–3.8	0.05
WT	124	1			1		
Non-direct DNA	43	1.6	1.0–2.5	0.05	1.5	0.93–2.6	n.s.
Direct DNA contact	10	2.7	1.2–5.8	0.02	3.4	1.5–7.6	0.002

WT, wild-type *TP53*. RHR, relative hazard rate. 95% CL, 95% confidence interval. For the explanation of codons involved in silent mutation, conserved regions, loops L2 and L3 and direct DNA contact see Figure 1 and Results.

contact DNA (Table 3) and by occurrence of silent mutations. Cox univariate analysis, at 5 years, showed that both the relapse-free and overall survival of patients with *TP53* mutations in either the conserved regions, within L2 and L3, or the codons that directly contact DNA is significantly worse (relative hazard rate (RHR) for RFS: 1.9, 2.0, 2.7 and 4.1 respectively; see Table 3) than those patients without mutations (WT, wild type), or with mutations in codons that are not conserved or are outside L2 and L3.

Actuarial relapse-free and overall survival curves stratified by *TP53* status revealed that the *TP53* gene mutation was significantly ($P = 0.02$) associated with an increased risk of relapse with a RHR of 1.6 [95% confidence interval (CI): 1.1–2.4], but not with the rate of death [$P = 0.07$; RHR = 1.5 (CI = 0.97–2.2), shown in Figure 2A and B]. When stratified as the function of type of *TP53* mutation, only mutations at residues that directly contact DNA retained significance (RFS: $P < 0.05$ and OS: $P < 0.02$, respectively, shown in Figure 2C and D).

In a multivariate Cox regression analysis, which included age, menopausal status, lymph node status, tumour size, steroid hormone receptor status and c-*MYC* amplification, *TP53* gene mutation independently predicts poor RFS and OS with relative hazard rates of 1.8 ($P < 0.01$) and 1.6 ($P = 0.04$) respectively.

DISCUSSION

The present prevalence of *TP53* mutations is in the range of the overall rate of *TP53* mutations of 15–71% (mean 25%; examined in 1452 breast tumour samples worldwide, by SSCA of exon 5–8, and reviewed by Hartmann et al, 1997). Evaluation of only exons 5 through 8 may, however, underestimate the overall prevalence of *TP53* mutations by 10–20% (Bergh et al, 1995; Hartmann et al, 1995). The mutations observed in this study resided mainly (41%) in exon 7. This high incidence is in accordance with the study of Anderson et al (1993) who also described a predominance of mutations in exon 7. The mutational 'hotspots' described in breast cancer (Greenblatt et al, 1994), i.e. codons Arg-175, Arg-248 and Arg-273 accounted for 3%, 17% and 7%, respectively, in this study. This differs from the prevalence of 6%, 7%, and 7%, respectively, summarized in the database established by Cariello et al (1994) on *TP53* gene mutations ($n = 365$) in human primary breast tumours. Interestingly, Børresen et al (1995) also showed a relatively lower frequency (3%) of mutations in codon 175 and this could imply a lower prevalence of codon 175 mutations in European women. When stratifying the mutations according to the evolutionarily conserved regions or functional domains, we observed that 41 out of 59 mutations (69%) were restrained to the conserved regions, which is in concordance with the percentage of mutations (73%) in this region summarized by Cariello et al (1994). Bergh et al (1995), who studied mainly tumours from node-negative patients, observed a smaller number of mutations in these conserved regions, i.e. 30 out of 65 (46%). In our smaller number of node-negative patients 13 out of 21 mutations were in the conserved regions (62%). Twenty-five mutations (42%) in this study were within the structural regions L2 and L3, which is in accordance with data observed by Børresen et al (1995).

As expected, the median TP53 level of tumours with missense mutations was higher (eightfold) than levels of tumours with a silent mutation or without a gene alteration, but also with four tumours with deletions/insertions. This last result shows that low levels of TP53 measured by LIA, and probably also by immunohistochemistry, are not always indicative of a normal *TP53* gene status and the data should be interpreted with care.

An unexpected finding was that the median RFS in the six patients with a neutral polymorphism at codon 213 or a silent mutation was shorter (median RFS = 11 months) than the RFS of the 47 patients with a mutation (median RFS = 34 months) and with the median RFS of 124 patients with wild-type *TP53* (median RFS = 48 months). Shiao et al (1995) also reported that two patients with the A→G transition in codon 213 experienced a very poor survival, but more studies on this silent third base mutation in Arg-213 will be needed to clarify these observations. We also observed that the different *TP53* gene mutations in primary breast cancer could be related to differences in (relapse-free) survival, for example our study shows that mutations that directly contact DNA are related with a poor (relapse-free) survival of breast cancer patients. This does not agree with data from Børresen et al (1995). These authors reported that, in a multicentre study, patients with TP53 mutations in the zinc-binding domains (L2 and L3) had a poor prognosis. One possible explanation for the observed difference could be the shorter follow-up time in their study (median: 40 months vs 115 months in this study). In conclusion, the analysis of type and location of *TP53* alterations can be used to select residues

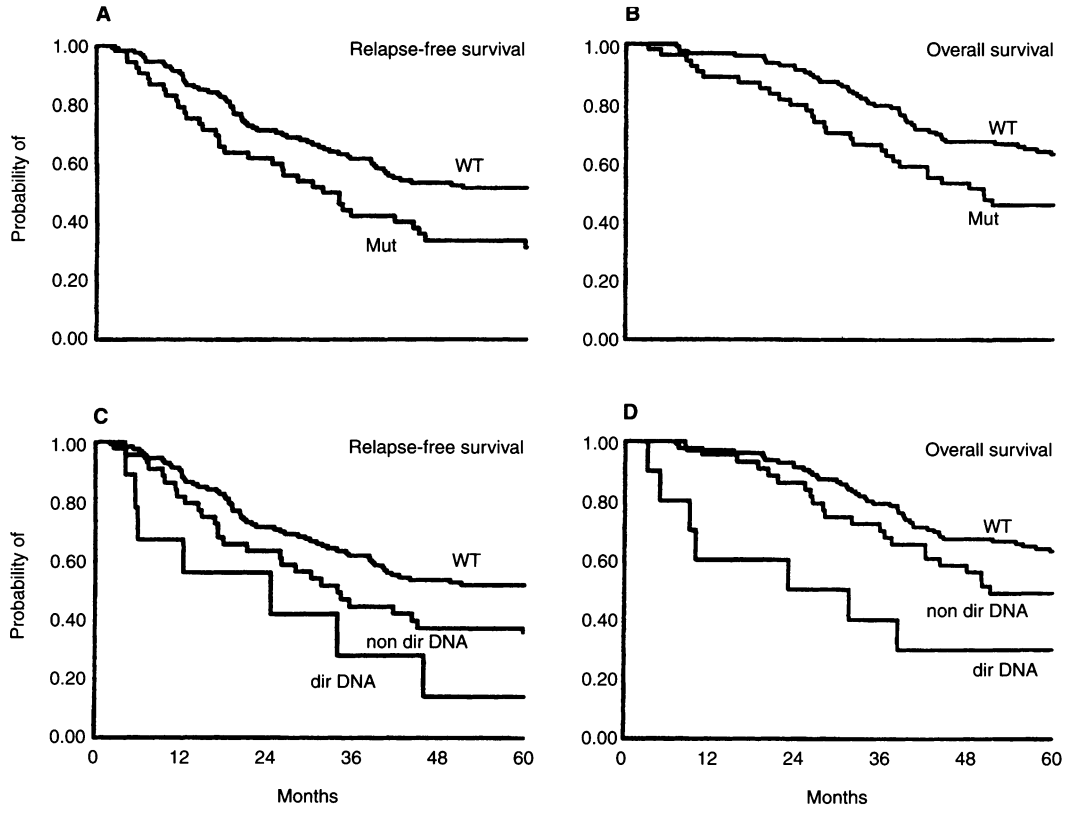

Figure 2 Actuarial relapse-free (**A** and **C**) and overall (**B** and **D**) survival as a function of *TP53* gene mutation. **A** and **B** WT, wild-type (n = 124 patients); mut, mutated in exons 5 to 8 (n = 53 patients). **C** and **D** non dir DNA, no direct contact of the affected codon with the DNA strand (n = 43 and ten patients respectively).

that could be of prognostic value. If these data are confirmed by other investigators in larger studies, the use of artificially or naturally created restriction sites or allele-specific oligo techniques can facilitate the detection of these DNA contact residues.

ACKNOWLEDGEMENTS

The authors wish to thank Henk Portengen, Doorlene van Tienoven and Drs Hans de Witte for excellent contribution to this project, and Drs Marion Meijer-van Gelder for clinical follow-up data. This work was supported through grants of the Dutch Cancer Society (DDHK 92-4/96–1234).

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