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ORIGINAL ARTICLE

A comparison between p53 accumulation determined by immunohistochemistry and *TP53* mutations as prognostic variables in tumours from breast cancer patients

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Abstract

Background. p53 accumulation and *TP53* mutations are known prognostic markers for breast cancer. To clarify their interrelationship and the importance of different *TP53* mutation types, these markers were investigated in tumours from 630 patients with breast cancer. *Materials and methods*. Tumour sections were stained for p53 and scored based on staining intensity and percentages of invasive tumour cells with nuclear staining. *TP53* mutations were identified by sequencing. Patient cohorts were from the DBCG (Danish Breast Cancer Cooperative Group) protocols DBCG82 and DBCG89. *Results*. *TP53* was mutated in 29% of the patients. The disease-specific survival (DSS) at 15 years of follow-up for patients with missense mutations directly involved in DNA or zinc binding was $21 \pm 8\%$. Patients with the remaining missense mutations within the structural/conserved domains and patients with null mutations had a DSS of $36 \pm 6\%$ and $31 \pm 17\%$, respectively. For patients without *TP53* mutations and patients with mutations affecting amino acids outside these domains, the 15 year DSS was $51 \pm 3\%$ and $71 \pm 10\%$, respectively. p53 accumulation was successfully scored in 567 patients and categorized into three groups. Tumours with no p53 expression had a high frequency of null mutations (37% compared to 10% in the whole cohort), and tumours with high p53 expression contained 82% of the missense mutations inside structural/conserved domains including those directly involved in DNA or zinc binding. *Conclusion*. The clinical outcome for breast cancer patients is significantly different for different *TP53* mutation types, but further functional studies are required to clarify the exact role of these mutation types. Most of the mutations that lead to mutant p53 protein accumulation can be detected by immunohistochemistry but the specificity is low. Samples showing lack of detectable p53 protein should be considered as an indication of a possible null mutation.

Several studies have evaluated the prognostic role of *TP53* mutations and p53 accumulation (determined by immunohistochemistry) in breast cancer. Some of the largest studies are performed by Olivier et al. [1] on *TP53* mutations in 1 794 patients with primary breast cancer and by Silvestrini et al. [2] on p53 accumulation in 1 400 node-negative breast cancer patients. Although these and other studies have confirmed that *TP53* mutation, and to a lesser extent p53 accumulation, are independent prognostic markers, there are still a number of open questions regarding the role of p53 in breast cancer [3,4]. Recent studies have shown that different *TP53* mutations can influence tumour phenotype and clinical outcome differently, but the biological mechanism behind this is only beginning to be elucidated. How this affects *TP53* as a clinical useful marker, is still unclear [3]. Similarly, recent knowledge has questioned the mechanisms behind the accumulation of mutant p53 protein [4].

In the present study, the role of *TP53* mutations is explored in further details and correlated with p53 accumulation as determined by immunohistochemistry. Two patient cohorts are investigated: a consecutive cohort of 401 patients treated according to the DBCG89 guidelines [5,6], and a subgroup of

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229 high-risk patients from the DBCG82 b&c trials [7,8].

Materials and methods

Patients and tumour samples

Tumour material was collected from two series. The first cohort consisted of 401 consecutive patients with early breast cancer diagnosed from January 1990 to 1994 and fulfilled the following criteria: having primary unilateral breast carcinoma with no clinical evidence of metastasis; availability of complete clinical, histopathological and biological information; having no other malignancies; having received radical surgical therapy according to the DBCG89 criteria (for details, see Offersen et al. [5,6]).

The second cohort consisted of 229 patients from the DBCG82 b&c studies diagnosed from 1982 to 1989 and fulfilled the following criteria: having high-risk breast cancer (defined as either positive lymph nodes and/or tumour size larger than 5 cm and/or invasion of tumour to surrounding skin or pectoral fascia); availability of complete clinical, histopathological and biological information (from frozen tissue); having no other malignancies; having received total mastectomy and a partial axillary dissection; having been randomized to CMF ± radiotherapy (DBCG82b, pre-menopausal women) or Tamoxifen 1 year ± radiotherapy (DBCG82c, post-menopausal women). For details, see Overgaard et al. [7–9] and Nielsen et al. [10].

TP53 evaluation for gene mutations by DNA sequencing

In the DBCG89 cohort, DNA was extracted from the pellet left over from estrogen receptor analysis [11]. The entire coding region and all exon/intron boundaries of *TP53* were analyzed by DGGE (denaturing gradient gel electrophoresis) [12]. Mutant heteroduplex and homoduplex bands were excised and reamplified. Sequencing of PCR products was performed either with ^{33}P-end-labeled primers using ThermoPrime Cycle Sequencing Kit (Amersham/GE Healthcare) or with the BigDye DyeTerminator Cycle Sequencing Kit and analyzed on an ABI PRISM™ 310 (Applied Biosystems). Only excised bands were sequenced.

In the DBCG82 cohort, DNA was extracted from frozen tumour samples using chloroform/phenol extraction followed by ethanol precipitation (Nuclear Acid Extractor 340A; Applied Biosystems) according to standard procedures. The entire coding region and all exon/intron boundaries of *TP53* were analyzed by direct sequencing using an ABI PRISM™ 3700 DNA Sequencer (Applied Biosystems).

TP53 mutation types and domain structure of p53 are defined as described by Alsner et al. [12].

p53 evaluation by immunohistochemistry

In the DBCG89 cohort, immunohistochemical staining of the p53 antigen was performed on 4 μm sections from the paraffin-embedded tumours. Antigen retrieval was carried out by microwaving the tissue sections in T-EG buffer (Tris 10 mM + EGTA 0.5 mM, pH 9.0) twice for 5 minutes followed by incubation with a monoclonal anti-p53 antibody (p-53 protein DO-7 code M 7001, Dako, Glostrup, Denmark) diluted 1:600 in Antibody Diluent (Dako, Glostrup, Denmark) overnight at 4°C. Primary antibody was detected by EnVision™+/HRP Mouse code K4001 (Dako, Glostrup, Denmark) and visualized by Dako Liquid DAB+. Counter staining was done with Mayers hematoxylin. Nuclear p53 staining intensity was defined as low or high. Tumours were semi-quantitatively categorized into three groups as follows: $<10\%$ (regardless of intensity), $>50\%$ (high intensity only), and 10–49% (regardless of intensity) plus $>50\%$ (low intensity) of nuclei staining positive.

In the DBCG82 cohort, tissue microarrays (TMAs) [13] were used for immunohistochemical staining of the p53 antigen [14]. Antigen retrieval was carried out by microwaving the TMA sections in T-EG buffer (Tris 10 mM + EGTA 0.5 mM, pH 9.0) twice for 5 minutes followed by incubation with a monoclonal anti-p53 antibody (p-53 protein DO-7 code K4001, Dako, Glostrup, Denmark) diluted 1:600 in Antibody Diluent (Dako, Glostrup, Denmark) overnight at 4°C. Primary antibody was detected by EnVision™+/HRP Mouse code K4001 (Dako, Glostrup, Denmark) and visualized with Novared (Vektor, SK-4800). Counter staining was done with Mayers hematoxylin. Nuclear p53 staining intensity was scored on a scale from 0 to 3 and percentages invasive tumour cells with nuclear staining were recorded. Tumours were categorized into three groups as follows: 0% (intensity 0), $>50\%$ (intensity 3), and 1–49% (regardless of intensity) plus $>50\%$ (intensity 1–2) of nuclei staining positive.

Statistical analysis

A χ^2 test was used to investigate the correlations among variables and known clinicopathological parameters. The probability of treatment failure were calculated for the endpoint of disease-specific survival [5] according to the Kaplan-Meier method

and the differences among the survival curves were calculated with a log-rank test with a test for trend. Follow-up time was calculated using the date of primary operation as initial value.

Results

p53 accumulation and TP53 mutations, correlations with clinicopathological parameters

The analysis for *TP53* mutations was performed in 630 tumours and *TP53* was mutated in 180 cases (29%). *TP53* mutations were categorized into four groups as described previously [12]. Missense mutations affecting amino acids directly involved in DNA or zinc binding accounted for 16% of all mutations. The remaining missense mutations within the structural/conserved domains, null mutations, and mutations affecting amino acids outside structural/conserved domains accounted for 34%, 37%, and 13% of all mutations, respectively. p53 accumulation measured by immunohistochemistry was successfully scored in 567 patients. As described in further details below, p53 accumulation was categorized into three groups: tumours with no expression (9%), high expression (46%) or intermediate expression (45%).

Table I shows the correlations between p53 accumulation, *TP53* mutations and classical prognostic markers in breast cancer in the whole cohort and when separated in the DBCG89 cohort and the DBCG82 cohort. The DBCG82 cohort consisted of high-risk patients. Compared to the DBCG89 cohort, there were significant differences in patient age and menopausal status, tumour size, histological malignancy grade, and lymph node status. p53 accumulation correlated significantly with histological malignancy grade, oestrogen receptor status, and lymph node status. *TP53* mutations correlated significantly with patient age, histological malignancy grade, and oestrogen receptor status.

TP53 mutation patterns and prognosis

Of the 401 DBCG89 patients presented here, 243 were included in a previous study presented with 5 year follow-up on the heterogeneity in clinical phenotype of different *TP53* mutation types [12]. Data on the extended cohort presented with 10 year follow-up are shown in Figure 1 (left). In full agreement with the first report, patients with tumours containing missense mutations directly involved in DNA or zinc binding had the worst outcome with a disease-specific survival (DSS) of $25 \pm 10\%$. Patients with null mutations and patients with the remaining missense mutations within the structural/conserved domains had an intermediate DSS of $49 \pm 8\%$ and $41 \pm 8\%$, respectively. Patients without *TP53* mutations had a DSS of $67 \pm 3\%$. Finally, a small group of patients with mutations affecting amino acids outside these domains had a DSS of $86 \pm 10\%$. In the study by Offersen et al. [5] comparing a number of biological variables as prognostic markers in the same patient cohort, *TP53* mutations were reduced to two groups. As depicted in Figure 1, "*TP53* wt" contains patients without mutations and patients with mutations affecting amino acids outside structural/conserved domains. "*TP53* mutation" contains the remaining three groups.

A similar association between *TP53* mutation type and outcome was observed in 229 DBCG82 patients presented with 15 year follow-up in Figure 1 (right). Patients with missense mutations directly involved in DNA or zinc binding had again the worst outcome with a DSS of $11 \pm 10\%$. Patients with null mutations and patients with the remaining missense mutations within the structural/conserved domains had an intermediate DSS of $25 \pm 8\%$ and $25 \pm 10\%$, respectively. Patients without *TP53* mutations had a DSS of $44 \pm 4\%$. Finally, patients with mutations affecting amino acids outside these domains had a DSS of $36 \pm 20\%$.

Figure 2 illustrates the associations between *TP53* mutation type and outcome in the combined cohort. For patients without *TP53* mutations and patients with mutations affecting amino acids outside these domains, the 15 year DSS was $51 \pm 3\%$ and $71 \pm 10\%$, respectively. Patients with missense mutations directly involved in DNA or zinc binding, null mutations, and patients with the remaining missense mutations within the structural/conserved domains had a DSS of $21 \pm 8\%$, $31 \pm 17\%$ and $36 \pm 6\%$, respectively.

p53 accumulation and prognosis

The 401 DBCG89 patients were originally semi-quantitatively scored in four groups ($<10\%$, 10–49%, 50–79%, $\geq 80\%$) based on the percentage of positive tumour nuclei in single sections. The staining intensity was scored as 'low' or 'high'. Initial analysis showed that the $<10\%$ group was associated with the worst outcome, whereas the 10–49% group had the best outcome (data not shown). Based on the associations between *TP53* mutations and p53 accumulation (see below), the final analysis presented in Figure 1 (left) includes three groups: $<10\%$ (regardless of intensity); $>50\%$ (high intensity only); and 10–49% (regardless of intensity) plus $>50\%$ (low intensity) of nuclei staining positive. In the DBCG89 cohort, these groups were associated

Table I. p53 accumulation and *TP53* mutation in relation to clinicopathological parameters in 630 patients diagnosed with breast cancer.

				p53 accumulation (IHC)†			*TP53* mutation				
	DBCG89 cohort	DBCG82 cohort	All	No staining	Intermediate	High staining	WT	Miss. outside	Null	Miss. inside	Direct contact
Variable, N (%)											
All	401 (100)	229 (100)	630 (100)	52 (9)	257 (45)	258 (46)	450 (71)	23 (4)	67 (11)	61 (10)	29 (5)
Age (years)											
<40	21 (5)	19 (8)	40 (100)	3 (9)	13 (38)	18 (53)	25 (63)	0 (0)	8 (20)	5 (13)	2 (5)
40–49	97 (24)	57 (25)	154 (100)	12 (8)	64 (45)	66 (46)	110 (71)	5 (3)	12 (8)	21 (14)	6 (4)
50–59	118 (29)	67 (29)	185 (100)	13 (8)	73 (45)	78 (48)	125 (68)	9 (5)	18 (10)	15 (8)	18 (10)
60–69	85 (21)	86 (38)	171 (100)	17 (12)	73 (50)	57 (39)	127 (74)	9 (5)	18 (11)	15 (9)	2 (1)
≥70	80 (20)		80 (100)	7 (9)	34 (43)	39 (49)	63 (79)	0 (0)	11 (14)	5 (6)	1 (1)
	P<0.0001				*P=0.8*				*P=0.004*		
Menopausal status											
Pre-	129 (32)	103 (45)	232 (100)	19 (9)	92 (45)	95 (46)	161 (69)	6 (3)	27 (12)	26 (11)	12 (5)
Post-	272 (68)	126 (55)	398 (100)	33 (9)	165 (46)	163 (45)	289 (73)	17 (4)	40 (10)	35 (9)	17 (4)
	P=0.001				*P=1.0*				*P=0.6*		
Tumour size (mm)¶											
≤20	150 (37)	69 (30)	219 (100)	13 (6)	103 (50)	90 (44)	167 (76)	11 (5)	17 (8)	19 (9)	5 (2)
21–50	218 (54)	128 (56)	346 (100)	36 (12)	125 (41)	146 (48)	236 (68)	12 (3)	44 (13)	34 (10)	20 (6)
>50	33 (8)	30 (13)	63 (100)	3 (6)	28 (53)	22 (42)	46 (73)	0 (0)	5 (8)	8 (13)	4 (6)
	P=0.03				*P=0.08*				*P=0.1*		
Histological malignancy grade‡											
Duct. I	76 (19)	38 (17)	114 (100)	8 (8)	55 (55)	37 (37)	105 (92)	1 (1)	1 (1)	4 (4)	3 (3)
Duct. II	138 (34)	103 (46)	241 (100)	17 (8)	109 (51)	88 (41)	171 (71)	10 (4)	25 (10)	23 (10)	12 (5)
Duct. III	131 (32)	43 (19)	174 (100)	18 (11)	43 (26)	107 (64)	89 (51)	9 (5)	33 (19)	31 (18)	12 (7)
	P=0.001				*P<0.0001*				*P<0.0001*		
Nonduct.	56 (14)	41 (18)	97 (100)	8 (10)	50 (60)	26 (31)	81 (84)	3 (3)	8 (8)	3 (3)	2 (2)
Oestrogen receptor status§											
Positive	286 (71)	129 (72)	415 (100)	31 (8)	212 (53)	160 (40)	339 (82)	10 (2)	24 (6)	29 (7)	13 (3)
Negative	115 (29)	51 (28)	166 (100)	21 (13)	44 (27)	98 (60)	75 (45)	12 (7)	36 (22)	28 (17)	15 (9)
	P=0.9				*P<0.0001*				*P<0.0001*		
Lymph node status											
None	190 (47)	14 (6)	204 (100)	18 (9)	78 (39)	104 (52)	155 (76)	10 (5)	18 (9)	15 (7)	6 (3)
1–3	107 (27)	118 (52)	225 (100)	23 (12)	86 (46)	78 (42)	165 (73)	7 (3)	23 (10)	23 (10)	7 (3)
>3	104 (26)	97 (42)	201 (100)	11 (6)	93 (52)	76 (42)	130 (65)	6 (3)	26 (13)	23 (11)	16 (8)
	P<0.0001				*P=0.04*				*P=0.08*		

Data missing on 2¶, 4‡, 49§, and 63† patients, respectively.

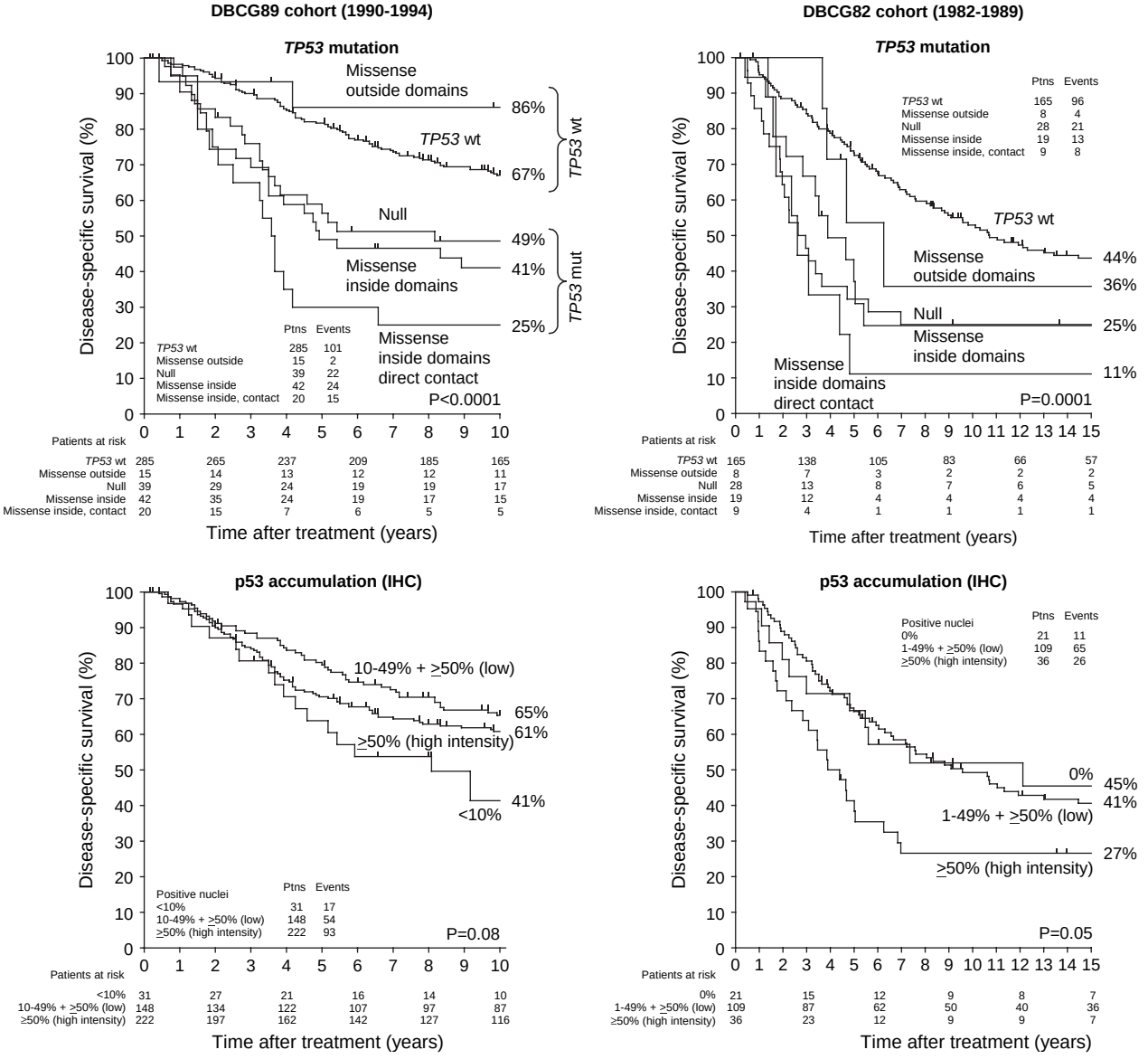

Figure 1. Disease-specific survival as a function of *TP53* mutation types (top) and p53 accumulation (bottom) in breast cancer patient cohorts from DBCG89 (left) and DBCG82 (right).

with a 15-year DSS of 41±9%, 61±3%, and 65±4%, respectively.

Of the 229 DBCG82 patients, 166 were analysed for p53 accumulation using tissue microarrays. The percentage of positive tumour nuclei was recorded and the intensity was scored on a scale from 0–3. Only samples with 0% positive nuclei were scored with an intensity of 0. For the analysis in Figure 1 (right), the samples were then categorized into three groups: 0% (intensity 0); >50% (intensity 3 only); and 10–49% (regardless of intensity) plus >50% (intensity 1–2) of nuclei staining positive. In the DBCG82 cohort, these groups were associated with a 15-year DSS of 45±11%, 41±5%, and 27±8%, respectively.

Figure 2 illustrates the associations between p53 accumulation and outcome in the combined cohort. Patients without staining, intermediate staining, and high staining had a 15-year DSS of 41±7%, 50±4% and 44±8%, respectively.

TP53 mutation patterns and p53 accumulation

Table II presents the associations between *TP53* mutations and p53 accumulation in the two breast cancer cohorts and in the combined group. A high frequency of null mutations leading to lack of protein expression was observed in the two groups with very low percentage of positive tumour nuclei. Overall, 10% of the patients had a null mutation. In the

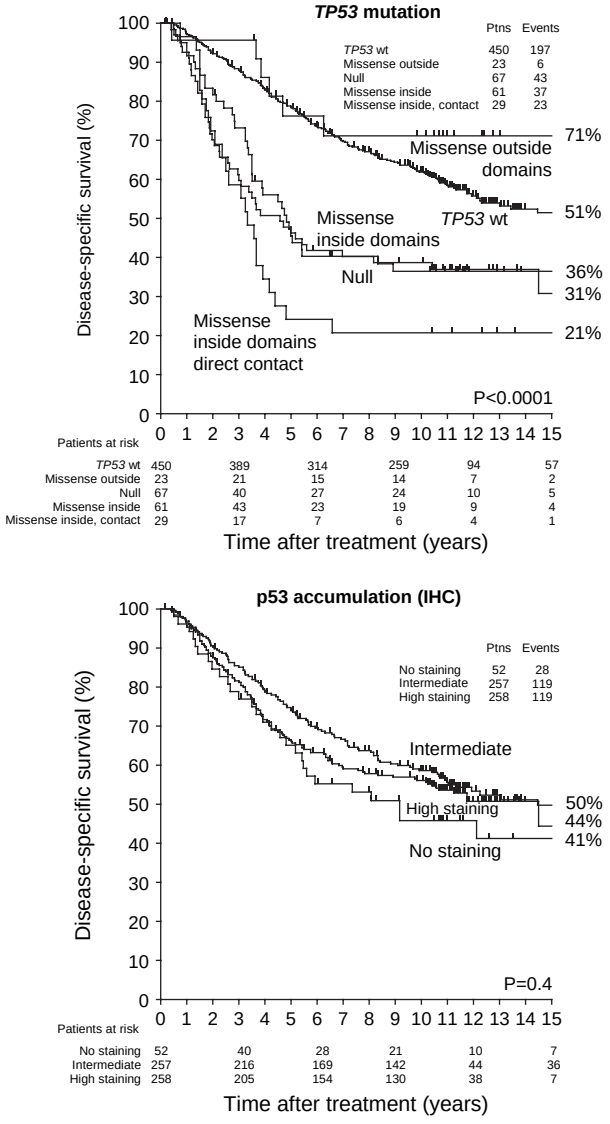

Figure 2. Disease-specific survival as a function of *TP53* mutation types (top) and p53 accumulation (bottom) in breast cancer patients.

group of very low percentage positive cells (no staining) that number was increased to 37%. In contrast, the majority of missense mutations inside structural/conserved domains including those directly involved in DNA or zinc binding were found in patients with >50% positive tumour nuclei (high intensity staining). In the combined cohort, 82% (69 of 84 total) of these missense mutations were associated with the >50% (high intensity) group.

Discussion

The present study including a total of 630 patients demonstrate that different *TP53* mutation types are associated with different clinical phenotypes. The distribution and frequency of different mutation types were similar in the two cohorts consisting of 401 consecutive DBCG89 patients and 229 DBCG82 patients, respectively. As some of the patient and tumour characteristics were significantly different, the cohorts were analysed both separately and combined. The same associations between *TP53* mutation type and outcome were observed in both cohorts. Missense mutations within structural/conserved domains (excluding missense mutations directly involved in DNA or zinc binding) and null mutations were associated with similar but significantly worse outcome compared to patients without mutations. Missense mutations affecting amino acids directly involved in DNA or zinc binding were associated with the worst outcome. Finally, missense mutations not affecting structural/conserved domains were associated with an outcome similar to patients without mutations.

The associations between *TP53* mutation types and outcome presented here are in agreement with the largest study published on *TP53* mutation types and associations with clinical phenotypes, a multicentre analysis by Olivier et al. on 1 794 patients screened for mutations within exons 5–8 [1]. Thus, Olivier et al. also found poor outcomes in patients with missense mutations within structural/conserved domains and null mutations. Although only exons 5–8 were analysed in the multi-centre analysis, missense mutations not affecting structural/conserved domains were also associated with an outcome more similar to patients without mutations. Missense mutations directly involved in DNA or zinc binding also seemed to be associated with a very poor outcome.

Even though there seems to be clear associations between clinical outcome and the different *TP53* mutation types described here and by Olivier et al. [1], there is still a long way before the impact of *TP53* mutations on breast cancer prognosis and outcome is well understood. Some of the open questions include a better understanding of the functional consequences of mutations (loss of transactivation activities, dominant-negative effects, gain-of-function activities), the effect of *TP53* haplotypes and loss of alleles, the effect on protein/protein interactions, tissue specificity, and the effect of genetic variation or alteration in other genes in the p53 pathway [3,4,15]. In a study on different biological factors and their potential as prognostic factors in 408 consecutive DBCG89 patients, *TP53* mutation status (recorded in the 401 patients further described here) was therefore reduced to either 'wt' or 'mutant' [5].

TP53 mutation status can also be assessed by immunohistochemical (IHC) detection of mutant p53 accumulation, but this association is neither very solid nor fully understood [4]. Besides the

Table II. Correlation between p53 accumulation (% positive nuclei) and *TP53* mutation types.

	All	All			DBCG89 cohort				DBCG82 cohort			
	All	No staining	Intermediate	High staining	All	<10%	10–49%	>50%, intense	All	0%	1–49%	>50%, intense
All	567 (100)	52 (100)	257 (100)	258 (100)	401 (100)	31 (100)	148 (100)	222 (100)	166 (100)	21 (100)	109 (100)	36 (100)
TP53 mutation												
WT	404 (71)	31 (60)	217 (84)	156 (60)	285 (71)	20 (65)	127 (86)	138 (62)	119 (72)	11 (52)	90 (83)	18 (50)
Miss. Outside	21 (4)	1 (2)	3 (1)	17 (7)	15 (4)	1 (3)	1 (1)	13 (6)	6 (4)	0 (0)	2 (2)	4 (11)
Null	58 (10)	19 (37)	23 (9)	16 (6)	39 (10)	10 (32)	14 (9)	15 (7)	19 (11)	9 (43)	9 (8)	1 (3)
Miss. inside	56 (10)	0 (0)	11 (4)	45 (17)	42 (10)	0 (0)	6 (4)	36 (16)	14 (8)	0 (0)	5 (5)	9 (25)
Direct contact	28 (5)	1 (2)	3 (1)	24 (9)	20 (5)	0 (0)	0 (0)	20 (9)	8 (5)	1 (5)	3 (3)	4 (11)

biological uncertainty, the stability of the p53 protein is also affected by different fixation methods [16], and the use of antigen retrieval will lower the detection threshold for p53 staining [17]. Thus, no consensus exists regarding how to score p53 accumulation. One of the problems recognized in the early reports comparing *TP53* mutation and p53 accumulation was the observation of gene mutations in samples that were negative by IHC [18]. In the present report, the percentage of positive tumour nuclei was recorded together with an estimate of staining intensity. As expected, the majority of missense mutations were identified in the group of patients with a high percentage of intensely stained tumour nuclei, but the specificity was low. Finally, it was also possible to define a small group of patients with undetectable levels of p53, in which a high proportion (37%) had a null mutation. Thus, future studies on p53 accumulation should not only consider intensely stained samples as indicators of *TP53* mutations, but also include samples without any indications of p53 protein.

In conclusion, this study demonstrates that clinical outcome for breast cancer patients are associated with different *TP53* mutation types. However, further functional studies are required to clarify the exact role of these mutation types. The study also confirms that many of the mutations will lead to mutant p53 protein accumulation detectable by immunohistochemistry, but that samples showing lack of detectable p53 protein should be considered as an indication of a possible null mutation.

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