Influence of mutation type on prognostic and predictive values of *TP53* status in primary breast cancer patients

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Abstract. High rates of mutation in the *TP53* tumor suppressor gene have been found in many human cancers, including breast tumors, making p53 one of the most studied proteins in oncology. However, the prognostic and predictive value of alterations in this gene remains ambiguous. To analyze the clinical value of somatic *TP53* mutations, we collected clinical and molecular data on 210 women with primary breast cancer. We found significant associations of p53 mutations with tumor grade, metastasis, molecular subtype, Her2 status and inverse correlations with estrogen and progesterone receptor status. Cox proportional hazard analysis confirmed a strong prognostic value of p53 mutation for overall survival rate and highlighted significant interactions with lymph node involvement and tumor size. In relation to treatment options, *TP53* mutations were associated with poor response to anthracyclines and radiotherapy. Categorization of *TP53* mutations according to their type and location revealed that patients with nonsense mutation have the poorest prognosis in comparison with wild-type cases and other types of mutations in this gene. Classification of *TP53* mutations with respect to the degree of disturbance of protein structure showed association of disruptive mutations with poorer patients' outcome in contrast to wild-type and non-disruptive mutations. In conclusion, the present study confirms p53 as a potential predictive and prognostic factor in oncology practice and highlights the growing evidence that distinct types of mutations have different clinical impacts.

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Introduction

Breast cancer is the most frequent invasive tumor diagnosed in women, showing growing incidence primarily in advanced countries. In the Czech Republic the average incidence of breast cancer in the population of women during the period 2005-2010 was 65.9 cases per 100,000, with an average mortality of 16.4. In contrast, in the years 1985-1990 the average incidence was 42.9, with average mortality 19.7 (data retrieved from The Czech National Cancer Registry) (1). As indicated, although the incidence has increased by more than one-third in the respective time periods, mainly due to improved screening programs and diagnostics, conversely, mortality has decreased due to earlier detection and more effective treatment. A similar trend is also observed in other European countries; for example, Cancer Research UK reports for the same time periods show an increase in the incidence rate by ~36%, however mortality rate decreased by almost one third (2). Although the success in breast cancer screening, treatment and familial cancer diagnosis highlight the effort of the international cancer research community, the cause of a large majority of breast cancers is unknown and the prospects for curing invasive or chemoresistant breast cancers remains relatively low.

Breast cancer has several important markers with worldwide clinical utility: the estrogen receptor, progesterone receptor and ErbB2 status. These three gene products play an important role in driving breast cancer development and anti-estrogen or anti-ErbB2 therapies are proving to be useful in treating many breast tumors. Nevertheless, successful breast cancer treatment is complicated by the fact that significant chemoresistance and metastasis can obstruct the current treatments. Thus, although therapeutic success is highly related to expression of hormonal receptors, it remains difficult to predict those individuals who will not respond to conventional therapy and/or will show metastatic progression. Significant efforts have been made to link the common oncogenic or tumor suppressor mutations in cancers to predict patient prognosis and/or tumor responses. One of the most studied genes in oncology for the last 35 years is *TP53*. The product of this gene, p53 protein has been named 'guardian of the genome'. This protein mediates the response to various forms of genotoxic stress, and is involved in cell cycle regulation (3), DNA repair (4), senescence and

apoptosis (3), and substantially prevents cells from malignant transformation. Its role as a tumor suppressor is reflected in its high rate of mutations in human cancers and there have been many studies evaluating the prognostic role of p53 for the outcome of different types of cancer (5). It was shown that genetic alterations leading to p53 inactivation cause abnormal pathological behavior (6) and even that mutations in p53 by dominant negative manner suppress the normal functions of the wild-type (wt) protein (7).

Considerable data indicate that mutant p53 proteins not only lose their tumor suppressive functions, but also gain new oncogenic properties that are independent of wt p53. This 'gain of function' received its first support when transfection of mutant p53 into *TP53* null cells was shown to enhance their ability to form tumors in mice. Then, a number of studies demonstrated a wide range of oncogenic properties for p53 mutants and provided key mechanistic insights to explain these phenomena (8).

p53 is composed of three domains: i) the trans-activation domain [amino acids (AAs) 1-95], ii) the DNA binding domain (DBD) (AA 102-292), and iii) the oligomerization domain (AA 300-393) (9). The mutation rate of p53 in breast cancer is ~30% (10) and the most common p53 mutations for breast cancer, similar to other types of cancer, are missense mutations in the DBD (11). A closer look at missense mutations shows they can be separated on DNA-binding motif (DBM) mutations involving: i) AAs from L2 and L3 loops (AAs 164-194 and 237-250) and ii) mutations in LSH (loop-sheet-helix) motif (AAs 119-135 and 272-287), and non-DBM mutations including other AAs (12). Missense mutations in DBM were shown to be associated with poorer prognosis (12-15) in comparison to missense mutations in non-DBM regions. Another method to sub-divide *TP53* mutations that have clinical value is the classification into 'nondisruptive' and 'disruptive' mutations, which include either truncating mutations or produce dissimilar AAs in the L2 or L3 loops (13).

In the present study, we investigated the role of *TP53* mutations for hospital-based study in breast cancer and we correlated them with standard clinicopathological characteristics. Furthermore, we compared specific groups of mutations in *TP53* with clinicopathological variables and patient outcome. We found that the prognostic significance of *TP53* mutation status in breast cancer is dependent on the specific mutation type, and that nonsense mutations in contrast to mutations in the DNA-binding domain are the most important for estimating the prognosis of breast cancer patients.

Materials and methods

Clinical samples and processing. Our hospital-based study included 210 primary breast tumors with determined p53 status from patients treated at the Masaryk Memorial Cancer Institute (MMCI). The median age of patients was 59 years (mean 59 with standard deviation 12). Samples were collected within 20 min of surgical removal and immediately evaluated by a pathologist according to standardized hospital protocol. Tissue pieces of ~3x3x8 mm were cut from redundant tumor tissue after standard surgicopathological processing and stored in RNA later for 3-5 days at 4˚C and then frozen at -80˚C. Subsequently, total cellular RNA was extracted using RNeasy Isolation kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were controlled by UV spectrophotometry (A_{260}:A_{280}>2.0; A_{260}:A_{230}>1.8). RNA integrity was checked using Agilent 2100 Bioanalyzer and only non-degraded RNA characterized by RNA integrity number (RIN) ≥7 with no DNA contamination signs was processed. *TP53* mRNA was then amplified using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen), as previously described (14). PCR products were purified by MinElute™ PCR Purification kit (Qiagen) and sequenced using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit on an ABI 3130 genetic analyzer (Applied Biosystems).

The main clinicopathological variables including tumor type, grade and nuclear grade, were determined according to Elston and Ellis (15); estrogen receptor (ER), progesterone receptor (PR) and Her2/neu status were extracted from pathological records obtained from the MMCI database. The tumors were also categorized according to IHC subtype with respect to ER, PR, Her2 and Ki67-expression. Her2 positivity was primarily determined by immunohistochemistry and in equivocal cases Her2 status was analyzed for gene amplification by FISH.

Patients and evaluation and treatment regimens. The present study was approved by the local ethics committee in the MMCI and informed consent was obtained from each patient. Clinical data including patient follow-up were evaluated by an oncologist from the hospital's patient records. The initial staging consisted of chest X-rays, radionuclide bone scans, abdominal ultrasound or computed tomography, bilateral mammography and determination of carcinoembryonic antigen (CEA) and CA15-3 (Mucin 1). All patients lacked evidence of distant metastases at the time of surgery. Primary treatment followed approximately up to one month post-surgery (partial/total mastectomy) and was administered with respect to clinicopathological parameters. With respect to other clinical indications, hormonal therapy was applied either in monotherapy or in combination with other treatment modalities. Adjuvant chemotherapy was administered with respect to expected response to systematic treatment according to standard protocols at MMCI and mainly consisted of a combination of taxanes, anthracyclines and cyclophosphamide. In total, 156 patients received radiation following completion of chemotherapy.

Classification of TP53 mutations. Missense mutations represent single base-pair substitutions that result in the translation of a different AA in that position in the context of the full-length protein. Nonsense mutations are created by single base substitutions (stop codon) or small insertions and deletions leading to a frameshift and creating a truncated protein. *TP53* mutations can also be categorized according to other criteria such as the location of the mutation and the predicted AA alterations. Following a study by Poeta *et al* (13), we also classified mutations as disruptive and nondisruptive, where disruptive mutations are DNA sequence alterations that i) introduce a stop codon or ii) occur within the L2, or L3 binding domains and replace an AA from one polarity/charge category with an AA from another category. Conversely,

nondisruptive mutation is any mutation occurring outside the L2 or L3 binding domain (except stop mutations) or mutations within the L2 or L3 binding domains that result in replacement of an AA with another from the same polarity/charge category.

Statistical analysis. Statistical analysis was performed using IBM SPSS Statistics 20.0. χ^2 test was used to evaluate associations of molecular and clinicopathological variables with *TP53* status. Both disease-free survival (DFS) and overall survival (OS) curves were generated by the Kaplan-Meier method. DFS was defined as the time from the date of surgery to the date of death or relapse of disease. OS was defined as the time from diagnosis to death or last record. Patients who had not died or who were lost to follow-up were censored when they were last known to be alive. The log-rank test was used for comparison of statistical significance for differences among the survival curves for both DFS and OS. A value of $p<0.05$ was evaluated as statistically significant. Multivariate Cox regression models with backward selection were used to test the prognostic effect of *TP53* status after adjusting for other important prognostic variables.

Results

Relationship between p53 status and clinicopathological variables. Mutational status of p53 was determined by direct sequencing in a group of 210 breast cancer patients. In parallel all clinicopathological variables available from patient records were statistically evaluated in relation to p53 status. The frequency of p53 mutations in our cohort of breast cancer patients was 25.7% which is consistent with other reports (10). Cases with mutations that did not change the resulting AA (silent mutations) were counted as wt p53. Table I shows the associations of clinicopathological parameters with p53 status. We found that mutant p53 was significantly associated with tumor grade ($p<0.001$), metastasis ($p=0.016$), molecular subtype ($p<0.001$), Her2 status ($p=0.004$) and inversely correlated with ER- and PR-positivity ($p<0.001$ for both). In parallel, the proportions of *TP53* missense, nonsense and silent mutations were determined and calculated with respect to clinicopathological variables (Table I). No significant changes were observed in statistical associations of clinicopathological parameters and type of p53 mutations.

Mutations in p53 and prognosis. To study the impact of p53 mutational status on patient outcome, DFS and OS were determined (Fig. 1). We found that the presence of mutations resulting in change in p53 protein significantly decreased both DFS and OS ($p=0.015$ and $p<0.001$, respectively). Grade, tumor size, lymph node involvement, metastasis and Her2 status also showed significant impact on both DFS and OS (data not shown).

Multivariate analysis including the most important patient characteristics (histological type, grade, pT, pN, ER, PR, Her2, p53 status), identified only p53 mutational status, pT ($p=0.013$) and pN as statistically significant factors for DFS (Table II), indicating that the presence of p53 mutation has independent prognostic value. Only p53 mutational status and pT showed statistical significance in relation to OS (Table II).

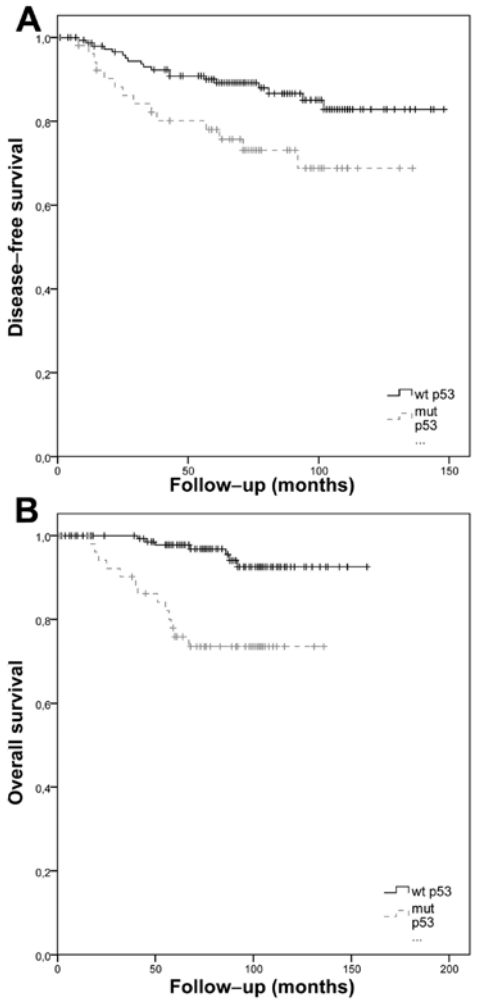

Figure 1. Disease-free and overall survival according to p53 mutational status. Kaplan-Meier plots of wt p53 vs. mut p53 for (A) disease-free survival ($p=0.015$) and (B) overall survival ($p<0.001$). wt p53 (wild-type p53), mut p53 (mutant p53).

Clinical utility of p53 status. Retrospective analysis of the impact of p53 mutations on patient response to first round adjuvant therapy revealed significantly better DFS of patients with wt p53 ($p=0.046$; Fig. 2A). On the other hand, p53 mutations had no effect on DFS in patients who did not receive adjuvant treatment ($p=0.235$; Fig. 2B). Notably, the presence of p53 mutations showed significant association with poorer OS for both treated and untreated patients (Fig. 2C and D).

The predictive values of p53 status were also retrospectively analyzed in relation to individual specific treatment options represented by anthracyclines, taxanes, tamoxifen, trastuzumab and radiotherapy. Better outcome was found in wt p53 patients treated with anthracyclines ($p=0.060$) and patients who underwent radiotherapy ($p=0.002$) compared to patients with p53 mutation (Fig. 3).

The effect of different types of TP53 mutations. Since our data clearly demonstrated the impact of *TP53* mutations on prognosis of breast cancer patients and their treatment response, we focused only on cases with mutation in *TP53* gene to categorize mutations according to type and location. In this group of 63 tumors, 63.5% contained missense mutations, 20.6% nonsense

Table I. *TP53* mutations in relation to clinicopathological parameters.

Parameters	N	Wild-type p53	Mutant p53	P-value	Missense mutation	Nonsense mutation	Silent mutation	Wild-type	P-value
Tumor histology				0.079					0.229
Ductal NOS	155	110	45		37	8	8	102	
Other	12	9	3		1	2	0	9	
Lobular	29	26	3		1	2	1	25	
Medullary	5	2	3		2	1	0	2	
Tubular	3	3	0		0	0	0	3	
Total	204	150	54		41	13	9	141	
Tumor grade				<0.001					<0.001
G1	69	66	3		2	1	2	64	
G2	42	37	5		2	3	1	36	
G3	98	52	46		37	9	7	45	
Total	209	155	54		41	13	10	145	
Tumor size				0.468					0.616
pT1	126	97	29		24	5	6	91	
pT2	67	47	20		14	6	4	43	
pT3	15	10	5		3	2	0	10	
Total	208	154	54		41	13	10	144	
Nodal status				0.994					0.861
pN0	85	63	22		18	4	5	58	
pN1	120	89	31		23	8	5	84	
Total	205	152	53		41	12	10	142	
Metastasis				0.016					0.017
No	174	135	39		32	7	8	127	
Yes	36	21	15		9	6	2	19	
Total	210	156	54		41	13	10	146	
Duplicity				0.544					0.349
0	178	135	43		33	10	9	126	
1	17	11	6		3	3	0	11	
2	13	9	4		4	0	1	8	
Total	208	155	53		40	13	10	145	
ER status				<0.001					<0.001
ER^-	45	19	26		21	5	3	16	
ER^+	164	136	28		20	8	7	129	
Total	209	155	54		41	13	10	145	
PR status				<0.001					<0.001
PR^-	56	29	27		22	5	4	25	
PR^+	151	124	27		19	8	6	118	
Total	207	153	54		41	13	10	143	
Her2 status				0.004					0.004
$Her2^-$	161	126	35		24	11	7	119	
$Her2^+$	44	25	19		17	2	3	22	
Total	205	151	54		41	13	10	141	
IHC based subtype				<0.001					<0.001
ER^- $Her2^+$	14	5	9		8	1	1	4	
ER^+ $Her2^-$ Ki67[a] low	63	59	4		2	2	3	56	
ER^+ $Her2^-$ Ki67 not low	52	37	15		10	5	2	35	
ER^+ $Her2^+$	30	20	10		9	1	2	18	
Triple-negative	27	12	15		11	4	2	10	
Total	186	133	53		40	13	10	123	

[a]Ki67 threshold was 15%.

Table II. Multivariate Cox proportional hazards models of disease-free survival and overall survival.

DFS	HR (95% CI)	P-value	OS	HR (95% CI)	P-value
p53 protein status			p53 protein status		
Wild-type p53	1		Wild-type p53	1	
Mutant p53	2.453 (1.219-4.935)	0.012	Mutant p53	5.378 (2.138-13.524)	0.000
Tumor size (pT)			Tumor size (pT)		
pT1	1		pT1	1	
pT2	1.890 (0.887-4.029)	0.099	pT2	2.947 (1.067-8.135)	0.037
pT3	4.755 (1.655-13.658)	0.004	pT3	11.238 (3.094-40.813)	0.000
Nodal status					
Negative	1				
Positive	2.890 (1.165-7.166)	0.022			

CI, confidence interval; HR, hazard ratio; DFS, disease-free survival; OS, overall survival.

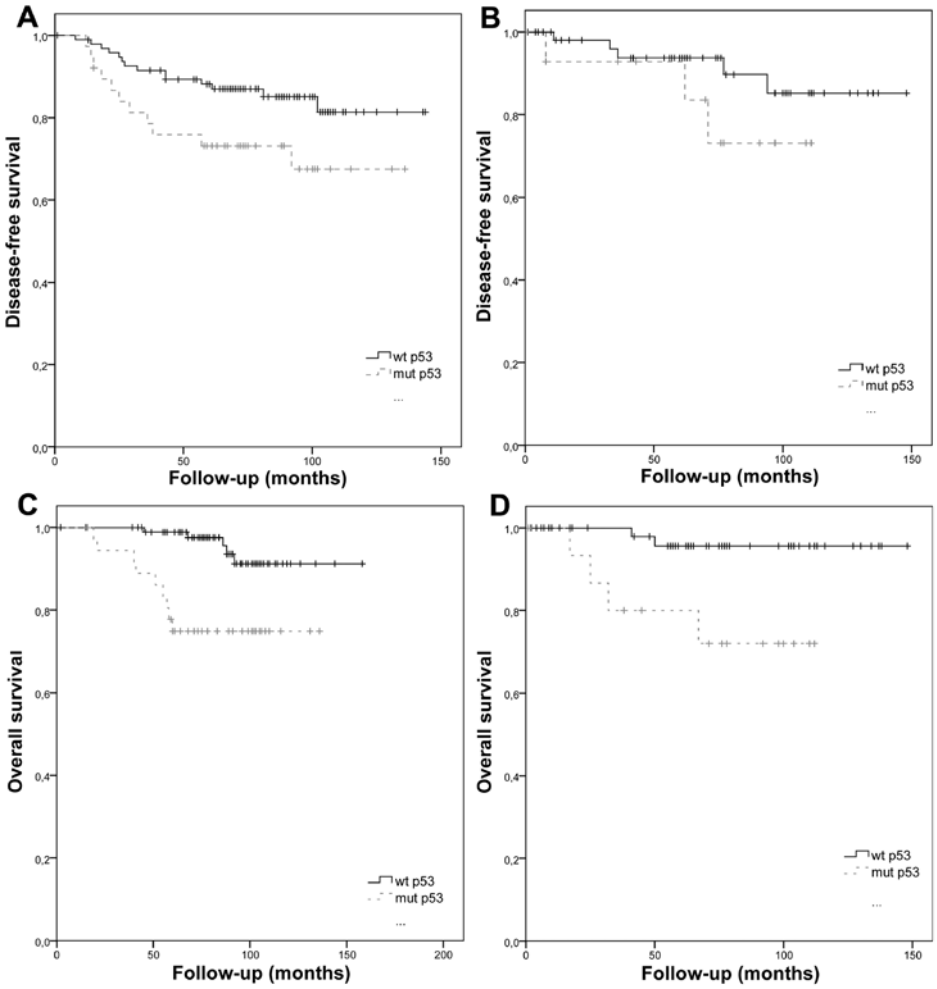

Figure 2. Disease-free and overall survival according to adjuvant treatment with respect to the presence of *TP53*; Kaplan-Meier plots of: (A) disease-free survival of patients who received adjuvant treatment in relation to wt p53 vs. mut p53 (p=0.046); (B) disease-free survival of patients who did not receive adjuvant treatment with respect to wt p53 vs. mut p53 (p=0.235); (C) overall survival of patients who received adjuvant treatment for wt p53 vs. mut p53 (p=0.001); (D) overall survival of patients who did not received adjuvant treatment with respect to wt p53 vs. mut p53 (p=0.008).

mutations and 15.9% silent mutations. Kaplan-Meier analysis clearly showed that patients with nonsense mutation have the poorest prognosis in comparison with other types of mutants, as well as wt cases (Fig. 4A and B). No significant differences in either DFS and OS were found when comparing missense mutations to DBM (L2/L3 and LSH) with missense mutations

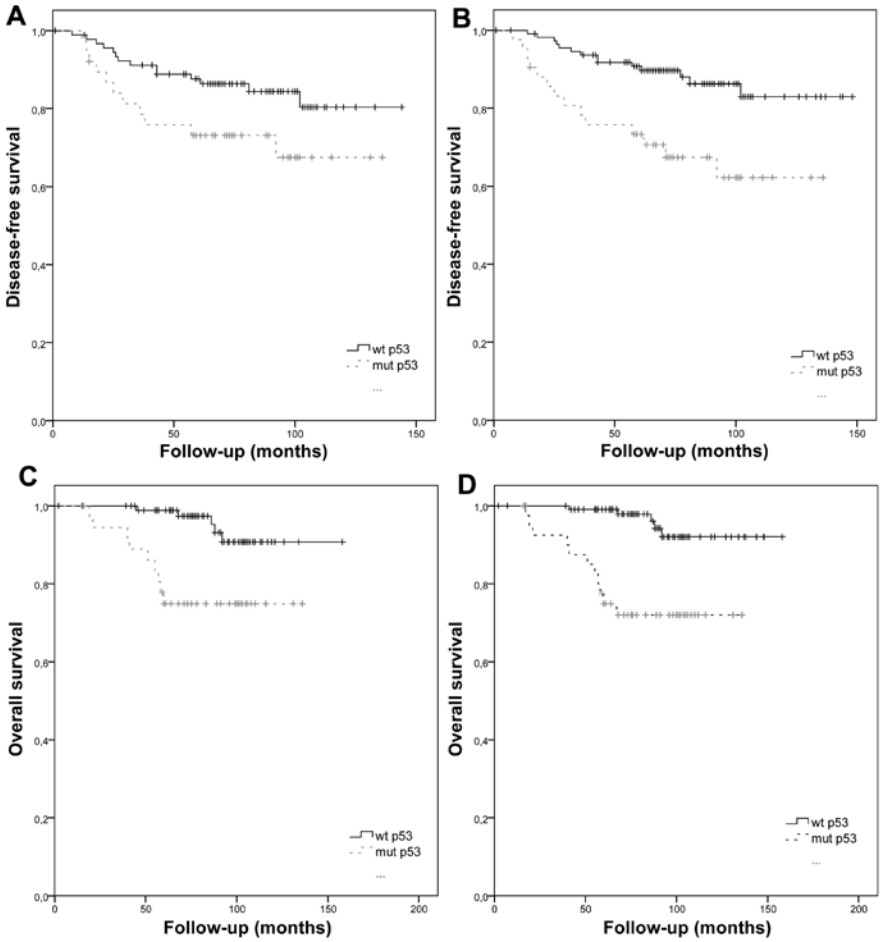

Figure 3. Disease-free and overall survival in response to anthracycline treatment and radiotherapy with respect to the presence of *TP53*. (A) Disease-free survival in response to anthracycline treatment for wt p53 vs. mut p53 (p=0.060); (B) disease-free survival of patients who underwent radiotherapy for wt p53 vs. mut p53 (p=0.002); (C) overall survival of patients who received anthracycline treatment with respect to wt p53 vs. mut p53 (p=0.002); (D) overall survival of patients who underwent radiotherapy for wt p53 vs. mut p53 (p<0.001).

outside of the DBM (Fig. 4C and D). Patients with missense *TP53* mutations in DBM showed significantly poorer OS compared to breast cancer patients bearing wt *TP53* (p=0.016), while patients with missense mutations outside of p53 DBM showed only marginal significance (p=0.077) (Fig. 4D).

Mutations were also classified into two groups, disruptive and nondisruptive, according to the degree of disturbance of protein structure predicted from the crystal structure of the p53-DNA complexes as described previously in patients with squamous-cell carcinoma of the head and neck (13). The comparison of disruptive and nondisruptive p53 mutations showed no statistically significant impact on patient DFS as well as OS (Fig. 5). On the other hand, in comparison with wt p53, only disruptive mutations in case of DFS (p=0.015) and both disruptive and nondisruptive p53 mutations for OS (p=0.009 and p<0.001) showed significantly poorer outcome (Fig. 5).

Discussion

Breast cancer represents the most common female malignancy, responsible for over 400,000 deaths yearly worldwide. This is a disease with a complex, heterogeneous genetic and biochemical background and no single genomic or metabolic condition may be considered as decisive for its formation and progression. However, a few key factors can be pointed out including *TP53* tumor suppressor gene. Mutant p53 is known to affect multiple oncogenic processes during breast tumorigenesis including tumor formation and development, growth and metastasis. Due to loss of tumor suppressor function and pro-oncogenic properties of mutant p53, many studies focused on analysis of *TP53* mutations as a prognostic and/or predictive biomarker (8). Mutations in the *TP53* gene were found to be more frequent in high-grade, large-size, node-positive breast tumors that typically lack functional ER and PR (12). In accordance to these findings, recent analysis of ~2,000 breast tumors found the lowest frequency of *TP53* mutations in luminal subtype A ~5%, followed by subtypes luminal B ~13%, Her2 ~22% and basal-like ~34% (16). The present study is compatible with those data (Table I).

To date, only limited information is available on the value of mutant p53 in breast cancer. Since p53 mutations occur mostly in ER- and PR-negative tumors, where chemotherapy usually combined with radiotherapy is applied, it is reasonable to study the impact of p53 status on these treatment regimens. In agreement with our data, several reports demonstrated that breast carcinomas expressing mutant p53 show lower sensitivity to treatment by anthracyclines (10,17,18). On the other

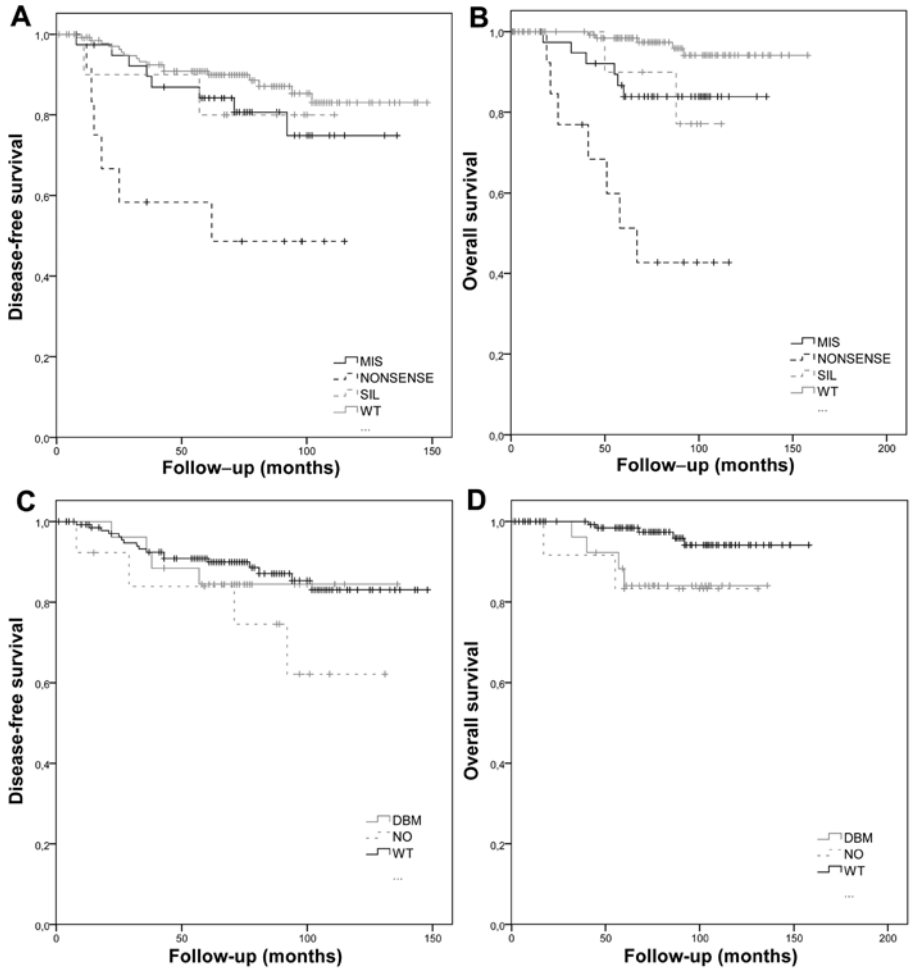

Figure 4. Disease-free and overall survival according to types of mutations in *TP53*. (A) Disease-free survival according to types of mutations in *TP53* (NONSENSE vs. MIS p=0.021; NONSENSE vs. SIL p=0.153; NONSENSE vs. WT, p<0.001); (B) overall survival according to types of mutations in *TP53* (NONSENSE vs. MIS p=0.003; NONSENSE vs. SIL p=0.062; NONSENSE vs. WT p<0.001); (C) disease-free survival according to types of mutations in *TP53* (DBM vs. NO, p=0.301; DBM vs. WT p=0.692; and NO vs. WT, p=0.080); (D) overall survival according to types of mutations in *TP53* (DBM vs. NO, p=0.907; DBM vs. WT, p=0.016; NO vs. WT, p=0.077). NONSENSE (nonsense mutation), MIS (missense mutation), SIL (silent mutation), WT (wild-type), DBM (missense mutation in DNA-binding motif), NO (missense mutation outside DBM).

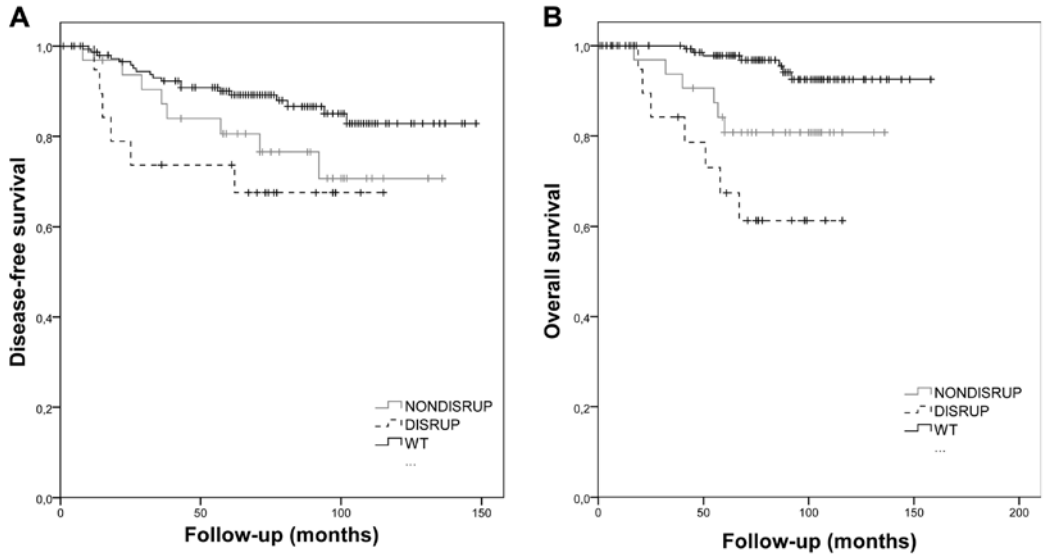

Figure 5. Disease-free and overall survival according to types of mutations in *TP53*. Kaplan-Meier estimation of (A) disease-free survival according to types of mutations in *TP53* (NONDISRUP vs. DISRUP, p=0.473; NONDISRUP vs. WT p=0.103; DISRUP vs. WT, p=0.015); (B) overall survival according to types of mutations in *TP53* (NONDISRUP vs. DISRUP, p=0.137; NONDISRUP vs. WT, p=0.009; DISRUP vs. WT, p<0.001). WT (wild-type), DISRUP (disruptive mutation), NONDISRUP (nondisruptive mutation).

hand, treatment by taxanes with respect to p53 status does not exhibit convincing data. Despite promising preliminary data (19,20), a recent large phase-3 clinical trial has shown similar sensitivity to taxanes in breast tumors bearing both wt and mutant p53 (21).

Increasingly more studies are focused on the localization and/or type of *TP53* mutations and their impact on patient prognosis. Due to the function of p53 as a transcriptional factor, many studies focus on missense mutations located in DBM (chiefly in the L2/L3 loops) showing association with a poorer outcome compared to mutations outside these motifs (17,22,23). Similarly in a large cohort of unselected breast tumors, Olivier *et al* found that missense mutations in DBM are associated with poorer survival than missense mutations outside DBM (12). Notably, they also found that truncating (nonsense) mutations compared to missense mutations in DBM have fairly similar prognostic value. However, retrospective study on the prognosis and predictive values of *TP53* somatic mutations in the BIG 02-98 randomized phase III trial, led by the same group, concluded that i) missense mutations are not associated with poor survival, ii) discrimination of missense mutations according to location inside DBM and outside DBM does not significantly affect patient outcome, and iii) only truncated (nonsense) mutations may serve as an independent predictor of poor DFS and OS (24). Our findings are consistent with their results showing significantly poorer effect of nonsense p53 mutations on patient outcome in comparison with both all *TP53* missense mutations and missense mutations located in the DBM only. Our results are also consistent with a study by Lai *et al* showing significant association of nonsense mutations with breast cancer-specific mortality (HR with 95% CI: 9.43, 1.29-69.12) (25). Our data is also indirectly supported by Geisler *et al* showing that mutations in DBM are associated with poorer prognosis; nevertheless, approximately half of the mutations classified in this manner are represented by truncating mutations (17,22). In contrast, categorization of p53 mutations according to the location and the predicted amino acid alterations showed no significant difference in impact on patient survival between disruptive and nondisruptive p53 mutations.

In conclusion, our results in general confirm a negative prognostic value of p53 mutations. However, the limitations of assessing the predictive value of a molecular marker in treatment regimens that combine drugs with different modes of action regarding this marker should be considered. With this in mind, our study demonstrates that clinical outcome for breast cancer patients is associated with different *TP53* mutation types and indicates potential utilization of *TP53* mutational status to predict response to treatment by anthracyclines and radiotherapy. However, further functional studies are required to clarify the impact of particular p53 mutations on patient outcome and response to specific therapy.

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