

# Intronic polymorphisms in TP53 indicate lymph node metastasis in breast cancer

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**Abstract.** Recent studies have suggested that genetic polymorphisms in the *TP53* pathway influence tumour formation, progression and response to therapy. We analysed the three most common *TP53* gene polymorphisms as potential genetic markers to predict the development and prognosis of breast cancer. The incidence of R72P, PIN3 Ins 16bp and PIN6 G13494A polymorphisms was determined in a cohort of 117 breast cancer tissues and 108 control specimens by PCR-RFLP. No significant difference was observed in the polymorphism variants in breast cancer specimens compared to controls. Furthermore, no statistically significant association of these polymorphisms with the outcome of the patients was observed. On the other hand we found positive correlation of lymph node metastases with both PIN3 Ins 16bp and PIN6 G13494A polymorphisms. The association of intronic *TP53* variants with an aggressive breast cancer phenotype may represent a useful predictive biomarker, particularly in patients of clinical stage I with low or intermediate risk.

## Introduction

Breast cancer is the most commonly diagnosed cancer and a predominate cause of death from malignant neoplasms in Czech female population. Although a substantial proportion of breast cancer cases are explained by well-established risk factors (i.e., later age at first pregnancy, nulliparity and first-degree family history of breast cancer), the reason for the observed worldwide increase in breast cancer incidences is still largely unknown. The molecular biology approaches in a population-based study will provide better mechanistic insights into breast cancer aetiology, prognosis and treatment.

The *TP53* gene represents one of the most studied anti-oncogenes in tumour biology. In response to stress signals, p53 protein is activated and directs stress-specific transcriptional response programs, leading to i) cell cycle arrest, ii) induction of cell senescence or iii) cellular apoptosis (1,2). *TP53* is the most commonly mutated gene found in human cancer (3,4) and selected mutations have already been correlated to specific clinical phenotypes (5). It is therefore feasible that the existence of natural variants of *TP53* is linked to the development of specific diseases and they could represent predictive markers for preventive and early intervention strategies. Natural genetic variants of *TP53* appear also as good resources to study inter-individual differences in cancer risk and therapeutic response.

A number of polymorphisms have been identified in the *TP53* gene (6). Most of these polymorphisms are single-nucleotide polymorphisms (SNPs) affecting a single base and localised within either introns or exons of *TP53*. Among the polymorphisms found in the coding regions of *TP53*, only two alter the amino-acid sequence of the protein, proline (P) to serine (S) at residue 47 and arginine (R) to proline (P) at residue 72. The codon 72 SNP results in a non-conservative change of an arginine (R72) to a proline (P72) that results in a structural change of the protein (7,8) while the polymorphism P47S was identified by Felley-Bosco *et al* (9) as very rare and undetectable in Caucasians. The frequency of 72 SNP in the population varies from the equator to higher latitudes, suggesting a selection pressure upon these two forms of p53 protein (10). Moreover, several lines of evidence suggest that this polymorphism can play a role in apoptosis and cancer formation in humans (11-13).

The IARC *TP53* Mutation Database describes 29 common polymorphisms in the non-coding region of *TP53*, of which two have been suggested to affect the level of expression of p53 as well as its function: i) a 16bp duplication in intron 3 localised at nucleotide 11951 (PIN3 Ins 16bp) (14), and ii) a G to A transversion in intron 6 at nucleotide 13494 (PIN6 G13494A) often reported as *MspI* (15) or *BstNI/NciI* polymorphism (16).

We performed a hospital-based study in breast cancer patients to evaluate the potential modifying role of the three highly common genetic polymorphisms in the *TP53* gene.

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We also took into account the potential interaction between these polymorphisms and the known clinicopathological features that are important prognostic markers in breast tumours. The data we present in this work suggest that analysis of polymorphisms in *TP53* gene can represent an additional useful tool for prognosis prediction.

## Materials and methods

**Clinical samples.** One hundred and seventeen breast cancer tissue samples were obtained from female patients in clinical stages I or II without previous therapy, diagnosed and treated at Masaryk Memorial Cancer Institute during the period 2004 to 2005. The lumpectomy or mastectomy resection specimens were received within 20 min of surgical removal and immediately evaluated by a pathologist. Tissue pieces of approximately 3x3x8 mm were cut from redundant tumour tissue after standard surgicopathological processing, snap frozen in liquid nitrogen and stored at -80°C. These specimens were subsequently used for DNA purification by DNeasy tissue kit (Qiagen). Routinely prepared formalin-fixed-paraffin-embedded (FFPE) tissue blocks taken in parallel, were fixed in 4% neutral formaldehyde for 24 h. Sections were cut at a thickness of 4 µm and collected onto positively charged slides for immunohistochemistry. The main clinicopathological variables including tumour type, grade and nuclear grade according to Elston-Ellis (17), estrogen receptor (ER), progesterone receptor (PR) and HER2/*neu* status, were extracted from pathological records obtained from the Masaryk Memorial Cancer Institute database. Ethical permission was granted following review at the Masaryk Memorial Cancer Institute and all patients gave written consent. DNA from one hundred and eight control samples was extracted from peripheral blood of healthy female volunteers with no oncological diagnosis to date.

**Immunohistochemistry and FISH.** Additional immunohistochemistry and fluorescence *in situ* hybridization (FISH) were performed to estimate cyclin D1 overexpression and amplification, and Ki67 expression. The antibodies used in this study are listed below: rabbit monoclonal cyclin D1 antibody (Lab Vision) and MIB1 mouse monoclonal antibody which recognises Ki67 (DakoCytomation). After removal of paraffin wax and rehydration, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate buffered saline (PBS), pH 7.5 for 15 min. Antigen retrieval was performed by heating sections in 1 mM EDTA-NaOH buffer (pH 8.0) for 40 min at 93°C. Primary antibodies were diluted in antibody diluent (DakoCytomation) and applied overnight at 4°C. Reactive sites were identified with biotinylated anti-mouse or anti-rabbit secondary antibodies and peroxidase ABC reagents (Vector-Elite) according to the manufacturer's instructions and peroxidase activity was visualised with DAB+ reagents (DakoCytomation). Sections were counterstained with Gills haematoxylin. FISH evaluations were performed using Vysis LSI Cyclin D1/CEP11 and PathVysion HER-2 DNA Probe Kits (Abbott Laboratories) according to the manufacturer's instructions.

**TP53 sequencing.** Total cellular RNA was extracted using TRI Reagent (MRC). *TP53* mRNA from tumour tissue was

amplified using the SuperScript™ III One Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen), sense primer: 5'-TCCCCTCCCATGTGCTCAAGACTG-3' and antisense primer: 5'-GGAGCCCCGGGACAAAGCAAA TGG-3'. PCR products were purified by MinElute™ PCR Purification kit (Qiagen) and sequenced using the ABI PRISM BigDye® Terminator v 3.1 Cycle Sequencing Kit on an ABI 3130 genetic analyser (Applied Biosystems).

**Determination of TP53 polymorphisms.** R72P polymorphism was assessed by PCR-RFLP technique as described previously (18). The codon 72 SNP determination was compared to *TP53* sequencing and resulted with 100% hit rate. The PIN3 Ins 16bp was genotyped by a simple PCR method, as performed previously (14) and PIN6 G13494A polymorphism was detected by PCR amplification of genomic DNA followed by *Bst*NI digestion according to (16).

**Statistical methods.** Statistical analysis was done using Statistica 8.0 (StatSoft).  $\chi^2$  test was used to evaluate association of *TP53* polymorphisms with breast cancer risk. The relationship between particular genotypes and multiple clinicopathological variables was determined using Kruskal-Wallis test. The relationship between allelic frequencies of polymorphisms and lymph node metastases was assessed using Mann-Whitney U test. Disease-free survival (DFS) curves were generated by the Kaplan-Meier method and verified by the log-rank test.

## Results

**TP53 genotyping.** The distribution of polymorphisms R72P, PIN3 Ins 16bp and PIN6 G13494A in *TP53* gene was assessed in a total of 117 female patients with breast cancer (mean age of 59.5 years, with an age range of 22-84 years) and compared to 108 healthy controls (mean age of 58.86 years, with an age range of 24-88 years) with respect to possible association with increased risk of tumour development. According to  $\chi^2$  test, no significant differences between the genotypes of patients and controls or allele frequencies were found for all three polymorphisms analysed in this study, except PIN6 G13494A heterozygotes (Table I). Additionally we analysed genotype effects of these polymorphisms on breast cancer risk (Table II), where only genotypes with frequency at least 5% were calculated. Comparing the common *TP53* R/R-A1/A1-G/G genotype with the other observed genotypes we did not find statistically significant difference between the breast cancer cases and control group.

**Relationship between p53 polymorphisms and clinicopathological variables.** The age of patients at diagnosis ranged from 22 to 84 years, with a mean age of 59.5 years. The relationship between age at onset and different genotype polymorphisms analysed using non-parametric Kruskal-Wallis test also showed no statistically significant associations between any genotype and an earlier age at onset. The relationships between studied *TP53* polymorphisms and other various clinicopathological parameters determined in our group of patients are shown in Table III, where 79 patients (67.5%) had infiltrative ductal carcinoma, 23 (19.7%) had lobular carcinoma and 15 patients (12.8%) had other types of breast cancer.



<i>TP53</i> polymorphism	Genotype	Frequency (%)		Breast cancer risk	
		Tumours	Controls	$\chi^2$ (p-value)	OR (95% CI)
R72P					
	R/R	62 (53.0)	55 (50.9)	Ref.	Ref.
	R/P	15 (12.8)	8 (7.4)	0.281	1.66 (0.66-4.22)
	P/P	40 (34.2)	45 (41.7)	0.405	0.79 (0.45-1.38)
	Alleles				
	R	139 (59.4)	118 (54.6)	Ref.	Ref.
	P	95 (40.6)	98 (45.4)	0.307	0.82 (0.57-1.20)
PIN3 Ins 16bp					
	A1/A1	81 (69.2)	81 (75.0)	Ref.	Ref.
	A1/A2	32 (27.4)	24 (22.2)	0.356	1.33 (0.72-2.46)
	A2/A2	4 (3.4)	3 (2.8)	0.711	1.33 (0.29-6.15)
	Alleles				
	A1	194 (82.9)	186 (86.1)	Ref.	Ref.
	A2	40 (17.1)	30 (13.9)	0.349	1.28 (0.76-2.14)
PIN6 G13494A					
	G/G	76 (65.0)	83 (76.85)	Ref.	Ref.
	G/A	39 (33.3)	23 (21.3)	0.044	1.85 (1.01-3.38)
	A/A	2 (1.7)	2 (1.85)	0.931	1.09 (0.15-7.95)
	Alleles				
	G	191 (81.6)	189 (87.5)	Ref.	Ref.
	A	43 (18.4)	27 (12.5)	0.086	1.58 (0.94-2.65)

A1, wt variant of intron 3; A2, 16bp insertion in intron 3.

Table II. Genotype frequencies between R72P, PIN3 Ins16bp and PIN6 G/A polymorphisms.

Genotypes	Frequency (%)		Breast cancer risk	
	Tumours	Controls	$\chi^2$ (p-value)	OR (95% CI)
R/R-A1/A1-G/G	52 (44.4)	51 (47.2)	Ref.	Ref.
R/P-A1/A2-G/A	20 (17.1)	15 (13.9)	0.496	1.31 (0.60-2.83)
R/P-A1/A1-G/A	6 (5.1)	1 (0.9)	0.071	5.88 (0.68-50.62)
R/P-A1/A1-G/G	14 (12.0)	26 (24.1)	0.096	0.53 (0.25-1.12)
P/P-A1/A2-G/A	6 (5.1)	5 (4.6)	0.798	1.18 (0.34-4.1)
P/P-A2/A2-A/A	2 (1.7)	1 (0.9)	-	
P/P-A1/A1-G/A	1 (0.9)	-	-	
P/P-A1/A1-G/G	6 (5.1)	2 (1.9)	0.181	2.94 (0.57-15.26)
R/R-A1/A2-G/A	3 (2.6)	-	-	
R/R-A1/A2-G/G	3 (2.6)	3 (2.8)	-	
R/R-A2/A2-G/A	1 (0.9)	-	-	
R/R-A2/A2-G/G	1 (0.9)	-	-	
R/R-A1/A1-G/A	2 (1.7)	1 (0.9)	-	
R/P-A1/A2-G/G	-	1 (0.9)	-	
R/P-A2/A2-G/A	-	1 (0.9)	-	
R/P-A2/A2-A/A	-	1 (0.9)	-	

A1, wt variant of intron 3; A2, 16bp insertion in intron 3.

Table III. Association of clinicopathological variables with different *TP53* genotypes.

Variable	Total of samples	R72P p-level	PIN3 Ins 16bp p-level	PIN6 G13494A p-level
Histologic grade <sup>a</sup>				
G1	33			
G2	41	0.1120	0.6418	0.3339
G3	43			
Nodal status <sup>a</sup>				
Negative	47	0.3869	0.0193	0.0293
Positive	70			
Tumour size <sup>b</sup>	117	0.7165	0.9760	0.9711
ER status <sup>a</sup>				
Negative	20	0.4961	0.8926	0.7456
Positive	97			
PgR status <sup>a</sup>				
Negative	26	0.8469	0.3662	0.4969
Positive	91			
Her2 amplification <sup>a</sup>				
Negative	91	0.9369	0.7158	0.7944
Positive	14			
CD1 amplification <sup>a</sup>				
Negative	87	0.2274	0.8246	0.7128
Positive	15			
CD1 expression <sup>b</sup> (%)	117	0.6848	0.3808	0.6312
Ki67 expression <sup>b</sup> (%)	117	0.1069	0.9196	0.5551

<sup>a</sup>Pearson  $\chi^2$  test; <sup>b</sup>Kruskal-Wallis ANOVA test.

These data reveal neither oestrogen receptor expression nor progesterone receptor expression associated with presence of a specific genotype. Similarly no relationship was found between genotype frequency and tumour size, grade, histological type of tumour, Ki67 and cyclin D1 expression. Evaluation of the lymph node status revealed a statistically significant relationship between 16bp duplication in intron 3 and development of lymph node metastases ( $p=0.019$ ,  $\chi^2$  test). The frequencies for particular intron 3 genotypes with respect to lymph node involvement are summarized in Table IV. The allelic frequencies were also strongly associated with node metastases ( $p=0.025$ , Mann-Whitney U test). A statistically significant association was also found between patients with lymph node involvement and PIN6 G13494A

Table IV. Observed frequencies.

Nodal status	PIN3 Ins 16bp			PIN6 G13494A		
	A1/A1	A1/A2	A2/A2	G/G	G/A	A/A
Negative	39	8	0	37	10	0
Positive	42	24	4	39	29	2

polymorphism ( $p=0.029$ ,  $\chi^2$  test). Similarly, allelic frequencies were also significantly associated with node metastases ( $p=0.030$ , Mann-Whitney U test).



Variable	Total of samples	R72P p-level	PIN3 Ins 16bp p-level	PIN6 G13494A p-level
Histologic grade <sup>a</sup>				
G1	28			
G2	34	0.5836	0.6835	0.4430
G3	20			
Nodal status <sup>a</sup>				
Negative	32	0.4016	0.0402	0.0269
Positive	50			
Tumour size <sup>b</sup>	82	0.4509	0.2228	0.9347
ER status <sup>a</sup>				
Negative	7	0.7169	0.9448	0.6453
Positive	75			
PgR status <sup>a</sup>				
Negative	10	0.5013	0.9302	0.6445
Positive	72			
Her2 amplification <sup>a</sup>				
Negative	63	0.3049	0.8880	0.9563
Positive	8			
CD1 amplification <sup>a</sup>				
Negative	61	0.3043	0.8962	0.4821
Positive	8			
CD1 expression <sup>b</sup> (%)	68	0.8452	0.2512	0.6718
Ki67 expression <sup>b</sup> (%)	82	0.7580	0.2708	0.5665

<sup>a</sup> $\chi^2$  test; <sup>b</sup>Kruskal-Wallis ANOVA test.

*TP53 polymorphisms, mutations and prognosis.* *TP53* gene mutations were identified in 29.9% tumours and only specimens bearing wild-type p53 have been used for further analysis to determine the role of the three studied polymorphisms in cells with functional p53 protein. Analogous to analysis of all specimens regardless of p53 status, no significant associations between the analysed polymorphisms and other clinicopathological variables were found, except development of lymph node metastases (Table V).

Interestingly, a significant relationship between allelic frequency of intronic polymorphism PIN3 Ins 16bp and lymph node involvement was also found ( $p=0.0169$  for PIN3 A2 allele,  $\chi^2$  test) in the group of wt p53 carriers. Determination of PIN6 G13494A allelic frequency revealed only

Table VI. Observed frequencies in wt p53 carriers.

Nodal status	PIN3 Ins 16bp			PIN6 G13494A		
	A1/A1	A1/A2	A2/A2	G/G	G/A	A/A
Negative	27	5	0	25	7	0
Positive	29	20	1	27	23	0

marginal statistical significance ( $p=0.0513$  for PIN6 13494A allele,  $\chi^2$  test) respectively. Observed frequencies are shown in Table VI.

The analysis of the link between SNPs and breast cancer survival found no association between all three polymorphisms and disease-free survival (DFS) (data not shown).

## Discussion

Cancers harbour germ line and/or somatic mutations in selected genes, resulting in disruption of signalling pathways involved in regulation of the homeostatic mechanisms in the cell. In this respect, the most interesting candidate genes include those that mediate a wide range of functions. The major risk factor for breast cancer can be linked to reproductive events that influence the lifetime levels of hormones. However, a large percentage of breast cancer cases cannot be explained by these risk factors. The identification of susceptibility factors that predispose individuals to this type of cancer will give further insight into the aetiology of this malignancy and provide targets for the future development of therapeutic approaches. Polymorphisms in the *TP53* gene as the frequent site of mutations are considered as one of those potential factors (19). A large number of studies have assessed the prognostic and predictive role of *TP53* polymorphisms in breast cancer yielding conflicting results (20).

We analysed R72P, PIN3 Ins 16bp and PIN6 G13494A polymorphisms in *TP53* gene and their association with an increased risk of tumour development, clinicopathological variables and prognosis in sporadic breast cancers. Concerning the R72P polymorphism, we did not find any association between this polymorphism and breast cancer risk in our set of samples. Additionally no relation was observed between R72P variants and other clinicopathological variables including DFS. These results are in agreement with other studies (21-23), nevertheless there are other reports showing important role for this polymorphism in breast cancers (24,25). These differences in findings can be explained by a more complex role of p53 R72P polymorphism in carcinogenesis (26). Polymorphisms in the non-coding region of *TP53* gene could also play an important role in the regulation of gene expression. Boldrini *et al* (27) analysed combined effect of the *TP53* codon 72 and PIN3 polymorphisms in patients with non-small cell lung cancer and showed evidence for dosage-effects of these polymorphisms. Patients ranging from zero to two *TP53* variant alleles tended to exhibit a better prognosis, compared to patients with three or four variants. We also analysed combined genotypes presented in individuals with respect to increased breast cancer risk (Table II) as well as DFS (data not shown). However, we did not find significant association of any genotype with both breast cancer risk and DFS, possibly due to short follow-up of our set of patients. Other published data suggest that combination of rare PIN3 A2 and PIN6 13494A alleles may modify the risk for breast cancer (28-30). Our results revealed only marginal association between PIN6 allele A and breast cancer incidence ( $p=0.086$ ). Nevertheless, predisposition of particular intronic haplotypes to breast cancer incidence was not confirmed in our study (data not shown).

Costa *et al* (23) show PIN3 Ins16bp polymorphism as a real risk modifier in breast cancer disease, either in sporadic and familial breast cancer, and moreover reveal association of this polymorphism with higher incidence of lymph node

metastases. These data are in agreement with Gemignani *et al*, who showed that 16bp duplication in intron 3 is associated with increased risk of colorectal cancer and with reduced levels of *TP53* mRNA, suggesting that the PIN3 A2 allele has reduced mRNA stability (31). However, the molecular mechanism as well as biological effect of this polymorphism has not been fully elucidated to date. Interestingly, we found that both intronic polymorphisms PIN3 Ins 16bp and PIN6 G13494A are significantly associated with higher incidence of lymph node metastases. These findings provide further evidence that these genotype variants are associated with a more aggressive tumour phenotype.

In summary, our results show no association of breast cancer risk with R72P, PIN3 Ins 16bp and PIN6 G13494A polymorphisms in *TP53* gene. On the other hand we found significant association between the presence of lymph node metastases and both intron 3 16bp duplication and intron 6 13494A allele variant. These findings provide support for potential prognostic effects of these two intronic polymorphisms in breast cancer.

Despite our best efforts, a significant proportion of patients suffering from breast carcinoma will develop advanced disease and we do not currently have sufficient reliable tools to predict who these patients are. For this reason, additional independent predictive bio-markers are required to select patients that will benefit from more intensive treatment and monitoring to prevent tumour progression. This applies especially to the group of patients of clinical stage I with low or intermediate risk according to the current NCI or St. Gallen criteria. In our study, *TP53* intron 3 16bp duplication and intron 6 13494A allele variant are significantly related to the presence of lymph node involvement, which is the strongest known prognostic indicator in low grade ER positive tumours. Further studies focused on the prognostic impact of these polymorphisms in representative datasets should be performed to elucidate their potential to serve as predictors of advanced disease development.

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