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ORIGINAL PAPER

Alterations in *p53*, *BRCA1*, *ATM*, *PIK3CA*, and *HER2* genes and their effect in modifying clinicopathological characteristics and overall survival of Bulgarian patients with breast cancer

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Abstract

Purpose Though *p53*, *BRCA1*, *ATM*, *PIK3CA*, and *HER2* genes are shown to be involved in various aspects of breast carcinogenesis, their functional relationship and clinical value are still disputable. We investigated the genetic status or expression profile of these genes to further elucidate their clinical significance.

Methods PCR-SSCP-Sequencing of *p53*, *BRCA1*, *ATM*, and *PIK3CA* was performed in 145 Bulgarian patients with sporadic breast cancer. Expression profiles of *HER2* were determined by ICH and CISH. Relationship between mutations and clinicopathological characteristics was evaluated by Chi-squared and Fisher's exact tests. Multivariate Cox proportional hazard test and Kaplan–Meier analysis were used to evaluate differences in overall survival between groups.

Results The frequency of *p53* (22.07%), *BRCA1* (0.69%), *ATM* (7.59%), and *PIK3CA* (31.25%) alterations and HER2 (21.21%) overexpression was estimated. Mutated *p53* was associated with tumor size ($P = 0.033$) and grade of malignancy ($P = 0.001$), *ATM*—with grade of malignancy ($P = 0.032$), and *PIK3CA*—with PR-positive tumors ($P = 0.047$). HER2 overexpression correlated with age of diagnosis ($P = 0.009$), tumor size ($P = 0.0004$), and ER expression ($P = 0.011$). Univariate survival analysis showed that mutated *p53* is an indicator for worse outcome ($P = 0.041$). Combination of two genetic abnormalities did not correlate with more aggressive carcinogenesis and worse overall survival.

Conclusions Our data indicated that *p53*, *BRCA1*, *ATM*, *PIK3CA*, and *HER2* alterations specifically correlate with clinicopathological characteristics of Bulgarian patients with breast cancer. Of these genes, only mutated *p53* showed significant, though not independent, negative effect on overall survival.

Keywords Breast cancer · Tumor suppressor genes · Proto-oncogenes · Mutations · Clinicopathological characteristics · Overall survival

Abbreviations

BC	Breast cancer
IHC	Immunohistochemical analysis
CISH	Chromogenic in situ Hybridization
OS	Overall survival
T	Tumor size
N	Nodal status
G	Grade of malignancy
ER	Estrogen receptor
PR	Progesterone receptor
HR	Hazard ratio
CI	Confidence interval

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Introduction

Breast cancer (BC) is the most common malignancy among women worldwide and the second leading cause of cancer death in women today. In Bulgaria, the incidence of BC amounts to approximately 25% of all female cancers, corresponding to ~3,500 new cases each year (Danon et al. 2006). The major risk factors are sex, age, family predisposition, early menarche, late menopause, late first childbirth, shorter breastfeeding period, use of oral contraceptives, and hormone replacement therapy. Expanding knowledge on BC revealed that several tumor suppressor genes and proto-oncogenes are implicated in its pathogenesis. Some of them are *BRCA1*, *BRCA2*, *p53*, *ATM*, *Chek1, Chek2*, *p65, PTEN, HER2*, and *PIK3CA*. The coordinated function of these genes enables maintenance of genome stability in cells. The disruption in their normal activities may stimulate carcinogenetic initiation and/or progression and correlates with the clinical characteristics of the patients. A direct functional relationship has been found between some of the key BC genes (Bartkova et al. 2005), though the exact mechanisms underlying these interactions remain unknown. Currently, the clinical management of BC is based mainly on classical biological indicators. It is expected however the genetic profiling of tumors to provide new molecular-genetic biomarkers, which will support cancer prevention and treatment.

Mutations in *p53* tumor suppressor gene are the most frequent event in carcinogenesis. Genetic alterations in *p53* were found in tumors of almost any type with a frequency ranging from 5.6 to 48.3% (*IARC p53 data base*). The majority of tumorigenic *p53* mutations are located in the conservative regions of exons 5 to 8. Between 5 and 10% of BC cases are result of inheriting a mutant gene or genes. It is known that most of them are due to mutations in a BC-specific tumor suppressor gene—*BRCA1* (Ford et al. 1998). Mutations in *BRCA1* are extremely rare in the sporadic form of the disease. Recently, a new tumor suppressor gene with a marked role in BC pathogenesis was discovered—*ATM*. Heterozygous carriers of *ATM* gene mutation exhibit higher predisposition to BC and other malignant diseases. Approximately 21% of these women develop BC below the age of 50 (Thompson et al. 2005). *p53, BRCA1*, and *ATM* are engaged in a cell signaling pathway, which enables maintaining of the genome stability by regulation of cell cycle, DNA repair, and apoptosis. In response to DNA damage, ATM directly phosphorylates *p*53, BRCA1, and other proteins, involved in the cellular response to genotoxic stress.

Recently, it was established that the proto-oncogene *PIK3CA* is mutated in more than 30% of breast tumors, which makes it the most frequently mutated gene in BC identified so far (Campbell et al. 2004). PIK3CA is a lipid kinase, and its oncogenic activity is due to missense mutations in hot-spots clustered predominantly in exons 9 and 20. The mutant PIK3CA activates AKT protein kinase, which stimulates cell proliferation and suppresses apoptosis thus inducing a tumorigenic process (Sansal et al. 2004). All of the discovered mutations are somatic and are present only in the tumor tissue. Some authors demonstrated a correlation between *PIK3CA* status and other cancer-related genes. Thus, Singh et al. (2002) reported that *PIK3CA* mutations and *p53* mutations are mutually exclusive. Saal et al. (2005) found a significant positive association between *PIK3CA* mutations and *HER2* overexpression.

Another proto-oncogene with a significant role in BC pathogenesis is *HER2* (*c-erbB-2*). HER2 is overexpressed in 25–30% of primary BC (Slamon et al. 1987). When activated, the HER2 tyrosine kinase receptor forms heterodimers with other members of the HER family, thus triggering a cascade of events, which transmit the growth signal to the nucleus of the cell through the process of signal transduction. Current studies demonstrated a cumulative effect of *HER2* and *p53* alterations on disease aggressiveness and clinical outcome (Yamashita et al. 2004; Bull et al. 2004; Rahko et al. 2003). Hence, the coexistence of HER2 overexpression and p53 protein accumulation was proposed as a potential prognostic molecular marker in BC. However, other studies have shown no significant correlation (Sawaki et al. 2006) or even a better prognosis (Rosen et al. 1995) in BC patients with both p53 protein accumulation and HER2 overexpression.

In this study, we investigated the genetic status or expression profiles of five BC-related genes to further elucidate the relationship between molecular characteristics and clinical outcome. So far, the status of *p53*, *BRCA1*, *ATM, PIK3CA*, and *HER2* genes was not comparatively studied. No data are available on the frequency and spectrum of mutations in these genes in Bulgarian patients with BC. Gene status of *p53*, *BRCA1*, *ATM*, and *PIK3CA* in a group of 145 patients was determined using PCR followed by single-stranded conformation polymorphism (SSCP) and sequencing analysis. HER2 expression profiles were established by immunohistochemical (IHC) analysis and chromogenic in situ hybridization (CISH). Hot-spot regions of the genes were screened. Clinical significance of the studied genes for BC pathogenesis was further estimated. Statistically significant correlations with clinical characteristics and overall survival were found.

Materials and methods

Patients

The study group included 145 Bulgarian female patients with primary invasive breast carcinoma, who were treated

Table 1 Patient characteristics and therapy

		n = 145 (%)
Tumor size	1	65 (45%)
	2	62 (43%)
	3	7 (5%)
	4	11 (7%)
Grade	1	6 (4%)
	2	103 (71%)
	3	36 (25%)
Nodal status	N+	53 (37%)
	N−	92 (63%)
Histological type	Ductal	130 (90%)
	Lobular	15 (10%)
Estrogen receptor status	ER+	81 (56%)
	ER−	64 (44%)
Progesterone receptor status	PR+	81 (56%)
	PR−	64 (44%)
HER2 status	HER2+	21 (14%)
	HER2−	78 (54%)
	Unknown	46 (32%)
Median age (min.–max.) years		54,4 (29–88)
Radiation therapy	+	115 (79%)
	−	30 (21%)
Chemotherapy	+	87 (60%)
	−	58 (40%)
Hormonal therapy	+	114 (79%)
	−	31 (21%)

at the Thoracic Clinic of the Bulgarian National Oncological Centre Hospital, Sofia between 2000 and 2003. They were staged according to the TNM classification of Union International Contre le Cancer (UICC). Age ranged from 29 to 88 years. Patients underwent adjuvant therapy according to accepted practice guidelines at that time. Clinical characteristics and therapy are presented in Table 1.

We followed the patients for a median period of 69 months (range from 11 to 96 months). The follow-up time began at the time of surgery and ended in November 2008 or at the date of death. Information on the date of death was obtained from the Bulgarian national population registry.

Blood specimens of clinically healthy persons were used as negative controls. All participants gave an informed consent. Clinical information was obtained from the existing medical records and presented in such a way that patients could not be identified directly or through linked identifiers.

DNA isolation

High molecular weight genomic DNA was isolated from fresh frozen breast tumors using a standard Proteinase K/Phenol procedure. Briefly, the tumor tissues were homogenized in lysis buffer (10 mM Tris/HCl pH 8.3, 400 mM NaCl, 2 mM EDTA-Na, 0.14 mg/ml Proteinase K, 1% SDS) at 37°C for 48 h and then subjected to phenol/chloroform/isoamyl alcohol purification and ethanol precipitation. DNA was isolated from blood specimens of healthy controls using a standard procedure.

PCR

To amplify exons 4 to 9 of p53 gene, PCRs were performed in a total volume of 25 µl containing 150 ng of template DNA, 1× PCR buffer, 1.75 mM MgCl2, 0.2 mM of each dNTP, 2.5 pmol of each primer, and 0.3 U of Taq DNA polymerase (Krasteva et al. 2003). The cycling reactions included 32 cycles of denaturation at 94°C for 30 s. (first cycle—5 min.), annealing at the appropriate temperature (exon 4, 64°C; exon 5, 59°C; and exons 6–9, 63°C) for 30 s, and elongation at 72°C for 30 s. (last cycle—5 min.).

Exons 2, 5, 11A, 11B, 11C, 11D, 13, 16, and 20 of *BRCA1* were analyzed in 25 µl PCRs containing 50 ng of template DNA, 1× PCR buffer, 2.25 mM $MgCl_2$, 0.2 mM of each dNTP, 100 nM of each primer, and 0.5 units of Taq DNA polymerase. The primers used for the amplification of exons 2, 5, 11A, 11B, 13, and 20 were according to Friedman et al. (Friedman et al. 1994). Exons 11C, 11D, and 16 were analyzed with primers designed by us. PCR consisted of 34 cycles of 30 s at 94°C denaturation (first cycle—5 min.), 30 s annealing (at 57, 60, 60, 53, 54, 66, 50, 60, and 60°C, respectively) and 1 min elongation at 72°C (last cycle—10 min).

PCRs for analysis of *ATM* gene were conducted in a total volume of 25 µl by the use of oligonucleotide primers for 17 exons—15, 30, 36, 54, 62, 65, 51, 5, 10, 19, 24, 31, 33, 39, 49, 52, and 59. Primer sequences and optimal PCR conditions were kindly provided by Prof. Patrick Concannon from the University of Washington School of Medicine, Seattle, Washington. The products were obtained after 35 cycles of amplification, consisting of denaturation for 30 s at 94°C (first cycle- 5 min.), annealing for 30 s at 54°C, and elongation for 1 min at 72°C (last cycle—10 min).

PCR amplification of *PIK3CA* exons 9 and 20 was carried out using 40 ng of genomic DNA in a reaction volume of 20 µl containing specific oligonucleotide primers (Samuels et al. 2004) at a final concentration of 5 µM and HotStar Taq Master Mix (Qiagen, Valencia, CA). After an initial denaturation step of 95°C for 10 min., 32 cycles of reaction were performed—94°C for 30 s., 61°C annealing temperature for 30 s, and 72°C for 45 s. This was followed by a final extension step of 72°C for 7 min. Unlike the other studied genes, in *PIK3CA* analysis PCR was followed by direct sequencing.

SSCP analysis

The amplified exons of *p53*, *BRCA1*, and *ATM* genes were analyzed on SSCP PAA gel electrophoresis. The products were denatured in 95% formamide, 0.01 N NaOH (1:1.4) at 95°C for 10 min, and separated on native polyacrylamide gels in 1xTBE buffer at 15 mA, 4°C. The polyacrylamide gel concentration varied between 8 and 18% depending on the tumor suppressor gene and the exon. Modified silver staining method was applied for visualization. Briefly, the gels were soaked in 50% methanol for 1 h, then transferred to ammonium–silver solution, containing 0.02 N NaOH, 0.375% NH_4OH, and 0.8% $AgNO_3$ for 15 min, and washed with 500 ml dH_2O for 10 min. The staining reaction was developed by the use of 0.01% citric acid, 0.035% formaldehyde, and the gels were fixed in 0.5% acetic acid.

Sequencing analysis

Amplified products with altered SSCP electrophoretic mobility were purified and sequenced. To validate wild-type status, a subset of samples lacking mobility shifts was also subjected to sequencing.

To purify PCR products GFX PCR DNA and Gel Band Purification Kit, Amersham Biosciences was used. The sequencing reactions were done using DYEnamic™ ET Terminator Cycle Sequencing Kit, Amersham Biosciences on ABI Prism™ 310 fluorescent sequencing analyzer following the manufacturer's instructions. To confirm the mutation, all mutant samples were subsequently sequenced in the reverse direction.

The sequence data of *p53*, *BRCA1*, *ATM*, and *PIK3CA* genes were obtained from GenBank.

Immunohistochemical analysis

Serial sections of primary breast tumor paraffin-embedded blocks (4-µm) were mounted on adhesive-coated glass slides for HER2 staining. Primary monoclonal rabbit anti-human c-erbB2 oncoprotein antibody was used at 1:300 dilution (DAKO c-erbB-2 oncoprotein). DAKO EnVision™ system was used for detection, following the manufacturer's instructions. The entire immunostained slides were evaluated by light microscopy and scored. HER2 immunostaining was evaluated using the grading system employed by the HercepTest™ (DAKO).

Chromogenic in situ hybridization (CISH)

HER2 gene amplification in formalin-fixed, paraffin-embedded tissue section was detected using Chromogenic In Situ Hybridization (Zymed's SPoT-Light^R HER2 CISH™ Kit) following the manufacturer's instructions.

Statistical analysis

The relationship between *p53*, *BRCA1*, *ATM, PIK3CA*, and HER2 alterations and clinicopathological characteristics of the patients was evaluated using Fisher's exact test and the Chi-squared test. Overall survival (OS) was estimated by the Kaplan–Meier method, and differences between survival curves were assessed for statistical significance using the log-rank test. Cox proportional hazards regression model was used to calculate the hazard ratios (HR) and their 95% confidence intervals (95% CI) for each variable in the univariate and multivariate analyses. All *P*-values were two sided, and results were considered statistically significant at *P* less than 0.05. Analyses were done using the SPSS software package (SPSS Inc., Chicago, IL, USA).

Results

Mutational analysis

Of the 145 patients analyzed, 17 had only *p53* aberration, 2—only *ATM*, 26—only *PIK3CA*, and 7—only *HER2*. Seventeen patients had aberrations in two genes, of which 4 in *PIK3CA* and *p53*, 3 in *PIK3CA* and *ATM*, 4 in *PIK3CA* and *HER2*, 1 in *ATM* and *HER2*, 2 in *p53* and *ATM*, and 3 in *p53* and *HER2*. Eight patients had three of the genes mutated and were divided into the following subgroups: 2 patients with aberrations in *PIK3CA*, *p53*, and *ATM;* 4—in *PIK3CA*, *p53*, and *HER2*; 1—in *PIK3CA, ATM*, and *HER2*; and 1 in *PIK3CA*, *HER2*, and *BRCA1*. None of the patients had the five studied genes mutated. Totally, 77 patients (53.1%) had an aberration in either of the genes and 68—in none (46.9%).

p53 gene mutations

The primers used to screen for *p53* mutations in exons 4 to 9 cover the whole DNA-binding domain (where more than 90% of all *p53* mutations were found), most of the proline-rich domain necessary in induction of apoptosis and a part of the C-terminal regulatory domain. Alterations in electrophoretic mobility were found in 32 of the patients, corresponding to a frequency of 22.07% (32/145) (Table 2). Mutations mapped to codons 97, 107, 108, 125, 140, 142, 175, 198, 205, 213, 220, 245, 248, 268, 273, 283, and 330. Ten mutations were missense, three were deletion frameshifts, one was an insertion frameshift, one was a nonsense mutation, and one was a splice-site mutation. Two silent substitutions, seven codon polymorphisms, and eight intronic polymorphisms were found. Both deletion frameshifts delACGGTTTCCG (12,244–12,253) and delGTTT CCG (12,247–12,253) contained a DNA region GTTT

Table 2 Type and number of mutations and polymorphisms in Bulgarian patients with breast cancer patients

Gene	*n* of patients	Nucleotide position/change	Location	Codon	Codon change	AA change	Effect
Mutations							
p53	1	12,215 delC	Exon 4	97	–	–	Frameshift
	1	12,244–12,253 delACGGTTTCCG	Exon 4	107–110	–	–	Frameshift
	1	12,247–12,253 delGTTTCCG	Exon 4	108–110	–	–	Frameshift
	1	12,299 G > A	Exon 4	125	ACG–ACA	Thr–Thr	Splice
	1	13,098 C > G	Exon 5	140	ACC–AGC	Thr–Ser	Missense
	1	13,104–13,105 insGC	Exon 5	142	–	–	Frameshift
	1	13,202 C > G	Exon 5	175	CGC–GGC	Arg–Gly	Missense
	1	13,352 G > T	Exon 6	198	GAA–TAA	Glu–STOP	Nonsense
	1	13,374 A > G	Exon 6	205	TAT–TGT	Tyr–Cys	Missense
	1	13,419 A > G	Exon 6	220	TAT–TGT	Tyr–Cys	Missense
	1	13,990 A > G	Intron 6	–	–	–	Int. polymorphism
		14,060 G > A	Exon 7	245	GGC–AGC	Gly–Ser	Missense
	1	14,069 C > T	Exon 7	248	CGG–TGG	Arg–Trp	Missense
	1	14,487 G > A	Exon 8	273	CGT–CAT	Arg–His	Missense
	1	14,487 G > T	Exon 8	273	CGT–CTT	Arg–Leu	Missense
	1	14,516 C > T	Exon 8	283	CGC–TGC	Arg–Cys	Missense
	1	14,750 T > C	Exon 9	330	CTT–CCT	Leu–Pro	Missense
ATM	2	2,119 T > C	Exon 15	707	TCT–CCT	Ser–Pro	Missense
	5	5,557 G > A	Exon 39	1,853	GAT–AAT	Asp–Asn	Missense
	4	5,557 G > A	Exon 39	1,853	GAT–AAT	Asp–Asn	Missense
		5,489 T > C	Intron 38	–	–	–	Int. polymorphism
PIK3CA	6	1,624 G > A	Exon 9	542	GAA–AAA	Glu–Lys	Missense
	9	1,633 G > A	Exon 9	545	GAG–AAG	Glu–Lys	Missense
	2	1,636 C > A	Exon 9	546	CAG–AAG	Gln–Lys	Missense
	1	1,637 A > G	Exon 9	546	CAG–CGG	Gln–Arg	Missense
	25	3,140 A > G	Exon 20	1,047	CAT–CGT	His–Arg	Missense
	1	3,140 A > T	Exon 20	1,047	CAT–CTT	His–Leu	Missense
	2	3,145 G > C	Exon 20	1,049	GGT–CGT	Gly–Arg	Missense
Polymorphisms							
p53	7	13,399 A > G	Exon 6	213	CGA–CGG	Arg–Arg	Polymorphism
	2	14,473 C > T	Exon 8	268	AAC–AAT	Asn–Asn	Polymorphism
	7	14,766 T > C	Intron 9	–	–	–	Intronic polymorphism
BRCA1	1	19,479 A > T	Intron 4	–	–	–	Intronic polymorphism

n number of patients with the respective mutation, *AA* amino acid

(12,247–12,250), which was found to be deleted (alone or along with a varying in size flanking sequence) also in three patients with cancer, all of BC, which were reported in Thierry Soussy and IARC p53 data bases. All seven polymorphisms in the coding regions were codon 213 (A > G) polymorphisms (Arg213Arg) and were found with a frequency of 0.0483. Of the eight intronic polymorphisms, seven were 14,766 T > C substitutions mapping 12 bp downstream of the 3′ end of exon 9.

Five of the *p53* mutations we found (the three deletions delACGGTTTCCG, delGTTTCCG, and delC; the insertion insGC, and the missense substitutions Thr140Ser) and one intronic polymorphism (13990A > G) were novel and have never been so far reported in any type of human cancer. Six of the *p53* mutations mapped to known hot-spots: 175, 220, 245, 248 and 273. Codons 175, 248, and 273 are hot-spots in almost all types of cancer, while codon 220 is a hot-spot, specific for BC.

BRCA1 gene mutations

All 145 patients were screened for mutations in *BRCA1* exons 2, 5, 11A, 11B, 11C, 11D, 13, 16, and 20. Amplified fragments cover the 20 most frequent *BRCA1* mutations according to BIC (Breast Cancer Information Core) database. Two of them (exons 2 and 5) map to the N-terminal

RING domain, one (exon 11A)—to the NLS (Nuclear Localization Signals) and two (exons 16 and 20)—to the BRCT domains. Aberrant SSCP pattern was found only in one patient (1/145, 0.69%). The sequencing data showed A to T substitution at a position 19,479 in intron 4 (Table 2).

ATM gene mutations

Mutation detection at the *ATM* locus is difficult because of the large size of the gene and the lack of mutation hotspots. Therefore, we selected 17 *ATM* exons, which were found to be mutated with a higher frequency according to the available data. The analyzed sequences cover parts of all functional domains of the protein. Mutations in *ATM* were found in 11 patients overall (11/145 i.e., 7.59%) mapping to exons 15 and 39 only (Table 2). All of the discovered *ATM* mutations were missense. In exon 15, we found two T > C base substitutions at a position 2,119 bp, leading to the Ser707Pro amino acid change.

In exon 39, we detected nine mutations, of which five were G to A substitutions at a position 5,557 bp altering the amino acid composition of ATM protein in codon 1,853 (Asp1853Asn). In the other four patients, we found the substitution Asp1853Asn (G > A) in combination with an intron 38 variant—IVS38-8 T > C (Asp1853Asn G > A—IVS38-8 T > C).

PIC3CA gene mutations

PIK3CA genetic status was evaluated in 144 of the breast carcinomas by direct sequencing of the mutational hotspot gene regions encoding the helical domain (exon 9) and the catalytic domain (exon 20). Forty-five (31%) of the investigated breast tumors harbored a missense mutation in the *PIK3CA* oncogene (Table 2). Eighteen mutations were located in exon 9 and 28 in exon 20 (one of the patients had mutations in both exons). Six (33%) of the 18 mutations found in exon 9 were of the E542K type, and 9 (50%) were of the E545K type. Twenty-five (89%) of the 28 mutations in exon 20 were H1047R substitutions.

HER2 expression level

Primary breast tumor paraffin-embedded blocks were available for 99 of the studied patients. HER2 status was determined by immunohistochemical analysis and CISH. Based on the obtained results, the patients were classified in two groups: HER2-positive (overexpressing HER2)—21 (21.21%) and HER2-negative—78 (78.78%). The level of HER2 protein expression was scored on a scale of 0 to 3 + using the grading system employed by the HercepTest™ (DAKO). Fifty-five patients had a score of 0 (55.55%), 22 − 1 + (22.22%), 7 − 2 + (7.07%), and 15 − 3 + (15.15%). The seven patients with a score of 2+ were further analyzed by CISH to confirm gene amplification. The CISH analysis revealed that with respect to *HER2* gene amplification, six patients were positive and one was negative.

Mutational gene status in relation to clinicopathological and molecular characteristics

To estimate the clinical impact of the studied genes, individually or in combinations, we compared the genetic status of the patients with the standard prognostic factors including age of diagnosis, tumor size (T), nodal (N) status, grade of malignancy (G), histological type, and estrogen (ER) and progesterone receptor (PR) status (Table 3).

p53

Sequence variants in *p53* were placed into two categories: (1) *deleterious mutations* (sequence changes that produce truncated p53 protein or alter amino acids critical for p53 function—deletions, insertions, and missense, nonsense, splice-site mutations)—16 patients and (2) *neutral variants* (silent substitutions and polymorphisms)—19 patients. In the statistical analysis, only deleterious mutations were considered.

The results showed that *p53* mutations were significantly associated with tumor size ($P = 0.033$) and grade of malignancy ($P = 0.001$) (Table 3). Tumors with mutation in *p53* ($p53^{+}$) were larger in size (predominantly at T2–T4) and poorly differentiated (G3). In addition, a tendency of later age expression in *p53*$^{+}$ patients was found ($P = 0.052$) as the majority (87.5%) of p53-positive women were over or at the age of 50.

BRCA1

Statistical analysis according to *BRCA1* gene was not done due to the low *BRCA1* mutational frequency. The tumor of the only patient with aberration in *BRCA1* was G2 and T3 staged, N^{+} and ER^{+}, and PR^{-}. The patient was over the age of 50, *PIK3CA*$^{+}$, *HER2*$^{+}$ and had ductal type of breast carcinoma. The woman died 19 months after surgery.

ATM

ATM mutational status was significantly associated with the grade of malignancy ($P = 0.032$) (Table 3). Tumors with *ATM* mutation (*ATM*$^{+}$) were mainly (72.7%) moderately differentiated (G2). There was also a trend for increased frequency of lobular type of breast carcinoma in *ATM*$^{+}$ patients ($P = 0.09$). A tendency for a higher frequency of

Table 3 Gene mutations in relation to clinicopathological and molecular characteristics

Variable		p53				ATM				PIK3CA				HER2			
		Total	Mutant	WT	*P*	Total	Mutant	WT	*P*	Total	Mutant	WT	*P*	Total	OE	Normal	*P*
		n = 145	*n* = 16	*n* = 129		*n* = 145	*n* = 11	*n* = 134		*n* = 144	*n* = 45	*n* = 99		*n* = 99	*n* = 21	*n* = 78	
Age	Years	54.4	57.3	54		54.4	55.5	54.3		54.4	54.7	54.2		54.7	58.8	53.6	
	Range	29–88	29–88	32–82		29–88	42–74	29–88		29–88	37–78	29–88		29–88	32–78	29–88	
	<50	52	2	50	0.052	52	2	50	0.33	52	18	34	0.58	34	2	32	**0.009**
	≥50	93	14	79		93	9	84		92	27	65		65	19	46	
T	T1	65	3	62	**0.033**	65	5	60	0.603	65	20	45	0.615	42	1	41	**0.00004**
	T2–T4	80	13	67		80	6	74		79	25	54		57	20	37	
N	N0	92	11	81	0.786	92	8	84	0.75	91	31	60	0.36	61	10	51	0.21
	N+	53	5	48		53	3	50		53	14	39		38	11	27	
G	G1	6	0	6	**0.001**	6	2	4	**0.032**	6	0	6	0.07	5	1	4	0.96
	G2	103	6	97		103	8	95		102	37	65		68	14	54	
	G3	36	10	26		36	1	35		36	8	28		26	6	20	
HT	Lobular	15	1	14	1	15	3	12	0.09	14	6	8	0.37	10	1	9	0.68
	Ductal	130	15	115		130	8	122		130	39	91		89	20	69	
ER	+	81	10	71	0.61	81	6	75	1	80	24	56	0.72	60	18	42	**0.011**
	–	64	6	58		64	5	59		64	21	43		39	3	36	
PR	+	81	9	72	1	81	7	74	0.76	81	31	50	**0.047**	56	13	43	0.63
	–	64	7	57		64	4	60		63	14	49		43	8	35	
p53	Mutant	–	–	–	–	16	2	14	0.35	16	4	12	0.78	14	3	11	1
	WT	–	–	–		129	9	120		128	41	87		85	18	67	
ATM	Mutant	11	2	9	0.35	–	–	–	–	11	6	5	0.098	7	2	5	0.64
	WT	134	14	120		–	–	–		133	39	94		92	19	73	
PIK3CA	Mutant	45	4	41	0.78	45	6	39	0.098	–	–	–	–	36	10	26	0.19
	WT	99	12	87		99	5	94		–	–	–		62	10	52	
HER2	OE	21	3	18	1	21	2	19	0.64	20	10	10	0.19	–	–	–	–
	Normal	78	11	67		78	5	73		78	26	52		–	–	–	

WT wild type, *OE* overexpression, *T* tumor size, *N* nodal status, *G* grade of malignancy, *HT* histological type, *ER* estrogen receptor, and *PR* progesterone receptor

Significant *P* values are in bold

ATM mutations in *PIK3CA*$^{+}$ tumors was also observed ($P = 0.098$).

To estimate whether the different type of *ATM* mutation has a specific clinical impact, we compared the characteristics of *ATM*$^{+}$ patients from the three genotypes (*data not shown*). No statistically significant correlation was found.

PIK3CA

The frequency of *PIK3CA* mutations in PR-positive tumors was significantly ($P = 0.047$) higher than that in PR-negative tumors (Table 3). We observed also a trend for more frequent mutations in moderately differentiated (G2) tumors ($P = 0.071$) and in tumors with *ATM* mutation ($P = 0.098$). There was no significant correlation with the clinicopathological features when patients with mutations in exon 9 or patients with mutations in exon 20 were analyzed separately (*data not shown*).

HER2

We found a strong correlation of HER2 overexpression with age of diagnosis ($P = 0.009$), tumor size ($P = 0.0004$), and ER expression ($P = 0.011$) (Table 3). There were 90.5% of HER2-positive patients over or at the age of 50. Most HER2^{+} tumors (95.2%) were T2–T4 staged and were mainly ER-positive (85.7%).

Relationship between mutational status and molecular type of breast cancer

The possible association between the type of breast cancer and mutational status of *p53*, *ATM*, and *PIK3CA* was also analyzed. Patients were classified into four groups according to their receptor status: luminal A ($n = 42$), luminal B ($n = 19$), HER2-positive ($n = 21$), and triple negative ($n = 17$). No statistically significant correlations were

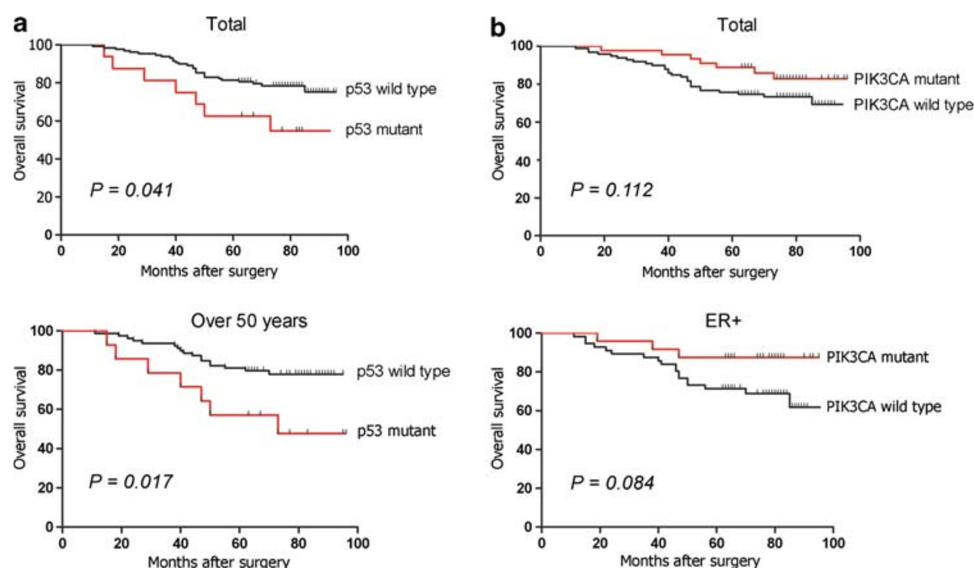

Fig. 1 Kaplan–Meier survival curves. **a** OS rates of total patients with *p53* mutation and the subgroup of patients with *p53* mutation over 50 years. **b** OS rates of total patients with *PIK3CA* mutation and the subgroup of patients with *PIK3CA* mutation and positive ER

found—*p53* ($P = 0.944$), *ATM* ($P = 0.823$), and *PIK3CA* ($P = 0.112$).

Mutational gene status and overall survival

OS of the investigated group was estimated to 75.86% (110/145). Kaplan–Meier analysis showed that of all studied genes, only *p53* significantly correlated with OS. Patients with mutation in *p53* had reduced survival compared to the wild-type *p53* patients ($P = 0.041$) (Fig. 1a). This correlation was even stronger in women over 50 ($P = 0.017$) (Fig. 1a) and in women with T1 tumors ($P = 0.014$). The effect of mutant *p53* on survival was apparent in ER-negative ($P = 0.046$) and PR-positive ($P = 0.026$) tumors. We also observed a trend for a worse OS of *p53*-positive patients who were negative with respect to *ATM* status compared to $p53^{-}ATM^{-}$ patients ($P = 0.051$).

Though mutated *ATM* itself did not show significant correlation with OS, the $ATM^{+}ER^{-}$ subgroup had reduced survival compared to $ATM^{-}ER^{-}$ ($P = 0.021$). It is worth mentioning that all ATM^{+} women who did not survive the follow-up period were of the genotype pAsp1853Asn (G > A) ($P = 0.081$).

PIK3CA-positive patients had a trend for a better OS ($P = 0.112$) (Fig. 1b), which increased in the $PIK3CA^{+}ER^{+}$ ($P = 0.084$) (Fig. 1b) and $PIK3CA^{+}ATM^{-}$ subgroups ($P = 0.068$).

Univariate and multivariate Cox proportional hazards model was used to estimate the hazard ratio for carriers of the mutation, when compared with noncarriers. In a multivariate model that included traditional prognostic factors such as age of diagnosis, tumor size, nodal status, grade of malignancy, and estrogen and progesterone receptor status, none of the genes reached significance as independent prognostic factors. The only independent prognostic factor that contributed significantly to a decrease in OS was tumor size with a fourfold increased risk of death (HR = 4.43, 95% CI 1.47–13.38, $P = 0.008$). Univariate analysis showed that patients with *p53* mutations have 2.31-fold increased risk of dying from breast cancer (95% CI 1.01–5.29, $P = 0.048$); however, multivariate analysis failed to show an independent effect (Table 4). We found a protective effect of *PIK3CA* mutations with HR of 0.51 though not statistically significant (95% CI 0.19–1.34, $P = 0.169$).

Correlation of genetic aberrations

We further analyzed the combined clinical impact of the studied gene aberrations on breast tumor parameters. Patients were divided into subgroups according to their genotype characteristics: $PIK3CA^{+}p53^{+}$; $PIK3CA^{+}ATM^{+}$; $PIK3CA^{+}HER2^{+}$; $ATM^{+}HER2^{+}$; $p53^{+}ATM^{+}$; and $p53^{+}HER2^{+}$. We carefully studied the patients with these composite genotypes with respect to the clinical parameters and OS but found no statistically significant differences between the subgroups.

Discussion

Though *p53, BRCA1, ATM, PIK3CA*, and *HER2* genes are involved in various aspects of BC development and progression, their direct functional relationship and clinical value are still disputable. Until now, BC research in Bulgaria is limited mainly to clinical, morphological, and epidemiological aspects of the disease. Except one previous investigation of our research group (Krasteva et al. 2003) and another study including 20 patients (Markoff et al. 1998), there are no other data on the genetic status of

Table 4 Univariate and multivariate survival analysis

Factor	Univariate			Multivariate		
	HR	95% CI	*P*	HR	95% CI	*P*
p53, mutated vs. wild type	2.31	1.01–5.29	**0.048**	1.08	0.38–3.09	0.889
PIK3CA, mutated vs. wild type	0.50	0.22–1.15	0.103	0.51	0.19–1.34	0.169
ATM, mutated vs. wild type	1.08	0.33–3.54	0.894	1.85	0.39–8.83	0.439
HER2, overexpression vs. normal	1.27	0.51–3.17	0.616	0.83	0.29–2.39	0.735
Age, < 50 vs. >=50 years	0.99	0.97–1.02	0.692	0.98	0.94–1.01	0.220
G, G1 vs. G2 vs. G3	0.66	0.33–1.35	0.259	1.49	0.63–3.59	0.364
T, T1 vs. T2–T4	1.96	0.96–3.99	0.065	4.43	1.47–13.38	**0.008**
N, positive vs. negative	0.98	0.49–1.96	0.963	0.87	0.35–2.19	0.771
ER, positive vs. negative	1.44	0.73–2.86	0.297	1.03	0.42–2.55	0.943
PR, positive vs. negative	0.53	0.27–1.03	0.060	0.53	0.22–1.27	0.153

HR hazard ratio, *CI* confidence interval, *G* grade of malignancy, *T* tumor size, *N* nodal status, *ER* estrogen receptor, and *PR* progesterone receptor

Significant *P* values are in bold

Bulgarian patients with BC. For this reason, here we investigated the mutation spectra of *p53, BRCA1, ATM*, and *PIK3CA* and the expression profiles of *HER2* proto-oncogene in Bulgarian patients with sporadic BC. The individual clinical significance of the studied genes and their combined clinical impact on BC pathogenesis were further estimated.

In breast tumors, *p53* mutations occur with the frequency of 20–40%, depending on the tumor size and stage of disease (Borresen-Dale. 2003) and are often associated with more aggressive carcinogenesis and worse overall survival (Petitjean et al. 2007; Olivier et al. 2006, Langerød et al. 2007). Specific types of *p53* mutations have been associated with resistance to radiotherapy and different chemotherapeutic agents (Borresen-Dale. 2003; Lacroix et al. 2006). The relatively low frequency of *p53* mutations in Bulgarian population is not so unusual. Similar frequencies were found by other authors as well (Buzin et al. 2001; Elledge et al. 1993). Mutational spectrum of *p53* differs significantly in breast tumors from geographically and ethnically diverse populations. Differences in frequencies and patterns of somatic *p53* mutations presumably reflect exposure to different environmental mutagens. Thus, the low *p53* mutational frequency might be an indication of either a predominance of environmental effects or utilization of a different cancer pathway, in which different cancer-related genes are engaged. The mutational spectrum of *p53* was rather heterogeneous as missense and nonsense mutations, deletion and insertion frameshifts, splice-site mutation, silent substitutions, exon and intron polymorphisms were found. Six *p53* mutations mapped to known mutational hot-spots. Five of the *p53* mutations were novel and have never been so far reported in any type of human cancer. Two of the novel mutations contained an identical DNA region GTTT (12,247–12,250), which alone or along with a varying in size flanking sequence was previously found to be deleted in three patients with BC. This gives us reason to speculate that the GTTT (12,247–12,250) is a rather mutable region in BC. Another specificity of *p53* mutational spectrum in Bulgarian patients with BC is the high frequency of the 14,766 T > C intronic polymorphisms accounting to 0.0483. The same polymorphic variant was previously found in three Bulgarian patients with Balkan Endemic Nephropathy (Krasteva and Georgieva 2006) and in one patient with colorectal carcinoma (Georgieva et al. 2008).

Comparison with clinical parameters showed that mutated *p53* predicts an aggressive clinical course of breast carcinomas. In support to this is the observation that $p53^{+}$ women had larger and poorly differentiated tumors. The finding that *p53* mutations are present predominantly in G3, compared to *ATM* and *PIK3CA* ($P < 0.0001$), signifies that this mutational event is strongly engaged in tumor dedifferentiation. Furthermore, mutated *p53* decreases OS of patients with breast cancer (HR = 2.31, 95% CI 1.01–5.29, $P = 0.048$), though multivariate analysis failed to show an independent effect. *p53*-positive patients tended to be over 50, and they had worse OS compared to p53-negative patients over 50. This presumes a stronger effect of mutated *p53* on survival of older patients. We found also that the negative effect of mutant *p53* on survival is apparent in ER^{-} patients. The clinical importance of ER relates principally to the fact that its presence identifies hormone-sensitive tumors. About 50 to 60 percent of patients with significant amounts of ER in their tumors respond favorably to hormone or endocrine therapy. In the absence of endocrine therapy, however, it is known that a positive ER status is not a good prognostic marker. We could speculate that the negative effect of mutated *p53* on survival is stronger in case of ER^{-}, probably because in $p53^{+}ER^{+}$ patients, $p53^{+}$ effect could be masked as a consequence of beneficial hormone therapy.

It is known that familial BC often results from germ-line mutations in the *BRCA1* gene (Bartkova et al. 2005).

However, *BRCA1* mutations are very rare in the sporadic form of the disease. Our data showed extremely low frequency of *BRCA1* genetic alterations in the studied Bulgarian patients with sporadic BC. The lack of *BRCA1* mutations in sporadic BC presumes that the gene could be inactivated through epigenetic mechanisms. In support to this are some data indicating for inactivation of *BRCA1* gene by hypermethylation in the promoter region (Esteller et al. 2000).

As a key activator of the cellular responses to DNA double-strand breaks, the ATM kinase has an essential role in maintaining genomic integrity (Khanna et al. 2001). In A-T (ataxia-telangiectasia) families, a higher frequency of BC was found, which indicated an association between A-T and BC (Swift et al. 1991). Though it was initially suggested that *ATM* heterozygosity may account for 5% of all BC (Easton 1994; Gatti et al. 1999), the role of *ATM* as a BC susceptibility gene outside the A-T families has remained controversial (Khanna and Chenevix-Trench 2004). The data so far available on the clinical significance of *ATM* gene for BC prognosis and therapy are limited and controversial. The here-revealed mutational frequency in the *ATM* gene of 7.59% is the first data on *ATM* gene status in Bulgarian patients with BC. In contrast to *p53*, *ATM* mutational spectrum was rather homogeneous as all mutations were missense and were clustered in only two exons. The Ser707Pro substitution found here was predicted to abolish a putative glycosylation site and was previously associated with high-risk breast carcinoma, characterized by a positive axillary N status and an increased risk of contralateral BC (Dork et al. 2001; Izatt et al. 2000). The Asp1853Asn substitution was described in other populations, and previous studies suggested a functional role for the mutant variant (Maillet et al. 2000; González-Hormazábal et al. 2008). Combination between the mutation (Asp1853Asn) and the intronic variant (IVS38-8 T > C), when occurring in *cis* position, was previously shown to be associated with cancer risk modifying effect (Heikkinen et al. 2005). We found that neither mutated *ATM* as a whole nor different mutational subtypes had noticeable clinical significance, which is in accordance with data previously reported by Tommiska et al. (2006). However, though not statistically significant, all patients with an *ATM* mutation who did not survive the follow-up period had mutation of the type Asp1853Asn. Though not associated with more aggressive disease or worse survival by itself, in combination with ER^- status, mutated *ATM* reduced survival. We suppose that the effect of mutated *ATM* is dominating in ER^- patients, where hormone therapy is inadequate. Compared to *p53* and *PIK3CA*, *ATM* mutations prevail in the early stages of the carcinogenic process, which implies that they may be involved in the initiation of breast carcinogenesis. Therefore, dysfunction in *ATM* may lead to accumulation of mutations in other cancer-related genes, thus provoking a disruption in the signaling mechanisms, which control the normal cellular pathways.

As a part of PI3K–AKT signaling pathway, *PIK3CA* has been shown to play an important role in cancer development and progression (Bader et al. 2006; Vogt et al. 2007; Samuels et al. 2004). It has been recently reported that the hot-spot (in exons 9 and 20) activating mutations in *PIK3CA* lead to increased phosphorylation of AKT, which results in transformation of normal breast epithelial cells to tumor cells (Ikenoue et al. 2005; Samuels et al. 2005). In our study, we found that the frequency of *PIK3CA* mutations in Bulgarian patients with breast cancer is 31% (45/144), which is similar to other studied populations (Campbell et al. 2004; Bachman et al. 2004; Saal et al. 2005; Maruyama et al. 2007; Barbareschi et al. 2007). This mutational frequency is much higher compared to the other studied genes in this group, indicating the importance of the PI3K–AKT signaling pathway in breast cancer development. Unlike other authors who detected more mutations in exon 9 (Campbell et al. 2004; Barbareschi et al. 2007; Liedtke et al. 2008), our results showed that *PIK3CA* mutations are located predominantly in exon 20 (61%) than in exon 9 (39%). Similar exon distribution was registered by Li et al. (Li et al. 2006) and Saal et al. (Saal et al. 2005).

Only PR status was found to be significantly associated with *PIK3CA* mutations. Patients harboring *PIK3CA* mutations had more frequently PR^+ tumors compared to patients with the wild-type gene. Data on the steroid receptor correlation with *PIK3CA* status are contradictory. Some authors showed significant correlations with one (Maruyama et al. 2007) or with both receptors (Saal et al. 2005; Li et al. 2006), while others failed to detect such correlations. A possible explanation of a correlation between *PIK3CA* and steroid receptor status could be their engagement in a same signaling pathway. In support to this is a recent report of a direct functional link between the target of PIK3CA—AKT kinase and ER (Yamashita et al. 2005). We also found a trend ($P = 0.07$) for the association of *PIK3CA* mutations with the tumor grade. Most *PIK3CA* mutations (82.3%) were found in G2 tumors, and no mutations were present in G1. This is indicative that a *PIK3CA* mutation in the breast tumor possibly occurs at the later stages of the carcinogenic process.

We analyzed the potential relationship between mutated *PIK3CA* and genetic status of the other studied genes. We observed a tendency for a higher frequency of *PIK3CA* mutations in ATM^+ tumors. This presumes a preferential association between *PIK3CA* and *ATM* aberrations. No other correlations were found.

There are conflicting results in the literature concerning the contribution of *PIK3CA* mutations to the clinical outcome (Maruyama et al. 2007; Li et al. 2006). In our study, we found that $PIK3CA^+$ patients have a trend for better OS.

The potential protective effect of mutated *PIK3CA* was demonstrated both by univariate (HR = 0.50, 95% CI 0.22–1.15, $P = 0.103$) and multivariate analysis (HR = 0.51, 95% CI 0.19–1.34, $P = 0.169$). This effect was even stronger within the ER^+ subgroup. A way to explain this effect is a recent finding that breast cancer cells with *PIK3CA* mutations have an increased ER expression compared to $PIK3CA^-$ (Whyte and Holbeck 2006). As already known, patients with ER^+ tumors are more responsive to tamoxifen and thus have a better chance of survival. Another correlation we observed between *PIK3CA* mutations and *ATM* status. The beneficial effect of $PIK3CA^+$ on survival increased in ATM^- patients.

HER2 gene amplification and protein overexpression occurs in approximately 25–30% of breast cancers and generally is associated with poor prognosis (Slamon et al. 1987). Amplification/overexpression of HER2 is shown to correlate with several negative prognostic variables (Ross et al. 2003) and is associated with shorter survival, higher recurrence rates, and lower response to chemotherapy and hormone therapy (Carr et al. 2000). From a clinical point of view, HER2 has become important mainly as a target for antibody-based therapy with trastuzumab. Adjuvant treatment of primary, HER2-positive breast cancers with trastuzumab has been shown to improve markedly patients' outcome (Tuma 2005). At present, determination of HER2 status in every breast cancer patient to select for adjuvant treatment with trastuzumab is becoming a standard in BC clinical practice worldwide.

Our studies revealed that 21.21% of tumor samples overexpress the proto-oncogene. We found that HER2 overexpression is related to an aggressive clinical course of breast carcinomas. Consistent with previous data, $HER2^+$ tumors were significantly larger than $HER2^-$. However, conversely, they were mainly ER^+. Though related to aggressive breast carcinogenesis, $HER2^+$ did not correlate significantly with survival. In contrast to previously reported data (Rudolph et al. 1999), we found that HER2 overexpression occurred in older patients, over or at the age of 50.

Given the functional interactions between *p53, BRCA1, ATM, PIK3CA*, and *HER2* genes and preliminary data on the clinical significance of these interactions, we sought to determine whether tumors containing genetic abnormalities in at least two genes correlate with more aggressive carcinogenetic process, worse prognosis, and overall survival. The analysis of their combined clinical influence however did not provide convincing data supporting significant interdependence of the studied genetic factors with respect to clinical parameters and survival. A way to explain the lack of such a correlation is the limited number of the available cases with composite mutational genotypes. Therefore, we can not exclude the possibility of a shared effect of the studied genes in modifying breast carcinogenesis predetermined on the basis of their functional correlations.

Conclusions

This study provided the first elaborate data on genetic status of Bulgarian patients with breast cancer. The frequency and spectrum of alterations in *p53*, *BRCA1*, *ATM*, *PIK3CA*, and *HER2* genes were similar to other studied populations; however, novel mutations in *p53* gene were registered. Our data indicated specific impact of the studied genes on clinicopathological characteristics of patients with breast cancer. Only mutated *p53* showed significant, though not independent, negative effect on overall survival.

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Conflict of interest statement We declare that we have no competing interests.

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