CANCER GENOMICS & PROTEOMICS *17*: 181-193 (2020)
doi:10.21873/cgp.20179

Tumor Mutational Patterns and Infiltrating Lymphocyte Density in Young and Elderly Patients With Breast Cancer

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Key Words: Age, genotype, stromal TIL density, co-mutation, NGS.

Abstract. *Background/Aim: Age may pertain to different tumor genotype characteristics which may interfere with treatment efficacy and prognosis. We investigated the distribution and prognostic effect of mutations and tumor infiltrating lymphocyte (stromal TIL density) in young (≤35 years) and elderly (>65 years) early breast cancer patients. Materials and Methods: Paraffin tumor genotypes of all clinical subtypes from 345 patients were examined. Results: A total of 638 mutations were detected in 221 patients (64.1%). Compared to young, elderly patients presented with lower TIL density (p<0.001) but more*

TILs in TP53 mutated tumors (p=0.042). Mutation in one, rather than in 2 or more genes, conferred better outcome (DFS: HR=0.51, p=0.016; OS: HR=0.47, p=0.015) but the effect was age-independent. Conclusion: There are fewer TILs and different mutations patterns in tumors from elderly patients compared to young. Age and TIL-independent gene agnostic co-mutations affect patient outcome.

Breast cancer (BC) is known to be a heterogeneous disease and besides the classic biological predictors, age at diagnosis has been proven as an independent prognostic factor in several studies (1, 2). Age is confirmed as an independent prognostic variable for locoregional-free interval, distant metastasis-free interval and breast cancer specific survival (3-6). Among women diagnosed with breast cancer during 1996-2000, 44.2% were aged 65 or more years and only 2% were <35 years (7), although the latter incidence may be higher in specific ethnic groups (8). Compared to elderly, postmenopausal patients, young breast cancer patients more often have a family history of malignancy, deleterious germline mutations in *BRCA1/2* and other highly penetrant cancer predisposing genes, such as *TP53*, and they also present with more aggressive disease: poorly differentiated, HER2-positive or triple negative (TNBC) tumors (9). Breast cancer in the young has been associated with increased likeliness for metastases (10, 11), with poor overall prognosis (10, 12), even for stage I-II disease (1, 13), while the underlying biology of tumors in this age group has seldom been described in a context other than inherited breast cancer predisposition (14).

TP53 and *PIK3CA* mutations are the most frequent genetic alterations in breast cancer, with a similar prevalence for both genes, observed in 26-36% of breast carcinomas based on data from COSMIC and cBioPortal. *TP53* mutant genotypes are unfavorable prognosticators in Luminal A/B and TNBC patients (15). *TP53* and *PIK3CA* mutations appear to have diverse effects on the outcome of early BC patients, according to whether these genes are co-mutated or not, and for *TP53* according to stromal TIL density and ER/PgR-status (16, 17).

In this study, we assumed that differences in the prognosis of young and elderly patients with BC may be associated with different genotype characteristics and with the efficiency of immune response in the two age groups. We investigated the distribution and prognostic effect of tumor mutations and TIL density in young, ≤35 years old and elderly >65 years old early breast cancer patients, treated with adjuvant chemotherapy in the context of clinical trials.

Materials and Methods

Paraffin tumors (FFPE) from 345 out of 1,502 early breast cancer patients with next generation sequencing (NGS) informative data were examined. All patients had been treated with adjuvant chemotherapy in the setting of four prospective trials conducted by the Hellenic Cooperative Oncology group (HE10/97; HE10/00; HE10/05; HE10/08) as previously described (18). In the HE10/05 and HE10/08 studies, patients had received trastuzumab treatment for HER2-positive disease sequentially for one year upon completion of chemotherapy (post-trastuzumab era), while they did not in earlier studies (HE10/97 and HE10/00, pre-trastuzumab era). All patients had provided written consent for the use of their biologic material for research purposes and the study was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Health Sciences, Faculty of Medicine (#77/10June2014) and by the Institutional Review Board of the Papageorgiou Hospital of Thessaloniki (#725/10May2013). Patients had received adjuvant hormone therapy and trastuzumab based on ER/PgR/HER2 immunohistochemistry (IHC) subtyping at local pathology laboratories following initial diagnosis. Subsequently, FFPE tumors were centrally processed at the Laboratory of Molecular Oncology (Hellenic Foundation for Cancer Research/Aristotle University of Thessaloniki) for histology review and subtyping with ER/PgR/HER2/Ki67 IHC and fluorescent *in situ* hybridization for HER2 (15), for stromal tumor infiltrating lymphocyte (TIL) density, as previously described (18), and for DNA extraction and next-generation sequencing (NGS) genotyping.

NGS genotyping. FFPE tumor genotypes were obtained by semiconductor sequencing with a panel covering 34845 nucleotides in coding regions of 59 genes (19). The examined 345 samples contained >50% cancer cell DNA in >90% of the cases, exhibited on average 18 variants (min 5 – max 182), their average mean depth was 1605.7 (min 106.3 – max 22852), and their average uniformity was 75.3% (min 50.3% – max 92.1%). Variants were accepted if allele frequency ≥5%, quality of position reads $p<0.0001$, lack of GC-stretches, position coverage >100 and variant coverage >40. Amino acid and splice-site changing variants with population minor allele frequencies <0.1% (dbSNP, 5000Exomes) were considered as mutations. Pathogenic mutations were not distinguished, since the majority can still only be presumed, and this with an accuracy of ~70% (20), while their coexistence in tumors may not always result in the expected pathogenic effect (21).

Statistical analysis. Frequencies with the corresponding percentages were used to summarize categorical data, while medians and range were used to describe continuous variables. Associations between selected genes' mutational status and several clinicopathological parameters were assessed with the chi-square or Fisher's exact test (where appropriate). The non-parametric Wilcoxon rank-sum test was used to detect differences in continuous variables between young and elderly patients and in the groups defined by the selected genes' mutational status.

Disease-free survival (DFS) was estimated from the time of breast cancer diagnosis to the date of first documented progression, death (from any cause) or last contact (whichever occurred first). Overall survival (OS) was also estimated from the date of diagnosis to the date of patient's death. Alive patients were censored at the date of last contact. Time-to-event distributions were estimated with the Kaplan–Meier product limit method and compared among groups with the two-sided log-rank test. Time-dependent covariates were used to evaluate proportionality for all parameters.

The effect of mutations in several genes and other clinicopathological parameters of interest on patients' DFS and OS were examined with hazard ratios (HR) estimated with univariate and multivariate Cox proportional regression models in the entire cohort (N=345) and separately in young (N=88) and old patients (N=257).

In multivariate analysis, in the entire cohort, a backwards selection procedure with a removal criterion of $p>0.10$ was used with the following parameters in the initial step of the model: number of positive nodes (0-3, ≥4), *PIK3CA*/*TP53* mutational status (mutations in both genes, none, *PIK3CA* mutations only, *TP53* mutations only, no mutations), number of mutated genes per tumor (none mutated, 1 mutated, ≥2 mutated), TILs (10% increments) and age group (young, elderly) with respect to DFS and number of positive nodes (0-3, ≥4), *PIK3CA*/*TP53* mutational status (mutations in both genes, none, *PIK3CA* mutations only, *TP53* mutations only, no mutations), number of mutated genes per tumor (none mutated, 1 mutated, ≥2 mutated) and age group (young, elderly) with respect to OS. Except for the two age groups that were study objectives included in both models, despite non-significant differences in outcome, the parameters selected for inclusion in the first step of each model were variables that showed (marginal) significance ($p<0.050$) in the univariate analyses.

All tests were two-sided and the level of significance was set at 5%. Adjustment for multiple comparisons was not applied given that this study was exploratory and mainly hypothesis generating with predefined parameters. The SAS version 9.3 (SAS Institute) was used for data manipulation and statistical analyses. The R studio version 3.5.0 was used to produce maps with the mutation pattern of genes and violin plots.

Results

The 345 patients included in this study were categorized in two groups based on their age at diagnosis (88 young *vs*. 257 elderly). In total, 109 women were treated in the pre-trastuzumab era (32 young and 77 elderly), while the rest 236 patients (68.4%) received adjuvant chemotherapy in the post-trastuzumab era (56 young and 180 elderly). Basic patient and tumor characteristics for the entire cohort and by age group are presented in Table I. Elderly women presented with lower TIL density and Ki67 labeling (both Wilcoxon rank sum $p<0.001$, Figure 1A) and were more frequently of the invasive ductal histological type (chi-square $p=0.032$), while no further differences were detected in the examined clinicopathological parameters by age group. Of the 20 young patients with HER2-positive tumors, only 7 were treated in the pre-trastuzumab era, whereas 19 of the 53 elderly patients with HER2-positive tumors received adjuvant treatment in the pre-trastuzumab era.

In total, 638 mutations (median 1 per tumor; range=0-58) were observed in the tumors of 221 patients (64.1%) and concerned 48 genes out of 59 targeted with the panel. Fifty-four of the 88 tumors from young patients (61.4%) carried 120 mutations in 30 genes; respectively, 167 of the 257 tumors from elderly patients (65.0%) carried 518 mutations in 47 genes. Neither the number of mutations nor the number of mutated genes per tumor differed between young and older women (Table I). *TP53* and *PIK3CA* were the most commonly mutated genes in the entire cohort of patients in both age groups, as depicted in Figure 1B. In total, 27% of the study population carried mutations in *PIK3CA* and 25.2% in *TP53*. In elderly patients, 29.2% and 22.6% of the tumors had mutations in *PIK3CA* and *TP53*, respectively. In comparison, the respective prevalence was inverted in young patients, with 20.5% and 33.0% of the tumors carrying mutations in *PIK3CA* and *TP53*, but this difference did not reach statistical significance. The mutant allele fraction and the number of tumors with hotspot mutations in these genes did not differ in the two age groups.

GATA3 mutations were observed in 10.2% and 5.4% in young and elderly patients, respectively, again a difference that did not reach statistical significance ($p=0.12$). Interestingly though, in 7 out of 9 tumors from young patients with *GATA3* mutations, these variants were missense or frameshifts at p.Pro409, pathogenic according to COSMIC, and were present at a high fraction (>25%) in the examined samples. Variants at a high allelic fraction at the same codon and overall pathogenic/deleterious variants in the same coding region were observed in only 3 out 14 *GATA3* mutated tumors from older patients. All 11 *EGFR* mutated tumors (3.2% of all tumors) were detected in elderly patients (Fisher's $p=0.049$) but none of these *EGFR* mutations was actionable. In addition, 24 of the 26 tumors (92.3%) with mutations in *MAP3K1* and 17 of the 18 (94.4%) with mutations in *PTEN* were from patients of older age ($p=0.030$ and $p=0.046$, respectively). The single young patient with a *PTEN* mutated tumor carried one frameshift (p.Ile253Asn/c.756_757insA) variant of unknown significance (VUS) at an allele frequency of 46%, and an additional stop-gain variant (p.Arg233*/c.697C>T) at an allele frequency of 56%. *MAP3K1* variants were of low allele frequency in tumors from young patients (<10%) and occurred at high frequency in tumors from 3 older patients only.

As can be inferred from Figure 1B, >50% of the tumors with *PIK3CA* or *TP53* mutations also carried mutations in one or more additional genes; however, these two genes were not preferentially co-mutated. Seventy-one patients (20.6%) had only *PIK3CA* mutations (11 young, 12.5%; 60 elderly, 23.3%), 65 patients (18.8%) had only *TP53* mutations (22 young, 25.0%; 43 elderly, 16.7%), while 22 patients carried mutations in both genes (7 young, 8.0%; 15 elderly, 5.8%) and 18.3% of the study population had mutations in genes other than *TP53* and *PIK3CA*.

The associations of selected clinicopathological parameters with the mutational status of *TP53* and *PIK3CA* in the entire cohort and in the subgroups of young and elderly patients are presented in Table II. In the entire cohort, tumors with *PIK3CA* mutations were less frequently HER2-positive (chi-square $p<0.001$), while tumors with *TP53* mutations presented with higher TIL density and Ki67 levels (Wilcoxon rank-sum $p=0.004$ and $p<0.001$, respectively; Figure 2A), and were less frequently of lower grade (chi-square $p<0.001$), HER2-negative ($p=0.005$) and ER/PgR positive ($p=0.001$), compared to those without mutations in these genes. In elderly patients, *TP53* mutated tumors had higher TIL density and Ki67 levels (Wilcoxon rank-sum $p=0.042$ and $p<0.001$,

Table I. *Selected patient and tumor characteristics.*

	Total (N=345)	Young (N=88)	Elderly (N=257)	*p*-Value
TILs	5.0 (0.00,95.0)	10.0 (0.00,85.0)	5.0 (0.00,95.0)	**<0.001**[a]
Ki67	25.0 (0.00,98.0)	35.0 (0.00,97.0)	20.0 (0.00,98.0)	**<0.001**[a]
N of mutations per tumor	1.00 (0.00,58.0)	1.00 (0.00,13.0)	1.00 (0.00,58.0)	0.42[a]
N of mutated genes per tumor	1.00 (0.00,23.0)	1.00 (0.00,10.0)	1.00 (0.00,23.0)	0.35[a]
N of positive nodes	2.0 (0.00,47.0)	2.0 (0.00,31.0)	3.0 (0.00,47.0)	0.14[a]
Tumor size	2.5 (0.00,10.0)	3.0 (0.20,8.0)	2.5 (0.00,10.0)	0.099[a]
	N (%)	N (%)	N (%)	
N of mutations per tumor				0.72[b]
1 mutation	128 (37.1)	33 (37.5)	95 (37.0)	
≥2 mutations	93 (27.0)	21 (23.9)	72 (28.0)	
no mutation	124 (35.9)	34 (38.6)	90 (35.0)	
N of mutated genes per tumor				0.53[b]
1 mutation	135 (39.1)	36 (40.9)	99 (38.5)	
≥2 mutations	86 (24.9)	18 (20.5)	68 (26.5)	
no mutation	124 (35.9)	34 (38.6)	90 (35.0)	
N of positive nodes				0.62[b]
0-3	200 (58.0)	53 (60.2)	147 (57.2)	
≥4	145 (42.0)	35 (39.8)	110 (42.8)	
Tumor size				0.67[b]
≤2	128 (37.1)	31 (35.2)	97 (37.7)	
>2	217 (62.9)	57 (64.8)	160 (62.3)	
Histology Grade*				0.096[b]
I	12 (3.5)	2 (2.3)	10 (3.9)	
II	160 (46.6)	33 (37.9)	127 (49.6)	
III	171 (49.9)	52 (59.8)	119 (46.5)	
Histological type				0.032[b]
Invasive ductal	280 (81.2)	70 (79.5)	210 (81.7)	
Invasive lobular	27 (7.8)	3 (3.4)	24 (9.3)	
Mixed	12 (3.5)	3 (3.4)	9 (3.5)	
Other	26 (7.5)	12 (13.6)	14 (5.4)	
HER2 status*				0.72[b]
Negative	257 (77.9)	65 (76.5)	192 (78.4)	
Positive	73 (22.1)	20 (23.5)	53 (21.6)	
ER/PgR status*				0.92[b]
Negative	59 (18.2)	15 (17.9)	44 (18.3)	
Positive	265 (81.8)	69 (82.1)	196 (81.7)	
Subtypes*				0.059[b]
HER2-Enriched	18 (5.6)	4 (4.8)	14 (5.8)	
Luminal A	101 (31.2)	16 (19.0)	85 (35.4)	
Luminal B	112 (34.6)	37 (44.0)	75 (31.3)	
Luminal HER2	52 (16.0)	16 (19.0)	36 (15.0)	
TNBC	41 (12.7)	11 (13.1)	30 (12.5)	

*Data not available for all subjects. Missing values: Histology Grade=2, HER2 status=15, ER/PgR status=21, Subtypes=21. Values presented as Median (min, max) or N (column %). *p*-Values: [a]Wilcoxon-rank sum test, [b]Pearson's chi-square/Fisher's exact test. Bold values show significance.

respectively), while *PIK3CA* or *TP53* mutated tumors were less frequently HER2-negative (chi-square p=0.002 and p=0.004, respectively). In young patients, tumors with *TP53* mutations were less frequently of lower-grade (p=0.030). Neither TIL density nor any other clinicopathological parameter was associated with the number of mutations per tumor or the number of mutated genes per tumor in the entire cohort or in the subgroups defined by age.

At a median follow-up of 7.0 years (95%CI=6.8-7.2), a total of 88 events of progression or death (DFS events) had occurred (25 in young patients, 28.4% and 63 in elderly patients, 24.5%), while 69 patients had died (18 young patients, 20.5% and 51 elderly patients, 19.8%). In the entire cohort and in the subgroup of elderly women, the median DFS and OS was 14.5 years, while the median DFS and OS had not been reached yet among young women.

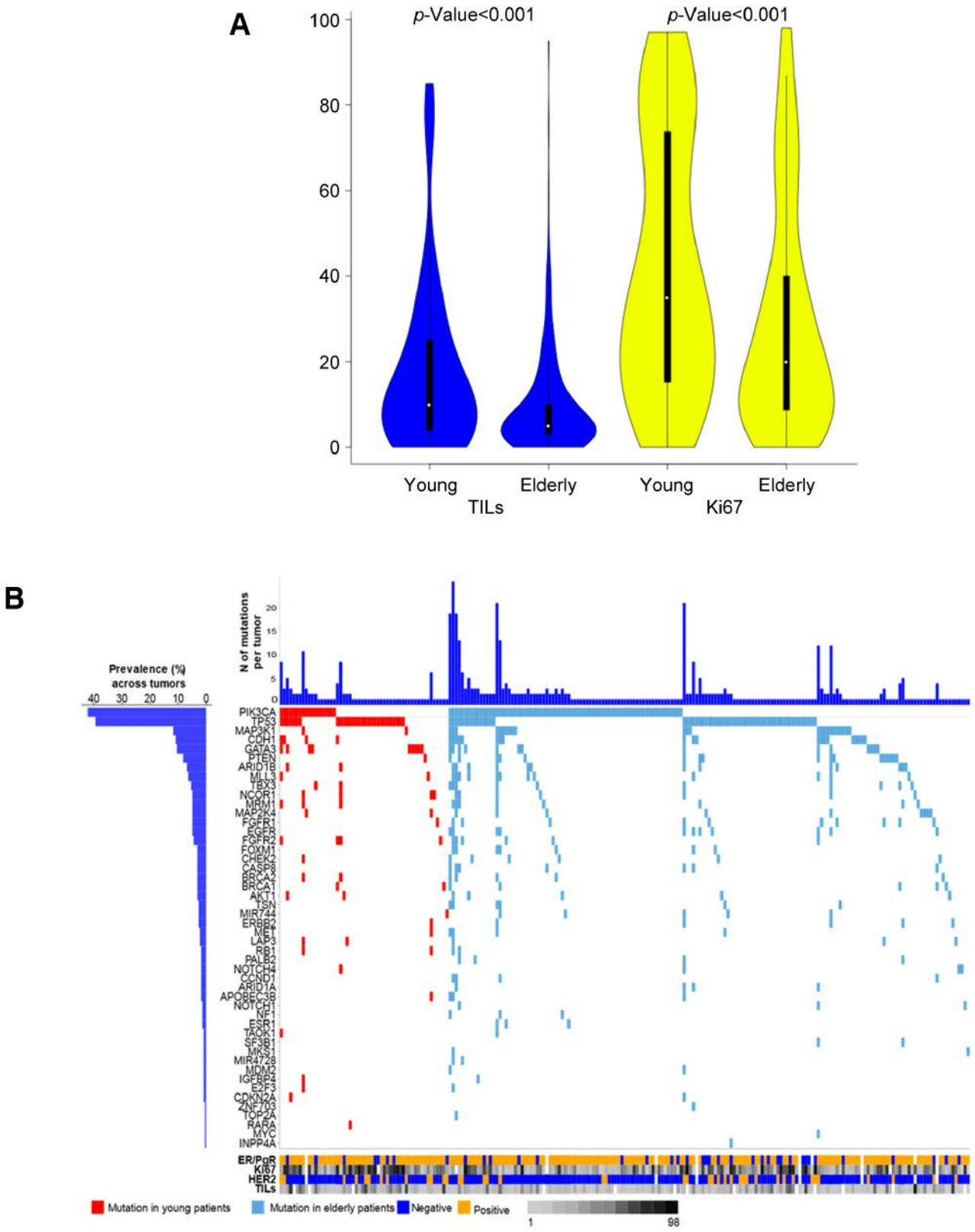

Figure 1. *TILs and mutations in tumors from young and elderly patients with early breast cancer. (A) Violin plots of TIL density and Ki67 by age group in the entire cohort of patients, (B) Map showing the distribution of mutations per tumor by age group.*

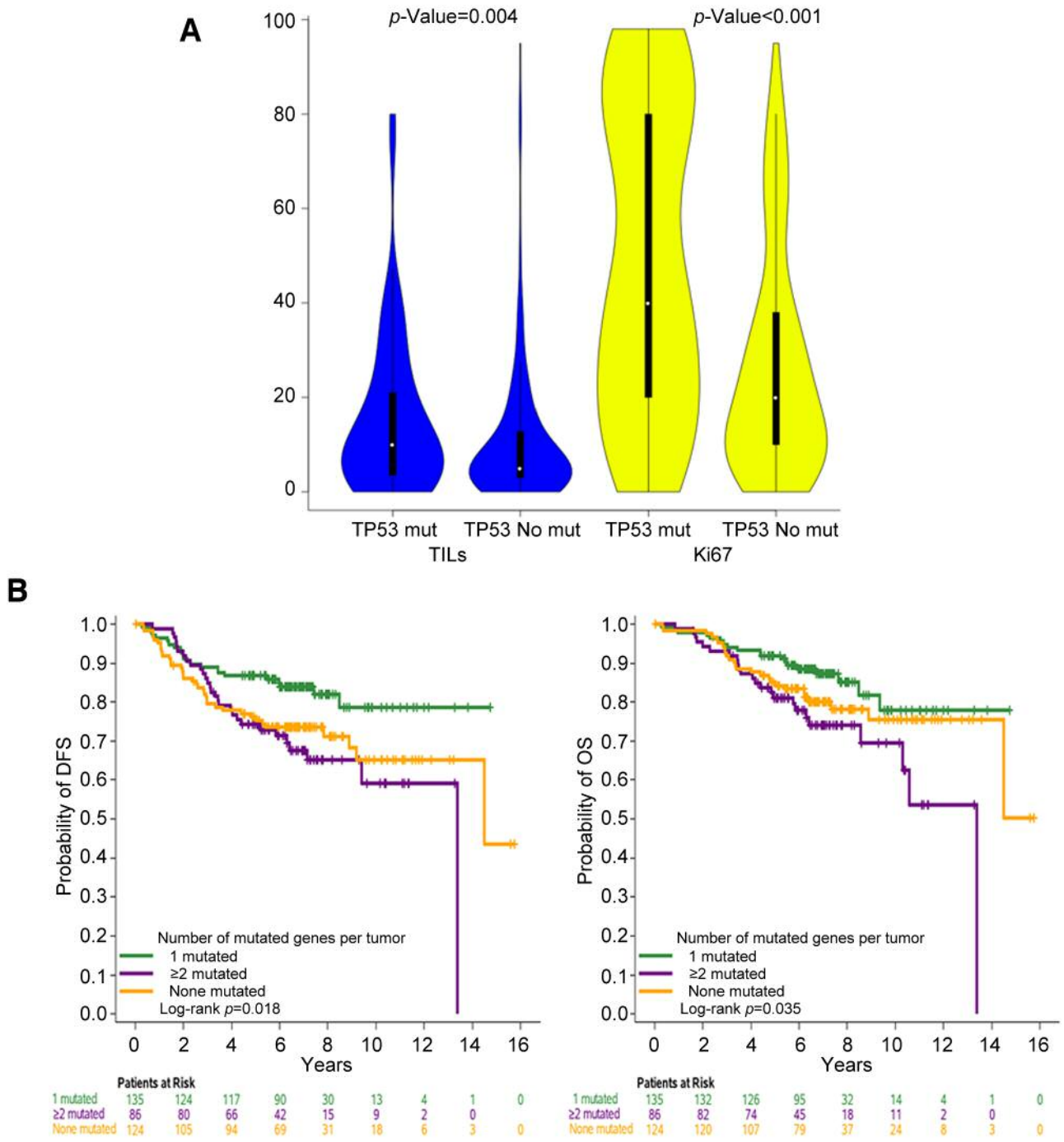

Figure 2. *Genotype features associated with clinicopathological characteristics and patient outcome. (A) Violin plots of TIL density and Ki67 by TP53 mutational status, (B) Kaplan–Meier curves based on the number of mutated genes per tumor with respect to DFS and OS in the entire study cohort.*

In the total cohort of patients (N=345), age was not associated with either DFS or OS (Wald's p=0.60 and p=0.70, respectively). Patients with mutations only in *PIK3CA* were at lower risk of progression and death compared to those carrying mutations in both *PIK3CA* and *TP53* genes (HR=0.33, p=0.013 and HR=0.37, 95% p=0.046, respectively, Table III). Additionally, patients carrying only one mutation as well as those with mutations in only one gene had longer DFS and OS compared to those with two or more mutations or mutated genes. Increased TILs conferred marginally significantly lower

Table II. *Associations of TP53 and PIK3CA mutational status with several clinicopathological parameters in the entire cohort and in the subgroups of patients defined by age.*

	Entire cohort PIK3CA			Young PIK3CA			Elderly IK3CA			Entire cohort TP53			Young TP53			Elderly TP53		
	Wild-type (N=252)	Mutated (N=93)	*p*-Value	Wild-type (N=70)	Mutated (N=18)	*p*-Value	Wild-type (N=182)	Mutated (N=75)	*p*-Value	Wild-type (N=258)	Mutated (N=87)	*p*-Value	Wild-type (N=59)	Mutated (N=29)	*p*-value	Wild-type (N=199)	Mutated (N=58)	*p*-value
Ki67	26.0 (0.00, 98.0)	16.0 (0.00, 95.0)	**0.019**[a]	35.0 (0.00, 97.0)	30.0 (3.0, 85.0)	0.49[a]	25.0 (0.00, 98.0)	15.0 (0.00, 95.0)	0.060[a]	20.0 (0.00, 95.0)	40.0 (0.00, 98.0)	**<0.001**[a]	28.0 (2.0, 95.0)	80.0 (0.00, 97.0)	**<0.001**[a]	17.5 (0.00, 95.0)	35.0 (0.00, 98.0)	**<0.001**[a]
TILs	5.0 (0.00, 95.0)	5.0 (0.00, 80.0)	0.62[a]	10.0 (0.00, 85.0)	7.5 (0.00, 80.0)	0.41[a]	5.0 (0.00, 95.0)	5.0 (0.00, 40.0)	0.69[a]	5.0 (0.00, 95.0)	10.0 (0.00, 80.0)	**0.004**[a]	10.0 (0.00, 85.0)	15.0 (0.00, 80.0)	0.14[a]	5.0 (0.00, 95.0)	8.0 (0.00, 75.0)	**0.042**[a]
Positive nodes	2.0 (0.00, 47.0)	2.0 (0.00, 31.0)	0.90[a]	2.0 (0.00, 29.0)	1.5 (0.00, 31.0)	0.97[a]	2.5 (0.00, 47.0)	3.0 (0.00, 28.0)	0.98[a]	2.0 (0.00, 47.0)	2.0 (0.00, 43.0)	0.24[a]	2.0 (0.00, 20.0)	2.0 (0.00, 31.0)	0.71[a]	3.0 (0.00, 47.0)	2.0 (0.00, 43.0)	0.14[a]
Mutated genes per tumor	1.00 (0.00, 19.0)	2.0 (1.00, 23.0)	**<0.001**[a]	1.00 (0.00, 8.0)	2.0 (1.00, 10.0)	**<0.001**[a]	1.00 (0.00, 19.0)	2.0 (1.00, 23.0)	**<0.001**[a]	1.00 (0.00, 19.0)	1.00 (1.00, 23.0)	**<0.001**[a]	0.00 (0.00, 10.0)	1.00 (1.00, 8.0)	**<0.001**[a]	1.00 (0.00, 19.0)	2.0 (1.00, 23.0)	**<0.001**[a]
Mutations per tumor	1.00 (0.00, 21.0)	2.0 (1.00, 58.0)	**<0.001**[a]	1.00 (0.00, 13.0)	2.0 (1.00, 12.0)	**<0.001**[a]	1.00 (0.00, 21.0)	2.0 (1.00, 58.0)	**<0.001**[a]	1.00 (0.00, 46.0)	2.0 (1.00, 58.0)	**<0.001**[a]	0.00 (0.00, 11.0)	1.00 (1.00, 13.0)	**<0.001**[a]	1.00 (0.00, 46.0)	2.0 (1.00, 58.0)	**<0.001**[a]
Tumor size	2.6 (0.00, 10.0)	2.5 (1.00, 7.0)	0.78[a]	2.8 (0.20, 8.0)	3.6 (1.2, 7.0)	0.14[a]	2.5 (0.00, 10.0)	2.5 (1.00, 7.0)	0.33[a]	2.5 (0.00, 10.0)	2.7 (1.00, 8.0)	0.14[a]	2.8 (0.20, 8.0)	3.0 (1.5, 8.0)	0.34[a]	2.5 (0.00, 10.0)	2.6 (1.00, 8.0)	0.36[a]
Histology grade			0.074[b]			0.34[b]			0.18[b]			**<0.001**[b]			**0.030**[b]			**<0.001**[b]
I-II	118 (47.2)	54 (58.1)		26 (37.7)	9 (50.0)		92 (50.8)	45 (60.0)		147 (57.4)	25 (28.7)		28 (48.3)	7 (24.1)		119 (60.1)	18 (31.0)	
III	132 (52.8)	39 (41.9)		43 (62.3)	9 (50.0)		89 (49.2)	30 (40.0)		109 (42.6)	62 (71.3)		30 (51.7)	22 (75.9)		79 (39.9)	40 (69.0)	
Histological type			0.31[b]			**0.045**[b]			0.88[b]			0.060[b]			0.86[b]			**0.028**[b]
Invasive ductal	202 (80.2)	78 (83.9)		55 (78.6)	15 (83.3)		147 (80.8)	63 (84.0)		209 (81.0)	71 (81.6)		46 (78.0)	24 (82.8)		163 (81.9)	47 (81.0)	
Invasive lobular	19 (7.5)	8 (8.6)		2 (2.9)	1 (5.6)		17 (9.3)	7 (9.3)		23 (8.9)	4 (4.6)		3 (5.1)	0 (0.0)		20 (10.1)	4 (6.9)	
Mixed	8 (3.2)	4 (4.3)		1 (1.4)	2 (11.1)		7 (3.8)	2 (2.7)		11 (4.3)	1 (1.1)		2 (3.4)	1 (3.4)		9 (4.5)	0 (0.0)	
Other	23 (9.1)	3 (3.2)		12 (17.1)	0 (0.0)		11 (6.0)	3 (4.0)		15 (5.8)	11 (12.6)		8 (13.6)	4 (13.8)		7 (3.5)	7 (12.1)	
HER2 status			**<0.001**[b]			0.20[b]			**0.002**[b]			**0.005**[b]			0.53[b]			**0.004**[b]
Negative	175 (73.2)	82 (90.1)		50 (73.5)	15 (88.2)		125 (73.1)	67 (90.5)		200 (81.6)	57 (67.1)		44 (78.6)	21 (72.4)		156 (82.5)	36 (64.3)	
Positive	64 (26.8)	9 (9.9)		18 (26.5)	2 (11.8)		46 (26.9)	7 (9.5)		45 (18.4)	28 (32.9)		12 (21.4)	8 (27.6)		33 (17.5)	20 (35.7)	
ER/PgR status			0.083[b]			0.46[b]			0.11[b]			**0.001**[b]			0.62[b]			**<0.001**[b]
Negative	48 (20.5)	11 (12.2)		13 (19.4)	2 (11.8)		35 (21.0)	9 (12.3)		34 (14.2)	25 (29.8)		9 (16.4)	6 (20.7)		25 (13.5)	19 (34.5)	
Positive	186 (79.5)	79 (87.8)		54 (80.6)	15 (88.2)		132 (79.0)	64 (87.7)		206 (85.8)	59 (70.2)		46 (83.6)	23 (79.3)		160 (86.5)	36 (65.5)	
Subtypes			**0.014**[b]			0.42[b]			**0.047**[b]			**<0.001**[b]			0.12[b]			**<0.001**[b]
HER2-enriched	17 (7.3)	1 (1.1)		4 (6.0)	0 (0.0)		13 (7.8)	1 (1.4)		9 (3.8)	9 (10.7)		2 (3.6)	2 (6.9)		7 (3.8)	7 (12.7)	
Luminal A	44 (18.8)	8 (8.9)		14 (20.9)	2 (11.8)		30 (18.0)	6 (8.2)		34 (14.2)	18 (21.4)		10 (18.2)	6 (20.7)		24 (13.0)	12 (21.8)	
Luminal B	65 (27.8)	36 (40.0)		10 (14.9)	6 (35.3)		55 (32.9)	30 (41.1)		89 (37.1)	12 (14.3)		15 (27.3)	1 (3.4)		74 (40.0)	11 (20.0)	
Luminal HER2	77 (32.9)	35 (38.9)		30 (44.8)	7 (41.2)		47 (28.1)	28 (38.4)		83 (34.6)	29 (34.5)		21 (38.2)	16 (55.2)		62 (33.5)	13 (23.6)	
TNBC	31 (13.2)	10 (11.1)		9 (13.4)	2 (11.8)		22 (13.2)	8 (11.0)		25 (10.4)	16 (19.0)		7 (12.7)	4 (13.8)		18 (9.7)	12 (21.8)	
Positive nodes			0.32[b]			0.53[b]			0.39[b]			0.69[b]			0.25[b]			0.25[b]
0-3	142 (56.3)	58 (62.4)		41 (58.6)	12 (66.7)		101 (55.5)	46 (61.3)		148 (57.4)	52 (59.8)		38 (64.4)	15 (51.7)		110 (55.3)	37 (63.8)	
≥4	110 (43.7)	35 (37.6)		29 (41.4)	6 (33.3)		81 (44.5)	29 (38.7)		110 (42.6)	35 (40.2)		21 (35.6)	14 (48.3)		89 (44.7)	21 (36.2)	
Tumor size			0.38[b]			0.46[b]			0.18[b]			0.40[b]			0.56[b]			0.56[b]
≤2	90 (35.7)	38 (40.9)		26 (37.1)	5 (27.8)		64 (35.2)	33 (44.0)		99 (38.4)	29 (33.3)		22 (37.3)	9 (31.0)		77 (38.7)	20 (34.5)	
>2	162 (64.3)	55 (59.1)		44 (62.9)	13 (72.2)		118 (64.8)	42 (56.0)		159 (61.6)	58 (66.7)		37 (62.7)	20 (69.0)		122 (61.3)	38 (65.5)	

[a]Wilcoxon-rank sum test; [b]Pearson's chi-square/Fisher's exact test; N: Number. Bold values show significance.

Table III. *Hazard ratios and 95%CIs estimated by univariate Cox regression with respect to disease-free survival (DFS) and overall survival (OS) in the total cohort of patients (N=345).*

	Event/Total	Hazard ratio (95%CI)	*p*-Value	Event/Total	Hazard ratio (95%CI)	*p*-Value
	DFS			OS		
Age group						
Young (≤35)	25/88	1.13 (0.71-1.80)	0.60	18/88	0.90 (0.52-1.54)	0.70
Elderly (>65)	63/257	Reference	--	51/257	Reference	--
Positive nodes						
0-3	35/200	Reference	--	21/200	Reference	--
≥4	53/145	2.22 (1.44-3.40)	**<0.001**	48/145	3.17 (1.89-5.30)	**<0.001**
PIK3CA						
Mutated	20/93	0.78 (0.47-1.29)	0.34	16/93	0.87 (0.50-1.53)	0.63
Wild-type	68/252	Reference	--	53/252	Reference	--
TP53						
Mutated	24/87	1.07 (0.67-1.72)	0.76	19/87	1.09 (0.64-1.85)	0.75
Wild-type	64/258	Reference	--	50/258	Reference	--
PIK3CA/TP53						
No mutation	36/124	0.65 (0.31-1.35)	0.25	26/124	0.57 (0.24-1.31)	0.18
PIK3CA mutation only	11/71	0.33 (0.14-0.79)	**0.013**	9/71	0.37 (0.14-0.98)	**0.046**
TP53 mutation only	15/65	0.48 (0.21-1.09)	0.080	12/65	0.48 (0.19-1.23)	0.13
Both	9/22	Reference	--	7/22	Reference	--
None mutated[1]	17/63	0.63 (0.28-1.42)	0.27	15/63	0.72 (0.30-1.78)	0.48
TILs*		0.82 (0.67-1.00)	**0.048**		0.83 (0.66-1.03)	0.096
Ki67^		1.00 (0.99-1.01)	0.99		1.00 (0.99-1.01)	0.51
Mutations per tumor^		0.99 (0.95-1.00)	0.72		0.98 (0.92-1.05)	0.57
Mutated genes per tumor^		1.00 (0.92-1.08)	0.91		0.99 (0.91-1.09)	0.90
Mutations per tumor						
1 mutation	22/128	0.49 (0.29-0.86)	**0.012**	18/128	0.47 (0.26-0.87)	**0.016**
≥2 mutations	30/93	Reference	--	25/93	Reference	--
No mutation	36/124	0.88 (0.54-1.43)	0.61	26/124	0.69 (0.40-1.21)	0.19
Mutated genes per tumor						
1 mutated	23/135	0.47 (0.27-0.82)	**0.008**	19/135	0.46 (0.25-0.84)	**0.011**
≥2 mutated	29/86	Reference	--	24/86	Reference	--
None mutated	36/124	0.85 (0.52-1.39)	0.51	26/124	0.67 (0.38-1.17)	0.16

[1]None of the *PIK3CA* and *TP53* were mutated; ^continuous variable; *10% increments. Bold values show significance.

risk of progression (HR=0.82, 95%CI=0.67-1.00 for each 10% TIL increment, p=0.048) (Table III). Likewise, elderly patients with only one mutation or with mutations in only one gene were at lower risk of progression and death compared to those with multiple mutations or multiple mutated genes (Table IV). Of note, tumors with multiple mutated genes and tumors without detected mutations with the applied panel were associated with similar outcomes (Figure 2B). No significant associations were detected between any of the examined parameters and DFS or OS among young women (Table V). Regarding the effect of clinicopathological parameters on patient outcome, only the number of positive nodes was found to be of prognostic significance for both DFS and OS. Women with four or more positive nodes were at higher risk of progression and death in the entire cohort of patients as well as in the subgroups of young (DFS: HR=2.79, 95%CI=1.23-6.33, p=0.014 and OS: HR=2.52, 95%CI=0.94-6.78, p=0.066, respectively) and elderly patients (DFS: HR=2.02, 95%CI=1.22-3.35, p=0.006 and OS: HR=3.39, 95%CI=1.85-6.21, p<0.001, respectively).

Upon multivariate analysis, in the entire cohort, the presence of mutations only in one gene retained its favorable prognostic significance for both DFS and OS (HR=0.51, 95%CI=0.29-0.88, p=0.016 and HR=0.47, 95%CI=0.26-0.86, p=0.015, respectively) but was independent of age (Table VI).

Discussion

Analyses comparing breast cancer in young *vs*. old women have as yet focused on the worse prognosis usually reported for younger women and on the increased frequency of inherited disease in this group of patients. Here, we investigated the impact of age on breast tumor genotype characteristics and TIL density, two parameters that are used for predicting patient prognosis and for treatment decision

Table IV. *Hazard ratios and 95%CIs estimated by univariate Cox regression with respect to DFS and OS in elderly patients (N=257).*

	Event/Total	Hazard ratio (95%CI)	*p*-Value	Event/Total	Hazard ratio (95%CI)	*p*-Value
	DFS			OS		
PIK3CA						
Mutated	16/75	0.81 (0.46-1.44)	0.48	13/75	0.88 (0.47-1.65)	0.69
Wild-type	47/182	Reference	--	38/182	Reference	--
TP53						
Mutated	15/58	1.06 (0.59-1.90)	0.84	12/58	1.05 (0.55-2.01)	0.88
Wild-type	48/199	Reference	--	39/199	Reference	--
PIK3CA/TP53						
No mutation	23/90	0.70 (0.26-1.85)	0.47	17/90	0.59 (0.20-1.78)	0.35
PIK3CA mutation only	11/60	0.50 (0.17-1.43)	0.19	9/60	0.52 (0.16-1.69)	0.28
TP53 mutation only	10/43	0.63 (0.21-1.83)	0.39	8/43	0.60 (0.18-2.01)	0.41
Both	5/15	Reference	--	4/15	Reference	--
None[1]	14/49	0.86 (0.31-2.39)	0.77	13/49	0.99 (0.32-3.05)	0.99
TILs*		0.74 (0.54-1.01)	0.058		0.75 (0.53-1.06)	0.10
Ki67^		1.00 (0.99-1.01)	0.93		1.00 (0.99-1.01)	0.58
Mutations per tumor^		1.00 (0.95-1.04)	0.83		0.99 (0.94-1.05)	0.78
Mutated genes per tumor^		1.01 (0.93-1.09)	0.83		1.02 (0.93-1.11)	0.74
Mutations per tumor						
1 mutation	16/95	0.47 (0.25-0.88)	**0.018**	13/95	0.43 (0.22-0.86)	**0.017**
≥2 mutations	24/72	Reference	--	21/72	Reference	--
No mutation	23/90	0.71 (0.40-1.28)	0.26	17/90	0.55 (0.29-1.06)	0.074
Mutated genes per tumor						
1 mutated	16/99	0.42 (0.22-0.80)	**0.008**	13/99	0.39 (0.20-0.79)	**0.008**
≥2 mutated	24/68	Reference	--	21/68	Reference	--
None mutated	23/90	0.67 (0.38-1.20)	0.18	17/90	0.52 (0.27-1.00)	0.051

[1]None of the *PIK3CA* and *TP53* were mutated; ^continuous variable; *10% increments. Bold values show significance.

making. This is the first study addressing differences in tumor genotypes and immune response characteristics in extreme age groups in breast cancer, in an effort to approach the recently highlighted paucity on genomic data that may shed further insights into the biology of breast cancer in young compared to older patients (22).

We observed the expected (23) prevalence of top mutated genes in breast cancer, *i.e.*, *TP53* and *PIK3CA* each >25% followed by <10% of each *GATA3, CDH1, MAP3K1* and *PTEN*. Mutations in the latter two genes characterized tumors of older age, with the exception of one young patient with a *PTEN* mutated tumor that exhibited two variants described in the context of the *PTEN* hamartoma tumor syndrome (24). Worth mentioning, the herein identified *GATA3* mutations with driver characteristics (abrogative for the corresponding protein, pathogenic in breast cancer according to COSMIC, high allelic fraction) in tumors from younger patients pertained to the same proline at codon 409, while *GATA3* mutations in the older age group did not share these characteristics. *GATA3* is recurrently mutated in breast cancer, mostly in Luminal A/B tumors (25). High expression of the *GATA3* protein (26) and *GATA3* mutations (27) have traditionally been considered as favorable prognosticators in early breast cancer and do not seem to interfere with benefit from endocrine therapy. It is reported though, that the impact of *GATA3* mutations on prognosis is diverse (favorable and unfavorable) depending on the affected gene domain (28). Clearly, the number of patients carrying *GATA3* mutations did not allow for comparisons against outcome in the two study groups. However, the described unique features of *GATA3* and *PTEN* mutations in tumors from extreme age groups and their anticipated diverse impact on outcome prompt for personalizing the interpretation of such alterations for the individual patient.

As previously described, *TP53* mutations are unfavorable prognosticators in non-HER2-positive breast cancer, while *PIK3CA* mutations are favorable in Luminal A/B tumors (15), particularly if occurring in the absence of *TP53* mutations (16). For these genes, however, we observed an inverse distribution of mutation patterns in tumors from the two study groups; more *TP53* in the young, more *PIK3CA* in the older patients. This pattern, in association with the expected aggravated characteristics of *TP53* and the more favorable ones of *PIK3CA* mutated tumors (23) is theoretically compatible with the worse outcome traditionally described for breast cancer in young patients (1, 2, 10-13). However, the expected associations of *TP53* with ER-negative, HER2-positive, high grade, highly proliferating tumors, and of *PIK3CA* with ER-positive tumors (23), were

Table V. *Hazard ratios and 95% CIs estimated by univariate Cox regression with respect to DFS and OS in young patients (N=88).*

	Event/Total	Hazard ratio (95%CI)	*p*-Value	Event/Total	Hazard ratio (95%CI)	*p*-Value
	DFS			OS		
PIK3CA						
Mutated	4/18	0.71 (0.24-2.06)	0.53	3/18	0.81 (0.23-2.79)	0.73
Wild-type	21/70	Reference	--	15/70	Reference	--
TP53						
Mutated	9/29	1.11 (0.49-2.52)	0.80	7/29	1.26 (0.49-3.25)	0.64
Wild-type	16/59	Reference	--	11/59	Reference	--
PIK3CA/TP53			0.21		0.41	
Wild-type	13/34	0.56 (0.18-1.72)	0.31	9/34	0.50 (0.14-1.86)	0.30
PIK3CA mut only	0/11	0.05 (0.00-1.15)	0.061	0/11	0.08 (0.00-1.84)	0.11
TP53 mut only	5/22	0.32 (0.09-1.17)	0.085	4/22	0.34 (0.08-1.51)	0.16
Both	4/7	Reference	--	3/7	Reference	--
None	3/14	0.35 (0.08-1.53)	0.17	2/14	0.32 (0.06-1.80)	0.19
GATA3						
Mutated	0/9	0.15 (0.01-2.67)	0.20	0/9	0.25 (0.01-4.43)	0.34
Wild-type	25/79	Reference	--	18/79	Reference	--
TILs*		0.87 (0.67-1.12)	0.27		0.91 (0.69-1.21)	0.53
Ki67^		1.00 (0.99-1.01)	0.91		1.01 (0.99-1.02)	0.57
Mutations per tumor^		0.96 (0.80-1.16)	0.67		0.80 (0.54-1.19)	0.28
Mutated genes per tumor^		0.92 (0.71-1.19)	0.50		0.78 (0.50-1.21)	0.27
Mutations per tumor^						
1 mutation	6/33	0.62 (0.20-1.92)	0.41	5/33	0.79 (0.21-2.93)	0.72
≥2 mutations	6/21	Reference	--	4/21	Reference	--
No mutation	13/34	1.42 (0.54-3.73)	0.48	9/34	1.43 (0.44-4.65)	0.55
Mutated genes per tumor^						
1 mutation	7/36	0.71 (0.23-2.24)	0.56	6/36	0.99 (0.25-3.98)	0.99
≥2 mutations	5/18	Reference	--	3/18	Reference	--
No mutation	13/34	1.50 (0.53-4.20)	0.44	9/34	1.64 (0.44-6.06)	0.46

^Continuous variable; *10% increments.

observed in elderly but not in young patients. In the latter group, *PIK3CA* mutations were seldom detected in the absence of *TP53* mutations. These profiles may be partly attributed to the subtype distribution in the young group, which was shifted towards Luminal B and Luminal HER2 tumors, in contrast to the elderly group, where Luminal A tumors prevailed. The subtype profile in the young is in line with previous observations in this age group (9). Breast cancers arising in carriers of pathogenic *BRCA1* variants would be more likely to bear *TP53* mutations and *PIK3CA* amplification (29) but not the hotspot missense single nucleotide variants (SNVs) observed here. Unfortunately, information on germline status was not available for our patients. It is expected though that part of the examined tumors developed on an inherited background in our young patients, which would further justify the observed *TP53* prevalence and distinct *PIK3CA* mutation profiles. Importantly, other than expected, we did not observe any age-associated difference in prognosis in the examined cohort. Tumors in the two groups were balanced for nodal status and tumor size; hence, these parameters that were previously associated with worse outcome in the young (1, 9), could not have accounted for this controversial finding. Given all the above, our data are in line with more recent observations attributing the worse prognosis of breast cancer in young patients to the intrinsic biology of the tumors, and especially to the Luminal A subtype, while prognosis for all other subtypes is comparable to that observed in elderly patients (30). Overall, our data highlight the difference of *TP53* and *PIK3CA* associated genotypes in young and older patients. This suggests that in young patients (a) mutations in these genes should be examined along with germline data, and (b) the mere presence of *PIK3CA* mutations may not be helpful in assessing patient prognosis and management.

An additional novel piece of data presented here pertains to anti-tumor host immune response in the extreme age groups of patients with breast cancer. TIL density was more intense in tumors from young compared to elderly patients, which may be attributed to the generally accepted decline in the immune system efficiency with progressing age. A further reason for tumors in the young being more TIL-rich could be the underlying germline *BRCA1* background in some of the

Table VI. *Hazard ratios and 95%CIs estimated by multivariate Cox regression with respect to DFS and OS in the entire cohort of patients; results of backwards selection models.*

	Event/Total	Hazard ratio (95%CI)	*p*-Value
		DFS*	
Positive nodes			
0-3	35/200	Reference	--
≥4	53/145	2.17 (1.41-3.33)	**<0.001**
Mutated genes per tumor			
1 mutated	23/135	0.51 (0.29-0.88)	**0.016**
≥2 mutated	29/86	Reference	--
None mutated	36/124	0.82 (0.50-1.34)	0.43
TILs^		0.83 (0.69-1.02)	0.071
OS**			
Positive nodes			
0-3	21/200	Reference	
≥4	48/145	3.15 (1.88-5.28)	**<0.001**
Mutated genes per tumor			
1 mutated	19/135	0.47 (0.26-0.86)	**0.015**
≥2 mutated	24/86	Reference	
None mutated	26/124	0.62 (0.35-1.08)	0.093

^10% increments. **PIK3CA/TP53* was removed from the model with p=0.23; age (young *vs*. elderly) was removed from the model with p=0.19. ***PIK3CA/TP53* was removed from the model with p=0.14; age (young *vs*. elderly) was removed from the model with p=0.75. Bold values show significance.

patients (31), which, although suspected, cannot be proven in our case. The diverse association of *TP53* mutations with TIL density, which was prominent in the elderly but not in the young patients, may be related to different mutagenic processes operating in the two age groups (32). The result might be the same mutations, as we observe them with a targeted panel; however, these will operate in a different genomic and hence molecular environment, and may elicit different anti-tumor immune response. These data are new and intriguing for further research and elucidation, particularly with respect to immunotherapy research trials and applications in breast cancer.

Lastly, we observed an adverse prognostic impact of multiple mutated genes on patient outcome, irrespectively of patient age. Because we did not assess pathogenic/deleterious mutations in particular, because the vast majority of the examined mutations are anticipated to be acquired (somatic), and although the size of the applied panel is evidently too small for counting mutations per megabase, this finding may be related to tumor mutational load or burden (TMB). This parameter has only recently been investigated in breast cancer (33-35) with respect to response to immunotherapy, in the metastatic setting only (35). This gene- and mutation-agnostic parameter has been associated with underlying alterations in particular nucleic acid repair genes (34). Based on the aforementioned reports, a higher number of mutations and mutated genes would be expected to associate with a higher TIL density and better survival. We observed such features with the same panel in a previous report by our Group in *de novo* but not in relapsed metastatic breast cancer (36). The impact of TMB on the outcome of patients with early breast cancer treated with adjuvant chemotherapy, as is the case for the present cohort, may not necessarily be the same as in various metastatic settings and needs to be elucidated.

Limitations of the present study include (a) the small size of the young age group, which precluded statistical analysis for most individual genes, a concern that has been highlighted already in the first presentation on breast cancer by The Cancer Genome Atlas (TCGA) (23); (b) the fact that information on patient germline status was not available. For the above reasons, our study should be considered as hypothesis generating and all findings should be pursued in larger studies.

In conclusion, here, we presented differences in the mutational patterns of tumors in young compared to elderly patients, pertaining to genes frequently mutated in breast cancer, such as *TP53, PIK3CA, GATA3* and *PTEN*. We also identified differences in the two age groups with respect to anti-tumor immune response in general and to *TP53* mutations in particular. If further validated, these findings, along with the novel finding on the adverse prognostic effect of gene-agnostic multiple mutations will aid in understanding molecular markers and underlying mechanisms for testing existing and new drugs in the context of personalized treatment of early breast cancer.

Funding Information

This study was supported by an internal Hellenic Cooperative Oncology Group (HeCOG) translational research grant (HE TRANS_BR). The funders played no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This study was also partly supported by the Greek General Secretary for Research and Technology (GSRT) Program, Research in Excellence II, funded by 75% from the European Union and the Operational Program ''Education & Lifelong Learning'' ESPA-THALIS#266 of the Ministry of Education, Lifelong Learning & Religious Affairs.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

Authors' Contributions

Conceptualization: AN, VK, GF; Formal Analysis: GAK, EG; Investigation: KP, MB, KC; Resources: AN, FZ, GP, HG, GO, DP, ES, NA, IN, PP, IB, GA, IA, GF; Writing – editing: AN, VK, GAK, KP, GF; Writing – review and editing: All Authors.

Acknowledgements

The Authors are indebted to all patients and their families for their trust and participation in the HeCOG trials and for the provision of biological material for research purposes. The Authors wish to thank Ms Emily Daskalaki for excellent technical assistance with NGS and for NGS library construction; Ms Sofia Chrisafi, MSc for performing IHC; and Ms Helen Sgouramalli, MSc for block sectioning. Part of this work was presented at St. Gallen International Breast Cancer Conference 2019.

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Received December 3, 2019
Revised December 16, 2019
Accepted December 17, 2019