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Original Article

Disease evolution and heterogeneity in bilateral breast cancer

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Abstract: Bilateral breast cancers (BBC) are currently treated as independent tumors arising in the same patient. Herein, we investigated whether BBC indeed evolve independently at the genomic level. We examined paired targeted next generation sequencing genotypes from 155 paraffin tumors corresponding to 76 BBC patients (75 women and one man; 52 concurrent and 24 metachronous), for coding mutations (amino acid changing, minor allele frequency <0.1%) and single nucleotide polymorphism (SNP) zygosity. Germline genotypes were available for 29 patients. Mutations were present in 80 tumors (54/76 patients; 71%), were mostly tumor-private (90%), more frequent in TP53 (19%), PIK3CA (14%), CDH1, GATA3, MLL3. TP53 mutations were more frequent in metachronous tumors (P<0.001); hormone receptor negative (P<0.001); with higher Ki-67 (P=0.002); and, in younger patients (P=0.01). Hypermutated tumors, all TP53 mutated, were diagnosed as the first incidence in 5 patients; their metachronous counterparts were mutation poor without TP53 involvement. Paired tumors shared common mutations at intratumoral frequency >20% in 10/54 comparable BBC (18.5%), 8/10 concurrent. SNP zygosity status was less preserved in metachronous, compared to concurrent disease. Pathogenic germline mutations were present in 10/29 patients, 9 in BRCA1 and one in TP53 (p.Phe341Val, first report in the germline). BBC demonstrated extensive inter- and intrapatient heterogeneity in the present thus far largest series of corresponding paired genotypes. The majority evolve independently and unpredictably, supporting current clinical practice. A considerable minority though, retains clonal origin and may be regarded as a distinct group for therapeutic interventions among concurrent BBC.

Keywords: Bilateral, breast cancer, targeted next generation sequencing, coding mutations, concurrent, metachronous, contralateral, clonality, hypermutation

Introduction

Breast cancer is the most common type of cancer among women, with 231,840 new patients diagnosed every year in the United States [1]. Due to novel diagnostic and therapeutic approaches breast cancer prognosis and survival have significantly improved. More than

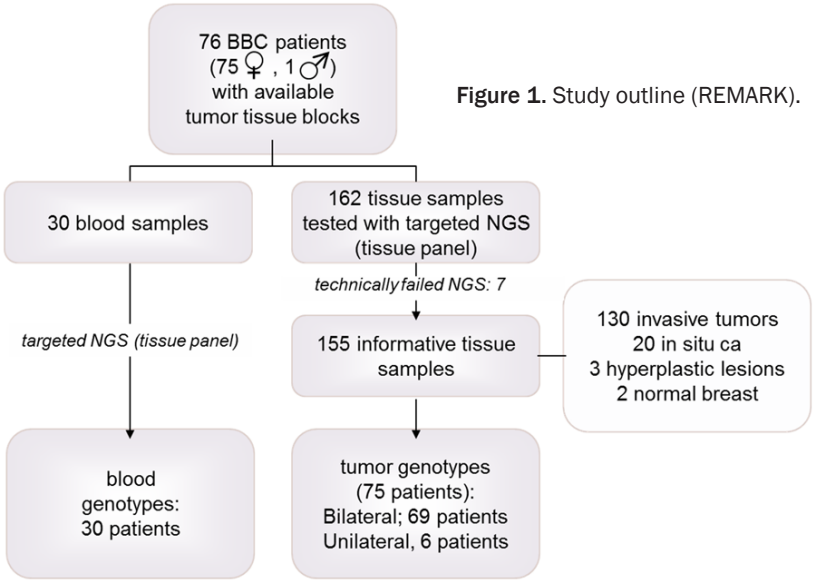

Figure 1. Study outline (REMARK).

90% of breast cancer patients are alive at 5 years and 78% at 15 years after diagnosis. An important issue in these women is the risk of developing a second primary cancer.

Bilateral breast cancer has an incidence of 4-20% in women diagnosed with breast cancer [2, 3]. Bilateral tumors are diagnosed simultaneously or within three months after the initial diagnosis (concurrent) in 0.3-12% of patients, while in 5-10% of patients the contralateral tumor is diagnosed more than three months after the first one (metachronous) [4, 5].

It is of great clinical importance to identify whether contralateral tumors represent a second primary or a metastatic spreading of the initial breast tumor. Such a distinction alters the physician's treatment plan and ultimately the prognosis of the disease. To date, metachronous contralateral tumors are treated as second primary tumors; concurrent tumors are evaluated in parallel and treatment decisions are made upon the characteristics of the more aggressive tumor.

Different studies attempting to define the origin of the contralateral tumor based on clinical and histopathologic criteria [2, 4, 6] did not reach definitive conclusions. Further studies explored the molecular characteristics of bilateral breast cancers. Few of these studies indicated that the contralateral tumor might represent a metastatic lesion [7, 8], while the majority demonstrated a high degree of discordance and advocated for a separate tumor origin [9-12]. Due to limited sample size, however, the results of these studies need to be reviewed with caution.

This is the first study to investigate large-scale genomic characteristics of bilateral breast cancer. Using targeted next generation sequencing (NGS), we explored the mutational profile of bilateral breast cancers, both concurrent and metachronous in 76 patients. We demonstrated that the majority of the tumor pairs do not share the same genetic alterations, supporting the independent nature of the contralateral tumor.

Methods

Study outline, patients and tumors

Clinical data and formalin-fixed paraffin-embedded (FFPE) tumor tissue samples from 76 patients with operable breast cancer were retrospectively retrieved from the Clinical Data Bank and Tumor Repository of the Hellenic Cooperative Oncology Group (HeCOG). Patients (75 women, 1 man) had been treated in Oncology departments of HeCOG-affiliated hospitals from 2000 to 2015. Bilateral tumors had been characterized at local pathology laboratories for histological type, tumor size, grade, nodal status, multifocality, and ER/PgR/HER2 expression with immunohistochemistry (IHC). Central pathology review of FFPE hematoxylin and eosin (H&E) sections was undertaken at the Laboratory of Molecular Oncology (MOL, Hellenic Foundation for Cancer Research/HeCOG/Aristotle University of Thessaloniki [AUTH]) by an experienced breast pathologist (M.B.) who recorded the presence of infiltrative carcinoma, in situ carcinoma, pre-cancerous lesions and normal tissue. Marked tissue areas with known tumor cell content (TCC%) were manually macrodissected and processed for DNA extraction and targeted next generation sequencing (NGS) genotyping with a previously validated custom tissue panel [13, 14]. The same panel was used for genotyping 30 blood

samples from the above patients. The study was approved by the Bioethics Committees of Aristotle University of Thessaloniki (2./4.2.2015). Written informed consent had been obtained from all patients allowing the use of their biologic material for research purposes. The study outline with respect to the performed tissue panel investigations is shown in the REMARK diagram in **Figure 1**.

Tumors were also centrally assessed for stromal tumor infiltrating lymphocytes (TILs) density based on Salgado et al [15] as previously described [16] on whole H&E sections; and, for clinical subtypes with ER/PgR/HER2/Ki67 IHC and HER2 FISH [17] on in-house low-density tissue microarrays (TMA) that contained two 1.5 mm cores per tumor. Ki67 cut-off at 20% was applied [18] to distinguish Luminal A and Luminal B tumors.

In addition, germline status was available for 29 of the above patients from NCSR "Demokritos". These data were obtained in the frame of a separate study that was locally approved (240/EHΔ/11.3) and was in agreement with the 1975 Helsinki statement, revised in 1983. Peripheral blood DNA had been tested as previously described [19] for the five Greek founder and one recurrent BRCA1 mutations; if found wild-type, samples were further tested by Sanger sequencing for BRCA1 and BRCA2 mutations or by massively parallel sequencing with the Trusight Cancer panel on Illumina MiSeq (Illumina, San Diego, USA). The median read depth was ~200×, with 50-fold being the minimum cut-off for variant calling.

Detailed patient clinicopathologic characteristics, including germline data for cancer predisposition genes can be found at (https://figshare.com/s/39adae1d323bde0884d5, Table S1, in file: BBC supplementary data).

Targeted NGS genotyping

The majority of tumor samples (78%) had TCC ≥50%, but samples with as low as 15% TCC were also processed based on our previous experience [14]. DNA was extracted from macrodissected tissue fragments with the QIAamp® DNA Mini kit (Hilden, Germany) according to the manufacturer's instructions; quantity was measured with the Qubit fluorometer (Life Technologies, Paisley, UK). Criteria for processing FFPE samples for NGS genotyping were ≥2 ng/ul DNA amplifiable at Ct≤32 for two control qPCR assays. Peripheral blood DNA had been isolated based on the salt-extraction procedure [20].

The tissue panel [14] targeted coding regions and single nucleotide polymorphisms (SNPs) in 61 genes most frequently implicated in breast cancer [21, 22] and was applied for library construction and NGS on an Ion Proton System (Ion Torrent/Invitrogen, Paisley, UK). Samples were accepted for further evaluation if all amplicons had been read >100 times. Variants obtained from Ion Reporter v.4 were filtered out if not annotated; if indels with GC-stretches (reading artifacts with semiconductor sequencing); and, if p (system quality metric including false discovery rate) >0.0001. Variants were accepted for analysis when position and variant coverage were higher than 100 and 40, respectively. In order to avoid false negative calls, and hence, false heterogeneity interpretation, we evaluated amplicon read efficiency in matched sample pairs prior to assessing common (shared) and private variants. As above, variants were compared among samples if corresponding amplicons had been read >100 times in each sample under comparison.

Bioinformatics-statistics

We examined the presence and pattern of coding mutations and the preservation of SNP zygosity in paired samples from the same patient. Coding mutations corresponded to amino acid changing variants with minor allele frequency (MAF) <0.1% or not reported in dbSNP from NCBI and in 5000 Exomes. Initially, we assessed mutation pathogenicity according to available information in COSMIC and by using ANNOVAR [2]. However, because this and any available algorithm prioritize genomic variants in the germline for their disease relevance and may, therefore, not adequately predict pathogenicity and functional implications of the same mutations in tumors [3], we did not further pursue with characterization of these features. We assessed SNP zygosity in matched sample pairs (bilateral tumors, tumor-blood) as an indicator for genomic stability. For this, we evaluated matched samples with ≥5 SNPs (range: 5-25 SNPs per case). Differences of ≤|20| in variant allele frequencies (VAF) at SNP posi-

Table 1. Patients, disease and tumor characteristics

All cases:	76					
Age						
N cases	76					
Mean ± SD	57.2 ± 15.4					
Median	57.2					
Min-max	30-87.5					
	N (%)					
Disease presentation						
N cases	76					
Concurrent	52 (68.4)					
Metachronous	24 (31.6)					
Menopausal status						
N cases	71					
Pre	25 (40)					
Post	46 (60)					
	SIDE 1	SIDE 2	TOTAL			
Positive nodes						
N cases	60	51	111			
Mean ± SD	3.1 ± 5.8	1.1 ± 1.9	2.5 ± 3.0			
Median	0	0.5	0			
Min-max	0-23	0-9	0-23			
Tumor size						
N cases	71	70	141			
Mean ± SD	2.3 ± 1.9	2.5 ± 3.0	2.4 ± 2.5			
Median	1.8	1.8	1.8			
Min-max	0.1-11	0.2-21	0.1-21			
Ki67						
N cases	68	68	136			
Mean ± SD	26.6 ± 25.4	29 ± 24.5	27.9 ± 24.8			
Median	18	23	20			
Min-max	1-90	1-95	1-95			
Tumor infiltrating lymphocytes (TILs)						
N cases	68	63	131			
Mean ± SD	9.5 ± 15.5	11.1 ± 17.2	10.3 ± 16.3			
Median	3	4	3			
Min-max	1-75	1-80	1-80			
	SIDE 1	SIDE 2	TOTAL			
	N	%	N	%	N	%
Main lesion, carcinoma						
In situ	7	9.2	7	9.3	14	9.2
Invasive	69	90.8	68	90.7	137	90.8
Concordance* invasive ca: 64/75 (85.3%)						
Histology						
Ductal, NST	51	67.1	48	64	99	65.1
Other	25	32.9	27	36	52	34.9
Concordance histology: 44/75 (61.3%)						
Grade						
I	11	15.7	8	11.9	19	13.9
II	27	39.7	27	39.7	54	39.4

III	32	45.7	32	47.8	64	46.7
Concordance grade: 37/63 (58.7%)						
In situ lesion						
Present	47	61.2	43	57.3	90	59.6
Absent	29	38.2	32	42.7	61	40.4
Concordance in situ presence: 39/76 (51.3%)						
Clinical subtype						
HER2-Enriched	5	6.9	3	4.2	8	5.5
Luminal A	34	45.9	31	43.1	65	44.5
Luminal B	21	28	21	29.2	42	28.8
Luminal-HER2	5	6.8	8	11.1	13	8.9
TNBC	9	12.2	9	12.5	18	12.3
Concordance, clinical subtype: 37/72 (51.4%)						
TILs						
Low (≤5%)	50	73.6	41	65.1	91	69.5
High (5-50%)	16	23.5	19	30.1	35	26.7
LPBC (>50%)	2	2.9	3	4.8	5	3.8
Concordance, TILs: 43/59 (72.9%)						
Multifocality						
No	54	72	49	67.1	103	69.6
Yes	21	28	24	32.9	45	30.4
Concordance, multifocality: 48/72 (66.7%)						
Size, categorical						
<2 cm	38	52.1	42	59.3	80	55.5
≥2 cm	35	47.9	29	39.7	64	44.5
Concordance, size: 36/69 (52.2%)						
Nodal status						
0-3 LN	49	80.3	46	90.2	95	84.8
≥4 LN	12	19.7	5	0.8	17	15.2
Concordance, nodal status: 35/44 (79.5%)						

*: concordance between Side 1 and Side 2.

tions were considered as corresponding to stable, the rest to altered zygosity status. Cases were considered as (a) stable, if >90% of SNPs retained their zygosity status upon paired comparisons, and (b) of intermediate stability, for zygosity preservation in 50-90% of SNPs. Only adequately covered positions were considered for matching mutations and SNP comparisons among samples from the same patient.

In order to avoid statistical bias, we divided concurrent breast cancer tumors based on location (right or left) as previously done. Our two groups were well balanced between different histopathologic parameters, as expected. Metachronous tumors were registered based on their presentation; side 1 included all 1st incidence tumors, while side 2 the respective metachronous ones.

Frequencies and percentages were used to present categorical variables, while various measures (mean, SD, median, min and max) were used for continuous variables. Classic statistics for associations between variables included chi-square and Mann-Whitney or Kruskal-Wallis tests, where appropriate. Correlations were calculated using the Spearman's rank correlation coefficient (Rho). Wilcoxon singed-rank test was used for comparing equal distributions of paired samples. All tests were 2-sided with the significance level set at α=0.05. Contingency tables were created with JMP v.10; descriptive statistics for parameter associations and correlation of continuous variables were performed by using the SPSS v.15 and the SAS software for statistical analysis (SAS for Windows, version 9.3, SAS Institute Inc., Cary, NC, USA).

Table 2. Comparison of clinicopathological and genomic characteristics in concurrent and metachronous BBC

	Concurrent	Metachronous	*p*-value		
All patients: 75	51	24			
Age			<0.001		
N (75)	51	24			
Mean ± SD	62.4 ± 14.2	45.9 ± 12.4			
Median	64.7	40.8			
Min-max	30-87.5	33.2-74.4			
N mut per case (N=75)			0.19		
Mean ± SD	1.65 ± 1.64	7.3 ± 14.2			
Median	3	1.5			
Min-max	0-6	0-56			
N genes per case (N=75)			0.23		
Mean ± SD	1.4 ± 1.4	4.9 ± 8.9			
Median	1	1			
Min-max	0-6	0-33			
	N	%	N	%	
Menopausal status (N=72)					<0.001
Premenopausal	9	18.4	17	73.9	
Postmenopausal	39	79.6	6	26.1	
N/A	1	8	0	0	
Concordant histology (N=75)					0.25
Yes	29	56.9	17	70.8	
No	22	43.1	7	29.2	
Concordant grade (N=75)					0.84
Yes	24	47	13	54.2	
No	19	37.3	8	33.3	
N/A	8	15.7	3	12.5	
Concordance for in situ (N=75)					0.12
Yes	29	56.9	9	37.5	
No	22	43.1	15	62.5	
Concordant ER/PR status (N=73)					0.013
Yes	46	92	16	69.6	
No	4	8	7	30.4	
Clinical subtype (N=147)					<0.001
ER+(LUMA,LUMB, LUM-HER2	92	92	30	63.8	
ER-(HER2-enriched, TNBC)	8	8	17	36.2	
Both ER/PgR+ vs. both TNBC (N=73)					<0.001
Both ER/PgR+	44	88	11	47.8	
Both TNBC	1	2	5	21.8	
Other	5	10	7	30.4	
Concordant size (N=63)					0.78
Yes	24	51.1	12	54.5	
No	23	48.9	10	45.5	
Multifocality (N=75)					0.31
Yes	13	72.5	9	37.5	
No	38	27.5	14	58.3	
N/A	0	0	1	4.2	

TP53 mutations (N=75)					<0.001
Mutated	12	23.5	16	66.7	
WT	39	76.5	8	33.3	
PIK3CA mutations (N=75)					0.62
Mutated	20	39.2	8	33.3	
WT	31	60.8	16	66.7	
GATA3 mutations (N=75)					0.88
Mutated	7	13.7	3	12.5	
WT	44	86.7	21	87.5	
MLL3 mutations (N=75)					0.16
Mutated	2	3.9	3	12.5	
WT	49	96.1	21	87.5	
Hypermutated samples (N=75)					<0.001
Hyper	0	0	5	20.8	
No	51	100	19	79.2	
Preserved SNPs>90%* (N=64)					0.1
Yes	37	82.2	12	63.2	
No	8	17.8	7	36.8	
Preserved zygocity in>90% of SNPs* (N=64)					0.042
Yes	29	64.4	7	36.8	
No	16	35.6	12	63.2	
T1 vs. T2 stability (N=64)			0.058		
Stable	26	57.8	5	26.3	
Intermediate	14	31.1	9	47.4	
Unstable	5	11.1	5	26.3	

N in parentheses: number of patients compared in each case; *: T1 vs. T2 comparisons.

Eligible samples

We excluded one pair of bilateral tumors (2 samples) due to inadequate DNA template, 1 sample with no tumor tissue and 4 samples identified as technical outliers based on the criteria mentioned above. Our final cohort consisted of 185 samples (155 FFPE and 30 peripheral blood) corresponding to 75 patients (**Figure 1**); matched bilateral tumor samples from 69 patients were eligible for analysis. The obtained values for mean depth of the eligible samples were median: 1638; mean ± SD: 3021 ± 2760; min-max: 350-21500. Despite outlier differences, mean depth did not significantly differ between tissue and blood samples (Mann-Whitney P=0.6328).

Results

Clinicopathological characteristics of BBC

Concurrent disease was noticed in 52 patients (**Table 1**). The time interval for manifestation of metachronous disease in 24 patients ranged between 0.5-19 yrs (mean ± SD: 6.3±4.8 yrs; median: 5.6 yrs). Menopausal status of the 75 female patients and age of all patients at first diagnosis are shown in **Table 1**. Twenty-eight patients (36.8%) were <50 yrs. The single male patient was 78y.o.

In total, 76 bilateral breast tissue surgical specimens were analyzed (152 unilateral specimens), involving 65 cases with bilateral invasive carcinomas, 3 cases with bilateral CIS and 8 with unilateral invasive and contralateral carcinoma in situ (CIS). CIS were observed in 90 specimens (59.6%), 89 of them of ductal origin (DCIS). This incidence is significantly higher compared to the approximately 26% reported for DCIS diagnoses [23]. Incidence and bilateral heterogeneity of clinicopathological parameters are described in **Table 1**. Histological parameters and their concordance status were not associated with patient age, disease presentation, tumor size or nodal status.

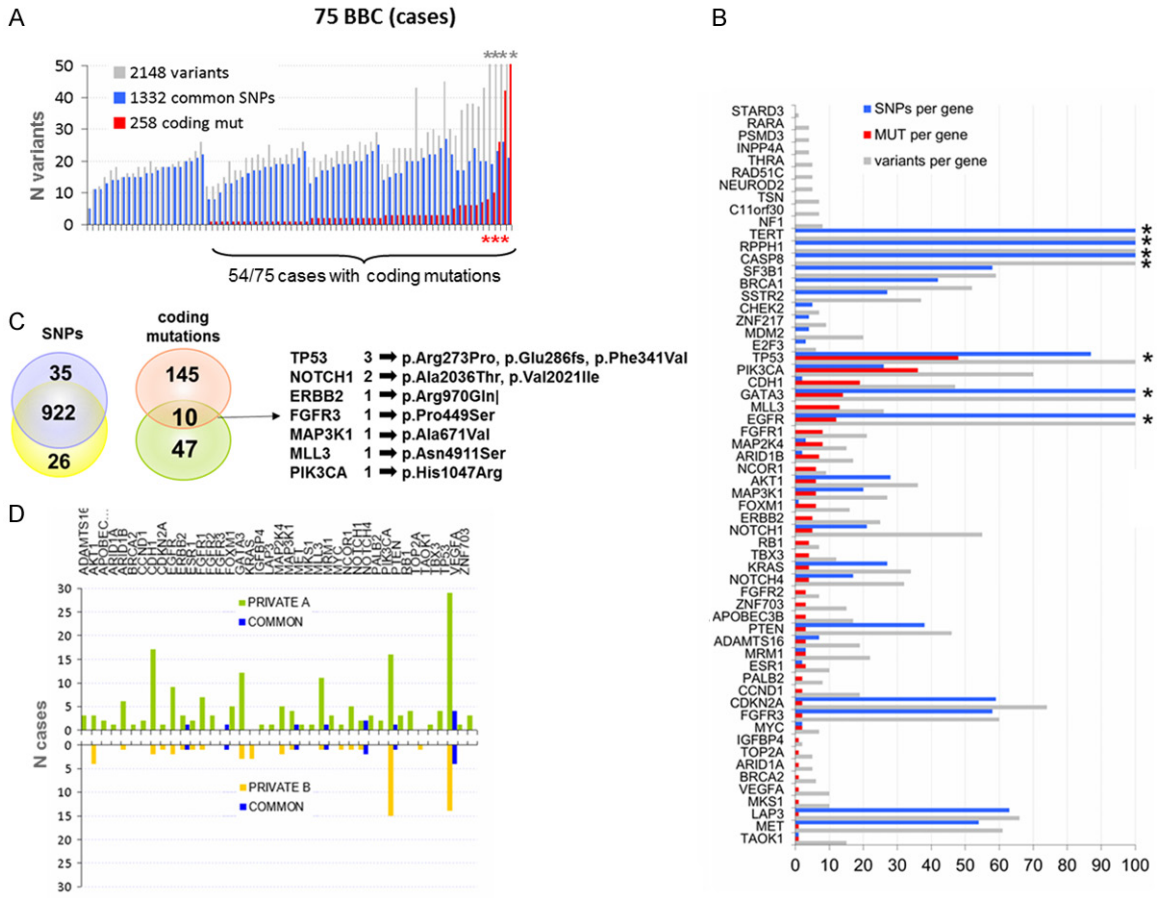

Figure 2. Distribution of NGS variants in BBC. A. Distribution of variants of any type in the study cases. All variants, SNPs and coding mutations are shown per case. For similar sequencing efficiency metrics (total reads, on target reads, uniformity of reads), 5-214 variants were identified per case (mean ± SD: 23 ± 30; median: 22), up to 54 in the same gene (mean ± SD: 15 ± 7; median: 12). Asterisks: truncated Y-axis for cases with >50 variants. B. Distribution of variants per gene. Grey bars: total number of variants of any type. Coding mutations were identified in 41 out of 61 genes in the MPS panel (red bars). The total number of mutations per gene varied from 1 to 49 (median: 3 mutations per gene). In additional 10 genes, only SNPs were present (blue bars). The number of SNPs per sample ranged from 10-20. Asterisks: >100 variants for these genes. C. Common and private SNPs and coding mutations. SNPs were preserved at 94% in both sides (922/983 comparable SNPs); by contrast, only 4.9% common coding mutations were observed (10/202 comparable mutations). The remaining 192 comparable mutations were present unilaterally, as shown. D. Distribution of gene coding mutations on either side; common, private A and private B mutations represented by blue, green and orange bars, respectively.

Comparisons of clinicopathological parameters, including disease presentation characteristics, are shown in **Table 2**. Compared to concurrent BBC, metachronous disease was significantly associated with younger patient age and premenopausal status, in line with previous reports [4, 24]. Metachronous BBC were rich in TNBC and HER2-enriched clinical subtypes (36%); the rate of ER/PgR-positive tumors was similar in the 1st (61%) and 2nd (61%) incidence but concordance of bilateral ER/PgR phenotypes was approximately 70%. The majority of concurrent BBC (96%) included at least one ER/PgR-positive tumor and this phenotype was preserved bilaterally in 92% of the cases. The overall incidence and bilateral concordance of the ER/PgR phenotype was significantly lower in metachronous compared to concurrent BBC (**Table 2**).

Stromal TILs density was assessable in 133 tumors including 60 matched bilateral; it was low (median: 4%) and it was not associated with ER/PgR status, other than previously reported [16, 25], probably due to over-representation of ER/PgR-positive tumors in this cohort. TILs

Table 3. Description of shared coding mutations identified in matched bilateral tumors and in peripheral blood samples (tissue panel)

Sample id	Gene	Mutation location	Coding	Protein	Function	MUT allele freq blood	MUT allele freq T1	MUT allele freq T2	COSMIC for the present mutation
BIL-006	FGFR3	T1, T2	c.1345C>T	p.Pro449Ser	Missense		0.52	0.5	Not registered
BIL-013*,$	TP53	T1, T2	c.818G>C	p.Arg273Pro	Missense		0.35	0.76**	COSM165077
BIL-017	PIK3CA	T1, T2	c.3140A>G	p.His1047Arg	Missense		0.3	0.22	COSM94986
BIL-028	ERBB2	T1, T2	c.2909G>A	p.Arg970Gln	Missense		0.63	0.55	Not registered
BIL-033	NOTCH1	T1, T2	c.6106G>A	p.Ala2036Thr	Missense		0.23	0.28	COSMIC: p.A2036V
BIL-035	MAP3K1	T1, T2	c.2012C>T	p.Ala671Val	Missense		0.41	0.48	COSM3674469
BIL-042*,$	TP53	BL, T1, T2	c.1021T>G	p.Phe341Val^	Missense	0.51	0.58	0.84**	Not registered, p.R342*; p.R342P
BIL-062	TP53	T1, T2	c.6061G>A	p.Val2021Ile	Missense		0.43	0.4	Not registered
BIL-064	MLL3	T1, T2	c.14732A>G	p.Asn4911Ser	Missense		0.51	0.64	Not registered
BIL-067$	TP53	T1, T2	c.856_857delGA	p.Glu286fs	Frameshift/Deletion		0.26	0.31	Not registered
BIL-020	FOXM1	BL	c.490C>T	p.Arg164Trp	Missense	0.47			COSM1476487
BIL-020	APOBEC3B	BL	c.568A>G	p.Arg190Gly	Missense	0.52			Not registered
BIL-048	EGFR	BL^^	c.3244A>T	p.Ile1082Leu	Missense	0.49			Not registered

^: shared TP53 mutation in blood (BL) and in both tumors (T1, T2). *: metachronous cases, one with common blood/tumor TP53 mutation. $: cases with germline mutations found upon testing for cancer predisposing genes. **: increased VAF indicating loss of the wild-type allele in the metachronous tumor. ^^: non-informative amplicons and positions in the tumors of this case.

were mostly homogeneous bilaterally (Fisher's exact P=0.018 for comparisons with the median 4% as a cut-off) and did not differ between concurrent and metachronous tumors.

Mutation analysis

In the informative 185 samples of our study, NGS revealed 2148 variants eligible for analysis (**Figure 2A**); among these, 258 (34.2%) were coding mutations, the rest being synonymous changes in coding regions or SNPs (**Figure 2B**). A detailed list of the identified coding mutations can be found at (https://figshare.com/s/39adae1d323bde0884d5, Table S2, in file: BBC supplementary data). Coding mutations were mostly missense (89%), 4% were nonsense and 7% were frameshift indels. Most frequently mutated genes were TP53 (19%) and PIK3CA (14%), followed by CDH1 (7%), GATA3 (6%) and MLL3 (5%). Recurrent coding mutations present in >2 tumors were observed only in PIK3CA hotspots (6x p.Glu542Lys; 6x p.Glu542Lys; 12x p.His1047Arg). Out of 197 unique mutations, 50.1% were characterized as probably damaging with ANNOVAR; 15.2% as possibly damaging; and 34% as benign (Table S3, in file: BBC supplementary data, https://figshare.com/s/39adae1d323bde0884d5).

Coding mutations were found in 80/130 invasive tumors (62%), in 8/20 (40%) DCIS, in all 3 hyperplastic and in the 2 normal informative samples. Mutations were identified in 72% of the cases (**Figure 2A**).

The 69 cases with matched bilateral tumor samples shared 50% of all variants. Among these, SNP variants were up to 94% common bilaterally, whereas mutations were mostly private in either side (**Figure 2C**). Out of 202 mutations that were comparable in both sides only 10 were bilaterally common (**Figure 2C** and **2D**; **Table 3**). The rate of BBC with common mutations was 18.5% among the 54 cases with mutant tumors, and 13.3% among patients with informative tumors in both sides.

Comparison of bilateral mutation profiles

Metachronous tumors had more than double mutations as compared to concurrent ones (174 vs. 84, respectively) but this difference did not reach statistical significance (chi-square, P=0.155). The majority of mutations concerned 5 tumors unilaterally (**Figure 3A**), which also carried multiple mutations in ≥2 genes (**Figure 3A** and **3C**), a feature that was absent in all other tumors. Based on the characteristics, we called these 5 tumors hypermutated. Sequencing performance of these samples did not differ from samples with lower mutation numbers or without mutations (**Figure 3A**). All 5 hypermutated tumors were the 1st incidence of metachronous disease (**Figure 3A** and **3C**). TP53 had a significantly higher mutation rate in metachronous than in concurrent tumors (**Figure 3B** and **3C**; **Table 2**). The 39.2% incidence of PIK3CA mutations (57% if examined among tumors with mutations) in the mostly ER/PgR positive concurrent tumors (**Figure 3B**) was in line with previous observations for this phenotype [26, 27]. There was no difference in mutation prevalence of the remaining recurrently mutated genes between the two subgroups.

Mutation rate in different genes was associated with various clinicopathological parameters (**Table 2**). TP53 mutations in tumors were associated with younger age (Kruskal-Wallis, P=0.013), higher Ki67 labeling (chi-square, P=0.002) and ER/PgR-negative clinical subtypes (chi-square, P<0.001). TP53 mutations were present in all 5 hypermutated tumors but in none of the lobular or mucinous types.

Stromal TILs, as a continuous variable, did not correlate with any clinicopathological or mutation parameter. However, all hypermutated tumors had <4% TILs. This feature was present in the metachronous tumors of the same patients as well. High stromal TILs (median cut-off) were often present in tumors with TP53 mutations (23/40 tumors in both sides; 57%), while tumors without TP53 mutations more frequently had low TILs (65/82; 79%); this pattern was observed in both sides but was statistically significant unilaterally (chi-square, P=0.008). The opposite pattern applied for PIK3CA mutations, which were infrequent in tumors with high TILs (9/40 tumors in both sides; 22%); significance was again obtained unilaterally only (P=0.041) probably due to small group numbers.

Variant allele frequencies (VAF) and SNP zygosity status

VAF distribution significantly differed between SNPs and coding mutations (P<0.0001), while

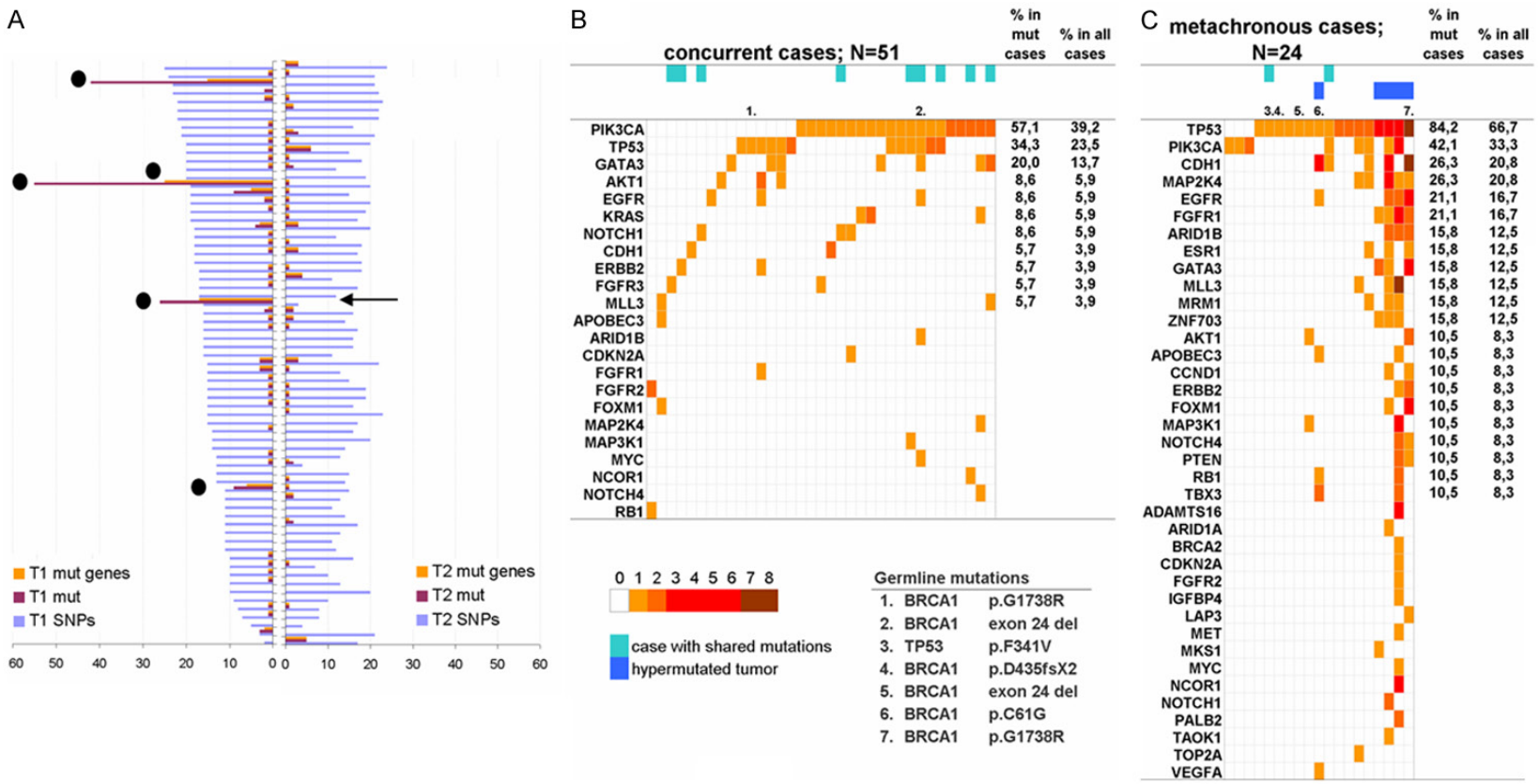

Figure 3. Mutation patterns in concurrent and metachronous BBC. (A) Distribution of mutations and mutated genes in tumors of both sides. SNP numbers are shown for comparison of sequencing performance of the samples; these did not differ for samples in side 1 vs. side 2 (Mann-Whitney P=0.5656) and showed a good correlation upon bivariate comparisons (Spearman's rho 0.641; P<0.0001). The matched tumor was not available in one of the hypermutated cases (arrow). (B and C) mutation maps in concurrent (B) and metachronous (C) cases. The number of mutations per gene is indicated on the color scale. Indicated are germline mutations; hypermutated tumors; tumors with shared mutations. Most frequently mutated gene in concurrent samples is PIK3CA, followed by TP53 and GATA3. Metachronous tumors carry more frequently mutations in TP53, followed by PIK3CA and CDH1.

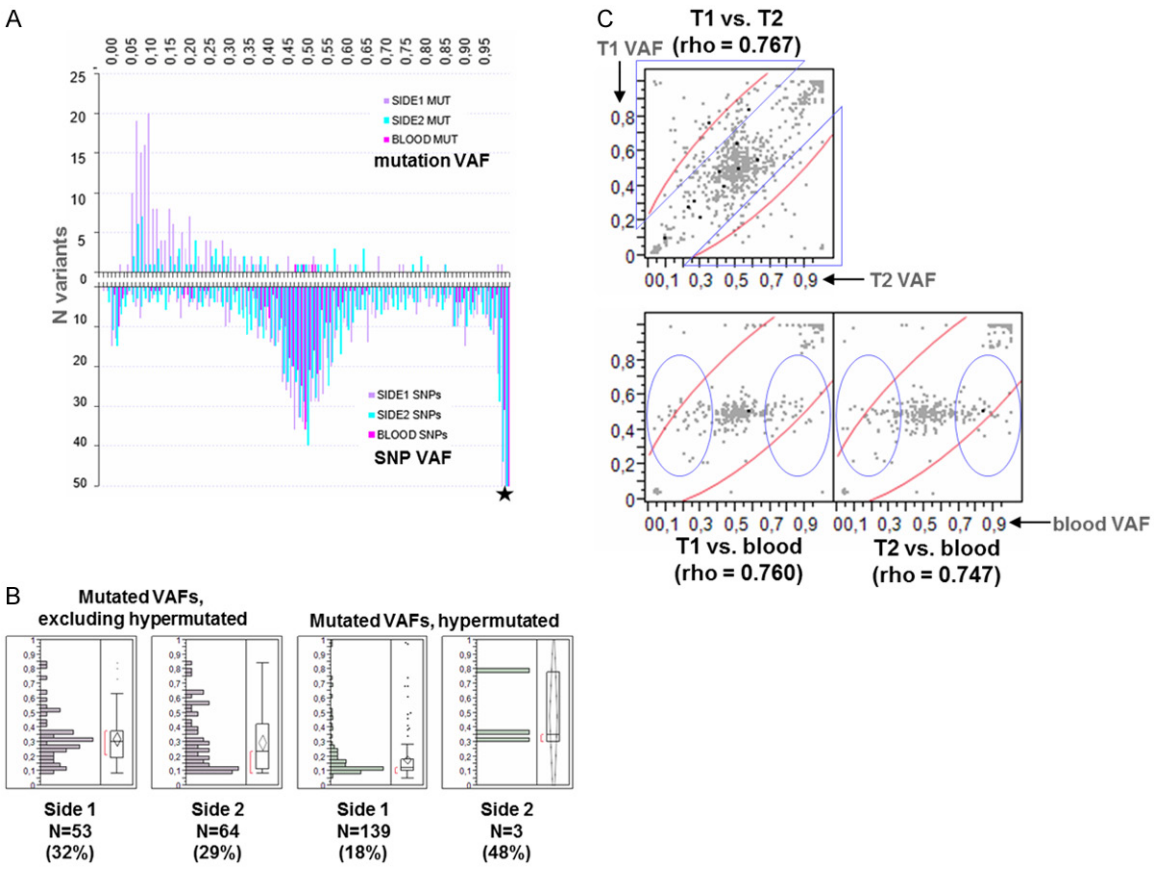

Figure 4. Bilateral comparisons of Variant Allele Frequencies (VAFs). A. VAF distribution for coding mutations and SNPs in both sides and in the germline. Germline variants as identified with the tissue panel. VAF distribution for coding mutations was skewed towards 0, with mean (median) values of 21% (14%) in side 1 and 30% (21%) in side 2. Most mutation VAFs were <25%, particularly in Side 1. B. Coding mutation VAFs are significantly lower in hypermutated as compared to non-hypermutated tumors (Mann-Whitney P<0.001). Numbers in parentheses: mean values for VAFs. N: number of mutations. Note that the contralateral tumors paired to hypermutated ones exhibited only one mutation in 3 out of 4 comparable cases, although they were diagnosed years later after the hypermutated tumors. C. Bilateral correlations of SNP VAFs in matched samples. Tumors in side 1 (T1) and in side 2 (T2) were compared with each other and with germline. SNPs were identified with the tissue panel. Triangles and circles: altered zygosity for these SNPs. Spearman's rho values yielded P's <0.0001 in all comparisons.

mutation VAFs were significantly lower in side 1 compared to side 2 (P=0.0001) (**Figure 4A**). Excluding the hypermutated tumors in side 1 resulted in similar mutation VAFs for both sides with mean values around 30% (**Figure 4B**). Distribution of SNP VAF's peaked around 50% and towards 100% (**Figure 4A**), following the expected germline zygosity pattern. SNP VAFs fairly correlated between tumors and between germline and each tumor (**Figure 4C**). In cases with hypermutated tumors, SNP VAFs between metachronous tumors showed a weaker but still fair correlation (Spearman's rho=0.700).

SNPs were compared for preserved incidence, zygosity and overall stability in 64 out of 76 cases. SNPs were shared bilaterally at a high rate in both concurrent (82.2%) and metachronous (63%) tumors. SNP zygosity status was less preserved in metachronous as compared to concurrent cases, but SNP stability reached only a trend of statistical significance (**Table 2**). In 25/30 cases with available peripheral blood samples, concurrent and metachronous disease did not differ in terms of germline SNP preservation. SNP zygosity was more frequently preserved (>90%) in ER/PgR-positive bilateral tumors, as opposed to bilateral TNBC or cases with different subtypes (chi-square, P=0.005). Stromal TILs did not associate with SNP zygosity and stability.

Table 4. Amino acid changing mutations in ductal carcinoma in situ (DCIS), hyperplastic lesions and normal breast tissue

Patient	sample histology	mut 1	mut 2	mut 3
BIL-016	DCIS	TP53 p.Tyr220Cys	PIK3CA p.His1047Arg	
BIL-034	DCIS	PIK3CA p.His1047Arg		
BIL-035	DCIS	MAP3K1 p.Ala671Val		-
BIL-042	DCIS	TP53 p.Phe341Val^		
BIL-057*	DCIS	RB1 p.Arg90*	FGFR2 p.Pro781Leu;	FGFR2 p.Pro784Leu
BIL-068	DCIS	AKT1 p.Glu17Lys	MAP3K1 p.Gly1372Asp	-
BIL-073	DCIS	TP53 p.Arg337fs		
BIL-074	DCIS	TP53 p.Gln167fs		
BIL-076	DCIS	GATA3 p.Ter445fs		
BIL-029	Hyperplasia	PIK3CA p.His1047Arg		
BIL-041	Hyperplasia, left	PIK3CA p.Pro17Leu	KRAS p.Gly12Cys	MAP2K4 p.Gly166Ser
BIL-041	Hyperplasia, right	PIK3CA p.IleHis13del_insIleTyr	NOTCH4 p.Val1806Ile	
BIL-078	Normal, left & right	TP53 p.Arg273Pro		
	Mutation present in DCIS and in the ipsilateral tumor			
	Mutation present in DCIS or hyperplastic lesion, absent in tumor			
	Mutation present in normal bilaterally, absent in both tumors			

*: two DCIS, one in each side. ^: germline variant.

Mutations in non-cancer samples and in peripheral blood DNA

We identified 13 coding mutations in 10 DCIS (**Table 4**). Three out of the four TP53 and one MAP3K1 mutation were preserved in the ipsilateral tumors, while the rest were DCIS-private. VAFs of the shared mutations in DCIS and matched tumors were >25%. Coding mutations were observed in the examined 3 hyperplastic and 2 normal samples (**Table 4**) with VAFs <20%. All hyperplastic lesions carried mutations in PIK3CA, 2 of them in other genes as well. All identified mutations in the hyperplastic lesions were private. The 2 normal bilateral samples were obtained from our male patient. Interestingly, they were both positive for the TP53 p.Arg273Pro mutation, which was absent from the matched tumors. The TP53 p.Arg273Pro missense mutation has been previously characterized as gain of function mutation providing cancer cells with growth and survival advantage [28]. The genotypes of all samples for these cases are presented in **Figure 5**.

We also tested 30 peripheral blood DNA samples with the tissue panel that, of note, was not designed for interrogating the status of cancer predisposing genes [14]. This step was undertaken for assessing the somatic nature of tissue mutations. Out of the 1080 variants in these samples, 414 and 385 were shared in tumors of either side, approximately 37%. Four heterozygous mutations were identified in blood samples from 3 patients, affecting TP53 (p.Phe341Val), FOXM1, APOBEC3B and EGFR, at the expected germline VAF, around 50% (**Table 3**). TP53 Phe341Val was present in tumors bilaterally. The EGFR mutation was not informative in the matched tumors, while FOXM1 and APOBEC3B mutations were not preserved in the tumors of the third patient.

As previously described, 29 patients had been tested within the frameworks of a separate protocol for germline mutations in cancer predisposing genes. Among them 9 harbored BRCA1 germline mutations, all described as pathogenic, while one patient harbored the aforementioned cross-validated TP53 p.Phe341Val (germline genotype (details in Table S1 in file: BBC supplementary data; https://figshare.com/s/39adae1d323bde0884d5). Seven out of 10 germline mutation carriers had tumors with mutations (**Figure 3B** and **3C**). Six out of the 9 BRCA1 mutation carriers developed metachronous tumors with a median time interval of 6 years between diagnoses. Median age at diagnosis was 37 years. The majority of the tumors were high-grade ductal carcinomas

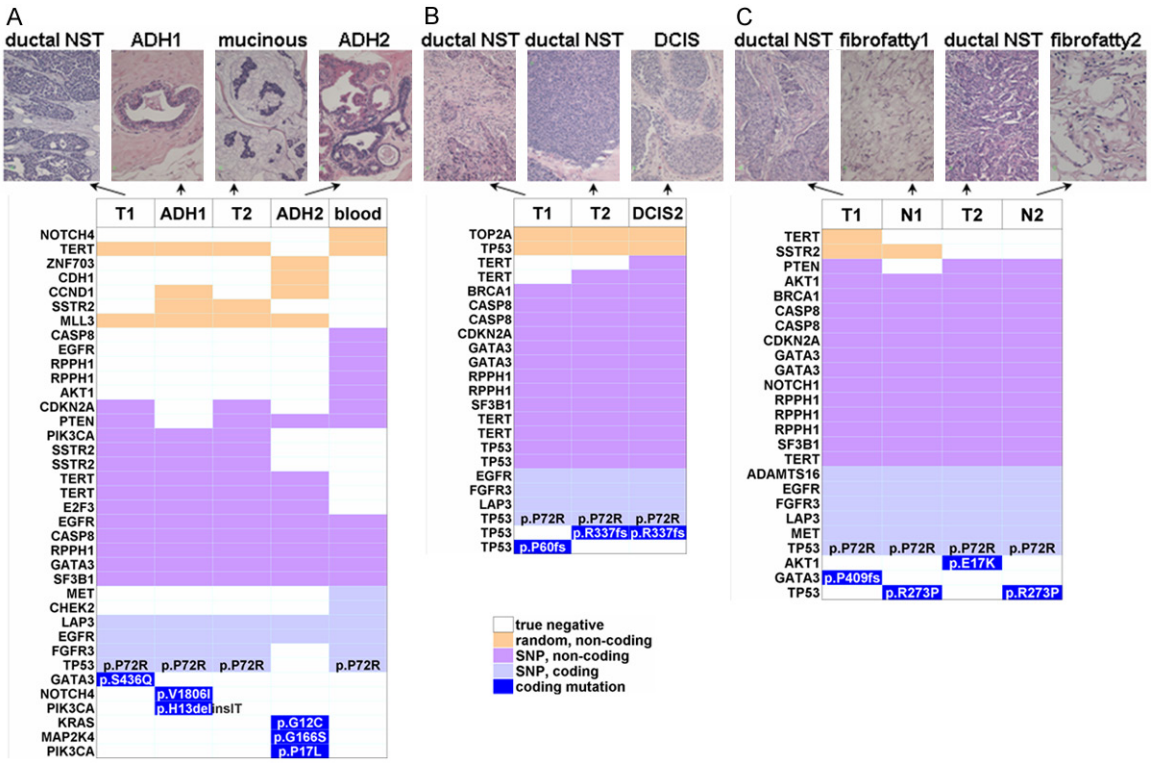

Figure 5. Example bilateral breast cancer (BBC) genotypes with corresponding histology. Three different cases are shown: BIL-041 (A), BIL-073 (B), BIL-078 (C), with case IDs as in Table S1. (A and C) Concurrent; (B) Metachronous. Note the different bilateral tumor genotypes in all cases; different mutations in hyperplastic lesions (ADH) in (A); shared mutations in the in situ carcinoma (DCIS) and in the unilateral tumor in (B), which were also morphologically consistent; shared TP53 mutation in the bilateral normal samples in the male BBC case in (C). SNPs were consistently shared among samples in (B) and (C) but less so in the case in (A). NST: non-specific type.

(NST), 2 were medullary and 1 was sarcomatous carcinoma. BRCA1 carriers had an increased rate of TNBC (8 out of 17 comparable tumors), 8 tumors were Luminal B and one was Luminal-HER2. Two out of the five hypermutated tumors were identified in BRCA1 mutation carriers. The rest of the patients with such tumors were not tested for germline mutations in BRCA1.

Discussion

To date contralateral tumors are treated as independent primary tumors. It is of great clinical importance to ascertain whether the contralateral tumor is indeed independent or whether it represents a metastatic lesion, since appropriate treatment modalities would be modified. To approach this issue, we compared genomic data obtained by multigene sequencing in bilateral tumors. The number of the examined patients may appear small but the series is currently the largest that has been extensively genotyped for this disease.

The majority of bilateral breast tumors did not share common mutations that would indicate a common origin for the development of the second tumor, as has been shown for metastatic lesions [29] and for the clonal evolution of TNBC [30]. A limitation of our approach is that targeted NGS used for genotyping may have missed common origin mutations in genes not included in the panel. As shown for multifocal breast cancer [31], wide genome sequencing revealed common unique structural alterations in multiple cancer foci from the same patient, indicating that they developed on the basis of a common defective genetic background. These lesions did not share common mutations when assessed by targeted NGS. As reported, the higher the distance between multifocal lesions, the higher the genomic diversity. Our findings in bilateral breast cancer fit this model, based on

the mutation diversity in the majority of cases. Nevertheless, at least 15% of the examined bilateral tumors shared common mutations indicative of clonal origin in both sides. The affected genes were previously reported as mutated in breast cancer [22] and were also found in the clonal trunk in breast tumor phylogenetics [30, 32]. The rate of such clonal tumors was 4:1 in concurrent compared to metachronous disease. The high mutant allele frequency in concurrent tumors and the increased such frequency in metachronous tumors that developed years later indicate a driver role for these shared mutations. Whether bilateral tumors with shared mutations should be considered as metastatic to the contralateral breast remains questionable. Such tumors may also develop independently as a result of locally operating mutational processes on a cancer predisposing genetic background [33]. Whether individualized treatments would benefit this minority of patients is still unexplored.

Bilateral breast cancers have classically been associated with genetic predisposition [34, 35]. Correspondingly, 1/3 of our patients tested for mutations in cancer predisposing genes carried pathogenic BRCA1 and, in one case, TP53 mutations. All were younger than 50 years at first diagnosis. The incidence of BRCA1 carriers was higher in metachronous cases as reported in large series, with a rate higher than 2:1 for metachronous vs. concurrent disease [35]. TP53 p.Phe341Val is a novel germline mutation, since it has not been reported before either in IARC TP53 or COSMIC databases. This mutation was predicted as benign by all pathogenicity prediction algorithms used by ANNOVAR. *However, based on the functional aspects of TP53 codon 341 [36], a disruptive effect cannot be excluded.* TP53 p.Phe341Val was preserved in the metachronous tumor that developed years apart in the same patient, further supporting its possible pathogenic role.

Beyond the above mutations in traditional cancer predisposing genes we also observed changes in blood samples at heterozygote germline frequencies, in genes usually reported as somatically mutated, such as FOXM1, APOBEC3B and EGFR. All these mutations were predicted as benign by ANNOVAR and older patient age at first diagnosis in these cases may support this feature. However, since none of the three genes is included in cancer predisposition panels, the role of the corresponding mutations in the development of the bilateral tumors in these patients remains unknown. To this end, such mutations are useful to report for future comparisons.

Three main genotype features characterized metachronous as opposed to concurrent disease: prevalence of TP53 mutations; presence of hypermutated tumors; and altered zygosity for common SNPs in the 2nd occurrence. Starting with the latter, altered zygosity status at SNP positions and genomic stability of metachronous tumors may just reflect the temporal instability changes that are established in cells following a greater number of divisions as revealed with mathematical models [37].

The prevalence of TP53 mutations may be related to the TNBC phenotypes more frequently observed in metachronous tumors. Compared to concurrent tumors, which were mostly ER/PgR-positive, metachronous tumors were significantly more frequently hormone receptor negative demonstrating significantly lower ER/PgR concordance, as previously described [2]. Concurrent and metachronous tumors demonstrated luminal-like and TNBC-like mutation patterns, respectively, in accordance with TCGA data [22, 38]. The luminal-like profile included a high rate of PIK3CA mutations [38, 39] in the usually described hotspots [27], followed by TP53 and GATA3. The TNBC-like profile of metachronous tumors was marked by TP53 followed by PIK3CA and CDH1 mutations [40-42].

The high rate of TP53 mutations in metachronous tumors may be related to mutations in cancer predisposing genes in such cases. Germline BRCA1 mutations are associated with tumor TP53 mutations in TNBC [43]. This condition may also be related to the hypermutated tumors that we only observed in patients with metachronous disease. Hypermutated tumors develop in BRCA1 mutation carriers [44], while the combination of inherited BRCA1 and acquired replication repair defects may result in ultramutated tumors [45]. The present hypermutated tumors had TP53 mutations, while three of them also had germline BRCA1 and/or somatic APOBEC3B mutations. Whether these APOBEC3B mutations contributed to the high mutation load in the two affected tumors, as published for this deaminase [46], needs func-

tional proof. Intriguingly, in the 5 patients with hypermutated tumors, we observed 0-1 mutations in the contralateral tumor that developed years later. This is of clinical importance, since it indicates that whatever the cause releasing hypermutation, it may have acted locally and perhaps temporarily.

Alterations in normal and pre-invasive lesions in the breast have been described at the genomic level [47-49], as has a shared susceptibility for infiltrative carcinomas and DCIS [50]. Our findings are similar to those reports except that we did not observe any PIK3CA mutations in DCIS, probably because of the small sample number. Mutations in the normal or hyperplastic breast or in DCIS were not necessarily preserved in the infiltrative tumor in the ipsilateral or contralateral breast. A characteristic example was the COSMIC registered TP53 p. Arg273Pro mutation that was present bilaterally in the normal breast of our male patient but in none of the corresponding tumors; the same mutation was present in both tumors of a female patient in the present series. These data are in line with recently proposed models on the genomic dynamics during cancer development and evolution that have been described for different types of cancer [51] and on the selective pressure for the preservation of mutations during tumor evolution [32].

Conclusions

Based on their histopathological and particularly genomic characteristics, bilateral breast carcinomas can be considered as two separate primary tumors arising in the same environmental and genetic background. Bilateral breast cancer may represent a model for studying the development of different cancers in the same organ and individual. In the majority of cases, bilateral breast tumors do not share mutations in recurrently mutated breast cancer related genes. Until the common genetic and/or environmental basis of these cancers is fully revealed it seems appropriate to consider the characteristics of both sides for clinical decision making. Assessing the clinicopathological and genetic characteristics of one side does not necessarily reflect those in the contralateral side, in either concurrent or metachronous disease, the latter more often exhibiting tumor-private mutation profiles. These findings support the management of bilateral breast cancers as independent tumors, as currently practiced.

Disclosure of conflict of interest

None.

Authors' contribution

Elena Fountzilas and Vassiliki Kotoula designed experiments, analyzed data and wrote the paper. Eleni Giannoulatou and George Kouvatseas: provided technical support and aided the statistical analysis, data analysis and interpretation. Triantafyllia Koletsa and Mattheos Bobos reviewed all histology parameters. Kyriaki Papadopoulou performed NGS experiments. Florentia Fostira performed germline testing for cancer susceptibility genes. Epaminontas Samantas, Efterpi Demiri, Spyros Miliaras, Christos Christodoulou, Evangelia Razis, Dimitrios Pectasides, George Zografos, Flora Zagouri, George Pentheroudakis provided patient samples and clinical data. Sofia Chrisafi collected the data. George Fountzilas had the conception and design of this project, provided the majority of patient samples and clinical data, and the funding. All authors commented on the manuscript and accepted it in its final form.

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