Circulating tumor cells and breast cancer-specific mutations in primary breast cancer

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Abstract. Circulating tumor cells (CTCs) play a pivotal role in tumor dissemination and progression, and are considered to be a critical part of the metastatic cascade. The aim of the present research article was to examine breast cancer-specific mutations in primary breast cancer (PBC) using targeted resequencing. A total of 78 patients with PBC were enrolled into this translational study. Reverse transcription-quantitative PCR assay for the expression of epithelial markers (*CK19*) or epithelial-to-mesenchymal transition (EMT)-related genes (*TWIST1*, *SNAIL1*, *SLUG* and *ZEB1*) was applied for identification of CTCs prior to surgery. Total DNA was isolated from fresh frozen primary tumors. Sequencing was performed by Agilent SureSelect target enrichment and Illumina paired-end sequencing on the MiSeq platform. The most commonly affected genes were *TP53* (mutated in 21 tumors; 26.9%), followed by *PIK3CA* (mutated in 16 tumors; 20.5%) and *BRCA1/2* (mutated in 7 tumors, *BRCA1* n=2 and *BRCA2* n=5; 9.0%). In our cohort, a significantly higher proportion of patients with epithelial CTCs harbored mutations in the *BRCA1/2* genes in the tumor tissue. There were no mutations in specific genes associated with CTCs with the EMT phenotype. To the best of our knowledge, this study is the first to report a correlation between the presence of epithelial CTCs in the peripheral blood and mutations of the *BRCA1/2* genes in primary tumor tissue.

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Abbreviations: CTCs, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; EP_CTCs, epithelial CTCs; ER, estrogen receptor; HR, hazard ratio; HD, healthy donor; LVI, lymphovascular invasion; PBMCs, peripheral blood mononuclear cells; PBC, primary breast cancer; PR, progesterone receptor; RT-PCR, reverse transcription-polymerase chain reaction

Key words: circulating tumor cells, primary breast cancer, targeted resequencing, epithelial-to-mesenchymal transition, BRCA1/2

Introduction

Breast cancer is the most commonly diagnosed malignancy among women, with 2,088,849 new cases of invasive breast cancer and 626,679 deaths reported in 2018 (1). Metastatic disease is typically an incurable condition associated with significant morbidity and mortality in breast cancer patients (2). The metastatic cascade is characterized as a multi-step process that includes escape of cancer cells from the primary tumor to a distant location, where they can potentially form new tumor colonies (3). To successfully complete the metastatic cascade, epithelial tumor cells detached from the primary tumor must penetrate into the peripheral circulation and ultimately undergo extravasation at the distant site and establish a new tumor focus. Previous findings have demonstrated that cancer cells undergoing epithelial-to-mesenchymal transition (EMT) acquire the characteristics necessary to go through the multiple steps of metastasis (4).

Circulating tumor cells (CTCs) essentially contribute to tumor dissemination and progression and are a crucial factor in the metastatic cascade (3,5). The prognostic and predictive value of CTCs was consistently established by numerous trials, not only in metastatic, but also in primary breast cancer (PBC) (6-11). CTCs are closely associated with several biological processes, involving EMT, 'self-seeding', which is characterized as re-infiltration of the primary tumor by CTCs, as well as establishment of metastasis by more aggressive CTCs. Therefore, CTCs have been suggested to represent an indicator of treatment failure and disease progression (12,13). Available experimental and translational research data

indicate that the generation of CTCs is a continuous process spanning from one end of the spectrum (epithelial phenotype) to the other end (mesenchymal phenotype), and involves those with a partial EMT phenotype (14,15). Accordingly, CTCs may be considered a heterogeneous population of cells, including CTCs with partial or complete EMT phenotype, and these subpopulations have different clinical and biological properties (5). In addition, CTCs display dynamic changes in epithelial and mesenchymal composition (15).

The most common somatic mutations in breast cancer are *TP53*, *PIK3CA* and *GATA3*, which are present in >10% of all breast cancers (16). In a study including 216 patients, 12 genes (*TP53*, *PIK3CA*, *GATA3*, *ESR1*, *MAP3K1*, *CDH1*, *AKT1*, *MAP2K4*, *RB1*, *PTEN*, *CBFB* and *CDKN2A*) were established as significantly mutated in metastatic breast cancer (mBC), while 8 genes (*ESR1*, *FSIP2*, *FRAS1*, *OSBPL3*, *EDC4*, *PALB2*, *IGFN1* and *AGRN*) were more frequently mutated in mBC as compared to early breast cancer (17). Several published studies have investigated the mutational status of CTCs (18-21); however, data on the association between gene mutations in primary tumor tissue and the presence of CTCs in the peripheral blood are lacking.

The aim of the present study was to identify the breast cancer-specific mutation status in PBC in association with the presence of various subpopulations of CTCs using targeted resequencing.

Patients and methods

Study patients. This translational study (Protocol TRU-SK 002; Chair: M. Mego) included 78 PBC patients with stages I-III after definitive surgery. Patients enrolled in this study were selected from a cohort of 427 PBC patients analyzed in a previous study detecting CTCs with EMT phenotype in 77 (18.0%) patients with early breast cancer (22). Fresh frozen tumor tissue and status of CTCs in peripheral blood were available for all enrolled participants. To exclude the presence of distant metastasis, each patient underwent a full diagnostic evaluation. Patients with concurrent malignancies other than non-melanoma skin cancer in the previous 5 years were excluded. Patients' data regarding age, tumor stage, histological type, regional lymph node involvement, hormone receptor status, and human epidermal growth factor receptor 2 (HER2) status were also tabulated and statistically analyzed.

The study was performed between March 2012 and February 2015. Healthy donors (n=60) were age-matched women without breast cancer who were recruited according to the Institutional Review Board (IRB)-approved protocol. The present study was approved by the Institutional Review Board (IRB) of the National Cancer Institute of Slovakia (Bratislava, Slovak Republic). Written informed consent was obtained from each participant prior to study enrollment.

Detection of CTCs in the peripheral blood. The peripheral blood samples used for CTC detection were collected into Vacutainer® Blood Collection Tubes EDTA (BD Biosciences) in the morning on the day of surgery. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay using $CD45^+$ cell depletion for CTC enrichment was employed for CTC identification in the peripheral blood, as described previously (23,24).

RNA extraction and cell lines. A RossetteSep™ kit (Stemcell Technologies, Inc.) was used for CD45 depletion in peripheral blood samples, according to the manufacturer's instructions. CD45-depleted cells were mixed with 500 μl TRIzol LS reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and stored at -80˚C until RNA was extracted according to the manufacturer's instructions. The precipitated pellet containing RNA was dissolved in 50 μl nuclease-free water. Absorbance readings at 260 nm (median, 5.95 ng/μl; range, 1.7-38.3 ng/μl) were used to determine RNA concentration. RNA extracted from HeLa, HCT116, MCF-7 and MDA-MB-231 cells served as the positive control.

Identification of gene transcripts in CD45-depleted subsets. EMT-inducing TF gene transcripts (*TWIST*, *SNAIL1*, *SLUG* and *ZEB1*) and epithelial antigen (*CK19*) were identified by RT-qPCR. In brief, the reaction was carried out in 25 μl reaction volume containing 12.5 μl QuantiFast Probe RT-PCR kit Master Mix, 0.25 μl QuantiFast RT mix, 8.5 μl water and 1.25 μl primers (Qiagen GmbH). Isolated RNA was subsequently added (in a volume of 2.5 μl) to the reaction mix. The following TaqMan assays were obtained from Thermo Fisher Scientific, Inc.: TWIST1: Hs00361186_m1; SNAIL1: Hs00195591_m1; SLUG: Hs00161904_m1; ZEB1: Hs01566408_m1; GAPDH Hs99999905_m1; and CK19 Hs00761767_s1. Amplicons or probes spanned intron-exon boundaries, with the exception of CK19. Amplification was performed on a Roche LightCycler 480 II Real-Time PCR system (Roche Diagnostics GmbH) using the following cycling program: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and at 60˚C for 60 sec. All the samples were analyzed in triplicate. Calibrator samples were run with every plate to ensure consistency of the PCR. For all fluorescence-based RT-PCR, fluorescence was detected between 0 and 40 cycles for the control and marker genes in single-plex reactions, which allowed for the deduction of the cycle quantification (Cq) value for each product. Establishment of *GAPDH* housekeeping gene expression was used as the expression control in the identification of the genes of interest. Target cDNA was measured by the ΔCq method with the formula: 1=2 Cq (target-GAPDH) (25).

CTC definition. Patient samples with elevated *CK19* gene transcripts compared with healthy donors were categorized as epithelial CTC-positive (CTC_EP), while patient samples with increased EMT-TF (*TWIST1*, *SNAIL1*, *SLUG* and *ZEB1*) gene transcripts compared with those of healthy donors were categorized as CTC_EMT-positive. To categorize a patient sample as CTC-positive, expression of at least one of the markers (either epithelial or mesenchymal) at levels above the defined cut-off was required. The highest expression levels of the *CK19* and EMT-inducing TF gene transcripts relative to that of *GAPDH* were $3.4x10^{-3}$ (median, $2.8x10^{-6}$; range, 0-$3.4x10^{-3}$) for *CK19*, $7.5x10^{-4}$ (median, 0; range, 0-$7.5x10^{-4}$) for *TWIST1*, $3.8x10^{-2}$ (median, 0.003135; range, $5.0x10^{-4}$-$3.8x10^{-2}$) for *SNAIL1* and $1.7x10^{-1}$ (median, $1.4x10^{-2}$; range, $2.2x10^{-3}$-$1.7x10^{-1}$) for *ZEB1*, while *SLUG* transcripts were not detected in any of the samples from healthy donors. The cut-off value determining CTC positivity was established according to the highest expression values in healthy donors (14).

Table I. Patient characteristics.

Variable	N	%
All	78	100.0
T-stage		
1	52	66.7
>1	26	33.3
N-stage		
0	48	61.5
>1	30	38.5
Grade		
1 and 2	50	64.1
3	28	35.9
TNM classification		
Stage I	34	43.6
Stage II	24	30.8
Stage III	14	17.9
Histology		
Invasive ductal carcinoma	70	89.7
Other	8	10.3
Hormone receptor status		
Negative for both	12	15.4
Positive for either	66	84.6
HER2 status		
Positive	16	20.5
Negative	62	79.5
Ki 67 (cut-off 20%)		
Low	48	61.5
High	30	38.5
Epithelial CTC		
Present	20	25.6
Absent	58	74.4
CTC_ EMT		
Present	28	35.9
Absent	50	64.1
Any CTC		
Present	48	61.5
Absent	30	38.5

DNA isolation from the tumor. Tumor tissues were homogenized in liquid nitrogen and DNA was isolated from disintegrated cells using a DNeasy blood and tissue kit (Qiagen GmbH) according to the original protocol. The most representative central part of the primary tumor was used for extraction of tumor DNAs.

Targeted resequencing

Panel of analyzed genes. The gene panel contained well-known genes associated with clinically relevant characteristics in breast cancer patients: *ACVR1B*,*AKT1*,*ARID1A*,*ATM*,*BARD1*, *BRCA1*, *BRCA2*, *BRIP1*, *CASP8*, *CBFB*, *CDH1*, *CDKN1B*, *CHEK2*, *CTCF*, *CUL4*, *EP300*, *EPCAM*, *ERBB2*, *ERBB3*, *FANCC*, *FRFR2*, *FOXA1*, *GATA3*, *GNPTAB*, *HIST1H3B*, *KMT2A*, *KRAS*, *MAP2K4*, *MAP3K1*, *MED23*, *MLH1*, *MLL3*, *MSH2*, *MSH6*, *MYB*, *NBN*, *NCOR1*, *NF1*, *PALB2*, *PIK3CA*, *PIK3R1*, *PMS2*, *PTEN*, *RAB40A*, *RAD51C*, *RAD51D*, *RB1*, *RUNX1*, *SF3B1*, *SPEN*, *STAG2*, *STK11*, *TBL1XR1*, *TBX3*, *TP53* and *XRCC2*. The customized SureSelect XT (Agilent Technologies, Inc.) probe-based enrichment strategy was used.

Genomic library preparation. DNA libraries were prepared by SureSelect QXT Target Enrichment Kit (Agilent Technologies, Inc.) according to the protocol for Illumina Multiplex Sequencing. Final DNA libraries were diluted to 13-20 pmol/l pools with 10-11 samples analyzed in parallel per one MiSeq sequencing run using a MiSeq sequencing reagent kit v3 150 cycles (Illumina, Inc.), set to run in the 2x100 mode.

Genomic data analysis. Following standard BaseSpace-based sequencing data analysis, read mapping and variant calling sequencing data were annotated and filtered using Ingenuity Variant Analysis (Qiagen GmbH), which was set up to search for pathogenic variants associated with breast cancer. The identified pathogenic and likely pathogenic variants were verified by visualization in IGV and subsequently confirmed by Sanger sequencing.

Statistical analysis. The patients' characteristics were tabulated and summarized as the median (range) for continuous variables and frequency (percentage) for categorical variables. Categorical data were tested by Fisher's exact test or Chi-squared test. All P-values were two-sided, and $P \leq 0.05$ was considered to indicate statistically significant differences. Statistical analyses were performed using NCSS 2007 statistical software (NCSS LLC) (26).

Results

Patient characteristics. The analyzed cohort included 78 patients with PBC. The median age of the enrolled patients was 57 years (range, 33-83 years). The clinicopathological characteristics of the patients are summarized in Table I. The majority of the patients had invasive ductal (89.7%), hormone receptor-positive (84.6%) tumors and T1 stage disease (66.7%). HER2/neu amplification was observed in 20.5% of the tumors.

In the present analysis, CTCs in PBC were detected in 44 (61.1%) patient tumor samples, while no CTCs were detected in 28 (38.9%) of the analyzed tumors. In order to improve clarity of published results only CTCs with epithelial or EMT phenotype were taken into consideration. In subgroup of CTCs-positive patients, 38.6% (17/44) samples were characterized as epithelial CTCs (EP_CTC)-positive, while CTCs EMT (CTC_EMT)-positive phenotype was actually identified in 61.4% (27/44) of samples Table II.

Identification of mutations in breast cancer-related genes in the primary tumor and its association with the presence of CTCs in the peripheral blood. Six of the 78 samples (7.7%) were excluded due to poor quality of DNA for analysis. Mutations were detected in 68.1% (49/72) of tumor samples, 36.1% (26/72) were classified as likely pathogenic and 61.1% (44/72) as pathogenic while in 27.8% (20/72) of tumors no

Table II. Association between mutation status and clinicopathological characteristics.

		Any mutation vs. no mutation		*TP53*		*PIK3CA*		*BRCA1/2*		Other than *TP53*, *PIK3CA*, *BRCA*		Double/triple hit vs. zero/single	
Variables	No.	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
T-stage													
1	49	38	77.6	13	26.5	12	24.5	4	8.2	28	57.1	12	24.5
>1	23	13	56.5	8	34.8	4	17.4	2	8.7	10	43.5	6	26.1
P-value			0.10		0.58		0.56		1.00		0.32		1.00
N-stage													
0	45	33	73.3	13	28.9	11	24.4	4	8.9	26	57.8	10	22.2
>1	27	18	66.7	8	29.6	5	18.5	2	7.4	12	44.4	8	29.6
P-value			0.60		1.00		0.77		1.00		0.33		0.58
Histology													
Invasive ductal cancer	69	47	68.1	20	29.0	15	21.7	6	8.7	36	52.2	15	21.7
Other	5	4	80.0	1	20.0	1	20.0	0	0.0	2	40.0	3	60.0
P-value			1.00		1.00		1.00		1.00		0.66		0.10
Grade													
1 and 2	42	29	69.0	9	21.4	13	31.0	3	7.1	23	54.8	9	21.4
3	25	18	72.0	11	44.0	2	8.0	3	12.0	13	52.0	6	24.0
P-value			1.00		0.06		**0.04**		0.66		1.00		1.00
Hormone receptor status													
Negative for both	12	11	91.7	9	75.0	1	8.3	2	16.7	11	91.7	4	33.3
Positive for either	60	40	66.7	12	20.0	15	25.0	4	6.7	27	45.0	14	23.3
P-value			0.16		**0.0004**		0.28		0.26		**0.004**		1.00
HER2 status													
Positive	16	13	81.3	9	56.3	1	6.3	1	6.3	10	62.5	4	25.0
Negative	56	38	67.9	12	21.4	15	26.8	5	8.9	28	50.0	14	25.0
P-value			0.37		**0.01**		0.10		1.00		0.41		1.00
Ki 67 (cut-off 20%)													
Low	42	29	69.0	5	11.9	13	31.0	2	4.8	18	42.9	10	23.8
High	30	22	73.3	16	53.3	3	10.0	4	13.3	20	66.7	8	26.7
P-value			0.80		**0.0002**		**0.05**		0.23		0.06		0.79
CTC epithelial													
Negative	**28**	19	67.9	8	28.6	4	14.3	0	0.0	11	39.3	7	25.0
Positive	**17**	13	76.5	3	17.6	4	23.5	4	23.5	11	64.7	3	17.6
P-value			0.74		0.49		0.45		**0.02**		0.13		0.72

Table II. Continued.

Variables	No.	Any mutation vs. no mutation		*TP53*		*PIK3CA*		*BRCA1/2*		Other than *TP53*, *PIK3CA*, *BRCA*		Double/triple hit vs. zero/single	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
CTC EMT													
Negative	**28**	19	67.9	8	28.6	4	14.3	0	0.0	11	39.3	7	25.0
Positive	**27**	19	70.4	10	37.0	8	29.6	2	7.4	16	59.3	8	29.6
P-value			1.00		0.57		0.20		0.24		0.18		0.77
CTC Any	N												
Negative	28	19	67.9	8	28.6	4	14.3	0	0.0	11	39.3	7	25.0
Positive	44	32	72.7	13	29.5	12	27.3	6	13.6	27	61.4	11	25.0
P-value			0.79		1.00		0.25		0.08		0.09		1.00

Bold, statistically significant.

mutation was detected. In 43.1% (31/72) of tumor samples the patient's single pathogenic or likely pathogenic mutation was detected, in 18.1% (13/72) patient mutations in two genes were found, while in 4.2% (3/72) of tumor samples patient mutations in 3 genes were detected.

The most commonly affected genes were *TP53*, mutated in 25.0% (18/72) tumors, followed by *PIK3CA* mutated in 22.2% (16/72) tumors, *BRCA1/2* in 9.7% (7/72) tumors (2 for *BRCA1* and 5 for *BRCA2*), *CDH1* and *GATA3* in 6.9% (5/72) tumors. *RUNX1* and *PTEN* were mutated in 4.2% (3/72) tumors, *NF1*, *BRIP1* and *ATM* in 2.8% (2/72) tumors while *BARD1*, *CDKN1B*, *GNPTAB*, *KRAS*, *PIK3R1* and *PMS2* were mutated in 1.4% (1/72) tumor (Table III).

There were no differences in the number of tumors with pathogenic or likely pathogenic mutations between participants with detectable CTCs in the peripheral blood compared with patients with non-detectable CTCs (67.9 vs. 72.7%, respectively; P=0.79) (Table II). This difference remained unchanged when likely pathogenic mutations were excluded and no mutations vs. pathogenic mutations were compared (46.4 vs. 52.3%, respectively; P=0.78) (data not shown). Similarly, there were no differences in CTC status regarding mutations of *TP53* and *PIK3CA* and/or between tumors with single mutations vs. those with double/triple mutations. However, no BRCA1/2 mutations were detected in CTC-negative tumors compared with 9.7% of BRCA1/2 mutations (P=0.08) in CTCs-positive tumors. Moreover, 4 (23.5%) patients with epithelial CTCs in peripheral blood had *BRCA1/2* mutations compared to 0 (0%) patients without *BRCA1/2* mutations (P=0.02), while there was no significant correlation between mutation in the specific gene and presence of CTC_EMT-positive cells. Similarly, there was a trend for an increased mutation rate of genes other than *TP53*, *PIK3CA* and *BRCA1/2* in CTC-positive compared with CTC-negative patients (61.4 vs. 39.3%, respectively; P=0.09) (Table II).

Examination of specific mutations revealed a significant association between *TP53* mutation and HER2-positive status, hormone receptor negativity, high grade and increased tumor cell proliferation, as determined by the expression of Ki67. *PIK3CA* mutations were associated with lower grade and low proliferation rate, as determined by Ki67 (Table II).

Discussion

To the best of our knowledge, the present study was the first to reveal the association between the presence of epithelial CTCs in the peripheral blood and mutations of the *BRCA1/2* genes in primary tumor tissue. We observed a numerically higher mutation rate in genes other than *TP53* and *PIK3CA* and *BRCA1/2* in patients with CTC-positive compared with CTC-negative breast tumors; however, the differences did not reach statistical significance. The most commonly mutated genes in our patient cohort included *TP53*, *PIK3CA*, *BRCA1/2*, *CDH1* and *GATA3*, corresponding to the observed incidence in published datasets (16,17). Similarly, Stephens *et al* detected somatic driver substitutions and small insertions/deletions (indels) in cancer genes previously implicated in breast cancer development, including *AKT1*, *BRCA1*, *CDH1*, *GATA3*, *PIK3CA*, *PTEN*, *RB1* and *TP53* (27). Cancer genes *TP53*, *PIK3CA*, *ERBB2*, *MYC*, *FGFR1/ZNF703*, *GATA3* and *CCND1*

Table III. Panel of specific mutations identified in PBC patients (n=79).

Patient no.	Category	Gene	Mutation effect
1	No mutation	No mutation	No mutation
3	Pathogenic	*CDH1*	Stop gain
4	No mutation	No mutation	No mutation
5	Likely Pathogenic	*BARD1*	Frameshift
	Likely Pathogenic	*TP53*	Frameshift
6	NA	NA	NA
7	Likely Pathogenic	*GATA3*	Frameshift
8	Likely Pathogenic	*RUNX1*	Frameshift
9	Likely Pathogenic	*PIK3R1*	Frameshift
10	Pathogenic	*PIK3CA*	Missense
	Likely Pathogenic	*TP53*	Frameshift
11	Likely Pathogenic	*TP53*	Frameshift
12	Likely Pathogenic	*CDH1*	Frameshift
	Pathogenic	*TP53*	Missense
13	Pathogenic	*NF1*	Stop gain
	Pathogenic	*TP53*	Missense
14	Pathogenic	*TP53*	Missense
15	Pathogenic	*ATM*	Frameshift
16	No mutation	No mutation	No mutation
17	Pathogenic	*PIK3CA*	Missense
18	No mutation	No mutation	No mutation
19	Likely Pathogenic	*BRIP1*	Missense
	Likely Pathogenic	*TP53*	Frameshift
20	Pathogenic Likely	*PIK3CA*	Missense
	Pathogenic	*GATA3*	Frameshift
	Pathogenic	*ATM*	Stop gain
21	Pathogenic	*TP53*	Missense
22	No mutation	No mutation	No mutation
23	No mutation	No mutation	No mutation
24	No mutation	No mutation	No mutation
25	NA	NA	NA
26	Pathogenic	*GATA3*	Frameshift
27	Pathogenic	*PIK3CA*	Missense
28	Pathogenic	*PIK3CA*	Missense
29	No mutation	No mutation	No mutation
30	No mutation	No mutation	No mutation
31	Pathogenic	*PIK3CA*	Missense
33	Pathogenic	*PIK3CA*	Missense
	Pathogenic	*TP53*	Missense
35	Pathogenic	*CDH1*	Stop gain
36	Likely Pathogenic	*GATA3*	Frameshift
37	Pathogenic	*TP53*	Missense
38	Pathogenic	*TP53*	Missense
40	Pathogenic	*BRCA1*	Frameshift
	Pathogenic	*TP53*	Missense
	Pathogenic	*RUNX1*	Frameshift
43	Pathogenic	*PIK3CA*	Missense
44	No mutation	No mutation	No mutation
45	Pathogenic	*PIK3CA*	Missense
46	Likely Pathogenic	*TP53*	Frameshift
47	Pathogenic	*BRCA2*	Frameshift
	Pathogenic	*BRCA2*	Stop gain

Table III. Continued.

Patient no.	Category	Gene	Mutation effect
48	Pathogenic	*TP53*	Missense
49	No mutation	No mutation	No mutation
50	No mutation	No mutation	No mutation
51	Pathogenic	*TP53*	Missense
52	No mutation	No mutation	No mutation
53	Likely Pathogenic	*GATA3*	Frameshift
54	Likely Pathogenic	*BRCA2*	Missense
55	Likely Pathogenic	*NF1*	Frameshift
	Likely Pathogenic	*TP53*	Frameshift
56	Likely Pathogenic	*TP53*	Frameshift
57	Pathogenic	*GNPTAB*	Frameshift
	Pathogenic	*BRCA2*	Frameshift
58	NA	NA	NA
59	No mutation	No mutation	No mutation
60	Pathogenic	*PIK3CA*	Missense
61	Pathogenic	*PIK3CA*	Missense
	Pathogenic	*BRIP1*	Frameshift
62	No mutation	No mutation	No mutation
63	Pathogenic	*PIK3CA*	Missense
	Pathogenic	*TP53*	Stop gain
	Likely Pathogenic	*PMS2*	Frameshift
64	No mutation	No mutation	No mutation
65	No mutation	No mutation	No mutation
66	Pathogenic	*PIK3CA*	Missense
	Likely Pathogenic	*BRCA2*	Missense
67	Likely Pathogenic	*TP53*	Missense
68	Likely Pathogenic	*CDH1*	Frameshift
	Likely Pathogenic	*RUNX1*	Frameshift
69	Pathogenic	*KRAS*	Missense
70	No mutation	No mutation	No mutation
71	Pathogenic	*PIK3CA*	Missense
72	No mutation	No mutation	No mutation
73	No mutation	No mutation	No mutation
74	Likely Pathogenic	*PTEN*	Frameshift
	Likely Pathogenic	*CDKN1B*	Frameshift
75	Pathogenic	*PTEN*	Stop gain
	Pathogenic	*PTEN*	Frameshift
76	Pathogenic	*BRCA1*	Frameshift
77	Pathogenic	*PIK3CA*	Missense
	Likely Pathogenic	*CDH1*	Frameshift
78	Pathogenic	*PIK3CA*	Missense

PBC, primary breast cancer.

were mutated in more than 10% of the analyzed cohort (27). *CDKN1B* (also known as p27 or KIP1) normally inhibits the activation of cyclin E/CDK2 and cyclin D/CDK4 complexes, thus preventing cell cycle progression at phase G1 (28). Our analysis revealed one frameshift mutation in CDKN1B. These data confirm previously published results reporting *CDKN1B* gene as a cancer gene (27). Stephens *et al* also identified 73 different combinations of mutated cancer genes. In our analyzed group we found 18 different combinations of mutated cancer genes. Based on these results, we determined that most breast cancers differed from all others (27). Significant correlations between *TP53* mutational status and certain clinicopathological characteristics of the primary tumor were in concordance with literature data (29). *BRCA1/2* are tumor suppressor genes that are involved in DNA repair pathways associated with hereditary breast and ovarian cancer

syndrome (30). A study by Erturk *et al* demonstrated that in *BRCA*-mutated triple-negative breast cancer, increased invasion and metastasis of cancer cells was mediated through the *BRCA* mutation-associated decreased expression of miR-200c (31). Therefore, our results may be partially explained by *BRCA1/2* mutation-mediated increased invasion of breast cancer cells. Germline mutations of *BRCA* are not associated with a higher rate of lymphovascular invasion (LVI) compared with non-mutated tumors (32); however, previous studies showed a correlation between LVI and the presence of CTCs (33,34).

Data regarding the association between mutations in the primary tumor and the presence of CTCs in the peripheral blood are limited. While there are some studies that investigate the correlations between mutations in specific genes in CTCs and corresponding tumor tissue, trials addressing the association between mutational status of the primary tumor and the presence of CTCs are currently lacking. For example, in a study by Bredemeier *et al*, no correlation was observed between *PI3KCA* mutations in cancerous tissue and the presence of CTCs in peripheral blood (19). In another study, there was a high match rate between specific mutations in CTCs and corresponding tumor tissue, but no data regarding the association between a specific mutation and the number of CTCs were reported (18). The cell-cell adhesion molecule CDH1 (E-cadherin) as well as CTNNB1 (beta-catenin), are involved in the process of cancer cell detachment from the primary tumor. Their association with EMT was revealed through several studies (35-37). The mechanism of EMT is predominantly activated by epigenetic events; however, data explaining the role of genetic are still limited. The study of Busch *et al* suggested that somatic mutations in *CDH1* and *CTNNB1* genes do not represent a major contributor to cancer cell detachment, and therefore presumably play a limited role in the etiology of tumor metastasis as well as in EMT (38). The association between CTC_EMT status and RFS (relapse-free survival) was evaluated in another study (22). This study including 427 PBC patients revealed the prognostic value of CTCs with EMT phenotype in all analyzed subgroups of patients. Patients enrolled into the present study were selected from the abovementioned cohort of PBC patients. In addition, CTCs with epithelial phenotype had no prognostic role in the abovementioned cohort (data not shown). It may be suggested that CTCs reflect specific biological characteristics of the tumor, similar to previous studies that showed no association between CTCs and common clinicopathological characteristics (6,9,23). Due to intratumoral heterogeneity, we cannot exclude an association between mutational status in specific parts of the tumor (such as the tumor edge) and/or involvement of genes other than the ones examined in CTC generation.

There were certain limitations to the present study, such as the limited sample size with decreased statistical power of analyses, as well as the fact that we only assessed 56 breast cancer-associated genes. It was not possible to assess the impact of total mutational burden on CTC positivity. In addition, the study population was a homogenous cohort of patients, treatment-naive, without metastatic disease, in order to avoid the effect of the metastatic site heterogeneity factor on analyzed variables. Furthermore, the evaluation of epithelial and mesenchymal phenotype changes via *BRCA* gene silencing *in vitro* was not performed in present study.

In conclusion, a correlation between the presence of epithelial CTCs in the peripheral blood and mutations of *BRCA1/2* genes in primary tumor tissue was identified, while there was no mutation in specific genes associated with CTC_EMT. The number of mutated breast cancer-associated genes was not associated with the presence of CTCs or the mutation of genes other than *BRCA1/2*, suggesting that different factors may be involved in the generation and migration of CTCs. These data support the concept that CTCs are of high biological and clinical value in breast cancer.

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Availability of data and materials

All datasets generated and analyzed during the present study are included in this published article.

Authors' contributions

MM, JMar and GM participated in the conception and design of this study. MM performed statistical analysis. GM, TS and KK were involved in CTC detection. GR, LK and GM performed next-generation sequencing. MK, JB and DP were involved in patient accrual and performed breast surgery. GS and JMac performed pathological examination. SJ was involved in patient accrual. MM drafted the article and all authors reviewed it critically for important intellectual content. All the authors participated in the acquisition, analysis and interpretation of data. All the authors have read and approved the final version of the manuscript for publication.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board (IRB) of the National Cancer Institute of Slovakia (Bratislava, Slovak Republic). Written informed consent was obtained from each participant prior to study enrollment.

Patient consent for publication

Not applicable.

Competing interests

Authors declare they have no competing interests.

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