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Prognostic Impact of Src, CDKN1B, and JAK2 Expression in Metastatic Breast Cancer Patients Treated with Trastuzumab

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Abstract

BACKGROUND: Src, CDKN1B, and JAK2 play a crucial role in the coordination of cell signaling pathways. In the present study, we aim to investigate the prognostic significance of these biomarkers in HER2-positive metastatic breast cancer (MBC) patients treated with trastuzumab (T). *METHODS:* Formalin-fixed paraffin-embedded tumor tissue samples from 197 patients with HER2-positive MBC treated with T were retrospectively collected. All tissue samples were centrally assessed for ER, PgR, Ki67, HER2, and PTEN protein expression; EGFR gene amplification; PI3KCA mutational status; and tumor-infiltrating lympocytes density. Src, CDKN1B, and JAK2 mRNA expression was evaluated using quantitative reverse transcription-polymerase chain reaction. *RESULTS:* Only 133 of the 197 patients (67.5%) were found to be HER2-positive by central assessment. CDKN1B mRNA expression was strongly correlated with Src (rho = 0.71) and JAK2 (rho = 0.54). In HER2-positive patients, low CDKN1B conferred higher risk for progression [hazard ratio (HR) = 1.58, 95% confidence interval (CI) 1.08-2.32, $P = .018$]. In HER2-negative patients, low Src was associated with longer survival (HR = 0.56, 95% CI 0.32-0.99, $P = .045$). Upon multivariate analyses, only low CDKN1B and JAK2 mRNA expression remained unfavorable factors for PFS in *de novo* and relapsed (R)-MBC patients, respectively (HR = 2.36, 95% CI 1.01-5.48, $P = .046$ and HR = 1.76, 95% CI 1.01-3.06, $P = .047$, respectively). *CONCLUSIONS:* Low CDKN1B and JAK2 mRNA expressions were unfavorable prognosticators in a cohort of T-treated MBC patients. Our results suggest that CDKN1B and JAK2, if validated, may serve as prognostic factors potentially implicated in T resistance, which seems to be associated with distinct pathways in *de novo* and R-MBC.

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Introduction

Breast cancer (BC) is the most commonly diagnosed female cancer and the second leading cause of cancer-related death in women in the United States [1]. In clinical practice, evaluation of biomarkers that may potentially play a role in tumor pathology allows physicians to prospectively select BC patients for specific therapies. In this context, approximately 20% of human BCs overexpress the epidermal growth factor receptor 2 (HER2), which participates in a series of processes promoting oncogenesis. HER2 can heterodimerize with any of the other three receptors of the ERBB family (EGFR, HER3, and HER4), thereby inducing and stimulating dimer transphosphorylation and consequent activation of various signaling pathways, such as phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinases (MAPK)/extracellular signal–regulated kinases (ERK) [2,3].

Src kinase is a member of a nonreceptor tyrosine kinase family, which is involved in a variety of cellular functions (proliferation, motility, and invasion) through its interaction with mediators, including steroid hormone receptors (HoRs), integrins, growth factors, such as platelet-derived growth factor receptors (PDGFR), HER family members (EGFR, HER2 and HER3) and insulin-like growth factor 1 receptor (IGF1R), signal transducers and activators of transcription (STAT) family members, and several other receptors and intracellular proteins [4–6]. Once activated, Src acts as a common upstream regulator of the MAPK/ERK and PI3K pathway inducing malignant transformation [7]. There is substantial evidence that Src plays an important role in the development of BC and is a key modulator of trastuzumab response [3,8,9].

On the other hand, mammalian cell proliferation is tightly regulated by the sequential activation of cyclin dependent kinases (CDKs). The CDKN1B gene encodes for the p27 protein, which inhibits the enzymatic activity of cyclin-CDK complexes and plays an important role in the progression from the G1 to the S phase of the cell cycle [10]. Oncogenic activation of receptor tyrosine kinases (RTK), PI3K, Src, or MAPK pathways cooperates to inactivate p27 or accelerate its proteolysis in human cancers, including BC [11]. In addition, p27 downregulation has been shown to correlate with trastuzumab resistance in preclinical models [12]. In addition to Src and CDKN1B, Janus kinase 2 (JAK2) and its main substrate STAT5 are critical for breast cell growth and differentiation [13]. Disturbances to the state of equilibrium of the JAK2/STAT5 pathway have been shown to contribute to mammary carcinogenesis [14]. Although the available preclinical data are promising, results in clinical series regarding the clinical utility of these biomarkers have been inconsistent.

Studies of mRNA levels in breast tumors have been useful for classifying breast cancers into subtypes that correlate with prognosis and drug responsiveness, for predicting recurrence, and for describing gene expression signatures associated with prognosis [15–18]. Herein, we seek to determine the prognostic significance, clinical utility, and association with trastuzumab response of Src, JAK2, and CDKN1B in HER2-possitive metastatic breast cancer (MBC) patients. To accomplish our goal we analyzed the mRNA expression of these biomarkers in correlation with patients' characteristics and outcomes in a series of HER2-possitive MBCs treated with trastuzumab.

Materials and Methods

The study was conducted on a previously published group of patients [19] enriched with new cases. Eligibility criteria for the study were a) histologically confirmed MBC; b) adequacy of clinical data on

patient's history, demographics, tumor characteristics, treatment details (drug dosages, schedule of administration, serious toxicities), and clinical outcome; c) availability of adequate tumor tissue for biological marker evaluation; and d) trastuzumab treatment for metastatic disease [19–23].

Formalin-fixed paraffin-embedded (FFPE) tumor tissue samples were retrospectively collected from patients treated with trastuzumab-based regimens in the metastatic setting, as previously described in detail [19–23]. All carcinomas had initially been diagnosed as HER2-positive, and all patients had therefore been treated with trastuzumab. The translational research protocol has been approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine (Protocol # 4283; January 14, 2008) under the general title "Investigation of major mechanisms of resistance to treatment with trastuzumab in patients with metastatic breast cancer." All patients included in the study after 2005 provided written informed consent for the provision of biological material for future research studies before receiving any treatment. Waiver of consent was obtained from the Bioethics Committee for patients included in the study before 2005.

All tumor samples were reevaluated by immunohistochemistry (IHC) for estrogen receptors (ER), progesterone receptors (PgR), HER2, and the expression of the proliferation marker Ki67, while HER2 status was reexamined by fluorescence *in situ* hybridization (FISH).

Data from earlier publications by our group, such as Ki67 and PTEN examined by IHC and single nucleotide polymorphism (SNP) genotyping performed for the evaluation of *PIK3CA* mutations, were used for associations with the present findings.

Tissue Microarrays (TMAs)

Representative hematoxylin and eosin (H&E)–stained sections from the tissue blocks were reviewed by a pathologist (M.B.). Seventeen TMA blocks were constructed from the 197 eligible cases using a manual tissue microarrayer (Beecher Instruments, Sun Prairie, WI), as previously described [19]. For the construction of the TMA blocks, two core samples (1.5 mm in diameter) were obtained from representative regions of each tumor in the donor blocks. Each TMA block also contained cores from various neoplastic, non-neoplastic and reactive tissues, serving as assay controls.

HC Tumor-Infiltrating Lymphocytes (TILs)

Serial 2.5-μm–thick TMA sections or whole tissue sections were stained for ER (clone 6F11, Leica Biosystems, Newcastle Upon Tyne, UK), PgR (clone 1A6, Leica Biosystems), HER2 (polyclonal Ab, code A0485, Dako, Glostrup, Denmark), Ki67 (clone MIB-1, code M7240, Dako), and stPTEN (clone 6H2.1, code M3627, Dako, at 1:200 dilution, for 30 minutes) using the Bond Max autostainer (Leica Microsystems, Wetzlar, Germany), as previously described in detail [23]. All sections were stained in one run for each antibody and were evaluated by pathologists experienced in breast cancer and blinded as to the patient's clinical characteristics and survival data. Positive controls were used for all antibodies from known positive breast cancer cases, while negative controls were obtained by omitting the primary antibody. All IHC stains were evaluated according to formerly outlined interpretation [24]. Stromal TILs density was assessed on whole H&E sections according to the guidelines by the International TILs Working Group [25] and was used as a continuous variable.

FISH

TMA sections or whole tissue sections (5 μm thick) were used for FISH analysis using the ZytoLight SPEC *HER2/TOP2A/CEN17* triple-color probe kit for HER2 (code Z-2093, ZytoVision, Bremerhaven, Germany) and ZytoLight SPEC EGFR/CEN 7 dual-color probe (code Z-2033).

FISH was performed according to the manufacturer's protocol with minor modifications in all cases and not only the HER2 IHC 2+ cases. Digital images were constructed using specifically developed software for cytogenetics (XCyto-Gen, ALPHELYS, Plaisir, France) and evaluated as previously described [19,23]. For assessing HER2 status, we used the 2007 ASCO/CAP guidelines [26] with the addition of the ≥6-HER2-copies criterion [27] because patients had locally received trastuzumab based on this classification.

EGFR gene status was assessed in 60 nonoverlapping nuclei from the invasive part of the tumor and evaluated as previously described [28].

Dual Nucleic Acid Extraction

Simultaneous isolation of DNA and RNA from whole or, in cases with <50% tumor cell content, macrodissected 10-μm paraffin sections was performed for the 197 tumors with silica-coated magnetic beads (Versant Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY) [23,29]. Each nucleic acid extract was divided into two aliquots, one for use in mutation analyses and the other for mRNA profiling, following treatment with DNase I to remove DNA and ensure the presence of pure RNA.

DNA Analysis for PIK3CA Mutations

Mutation testing for hotspot PIK3CA mutations E542K and E545K (coding exon 9) and H1047R (coding exon 20) was accomplished with custom Taqman-MGB-SNP genotyping assays, as described before [19].

mRNA Expression Analysis

cDNA synthesis was performed with random primers and SuperScript III Reverse Transcriptase (Invitrogen, cat. no. 48190011 and 18080044), according to the manufacturer's instructions. cDNAs were assessed in duplicates in the 7900HT system for 45 cycles of amplification (default conditions). mRNA expression analysis was performed with premade exon-spanning Taqman-MGB assays (Applied Biosystems/Life Technologies) targeting the following transcripts: Src exons 7-8 (assay ID Hs00178494_m1; 70 bp), CDKN1B exons 1-2 (Hs00153277_m1; 71 bp), and JAK2 exons 23-24 with NM_004972.3 (Hs00234567_m1; 101 bp). A Taqman-MGB expression assay targeting β-glucuronidase (Hs99999908_m1) was used as the endogenous reference for relative quantification. The commercially available TaqMan Control Total RNA (cat. no 4307281, Applied Biosystems) was applied as a positive control for interrun evaluation of polymerase chain reaction (PCR) assay efficiency, together with no-template controls. To obtain linear relative quantification (RQ) values, relative expression was assessed as 40-dCT, as previously described [23,29]. Samples were considered eligible for GUSB CT <36 and deltaRQ for each duplicate pair (intrarun variation) of <1. Based on the above criteria, 173 (88%) RNA samples yielded informative results for all examined targets, whereas all samples were informative for at least one target and were considered eligible for relative mRNA expression analysis.

Statistical Analysis

Categorical data are presented as frequencies with corresponding percentages, while the median and range are presented for the continuous

variables. Comparisons between categorical variables were performed using the chi-square or Fisher's exact (where appropriate) tests, while Wilcoxon rank-sum tests were used to detect differences between categorical and continuous variables. Spearman correlations were performed to examine the association between continuous variables.

The 50th percentile (median value) of the markers of interest was used as the optimal cutoff to examine their prognostic value as well as the associations between them and with several clinicopathological parameters. In addition, the upper and lower quartiles of the mRNA distribution were assessed as possible thresholds.

Progression-free survival (PFS) was defined as the time interval from the initiation of trastuzumab treatment for metastatic disease (with or without concurrent use of chemotherapy/hormonal therapy) to the date of disease progression, death from any cause, or the date of last contact, whichever occurred first. Survival was calculated from the initiation of trastuzumab treatment to the date of death. Alive patients were censored at the date of their last contact. Time to event data were analyzed using the Kaplan-Meier product limit method and compared across groups with the log-rank test. The associations between factors of interest and progression/mortality rates were assessed using hazard ratios (HRs) estimated with univariate and multivariate Cox proportional-hazard regression models.

In the multivariate analyses, a backward selection procedure with a removal criterion of $P > .10$ was used including the following clinicopathological parameters in the initial step: menopausal status (premenopausal vs. postmenopausal), performance status (1-3 vs. 0), PIK3CA status (mutant vs. wild-type), PTEN status (no loss vs. loss), Ki67, as well as each of the markers that was found to be significant or revealed a trend towards significance in the univariate analyses ($P < .10$).

The PFS and survival analyses were performed separately in HER2-positive, HER2-negative, relapsed metastatic breast cancer (R-MBC), and *de novo* patients, while all other analyses were performed in the entire study population.

Results of this study are presented according to reporting recommendations for tumor marker prognostic studies [30]. All tests were two-sided at an alpha 5% level of significance. No adjustment for multiple comparisons was performed since this study was exploratory and mainly hypothesis generating with predefined parameters. Analyses were conducted using the SAS (version 9.3, SAS Institute Inc., Cary, NC) software. R studio version 3.5.0 was used to produce violin and survival plots.

Results

A total of 197 women with metastatic breast cancer treated with trastuzumab were included in the current study. Even though all patients had initially been diagnosed with HER2-positive disease, only 133 of them (67.5%) were found to have HER2 gene amplification and/or 3+ HER2 protein expression (by FISH and IHC, respectively) per central HER2 assessment. Sixty-four patients (32.5%) had therefore been treated with trastuzumab therapy for metastatic disease despite truly having HER2-negative tumors. It is of note that in only 29.7% of the HER2-negative population was trastuzumab initiated after 2006, while in 45 of the 64 truly HER2-negative patients (70.3%), trastuzumab treatment was administered earlier. Selected patient and tumor characteristics by HER2 status are presented in Table 1. Patients diagnosed with stage IV were characterized as *de novo* metastatic breast cancer patients, while the rest of the patients, diagnosed at earlier stages of the disease, were considered as relapsed metastatic breast cancer patients (R-MBC).

In 176 patients (89.3%), trastuzumab was administered as first-line treatment, while 17 patients (8.6%) received trastuzumab as second-line therapy. In two of the remaining four patients trastuzumab therapy was initiated latter in the third and seventh-line of treatment, respectively. It is of note that 178 patients (90.4%)

Table 1. Basic Patient and Tumor Characteristics by HER2 Status

	HER2 Status	
	Negative	Positive
	(*N* = 64)	(*N* = 133)
	N (%)	*N* (%)
Age * **(years)**		
Median (range)	58.9 (32.5-75.8)	55.0 (28.3-95.0)
***De novo* MBC**	21 (32.8)	53 (39.8)
Type of surgery †		
Biopsy only	10 (47.6)	16 (30.2)
Modified radical	4 (19.0)	20 (37.7)
Partial mastectomy	6 (28.6)	13 (24.5)
Simple mastectomy	1 (4.8)	4 (7.5)
R-MBC	43 (67.2)	80 (60.2)
Adjuvant CT ‡	39 (90.7)	63 (78.8)
Anthracycline-based adjuvant CT ‡	24 (55.8)	51 (63.8)
Taxane-containing CT ‡	11 (25.6)	35 (43.8)
CMF-based CT ‡	24 (55.8)	32 (40.0)
Adjuvant HT ‡	32 (74.4)	53 (66.3)
Adjuvant RT ‡	24 (55.8)	44 (55.0)
ER/PgR status		
Negative	11 (17.2)	51 (38.3)
Positive	53 (82.8)	82 (61.7)
Subtypes		
HER2-enriched	0 (0.0)	51 (38.3)
Luminal A	10 (15.6)	0 (0.0)
Luminal B	41 (64.1)	0 (0.0)
Luminal HER2	0 (0.0)	82 (61.7)
TNBC	11 (17.2)	0 (0.0)
Unknown	2 (3.1)	0 (0.0)
Histological grade		
I-II	25 (39.1)	46 (34.6)
III	35 (54.7)	82 (61.7)
Unknown	4 (6.3)	5 (3.8)
Menopausal status *		
Premenopausal	16 (25.0)	33 (24.8)
Postmenopausal	48 (75.0)	100 (75.2)
Performance status *		
0	47 (73.4)	98 (73.7)
1-3	15 (23.4)	34 (25.6)
Unknown	2 (3.1)	1 (0.8)
Number of trastuzumab lines		
1	27 (42.2)	49 (36.8)
2	15 (23.4)	31 (23.3)
3	9 (14.1)	21 (15.8)
≥4	13 (20.3)	32 (24.1)
Number of metastatic sites		
≤3	54 (84.4)	122 (91.7)
>3	8 (12.5)	10 (7.5)
Unknown	2 (3.1)	1 (0.8)
Bone metastases	28 (45.2)	46 (34.8)
Nodal metastases	11 (17.7)	26 (19.7)
Visceral metastases	42 (68.9)	96 (72.7)

HER2, human epidermal growth factor receptor; *MBC*, metastatic breast cancer; *R-MBC*, relapsed metastatic breast cancer; *CT*, chemotherapy; *HT*, hormonal therapy; *RT*, radiotherapy.

* At initiation of trastuzumab treatment.

† Only for patients with *de novo* metastatic breast cancer.

‡ Only for R-MBC patients.

Table 2. Distribution of CDKN1B, JAK2, and Src by Disease Presentation Status

		Disease Presentation Status		
		R-MBC	*De Novo* MBC	*P* Value
CDKN1B mRNA expression	Median (range)	41.6 (36.6-45.1)	40.9 (36.0-45.1)	<.001
CDKN1B mRNA expression	High	101 (83.5)	43 (60.6)	<.001
(lower quartile as cutoff)	Low	20 (16.5)	28 (39.4)	
CDKN1B mRNA expression	High	72 (59.5)	24 (33.8)	.001
(median value as cutoff)	Low	49 (40.5)	47 (66.2)	
CDKN1B mRNA expression	High	37 (30.6)	11 (15.5)	.020
(upper quartile as cutoff)	Low	84 (69.4)	60 (84.5)	
JAK2 mRNA expression	Median (range)	37.0 (27.9-40.2)	36.7 (27.5-41.0)	.19
JAK2 mRNA expression	High	86 (77.5)	48 (70.6)	.30
(lower quartile as cutoff)	Low	25 (22.5)	20 (29.4)	
JAK2 mRNA expression	High	60 (54.1)	29 (42.6)	.14
(median value as cutoff)	Low	51 (45.9)	39 (57.4)	
JAK2 mRNA expression	High	28 (25.2)	16 (23.5)	.80
(upper quartile as cutoff)	Low	83 (74.8)	52 (76.5)	
Src mRNA expression	Median (range)	40.1 (36.0-43.7)	39.7 (36.7-43.4)	.003
Src mRNA expression	High	96 (80.7)	47 (65.3)	.017
(lower quartile as cutoff)	Low	23 (19.3)	25 (34.7)	
Src mRNA expression	High	67 (56.3)	28 (38.9)	.020
(median value as cutoff)	Low	52 (43.7)	44 (61.1)	
Src mRNA expression	High	38 (31.9)	9 (12.5)	.003
(upper quartile as cutoff)	Low	81 (68.1)	63 (87.5)	

received trastuzumab with concurrent chemotherapy, 15 patients (7.6%) were treated with hormonal therapy at the time of trastuzumab initiation, while 4 patients received trastuzumab as a monotherapy. In addition, 11 patients (5.6%) had been treated with trastuzumab in the adjuvant and/or neoadjuvant setting as well.

The median follow-up time for the entire study population was 120.9 months [95% confidence interval (CI) 109.4-130.2]. At that time, 150 deaths had been reported (97 in HER2-positive and 53 in HER2-negative patients). The median PFS was 13.5 months (95% CI 10.7-16.2) for all patients included in the study. Patients with HER2-positive tumors presented with longer PFS compared to those with HER2-negative disease [median PFS: 14.4 (95% CI 12.4-19.5) vs. 9.0 months (95% CI 6.9-14.5), log-rank $P = .035$], while no difference was detected between R-MBC and *de novo* MBC patients in terms of PFS [median PFS: 11.4 (95% CI 8.9-14.4) vs. 16.0 (95% CI 13.1-22.9), log-rank $P = .30$]. The median survival was 41.9 months (95% CI 35.3-50.4) in the entire study population, while a significantly longer survival was observed for HER2-positive as compared to HER2-negative patients [median survival: 48.5 (95% CI 37.2-56.5) vs. median survival: 35.3 (95% CI 25.8-45.6), log-rank $P = .034$]. In contrast, no significant difference was detected in the survival of R-MBC and *de novo* MBC patients [median survival: 37.6 (95% CI 31.9-48.5) vs. 48.5 (95% CI 35.7-63.2), log-rank $P = .19$]. Among the 197 women included in the analysis, mRNA expression data for CDKN1B, JAK2, and Src were available for 192 (97.5%), 179 (90.9%), and 191 (97.0%) patients, respectively. The distribution of CDKN1B, JAK2, and Src (using all three quartiles as possible cutoffs) by disease presentation status is presented in Table 2, while Supplemental Table 1 presents the distribution of the examined markers by HER2 status. No significant differences were observed between HER2-positive and HER2-negative patients in terms of CDKN1B, JAK2, and Src mRNA expression (Figure 1*A*), while CDKN1B and Src mRNA expression was higher in R-MBC as compared to *de novo* MBC patients (Figure 1*B*) (Wilcoxon rank-sum $P < .001$ and $P = .003$, respectively).

As presented in Supplemental Table 2, women with low CDKN1B mRNA expression (using the median value as a cutoff) were more frequently of older age (>50 years) and of zero performance status compared to those with high CDKN1B mRNA expression (chi-square $P = .007$ and $P = .042$, respectively), while patients with high CDKN1B mRNA expression (using the median value as a cutoff) had more frequently positive ER/PgR tumors ($P = .013$). Patients with low JAK2 mRNA expression (using the median value as a cutoff) had more frequently higher histological grade (grade III) compared to those with high JAK2 mRNA expression, while low Src mRNA expression (using the median value as a cutoff) was more frequently observed in patients with a performance status of zero ($P = .021$ and $P = .024$, respectively).

The associations among CDKN1B, JAK2, and Src as well as their associations with other markers of interest are presented in Supplemental Table 3. Low CDKN1B mRNA expression (using the median value as a cutoff) was associated with low JAK2 mRNA expression (using the median value as a cutoff) and low Src mRNA expression (using the median value as a cutoff) ($P < .001$ and $P < .001$, respectively), while high CDKN1B mRNA expression (using the median value as a cutoff) was associated with wild-type PIK3CA ($P = .005$). Low JAK2 mRNA expression (using the median value as a cutoff) was associated with low Src mRNA expression (using the median value as a cutoff) ($P < .001$), while high Src mRNA expression (using the median value as a cutoff) was associated with wild-type PIK3CA ($P = .026$). No associations were observed between PTEN protein expression and any of the examined markers. Spearman correlations were also performed to examine the association between the markers of interest as well as with TILs and Ki67. A strong, positive correlation was observed between CDKN1B and JAK2 (Spearman rho = 0.54, $P < .001$), as well as between CDKN1B and Src (rho = 0.71, $P < .001$). In addition, Src was positively correlated with JAK2 (rho = 0.47, $P < .001$) and Ki67 (rho = 0.21, $P = .004$). However, none of these correlations was significantly strong. TILs density was not associated with any of the examined markers (Supplemental Table 4).

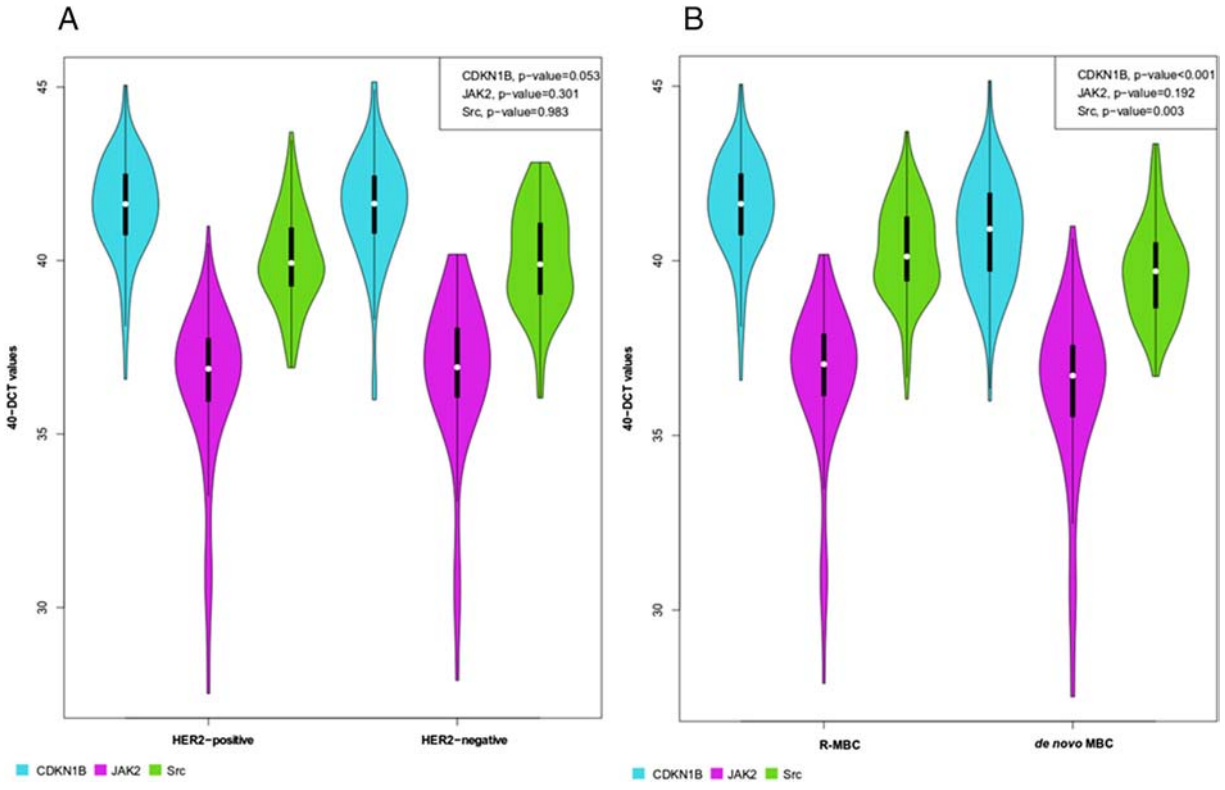

Figure 1. Violin plots of CDKN1B, JAK2, and Src distribution by (A) HER2 status and (B) disease presentation status.

Association of CDKN1B, JAK2, and Src mRNA Expression with Clinical Outcome

In the univariate analysis, with respect to PFS, HER2-positive patients with low CDKN1B mRNA expression (using the median value as a cutoff) were at higher risk of disease progression as

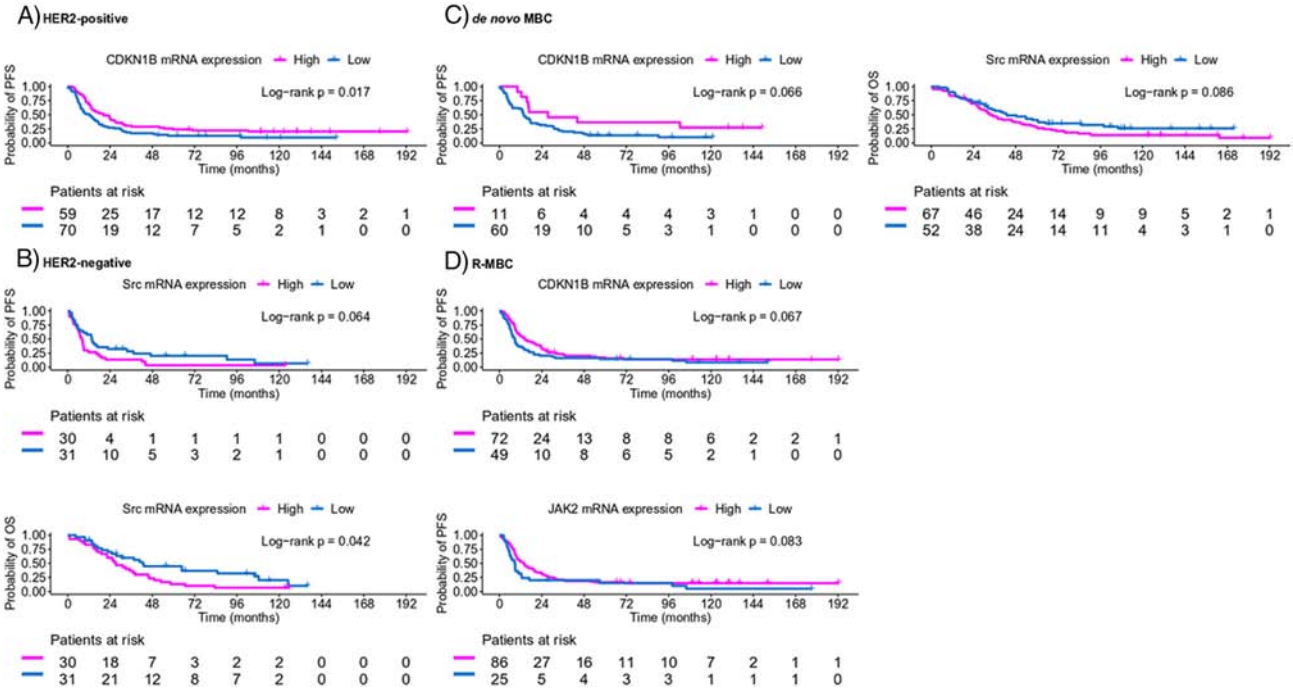

Figure 2. Kaplan-Meier plots according to (A) CDKN1B mRNA expression (using the median value as a cutoff) with respect to PFS in HER2-positive patients, (B) Src mRNA expression (using the median value as cutoff) with respect to PFS and OS in HER2-negative patients, (C) CDKN1B mRNA expression (using the upper quartile as cutoff) with respect to PFS in *de novo* MBC patients, and (D) CDKN1B and JAK2 mRNA expression with respect to PFS and Src mRNA expression with respect to OS in R-MBC patients.

compared to HER2-positive patients with high CDKN1B mRNA expression (HR = 1.58, 95% CI 1.08-2.32, Wald's P = .018) (Figure 2*A*), while neither Src nor JAK2 mRNA expression was of prognostic significance for survival in this population subgroup (Supplemental Table 5). Among HER2-negative patients, a trend towards significance associated with lower risk of progression was observed for patients with low Src mRNA expression (using the median value as a cutoff) (HR = 0.61, 95% CI 0.35-1.04, P = .067), while patients with HER2-negative and of low Src mRNA expression (using the median value as a cutoff) tumors were also at marginally significantly lower risk of death compared to those with high Src mRNA expression (HR = 0.56, 95% CI 0.32-0.99, P = .045) (Supplemental Table 6, Figure 2*B*). A trend for increased risk of progression was detected for *de novo* MBC patients with low CDKN1B mRNA expression (using the upper quartile as a cutoff) (HR = 2.00 95% CI 0.94-4.26, P = .072) (Supplemental Table 7, Figure 2*C*). In the subpopulation of R-MBC patients, low CDKN1B (using the median value as a cutoff) and low JAK2 mRNA expression (using the lower quartile as a cutoff) revealed a trend for higher risk of progression (HR = 1.44, 95% CI 0.97-2.12, P = .068 and HR = 1.51, 95% CI 0.94-2.42, P = .086, respectively) (Figure 2*D*). In terms of survival, a trend for lower risk of death was observed for R-MBC patients with low Src mRNA expression (using the median value as a cutoff) (HR = 0.70, 95% CI 0.46-1.05, P = .087) (Supplemental Table 8).

In the multivariate analyses, a predefined set of clinicopathological parameters was used for all comparisons, and backwards selection models were applied as described in the "Statistical Analysis" section.

Low CDKN1B mRNA expression did not retain its unfavorable prognostic significance for PFS in HER2-positive patients (P = .37), and only mutated PIK3CA remained a significant prognostic factor associated with increased risk of progression in HER2-positive patients (HR = 3.37, 95% CI 1.98-5.73, P < .001) (Table 3). Similarly, low Src mRNA expression did not retain its favorable significance for either PFS or survival in the subgroup of patients with HER2-negative tumors (P = .96 and P = .58, respectively). Among *de novo* patients, low CDKN1B mRNA expression was an unfavorable factor for PFS (HR = 2.36, 95% CI 1.01-5.48, P = .046) along with mutated PIK3CA and worse (1-3) performance status (P = .005 and P = .042, respectively). However, the significance of this result is limited by the small number of *de novo* patients with available CDKN1B data included in the multivariate model and should therefore be interpreted with caution. In R-MBC patients, only low JAK2 mRNA expression showed unfavorable significance for PFS (HR = 1.76, 95% CI 1.01-3.06, P = .047).

Table 3. Multivariate Analyses (Results of Backwards Selection Models) for (A) HER2-Positive, (B) HER2-Negative, (C) *De Novo* MBC, and (D) R-MBC Patients with Respect to PFS and OS

Parameter/Categories	*N* of Patients	*N* of Events	HR	95% CI	*P* Value
(A) HER2-positive					
PFS					
PIK3CA status *					
Mutated vs. *wild-type*	20 vs. 87	20 vs. 70	3.37	1.98-5.73	<.001
(B) HER2-negative					
PFS					
Menopausal status †					
Premenopausal vs. *postmenopausal*	10 vs. 31	10 vs. 28	0.40	0.16-1.01	.053
Ki67			1.02	1.00-1.04	.015
OS					
Menopausal status ‡					
Premenopausal vs. *postmenopausal*	10 vs. 31	9 vs. 28	0.43	0.17–1.08	.074
PS					
1-3 vs. *0*	10 vs. 31	9 vs. 28	3.28	1.41-7.61	.006
Ki67			1.02	1.00-1.04	.013
(C) *De novo* MBC					
PFS					
PIK3CA status					
Mutated vs. *wild-type*	9 vs. 44	9 vs. 36	3.07	1.41-6.72	.005
PS					
1-3 vs. *0*	13 vs. 40	12 vs. 33	2.06	1.03-4.12	.042
CDKN1B mRNA expression (upper quartile as cutoff)					
Low vs. *high*	43 vs. 10	38 vs. 7	2.36	1.01-5.48	.046
(D) R-MBC					
PFS					
1st model					
PIK3CA status					
Mutated vs. *wild-type*	24 vs. 74	22 vs. 64	1.61	0.96-2.69	.070
PTEN status					
No loss vs. *loss*	44 vs. 54	37 vs. 49	0.65	0.41-1.03	.068
Ki67			1.02	1.01-1.03	<.001
CDKN1B mRNA expression (median value as cutoff)					
Low vs. *high*	35 vs. 63	32 vs. 54	1.52	0.94-2.44	.086
2nd model					
PIK3CA status					
Mutated vs. *wild-type*	23 vs. 66	21 vs. 57	1.78	1.06-2.97	.028
Ki67			1.02	1.01-1.03	<.001
JAK2 mRNA expression (lower quartile as cutoff)					
Low vs. *high*	17 vs. 72	16 vs. 62	1.76	1.01-3.06	.047
OS					
Ki67 §			1.01	1.00-1.02	.022

* CDKN1B mRNA expression (median value as cutoff) was removed from the model with P = .37.
† Src mRNA expression (median value as cutoff) was removed from the model with P = .96.
‡ Src mRNA expression (median value as cutoff) was removed from the model with P = .58.
§ Src mRNA expression (median value as cutoff) was removed from the model with P = .12.

Discussion

In the present study, we sought to determine the prognostic significance and association with clinical outcome and trastuzumab response of Src, JAK2, and CDKN1B mRNA expression in archived samples from patients with HER2-positive MBC treated with trastuzumab. Of note, among them, only 133 (67.5%) were found to have centrally assessed HER2 gene amplification by FISH and/or 3+ HER2 protein expression by IHC. Therefore, 64 patients (32.5%) had been treated with trastuzumab despite being HER2-negative. Our findings support a prognostic role of these biomarkers and possible involvement in mechanisms of trastuzumab resistance in MBC.

Src protein-tyrosine kinase activity is elevated in several types of human cancer, and this has been attributed to both elevated Src expression and increased specific activity [31]. Activated Src expression has been reported in 18%–39% of breast carcinomas [8,32,33]. In MBC, Src expression rate has been shown to be slightly higher (54.9%) [34]. In agreement, we found that 50% of our tumors showed high Src mRNA expression. No difference was observed based on HER2 status. In addition, high Src mRNA expression was more frequent in R-MBC patients as compared to *de novo* MBC patients (chi-square P = .001 and P = .020, respectively). However, it was not statistically correlated with aggressive phenotypes, as previously reported [8]. On the other hand, we found a positive correlation between Src and both CDKN1B and JAK2 expression. In contrast, there were no differences in PTEN status or EGFR expression between patients with high or low Src expression. These results support that Src activation in cancers is a complex phenomenon mediated by oncogenic activation of RTKs, including

EGFR, HER2, and IGF-1R, but also by other independent pathways.

In our study, univariate analysis showed an association of decreased Src mRNA expression with longer survival and reduced risk for progressive disease in MBC. In agreement, Zhang et al. discovered that high Src expression measured by IHC was associated with poor PFS and disease-specific survival (DSS) in patients with MBC (P = .027 and P = .024, respectively), predominantly in patients with bone metastases [34]. Moreover, both elevated cytoplasmic Src expression and activated (phosphorylated) Src have been associated with decreased DSS in BC tumor samples [35,36]. On the other hand, Zhang et al. showed that high levels of phosphorylated Src were associated with lower clinical response rates and higher progressive disease rates after trastuzumab treatment in BC tumor samples [37]. Similarly, Peiro et al. demonstrated that Src correlated with poorer outcome in patients under trastuzumab treatment [8]. Overall, our finding is in line with previously reported preclinical data supporting a role of activated Src in trastuzumab resistance [38,39]. However, this was not confirmed in multivariate analysis. Of note, a phase II study evaluating the role of Src inhibitor dasatinib in HER2-positive MBC has shown limited single agent activity in this population [40].

Quiescent normal epithelia of breast, prostate, ovary, lung, and other sites express high levels of nuclear p27 [11]. In our study, high CDKN1B mRNA expression was observed in 45.7% of HER2-possitive carcinomas and was associated with HR-positive status, contrary to published data showing correlation with HR-negative status [11]. Most importantly, reduced CDKN1B mRNA expression was shown to be a statistically significant unfavorable prognostic factor for PFS for patients with *de novo* MBC treated with trastuzumab. This finding is in line with preclinical evidence demonstrating an association between decreased p27 levels and trastuzumab resistance [12]. However, this was not confirmed in multivariate analysis for the subgroup of patients with HER2-positive disease. While p27 protein expression has been consistently shown to be an independent prognostic factor in early BC [41–47], this is the first study, to our knowledge, to demonstrate prognostic significance for CDKN1B/p27 in MBC. More specifically, in a large retrospective analysis of tissue specimens from a randomized adjuvant clinical trial in early BC, reduced p27 was shown to be independently significant for reduced OS (HR = 1.31, 95% CI 1.05-1.64), especially among patients with HR-positive disease (HR = 1.42, 95% CI 1.05-1.94) [45]. Similarly, low p27 expression correlated with decreased relapse-free survival (P = .04) in a retrospective analysis of tumor specimens derived from early-stage BC patients enrolled in the ABCSG 5 trial [47]. In a recent report, p27 failed to show prognostic significance in HER2-positive MBC [48].

In the present study, low JAK2 mRNA expression was shown to be a statistically significant unfavorable prognostic factor for PFS in R-MBC. This finding might be unexpected because IL6/JAK/STAT is a crucial regulatory pathway essential for growth of HER-positive BC; in addition, genetic studies in mouse models support a role for STAT5, the main substrate of JAK2, as a proto-oncogene in mammary tumor initiation [49]. Indeed, preclinical data show that the combination of JAK inhibitors plus trastuzumab is synergistic in tumor growth inhibition in mouse xenografts of HER2-positive BC cell lines, and JAK inhibitors are currently evaluated in combination with trastuzumab in patients with HER2-positive BC [50]. Nonetheless, a differentiation-promoting and invasion-suppressive role of STAT5 in advanced human breast cancers has also been proposed [13,14]. Consistent with this notion, the levels of active STAT5 are significantly reduced in primary breast cancers with unfavorable prognosis and in metastatic lesions. Of note, in agreement with our findings, elevated JAK2 mRNA levels have been associated with improved breast cancer outcomes in archived early BC tumor samples [51]. The association of low JAK2 mRNA with unfavorable prognosis may also represent a lack of concordance between levels of JAK2 mRNA and total JAK2 protein and the active phospho-JAK2 in breast tumor cells [51].

Our study has several strengths. There were strong laboratory and clinical data supporting our interest in exploring Src, CDKN1B, and JAK2 as biomarkers in HER2-positive MBC. The patient population was well defined, and HER2 status was centrally assessed in all patients included. All important patient and tumor characteristics were presented and were also included in the multivariate analyses. A major limitation of our study is the small sample size of the analyzed subgroups, especially of the *de novo* MBC subgroup. Moreover, it is a retrospective study; our results should therefore be confirmed in validation cohorts. Furthermore, a significant proportion of analyzed carcinomas were treated with trastuzumab despite being HER2-negative. In this population, it is difficult to draw definite conclusions about disease outcome in association with analyzed biomarkers. In addition, all tumors analyzed are primary tumors. This means that for R-MBC patients, the status of the analyzed markers may not be the same when patients relapse. By contrast, for *de novo* patients, the marker status may presumably directly affect outcome. Finally, protein expression of all markers examined should also be evaluated by IHC in additional translational studies.

Conclusions

In conclusion, we demonstrated that low CDKN1B and JAK2 mRNA expressions are unfavorable prognostic factors for PFS in a large cohort of metastatic breast cancer patients treated with trastuzumab. In addition, low Src mRNA expression was associated with reduced risk for progressive disease in univariate analysis. These findings have implications for the prognostic classification of BC patients and suggest that these biomarkers might be involved in mechanisms of trastuzumab resistance. Therefore, our results indicate that these biomarkers might serve as a potential molecular targets in the future. Moreover, considering that different resistance mechanisms may coexist in the same tumor, combination strategies with other targeted agents might result in synergistic activity, which might possibly help to restore sensitivity to trastuzumab.

Conflict of Interest

Christos Christodoulou: Advisory Role: Roche, Honoraria: Roche.
George Pentheroudakis: Advisory Role: Roche, Honoraria: Roche, Speaker bureau: Roche.
Georgios Lazaridis: Honoraria: Roche.
Angelos Koutras: Advisory Role: Roche.
Evangelia Razis: Travel: Roche.
Gerasimos Aravantinos: Advisory Role: Roche.
Epaminondas Samantas: Advisory Role: Roche.
Amanta Psyrri: Consultation Fees: Roche, Honoraria: Roche.
George Fountzilas: Advisory Role: Roche.

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Appendix A. Supplementary data

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