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Original Article

Expression analysis elucidates the roles of Nicastrin, Notch4, and Hes1 in prognosis and endocrine-therapy resistance in ER-positive breast cancer patients

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Abstract

Background and purpose: Although some proposed mechanisms responsible for tamoxifen resistance have already been present, further study is needed to determine the mechanisms underlying tamoxifen resistance more clearly. The critical role of Notch signaling has been described in promoting resistance in therapeutics, but there is little information about its role in tamoxifen resistance progression.
Experimental approach: In the present study, the expression of Notch pathway genes, including *Notch4*, *nicastrin*, and the Notch downstream target *Hes1* was evaluated using quantitative RT-PCR in 36 tamoxifen-resistant (TAM-R) and 36 tamoxifen-sensitive (TAM-S) patients. Expression data were correlated with the clinical outcome and survival of patients.
Findings/Results: mRNA levels of *Notch4* (fold change = 2.7), *nicastrin* (fold change = 6.71), and *Hes1* (fold change= 7.07) were significantly higher in TAM-R breast carcinoma patients compared to sensitive cases. We confirmed all these genes were co-expressed. Hence, it seems that Notch signaling is involved in tamoxifen resistance in our TAM-R patients. Obtained results showed that *Hes1*, *nicastrin*, and *Notch4* mRNA upregulation was correlated with the N stage. The extracapsular nodal extension was associated with *nicastrin* and *Notch4* overexpression. Moreover, *nicastrin* overexpression was correlated with perineural invasion. *Hes1* upregulation was also associated with nipple involvement. Finally, the Cox regression proportional hazard test revealed that overexpression of *nicastrin* was an independent worse survival factor.
Conclusion and implications: Presumably, upregulation of the Notch pathway may be involved in tamoxifen resistance in breast cancer patients.

Keywords: Breast cancer; Hes1; Nicastrin; Notch4; Tamoxifen resistance.

INTRODUCTION

Breast cancer is the most prevalent heterogeneous and malignant cancer in women (1). Approximately 70% of breast carcinoma are estrogen receptor-positive (ER^+), hence, numerous drugs are designed for the treatment of ER^+ patients. Tamoxifen (TAM) is the most routine treatment component against ER^+ breast tumors (2). TAM is considered a selective ER modulator that competes with estrogen to bind to estrogen receptors and repress carcinogenic and estrogenic effects.

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Despite decreasing the relapse rate, about one-third of breast cancer patients experience resistance to this therapy. Multiple molecular mechanisms have been described as responsible for TAM resistance. However, due to the complexity of cellular behaviors in the tumor environment, many aspects of resistance are still unclear (3).

It seems that epithelial-to-mesenchymal transition (EMT) and cancer stem cells (CSCs) have a key role in developing resistance in TAM-treated patients (4,5). EMT reflects the trans-differentiation of epithelial cells to acquire migratory, invasive, metastatic, and fibroblast-like properties. TAM-resistant (TAM-R) cells express mesenchymal-like phenotypes. In these cells, the expression of epithelial markers such as E-cadherin is reduced, and mesenchymal characteristics such as the expression of vimentin and N-cadherin are increased (4,6). CSCs are a subgroup of cancer cells that possess self-renewal and multi-lineage differentiation features that initiate malignancy, promoting drug resistance, and tumor recurrence. Most chemotherapeutic and radiotherapy approaches could successfully eradicate cancer cells; however, CSCs survive and promote resistance to therapy (5).

Notch signaling is one of the highly conserved signaling pathways in metazoans and makes communication between two neighboring cells feasible. In mammals, Notch consists of four paralogs (Notch1-4) cleaved by either γ-secretase (GS) complex or ADAM metalloproteases to release the Notch intracellular domain (NICD). NICD enters the nucleus and regulates the transcription of stemness genes (7). Several studies have reported the Notch signaling pathway upregulation in cancer therapy (7,8). In particular, the Notch pathway contributes to TAM resistance in ER^+ breast carcinoma cells. Notch induces CSCs and promotes the EMT process. Inhibition of Notch sensitized TAM-R cells. Furthermore, Notch receptors stimulate the proliferation of ER^+ and ER^- *via* canonical and non-canonical mechanisms (9,10). Single-pass transmembrane protein Notch4 is a fourth member of Notch receptors (11). Notch4 protects the haphazard apoptotic process, increases cellular survival in response to a wide range of anticancer agents, and promotes poor prognosis in breast cancer patients (12,13). Investigation on long-term TAM-treated breast cancer cells showed that these cells underwent resistance through JAG1-Notch4 receptor activity. Conversely, Notch4 inhibition decreased breast CSCs population and cell malignancy (14). In TAM-R MCF-7 cells, and MDA-MB-231 breast cancer cells, inhibition of Notch4 by siRNA increased TAM sensitivity and reduced EMT signaling (15,16).

Type I transmembrane glycoprotein *nicastrin* is the largest subunit of the GS. Nicastrin protects the GS complex from large proteins from reaching the catalytic center. Moreover, nicastrin increases the steadiness, assembly, and catalytic activity of the GS complex and, with the help of other members, cleaves a variety of sending-signal proteins, including Notch receptors (17). It has been demonstrated that *nicastrin*'s overexpression led to an enrichment of the CSCs population and enhanced EMT *via* activation of PI3K/Akt and Notch signaling pathways. Upregulation of *nicastrin* is important for the development of various cancers, including hepatocellular carcinoma and breast cancer (12,18). In MCF-7 breast cancer cells, *nicastrin* expression conferred worse overall survival. It also has been shown that Notch signaling is significantly inhibited by nicastrin knockdown in basal-like breast neoplasms (12).

The transcriptional repressor hairy enhancer of split (*Hes1*) is an evolutionarily conserved transcription factor that is the Notch signaling's final target gene. Hes1 has an autoregulatory expression mechanism that represses its gene. Noteworthy, NICD binds to Hes1 and activates downstream pathways (19).

Although there are many reports about the Notch signaling association in breast cancer, no direct description has investigated the role of nicastrin, Notch4, and Hes1 in ER^+ breast cancer patients. Also, little is known about the association of the expression level of these genes with clinicopathological features of breast carcinoma patients. Therefore, in the present study, we studied the role of Notch signaling in promoting TAM resistance by measuring mRNA expression of *Notch4*, *nicastrin*, and *Hes1* in TAM-sensitive

and resistant patients. We also evaluated their potential role in patient survival.

MATERIALS AND METHODS

Patients

Our previous publication mentioned a comprehensive approach for tissue selection and patients' features (6). In brief, Iran National Tumor Bank granted 72 frozen breast cancer tissues from patients who had undergone breast surgery with lymph node dissection and had complete clinicopathological records at Iran's tumor bank. ER^+ breast carcinoma patients undergoing surgery received adjuvant radiotherapy and chemotherapy and eventually acquired TAM for six months to five years or more were entered in this study. Patients still responsive to TAM for at least five years were regarded as TAM sensitive (TAM-S). Patients experiencing tumor recurrence while receiving TAM treatment for at least six months (median time recurrence = 25 months) were considered TAM-R (6). All study patients were followed up for 85 months. Informed written consent was obtained from each participant. The clinicopathological features of the recruited breast cancer patients are summarized in Table 1.

This study was conducted according to the ethical standards of the local ethical committee at Mashhad University of Medical Sciences (MUMS) and obtained the ethical code IR.MUMS.MEDICAL.REC.1398.600.

RNA purification

Total RNA extraction from tumor tissues was performed utilizing RiboEx Total RNA kit (GeneAll, Korea South). Extracted RNAs were eluted in diethylpyrocarbonate-treated water. Concentration and purity (260/280 and 260/230 ratio) were measured in duplicate by the NanoDrop™ 2000c (Thermo Scientific, USA) spectrophotometer. In order to confirm RNA integrity, aliquots of the RNA samples were electrophoresed in agarose gel. Bands of 28s, 18s, and 5s rRNAs were observed, which indicates RNA integrity.

cDNA synthesis

Reverse transcription of total RNA into cDNA was performed using Yekta Tajhiz Azma cDNA synthesis kit (Iran). Following incubation of 2 µg of total RNA and random hexamer primers for 5 min at 70 °C and then replacement on ice, a reaction mixture consisting of 10 mM dNTPs, 40 unit/µL RNase inhibitor, 200 unit/µL reverse transcriptase, and first-strand buffer × 5 was added according to the manufacturer's instructions (the mixture was incubated for 5 min at 70 °C, followed by 60 min at 37 °C, then 5 min at 70 °C).

Table 1. Clinical characteristics of breast cancer patients.

Features	Categories	Number of tissues	Tamoxifen sensitive	Tamoxifen resistant	*P*-value
Age (average)		72	43.38 ± 4.38	49.21 ± 10.24	0.118
T Stage	T1, T2 T3, T4	53 19	24 (66.66%) 12 (33.33%)	29 (80.5%) 7 (19.5%)	0.494
N Stage	N0, N1 N2, N3	44 28	24 (66.66%) 12 (33.33%)	20 (55.5%) 16 (44.5%)	0.021
PR status	Positive Negative	47 25	24 (66.67%) 12 (33.3%)	23 (63.9%) 13 (36.1%)	> 0.99
HER-2 status	Positive Negative	19 53	11 (30.6%) 25 (69.4%)	8 (22.2%) 28 (77.8%)	0.594
P53 status	Positive Negative	23 49	14 (38.9%) 22 (61.1%)	9 (25.0%) 27 (75.0%)	0.312
Ductal carcinoma *in situ*	Comedo type Non-Comedo type	9 63	4 (11.1%) 32 (88.9%)	5 (13.9%) 31 (86.1%)	0.5
Nipple involvement	Present Absent	13 59	6 (16.7%) 30 (83.3%)	6 (16.7%) 30 (83.3%)	> 0.99
Lymphatic invasion	Present Absent	55 17	25 (69.4%) 11 (30.6%)	30 (83.3%) 6 (16.7%)	0.267
Perineural Invasion	Present Absent	30 42	10 (27.8%) 26 (72.2%)	20 (55.6%) 16 (44.4%)	0.031
Extracapsular nodal extension	Present Absent	15 57	4 (11.1%) 32 (88.9%)	11 (30.6%) 25 (69.4%)	0.079

PR, Progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table 2. List of primers used in a quantitative real-time polymerase chain reaction.

Genes	Forward (5′ to 3′)	Reverse (5′ to 3′)	Reference
β-actin	TCATGAAGTGTGACGTGGACATC	CAGGAGGAGCAATGATCTTGATCT	(6)
Nicastrin	GGAGTAAACACCAAACCCA	GGAGAACCAGCCGAATTG	(19)
Notch4	AACTCCTCCCCAGGAATCTG	CCTCCATCCAGCAGAGGTT	(20)
Hes1	CCCAACGCAGTGTCACCTTC	TACAAAGGCGCAATCCAATATG	(21)

Quantitative real-time polymerase chain reaction

By employing specific primers (Table 2), the quantitative real-time polymerase chain reaction (qRT-PCR) was performed on synthesized cDNAs using the SYBR Green protocol. Based on the manufacturer's instructions (YTA SYBR Green qPCR master mix 2X, Iran), the PCR experiment was conducted under the following condition: 95 °C for 5 min to polymerase activation and initialize denaturation, then 95 °C for 5 s and 60 °C for 30 s for 40 cycles. The melting curves of PCR products were monitored at the end of each reaction to evaluate the specificity of PCR reactions. The outcomes were drawn as the target/reference ratio of the TAM-R specimens divided by the target/reference ratio of the calibrators (TAM-S specimens). β-actin was used as an internal control.

Statistical analysis

Data extracted from this research were analyzed by SPSS software version 26 and GraphPad Prism9. Shapiro-Wilk test was used for the normality test. T-test was used to analyze the differences between TAM-R and TAM-S breast carcinoma cases. Spearman's correlation coefficient was used to analyze the association between genes. Logistic regression was applied to evaluate the correlation between gene expression and clinicopathological features. Kaplan-Meier and Cox regression statistical methods were conducted to determine the association between mRNA expressions of studied genes and the hazard of tumor recurrence or death. Considering the mean levels of expression, patients were divided into two groups: high expression versus low expression. Hence, Cox regression analysis was used for evaluating the effect of the expression of desired genes when added to the base model of other elements. Disease-free survival (DFS) was considered the time interval between the date of primary treatment and the date of first proven tumor recurrence. In this approach, both regional and distant metastasis were regarded as an event. In order to estimate the patient's prognosis, overall survival (OS) was reported. OS is the period between surgery and death. In OS analysis, death was considered an event. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of mRNA expression of nicastrin, Notch4, and Hes1 genes between TAM-S and TAM-R breast cancer patients

Levels of *Notch4*, *nicastrin*, and *Hes1* mRNA expression were assessed by qRT-PCR conducted on cDNA samples of TAM-S and TAM-R patients. qRT-PCR analysis confirmed that there was a statistically significant upregulation of *Notch4*, *nicastrin*, and *Hes1* in TAM-R compared to TAM-S tumor samples (Fig. 1). Mean fold changes of *Notch4*, *nicastrin*, and *Hes1* in TAM-R compared to TAM-S were 2.71, 6.71, and 7.07, respectively.

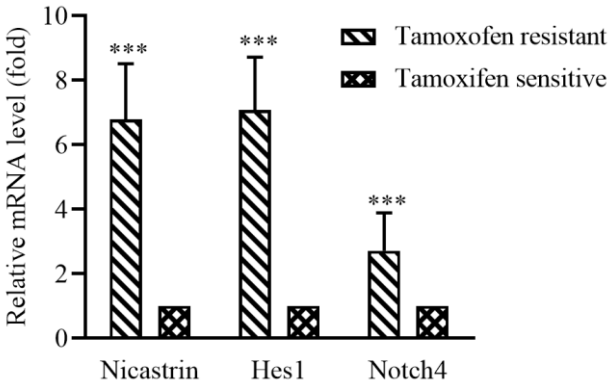


Fig. 1. Expression of *nicastrin, Notch4, and Hes1* genes was assessed by quantitative real-time polymerase chain reaction analysis and normalized by β-actin. Data were scrutinized by a t-test Data are delineated as mean ± SD. ***$P < 0.001$ indicates the significant differences between the two groups regarding each gene.

Table 3. Correlation among mRNA expression of ***nicastrin, Notch4, and Hes1*** genes.

Genes	r	95% CI	*P*-value
Nicastrin *vs* Notch4	0.3704	0.1449 to 0.5593	0.0014
Nicastrin *vs* Hes1	0.2451	0.007263 to 0.4567	0.0190
Notch4 *vs* Hes1	0.2695	0.03338 to 0.4771	0.0110

Table 4. Association of the mRNA expression level of *nicastrin* with clinicopathological characteristics of patients.

Variables	Expression of *nicastrin*		OR	95% CI	*P*-value
	Low (%)	High (%)			
Grade			1.307	0.501-3.406	0.584
Grade 1	15 (40%)	12 (34%)			
Grades 2 & 3	22 (60%)	23 (66%)			
N stage			18.00	5.109-63.419	0.0001
N0 & N1	33 (89%)	11 (31%)			
N1 & N2	4 (11%)	24 (69%)			
T stage			1.661	0.576-4.792	0.576
T1 & T2	8(21%)	11(31%)			
T3 & T4	29(79%)	24(69%)			
Extracapsular nodal extension			0.169	0.43-0.666	0.011
Yes	34 (92%)	23 (65%)			
No	3 (8%)	12 (35%)			
DCIS histology			0.422	0.564-3.922	0.422
Comedo type	15 (40%)	11 (31%)			
Non comedo	22 (60%)	24 (69%)			
Nipple involvement			1.058	0.858-1.304	0.599
No	30 (81%)	30 (85%)			
Yes	7 (11%)	5 (15%)			
Lymphatic invasion			0.754	0.221-2.984	0.754
No	10 (27%)	7 (20%)			
Yes	27 (73%)	28 (80%)			
Perineural invasion			7.778	1.623-37.283	0.010
No	23 (62%)	19 (54%)			
Yes	14 (38%)	16 (46%)			
PR status			2.007	0.578-6.976	0.273
Positive	24 (65%)	21 (60%)			
Negative	13 (35%)	14 (40%)			
HER-2 status			1.220	0.365-4.079	0.747
Positive	6 (16%)	13 (37%)			
Negative	31 (84%)	22 (63%)			
P53 status			2.370	0.772-7.280	0.132
Positive	11 (30%)	12 (34%)			
Negative	26 (70%)	23 (66%)			

OR, Odd ratio; DSCI, ductal carcinoma *in situ*; PR, progesterone receptor.

Association between nicastrin, Notch4, and Hes1 genes expression

Spearman's correlation coefficient showed a significant association between *nicastrin/Notch4* and *nicastrin/Hes1* expression (Table 3). We used *Sox2*, *Nanog*, and *Oct4* expression results from our previous study to analyze the correlation between *nicastrin*, *Notch4*, and *Hes1* expression with mentioned genes. These results demonstrated a notable correlation between stemness factors *Sox2* and *Oct4* expression with the mRNA level of *nicastrin*, *Notch4*, and *Hes1*. We could not find any conclusive result related to the correlation of *Nanog* with genes assessed in this study. (Data not shown) (6).

Correlation analysis of nicastrin, Notch4, and Hes1 expression with clinicopathological features of patients

To discover any association between gene expression results and clinicopathological features, various clinicopathological variables were evaluated. Our findings showed that higher expression of *nicastrin* (Table 4), *Notch4* (Table 5), and *Hes1* (Table 6) were associated with the N stage. Likewise, *nicastrin* and *Notch4* showed significant association with extracapsular nodal extension (ECE).

Moreover, perineural invasion (PNI) was only correlated with *nicastrin* expression. It was also found that involvement of the nipple was solely associated with *Hes1* upregulation.

Association of nicastrin, Notch4, and Hes1 expression with clinical outcome

To estimate the survival function of the genes in TAM-treated breast cancer patients, we conducted a Kaplan-Meier statistical test. Results indicated that higher expression of *Notch4* ($P = 0.001$), *nicastrin* ($P < 0.0001$), and *Hes1* ($P = 0.047$) were associated with worse prognosis in DSF patients. Furthermore, data analysis showed *nicastrin* expression was correlated with OS ($P = 0.013$) (Fig. 2).

Univariate and multivariate Cox regression analysis

In order to investigate the association between the time of disease recurrence in TAM-treated patients and one predictor variable, a univariate Cox survival analysis was conducted (Table 7). The results demonstrated that ECE, PNI, and overexpression of *nicastrin* could be critical predictors for DFS. In addition, co-overexpression of *Notch4* and *nicastrin* could be considered unfavorable factors in OS. Significant data from univariate Cox regression analysis were included in multivariate Cox regression (Table 8). After adjustment, in DFS, it was observed that PNI and *nicastrin* expression were still significant and they could be contemplated as independent survival factors. In OS, exclusively overexpression of *nicastrin* indicated a significantly worse predictor of survival in TAM-treated breast cancer patients.

Table 5. Association of the mRNA expression level of ***Notch4*** with clinicopathological characteristics of patients.

Variables	Expression of *Notch4*		OR	95% CI	*P*-Value
	Low (%)	High (%)			
Grade			0.640		
Grade 1	12 (34.3%)	20 (57.1%)		0.245-1.672	0.362
Grades 2 & 3	25 (67%)	15 (42.9%)			
N stage			12.267	3.769-39.634	0.0001
N0 & N1	32 (86.5%)	12 (34.3%)			
N1 & N2	5 (13.5%)	23 (65.7%)			
T Stage			2.236	0.760-6.577	0.144
T1 & T2	30 (81.1%)	23 (65.7%)			
T3 & T4	7 (18.9%)	12 (34.3%)			
Extracapsular nodal extension			0.264	0.075-0.932	0.038
Yes	33 (89.2%)	24 (68.6%)			
No	14 (37.8%)	11 (31.4%)			
DCIS histology			1.167	0.445-3.058	0.754
Comedo Type	23 (62.2%)	12 (34.3%)			
Non Comedo	34 (91.9%)	23 (65.7%)			
Nipple involvement			0796	0.630-1.006	0.056
No	34 (91.9%)	26 (74.3%)			
Yes	7 (8.1%)	9 (25.7%)			
Lymphatic invasion			0.922	0.310-2.740	0.884
No	9 (24.3%)	8 (22.9%)			
Yes	28 (75.7%)	27 (77.1%)			
Perineural invasion			0.723	0.282-1.851	0.499
Yes	23(62.2%)	19(54.3%)			
No	14(37.8%)	16(45.7%)			
PR status			1.038	0.39-2.741	0.940
Positive	24 (64.9%)	23 (65.7%)			
Negative	13 (35.1%)	12 (34.3%)			
HER-2 status			1.244	0.436-3.555	0.683
Positive	9 (24.3%)	10 (28.6%)			
Negative	28 (75.7%)	25 (71.4%)			
P53 status			1.595	0.588-4.329	0.359
Positive	10 (27%)	13 (37.1%)			
Negative	27 (73%)	22 (92.9%)			

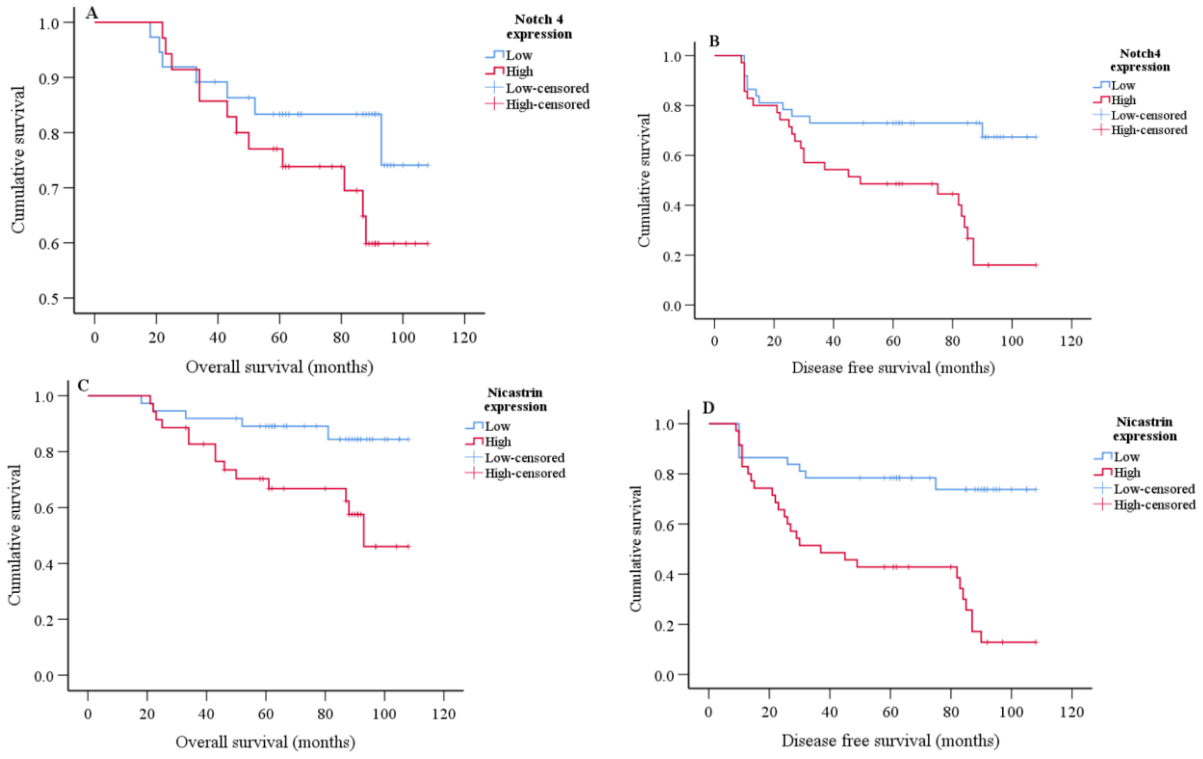
OR, Odd ratio; DSCI, ductal carcinoma *in situ*; PR, progesterone receptor.

Table 6. Association of mRNA expression level of ***Hes1*** with clinicopathological characteristics of patients.

Variables	Expression of *Hes1*	OR	95% CI	P-Value

	Low (%)	High (%)			
Grade			1.326	0.683-2.575	0.405
Grade 1	12 (36.3%)	13 (33.3%)			
Grade 2 & 3	21 (63.7%)	26 (66.7%)			
N stage			3.289	1.193-9.067	0.021
N0 & N1	25 (75.8%)	19 (48.7%)			
N1 & N2	8 (24.2%)	20 (51.3%)			
T Stage			0.920	0.322-2.629	0.876
T1 & T2	24 (72.7%)	29 (74.4%)			
T3 & T4	9 (27.3%)	10 (25.6%)			
Extracapsular nodal extension			0.518	0.157-1.707	0.518
Yes	28 (84.8%)	29 (74.4%)			
No	5 (15.2%)	10 (25.6%)			
DCIS histology			1.020	0.389-2.678	0.967
Comedo Type	12 (36.4%)	14 (35.9%)			
Non Comedo	21 (63.6%)	25 (64.1%)			
Nipple involvement			1.285	1.017-1.624	0.036
No	9 (27.3%)	3 (7.7%)			
Yes	24 (72.7%)	36 (92.3%)			
Lymphatic invasion			1.281	0.426-3.853	0.660
No	7 (21.2%)	29 (74.3%)			
Yes	26 (78.8%)	10 (25.6%)			
Perineural invasion			0.526	0.202-1.372	0.189
No	22 (66.6%)	20 (51.3%)			
Yes	11 (33.3%)	19 (48.7%)			
PR status			1.462	0.552-2.876	0.445
Positive	20 (60.6%)	27 (69.2%)			
Negative	13 (39.4%)	12 (30.8%)			
HER-2 status			1.228	0.426-3.538	0.704
Positive	8 (24.2%)	11 (28.2%)			
Negative	25 (75.8%)	28 (71.8%)			
P53 status			0.435	0.545-4.090	0.435
Positive	9 (27.3%)	14 (25.9%)			
Negative	24 (72.7%)	25 (64.1%)			

OR, Odd ratio; DSCI, ductal carcinoma *in situ*; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.



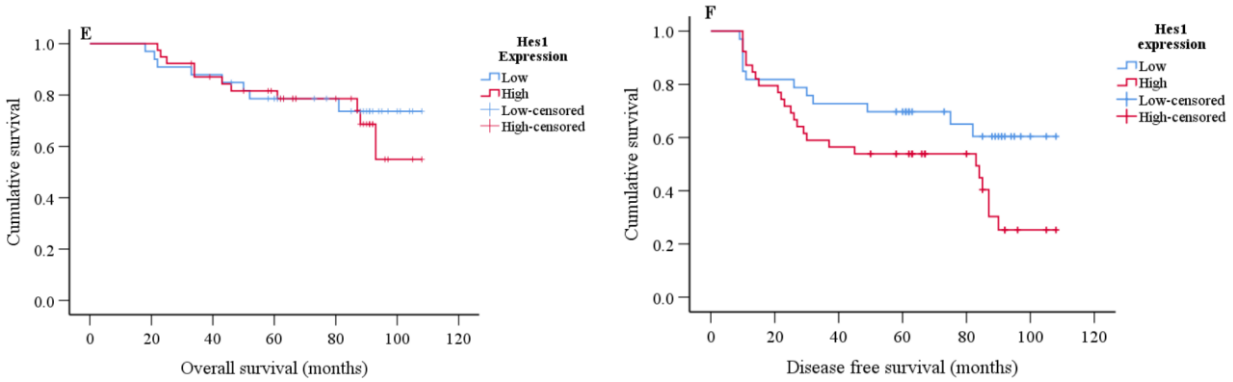


Fig. 2. Kaplan-Meier cumulative survival curves of tamoxifen-resistant and tamoxifen-sensitive patients have been illustrated in two modes of disease-free survival and overall survival for (A and B) *Notch4*, (C and D) *nicastrin*, and (E and F) *Hes1*.

Table 7. Univariate Cox regression for tamoxifen-treated in estrogen receptor-positive breast carcinoma patients, N = 72.

Factor base model	Disease-free survival			Overall survival		
	HR	**CI 95%**	***P*-value**	**HR**	**CI 95%**	***P*-value**
Histological grade		0.915-4.530		3.969	1.104-14.261	0.035
Grade I	20.34		0.081		0.715-14.328	0.128
Grade II and III	20.16		0.149			
T stage	1.63	0.816-3.270	0.166	1.835	0.720-4.672	0.203
T1 and T2						
T3 and T4						
N stage	2.63	1.355-5.128	0.004	2.132	0.850-5.287	0.103
N0 and N1						
N0 and N1						
Extracapsular nodal extension	2.17	1.20-4.06	0.012	2.002	0.76-5.274	0.160
Absent						
Present						
Ductal carcinoma *in situ* histology	1.01	0.395-2.620	0.973	1.840	0.609-5.557	0.280
Comedo						
Non-Comedo						
Absent of nipple involvement	1.41	0.585-3.434	0.44	1.099	0.317-2.810	0.881
Lymphatic invasion	1.78	0.742-4.296	0.196	1.828	0.531-6.268	0.339
Perineural invasion	2.32	1.99-4.499	0.013	0.787	0.310-1.999	0.614
Absent						
Present						
PR status	1.15	0.585-2.283	0.677	2.466	0.997-6.102	0.051
Positive						
Negative						
HER2 status	1.14	0.519-2.52	0.739	1.223	0.403-3.706	0.723
Positive						
Negative						
***Nicastrin* expression**	4.336	2.027-9.275	< 0.001	3.4	1.222-9.545	0.019
***Notch4* expression**	1.86	0.733-4.763	0.191	3.122	1.521-6.409	0.002
***Hes1* expression**	1.98	0.987-3.972	0.054	1.217	0.488-3.032	0.674

HR, Hazard ratio; HER2, Human epidermal growth factor receptor 2; Hes1, hairy enhancer of split, PR, progesterone receptor.

Table 8 Multivariate Cox regression for tamoxifen response in estrogen receptor-positive breast, N = 72.

Factor of the base model	Disease-free survival			Overall survival		
	HR	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
N stage N0 & N1 N2 & N3	1.240	0.540-2.849	0.612	-	-	-
Extracapsular nodal extension	0.843	0.360-1.974	0.694	-	-	-
Perineural invasion	0.456	0.360-1.974	0.023	-	-	-
Nicastrin	3.735	1.605-8.692	0.002	3.368	1.050-10.802	0.041
Notch4	-	-	-	1.018	0.351-2.958	0.973

HR, Hazard ratio.

DISCUSSION

In this study, we demonstrated that *Notch4*, *nicastrin*, and *Hes1* were overexpressed in TAM-R patients in comparison to those in TAM-S group. It was also highlighted that over-expression of *nicastrin*, *Notch4*, and *Hes1* were correlated with some of the clinicopathological features such as N stage and ECE. Moreover, it was shown that they could have an essential role in the worse prognosis of our breast carcinoma patients.

Lombardo *et al*. reported that the inhibition of *nicastrin* mRNA by shRNA in HCC1806 (triple-negative) and MCF10A (non-transformed) breast cancer cell lines were able to disrupt GS complex and consequently the production of ICD from Notch1 and Notch4 significantly reduced. They found that there might be a positive correlation between *nicastrin* and *Notch* expression. In addition, they reported that nicastrin promotes the expression of EMT genes such as vimentin, twist1, SIP1, snail, MMP2, and MMP9 in breast carcinoma cells (19). In another study, Filipović *et al*. blocked nicastrin protein by anti-nicastrin monoclonal antibodies in triple-negative breast cancer cell lines and *in vivo* as well. Subsequently, the uncontrollable proliferation of cancer cells was reduced, and multiple critical steps emerged: first, the invasive potential was significantly decreased. Second, the ability of diapedesis was impeded. Third, cancer cells were not able to degrade the extracellular matrix *via* invadopodia extension (22). In their previous research, they found that *nicastrin* was upregulated in human breast cancer tissues compared to normal tissues and positively correlated with expression levels of Era, progesterone receptor, and cytokeratin 18, and negatively with the expression of cytokeratin 5/6 (23). In line with these studies, we also observed overexpression of *nicastrin* in TAM-R compared to TAM-S patients. Moreover, it was shown that a higher level of *nicastrin* was associated with N stage, ECE, and PNI, and more importantly, its higher level of expression was correlated with worse survival in OS and DFS.

Zhou and colleagues claimed that *Notch4* aberrantly overexpressed in triple-negative breast cancer cells and was correspondent with the decreased OS. They proved that Notch4 could effectively increase CSCs subpopulation and the EMT phenomenon. In the MCF-7 cell line, Notch4 acted as a tumor suppressor which caused cell differentiation and reduced metastasis (24). In a study conducted by wang *et al*. *Notch4* expression and its relation to clinicopathological features, survival, and prognostic value were investigated in different subtypes of breast cancer. Survival analysis showed that *Notch4* expression did not reveal prognostic significance in the *Her-2* overexpression patients. OS rates were lower in luminal breast cancer patients with elevated expression of *Notch4* compared to patients with a low expression level of *Notch4*. Due to the fact that Notch4 had a worse prognosis value in luminal breast cancer, it was deemed that Notch might cause resistance to hormone therapy. However, their results showed that Notch4 was not an independent prognosis factor in breast cancer patients (25). In agreement with previous studies, our data suggested that in ER^+ TAM-R patients similar to triple-negative breast cancer, the mRNA level of *Notch4* was significantly increased. In addition, Notch4 was responsible for poor prognosis in DFS and was an independent survival factor in OS.

Hes1, which involves morphological processes, such as cell cycle, apoptosis, and

pluripotency, is a downstream target of the Notch signaling pathway. Hes1 expression promotes proliferation, tumor recurrence, and cell survival in different cancer cell lines. In breast cancer, high levels of Hes1 bring about EMT-like features, invasiveness, lymph node metastasis, and advanced TNM (26). Moreover, the upregulation of Hes1 had a crucial role in chemoresistance, higher recurrence rate, and worse survival in colorectal cancer patients. It was demonstrated that Hes1 downregulated the E-cadherin and upregulated N-cadherin and ABC transporters. Hence, Hes1 was able to prompt EMT and was correlated with tumor recurrence (27).

Similar to these findings, our results showed that TAM-R patients exhibited higher *Hes1* overexpression compared with TAM-S patients. In addition, in parallel with the previous studies (26,27), it was indicated that *Hes1* expression correlated with clinicopathological features and influenced N-stage nipple involvement and also was an unfavorable prognosis factor in TAM-treated breast cancer patients.

Our findings showed that *Notch4*, *nicastrin*, and their downstream target *Hes1* were concomitantly upregulated in TAM-R patients. In addition, analyzing previous data revealed that overexpression of *Notch4*, *nicastrin*, and *Hes1* had a significant correlation with stemness factors Oct4 and Sox2 (6). Altogether, these genes, all of which are involved in promoting CSCs and EMT, might promote TAM resistance in TAM-treated patients.

CONCLUSION

According to the findings of the current study and considering the results of other related research, the Notch signaling pathway is upregulated in TAM-R patients compared to TAM-S patients, which can lead to CSCs and EMT promotion. Furthermore, evaluating *Notch4*, *nicastrin*, and *Hes1* concomitant mRNA expression showed that their concomitant expression might have a crucial role in poor prognosis, TAM resistance, and tumor recurrence in ER^+ breast cancer patients. However, further studies are needed to elucidate the exact role of these genes in TAM resistance and their potential function in prognostic or even diagnostic applications.

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Conflict of interest statements

The authors declared no conflict of interest in this study.

Authors' contributions

A. Boustan contributed to experimental procedures, statistical analysis, writing, and initial draft preparation of the manuscript; A. Dahmardeh and M. Khorsandi wrote, reviewed, and edited the article; R. Jahangiri contributed to sample preparation and data collection; F. Mosaffa and Kh. Jamialahmadi contributed to resources, conceptualization, and supervision. The finalized article was approved by all authors.

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