

Original Article

Increased expression of ASRGL1 in invasive ductal carcinoma and its association with estrogen-progesterone receptor status of tumors

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Abstract: Aims: Human asparaginase-like protein 1 (ASRGL1) is closely related to tumor growth. ASRGL1 can significantly promote cell proliferation and suppress apoptosis. To date, high levels of expression of ASRGL1 have been reported in various tumors, but the function of ASRGL1 in carcinogenesis is still not well understood. In this study, we aimed to immunohistochemically investigate the expression of ASRGL1 in non-neoplastic breast tissue and invasive ductal carcinoma. Methods and results: ASRGL1 was evaluated immunohistochemically in 148 invasive ductal carcinomas and 105 nonneoplastic breast tissue samples to assess the impact on breast cancer development and its association with clinicopathologic features. ASRGL1 was observed positive in 63 (42.6%) and negative in 85 (57.4%) invasive ductal carcinoma. In nonneoplastic breast tissue, 24 (22.9%) cases were ASRGL1 positive and 81 (77.1%) were negative. A significant difference was observed between invasive ductal carcinoma and nonneoplastic breast tissue in terms of ASRGL1 expression, and ASRGL1 expression was increased in invasive ductal carcinoma ($P = .001$). Most estrogen receptor-negative tumors and progesterone receptor-negative tumors were also negative with ASRGL1 and the difference was significant ($P = .006$ and $P = .001$, respectively). The correlation between the ASRGL1 expression of the tumors and event-free survival or overall survival was not significant ($P > .05$). Conclusions: ASRGL1 may play a role in increasing cell proliferation and breast cancer development. ASRGL1 expression in breast cancer closely correlates with the hormone receptor status of the tumor. In breast cancer, ASRGL1 expression does not contribute to predicting tumor behavior.

Keywords: Asparaginase-like protein 1, ASRGL1, breast, breast cancer, immunohistochemistry, invasive ductal carcinoma

Introduction

Human asparaginase-like protein 1 (ASRGL1) is an enzyme and is one of the N-terminal nucleophile hydrolases first described as a sperm autoantigen in rats [1]. ASRGL1 is a β -aspartyl peptidase and its function is the degradation of isoaspartyl peptides [2]. Isoaspartyl-dipeptides are produced by non-enzymatic damage in proteins and cause misfolding, dysfunction, and reduced degradation [3]. Loss of function of ASRGL1 results in increased levels of asparagine and isoaspartyl-dipeptide in cells, thereby leading to an accumulation of dysfunctional proteins [4]. A mutation in ASRGL1 and an accumulation of isoaspartyl peptides in the

retina and brain can cause retinal degeneration, aging, and neuropathology [5-7].

ASRGL1 has been closely related to tumor growth [8]. ASRGL1 could significantly promote cell proliferation and suppress apoptosis [9]. To date, high expression levels of ASRGL1 have been reported in various tumors, including breast, ovarian, and cervical cancers, but the function of ASRGL1 in carcinogenesis is still not well known [8-10]. In a previous publication that reported that ASRGL1 was increased in breast carcinoma, the number of cases was small and the relationship to ASRGL1 expression and clinicopathologic features were not investigated [10].

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Breast cancer is the most common cancer in women worldwide and the second most common cause of cancer-related death in women. Many mechanisms in breast cancer development are known and successful treatments are used, but new studies on breast cancer show that many mechanisms in breast cancer development remain unknown. In recent years, down-regulation of various genes and overexpression of various proteins have been identified in invasive ductal carcinoma (IDC). They are thought to play a role in the development of breast cancer and may be therapeutic targets [11-13].

In this study, we aimed to investigate the immunohistochemical expression of ASRGL1 in non-neoplastic breast tissue (NNBT) and IDC and to analyze the association between clinicopathologic data and ASRGL1 expression in IDC.

Materials and methods

Patients' specimen and clinicopathologic data

After obtaining ethical approval (Ethics Committee of Selcuk University, Faculty of Medicine; 2018/146), specimens of IDC diagnosed and treated by surgical resection (total mastectomy or lumpectomy) in the Department of Pathology, Konya Education and Research Hospital between 2011 to 2018 were retrieved and used in this study. Patients with histopathologically diagnosed IDC, females, patients who underwent surgical resection, and patients who did not receive neoadjuvant therapy or who did not respond to neoadjuvant therapy were included in the study. Patients diagnosed as having special types of breast carcinomas, with partial or complete response to neoadjuvant chemotherapy, males, and those with unavailable archival tissue blocks were excluded from the study. One hundred forty-eight patients who were diagnosed as having invasive breast carcinoma of no special type were included in the study. One hundred five breast reduction materials that were examined in the Department of Pathology, Konya Education and Research Hospital, between 2008 to 2018 were used for the study to examine the non-neoplastic breast tissue. Informed consent was signed by the patients.

Clinicopathologic features were obtained from the patient files. Tumors were graded according

to the Bloom & Richardson grading system, modified by Elston and Ellis, and they were staged according to the tumor, node, metastasis (TNM) staging system [14, 15]. The estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2) expression status, as well as the Ki67 proliferation indices of the tumors were evaluated immunohistochemically. Cases that were scored 2 for Her2 immunohistochemically were examined using fluorescence *in situ* hybridization to evaluate the presence of Her2 amplification. To determine the Ki67 proliferation index, 1000 cells were evaluated in hot spot areas in immunohistochemically stained preparations. Positive nuclear-stained cells were counted, and the percentage of positively-stained cells was calculated. The Ki67 proliferation index was considered high if it was $\geq 14\%$ and low if it was $< 14\%$. Molecular subtypes of the cases were determined according to their ER, PR, and Her2 expression status [15].

Immunohistochemical staining

Four-micrometer formalin-fixed paraffin-embedded (FFPE) tissue sections were subjected to immunohistochemistry using a Leica Bond-Max fully automated IHC & ISH instrument (Leica Biosystems Melbourne Pty Ltd., Bondmax, M212536, 2014, Melbourne Australia). An ASRGL1 antibody for immunohistochemistry (HPA055572; Atlas Antibodies, Stockholm Sweden) and the Bond Polymer Refine Detection Kit (Leica Biosystems, Newcastle Upon Tyne, UK) was used. The exhaustive protocol was attained from the anti-ASRGL1 product datasheet, and slides were stained with ASRGL1 (1/200 concentration) in accordance with the protocol. Diaminobenzidine (DAB) and hematoxylin counterstaining were performed for visualization. The stained preparations were then sealed with a coverslip using Entellan.

Immunohistochemistry evaluation

A semi-quantitative scoring system according to the intensity and percentage of positive staining was used to evaluate ASRGL1 expression. The scores for the percentage of positive cells were: 0 = no positive cells; 1 = $\leq 25\%$ positive cells; 2 = 26-50% positive cells; 3 = 51-75% positive cells; and 4 = $> 75\%$ positive cells. For staining intensity, the scores were: 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. If

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Table 1. Immunohistochemical ASRGL1 staining in IDC and NNBT

ASRGL1	Positive	Negative	P value
IDC (n = 148) (%)	63 (42.6%)	85 (57.4%)	.001
NNBT (n = 105) (%)	24 (22.9%)	81 (77.1%)	

IDC: Invasive ductal carcinoma, NNBT: Non-neoplastic breast tissue.

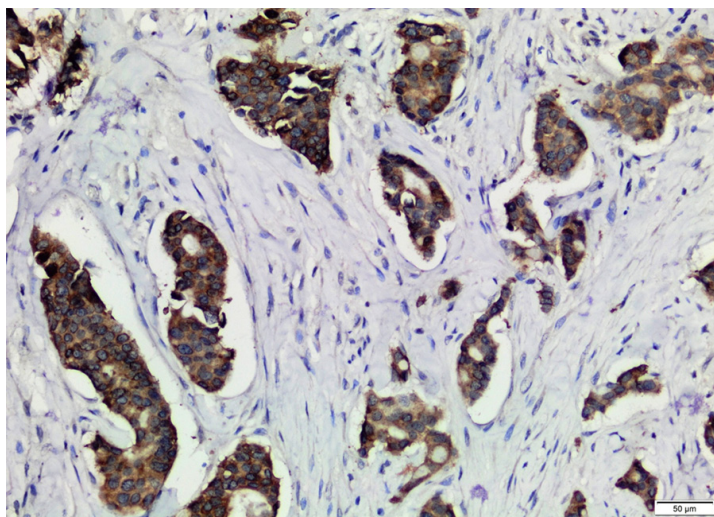


Figure 1. Cytoplasmic ASRGL1 positivity in invasive ductal carcinoma. ASRGL1×200.

the product of the percentage and scoring intensity scores was >3 , the specimen was considered to be immunohistochemically positive for ASRGL1 [16]. Cytoplasmic staining was considered positive. Each tissue section was independently analyzed by two pathologists who were blinded to the clinicopathologic data.

Statistical analyses

Statistical analyses were performed using SPSS 22.0 for Windows software (SPSS, Chicago, IL, USA). The Shapiro-Wilk test was used to examine continuous variables with normal and abnormal distributions, and one-way analysis of variance (ANOVA) was used for normally distributed continuous variables. The Kruskal-Wallis test was used for abnormally distributed continuous variables. When the Kruskal-Wallis test indicated statistically significant differences, the causes of the differences were determined using a Bonferroni-adjusted Mann-Whitney U test. Nominal variables were analyzed using Pearson's Chi-square or Fisher's exact test, when applicable. Continuous variables are presented as mean \pm

standard deviation or median (min-max), and categorical variables are presented as the number of cases and percentages. For all possible multiple comparisons, Bonferroni-adjustment was performed to control for type I errors. Pearson's test was used for normally distributed variables and Spearman's rho test was used for abnormally distributed variables for correlation analysis. Survival was assessed using Kaplan-Meier analysis. Statistical significance was set at $P < 0.05$.

Results

Immunohistochemical ASRGL1 staining results in IDC and NNBT

One hundred forty-eight IDCs and 105 NNBTs were available for analysis. The mean age of the patients with IDC was 52.54 ± 12.21 years. The mean tumor diameter was 2.79 ± 1.54 cm.

Immunohistochemical ASRGL1 staining in IDC and NNBT is shown in **Table 1**. Cytoplasmic ASRGL1 staining was observed in 63 (42.6%) IDCs and ASRGL1 was negative in 85 (57.4%) IDC (**Figure 1**). In NNBTs, 24 (22.9%) cases were ASRGL1-positive and 81 (77.1%) were negative (**Figure 2**). A difference was observed between IDC and NNBT in terms of ASRGL1 expression, and it was revealed that ASRGL1 expression was increased in IDC ($P = .001$).

Results of the relationship with ASRGL1 staining and the clinicopathologic data of the cases

The clinicopathologic data of the cases and the relationship with ASRGL1 staining are shown in **Table 2**.

Most of the ER-negative and PR-negative tumors were also negative with ASRGL1 ($P = .006$ and $P = .001$, for differences respectively). A significant difference was observed between the molecular subtype of the tumors and ASRGL1 staining intensities ($P = .019$). ASRGL1 was positive in a significant proportion of luminal B tumors, but it was observed that the majority of Her2-like tumors and basal-like

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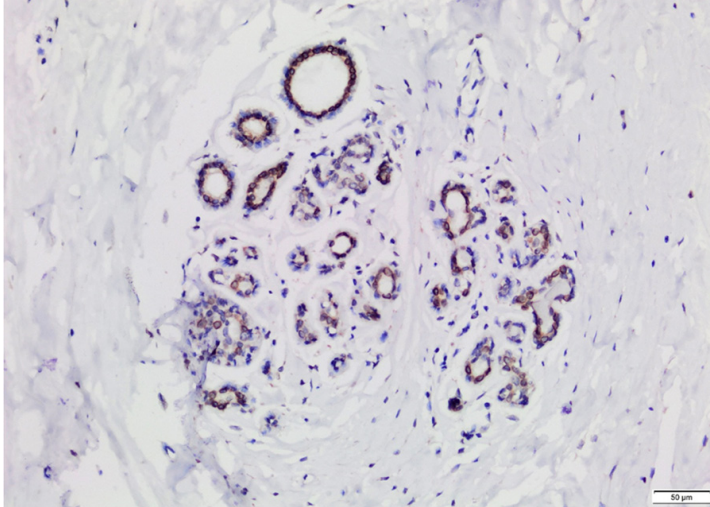


Figure 2. Cytoplasmic ASRGL1 positivity in non-neoplastic breast tissue. ASRGL1×200.

tumors were ASRGL1-negative. There was no significant relationship between the ASRGL1 expression of the tumors and the age of the patients, tumor diameter, number of metastatic lymph nodes, presence or absence of distant metastases, stage, grade, Her2 status, and Ki67 proliferation indices of the tumors ($P>.05$).

Correlation between the ASRGL1 expression of tumors and event-free survival or overall survival

The median follow-up time of the patients was 4.49 years for event-free survival (EFS) and 4.78 years for overall survival (OS). The correlation between the ASRGL1 expression of the tumors and EFS or OS was not significant (Log-rank, $P = .930$ and $P = .730$, respectively) (Figures 3, 4).

Discussion

CRASH is a human asparaginase-like protein and is a viable candidate as a new target in the diagnosis and therapy of cancer. Weidle et al. investigated the expression of CRASH using immunohistochemical methods in several normal and tumor tissues of humans. Normal tissues showed strong expression in the testis, prostate, brain, and esophagus, and weaker expression in the fallopian tube, lung, and kidney. Although there was no staining of normal tissue in the endometrium, breast, colon and ovary, they observed CRASH expression in tumors of these organs, reporting

CRASH expression in 16 of 68 breast carcinomas [10].

ASRGL1 has been found closely associated with tumor growth, and the loss of ASRGL1 suppressed proliferation in various carcinomas [8-10]. When ASRGL1 expression is inhibited, the expression of CDK2 and Cyclin A2 proteins is markedly decreased. Thus, the transition of cells from S phase to G2 and M phases in the cell cycle is prevented, cells are captured in S phase, and cell proliferation is inhibited [9, 17]. ASRGL1 could act as an anti-apoptotic factor and the loss of ASRGL1 may promote apoptosis. Lv et al. reported that significantly enhanced ASRGL1 expression was present in cervical carcinoma compared with paracancerous tissue. Moreover, they revealed that the knockdown of ASRGL1 resulted in inhibition of proliferation, promoting apoptosis. The authors suggested that ASRGL1 downregulation could be a treatment in cervical carcinoma [9].

More

In this study, it was revealed that ASRGL1 expression in IDC was distinctly increased in comparison with NNBT. This indicates that ASRGL1 increases cell proliferation in breast tissue. Increased ASRGL1 expression in IDC compared with NNBT may be useful in distinguishing benign lesions of the breast that can be confused with IDC, and increased ASRGL1 expression can be considered as a finding in favor of IDC.

In endometrioid endometrial adenocarcinoma, ASRGL1 is an independent factor for predicting survival, and loss of ASRGL1 is associated with lymph node metastasis and poor prognosis [17-20]. ASRGL1 levels have been stated to correlate with tumor progression and the metastatic tendency of human colorectal and pancreatic carcinomas [8]. Although previous studies reported that ASRGL1 expression and asparagine levels were associated with metastasis in breast cancer and suggested that ASRGL1 was high in metastatic breast tumors, we observed no correlation between ASRGL1 expression and regional lymph node metastasis or distant metastasis [10, 21].

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Table 2. Relationship between ASRGL1 expression and clinicopathologic characteristics of IDCs

Characteristic	Total Cases (n = 148)	ASRGL1 in IDC		P value	R value	
		Positive	Negative			
Age	years <40	20 (13.5%)	11 (55.0%)	9 (45.0%)	.227	-.099
	years ≥40	128 (86.5%)	52(40.6%)	76 (59.4%)		
Tumor (T)	T1	53 (35.8%)	22 (41.5%)	31 (58.5%)	.959	.10
	T2	85 (57.4%)	37 (43.5%)	48 (56.5%)		
	T3	10 (6.8%)	4 (40.0%)	6 (60.0%)		
Lymph node status (N)	N0	55 (37.2%)	20 (36.4%)	35 (63.6%)	.597	.105
	N1	55 (37.2%)	24 (43.6%)	31 (56.4%)		
	N2	27 (18.2%)	14 (51.9%)	13 (48.1%)		
	N3	11 (7.4%)	5 (45.5%)	6 (54.5%)		
Distant metastasis (M)	M0	142 (95.9%)	4 (66.7%)	2 (33.3%)	.223	.064
	M1	6 (4.1%)	59 (41.5%)	83 (58.5%)		
Stage	I	26 (17.6%)	11 (42.3%)	15 (57.7%)	.466	.085
	II	81 (54.7%)	31 (38.3%)	50 (61.7%)		
	III	35 (23.6%)	17 (48.6%)	18 (51.4%)		
	IV	6 (4.1%)	4 (66.7%)	2 (33.3%)		
Grade	1	17 (11.5%)	8 (47.1%)	9 (52.9%)	.922	-.016
	2	79 (53.4%)	33 (41.8%)	46 (58.2%)		
	3	52 (35.1%)	22 (42.3%)	30 (57.7%)		
Estrogen Receptor	Positive	110 (74.3%)	54 (49.1%)	56 (50.9%)	.006	.224
	Negative	38 (25.7%)	9 (23.7%)	29 (76.3%)		
Progesterone Receptor	Positive	106 (71.6%)	54 (50.9%)	52 (49.1%)	.001	.269
	Negative	42 (28.4%)	9 (21.4%)	33 (78.6%)		
Cerb-2	Positive	32 (21.6%)	16 (50.0%)	16 (50.0%)	.337	.079
	Negative	116 (78.4%)	47 (40.5%)	69 (59.5%)		
Ki-67 index (%)	<14	58 (39.2%)	28 (48.3%)	30 (51.7%)	.260	-.093
	≥14	90 (60.8%)	35 (38.9%)	55 (61.1%)		
Molecular Subtype	Luminal A	90 (60.8%)	40 (44.4%)	50 (55.6%)	.019	-.106
	Luminal B	19 (12.8%)	13 (68.4%)	6 (31.6%)		
	Her-2 like	14 (9.5%)	4 (28.6%)	10 (71.4%)		
	Basal like	25 (16.9%)	6 (24.0%)	19 (76.0%)		

IDC: Invasive ductal carcinoma; ASRGL1: Asparaginase-like protein 1.

It was proposed that CRASH expression was directly related to the progression of endocrine-sensitive tumors [9]. It was reported that the expression of ASRGL1 was increased in breast, ovarian, endometrium, and small cell lung carcinomas among endocrine-related tumors. It is noteworthy that in this study, ASRGL1 was observed as negative in IDCs with negative ER and PR. ASRGL1 has been detected positive in ER and PR-positive IDC. It is known that the prognosis is better in patients with ER and PR-positive IDCs [15]. Although the absence of ASRGL1 expression in ER and PR-negative tumors could suggest that the

prognosis may be slightly worse in ASRGL1-negative IDC, we found no significant relationship between ASRGL1 expression and the EVS or OS of patients with IDC. There was no significant relationship between other known prognostic parameters of IDC and the expression of ASRGL1. These results indicate that ASRGL1 expression cannot be recommended as a prognostic parameter for IDC.

In summary, ASRGL1 expression is higher in IDCs than in NNBTs. Furthermore, ASRGL1 expression is decreased in ER and PR-negative IDCs. ASRGL1 expression in IDC is not associated with EFS or OS in patients with IDC.

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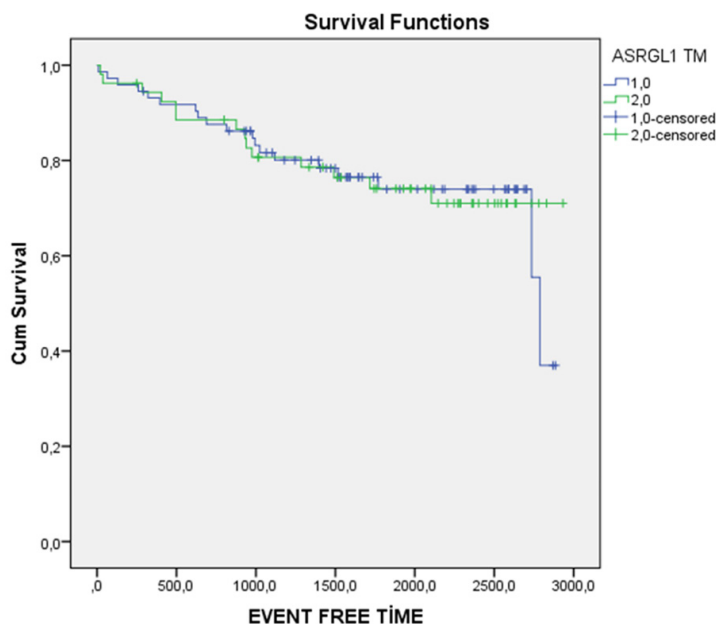


Figure 3. Event-free survival analysis diagram of invasive ductal carcinoma patients.

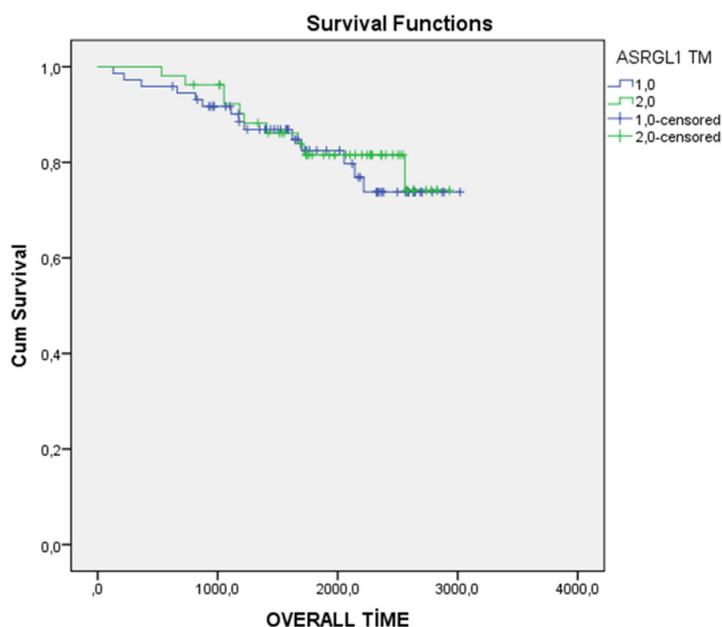


Figure 4. Overall survival analysis diagram of invasive ductal carcinoma patients.

In conclusion, our study demonstrates that ASRGL1 may play a role in increasing cell proliferation and breast cancer development. ASRGL1 expression in breast cancer closely correlates with the hormone receptor status of the tumor. In breast cancer, ASRGL1 expression does not contribute to predicting

tumor behavior. ASRGL1 may serve as a therapeutic target in breast cancer, but further studies are needed to clarify this issue.

Acknowledgements

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Abbreviations

ASRGL1, Asparaginase-like protein 1; FFPE, Formalin-fixed paraffin-embedded; IDC, Invasive ductal carcinoma; NNBT, Nonneoplastic breast tissue.

Disclosure of conflict of interest

None.

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