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ORIGINAL ARTICLE

Expression levels of genes involved in cell adhesion and motility correlate with poor clinicopathological features of epithelial ovarian cancer

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Summary

Purpose: *Changes in the expression levels of genes involved in cancer cell adhesion and motility have been reported to have an important role in tumor progression. In this study, we aimed to investigate the clinical significance of ITGAV and CALD1 gene expression in epithelial ovarian cancer (EOC), the most lethal gynecological malignancy.*

Methods: *Reverse transcription quantitative polymerase chain reaction was used to evaluate ITGAV and CALD1 expression levels in 47 EOC and 19 benign formalin-fixed paraffin-embedded samples. We used Spearman's test to determine the association between ITGAV and CALD1 expression and Wilcoxon test to compare expression levels between malignant and benign ovarian tumor specimens as well as to determine their association with clinicopathological characteristics of EOC. Survival analysis was done by the Kaplan-Meier method and the log-rank test. P ≤ 0.05 was considered statistically significant.*

Results: *CALD1 and ITGAV showed significantly lower expression in EOC than in benign ovarian samples (p<0.001). Furthermore, CALD1 was significantly lower expressed in high-grade tumors (p=0.037) while there was a trend for a lower expression of ITGAV in tumors with high histological grade (p=0.043), in tumors with ascites (p=0.055), and in tumors of patients who relapsed (p=0.083). We also found a significant positive association between ITGAV and CALD1 expression (ρ=0.640, p<0.001) in EOC samples. Kaplan-Meier analysis showed no significant impact of ITGAV and CALD1 expression levels on overall survival of EOC patients (p=0.149 and p=0.430, respectively).*

Conclusion: *Our findings indicate that CALD1 and ITGAV gene expression levels correlate with poor clinicopathological features of the EOC.*

Key words: *CALD1, epithelial ovarian cancer, epithelial mesenchymal transition, ITGAV*

Introduction

Epithelial ovarian cancer (EOC), typically diagnosed when the disease has spread beyond the ovaries in which case the majority of patients relapse after the good initial response to the treatment [1], remains the most lethal gynecological malignancy [2]. The current standard of care for EOC is surgery followed by platinum-based chemotherapy [3] and although certain improvements in the disease management have improved progression-free survival (PFS), there was no significant change in overall survival (OS) [4,5]. These facts highlight the importance of ovarian cancer research, including the need for novel and more effective therapies, as well as better prognostic and predictive biomarkers. Recently, it has been proposed that the process of epithelial-mesenchymal transition (EMT), involved

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in embryonic development and wound healing, is also associated with the progression, metastasis and treatment resistance of many tumors of epithelial origin [6], including ovarian [7]. This transient, multi-step process enables epithelial cancer cells to acquire motile and invasive characteristics of mesenchymal cells, allowing them to detach from the primary tumor and to invade distant sites in the body [8]. The main features of epithelial cells exhibited during this process are the loss of cell-cell junctions, altered interaction of cells with the components of extracellular matrix (ECM) and changes in the cytoskeletal organization [6]. Therefore, proteins involved in cell adhesion and motility have emerged as potential regulators of tumor invasion and metastasis [9].

Table 1. Clinicopathological characteristics of EOC patients and association with ITGAV and CALD1 expression

Characteristics	*No. of patients (%)*	*ITGAV relative expression*		*CALD1 relative expression*	
	N=57	*Median (range)*	*Wilcoxon rank sum test*	*Median (range)*	*Wilcoxon rank sum test*
Age, years			p=0.7491		p=0.7140
≤60	33 (57.90)	0.12 (0.002-47.120)		0.26 (0.006-409)	
>60	24 (42.10)	0.20 (0.004-75.99)		0.441 (0.004-19.93)	
Menopause			p=0.337		p=0.443
Yes	36 (63.20)	0.12 (0.002-75.99)		0.29 (0.004-409)	
No	15 (31.91)	0.10 (0.005-1.57)		0.197 (0.006-7.07)	
N/A	4-5 (8.70)				
Histological type			p=0.7017		p=0.8357
Serous	42 (76.60)	0.14 (0.002-47.12)		0.26 (0.004-409)	
Other	11 (23.40)	0.108 (0.02-75.99)		0.242 (0.022-16.59)	
FIGO stage			p=0.2901		p=.858
I+II	22 (39.60)	0.28 (0.005-75.99)		0.33 (0.007-13.21)	
III+IV	34 (59.70)	0.12 (0.002-47.12)		0.20 (0.004-409)	
Histological grade			p=0.054		p=0.037
1	17 (31.91)	0.43 (0.02-75.99)		1.67 (0.021-409)	
2+3	28 (59.70)	0.10 (0.002-26.06)		0.13 (0.004-19.93)	
N/A	64 (10.5)				
Lymph node metastasis			p=0.49		p=0.92
Yes	9 (15.80)	0.11 (0.002-47.12)		0.24 (0.007-409)	
No	34 (59.70)	0.1549 (0.004-75.99)		0.26 (0.004-16.59)	
N/A	14 (24.50)				
Peritoneal metastasis			p= 0.332		p=0.442
Yes	36 (63.20)	0.12 (0.002-47.12)		0.224 (0.004-409)	
No	18 (27.66)	0.28 (0.02-75.99)		0.94 (0.01-16.59)	
N/A	2 (4.26)				
Distant metastasis			p=0.523		p=0.276
Yes	8 (14.00)	0.08 (0.002-1.32)		0.16 (0.013-9.732)	
No	47 (65.96)	0.1546 (0.004-7.99)		0.37 (0.004-409)	
N/A	2 (3.50)				
Ascites			p=0.055		p=0.186
Yes	24 (42.40)	0.11 (0.002-1.32)		0.17 (0.004-16.59)	
No	31 (54.404)	0.17 (0.02-75.99)		0.814 (0.007-409)	
N/A	2 (3.50)				
Residual disease			p=0.690		p=0.8896
Yes	20 (35.10)	0.15 (0.003-0.64)		0.33 (0.002-19.93)	
No	24 (42.10)	0.10 (0.002-10.33)		0.20 (0.01-13.12)	
N/A	13 (22.80)				
Relapse			p=0.083		p=0.766
Yes	15 (26.30)	0.10 (0.002-26.06)		0.20 (0.004-19.93)	
No	31 (54.40)	0.30 (0.005-75.99)		0.32 (0.007-409)	
N/A	9 (19.15)				

Integrins constitute a large family of transmembrane cell surface receptors composed of α and β subunits which are crucial for establishing and modulating both cell-cell and cell-extracellular matrix (ECM) interactions, providing a signal in both directions [10]. The extracellular domain of integrins binds to different ECM or cell surface proteins, while the intracellular domain interacts with the actin cytoskeleton through different signaling and adaptor molecules [11]. Changes in the expression patterns of integrins have been reported in many different malignancies and have been shown to be highly dependent on tumor cell of origin [12]. Integrin alpha V (*ITGAV*) gene is a protein-coding gene [10] found to be over-expressed in many cancers both in tumor cells and activated endothelial cell during neoangiogenesis [13]. Preclinically, targeting *ITGAV* was shown to inhibit angiogenesis, tumor growth and metastasis in several solid malignancies [14-17].

Changes in the cytoskeleton have also been identified as the key component involved in the process of tumor progression [18]. *CALD1* gene encodes caldesmon 1, a cytoskeleton-associated protein which has a major role in the regulation of the actomyosin contractile system, thereby influencing numerous cell functions, such as motility, migration, invasion and proliferation [19]. With binding sites for actin, myosin, tropomyosin, Ca-calmodulin and phospholipids [20], it acts as a cell motility suppressor that inhibits actin-mediated myosin ATPase activity by blocking the interaction between actin and myosin [21]. Aberrant expression of *CALD1* has been implicated in several malignancies, though with conflicting results [18,22,23].

To our knowledge, data regarding *ITGAV* and *CALD1* expression status in clinical specimens of EOC have been scarce. In this study, we aimed to evaluate the differences in the expression patterns of these genes between benign and malignant epithelial ovarian tumor samples as well as their correlation with clinicopathological parameters and overall survival OS of EOC patients.

Methods

Patients and samples

This study was performed on formalin-fixed paraffin-embedded (FFPE) tissue blocks comprising 57 EOC and 19 benign ovarian tumor samples, obtained from patients who had undergone primary debulking surgery at the Institute for Oncology and Radiology of Serbia between May 2007 and December 2014 and were clinically staged according to the International Federation of Gynecologists and Obstetricians (FIGO) staging system. None of the EOC patients included in this study had received neoadjuvant therapy prior to surgery. The median patient's age was 57 years in the EOC group and 56 in the benign group, with an age range of 25-79 and 17-79 years, respectively. The majority of malignant tumors were of advanced FIGO stage (59.70%) and serous histological subtype (73.70%). Detailed clinicopathological features of EOC patients are shown in Table 1. OS was defined as the time between disease diagnosis (date of surgery) and the date of death or last follow up. The median follow-up period for OS was 42 months (range 4-108). This retrospective study was approved by the Ethics Committee of the Institute for Oncology and Radiology of Serbia and written informed consent was collected from each patient.

Reverse transcription and quantitative real-time PCR (RT-qPCR)

The expression levels of *ITGAV* and *CALD1* gene were analyzed using reverse transcription and quantitative real time PCR. Total RNA was extracted using the RNeasy FFPE kit (Qiagen, Germany) according to the manufacturer's instructions. All samples were pretreated with RNase-free DNase I. For assessing the concentration and purity of RNA, BioSpecNano spectrophotometer was used (Shimadzu, Japan). RNA (2μg) was reversely transcribed into complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) while the expression levels were determined using commercially available TaqMan Gene Expression Assays (Thermo Fisher Scientific, USA) for *ITGAV* (Hs00233808_m1), *CALD1* (Hs00921982_m1) and GAPDH (Hs02758991_g1) genes. Gene expression analyses were performed on 96-well plates in duplicates and quantified on the 7500 Real-Time PCR system (Applied Biosystems, USA). PCR reactions were set to 20 μl and contained TaqMan® Universal PCR Master Mix (2X), TaqMan Gene Expression Assay (20X), cDNA template and nuclease-free water. The PCR conditions were as follows: hold at 50°C for 2 min, 1 cycle of denaturation at 95°C for 10 min and 40 cycles at 95°C for 15 sec and annealing at 60°C for 1 min. The expression levels of target genes were normalized to the amount of GAPDH as the reference gene and calculated using comparative delta-delta Ct method.

Statistics

The Wilcoxon rank sum test was used to compare *ITGAV* and *CALD1* expression levels in the malignant and benign ovarian tissue as well as to determine whether these expression levels were correlated with different clinicopathological parameters of EOC patients. The association between gene expression status and age, preoperative CA125 status, and the correlation between *ITGAV* and *CALD1* expression levels were evaluated by Spearman's rank correlation. The OS was estimated by the Kaplan-Meier method and comparison was done using the log-rank test. P value <0.05 was considered statistically significant. All statistical analyses were performed using the program R (version 3.3.2), except for the survival curves, which were analyzed using SigmaStat (version 3.5).

Results

We evaluated 57 EOC and 19 benign ovarian specimens using RT-qPCR to analyze the differences in the *ITGAV* and *CALD1* expression levels and to correlate this data with clinicopathological characteristics and OS of EOC patients. *CALD1* and *ITGAV* showed significantly lower expression in malignant than in benign ovarian tissue (p<0.001). We also found that *CALD1* was significantly lower expressed in high-grade tumors (grade 2 and 3) in comparison with tumors with grade 1 (p=0.037). No significant correlations were found between *CALD1* and *ITGAV* expressions levels and patient age, FIGO, histological subtype, lymph node status, peritoneal and distant metastases, ascites, menopausal status, CA125 level, residual tumor and relapse. However, there was a trend for a lower expression of *ITGAV* in tumors with high histological grade (grade 2 and 3) (p=0.053), in tumors with ascites (p=0.055) and in tumors of patients who relapsed (p=0.083). Relationships between *ITGAV* and *CALD1* expression levels and clinicopathological characteristics are shown in Table 1. For survival analysis, patients were divided into high and low expression groups based on the median fold change value for *ITGAV* and *CALD1* relative expression levels. Kaplan-Meier analysis and log-rank test found no significant impact of *ITGAV* and *CALD1* expression levels on OS of EOC patients (p=0.149 and p=0.430, respectively) (Figure 1 and 2). The association between *ITGAV* and *CALD1* expression levels in EOC, analyzed using the Spearman's rank correlation coefficient, showed significant positive correlation (ρ=0.64, p<0.001)

Discussion

Epithelial ovarian cancer is the deadliest and most common type of ovarian cancer, with a 5-year survival rate of only 39% and 17% for stage III and IV, respectively [3]. Due to the absence of early symptoms of the disease, the complexity of ovarian embryonic development and its endocrine function, most epithelial ovarian cancer cases are diagnosed at an advanced stage [24]. Unlike most epithelial malignancies that mainly spread hematogenously, late-stage EOC is usually characterized by the metastases that originate from the cancer cells that are shed from the primary tumor and transported by the physiological peritoneal fluid, and adhere to the abdominal peritoneum and/or omentum [25]. This feature indicates that the spread of EOC is mediated by cell-cell or cell-matrix adhesion mechanisms [13]. Since the main link between a cell and the ECM are integrins, it is believed that they have an important role in the process of cancer invasion [26]. Integrins are not only important for cell adhesion, but they also activate many intracellular signaling pathways that are involved in cell migration, invasion, proliferation and survival, after binding different extracellular ligands [27]. Altered expression of certain integrins, including *ITGAV*, has been detected in the majority of malignant tumors and it has been shown to vary between cancer types [13,28,29].

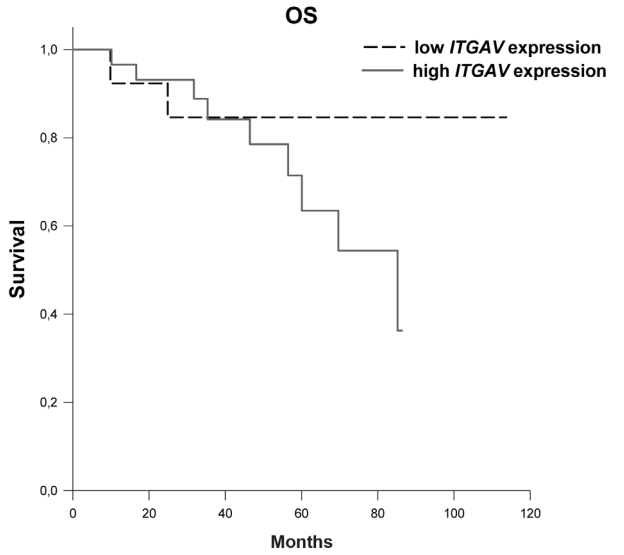

Figure 1. Kaplan-Meier survival curve of EOC patients according to *ITGAV* expression. No significant difference was observed between patients with high and low *ITGAV* expression (p=0.149).

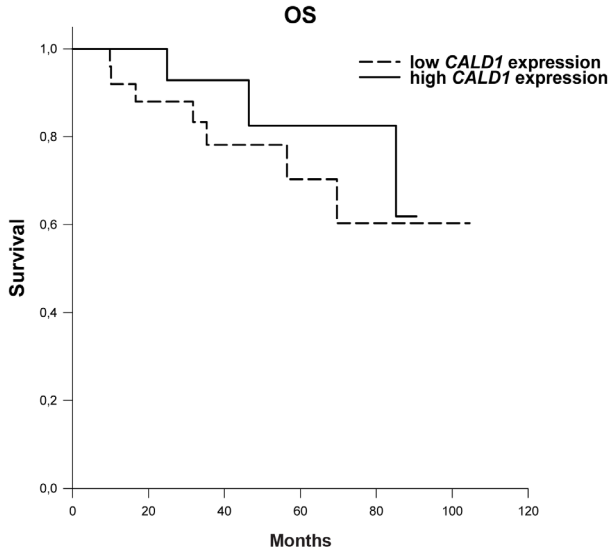

Figure 2. Kaplan-Meier survival curve of EOC patients according to *CALD1* expression. No significant difference was observed between patients with high and low *CALD1* expression (p= 0.430).

We demonstrated significantly lower expression levels of *ITGAV* in malignant compared with benign ovarian tumor samples. Moreover, there was a trend for lower *ITGAV* expression in high-grade tumors, in tumors of patients with ascites and patients who relapsed, indicating that low *ITGAV* expression might be associated with more aggressive ovarian tumor phenotype. No statistically significant differences were found between *ITGAV* gene expression levels and OS of EOC patients. These results are not consistent with the majority of studies evaluating the role of *ITGAV* gene expression in different malignancies in which the over-expression of *ITGAV* was mostly associated with poor clinicopathological characteristics and patient survival. To our knowledge, the only study focusing on the role of *ITGAV* expression in clinical specimens of ovarian cancer revealed that positive *ITGAV* expression was associated with decreased OS in advanced stage EOC patients [12]. Many studies examining the role of *ITGAV* expression levels were performed on colorectal carcinoma (CRC). A study by Denedai et al [30] found that *ITGAV* gene and protein levels were over-expressed in tumors with perineural invasion, while tissue microarray analysis additionally showed over-expression of *ITGAV* protein levels in tumors of the advanced TNM stage and in the presence of venous invasion. Moreover, over-expression of *ITGAV* was associated with tumors with lymph node metastases. These results are consistent with another study investigating the prognostic role of *ITGAV* expression in CRC by immunohistochemistry. Increased *ITGAV* expression was more frequently found in adenocarcinoma compared to adenoma and normal mucosa specimens, and it was also correlated with poorly differentiated tumors, high TNM stage as well as shorter OS and DFS of CRC patients [31]. Linhares et al [32] also demonstrated that increased *ITGAV* expression was an independent unfavorable prognostic factor in CRC. Taken together, these results indicate that *ITGAV* over-expression is associated with adverse clinicopathological characteristics and poor outcome of CRC patients, suggesting that it might be involved in the progression and dissemination of this malignancy.

Regarding the role of *ITGAV* in other malignancies, several studies addressed the role of *ITGAV* in lung cancer, though with conflicting results. Clarke et al [33] reported an association between *ITGAV* protein expression and nodal metastasis, whereas Smyth et al [34] showed a significant association between loss of expression of this integrin and nodal metastasis.

One of the possible explanations for the discrepancy between our results and the literature data is that integrins, based on the tissue type and different receptors they form by interacting with different subunits, might have different roles in tumor progression. It has been shown that cellular integrin expression pattern highly varies between cancer types, in individual tumors, and even in different parts of a tumor cell within a single tumor [35]. Furthermore, it has been hypothesized that the function of an integrin may change during tumor progression. Certain integrins induce the expression of different ECM degrading proteases [35], while integrins containing the alpha V subunit can activate latent TGF-β, resulting in TGF-β-induced EMT. In turn, TGF-β-signalling can activate integrins and also up-regulate their expression [36]. However, it is also hypothesized that an integrin may be down-regulated during transformation because it supports a normal cell phenotype [35].

Dysregulation of cell migration is crucial for the metastatic cascade, so along with the changes in the integrin expression pattern, malignant cells also exhibit changes in the cytoskeletal organization [11]. There is a significant amount of data that supports the role of cytoskeletal proteins, especially the ones that are actin-associated as candidates for cancer progression [37-39]. *CALD1* is a major actomyosin binding protein that acts as an inhibitor of myosin ATPase activity, thus blocking cell contraction and migration [20]. In our study, *CALD1* expression was significantly lower in malignant compared to benign ovarian tumor tissue. When analyzing different clinicopathological features of EOC, we found that *CALD1* expression was significantly lower in high-grade tumors. We also report that lower *CALD1* expression was observed in higher FIGO stage, tumors with metastases and ascites as well as in tumors of patients who relapsed, though the differences were not statistically significant. Furthermore, Kaplan-Meier analysis and log-rank test showed no statistically significant difference between *CALD1* gene expression levels and OS of EOC patients. This is in concordance with the study which identified *CALD1* as a potential biomarker for gastric cancer metastasis, showing that the expression of *CALD1* gene was reduced in metastasis and ascites-derived gastric cell lines compared to primary cancer-derived gastric cell lines. These findings were further confirmed by immunohistochemistry on gastric cancer tissues [40]. Moreover, Lee et al [41] showed that *CALD1* had a significantly lower expression in bladder cancer tissue compared to normal bladder tissue. Moreover, positive expression of *CALD1* was significantly associated with adverse clinicopathological characteristics, including large tumor size, the presence of lymphovascular invasion, and higher

tumor stage and grade. Survival analysis showed that positive *CALD1* expression was significantly associated with lower recurrence-free survival (RFS) and progression-free survival (PFS). This is in line with functional studies showing that knockdown of caldesmon 1 in breast and colon cancer cell lines promoted cell migration and invasion [23,40], while its over-expression in cancer cells decreased cell invasion, indicating its possible role as a metastasis repressor [39].

However, in oral cavity carcinoma, *CALD1* expression was significantly higher in metastatic lymph nodes compared with the primary tumor and was almost undetected in normal oral epithelia. Furthermore, higher *CALD1* expression was correlated with poor prognostic factors such as lymph node metastasis, tumor dedifferentiation, perineural invasion and tumor depth. Additionally, survival analyses showed that patients with higher *CALD1* expression had shorter disease-specific and disease-free survival [18]. This is in concordance with the study by Kim et al which showed that high *CALD1* expression was associated with increased malignancy of CRC [22]. *CALD1* was over-expressed in both CRC and liver metastasis compared to normal colorectal tissue. All this data indicate that *CALD1* over-expression is associated with tumor progression and poor prognosis. However, conflicting results found in different malignancies might be due to different caldesmon 1 isoforms, produced by alternative splicing [22], which may have different functions depending on the tissue type.

The positive correlation we observed between *ITGAV* and *CALD1* gene expression levels might suggest an interaction between these two signaling pathways. This would not be surprising knowing that integrins can modulate the actin cytoskeleton by interacting with different signaling molecules and adaptor proteins [11].

Conclusion

We found statistically significant differences in *ITGAV* and *CALD1* gene expression levels between benign and malignant ovarian tissue, suggesting the potential roles of these genes in the pathogenesis of EOC. Furthermore, our data demonstrated that low expression levels of *ITGAV* and *CALD1* are associated with poor prognostic factors of the disease. However, our results should be taken with caution due to the small number of enrolled patients and the fact that the role of *ITGAV* and *CALD1* gene expression levels in EOC pathogenesis remains to be fully elucidated.

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Conflict of interests

The authors declare no conflict of interests.

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