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Authors: Sebastian Szubert, Wojciech Jozwicki, Lukasz Wicherek, Krzysztof Koper

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Cytoplasmic and membranous receptor-binding cancer antigens expressed on SiSo cells (RCAS1) immunoreactivity in epithelial ovarian cancer cells represent differing biological function of RCAS1

Sebastian Szubert¹,², Wojciech Jozwicki³, Lukasz Wicherek¹, Krzysztof Koper⁴
¹2nd Department of Obstetrics and Gynecology, Medical Centre of Postgraduate Education, Warsaw, Poland
²Clinical Department of Gynecological Oncology, Franciszek Lukaszczyk Oncological Center, Bydgoszcz, Poland
³Department of Tumor Pathology and Pathomorphology, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, Torun, Poland
⁴Department of Chemotherapy, Franciszek Lukaszczyk Oncological Center, Bydgoszcz, Poland

RCAS1 in ovarian cancer

Correspondence address: Krzysztof Koper, MD, PhD
Department of Chemotherapy, Franciszek Lukaszczyk Oncological Center
Romanowska Street 2,
Bydgoszcz 85–796, Poland
e-mail: krzyskoper@gmail.com
Abstract

Introduction. Receptor-binding cancer antigen expressed on SiSo cells (RCAS1) is a selective suppressor of the immune response that has been linked to the evasion of immune surveillance by cancer cells. However, the exact prognostic impact of RCAS1 on epithelial ovarian cancer (EOC) has not been fully elucidated. The main aim of our study was to evaluate the influence of RCAS1 immunoreactivity (RCAS1-Ir) in EOC cells and in tumor stroma cells on patient overall survival. We also focused on RCAS1-Ir and the structure of the tumor stroma.

Material and methods. RCAS1-Ir was evaluated by means of immunohistochemistry in 67 patients with EOC. We distinguished cytoplasmic and membranous immunoreactivity patterns.

Results. We found that high cytoplasmic RCAS1-Ir in cancer cells was associated with more than a two-time shortened period of overall survival. Membranous RCAS1-Ir in cancer cells, as well as in tumor stroma macrophages and fibroblasts, did not correlate with patient survival. RCAS1-Ir in the cytoplasm of cancer cells was positively correlated with the degree of tumor stroma infiltration by fibroblasts and macrophages, but not with RCAS1-Irin these cells. On the other hand, membranous RCAS1-Ir in cancer cells was positively correlated with RCAS1-Ir in fibroblasts and macrophages, but not with their quantity.

Conclusions. Due to their different impacts on patient prognosis and tumor stroma structure, it seems that cytoplasmic and membranous RCAS1-Ir in EOC cells may have different biological functions.

Key words: RCAS1; receptor-binding cancer antigen expressed on SiSo cells; epithelial ovarian cancer; tumor stroma; tumor microenvironment
Introduction

Epithelial ovarian cancer (EOC) is still the leading cause of death from gynecological malignancies in the Western World [1]. Patient prognosis depends on the stage of the disease, patient age at diagnosis, the histological type of the tumor, and the first-line chemotherapy [2]. Current data suggests that the residual disease after primary debulking surgery is the most important prognostic factor in EOC [3, 4]. However, patient prognosis may be influenced by the structure and function of the tumor stroma [5, 6]. Chen et al. showed that stroma-rich ovarian cancer patients had a worse prognosis and higher risk of relapse compared to patients who had tumors with poorly developed stroma [7].

In 1996, Sonoda et al. discovered receptor-binding cancer antigen expressed on SiSo cells (RCAS1) as a membrane protein on uterine cervix cancer cells [8, 9]. RCAS1 has strong immunosuppressive activity and is linked with the evasion of immune surveillance by cancer cells. RCAS1 is responsible for the inhibition of both cytotoxic lymphocytes and natural killer (NK) cell activity [10]. Furthermore, RCAS1 may induce Fas-independent apoptosis of both T-cells and NK cells infiltrating the tumor microenvironment [11]. RCAS1 staining was also found on numerous noncancerous cells infiltrating the tumor stroma, such as tumor-associated macrophages (TAMs) and cancer-associated fibroblast (CAFs) [12]. Therefore, RCAS1 expression on cancer stroma cells may be responsible for the immune evasion of cancer cells and the development of a more cancer-permissive microenvironment facilitating tumor growth [12, 13]. By stimulating the expression of vascular endothelial growth factor (VEGF), RCAS1 also indirectly stimulates angiogenesis [14]. RCAS1 expression was also associated with the increased secretion of matrix metalloproteinase 1 (MMP-1) and laminin-5 in cervical cancer [15]. Additionally, in their study, Sonoda et al. found that RCAS1 expression was negatively correlated with the number of vimentin-positive stromal cells in EOCs [16, 17].

Taken together, such evidence suggests that RCAS1 may participate in the rebuilding of the tumor stroma.

RCAS1 expression in cancer cells has been shown to be a negative prognostic factor in patients with numerous types of human neoplasms, including gynecological malignancies [9, 18–20]. However, the exact role of RCAS1 in the survival of EOC patients is not fully understood. Furthermore, although RCAS1 expression has been observed in tumor stroma macrophages and fibroblasts, there is sparse data concerning its influence on patient overall survival. Therefore, the main aim of our study was to evaluate the impact of RCAS1 immunoreactivity in both cancer and tumor stroma cells on EOC patient survival. Secondly, our goal was to observe whether RCAS1 immunoreactivity influenced select features of the
tumor stroma, such as fibroblast, macrophage, and lymphocyte infiltration or tumor stroma cellularity.

**Material and methods**

**Characteristics of patients.** The study group consisted of 67 samples of primary EOCs collected from women treated in the Clinical Department of Gynecological Oncology of the Lukaszczyk Oncological Center, Bydgoszcz, Poland, from 2005 through 2010. The median patient age was 58 years (range 38–86). The median age of pre- and postmenopausal women was 49 years (range 39–56) and 59 yrs (48–84), respectively. The samples were obtained during primary cytoreductive surgery, and all patients achieved optimal cytoreduction (residual tumors each measuring 1 cm or less in maximum diameter). The study group included 52 high-grade serous adenocarcinomas, 4 clear cell adenocarcinomas, and 11 endometrioid adenocarcinomas. Twelve tumors were graded as G2, and 55 as G3 cancers. EOCs were classified according to the then-current International Federation of Gynecology and Obstetrics (FIGO) system. Patients were subdivided according to the FIGO stage of the disease as follows: 16 stage II patients, and 51 stage III patients. The median follow-up for our patients was 35 months (range 0–160 months). This study received the Jagiellonian University Ethical Committee approval (KBET/89/B/2005 and DK/KB/CM/0031/447/2010) and all patients gave written informed consent.

**Immunohistochemistry.** Immunohistochemical (IHC) stainings were performed using anti-RCAS1 (human) mAb (dilution 1:1000) purchased from Medical & Biological Laboratories Co., Ltd., Nagoya, Japan, Code No.: D060-3. All IHC studies were performed on 4 μm-thick sections taken from cancerous tumors fixed in 4% buffered-formalin and embedded in paraffin blocks. The specimens for IHC staining were selected according to routine histopathological protocols. Thus, among multiple tumor sections evaluated in hematoxylin and eosin (H&E) stain we selected the most representative specimen with the highest tumor volume and without necrosis. Paraffin sections were placed on Knittel Glass adhesive slides and incubated for 2 h in a chamber thermostat at 60°C. Prior to the automatic performance of the study, tissue sections were subjected to the dewaxing procedure followed by the thermal epitope detection (HIER) in a PT Link device using EnVision™ FLEX Target Retrieval Solution, High pH (50x) (K8002) (Dako, Carpinteria, CA, USA). Finally, the preparations
were dehydrated in a series of alcohols and enclosed in a medium (Consul Mount, Thermo Fisher Scientific Inc. Waltham, MA, USA). This method was performed at room temperature (RT). In each instance, a control preparation was added to a series of patient samples. According to the recommendation by the antibody manufacturer breast cancer sections were used as a positive control. The sections were viewed in Nikon Eclipse 80i microscope (Nikon Instruments Europe BV, Badhoevedorp, The Netherlands).

**Evaluation of RCAS1 immunoreactivity.** The RCAS1 immunoreactivity (RCAS1-Ir) in cancer cell cytoplasm and cancer cell membranes was evaluated separately. The evaluation of RCAS1-Ir was based on the Immunoreactive Score (IRS) and included the simultaneous assessment of the number of RCAS1-positive cells and the intensity of the immunoreactivity. Staining intensity was evaluated as negative (0) or positive with a grade of 1+ (pale brown), 2+ (brown), or 3+ (dark brown). The percentage of stained cells was evaluated using the subjective method of the succeeding approximations as previously described [21]. The IRS with the simultaneous assessment of staining intensity and the percentage of positive cells was conducted as follows: If the intensity of RCAS1-Ir in the cancer cell cytoplasm was assessed as 3+ in 70% of cells, while 30% had RCAS1-Ir evaluated as 2+, we calculated the immunoreactivity score (IRS) according to the formula: \[[(3 \times 70) + (2 \times 30)]/100 = 2.7,\]
where one hundred refers to 100 analyzed cells. The combined result of the staining intensity and the percentage of positive cells equaled 2.7 (range 0 to 3.0). The cut-offs to separate “low” and “high” RCAS1-Ir, were determined following Receiver Operating Characteristic (ROC) curve analysis with the endpoint (death) as a classification variable. Therefore, the cut-offs for cancer cell cytoplasm and membranous RCAS1-Ir were 1.125 and 0.5, respectively.

The evaluation of stromal cellularity was done by counting the number of tumor stroma cells in the microscopic High Power Field (HPF). The cellularity was assessed as low (1+), moderate (2+), or high (3+). Similarly, using HPF, we have evaluated the number of fibroblasts, macrophages, and the degree of lymphocyte infiltration. The tumor stroma cells were differentiated solely based on cell morphology. The parameters mentioned above were quantified as follows: absent (lack of examined cells), low number (1+), moderate number (2+), or high number (3+). The RCAS1-Ir in macrophages and fibroblasts was very homogenous; thus we used only staining intensity for the evaluation. Staining intensity was evaluated as negative (0) and positive staining was graded as 1+ (pale brown), 2+ (brown), or 3+ (dark brown). The number of cells (macrophages or fibroblasts) was evaluated separately.
from the RCAS1-Ir in these cells. Representative images of RCAS1 staining are presented in the Figure 1.

**Statistical analysis.** The nonparametric Mann-Whitney test was used to compare RCAS1-Ir within the subgroups studied. Correlations were calculated using the nonparametric Spearman’s rho test. RCAS1-Ir in macrophages and fibroblasts relative to the patient’s menopausal status was studied using the Fisher-exact test. Survival analyses were conducted using the Kaplan-Meier survival curves and the differences in patient survival were compared using log-rank test. Multivariate survival analysis was conducted using Cox proportional-hazards regression with the stepwise entering method. Statistical analysis was conducted using MedCalc 11.4.2.0, MedCalc Software, Seoul, Republic of Korea, and GraphPad InStat 3.06, GraphPad Software Inc., San Diego, CA, USA.

**Results**

**Immunoreactivity of RCAS1 in cancer cells and tumor stroma**

We found significantly higher RCAS1-Ir in the cytoplasm of the EOC cells of premenopausal patients compared to postmenopausal patients (P = 0.01); however, there were no differences between pre- and postmenopausal patients with respect to membranous RCAS1-Ir in cancer cells (P = 0.88) (Table 1). There were no differences in cytoplasmic RCAS1-Ir in tumor stroma macrophages and fibroblasts corresponding to menopausal status. In the case of macrophages, among the premenopausal women, 8 cases showed absent or low immunoreactivity while 9 showed moderate or high RCAS1-Ir; the results for postmenopausal women were 29 and 24, respectively (P = 0.78). In relation to the cytoplasmic RCAS1-Ir in fibroblasts, the results were as follows: 15 (absent or low) and 2 (moderate or high) for premenopausal, and 41 (absent or low) and 12 (moderate or high) for postmenopausal, P = 0.49.

The detailed results of RCAS1-Ir according to the cell types studied and the results of tumor stroma analyses are summarized in Table 1.
**Immunoreactivity of RCAS1 and patients survival**

Patients with high RCAS1-Ir in the cytoplasm of cancer cells had significantly worse median OS compared to patients with low RCAS1-Ir in the cytoplasm of the tumors (OS 31 months, range 0–160 vs. 73 months, range 1–160, P = 0.04). By contrast, we found no differences in patient survival with respect to high and low membranous RCAS1-Ir in cancer cells within the analyzed tumors. We identified improved survival in patients with a low quantity of fibroblasts within tumor stroma compared to patients with a moderate or high quantity of fibroblasts (median OS 91 months, range 6–160 vs. 32 months, range 0–160; P = 0.03).

Patient survival was unaffected by RCAS1 cytoplasmic immunoreactivity in macrophages, RCAS1 cytoplasmic immunoreactivity in fibroblasts, stromal macrophage quantity, stromal cellularity, or the degree of lymphocytic infiltration. The survival curves are presented in Figure 2. In the multivariate survival analysis including cytoplasmic RCAS1-Ir, the stage of the disease, tumor grade and histopathological type of the tumor, only cytoplasmic RCAS1-Ir remained independent predictor of patients’ overall survival (P = 0.026).

**Immunoreactivity of RCAS1 and tumor stroma characteristics**

Cytoplasmic RCAS1-Ir in cancer cells did not correlate with membranous RCAS1-Ir in cancer cells (R = 0.09, P = 0.44). RCAS1-Ir in the cytoplasm of fibroblasts was positively correlated with fibroblast quantity (R = 0.56, P < 0.0001). Similarly, RCAS1-Ir in macrophages was positively correlated with macrophage quantity (R = 0.75, P < 0.0001).

Stroma cellularity was positively correlated with fibroblast quantity (R = 0.27, P = 0.02). On the other hand, there was no correlation between stroma cellularity and macrophage quantity (R = 0.22, P = 0.06) and the degree of lymphocyte infiltration (R = −0.01, P = 0.93).

We found significantly higher RCAS1-Ir in the cytoplasm of cancer cells in EOC patients with a large number of macrophages and fibroblasts within the tumor stroma (Fig. 3).

Cytoplasmic RCAS1-Ir in cancer cells did not differ depending on the lymphocyte infiltration rate and stroma cellularity. Similarly, the RCAS1-Ir in cancer cell cytoplasm was not associated with RCAS1 cytoplasmic immunoreactivity in tumor stroma macrophages and fibroblasts (Fig. 3).

Median membranous RCAS1-Ir in cancer cells was significantly higher in tumors with high and moderate RCAS1-Ir in stromal macrophages compared to tumors with absent or low membranous RCAS1-Ir in cancer cells (Fig. 3). A similar observation was noted in the case of RCAS1-Ir in tumor stroma fibroblasts. Membranous RCAS1-Ir in tumor cells was not associated with the quantity of fibroblasts and macrophages within the tumor.
microenvironment. Moreover, membranous RCAS1-Ir in cancer cells did not differ according to lymphocyte infiltration and tumor stroma cellularity (Fig. 3).

Discussion
In this study, we observed the relationships between the patterns of RCAS1 immunoreactivities and the long-term outcomes of EOC patients. We found that high RCAS1-Ir in cancer cell cytoplasm resulted in more than two-times shortened overall survival of EOC patients. On the other hand, membranous RCAS1-Ir in cancer cells did not influence patient survival. Several papers have revealed cytoplasmic and membranous RCAS1-Ir in cancer cells [22–24]. However, to the best of our knowledge, our observation is the first to separate cytoplasmic and membranous staining showing the different impact of these staining patterns on patient survival. This data suggests that RCAS1 located in the cytoplasm of cancer cells plays a different role than the protein within the membrane or that it may represent a different subpopulation of cancer cells.

Tumor RCAS1 expression has already been studied as a prognostic factor in various neoplasms, including gynecological malignancies [15, 25, 26]. Three separate studies have investigated RCAS1-Ir in patients with EOCs, linking it with the prognosis for ovarian cancer patients [16, 23, 24]. The first report was done by Akahira et al. [23]. The authors showed that the RCAS1-Ir is higher in more advanced stages of the disease. However, no significant relationship was detected between RCAS1-Ir and patient OS. Although the authors detected cytoplasmic RCAS1-Ir, they did not distinguish it from membranous immunoreactivity. Additionally, Akahira et al. used a different method of assessment than we did; they merely divided the tumors into RCAS1-positive and RCAS1-negative groups according to subjective assessment [23]. We used a broader assessment involving both the evaluation of the percentage of RCAS1-positive cells and the immunoreactivity intensity. Similarly, Ali-Fehmi et al. investigated the immunoreactivity of RCAS1 and other antigens in EOC [24]. The authors did not observe any influence of RCAS1 immunoreactivity on patient survival. Like Akahira et al. [23], the authors detected cytoplasmic RCAS1-Ir, but did not distinguish it from the RCAS1-Ir on the cell surface. Furthermore, Ali-Fehmi et al. also used a different method of assessment than we did; they regarded tumors as RCAS1-positive when immunoreactivity was found in more than 5% of the tumor cells [24]. In their study, Sonoda et al. found no significant relationship between RCAS1-Ir and overall survival; however, the difference was very close to the level of significance (P = 0.06) [16]. Similarly, the method of assessment
was different from the one we used; the authors classified tumors with high RCAS1-Ir when more than 25% of cells were RCAS1 positive [16]. Consequently, the discrepancy between our study and the studies mentioned above could be explained by the different methods of RCAS1-Ir assessment. We consider our method of assessment to be more accurate because it includes both the number of RCAS1-positive cells and the intensity of immunostaining. Additionally, we have distinguished cytoplasmic from membranous immunoreactivity, even though only cytoplasmic immunoreactivity had an impact on patient survival.

RCAS1 expression was also observed on noncancerous cells infiltrating the tumor microenvironment, such as macrophages and fibroblasts; however, the clinical significance of this observation is still unknown [12, 27, 28]. Due to its selective immunosuppressive activity, RCAS1 expression on tumor stroma cells could potentially be responsible for creating tumor-pervasive stroma which could in turn result in a shortened patient survival period. However, this association has not yet been proven. Jozwicki et al. have also showed that higher RCAS1-Ir in tumor stroma macrophages and fibroblasts was present in ovarian neoplasms with lymph node metastases [29]. Galazka et al. demonstrated that RCAS1 expression in macrophages and fibroblasts within cervical cancer stroma did not influence the FIGO stage of the disease and lymph node metastases; it also was not related to the grade of the tumor [12]. In our study, we did not find that RCAS1-Ir in macrophages and fibroblasts had an impact on patient survival. However, because of the positive correlation between cancer cell membranous RCAS1-Ir and RCAS1 presence in macrophages and fibroblasts, an interaction based on RCAS1 activity between these cells could exist. Further studies in this field are therefore needed.

We found significantly higher RCAS1 immunoreactivity in the cytoplasm of EOC cells of the patients who were premenopausal compared to those who were postmenopausal. Although the exact mechanism of this association is not fully understood, we speculate that RCAS1 expression may be influenced by hormonal status. RCAS1 is identical with the estrogen-responsive protein EBAG9 (estrogen receptor-binding fragment-associated gene 9), which is an estrogen-responsive gene, and the regulation of transcription is mediated by estrogen receptors [30]. Moreover, in their study, Akahira et al. found a highly significant positive correlation between RCAS1-Ir and estrogen receptor alpha expression [23]. Thus, we speculate that RCAS1 expression is related to higher estrogen levels.

Previous data has suggested that RCAS1 can participate in tumor stroma rebuilding through interaction with noncancerous stromal cells (CAFs and TAMs), induction of apoptosis of tumor-infiltrating lymphocytes, and facilitating angiogenesis [12, 13, 26, 31]. We focused on
stroma infiltration by macrophages, fibroblasts, and lymphocytes and evaluated tumor stroma cellularity as a whole. We found that RCAS1-Ir in the cytoplasm of cancer cells was positively correlated with the degree of tumor stroma infiltration by both fibroblasts and macrophages, but did not correlate with the RCAS1-Ir in these cells. On the other hand, RCAS1 membranous immunoreactivity in cancer cells positively correlated with RCAS1-Ir in fibroblasts and macrophages, but not with their quantity. Our results suggest that RCAS1 may participate in tumor stroma remodeling through the modification of tumor stroma infiltration by immune cells and fibroblasts. Additionally, this data supports the possibility of a different biological role for cytoplasmic as opposed to membranous RCAS1 localization.

The main weakness of this study was limited study group. However, we included only type-II tumors according to Shih and Kurman model [32]. Furthermore, all of our patients underwent optimal cytoreduction defined as the largest residual tumor nodule measuring 1 cm or less. Thus in respect of patient prognosis, our study group was highly homogeneous. The homogenous character of our study group is supported by the results of multivariate survival analysis, where known risk factors were not linked with patient prognosis.

In conclusion, our results suggest that the presence of RCAS1 in cytoplasm of epithelial ovarian cancer cells seems to have a different biological function from the RCAS1 present on the membrane, as only cytoplasmic RCAS1-Ir correlated with patient overall survival. Additionally, the exact role of RCAS1-positive fibroblasts and macrophages within EOC stroma needs further elucidation, even though the presence of these cells is not associated with patient survival. Furthermore, we suggest that RCAS1 may participate in the rebuilding of the tumor microenvironment by the influence on tumor stroma infiltration by macrophages and fibroblasts.

Acknowledgments
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References


Table 1. RCAS1 immunoreactivity (RCAS1-Ir) and tumor stroma characteristics in ovarian epithelial cancer

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<th>Immunological score (IRS) (Median and range)</th>
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<tr>
<td><strong>Cytoplasmic RCAS1-Ir in cancer cells of premenopausal patients</strong></td>
<td>1.80 (1.025–2.90)</td>
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<tr>
<td><strong>Cytoplasmic RCAS1-Ir in cancer cells of postmenopausal patients</strong></td>
<td>1.60 (0.35–2.70)</td>
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<tr>
<td><strong>Membranous RCAS1-Ir in cancer cells of premenopausal patients</strong></td>
<td>0.00 (0.00–2.00)</td>
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<tr>
<td><strong>Membranous RCAS1-Ir in cancer cells of postmenopausal patients</strong></td>
<td>0.06 (0.00–2.1)</td>
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<tr>
<td><strong>RCAS1-Ir in stromal macrophages</strong></td>
<td>Number (%)</td>
</tr>
<tr>
<td>Absent</td>
<td>10 (14%)</td>
</tr>
<tr>
<td>Low</td>
<td>27 (39%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>22 (31%)</td>
</tr>
<tr>
<td>High</td>
<td>11 (16%)</td>
</tr>
<tr>
<td><strong>RCAS1-Ir in stromal fibroblasts</strong></td>
<td>Number (%)</td>
</tr>
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<td>Absent</td>
<td>2 (3%)</td>
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<tr>
<td>Low</td>
<td>54 (77%)</td>
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<tr>
<td>Moderate</td>
<td>11 (16%)</td>
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<tr>
<td>High</td>
<td>3 (4%)</td>
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<tr>
<td><strong>Stromal fibroblast quantity in HPF</strong></td>
<td>Number (%)</td>
</tr>
<tr>
<td>Absent</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Low</td>
<td>12 (18%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>31 (47%)</td>
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<tr>
<td>High</td>
<td>23 (35%)</td>
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<tr>
<td><strong>Stromal macrophage quantity in HPF</strong></td>
<td>Number (%)</td>
</tr>
<tr>
<td>Absent</td>
<td>10 (14%)</td>
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<tr>
<td>Low</td>
<td>21 (30%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>26 (37%)</td>
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<tr>
<td>High</td>
<td>13 (19%)</td>
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<tr>
<td>Lymphocytic infiltration</td>
<td>Number (%)</td>
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<td>--------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Absent</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Low</td>
<td>20 (29%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>13 (19%)</td>
</tr>
<tr>
<td>High</td>
<td>36 (52%)</td>
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<table>
<thead>
<tr>
<th>Stroma cellularity</th>
<th>Number (%)</th>
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<tbody>
<tr>
<td>Low</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>46 (66%)</td>
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<tr>
<td>High</td>
<td>23 (33%)</td>
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</table>

Cytoplasmic RCAS1-Ir and membranous RCAS1-Ir are expressed as Immunoreactivity Score (IRS) that was based on the simultaneous assessment of the number of RCAS1-positive cells and the intensity of the immunoreactivity as described in Methods. Stromal fibroblasts and macrophages quantity refer to the number of fibroblasts and macrophages, respectively, identified in tumor stroma under microscopic High-Power-Fields (HPFs) as described in Methods.

**Figure 1.** Representative microphotographs of RCAS1 immunoreactivity evaluated as Immunoreactive Score (RCAS1-IRS) in cancer cells and tumor stroma cells in ovarian serous adenocarcinoma.  
**A.** Homogeneous cytoplasmic RCAS1 immunoreactivity (RCAS1-Ir) in cancer cells assessed as 1+; and no RCAS1-Ir in cancer cell membrane (0+).  
**B.** Moderate (2+) and homogeneous RCAS1-Ir in cancer cells; no RCAS1-Ir in cancer cell membrane (0+). Tumor stroma contained large number (3+) of cancer-associated fibroblasts (CAFs) and a low number (1+) of tumor-associated macrophages (TAMs). Low (1+) RCAS1-Ir in TAMs.  
**C.** Heterogeneous RCAS1-Ir in both cancer cell cytoplasm and cell membrane. RCAS1-Ir in cancer cell cytoplasm was assessed as high (3+) in 10% of the cancer cells, moderate (2+) in 80% of the cancer cells, and low (1+) in 10% of the cells. Thus, RCAS1-IRS in this case was equal to 2 since [(3 × 10) + (2 × 80) + (1 × 10)]/100 = 2. RCAS1-Ir in cancer cell membrane was assessed as high (3+) in 25% of the cancer cells, moderate (2+) in 5% of the cells, low (1+) in 60% of the cells and no reactivity (0) was found in 10% of the cells. Thus, RCAS1-IRS in cancer cell membrane was equal to 1.45 since (3 × 25) + (2 × 5%) + (1 × 60) + (0 × 10)]/100 = 1.45.  
**D.** The RCAS1-IRS in cancer cell cytoplasm and cell membrane was 1.8 and 1.45, respectively.  
**E.** The quantities of both TAMs
and CAFs in the tumor stroma were assessed as moderate (2+). Similarly, RCAS1-Ir in TAMs and CAFs was assessed as high (3+). F. Tumor stroma presented with a low quantity (1+) of TAMs and a moderate (2+) quantity of CAFs. RCAS1-Ir in TAMs cytoplasm was assessed as low (1+), no RCAS1-Ir (0) in CAFs. G. Tumor stroma with a moderate (2+) quantity of TAMs and a low (1+) quantity of CAFs. RCAS1-Ir in TAMs cytoplasm was assessed as high (2+), RCAS1-Ir in CAFs cytoplasm was assessed as low (1+). H. Tumor stroma with a high (3+) quantity of TAMs and a moderate quantity of CAFs (2+). RCAS1-Ir in the macrophages was assessed as high (3+), and RCAS1-Ir in CAFs as low (1+). Magnifications: A, B, E, F ×200; C, D, G, H ×400.

Figure 2. Survival analyses according to RCAS1 immunoreactivity (RACS1-Ir) expressed as Immunoreactive Score (RCAS1-IRS) and analyzed tumor stroma features in ovarian cancer. A. Group 1: low RCAS1-Ir (n = 12, median overall survival (mOS) 73 months, range 1–160) vs. Group 2: High RCAS1-Ir (n = 55, mOS 31 mo, range 0–160), P = 0.04. B. Group 1: Low RCAS1-Ir (n = 22, mOS 38 mo, range 0–160) vs. Group 2: High RCAS1-Ir (n = 48, mOS 32 mo, range 0–160), P = 0.85. C. Group 1: absent or low RCAS1-Ir (n = 31, mOS 31 mo, range 0–160) vs. Group 2: High and moderate RCAS1-Ir (n = 36, mOS 38 mo, range 0–156), P = 0.44. D. Group 1: absent or low RCAS1-Ir (n = 35, mOS 34 mo, range 0–160) vs. Group 2: High and moderate RCAS1-Ir (n = 32, mOS 38 mo, range 0–160), P = 0.73. E. Group 1: low RCAS1-Ir (n = 12, mOS 91 mo, range 6–160) vs. Group 2: moderate and high RCAS1-Ir (n = 55, mOS 32 mo, range 0–160), P = 0.03. F. Group 1: absent or low RCAS1-Ir (n = 53, mOS 32 mo, range 0–160) vs. Group 2: moderate and high RCAS1-Ir (n = 14, mOS 38 mo, range 0–160), P = 0.32. G. Group 1: absent or low RCAS1-Ir (n = 20, mOS 31 mo, range 0–160) vs. Group 2: moderate and high (n = 47, mOS 40 mo, range 0–160), P = 0.13. H. Group 1: low and moderate RCAS1-Ir (n = 44, mOS 32 mo, range 0–160) vs. Group 2: high RCAS1-Ir (n = 23, mOS 36 mo, range 5–160), P = 0.28). n, refers to the number of patients.
**Figure 3.** The relationships between Immunoreactivity Score of RCAS1 (RCAS1-IRS) in ovarian cancer cells and the analyzed tumor stroma features. 

A. For the patients with absent and low immunoreactivity the median RCAS1-IRS was 1.5 (range 0.35–2.6) and for moderate and high immunoreactivity IRS was 1.8 (range 0.5–2.9).  

B. For the patients with absent and low immunoreactivity the median RCAS1-IRS was 0.00 (range 0.00–2.00) and for moderate and high immunoreactivity IRS was 0.30 (0.00–2.10).  

C. For the patients with absent and low immunoreactivity the median RCAS1-IRS was 1.6 (range 0.35–2.9) and for moderate and high immunoreactivity IRS was 1.8 (0.5–2.7).  

D. For the patients with absent and low immunoreactivity the median RCAS1-IRS was 0.00 (range 0.00–1.9) and for moderate and high IRS was 0.6 (0.0–2.1).  

E. For the patients with absent and low quantity of macrophages within tumor stroma the median RCAS1-IRS was 1.5 (range 0.00–2.60) and for moderate and high IRS was 1.8 (0.50–2.90).  

F. For the patients with absent and low quantity of macrophages within tumor stroma the median RCAS1-IRS was 0.00 (range 0.00–2.00) and for moderate and high IRS was 0.25 (0.00–2.10).  

G. For the patients with low quantity of fibroblasts within tumor stroma the median RCAS1-IRS was 1.5 (range 0.35–2.70) and for moderate and high IRS was 1.95 (0.90–2.90).  

H. For the patients with low quantity of fibroblasts within tumor stroma the median RCAS1-IRS was 0.00 (range 0.00–2.10) and for moderate and high IRS was 0.20 (0.00–2.10).  

I. For the patients with absent and low tumor stroma lymphocytic infiltration the median RCAS1-IRS was 1.65 (range 0.50–2.70) and for moderate and high IRS was 1.725 (0.35–2.90).  

J. For the patients with absent and low tumor stroma lymphocytic infiltration the median RCAS1-IRS was 0.00 (range 0.00–2.10) and for moderate and high IRS was 0.23 (0.00–2.10).  

K. For the patients with low and moderate tumor stroma cellularity the median RCAS1-IRS was 1.65 (range 0.50–2.70) and for moderate and high IRS was 1.70 (0.35–2.90).  

L. For the patients with low and moderate tumor stroma cellularity the median RCAS1-IRS was 0.00 (range 0.00–2.10) and for moderate and high IRS was 0.3 (0.00–2.10).