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## The differences in RCAS1 and DFF45 endometrial expression between late proliferative, early secretory, and mid-secretory cycle phases

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**Abstract:** RCAS1 expression is related to the regulation of activated immune cells and to connective tissue remodeling within the endometrium. DFF45 seems to play an important role in the apoptotic process, most likely by acting through the regulation of DNA fragmentation. Its expression changes within the endometrium seem to be related to the resistance of endometrial cells to apoptosis. The aim of the present study was to evaluate RCAS1 and DFF45 endometrial expressions during ovulation and the implantation period. RCAS1 and DFF45 expression was assessed by the Western-blot method in endometrial tissue samples obtained from 20 patients. The tissue samples were classified according to the menstrual cycle phases in which they were collected, with a division into three phases: late proliferative, early secretory, and mid-secretory. The lowest level of RCAS1 and the highest level of DFF45 endometrial expression was found during the early secretory cycle phase. Statistically significantly higher RCAS1 and statistically significantly lower DFF45 endometrial expression was identified in the endometrium during the late proliferative as compared to the early secretory cycle phase. Moreover, statistically significantly higher RCAS1 and statistically significantly lower DFF45 expression was found in the endometrium during the mid-secretory as compared to the early secretory cycle phase. The preparation for implantation process in the endometrium is preceded by dynamic changes in endometrial ECM and results from the proper interaction between endometrial and immune cells. The course of this process is conditioned by the immunomodulating activity of endometrial cells and their resistance to immune-mediated apoptosis. These dynamic changes are closely related to RCAS1 and DFF45 expression alterations.

**Key words:** DFF45 - RCAS1 - Menstrual cycle phases - Endometrium

### Introduction

The endometrium represents a unique kind of tissue with immunomodulating activity that enables the accumulation of active cytotoxic cells during the implantation window. In order to evaluate more close-

ly the dynamic changes that take place during the menstrual cycle phases, the menstrual cycle has been further divided into six sub-phases: early-, mid-, and late-proliferative, and early-, mid-, and late-secretory [1,2]. The cyclic alterations of the immunomodulating factor expressions such as interleukins (IL-1, IL-11, IL-13, IL-15, IL-18) leukemia inhibitory factor (LIF), mRNA transcripts encoding regulated upon activation normal T-cell-expressed and -secreted (RANTES), Fas, Fas-L, tumor necrosis factor-alpha (TNF-alpha), HOXA10 and others have been observed in the

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endometrium [3-18]. Recently, RCAS1 (receptor-binding cancer antigen expressed on SiSo cells) protein has been shown to participate in the regulation of immune cell activity in the endometrium [19-24]. RCAS1 has been observed as a factor responsible for tumor escape from host immunological surveillance [19,20,26]. RCAS1 may induce apoptosis of cytotoxic lymphocytes and mainly leads to strong suppression of lymphocyte growth [19,25,26]. Moreover, it has been suggested that RCAS1 also induces connective tissue remodeling [25,27]. A dispersion of extracellular matrix (ECM) during the mid-secretory phase of the cycle has been found to be crucial for ovum implantation [28]. Besides the endometrial immunomodulating activity, its proper function is preserved by the protection of endometrial cells from immune-mediated apoptosis through the disturbance of apoptotic signal transduction. This endometrial ability has been named resistance to immune-mediated apoptosis and seems to be linked with DNA fragmentation factor (DFF) expression changes [29]. DFF is composed of a 40 kDa protein DFF40, with a nuclease activity, and a 45 kDa protein inhibitory sub-unit DFF45 [30]. DFF45 seems to play an important role in the onset of the apoptotic process, most likely by acting through the regulation of DNA fragmentation [30-33]. The aim of the present study was to evaluate RCAS1 and DFF45 endometrial expressions with respect to the menstrual cycle phase (late proliferative, early secretory, and mid-secretory) as these factors have not been estimated simultaneously in the endometrium during ovulation and the implantation period.

## Materials and methods

**Tissue samples.** Human endometrial tissues were obtained from 20 non-menopausal fertile women, aged 20-47 years. These patients had all undergone hysterectomies due to benign gynecological indication (leiomyomas). No patient included in our study received any hormonal treatment. The surgical procedure was performed in the Gynecology and Obstetrics Department, headed by Professor Antoni Basta, of the Jagiellonian University in Krakow, Poland. Tissue samples were classified according to the menstrual cycle phase in which they were collected, with division into the three phases: late proliferative (9 cases), early secretory (5 cases), and mid-secretory (6 cases).

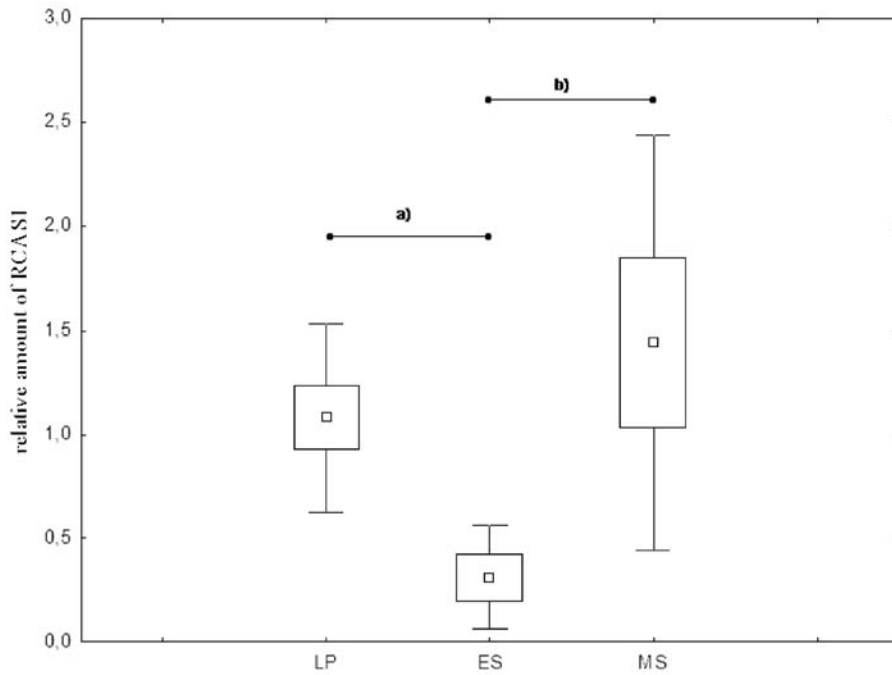
**Preparation of tissue extracts and Western blotting.** The specimens were mixed with a complete proteinase inhibitor cocktail (Roche, Germany) and homogenized in an ice-bath in a glass-glass Potter-Elvehjem homogenizer. The resulting suspensions were mixed with equal volumes of SDS sample lysis buffer (4% SDS, 20% glycerol, 125 mM Tris-HCl pH=6.8) and boiled in a water bath for 5 minutes. The chilled samples were then centrifuged at 16,000 g for 15 min at room temperature. The collected supernatants were used for further analysis. Total protein content in the obtained supernatants was measured using a BCA assay kit, and different sample volumes (usually in the range of 2-10  $\mu$ l), equivalent to 50  $\mu$ g of total protein were then loaded on SDS-PAGE tris-tricine peptide-separating gels. A pre-stained broad range molecular weight proteins standard (Bio Rad, USA) was used in the gel

marker lane. Following electrophoresis, the gels were electro-transferred on Immobilon-P polyvinylidene difluoride (PVDF) 0.45  $\mu$ m membrane (Millipore, USA) in the buffer containing 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) pH=11, supplemented with 10% methanol. The obtained membrane blots were blocked overnight by gentle agitation in 5% bovine albumin in TST buffer (0.01 M Tris-HCl, pH=7.4, 0.9% NaCl), 0.5% Tween-20). All described procedures were performed at room temperature. The albumin solution was discarded and the membranes were then agitated for 2 hrs in TST with the mouse monoclonal anti-RCAS1 IgM-class antibody, 1: 4000 dilution (Medical and Biological Laboratories, Japan) and, in the second step, monoclonal antibody against DFF-45 (Abcam, USA). The membranes were then subjected to 4 cycles of washings in TST, 5 min each, and immersed for agitation in the 1: 2 000 dilution of SIGMA biotinylated anti-mouse IgM  $\mu$ -chain specific antibodies for a 2-hour period. After 4 cycles of washings, the membranes were treated for an additional 2 hours in 1: 2 000 dilution of Extravidin alkaline phosphatase conjugate (SIGMA, USA), and finally washed 2 times in TST and 2 times in TST without Tween-20. Color reaction was developed with the use of Fast Red TR/Naphthol AS-MX tablet set (SIGMA, USA). A sufficient band intensity was obtained, usually after a 5 min period of developing. The obtained immunoblots were then rinsed with distilled water and air-dried. The RCAS1 antigen was identified as a 32 kDa band, DFF45 as a 45 kDa band, and  $\beta$ -actin represented a 42 kDa band [29,30,34].

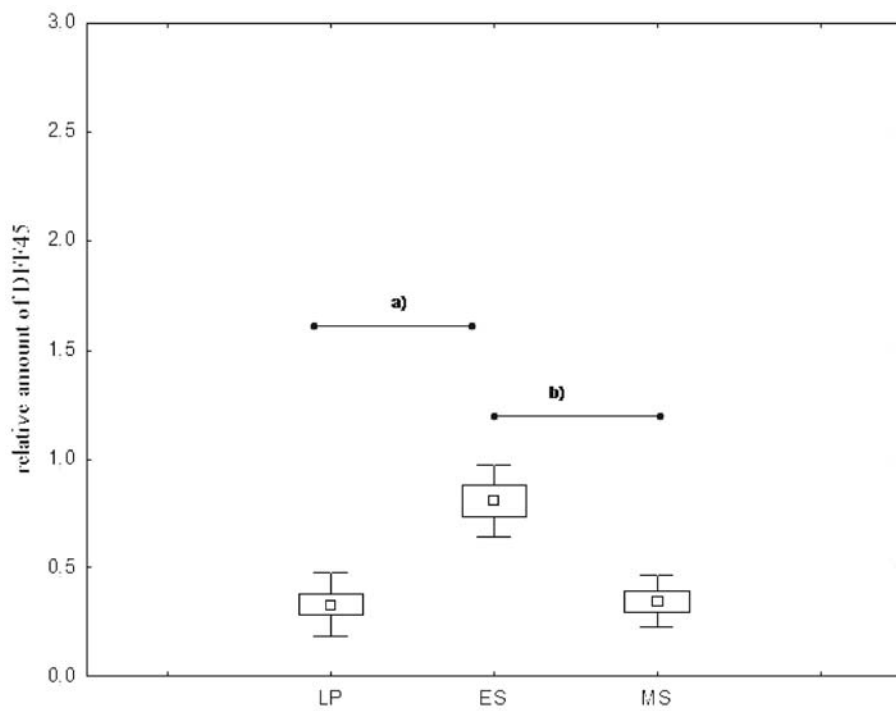
**Computer aided image analysis.** Fluor-S MultiImager (BioRad, USA) was used to scan immunoblotted membranes and a QuantityOne software (BioRad, USA) was used to quantitate band intensities. All calculations were performed on RCAS1 antigen band having a molecular mass of about 32 kDa, DFF-45 of 45 kDa. The intensities of this band were expressed in arbitrary relative units, and one unit (U) was defined as band intensity produced in the reference sample. This reference sample was randomly chosen, but was strictly the same on all blots and was applied in the same amount each time. The typical procedure of RCAS1 and DFF45 quantitation was as follows: scanned immunoblot membrane contained one lane of molecular mass standards, while one lane of sample was used as a reference to calibrate RCAS1 and DFF45 amounts and 12 lanes containing unknown samples. The location of 32 kDa RCAS1 and 45kDa bands in reference and unknown lanes were identified according to the lane containing molecular mass standard. The bands' intensities in reference and unknown lanes were then calculated and expressed in pixel number units. These units were divided by pixel number of reference lane band which resulted in relative-intensity units "U". The resulting intensity of reference lane band was always 1.00 U while the intensities of bands from 12 unknown sample lanes on the same membrane changed according to RCAS1 and DFF45 levels (*e.g.*, if RCAS1 amount in a given sample was 2 times higher than this in the reference sample, the result was 2.00 U. If the RCAS1 amount was 2 times lower, then the result was 0.50 U). As mentioned earlier, because all immunoblots contained the same RCAS1 and DFF45 quantity standard and all lanes were loaded with the same amount of total protein (50  $\mu$ g), the determined values were highly repetitive and independent of the experiment conditions. The results consistently show a relative amount of RCAS1 32 kD antigen and DFF45 of 45 kDa and beta-Actin 42 kDa in 50  $\mu$ g of total sample protein [29,30,34].

**Quantity control.** Using the Western-blot method and monoclonal anti- $\beta$ -Actine mouse antibody, the relative quantity of  $\beta$ -Actine was estimated as a control [34].

**Statistics.** The statistical significance between the groups was determined by the analysis of variance (ANOVA) test. Post-hoc Fisher test was used as applicable.



**Fig. 1.** Presents a comparison of RCAS1 endometrial expression changes between the late proliferative (LP), early secretory (ES), and mid-secretory (MS) cycle phases. Each box represents the 25-75% range of data. The median (□) was presented for each group. Statistically significant differences were observed in RCAS1 relative amount: <sup>a</sup>LP versus ES (p=0.04); <sup>b</sup>ES versus MS (p<0.01).



**Fig. 2.** Presents a comparison of DFF45 endometrial expression changes between the late proliferative (LP), early secretory (ES), and mid-secretory (MS) cycle phases. Each box represents the 25-75% range of data. The median (□) was presented for each group. Statistically significant differences were observed in the DFF45 relative amount: <sup>a</sup>LP versus ES (p<0.001); <sup>b</sup>ES versus MS (p<0.001).

**Ethical issues.** The patient's consent was obtained in all cases. Prior to the study, the approval of the research program by the Jagiellonian University Ethical Committee was also obtained (KBET/89/B/2005).

**Results**

The amount of β-Actin level in all the groups of examined endometrial tissue was found to be identical; this indicates that the loading of protein was equal in all the examined samples and allows the performance of a

comparative study of RCAS1 and DFF45 expression between the examined groups.

The lowest level of RCAS1 and the highest level of DFF45 endometrial expression was found during the early secretory cycle phase. Statistically significantly higher RCAS1 and statistically significantly lower DFF45 endometrial expression was identified in the endometrium during the late proliferative as compared to the early secretory cycle phase. Moreover, statistically significantly higher RCAS1 and statistically sig-

nificantly lower DFF45 expression was found in the endometrium during the mid-secretory as compared to the early secretory cycle phase.

## Discussion

Dynamic changes in DFF45 and RCAS1 expression were observed in the endometrium during the late proliferative, early secretory and mid-secretory cycle phases. To our knowledge, this is the first investigation to focus on RCAS1 and DFF45 expression in the endometrium during the periovulatory period. Until now, a simultaneous analysis of RCAS1 and DFF45 expressions had only been performed on nasal polyps [35].

Changes in apoptotic levels corresponding to the menstrual cycle phases have been observed in the endometrium. Apoptosis is more prominent during the secretory cycle phase than the proliferative, and its level increases insignificantly during menstruation [36]. The disturbance of apoptosis leads to either a disabling of apoptotic signal transduction or to an inability to receive such a signal [37]. Watanabe *et al.* have therefore suggested that endometrial cell apoptosis may not depend on the expression of Bcl-2 level and the Fas expression [37]. The initiation of apoptosis seems result from the activity of various factors. In the final stage of this process, DNA fragmentation related to the level of DNA-nuclease (DFF40) activity can be observed [30-33]. The activation of this enzyme is processed by the fragmentation of DFF45/DFF40 complex by caspase-3 [30]. Since DFF45 is able to interact with this nuclease as a molecular chaperone and ensures its correct folding, it was shown to be necessary for generating functional DFF40 nuclease [31,38]. It has also been demonstrated that DFF45-deficient thymocytes were more resistant to programmed cell death [32]. Moreover, in esophageal and colon carcinoma cells characterized by an impairment of apoptosis, a decrease of DFF45 level has been observed [33]. Its expression changes within the endometrium seem to be related to the resistance of endometrial cells to immune-mediated apoptosis. In our recent study, an increase of DFF45 expression was found in the secretory cycle phase in comparison to the proliferative [29]. As in this study the more detailed division of the cycle into the six sub-phases was not considered, the differences between the periovulatory and implantation period were not able to be shown. The dispersion of ECM begins during the implantation window [28,39-41]. This is the process that is most likely related to the changes in the resistance to apoptosis level and that seems to be crucial for ovum implantation. Nevertheless, the increase in the level of endometrial cell resistance to apoptosis appears earlier, during ovulation, and alters during the early secretory cycle phase. This finding was confirmed in the

present study by the changes in DFF45 level. Moreover, Tabibzadeh [28] has stated that the balance between the grade of TGF-beta (transforming growth factor - beta) activity and LEFT protein expression is essential for endometrial ECM status [42-44]. LEFTY might affect matrix metalloproteinases (MMP) endometrial activity [44], while MMP is responsible for endometrial ECM disintegration [45]. Furthermore, Sonoda has shown the association between RCAS1 expression and MMP-1 expression in the tumor micro-environment of the uterine cervix [27]. RCAS1 seems also to be responsible for tissue remodeling [20,25,27]. The status of endometrial ECM is regulated as well by the infiltrating immune cells [46,47]. Immune cells constitute 7% of all endometrial cell population during the proliferative menstrual cycle phase; during the secretory cycle phase, their percentage increases to 30 [48]. The increase in immune cell infiltration starts during the late proliferative cycle phase and peaks during the mid-secretory cycle phase. Immune cell status depends on the concentration of immunomodulating factors derived from endometrial cells, including RCAS1 [49]. So far the evaluated results in RCAS1 expression level in the endometrium with respect to menstrual cycle phases have been controversial. On the one hand, it has been demonstrated that RCAS1 expression level does not differ with respect to menstrual cycle phases [21,25,50]. On the other hand, it has been observed that RCAS1 expression during the secretory cycle phase was statistically significantly higher than during the proliferative phase [22,23]. These obtained various results in RCAS1 expression could derive from the dynamic changes in RCAS1 expression found between late proliferative, early secretory, and mid-secretory cycle phases.

The preparation for the implantation process in the endometrium is preceded by dynamic changes in endometrial ECM and results from the proper interaction between endometrial and immune cells. The course of this process is conditioned by the immunomodulating activity of endometrial cells and their resistance to immune-mediated apoptosis. These dynamic changes are closely related to those observed in the current study of RCAS1 and DFF45 expression alterations.

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