Expression of LIMS-1 Protein in Selected Cancers of the Genital Organ in Women

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Abstract: *Aim*: The study aimed at immunohistochemical evaluation of LIMS-1 (PINCH-1-cysteine-histidine-rich) protein expression and localization in squamocellular cancer of uterine cervix, grades IB1 (T1b) and IIIB, in endometrial cancer, grades IB (T2b, NO, MO) and IIIB (T1, T2, T3, N1, MO) and in serous ovarian cancer, grades IB (pT1b) and IIIB (pT3b).

Methods: The immunohistochemical studies were conducted in paraffin sections, using rabbit polyclonal antibodies specific for LIMS-1 (Sigma^{R}), diluted 1/200. The reaction was developed using EnVision^{IM} FLEX DAB^{+} Chromogen DAB solution. The expression was evaluated using modified IRS scale according to Remmele. Statistical analysis involved application of Kolmogonov–Smirnov test of normal distribution, Kruskall–Wallis test, analysis of K–means cluster and R-Spearman test.

Results: there was no relationship between the expression of LIMS-1 and the advancement of neoplastic disease according to FIGO classification in squamocellular cancer of uterine cervix: the significance coefficient in the chi-square test amounted to p = 0.086. In cases of endometrial cancer and ovarian cancer, the relationship between these variables showed the level of significance to be p = 0.045 and p = 0.041, respectively. In the cases where the chi-square test demonstrated a relationship between the stage of FIGO classification and the LIMS-1 expression, the R correlation coefficients of Spearman presented values of 0.66 (p=0.003) for endometrial cancer and 0.88 (p=0.005).

Conclusion: Increase in expression level of LIMS-1 protein in studied neoplasms was demonstrated to manifest positive correlation with degree of histological malignancy according to FIGO, or with neoplastic progression.

Keywords: Endometrial cancer, FIGO classification, LIMS-1 (PINCH-1), serous ovarian cancer, squamocellular cancer of uterine cervix.

INTRODUCTION

For a clinician, the ability to differentiate a malignant tumor and determine the traits predisposing it to metastases seems much more important than the histogenesis of the cancer. Histologic grade significantly improves both the prognosis and the planning of an effective therapy. Knowing the histologic grade and knowing how it translates on clinical stage of progression of the disease, it can be faster and more accurately predict the natural course of neoplastic disease. Frequently, the histological examination of neoplasms aiming at establishing a correct diagnosis has to be supplemented by histochemical tests, which examine the presence of biological markers of neoplastic cells, and have a prognostic or predictive significance.

Recently, the list of factors involved in oncogenesis, which can potentially facilitate neoplastic metastases has been extended to include the LIM group of proteins, together with the LIMS-1 (PINCH-1-cysteine-histidine–rich) protein [1,2]. This protein, rich in cystein and histidine junctions, is coded by a gene located in chromosome 2q12.2 and mediates the transmission of signals from the extracellular matrix (ECM) to intracellular effectors [3]. Fukuda *et al.* demonstrated overexpression of LIMS-1 in rectal cancer, squamocellular cancer of the esophagus, endometrial cancer and in a typical hyperplasia of the endometrium [4]. Moreover, the protein was found to be capable of participation in the induction of stromal tumors and their progression. It was also found to take affect the ability of stromal tumors to infiltrate tissues and metastasize [5,6].

The research for novel markers of oncogenesis and attempts to evaluate their usefulness if establishing of prognosis carries particular significance in tumors of

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similar morphological parameters but of a distinct clinical course. Therefore, we undertook our studies aimed at determining the localization and expression of the LIMS-1 protein in cancers of the uterine cervix, uterine body and ovary in women. To date, no such investigations had been performed.

MATERIAL AND METHODS

The study material included samples of cancers of human female genital organ. The International Federation of Gynecology and Obstetrics (FIGO) was used to evaluate the staging of each cancer. This surgical classification allows to assign cancer depending on the depth of infiltration and presence of distant metastases into one of the four stages, where stage I means the smallest cancer invasion and stage IV means deep infiltration with distant metastases. We compared tumors restricted to the lesion site (stage I in FIGO classification) with those manifesting in stage III FIGO classification. Such a comparison was made in an attempt to differentiate the expression level of the studied protein depending on the spread of the neoplastic process, which may be of prognostic significance. The studies included:

1. Squamocellular cancer of uterine cervix, grades IB (T1b) (13 cases) and IIIB (12 cases).

2. Endometrial cancer, grades IB (T2b, N0, M0) (12 cases) and IIIB (T1, T2, T3, N1, M0) (13 cases).

3. Serous ovarian cancer, grades IB (pT1b) (13 cases) and IIIB (pT3b) (12 cases).

The samples were fixed in 7% buffered formalin for 24 hours, routinely embedded in paraffin blocks and cut into 4μm-thick sections. Based on the histopathological examination of the hematoxylin and eosin-stained preparations, the tumors were classified according to FIGO classification.

Immunohistochemical studies were conducted on 4μm-thick paraffin sections, placed on Superfrost Plus silanized slides (Menzel Gläser, Germany), which were cleared in xylene and passed through a row of alcohols of a decreasing concentration to water. Antigens of the formalin-fixed tissues were retrieved in the EnVision™ FLEX Target Retrieval Solution, High pH (50x) DAKO® catalogue No. K8004, by heating the samples in a water bath at 96ºC for 20 minutes. Endogenous peroxidase was blocked in EnVision™ FLEX Peroxidase- Blocking Reagent for 5 minutes. Subsequently, the sections were overlayed with primary antibodies: polyclonal rabbit Anti-RGS5, catalogue No. HPA001821 (SIGMA®), diluted to 1/50, polyclonal rabbit Anti-LIMS1, catalogue No. Sab2701201 (SIGMA®), diluted to 1/200.

Subsequently, the sections were washed in the EnVision™ FLEX Wash Buffer (20x) and overlayed with the visualization system of EnVision™ FLEX /HR SM802. They were then incubated for 30 minutes at room temperature. An immunocytochemical reaction was developed using a solution of 3,3diaminobenzidine tetrahydrochloride (DAB), EnVision™ FLEX DAB+ Chromogen (DAKO). Next, the sections were washed in distilled water. Cell nuclei were counterstained with hematoxylin, and the sections were dehydrated in a range of alcohols. The material was cleared in xylene and closed in Canada balsam.

The expression of the studied antigens was evaluated using a modified semiquantitative IRS scale of Remmele (Immunoreactive Remmele Score - IRS), which takes into account both the intensity of the color reaction and the percentage of positive cells. [7] (Table 1). The method takes into account both the proportion of the stained cells and the intensity of the colour reaction, while the final results reflect the product of both parameters with the manifesting values ranging from 0 to 12 points: no reaction = 0 points (-), weak reaction = 1-2 points (+), moderate reaction = 3-4 points (++), intense reaction = 6-12 points (+++).

Microphotographs of all studied tumors were subjected to a computer-assisted image analysis employing a computer coupled to an Olympus BX53 (Olympus, Japan) optical microscope. This set-up can record images and perform their digital analysis. The measurements were conducted using the cell^A software (Olympus Soft Imaging Solution GmbH, Germany).

Statistical Analysis

The data were subjected to statistical analysis using the Statistica 10.0 software. The Kolmogorov-Smirnov test verifying a normal distribution of variables and a non-parametric alternative of the analysis of variance in the form of the Kruskall-Wallis test were chosen as the most suitable analysis tools.

The analysis of the K-means cluster was performed in order to examine the data and to distinguish their clusters. Using the semiquantitative IRS scale allowed

variable expressions of the LIMS-1 protein to be classified into appropriate FIGO classification stages.

In order to analyze the relationship between LIMS-1 values and the groups earlier classified in line with FIGO classification (stage I and III), the chi-square test was employed. The correlation between the variables was calculated using Spearman's R test.

RESULTS

Expression of studied antigens was evaluated using a modified semiquantitative IRS scale of Remmele (Table **1**).

Table 1. Expression of the studied antigens evaluated using the modified semiquantitative IRS scale according to Remmele [7]

Number of cells with positive reaction	Intensity of color reaction
0 points – absence	0 points – absence
1 points – up to 10%	1 points – low intensity
2 points – 11 to 50 %	2 points – moderate intensity
3 points – 51 to 80%	3 points – intense
4 points – above 80%	4 points – high intensity

Statistical analysis of the LIMS-1 protein activity in cancer of the uterine cervix, uterus and in ovarian cancer was presented in Table **2**.

The analysis of clusters permitted classification of the expression of LIMS-1 into groups of stage I and stage III FIGO classification using the following criteria: values less than or equal to three in the IRS scale were assigned to stage I FIGO classification while those above three were allocated to stage III.

The evaluation of the relationships between the variables demonstrated that there was no relationship between the expression of LIMS-1 and the advancement of neoplastic disease according to FIGO classification in squamocellular cancer of uterine cervix: the significance coefficient in the chi-square test amounted to p = 0.086. In cases of endometrial cancer and ovarian cancer, the relationship between these variables showed the level of significance to be p = 0.045 and p = 0.041, respectively. In the cases where the chi-square test demonstrated a relationship between the stage of FIGO classification and the LIMS-1 expression, the R correlation coefficients of Spearman presented values of 0.66 (p=0.003) (a significant correlation) for endometrial cancer and 0.88 (p=0.005) (a very significant correlation), respectively.

Results of Histochemical Studies

The expression of the LIMS-1 protein was located in the cytoplasm of the tumor cells, particularly in regions bordering the nuclear envelope and in blood vessels. In the immunohistochemical analysis of serous ovarian cancers, an intense (+++) expression of LIMS-1 was detected in over 21% of cases, a moderate one (++) in 53% of cases and a weak expression (+) was present in 26% of cases (Figures **1** and **2**). In cases of

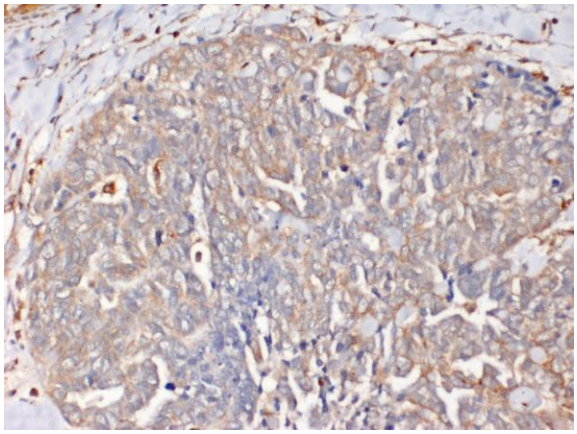

Figure 1: Serous ovarian cancer of stage I according to FIGO classification. Cytoplasmic expression of LIMS-1(+). Magnification 200x.

Table 2. Value of the LIMS-1 protein expression in the studied cancers

Squamocellular cancer of uterine cervix	N	Mean	Median	Minimum	Maximum	Lower quartile (25%)	Upper quartile (75%)	Standard deviation
LIMS-1	10	3.7	3	1	8	2	6	2.31

Endometrial cancer	N	Mean	Median	Minimum	Maximum	Lower quartile	Upper quartile	Standard deviation
LIMS-1	10	2.3	1.5	0	6	1	4	1.89

Serous ovarian cancer	N	Mean	Median	Minimum	Maximum	Lower quartile	Upper quartile	Standard deviation
LIMS-1	10	3.5	4	1	6	2	4	1.78

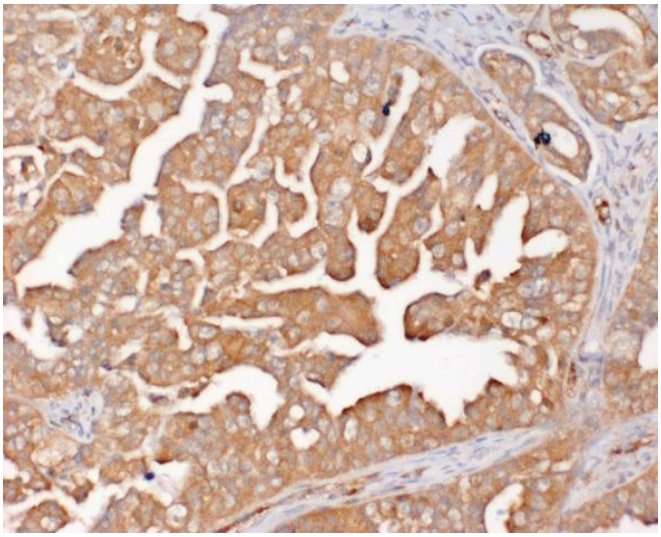

Figure 2: Serous ovarian cancer of stage III according to FIGO classification. Cytoplasmic expression of LIMS-1(+++). Magnification 200x.

endometrial cancer an intense expression of the protein was detected in only 15% of examined tumors, a moderate expression was seen in 30% of the tumors and a weak expression was noted in over 45% of the tumors (Figures **3** and **4**). No LIMS -1 expression was detected in 10% of the endometrial cancers in the uterine body. In the cases of squamocellular cancer of uterine cervix an intense expression of LIMS-1 was detected in almost 30% of cases, a moderate expression was seen in 38% of cases and a weak expression in 32% of cases (Figures **5** and **6**).

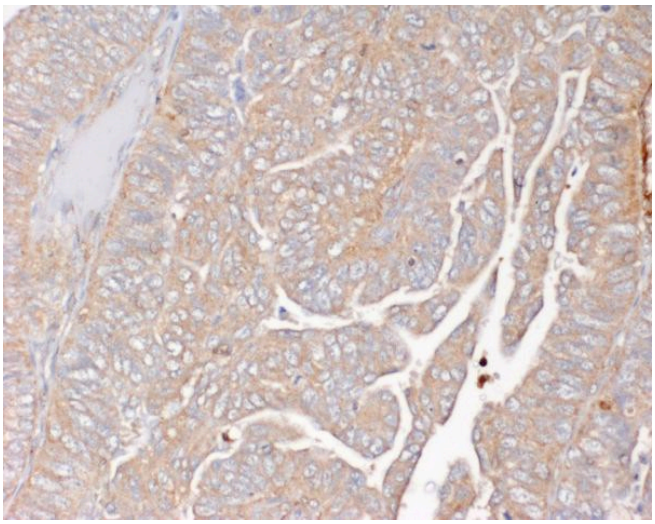

Figure 3: Endometrial cancer of stage I according to FIGO classification. Cytoplasmic expression of LIMS-1(+). Magnification 200x.

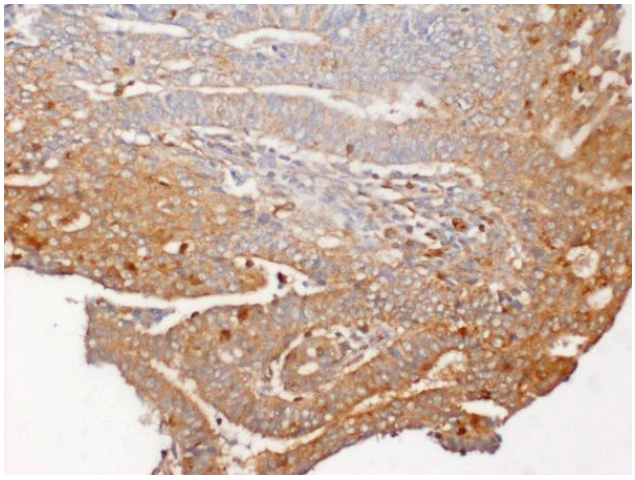

Figure 4: Endometrial cancer of stage III according to FIGO classification. Cytoplasmic expression of LIMS-1(+++). Magnification 200x.

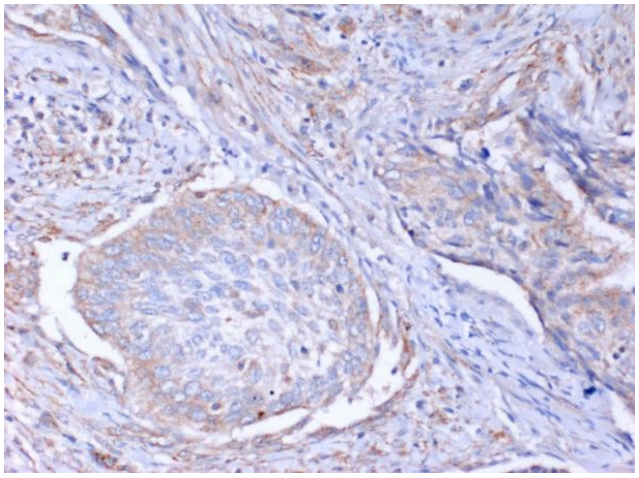

Figure 5: Squamocellular cancer of uterine cervix of stage I according to FIGO classification. Cytoplasmic expression of LIMS-1(+). Magnification 200x.

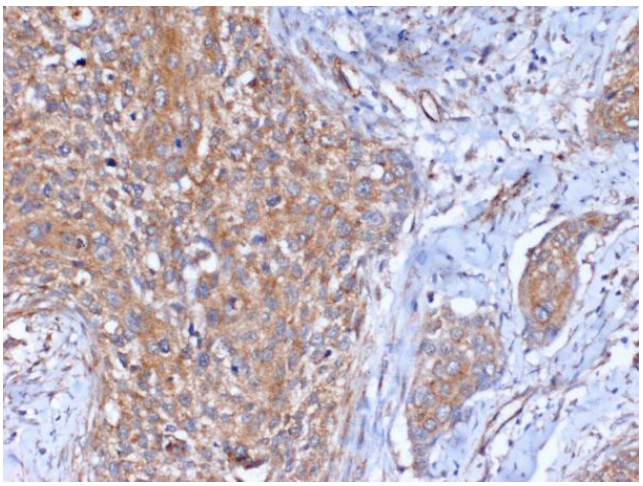

Figure 6: Squamocellular cancer of uterine cervix of stage III according to FIGO classification. Cytoplasmic expression of LIMS-1(+++). Magnification 200x.

DISCUSSION

In neoplastic cells, the LIMS-1 protein undergoes the phenomenon of up–regulation, which is manifested by its increased concentration. This is supposed to result in an intensified proliferation of neoplastically transformed cells. Accordingly, patients with rectal cancer demonstrate a pronounced expression of the LIMS-1 protein and an intensified progression of the cancer independently of the tumor advancement in the TNM scale as compared to patients with weak expression of the marker [8]. Similar observations were made on patients with colorectal cancer, previously subjected to radiotherapy [9]. It seems that such findings will be a useful tool for qualifying patients for pre-operative radiotherapy. Apart from the already mentioned increased expression of LIMS-1 in the genital system (endometrial cancer, an atypical proliferation of the endometrium), increased levels of the protein were also detected in patients with arterial hypertension and women using oral contraceptive agents for a period of 30 days, compared to healthy women [10]. At the same time, the activity of LIMS-1 proved not to be linked to menopause, pregnancy, blood concentrations of sugar/lipids, TNM advancement of tumors, metastases to lymph nodes or the presence of estrogen/progesterone receptors in tumor cells [10]. The relations between the expression of the LIMS-1 protein and the tumor relapse, the process of angiogenesis, presence of inflammatory infiltrate and apoptosis were also examined [11]. Moreover, the protein was found to be capable of inducing lymphangiogenesis as a reaction to cell injury during radiotherapy, which may intensify penetration of neoplastic cells to lymphatic vessels and lead to subsequent metastases [11,12]. Nevertheless, colorectal cancers with high expression of LIMS-1 are accompanied by a negligible inflammatory reaction, which points to poor anti-neoplastic cell-mediated immunity [12]. This has been confirmed by our own investigations, in which no inflammatory infiltrate was observed within the uterine cervix or uterus.

Apoptosis or programmed cell death involves, inter alia, a mechanism controlling the number of tumor cells, while its reduction leads to an increase in the tumor growth [3,13]. The expression of LIMS-1 was associated with reduced apoptosis, whereas the localization of the protein at the periphery of a neoplastic tumor promoted penetration of the tumor cells to the vascular lumen. The protein may induce cell proliferation and simultaneously, intensify cell ischemia and necrosis, which may be recognized as a process of self-limitation in neoplasia [14]. LIMS-1 is thought to be involved in the determination of the intensity of necrosis induced by an excessively rapid tumor growth or radiotherapy. In summary, the currently available literature data indicate that the LIMS-1 protein may provide a valuable prognostic marker in patients with malignant tumors.

In our investigations, we demonstrated the expression of the LIMS-1 protein in the cytoplasm of tumor cells, particularly in the regions neighboring the nuclear envelope. The reaction was also present in blood vessels. Thus, immunohistochemical reactions revealed that the expression of the LIMS-1 protein increased in parallel with neoplastic progression and this pertained in all evaluated tumors. Therefore, the protein may be engaged in a progressive transformation of cells in the uterine cervix, uterus and ovary. Unfortunately, despite the evident expression of LIMS-1 in neoplastic cells, the role of the protein in oncogenesis remains to be fully clarified, pointing to the need for further studies, particularly as regards pre- and post-operative therapy of the tumors.

CONCLUSION

An increased expression level of the LIMS-1 (PINCH-1) protein in squamocellular cancer of uterine cervix, endometrial cancer and ovarian cancer manifests a positive correlation with the stages of FIGO classification of their histological malignancy and with the neoplastic progression.

The immunohistochemical evaluation of the localization and expression level of the LIMS-1 protein in tumors of the female genital system seems to provide a useful marker of their growth and metastasizing potential.

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