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Original Research

Over expressions of neutrophil gelatinase-associated lipocalin and kidney injury molecule-1 in human uterine cervical neoplasms enhance tumor invasion

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Objective: Accumulating evidence has demonstrated upregulation of neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) in multiple malignancies which play a critical role in tumor progression. However, up to now little is known about the role played by NGAL in human uterine cervical carcinomas. In this retrospective study we aimed to evaluate the role of tissue expressions of NGAL and KIM-1 in a spectrum of uterine invasive and noninvasive cervical neoplasms. *Methods*: Immunohistochemical NGAL and KIM-1 expressions were investigated in a total of 107 formalin-fixed, paraffin-embedded uterine cervical tumor specimens and their association with different clinicopathologic parameters was evaluated. *Results*: In this series, cases with 30 low- and 29 high- grade cervical squamous intraepithelial lesion (SIL), 27 squamous cell carcinoma (SCC), 15 adenosquamous carcinoma (ASC) and 6 adenocarcinoma (ACs) were detected. Positive NGAL expression was detected in only inflammatory cells of cases, whereas KIM-1 expression was detected in tumor cells. Statistically it was determined that the positivity rate of strong NGAL and KIM-1 expression was prominently higher in invasive carcinomas when compared with non-invasive squamous cell neoplasms ($P < 0.01$). KIM-1 expressions were not detected in any of the few cases with adenocarcinoma. *Conclusion*: Our findings have showed the presence of a correlation between membranous and cytoplasmic expressions of NGAL and KIM-1 and the invasive characteristics of uterine cervical neoplasms. As a result, expressions of NGAL and KIM-1 may be important in foreseeing the invasion and tumor progression.

Keywords

Adenosquamous carcinoma; Adenocarcinoma; Low- grade squamous intraepithelial lesions; High- grade squamous intraepithelial lesions; NGAL; KIM-1; Squamous cell carcinoma; Uterine cervix

1. Introduction

As a member of the lipocalin superfamily, neutrophil gelatinase-associated lipocalin (NGAL, also called lipocalin 2 or 24p3), was first isolated as a 25 kDA glycoprotein covalently bound to matrix metalloproteinase 9 (MMP9) in neutrophils [1–5]. NGAL is an acute-phase protein, and it is rapidly released from both neutrophils and a variety of cell types as a response to inflammation and tissue injury [5]. NGAL is also expressed in kidney tubular cells in response to various stimuli including ischemia, infections, and toxicity [2–4]. Higher serum and urine levels of NGAL have been detected in renal injuries such as renal ischemia, in cases with several kidney parenchymal diseases, and post-transplantation rejection [1–4]. NGAL is expressed in neutrophils, and as recently discovered in most epithelial cells, and involves in diverse processes of growth, development, and tumorigenesis [6, 7].

Firstly, lchimura revealed in I998 that kidney injury molecule-1 (KIM-1) was a sensitive and specific biomarker in predicting injury of the proximal tubules [8]. KIM-1 is a type 1 membrane protein and consists of a novel six-cysteine immunoglobulin-like domain and a mucin domain. As a member of the immunoglobulin gene superfamily, structurally KIM-1 mostly resembles mucosal addressin cell adhesion molecule-1 (MAdCAM-1). A homology also exists between human KIM-1 and the monkey hepatitıs A virus cell receptor-1 (HAVcr-1) [91]. KIM-1 is expressed at a low level in the normal kidney, however its expression increases dramatically in the post-ischemic kidney [8–10].

Despite frequently performed screening tests, malignancies of uterine cervix still remains to be among the predominant causes of cancer mortality in women worldwide [11]. Its most frequently encountered histological type is squamous cell carcinoma (SCC) detected in 75-80% of the cases with invasive uterine cervical carcinomas [12]. Adenosquamous carcinoma (ASC) is composed of a mixture of malig-

nant squamous and glandular cells. As the second most frequently reported cervical cancer, the incidence of ASC ranges between 3.6% and 25% among all cervical cancers. The prevalence of ASC is higher particularly in young women. ASC metastasizes to pelvic lymph nodes twice as often as SCC or adenocarcinomas [12, 13]. ASCs and pure adenocarcinoma (AC) of the uterine cervix have been most often associated with a poorer prognosis. The presence of a close relationship between the human papilloma virus (HPV) and cervical cancers has been reported in early epidemiological studies concerning cervical neoplasia. Therefore, conduction of screening studies among patients with cervical dysplasia associated with HPV carries utmost importance [11–15].

Hitherto, as relevant markers for assessing the proliferative activity and tumor cell dynamics of cervical neoplasms, many parameters have been suggested. However, among these parameters NGAL and KIM-1 have not been investigated extensively [6+7]. In this study we aimed to explore the diagnostic significance of these two markers in cervical neoplasms.

2. Material and methods

High- and low- grade cervical squamous intraepithelial lesions (SILs) and invasive squamous or adenosquamous carcinomas (SCCs) and adenocarcinomas (ACs) were identified in 107 patients recently diagnosed as cervical neoplasms at the Hospital. The study was approved by the Local Ethics Committee of the Hospital.

For the selection of appropriate paraffin blocks to be examined, and identification of viable tumor areas immunohistochemical (IHC) analysis was performed with hematoxylin and eosin (H&E) stained slides. IHC was performed using the streptavidin- biotin peroxidase staining technique (Invitrogen, Camarillo, 85-9043, USA). Serial 5-μm sections were prepared, and their slides were baked overnight at 60 °C, dewaxed in xylene, and hydrated with distilled water through decreasing concentrations of alcohol. All slides were subjected to heat-induced epitope retrieval (HIER) procedure in the microwave (in 10 mM/L citrate buffer, pH 6.0, for 20 minutes, followed by cooling at room temperature for 20 minutes) and then blocked to determine the presence of endogenous peroxidase and biotin. Affinity-purified monoclonal mouse antibodies against NGAL (Novus Biologicals, Littleton, USA, NDP1- 90331) and KIM-1 (Bioss, Philadelphia, USA, HAVCRI) were used at a dilution of 1 : 300. As positive control for KIM-1, renal tissue damaged by acute tubular necrosis, and for NGAL, splenic tissue were used. The pathologist who was blinded to the clinical features of the patients examined the slides and staining patterns were classified according to the intensity of staining. KIM-1 positivity was defined as cytoplasmic staining intensity comparable to that of renal proximal tubules in control tissues. Focal staining occupying less than 5% of the high-power field (HPF) of view or diffuse weak staining visible under microscope was considered as KIM-1 negativity. In previous studies, authors have indicated the presence of cytoplasmic and membranous expressions of NGAL in most tissues. Contrary to the other studies, in our study expression of NGAL was not detected in tumor cells. To assess the intensity of NGAL expression, we counted the NGAL-positive neutrophils that infiltrated the tumor cells in every high-power field (HPF) view. Statistical analysis was performed using statistical package of SPSS 25.0. *P* values less than 0.05 was statistically significant.

3. Results

In this series, 30 low- (LSIL) and 29 high- grade SIL (HSIL), 27 squamous cell carcinomas (SCC), 15 adenosquamous carcinomas (ASC) and 6 adenocarcinomas (AC) were detected. Mean age of the patients was 47.8 ± 13 years (range, 20-80 years). The cases with invasive carcinomas (52.8 ± 13.7 years; range, 30-80 years) were older than those with intraepithelial neoplasms (44.1 ± 11.2 years; range, 20-66 years). Mean ages of the women with SCC (52.8 ± 14.8), ASC (52.9 ± 12.1) and AC (51.5 ± 9.3) had a similar distribution pattern.

Distribution of NGAL and KIM-1 expressions among patients are shown in Tables 1,2. Inflammatory cells with NGAL expression were detected in indicated number (%) of cases with LSIL (30%: n = 9), HSIL (48.3%: n = 14), SCC (88.9%: n = 24), ASC (93.3%: n = 14) and AC (100%: n = 6) (Fig. 1). KIM-1 expression was also detected in cases with LSIL (10%: n = 3), HSIL (10.3%: n = 3), SCC (48.1%: n = 13), and ASC (60%: n = 9) (Fig. 2). KIM-1 expression was not detected in any of the few cases with adenocarcinoma (Fig. 3). NGAL expression was not detected in tumor cells (Fig. 4). It was statically determined that the positivity rates of NGAL and KIM-1 expressions were significantly higher in invasive carcinomas when compared with intraepithelial neoplasms ($P < 0.01$). Expressions of KIM-1 and NGAL were found to be similar in invasive tumors. Only KIM-1 was not expressed in AC. However, this finding was not statistically significant because of the small number of cases examined.

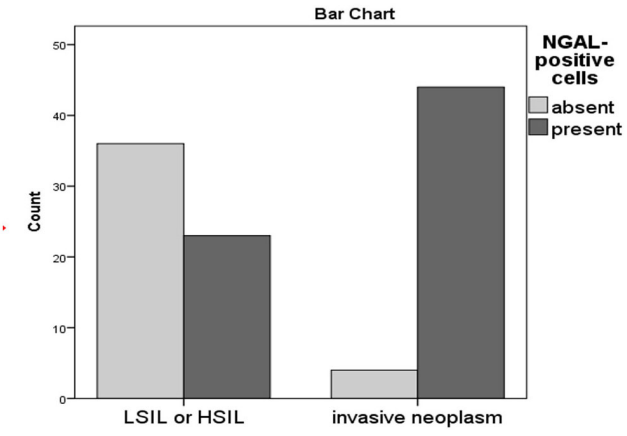

Fig. 1. NGAL expressing inflammatory cells were present in most of the invasive neoplasms ($P < 0.01$).

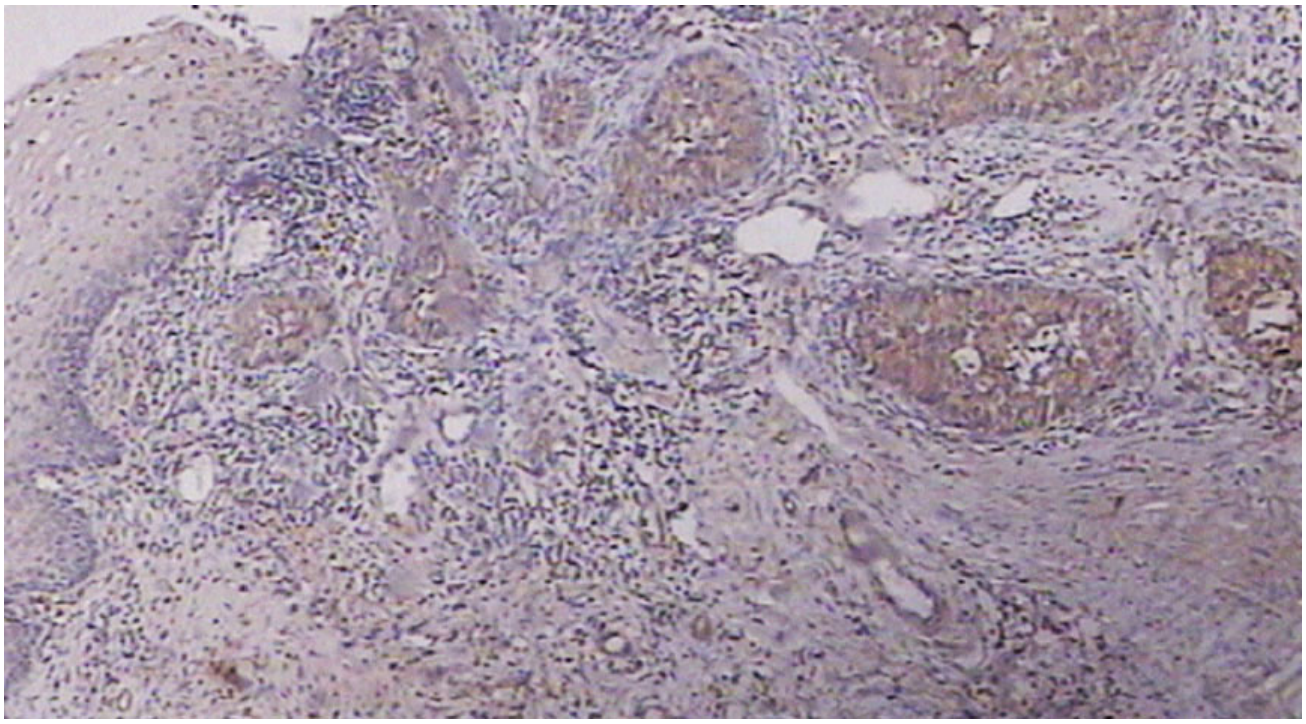

Fig. 2. KIM-1 negative in normal epithelium (at left) and positive in invasive tumor area (at right).

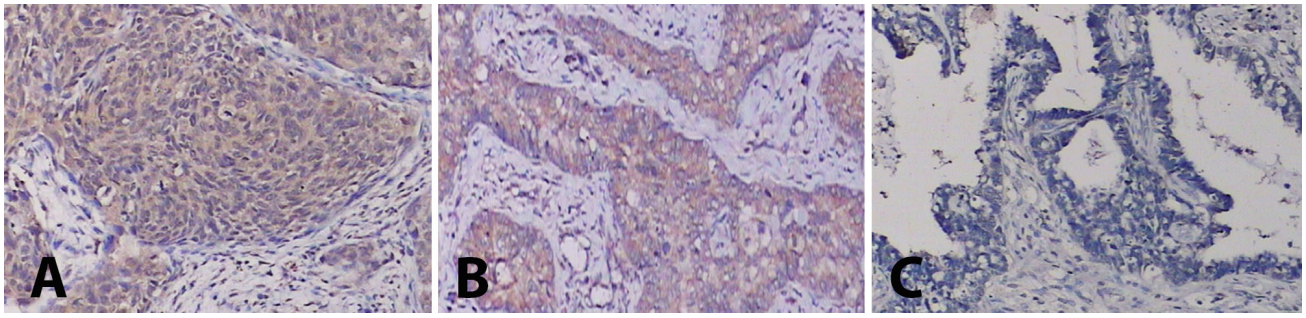

Fig. 3. Immunohistochemical expression of KIM-1: (A) positivity of Squamous cell carcinoma and (B) Adenosquamous carcinoma (C) negativity of Adenocarcinoma.

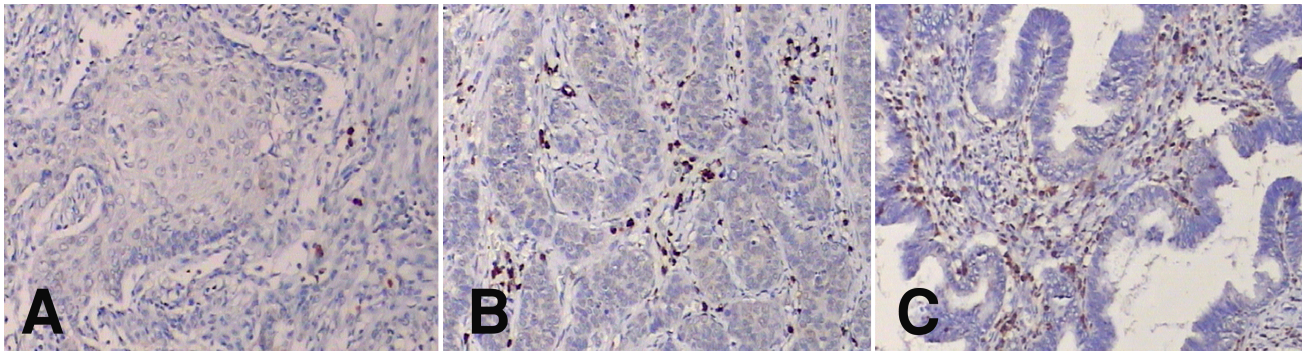

Fig. 4. Immunohistochemical expression of NGAL in inflammatory cells: (A) Squamous cell carcinoma (B) Adenosquamous carcinoma and (C) Adenocarcinoma.

4. Discussion

So far, NGAL has been studied only as an inflammatory marker of renal damage [1–4]. However, recent studies have shown the potential roles of NGAL in carcinogenesis and its likely oncogenic or anti-oncogenic implications [6, 7]. Recent reports have demonstrated critical roles played by NGAL

Table 1. Expressions of NGAL in inflammatory cells in various cervical neoplasms

Disease	Frequency (n)	Percent (%)
LSIL absent	21	70.0
present	9	30.0
total	30	100.0
HSIL absent	15	51.7
present	14	48.3
total	29	100.0
SCC absent	3	11.1
present	24	88.9
total	27	100.0
ASC absent	1	6.7
present	14	93.3
total	15	100.0
AC absent	6	100.0
present	0	0.0
total yes	0	0.0

Abbreviations: L-SIL: Low- grade squamous intraepithelial lesions; H-SIL: High- grade squamous intraepithelial lesions; SCC: Squamous cell carcinoma; ASC: Adenosquamous carcinoma; AC: Adenocarcinoma.

Table 2. Expressions of KIM-1 in various cervical neoplasms

Disease	Frequency (n)	Percent (%)
LSIL absent	27	90.0
present	3	10.0
total	30	100.0
HSIL absent	26	89.7
present	3	10.3
total	29	100.0
SCC absent	14	51.9
present	13	48.1
total	27	100.0
ASC absent	6	40.0
present	9	60.0
total	15	100.0
AC absent	6	100.0
present	0	0.0
total	0	0.0

Abbreviations: L-SIL: Low- grade squamous intraepithelial lesions; H-SIL: High- grade squamous intraepithelial lesions; SCC: Squamous cell carcinoma; ASC: Adenosquamous carcinoma; AC: Adenocarcinoma.

and NGALR, the cell surface receptor in various tumors [16–20]. Besides, an inverse relationship between high NGAL expression and mean survival in pulmonary adenocarcinomas was determined. Therefore NGAL has been proposed to be a prognostic factor unrelated to the stage of the tumor [17–20]. Based on immunohistochemical analysis, in cancers of bladder, colorectal region, liver, lung, ovary, and pancreas significant elevations of NGAL, NGAL mRNA gene and protein expressions were detected. In most hematological malignancies, up-regulation of NGAL was determined and tissue NGAL protein expression was lower than NGAL mRNA expression. In short, there is no definite correlation between NGAL's gene and protein expression. As an important limitation of this study, NGAL gene status was not evaluated.

NGAL is also normally expressed by immature CD34-positive bone marrow progenitor cells [21, 22]. During the maturation process of granulocyte precursors in bone marrow, NGAL is expressed almost entirely in myelocytes and metamyelocytes [22]. In many studies NGAL and KIM-1 expressions in blood and/or urine have been indicated as predictors for early detection and subsequent confirmation of kidney damage in high-risk patients [6–10]. However, very limited number of studies have been performed on the presence and prognostic significance of NGAL and KIM-1 expressions in neoplastic cells [22, 23]. In a study evaluating patients with multiple myeloma, lower renal failure and mortality rates were detected in patients with NGAL-expressing myeloid cells. However, the authors could not obtain statistically significant data due to the small number of cases in their series. In our study, we only evaluated tissue expressions of NGAL and KIM-1 and found a relationship between invasion of uterine cervical tumors and expression of these two proteins [21]. If the similar relationship will be confirmed in larger patient groups, it may be possible to interpret the invasiveness of cervical tumors by measuring the urinary or blood levels of NGAL and/or KIM-1.

Currently, multiple number of biomarkers have been started to be used in several benign and malignant diseases. Due to up-regulation of NGAL, the invasive potential of mammary, bladder, stomach, gynecological, thyroid, lung, esophageal, and colon cancers, and chronic myelogenous leukemia increases but invasiveness of pancreatic and oral cancers decreases [16, 20, 24–26]. However, the expression pattern of NGAL and its biological relationship in uterine tumors remain unclear [16–20]. Wang *et al.* [27] reported that, up-regulation of NGAL enhances proliferation of cancer cells in uterine cervix, while downregulation suppresses cell proliferation [27]. As is shown in many studies NGAL gene expression increases in cervical carcinoma compared to high- and low-grade dysplasia. This finding implies that NGAL expression is associated with proliferation of the malignant cells in cervical cancer tissues during the cervical carcinogenesis [27, 28]. Similarly Syrjanen *et al.* revealed the association between up-regulation of NGAL expression, high-risk human papillomavirus infection and grade of cervical lesion [28]. Interestingly, in our series, NGAL expression was not detected in tumor cells. In our previous study about the Wilms tumor, we detected that NGAL expression was also confined in the inflammatory cells [29]. İn fact, when the microfilms presented in the study of Syrjanen *et al.* are examined, it is seen that the NGAL expression is generally focal and localized on the surface of the epithelium. In addition, since the primary antibodies used in these studies are different, differences in

expression rates can be expected. It can even be thought this difference may be due to cross reactions between viral antigens and NGAL. In our study, significantly higher number of NGAL-positive leucocytes were found in invasive carcinomas contrary to low- and high- grade squamous intraepithelial lesions of the cervix. So, it may be suggested that the presence of NGAL-positive inflammatory cells was associated with tumor invasion in our study.

The most important limitation of our study is the lack of data concerning follow-up and clinical characteristics of the patients. In summary, our study is basically a histopathological study. In addition, especially scarce number of adenocarcinoma cases were detected in our study. However, due to the rare occurrence of the presented tumor types, all data retrieved were evaluated in our study in the hope to contribute to the literature.

This study is one of the rare studies concerning KIM-1 expression in cervical carcinomas. Although the relationship between NGAL expression and cancer has been established, KIM-1 expression has been quantified only to detect renal damage caused by malignancy or chemotherapeutic drugs [10, 20, 27]. In our study, very low levels of KIM-1 expression were detected in cervical intraepithelial neoplasms, while higher rates of expression were noted in SCC and ASC rather than AC. In the present study, we suggested that KIM-1 can be used to predict the extent of invasion in cervical neoplasms.

In conclusion, we think that NGAL and KIM-1 may be potential determinants for predicting the behavior of cervical tumors. However, these findings need to be confirmed in larger series.

Author contributions

DSK: Data Collection, Manuscript writing; GD: Project development, Manuscript writing, Data analysis; SS: Protocol/ project development, Data collection; SE: Data collection or management; DA: Protocol/ Data collection; EK: Data collection or management; IG: Protocol/project development, Data collection.

Ethics approval and consent to participate

The study was approved by the Local Ethics Committee of the Hospital (2015/21/2- 19 March 2015).

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Conflict of interest

The authors report no conflicts of interest.

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