Interstitial collagenase MMP-1 and EMMPRIN in cell lines transformed with HPV16 and HPV18, and clinical specimens of cervical squamous cell carcinoma

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Research Article

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**Abstract**

**Background:** The aim of this study was to elucidate the features of expression of the inducer of expression of matrix metalloproteinases – EMMPRIN (EMN) and matrix metalloproteinase - MMP-1 in cell lines transformed with HPV16 and HPV18, and clinical samples of squamous cell carcinoma of the cervix uteri (SCC), as factors of tumor invasion.

**Material and methods:** The study was carried out using RT-PCR and immunohistochemical studies (IHC).

**Results:** The data obtained indicate that the expression of mRNA EMN and MMP-1 occurs in all cell lines. No direct correlation was found between the expression of EMN and MMP-1. Expression of mRNA of both EMN and MMP-1 in the HPV18 cell lines was more pronounced than in HPV16. The expression of MMP-1 in tumor tissue of SCC, as a rule, was significantly higher than in normal tissue, according to densitometry data by 5-7 times. The high MMP-1 mRNA expression was found in 90% of tumor samples. Expression of EMN mRNA was found in tumor and normal tissues. In most tumor samples, expression was at or below normal tissue level, and an increase in expression was detected in only isolated cases of SCC.

**Conclusion:** The obtained data are important for understanding the process of tumor development and may have prognostic value. MMP-1 can serve as a marker of the invasive potential of SCC.

**Introduction**

In the multistage process of carcinogenesis, one of the main stages is the process of tissue destruction and tumor invasion. A key role in this process is played by matrix metalloproteinases (MMPs), which specifically hydrolyze all main components of connective tissue, performing both destructive and regulatory functions [1-5]. MMPs are zinc calcium dependent proteinases that play a decisive role in the destruction of connective tissue (CT). In tissues of humans and animals, 25 MMPs were found, which, according to their structure and specificity, are divided into the following families: collagenases, gelatinases, stromelysins, matrilisins, membrane-bound and unclassified MMPs. MMP-1 belongs to the family of tissue collagenases, which are represented in human tissues by three MMPs: MMP-1, MMP-8, MMP-13. The destructive function of these enzymes, and first of all MMP-1, as the most widespread and studied, is associated with the hydrolysis of fibrillar collagens of types I, II, III, V and ensure the development of invasive processes [1, 2, 5-7]. Tissue collagenases, along with other MMPs, perform important regulatory functions by activating, inactivating and modifying the properties of a number of biologically active molecules, such as growth factors, interleukins, various pro-enzymes, etc. This leads to the generalization of the oncological process [1, 5, 7]. MMP-1 can promote tumor growth, hydrolyze collagens, thereby participating in the development of destructive processes, and affect the prognosis and survival of patients with a number of oncological diseases [6-8].

Tissue collagenases are inducible enzymes with very little or no activity under normal conditions. Induction of MMP expression occurs during the development of physiological processes such as
morphogenesis, metamorphosis, tissue remodeling [1, 5], angiogenesis [9, 10], as well as in a number of pathological processes, such as inflammation and carcinogenesis. Expression factors can be hormones, oncogenes, chemical agents, as well as growth factors, interleukins and other agents, in particular EMN, whose expression increases sharply during carcinogenesis [1, 4, 5, 9-11].

The function of EMN as an inducer of MMPs is associated with an increase in the expression and secretion of MMPs, in particular such as MMP-1, MMP-3, MMP-9, MMP-14, which leads to the destruction of basement membranes and extracellular matrix (ECM) [13-17], stimulation of angiogenesis in the tumor [18, 19] as well as activation of a number of regulatory growth factors, in particular VEGF, the main inducer of angiogenesis [20]. The interaction of EMN with integrins alpha3 beta1 and alpha5 beta1 is aimed at regulating adhesion processes in ECM, as well as promoting the processes of invasion and metastasis [21, 22]. EMN can participate in the processes of proliferation and promotion of tumor invasion with the participation of MMPs [23, 24]. It should be emphasized that often in the stroma of tumor tissue, a higher expression of MMPs is observed in a wider spectrum [25].

EMN or cluster of differentiation 147 (CD 147) is a polyfunctional lipoprotein that belongs to the immunoglobulin family [26]. EMN exists in both membrane-bound and soluble forms, with the membrane-bound form being the main one, which consists of an extracellular domain, two immunoglobulin, a transmembrane and a short cytoplasmic domain. Immunoglobulin domains induce MMP expression [27]. EMN is widely expressed in a variety of tissues and cells. EMN can associate with other membrane proteins that activate signaling pathways involved in the regulation of physiological functions, for example, with integrins alpha3beta1 and alpha5beta1 [21, 22] or with monocarboxylate transporters – MCTs [28, 29]. In the development of normal physiological processes, as well as in diseases not associated with a tumor nature, the action of EMN is functionally associated with MCT [29]. Special attention is paid to the study of EMN expression in oncological processes, since its high expression is associated with the progression of the oncological process and poor survival [13-15]. It has been established that the expression of EMN promotes the expression of MMPs, as well as the development of such processes as proliferation, migration, invasion and metastasis, and angiogenesis [17-19, 23, 24]. The molecular mechanisms of these processes are being studied.

The present work is devoted to the study of the relationship between the expression of EMN and MMP-1, as factors of destruction and invasion, in commercial cell lines transformed with the HPV16 and HPV18 oncogenes, as well as in clinical samples of SCC and normal tissue adjacent to the tumor. Cervical cancer ranks second, after breast cancer, in terms of incidence and mortality from cancer in women. Papilloma viruses (HPV) are the etiological factors of cervical cancer. Currently, more than 120 types of HPV are known, among which about 30 types can be associated with SCC. Viruses HPV16 and HPV18 types are the most common and aggressive [30, 31]. In Russia, the distribution of HPV genotypes is as follows: HPV-16 – 62%, HPV18 – 11%, HPV45 – 6,5%, HPV31– 10% [32]. It has been established that the main transforming HPV genes are gags E6 and E7, which induce immortalization and transformation of cells, while inactivating the p53 tumor suppressor genes and Rb105 retinoblastoma genes, that is, they are oncogenes. Transcripts of these genes are found in 90% of patients with SCC [33-36]. The products of
these genes are polyfunctional. With SCC, increased expression of a number of MMPs (MMP-1, 2, 9, 14) in tumor and stromal tissue was found. Data on the expression of tissue inhibitors of MMPs – TIMP-1 and TIMP-2 were obtained [34, 36-38]. The data on the expression of EMN in SCC indicate its participation in the pathological process and allow us to consider it as a prognostic factor [39, 40]. The effect of EMN expression on the expression of individual MMPs has hardly been studied. The aim of this study is to investigate the features of EMN expression and its correlation with the expression of MMP-1, as factors of invasion and metastasis, in commercial cell lines transformed with HPV16 and HPV18 oncogenes, as well as in clinical samples of SCC and morphologically normal tissue adjacent to the tumor using RT-PCR and IHC.

**Material And Methods**

*Cell lines.*

Experiments were performed using cervical carcinoma cell lines (American Type Culture Collection, USA) kindly supplied from the Cell Culture Bank ("N. N. Blokhin National Medical Research Centre of oncology" of the Health Ministry of Russia (N.N. Blokhin NMRCO)). The cell lines differed by HPV types: SiHa, Caski had HPV16, Hela, C4-1 – HPV18. As a control of the effect of HPV virus on the expression of MMP 1 and EMN, a cell line C33A, in which HPV copies are absent, was used.

*Clinical material.*

The study used surgical material obtained after uterine extirpation in patients diagnosed with squamous cell carcinoma of the cervix (SCC), who were treated at the N.N. Blokhin National Medical Research Center of Oncology. Consent to use the material for scientific research was obtained from all patients. The research was conducted in accordance with the principles outlined in the Helsinki Declaration. The study used samples of cervical squamous cell carcinoma and morphologically normal tissue adjacent to the tumor. All cases were classified according to the TNM clinical classification of tumors in accordance with the requirements of the Union for International Cancer Control (UICC). The tissues were histologically identified. All carcinoma samples expressed the E7 HPV16 gene.

*Cultivation of cell cultures.*

Cell lines SiHa ,Caski, Hela and C33A were cultivated for 48 h in a serum-free DMEM medium containing 0.5% lactalbumin hydrolysate (1 : 1) and supplemented with a vitamin solution (10 mkl/mL) and gentamicin (100 U/mL). Cells were harvested by treatment with 0.0002% chymopsin followed by wash with Hanks solution (4—5 times) and sedimentation by centrifugation at 500 g for 10 min. Cells were collected and stored at -70°C.

*RT-PCR.*
Total RNA was extracted using the «SV Total RNA Isolation System» (Promega), and reverse transcription was carried out using the «MMLV RT kit» (Evrogen) according to the manufacturer’s instructions.

Gene expression was studied by semiquantitative RT-PCR [41], the housekeeping genes HPRT and GAPDH were used as the control of cDNA quantity.

The following primers have been used:

EMMPRIN, forward: 5’GGC CAG AAA ACG GAG TTC AA 3’, reverse: 5’ GCG CTT CTC GTA GAT GAA GA 3’ (492 bp, 60°C, 28 cycles, 60 s); ММП-1, forward: 5’ GGA CAA ACA CAT CTG ACE TAC AGG A 3’, reverse: 5’ TTG TCC EGA TGA TCT CCC CTG ACA 3’ (185 bp, 62°C, 34 cycles, 30 s); GAPDH, forward: 5’ ACC ACA GTC CAT GCC ATC AC 3’, reverse: 5’ TCC ACC ACC CTG TTG CTG TA 3’ (450 bp, 60°C, 28 cycles, 30 s); HPRT, forward: 5’ CTG GAT TAC ATC AAA GCA CTG 3’, reverse: 5’ GGA TTA TAC TGC CTG ACC AAG 3’ (230 bp, 60°C, 30 cycles, 30 s).

Specific primers were selected using GeneBank Nucleotide Sequence Database. The structure of the primer was evaluated by means of the Oligo 4.1 Primer Analysis Software.

The PCR conditions for each gene ensured the performance of the reactions in the zone of linear dependence of amplification on the number of cycles. The reaction was performed in 25 µl of incubation medium which contained 60 mM Tris-HCl (pH 8.3), 1.6 mM (NH4)2SO4, 2.5 mM MgCl2, 10% glycerin, 100 µM each of four deoxynucleoside triphosphates, 20pM of each primer, and 1-2.5 unit Taq polymerase (Sintol, Russia) and 20-100 ng of cDNA. The amplification conditions were as follows: 94°C, 30 sec; 60-62°C, 30 sec; 72°C, 1 min, n - cycles (the number of cycles for each pair of primers was selected individually). Before the start of the first cycle, the DNA was denatured at 940 C for 5 minutes.

The amplification products were separated in 1.5% agarose gel at the ethidium bromide concentration of 0.5 µg/ml, and electric field strength of 10-15 V/cm. The results were recorded by fluorescence in ultraviolet light and photographed with a video system DNA Analyzer (Litekh, Russia). Densitometric analysis of theograms was performed using the ImageJ program.

Immunohistochemistry.

Postoperative material was fixed in 10% neutral formalin for 24 h, dehydrated and embedded in paraffin using automated mode of an STP120 tissue processor (Germany) [42]. The paraffin embedded material was used for preparation of sections (4 µm thick), which were layered onto highly adhesive glasses and dried at 37°C for 18 h. Reduction of antigenic activity was performed at 121°C for 20 min in citrate buffer, pH 6.0 (for antibodies to MMP-1), or in standard Tris-EDTA buffer, pH 9.0 (for antibodies to EMMPRIN). Rabbit antihuman monoclonal antibodies to MMP-1 and EMMPRIN (LabVision, USA) (ready to use dilution) were used. Immunohistochemical reactions were performed in automated mode using an immunohistostainer (Dako, Denmark), diaminobenzidine as a chromogen and an Envision system (Dako, Denmark) as the detection system. After staining with hematoxylin quantitative imaging was carried out using a Leica Q 550 workstation. Reaction intensity was evaluated semiquantitatively using a score
system from 0 to 3 and taking into consideration manifestation of the reaction and its localization: 0 – lack of reaction, 1 – weak reaction 2 – moderate reaction, 3 – strong reaction.

Results

Cell lines.

The study of the expression of EMN and MMP-1 was carried out on commercial cell lines of SCC, which differed in the type of HPV and the number of copies of the virus. The following cell lines were used in the work: HPV16 type: SiHa (1-2 copies of HPV16), Caski (500-600 copies of HPV16); HPV18 type: Hela (10-50 copies of HPV18) and a C33A cell line with no HPV copies was used as a control. The work was carried out using the RT-PCR method. The data obtained (Fig. 1 A and B) indicate that the expression of the EMN and MMP-1 genes occurs in all cell lines, including the line of transformed cells – C33A. Gene expression levels in cell lines differed by a factor of 2-3 (Fig. 1 A and B). The ratio of gene expression levels, according to densitometry data, can be represented as follows (Fig. 1 A and B): in the case of EMN – Siha: Caski: Hela as 2.3: 1: 2: 2.8; in the case of MMP-1 as 1: 2.3: 1.6: 3.8. Expression of both EMN and MMP-1 genes in the Hela cell line transformed with oncogeae HPV18 was most pronounced. The number of copies of HPV genes had no effect on the degree of expression of both EMN and MMP-1. No direct correlation was found between the expression of EMN and MMP-1.

Paired samples (tumor and normal tissue adjacent to the tumor).

The study of the expression of genes EMN and MMP-1 was carried out on 34 samples of SCC and 10 samples of morphologically normal tissue adjacent to the tumor. Gene expression data (Fig. 2 A and B) indicate that EMN expression was found in both tumor and normal tissues, and in normal tissue the expression in some cases exceeded the expression in the tumor (Fig. 2 A). The expression of MMP-1 in the tumor, as a rule, was significantly increased (Fig. 2 A and B). The quantitative assessment of EMN and MMP using densitometry showed that the expression of MMP-1 in the tumor was 2-3 times higher than the expression in the normal tissue adjacent to the tumor, and in rare cases it was comparable to the norm. An increase in MMP-1 expression was found in 90% of tumor samples. Expression of EMN occurred in most tumor samples at the level of normal tissue or below it, an increase in expression by 20-30% was detected in 30% of tumors (Fig. 3 B). No direct correlation was found between the expression of EMN and MMP-1.

IHC studies have shown (Fig. 3 A and B) that high expression of MMP-1 was found in most samples of tumor tissue and stroma (2+ - 3+) and at a much lower level in normal tissue (1+) (Fig. 4). Expression of EMN in tumor tissue of SCC was detected in 76% of samples, and in 38% of cases it was at a high level (2+ - 3+), and in 24% of samples it was absent. In normal tissue, EMMPRIN expression was found in 43% of the samples.

Discussion
MMPs are regarded as key enzymes in the destruction of connective tissue [1, 5]. Numerous data indicate the leading role of MMP in carcinogenesis [2-4]. The works carried out by a number of authors have shown that MMP-1 plays an important role in various types of human tumors [6-8]. Increased expression of MMP-1 in humans is found in cancers: prostate [7], colon [8], stomach [11], bladder [12], and others [4, 6, 7]. These studies have shown that MMP-1 is an independent predictor of tumor development. It was found that MMP-1 can promote angiogenesis [9, 10] as well as participate in the development of adhesion processes [17, 18], invasion and metastasis of cells [2, 3]. In SCC, there is a high expression of MMPs, which can serve as a prognostic marker for patients with cervical cancer [36-38]. The interaction of MMPs with tissue regulators of their activity, tissue inhibitors and activators, is being intensively studied [36, 38]. However, the molecular mechanisms of induction of MMP expression at the gene level remain unclear.

In the present work, we investigated the relationship between the inducer of MMP expression - EMN and MMP-1, as factors of destruction and invasion, in cell lines transformed with HPV16 and HPV18 oncogenes, as well as in clinical samples of SCC and normal tissue adjacent to the tumor.

Previously, we found that the transformation of fibroblasts with the E7 HPV16 oncogene was accompanied by the induction of MMP-1 expression [42, 45]. The study of MMP expression, carried out by us on E7 HPV16-associated samples of PCSM tumors, showed that the main contribution to the invasive and metastatic potential of the tumor is made by an increase in the expression of collagenases and, first of all, MMP-1 along with MMP-9, which can serve as markers of invasive and metastatic potential tumors [44-46]. In previous our studies, the expression of MMP-1 and EMN in SCC was found both in the cervical tumor and in the stroma of the tumor and uterine tissue, and the expression of MMP-1 in normal tissue was at a lower level than in the tumor, and the level of EMN expression did not change significantly. Increased expression of MMP-1 in the uterine body may additionally contribute to the destructive potential in SCC, which is of therapeutic and prognostic significance [44, 45].

Our data on the increased expression of MMP-1 in clinical samples of SCC confirm the results obtained by other authors on a significant increase in the expression of MMP-1 in tumor tissue and stroma [6, 37]. It was shown that the expression of MMP-1 has a prognostic value, and MMP-1 can serve as a promoter and marker of the destructive potential of a tumor [6, 37, 44, 45].

A study of EMN expression in SCC, carried out by a number of authors, showed that the inducer of MMP expression plays a significant role in modulating cell adhesion, invasion, metastasis, tumor growth, angiogenesis, which is accompanied by the induction of the production of a number of biologically active molecules, including MMP and endothelial growth factor. A number of authors believe that the high expression of EMN allows us to consider the possibility of using it as a diagnostic marker of cervical cancer [39, 41]. Our data on the expression of EMN genes in tissues with SCC indicate that, in most tumors, EMN expression occurred at the level of normal tissue; a slight increase in expression was observed in 30% of cases. IHC data are consistent with gene expression data. The level of MMP-1 expression did not correspond to the level of EMN expression. No direct correlation was found between
the expression of EMN and MMP-1. Our results do not allow us to recommend EMN as a diagnostic marker for SCC [13, 17, 39, 40].

The data on the expression of MMP-1 and EMN in cell lines HPV16 (SiHa and Caski) and HPV18 (Hela), obtained by us, coincide with the results obtained earlier and indicate that the expression of this enzyme occurs in all cell lines. Activity of MMP-1 was previously found by us only in a conditioned medium, and not in cell lysates [44]. It should be emphasized that MMP-1 is a secreted MMP.

A number of authors believe that inhibition of MMP1 in cervical cancer cell lines SiHa and HeLa led to a significant decrease in the ability of cells to proliferate, migrate, and invade [44]. In our study, the expression of both EMN and MMP-1 genes in the Hela cell line transformed with oncogae HPV18 was most pronounced. No correspondence was found in EMN and MMP-1 expression levels in cell line HPV16.

Conclusions

The obtained data are important for understanding the mechanism of MMP-1 expression, which is considered as a marker of the destructive potential of a tumor in SCC. Our data indicate that in SCC, both in cell lines HPV16 and in tumor tissue, there is a significant increase in gene expression of MMP-1, while the expression of EMN mainly occurred at the level of normal tissue or below it, a slight increase in expression was observed in 20% of cases. The level of MMP-1 expression did not correspond to the level of EMN expression. Our results do not allow us to recommend EMN as a diagnostic marker in SCC. In the activation of pro-MMP-1 expression in SCC, other components, for example, such as growth factors and other cytokines, apparently play an important role.

Declarations

Acknowledgment

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Availability of data and material
All the data on the basis of which the conclusions in this article are made are presented in this manuscript.

**Code availability**

Adobe Photoshop Elements11, ImageJ1.48V, World2010, Excel2010

**Authors’ contributions**

Nina I. Solovyeva - the concept of the research, data analysis, writing articles;

Olga S. Timoshenko - isolation RNA from cells and tissues, RT-PCR, separation of products in agarose gel;

Elena V. Kugaevskaya - preparation of experimental samples from postoperative tissues, carrying out an immunohistochemical reaction;

Tatiana A. Gureeva – cultivation of cell cultures.

All authors read and approved the manuscript.

**Ethics approval**

Samples of squamous cell carcinomas (SCC) of the cervix were obtained from tumor tissue bank of N.N. Blokhin National Medical Research Center of Oncology under the approval of the Ethical Board of the Center. Informed consent was obtained from all patients.

**Consent to participate and publication**

Informed consent to participate and publication was obtained from all patients.

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Figures
Figure 1

mRNA expression of EMN and MMP-1 evaluated by RT-PCR (a) and densitometry (b) in SiHa, Caski, C33A and Hela cell lines. The amount of cDNA added to the reaction mixture corresponded to the equal amount of products of GAPDH and HPRT genes for each clone of cells.
mRNA expression of EMN and MMP-1 evaluated by RT-PCR (a) and densitometry (b) in cervical cancer specimens and morphologically normal tissue adjacent to the tumor. The amount of cDNA added to the reaction mixture corresponded to the equal amount of products of GAPDH and HPRT genes for each specimen.

Figure 2
Figure 3

mRNA expression of EMN and MMP-1 in cervical cancer samples and morphologically normal tissue adjacent to the tumor (paired samples of tumor and normal tissues are connected by vertical lines) The average norm was calculated as the mean value of EMN and MMP-1 expression in normal tissue samples.
Figure 4

Expression of EMN and MMP-1 in cervical squamous cell carcinomas specimen. Magnification, ×400