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Regulation of Matrix Metalloproteinase-9 Transcription in Squamous Cell Carcinoma of Uterine Cervix: the Role of Human Papillomavirus Gene *E2* Expression and Activation of Transcription Factor NF-κB

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Abstract—Matrix metalloproteinase-9 (MMP-9) plays an important role in initiation and progression of squamous cell carcinoma (SCC) of human uterine cervix. Regulation of MMP-9 expression in such tumors is insufficiently studied. Involvement of the human papillomavirus (HPV) gene *E2* and transcription factor NF-κB in the regulation of *MMP-9* transcription has been shown in some model systems and types of malignant tumors. The present work was mainly designed to reveal a possible role of the HPV gene *E2* and transcription factor NF-κB in the induction of MMP-9 expression in SCC. Specimens of tumor and corresponding adjacent normal tissue from 26 patients with SCC of the uterine cervix were studied. The intact *E2* frame was observed in 19 of 26 (73.1%), the *E2* gene mRNA was expressed in 10 of 15 (66.7%), NF-κB was activated in 17 of 23 (73.9%), and the expression of *MMP-9* mRNA was recorded in 10 of 20 (50%) of the informative cases. The *MMP-9* transcription did not correlate with gene *E2* status, but in all cases correlated with the activation of NF-κB transcription factor (10 of 10 vs. 5 of 10 MMP-9-negative cases, $p = 0.016$). Thus, the NF-κB role has been proved in the regulation of *MMP-9* transcription in SCC. There was no correlation of the *E2* status and MMP-9 expression with clinical/morphological characteristics of the tumors: size, local invasiveness, metastasizing into regional lymph nodes, and level of differentiation. The high intensity of NF-κB activation correlated with low degree of differentiation of the tumors studied ($p = 0.044$). These findings suggested that NF-κB should be a molecular factor of the poor prognosis of human SCC.

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Key words: cervical cancer, *E2* gene, papillomavirus, MMP-9, NF-κB

Initiation of human squamous cell cervical cancer (SCC) is associated with infection of the uterine cervix by high risk human papillomaviruses (HPV). Activities of viral oncogenes (mainly *E6* and *E7*) cause a number of crucial changes in transmission of molecular signals in epithelial cells, which results in disorders in the cell cycle regulation, inhibition of apoptosis, induction of telomerase, and aneuploidy [1, 2]. Realization of the HPV oncogenic potential significantly depends on the intact frame and expression of the early viral gene *E2* whose product is involved in transcription of viral proteins and replication of the viral genome [3]. The intact *E2* frame and expression of this gene determine the replication and persistence of the viral episomal form in the cells [4]. Moreover, E2 can induce activities of a number of cellular genes whose products are important for initiation and progression of SCC. Matrix metalloproteinase-9 (*MMP-9*) is one such gene. Matrix metalloproteinases are involved in degradation and rearrangement of extracellular matrix accompanied by release of growth factors [5]. Their activities are important during stromal invasion and subsequent metastasizing. Induction of MMP-9 expression is recorded even during early stages of SCC development [6]. It was shown experimentally that the E2 activity induced MMP-9 expression via stimulation of the MAP kinase cascade and activation of transcription factor AP-1 [7]. Transcription factor NF-κB is also involved in the regulation of *MMP-9* transcription [8]. NF-κB activity was initially thought to be important only for functioning of the immune system, but during the last 15 years many data

Abbreviations: HPV) human papillomavirus; MMP-9) matrix metalloproteinase-9; SCC) squamous cell carcinoma; TAE buffer) Tris-acetate buffer with EDTA; TBE buffer) Tris-borate buffer with EDTA.

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have been accumulated about the role of this transcription factor in initiation and progression of malignant tumors [9]. NF-κB is a homo- or heterologous complex of the Rel family proteins as subunits. In the majority of cell types there are two main variants of NF-κB: p65 (RelA)/p50 and p50/p50. NF-κB is activated in response to different stresses (exposure to cytokines and growth factors, ionizing radiation, hypoxia, carcinogens, cytostatics, bacteria, and viruses) and is responsible for survival of normal and tumor cells under nonpermissive conditions. In addition to regulation of transcription of *MMP-9* and some other genes involved in the regulation of invasion, metastasizing, and tumor-associated angiogenesis, NF-κB also stimulates expression of proteins which activate cell proliferation and prevent programmed cell death: apoptosis and necrosis [9, 10]. NF-κB is constitutively active in various human epithelial tumors [11], including uterine cervix carcinomas [12]. The mechanism of this activation is unknown. Data on the role of papillomavirus in NF-κB activation are contradictory. Thus, the HPV16 oncogenes *E6* and *E7* were shown to enhance the expression of NF-κB-regulated genes in differentiated epithelial cells of uterine cervix [13]. The nuclear translocation of NF-κB correlated with the level of HPV16 *E7* expression in laryngeal carcinomas [14]. On the other hand, in model systems the *E6* and *E7* oncogenes downregulated NF-κB activation [15, 16]. The role of *E2* intact frame and expression of this viral gene in the NF-κB activation is unknown.

The present work was designed to reveal a possible influence of gene *E2* expression and the associated episomal persistence of HPV on the activation of NF-κB transcription factor and regulation of *MMP-9* transcription in uterine cervix carcinoma. The status of these molecular factors was analyzed in specimens of uterine cervix primary carcinomas. The *E2* status, activation of NF-κB, and expression of *MMP-9* mRNA were also compared with clinical/morphological characteristics of the tumors, such as size, invasiveness, and metastasizing into the regional lymph modes, to find a possible correlation between status of the molecular factors under study and the disease progression.

MATERIALS AND METHODS

Tumor specimens. Twenty-six patients with invasive squamous cell uterine cervix carcinoma treated in the Blokhin Cancer Research Center (CRC) of the Russian Academy of Medical Sciences (RAMS) were studied. The clinical material was collected in accordance with the CRC Ethics Committee rules. None of the patients involved in this study was subjected to chemo- or radiotherapy before surgery. Immediately after the tumor removal, the tissue specimens were frozen and stored in liquid nitrogen. All cases were TNM classified according to the rules of the International Union against Cancer (UICC). The tissues were histologically examined in the Department of Tumor Pathomorphology, Institute of Clinical Oncology (CRC, RAMS) where serial sections of the frozen tumor specimens were prepared. The sections stained with hematoxylin and eosin were used to verify the clinical diagnosis, the tumor tissue differentiation degree, and the cell number in the specimen. Only specimens with tumor cell number per section no less than 75% of the total cell number were included in the study.

Isolation of nucleic acids and preparation of protein extracts. Specimens of the frozen tissues 50-100 mg in weight were minced in liquid nitrogen to powder using a microdismembrator U (B. Braun, Biotech International, Inc., Germany). Then about two thirds of each specimen was used for isolation of total RNA and DNA by centrifugation through a cesium chloride cushion with a subsequent purification of DNA from protein by extraction with chloroform [17]; one third was used for extraction of total protein.

To isolate the protein, the unfrozen tissue powder immediately after the mincing in the microdismembrator was placed into a Dounce homogenizer which contained 2 ml of cooled to 4°C buffer of the following composition: 20 mM Tris-HCl (pH 7.0), 300 mM NaCl, 10 mM NaF, 0.5 mM Na_3PO_4, 0.1% Nonidet P-40, double quantity of protease inhibitor mixture according to the producer's protocol (Roche Applied Science, Inc., USA), 1 mM dithiothreitol (DTT); the homogenization was performed by four or five movements of the plunger. The tissue homogenate was incubated at 4°C for 20 min. Then the lysate was clarified by centrifugation on a minicentrifuge (15,000 rpm, 10 min, 4°C). The protein concentration in the resulting extract was determined by Bradford's method with a kit from Bio-Rad Laboratories (USA).

NF-κB and Oct-1 binding with consensus oligonucleotide (Electrophoretic Mobility Shift Assay). The binding reaction was performed as follows: 10 μg protein was incubated with 20 μl of binding buffer: 10 mM HEPES (pH 7.5), 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 0.5 μg poly(dIdC), and the consensus oligonucleotide labeled by the 5′-end with ^{32}P ($(1\text{-}3)\cdot10^5$ cpm). The labeling was performed according to the protocol of the consensus nucleotide producer (Promega Corp., USA). To verify the binding specificity, the shift assay was performed using antibodies to p65 (Santa Cruz Biotechnology, Inc., USA) as described in [18]. The protein–oligonucleotide complexes were analyzed in 6% native polyacrylamide gel containing 0.25-fold TBE buffer. The dried gel was exposed with an X-ray film (GE Healthcare, USA) at –80°C for 1-7 days.

Determination of HPV gene *E2* intact frame. The intact frame of the HPV gene *E2* was determined by amplification of 200 ng total DNA isolated from the tumor tissue specimens. The reaction was performed in 25 μl of incubation medium which contained 60 mM Tris-HCl (pH 8.3), 16 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$,

Table 1. Conditions of PCR

Gene	Sequence of primers	Program	Product size (bp)
E2	F 5'-GACATGAAAATGATAGTACA R 5'-CCAGTAGACACTGTAATAG	94°C – 2 min, 40 cycles: 94°C – 0.5 min, 48°C – 0.5 min, 72°C – 2 min, 72°C – 6 min, 4°C – 20 min	1026
MMP-9	F 5'-AGTTTGGTGTCGCGGAGCAC R 5'-TACATGAGCGCCTCCGGCAC	94°C – 2 min, 32 cycles: 94°C – 0.5 min, 60°C – 0.5 min, 72°C – 1 min, 72°C – 10 min, 4°C – 20 min	754
HPRT	F 5'-CTGGATTACATCAAAGCACTG R 5'-GGATTATACTGCCTGACCAAG	similar with the program for *MMP-9*	230

200 μM each of four deoxynucleoside triphosphates, 50 pM each primer, and 1 unit *Taq* polymerase (Institute of Bioorganic Chemistry, Russia). Sequences of the primers used for the reaction, its conditions, and product size are presented in Table 1. The primers were chosen to completely overlap the DNA sequence encoding the *E2* gene in the HPV genome. The *E2* intact frame was determined by the presence of the specific PCR product.

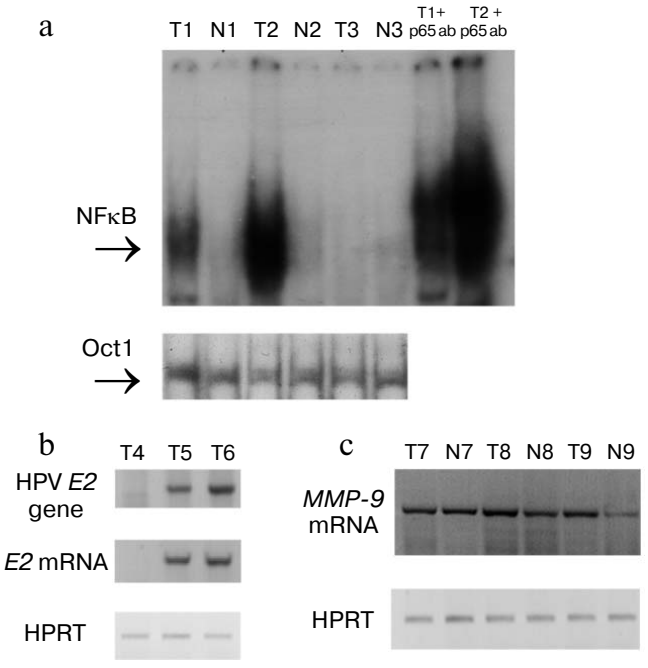
Reversed transcription and semiquantitative PCR. cDNA was prepared from 2 μg total RNA pretreated with DNase I (Promega) using Superscript II RNase H^- reverse transcriptase (Invitrogen Corp., USA) and a hexaprimer according to the producer's protocol. Then cDNA was amplified by PCR with specific primers (Table 1). The cDNA quantity was monitored using the housekeeping gene *HPRT1*. For each gene, the PCR conditions were chosen to perform the reaction in the zone of the amplification linear dependence on the number of cycles (Table 1). In all cases, no amplification of DNA admixtures by RT-PCR was observed (data not presented).

Electrophoresis in agarose gel. The amplification products were separated in 2% agarose gel in the presence of ethidium bromide (0.5 μg/ml), one-fold TAE buffer, and electric field strength of 10-15 V/cm for 30-45 min. The results were fixed by fluorescence in UV and photographed using a DNA Analyzer video system (Litex, Russia).

Data processing and statistical analysis. Photographs of PCR products and films with EMSA radioautographs were analyzed using computer programs Molecular Dynamics ImageQuant (v. 3.3) and Microsoft® Excel 2002. For each case, the fluorescence intensity of PCR product or the consensus oligonucleotide binding (EMSA) in the tumor was referred to value of the corresponding parameter in the normal tissue specimen. The MMP-9 expression and NF-κB activation were considered to be enhanced if this parameter was increased twofold and more. Because we studied small samples, the significance of differences between the groups under comparison was assessed with Fisher's exact test using the StatSoft Statistica computer program, v. 6.0. The difference was considered significant if the error probability was lower than 0.05 ($p < 0.05$).

RESULTS

The figure exemplifies molecular characteristics of specimens of the tumor tissue and histogenetically related adjacent normal tissue from three patients with SCC: the papillomavirus gene *E2* intact frame and expression of



a) NF-κB binding with consensus oligonucleotide in specimens of tumor (T) and normal (N) tissues from three patients with SCC. The arrow indicates enhanced binding of NF-κB in the tumor. The complex specificity was verified by their shift upon the interaction with antibodies (ab) to p65 (two right lanes). To monitor application of equal amounts of the protein, the binding with the transcription factor Oct-1 was used. b) Examples of HPV gene *E2* intact frame (tracks T5 and T6) and the absence of this gene frame (T4). Expression of the *E2* gene mRNA correlates with the gene *E2* intact frame (middle panel). c) Expression of *MMP-9* mRNA in specimens of the tumor (T) and normal (N) tissues from three patients with SCC. *HPRT* gene expression was used to monitor equal amounts of cDNA in the course of semiquantitative PCR

Table 2. NF-κB activity and expression of *MMP-9* mRNA in groups with different status of the HPV *E2* open reading frame

E2 intact frame	NF-κB active ($n = 17$)	NF-κB inactive ($n = 6$)	*MMP-9* mRNA present ($n = 10$)	*MMP-9* mRNA absent ($n = 10$)
Present	12	5	6	8
Absent	5	1	4	2
E2 expression	$n = 15$	$n = 5$	$n = 10$	$n = 9$
mRNA present	7	3	4	5
mRNA absent	8	2	6	4

the *E2* gene mRNA (b), activation of NF-κB transcription factor (a), expression of the *MMP-9* gene mRNA (c). The *E2* gene intact frame was recorded in 19 of 26 patients (73.1%). The presence of the *E2* frame indicated the HPV episomal persistence in the tumor cells. And expression of the *E2* gene mRNA was observed in 10 of 15 (66.7%) cases with the intact frame. The NF-κB transcription factor was activated in 17 of 23 (73.9%) of the cases studied. Expression of the *MMP-9* gene mRNA was observed in tumors from 10 of 20 patients (50%).

We studied whether there was a correlation between the HPV gene *E2* intact frame, expression of mRNA of this gene, activation of NF-κB, and expression of the *MMP-9* gene mRNA. Because HPV infection is a key factor in malignant transformation of the cervical epithelium cells, it determines the earliest molecular events of SCC carcinogenesis. NF-κB activation and induction of *MMP-9* appear later. Moreover, as mentioned above, *E2* and NF-κB can positively and independently influence *MMP-9* transcription [7, 8], and HPV can also modulate NF-κB activity [13-16]. Thus, MMP-9 expression can be induced either as a result of the viral genome activity or of NF-κB transcription factor activation. To evaluate a possible influence of the HPV gene *E2* frame status on NF-κB activation and MMP-9 expression, the data on the NF-κB and *MMP-9* status were grouped depending on the *E2* frame status and expression of this viral gene (Table 2). The data indicated that the *E2* frame was intact in 12 of 17 cases (70.6%) with NF-κB activation and in five of six cases (83.3%) with inactive NF-κB. Thus, the *E2* intact frame and associated persistence of HPV in the episomal form did not correlate with the NF-κB activation in the analyzed specimens ($p >> 0.05$). *E2* transcription was observed in 7 of 15 cases (46.7%) with NF-κB activation and in three of five cases (60.0%) with inactive NF-κB. These findings have shown the absence of correlation between *E2* transcription and NF-κB activation in the sample under study ($p >> 0.05$).

The *E2* frame was intact in 6 of 10 cases (60%) with the *MMP-9* gene expression and in 8 of 10 cases (80%) without it. *E2* expression was observed in 4 of 10 (40%) *MMP-9*-positive cases and in 5 of 10 (50%) *MMP-9*-negative cases. Thus, there was no correlation between the *E2* intact frame (virus episomal persistence), the gene expression, and induction of *MMP-9* transcription ($p >> 0.05$).

NF-κB activation was observed in all ten tumors expressing MMP-9 (100%) (Table 3), whereas in the *MMP-9*-negative tumors NF-κB was activated in only 5 of 10 cases (50%). These differences between the groups are significant ($p = 0.016$). These findings indicate an important role of NF-κB in the induction of *MMP-9* transcription.

Then we studied possible correlations between the status of the above-described molecular factors and clinical/morphological characteristics of the tumors: size, invasiveness (T1b vs. T2b), metastasizing into the regional lymph nodes (N0 – metastases are absent, N1 – metastases are present), and tumor differentiation degree (low vs. high and moderate). The data are shown in Table 4. The data analysis according to the above-described principle did not reveal correlations between the status of molecular characteristics and clinical/morphological features of the tumors under study. However, the separation in the group of NF-κB-positive cases of a subgroup with more than threefold enhanced activation of this factor in the tumor with respect to normal allowed us to establish that low-differentiated tumors in this subgroup occurred markedly more often (six of eight cases (75%) vs. two of nine cases (22.2%) in the subgroup with the NF-κB acti-

Table 3. Expression of *MMP-9* mRNA in groups with different NF-κB status

NF-κB status	*MMP-9* mRNA present ($n = 10$)	*MMP-9* mRNA absent ($n = 10$)
Active	10	5
Inactive	0	5

Table 4. Status of *E2*, NF-κB, and *MMP-9* in different clinical/morphological groups of SCC

Clinical/ morphological groups	*E2* frame present, $n = 19$	*E2* frame absent, $n = 7$	*E2* mRNA present, $n = 10$	*E2* mRNA absent, $n = 12$	NF-κB active, $n = 17$	NF-κB inactive, $n = 6$	*MMP-9* present, $n = 10$	*MMP-9* absent, $n = 10$
T1b	13	6	6	10	11	6	6	8
T2b	6	1	4	2	6	0	4	2
N0	10	5	4	8	12	3	5	4
N1	9	2	6	4	5	3	5	6
High differentiation	11	4	5	9	9	4	7	5
Low differentiation	8	3	5	3	8	2	3	5

vation lower than 1.5-fold above the normal) ($p = 0.044$). And neither the tumor size and invasiveness, nor metastasizing into the regional lymph nodes correlated with the NF-κB enhanced activity.

DISCUSSION

The HPV gene *E2* intact frame and expression of this gene were shown in 73.1 and 66.7%, respectively, of the cases studied. And the expression was observed only in the cases with the intact frame. The absence of detectable mRNA of this gene in some cases with the intact frame might be explained by the low level of transcription and a possible negative influence of epigenetic factors (methylation, absence of transcription regulators necessary for *E2* expression). In all cases, studied HPV16 had been detected earlier. And in about 70% of these cases the intact frame and *E2* expression were observed, and this was associated with the viral episomal persistence. The activation of NF-κB transcription factor was observed in a similar percent of cases (73.9%). Other authors have observed that NF-κB activation begins in early stages of cervical epithelium carcinogenesis and reaches high values in invasive carcinomas [12, 19]. Thus, the high frequency of NF-κB activation in invasive carcinomas correlates with data of other researchers. The enhanced transcription of matrix metalloproteinase-9 was recorded in 50% of the cases studied. Other authors have observed MMP-9 induction even in early stages of carcinogenesis in cervical intraepithelial neoplasia (CIN) and to 30-80% in invasive carcinomas [20, 21]. Thus, our data on the MMP-9 induction frequency do not contradict results of the other authors. Because the number of cases with MMP-9 induction was not critically different from the number of cases with the intact frame and E2 expression and from the number of cases with the NF-κB activation, theoretically both E2 expression and NF-κB activity could influence *MMP-9* transcription. Nevertheless, the statistical analysis has shown the lack of correlation between the intact frame, E2 expression (corresponding to the viral episomal persistence), and increased level of *MMP-9* transcription.

Despite contradictory data on the role of HPV oncogenes in the modulation of the NF-κB transcription factor activity [13-16], one might theoretically expect that the active virus persistence in the episomal form should lead to NF-κB activation. This suggestion is based on a strictly established ability of HPV to inhibit activity of the known tumor suppressor p53, which in most cases is an antagonist of NF-κB, and its inactivation results in activation of the latter [22-24]. However, the NF-κB activation in the cases studied did not correlate with the *E2* intact frame or expression. Thus, the episomal form of HPV persistence is not essential for activation of this transcription factor.

The activation of NF-κB correlated with the overexpression of *MMP-9* mRNA. These findings are in agreement with the data of other authors about the important role of NF-κB in the regulation of *MMP-9* transcription [8]. We have compared the status of molecular factors studied with clinical/morphological features of the tumors, and this revealed the correlation between high level of NF-κB activation and low level of the tumor tissue differentiation. These findings accord well with the known role of NF-κB as a stimulator of cell proliferation [9, 10] (low-differentiated tumors are characterized by a high proliferation index).

The lack of correlation between the enhancement of *MMP-9* transcription and the invasion/metastasizing type is rather unexpected. It is necessary to study expression of the protein and enzymatic activity of this metalloproteinase to determine reasons for its use as a molecular marker of SCC progression.

Note in conclusion that we failed to reveal correlations between the HPV episomal persistence, NF-κB activation, and transcriptional regulation of the MMP-9 activity. But this does not suggest that activities of other viral determinants have no influence on the induction of NF-κB transcription factor and MMP-9 *in vivo* expression. The studies in this line will be continued.

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