SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH.D.)

EFFECT OF HUMAN PAPILLOMAVIRUS INFECTION ON EPIGENETIC REGULATORY MECHANISMS AND SIGNAL TRANSDUCTION IN KERATINOCYTES

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The Ph.D. Defense takes place at the Lecture Hall of Bldg ″A″, Department of Internal Medicine, Medical and Health Science Center, University of Debrecen, 13:00, 04. 06. 2013.

BACKGROUND

Human papillomavirus (HPV) is considered as one of the most prevalent sexually transmitted infectious agents, and it is well established that persistent infection with high oncogenic risk HPV types is the main etiological factor of uterine cervical cancer and its premalignant lesions. Malignant progression is a rare event in anogenital HPV infections. In the majority of the cases, even in persistent HPV infections, the epithelial lesions regress to normal state or stop progressing. However, due to the high prevalence of HPV infection, the HPV-associated malignancies, including cervical cancer, are among the most common cancers. Thus, cervical cancer is the second most prevalent malignancy in women worldwide, with approximately 450000 new cases diagnosed each year.

Although high-risk HPV infection is a necessary cause of cervical cancer, besides the presence of HPV, other co-factors can influence the development and progression of cervical malignancies. Thus, the sexual behavior, genetic background, and immunologic status of the infected individual can support the development of persistent HPV infection, and the epigenetic and intracellular signaling regulatory mechanisms in association with high-risk HPV oncoproteins can induce or promote the malignant transformation of the host cell.

Previous studies of our research group focused on the co-factor role of interleukin-10 (IL-10) polymorphism in the susceptibility for high-risk HPV infection and in the risk for the development of cervical neoplasias. Immunoregulatory IL-10 plays a critical role in limiting the intensity and duration of immune and inflammatory responses. In the normal transformation zone of the uterine cervix, where most cervical neoplasias arise from, there is an increased expression of IL-10, which further increases in HPV-induced epithelial lesions. Local IL-10 production can suppress cell mediated immune defense, therefore it can support the survival of pathogens and subsequently promote the development of persistent virus infection. This theory is supported by the notion that during carcinogenesis of the uterine cervix, increased IL-10 expression is associated with cancer progression. According to recent studies investigating IL-10 expression, IL-10 transcripts are absent from keratinocytes and epithelial cells, the host cells of HPV. Established cervical cancer cell lines, do not produce IL-10 either, suggesting the existence of lineage specific silencing mechanism. Transcription factors regulating the IL-10 promoter activity are active also in epithelial cells. The access of

these transcription factors to their binding sites can be influenced, however, by the local chromatin status, which in turn is subjected to epigenetic regulation. Therefore, an epigenetic analysis was performed to demonstrate that normal and neoplastic epithelial cells do not serve as the source of IL-10 during cervical carcinogenesis. Furthermore, we investigated whether the presence of high-risk HPV genome sequences in certain cervical cancer cell lines, which are also of epithelial origin, can influence the epigenetic mechanisms silencing the IL-10 expression.

Signal transducer molecules regulating intracellular pathways that influence cell survival, proliferation, and migration may exhibit oncogenic activity when they become over-activated, thus, they can serve as cellular factors promoting cervical carcinogenesis. It is well known, that persistent HPV infection results in increased expression of E6 and E7 viral oncoproteins which are able to induce cell-cycle progression and hinder cellular differentiation, thereby promote cell proliferation and neoplastic transformation, via associating with several regulator molecules of cell proliferation, migration, and viability. The identification of signal transducer molecules influenced by papillomaviral oncoproteins is important because some of them can be targeted by small molecule inhibitors for suppression of neoplastic cell proliferation and invasive capacity.

Overactivation of Src family cytoplasmic kinases is frequently detected in solid tumors where it has been associated with advanced disease stages and metastatic potential. Recent studies have demonstrated elevated Src activity also in cervical cancer tissue samples. Furthermore, treatment with Src kinase inhibitors resulted in decreased cell motility and invasion abilities of cervical cancer cell lines. However, it is not known whether the activation of Src in cervival cancer tissues and established cell lines of cervical cancer origin is a downstream effect of papillomaviral oncoproteins. Therefore, we investigated both the expression and the activation by phosphyorylation of the ubiquitously expressed Src family kinases, namely Src, Yes, and Fyn in keratinocytes expressing HPV 16 E6, E7 or both oncoproteins.

AIMS

Among the co-factors that can promote persistent HPV infection, I investigated the epigenetic mechanisms regulating the immunomodulatory IL-10 expression. Furthermore, I studied the activation of Src family cytoplasmic kinases which can support the malignant transformation of HPV infected epithelial cells.

Role of epigenetic regulatory mechanisms in lineage-specific silencing of IL-10 gene expression in human epithelial cells:

- Analyse the CpG methylation pattern of the IL-10 promoter and the histone acetylation in human keratinocytes, in established cell lines of epithelial origin, and in peripheral blood mononuclear cells (PBMC).
- In cervical cancer cell lines of epithelial origin, investigate the effect of high-risk HPV genomic sequences on epigenetic mechanisms influencing IL-10 expression.
- Demonstrate the relationship between promoter CpG methylation and expression activity using an *in vitro* model system.

Effect of high-risk papillomaviral oncoproteins on the expression and activity of cytoplasmic Src family kinases in keratinocytes:

- Study the separate and combined effects of HPV 16 E6 and E7 oncoproteins on the mRNA and protein expression of ubiquitously expressed Src family kinases (Src, Yes, Fyn) in human keratinocites.
- Investigate the active state of Src family kinases in the presence of HPV 16 E6 and/or E7 oncoproteins.
- Study the effect of keratinocyte differentiation on the expression and activity of Src kinases.

MATERIALS AND METHODS

Study of mRNA expression

Total RNA was isolated from cells using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions.

Qualitative analysis of IL-10 mRNA expression was performed from PBMC isolated from peripheral vein blood buffy coat preparations of healthy blood donors, established cervical cancer cell lines of epithelial origin (HeLa, SiHa, CaSki, HT-3 és C-33A), *in vitro* spontaneously transformed keratinocytes from histologically normal skin (HaCaT), and primary human keratinocytes.

Quantitative analysis of Src, Yes and Fyn mRNA expression was performed from neonatal human foreskin keratinocytes transduced with control LXSN retroviral vector or LXSN-based vectors expressing HPV 16 E6, HPV 16 E7 or both E6 and E7, using custom TaqMan gene expression assays and ABI Prism 7500 Sequence Detection System (Life Technologies).

Analysis of IL-10 promoter CpG methylation

Genomic DNA was isolated by using phenol-chloroform extraction from PBMC, established cervical cancer cell lines of epithelial origin (HeLa, SiHa, CaSki, HT-3 és C-33A), immortalized keratinocytes (HaCaT), and primary human foreskin keratinocytes. Archived DNA samples from primary cervical tumors (n=10) and exfoliated normal cervical epithelial cells (n=3) were obtained from previous studies.

Automated genomic sequencing of sodium bisulfite-treated DNA was performed. The IL-10 proximal promoter region was amplified from bisulfite-modified DNA by nested PCR. The reverse nested primer was biotin labeled at the 5' end and the forward primer carried 15 bases of M13 universal primer at its 5' end. The product of the nested PCR reaction was bound to streptavidin-coated magnetic beads (Dynal) and the purified, biotin-labeled DNA was sequenced using the Alfexpress Autoread Sequencing kit (Amersham Pharmacia Biotech). The reaction products were separated on acrylamide gel using an automated DNA sequencer (Amersham Pharmacia Biotech).

Chromatin immunoprecipitation assay

Cultured PBMC, HaCaT, HeLa and primary human keratinocyte cells were fixed with formaldehyde, then cells were lysed and DNA was sheared by sonication. Cell lysates were precleared by using salmon sperm DNA/protein G agarose beads (Upstate), then incubated with anti-acetyl-histone 3 (H3), anti-acetyl-histone 4 (H4) antibodies (Upstate), and normal mouse IgG. DNA-protein complexes were collected with salmon sperm DNA/protein G agarose. The washed beads were treated with RNase and Proteinase K, and the DNA was purified by using PCR purification kit (Qiagen). Quantitations of immune precipitates were performed by real-time PCR using ABI Prism 7500 Sequence Detection System (Life Technologies). Primers were designed to amplify 150-250 bp amplicons of proximal IL-10 promoter harboring known transcription factor binding sites. PCR analyses were performed by using SYBR Green Master Mix (Life Technologies).

Methylation cassette assay

Proximal 1089 bp and 618 bp IL-10 promoter fragments were ligated into pGL2 reporter plasmid vectors (pGL2-Basic Luciferase Reporter Vector, Promega) by using T4 DNA ligase (Promega) and expanded in competent XL-1 *E. coli* strain. Plasmid constructs were isolated from bacteria by using Wizard Plus Midipreps DNA Purification System (Promega) and were confirmed by sequencing.

Next, the pGL2 plasmid contructs containing IL-10 promoter fragments were digested by FastDigest^{TM} *Kpn*I and *Bgl*II (Fermentas) restriction endonucleases. One aliquot was methylated *in vitro* with *Sss*I CpG methylase (NEB) using S-adenosyl-methionine as methyl group donor. The other aliquot (mock methylated control) was treated int he same way except that the *Sss*I methylase was omitted. The methylated and unmethylated cassette fragments were gel purified using the Qiaquick gel extraction kit (Qiagen). The methylated or mock-methylated cassette fragments were then ligated back into unmethylated pGL2-Basic vector in a 1:1 molar ratio with T4 DNA ligase.

Transient transfection and luciferase assy

HeLa cells of cervical epithelial origin were transiently transfected with reporter pGL2 reporter plasmid constructs containing methylated and unmethylated IL-10 promoter sequences by using Lipofectamine^{TM} 2000 reagent (Invitrogen) according to the

manufacturer's protocol. Transgene expression was investigated 24 hours following transfection by using Luciferase Reporter Assay (Promega).

Western blot

For the study of protein expression and phosphorylation of Src family kinases, whole cellular protein extracts were obtained from proliferating and differentiating primary human foreskin keratinocytes transduced with control LXSN retroviral vector or LXSN-based vectors expressing HPV 16 E6, HPV 16 E7 or both HPV 16 E6 and E7. The total protein concentration of the lysates was estimated using Bradford protein assay. The extracts were mixed with Laemmli buffer, and electrophoresed on SDS-polyacrilamide gel by using Bio-Rad Mini Protean II System. The separated proteins were electrotransferred onto nitrocellulose membrane (GE Healthcare) and after transfer, the membrane was blocked using 3% BSA in phosphate-buffered saline 0.05% Tween20 (PBST). Membrane was probed with primary antibodies diluted in 3% BSA in PBST. After incubation with HRP-conjugated secondary antibodies, the signals were detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce) followed by exposure to X-ray film (Thermo Scientific).

Phospho-kinase array

Protein phosphorylation was detected using the Human Phospho-Kinase Array Kit (Proteome Prolifer Array, R&D Systems). Total protein extracts were prepared from HFK cells transduced by recombinant retroviruses carrying either the control vector (LXSN) or vectors encoding HPV 16 E6, E7, or E6/E7. The signals were detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce) followed by exposure to X-ray film (Thermo Scientific). The obtained signals were quantified with ImageJ software.

RESULTS

ROLE OF EPIGENETIC REGULATORY MECHANISMS IN LINEAGE-SPECIFIC SILENCING OF IL-10 GENE EXPRESSION IN HUMAN KERATINOCYTES AND EPITHELIAL CELLS

Methylation status and activity of the human IL-10 promoter

There are eight CpG sites in the analyzed IL-10 promoter region, and the six proximal CpG sites were highly methylated both in human keratinocytes (primary keratinocytes and HaCaT) and immortalized epithelial cell lines of cervical cancer origin (HeLa, HT-3, C33-A, HeLa, CaSki, SiHa). In contrast, the proximal IL-10 promoter was unmethylated in PBMCs regardless to immunological maturation. Using quantitative RT-PCR, we detected IL-10 transcripts in peripheral blood mononuclear cells but not in primary keratinocytes or HaCaT or cervical cancer cell lines.

Among the studied cervical cancer cell lines of epithelial origin, HeLa contains HPV 18 sequences, SiHa and CaSki harbors HPV 16 genomes in different copy number. Regardless the presence of high-risk oncogenic HPV genomes, the lineage-specific epigenetic silencing of IL-10 expression was maintained uniformly in these cell lines.

CpG methylation pattern of the proximal IL-10 promoter in normal exfoliated cervical cells and in cervical cancer tissue specimens

The methylation state of the two most proximal CpG sites in the IL-10 promoter (-110 and -185) differed most markedly between IL-10 producing and non-producing cell lines. Therefore, we determined the methylation status of these two CpG sites also in normal exfoliated cervical cells (n=3) and in cervical cancer tissue specimens (n=10). CpG site -110 was uniformly methylated in cervical cancer biopsies and in normal cervical epithelial cells. CpG site -185 also tended to be highly methylated in the clinical samples, although partial demethylation at this site was detected in two cervical cancer biopsies.

Effect the *in vitro* CpG methylation of IL-10 promoter regulatory sequences on promoter activity

The approximately 1 kb and 0.6 kb proximal IL-10 promoter fragments were cloned into PGL2-Basic reporter plasmids, and these recombinant plasmids were used to study *in vitro* the effect of CpG methylation of IL-10 promoter regulatory sequences on promoter activity by using methylation cassette assay. The IL-10 promoter inserts (”cassettes”) were excised from the constructs and methylated *in vitro* with SssI methylase. Methylated or mock-methylated casettes were ligated back into the unmethylated pGL2-Basic vector. The pGL2 constructs carrying CpG methylated or mock- methylated IL-10 promoter regulatory sequences were used for transient transfections of HeLa cells and tested for their ability to drive luciferase expression. Luciferase activity was normalized to total cellular protein content of transfected HeLa cell cultures. Similar significant levels of inhibition in promoter activity were detected after CpG methylation of both of the 1 kb and the 0.6 kb cassettes.

Histone acetylation status adjacent to IL-10 promoter in IL-10 expressing and non-expressing cells

To determine whether the IL-10 gene resides in the transcriptionally active euchromatin or in the inactive heterochromatin, we compared the acetylation state of histones H3 and H4 bound to the IL-10 proximal promoter region in the IL-10 expressing PBMCs and three IL-10 non-expressing epithelial cell lines (pHKC, HaCaT, HeLa). In the immunoprecipitated chromatin, we quantified the relative amount of two IL-10 promoter regions, one (-669 to -531) carrying Ets-1 and Sp1 transcription factor binding sites and two CpGs, and the other (-233 to -70) carrying CCAAT-box and STAT-3 transcription factor binding sites and the two most proximal CpGs.

Acetylated histones were associated to the IL-10 promoter only in the IL-10 expressing PBMCs and in none of the IL-10 non-expressing epithelial cell lines. The uniform histone acetylation status along the examined IL-10 promoter region in PBMC suggests that the enrichment of acetylated histones H3 and H4 in the IL-10 proximal promoter region is associated with open chromatin and transcriptional activation in IL-10 expressing cells.

EFFECT OF HIGH-RISK PAPILLOMAVIRAL ONCOPROTEINS ON THE EXPRESSION AND ACTIVITY OF CYTOPLASMIC SRC FAMILY KINASES IN HUMAN KERATINOCYTES

Effect of HPV 16 E6 and E7 on the protein expression of Src family kinases in human keratinocytes

In light of recent publications indicating that certain Src family cytoplasmic kinases might be overactivated during the process of HPV-associated cervical carcinogenesis, we examined both the individual and the combined effects of HPV 16 E6 and E7 oncoproteins on the expression and activity of ubiquitously expressed Src family kinases, namely Src, Yes, and Fyn, in transduced human keratinocytes. The presence of functionally active E6 and E7 oncoproteins were confirmed by the decreased levels of p53 or pRb proteins, respectively.

Western blot analysis revealed that in proliferating keratinocytes, there was a significant increase in Src and Yes protein levels in the presence of both E6 and E7, compared to the cells containing the control LXSN vector or only one of the oncogenes. A constitutively high level of Fyn protein was observed in all studied cell lines suggesting that HPV 16 E6 and E7 oncoproteins have no significant effect on Fyn expression in human keratinocytes. Western blot analysis using an antibody specific to the Src family kinases phosphorylated on a tyrosine residue in their activation site revealed that Src family kinase activation by phosphorylation significantiy increased in the presence of HPV 16 E7 but was not affected by HPV 16 E6.

Effect of HPV 16 E6 and E7 on the mRNA expression of Src family kinases in human keratinocytes

Since both the protein level and the activation state of two Src family kinases, Src and Fyn were affected by HPV 16 oncoproteins, we also quantitated the RNA transcripts of the Src family kinases. However, the quantitative RT-PCR analysis could not reveal transcriptional mechanisms behind the different protein levels, thus, the HPV 16 oncoproteins did not alter significantly the transcription of either Src family kinases (Src, Yes) with inducible protein level, and the HPV related transcriptional pattern of Src was very similar to that of Fyn with constitutively high protein level.

Effect of HPV 16 E6 and E7 on the phosphorylation of Src family kinases in human keratinocytes

Since western blot analysis revealed that the presence of HPV 16 E7 is associated with elevated activating tyrosine phosphorylation of Src family kinases in human keratinocytes, next we determined the effect of HPV 16 E6 and E7 oncoproteins on the phosphorylation state of the individual members of the Src family on those tyrosin residues that are phosphorylated upon activation and therefore indicating increased kinase activity. To this end, human phospho-kinase array was performed from whole cell lysates. The results showed that in the presence of E7 there was a significant increase in the phosphorylation levels of all three ubiquitously expressed Src family cytoplasmic kinases.

Cellular differentiation alters expression of Src family kinases in keratinocytes expressing HPV16 E6, E7 or both oncoproteins

Next, we studied whether the effect of HPV 16 E6 and E7 oncoproteins on the expression and activity of Src, Yes and Fyn could be affected by cellular differentiation. To induce cellular differentiation, human keratinocytes transduced by HPV 16 E6, E7 or both oncogenes were cultured in the presence of serum and high calcium. First we confirmed that the activities of E6 and E7 oncoproteins were maintained in differentiating cells as well. Concomitant with the presence of functionally active E6 or E7, decreased levels of p53 or pRb proteins could be observed, respectively.

In differentiating keratinocytes, the presence of E7 alone was sufficient to induce upregulation of the Src protein level. However, neither differentiation, nor the presence of papillomaviral oncoproteins could alter significantly Src mRNA levels. On the contrary, differentiation upregulated Yes protein expression to a level not influenced by the viral oncoproteins. Yes upregulation involved a transcriptional mechanism, since differentiation itself resulted in significant increase in Yes mRNA levels. Fyn protein level, as in proliferating keratinocytes, was constitutively high and unaffected by the papillomaviral oncoproteins. Fyn mRNA level was also significantly increased in differentiating keratinocytes but it was not significantly altered in the presence papillomaviral oncoproteins.

DISCUSSION

Persistent infection caused by oncogenic HPV types is considered as the initiating and maintaining event during the multistep process of cervical carcinogenesis. In the HPV-infected epithelium, local production of immunosuppressive cytokines can support the development of persistent HPV infection and subsequently the progression of certain HPV-associated neoplasms by inhibiting cell mediated immune defenses. In cervix carcinoma and in premalignant lesions of the uterine cervix, locally elevated levels of the anti-inflammatory and immunomodulatory IL-10 was detected. In solid tumors, infiltrating inflammatory cells can serve as potential sources of IL-10. In a murine model of high-risk HPV 16 infection-associated carcinogenesis it was observed that tumor infiltrating macrophages secrete IL-10 and subsequently induce the development of regulatory T cell phenotype thereby promoting immunotolerance and malignant progression. In the normal transformation zone of the uterine cervix, where most cervical neoplasias arise from, there is an increased expression of IL-10 and in premalignant cervical lesions IL-10 expression is further increased. However, cell lines of cervical cancer origin consistently lack IL-10 expression despite the fact that they express the major transcription factors involved in the regulation of IL-10 promoter activity. A uniform lack of IL-10 transcription in cells of cervical epithelial origin suggests that IL-10 promoter is permanently repressed by a lineage specific manner.

Study of epigenetic mechanisms playing crucial roles in the regulation of gene expression may explain the lineage-specific production of certain cytokines. Recent studies demonstrated that CpG methylation of promoter regions control the expression of certain human cytokine genes, such as the IFN-gamma, IL-4, and IL-2, where site-specific methylation of promoter proximal CpG dinucleotides strongly correlates with transcriptional activity. Noteworthy, promoter regions of cytokines produced by a lineage-specific manner usually have a low CpG content. The human IL-10 promoter also has a low CpG content, there are only 8 CpG sites in the 700 bp long proximal promoter region that harbors the binding sites for critical transcription factors regulating promoter activity.

First, we investigated the CpG methylation pattern of the proximal IL-10 promoter region in keratinocytes (pHKC, HaCaT), in established cervical cancer cell lines (C-33A, HeLa, HT-3, CaSki, SiHa), in exfoliated normal cervical epithelial cells, in cervical cancer biopsies, and in PBMCs. We found the IL-10 promoter highly methylated both in human keratinocytes and immortalized epithelial cell lines of cervical cancer origin. In contrast, the

proximal IL-10 promoter was unmethylated in PBMCs. Furthermore, we found that the methylation state of the most proximal CpG site in IL-10 promoter regulatory sequences correlated best with IL-10 transcription. We detected IL-10 transcripts in PBMCs but not in keratinocytes or epithelial cell lines.

Demethylated state of critical CpG sites provides a basis for further fine regulation of chromatin structure through histone modifications. We showed histone H3 acetylation along the regulatory region of human IL-10 promoter in mononuclear blood cells. Our results suggest that cells with IL-10 producing potential have demethylated promoter proximal CpG sites, and the commitment to IL-10 production is mediated by loosening the chromatin structure, making the promoter accessible for transcriptional activators.

Although there is a marked IL-10 production in cervical cancer, the results of this and other studies suggest that the cancerous cells themselves do not serve as the source of the local IL-10 production.

The functional tests in this study were based on transient reporter expression assays. Using methylation casette assay, we demonstrated that CpG methylation of IL-10 promoter regulatory sequences inhibits the expression of the reporter luciferase gene. From the shorter plasmid construct containing the proximal 0.6 kb promoter sequence, transcription was initiated at a similar level as from the 1 kb plasmid construct spanning the entire proximal IL-10 promoter, suggesting that the sequences adjacent to the transcriptional start site play an essential role in IL-10 transcription by binding crucial transcription factors. It is important to note that the exogenous IL-10 promoter constructs were actively transcribed in HeLa cells, thus, indicating that an epithelial cell line can express all transcription factors necessary to activate the IL-10 promoter.

The most common high-risk HPV type that infects the genital area is HPV 16. This type is detected from about half of the carvical cancer cases and premalignant lesions of the uterine cervix. Cervical carcinogenesis has the unique feature of requiring exogeneous papillomaviral oncoproteins from initiation to final stage. The E6 and E7 oncoproteins of the high-risk HPV types can cause abnormal proliferation of the host cell and subsequently promote viral replication by interacting with cellular regulatory proteins. More specifically, high-risk E7 oncoprotein is able to promote neoplastic transformation mainly by binding and subsequently promoting the proteasome-mediated degradation of the hypophosphorylated, growth suppressive form of the retinoblastoma tumor suppressor protein and related proteins, thereby, causing aberrant S-phase entry in cells that would have normally withdrawn from the

cell division cycle. Moreover, E7 can also associate and interfere with the activities of multiple cellular factors playing important roles in the regulation of cell proliferation, survival and adhesion. Association of high-risk E6 with tumor suppressor protein p53 leads to the proteasomal degradation of p53 via recruitment of an ubiquitin ligase, thus, inhibiting apoptosis of the abnormally proliferating host cells. In addition, p53-independent activities of high-risk E6 such as telomerase activation, association with PDZ proteins and other cellular target proteins may also contribute to its oncogenic activities.

Enhanced activity of SFKs has been detected in a wide range of malignancies including cervical cancer, where the most studied member of the family, Src was shown to be overactive based on the presence of activating phosphorylation at Y416 in its catalytically active subunit. Besides Src, this kinase family involves two other ubiquitously expressed non-receptor tyrosine kinases, namely Yes and Fyn, which are expressed in various cell types including keratinocytes. Although Yes and Fyn have not yet been studied in cervical cancer, they are overactivated in several malignancies.

The key role of papillomaviral oncoproteins in cervical carcinogenesis, the ubiquitous availability of Src, Fyn and Yes non-tyrosine receptor kinases and the observed Src activation in cervical cancer suggest a link between these important oncogenic factors and the papillomaviral oncoproteins. Indeed, increased Src Y416 phosphorylation has been observed in both established cell lines (SiHa, HeLa) and biopsies of cervical carcinoma origin.

Therefore, we used primary human keratinocytes transducted with HPV 16 E6, E7 or both oncoproteins to study the downstream effects of HPV 16 E6 and E7 oncoproteins on the activity of Src family kinases. Keratinocytes were cultured under conditions promoting proliferation or differentiation.

We observed that Src and Yes kinases had similar requirement for both E6 and E7 to be upregulated in proliferating but not in differentiating keratinocytes. Cellular differentiation itself made all transduced cell lines upregulate Yes protein expression with no further effect by HPV 16l oncoproteins. For Src upregulation, differentiation maintained the necessity of E7 activity but abrogated that of E6. Fyn protein expression was not influenced by the studied papillomaviral oncoproteins at all. Noteworthy, alterations in mRNA expression could not be identified in the background of HPV 16 oncoprotein-mediated upregulation of Src family kinases, suggesting the importance of posttranscriptional regulatory mechanisms.

Despite the heterogenous effects on the protein expression, activation of Src, Yes and Fyn by phosphorylation was uniformly dependent on the presence of HPV 16 E7. Thus, the HPV 16 E7 oncoprotein, assisted also by the E6, might have dual effect on Src kinase activity.

It can activate constitutively available non receptor tyrosine kinase Fyn and it can also elevate the intracellular level of others such as Src and Yes. Being available, Src, Yes and Fyn are uniformly activated by phosphorylation on Y416 and homologous tyrosine residues, respectively, in the presence of HPV 16 E7. It is important to note that we observed HPV related alterations in the protein expression and activation of Src family kinases as a phenomenon. Underlying mechanisms can be proposed based on further studies.

We believe that our work revealed an important mechanism of high-risk HPV oncoproteins which might contribute to the development of malignant phenotype of the host keratinocyte. Since functions of the HPV oncoproteins are necessary to maintain the malignant phenotype of cervical cancer cells, the HPV related alterations such as the elevated expression and activity of certain Src kinases, can be regarded as both initiating and maintaining oncogenic mechanisms during cervical carcinogenesis.

SUMMARY

Cervical cancer induced and maintained by oncogenic human papillomavirus infection. Among various cofactors promoting cervical carcinogenesis, my research work targeted the mechanism of intratumoral IL-10 production and the activation of the Src-family non-receptor tyrosine kinases.

Our results can be summarized as follows:

- Epigenetic analysis revealed that in normal and neoplasitic epithelial cells the IL-10 promoter is repressed by epigenetic regulatory mechanisms in a lineage specific manner.
- CpG methylation of the proximal promoter region and absence of acetylated histones indicating closed chromatin state are the major determinants of transcriptional silencing of IL-10 expression in normal and neoplastic human epitelial cells.
- Regardless the presence of high-risk oncogenic HPV genomes, the lineage-specific epigenetic silencing of IL-10 expression is maintained uniformly in keratinocytes and in epitelial cell lines of cervical cancer origin.

Our results support the notion that during cervical carcinogenesis, epithelial cells do not serve as the source of the locally elevated IL-10 production. Thus, possible sources of topical IL-10 secretion are the leukocytes infiltrating the uterine cervix.

- HPV 16 E6 and E7 upregulate Src family kinases Src and Yes via posttranscriptional mechanisms in keratinocytes expressiong HPV 16 oncoproteins.
- HPV 16 E7 enhanced the activating phosphorylation of all expressed Src-family kinases in keratinocytes.

Thus, high-risk HPV oncoproteins might influence the activation of Src family kinases, and the altered expression or activity of Src-family kinases can serve as both initiating and maintaining oncogenic mechanisms during HPV-associated malignancies.

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List of publications related to the dissertation

1. **Szalmás, A.**, Gyöngyösi, E., Ferenczi, A., László, B., Karosi, T., Csomor, P., Gergely, L., Veress, G., Kónya, J.: Activation of Src, Fyn and Yes non-receptor tyrosine kinases in keratinocytes expressing human papillomavirus (HPV) type 16 E7 oncoprotein.
Virol. J. 10 (1), 79, 2013.
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IF:2.343 (2011)

2. **Szalmás, A.**, Bánáti, F., Koroknai, A., László, B., Fehér, E., Salamon, D., Gergely, L., Minárovits, J., Kónya, J.: Lineage-specific silencing of human IL-10 gene expression by promoter methylation in cervical cancer cells.
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3. Gyöngyösi, E., **Szalmás, A.**, Ferenczi, A., Kónya, J., Gergely, L., Veress, G.: Effects of human papillomavirus (HPV) type 16 oncoproteins on the expression of involucrin in human keratinocytes.
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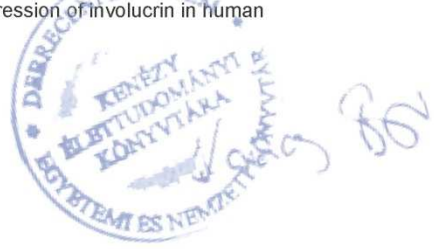

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