EXPRESSION OF *S100P* GENE IN CERVICAL CARCINOMA CELLS IS INDEPENDENT OF *E7* HUMAN PAPILLOMAVIRUS ONCOGENE

L. JAKUBÍČKOVÁ, M. BARÁTHOVÁ, S. PASTOREKOVÁ, J. PASTOREK, A. GIBADULINOVÁ*

Centre of Molecular Medicine, Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 05 Bratislava, Slovak Republic

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Summary. – High-risk human papillomaviruses (HPV) significantly contribute to development of cervical cancer. HPV E7 oncoprotein interferes with the control of cell growth via functional inactivation and/or regulation of multiple molecular targets. Induction of ectopic E7 in breast carcinoma cells has been proposed to decrease transcription of *S100P* gene, which encodes a calcium-binding protein associated with different types of tumors. We examined a possible relationship between *E7* and *S100P* genes in cervical cell lines. RT-PCR analysis revealed that all HPV-positive cell lines expressed approximately equal levels of *E7*. Out of them, HeLa, CGL3 and SiHa carcinoma cells as well as HCE16/3 immortalized cells expressed also *S100P* gene. Inhibition of a DNA methylation by 5-aza-2'-deoxycytidine (5-aza-dC) in *S100P*-negative cell lines CGL1 and Caski resulted in induced transcription of *S100P*, but the normal *S100P* level in SiHa cells was not further increased. Our results suggest that *S100P* gene expression is independent of E7 in cervical cell lines and that at least in some cases it is subjected to regulation by DNA methylation.

Key words: human papillomaviruses; *E7* oncogene; *S100P* gene; cervical carcinoma

Introduction

High-risk HPV are causally linked to cervical carcinoma, the second most common cancer in women worldwide (zur Hausen, 2000). They operate via two early viral proteins E6 and E7, which are essential for the induction and maintenance of the malignant phenotype of carcinoma cells. E6 functionally inactivates the p53 tumor suppressor protein and abrogates its apoptosis-promoting and antiproliferative activities (Scheffner *et al*., 1990). E7 binds to the tumor suppressor retinoblastoma protein pRb, promotes its degradation and interferes with its capacity to sequester E2F transcription factor and block the expression of cell cycle-related genes (Dyson *et al*., 1989). As a result, E7 permits premature S-phase entry, induces DNA synthesis and cellular proliferation (Münger *et al.*, 2001). E7 interacts with a number of additional cellular proteins such as pRb-related "pocket proteins", cell cycle regulators $p27^{kip1}$ and $p21^{cip1}$, insulin-like growth binding protein IGFBP-3 and other regulatory molecules. Moreover, E7 binds different transcription factors and chromatin modulators including AP-1, TATA box-binding protein, Oct-4, Mi2β histone deacetylase, and thereby influences the gene expression profiles of HPV-infected cells (Duensig *et al*., 2001; Münger and Howley, 2002).

It has been reported that ectopic expression of E7 oncoprotein in MCF-7 breast carcinoma cell line leads to a decreased transcription of the gene coding for S100P protein (Hellung Schønning *et al*., 2000). S100P belongs to a family of low-molecular weight calcium-binding S100 proteins, which are expressed in various cell types and act as intracellular and extracellular signal molecules with a regulatory role in diverse cellular processes including differentiation, proliferation, immortalization and transformation (Donato, 2003; Marenholz *et al*., 2004). S100P is an 11 kDa protein composed of 95 amino acids, which

*Corresponding author. E-mail: virugiba@savba.sk; fax: +4212-54774284.

Abbreviations: 5-aza-dC = 5-aza-2'-deoxycytidine; *ß-act* = ß-actin; FCS = fetal calf serum; HPV = human papillomaviruses**;** HPV-16 and HPV-18 = HPV types 16 and 18

was originally isolated from placenta (Emoto *et al.*, 1992; Becker *et al.*, 1992). Distribution of S100P in tumor cell lines and in the surgical tumor specimens strongly supports the link between S100P and neoplastic transformation. In the epithelial breast cells, S100P expression is associated with immortalized phenotype *in vitro* and with tumor tissues *in vivo* (da Silva *et al.*, 2000) and correlates with the overexpression of c-ErbB2/HER-2 protein (Mackay *et al.*, 2003). In two independent gene profile analyses, S100P has been identified as a gene selectively expressed in the neoplastic epithelium of pancreatic adenocarcinoma (Logsdon *et al.*, 2003; Crnogorac-Jurcevic *et al.*, 2003). In the prostate tumors, expression of S100P correlates with the loss of hormonal dependence and disease progression (Amler *et al.*, 2000; Mousses *et al.*, 2002). Level of S100P is increased in the colorectal cell lines resistant to chemotherapeutic drug doxorubicin (Bertram *et al.*, 1998). The role of S100P in the control of cellular processes has remained largely unknown. S100P binds ezrin, a cytoskeletal protein that is involved in the regulation of cell adhesion and motility (Koltzscher *et al.*, 2003), and interacts with CacyBP/SIP protein, which is a component of the ubiquitin pathway leading to degradation of the cell adhesion molecule ß catenin (Filipek *et al.*, 2002). S100P exists also as an extracellular soluble molecule that binds to RAGE receptor, a member of the immunoglobulin superfamily, and thereby affects cell proliferation and survival *in vitro* (Arumugam *et al.*, 2004).

In this paper, we wanted to evaluate the expression of *S100P* gene in cervical carcinoma cell lines and examine its proposed relationship to HPV E7 oncoprotein.

Materials and Methods

Cell culture and 5-aza-dC treatment. HPV-positive cervical carcinoma cell lines HeLa, HeLa-R (with reduced tumorigenicity), CGL1 and CGL3 (somatic cell hybrids between HeLa and normal human fibroblasts, Stanbridge *et al*., 1981), SiHa, CaSki and HPV-negative cell line C33a were grown in DMEM (Bio-Whittaker) supplemented with 10% of fetal calf serum (FCS) and antibiotics, at 37°C in 5% CO_2. Human ectocervical epithelial cells HCE16/3 immortalized by HPV-16 DNA (kindly provided by Prof. Antti Vaheri, Haartman Institute, Helsinki, Finland) were cultured in a 1:1 mixture of DMEM and Ham's F12 medium with 2% of FCS and growth factors and supplements as described (Zheng *et al*., 1994). For the expression analyses, the cells were grown on 6 cm-plates in monolayer for 3 days. To block the DNA methylation, the cells were seeded 16 hrs prior to the experiment at a density of 1.5 x 10^4 cells per cm^2 and then incubated for 6 days in the culture medium containing a freshly prepared 5-aza-dC (Sigma) in a 2 µM final concentration. The culture medium was replaced every 48 hours.

RNA extraction and reverse transcription of cDNA. Total RNA was extracted from the cells with the INSTAPURE solution (Eurogentech). RNA was precipitated by isopropanol and dissolved in diethylpyrocarbonate (Sigma)-treated water. One µg of total RNA was added to a reaction mixture (20 µl) composed of 0.5 mmol/l dNTPs, random heptanucleotides (400 µg/ml), and a reverse transcriptase buffer. The mixture was heated for 10 mins at 70°C, cooled on ice, supplemented with 200 U of MLV reverse transcriptase, incubated for 1 hr at 42°C, heated for 10 mins at 70°C and stored at -80°C until used. Unless otherwise stated all the reagents were from Finnzymes.

PCR amplification was performed with an automatic DNA thermal cycler (Eppendorf) using the cDNA-specific primers for the genes for E7 of HPV-16 and HPV-18, respectively, S100P, and ß-actin (*ß-act*) that served as internal standard. The nucleotide sequences of the primers, with relevant positions and database accession numbers in parentheses, were as follows (s = sense, a = antisense): for ß-act (BC 002409.2): ß-acts 5'-CCAACCGC GAGAAGATGACC-3' (nts 412–431) and ß-acts 5'-GATCTTCAT GAGGTAGTCAGT-3' (nts 627–647); for S100P (NM 005980.2): S100Ps 5'-AAGGGGGAGCTCAAGGTGCTGA-3' and S100Pa 5'-ATCTGTGACATCTCCAGGGCATC-3'; for HPV16 E7 (AY 262282): E7-16s 5'-GAGATACACCTACATTGCATGAAT-3' and E7-16a 5'-ACGTGTGTGCTTTGTACGC ACA-3'; for HPV18 E7 (AF 536179.1): E7-18s 5'-AGACATTGTATTGCATTTAGAGCC -3' and E7-18a 5'-GTGTTCAGAAACAGCTGCTGGA-3'. The amplification program was set to initial denaturation at 95°C for 3 mins, denaturation at 95°C for 30 secs, annealing at primer-specific temperature for 30 secs, and extension at 72°C for 40 secs for a total of 30 cycles, and final extension at 72°C for 5 mins. Resulting PCR fragments were run on 2% agarose gels.

Results

Lack of correlation between expression of E7 and S100P

In order to compare the expression of *E7* and *S100P* genes in cells derived from the cervical carcinoma, we performed their RT-PCR analysis in established cell lines that contain one or more integrated HPV genomes and are known to transcribe the viral oncogenes. In accord with the literature, HeLa cells, their HeLa-R derivative with reduced tumorigenicity, non-tumorigenic CGL1 HeLa x fibroblast hybrids and their tumorigenic segregant CGL3 were proven to express E7. They all contained HPV18 E7-specific amplification product of 251 bp (Fig. 1). SiHa and Caski carcinoma cell lines and immortalized HCE16/3 ectocervical cells showed HPV16 E7-specific RT PCR product of 213 bp, whereas control C33a cervical carcinoma cells were E7-negative due to the absence of HPV genome from this cell line. Levels of the E7 transcript in the HPV-positive cell lines were approximately equal as could be judged from the amount of *ß-act* internal standard. S100P expression was either minimal or not present in three HPV-infected cell lines HeLa-R, CGL1 and Caski as well as in HPV-negative C33a cells. However,

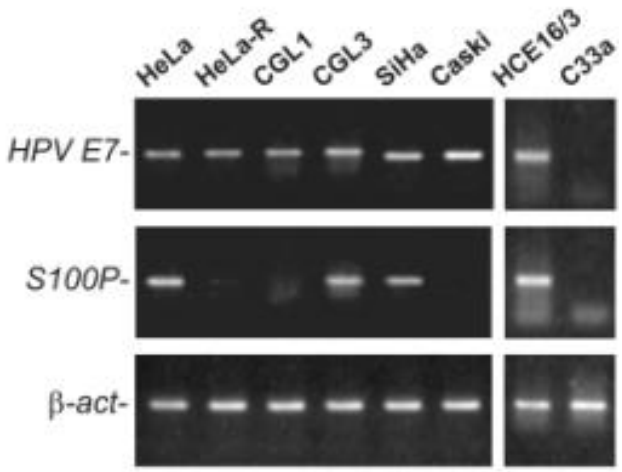

Fig. 1

Expression of HPV *E7* and *S100P* genes in the cervical cell lines

The cells were grown in monolayer and subjected to RT-PCR analysis using the gene-specific primers for HPV 18 *E7* (lanes 1–4), HPV 16 *E7* (lanes 5–8), *S100P* and *β-act* that served as internal standard.

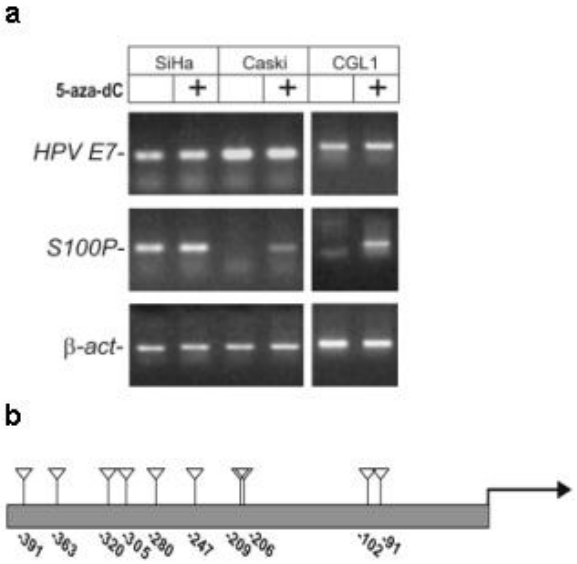

Fig. 2

Effect of inhibition of DNA methylation on *S100P* gene expression

(a) Effect of 5-aza-dC on *S100P* gene expression. The cells were treated for 6 days with 5-aza-dC. The cells grown in the absence of 5-aza-dC were used as negative control. Expression of *S100P* in comparison with HPV *E7* and *β-act* was examined by RT-PCR and agarose gel electrophoresis.

(b) Schematic drawing of the 5'-upstream region of *S100P* gene with the CpG dinucleotides indicated by triangular marks. Positions of these potential methylation sites relative to the transcription start are indicated below the scheme.

it was clearly detectable in several HPV-positive cell lines including HeLa, CGL3, SiHa and HCE16/3 (Fig. 1), These data do not fit the down-regulation of *S100P* transcription by E7 anticipated on the basis of the results obtained in MCF breast carcinoma cells (Hellung Schønning *et al*., 2000) and rather indicate lack of a direct relationship between these two genes in cervical carcinoma cells.

Induction of S100P expression by inhibition of methylation

S100P gene has been recently identified as a hypomethylation target in pancreatic cancer (Sato *et al*., 2004). Therefore, we decided to examine a possible involvement of methylation in a variable expression of *S100P* observed above in cervical carcinoma cell lines. The cell lines SiHa, Caski and CGL1 that differ by expression of *S100P* were treated by 5-aza-dC methylation-blocking agent for 6 days. Negative control cells were maintained in parallel for the same time period without 5-aza-dC. Interestingly, inhibition of methylation resulted in expression of *S100P* in Caski and CGL1 cells that were both found negative for *S100P* amplicon in the control conditions, whereas the level of *S100P* RT-PCR product did not show any marked change in SiHa cells that expressed *S100P* already in the absence of 5-aza-dC indicating its hypomethylation (Fig. 2a). On the other hand, HPV E7 levels were not affected by the 5-aza-dC-mediated inhibition of methylation in any of the examined cell lines. The same was true for *β-act* internal standard. These results harmonize with the observation of Sato *et al*. (2004) and support the involvement of methylation in the control of *S100P* expression also in cervical carcinoma cells.

Discussion

HPV E7 oncoprotein expressed from an early viral genomic region is strongly implicated in cervical carcinogenesis. Its molecular strategy is principally related to a suppression of the growth-restrictive genes and induction of the growth-promoting genes (Münger and Howley, 2002). Attempts to better understand its molecular targets led to a suggestion that E7 can down-regulate the gene coding for S100P calcium-binding protein (Hellung Schønning *et al*., 2000). This suggestion was based on a differential display analysis of the breast carcinoma cells MCF7 and the same cells with an inducible ectopic expression of HPV16 E7 oncoprotein. However, the idea of S100P as a target of negative regulation by E7 seems inherently paradoxical because *E7* and *S100P* genes are both significantly and positively linked with tumorigenesis. S100P expression has been detected in only few normal

tissues, but is readily present in immortalized and/or neoplastic cells derived from different types of tumors and is associated with the features of tumor progression. Although the precise role of S100P in cancer is not fully understood, there are several evidences in favor of its direct involvement in metastasis, survival, hormone-independence, chemoresistance and generally in signal transduction (Diederichs *et al*., 2004; Arumugam *et al*., 2003; Marenholz *et al*., 2004). It is quite conceivable that the down-regulation of this type of gene would work rather against the tumor development then towards it. Moreover, the breast epithelium does not belong to tissues naturally infected by HPVs and thus MCF7 cells represent a model that might not be fully relevant for HPV-triggered carcinogenesis. Indeed, our results obtained with the cervical carcinoma cell lines do not support any correlation between *E7* and *S100P*. Transcription pattern of these two genes evaluated here by RT-PCR suggests that these genes are mutually independent at least in our experimental settings. Furthermore, our data showing expression of *S100P* in consequence to inhibition of methylation agree with the role of methylation in the regulation of *S100P* transcription and harmonize with the recent view that hypomethylation of the growth-promoting genes may contribute to tumorigenesis (Ehrlich, 2002). In fact, upstream genomic region of *S100P* gene contains several CpG dinucleotides that may be potentially modified by methylation (Fig. 2b), but the precise position of the actual methylation site(s) remains to be determined. Altogether, we can conclude that *S100P* expression is independent of HPV E7 in cervical carcinoma cell lines and that it is subjected to methylation control.

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