Analysis of expression and structure of the *TSG101* gene in cervical cancer cells

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Abstract. Human papillomavirus (HPV)-mediated transformation of human epithelial cells has been recognized as a multi-step process in which additional unknown factors and (epi)genetic events are required. *The tumor susceptibility gene 101* (*TSG101*) was discovered in mouse NIH3T3 fibroblast cells as a gene whose functional knockout leads to transformation. TSG101 protein is involved in a variety of important biological functions, such as ubiquitination, transcriptional regulation, endosomal trafficking, virus budding, proliferation and cell survival. It is suggested that TSG101 is an important factor for maintaining cellular homeostasis and that perturbation of TSG101 functions leads to cell transformation. Interestingly, a recent report showed up- or down-regulation of TSG101 in several human malignancies. At present, the role of TSG101 in cervical tumorigenesis is unexplained. TSG101 expression in tumors, where carcinogenesis is connected with viral infection, and a mechanism of TSG101 expression regulation in cancer cells are also unknown. The aim of our study was to estimate the *TSG101* mRNA and protein level in cervical cancer and non-tumor epithelial cells. We also analyzed the *TSG101* coding and promoter sequence using the PCR-SSCP technique and methylation pattern of the *TSG101* promoter. Our real-time PCR and Western blot analysis showed decreased *TSG101* mRNA and protein level in cervical cancer tissue in comparison to normal (non-tumor) HPV(-) and HPV16(+) epithelial cells. Our results suggest that *TSG101* down-regulation in cervical cancer cells is not regulated by genetic or epigenetic events. However, we detected novel single nucleotide polymorphisms in the promoter of this gene.

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Introduction

Development of cervical cancer is strongly linked to infection by high-risk human papillomaviruses (HPV), e.g. HPV16, and HPV18 (1). HPV16 is the most common type in cervical cancer and also among cytologically normal women. Interaction between high-risk HPV16 viral E6 and E7 proteins and human tumor suppressor gene product P53 and retinoblastoma (RB) result in functional inactivation of these critical cell regulatory proteins and therefore initiation of the tumorigenesis process. Despite relatively high HPV prevalence in the cervix, the development of cervical cancer is a rare event occurring after a long period of viral persistence. This suggests that additional genetic and epigenetic alterations as well as other factors may be required to maintain a malignant phenotype in the cervix (2).

The *TSG101* gene was discovered in mouse NIH3T3 fibroblast cells as a gene whose functional knockout leads to transformation and the ability to form metastatic tumors in nude mice (3). The human homologue of *TSG101* was mapped to chromosome 11.p15.1-p15.2. This region of the chromosome is known to be associated with a loss of heterozygosity (LOH) in several tumor types, e.g. breast cancer as well as in cervical cancer (4,5).

TSG101 might be constitutively expressed in many human tissues (6). The 5' sequence of this gene has common features of housekeeping gene promoters. It lacks both TATA and CAAT boxes, has a high GC content and includes several potential Sp1 and AP2 consensus sites and >20 possible transcriptional start sites (6,7). However, up-regulation of *TSG101* was found in thyroid papillary carcinomas, breast, ovarian and gastrointestinal tumors while down-regulation of *TSG101* was observed in endometrial cancers (8-10).

TSG101 encodes a multi-domain protein at a molecular weight of 43 kDa involved in a variety of important biological functions, such as ubiquitination, transcriptional regulation, endosomal trafficking, viruses budding, proliferation and cell survival (11-23). It has been suggested that TSG101 is an

Table I. Position and frequency of single nucleotide polymorphism identified in TSG101 promoter sequence.

Diagnosis	Number of patients	SNP G>A [-1801]		SNP C>T [-1861]		SNP T>C [-185]			SNP A>C [+181]		
		G	A	C	T	T	T/C	C	A	A/C	C
Control HPV16(-)	20	19	1	20	-	19	1	-	13	7	-
Control HPV16(+)	20	19	1	19	1	20	-	-	9	10	1
Cervical cancer	47	47	-	45	2	35	9	3	18	21	8
Total	87	85	2	84	3	74	10	3	40	38	9

important factor for maintaining cellular homeostasis and that perturbation of TSG101 functions lead to transformation (24). At present, the role of TSG101 in cervical tumorigenesis is unknown. Furthermore, mechanisms of TSG101 expression regulation in cancer cells are still unexplained.

The aim of this study was to evaluate the transcript and protein level of TSG101, analyze coding and promoter sequence of this gene and methylation pattern of promoter sequence in cervical cancer and normal (non-tumor) HPV(-) and HPV16(+) epithelial cells.

Materials and methods

Tissue specimens. Samples were collected from patients (median age 54.3, range 24-72) undergoing gynecological surgical procedures at the Department of Gynecology, Poznan University of Medical Sciences and Department of Obstetrics and Gynecology, University School of Medicine of Lublin, Poland. Cervical samples (n=47) and adjacent non-tumor specimens (n=40) were collected following approval by the local ethics committee. For localization, cancer cells and all specimens underwent initial H&E staining followed by reviewing by a pathologist. Cervical sections with cancer cells were used as cancer samples. The same criteria were applied to identify cancer free 'normal' cervical tissue samples from the same patients or from other patients undergoing gynecological surgical procedures.

After surgical removal, tissue samples were frozen immediately in liquid nitrogen and stored at -80˚C until used. All 47 studied patients had squamous cell carcinoma (SCC). In these groups, there were 33 cases of SSC keratinizing, 10 cases of SSC non-keratinizing, and 4 cases of SSC not specified otherwise. Staging was also reviewed based on International Federation of Gynecology and Obstetrics (FIGO) classification system. Among 47 patients with SCC, 42 patients were classified as stage IB and 5 as stage IIA.

DNA, RNA and protein extraction. Genomic DNA, RNA and protein from cervical cancer and non-tumor epithelial tissue were isolated using All Prep Kit (Qiagen, Cat. No. 80004) according to the manufacturer's instruction.

HPV16 detection. Genomic DNA was used for amplification of appropriate HPV genes by polymerase chain reaction (PCR) with two specific primer pairs, complementary to genomes of at least 33 types of HPV viruses as described previously (25,26). The PCR reaction was performed using Taq polymerase (Fermentas, Cat. No. EP0401) according to the manufacturer's protocol.

Reverse-transcription and real-time PCR analysis of TSG101 cDNA. Total RNA (1 μg) was isolated from study tissues and reverse transcribed into cDNA using MMLV reverse transcriptase and oligo-dT primers (Invitrogen Ltd., UK). Real-time PCR was performed in a Light Cycler 1.0 (Roche, Mannheim, Germany) using glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) as a reference gene (NCBI Accession No. NM_002046). Of total (20 μl) cDNA 2 μl were added to 18 μl of QuantiTect® SYBR® Green PCR Master Mix (Roche Applied Sciences) and primers for *TSG101* transcripts (Table II). Since the amplification efficiency of target and reference genes differed, quantification of copy number of these genes was respectively derived from a different standard curve for target and reference genes. One RNA sample of each preparation was processed without RT-reaction to provide a negative control in the subsequent PCR.

Western blot analysis of the TSG101 protein. Protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (Sigma). Protein (30 μg) was resuspended in sample buffer and separated on 12% polyacrylamide gel (SDS-PAGE). Next, gel proteins were semi-dried and transferred onto PVDF membrane (Amersham Biosciences) and subsequently blocked with 5% non-fat milk in 1x TBST buffer (0.1 M Trizma Base, 0.15 M NaCl, 0.05% Tween-20, pH 7.4). Detection of the protein was performed with mouse monoclonal anti-TSG101 antibody (Santa Cruz Biotechnology) followed by incubation with anti-mouse HRP-conjugated antibody. To ensure equal protein loading of the lanes, the membranes were reblotted and incubated with mouse monoclonal anti-tubulin antibody (Sigma). Bands were revealed using ECL Plus Western blot detection reagents (Amersham Biosciences) according to the manufacturer's protocol. The Western blot quantification was determined by densitometry analysis using Photo-print system Vilber-Lourmat (Marne-La Vallee Cedex, France) and the Bio-1D Software (version 5.01, Vilber Lourmat, France).

PCR-SSCP analysis of TSG101 coding and promoter sequence. Fragments of coding and promoter sequence of the *TSG101* gene were amplified using primers described in Table I and Taq polymerase according to the manufacturer's instruction. The mixture was diluted with formamide dye solution, heated at 98˚C for 10 min and applied to 8% non-denaturing

Table II. Primers used in PCR and real-time PCR analysis.

Primer pairs	Primer sequence (5'-3')	Target sequence	Amplicon length (bp)	Annealing temperature (˚C)
TSG101F	GGCTGTCCTTACCCACCTG	Exon 7 and 8	181	62.0
TSG101R	CCATTTCCTCCTTCATCCGC	*TSG101* gene		
GAPDHF	CTGCACCACCAACTGCTTAG	Exon 7 and 8	105	60.0
GAPDHR	TTCTGGGTGGCAGTGATG	*GAPDH* gene		
TSG1F	ATTGTGTGGGACGGTCTG	Exon 1	243	56.8
TSG1R	AGGTCGCTAAGGACTGCA	*TSG101* gene		
TSG2F	TAAACTAAATTCTTATGCGAT	Exon 2	185	53.0
TSG2R	TACAATCATTAACAAAGAGAGTA	*TSG101* gene		
TSG3F	TGGACAGGATGGATGATACA	Exon 3	251	54.4
TSG3R	ATGCTGGAAACCTAATAAGACA	*TSG101* gene		
TSG4F	AATAAAAATAGAGTAACTGAAGT	Exon 4	202	48.5
TSG4R	TCACAGAACACTATGAATACT	*TSG101* gene		
TSG5F	CAACTCTGTAGCTGACTTG	Exon 5	188	54.4
TSG5R	AAAGTAAAATCTCAATTCTACT	*TSG101* gene		
TSG6F	TATTCAGTAGCCTACATATTCAG	Exon 6	281	51.4
TSG6R	ATAAATAAATTCCAGGAATAAAC	*TSG101* gene		
TSG7F	ATACTATAAGGTTGGGTCATC	Exon 7	196	54.8
TSG7R	GTTAGACTTTGCTTATATGG	*TSG101* gene		
TSG8F	CTGTGAGACAAATAGAAGGAACT	Exon 8	325	59.0
TSG8R	ACATATAGAACACTCTGCCATG	*TSG101* gene		
TSG9F	TTAATGCTTGGACTGTTCTG	Exon 9	280	52.2
TSG9R	TCTTGGAACGTAAAATGAAG	*TSG101* gene		
TSG10F	GCCATTCTAAACTATTCAAACT	Exon 10	190	54.3
TSG10R	TGATAAAAGGAAGAGAAGAATAC	*TSG101* gene		
TSGPR1F	GTTCCAATGAAATAGATAAGAAG	-2276 to -2040	235	58.0
TSGPR1R	AGATGAGGTCTTACTTTGTTGC	*TSG101* promoter		
TSGPR2F	GCAACAAAGTAAGACCTCATC	-2062 to -1756	306	62.0
TSGPR2R	GTAGAGACAGGATTTCACTATG	*TSG101* promoter		
TSGPR3F	CATAGTGAAATCCTGTCTCTAC	-1778 to -1596	183	58.4
TSGPR3R	GAGTTTTGCTCTTGCTTCCCA	*TSG101* promoter		
TSGPR4F	CCGATTCGTCCTGCTGATTC	-267 to +21	287	61.0
TSGPR4R	CTGGCTCTCCGACACCGC	*TSG101* promoter		
TSGPR5F	GCGGTGTCGGAGAGCCAG	-18 to +179	176	61.0
TSGPR5R	TGAGGAGGTCGCTAAGGACT	*TSG101* promoter		
TSGPR6F	GTCCTTAGCGACCTCCTC	+158 to +401	243	61.0
TSGPR6R	ATCTACAACTAAGTATGATCC	*TSG101* promoter		
TSGmetF	GTTGGAATTTATTTTATTAGGT	-226 to +418 *TSG101*	645	62.0
TSGmetR	ATAAAAAATTACCTCAAATCTAC	promoter		

acrylamide gels. In order to increase sensitivity of PCR/SSCP technique, gels were run with and without 5% glycerol. DNA strands were subsequently visualized by standard silver staining procedure. Fragments with different migration pattern were analyzed by sequencing.

Methylation pattern of TSG101 promoter sequence. Genomic DNA (100 ng) from cancer and normal (non-tumor) epithelial cells were treated with sodium bisulfite at 50˚C for 16 h using EZ DNA Methylation Kit™ (Zymo Research). Then, DNA was amplified with the primers complementary to the promoter region of the *TSG101* gene (Table II) using FastStart Taq DNA polymerase (Roche Applied Science, Cat. No. 12032929 001) according to the manufacturer's instruction. PCR products were cloned into TA cloning vector (pGEM T Easy Vector System I Promega) and analyzed by DNA sequencing.

DNA sequencing. Plasmids and PCR products were sequenced at the DNA Sequencing Laboratory of Adam Mickiewicz University (Poznan, Poland). Nucleotide sequences were identified and compared with *TSG101* genomic sequence from GenBank, Accession No., NT_009237.17 using the Blast service at NCBI (Bethesda, USA).

Statistical analysis. Statistical analysis was performed using ANOVA software. CISTER (27) and Alibaba 2.1 software

(28) were used for calculating the presence of transcriptional factors binding elements in *TSG101* promoter sequence (http://www.ensembl.org; ENST00000251968).

Results

Detection of HPV viruses in clinical samples. Before molecular analysis, clinical samples were screened for the presence of 33 types of HPV and HPV16 virus. For further study, only 47 specimens with SCC infected with HPV16 viruses were chosen. HPV16 virus was also detected in 20 (50%) of the 40 non-tumorous tissues.

Analysis of TSG101 transcripts and protein level. Total RNA was extracted from cervical cancer tissues (n=47) and non-tumorous samples, HPV negative (n=20) and HPV16 positive (n=20) then reverse transcribed using oligo(dT) primers. Real-time PCR analysis showed decreased level of *TSG101* transcript in cervical cancer samples. We observed 74 and 56% *TSG101* transcript level decrease ($p<0.05$) in cervix cancer samples as compared to non-tumor samples HPV(-) and HPV16(+), respectively (Fig. 1). Transcript level was normalized to GAPDH gene expression. The decrease in transcript level was associated with TSG101 protein content. In 10 randomly selected patients, we detected reduced TSG101 protein level. The mean level of TSG101 protein from the tumors was 60% decreased ($p<0.05$) as compared to normal HPV16(+) epithelial cells (Fig. 2).

PCR-SSCP analysis of TSG101 promoter and coding sequence. To determine the possible mechanism of decreased *TSG101* expression, we analyzed promoter and coding sequence of this gene to detect the possible mutations. For PCR-SSCP analysis cervical cancer tissue (n=47) and non-tumor samples, HPV(-) (n=20) and HPV16 positive (n=20) were used. On the basis of bioinformatics analysis of the promoter sequence, we selected three regions of high content of transcription factor binding sites (TFBs), A [-4,269; -3,779], B [-2273; -1,579], C [-267; +401] (Fig. 3). Region C includes ATG codon for translation start and regions A and B are located upstream translation initiation site. In the B and C regions, we detected Sp1, ERE, ETS, CRE, NF1, E2F and Myc TFBs using CISTER and AliBaba 2.1 software (27,28). We also detected a new, never described before, single nucleotide polymorphisms (SNP) in promoter sequence at position -185T>C, -1861C>T, +181A>C and -1801G>A (Table I). Three of these SNPs were located in Sp1 (-1861C>T; +181A>C) and ETS (-1801G>A) TBFs (Fig. 3). The above described SNPs are not associated with a decreased level of *TSG101*. These changes were observed in both tumor and non-tumor tissues ($p>0.05$). We did not observe any mutations in *TSG101* coding sequence.

Methylation analysis of TSG101 promoter fragment. To explain whether methylation of the *TSG101* promoter region is associated with a decreased expression of this gene, we analyzed the methylation status of CpG using bisulfite modification genomic and DNA sequencing analysis. We analyzed TSG101 promoter region from -226 to +418 bp, which comprise 48 CpGs motifs. Preliminary epigenetic analysis based on the samples of cervical SCC tissues (n=10) and their paired normal (non-tumor) HPV16-positive tissues (n=10) indicated only one CpG methylation event in a tumor sample at position +198 bp.

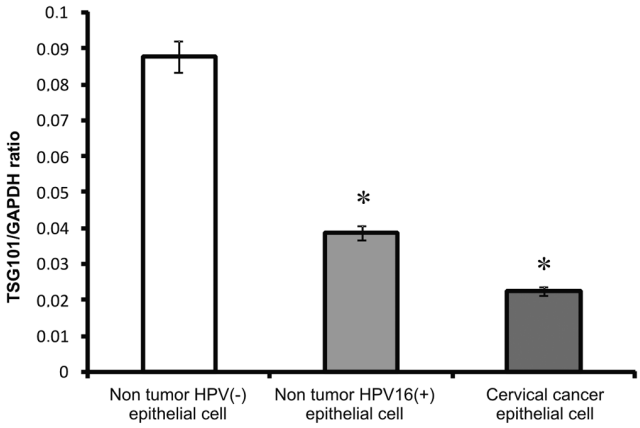

Figure 1. *TSG101* transcripts level in non-tumor HPV(-), HPV16(+) and cervical cancer epithelial cells. The *TSG101* transcript levels were determinated by RQ-qPCR analysis of cDNA. Copy number of *TSG101* transcripts was normalized to GAPDH expression level. *$p<0.05$.

Discussion

HPV-mediated transformation of human epithelial cells is a multi-step process. The presence of the virus is necessary but not sufficient, and additional unknown factors and epigenetic or genetic events are important for cell transformation.

The function of TSG101 is essential for cell proliferation, survival and embryonic development under physiological conditions. However, the role of TSG101 in tumor formation and development has proved to be complex and remains controversial (21-24). TSG101 was initially described as a potential tumor suppressor, and the expression of TSG101 has been shown to decrease in endometrial cancer (9). Other studies showed that TSG101 level is elevated in thyroid, gastrointestinal and ovarian tumors (8,24). It is suggested that TSG101 is an important factor for maintaining cellular homeostasis. This hypothesis is consistent with the observation that steady-state TSG101 level is tightly controlled in normal cells, keeping the protein concentration within a narrow range (24,29). At present, the role of TSG101 protein in cervical carcinogenesis is not known, there is also not much information about TSG101 expression in tumors where carcinogenesis is connected with viral infection and about the mechanism of TSG101 expression regulation in cancer cells.

Our results showed significant difference in TSG101 expression between non-tumor and cervical cancer epithelial cells ($p<0.05$) (Figs. 1 and 2). We also noted that TSG101 expression is lower in non-tumor HPV16(+) epithelial cells than in HPV(-) ($p<0.05$) (Fig. 1). Furthermore, we observed that TSG101 level is decreasing in correlation with cervical cancer development (data not shown). However, further analysis is required to clarify this correlation and explain the precise function of TSG101 in cervical cancer development. It is also unknown if TSG101 deficiency leads to change in a viral life cycle and initiation of tumorigenesis or the presence of viruses in the cervix cells and viral protein expression is

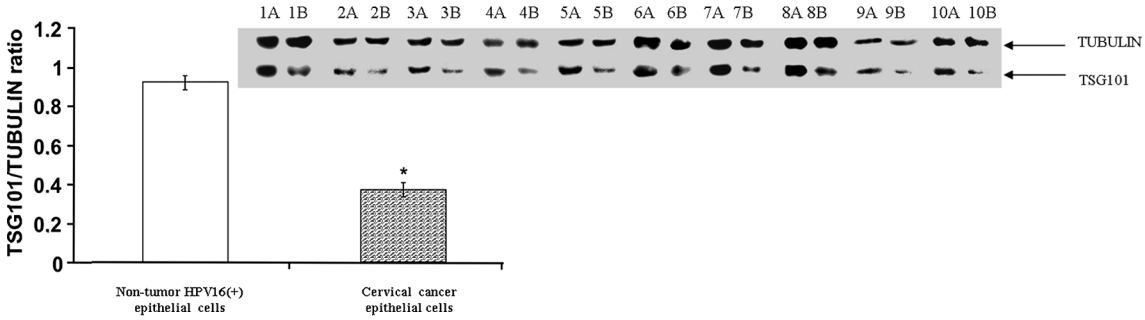

Figure 2. Western blot analysis of TSG101 protein level in HPV 16(+) non-tumor (1A-10A) and cervical cancer epithelial cells (1B-10B). For Western blot analysis, proteins were separated by SDS-PAGE and transferred to a membrane that was then immunoblotted with mouse monoclonal anti-TSG101, followed by incubation with anti-mouse HRP-conjugated Ab, and to equalize protein loading, reblotted with anti-tubulin HRP-conjugated Ab. The quantification of Western blotting was determined by densitometry analysis of bands intensity in the autoradiogram using Photo-print system Vilber-Lourmat and the Bio-1D Software (version 5.01). *p<0.05.

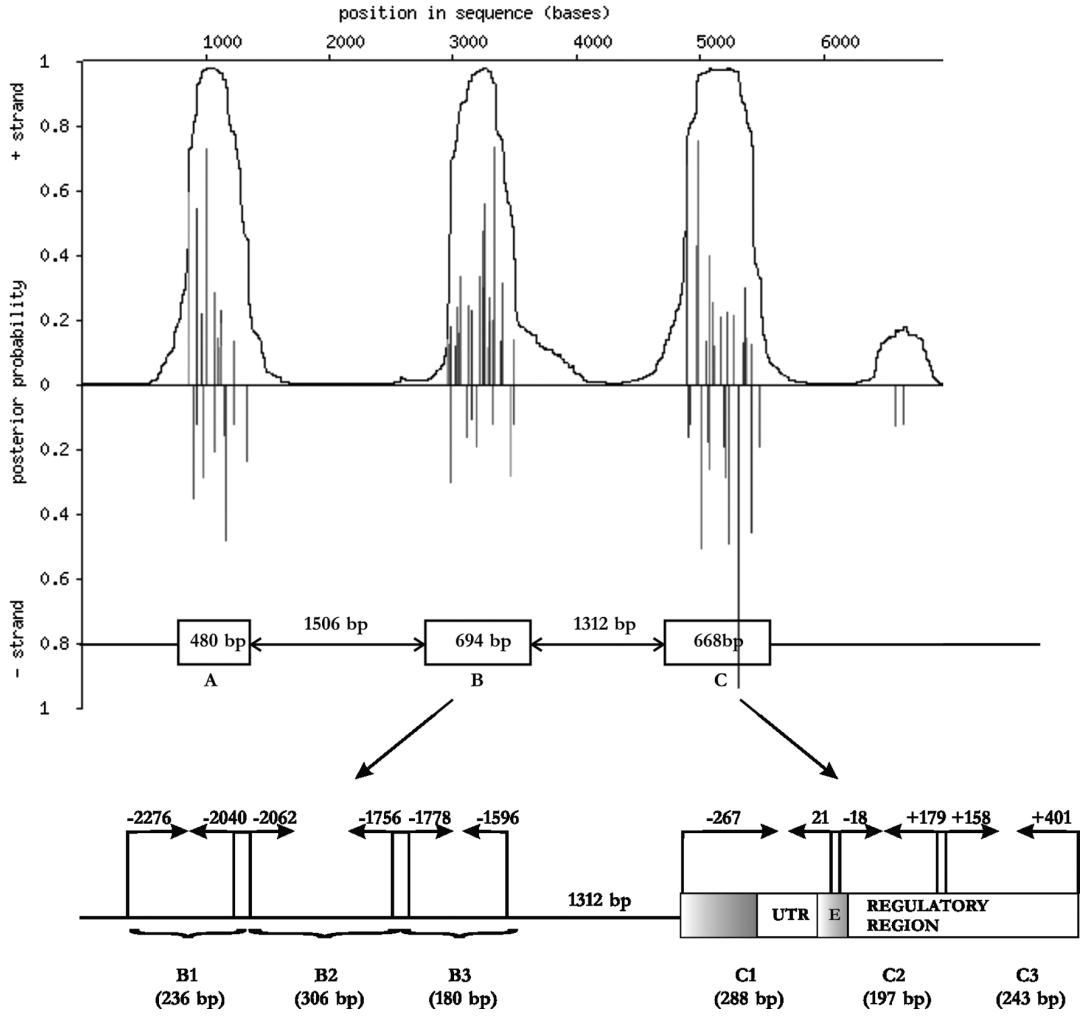

Figure 3. Bioinformatics analysis of *TSG101* promoter sequence using CISTER Cis Element Cluster Finder. Based on this analysis three regions of high contents of transcription factor binding sites were selected, A [-4269; -3779], B [-2273; -1579], C [-267; +401]. For PCR-SSCP analysis of *TSG101* promoter B (676 bp) and C (668 bp) regions were chosen, which were divided into six fragments, B1 [-2273; -2040], B2 [-2062; -1756]; B3 [-1778; -1597], C1 [-267; +21]; C2 [-18; +179]; C3 [+158; +401]. In these regions there are located binding sites for such transcription factors such as, Sp1 (12 sites), ERE (7), ETS (6), CRE (3), NF1 (2), E2F (1), Myc (3).

responsible for TSG101 down-regulation. More studies are needed to explain if TSG101 is a factor important for initiation or supporting cervical carcinogenesis.

TSG101 expression is crucial for tumor cell growth, survival and maintenance of tumor cell parameters that may contribute to the malignant potential (30). Moreover, TSG101

protein is essential for endosomal trafficking, transcriptional regulation and other cellular processes in addition to the regulation of ubiquitination (10-23). It is therefore very likely that TSG101 deficiency will cause some negative impact on a number of biological processes, leading to cell transformation (24). Depletion of TSG101 inhibits multi-vesicular body formation (MVB) and down-regulation of activated EGFR by disrupting receptor trafficking from early to late endosomes (31,32). Interestingly, it was previously demonstrated that HPV16-immortalized human cervical epithelial cells and cells derived from tumors which HPV16 oncogenes express high levels of epidermal growth factor receptor (EGFR) compared to normal cervical cells (33).

Considering the high correlation between TSG101 expression levels, HPV genome presence in cells and cervical cancer development, it is important to discover the mechanism of TSG101 down-regulation in cervical cancer cells. Decreased protein expression during tumor progression could be the result of changes in coding and promoter nucleotide sequence or in promoter silencing by methylation.

Our results confirm an earlier hypothesis which suggested that nucleotide changes in *TSG101* coding sequence occur rarely, if ever, in cervical cancer cells in spite of the fact that *TSG101* was mapped to the region of the chromosome (11.p15.1-p15.2.), which is known to be associated with a loss of heterozygosity (LOH) in cervical cancer (4-5,34).

In promoter sequence, we detected new SNPs -1861C>T, -1801G>A, -185T>C and +181A>C. Three of them -1861C>T, +181A>C and 1801G>A are located in Sp1 and ETS sites, respectively. The above described new SNPs are not associated with a decreased level of *TSG101*, because these changes are observed in both tumor and non-tumor tissues. However, TSG101 elevated expression in cervical cancer cells could be also related with changes in transcription factor profile in epithelial cells during cervical cancer progression (35). TSG101 promoter sequence includes several Sp1 binding sites. Interestingly, it is known that Sp1 transcript level and Sp1 protein affinity to binding DNA increased in epithelial tumors (36) and Sp1 also is up-regulated in keratinocytes (37).

Epigenetic silencing of genes is known in many tumor types (38,39). Pattern of promoter sequence hyper-methylation vary in different genes and tumor types (40,41). The first described hyper-methylated and silenced tumor suppressor gene was RB (42). To this time, about 20 different tumor suppressor genes are epigenetically regulated in cervical cancer (43). Our preliminary analysis, based on group of 10 patients, showed the presence of one CpG methylation at position +198. This suggests that TSG101 expression is not regulated by epigenetic events.

Our results suggest that TSG101 down-regulation in cervical cancer cells is not regulated by changes in coding and promoter sequence or epigenetic events. Protein expression in cervical cells might be also regulated by viral proteins. This hypothesis is supported by the decreased TSG101 level in non-tumor cervical cell infected by HPV16. However, further analyses are required to clarify this approach and explain the precise mechanism of TSG101 down-regulation in cervical cells. Because of high correlation between TSG101 deficiency, HPV genome presence in the cells and cervical cancer development, it is crucial to identify factors responsible for decreased TSG101 expression in cervical cancer cells. Clarification of TSG101 down-regulation mechanisms and the role of TSG101 in cervical carcinogenesis might have not only cognitive, but also therapeutic significance.

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