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High SOX2 expression as an indicator of the potential involvement of cancer stem cells in the development of cervical cancer among females with HPV infection

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Abstract

Objective: **Cervical cancer ranks fourth for both incidence and mortality among females. Human papillomavirus (HPV) infection plays an important role in the pathophysiology of cervical cancer. However, the evidence that cervical cancer develops only in a small cohort of HPV-positive females suggests that additional factors may also contribute to the pathophysiology of this disease. We hypothesized that one of the possible factors is the activation of cancer stem cells (CSCs) in HPV-positive subjects. Our study aimed to evaluate the abovementioned hypothesis by determining the expression of SOX2 gene as well as the status of TP53 in HPV-positive subjects.**

Patients and Methods: **150 HPV-positive subjects were recruited for assessment of: a) SOX2 gene expression by ELISA method and b) status of the TP53 gene by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).**

Results: **The level of SOX2 expression was elevated (7.5 ng/mL; p=0.03) in 124 (82.7%) and high (9.2 ng/mL; p=0.01) in 26 (17.3%) HPV-positive subjects, respectively. In 142 (94.7%) HPV-positive subjects, TP53 gene codon 72 polymorphisms (Pro/Pro homozygote in 102 subjects; and Arg/Pro heterozygote in 40 subjects) have been identified. Furthermore, all 26 HPV-positive subjects with high SOX2 expression level showed the TP53 codon 72 Pro/Pro homozygote polymorphism.**

Conclusions: **The high expression of SOX2 and failure of the regulatory function exerted by p53 tumor suppressor protein may suggest the activation of CSCs in HPV-positive subjects.**

Introduction

Cervical cancer ranks fourth for both incidence and mortality among females[1]. Despite the global availability of the screening programs, this cancer still represents an important public health problem[2]. Human papillomavirus (HPV) infection plays an important role in the pathophysiology of cervical cancer[3,4]. However, the evidence that cervical cancer develops only in a small cohort of HPV-positive females suggests that additional factors may also contribute to the pathophysiology of this disease[5]. We hypothesized that one of the possible factors is the activation of cancer stem cells (CSCs) in HPV-positive subjects. We expected that the activation of CSCs correlates with the expression of specific biomarkers and with the failure of the regulatory function of p53 tumor suppressor protein. This study aimed to assess the abovementioned hypothesis by determining the expression of the sex-determining region Y-box 2 (SOX2) gene as well as the status of TP53 gene in HPV-positive subjects.

One of the CSC-related markers is Sox2 protein, which is encoded by the SOX2 gene[6]. This protein is a transcription factor, which has an important role in pluripotent and multipotent stem cell biology and tissue regeneration[7]. Yet, in some cancers (including cervical cancer) a stem cell-like state correlates with the expression of SOX2 gene[8,9].

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The expression of SOX2 gene is regulated by p53 tumor suppressor protein, which is encoded by the TP53 gene[10,11]. The failure of the regulatory function exerted by p53 is a common phenomenon in several cancers and might be due to the TP53 mutations or single nucleotide polymorphisms (SNPs). One of the most frequent TP53 gene SNPs is located in exon 4 and results in the presence of either arginine or proline at amino acid position 72 in p53. We expected that two genetic variants (Pro 72 and Arg 72) of TP53 have different functional activities and that polymorphisms of the codon 72 alter SOX2 gene expression in HPV-positive subjects.

Patients and Methods

150 HPV-positive subjects were recruited for determination of: a) SOX2 gene expression by an enzyme-linked immunosorbent assay (ELISA) method and b) status of the TP53 gene by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

150 blood samples were collected from females of reproductive age (18-65 years) positive for high-risk HPV types (16/18/31/33/35). The status of HPV has been determined by genotyping according to the manufacturer's instructions (Norgen Biotek Inc, Thorold, Canada). 50 blood samples from females of reproductive age (18-65 years) negative for HPV were used as control. For study and control group participants, we performed the liquid-based cytology for cervical cancer screening. Data on the HPV status and liquid-based cytology for cervical cancer screening are listed in Table 1.

The expression of the SOX2 gene has been determined by the Human Transcription factor SOX-2 ELISA kit (MyBioSource, San Diego, CA, USA). The kit has been employed for research purposes only and not for use in diagnostic procedures. The SOX-2 ELISA kit was based on sandwich ELISA technology. Anti-SOX2 antibody was pre-coated onto 96-well plates; the biotin-conjugated anti-SOX2 antibody was used as detection antibody. The ELISA microplate and reagents [standards, sample/standards dilution buffer, biotin-conjugated detection antibody, antibody dilution buffer, HRP-Streptavidin conjugate (SABC), SABC dilution buffer, TMB substrate, stop solution, and wash buffer] essential for the performance of the ELISA reaction were provided with the kit. The ELISA test was performed following the manufacturer's instructions, and the results were obtained spectrophotometrically at 450 nm in a microplate reader. The detection range was 0.156-10 ng/mL.

The PCR-RFLP and BstUI restriction endonuclease (Fermentas, Vilnius, Lithuania) were used for determination of the TP53 Arg72Pro polymorphism. DNA extraction (DNeasy Blood & Tissue Kit; Qiagen, Hilden, Germany) was performed following the manufacturer's instructions, and the extracted DNA was stored at -18°C. A 296 base pair (bp) targeted fragment has been amplified by using the following primers:

Forward primer:
5' – TTGCCGTCCCAAGCAATGGATGA – 3'
Reverse primer:
5' – TCTGGGAAGGGACAGAAGATGAC – 3'

Each PCR (50 μL) reaction contained: 0.1 μg genomic DNA; 1 U Taq DNA polymerase; 10 pmol of each primer; 200 Mm of each deoxynucleotide triphosphate (dNTP); 1.5 mM magnesium chloride (MaCl2). The primers and reagents essential for the PCR reaction were purchased from Norgen Biotek Inc., (Thorold, Canada).

Table 1. Data on the HPV status and liquid-based cytology for cervical cancer screening from the study groups.

Liquid-based cytology for cervical cancer screening (The 2001 Bethesda System)	Subjects (n)	HPV status: positive for high-risk HPV types (16/18/31/33/35)
NILM: Negative for intraepithelial lesion or malignancy	42	No
ASC-US: Atypical squamous cells of undetermined significance	8	No
	57	Yes
LSIL: Low-grade squamous intraepithelial lesion	36	Yes
HSIL: High-grade squamous intraepithelial lesion	32	Yes
ASC-H: Atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion	25	Yes

Table 2. Data on SOX2 gene expression and TP53 gene 72 codon polymorphism. Abbreviations: SNPs, single nucleotide polymorphisms.

TP53 72 SNPs	HPV-positive subjects		HPV-negative subjects (control group)	
	Subjects (n)	SOX2 expression level and *p*-values	Subjects (n)	SOX2 expression level and *p*-values
72 Arg/Arg	8 (5.4%)	7.5 ng/mL (p=0.03)	40 (80%)	0.2 ng/mL (p=0.02)
72 Arg/Pro	40 (26.7%)	7.5 ng/mL (p=0.03)	10 (20%)	0.2 ng/mL (p=0.02)
72 Pro/Pro	76 (50.6%)	7.5 ng/mL (p=0.03)	--	--
	26 (17.3%)	9.2 ng/mL (p=0.01)	--	--

The reaction was carried out under the following PCR conditions: 5 minute-denaturation at 95°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 57°C, and 1 minute at 72°C with a final step at 72°C for 7 minutes. The resulting 312 bp PCR products of TP53 exon 4 were digested with BstUI following manufacturer's instructions (Fermentas, Vilnius, Lithuania). 10 μL PCR product has been mixed with 10 U BstUI restriction enzyme in 1xbuffer, containing 10 mM trisaminomethane, 10 mM MgCl2, 100 mM potassium chloride (KCl), and 0.1 mg/mL bovine serum albumin (BSA), pH 8.5. After separation in 4% agarose gel electrophoresis containing ethidium bromide, digestion products were visualized under ultraviolet (UV) transilluminator. We revealed three variants:

1.two bands with size 259 bp and 53 bp were indicative of codon 72 wild-type Arg/Arg homozygous variant;
2.a single 312 bp size was indicative of codon 72 Pro/Pro homozygous variant;
3. all three bands (259 bp, 53 bp and 312 bp) were indicative of codon 72 heterozygous variant.

Statistical Analysis

Statistical analysis has been performed by using SPSS v.21.0 software (SPSS Inc., Chicago, IL, USA). The intra-group comparisons were used for *p*-value. A *p*-value <0.05 was considered statistically significant.

The present study was approved by the Bioethics Committee of the Petre Shotadze Tbilisi Medical Academy (identification code: 20042019/2, Tbilisi, Georgia). All procedures performed in the present study were in accordance with the Helsinki Declaration of 1975, as revised in 2013. The patients were informed about the design and objectives of the study. All participants provided written informed consent for inclusion before they participated in the study.

Results

As it was mentioned above, the study aimed to determine the expression of SOX2 gene and the status of TP53 gene in HPV-positive subjects, as compared to HPV-negative subjects (control group). Based on the data of the ELISA analysis, the standard curve was generated and it has been used for the determination of SOX2 expression. We revealed that the SOX2 expression is low (0.2 ng/mL; *p*=0.02) in control group (48 subjects, 96%), elevated (7.5 ng/mL; *p*=0.03) and high (9.2 ng/mL; *p*=0.01) in 124 (82.7%) and 26 (17.3%) HPV-positive subjects, respectively. In 142 (94.7%) HPV-positive subjects, the TP53 gene codon 72 polymorphism (Pro/Pro homozygote – 102 subjects, 68.0 %; and Arg/Pro heterozygote – 40 subjects, 26.7%) has been identified. However, in 26 HPV-positive subjects high SOX2 expression correlated with TP53 codon 72 Pro/Pro homozygous condition. In the control group, TP53 gene codon 72 Arg/Arg homozygote has been determined in 40 subjects (80%), whereas Arg/Pro heterozygote has been determined in 10 subjects (20%). The SOX2 gene expression and TP53 gene 72 codon polymorphism data are presented in Table 2.

The SOX2 gene expression has been revealed in subjects cytologically diagnosed as low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) and atypi-

Table 3. Liquid-based cervical cytology and SOX2 gene expression data.

Liquid-based cytology cervical screening (the 2001 Bethesda System)	Subjects (n)	SOX2 expression level and *p*-values
NILM: Negative for intraepithelial lesion or malignancy	42	0.2 ng/mL (p=0.02)
ASC-US: Atypical squamous cells of undetermined significance	8	0.2 ng/mL (p=0.02)
	57	7.5 ng/mL (p=0.03)
LSIL: Low-grade squamous intraepithelial lesion	36	7.5 ng/mL (p=0.03)
HSIL: High-grade squamous intraepithelial lesion	32	7.5 ng/mL (p=0.03)
ASC-H: Atypical squamous cells, cannot exclude high grade intraepithelial lesion	25	9.2 ng/mL (p=0.01)

cal squamous cells, cannot exclude high-grade intraepithelial lesion (ASC-H; which may be a sign of HSIL). These cytological diagnoses correspond to elevated and high expression of SOX2 (Table 3). However, among the subjects with atypical squamous cells of undetermined significance (ASC-US; which can represent the reactive, reversible changes of the squamous cells) the diagnosis can be distinguished into two categories: a) subjects with low SOX2 expression; and b) subjects with elevated SOX2 expression. We revealed that TP53 gene 72 codon Pro/Pro homozygous variant is present in subjects who were diagnosed as LSIL, HSIL and ASC-H.

Discussion

The mechanisms of CSC activation and the identification of their potential biomarkers are the subject of intensive research[12]. One of such candidates is the transcription factor Sox2 encoded by the SOX2 gene which belongs to the SRY-related HMG-box (SOX) gene family. This gene contributes to the stimulation of adult cells by reprogramming them into induced pluripotent stem cells and maintains stem cell-like properties in cancer. It has been revealed that SOX2 is highly expressed in premalignant lesions (i.e., squamous dysplasia of lung and carcinoma in situ of the lung)[13]. The expression of SOX2 gene is regulated by p53[10,11]. Although the role of SOX2 gene in tumorigenesis is still controversial[14], we aimed to determine the expression of SOX2 gene in HPV-positive subjects and to access the functionality of p53 through determination of the SNP in codon 72 of TP53 gene. Our study revealed elevated (7.5 ng/mL; p=0.03) and high (9.2 ng/mL; p=0.01) expression rates of the SOX2 gene in HPV-positive subjects. The TP53 gene codon 72 polymorphisms are located in a gene region which encodes proline-rich domain of p53 essential for its DNA-binding ability and induction of apoptosis and is of decisive importance for p53 functionality[10,11]. These polymorphisms are highly frequent (94.7%) in HPV-positive subjects. Furthermore, our study revealed that cytological diagnosis of atypical squamous cells (LSIL, HSIL and ASC-H) correlates with high expression level of SOX2 as well as with the presence of TP53 gene 72 codon polymorphisms. However, the abovementioned parameters should be further investigated in subjects with cytological diagnosis of ASC-US. Furthermore, further research is needed to identify other potential biomarkers (i.e., OCT4)[15] and TP53 polymorphisms involved in cervical cancer pathophysiology.

Conclusions

Elevated (7.5 ng/mL; p=0.03) and high (9.2 ng/mL; p=0.01) expression rates of the SOX2 gene in HPV-positive subjects have been revealed by our study. These data indicate that HPV-positive status correlates with elevated expression of SOX2, which may be the reason behind the failure of the regulatory function of p53 due to the TP53 gene codon 72 polymorphism.

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Author Contributions:

Tornike Metreveli analyzed and interpreted the data regarding the SOX2 gene expression. Saba Iordanishvili performed the PCR-RFLP activities. Ekaterina Kldiashvili wrote the manuscript and was a major contributor of the present study and. All authors equally contributed to the preparation of the present article. All authors read and approved the final manuscript.

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Conflict of Interest:

The authors declare that they have no conflict of interest to disclose.

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