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Differences in the Expression of Human Papillomavirus Type 16 (HPV-16) E6 Oncogene mRNA in SiHa Cell Line Inoculated with CMV, HSV or Ureaplasmas*

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One of the factors associated with an increased risk of HPV-related malignant transformation may be bacterial and/or viral infections. The aim of our study was to examine whether the presence of infectious agents commonly detected in the genitourinary tract such as herpesviruses (HSV, CMV), and ureaplasmas (*Ureaplasma urealyticum, Ureaplasma parvum)* may lead to alterations in the expression of the HPV-16 E6 oncogene. Quantitative RT-PCR analysis was used to assess the level of HPV-16 E6 mRNA expression in SiHa cells. The presence of HSV-1 or HSV-2 in SiHa cells caused a 1.5-fold increase in HPV-16 E6 mRNA expression as compared with non-inoculated SiHa cells. *Ureaplasma urealyticum* presence but not *Ureaplasma parvum* stimulated the expression of HPV-16 E6 resulting in a nearly five-fold (4.8) up-regulated E6 mRNA level in SiHa cells. Our study is the first to suggest that infection of *Ureaplasma urealyticum* in an urogenital tract could increase the risk of cervical cancer by overexpression of the HPV E6 oncogene.

Key words: HPV-16 oncogene E6 mRNA, cytomegalovirus, *Herpes simplex*, ureaplasma, quantitative real-time PCR.

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Human cervical cancer is the second major type of cancers for women worldwide. Persistent infection with human papillomavirus, especially HPV-16, is considered to be a very important risk factor (ZUR HAUSEN 2002). HPV-mediated tumorigenesis is mainly due to the activities of two viral oncoproteins: E6 and E7. The expression of E6 and E7 is controlled during the normal viral life cycle when viral DNA replicates extrachromosomally. HPV E6 and E7 oncoproteins are overexpressed when the viral genome integrates into the host DNA (ZUR HAUSEN 2002; SZOSTEK *et al*. 2008). Deregulated overexpression of E6 and E7 oncoproteins can cause several changes in cellular pathways and functions leading to malignant transformation of cells and tumorigenesis (GANGULY & PARIHAR 2009). HPV E7 binds to hypophosphorylated pRb, preventing its interaction with the E2F transcription factor (DYSON *et al.* 1998; MUNGER & HOWLEY 2002). During the normal cell cycle, the pRb protein is active in its hypophosphorylated form and binds to E2F to prevent S-phase entry. HPV E6 is able to induce the degradation of p53 by direct binding to the cellular protein ubiquitin ligase E6AP (E6-associated protein) (SCHEFFNER *et al.* 1993). p53 is a well-known cellular tumor suppressor protein involved in processes such as DNA repair, chromatin remodeling, differentiation, and apoptosis. Neutralization of p53 tumor suppressor pathways by increased expression of HPV E6 as well as E7 oncogenes can cause disturbances in fundamental cell-

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cycle proteins and may contribute to tumorigenesis (SCHEFFNER *et al.* 1990; WERNESS *et al.* 1990). Moreover, the high-risk HPV-16 E6 protein has been reported to prevent apoptosis by a p53-independent mechanism which involves inhibition of *Bax* gene expression and degradation of Bax protein in human keratinocytes (MAGAL *et al.* 2005). Inhibition of the pro-apoptotic protein Bax results in inhibition of apoptosis and therefore cells accumulate mutations in their DNA (GANGULY & PARIHAR 2009). Although the E6 and E7 genes are necessary for immortilization, and malignant transformation, they are not sufficient.

A number of factors are associated with an increased risk of HPV-related malignant transformation, including UV and X-ray radiations, smoking tobacco, steroid hormones, and probably bacterial and viral infections. It is worth noting that studies showing that HSV infection, in conjunction with HPV infection, may increase the risk of invasive cervical carcinoma have received much attention over the years (HILDESHEIM *et al.* 1991; DIPAOLO *et al.* 1998; SMITH *et al*. 2002; ZHAO *et al*. 2012). SMITH *et al*. (2002) performed a pooled analysis that found prior exposure to HSV-2 was associated with a 2-fold increased risk of squamous cell carcinoma of the cervix in patients with HPV (SMITH *et al*. 2002). Whereas *in vitro* studies suggest that the XhoII subfragment of the HSV-2 genome is capable of inducing the tumorigenic conversion of HPV-immortalized cervical keratinocytes (DIPAOLO *et al.*1998).

The immediate-early gene products of cytomegalovirus (CMV) can transactivate other viral and cellular genes (TENNEY & COLBERG-POLEY 1991). At least four HCMV proteins can modulate cell cycle progression by binding to the retinoblastoma family members, inducing their degradation. It has been suggested that concurrent genital infection with CMV and HPV might increase the risk for cervical cancer (SHEN *et al.* 1993; SZOSTEK *et al.* 2009).

Ureaplasmas represent atypical bacteria that lack cellular membranes, closely adhere to human epithelial cells and may be associated with urogenital diseases (ABELE-HORN *et al.*1997; KIM *et al.* 2003; ZDRODOWSKA-STEFANOW *et al.* 2006; EKIEL *et al.* 2009; BIERNAT-SUDOLSKA *et al.* 2011).

The aim of our study was to examine whether the presence of infectious agents commonly detected in the human genitourinary tract such as herpesviruses (HSV, CMV), and ureaplasmas (*Ureaplasma urealyticum, Ureaplasma parvum)* may lead to alterations in the expression of HPV-16 E6 in the SiHa cell line.

Material and Methods

Cell cultures

The SiHa cell line is reported to contain an integrated human papillomavirus type 16 (HPV-16) in 1-2 copies per cell. SiHa cells (ATCC: HTB-35) were cultured in Eagle medium (MEM) (CytoGen, Germany) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Invitrogen), 2 mM L-glutamine, 200 U/mL penicillin, 150 μg/mL streptomycin (Polfa Tarchomin SA, Poland). Rabbit kidney cells (RK-13, ATCC: CCL-37) and human diploid fibroblast cells (MRC-5, ATCC:CCL-171) were maintained in the same MEM and were used for propagation of herpesviruses.

Amounts of $1x10^6$ cells were placed in the flasks and were incubated in a humidified incubator with 5% CO_2 at 37°C.

Preparation of viral and ureaplasma strains

HSV-1 (McIntyre strain; ATCC: VR-539) and HSV-2 (ATCC VR-734) were grown in a RK-13 cell monolayer. Infected cells were subjected to three cycles of freezing and thawing and were kept at -80°C prior to use. Virus titers were determined in RK-13 cell culture and are expressed as tissue culture infective dose ($TCID_{50}$). The CMV strains Town (ATCC VR-977) and AD169 (ATCC VR-538) were grown in MRC-5 monolayers. CMV titer was assessed in MRC-5 cells by the IF method with the use of monoclonal antibodies CCH2+DDG9 (Dako, Denmark) specific for viral immediate early and early proteins and expressed as cell forming unit (CFU).

Ureaplasma species (*U. urealyticum*; ATCC 27816, *U. parvum*; ATCC 27815) were cultured in liquid and solid PPLO media according to a procedure described earlier (BIERNAT-SUDOLSKA *et al.* 2006). Liquid media were incubated for 72 hours at 37°C, while solid media for 5-7 days. The growth of microorganims on liquid PPLO media caused a colour change of the medium (hydrolysis of urea with the release of ammonia, signalled by a colour change of a pH indicator), while on solid media it was the presence of characteristic brownish colonies of ureaplasmas (magnification 125x). Ureaplasma titers were determined in liquid PPLO media and expressed as colour changing units (CCU).

Inoculation of SiHa cells

SiHa cells were inoculated separately with six different pathogens, i.e. one flask of SiHa cell monolayer for each pathogen was inoculated with defined doses of studied microorganisms: HSV-1

at dose of 100 TCID50, HSV-2 at dose of 100 TCID50, CMV Town strain at dose of 100 CFU, CMV AD169 strain at dose of 100 CFU, $2x10^5$ CCU *Ureaplasma parvum*, $2x10^5$ CCU *Ureaplasma urealyticum*. A non-inoculated SiHa cell monolayer was used as a control. Each experiment was performed in five repetitions.

After adsorption of microorganisms to SiHa cells (60 min. 20°C), unadsorbed viruses or ureaplasmas were removed by washing of cell monolayers with phosphate-buffered saline (PBS). The cells were covered with maintained medium and incubated in a humidified incubator with 5% CO_2 at 37°C. After 24 hours of incubation, the cells were harvested and immediately used for RNA isolation.

Reverse transcription and real-time quantitative PCR (qRT-PCR)

Total RNA was extracted using Trizol (Invitrogen) according to the method of CHOMCZYNSKI & SACCHI 1987. RNA integrity was judged by electrophoresis and RNA concentration was quantified by measuring the optical density (OD) at 260 nm. Measuring the A260 nm/A280 nm ratio (always >1.8) assessed the nucleic acid purity. RNA samples were treated with DNase I and reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Sigma) with oligo-(dT) primer (Sigma). Quantitative real-time PCR (qRT-PCR) was performed using Fast SYBR Master Mix as a detection dye in an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA) and was quantified using the relative quantification method. The quantity of HPV-16 E6 transcripts in each sample was standardized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript level. For HPV-16 E6, the primers were 5'-GACCCAGAAAGTTACCACAG-3' (forward) and 5'-CATAAATCCCgAAAAGCAAAG-3' (reverse) and for GAPDH the primers were 5'-CTGCACCACCAACTGCTTAG-3' (forward) and 5-TTCTGGGTGGCAGTGATG -3' (reverse) (LUCZAK & JAGODZINSKI 2008). For amplification, 2 μl of total (20 μl) cDNA solution was added to 18 μl of Fast SYBR Master Mix (Life Technologies). Thermal cycling was performed with an initial denaturation step of 10 s at 95°C, followed by 40 cycles of 3 s at 95°C, and 60°C for 30 s. Real-time PCR reactions were always set up in triplicate in order to estimate PCR variation. The qRT-PCR specificity was assessed on the basis of the melting temperature for each amplimer. The quantity of HPV-16 E6 transcripts in each sample was standardized to GAPDH transcript level. Relative quantitation of gene expression was performed as previously described (KANG *et al.* 2010). Since the amplification efficiency of HPV-16 E6 and GAPDH transcripts (as a reference gene) differed, quantification of copy number of these genes was, respectively, derived from a different standard curve for HPV-16 E6 and the reference gene.

Statistical analysis

Serial dilutions from the cDNA SiHa cells were used to produce standard curves, the quality of which was assessed by the slope of the standard curve and the square of the Pearson correlation coefficient (R^2). The efficiency of the target amplification and threshold cycle values (Ct) were automatically determined by ABI 7500 Fast Real-time PCR software. Relative expression levels for E6 HPV-16 versus GAPDH were assessed as means of five measurements ± 0.95 confidence interval. Expression of E6HPV-16 transcripts in non-inoculated SiHa cells was assigned a value of 1. All data were analyzed using STATA 10.0 software. Correlations were calculated by using an ANOVA test. A p-value of <0.05 was considered significant.

Results

The qRT-PCR analysis showed the presence of HPV-16 E6 mRNA in the examined SiHa cells 24 h after inoculation with HSV-1, HSV-2, CMV Town strain, CMV AD169 strain, *Ureaplasma parvum* or *Ureaplasma urealyticum.* Expression levels for HPV-16 E6 mRNA were calculated relative to GAPDH transcripts according to a method described previously by PFAFFL (2001) using the standard curves for target and reference genes. Standard curves of Ct for HPV-16 E6 and GAPDH versus mRNA copy number indicated linearity and the R^2 values were more than 0.99 (Fig. 1a, 1b).

Figure 2 shows the results obtained from five repeated experiments, for each pathogen the level of E6 HPV-16 expression was assessed 24 hours after inoculations and it was compared with the level of E6 HPV-16 mRNA in non-inoculated SiHa cells. The presence of the pathogens in SiHa cells together with maintained culture medium after 24 hours post inoculation was confirmed using methods described in Material and Methods. Although the titer of each pathogen after 24 hours post inoculation was lower by approximately 1-2 log than at the time of inoculation, all studied microorganisms were still viable. The level of HPV-16 E6 mRNA expression in the examined SiHa cells 24h after inoculation by HSV-1, HSV-2, *Ureaplasma parvum* or *Ureaplasma urealyticum* was higher, 1.4 ± 0.25, 1.5 ± 0.32, 1.1 ± 0.16 and 4.8 ±1.31 fold respectively, as compared to non-inoculated SiHa cells, but the difference was statistically significant

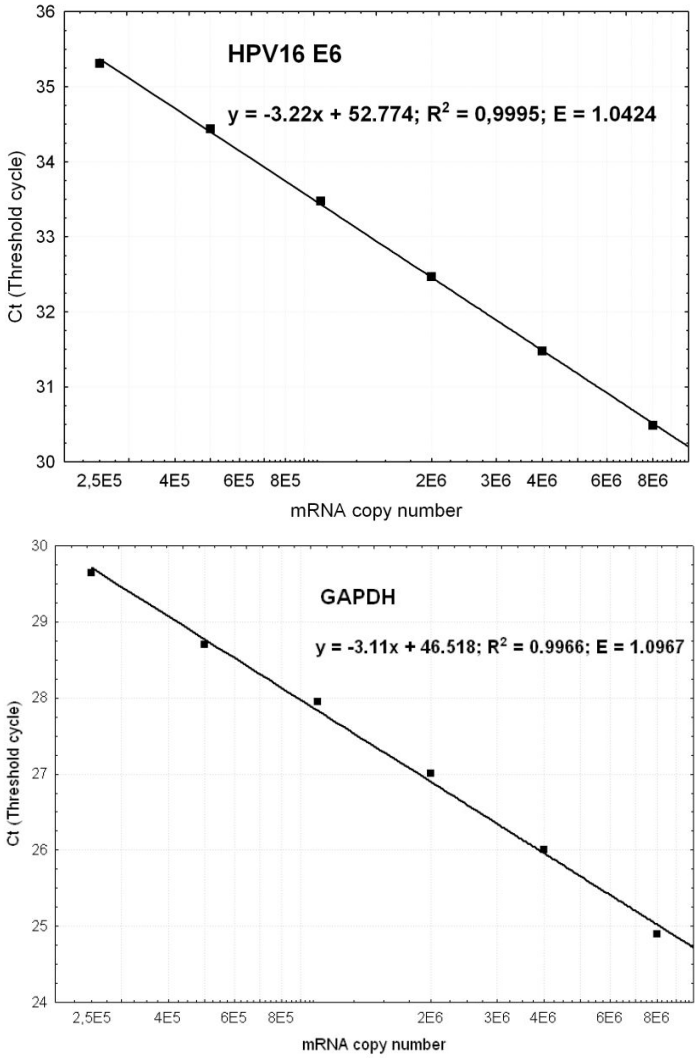

Fig. 1. Standard curves of quantitative real-time PCR amplification transcripts of: A. HPV-16 E6 oncogene; B. glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Serial dilutions of cDNA SiHa cells (containing an integrated HPV-16) were performed for quantification experiments. The curve equations, efficiency values (E) and square of the Pearson correlation coefficient (R2) were plotted.

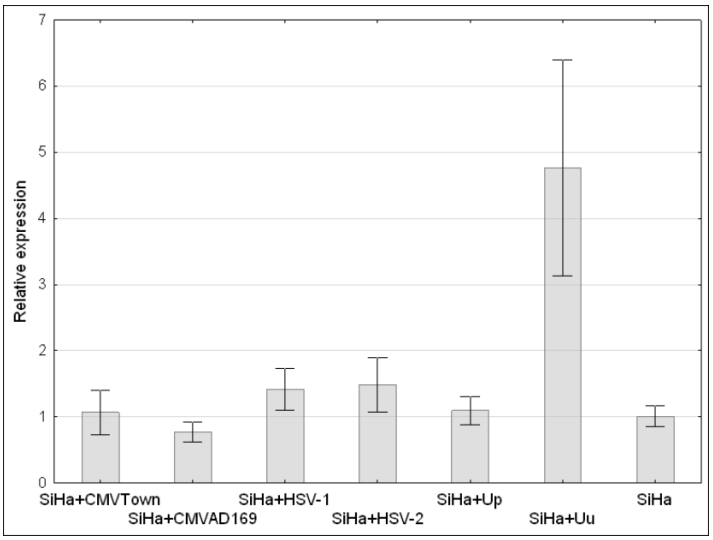

Fig. 2. Relative expression of HPV-16 E6 mRNA in SiHa cells before and 24h after inoculation with HSV-1, HSV-2, CMV Town strain or CMV AD169 strain, *Ureaplasma parvum* (Up), *Ureaplasma urealyticum* (Uu) in non-inoculated SiHa cells (control). Gene expression levels were calculated by the relative standard curve method. Values are means ± 0.95 confidence interval. The significance of difference for the amount of HPV-16 E6 mRNA expression was determined by ANOVA (P=0.02).

only for *Ureaplasma urealyticum* (Kruskal-Wallis test, P=0.02). The qRT-PCR results showed a reduction of HPV-16 E6 mRNA transcript levels after incubating the SiHa cells with CMV AD-169 strain but this effect was not statistically significant (Fig. 2).

Discussion

As early as the late sixties of the 20th century, it was suggested that HSV is associated with cervical cancer (RAWLS *et al.* 1968). Women testing positive for HSV-2 antibodies were found to have a 60% increased risk of cervical cancer compared with seronegative women (HILDESHEIM *et al.* 1991).

Our results showed that the presence of HSV-1 or HSV-2 in SiHa cells increases (1.5-fold) in HPV-16 E6 mRNA expression, however, compared to non-inoculated SiHa cells, it is not statistically significant. The results obtained by other authors in another model system demonstrated that the level of transcription of E1, E2 and E6 genes was up to 3-fold enhanced after HSV-2 inoculation (PISANI *et al*. 2004). The authors used CaSki cells which contained 60 to 600 copies of HPV-16 DNA per cell and perhaps more copies of the virus genome in the cell is affected by the differences in our results. The same authors in a previous study in HeLa cells revealed an increase of HPV18 E1 and E6 mRNA resulting in 9-fold and 3-fold increased transcription, respectively, twenty hours post HSV-2 infection (PISANI *et al.* 2002). However, *in vitro* double infection in A431 and CaSki cells and also vaginal epithelial xenograft model analyses demonstrated that HSV-2 infection down-regulates HPV-11 and HPV-16 transcripts (FANG *et al.* 2003). To date, a few studies have investigated the effect of HSV-1 infection on HPV gene expression *in vitro* and/or *in vivo* models (KARLEN *et al*. 1993; MEYERS *et al.* 2003). HSV-1 infection down-regulated HPV-18 E6 transcripts in HeLa cells (KARLEN et al. 1993) and HPV-31b E2, E6, and E7 mRNA in CIN-6129E raft tissue (MEYERS *et al.* 2003).

A study of the cervical exfoliative cells by real-time PCR revealed that the presence of HSV-2 and HPV coinfection was much higher in cervical intraepithelial neoplasia and squamous cell carcinoma than in healthy women, OR 34.2 and OR 61.1 respectively. The authors conclude that HSV-2 infection or coinfection with HPVs may be of importance in the development of cervical neoplasias (ZHAO *et al.* 2012). However, others do not confirm the above conclusions (TRAN-THANH *et al.* 2003; ZEREU *et al.* 2007).

In our study the presence of *Ureaplasma urealyticum* stimulated the expression of HPV-16 E6 resulting in almost five-fold (4.8) increases in E6 mRNA levels in SiHa cells. To our knowledge these results are the first to suggest that U*reaplasma urealyticum* infection of the urogenital tract could increase the risk of cervical cancer by overexpression of the HPV E6 oncogene.

Ureaplasmas as cervicovaginal microorganisms have been studied as risk factors interacting with HPV in the development of precancerous and cancerous lesions of the uterine cervix (LUKIC *et al.* 2006; BIERNAT-SUDOLSKA *et al.* 2011). It is known that as a member of the *Mycoplasmataceae* family, *Ureaplasma* is comprised of two species and 14 serovars. Serovars 1, 3, 6 and 14 were classified as *Ureaplasma parvum* species and serovars 2, 4, 5, 7-13 as *Ureaplasma urealyticum*. All ureaplasmas, the smallest self-replicating organisms capable of a cell-free existence and lacking cell walls, are characterized by the possibility of urea hydrolysis to generate ATP and by adherence to human mucous membranes. *Ureaplasma urealyticum* and *Ureaplasma parvum* are commensals and pathogens of the human urogenital tract and of newborn infants. In a previous study we showed that with concomitant *Ureaplasma urealyticum* infection, the risk of HPV infection was 4.7-fold ($p<0.001$, 95 % CI: 1.362031-3.507517) greater than in its the absence. However in the case of *Ureaplasma parvum* we did not observe such a relationship (BIERNAT-SUDOLSKA *et al.* 2011). It was reported that *Ureaplasma urealyticum* was significantly more common in women with high oncogenic HPV infection and occurred more frequently in women with squamous intraepithelial lesions (LUKIC *et al.* 2006; EKIEL *et al.* 2009). Interestingly, a study on the most numerous probiotic, *Bifidobacterium adolescentis* SPM1005-A, showed a reduction of the level of HPV-16 E6 and E7 mRNA expression in examined SiHa cells. The authors suggested that *Bifidobacterium adolescentis* SPM1005-A had antiviral activity through suppression of E6 and E7 oncogene expression and could potentially be applied in HPV-associated cervical cancer prevention (CHA *et al.* 2012).

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