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.

Human cervical cancer is the second major type of cancers for women worldwide. Frequent 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 tumorigene-
sis (Scheffner et al. 1990; Werness et al.
1990). Moreover, the high-risk HPV-16 E6 pro-
tein has been reported to prevent apoptosis by a
p53-independent mechanism which involves in-
hbition 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 immortalization, and malignant
transformation, they are not sufficient.

A number of factors are associated with an in-
creased risk of HPV-related malignant transfor-
mation, 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 pa-
inents 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 tu-
morogenic conversion of HPV-immortalized cervi-
cal keratinocytes (Dipaolo et al. 1998).

The immediate-early gene products of cyto-
maelovirus (CMV) can transactivate other viral and
At least four HCMV proteins can modulate cell cy-
cle 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-Sudol ska 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 her-
pesviruses (HSV, CMV), and ureaplasmas (Urea-
plasma 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 inte-
grated human papillomavirus type 16 (HPV-16) in
1-2 copies per cell. SiHa cells (ATCC: HTB-35,
germany) supplemented with 10% heat-
activated 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
two 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 infectious 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 PPL0 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 mi-
croorganisms on liquid PPL0 media caused a col-
our 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 colo-
nies of ureaplasmas (magnification 125x). Urea-
plasma titers were determined in liquid PPL0 me-
dia 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, 2x10^3 CCU Ureaplasma parvum, 2x10^3 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% CO2 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. The quantity of HPV-16 E6 transcripts in each sample was standardized by 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 (R²). 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 PFAYFL (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 R² 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 24 h 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).
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 realtime 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 HPV's may be of importance in the development of cervical neoplasias (ZHAO et al. 2012). However, others do not confirm the above conclusions (TRAN-TANH 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 Ureaplasma 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 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).

References


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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).


