

## TT Virus in Peripheral Blood Cells From Patients with Human Papillomavirus (HPV): Investigating Association with Cervical Carcinoma

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### ABSTRACT

*Torque Teno Virus (TTV) presence was investigated in peripheral blood of 117 Brazilian women by nested polymerase chain reaction. TTV DNA was observed in 18.6% of healthy donors and in 24.32% Human Papillomavirus (HPV) patients. TTV presence was also investigated in the HPV positive group for comparison between the cervical cancer and noncancerous patients. TTV DNA prevalence was significantly higher among the HPV positive patients with cervical cancer (57.14%) than in HPV noncancerous patients (16.67%). Thus, the presence of TTV infection could be a risk factor for cancer development in the patients presenting HPV-TTV coinfection. Further studies are required to clarify the TTV influence in HPV pathogenesis.*

**Key words:** TTV DNA, HPV, cervical cancer

### INTRODUCTION

Cervical cancer is the second most common cause of cancer among the middle-aged women (40-60 years old). Current estimates indicate that every year 19,603 women are diagnosed with cervical cancer and 8,286 die from the disease in Brazil (WHO/ICO 2010). Human Papilloma Virus (HPVs) are small circular double-stranded DNA viruses, with approximately 8000 base pair (bp) genome encased in a naked icosahedral capsid of 55 nm diameters (Sinal and Woods 2005). Two major classes of genital HPV types have been identified, depending upon their association with cervical cancer. The “low-risk” types, especially

HPV6 and HPV11, are almost never found in cervical malignancies. In contrast, viral DNAs from the “high-risk” types are identified in most cervical cancer cases, although the vast majority of lesions in which they are found are nonmalignant. HPV16 and HPV18 are the two most carcinogenic HPV types and are responsible for 70% of cervical cancer and about 50% of cervical intraepithelial neoplasia (CIN) grade 3 (CIN3) (Smith et al. 2007), while HPV6 and HPV11 are responsible for about 90% of genital warts (Schiffman et al. 2007).

Cervical cancer development is a multi-step process. The major steps are HPV infection and persistence for over one year, followed by slow

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progression to precancerous lesions and, eventually, to invasive cancer. Most HPV infections resolve spontaneously in 6-12 months and the majority of precancerous lesions regress due to immune response. Since only a small proportion of HPV infections eventually might lead to cervical cancer, other cofactors are required for cervical cancer development (Schiffman and Kjaer 2003). Cofactors that modify the risk in HPV DNA positive women include the use of oral contraceptives for five or more years, smoking, high parity and previous exposure to other sexually transmitted diseases (Bosch and de Sanjose 2007). Szladek et al. (2005) reported that coinfection with a virus named Torque Teno Virus (TTV), belonging to genogroup 1 and HPV promoted the progression of squamous cell carcinoma of the larynx and was associated with an unfavorable prognosis. This TT Virus was first described in the patients with posttransfusion hepatitis by Nishizawa et al. (1997) who designated this agent as TTV after the initials of the patient in whom it was discovered.

Several studies have shown that TTV is distributed widely throughout the world (Abe et al. 1999; Vasconcelos et al. 2003; Asim et al. 2010; Maggi et al. 2010). The viral genome is a circular molecule of negative-sense single stranded DNA of approximately 3.8 kb and is organized in at least two open reading frames and a noncoding region (UTR) containing regulatory elements believed to be involved in virus replication (Bendinelli et al. 2001). No pathological role for TTV infection has been established; however, the high prevalence of the virus in the general population leads to frequent coinfections with other viruses (Thom and Petrik 2007; Nasser et al. 2009; Alavi et al. 2011). The aim of the present study was to investigate the TTV presence in HPV positive women and in controls in order to elucidate any possible influence on cancer development.

## MATERIALS AND METHODS

### Patients

Following the approval from the Human Ethics Committee of the State University of Londrina and State University of Maringá, peripheral blood cells were collected from 117 Brazilian women, including 74 HPV positive patients and 43 healthy women blood donors. The samples were collected from the women following Pap smears routine

proceedings for screening of cervical cancer in the University Hospital of State University of Londrina and University Hospital of State University of Maringá, Parana, Brazil. Molecular analyses for HPV were performed at the Molecular Genetics and Immunology Laboratory, State University of Londrina, Parana, Brazil.

### TTV DNA Detection

Genomic DNA was isolated from the peripheral blood cells (Kirby 1993) in the presence of 0.2 M NaCl and 0.25% SDS at 37°C for 4 h. After precipitation with ethanol, the pellet was dried and re-suspended in 50 µL of ultra pure water. The presence of TTV DNA was determined by the nested PCR (Polymerase Chain Reaction) using a set of four primers: in the first PCR, RD037 sense: 5' GCA GCA GCA TAT GGA TAT GT 3' and RD038 anti-sense: 5' TGA CTG TGC TAA GGC CTC TA 3' were used and; in the second PCR, RD051 sense: 5' CAT ACA CAT GAA TGC CAG GC 3' and RD052 anti-sense: 5' GTA CTT CTT GCT GGT GAA AT 3' were used. The PCR product in this system was a DNA fragment of 197 bp located in a region of the large open reading frame (ORF-1) that has been named region N22. Reaction conditions for the two PCR rounds were the same [20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTP and 1.25 units of Taq polymerase (Invitrogen Life Technologies, São Paulo, SP, Brazil)] and consisted of an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension of 72°C for 10 min using a thermocycler (PCR Sprint, ThermoHyaid, Ashford, Middlesex, UK). The PCR products of 197 bp were analyzed by electrophoresis on 10% acrylamide gel and detected by a nonradioisotopic technique using a commercially available silver staining method.

### Sequencing

The PCR products were purified using the QIAquick Gel Extraction Kit Protocol (QIAGEN, Hilden, Germany). After purification, PCR<sup>®</sup>4-TOPO<sup>®</sup> Vector plasmid (Carlsba, California, USA) was used for linked reaction and transformation into *Escherichia coli* DH5 alpha in accordance to the manufacturer's instructions. Plasmid DNA was obtained using the GFX Micro Plasmid Prep Kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA). DNA was sequenced using the DYEnamic<sup>™</sup> ET dye terminator cycle sequencing kit (Amersham Pharmacia Biotech Inc)

in MegaBace™ sequencer (Amersham Pharmacia Biotech Inc). Sequence analysis of TTV was performed and compared with data in the NCBI-NIH database (Blastn).

### Statistical analysis

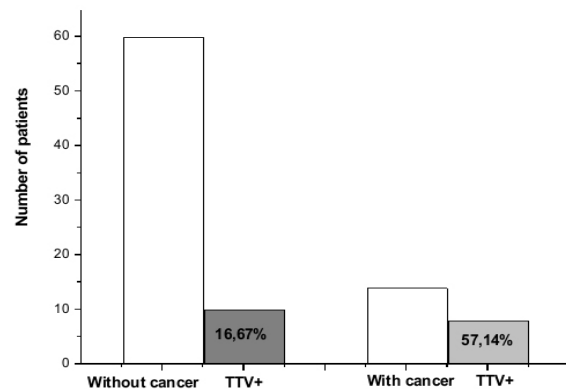
The data were analyzed by the Chi square ( $\chi^2$ ) test, with the level of significance set at  $p < 0.05$ . Demographic characteristics were evaluated by ANOVA using Microcal Origin™ 4.1 software.

## RESULTS AND DISCUSSION

It is well established that high-risk HPV infection causes cervical cancer. HPV is the most prevalent sexually transmitted viral infection among both men and women. It is estimated that 80% of sexually active adults have been infected with at least one HPV type (Baseman and Koutsky 2005). The present study analyzed the DNA samples from 117 Brazilian women, aged 13-81 years-old, including 74 HPV positive patients and 43 individuals negative for HPV. Among the HPV positive women, an age prevalence of 29-44 years-old was found, whereas among the women negative for HPV an age prevalence of 45-60 years-old was found, independent of subtypes for HPV.

Cancer progression was observed in 19% (14/74) of HPV infected women, of which 10 developed invasive epidermoid cancer. No difference in age range was observed between the HPV positive women who presented no cancer and those that developed cervical cancer, with age ranging from 13-81 and 29-74 years-old, respectively. Even though infection with oncogenic HPV genotypes is frequent among the sexually active women, most of cases are self-limiting; the development of malignant cervical lesions only occurs in a small proportion of infected women that harbor persistent infections involving oncogenic genotypes. Feher et al. (2009) investigated the occurrence of torque teno virus in malignant and potentially malignant disorders associated with human papillomavirus. The results suggested that genogroup 1 of TTV could be associated specifically with some head and neck mucosal disorders, but disproved a (co)carcinogenic role in oral cancer or cervical cancer as well as in association with HPV or with malignancies associated with it. Salakova et al. (2009) proposed that high prevalence of TTV in cervical smears suggested that sexual transmission was another

mode of expansion of TTV infection among the population. The higher viral load in cervical smears than in the respective serum samples might indicate active TTV replication in the female genital tract. The HPV infection alone is not sufficient to elicit cervical cancer development. Certain endogenous and exogenous factors can act as cofactors by influencing the risk of HPV persistence and cancer progression. TTV replication has been demonstrated in hepatocyte and leukocyte cell lines (Desai et al. 2005). Barril et al. (2000) observed the presence of TTV in serum and peripheral mononuclear cells from continuous ambulatory peritoneal dialysis using primers for the ORF1 and ORF 2 regions of TTV. It has been suggested that while concealed like a “Trojan horse”, TTV in peripheral blood cells might serve as a TTV reservoir, inducing infection and transmission chronicity in certain clinical and epidemiological settings (Zhong et al. 2002). In this work, TTV presence in peripheral blood cells was assessed in all samples, using primers for the N22 region of the large ORF-1 that amplified a 197 bp DNA fragment. Figure 1 showed the TTV DNA detection, which was amplified by the nested PCR for healthy donors and HPV positive patients.



**Figure 1** - Detection of Torque Teno Virus (TTV) DNA in Human Papillomavirus positive patients. TTV detection was realized by Polymerase Chain Reaction. A lower prevalence of TTV DNA occurred among noncancerous patients when compared to patients that developed cervical cancer. ( $\chi^2 = 10.07$ ; 1 degree of freedom;  $p < 0.01$ ).

According to the present data, TTV DNA was present in the peripheral blood cells of 18.6% (8/43) healthy individuals and 24.32% (18/74) patients. Despite the fact that no statistically significant differences occurred between these groups, TTV DNA was higher in the patient

group. Normally, RT-PCR products were detected in agarose gels, but all the products of this work were detected in acrylamide gels, since these were more sensitive than agarose for this type of detection. The cloned positive PCR samples were compared with the GenBank database and the resulting sequences revealed confirmed identification with GenBank TTV sequences.

For TTV DNA detection, N22 primer sets were used. It has been observed that when using PCR with UTR primers, TTV DNA was detected in a very high percentage of samples. TTV DNA was detected by the UTR PCR at a high frequency (93%), with no difference in prevalence between the blood donors presenting elevated ALT levels and those presenting normal levels. The results demonstrated by Itoh et al. (1999) indicated that the selection of PCR primers influenced the TTV DNA detection. These data suggested that restricted TTV genotypes detected by the N22 PCR could be associated with liver damage among the blood donors. The use of PCR with N22 primers was shown to detect the viral strains associated with hepatitis of unknown etiology. Hu et al. (2005) investigated the ability of specific primer sets to detect the known TTV genotypes and observed that the set of primers RD037/038/051/052 only amplified PCR products for genotype 1. Therefore, in the present study the presence of TTV DNA in the peripheral blood cells was investigated using N22 primers specific for TTV genotype 1. Other factors pertaining to HPV infection, such as variants, viral load, viral integration and multiple infections may modify viral-host biological interaction and play a role in the development of cervical cancer.

TTV was originally thought to be a candidate for a new hepatitis virus and although its role in liver disease remains controversial, until its pathogenic potential is fully characterized, it will remain relevant to investigate its implications in individuals presenting coinfections with other viruses (Thom and Petrik 2007). The irregular regeneration of hepatocytes in TTV positive patients has been described as significantly higher than that found in TTV negative patients (Moriyama et al. 2001). Kooistra et al. (2004) proposed that TT virus-derived apoptosis-inducing protein preferentially induced apoptosis in hepatocellular carcinoma-derived cells.

Although many reports describe that TTV does not appear to contribute to the development of human hepatocellular carcinoma from the chronic liver disease (Pineau et al. 2000; Yoshida et al. 2000);

Camci et al. (2002) described a high prevalence of TTV in the patients with hematological and solid malignancies. Feher et al. (2009) investigated the occurrence of torque tenovirus in malignant and potentially malignant disorders associated with human papillomavirus. Therefore, the presence of TTV was also investigated regarding the HPV patient group presenting cancer. TTV was significantly more prevalent among the HPV patients with cancer (57.14%; 8/14) than among detected HPV infected noncancerous patients (16.67%; 10/60) ( $p < 0.01$ ). TTV and HPV coinfection in cervical smears of patients with cervical lesions has been investigated by Salakova et al. (2009). These authors observed that symptomatic women had significantly higher prevalence of TTV DNA in cervical smears (74.7%) than healthy controls and the TTV DNA prevalence in patient serum samples was 51%. The phylogenetic groups of TTV serum isolates were concordant with those of TTV from cervical smears of the same subjects. In cervical smears, a wider variety of TTV isolates was found.

## CONCLUSION

TTV was more prevalent in HPV patients presenting cervical cancer. Although the pathogenesis of TTV remains unknown, it may be a risk factor for cancer development in the patients infected by HPV that present TTV coinfection. Further studies are required to clarify the TTV influence in HPV pathogenesis.

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