ORIGINAL PAPER

# Prevalence of Polyoma BK Virus ( BKPyV), Epstein-Barr Virus (EBV) and Human Papilloma Virus (HPV) in Oropharyngeal Cancer

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A b s t r a c t

The aim of this study was to analyze the prevalence of BK virus, Human Papillomavirus and Epstein-Barr virus in oropharyngeal cancer, and to test our hypothesis that BKV/HPV/EBV co-infection plays a role in oropharyngeal squamous cell carcinoma. The correlation between viral infection, OSCC, anatomic location, pre-treatment staging, evidence of metastases to lymph nodes, and grading was also investigated. The examination samples were collected from 62 patients from paraffin tissue blocks. Males (90.3%) with, smoking (83.9%) and alcohol abuse (67.7%) problems prevailed in the studied group. G2 histological type was recognized in 80.6% cases. T4 (77.4%) and N2 (56.5%) traits occurred in the majority of patients. No cases of metastasis were observed (M0 100%). HPV – 24.2%, EBV – 27.4% and BKV 17.7% were detected in the studied samples. We observed co-infection EBV/BKV in 8% of cases, HPV/BKV in 4.8%, and HPV/EBV in 9% cases. Only in two cases co-infection of all three viruses was found.

K e y  w o r d s: BKPyV, Epstein-Barr virus (EBV), Human Papilloma virus (HPV), oral squamous cell carcinoma (OSCC), oropharynx

## Introduction

Head and neck cancer is a very important problem in public health worldwide and as well as in Poland. In Poland oral and oropharyngeal cancer constitutes 3.8% cancers among men and 1.3% cancers among women (Didkowska *et al.*, 2013). Squamous cell carcinoma (SCC) constitutes 90% of all cases of cancers localised in the oral cavity and the oropharynx. The etiology of OSCC is considered to be multifactorial. Oncogenic viruses have also been involved in OSCC development (Hillbertz *et al.*, 2012; Scully, 2011; Alibek *et al.*, 2013). The cancerogenicity of HPV in humans was conducted by the International Agency for Research on Cancer (IARC) in 2007 and 2012. It was concluded that there is evidence indicating that HPV 16 can cause oral cancer (IARC, 2007; IARC, 2012). Ten to thirty percent of oral cancers are associated with HPV.

A number of studies also suggest the participation of Epstein-Barr virus (EBV) in oral cancer (Acharya *et al.*, 2014; Kis *et al.*, 2009; Jalouli *et al.*, 2010; Jalouli *et al.*, 2012). It is one of the most common viruses in humans (Alipov *et al.*, 2005). EBV was the first human virus to which oncogenic potential was attributed, and it is classified as group 1 carcinogen by the World Health Organization's International Agency for Research on Cancer (IARC, 1997). EBV is correlated with naso-pharyngeal and gastric carcinoma, squamous cell carcinoma, Hodgkin's lymphoma and Burkitt's lymphoma (Peh *et al.*, 2003; Slots, 2005; Slots *et al.*, 2006; Tiwawech *et al.*, 2008; Perera *et al.*, 2010; Chen, 2011).

The BK polyomavirus (BKPyV,) is a small, non-enveloped, circular double-stranded DNA virus, belonging to the *Polyomaviridae* family, genus *Orthopolyomavirus*. The *Orthopolyomavirus* genus includes the JC virus (JCPyV), BK virus (BKPyV), the Simian virus SV40, Merkel cell polyomavirus (MCV, MCPyV) and trichodysplasia spinulosa-associated polyomavirus (Benett *et al.*, 2012). All polyomaviruses encode regulatory proteins, named large and small tumor antigens (LT-ag and ST-ag). The LT-ag has oncogenic potential in cell culture and animal models. It is estimated that up to 90% of the general population may be BKV infected, mainly during childhood (between 5 and 10 years of age). The transmission route is not clearly defined; however, the orofecal and respiratory route transmission is most possible as the upper respiratory tissues are more susceptible to infections. Additionally, BKV-induced

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nephropathy is a well-known problem among kidney transplant recipients. What is more, the BKV DNA was detected in human brain tumors, in neuroblastoma, in urinary tract tumors, in carcinomas of the uterine cervix, vulva, lips and tongue, and in Kaposi's sarcoma. (Tognon *et al.*, 2003; Neirynck *et al.*, 2012; Schowalter *et al.*, 2012; Raeesi *et al.*, 2012; Konietzny *et al.*, 2012). The correlation between BKV and metastatic bladder carcinoma among immunosuppressed transplant recipients, and among BKV and prostate and bladder carcinoma was analyzed. It is probably consequence of the kidneys being the main site of BKV latency (Tognon *et al.*, 2003). On the other hand, salivary glands are described as a potential location of the virus as the presence of BKV genetic material was detected in saliva (Jeffers *et al.*, 2009). The BKV virus is detected in the oral cavity, and probably has tropism to squamous cells.

The aim of this study was to analyze the prevalence of BK virus, Human Papillomavirus and Epstein-Barr virus in oropharyngeal cancer, and to test our hypothesis that BKV/HPV/EBV co-infection plays a role in oropharyngeal squamous cell carcinoma.

## Experimental

### Materials and Methods

The present study comprised a group of 62 patients with a diagnosed and histopathologically confirmed OSCC who were hospitalized between 1995–2005 at the Chair and Department of Otolaryngology and Laryngological Oncology of the Medical University of Lublin, Poland. The patients had not received radiotherapy or chemotherapy before. TNM classification was done according to the criteria of the Union Against Cancer (UICC) (Sobin *et al.*, 2009). Histological grading was performed according to the World Health Organization criteria, which divide tumors into three types: well differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3) (Cardesa *et al.*, 2010).

**DNA extraction.** DNA was extracted from three, 10-μm thick sections of formalin-fixed and paraffin-embedded tissue. The samples were transferred to Eppendorf tubes after cutting deep into the block. The microtome blade was changed after each time. One milliliter of xylene was added into test-tubes, mixed using puls-vortexing, and later centrifuged (3 min, 8000 rpm). Then the supernatant was removed. One milliliter of 96% ethanol was added to remove the residual xylene. The samples were vortexed and then centrifuged at 800 rpm for 3 minutes. The supernatant was carefully removed. Opened tubes were incubated at 37°C until residual ethanol evaporated.

**DNA measuring.** Measurement of DNA concentration was made using spectrophotometry, and β-glo-

bin gene was amplified to evaluate the DNA extraction process and the presence of amplification reaction inhibitors.

**Detection of BKV.** The polymerase chain reaction (PCR) method was used to detect the BK/JC virus in the specimens. With the aim of detecting the genetic material of the BK/JC virus, the primers described for the first time by Arthur *et al.* (1989) were used (Table I). The oligonucleotides attach to a highly conservative region of early coding T-Ag. Because of a high homology of BKV and JCV genomes (75%), polymers are complimentary to the DNA of both viruses. The described primer pair can therefore be used for detecting both BK and JC viruses. The PCR product sequence is specific for a given virus. Primers amplify a 176 bp fragment of BKV genetic material, and 173 bp fragment of JCV genetic material. The final concentrations of the PCR reaction mixture were as follows: 2.0 mM $MgCl_2$, 200 μM dNTPs, 0.25 μM of each primer, 0.5 U Hot Start Taq DNA polymerase (Qiagen/Hilden/Germany). Amplification was performed under the following conditions: initial denaturation 94°C 15 min., followed by 40 cycles: 94°C 1 min., 55°C 1 min., 72°C 1 min.; final extension: 72°C 10 min. During each PCR

Table I
Epidemiological and clinical characteristics of patients.

|  |  | N = 62 | % |
|---|---|---|---|
| sex | female | 6 | 9.7 |
|  | male | 56 | 90.3 |
| age | 40–49 | 12 | 19.4 |
|  | 50–59 | 46 | 74.2 |
|  | 60 ≤ | 4 | 6.4 |
| place of residence | urban | 37 | 59.7 |
|  | rural | 25 | 40.3 |
| smoking | yes | 52 | 83.9 |
|  | no/no answer | 10 | 16.1 |
| alcohol abuse | yes | 42 | 67.7 |
|  | no/no answer | 20 | 32.3 |
| histology stage G | 1 | 2 | 3.2 |
|  | 2 | 50 | 80.6 |
|  | 3 | 10 | 16.2 |
| T stage | 1 | – | – |
|  | 2 | 2 | 3.2 |
|  | 3 | 12 | 19.4 |
|  | 4 | 48 | 77.4 |
| N stage | No | 11 | 17.7 |
|  | N1 | 14 | 22.6 |
|  | N2 | 35 | 56.5 |
|  | N3 | 2 | 3.2 |
| M stage | 0 | 62 | 100.0 |
|  | 1 | 0 | 0 |

Table II
The primer sequences used for the studied viruses.

| Virus | Primer sequences | Product size |
|---|---|---|
| BKPyV | PEP-1 (5'-AGTCTTTAGGGTCTTCTACC-3') <br> PEP-2 (5'-GGTGCCAACCTATGGAACAG-3') | 176 bp |
| Epstein-Barr virus | Outer <br> 5'-GTC ATC TAC GGG GAC ACG GA-3' <br> 5'-AAG AAG AGA TAT GTG GGG GT-3' <br> Inner <br> 5'-ACC CGG AGC CTG TTT GTG GC-3' <br> 5'-GGA GAA GGT CTT CTC GGC CTC-3' | 54 bp |

run, the samples were tested, together with one negative and one positive control. DNA from urine of a kidney transplant patient was used as a positive PCR control to assess the success of amplification (ATCC VR-837). PCR reagents without template DNA served as a negative control. The PCR products were analyzed using electrophoresis in 2% agarose gel.

**Detection of EBV.** Nested PCR was carried out for detection of EBV DNA (54 bp). The primer sequences are shown in Table II.

All PCR reactions were carried out in the final volume of 25 µl using Taq PCR Core Kit (Qiagen/Hilden/Germany). The concentrations of PCR reaction components were prepared as follows: 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each forward and reverse primer and 0.5 U of Taq DNA polymerase. During each run, the samples were analyzed together with one negative and positive control. The negative control consisted of nuclease-free water, while the positive control for EBV was EBV-positive cell line, Namalwa, ATCC-CRL-1432. The reaction mixture containing 5 µl of extracted DNA was amplified under the following conditions: 94°C for 3 minutes of initial denaturation, then 35 cycles of 94°C for 30 seconds, 55°C for 40 seconds, 72°C for 1 minute with the final extension at 72°C for 5 minutes. The second round amplification was performed with 1 µl of the first run product in the same conditions. The final PCR products were analyzed on 3% agarose gel.

**HPV detection and genotyping**. HPV detection and genotyping was performed using the INNO-LiPA HPV Genotyping Extraassay (Innogenetics/Gent/Belgium). The kit is based on the amplification of a 65 bp fragment from the L1 region of the HPV genome with SPF10 primer set. PCR products are subsequently typed with the reverse hybridization assay. PCR products are subsequently typed with the reverse hybridization assay. This kit identifies 28 HPV genotypes: HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 52, 52, 53, 54, 56, 58, 59, 66, 68, 69, 70,71, 73, 74, and 82.

**Statistical analysis.** Statistical analysis was performed to investigate the relationship between EBV and HPV presence and clinical and demographical characteristics of patients were determined by means of Pearson's chi-square test and with Kruskal-Wallis test for small groups. Statistical significance was defined as $p < 0.05$.

This research was approved by the Ethics Committee and is in accordance with the GCP regulations (no. KE-0254/133/2013).

## Results

Males (90.3%) with, smoking (83.9%) and alcohol abuse (67.7%) problems prevailed in the studied group. G2 histological type was recognized in 80.6% of cases. T4 (77.4%) and N2 (56.5%) traits occurred in the majority of patients. No cases of metastasis were observed (M0 100%). Characteristics of patients with oropharyngeal carcinoma are shown in Table I.

In the studied samples HPV was detected in 27.4%, EBV in 29%, and BKV in 17.7%. We observed co-infection EBV/BKV in 8% of cases, HPV/BKV in 4.8%, and HPV/EBV in 9.7%. Only in two cases co-infection all three viruses was detected. The presence of JCPyV was observed in none of the studied samples. There isn't statistical significance between HPV, EBV, BKV infection and sex, age, place of residence, tobacco smoking, alcohol use, G, T, N features. Detailed results are shown in Table III.

## Discussion

Literature on the role of HPV virus, and especially HPV 16 in oral squamous cell carcinoma is abundant (Syrjanen *et al.*, 1983; Szostek *et al.*, 2009; Jalouli *et al.*, 2010; Jalouli *et al.*, 2012; Syrjanen and Syrjanen, 2013; Metgud *et al.*, 2014; Sand and Jalouli, 2014; Sathish *et al.*, 2014). The frequency of HPV occurrence varies in different populations and is dependent on the kind of the clinical material and the applied research methods. According to the available data, the prevalence of HPV in squamous cell carcinoma of the oral cavity

Table III
Correlation between HPV, EBV, BKV and epidemiological and histological grades and TN classification.

| | | HPV + (N =17) | | | EBV + (N = 18) | | | BKV + (N = 11) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | n | % | p | n | % | p | n | % | p |
| sex | female | 2 | 88.2 | 0.6234 | 15 | 83.3 | 0.2338 | 10 | 90.9 | 0.9422 |
| | male | 15 | 11.8 | | 3 | 16.7 | | 1 | 9.1 | |
| age | 40–49 | 6 | 35.3 | 0.0698 | 1 | 5.6 | 0.1954 | 3 | 27.3 | 0.6775 |
| | 50–59 | 10 | 58.8 | | 16 | 88.8 | | 7 | 63.6 | |
| | 60 ≤ | 1 | 5.9 | | 1 | 5.6 | | 1 | 9.1 | |
| place of residence | urban | 9 | 52.9 | 0.4725 | 12 | 66.7 | | 7 | 63.6 | 0.7679 |
| | rural | 8 | 47.1 | | 6 | 33.3 | | 4 | 36.4 | |
| smoking | yes | 13 | 76.5 | 0.7962 | 13 | 72.2 | 0.0595 | 10 | 90.9 | 0.1527 |
| | no/no answer | 4 | 23.5 | | 5 | 27.8 | | 1 | 9.1 | |
| alcohol | yes | 11 | 64.7 | 0.7860 | 11 | 61.1 | 0.6593 | 9 | 81.8 | 0.1527 |
| | no/no answer | 6 | 35.3 | | 7 | 38.9 | | 2 | 18.2 | |
| G | G1 | 0 | 0 | 0.7838 | 1 | 5.6 | 0.7838 | 1 | 9.1 | 0.3987 |
| | G2 | 15 | 88.2 | | 13 | 72.2 | | 9 | 81.8 | |
| | G3 | 2 | 11.8 | | 4 | 22.2 | | 1 | 9.1 | |
| T | T2 | 2 | 11.8 | 0.4855 | 0 | 0 | 0.1527 | 10 | 90.9 | 0.4786 |
| | T3 | 0 | 0 | | 6 | 33.3 | | 1 | 9.1 | |
| | T4 | 15 | 88.2 | | 12 | 66.7 | | 0 | 0 | |
| N | N0 | 5 | 29.4 | 0.3939 | 3 | 16.7 | 0.3144 | 3 | 27.3 | 0.1649 |
| | N1 | 4 | 23.5 | | 4 | 22.2 | | 1 | 9.1 | |
| | N2 | 8 | 47.1 | | 11 | 61.1 | | 7 | 63.6 | |
| | N3 | 0 | 0 | | 0 | 0 | | 0 | 0 | |

and oropharynx varies and ranges from 8% to 74% (Castro and Busolotti, 2006; Ritchie *et al.*, 2003; Sathish *et al.*, 2014). In the present study, HPV DNA was detected in formalin-fixed and paraffin-embedded tissue in 24.2% cases. In our earlier studies from fresh frozen tissue, HPV was detected in 32.5% (HPV 16 composed 22.5%) (Polz-Gruszka *et al.*, 2015). The presence of EBV was found in 27.4% of the studied patients. The role of EBV virus in OSCC was first observed by zur Hausen (1976), while in Poland by Szkaradkiewicz *et al.* (2002). Other authors also emphasize the role of EBV in the development of OSCC (Kis *et al.*, 2009; Jaloluli *et al.*, 2010; Jaloluli *et al.*, 2011; Senyuta *et al.*, 2013). Jaloluli *et al.* (2012) detected the presence of EBV In 55% of samples from 8 different countries. A number of studies point to the co-infection by HPV and EBV in cervix cancer (Szkaradkiewicz *et al.*, 2004) and in oral squamous cell carcinoma (Al Mustafa *et al.*, 2009; Achayra *et al.*, 2014; Jiang *et al.*, 2015). Several articles indicate that co-infection by multiple oncogenic viruses may be an important risk factor in the development of OSCC (Jalouli *et al.*, 2011; Achayra *et al.*, 2014; Jiang *et al.*, 2014; Sand *et al.*, 2014). Al Moustafa *et al.* (2009) showed that high-risk HPV and EBV co-infections play an important role in initiating neoplastic transformation of human oral epithelial cells.

In our study co-infection HPV/EBV was identified in 9.7%. Jiang *et al.* (2014) suggest that co-infected cells can have a higher tumorigenic potential than normal cells.

BKV DNA was also detected (17.7%) in the studied material from oropharyngal cancer. In our earlier studies of the clinical material from oral cavity carcinoma BKV DNA was detected in 18.5% cases (Polz *et al.*, 2015). Recent data suggested a correlation between BK virus and various types of human cancers, Kaposi's sarcoma, brain tumors, and tumors of the urinary tract (Hachana *et al.*, 2012). The role of BKV in oral squamous cell carcinoma is controversial. What is more, BKV DNA presence was confirmed in high-grade squamous intraepithelial cervical lesions (precancerous lesions) (Comar *et al.*, 2011). Some authors suggest that BK virus may be a potential co-factor for HPV in the development of cervical neoplasia (Fraase *et al.*, 2011), especially together with the HPV genotype 16 (Comar *et al.*, 2011). Burger-Calderon *et al.* (2014) suggests a connection between BKPyV and the oral compartment. BKV DNA was detected in tonsilar biopsy specimens and nasopharyngeal aspirates. Besides, Moens *et al.* (2014) suggest that polyomaviruses, including those induced by other oncogenic viruses, may be a co-factor in the development of cancer. In our studies HPV/BKV co-infection was detected In 4.8% cases, while

EBV/BKV in 8%. In the light of our research results and the opinions of other researchers, we cannot exclude the role of BKV in oral SCC, taking into account the fact that the genetic material of BKV was detected in saliva (Jeffers *et al.*, 2009). Double or mixed infection with other oncogenic viruses may induce transformation. The question remains open whether BKPyV play a role in oral squamous cell carcinoma or it is a co-factor for cancers induced by other oncoviruses. It is well-known that chronic infection affects the immunological response of the host. Primary infection with a nononcogenic virus may promote superinfection with oncogenic virus capable of neoplastic transformation. The oncogenic potential of HPV is related to the expression of E6 and E7, whereas the oncogenic potential of EBV – to the expression of LMP-1 and LMP-2 and of BKV-LTag. Toll-like receptors (TLRs) play a critical role in the early innate immune response to invading pathogens by sensing microorganism and they are involved in sensing endogenous danger signals. LTag of virus BKPyV as well as the protein of LMP-1 of virus EBV lowers the expression of TLR9, which can favour the aforementioned superinfection (Moens *et al.*, 2014). According to Siennicka *et al.* (2013), TRLs simulation with microbial ligands influences EBV replication.

These observations provide insights for future studies of EBV and BKPyV pathogenesis and the association with oral squamous cell carcinoma. Future studies on the mechanisms of co-infection and/or superinfection and their role in oral squamous cell carcinoma are necessary. The knowledge about these viruses may provide targets for therapy and for diagnostic methods.

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