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The significance of mannose-binding lectin gene polymorphisms on the risk of BK virus coinfection in women with human papillomavirus–positive cervical lesions

Manola Comar [a], Ludovica Segat [b,*], Sergio Crovella [b], Massimo Bovenzi [c], Enzo Cortini [a], Mauro Tognon [d]

[a] Department of Reproductive, Developmental, and Public Health Sciences, University of Trieste, and Clinical Unit of Hygiene and Preventive Medicine, IRCCS Burlo Garofolo, Trieste, Italy
[b] Genetic Service, IRCCS Burlo Garofolo, Trieste, Italy
[c] Department of Reproductive, Developmental, and Public Health Sciences, Clinical Unit of Occupational Medicine, University of Trieste, Trieste, Italy
[d] Department of Morphology and Embryology, Section of Cell Biology and Molecular Genetics, University of Ferrara, Ferrara, Italy

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ABSTRACT

The simultaneous detection of oncogenic human papillomavirus (HPV) and BK virus (BKV) has been recently reported in cervical cancers, suggesting that these viruses may act together in the process of cell transformation; host genetic polymorphisms may also influence virus persistence/reactivation. To disclose a possible role of the gene encoding for the mannose-binding lectin, *MBL2*, in susceptibility to BKV infection, we analyzed functional polymorphisms in the first exon of *MBL2* in women stratified for the presence/absence of BKV and affected by different grades of HPV-induced cervical precancerous lesions. All BKV-positive samples were also HPV positive (HPV 16), and all presented with high-grade squamous intraepithelial lesions. The *MBL2* A allele was significantly more frequent in BKV-negative patients than in BKV-positive patients. These data indicate a possible role for the A allele in conferring protection to BKV infection in high-risk HPV-positive women (odds ratio 0.40, 95% confidence interval 0.20 – 0.85, $p = 0.01$).

1. Introduction

Human papillomavirus (HPV) is a well-established cause of cervical cancer. Indeed, oncogenic high-risk HPV genotypes are closely associated with more than 95% of cases of squamous cell carcinoma of the cervix [1]. Recent studies indicate that infection with other DNA viruses also possessing oncogenic potential may occur in HPV-positive cervical cancers [2–4]. Sequences of the polyomaviruses BK (BKV), JC, and SV40 have been detected in several different human tumors [5–8]. Among polyomaviruses, simultaneous detection of BKV and oncogenic HPVs was reported in cervical cancers and in the surrounding normal tissues of the cervix [9]. HPV and BKV, after primary infection, can remain in a latent/persistent phase or reactivate in the human host, a characteristic that may account for the long period before the onset of cell transformation and neoplasia [10]. It has been speculated that BKV could influence the progression of the tumorigenic process by acting with HPV in the early steps of cell transformation [9]. Indeed, different tumorigenic agents determine the multistep mechanism of malignant cell transformation. Nevertheless, host factors and host immune surveillance are likely to play a key role in the control of the cell transformation process [11].

Host genetic polymorphisms are certainly associated with virus persistence/reactivation and contribute both to the control and to the outcome of infection. Recently, it has been suggested that there is a critical role for some plasma proteins such as mannose-binding lectin (MBL), which contribute to the innate immune first line of defense, providing nonspecific protection against a variety of infectious pathogens and enhancing the adaptive immune response [11,12]. MBL contributes to innate immune defense by activating the complement system and promoting complement-independent opsonophagocytosis, inflammation, and apoptosis [12]. MBL deficiency has been demonstrated to be associated with increased susceptibility to many infectious agents, including human immunodeficiency virus as well as other viruses [12]. Moreover, several reports suggest that MBL can also modulate disease severity [13,14]. MBL levels within an individual can vary constantly because of its consumption in inflammatory responses as a reactant phase protein. In addition, polymorphisms in the *MBL2* gene influence MBL serum level [15–17].

To our knowledge, no association study has yet been performed to disclose the putative role of *MBL2* polymorphisms in susceptibility to BKV infection nor has the role been considered alone in the

* Corresponding author.
E-mail address: segat@burlo.trieste.it (L. Segat).

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context of HPV-positive cervical specimens. In our study we analyzed functional polymorphisms in the first exon of the *MBL2* gene in a series of women stratified according to the presence/absence of BK polyomavirus and affected by different grades of HPV-induced cervical precancerous lesions.

2. Subjects and methods

2.1. Specimens

A total of 173 cytologic swab samples, 93 classified as high-grade squamous intraepithelial lesions (HSIL) and 80 as low-grade squamous intraepithelial lesion (LSIL), were collected from women enrolled at the Gynecological Service of the Children's Hospital, Burlo Garofolo (Trieste, Italy), in a secondary care setting for cervical cancer prevention. The classification LSIL encompassed mild dysplasia and cervical intraepithelial neoplasia 1, condyloma, whereas HSIL encompassed moderate dysplasia, severe dysplasia, carcinoma *in situ*, cervical intraepithelial neoplasia 2, and cervical intraepithelial neoplasia 3, following the diagnostic criteria of the Bethesda System 2001 [18].

Moreover, 105 normal cervical samples from healthy HPV-negative women were also collected as controls. HPV infection and the presence of cervical lesions were exclusion criteria for controls.

All subjects were Caucasian from northern Italy. The significance of the study was explained to the patients and informed consent was obtained. The study was approved by the IRCCS Burlo Garofolo Ethical Committee (CIB 118/10 09/02/2010).

2.2. DNA and polymerase chain reaction analyses

Genomic DNA was extracted from cervical swab samples using the GenomePrep extraction kit (Amersham-Pharmacia, Buckinghamshire, UK). Detection of BKV sequences was performed, as previously described [19], by the real-time polymerase chain reaction method using the ABI Prism 7900 sequence detection system (Applied Biosystems, Milan, Italy) following the manufacturer's recommendations. Briefly, 10 μl of DNA and 10 μl of the specific standard scale dilution, from 10^7 to 10_0 copies, were added to a final volume of 50 μl of reaction mix in duplicate. Primers and probes detected sequences in the conserved N-terminal region of the large Tag gene, whereas BKV DNA, Dunlop-1 strain, cloned in plasmid vector was used in positive controls.

All samples were analyzed in duplicate. Cell positivity for BKV infection was considered on the assumption of 1 viral genome copy/infected cell. Viral copy quantification was performed using the single copy RNAse P gene as a reference in real-time polymerase chain reaction following the manufacturer's instructions (Applied Biosystems, Milan, Italy).

Detection and genotyping of HPV DNA were performed by linear hybridization assay (linear array detection HPV, Roche Molecular Systems, Milan, Italy). The master mix reagent used contains primers for the DNA amplification of the most significant HPV genotypes, 13 high risk and 24 low risk, and for amplification of the β-globin human gene as a genomic DNA control. This test leads to the independent identification of HPV singular genotype. β-Globin amplification–negative samples were excluded from virus evaluation.

MBL2 single nucleotide polymorphism genotyping was performed by melting temperature assay as previously described [20] using the ABI Prism 7900 sequence detection system (Applied Biosystems, Milan, Italy). The 3 *MBL2* polymorphisms, at codons 52, 54, and 57 in exon 1, were grouped together in 1 category (allele O) because they have a similar functional effect on serum MBL levels, whereas the combination of 3 wild-type alleles was grouped as allele A [12]. Melting temperature assay results have been double checked by direct sequencing of the amplicons, performed blind in 100 randomly selected samples. Sequencing reactions have been performed using the Big Dye Terminator 3.1 sequencing kit (Applied Biosystems, Milan, Italy) and run on an ABI 3130 genetic analyzer (Applied Biosystems, Milan, Italy).

2.3. Statistical analysis

Statistical analysis was performed using "R" software version 2.11.1 (http://www.r-project.org/). *MBL2* allele and genotype frequencies were calculated by direct gene counting. Fisher's exact test was used to assess the difference between proportions. Relative risk was expressed in terms of odds ratios (OR) and 95% confidence intervals (95% CI). p values < 0.05 were considered statistically significant.

3. Results

3.1. HPV and BKV analyses

DNA from 173 cytologic swab samples, composed of 93 HSIL and 80 LSIL, together with 105 normal specimens for a total of 278 DNA samples, was investigated for the presence of 37 (13 high-risk and 24 low-risk types) HPV type-specific genotypes. All 93 HSIL specimens tested positive for high-risk HPV genotypes, with the most frequent genotypes being HPV 16 (56/93; 60%), HPV 18 (13/93; 14%), and HPV 31 (11/93; 12%). In the LSIL group, HPV DNA sequences belonging to high-risk types were detected in 40 of 80 (50%) specimens analyzed; of these, 24/40 (60%) were HPV 16, 6/40 (15%) HPV 18, and 10/40 (25%) HPV 31. This different rate of positivity for high-risk HPV genotypes between HSIL and LSIL was statistically significant (100% vs 50%, respectively, $p < 2.2e\text{-}16$).

The same samples under analysis were also investigated for the presence of BKV DNA: 31 of the 93 HSIL samples (33.3%) were positive for BKV DNA sequences, whereas all LSIL samples tested BKV negative. This different distribution was statistically significant ($p = 3.19e\text{-}10$), which means that all BKV-positive samples were also HPV positive (in particular, they all were HPV 16) and all presented HSIL; none of the HPV 16–positive LSIL sammpels (24 samples) was BKV positive.

BKV DNA load in the 31 HSIL BKV-positive samples ranged from 2.8×10^3 to 1.8×10^4 copies. All samples were successfully amplified with RNAse P primers within a range from 4.5×10^5 to 1.6×10^6copies/reaction.

DNA samples from the control group all tested negative for BKV sequences. The rate of positivity for BKV was significantly higher in HSIL compared with controls (33% vs 0%, $p = 2.793e\text{-}12$) but was the same in LSIL and controls (0 in both groups).

3.2. MBL2 gene polymorphism

The *MBL2* polymorphism frequency distribution in patients positive for high-risk HPV genotype stratified for the presence/absence of BKV is reported in Table 1. The *MBL2* O allele was significantly more frequent in BKV-positive patients (29%) than in BKV-negative patients (14%; $p = 0.01$). These data indicate a possible role for the O allele in conferring susceptibility to BKV infection in high-risk HPV-positive women (OR = 2.46, CI 1.17–5.07) and for the A allele to be protective (OR = 0.40, CI 0.20–0.85). When testing a dominant and recessive model for *MBL2* genotype association with BKV, there was a significant association for *MBL2* genotypes and BKV infection in the recessive model for the A allele, which means that the presence of the A allele is able to confer protection when present in homozygosis (AA vs AO + OO, OR = 0.31, CI 0.12–0.77, $p = 0.007$; AO + AA vs OO, $p = 0.6$).

When BKV patients were divided according to the degree of the lesions, we could observe the following: (i) no difference in *MBL2* allele or genotype frequencies was evident between HSIL and LSIL in BKV-negative patients; (ii) *MBL2* A allele and AA genotype frequencies were significantly more frequent in BKV-negative/HSIL women than in BKV-positive/HSIL women ($p = 0.016$ for alleles and