DNA Sequences of Small DNA Tumor Viruses, Merkel Cell Polyomavirus and Human Papillomavirus, in Spontaneous Abortion Specimens

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Abstract:

Background. The role of viruses in spontaneous abortion (SA) is not completely known.

Methods. Two small DNA tumor viruses, Merkel cell polyomavirus (MCPyV) and Human papillomavirus (HPV), were investigated in SA. MCPyV/HPV DNAs were investigated by PCR and droplet-digital/quantitative PCRs (ddPCR/qPCR) in chorionic villi and peripheral blood mononuclear cells (PBMCs) taken from SA females (n=100), the cases, and voluntary interruption (VI, n=100) from pregnant females, the controls.

Results. MCPyV DNAs were detected in 4% and 5% of SA and VI chorionic villi, respectively, with a mean DNA load of 1.99 copy/10^4 cells in SA and 3.02 copy/10^4 cells in VI (p>0.05). HPV DNAs were detected in 2% of VI chorionic villi, with a mean DNA load of 7.12 copy/cell. Two cases in the VI samples tested HPV-45 positive. In PBMCs, MCPyV DNA was detected in 9% and 14% of SA and VI samples, with a mean viral DNA load of 2.09 and 4.70 copy/10^4 cells in SA and VI samples, respectively (p>0.05). None of the PBMCs samples tested HPV-positive.

Conclusions. MCPyV and HPV DNAs were quantified, for the first time, using ddPCR/qPCR in SA and VI chorionic villi and PBMCs. Data from the present study may help to better understand the role of MCPyV/HPV in SA.

Keywords: Pregnancy; Abortion; Virus; Cell; DNA; Sequence.
1. Introduction

Spontaneous abortion (SA) is the most common complication during pregnancy. The estimated incidence of SA ranges from 15-20% in all pregnancies [1]. The factors behind miscarriage events are not completely known. However, SA has been associated with a large number of causes, including age, ethnic origin, stress, occupational/chemical exposures lifestyle factors, such as obesity, smoking and alcohol [2]-[4]. Other causes including endocrine, hormonal, autoimmune and immunological disorders, as well as anatomical and genetic abnormalities, which have also been found to be associated with SA [5]. Male factors, including epigenetics deregulations of sperm DNA, are also related to abortive events [6].

Pathogenic infections may account for nearly 15% and 66% of early and late SAs, respectively [1]. However, the role of infections in adverse pregnancy outcome has been poorly studied [7]. Viral infections have been reported to be associated with SA [1]. Indeed, viruses may cause complications during pregnancy, ending, in the worst cases, in the loss of the embryo. The placenta and chorionic tissues can be infected by different viruses, such as, cytomegalovirus (HCMV), herpes simplex virus (HSV), dengue virus and adeno-associated virus (AAV). In addition, other viruses, including human immunodeficiency (HIV), influenza and Zika viruses are considered SA-risk factors [1]. Discordant results have been published about HSV, parvovirus and Hepatitis B virus (HBV) and pregnancy loss. While, other viruses such as Bocavirus and Hepatitis C virus (HCV) seems not to be associated with SA [1].

The role of human polyomaviruses (HPyVs) upon complications during pregnancy remains unclear [1]. Serum antibodies against Simian virus 40, JC (JCPyV) and BK (BKPyV) polyomaviruses, which are small DNA tumor viruses [8], [9], have been detected in <1 month-1 year children and in pregnant women, respectively [10]-[12]. These data suggest that HPyVs could be potentially transmitted from mother to fetus. However, only a few case-control studies investigated the association between HPyVs and SA. Moreover, these studies were conducted with a small sample size, providing qualitative data about viral DNA, only [1].

Merkel cell polyomavirus (MCPyV) is the viral agent associated with the Merkel cell carcinoma (MCC), which is a rare but aggressive tumor of the skin [13]. Recently, IgG antibodies against MCPyV were detected at a high prevalence, of up to 80%, in healthy subjects and patients affected by MCC [14]. MCPyV DNA sequences were detected in buffy coats and sera of healthy subjects, with a prevalence of 22% and 2.6%, respectively [15], [16]. MCPyV footprints have also been detected in uterine cervical cancer specimens [17], suggesting that this virus, after infecting the uterus, could participate as a risk factor in SA. A recent study investigated MCPyV DNA in fetal autopsy samples, aborted tissues, and tissues from voluntary abortions [18].

The data regarding the potential role of HPV in SA are discordant [1]. Oncogenic high-risk HPVs (HR-HPVs) are one of the most important factors in the onset/progression of gynecological malignancies [19]. Immunological data show that approximately 60% of females have serum anti-HPV antibodies [20]. Indeed, HPVs have been detected in cytological samples from normal females worldwide with a prevalence of 12% [21]. In addition, HPV DNA has been reported in sperm fluids from males belonging to miscarriage-affected couples. HPV DNA sequences have been detected with a large range of prevalence (9.9%-60%) in SA samples, and in 20-24% of specimens from voluntary pregnancy interruption (VI) of pregnancy [22]-[24].

In this study, we investigated the association between the MCPyV and HPV infections and SA. MCPyV/HPV DNA sequences were qualitatively assayed, by PCR in chorionic villi and PBMCs belonging from females affected by SA, the cases, and females who underwent voluntary pregnancy interruption (VI), the control group. Subsequently, the quantitative droplet-digital polymerase chain reaction (ddPCR) and quantitative PCR (qPCR) methods were used to quantify the amount MCPyV and HPV DNAs, respectively, in samples from SA and VI.
2. Materials and Methods

2.1 Samples

Chorionic villi specimens (n=200) and corresponding PBMCs (n=200) were from our collection [7]. The samples were taken from two different cohorts: (i) females affected by spontaneous abortion (SA, n=100, mean age ± standard deviation of mean [±SD], 36.5 yrs), the cases; (ii) females who underwent voluntary pregnancy interruption (VI, n=100, mean age ±SD, 32±6 yrs), the controls. Samples were collected by Drs. Roberta Capucci and Alice Poggi, Obstetrics and Gynecology Clinic, University Hospital of Ferrara, as published previously [7]. Written informed consents was obtained from all females. The study was approved by the County Ethics committee of Ferrara, Italy (approval, December 12, 2017; ID: 151078). The two cohorts had a gestational age ≤ 12 weeks and a similar number of pregnancies (between 1 and 6).

2.2 DNA isolation

Total DNA was purified from Chorionic villi tissues as reported [7]. PBMCs were isolated from SA and VI blood samples with a density gradient using Histopaque-1077 (Sigma-Aldrich). DNA was then extracted from PBMCs using the QIAmp DNA Blood and Tissue Extraction Kit (Qiagen, Milan, Italy) according to manufacturer’s instructions [26]. Each DNA sample was quantified and evaluated for its PCR suitability using a spectrophotometric reading (NanoDrop 2000, Thermo Scientific, Monza, Italy) and by amplifying the β-globin, respectively [27]. DNAs were isolated with a sample of salmon sperm DNA and sample lacking DNA (H2O), and subjected to PCR techniques [13].

2.3 Viral DNA detection

MCPyV and HPV genomic sequences in SA and VI chorionic villi and PBMCs were investigated using qualitative PCR. DNA from samples was analyzed by PCR with primers targeting MCPyV large T antigen (LT) region (Table 1) [13].
Table 1. Primer sets employed using PCR techniques to detect and quantify MCPyV and HPV in chorionic villi tissue specimens and PBMCs from SA and VI groups.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Primers names</th>
<th>Primers sequence (5’→3’)</th>
<th>Amplicon size (bp)</th>
<th>Annealing temp. (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCPyV</td>
<td>MCPyV-LT1709F</td>
<td>CAGGCACTGCCTGTGAATTAGGATG</td>
<td>138</td>
<td>53</td>
<td>Rotondo et al, 2017</td>
</tr>
<tr>
<td></td>
<td>MCPyV-LT1846R</td>
<td>TCAGGCACTCTTATTCTACTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV</td>
<td>GP5</td>
<td>TTTGTTACTGTGGTAGATAC</td>
<td>139-145</td>
<td>48</td>
<td>Rotondo et al, 2015</td>
</tr>
<tr>
<td></td>
<td>GP6</td>
<td>GAAAAATAAAACTGAAATCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ddPCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCPyV</td>
<td>RQMCPyV_LT_1F</td>
<td>CACAGCCAGAGCTCTTCTCTCTCTTTTCTCTCTCTGCTACTG</td>
<td>156</td>
<td>60</td>
<td>This paper</td>
</tr>
<tr>
<td></td>
<td>RQMCPyV_LT_1R</td>
<td>TGGTGGTCTCTTCTCTGCTACTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RQMCPyV Probe</td>
<td>FAM-TCCCTCTAGCGTCCAGGCTCTTA-TAMRA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>qPCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV</td>
<td>GP5+</td>
<td>TTTGTTACTGTGGTAGATACACTAC</td>
<td>139-145</td>
<td>60</td>
<td>Jacobs et al, 1995</td>
</tr>
<tr>
<td></td>
<td>GP6+</td>
<td>GAAAAATAAAACTGAAATCATATTC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The pUC57MC1 recombinant plasmid carrying DNA from the MCC350 strain (GenBank sequence EU375803) was used as a positive control [15]. HPV DNA was investigated by PCR amplifying the highly conserved HPV L1 genomic region (Table 1) [19]. Each HPV reaction was conducted with recombinant plasmid vectors containing complete HPV DNA (NC_001526.4), as positive control [19]. PCRs were carried out with 500 ng human genomic DNA [19]. Each reaction included two DNA extraction negative controls and an additional PCR negative control (H₂O). DNAs were analyzed three times by different operators with the same results. PCR amplicons were analyzed with 2-2.5% gel electrophoresis.

2.4 Viral DNA load quantification and HPV genotyping

MCPyV DNA load was determined using the ddPCR method with the QX200™ Droplet Digital™ PCR System (Bio-Rad, Segrate, Milan, Italy) [15]. A qPCR assay, using SYBR green, was performed with the CFX96 Touch™ RT-PCR Detection System (Bio-Rad, Segrate, Milan, Italy) for HPV DNA load quantification. The Primers/probes sets used in ddPCR/qPCR are shown in Table 1 [15], [28]. Recombinant plasmids containing HPV-16/18/6/11/31/33/45 DNAs were used as positive controls [19]. DNA amplification was carried out with 50 ng human genomic DNA. Standard calibration curves were generated using 10-fold dilutions, from 10⁷ to 10¹ copies, of pUC19 [29]. Human EIF2C1 and RNase P genes were used to determine the human cell equivalents for each sample under ddPCR and qPCR analysis, respectively. MCPyV and HPV DNA load values were reported as viral copies per 10⁴ human cell equivalents (copy/10⁴ cells) and per human cell equivalent (copy/cell), respectively. DNAs were analyzed three times by different operators. Each ddPCR/qPCR experiment included two DNA extraction negative controls and an additional negative control containing H₂O. HPV genotypes were determined on the basis of differential Tm obtained in qPCR [30]. Briefly, after qPCR amplification, a melting curve was performed between a 65°C and 95°C temperature range, with an increasing rate of 0.5°C s⁻¹. The melting temperature (Tm) range was identified to discriminate GP5+/6+ amplicons between 75.4-79.5±0.2°C.

2.5 Statistical analysis

Chi-square test was employed to statistically analyze the prevalence of viral DNAs in SA and VI chorionic tissues and PBMCs. Viral load values were analyzed with the D’Agostino-Pearson test for normality and means were compared with the non-parametric Mann–Whitney-U test. Statistical analyses were carried out using Graph Pad Prism version 5.0 for Windows (Graph Pad, La Jolla, CA, USA). P-values <0.05 were considered statistically significant.

3. Results

3.1 MCPyV and HPV DNA sequences analyses

MCPyV and HPV DNA sequences were investigated in chorionic villi and PBMCs, employing qualitative PCR techniques. DNA sequences belonging to MCPyV were detected in 4% (4/100) of SA chorionic tissue specimens, and in 5% (5/100) of VI samples. HPV DNA was detected in 2% (2/100) of chorionic tissue specimens belonging to the VI group, whereas none of the SA samples tested HPV-positive (Table 2; p>0.05).
**Table 2.** Prevalence of MCPyV and HPV DNA sequences in Chorionic Villi and PBMC samples, from females affected by spontaneous abortion (SA) and females who underwent voluntary pregnancy interruption (VI).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of positive samples/samples analyzed (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCPyV</td>
<td>HPV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chorionic Villi/ PBMCs</td>
<td>Chorionic Villi/ PBMCs</td>
<td></td>
</tr>
<tr>
<td>SA</td>
<td>4/100 (4)</td>
<td>0/100 (0)</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>5/100 (5)</td>
<td>2/100 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.7561</td>
<td>0.4975</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.3757</td>
<td>1.000</td>
<td></td>
</tr>
</tbody>
</table>

HPV genotyping by qPCR Tm comparison with positive controls, i.e. the recombinant plasmids carrying HPV-16/-18/-6/-11/-31/-33/-45, showed that 2 samples from the VI group were positive for HPV-45. MCPyV DNA was revealed, in 9% (9/100) of SA PBMCs and in 14% (14/100) of VI samples (Table 2; p>0.05). Furthermore, three females from the VI group resulted positive for MCPyV DNA in both chorionic tissues and PBMCs. All PBMC samples from both SA and VI cohorts were found to be HPV-negative.

### 3.2 Viral co-infection determination

Chorionic villi and PBMCs from SA and VI cohorts were previously investigated in our laboratory for the presence of two other polyomaviruses, such as JCPyV and BKPyV DNAs (submitted) using PCR techniques Co-infections with MCPyV, BKPyV, JCPyV and HPV were evaluated (Table 3).
Table 3. MCPyV, HPV, JCPyV and BKPyV co-infection in chorionic villi samples from females affected by spontaneous abortion (SA) and females who underwent voluntary pregnancy interruption (VI).

<table>
<thead>
<tr>
<th>Viral Co-infection(s)</th>
<th>Number of positive samples/ total of samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SA</td>
</tr>
<tr>
<td>MCPyV/JCPyV</td>
<td>1/100 (1)</td>
</tr>
<tr>
<td>MCPyV/JCPyV/BKPyV</td>
<td>1/100 (1)</td>
</tr>
<tr>
<td>HPV/JCPyV</td>
<td>-</td>
</tr>
<tr>
<td>JCPyV/BKPyV</td>
<td>9/100 (9)</td>
</tr>
<tr>
<td>Total</td>
<td>11/100 (11)</td>
</tr>
</tbody>
</table>

Viral co-infections were revealed in 11% (11/100) and in 14% (14/100) of SA and VI chorionic villi samples, respectively. Specifically, one (1%) and two (2%) samples from SA and VI groups, respectively, were found to be MCPyV and JCPyV-positive. In one (1%) SA sample, MCPyV DNA was detected together with JCPyV and BKPyV DNA sequences. Both chorionic villi (n=2; 2%) from VI were HPV and JCPyV-positive. Furthermore, 9% (9/100) and 10% (10/100) of SA and VI samples, respectively, tested JCPyV and BKPyV-positive. Data within groups were not statistically significant (p>0.05). SA and VI specimens JCPyV DNA sequences were more prevalent compared to the other HPyVs, such as BKPyV and MCPyV, and HPV (p<0.0001). Lastly, one PBMC sample from VI was found to be co-infected with both MCPyV and JCPyV.

3.3 Quantitative determination of viral DNA load

MCPyV DNA load in DNA from chorionic tissues and PBMCs was determined using the ddPCR method (Figure 1). Viral DNAs from both MCPyV and HPV, detected earlier by qualitative PCR assays in chorionic tissues, were confirmed using the ddPCR approach. The mean MCPyV DNA load observed in chorionic tissues was 1.99 copy/10^4 cells (range 1.25-3.35 copy/10^4 cells) in SA group (n=4) and 3.02 copy/10^4 cells (range 1.42-6.05 copy/10^4 cells) in VI group (n=5) (Figure 1, panel A).

![Figure 1](image-url)
load (viral DNA copy/cell) in chorionic villi specimens from VI (n=2). All panels: error bars represent standard mean deviation. The difference in viral load between the SA and VI groups was not statistically significant (P>0.05).

The different MCPyV DNA loads revealed in the SA and VI specimens was not statistically significant (p>0.05). MCPyV DNA sequences detected using qualitative PCR assays in PBMCs, were confirmed by ddPCR (Figure 1). The mean MCPyV DNA load observed in PBMCs was 2.09 copy/10⁴ cells (range 1.15-4.66 copy/10⁴ cells) in SA (n=9) and 4.70 copy/10⁴ cells (range 1.5-17.55 copy/10⁴ cells) in VI samples (n=14) (Figure 1, panel B). Differences in DNA loads between the SA and VI groups were not statistically significant (p>0.05). The mean HPV DNA load observed in chorionic tissues from the VI group (n=2) was 7.12 copy/cell (range 14.13-0.11 copy/cell) (Figure 1, panel C).

4. Discussion

SA represents the most common pregnancy adverse outcome. Viral infections are considered to be SA-risk factors [1]. Herein, the association between MCPyV/HPV infections and SA was investigated.

In this study, MCPyV DNA was analyzed in chorionic villi from SA and VI females with a prevalence of 4% and 5%, respectively. MCPyV DNA load, investigated for the first time in these kinds of specimens by ddPCR, was low in both SA and VI groups. Until now, only one work has reported on MCPyV DNA sequences in miscarriage samples. With the exception of one placenta sample, all tissues tested MCPyV-negative [18].

Our data, together with the previously reported results, indicate that MCPyV is not related to SA. Indeed, MCPyV detected at such a low rate suggests that this virus rarely infects the chorionic villi. In addition, quantitative data indicate that chorionic cells are very likely non-permissive for this virus, whereas host cells in which the virus establishes a persistent/latent infection are still unknown [31]. Interestingly, MCPyV DNA was detected with a low viral DNA load in PBMCs from both SA (9%) and VI (14%) specimens. These data are in agreements with the results reported in previous investigations where a low viral DNA load was detected in Buffy coats (22%) and sera (2.6%) from healthy subjects [15], [16]. Previous publications have established that healthy subjects carry a high prevalence (80%) anti-MCPyV IgG antibodies [14]. After the MCPyV primary infection, which occurs early in life, MCPyV remains lifelong in a latent phase [31]. We may speculate that the low MCPyV DNA prevalence and the low viral DNA load revealed in the SA and VI indicate a latent infection, which does not impact on embryo development.

In order to establish whether HPV might be associated with early pregnancy loss, DNA sequences of this virus were assayed in chorionic villi and PBMCs from SA and VI females. Few copies of HPV DNA were detected at a low rate in chorionic villi from VI (2%) suggesting that this viral agent is not associated with the SA. Accordingly, all PBMCs investigated were HPV-negative, indicating the absence of a systemic HPV infection. Previous works focusing on HPV and SA are contradictory. No difference in miscarriage rates were observed between HPV-positive and -negative women with abnormal cervical cytology [32]. In another study, HPV DNA was not detected in aborted placentae [33]. Other research reported that HPV DNA ratio was 2:1 in SA/VI aborted tissues, the [24, 25].

In our data indicate that there was no correlation between HPV and SA. However, additional studies are needed to verify if HPV has a role in SA.

Interestingly, we observed that chorionic villi from two cases, belonging to VI group, were HPV45-positive. It is plausible that these females were administered with the pre-pubertal quadrivalent vaccine, which is active against the oncogenic HR-HPVs, 6/-11/-16/-18 [34]. Our results
seem to indicate that this vaccine is unable to prevent infections from other oncogenic HPVs, such as HPV-45. However, the potential benefit of the new large-spectrum nonavalent vaccine, which is active against HPV -6/-11/-16/-18/-31/-33/-45/-52/-58, is advocated [34].

Since SA and VI chorionic villi were investigated, in a previous study from our groups, for the presence of JCPyV and BKPyV DNA sequences (submitted), herein HPyVs/HPVs co-infections was evaluated. Chorionic tissues from SA (11%) carried at least two different HPyVs, whereas samples from VI (14%) carried at least two different HPyVs and/or one HPyV and one HPV. The simultaneous presence of different viruses and SA has been poorly studied [35]. Notably, this is the first evaluation of HPyVs/HPV co-infection in chorionic tissues from SA and VI females. The absence of a statistically significant association between HPyVs/HPV and SA, reported in this study, our data indicate that the viral co-infections in SA have not clinical relevance. Another interpretation is that this kind of study needs a larger sample size.

5. Conclusions

This investigation shows no evidence of an association between MCPyV/HPV and SA. MCPyV and HPV DNAs were identified with a low viral load in chorionic villi from SA and VI. The few copies of MCPyV DNA detected in both chorionic tissues and PBMCs may suggest that this virus might be present in a latent/persistent phase. Negative data on PBMC samples, which tested HPV-negative, indicate the absence of an active infection. In conclusion, the results of this study indicated that HPyV/HPV infections have neither pathogenic nor clinical role in SA.

Author Contributions:

Study concept, design and supervision: Fortunato Vesce, Carlo Contini, Fernanda Martini and Mauro Tognon. Sample analysis and validation, investigation, interpretation of data, experiments execution, methodology: Andrea Tagliapietra, John Charles Rotondo, Federica Magagnoli, Elisa Mazzoni, Lucia Oton Gonzalez. Data curation, statistical analysis with software: John Charles Rotondo, Elisa Mazzoni, Lucia Oton Gonzalez, Federica Magagnoli. Writing of the manuscript, original draft preparation, data visualization: John Charles Rotondo, Andrea Tagliapietra. Critical revision/discussion of the manuscript, formal analysis, writing - review and editing: Fortunato Vesce, Carlo Contini Fernanda Martini, and Mauro Tognon. Administrative, technical, or material support: Ilaria Bononi. Resources acquisition: Ilaria Bononi. Funding acquisition, project administration: Carlo Contini, Fortunato Vesce, Mauro Tognon and Fernanda Martini.

Funding: This research was funded by the University of Ferrara, FAR grants (2017 and 2018 to CC, MT and FM); University Hospital of Ferrara-University of Ferrara joint grant (3506 to FV and MT). Associazione Italiana per la Ricerca sul Cancro (AIRC), Milan, Contract grant number: IG 16046 to MT.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.
References


