Quantitative analysis of Merkel cell polyomavirus (MCPyV) genome in non-melanoma skin cancer and normal tumor margins

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Abstract
Merkel cell polyomavirus (MCPyV) is the cause of approximately 80% of Merkel cell carcinomas (MCC). The common types of non-melanoma skin cancer (NMSC) including squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) are histologically similar to MCC. In the present study, 58 NMSC formalin-fixed paraffin-embedded tissue (FFPE) samples including 12 SCC, 46 BCC, and 58 FFPE samples of adjacent non-tumoral margins as the control were included. Determination of large tumor antigens (LTAg) copy number was performed by qReal-Time PCR as a viral copy number per cell to elucidate MCPyV carcinogenic role in non-melanoma skin cancer. Out of 58 samples, 36 (62%) cancerous and 22 (37.9%) normal tumor margins were positive for MCPyV LTAg. Median copy numbers of MCPyV LTAg among all NMSC samples and non-tumoral margins were 0.308×10−2 and 0.269×10−3 copies per cell respectively (P=0.001). In addition, although the viral load in the majority of samples was detected to be lower than one copy per cell, in 4 BCC samples, a viral load higher than one LTAg copy per cell was detected. The present study revealed that the detection of higher levels of MCPyV LTAg viral load in some BCC and SCC samples may be correlated with the role of MCPyV in some cases of BCC and SCC skin cancer.

Keywords Merkel cell polyomavirus (MCPyV) · Large T-antigen (LTAg) · Non-melanoma skin cancer (NMSC) · Basal cell carcinoma (BCC) · Squamous cell carcinoma (SCC) · Skin cancer

Introduction
Skin cancer is one of the most common types of cancer in fair-skinned populations around the world. The incidence and mortality rates of skin cancers are increasing dramatically and this poses a threat to public health [1]. Non-melanoma skin cancer (NMSC) is one of the most common types of cancer worldwide and comprises approximately 40% of all malignancies [2]. Non-melanoma skin cancer is a collective term for basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Basal cell carcinoma is the common form of skin cancer in white individuals in temperate climates and accounts for about 80% of NMSCs. Squamous cell carcinoma is the second common form of NMSC with 20% prevalence rate [3]. Squamous cell carcinoma is more malignant than BCC and has a greater ability for progression and metastasis [4]. Prolonged exposure to ultraviolet (UV) radiation from sunlight is known to be the most important cause of NMSCs, although other risk factors are proposed such as frequent medical or occupational exposure
to ionizing radiation, smoking, hereditary predisposition, immunosuppression, and infectious agents [1]. Recent epidemiological and experimental studies have shown the role of carcinogenic viruses in skin cancer [5]; in particular, Merkel cell polyomavirus (MCPyV) was detected in 80% of Merkel cell carcinoma (MCC) [6]. Merkel cell polyomavirus is a circular dsDNA virus, which belongs to the Polyomaviridae family. Merkel cell polyomavirus large tumor antigens (LTAg) and small tumor antigens (STAg) have oncogenic potential through interaction with cell cycle regulatory proteins including pRb, p53, Hsc70, and PP2A [7–9]. Primary infection with MCPyV may occur in childhood and is mostly subclinical [10], but MCC, BCC, and SCC usually occur in adulthood and aging [11, 12]. In MCC, viral genome was integrated within the tumor genome in a clonal pattern, suggesting that MCPyV infection and integration preceded the clonal expansion of the tumor cells [6, 13]. Also, sequence analysis demonstrated that integrate LTAg in all MCC cases have been truncated, whereas Rb binding LXCXE and DnaJ motifs were preserved but there were truncation mutations in exon 2 encoding the LT helicase which eliminated MCPyV replication capacity, but the oncogenic property remained [14]. The MCPyV carcinogenic association in MCC has been proven in several studies [15] that the MCPyV copy number in each MCC cell varies from one copy to more than a hundred copies [16, 17]. However, MCPyV status in SCC and BCC cancers has not been definitively proved and needs further studies regarding detection and especially viral genome quantification. Therefore, the current study aims to investigate MCPyV infection and its viral load in BCC and SCC and compare normalized viral levels in tumor and normal margins.

Material and methods

Clinical samples

In this cross-sectional study, a total of 116 formalin-fixed paraffin-embedded (FFPE) resection specimens were collected from one of the referral pathology centers in Mazandaran province, Northern Iran (Pathology Department of Shahid Beheshti Hospital, affiliated to Babol University of Medical Sciences, Babol, Iran). All cases with a history of immunosuppression or genetic susceptibility to tumors were excluded from the current study. Out of 116 specimens, 58 samples had NMSC (including 46 BCC and 12 SCC) and 58 samples were retrieved from normal tumor margins of the same patients. The gender distribution of the samples included 36 men and 22 women, and the minimum age of the patient was 39 years and the maximum was 96 years (mean 68.8 years old). The location of tumors was mostly on the face, scalp, and surrounding areas, which is mentioned in Table 1. The BCC samples based on histological classification mostly were nodular types (39 samples), and other types such as infiltrative, basosquamous, infundibulocystic, and superficial were also obtained. The SCC samples were categorized based on tumor differentiation grade as follows: three samples with moderate differentiation and nine samples with good differentiation. The study protocol was approved by the Ethics Committee of Golestan University of Medical Sciences (number: IR.GOUMS.REC.1398.123).

DNA extraction

Five tissue sections (5 μm thick) were cut by microtome and placed inside a separate microtube. After deparaffinization of samples by xylene and ethanol, DNA was extracted from each tissue sample using the DNA Extraction Mini Kit from Tissue (Yekta TajhizAzma, Tehran, Iran) according to the manufacturer’s instructions. To rule out the possibility of contamination in DNA extraction, along with the tissue samples, negative controls (sterile microcentrifuge tubes containing only reaction mixtures) were also included.

MCPyV detection and quantitation

A real-time PCR technique was utilized for quantitative detection of MCPyV DNA. The normalized viral DNA load was determined by dividing the MCPyV copy number by half of the RNase P copy number (a proven single copy gene), which described the copy number per cell. Preparation of plasmids containing cloned target sequences of MCPyV LTAg and human RNase P gene (real-time PCR standards) as described previously [18]. Quantitative real-time PCR was conducted using a Rotor Gene Q real-time PCR system (QIAGEN GmbH, Hilden, Germany) with the primer sets and TaqMan probe specified for the human RNase P gene and MCPyV LTAg gene [19, 20]. The total volume of each reaction was 25 μl containing 500 ng of extracted DNA, 12.5 μl YTA2X Multiplex Real-Time PCR Smart mix (Yekta Tajhiz Azma, Tehran, Iran), 0.3 μl each primer, and 0.2 μl dual-labeled probe. To create standard curves, real-time PCR was conducted on a tenfold dilution series of each purified plasmid pMCPyV LTAg ranging from 19×10⁹ to 19×10⁻¹ copy/μl and pRNase P ranging from 13×10⁹ to 13×10⁻¹ copy/μl. Each run of real-time PCR included reaction mixtures without DNA template as a non-template control.

Statistical analysis

The data were analyzed using R version 4.0.1. Descriptive statistics such as median and interquartile range (IQR) were used to present the quantitative variable. These variables were compared between two groups by the Wilcoxon
| Characteristics | Samples no. (%) | MCPyV LTAg Positive no. (%) | Median MCPyV LTAg (copy/cell) | P value |
|-----------------|----------------|----------------------------|-------------------------------|--------|
|                 | BCC  | SCC  | Cancer Total | Control Total | Cancer  | Control  |        |        |        |
| Total           | 46 (100) | 12 (100) | 58 (100) | 58 (100) | 36 (100) | 22 (100) | 0.086 | 0.308×10⁻² (0.607×10⁻³, 0.613×10⁻¹) | 0.269×10⁻³ (0, 0.187×10⁻²) | 0.001 |
| Gender          |      |      |             |               |         |         |        |        |        |
| Male            | 27 (58.7) | 9 (75) | 36 (62.1) | 36 (62.1) | 21 (58.3) | 15 (68.2) | 0.405 | 0.247×10⁻² (0.553×10⁻³, 0.657×10⁻²) | 0.437×10⁻³ (0, 0.25×10⁻²) | 0.046 |
| Female          | 19 (41.3) | 3 (25) | 22 (37.9) | 22 (37.9) | 15 (41.7) | 7 (31.8) | 0.133 | 0.371×10⁻² (0.833×10⁻², 0.72×10⁻¹) | 0 (0, 0.788×10⁻³) | 0.005 |
| Location of tumors |      |      |             |               |         |         |        |        |        |
| Scalp           | 14 (30.4) | 3 (25) | 17 (29.3) | 17 (29.3) | 12 (33.3) | 8 (36.4) | 0.503 | 0.277×10⁻² (0.58×10⁻², 0.562×10⁻²) | 0.193×10⁻³ (0, 0.739×10⁻²) | 0.099 |
| Ear             | 10 (21.7) | 1 (8.3) | 11 (19.0) | 11 (19.0) | 8 (22.2) | 4 (18.2) | 0.387 | 0.298×10⁻¹ (0.235×10⁻², 0.16) | 0.362×10⁻² (0, 0.19×10⁻²) | 0.017 |
| Lip             | 3 (6.5) | 0 (0.0) | 3 (5.2) | 3 (5.2) | 2 (5.6) | 2 (9.1) | 1 | 0.328×10⁻⁵ (0.24×10⁻³, 0.416×10⁻³) | 0.166×10⁻² (0, 0.597×10⁻³, 0.273×10⁻²) | 0.18 |
| Nose            | 10 (21.7) | 1 (8.3) | 11 (19.0) | 11 (19.0) | 8 (22.2) | 4 (18.2) | 0.387 | 0.298×10⁻¹ (0.235×10⁻², 0.16) | 0.362×10⁻² (0, 0.19×10⁻²) | 0.017 |
| Face            | 3 (6.5) | 0 (0.0) | 3 (5.2) | 3 (5.2) | 2 (5.6) | 2 (9.1) | 1 | 0.328×10⁻⁵ (0.24×10⁻³, 0.416×10⁻³) | 0.166×10⁻² (0, 0.597×10⁻³, 0.273×10⁻²) | 0.18 |
| Forehead        | 4 (8.7) | 1 (8.3) | 5 (8.7) | 5 (8.7) | 2 (5.6) | 2 (9.1) | 1 | 0.328×10⁻⁵ (0.24×10⁻³, 0.416×10⁻³) | 0.166×10⁻² (0, 0.597×10⁻³, 0.273×10⁻²) | 0.18 |
| Cheek           | 2 (4.3) | 1 (8.3) | 3 (5.2) | 3 (5.2) | 2 (5.6) | 1 (4.5) | 1 | 0.168 (0.573×10⁻³, 0.279) | 0.218×10⁻³ (0, 0.437×10⁻²) | 0.18 |
| Neck            | 1 (2.2) | 0 (0.0) | 1 (1.7) | 1 (1.7) | 1 (2.8) | 0 (0.0) | - | - | - | - |
| Eyelid          | 1 (2.2) | 0 (0.0) | 1 (1.7) | 1 (1.7) | 1 (2.8) | 1 (4.5) | 1 | - | - | - |
| Others          | 6 (13.0) | 0 (0.0) | 6 (10.3) | 6 (10.3) | 3 (8.3) | 1 (4.5) | 0.625 | 0.476×10⁻² (0.472×10⁻², 0.376×10⁻¹) | 0 (0, 0.526×10⁻²) | 0.109 |
| Age             |      |      |             |               |         |         |        |        |        |
| 60 >            | 10 (21.7) | 2 (16.7) | 12 (20.7) | 12 (20.7) | 7 (19.4) | 4 (18.2) | 0.548 | 0.657×10⁻² (0.307×10⁻², 0.573×10⁻¹) | 0.247×10⁻³ (0, 0.181×10⁻²) | 0.011 |
| 60 ≤            | 36 (78.3) | 10 (83.3) | 46 (79.3) | 46 (79.3) | 29 (80.6) | 18 (81.8) | 0.143 | 0.178×10⁻² (0.399×10⁻², 0.351×10⁻¹) | 0.292×10⁻³ (0, 0.155×10⁻²) | 0.029 |
signed-rank test. The binomial exact test was used for the proportions of different groups. \( P \)-value less than 0.05 was set as a significant level. The analysis of location parameters including the median and IQR between two groups, positive samples with viral copy per cell, was considered.

**Results**

**Patient’s demographic and clinical characteristics**

Study subjects were divided into two groups: patients with diagnosed BCC or SCC \( (n = 58) \) and their adjacent non-tumoral margins \( (n = 58) \). Gender distribution is given in Table 1, the samples from men were more than women, and no statistically significant difference between the distribution of two genders in BCC \( (P=0.3) \) and SCC groups were observed \( (P=0.14) \). However, there was a statistically significant difference between the distribution of the two age groups \( (60 > \) and \( 60 \leq \) years old) in BCC \( (P<0.001) \) and SCC patients \( (P=0.04) \) (Table 1).

**Detection of MCPyV LTAg**

Of the 58 cancerous samples, MCPyV LTAg was identified in 36 (62%) cases, of which 31 and 5 positive samples belonged to BCC and SCC groups, respectively. Of the 58 adjacent non-tumoral margins, MCPyV LTAg was identified in 22 (37.9%) samples, of which 18 and 4 positive samples belonged to BCC and SCC normal tumor margins, respectively. It is noteworthy that regarding all positive normal tumor margin samples, between their cancerous samples were also positive for MCPyV LTAg. There was no statistically significant difference between positivity for MCPyV LTAg in total cancerous and control groups \( (P = 0.086) \). As shown in Table 2, there was no statistically significant difference between positivity for MCPyV LTAg in BCC \( (67.3\%) \) and BCC normal tumor margins \( (39.1\%) \) \( (P = 0.085) \). Also, there was no significant difference between positivity for MCPyV LTAg in SCC \( (41.6\%) \) and SCC normal tumor margins \( (33.3\%) \) \( (P=1) \). In addition, there was no significant difference between positivity for MCPyV LTAg in males \( (P=0.405) \) and females \( (P=0.133) \) and the two age groups \( (<60 \) and \( \geq 60) \) \( (P=0.548 \) and \( P=0.143) \). Regarding the location of tumors, a high frequency of MCPyV LTAg was detected in the scalp and nose in cancerous samples compared with normal tumor margins. But, there was no significant difference between positivity for MCPyV LTAg and location of tumors in the scalp \( (P=0.503) \) and nose \( (P=0.387) \). Details regarding the detection of MCPyV LTAg in NMSC \( (BCC \) and SCC) samples and non-tumoral margins in two genders, locations of the tumor, and the age groups are shown in Table 1.

**Normalized MCPyV viral load**

The median MCPyV LTAg copy number per cell was higher in NMSC samples \( [0.308\times10^{-2} \ (0.607\times10^{-3}, 0.613\times10^{-1})] \) compared to the adjacent non-tumoral margins \( [0.269\times10^{-3} \ (0, 0.187\times10^{-2})] \). In addition, median MCPyV LTAg normalized viral load was higher in BCC \( [0.309\times10^{-2} \ (0.553\times10^{-2}, 0.704\times10^{-1})] \) and SCC \( [0.247\times10^{-2} \ (0.593\times10^{-3}, 0.572\times10^{-2})] \) samples compared with normal margins of BCC \( [0.247\times10^{-3} \ (0, 0.117\times10^{-2})] \) and SCC \( [0.193\times10^{-2} \ (0.534\times10^{-4}, 0.458\times10^{-2})] \). There was a statistically significant difference between the median MCPyV LTAg copies per cell in total cancerous and adjacent non-tumoral margins \( (P=0.001) \). Moreover, there was a significant difference between the median of MCPyV LTAg copies per cell in BCC and normal margins of BCC \( (P=0.001) \), but no statistical difference was observed between the median of MCPyV LTAg copies per cell in SCC and normal margins of SCC \( (P=0.686) \). Moreover, the median MCPyV LTAg copies per cell were significantly different between cancerous and non-tumoral margins in males \( (P=0.046) \) and females \( (P=0.005) \). Also, median MCPyV LTAg copies per cell were significantly different between cancerous and non-tumoral margins in terms of two age groups \( (<60 \) and \( \geq 60) \) \( (P=0.011 \) and \( P=0.029) \). In addition, the median MCPyV LTAg copies per cell were significantly different between BCC and non-tumoral margins of SCC \( (P=0.039) \) and females \( (P=0.009) \). Details regarding median copies per cell of MCPyV LTAg in NMSC \( (BCC \) and SCC) samples and non-tumoral margins in two genders, locations of the tumor, and the age groups are shown in Tables 1 and 2. The median MCPyV LTAg copies per cell were significantly different between BCC and non-tumoral margins of BCC in males \( (P=0.039) \) and females \( (P=0.009) \). Also, median MCPyV LTAg copies per cell were significantly different between BCC and non-tumoral margins of SCC in terms of two age groups \( (<60 \) and \( \geq 60) \) \( (P=0.028 \) and \( P=0.018) \). In addition, the median MCPyV LTAg copies per cell were significantly different between cancerous and non-tumoral margins of BCC in nose samples \( (P=0.017) \) but not significant in scalp samples \( (P=0.099) \). Details regarding median copies per cell of MCPyV LTAg in BCC \( (P=0.011) \). Regarding median MCPyV LTAg copies per cell, there were none significantly different between SCC and non-tumoral margins of SCC in any characteristics.

In the present study, the MCPyV LTAg copy number in most cancerous samples was higher than non-tumoral margins and the majority of samples had normalized viral load lower than one copy per cell, but some BCC and SCC samples had high copies per cell, particularly four BCC samples had more than one copy per cell of MCPyV LTAg (Table 3).
Discussion

Merkel cell polyomavirus is causally associated with a rare human neuroendocrine origin skin cancer, MCC [18]. Generally, in most tumors caused by polyomaviruses, viral abortive infection does occur; it is defined by virus penetration and early gene expression (viral oncopogenes) but no late gene expression (viral structural genes) and virion production. At first, all cell populations can become infected, but after subsequent cell divisions, only rare cells carrying an integrated viral oncogene proliferate [21, 22]. Therefore, the presence and high copy number of polyomavirus oncogene provided important evidence in the virus’s carcinogenic role in human cancers [23]. The normalized copy number of the MCPyV LTAg gene in MCC is usually high and more than one copy per cell [6]. The main objective of the current study was to investigate the presence and quantitate MCPyV viral load in patients with NMSC and compare it with adjacent non-tumoral margins. In addition, we used the human RNase P internal control gene as a normalizer to compare the level of infection in skin tumor cells with adjacent normal tissues.

In the present investigation, the frequency and median copy number of MCPyV LTAg in cancerous samples (both BCC and SCC groups) were higher than normal tumor margins. Viral LTAg was detected in 67.3% of BCC tumors and 39.1% of normal margins. Moreover, the MCPyV genome was observed in 41.6% of SCC compared with 33.3% of normal margins. The frequency of the MCPyV LTAg gene in non-MCC skin cancers (BCC and SCC) was evaluated in several studies across the globe. A large number of BCC (n = 114) and SCC (n = 53) samples by Kassem et al.’s study in Germany revealed the presence of MCPyV genomic sequences (LTAg or VP1) in 72.2% and 37.5% of BCCs and 52% and 25% of SCCs in immunosuppressed and immunocompetent patients respectively [24]. According to this study, MCPyV was 2-fold more frequent in immunosuppressed compared with immunocompetent BCC and SCC patients. To determine the direct carcinogenic role of the virus, we exclude immunosuppressed patients in our investigation. The lower frequency of MCPyV in Kassem et al.’s BCC and SCC immunocompetent patients compared to ours may be explained by different sensitivity of PCR techniques (end-point PCR in Kassem et al.’s study vs real-time PCR in our study). In another study done by Mertz et al., they detected MCPyV LTAg in 40.9% BCC and 21.3% SCC, by PCR techniques. In comparison to the present study, they used two type of primers, in addition immunosuppressed patient’s samples included in study [25]. In addition, the results of the present study are inconsistent with recent reports from Iran [26], which demonstrated no MCPyV detection in SCCs and only 10% positivity in BCC samples, in comparison to our study in addition of climate difference, that study detection MCPyV by conventional PCR using 2 primer sets (VP1 and LT3). Moreover, in the other two studies from Japan, low frequency of MCPyV genome was detected in BCCs and SCCs. In a study done by Satoshi Ota et al., only 2.2% of BCC cases were positive [27], also in the Murakami study, 13% of SCC samples had MCPyV genomic sequence and no MCPyV detection in BCC samples [28]. It should be noted that in these studies similar to Kassem et al.’s investigation, the presence of MCPyV was evaluated by conventional PCR, and normal tumor margins were not evaluated for virus sequences. It is noteworthy that climate, lifestyle, and genetic differences between studies might have an effect on MCPyV infection rate and carcinogenic role in non-melanoma skin cancer which need a more specific and comprehensive study between nations.

In terms of MCPyV quantitative analysis, several studies were done on MCC and non-MCC samples throughout the world. Regarding MCC samples, the investigations conducted by Shuda [16], Loyo [17], Bhatia [29], and Satoshi Ota [27] showed an MCPyV normalized viral load between one to over one hundred copies per cell. Unfortunately, in the present study, there was no access to the MCC samples for viral load determination and quantitative analysis. In the current investigation, the MCPyV LTAg normalized viral load in cancerous and normal tumor margins had a wide range; the lowest viral load was 10^-4 and the highest was 50.53 copies per cell. It is worth noting that we had 4 BCC samples with more than 1 copy per cell normalized MCPyV LTAg viral load and 2 BCC samples with moderate viral load (compared to observed in the MCCs). This finding was in agreement with Satoshi Ota’s report [27] that revealed a moderate MCPyV viral load compared to observed in the MCC (0.662 viral copies per haploid human genome) in 1 BCC case and recommended that MCPyV could be a factor in development of the minority of BCC skin tumors in addition to MCC. [27] The results of the current investigation are consistent with two reports indicating low MCPyV viral load in the majority of SCC and BCC samples [30, 31]. Regarding MCPyV LTAg low viral load in adjacent non-tumoral margins, generally, low level of MCPyV is present on all skin surfaces of most healthy individuals, and in rare part of skin surfaces especially those exposed to sunlight could be high level of MCPyV which had long-term persistence. Overall this virus is considered as a part of physiological skin microbiota [32, 33].

In non-MCC samples, the detection of low level of MCPyV LTAg might be explained by several factors. First, MCPyV could be played as a passenger virus without clear pathological consequences or viral-containing lymphocytes that infiltrate within epidermis in SCC and BCC. Second, low viral load suggests the hypothesis that the virus may be involved in the early stages of tumor progression through
### Table 2 Multivariate analysis of factors associated with BCC, SCC, and control groups

| Characteristics | MCPyV LTAg positive no. (%) | Median MCPyV LTAg (copy/cell) | P value | BCC control | SCC control | P value | SCC control | P value |
|-----------------|-----------------------------|-----------------------------|---------|-------------|-------------|---------|-------------|---------|
| Gender          |                             |                             |         |             |             |         |             |         |
| Male            | 17 (54.8)                   | 0.344                       | 0.085   | 11 (61.1)   | 4 (100)     | 0.085   | 4 (100)     | 1 0.085 |
| Female          | 14 (45.2)                   | 0.189                       | 0.085   | 7 (38.9)    | 1 (20)      | 1 0.085 |
| Location of tumors |                         |                             |         |             |             |         |             |         |
| Scalp           | 10 (32.3)                   | 0.454                       | 0.085   | 6 (33.3)    | 2 (40)      | 0.085   | 2 (50)      | 1 0.085 |
| Ear             | 3 (9.7)                     | 0.242                       | 0.085   | 2 (11.1)    | 0 (0.0)     | 0.085   | 1 0.085     | 0.085   |
| Lip             | 0 (0.0)                     | -                           | 0.085   | 0 (0.0)     | -           | 0.085   | 2 (40)      | 1 0.085 |
| Nose            | 7 (22.6)                    | 0.531×10⁻¹ (0.235×10⁻², 0.16) | 0.085   | 3 (16.7)    | 1 (20)      | 0.085   | 1 (25)      | 1 0.085 |
| Face            | 2 (6.5)                     | 0.328×10⁻³ (0.24×10⁻³, 0.16) | 0.085   | 2 (11.1)    | 0 (0.0)     | 0.085   | 1 0.085     | 0.085   |
| Forehead        | 2 (6.5)                     | 1.89×10⁻² (0.38×10⁻³, 0.16) | 0.085   | 2 (11.1)    | 0 (0.0)     | 0.085   | 1 0.085     | 0.085   |
| Cheek           | 2 (6.5)                     | 0.168 (0.57×10⁻², 0.279)    | 0.085   | 1 (5.6)     | 0 (0.0)     | 0.085   | 1 0.085     | 0.085   |
| Neck            | 1 (3.2)                     | 0 (0.0)                     | 0.085   | 0 (0.0)     | 0 (0.0)     | 0.085   | 0 (0.0)     | 0.085   |
| Eyelid          | 1 (3.2)                     | -                           | 0.085   | 1 (5.6)     | 0 (0.0)     | 0.085   | -           | -       |
| Others          | 3 (9.7)                     | 0.476×10⁻² (0.47×10⁻², 0.376×10⁻¹) | 0.085   | 1 (5.6)     | 0 (0.0)     | 0.085   | 0 (0.0)     | 0.085   |
| Age            | 60 >                         | 0.548                       | 0.085   | 7 (22.6)    | 2 (40)      | 0.085   | 1 (25)      | 1 0.085 |

The numbers in parentheses are the 95% confidence intervals.
indirect carcinogenic mechanisms such as hit and run had no role in the complete progression of malignancy [34, 35].

The findings of the present study should be interpreted with caution because of some limitations such as the lack of fresh and non-paraffin samples, the small sample size in SCC and BCC groups, and the possible presence of some normal cells in SCC and BCC sections that could reduce MCPyV LTAg genome copy number per cell. Further complementary studies can shed more light on the possible role of the virus in SCC and BCC skin tumors, including the analysis of the integration of the viral genome (especially the LTAg region), identification of nonsense or stop mutations in the LTAg region, evaluation of MCPyV oncogenes expression, and investigation of the interaction between MCPyV oncoproteins and human tumor suppressor proteins.

In conclusion, the present study revealed the detection of MCPyV LTAg sequences at low viral copy numbers (less than 1 viral copy per cell) in the majority of NMSCs and the adjacent non-tumoral margins of the same patients weakening the hypothesis of the pathogenic role of MCPyV in SCC and BCC tumorigenesis. Higher levels of MCPyV LTAg normalized viral load in some BCC and SCC samples may be correlated with the role of the virus in some cases of BCC and SCC skin cancer. These findings should encourage further investigations to determine the possible role of MCPyV in NMSC.

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| Table 2 (continued) | Characteristics | MCPyV LTAg positive no. (%) | Median MCPyV LTAg (copy/cell) | Median MCPyV LTAg (copy/cell) | Median MCPyV LTAg (copy/cell) | Median MCPyV LTAg (copy/cell) |
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Data availability The data used to support the findings of this study are included in the article.

Declarations

Ethics approval All procedures performed in studies were in accordance with the ethical standards and informed consent was obtained. This project was approved by the Ethics Committee of Golestan University of Medical Sciences (Ethics code: ir.goums.rec.1398.123).

Informed consent Informed consent was obtained from all individual participants included in the study. Individuals participate this study consent to publish article in a journal.

Competing interests The authors declare no competing interests.

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