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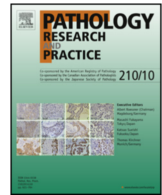

Original Article

No evidence for a role of Merkel cell polyomavirus in small cell lung cancer among Iranian subjects

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A B S T R A C T

Merkel cell polyomavirus (MCPyV), as a new member of polyomaviruses, has recently been discovered as a possible etiologic factor for human cancer. It was first detected in Merkel cell carcinoma (MCC). Small cell lung cancer (SCLC) is a malignant lung tumor which shares histopathological and genetic features with MCC, as both are of neuroendocrine origin. In this study, we investigated the presence of MCPyV DNA in SCLC specimens by real-time PCR. Our null hypothesis was that MCPyV is an etiologic factor in SCLC, as previously seen in MCC. Formalin-fixed and paraffin-embedded (FFPE) specimens were obtained from 50 patients, who underwent bronchoscopic biopsy and were diagnosed with SCLC between March 2010 and March 2012. Similarly, we obtained bronchoscopic biopsy specimens from 29 patients, who were diagnosed with non-small cell lung cancer (NSCLC). All samples were obtained at a single center (Masih Daneshvari Hospital, Tehran, Iran). Real-time PCR was done to detect the presence of MCPyV DNA. After excluding one specimen from the SCLC group due to loss of tumor tissue, we did not detect MCPyV DNA in samples from patients with either SCLC (the mean age 58.9 years, male/female ratio: 7.3/1) or NSCLC. Our results suggest that MCPyV does not play a role in the pathogenesis of SCLC, which is in accord with the results from other prior investigations.

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Introduction

Cancer is a major public health problem in many parts of the world. Currently, one in four deaths in the United States is due to cancer [1]. With more than 1.1 million deaths annually worldwide, lung cancer is the most common, and one of the deadliest types of cancer [2–4]. Patients with SCLC are different from those with NSCLC in that their presenting symptoms are frequently caused by distant metastases [2]. Unfortunately, because of the poor survival in patients with SCLC, most studies continue to focus on other types of lung cancer.

Polyomaviruses are small double-stranded DNA viruses that are suspected to be the etiologic factors in many different human malignancies. Four of these viruses, BKV (first isolated in 1971 from the urine of a renal transplant patient, initials B.K), JCV (John Cunningham virus), KIV (Karolinska Institute virus), and WUV (Washington University virus), belong to the SV40 (Simian vacuolating virus 40) subgroup [5,6]. However, a direct oncogenic role in humans has never been proven [7]. Recently, Feng et al. reported the identification of a fifth human polyomavirus, which was designated Merkel cell polyomavirus (MCPyV) based on its detection in Merkel cell carcinoma (MCC), an aggressive, metastatic form of

Abbreviations: MCPyV, Merkel cell polyomavirus; MCC, Merkel cell carcinoma; SCLC, Small cell lung cancer; PCR, Polymerase chain reaction; FFPE, Formalin-fixed and paraffin-embedded; NSCLC, Non small cell lung cancer; IHC, Immunohistochemistry; H&E, Hematoxylin and eosin; COPD, Chronic Obstructive Pulmonary Disease.

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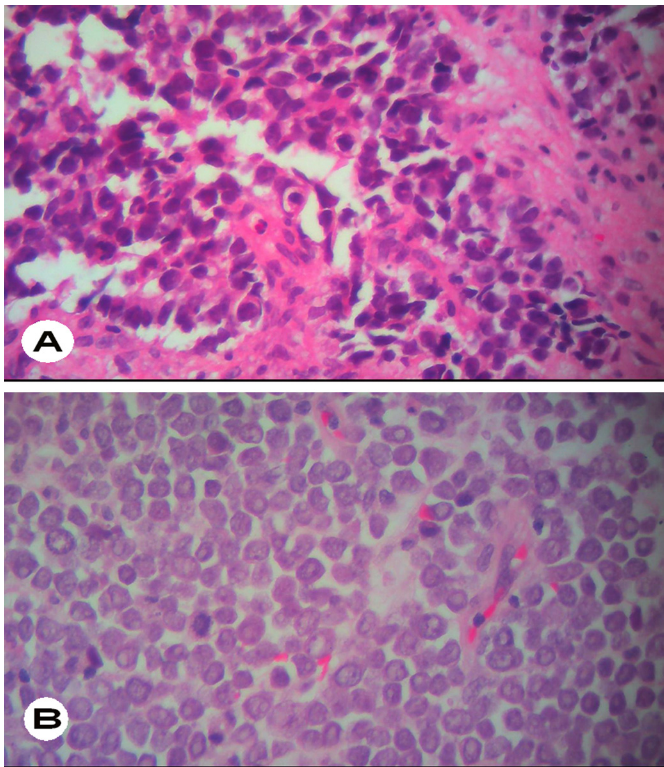

Fig. 1. Histologic slides of small cell lung cancer (A) and Merkel cell carcinoma (B), both characterized by small round to oval cells with fine granular nuclei and scanty cytoplasm (H&E, 400×). These diagnoses were confirmed by IHC studies.

skin cancer [8]. Merkel cell polyomaviurs is detected in 39%–80% of patients with MCC [8–10]. However, its association with malignancies that are histologically similar to MCC, specifically SCLC, and other high-grade neuroendocrine tumors, has yet to be investigated [11].

Merkel cell carcinoma and SCLC are neuroendocrine tumors with remarkable histopathological similarities (Fig. 1). They are made of small round cells that often contain dense core granules, and express several identical immunohistochemical markers [12]. Once a diagnosis of MCC has been made, it is recommended that patients should undergo screening for metastatic SCLC [13].

Knowing the unfavorable outcome of SCLC, and a 5-year survival rate less than 5% [4,14], few etiologic factors for this cancer have been identified. However, a viral etiology for a subset of patients developing lung cancer has been suggested [15].

Although there has been a reduction in the prevalence of smoking, and a decrease in the incidence of SCLC, there has been loss of a decline noted among women and nonwhites [16]. Interestingly, females with lung cancer have a better survival rate than males with lung cancer, despite the higher incidence of SCLC in women, which has a worse prognosis than the other types of lung cancer [3]. On the other hand, MCC with very low or undetectable levels of MCPyV DNA has a tendency for poorer survival [17].

Herein we investigated the prevalence of MCPyV as a possible etiologic factor in patients with SCLC.

Materials and methods

Design

This was a single center observational case–control study, conducted between March 2010 and March 2012 at Masih Daneshvari Hospital, Tehran, Iran. The prevalence of MCPyV in FFPE samples of bronchoscopic biopsies diagnosed as SCLC was determined by PCR. Additionally, we included FFPE samples from NSCLC with relatively similar characteristics to those of the SCLC group as our control group. Subsequently, patients' clinical information were correlated with radiologic studies and pathologic reports.

All previously performed immunohistochemistry (IHC) studies for thyroid transcription factor 1 (TTF1), and cytokeratin (CK), along with the hematoxylin and eosin (H&E) stained slides, were reviewed by two expert pathologists to confirm the diagnosis.

Studied variables

Demographic and histological data collected included sex, age, smoking status (at the time of diagnosis), histopathological diagnosis after re-examination of H&E stained slides, and presence or absence of MCPyV DNA, as detected by PCR. Treatment status of the patients and their survival were obtained from medical records.

Subjects and data collection

We used 50 FFPE samples from patients diagnosed with SCLC as the case group, and 29 FFPE samples from patients with NSCLC as our control group. These samples were obtained from the archives of the Pathology Department of the Masih Daneshvari Hospital, a teaching affiliate of Shahid Beheshti University of Medical Sciences, Tehran, Iran. All samples were collected for diagnostic purposes between 2010 and 2012. In all cases the diagnosis of either SCLC or NSCLC was confirmed after re-examination of H&E stained slides by two expert pathologists. Of the 50 SCLC patients, samples from 49 patients underwent PCR (one sample was excluded due to loss of tumor tissue after re-examination of H&E slides). Similarly, all 29 samples from patients with NSCLC underwent PCR. After re-examining H&E slides, samples that included evidence of a different malignancy, and those with necrosis of more than 50% of the tissue were excluded.

DNA extraction

Paraffin was removed from the FFPE samples after undergoing xylene treatment, followed by two washes with pure ethanol. Total DNA was extracted from the air-dried pellet tissues according to the QIAamp DNA Mini Kit procedure. The extracted DNA was stored at −20 °C until it underwent PCR.

PCR

To assess the quality of the extracted DNA, as well as inhibition of PCR test, all extracted and stored DNA underwent beta-globin PCR, using the PCO3/PCO4 primer set, as described previously [18]. To detect MCPyV, real-time PCR was performed using VP2 primer sets amplifying 82 bp gene region of the virus genome (forward primer: 5′-GCCTAGAGGTAGGAGATAAAGAATTAAAAA-3′, reverse primer: 5′-CTAGATCCTCTGCAGTGGGAAAA-3′, with an internal probe 5′FAMTTGCCCCACAGAATGCAGCAAGC-TAMRA3′) [19]. Amplification of MCPyV VP2 genes was performed in reaction volumes of 20 μL with under the following conditions: first the samples underwent denaturation at 95 °C for 10 min, followed by denaturation at 95 °C for 10 s, followed by annealing and extension at 60 °C for 1 min, 50 cycles. Real-time PCR System CFX-96 (BIO-Rad) with *Maxima Probe* qPCR *Master Mix* TaqMan reagent (Thermo Scientific) was used in Real-time PCR assay. The limit detection of five genome equivalent of MCPyV per reaction, was determined by the real-time assay, using the serial dilutions of cloned VP2 PCR fragment plasmid. In addition, we used the genome extracted from the two different FFPE MCC tissues (diagnosed between 2009 and 2011), as well as sputum samples from four patients with chronic

Table 1
The characteristics of SCLC and NSCLC patients.

Characteristic	SCLC[a] n (%)	NSCLC[b] n (%)
Gender		
Male	43 (87.8)	23 (79.3)
Female	6 (12.2)	6 (20.7)
Age		
The mean age (years)	58.9	62.7
<70 years	41 (83.7)	19 (65.5)
≥70 years	8 (16.3)	10 (34.5)
Positive smoking history		
Total	43 (87.7)	20 (69)
Male	39 (90.7)	18 (78.3)
Female	4 (66.7)	2 (33.3)

[a] 49 samples after excluding one case due to the loss of tumoral tissue.
[b] 29 samples consisting of 13 (44.8%) squamous cell carcinoma, 4 (13.8%) adenocarcinoma and 12 (41.4%) undifferentiated carcinoma.

obstructive pulmonary disease exacerbation, which were positive for MCPyV, as positive control in real-time PCR.

Because of the rarity of MCC, and presence of minimal amounts of tissue in FFPE blocks, we used previously extracted genome from two samples as positive controls.

Results

Patient characteristics

Forty-nine FFPE samples from patients diagnosed with SCLC and 29 FFPE samples from patients with NSCLC were included in the study. Re-examination of H&E slides confirmed the previous diagnosis. All SCLC tumors were primary in site. The mean age of patients was 58.9 years. Eighty-eight percent of the patients were males, with the mean age 59 years. The mean age of the female patients in this study was 58 years. Among 29 NSCLC samples, 13 (44.8%) were squamous cell carcinoma (SCC), 12 (41.4%) were undifferentiated carcinoma, and four (13.8%) were adenocarcinoma. Of the 29 NSCLC samples, 79% were from male patients, with the mean age of 63 years, and 21% were from female patients, with the mean age 62 years. Smoking was prominent in the SCLC group, 88% (43/49) of the subjects being smokers, with a mean smoking history of 32 packs/year.

In the SCLC group, 91% (39/43) of the men were smokers, compared with 67% (4/6) of the women. In the NSCLC group, 69% (20/29) of the patients were smokers, with a mean smoking history of 25 packs/year. In the NSCLC group, 78% (18/23) of men were smokers, compared to 33% (2/6) of the women (Table 1). After undergoing tumor resection, NSCLC patients mainly received gemcitabine and carboplatin as adjuvant chemotherapy. Patients with SCLC were mostly treated with etoposide and cisplatin. Whole brain radiotherapy was performed when indicated.

The frequency of MCPyV DNA in selected specimens

Beta-globin was present in the extracted DNA from all samples. All subjects were tested for the MCPyV DNA using VP2 primer set. Real-time PCR did not detect MCPyV in any of the samples from patients with SCLCs or NSCLCs.

Discussion

The causal role of viruses has been well documented in many different human cancers, including Burkitt's lymphoma, nasopharyngeal carcinoma, hepatocellular carcinoma, cervical cancer, T-cell leukemia, Kaposi's sarcoma, and lung and breast cancers [20–22]. Furthermore, the list of human oncogenic viruses continues to grow considerably due to advances in modern molecular technology.

After Feng et al. reported the causative role of MCPyV in MCC [8]; several other studies similarly reported the expression of this virus in MCCs. The MCPyV genome displays features that are found in other polyomaviruses as well [23,24]. The MCPyV genome is divided into early and late gene regions by a non-coding regulatory region. The early region encodes for alternatively spliced, overlapping RNAs, that generate large T (LT), small T (ST), and 57k T antigens [24], and share a common 78 amino acid N terminal encoded by exon 1 [23,24].

Regardless of how viral integration occurs, expression of T antigen will lead to unlicensed DNA replication from a viral origin fused into the human genome – a potential catastrophe for the nascent tumor cell [25].

Further studies on the presence of MCPyV in other human neuroendocrine tumors are warranted. One of the most interesting tumor entities to test for the presence of MCPyV certainly will be small cell lung cancer, which is also a neuroendocrine human malignancy, and with regard to histopathological findings, is nearly indistinguishable from MCC [26].

Andres et al. performed a molecular pathology study in 31 patients diagnosed with SCLC, analyzing the presence of MCPyV DNA by PCR and chemiluminescence southern blot hybridization of PCR products. After excluding the sample from one patient due to absence of beta globin product in DNA quality confirmation test, MCPyV sequences were detected in 2 out of the 30 patients (7.5%) [27].

Helmbold et al. investigated the presence of MCPyV in 18 samples from patients diagnosed with SCLC and found MCPyV in 39% (7 of 18) of the tissues but did not detect it in any of the control samples. Similarly, they did not detect SV40 in any of the tumor tissues [9].

In another study by Busam et al., 17 frozen samples from patients with MCC were examined for presence of MCPyV, using quantitative PCR. Fifteen samples (88%) were positive for MCPyV DNA. Subsequently, frozen sections from these samples underwent IHC examination, with the novel monoclonal antibody CM2B4, generated against an antigenic epitope on the MCV T antigen. Sufficient material for IHC was available in only 15 of the 17 cases. Of the 15 samples analyzed, 10 (67%) showed positive labeling with CM2B4. Twenty-six samples from pulmonary carcinomas of neuroendocrine origin were also examined by the same method and none reacted with CM2B4 [28]. Wetzels et al. studied the prevalence of MCPyV in patients with MCC and SCLC. They studied samples from 5 patients with MCC and 10 patients with SCLC and detected MCPyV in 2 samples from patients with MCC. Similar to our study, they did not detect MCPyV in any of the samples from patients with SCLC [29]. Our study included a larger sample size and we did not detect MCPyV in any of the 49 samples with SCLCs or the 29 samples with NSCLC.

Based on recent epidemiologic studies, SCLC used to be the predominant lung cancer in males (51.2% in males versus 48.8% in females). However, this has changed and according to a report in 2007, SCLC has become more prevalent among females (49.6% in males versus 50.4% in females) [30]. In our study, SCLC was more prevalent in the biopsy specimens from male patients (88% in males versus 12% in females). According to the same epidemiologic report from 2007, 61% of the patients with SCLC were younger than 70 years old, and 39% were 70 years old or older. In our study only 16% of the patients with SCLCs were 70 years or older.

Several recent studies have attempted to evaluate the presence of MCPyV in non-neuroendocrine tumors. Non-small cell lung cancer is one of these tumors. In a study published by Gheit et al., 86 samples from patients with NSCLC, including adenocarcinomas, and squamous cell lung carcinomas, were analyzed for the

presence of MCPyV, using PCR. Four out of the 86 samples (4.7%) were positive for MCPyV [15]. We examined 29 samples from patients with NSCLC, including 13 (44.8%) SCCs, 4 (13.8%) adenocarcinomas, and 12 (41.4%) undifferentiated carcinomas. All 29 samples were negative for MCPyV.

All of our subjects were newly diagnosed with cancer and had not undergone treatment. Therefore, treatment could not have possibly influenced our results. In summary, it appears that parallel to the rapid progression of genetic pathogenesis in cancer, discovery of new human viruses has provided fundamental insight into pathogenesis of cancer, as well as new treatment options. Further studies are necessary to better understand cancer pathophysiology, and come up with new treatment options, and prevention methods.

Conclusion

Our results demonstrate that despite MCPyV's presence and role in the pathogenesis of MCC, it is not present in patients diagnosed with SCLC. The detection rates of MCPyV in SCLC are subject to wide variations, and perhaps reflect the possible geographic variability in infection with MCPyV. We did not detect MCPyV DNA in the non-neuroendocrine tumors (NSCLC samples) in our study. Further studies with larger sample sizes are recommended to confirm our results. Detection of MCPyV by gene targeting primers is also suggested. Finally, MCPyV detection sensitivity in tumor tissues may be improved by using both PCR and IHC methods, especially with the development of IHC techniques using novel monoclonal antibodies that target MCPyV large T antigen.

Conflicts of interest

The authors have no conflicts of interest to declare.

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