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ORIGINAL ARTICLE

Epidemiological, Clinicopathological and Virological Features of Merkel Cell Carcinomas in Medical Center of University of Pécs, Hungary (2007–2012)

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Abstract Merkel cell carcinoma (MCC) is a rare, highly aggressive skin tumour. In 2008, a Merkel cell polyomavirus (MC) was identified in MCCs as a potential etiological factor of MCC. The aims of this retrospective study were to investigate the epidemiological, clinicopathological and virological features of MCCs. Between 2007 and 2012, 11 patients had been diagnosed with MCC by histological methods in University of Pécs, Hungary. In eight MCC cases MC was tested by PCR (in primary skin lesions, lymph nodes/cutan metastases, MCC neighboring carcinomas). Clinicopathological characteristics (age, histological pattern, lymphovascular invasion, co-morbidities) of MC-positive and MC-negative cases were compared. MC was detected in three (37.5 %) out of eight patients' primary tumour or metastasis. The average age was 73.8 (64.3 in MC-positive group). Except the youngest, 55 year-old patient (the primary tumour appeared on his leg), all tumours were found at the head and neck region. Immunosuppression (steroid therapy, chronic lymphoid leukaemia, chronic obstructive pulmonary disease) and/or old age were characteristic for all cases. Histological pattern was different in MC-positive and in MC-negative groups: MCCs with MC showed more homogeneous histological pattern, lack of lymphovascular invasion and were associated with better prognosis (mortality rate: 33 % versus 80 %). MCC associated with oncogenic virus is a newly recognized clinical entity. However, MC could not be detected in all histologically proven MCCs. The well-defined selection of patients/disease groups and better characterization of differences between MC-positive and negative cases is an important step towards the recognition of the etiology and pathogenesis of all MCCs.

Keywords Merkel cell polyomavirus · Merkel cell carcinoma · Skin tumour · DNA tumour virus

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Introduction

The pathogenic role of infectious agents has already been proved in ~20 % of the human tumours. Merkel cell carcinoma (MCC) is a rare and highly aggressive type of skin cancer. Its origin is uncertain, but it is thought that this (neuroendocrine?/epithelial?) carcinoma arises from the stratum basale [1, 2]. MCC affects mostly elderly and immunosuppressed patients, and it frequently occurs on sun-exposed areas of the skin, especially at the head and neck region [3]. A DNA tumour virus, a Merkel cell polyomavirus (MC), was identified in MCC in 2008 [4]. The discovery opened new perspectives for uncovering the etiology of the disease. The suggested infectious – viral – origin of the disease results in attitude change not only concerning the etiology, but also pathogenesis, diagnostics, and then in the future, prevention and therapy of MCC. The importance of the prognosis is further emphasized by the fact that the lethality of MCC is more than three times higher (46 %) than that of malignant melanoma (12 %) related to all diagnoses [5]. Since its discovery, MC polyomavirus is detected between 71 and 94 % of the MCCs by PCR, worldwide [6]. The MC polyomavirus is not well studied in Hungary. Recently, MC polyomavirus was detected by molecular methods in seven of the eight MCC lesions both in primary

tumors and in recurrent lesions in University of Szeged, Szeged, Hungary [7].

The aim of this study was the detection of MC polyomavirus in our series of MCC samples in Southwest Hungary, and the investigation of the epidemiological and clinicopathological characteristics.

Materials and Methods

Sample Selection

Archived skin samples were collected retrospectively from the period between 2007 and 2012 in the Department of Pathology, University of Pécs. The histological diagnosis relied on haematoxylin-eosin morphology and immunophenotype, using monoclonal antibodies as CK20, CD56, Synaptophysin, ChromograninA and TTF1 [8]. All samples were reanalysed histologically in 2012. Beside the primary MCC, the available skin and lymph node metastases, and the basal and squamous cell carcinomas adjacent to some MCCs were also tested. Samples with inadequate DNA quality were excluded from the study.

Viral DNA Isolation and PCR

Viral DNA was isolated from native (N = 6) or in paraffin embedded (N = 9) tissue samples after deparaffinization using commercial extraction buffer (BK virus PCR kit, Shanghai Biotech, Brussels, Belgium) [9]. MC polyomavirus was detected by PCR method as published previously [4]. In one case, the total genome sequence of the MC polyomavirus was determined by primer walking method.

Detection of non-MC Polyomaviruses in MCC Samples

Newly discovered polyomaviruses, polyomavirus 6, 7, 9 and trichodysplasia spinulosa associated polyomavirus were also screened by PCR method as published previously in MCCs samples [10–12].

Epidemiological and Clinical Data

The patients' data have been collected from the electronic medical reports with special attention to the case history, comorbidities, potential immunosuppressive factors, location of MCC and the time elapsed between the appearance of symptoms and the diagnosis. Clinicopathological characteristics (age, histological picture and lymphovascular invasion, comorbidities) have been evaluated in MC-positive and MC-negative cases.

Results

Altogether, 11 patients have been diagnosed histologically with MCC in the Department of Pathology, University of Pécs between 2007 and 2012. A total of 15 histological samples of the 11 patients were available. Three patients and their samples were excluded from the study because of inadequate sample quality.

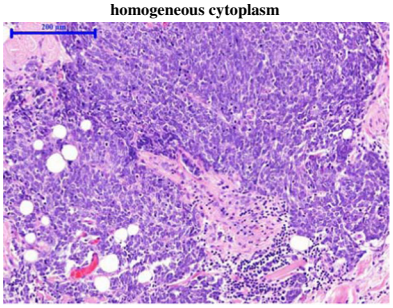
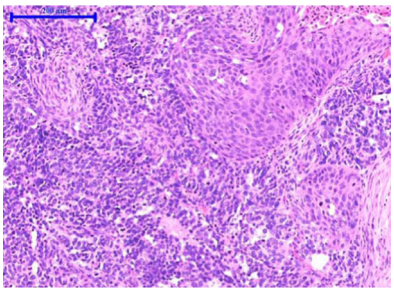
Out of the eight patients, two had three different samples, three had two different samples and three patients had one sample each (Table 1). MC polyomavirus was successfully detected by PCR in samples from three (37.5 %) patients' primary tumours (2 samples), skin (1 sample) and lymph node (2 samples) metastases (Table 1). In one patient (No1), the viral genome was present both in the primary skin tumour, and in the skin and lymph node metastases (Table 1). The histologically non-metastatic lymph node (patient No3) was negative for MC by PCR. None of the 15 samples were positive for polyomavirus 6, 7 and 9 by PCR.

Table 1 summarizes the epidemiological, clinical, virological and histopathological characteristics of the eight patients investigated (Table 1). None of the preliminary clinical diagnoses was MCC before the histological examination. Pyogenic granuloma, keratoacanthoma, basal cell carcinoma and squamous cell carcinoma were established as primary clinical diagnoses. Out of the eight patients, 6 (75 %) were male and 2 (25 %) were female. In the case of the MC PCR-positive patients, 66 % percent were female and 33 % were male. The year-wise distribution of the histological diagnoses was as follows: one patient in 2007, 2008, and 2012 in each, two patients in 2010, and three patients in 2011. The youngest patient was 55 year old, the oldest was 86. The patients' average age was 73.8 (this was 64.3 in the case of the MC PCR-positive patients). With the exception of the youngest patient (the MC-positive primary tumour appeared on his leg), all tumours were found at the head and neck region (face, forehead, nostril and neck). Factors predisposing to MCC such as immunosuppression (e.g. prolonged oral steroid therapy, chronic lymphoid leukaemia, chronic obstructive pulmonary disease) and/or old age were found in all cases.

Morphological differences could be found in the histological pattern of the MC-positive and MC-negative groups (Table 1). In the MC polyomavirus-associated cases, the histological samples have much more ordered structure (round or oval cells with vesicular nuclei and homogeneous cytoplasm), while the structure of MC-negative cases was much more disordered (irregular, heterogeneous tumour cells having polygonal nuclei and heterogeneous cytoplasm).

Additionally, there were differences regarding lymphovascular invasion of the primary tumour (Fig. 1). While lymphovascular invasion could be observed at the edge of the MC-negative primary tumours, this could not be observed in the MC-positive cases.

Table 1 Epidemiological, clinicopathological, and virological characteristics of the Merkel cell carcinoma including histopathological characteristics of primary skin tumours (Merkel cell carcinoma) in histological samples (haematoxylin-eosin; 14-fold magnification): (A) Merkel cell polyomavirus can be detected by PCR; (B) Merkel cell polyomavirus can not be detected by PCR. In picture (B), squamous cell carcinomas can also be observed

Epidemiological data				Clinical data			Virological results	Histopathological results	
Patients (sample)		Age (years)	Date of the (histological) diagnosis († death)	Type of the sample	Localization	Co-morbidities	MC-polyomavirus (PCR)	Lymphovascular invasion of primary MCC	Histological pattern
No1 ♂	sample 1	55	June, 2011 († May, 2013)	MCC	leg	COPD, psoriasis, (PUVA therapy)	+	-	A: round or oval cells with vesicular nuclei and homogeneous cytoplasm 
	sample 2			skin metastasis	leg (native)		+		
	sample 3			lymph node	groin (native)		+		
No2 ♀		68	May, 2011	MCC	nostril	pemphigus vulgaris, steroid therapy	+	-	
No3 ♀	sample 1	70	December, 2010	lymph node	neck (native)	severe coronary artery disease	+	-	
	sample 2			lymph node (histology revealed no metastasis)	neck		-		
No4 ♂		86	November, 2012	MCC	face (native)	stroke basal cell carcinoma	-	+	B: irregular, heterogeneous tumour cells with polygonal nuclei, and heterogeneous cytoplasm 
No5 ♂		77	November, 2010 († January 2011)	MCC	forehead (native)	seborrheic keratosis, CLL	-	+	
No6 ♂	sample 1	80	July, 2011 († April 2012)	MCC	face (native)	COPD, diabetes, basal cell carcinoma, hypertonia	-	+	
	sample 2			lymph node	face		-		
	sample 3			basal cell carcinoma	face		-		
No7 ♂	sample 1	77	October, 2007 († June 2010)	MCC	face	COPD basal cell carcinoma	-	+	
	sample 2			lymph node	neck		-		
No8 ♂	sample 1	78	November, 2008 († January 2009)	MCC	neck	squamous cell carcinoma	-	+	
	sample 2			squamous cell carcinoma (*in situ*)	neck		-		

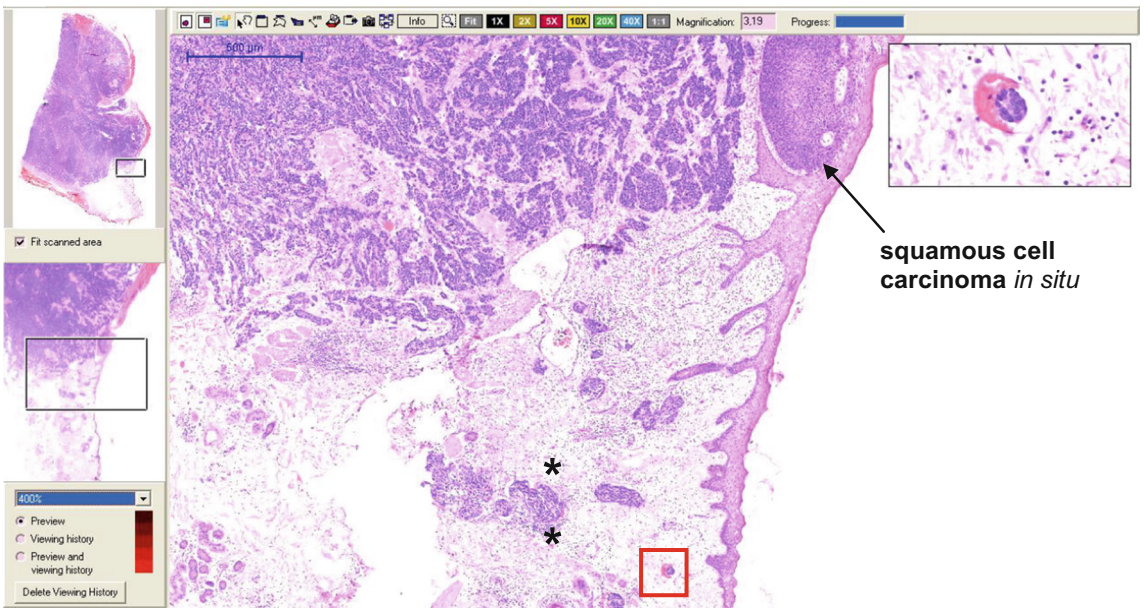


Fig. 1 MC polyomavirus-negative Merkel cell carcinoma (haematoxylin-eosin, 3.19-fold magnification). The invasion of lymphovascular spaces by the tumour cells can be observed at the areas marked by *asterix* (*); invasion into a small vascular structure can be seen in the small red square (12.76-fold magnification in the right upper corner); and an in situ squamous cell carcinoma can also be seen

Detailed Characteristics of the Cases

The medical history of the youngest patient (No1) revealed end stage COPD and psoriasis vulgaris (Table 1) treated with PUVA for 8-years. The primary tumour appeared on his left lower leg, which was incompletely excised surgically in June 2, 2011, 2 months after the first appearance. After the histological diagnosis of MCC, the patient experienced a rapid re-growth of the tumour in the primary tumour site and enlarged left inguinal lymph nodes were palpable upon physical examination. In November 2011, primary tumour site was re-operated with wide surgical margins and left inguinal block dissection was performed (Fig. 2). Radiotherapy was introduced to the left groin region. The dissemination of the disease was detected on January 14, 2013. CDDP/EPI chemotherapy was started according to the established guidelines. The control CT-scan showed progression after the 4th treatment cycle. After 6 cycles of chemotherapy the patient died of progressive carcinoma in May 17, 2013. MC polyomavirus could be detected in the paraffin-embedded sample of his primary tumour and the native samples of the skin and lymph node metastases.

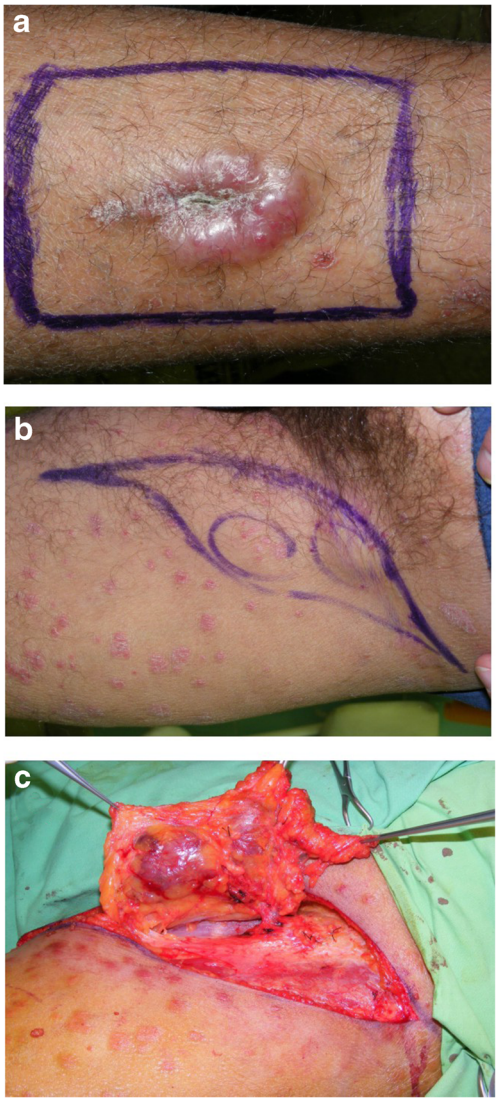


Fig. 2 Recurrent MCC at the primary site of the tumour (**a**), inguinal lymph nodes metastasis (**b**) and its surgical excision (**c**) (Patient No1). Erythro-squamous lesions due to psoriasis were also visible on the skin

Patient No2 was 68 when she was diagnosed with MCC in May 2011. She was diagnosed with pemphigus vulgaris in 2004, and therefore she was administered methylprednisolone and azathioprine daily. In March 19, 2011, there was a growing lesion on her left nostril for about 1 month, which was diagnosed as pyogenic granuloma. She got a surgery date for June 6, 2011 but due to the rapid tissue growth the surgery was done on May 24, 2011. MC-polyomavirus could be detected in the paraffin-embedded sample. Since the operation, the patient is under close observation, and no progression was found to date.

Patient No3 with ischaemic heart disease had an acute myocardial infarction in 1994. She was diagnosed with paroxysmal atrial fibrillation in 2002. She had triple coronary bypass surgery in December 2004 and pacemaker implantation in July 2011. A firm, elastic nodule 10 mm in diameter was removed from her nostril in a city hospital in December 6, 2010. By then, the nodule has already grown for 1 month. The neck ultrasound and the ultrasound-guided fine-needle aspiration biopsy (FNAB) revealed right-sided submandibular lymph node metastasis in March 30, 2011. The neck block dissection was carried out in May 9, 2011. The primary tumour was not available for further analysis. MC polyomavirus could be detected by PCR in the metastatic lymph node. Since the operation, the patient is under close follow-up, and no progression was found to date. In the native metastatic lymph node sample the complete 5392 nucleotide long genome of MC polyomavirus (KC202810) could be determined. The polyomavirus differed from the prototype MC (EU375803) in 10 nucleotides (0.19 %). Truncating point mutation (cytosine to thymine) could be detected at nucleotide position 1461th of the large T (LT)-protein-coding part of the MC polyomavirus genome.

Patient No5 was 77, when he was diagnosed with MCC in November 13, 2010. Previously, he was diagnosed with chronic lymphocytic leukaemia in 2005 and type-II diabetes in 2007. The MCC appeared on his forehead and was rapidly growing towards the vertex. The tumour was 3 × 3 × 2 cm in size, bleeding, and ulcerated by the time of the surgery in November 28, 2010 (Fig. 3). At the same time, a pigmented, reticular type seborrhoeic keratosis 5 × 5 mm in size was also excised left and caudally from the MCC. MC polyomavirus could not be detected in the paraffin-embedded sample. The patient died 1.5 month after the surgery.

Neighbouring MCC, squamous cell carcinoma in situ could be observed in patient No8, and basal cell carcinoma in patients No4, No6 and No7. In these cases, MC

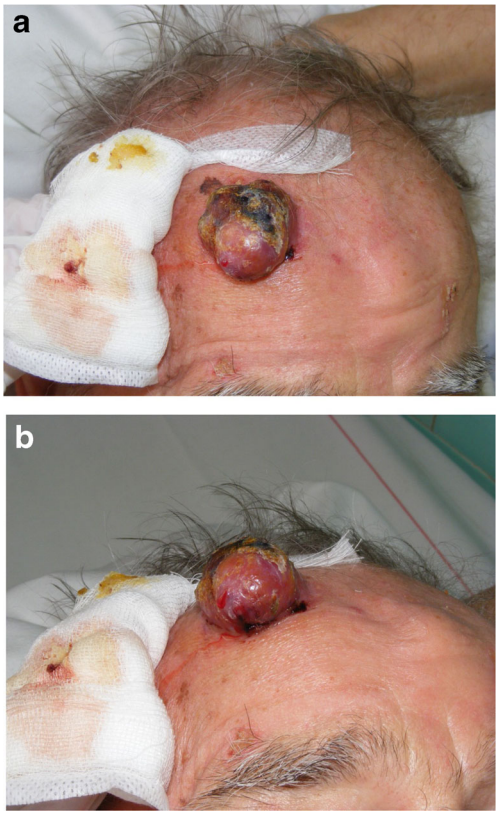


Fig. 3 Merkel cell carcinoma in the right frontal region with 1 month history of appearance (Patient No5). Pigmented seborrhoeic keratosis is visible at left and caudally from the MCC

polyomavirus could not be detected by PCR neither in MCC, nor in associated tumours and metastatic lymph nodes (three native and seven paraffin-embedded samples). Three out of the four patients died meanwhile.

Discussion

Merkel cell carcinoma of the skin was first described by Toker in 1972 as "trabecular carcinoma of the skin" [13]. As an oncogenic virus associated cancer, it is a novel clinical entity. Although this previously rare disease has been known for a long time, the increasing number of the acquired immunosuppressive conditions has led to the increasing occurrence of the disease, and later to the confirmation of the infectious origin [3, 14]. In this study, MC polyomavirus was detected partly in histological samples of MCC by molecular techniques, and in one case the location of the truncating point mutation having crucial role in tumour formation was identified.

None of the preliminary clinical diagnoses raised the suspicion of MCC before the histological examination in cases above, emphasizing the differential diagnostic problem leading to incorrect or incomplete diagnoses in clinical practice. Some of the conditions do not require urgent, wide surgical excision as opposed to the rapidly progrediating MCC. The early recognition of MCC is especially important, since the expected 5-year survival is 79 % in the early stage (I/A), but it is only 18 % by the time of the appearance of the first distant metastasis (stage IV) [5].

The improvement of knowledge, minimization of risk factors, early recognition and effective treatment highly contribute to the increase in MCC patients' survival. We consider the case of the young 55-year-old patient especially instructive, since the development of the MCC and the patient's (early) death was probably activated by a medical treatment (PUVA) of a basically non-lethal disease (psoriasis), which therapy increases the risk of MCC nearly 100-fold [15]. This raises the attention to the fact that risk factors should be known and avoided as much as possible. Patients treated by any means of immunosuppressive method should be followed up closely, and in case of suspicious skin changes, an early intervention is necessary. This also means that similarly to other tumour types, MCC also requires cooperation of different specialists. Close co-operation of the observing physician and the dermatologist, pathologist, oncologist, radiologist, surgeon and in some cases the microbiologist may also be needed.

Although we worked with a relatively small cohort, and our results are based on retrospective data and archival histological samples, we consider our findings important. Considering the epidemiological characteristics of our patients, we should highlight that the average age (73.8 years) was in accordance with the published data (69 years), as was gender distribution (66 % of our patients are male similarly to known international data demonstrating a higher ratio – 59 % - of MCC patients being males) [3]. Considering the localization of the tumour, there was no difference between the virus-associated and virus non-associated cases. Similarly to the literature data, the tumour appeared at the head and neck region in all but one case. In our series in case of the youngest patient the primary tumour developed on a naturally sun-protected area of the leg. All patients' history revealed some factors predisposing to MCC, like immunosuppression and/or old age. In our patients, the most frequent locations of the metastases were the skin and the lymph nodes. This could be observed in three quarter of the cases, which can be partially explained by the fact that the tumours were diagnosed in late stages, and the treatments were ineffective.

Our results can be useful not only for clinical practice but also for laboratory diagnostics. The well-defined selection of patients/disease groups and better characterization of differences between MC-polyomavirus positive and negative cases is an important step towards the recognition of the etiology and pathogenesis of all MCCs. The diagnosis of MCC is based on histological examination. According to our observations, the presence of MC polyomavirus can already be suggested based on the histological examination. The histological pattern of the virus-negative tumour tissues shows much more irregular, heterogeneous tumour cells with polygonal nuclei

and sometimes light-coloured, less uniform cytoplasm. The MC-positive tumour cells are round or oval; they have vesicular nuclei and homogeneous cytoplasm. This study supports the theory that among the histologically proven MCC (more homogeneous histological pattern, lack of lymphovascular invasion) the presence of MC polyomavirus is associated with better prognosis [16–19]. Based on earlier experiences, it seems certain that the virus non-associated cases might represent independent clinicopathological entity, that is similar to the virus-associated type in its clinical manifestation, but differ from it histologically and development. Other types of skin cancer are also commonly found in MC-negative cases, of which the pathomechanism may be better understood in the light of the recent discovery that Merkel cells have epidermal (and not neural crest) origin. So, theoretically, the epidermal premalignant progenitor cells may develop into epithelial or neuroendocrine structures [1, 2, 20]. In MC polyomavirus-negative MCC cases, other factors, such as ultraviolet irradiation alone, immunosenescence and use of immunosuppressants may be involved in the carcinogenesis [3]. Ionizing radiation may also be related to the occurrence of MCC. Consequently, the tumour presumably originates from immature cells in these cases, which may explain the unfavourable prognosis of the MC-negative cases by itself. Although the presence of the MC polyomavirus and the unequivocal integration of its genome into the genome of the MCC cells seem to prove its role in the development of the tumour. However, the exact pathomechanism still raises many questions and requires further investigations [21].

It also deserves attention that the detection of the nucleic acid of MC polyomavirus is not equal to the diagnosis of the carcinoma. According to the present knowledge, only viral nucleic acid integrated in the host cell's genome can be responsible for the development of a malignant tumour [4, 21]. This phenomenon is similar to the carcinogenetic mechanism of human papillomavirus, the closest relative of MC polyomavirus. The virus may be present "innocently" (i.e. it can be detected) e.g. in the cervix, but only human papillomavirus integrated into the genome of the infected host cell can be hold responsible for cervix carcinoma.

There are only small nucleotide differences in the genome of the MC polyomaviruses investigated in different geographical areas. Only every 540th nucleotide of MC polyomavirus found in our patient differs from those of the prototype virus (including the truncating point mutation that results in a stop codon which has a prominent role in the viral oncogenesis). This stops the viral replication and induces the pathological cell proliferation. The former indicates that the evolutionary mutation rate of these viruses having double-stranded DNA genome – related to the whole genome – may be low. This may also mean that the difficulties in the molecular diagnostics originating from the genetic variability should be less considered than that of the RNA viruses characterized by high mutation rate. Well-developed, sensitive, differentiating molecular biology techniques (based on either quantitative or mutational measurements) will be of significant diagnostic and prognostic importance in the detection of both the virus and the antibodies against the large T antigen (used as a biomarker) in MCC diagnostics in the future.

MC polyomavirus is the first known cancer-causing polyomavirus in human, which has an association with MCC [4]. The potential infectious origin of this aggressive carcinoma leads to a change in attitude in the field of its pathogenesis and diagnostics. However, only detailed disclosure of the pathomechanism of the MCC may lead to effective prevention and specific treatment modalities. MCC with viral origin – similarly to tumours caused by hepatitis B and papillomaviruses – may be preventable by vaccines in the future.

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Conflict of Interest The authors declare that they have no conflict of interest.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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