Virchows Archiv (2018) 472:825–837
https://doi.org/10.1007/s00428-018-2342-0

ORIGINAL ARTICLE

Detection of the Merkel cell polyomavirus in the neuroendocrine component of combined Merkel cell carcinoma

Thibault Kervarrec[1,2,3] · Mahtab Samimi[2,4] · Pauline Gaboriaud[2] · Tarik Gheit[5] · Agnès Beby-Defaux[6,7] · Roland Houben[3] · David Schrama[3] · Gaëlle Fromont[1] · Massimo Tommasino[4] · Yannick Le Corre[8] · Eva Hainaut-Wierzbicka[9] · Francois Aubin[10] · Guido Bens[11] · Hervé Maillard[12] · Adeline Furudoï[13] · Patrick Michenet[14] · Antoine Touzé[2] · Serge Guyétant[1,2]

Received: 13 November 2017 / Revised: 15 February 2018 / Accepted: 19 March 2018 / Published online: 28 March 2018

Abstract

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine carcinoma of the skin. The main etiological agent is Merkel cell polyomavirus (MCPyV), detected in 80% of cases. About 5% of cases, called combined MCC, feature an admixture of neuroendocrine and non-neuroendocrine tumor cells. Reports of the presence or absence of MCPyV in combined MCC are conflicting, most favoring the absence, which suggests that combined MCC might have independent etiological factors and pathogenesis. These discrepancies might occur with the use of different virus identification assays, with different sensitivities. In this study, we aimed to determine the viral status of combined MCC by a multimodal approach. We histologically reviewed 128 cases of MCC and sub-classified them as "combined" or "conventional." Both groups were compared by clinical data (age, sex, site, American Joint Committee on Cancer [AJCC] stage, immunosuppression, risk of recurrence, and death during follow-up) and immunochemical features (cytokeratin 20 and 7, thyroid transcription factor 1 [TTF1], p53, large T antigen [CM2B4], CD8 infiltrates). After a first calibration step with 12 conventional MCCs and 12 cutaneous squamous cell carcinomas as controls, all eight cases of combined MCC were investigated for MCPyV viral status by combining two independent molecular procedures. Furthermore, on multiplex genotyping assay, the samples were examined for the presence of other polyoma- and papillomaviruses. Combined

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00428-018-2342-0) contains supplementary material, which is available to authorized users.

✉ Thibault Kervarrec
thibaultkervarrec@yahoo.fr

[1] Department of Pathology, Université de Tours, Centre Hospitalier Universitaire de Tours, 37044 Tours Cedex 09, France

[2] Biologie des infections à polyomavirus team, UMR INRA ISP 1282, Université de Tours, 31, Avenue Monge, 37200 Tours, France

[3] Department of Dermatology, Venereology and Allergology, University Hospital Würzburg, Josef-Schneider-Straße 2, 97080 Würzburg, Germany

[4] Department of Dermatology, Université François Rabelais, Centre Hospitalier Universitaire de Tours, 37044 Tours Cedex 09, France

[5] Infections and Cancer Biology Group, International Agency for Research on Cancer, 150 Cours Albert Thomas, 69008 Lyon, France

[6] Université de Poitiers, 2RCT "Récepteurs et régulation des cellules tumorales" team, 1 rue Georges Bonnet, 86073 Poitiers, France

[7] Department of Virology, Université de Poitiers, Centre Hospitalier Universitaire de Poitiers, 2 rue de la Milétrie, 86021 Poitiers, France

[8] Department of Dermatology, LUNAM Université, Centre Hospitalier Universitaire d'Angers, 4 rue Larrey, 49933 Angers, France

[9] Department of Dermatology, Université de Poitiers, Centre Hospitalier Universitaire de Poitiers, 2 rue de la Milétrie, 86021 Poitiers, France

[10] Department of Dermatology, Université de Franche Comté, Centre Hospitalier Universitaire de Besançon, EA3181, IFR133, 2 boulevard Fleming, 25030 Besançon, France

[11] Department of Dermatology, Centre Hospitalier Régional d'Orléans, 14 avenue de l'Hôpital, CS 86709, 45067 Orléans cedex 2, France

[12] Department of Dermatology, Centre Hospitalier Régional du Mans, 194 avenue Rubillard, 72037 Le Mans, France

[13] Department of Pathology, Université de Bordeaux, Centre Hospitalier Universitaire de Bordeaux, Avenue de Magellan, 33604 Pessac, France

[14] Department of Pathology, Centre Hospitalier Régional d'Orléans, 14 avenue de l'Hôpital, CS 86709, 45067 Orléans cedex 2, France

MCC differed from conventional MCC in earlier AJCC stage, increased risk of recurrence and death, decreased CD8 infiltrates, more frequent TTF1 positivity (5/8), abnormal p53 expression (8/8), and frequent lack of large T antigen expression (7/8). With the molecular procedure, half of the combined MCC cases were positive for MCPyV in the neuroendocrine component. Beta papillomaviruses were detected in 5/8 combined MCC cases and 9/12 conventional MCC cases. In conclusion, the detection of MCPyV DNA in half of the combined MCC cases suggests similar routes of carcinogenesis for combined and conventional MCC.

Keywords Merkel cell carcinoma · Merkel cell polyomavirus · Combined merkel cell carcinoma · Squamous carcinoma · Polyomavirus · Papillomavirus

Introduction

Merkel cell carcinoma (MCC) is a rare tumor of the skin that features an aggressive course, with overall 5-year survival estimated at 40% [1]. MCC occurs essentially in older people and the two main risk factors are sun exposure and immunodeficiency [1]. The diagnosis is based on histology, which reveals high-grade neuroendocrine morphological features close to small cell carcinoma and expression of neuroendocrine markers and/or cytokeratin 20 [2].

In 2008, Moore et al. described Merkel cell polyomavirus (MCPyV) as the major etiological agent of MCC [3]. MCPyV establishes a latent chronic infection in the dermis of most healthy individuals, and the mechanisms leading to oncogenesis are still under investigation. In about 80% of cases, tumor cells harbor integrated virus. Of note, MCPyV large T antigen in tumor cells is characterized by non-sense mutations leading to the loss of replicative abilities of the virus and the lack of late protein synthesis [4]. MCPyV genome expression in tumors is restricted to the early oncogenic proteins (small and large T antigens), and expression of T antigens is required for tumor proliferation [5]. These two viral proteins are probably the main early determinants of MCC oncogenesis.

Whether MCPyV-negative tumors should be considered a specific biological entity is debated. Indeed, MCPyV-negative MCC is thought to be essentially due to UV exposure [6] because the rates of somatic genetic mutations with a predominant UV signature are higher than in MCPyV-positive tumors. Moreover, levels of intratumoral CD8 infiltrates are lower, which suggests decreased immunogenicity [7] and a worse outcome [8].

Combined MCC involves rare MCC variants that represent 5 to 10% of MCC cases [9]. Combined MCC cases are characterized by the association of a main component of MCC with one or more other tumor components harboring non-neuroendocrine differentiation, including an epidermic orthologous component [2] (squamous, basal-cell-like, adnexial, and melanocytic) or a heterologous component [10] (glandular and sarcomatous).

Three main studies detected no MCPyV in combined MCC [9–11]. Conversely, a few recent case reports have demonstrated the presence of MCPyV in the MCC component of combined MCC [12], associated with papillomavirus infection in one case [13].

Papillomaviruses and polyomaviruses are closely related double-strand DNA viruses with similar oncogenic abilities. Indeed, beta human papillomaviruses are involved in the oncogenesis of cutaneous squamous cell carcinoma, and alpha-human papillomaviruses involved in cutaneous Bowen disease and combined tumors in the oropharynx (associating neuroendocrine and squamous components) [14]. Hence, human papillomavirus might be an etiological agent of combined carcinomas.

Whether combined MCC belongs to the spectrum of virus-induced tumors or should be considered a non-MCPyV MCC induced by an alternative oncogenetic pathway remains unclear. In this study, we compared the clinical features of conventional and combined MCC and determined the viral status of combined MCC by systematic assessment of MCPyV, 9 other polyomaviruses and 46 papillomaviruses.

Methods

Study period, data, and settings

MCC cases were selected from an ongoing historical/prospective cohort of 223 patients with MCC from six French hospital centers. The diagnosis of MCC was established between 1998 and 2015 (local ethics committee approval, Tours, France, no. RCB2009-A01056-51). The cohort inclusion criteria were previously reported [15]. All tumors were submitted to histological review by an endocrine pathologist (SG), based on the identification of morphological features of high-grade neuroendocrine carcinoma and immunohistochemical expression of epithelial and neuroendocrine markers. Only cases with available formalin-fixed paraffin-embeded (FFPE) samples and sufficient tumor material for tissue microarray inclusion were included in the study ($n = 107$).

Design of the study

Tumors were classified as combined MCC when the following criteria were met on pathological examination: presence in the same tumor mass of a conventional MCC component and an additional subpopulation of tumor cells showing non-neuroendocrine differentiation in contact with MCC. Both

conventional and combined MCC were compared on clinical and immunochemical features.

All cases of combined MCC, 12 randomly selected conventional MCC cases from the cohort and 12 cutaneous squamous cell carcinoma cases from the pathology department of the hospital center of Tours were considered for molecular analysis.

Clinical and histological data

The following data were collected from patient files: age, sex, tumor site, tumor extension at the time of diagnosis (reported in accordance with American Joint Committee on Cancer [AJCC] staging), immunosuppression (HIV infection, organ transplant recipients, hematological malignancies) [16], and follow-up data. On histology, the characteristics of the non-MCC component were noted: histological type confirmed by immunochemistry, notable expression of squamous cell carcinoma markers, degree of cytological atypia, and presence of keratinization. In addition, the relation between the two tumoral components was investigated: the tumor was considered admixed when one component surrounded multiple small foci of another component and distinct when the two components were located in two distinct areas of the tumor. Finally, the characteristics of the epidermis (connection with the tumor or not, presence of an ulceration) were noted.

Immunohistochemistry

FFPE tumor samples were included in a tissue microarray. Briefly, representative areas were selected on hematoxylin/eosin-stained sections (representative of the two tumor components in combined MCC), extracted by using a 1-mm tissue core and mounted by using a semi-motorized tissue arrayer (MTA booster OI v2.00, Alphelys). For each patient, five tumor cores were placed adjacent to each other on the tissue microarray.

Tumors were screened with a panel of antibodies including conventional MCC markers used for diagnosis (pan-cytokeratin AE1-AE3, chromogranin A, synaptophysin, cytokeratin 20), several markers rarely expressed by MCC (thyroid transcription factor 1 (TTF-1), cytokeratin 7), squamous differentiation markers (cytokeratin 5/6, p40), MCPyV large T antigen, and p53. Antibodies and dilutions are available in Supplemental Table S1. Staining was performed on a Benchmark platform, except for CM2B4 staining, which was manually performed, as previously described [5]. Immunohistochemical viral status was interpreted by using the Allred score [8]: intensity and percentage of positive cells were assessed by an 8-point semi-quantitative score. A score > 2 was considered MCPyV-positive. In the same way, p53 expression was evaluated according to the Allred score, considering scores 0, 7, and 8 as abnormal expression, predictive for loss of active p53 [17]. Intratumor CD8 infiltrate was scored as previously described [7]. For all immunohistochemical analyses, the number of uninterpretable samples (mainly due to failure of tissue microarray inclusion) is mentioned in the figures.

DNA extraction

Three 10-μm-thick FFPE sections of representative tumor areas were used for molecular analysis. In addition, for MCPyV-positive combined MCC cases showing "distinct patterns" (defined as a large distinct area of both components), each component underwent specific sample coring, followed by a morphological control on HE slides. Genomic DNA was isolated from FFPE tissue samples by using a Maxwell 16 instrument (Promega) with the Maxwell 16 FFPE Plus LEV DNA purification kit (Promega) according to the manufacturer's instructions.

VP1 gene PCR assay

MCPyV VP1 coding sequence was detected by nested PCR as previously described [4]. Primer sequences are listed in Supplemental Table S2.

Large T antigen quantitative PCR assay

Quantitative PCR assay was performed as reported previously [18]. Briefly, 100 ng DNA was mixed with 0.2 μM primers (Supplemental Table S2), 0.1 μM DNA probe, and 2xTaqman Universal PCR Master Mix (Applied Biosystems) in a final volume of 25 μl. PCR reaction involved use of Applied Biosystems 7500 Real-Time PCR Systems programmed for 50 °C × 2 min with an initial denaturation at 95 °C × 15 min, followed by 45 cycles at 95 °C × 15 s and 60 °C × 60 s. Normalization was to human albumin gene level under the same conditions. DNA range was determined by using the MKL-1 cell line as a reference (6 points: 100 copies to 10,000,000 copies).

MCPyV viral status determination

Because MCPyV is a ubiquitous virus of the skin infecting a large part of the population and the papillary dermis is the site of replication of wild-type episomal MCPyV [19], low viral load detection may be expected in the dermis of healthy people in the absence of MCC and in non-MCC skin neoplasms when using ultrasensitive methods [19–21]. To avoid detection of wild-type episomal MCPyV of the dermis unrelated to MCC tumors, a first validation step of the MCPyV detection procedures (VP1 PCR, large T antigen quantitative PCR) was performed with 12 conventional MCC and 12 non-MCC tumor samples as positive and negative controls, which

confirmed the high sensitivity and specificity of the procedures. Therefore, MCPyV-positive status was retained only in cases positive with both validated molecular procedures.

Type-specific multiplex genotyping assays

In total, 76 different polyomaviruses and papillomaviruses were investigated by type-specific multiplex genotyping (TS-MPG) assay, a validated, highly sensitive procedure [22] designed to detect low load of episomal viruses in the skin [23] and the environment [24].

The procedure combines multiplex PCR and bead-based Luminex technology (Luminex Corp., Austin, TX, USA), as previously described [22, 25]. Multiplex type-specific PCR involved use of specific primers for detecting 9 polyomaviruses (BKV, KIV, JCV, WUV, TSV, HPyV6, HPyV7, HPyV9, and SV40), 19 high-risk alpha-human papillomaviruses (types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68a, 68b, 70, 73, and 82), 2 low-risk alpha-human papillomaviruses (types 6, 11), and 46 beta human papillomaviruses (types 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93, 96, 98, 99, 100, 104, 105, 107, 110, 111, 113, 115, 118, 120, 122, 124, 143, 145, 150, 151, 152, 159 and 174). Two primers for amplifying beta-globin were added to provide a positive control for determining quality of the template DNA. Of note, the original set of primers included MCPyV sequences, which were excluded from the present study because of detection of the episomal virus in healthy skin as well as non-MCC tumors [21] as described previously.

Statistical analysis

Continuous data are described as median and range and categorical data as number and percentage of cases for which data were available. Proportional analysis was assessed by two-tailed Fisher's exact test. Continuous variables were compared by non-parametric Mann-Whitney *U* test. Recurrence-free survival and overall survival related to patient characteristics were analyzed by log-rank test and represented by Kaplan-Meier curves. Univariate and multivariate Cox proportional-hazards regression was used to identify factors associated with MCC recurrence and death, estimating hazard ratios (HRs) and 95% confidence intervals (CIs). Overall deaths were considered as events and living patients were censored on the date of last follow-up. AJCC stage [26], immunosuppression [16] and covariates with $p \leq 0.20$ on Cox univariate analysis were included in the multivariate Cox analysis as potential prognostic confounders. Statistical analysis involved use of XL-Stat-Life (Addinsoft, Paris, France). $p < 0.05$ was considered statistically significant.

Results

Characterization of the MCC population

Among 128 MCC cases with available FFPE samples, 8 (6%) met combined MCC criteria (Fig. 1), exhibiting squamous cell carcinoma differentiation in all cases. Characteristics of these cases are shown in Table 1. The 120 other tumors were classified as conventional MCC; the 8 combined MCC and 99 conventional cases could be included in the tissue microarray and were considered in this study.

Comparison between combined and conventional MCC

Clinical and immunohistochemical data of both groups are summarized in Table 2.

Combined MCC cases were more often diagnosed at localized stages (stage I: $n = 5$, 63%; stage II: $n = 3$, 37%) than conventional MCC cases (stage I: $n = 23$, 28%; stage II: $n = 26$, 32%, stage III: $n = 29$, 36%; stage IV: $n = 3$, 4%; localized (stages I–II) vs metastatic diseases (stages III–IV), $p = 0.046$). In addition, most combined MCC cases ($n = 5$, 62%) occurred in the head and neck area, with no significant difference from conventional MCC cases ($n = 27$, 34%) ($p = 0.3$).

On immunohistochemistry, combined MCC cases showed frequent expression of TTF1 ($n = 5$, 62%), which was rarely expressed in the other tumors ($n = 10$, 11%; $p = 2.10^{-3}$). Furthermore, all combined MCC cases but only 15 (16%) conventional MCC cases demonstrated abnormal p53 staining, possibly reflecting p53-inactivating mutation ($p = 3.10^{-6}$). Intratumoral CD8 infiltrates were absent in 6 (75%) combined MCC cases and was brisk (score 1) in the other cases. In contrast, CD8 intratumoral cells were observed in 64 (67%) conventional MCC cases ($p = 0.03$), with high density (scores 2–5) in 12 (13%). Representative illustrations of immunohistochemical staining are in Fig. 2. Only one combined MCC case presented weak large T antigen positivity (Fig. 3), whereas the viral protein was detectable by immunohistochemistry in 56 (62%) conventional MCC cases ($p = 0.01$).

Follow-up data were available for 86 patients including 7 combined MCC cases. Median duration of follow-up was 17 months (ranges 2–209) and 36 recurrences and 33 deaths were reported during follow-up. On univariate analysis, combined MCC patients harbored a trend towards increased risk of recurrence (HR 2.44, 95% CI 0.95–6.29, $p = 0.065$) as shown in Fig. 4. Only male sex was associated with a decreased risk of recurrence (HR 2.83, 95% CI 1.44–5.56, $p = 0.002$) (Supplemental Table S3) whereas male sex and older age were associated with death (HR 2.27, 95% CI 1.13–4.58, $p = 0.022$ and HR 2.07, 95% CI 1.02–4.19, $p = 0.043$, respectively).

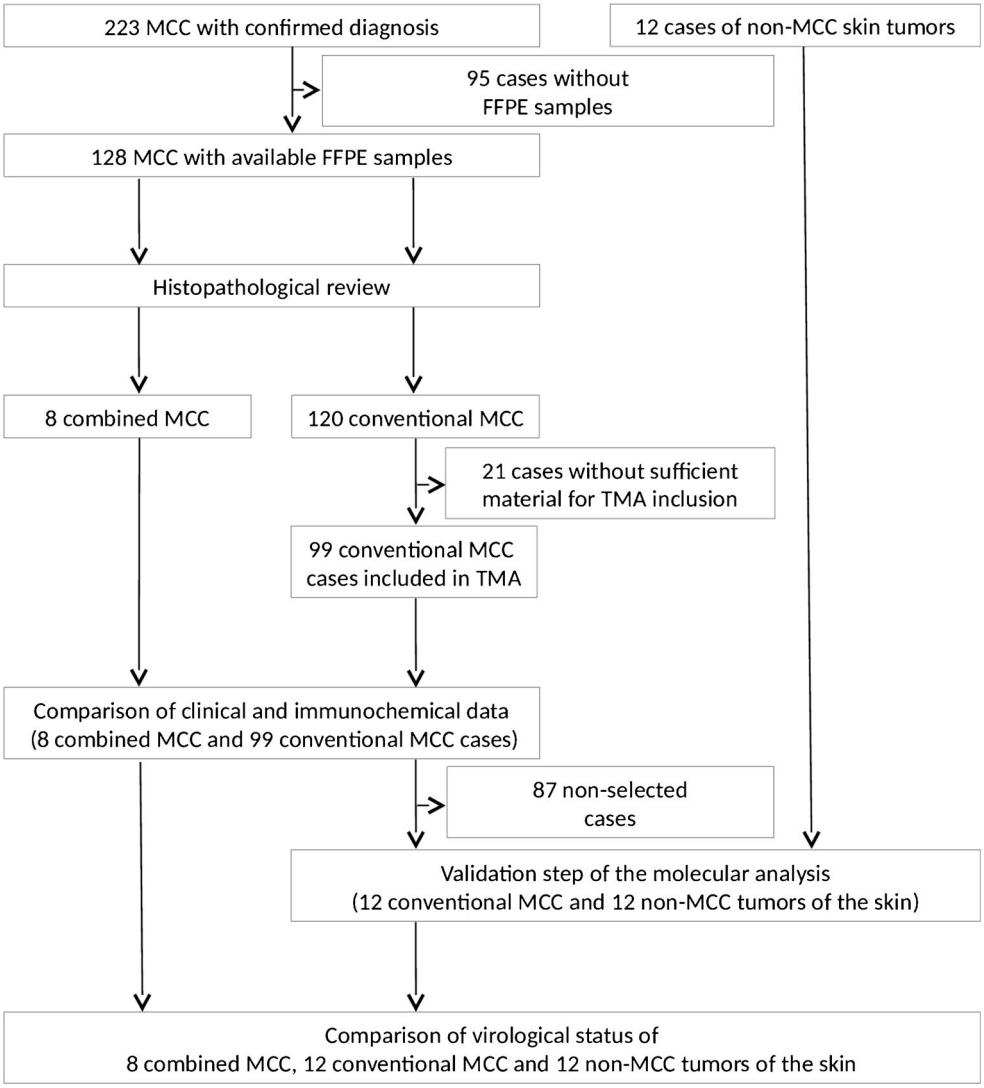

Fig. 1 Flow chart of cases in the study. *MCC* Merkel cell carcinoma, *FFPE* formalin-fixed paraffin-embedded, *TMA* tissue microarray

A multivariate Cox analysis model including age, sex, immunosuppression, and AJCC stage (Table 3) revealed increased risk of recurrence (HR 4.15, 95% CI 1.37–12.57, $p = 0.012$) and death (HR 4.15, 95% CI 1.22–14.16, $p = 0.023$) with combined MCC.

MCPyV genome detection

The preliminary validation step of the MCPyV detection procedures (VP1 PCR, Large T antigen quantitative PCR) allowed us to detect MCPyV in all 12 conventional MCC cases except one by quantitative PCR (Table 2). All non-MCC tumors were negative for MCPyV with the two procedures, which led to the validation of this bimodal strategy for MCPyV status characterization.

This bimodal approach revealed MCPyV-positive status in 4/8 combined MCC cases, as shown in Table 2. Median MCPyV load was lower in combined MCC than MCC control cases (5.7 [range 0.13–28] vs 58 [4–313] copies/cell) ($p = 0.04$). Three of the four MCPyV-positive combined tumors were eligible for specific sampling independently targeting the two tumor components, and the remaining case consisted of closely intermixed components, which ruled out reliable specific separation. MCPyV DNA could be detected in the MCC component of the three tumors but was consistently absent in the other non-MCC part of the tumor.

Clinical, histological, and immunochemical features of MCPyV-positive and MCPyV-negative combined MCC cases are summarized in Table 1, and representative histological features of both subgroups are in Fig. 5. MCPyV was detected in combined MCC cases with basaloid squamous cell carcinoma ($n = 2$) and conventional squamous cell carcinoma ($n = 2$).

Other polyomaviruses and papillomaviruses

Beta papillomavirus DNA was detected in 5/8 combined MCC cases (63%), 9/12 conventional MCC cases (75%), and 9/12 (75%) squamous cell carcinoma cases. No

Table 1 Features of combined Merkel cell carcinoma (MCC) cases

Case number	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Clinical data								
Age	85	95	81	80	68	88	92	79
Sex	M	F	F	F	F	F	F	M
Tumor location	Neck	Ear	Leg	Leg	Eyelid	Face	Leg	Lip
AJCC stage	II	II	I	I	I	I	II	I
Immunosuppression	–	UD	–	–	–	–	–	–
Morphologic features SCC component								
Morphology	Basaloid	Basaloid	Conventional	Conventional	Conventional	Bowen	Bowen	Conventional
Atypia	+	+++	++	+++	++	++	++	++
Keratinization	–	–	+	+	+	–	+	+
Architecture epidermis	Admixed	Distinct	Distinct	Distinct	Admixed	Distinct	Distinct	Admixed
Connection	+	+	+	+	+	+	+	UD
Ulceration	+	–	+	+	–	–	+	UD
References (Fig. 5)	a	b	c	d	e	f	g	h
Immunochemical features: MCC component								
CK20	+	+	+	+	–	+	+	+
CK7	–	–	–	–	–	–	–	–
TTF1	–	–	Low	Low	Low	High	Low	–
p53 (status/Allred score)	PMS/0	PMS/8	PMS/8	PMS/0	PMS/0	PMS/8	PMS/8	PMS/8
LTAg	–	+	–	–	–	–	–	–
CD8 (score)	0	1	0	0	0	1	0	0
SCC component								
CK5/6	+	+	+	+	+	+	+	+
p40	+	+	+	+	+	+	+	+
Virologic features polyomavirus detection								
VP1 PCR	+	+	+	+	–	–	–	–
qPCR (Nb of copy/cell)	0,13	11	28	0,38	–	–	–	–
Other HPyV	–	–	–	–	–	–	–	–
Papillomavirus detection								
α type	–	–	–	–	–	–	–	–
β type	–	–	98	9/12/98/110	–	5/38	107	21/122

AJCC American Joint Committee on Cancer, *CK* cytokeratin, *LTAg* large T antigen, *MCC* Merkel cell carcinoma, + positive, − negative, *MCPyV* Merkel cell polyomavirus, *PCR* polymerase chain reaction, *PMS* predicted mutated status (i.e., Allred score = 0, 7, or 8), *PWTS* predicted wild-type status (i.e., Allred score = 1 to 6), *SCC* squamous cell carcinoma, *TTF1* thyroid transcription factor, *UD* unavailable data

recurrence considering genotypes was observed in the combined MCC group, except for human papillomavirus 98, which was present in 2 cases (Supplemental Table S4). In addition, we detected human polyomavirus 6 in one conventional MCC case and human papillomavirus 16 in 2 squamous cell carcinoma cases.

Discussion

We identified 8 cases of combined MCC in a series of 128 MCC tumors. Combined MCC often expressed TTF1, which is almost always absent in conventional MCC cases and often showed p53-aberrant expression. In addition, multivariate analysis revealed an increased risk of recurrence and death in this population. Using two independent validated molecular procedures, we detected MCPyV in half of our combined MCC cases, only in the MCC component. Moreover, combined MCC cases showed lower MCPyV load as compared with conventional MCC tumors and frequent large T antigen negativity on immunochemistry. Finally, beta papillomaviruses were frequent in combined and conventional MCC and non-MCC tumor samples.

In accordance with previous reports, our series shows that combined MCC is a rare tumor, representing 6% of our cohort. Combined MCC tumors occurred preferentially on the head, the main sun-exposed area in our country. On immunohistochemistry, the neuroendocrine component of these

Table 2 Clinical, immunochemical, and virological features of combined and conventional MCC cases

Clinical data	Combined MCC ($n = 8$)	Conventional MCC ($n = 99$)	p value
Age, y, median, range	83 (68–95)	76 (45–96)	0.4
Sex			0.5
Female	6	50	
Male	2	35	
Missing data	0	14	
Location			0.3
Head	5	27	
Trunk	0	7	
Upper limb	0	8	
Lower limb	3	24	
Unknown primary	0	13	
Missing data	0	20	
Disease extension at diagnosis			0.046
Localized (stages I–II)	8	49	
Metastatic (stages III–IV)	0	32	
Missing data	0	18	
Immunodepression			0.9
Yes	0	9	
No	7	68	
Missing data	1	22	
Immunochemical features	($n = 8$)	($n = 99$)	p value
Cytokeratin 20			0.12
Positive	6	89	
Negative	2	6	
Uninterpretable cases	0	4	
Cytokeratin 7			0.9
Positive	0	9	
Negative	8	80	
Uninterpretable cases	0	10	
TTF1			2.10^{-3}
Positive	5 (4 low)	10	
Negative	3	80	
Uninterpretable cases	0	9	
p53			3.10^{-6}
PMS	8	15	
PWTS	0	76	
Uninterpretable cases	0	8	
Large T antigen (CM2B4)			0.01
Positive	1	56	
Negative	7	35	
Uninterpretable cases	0	8	
CD8			0.03
Absent (score 0)	6	31	
Present (score 1–5)	2	64	
Uninterpretable cases	0	4	
Virological features	($n = 8$)	($n = 12$)	p value
MCPyV detection (VP1 PCR)			0.01
Positive cases	4	12	
Negative cases	4	0	
Uninterpretable cases	0	0	
MCPyV detection (LTAg qPCR)			0.1
Positive cases	4	11	
Negative cases	4	1	
Uninterpretable cases	0	0	
Other polyomavirus detection			0.9
Positive cases	0	0	
Negative cases	8	12	
Uninterpretable cases	0	0	
Papillomavirus detection			0.6
Positive cases	5	9	
Negative cases	3	3	
Uninterpretable cases	0	0	

AJCC American Joint Committee on Cancer, *CMCC* combined MCC, *LTAg* large T antigen, *MCC* classical MCC, + positive, − negative, *MCPyV*(+) Merkel cell polyomavirus-positive MCC, *MCPyV*(−) Merkel cell polyomavirus-negative MCC, *PMS* predicted mutated status (i.e., Allred score = 0, 7, or 8), *PWTS* predicted wild-type status (i.e., Allred score = 1 to 6), *SCC* squamous cell carcinoma, *TTF1* thyroid transcription factor 1

tumors harbored the same phenotypical markers as conventional MCC, notably cytokeratin 20, but with unusual expression of TTF1 [27]. In addition, aberrant expression of p53, suggesting p53 mutation [9, 28], was common. This finding agrees with genomic analysis performed by Pullitzer et al. reporting higher somatic gene mutation rate in combined than conventional MCC [29].

In accordance with the Martin et al. study, which found 61% mortality[3], we found combined MCC an aggressive neoplasm with decreased recurrence-free survival ($p = 0.012$) and overall survival ($p = 0.023$) as compared with conventional MCC, which highlights the importance of this morphological distinction in current practise.

Three studies [9–11] by Busam (in 2009), Martin (in 2013), and Carter (in 2017) reported the absence of MCPyV in combined MCC (7 and 15 cases, respectively). As a result, the authors proposed that combined MCCs should be included in the MCPyV-negative cutaneous tumor group. However, two case reports have given contradictory results, identifying MCPyV in two combined MCC cases by molecular biology [13, 30]. In addition, MCPyV was identified in the neuroendocrine component of a combined MCC case by high-sensitive immunofluorescence staining [12] and in another case by conventional immunochemistry [31]. Here, using a multimodal approach for MCPyV detection, we confirm these findings in a large series of combined MCC.

Weak expression of MCPyV large T antigen was detectable by immunochemistry in only one case with high MCPyV viral load (11 copies/cell), and MCPyV DNA was identified in half of the cases with a lower viral load. Variations in viral load offer an explanation for the previous discordant results. Indeed, immunochemical procedures used in most studies have a sensitivity of 80% as compared with amplification techniques, which probably reflects the influence of viral load on the sensitivity of the different identification approaches [32].

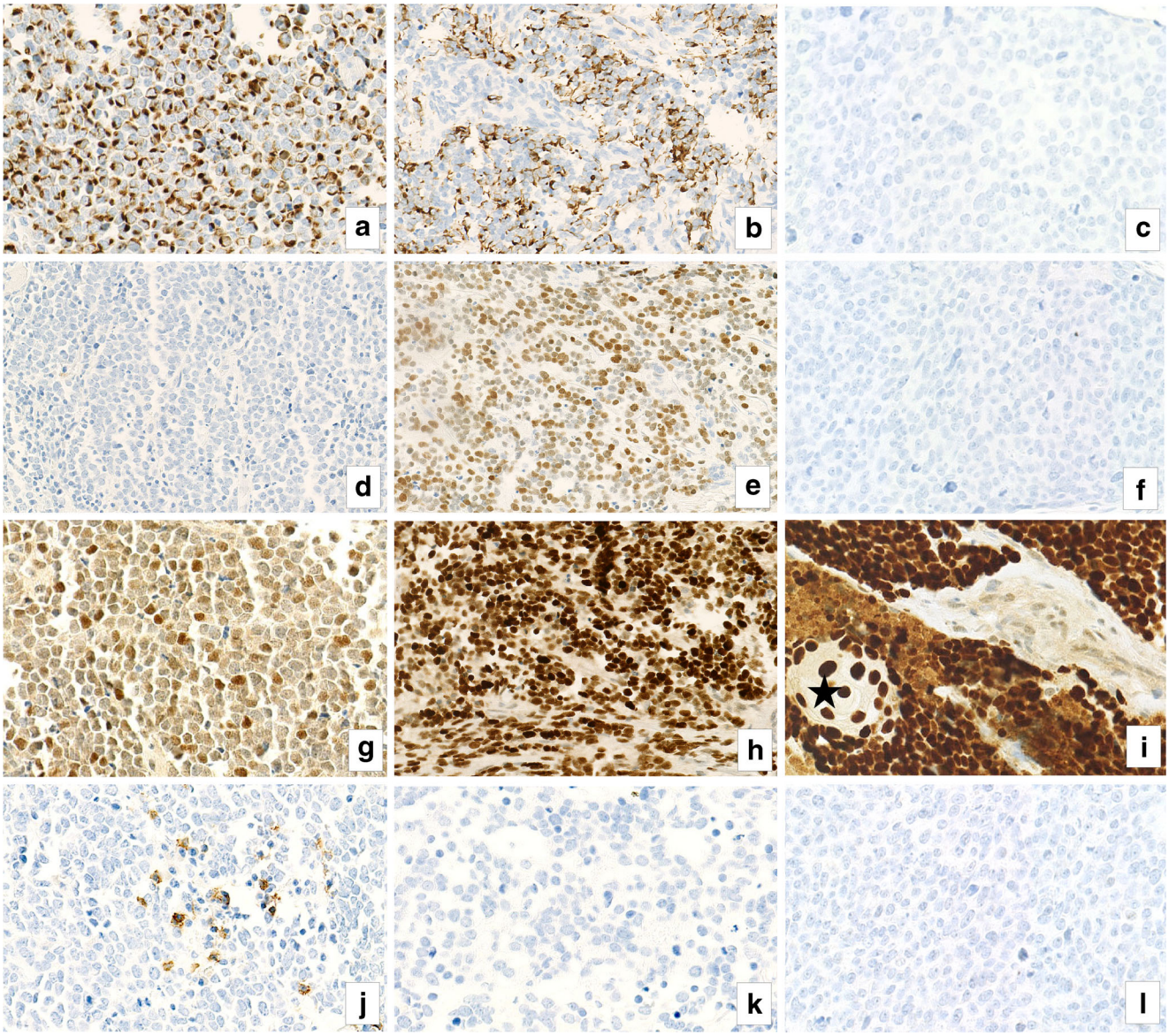

Fig. 2 Representative microphotographs of the immunohistochemical study for cytokeratin 20 (**a**–**c**), TTF-1 (**d**–**f**), p53 (**g**–**i**), and CD8 immune infiltrate (**j**–**l**) in conventional (**a**, **d**, **g**, **j**) and combined MCC (MCC (**b**, **e**, **h**, **k**) and squamous (**c**, **f**, **i**, **l**) component. To note, both components of combined cases harbored same immunochemical p53 profile as illustrate in (**i**): overexpresion of p53 in a small islet of squamous carcinoma (black star) surrounding by the MCC component

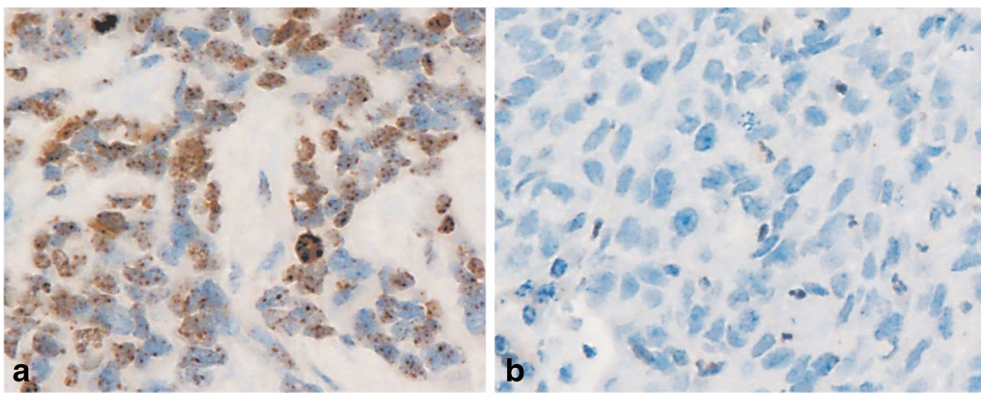

Fig. 3 Immunohistochemical detection of Merkel cell polyomavirus (MCPyV) large T antigen in a combined MCC sample: **a** moderate nuclear large T antigen expression in the MCC component and **b** lack of large T antigen expression in the squamous carcinoma component

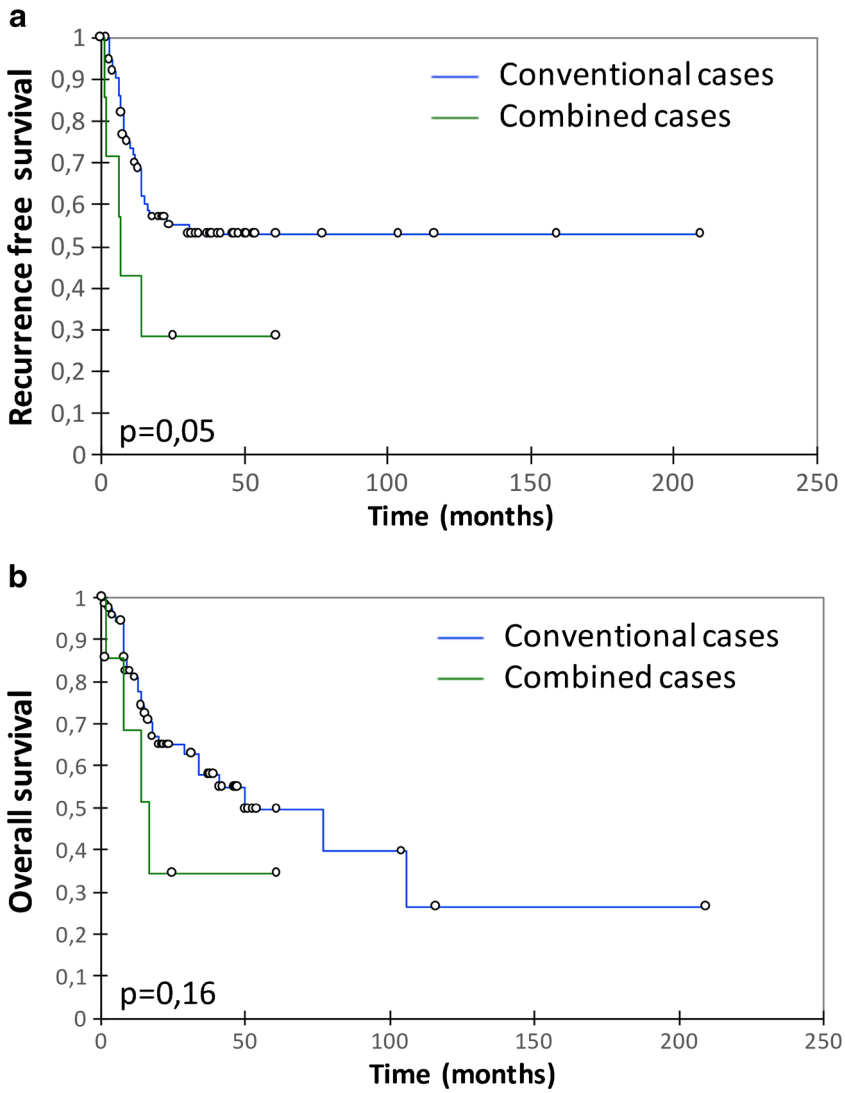

Fig. 4 Kaplan-Meier survival curves for the Merkel cell carcinoma population by the MCC type (combined vs conventional cases): **a** recurrence-free survival and **b** overall survival

Considering the high prevalence of the MCPyV in normal skin, the use of highly sensitive molecular biology procedures might represent a pitfall in the identification of an MCPyV-related tumor, introducing a risk of false-positive results, which justifies our multimodal approach. Although MCPyV is considered a hallmark of MCC, episomal MCPyV can be detected with very low load in the papillary dermis of normal skin in many healthy adults [19] and in non-MCC skin tumors [20]. With the lack of an international gold standard for MCPyV quantification, arbitrary cutoffs have been used [33] to distinguish MCPyV-negative from MCPyV-positive tumors. To avoid this bias, we introduced a first step of calibration of our molecular MCPyV

Table 3 Multivariate Cox proportional-hazard analysis of factors associated with MCC recurrence and death

Covariate	Recurrence		Death	
	Adjusted HR (95% CI)	*p*	Adjusted HR (95% CI)	*p*
Age (≥ 77 vs < 77 years)	2.14 (1.04–4.41)	0.040	2.87 (1.27–6.52)	0.011
Sex (male vs female)	4.67 (2.12–10.32))	0.0001	5.53 (2.26–13.57)	0.0002
AJCC stages (3–4 vs 1–2)	1.64 (0.76–3.52)	0.206	2.10 (0.96–4.60)	0.065
Immunosuppression (yes vs no)	0.93 (0.32–2.73)	0.901	2.23 (0.93–5.34)	0.072
MCC status (combined vs conventional)	4.15 (1.37–12.57)	0.012	4.15 (1.22–14.16)	0.023

HR hazard ratio, *CI* confidence interval, *MCC* Merkel cell carcinoma

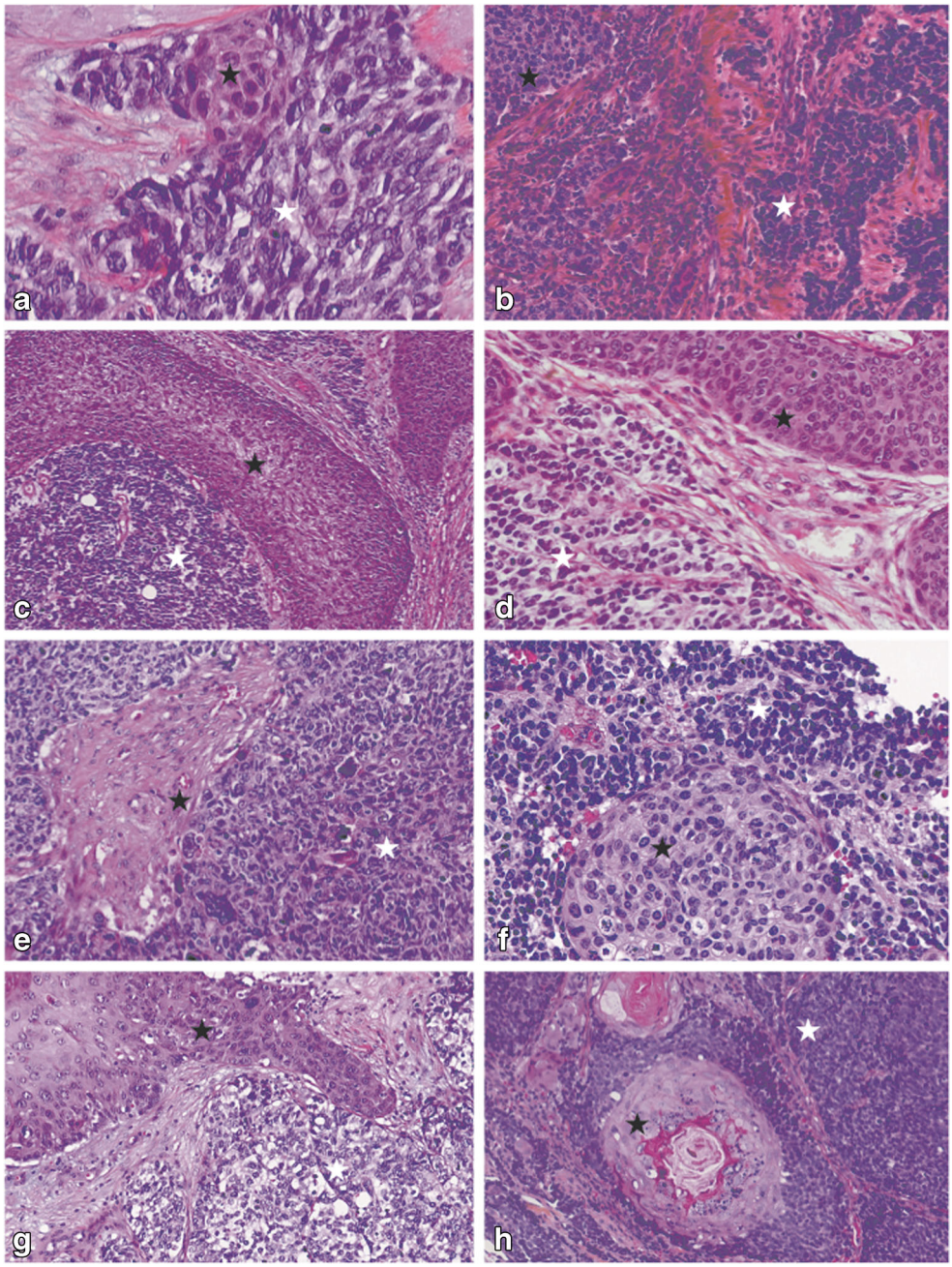

Fig. 5 Representative microphotographs of MCPyV-positive (**a**–**d**) and MCPyV-negative (**e**–**h**) combined MCC samples. The neuroendocrine component is characterized by sheet of small uniform cells with high nucleocytoplasmic ratio (white star). It is associated with another carcinomatous component (black star): basaloid squamous cell carcinoma (**a**, **b**), conventional squamous cell carcinoma (**c**, **d**, **e**, **h**), and Bowen disease (**f**, **g**)

detection test in the study, using conventional MCC and non-MCC skin tumors as controls. Indeed in our setting, the sensitivity of the MCPyV detection was carefully managed to detect only significant MCPyV loads associated with MCC.

The significance of MCPyV detection in tumors and its relation with somatic mutations is still unclear. According to the "hit and run" phenomenon [34], MCC could feature loss of large T antigen dependence. Indeed, accumulation of somatic mutations—notably p53 mutations—could cause an empowerment process and might finally cause loss of MCPyV sequence by a selection process. In this way, the low viral load that we detected in combined MCC cases could be due to the presence of a minor MCPyV-positive tumor subpopulation. The divergent differentiation component that we found was squamous cell carcinoma in all cases. Because squamous cell carcinoma is related to papillomavirus infection [35], papillomaviruses could represent a possible etiological agent for combined MCC. Beta human papillomaviruses were detected in 63% of our combined MCC cases and 75% of conventional MCC cases, which rules out the possibility of a specific association between human papillomavirus and combined MCC. However, the frequent detection of cutaneous human papillomavirus in MCC in our study raises the question of their potential impact as a co-carcinogen.

Because specific morphological features—notably non-keratinizing basaloid morphology—have been described in virus-induced tumors [36], we compared morphological features of MCPyV-positive and MCPyV-negative tumors (data not shown). Although we found no statistically significant difference because of the small number of cases, we detected MCPyV in the two tumors containing squamous components with basaloid morphology, which suggests possible interactions between viral status and morphology.

MCPyV integration has been found in the main oncogenic event in MCC, but the nature of the cell in which this integration occurs is unknown. Of note, in contrast to conventional MCC, combined MCC with squamous differentiation showed an epidermal connection in all of our cases. The development of combined tumors implicates a common progenitor cell; as previously mentioned, Bowen's disease is an epidermic intraepithelial neoplasm often present in combined MCC. Considering these two findings, combined MCC might originate from an epidermal cell. In addition, keratinocytes have been suggested as precursors of Merkel cells [37]. These considerations led us to hypothesize that MCPyV infection in a keratinocyte could be the first oncogenic event in combined MCC. Premature occurrence and accumulation of UV-induced somatic mutations could secondarily lead to the loss of the virus in a portion of the cell population and to squamous differentiation in the intraepithelial component. At the same time, the MCC component could invade the dermis and further undergo progressive loss of MCPyV. Hence, advanced combined MCC would feature very low load or lack of MCPyV, high somatic mutation rate, lack of T cell response and impaired outcome. In this way, our results do not rule out that combined MCC shares the same genetic background and behavior as other MCC types with negative-status or low-load MCPyV but may improve our understanding of their oncogenesis.

Our study has some limitations, owing to the low number of combined MCC cases, which is inherent to the low incidence of the tumor. The frequent MCPyV negativity found on immunostaining in combined MCC—currently considered the main tool for viral status determination [8]—led us to use molecular detection procedures, which implies sensitivity bias management, as discussed previously. Nonetheless, our results were validated by bimodal molecular procedures and positive and negative relevant controls.

To conclude, we detected MCPyV in half of our combined MCC cases, with a lower viral load than with conventional MCC, which suggests a shared oncogenesis between both MCC variants. The impact of MCPyV on the oncogenesis and behavior of the tumor remains to be determined.

Funding This work was funded by project POCAME, Axe Immunothérapies, Cancéropole Grand Ouest-Région Centre Val de Loire (France) and by the Fond de recherche de la société française de Pathologie 2016.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Institutional review board The local Ethics Committee of Tours (France) approved the study (no. RCB2009-A01056-51)

References

1. Lemos BD, Storer BE, Iyer JG, Phillips JL, Bichakjian CK, Fang LC, Johnson TM, Liegeois-Kwon NJ, Otley CC, Paulson KG, Ross MI, Yu SS, Zeitouni NC, Byrd DR, Sondak VK, Gershenwald JE, Sober AJ, Nghiem P (2010) Pathologic nodal evaluation improves prognostic accuracy in Merkel cell carcinoma: analysis of 5823 cases as the basis of the first consensus staging system. J Am Acad Dermatol 63:751–761. https://doi.org/10.1016/j.jaad.2010.02.056
2. LeBoit PE, World Heath Organisation, International Agency for Research on Cancer (eds) (2007) Pathology and genetics of skin tumours: [... reflects the views of a working group that convened for an editorial and consensus conference in Lyon, France, September 22 - 25, 2003]. Reprinted. IARC Press, Lyon
3. Feng H, Shuda M, Chang Y, Moore PS (2008) Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science 319:1096–1100. https://doi.org/10.1126/science.1152586
4. Touzé A, Gaitan J, Maruani A, Le Bidre E, Doussinaud A, Clavel C et al (2009) Merkel cell polyomavirus strains in patients with Merkel cell carcinoma. Emerg Infect Dis 15:960–962. https://doi.org/10.3201/eid1506.081463
5. Houben R, Adam C, Baeurle A, Hesbacher S, Grimm J, Angermeyer S, Henzel K, Hauser S, Elling R, Bröcker EB, Gaubatz S, Becker JC, Schrama D (2012) An intact retinoblastoma protein-binding site in Merkel cell polyomavirus large T antigen is required for promoting growth of Merkel cell carcinoma cells. Int J Cancer 130:847–856. https://doi.org/10.1002/ijc.26076
6. González-Vela MDC, Curiel-Olmo S, Derdak S, Beltran S, Santibañez M, Martínez N et al (2017) Shared oncogenic pathways implicated in both virus-positive and UV-induced Merkel cell carcinomas. J Invest Dermatol 137:197–206. https://doi.org/10.1016/j.jid.2016.08.015
7. Paulson KG, Iyer JG, Tegeder AR, Thibodeau R, Schelter J, Koba S, Schrama D, Simonson WT, Lemos BD, Byrd DR, Koelle DM, Galloway DA, Leonard JH, Madeleine MM, Argenyi ZB, Disis ML, Becker JC, Cleary MA, Nghiem P (2011) Transcriptome-wide studies of merkel cell carcinoma and validation of intratumoral CD8+ lymphocyte invasion as an independent predictor of survival. J Clin Oncol Off J Am Soc Clin Oncol 29:1539–1546. https://doi.org/10.1200/JCO.2010.30.6308
8. Moshiri AS, Doumani R, Yelistratova L, Blom A, Lachance K, Shinohara MM, Delaney M, Chang O, McArdle S, Thomas H, Asgari MM, Huang ML, Schwartz SM, Nghiem P (2016) Polyomavirus-negative Merkel cell carcinoma: a more aggressive subtype based on analysis of 282 cases using multimodal tumor virus detection. J Invest Dermatol 137:819–827. https://doi.org/10.1016/j.jid.2016.10.028.
9. Busam KJ, Jungbluth AA, Rekthman N, Coit D, Pulitzer M, Bini J, Arora R, Hanson NC, Tassello JA, Frosina D, Moore P, Chang Y (2009) Merkel cell polyomavirus expression in merkel cell carcinomas and its absence in combined tumors and pulmonary neuroendocrine carcinomas. Am J Surg Pathol 33:1378–1385. https://doi.org/10.1097/PAS.0b013e3181aa30a5
10. Martin B, Poblet E, Rios JJ, Kazakov D, Kutzner H, Brenn T, Calonje E (2013) Merkel cell carcinoma with divergent differentiation: histopathological and immunohistochemical study of 15

cases with PCR analysis for Merkel cell polyomavirus. Histopathology 62:711–722. https://doi.org/10.1111/his.12091

11. Carter MD, Gaston D, Huang W-Y, Greer WL, Pasternak S, Ly TY, Walsh NM (2017) Genetic profiles of different subsets of Merkel cell carcinoma show links between combined and pure MCPyV-negative tumors. Hum Pathol 71:117–125. https://doi.org/10.1016/j.humpath.2017.10.014.
12. Chou T-C, Tsai K-B, Wu C-Y, Hong C-H, Lee C-H (2016) Presence of the Merkel cell polyomavirus in Merkel cell carcinoma combined with squamous cell carcinoma in a patient with chronic arsenism. Clin Exp Dermatol 41:902–905. https://doi.org/10.1111/ced.12954
13. Mitteldorf C, Mertz KD, Fernández-Figueras MT, Schmid M, Tronnier M, Kempf W (2012) Detection of Merkel cell polyomavirus and human papillomaviruses in Merkel cell carcinoma combined with squamous cell carcinoma in immunocompetent European patients. Am J Dermatopathol 34:506–510. https://doi.org/10.1097/DAD.0b013e31823b9b4e
14. Bishop JA, Westra WH (2011) Human papillomavirus-related small cell carcinoma of the oropharynx. Am J Surg Pathol 35:1679–1684. https://doi.org/10.1097/PAS.0b013e3182299cde
15. Gardair C, Samimi M, Touzé A, Coursaget P, Lorette G, Caille A et al (2015) Somatostatin receptors 2A and 5 are expressed in Merkel cell carcinoma with no association with disease severity. Neuroendocrinology 101:223–235. https://doi.org/10.1159/000381062
16. Asgari MM, Sokil MM, Warton EM, Iyer J, Paulson KG, Nghiem P (2014) Effect of host, tumor, diagnostic, and treatment variables on outcomes in a large cohort with Merkel cell carcinoma. JAMA Dermatol 150:716–723. https://doi.org/10.1001/jamadermatol.2013.8116
17. Watanabe G, Ishida T, Furuta A, Takahashi S, Watanabe M, Nakata H, Kato S, Ishioka C, Ohuchi N (2015) Combined immunohistochemistry of PLK1, p21, and p53 for predicting TP53 status: an independent prognostic factor of breast cancer. Am J Surg Pathol 39:1026–1034. https://doi.org/10.1097/PAS.0000000000000386
18. Rodig SJ, Cheng J, Wardzala J, DoRosario A, Scanlon JJ, Laga AC, Martinez-Fernandez A, Barletta JA, Bellizzi AM, Sadasivam S, Holloway DT, Cooper DJ, Kupper TS, Wang LC, DeCaprio JA (2012) Improved detection suggests all Merkel cell carcinomas harbor Merkel polyomavirus. J Clin Invest 122:4645–4653. https://doi.org/10.1172/JCI64116
19. Liu W, Yang R, Payne AS, Schowalter RM, Spurgeon ME, Lambert PF, Xu X, Buck CB, You J (2016) Identifying the target cells and mechanisms of Merkel cell polyomavirus infection. Cell Host Microbe 19:775–787. https://doi.org/10.1016/j.chom.2016.04.024
20. Rollison DE, Giuliano AR, Messina JL, Fenske NA, Cherpelis BS, Sondak VK, Roetzheim RG, Iannacone MR, Michael KM, Gheit T, Waterboer T, Tommasino M, Pawlita M (2012) Case-control study of Merkel cell polyomavirus infection and cutaneous squamous cell carcinoma. Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol 21:74–81. https://doi.org/10.1158/1055-9965.EPI-11-0764
21. Hampras SS, Michel A, Schmitt M, Waterboer T, Kranz L, Gheit T, Fisher K, Sondak VK, Messina J, Fenske N, Cherpelis B, Tommasino M, Pawlita M, Rollison DE (2015) Merkel cell polyomavirus (MCV) T-antigen seroreactivity, MCV DNA in eyebrow hairs, and squamous cell carcinoma. Infect Agent Cancer 10:35. https://doi.org/10.1186/s13027-015-0030-0
22. Gheit T, Billoud G, de Koning MNC, Gemignani F, Forslund O, Sylla BS, Vaccarella S, Franceschi S, Landi S, Quint WGV, Canzian F, Tommasino M (2007) Development of a sensitive and specific multiplex PCR method combined with DNA microarray primer extension to detect Betapapillomavirus types. J Clin Microbiol 45:2537–2544. https://doi.org/10.1128/JCM.00747-07
23. Iannacone MR, Gheit T, Pfister H, Giuliano AR, Messina JL, Fenske NA, Cherpelis BS, Sondak VK, Roetzheim RG, Silling S, Pawlita M, Tommasino M, Rollison DE (2014) Case-control study of genus-beta human papillomaviruses in plucked eyebrow hairs and cutaneous squamous cell carcinoma. Int J Cancer 134:2231–2244. https://doi.org/10.1002/ijc.28552
24. Di Bonito P, Libera SD, Petricca S, Iaconelli M, Accardi L, Muscillo M et al (2015) Frequent and abundant Merkel cell polyomavirus detection in urban wastewaters in Italy. Food Environ Virol 7:1–6. https://doi.org/10.1007/s12560-014-9168-y.
25. Gheit T, Landi S, Gemignani F, Snijders PJF, Vaccarella S, Franceschi S, Canzian F, Tommasino M (2006) Development of a sensitive and specific assay combining multiplex PCR and DNA microarray primer extension to detect high-risk mucosal human papillomavirus types. J Clin Microbiol 44:2025–2031. https://doi.org/10.1128/JCM.02305-05
26. Harms KL, Healy MA, Nghiem P, Sober AJ, Johnson TM, Bichakjian CK, Wong SL (2016) Analysis of prognostic factors from 9387 Merkel cell carcinoma cases forms the basis for the new 8th edition AJCC staging system. Ann Surg Oncol 23:3564–3571. https://doi.org/10.1245/s10434-016-5266-4
27. Czapiewski P, Majewska H, Kutzner H, Kazakov D, Renkielska A, Biernat W (2016) TTF-1 and PAX5 are frequently expressed in combined Merkel cell carcinoma. Am J Dermatopathol 38:513–516. https://doi.org/10.1097/DAD.0000000000000464
28. Lai JH, Fleming KE, Ly TY, Pasternak S, Godlewski M, Doucette S, Walsh NM (2015) Pure versus combined Merkel cell carcinomas: immunohistochemical evaluation of cellular proteins (p53, Bcl-2, and c-kit) reveals significant overexpression of p53 in combined tumors. Hum Pathol 46:1290–1296. https://doi.org/10.1016/j.humpath.2015.05.008
29. Pulitzer MP, Brannon AR, Berger MF, Louis P, Scott SN, Jungbluth AA, Coit DG, Brownell I, Busam KJ (2015) Cutaneous squamous and neuroendocrine carcinoma: genetically and immunohistochemically different from Merkel cell carcinoma. Mod Pathol Off J U S Can Acad Pathol Inc 28:1023–1032. https://doi.org/10.1038/modpathol.2015.60
30. Chen C-H, Wu Y-Y, Kuo K-T, Liau J-Y, Liang C-W (2015) Combined squamous cell carcinoma and Merkel cell carcinoma of the vulva: role of human papillomavirus and Merkel cell polyomavirus. JAAD Case Rep 1:196–199. https://doi.org/10.1016/j.jdcr.2015.04.005
31. Iwasaki T, Kodama H, Matsushita M, Kuroda N, Yamasaki Y, Murakami I, Yamamoto O, Hayashi K (2013) Merkel cell polyomavirus infection in both components of a combined Merkel cell carcinoma and basal cell carcinoma with ductal differentiation; each component had a similar but different novel Merkel cell polyomavirus large T antigen truncating mutation. Hum Pathol 44:442–447. https://doi.org/10.1016/j.humpath.2012.08.022
32. Leroux-Kozal V, Lévêque N, Brodard V, Lesage C, Dudez O, Makeieff M, Kanagaratnam L, Diebold MD (2015) Merkel cell carcinoma: histopathologic and prognostic features according to the immunohistochemical expression of Merkel cell polyomavirus large T antigen correlated with viral load. Hum Pathol 46:443–453. https://doi.org/10.1016/j.humpath.2014.12.001
33. Eid M, Nguyen J, Brownell I (2017) Seeking standards for the detection of Merkel cell polyomavirus and its clinical significance. J Invest Dermatol 137:797–799. https://doi.org/10.1016/j.jid.2016.12.024
34. Houben R, Grimm J, Willmes C, Weinkam R, Becker JC, Schrama D (2012) Merkel cell carcinoma and Merkel cell polyomavirus: evidence for hit-and-run oncogenesis. J Invest Dermatol 132:254–256. https://doi.org/10.1038/jid.2011.260

35. Tommasino M (2017) The biology of beta human papillomaviruses. Virus Res 2016. https://doi.org/10.1016/j.virusres.2016.11.013
36. Mehrad M, Carpenter DH, Chernock RD, Wang H, Ma X-J, Luo Y, Luo J, Lewis JS Jr, el-Mofty SK (2013) Papillary squamous cell carcinoma of the head and neck: clinicopathologic and molecular features with special reference to human papillomavirus. Am J Surg Pathol 37:1349–1356. https://doi.org/10.1097/PAS.0b013e318290427d
37. Ostrowski SM, Wright MC, Bolock AM, Geng X, Maricich SM (2015) Ectopic Atoh1 expression drives Merkel cell production in embryonic, postnatal and adult mouse epidermis. Dev Camb Engl 142:2533–2544. https://doi.org/10.1242/dev.123141