Original paper

Prognostic and predictive value of EZH2 expression and the tumor immune microenvironment in Merkel cell carcinoma

Arbil Acikalin[1], Emine Bagir[1], Semra Paydas[2]

[1]Pathology Department, Medical Faculty, Cukurova University, Adana, Turkey
[2]Oncology Department, Medical Faculty, Cukurova University, Adana, Turkey

Merkel cell carcinoma (MCC) is a rare and lethal type of skin cancer characterized by frequent recurrences and metastases. In view of the lack of a proven treatment option for MCC, we immunochemically evaluated the presence of Merkel cell polyomavirus (MCPyV), PD-1, PD-L1, CD8, and EZH2 on slides prepared from tumor tissues of 13 patients with MCC, and examined their association with disease progression and overall survival. PD-1 was expressed on tumor infiltrating lymphocytes (TILs) in 92.3% of the patients. None of the tumor cells expressed PD-L1. CD8 levels were higher in MCPyV-positive tumors. Interestingly, higher CD8 levels correlated with better overall survival ($p = 0.025$), while higher EZH2 expression correlated with metastasis/recurrence ($z = -1.396$, $p = 0.089$). However, low EZH2 expression was associated with poor overall survival ($\chi^2 = 3.745$, Cramer $V = 0.537$, $p = 0.086$). These findings suggest that EZH2 plays a significant role in MCC and may be a promising therapeutic target.

Key words: CD8, EZH2, Merkel cell carcinoma, PD-1, PD-L1.

Introduction

Merkel cell carcinoma (MCC) or primary cutaneous neuroendocrine carcinoma is an aggressive neoplasm of the skin with a low but increasing incidence [1]. Mortality rate in MCC is higher than in stage-matched melanoma, with death mainly associated with metastases and recurrences [2]. The median age of onset is 75 years.

Even though MCC owes its eponym to the ultrastructural and immunophenotypical resemblance to normal Merkel cells [3], its actual cellular origin is unclear, as recent studies presented data suggesting that MCC may arise from keratinocytic progenitor cells (either epidermal or hair follicle cells), dermal fibroblasts/stem cells, or pre/pro B cells [4]. Ultraviolet radiation (UV) and Merkel cell polyomavirus (MCPyV) are the known etiologic factors of MCC. Due to the observed dichotomy in clinical and genetic profiles, MCC is often classified as "virus-positive MCC" (VP-MCC) or "virus-negative (also called "UV-driven") MCC" (VN-MCC). The latter has a higher tumor mutation burden, with recurrent mutations in TP53 and RB1, which are considered UV radiation signature mutations [5].

Treatment of MCC remains a challenge and recurrence following surgical excision has been reported in one-third of patients [6]. Moreover, most patients develop metastases. The 5-year survival rates for cases with localized tumors, regional metastasis, and distant metastasis are 51%, 35%, and 14%, respectively [7]. To date, there is no therapy shown to be effective in prolonging the survival of patients with MCC [8]. Besides the extent of the disease, variable prognostic markers, such as MCPyV status, anti-tumor immune response (ascertained by the presence of $CD8^+$ T cells

and tumor PD-L1 expression), and the expression of biomarkers such as p63, bcl2, EZH2, survivin, and TP53, have been described [9, 10, 11, 12].

VP-MCC is highly immunogenic and correlates with a high density of $CD8^+$ tumor infiltrating lymphocytes (TILs). Even though patients with higher $CD8^+$ TIL ratios have improved overall survival [13], these tumors are more prone to escaping host immune attack because they aberrantly express PD-L1, a peptide that binds to the PD1 receptor on T lymphocytes. There is a growing interest in the expression of PD-L1 by tumor cells in many cancers, including MCC. The prognostic role of PD-L1 seems to vary among cancer types; it has been reported to be correlated with better prognosis in MCC [8, 9, 14, 15].

EZH2, a histone methyltransferase with a vital role in epigenetic gene silencing [16], may function as an oncogene or a tumor suppressor gene depending on the tumor type [17]. Oncogenic function of EZH2 has been described in cancers such as breast, prostate, endometrial, bladder, liver, lung and ovarian cancer, as well as melanoma, glioblastoma, and non-Hodgkin lymphoma (NHL) [16]. Loss-of-function mutations have been reported in a subset of myelodysplastic syndromes, myeloproliferative neoplasm, and T-acute lymphoblastic leukemia [16]. Association of EZH2 with disease progression and worse outcome has been shown in melanoma and some other cancers. In the case of MCC, EZH2 overexpression and its prognostic role have been reported in a few studies [11, 17, 18].

New-age drugs targeting immune checkpoints, such as PD1 and PD-L1, or EZH2 are promising agents for the treatment of various cancer types at advanced stages. However, more data are needed to understand the prognostic and therapeutic role of these biomarkers in MCC. In the current study, we examined the status of PD1, PD-L1, CD8, and EZH2 in a series of MCC cases from our institute and correlated the results to clinical follow-up data.

Material and methods

Patient data

The pathology archive database of the of our institute was scanned retrospectively for diagnosed cases of MCC. Fourteen cases diagnosed between 2005 and 2020 were identified, for which clinical follow-up and treatment data were extracted from the hospital record system and pathology reports, as well as by directly contacting clinicians or patients. One case was removed from the study group because of lack of both follow-up data and paraffinized tumor tissue sample. The mean overall follow-up time for the remaining 13 cases was 28.5 months (range: 4-103 months). Eligible paraffin blocks from 13 cases were selected from Hematoxylin and Eosin (HE) staining.

Ethical committee approval was not an obligation institutionally, hence the research was not directly realized on human subjects, but on the human tissue samples preserved in the archieve of the department.

Immunohistochemistry

Immunohistochemical staining was performed on 13 slides prepared from FFPE (formalin-fixed, paraffin-embedded) tumor tissues on a Ventana Benchmark XT automated stainer (Ventana Medical Systems, Oro Valley, AZ, USA), according to the manufacturer's protocols, for the following biomarkers: Merkel cell polyoma virus large T-antigen (CM2B4) (sc-136172; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PD1 (MRQ-22; Ventana Medical Systems), PD-L1 (Ventana SP263; Roche Tissue Diagnostics and ab205921[28-8]; Abcam), CD8 (NCL-L-CD8-4B11; Novo castra, Sheffield, UK), and EZH2 (Cell Marque, Rocklin, CA, USA).

PD1 and PD-L1 staining were evaluated separately on tumor cells and TILs. PD1 staining on tumor cells and TILs, as well as PD-L1 staining on TILs, were evaluated as "absent" or "present" regardless of the percentage of stained cells. On the other hand, PD-L1 staining on tumor cells was considered "positive" when the protein was detected in the membranes of more than 1% of tumor cells. Plasenta sections were stained as external positive control for both anti-PD-L1 (according to the manufacturers' recommendations) antibodies. Additionally, histiocyte staining was accepted as internal control in available tissues. In addition, intra-tumoral and peritumoral $CD8^+$ T cells were categorized as "low-" or "high-density" using a cut-off point of approximately 60 $CD8^+$ cells per typical HPF (high power field), as described previously [8]. Finally, the semi-quantitative method of H-score (Histoscore algorithm) was used for EZH2 assessment. Specifically, EZH2 expression was calculated for each tumor by multiplying each level of staining intensity present in a tumor (ranked 0-3) with the percentage of cells displaying that intensity. Then, tumors were classified into weak EZH2 expressers (H-score: 0-155) and moderate/strong EZH2 expressers (H-score: 155-300), according to the threshold obtained by the ROC (Receiver operating characteristic) analysis for predicting unfavorable outcome performed by Harms *et al.* [17].

Statistical analysis

Quantitative variables were summarized with median 1st and 3rd quarter statistics, while qualitative variables were summarized with numbers and percentages. Comparison of quantitative variables between two groups was performed using the Mann-

Table I. Merkel cell carcinoma patients demographics

	Age, gender	Location	Metastasis	Status	Follow-up time (months)	MCPyV*
Case 1	55, F	Right posterior leg skin	Inguinal, pelvic, iliac lymph nodes, spleen hilus, lung	Deceased	31	Positive
Case 2	79, M	Left malar skin	Submandibular and mediastinel lymph nodes, lung, liver, bone marrow	Deceased	8	Negative
Case 3	35, F	Auricular skin	Over metastasis (at 17 months)	Alive	103	Positive
Case 4	60, M	Left proximal arm skin	None	Alive	75	Positive
Case 5	62, M	Right inguinal skin	Right parailiac, right perirenal, liver	Deceased	17	Negative
Case 6	78, F	Left upper eyelid	None	Alive	52	Negative
Case 7	84, M	Left nasolabial skin	Local recurrence (at 6 months)	Deceased	10	Negative
Case 8	80, M	Gluteal skin	None	Deceased	4	Positive
Case 9	69, F	Left malar skin	None	Deceased	13	Negative
Case 10	62, F	Frontotemporal skin	Lumbar skin, mediastinal lymph node	Deceased	18	Negative
Case 11	68, F	Right upper eyelid	None	Alive	26	Positive
Case 12	79, F	Right upper eyelid	Cervical lymph node	Alive	9	Positive
Case 13	60, M	Right arm	Local lymph node	Alive	4	Positive

**Merkel Cell Polyoma Virus-LT antigen*

Whitney U-test, whereas the significance of the relationships between qualitative variables was assessed using the Chi-square test and the level of relationship was calculated using the Cramér V statistic. In all statistical analyses, one-way exact p-value was calculated, with $p < 0.10$ considered as significant. IBM SPSS statistics for windows v25 (IBM Corp, Armonk, NY, USA) was used.

Results

Patient demographics and clinical follow-up

Detailed data of the patients are presented in Table I.

The selected patients (n = 13) were mainly older adults with a median age of 68 years (range: 35-84 years) and a slight women dominance (53.8%). With respect to tumor location, the vast majority of cases (61.5%) were head and neck, followed by extremities (23.1%) and trunk (15.4%). Two patients had chronic lymphocytic leukemia/small lymphocytic lymphoma before MCC diagnosis. At the time of the study, 7 out of the 13 cases (53.9%) had passed away due to disease progression. The mean overall survival (OS) for these patients was 14.4 months (range: 4-31 months). The six remaining patients (46.1%) have been followed-up for a mean time of 32.3 months (range: 4-103 months). With respect to metastasis, 3 out of the 6 living patients (50%) were non-metastatic, whereas 5 out of the 7 (71.4%) deceased cases were metastatic at time of diagnosis (n = 4) or at recurrence (local recurrence at 6 months after first diagnose due to close surgical margin, n = 1). The selected treatment approach was mainly total excision and lymphadenectomy in non-metastatic cases, while radiotherapy (RT) and chemotherapy (CT), alone or in combination, were applied to metastatic patients.

Immunohistochemistry results

MCPyV-LT antigen was positive in 7 cases (53.9%) and negative in 6 cases (46.1%). VP-MCC tumors were distributed as extremities (42.3%), head and neck (42.3%), and trunk (14.3%), whereas VN-MCC tumors were predominantly located on head and neck (83.3%), followed by trunk (16.7%) (Table I).

Virus positivity was not statistically related to metastasis/recurrence status ($\chi^2 = 0.124$, $p = 0.587$). Patients with VP-MCC tumors were mostly alive (71.4%) with a mean follow-up of 43.4 months. In

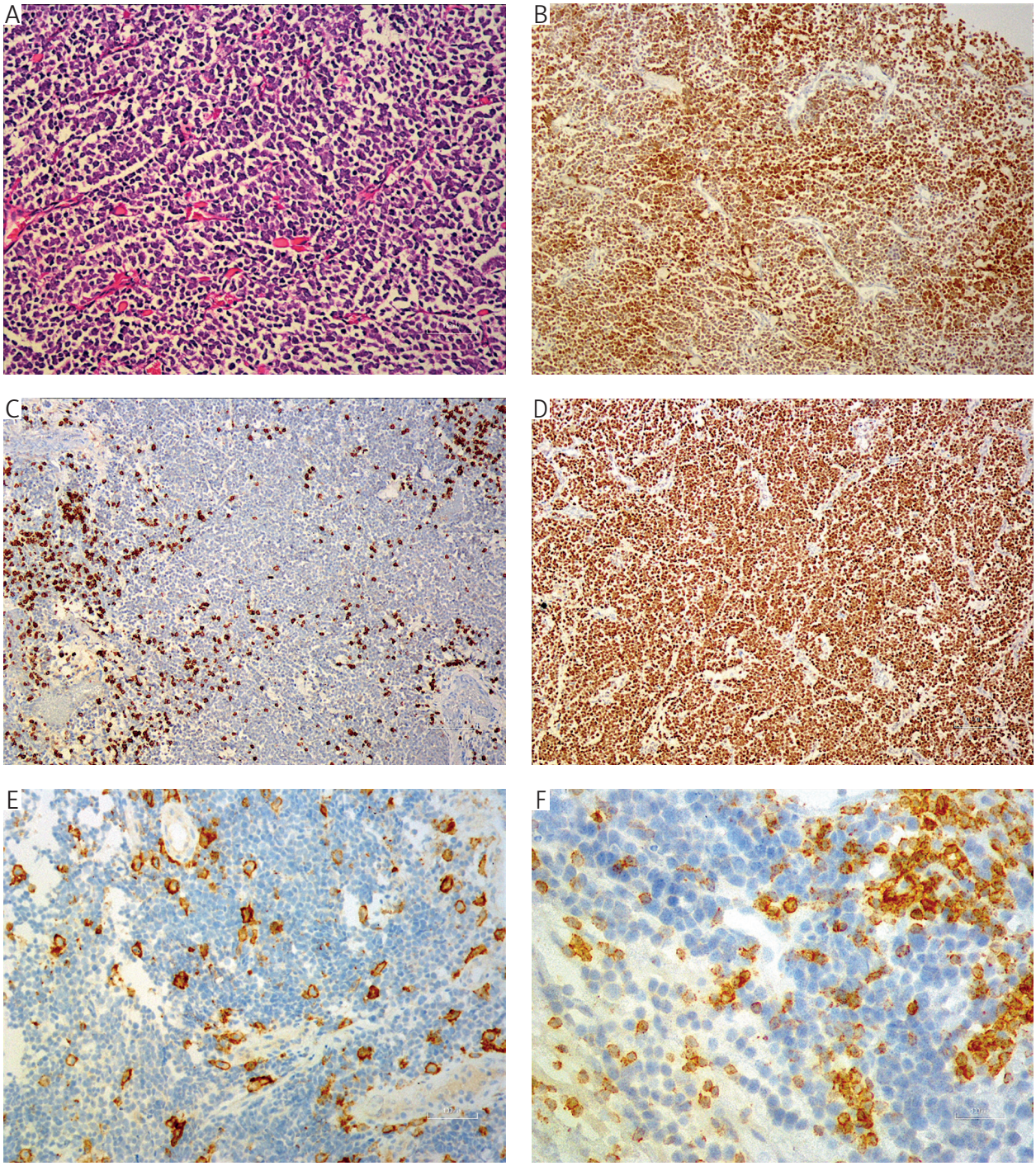

Fig. 1. Virus-positive Merkel cell carcinoma (VP-MCC) case. A) small round tumor cells with coarse chromatin (hematoxylin eosin (HE), 200×); B) positive staining with MCPyV-LT antigen within tumor cells (immunohistochemistry (IHC), 100×); C) high density $CD8^+$ TILs are present (IHC, CD8, 100×); D) strong nuclear positivity in all tumor cells with EZH2 (IHC, EZH2, 100×); E) PD-L1 positivity in tumor infiltrating macrophages but not in tumor cells (IHC, PD-L1, 200×); F) PD-1 positivity in tumor infiltrating lymphocytes, but not tumor cells (IHC, PD-1, 100×)

contrast, those with VN-MCC tumors were mostly deceased (83.3%) with a mean OS of 13.2 months ($\chi^2 = 3.899$, Cramér V = 0.548, p = 0.078).

When we compared MCPyV-LT antigen positivity with CD8 density, the majority of VP-MCC tumors (83.3%) had high-density $CD8^+$ TILs (Fig. 1). This result was statistically significant ($\chi^2 = 3.899$, Cramér V = 0.548, p = 0.078) (Table II). These findings suggest that MCPyV positivity is correlated with a stronger immune response and longer survival.

Regardless of MCPyV status, CD8 density was high in 83.3% of all living patients and in 14.3%

of deceased patients. The association between high CD8 density and continued survival was statistically significant ($\chi^2 = 6.198$, Cramér V = 0.690, p = 0.025).

EZH2 expression was variable. Eight cases (61.5%) had weak expression (H-score: 0–155) and five cases (38.5%) moderate/strong expression (H-score > 155) (Figs. 1, 2). Of note, H-score was null in two cases. EZH2 was not found to be related with MCPyV presence ($\chi^2 = 2.236$, p = 0.179). EZH2 expression (based on two-tiered categorization) and metastasis/recurrence status were not statistically related ($\chi^2 = 1.170$, p = 0.315). Importantly, when we repeated the analysis using the separate H-scores themselves for EZH2 instead of the two-tiered categorization, EZH2 values were found to be higher (median H-score = 140) in metastasis/recurrence group compared to the non-metastasis/recurrence group (median H-score = 60). That was statistically significant (z = –1.396, p = 0.089). These findings suggest that higher levels of EZH2 plays a significant role in disease progression. In terms of OS, there was a significant relation between moderate/strong EZH2 expression and continued survival (Chi-sqr = 3.745, Cramér V = 0.537, p = 0.086) (Table III). Alive patients with moderate /strong expression were all MCPyV positive and all with high CD8 levels. Similarly; among the weak EZH2 expressors (n = 8), 83.3 % of the deceased cases (5/6) were with low CD8 levels. Therefore; these results make us to conclude that higher EZH2 levels alone may not have a role on OS in VP-MCC cases, and CD8 levels seem to be more significative on OS in MCC. However, studies employing larger cohorts are needed in order to verify this result and also help determine a better cut-off value.

PD1 expression in TILs was seen in all cases, while no expression was detected in tumor cells (Fig. 2). PD-L1 expression was not present in tumor cells of any cases for both clone types. Besides, nearly half of VP-MCC cases (42.3%) revealed PD-L1(SP263) expression on TILs (Fig. 1). The universally negative PD-L1 staining in tumor cells may be associated with the clone types of the antibody (Ventana SP263 and Abcam ab205921[28-8]) used in our study.

Table II. Correlation of MCPyV presence with immune response and overall survival

	MCPyV		
	Negative	Positive	p*
CD8			0,078
Low, n (%)	5 (71.4)	2 (28.6)	
High, n (%)	1 (16.7)	5 (83.3)	
Status			0.078
Alive, n (%)	1 (16.7)	5 (83.3)	
Deceased, n (%)	5 (71.4)	2 (28.6)	

** χ^2 test, 1-sided exact p value*

Discussion

MCC is an aggressive neoplasm and there are no optimized treatment approach and undisputed prognostic parameters for it currently. The discovery of the carcinogenic role of MCPyV in MCC has provided new perspectives, such as a focus on the importance of the immune response and the immune checkpoints, as well as the possibility of treatment with immune checkpoint inhibitors, such as pembrolizumab, nivolumab, and avelumab. With that in mind, we aimed to analyze the PD-1 and PD-L1 statuses in MCC and assess their prognostic role in our MCC cohort.

MCPyV, whose prevalence among MCC cases has been reported to be as high as 80%, was detected in 53.9% of the patients in our cohort [8, 19]. VP-MCCs are associated with an increased presence of infiltrating $CD8^+$ T cells, which predominantly co-express PD-1 (71%) [14]. In our study, all (100%) VP-MCCs co-expressed PD-1within TILs (Fig. 1).

With respect to the expression of the immunosuppressive ligand PD-L1 by tumor cells, the ratios

Table III. Correlation of EZH2 overexpression with disease progression and overall survival

	EZH2		
	Weak (H score < 155)	Moderate/Strong (H score > 155)	p*
Metastasis/recurrence			0.315
Absent, n (%)	4 (80)	1 (20)	
Present, n (%)	4 (50)	4 (50)	
Status			0.086
Alive, n (%)	2 (33.3)	4 (66.7)	
Deceased, n (%)	6 (85.7)	1 (14.3)	

** χ^2 test, 1-sided exact p value*

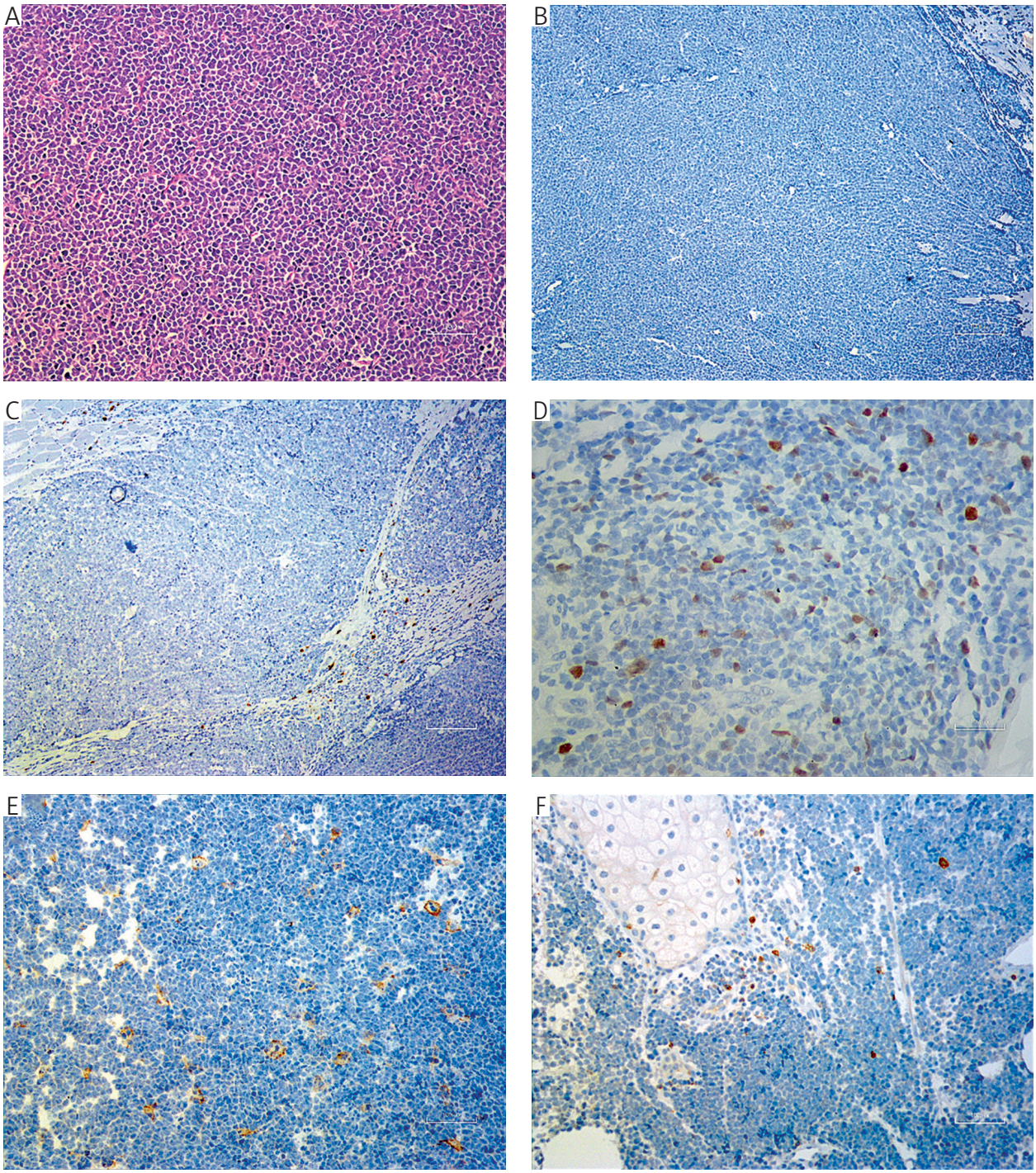

Fig. 2. Virus-negative Merkel cell carcinoma (VN-MCC) case. A) typical histomorphology of MCC (HE, 200×); B) negative staining with MCPyV-LT antigen within tumor cells (IHC, 100×); C) low density CD8+ TILs are present (IHC, CD8, 100×); D) weak nuclear positivity in most of the tumor cells with EZH2 (IHC, EZH2, 200×); E) PD-L1 negativity in tumor cells (IHC, PD-L1, 200×); F) PD-1 positivity in small numbers of tumor infiltrating lymphocytes, but not tumor cells (IHC, PD-1, 200×)

reported by the literature vary between 20% and 70% [8, 14, 15]. In our study, staining with PD-L1 (Ventana SP263 and Abcam ab205921[28-8]) did not produce any positive tumor cell staining. This discrepancy may be associated with variations in antibody clones and/or a lack of standardized criteria for analysis. However, 42.3% of VP-MCC cases demonstrated PD-L1 (SP263) positivity in the tumor microenvironment, which contained lymphocytes and macrophages.

PD-L1 is expected to be related with worse prognosis, as it inhibits the T cell response. Indeed, PD-L1

expression is associated with poor prognosis in many cancers such as hepatocellular carcinoma, pancreatic carcinoma, gastric carcinoma, renal cell carcinoma, and ovarian cancer. On the other hand, both positive and negative predictive effects of PD-L1 expression have been reported in lung cancer, melanoma, and colorectal carcinoma [20], whereas in some tumors, such as thymoma and thymic carcinoma, squamous lung carcinoma, cervical cancer, and MCC, PD-L1 expression per se was not found to be a reliable prognostic marker, but acquired statistically significant predictive value when combined with variables related to other immune mediators such as the ratio of $CD8^+$ $Foxp3^+$ T cells [21]. Additionally, the expression of PD-L1 in tumor cells but not TILs was associated with improved OS in a recent study [8]. Absence of MCPyV and lower $CD8^+$ T cell infiltration have been reported to be related with worse prognosis [22]. In our group, the universal PD-L1 negativity in tumor cells prevented us to evaluate the prognostic value of PD-L1. However, we must note that VP-MCC cases showed significantly increased $CD8^+$ T cells, and higher levels of $CD8^+$ T cells significantly correlated with better OS ($p = 0.078$). This finding suggests that the immune response level has a definite prognostic value in MCC, and should thus be mentioned in the pathology report, as Naseri *et al.* proposed [22].

For the last two decades, epigenetic mechanisms have been the focus of intense studies in the field of cancer research and have become a promising target for new treatment options. EZH2 is an enzymatic subunit of Polycomb Repressor Complex 2 (PCR2) that mediates epigenetic gene silencing by Histone 3 K27 (H3K27) trimethylation [16]. In the epidermis, PCR2 activity opposes the differentiation of epidermal progenitor cells into Merkel cells [23]. It has been suggested that EZH2 plays a role in maintaining cancer stem cell properties in various tumors [17]. In melanoma patients, EZH2 was shown to correlate with poor survival. Moreover, it has been found to promote the initiation and progression of melanoma in mouse models, while downregulation of EZH2 in human melanoma cells reduced their proliferation and invasiveness [24]. To our knowledge, this is the third study examining EZH2 expression in MCC and the second study to present a correlation analysis between EZH2 expression and clinical parameters in patients with MCC [11, 17]. After performing a detailed analysis of EZH2 expression in MCC, Harms *et al.* reported moderate to strong expression in 54% of cases, which is slightly higher than the percentage reported in the present study (38.5%). Moreover, they showed that lower expression of EZH2 in the primary tumor was associated with improved prognosis and longer disease-free survival, whereas a higher expression in lymph node metastasis was concluded to have a role in disease progression [17]. Similarly, we found higher EZH2 levels (median H-score = 140) in metastatic patients compared to non-metastatic ones (median H-score = 60), with the difference being statistically significant ($p = 0.089$). Interestingly, this correlation was not observed ($p = 0.315$) when the EZH2 levels of the individual patients were not processed as separate H-score values but as belonging to a "low" and a "moderate/strong" H-score group with a cut-off value of 155. Moreover, in contrast to what was reported by Harms *et al.*, OS was better in moderate/strong expressers compared to weak expressers in the present study ($p = 0.086$). Regarding this group patients' completely MCPyV positivity and high CD8 levels we conclude that EZH2 alone may not be a predictive parameter on OS. As a whole, our findings regarding EZH2 strongly suggest that it may play a role in MCC tumor progression, but not on OS alone. MCPyV and CD8 status seem to be more significative on OS. However, determination of the most informative, with respect to prognosis, cut-off value for EZH2 expression requires additional studies with larger cohorts.

The limitations of this study include the small number of cases and the fact that the detection of MCPyV was performed immunohistochemically and not by molecular analysis using PCR. Despite these limitations, our results provide an insight into the role of EZH2, PD-1, and $CD8^+$ TILs in VP- and VN-MCC patients. We present evidence that a high density of $CD8^+$ T cells is a good prognostic factor in VP-MCC patients. Moreover, even though high EZH2 expression was correlated with tumor progression, EZH2 alone was not predictive on OS. As a whole, our results suggest that immune checkpoint inhibitors and EZH2 inhibitors should be included in future clinical studies on MCC treatment.

The authors declare no conflict of interest.

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Address for correspondence

Arbil Acikalin
Pathology Department
Medical Faculty
Cukurova University
01130 Adana, Turkey
e-mail: arbilacikalin@gmail.com