Higher Expression of Activation-induced Cytidine Deaminase Is Significantly Associated with Merkel Cell Polyomavirus-negative Merkel Cell Carcinomas

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ABSTRACT

Background Merkel cell carcinomas (MCCs), clinically aggressive neuroendocrine skin cancers, are divided into Merkel cell polyomavirus (MCPyV)-positive and -negative tumors, which show different clinicopathological features and may develop through different mechanisms of carcinogenesis. Aberrant expression of activation-induced cytidine deaminase (AID) as a genomic modulator was demonstrated through pathogen-related NF-κB signal in *Helicobacter pylori*-associated gastric cancer, adult T cell leukemia/lymphoma (HTLV-1), hepatoma (HCV), and Burkitt lymphoma (EBV).

Methods To elucidate the relation of aberrant AID expression in MCPyV-positive and -negative MCCs, we evaluated immunohistochemical expressions of AID and AID-regulating factors between 24 MCPyV-positive and 17 MCPyV-negative MCCs.

Results AID expression was significantly higher in MCPyV-negative MCCs than MCPyV-positive ones ($P = 0.026$), although expression of NF-κB p65 (phospho S536) (AID-enhancer) was significantly higher in MCPyV-positive MCCs than MCPyV-negative ones ($P = 0.034$). Expressions of PAX5 and c-Myb were not significantly different between these subgroups. Expressions of AID and AID-regulating factors were not correlated to prognosis of MCC patients.

Conclusion Our findings suggest that although pathogen-induced AID expression through upregulation of NF-κB may be relevant to carcinogenesis of MCPyV-positive MCCs, the significantly higher aberrant AID expression in MCPyV-negative MCCs is consistent with the fact that MCPyV-negative MCCs have an extremely higher mutation burden than MCPyV-positive ones.

Key words activation-induced cytidine deaminase; Merkel cell carcinoma; Merkel cell polyomavirus

Merkel cell carcinoma (MCC) is a clinically aggressive neuroendocrine skin cancer and Merkel cell polyomavirus (MCPyV) is monoclonally integrated into the genome of approximately 80% of MCCs.[1] Recently, we demonstrated clinical and pathogenetic differences between MCPyV-positive MCCs and MCPyV-negative MCCs; MCPyV-positive MCCs have a more round and narrow shape than MCPyV- negative MCCs,[2] and MCPyV-positive MCC showed a better prognosis than MCPyV-negative MCCs.[3–5] However, it has not been fully elucidated how the tumorigenic mechanism or pathway is different between MCPyV-positive and -negative MCCs.

Activation-induced cytidine deaminase (AID), a nucleotide-editing enzyme, is essential for the somatic hypermutation (SHM) and class-switch recombination (CSR) of the immunoglobulin gene[6] and evidence of AID's involvement in carcinogenesis has been accumulated in not only B-cell lymphoma but non-B cell malignancy.[6, 7] AID is a APOBEC family protein and induces off-target deamination of cytosine to uracil in DNA, and this AID-induced mutagenic U:G mismatch is considered as a common mutagenic mechanism in carcinogenesis.[8] AID is also important as a genomic modulator in inflammation-associated cancer development in digestive organs including *Helicobacter pylori* (*H. pylori*)-associated gastric cancer, hepatitis C virus-positive hepatocellular carcinoma, colitis-associated colon cancers[9] and pancreatic cancer.[10] Nuclear factor-κB (NF-κB) activation in epithelial cells and malignant cells is involved in generating genomic instability through aberrant AID expression, cell growth, proliferation, survival, angiogenesis, and epithelial-to-mesenchymal transition (EMT).[11] Transcription and expression of *AID*

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Abbreviations: AID, activation-induced cytidine deaminase; CSR, class-switch recombination; DSS, disease-specific survival; HR, hazard ratio; MCC, Merkel cell carcinomas; MCPyV, Merkel cell polyomavirus; NF-κB, Nuclear factor-κB; OS, overall survival; PAX5, paired box gene 5; Rb, retinoblastoma; SHM, somatic hypermutation; UV, ultraviolet light

gene are controlled by many elements and transcription factors including NF-κB, Stat6, C/EBP, Smad3/4, Myb, Pax5, E2A, E2f and BATF, bind to the AID regulatory regions.[12] The expression and activity of AID are tightly regulated at the levels of transcription, post-transcription, and enzymatic function. Four distinct DNA regions (region I to IV) of the *AID* gene locus contain binding sites for multiple transcription factors. Region I functions as a promoter containing the binding site for NF-kB, a transcriptional activator. In B and non-B cells, enhancer elements in region II bind to the enhancer proteins PAX5 (paired box gene 5) and E2A, while silencer elements in region II bind to the silencer proteins c-Myb and E2f in order to counter the activities of transcriptional enhancers.[12] Honjo et al. described pathogen-induced AID expression in gastric cancer (*H. pylori*), adult T cell leukemia/lymphoma (ATLL) (HTLV-1), hepatoma (HCV), and Burkitt lymphoma (EBV), but not in classical Hodgkin's lymphoma (EBV).[6] However, aberrant AID expression in MCPyV-associated MCC has not yet been elucidated. MCPyV is an oncogenic agent in a set of MCCs.[3] In this study, we evaluated and compared the expression of AID and AID-regulating factors such as NF-κB (enhancer), PAX5 (enhancer), and c-Myb (silencer) between MCPyV-positive and –negative MCCs in order to elucidate the association of MCPyV infection with expressions of AID and AID-regulating factors in the molecular pathogenesis of MCCs and clarify the question whether or not MCPyV-induced AID expression is a causative factor of carcinogenesis in MCPyV-associated MCC.

MATERIALS AND METHODS

Samples

This study was approved by the Institutional Review Board of the Faculty of Medicine at Tottori University (No.1216). We used 24 MCPyV-positive and 17 MCPyV-negative formalin-fixed paraffin- embedded (FFPE) MCC samples.

MCPyV detection

To detect MCPyV infection, real-time quantitative polymerase chain reaction (q-PCR) and immunohistochemistry with antibody to MCPyV-Large T protein (CM2B4) were performed according to our previous studies.[5, 13]

Immunohistochemistry

Immunohistochemistry staining was performed on 4-μm-thick paraffin sections as described previously.[14] Primary antibodies were used with the following dilutions: mouse monoclonal anti-AID (clone ZA001, dilution 1/200; Thermo Fisher Scientific, Waltham, MA), rabbit polyclonal anti-NF-κB p65 (phospho S536) (1/500; Abcam, Cambridge, UK), rabbit monoclonal anti-c-Myb (clone EP769Y, dilution 1/150; LSBio, Seattle, WA), mouse monoclonal anti-PAX5 (clone DAK-Pax5, dilution 1/30; Agilent Technologies, Santa Clara, CA). Antigen retrieval of a rabbit polyclonal anti-NF-κB p65 (phospho S536) was performed by incubating the sections with target retrieval solution (pH 9.0) for 20 minutes, other antibodies were performed by incubating the sections with citrate buffer (pH 6.0) for 10 minutes using pressure cooker, then the immune complex was detected with an anti-mouse or anti-rabbit EnVision detection system and 3, 3′-diaminobenzidine and chromogenic substrate (DAKO).

For evaluating immunohistochemistry, H-score was used as described previously.[15]

The H-score is performed as the sum of the percentage of staining multiplied by an ordinal value corresponding to the intensity level (0 = none, 1 = weak, 2 = moderate, 3 = strong). The resulting score ranged from 0 (no staining in the tumor) to 300 (100% diffuse strong staining of the tumor).

Statistical analysis

Immunohistochemical findings were analyzed in relation to MCPyV status using the Mann–Whitney *U* test. To identify baseline patient and AID, NF-κB p65 (phospho S536), c-Myb and PAX5 associated with prognosis, univariate hazard ratios were calculated with 95% confidence intervals (CIs) using the Cox proportional-hazards model. The hazard ratio (HR) and 95% CI were estimated using Cox hazard regression analysis for overall survival (OS), disease-specific survival (DSS) and stratification for MCPyV status, AID, NF-κB p65(-phospho S536), c-Myb and PAX5. The goodness of fit of each Cox model was evaluated using the likelihood ratio test, and the association between the individual variables and outcome was assessed using the forward Wald test selection ($P \leq 0.05$ was selected for entry into the model, and $P > 0.1$ was selected for removal).

RESULTS

MCPyV detection

MCPyV infection in MCCs was confirmed by the presence of MCPyV-DNA with q-PCR and positive staining of MCPyV-Large T antigen (CM2B4) in tumor cells. And the clinicopathological characteristics of 24 MCPyV-positive and 17 MCPyV-negative MCC cases are summarized in Table 1.

Immunohistochemical staining

The summary of immunohistochemical H-score for

Table 1. Clinical information of the patients in this study

Sample No.	MCPyV (q-PCR)	Age (years old)	Sex	Tumor size (cm)	Initial treatment	Organ	Site	Diagnosis	Month
UK-M-1	–	81	F	4	RE	Dermis	Rt. shin	Combined MCC & BD	7
UK-M-2	–	81	F	3.4 × 3 × 1.2	RE	Dermis	Rt. leg	Combined MCC & BD	44
UK-M-3	–	85	F	2.1	RE	Dermis	Lower leg	Combined MCC & SqCC	21
UK-M-4	–	93	F	5	RE	Dermis	Lt. cheek	Combined MCC & SqCC	6
UK-M-8	–	87	F	1.4	RE	Dermis	Forehead	Pure MCC	12
UK-M-10	–	94	F	6.5 × 5 × 2.5	PE	Dermis	Rt. lateral leg with multiple metastasis in the resion	Pure MCC	16
UK-M-13	–	61	M	1.8 × 1.5	RE	Dermis	Rt. shin	Pure MCC	23
UK-M-14	–	86	F	1.5 × 1.5	RE	Dermis	Lt. dorsam foot	Combined MCC & BD	11
UK-M-20	–	82	M	3.5	RE, RD	Dermis	Rt. knee	Combined MCC & BD	31
UK-M-5	–	81	F	3.5	PE	Dermis	Lt. leg with multiple satellites	Combined MCC & SqCC	18
UK-M-23 (meta)	–					LN	Lt. groin		
UK-M-17	–	83	F	1.7 × 1.4	PE	Dermis	Lt. lower eyelid	Combined MCC & BCC	39
UK-M-17 (meta)	–					Dermis	Conjunctiva		
UK-M-18	–	94	F	5 × 4 × 2	RD, RE	Dermis	Rt. temple	Pure MCC	6
UK-M-31 (meta)	–					LN	Rt. parotid		
MCC50	–	82	M	1.5	RE	Dermis	Dorsum of the hand	Combined MCC & BD	13
MCC51 (meta)	–					LN	Axilla		
UK-M-7	+	68	M	2.7 × 1.7 × 0.8	RE	Dermis	Rt. knee	Pure MCC	59
UK-M-9	+	69	M	5 × 5 × 4	RE	Dermis	Rt. groin	Pure MCC	55
UK-M-11	+	61	F	1.2	RE	Dermis	Rt. cheek	Pure MCC	18
UK-M-16	+	63	F	2.5 × 2.5	RE, RD	Dermis	Lt. upper arm	Pure MCC	70
UK-M-19	+	85	F	4 × 2.8 × 1.3	RE	Dermis	Rt. forearm	Pure MCC	21
UK-M-22	+	74	F	6 × 5.5 × 2	RE	Dermis	Lt. elbow	Pure MCC	72
MCC36	+	87	F	2 × 2.3	RE	Dermis	Nasal ala	Pure MCC	3
MCC37	+	73	F	0.9	RE	Dermis	Cheek	Pure MCC	29
MCC38	+	83	F	1.1 × 1.0 × 0.8	RE	Dermis	Cheek	Pure MCC	2
MCC45	+	64	F	2 × 3	Unknown	Dermis	Lower thigh	Pure MCC	Unknown
MCC46	+	67	F	2 × 1.5	Unknown	Hypodermis	Buttock	Pure MCC	Unknown
MCC47	+	90	M	2	Unknown	Dermis	Humerus	Pure MCC	Unknown
MCC53	+	61	F	1.9	RE	Dermis	Earflap	Pure MCC	28
MCC55	+	75	M	1.9 × 1.7	Unknown	Dermis	Cheek	Pure MCC	Unknown
MCC57	+	77	F	Hen's egg size	CT, RE	Hypodermis	Knee	Pure MCC	53
MCC61	+	59	F	1.4 × 1.1	RE	Dermis	Lower thigh	Pure MCC	35
MCC66	+	86	F	10	RD	Dermis	Superior eyelid	Pure MCC	1
MCC70	+	NA	M	3	RE	Dermis	Thigh	Pure MCC	34
MCC73	+	90	F	2	RD	Dermis	Cheek	Pure MCC	3
MCC83	+	71	F	2	RE, RD	Dermis	Lt. thigh	Pure MCC	31
UK-M-21	+	46	F	3 × 2.5	RE	Dermis	Lt. buttock	Pure MCC	59
UK-M-21 (meta)	+					Dermis			
MCC32	+	66	M	2 × 2.5	RE	Dermis	Thigh	Pure MCC	2
MCC33 (meta)	+					LN	Inguina		

BD, Bowen's disease; CT, chemotherapy; F, female; LN, lymph node; Lt., left; M, male; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; meta, metastasis; NA, not available; PE, palliative excision; q-PCR, quantitative polymerase chain reaction; RD, radiation; RE, radical excision; Rt., right; SqCC, squamous cell carcinoma.

AID, NF-κB p65 (phospho S536), PAX5 and c-Myb, and the representative immunostainings in the MCC subgroups are shown in Table 2, Figure 1, respectively. NF-κB p65 (phospho S536) was expressed only in the nuclei.

AID expression was significantly lower in MCPyV-positive MCCs than MCPyV-negative ones (P = 0.026), although expression of NF-κB p65 (phospho S536) (AID-enhancer) was significantly higher in MCPyV-positive MCCs than MCPyV-negative ones (P = 0.034) (Table 3, Fig.2). There were no statistically significant differences between MCPyV-positivity in MCCs

and the other AID-regulating factors (PAX5 and c-Myb) (Table 3, Fig.2).

In addition, there was a statistically significant association between AID and NF-κB p65 (phospho S536) expressions ($P < 0.05$) but no statistically significant correlations between AID and other AID-regulating factors (PAX5 or c-Myb) in MCCs.

Table 2. Summary of immunohistochemical H-scores for AID and AID-regulating factors in MCPyV-positive and -negative MCCs

	q-PCR	IHC	IHC (H-score)			
Sample No.	*MCPyV*-DNA	MCPyV-LT (CM2B4)	AID	NF-κB p65 (phospho S536) (AID-enhancer)	PAX5 (AID-enhancer)	c-Myb (AID-silencer)
UK-M-1	–	–	180	0	150	200
UK-M-2	–	–	225	0	125	84
UK-M-3	–	–	245	0	200	165
UK-M-4	–	–	180	15	150	140
UK-M-8	–	–	150	20	170	235
UK-M-10	–	–	205	120	142	190
UK-M-13	–	–	220	100	100	250
UK-M-14	–	–	185	200	100	270
UK-M-20	–	–	185	0	190	175
UK-M-5	–	–	90	10	170	110
UK-M-23 (meta)	–	–	190	39	150	265
UK-M-17	–	–	150	10	160	140
UK-M-17 (meta)	–	–	300	20	190	235
UK-M-18	–	–	180	15	56	130
UK-M-31 (meta)	–	–	100	90	205	120
MCC50	–	–	195	20	85	170
MCC51 (meta)	–	–	228	35	173	80
UK-M-7	+	+	175	40	155	260
UK-M-9	+	+	145	31	140	210
UK-M-11	+	–	140	99	130	160
UK-M-16	+	+	190	85	205	190
UK-M-19	+	+	205	225	160	255
UK-M-22	+	+	150	60	180	200
MCC36	+	+	135	165	200	205
MCC37	+	+	240	53	105	225
MCC38	+	+	125	60	110	230
MCC45	+	+	165	66	105	205
MCC46	+	+	108	14	115	5
MCC47	+	+	165	40	65	103
MCC53	+	+	100	120	110	126
MCC55	+	+	80	393	105	260
MCC57	+	+	195	80	170	245
MCC61	+	+	170	130	200	NA
MCC66	+	+	70	40	200	245
MCC70	+	+	190	30	180	87
MCC73	+	+	215	240	240	93
MCC83	+	+	185	190	170	150
UK-M-21	+	+	250	10	90	150
UK-M-21 (meta)	+	+	280	11	170	260
MCC32	+	+	120	30	180	130
MCC33 (meta)	+	+	90	55	90	145

AID, activation-induced cytidine deaminase; c-Myb, a member of MYV family; IHC, immunohistochemistry; LT, Large T antigen; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; meta, metastasis; NA, not available; NE, not examined; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PAX5, Paired Box 5; q-PCR, quantitative polymerase chain reaction.

Table 3. Comparison of expressions of AID and AID-regulating factors in MCCs based on MCPyV status

Clinicopathological data	MCPyV-positive MCCs	MCPyV-negative MCCs	*P*-value†
Age (y.o.; mean ± SD)	74.6 ± 9.8	83.3 ± 9.1	0.065
Sex (male/female)	6/16	3/10	0.56
Stage (I/ II/ III)	9/11/2	2/7/2	0.835
AID H-score (mean ± SD)	157.4 ± 44.0	197.2 ± 27.4	0.026*
NF-κB p65 (phospho S536) H-score (mean ± SD)	108.1 ± 91.8	50.6 ± 68.3	0.034*
PAX5 H-score (mean ± SD)	152.3 ± 44.4	147.4 ± 33.5	0.627
c-Myb H-score (mean ± SD)	181.8 ± 69.2	189.9 ± 54.5	1

*Statistically significant. †Mann–Whitney *U* test.

AID, activation-induced cytidine deaminase; c-Myb, a member of MYV family; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PAX5, Paired Box 5; y.o., years old.

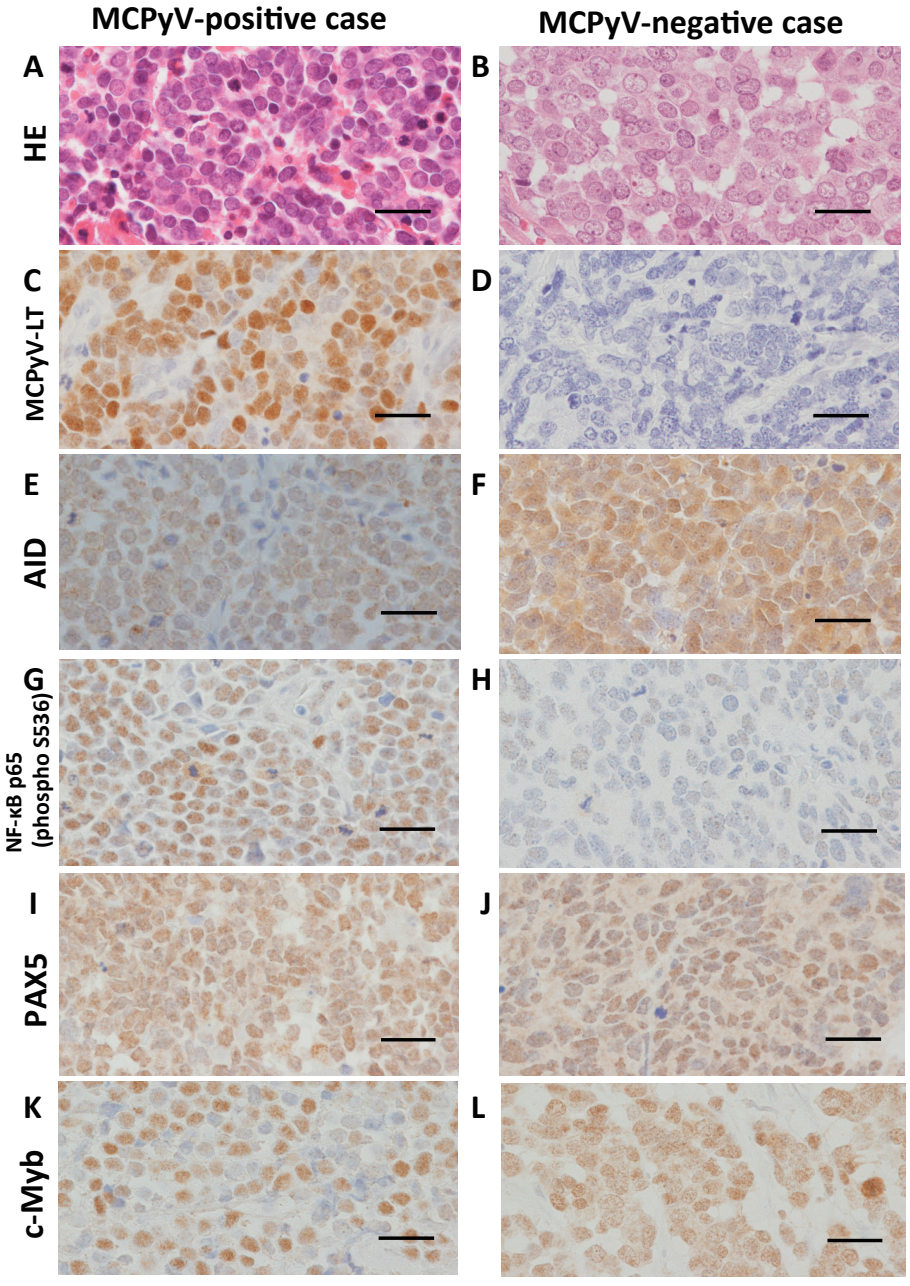

Fig. 1. Representative images of immunostaining of MCPyV-positive and MCPyV-negative MCCs. Representative images of IHC of AID and AID-regulating factors [NF-κB p65 (phospho S536) (AID-enhancer), PAX5 (AID-enhancer), and c-Myb (AID-silencer)] in MCPyV-positive MCCs (**A, C, E, G, I,** and **K**) and MCPyV-negative MCCs (**B, D, F, H, J,** and **L**) are shown. MCPyV-negative MCC tumor cells (**B**) had more irregular nuclear shapes and more abundant cytoplasm than did MCPyV-positive MCC cells (**A**). MCPyV DNA-positive MCCs showed strong positive nuclear immunoreactivity for MCPyV-LT (**C**), whereas MCPyV DNA–negative MCCs were negative for MCPyV-LT (**D**). AID expression was observed with significantly higher H-score in MCPyV-negative MCCs (**F**) than in MCPyV-positive MCCs (**E**) (H-score, mean ± SD, 197.2 ± 27.4 versus 157.4 ± 44.0; P = 0.026). NF-κB p65 (phospho S536) expression was significantly higher in MCPyV-positive MCCs (**G**) than in MCPyV-negative MCCs (**H**) (H score: mean ± SD, 108.1 ± 91.8 versus 50.6 ± 68.3; P = 0.034). However, no significant differences were observed in the H-scores of PAX5 and c-Myb between MCPyV-positive and –negative MCCs.

A and **B**, hematoxylin and eosin stain; **A** through **L**, IHC, scale bar = 25μm. AID, activation-induced cytidine deaminase; c-Myb, a member of MYV family; HE, hematoxylin-eosin; LT, Large T antigen; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PAX5, Paired Box 5.

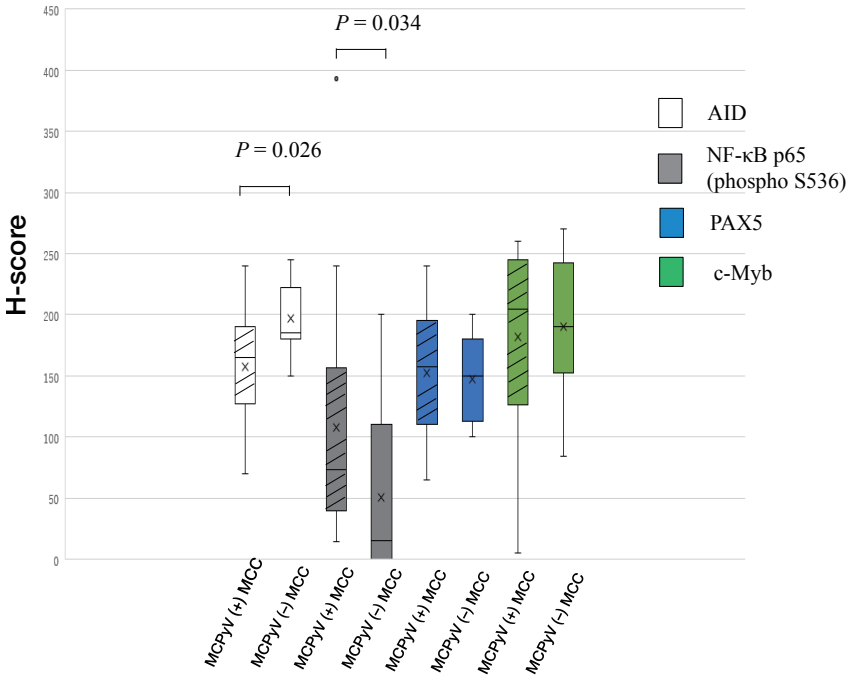

Fig. 2. Comparison of the immunohistochemical H-score for AID, NF-κB p65 (phospho S536), PAX5 and c-Myb between MCPyV-positive and -negative MCCs. AID expression was significantly lower in MCPyV-positive MCCs than MCPyV-negative ones (P = 0.026) while NF-κB p65 (phospho S536) expression was significantly higher in MCPyV-positive MCCs than MCPyV-negative ones (P = 0.034). No statistically significant difference was observed on H-scores of PAX5 and c-Myb expression between MCPyV-positive and -negative MCCs. AID, activation-induced cytidine deaminase; c-Myb, a member of MYV family; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PAX5, Paired Box 5.

Statistical analysis for survival

Results of univariate and multivariate Cox regression analyses for all patients can be seen in Table 4. In univariate analysis, MCPyV-positivity was a favorable parameter associated with a significant increase of both OS (HR: 0.101, P = 0.001) and DSS (HR: 0.090, P = 0.003), while male gender was significantly unfavorable in DSS (HR: 0.090, P = 0.003) but not in OS (HR: 2.399, P = 0.250). With multivariate analysis, only the presence of MCPyV was found to be a significantly favorable prognostic factor for both OS (HR: 0.03, P < 0.001) and DSS (HR: 0.025, P = 0.006), while male gender and advanced stage (stage II and III) were significantly unfavorable only for OS (HR: 10.89, P = 0.045; HR: 14.39, P = 0.026, respectively). Expressions of AID and AID-regulating factors were not significantly associated with OS and DSS.

DISCUSSION

AID expression of Merkel cell carcinoma with lymph-node metastasis was reported in a case report.[16] However, for the first time, we focused to study on the association of expression of AID, a genomic mutator, with pathogenetic differences between MCPyV-positive and –negative MCCs and demonstrated that MCPyV-negative MCCs showed a significantly higher expression of AID than MCPyV-positive ones. This finding is consistent with the reported facts that MCPyV-negative MCCs displayed higher overall mutation burden than MCPyV-positive ones.[17, 18]

Our previous studies showed some clinicopathological differences between MCPyV-positive and -negative MCCs; MCPyV-positive MCCs showed a significantly higher expression of retinoblastoma protein and less p53 expression compared to MCPyV-negative MCCs, and frequency of *TP53* non-ultraviolet signature mutation was significantly higher in MCPyV-negative MCCs than in MCPyV-positive MCCs.[4] And we also reported that Akt phosphorylation at T308 in activation of the Akt/mammalian target of rapamycin (mTOR)/4E-binding protein 1 (4E-BP1) signaling pathway, was significantly greater in MCPyV-negative than in MCPyV-positive MCCs[19] and that lower expression of CADM1 and higher expression of MAL in MCCs are associated with MCPyV infection and better prognosis.[15] It can be considered that MCPyV-positive and MCPyV-negative MCCs have different tumorigenic pathways; the integrated-mutated form of MCPyV is directly involved in "virus-mediated" tumorigenesis, whereas the accumulation of more complicating genetic aberrations is required for "nonviral" tumorigenesis; and both may develop under systemic/local impairment of host immune surveillance caused by UV irradiation, immunosenescence, use of immunosuppressants, and other factors.[3]

Cancer cells are considered to be generated from the stepwise accumulation of genetic alterations in various genes in inflammation-associated carcinogenesis.[9] AID, a nucleotide-editing enzyme that is essential for SHM and CSR of the immunoglobulin gene, is also known to play a role as a genomic mutator in carcinogenesis.[11]

Table 4. Univariate and multivariate cox proportional hazard analysis for mortality in MCC cases

Factors	Overall survival			MCC specific survival		
Univariate analysis	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (> 75/≤ 75 y.o.)	4.250	0.559–32.312	0.162	2.919	0.371–22.970	0.309
Sex (male/female)	2.399	0.540–19.664	0.250	0.090	0.180–0.441	0.003
Stage (stageII, III/stageI)	2.106	0.462–9.595	0.336	1.692	0.205–13.957	0.625
MCPyV-positive/-negative	0.101	0.028–0.370	0.001	0.090	0.018–0.441	0.003
AID H-score (> 160 vs ≤ 160)	1.174	0.400–3.444	0.770	0.993	0.283–3.491	0.992
NF-κB H-score (> 65 vs ≤ 65)	0.834	0.287–2.423	0.739	0.718	0.188–2.742	0.628
PAX5 H-score (> 130 vs ≤ 130)	1.448	0.465–4.512	0.523	2.147	0.462–9.985	0.330
c-Myb H-score (> 160 vs ≤ 160)	1.392	0.504–3.848	0.524	1.051	0.319–3.463	0.935

Factors	Overall survival			MCC specific survival		
Multivariate analysis	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (> 75/≤ 75 y.o.)	0.23	0.010–5.166	0.356	0.064	0.001–2.866	0.156
Sex (male/female)	10.89	1.051–112.832	0.045	2.665	0.252–28.180	0.415
Stage (stageII,III/stageI)	14.39	1.369–151.338	0.026	6.632	0.197–222.796	0.291
MCPyV-positive/-negative	0.03	0.004–0.207	< 0.001	0.025	0.002–0.346	0.006
AID H-score (> 160 vs ≤ 160)	1.04	0.208–5.162	0.966	2.467	0.273–22.286	0.421
NF-κB p65 (phospho S536)H-score (> 65 vs ≤ 65)	1.43	0.324–6.315	0.636	1.401	0.200–9.798	0.734
PAX5 H-score (> 130 vs ≤ 130)	2.40	0.426–13.536	0.321	18.006	0.938–345.730	0.055
c-Myb H-score (> 160 vs ≤ 160)	4.43	0.697–28.102	0.115	0.846	0.078–9.209	0.891

AID, activation-induced cytidine deaminase; CI, confidence interval; c-Myb, a member of MYV family; HR, hazard ratio; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PAX5, Paired Box 5; y.o., years old.

Pathogenic bacterial or viral factors and subsequent inflammatory reactions in *H. pylori*-related gastritis, chronic viral hepatitis, Barrett's esophagus, and inflammatory bowel disease lead to the aberrant expression of AID in various epithelial cells via NF-κB activation, which causes the accumulation of genetic alterations in tumor-related genes.[11]

Most MCCs are MCPyV-infected tumor. Our study revealed that the aberrant expression of AID was significantly lower in MCPyV-positive MCCs than in MCPyV-negative MCCs ($P = 0.026$), although aberrant NF-κB p65 (phospho S536) expression (an AID-enhancer) was significantly higher in MCPyV-positive MCCs than in MCPyV-negative MCCs ($P = 0.034$). Aberrant expression of PAX5 (an AID-enhancer) and c-Myb (an AID-silencer) was not significantly different between MCPyV-positive and –negative MCCs, and also that MCPyV was likely to be involved in pathogen-induced AID expression via NF-κB activation in MCPyV-positive MCCs. These results suggest that the genomic modulation of tumor-related genes by MCPyV-induced AID expression is a causative mechanism in the carcinogenesis of MCPyV-positive MCCs. However, Unexpectedly, the aberrant expression of AID was higher in MCPyV-negative MCCs with lower NF-κB p65 (phospho S536) expression than in MCPyV-positive MCCs with higher NF-κB p65 (phospho S536) expression ($P = 0.026$). This finding indicates that additional factors may modulate expression level of AID; the other enhancers and/or silencers of AID may influence the expression of AID in MCCs. Recently, in fact, it is known that not only NF-κB, but also Homeobox protein C4 may promote the transcription of AID and E2f, a transcription factor targeted by Rb protein, is also a silencer for AID.[12]

Cimino PJ et al[20] performed whole exome sequencing on five MCPyV-positive cases and three MCPyV-negative cases and the retinoblastoma gene (*RB1*) was found to have nonsense truncating gene mutations in all three MCPyV-negative cases, whereas no such mutations were found in the MCPyV-positive cases. While MCPyV-positive MCCs is believed to undergo retinoblastoma dysregulation through viral large T antigen expression, their findings demonstrate that somatic mutations of *RB1* in MCPyV-negative MCCs lead to retinoblastoma dysregulation through an alternative pathway. Harms PW et al performed integrative sequencing on 16 cases of MCC and reported

that MCPyV-negative tumors displayed high overall mutation burden (10.09 ± 2.32 mutations/Mb) and were characterized by a prominent UV-signature pattern with C > T transitions comprising 85% of mutations, while mutation burden was low in MCPyV-positive tumors (0.40 ± 0.09 mutations/Mb) and lacked a UV signature. Recent exome sequencing studies on 49 MCCs[18] also reconfirmed the dramatical differences of gene mutations between MCPyV-positive and –negative MCCs; MCPyV-negative MCCs have a high mutation burden (median of 1121 somatic single nucleotide variants (SSNVs) per-exome) with frequent mutations in *RB1* and *TP53* and additional damaging mutations in genes in the chromatin modification (*ASXL1, MLL2,* and *MLL3*), JNK (*MAP3K1* and *TRAF7*), and DNA-damage pathways (*ATM, MSH2,* and *BRCA1*). In contrast, MCPyV-positive MCCs harbor few SSNVs (median of 12.5 SSNVs/tumor) with none in the genes listed above. Goh et al.[18] also reconfirmed that MCPyV-negative MCCs were significantly enriched for C > T transitions (median of 86% of SSNVs in MCPyV-negative MCCs vs. 47% of SSNVs in MCPyV-positive MCCs; P = 3.1E-7; two-sided Mann-Whitney test) and hypothesized that the enrichment of C > T transitions were a result of ultraviolet light (UV), because MCCs develop on the skin. However, they also pointed that C > T transitions can be caused by other mechanisms, e.g. such as aging and impaired mismatch repair[21] and reported that a median of 66% of SSNVs per MCPyV-negative MCC could be attributed to UV exposure.

Recent high-throughput sequencing of large numbers of human cancer genomes showed that mutations at cytosine residues, particularly C to T transitions, are the most prevalent mutations in human cancer, highlighting enzymatic deamination of cytosine to uracil like AID-induced U:G mismatches in DNA as a potential source of mutagenesis.[21] AID-induced U:G mismatches from C:G in DNA are partially transited by replication over deoxyuridines to T:A, resulting in AID-induced C > T transition (C:G to T:A transition). Despite AID's important physiological functions for SHM and CSR in Ig gene, these host defense mechanisms entail a high risk of potentially carcinogenic off-target genomic mutagenesis. Our finding of the significantly higher aberrant AID expression in MCPyV-negative MCCs than in MCPyV-positive MCCs (P = 0.026) is, compatible with the reported exome sequencing data[17, 18] that MCPyV-negative MCCs have an extremely higher mutation burden than MCPyV-positive ones, although UV looks like a major mutagenic factor in MCPyV-negative MCCs.

In conclusion, lower expression of AID and higher expression of NF-κB p65 (phospho S536) were significantly associated with MCPyV-positive MCCs. While pathogen (MCPyV)-induced AID expression through upregulation of NF-κB may be relevant to carcinogenesis of MCPyV-positive MCCs, the other regulating factors for AID may influence the higher AID expression in MCPyV-negative MCCs. The higher aberrant expression of AID, a mutagenic enzyme, and more frequent AID-related mutation signatures observed in MCPyV-negative MCCs associated with poor prognosis in this study is compatible with the fact that MCPyV-negative MCCs have an extremely higher mutation burden than MCPyV-positive ones.

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