ANTICANCER RESEARCH *39*: 319-329 (2019)
doi:10.21873/anticanres.13114

Expression of Notch 3 and Jagged 1 Is Associated With Merkel Cell Polyomavirus Status and Prognosis in Merkel Cell Carcinoma

LUSI OKA WARDHANI[1,2], MICHIKO MATSUSHITA[1,3], SATOSHI KUWAMOTO[1], DAISUKE NONAKA[4,5], KEIKO NAGATA[1], MASAKO KATO[1], YUKISATO KITAMURA[3] and KAZUHIKO HAYASHI[1]

[1]*Division of Molecular Pathology, Department of Pathology, Tottori University Faculty of Medicine, Yonago, Japan;*
[2]*Clinical Pathology Department, Medical Faculty of Sebelas Maret University, Surakarta, Indonesia;*
[3]*Department of Pathobiological Science and Technology, School of Health Science, Tottori University Faculty of Medicine, Yonago, Japan;*
[4]*Department of Cellular Pathology, The Guy's and St Thomas' NHS Foundation Trust, London, U.K.;*
[5]*The Christie Hospital and University of Manchester, Manchester, U.K.*

Abstract. *Background/Aim: Merkel cell carcinoma (MCC) is a rare, aggressive, neuroendocrine skin cancer and most MCCs are related to infection with Merkel cell polyomavirus (MCPyV). Notch signaling modulates cell fate in various tissues including the skin during development and homeostasis, and its aberrant activity relates to onset and progression of various malignancies. Therefore, association of NOTCH1/ NOTCH2/NOTCH3/jagged 1 (JAG1) expression with MCPyV status and prognosis in MCC was investigated. Materials and Methods: A total of 19 MCPyV-positive and 19 MCPyV-negative MCC samples from patients were stained immunohistochemically with antibodies against NOTCH1, NOTCH2, NOTCH3, and JAG1 and analyzed. Results: Expression of NOTCH1 and NOTCH2 was not associated with MCPyV status or prognosis. However, higher JAG1 expression was found in MCPyV-negative than in MCPyV-positive MCC ($p<0.001$), and NOTCH3 expression was higher in MCPyV-positive MCC ($p=0.062$). Kaplan–Meier and multivariate analyses showed that patients with MCC with higher NOTCH3 expression had better overall survival than otherwise ($p=0.001$ and $p=0.033$, respectively). Conclusion: Expression of NOTCH3, as a tumor suppressor, is an independent predictor of MCC outcome.*

Correspondence to: Lusi Oka Wardhani, MD, and Kazuhiko Hayashi, MD, Ph.D., Division of Molecular Pathology, Department of Pathology, Tottori University Faculty of Medicine, Nishi-cho 86, Yonago, Tottori 683-8503, Japan. E-mail: L.O. Wardhani: lusioka@gmail.com, K. Hayashi: hayashik@med.tottori-u.ac.jp

Key Words: Merkel cell carcinoma, Merkel cell polyomavirus, notch 3, jagged 1.

Merkel cell carcinoma (MCC) is an aggressive yet rare cutaneous cancer with neuroendocrine features associated with risks factors including advanced age, immunosuppression, and chronic sun exposure (1). The incidence rate of MCC in the United States has continued to rise, by approximately fivefold over the past 30 years: from 0.22 per 100,000 in 1986 to 0.79 per 100,000 in 2011 (2).

In 2008, it was found that about 80% of MCC cases were associated with Merkel cell polyomavirus (MCPyV), in which MCPyV had been integrated into the tumor genome (3). In Northern areas, the majority of cases of MCC are caused by MCPyV, whereas in areas with higher ultraviolet (UV) exposure, carcinogenesis is driven predominantly by UV. On the other hand, UV exposure can also induce local immunosuppressive condition in viral tumorigenesis (4).

The existence of MCPyV infection is related to histological distinction in MCC: tumor cells in MCPyV-positive MCC have homogeneous round nuclei and less cytoplasm, whereas MCPyV-negative MCC tumor cells have pleomorphic nuclei and a plentiful cytoplasm (5). As well as histological differences, MCPyV integration into tumor cells renders different survival prognoses. MCPyV-negative MCC usually leads to shorter survival and worse prognosis compared with MCPyV-positive MCC (6-10).

The Notch signaling pathway regulates both embryonic and adult tissues, and affects the establishment, growth, and regenerative potential of multiple tissues by determining cell fate (11). Its roles in regulation include maintenance and differentiation of stem cells, cell fate determination and cell-cycle regulation, and other functions are being unveiled as investigations continue; it is clear that the role of notch signaling is highly complex and multifaceted (12). Notch signaling is important in regulating cellular behavior, therefore,

unsurprisingly, Notch plays an important role in many types of cancers, particularly as it plays such a prominent part in regulation of stem and progenitor cells. Its aberrant activity relates to initiation and progression of various malignancies, and it can play a role either as oncogene or tumor suppressor depending on the tissue and cellular type (13, 14).

There are four receptors (NOTCH1-4) in mammals, and five notch ligands are delta-serrate-lag (DSL) family [jagged 1 (JAG1), JAG2, delta-like 1 (DLL1), DLL3, and DLL4)]. Structurally, Notch receptors have a single-pass transmembrane heterodimers consisting of an extracellular ligand-binding domain and an intracellular domain comprising a transmembrane region and an intracellular portion that mediates signaling upon receptor ligation. The same as Notch receptors, Notch ligands structurally are also transmembrane proteins containing epidermal growth factor (EGF)-like repeats (13, 15). Interaction between the extracellular portion of Notch receptor and the Notch ligand results in a series of events converting the transmembrane form of Notch into a nuclear transcriptional co-activator and activates transcription of target genes, including hairy/enhancer of split (HES) and HES-related with YRPW motif (HEY) family genes (16).

Studies in mice and human tissue indicate that Notch receptors and ligands are distributed in spatially restricted expression patterns throughout the epidermis and its appendages. In the interfollicular epidermis, NOTCH1-4 are expressed at the highest levels in the suprabasal cells of the spinous and granular layers, where cells are undergoing differentiation, whereas Notch ligands JAG1 and JAG2 are expressed in the interfollicular epidermis, predominantly in the suprabasal layers, with limited expression in basal cells (12).

Several studies have been conducted exploring carcinogenesis in MCPyV-positive and MCPyV-negative MCC. Harms *et al*. performed transcriptome analysis of 30 MCC cases and found that MCPyV-negative tumors displayed a relative up-regulation of mRNA of gene groups associated with Notch signaling (17). Another study performed whole-exome sequencing of 16 MCC cases, nine MCPyV-negative and seven MCPyV-positive, and identified previous known mutations in *TP53*, retinoblastoma 1 (*RB1*) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) along with novel activating mutations in oncogenes such as *HRAS*, loss-of-function mutations in prune homolog 2 with BCH domain (*PRUNE2*) and *NOTCH* (*NOTCH1-4*) family genes in MCPyV-negative cases (18). A larger scale exome sequencing study of 49 MCCs confirmed the previous report that MCPyV-negative MCCs have a higher mutation burden, frequent mutations in *TP53* and *RB1* and additional mutations in genes involved in chromatin modification and DNA damage pathways, and interestingly, both MCPyV-positive and -negative tumors were found to have mutations inactivating the Notch signaling pathway (*NOTCH1, NOTCH2*) (19).

Panelos *et al*. performed the first immunohistochemical studies of NOTCH1 expression in MCC regardless of MCPyV status and found 30/31 cases had cytoplasmic and membranous NOTCH1 expression in more than 50% of cells (20). Wong *et al*. reported that MCC with mutant *NOTCH1* showed low or absent nuclear NOTCH1 expression (21). As there are no other studies evaluating Notch signaling expression in MCC using immunohistochemistry, in this study, we evaluated the nuclear expression of activated NOTCH1 and NOTCH2 and membranous/cytoplasmic expression of NOTCH3 and JAG1 in tumor cells of MCPyV-positive and -negative MCC, and examined the association of expression of these markers with clinicopathological factors and the prognosis of MCC.

Materials and Methods

In this study, 38 formalin-fixed paraffin-embedded MCC samples were prepared. These included 19 MCPyV-positive MCCs (15 samples from the United Kingdom and four samples from Japan) and 19 MCPyV-negative MCCs (14 samples from the United Kingdom and five from Japan). The MCPyV-negative MCC samples included 13 of MCC combined with squamous cell carcinoma (SCC) or Bowen disease. A summary of clinicopathological data is listed in Table I. This study was approved by the Institutional Review Board of Medical Faculty, Tottori University, Japan.

Immunohistochemistry. Formalin-fixed paraffin-embedded samples were sectioned into 4-μm-thick pieces, followed by deparaffinization and rehydration. Antigen retrieval was performed by incubating the sections for 40 min at 100˚C in Nichirei Heat Pro II (Tokyo, Japan). After blocking endogenous peroxidase activity for 5 minutes, sections were incubated for 60 min with primary antibody, and then incubated with the secondary antibody for 30 min. Sections were then incubated with diaminobenzidine for 10 min; all these processes used a Nichirei Histo Stainer (Tokyo, Japan). After washing the sections using phosphate-buffered saline, they were counterstained with hematoxylin for 5 sec, and then rehydrated and mounted. The primary antibodies (against NOTCH1, NOTCH2, NOTCH3, and JAG1) used in this study and the tissues used as positive controls are listed in Table II.

Immunohistochemical evaluation and scoring system. The stained tissue slides were evaluated by pathologists and researchers who were blinded to the patients' clinical data. The nuclear expression of NOTCH1 or, NOTCH2, and membranous/cytoplasmic expression of NOTCH3 and JAG1 in the stained tumor cells of MCC and combined tumor were evaluated using the modified H-score. The percentage of cells stained was summed and multiplied by values according to the staining intensity level (0=not stained, 1=weakly stained, 2=moderately stained, and 3=strongly stained), and the H-score ranged from 0 to a maximum of 300 (22). The internal negative controls used were non-neoplastic skin and subcutaneous tissues from MCC samples. A summary of H-score data of NOTCH1, NOTCH2, NOTCH3 and JAG1 is listed in Table III.

Statistical analysis. All clinicopathological parameters, such as age, sex, race, and immunohistochemical results, were partitioned based on the MCPyV status and analyzed using the Mann–Whitney *U*-test.

Table I. *Summary of clinicopathological features of 38 Merkel cell carcinoma cases*.

Sample no.	MCPyV status	Age (years)	Gender	Primary site	Diagnosis	Tumor size (cm)	Clinical stage	Initial treatment	Local recurrence (months)[1]	Regional nodal metastasis (months)[1]	Distant metastasis (months)[1]	Outcome (months)[2]
UK-M-7	+	68	M	Rt. knee	Pure MCC	2.7×1.7×0.8	IIA	RE	No	No	No	NED (59)
UK-M-9-7	+	69	M	Rt. Groin	Pure MCC	5×5×4	IIA	RE	No	No	No	NED (55)
UK-M-11	+	61	F	Cheek	Pure MCC	1.2	IA	RE	No	No	No	NED (18)
UK-M-16-2	+	83	F	Upper arm	Pure MCC	2.5×2.5	IIB	RE, RD	No	Yes (6)	No	NED (70)
UK-M-19	+	85	F	Forearm	Pure MCC	4×2.8×1.3	IIB	RE	No	No	No	DOC (31)
UK-M-21	+	46	F	Lt. buttock	Pure MCC	3×2.5	II	RE	N.A.	N.A.	N.A.	NED (59)
UK-M-30-7	+	74	F	Lt. elbow	Pure MCC	6×5.5×2	IIA	RE	Unknown	Yes (33)	Unknown	NED (57)
UK-M-34-2	+	Unknown	M	Rt. Elbow	Pure MCC	6	IIIB	PE+RD	Yes (16)	Yes (0)	Unknown	DOC (36)
UK-M-36-1	+	84	F	Lt. thigh	Pure MCC	7.5	IIA	RE	Unknown	Yes (1)	No	DOC (3)
UK-M-37-1	+	76	M	Rt. forearm	Pure MCC	2.5	IIA	RE	Unknown	Yes (5)	No	NED (48)
UK-M-40-1	+	76	M	Lt. lower leg	Pure MCC	2	IA	RE	No	No	No	NED (43)
UK-M-42-1	+	76	F	Lt. knee	Pure MCC	3.8	IIA	PE	Yes (4)	Yes (4)	No	DOD (9)
UK-M-48-1	+	83	F	Rt. knee	Pure MCC	1.9	IA	RE	No	No	No	DOC (15)
UK-M-51-2	+	83	F	Scalp	Pure MCC	3	IIA	RE, RD	No	Yes (6)	No	DOD (8)
UK-M-53-1	+	Unknown	F	Rt. Lower leg	Pure MCC	7	IIA	RE	No	No	No	NED (12)
MCC32	+	66	M	Forearm	Pure MCC	2×2.5	IIIB	RE	No	Yes (0)	No	NED (2)
MCC93	+	84	F	Lt. cheek	Pure MCC	1.2×1.0	IA	RE	No	No	No	NED (12)
MCC94	+	86	F	Rt. Cheek	Pure MCC	4.5×4.0	IIB	RE	Yes (3)	No	No	NED (4)
MCC95	+	76	F	Lower jaw	Pure MCC	3.5×3	II	RE, RT	No	No	No	NED (4.5)
UK-M-3-3	–	85	F	Lower leg	Combined MCC+SCC	2.1	IIB	RE	No	No	No	DOC (21)
UK-M-5-2	–	81	F	Lt. leg with multiple satellites	Combined MCC+SCC	3.5	II	PE	No	No	No	DOD (18)
UK-M-6-1	–	82	F	Forehead	Combined MCC+BD	3.5×3×2	II	RE	No	No	No	DOC (4)
UK-M-10	–	94	F	Lateral leg with multiple metastasis in the region	Pure MCC	6.5×5×2.5	IIB	PE	Yes (12)	No	Yes (12)	DOD (12)
UK-M-13	–	61	M	Rt. Shin	Pure MCC	1.8×1.5	I	RE	No	Yes (14)	Yes (18)	DOD (23)
UK-M-14	–	86	F	Lt. dorsum foot	Combined MCC+SCC	1.5×1.5	I	RE	No	No	No	DOC (11)
UK-M-15	–	83	F	Lt. dorsum foot	Combined MCC+SCC	1.5×1.2×0.6	II	PE	No	No	No	DOC (2)
UK-M-18-6	–	94	F	Temple	Pure MCC	5×4×2	IIIB	RD, RE	No	Yes (0)	No	DOC (6)
UK-M-35-2	–	78	F	Lt. temple	Combined MCC+SCC	3.5	IIA	RE	No	Yes (2)	Yes (2)	DOC (10)
UK-M-41	–	85	M	Rt. forehead	Combined MCC+ superficial squamous cell atypia	0.5	IA	RE (deep margin +)	No	No	No	DOC (40)
UK-M-44-2	–	86	F	Rt.cheek	Combined MCC+BD	2	IA	RE, RD	No	No	No	NED (35)
UK-M-45-2	–	84	F	Lt. calf	Pure MCC	0.5	III	RE	No	Yes (1)	No	NED (31)
UK-M-50-1	–	85	F	Rt. shin	Combined MCC+BD	2	I	RE	No	No	No	NED (16)
UK-M-54	–	68	M	Rt. Axilla LN	Pure MCC	1.2	III	CT	No	No	Yes (N.A.)	AWD (14)
MCC81	–	85	M	Lt. hand	Combined MCC+BD	1×0.3	I	N.A.	N.A.	N.A.	N.A.	N.A. (13)
MCC99	–	102	F	Rt.cheek	Combined MCC+SCC	3×2.5	II	PE	Yes (N.A.)	Yes (14)	No	DOD (19)
MCC100	–	90	F	Lt. temple	Combined MCC+SCC	0.3×0.7	I	N.A.	N.A.	N.A.	N.A.	N.A. (19)
MCC102	–	86	F	Orbit	Pure MCC	1.5×0.3	I	RE, RD	No	Yes (3)	Yes (7)	DOD (9)
MCC107-1	–	100	M	Rt. Ear	Combined MCC+BD	1×0.4	I	N.A.	N.A.	N.A.	N.A.	N.A. (6)

AWD: Alive with disease; BD: Bowen's disease; CT: chemotherapy; DOC: dead from other causes; DOD: dead of disease; F: female; Lt: left; M: male; MCPyV: Merkel cell polyomavirus; N.A.: not available; NED: no evidence of disease; NT: no treatment; PE: palliative excision; RD: radiation; RE: radical excision; SCC: squamous cell carcinoma; +: positive; –: negative. [1]From diagnosis; [2]of observation period.

Table II. *List of antibodies and positive controls used for immunohistochemistry.*

Antibodies	Host and type	Source/clone	Catalog number	Dilution ratio	Positive control	Site of expression evaluation
NOTCH1 (activated form)	Rabbit polyclonal	Abcam (Cambridge, MA, USA)	ab8925	1:50	Lymph node	Nucleus
NOTCH2	Rabbit polyclonal	Abcam (Cambridge, MA, USA)	ab99404	1:100	Breast cancer	Nucleus
NOTCH3	Rabbit polyclonal	Abcam (Cambridge, MA, USA)	ab23426	1:300	Breast cancer	Membranous/ cytoplasmic
JAG1	Rabbit monoclonal	Cell Signaling Technology (Danvers, MA, USA); clone D4Y1R	#70109S	1:300	Lung cancer	Membranous/ cytoplasmic

JAG1: Jagged 1.

Survival was measured from diagnosis of MCC. The survival analysis was evaluated using the Kaplan–Meier method in accordance with NOTCH1/NOTCH2/NOTCH3/JAG1 expression using the mean H-score to divide patients into groups with low and high expression. The significant differences were examined by the log-rank test. The Cox proportional hazards regression model was applied to perform univariate and multivariate analyses, and those variables that achieved statistical significance in the univariate analysis were included in the multivariable analysis. All data were analyzed statistically using SPSS software (version 21.0J; SPSS Japan Inc., Tokyo, Japan), and a *p*-value of less than 0.05 was considered statistically significant.

Results

Characteristics of clinical background. The 38 cases of MCC used in this study, comprising 19 MCPyV-positive MCCs and 19 MCPyV-negative MCCs, are described in Tables I and IV. There was a significant difference in the ages of both MCC groups: MCPyV-negative cases (mean=85 years) were older than MCPyV-positive cases (mean=75.06 years), as shown in Table IV (p=0.002). The other clinical parameters, such as sex, race, and staging, were not different between the two groups.

Histological findings in NOTCH1, NOTCH2, NOTCH3 and JAG1 expressions. Immunohistochemical staining was performed to evaluate the expression of NOTCH1, NOTCH2, NOTCH3, and JAG1 in MCC tumor cells and the results are summarized in Tables III-V. Representative immunostaining features of NOTCH1, NOTCH2, NOTCH3 and JAG1 in MCPyV-positive and MCPyV-negative MCC cases are shown in Figure 1. The H-scores for NOTCH1 and NOTCH2 were similar between MCPyV-positive and MCPyV-negative MCCs (Figure 1E and F, Table IV; p=0.51, and Figure 1G and H, Table IV; p=0.173, respectively). NOTCH3 H-score was higher in MCPyV-positive than MCPyV-negative MCC (Figure 1I and J), even though not statistically significant (p=0.062). The H-score for JAG1 was significantly higher in MCPyV-negative (Figure 1L) than in MCPyV-positive MCC (Figure 1K) (Table IV; p<0.001). All MCPyV-positive MCCs, except one focally positive case, showed no JAG1 expression.

In 13 cases of combined MCC and SCC, tumor cells in the MCC component had a significantly higher H-score for NOTCH2 and NOTCH3 expression than did those in the SCC component (Table V; p<0.001, and p=0.021, respectively). There were trends that NOTCH1 and JAG1 expression of tumor cells in the MCC component were higher than those in the SCC component in combined MCC and SCC cases but not statistically significant (Table V; p=0.071 and p=0.877, respectively).

Prognostic analysis of NOTCH1, NOTCH2, NOTCH3 and JAG1 in MCC. The Kaplan–Meier method with log-rank test was used to analyze prognostic survival in this study, and the results are summarized in Table VI. High NOTCH3 expression in MCC tumor cells (H-score ≥199) significantly corresponded to more favorable overall survival (OS) (Figure 2A; p=0.001), but not disease-specific survival (DSS) (Figure 2B; p=0.371).

Clinicopathological parameters and NOTCH1, NOTCH2, NOTCH3, and JAG1 expressions in tumor cells for the prediction of OS and DSS were further investigated by univariate and multivariate analyses with the Cox proportional hazards regression model. The results are shown in Table VII. Results from the univariate analysis indicated that MCPyV negativity [hazard ratio (HR)=3.559; p=0.012] and advanced age (HR=8.918; p=0.034) were unfavorable factors for OS. Female patients had shorter survival than male patients even though not statistically significant [OS (HR=2.3; p=0.118) and DSS (HR=3.735; p=0.226)]. Caucasian patients had better survival than Japanese patients but not significantly [OS (HR=0.413; p=0.105) and DSS (HR=0.325; p=0.198)]. Patients with stage III/IV disease had longer survival than those with stage I/II, although not statistically significant [OS (HR=0.803; p=0.768) and DSS (HR=0.041; p=0.541)]. High

Table III. *Summary of immunohistochemistry (H-score) of tumor cells for NOTCH1, NOTCH2, NOTCH3, Jagged 1 (JAG1) in Merkel cell polyomavirus (MCPyV)-positive and MCPyV-negative Merkel cell carcinomas (MCCs).*

Sample No.	MCPyV status	NOTCH1	NOTCH2	NOTCH3	JAG1
UK-M-7	+	230	50	220	0
UK-M-9-7	+	260	70	245	0
UK-M-11	+	295	165	285	0
UK-M-16-2	+	260	130	275	0
UK-M-19	+	140	10	35	0
UK-M-21	+	93	20	205	0
UK-M-30-7	+	275	100	200	0
UK-M-34-2	+	280	105	150	0
UK-M-36-1	+	285	90	295	0
UK-M-37-1	+	270	75	250	6
UK-M-40-1	+	295	95	260	0
UK-M-42-1	+	270	80	160	0
UK-M-48-1	+	295	95	180	0
UK-M-51-2	+	270	40	240	0
UK-M-53-1	+	280	90	245	0
MCC32	+	190	20	145	0
MCC93	+	280	125	140	0
MCC94	+	260	90	215	0
MCC95	+	285	90	270	0
UK-M-3-3	–	290	90	185	0
UK-M-5-2	–	270	99	180	21
UK-M-6-1	–	255	110	195	7
UK-M-10	–	260	110	220	0
UK-M-13	–	240	95	110	0
UK-M-14	–	290	100	230	2
UK-M-15	–	270	95	165	4
UK-M-18-6	–	230	75	140	105
UK-M-35-2	–	215	95	150	200
UK-M-41	–	230	122	240	9
UK-M-44-2	–	240	95	250	30
UK-M-45-2	–	280	90	195	2
UK-M-50-1	–	295	60	200	9
UK-M-54	–	295	110	190	25
MCC81	–	290	85	175	215
MCC99	–	215	85	210	7
MCC100	–	265	60	190	9
MCC102	–	240	99	190	2
MCC107-1	–	250	120	120	7
Mean of positive cases		253.32	81.05	211.32	0.32
Mean of negative cases		258.95	94.47	186.05	34.42
Mean in MCC overall		256.13	87.76	198.68	17.37

NOTCH3 expression (H-score ≥199) significantly lengthened OS (HR=0.213; p=0.003). Only radical excision (HR=0.154; p=0.017) significantly extended DSS. With multivariate analysis, only high expression of NOTCH3 was a significantly favorable prognostic factor for OS (HR=0.313; p=0.033), whereas only radical excision was a significant favorable prognostic factor for DSS (HR=0.107; p=0.019).

Discussion

MCC can be caused either by MCPyV infection, as in MCPyV-positive MCC, or by UV exposure-driven carcinogenesis, as in MCPyV-negative MCC, even though UV exposure can also induce local immunosuppression in viral tumorigenesis. Evaluation of the difference between MCPyV-positive and MCPyV-negative MCC showed that MCPyV-negative MCC has a high frequency of DNA mutations associated with UV damage, disruption of *RB1* and *TP53*, presence of a high degree of aneuploidy, and mutations in genes related to responses to DNA impairment and repair, whereas MCPyV-positive MCC generally has few somatic mutations and little evidence of UV damage, and most MCPyV-positive cases have intact *RB1* and wild-type *TP53* (4, 19, 21).

Investigation using whole-exome sequencing of 16 MCC cases identified loss-of-function mutations in one or more *NOTCH* family genes (*NOTCH1-4*) in six out of eight (75%) MCPyV-negative cases, and *NOTCH* mutations in MCC were mainly located in EGF or ankyrin repeat regions, which is consistent with a loss-of-function event, and the authors suggested that Notch signaling plays a tumor-suppressive role in MCC similarly to other neuroendocrine malignancies (18).

Wong *et al*. evaluated targeted capture and massively parallel DNA sequencing of 619 cancer genes to compare the gene mutations and copy number alterations in MCPyV-positive (n=13) and MCPyV-negative (n=21) MCC tumors and cell lines. All MCPyV-negative tumors harbored a high frequency of mutations in *NOTCH1*, and immunohistochemistry with NOTCH1 antibody revealed that MCC with mutated *NOTCH1* showed a marked reduction of NOTCH1 expression (21).

Another larger scale exome sequencing of 49 MCCs by Goh *et al*. confirmed the previous report and found that both MCPyV-positive and MCPyV-negative MCCs had mutations inactivating the Notch signaling pathway (*NOTCH1* and *NOTCH2*). MCPyV-negative MCC had sporadic somatic single nucleotide variants affecting *NOTCH1* and *NOTCH2* that were also seen in small-cell lung cancer and they suggested that dysregulation of these genes and pathways were required for the neuroendocrine differentiation of epithelial cells, a common feature of both MCCs and small-cell lung cancer (19).

Whereas the previous studies by Harms *et al*. (18) and Goh *et al*. (19) showed that *NOTCH* genes had loss-of-function events and they suggested that Notch signaling played a tumor-suppressive role in MCC similarly to other neuroendocrine malignancies, we found that there was no difference in nuclear expression of NOTCH1 nor NOTCH2 in both MCPyV-positive and MCPyV-negative MCC (p=0.51 and p=0.173, respectively). Moreover, our finding of high NOTCH1 expression observed in most MCC cases (mean H-score of all cases: 256.13 in Table III) is similar to the result of Panelos *et al*., as they showed cytoplasmic and membranous NOTCH1

Table IV. *Comparison of clinicopathological parameters of Merkel cell carcinoma based on Merkel cell polyomavirus (MCPyV) status.*

Clinicopathological parameters	MCPyV-positive	MCPyV-negative	*p*-Value
Gender, n (%)			
Male	6 (31.6%)	5 (26.3%)	0.724
Female	13 (68.4%)	14 (73.7%)	
Age (years)			
Mean±SD	75.06±10.56	85±9.57	**0.002**
Race, n (%)			
Japanese	4 (21.1%)	5 (26.3%)	0.707
Caucasian	15 (78.9%)	14 (73.7%)	
Staging, n (%)			
I/II	17 (89.5%)	16 (84.2%)	0.636
III/IV	2 (10.5%)	3 (15.8%)	
Radical excision, n (%)			
No	2 (10.5%)	5 (31.2%)	0.132
Yes	17 (89.5%)	11 (68.8%)	
NOTCH1 H-score			
Mean±SD	253.32±54.72	258.95±26.80	0.510
NOTCH2 H-score			
Mean±SD	81.05±39.67	94.47±16.97	0.173
NOTCH3 H-score			
Mean±SD	211.32±64.59	186.05±37.44	0.062
JAG1 H-score			
Mean±SD	0.32±1.38	34.42±65.46	**<0.001**

JAG1: Jagged 1; SD: standard deviation. Statistically significant results are shown in bold (Mann-Whitney *U*-test, $p<0.05$).

Table V. *Comparison of NOTCH1, NOTCH2, NOTCH3 and Jagged 1 (JAG1) expression by immunohistochemistry of tumor cells in 13 cases of combined Merkel cell carcinoma (MCC) and squamous cell carcinoma (SCC, including Bowen's disease).*

	H-score (mean±SD)		
Factor	MCC component	SCC component	*p*-Value
NOTCH1	259.62±28.32	208.46±66.69	0.071
NOTCH2	93.54±18.86	53.46±22.95	**<0.001**
NOTCH3	191.54±36.14	117.69±90.50	**0.021**
JAG1	40±74.82	31.08±34.17	0.877

JAG1: Jagged 1; SD: standard deviation. Statistically significant results are shown in bold (Mann-Whitney *U*-test, $p<0.05$).

expression in more than 50% of cells of in 30/31 MCC cases regardless of MCPyV status (20). However, we investigated the nuclear expression of NOTCH1 (activated NOTCH1 expression) rather than cytoplasmic/membrane expression reported by Panelos *et al*. because nuclear NOTCH1 expression reflects the translocation of the activated form of NOTCH1 from the cytoplasm to the nucleus. Our data is different from that for other neuroendocrine tumors such as gut carcinoids, medullary thyroid carcinoma (MTC), and pulmonary typical and atypical carcinoids which show minimal or absence of NOTCH1 signaling (23), and from previous data in MCCs which suggest *NOTCH1* is a tumor suppressor in MCCs (18, 19, 21).

What is a reasonable explanation for the discrepancy between our finding of high nuclear NOTCH1 expression in MCC suggesting *NOTCH1* to be an oncogene and the suggestion that it is a suppressor by the previous genome-wide studies showing inactivating *NOTCH1* and *NOTCH2* mutation with somatic single nucleotide variants? It is well known that P53 is often overexpressed or occasionally not expressed immunohistochemically in the nuclei of cancer cells due to mutation of *TP53* as a suppressor gene. Overexpression of NOTCH1 and NOTCH2 in our study may reflect the accumulation of mutation-induced abnormal NOTCH1 or NOTCH2 protein in the nuclei of Merkel cell tumor cells, like P53. The antibody used for immunohistochemistry in our study (Abcam, ab8925) was developed for detecting the activated form of NOTCH1 and different from those of previous reports (20, 21). This may be an important reason for our different results of immunohistochemistry for NOTCH1.

In contrast to NOTCH1 and NOTCH2, NOTCH3 expression in MCPyV-negative cases (mean H-score=186.05) was found to be lower than in MCPyV-positive ones (mean H-score=211.32) ($p=0.062$). In addition, high NOTCH3 expression of tumor cells (H-score ≥199) was associated with more favorable OS. This suggests that NOTCH3 has activity as a tumor suppressor. NOTCH3 dysregulation has been associated with a wide variety of malignancies as it has been shown to affect tumor aggressiveness, maintenance and resistance to chemotherapy, it has roles both as tumor suppressor and oncogene (24). In this study, NOTCH3 appeared to function as a tumor suppressor, and this same function was shown in MTC and ovarian cancer as NOTCH3 induced apoptosis and had an antiproliferative function (24, 25), whereas in breast cancer, NOTCH3 increased chemosensitivity of doxorubicin-resistant breast cancer (24).

Harms *et al*. used transcriptome analysis of 30 MCC cases and found that MCPyV-negative MCC had relative up-regulation of *DLL1*, *C*-terminal binding protein 2 (*CTBP2*), hairy/enhancer of split 1 (*HES1*), *JAG2* and *JAG1* mRNA compared with MCPyV-positive MCC (17). In accordance with their result for up-regulation of *JAG1*, we showed that JAG1 expression was significantly higher in membranous/cytoplasmic of tumor cells in MCPyV-negative than in MCPyV-positive MCCs ($p<0.001$), 16 of 19 MCPyV-negative cases expressed JAG1 and no MCPyV-positive case had JAG1 expression, except in one case. In survival analysis, there was no difference in survival according to JAG1 H-score (cut-off ≥17). Notch ligand, JAG1, is overexpressed in many cancer types, and plays an important role in several aspects of tumor biology. JAG1-stimulated Notch activation is directly implicated in tumor growth through maintaining cancer stem cell populations, promoting cell

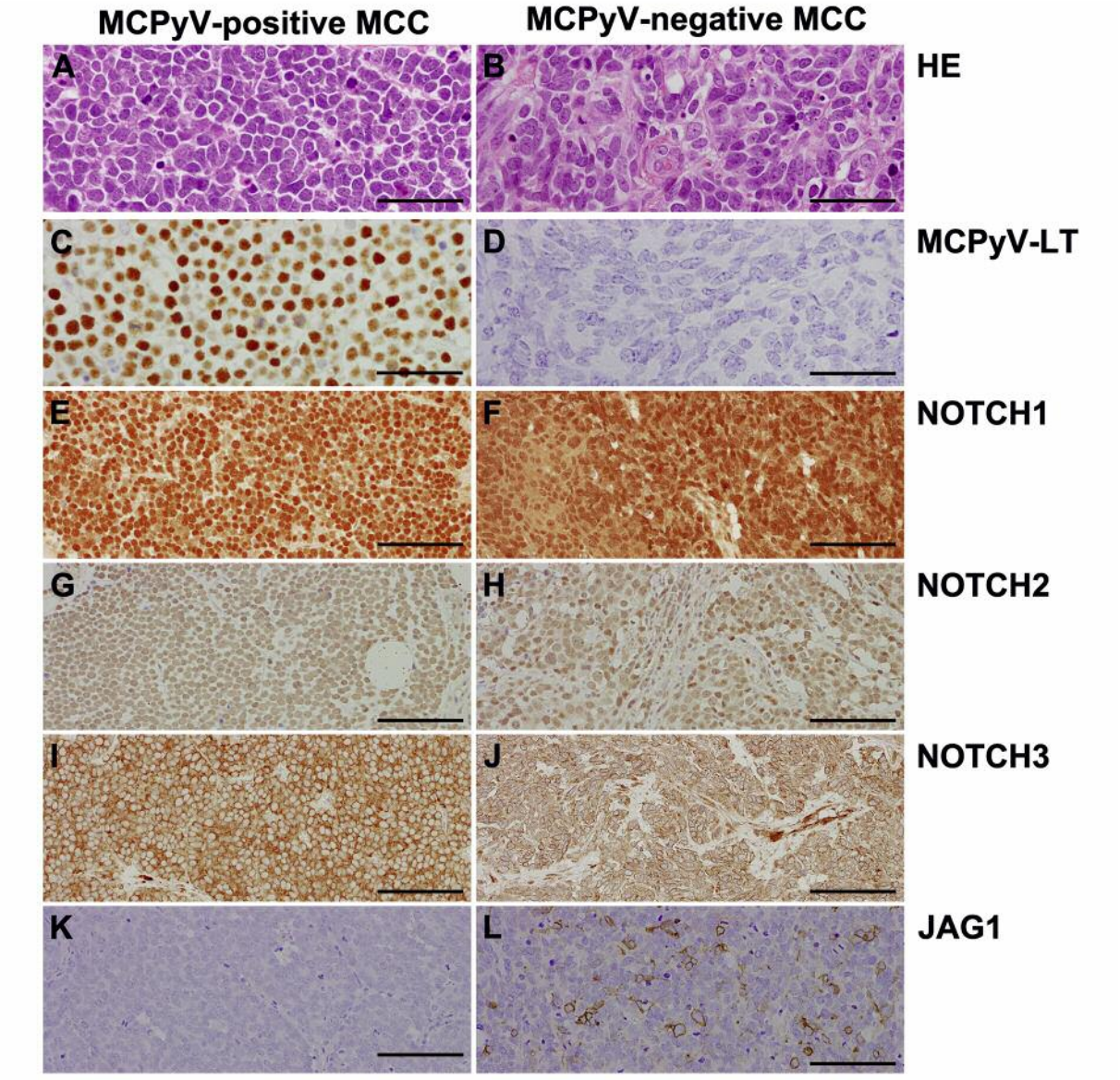

Figure 1. *Representative images of immunohistochemical staining of Merkel cell polyomavirus (MCPyV)-positive and -negative Merkel cell carcinoma (MCC). The morphology and immunostaining of MCPyV-positive MCC (A, C, E, G, I and K) and MCPyV-negative MCC (B, D, F, H, J, and L) are shown. MCPyV-positive MCC tumor cells (A) had nuclei with a regular shape and less cytoplasm than MCPyV-negative MCC cells (B). Positivity for MCPyV-large T-antigen (LT) was shown as a dense or moderate nuclear reactivity in all MCPyV-positive MCC tumor cells (C), but not in MCPyV-negative MCC (D). The bar represents 50 μm. The expressions of NOTCH1 and NOTCH2 in MCC tumor cells were similar in MCPyV-positive (E and G, respectively; bar, 100 μm) and MCPyV-negative (F and H, respectively; bar, 100 μm) tumor cells. NOTCH3 expression was more prominent in MCPyV-positive tumor cells (I) than in MCPyV-negative tumor cells (J) but not statistically significantly (H-score: mean±SD, 211.32±64.59 and 186.05±37.44, respectively; p=0.062); bar, 100 μm. Jagged 1 (JAG1) expression was significantly more frequent in MCPyV-negative MCC (L) than with only one focal positive case in MCPyV-positive MCC (K) (H-score: mean±SD, 34.42±65.46 versus 0.32±1.38; respectively, p<0.001; bar, 100 μm). A and B, Hematoxylin-eosin (HE) stain; C-L, immunostain.*

survival, inhibiting apoptosis, and driving cell proliferation and metastasis. In addition, JAG1 can indirectly affect cancer by influencing tumor microenvironment components such as tumor vasculature and immune cell infiltration (26).

In this study, we reconfirmed the findings of our previous studies that patients with MCPyV-positive MCC have a favorable survival (8, 10, 22, 27), elderly patients have worse survival (10, 22), and radical excision significantly extend OS

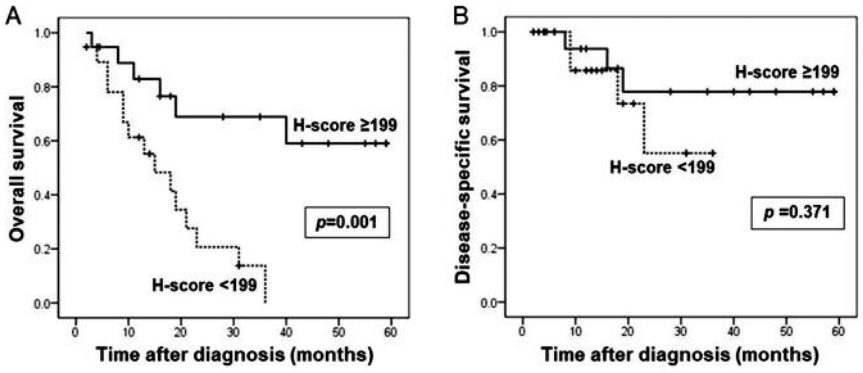

Figure 2. *Overall survivaI (OS; A) and disease-specific survival (DSS; B) classified by mean expression of NOTCH3 in Merkel cell carcinoma (MCC) tumor cells. Kaplan–Meier with log-rank test evaluated the statistical significance. A and B, Patients with high NOTCH3 expression in MCC tumor cells (H-score ≥199) survived significantly longer than did those with low NOTCH3 expression (H-score <199) regarding OS (A), but there was no significant difference in DSS (B).*

and DSS (10). MCC can occur in both female and male patients (1), although it is almost twice as frequent in males (62.1%) than in females (37.9%) (28). In this study, female patients were the majority (71.1%). This study revealed that female patients had shorter survival (in OS and DSS) compared to male patients, although this was not statistically significant. A previous study supported our result that male patients had significantly improved OS, but not DSS by Kaplan–Meier analysis (27). On the contrary, another study showed that male patients had non-significantly shorter OS and DSS compared to female patients (22), and a large cohort study (n=351) revealed that male sex was an independent prognostic factor of unfavorable outcome in OS and DSS (29).

MCC mainly affects Caucasians (96.4%) and is very rare among individuals of African American (1.2%), and Asian and Pacific Islander (0.8%) descent (28). In this study, Caucasian patients with MCC were the majority (76.3%), whereas only 23.7% were Japanese. Kuromi *et al.* reported that Japanese ethnicity was linked to significantly longer OS but not linked with DSS by Kaplan–Meier analysis (27). In contrast, this study showed that Caucasians had longer OS and DSS than did Japanese, but not statistically significant. Another study comparable with our result showed that Caucasian patients had better survival than those of Japanese ethnicity, which was also not statistically significant (10).

Harms *et al.* showed that the extent of MCC was predictive of 5-year OS, with estimated OS of 51, 35, and 14% for those with local, nodal and distant disease, respectively (28). In this study, the majority of patients had stage I/II disease (33/38, 86.8%), and advanced stage was not a predictor of worse OS or DSS. Our previous study showed that patients with stage III/IV disease had shorter OS and DSS compared with those with stage I/II disease, even though not statistically significant (10). However, unexpected result of longer OS or DSS in stage III/IV in this

Table VI. *Comparison between clinicopathological parameter including immunohistochemistry (H-score) and overall survival (OS) or disease-specific survival (DSS).*

Factor	Mean OS (months)	OS (*p*-Value)	Mean DSS (months)	DSS (*p*-Value)
NOTCH1 H-score in MCC				
<256	26.56	0.517	43.79	0.726
≥256	30.96		46.57	
NOTCH2 H-score in MCC				
<88	32.05	0.584	45.14	0.794
≥88	27.25		44.80	
NOTCH3 H-score in MCC				
<199	17.08	**0.001**	27.55	0.371
≥199	42.52		49.25	
JAG1 in H-score in MCC				
<17	31.21	0.328	46.31	0.872
≥17	18.08		26.50	
MCPyV				
Positive	41.51	**0.008**	52.27	0.172
Negative	18.47		28.50	
Age				
<75 Years	51.80	**0.010**	51.80	0.327
≥75 Years	21.49		36.03	
Gender				
Male	40.19	0.107	53.86	0.194
Female	26.05		42.83	
Race				
Japanese	13.83	0.092	17.00	0.172
Caucasian	32.55		48.28	
Staging				
I/II	29.32	0.766	44.92	0.340
III/IV	28.50		*	
Radical excision				
No	17.46	**0.046**	20.04	**0.006**
Yes	35.91		51.68	

JAG1: Jagged 1; MCC: Merkel cell carcinoma; MCPyV: Merkel cell polyomavirus. *No statistics are computed because all cases are censored (no death events occurred in patients with stage III/IV). Statistically significant results are shown in bold (Kaplan–Meier method, $p<0.05$).

Table VII. *Univariate and multivariate Cox proportional hazard regression analysis of prognostic factors for mortality in Merkel cell carcinoma (MCC) cases.*

Factor		OS			DSS		
	Comparison *vs*. reference	HR	95% CI	*p*-Value	HR	95% Cl	*p*-Value
Univariate							
MCPyV	Negative *vs*. positive	3.559	1.323-9.576	**0.012**	2.994	0.572-15.686	0.194
Gender	Female *vs*. male	2.300	0.81-6.527	0.118	3.735	0.442-31.581	0.226
Age	≥75 *vs*. <75 years	8.918	1.181-67.338	**0.034**	2.76	0.329-23.145	0.349
Race	Caucasian *vs*. Japanese	0.413	0.142-1.203	0.105	0.325	0.059-1.796	0.198
Staging	III/IV *vs*. I/II	0.803	0.186-3.467	0.768	0.041	0-1113	0.541*
Radical excision	Yes *vs*. no	0.372	0.135-1.023	0.055	0.154	0.033-0.714	**0.017**
NOTCH1 H-score	High *vs*. low	0.752	0.315-1.796	0.521	0.765	0.169-3.46	0.728
NOTCH2 H-score	High *vs*. low	1.288	0.516-3.212	0.587	0.82	0.183-3.676	0.796
NOTCH3 H-score	High *vs*. low	0.213	0.075-0.601	**0.003**	0.51	0.113-2.31	0.382
JAG1 H-score	High *vs*. low	1.729	0.565-5.287	0.337	1.191	0.14-10.104	0.873
Multivariate							
MCPyV	Negative *vs*. positive	1.684	0.564-5.028	0.351	1.173	0.194-7.108	0.862
Age	≥75 *vs*. <75 years	6.116	0.796-46.972	0.082	1.863	0.213-16.308	0.574
Radical excision	Yes *vs*. no	0.427	0.135-1.352	0.148	0.107	0.017-0.689	**0.019**
NOTCH3 H-score	High *vs*. low	0.313	0.107-0.913	**0.033**	0.46	0.096-2.192	0.330

CI: Confidence intervaI; DSS: disease-specific survival; HR: hazard ratio; JAG1: Jagged 1; MCC: Merkel cell carcinoma; MCPyV: Merkel cell polyomavirus; OS: overall survival. *There are no death events due to MCC in patients with stage III/IV. Statistically significant results are shown in bold.

study can be attributed to the small number of total samples (n=38) and small number of stage III/IV samples (n=5, only 13.2%) compared to stage I/II samples (n=33). On the other hand, patient age can influence survival even when diagnosed with early-stage MCC.

This study has a limitation given that we used relatively few samples (38 samples). In order to get reliable results statistically, for example in evaluating the association of clinicopathological variables and Notch signaling markers with patients' survival, in the future, our findings should be confirmed in a larger sample population cohort.

NOTCH3 has roles as an oncogene and a tumor-suppressor gene. Therapy targeting NOTCH3 as an oncogene aims to inhibit the level of NOTCH3 expression, with use of agents such as gamma secretase inhibitors, which are not specific to Notch subtypes; furin-like convertase and a disintegrin and metalloprotease (ADAM) inhibitors for inhibition notch enzymatic processing; monoclonal antibodies to block NOTCH3 to avoid pan-Notch blockage; and microRNA targeting specific Notch subtypes. In contrast, in therapy targeting NOTCH3 as a tumor suppressor, it would be beneficial to induce expression of NOTCH3 rather than to inhibit it (24). Several histone deacetylase inhibitors (HDACi) have been shown to increase Notch expression in a variety of malignancies in which Notch plays a tumor-suppressive role (24). Jaskula-Sztul *et al*. investigated the role of NOTCH3 signaling as a tumor suppressor in MTC using doxycycline-inducible NOTCH3 intracellular domain (NICD3, the post-γ-secretase cleavage product of NOTCH3) and HDACi AB3, and showed that induction of NOTCH3 signaling can inhibit tumor proliferation and suppress neuroendocrine markers (30). Comparable to that of Jaskula-Sztul *et al*., another study revealed that NOTCH3 expression declined as thyroid cancers became less differentiated and more malignant, resembling its conserved pattern in development, therefore predicting disease prognosis, and NOTCH3 activation in a gain-of-function follicular thyroid carcinoma cell line inhibited cell proliferation and migration, activated the intrinsic apoptotic cascade, and reduced tumor burden *in vivo* (25).

Several clinical trials of immune checkpoint inhibition using an antibody to programmed cell death protein 1 (PD1) or PD-ligand 1 in patients with advanced-stage MCC show higher and more durable response rates than conventional chemotherapy. However, not all patients have durable responses to immune checkpoint inhibitors (31-33). Other immunotherapies for MCC that act through mechanisms other than inhibition of PD1 or PD-ligand 1 including therapeutic combinations of anti-cytotoxic T-lymphocyte antigen 4 are still under investigation (34). Combination therapy of immune-checkpoint inhibitors and HDACi for increasing NOTCH3 expression as a tumor suppressor might be a candidate for novel therapy for MCC.

In conclusion, we showed that MCPyV-negative MCC is significantly associated with higher JAG1 expression in tumor cells than MCPyV-positive MCC. NOTCH3 expression is associated with a significantly longer OS by using Kaplan–

Meier analysis, and high NOTCH3 expression is an independent predictor of favorable OS in multivariate analysis. This study suggests that NOTCH3 and JAG1 may play a role in MCC tumorigenesis.

Competing Interest

There is no conflict of interests.

Funding/support

This study was supported, in part, by grants from Japan Society for the Promotion of Science [no. 26460433 (K.H.) and no. 26860238 (M.M.)].

Acknowledgements

This study was supported, in part, by grants from the Japan Society for the Promotion of Science [no. 26460433 (K.H.) and no. 26860238 (M.M.)], and the Authors thank the laboratory staff at the Division of Molecular Pathology, Department of Pathology, Tottori University, Faculty of Medicine, particularly Mr. H. Sugihara for the skillful preparation of tissue slides.

References

1 Kuwamoto S: Recent advances in the biology of Merkel cell carcinoma. Hum Pathol *42(8)*: 1063-1077, 2011.

2 Fitzgerald TL, Dennis D, Kachare SD, Vohra NA, Wong JH and Zervos EE: Dramatic increase in the incidence and mortality from Merkel cell carcinoma in the United States (Abstract). Am Surg *81(8)*: 802-806, 2015.

3 Feng H, Shuda M, Chang Y and Moore PS: Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science *319(5866)*: 1096-1100, 2008.

4 Becker JC, Stang A, DeCaprio JA, Cerroni L, Lebbe C, Veness M and Nghiem P: Merkel cell carcinoma. Nat Rev Dis Primers *3*: 17077, 2017.

5 Kuwamoto S, Higaki H, Kanai K, Iwasaki T, Sano H, Nagata K, Kato K, Kato M, Murakami I, Horie Y, Yamamoto O and Hayashi K: Association of Merkel cell polyomavirus infection with morphologic differences in Merkel cell carcinoma. Hum Pathol *42(5)*: 632-640, 2011.

6 Bhatia K, Goedert JJ, Modali R, Preiss L and Ayers LW: Immunological detection of viral large T antigen identifies a subset of Merkel cell carcinoma tumors with higher viral abundance and better clinical outcome. Int J Cancer *127(6)*: 1493-1496, 2010.

7 Sihto H, Kukko H, Koljonen V, Sankila R, Böhling T and Joensuu H: Merkel cell polyomavirus infection, large T antigen, retinoblastoma protein and outcome in Merkel cell carcinoma. Clin Cancer Res *17(14)*: 4806-4813, 2011.

8 Higaki-Mori H, Kuwamoto S, Iwasaki T, Kato M, Murakami I, Nagata K, Sano H, Horie Y, Yoshida Y, Yamamoto O, Adachi K, Nanba E and Hayashi K: Association of Merkel cell polyomavirus infection with clinicopathological differences in Merkel cell carcinoma. Hum Pathol *43(12)*: 2282-2291, 2012.

9 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 and Nghiem P: Polyomavirus-negative Merkel cell carcinoma: a more aggressive subtype based on analysis of 282 cases using multimodal tumor virus detection. J Invest Dermatol *137(4)*: 819-827, 2017.

10 Wardhani LO, Matsushita M, Iwasaki T, Kuwamoto S, Nonaka D, Nagata K, Kato M, Kitamura Y and Hayashi K: Expression of the IDO1/TDO2-AhR pathway in tumor cells or the tumor microenvironment is associated with MCPyV status and prognosis in Merkel cell carcinoma. Hum Pathol pii: S0046-*8177(18)*: 30361-30367, 2018.

11 Liu J, Sato C, Cerletti M and Wagers A: Notch signaling in the regulation of stem cell self-renewal and differentiation. Curr Top Dev Biol *92*: 367-409, 2010.

12 Nowell C and Radtke F: Cutaneous Notch signaling in health and disease. Cold Spring Harb Perspect Med *3(12)*: a017772, 2013.

13 Nowell CS and Radtke F: Notch as a tumor suppressor. Nat Rev Cancer 17(3): 145-159, 2017.

14 Radtke F and Raj K: The role of Notch in tumorigenesis: Oncogene or tumour suppressor? Nat. Rev. Cancer *3(10)*: 756-767, 2003.

15 Ntziachristos P, Lim JS, Sage J and Aifantis I: From fly wings to targeted cancer therapies: A centennial for Notch signaling. Cancer Cell *25(3)*: 318-334, 2014.

16 Watt FM, Estrach S and Ambler CA: Epidermal Notch signalling: differentiation, cancer and adhesion. Curr Opin Cell Biol *20(2)*: 171-179, 2008.

17 Harms PW, Patel RM, Verhaegen ME, Giordano TJ, Nash KT, Johnson CN, Daignault S, Thomas DG, Gudjonsson JE, Elder JT, Dlugosz AA, Johnson TM, Fullen DR and Bichakjian CK: Distinct gene expression profiles of viral- and nonviral-associated Merkel cell carcinoma revealed by transcriptome analysis. J Invest Dermatol *133(4)*: 936-945, 2013.

18 Harms PW, Vats P, Verhaegen VE, Robinson DR, Wu YM, Dhanasekaran SM, Palanisamy N, Siddiqui J, Cao X, Su F, Wang Rui, Xiao H, Kunju LP, Mehra R, Tomlins SA, Fullen DR, Bichakjian CK, Johnson TM, Dlugosz AA and Chinnaiyan AM: The distinctive mutational spectra of polyomavirus-negative Merkel cell carcinoma. Cancer Res *75(18)*: 3720-3727, 2015.

19 Goh G, Walradt T, Markarov V, Blom A, Riaz N, Doumani R, Stafstrom K, Moshiri A, Yelistratova L, Levinsohn J, Chan TA, Nghiem P, Lifton RP and Choi J: Mutational landscape of MCPyV-positive and MCPyV-negative Merkel cell carcinomas with implications for immunotherapy. Oncotarget *7(3)*: 3403-3415, 2016.

20 Panelos J, Batistatou A, Paglierani M, Zioga A, Maio V, Santi R, Pimpinelli N, De Giorgi V, Santucci M and Massi D: Expression of NOTCH-1 and alteration of the E-cadherin/beta-catenin cell adhesion complex are observed in primary cutaneous neuroendocrine carcinoma (Merkel cell carcinoma). Mod Pathol *22(7)*: 959-968, 2009.

21 Wong SQ, Waldeck K, Vergara IA, Schröder J, Madore J, Wilmott JS, Colebatch AJ, De Paoli-Iseppi R, Li J, Lupat R, Semple T, Arnau GM, Fellowes A, Leonard JH, Hruby G, Mann GJ, Thompson JF, Cullinane C, Johnston M, Shackleton M, Sandhu S, Bowtell DD, Johnstone RW, Fox SB, McArthur GA, Papenfuss AT, Scolyer RA, Gill AJ, Hicks RJ and Tothill RW: UV associated mutations underlie the etiology of MCV-negative Merkel cell carcinomas. Cancer Res *75(24)*: 5228-5234, 2015.

22 Iwasaki T, Matsushita M, Nonaka D, Nagata K, Kato M, Kuwamoto S, Murakami I and Hayashi K: Lower expression of CADM1 and higher expression of MAL in Merkel cell carcinomas are associated with Merkel cell polyomavirus infection and better prognosis. Hum Pathol *48*: 1-8, 2016.
23 Crabtree JS and Miele L: Neuroendocrine tumors: Current therapies, notch signaling, and cancer stem cells. J Cancer Metastasis Treat *2*: 279-293, 2016.
24 Aburjania Z, Jang S, Whitt J, Jaskula-Stzul R, Chen H and Rose JB: The role of NOTCH3 in cancer. Oncologist *23(8)*: 900-911, 2018.
25 Somnay YR, Yu XM, Lloyd RV, Leverson G, Aburjania Z, Jang S, Jaskula-Sztul R and Chen H: NOTCH3 expression correlates with thyroid cancer differentiation, induces apoptosis, and predicts disease prognosis. Cancer *123(5)*: 769-782, 2017.
26 Li D, Masiero M, Banham AH and Harris AL: The Notch ligand JAGGED1 as a target for anti-tumor therapy. Front Oncol *4*: 254, 2014.
27 Kuromi T, Matsushita M, Iwasaki T, Nonaka D, Kuwamoto S, Nagata K, Kato M, Akizuki G, Kitamura Y and Hayashi K: Association of expression of the hedgehog signal with Merkel cell polyomavirus infection and prognosis of Merkel cell carcinoma. Hum Pathol *69*: 8-14, 2017.
28 Harms KL, Healy MA, Nghiem P, Sober AJ, Johnson TM Bichakjian CK and Wong SL: 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(11)*: 3564-3571, 2016.
29 van Veenendaal LM, van Akkooi ACJ, Verhoef C, Grünhagen DJ, Klop WMC, Valk GD and Tesselaar MET: Merkel cell carcinoma: clinical outcome and prognostic factors in 351 patients. J Surg Oncol *117(8)*: 1768-1775, 2018.
30 Jaskula-Sztul R, Eide J, Tesfazghi S, Dammalapati A, Harrison AD, Yu XM, Scheinebeck C, Winston-McPherson G, Kupcho KR, Robers MB, Hundal AK, Tang W and Chen H: Tumor-suppressor role of NOTCH3 in medullary thyroid carcinoma revealed by genetic and pharmacological induction. Mol Cancer Ther *14(2)*: 499-512, 2015.
31 Kaufman HL, Russel J, Hamid O, Bhatia S, Terheyden P, D'Angelo SP, Shih KC, Lebbé C, Linette GP, Milella M, Brownell I, Lewis KD, Lorch JH, Chin K, Mahnke L, von Heydebreck A, Cuillerot JM and Nghiem P: Avelumab in patients with chemotherapy-refractory metastatic Merkel cell carcinoma: A multicentre, single-group, open-label, phase 2 trial. Lancet Oncol *17(10)*: 1374-1385, 2016.
32 Ngiem PT, Bhatia S, Lipson EJ, Kudchadkar RR, Miller NJ, Annamalai L, Berry S, Chartash EK, Daud A, Fling SP, Friedlander PA, Kluger HM, Kohrt HE, Lundgren L, Margolin K, Mitchell A, Olencki T, Pardoll DM, Reddy SA, Shanta EM, Sharfman WH, Sharon E, Shemanski LR, Shinohara MM, Sunshine JC, Taube JM, Thompson JA, Townson SM, Yearley JH, Topalian SL and Cheever MA: PD-1 blockade with pembrolizumab in advanced Merkel-cell carcinoma. N Engl J Med *374(26)*: 2542-2552, 2016.
33 Topalian SL, Bhatia S, Hollebecque A, Awada A, De Boer JP, Kudchadkar RR, Goncalves A, Delord JP, Martens UM, Picazo JML, Oaknin A, Spanos WC, Aljumaily R, Sharfman WH, Rao S, Soumaoro I, Cao A, Nghiem P and Schadendorf D: Non-comparative, open-label, multiple cohort, phase 1/2 study to evaluate nivolumab (NIVO) in patients with virus-associated tumors (Checkmate 358): Efficacy and safety in Merkel cell carcinoma (MCC) (Abstract). In: Proceedings of the American Association for Cancer Research Annual Meeting. Cancer Res *77(13 Suppl)*: CT074, 2017.
34 Harms PW, Harms KL, Moore PS, DeCaprio JA, Nghiem P, Wong MKK and Brownell I; on behalf of the International Workshop on Merkel Cell Carcinoma Research (IWMCC) Working Group: The biology and treatment of Merkel cell carcinoma-current understanding and research priorities. Nat Rev Clin Oncol *4*: 1-14, 2018.

Received November 18, 2018
Revised December 3, 2018
Accepted December 12, 2018