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Original contribution

Association of expression of the hedgehog signal with Merkel cell polyomavirus infection and prognosis of Merkel cell carcinoma☆,☆☆

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Summary Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer that mostly occurs in the elderly. Merkel cell polyomavirus (MCPyV) is detected in approximately 80% of MCCs and is associated with carcinogenesis. Hedgehog signaling pathway plays a role in human embryogenesis and organogenesis. In addition, reactivation of this pathway later in life can cause tumors. Twenty-nine-MCPyV-positive and 21 MCPyV-negative MCCs were immunohistochemically stained with primary antibodies for hedgehog signaling (SHH, IHH, PTCH1, SMO, GLI1, GLI2, and GLI3) and evaluated using H-score. Polymerase chain reaction and sequence analysis for *SHH* and *GLI1* exons were also performed. Expression of SHH was higher in MCPyV-positive MCCs than in MCPyV-negative MCCs ($P < .001$). Higher expression of GLI1, MCPyV infection, male sex, and Japanese ethnicity were associated with better overall survival ($P = .034$, $P = .001$, $P = .042$, and $P = .036$, respectively). Higher expression of SHH and MCPyV infection were associated with improved MCC-specific survival ($P = .037$ and $P = .002$, respectively). The mutation analysis of prognosis-related *GLI1* and *SHH* genes in our study revealed a low frequency of mutations in the 10 exons examined, except *GLI1* exon 5 (18/22 cases), all having the same silent mutation of c.576G > A. Only 2 mutations with amino acid changes were detected in MCPyV-negative MCCs only: 1 missense mutation in *GLI1* exon 4 and 1 nonsense mutation in *SHH*-3B. Expression of SHH and GLI1 may be useful prognostic markers of MCC because increased expression was associated with better prognosis. The high rate of c.576G > A silent mutation in *GLI1* exon 5 was a feature of MCC.

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1. Introduction

Merkel cell carcinoma (MCC) is a rare and aggressive neuroendocrine skin cancer that mostly occurs in sun-exposed areas of the elderly and immunosuppressed individuals [1,2]. Approximately 80% of MCC cases are associated with Merkel cell polyomavirus (MCPyV), and viral DNA is integrated within the tumor genome in a clonal pattern [1,3,4]. MCPyV-encoded large T antigen is expressed by infected cells and inactivates Rb tumor suppressor protein [3,4]. Significant differences in histological appearance, oncogene expression patterns, and prognosis between MCPyV-positive and -negative MCCs suggest different mechanisms of carcinogenesis [2,5]. Some studies have shown that patients with MCPyV-positive MCC have a better prognosis than those with MCPyV-negative MCC [2,6,7].

The hedgehog (HH) signaling pathway is the main developmental pathway involved in human embryogenesis and organogenesis [8]. In addition, recent studies have demonstrated that aberrant overexpression of HH pathway signals later in life can play a critical role in the development of malignant tumors [8,9]. Binding of HH proteins, like Sonic HH (SHH) or Indian HH (IHH), to their receptor Patched 1 (PTCH1) results in the activation of Smoothened (SMO) and the subsequent activation of the signaling cascade of GLI proteins. It is thought that PTCH1 blocks the function of SMO and the following signaling cascade when HH proteins are not present [10,11]. GLI1 and GLI2 can mediate HH signals and have been implicated in tumorigenesis. GLI1 is an HH response gene product that exists only as a transcriptional activator and functions via a positive feedback loop upon pathway activation. GLI2 functions as a transcriptional activator, whereas GLI3 is the primary transcriptional repressor [12]. Mutations causing inappropriate reactivation of SHH signaling pathway have been linked to development of basal cell carcinoma (BCC) [13] as well as lung, colon, cervical, and breast cancers [9,10]. The aberrant activation of HH signaling pathway also plays an important role in squamous cell carcinoma (SCC), malignant melanoma (MM) of the skin [9], and malignant hematological neoplasms [14].

Frequent and intense overexpression of HH signaling molecules was observed in tissues from 25 patients with MCC analyzed by microarray, and high levels of PTCH and IHH were significantly associated with an increase in overall and recurrence-free survival, respectively [10]. However, there are no data on the MCPyV infection status in these patients. In this study, we investigated and discussed the association of expression of HH signaling molecules with MCPyV infection and prognosis in 50 cases of MCC.

2. Materials and methods

2.1. Samples

This study was approved by the institutional review board of Tottori University Faculty of Medicine. We included 29 MCPyV-positive samples (13 Japanese and 16 whites in the United Kingdom) and 21 MCPyV-negative samples (1 Japanese and 20 whites in the United Kingdom) in the study. All samples were formalin-fixed and paraffin-embedded (FFPE). Clinicopathological characteristics of the patients are summarized in Supplementary Table S1.

2.2. Immunohistochemistry

Each sample was stained by polymer-based immunohistochemistry using EnVision + System-HRP Labeled Polymer Anti-Rabbit (Dako, Glostrup, Denmark) and Liquid DAB+ Substrate Chromogen System (Dako). Primary antibodies used in this experiment are listed in Table 1. After staining, pathologists and researchers who were blinded to the clinical details of the patients evaluated the stained tissues. Cytoplasmic (SHH, IHH, PTCH1, and SMO) and nuclear (GLI1, GLI2, and GLI3) immunostains were evaluated using the H-score [15]. Tissues used as positive controls were also stained and are shown in Table 1. Nonneoplastic skin and subcutaneous tissues from MCC samples were used as internal negative controls.

2.3. DNA extraction

DNA was extracted from each FFPE tissue sample using the QIAamp DNA FFPE Tissue Kit following the manufacturer's protocol (Qiagen, Hilden, Germany).

2.4. Mutation analysis by polymerase chain reaction and sequencing

Polymerase chain reaction (PCR) and sequencing for *SHH* and *GLI1* exons were performed because expression levels of SHH and GLI1 were significantly associated with

Table 1 List of antibodies and positive controls used for immunohistochemistry

Antibodies	Host and type	Sources	Dilution ratio	Positive controls
SHH	Rabbit monoclonal	Abcam	1:100	Kidney
IHH	Rabbit polyclonal	Santa Cruz Biotechnology	1:80	Colon
PTCH1	Rabbit polyclonal	Santa Cruz Biotechnology	1:100	Breast Cancer
SMO	Rabbit polyclonal	Abcam	1:150	Brain
GLI1	Rabbit polyclonal	Santa Cruz Biotechnology	1:40	Testis
GLI2	Rabbit polyclonal	GeneTex, Inc	1:250	Testis
GLI3	Rabbit polyclonal	Atlas Antibodies	1:50	Testis