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Original contribution

Comparison of Akt/mTOR/4E-BP1 pathway signal activation and mutations of *PIK3CA* in Merkel cell polyomavirus–positive and Merkel cell polyomavirus–negative carcinomas☆,☆☆

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Summary Merkel cell polyomavirus (MCPyV) integrates monoclonally into the genomes of approximately 80% of Merkel cell carcinomas (MCCs), affecting their clinicopathological features. The molecular mechanisms underlying MCC development after MCPyV infection remain unclear. We investigated the association of MCPyV infection with activation of the Akt/mammalian target of rapamycin (mTOR)/4E-binding protein 1 (4E-BP1) signaling pathway in MCCs to elucidate the role of these signal transductions and to identify molecular targets for treatment. We analyzed the molecular and pathological characteristics of 41 MCPyV-positive and 27 MCPyV-negative MCCs. Expression of mTOR, TSC1, and TSC2 messenger RNA was significantly higher in MCPyV-negative MCCs, and Akt (T308) phosphorylation also was significantly higher (92% *vs* 66%; $P = .019$), whereas 4E-BP1 (S65 and T70) phosphorylation was common in both MCC types (92%-100%). The expression rates of most other tested signals were high (60%-100%) and not significantly correlated with MCPyV large T antigen expression. *PIK3CA* mutations were observed more frequently in MCPyV-positive MCCs (6/36 [17%] *vs* 2/20 [10%]). These results suggest that protein expression (activation) of most Akt/mTOR/4E-BP1 pathway signals was not significantly different in MCPyV-positive and MCPyV-negative MCCs, although these 2 types may differ in tumorigenesis, and MCPyV-negative MCCs showed significantly

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more frequent p-Akt (T308) activation. Therefore, certain Akt/mTOR/4E-BP1 pathway signals could be novel therapeutic targets for MCC regardless of MCPyV infection status.

1. Introduction

Merkel cell carcinoma (MCC) is a clinically aggressive neuroendocrine skin cancer that often occurs in the elderly and in immunosuppressed individuals. Approximately 80% of MCCs harbor a novel polyomavirus named Merkel cell polyomavirus (MCPyV), which is believed to be a carcinogenic agent [1]. We recently demonstrated that MCPyV-positive MCC from a case with spontaneous regression differed in morphology and showed a better prognosis and significantly higher expression of the retinoblastoma protein and lower expression of p53 than MCPyV-negative MCCs [1–3]. The tumorigenic pathway of MCPyV-negative MCCs is hypothesized to be different from that of MCPyV-positive MCCs, but the differences have not been clearly elucidated.

Generally, mammalian DNA viruses, including polyomaviruses, activate the phosphoinositide 3-kinase (PI3K)/serine-threonine protein kinase Akt (also known as protein kinase B)/mammalian target of rapamycin (mTOR) pathway at certain points in their life cycle to benefit from the growth, metabolic, antiapoptotic, and translational functions the pathway controls [4]. The PI3K/Akt/mTOR pathway is highly activated in various malignant tumors, including MCCs [5,6]. In this pathway, PI3K activates the downstream serine-threonine kinase Akt. Akt, in turn, phosphorylates many downstream molecules involved in the regulation of cellular functions. mTOR, an Akt substrate, forms 2 distinct protein complexes, mTORC1 and mTORC2, which affect protein synthesis. The mTORC1 complex controls downstream targets, ribosomal protein S6 kinase 1, and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1).

Recently, Shuda et al [7] revealed the activation of some PI3K signaling molecules in MCC and showed that MCPyV small T antigen (MCPyV-ST) is an oncoprotein that targets 4E-BP1, a regulator of translation. However, the association of MCPyV infection and PI3K/AKT/mTOR/4E-BP1 pathway signal activation in MCCs has not been completely elucidated.

In this study, we investigated the association of MCPyV infection with activation of the PI3K/AKT/mTOR/4E-BP1 signaling pathway in MCCs to elucidate the mechanisms involved in the molecular pathogenesis of and signal transduction in MCCs and to identify novel molecular targets for MCC treatment.

2. Materials and methods

2.1. Samples

This study was approved by the institutional review board of the Faculty of Medicine at Tottori University. We used 41 MCPyV-positive (34 samples from 32 patients in Japan, and 7 samples from 7 patients in the United Kingdom) and 27 MCPyV-negative (Japan: 12 samples from 11 patients; United Kingdom: 15 samples from 15 patients). All 22 patients from the United Kingdom were whites. All samples had been formalin fixed and paraffin embedded (FFPE). Four cases were combined MCC and squamous cell carcinoma (MCC + SCC); 5 cases were combined MCC and Bowen disease (MCC + BD); and 1 case was MCC combined with a benign adnexal tumor. These FFPE samples were used in previous studies [2,8]. The clinicopathological characteristics of the patients are summarized in Supplementary Table S1.

2.2. DNA and RNA extraction

Four-micrometer-thick sections were cut from FFPE tissue samples and mounted on glass slides. The MCC components were dissected away from the supporting tissue using microscopic views and razor blades. The DNA was extracted with the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany), and RNA was extracted using the RNeasy FFPE kit (Qiagen) according to the manufacturer's protocols. To determine the MCPyV copy number for each case, real-time quantitative polymerase chain reaction (PCR) was performed as reported previously [2].

2.3. Nucleotide expression

The messenger RNA (mRNA) expression analyses of the complementary DNA of the PI3K/Akt/mTOR/4E-BP1 signaling pathway genes were done using ReverTraAce qPCR RT Master Mix with gDNA Remover (Toyobo, Tokyo, Japan) according to the manufacturer's protocols. The amounts of mRNA from the PIK3CA/Akt/mTOR/4E-BP1 genes were measured by quantitative real-time PCR using *Taq*Man gene expression assays and on-demand probes (Life Technologies, Carlsbad, CA) for PIK3CA (Hs00907957_m1), PDK1 (Hs01561850_m1), PTEN (Hs02621230_s1), PIK3R5 (Hs01046353_m1), PPP2R1A (Hs00204426_m1), mTOR (Hs00234508_m1), RPS6KB1 (Hs00177357_m1), EIF4E (Hs00854166_g1), EIF4EBP1 (Hs00854166_g1), TSC1 (Hs01060648_m1), and TSC2 (Hs00241068_m1) with the ABI PRISM 7900HT sequence detection system (Life Technologies). Results were expressed with $2^{-\Delta\Delta Ct}$, the reference or internal control was Universal Probe Library Human TBP (TATAA-box-binding protein) Gene (Roche Diagnostics KK, Tokyo, Japan), and the calibrator was the positive samples. We performed heat map analysis with average linkage as the clustering method and Euclidean distances as the distance measure using DataAssist software (Life Technologies).