

# 主 論 文

Melanoma cell adhesion molecule is the driving force behind the dissemination of melanoma upon S100A8/A9 binding in the original skin lesion

(皮膚原発巣におけるメラノーマ浸潤を誘発する S100A8/A9-MCAM シグナルの本態解明)

## 【緒言】

Metastatic melanoma is one of the most serious diseases in Caucasian (white people). More than 90% of such patient die of metastasis, a process by which cancer cells depart from their tumor of origin, spread systemically, and colonize at distant organs. Even though a lot of methods have been developed to treat with this disease, it is still quite difficult to effectively treat melanoma when it comes into a highly metastatic phenotype as a late malignant stage. Therefore, a better understanding of how transformation onset toward metastatic phenotype systematically occurs at the molecular level is needed to establish a novel strategy that effectively regulates cancer metastasis.

Now, S100A8/A9, a heterodimer complex of S100A8 and S100A9, is an attractive molecule involved in cancer metastasis. This protein has an interesting feature of high secretion in the lung. Even though melanoma present at distant place far from the lung, the secreted S100A8/A9 attracts melanoma from the original skin lesion to the metastatic site in the lung. What receptor(s) do cancers use to sense the S100A8/A9 signal? TLR4 (Hiratsuka et al., Nature Cell Biol 2006 & 2008) and RAGE (Saha et al., J Biol Chem 2010) have been known as S100A8/A9 receptors. However, the expression of these receptors is not common in metastatic melanoma. We previously succeeded to identify other important receptors, EMMPRIN (Hibino et al., Cancer Res 2013) and Melanoma cell adhesion molecule (MCAM) (Ruma et al., Clin Exp Metastasis 2016; Sakaguchi, Theranostics 2017), for melanoma lung metastasis. In comparison to EMMPRIN studies, MCAM studies on how to acquire the driving force of metastasis upon S100A8/A9 binding is poorly understood. In this study, we aim to clarify an unidentified MCAM downstream signal. This novel finding will contribute to a deep study of the metastatic feature in melanoma.

Here we first show the unveiled S100A8/A9-MCAM-mediated signal pathway that leads to an aggressive metastasis of melanoma to the lung in melanoma bearing mouse. The identified molecules activated one after another through the stimulated MCAM with S100A8/A9 are all novel findings and the revealed signal pathway consisting of those molecules plays a critical role in melanoma metastasis. We hope that our findings will lead to a better understanding of the physiological and pathological studies in melanoma lung-tropic metastasis and suppression of the identified pathway might be an effective approach for prevention of metastasis by targeting the S100A8/A9-MCAM axis in melanoma and probably other cancers.

## 【材料と方法】

### Cell lines and reagents

Two human melanoma cell lines established from the same patient—i.e., WM-115 (derived from the primary tumor, ATCC, Rockville, MD) and WM-266-4 (derived from a metastatic site, ATCC)—and mouse melanoma cell line B16-BL6, a highly invasive variant of the mouse melanoma B16 cell line (a kind gift from Dr. Isaiah J. Fidler, M.D., Anderson Cancer Center, Houston, TX). The stable clones were established by electroporation and followed by puromycin selection.

### Quantitative real-time PCR analysis

Real-time RT-PCR was performed using a LightCycler rapid thermal cycler system (ABI

7900HT; Applied Biosystems, Foster City, CA) with LightCycler 480 SYBR Green I Master (Roche Diagnostics, Indianapolis, IN).

#### Migration and invasion assays

Migration and invasion assays were performed by the Boyden chamber method. In the case of the invasion assay, we sealed the 8- $\mu$ m pore filters set in the Transwell culture inserts (BD Biosciences, Bedford, MA) with Matrigel. Cells were seeded in the upper chamber in a low serum medium, D/F (0.5% FBS), and the lower chamber was filled with high serum medium, D/F (10% FBS), in either the presence or absence of S100A8/A9 recombinant protein as a chemoattractant.

#### Co-immunoprecipitation and Western blotting

Monoclonal Anti-HA tag (clone HA-7) agarose (Sigma-Aldrich, St. Louis, MO) was used for the co-immunoprecipitation experiments. The tag-agarose beads were mixed with various cell extracts expressing an excess amount of foreign kinases (MAPK cascade upstream kinases) and incubated for 3 h at 4 °C. After incubation of the samples, bound proteins were pulled down by centrifugation and the precipitates were subjected to SDS-PAGE followed by Western blotting

#### Protein/DNA array and electrophoretic mobility shift assay (EMSA)

Screening of activated TFs through MCAM overexpression in HEK293T cells ( $1 \times 10^6$  cells) was performed by using a protein/DNA array I (Thermo Fisher Scientific) according to the manufacturer's instructions. Electrophoretic mobility shift assay (EMSA) was performed using a LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific) to confirm the activation of ETV4 in the stimulation of S100A8/A9.

#### Animal experiments

Experimental protocols were approved by the Animal Experiment Committee in Okayama University (approval No. OKU-2014011). All mouse procedures and euthanasia, including cell transplantations, were done painlessly or under inhalation anaesthesia with isoflurane gas according to the strict guidelines of the Experimental Animal Committee of Okayama University.<sup>2</sup>

### **[結果]**

#### MCAM stimulates cellular migration in response to S100A8/A9 in melanoma cells

To begin with, we attempted to determine the significance of the S100A8/A9-MCAM axis in metastatic mortality of WM-115 and WM-266-4 cells. When foreign MCAM was overexpressed in WM-115 cells, the migration ability in response to S100A8/A9 stimulation was upregulated. We also used the siRNA technique to attenuate the intrinsic MCAM function. The two siRNAs induced downregulation of the migration ability of WM-266-4 cells at not only basal activity but also S100A8/A9-responded activity.

#### Identification of ETV4, which plays a pivotal role in S100A8/A9-MCAM-mediated upregulation of cellular motility

Our screening for candidate transcription factors that produce metastatic driving force and are regulated by an MCAM downstream signal provided four potential candidates, ETS, GATA, NF and PPAR. Among these, we focused on the significance of ETS since an ETS decoy oligonucleotide had a preventive effect of migration activity in WM-266-4 cells. That spurred us to try to identify the ETS protein producing the metastatic force in melanoma from ETS family proteins. Quantitative real-time PCR analysis showed marked expression of ETV1, ETV4, ETV5 ELK, EHF, ERG, ELF, FEV and SPDEF in many melanoma cell lines in a high

and specific manner. When we forced WM-115 cells to overexpress these proteins, we found that the expression of ETV4 resulted in acquisition of much higher activity for cellular migration, while in WM-266-4 cells, ETV4 dn led to a marked reduction of both innate migration activity and the stimulation by S100A8/A9. We verified this result by EMSA and further confirmed the expression of MCAM and ETV4 in clinical human samples by immunohistochemistry. Furthermore, ETV4 induced lung metastasis also confirmed by in vivo animal experiments. After establishing the stable clones by electroporation, the lung metastasis ability was examined by tail-vein injection. The number of lung tumor nodules derived from MCAM-ETV4wt group was larger than that from control group.

#### TPL2 is a critical activator of ETV4 in the S100A8/A9-MCAM signaling cascade

To investigate whether MAPKs family contribute to the S100A8/A9-MCAM-ETV4 pathway, we examined the potential interactions between MCAM and MAPKs. Our candidate screening method based on protein-protein interactions provided novel partners of MCAM for its downstream signaling, i.e., four MAP3Ks (DLK, TPL2, ASK1 and MLK1) and one MAP2K (MKK5). Among these, TPL2 alone possessed a high ability to induce upregulation of cellular motility in WM-115 cells. Moreover, TPL2 wt significantly enhanced ETV4-mediated migration, and this was not observed when ETV4 dn was overexpressed in WM-115 cells. TPL2 mut (KD) could block MCAM-induced cell migration, further suggest that MCAM mediated ETV4 activity in TPL2 depended manner.

#### MMP25, a key molecule for the regulation of melanoma motility and invasiveness, is strongly induced by ETV4 activation through the S100A8/A9-MCAM axis

As a transcription factor, ETV4 is reported to regulate numerous genes in different cancers. We found that among all the matrix metalloproteinases (MMPs), only MMP25 was remarkably increased by overexpress ETV4 wt, but not ETV4 dn, in both WM-115 and WM-266-4 cells. The results were also confirmed from MMP25 promoter activity assay and immunohistochemistry in clinical human melanoma samples. Interestingly, we found that Mmp25 siRNA strongly decreased the metastatic ability of mouse melanoma B16-BL6 cells to the lung in the in vivo study on mice. These results further supported our conclusions.

#### [考察]

Melanoma cell adhesion molecule, MCAM (synonym: CD146, Mel-CAM, MUC18, S-endo1), was first reported in malignant melanomas where a high level of MCAM expression was associated with a poor prognosis. The presence of several protein kinase recognition motifs in the cytoplasmic domain suggests the involvement of MCAM in downstream cell signaling. Our study identified that TPL2, which is one of the mitogen-activated protein kinase kinase kinase (MAP3K) directly binds to MCAM cytoplasmic tail, plays a key role in leading the activation of malignant melanoma metastasis. This finding revealed a distinctive function of MCAM other than adhesion. To determine the mechanism(s) by which MCAM modulates metastasis, we found that ETV4 transcription factor, which is positively regulated by MCAM, may be one of the key nuclear effectors of oncogenic MAPK signaling. Indeed, the results of a prior study suggested that ETV4 activates a RAS and MAPK transcriptional program in the absence of the MAPK pathway. ETV4 is known to be involved in events participating in tumor development and progression, indicating a function similar to that of MCAM. Since robust ERK1/2 activation was observed in S100A8/A9-MCAM-induced melanoma metastasis, we postulated that ETV4, a positive regulator from ERK/MAPK, is involved in the migration of cancer cells. This speculation is also supported by the results of another recent study. Fung reported a novel signal pathway, ERK-MAPK-ETV4-MMP-E-cadherin, in driving malignance

in oesophageal carcinoma. Consistently, we clearly demonstrated that MCAM exacerbated melanoma migration, which was associated with the enhanced ERK1/2-ETV4 activation.

In addition, we should not rule out the importance of matrix metalloproteinases (MMPs) in cancer metastatic progression. We therefore examined whether these genes are affected by ETV4 at the transcription level in melanoma cells. We found that only MMP25 was remarkably upregulated by forced expression of ETV4 wt, but not that of ETV4 dn, in both WM-115 and WM-266-4 cells. MMP25 is a unique membrane-anchored matrix metalloproteinase that is located on the cell surface via a glycosyl-phosphatidyl inositol (GPI) anchor. Increased expression of MMP25 has been found in several types of invasive cancer cells, including glioma, colon and prostate cancer cells. In addition, when colon cancer cells were transplanted subcutaneously in mice, MMP25 showed a specific distribution at the infiltrative rim of the cancer nugget, suggesting an important role of MMP25 in dissemination onset of cancer cells at the interface region in the cancer primary site. A similar phenomenon was also observed in our melanoma study. Collectively, our results support the idea that MMP25 plays an unusual role in the onset of melanoma dissemination in skin.

#### **[結論]**

In this study, we unveiled a critical role of MCAM in melanoma metastasis upon S100A8/A9 binding. Downstream signaling analysis showed that MCAM promotes activation of ETV4 through TPL2-ERK1/2 pathway, which further modulates MMP25 and induce the dissemination of cancer cells. Inhibition of this pathway would be a promising therapeutic alternative in melanoma treatment