Functional Analysis of glioma-associated homolog 1 in maintaining invasive and mesenchymal-like properties of melanoma cells

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(メラノーマ細胞の浸潤及び間葉系様細胞の性質維持における転写因子 **GLI1** の機能解析**)**

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The ability to reversibly switch between invasive and non-invasive phenotype has been considered to be critical for the high malignancy of melanoma. Increasing evidence suggests that this ability is determined by the activities of microphthalmia-associated transcription factor (MITF) and EMT-related transcription factors (EMT-TFs). GLI1 and GLI2, the components of Hedgehog (HH) pathway, are associated with progression and invasiveness of melanoma. Although GLI2 is known to suppress MITF expression and to promote invasive phenotype of melanoma, the role of GLI1 remains elusive. Here I show that inhibition of Shh-pathway by cyclopamine or GLI1 knockdown (*Gli1* KD) decreased migration and invasion activity of mouse and human melanoma without affecting MITF levels. I observed that *Gli1* KD B16F10 melanoma cells exhibited a loss of mesenchymal-like characteristic as indicated by an increased expression of E-cadherin and a decreased expression of mesenchymal markers. To gain insight into the molecular mechanism, I analyzed several EMT-TFs that are known to regulate melanoma invasiveness. My analysis suggested that the expression of SNAIL1, ZEB1, and TWIST1, but not SNAIL2 and ZEB2, are directly regulated by GLI1. Collectively my findings suggest a regulatory role of GLI1 on a subset of EMT-TFs to promote invasive phenotype of melanoma independent of MITF.

Melanoma incidence is increasing over past 50 years. It represents a small percentage of skin cancers, but is the major cause of mortality from these cancers. The primary source of melanoma is melanocytes (pigment cells) that underwent a mutation that lead to the constitutive activation of MAPK signaling pathway. Approximately 50% of melanomas harbor the activating $BRAF^{V600E}$ mutation. Current therapies for metastatic melanoma provides only transient response and modest disease-free survival.

Tumor heterogeneity poses a major challenge for the effective treatment of cancer. Accumulating evidence indicates that the heterogeneity in melanoma can be driven through phenotypic plasticity. That is, the aggressiveness of melanoma appears to be due to the cancer cells' ability to reversibly switch between different phenotypes with non-invasive and invasive potentials. Microphthalmia-associated transcription factor (MITF) plays an essential role in determining the melanocyte lineage and has been proposed to act as a rheostat for the cellular heterogeneity in melanoma. In the rheostat model, low levels of MITF generate invasive, stem-like cells, whereas high MITF levels stimulate proliferation and inhibit invasion.

Acquisition of invasive phenotype is an important step for metastasis of melanoma which involves downregulation of melanocyte differentiation program accompanied by decreased expression of E-cadherin, increase expression of mesenchymal markers, and enhanced invasion. This process resembles the epithelial-mesenchymal transition (EMT) in carcinoma. Epithelial-to-mesenchymal transition (EMT**)** is a dynamic and reversible phenotypic switching process from polarized epithelial cells to motile mesenchymal cells; this process is essential for normal development and is widely thought to be a critical switch for tumor-cell invasiveness. EMT is driven by an interconnected signaling network of EMT-inducing transcription factors (EMT-TFs), including SNAIL, TWIST, and ZEB. Recent studies have shown that some EMT-TFs play important roles in malignant melanoma, but their regulation and function are different from those in epithelial cancers.

These studies also suggest that the EMT-TFs SNAIL2 and ZEB2 act as tumor-suppressor proteins by activating an MITF-dependent melanocyte differentiation program.

Sonic hedgehog (Shh) signaling has critical roles in embryonic patterning, and aberrant Shh-signaling activation is implicated in various cancer types, including skin cancer. The Shh signal is transduced by a receptor complex composed of two proteins, Patched (PTCH) and Smoothened (SMO). The binding of Shh to PTCH relieves PTCH's repression of SMO. The transcription factor glioma-associated oncogene homolog 1 (GLI1) acts as a terminal, positive effector of Shh signaling, and *Gli1* itself is a Shh-target gene. GLI1's expression and activity are also regulated through a non-canonical Shh pathway, such as those involving hypoxia or transforming growth factor (TGF)-β. GLI1 has been suggested to be involved in melanoma progression, although its precise role and the mechanism underlying invasion remain unclear. In this study, we show that GLI1 has a role in maintaining the invasive and mesenchymal-like properties of melanoma cells.

To examine the role of Shh signaling in maintaining the invasive phenotype of melanoma cells, I blocked the Shh-GLI signaling pathway using cyclopamine, an inhibitor of SMO. B16F10 cells were treated with cyclopamine at relatively low concentrations, 2.5 μM and 5 μM. The inhibition of Shh-GLI signaling pathway was confirm as shown by decreased levels of GLI1 protein and of GLI-mediated transcriptional activity, in a dose-dependent manner. GLI2 protein levels was decreased by cyclopamine, but to a lesser degree than GLI1. The processed, repressive form of GLI3 (GLI3-R) was slightly increased, and full-length GLI3 (GLI3-FL) was decreased, in cyclopamine-treated B16F10 cells in a dose-dependent manner. Blocking Shh signaling with cyclopamine caused a dose-dependent decrease in invasion activity.

The invasive phenotype of melanoma cells is often characterized by low levels of MITF. Therefore, I examined the expression levels of MITF in cyclopamine-treated B16F10 cells at 2.5 μM and 5 μM. The protein and mRNA levels of MITF were comparable among these cells. There were also no significant differences in the mRNA

levels *Tyrosinase* (*Tyr*), a MITF-target gene, in these cells. These results indicated that blocking the Shh signaling by cyclopamine at low concentration inhibits the invasion ability of B16F10 melanoma cells without affecting their MITF expression and activity.

I next asked whether GLI1 is important for the invasive phenotype of melanoma cells. I knockdown *Gli1* in B16F10 cells using two *Gli1*-targeting lentiviral shRNAs (shGli1-1 and shGli1-2), and then examined the invasion ability and migration of the *Gli1*-knockdown (*Gli1* KD) cells. The invasion ability of the KD cells was substantially reduced compared to that of control B16F10 cells expressing a *Luc*-targeting lentiviral shRNA (shLuc). The cell migration activity was also inhibited by knocking down *Gli1*. Furthermore, exogenous expression of GLI1 in B16F10 cells significantly increased the invasion activity. Then I analyzed the expression levels of MITF (protein and mRNA) and its target gene *Tyr* in the *Gli1* KD B16F10 cells, and found that the MITF and *Tyr* levels were unchanged, as seen in the cyclopamine-treated B16F10 cells. I also observed similar effects on the cell migration and invasion abilities and the MITF expression and activity in *GLI1* KD MeWo (wild-type BRAF/NRAS) and G361 ($BRAF^{V600E}$) cells, metastatic human melanoma cell lines. Taken together, these results strongly suggested that GLI1 plays a crucial role in maintaining the invasive phenotype of melanoma cells, independently of the regulation of MITF expression and activity.

The decreased invasion and migration after *Gli1* KD prompted us to examine the metastatic ability of the KD cells *in vivo*. I performed an experimental *in vivo* metastasis assay, in which B16F10 cells expressing shLuc (control) or shGli1 were injected into the mouse tail vein, and lung metastasis was evaluated. There were many fewer metastatic nodules in the lungs of mice injected with shGli1-expressing cells than in the lungs of control B16F10-injected mice, indicating that *Gli1* KD decreases the lung metastasis ability of B16F10 cells.

Gli1 activity has been associated with EMT in various cancer. I question whether the decrease invasion and migration is also associated with this function in melanoma.

Further examination by phalloidin staining showed that stress fiber formation was severely inhibited in the *Gli1* KD cells. Consistent with the changes in morphology and cytoskeletal structures, the expression levels of E-cadherin (protein and mRNA) were substantially increased, with the E-cadherin protein being predominantly localized to areas of cell-cell contact, in the *Gli1* KD cells. Furthermore, the mRNA expression levels of mesenchymal markers, such as N-cadherin and vimentin were significantly decreased in the *Gli1* KD cells compared to the shLuc-expressing control cells. Similar expression profiles of E-cadherin and mesenchymal markers were obtained using MeWo and G361 cells, although there were no increased cell-cell adhesion in both cell lines as observed in B16F10. These results may indicate that GLI1 regulates a subset of EMT-TFs to prevent the reverse transition from a mesenchymal-like to an epithelial-like phenotype.

To gain insight into the molecular mechanism, I investigated whether GLI1 modulates the expression of key EMT-TFs, including SNAIL and ZEB family members. The mRNA levels of *Snail1, Zeb1,* and *Twist1* were significantly decreased in the *Gli1* KD B16F10 cells compared to the control B16F10 cells, whereas no significant differences in the *Snail2* or *Zeb2* mRNA levels were observed between the *Gli1* KD and control cells. Using the MatInspector software, I identified several putative GLI-binding sites within the one-kilobase (1-kb) upstream region of the transcriptional start sites of *Snail1, Zeb1*, and *Twist1*. I then conducted ChIP assays with an anti-GLI1 Ab in B16F10 cells. The precipitated DNAs were analyzed by qPCR using primers specific for the GLI1-binding sites. The ChIP results revealed that GLI1 bound significantly to upstream region of *Snail1, Zeb1,* and *Twist1*. I then examined the effect of GLI1 overexpression on the potential promoters of *Snail1, Zeb1*, and *Twist1* using reporter assays, and found that GLI1 overexpression substantially enhanced the promoter activities of the 1-kb regions. The activities were not significantly increased when the regions' corresponding deletion derivatives were used. Collectively, these results strongly suggest that GLI1 binds directly to the promoters of *Snail1, Zeb1*, and *Twist1*, and regulates their expression. In

addition, I found a significant co-occurrence between *Gli1* and *Zeb1* (P = 0.002) and between *Gli1* and *Sail1* (P = 0.004) by analyzing the TCGA cutaneous melanoma dataset (287 samples with RNA sequencing expression data) through the cBioPortal for cancer genomic data using mutual exclusivity analysis.

The expression level of MITF is thought to be the key factor in determining the proliferative or invasive state of melanoma according to MITF-dependent phenotype switching model. MITF is a key molecule that regulates heterogeneity in melanoma, and the MITF rheostat model has become widely accepted in melanoma biology. In the present study, I demonstrated for the first time that the transcription factor GLI1 plays an important role in maintaining the invasive phenotype of melanoma cells without affecting the MITF expression and activity. I also showed that GLI1 prevents the reversal of the mesenchymal-like phenotype of melanoma cells, most likely by modulating a subset of EMT-TFs. These findings provide new insight into how a high degree of heterogeneity and plasticity is achieved and regulated in melanoma.

Recent studies have shown that a switch in the EMT-TF expression pattern from SNAIL2^{high}/ZEB2^{high}/TWIST1^{low}/ZEB1^{low} to SNAIL2^{low}/ZEB2^{low}/TWIST1^{high}/ZEB1^{high} occurs during melanoma progression. In this study, I found that *Gli1* KD induced a mesenchymal-epithelial-like transition in melanoma cells, which was accompanied by severely decreased invasive and migratory properties, and by an increased expression of E-cadherin and downregulation of mesenchymal markers. I also observed decreased mRNA levels of *Snail1*, *Zeb1*, and *Twist1*, but not of *Snail2* or *Zeb2*, in the *Gli1* KD melanoma cells. It is reported that SNAIL1 and TWIST1 cooperatively control *Zeb1* expression during EMT in epithelial cells. Taken together with the results of my ChIP and Luc reporter assays, it is conceivable that GLI1 directly regulates the transcriptional expression of a subset of EMT-TFs, including *Snail1* and *Twist1*, as in non-melanoma cancer cells, to maintain the invasive activity of melanoma cells through MITF-independent mechanisms. Further studies are needed to clarify this issue.

An increased expression of *Gli1* has been observed in BRAFi-resistant melanoma cells and patient samples, as well as during melanoma progression. Taken together with my present results, GLI1 may play a role in generating a high level of intratumor heterogeneity in melanoma. Targeting GLI1 may therefore be an effective approach for melanoma therapy. Indeed, accumulating evidence suggests that GLI antagonists, of which GANT61 has been most extensively studied *in vitro* and in animal models, are promising therapeutic candidates for a wide range of cancers, including melanoma.

平成２９年８月３日

学位論文審査報告書（甲）

１．学位論文題目（外国語の場合は和訳を付けること。）

Functional Analysis of glioma-associated homolog 1 in maintaining invasive and mesenchymal-like properties of melanoma cells

（メラノーマ細胞の浸潤能の維持及び間葉系様細胞としての特性維持における GLI1 の機能解析）

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３．審査結果の要旨（600～650 字）

メラノーマは皮膚がんの中で悪性度が最も高い悪性腫瘍である。その高悪性度は、これまでの多くの研究から、メラノーマ細胞がもつ可塑性、すなわち浸潤性から非浸潤性への変換（あるいは、その逆の変換）が可逆的であることに起因すると強く示唆されている。また転写因子 MITF は、メラノーマ細胞の可塑性を担う中心的な分子であり、いわゆる MITF モデルも提唱されている。一方、転写因子 GLI1 は、古典的および非古典的ソニックヘッジホッグ経路の構成要素の１つで、メラノーマの進展制御に関わることが知られている。しかし、その詳細な分子機構については不意な点が多い。そこで研究では、先ず、GLI1 がメラノーマ細胞の浸潤に関与するかを検討した。その結果、GLI1 mRNA に対する shRNA（shGLI1）を発現する GLI1 ノックダウン（KD）メラノーマ細胞の浸潤能が低下することを見出した。一方で、GLI1 KD メラノーマ細胞における MITF の発現レベルや活性において、shGLI1 の影響は認められなかった。次に、GLI1 KD 細胞をマウス尾静脈に投与して、肺への転移を調べ、GLI1 KD 細胞では転移能が有意に低下することを明らかにした。さらに、GLI1 KD 細胞では間葉上皮移行に類似した細胞転換が促進され、それに伴って上皮間葉移行を担う転写因子 Snail1, Zeb1 および Twist1 の発現が低下することを見出した。以上の結果から、GLI1 はメラノーマ細胞の浸潤能の維持および間葉系様細胞としての特性維持に関わる重要な因子であることが示唆された。

本論文は、メラノーマ細胞の可塑性における GLI1 の役割とその分子機構の一端を明らかにした労作であり、学位に値すると評価された。

４．審査結果　(1) 判　　定（いずれかに○印）　（合　格）・　不合格

(2) 授与学位　　博　士（　理学　）