Activation of the Hedgehog and Wnt/β-catenin Signaling Pathways in the Immunohistochemistry of Basal Cell Carcinoma

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Abstract

Immunohistochemical studies have revealed that the tumor of a patient with basal cell carcinoma exhibited activation of the Hedgehog and Wnt/β-catenin signaling pathways. The glioma-associated oncogene1 overexpression leads to transcriptional activation making it an attractive molecular target in anticancer therapies due to the main downstream effectors of the cascade.

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Running head:

Overexpression of Glioma-associated oncogene 1 and coding region determinant binding protein

Abstract

Immunohistochemical studies have revealed that the tumor of a patient with basal cell carcinoma exhibited activation of the Hedgehog and Wnt/β-catenin signaling pathways. The *glioma-associated oncogene1* overexpression leads to transcriptional activation making it an attractive molecular target in anticancer therapies due to the main downstream effectors of the cascade.

Keywords

smoothened protein, glioma-associated oncogene1, coding region determinant binding protein, basal cell carcinoma, hedgehog signaling pathway.

Key Clinical Message

The pathogenesis of basal cell carcinoma is very complex. The positive expressions of the smoothened protein, glioma-associated oncogene1, coding region determinant binding protein, p53, and $p16^{INK4a}$ suggest numerous unknown interactions between multiple signaling pathways.

1 INTRODUCTION

Basal cell carcinoma (BCC) is the most common human cancer, characterized

by aberrant activation of the hedgehog (Hh) signaling pathway resulting from the mutations in the *patched 1 (PTCH1)* or *smoothened (SMO)* genes.[1] Human sporadic BCCs consistently express glioma-associated oncogene 1 (GLI1) which acts as a target and mediator of the Sonic hedgehog (SHH) signaling. Any mutations leading to the expression of GLI1 in basal cells are predicted to induce BCC formation.[2] The Wnt/beta-catenin (Wnt) signaling pathway stimulates the transcriptional output of Hh signaling. The Wnt signaling induces the expression of an RNA-coding region determinant binding protein (CRD-BP) or

insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) which in turn binds and stabilizes *GLI1* mRNA, causing an elevation of GLI1 expression and transcriptional activity.[3, 4] Presently, the Food and Drug Administration (FDA)-approved Hh pathway inhibitors include vismodegib and sonidegib as SMO inhibitors.[5] In the present study, we analyzed the expression patterns of GLI1, SMO, and IGF2BP1, a target molecule of the Hh signaling pathway and Wnt signaling pathway, respectively in a tumor from a patient with sporadic BCC and adjacent tissue using immunohistochemistry (IHC).

2 CASE PRESENTATION

2.1. Case history and examination

An 80-year-old man presented with a 5.0 × 4.0 mm, waxy black spot detected over 1 year ago on the left cheek. The limbus was slightly irregular, the concavity and convexity in the surface were recognized. The Dermoscopy (DZ-100; Yamagata Casio Co., Ltd., Higashine City, Japan) showed the presence of leaf-like structures and multiple blue-gray globules. There were few findings suggestive of melanocytic and vasculature lesions via dermoscopy (Figure 1, A). On histopathological examinations using hematoxylin and eosin(H&E)staining an elevated lesion with some ulceration and crusting was observed in the dermis, hair blast-like cells with densely-stained nuclei, and few cytoplasmic bodies increased in a nodular or cord-like pattern. There was a palisade arrangement of the nuclei at the margin of these tumors. The mucin deposition was evident around the tumor by Alcian blue staining. Some of the intradermal tumors showed continuity with the epidermis (Figure 2, A–B).

2.2. Differential diagnosis, investigation, and treatment

A clinical diagnosis of BCC was made, and surgical resection with flap creation of the affected tissue was performed at a 4 mm margin of normal (Figure 1, B). Written informed consent was obtained from the patient for the use of the tissue samples in this study. The final pathology was diagnosis as BCC, Solid type. IHC was performed with the monoclonal mouse anti-human Ki-67 antibody (IR626; Dako Denmark A/S), monoclonal mouse anti-human p53 protein (M705-4713; Dako Denmark A/S), CINtec p16 antibody (705-4713; Ventana Medical System Inc.), anti-Gli1 antibody (ab217326; Abcam plc.), SMO polyclonal antibody (20787-1-AP; ProteinTech), and IGF2BP1 Polyclonal antibody(22803-1-AP; ProteinTech). The staining evaluation followed the standard scores of the World Health Organization: <20% (nucleus) or <25% (cytoplasm) stained cells indicated negative/low expression; 20–50% (nucleus) or 25–50% (cytoplasm) indicated moderate expression; and >50% (nucleus, cytoplasm) indicated high expression. Staining for Ki-67, p53, and p16 show moderate nuclear expression in the tumor tissue and negative nuclear expression in the adjacent normal skin tissues. None of the proteins demonstrated cytoplasmic expression. The Ki-67 and p53 revealed low nuclear expression in only the basal cell in the adjacent normal tissues (Figure 3, A–B). The GLi-1 expression was high both in the nucleus as well as cytoplasm of the tumor tissue. In the adjacent normal skin tissues, there were the high nucleus and low cytoplasmic expression. The expression of SMO was high both in the nucleus and cytoplasm in the tumor tissues. In the adjacent normal skin tissue, there was high nuclear and low cytoplasmic expression. The IGF2BP1 (CRD-BP) protein shows high expression in both the nucleus and cytoplasm of the tumor tissues. In the adjacent normal skin tissue, there were moderate nuclear and cytoplasmic expressions (Figure 4, A–C).

2.3. Outcome and follow-up

The tissues were finally diagnosed Histopathologically as BCC, which was completely and curatively resected. The postoperative course was encouraging showing no evidence of a local recurrence. There were no findings of recurrence, 6 months after the operation, and careful postoperative follow-up would be planned in the future. To avoid recurrence in the future, local therapy without invasion such as palliative surgery has been planned.

3 DISCUSSION

BCC is the most common human cancer worldwide and is a subtype of non-melanoma skin cancer, characterized by a constantly increasing incidence due to an aging population and widespread exposure to the

sun. Although the mortality from BCC is negligible, this tumor can be associated with significant morbidity and expenditure.[6] BCC displays a different behavior, compared with other neoplasms, has a slow evolution, and metastasizes very rarely, but sometimes it causes important local destruction. Chronic ultraviolet exposure along with genetic factors are the most important risk factors for the development of BCC.[7]The whole-exome sequencing to characterize the mutational landscape of sporadic BCCs identified only *PTCH1 (Patched 1)* as having a significant functional mutation burden. These findings support the central role of *PTCH1* mutations in BCC genesis.[8] Mutations in the *PTCH1* gene are associated with Gorlin syndrome, an autosomal dominant disorder characterized by the occurrence of multiple BCCs, but are also the most frequent mutations observed in sporadic BCCs. *PTCH1* encodes for the PTCH1 protein, the most important negative regulator of the Hh pathway. Numerous studies are confirming the involvement of the Hh pathway in BCC pathogenesis. Although the Hh pathway has been intensively investigated, it remains incompletely elucidated. Recent studies on BCC tumorigenesis have shown that in addition to the Hh pathway, there are other signaling pathways involved in BCC development. The pathogenesis of BCC is very complex. *PTCH1* mutations play a crucial role in activating the Hh pathway; however, additional mutations that promote BCC carcinogenesis have been identified. Recent studies have shown that there is a significant cross-talk between Hh signaling pathway and other signaling pathways, including Wnt, Notch, EGFR, p53, PI3K/mTOR, and vitamin D. A further argument for the involvement of other pathways in the development of BCC could be the tumor resistance to the Hh inhibitors.[7] Activation of the Hh signaling pathway appears to be a key driver of BCC development. Studies involving mouse models have provided evidence that activation of the glioma-associated oncogene (GLI) family of transcription factors is a key step in the initiation of the tumorigenic program leading to BCC. Activation of the Wnt pathway is also observed in BCCs. In addition, the Wnt signaling pathway is required in the Hh pathway-driven development of BCC in a mouse model. Cross-talks between Wnt and Hh pathways have been observed at different levels, yet the mechanisms of these cross-talks are not fully understood. Recent studies have identified IGF2BP1 also known as IMPI1, CRD-BP, and ZBP1, a direct target of the Wnt signaling, as the factor that binds to *GLI1* mRNA and upregulates its levels and activities. This mode of regulation of GLI1 appears important in BCC tumorigenesis and could be explored in the treatment of BCCs.[3] A novel mechanism has been previously identified by which the Wnt signaling regulates the transcriptional outcome of the Hh signaling pathway. It was demonstrated that CRD-BP, a direct target of the Wnt signaling, binds to *GLI1*mRNA, stabilizes it, and consequently upregulates its levels (mRNA and protein) and activities. It was hypothesized that Wnt-induced and CRD-BP-dependent regulation of GLI1 expression and activities is important for the development of BCC. It showed that CRD-BP is over-expressed in BCC and that its expression positively correlates with the activation of both the Wnt and Hh signaling pathways. It also described the generation and characterization of a human BCC cell line utilized to demonstrate the importance of CRD-BP-dependent regulation of GLI1 expression and activities in the development of BCC.[9] The primary goal of the treatment of BCC is the complete removal of the tumor and the maximal prevention of function and cosmesis. All treatment decisions should be customized to account for the particular factors present in the individual case and the patient's preference. Primary treatment is a standard excision with 4 mm clinical margins and postoperative margin assessment and second intention, linear repair, or a skin graft. In case of negative margins, the follow-up included a complete skin examination every 6–12 months for the first 5 years, and then at least annually for life and patient education: sun protection and self-examination. Systemic therapy may be considered for locally advanced BCC and metastatic BCC. The present Food and Drug Administration (FDA)-approved Hh pathway inhibitors include vismodegib and sonidegib. Vismodegib is FDA approved for the treatment of adults with metastatic BCC or locally advanced BCC that has recurred following surgery, or who are not candidates for surgery and who are not candidates for the radiation therapy (RT). Sonidegib was FDA-approved for the treatment of adult patients with locally advanced BCC that has recurred following surgery or RT, or those who are not candidates for surgery or RT. Sonidegib was not FDA-approved for metastatic BCC. Cemiplimab-rwic was FDA-approved for patients with locally advanced or metastatic BCC previously treated with a Hh pathway inhibitor (HhI) or for whom an HhI is not appropriate.[5] It has been shown that *p53* is overexpressed in BCC samples and suggested that*p53* mutations following chronic UV exposure might be an important factor in BCC development. [10] One of the mechanisms by which the Hh pathway activation induces tumorigenesis by

evasion of p53-mediated tumor-suppressive activity and G2/M cell cycle checkpoints.[11] The role of *p53* mutations in BCC pathogenesis shows an increased expression of some major proteins involved in p53-mediated cellular signaling associated with the downregulation of MDM-2 in an early stage of DNA damage.[12] Aberrant Hh signaling activates p53 via Arf. The stress-induced by oncogenes results in Arf activation which induces an increased p53 expression.[13] BCC patients have cell cycle abnormalities of different parts of the signaling pathway. The retinoblastoma regulatory pathway is important in cell cycle arrest. In this pathway, $p16^{INK4a}$ (p16), an inhibitor of the Rb pathway, binds to CDK4 and CDK6 competitively with cyclin D1 to prevent phosphorylation of tumor suppressor, *pRB* gene. This study analyzed mRNA expression using *in situ* RT-PCR and the role of immunohistochemical expression of p16 in BCC, nuclear and cytoplasmic staining intensity of samples within tumor cells and normal skin tissue illustrates different mRNA and protein expression of the *p16*gene. The mRNA of the *p16* gene and the expressed protein induces cell cycle proliferation and involves both tumor tissues as well as normal skin tissues. *P16* gene is involved in the pathogenesis of human skin BCC given increased *p16* mRNA and expressed protein within tumor cells. In BCC mutation of *p16* connections to the Hh-Gli pathway has not yet been elucidated.[14] A*SHH* and *Gli* genes are normally expressed in the hair follicles, and human sporadic BCCs consistently express Gli1. The expression of Gli1 in basal cells induces BCC formation. Any mutations leading to the expression of Gli1 in basal cells are predicted to induce BCC formation.[2] BCC is characterized by aberrant activation of the Hh signaling pathway resulting from mutations in the*PTCH1* or *SMO* genes. The expression profile of Hh signaling-related molecules, the mRNA and protein expression levels of six molecules including GLI1, GLI2, PTCH1, PTCH2, SHH, and SMO in BCC and various other cutaneous tumors demonstrated that BCC showed remarkably enhanced mRNA expression of all Hh molecules, except SMO compared to other skin tumors. Immunohistochemical analysis revealed that only GLI1 protein was specifically upregulated in BCC, while the other Hh-related proteins did not show any significant differences between the tumors. There was no difference in GLI1 expression between the BCC subtypes. An interaction between the Hh and Wnt

signaling pathways may be involved in the development of BCCs.[1] The Wnt and Hh signaling pathways play central roles in embryogenesis, stem cell maintenance, and tumorigenesis. The Wnt signaling pathway stimulates the transcriptional output of Hh signaling. The Wnt signaling induces the expression of an RNA-binding protein, CRD-BP, which in turn binds and stabilizes *GLI1* mRNA, causing an elevation of GLI1 expression and transcriptional activity.[4] The stabilization of glioma-associated oncogene 1, *GLI1* mRNA by coding region determinant binding protein, CRD-BP through the Wnt signaling pathway is implicated in the proliferation of colorectal cancer and basal cell carcinoma. A specific oligonucleotide is found to be effective in blocking *CRD-BP-GLI1*RNA interaction.[15] A cellular screen for small-molecule antagonists of GLI-mediated transcription, which constitutes the final step in the Hh pathway, revealed two molecules that can selectively inhibit GLI-mediated gene transactivation. Mechanistically, both the inhibitors act in the nucleus to block GLI function, and one of them interferes with GLI1 DNA-binding in living cells.[16] By computational screening approach to identify small molecules that directly bind GLI1 for potential development as inhibitors of GLI-mediated transcription, compound 1, which is an 8-hydroxyquinoline, as a high-affinity binder of GLI1 is identified. These results strongly suggest that binding of compound1 to GLI1 does not prevent GLI1/DNA binding nor disrupt the GLI1/DNA complex, but rather, it induces specific conformational changes in the overall complex that prevents proper GLI function.[17]Inhibitors targeting the Hh signal transducer SMO are widely used and display a good initial efficacy in patients suffering from BCC; however, a large number of patients relapse. Though *SMO* mutations may explain acquired therapy resistance, a growing body of evidence suggests that the non-canonical, SMO-independent activation of the Hh pathway in BCC patients can also account for this adverse effect. It is highlighted that the importance of GLI transcription factors (the main downstream effectors of the canonical and the non-canonical Hh cascade) and their putative role in the regulation of multiple oncogenic signaling pathways.[18]

4 **CONCLUSION**

By IHC, within the same BCC tissue specimen, GLI1 and SMO in Hh signaling pathway and IGF2BP1 (CRB-BP) in the Wnt signaling pathway were overexpressed. The knowledge and characterization of the BCC signaling pathways and the interactions between them could underlie the development of new therapies in

BCC. Numerous lines of evidence support that as a therapeutic strategy for cancer small-molecule antagonists of GLI1-mediated transcription constitute the final step in the Hh signaling pathway.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

TT: Collected the data and wrote the manuscript.

ETHICAL APPROVAL

Written informed consent was obtained from the patient for publication of this case report and accompanying image.

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Figure Legends

FIGURE 1 Clinical presentation. A, Nodular basal cell carcinoma on the left cheek. Dermoscopy showed the presence of leaf-like structures and multiple blue-gray globules (inset outlined by a black box). B, Postoperative clinical photograph. Surgical resection with flap creation was performed. The resected specimen with a 4 mm margin of normal tissue (inset outlined by a black box).

FIGURE 2 Histologic findings. A, Nodular tumor aggregates may be of varying sizes. Nodular basal cell carcinoma, the most common type, generally consists of large, round or oval tumor islands within the dermis, often with an epidermal attachment. The peripheral cell mass is in a palisade arrangement that resembles the basal layer of the epidermis. The connective tissue stroma surrounding the tumor islands is arranged in parallel bundles. Melanin is also present within the tumor and in the surrounding stroma. Increased mucin is present in the surrounding dermal stroma. Hematoxylin-eosin stain. Original magnification, x30; scale bar, 500 μm. Inset (black box): x 400; scale bar, 50 μm. B, Mucin deposition can be seen blue color around the tumor. Alcian blue stain. Original magnification, x30; scale bar, 500 μm.

FIGURE 3 Immunohistochemical staining of Ki-67, p53, and p16. Staining for Ki-67, p53, and p16 showed moderate nuclear expression in the tumor tissue and negative nuclear expression in the adjacent normal skin tissue. All showed no cytoplasmic expression. Ki-67 and p53 revealed low nuclear expression in only the basal cell in the adjacent normal tissue. A: Ki-67stain, B: p53 stain, and C:p16 stain. Original magnification, x30; scale bar, 500 μm.

FIGURE 4 Immunohistochemical staining of GLi-1, SMO, and CRD-BP. A, GLi-1 expression was high both in the nucleus and cytoplasm of the tumor

tissue. In the adjacent normal skin tissue, there were a high nucleus and low cytoplasmic expression. GLi-1 stain, Original magnification, x30; scale bar, 500 μm.

B, SMO expression was high both in the nucleus and cytoplasm in the tumor tissue. In the adjacent normal skin tissue high nuclear and low cytoplasmic

expression was observed. SMO stain, Original magnification, x30; scale bar, 500 μm.

C, CRD-BP (IGF2BP1) expression showed high expression in both the nucleus and cytoplasm of the tumor tissue sites. In the adjacent normal skin tissue moderate nuclear and cytoplasmic expression. IGF2BP1 stain, Original magnification, x30; scale bar, 500 μm.