Downregulation of *VANGL1* Inhibits Cellular Invasion Rather than Cell Motility in Hepatocellular Carcinoma Cells Without Stimulation

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Aims: The Wnt planar cell polarity (PCP) pathway is one of the Wnt pathways which plays a critical role in cell proliferation and fate. The VANGL1 protein is one of Wnt-PCP pathway components. It is known that Wnt-PCP pathway has major roles in cell motility but its role in hepatocellular carcinoma (HCC) progression through invasion and metastasis needs to be clarified. *Methods:* We silenced *VANGL1* gene expression in the HepG2 HCC cell line by stable transfection with a vector containing siRNA template for *VANGL1* and investigated the change in cell invasion and motility. *Results:* Transfected cells with the siRNA template showed significantly suppressed invasive capacity when compared to controls although cellular motility was only slightly affected. *Conclusion:* Our study showed a basal role for *VANGL1* with respect to the invasive capacity of HCC cells. This suggests that the Wnt-PCP pathway may play a role in progression of HCC through cellular invasion but further studies are needed to clarify its role in cell motility.

Introduction

HEPATOCELLULAR CARCINOMA (HCC) is the fifth most frequent cancer worldwide and is responsible for an estimated 600,000 deaths annually (Llovet *et al.*, 2003). There is no effective treatment for this malignancy and a number of molecular changes, including hypermethylation or hypomethylation changes, mutations, and loss of heterozygosity of some genes, were thought to be associated with its development; however, there is no specific mechanism implicated in HCC (Moeini *et al.*, 2012).

The Wnt proteins are a large family of highly conserved glycoproteins that have multiple roles in the cell fate and development of several organisms. Wnt pathways regulate different cellular functions, such as proliferation, differentiation, polarity, survival, and migration, and affect the organization of body plan, organogenesis, and tissue patterning during development (Gómez-Orte *et al.*, 2013; Rosenbluh *et al.*, 2014). The activation of Wnt signaling contributes to the etiology of several human cancers and may participate in malignant metastasis (An *et al.*, 2013; Gao *et al.*, 2014). While the Wnt/β-catenin pathway (known as canonical) plays a major role in Wnt signaling, there are also alternative (noncanonical) pathways, including the Wnt-planar cell polarity (PCP) and Wnt-Ca^{+2} pathways (Takahashi-Yanaga and Kahn, 2010). The Wnt system regulates motility and

invasion not only by the canonical pathway but also by the PCP pathway in neoplasia as well as in physiologic processes. Although alterations in the Wnt/β-catenin pathway have been reported to be involved in hepatocarcinogenesis, there are limited data related to the Wnt/PCP pathway in HCC (Ikenoue *et al.*, 2002; Pez *et al.*, 2013).

It is well known that the components of the noncanonical Wnt pathway are essential for the regulation of PCP during embryonic differentiation. The core components of this pathway are Frizzled (Fz), Van Gogh (Vang), (Strabismus [Stbm]), Flamingo (Fmi), Disheveled (Dsh), Prickle (Pk), and Diego proteins. The PCP is established by both the Fz gradient and the asymmetric distribution of core PCP proteins in the surface of cells (Rosso and Inestrosa, 2013). Convergent extension movement of embryonic cells is also mediated by the noncanonical Wnt pathway (Walck-Shannon *et al.*, 2014). Although various other signaling pathways besides the Wnt pathway have been implicated in cell motility, several data indicate that the noncanonical Wnt signaling pathway has the major role in migration during embryogenesis (Walck-Shannon and Hardin, 2014).

The *VANGL1* gene is located on human chromosome 1p13. It encodes a transmembrane protein that interacts with PCP core proteins Disheveled (Dvl), Prickle (Pk), and Frizzled (Fz), a tumor metastasis suppressor KAI1 protein, and an intestinal epithelial restitution factor ITF protein (Jenny

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et al., 2003). The role of VANGL1 in normal and malignant human cells needs to be further investigated to better understand the mechanisms of cell invasion and motility. In this study, we investigated the effects of the inhibition of *VANGL1* gene expression on cellular motility and invasion on human HCC cells by the siRNA method.

Materials and Methods

Cell lines and cell culture

Human HCC cell lines HEP-G2, HEP-3B, and SK-HEP-1, the colon adenocarcinoma cell line SW-480, and the chronic myeloid leukemia cell line K-562 were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), and 100 IU/mL penicillin (Sigma-Aldrich), and 100 μg/mL streptomycin (Sigma-Aldrich) and cultured at 37°C humid air containing 5% CO_2.

Real-time reverse transcriptase-polymerase chain reaction

Total RNA was extracted from the cell lines with the Nucleospin® RNA II kit (Macherey Nagel), and first-strand cDNA synthesis was performed with the RevertAid™ First Strand cDNA synthesis Kit (Fermentas) according to the manufacturer's protocols. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in LightCycler 2.0 (Roche) using the FastStart DNA Master SYBR Green I Kit (Roche). The PCR conditions were 95°C for 10 min followed by 28 cycles of 95°C for 10 s, 59°C for 10 s, and 72°C for 20 s. In real-time PCR, the *HPRT1* gene was used as a housekeeping gene and relative quantification of the products was carried out using the LightCycler Software 4.0. Primer sequences were as follows: for *VANGL1* (179 bp product), forward 5′-ATCACCAACGGCATGACC-3′ and reverse 5′-AGGCTGAAGTCCAAGCAC-3′ and for *HPRT1* (262 bp product), forward 5′-GTGGAGATGATCTCTCAACT-3′ and reverse 5′-ACATGATTCAAATCCCTGAAG-3′.

Construction of shRNA expression plasmid and transfection

The shRNA template for *VANGL1* gene silencing was designed using a web-based tool (https://rnaidesigner.life-technologies.com/rnaiexpress/). The sequence of the shRNA template for *VANGL1* gene was sense strand 5′-GATC CGCCACAACGAGTTGTATTA TTCAAGAGATAATAC AACTCGTTGTGGCTTA-3′ and antisense strand 5′-AG CTT AAGCCACAACGAGTTGTATTATCTCTTGAATA ATACAACTCGTTGTGGCG-3′. The oligonucleotides were annealed and inserted into p*Silencer*™ 4.1-CMV-Neo plasmid (Ambion) and maxipreparation of the plasmid was performed; then, the plasmid was controlled by the sequence analysis. To create stable clones of HEP-G2 cells carrying p*Silencer* 4.1-CMV Neo plasmid, HEP-G2 cells were transfected with the plasmid using Tfx™ 50 reagent (Promega). Selection of transfected HEP-G2 cells was performed with culturing the cells in complete DMEM supplemented with 1000 μg/mL Geneticin as a selection agent. HEP-G2 cells were incubated for 3 weeks and the cell culture medium was replaced every 48 h. At the end of the incubation period, colonies of the stably transfected cells were picked up by cloning disks (Sigma-Aldrich).

Western blot analysis

Proteins were subjected to SDS-PAGE under reducing conditions and then electrophoretically transferred to the nitrocellulose membrane. After blocking with 5% nonfat dried milk in the TBS-Tween 20 buffer at room temperature for 30 min, nitrocellulose membranes were sequentially blotted at 4°C for overnight with the anti-VANGL1 antibody (Sigma-Aldrich) and for 45 min at room temperature with horseradish peroxidase-conjugated anti-rabbit IG (Amersham). Protein bands were visualized with enhanced chemiluminescence.

Motility and invasion assays

Motility and invasion assays were performed in modified Boyden chambers with 8.0 μm pore size (BD Clontech). Motility chambers coated with the Matrigel matrix (BD Clontech) were used as invasion chambers. Cells were seeded into the upper reservoir of motility or invasion chambers and were cultured in the complete DMEM supplemented with 2% FBS for 48 h. The cells that migrated through the pores of the chambers were stained and then counted with an inverted microscope.

Statistical analyses

The GraphPad Prism 2.0 software (GraphPad Software, Inc.) was used for statistical analyses. The Mann–Whitney *U* test was performed to analyze the significance of the differences between the cells.

Results

Relative quantification of *VANGL1* gene expression in HEP-G2, HEP-3B, and SK-HEP-1 cell lines was determined by quantitative RT-PCR, while SW-480 and K-562 cell lines were used as controls. HEP-G2 cells were found to express *VANGL1* more than HEP-3B and SK-HEP-1 cells (Fig. 1) and subsequent experiments were carried out with the HEP-G2 cell line.
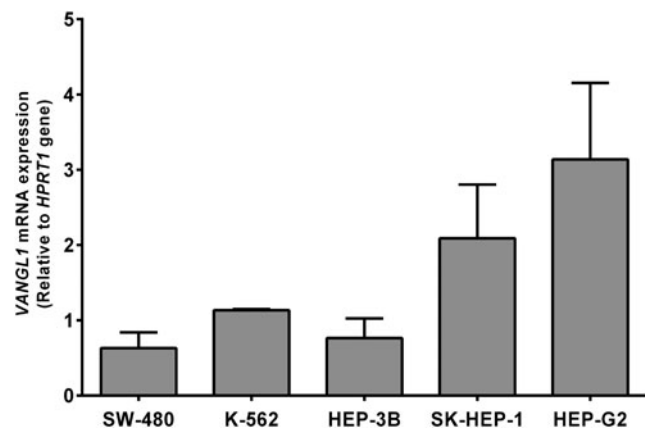
FIG. 1. Relative quantification of *VANGL1* expression with real-time polymerase chain reaction (PCR) in hepatocellular cancer cell lines.
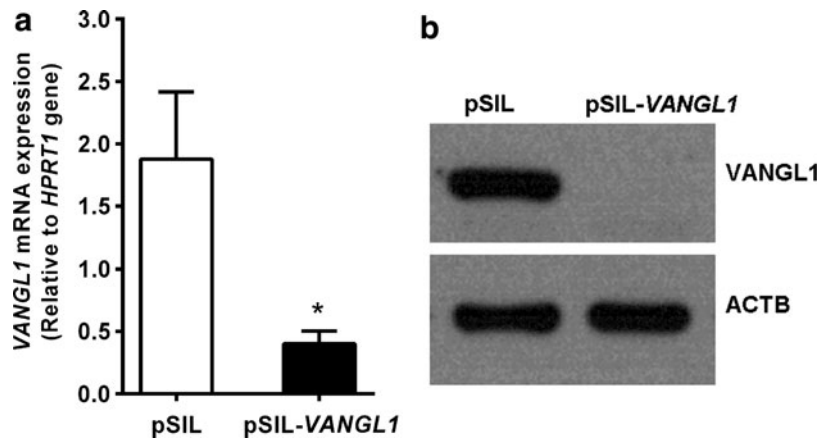
FIG. 2. **(a)** *VANGL1* expression of HEP-G2 cells transfected with pSilencer plasmid. pSIL-*VANGL1* cells carry the shRNA template for *VANGL1* and pSIL where the control cells transfected with the control pSilencer vector. Relative quantification of *VANGL1* expression with real-time PCR in transfected cells showing the degree of gene silencing ($p = 0.0009$). *Indicates the significance. **(b)** Western blot analysis of *VANGL1* expression in the cells transfected with siRNA.

To silence the *VANGL1* gene, HEP-G2 cells were transfected with the pSilencer 4.1 vector containing the template for shRNA expression. To detect the degree of gene expression at the mRNA level after shRNA transfection, relative quantification was performed by real-time PCR (Fig. 2a). Nearly 70% *VANGL1* gene silencing at the mRNA level was achieved and western blot analysis confirmed the gene silencing at the protein level (Fig. 2b).

Motility and invasion assays to assess the invasion capacity of the cells after *VANGL1* silencing were carried out with HEP-G2 cells with the silenced *VANGL1* gene (pSIL-*VANGL1*) and a control colony transfected with the control plasmid (pSIL). When compared to the control cells, the cells with the silenced *VANGL1* gene showed a more suppressed invasive capacity (Fig. 3a). Motility assay results showed that there is minimal difference between the control cells and the *VANGL1* gene-silenced HEP-G2 cells (Fig. 3b).

Discussion

The effects of *VANGL1* gene silencing on the cellular invasion and motility were investigated in this study. The shRNA application method of stable gene silencing was performed to inhibit the expression of the *VANGL1* gene in the HEP-G2 hepatocellular carcinoma cell line. Invasion assays revealed a significantly reduced invasive capacity of the cells with the silenced *VANGL1* gene, whereas motility assays showed a slight but insignificant difference compared to control cells. These results implicated that the VANGL1 protein was mostly involved in cell invasion rather than cell motility.

Transient siRNA silencing of the *VANGL1* gene in various cancer cell lines was reported to regulate cellular invasion and motility. In gastric carcinoma, colorectal cancer, and oral cancer cell lines, fibronectin-stimulated cellular invasion of gelatin was shown to be decreased, and motility of the cells was found to be reduced with the scratch wound assay following transient siRNA silencing of the *VANGL1* gene (Ryu *et al.*, 2010; Lee *et al.*, 2011; Yoon *et al.*, 2013). Although our invasion assay results were similar, our motility assay results were not similar to these reports and there were differences in the assay methods used to analyze cellular motility and invasion. In our assays, invasion was measured with Matrigel matrix-coated transwell chambers and there was no stimulating agent to induce cell invasion. While the Matrigel matrix invasion assay has a superior capacity to analyze cellular invasion since it contains numerous molecules, including laminin, collagen IV, enactin, and fibronectin, only the gelatin matrix has collagen molecules (Kleinman and Martin, 2005). Associated with the multimolecular composition of
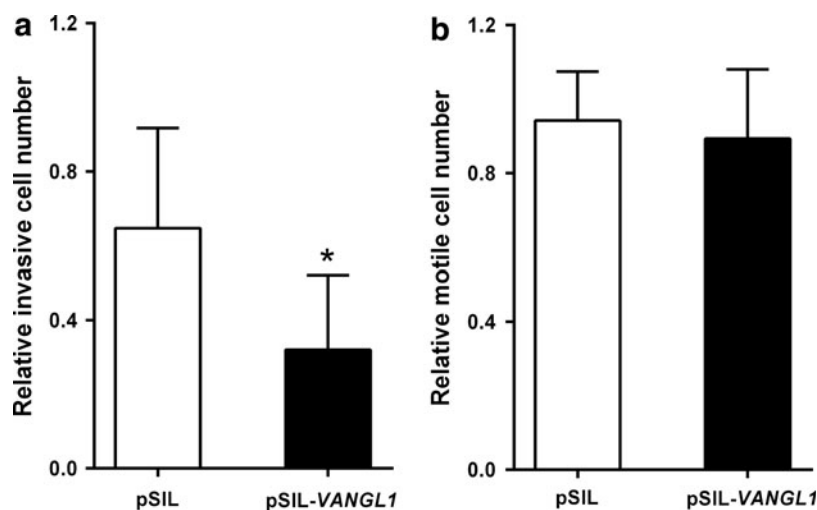
FIG. 3. The change in the number of **(a)** invasive ($p = 0.03$) and **(b)** motile HEP-G2 cells after transfection with *VANGL1* siRNA. *Indicates the significance. pSIL-*VANGL1* cells carry the shRNA template for *VANGL1* and pSIL where the control cells transfected with the control pSilencer vector.

the Matrigel matrix, cellular response to invade the Matrigel matrix is more complex than the gelatin matrix (Brinckerhoff *et al.*, 2000). Therefore, we preferred to use the Matrigel matrix assay method to analyze basal invasion capacity of cells without stimulation.

In the previous studies, motility assays were performed with the scratch wound assay, but in our experiments, transwell chambers were used to investigate cell motility (Ryu *et al.*, 2010; Lee *et al.*, 2011; Yoon *et al.*, 2013). Since the cytoskeletal movement of the cell has a similar pattern in both transwell migration and invasion experiments, we preferred to use the transwell migration assay instead of the wound closure assay (Albini *et al.*, 1987; Mierke, 2013). In one study, the effect of *VANGL1* silencing on cellular migration was analyzed with the transwell assay as in our experiments, and their results showed that motility of the breast cancer cells reduced with *VANGL1* silencing (Anastas *et al.*, 2012). Our findings are not concordant with this report, and we suppose that *VANGL1* silencing may be resulting with a different effect on cellular motility depending on the cell type. In addition, the influence of the VANGL1 protein on cellular motility might be more pronounced under specific conditions, which are stimulating the cellular motility response. It has been shown that *VANGL1* silencing reduced the ITF-stimulated migration of intestinal epithelial cells, but did not affect the basal motility of these cells (Kalabis *et al.*, 2006). Moreover, the discrepancy of our motility assay results with the previous studies may be related with the siRNA silencing method whether transient or stable.

The mechanism of the inhibition of cell invasion with transient *VANGL1* silencing was reported to be associated with the regulation of the extracellular matrix. Results showed that the silencing of the *VANGL1* gene inhibits the transcription of metalloproteinase enzymes, which are degrading the extracellular matrix proteins with their proteolytic enzyme activity. The association of Vangl1 protein with cell invasion and motility might also be related by its effect on the cell adhesion. A number of molecules, which are located downstream of the VANGL1 on the Wnt-PCP pathway, have a role in the regulation of both cell to cell and cell to extracellular matrix adhesion. Particularly, the activity of small GTPases, like Rac and RhoA proteins, contributes to the inactivation of cadherin-mediated cell to cell adhesion in human cancer (Evers *et al.*, 2000; Fort and Théveneau, 2014). Moreover, Rac and RhoA proteins are involved in cell motility and the invasion processes with their role in the formation of lamellipodia, membrane ruffles, focal contacts, and cytoskeletal changes (Hall, 1998). Both the loss of intercellular adhesion and the stimulation of migration are well-known characteristic features of invasive cancers. Recently, studies with breast cancer and HCC tissues showed that cancer cells have a higher expression level of the *VANGL1* gene compared to normal cells (Cho *et al.*, 2011; Anastas *et al.*, 2012). Furthermore, both in colon adenocarcinomas and gastric tumors, metastatic cancer tissues were found to have an elevated VANGL1 protein expression (Kho *et al.*, 2009; Ryu *et al.*, 2010). These data are implying that the increased levels of the VANGL1 protein might be associated with the invasion and metastatic progression of cancer cells.

Taken together, we propose that VANGL1 is associated to the natural invasion capacity of HCC cells rather than motility, and further studies need to be performed to understand both basal and stimulated cellular invasion related with the VANGL1 protein in distinct types of cancer.

Acknowledgments

Author Disclosure Statement

No competing financial interests exist.

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