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GLI1 and *CTNNB1* Knockdown Activates NOTCH and mTOR Signalling in NB4 Myeloid Leukaemia Cells

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Abstract. *Background: Hedgehog (HH), WNT, NOTCH, and mechanistic target of rapamycin (mTOR) signalling pathways are known to regulate the progression of cancer; however, their interaction in leukaemia cells is not fully clarified. Materials and Methods: Myeloid and T-lymphoblastic leukaemia cell lines (NB4, THP-1, Jurkat, and DND-41) were transfected with small interfering RNAs targeting the glioma-associated oncogene homolog 1 (GLI1) and catenin beta-1 (CTNNB1) genes involved in the regulation of HH and WNT pathways, respectively, and we examined cell proliferation and gene expression. Results: The knockdown of GLI1 and CTNNB1 did not significantly affect proliferation of any cell line; however, it up-regulated the expression of NOTCH1, cleaved NOTCH1 fragment, and phosphorylated mTOR in NB4 cells, but not in the other cell lines. Conclusion: Our data suggest that HH and WNT act upstream of NOTCH and mTOR pathways and negatively regulate them in myeloid NB4 cells. Further studies are required to determine the biological significance of this signalling crosstalk in leukaemia.*

Hedgehog (HH) and WNT signalling pathways are known to play an important role in regulating cell proliferation and differentiation (1-4). When HH proteins are bound to their receptor Patched 1, glioma-associated oncogene homolog (GLI) is translocated into the nucleus, where it regulates the transcription of target genes involved in cell growth (5-8). However, the effects of HH ligands in leukaemia cells have not been fully elucidated.

WNT proteins activate the canonical WNT signalling cascade by inducing nuclear translocation of β-catenin and regulating gene expression. WNT proteins also activate the non-canonical WNT signalling cascade, which is independent of nuclear translocation of β-catenin (9, 10).

Previously, we reported that HH inhibitor cyclopamine and WNT inhibitor quercetin suppressed the growth of leukaemia cells (11, 12); however, these compounds have off-target effects. We also showed that small interfering (si)RNA-mediated knockdown of *NOTCH1* and *NOTCH2* suppressed the growth of T-cell acute lymphoblastic leukaemia (T-ALL) cell lines (13), suggesting that specific inhibition of HH and WNT pathways with siRNAs might be an effective approach to investigating the role of HH and WNT signalling in leukaemia cells.

The interplay among HH, WNT, NOTCH, and mechanistic target of rapamycin (mTOR) pathways is known to regulate cell stemness (14); however, their relationship in leukaemia should be clarified. To address this question, in this study, the expression of *GLI1* and catenin beta-1 (*CTNNB1*) gene, which mediate HH and WNT signalling, respectively, were knocked-down in acute myeloid leukaemia (AML) and T-ALL cell lines and the effects on cell proliferation and NOTCH and mTOR pathways were assessed.

Materials and Methods

Cell culture and siRNA transfection. Two AML cell lines (NB4 and THP-1) and two T-ALL cell lines (Jurkat and DND-41) were used. NB4 derived from a patient with acute promyelocytic leukaemia was kindly provided by Dr. M. Lanotte (France), THP-1 derived from a patients with acute monocytic leukaemia was obtained from the Japanese Cancer Research Resources Bank, and Jurkat and DND-41 cells were donated by Drs. Harashima and Orita (Fujisaki Cell Centre, Japan). All cells were cultured in RPMI-1640 supplemented with 10% foetal calf serum (FCS).

Three sets of pre-designed siRNAs targeting *GLI1* (siGL: HSS104170, HSS178441, and HSS178442), *CTNNB1* (siCT: HSS102460, HSS102461, and HSS102462), and *NOTCH1* (siN1: HSS107248, HSS107249, and HSS181550) were purchased from Life Technologies (Carlsbad, CA, USA). Stealth RNAi negative control Duplex was used as a control siRNA (siCont). Cells (7×10^5 per 10-μl tip) were transfected with 40 nM of each siRNA using the Neon™ Transfection system (Life Technologies) according to the manufacturer's instructions, and immediately transferred to cell culture medium.

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Quantitative reverse transcription-polymerase chain reaction (RT-PCR). Cells transfected with siRNAs were cultured for 24 h and mRNA expression of *GLI1, CTNNB1*, and *NOTCH1* was measured by quantitative RT-PCR using the FastStart DNA Master SYBR Green I kit with LightCycler primer sets (Roche Diagnostics, Mannheim, Germany). Expression of the target genes was normalized to that of the β-actin-encoding gene (*ACTB*) and presented as the mean percentage of expression in siCont-transfected cells.

Cell growth assay. Short-term cell growth was examined using a colorimetric WST-8 assay (Dojindo Laboratories, Kumamoto, Japan). Cells transfected with siRNAs were plated in 0.1 ml of 10% FCS-supplemented RPMI-1640 in 96-well culture plates (5×10^4 cells/well) and cultured for 3 days. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] was then added to the wells and optical density (OD) was measured in an enzyme-linked immunosorbent assay plate reader. Cell growth was expressed as the mean percentage of the OD value for siCont-transfected cells.

Immunoblotting. Cells transfected with siRNAs were harvested 2 days later and lysed. Cell lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with antibodies against GLI1, phospho-β-catenin (Ser675), β-catenin, NOTCH1, cleaved NOTCH1 (Val1744), MYC, and phospho-mTOR (Ser2481) (Cell Signaling Technology, Danvers, MA, USA); anti-α-tubulin antibody (Abcam, Cambridge, MA, USA) was used to ensure equal loading. Three independent experiments were performed to verify reproducibility. Qualitative assessment of the immunoblot assay was performed by comparing the density of bands from the target siRNA-transfected cells and control siRNA-transfected cells.

Statistical analysis. The data are expressed as the mean±standard deviation (SD). Student's *t*-test was used to determine statistical significance of the differences between siCont- and target siRNA-treated cells; values of $p<0.05$ was considered significant.

Results

Effects of siRNAs on GLI1, CTNNB1, and NOTCH1 expression. The most potent among siGL siRNAs were HSS104170 for T-ALL and HSS178441 for AML cell lines; among siCT siRNAs, HSS102460 for all cell lines, and among siN1 siRNAs, HSS107248 for T-ALL and HSS107249 for AML cell lines. The knockdown effects of these siGL, siCT, and siN1 siRNAs on the expression of *GLI1, CTNNB1*, and *NOTCH1* mRNA are shown in Table I. Transfection with siGL, siCT, and siN1 selectively suppressed the mRNA expression of *GLI1, CTNNB1*, and *NOTCH1*, respectively.

Effect of GLI1 and CTNNB1 knockdown on short-term cell growth. The results indicated that the knockdown of *GLI1* and *CTNNB1* genes had no significant effects on short-term cell proliferation (Figure 1).

Effects of GLI1, CTNNB1, and NOTCH1 knockdown on the expression of signalling proteins. Figure 2 shows the results of protein expression analysis for NB4 and Jurkat chosen as representative cell lines. Transfection with siN1, siGL, and siCT suppressed the expression of NOTCH1, GLI1, and β-catenin proteins in NB4 and Jurkat cells. Interestingly, *GLI1* and *CTNNB1* knockdown in NB4 cells increased the expression levels of NOTCH1, cleaved NOTCH1 fragment (active form of NOTCH1), and phosphorylated mTOR, whereas NOTCH1 knockdown slightly increased phosphorylated mTOR (Figure 2, left panel). However, no such effects on protein expression were observed in transfected Jurkat cells (Figure 2, right panel) nor in DND-41 and THP-1 cells (data not shown). The combined transfection with siGL and siCT siRNAs did not increase the up-regulatory effects on protein expression in NB4 cells (Figure 2, left panel).

Table I. *Efficiency of glioma-associated oncogene homolog 1 (GLI1), catenin beta-1 (CTNNB1), and NOTCH1 knockdown by specific siRNAs. The numbers in siCont columns indicate the ratio obtained by dividing the expression level of each mRNA by that of β-actin mRNA in control siRNA (siCont)-transfected cells. Effects of specific siRNAs on gene expression are presented as the mean percentage of that in siCont-transfected cells.*

	GLI1		*CTNNB1*		*NOTCH1*	
Cell line	siCont	siGL	siCont	siCT	siCont	siN1
NB4	3.01.E-03	24.2%	5.71.E-02	27.5%	4.59.E-01	24.6%
THP-1	4.60.E-01	48.0%	5.56.E-01	19.8%	6.32.E-02	9.3%
Jurkat	1.12.E-02	51.7%	3.11.E-02	30.8%	1.70.E-01	30.5%
DND-41	1.20.E-02	17.0%	1.88.E-02	26.9%	8.59.E-02	47.6%

Discussion

The results of our siRNA-mediated knockdown experiments indicate that HH and canonical WNT signalling did not significantly affect proliferation of the four leukaemia cell lines. Interestingly, we found that *GLI1* and *CTNNB1* knockdown up-regulated the expression of NOTCH1, cleaved NOTCH1 fragment, and phosphorylated mTOR in NB4 cells. These findings indicate that inhibition of WNT and HH signalling activates NOTCH and mTOR pathways in some AML cells, suggesting a possibility that WNT and HH function upstream of NOTCH and mTOR pathways (Figure 3). The knockdown of *NOTCH1* in NB4 cells did not inhibit mTOR phosphorylation, suggesting that mTOR signalling is not located downstream of NOTCH1. However, these effects were not observed in the other cell lines.

Previous studies investigating the relationship among HH, WNT, NOTCH, and mTOR pathways found that NOTCH receptor activation induced HH and mTOR signalling (15) and that the NOTCH pathway positively regulated mTOR phosphorylation (16), whereas mTOR directly activated GLI1

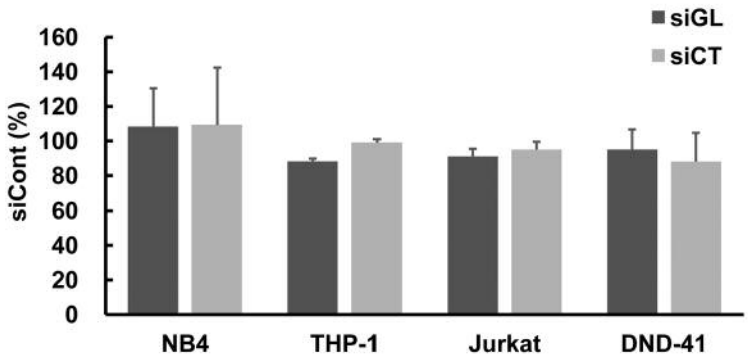

Figure 1. *Effect of glioma-associated oncogene homolog 1 (GLI1) and catenin beta-1 (CTNNB1) knockdown on short-term cell growth. Leukaemia cells were transfected with siRNAs targeting GLI1 (siGL) or CTNNB1 (siCT) and analyzed for proliferation after 3 days using a colorimetric assay. Cell growth is presented as the percentage of the mean OD value normalized to that of control siRNA (siCont)-transfected cells.*

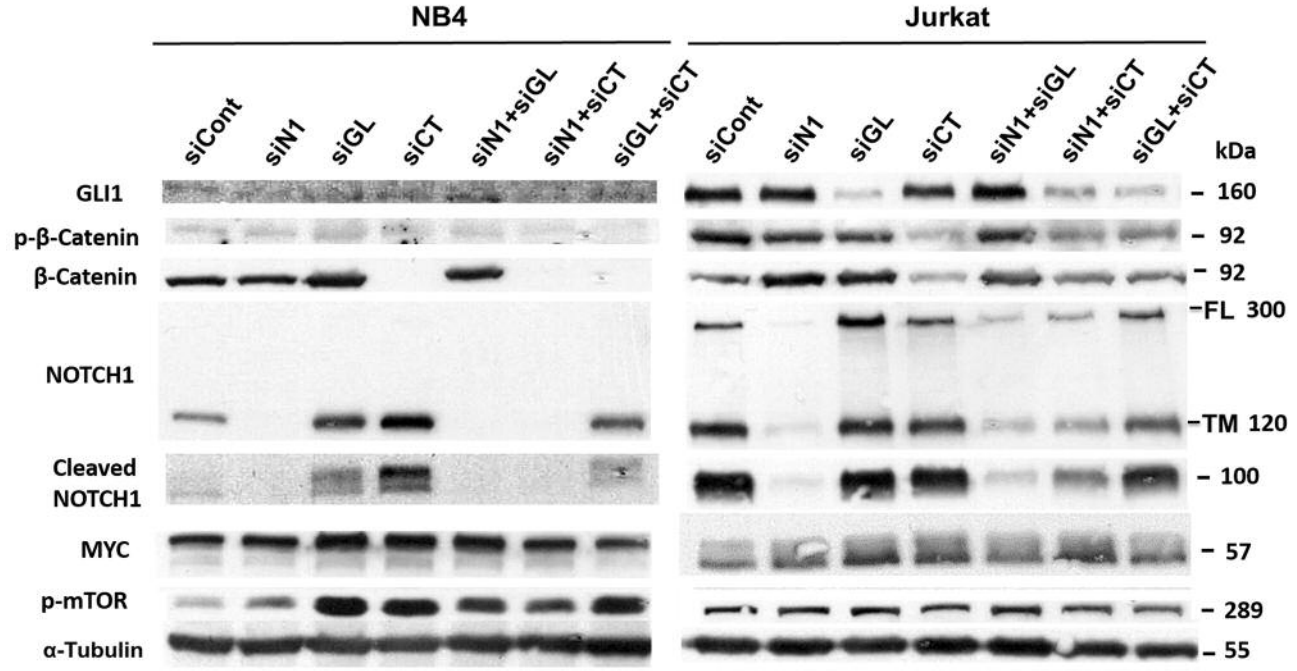

Figure 2. *Effects of glioma-associated oncogene homolog 1 (GLI1), catenin beta-1 (CTNNB1), and NOTCH1 silencing on the expression of signalling proteins. Cells were transfected with the indicated siRNAs or their combinations for 24 h and analyzed for protein expression by immunoblotting. FL: Full-length NOTCH1; p-: phosphorylated; TM: transmembrane subunit of NOTCH1.*

and smoothened (SMO), effectors of the Sonic HH pathway, in oesophageal adenocarcinoma (17). Furthermore, WNT3A induced the expression of GLI1 and GLI3 and Sonic HH transcription in patients with chronic myeloid leukaemia (18). These studies suggest that NOTCH regulates mTOR and HH signalling and acts upstream of these pathways.

Thus, our study revealed a novel mode of interaction among the four signalling cascades; however, it had limitations. Firstly, the novel relationship was observed only in one out of the four cell lines investigated; secondly, gene silencing by siRNAs might be too weak to be translated to the cellular level; and thirdly, the underlying molecular mechanisms and significance of the observed effect have not been elucidated. Further experiments are needed to clarify the functional role of the proposed crosstalk among HH, WNT, NOTCH, and mTOR pathways in leukaemia, which

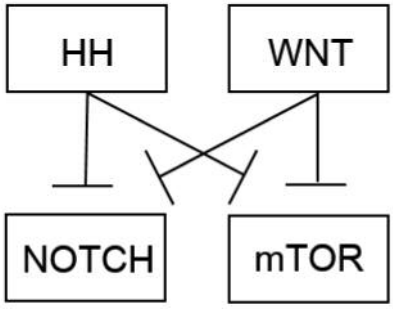

Figure 3. *Schematic representation of a possible crosstalk among Hedgehog (HH), WNT, NOTCH and mechanistic target of rapamycin (mTOR) signalling pathways in NB4 cells.*

may promote the development of HH- and WNT-targeting therapeutic approaches.

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