GLI1 Transcription Factor Affects Tumor Aggressiveness in Patients With Papillary Thyroid Cancers

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Abstract: A significant proportion of patients with papillary thyroid cancer (PTC) present with extrathyroidal extension (ETE) and lymph node metastasis (LNM). However, the molecular mechanism of tumor invasiveness in PTC remains to be elucidated. The aim of this study is to understand the role of Hedgehog (Hh) signaling in tumor aggressiveness in patients with PTC. Subjects were patients who underwent thyroidectomy from 2012 to 2013 in a single institution. Frozen or paraffin-embedded tumor tissues with contralateral-matched normal thyroid tissues were collected. Hh signaling activity was analyzed by quantitative RT-PCR (qRT-PCR) and immunohistochemical (IHC) staining. Datasets from Gene Expression Omnibus (GEO) (National Center for Biotechnology Information) were subjected to Gene Set Enrichment Analysis (GSEA). BRAFV1799A and telomerase reverse transcriptase promoter mutation C228T were analyzed by direct sequencing. Among 137 patients with PTC, glioma-associated oncogene homolog 1 (GLI1) group III (patients in whom the ratio of GLI1 messenger ribonucleic acid (mRNA) level in tumor tissue to GLI1 mRNA level in matched normal tissue was in the upper third of the subject population) had elevated risk for ETE (odds ratio [OR] 4.381, 95% confidence interval [CI] 1.414–13.569, \( P = 0.031 \)) and LNM (OR 5.627, 95% CI 1.674–18.913, \( P = 0.005 \)). Glioma-associated oncogene homolog 2 (GLI2) group III also had elevated risk for ETE (OR 4.152, 95% CI 1.292–13.342, \( P = 0.017 \)) and LNM (OR 3.924, 95% CI 1.097–14.042, \( P = 0.036 \)). GSEA suggested that higher GLI1 expression is associated with expression of the KEGG gene set related to axon guidance (\( P = 0.031, \text{false discovery rate } < 0.05 \)), as verified by qRT-PCR and IHC staining in our subjects.

Abbreviations: ABLIM3 = actin binding LIM protein family, member 3, ATC = anaplastic thyroid carcinoma, BCC = basal cell carcinoma, BRAF = v-raf murine sarcoma viral oncogene homolog B, CDH1 = E-cadherin, CLND = central lymph node dissection, DHH = Desert Hedgehog, EMT = epithelial-mesenchymal transition, EPHA8 = EPH receptor A8, ETE = extrathyroidal extension, E5 = feline sarcoma oncogene, FIA = follicular adenoma, GLI1 = glioma-associated oncogene homolog 1, GLI2 = glioma-associated oncogene homolog 2, GSEA = Gene Set Enrichment Analysis, HHIP = Hedgehog-interacting protein, IHC = immunohistochemical, IHH = Indian Hedgehog, LICAM = L1 cell-adhesion molecule, LNM = lymph node metastasis, MB = medulloblastoma, NEAT1-4 = nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4, PLXNB3 = plexin B3, PTC = papillary thyroid cancer, PTCH = patched, ROBO3 = roundabout, axon guidance receptor, homolog 3, SEMA3D = sema domain, short basic domain, secreted, (semaphorin) 3D, SEMA6A = sema domain, cytoplasmic domain, (semaphorin) 6A, SHH = Sonic Hedgehog, TERT = telomerase reverse transcriptase, TGFβ = transforming growth factor-β, VIM = vimentin.

INTRODUCTION

Papillary thyroid cancer (PTC) is generally associated with favorable outcomes. However, lymph node metastasis (LNM) occurs in 20% to 90% of patients with PTC.1–3 LNM is commonly associated with locoregional recurrence of PTC.4,5 Therapeutic central lymph node dissection (CLND) in patients with clinically diagnosed metastatic lymph nodes has been accepted as a standard therapy. However, prophylactic CLND in patients with no clinically detectable metastatic lymph nodes has remained controversial because of the limited therapeutic benefit and the risk of surgical complications.6–8 Previous reports have attempted to resolve this controversy by proposing clinical or molecular markers that might predict LNM and locoregional recurrence, such as large tumor size, extrathyroidal extension (ETE), BRAFV600E mutation, and telomerase reverse transcriptase (TERT) promoter mutations.8–10

The Hedgehog (Hh) signaling pathway is an evolutionarily conserved system that is essential for patterning and organogenesis during embryonic development.11–14 The central components of the mammalian Hh pathway include 3 secretory ligands, Sonic Hedgehog (SHH), Desert Hedgehog (DHH), and Indian Hedgehog (IHH); patched (PTCH), a receptor involved
in the negative regulation of the pathway; Hedgehog-interacting protein (HHIP), a negative regulation factor; and the glioma-associated oncogene homolog 1 (GLI1), glioma-associated oncogene homolog 2 (GLI2), and GLI family zinc finger 3 (GLI3). Recently, aberrant activation of the Hh signaling pathway has been linked to tumorigenesis in medulloblastoma (MB) and basal cell carcinoma (BCC). In addition, hyperactive Hh signaling has been reported in pancreatic, colon, gastric, lung, breast, and prostatic cancers, as well as leukemia and multiple myeloma.

In this study, we measured the messenger ribonucleic acid (mRNA) expression levels of the central components of the Hh signaling pathway in patients with PTC. Mean mRNA levels of Hh signaling components in PTC were lower than the corresponding levels in matched normal thyroid tissues. However, patients with PTC expressing higher levels of GLI1 and GLI2 mRNA frequently presented with ETE and LNM. In addition, Gene Set Enrichment Analysis (GSEA) revealed that GLI1 expression was coordinately regulated with genes involved in axon guidance, as verified by quantitative RT-PCR (qRT-PCR) and immunohistochemical (IHC) staining.

MATERIALS AND METHODS

Subjects and Clinical Data
We enrolled 137 patients (21 men and 116 women) who underwent thyroidectomy for the management of conventional PTC between January 2012 and December 2013 at Severance Hospital, Yonsei Cancer Center, Seoul, South Korea. For a validation set, we also collected 103 patients independently (21 men and 82 women). The baseline clinicopathological characteristics of derivation and validation cohort are summarized in supplementary Table 1.

DNA Isolation and Sequencing
Genomic DNA from paraffin-embedded thyroid tissue specimens was prepared using QIAamp DNA Formalin-fixed, paraffin-embedded Tissue Kit (Qiagen, Venlo, The Netherlands). Exon 15 of the BRAF gene was amplified by Polymerase chain reaction (PCR) with the following primers: forward 5‘-ATG CTT GCT CTG ATA GGA AA-3 and reverse 5‘-AGT GGA TTC GCG GGC ACA GA-3. For TERT promoter containing the hotspots of C228T and C250T, mutations were amplified using primers: forward 5‘-AGT GTA TCT GCG GGC ACA GA-3 and reverse 5‘-CAG GCC CGC TTC GTG AAA CTA CTC-3. The purified PCR products were sequenced on an ABI PRISM 3100 automated capillary DNA Sequencer, Foster City, CA using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA).

RNA Isolation and Real-Time PCR
Total RNA was extracted from fresh frozen tissues using Trizol (Invitrogen, Carlsbad, CA), and the RNA quality was verified by using Agilent’s 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA) as shown in supplementary Figure 1. Complementary DNA (cDNA) was prepared from total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) and oligo-dT primers (Promega, Madison, WI). qRT-PCR was performed on the cDNA using the Quantitect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA). Primers used in qRT-PCR are listed in supplementary Table 2. Relative expression was calculated using the StepOne Real-Time PCR System (Applied Biosystems). qRT-PCR experiments were repeated 3 times, and each experiment was performed in triplicate.

GSEA of GLI1-Correlated Genes
Microarray data from the Gene Expression Omnibus (GEO) of National Center for Biotechnology Information (NCBI) (Data-Set Record GDS1732 and GSE33630 for PTC, GSE16515 for pancreatic cancer) were subjected to GSEA. Genes whose expression was strongly correlated to GLI1, or that were interesting for other reasons, were selected for validation by qRT-PCR using cDNA derived from our subjects. To verify our GSEA, the public data from The Cancer Genome Atlas Research Network has been subjected to statistical analyses [The Cancer Genome Atlas (TCGA), http://cancergenome.nih.gov].

IHC Analysis
Paraffin-embedded tissue sections (4 μm thick) were deparaffinized in xylene and rehydrated in a graded series of ethanol (100%–80%). Antigens were retrieved in 0.01 M citrate buffer (pH 6.0) by heating the tissue sections in an autoclave (CHS-ACCE-860, Choong Wae Medical Corporation, Korea). The tissue sections were then placed in 3% hydrogen peroxide for 5 minutes to inactivate endogenous peroxidases and then blocked for 10 minutes with normal horse serum (UltraTech HRP kit; Immunotech, Marseille, France). The primary antibodies used for this study were anti-Gli1 (sc-20687; Santa Cruz Biotechnology, Santa Cruz, CA), anti-NFATc4 (sc-32985; Santa Cruz Biotechnology, Santa Cruz, CA), anti-ABLIM3 (HPA-001376, Sigma), anti-FES (HPA-003245, Sigma, St Louis, MO), and anti-FES (HPA-001376, Sigma). Sections were incubated for 60 minutes with appropriately diluted primary antibodies, incubated with biotinylated secondary antibody for 20 minutes, and then visualized with UltraTech HRP, streptavidin peroxidase (Immunotech) and aminoethylcarbazole solution for a further 10 minutes. Finally, the tissue sections were counterstained with hematoxylin for 10 seconds. All incubations were performed at room temperature.

Statistical and Graphical Analysis
Differences between means were analyzed by Mann–Whitney U test, paired t test, independent samples t test, or 1-way Analysis of variance, as indicated in figure and table legends. Comparisons between groups were performed by χ² or linear-by-linear association. All analyses were performed using SPSS version 18.0 for Windows (SPSS Inc, Chicago, IL) or GraphPad Prism (GraphPad Software, Inc, San Diego, CA). All reported P values are 2-sided.

RESULTS

mRNA Expression of Central Components of the Hh Pathway is Reduced in PTC
Initially, we performed qRT-PCR and calculated the relative mRNA expression of each central component of the
mammalian Hh pathway, normalized to the expression of the internal control gene GAPDH. In this step, we used tumor and contralateral matched normal samples from 137 patients with PTC. As shown in Figure 1A, relative mRNA expression of GLI1 in normal thyroid tissue was 1.73 ± 0.06. In PTC, GLI1 mRNA expression in PTC was statistically significantly reduced (0.88 ± 0.04; paired t test, P < 0.001). The other central components of the Hh pathway, including GLI2, GLI3, HHIP, PTCH1, SHH, DHH, and IHH, exhibited the same expression patterns, with significantly lower levels in PTC than in matched normal tissues (Figure 1B–H). Thus, our qRT-PCR data suggested that the Hh pathway activity might be generally reduced in PTC.

Relationship Between GLI1/GLI2 and HHIP Expression in Normal Thyroid Tissues and PTC

Three of the genes analyzed, GLI1, GLI2, and HHIP, are regarded as targets of the Hh pathway.14 To verify whether the GLI1-dependent transcriptional activities of these target genes are coordinately regulated in normal thyroid tissue and PTC, we analyzed the correlation between the mRNA expression levels of HHIP and GLI1 or GLI2. In normal thyroid tissues, we found a strong positive correlation between levels of GLI1 and HHIP mRNA levels (r = 0.55, P < 0.001), indicating that the negative-feedback loop of Hh pathway is functioning normally in normal thyroid tissues via expression of HHIP (Figure 2A). However, the correlation between GLI1 and HHIP mRNAs disappeared in PTC (Figure 2B). Likewise, expression of GLI2 (Figure 2C and D) was also positively correlated with that of HHIP in normal tissues (r = 0.17, P = 0.05) but not in PTC (r = 0.12, P = 0.17). Taken together, these data suggest that GLI1, GLI2, and HHIP are coordinately regulated by a negative-feedback loop in normal thyroid tissues. By contrast, in

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| Extrathyroidal Extension | Absent (n = 68) | Present (n = 69) | P      |
|--------------------------|----------------|----------------|--------|
| Age, y, mean ± SD        | 46.1 ± 11.7    | 47.5 ± 14.9    | 0.528  |
| Sex (female)             | 58 (85.3)      | 58 (84.1)      | 0.841  |
| Tumor size, cm, mean ± SD| 2.2 ± 1.0      | 2.2 ± 0.8      | 0.840  |
| Multifocality             |                |                | 0.001  |
| Absent                   | 62 (91.2)      | 51 (73.9)      |        |
| Unilateral               | 6 (8.8)        | 4 (5.8)        |        |
| Bilateral                | 0 (0)          | 14 (20.3)      |        |
| LNM                      |                |                | 0.001  |
| Absent                   | 37 (54.4)      | 22 (31.9)      |        |
| Central                  | 30 (44.1)      | 37 (53.6)      |        |
| Lateral                  | 1 (1.5)        | 10 (14.5)      |        |
| Distant metastasis       |                |                | 0.321  |
| Absent                   | 68 (100)       | 68 (98.6)      |        |
| Present                  | 0 (0)          | 1 (1.4)        |        |
| TNM stage                |                |                | 0.001  |
| I                        | 35 (55.9)      | 28 (40.6)      |        |
| II                       | 15 (22.1)      | 1 (1.4)        |        |
| III                      | 1 (1.5)        | 7 (10.1)       |        |
| IV                       | 14 (20.6)      | 33 (47.8)      |        |
| GLI1                     |                |                | 0.002  |
| I                        | 31 (45.6)      | 15 (21.7)      |        |
| II                       | 21 (30.9)      | 25 (36.2)      |        |
| III                      | 16 (23.5)      | 29 (42.0)      |        |
| GLI2                     |                |                | 0.005  |
| I                        | 31 (45.6)      | 15 (21.7)      |        |
| II                       | 20 (29.4)      | 26 (37.7)      |        |
| III                      | 17 (25.0)      | 28 (40.6)      |        |
| GLI3                     |                |                | 0.250  |
| I                        | 26 (38.2)      | 20 (29.0)      |        |
| II                       | 22 (32.4)      | 24 (34.8)      |        |
| III                      | 20 (29.4)      | 25 (36.2)      |        |
| HHIP                     |                |                | 0.916  |
| I                        | 22 (32.4)      | 24 (34.8)      |        |
| II                       | 25 (36.8)      | 21 (30.4)      |        |
| III                      | 21 (30.9)      | 24 (34.8)      |        |
| PTCCH1                   |                |                | 0.755  |
| I                        | 23 (33.8)      | 23 (33.3)      |        |
| II                       | 21 (30.9)      | 25 (36.2)      |        |
| III                      | 24 (35.3)      | 21 (30.4)      |        |
| SHH                      |                |                | 0.920  |
| I                        | 22 (32.4)      | 26 (37.7)      |        |
| II                       | 27 (39.7)      | 21 (30.4)      |        |
| III                      | 19 (27.9)      | 22 (31.9)      |        |
| DHH                      |                |                | 0.465  |
| I                        | 21 (30.9)      | 25 (36.2)      |        |
| II                       | 23 (33.8)      | 23 (33.3)      |        |
| III                      | 24 (35.3)      | 21 (30.4)      |        |
| HHH                      |                |                | 0.916  |
| I                        | 22 (32.4)      | 24 (34.8)      |        |
| II                       | 25 (36.8)      | 21 (30.4)      |        |
| III                      | 21 (30.9)      | 24 (34.8)      |        |
| BRAF T1799A mutation     |                |                | 0.119  |
| Absent                   | 24 (35.3)      | 16 (23.2)      |        |
| Present                  | 44 (64.7)      | 53 (76.8)      |        |
GLI1 Expression in Papillary Thyroid Cancers

**Table 2.** Multivariate Analysis of the Association of ETE With GLI1 and GLI2 mRNA Expression Level

|     | ETE          | OR  | 95% CI       | P     |
|-----|--------------|-----|--------------|-------|
| GLI1 group III | 3.701  | 1.550–8.837 | 0.003 |
| GLI1 group III | 3.340  | 1.204–9.269 | 0.021 |
| GLI1 group III | 3.989  | 1.345–11.836 | 0.013 |
| GLI1 group III | 4.381  | 1.414–13.569 | 0.01  |
| GLI2 group III | 3.926  | 1.430–8.043 | 0.006 |
| GLI2 group III | 3.496  | 1.242–9.839 | 0.018 |
| GLI2 group III | 4.028  | 1.276–12.720 | 0.018 |
| GLI2 group III | 4.152  | 1.292–13.342 | 0.017 |

**Impact of the Central Components of the Hh Pathway on ETE and LNM**

To determine the factors affecting ETE and LNM in our study patients, we performed statistical analyses of clinicopathological parameters and mRNA expression of the central components of the Hh pathway, according to the presence or absence of ETE and LNM. As shown in Table 1, ETE was associated with multifocality (P = 0.001), LNM (P = 0.001), TNM Classification of Malignant Tumours (TNM) stage (P = 0.001), GLI1 expression (P = 0.002), and GLI2 expression (P = 0.005). Remarkably, GLI1 group III was a strong positive predictor of ETE after adjusting for clinicopathological parameters, expression of Hh pathway components, the mutation status of BRAF T1799A and TERT promoter (Table 2; odds ratio [OR] 4.381, 95% confidence interval [CI] 1.414–13.569, P = 0.01) compared with group I. In the same multivariate analyses, GLI2 group III was also a positive predictor of ETE (Table 2; OR 4.152, 95% CI 1.292–13.342, P = 0.017).

In the analyses of LNM (Table 3), several factors were identified as predictors: ETE (P = 0.003), TNM stage (P = 0.005), GLI1 (P < 0.001), GLI2 (P = 0.005), and HHIP (P = 0.025); all except HHIP were positive predictors. In multivariate analyses (Table 4), GLI1 group III was the strongest positive predictor of LNM (P = 0.005, OR 5.627, 95% CI 1.674–18.913) compared with group I, and GLI2 group III was also a strong positive indicator (P = 0.036, OR 3.924, 95% CI 1.097–14.043). Additionally, HHIP, group III was a statistically significant negative predictor of LNM, even after adjusting for clinicopathological parameters (P = 0.012, OR 0.278, 95% CI 0.102–0.759).

To validate our data, we performed an independent analysis for 103 patients with conventional PTC. In this
### TABLE 3. Analyses of Clinicopathological Parameters and mRNA Expression of the Central Components of Hh Pathway, According to Presence or Absence of LNM

| LNM                  | Present (n = 78) (%) | Central LN (n = 67) (%) | Lateral LN (n = 11) (%) | P     |
|----------------------|----------------------|-------------------------|-------------------------|-------|
| **No. (n = 59) (%)** |                      |                         |                         |       |
| Age, y, mean ± SD    | 46.1 ± 12.7          | 48.0 ± 12.6             | 43.6 ± 20.7             | 0.517⁷|
| Sex (female)         | 54 (91.5)            | 53 (79.1)               | 9 (81.8)                | 0.098¹|
| Tumor size, cm, mean ± SD | 2.4 ± 0.9    | 2.1 ± 0.9               | 2.2 ± 0.9               | 0.217⁷|
| Multifocality        |                      |                         |                         | 0.095⁵|
| Absent               | 51 (86.4)            | 54 (80.6)               | 8 (72.7)                |       |
| Unilateral           | 2 (3.4)              | 8 (11.9)                | 0 (0)                   |       |
| Bilateral            | 6 (10.2)             | 5 (7.5)                 | 3 (27.3)                |       |
| ETE                  |                      |                         |                         |       |
| Limited to thyroid   | 37 (62.7)            | 30 (44.8)               | 1 (9.1)                 | 0.003³|
| Presence             | 22 (37.3)            | 37 (55.2)               | 10 (90.9)               |       |
| Distant metastasis   |                      |                         |                         | 0.514⁴|
| Absent               | 58 (98.3)            | 67 (100)                | 11 (100)                |       |
| Present              | 1 (1.7)              | 0 (0)                   | 0 (0)                   |       |
| TNM stage            |                      |                         |                         | 0.005⁵|
| I                    | 32 (54.2)            | 27 (40.3)               | 7 (63.6)                |       |
| II                   | 15 (25.4)            | 23 (34.3)               | 2 (18.2)                |       |
| III                  | 7 (11.9)             | 0 (0)                   | 1 (9.1)                 |       |
| IV                   | 5 (8.5)              | 39 (58.2)               | 3 (27.3)                |       |
| **GLI1**             |                      |                         |                         | <0.001¹|
| I                    | 26 (44.1)            | 20 (29.9)               | 0 (0)                   |       |
| II                   | 21 (35.6)            | 23 (34.3)               | 2 (18.2)                |       |
| III                  | 12 (20.3)            | 24 (35.8)               | 9 (81.8)                |       |
| **GLI2**             |                      |                         |                         | 0.005⁵|
| I                    | 24 (40.7)            | 21 (31.3)               | 1 (9.1)                 |       |
| II                   | 21 (35.6)            | 23 (34.3)               | 2 (18.2)                |       |
| III                  | 14 (23.7)            | 23 (34.3)               | 8 (72.7)                |       |
| **GLI3**             |                      |                         |                         | 0.466⁴|
| I                    | 19 (32.2)            | 23 (34.3)               | 4 (36.4)                |       |
| II                   | 17 (28.8)            | 26 (38.8)               | 3 (27.3)                |       |
| III                  | 23 (39.0)            | 18 (26.9)               | 4 (36.4)                |       |
| **HHIP**             |                      |                         |                         | 0.025³|
| I                    | 14 (23.7)            | 26 (38.8)               | 6 (54.5)                |       |
| II                   | 20 (33.9)            | 25 (37.3)               | 1 (9.1)                 |       |
| III                  | 25 (42.4)            | 16 (23.9)               | 4 (36.4)                |       |
| **PTCH1**            |                      |                         |                         | 0.574⁴|
| I                    | 21 (35.6)            | 22 (32.8)               | 3 (27.3)                |       |
| II                   | 15 (25.4)            | 25 (37.3)               | 6 (54.5)                |       |
| III                  | 23 (39.0)            | 20 (29.9)               | 2 (18.2)                |       |
| **SHH**              |                      |                         |                         | 0.939³|
| I                    | 15 (25.4)            | 29 (43.3)               | 4 (36.4)                |       |
| II                   | 29 (49.2)            | 18 (26.9)               | 1 (9.1)                 |       |
| III                  | 15 (25.4)            | 20 (29.9)               | 6 (54.5)                |       |
| **DHH**              |                      |                         |                         | 0.074¹|
| I                    | 25 (42.4)            | 19 (28.4)               | 2 (18.2)                |       |
| II                   | 19 (32.2)            | 21 (31.3)               | 6 (54.5)                |       |
| III                  | 15 (25.4)            | 27 (40.3)               | 3 (27.3)                |       |
| **HHH**              |                      |                         |                         | 0.821⁴|
| I                    | 20 (33.9)            | 22 (32.8)               | 4 (36.4)                |       |
| II                   | 19 (32.2)            | 23 (34.3)               | 4 (36.4)                |       |
| III                  | 20 (33.9)            | 22 (32.8)               | 3 (27.3)                |       |
| **BRAF T1799A mutation** |            |                         |                         |       |
| Absent               | 17 (28.8)            | 20 (29.9)               | 3 (27.3)                | 0.996⁴|
| Present              | 42 (71.2)            | 47 (70.1)               | 8 (72.7)                |       |
GLI1 group III promoter C228T mutation.

C228T mutations.

hog, TERT

¼ PTCH

interacting protein, IHH = Indian Hedgehog, LN = Lymph node, LNM = lymph node metastasis, mRNA = Messenger ribonucleic acid, PTCH = patched, SHH = Sonic Hedgehog, TERT = telomerase reverse transcriptase, TNM = TNM Classification of Malignant Tumours.

Unless stated otherwise, data represent and followed by the corresponding percentage of total (in parentheses). ANOVA = Analysis of variance, DHH = Desert Hedgehog, ETE = extrathyroidal extension, GLI1 = glia-associated oncogene homolog 1, Hh = Hedgehog, HHIP = Hedgehog-interacting protein, IHH = Indian Hedgehog, LN = Lymph node, LNM = lymph node metastasis, mRNA = Messenger ribonucleic acid, PTCH = patched, SHH = Sonic Hedgehog, TERT = telomerase reverse transcriptase, TNM = TNM Classification of Malignant Tumours. § In addition to adjustment.

validation set, GLI1 group III was consistently related to ETE (P < 0.001) and LNM (P < 0.001) (supplementary Table 6, http://links.lww.com/MD/A305).

Strong Correlation of GLI1 Expression With Axon Guidance Gene Set

To obtain further insight into the mechanisms of GLI1 action, we performed GSEA using data from a public repository, NCBI GEO. The dataset GDS1732 contained expression profiles from 7 normal thyroid tissues and matched PTC specimens (14 total samples). In this dataset, GLI1 expression was quite heterogeneous in normal and tumor tissues (supplementary Figure 4A, http://links.lww.com/MD/A305); moreover, GSEA revealed no enrichment in genes associated with the Hh pathway in normal or PTC (supplementary Figure 4B, http://links.lww.com/MD/A305). However, when we performed GSEA on the samples with the lowest GLI1 expression (group 1: GSM85223 and GSM85225) and those with the highest GLI1 expression (group 2: GSM85222 and GSM85224), we observed that the top 20 KEGG gene sets enriched in group 2 included sets related to cell interaction and migration, for example, neuroactive ligand-receptor interaction, leukocyte transendothelial migration, cytokine and cytokine receptor interaction, and the Notch signaling pathway (supplementary Table 7, http://links.lww.com/MD/A305). In addition, the gene set ‘axon guidance’ was significantly enriched in group 2 (supplementary Figure 5, http://links.lww.com/MD/A305; ES = −0.38238, nominal P value = 0.005165, false discovery rate q-value = 0.235033), indicating that GLI1 might increase the migration and invasion abilities of PTC by inducing expression of genes related to axon guidance. Furthermore, the dataset GSE33630 contained expression profiles from 45 normal thyroid tissues; 49 PTC and 11 anaplastic thyroid carcinoma (ATC) specimens (105 total samples) showed the enrichment of Hh signaling pathway in normal thyroid tissues (supplementary Figure 6, http://links.lww.com/MD/A305). However, when we selected 16 cases of high GLI1 expression (high GLI1 group) and 16 cases of low GLI1 expression (low GLI1 group) and performed the GSEA using those 2 groups, the gene sets related to axon guidance were consistently enriched in high GLI1 group (Figure 3A). Because the classic targets of Hh pathways such as GLI1, GLI2, and HHIP have been discovered in human pancreatic cancers, we performed GSEA using GSE16515 derived from pancreatic cancers.53 Compatible with our GSEA data using GDS1732 and GSE33630, the gene set encompassing the axon guidance was significantly enriched in pancreatic cancers with high GLI1 expression (supplementary Figure 7, http://links.lww.com/MD/A305). To validate the results of our GSEA, we performed qPCR, using cDNA derived from GLI1 groups I (n = 7) and III (n = 7), to detect mRNAs encoding actin binding LIM protein family, member 3 (ABLIM3); nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4 (NFATc4); L1 cell-adhesion molecule (L1CAM); sema domain, immunoglobulin domain, short basic domain, secreted, (sema)phorin) 3D (SEMA3D); feline sarcoma oncogene (FES); plexin B3 (PLXNB3); sema domain, transmembrane domain, and cytoplasmic domain, (sema)phorin) 6A (SEMA6A); roundabout, axon

### TABLE 4. Multivariate Analysis of the Association of LNM With GLI1, GLI2, and HHIP mRNA Levels

| GLI1 group III | OR     | 95% CI | P     |
|---------------|--------|--------|-------|
| GLI1 group III  | 3.723  | 1.515–9.148 | 0.004 |
| GLI1 group III† | 4.568  | 1.603–13.111 | 0.004 |
| GLI1 group III‡ | 5.780  | 1.779–18.779 | 0.004 |
| GLI1 group III§ | 5.627  | 1.749–18.913 | 0.005 |
| GLI1 group III∥ | 2.423  | 1.013–5.793 | 0.047 |
| GLI1 group III¶ | 1.942  | 0.694–4.890 | 0.220 |
| GLI1 group III‖ | 5.780  | 1.779–18.779 | 0.004 |
| GLI1 group III|| | 3.949  | 1.105–14.107 | 0.034 |
| GLI1 group III| 3.924  | 1.097–14.043 | 0.036 |
| HHIP group III*| 0.351  | 0.145–0.850 | 0.02  |
| HHIP group III†| 0.278  | 0.102–0.759 | 0.012 |
| HHIP group III‡| 0.239  | 0.049–1.158 | 0.075 |
| HHIP group III§| 0.214  | 0.043–1.072 | 0.061 |

| GLI2 group III | OR     | 95% CI | P     |
|---------------|--------|--------|-------|
| GLI2 group III | 3.949  | 1.105–14.107 | 0.034 |
| HHIP group III | 0.351  | 0.145–0.850 | 0.02  |
| HHIP group III†| 0.278  | 0.102–0.759 | 0.012 |
| HHIP group III‡| 0.239  | 0.049–1.158 | 0.075 |
| HHIP group III§| 0.214  | 0.043–1.072 | 0.061 |

CI = confidence interval, DHH = Desert Hedgehog, ETE = extrathyroidal extension, GLI1 = glia-associated oncogene homolog 1, IHH = Indian Hedgehog, LN = Lymph node, LNM = lymph node metastasis, mRNA = Messenger ribonucleic acid, OR = odds ratio, PTCH = patched, SHH = Sonic Hedgehog, TERT = telomerase reverse transcriptase. Adjusted for age and sex. 

In addition to adjustment, adjusted for ETE, multiplicity, tumor size and TNM stage.

In addition to adjustment, adjusted for BRAF 1799A and TERT C228T mutations.

In addition to adjustment, adjusted for GLI1, GLI3, HHIP, PTCH1, SHH, DHH, and IHH mRNA levels.

In addition to adjustment, adjusted for BRAF 1799A and TERT C228T mutations.

In addition to adjustment, adjusted for BRAF 1799A and TERT C228T mutations.

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In addition to adjustment, adjusted for BRAF 1799A and TERT C228T mutations.
guidance receptor, homolog 3 (ROBO3); EPH receptor A8 (EPHA8). As expected, expression of all those axon guidance-related mRNAs was higher in group III than in group I (Figure 3B and C). In addition, IHC staining of paraffin-embedded tumor samples from the same patients used for the qRT-PCR analysis revealed that NFATc4, ABLIM3, and FES expressions are increased in high GLI1 PTC (Figure 4). Taken together, these data indicate that GLI1 expression is consistently associated with elevated aberrant expression of axon guidance genes.24,25 In addition, the correlation analyses using GSE33630, GLI2 mRNA expression showed a negative relationship with E-cadherin (CDH1) mRNA expression and a positive relationship with vimentin (VIM). Moreover, HHIP mRNA expression showed a negative relationship with VIM (data not shown) indicating GLI and HHIP expression might be also linked to these informative markers of the epithelial-mesenchymal transition (EMT).

Validation Analyses Using the Public Data From the Cancer Genome Atlas Research Network

To verify our GSEA results, we performed additional analyses using the TCGA public data. As shown in Figure 5, the GLI1 and GLI2 mRNA expression showed a negative relationship with E-cadherin (CDH1) mRNA expression and a positive relationship with vimentin (VIM). Moreover, HHIP mRNA expression showed a negative relationship with VIM (data not shown) indicating GLI and HHIP expression might be also linked to these informative markers of the epithelial-mesenchymal transition (EMT).

FIGURE 3. Relationship between expression levels of GLI1 and the KEGG axon guidance gene set. (A) GSEA using gene expression profiles selected from NCBI GEO Record GSE33630. (B and C) qRT-PCR analysis for representative axon guidance genes in 7 patients each from GLI1 groups I (ratio of GLI1 expression in PTC vs normal tissues in the lowest one third of all patients) and III (ratio in the highest one third). Means were compared and analyzed by Mann–Whitney U test. All data are means ± SEM, *P < 0.05, **P < 0.01, and ***P < 0.001. GEO = Gene Expression Omnibus, GLI1 = glioma-associated oncogene homolog 1, GSEA = Gene Set Enrichment Analysis, PTC = papillary thyroid cancer, qRT-PCR = quantitative RT-PCR.

DISCUSSION

ETE and LNM are important clinical and histological indicators that predict locoregional recurrence in patients with PTC. To date, however, the molecular details of the roles played by these 2 factors in PTC have remained unclear. One of the signature molecular mechanisms of tumor progression is EMT.26 Indeed, stem-like properties of cancer cells have been implicated in local invasion and metastasis.27 Consistent with this, expression levels of the EMT-associated factors Slug and Twist1 are elevated in thyroid cancers.28–31 Over-expression of VIM, transforming growth factor-β (TGFβ), NF-κB, and integrin has been observed in invasive fronts of PTC.32–33 However, most studies of EMT have been performed in cell lines or tissues derived from ATC, and extensive verification of new EMT markers using clinical samples has not been performed.

Over the last 2 decades, many studies have mechanistically investigated the roles of aberrant activation or constitutive activation of the Hh pathway in cancer progression.14
Loss-of-function mutations in PTCH are frequently identified in BCC and MB.\textsuperscript{17,34} In addition, gain-of-function mutations in smoothened, frizzled class receptor (SMO), a G-protein-coupled receptor-like molecule that positively regulates Hh signaling, have also been detected in BCC, MB, and even in meningiomas.\textsuperscript{35} Ligand-dependent activation mechanism may also be important. For example, autocrine activation of the Hh pathway in tumor cells has been reported in cancers of the lung, breast, stomach, and prostate.\textsuperscript{36–39} Moreover, the Hh pathway may contribute to the maintenance and differentiation of cancer stem cells through ligand-dependent mechanisms.\textsuperscript{40}

In this study, we compared relative mRNA expression of central components of the Hh pathway between PTC and matched normal tissues. These experiments revealed that PTC specimens expressed lower levels of the \textit{GLI1}, \textit{GLI2}, \textit{GLI3}, \textit{HHIP}, \textit{PTCH1}, \textit{SHH}, \textit{DHH}, and \textit{IHH} mRNAs than the corresponding matched normal tissues, suggesting that Hh pathway activity is reduced in PTC. In general, the zinc-finger transcription factor \textit{GLI1} and \textit{GLI2} act as the final effectors of upstream signaling pathways by inducing the expression of various genes in a context-specific manner. Hh target genes include \textit{GLI1}, \textit{PTCH1}, and \textit{HHIP}. Although our qRT-PCR data did not demonstrate any relationship between \textit{GLI1} and \textit{PTCH1} expression levels in normal thyroid tissues (data not shown), we did observe a strong positive correlation between \textit{GLI1} and \textit{HHIP} levels, indicating that the physiologically relevant negative-feedback loop is operational in normal follicular cells. However, this relationship disappeared in PTC, suggesting that the negative-feedback loop might be dysregulated in these cells, or alternatively that \textit{GLI1} expression is transcriptionally regulated independently of Hh signaling in these tumors PTC.\textsuperscript{41} Supporting our data, 2 NCBI DataSet Records (GDS1732 and GSE33630) did not show any correlation between \textit{GLI1} and \textit{HHIP} expression in thyroid tumor tissues (data not shown).

Recently, BRAFV600E and \textit{TERT} promoter mutations have been suggested to cooperatively identify recurrent PTC.\textsuperscript{42} In our study, \textit{GLI1} group III has more frequent \textit{TERT} promoter C228T mutation. However, in our study, the prevalence (6.6%) of \textit{TERT} promoter C228T mutation was relatively lower than previous western studies.\textsuperscript{10,43} In addition, \textit{TERT} promoter C250T mutation was not detected. In fact, all of the PTCs in our study subjects were conventional PTC, which might be the reason of low frequency in \textit{TERT} promoter mutations.\textsuperscript{10}

A previous study suggested that the Hh pathway is activated in PTC, ATC, and follicular adenoma, as demonstrated by IHC staining to detect \textit{SHH}, \textit{PTCH}, \textit{SMO}, and \textit{GLI1}.\textsuperscript{44–46} However, those authors did not detect a correlation between Hh activation and any clinicopathological parameter. By contrast, in this study, we used qRT-PCR to quantitate mRNA levels in normal and tumor tissues, and then applied the resultant data to direct comparisons between paired samples. Through this approach, we could observe changes in Hh pathway-related gene expression separately in samples from individual patients, ultimately allowing us to categorize our study subjects into 3 groups according to Hh pathway activity. In particular, we divided our subjects into 3 groups, with group III expressing the highest ratio of \textit{GLI1} or \textit{GLI2} mRNA in PTC.
relative to normal tissue. Multivariate analyses revealed that patients in group III for GLI1 and GLI2 exhibited the highest risk of ETE and LNM, even after adjusting for clinicopathological parameters. Furthermore, HHIP group III was negatively associated with LNM.

However, genes encoding Hh pathway ligands, such as SHH, DHH, and IHH, were not valuable as prognostic indicators, and their expression levels were not related to those of GLI1, GLI2, and HHIP (data not shown). We postulated that these negative results reflect the role of the noncanonical GLI signaling in PTC. Moreover, these negative results reflect the role of the noncanonical GLI signaling.47,48 Because the mRNA expressions of any correlation of the GLI2 expressions were decreased in PTC compared with matched normal tissue, indicating downregulation of Hh pathway might be occurred in carcinogenic process in PTC. Whereas, as our qRT-PCR data indicated, GLI1 and GLI2 mRNA expressions were decreased in PTC compared with matched normal tissue indicating downregulation of Hh pathway might be occurred in carcinogenic process in PTC. Additionally, our qRT-PCR data indicated, GLI1 and GLI2 mRNA expressions were decreased in PTC compared with matched normal tissue indicating downregulation of Hh pathway might be occurred in carcinogenic process in PTC. Indeed, although our correlation analyses using the TCGA data did not any correlation of the GLI1/2 mRNA expressions with AKT1 and mTOR1 expressions, recent molecular biological studies have revealed crosstalk between GLI1 signaling and the PI3K-AKT/mTOR pathways.49,50 Because the mRNA expressions of AKT1 and mTOR1 cannot be representative makers for AKT1 and mTOR1 activities, future studies need to be conducted to investigate the relationship of GLI signaling with the PI3K-AKT/mTOR pathways in PTC. Furthermore, TGFβ and constitutively active Kirsten rat sarcoma viral oncogene homolog have been reported to activate Hh signaling.51

In addition, our GSEA using data derived from a public repository revealed that GLI1 expression is coordinately regulated along with genes involved in axon guidance. Consistent with this, qRT-PCR data from our patients consistently indicated that GLI1 expression was closely related to expression of axon guidance genes such as ABLIM3, NFATc4, L1CAM, SEMA3D, FES, PLXNB3, SEMA6A, SEMA6B, ROBO3, and EPHA8 as further validated by IHC staining and the TCGA data analyses. These data suggest that GLI1 might be able to facilitate the EMT by inducing genes related to axon guidance. Supporting our idea, the mRNA expressions of the EMT markers such as CDH1 and VIM were accordingly related to the GLI1/2 expressions. Furthermore, our GSEA indicated that gene sets involved in neuroactive ligand–receptor interactions, cytokine–cytokine receptor interactions, and the Notch signaling pathways were also coordinately upregulated in association with GLI1 expression, suggesting these pathways might be involved in ETE and LNM in the context of GLI1-driven EMT. In the case of GLI2, the GSEA analysis using same data sets presented coordinately enrichment of gene sets involved in ribosome, DNA replication and pentose phosphate pathways suggesting sustained proliferative signaling (data not shown). Taken together, although we could not investigate the GLI1 and GLI2 expressions in the invasive front because of the ethical issues of our institute,33,52 all of our data from qRT-PCR, IHC, GSEA, statistical analyses using the TCGA data and our cohort...
In addition, the noncanonical mechanisms of Hh pathway and the GLI proteins should be validated as novel drug targets. Underlying activation of GLI-mediated transcription in PTC, future studies should investigate the molecular mechanisms and can therefore be considered a marker of poor prognosis.

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