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Relationship of Focally Amplified Long Noncoding on Chromosome 1 (*FAL1*) lncRNA with E2F Transcription Factors in Thyroid Cancer

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Abstract: Recent functional genomic studies revealed that the oncogenic activity of focally amplified lncRNA on chromosome 1 (*FAL1*, ENSG00000228126) contributes to tumor growth by p21 repression in human cancers. However, the expression of *FAL1* was not investigated in papillary thyroid cancer (PTC).

We aimed to determine if *FAL1* was up-regulated in PTC compared to paired contralateral normal thyroid tissues, and to investigate the potential targets of this lncRNA and its clinicopathological significance in PTC.

We analyzed *FAL1* and p21 expression levels in 100 PTC samples and matched normal thyroid tissue by qRT-PCR. Using lncRNA microarray data from the Gene Expression Omnibus (accession no. GSE61763), we explored potential targets of *FAL1* by Gene Set Enrichment Analysis, followed by verification by qRT-PCR in our PTC samples. A cross-sectional observational study was conducted to investigate the relationship between patients' clinicopathological features and *FAL1* expression.

FAL1 expression was significantly higher in PTC than in paired normal thyroid tissues (paired *t* test, $P < 0.001$). p21 mRNA expression was also increased, not decreased, in PTC, and had no correlation with *FAL1* expression ($r = 0.0897$, $P = 0.4002$). Gene Set Enrichment Analysis, using publicly available microarray data, indicated that a gene set related to the cell cycle, including E2F transcription factors 1 and 2, and cyclin D1, was coordinately enriched among samples with high *FAL1* expression. A volcano plot showed that *E2F1*, *E2F2*, and *VEGFA* mRNAs were increased in the high *FAL1* samples. In clinicopathological analyses,

multifocality was more frequently observed in PTC patients with high *FAL1* ($P = 0.018$). Multivariate analysis showed that high *FAL1* expression increased the risk of multifocality (after adjustment for clinical variables, OR = 4.019, CI = 1.041–11.020, $P = 0.043$).

FAL1 may have a role in cell-cycle progression and may be associated with aggressive tumor behavior in PTC.

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Abbreviations: BANC1 = BRAF-activated lncRNA, BCPAP = BCPAP cell, BMI1 = BMI1 proto-oncogene, BTG2 = BTG family member 2, CDKN1A = cyclin-dependent kinase inhibitor 1A, CYFIP2 = cytoplasmic FMR1 interacting protein 2, DTC = differentiated thyroid cancer, E2F1 = E2F transcription factor 1, E2F2 = E2F transcription factor 2, *FAL1* = focally amplified long noncoding on chromosome 1, FAS = Fas cell surface death receptor, FBXW7 = F-box and WD repeat domain containing 7, GEO = Gene Expression Omnibus, GSEA = Gene Set Enrichment Analysis, H2AK119 = Histone H2A (Lys119), IHC = immunohistochemical, lncRNA = long noncoding ribonucleic acid, LOH = loss of heterozygosity, mTOR = mechanistic target of rapamycin, NAMA = noncoding RNA associated with MAP kinase pathway and growth arrest, NCBI = National Center for Biotechnology Information, PRC1 = polycomb repressive complex 1, PTC = papillary thyroid cancer, PTMC = papillary thyroid microcarcinoma, PTSC2 = papillary thyroid cancer susceptibility candidate 2, PTSC3 = papillary thyroid cancer susceptibility candidate 3, RB = retinoblastoma protein, Ring1B = ring finger protein 2, RT-PCR = reverse transcription polymerase chain reaction, SCNAs = somatic copy-number alterations, TP53IP3 = tumor protein p53 inducible protein 3, TPC1 = TPC1 cell, VEGFA = vascular endothelial growth factor A.

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INTRODUCTION

Thyroid cancer of follicular cell origin is the most common endocrine malignancy, and its incidence has been continuously increasing worldwide.^{1,2} The most effective therapy for differentiated thyroid cancer (DTC) is thyroidectomy, followed by ablation with radioactive iodine, which permits 5-year survival rates above 95% in the USA.^{3,4} However, the treatment of persistent or recurrent thyroid cancer is problematic because DTC is resistant to conventional chemotherapy or radiotherapy.^{5–7} As a result, the survival rate among patients with persistent DTC is approximately 60%, which means that a significant proportion of DTCs are life-threatening diseases.⁸ Even more problematic is that diagnostic markers to predict persistence or recurrence of DTC are not available.^{4,9}

A noncoding RNA (ncRNA) is an RNA molecule that is not translated into a protein.^{10,11} Nonetheless, ncRNAs are

abundant and functionally important and include transfer RNAs, ribosomal RNAs, small nuclear RNAs, microRNAs, piwi-interacting RNAs, and long ncRNAs (lncRNAs).¹² By definition, lncRNAs are nonprotein-coding transcripts that are longer than 200 nucleotides.^{13,14} lncRNAs may have an important role in the development of thyroid cancers. For example, the papillary thyroid cancer (PTC) susceptibility candidate 2 (*PTSC2*) gene encodes a lncRNA that is down-regulated in PTC, affecting the expression of genes functionally related to the cell cycle and cancer.¹⁵ In addition, the polymorphism rs944289 on the PTC susceptibility candidate 3 (*PTSC3*) gene, which also encodes a lncRNA, predisposes to PTC, indicating that *PTSC3* functions as a tumor suppressor.¹⁶ Moreover, the BRAF-activated lncRNA (BANCR) is up-regulated in PTC compared to matched normal tissue, and BANCR over-expression induces cell proliferation via autophagy regulation.¹⁷

Functional genomic studies recently revealed that the oncogenic activity of focally amplified lncRNA on chromosome 1 (*FALI*, ENSG00000228126) contributes to the repression of p21 expression in different human cancers such as ovarian cancers.^{18,19} Mechanistically, *FALI* binds to the BMI1 proto-oncogene (BMI1), a component of the polycomb repressive complex 1 (PRC1). This direct interaction between *FALI* and BMI1 increases BMI1 stability, changes the levels of H2AK119 ubiquitination, and finally represses the expression of a wide range of genes, such as cyclin-dependent kinase inhibitor 1A (CDKN1A, p21, Cip1), Fas cell surface death receptor (FAS), BTG family, member 2 (BTG2), tumor protein p53 inducible protein 3 (TP53I3), F-box and WD repeat domain containing 7 (FBXW7), and cytoplasmic FMR1 interacting protein 2 (CYFIP2).¹⁹ Among those targets, CDKN1A, also called p21, inhibits cyclin-dependent kinase (CDK) 1, 2, and 4/6 complexes, thereby inhibiting cell cycle progression at the G1/S transition.²⁰ In untransformed cells, inactivation of CDK4/6 results in de-phosphorylation of retinoblastoma protein (RB), the first tumor suppressor to be identified. De-phosphorylated RB binds to E2F transcription factors (E2F) such as E2F1, E2F2, and E2F3a, which represses E2F transcriptional activity.²¹ Taken together, the results suggest that repression of p21 expression by *FALI* may increase CDK activity, promote RB phosphorylation and E2F transactivation, and finally promote the G1/S transition.

However, the role of *FALI* expression has never been studied in PTC. Therefore, we have investigated *FALI* expression in PTC and in paired contralateral normal thyroid tissues. In addition, we explored potential targets of *FALI* in PTC using Gene Set Enrichment Analysis and analyzed the clinicopathological significance of this lncRNA in patients with PTC.

METHODS

Study Subjects and Clinical Data

The study enrolled 100 patients who underwent thyroidectomy for PTC between July and October 2014 at Yonsei Cancer Center (Seoul, South Korea). Samples were collected from the central part of the cancer and contralateral histologically normal tissue. Thyroid samples were examined microscopically immediately after surgery and stained with hematoxylin–eosin. On histological examination, >80% of the cells from the central part of the cancer were thyroid cancer cells. The study protocol was approved by the Institutional Review Board of Severance Hospital, and all patients provided informed consent before study participation.

Cell Culture and Plasmid

Human thyroid cancer cell lines BCPAP, 8505C, C643, HTH63, and SW1736 were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA). The cell lines TPC-1 and FTC-133 were cultured in high-glucose DMEM (Invitrogen) with 10% FBS at 37°C in a humidified incubator with 5% CO₂. To generate plasmid expressing *FALI*, sense (5'-CTC GGATCC GCG CAT CTC CTA CGG CCT CCA GGA CAG AGG-3') and anti-sense (5'-CGA GCG GCC GCA GAC ATC CAA GTG TCC TGT GTA ATA GGC TG-3') primers were used. PCR was performed by the standard PCR protocol (PrimeSTAR[®] HS Polymerase Takara Bio, Shiga, Japan). The PCR product was cloned into pcDNA3.1 vector (Thermo Fisher Scientific, Waltham, MA).

RNA Isolation, Quantitative RT-PCR, and BRAF Sequencing

Total RNA from thyroid tissue and cells was extracted using TRIzol reagent according to the manufacturer's protocol (Invitrogen). Total RNA (2 µg) was converted into cDNA with Superscript II reverse transcriptase (Invitrogen) and used in quantitative RT-PCR (qRT-PCR) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). The SYBR green RT-PCR kit (Life Technologies) and the Solg 2X PCR Smart Mix kit (Solgent, Daejeon, South Korea) were used. The primers were as follows: *FALI*, 5'-GCA AGC GGA GAC TTG TCT TT-3' and 5'-TTG AAC TCC TGA CCT CGT GA-3'; p21 (*CDKN1A*), 5'-CCT CAT CCC GTG TTC TCC TTT-3' and 5'-GTA CCA CCC AGC GGA CAA GT-3'; E2F transcription factor 1 (*E2F1*), 5'-GGA CCT GGA AAC TGA CCATCA G-3' and 5'-CAG TGA GGT CTC ATA GCG TGA C-3'; E2F transcription factor 2 (*E2F2*), 5'-CGT CCC TGA GTT CCC AAC C-3' and 5'-GCG AAG TGT CAT ACC GAG TCT T-3'; vascular endothelial growth factor A (*VEGFA*), 5'-AGG GCA GAA TCA TCA CGA AGT-3' and 5'-AGG GTC TCG ATT GGATGG CA-3'; *U6*, 5'-CTC GCT TCG GCA GCA CA-3' and 5'-AAC GCT TCA GGA ATT TGC GT-3'; and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-GCC GTC TCA AAA AAC CTG CC-3 and 5'-ACC ACC TGG TGC TCA GTG TA-3'. Relative expression was calculated by the StepOne[™] Real-Time PCR System (Applied Biosystems). To detect BRAFV600E mutation, BRAF exon 15 was amplified by PCR with the following primers: forward 5'-TGT TAC CCA GTG GTG TGA GG-3' and reverse 5'-CAT CTG ACT GAA AGC TGT ATG GA-3'. The PCR products were sequenced on an ABI 3730XL DNA Analyzer with BigDye[®] Terminator v3.1 cycle sequencing kits (Applied Biosystems).

Western Blot Analysis and Immunohistochemistry

Western blot analysis was performed according to previously described protocols with commercially available antibodies: p21 mouse monoclonal antibody (sc-6246, Santa Cruz Biotechnology, Inc., Dallas, TX) and anti-β-Actin Antibody (#4967, Cell Signaling, Danvers, MA).⁵ The signal intensities on Western blots were quantified by ImageJ software. Immunohistochemical staining for cyclin D1 was performed on 10 PTC samples with the lowest and 10 with the highest *FALI* expression ratios compared to internal control (*GAPDH*) by the protocols previously described.⁵ The primary antibody in our IHC study was rabbit anti-human cyclin D1 monoclonal antibody (Thermo Scientific, Fremont, CA).

Bioinformatics and Statistical Analyses

The secondary mRNA structure of *FAL1* was predicted using the web server CentroidFold (<http://www.ncrna.org/centroid-fold>).²² lncRNA microarray data (accession no. GSE61763, 15 corresponding normal and malignant tissues) were obtained from the Gene Expression Omnibus (GEO) of NCBI and subjected to Gene Set Enrichment Analysis.²³ Analysis of public repository data for p21 expression in thyroid cancers was performed using the Human Protein Atlas (<http://www.proteinatlas.org/>). Statistical analysis was performed using Prism (GraphPad Software, San Diego, CA) or SPSS version 18.0 for Windows (SPSS, Chicago, IL). All *P* values are 2-sided.

RESULTS

Expression of *FAL1* in PTC *FAL1* is a lncRNA-coding gene located in a focal amplicon on chromosome 1q21.2. The coding region of *FAL1* starts from 150,515,757 and ends at 150,518,032 on chromosome 1 and has 2 exons of 306 bp and 260 bp (Supplementary Figure 1A, <http://links.lww.com/MD/A664>). The predicted RNA secondary structure of *FAL1* suggests the presence of functional “modules” (Supplementary Figure 1B, <http://links.lww.com/MD/A664>). Based on this structural prediction, Hu et al¹⁹ postulated that *FAL1* interacts with other nucleotides and proteins.

To determine if *FAL1* expression was increased in PTC, we performed qRT-PCR using cDNA from PTC and paired normal thyroid tissues (n = 100). As shown in Figure 1A, *FAL1* expression was significantly higher in PTC than in paired normal thyroid tissues (paired *t* test, *P* < 0.001). Because *FAL1* was

reported to repress p21 expression in cancer through the BMI1 proto-oncogene (BMI1) and ring finger protein 2 (RNF2, Ring1B),¹⁹ we compared p21 mRNA expression between the 2 thyroid tissue types. Contrary to that reported for other cancers, p21 mRNA expression was increased, not decreased, in PTC (paired *t* test, *P* < 0.0001; Figure 1B), suggesting that *p21* expression is not negatively regulated by *FAL1* in PTC. In addition, we found no evidence of a correlation between *FAL1* and p21 expression in PTC (Pearson *r* = 0.0897, *P* = 0.4002; Supplementary Figure 2A, <http://links.lww.com/MD/A664>). This finding was supported by qRT-PCR using cDNA from thyroid cancer cell lines, which also indicated the absence of a negative relationship between *FAL1* and p21 expression (Supplementary Figure 2B, <http://links.lww.com/MD/A664>). The increased p21 mRNA expression raised the possibility that p21 mRNA levels might not reflect precisely p21 protein levels. To verify p21 expression at the protein level, we conducted Western blot analyses using PTC samples showing high *FAL1* expression. Consistently, p21 expression was not lower in these PTC samples than in matched normal tissues (Figure 1C and D). Furthermore, immunohistochemical staining data from Human Protein Atlas indicated that p21 expression is easily observable in PTCs but not in Follicular neoplasm (FN), supporting the notion that p21 is active in PTC tumor cells (Supplementary Figure 2C and D, <http://links.lww.com/MD/A664>).

Clinicopathological Implications of *FAL1* Expression in PTC

To understand the clinicopathological significance of *FAL1* expression, we compared the 30 patients with the highest

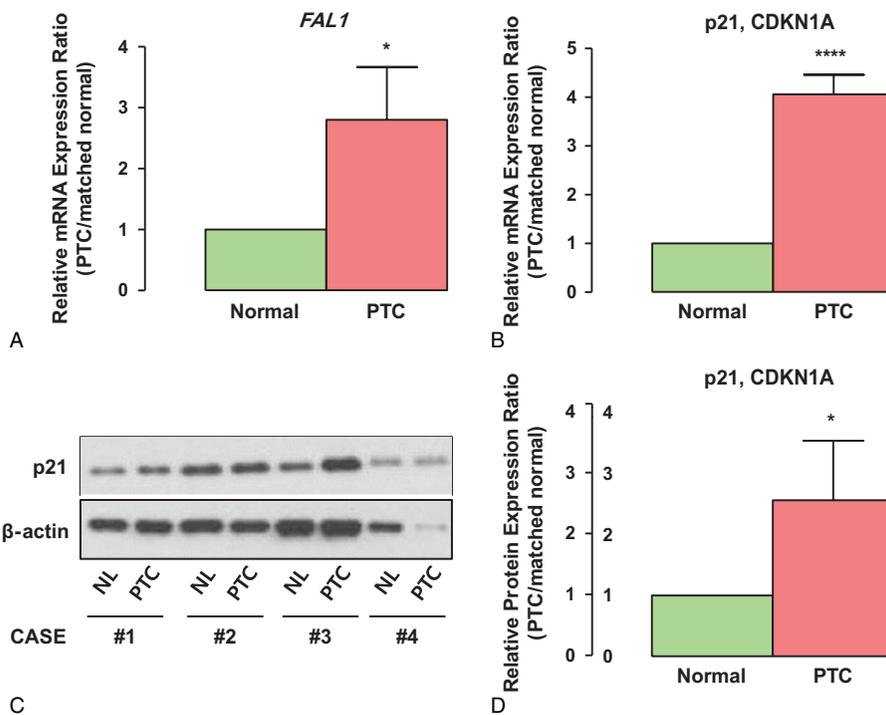


FIGURE 1. Relative RNA expression *FAL1* in PTC. (A, B) Relative RNA-expression ratios of *FAL1* and p21 between PTC and paired normal thyroid tissue (n = 100). Average ratios were compared with the 1-sample *t* test. **P* < 0.05, *****P* < 0.0001. The experiments were performed in triplicate and repeated 3 times. (C) Comparisons of p21 protein expression status between PTC with high *FAL1* expression and paired normal thyroid tissue. (D) Quantitative analysis of p21 expression assessed by ImageJ. The experiments were repeated 3 times. **P* < 0.05. PTC = papillary thyroid cancer.

FALI expression to the 30 with the lowest *FALI* expression (Table 1). No association was found for age, sex, tumor size, or tumor stage. Remarkably, patients with high expression were more likely to have multifocality ($P=0.018$). Univariate analyses (Supplementary Table 1, <http://links.lww.com/MD/A664>) and multivariate analyses indicated that high *FALI* expression increased the risk of multifocality (after adjustment for clinical characteristics, OR = 4.019, CI = 1.041–11.020, $P=0.043$; Table 2). Taken together, our qRT-PCR data and analysis of clinical data suggest that high *FALI* expression may affect tumor behavior and generate aggressive features of PTC.

Discovery of *FALI* Target Genes

Because we did not find a negative relationship between *FALI* and p21, we aimed to find new *FALI* targets that promote tumor aggressiveness. We therefore performed Gene Set Enrichment Analysis. This analysis indicated that a gene set related to the cell cycle, including genes encoding *E2F1*, *E2F2*, and cyclin D, was coordinately enriched in *FALI* up-regulation group (Figure 2A). In addition, the mTOR signaling pathway was also related to *FALI* up-regulation (Supplementary Figure 3A, <http://links.lww.com/MD/A664>). A volcano plot also presented that

E2F1, *E2F2*, *cyclin D1*, *cyclin D2*, and *VEGFA* expression were related to *FALI* up-regulation (Figure 2B). To determine if these findings were reproducible in our own set of 100 PTCs, we performed qRT-PCR using cDNA from the 10 samples with the highest and 10 with the lowest *FALI* expression. Compatibly, mRNA expression ratios between PTC and matched normal thyroid tissue were higher for *E2F1*, *E2F2*, and *VEGFA* among samples with high *FALI* expression (Figure 2C). Furthermore, immunohistochemical staining also showed cyclin D1 up-regulation among PTC samples with the highest *FALI* expression (Figure 2D). To verify the *FALI* targets in the GSEA and qRT-PCR data, we transfected TPC1 (RET/PTC1 rearrangement) and BCPAP (BRAFV600E) thyroid cancer cell lines with the pcDNA3.1-*FALI* plasmid (Figure 3A). Consistently with the GSEA and qRT-PCR data, we confirmed that *E2F1*, *E2F2*, *VEGFA*, and *cyclin D1* were significantly induced by *FALI* overexpression in both cell lines (Figure 3B–E).

In fact, a gain of 1q (SCNA-low-1q-amp) is a causative genetic event of *FALI* up-regulation. To investigate the association of SCNA-low-1q-amp with cell cycle regulators, we performed GSEA using TCGA (The Cancer Genome Atlas, <http://cancergenome.nih.gov/>) data. Remarkably, high

TABLE 1. Clinicopathological Characteristics of Patients with Papillary Thyroid Cancer According to Relative *FALI* Expression

	<i>FALI</i> Expression		<i>P</i> Value
	Lowest (n = 30), n (%)	Highest (n = 30), n (%)	
Relative <i>FALI</i> expression	0.21 ± 0.11	3.69 ± 3.29	<0.001*
Age (y), mean ± SD	41.5 ± 13.8	40.7 ± 14.5	0.994*
Gender (F/M)	21/9	22/8	0.774†
Tumor size, cm	2.33 ± 0.87	2.23 ± 0.94	0.696*
T-stage			
T1	6 (20.0)	4 (13.3)	0.097†
T2	0	4 (13.3)	
T3	22 (73.3)	17 (56.7)	
T4	2 (6.7)	5 (16.7)	
Extrathyroidal extension			
No	6 (20.0)	8 (26.7)	0.542†
Yes	24 (80.0)	22 (73.3)	
Multifocality			
No	22 (73.3)	13 (43.3)	0.018†
Yes	8 (26.7)	17 (56.7)	
Lymph node metastasis			
N0	10 (33.3)	6 (20.0)	0.272†
N1a	10 (33.3)	8 (26.7)	
N1b	10 (33.3)	16 (53.3)	
Distant metastasis			
M0	30 (100)	28 (93.3)	0.150†
M1	0	2 (6.7)	
TNM stage group			
I	20 (66.7)	21 (70.0)	0.554†
II	0	1 (3.3)	
III	6 (20.0)	4 (13.3)	
IV	4 (13.3)	4 (13.3)	
BRAF T1799A mutation			
Absent	6 (20.0)	7 (23.3)	0.754†
Present	24 (80.0)	23 (76.7)	

SD = standard deviation.

* *P* values calculated by Student *t* test. Data are mean ± SD.

† *P* values calculated by χ^2 test or linear-by-linear association.

TABLE 2. Multivariate Analysis of the Association of Multifocality with Highest *FAL1* Expression

	Multifocality		
	Odds Ratio	95% CI	P Value
FAL1 Group III*	3.682	1.227–11.050	0.020
FAL1 Group III†	3.552	1.086–11.619	0.036
FAL1 Group III‡	3.610	1.098–11.861	0.034
FAL1 Group III§	4.109	1.041–11.020	0.043
FAL1 Group III	3.314	1.033–10.639	0.044

Adjusted for age and sex.
 CI = confidence interval.
 * Adjusted for age and sex.
 † In addition to adjustment*, adjusted for symptom/sign and pathologic tumor size.
 ‡ In addition to adjustment†, adjusted for extrathyroidal invasion.
 § In addition to adjustment‡, adjusted for T-, N-, M-, and TNM-stage.
 || In addition to adjustment§, adjusted for BRAF T1799A mutation.

expression of *E2F1* in PTC was significantly correlated with coordinately enrichment of positional gene set related to chromosome 1q21 which is the coding region of *FAL1* (Supplementary Figure 3B, <http://links.lww.com/MD/A664>). This correlation of *E2F1* expression with positional gene set of Chr. 1q21 suggested that 1q amplification might be related to high *E2F1* expression.

DISCUSSION

Given the increase in thyroid cancer incidence,¹ the proper management of PTC has become a serious concern.²⁴ Although PTC has a favorable outcome, a small proportion of PTC can progress and finally undergo fatal outcomes.^{4,25} In fact, to improve the clinical outcome of recurrent or persistent PTC, novel therapeutic modalities such as small molecular inhibitors have been investigated rigorously.^{26,27} Moreover, in line with the development of new therapeutic modalities, novel biomarkers to predict clinical outcomes have also been explored.^{28–32}

Recently, several lncRNAs were reported to be genetic predisposition factors or disease progression markers for PTC.³³ For example, loss of *PTCS2* and *PTCS3*, as the names indicate, predisposes to PTC.^{15,16} Loss of heterozygosity in genomic regions harboring NAMA (ncRNA associated with MAP kinase pathway and growth arrest) or up-regulation of *BANCR* contributes to tumor progression.^{17,34} In this study, we investigated the expression of *FAL1* in PTC, as this lncRNA is increased in various cancers such as breast, colon, and ovarian cancers by somatic copy-number alterations (SCNAs).¹⁹ Compatible with this previous report, our qRT-PCR data indicate that *FAL1* is up-regulated in PTC compared to paired contralateral normal thyroid tissues. However, in contrast to the previous report, our data did not show a negative relationship between *FAL1* and p21 in PTC, indicating that the regulatory target of *FAL1* in PTC might be different from that in other cancers. This possibility is supported by the observation that lncRNAs are expressed in a

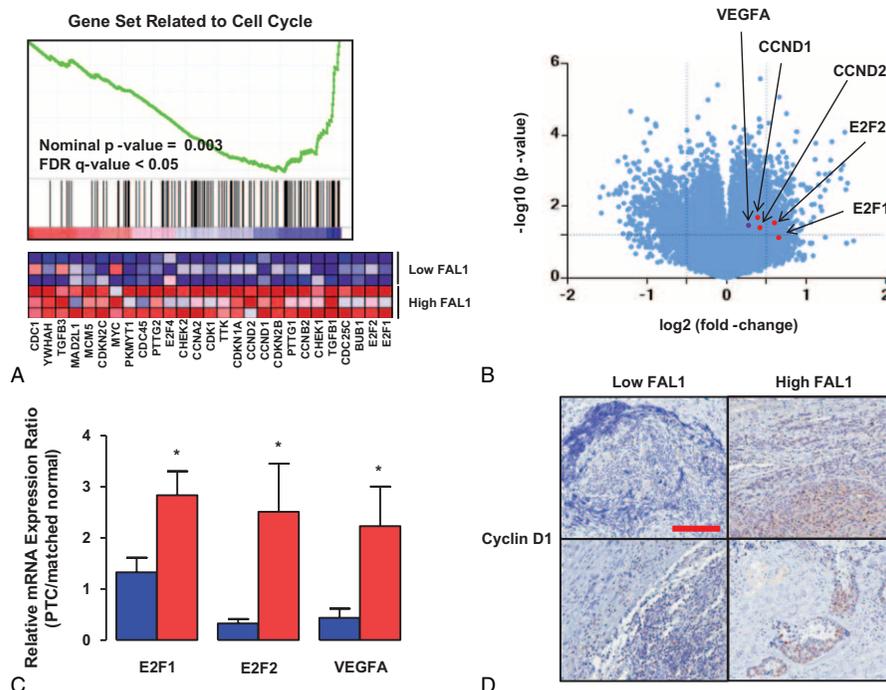


FIGURE 2. Relation of *FAL1* expression with cell-cycle regulators. (A) Gene Set Enrichment Analysis revealed that samples (GSE61763) with high *FAL1* expression ratio (*FAL1/GAPDH*) had up-regulation of genes involved in the cell cycle. (B) Volcano plot of the same data showing protein log₂ fold changes (on the X axis) and the corresponding tempered log₂ P values (on the Y axis). (C) mRNA expression ratios for possible *FAL1* target genes in PTC samples with the lowest (blue) and highest (red) *FAL1* expression in our study population (each group, n = 10). Average ratios were compared with the unpaired *t* test with Welch correction. All data are means ± SEM. **P* < 0.05. The experiment was performed in triplicate and repeated 3 times. (D) Representative photomicrographs of immunohistochemical staining for cyclin D1 among PTC samples with low or high *FAL1* expression (original magnification, ×40; scale bar, 300 μm). PTC = papillary thyroid cancer, SEM = standard error of mean.

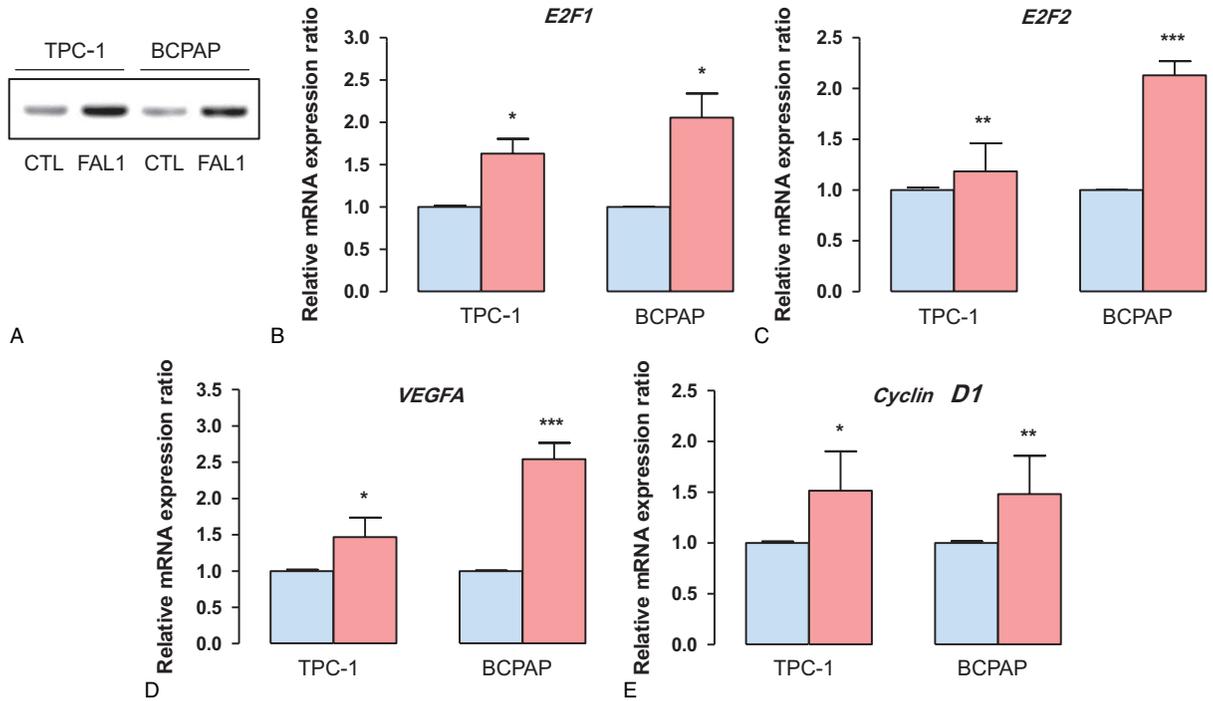


FIGURE 3. Induction of cell-cycle regulators by FAL1 overexpression in thyroid cancer cell lines. (A) TPC1 (PTC cell line harboring RET/PTC1 rearrangement) and BCPAP (PTC cell line harboring BRAFV600E mutation) cells transfected with pcDNA3.1-FAL1 plasmid for 48 h. (B–E) mRNA expression ratios of potential *FAL1* target genes in control (CTL, blue) and FAL1 overexpressed (red) TPC1 and BCPAP cells. Average ratios were compared using the unpaired *t* test with Welch correction. All data are means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. The experiment was performed in triplicate and repeated 3 times. PTC = papillary thyroid cancer, SEM = standard error of mean.

tissue-specific manner.³⁵ In fact, high mRNA expression of p21 was not expected. However, in other types of cancer such as colon, breast, and ovary cancers, p21 positive tumors presented less aggressive tumor behaviors.^{36–38} The biological relevance and prognostic value of p21 in PTC will be necessary to elucidate in future studies.

To understand the clinicopathological significance of *FAL1* expression in PTC, we analyzed the association between *FAL1* expression levels and the patients’ characteristics. Interestingly, these analyses showed that *FAL1* expression increases the risk of tumor multifocality. In fact, multifocality is a predictor of aggressiveness such as intrathyroidal metastasis or disease recurrence.^{39,40} Supporting this idea, we found that a gene set related to the cell cycle was coordinately enriched in high *FAL1* expression group and that 3 genes from this set, namely, *E2F1*, *E2F2*, and *VEGFA*, were up-regulated in our PTC samples. Moreover, PTC with high *E2F1* expression showed coordinately up-regulated expression of genes located on Chromosome 1q21 expression, suggesting 1q amplification might be related to high *E2F1* expression.

E2F1 and *E2F2* are members of the E2F family of transcription factors and bind specifically to RB, indicating that these transcription factors might function as tumor suppressors.^{41,42} However, as they function in a cell-cycle-dependent manner, *E2F1*- and *E2F2*-dependent transactivation induces the expression of genes related to cell-cycle progression.^{43,44} Compatibly, our qRT-PCR and immunohistochemical staining data clearly show that PTC with high *FAL1* expression have not only high *E2F1* and *E2F2* mRNA expression but also high *VEGFA* mRNA and high cyclin D1 protein levels. *VEGFA* and

cyclin D1 are important molecular markers of cellular proliferation in PTC.⁴⁵ In fact, most lncRNAs including *FAL1* are located in the nucleus and work as chromosome modifiers, ribonucleoprotein components, miRNA sponges, or mRNA stabilizers.⁴⁶ It was previously shown that *FAL1* associates with BMI1 and regulates its stability to repress p21.¹⁹ In line with these actions of this lncRNA, we postulate that *FAL1* in PTC integrates with protein complexes such as chromatin-modifying complexes or ribonucleoprotein components. Therefore, our future work should investigate a possible role of *FAL1* in cell-cycle regulation.

Recently, several small molecular inhibitors, such as vemurafenib, dabrafenib, and trametinib, have been developed to treat recurrent or persistent PTC. However, because de novo and acquired drug resistance has emerged, new investigations are needed to determine the therapeutic efficacy of broad-range RAF inhibitors or combination therapies employing 2 drugs with different action mechanisms.^{5,47–49} Although the study of lncRNA is in its infancy, we are cautiously anticipating that lncRNAs such as *FAL1* will become a new therapeutic target. Unfortunately, to the best of our knowledge, lncRNA inhibitors have not yet been used in clinical practice. However, strategies for therapeutic manipulation of lncRNAs have been developed. For example, targeting natural antisense transcripts (NATs) with single-stranded therapeutic oligopeptides termed “antagonAT” can cause mRNA de-repression.^{50,51}

The limitations of our study include its retrospective nature and the small number of study patients. In addition, the mechanism by which *FAL1* induces mRNA expression of *E2F1*, *E2F2*, *VEGFA*, and *cyclin D* remains to be elucidated.

In summary, PTC samples had increased expression of *FAL1* compared to paired normal thyroid tissues. Up-regulation of *FAL1* is related to an increase in expression of genes such as *E2F1*, *E2F2*, *VEGFA*, and *cyclin D1*, which can promote cellular proliferation and make the tumor more aggressive. In the clinical setting, up-regulation of *FAL1* is associated with aggressive tumor features such as multifocality. Taken together, our data suggest that *FAL1* is a potential biomarker to predict poor clinical outcomes and a molecular target for the treatment of aggressive thyroid cancer.

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