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

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Abstract: Recent functional genomic studies revealed that the oncogenic activity of focally amplified IncRNA 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 IncRNA 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 IncRNA 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.

Editor: Shihan He.
Received: July 15, 2015; revised: December 8, 2015; accepted: December 29, 2015.
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Supplemental Digital Content is available for this article. SJ and JL contributed equally to this work.
This study was supported by National Research Foundation of Korea (NRF) grants funded by the Korean Government (MEST) (2014R1A1A2059343).
The authors have no conflicts of interest to disclose.
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ISSN: 0025-7974
DOI: 10.1097/MD.000000000002592

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.5,8 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 non-coding RNAs (lncRNAs). By definition, lncRNAs are nonprotein-coding transcripts that are longer than 200 nucleotides. 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 an lncRNA that is downregulated in PTC, affecting the expression of genes functionally related to the cell cycle and cancer. In addition, the polymorphism rs944289 on the PTC susceptibility candidate 3 (PTSC3) gene, which also encodes an lncRNA, predisposes to PTC, indicating that PTSC3 functions as a tumor suppressor. Moreover, the BRAF-activated lncRNA (BANCR) is upregulated in PTC compared to matched normal tissue, and BANCR overexpression induces cell proliferation via autophagy regulation.

Functional genomic studies recently revealed that the oncogenic activity of focally amplified lncRNA on chromosome 1 (FAL1, ENSG00000228126) contributes to the repression of p21 expression in different human cancers such as ovarian cancers. Mechanistically, FAL1 binds to the BMI1 proto-oncogene (BMI1), a component of the polycomb repressive complex 1 (PRC1). This direct interaction between FAL1 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 (CFIP2). Among those targets, CDKN1A, also called p21, inhibits cyclin-dependent kinase (CDK) 1, 2, and 4/6 complexes, thereby inhibiting cell cycle progression and promoting the G1/S transition. 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. Taken together, the results suggest that repression of p21 expression by FAL1 may increase CDK activity, promote RB phosphorylation and E2F transactivation, and finally promote the G1/S transition.

However, the role of FAL1 expression has never been studied in PTC. Therefore, we have investigated FAL1 expression in PTC and in paired contralateral normal thyroid tissues. In addition, we explored potential targets of FAL1 in PTC using Gene Set Enrichment Analysis and analyzed the clinicopathological significance of this lncRNA in patients with PTC.

**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 MEM (Invitrogen) with 10% FBS at 37 °C in a humidified incubator with 5% CO2. To generate plasmid expressing FAL1, sense (5′-CTG CTG ATT GGC CGG TTC GCT GG-3′) and anti-sense (5′-CGG GGC GCA GCC GAC GCC ATC ACA GTA TGC TCC TGT GTA ATA GCC 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 Green RT-PCR kit (Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) were used to culture the cell lines TPC-1 and FTC-133. The 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 MEM (Invitrogen) with 10% FBS at 37 °C in a humidified incubator with 5% CO2. To generate plasmid expressing FAL1, sense (5′-CTG CTG ATT GGC CGG TTC GCT GG-3′) and anti-sense (5′-CGG GGC GCA GCC GAC GCC ATC ACA GTA TGC TCC TGT GTA ATA GCC 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).

**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.

**METHODS**

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**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-p-Actin Antibody (#4967, Cell Signaling, Danvers, MA). 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 FAL1 expression ratios compared to internal control (GAPDH) by the protocols previously described. 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). IncRNA 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. 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 an IncRNA-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 al 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), 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
FAL1 expression to the 30 with the lowest FAL1 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 FAL1 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 FAL1 expression may affect tumor behavior and generate aggressive features of PTC.

### Discovery of FAL1 Target Genes

Because we did not find a negative relationship between FAL1 and p21, we aimed to find new FAL1 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 FAL1 up-regulation group (Figure 2A). In addition, the mTOR signaling pathway was also related to FAL1 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 FAL1 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 FAL1 expression. Compatibly, mRNA expression ratios between PTC and matched normal thyroid tissue were higher for E2F1, E2F2, and VEGFA among samples with high FAL1 expression (Figure 2C). Furthermore, immunohistochemical staining also showed cyclin D1 up-regulation among PTC samples with the highest FAL1 expression (Figure 2D). To verify the FAL1 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-FAL1 plasmid (Figure 3A). Consistently with the GSEA and qRT-PCR data, we confirmed that E2F1, E2F2, VEGFA, and cyclin D1 were significantly induced by FAL1 overexpression in both cell lines (Figure 3B–E).

### TABLE 1. Clinicopathological Characteristics of Patients with Papillary Thyroid Cancer According to Relative FAL1 Expression

| Relative FAL1 expression | Lowest (n = 30), n (%) | Highest (n = 30), n (%) | $P$ Value |
|--------------------------|-----------------------|------------------------|-----------|
| Relative FAL1 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$^1$ |
| Tumor size, cm           | 2.33 ± 0.87           | 2.23 ± 0.94            | 0.696$^*$ |
| Extrathyroidal extension | 6 (20.0)              | 8 (26.7)               | 0.542$^1$ |
| Lymph node metastasis    | 10 (33.3)             | 6 (20.0)               | 0.272$^1$ |
| Distant metastasis       | 10 (33.3)             | 8 (26.7)               | 0.150$^1$ |
| BRAF T1799A mutation     | Absent                | Present                | 0.754$^1$ |

$SD$ = standard deviation.

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

$^1$ $P$ values calculated by $\chi^2$ test or linear-by-linear association.
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.

**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.
* 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.

**DISCUSSION**

Given the increase in thyroid cancer incidence,1 the proper management of PTC has become a serious concern.2,4 Although PTC has a favorable outcome, a small proportion of PTC can progress and finally undergo fatal outcomes.3,25 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.17,18 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...
Interestingly, these analyses showed that expression levels and the patients’ characteristics. Inter-
FAL1 expression in PTC, we analyzed the association between and prognostic value of p21 in PTC will be necessary to elucidate in future studies.

p21 positive tumors presented less aggressive tumor behaviors.36–38 The biological relevance of genes related to cell-cycle progression.43,44 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 predictor of aggressiveness such as intrathyroidal metastasis or disease recurrence.39,40 Supporting this idea, we found that a

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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 IncRNAs 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 IncRNA, 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.

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 D1 remains to be elucidated.
In summary, PTC samples had increased expression of \textit{FAL1} compared to paired normal thyroid tissues. Up-regulation of \textit{FAL1} is related to an increase in expression of genes such as \textit{E2F1}, \textit{E2F2}, \textit{VEGFA}, and \textit{cyclin D1}, which can promote cellular proliferation and make the tumor more aggressive. In the clinical setting, up-regulation of \textit{FAL1} is associated with aggressive tumor features such as multifocality. Taken together, our data suggest that \textit{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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