이학박사 학위논문

Driver Roles of a Novel Fusion Gene Discovered in Thyroid Carcinomas

갑상선암에서 발견한 융합유전자의 원인유전자로서의 역할 연구

2014 년 2 월

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협동과정 종양생물학전공

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Driver Roles of a Novel Fusion Gene Discovered in Thyroid Carcinomas

by

Hyeon-Gun Jee

A thesis submitted to the Interdisciplinary Graduate Program in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Tumor Biology at Seoul National University College of Medicine

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논문 제목: Driver Roles of a Novel Fusion Gene Discovered in Thyroid Carcinomas

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ABSTRACT

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Introduction: Thyroid cancer incidence is increasing. Follicular variant papillary thyroid carcinomas (fvPTC) are hard to diagnose and genetic alterations are largely unknown. Discovery of novel alteration(s) might aid diagnosis of fvPTCs.

Methods: RNA sequencing was performed on 70 cases of thyroid carcinoma tissues, including 9 fvPTCs. Findings of fusion transcripts were validated by RT-PCR in additional 20 cases of fvPTC samples. Structural analysis was performed to determine the fusion transcript's driver role in oncogenesis. A cell line model has been generated and its transforming phenotype was examined in soft agar assays. Phospho-kinase assay was performed to reveal the underlying molecular mechanisms.

Results: A novel EZR-ERBB4 fusion has been discovered. It

was mutually exclusive with previously known thyroid oncogenes, and structurally a driver function has been expected. In a cell line model the fusion gene showed oncogenic phenotype. Phosphorylation of STAT3 was associated with the transformation.

Conclusion: Based on the results a driver role of EZR-ERBB4 might be considered in fvPTCs.

Keywords: Fusion gene, Thyroid cancer, Follicular variant papillary thyroid carcinomas, Driver alteration, Diagnosis
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LIST OF ABBREVIATIONS

cDNA, complementary deoxyribonucleic acid

DNA, deoxyribonucleic acid

FTC, follicular thyroid carcinoma

fvPTC, follicular variant papillary thyroid carcinoma

EGFR, epidermal growth factor receptor

PTC, papillary thyroid carcinoma

RNA, ribonucleic acid

RT-PCR, reverse transcription polymerase chain reaction

INTRODUCTION

World-wide incidence of thyroid carcinomas is increasing and the increase is also eminent in Korea where thyroid carcinoma incidence is ranked as number one since 2009 [1]. In the USA, the incidence of thyroid carcinomas tripled from 5 to 15 per 100,000 during the last three decades [2]. Advances in diagnostic tools such as ultrasound imaging may explain this phenomenon to some extent, but studies suggest that large sized thyroid tumors are also becoming more prevalent, implying overall increase of the thyroid malignancy prevalence itself [3]. The most prevalent form of thyroid cancer is papillary thyroid carcinoma (PTC), and a number of genetic alterations are reported to play driver functions of the malignancy. The most often found is BRAF T1799A point mutation found in 40-60% of PTC patients, followed by RET/PTC1 fusion (10-20%), and RAS point mutation (5-10%) [2-6]. However, the driving mutations, or molecular events, of approximately 10-45% of PTC are still unknown (Figure 1).

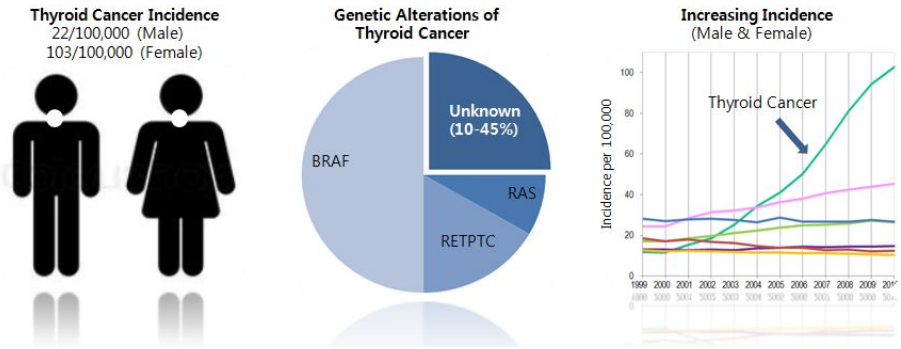

Figure 1. Thyroid Carcinoma Overview

The incidence of thyroid carcinomas is increasing and 10-45% of them are without known genetic alterations. Statistics are from National Cancer Information Center of Korea.

There are several subtypes of PTCs including follicular, tall-cell, and diffuse sclerosing variants. The follicular variant papillary thyroid carcinomas (fvPTC) are the second prevalent subtype after classical PTCs and it composes 9-32% of all PTCs [4]. Although still controversial, fvPTCs are thought to have less aggressive clinical outcomes when compared to that of classical PTCs [5]. As the name implies, fvPTCs are PTCs which tend to also have characters related to follicular thyroid carcinomas (FTC), which are often derived from cells with RAS mutation or PAX8-PPARg fusion. Genetic alterations typical of classical PTC such as BRAF mutations or RET/PTC fusions are less prevalent in fvPTCs and higher percentage of RAS mutations or PAX8-PPARg fusions are found [5]. Still, a large number of fvPTCs are without known genetic alterations, and these are yet to be discovered (Table 1).

Table 1. Genetic alterations in follicular variant papillary thyroid carcinomas

Author	# of fvPTC cases	BRA T1799A	RET/PTC	RAS	PAX8-PPARg
Zhe et al. (2003)	30	ND	3%	43%	0%
Adeniran et al. (2006)	30	7%	3%	47%	ND
Castro et al. (2006)	40	10%	ND	25%	38%
Lupi et al. (2007)	113	19%	ND	ND	ND
Oler et al. (2007)	47	19%	ND	ND	ND
Santarpia et al. (2009)	30	13%	ND	ND	ND
Rivera et al. (2010)	47	10.6%	4.2%	25.5%	4.2%
Santarpia et al. (2010)	30	10%	20%	17%	17%
Park et al. (2013)	132	33%	ND	27%	ND

Abbreviation: ND, not determined. Data is modified from a previous article by Daniels et al. [5]

The diagnosis of thyroid carcinomas is largely dependent of fine needle aspiration (FNA) biopsy, where isolated cells are cytologically examined to give diagnosis [6]. As is with all diagnostic tools, quite a number of cases are undeterminable by FNA biopsy. Recent approaches are molecular diagnosis of these hard-to-decide cases, and genetic alterations specific to thyroid carcinomas play an important role. Molecular diagnosis from FNA samples is a rising field of translational medicine both academically and industrially [7]. Presence of a thyroid specific genetic alteration in DNA/RNA isolated from an FNA biopsy sample helps clinicians to clarify the malignant status of the nodule, resulting in extent of surgery and post-operational treatment [7]. The problem is, however, indeterminate rate of fvPTCs is much higher than that of classical PTCs. The overall diagnosis rate of classical PTC ranges between 58-93%, but that of fvPTC is only 25-37% [8-11]. This is because fvPTCs do not have a cytologically distinguishable morphology and there is still approximately 30-50% of fvPTCs with no molecular marker described [5]. Considering the large proportion of fvPTCs among the thyroid carcinoma patients, discovery and

development of a molecular marker for fvPTCs may be of great clinical importance.

MATERIALS AND METHODS

1. Patients and specimen (for RNA sequencing)

Patients who were diagnosed with thyroid carcinoma and underwent thyroidectomy in Seoul National University Hospital (SNUH) were asked to participate in the study. Fifty-four cases of classical PTCs, 9 cases of fvPTCs, 5 cases of FTCs, and 2 cases of follicular adenoma specimens were collected and used for RNA sequencing (Table 2). Core part of carcinoma tissue was collected from surgically a removed thyroid tumor and was immediately frozen in liquid nitrogen. Entire process of specimen handling, including selection of patients, collection of tissue, labeling of each sample, and storage in liquid nitrogen container was performed in accordance to standard operating procedure and was approved by the Institutional Review Board of SNUH (IRB approval number: H-0809-097-258). Informed consent was acquired from all participants.

Table 2. Thyroid carcinoma tissue subtypes used in the study

Tissue Subtype	Number
PTC, classical	54
PTC, follicular variant	9
Follicular Thyroid Carcinoma	5
Follicular Adenoma	2

Abbreviation: PTC, Papillary Thyroid Carcinoma

2. Patients and specimen (for RT-PCR validation)

Additional 20 cases, all BRAF wild type fvPTCs, were collected for reverse transcription polymerase chain reaction (RT-PCR) validation. BRAF T1799A mutation analysis, upon informed consent, is routinely performed at Seoul National University Hospital to supplement the permanent diagnosis of surgically dissected thyroid carcinoma tissues.

3. RNA sequencing and fusion transcript analysis

Isolation of RNA from frozen tissues was performed using the QIAcube and RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was assessed for quality and concentration using an RNA 6000 Nano LabChip on a 2100 Bioanalyzer (Agilent Inc., Palo Alto, CA). The sequencing libraries were prepared as described in previous reports and sequenced through HiSeq 2000 (Illumina, San Diego, CA) [12]. (Experimental procedures were performed through the courtesy of Seungbok Lee)

4. Fusion transcript analysis and ERBB4 mRNA expression analysis

Two independent fusion analysis programs TopHat-Fusion and deFuse were used for discovery of fusion transcripts in the specimen [13, 14]. The sequenced reads were manually aligned to ERBB4 of a human reference genome (hg19) using the TopHat2 alignment program. (Experimental procedures were performed through the courtesy of Seungbok Lee)

5. cDNA preparation for validation

From the 20 cases of BRAF wild type fvPTC carcinoma tissue samples RNA was extracted as is described above. Isolated RNA was reverse transcribed using 1:1 mix of oligo dT and random hexamer priming and an MMLV reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA).

6. Reverse transcription polymerase chain reaction (RT-PCR)

Forward and reverse primers were designed using PerlPrimer (version 1.1.21, http://perlprimer.sourceforge.net) Primer sequences were as follows: forward, 5'-GGA GTT GAT GCC CTT GGA C-3'; reverse, 5'-AAA CCG TTC CAA AAG CAC CT-3'. The primer set amplifies an 808 base pair fragment.

AmpliTaq Gold polymerase was used (Perkin-Elmer, Foster City, CA, USA). The reaction was performed at 2 mM MgCl2, 0.2 μM dNTP, and 0.2 μM of each primer. Annealing temperature for the templates was 60 ℃ and the 40 cycles were run. The RT-PCR products were visualized by a DNA dye (LoadingStar, DyneBio, Seoul, Korea) on a 2% agarose gel (SeaKem, Lonza, Basel, Switzerland) using Tris-borate-EDTA buffer system. Gel electrophoresis was performed on an electrophoresis system (Mupid, Tokyo, Japan) A plasmid containing EZR-ERBB4 fusion transcript was used as a positive control.

7. Sanger sequencing

PCR products were isolated from agarose gel using a DNA purification kit (HiYield DNA fragment extraction kit, Real Genomics, New Taipei City, Taiwan). When necessary, purified DNA was inserted into a T vector for TA cloning (TOPO TA cloning kit, Invitrogen, Carlsbad, CA, USA). DH5alpha E. coli containing the PCR products were grown in lysogeny broth (LB) and plasmid DNA was isolated for Sanger sequencing (Qiagen Plasmid Mini kit, Hilden, Germany).

8. Cell culture

Human thyroid carcinoma cell line TPC1 was generous gift from Dr. Orlo Clark. Another thyroid carcinoma cell line 8505c was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Immortalized human thyrocyte Nthy-ori 3-1 (Nthy) was purchased from European Collection of Cell Cultures (ECACC, Salisbury, UK). These cell lines were cultured in RPMI 1640 media (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Nthy cells expressing exogenously incorporated target genes were cultured in the same media composition. HEK293FT cells for lentivirus production were from Invitrogen (Invitrogen, Carlsbad, CA, USA). HEK293FT was cultured in DMEM (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell lines were maintained in 37 °C humidified incubator supplied with 5% CO_2.

9. Cloning of EZR-ERBB4 fusion transcript

The complete sequence of EZR-ERBB4 fusion transcript was derived from the RNA sequencing data. There are 2 transcript variants of ERBB4 gene, a component of the fusion transcript, the shorter one missing exon 26. Exonal mRNA expression analysis revealed exon 26 expression was similar to that of adjacent exons, implying the major form of the fusion transcript is composed of the longer transcript variant of ERBB4 (Figure 2). The whole fusion transcript was synthesized from a nucleotide synthesis company (Bioneer, Daejeon, Korea). Synthesized gene was inserted into pCDH-MCS-T2A-copGFP-MSCV plasmid vector (System Biosciences, CA, USA) using restriction enzymes (EcoRI and NotI) and T4 ligase (Enzynomics, Daejeon, Korea). pCDH-MCS-T2A-copGFP-MSCV plasmid vector contains sequences necessary for lentivirus production such as 5' and 3' LTR, cPPT, and WPRE. The target transcript sequence was inserted into the multiple cloning site which is connected to a T2A and copGFP sequences. The entire fusion sequence was confirmed by Sanger sequencing after insertion into the plasmid.

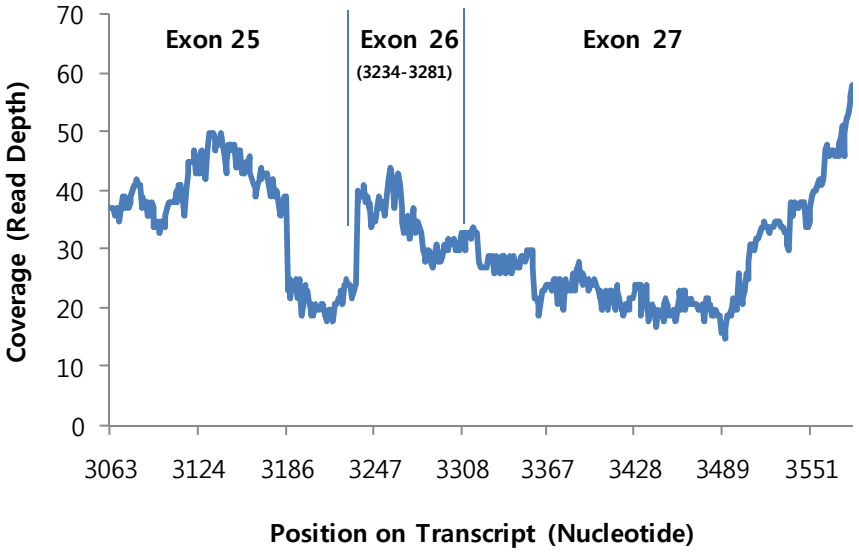

Figure 2. ERBB4 transcript variants

Messenger RNA expression level of exon 26, an exon often missing in the alternative isoform of ERBB4, was analyzed based on sequence coverage data to determine the major isoform of ERBB4 used as a fusion partner of EZR.

10. Cloning of BRAF T1799A and RET/PTC1

Protein coding sequences of BRAF gene was amplified by RT-PCR from 8505c cells. 8505c is a thyroid undifferentiated carcinoma cell line with BRAF T1799A point mutation. Each end of primers contained restriction enzyme sites (NheI and NotI) for insertion into plasmid. The amplified products were ligated into pCDH-MCS-T2A-copGFP-MSCV plasmid vectors. Cloning of RET/PTC1 followed similar procedures, only that TPC1, a poorly differentiated papillary thyroid carcinoma cell line, was used. The entire protein coding sequence was confirmed by Sanger sequencing for each gene after insertion into the plasmid. Lentivirus production followed identical steps to that of EZR-ERBB4.

11. Production of lentivirus particles

Packaging plasmid psPAX2 encodes factors necessary for lentivirus production. Envelope plasmid pMD2.G encodes vesicular stomatitis virus G glycoprotein. psPAX2 and pMD2.G plasmids are from a non-profit plasmid repository (Addgene, Cambridge, MA, USA) and are originally developed by Dr. Didier Trono. HEK293FT is a fast growing clone of HEK293

and is widely used for lentivirus generation. HEK 293FT cells were purchased from Invitrogen (Carlsbad, CA, USA). HEK293FT was cultured on poly-L-lysine coated T75 flasks until 80% confluence was reached. The optimized coating concentration of poly-L-lysine was 7.5 μg/cm² and coating time was 30 minutes at room temperature. Mixture of the above-mentioned 3 plasmids was co-transfected into HEK293FT using Lipofectamine™ 2000 according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). The culture supernatant with lentiviral particles containing the target gene sequence was collected twice at 48 h and 72 h. After removal of floating cells and debris by a brief centrifugation, the lentivirus containing supernatant was ultra-centrifuged at 40,000 xg for 3 h on a 20% sucrose cushion. The purified lentivirus was suspended in a phenol-red free RPMI media and was stored frozen until use. All lentivirus handling procedures were performed in a Bio-Safety Level-II facility according to the guidelines of Ministry of Education and Science of Korea (BSL-II permit number LML08-986).

12. Generation of stable cells expressing target genes

The produced lentivirus containing target genes EZR-ERBB4, BRAF T1799A or RET/PTC1 were treated for 6-24 h on human normal thyrocyte Nthy-ori 3.1. Multiple lentiviral titers were tested and the titer which produced approximately 90% GFP positive cells at day 7 was selected for further analysis. The transfected cells were sorted between day 7 and 14 based on GFP fluorescence intensity using FACS Aria (BD Biosciences, San Jose, CA, USA). Sorting conditions were adjusted so that all cell lines possess similar expression level of the marker protein copGFP.

13. Soft agar transformation assay

For soft agar transformation assay, a commercial kit was used and 5,000 cells were seeded on a 48-well culture plate in a soft agar (Cell Biolabs, San Diego, CA, USA). Base agar was plated and solidified before seeding of the cells to prevent attachment to the bottom of the plate. Top agar, which contain cells, were placed on top of the bottom agar. After solidification of top agar, complete culture media was added on top. The media was replaced at every 3-4 days until day 14 and colonies formed within the agar matrix were counted.

Photographs were taken using a digital camera (Canon IXUS 860, Tokyo, Japan) connected to an inverted microscope (Olympus CKX41, Tokyo, Japan).

14. Phospho-kinase assay

The assay is a mixture of immunoblotting and ELISA techniques, with phosphorylation site specific antibodies are bound on a membrane and target proteins are bound and detected by chemiluminescence in a way similar to sandwich-ELISA. Phospho-kinase antibody assay was performed following the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). For detection of chemiluminescence signals, an image analyzer LAS-3000 was used (BioRad, Hercules, CA, USA).

15. Statistical analysis

IBM® SPSS® Statistics version 20 was used for statistical analyses (SPSS, Chicago, IL, USA). Independent sample t-test was performed and P value of less than 0.05 was considered as statistically significant.

RESULTS

A novel fusion transcript was discovered through RNA sequencing

The sequencing data was analyzed for the fusion transcripts using DeFuse and TopHat-Fusion programs. The fusion candidates were considered significant only when noted by both of fusion analysis programs, finally yielding 3 in-frame fusion transcripts (Table 3). Among them, CCDC6-RET of a classical PTC and PAX8-PPARg of a FTC were previously reported fusions. A novel fusion of EZR and ERBB4, however, was discovered in 1 out of 9 fvPTC cases and was chosen for the further study (Figure 3). The fusion transcript was created by rearrangement of chromosome 2 and chromosome 6. EZR Exon 11 and ERBB4 exon 18 was fused in-frame to create a 3.2 kb transcript. An increased mRNA expression of ERBB4 was observed from exon 18, implying the increase was caused by the fusion event (Figure 4).

Table 3. Fusion transcripts discovered by RNA sequencing

Sample	Gene A	Chromosome A	Gene B	Chromosome B	TopHat-Fusion		deFuse	
					spanning reads, No.	spanning mate pairs, No.	spanning reads, No.	spanning mate pairs, No.
T23	*CCDC6*	chr10	*RET*	chr10	63	12	68	42
T51	*PAX8*	chr2	*PPARG*	chr3	446	88	524	270
T59	*EZR*	chr6	*ERBB4*	chr2	9	7	17	19

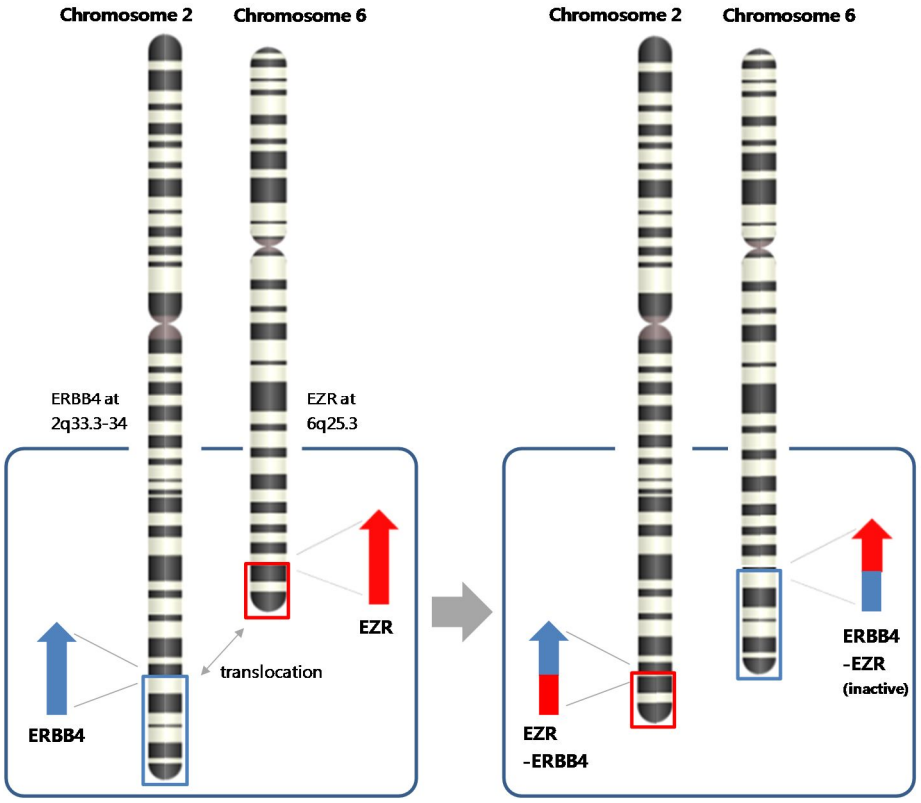

Figure 3. EZR-ERBB4 fusion overview

Interchromosomal rearrangement between chromosome 6 and chromosome 2 occurred resulting in an in-frame fusion of EZR and ERBB4 genes (predicted from transcriptome analysis data).

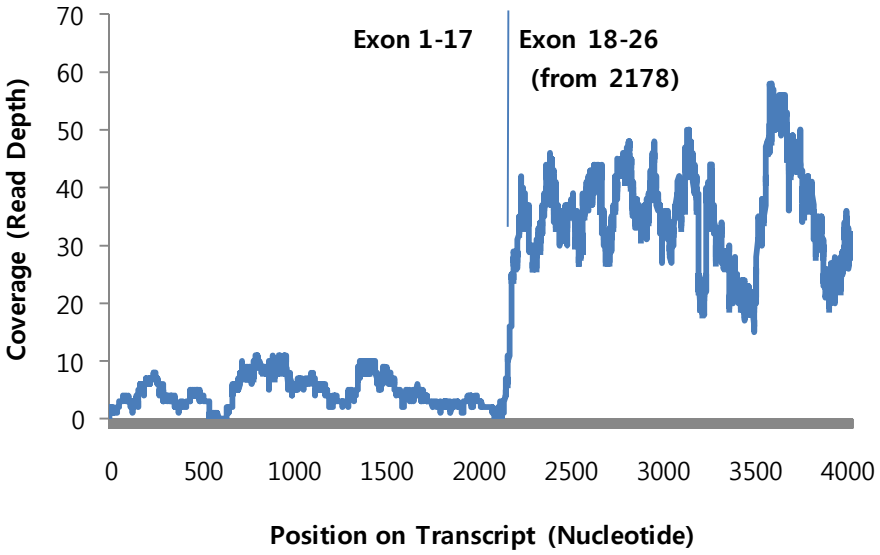

Figure 4. Exonic mRNA expression of ERBB4

Messenger RNA expression level of exons adjacent to the fusion site of ERBB4 was depicted based on sequence coverage.

Validation of EZR-ERBB4 presence in fvPTC samples

The presence of EZR-ERBB4 was validated in 20 additional cases of snap-frozen fvPTC specimens by RT-PCR and Sanger sequencing. These fvPTCs were all BRAF wild type. Among the 20 cases examined, 4 yielded positive PCR amplification for the fusion transcript (Figure 5). The PCR products were purified and the inclusion of the fusion sequence was confirmed by Sanger sequencing (Figure 6).

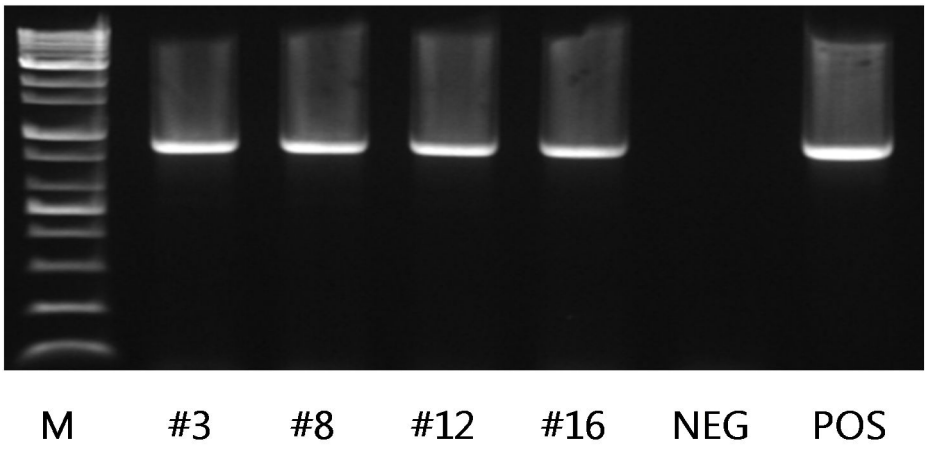

Figure 5. Validation of EZR-ERBB4 by RT-PCR

Fusion transcript specific primers were designed and the presence of fusion transcripts was analyzed by RT-PCR in follicular variant papillary thyroid carcinoma tissue samples. Forward primer is located in the EZR gene, and the reverse primer is located in the ERBB4 yielding an 808 bp PCR product. Lanes 2-5 indicate sample IDs used for validation. A plasmid containing EZR-ERBB4 was used as a positive control. M, size marker; NEG, negative control (no template); POS, positive control (plasmid)

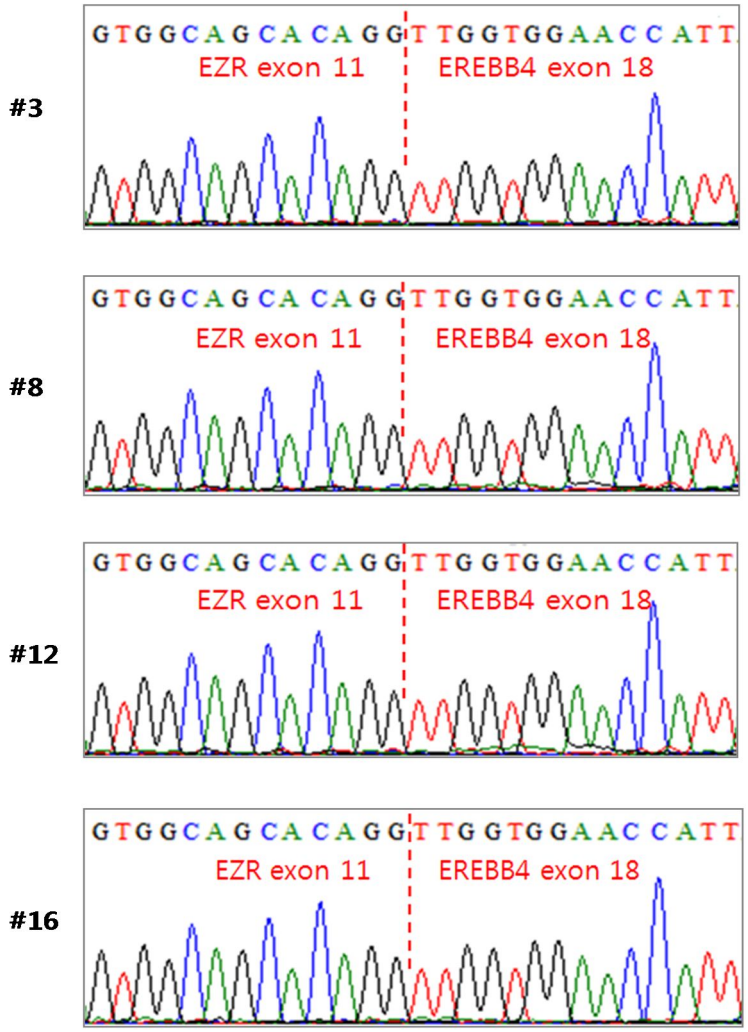

Figure 6. Sanger sequencing confirmation of RT-PCR products

RT-PCR products were purified and were the fusion transcript sequence was confirmed by Sanger sequencing.

Possibility of EZR-ERBB4 fusion as a driver alteration: mutual exclusivity

Mutual exclusivity is an important concept when the driver role of a genetic alteration is considered. Previously reported genetic alterations of thyroid carcinomas include BRAF point mutations, RAS point mutations, RET/PTC fusions, PAX8-PPARg fusions, and a number of minor alterations. All these oncogenes are mutually exclusive. Among the RNA sequenced cases, the one with EZR-ERBB4 fusion transcript was without any of the previously known thyroid carcinoma specific alteration, and none of the cases with other genetic alterations contained the fusion transcript (data included in detail in a separate manuscript in preparation,).

Possibility of EZR-ERBB4 fusion as a driver alteration: inclusion of a kinase domain

In case of fusion transcripts, the preservation of a kinase domain which activates survival signals is important for its driver role. For the newly discovered EZR-ERBB4 fusion, the ERBB4 gene was fused in-frame and the amino acid sequences were unchanged. ERBB4 contains a tyrosine kinase domain and

it is included in the EZR-ERBB4 fusion transcript without any disruption of the sequence (Figure 7). Furthermore, EZR protein is known to form homo-dimers and in EZR FERM-C domain a predicted coiled-coil sequence is present. The domain is known to be responsible for homo-dimerization of several proteins including RET/PTC fusion proteins [15]. It is plausible to expect that the novel EZR-ERBB4 fusion might be a driver alteration of fvPTC owing to constitutive activation of its protein kinase domain by homo-dimerization in a way similar to that of RET/PTC fusions, at least when the protein domain structure is considered.

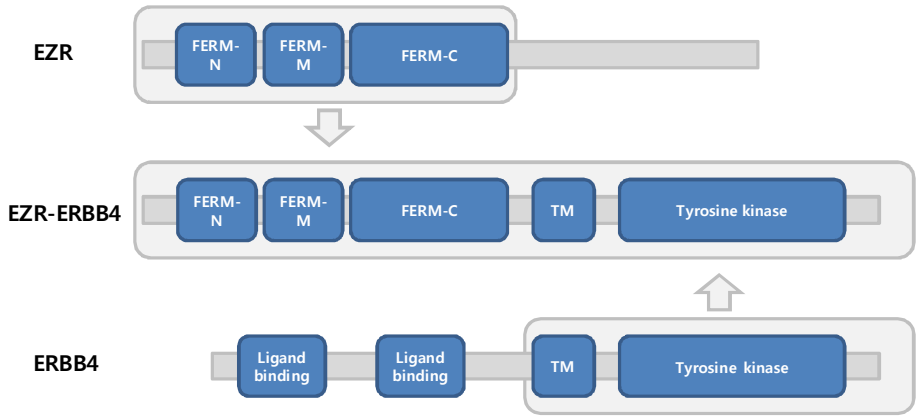

Figure 7. Protein domains of the EZR-ERBB4 fusion gene

Protein domains of EZR and ERBB4 are depicted. FERM-C domain contains a predicted coiled-coil sequences used for homodimerization. A tyrosine kinase is preserved in ERBB4. FERM, F for 4.1 protein, E for ezrin, R for radixin and M for moesin; TM, transmembrane domain.

EZR-ERBB4 cells show anchorage-independent growth in soft agar

To determine the driver role of the fusion transcript, a cell line model ectopically expressing the EZR-ERBB4 has been generated. When EZR-ERBB4 expressing cells (EEt) and vector only control cells (Vt) were compared, EEt showed a smaller cytosol to nucleus ratio upon observation on a microscope. EEt cells also showed a tendency to tightly aggregate with each other. Vt cells did not have any morphologic difference with the parental Nthy cells (Figure 8). Anchorage-independent growth capability, which is a prerequisite factor for oncogenesis, of Vt and EEt was measured by soft agar transformation assay. EEt cells had more number of colonies proliferating in soft agar and the size of each colony was larger than that of Vt cells, implying that EEt cells are more adaptable to anchorage independent growth (Figure 9). This finding supports, at least partially, the driver role of EZR-ERBB4 fusion for thyroid carcinoma development.

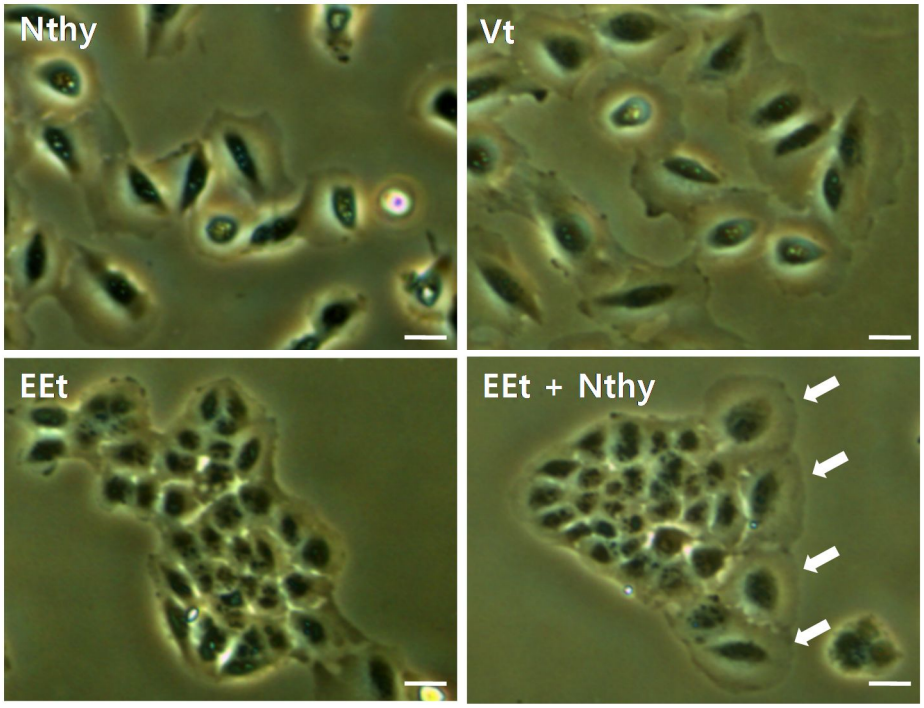

Figure 8. Morphology of cells with EZR-ERBB4 fusion

Cells stably transduced with EZR-ERBB4 fusion transcripts, as well as its vector-only control, were photographed using a inverted microscope. Scale bar indicates 50 μm. Arrows indicate Nthy cells co-cultured with EEt cells. Nthy, Nthy-ori 3.1; Vt, vector only control; EEt, EZR-ERBB4 expressing cells

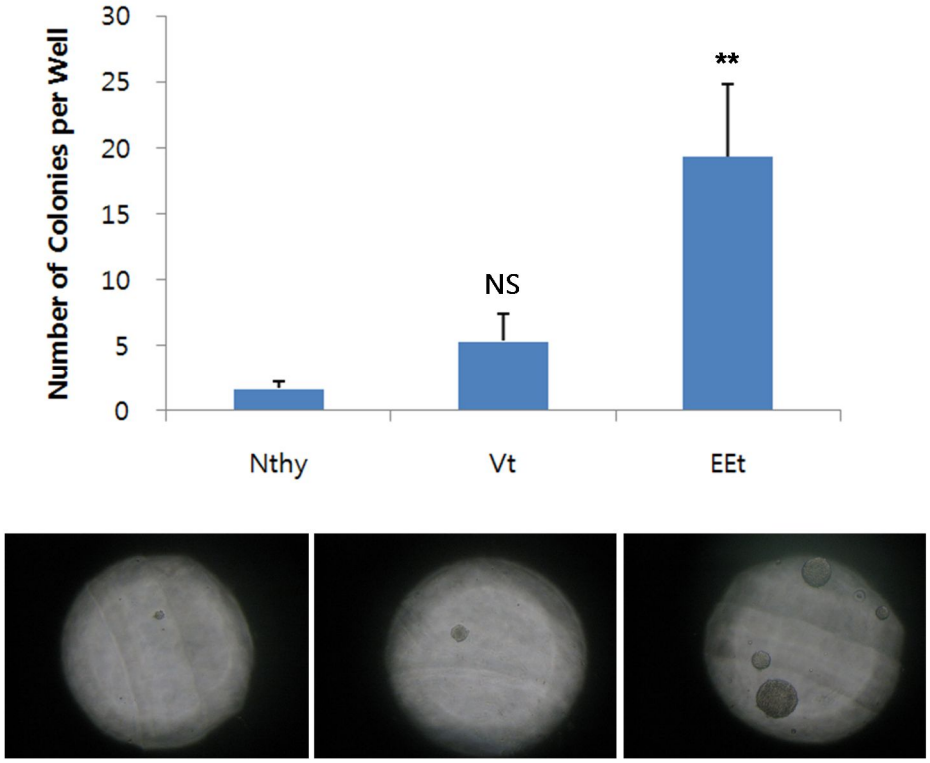

Figure 9. Soft agar transformation ability of EZR-ERBB4 expressing cells

The parental cells (Nthy), vector-only control cells (Vt), and EZR-ERBB4 expressing cells (EEt) were grown in soft agar for 14 days. Number of the colonies was counted and photographs were taken using a digital camera connected to an inverted microscope. (NS, not significant; **, $p < 0.01$ vs Nthy)

STAT3 was phosphorylated in EZR-ERBB4 cells

To examine underlying mechanisms of cellular transformation, phosphorylation status of kinases involved in oncogenesis was screened using a human phospho-kinase antibody array (R&D Systems, Minneapolis, MN, USA). Vt and EEt were compared, in the presence and absence of serum, and significantly increased phosphorylation of STAT3 was observed at serine 727 (S727) (Figure 10). There was no increase in phosphorylation status of tyrosine 705 (Y705) of STAT3. No significant change in STAT2 (Y689), STAT5a (Y694), STAT5b (Y699), or STAT6 (Y641) was found. Phosphorylation of STAT3 (S727) was also eminent in the serum free condition. There was a minimal phosphorylation of ERK1/2 or AKT1/2/3 levels which are considered to be important in thyroid carcinogenesis. These data raises a possibility that EZR-ERBB4 drives thyroid oncogenesis by phospho-STAT3 dependent mechanisms.

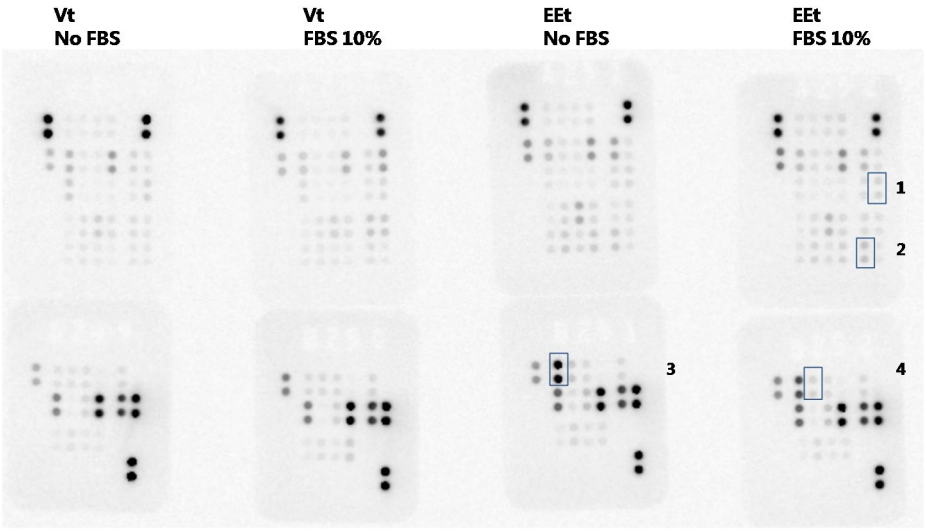

Figure 10. Phospho-kinase assay reveals phosphorylation of STAT3

Protein lysates of vector-only control cells (Vt) and EZR-ERBB4 expressing cells (EEt) were subjected for a phospho-kinase antibody assay. 1, ERK1/2; 2, AKT1/2/3; 3, STAT3 serine 727; 4, STAT3 tyrosine 705.

DISCUSSION

Follicular variant PTCs are called “the bane of the pathologist” [16, 17]. There is yet no consensus for pathologic diagnosis and it vexes the estimation of the percentage of genetic alterations of fvPTCs. It is generally accepted that less BRAF and RET/PTC alterations are prevalent compared to classical PTCs, and more RAS and PAX8-PPARg alterations than classical PTCs. To our knowledge, only 2 articles analyzed the above-mentioned 4 major alterations of thyroid carcinoma in fvPTCs (BRAF, RET/PTC, RAS, and PAX8-PPARg). In a study by Rivera et al., genetic alterations of 47 cases of fvPTCs were BRAF 10.6%, RET/PTC 4.2%, RAS 25.5%, PAX8-PPARg 4.2% and alteration unknown was 55.5% [18]. In Santarpia el al.’s work with 30 cases of fvPTCs, BRAF 10%, RET/PTC 20%, RAS 17%, PAX8-PPARg 17% and unknown was 36% [19]. These articles demonstrate that still a large portion of fvPTCs are without clarified molecular mechanisms.

Our work is the first report of the fusion of EZR and ERBB4. In a validation test with additional 20 cases of BRAF

wild type fvPTCs, 20% of BRAF wild type fvPTCs were revealed to have EZR_ERBB4 alteration (4 out of 20). Considering a previous report that BRAF T1799A prevalence of fvPTCs in Korea is 32.6% [20], it is plausible to estimate roughly that approximately 15% of fvPTCs possess the EZR-ERBB4 fusion transcript (1/1.326). A more detailed analysis with a larger number of patients is necessary for more accurate information.

High expression of EZR is correlated pancreatic carcinoma metastasis [21] and EZR relationship with clinical stage of rhabdomyosarcoma has been suggested [22]. In the same context, EZR expression in a thyroid carcinoma is increased, as reported by a mouse model study mimicking FTC by a thyroid hormone receptor mutation [23]. A recent article demonstrating increased EZR expression in ovarian cancer by estradiol treatment implies an estrogen-related function of EZR [24]. Considering the fact that most of the thyroid carcinoma patients are women and its incidence is peaked at midlife, which suggest a role of sex-hormones, a possible molecular

connection of EZR with estrogen needs further investigation [25].

In most tumor types ERBB4 expression is related with favorable prognosis of the disease [26]. The concept also applies to thyroid carcinomas, and there are reports that ERBB4 expression correlates with small tumor size and lower T stage of thyroid carcinomas [27, 28]. Mutated ERBB4, however, might promote cancer growth and EZR-ERBB4 may have a similar function with the mutant form different from wild type. In a kinome sequencing analysis of 79 melanomas, mutated ERBB4 was discovered in 19% of them. The mutated sites were spread all over the ERBB4 sequence, and functional studies on 7 of the mutations resulted in transforming phenotypes. One important thing to note is that the kinase activity was increased in all 7 cases [29]. Whether kinase activity is actually activated and is associated with oncogenic role of the fusion protein awaits deeper analysis. Regarding estrogen response, ERBB4 was found to be a co-regulator of estrogen receptor and promotes proliferation of estrogen receptor positive breast carcinoma cells [30]. Functions of ERBB4 in response to

estrogen in thyroid carcinomas, a malignancy much more prevalent in women than in men, has not been studied yet. ERBB4 is one of the first receptor protein kinase (RTK) that in addition to its canonical indirect regulation of target genes, regulated intramembrane proteolysis occurs and intracellular domains directly activate target genes [31, 32]. EZR-ERBB4 fusion, however, lacks proteolytic cleavage site at exon 16 and direct activation of target genes by intracellular domain following intramembrane proteolysis is unlikely to occur.

Most genetic alterations, including mutations and chromosomal rearrangements, are thought to be passengers rather than drivers in the course of carcinogenesis [33]. Therefore it is of a great interest to develop a method of distinguishing drivers from passenger alterations, especially *in silico*, since functional analysis in *in vitro* models is not high-throughput. Screening out driver alterations by their mutual exclusivity with previously known genetic alterations is a widely-used method [34]. In our RNA sequencing analysis of 70 cases of thyroid tumors, the EZR-ERBB4 fusion transcript showed no co-occurrence with any of the previously known

genetic alterations of thyroid carcinoma. There are limitations of this computational approach, however, since not all genetic alterations are mutually exclusive and there are exceptions such as CBF translocations and kinase mutations in acute myeloid leukemias [35] and VHL/SETD2/PBRM1 mutations in renal carcinomas [36]. There is no previous report of EZR-ERBB4 and mutual exclusivity with other thyroid carcinoma driver alterations is to be clarified by further studies, ERBB4 is reported to be mutated in some melanoma cases and the mutation is mutually exclusive with BRAF or RAS mutations [37]. Mutations in epidermal growth factor receptor (EGFR), a more well-known member of ERBB family genes, are also mutually segregated BRAF T1799A and RAS mutations [38]. From these observations, it was carefully expected that the EZR-ERBB4 is a driver genetic alteration of thyroid carcinoma, especially fvPTCs.

The driver function of EZR-ERBB4 was also interrogated by a structural analysis. RET/PTC fusions are typical alterations of PTCs, with chromosomal rearrangement of RET gene with various fusion partners [39]. RET is originally a

membrane-bound receptor tyrosine kinase protein which is activated by dimerization upon ligand binding [40]. The kinase domain of RET is preserved in the all fusion alterations regardless of its fusion partners. In the case of RET/PTC1, a fusion of CCDC6 and RET, the fusion protein forms a homo-dimer and RET kinase is constitutively activated giving a survival signal to the thyroid cell. The coiled-coil domain of CCDC6 is thought to play an important role in fusion protein dimerization, as revealed by knock-down studies [41]. ERBB4 contains a kinase domain in a way very similar to RET of RET/PTC fusions. ERBB4, upon ligand interaction, also dimerizes to itself, resulting in homo-dimerization, or with other ERBB family members forming hetero-dimers [42]. EZR, a fusion partner of ERBB4, also contains a coiled-coil domain [43]. This structural similarity of RET/PTC1 and EZR-ERBB4 implies oncogenic role of EZR-ERBB4 in a way very similar to that of RET/PTC fusions.

In accordance with the driver characteristics of EZR-ERBB4 discussed above, our *in vitro* model demonstrated oncogenic ability of the fusion transcript in soft agar colony

formation assay. Soft agar assay, or anchorage-independent colony formation assay, is a tool widely used to verify oncogenic phenotype of a genetic alteration [44]. This technique has also been recently used to clarify the driver role of a novel fusion gene found in lung adenocarcinomas [45]. Cells with EZR-ERBB4 expression formed more number of colonies and individual colony size was larger compared to the vector-only control, implicating enhanced survival on anchorage-independent condition often a carcinoma cell faces. There are limitations of soft agar assay, however, and a further study in an animal model is necessary to determine the tumorigenicity of the EZR-ERBB4 cells. When all of the above-mentioned aspects are considered, including mutual exclusivity with other alterations, structural similarity with RET/PTC fusions, and ability to form colonies in soft agar, it can be carefully expected that EZR-ERBB4 may play a driver role in fvPTC oncogenesis.

In our EZR-ERBB4 expressing cell line model phosphorylation of ERK1/2 or AKT1/2/3, which are typically activated in the course of thyroid oncogenesis, was not

observed. It might be expected that the fusion transcript does not share the MAPK or PI3K signal pathways typical of PTCs and FTCs, respectively. On the contrary, phosphorylation of serine 727 (S727) of STAT3 was observed even in the absence of serum in EZR-ERBB4 expressing cells and this implies activation of an alternative signal pathway. Increased or constitutive phosphorylation of STAT3 has been reported in various carcinoma types, but most of them are related to phosphorylation of tyrosine 705 (Y705) [46, 47]. Albeit some conflicting results, S727 phosphorylation of STAT3 is generally thought to play a similar role with Y705 [48]. In our experiment condition, Y705 was not phosphorylated regardless of serum status in EZR-ERBB4 expressing cells. To our knowledge, the only gene studied so far to phosphorylate STAT3 S727 but not Y705 is the RAS oncogene (N-, H-, and K- RAS) [49]. In a recent study by Gough et al., ERK2 was suspected to be a mediator of RAS-induced phosphorylation of STAT3 S727, but there are also other serine kinases which might be responsible for S727 phosphorylation [50]. Furthermore, S727 phosphorylation of STAT3 was reported to play an important role in proliferation and transformation of NIH-3T3 cells

exogenously expressing NRAS oncogene [51]. The role of EZR-ERBB4 on constitutive STAT3 S727 phosphorylation and its relationship with fvPTC oncogenesis requires a further analysis, especially on its connection with RAS -mediated signal pathways.

In conclusion, a novel fusion of EZR and ERBB4 in fvPTCs was discovered and validated in 20 cases. The estimated prevalence in fvPTCs was 15%. The fusion was mutually exclusive with previously described genetic alterations of thyroid carcinomas. In a structural analysis, the fusion gene contains domains necessary for homo-dimerization and protein kinase activation in a way resembling that of RET/PTCs. *In vitro* model showed transforming phenotype. The constitutive phosphorylation of STAT3 S727 seems to be an underlying mechanism. The findings, at least partially, support the driver role of EZR-ERBB4 in fvPTC oncogenesis.

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국문 초록

서론: 갑상선암이 증가하고 있으며 특히 진단이 어려운 갑상선 여포상 유두암은 원인이 되는 유전자 변이가 알려져 있지 않은 경우가 많다. 새로운 유전자 변이를 발굴하는 것은 여포상 유두암의 진단에도 도움이 된다.

방법: 70 례의 갑상선암 조직에 대하여 RNA 시퀀싱을 시행하였다. 새로운 융합유전자가 발견된 갑상선암 아형인 여포상 유두암에 대해 20 례의 추가적인 냉동조직 샘플을 이용, RT-PCR 로 빈도를 확인하였다. 구조적 예측을 통해 종양의 원인유전자로서의 가능성을 타진한 후 세포주 모델을 만들어 soft agar assay 를 시행하여 검증하였다. 종양개시의 바탕이 되는 분자기전을 조사하기 위해 phospho-kinase assay 를 시행하였다.

결과: EZR-ERBB4 융합유전자를 발굴하였다. 기존의 갑상선암 발암유전자와 상호배제적이었으며 구조적으로 종양개시자의 가능성이 농후하였다. 세포주 모델을 이용한 실험에서 soft agar 에서 종양형성능을 보였다. 여기에는 STAT3 의 인산화가 수반되었다.

결론: 일련의 결과를 토대로 EZR-ERBB4 는 갑상선 여포상 유두암의 원인유전자로서 작용할 가능성이 있음을 생각할 수 있다.

주요어 : 융합유전자, 갑상선암, 여포상 유두암, 원인유전자, 진단

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