Identification of Three Novel Fusion Oncogenes, SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET in Thyroid Cancers of Young Patients in Fukushima

Keita Iyama1,3, Michiko Matsuse1, Norisato Mitsutake1*, Tatiana Rogounovitch1, Vladimir Saenko2, Keiji Suzuki1, Mai Ashizawa4, Chiyo Ookouchi4, Satoshi Suzuki4, Hiroshi Mizunuma4, Toshihiko Fukushima4, Shinichi Suzuki4*, Shunichi Yamashita1,2

1Department of Radiation Medical Sciences, 2Department of Radiation Molecular Epidemiology, Atomic Bomb Disease Institute, Nagasaki University
3Nagasaki University Graduate School of Biomedical Sciences
4Department of Thyroid and Endocrinology, Fukushima Medical University

Running title: Novel Fusions in young PTCs in Fukushima

Key words: fusion gene, rearrangement, oncogene, NTRK3, RET, papillary thyroid carcinoma

Authors’ contact information:

Keita Iyama, MD
Department of Radiation Medical Sciences, Atomic Bomb Disease Institute, Nagasaki University
1-12-4 Sakamoto, Nagasaki 852-8523, Japan
Tel: +81-95-819-7116
Fax: +81-95-819-7117
E-mail: keita.i0829@gmail.com

Michiko Matsuse, PhD
Department of Radiation Medical Sciences, Atomic Bomb Disease Institute, Nagasaki
Fax: +81-95-819-7117
E-mail: kzsuzuki@nagasaki-u.ac.jp

Mai Ashizawa, MD PhD
Department of Thyroid and Endocrinology
Fukushima Medical University
1 Hikarigaoka, Fukushima 960-1295, Japan
Tel: +81-24-547-1427
Fax: +81-24-548-3249
E-mail: ashimai@fmu.ac.jp

Chiyo Ookouchi, MD PhD
Department of Thyroid and Endocrinology
Fukushima Medical University
1 Hikarigaoka, Fukushima 960-1295, Japan
Tel: +81-24-547-1427
Fax: +81-24-548-3249
E-mail: ookouchi@fmu.ac.jp

Satoshi Suzuki, MD PhD
Department of Thyroid and Endocrinology
Fukushima Medical University
1 Hikarigaoka, Fukushima 960-1295, Japan
Tel: +81-24-547-1427
Fax: +81-24-548-3249
E-mail: satop@fmu.ac.jp
Hiroshi Mizunuma, MD PhD  
Department of Thyroid and Endocrinology  
Fukushima Medical University  
1 Hikarigaoka, Fukushima 960-1295, Japan  
Tel: +81-24-547-1427  
Fax: +81-24-548-3249  
E-mail: hiromizunuma@yahoo.co.jp

Toshihiko Fukushima, MD PhD  
Department of Thyroid and Endocrinology  
Fukushima Medical University  
1 Hikarigaoka, Fukushima 960-1295, Japan  
Tel: +81-24-547-1427  
Fax: +81-24-548-3249  
E-mail: t-fuku@xa2.so-net.ne.jp

Shunichi Yamashita, MD PhD  
Department of Radiation Medical Sciences, Atomic Bomb Disease Institute, Nagasaki University  
1-12-4 Sakamoto, Nagasaki 852-8523, Japan  
Tel: +81-95-819-7116  
Fax: +81-95-819-7117  
E-mail: shun@nagasaki-u.ac.jp

*Correspondence and reprint requests:*

Norisato Mitsutake, MD PhD  
Department of Radiation Medical Sciences, Atomic Bomb Disease Institute, Nagasaki
University.

1-12-4 Sakamoto, Nagasaki 852-8523, Japan

Tel: +81-95-819-7116

Fax: +81-95-819-7117

E-mail: mitsu@nagasaki-u.ac.jp

Shinichi Suzuki, MD PhD

Department of Thyroid and Endocrinology

Fukushima Medical University

1 Hikarigaoka, Fukushima 960-1295, Japan

Tel: +81-24-547-1427

Fax: +81-24-548-3249

E-mail: shsuzuki@fmu.ac.jp
Abstract

Background: The $BRAF^{V600E}$ mutation is the most frequent genetic abnormality in adult papillary thyroid carcinomas (PTCs). On the other hand, various chromosomal rearrangements are more prevalent in childhood and adolescent PTCs. The aim of the present study was to identify novel rearrangements in PTCs from young patients.

Methods: Among 63 postoperative specimens of childhood and adolescent PTCs, which had been discovered by the thyroid ultrasound screening program in Fukushima, 9 samples without prevalent known oncogenes, $BRAF^{V600E}$, $RAS$, $RET/PTC1$, $RET/PTC3$, and $ETV6/NTRK3$ were analyzed in the current study by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) to screen for novel fusion genes by comparing transcript expression between extracellular and kinase domains of $ALK$, $NTRK1$, $NTRK3$, and $RET$.

Results: Of the above nine samples, five samples were suspected to harbor a fusion, and using subsequent 5’ rapid amplification of cDNA end (RACE), we identified two already reported fusion oncogenes, $STRN/ALK$ and $TPR/NTRK1$, and three novel fusions, $SQSTM1/NTRK3$, $AFAP1L2/RET$, and $PPFIBP2/RET$. We performed functional analyses of these three chimeric genes and confirmed their transforming abilities through the activation of mitogen-activated protein kinase (MAPK).

Conclusions: we have identified three novel fusion oncogenes in young PTC patients in Fukushima, suggesting that rare fusions may be present among the cases negative for known oncogenes in this age group and that such rearrangements can play a significant role in thyroid carcinogenesis.
**Introduction**

Papillary thyroid carcinoma (PTC) is the most frequent malignant tumor in endocrine organs, and its global incidence has rapidly increased in recent decades (1-3). Gene rearrangements such as RET/PTC or point mutations in the RAS or BRAF genes are detected in approximately 80% of PTCs (4). These genetic abnormalities lead to constitutive activation of the mitogen-activated protein kinase (MAPK) pathway, and a mutually exclusive fashion of the presence of these oncogenes strongly suggests the importance of the activated MAPK pathway for PTC development (3, 5).

In adult sporadic PTCs, a point mutation in BRAF, especially BRAF\(^{V600E}\), is the most prevalent genetic abnormality (4, 6). In children, however, it has been reported that the prevalence of BRAF\(^{V600E}\) is much lower (0–37%) (7, 8), and various chromosomal rearrangements including RET/PTC are more frequent (7, 9-15). The accumulating knowledge of genetic abnormalities in different age groups has increased our understanding of carcinogenic mechanisms of PTCs; however, the reason of different oncogenic profiles between adults and childhood PTCs is still unclear.

After the accident at the Fukushima Daiichi Nuclear Power Plant, the Thyroid Ultrasound Screening Program was started beginning in October 2011 for all children aged 0–18 years old at the time of the accident (16, 17). Although many PTCs were found during the first round of screening, those are thought to be spontaneous, because 1) the estimated thyroid dose of radiation exposure was quite low, 2) the latency period was too short, and 3) most of the patients were teenagers (17). Indeed, the purpose of the first round screening was to understand background ultrasound findings in thyroids of this age group. Presumably, these PTCs all reflect naturally occurring tumors including latent cancers in the young population, and many of them could be silent or slowly growing until middle age. However, they were discovered by mass screening.
using highly sensitive ultrasound instruments. Therefore, it is highly important to perform detailed analysis of these cases, because it may help to understand the etiology, mechanisms, and natural course of PTCs in children and other age groups. We have reported that approximately 85% of the PTCs in the Fukushima area harbor known driver mutations such as the \textit{BRAF}^{V600E} mutation and the \textit{RET/PTC} rearrangement (18). However, the oncogenic drivers in the remaining 15% remained to be identified. In the present study, we screened these cases for novel fusion oncogenes to further clarify the oncogenic profile of these PTCs.

\textbf{Materials and Methods}

\textit{\textbf{Samples}}

In our previous work (18), a total of 63 childhood and adolescent PTC patients operated at Fukushima Medical University Hospital were examined for the presence of the following known genetic abnormalities: \textit{BRAF}^{V600E}, \textit{RAS}, \textit{RET/PTC1}, \textit{RET/PTC3}, and \textit{ETV6/NTRK3}. Mean age at the time of operation was 17.3±2.8 y.o.; range, 9–22 y.o.; Sex distribution, male: 22/63 (34.9%), female: 41/63 (65.1%). The size of the tumors varied from 6 to 40.5 mm, mean 14.1±8.2 mm. After surgery, all tissue samples were reviewed by a thyroid pathologist to confirm diagnosis. The study was approved by the ethics committees of Nagasaki University and Fukushima Medical University. Written informed consent was obtained from each patient.

In the current study, nine samples which did not harbor any of the mutations listed above were included. We used RNA/cDNA samples, which were generated in the previous work (18). These samples were screened for possible rearrangements of \textit{ALK}, \textit{NTRK1}, \textit{NTRK3}, and \textit{RET} by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR).
Fusion gene screening by qRT-PCR and 5'-RACE

To detect rearrangements, we screened samples for the imbalance in expression of exons 3–4 relative to exons 27–29 of ALK, exons 1–3 relative to exons 15–16 of NTRK1, exons 8–9 relative to exons 15–16 of NTRK3, and exons 2–3 relative to exons 17–18 of RET. We performed quantitative PCR in a Thermal Cycler Dice Real-time system (TaKaRa Bio) using SYBR Premix Ex Taq II (Takara Bio). The following PCR primers were used: for ALK ex3-4, 5'-GGGCAGAGCGTTCTAAGGAGATG-3' (forward) and 5'-CGGCCAGTGTGCAGTGCT-3' (reverse); for ALK ex27-29, 5'-CCCTGGGCTGTATACCGGATA-3' (forward) and 5'-TACATCCGGGTCCTGGGTGC-3' (reverse); for NTRK1 ex1-3, 5'-CCCTGGATAGCCTCCACCAC-3' (forward) and 5'-ACGGAGACCACTCTTCACGATG-3' (reverse); for NTRK1 ex15-16, 5'-CGAGAGCATCCTGTACCGTAAGTTC-3' (forward) and 5'-CCCTGCGTGATGCAGTCG-3' (reverse); for NTRK3 ex8-9, 5'-CAACCTGACCCTCGAGAGGGT-3' (forward) and 5'-GATGGCATGAACATTGGTCCAG-3' (reverse); for NTRK3 ex15-16, 5'-CCGACCAAGGACAAGATGCTTG-3' (forward) and 5'-GCCGCACACTCCATAGAACATGAC-3' (reverse); for RET ex2-3, 5'-AAGCTCAGTGTCCGCAACC-3' (forward) and 5'-ATGTGGGTGACAGGAAGACC-3' (reverse); for RET ex17-18, 5'-TGCGCCAGGAGATGCTACC-3' (forward) and 5'-CCAGGTCTTTTGCTGATGTCC-3' (reverse).

The 5’ rapid amplification of cDNA end (RACE) was then performed for samples suspected to have fusion genes using a 5’-Full RACE Core Set (Takara Bio) according to manufacturer’s protocol. Briefly, first strand cDNA was synthesized by reverse transcription using 5’ end-phosphorylated RT primers. Sequences of each primer were as
follows: for **ALK**, 5'-CACCTCCTTCAGG-3'; for **NTRK1**, 5'-GTACAGGATGCTCTC-3'; for **NTRK3**, 5'-CACACTCCATAGAAC-3'; for **RET**, 5'-TCTCGCGAGGAAGC-3'. Next, DNA-RNA hybrid was treated with RNase H to degrade RNA. Then the single strand DNA was incubated with T4 RNA Ligase to circularize it or to form concatemers. These are amplified by nested PCR using primers located in the region encoding the kinase domain of each target. First PCR primers were as follows: for **ALK**, 5'-CTGAGCAAGCTCCGCACCTCGAC-3' (forward) and 5'-GCTCTGCAGCTCCATCTGCATGG-3' (reverse); for **NTRK1**, 5'-AGCAGGGGATATCTACAGCACCGA-3' (forward) and 5'-CCCACTAGACAGTTGCGTGTGG-3' (reverse); for **NTRK3**, 5'-GGCTGTGAAGGCCCTGAAGG-3' (forward) and 5'-GCACTCGGCCAGGAAGACCT-3' (reverse); for **RET**, 5'-GTCTGTGAAGGCCCTGAAGG-3' (forward) and 5'-GCCTCGGCCAGGAAGACCT-3' (reverse). Second PCR primers are: for **ALK**, 5'-GCTGGCAAGACCTCCTCCATCAGTG-3' (forward) and 5'-GCTCCTGGTGCTTCCGGCGGTAC-3' (reverse); for **NTRK1**, 5'-CACCATGCTGCCCATTCGCT-3' (forward) and 5'-CAGCCAGCAGCTTGGCATCA-3' (reverse); for **NTRK3**, 5'-CTGCAGCATGAGCACATTGTCA-3' (forward) and 5'-CTCACCCAGTTCTCGTTCAGC-3' (reverse); for **RET**, 5'-CGCTCTCTCCTCATCGTGAGTACG-3' (forward) and 5'-AAAGAACCAAGTTCTTCCGAGGAATTCC-3' (reverse). The PCR amplicons were checked by agarose gel electrophoresis and subjected to Sanger sequencing using the above second PCR primers.

**Cell lines**

The murine fibroblast NIH3T3 cell line was maintained in Dulbecco’s modified Eagle
medium (DMEM) (Wako Pure Chemicals) supplemented with 5% bovine serum (BS) (Gibco, Thermo Fisher) and 1% Penicillin/Streptomycin (Wako Pure Chemicals). The 293FT packaging cell line (Invitrogen, Thermo Fisher) was maintained according to the manufacturer’s protocol. For serum starvation, DMEM with 0.5% fetal bovine serum was used for 293FT cells.

- **Retrovirus vectors**

Full length coding portions of SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET were amplified from each cDNA sample generated in our previous work (18) and subcloned into pDON-AI (Takara Bio) with a C-terminal V5-tag. The accuracy of the sequence of the inserts was confirmed by Sanger sequencing. Recombinant retroviruses were produced by introduction of the above plasmids into 293 10A-1 cells. The titer of the virus vector was checked using NIH3T3 cells.

- **Focus formation assay**

NIH3T3 cells were infected with the appropriate retrovirus at multiplicity of infection (MOI) of 0.1 in the presence of 4 μg/ml polybrene (Sigma–Aldrich). Infected cells were cultured for two weeks in the presence of 0.75 mg/ml G418 (Wako Pure Chemicals), and the number of transformed foci was counted under an inverted phase-contrast microscope.

- **Cell growth**

NIH3T3 cells stably expressing EGFP-V5, SQSTM1/NTRK3-V5, AFAP1L2/RET-V5 and PPFIBP2/RET-V5 were plated in 6-well plates and grown in medium supplemented with 0.5% BS (Gibco, Thermo Fisher), 1% Penicillin-Streptomycin, and 0.75 mg/ml G418. At the indicated time points, cells were counted using a TC20 Automated Cell Counter (Bio Rad).
Western blotting

Protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). After blocking and incubation with an appropriate primary antibody, the antigen-antibody complexes were visualized using a HRP-conjugated secondary antibody (Cell Signaling Technology) and a chemiluminescence system (Nacalai Tesque). Detection was performed using a LAS-3000 imaging system (Fujifilm). Primary antibodies were obtained from the following sources: anti-phospho-ERK (Thr202/Tyr204), anti-ERK, anti-phospho-MEK (Ser217/221), and anti-MEK from Cell Signaling Technology; anti-β-actin from Santa Cruz Biotechnology; anti-V5 from Invitrogen.

Statistical analysis

Differences between groups were examined for statistical significance with ANOVA followed by Tukey’s post test. A $p$-value not exceeding 0.05 was considered statistically significant.

Results

Cases and genetic analysis

In our previous study (18), we performed mutational analysis for the presence of the following well-established oncogenes, $BRAF^V_{600E}$, $RAS$, $RET/PTC1$, $RET/PTC3$, and $ETV6/NTRK3$, in 63 Fukushima PTC samples. We found 43 (68.3%) cases with the $BRAF^V_{600E}$ mutation, 6 (9.5%) with $RET/PTC1$, 1 (1.6%) with $RET/PTC3$, and 4 (6.3%) with $ETV6/NTRK3$ rearrangements. There were nine samples, however, in which no mutation could be identified. We then performed qRT-PCR to screen for novel fusion
genes by comparing the expression levels between the extracellular domain (5’) and the kinase domain (3’) of ALK, NTRK1, NTRK3, and RET. A higher expression of the kinase domain suggests the possibility of a gene rearrangement. Representative images are shown in Supplementary Fig. S1 online. Among the nine samples, five displayed a higher expression of the 3’ exons of ALK, NTRK1, NTRK3, or RET. We then performed subsequent 5’-RACE in these samples. Two of the five cases were found to have already reported fusion oncogenes, TPR/NTRK1 and STRN/ALK (19, 20). Sequencing of the remaining three samples resulted in the discovery of SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET rearrangements as shown in Fig. 1a–c.

All three cases with novel fusion genes were histologically confirmed to be classical papillary adenocarcinomas without specific pathomorphological features or multifocality. The characteristics of the three patients were as follows: 1) SQSTM1/NTRK3: 18 years old (yo) female, hemithyroidectomy, 7.0 mm in diameter, pT1a pN0 M0, no extrathyroidal extension; 2) AFAP1L2/RET: 18 yo male, total thyroidectomy, 40 mm in diameter, pT2 pN1b M1 (lung), no extrathyroidal extension; 2) PPFIBP2/RET: 13 yo female, hemithyroidectomy, 15 mm in diameter, pT1b pN1a Mo, no extrathyroidal extension. None of the three patients has developed a recurrence.

**Characteristics of the three novel fusion genes**

SQSTM1/NTRK3 is a result of an interchromosomal translocation t(5;15)(q35.3;q25.3), which juxtaposes exons 1–5 of SQSTM1 to exons 14–19 of NTRK3 (Fig. 1a). The fusion point in NTRK3 is localized at the same position as in ETV6/NTRK3 (14). As the lengths of intron 5 of SQSTM1 and intron 13 of NTRK3 are 7,805 bp and 93,252 bp, respectively, we did not search a breakpoint on genomic DNA.

AFAP1L2/RET is a result of an intrachromosomal translocation t(10)(q25.3;q11.2), which fuses exons 1–2 and a part of intron 2 (38-bp) of AFAP1L2 to the 3’ part of exon
11 (33-bp) and exons 12–20 of *RET* in frame (Fig. 1b). This created a new exon, and genomic breakpoints are located in intron 2 and exon 11 of *AFAP1L2* and *RET*, respectively. As an acceptor site at the 5’ end of exon 11 was missing, the genomic sequence TTTTTTTTTTGGAG-A in intron 2 was presumably recognized as an acceptor (Fig. 1b).

*PPFIBP2/RET* is a result of an interchromosomal translocation t(11;10)(p15.4;q11.2), which juxtaposes exons 1–9 of *PPFIBP2* to exons 12–20 of *RET* (Fig. 2c). Although breakpoints are located in intron 9 and in the middle of exon 11, the part of exon 11 was spliced out. In both latter cases, the kinase domain of *RET* is fully preserved as in conventional *RET/PTC* rearrangements.

As these types of fusion proteins are usually activated through dimerization by a domain such as a coiled-coil domain in the N-terminal partner protein, we performed *in silico* prediction analysis using the COILS Server (http://embnet.vital-it.ch/software/COILS_form.html) (21). SQSTM1/NTRK3 seemed not to have a coiled-coil domain (Fig. 2). On the other hand, *PPFIBP2/RET* highly likely possesses a coiled-coil domain (Fig. 2). For the *AFAP1L2/RET* fusion, the signal was moderate (Fig. 2).

**Functional studies**

First, we examined the effects of SQSTM1/NTRK3, AFAP1L2/RET, and *PPFIBP2/RET* on the MAPK signaling pathway. For this purpose, these fusion proteins were overexpressed in 293FT cells (Fig. 3a), and the phosphorylation status of MEK and ERK was assessed by immunoblotting. As shown in Fig. 3b, ERK phosphorylation was induced by all three novel rearrangements, indicative of their ability to constitutively activate the MAPK pathway. We also checked AKT phosphorylation but did not observe a significant change induced by these fusion proteins.
Next, we compared cell growth under low serum concentration. In this condition, EGFP-expressing control cells exhibited modest growth. In contrast, transduction with SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET significantly promoted cell growth (Fig. 4a). Note that the cells with SQSTM1/NTRK3 grew faster than the other two RET-carrying cells (Fig. 4a).

We then performed a focus formation assay to confirm the transforming potential of each of the novel fusion genes. A number of foci were successfully observed in NIH3T3 cells stably transduced by SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET (Fig. 4b, c). In contrast, overexpression of EGFP (control) did not result in the formation of any foci (Fig. 4b, c) and the number of foci was statistically different for all fusions compared to the control (Fig. 4b). Again, note that the number of foci after transduction with SQSTM1/NTRK3 was greater than with AFAP1L2/RET and PPFIBP2/RET (Fig. 4b).

These results indicate that the newly discovered three fusions, SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET represent novel driver oncogenes.

**Discussion**

In this study, we discovered three novel fusion oncogenes, SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET, which have a transforming ability through MAPK activation according to the *in vitro* studies. Taking into consideration our previous results of molecular analysis in the 63 young PTC patients from Fukushima (18), in which 43 cases with BRAFV600E (68.3%) and 11 (17.5%) with fusion genes were identified, the number of gene rearrangement-positive cases is now increased to 16 cases (25.4%). In total, 59 out of 63 cases (93.7%) were confirmed to have a driver mutation; only 4 cases (6.3%) remain negative for any oncogene so far.
We analyzed the oncogenic potential of the newly discovered fusion genes. First, $SQSTM1/NTRK3$ is a fusion between $SQSTM1$ on chromosome 5 and $NTRK3$ on chromosome 15. $NTRK3$ is a transmembrane receptor tyrosine kinase, whose ligand is neurotrophin-3. Recently, other $SQSTM1$ fusion genes have been identified in hematological malignancies: $SQSTM1/ALK$ in large B-cell lymphoma, $SQSTM1/NUP214$ in T-cell acute lymphoblastic leukemia, and $SQSTM1/FGFR1$ in myelomonocytic leukemia (22-25). $SQSTM1/ALK$ has also been shown to have a transforming ability by focus formation assay using 3T3 fibroblasts. Although $SQSTM1$ does not have a coiled-coil domain, there is a Phox and Bem1p (PB1) domain at the N-terminus which enables formation of heteromeric and homomeric complexes (26). Presumably, $SQSTM1/NTRK3$ is constitutively activated through dimerization mediated by the PB1 domain.

The $SQSTM1$ gene product is involved in several intracellular signal transduction cascades comprising a signaling node for multiple pathways maintaining cellular homeostasis. $SQSTM1$ may functionally contribute to aging, autophagy and the development of degenerative diseases (27). How the disruption of one copy of $SQSTM1$ may contribute to tumor growth remains unclear. Note that mutations in the C-terminus of $SQSTM1$ have been reported to cause Paget’s bone disease (28). Although the $SQSTM1/NTRK3$-expressing NIH3T3 cells showed a higher growth rate and transformation than the other two RET fusions, we cannot definitely conclude that this fusion has a distinct malignant potential compared to the other two rearrangements because of the intrinsic limitations of our assay systems.

The two other fusion oncogenes, $AFAP1L2/RET$ and $PPFIBP2/RET$, are new variants belonging to the $RET/PTC$ family. The $RET$ gene was originally identified as a
proto-oncogene more than 30 years ago (29), and then a rearrangement named RET/PTC was found in PTC (30, 31). At present, more than 15 types of RET/PTC rearrangements with different partner genes are known (32), to which our present work adds two more. RET encodes a transmembrane receptor tyrosine kinase; binding of a ligand, glial cell line-derived neurotrophic factor (GDNF), stimulates receptor dimerization, which is a critical step for gaining tyrosine kinase activity. Although the expression of RET in thyroid follicular cells is very limited, the fusion proteins are commonly expressed in thyroid follicular cells and possess coiled-coiled, leucine zipper, lis homology (LisH), or other domains that enable homo-dimerization of RET/PTC fusion proteins. As a result, RET/PTC gene products are constitutively activated without ligand binding in thyroid cells.

The novel partner gene AFAP1L2 is located on chromosome 10 and belongs to the actin filament-associated protein (AFAP) family. Although AFAP1L2 is expressed in the human thyroid as well as in other organs, the role of AFAP1L2 in thyroid carcinogenesis is not yet formally clarified (33). In AFAP1L2/RET, we found that only exons 1–2 were fused to RET, but according to our in silico analysis, a coiled-coil domain likely exists in this portion and enables dimerization, leading to constitutive activation of the RET kinase. All of the results of our functional study suggest that AFAP1L2/RET is a novel oncoprotein. However, as the breakpoint of this fusion is very unique (at intron and exon), this is probably a rare event.

Finally, another newly discovered partner gene is PPFIBP2. It is located on chromosome 11, and encodes the protein-tyrosine phosphatase receptor-type f-polypeptide (PTPRF)-binding protein. The PPFIBP2 product plays a role in axon guidance and neuronal synapse development; its function in the thyroid is unknown. Of note, rs12791447 SNP in the PPFIBP2 intron was found to be a susceptibility marker.
for prostate cancer (34). Also, elevated PPFIBP2 mRNA expression was detected in endometrial cancer (35), suggesting that it may be associated with cancer pathogenesis. Again, the in silico analysis revealed that exons 1–9 of PPFIBP2 highly likely harbor a coiled-coil domain and promote dimerization, leading to constitutive activation of RET.

The limitation of the present work consists in the lack of in vivo tumor formation studies. However, all driver oncogenes that have a focus-forming ability in NIH3T3 cells also formed tumors in immunodeficient mice.

In summary, three novel fusion oncogenes, SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET were identified in the PTCs from one child and two adolescents from a cohort consisting of 63 members from Fukushima. The findings suggest that rare fusions may be present among the cases negative for known oncogenes in this age group and that they play a significant role in thyroid carcinogenesis. Our results also indicate that almost all PTCs in young patients (93.7%) have oncogenic driver mutations activating the MAPK intracellular signaling pathway.

References


120:799-807.


16. Homepage FRS Fukushima Revitalization Station Homepage. Available at: https://www.pref.fukushima.lg.jp/site/portal/list279-884.html.


Acknowledgements

This work was supported in part by JSPS KAKENHI, grant number: 25257508 (SY), 26293222 (SY), 26293142 (NM), and 16K09804 (MM). This study was also supported in part by Cooperative Research Grant of Atomic Bomb Disease Institute, Nagasaki University (ShS). We thank the members of the Departments of Thyroid and Endocrinology, and Organ Regulatory Surgery, Fukushima Medical University, the Thyroid Examination Unit of the Radiation Medical Centre for the Fukushima Health Management Survey, and the Consensus Meeting of Pathological Diagnosis of Fukushima Thyroid Cancers, who participate in the thyroid ultrasound screening program in Fukushima Prefecture.

Competing financial interests

We declare no competing interests.

Figure Legends

Figure 1. Genomic location, mRNA structure, sequence chromatogram of the breakpoint, and genomic DNA structure around the breakpoint of (a) SQSTM1/NTRK3, (b)
AFAP1L2/RET, and (c) PPFIBP2/RET. Exons of a partner gene are indicated in blue numbers, and those of a kinase gene are in red. The breakpoints are indicated by arrowheads.

Figure 2. *In silico* prediction of a coiled-coil domain. Amino acid sequences of SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET were entered into the COILS server. Plotted is the probability of a coiled-coil domain existence at three different scanning windows. The breakpoints are indicated as arrowheads and dotted lines.

Figure 3. Effect of the novel fusions on signaling pathways. (a) 293FT cells were transiently transfected with mock control or expression vectors of the V5-tagged fusion genes. After 48 h of transfection, Western blot was performed using an anti-V5 antibody. (b) 293FT cells were transiently transfected with mock control or expression vectors of the V5-tagged fusion genes. After 6–8 h incubation, the cells were serum-starved. After additional 40 h incubation, whole cell lysates were subjected to Western blot using indicated primary antibodies. Experiments were repeated at least twice with similar results.

Figure 4. Transforming potential of the novel fusions. NIH3T3 cells were transduced with the indicated retroviruses. (a) 5 × 10^4 of the cells were plated, and the number of cells were counted after the indicated time points. Each point indicates the mean and standard error of 3–6 wells of a 6-well plate. *p<0.05 vs. EGFP. (b) The cells were cultured in a 6-well plate for two weeks, and the number of foci was counted. The bars represent the mean and standard error of three wells of a 6-well plate. *p<0.01, #p<0.05 vs. EGFP (c) Representative images of the foci induced by the indicated fusions. These data are representative of at least two independent experiments.
Figure 1
Figure 2

![Graphs showing SQSTM1/RET, AFAP1L2/RET, and PPFIBP2/RET with different window sizes.](image)
Figure 3

(a) Western blot analysis showing the expression levels of p-AKT, AKT, p-MEK, MEK, p-ERK, ERK, and β-actin in control, EGFP, SOSTM1/NTRK3, APP1/2/RET, and PPRIBP2/RET cells. The molecular weights of the proteins are indicated at 80 kDa, 50 kDa, and 30 kDa.

(b) Table summarizing the expression levels of the proteins mentioned in (a).
Figure 4

(a)  Graph showing the cell number over time for different proteins: EGFP, SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET.

(b) Bar graph showing the number of foci per plate for different proteins: EGFP, SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET.

(c) Images of foci under a microscope for EGFP, SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET.
Identification of Three Novel Fusion Oncogenes, SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET in Thyroid Cancers of Young Patients in Fukushima

Keita Iyama1,3, Michiko Matsuse1, Norisato Mitsutake1, Tatiana Rogounovitch1, Vladimir Saenko2, Keiji Suzuki1, Mai Ashizawa4, Chiyo Ookouchi4, Satoshi Suzuki4, Hiroshi Mizunuma4, Toshihiko Fukushima4, Shinichi Suzuki4, Shunichi Yamashita1,2

1Department of Radiation Medical Sciences, 2Department of Health Risk Control, Atomic Bomb Disease Institute, Nagasaki University
3Nagasaki University Graduate School of Biomedical Sciences
4Department of Thyroid and Endocrinology, Fukushima Medical University

Supplementary Figure S1

Supplementary Figure S1. Examples of amplification curves by qRT-PCR. (a) Upper and middle, the samples with RET fusion genes. The cycle threshold of the amplicon located at the tyrosine kinase (TK) domain was far smaller than that at the extracellular (EC) domain. The TK domain was abundantly expressed. Lower, in the fusion gene negative sample, both cycle thresholds were similarly large, indicating that both domains were rarely expressed. (b) Upper, the sample with NTRK3 fusion gene, both domains were abundantly expressed. Lower, the representative data of the NTRK3 fusion gene negative samples. Both domains were rarely expressed. The cycle threshold of TK domain was always larger than that of EC domain, presumably due to lower amplification efficiency.