1 **Identification of Three Novel Fusion Oncogenes, *SQSTM1/NTRK3*, *AFAP1L2/RET*,**

2 **and *PPFIBP2/RET* in Thyroid Cancers of Young Patients in Fukushima**

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122

123   **Abstract**

124   **Background:** The $BRAF^{V600E}$ mutation is the most frequent genetic abnormality in adult

125   papillary thyroid carcinomas (PTCs). On the other hand, various chromosomal

126   rearrangements are more prevalent in childhood and adolescent PTCs. The aim of the

127   present study was to identify novel rearrangements in PTCs from young patients.

128   **Methods:** Among 63 postoperative specimens of childhood and adolescent PTCs,

129   which had been discovered by the thyroid ultrasound screening program in Fukushima,

130   9 samples without prevalent known oncogenes, $BRAF^{V600E}$, *RAS*, *RET/PTC1,*

131   *RET/PTC3, and ETV6/NTRK3* were analyzed in the current study by quantitative

132   real-time reverse transcription polymerase chain reaction (qRT-PCR) to screen for novel

133   fusion genes by comparing transcript expression between extracellular and kinase

134   domains of *ALK*, *NTRK1*, *NTRK3*, and *RET*.

135   **Results:** Of the above nine samples, five samples were suspected to harbor a fusion,

136   and using subsequent 5' rapid amplification of cDNA end (RACE), we identified two

137   already reported fusion oncogenes, *STRN/ALK* and *TPR/NTRK1*, and three novel

138   fusions, *SQSTM1/NTRK3*, *AFAP1L2/RET*, and *PPFIBP2/RET*. We performed

139   functional analyses of these three chimeric genes and confirmed their transforming

140   abilities through the activation of mitogen-activated protein kinase (MAPK).

141   **Conclusions:** we have identified three novel fusion oncogenes in young PTC patients in

142   Fukushima, suggesting that rare fusions may be present among the cases negative for

143   known oncogenes in this age group and that such rearrangements can play a significant

144   role in thyroid carcinogenesis.

145

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

7

173 using highly sensitive ultrasound instruments. Therefore, it is highly important to

174 perform detailed analysis of these cases, because it may help to understand the etiology,

175 mechanisms, and natural course of PTCs in children and other age groups. We have

176 reported that approximately 85% of the PTCs in the Fukushima area harbor known

177 driver mutations such as the $BRAF^{V600E}$ mutation and the RET/PTC rearrangement (18).

178 However, the oncogenic drivers in the remaining 15% remained to be identified. In the

179 present study, we screened these cases for novel fusion oncogenes to further clarify the

180 oncogenic profile of these PTCs.

181

182

183 **Materials and Methods**

184 ・*Samples*

185 In our previous work (18), a total of 63 childhood and adolescent PTC patients operated

186 at Fukushima Medical University Hospital were examined for the presence of the

187 following known genetic abnormalities: $BRAF^{V600E}$, *RAS*, *RET/PTC1*, *RET/PTC3*, and

188 *ETV6/NTRK3*. Mean age at the time of operation was 17.3 ± 2.8 y.o.; range, 9–22 y.o.;

189 Sex distribution, male: 22/63 (34.9%), female: 41/63 (65.1%). The size of the tumors

190 varied from 6 to 40.5 mm, mean 14.1 ± 8.2 mm. After surgery, all tissue samples were

191 reviewed by a thyroid pathologist to confirm diagnosis. The study was approved by the

192 ethics committees of Nagasaki University and Fukushima Medical University. Written

193 informed consent was obtained from each patient.

194

195 In the current study, nine samples which did not harbor any of the mutations listed

196 above were included. We used RNA/cDNA samples, which were generated in the

197 previous work (18). These samples were screened for possible rearrangements of *ALK*,

198 *NTRK1*, *NTRK3*, and *RET* by quantitative real-time reverse transcription polymerase

199 chain reaction (qRT-PCR).

200

201 · *Fusion gene screening by qRT-PCR and 5'-RACE*

202 To detect rearrangements, we screened samples for the imbalance in expression of

203 exons 3–4 relative to exons 27–29 of *ALK*, exons 1–3 relative to exons 15–16 of *NTRK1*,

204 exons 8–9 relative to exons 15–16 of *NTRK3*, and exons 2–3 relative to exons 17–18 of

205 *RET*. We performed quantitative PCR in a Thermal Cycler Dice Real-time system

206 (TaKaRa Bio) using SYBR Premix Ex Taq II (Takara Bio). The following PCR primers

207 were used: for *ALK ex3-4*, 5'-GGGCAGAGCGTTCTAAGGAGATG-3' (forward) and

208 5'-CGGCCAGTGTGCAGTGCT-3' (reverse); for *ALK ex27-29*,

209 5'-CCCTGGGCCTGTATACCGGATA-3' (forward) and

210 5'-TACATCCGGGTCCTGGGTGC-3' (reverse); for *NTRK1 ex1-3*,

211 5'-CCCTGGATAGCCTCCACCAC-3' (forward) and

212 5'-ACGGAGACCACTCTTCACGATG-3' (reverse); for *NTRK1 ex15-16*,

213 5'-CGAGAGCATCCTGTACCGTAAGTTC-3' (forward) and

214 5'-CCCTGCGTGATGCAGTCG-3' (reverse); for *NTRK3 ex8-9*,

215 5′-CAACCTGACCGTACGAGAGGGT-3′ (forward) and

216 5′-GATGGCATGAACATTGGTCCAG-3′ (reverse); for *NTRK3 ex15-16*,

217 5′-CCGACCAAGGACAAGATGCTTG-3′ (forward) and

218 5′-GCCGCACACTCCATAGAACTTGAC-3′ (reverse); for *RET ex2-3*,

219 5'-AAGCTCAGTGTCCGCAACC-3' (forward) and

220 5'-ATGTGGGTGACAGGAAGACC-3' (reverse); for *RET ex17-18*,

221 5'-TGCAGCGAGGAGATGTACC-3' (forward) and

222 5'-CCAGGTCTTTGCTGATGTCC-3' (reverse).

223 The 5' rapid amplification of cDNA end (RACE) was then performed for samples

224 suspected to have fusion genes using a 5'-Full RACE Core Set (Takara Bio) according

225 to manufacturer's protocol. Briefly, first strand cDNA was synthesized by reverse

226 transcription using 5' end-phosporylated RT primers. Sequences of each primer were as

227 follows: for *ALK*, 5'-CACCTCCTTCAGG-3'; for *NTRK1*,

228 5'-GTACAGGATGCTCTC-3'; for *NTRK3*, 5'-CACACTCCATAGAAC-3'; for *RET*,

229 5'-TCTCGCGGAGGAAGC-3'. Next, DNA-RNA hybrid was treated with RNase H to

230 degrade RNA. Then the single strand DNA was incubated with T4 RNA Ligase to

231 circularize it or to form concatemers. These are amplified by nested PCR using primers

232 located in the region encoding the kinase domain of each target. First PCR primers were

233 as follows: for ALK, 5'-CTGAGCAAGCTCCGCACCTCGAC-3' (forward) and

234 5'-GCTCTGCAGCTCCATCTGCATGG-3' (reverse); for NTRK1,

235 5'-AGCAGGGATATCTACAGCACCGA-3' (forward) and

236 5'-CCCACTAGACAGTTGCGTGTGG-3' (reverse); for NTRK3,

237 5'-GGCTGTGAAGGCCCTGAAGG-3' (forward) and

238 5'-GCACTCGGCCAGGAAGACCT-3' (reverse); for RET,

239 5'-GTCCTGAAGCAGGTCAACCACCCA-3' (forward) and

240 5'-GACAGCAGGTCTCGCAGCTCACTC-3' (reverse). Second PCR primers are: for

241 ALK, 5'-GCTGGCAAGACCTCCTCCATCAGTG-3' (forward) and

242 5'-GCTCCTGGTGCTTCCGGCGGTAC-3' (reverse); for NTRK1,

243 5'-CACCATGCTGCCCATTCGCT-3' (forward) and

244 5'-CAGCCAGCAGCTTGGCATCA-3' (reverse); for NTRK3,

245 5'-CTGCAGCATGAGCACATTGTCA-3' (forward) and

246 5'-CTCACCCAGTTCTCGCTTCAGC-3' (reverse); for RET,

247 5'-CGCTCCTCCTCATCGTGGAGTACG-3' (forward) and

248 5'-AAGAACCAAGTTCTTCCGAGGGAATTCC-3' (reverse). The PCR amplicons

249 were checked by agarose gel electrophoresis and subjected to Sanger sequencing using

250 the above second PCR primers.

251

252 ・*Cell lines*

253 The murine fibroblast NIH3T3 cell line was maintained in Dulbecco's modified Eagle

254 medium (DMEM) (Wako Pure Chemicals) supplemented with 5% bovine serum (BS)

255 (Gibco, Thermo Fisher) and 1% Penicillin/Streptomycin (Wako Pure Chemicals). The

256 293FT packaging cell line (Invitrogen, Thermo Fisher) was maintained according to the

257 manufacturer's protocol. For serum starvation, DMEM with 0.5% fetal bovine serum

258 was used for 293FT cells.

259

260 ・*Retrovirus vectors*

261 Full length coding portions of *SQSTM1/NTRK3*, *AFAP1L2/RET*, and *PPFIBP2/RET*

262 were amplified from each cDNA sample generated in our previous work (18) and

263 subcloned into pDON-AI (Takara Bio) with a C-terminal V5-tag. The accuracy of the

264 sequence of the inserts was confirmed by Sanger sequencing. Recombinant retroviruses

265 were produced by introduction of the above plasmids into 293 10A-1 cells. The titer of

266 the virus vector was checked using NIH3T3 cells.

267

268 ・*Focus formation assay*

269 NIH3T3 cells were infected with the appropriate retrovirus at multiplicity of infection

270 (MOI) of 0.1 in the presence of 4 μg/ml polybrene (Sigma–Aldrich). Infected cells were

271 cultured for two weeks in the presence of 0.75 mg/ml G418 (Wako Pure Chemicals),

272 and the number of transformed foci was counted under an inverted phase-contrast

273 microscope.

274

275 ・*Cell growth*

276 NIH3T3 cells stably expressing EGFP-V5, SQSTM1/NTRK3-V5, AFAP1L2/RET-V5

277 and PPFIBP2/RET-V5 were plated in 6-well plates and grown in medium supplemented

278 with 0.5% BS (Gibco, Thermo Fisher), 1% Penicillin-Streptomycin, and 0.75 mg/ml

279 G418. At the indicated time points, cells were counted using a TC20 Automated Cell

280 Counter (Bio Rad).

281

282   ・*Western blotting*

283   Protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel

284   electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF)

285   membrane (Millipore). After blocking and incubation with an appropriate primary

286   antibody, the antigen-antibody complexes were visualized using a HRP-conjugated

287   secondary antibody (Cell Signaling Technology) and a chemiluminescence system

288   (Nacalai Tesque). Detection was performed using a LAS-3000 imaging system

289   (Fujifilm). Primary antibodies were obtained from the following sources:

290   anti-phospho-ERK (Thr202/Tyr204), anti-ERK, anti-phospho-MEK (Ser217/221), and

291   anti-MEK from Cell Signaling Technology; anti-β-actin from Santa Cruz

292   Biotechnology; anti-V5 from Invitrogen.

293

294   ・*Statistical analysis*

295   Differences between groups were examined for statistical significance with ANOVA

296   followed by Tukey's post test. A *p*-value not exceeding 0.05 was considered statistically

297   significant.

298

299

300   **Results**

301   *Cases and genetic analysis*

302   In our previous study (18), we performed mutational analysis for the presence of the

303   following well-established oncogenes, $BRAF^{V600E}$, *RAS*, *RET/PTC1, RET/PTC3*, and

304   *ETV6/NTRK3*, in 63 Fukushima PTC samples. We found 43 (68.3%) cases with the

305    $BRAF^{V600E}$ mutation, 6 (9.5%) with *RET/PTC1*, 1 (1.6%) with *RET/PTC3*, and 4 (6.3%)

306   with *ETV6/NTRK3* rearrangments. There were nine samples, however, in which no

307   mutation could be identifed. We then performed qRT-PCR to screen for novel fusion

308 genes by comparing the expression levels between the extracellular domain (5') and the

309 kinase domain (3') of *ALK*, *NTRK1*, *NTRK3*, and *RET*. A higher expression of the

310 kinase domain suggests the possibility of a gene rearrangement. Representative images

311 are shown in Supplementary Fig. S1 online. Among the nine samples, five displayed a

312 higher expression of the 3' exons of *ALK, NTRK1, NTRK3*, or *RET*. We then performed

313 subsequent 5'-RACE in these samples. Two of the five cases were found to have

314 already reported fusion oncogenes, *TPR/NTRK1* and *STRN/ALK* (19, 20). Sequencing of

315 the remaining three samples resulted in the discovery of *SQSTM1/NTRK3*,

316 *AFAP1L2/RET*, and *PPFIBP2/RET* rearrangements as shown in Fig. 1a–c.

317

318 All three cases with novel fusion genes were histologically confirmed to be classical

319 papillary adenocarcinomas without specific pathomorphological features or

320 multifocality. The characteristics of the three patients were as follows: 1)

321 *SQSTM1/NTRK3*: 18 years old (yo) female, hemithyroidectomy, 7.0 mm in diameter,

322 pT1a pN0 M0, no extrathyroidal extension; 2) *AFAP1L2/RET*: 18 yo male, total

323 thyroidectomy, 40 mm in diameter, pT2 pN1b M1 (lung), no extrathyroidal extension;

324 2) *PPFIBP2/RET*: 13 yo female, hemithyroidectomy, 15 mm in diameter, pT1b pN1a

325 Mo, no extrathyroidal extension. None of the three patients has developed a recurrence.

326

327 ***Characteristics of the three novel fusion genes***

328 *SQSTM1/NTRK3* is a result of an interchromosomal translocation t(5;15)(q35.3;q25.3),

329 which juxtaposes exons 1–5 of *SQSTM1* to exons 14–19 of *NTRK3* (Fig. 1a). The fusion

330 point in *NTRK3* is localized at the same position as in *ETV6/NTRK3* (14). As the lengths

331 of intron 5 of *SQSTM1* and intron 13 of *NTRK3* are 7,805 bp and 93,252 bp,

332 respectively, we did not search a breakpoint on genomic DNA.

333 *AFAP1L2/RET* is a result of an intrachromosomal translocation t(10)(q25.3;q11.2),

334 which fuses exons 1–2 and a part of intron 2 (38-bp) of *AFAP1L2* to the 3' part of exon

335 11 (33-bp) and exons 12–20 of *RET* in frame (Fig. 1b). This created a new exon, and

336 genomic breakpoints are located in intron 2 and exon 11 of *AFAP1L2* and *RET*,

337 respectively. As an acceptor site at the 5' end of exon 11 was missing, the genomic

338 sequence TTTTTTTTTGAG-A in intron 2 was presumably recognized as an acceptor

339 (Fig. 1b).

340 *PPFIBP2/RET* is a result of an interchromosomal translocation t(11;10)(p15.4;q11.2),

341 which juxtaposes exons 1–9 of *PPFIBP2* to exons 12–20 of *RET* (Fig. 2c). Although

342 breakpoints are located in intron 9 and in the middle of exon 11, the part of exon 11 was

343 spliced out. In both latter cases, the kinase domain of *RET* is fully preserved as in

344 conventional *RET/PTC* rearrangements.

345

346 As these types of fusion proteins are usually activated through dimerization by a domain

347 such as a coiled-coil domain in the N-terminal partner protein, we performed *in silico*

348 prediction   analysis   using   the   COILS   Server

349 (http://embnet.vital-it.ch/software/COILS_form.html) (21). SQSTM1/NTRK3 seemed

350 not to have a coiled-coil domain (Fig. 2). On the other hand, PPFIBP2/RET highly

351 likely possesses a coiled-coil domain (Fig. 2). For the AFAP1L2/RET fusion, the signal

352 was moderate (Fig. 2).

353

354 ***Functional studies***

355 First, we examined the effects of SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET

356 on the MAPK signaling pathway. For this purpose, these fusion proteins were

357 overexpressed in 293FT cells (Fig. 3a), and the phosphorylation status of MEK and

358 ERK was assessed by immunoblotting. As shown in Fig. 3b, ERK phosphorylation was

359 induced by all three novel rearrangements, indicative of their ability to constitutively

360 activate the MAPK pathway. We also checked AKT phosphorylation but did not observe

361 a significant change induced by these fusion proteins.

362

363   Next, we compared cell growth under low serum concentration. In this condition,

364   EGFP-expressing control cells exhibited modest growth. In contrast, transduction with

365   *SQSTM1/NTRK3*, *AFAP1L2/RET*, and *PPFIBP2/RET* significantly promoted cell

366   growth (Fig. 4a). Note that the cells with SQSTM1/NTRK3 grew faster than the other

367   two RET-carrying cells (Fig. 4a).

368   We then performed a focus formation assay to confirm the transforming potential of

369   each of the novel fusion genes. A number of foci were successfully observed in NIH3T3

370   cells stably transduced by *SQSTM1/NTRK3*, *AFAP1L2/RET*, and *PPFIBP2/RET* (Fig.

371   4b, c). In contrast, overexpression of EGFP (control) did not result in the formation of

372   any foci (Fig. 4b, c) and the number of foci was statistically different for all fusions

373   compared to the control (Fig. 4b). Again, note that the number of foci after transduction

374   with *SQSTM1/NTRK3* was greater than with *AFAP1L2/RET* and *PPFIBP2/RET* (Fig.

375   4b).

376   These results indicate that the newly discovered three fusions, *SQSTM1/NTRK3*,

377   *AFAP1L2/RET*, and *PPFIBP2/RET* represent novel driver oncogenes.

378

379

380   **Discussion**

381   In this study, we discovered three novel fusion oncogenes, *SQSTM1/NTRK3*,

382   *AFAP1L2/RET*, and *PPFIBP2/RET*, which have a transforming ability through MAPK

383   activation according to the *in vitro* studies. Taking into consideration our previous

384   results of molecular analysis in the 63 young PTC patients from Fukushima (18), in

385   which 43 cases with $BRAF^{V600E}$ (68.3%) and 11 (17.5%) with fusion genes were

386   identified, the number of gene rearrangement-positive cases is now increased to 16

387   cases (25.4%). In total, 59 out of 63 cases (93.7%) were confirmed to have a driver

388   mutation; only 4 cases (6.3%) remain negative for any oncogene so far.

389

390   We analyzed the oncogenic potential of the newly discovered fusion genes. First,

391   *SQSTM1/NTRK3* is a fusion between *SQSTM1* on chromosome 5 and *NTRK3* on

392   chromosome 15. *NTRK3* is a transmembrane receptor tyrosine kinase, whose ligand is

393   neurotrophin-3. Recently, other *SQSTM1* fusion genes have been identified in

394   hematological malignancies: *SQSTM1/ALK* in large B-cell lymphoma,

395   *SQSTM1/NUP214* in T-cell acute lymphoblastic leukemia, and *SQSTM1/FGFR1* in

396   myelomonocytic leukemia (22-25). SQSTM1/ALK has also been shown to have a

397   transforming ability by focus formation assay using 3T3 fibroblasts. Although SQSTM1

398   does not have a coiled-coil domain, there is a Phox and Bem1p (PB1) domain at the

399   N-terminus which enables formation of heteromeric and homomeric complexes (26).

400   Presumably, SQSTM1/NTRK3 is constitutively activated through dimerization

401   mediated by the PB1 domain.

402

403   The *SQSTM1* gene product is involved in several intracellular signal transduction

404   cascades comprising a signaling node for multiple pathways maintaining cellular

405   homeostasis. SQSTM1 may functionally contribute to aging, autophagy and the

406   development of degenerative diseases (27). How the disruption of one copy of *SQSTM1*

407   may contribute to tumor growth remains unclear. Note that mutations in the C-terminus

408   of *SQSTM1* have been reported to cause Paget's bone disease (28). Although the

409   SQSTM1/NTRK3-expressing NIH3T3 cells showed a higher growth rate and

410   transformation than the other two RET fusions, we cannot definitely conclude that this

411   fusion has a distinct malignant potential compared to the other two rearrangements

412   because of the intrinsic limitations of our assay systems.

413

414   The two other fusion oncogenes, *AFAP1L2/RET* and *PPFIBP2/RET*, are new variants

415   belonging to the *RET/PTC* family. The *RET* gene was originally identified as a

416   proto-oncogene more than 30 years ago (29), and then a rearrangement named

417   *RET/PTC* was found in PTC (30, 31). At present, more than 15 types of *RET/PTC*

418   *rearrangements* with different partner genes are known (32), to which our present work

419   adds two more. *RET* encodes a transmembrane receptor tyrosine kinase; binding of a

420   ligand, glial cell line-derived neurotrophic factor (GDNF), stimulates receptor

421   dimerization, which is a critical step for gaining tyrosine kinase activity. Although the

422   expression of *RET* in thyroid follicular cells is very limited, the fusion proteins are

423   commonly expressed in thyroid follicular cells and possess coiled-coiled, leucine zipper,

424   lis homology (LisH), or other domains that enable homo-dimerization of RET/PTC

425   fusion proteins. As a result, *RET/PTC* gene products are constitutively activated without

426   ligand binding in thyroid cells.

427

428   The novel partner gene *AFAP1L2* is located on chromosome 10 and belongs to the actin

429   filament-associated protein (AFAP) family. Although *AFAP1L2* is expressed in the

430   human thyroid as well as in other organs, the role of *AFAP1L2* in thyroid carcinogenesis

431   is not yet formally clarified (33). In *AFAP1L2/RET*, we found that only exons 1–2 were

432   fused to *RET*, but according to our *in silico* analysis, a coiled-coil domain likely exists

433   in this portion and enables dimerization, leading to constitutive activation of the RET

434   kinase. All of the results of our functional study suggest that AFAP1L2/RET is a novel

435   oncoprotein. However, as the breakpoint of this fusion is very unique (at intron and

436   exon), this is probably a rare event.

437

438   Finally, another newly discovered partner gene is *PPFIBP2*. It is located on

439   chromosome 11, and encodes the protein-tyrosine phosphatase receptor-type

440   f-polypeptide (PTPRF)-binding protein. The *PPFIBP2* product plays a role in axon

441   guidance and neuronal synapse development; its function in the thyroid is unknown. Of

442   note, rs12791447 SNP in the *PPFIBP2* intron was found to be a susceptibility marker

443 for prostate cancer (34). Also, elevated *PPFIBP2* mRNA expression was detected in

444 endometrial cancer (35), suggesting that it may be associated with cancer pathogenesis.

445 Again, the *in silico* analysis revealed that exons 1–9 of *PPFIBP2* highly likely harbor a

446 coiled-coil domain and promote dimerization, leading to constitutive activation of RET.

447

448 The limitation of the present work consists in the lack of *in vivo* tumor formation

449 studies. However, all driver oncogenes that have a focus-forming ability in NIH3T3

450 cells also formed tumors in immunodeficient mice.

451

452 In summary, three novel fusion oncogenes, *SQSTM1/NTRK3*, *AFAP1L2/RET*, and

453 *PPFIBP2/RET* were identified in the PTCs from one child and two adolescents from a

454 cohort consisting of 63 members from Fukushima. The findings suggest that rare

455 fusions may be present among the cases negative for known oncogenes in this age

456 group and that they play a significant role in thyroid carcinogenesis. Our results also

457 indicate that almost all PTCs in young patients (93.7%) have oncogenic driver

458 mutations activating the MAPK intracellular signaling pathway.

459

460

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Competing financial interests

593 We declare no competing interests.

594

595

Figure Legends

597 Figure 1. Genomic location, mRNA structure, sequence chromatogram of the breakpoint,

598 and genomic DNA structure around the breakpoint of (a) *SQSTM1/NTRK3*, (b)

599 *AFAP1L2/RET*, and (c) *PPFIBP2/RET*. Exons of a partner gene are indicated in blue

600 numbers, and those of a kinase gene are in red. The breakpoints are indicated by

601 arrowheads.

602

603 Figure 2. *In silico* prediction of a coiled-coil domain. Amino acid sequences of

604 SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET were entered into the COILS

605 server. Plotted is the probability of a coiled-coil domain existence at three different

606 scanning windows. The breakpoints are indicated as arrowheads and dotted lines.

607

608 Figure 3. Effect of the novel fusions on signaling pathways. (a) 293FT cells were

609 transiently transfected with mock control or expression vectors of the V5-tagged fusion

610 genes. After 48 h of transfection, Western blot was performed using an anti-V5 antibody.

611 (b) 293FT cells were transiently transfected with mock control or expression vectors of

612 the V5-tagged fusion genes. After 6–8 h incubation, the cells were serum-starved. After

613 additional 40 h incubation, whole cell lysates were subjected to Western blot using

614 indicated primary antibodies. Experiments were repeated at least twice with similar

615 results.

616

617 Figure 4. Transforming potential of the novel fusions. NIH3T3 cells were transduced

618 with the indicated retroviruses. (a) 5×10^4 of the cells were plated, and the number of

619 cells were counted after the indicated time points. Each point indicates the mean and

620 standard error of 3–6 wells of a 6-well plate. *$p<0.05$ *vs*. EGFP. (b) The cells were

621 cultured in a 6-well plate for two weeks, and the number of foci was counted. The bars

622 represent the mean and standard error of three wells of a 6-well plate. *$p<0.01$, #$p<0.05$

623 *vs*. EGFP (c) Representative images of the foci induced by the indicated fusions. These

624 data are representative of at least two independent experiments.
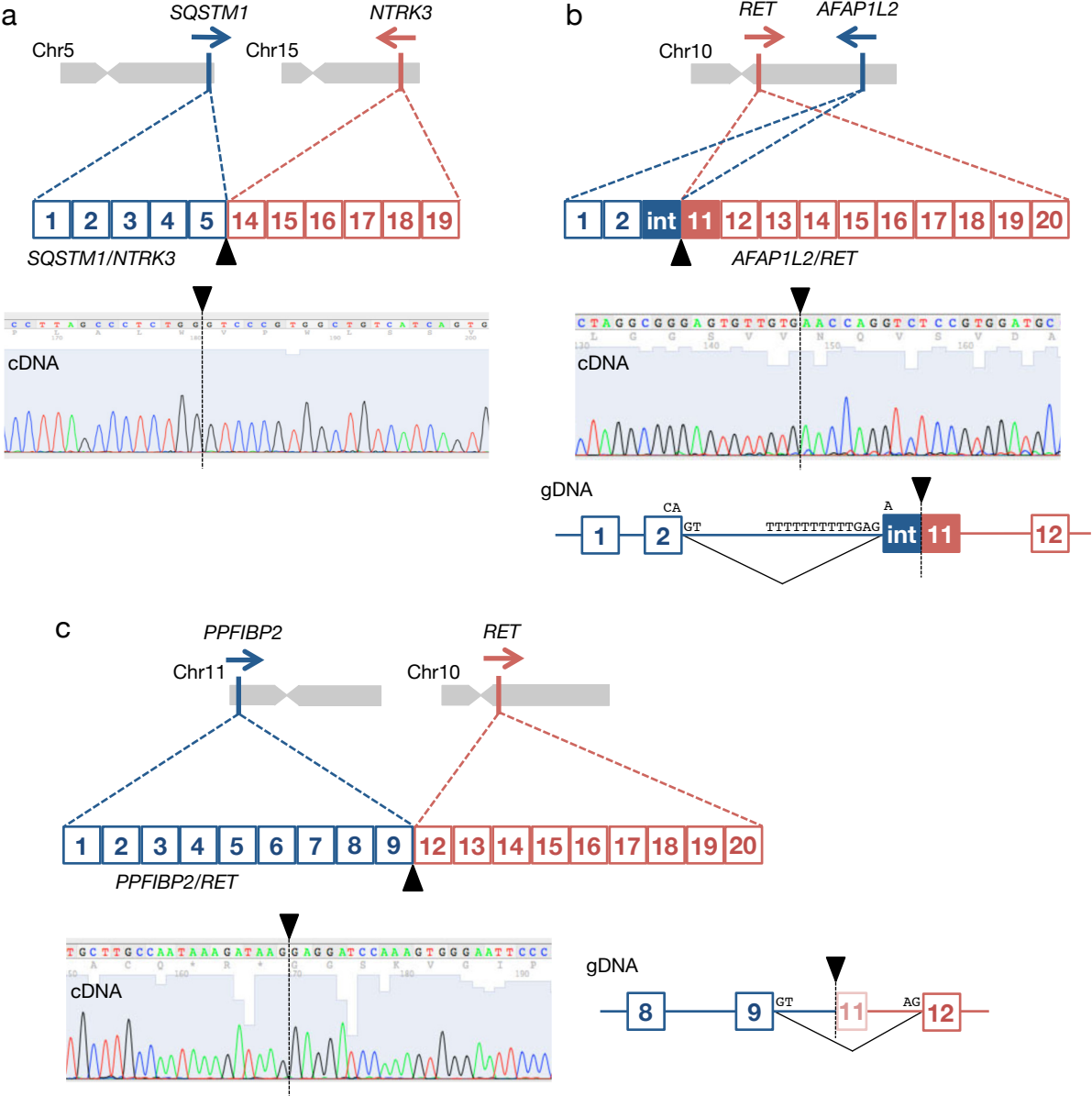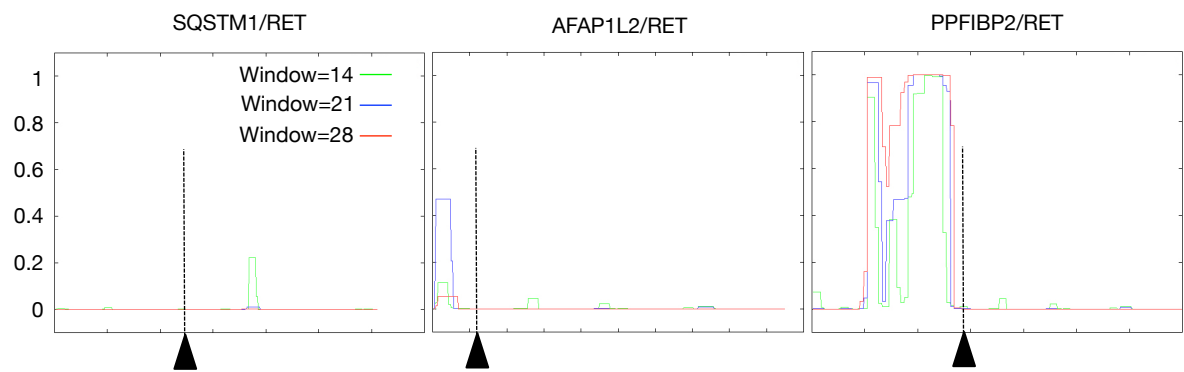
625

Figure 1

Figure 2

SQSTM1/RET   AFAP1L2/RET   PPFIBP2/RET

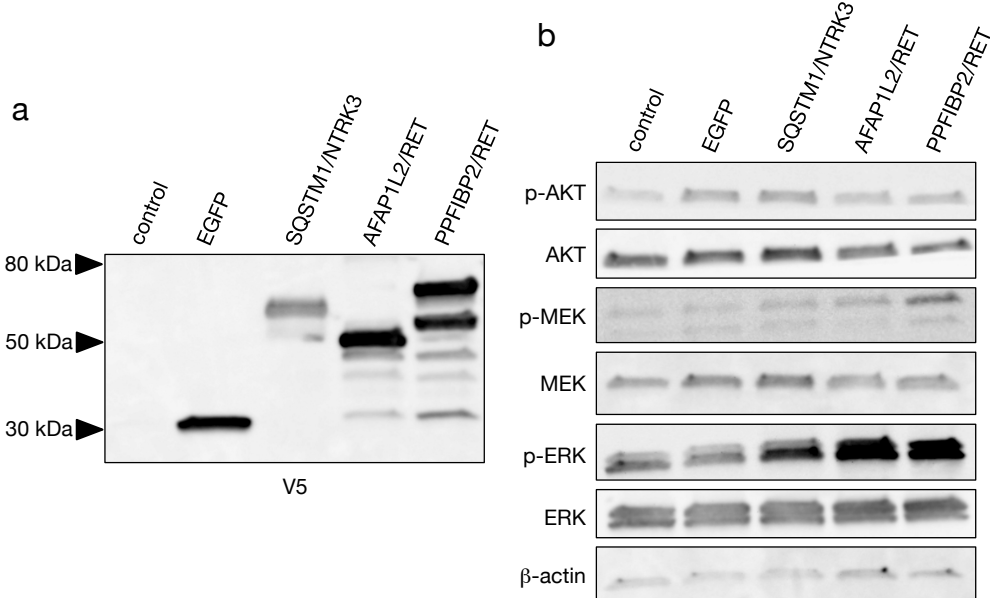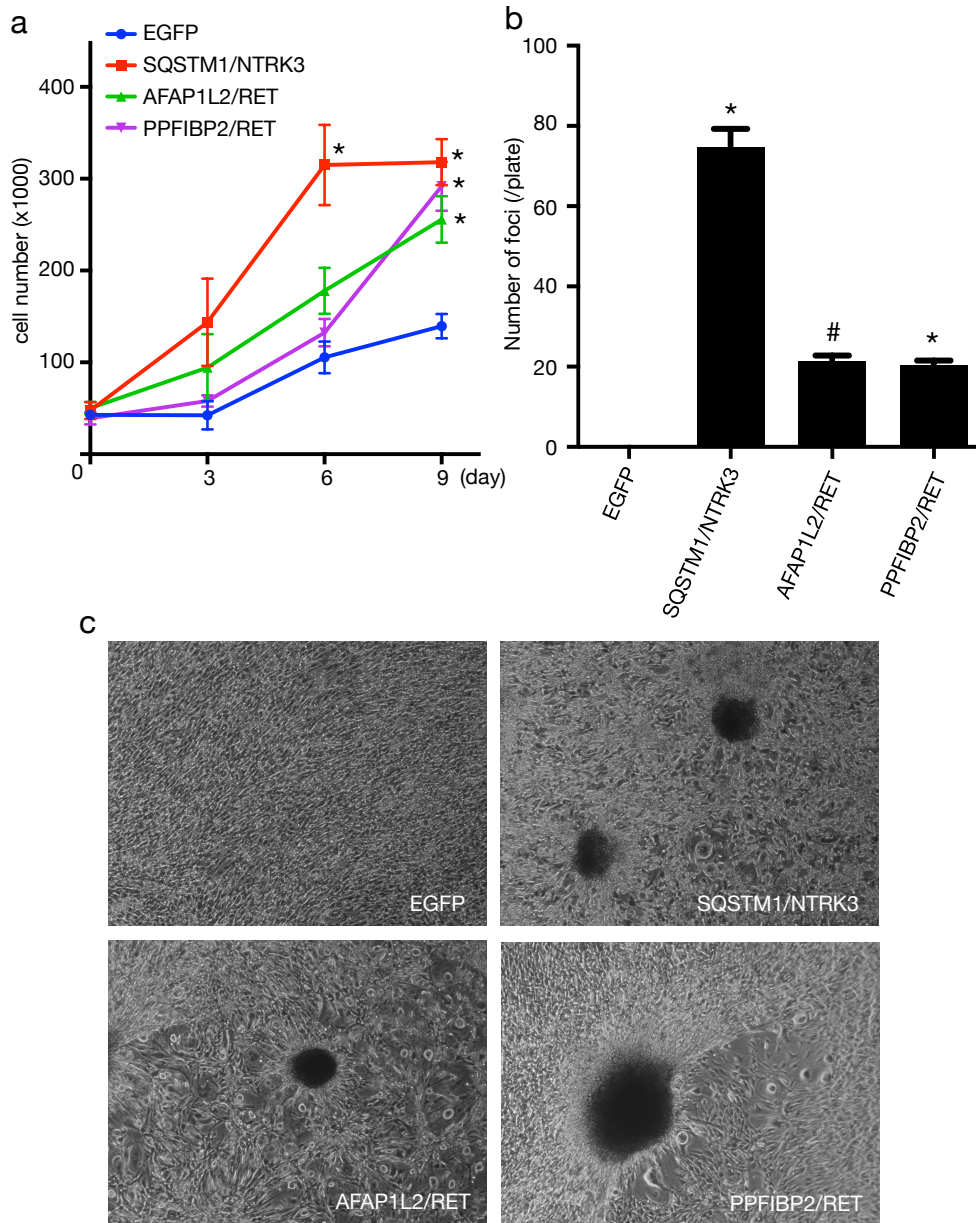Window=14
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Figure 3

Figure 4

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

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Supplementary Figure S1
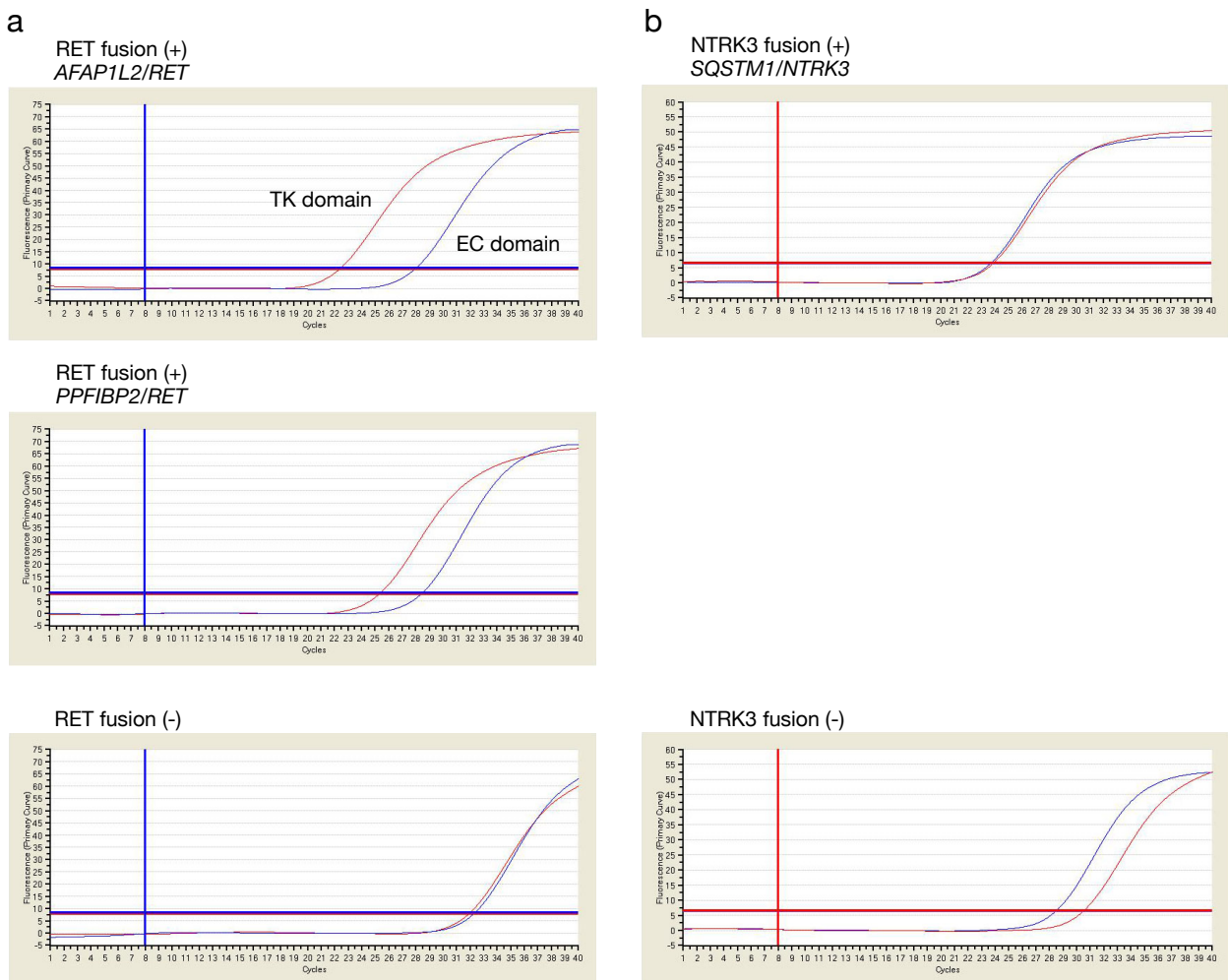
Supplementary Figure S1. Examples of amplification curves by qRT-PCR. (a) Upper and middle, the samples with *RET* fusion genes. The cycle threshold of the amplicon located at the tyrosine kinase (TK) domain was far smaller than that at the extracellular (EC) domain. The TK domain was abundantly expressed. 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.