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A multicenter analysis of the FIP1L1-αPDGFR fusion gene in Japanese idiopathic hypereosinophilic syndrome: An aberrant splicing skipping the αPDGFR exon 12

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Abstract

To study the clinical characteristics of hypereosionophilic syndrome and chronic eosinophilic leukemia (HES/CEL) in Japan, the clinical data of 29 HES/CEL patients throughout the country were surveyed. Moreover, the involvement of the ***FIP1L1-αPDGFR*** fusion gene resulting from a cryptic del (4)(q12q12) was examined in 24 cases. The ***FIP1L1-αPDGFR*** mRNA was detected in three patients (13 % of patients fulfilled WHO criteria and 17 % of Chusid criteria). One had a novel fusion transcript which skipped the exon 12 of αPDGFR. The transcript appears to be generated by a splicing mechanism that is different from the previously reported splicing patterns. ***In Silico*** analysis, the exon skipping was not related to a disruption of the exonic splicing enhancers within the exon, but strongly associated with the loss of the vast majority of the ***FIP1L*** intron 8a where intronic splicing enhancers were accumulated. Unexpectedly, pseudo-chimera DNA fragments with some shared characteristic features were occasionally generated from healthy control samples by RT-PCR. Considering the relatively low incidence of the ***FIP1L1-αPDGFR*** transcript positive case, extreme care must

therefore be taken when making a diagnosis using RT-PCR before imatinib therapy.

Key words

hypereosionophilic syndrome, imatinib, *FIP1L1-αPDGFR*, RT-PCR, exon skipping, alternative splicing, CEL

Introduction

Hypereosinophilic syndrome (HES) and chronic eosinophilic leukemia (CEL) are heterogeneous disorders characterized by prolonged eosinophilia. There are two commonly used diagnostic criteria proposed by Chusid in 1975 [1] and by the World Health Organization (WHO) in 2001 [2]. The former includes (1) eosinophilia exceeding 1.5×10^9/l, and persisting for more than 6 months, (2) no secondary cause such as allergies or helminthic infection and (3) evidence of eosinophil-mediated tissue damage. The latter consists of (1) persistent eosinophilia exceeding 1.5×10^9/l, (2) increased numbers of bone marrow eosinophils, and myeloblasts under 20% in blood or bone marrow and (3) exclusion of known causes of eosinophilia, including clonal or abnormal T-cell populations. If clonal proliferation of eosinophils can be demonstrated, HES is reclassified as CEL [2].

Most HES patients exhibit normal karyotypes by conventional cytogenetics. Recently, an 800 kb interstitial deletion on 4q12 resulting in the fusion of the FIP1-like-1 gene (***FIP1L1***) and the alpha-type platelet-derived growth factor receptor gene

(*αPDGFR*) was identified to be a cause of HES/CEL [3, 4]. Platelet-derived growth factor (PDGF) was first identified as a major connective tissue cell mitogen that plays a role in atherosclerosis and wound healing. Thereafter, the finding that the PDGF B-chain gene is a human homologue of a viral oncogene, *v-sis*, expedited understanding the molecular mechanisms of human oncogenesis. The autophosphorylated alpha- as well as beta-type PDGFR (βPDGFR) stimulated by dimeric PDGF isoforms transduce the intracellular signaling for cell proliferation [5]. Moreover, the mitogenic signaling pathways in mesenchymal stromal cells can be reconstituted by the ectopic expression of these receptor genes in hematopoietic cells [6]. Since the 1990s, genomic mutations of *α and βPDGFR* had been found in various types of human leukemia, such as chronic myelogenous leukemia with t(4;22) or t(5;10) [7] [8], chronic myelomonocytic leukemia with t(5;12), t(5;7)or t(5;17) [9-11], acute myelogenous leukemia with t(5;14) [12]. Among them, FIP1L1-αPDGFR is a constitutively activated tyrosine kinase, which does not depend on dimerization but on the disruption of an autoinhibitory juxtamembrane domain encoded by exon 12 of *αPDGFR* [13]. A small molecule kinase

inhibitor, imatinib is active not only for bcr/abl kinase but also for the PDGF receptor-family kinases. Therefore, most HES patients with *FIP1L1-αPDGFR* show a dramatic response to imatinib [4,14,15]. Deletion of 4q12 with *FIP1L1-αPDGFR* is cytogenetically cryptic, but can be detected by reverse transcriptase-PCR (RT-PCR) [3, 4] or fluorescence *in situ* hybridization (FISH) [16].

The prevalence of *FIP1L1-αPDGFR* varies from 11% to 56% in each country reported. Cools *et al.* first reported that the fusion gene was identified in 9 (56%) of 16 patients [4], whereas subsequent reports showed much lower prevalence; 11/81 (14%), 6/35 (17%) and 40/376 (11%) from the USA, France and the UK, respectively [17, 18, 19]. So far, no nationwide survey data from Asia, including Japan, has yet been reported.

The first aim of this study was to examine the prevalence of *FIP1L1-αPDGFR* among Japanese HES/CEL by RT-PCR, and examine the possible association between clinical characteristics and the expression of the fusion gene. During this study, a novel fusion transcript generated by skipping the exon 12 of *αPDGFR* was found.

Thereafter, a novel mechanism of the aberrant splicing was also analyzed *in Silico*.

Materials and methods

Patients

The clinical data of 29 Japanese patients with HES/CEL diagnosed by WHO or Chusid criteria between January 1995 and February 2007 was collected by questionnaires. Twenty-one patients were enrolled from the Japanese Elderly Leukemia and Lymphoma Study Group (JELLSG; http://www.jellsg.umin.jp), and eight patients were directly from a variety of nationwide hospitals. Peripheral blood (PB) samples were obtained from 24 cases among these patients. The gene analysis was approved by the Institutional Review Board of Kobe University Graduate School of Medicine (Protocol number 150023). Written informed consent was obtained from all patients.

RT-PCR

Ten ml of PB were collected in a tube containing EDTA as an anti-coagulant from each patient. White blood cells (WBC) obtained by red cell-lysis and/or mononuclear cells (MNC)

isolated by density gradient centrifugation were stored at -80°C until RNA/DNA extraction.

Total RNA was isolated from WBC or MNC using the RNeasy kit (Qiagen) according to the manufacturer's recommendation including a step of DNase-1 treatment. cDNA was synthesized from 0.4-1.7 μg of total RNA using the Superscript first-strand synthesis system (Invitrogen) with oligo (dT) primers. The *FIP1L1-αPDGFR* fusion transcript was confirmed by nested PCR using two primer sets. The primer set 1 was the same as described by Cools *et al.* [4]; FIP1L1-F1, 5' acctggtgctgatctttctgat 3' and αPDGFR-R1, 5' tgagagcttgtttttcactgga 3' for the first step PCR, and FIP1L1-F2, 5' aaagaggatacgaatgggacttg 3' and αPDGFR-R2, 5' gggaccggcttaatccatag 3' for the second step PCR. The forward primers of primer set 2 were modified as described by Score *et al.* [20]: FIP1L1-F3, 5' cacctggaagcattaatggag 3' and FIP1L1-F4, 5' agttccactcttagaggtag 3' for the first and second PCR, respectively.

A master PCR mixture was freshly prepared with final concentrations of 0.2 mM dNTP, 1.5 mM $MgCl_2$, 5 μl of 10× PCR buffer

containing 10 mM Tris-HCl (pH8.0), 100 mM NaCl, 0.1 mM EDTA, 1mM DTT, 50% glycerol and 1% TritonX-100, 1 μl of DMSO, 2.5 U of Taq polymerase and mixed with 30 pmol of each primer in a total volume of 50 μl. PCR was performed with an initial denaturation step of 3 min at 94°C followed by 35 cycles for first step at 94°C for 30 sec, at 60°C for 20 sec and at 72°C for 1 min, and final elongation step of at 72°C for 7 min. Five μl of each PCR product was used for next nested step for 20 cycles in a total volume of 50 μl.

Genomic PCR

Cell pellets of MNC or WBC are digested with 0.5 ml of lysis buffer (10 mM Tris-HCl [pH 8.5], 5 mM EDTA [pH 8.0], 200 mM NaCl, 0.2% [w/v] SDS) containing proteinase K to a final concentration of 0.12 mg/ml overnight in 55°C, followed by treatment with RNase A for one hour at room temperature at a final concentration of 50 μg/ml. Genomic DNA was precipitated with an equal volume of isopropanol, extracted with a 100-μl pipette tip or pelleted, and then dissolved in 0.2 ml of distilled water. Amplification

of 5 μl of digested DNA was performed using the Expand Long Template PCR System (Roche) in a total volume of 50 μl. The PCR cycling profile was as follows; denaturation at 94°C for 2 min followed by 25 cycles at 94°C for 10 sec, 60°C for 30 sec, 68°C for 2 min and a final extension at 68°C for 7 min. Nested PCR was performed with 5 μl of first PCR products under the same conditions as the first.

Oligonucleotide primers used for the genomic PCR for Patient #005 were; FIP1L1-F5, 5'-aagcatctaattaggtgaaactg 3' and αPDGFR-R3 5' aagttgtgtgcaagggaaaaggg 3' for the first step, FIP1L1-F6, 5' cagactataactatcagccg 3' and αPDGFR-R4, 5' gtccatctcttggaaactcc 3' for the second step. Primers for Patients #017 and #038 were: FIP1L1-F7, 5' cagcacttcttctcagtctca 3' and αPDGFR-R5, 5' tgagagcttgtttttcactgga 3' for the first step, FIP1L1-F8, 5'-gacaagtactgcctccagaa 3' and αPDGFR-R6, 5' gggaccggcttaatccatag 3' for the nested step. Primers for #058 were: FIP1L1-F9, 5' catgggctttatcagtctcttta3' and αPDGFR-R5 for the first step, FIP1L1-F10, gtgcttgtggaagtaaaacgta and αPDGFR-R6 for the second step

Nucleotide Sequencing

After agarose gel electrophoresis, amplified DNA fragments were purified (QIAEX II Gel Purification kit, Qiagen), and sequenced either directly, using an ABI Prism 310 Genetic Analyzer (Applied Biosystems) and a Big Dye Terminator DNA sequencing kit (Applied Biosystems). Homology searches were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Results

Patient characteristics

The patient characteristics are summarized in **Table 1**. Twenty-seven of the total 29 diagnostic HES/CEL patients fulfilled the WHO criteria, and 16 patients fulfilled the Chusid criteria, including 14 that overlapped. Two of the 16 patients diagnosed by Chusid criteria did not meet the WHO criteria because of insufficient examination of their bone marrow. Thirteen of 27 patients diagnosed by WHO criteria did not satisfy Chusid criteria, they did not demonstrate any specific symptoms or the eosinophilia persisted less than six months. In the patients who met the WHO criteria, the male/female ratio was 1.7 : 1 with a median age at diagnosis of 50 years (range 17-87. The median eosinophil count was 11,900/μl (range, 2,200 to 60,700. Two patients with abnormal karyotype were classified as CEL; one had t(2;5)(p23;q31), and the other had der(1)t(1;12)(q32;q13), inv(7)(q22;q31). Twenty-three patients were symptomatic with fever of unknown origin, cough, hepatomegaly, skin lesions and/or splenomegaly. Twenty-three were treated with corticosteroids (19 cases) or imatinib (4 cases). Three

patients treated with imatinib achieved a complete response. Another patient discontinued imatinib within two days, because of severe diarrhea and vomiting.

FIP1L1-αPDGFR mRNA positive HES/CEL

The expression of the ***FIP1L1-αPDGFR*** fusion transcript was assayed by RT-PCR using primer sets 1 and 2 in 24 cases. ***FIP1L1-αPDGFR*** mRNA was reproducibly detectable in three patients (#005, #017 and #058) by both primer sets (**Figure 1**). Patients #005 and #017 were males, age 43, with normal karyotypes by the conventional G-banding method. The clinical characteristics of #005 were described previously [21]. Briefly, he had a fever of unknown origin, cardiac murmur, hepatosplenomegaly, leukocytosis (WBC 17.2×10^9/l, 60% eosinophils, 5% blasts), anemia (hemoglobin 6.9 g/dl) and thrombocytopenia (51.9×10^9/l). The bone marrow aspirate showed a nucleated cell count of 130×10^9/l, 3.6% blasts and 65% eosinophils. Patient #017 did not have significant symptoms except for a persistent cough. A blood examination showed peripheral blood leukocytosis (WBC count of 35.8×10^9/l with 35% eosinophils and 0% blasts) and mild anemia

(hemoglobin 12.8 g/dl) accompanied by bone marrow eosinophilia (37%). These two patients were administered imatinib therapy, and achieved a complete response. Patient #058 was a male at age 26 with a fever of unknown origin, cough, cardiac murmur, congestive heart failure with mitral-valve disorder and splenomegaly. A blood examination showed leukocytosis (WBC count of 21.5×10^{9}/l with 59% eosinophils and 0% blasts) and anemia (hemoglobin 9.2 g/dl) accompanied by bone marrow eosinophilia (36%) with normal karyotype by the G-banding method. While the administration of prednisolone had no significant effect on eosinophilia, a low dose of imatinib (100mg/day) was found to induce a complete remission. This case met the WHO criteria but not the Chusid criteria, because the diagnosis was done at only two months from the onset. It was difficult to distinguish FIP1L1-αPDGFR positive cases from negative ones by physical appearance and/or by other laboratory findings (**Table 1**).

Meanwhile, a ***FIP1L1-αPDGFR*** chimera cDNA fragment of patient #038 was amplified by primer set 2 but not by primer set 1 (**Figure 1A**). It appeared to be pseudo positive, because a chimera gene was not amplified from the patient's genomic DNA as described

below (**Figure 1B**).

Nucleotide Sequences of the chimera transcripts

In case #005, ***FIP1L1*** exon 9 was fused to a truncated exon 12 of αPDGFR with a breakpoint at nucleotide numbers #1160 of FIP1L1 mRNA (GenBank accession number NM030917) and #1877 of αPDGFR (M21574) (Figure 2, #005 type 1). There was another splice variant which lacked the ***FIP1L1*** exon 8a, but had the same breakpoint (#005 type 2). In #017, the ***FIP1L1*** exon 8a was fused to the ***αPDGFR*** exon 13 with a breakpoint at nucleotide #1066 and #1926, respectively. In #058, the truncated intron 8a of ***FIP1L1*** at nucleotide #1627077 of the human chromosome 4 genome (GenBank accession number NT22853) was fused to truncated exon 12 of ***αPDGFR*** at nucleotide #1864. Case #058 also had splice variant without the ***FIP1L1*** exon 7 (#058 type 2). All of the transcripts were in frame.

The cDNA fragment amplified by primer set 1 in #038 had ***FIP1L1-αPDGFR*** chimera sequences similar to those of #005, #017 and #058 (**Figure 2**). In #038, however, two additional nucleotides

'TA' of unknown origin were found at the breakpoint of the *FIP1L1* exon 8a (nucleotide #1066) and of the truncated exon12 of *αPDGFR* (nucleotide #1863). The characteristic nucleotide insertion suggested that the DNA fragment amplified from #038 was not a contamination from other *FIP1L1-αPDGFR* positive samples.

Genomic analysis of the FIP1L1-αPDGFR chimera gene

The *FIP1L1-αPDGFR* fusion gene was amplified by genomic PCR in three patients (#005, #017, and #058), but not in #038 or healthy controls (**Figure 1B**). The genomic breakpoints of the fusion genes were shown in **Figure 3**. In #005, the *FIP1L1* intron 9 at nucleotide #1632015 (NT22853) fused to the truncated αPDGFR exon 12 at nucleotide #2480965. In #017, the *FIP1L1* intron 8a at #1622282 fused to the *αPDGFR* exon 12 at #2480961. In #058, the *FIP1L1* intron 8a at #1627077 fused to the *αPDGFR* exon 12 at #2480962. The splicing pattern was different in each patient. Two types of splicing pattern of the fusion gene have been reported [22]. The splice patterns of #005 and #058 were type II and I, respectively. The cryptic splice sites located at 10

and 31 bp from the breakpoint in #005 and #058, respectively. Those were close to the breakpoint, as those reported previously [4,15,18,19,22]. In contrast, the fusion transcript of #017 was generated by DNA splicing using a 5'donor splice site of the *FIP1L1* intron 8a and a 3' authentic acceptor site of the *αPDGFR* exon 13. This is the first case with the *FIP1L1-αPDGFR* transcript skipping the *αPDGFR* exon 12, which is not consistent with any types of splicing pattern reported [22].

The lack of amplification of the chimera gene by genomic PCR in case #038 also supports the speculation that the *FIP1L1-αPDGFR* fusion DNA fragment detected by RT-PCR was generated artificially. Therefore, we examined whether a pseudo-*FIP1L1-αPDGFR* chimera DNA fragment could be amplified in healthy individuals by RT-PCR. Surprisingly, chimera cDNA fragments were detected in two of seven healthy normal controls by using one of two primer sets occasionally (one of several PCR reactions in each sample), but not by both sets. The chimera cDNA fragments derived from the healthy controls showed the same characteristics of case #038 with 'TA' insertion adjacent to the breakpoint at nucleotide #1863 of the truncated *αPDGFR* exon

12 (**Figure 4**). Genomic PCR failed to show any rearrangements in healthy controls as well as in case #038.

In Silico analysis of the splicing mechanism skipping the αPDGFR exon 12

Because the cryptic splice sites were located close to the breakpoints of cases #005 and #058 as well as those of all cases reported previously [4,15,18,19,22], the sequences around the splice sites, approximately 100 bp from the breakpoint were analyzed using a web-based resource ASD [23]. There were several candidate sequences to substitute the classical splicing signals such as 3' splice site, branch site and polypyrimidine tract at the intron-exon boundary in #017 as well as in other cases (data not shown). Therefore, the nucleotide sequences upstream of the breakpoint of *αPDGFR* exon12 could not seem to explain the differences of the splicing pattern.

Next, the auxiliary motifs named exonic splicing enhancers (ESEs) that are used by the splicing machinery for recognizing exon-intron junctions were assessed using web-based resources, ESE-finder [24] and RESCUE-ESE [25]. The genomic breakpoints of

αPDGFR exon12 in three patients were closely located within a 4 bp distance. New ESE motifs were created at the breakpoint of *αPDGFR* exon 12 in all cases (data not shown). This means that no relationship was observed between the breakpoint and the splicing type.

Thereafter, the differences in intronic auxiliary motifs named intronic splicing enhancers (ISE) were further analyzed. The number of ISE motifs every 500 nucleotides were examined using web-based resource, RESCUE-ISE [26]. In *FIP1L1* intron 8a, there are three ISE motif peaks of over 0.1 in the frequency (the number of motif per nucleotide) (**Figure 5**). The first peak contained known ISE motifs, the repetitive dinucleotides $(CA)_{12}(CU)_{28}$ from nucleotides #1623790 to #1623869 of the human chromosome 4 genome (NT22853). The second and third peaks were composed of overlapped multiple divergent motifs, but they did not match any ISE motifs identified by mutational analysis [27]. The breakpoints of case #058 as well as other cases reported by Klion *et al.* [28] and Cools *et al.* [4] in the *FIP1L1* intron 8a were located between the second and third peaks, whereas case #017 lost all three peaks as shown in Figure 5. Taken together, the exon skipping of #017 was

probably due to the loss of a vast majority of ***FIP1L1*** intron 8a and the ISE motifs.

Discussion

The incidence of the *FIP1L1-αPDGFR* fusion gene in Japanese HES (13% in WHO, 17% in Chusid) was similar to those described in recent reports from Western countries (10-20%) [17-19]. All *FIP1L1-αPDGFR*-positive cases in this study are male. The male predominance is compatible with previous reports [4, 22]. There was no trend of difference in other clinical features listed in Table 1 between *FIP1L1-αPDGFR*-positive and -negative patients, although the number of positive cases is too low to compare statistically. Therefore, it is critical to examine the *FIP1L1-αPDGFR* fusion gene of HES, because imatinib therapy is expected to provide a complete response for the fusion gene positive cases [4, 14, 15, and 19].

This study found an aberrant fusion transcript skipping the *αPDGFR* exon 12. A comparative analysis of splicing mechanisms of exon inclusion and skipping revealed that the difference may depend on the site of the breakpoint in the *FIP1L1* intron. The exon skipping is possibly due to the loss of the dinucleotide repeats as known ISE sequences, and/or the loss of accumulated multiple hexameric ISE motifs. Simple repeats such as the

$(CA)_{12}(CU)_{28}$ deleted in #017 could affect splicing efficiency or splice site choice [29]. For example, the CA-repeat which was found in intron 13 of the human endothelial NO synthetase gene could function either as splicing enhancers or silencers, depending on their proximity to the alternative 5' splice site [30]. The CUCUCU element is required for splicing repression, and binds to the polypyrimidine tract binding protein or its neuronal homolog (PTB/nPTB). It is often found in the polypyrimidine tract of regulated 3′ splice sites [31]. Those are considered to be strong signals that are important for efficient splicing. Meanwhile, the distribution of the ISE motif such as the second and third peaks in ***FIP1L1*** intron 8a is composed of the accumulation of multiple hexameric motifs. These sites with a high distribution of ISE motifs may also be responsible to recognize the downstream sequence of the ***αPDGFR*** exon 12. This explanation is also consistent with the current exon recognition model; initial splice-site recognition is achieved by the accumulation of multiple weak signals, not by a few strong signals [27,32-34]. It is interesting to elucidate whether the latent exon 12 skipping of ***αPDGFR*** is rare or not in order to

understand the splicing mechanism of the FIP1L1-αPDGFR fusion gene.

These experiments also found an amplification of pseudo-*FIP1L1-αPDGFR* DNA fragments from healthy controls by RT-PCR. Sequence analysis revealed that the recombination of the pseudo-chimera occurred between *FIP1L1* exon 8a and *αPDGFR* exon 12, and that two nucleotides 'TA' were commonly inserted at the breakpoint. Therefore, this *in vitro* recombination is not random, but it instead seemed to mimic the genetic recombination *in vivo*.

Using a RT-PCR analysis, several aberrant mutations in normal controls have been reported in differential processing; in blood storage [35], during RT-PCR [36] and the PCR reaction [37]. These artificial mutations also occurred in mutational hotspots, and mimic a fusion formation. The artificial recombination between *FIP1L1* and *αPDGFR* transcripts is thought to be produced in the process of PCR amplification, because the artifact chimera formation was occasionally generated using a same cDNA sample. Several mechanisms of an artificial chimera formation during PCR have thus been proposed [38], although it is difficult to explain

the molecular mechanism of the pseudo *FIP1L1-αPDGFR* chimera formation. The *FIP1L1-αPDGFR* fusion gene/transcript was usually examined by nested PCR, because of the low expression of the fusion gene [4, 20], which may contribute to a sufficient amplification of the pseudo-chimera. The breakpoints of the atypical chronic myeloid leukemia with the *BCR-αPDGFR* fusion gene associated with t(4;22)(q12;q11) also fell in the *αPDGFR* exon12 [7, 39]. Therefore, the site at the *αPDGFR* exon 12 may be highly susceptible to become rearranged during *in vivo* DNA replication, and even *in vitro* during PCR. The molecular mechanism of *in vitro* recombination may offer the key to understand the DNA recombination in HES/CEL.

Meanwhile, these mutational hotspots i*n vitro* could easily lead to a misdiagnosis. It is therefore necessary to be careful when making a diagnosis of HES with *FIP1L1-αPDGFR* by RT-PCR. To rule out pseudo-positive cases, repeated RT-PCR with several primer sets, genomic PCR and/or a FISH analysis may be useful to confirm the presence of the *FIP1L1-αPDGFR* fusion gene.

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Figure Legends

Figure 1 Detection of *FIP1L1-αPDGFR* fusion transcript and gene

(A) *FIP1L1-αPDGFR* cDNAs of cases #005, #017 and #058 were detected by PCR using both primer set1 and 2. However, the fusion band of #038 was detected only by primer set2.

(B) The *FIP1L1-αPDGFR* fusion gene was amplified from genomic DNA samples of #005, #017 and #058, but not from those of #038 or normal control.

The nucleotide length(s) of the corresponding band(s) are shown below each lane. M; 100 bp DNA ladder size marker

Figure 2 The structure of *FIP1L1-αPDGFR* fusion transcripts

The structure of *FIP1L1-αPDGFR* mRNAs of #005, #017 and #058 are illustrated. There are alternative splice variants (type 2) without exon 8a and exon 7 in #005 and #058, respectively.

The exons of *FIP1L1* and *αPDGFR* are shown in green and red boxes, respectively. Gray boxes indicate a truncated intron 8a of *FIP1L1*. Arrows indicate the position of PCR primers used. The nucleotide numbers of each mRNA (The GenBank accession number is NM030917

for *FIP1L1*, M21574 for *αPDGFR*) are shown below the boxes. The nucleotide sequences around the breakpoint are also shown in each transcript.

The fusion-point of #038 was similar to that of #058, but an additional nucleotides 'TA' insertion (a white box) was found.

Figure 3 The splicing pattern of *FIP1L1-αPDGFR* fusion genes

A cryptic splice acceptor site close to the breakpoint of *αPDGFR* exon 12 or *FIP1L1* intron 8a was activated for exon inclusion in #005 or #058, while the *αPDGFR* exon12 was skipped in #017. Boxes and dotted lines represent exons and introns, respectively. The corresponding nucleotide numbers of the human chromosome 4 genome (GenBank accession number NT22853) are shown above each exon and introns. Colored solid lines represent transcripts. Vertical bars represent genomic breakpoints. Each blue box indicates a 5'donor splice site (5'SS) or a 3' acceptor splice site (3'ss). Inverted caret-like lines indicate splicing events. Type I and II indicate the corresponding splicing pattern described by Vandenberghe *et al* [22].

Figure 4 The structure of the FIP1L1-αPDGFR chimera DNA fragments amplified in normal individuals

The structures of pseudo-chimera DNA fragments detected in healthy individuals are shown. The ***FIP1L1-αPDGFR*** chimera was detected in two of seven healthy individuals: Normal 1 and Normal 2. The chimera DNA was detected only by primer set 2 in Normal 1, but only by primer set 1 in Normal 2. Both showed the breakpoints at ***FIP1L1*** exon 8a and ***αPDGFR*** exon 12, with all transcripts accompanied by an additional two nucleotide insertion (white boxes). Arrows indicate the position of PCR primers used. The nucleotide sequences around the breakpoint are shown. The corresponding nucleotide numbers of each mRNA are also shown as indicated in Figure 2.

Figure 5 Distribution of ISE motifs in FI1L1 intron 8a

The ISE motifs were examined using RESCUE-ISE [26]. The frequency of the ISE motifs (motifs number per one nucleotide) is shown on the Y-axis. The nucleotide numbers of the human chromosome 4 genome (GenBank accession number NT22853) corresponding to

FIP1L1 intron 8a are shown on the X axis. There are three peaks of ISE motifs distribution over 0.1 in frequency. The deleted nucleotide length is 9,639 bp in #017, while it is 4,844 bp in #058 (Total nucleotide length of intron 8a is 11,149 bp). The breakpoints of the other two patients reported by Cools ***et al.*** (case #1) [4] and by Klion ***et al.*** (case #5) [28] are also indicated by arrows.

Table 1. Clinical and biological characteristics comparing FIP1L1-αPDGFR positive and negative patients.

Characteristics	All*	Diagnosis of HES	
		Chimera negative	Chimera positive
No. of patients	29	21 (87.5%)	3 (12.5%)
Age, y, median (range)	52 (17 - 87)	52 (17-87)	37 (26 - 43)
M/F ratio	1.9 : 1	1.3 : 1	3 : 0
WBC count, $\times 10^9$ /l, median (range)	22.2 (5.9 - 70.6)	22.1 (5.9-70.6)	24.8 (17.2 - 25.8)
Eosinophil count, $\times 10^9$ /l, median (range)	12.7 (1.7 - 60.7)	12.3 (1.7-60.7)	12.5 (12.4- 12.7)
Abnormal karyotype	2	2	0
Marrow eosinophilia(%)	36.7 (9.9 - 68.0)%	36.9(9.9-68.0)	44.5 (36.2 - 60.0)%
Clinical features			
Symptomatic	25 (86%)	17 (81%)	3 (100%)
fever of unknown origin	7 (24%)	5 (24%)	2 (67%)
skin lesions	5 (17%)	5 (24%)	0 (0%)
cough	4 (14%)	1 (5%)	1 (33%)
hepatomegaly	4 (14%)	3 (14%)	1 (33%)
splenomegaly	4 (14%)	2 (10%)	2 (67%)
cardiac murmur	4 (14%)	1 (5%)	2 (67%)
central nervous system dysfunction	3 (10%)	2 (10%)	0 (0%)
extremity edema	2 (7%)	2 (10%)	0 (0%)
Treatments	23 (79%)	15 (71%)	3 (100%)
corticosteroids	21 (72%)	13 (62%)	3 (100%)
imatinib	4 (14%)	1 (5%)	3 (100%)
hydroxyurea	5 (17%)	1 (5%)	1 (33%)
other chemotherapeutic agents	4 (14%)	1 (5%)	0 (0%)

***HES/CEL were diagnosed according to the WHO and/or Chusid criteria. Among them, 24 cases were examined the presence of the FIP1L1/αPDGFR chimera gene.**

Fig.1

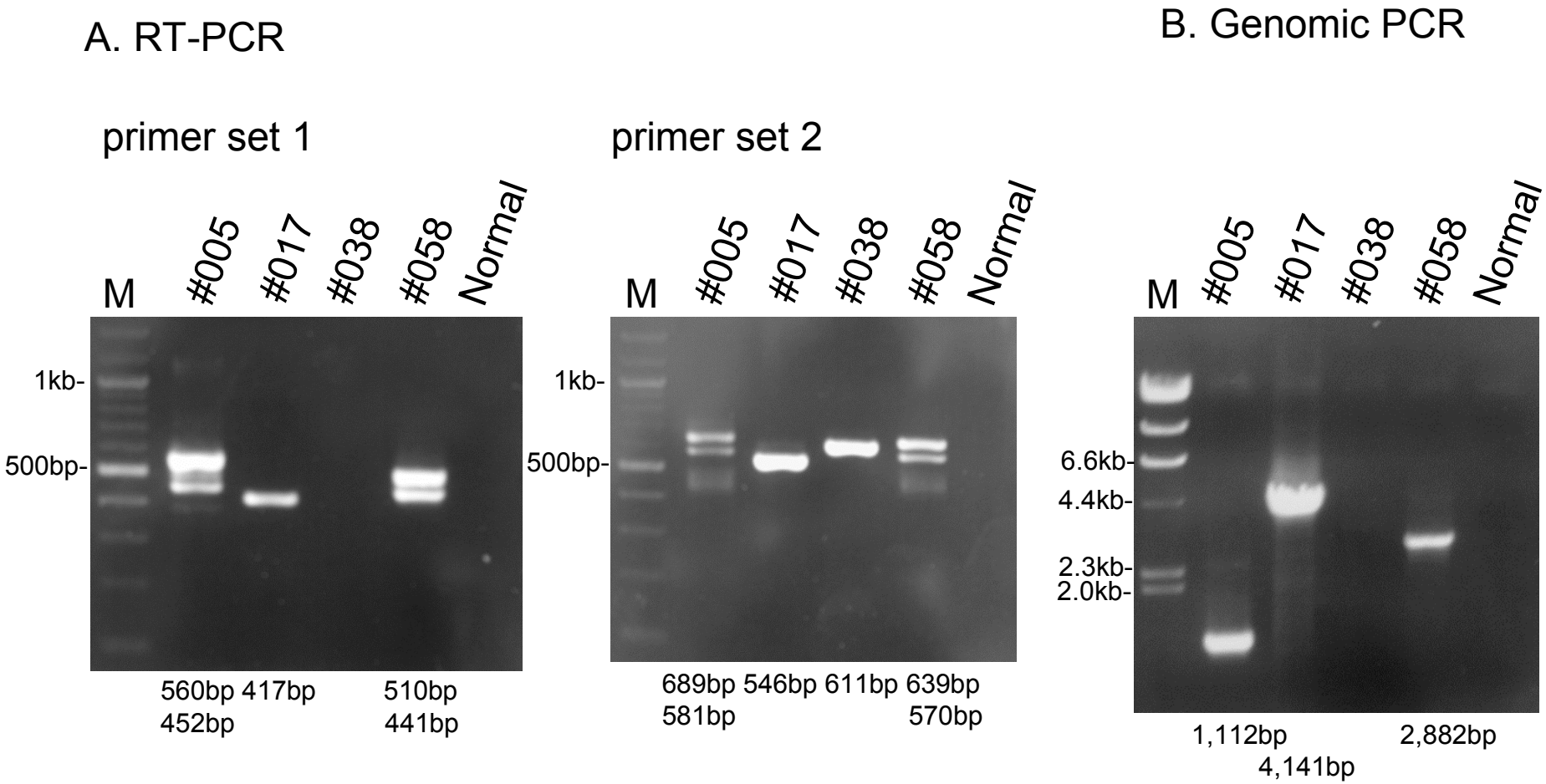

A. RT-PCR
primer set 1
M
#005
#017
#038
#058
Normal
1kb-
500bp-
560bp 417bp
452bp
510bp
441bp
primer set 2
M
#005
#017
#038
#058
Normal
1kb-
500bp-
689bp 546bp 611bp 639bp
581bp
570bp
B. Genomic PCR
M
#005
#017
#038
#058
Normal
6.6kb-
4.4kb-
2.3kb-
2.0kb-
1,112bp
2,882bp
4,141bp

Fig. 2

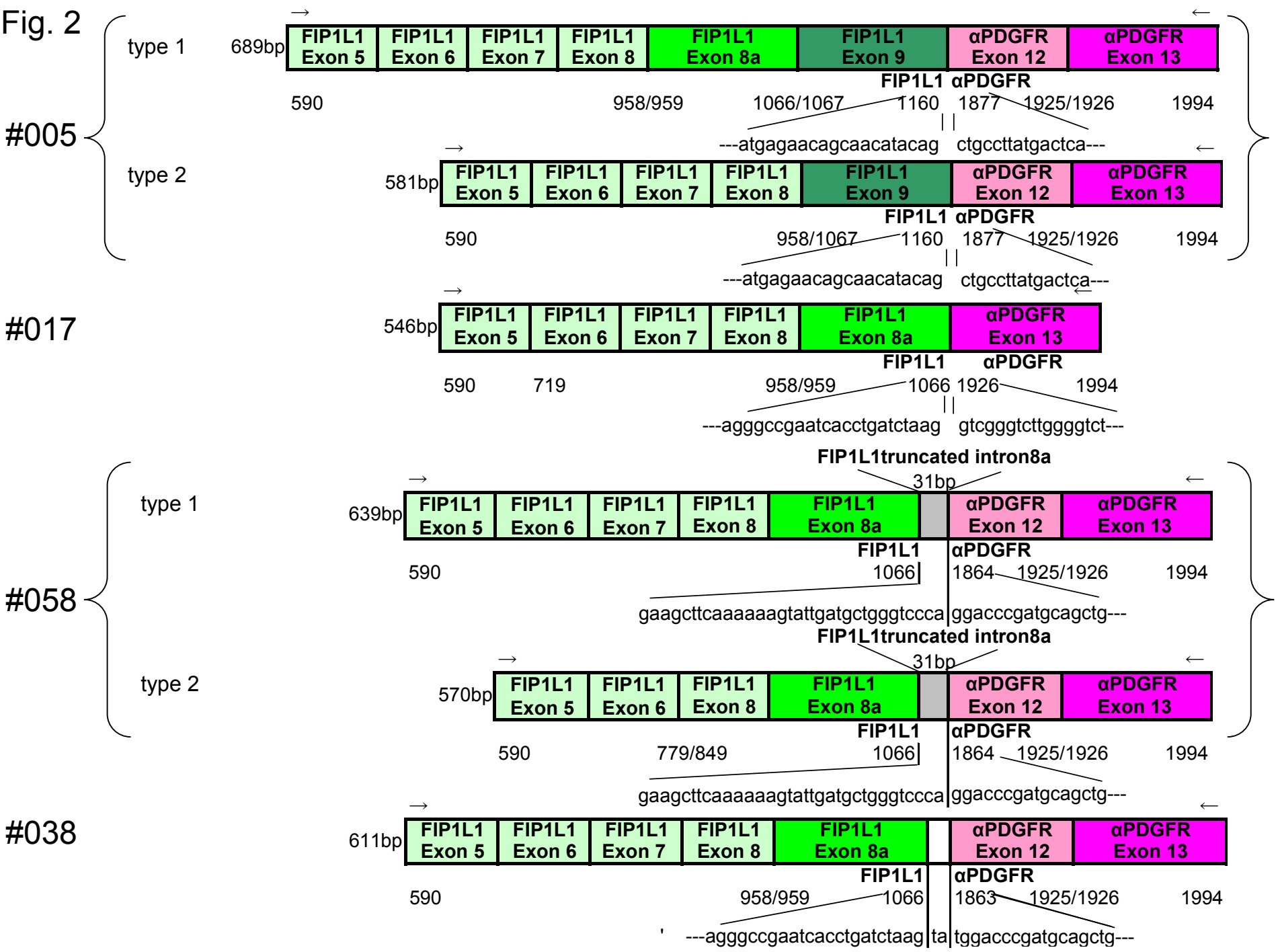

#005
type 1
689bp
FIP1L1 Exon 5
FIP1L1 Exon 6
FIP1L1 Exon 7
FIP1L1 Exon 8
FIP1L1 Exon 8a
FIP1L1 Exon 9
αPDGFR Exon 12
αPDGFR Exon 13
FIP1L1 αPDGFR
590
958/959
1066/1067
1160
1877
1925/1926
1994
---atgagaacagcaacatacag ctgccttatgactca---
type 2
581bp
FIP1L1 Exon 5
FIP1L1 Exon 6
FIP1L1 Exon 7
FIP1L1 Exon 8
FIP1L1 Exon 9
αPDGFR Exon 12
αPDGFR Exon 13
FIP1L1 αPDGFR
590
958/1067
1160
1877
1925/1926
1994
---atgagaacagcaacatacag ctgccttatgactca---
#017
546bp
FIP1L1 Exon 5
FIP1L1 Exon 6
FIP1L1 Exon 7
FIP1L1 Exon 8
FIP1L1 Exon 8a
αPDGFR Exon 13
FIP1L1 αPDGFR
590
719
958/959
1066
1926
1994
---agggccgaatcacctgatctaag gtcgggtcttggggtct---
#058
FIP1L1truncated intron8a
31bp
type 1
639bp
FIP1L1 Exon 5
FIP1L1 Exon 6
FIP1L1 Exon 7
FIP1L1 Exon 8
FIP1L1 Exon 8a
αPDGFR Exon 12
αPDGFR Exon 13
FIP1L1 αPDGFR
590
1066
1864
1925/1926
1994
gaagcttcaaaaaagtattgatgctgggtccca ggacccgatgcagctg---
FIP1L1truncated intron8a
31bp
type 2
570bp
FIP1L1 Exon 5
FIP1L1 Exon 6
FIP1L1 Exon 8
FIP1L1 Exon 8a
αPDGFR Exon 12
αPDGFR Exon 13
FIP1L1 αPDGFR
590
779/849
1066
1864
1925/1926
1994
gaagcttcaaaaaagtattgatgctgggtccca ggacccgatgcagctg---
#038
611bp
FIP1L1 Exon 5
FIP1L1 Exon 6
FIP1L1 Exon 7
FIP1L1 Exon 8
FIP1L1 Exon 8a
αPDGFR Exon 12
αPDGFR Exon 13
FIP1L1 αPDGFR
590
958/959
1066
1863
1925/1926
1994
' ---agggccgaatcacctgatctaag ta tggacccgatgcagctg---

Fig.3

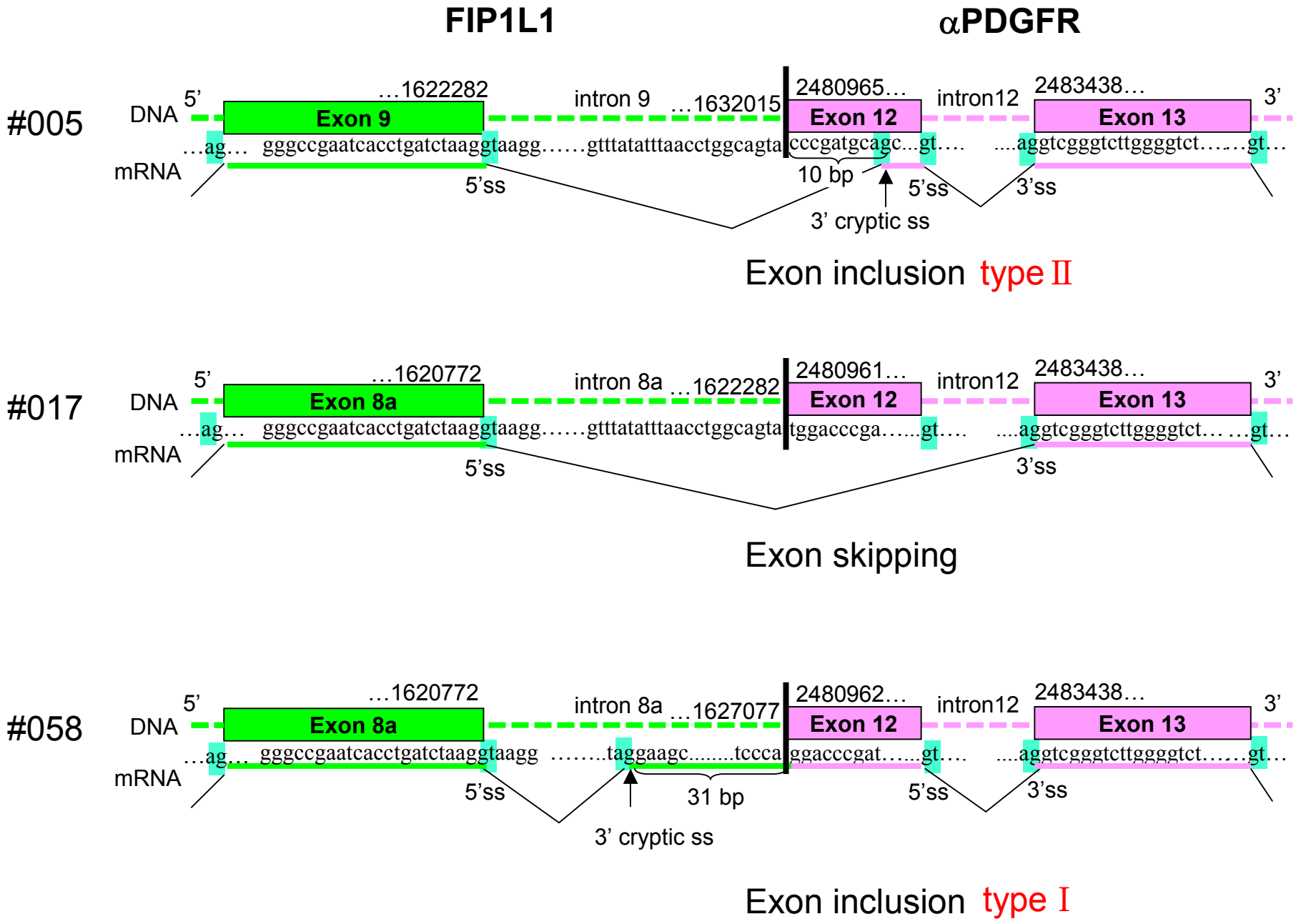

FIP1L1
αPDGFR
#005
DNA
mRNA
5'
…1622282
Exon 9
intron 9
…1632015
2480965…
Exon 12
intron12
2483438…
Exon 13
3'
…ag…
gggccgaatcacctgatctaaggtaagg……gtttatatttaacctgg
cagta
cccgatgcagc…gt….
….aggtcgggtcttggggtct……gt…
5'ss
10 bp
5'ss
3'ss
3' cryptic ss
Exon inclusion type Ⅱ
#017
5'
…1620772
Exon 8a
intron 8a
…1622282
2480961…
Exon 12
intron12
2483438…
Exon 13
3'
…ag…
gggccgaatcacctgatctaaggtaagg……gtttatatttaacctgg
cagta
tggacccga……gt….
….aggtcgggtcttggggtct… …gt…
5'ss
3'ss
Exon skipping
#058
5'
…1620772
Exon 8a
intron 8a
…1627077
2480962…
Exon 12
intron12
2483438…
Exon 13
3'
…ag…
gggccgaatcacctgatctaaggtaagg
……..taggaagc……..tccca
ggacccgat……gt….
….aggtcgggtcttggggtct……gt…
5'ss
31 bp
5'ss
3'ss
3' cryptic ss
Exon inclusion type Ⅰ

Fig.4

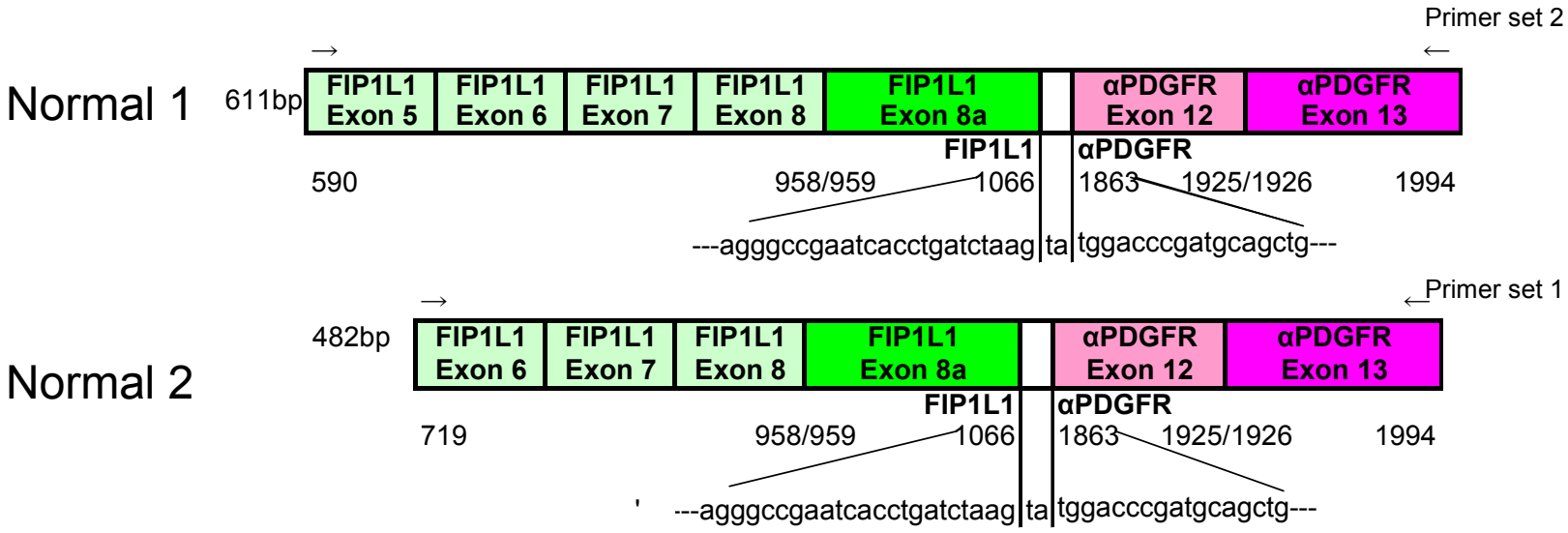
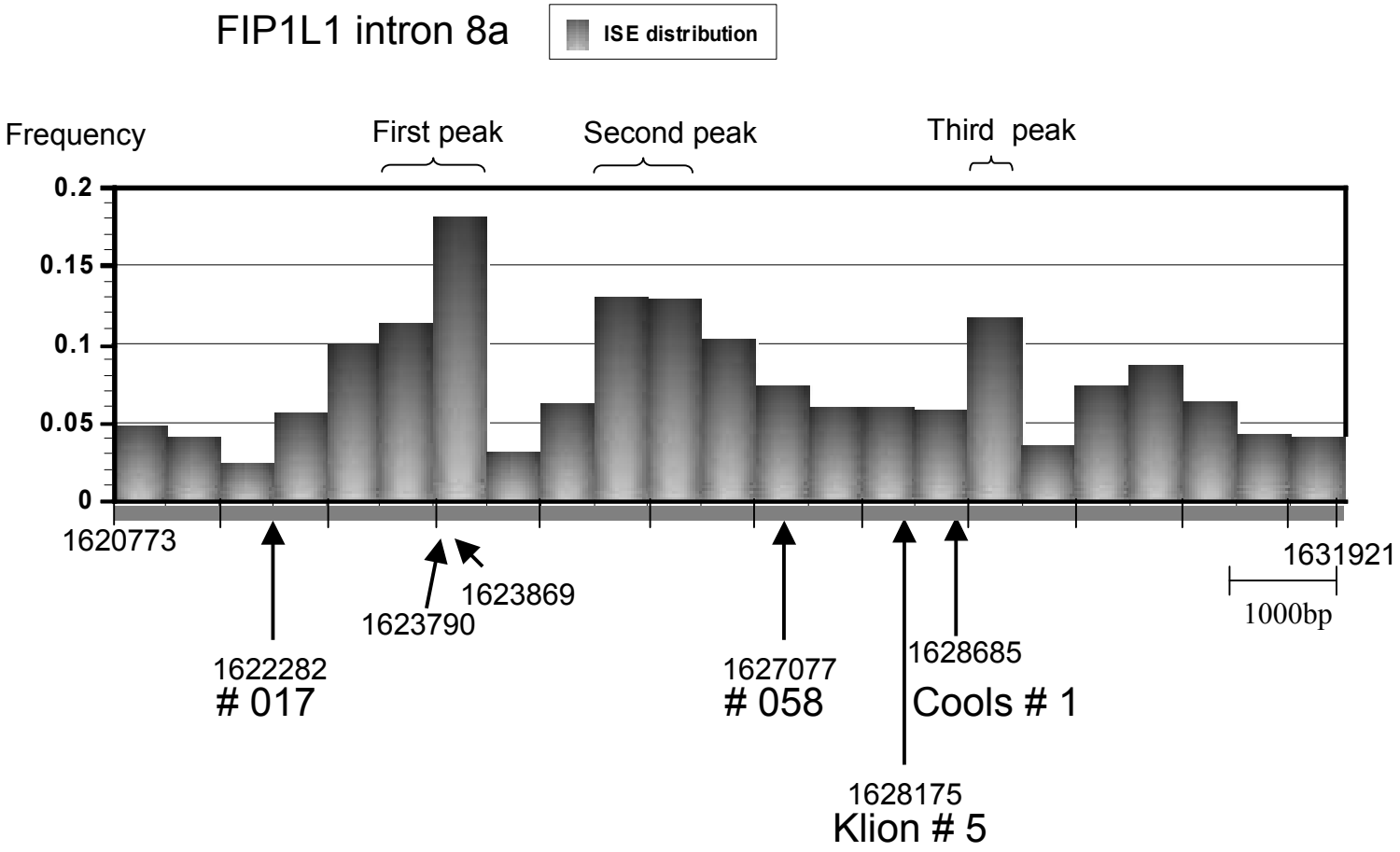

Primer set 2
Normal 1
611bp
FIP1L1 Exon 5
FIP1L1 Exon 6
FIP1L1 Exon 7
FIP1L1 Exon 8
FIP1L1 Exon 8a
αPDGFR Exon 12
αPDGFR Exon 13
FIP1L1
αPDGFR
590
958/959
1066
1863
1925/1926
1994
---agggccgaatcacctgatctaag ta tggacccgatgcagctg---
Primer set 1
Normal 2
482bp
FIP1L1 Exon 6
FIP1L1 Exon 7
FIP1L1 Exon 8
FIP1L1 Exon 8a
αPDGFR Exon 12
αPDGFR Exon 13
FIP1L1
αPDGFR
719
958/959
1066
1863
1925/1926
1994
---agggccgaatcacctgatctaag ta tggacccgatgcagctg---

Fig.5


FIP1L1 intron 8a
ISE distribution
Frequency
First peak
Second peak
Third peak
0.2
0.15
0.1
0.05
0
1620773
1631921
1000bp
1622282
017
1623790
1623869
1627077
058
1628685
Cools # 1
1628175
Klion # 5