中文摘要

此計劃的目的為尋找在未知原因的原發性 B 細胞免疫缺陷病童的致病機轉是否為 p110δ 基因突變所造成。但因根據外國資料, 原發性 B 細胞免疫缺陷的病童大部(約 85%)為 Btk 基因突變所引起, 所以應須先行檢查 Btk 基因； 不過, 因為目前台灣尚未有方便且可靠的分子生物學方法來在原發 B 細胞免疫缺陷病童中確定診斷為 XLA 疾病 , 也無 XLA 病童的 Btk 基因突變型本土資料。在國科會補助下 (NSC91-2314-13-002-189/NSC92-2314-B-002-211) , 我們已建立用 genomic DNA-PCR and direct sequencing 的方法來從患有原發 B 細胞免疫缺陷疾病的病童中篩檢及確定其 Btk 基因突變位置及多型式從而來證實診斷為 XLA 疾病。目前, 從所篩檢病童中, 已成功確立 4 個病童其 Btk 基因突變位置及型式以及其家族的基因篩檢。 同時, 亦已建立用 RT-PCR and direct sequencing 的方法來篩檢在 Btk 基因檢查確定無突變但仍不知原因的原發 B 細胞免疫缺陷病童的 PI3 kinase p110δ基因是否有多型性及突變以證實 p110δ基因的突變可能是某些未知原因的原發性 B 細胞免疫缺陷的致病機轉。目前, 在 3 位病人發現其 p110δ基因有一整段 Exon14 刪除。這段被刪除的基因位於 PI3 kinase 的 accessory domain, 它在 PI3 和 PI4-kinase 是 conserved 的,其功能為受胸質的呈現。

Abstract

This study is to explore the possibility that some patients with defects in B-cell immunodeficiency of unknown etiology might have mutations in PI3 kinase p110δ. Although, according to the foreign data, the majority of primary B-cell immunodeficiency (about 85%) is due to Btk mutation. We need to exclude the possibility of Btk gene mutation before we check the p110δ gene. However, at present, there is still no convenient and reliable molecular method for definite diagnosis of XLA in patients with primary B-cell immunodeficiency in Taiwan. There is no Btk gene mutation and polymorphism data for Taiwanese XLA patients, either. Under this grant support of NSC (NSC91-2314-13-002-189/NSC92-2314-B-002-211), we have established the methods to screen the patients with primary B-cell defect by genomic DNA-PCR and direct sequencing analysis to identify the Btk gene mutations and then establish the diagnosis of the XLA patients. Until now, we have already successfully identified 4 patients of primary B-cell immunodeficiency with Btk gene mutation and screened their family members. We also have established the RT-PCR and direct sequencing methods to screen the potential p110δ gene polymorphism and mutation in the remaining primary B-cell immunodeficiency patients without Btk mutations. Until now, we have identified three patient with 2 different sizes of his 3rd PCR amplified fragment. After cloning and comparison with human p110δ sequence, the nucleotides 2,007-2,150 were completely missed in the shorter band. These missed nucleotides coded for the entire Exon 14(in-reading frame deletion). The missed Exon 14 is located in PI3K accessory domain which is conserved in all PI3 and PI4-kinase with the function for substrate presentation.

Brief Background, Rationale and Purposes

Search for genes involved in the pathogenesis of primary immunodeficiency diseases in human by mouse knockout model is well established. Our recent study[1] using targeted gene disruption have shown that defects in PI3 kinase p110δ subunit result in a B-cell immunodeficiency that is very similar to that seen in Btk-deficient mice[2].

However, only about 85% of patients with primary B-cell immunodeficiency have mutations in Btk and manifested as XLA clinically, and some with the defects in the components of the B-cell signal transduction complex, “sigmosome”, which include μ heavy chain, the λ (5/14.1) component of the surrogate light chain, Ig α and the adaptor protein BLNK (Fig. 1). The remaining 10-15% of patients (mainly composed of common variable immunodeficiency(CVID)/ selective IgA deficiency (IgAD), the nature of the underlying defect is not yet known. Interestingly, our novel study showed not only the PI3 kinase p110δ fit this B cell signalosome transduction link, but the phenotype of its knockout mice model is also very similar to that of CVID/IgAD in term of lack of B-T cell interaction. We hypothesize mutations of p110δ may play the role of

pathogenesis for some primary B-cell immunodeficiency of unknown etiology (Fig. 1).

This study is to explore the possibility that some patients with defects in B-cell immunodeficiency of unknown etiology might have mutations in PI3 kinase p110 δ. Although, according to the foreign data, the majority of primary B-cell immunodeficiency (about 85%) is due to Btk mutation. We need to exclude the possibility of Btk gene mutation before we check the p110 δ gene. However, at present, there is still no convenient and reliable molecular method for definite diagnosis of XLA in patients with primary B-cell immunodeficiency in Taiwan. There is no Btk gene mutation and polymorphism data for Taiwanese XLA patients, either. The following are our recent novel results.

Results and Discussion

For Btk gene mutation and polymorphism:
Under the support of NSC grant (NSC91-2314-13-002-189/NSC92-2314-B-002-211), we have established the methods to screen the patients with primary B-cell defect by genomic DNA-PCR and direct sequencing analysis to identify the Btk gene mutations and then establish the diagnosis of the XLA patients. Until the end of this application, we have already successfully identified 4 patients of primary B-cell immunodeficiency with Btk gene mutation and screened their family members (Table 1 and Fig. 2-5).

The data identified are compatible with X-link heritage. The further genes investigation and comparison with foreign mutation bank are going and the novel mutations derived proteins will be characterized. For example, because the deletion of 569C (Fig. 3) was not found in foreign mutation bank (maybe a new mutation) and its deletion shifted reading frame premature stopped at amino acid 198 in TH domain. We are going to check its RNA transcript by Northern Blot Analysis and RT-PCR as well as its derived protein function by Btk kinase assay as mentioned in material and method section. The in vivo phenotype rechecking will be done as mentioned in material and method section to characterize the sequel of this new mutation, too.

For p110 δ gene mutation and polymorphism:
We also have established the RT-PCR and direct sequencing methods to screen the potential p110 δ gene polymorphism and mutation in the remaining primary B-cell immunodeficiency patients without Btk mutations(Fig. 6). Until now, we have identified three patients with 2 different sizes of PCR product(one was 1,111 bp, another was 967 bp) for his 3rd PCR amplified fragment. (Fig. 7). These two bands were then cloned, amplified and sequenced. After comparison with human p110 δ sequence (nm_005026) by NCBI Pairwise Blast, the nucleotides 2,007-2,150 were completely missed in the shorter band (Fig. 8). The further analysis of Human p110 δ genomic structure, these missed nucleotides coded for the entire Exon 14(in-reading frame deletion). The Exon 13 is the corresponding one for mouse p110 δ gene. These two exons have 92% identity in nucleotides and 96% identity in amino acids(Fig. 9). The analysis of the p110 δ protein conserved domain revealed the Exon 14 is located in PI3K accessory domain which is conserved in all PI3 and PI4-kinase with the function for substrate presentation(Fig. 10).

The shorter transcript in patient's fragment maybe a splicing variant, because the missed part was an entire exon. But, the signal for this shorter band was consistent and strong (Fig. 7(A,B,C)). Moreover, this band could not identified in all other patients' samples(as the patient 21, 24 and normal control shown in Fig. 7(A). Besides, as our knowledge, only the splicing variants for PI3-kinase P85 subunit had been published[3].

There was still another longer band coded for the normal transcript for patient's fragment. This means there were two different p110 δ transcripts coexistence in patient 23. Because, there were some mutant genes worked in the "dominant negative" model, we tentatively hypothesize this identified mutant maybe worked in this way for our further analysis. We currently are cloning the normal and mutant transcripts into PcDNA3 vector with different Tags(HA- and Flag-) for in vitro kinase assay. To clone the mutant transcript into MSCV retrovirus vector, then infect the wild type B-cells and check their functions such as mitogen

proliferative assay will be the next analysis to characterize its role in the pathogenesis of primary B-cell immunodeficiency.

In summery, we have established the method to definitely diagnosis of XLA (Btk gene mutations), to detect the maternal and sisters carrier and the method to identify the mutation of p110 δ gene. We will establish the database of Taiwanese Btk mutations, identify potential novel mutation and prenatal diagnosis. Moreover, this study would focus to identify the potential PI3 kinase p110 δ polymorphisms/mutations and to characterize their derived proteins to elucidate their roles in the pathogenesis of patients with defects in B-cell development with unknown etiology.

Self-Estimation

We have completed almost all of the expected goals. The analysis of the function of this deleted exon 14 is being studied. This work is very important to eluciate the function of the catalytic subunit p110δ of PI 3-kinase in the pathogenesis of primary B-cell immunodeficiency. This novel finding would be a highlight in the journal.

Reference

1. Jou ST, Carpino N, Takahashi Y, Piekorz R, Chao JR, Carpino N, Wang D, Ihle JN: An Essential, Non-redundant Role for the Phosphoinositide 3- Kinase p110δ in Signaling by the B Cell Receptor Complex. Mol Cell Biol. 2002 in press.

2. Khan WN, Alt FW, Gerstein RM, Malynn BA, Larsson I, Rathbun G, Davidson L, Muller S, Kantor AB, Herzenberg LA, et al. Defective B cell development and function in Btk-deficient mice. Immunity 1995 Sep;3(3):283-99

3. Suzuki H, Terauchi Y, Fujiwara M, Aizawa S, Yazaki Y, Kadowaki T, Koyasu S: Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. Science 1999 Jan 15;283(5400):390-2

Hypothesis

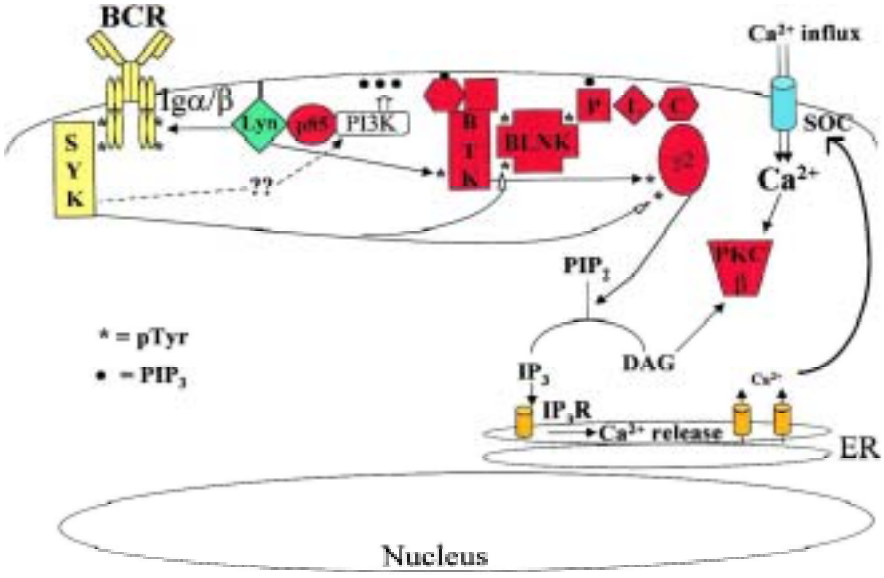


Figure 1. Schematic Diagram of the Signalosome Linking BCR Stimulation with Sustained Ca^{2+} Flux

BCR cross-linking leads to the activation of three tyrosine kinases (Lyn, Syk, and Btk) and a lipid kinase (PI3K). Production of PIP_3 and tyrosine phosphorylation of the BLNK adapter protein nucleates a complex that coordinates the activation of PLCγ2. Maximal activation of PLCγ2 is required to generate sufficient IP_3 to empty internal Ca^{2+} stores, which in turn is necessary for opening of SOC. Ca^{2+} and DAG are necessary cofactors for activation of PKCβ. Different colors represent proteins whose gene deletion results in a block at the pro-B to pre-B transition (yellow), *Xid*-like defects in development and function (red), or B cell hyperresponsiveness (green). The store-operated calcium channel mechanism is shown in blue. Arrows pointing to asterisks indicate phosphorylation events.

Immunity 2000; 13:1

P85 → mouse KO, XID-like but early lethal Science 1999

PLC2 → mouse KO, XID-like with FcR defect Immunity 2000

→ only polymorphism in human B-cell defect Immunogenetics 2001

Table 1. Four patients of primary B-cell immunodeficiency with Btk gene mutation and heterozygote in their family members.

Test No.	Name	Sex	Birthday	Btk gene mutation and sequel
#3483	黃 X 利	M	1986/12/03	1713→G(Y571X) at TK domain (Fig.2)
#3484	黃 X 利之姐	F		no mutation (Fig.2)
#3485	黃 X 利之母	F		no mutation (Fig.2)
#3486	鄒 X 彬	M	1980/03/03	del 569C(P190Q→198 stop), Premature stop at TH domain (Fig. 3) Polymorphism in 1899C→T, no amino acid change (Fig. 4)
#3762	鄒 X 怡 (鄒 X 彬之sister)	F		del 569C(P190Q→198 stop), Heterozygote carrier (Fig. 3)
#3976	鄭 X 聲(twin A)	M	2002/01/24	862C→T(R288W) at SH2 domain (Fig.5)
#3977	鄭 X 駿(twin B)	M	2002/01/24	862C→T(R288W) at SH2 domain (Fig.5)
#3979	鄭媽媽	F		862C→T Heterozygote carrier (Fig.5)

Btk gene mutation in Exon 17: 1713T→G (Y571X) at TK domain

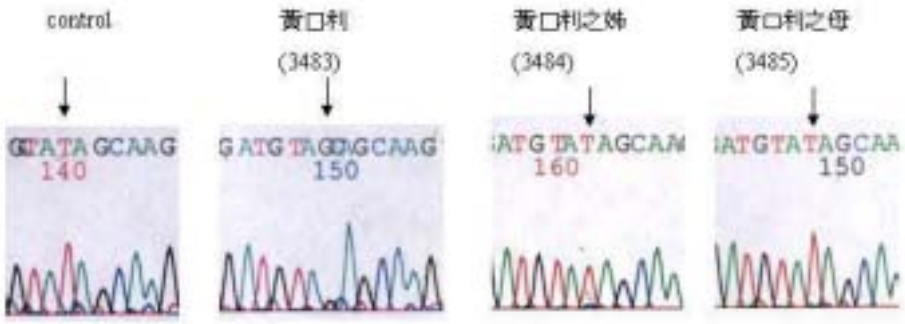


Fig. 2

Btk gene mutation in Exon 7: P190Q→198stop at TH domain

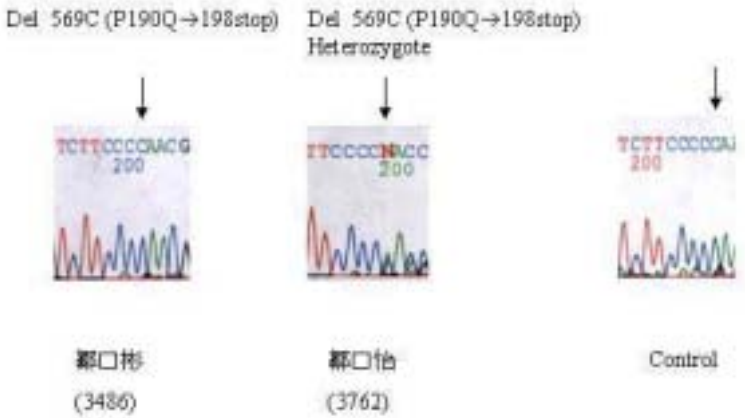


Fig. 3

Btk gene polymorphism in Exon 8: 1899C→T, not change amino acid

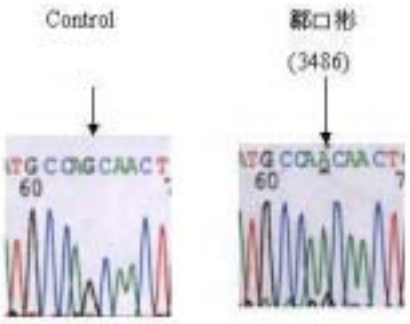


Fig. 4

Btk gene mutation in Exon 10: 862C→T (R288W) at SH2 domain

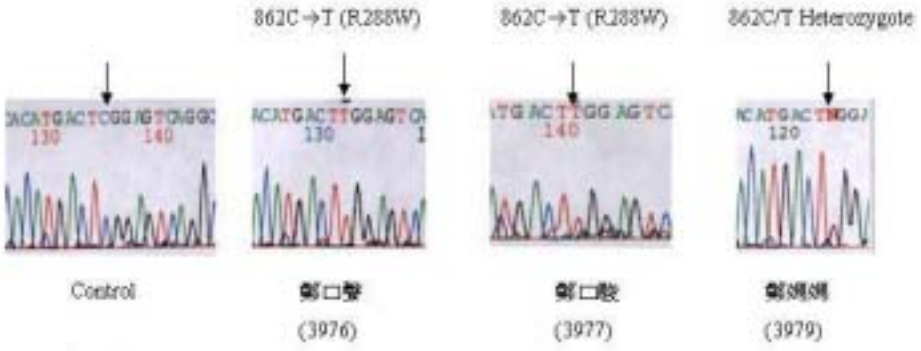
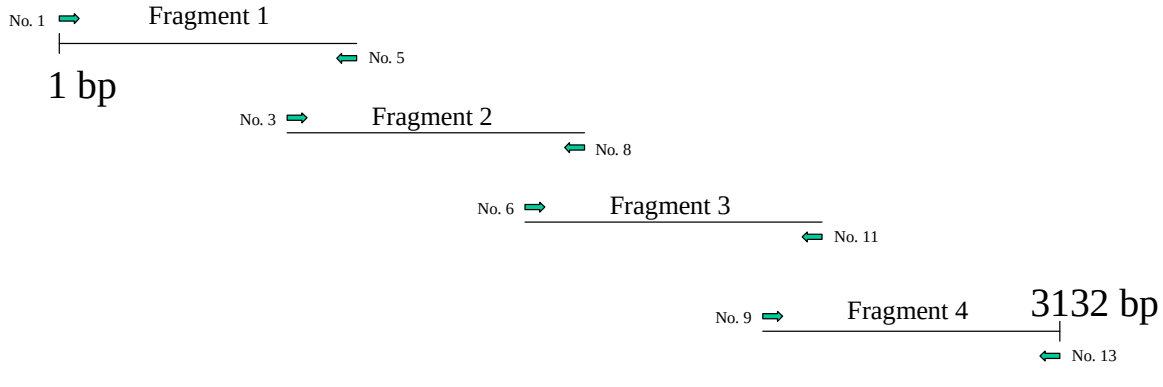


Fig. 5



No.	OligoName	Sequence (5' to 3')	Ser. no	Length	MW	TM
1	145	CTTGGACTATTCCAGAGAGC	22020021104004000	20	6138	52
3	515	ACCGCGTGAAGAAGCTCATC	33020021104004000	20	6082	54
3	905	AAGACTACACGCTGCAGGTG	22030021104004000	20	6132	54
4	1235	TTTTCCACGGCAACGAGATG	22020021104004000	20	6098	52
5	1353R	ATCTCGTTGCCGTCGAAAAG	22020021104004000	20	6138	52
6	1578	AGTAACCCCAACACCGATAG	22020021104004000	20	6085	52
7	1937	AGCTGCTAGACTTCAGCTTC	22020021104004000	20	6049	52
8	1955R	AAGCTGAAGTCTAGCAGCTC	22020021104004001	20	6107	52
9	2291	TGAAGCTGAGCTCTCAGAAG	22020021104004001	20	6107	52
10	2666	ATTGAGGTGGTACTCCGTTC	22020021104004001	20	6120	52
11	2686R	AACGCAGTACCACCTCAATG	22020021104004001	20	6076	52
12	3014	ATGTGATTCAGCAGGGGAAG	22020021104004001	20	6237	52
13	3299R	TAGGACGAATGTGCTTGGTC	22020021104004001	20	6129	52

Fig. 6 The human P110 δ cDNA contains 3,132 nucleotides. The P110 δ gene was amplified from RT-derived cDNA by using 4 sets of PCR primers (No.1 + No.5 primers for fragment 1, No.3 + No.8 primers for fragment 2, No.6 + No.11 primers for fragment 3, and No.9 + No.13 primers for fragment 4) as shown above.

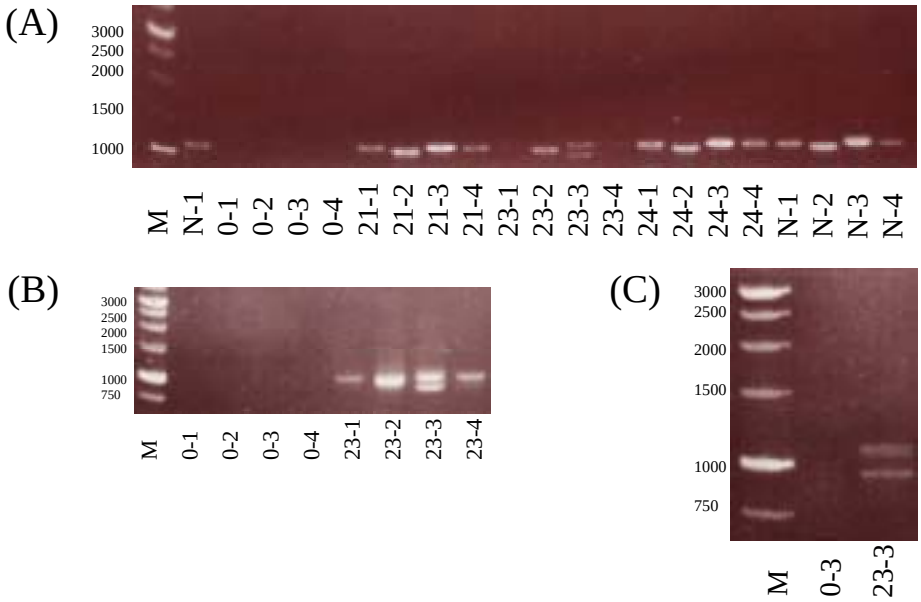


Fig. 7 The DNA gel electrophoresis for the RT-PCR products of patient 21, 23, 24, negative(0) and normal (N) control. (A) There are two bands (1,111 & 967 bp) for patient 23-3 fragment, but only one band for corresponding fragment for patient 21, 24 and normal control. The 23-1 and 23-4 fragments are weak but still visible in the original gel. (B) The repeated independent PCR reaction for confirmation.(C) Another independent PCR reaction showed two bands for patient 23-3 fragment amplification before TA cloning for further analysis.

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Blast 2 Sequences results VERSION BLASTN 2.2.5[Nov-16-2002] PubMedEntrezBLASTOMIMTaxonomyStructure
 Query Sequence 1 Length 5220(1 .. 5220)(sequence for human P110 D mRNA, nm_005026)
 Sbjct Sequence 2 Length  974(1 ..  974)(sequence for shorter band of patient 23-3 fragment)
Score =  829 bits (431), Expect = 0.0Identities = 431/431 (100%) Strand = Plus / Plus

Query: 1576 agtaaccccaacacggatagcgccgctgccctgctcatctgcctgcccgaggtggccccg 1635
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Sbjct: 43   agtaaccccaacacggatagcgccgctgccctgctcatctgcctgcccgaggtggccccg 102


Query: 1636 caccccgtgtactaccccgccctggagaagatcttggagctggggcgacacagcgagtgt 1695
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 103  caccccgtgtactaccccgccctggagaagatcttggagctggggcgacacagcgagtgt 162


Query: 1696 gtgcatgtcaccgaggaggagcagctgcagctgcgggaaatcctggagcggcgggggtct 1755
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Query: 1756 ggggagctgtatgagcacgagaaggacctggtgtggaagctgcggcatgaagtccaggag 1815
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Query: 1816 cacttcccggaggcgctagcccggctgctgctggtcaccaagtggaacaagcatgaggat 1875
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Sbjct: 283  cacttcccggaggcgctagcccggctgctgctggtcaccaagtggaacaagcatgaggat 342


Query: 1876 gtggcccagatgctctacctgctgtgctcctggccggagctgcccgtcctgagcgccctg 1935
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Score =  487 bits (253), Expect = e-133Identities = 315/332 (94%), Strand = Plus / Plus

Query: 2151 ctccgagatgcacgtgccgtcggtggccctgcgcttcggcctcatcctggaggcctactg 2210
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Query: 2211 caggggcagcacccaccacatgaaggtgctgatgaagcaggggaagcactgagcaaact 2270
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Sbjct: 534  caggggcagcacccaccacatgaaggtgctgatgaagcaggggaagcactgagcaaact 593
Fig.

Query: 2271 gaaggccctgaatgacttcgtcaagctgagctctcagaagacccccaagccccagaccaa 2330
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Query: 2451 ggactccaagatgaagcccctgtggatcatgt 2482
            ||||||| ||||||| |||||| |||||||||
Sbjct: 769  ggactcc-agatgaa-cccctggggatcatgt 798
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Fig. 8 The above NCBI Pairwise Blast data showed the 2,007-2,150 nucleotides were completely missed in the shorter band of patient 23-3 fragment. The missed nucleotides coded for Exon 14.

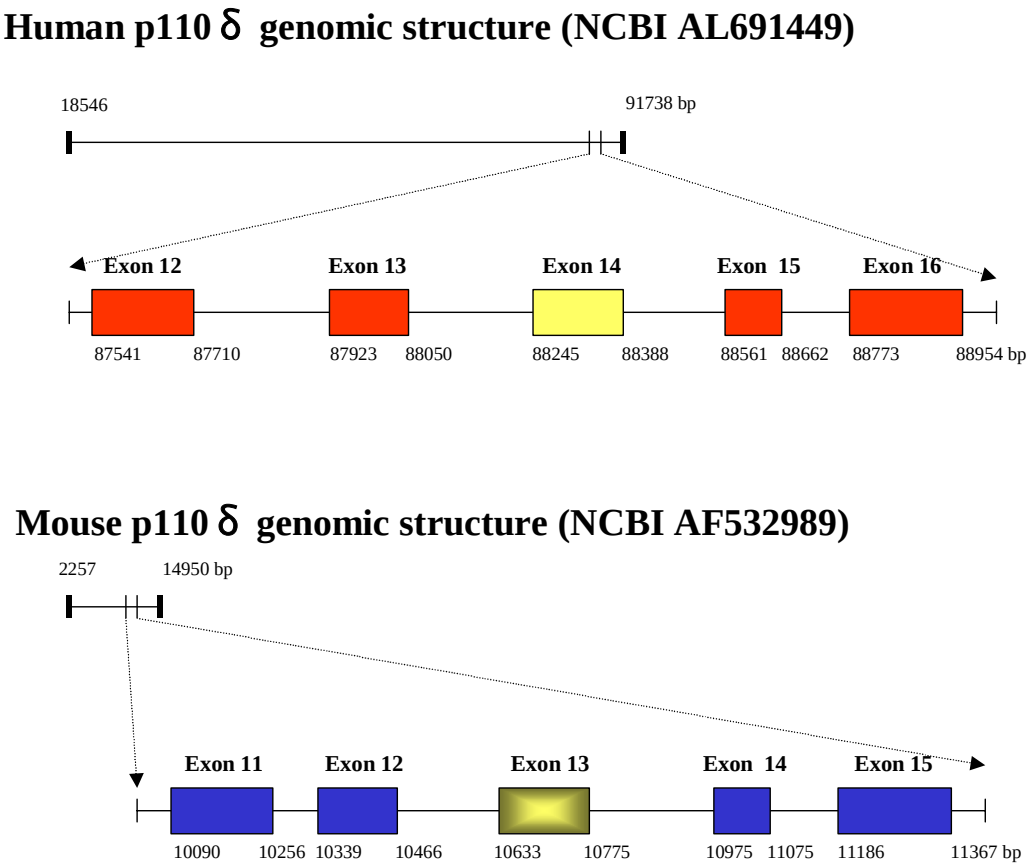
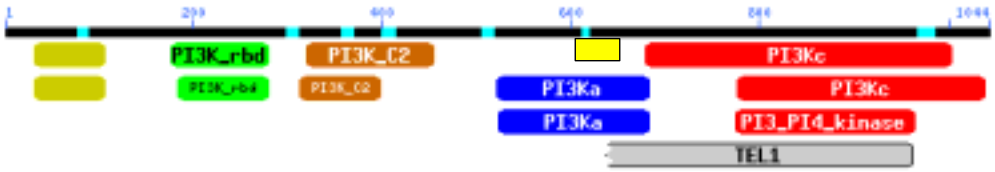


Fig. 9 Human and mouse p110 δ genomic structure. The patient 23 had an entire Exon 14 deletion-transcription in the extracted RNA from the blood mononuclear cells. The numbers indicate the nucleotide numbers. The corresponding Exons 11-15 structure in mouse is shown below.

NCBI

NCBI Conserved Domain Search

Human p110 δ protein



Mouse p110 δ protein

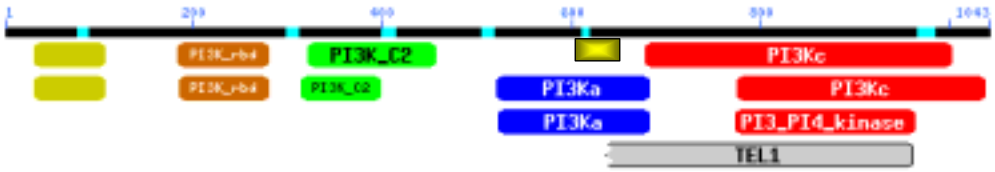


Fig. 10 The human Exon 14 and the corresponding mouse Exon 13 are located in PI3Ka domain, an accessary domain which is conserved in all PI3 and PI4-kinase. Its role is still unclear but it has been suggested to be involved in substrate presentation. The human Exon 14 and mouse Exon 13 have 92% identity in nucleotides and 96% identity in amino acids.