mRNA Analysis and Multiplex Ligation Probe Amplification for Hemophilia A Patients without Found Mutation or Suspected Exon(s) Deletion in Genomic DNA

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Introduction

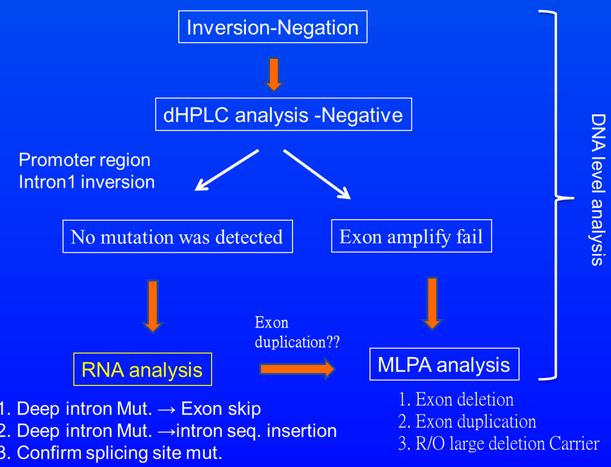
- Some methods such as denaturing high performance liquid chromatography (DHPLC), denaturant gradient gel electrophoresis (DGGE) and direct sequencing are sensitive to detect most FVIII mutations.
- There are still about 2-7% of HA patients whose genetic abnormality remains unidentified, particularly those with mild or moderate type hemophilia A (HA).

The aim of this study

- To confirm the skipped exon(s) caused by the known splice site mutation.
- To confirm skipped exon or alternative exon deletion in patients with no amplified exon(s) by PCR.
- To search for the deep intronic variations in patients without mutation in coding DNA of F8 gene.
- To search for the large deletion or duplication in patients without mutation in coding DNA of F8 gene.

Patients and methods

- This study has been approved by the ethics review board of our institution.
- 24 HA patients with or without inhibitors from 19 unrelated families from 1/1/2015 to 12/15/2015.
- Age: 8-78 y/o, median: 26 y/o
- Genetic defect of 19 unrelated families
  - 2 with splice site mutation
  - 8 with failed exon(s) amplification
  - 9 with no identified mutation in cDNA
- Reverse transcription-polymerase chain reaction (RT-PCR) can be used for the detection of disease related genes in patients without genetic mutations in the DNA coding regions.
- Multiplex Ligation-dependent Probe Amplification (MLPA) is a technique that is sensitive to the number of F8 gene copies (dosage analysis).



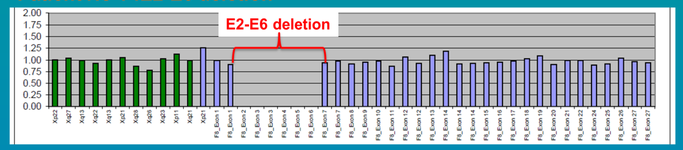
Results

Table 1. Severity and the detected defects by DNA defect, mRNA analysis and MLPA in 19 unrelated families with hemophilia A.

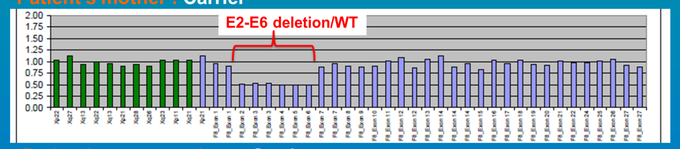
Pts	Severity	DNA defect	RNA analysis Result	MLPA
1	Inhibitor	No E2-E6	E2-7 deletion; E13ins122bpE14	E2-E6 deletion
2(3)	Severe/Inhibitor	No E4-E10	E4-10 deletion	
3	Inhibitor	No E1	nonconclusive	E1 deletion
4	Inhibitor	No E2-E4	E2-7 deletion	E2-E4 deletion
5	Severe	No E6	E6-7 deletion	E6 deletion
6	Inhibitor	No E7-E10	E7-E10 deletion	
7	Inhibitor	No E4-E10	E4-10 deletion / E4-10 deletion +36bp in E11	
8	Inhibitor	Exons Amplify Fail	nonconclusive	Exon 5-Exon22 deletion
9	Severe	IVS18-1G>A	E18-19 deletion	
10	Severe	IVS16-1C>T	nonconclusive	
11(2)	Mild	No finding	E18ins90bpE19	
12(2)	Moderate	No finding	E19 deletion	
13(2)	Mild	No finding	E18ins90bpE19	
14	Mild	No finding	E18ins90bpE19	
15	Severe	No finding	nonconclusive	wild type
16	Mild	No finding	E19 Skip	
17	Severe	No finding	nonconclusive	E8-E14 duplication
18	Mild	No finding	nonconclusive	wild type
19	Moderate	No finding	E18ins90bpE19	wild type

MLPA Analysis- analysis data

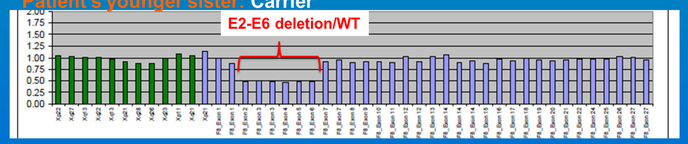
Patient No 1 :E2-E6 deletion



Patient's mother : Carrier



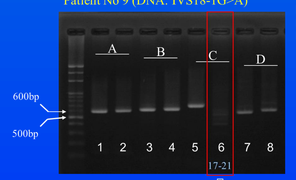
Patient's younger sister: Carrier



RNA Analysis Data

Patient No 2-B, severe type (DNA: E4-E10 amplify fail)

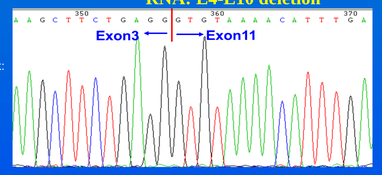
Patient No 9 (DNA: IVS18-1G>A)



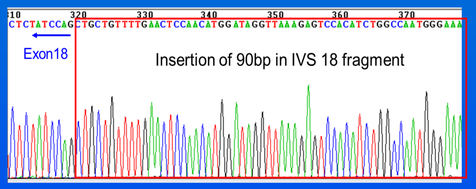
Primer using:
First PCR: AFBR
Nested PCR: 1F4R

Using other primers to amplify CFCR fragment and sequencing to confirm the deletion

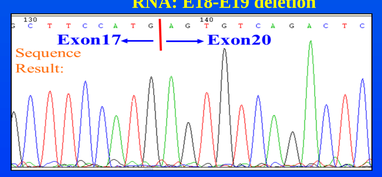
Patient No 2-B: DNA: E4-E10 amplify fail
RNA: E4-E10 deletion

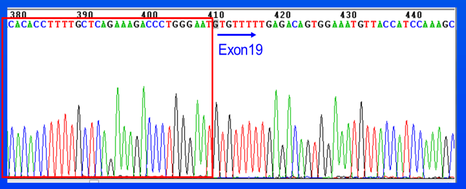


Patient No 13-A: RNA: E18ins90bpE19



Patient No 9: DNA: IVS18-1G>A
RNA: E18-E19 deletion





Discussion and Conclusion

- Combination of mRNA analysis and MLPA was effective assay for HA patient who were wild type or failed amplification of exon(s) in DNA level and 84% of detection rate was achieved.
- All 7 HA with inhibitor patients were found and confirmed to have exon(s) deletion.
- One hot spot mutation in deep intron: 90 base pairs insertion in intron 18, which occurred in four patients (1 moderate, 3 mild type).
- One severe HA patient was identified to carry E4-14 duplication by MLPA, which type mutation account for 0.5% of F8 gene defect.
- MLPA was effective in confirmation of exon(s) deletion in HA patients and female carrier.