CHAPTER V

DISCUSSION AND CONCLUSION

An early diagnosis of XLA with immediate initiation of therapy is crucial for ensuring good outcomes for the patients. In this study we described six unrelated Thai individuals clinically suspected of XLA. Mutation screening by sequencing of RT-PCR products followed by PCR-sequencing of the gDNA revealed five patients having different mutations including three novel ones. This molecular method is very useful for definite diagnosis of XLA in cases of overlapping clinical features with some disorders including common variable immunodeficiency, transient hypogammaglobulinemia, and mixed neutropenia situations [16]. According to the *BTK* gene database mutations (http:// bioinf.uta.fi/BTKbase/type_domains.heml), a total of 620 gene mutations from 974 unrelated families have *BTK* gene mutations in 90-95% of the cases and, if a mutation in *BTK* revealed, the diagnosis is confirmed.

Five different potential pathogenic mutations including three novel ones were identified in six unrelated patients. The novel mutation in patient 1 was observed in the PH domain, but in exon 3. A point deletion of T at nucleotide 400 would lead to a frameshift introducing 64 missense amino acid after residue 134 before terminating prematurely. The truncated protein, if transcribed and translated, would lack most of the C-terminal sequence of BTK protein and is likely to be severely dysfunctional as well as the known mutation in patient 2. Two of the six patient showed missense substitutions in two adjacent nucleotides. Though missense mutations affecting non-consearved residues in the amino acid sequence for each domain [17], the genotype-phenotype correlation has not been well-characterized [16].

One of six pathogenic mutations we found was a novel intronic point mutation in intron 3 of the *BTK* gene. With a C>A transversion at nucleotide 109 of intron 3 at position c.240 (+109) would result in the inclusion of intron 3. This mutation was lead to novel splicing and unusual transcripts. In this case, the mutation changes a C>A nucleotide base that can act as a new 5' splice site, an artificial splicing site, causing insertion of amino acids.

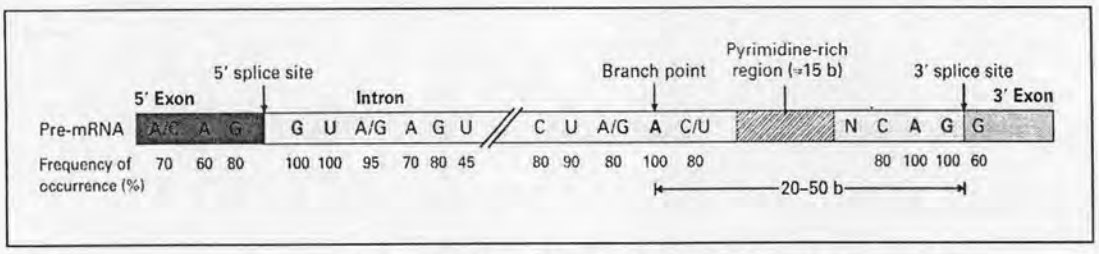

Figure 25: Consensus sequences around 5' splice sites, 3' splice sites, lariat branch point and its surrounding nucleotides in vertebrate pre-mRNAs [28]

Our study demonstrated that delivering AMOs into the patient's PBMCs was able to correct aberrant splicing caused by the c.240+109 C>A mutation. Basically, antisense oligonucleotides target pre-mRNA in a sequence-specific manner, stericcally blocking the target splice sites and redirecting the splicing machinery to a more appropriate nearby splice site. The splicing pathways can be modified *in vivo* in

a sequence specific manner by AMOs using the endo-portor reagent as a carrier. The AMOs were able to restore correctly spliced mRNA and their expression in the patient's PBMCs through skipping of the aberrant inclusion. AMOs influence splicing outcomes without inducing degradation of their target mRNAs. The predicted effect of repressing intron 3 inclusion was probably due to an influence of AMOs on RNA processing by annealing to pre-mRNA and blocking the access of splicing factors to splice sites or cis-acting regulatory elements [18].

In view of the nature of the splicing mechanisms, this approach could be applied in several conditions because the oligonucleotides were found to be effective in different cell types, including PBMCs in our study, and able to target splice sites in a variety of pre-mRNAs. Although the feasibility of therapy in XLA patients with antisense oligonucleotides has not yet to be explored, several previous observations in other diseases suggesting that this approach may be clinically promising. A clinical trial which has been successfully performed by intramuscular administration of these therapeutic tools to human patients has yielded promising results for patients with Duchenne muscular dystrophy [22].

The optimal concentration of AMOs we chose in this study was at 10 μM, a concentration achieved in PBMCs. Our study also revealed that the correctly spliced *BTK* mRNAs were quite stable as they persisted for about 30 days in PMBC cultures.

An effect on aberrant splicing correction with the use of a control AMOs (five-mispair AMOs) was also detected. These mispair control oligos determine the effective-and-specific range of concentrations for a target AMOs. The correction of a target mRNA's splicing by its five-mispair oligo is the indication which our target AMOs have low specificity, detrimental effects due to aberrant splicing correction of cellular mRNA be occur.

The invert AMOs which has the same length and base composition as target AMOs had no effect on aberrant splicing correction. Furthermore, our results showed no effect of different AMOs in unaffected cells.

Several reasons could explain the differences in AMOs activity in all previous studies, for example, differences in the strength of the cryptic acceptor/donor splice sites or variations in the extent to which AMOs accesse the pre-mRNA secondary structures. Different strategies could be used to improve normal splicing for the mutation with lowest correction rate, which include designing a new 5'ss AMOs, using AMOs at the 3'ss, using both 5'ss and 3'ss together or designing AMOs to target exon splicing enhancer (ESE) elements. It has been reported that the use of AMOs can, in some cases, be more efficient for one of the cryptic splice sites than the other [21].

This is the first experiment studying the treatment of AMOs in peripheral blood mononuclear cells which was effective in blocking the aberrant splice site and restoring correct splicing of *BTK* pre-mRNA. However, PBMC contain a variety of blood components. It is been hard to control the outcome by using these cells. Therefore, further studies should be done to demonstrate mRNA levels in the purified cell population.

In addition to our study showing that AMOs can be delivered into PBMCs, several studies have demonstrated that it is possible to effectively deliver AMOs to the nuclei of various cell types [19, 20]. These findings suggest that it should be feasible to find appropriate conditions and carriers for delivery of the AMOs into cells of the patients in clinical settings. There are some difficulties is AMOs possible attempts to interfere with the function of one gene may lead to unwanted side-effects on another and although conventional genetic screens are of inestimable value in understanding a particular process, the researchers cannot be guaranteed to target the desired gene nor can they be guaranteed to produce a null mutation[24]. Two published examples of such off-target effects come from work on sea urchins and zebrafish. Unfortunately, there are likely to be many other cases, many yet to be recognised, in which phenotypes caused by AMOs are non-specific.

In conclusion, our studies suggest that AMOs could be used as a therapeutic tool for a patient with XLA bearing an intronic mutation in the *BTK* gene causing a splicing defect. Similar therapeutic strategies could possibly be applied to other types of *BTK* splice site mutations as well as to other genetic disorders. As such, it would be interesting to examine the effects of similar antisense approaches on these types of mutations as well as to initiate *in vivo* experiments with animal models.