Arch. Immunol. Ther. Exp. (2014) 62:431–436
DOI 10.1007/s00005-014-0283-0

ORIGINAL ARTICLE

In vitro Correction of a Novel Splicing Alteration in the *BTK* Gene by Using Antisense Morpholino Oligonucleotides

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Received: 29 August 2013 / Accepted: 5 February 2014 / Published online: 23 March 2014

Abstract A novel sequence variant, c.240+109C>A, in the Bruton's tyrosine kinase (*BTK*) gene was identified in a patient with X-linked agammaglobulinemia. This alteration resulted in an incorporation of 106 nucleotides of *BTK* intron 3 into its mRNA. Administration of the 25-mer antisense morpholino oligonucleotide analog in the patient's cultured peripheral blood mononuclear cells was able to restore correctly spliced *BTK* mRNA, a potential treatment for X-linked agammaglobulinemia.

Keywords X-linked agammaglobulinemia · *BTK* · Mis-splicing · Antisense morpholino oligonucleotides

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Introduction

X-linked agammaglobulinemia (XLA; MIM 300300) is a primary immunodeficiency disease characterized by an aberration of B lymphocyte differentiation leading to a decreased number of mature B cells and reduction of immunoglobulin (Ig) production. After maternal Ig supplies have dissipated, the low to absent Ig levels make the infants with XLA more susceptible to severe bacterial infections including otitis, sinusitis, and pneumonia, which can be life threatening (Sideras and Smith 1995).

Approximately, 90 % of XLA are caused by alterations in the Bruton's tyrosine kinase (*BTK*) gene (Vihinen et al. 2000). It is involved in cell growth, survival, and migration to blood circulation (Buggy and Elias 2012; Tsukada et al. 1993). The *BTK* gene spans 37.5 kb on the Xq21.3–Xq22 region and contains 19 exons, encoding the 659-amino acid BTK enzyme. *BTK* alterations causing XLA are scattered throughout the gene. These are archived in public available databases including http://bioinf.uta.fi/BTKbase (Valiaho et al. 2006; Zhang et al. 2006) and Resource of Asian Primary Immunodeficiency Diseases in Asian populations (http://rapid.rcai.riken.jp/RAPID). However, there have been some patients whose alterations in *BTK* cannot be detected (Kanegane et al. 2000; Moschese et al. 2000). Autosomal recessive inheritance of agammaglobulinemia has also been described and accounts for 15 % of patients with agammaglobulinemia (Ferrari et al. 2007a). It is genetically heterogeneous. Alterations in other genes including *IGHM*, *IGLL1*, *CD79A*, *BLNK*, *LRRC8*, *CD79B*, and *PIK3R1* have been identified (Conley et al. 2012; Dobbs et al. 2007; Ferrari et al. 2007b; Minegishi et al. 1998, 1999a, 1999b, 1999a, b; Sawada et al. 2003).

The current treatment for XLA patients includes an intravenous infusion of immunoglobulins (IVIG) typically

every 3–4 weeks beginning at the age of 10–12 weeks and prophylactic antibiotics (D'Eufemia et al. 2000). Alternative treatments manipulating at the DNA or RNA levels have been proposed (Du and Gatti 2011). Gene-corrected stem cell-based therapy was studied in *Btk*-deficiency mouse models (Hendriks et al. 2011). Antisense morpholino oligonucleotides (AMOs) can be designed to restore normal splicing by blocking aberrant splice sites (Zhao et al. 2012). This strategy has been explored in several genetic diseases (Corey and Abrams 2001; Du et al. 2007; Rodriguez-Pascau et al. 2009; Vacek et al. 2003).

In this study, we identified and characterized a novel mis-splicing variant located in intron 3 of the *BTK* gene in a Thai patient with XLA. In addition, we demonstrated that AMOs could restore normal mRNA *BTK* splicing in the patient's peripheral blood mononuclear cells (PBMCs).

Materials and Methods

Case Report

A 4-year-old boy was diagnosed with XLA. He had recurrent bacterial sepsis since the age of 1 year. Immunological tests showed absence of circulating B cells, very low levels of serum immunoglobulins, and neutropenia. IVIG has been given since then. No other family members had clinical features consistent with XLA.

Genetic Analysis

After informed consent, his peripheral blood was obtained for RNA and DNA extraction and PBMC isolation. Both parental DNA was also obtained. Total mRNA was isolated by QIAamp® RNA blood mini kit (Qiagen, Hilden, Germany). Reverse transcription-PCR (RT-PCR) was performed using an RT-PCR kit (Promega, Madison, WI, USA). The entire coding region of the *BTK* gene was amplified by using a forward primer: 5′-CAAT GCATCTGGGAAGCTAC-3′ and a reverse primer 5′-AGCTTGGGATTTCCTCTGAG-3′. The 2,129-bp PCR product was treated by ExoSAP-IT (USP Corporation, Cleveland, OH, USA) prior to sending for direct sequencing at the Macrogen Inc., Seoul, Korea. Sequence analysis was performed by Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA).

To identify the variant in DNA, genomic DNA covering the intron 3 was PCR amplified and sequenced. The primers were 5′-CCTGGTGCCACCTCACTTTG-3′ and 5′-GATCCTGAGAGAACTGAGGG-3′ with the expected product size of 501 bp.

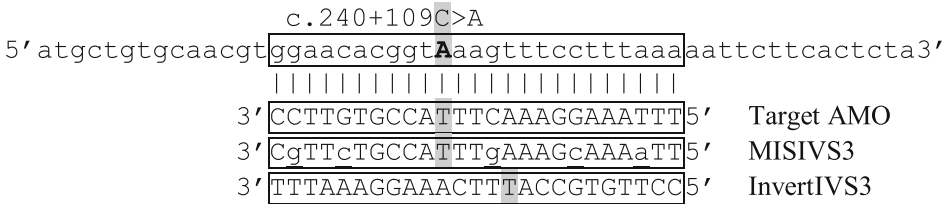
Pyrosequencing was used to screen for the presence of the identified variant in 120 healthy female controls.

AMO Administration

Isolation of PBMCs from peripheral blood specimens was performed using the standard protocol of Ficoll–Hypaque density gradient centrifugation. PBMCs were then diluted for cell counts and cultured in tissue culture medium RPMI 1640 supplemented with PenStrep and 10 % fetal bovine serum. PBMCs were cultured in a 24-well plate at 8×10^5 cells per well. To activate PBMCs, we added 50 µl of phytohemagglutinins (PHA) per well and PBMCs were incubated overnight in a 5 % CO_2 incubator.

Three specific 25-mer AMOs targeting the sequences containing the aberrant splicing variant and normal control sequences in intron 3 of the *BTK* gene were designed and synthesized by GeneTools® in accordance with the manufacturer's criteria. The target AMO contained reverse-complement sequences specific to "A" of the c.240+109C>A and was expected to block an access of the splicing machinery to the pre-mRNA (Table 1). The

Table 1 Nucleotide sequence of each AMO



AMOs	Nucleotide sequence 5′ → 3′
Target AMO	TTTAAAGGAAACTTTACCGTGTTCC
MISIVS3	TTaAAAcGAAAgTTTACCGTcTTgC
InvertIVS3	CCTTGTGCCATTTCAAAGGAAATTT

other two morpholino control oligos were MISIVS3, a mispaired control with reverse-complement sequences containing five nucleotide mismatches from the target AMO, and InvertIVS3—the inverted direction of the target AMO (Table 1).

Results

Sequence analysis of the entire coding region of the *BTK* gene revealed an insertion of the 106-bp (+1 to +106) into the 5′ end of the intron 3 (Fig. 1a). Sequencing of the patient's genomic DNA identified a c.240+109C>A (IVS3+109C>A) variant (Fig. 1b). This splice site variant has never been previously described. The change creates a new cryptic 5′ splice site resulting in an incorporation of the 106-bp pseudoexon into intron 3 of the mature mRNA (Fig. 1c). This unusual transcript is expected to result in a truncated protein of 118 amino acid residues. This mutant allele was inherited from the patient's mother. It was absent in 120 healthy female controls.

We tested if AMOs could restore normal mRNA *BTK* splicing in the patient's PBMCs. The target AMO was able to correct the aberrant splicing defect caused by the c.240+109C>A. While the InvertIVS3 with the same length and base composition as the target AMO but inverted direction had no effect on aberrant splicing correction, the MISIVS3 containing five nucleotide mismatches had an effect on correction of aberrant splicing (Fig. 2a).

To determine the optimal concentration and time course of AMO administration on *BTK* transcription, AMOs with different concentrations of 10–60 μM were delivered into the overnight-grown PBMCs which were diluted by using the peptide-based Endo-Porter system. RT-PCR was performed to measure the mRNA expression levels using the primers (BTKE3-mRNA_F1 GGAGAAGAGGCAGTAA GAAG and BTKE4-mRNA_R GGGATAAGGGAACC TTTCAA) that were located within cDNA sequences of *BTK* exons 3 and 4 and the flanking 106-bp insertion.

After 24 h of AMO incubation (day 1), the RT-PCR was carried out for 35 cycles. The 144-bp product which was

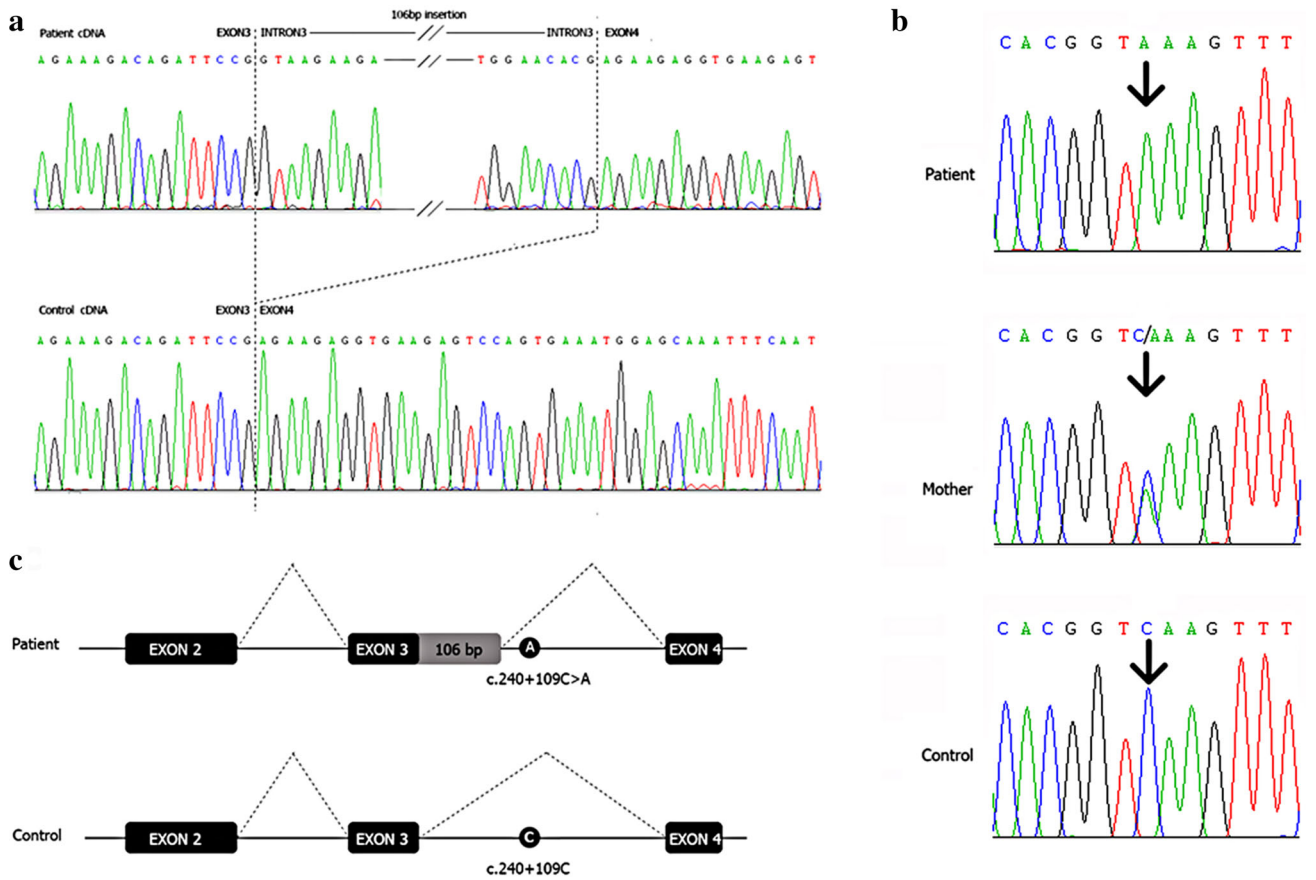


Fig. 1 The *BTK* sequence analysis. **a** Partial sequences of the cDNA of the patient showing an insertion of 106 bp (+1 to +106) into the 5′ end of intron 3; **b** gDNA of the patient showing a c.240 + 109C>A (IVS3 + 109C>A) variant inherited from the mother (*arrows*). **c** Schematic representation of the *BTK* coding region with the pseudoexon. Exons and pseudoexon are *boxed*. A splice site alteration at c.240 + 109C>A was located in intron 3

the corrected splicing was detected, along with the 250-bp band corresponding to the mis-spliced product in all AMO concentrations. At the 24-h incubation, no differences of the ratio between the aberrantly spliced RNA band and the normally spliced band were observed in different AMO concentrations (Fig. 2b).

We then explored the duration of effects of the AMOs. First, activated PBMCs with 10 and 20 μM of AMOs were cultured and harvested for RNA extraction at 24, 48, and 72 h. No differences of the ratio between the aberrantly and the normally spliced bands were observed at different durations (Fig. 2c).

PBMCs with 10 μM of AMO were further cultured and harvested at days 1, 2, 3, 4, 10, 14, and 30. We demonstrated that AMOs remained effective in restoring the normal splicing up to 30 days (Fig. 2d).

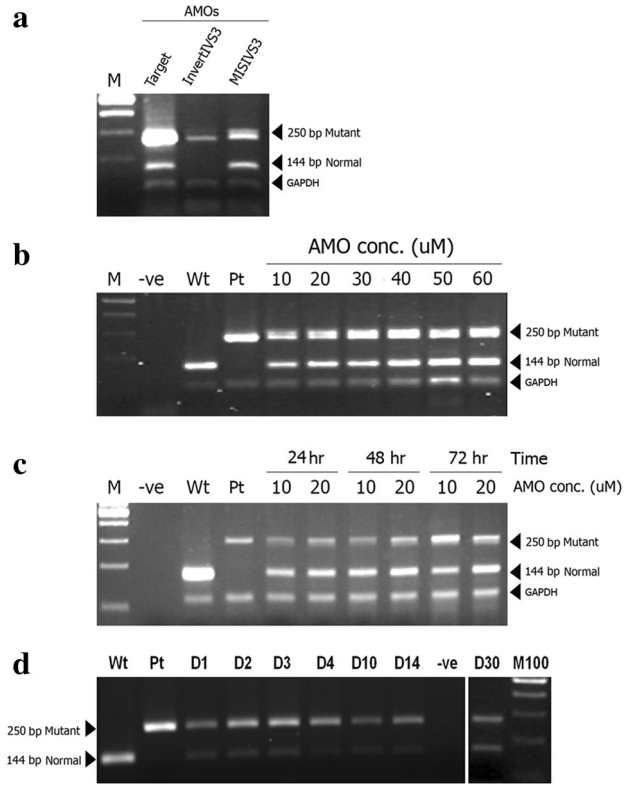


Fig. 2 **a** An RT-PCR analysis of the patient's PBMCs treated with each AMO. The target AMO contained reverse-complement sequences specific to "A" of the c.240 + 109C>A to block an access of the splicing machinery to the pre-mRNA. The InvertIVS3, the inverted direction of the target AMO, and MISIVS3, a mispaired control with reverse-complement sequences containing five nucleotide mismatches from the target AMO, were used as control oligos. The 144-bp corrected splicing product was detected when PBMCs were treated with the target AMO and MISIVS3. **b** An RT-PCR analysis of the *BTK*-mutated PBMCs treated with various concentrations of target AMOs (10–60 μM). *−ve* negative control (no template), *Wt* an unaffected control, *Pt* the patient's PBMCs without AMO treatment. **c** Correction of the aberrant splicing of the *BTK* mRNA in PBMCs after AMO administration. Cells were treated with two concentrations (10 and 20 μM) of the target AMOs. Total RNA samples were collected at 24, 48 and 72 h after AMO administration and analyzed by RT-PCR. *−ve* negative control (no template), *Wt* RNA sample from an unaffected control, *Pt* the patient's untreated PBMCs. **d** Duration of effect of the target AMOs on aberrant splicing correction. PBMCs derived from the patient were treated with mutation-specific AMOs with a concentration of 10 μM. RT-PCR analysis was performed in PBMCs treated with AMOs and harvested at days 1, 2, 3, 4, 10, 14, and 30. *Upper band* the mutant PCR product; *lower band* the wild-type PCR product

Discussion

In this study we described a Thai patient with XLA. Sequencing of the entire coding region of *BTK* revealed a novel c.240+109C>A variant in intron 3. Of note, it is located deep within an intron. Primers for PCR of the genomic DNA are usually designed to cover an exon with less than 60 bp of the franking introns. Therefore, if the sequence analysis was performed by PCR sequencing of the genomic DNA, this variant could have been missed. Some reported XLA patients with unidentified *BTK* variants might have alterations in introns. Our approach emphasizes the usefulness of sequencing complementary DNA, complementing with sequencing genomic DNA.

The identified C > A transversion at position 109 from the 5′ end of the intron 3 in our patient creates a new 5′ cryptic splice site. Of the 129 splicing defects from a total of 960 *BTK* alterations reported in the Asian populations (Keerthikumar et al. 2009), 9 were reported at G or T intronic splice sites (c.240+1G and c.240+2T) after the *BTK* exon-3 coding region and A or G intronic splice sites (c.241–1G and c.241–2A) before the *BTK* exon-4 coding region. The novel c.240+109C>A variant reported in this study did not affect the GT/AG splice sites directly, but it could act as a new cryptic splice site. It resulted in an aberrant 106-bp pseudoexon insertion. This leads to the insertion of extra amino acids and a premature stop producing a truncated 118-residue protein, instead of the normal 659 residues.

Our study demonstrated the feasibility of delivering specific AMOs into the patient's activated PBMCs in vitro to correct the aberrant splicing defect caused by the c.240+109C>A and restore the normal transcript. The AMOs were able to restore correctly spliced mRNA in the patient's PBMCs through skipping of the aberrant inclusion by annealing to pre-mRNA and blocking access of splicing factors or other cis-regulatory elements binding at the splice sites (Dominski and Kole 1993).

The concentrations of AMOs we selected in this study ranged 10–60 μM. No difference of their effectiveness was observed at 24 h. The 10 and 20 μM of AMO treatment also showed no difference at 72 h. We therefore used the least concentrations (10 μM) to determine the duration of

effects of the AMO. We found that 10 μM of AMO could have an effect up to 30 days.

Even with the highest concentration of AMOs (60 μM) we used in our studies, aberrant RNA remained. It has been demonstrated that only a small percent of factor VIII could improve clinical manifestations of patients with hemophilia A (Witmer and Young 2013). Therefore, a certain amount of normal BTK protein might be sufficient for a patient with XLA to have clinical improvement. Hence, AMOs could be developed as a potential therapeutic tool for patients with XLA caused by splicing defects even though all aberrations could not be completely corrected.

Because AMOs were found to be effective in different cell types, including activated PBMCs as in our study, and able to target splice sites in a variety of pre-mRNAs, this approach could be applied in several conditions. Clinical trials which have been successfully performed by intramuscular administration of these therapeutic tools to human patients have yielded promising results for patients with Duchenne muscular dystrophy and dystrophic epidermolysis bullosa (Goto et al. 2006; van Deutekom et al. 2007). These combined with several other observations in diseases such as cystic fibrosis and β-thalassemia (Friedman et al. 1999) suggested the possibility of this approach in the treatment of diseases with genetic defects.

Before delivery of the AMOs into the patients in clinical settings, there are some concerns including a possibility of the AMOs to interfere with the functions of other genes in any tissue, leading to unwanted side effects (Carvajal et al. 2001). The published examples of such off-target effects come from work on sea urchins, zebrafish, and *Xenopus* (Corey and Abrams 2001). The detection of an effect on aberrant splicing correction with the use of a control AMO, a five-mispair oligo, indicates that our target AMOs have low specificity and detrimental effects as aberrant splicing correction of cellular mRNA might occur.

In conclusion, our studies suggest that AMOs could be used as a therapeutic tool for a patient with XLA bearing an intronic alteration in the *BTK* gene causing a splicing defect. Similar therapeutic strategies could possibly be applied to other types of *BTK* splice site alterations 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 alterations as well as to initiate in vivo experiments using animal models.

Acknowledgments We would like to thank Dr. Surasawadee Ausavarat for commenting on the manuscript, and Ms. Supranee Buranapraditkun of the Faculty of Medicine, Chulalongkorn University, for excellent technical assistance in cell culture. This work was supported by the 90th Anniversary of Chulalongkorn University Fund, Ratchadaphiseksomphot Endowment Fund, Faculty of Medicine, Chulalongkorn University (RES560530177-HR), and the Thailand Research Fund.

Conflict of interest We have no conflict of interest to declare.

References

Buggy JJ, Elias L (2012) Bruton tyrosine kinase (BTK) and its role in B-cell malignancy. Int Rev Immunol 31:119–132

Carvajal JJ, Cox D, Summerbell D et al (2001) A BAC transgenic analysis of the Mrf4/Myf5 locus reveals interdigitated elements that control activation and maintenance of gene expression during muscle development. Development 128:1857–1868

Conley ME, Dobbs AK, Quintana AM et al (2012) Agammaglobulinemia and absent B lineage cells in a patient lacking the p85alpha subunit of PI3K. J Exp Med 209:463–470

Corey DR, Abrams JM (2001) Morpholino antisense oligonucleotides: tools for investigating vertebrate development. Genome Biol 2:REVIEWS1015

D'Eufemia P, Nigro G, Celli M et al (2000) Low-dosage immunoglobulins for an infant with hypogammaglobulinemia, maple syrup urine disease, and parvovirus B19-associated aplastic crisis. J Pediatr Hematol Oncol 22:485–487

Dobbs AK, Yang T, Farmer D et al (2007) Cutting edge: a hypomorphic mutation in Igbeta (CD79b) in a patient with immunodeficiency and a leaky defect in B cell development. J Immunol 179:2055–2059

Dominski Z, Kole R (1993) Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. Proc Natl Acad Sci USA 90:8673–8677

Du L, Gatti RA (2011) Potential therapeutic applications of antisense morpholino oligonucleotides in modulation of splicing in primary immunodeficiency diseases. J Immunol Methods 365:1–7

Du L, Pollard JM, Gatti RA (2007) Correction of prototypic ATM splicing mutations and aberrant ATM function with antisense morpholino oligonucleotides. Proc Natl Acad Sci USA 104:6007–6012

Ferrari S, Lougaris V, Caraffi S et al (2007a) Mutations of the Igbeta gene cause agammaglobulinemia in man. J Exp Med 204: 2047–2051

Ferrari S, Zuntini R, Lougaris V et al (2007b) Molecular analysis of the pre-BCR complex in a large cohort of patients affected by autosomal-recessive agammaglobulinemia. Genes Immun 8: 325–333

Friedman KJ, Kole J, Cohn JA et al (1999) Correction of aberrant splicing of the cystic fibrosis transmembrane conductance regulator (CFTR) gene by antisense oligonucleotides. J Biol Chem 274:36193–36199

Goto M, Sawamura D, Nishie W et al (2006) Targeted skipping of a single exon harboring a premature termination codon mutation: implications and potential for gene correction therapy for selective dystrophic epidermolysis bullosa patients. J Investig Dermatol 126:2614–2620

Hendriks RW, Bredius RG, Pike-Overzet K et al (2011) Biology and novel treatment options for XLA, the most common monogenetic immunodeficiency in man. Expert Opin Ther Targets 15:1003–1021

Kanegane H, Tsukada S, Iwata T et al (2000) Detection of Bruton's tyrosine kinase mutations in hypogammaglobulinaemic males registered as common variable immunodeficiency (CVID) in the Japanese Immunodeficiency Registry. Clin Exp Immunol 120: 512–517

Keerthikumar S, Raju R, Kandasamy K et al (2009) RAPID: resource of Asian primary immunodeficiency diseases. Nucleic Acids Res 37:D863–D867

Minegishi Y, Coustan-Smith E, Wang YH et al (1998) Mutations in the human lambda5/14.1 gene result in B cell deficiency and agammaglobulinemia. J Exp Med 187:71–77

Minegishi Y, Coustan-Smith E, Rapalus L et al (1999a) Mutations in Igalpha (CD79a) result in a complete block in B-cell development. J Clin Investig 104:1115–1121

Minegishi Y, Rohrer J, Coustan-Smith E et al (1999b) An essential role for BLNK in human B cell development. Science 286: 1954–1957

Moschese V, Orlandi P, Plebani A et al (2000) X-chromosome inactivation and mutation pattern in the Bruton's tyrosine kinase gene in patients with X-linked agammaglobulinemia. Italian XLA Collaborative Group. Mol Med 6:104–113

Rodriguez-Pascau L, Coll MJ, Vilageliu L et al (2009) Antisense oligonucleotide treatment for a pseudoexon-generating mutation in the NPC1 gene causing Niemann-Pick type C disease. Hum Mutat 30:E993–E1001

Sawada A, Takihara Y, Kim JY et al (2003) A congenital mutation of the novel gene LRRC8 causes agammaglobulinemia in humans. J Clin Investig 112:1707–1713

Sideras P, Smith CI (1995) Molecular and cellular aspects of X-linked agammaglobulinemia. Adv Immunol 59:135–223

Tsukada S, Saffran DC, Rawlings DJ et al (1993) Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. Cell 72:279–290

Vacek M, Sazani P, Kole R (2003) Antisense-mediated redirection of mRNA splicing. Cell Mol Life Sci 60:825–833

Valiaho J, Smith CI, Vihinen M (2006) BTKbase: the mutation database for X-linked agammaglobulinemia. Hum Mutat 27: 1209–1217

van Deutekom JC, Janson AA, Ginjaar IB et al (2007) Local dystrophin restoration with antisense oligonucleotide PRO051. N Engl J Med 357:2677–2686

Vihinen M, Mattsson PT, Smith CI (2000) Bruton tyrosine kinase (BTK) in X-linked agammaglobulinemia (XLA). Front Biosci 5:D917–D928

Witmer C, Young G (2013) Factor VIII inhibitors in hemophilia A: rationale and latest evidence. Ther Adv Hematol 4:59–72

Zhang YM, Tung CH, He J et al (2006) Construction of a novel chimera consisting of a chelator-containing Tat peptide conjugated to a morpholino antisense oligomer for technetium-99 m labeling and accelerating cellular kinetics. Nucl Med Biol 33:263–269

Zhao Y, Ishibashi S, Amaya E (2012) Reverse genetic studies using antisense morpholino oligonucleotides. Methods Mol Biol 917: 143–154