CASE REPORT

REVISED **Case Report: Whole exome sequencing identifies a novel frameshift insertion c.1325dupT (p.F442fsX2) in the tyrosine kinase domain of *BTK* gene in a young Indian individual with X-linked agammaglobulinemia [version 2; referees: 2 approved]**

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Abstract
X-linked agammaglobulinemia (XLA) is an extremely rare inherited primary immunodeficiency characterized by recurrent bacterial infections, decrease in number of mature B cells and low serum immunoglobulins. XLA is caused by mutations in the gene encoding Bruton's tyrosine kinase. We report a case of a young Indian boy suspected to have XLA. Immunophenotyping was performed for the affected child using CD20, CD19 and CD3 antibodies. Whole exome sequencing was performed using trio-based approach. The variants were further analyzed using capillary sequencing in the trio as well as maternal grandmother. Initial immunophenotyping in the affected child showed decreased count of CD19+ B cells. To strengthen the clinical findings and confirm the diagnosis of XLA, we performed whole exome sequencing. Our analysis identified a novel frameshift insertion (c.1325dupT) in the *BTK* gene, which was further validated by Sanger sequencing. Our approach shows the potential in using whole exome sequencing to pinpoint the molecular lesion, enabling timely diagnosis and genetic counseling, and potentially offering prenatal genetic testing for the family.

This article is included in the Rare diseases collection.

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Referee Status: ✔✔

	Invited Referees	
	1	**2**
REVISED version 2 published 31 Aug 2017		✔ report
	↑	
version 1 published 14 Nov 2016	✔ report	? report

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Introduction

Primary immunodeficiencies are congenital defects in the immune defence mechanisms of the host against invading pathogens. X-linked agammaglobulinemia (XLA) is a primary immunode-ficiency disorder (OMIM# 300755) characterized by recurrent infections causing pneumonia, conjunctivitis, gastrointestinal infections, otitis media and sinopulmonary infections[1], which may require frequent hospitalization. The disease is extremely rare with an estimated prevalence of 3–6 per 1,000,000 males[2] and is inherited in an X-linked recessive manner. The disease arises from genetic defects, due to which the mature B lymphocytes are either low in number or completely absent in the bloodstream while also exhibiting a complete absence of serum immunoglobulins[1]. The absence of immunoglobulins results in a compromised humoral immune response, which makes the affected individual extremely vulnerable to infections with encapsulated bacteria and enteroviruses. Molecular genetic studies have conclusively mapped the genetic locus of XLA to the gene encoding for the Bruton's tyrosine kinase (BTK)[3]. A comprehensive mutation database (BTKbase) lists over 700 unique mutations associated with XLA that affect the activity of BTK protein[4].

Case report

A five year old boy of north Indian origin, born out of a non-consanguineous marriage (Figure 1A) presented to the hospital with headache, fever and a history of recurrent infections requiring hospitalization. The antenatal and perinatal periods were unevent-ful. His clinical history revealed that the child had been hospital-ized for septicaemia and underwent treatment with intravenous (IV) antibiotics for 2 weeks at one year of age. Later, at 2.5 years, the child developed fever with swelling in right knee joint that was diagnosed as septic arthritis. The child was again hospitalized at 5 years of age for pyogenic meningitis. The culture of cerebrospinal fluid was found to be positive for *Pseudomonas aeruginosa*. On close examination, the tonsils were found to be absent and there was no peripheral lymphadenopathy. The immunoglobulin profile revealed serum level of IgG 50 mg/dl (200–700 mg/dl), IgA 5 mg/dl (40–200 mg/dl) and IgM 9 mg/dl (50–200 mg/dl). The hemogram showed hemoglobin con-centration of 9.5 g/dl (11.5–15.5 g/dl), total leukocyte count 14800/cumm (6000–13500/cumm), platelet count 931000/cumm (150000–400000/cumm) and erythrocyte sedimentation rate ESR 18mm/1st hr (0–14 mm/1st hr).

The blood flow cytometric analysis of the affected child was performed to evaluate the status and count of mature B cells. The patient (III.1) has only CD3+ lymphocytes as observed on CD19/CD3 dot plot and there was complete absence of CD19+ cells (0.02%) (Figure 1B). The staining for BTK pro-tein on CD14+ monocytes showed decreased (20.71%)

expression of BTK in proband as compared to control (72.07%) (Figure 1C). This observation was consistent with the diagnosis of XLA[5]. The patient had no family history of immunodeficiency and no such characteristics were present in any other family members.

The blood samples were collected and processed for genomic DNA isolation by salting out method[6]. We performed the whole exome sequencing using trio-based approach (patient, mother and father). In brief, the whole-exome library was prepared using Nextera rapid capture expanded exome kit (Illumina Inc., USA) according to manufacturer's standard protocol. Sequencing was performed on Illumina Hiseq2500 platform (Illumina Inc., USA) with 130bp paired-end reads. Reads were trimmed using Trim-momatic v0.33[7] and aligned to reference genome hg19 (GRCh37) by Stampy v1.0.23[8] along with BWA v0.7.12-r1039[9]. PCR dupli-cates were marked using Picard tools v1.127. Variations were called using Platypus v0.7.9[10] and annotated using ANNOVAR[11]. Analysis revealed a novel frameshift insertion c.1325dupT in exon 14 of the *BTK* gene. The mutation was found to be homozygous in child and heterozygous in mother. The identified mutation c.1325dupT has not yet been reported in the BTKbase[4] and absent in ExAC, 1000genome as well as internal control databases from South Asia and Middle East (http://clingen.igib.res.in/almena), which confirms the novelty of the variation. The mutation evalu-ation by SIFT Indel tool (http://sift.bii.a-star.edu.sg/www/SIFT_indels2.html, 12) was predicted to be damaging and caused nonsense mediated decay (confidence score 0.858). Further *in silico* analysis suggested that the mutation causes Isoleucine at 443 residue in BTK to be replaced by Histidine and introduces a premature stop codon at 444 residue, which lies in the kinase domain of the BTK protein (Figure 1D).

The variation was further validated by PCR amplification of region encompassing the variation using specific primer sets (Forward primer: 5'-CCCCAAATGCTACTGAGATGGT-3' and Reverse primer: 3'-CCTATTTCTACCCCAGTAGGGA-5') with the annealing temperature of 59°C using Brazilian *taq* polymerase (Invitrogen, USA) according to manufacturer instruction. PCR products were purified using Qiaquick PCR purification kit (QIAGEN, Germany). Capillary sequencing was performed using BigDye-terminator chemistry on 3130xl Genetic Analyzer (Applied Biosystems, USA). Analysis revealed that the mutation was homozygous in child (III.1), heterozygous in mother (II.3) and absent in father (II.2) and maternal grandmother (I.4) (Figure 1E).

Discussion

XLA is a primary immunodeficiency disorder characterized by recurrent infections causing pneumonia, conjunctivitis, gastrointes-tinal infections, otitis media and sinopulmonary infections[1]. Whole exome sequencing has been increasingly used to identify mutations in rare genetic diseases mainly due to the speed, cost and amenabil-ity as compared to traditional capillary sequencing[13]. Recent reports have suggested the application of whole exome sequencing for muta-tion detection in a variety of primary immunodeficiency cases[14,15].

In the present report, we performed whole exome sequencing using a trio-based approach for a child from an Indian family who presented to the clinic with the suspected diagnosis of XLA. The

lack of readily available specific gene sequencing assays coupled with absence of a next-generation sequences (NGS) based targeted gene panels for XLA provided the impetus for attempting exome sequencing.

Our exome sequencing analysis revealed a novel frameshift insertion c.1325dupT in exon 14 of the *BTK* gene. The mutation was found to be homozygous in patient and heterozygous in unaffected mother, which was further validated by capillary sequencing. This confirmed the X-linked inheritance and carrier status of the mother for the mutation. The mutation was found to be absent in unaffected father and maternal grandmother. The identified mutation c.1325dupT was found to be novel and damaging due to

truncation of the BTK at 444 residue of kinase domain. The flow cytometric analysis for BTK stained monocytes shows decreased expression of BTK in proband as compared to control (Figure 1C). The mutation excludes functionally well characterized active site residue Y551 of the protein. Additionally, nonsense mutation at the codon Y425X, E441X, Q459X and Q497X is known to cause loss of kinase activity of BTK, which has been previously demonstrated using *in vitro* kinase activity assay in Japanese individuals[16]. Since c.1325dupT (p.F442fsX2) lies in the vicinity of the above mentioned well studied codon positions, the effect of the mutation is expected to be damaging to BTK. Currently the patient is on intravenous immunoglobulin replacement therapy (15 g every 3–4 weekly) and is responding well. We could not avail the RNA samples to perform transcript analysis or functional studies.
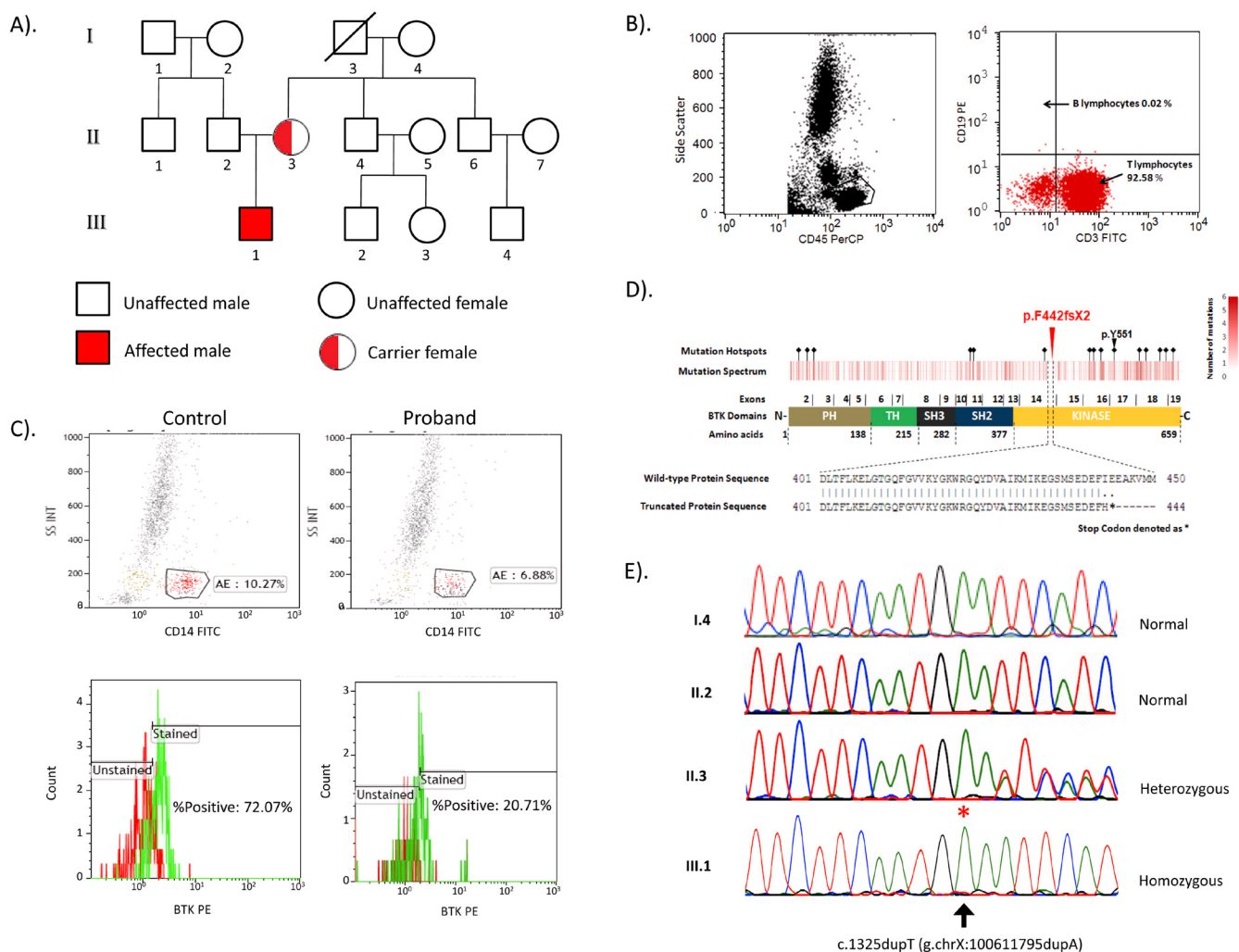

Figure 1. A). Pedigree of the family **B**). Flow cytometric immunophenotyping of peripheral blood lymphocytes gated on side scatter/CD45 plot. In CD19/CD3 plot, arrow depicts the count of B lymphocytes (CD19+) and T lymphocytes (CD3+). **C**). Flow cytometric estimation of BTK protein expression on CD14+ monocytes in control and proband. **D**). Heat map showing mutation spectrum corresponding to BTK protein structure and hotspots are marked with black diamond symbol. BTK domains (PH, TH, SH3, SH2 and Kinase) are marked in respective colors. The exons encoding specific region of domain and amino acids span for each domain is represented in schematic of BTK. A red triangle represents the novel variation p.F442fsX2, which lies in exon 14 and BTK kinase domain. The widely studied p.Y551 is also marked with black arrow. Pairwise alignment between wild type and truncated protein sequence is performed by using EMBOSS online software. **E**). The chromatogram depicts capillary sequencing results of c.1325dupT (p.F442fsX2) (marked with asterisks).

In summary, our flow cytometry data and exome sequencing analysis are well correlated for confirming the diagnosis of XLA. The outcome from the present study strongly supports the pathogenicity of identified novel mutation in *BTK* gene.

Consent

Written informed consent was obtained the parents of the child.

Data availability

The raw sequencing data are available at NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) with accession number SRR3439009.

Author contributions

AR, AG and SS clinically characterized the patient and collected blood samples for the study. SKV, AV and RJ isolated the DNA, prepared the exome enrichment and performed sequencing, analysis and validation. AC, RK performed the immuno-characterization of the family. SSivasubbu and VS designed the study and oversaw all the experiment and validation. AR, AV, SKV, SSivasubbu and VS contributed in writing the manuscript.

Competing interests

No competing interests were disclosed.

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    PubMed Abstract

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Current Referee Status: ✔ ✔

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Version 2

Referee Report 07 September 2017

doi:10.5256/f1000research.13113.r25554

✔ **Saharuddin Bin Mohamad**

Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

I am satisfied with the corrections and comments made by the authors.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Version 1

Referee Report 10 February 2017

doi:10.5256/f1000research.10201.r20086

❓ **Saharuddin Bin Mohamad**

Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

XLA is a rare disease which gave this case report high value for being indexed. However I have some concerns regarding the report as below:

1. Since the patient was suspected with XLA and BTK gene is well known as the cause of the condition. Why Whole Exome Sequencing (WES) was conducted for this case? Why not conduct targeted sequencing for BTK since that would be much cheaper and easier to analyse. Since WES was conducted, I think authors should provide other results from WES analysis such as variant analysis, etc.

2. The results in Figure 1D for heterogeneous needs further explanation. It seems like the heterogeneous is not having two peaks at the asterisks position, but having two peaks at 5 positions downstream to the asterisks.

3. I would like to suggest evidence to be provided for the truncated BTK gene due to the mutation (Reverse transcriptase-PCR).

4. I would like to suggest the authors to referred to other database with bigger number of samples such as Exome Aggregation Consortium and 1000 Genomes database (not only BTKbase).

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 02 Mar 2017

Amit Rawat, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Why Whole Exome Sequencing (WES) was conducted for this case?

The differential diagnosis in a case of hypogammaglobulinemia with absent B cells would include X-linked agammaglobulinemia and autosomal recessive forms of hypogammaglobulinemia. X-linked agammaglobulinemia is caused by mutations in the BTK gene, but there is wide list of genes implicated in autosomal recessive forms of hypogammaglobulinemia. These include *PIK3R1, GRB1, AGM7, IMD36, LRRC8A, KIAA1437, AGM5, BLNK, SLP65, AGM4 IGHM, MU, AGM1, CD79B, IGB, B29, AGM6, CD79A, IGLL1, IGO, IGL5, VPREB2, AGM2 AGMX2, XLA2, IMD6, IMD1, AGMX*1

Therefore, a whole exome sequencing was used as diagnostic strategy in this case

The variant found in the index case was also checked in the ExAC and not found to be reported there.

Competing Interests: No competing interests were disclosed.

Author Response 26 Jul 2017

Vinod Scaria, Institute of Genomics and Integrative Biology, Delhi, India

XLA is a rare disease which gave this case report high value for being indexed. However I have some concerns regarding the report as below:

1. Since the patient was suspected with XLA and BTK gene is well known as the cause of the condition. Why Whole Exome Sequencing (WES) was conducted for this case? Why not conduct targeted sequencing for BTK since that would be much cheaper and easier to analyse. Since WES was conducted, I think authors should provide other results from WES analysis such as variant analysis, etc.

Clarification 1: Targeted sequencing of BTK is not readily available in the India. In many cases, due to the unavailability of ready services, whole exome sequencing is an attractive alternative due to the speed, cost-effectiveness as well as the coverage of genes. Indeed the targeted sequencing for BTK was performed previously by another organization outside the country, which was reported negative, which prompted us to explore additional genes involved. In addition, number of genes have been previously implicated in agammaglobulinemia including PIK3R1, GRB1, AGM7, IMD36, LRRC8A, KIAA1437, AGM5, BLNK, SLP65, AGM4 IGHM, MU, AGM1, CD79B, IGB, B29, AGM6,

CD79A, IGLL1, IGO, IGL5, VPREB2, AGM2, AGMX2, XLA2, IMD6, IMD1, AGMX apart from BTK.

The whole exome sequencing generated a total of 67437 variants in the patient, and 2046 and 891 variants for the mother and father respectively. Analysis of the variants are summarized in Table 1.

Analysis revealed four variants in genes related to agammaglobulinemia. Out of which three were with an allele frequency quite common in the Indian population. The BTK variant was previously not described in the variant frequency databases. The variants are summarized in Table 2.

2. The results in Figure 1D for heterogeneous needs further explanation. It seems like the heterogeneous is not having two peaks at the asterisks position, but having two peaks at 5 positions downstream to the asterisks.

Clarification 2: The asterisk position represents the site where 'A' base is inserted. The frameshift starts to be noticed after 1 peak due to AAA repeats. Please see Figure.

3. I would like to suggest evidence to be provided for the truncated BTK gene due to the mutation (Reverse transcriptase-PCR).

Clarification 3: We could not avail the RNA sample for the case for further studies. We have added a sentence in the discussion to highlight this caveat. Ample evidence suggests that the nonsense mutation at the codon Y425X, E441X, Q459X and Q497X is known to cause loss of kinase activity of BTK, which has been previously demonstrated using *in vitro* kinase activity assay in Japanese individuals (Hashimoto et al. 1996) . Since c.1325dupT (p.F442fsX2) lies in the vicinity of the above mentioned well studied codon positions, the effect of the mutation may be damaging to BTK.

Ref: Hashimoto S, Tsukada S, Matsushita M, et al.: Identification of Bruton's tyrosine kinase (Btk) gene mutations and characterization of the derived proteins in 35 X-linked agammaglobulinemia families: a nationwide study of Btk deficiency in Japan. *Blood.* 1996;88(2):561–73. 8695804

4. I would like to suggest the authors to referred to other database with bigger number of samples such as Exome Aggregation Consortium and 1000 Genomes database (not only BTKbase).

Clarification 4: The suggestions have been incorporated in the revised manuscript. Databases such as ExAC, 1000genome and al-mena (Koshy et al. 2017) are mentioned in the manuscript.

Ref: Koshy R, Ranawat A, Scaria V. al mena: a comprehensive resource of human genetic variants integrating genomes and exomes from Arab, Middle Eastern and North African populations. Journal of Human Genetics. 2017 Jun 22.

Competing Interests: No competing interests were disclosed.

Author Response 04 Sep 2017

Vinod Scaria, Institute of Genomics and Integrative Biology, Delhi, India

There is bit correction in the total number of variations mentioned in comment box (See **Clarification 1)**. The whole exome sequencing generated a total of 67437 variants in the patient, and 61429 and 31074 variants for the mother and father respectively.

Competing Interests: No competing interests were disclosed.

Referee Report 20 January 2017

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✔ **Mohamed Badawy Hassan Tawfik Abdel-Naser**

Faculty of Medicine, Ain Shams University, Cairo, Egypt

- The case report is interesting.

- The authors unnecessarily repeated several sentences and phrases e.g. in the Introduction section the sentence "X-linked agammaglobulinemia (XLA) is a primary immunodeficiency disorder ...." has been repeated in the discussion section. Also, in the case report section the phrase "of the right knee joint".

- The findings of the whole exome sequencing analysis should be regarded as interesting findings because a causal relationship with X-linked agammaglobulinemia needs additional cases and animal studies.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.