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LETTER TO EDITOR

Damaging *BTK* Variant Demonstrated by Carrier, Allele-Specific *BTK* Expression in B Cells and Monocytes

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Abbreviations

BTK	Bruton's tyrosine kinase
FACS	Fluorescence activated cell sorting
NGS	Next generation sequencing
VUS	Variant of uncertain significance
XLA	X-linked agammaglobulinemia

To the Editor:

With the increased use of next generation sequencing (NGS), ascertaining the clinical significance of a variant of unknown significance (VUS) is a difficult and common problem. In silico protein modeling and pathway analysis is currently not definitive, and in vitro *or* in vivo evaluation of the function of the mutant protein can be challenging, expensive and time consuming. We report a novel approach to define the pathogenicity of a VUS in the *BTK* gene that led to the diagnosis of XLA. This approach may be useful in defining the pathogenicity of VUS in other X-linked disorders.

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We evaluated a 17-year-old (yo) male on IVIG previously diagnosed with CVID who presented with respiratory tract infections and hypogammaglobulinemia beginning in the first year of life. Laboratory evaluation of the proband demonstrated an absent/low levels of IgA/IgM, but with a normal level of IgE, and no B cells in the peripheral blood (Table 1). The IgG level prior to antibody replacement was 46 mg/dl. BTK protein expression in monocytes assessed by flow cytometry was slightly lower than controls (Table 1, Supp. Fig. 1).

Family history is significant for a maternal uncle with an uncharacterized antibody deficiency and a history of recurrent sinus infections and pneumonias treated with immunoglobulin replacement therapy. Although records are not available from the time of diagnosis, his B cell count was 0 cells/mm^3 tested at age 60 years. On Ig replacement he lacks serum IgA and IgM and did not respond to vaccination to *Salmonella typhi* (data not shown). Interestingly, similar to the proband, his IgE was within normal limits. Targeted high -throughput DNA sequencing using Illumina's TruSight One gene panel (4,813 genes) of both proband and the uncle revealed a novel missense variant [NM_000061.2:c. 1004T>A (Val335Asp); NC_000023.11:g.101,358,408 (GRch38.p7)] in exon 12 (Supplementary Fig. 1) of *BTK*, leading to the substitution of an aspartic acid residue for a valine (p.V335D.) Sanger sequencing of the proband's mother and sister demonstrated that they were carriers of the c. 1004T>A *BTK* variant. The sister had normal numbers of B cells and normal serum immunoglobulin levels (Table 1). Polyphen and SIFT both predicted the variant to be tolerated and benign, respectively. However, the p.V335D variant was in a highly conserved region of the SH2 domain of BTK, with a PhyloP score 2.8, indicative of evolutionary change that is slower than expected under the null hypothesis ($p < 0.0016$) [1]. The CADD score (combined annotation dependent depletion) of 28.7 for the p.V335D mutation was also predictive of a deleterious effect on the protein. Thus, in silico predictive programs, which are

Table 1 Summary of routine immunological studies

Subject	BTK	Ig (mg/dL)	B cell count (#/mm^3)	BTK expression (% of normal)
Proband (17 years)	Hemizygous variant	IgG 46* IgA < 6 IgM 6 IgE 40	0	69%
Maternal uncle (60 years)	Hemizygous variant	IgG 1070** IgA < 6 IgM < 5 IgE 29	0	73%
Sister (25 years)	Heterozygous	IgG 1080 IgA 240 IgM 92 IgE 9	174	100%

*At time of diagnosis; **on immunoglobulin replacement therapy

insufficient to predict the pathogenicity of a variant, had contradictory results.

BTK is required for the development and maturation of circulating B cells, but not other hematopoietic cells. Because *BTK* is located on the X-chromosome, X-inactivation (lyonization) of *BTK* usually results in expression of one allele in approximately 50% of the cells. Therefore, we hypothesized that if the c. 1004T>A *BTK* variant is pathogenic, circulating B cells in carriers would only express wild type (wt) BTK, whereas monocytes would express comparable amounts of wt and mutant forms of BTK. To address this hypothesis, B cells and monocytes from the carrier were purified by fluorescence activated cell sorting (FACS), cDNAs of *BTK* were synthesized by RT-PCR, and high-throughput sequencing of the *BTK* cDNAs was performed to a read depth of greater than 16,000 (Supplementary Fig. 2B). B cells purified from the carrier expressed only wt BTK (~99% wt BTK), whereas monocytes from the carrier expressed the wt and variant alleles at a comparable frequency (51.1 and 48.9%, respectively.) These data strongly suggest that the c. 1004T>A *BTK* variant is pathogenic and p.V335D BTK has an altered function that does not support B cell maturation and survival.

Currently, there are 891 unique variants in BTKbase, and of the 84 non-intronic variants in the SH2 domain, most are listed as pathogenic in ClinVar [2]. However, many of the other variants in the BTKbase are still considered VUS. Therefore, a determination of the functional significance of these variants is important for genetic counseling as well as an understanding of the genotype-phenotype relationship in XLA [3]. We found a novel c. 1004T>A *BTK* variant in the proband and affected uncle that minimally affects BTK expression. High-throughput sequencing of cDNAs in purified B cells and monocytes of the carrier demonstrated that B cells only expressed wt BTK while monocytes expressed both the wt and mutant BTK. These data suggest the p.V335D variant results in loss of function the BTK protein. Our in vivo functional studies, in conjunction with a known clinical phenotype associated with affected hemizygous affected males results in moderate to strong support for classification of this variant as pathogenic using the American College of Medical Genetics and Genomics (ACMG) guidelines [4].

The p.V335D substitution is found in the βD structural element of the SH2 domain of BTK that is part of a hydrophobic phosphotyrosine binding pocket [5]. A variant described in an XLA patient in the adjacent residue Y334S has been shown to alter protein conformation and decrease binding affinity to PLCϒ2 by roughly fourfold, reflected in the CADD score of 31, predictive of a deleterious effect on the protein [6]. Therefore, we hypothesize that p.V335D BTK may also fail to interact properly with PLCϒ2 leading to a loss of function.

Our studies do not address the expression of IgE in the proband and uncle despite the absence of B cells in the periphery and a functionally aberrant form of BTK. We hypothesize that p.V335D BTK has a small amount of residual activity leading to the production of a few B cells that are present outside of the peripheral compartment. These B cells may then be stimulated to produce IgE in a manner independent of the BCR and functional BTK, perhaps via engagement of TLRs in conjunction with CD154 (CD40L).

In summary, we used FACS to purify B cell and monocytes from a proband and carrier combined with high-throughput cDNA sequencing of *BTK* to demonstrate the pathogenicity of the c. 1004T>A *BTK* variant. We believe a similar approach may be useful to define the functionality of VUS in patients with novel variants in other X-linked disorders where the expression of either the wt or mutant allele confers a selective advantage or readily identifiable cellular phenotype.

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Compliance with Ethical Standards

Conflict of Interest Dr. Routes reports a grant and independent contractor from CSL Behring, a grant from Baxalta, grant from Bio Products Laboratory Limited, outside the submitted work. All other authors declare that they have no conflict of interest.

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