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ORIGINAL ARTICLE

Novel nonsense IL-12Rβ1 mutation associated with recurrent tuberculosis

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Abstract

The interleukin (IL)-12/interferon(IFN)γ axis plays an important role in the control of mycobacterial diseases as demonstrated by the increased susceptibility to mycobacterial species in patients with an inborn error of the IL-12-dependent IFNγ immunity. Here, we report a novel mutation in the *IL-12Rβ1* gene in a female Pakistani patient who was born in a consanguineous marriage and developed severe bacille Calmette–Guérin (BCG) infection and recurrent tuberculosis. After reviewing the patient's clinical records, she was investigated for IL-12/IFNγ defects using enzyme-linked immunosorbent assay (ELISA), flow cytometry, and DNA genetic Sanger sequencing. Quantification of secretory cytokines from the patient's peripheral blood mononuclear cells (PBMCs) revealed significantly reduced IFNγ production. Flow cytometric analysis revealed no surface expression of IL-12Rβ1 on PHA-activated T lymphocytes. In addition, IL-12-induced impaired STAT4 phosphorylation in the patient's lymphocytes when compared with those from five healthy controls. The genetic analysis of *IL-12Rβ1* gene identified a novel nonsense mutation c.199G>T/p.E67* within exon 3, which encodes part of the cytokine-binding region (CBR). In silico analysis indicates that this novel nonsense mutation generates a truncated protein with an apparent inactivating effect. Our data expand the genetic spectrum of IL-12Rβ1 deficiency. Moreover, our findings highlight the need for developing newborn screening for patients with primary immunodeficiency associated with mycobacterial infections in areas where BCG vaccination is mandatory in order to improve the treatment of patients, and consequently their quality of life.

Keywords IL-12Rβ1 · Novel mutation · IFNγ · Tuberculosis

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Abbreviations

TB	Tuberculosis
IL	Interleukin
IFN	Interferon
BCG	Bacille Calmette–Guérin
CBR	Cytokine-binding region
NTM	Non-tuberculous mycobacteria
TYK	Tyrosine-protein kinase
JAK	Janus kinase
PIDs	Primary immunodeficiencies
SCID	Severe combined immunodeficiency
G6PD	Glucose-6-phosphate dehydrogenase
NEMO	NF-kappa B essential modulator
MSMD	Mendelian susceptibility to mycobacterial disease
FNIII	Fibronectin type-III domain

Introduction

Tuberculosis (TB) is an infectious disease mainly caused by *Mycobacterium tuberculosis complex* (MTC) bacteria, representing a major health problem worldwide [1], with a high incidence in developing countries such as Pakistan [2]. Immunity to mycobacteria species such as non-tuberculous mycobacteria (NTM), *M. bovis bacillus Calmette–Guérin* (*BCG*) strains, and *M. tuberculosis complex* (MTC) bacteria depends on the functional integrity of interleukin (IL)-12 and interferon (IFN)γ signaling pathways. For instance, the binding of IL-12 to both subunits of its receptor (IL-12Rβ1 and IL-12Rβ2), which are expressed on cells such as natural killer cells and T lymphocytes, activates a variety of downstream signaling molecules (e.g., tyrosine-protein kinase or TYK2 and Janus kinase 2 or JAK2). This leads to the subsequent phosphorylation of signal transducer and activator of transcription (STAT)4, as well as formation of STAT4 homodimers which translocate to the nucleus and activate the transcription of genes such as *IFNG* [3, 4].

An increased susceptibility to severe mycobacterial disease has been observed in patients with inborn error of IL-12-dependent IFNγ immunity (also known as Mendelian susceptibility to mycobacterial disease or MSMD) [5] as well as in other inborn errors immunity (also called primary immunodeficiency disorders (PIDs) [6–9] such as severe combined immune deficiency (SCID), affecting the cellular immune response or congenital defects of phagocytes [6–9] such as severe combined immune deficiency [10], chronic granulomatous disease (CGD) [10], as well as glucose-6-phosphate dehydrogenase (G6PD) [11], CD40 ligand (CD40L) [12–14], and NF-kappa B essential modulator (NEMO) [15] deficiencies. However, a striking contrast between MSMD patients and the other aforementioned PIDs is that the former group is vulnerable to only a narrow spectrum of pathogens such as non-tuberculous mycobacteria (e.g., BCG, *Salmonella* spp., and *Candida* spp.). Importantly, infections caused by MTC are not frequently reported in MSMD patients [5, 16], however, they are more commonly developed by patients with other PIDs.

The genetic dissection of MSMD has identified mutations in seven autosomal (*STAT1*, *IL-12p40*, *IFNGR1*, *IFNGR2*, *IL-12Rβ1*, *IRF8*, *ISG15*) and two X-linked (*NEMO* and *CYBB*) genes [17]. The products of these genes are directly or indirectly involved in the IFNγ-dependent immunity to mycobacteria. Among the MSMD-causing genes, the autosomal recessive *IL-12Rβ1* deficiency accounts for more than 50% of the cases [5] and the majority of them belong to consanguineous marriages [18]. Here, we expand the molecular spectrum that confers susceptibility to mycobacterial diseases by characterizing a novel nonsense mutation in the *IL-12Rβ1* gene in a patient with recurrent TB from a consanguineous Pakistani family.

Materials and methods

Case report

An HIV-negative 11-year-old Pakistani female born from a consanguineous marriage was admitted to the hospital with a complaint of severe coughing, weight loss, and fever. There were no other reports of TB in her family history. She had a history of cervical adenopathy due to BCG vaccination and tuberculosis (TB) at the age of 3 months and 8 years old, respectively. To fully recover from TB, 10 months of anti-tuberculous chemotherapy was required while the standard anti-tuberculous treatment regimen is usually 6 months [19]. Recombinant human IFNγ was not administered to the patient who developed a second episode of TB at 11 years of age. The diagnosis was confirmed by the presence of characteristic TB lesions in chest X-ray and by sputum smear microscopy, culture, and GeneXpert. The drug susceptibility confirmed that MTC was not resistant to anti-tuberculous drug.

Ethics approval

The patient and healthy controls (all from Pakistan; mean age, 20 years old) provided written consent to participate in the study, and their blood sample was collected according to the institutional guidelines for functional and molecular characterization. The study was approved by the ethics committee of Kohat University of Science and Technology, Kohat, Pakistan.

Quantification of IL-12 and IFNγ

The quantification of IL-12 and IFNγ in response to stimulation with BCG + IFNγ and BCG + IL-12 respectively was

analyzed by enzyme-linked immunosorbent assay (ELISA) method using IFNγ/IL-12 ELISA kits according to manufacturer's instructions (STEMCELL Technologies). The quantification of IL-12 and IFNγ and all the other functional assays described below ("Flow cytometric analysis of IL-12Rβ1 expression on T lymphocytes", "STAT4 phosphorylation", and "Genetic analysis" sections) carried out to characterize the defective IL-12/IFNγ axis were performed as previously described [20].

Flow cytometric analysis of IL-12Rβ1 expression on T lymphocytes

Flow cytometric analysis of IL-12Rβ1 surface expression was performed as previously described [21]. Briefly, pre-activated peripheral blood mononuclear cells (PBMCs) (8 μg/mL phytohemagglutinin [PHA] for 3 days) were stained with anti-IL-12Rβ1/CD212 antibody (Extracellular Domain, PE, LifeSpan BioSciences, Inc.) and anti-CD3 antibody before the cells were fixed with 2% paraformaldehyde. The cells were evaluated by flow cytometry on a BD FACS Canto II cytometer (BD Biosciences, San Jose, CA, USA). Cells were gated for lymphocytes according to size (forward scatter, FSC) and granularity (side scatter, SSC). The data obtained were analyzed with FlowJo software (TreeStar, Ashland, OR).

STAT4 phosphorylation

Evaluation of STAT4 phosphorylation was carried out in T lymphocytes pre-activated with PHA and stimulated with 10 ng/mL recombinant human IL-12 (rhIL-12), as previously described [20]. After fixation, permeabilization, and staining with anti-CD3 antibody (abcam) and anti-pY693 STAT4 antibody (BD Biosciences), the STAT4 phosphorylation was analyzed in T lymphocytes by flow cytometry.

Genetic analysis

DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) from EDTA blood. All exons of IL-12Rβ1 gene were amplified by polymerase chain reaction (PCR) using IL-12Rβ1 gene–specific primers (available upon request). The PCR products were subjected to Sanger sequencing for mutational analysis through the genetic analyzer ABI 3500 (Thermo Fisher Scientific, Waltham, MA).

In silico analyses

MutationTaster was used for the pathogenic prediction of the novel mutation, p.E67*. In addition, a comparative three-dimensional (3D) structure of wild-type and mutant IL-12Rβ1 was constructed based on the crystal structure of the full ectodomain of human gp130 (IL6Rβ, pdb id 3L5H) [22] and IL-12Rβ1 p.E67* mutant's 3D structure was compared with that of wild-type IL-12Rβ1 by using the program MODELLER 9v15 [23, 24]. The best model, which consists of all non-hydrogen main-chain, side-chain atoms, was selected based on PROCHECK and ProSa evaluation protocols [11, 25, 26]. Structures were visualized and analyzed using Accelrys DS Visualizer 2.0 (Accelrys, USA). Multiple sequence alignment was performed by ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/).

Statistical analysis

The data obtained were analyzed using GraphPad Prism 5 statistical software (GraphPad Software, San Diego, CA). Unpaired *T* test (Mann–Whitney test) was applied on controls and patient group. Five control individuals were compared with three replicates of the patient's data and $P < 0.05$ was considered significant.

Results

Decreased IFNγ production by the patient's PBMCs

The IL-12/IFNγ axis plays a vital role in the protection of mycobacteria, and therefore the patient was screened for defects in the IL-12/IFNγ axis [27, 28]. The PBMCs from the patient responded to BCG and/or IFNγ demonstrating normal production of IL-12 when compared with the healthy controls (Fig. 1a), ruling out intrinsic IFNγ signaling defects. In contrast, the PBMCs from the patient secreted significantly less IFNγ than those from healthy subjects (Fig. 1b), suggestive of impaired IL-12 signaling.

Defective IL-12Rβ1 expression and impaired STAT4 phosphorylation

The patient was screened for defects in the IL-12 signaling pathway by analyzing the expression of IL-12Rβ1 on the surface of the patient's T lymphocytes. In accordance with the impaired IFNγ secretion, the patient's lymphocytes displayed defective expression of the IL-12Rβ1, while it was normally detected on the control lymphocytes (Fig. 2a, b). As the binding of IL-12 to its receptors (IL-12Rβ1 and IL-12Rβ2) leads to the phosphorylation of STAT4 [29], we evaluated the STAT4 phosphorylation. The patient's T lymphocytes showed impaired STAT4 phosphorylation upon IL-12 stimulation as compared with the healthy control (Fig. 2c, d).

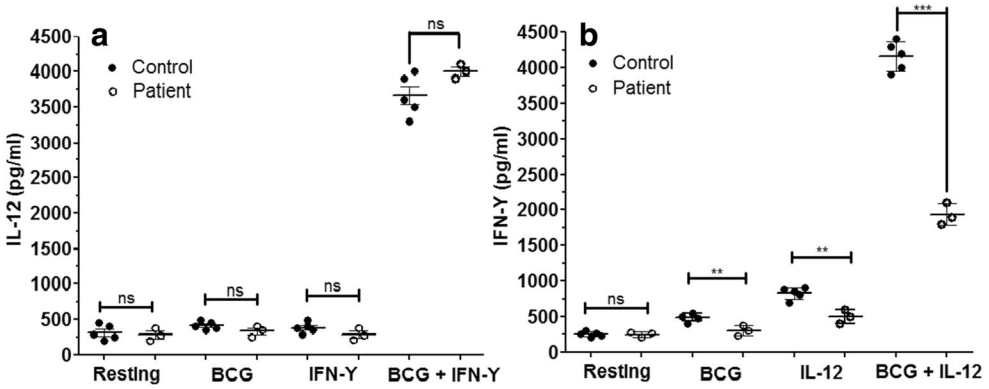

Fig. 1 Impaired IFNγ production by patient's PBMCs. **a** Normal IL-12 secretion by patient's PBMCs in response to BCG and/or IFNγ. **b** Reduced IFNγ synthesis by patient's PBMCs after stimulation with BCG and/or IL-12 when compared with 5 healthy controls. Each dot is a mean of an experiment performed in triplicate. Error bars denote mean with SD; $*p \leq 0.05$; *ns*, non-significant, ($n = 3$; Mann–Whitney test). All measurements were performed using ELISA

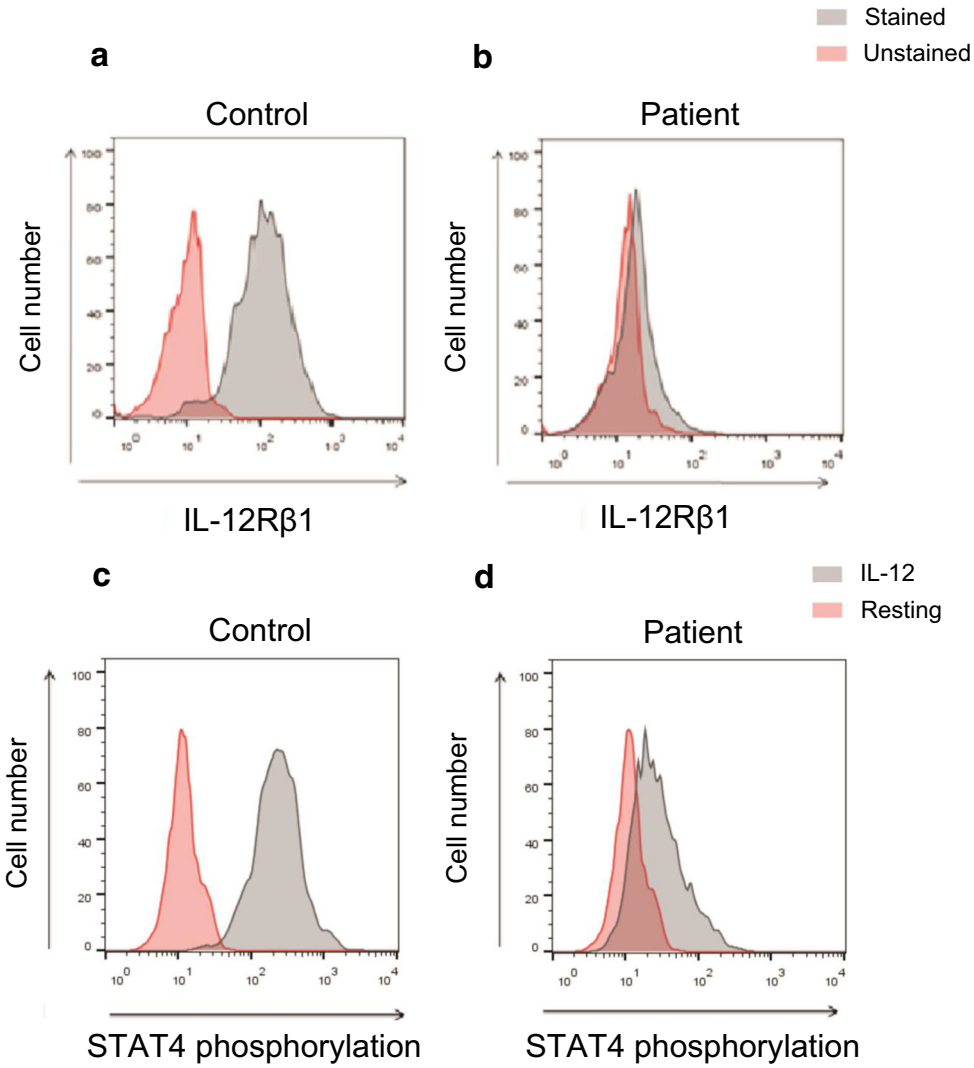

Fig. 2 Low IL-12Rβ1 expression and STAT4 phosphorylation by patient's T lymphocytes. Histograms compare the expression of IL-12Rβ1 on the surface of T lymphocytes from controls (**a**) and the patient (**b**). Histograms demonstrate induced STAT4 phosphorylation in T lymphocytes of healthy controls (**c**) while defective STAT4 phosphorylation in patient's (**d**) T lymphocytes. Both IL-12Rβ1 expression and STAT4 phosphorylation were assessed by flow cytometry using PHA-pre-activated PBMCs and the data was analyzed using the FlowJo software

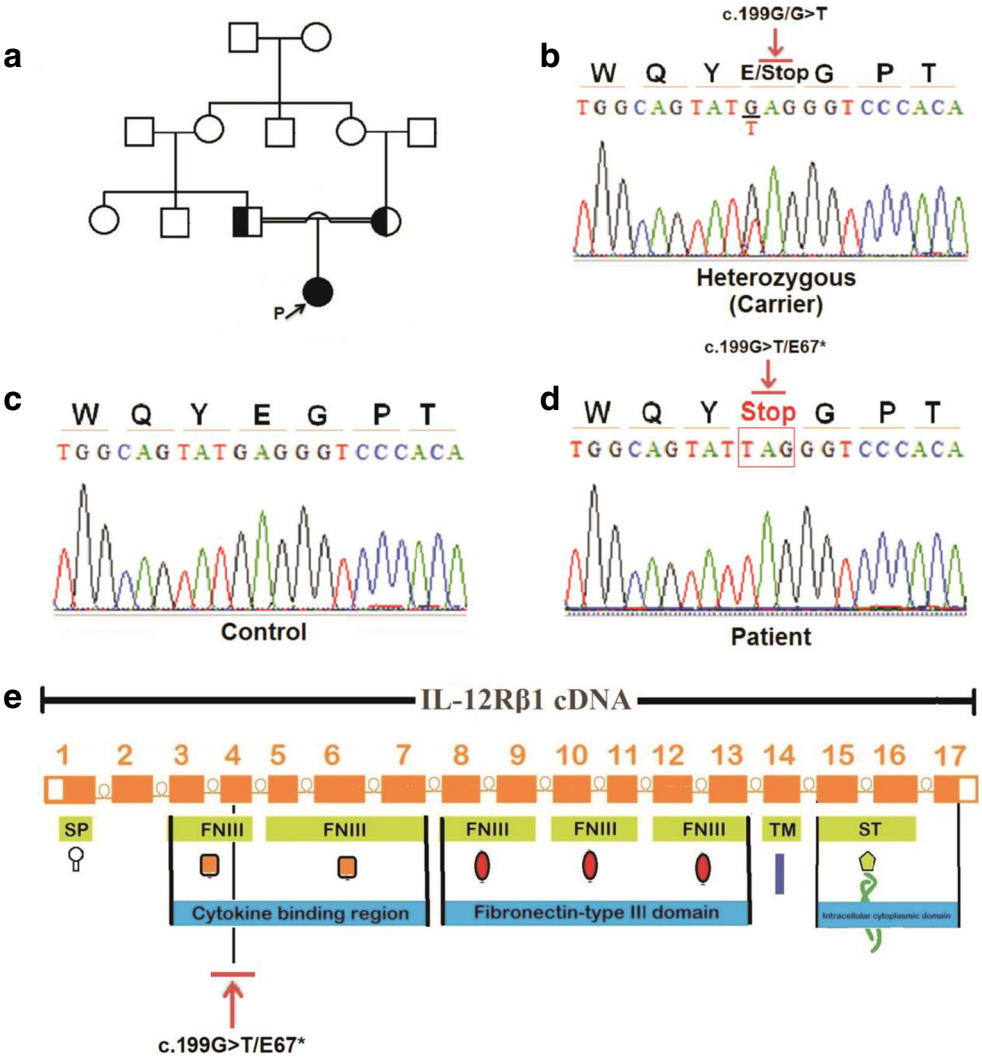

Fig. 3 Genetic analysis of *IL-12Rβ1* gene identifies a novel nonsense mutation. **a** The patient's pedigree is shown. Squares denote male; circles indicate female; the double line represents consanguineous marriage; and the filled circle shows the patient, while half-filled circle and square represent heterozygous (carrier). Chromatograms of the *IL-12Rβ1* gene showing heterozygous parents (**b**) harboring the c.199G/G>T mutation, control sequence (**c**), novel homozygous nonsense mutation (c.199G>T/p.E67*) (**d**). Exons encoding the IL-12Rβ1 protein were amplified by PCR and directly analyzed by Sanger sequencing (**e**). Schematic representation of the *IL-12Rβ1* gene shows where the c.199G>T/p.E67* mutation is located. *TM*, transmembrane; *ST*, signal transduction; *SP*, signal peptide; *FNIII*, fibronectin type-III domain

Identification of the novel nonsense mutation c.199G>T/p.E67*

Based on several indications (low production of IFNγ, lack of IL-12Rβ1 surface expression, and reduced STAT4 phosphorylation) and autosomal recessive IL-12Rβ1 deficiency (Fig. 3a), we genetically analyzed the *IL-12Rβ1* gene. This was of particular interest as this deficiency has previously been reported in children with severe TB [18] who were from consanguineous marriages [3, 30]. Sanger sequencing of *IL-12Rβ1* of our patient identified a novel nonsense mutation (c.199G>T/p.E67*) in exon 3, creating a stop codon (Fig. 3b–d). This mutation is located with the first fibronectin type-III (FNIII) repeat domain which forms the cytokine-binding region (CBR) of the *IL-12Rβ1* (Fig. 3e). The parents of the patient were heterozygous for the novel nonsense mutation c.199G>T/p.E67*.

In silico analysis predicts structural and functional impacts of the novel *IL-12Rβ1* disease-causing mutation

The novel nonsense mutation c.199G>T/p.E67* was not found in Ensemble, Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/), Human Gene Mutation Database (HGMD®, http://www.hgmd.cf.ac.uk/ac/index.php), 1000 Genomes Data (1000G, http://www.internationalgenome.org/data/), and online IL-12Rβ1 variation databases (www.LOVD.nl/IL12Rβ1). Therefore, suggesting the c.199G>T/p.E67* mutation is novel. The MutationTaster prediction tool indicated this mutation as disease causing (data not shown). Structurally, the human IL-12Rβ1 is composed of extracellular (further divided into five FNIII domains), transmembrane, and cytoplasmic domains and its functionality is attributed to these domains [3]. The 3D structure of the wild-type and mutant E67* IL-12Rβ1

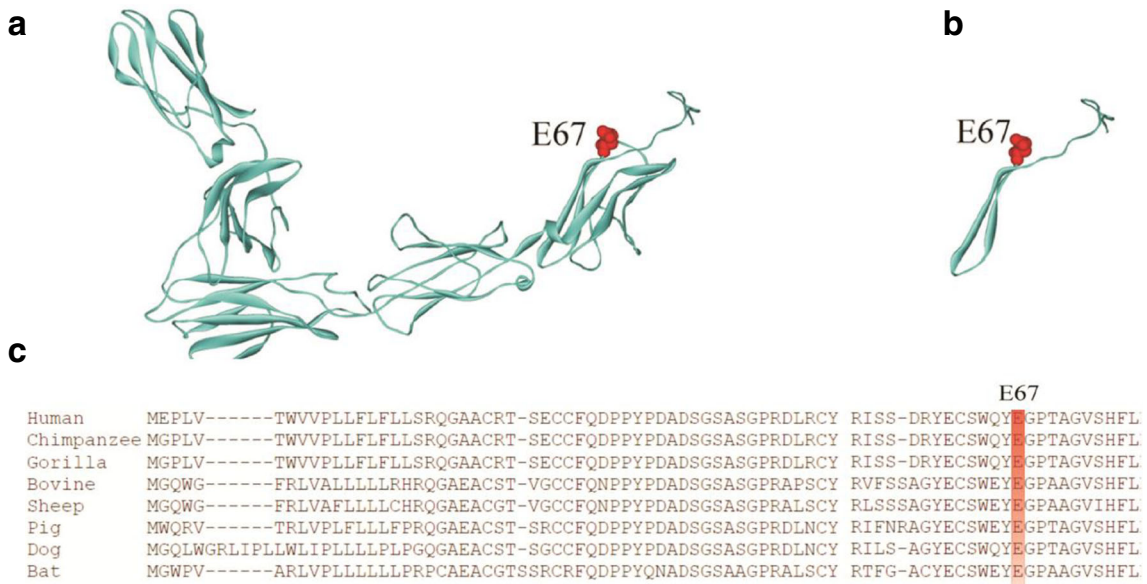

Fig. 4 In silico analyses of IL-12Rβ1 mutant. The structure of the wild type IL-12Rβ1 (**a**) and p.E67* mutant (**b**). The residue E67 is shown by the small red balls. **c** Multiple sequence alignments of the motif containing p. E67* mutation across different species

truncated proteins is shown in Figs. 4a and b, respectively. The mutant E67* IL-12Rβ1 truncated protein indicates a loss-of-function due to an abnormal IL-12Rβ1 structure. Furthermore, multiple sequence alignments showed that the c.199G>T/p.E67* is located in a highly conserved protein region across different species (Fig. 4c).

Discussion

We herein reported a Pakistani patient with IL-12Rβ1 deficiency that had a history of BCG and recurrent TB infections. Investigation of the IL-12/IFNγ axis demonstrated impaired production of IFNγ by patient's PBMCs, lack of IL-12Rβ1 surface expression, and reduced STAT4 phosphorylation upon IL-12 activation. Genetic evaluation revealed a novel nonsense mutation, c.199G>T/p.E67*, which affects a highly conserved IL-12Rβ1 sequence. These molecular and functional events associated with the nonsense mutation 199G>T/p.E67*, which affects highly conserved IL-12Rβ1 sequence thereby expanding the genetic spectrum of IL-12Rβ1 deficiency. Although we have not performed a site-directed mutagenesis study to prove that the mutation is of clinical significance, our data suggests that the absence of IL-12Rβ1 surface expression is due to the new stop codon created in the *IL-12RB1* gene of our patient. Considering the well-known role of IFNγ immunity in the control of mycobacterial infections [31, 32], our data indicated that the occurrence of recurrent mycobacterial infections developed by our Pakistani patient is, at least, partially attributed to the novel molecular alteration reported here. Since we did not perform whole-exome sequencing in our patient, we cannot exclude the existence of another PID simultaneously to the IL-12Rβ1 deficiency which could contribute to the susceptibility to mycobacterial infections.

Since the first patients with IL-12Rβ1 deficiency reported in 1998 [33, 34], approximately two hundred patients from different world regions have been characterized [3, 35]. IL-12Rβ1 deficiency is characterized by the susceptibility to poorly pathogenic non-tuberculous (environmental) mycobacteria or BCG as well as salmonellae [3]. Furthermore, other infections such as *Candida* and MTC are occasionally reported [35]. While both non-tuberculosis (BCG) and *M. tuberculosis* infections were presented by our patient, no events of candidiasis or salmonellosis were reported. Despite the high incidence of consanguineous marriages in Pakistan [36], this is the second Pakistani individual identified with IL-12Rβ1 deficiency to the best of our knowledge [35, 37]. This identifies a need to locally expand the availability of genetic analysis for Pakistani patients who developed mycobacterial infections. A recent study reported that two of fifty children with severe TB from Iran, Morocco, and Turkey [18] have IL-12Rβ1 deficiency. In light of this, we aim that our efforts to establish the genetic analysis of the IL-12/IFNγ axis can characterize more children with PID affected by TB infections in developing countries [10, 11, 13–15, 38–40]. Reaching this goal will considerably improve the mortality and morbidity associated with mycobacterial infections due to inborn errors of immunity at these geographic regions. For instance, this can be addressed by including recombinant human IFNγ in the treatment schedule of IL-12Rβ1-deficient patients [18, 41].

As we report here, most of the previously reported IL-12Rβ1-deficient patients belong to consanguineous families

[42], and family members who have only one affected allele usually do not develop clinical symptoms or any detectable immunological defect [3]. So far, approximately seventy pathogenic mutations have been identified across all exons of the *IL-12Rβ1* gene, except exons 16 and 17 [3]. A small mutation hotspot is suggested in exon 15 where three different mutations had been identified in more than sixty IL-12Rβ1-deficient patients [3]. While the 199G>T/p.E67* is located in exon 3 of the *IL-12Rβ1* gene, 5 different mutations in approximately 10 individuals have been identified so far. In this context, our Pakistani patient expands the genetic spectrum of IL-12Rβ1 deficiency, which has a high heterogeneity.

Moreover, most of the mutations in the *IL-12Rβ1* gene, including those affecting the CBR, which also contains the cytokine receptor signature (two Cys-Cys pairs and the [STGL]xWSxWS motif), are nonsense, frameshift, and splice site mutations. Consequently, they result in the complete lack of IL-12Rβ1 expression on the cell surface [3, 29], as the case of the novel mutation (c.199G>T/p.E67*) identified in our patient. In this context, the functional high-affinity IL-12R requires both β-type subunits (β1 and β2) for complete function. However, each subunit still exhibits a low affinity for IL-12 [43]. This fact might justify the residual production of IFNγ and STAT4 phosphorylation observed in our patient's PBMCs supernatant and T lymphocytes, respectively.

In conclusion, our data broadens the genetic spectrum of IL-12Rβ1 deficiency and reinforces that mutations in the *IL-12Rβ1* gene must be investigated in patients with BCG complications and/or severe TB. In addition, the consanguinity rate, as well as TB incidence, is high in Pakistan [2, 44], a country in which BCG vaccination is mandatory [45]. Therefore, it is urgent and of clinical importance to establish newborn screening programs in this region to improve the quality of life of families affected by PIDs associated with mycobacterial infections.

Compliance with ethical standards

The patient and healthy controls (all from Pakistan; mean age, 20 years old) provided written consent to participate in the study, and their blood sample was collected according to the institutional guidelines for functional and molecular characterization. The study was approved by the ethics committee of Kohat University of Science and Technology, Kohat, Pakistan.

Conflict of interest The authors declare that they have no conflict of interest.

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