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A novel missense mutation of the *CYLD* gene identified in a Hungarian family with Brooke–Spiegler syndrome

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Abstract: Brooke–Spiegler syndrome (BSS; OMIM 605041) is an autosomal dominant disease characterized by skin appendage tumors due to mutations in the cylindromatosis gene (*CYLD*). We investigated a Hungarian BSS pedigree with two affected members, father and daughter. Direct sequencing demonstrated a novel missense mutation (c.2613C>G; p.His871Gln) in exon 19 within the ubiquitin-specific protease domain of the encoded protein. We performed preliminary analysis to reveal the functional role of this novel mutation. Our data suggest that this novel *CYLD* mutation leads to increased ubiquitination of NEMO through influencing deubiquitinating activity of the CYLD protein and thus may result in enhanced NF-κB signalling.

Key words: appendage tumor, Brooke–Spiegler syndrome, *CYLD* gene, NEMO protein, ubiquitination

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Background

Brooke–Spiegler syndrome (BSS; OMIM 605041) has been described as an autosomal dominant disease characterized by the development of a wide variety of skin appendage tumors, such as cylindromas, trichoepitheliomas and spiradenomas (1,2).

The gene responsible for BSS, the cylindromatosis gene *(CYLD),* is localized at 16q12-q13 (2). So far 86 mutations have been identified, mainly at the 3′ end of the *CYLD* gene (exons 8–20; 3–9). These mutations have been identified in patients with phenotypic features of either BSS, familial cylindromatosis (OMIM 132700) or multiple familial trichoepithelioma type 1 (OMIM 601606; 10).

The *CYLD* gene codes for a cysteine protease type deubiquitinase, which directly interacts with and deubiqutinates TRAF2, TRAF6 (TNF receptor-associated factors) and NEMO (IKBKG; an inhibitor of the kappa light polypeptide gene enhancer in B cells, kinase gamma; 11–13). Thus, CYLD attenuates TNF-α-induced classical NF-κB signalling leading to programmed cell death (14,15). Reduced CYLD activity results in an elevated activity of NF-κB and hence increased resistance to apoptosis and carcinogenesis (14,15). Through the deubiquitination of the dishevelled protein, CYLD can also influence WNT/β-catenin signalling (16).

Questions addressed

This study reports a novel missense mutation on the *CYLD* gene in a Hungarian family with BSS, which by influencing the deubiquitination activity of CYLD leads to increased ubiquitination of NEMO.

Experimental design

A pedigree from the Southern part of Hungary (Szeged region) affected by BSS was investigated. The pedigree has two affected and five unaffected individuals spanning two generations. One of the affected individuals, a 60-year-old man (I-1), has numerous soft, hairless, skin-coloured papules around his nose, in his ears, on his scalp and on his shoulders (Fig. 1a–d). Some of the tumors in the head and neck region were surgically removed. His daughter (II-2), a 35-year-old woman, presented with milder symptoms. Routine histological examination of the excised tumors suggested the diagnosis of the BSS. Internal cancer, parotoid tumors or other malignancies were not present in the history of these patients. Written informed consents were obtained from the enrolled patients and controls according to a protocol approved by the Local Ethics Committee in adherence to the Helsinki guidelines.

Materials and methods can be found in the Supporting information.

Results and discussion

Mutation analysis with direct sequencing of the coding region of the *CYLD* gene revealed a novel missense mutation (c.2613C>G p.His871Gln), located in exon 19 in heterozygous form in both affected patients (Fig. 1e). This mutation could not be identified in any of the clinically unaffected family members or in the screened 95 Hungarian generally healthy controls (Fig. 1f). The mutation is situated in a highly conserved region of the *CYLD* gene (Fig. 2b), encoding a histidine-box structure, which is part of a larger structure forming the ubiquitin-specific protease (USP) domain. This novel mutation leads to a change from histidine to glutamine at position 871. Interestingly, this position in the amino acid sequence change is localized within an active site where phosphorylation takes place (www.uniprot.org; Accession No: Q9NQC7), suggesting a putative role for this amino acid change in the function of the USP domain. To note, another mutation at the same amino acid position (c.2611C>A p.His871Asn) induced by *in vitro* mutagenesis has been functionally characterized: the functional data indicated impairment in the deubiquitinase

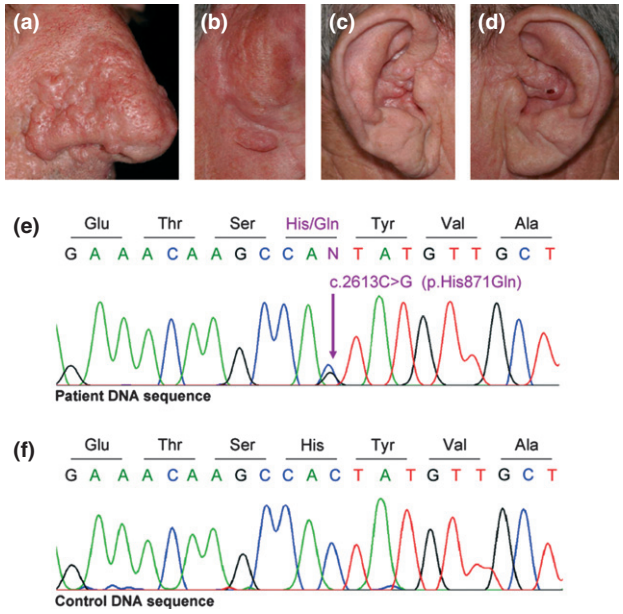

Figure 1. (a) Numerous skin-coloured, dome-shaped tumors are present around the nose, (b) in the retroauricular region (c, d) and in the ears of patient z-1. (e) The DNA sequence of patient I-1 shows a heterozygous single nucleotide substitution c.2613C>G, which converts codon 871 CAC for histidine to CAG for glutamine; this mutation is designated p.His871Gln. The same mutation was found in patient II-2. (f) Wild-type sequence of genomic DNA spanning codons 868–874 of the *CYLD* gene.

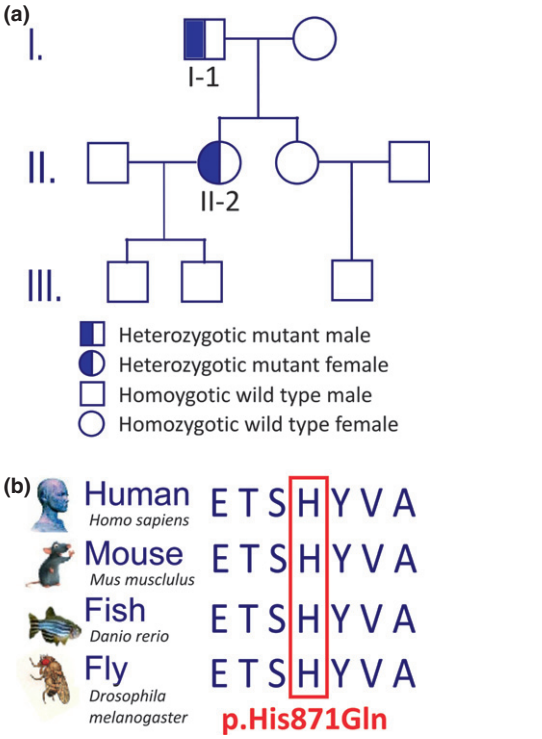

Figure 2. (a) The investigated Hungarian pedigree has two clinically affected individuals, who were heterozygous for the c.2613C>G p.His871Gln mutation; all the other family members were clinically unaffected and carried only wild-type alleles. (b) Comparison of the sequences of the *CYLD* gene among different species demonstrated that the c.2613C>G p.His871 mutation is located in a highly conserved region, which forms part of a histidine box.

activity of CYLD protein *in vitro*, demonstrated by its reduced enzymatic activity (14).

To reveal the function of this novel mutation, we studied the known CYLD-regulated pathways. As CYLD is known to directly interact with the NF-κB signalling NEMO protein, the expression of NEMO was determined and found to be significantly reduced in the $CD4^+$ T lymphocytes of the patients compared to healthy controls (data not shown). Our preliminary data suggest that decreased NEMO expression is associated with its altered deubiquitination, NEMO immunoprecipitated from fibroblasts carrying the CYLD mutation demonstrated significantly higher ubiquitination than NEMO immunprecipitated from control fibroblasts (Figure S1).

As it is known that CYLD protein is expressed in nearly every human tissue with the highest expression level in the $CD4^+$ and $CD8^+$ lymphocytes and CYLD deficiency can lead to defects in T-cell maturation and B-cell responses (17–25), however, others reported on normal T- or B-cell phenotype (24,26,27). We characterized peripheral blood lymphocytes in patients with BSS, but only minor alterations were observed (data not shown).

We also investigated whether the newly identified mutation affects the WNT/β-catenin pathway as it has been recently suggested as one of the functions of CYLD protein (16). For that, we performed immunohistochemical detection of the translocation of β-catenin in the tissue samples of patient I-1, but found no alteration in the intracellular distribution of β-catenin (data not shown).

We identified here a novel mutation (c.2613C>G p.His871Gln) of *CYLD* in a Hungarian BSS pedigree. Identification of the underlying mutation may have a considerable impact on family planning because it offers the possibility of prenatal mutation screening. Our functional studies revealed a decreased NEMO protein level in the $CD4^+$ T lymphocytes of the patients compared to healthy controls. As in the literature, there is evidence that CYLD can decrease the ubiquitination of NEMO, but there are no data suggesting that CYLD can influence the expression level of the NEMO protein, we have measured its ubiquitination after immunoprecipitation and found an increased level of ubiquitination of the NEMO protein in fibroblasts carrying the novel CYLD mutation. However, it is well known that CYLD removes only the Lys63-linked polyubiquitin chain taking part in the proteosomal-independent cellular processes (11), and it has been recently reported that 26S proteasome can degrade Lys63-linked ubiquitin substrates (27) raising the possibility that the Lys63-linked ones can also serve as a targeting signal for proteaseomal degradation.

Based on our results, we suppose that this novel mutation through the increased ubiquitination of NEMO leads to decreased NEMO expression and as a consequence may influence the NF-κB pathway. Further studies are needed to elucidate the exact mechanism of the development of BSS symptoms. As BSS displays only skin tumors, and mainly appendageal tumor formations, it may be an appropriate model for the development of novel gene therapy methods.

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Author contributions

NN, KF, ÁK, IBN performed the research; NN, MS designed the research study; ÁK, EK, JV contributed essential reagents and tools; NN, ZB-C, LK, MS analysed the data; NN, MS wrote the paper.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. NEMO was immunoprecipitated from fibroblasts of BSS patients ($n = 2$) and from fibroblasts of healthy controls ($n = 2$).

Data S1. Experimental design.

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Letter to the Editor

Attenuation of contact hypersensitivity by cell-permeable heat shock protein 70 in BALB/c mouse model

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Abstract: In contact hypersensitivity (CHS), multiple cells, inflammatory mediators and cytokines are known to be involved in the regulation of the immune response. Previously, we revealed the reactive oxygen species generation by 2, 4, 6-trinitrobenzene sulphonic acid (TNBS) *in vivo*, followed by heat shock protein 70 (Hsp70) carbonylation and the exogenous antioxidant role of cell-permeable Hsp70. Here, we demonstrate the role of Hsp70 using cell-permeable Hsp70 in the mouse CHS model. Pretreatment of cell-permeable Hsp70: (i) suppressed ear swelling; (ii) down-regulated phosphorylated p38, but up-regulated phosphorylated extracellular signal-regulated kinase; (iii) increased population of $CD4^+CD25^+Foxp3^+$ T cells; (iv) decreased secretion of tumor necrosis factor-α (TNF-α), IL-12, interferon-γ and IL-2 and (v) but up-regulated IL-4 and transforming growth factor beta (TGF-β) in the lymph nodes. In conclusion, cell-permeable Hsp70 attenuates CHS through modulation of MAPK pathway and regulation of Th1, Th2 and regulatory T cells.

Key words: allergic contact dermatitis, heat shock protein70, inflammatory cytokines, protein transduction domain

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Background

Allergic contact dermatitis (ACD) is related to the development of contact hypersensitivity (CHS). ACD is divided into a sensitization phase and a challenge phase (1). Recently, a few reports have revealed the generation of reactive oxygen species (ROS) in CHS by dendritic cells (DCs) (2) and keratinocytes (3). Previously, the ROS generation by 2, 4, 6-trinitrobenzene sulphonic acid (TNBS) in mouse DCs, XS-106 DCs followed by ATP synthase carbonylation was demonstrated (4). Furthermore, we verified the carbonylation of heat shock protein 70 (Hsp70) and an antioxidant effect