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Mutations of the CD40 ligand gene in 13 Japanese patients with X-linked hyper-IgM syndrome

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Abstract X-linked hyper-IgM syndrome (XHIM) is a rare primary immunodeficiency caused by a defective CD40 ligand. We identified mutations of the CD40 ligand gene in 13 unrelated Japanese XHIM patients. Of the four patients with missense mutations, one had a mutation within the transmembrane domain, and the three others had mutations affecting the TNF homology region of the extracellular domain. Two of the missense mutations resulted in the substitution of amino acids that are highly conserved in TNF family proteins. Three patients had nonsense mutations, all of which resulted in the truncation of the TNF homology domain of the CD40 ligand. Three patients had genomic DNA deletions of 2, 3 or 4 nucleotides, respectively. All of the deletions were flanked by direct repeat sequences, suggesting that these deletions were caused by slipped mispairing. Three patients had mutations within introns resulting in altered splicing, and multiple splicing products were found in one patient. Thus, each of the 13 Japanese patients had different mutations, 9 of them being novel mutations. These results indicate that mutations in XHIM are highly heterogeneous, although codon 140 seems to be a hot spot of the CD40 ligand gene since two additional point mutations were located at Trp 140, bringing the total numbers of mutations affecting codon 140 to six. In one XHIM family with a missense mutation, prenatal diagnosis was performed by single-strand conformation polymorphism analysis of genomic DNA of a male fetus.

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Introduction

X-linked hyper-IgM syndrome (XHIM) is a rare genetic disorder characterized by markedly decreased serum levels of IgG and IgA, elevated or normal levels of IgM and defective isotype switching. The gene responsible for XHIM encodes CD40 ligand, which is expressed by activated T cells and important for T/B interaction, B-cell activation, class switching and a normal antibody response to T-dependent antigens (Allen et al. 1993; Aruffo et al. 1993; DiSanto et al. 1993; Korthäuer et al. 1993). Mutations of the CD40 ligand gene have been reported in approximately 40 XHIM patients (Allen et al. 1993; Aruffo et al. 1993; Disanto et al. 1993; Korthäuer et al. 1993; Ramesh et al. 1993; Villa et al. 1994a, b; Macchi et al. 1995; Lin et al. 1996), and include missense and nonsense mutations, deletions, insertions and splicing defects. Finding additional mutations may identify mutational hot spots and regions critical for the function of CD40 ligand protein. Here we report mutations of the CD40 ligand gene in 13 unrelated Japanese XHIM patients. In one family, prenatal diagnosis of XHIM using single-strand conformation polymorphism (SSCP) analysis was successfully performed.

Materials and methods

Thirteen Japanese XHIM patients from 13 unrelated families were included in this study. They had low serum IgG and IgA concentrations and high or normal levels of serum IgM. Neutropenia was observed in ten patients (KO, TY, IN, KA, TA, MR, FK, US, KS and KK). Activated lymphocytes from all the patients failed to bind a CD40-Ig construct. Patients KS and FK have been reported elsewhere (Iseki et al. 1994; Iwata et al. 1995).

Purification of total RNA and synthesis of first-strand cDNA was performed as previously described (Shimadzu et al. 1995). cDNA was amplified by the polymerase chain reaction (PCR) using primers that permitted the amplification of the entire peptide-coding sequence of the CD40 ligand gene (Aruffo et al. 1993). PCR products were subsequently subcloned into pT7Blue T vector, and at least three clones were sequenced by the dideoxy chain-termination method with a Model 373A Automated DNA Sequencer (Perkin Elmer Japan Applied Biosystem Division). In some patients, genomic DNA was purified from peripheral blood lymphocytes, and exons of the CD40 ligand gene and flanking intron sequences were amplified by PCR using exon-specific primers (Shimadzu et al. 1995). PCR products were then subcloned and sequenced. SSCP analysis of the CD40 ligand gene was performed as previously reported (Shimadzu et al. 1995).

Table 1 Mutations of the CD40 ligand gene in 13 Japanese patients. (*del* deletion, *ins* insertion, *nt* nucleotide, *n.a.* not appilcable)

Family	cDNA mutation (nucleotide no.)	Amino acid change (codon no.)	Genomic DNA	Exon	Domain
MR	T128G	Met36Arg	n.a.	1	TM
KO	G441T	Trp140Cys	n.a.	5	EC
TY	T713C	Leu231Ser	n.a.	5	EC
IN	C782T	Thr254Met	C→T	5	EC
KS	G440A	Trp140stop	n.a.	5	EC
KK	C572A	Ser184stop	n.a.	5	EC
SS	C675A	Cys218stop	C→A	5	EC
SU	del 178-181 (ATAG)		4-nt deletion (ATAG)	2	EC
FK	del 657-658 (CA)		2-nt deletion (CA)	5	EC
US	del 699-701 (GGA)	Gly227 deletion	3-nt deletion (GGA)	5	EC
TA	del 178-309 (exon 2 skipping) ins 19 nt		g→a at +1 base of 5′ splice junction (intron 2)	2	EC
RA	del 368-430 (exon 4 skipping)		g→a at +1 base of 5′ splice junction (intron 4)	4	EC
KA	del 431-438		1-nt deletion at –1 base of 3′ splice junction (intron 4)	5	EC

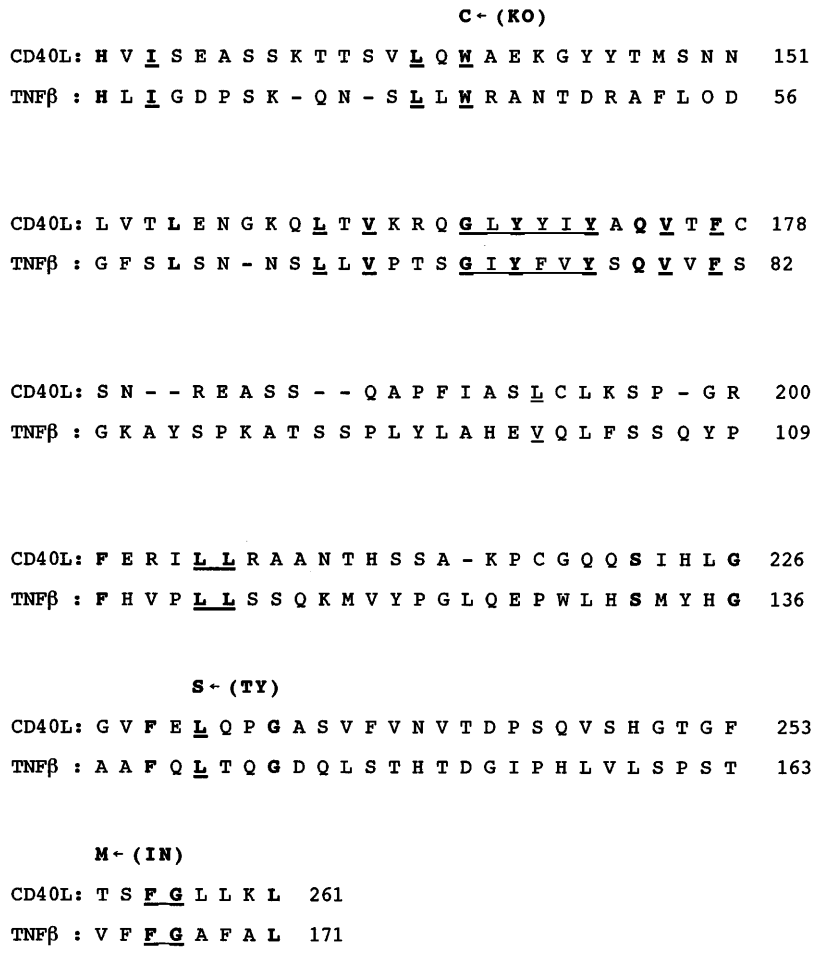

Fig. 1 Missense mutations observed in the X-linked hyper-IgM (XHIM) syndrome patients. Structure-based sequence alignment of extracellular domain of CD40 ligand and TNFβ is shown (Bajorath et al. 1996). Conserved residues are shown in *bold* and residues most conserved across the TNF family (Smith et al. 1994) are *underlined*. Amino acid substitutions found in the CD40 ligand obtained from KO, TY, and IN are indicated above the wild-type protein sequence

Results and discussion

Four different categories of mutations were detected in 13 unrelated Japanese XHIM patients: missense mutations, nonsense mutations, genomic deletions and splicing mutations. Results of the mutational analysis are summarized in Table 1.

Four patients (MR, KO, TY and IN) were found to have missense mutations. In patient MR, the mutation (M36R) affected the transmembrane domain. The same mutation within the transmembrane domain has been reported previously in an unrelated XHIM patient from Europe (Korthäuer et al. 1993); this mutation is considered to introduce a positive charge into the transmembrane domain and to abrogate cell-surface expression of CD40 ligand. The three other point mutations were located within the extracellular domain and affected the TNF homology region. Two of these mutations (W140C, L231S) affect residues that are most conserved across the TNF family (Fig. 1) (Bajorath et al. 1995). The three missense mutations of the extracellular domain are considered to be important for the packing of the hydrophobic core regions of CD40 ligand (Bajorath et al. 1996).

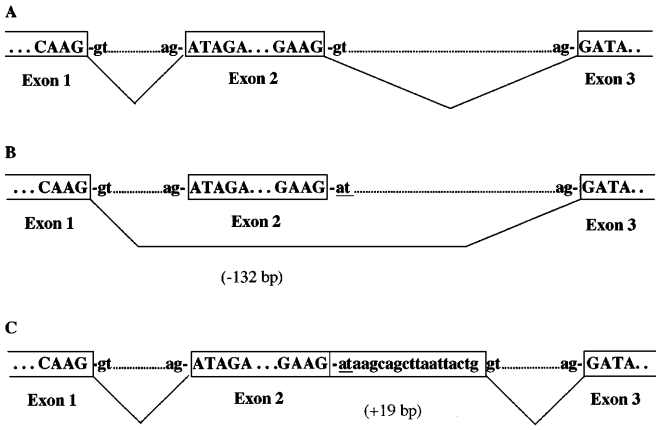

Fig. 2A–C Abnormal splicings of the CD40 ligand gene observed in an XHIM patient (TA). **A** Normal splicing of the CD40 ligand gene, **B** exon 2 skipping, and **C** insertion of 19 nucleotides observed in this patient. Exons are *boxed* and *numbered*. Introns are represented by *dotted lines*. *Boldface uppercase letters* indicate normal nucleotide sequence at exon boundaries. The number of nucleotides deleted or inserted in the mature transcripts is in *parentheses*. Point mutation abolishing a normal splice site is *underlined*

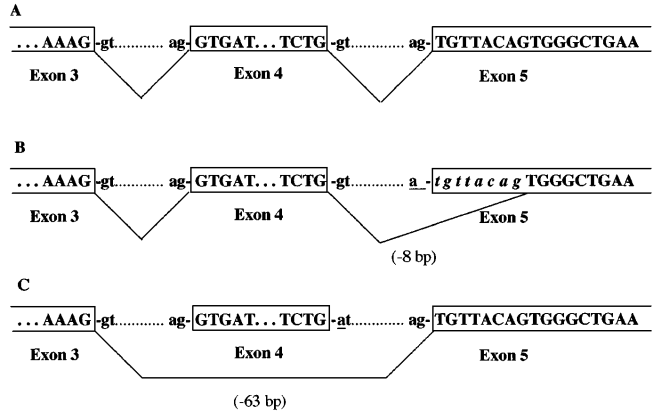

Fig. 3A–C Abnormal splicings of the CD40 ligand gene observed in two XHIM patients (KA, RA). **A** Normal splicing of the CD40 ligand gene, **B** 8-nucleotide deletion observed in patient KA caused by the g deletion at base –1 of the 3′ splice junction of intron 4, and **C** exon 4 skipping observed in patient RA caused by g to a transition at 5′ splice junction of intron 4. Exons are *boxed* and *numbered*. Introns are represented by *dotted lines*. *Boldface uppercase letters* indicate normal nucleotide sequence at exon boundaries. The number of nucleotides deleted or inserted in the mature transcripts is in *parentheses*. Point mutation abolishing normal splice site is *underlined*

Three patients were found to have nonsense mutations, at codon 140 (patient KS), codon 184 (patient KK) and codon 218 (patient SS). These mutations cause the truncation of the extracellular domain of CD40 ligand, and are expected to result in large deletions of the TNF homology region.

In three patients, deletions of 2 (patient FK), 3 (patient US) or 4 (patient SU) nucleotides were detected in CD40 ligand cDNA. The deletions were confirmed by analysis of genomic DNA. All three deletions were flanked by direct repeat sequences, i.e., direct repeat of CA (FK), GGA (US) and ATAG (SU), suggesting that these mutations were caused by slipped mispairing mechanisms. The deletions observed in FK and SU result in frameshifts and are expected to produce truncated CD40 ligand proteins.

Three patients (TA, KA, and RA) were found to have mutations within introns, resulting in alternative splicing. Patient TA had a single point mutation at a donor splice site (position +1) of intron 2. In this patient, two different sizes of cDNA were detected by reverse-transcription-PCR. cDNA sequencing demonstrated skipping of the entire exon 2 or the insertion of 19 nucleotides by utilizing a cryptic splice site (Fig. 2). Patient KA had a 1-nucleotide deletion at a splice acceptor site of intron 4. This generated the use of a cryptic splice site resulting in the deletion of 8 nucleotides from the cDNA (Fig. 3B). Patient RA had a g→a transition at the 5′ splice junction of intron 4, resulting in the skipping of exon 4 (Fig. 3C).

To date, approximately 40 unique mutations of the CD40 ligand gene have been reported, demonstrating the heterogeneous nature of the mutations in XHIM. Analysis of 13 unrelated Japanese XHIM patients confirms this trend: each of the 13 boys had a different mutation, 9 being as yet unreported novel mutations. As reported from the USA and Europe, only 1 of the Japanese patients had a missense mutation affecting the transmembrane domain. The other 3 missense mutations were located within a TNF homology region of the extracellular domain of he CD40 ligand, suggesting the functional importance of this region. Two of the point mutations described affected codon 140; one patient (KS) had the same mutation as previously described (TGG→TAG) (Korthäuer et al. 1993); the other patient (KO) had a novel mutation (TGG→TGT). This brings the total number of mutations affecting codon 140 to 6, further con-

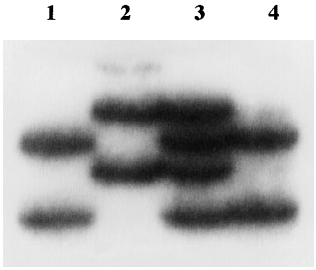

Fig. 4 Single-strand conformation polymorphism (SSCP) analysis of exon 5 of the CD40 ligand gene from an XHIM patient, his carrier mother and the fetus. Exon 5 of the CD40 ligand gene was analyzed by SSCP. *Lane 1* healthy control, *lane 2* XHIM patient (SS), *lane 3* his carrier mother, *lane 4* the fetus

firming that codon 140 is a hot spot for CD40 ligand gene mutation.

Using SSCP analysis, we were able to perform prenatal diagnosis in the pregnant mother of patient SS. SS had a C to A transversion at codon 218 in exon 5. Genomic DNA of the fetus isolated from amnionic cells was analyzed by SSCP for exon 5. As demonstrated in Fig. 4, the fetal DNA showed the same SSCP pattern as the normal control, while the patient's DNA showed a clearly different pattern. The mother had both normal and abnormal bands, indicating carrier status. Analysis of the SRY and amelogenin genes predicted that the fetus was male (data not shown). The fetus was delivered as a healthy boy, confirming the results of SSCP analysis.

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References

Allen RC, Armitage RJ, Conley ME, Rosenblatt H, Jenkins NA, Copeland NG, Bedell MA, Edelhoff S, Disteche CM, Simoneaux DK (1993) CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. Science 259:990–993

Aruffo A, Farrington M, Hollenbaugh D, Li X, Milatovich A, Nonoyama S, Bajorath J, Grosmaire LS, Stenkamp R, Neubauer M, Roberts RL, Noelle RJ, Ledbetter JA, Francke U, Ochs HD (1993) The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. Cell 72:291–300

Bajorath J, Marken JS, Jan Chalupny N, Spoon TL, Siadak AW, Gordon M, Noelle RJ, Hollenbaugh D, Aruffo A (1995) Analysis of gp39/CD40 interactions using molecular models and site-directed mutagenesis. Biochemistry 34:9884–9892

Bajorath J, Seyama K, Nonoyama S, Ochs HD, Aruffo A (1996) Classification of mutations in the human CD40 ligand, gp39, which are associated with X-linked hyper IgM syndrome. Protein Sci 5:531–534

DiSanto JP, Bonnefoy JY, Gauchat JF, Fischer A, de Saint Basile G (1993) CD40 ligand mutations in X-linked immunodeficiency with hyper-IgM. Nature 361:541–543

Iseki M, Anzo M, Yamashita N, Matsuo N (1994) Hyper-IgM immunodeficiency with disseminated cryptococcosis. Acta Paediatr 83:780–782

Iwata M, Nunoi H, Nonoyama S, Shimadzu M, Higuchi S, Yanabe Y, Migita M, Adachi N, Matsuda I (1995) Neutropenia in patient with X-linked hyper-IgM syndrome. Jpn J Clin Hematol 36:1223–1229

Korthäuer U, Graf D, Mages HW, Briere F, Padayachee M, Malcolm S, Ugazio AG, Notarangelo LD, Levinsky RJ, Kroczek RA (1993) Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. Nature 361:539–541

Lin Q, Rohrer J, Allen RC, Larché M, Greebe JM, Shigeoka AO, Gatti RA, Derauf DC, Belmont JW, Conley ME (1996) A single strand conformation polymorphism study of CD40 ligand: efficient mutation analysis and carrier detection for X-linked hyper IgM syndrome. J Clin Invest 97:196–201

Macchi P, Villa A, Strina D, Sacco MG, Morali F, Brugnoni D, Giliani S, Mantuano E, Fasth A, Andersson B (1995) Characterization of nine novel mutations in the CD40 ligand gene in patients with X-linked hyper IgM syndrome of various ancestry. Am J Hum Genet 56:898–906

Ramesh N, Fuleihan R, Ramesh V, Lederman S, Yellin MJ, Sharma S, Chess L, Rosen FS, Geha RS (1993) Deletions in the ligand for CD40 in X-linked immunoglobulin deficiency with normal or elevated IgM (HIGMX-1). Int Immunol 5:769–773

Shimadzu M, Nunoi H, Terasaki H, Ninomiya R, Iwata M, Kanegasaka S, Matsuda I (1995) Structural organization of the gene for CD40 ligand: molecular analysis for diagnosis of X-linked hyper-IgM syndrome. Biochim Biophys Acta 1260:67–72

Smith CA, Farrah T, Goodwin RG (1994) The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell 76:959–962

Villa A, Notarangelo LD, Di Santo JP, Macchi PP, Strina D, Frattini A, Lucchini F, Patrosso CM, Guliani S, Mantuano E, Agosti S, Nocera G, Kroczek RA, Fischer A, Ugazio AG, de Saint Basile G (1994a) Organization of the human CD40L gene; implications for molecular defects in X chromosome-linked hyper-IgM syndrome and prenatal diagnosis. Proc Natl Acad Sci USA 91:2110–2114

Villa A, Strina D, Macchi P, Patrosso MC, Vezzoni P, Tovo PA, Giliani S, Ugazio AG, Notarangelo LD (1994b) C to T mutation causing premature termination of CD40 ligand at amino acid 221 in a patient affected by hyper IgM syndrome. Hum Mutat 3:73–75