

## X-linked Hyper-IgM Syndrome Associated with *Cryptosporidium parvum* and *Cryptococcus neoformans* Infections: the First Case with Molecular Diagnosis in Korea

X-linked hyper-IgM syndrome (XHIM) is a rare primary immunodeficiency disorder, caused by mutations of the gene encoding CD40 ligand (CD40L; CD154). We report the clinical manifestations and mutational analysis of the *CD40L* gene observed in a male patient from a XHIM family. Having hypogammaglobulinemia and elevated IgM, the 3-yr-old boy exhibited the characteristic clinical features of XHIM. The patient suffered from frequent respiratory infections, and chronic enteritis caused by *Cryptosporidium parvum*. In addition, a lymph node biopsy and a culture from this sample revealed *C. neoformans* infection. Activated lymphocytes from the patient failed to express CD40L on their surface as assessed by flow cytometry and a missense mutation (W140R) was found at the XHIM hotspot in his *CD40L* cDNA to confirm the diagnosis. Genetic analysis of the mother and sister showed a heterozygote pattern, indicating carrier status. To our knowledge, this is the first report on the molecular diagnosis of an XHIM patient in Korea.

**Key Words :** CD40 Ligand; Mutation; Immunoglobulin M; Flow Cytometry; *Cryptosporidium parvum*; *Cryptococcus neoformans*

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## INTRODUCTION

X-linked hyper-IgM syndrome (XHIM) is a primary immunodeficiency syndrome, characterized by recurrent infections, hypogammaglobulinemia, and normal or elevated serum levels of IgM (1, 2). Most patients with the syndrome suffer from infections by opportunistic pathogens, such as *Cryptosporidium* and *Pneumocystis carinii* (2, 3). XHIM results from mutations in the gene encoding for CD40 ligand (CD40L) (4, 5).

The CD40L is a membrane-bound protein found on activated CD4<sup>+</sup> T lymphocytes (2), and plays critical roles in the generation of memory B cells and the formation of germinal centers (6). The CD40L defect in XHIM prevents B cells from undergoing isotype switching and explains the presence of IgM in the absence of other immunoglobulin classes (1). To date, about one hundred mutations in the *CD40L* gene have been described worldwide (7-9). However, little is known about Korean patients with XHIM in respect to the clinical

and immunologic characteristics, or the genetic polymorphism. Here, we present the first mutation identified in a Korean patient with XHIM along with the clinical and immunologic manifestations.

## CASE REPORT

A 3-yr-old boy visited Chonbuk University Hospital in 1991 due to recurrent upper respiratory infections, diarrhea, and fever over the previous 2 yr. The patient had a history of strong positive tuberculin skin test and was treated with isoniazid for 12 months. On physical examination, gross hepatosplenomegaly was evident and a complete blood count showed lymphocytosis, eosinophilia, neutropenia, and mild anemia. Peripheral blood mononuclear cells (PBMC) were obtained from the patient, and phenotypic expression of immune cells was evaluated by using fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies: CD3

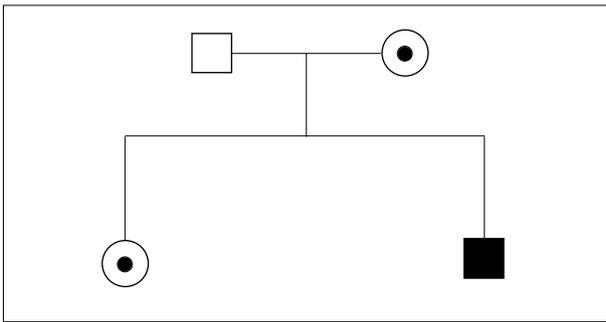


Fig. 1. Pedigree of the patient described in this study. Squares represent males and circles denote females. The shaded symbol represents patient with XHIM. Dot in the circle indicates that the individual is a carrier.

(85.9%); CD4 (60.0%); CD8 (16.1%); CD19 (13.6%); CD 16/56 (4.5%). Immunologic examinations revealed clinical XHIM, with a decreased serum IgG (291.0 mg/dL; reference, 700-1,600 mg/dL) and increased IgM (1,220.0 mg/dL; reference, 40-230 mg/dL). The serum IgA (117.0 mg/dL) and IgE (29.8 IU/mL) were within normal ranges. The patient had chronic neutropenia (lowest count, 220/mL). The clinical diagnosis was compatible with hyper-IgM syndrome and intravenous immunoglobulin (IVIg) therapy was started.

Subsequently, he had several episodes of bronchitis and chronic diarrhea. His fecal sample was processed using a quantitative centrifugation concentration flotation technique to identify *Cryptosporidium parvum*, which was enumerated using bright field and phase contrast microscopy. The *C.*

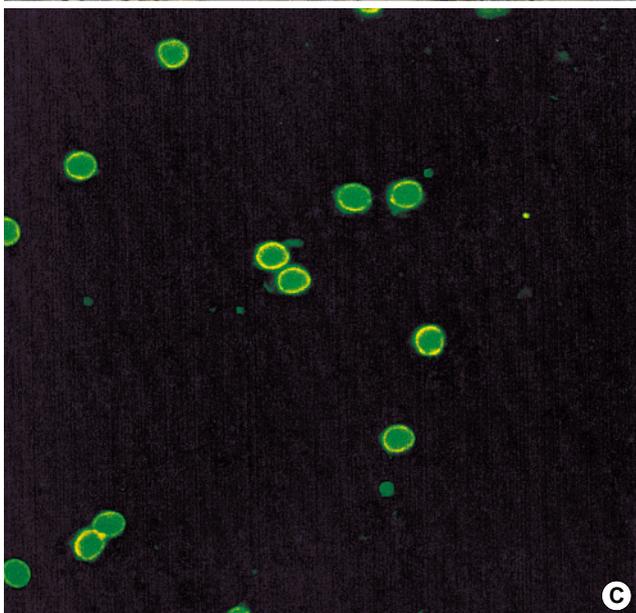
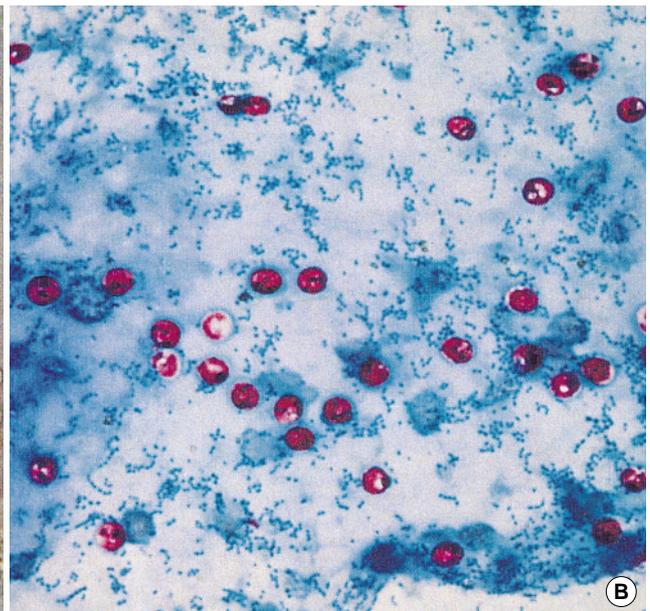
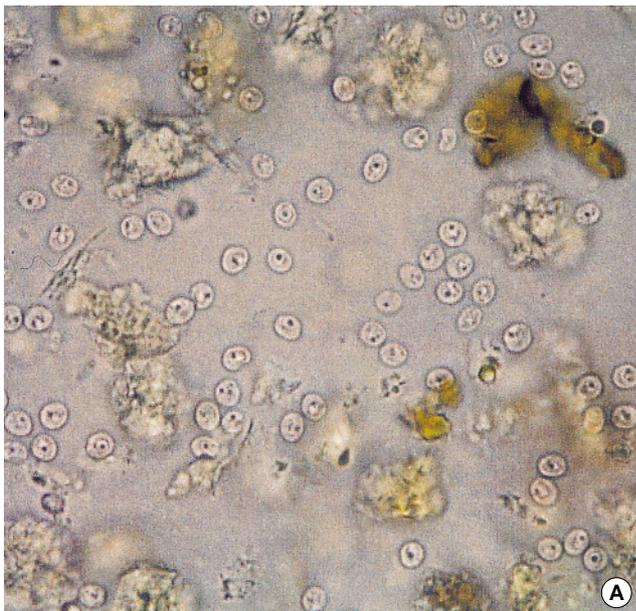


Fig. 2. *Cryptosporidium parvum* oocysts from a fecal specimen (arrows). A: Oocysts seen using a quantitative centrifugation concentration flotation technique. B: Oocysts in an acid-fast stain of a fecal smear. C: Oocysts in a fluorescent antibody-stained fecal smear ( $\times 500$ ).

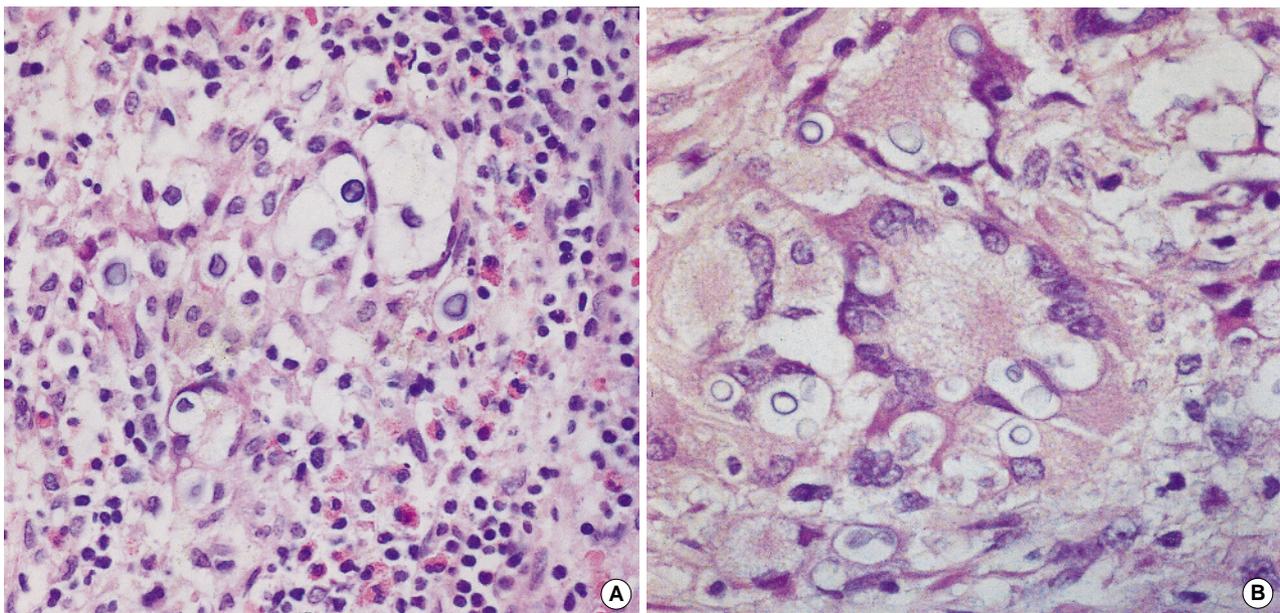


Fig. 3. A photograph of a cervical lymph node showing granulomatous inflammation involving epithelioid histiocytes, lymphocytes, eosinophils, and neutrophils (A, H&E stain,  $\times 200$ ). *Cryptococcus*-like organisms were found in the cytoplasm of multinucleated giant cells (B, H&E stain,  $\times 400$ ).

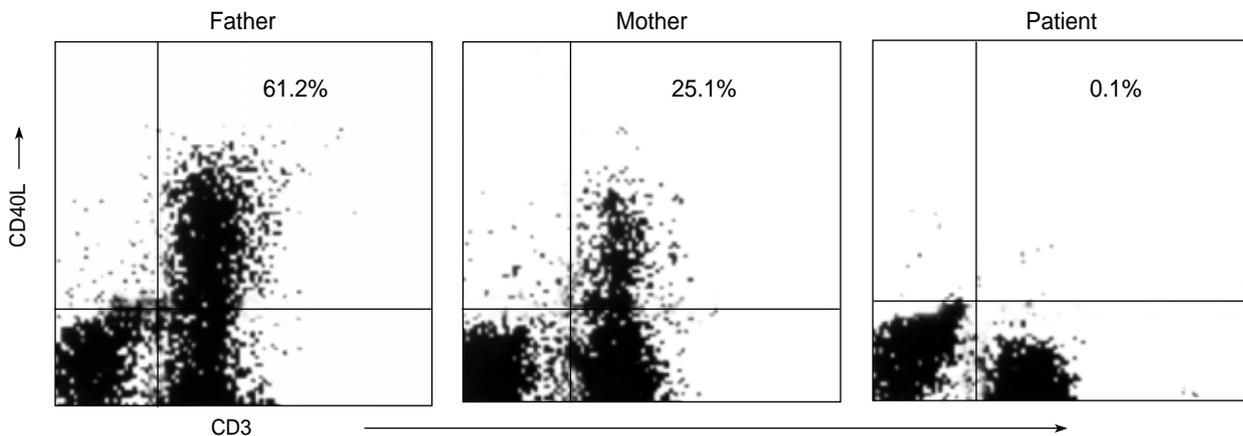


Fig. 4. CD40L expression by activated PBMC from the father (normal), mother (carrier), and the patient. After stimulation with ionomycin and PMA for 8 hours, CD40L expression was evaluated by FACS analysis after immunostaining cells using MoAb 5c8.

*parvum* oocysts were seen in sugar flotations as translucent, spherical bodies containing one to four dark granules (Fig. 2A). Each oocyst was visible with acid-fast stain (Fig. 2B) and fluorescent stain (Fig. 2C).

Nine years after the initial diagnosis, he visited the Department of Pediatrics to have a palpable lateral neck mass evaluated. Chest and abdominal CT scans revealed widespread lymphadenopathy and hepatosplenomegaly. Cytology of a fine needle aspiration biopsy of a cervical lymph node revealed some granulomatous inflammation due to fungal infection with both May-Grünwald-Giemsa and Pap stains. A sequential excision biopsy of the cervical lymph node was performed and samples were cultured. In the lower power view, we

observed the absence of germinal centers in the lymph node biopsy sample, a striking feature of the XHIM patient, suggesting the failure of generation of memory B lymphocytes (data not shown). Hematoxylin and eosin stain revealed numerous scattered multinucleated giant cells and granulomatous inflammation throughout the cortex and medulla of the lymph node (Fig. 3A). In the high power view, the giant cells contained anucleated, silver-colored, round to oval foreign material surrounded by clear halos in the cytoplasm. The largest measured about  $15 \mu\text{m}$  in diameter (Fig. 3B). These morphologic features suggested cryptococcal infection and a culture from the lymph node biopsy sample showed *C. neoformans*. The bone marrow examination revealed marked

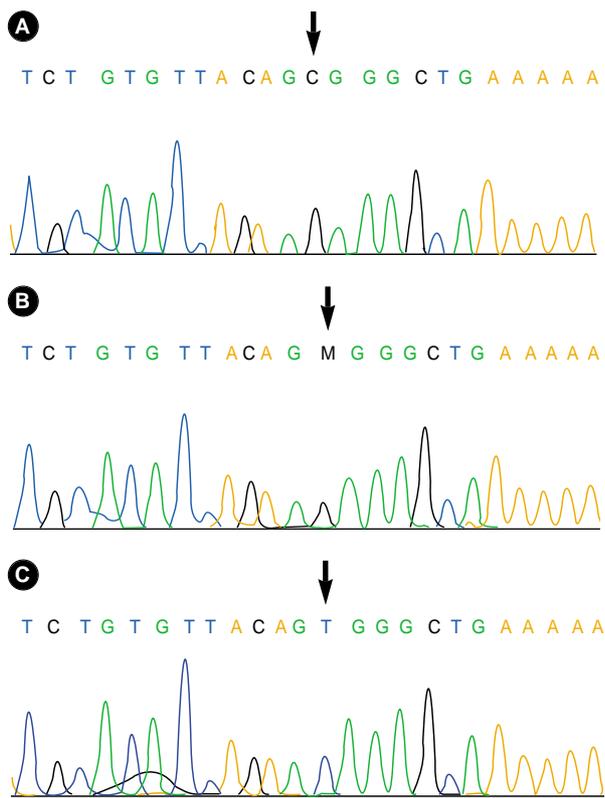


Fig. 5. Sequence analysis showing a missense mutation in the *CD40L* gene. A, Genomic sequence encompassing a point mutation (T to C) in exon 5 of the *CD40L* gene from the patient; B, mother (carrier); C, father (normal control).

eosinophilia with a left shift. No immature or abnormal cells were noted.

To evaluate CD40L expression, PBMC were prepared by Ficoll-Hypaque gradient centrifugation, and cultured with phorbol 12-myristate 13-acetate (10 ng/mL) (Sigma Chemical Co., St Louis, MO) and ionomycin (400 ng/mL) (Calbiochem-Novabiochem Corp, La Jolla, CA) for 8 hr. In an immunophenotypic analysis of CD40L expression, activated T cells from the patient did not react to monoclonal antibody 5c8 (mouse IgG2a, Biogen, Cambridge, MA) (Fig. 4). In addition, only a small percentage (25.1%) of the patient's mother's PBMC expressed functional CD40L, whereas his father showed normal expression of CD40L (61.2%) (Fig. 4).

For mutation analysis, total RNA and genomic DNA were extracted from PBMC by conventional methods, and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR), as described previously (11). We found a T-to-C transversion at nt 439, the first nucleotide of codon 140, by direct sequencing of the cDNA-PCR products (Fig. 5). This single base substitution resulted in a missense mutation from tryptophan to arginine (W140R). His mother and sister were heterozygotes in the mutation analysis, suggesting that they were obligate carriers (Fig. 5).

## DISCUSSION

We performed a molecular characterization of a case of XHIM, and presented the clinical findings and family history. The patient showed typical serum immunoglobulin profiles of XHIM, an elevated serum IgM with low IgG level. IgA and IgE were in normal ranges. Although mutations in the *CD40L* gene explain the inability of XHIM patients to switch from IgM to IgG production both in vitro and in vivo, variability in the serum IgM profile in XHIM patients is often seen (10).

Our patient had chronic *Cryptosporidium parvum* enteritis, diagnosed by a quantitative centrifugation concentration flotation technique. Chronic diarrhea and severe hepatobiliary disease are common and often associated with a poor prognosis in XHIM (2). In addition, *Cryptosporidium* infection is frequent and is associated with sclerosing cholangitis in some XHIM patients (2), as seen in patients with acquired immunodeficiency syndrome (11). It is noteworthy that XHIM patients show inadequate T cell function and are often susceptible to opportunistic infections typical of T cell deficiency such *Pneumocystis carinii* pneumonia and *Cryptosporidium* diarrhea (2). A recent study demonstrated that activated T cells from patients with XHIM produced markedly reduced levels of IFN- $\gamma$ , and failed to induce antigen-presenting cells to synthesize IL-12 and TNF- $\alpha$ , suggesting a basis for the increased susceptibility of XHIM patients to certain opportunistic infections (3).

Cryptosporidial infection causing chronic diarrhea and possibly cholangiopathy or liver cirrhosis (12), malignancies (1, 12), and autoimmune disorders (1) are also known to occur in XHIM patients. To our knowledge, this is the first report on the isolation of *C. parvum* in a XHIM patient in Korea. The patient in this case suffered from *C. parvum* enteritis in spite of the IVIG treatment. In agreement with our case, others have reported that some XHIM patients frequently suffer from infection with opportunistic pathogens, such as *Cryptosporidium* and *P. carinii*, despite IVIG treatment (2, 13).

In a previous study, we identified *Cryptococcus* in fine needle aspiration smears of the cervical lymph node using May-Grunwald-Giemsa stain (14). The characteristic features of cryptococci were identified using several staining methods, including Gomori methenamine silver stain and periodic acid Schiff stain (14). In this case, we confirmed the cryptococcal infection with cultures, which are important for the characterization and diagnosis of cryptococcosis (15). The cervical lymphadenopathy partially regressed three weeks after the initiation of antifungal medication. The patient also had chronic neutropenia, which might have predisposed the patient to cryptococcal infection, as has been reported in other children with XHIM (2, 16).

In the mutation analysis, we found a missense mutation at codon 140. Other patients with a mutation at codon 140 have been reported (4, 10, 17), confirming that codon 140

represents a hotspot for XHIM mutations, although the mutations in XHIM are highly heterogeneous. To date, about one hundred mutations in the *CD40L* gene have been described (7-9). The majority of mutations are missense mutations (7, 9), reading-frame terminations (7), or splicing defects that give rise to aberrant transcripts (5, 7, 9). In a recent report (9), activated PBMC from some patients had functional CD40L. These patients appeared to undergo milder clinical courses. Thus, nonfunctional CD40L is not an absolute diagnostic hallmark of XHIM, but molecular analysis of the *CD40L* gene may be required for the definitive diagnosis of XHIM (9). Using direct sequencing, we were able to confirm that the patient's mother and sister had both normal and abnormal bands. Identification of mutations in the *CD40L* gene has allowed unambiguous assignment of carrier status and offers the possibility of prenatal diagnosis in this family.

In this paper, we described the clinical characteristics and molecular identification of an XHIM patient in Korea. It emphasizes the need for genetic evaluation of functional CD40L expression in male patients with a low serum IgG and normal or high IgM levels and with a normal proportion of circulating B cells.

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