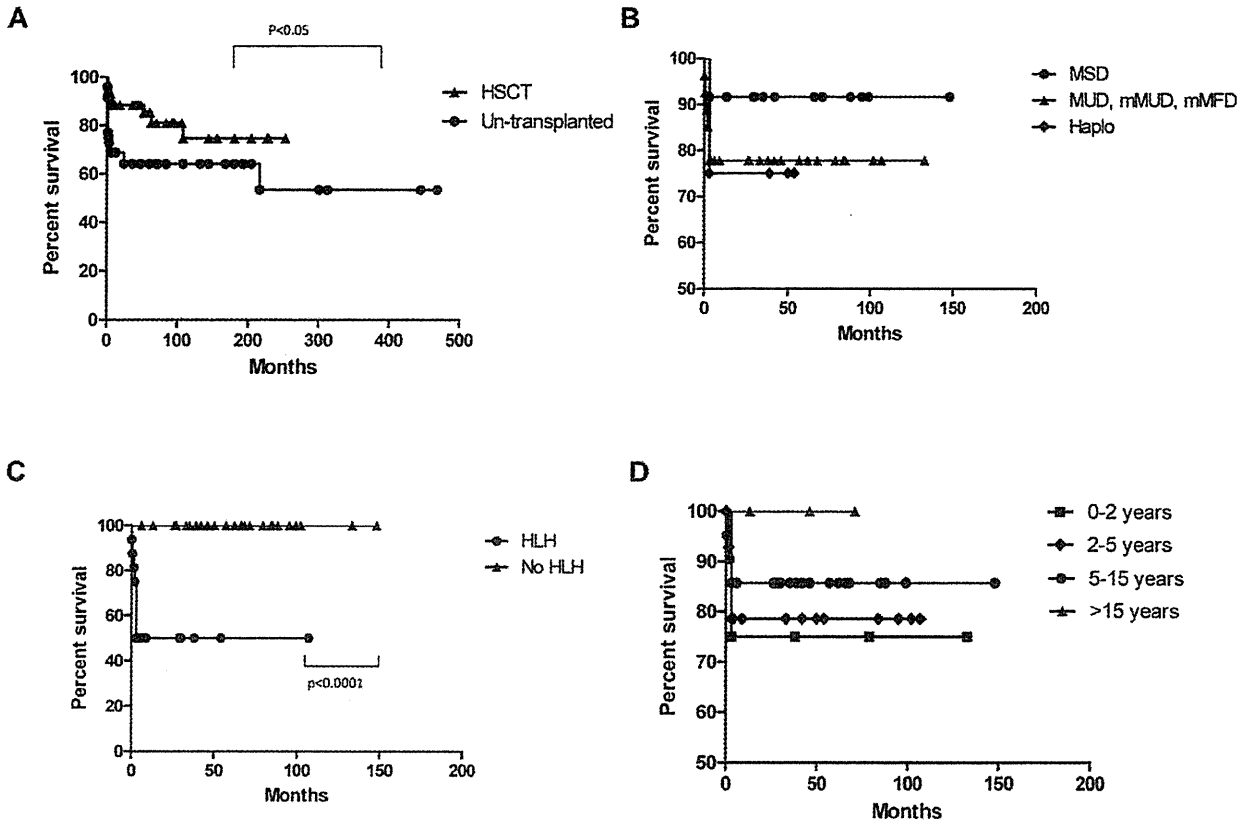


**Figure 2. Survival in XLP1 related to different variables.** (A) Overall survival of transplanted versus untransplanted patients. In the transplanted group this represents time from presentation and not transplant. (B) Survival according to donor source. (C) Survival after HSCT with relation to presence of HLH before transplant. (D) Survival according to age at transplant.

HLH/FIM was the most common feature in this group being seen in 35 patients (68.6%), with lymphoma present in 10 patients (19.6%), and dysgammaglobulinemia in 19 (37.2%). Nine EBV-positive patients had a family history of XLP1, and two others had a family history suggestive of an X-linked immunodeficiency. Of the 18 EBV-positive patients who died, the majority (14/18) died within 2 months of presentation due to disease progression. Three died in the early posttransplant period of infective complications and disease progression, and 1 died during treatment for lymphoma.

Twenty-eight patients were EBV negative at presentation or diagnosis. The median age of presentation for this group was 3 years (range birth to 31 years). Family history of XLP1 was the presenting feature for 12 patients, and a further 7 patients described a family history suggestive of an X-linked immunodeficiency or lymphoma. There was a higher rate of dysgammaglobulinemia (51.8%) in this group. Lymphoma was present in 7 patients. Fewer EBV negative patients presented with HLH/FIM, and this may suggest that at least for this manifestation a viral trigger is important. Information was sought on other viral infectious agents including cytomegalovirus and adenovirus, but data were not available for most patients. Other clinical features included aplastic anemia in 3 patients and vasculitis in 2 patients. The mortality for this EBV negative group was 28.6% (8/28); 3 patients died shortly after presentation before HSCT with central nervous system vasculitis (2) and HLH with enterococcal sepsis (1). One patient died 11 years after presentation following a complex course, and a further 4 patients died in the early posttransplant period (described in Table 5).

**Table 2. Characteristics of EBV-positive and EBV-negative XLP1 patients**

| | EBV positive (64.6%, n = 51) | EBV negative (35.4%, n = 28) |
|---|---|---|
| Median age at presentation | 4 y (8 mo-40 y) | 3 y (0-31 y) |
| Family history of XLP1 | 17.6% | 42.9% |
| HLH | 51% | 21.4% |
| FIM | 17.6% | |
| Lymphoma | 19.6% | 25% |
| Dysgammaglobulinemia | 37.2% | 51.8% |
| Mortality | 35.2% | 28.6% |
| Median age at death | 3 y 6 mo (14 mo-21 y) | 5 y 11 mo (20 mo-31 y) |

### HSCT for XLP1

HSCT was undertaken in 22 centers (range of patients/center: 1-7) between 1997 and 2009 (Table 3). Forty-six transplants were performed on 43 patients, and the median age at transplant was 6.25 years (range 8 months to 19 years); 1 patient who had undergone a haploidentical transplant received a $CD34^+$ selected boost 1 year after initial transplant. One patient received an allogeneic HSCT to treat lymphoma before a diagnosis of XLP1 was established. Most patients received bone marrow or peripheral blood stem cells, and only 2 patients received umbilical cord HSCT. Donor grafts were from human leukocyte antigen-matched family donors in 14 cases, mismatched family donors or matched unrelated grafts in 28 cases, and haploidentical donors in 4 cases. Half of the transplant procedures (23/46) were performed using myeloablative conditioning regimes including combinations of

**Table 3. Characteristics of XLP1 patients receiving allogeneic HSCT**

| | Percentage | Number | 1-y survival | HR | 95% CI | P |
|---|---|---|---|---|---|---|
| **XLP1 features** | | | | | | |
| Previous HLH | 37.2% | 16/43 | 50% | 23.93 | 5.31-108.0 | < .0001 |
| Previous NHL | 27.9% | 12/43 | 74.2% | 0.23 | 0.05-1.06 | .06 |
| Previous dysgammaglobulinemia | 46.5% | 20/43 | 80% | 1.2 | 0.29-4.96 | .77 |
| EBV+ | 51.2% | 21/41 | 75% | 1.37 | 0.36-5.3 | .65 |
| **Age at HSCT** | Mean 7 y (8 mo to 19 y 7 mo) | | | | | |
| 0-2 y | 9.3% | 4/43 | 75% | 5.75 | 0.11-302.1 | .38 |
| 2-5 y | 34.9% | 15/43 | 78.6% | 3.61 | 0.18-71.76 | .40 |
| 5-15 y | 48.8% | 21/43 | 85.7% | 3.16 | 0.11-90.83 | .50 |
| > 15 y | 7% | 3/43 | 100% | | | |
| **Year of HSCT** | | | | | | |
| < 2000 | 7.0% | 3/43 | 66.7% | | | |
| 2000-2005 | 37.2% | 16/43 | 87.5% | | | |
| 2005-2009 | 55.8% | 24/43 | 79.2% | | | |
| **Donor Type** | | | | | | |
| MSD, MFD | 30.4% | 14/46 | 91.77% | | | |
| MUD, mMFD, mMUD | 60.9% | 28/46 | 77.8% | 0.42 | 0.08-2.07 | .27 |
| Haplo | 8.7% | 4/46 | 75% | 0.24 | 0.01-6.58 | .4 |
| **Source** | | | | | | |
| Bone marrow | 58.5% | 24/41* | 82.6% | | | |
| Peripheral blood | 36.6% | 15/41* | 92.9% | | | |
| Umbilical cord | 4.9% | 2/41* | 50% | | | |
| **Conditioning** | | | | | | |
| MA | 50% | 23/46 | 82.9% | | | |
| NMA | 50% | 23/46 | 78.9% | 1.25 | 0.30-5.2 | .77 |
| Serotherapy | 30.4% | 14/46 | | | | |
| **GVHD** | 50% | 19/38 | | | | |
| Grade 1 | 18.4% | 7/38 | | | | |
| Grade 2-3 | 26.3% | 10/38 | | | | |
| Grade 4 | 5.3% | 2/38 | | | | |
| Chronic | 5.3% | 2/38 | | | | |
| **Chimerism** | | | | | | |
| Full (> 98%) | 92% | 35/38 | 100% | | | |
| Mixed | 8% | 3/38 | 88.8% | 2.98 | 0.06-151.0 | .59 |
| Replacement IVIg | 20% | 7/35† | | | | |
| **Alive** | 81.4% | 35/43 | | | | |
| Follow up | 6 wk to 148 mo | | | | | |

*Data missing on 5 transplants, 1 died during conditioning.

†Three patients < 1 year after transplant.

CI indicates confidence interval; HR, hazard ratio; MSD, matched sibling donor; MFD, matched family donor; MUD, matched unrelated donor; mMFD, mismatched family donor; mMUD, mismatched unrelated donor; Haplo, haploidentical transplant; MA, myeloablative; and NMA, nonmyeloablative.

busulfan 12-20 mg/kg, cyclophosphamide 50-200 mg/kg, and total body irradiation 5-12 Gy. The other half of procedures used nonmyeloablative conditioning regimes consisting of fludarabine (30 mg/kg), melphalan (70-140 mg/kg), busulphan (4-12 mg/kg), or total body irradiation (3-5 Gy). Twenty-six patients received additional serotherapy with alemtuzumab, anti-thymocyte globulin, anti-CD3 antibody, and anti-CD20 antibody (rituximab). Graft-versus-host disease (GVHD) prophylaxis regimes differed between centers, but mostly involved combinations of cyclosporin with methotrexate, mycophenolate mofetil, steroids, and tacrolimus. T-cell depletion of the graft was used in 1 case.

Outcome for XLP1 patients who received allogeneic HSCT was good with 81.4% surviving the procedure (35/43) with a median follow up of 52 months. The majority of these patients (28/35 survivors) required no ongoing immunoglobulin replacement therapy. Tables 3 and 4 highlight details of transplanted patients, and Figure 2 describes survival according to several factors.

Sixteen patients were diagnosed with HLH before transplant and 12 patients had some form of lymphoproliferative disease (lymphoma). Only 51.2% of the cohort had documented evidence of EBV infection (by polymerase chain reaction) with survival rates in EBV+ patients similar to those without EBV infection (75% vs 80%). Most patients experienced some delay from first symptoms to diagnosis (average delay 2 years 7 months) but once a diagnosis of XLP1 was established time to transplant was generally less than 1 year. Median age at transplant was 6.25 years with a range of 8 months to 19 years.

Univariate analysis was performed to identify the major risk factors for survival after HSCT. The most important risk factor was prior HLH, which significantly decreased the survival outcome to 50%. A previous diagnosis of lymphoma had a near significant effect, but other variables were not shown to have a significant effect including importantly, previous evidence of EBV infection, the age at transplant, donor type, or the conditioning regime. It is also important to note that only patients who had HLH at some point before or during transplant died. Conversely, all patients without HLH (n = 27) survived the transplant procedure.

Half of the patients underwent a nonmyeloablative conditioning regime before HSCT and this did not impact on survival (nonmyeloablative vs myeloablative, 78.9% vs 82.9%) or long-term chimerism. More than 90% of patients achieved full donor chimerism, and

**Table 4. Details of XLP1 patients surviving allogeneic HSCT**

| Year of HSCT | EBV | HLH | Age at HSCT | Donor | Conditioning/serotherapy/ graft manipulation | GVHD prophylaxis | GVHD | Chimerism | Follow up (mo) | Ig |
|---|---|---|---|---|---|---|---|---|---|---|
| 1997 | NK | | 7 y | MSD | Cy, TBI | MTX, CSA | 1 S* | 100% | 148 | |
| 1998 | | | 1 y | MUD | Bu, Cy, ATG | MTX, CSA, P | 1 S | 100% | 133 | |
| 2000 | + | | 4 y | MUD | Bu, Cy, Campath | CSA | 2 S | 100% | 102 | |
| 2000 | + | Yes | 3 y | mMUD | Bu, Cy | MTX, CSA | 2 S, L | 100% | 107 | |
| 2001 | | | 4 y | MUD | Flu, Melph, ATG, TBI | MMF, CSA | | 100% | 102 | |
| 2001 | + | | 10 y | MSD | Bu, Cy, VP-16 (NHL) | MTX, CSA | 2-3 GI | 100% | 99 | |
| 2001 | | | 4 y | MSD | Bu, Cy | CSA | 2 S | 100% | 95 | |
| 2002 | | | 13 y | MSD | Thio, Flu, ATG | CSA | | 100% | 88 | |
| 2002 | + | | 7 y | MUD | Bu, Cy, ATG | MTX, CSA | 1 S | 100% | 85 | |
| 2002 | | | 3 y | MUD | Bu, Cy, ATG | MTX, CSA | | 100% | 84 | |
| 2003 | | | 8 mo | mMUD | Flu, Melph, ATG, TBI | TAC, MTX, P | S | 100% | 79 | |
| 2003 | | | 19 y | MSD | Thio, Flu, ATG | CSA | | 100% | 71 | |
| 2003 | | | 11 y | mMUD | Flu, Melph, ATG | MMF, CSA | | 100% | 68 | |
| 2004 | + | | 5 y | MSD | Bu, Cy | MTX, CSA | | 20% PBMC | 66 | |
| 2004 | + | | 12 y | mMFD | Flu, Melph, Campath, $34^+$ | MMF, CSA | 4 S, L* | 100% | 62 | Y |
| 2004 | + | | 8 y | mMUD | Flu, Melph, Campath | CSA | 2-3 S, GI | 100% | 57 | |
| 2005 | + | Yes | 2 y | Haplo | Bu, Cy, ATG, $34^+$ | CSA | | 100% | 54 | Y |
| 2005 | | | 2 y | Haplo | Bu, Cy, ATG, $34^+$, top up 1 year | CSA | | 88% PBMC 97% M | 50 | Y |
| 2005 | | | 12 y | mMUD | Flu, Melph, Campath, $34^+$ | MMF, CSA | 3 S | 100% | 46 | |
| 2005 | | | 18 y | MUD | | NK | | NK | 46 | NK |
| 2006 | | | 5 y | MSD | Bu, Cy | CSA | 1 G, 3 S | 100% | 42 | |
| 2006 | NK | | 2 y | MUD | Bu, Flu, Campath | MTX | | 100% | 42 | |
| 2006 | + | | 7 y | Haplo | Flu, Melph, Thio, OKT3, ATG | | | 100%, 75% CD3 | 39 | |
| 2006 | + | Yes | 1 y | MUD | Flu, Melph, Ritux | CSA | 1 S | 5% | 38 | |
| 2006 | + | | 11 y | MSD | Bu, Cy, ATG | MTX, CSA | | 100% | 35 | |
| 2006 | | | 4 y | MUD | Bu, Cy, Campath | MMF, CSA | 1 S | 100% | 33 | |
| 2007 | + | Yes | 6 y | MSD | Bu, Cy | MTX, CSA | | 99% | 30 | |
| 2007 | + | Yes | 7 y | MSD | Bu, Cy | CSA | 3 S, L, GI | 100% | 29 | |
| 2007 | NK | | 7 y | MUD | Flu, Melph, TBI | TAC, MTX | | 98% | 27 | |
| 2007 | | | 7 y | MSD/mMUD | Bu, Cy | CSA | 2 S, GI | 100% | 26 | |
| 2008 | | | 17 y | MFD | Flu, Melp, Campath | MMF, CSA | | 100% | 13 | |
| 2008 | | Yes | 3 y | MUD | Bu, Flu | TAC, MTX | | 100% | 9 | Y |
| 2009 | + | | 7 y | MUD | Bu, Cy | | | 100% | 6 | Y |
| 2009 | + | Yes | 6 y | mMUD | Flu, Melph, Campath | CSA, MMF | 1 S | 100% | 5 | Y |
| 2009 | + | Yes | 3 y | MUD | Thio, Cy, ATG | CSA, P | | 100% | 4 | Y |

*Chronic GVHD.

PBMC indicates peripheral blood mononuclear cell; Flu, fludarabine; Melph, melphalan; $34^+$, $CD34^+$ stem cell infusion; Bu, busulfan; Cy, cyclophosphamide; Thio, thiotepa; TBI, total body irradiation; CSA, cyclosporin A; MMF, mycophenalate mofetil; MTX, methotrexate; P, prednisolone; TAC, tacrolimus; S, skin; GI, gastrointestinal; L, lung; and Ig, replacement immunoglobulin.

those with a mixed or falling chimerism remained well with 1 patient still receiving replacement immunoglobulin.

Data were also collected on common posttransplant complications such as GVHD, infectious complications and toxicity attributable to chemotherapy. Half of the patients (50%) suffered from some form of GVHD; the majority of cases were grade 1-3 affecting the skin, liver, and gut. Two patients suffered grade 4 disease (of skin and liver), and 1 of these children died. Only 2 patients went on to develop chronic GVHD (see Table 3). One patient experienced both veno-occlusive disease and renal toxicity due to conditioning (busulfan, cyclophosphamide, and antithymocyte globulin), and this patient succumbed shortly after a haploidentical transplant.

In 3 patients with mixed chimerism in peripheral blood mononuclear cells, this remained stable in all but 1 patient, in whom it fell from 92% to 5%. However, this patient remains well 3 years posttransplant and does not require replacement immunoglobulin therapy. From this series, there is little evidence of viral reactivation posttransplant. Thirty-five patients are alive with 5 suffering some long-term effects including EBV viremia (managed with rituximab), bronchiectasis, autoimmune disease, chronic psoriasis, and neutropenia.

Eight patients did not survive after HSCT (see Table 5). Seven patients who died presented with HLH before HSCT (4/7 $EBV^+$) compared with 8 of 35 survivors, but HLH was a feature of disease in all 8 nonsurvivors. The majority of nonsurvivors were ≤ 3 years old (5/8), and conditioning regime did not appear to play a role as 5/8 patients received a full myeloablative regime. The main cause of death in this group was sepsis, but disease progression accounted for 2 deaths. The 2 children dying with disease progression went into transplant with active disease; 1 died during conditioning and the other 3 days after HSCT. One further patient died 3 weeks after HSCT (7 months after presentation) from veno-occlusive disease (VOD), multiorgan failure, and renal toxicity attributable to chemotherapy. The remaining 5 patients died of sepsis (2 pseudomonal sepsis, 1 parainfluenza III infection, 1 with disseminated adenoviral infection, and 1 with EBV and fungal infection) within 3 months of HSCT.

### Untransplanted patients

Data were available for 48 patients who did not receive HSCT (Table 6); 30 are alive, 4 of whom are actively awaiting transplant, and 3 who refused HSCT. One patient had received an autologous HSCT before diagnosis with XLP1, and this patient's data were

**Table 5. Details of XLP1 patients not surviving allogeneic HSCT**

| EBV | HLH | Age at HSCT, y | Year of HSCT | Donor | Conditioning/serotherapy/ graft manipulation | GVHD prophylaxis | GVHD | Chimerism | Cause of death |
|---|---|---|---|---|---|---|---|---|---|
| + | Yes | 2 | 2005 | MMFD | Flu, TBI | N/A | | | Died during conditioning 6 wk from presentation |
| + | Yes | 3 | 2003 | MUD | Bu, Flu, Campath, Rituximab | CSA | | | Died 3 d after HSCT disease progression |
| | Yes | 6 | 2005 | MMFD | Bu, TBI | MMF. MTX, P | | | Died 14 d after HSCT MDR pseudomonal sepsis |
| + | Yes | 3 | 2009 | Haplo | Bu, Cy, ATG | TCD | | | Died 3 wk after HSCT VOD, MOF, renal toxicity |
| + (after HSCT) | Yes | 5 | 2008 | mMUD (cord plus PBSC 4 months later) | Bu, Flu, ATG then Flu, TBI | TAC, P | | 100% | Died 2 mo after second HSCT EBV, fungal, and ?PCP sepsis |
| | Yes | 3 | 1998 | MSD × 2 | Flu, Melph | CSA, P | | 100% | Died 3 mo after HSCT *Pseudomonas* sepsis |
| + | Yes | 12 | 2003 | MUD | Bu, Cy, Flu, Campath | | 4 S | 100% | Died 3 mo after HSCT disseminated adenovirus |
| | Yes | 1 | 2007 | MUD | Flu, Melph, ATG, $34^+$ | CSA | 2-3 S, L | 100% | Died 3 mo after HSCT paraflu III sepsis |

PBSC indicates peripheral blood stem cell; Flu, fludarabine; Melph, melphalan; $34^-$, $CD34^+$ stem cell infusion; Bu, busulphan; Cy, cyclophosphamide; Thio, thiotepa; TBI, total body irradiation; CSA, cyclosporine A; MMF, mycophenalate mofetil; MTX, methotrexate; P, prednisolone; TAC, tacrolimus; TCD, T-cell depletion; S, skin; L, lung; VOD, veno-occlusive disease; MOF, mullti-organ failure; MDR, multidrug resistant; and PCP, *Pneumocystis jiroveci*.

analyzed as though untransplanted. Less detailed information was available for this set of patients compared with those receiving HSCT. This may be because some patients died before EBV status and immune function could be established and any first symptoms may not have been recognized as a manifestation of XLP1. From data available, median age at presentation was 5 years, and delay in diagnosis ranged from a few weeks to 32 years.

Presentation was highly variable but as expected included HLH/FIM, dysgammaglobulinemia, and recurrent infection. More unusual presentations included 1 patient with central nervous system vasculitis, intracranial hemorrhage and myocardial fibrosis, and peripheral eosinophilia. The course of XLP1, both temporal and clinical, was extremely variable without any apparent correlation to family history or genetic mutation.

**Table 6. Characteristics of XLP1 patients not receiving HSCT**

| | | Number |
|---|---|---|
| Age at first symptom | 8 y 8 mo (6 mo-40 y) | |
| Age at death | 7.5 y (1-31 y) | |
| Time from presentation to death | 17.3 mo (1 NK) 9 d-18 y | |
| Time from first symptom (in those patients alive) | 12 y (1 NK) 1-39 y | |
| **Presenting symptom** | | |
| HLH | 31.3% | 15/48 |
| FIM | 10.4% | 5/48 |
| Lymphoma | 16.7% | 8/48 |
| Dysgammaglobulinemia | 29.2% | 14/48 |
| Other | 12.5% | 6/48 |
| **Features** | | |
| HLH | 33.3% | 16/48 |
| FIM | 12.5% | 6/48 |
| Lymphoma | 20.1% | 10/48 |
| Dysgammaglobulinemia | 56.3% | 27/48 |
| Gut | 8.3% | 4/48 |
| Other | 14.6% | 7/48 |
| **EBV status** | | |
| $EBV^+$ | 66.6% | 32/48 |
| $EBV^-$ | 14.6% | 7/48 |
| Unknown | 18.8% | 9/48 |
| **Mortality** | 37.5% | 18/48 (4 $EBV^-$) |
| Associated with HLH | 81.3% | 13/16 |
| Associated with FIM | 33.3% | 2/6 |
| Associated with lymphoma | 20% | 2/10 1 had previous HLH and died during chemotherapy; 1 had recurrent lymphoma and many other problems |
| **Immunoglobulin replacement** | | |
| Yes | 70% | 21/30 |
| No | 23.3% | 7/30 |
| Unknown | 6.7% | 2/30 |

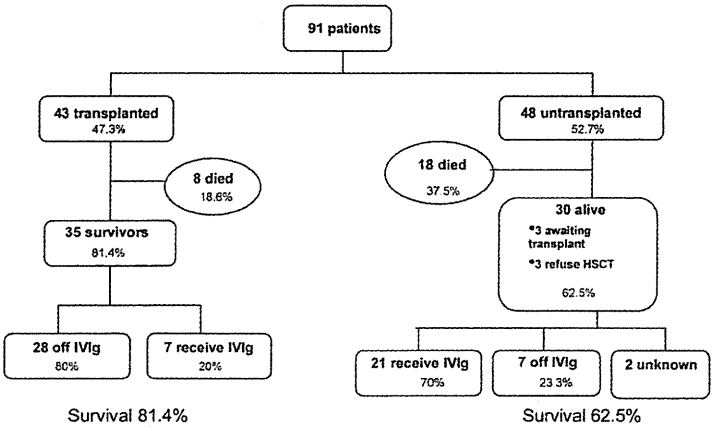


**Figure 3. Outcome of patients with SAP/*SH2D1A* mutations.**

As with transplanted patients the significant mortality associated with HLH is evident in untransplanted patients (81.3%). Presentation or manifestation of HLH (n = 15 and 16, respectively) was associated with a rapid decline and death within 6 weeks, especially in patients less than 5 years of age. Of the 48 patients, 32 did not have manifestations of HLH, and in this group 5 died, thereby giving a survival of 84.4% with a mean follow-up in this group of 11.6 years. For those untransplanted patients who survive, 70% received replacement immunoglobulin therapy, with few suffering from long-term complications. Only 5 patients have recorded complications, including 1 with recurrent infection, 1 with neutropenia, 1 with bronchiectasis, and 2 boys with gastrointestinal disease and growth delay.

Supplemental Table 2 compares the demographics between transplanted and untransplanted patients. No significant differences were seen between the 2 populations other than mortality, which was twice as high in the untransplanted cohort ($P < .05$). Age of death was lower in transplanted patients and may reflect the more severe course that may have led to the need for HSCT.

## Discussion

This report summarizes data on 91 patients from 64 families worldwide with a genetic diagnosis of XLP1 and provides information on outcome with and without allogeneic HSCT using current treatment protocols (summarized in Figure 3). This report is the first large-scale analysis of XLP1 patients since the report by the XLP1 registry in 1995 and has for the first time gathered patients who have confirmed SAP/*SH2D1A* mutations. Therefore this report represents a genetically homogeneous cohort and avoids possible phenotypic variability through inclusion of other patients with genetic defects such as *XIAP/BIRC* 4 mutations.

The clinical features of the disease are similar to those reported by the XLP1 Registry, with HLH and FIM remaining the most common and most lethal complication. With the advent of more accessible genetic screening and mutation analysis confirming the diagnosis, more patients have been diagnosed early on the basis of family history and increased awareness of the disease has also led to patients being diagnosed after presentation with immune dysregulation and more unusual presenting features such as vasculitis.

A diagnosis of XLP1 is still a difficult one to make, and it is possible that some patients mistakenly fall under the umbrella of common variable immunodeficiency, although previous genetic screening studies suggest that the incidence of XLP1 patients in common variable immunodeficiency cohorts is low.[33] It is also possible that there are older individuals who present in adulthood and have not been identified and included in this study, and this may result in a bias in the method of data collection as the majority of centers approached to contribute data were specialist pediatric centers. For example, a recent case report describes a 41-year-old man who presented with an EBV-induced central nervous system B-cell lymphoma and absent B cells.[34] The oldest surviving patient from this cohort presented at the age of 7 years with recurrent infections and hypogammaglobulinemia, but remains well without transplant and is receiving replacement immunoglobulin therapy at 46 years of age.

The prognosis for XLP1 has greatly improved since 1995, when Seemayer et al[2] reported an overall survival of 25% survival with 71.4% of patients in this cohort alive at the time of data analysis. Indeed, the mortality in untransplanted patients was lower than we expected, with 62.5% surviving, including 3 boys who presented with HLH, but the mortality in this group secondary to HLH remains high at 81.3%. It is also interesting to note that a considerable mortality of 28.6% is seen in EBV-negative patients who do not receive HSCT and is related to HLH, sepsis, and vasculitis, suggesting that underlying immunological abnormalities in XLP1, and not only EBV-driven disease, can be fatal. Few complications from recurrent infection and immune dysregulation were reported, suggesting that early diagnosis and good supportive care with replacement immunoglobulin and prophylactic antibiotics can improve the outcome for untransplanted patients. Although over 60% of patients survive without HSCT, it will be important to follow patients carefully, since there is the potential for more severe manifestations to arise, and the options for transplant should be explored.

The mortality associated with the different clinical phenotypes has changed over time, with an improved survival for both HLH (34.5% vs 4%) and lymphoma (91% vs 35%).[2] This most likely reflects improved treatment strategies for both HLH (especially the use of agreed protocols such as HLH 94[35] and 2004[36]) and malignancy. Although these figures represent survival with either HLH or lymphoma as features of XLP1 at any stage, they are very similar to the survival seen if patients present with these features (44.5% and 92% for HLH/FIM and lymphoma, respectively). A mortality of 13% in patients who exhibit dysgammaglobulinemia is associated with HLH, infection, vasculitis, and hemorrhage and highlights that although clinically this phenotype may be milder, it is not an innocuous phenotype, and progression to further fatal symptoms is not uncommon.

The outcome data following allogeneic HSCT from this report is encouraging. The outcome data presented is the largest ever

gathered and shows that approximately 80% of patients survive the procedure with complete cellular and humoral reconstitution in the large majority of cases. In this series, there is little evidence of problematic EBV reactivation adversely affecting transplant outcome and no increased incidence of long-term complicating features such as autoimmunity in comparison to transplant for other conditions.[37,38] Although donor chimerism in the majority of patients was complete, even low level chimerism in 2 patients with 5% and 20% donor chimerism was associated with good immune recovery. Conversely however, when the patients who required ongoing immunoglobulin support are analyzed, all but 1 have 100% donor engraftment. Further detailed lineage-specific analysis and study of T- and B-cell function in these patients is necessary to determine why humoral function has not been established. The availability of a fully matched donor is associated with an improved survival outcome (approximately 92%), although with the present low numbers this is not statistically significant. Haploidentical grafts show a good outcome in this cohort, but the numbers are extremely low (only 4 transplants performed), and therefore this information needs to be interpreted with caution.

The most important factor affecting survival after transplant is a manifestation of HLH, which significantly reduces survival to 50%. Indeed all 8 patients who died had a complication of HLH at some point in their clinical course. This may reflect the effects of HLH itself or HLH chemotherapy and immunosuppression on the transplant process, including increased organ related toxicity and increased susceptibility to pathogens. In comparison to data reported on cohorts of patients undergoing transplant for HLH associated with other gene defects (eg, perforin and munc 13-4)[39-41] it appears that the outcome for HLH associated with XLP1 is worse and may relate to the multiple immune deficits associated with SAP deficiency. By contrast all XLP1 patients who had no HLH manifestations (n = 27) survived the HSCT procedure.

These data may now allow more informed recommendations to be made regarding transplantation in XLP1. It is clear from this report that HLH in XLP1 has a very poor prognosis if left untransplanted. Therefore any individual with HLH as a manifestation of XLP1 should undergo allogeneic HSCT.

For patients who are newly diagnosed because of a family history but with no clinical features or for those who present with manifestations other than HLH/FIM, the decision to transplant a relatively well child has been more challenging. An important observation from this report is that all patients (n = 27) who went into transplant without prior HLH survived the procedure in comparison to 84.4% survival for those who are untransplanted and have not manifested with HLH. Since progression to HLH without transplant may occur at a later stage, there is a strong argument to transplant all individuals with a diagnosis of XLP1.

However, there is a counter argument to such a recommendation. As with other immunodeficiencies, the data collected and presented here may not give a complete picture of the natural course of XLP1 and is a historical cohort study conducted before the advent of recent improved therapies. Further, milder patients may also remain undiagnosed having been labeled with a diagnosis of common variable immunodeficiency. It is also the case that HLH is most often seen in younger patients (median age of presentation 3.2 years) and older individuals are less likely to manifest with HLH. There may also be reluctance on the part of families and physicians to undertake a transplant in a well child given that, even in the best-case scenario, there will be a certain mortality associated with any allogeneic transplant procedure.

A more pragmatic recommendation would be to undertake transplant in all patients presenting or manifesting with HLH. Similarly for newly diagnosed or young children without any HLH, if a well-matched donor is available, HSCT should be undertaken, since a manifestation of HLH may be catastrophic or may severely compromise transplant outcome. For older individuals, we would still recommend that HSCT be undertaken, but this decision to transplant should be based on available donor status, wellbeing of the patient, and the attitude of family and physician to the risk of transplant. If HSCT is not undertaken immediately, it is recommended that a donor source is identified and that all patients are followed very carefully in case of disease progression and onset of other manifestations, at which point HSCT could be performed rapidly.

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## Authorship

Contribution: C.B. designed the research, collected and acquired data, analyzed the data, and wrote the manuscript; H.B.G. assumed overall responsibility for the research, oversaw analysis, and revised the manuscript; and all authors contributed clinical data and reviewed the manuscript before submission.

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For a complete list of Inborn Errors Working Party participants, please see the supplemental Appendix.

Correspondence: Hubert B. Gaspar, Center for Immunodeficiency, Molecular Immunology Unit, Institute of Child Health, 30 Guilford St, London, WC1N 1EH, United Kingdom; e-mail: h.gaspar@ich.ucl.ac.uk.

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# Clinical similarities and differences of patients with X-linked lymphoproliferative syndrome type 1 (XLP-1/SAP deficiency) versus type 2 (XLP-2/XIAP deficiency)

Jana Pachlopnik Schmid,[1-3] Danielle Canioni,[4] Despina Moshous,[1-3] Fabien Touzot,[3] Nizar Mahlaoui,[3] Fabian Hauck,[1,2] Hirokazu Kanegane,[5] Eduardo Lopez-Granados,[6] Ester Mejstrikova,[7] Isabelle Pellier,[8,9] Lionel Galicier,[10] Claire Galambrun,[11] Vincent Barlogis,[11] Pierre Bordigoni,[12] Alain Fourmaintraux,[13] Mohamed Hamidou,[14] Alain Dabadie,[15] Françoise Le Deist,[16] Filomeen Haerynck,[17] Marie Ouachée-Chardin,[18] Pierre Rohrlich,[19-21] Jean-Louis Stephan,[22] Christelle Lenoir,[1] Stéphanie Rigaud,[1,2] Nathalie Lambert,[1,23] Michèle Milili,[24] Claudin Schiff,[24] Helen Chapel,[6] Capucine Picard,[2,23,25] Geneviève de Saint Basile,[1-3] Stéphane Blanche,[3] Alain Fischer,[1-3] and Sylvain Latour[1,2]

[1]Inserm Unité 768, Laboratoire du Développement Normal et Pathologique du Système Immunitaire, Hôpital Necker-Enfants Malades, Paris, France; [2]Université Paris Descartes, Institut Fédératif de Recherche Necker Enfants-Malades (IFR94), Paris, France; [3]Unité d'Immunologie et Hématologie Pédiatrique, Assistance Publique–Hôpitaux de Paris (AP-HP), Hôpital Necker Enfants-Malades, Paris, France; [4]Service d'Anatomie et de Cytologie Pathologiques, AP-HP, Hôpital Necker Enfants-Malades, Paris, France; [5]Department of Pediatrics, Graduate School of Medicine, University of Toyama, Toyama, Japan; [6]Department of Clinical Immunology, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom; [7]Department of Pediatric Hematology and Oncology, Teaching Hospital Motol and 2nd Medical School, Charles University, Prague, Czech Republic; [8]Unité d'Hématologie-Immunologie-Oncologie Pédiatrique, Centre Hospitalier Universitaire (CHU) Angers, Angers, France; [9]Inserm Unité 892, Centre de Recherche en Cancérologie Nantes-Angers, Nantes, France; [10]Service Immuno-Hématologie, AP-HP, Hôpital Saint-Louis, Paris, France; [11]Service d'Hématologie Pédiatrique, Hôpital Timone Enfants, Marseille, France; [12]Departement d'Hémato-Oncologie Pediatrique et de Transplantation Medullaire, CHU Nancy, Vandoeuvre, France; [13]Service de Néonatologie, Groupe Hospitalier Sud Réunion, Saint Pierre, La Réunion, France; [14]Médecine Interne, CHU Nantes, Nantes, France; [15]Service de Hépato-Gastro-Entérologie, CHU Rennes, Rennes, France; [16]Département de Microbiologie et d'Immunologie, et Département de Pédiatrie, Université de Montréal, CHU Sainte-Justine, Montréal, QC; [17]Département of Child Immunology, Ghent University Hospital, Ghent, Belgium; [18]Service d'Hématologie, AP-HP, CHU Hôpital Robert Debré, Paris, France; [19]Inserm UMR645, Besançon, France; [20]Université Franche-Comté, Besançon, France; [21]Service de Pédiatrie, CHU Besançon, Besançon, France; [22]Unite d'Hématologie et Oncologie Pédiatrique, Hôpital Nord, CHU Saint-Etienne, Saint-Etienne, France; [23]Centre d'Etude des Déficits Immunitaires, Hôpital Necker-Enfants Malades, Paris, France; [24]Centre d'é3'Centre d'Immunologie de Marseille-Luminy, Parc Scientifique de Luminy-Case 906, Marseille, France; and [25]Inserm Unité 980, GHMI, Hôpital Necker Enfants-Malades, Paris, France

**X-linked lymphoproliferative syndromes (XLP) are primary immunodeficiencies characterized by a particular vulnerability toward Epstein-Barr virus infection, frequently resulting in hemophagocytic lymphohistiocytosis (HLH). XLP type 1 (XLP-1) is caused by mutations in the gene *SH2D1A* (also named *SAP*), whereas mutations in the gene *XIAP* underlie XLP type 2 (XLP-2). Here, a comparison of the clinical phenotypes associated with XLP-1 and XLP-2 was performed in cohorts of 33 and 30 patients, respectively. HLH (XLP-1, 55%; XLP-2, 76%) and hypogammaglobulinemia (XLP-1, 67%; XLP-2, 33%) occurred in both groups. Epstein-Barr virus infection in XLP-1 and XLP-2 was the common trigger of HLH (XLP-1, 92%; XLP-2, 83%). Survival rates and mean ages at the first HLH episode did not differ for both groups, but HLH was more severe with lethal outcome in XLP-1 (XLP-1, 61%; XLP-2, 23%). Although only XLP-1 patients developed lymphomas (30%), XLP-2 patients (17%) had chronic hemorrhagic colitis as documented by histopathology. Recurrent splenomegaly often associated with cytopenia and fever was preferentially observed in XLP-2 (XLP-1, 7%; XLP-2, 87%) and probably represents minimal forms of HLH as documented by histopathology. This first phenotypic comparison of XLP subtypes should help to improve the diagnosis and the care of patients with XLP conditions. (*Blood*. 2011;117(5):1522-1529)**

## Introduction

X-linked lymphoproliferative syndrome (XLP) is a rare immunodeficiency condition characterized by an extreme vulnerability to Epstein-Barr virus (EBV) infection, frequently resulting in hemophagocytic lymphohistiocytosis (HLH) or virus-associated hemophagocytic syndrome (VAHS).[1-3] HLH is caused by overwhelming T-cell and macrophage activation, leading to fever, splenomegaly, cytopenia, hypofibrinogenemia, or hypertriglyceridemia, hyperferritinemia, and hemophagocytosis.[4]

XLP belongs to the group of familial hemophagocytic lymphohistiocytosis (FHL) as originally proposed by Purtilo et al.[1] In the original description, the term "lymphoproliferative disease" in the Duncan kindred[1] was used for benign or malignant lymphoproliferation but also for the diffuse organ "infiltrates composed of lymphocytes, plasma cells, and histiocytes, some containing erythrocytes," describing histologic features of HLH. Thus, the term "X-linked lymphoproliferative disease or syndrome" used thereafter to name this condition refers not only to malignant lymphomas but also to HLH. Two genetic causes are responsible for XLP. XLP type 1 (XLP-1) is caused by hemizygous mutations in the gene *SH2D1A* encoding the signaling lymphocyte activation molecule (SLAM)–associated protein (SAP) (MIM no. 308240).[5,6] Hemizygous mutations in the gene encoding the X-linked inhibitor of

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apoptosis protein (XIAP; also termed *BIRC4*; MIM no. 300635) have been discovered in a cohort of patients with clinical XLP without any identified mutations in *SH2D1A* and normal SAP protein expression.[7] Thus, mutations in *XIAP* define the XLP type 2 (XLP-2). These findings were confirmed by the identification of additional patients with XIAP deficiency.[8,9] After EBV infection in most (but not all) cases, patients bearing mutations in *SH2D1A* (hereafter denoted SAP-deficient patients) may experience variable manifestations such as fulminant infectious mononucleosis corresponding pathophysiologically to HLH, malignant lymphoma, and hypogammaglobulinemia.[2,10,11] Less common findings are dysgammaglobulinemia, bone marrow hypoplasia, especially aplastic anemia, and lymphocytic vasculitis.[12,13] However, although HLH is almost always triggered by EBV, the other manifestations can be present even in SAP-deficient patients who have never encountered EBV.[2,3,10,11] The clinical features of the 12 patients with mutations in *XIAP* (hereafter denoted XIAP-deficient patients) initially described, slightly differed from the features described above. In some XIAP-deficient patients, splenomegaly was noticed as the first clinical symptom, and chronic colitis occurred during the disease course in 2 patients.[7]

The gene product affected in XLP-1 patients, SAP, is a small SH2-containing adaptor protein that is expressed in T, natural killer (NK), and invariant NKT (iNKT) cells.[5,14] SAP binds with high affinity and specificity to tyrosine-based motifs located in the cytoplasmic domains of the transmembrane receptors of the SLAM family. SAP couples SLAM family receptors to downstream signaling pathways and thereby enables SLAM receptors to mediate an array of activating or regulatory signals. In SAP-deficient humans and mice, multiple cellular defects have been documented, including altered $CD8^+$ T- and NK-cell cytotoxicity responses, $CD4^+$ T helper cell cytokine production and function, block of CD1d-restricted iNKT-cell development, defective antibody production associated with reduced numbers of switched memory B cells and defects in germinal center formation.[11,14] Studies of SAP-deficient humans and mice support the notion that the immune dysfunctions seen in SAP-deficiency are mostly caused by alterations in the signal transduction of SLAM family receptors.

The XLP-2 gene product, XIAP, belongs to the family of inhibitor of apoptosis proteins and is well known to be a potent physiologic inhibitor of caspases 3, 7, and 9.[15] XIAP is ubiquitously expressed.[7] In addition to its antiapoptotic role, XIAP is also involved in multiple signaling pathways, including copper metabolism, activation of the nuclear factor κB and the mitogen-activated protein kinases pathways and the transforming growth factor-β–receptor and bone morphogenetic protein–receptor signal transduction.[16] In XIAP-deficient patients, lymphocytes are characterized by an increased susceptibility to apoptosis in response to CD95 and tumor necrosis factor receptor–related apoptosis-inducing ligand receptor stimulation as well as enhanced activation-induced cell death.[7] XIAP-deficient patients also display low but detectable numbers of iNKT cells in blood although a recent study indicated that they can have normal numbers of iNKT cells.[9] NK cell–mediated cytotoxicity is apparently normal in XIAP-deficient patients.[7,9]

Our knowledge of the immune dysfunctions underlying the clinical manifestations in SAP-deficient patients has been largely improved in the past decade. However, this is not the case for XIAP-deficient patients. A better characterization of the clinical similarities and the differences between XLP-1 and XLP-2 could provide hints for a better understanding of the pathogenesis of these conditions and, furthermore, improve diagnostic and therapeutic procedures for these patients. Therefore, we performed a retrospective analysis of the clinical features observed in cohorts of 33 SAP- and 30 XIAP-deficient patients.

## Methods

### Patients and diagnosis

We performed a retrospective analysis of the clinical and laboratory features of SAP- and XIAP-deficient patients in whom confirmative molecular diagnosis had been performed at the Necker Children's Hospital. Patient conditions were diagnosed as XLP-1 and XLP-2 on the basis of molecular results or on the basis of clinical features when disease had been molecularly proven in male relatives on the mother's side (supplemental Methods and Results, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Patients and families provided informed consent for genetic and immunologic studies in accordance to the 1975 Declaration of Helsinki, and the study was approved by the local ethics regulations (Necker-Enfants Malades Ethical Board Committee).

### Protein expression

Expression of SAP and XIAP was analyzed by Western blotting or flow cytometry or both after intracellular staining in phytohemagglutinin-induced T-cell blasts or peripheral blood mononuclear cells or both as described.[7] The monoclonal antibody (mAb) anti-SAP was kindly provided by Dr A. Veillette, IRCM, Montréal. Intracellular SAP was stained by fluorescein isothiocyanate– or phycoerythrin-coupled anti-SAP mAb and XIAP detected with noncoupled anti-XIAP mouse mAb (clone 48; BD Biosciences) revealed with fluorescein isothiocyanate–coupled anti–mouse antibodies (Jackson ImmunoResearch Laboratories Inc) after cell permeabilization with Perm 2 (BD Biosciences).

### Histology and immunohistochemistry

All diagnostic specimens were fixed in 10% buffered formalin and stained with hematoxylin and eosin, Giemsa, or trichrome dyes (for the liver). Immunohistochemistry was performed on fixed tissues with a peroxidase-based method (Dako). Antibodies used were raised against CD20, CD3, CD8, and latent membrane protein 1 (LMP-1) (Dako); CD25 (Novocastra); and T-cell intracellular antigen-1 (Immunotech). EVB-encoded RNA (EBER) was probed on some specimen with the use of in situ hybridization technique. Slides were observed using a Leica DM LB microscope with ×20, ×40, and ×100 objectives and a 10× eyepiece. Acquisition of images was with IM50 software (Leica Microsystems). All slides were analyzed by the same pathologist (D.C.), and an independent review was also performed (F.H.).

### Clinical assessment

The patients' clinical events and laboratory features were assessed retrospectively by retrieval of data from medical records.

### Statistical analysis

The statistical analyses were performed with Fisher exact tests or log-rank tests (for comparison of survival curves) with the use of the PRISM software (GraphPad Software Inc).

## Results

XLP-1 was diagnosed in 33 patients from 19 families, and mutations of *SH2D1A* were found in 18 families, and XLP-2 was

**Table 1. Characteristics of patients with mutations in *SH2D1A/SAP* (XLP-1)**

| Patient ID* | *SH2D1A/SAP* mutation | SAP protein | HLH (age in years at diagnosis) | EBV at first HLH | HLH relapses (age in years at relapse) | SM (age in years at diagnosis) | Hypo-γ (age in years at diagnosis) | Lymphoma (age in years at diagnosis) | Other manifestations (age in years at diagnosis) | Outcome (age in years) |
|---|---|---|---|---|---|---|---|---|---|---|
| S1.1 | E67G | | – | NA | | – | – | 13 | – | Alive, well (19) |
| S1.2 | E67G | – | 3 | + | + (25) | – | + (26) | 34 | – | Alive, under lymphoma treatment (34) |
| S1.3 | E67G | | 15 | ? | – | – | – | 7, 30 | – | Alive, under lymphoma treatment (30) |
| S1.4 | E67G | | – | NA | | – | + (4)† | – | – | Alive, well, IVIG (10) |
| S2.1 | I96X | – | 4 | ? | – | ? | ? | – | – | Died (4, HLH) |
| S3.1 | del. of exons 1-4 | | – | NA | | – | +† | – | Chronic gastritis, | Alive, well, IVIG (20) |
| S3.2 | del. of exons 1-4 | | – | NA | | – | +† | – | IM (2), chronic gastritis | Alive, well, IVIG (20) |
| S4.1 | R55X | | – | NA | | – | – | 40 | – | Alive, well (42) |
| S4.2 | ND | | 6 | + | – | – | – | – | – | Died (6, HLH) |
| S5.1 | del. of exon 2 | | 3.7 | + | – | ? | ? | – | – | Died (3.7, HLH) |
| S5.2 | ND | | – | NA | | – | ? | 5 | – | Died (5, lymphoma) |
| S6.1 | del. of exon 1 | | 2.2 | | – | – | ? | – | – | Died (2.2, HLH) |
| S7.1 | R55X | – | 2.5 | + | – | – | ? | – | Recurrent infections | HSCT (2.7), alive (11) |
| S8.1 | X129RfsX141 | – | 2.4 | + | + (9) | – | + (3)† | – | – | First HSCT (9); second HSCT (10); died (10.2) |
| S8.2 | ND | | 2 | + | – | – | ? | – | – | Died (2, HLH) |
| S9.1 | C42Y | +/– | – | NA | | – | – | 2 | – | Alive (18) |
| S9.2 | C42Y | | – | NA | | – | + (1)† | – | – | Alive, well, IVIG (16) |
| S10.1 | R55Q | | 14 | ? | – | ? | ? | – | – | Died (14, HLH) |
| S11.1 | X129R fsX141 | – | – | NA | | – | – | – | – | Alive, well, N+T, IVIG (22) |
| S11.2 | X129R fsX141 | | – | NA | | ? | ? | – | Recurrent pneumonia | Alive, well (66) |
| S11.3 | X129R fsX141 | | – | NA | | – | – | – | – | Alive, well, IVIG (15) |
| S11.4 | X129R fsX141 | | – | NA | | – | + (9) | 7 | – | Alive, well, IVIG (19) |
| S12.1 | del. of exon 3 | – | 19 | + | – | – | + (10)† | 11 | T (22) | Alive, T, IVIG (23) |
| S12.2 | del. of exon 3 | | 19 | ? | – | – | + (19)† | 20 | – | Died (21, lymphoma) |
| S13.1 | N82FfsX103 | ND | 10§ | – | + (12, EBV+) | + (9)‡ | ? | – | – | Died (12, HLH) |
| S14.1 | del. of exons 1-4 | | 3.5 | + | – | – | – | – | HUS (3.5) | Died (3.6, HLH) |
| S15.1 | A22P | – | – | NA | | – | + (13)† | – | – | Alive, well, IVIG (25) |
| S15.2 | ND | | 3.6 | ? | – | – | ? | – | – | Died (3.6, HLH) |
| S15.3 | ND | | – | NA | | + (45)‡ | ? | – | – | Died (69, myelodysplasia) |
| S16.1 | del. of exons 2-4 | – | 3.1 | + | – | – | ? | – | – | Died (3.1, HLH) |
| S17.1 | M1T | – | – | NA | – | – | + (4)† | – | IM (2.4) | Alive, N+T, IVIG (20) |
| S18.1 | No mutation | – | 16§ | ? | – | – | + (15)† | 9 | – | Died (17, HLH) |
| S19.1 | del. of exons 1-4 | – | 3.3 | + | | – | – | – | Hypopigmented hair | HSCT (3.7), died (3.8) |

SM indicates recurrent splenomegaly or hepatosplenomegaly; Hypo-γ, hypogammaglobulinemia; NA, not applicable; del., deletion; ?, unknown; IM, infectious mononucleosis; ND, not done; HSCT, hematopoietic stem cell transplantation; N, neutropenia; T, thrombocytopenia; and HUS, hemolytic uremic syndrome.

*Patient identification: S indicates SAP-deficiency, the first number corresponds to the family and the second to the individual patient.

†With recurrent respiratory infections; + indicates yes or positive; –, no or negative.

‡Recurrent splenomegaly or hepatosplenomegaly associated with intermittent fever, anemia, and cytopenia.

§Diagnosed as incomplete HLH.

diagnosed in 30 patients from 11 families (Tables 1 and 2). In one patient (PS18.1), no mutation in *SH2D1A* was found; however, no SAP protein expression was detected.[17] Six and 7 mutations in *SH2D1A* and *XIAP* were novel and not reported, respectively (supplemental Methods and Results).

Clinical manifestations included HLH, splenomegaly and incomplete forms of HLH, lymphoma, dysgammaglobulinemia, colitis, and rare clinical manifestations.

### HLH

The mean age at first episode of HLH was 7.35 years (range, 2.0-19.0 years) in SAP-deficient and 6.5 years (range, 0.1-23.0 years) in XIAP-deficient patients ($P = .89$). The occurrence of HLH in SAP-deficient (18 of 33, 55%) and in XIAP-deficient (22 of 29, 76%, one unknown) patients did not differ significantly ($P = .112$) (Figure 1A; Table 3). XIAP-deficient patients with null mutations (families X1 to X7 and X11) more frequently developed HLH (19 of 20, 95%) compared with XIAP-deficient patients expressing non-null mutations (families X8, X9, and X10; 3 of 9, 33%; $^{**}P = .0011$; supplemental Figure 1A).

Overall, 11 of the 33 SAP-deficient patients (33%) and 5 of 30 the XIAP-deficient patients (17%) succumbed to HLH ($P = .1563$). Among patients with HLH, HLH-associated lethality was significantly higher in SAP-deficient patients (11 of 18, 61%) than in XIAP-deficient patients (5 of 22, 23%) ($^{*}P = .0230$). HLH

**Table 2. Characteristics of patients with mutations in *XIAP* (XLP-2)**

| Patient ID* | *XIAP* mutation | XIAP protein | HLH (age in years at diagnosis) | EBV at first HLH | HLH relapses (age in years at relapse) | SM (age in years at diagnosis) | Hypo-γ (age in years at diagnosis) | Chronic colitis (age in years at diagnosis) | Other manifestations (age in years at diagnosis) | Outcome (age in years) |
|---|---|---|---|---|---|---|---|---|---|---|
| X1.1 | E99KfsX129 | | 5 | + | ? | ? | – | – | ? | Alive, well (8) |
| X1.2 | E99KfsX129 | – | 5.3 | + | ? | + (5) | – | – | ? | Alive, well (11) |
| X1.3 | E99KfsX129 | | 2.5 | + | ? | + (2.5) | + | – | ? | Alive, well, IVIG (14) |
| X1.4 | E99KfsX129 | – | 7.8 | + | + | + (6) | – | + (4) | Cholangitis (23) | Alive, ileitis (23) |
| X1.5 | E99KfsX129 | | 3 | + | + | + (3) | – | – | – | Alive, well (30) |
| X1.6 | E99KfsX129 | – | 0.8 | – (HHV-6+/–) | + (EBV+) | + (1)‡ | +(10) | – | – | HSCT (11), died (11) |
| X1.7 | ND | | 1.5† | ? | + | + (1.5)‡ | +(42) | + (41) | Cholangitis (41) | Died (42, colitis) |
| X2.1 | I397FfsX414 | – | 1.2 | + | + | + (1)‡ | – | – | – | HSCT (1.6), died (d+13, HLH) |
| X3.1 | E118X | – | 23 | + | + | + (22) | +(22) | – | – | Alive, well, IVIG (39) |
| X3.2 | ND | | 0.5 | ? | – | ? | ? | | – | Died (0.5, HLH) |
| X3.3 | ND | | 20 | + | – | ? | ? | | – | Died (20, HLH) |
| X3.4 | E118X | – | – | NA | – | + (7) | – | – | – | Alive, well, SM (10) |
| X4.1 | del. of exon 2 | – | 20 | + | + (21,EBV–) | + (1)‡ | – | – | – | Alive, well (28) |
| X4.2 | del. of exon 2 | – | 10 | ? | + (11, EBV+) | + (6)‡ | – | – | – | Alive, well (15) |
| X5.1 | D130GfsX140 | – | 2.5 | + | + (3.4-3.6) | + (1) | – | – | – | HSCT (3.6), died (4) |
| X5.2 | ND | | 0.1 | ? | – | ? | ? | – | – | Died (0.1, HLH) |
| X5.3 | ND | | 3.5 | + | – | ? | ? | – | – | Died (3.5, HLH) |
| X6.1 | R238X | – | 1.7 | + | + | +‡ | – | – | – | Alive, recurrent HLH (3) |
| X7.1 | I397NfsX405 | – | 2.7 | + | + (3.2-3.5, EBV+) | + (2.7) | – | – | – | Alive, recurrent HLH (3.5) |
| X8.1 | E434AfsX457 | +/– | 15.5 | + | – | – | – | – | – | Alive, well (16) |
| X9.1 | G466X | +/– | 8† | + | – | + (8)‡ | + (8)§ | – | – | Alive, well, SM (27) |
| X9.2 | G466X | +/– | – | NA | – | + (21) | + (21)§ | – | – | Alive, well, SM (30) |
| X9.3 | G466X | +/– | – | NA | – | + (4)‡ | – | + (12) | Recurrent infections | Alive, colitis (14) |
| X9.4 | G466X | +/– | – | NA | – | + (22)‡ | + (10)§ | – | Chronic liver failure (22) | Died (29, liver failure), IVIG, |
| X9.5 | G466X | +/– | – | NA | – | – | – | – | – | Alive, well (39) |
| X9.6 | ND | | – | NA | – | ? | ? | + | ? | Died (27, colitis) |
| X9.7 | ND | | – | NA | – | +‡ | ? | – | Recurrent infections | Died (52, pneumonia) |
| X10.1 | T470S | + | 8† | – (HSV-1+) | – | – | + (4)§ | – | Cryptococcosis (4) | Alive, well, IVIG (8) |
| X11.1 | R381X | – | 0.9† | – | NA | + (0.6)‡ | – | – | – | HSCT (1.2), died (1.4) |
| X11.2 | ND | | ? | ? | ? | ? | ? | +(4) | ? | Died (4, colitis) |

SM indicates recurrent splenomegaly or hepatosplenomegaly; Hypo-γ, hypogammaglobulinemia; ?, unknown; HSCT, hematopoietic stem cell transplantation; ND, not done; NA, not applicable; and del., deletion.

*Patient identification: X indicates XIAP deficiency, the first number corresponds to the family and the second to the individual patient; + indicates yes or positive; –, no or negative; and +/–, weakly positive.

†Diagnosed as incomplete HLH.

‡Recurrent splenomegaly or hepatosplenomegaly associated with intermittent fever, anemia, and cytopenia.

§With recurrent respiratory infections.

relapsed in 2 of 7 SAP-deficient HLH-survivors (29%), whereas 11 of 14 XIAP-deficient HLH-survivors (79%, 3 unknown) had ≥ 1 relapse of HLH ($P = .055$).

Six of the 18 SAP-deficient patients with HLH (33%) had proven neurologic involvement with mostly (5 of 6, 83%) lethal outcome, whereas 2 of 22 of XIAP-deficient patients with HLH (9%) had neurologic involvement with less mortality (1 of 2, 50%).

EBV infection was the most-frequent identified trigger of the first HLH episode in the SAP-deficient (11 of 12, 92%, 6 unknown) and XIAP-deficient (15 of 18, 83%, 4 unknown) patients ($P = .63$) (Table 3). Only PS13.1, PX1.6, PX10.1, and PX11.1 had a first HLH episode in the absence of a proven EBV-infection, whereas the EBV status of 6 SAP-deficient patients and 4 XIAP-deficient patients is not known. PX1.6 and PX4.2 subsequently experienced an HLH-relapse with positive EBV polymerase chain reaction. In 2 patients, herpes simplex virus type 1 (HSV-1) and human herpesvirus type 6 (HHV-6) were detected in the blood by polymerase chain reaction in the course of their first HLH episode. Of note, in several XIAP-deficient patients, other viruses than EBV were tested, including cytomegalovirus, parvovirus B19, HSV, HHV-6, HHV-8, HIV, human T-cell leukemia virus, adenovirus, and varicella-zoster virus. All were negative.

### Splenomegaly and incomplete forms of HLH

Recurrent splenomegaly occurring in the absence of systemic HLH and often associated with fever and cytopenia (consisting of pancytopenia, bicytopenia, thrombocytopenia, and anemia) was frequently observed in XIAP-deficient patients (20 of 23, 87%, 7 unknown), whereas it was only found in 2 of 29 SAP-deficient patients (7%, 4 unknown; $***P < .0001$; Table 3). In 8 XIAP-deficient patients, episodes of splenomegaly occurred before they developed HLH and were the first clinical sign of the disease. Overall, although 3 patients with splenomegaly up to now did not

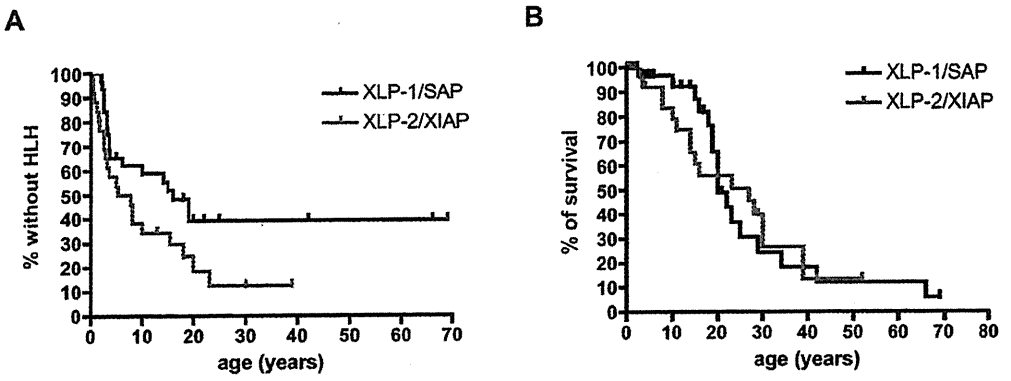


**Figure 1. Comparison of HLH phenotypes and survival curves of SAP-deficient (XLP-1) and XIAP-deficient (XLP-2) patients.** Kaplan-Meier survival curves were constructed on the basis of data presented in Table 1 and Table 2. Statistical analyses with log-rank tests. (A) Percentage of XLP-1/SAP and XLP-2/XIAP patients without HLH phenotype ($P$ = .099). (B) Overall survival curves for XLP-1/SAP and XLP-2/XIAP patients ($P$ = .948).

develop HLH, the others subsequently developed HLH within a period of time, varying from a few months to 19 years. In 2 XIAP-deficient patients, transient pancytopenia with splenomegaly was noticed after vaccinations against measles, mumps, and rubella or measles and rubella. Importantly, none of the XIAP-deficient patients developed B-cell lymphoproliferative disease.

PX4.1 underwent splenectomy at the age of 21 years, and histopathologic examination of the spleen showed reduced white pulp areas, and red pulp was extended with a mild fibrosis (supplemental Figure 2 top left). In the white pulp, most of the lymphocytes were $CD20^+$, whereas in the red pulp there was an accumulation of $CD3^+$ T cells that were mostly $CD8^+$ and cytotoxic (T-cell intracellular antigen-$1^+$; data not shown; supplemental Figure 2 bottom). Strikingly, features of hemophagocytosis were observed in the red pulp (supplemental Figure 2 upper right). Lymphocytes were negative for LMP-1 with very rare $EBER^+$ cells, suggesting that the infiltration was not related to EBV infection (data not shown). Altogether, these observations strongly suggest that these lymphoproliferative manifestations can be regarded as incomplete or attenuated forms of HLH.

In addition, 3 XIAP-deficient patients had liver disease (2 patients with cholangitis and 1 patient with chronic liver failure). In 2 of the patients, the cholangitis was associated with colitis, which are known to overlap.[18] For patient PX1.7, histopathologic examination of the liver showed granulomatous hepatitis in lobular areas with foci of macrophages around necrotic hepatocytes (supplemental Figure 3). Staining for LMP-1 was negative (data not shown). It is unclear whether these liver diseases should also be considered as an incomplete form of HLH.

**Table 3. Comparison of XLP-1 and XLP-2 phenotypes**

| | SAP-/Y, n (%) | XIAP-/Y, n (%) | P* |
|---|---|---|---|
| HLH | 18 of 33 (55) | 22 of 29 (76) | NS |
| HLH relapses (/HLH-survivors) | 2 of 7 (29) | 11 of 14 (79) | NS |
| EBV at first HLH | 11 of 12 (92) | 15 of 18 (83) | NS |
| Fatal HLH | 11 of 33 (33) | 5 of 30 (17) | NS |
| Fatal HLH (/HLH patients) | 11 of 18 (61) | 5 of 22 (23) | .0230 |
| Hypogammaglobulinemia | 14 of 21 (67) | 8 of 24 (33) | .0377 |
| Lymphoma | 10 of 33 (30) | 0 of 30 (0) | .0010 |
| Cytopenias (in the absence of full-blown HLH) | 4 of 33 (12) | 11 of 21 (52) | .0020 |
| Splenomegaly (in the absence of full-blown HLH) | 2 of 29 (7) | 20 of 23 (87) | <.0001 |
| Hemorrhagic colitis | 0 of 33 (0) | 5 of 30 (17) | .0203 |

*Calculated with Fisher exact tests.

### Lymphoma

Ten of 33 SAP-deficient patients (30%) and none of the 30 XIAP-deficient patients developed lymphoma (Tables 1-3; supplemental Figure 1B; ***$P$ = .001). Mean age at diagnosis of lymphoma was 15 years (range, 2-40 years). Diagnoses were non-Hodgkin lymphoma (n = 9), including EBV-positive Burkitt lymphoma (n = 6) and EBV-negative (n = 3). Lymphomas were localized in the ileocecal (n = 5), cerebral[19] (n = 1), cervical (n = 2), and spinal (n = 2) regions, and for one the origin was not known. One patient (PS1.3) had a second lymphoma at the age of 30 years, 23 years after the first one, and one patient (PS15.3) had myelodysplasia.

### Dysgammaglobulinemia

Hypogammaglobulinemia was documented in 14 SAP-deficient patients (14 of 21, 67%) and in 8 XIAP-deficient patients (8 of 24, 33%) (*$P$ = .0377) (Tables 1-3). Thirty percent (10 of 33) of SAP-deficient patients and 13% (4 of 30) of XIAP-deficient patients received intravenous immunoglobulin (IVIG) substitution ($P$ = .1357) (supplemental Figure 1C). Interestingly, hypogammaglobulinemia was transient in 2 of the 8 XIAP-deficient patients. PX3.1 was substituted with IVIG between the age of 23 and 35 years, currently, 4 years after stopping IVIG, immunoglobulin levels remain within the normal range, and the patient does not experience recurrent respiratory infections. Two XIAP-deficient patients developed hypergammaglobulinemia, with higher than normal IgA and IgM levels in PX9.3 and elevated IgG and IgM levels in PX11.1, respectively.

Severe infections were noted in several SAP- and XIAP-deficient patients with hypogammaglobulinemia before initiation of the IVIG substitution when treated. Ten of the 14 SAP-deficient and 4 of the 8 XIAP-deficient patients had recurrent respiratory tract infections. Rare severe infections caused by *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Cryptococcus neoformans* were also observed in SAP- and XIAP-deficient patients (supplemental Table 1).

### Colitis

Chronic colitis with hemorrhagic diarrheas or rectal bleeding or both evoking inflammatory bowel disease was observed in 5 of 30 XIAP-patients (17%) but in none of 33 SAP-deficient patients (*$P$ = .0203; Tables 1-3). In PX1.4, colitis initially responded to immunosuppressive treatment with corticosteroids and cyclosporine A. However, corticosteroids could not be withdrawn, and the

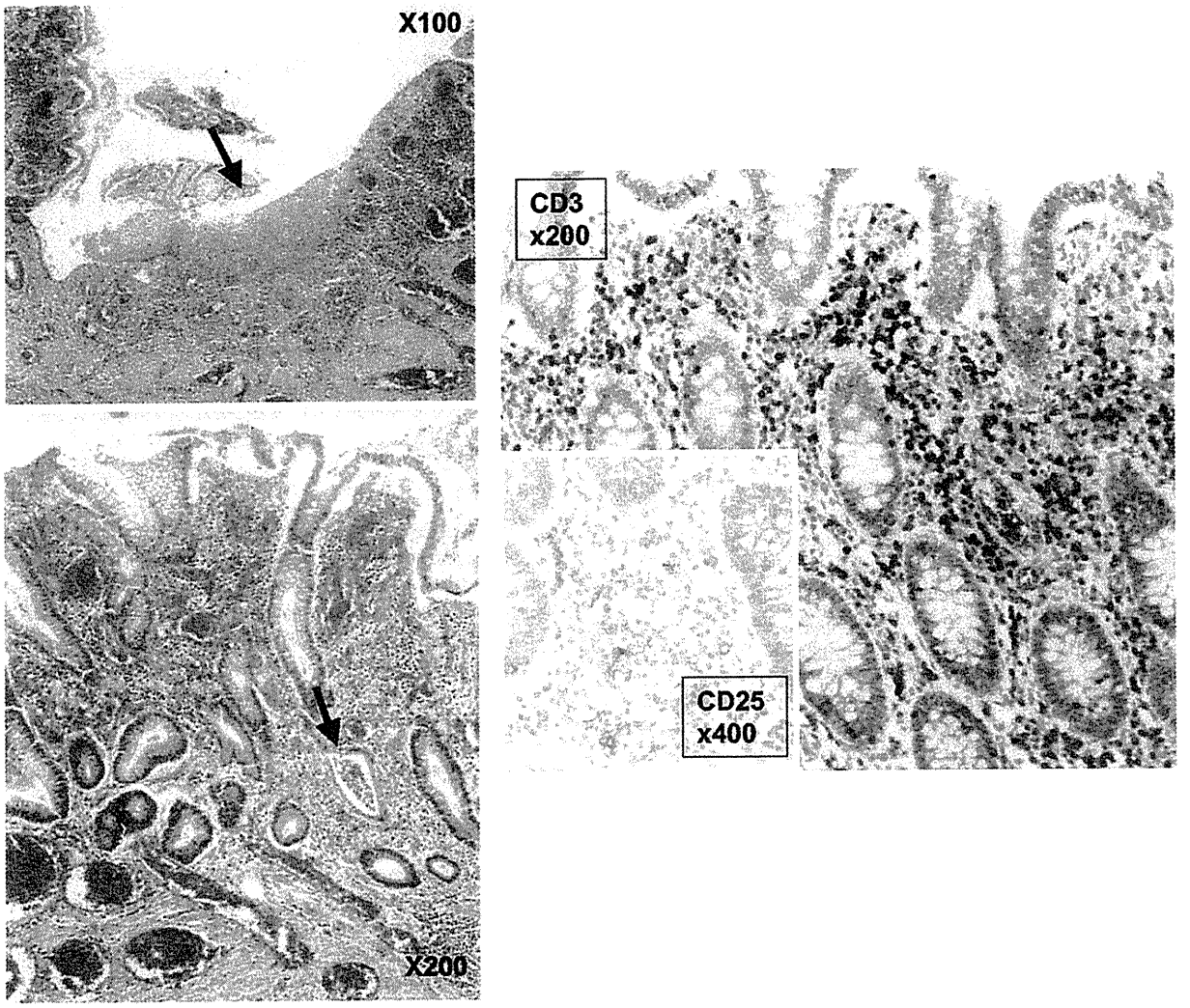


**Figure 2. Histology of the large bowel of PX1.7 with XIAP deficiency.** (Top left) On hematoxylin and eosin at low magnification (×100), a large ulceration is seen, indicated by an arrow. (Bottom left) Higher magnification (×200) shows a massive polymorphic inflammatory infiltrate associated with a crypt abscess (indicated by the arrow). (Central right) Immunostaining with anti-CD3 shows frequent lymphoid T cells (on the right, ×200), some of them express the activation marker CD25 (×400, inset).

addition of azathioprine could not prevent the recurrence of symptoms. Anti–tumor necrosis factor-α mAb treatment (infliximab) provided partial improvement. Recently, a colectomy was performed, but the patient now has terminal ileitis. In PX1.7, severe hemorrhagic colitis was associated with portal hypertension and massive gastroduodenal bleeding that lead to death of this patient. Patients PX9.6 and PX11.2 also suffered from chronic colitis and most probably died of intestinal hemorrhage.

Histopathologic examination of intestinal mucosa biopsy specimens was performed in 3 patients, PX1.4, PX1.7, and PX9.3. Representative images are shown in Figure 2. Hemorrhagic ulcerations of the colon associated with mononuclear infiltration consisting of lymphoid cells and plasma cells in the lamina propria were observed (Figure 2 left top). Crypt architecture was mostly preserved, except for rare crypt abscesses (Figure 2, left bottom), but frequent apoptotic crypt cells were seen (supplemental Figure 4). The lymphoid cells were mostly $CD3^+$ and $CD8^+$ with some lymphocytes expressing CD25 with numerous eosinophils (in PX1.4) (Figure 2; supplemental Figure 4). $CD20^+$ cells were rare, EBER staining was negative (not shown), and there was no granuloma formation. Microbiologic cultures were negative in all 3 cases.

### Rare clinical manifestations

Rare clinical features (supplemental Table 1), each observed in 1 SAP-deficient patient, were hemolytic uremic syndrome associated with HLH, vasculitis, and arthritis. Clinical features, each observed in 1 XIAP-deficient patient, were Kawasaki syndrome and psoriasis. Additional infections in patients without hypogammaglobulinemia were caused by *Pseudomonas aeruginosa* (1 SAP-deficient patient), recurrent measles (1 XIAP-deficient patient), and HSV-(1 XIAP-deficient patient). Of note, 2 SAP-deficient patients (PS3.1 and PS3.2) had chronic gastritis.[20]

### Survival and outcome

Sixteen of 33 SAP-deficient patients and 12 of 30 XIAP-deficient patients died at a mean age of 11 years (range, 2-69 years) and 16 years (range, 0.1-52 years), respectively. Survival rates did not differ between both patient groups ($P = .93$; Figure 1B), and the proportions of whom reached adulthood (age ≥ 16 years) were similar in both groups (17 of 33 SAP-deficient patients [52%] and

13 of 30 XIAP-deficient patients [43%]). Mortality was related to HLH (11 SAP- and 5 XIAP-deficient patients), lymphoma (2 SAP-deficient patients), myelodysplasia (1 SAP-deficient patient), colitis (3 XIAP-deficient patients), hepatitis (1 XIAP-deficient patient), complications of hematopoietic stem cell transplantation (2 SAP- and 4 XIAP-deficient patients), and pneumonia (1 XIAP-deficient patient). Mean age at last follow-up was 24.9 years (range, 10-66 years) for SAP-deficient patients and 17.5 years (range, 0.7- 39 years) for XIAP-deficient patients. Among the surviving 17 SAP-deficient patients, 4 are well without any treatment, 10 receive IVIG substitution, 2 are currently treated for a lymphoma, and 1 had successful hematopoietic stem cell transplantation. Among the surviving 17 XIAP-deficient patients, 10 are well without any treatment (among them 3 with splenomegaly), 2 received recently anti-CD20 antibody treatment because of EBV-related HLH, 2 are under IVIG substitution, 1 has terminal ileitis after colectomy, 1 has colitis treated with mesalazine and azathioprine, and 1 has recurrent HLH treated with cyclosporine A and dexamethasone. One XIAP-deficient and 2 SAP-deficient patients have never developed clinical signs and are considered to be asymptomatic.

## Discussion

We report the first comparison of the clinical phenotypes of SAP- and XIAP-deficient patients. The present study was based on a retrospective analysis with data from medical records on 33 SAP- and 30 XIAP-deficient patients. The relatively small size of both cohorts obviously implies that data should be interpreted with caution.

The overall clinical phenotypes of the affected persons matched with the phenotypes previously reported.[2,7,9,21] In accordance to previous studies, we did not observe any genotype-phenotype correlation in the SAP-deficient patients. However, in our cohort of XIAP-deficient patients, we noticed that XIAP-deficient patients carrying non-null mutations had a tendency to be less prone to develop HLH by contrast to patients with null mutations. However, other genetic or environmental factors may contribute to the variety of phenotypes observed in XLP-1 and XLP-2.

HLH occurred both in SAP- and in XIAP-deficient patients but with more frequent neurologic involvement and fatal outcome in SAP-deficient patients than in XIAP-deficient patients. Splenomegaly often associated with cytopenia and fever was more frequent in XIAP-deficient patients than in SAP-deficient patients. Histologic analysis of one spleen showed accumulation of activated $CD8^+$ T cells and hemophagocytosis without $EBV^+$ cells. These symptoms probably represent incomplete forms of HLH. In addition, HLH relapses seemed to be more common in XIAP- than in SAP-deficient patients who survived HLH. Together, these findings suggest that HLH has a less severe disease course in XIAP- deficient patients than in SAP-deficient patients.

In most of the patients from both groups, the trigger of HLH was an EBV infection (> 80%); EBV may favor HLH by eliciting a potent CD8 T-cell response. It is also postulated that SAP and possibly XIAP are associated with activation pathways that are more important in triggering selective cytotoxicity toward B cells.[22-27] HLH in most hereditary conditions such as FHL, Griscelli syndrome type II, and Chediak-Higashi syndrome shares common pathophysiologic mechanisms, that is, global impaired cytotoxicity responses that lead to the inability of effector lymphocytes to kill infected cells and antigen-presenting cells.[28] In mice and humans, SAP-deficient $CD8^+$ T and NK cells exhibit defective cytotoxicity responses caused by abnormal functions of SLAM receptors.[29] This could explain the occurrence of HLH in SAP-deficient patients.[22-27] In contrast, NK-cell and T-cell cytotoxic responses appear to be preserved in XIAP-deficient patients[7,9] (C. Synaeve and S.L., unpublished data, 2009 and 2010). This might account for the lower severity of the HLH in the XIAP deficiency. Hence, the precise immune defects responsible for HLH in XIAP deficiency remain to be elucidated.

Only XIAP-deficient patients were at risk for chronic colitis with often a lethal outcome. This phenotype seems that is may be even worse than HLH, because the mortality in the group of patients with colitis (3 of 5) has a tendency to be higher than in the group with HLH (5 of 22). Histopathologic analysis of intestinal mucosal biopsy specimens showed an inflammatory process with an accumulation of activated T cells (and eosinophils in one patient) that could evoke inflammatory bowel disease. Interestingly, a recent report indicates that XIAP is involved in nucleotide-binding oligomerization domain containing 2 (NOD2) activation which is an intracellular pattern recognition receptor of the NOD-like receptor family.[30] Importantly, *NOD2* is a key susceptibility gene for Crohn disease.[31] Thus, defects in XIAP might lead to defective NOD2 responses as an additive risk factor for colitis in some of these patients. Of note, however, *NOD2* was sequenced in 2 XIAP-deficient patients with colitis, and none had the genotype shown to be a risk factor for Crohn disease (J.P. Hugot and S.L., unpublished data, June 2006).

One striking difference between XLP-1 and XLP-2 was that only SAP-deficient patients developed lymphoma, although it could not be formally excluded that XIAP-patients might develop lymphomas in the future. In SAP-deficiency, the occurrence of lymphomas may be explained by defective immunosurveillance of hematopoietic cells, resulting from alterations in SLAM receptor-mediated NK- and T-cell cytotoxicity responses,[22-24,26] but also by the proapoptotic functions that have been assigned to SAP.[32,33]

Another common finding shared by XLP-1 and XLP-2 is the hypogammaglobulinemia. Interestingly, 2 XLP-2 patients recovered from hypogammaglobulinemia, which so far seems not to be the case for XLP-1 patients. Numerous studies in mice and humans have documented that impaired antibody production found in XLP-1 resulted from a block in germinal center formation, leading to defects in the differentiation of Ig-isotype–switched memory B cells.[34-36] In most of the XIAP-deficient patients, Ig-isotype–switched memory B cells are not found to be decreased[7] (S. Siberil and S.L., unpublished data, 2008 and 2009). In XIAP deficiency, hypogammaglobunemia could be the consequence of increased activation-induced cell death of B cells, a hypothesis that needs to be tested.

In conclusion, the present comparison of the clinical features of SAP- and XIAP-deficient patients shows that SAP deficiency and XIAP deficiency share a main phenotype, that is, EBV-induced HLH. This similarity raises the possibility of a functional/molecular link between SAP and XIAP proteins. Alternatively, impairment of 2 independent pathways, both important in EBV immunity, could lead to a shared phenotype. Nevertheless, we also demonstrate that XLP-1 and XLP-2 can be distinguished on several clinical aspects, which could be helpful for diagnosis and therapeutic decisions.

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## Authorship

Contribution: J.P.S. collected and analyzed the data and participated in study design, writing of the report, and patients' care; D.C. performed the immunohistochemistry experiments and analyzed histopathologic findings; F.H. participated in histopathologic analysis and writing of the report; C.L., N.L., and S.R. realized gene sequencing and protein expression tests; G.S.B. participated in data analysis; A.F. contributed to study design, data analysis, writing of the report, and patients' care; S.L. coordinated the study collected the data and contributed to sequencing, expression tests, data analysis, and wrote the report. The other authors provided and collected the clinical data on patients' status and contributed to the data analysis and patients' care.

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Correspondence: Sylvain Latour and Alain Fischer, Laboratoire du Développement Normal et Pathologique du Système Immunitaire, Inserm U768, Hôpital Necker-Enfants Malades, F-75015 Paris, France; e-mail: sylvain.latour@inserm.fr.

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ORIGINAL ARTICLE

# Genetic analysis of contiguous X-chromosome deletion syndrome encompassing the *BTK* and *TIMM8A* genes

Takashi Arai[1], Meina Zhao[2], Hirokazu Kanegane[2], Menno C van Zelm[3], Takeshi Futatani[2,4], Masafumi Yamada[5], Tadashi Ariga[5], Hans D Ochs[4], Toshio Miyawaki[2] and Tsutomu Oh-ishi[6]

**Patients with X-linked agammaglobulinemia (XLA) can present with sensorineural deafness. This can result from a gross deletion that not only involved the Bruton's tyrosine kinase (*BTK*) gene, but also *TIMM8A*, mutations in which underlie the Mohr-Tranebjærg syndrome (MTS). We analyzed the genomic break points observed in three XLA–MTS patients and compared these with deletions break points from XLA patients. Patient 1 had a 63-kb deletion with break points in intron 15 of *BTK* and 4 kb upstream of *TAF7L*. Patients 2 and 3 had 149.7 and 196 kb deletions comprising *BTK*, *TIMM8A*, *TAF7L* and *DRP2*. The break points in patients 1 and 3 were located in *Alu* and endogenous retrovirus (*ERV*) repeats, whereas the break points in patient 2 did not show involvement of transposable elements. Comparison of gross deletion sizes and involvement of transposable elements in XLA and XLA–MTS patients from the literature showed preferential involvement of *Alu* elements in smaller deletions (< 10 kb). These results show further insights into the molecular mechanisms underlying gross deletions in patients with primary immunodeficiency.**
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**Keywords:** Alu; BTK; Mohr-Tranebjærg syndrome; TIMM8A; X-linked agammaglobulinemia

## INTRODUCTION

X-linked agammaglobulinemia (XLA) is an inherited primary immunodeficiency characterized by early onset of recurrent bacterial infections, profound hypogammaglobulinemia and markedly reduced circulating B cells.[1] The gene responsible for XLA was identified in 1993,[2,3] and named Bruton's tyrosine kinase (*BTK*). The *BTK* gene is mapped to the Xq21.3–Xq22 region, encompasses 37.5 kb of genomic DNA and contains 19 exons (the initiation codon is in exon 2). Only 770 bp centromerically of the *BTK* gene is the gene *TIMM8A* (formerly *DDP1*) located; it consists of two exons and produces a 97 amino acid polypeptide.[4] Mutations in *TIMM8A* cause the rare X-linked neurodegenerative Mohr-Tranebjærg syndrome (MTS), which is clinically characterized by a progressive neurological deficits, including early onset of sensorineural deafness.[5–7]

A large number of *BTK* mutations, scattered over the entire gene, have been reported and deposited in an international mutation database (http://bioinf.uta.fi/BTKbase/). The most commonly found mutations are missense (34%), followed by nonsense mutations (20%). Mutations affecting splice sites were reported in 18% and small insertions and deletions in another 18% of XLA families. Disruption of the *BTK* gene by gross deletions occurs in about 3.5% of XLA families.[1] As *BTK* and *TIMM8A* are positioned in close genomic proximity, gross gene deletions can result in disruption of both genes causing a contiguous deletion syndrome of XLA and MTS, which has been observed previously in nine families.[4,8–10]

In this study, we describe three patients with gross deletions including the *BTK* and *TIMM8A* genes. We characterized the deletions and identified the genomic break points by combining comparative genomic hybridization (CGH) array, DNA fluorescence *in situ* hybridization (FISH) and long accurate (LA)-PCR analyses. The results of these investigations provide new insight into the genetic mechanisms causing the XLA–MTS deletion syndrome and allow accurate genetic analysis of patients and carriers.

## MATERIALS AND METHODS

### Patients

Patient 1 is a 15-year-old Japanese boy without a family history of immunodeficiency or neurological deficits. He was diagnosed with XLA at 7 years of age when he developed recurrent bacterial infections. Deafness was noticed 1 year earlier and gradually worsened.

Patient 2 is a 10-year-old Japanese boy, who has a history of recurrent otitis media and sinusitis since 12 months of age. He had been diagnosed with deafness and autism at 18 months of age. Agammaglobulinemia and lack of circulating B cells were recognized at 8 years of age, and was diagnosed with XLA.

Both patients are doing well on immunoglobulin replacement therapy. They have no dystonia, but their hearing losses are severe and progressive.

[1]Division of Clinical Research, Saitama Children's Medical Center, Saitama, Japan; [2]Department of Pediatrics, Graduate School of Medicine, University of Toyama, Toyama, Japan; [3]Department of Immunology, Erasmus MC, Rotterdam, The Netherlands; [4]Center for Immunity and Immunotherapies, Seattle Children's Research Institute, University of Washington, Seattle, WA, USA; [5]Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo, Japan and [6]Department of Infectious Diseases, Immunology and Allergy, Saitama Children's Medical Center, Saitama, Japan
Correspondence: Dr H Kanegane, Department of Pediatrics, Graduate School of Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan.
E-mail: kanegane@med.u-toyama.ac.jp
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Patient 3 corresponds to patient 6 who was described in a previous report.[9] He was diagnosed with XLA at 8 months of age, and was found to have sensorineural hearing loss at 3 years of age. Genetic analysis resulted in the identification of a gross deletion involved the entire coding regions of the *BTK*, *TIMM8A*, *TAF7L* and *DRP2* genes.

### Gene analysis of *BTK*

Informed consent for genetic analysis was obtained from the patients and their parents under a protocol approved by the Institutional Review Board of University of Toyama. *BTK* mutation analysis was performed by direct sequencing of complementary DNA and all 19 exons and exon–intron boundaries using genomic DNA as described previously.[11,12]

### Fluorescence *in situ* hybridization

Peripheral blood mononuclear cells from patients and controls were stimulated in culture with phytohemagglutinin for 72 h, followed by treatment with a 0.075 M. KCl solution and fixation with Carnoy's solution (3:1 methanol and acetic acid) for metaphase preparation. A *BTK–TIMM8A*-specific genomic probe (5231 bp long) was prepared using the LA-PCR Kit (Takara, Kyoto, Japan) with primer pairs reflecting exon 19 of *BTK* and exon 2 of *TIMM8A* (5′-AGCATTCTGGCATGAATGTTCCCTGAAC-3′ and 5′-ATCTCTCCGGGT TGCAGATAATAACTG C-3′, respectively). In addition, probes were designed to detect the *TAF7L* and *DRP2* genes, which are located centromerically of TMM8A. The *TAF7L*- and *DRP2*-specific genomic probes (5662 and 5975 bp) were prepared similarly by LA-PCR Kit (Takara) with primers derived from exons 2 and 5 of *TAF7L* (5′-GCTTAGGTAGCCACCAACGTGTTGTTGA-3′ and 5′-GACGTCCCTGTTTCACAAGGAATTAGGA-3′) and from exons 7 and 12 of *DRP2* (5′-CGTGACTGTATTAAAGGGCTCAACCATG-3′ and 5′-GCAG TACTTCCTGTCACAAAGCAGTTGC-3′), respectively. For FISH analysis, denatured metaphase spreads were hybridized with probes labeled with digoxigenin-11-deoxyuridine triphosphate using a nick translation kit (Roche Diagnostics KK, Tokyo, Japan). The Spectrum Green-labeled X-chromosome probe (DXZ1, Abbott Japan, Tokyo, Japan) was used as a control. The *BTK–TIMM8A*-, *TAF7L*- and *DRP2*-specific probes were detected with anti-digoxigenin rhodamine (Roche Diagnostics KK) providing a red signal, whereas DXZ1 was detected by a green signal. Metaphases were counterstained with 4′, 6-diamino-2-phenylindole dihydrochloride, and the images of the hybridization captured by fluorescence microscopy (Carl Zeiss Co., Tokyo, Japan). At least 20 metaphases were observed in each setting.

### Array CGH analysis

Array CGH analysis was performed using the Agilent kit (Agilent Technologies, Palo Alto, CA, USA) as described.[13] A total of 1 μg DNA from patients and male controls were double-digested with *Rsa*I and *Alu*I for 4 h at 37 °C. After column purification, each digested sample was labeled by random priming for 2 h using Cy3-deoxyuridine triphosphate for the patient DNA and Cy5-deoxyuridine triphosphate for the control DNA. Labeled products were purified by Microcon YM-30 filter units (Millipore, Billerica, MA, USA). After probe determination and pre-annealing with Cot-1 DNA (Invitrogen, Carlsbad, CA, USA), hybridization was performed at 65 °C with rotation for 40 h. The hybridized array was analyzed with the Agilent scanner and the Feature Extraction software (v9.5.3.1; Agilent). A graphical overview was obtained using the CGH analytics software (v3.5.14; Agilent). The UCSC Genome Browser was used to retrieve the reference genome sequence (http://www.genome.ucsc.edu).

### LA-PCR and sequencing

LA-PCR reaction was performed with the appropriate forward and reverse primers that were used to map the gross deletion boundaries as described previously.[14] PCR products were excised from the gel, purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), and sequenced on ABI Prism 3130XL sequence detection system (Applied Biosystems, Foster City, CA, USA).

## RESULTS

### *BTK* mutation analysis

PCR analysis of genomic DNA resulted in the amplification of *BTK* exons 1–15, but not of exons 16–19 in patient 1, and amplifications of exons 1–5, but not of exons 6–19 in patient 2.

### FISH analysis

To confirm a large deletion in the *BTK* gene and to investigate a possible deletion of the *TIMM8A* gene, we performed FISH analysis with a *BTK–TIMM8A*-specific probe. Cells from both patients 1 and 2 lacked signals of this probe (Figures 1a and b), indicating a contiguous

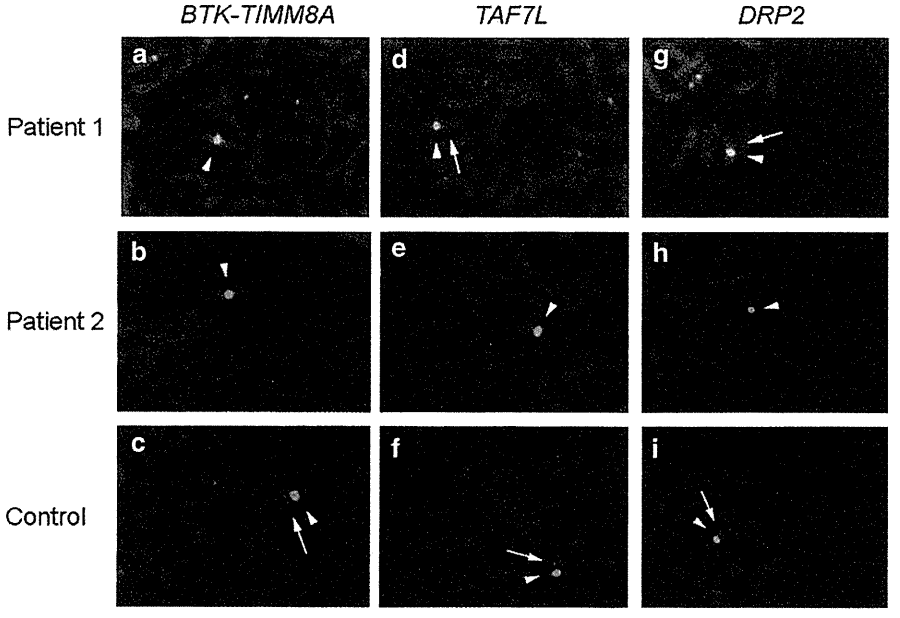


**Figure 1** Identification of multigene detections by fluorescence *in situ* hybridization analysis with Bruton's tyrosine kinase (*BTK*)–*TIMM8A*- (**a–c**), *TAF7L*- (**d–f**) and *DRP2*-specific probes (**g–i**) and the X-chromosome-specific DXZ1 probe in patient 1 (**a**, **d** and **g**), patient 2 (**b**, **e** and **h**) and healthy male control (**c**, **f** and **i**). The DZX1 probe is shown in green (arrow heads), whereas the *BTK–TIMM8A*, *TAF7L* and *DRP2* probes are shown in red (arrows).

gene deletion syndrome of XLA and MTS. To study the extent of the deletion further, we performed additional FISH experiments using *TAF7L*- and *DRP2*-specific probes. Lymphocytes from patient 1 showed normal signals for both probes (Figures 1d and g), but cells from patient 2 failed to give signals for either *TAF7L* or for *DRP2* (Figures 1e and h). We conclude that the genomic deletion in patient 1 is restricted to the *BTK* and *TIMM8A* genes, whereas the deletion in patient 2 is considerably larger involving *TAF7L* and *DRP2*.

### Array CGH analysis

To study the extent of the deletions in patients 1 and 2, we performed array CGH analysis of genomic DNA. In patient 1, we found an interstitial loss in copy number in the Xq22 region, involving the *BTK* and *TMM8A* genes, spanning a minimum of 63 kb (Figure 2). In patient 2, we observed a deletion of at least 138 kb including the *BTK*, *TIMM8A*, *TAF7L* and *DRP2* genes (Figure 2). These results confirmed the FISH analysis of patient 1 having a deletion of *BTK* and *TIMM8A*, and of patient 2 having a deletion involving the *BTK*, *TIMM8A*, *TAF7L* and *DRP2* genes, similar to what was found for patient 3.[9]

### Analysis of the break point junctions

On the basis of FISH and array CGH results, PCR primers were designed to span the putative break points. Sequence analysis of the PCR products from patient 1 revealed that recombination had occurred between a site in intron 15 of *BTK* and a site 4.4 kb upstream of *TAL7L* (Figure 3). It is an unequal crossover, which shares a 22-bp stretch of 100% homology and resulted in the deletion of 63 kb. To determine whether the break points were located in transposable elements, we analyzed the ± 1000 bp genomic sequences flanking the break point regions against reference collection repeats.[15] The results revealed that both the distal and proximal deletion break points of patient 1 are located within short interspersed element of the *Alu* subclass (Figure 4).

In patient 2, the deletion extended from a site 4.4 kb upstream of *DRP2* to a site in intron 5 of *BTK*, encompassing 149.7 kb (Figure 3). Break point junction analysis revealed 3 bp microhomology between the two break point regions. Neither of the break points was located in DNA sequences derived from transposable elements (Figure 4).

The gross deletion in patient 3 encompassed 196 kb, and the junction showed microhomology between the 5′ and 3′ break points (Figure 3). The 5′ break point was located upstream of the *DRP2* gene in a repeat derived from an endogenous retrovirus 2 (ERV2), whereas the 3′ break point in intron 1 of *BTK* was not located in a transposable element, but close to an *Alu* element (Figure 4).

Thus, two of the three break point regions of the XLA–MTS patients we studied showed involvement of transposable elements. Although six out of eight break points of *BTK* deletions were found to be located in an *Alu* element in previous studies,[14,16,17] we observed *Alu* element involvement only in patient 1.

### Transposable element involvement in XLA and XLA–MTS

It was previously shown that *BTK* gross deletion break points were frequently located in *Alu* elements. To study whether gross deletions resulting in XLA or XLA–MTS are derived from similar deletion mechanisms, we analyzed all reported gross deletions in XLA and XLA–MTS patients (Figure 5).[9,14,16,17] Including our patients, we collected data from five XLA and six XLA–MTS deletions. The deletion size clearly differed: XLA–MTS patients, as expected, had much larger disease-causing deletions. As *BTK* and *TIMM8A* are in close genomic proximity, a deletion < 10 kb can already disrupt both genes.

Interestingly, all deletions < 10 kb involved *Alu* elements, whereas only two out of six deletions > 10 kb had break points located in *Alu* elements (Figure 5). These results imply that *Alu* elements are more frequently associated with smaller deletions, whereas other elements, such as long interspersed elements and ERV elements, seem to be more frequently associated with larger deletions. As XLA–MTS deletions are on average larger than XLA deletions, they show less frequently involvement of *Alu* elements.

## DISCUSSION

The contiguous gene deletion syndrome involving *BTK* and *TIMM8A* has previously been described in 12 patients from nine unrelated families with deletion sizes ranging from 20–196 kb.[8–10] Of these, only three break point junctions have been cloned.[14,16] In this study, we describe three patients with 63, 150 and 196 kb large deletions at the Xq22 region, which included *BTK* and *TIMM8A*.

Short interspersed elements of the *Alu* subclass are the most frequently occurring interspersed repeat elements in the human genome: the 280-bp sequence occurs approximately every 4 kb in the human genome.[18] Mispairing between such repeats has been shown to be frequent causes of deletions and duplications. *Alu/Alu*-mediated genomic rearrangements are classical homologous recombination that result in a loss or gain in the number of nucleotide bases,

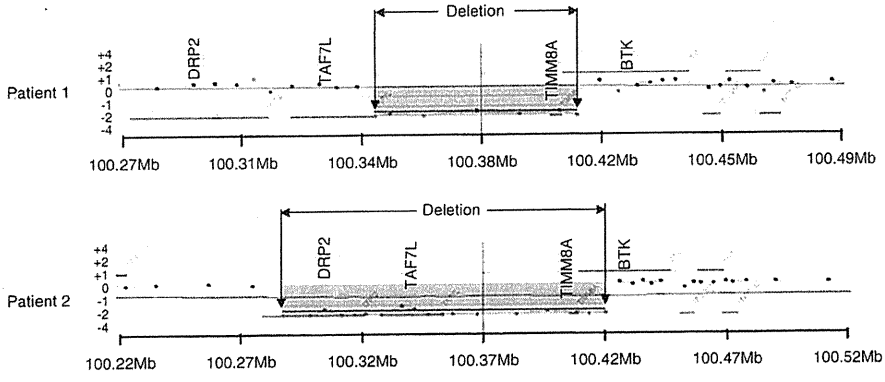


**Figure 2** Array comparative genomic hybridization analysis of the X-chromosomes of patient 1 (upper panel) and patient 2 (lower panel). Grey belts indicate regions of continuously reduced copy number around Xq22. Upper panel discloses a deletion of at least 65.8 kb including the Bruton's tyrosine kinase (*BTK*) and *TIMM8A* genes in patient 1, and lower panel discloses a deletion of at least 138 kb including the *BTK*, *TIMM8A*, *TAF7L* and *DRP2* genes in patient 2. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

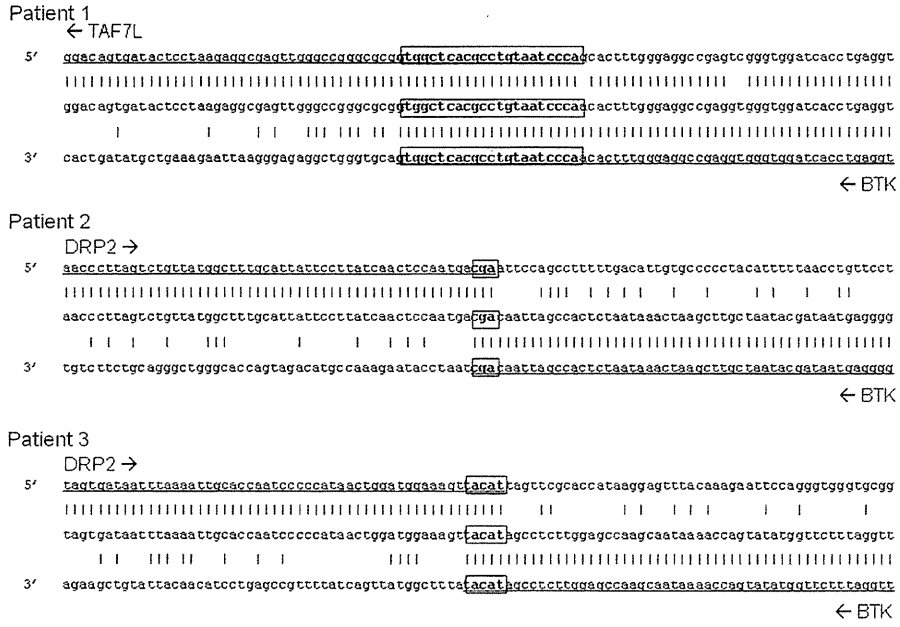


**Figure 3** Sequences of the gross deletion break point junctions of three patients compared with control sequences. The upper, middle and lower panels indicate patient 1, patient 2 and patient 3. Microhomology regions at the junctions are boxed. BTK, Bruton's tyrosine kinase.

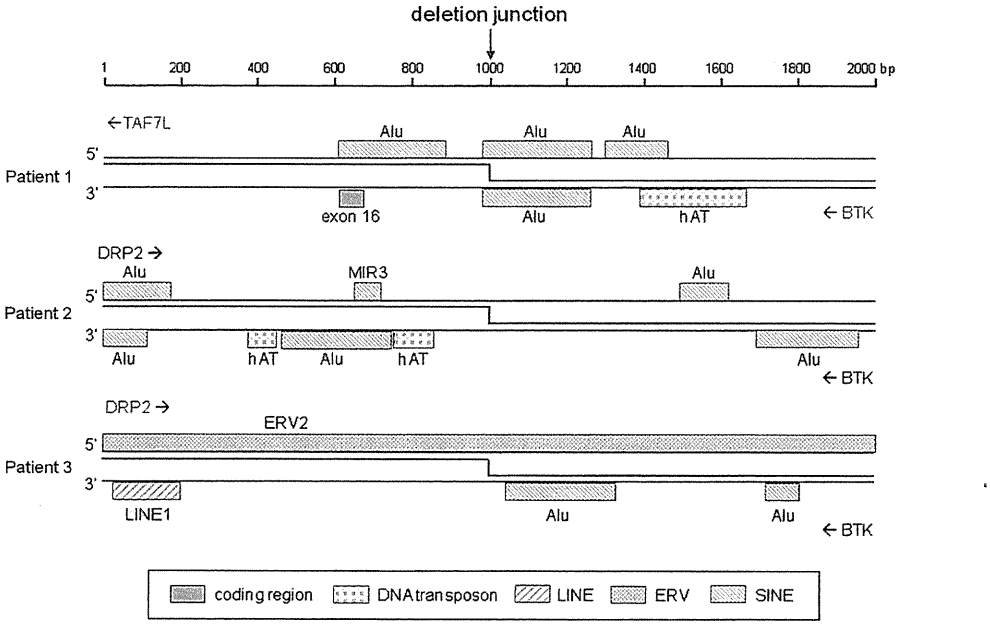


**Figure 4** A ±1000 bp flanking the gross deletion break points of patient 1 (upper panel), patient 2 (middle panel) and patient 3 (lower panel). Besides coding elements, four types of transposable elements are indicated: DNA transposons, endogenous retrovirus (ERV), long interspersed element (LINE) and short interspersed element (SINE). BTK, Bruton's tyrosine kinase.

causing approximately 0.3% of human genetic diseases.[19,20] Recently, the break point junctions of eight XLA patients with a *BTK* gross deletion were analyzed for the presence of transposon-derived repetitive elements. Most of the break points were located in *Alu* elements.[14] These observations have suggested a general role for *Alu* sequences in promoting recombination in the *BTK* gene. Besides deletions, *Alu*-mediated recombination may also promote genomic duplications in *BTK*, depending on how the break points are joined.[16] The 5′ and 3′ break points in patient 1 were located in *Alu* elements, similar to most XLA patients with gross deletions affecting the *BTK* gene.[14,16,17] The break points of patients 2 and 3, however, were not located in *Alu* elements, and, intriguingly, the homology regions were very small repeat fragments of only 3 or 5 bp nucleotides, implying that the deletion was not due to an unequal homologous recombination.

A comparison of the deletion size and the presence of transposable elements resulting in gross deletions causing XLA and XLA–MTS strongly suggests that *Alu* elements are mainly involved in <10 kb

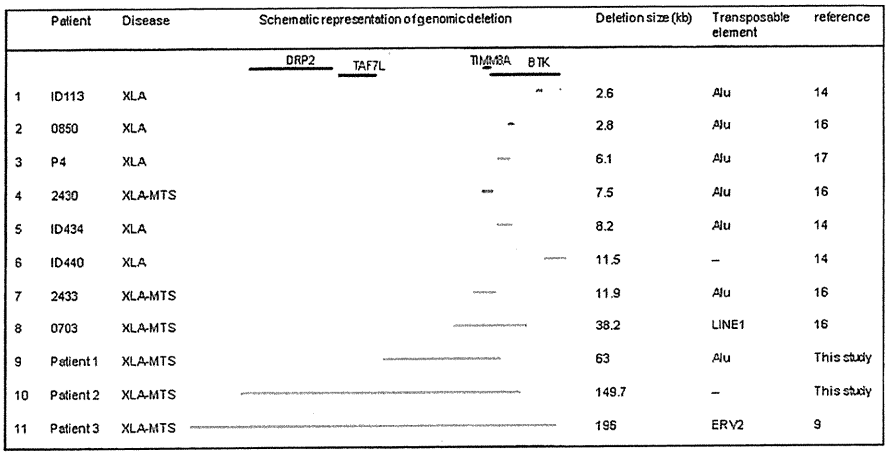


**Figure 5** Schematic representation of all molecularly characterized gross deletions in patients with XLA and XLA–MTS. The patients are listed according to the size of the gross deletion and for each patient, the disorder (XLA or XLA–MTS) is given as well as the involvement of transposable elements. BTK, Bruton's tyrosine kinase; ERV, endogenous retrovirus; LINE, long interspersed element; MTS, Mohr-Tranebjærg syndrome; XLA, X-linked agammaglobulinemia.

deletions. As most deletions underlying XLA–MTS are >10 kb, the involvement of *Alu* elements in deletions causing XLA–MTS is less frequent than in those associated with XLA. Thus, it is likely that the majority of the deletions underlying XLA–MTS arise from mechanisms that are different from those underlying XLA.

We used a combined approach of DNA FISH, array CGH and LA-PCR to characterize the break point regions in our patients and clone the break point regions. In doing so, we were able to establish a genetic basis of the disease. This is important for patient care with respect to genetic counseling and carriership analysis in family members. Wide use of DNA FISH and array CGH analysis to establish genetic abnormalities will support higher rates of gross lesions. This will not contribute to patient care, but to general understanding of complexity and frequency of these types of genetic lesions.

In patients 2 and 3, the *TAF7L* and *DRP2* genes were deleted in addition to *BTK* and *TIMM8A*. *TAF7L* encodes an RNA polymerase II TATA-box-binding protein-associated factor II protein, which is expressed only in male spermatogonia and may have a role in pre-meiotic stages of mammalian spermatogenesi.[21] The *DRP2* gene encodes the dystrophin-related protein 2, which is a member of the dystrophin family of proteins performing a critical role in the maintenance of membrane-associated complexes at points of intercellular contact in vertebrate cells.[22] It is expressed principally in the brain and spinal cord. Disruption of the DRP2-dystroglycan complex is followed by hypermyelination and destabilization of the Schwann cell-axon unit in *Prx* (−/−) mice.[23] However, no human pathology has been reported due to any mutation in these genes. Both patients presented with recurrent infections and hearing loss as typical symptoms of contiguous gene deletion syndrome of XLA and MTS, and showed no pathological findings which might be associated with the deletion of *TAF7L* and *DRP2* genes. Therefore, these genes might not have a critical function in humans or other proteins might compensate for their loss.

In conclusion, sensorineural hearing loss in XLA should raise concerns about a possible contiguous gene deletion syndrome. Array CGH analysis combined with FISH analysis can provide a more accurate diagnosis of large deletions. Not only transposable elements and microhomology, but also other mechanisms may contribute to gross rearrangements involving the *BTK* gene.

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