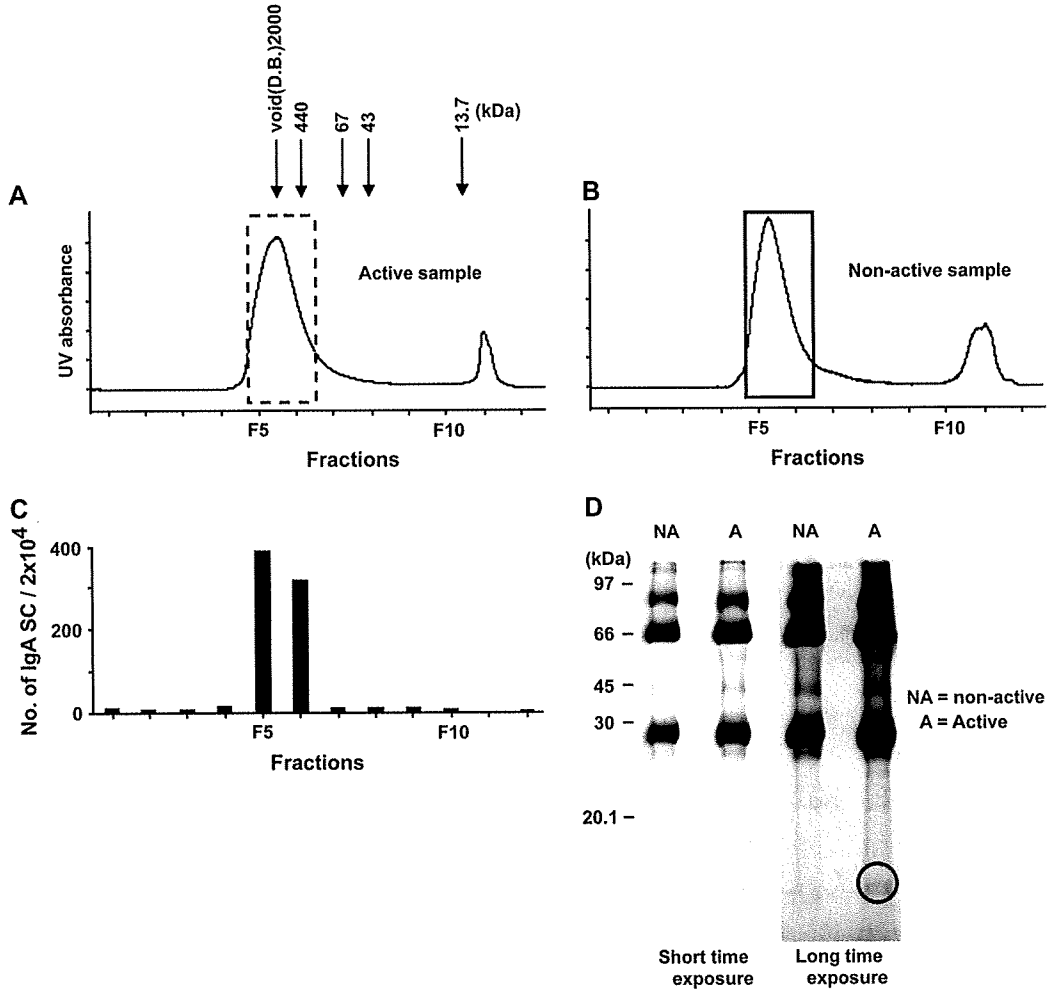

Fig. 4. Second gel filtration column chromatography of active and non-active colostrums. (A) Active fraction and (B) non-active fractions separated by anion exchange chromatography were applied to a second Superdex 200 10/30 GL column and eluted with 20 mM ammonium hydrogen carbonate. Elution of proteins was monitored by UV detector. The arrows indicate the positions where the molecular weight standards were eluted. The standards were blue dextran (2000 kDa), ferritin (440 kDa), albumin (67 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa). (C) IgA-inducing activity in the second GFC in (A) was tested as described in the legend of Fig. 1. The activity was found in fraction F5 and F6. (D) The IgA-inducing fractions (marked with the dashed line) and corresponding non-active fractions (marked with solid line) were pooled. The proteins in aliquots of both fractions were labeled with Cy5 and separated by SDS-PAGE. A and NA denotes the active and the non-active samples, respectively. With long time exposure to Cy5 (right) several minor bands including a low molecular weight band (marked with a circle) were observed selectively in the active sample.

spots significantly increased in active fraction under the conditions where standardized average spot volume ratios exceed two times and P values were $<10^{-2}$ in three parallel gels (Fig. 5A). To take into consideration of post-translational modification and ratio of fluorescence intensity of active spot to corresponding non-active spot, we divided the 37 spots into seven groups. One spot in each group was excised from gels and subjected to gel digestion with trypsin. Resulting peptides then were processed for LC-MS/MS analysis. In a search of the National Center for Biotechnology Information non-redundant database, peptide mass information identified seven proteins (Table 1, positions marked in Fig. 5A). Of the identified proteins, we focused on lactoferrin, lactadherin and syntenin-1. In the case of the small molecular mass protein band (long time exposure to Cy5) marked as a circle (Fig. 4D), protein band was excised and subjected to gel digestion with trypsin. According to MS analysis of the resulting polypeptides, the proteins were identified as Ig kappa, lambda and alpha-1 chain C region.

GST-syntenin-1 fusion protein induces preferentially IgA SC from naive B cells

GST-syntenin-1 fusion protein was expressed in *E. coli* and purified as described in Methods. The fusion protein was expressed upon IPTG induction as a major protein and was exclusively recovered in soluble form. GST-syntenin-1 was added to the B cell culture with different concentrations and also GST alone in the same concentrations. It was demonstrated that GST-syntenin-1 fusion protein was able to induce the generation of IgA SC from cord blood naive B cells in a dose-dependent manner, till saturation at the concentration of 5 μg ml^{-1}, but no IgA induction was detected

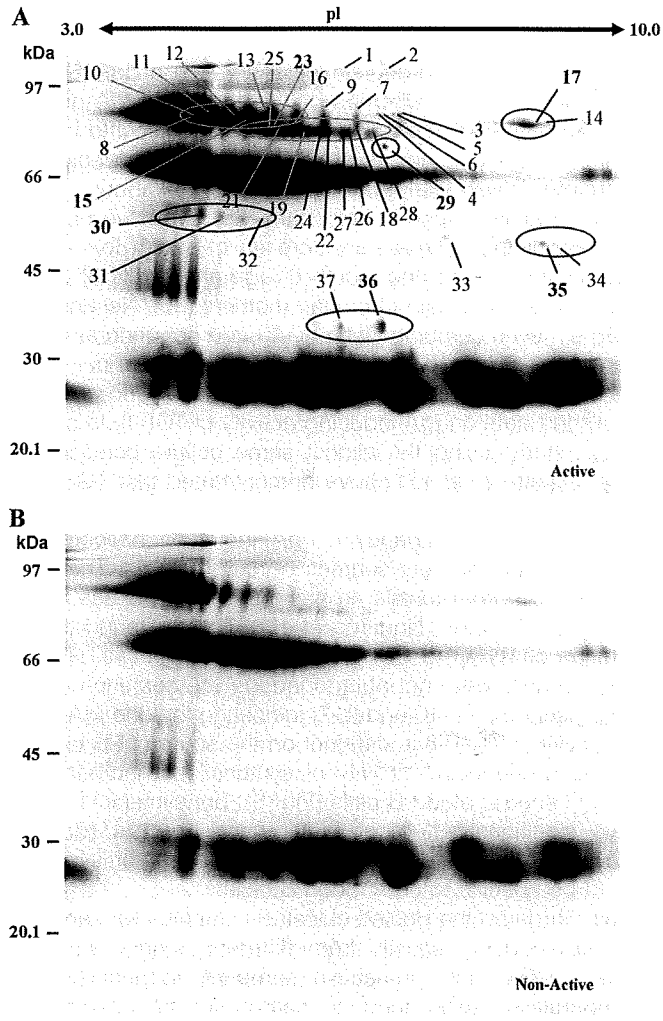

Fig. 5. Differential expressed protein spots displayed in two-dimensional difference gel electrophoresis (2-D DIGE) image. Proteins of active (A) and non-active (B) fractions were labeled with cyanine dyes and mixed together for 2-D DIGE analysis. A pool of the two fractions was also labeled another cyanine dye. The mixture was first separated by IEF (pH 3 to pH 10; horizontal axis) and further separated by 12% SDS–PAGE (vertical axis), which stretches from ~100 (top) to 15 kDa (bottom). (A) Proteins in active fraction labeled by Cy5. (B) Proteins in non-active fraction labeled by Cy3. 2-D gel electrophoresis of the active sample shows 37 spots that are, two times or more, higher in the active colostrum than in the non-active ones. Spots with similar molecular masses and different iso-electric points are grouped as marked by circles. The spots with bold numbers show the identified proteins by LC-MS/MS.

with GST (Fig. 6A). Importantly, the cultures in the presence of GST-syntenin-1 (5 μg ml^{-1}) did not generate IgG SC (Fig. 6B). To confirm the actual IgA induction by syntenin-1, the cells cultured with GST-syntenin-1 were checked for surface expression of IgA on B cells. As shown in Fig. 6(C), it was marked that the sizable number of B cells expressing surface IgA appeared in the cultures with GST-syntenin-1, though less than that with rIL-10. In the case of lactadherin, GST-lactadherin fusion protein expressed in *E. coli* was recovered in soluble fraction. Therefore, MBP-lactadherin was prepared instead of GST-lactadherin. The fusion protein was

Table 1. Proteins identified in partially purified fraction of active colostrum by two-dimensional difference gel electrophoresis followed by LC-MS/MS

Spot no.	Protein	pI	Accession no.	Molecular mass (kDa)	No. of peptides matched	Coverage (%)
15	Polymeric Ig receptor	5.58	gi|31377806	84	14	18
17	Lactoferrin	8.51	P02788	80	13	23
23	Ig mu chain C region	6.35	P01871	50	8	18
29	Complement C3 precursor	6.02	P01024	188	8	5
30	IGHA1 protein	6.21	gi|13543597	55	6	15
35	Lactadherin precursor	8.47	Q08431	43	4	15
36	Syntenin-1	7.05	O00560	32	2	9

Accession numbers are from NCBI or Swiss-Prot.

recovered in a soluble form. However, purified MBP-lactadherin as well as human milk lactoferrin (Sigma) did not generate IgA SC from naive B cells even in the same concentration as that described in human milk (data not shown).

To address whether syntenin-1 is required for the IgA class switching of naive B cells, the expression of AID mRNA was examined by RT–PCR. As shown in Fig. 6(D), AID mRNA was detected even in the control B cells cultured only with DC and CD40L-transfected L cells, although the message was not detected in the cultures of B cells alone (data not shown). This is consistent with the data that ~1.6% of B cells in the CD40L culture system expressed IgA (Fig. 6C). In addition, expression of AID mRNA did not increase by the addition of syntenin-1 or IL-10. These data suggest that syntenin-1 does not trigger IgA class switching but rather acts on post-switched B cells probably as a proliferation or survival factor.

IgA-inducing activity of colostrum is associated with the presence of syntenin-1

To investigate whether syntenin-1 was eluted in other fractions except the IgA-inducing activity fractions, we examined the void volume fractions of the first GFC and the remaining eluted fractions (other than the void volume) of both the active and the non-active samples (Fig. 7A and B) by western blot using anti-syntenin-1-specific antibody. Syntenin-1 (32 kDa) was detected in the void volume fractions (≥440 kDa) but not in the other eluted fractions. The higher concentration of syntenin-1 in the active sample than that of the non-active one was clearly noticed (Fig. 7C). Finally, we examined 10 colostrum samples for the relation between IgA-inducing activity evaluated by ELISPOT and syntenin-1 levels assessed by western blot (Fig. 8). It was clearly demonstrated that colostrum with IgA-inducing activity contained detectable levels of syntenin-1 but colostrum without IgA-inducing activity did not. The concentration of syntenin-1 in the active colostrums was estimated to be 87.3 ± 8.7 μg ml^{-1} by western blot analysis using recombinant synthenin-1 as a standard (data not shown).

Discussion

The aim of the present study was to identify unknown factors in colostrums regulating generation of IgA-producing cells from naive B cells. For this purpose, we used the CD40L system in which naive B cells were cultured together with DC and CD40L-transfected L cells (21). Using this culture system, we showed that the addition of some colostrum samples alone to the culture resulted in the appreciable generation of IgA SC from cord blood naive B cells (Fig. 1). IL-10 has been shown to promote production of IgA as well as IgG by CD40L-activated naive B cells (14). However, we demonstrated a marked difference between the cultures with colostrum and rIL-10. Namely, the cultures with colostrum alone induced the predominant generation of IgA SC from cord blood B cells, whereas rIL-10 generated IgA and IgG SC similarly (Fig. 2). TGF-β is a well-documented switch factor for IgA (24) and is abundantly present in colostrum. Therefore, it was likely that TGF-β might contribute to IgA-inducing activity seen in colostrum (25). We have observed the association between an increase of serum IgA in newborn infants during one month of age and levels of TGF-β1 and TGF-β2 in colostrum of their mothers (26). Nevertheless, rTGF-β1, even combined with rIL-10, did not generate significantly IgA SC from cord blood naive B cells in our culture system (Fig. 2). These results are contradictory to previously published data on IgA-inducing activity of TGF-β. In contrast to our study, using the almost same culture conditions as ours, Fayette *et al.* (21) have demonstrated that TGF-β can enhance IgA induction by human naive B cells. One possibility for this discrepancy in IgA induction between both studies is the different sources of naive B cells. They used IgD^+ B cells from tonsils as naive B cells, whereas we did cord blood B cells. Nagumo *et al.* (27) have found that the addition of TGF-β to CD40-stimulated cord as well as adult blood B cells were not able to induce IgA secretion even in the presence of IL-10 and IL-2, indicating that the IgA-inducing activity of TGF-β is different on the source of B cells. As for the IgA-inducing activity of syntenin-1, it increased the IgA SC in cord blood B cells (Fig. 6), but syntenin-1 did not have significant effect on adult naive B cells (data not shown). These data indicate that the effect of syntenin-1 could be specific to cord blood B cells.

We prepared the pooled colostrum samples with and without IgA-inducing activity from different donors, which we called active and non-active samples, respectively, and comparatively used them to search for the IgA-inducing factor in colostrum. We identified seven proteins to be higher (two times or more) in the active colostrum than in the non-active one with comparative quantitative proteomic analysis

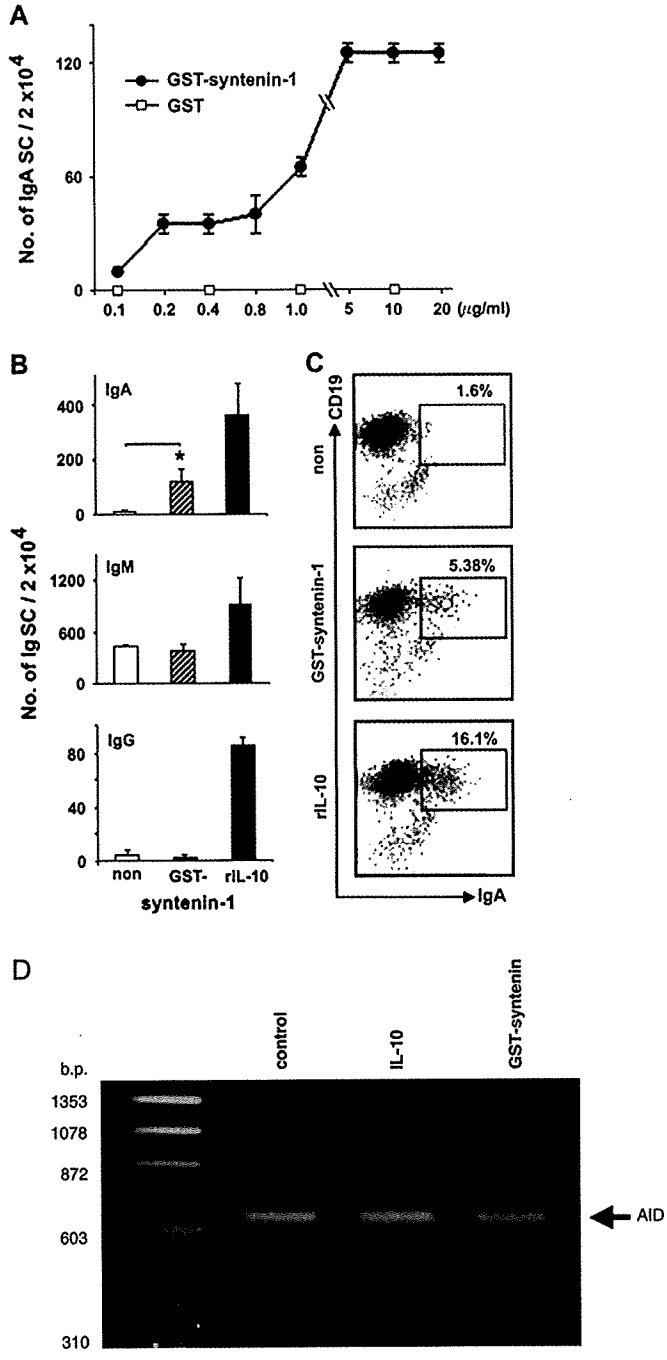

Fig. 6. IgA Induction by GST-syntenin-1. Generation of Ig SC in the CD40L culture system was evaluated as described in the legend of (Fig. 1A and B). (A) GST-syntenin-1 was added to the culture in increasing concentrations and also GST alone in the same concentrations. It was marked that GST-syntenin-1 induced the generation of IgA SC from naive B cells in a dose-dependent manner till saturation at the concentration of 5 μg ml^{-1}, whereas there was no IgA induction by GST alone with any of its concentrations. (B) Whereas rIL-10 (200 ng ml^{-1}) induced IgG, IgA and IgM SC considerably, GST-syntenin-1 (5 μg ml^{-1}) preferentially induced IgA SC. $^*P < 0.05$. (C) The cells cultured with GST-syntenin-1 fusion protein 1 (5 μg ml^{-1}) or rIL-10 (200 ng ml^{-1}) were examined for expression of surface IgA. After 5 days, the cells were harvested, stained with both anti-CD19-PE and anti-IgA-FITC and analyzed by a flow cytometer. The sizable number of B cells expressing surface IgA appeared in the cultures with GST-syntenin-1, though less than that with rIL-10. Values in the cultures with rIL-10 are means ± SEMs ($n = 3$). Results are representative of three separate experiments. (D) Evaluation of mRNA for AID by RT-PCR. Naive B cells in the CD40L culture system were cultured with IL-10 (200 ng ml^{-1}) or GST-syntenin-1 (5 μg ml^{-1}) for 6 days. At the end of this time, cells were harvested, RNA extracted, and semi-quantitative RT-PCR was performed to evaluate AID transcript. Control: naive B cells + DC + CD40L cells, rIL-10: naive B cells + DC + CD40L cells + rIL-10, syntenin-1: naive B cells + DC + CD40L cells + GST-syntenin-1.

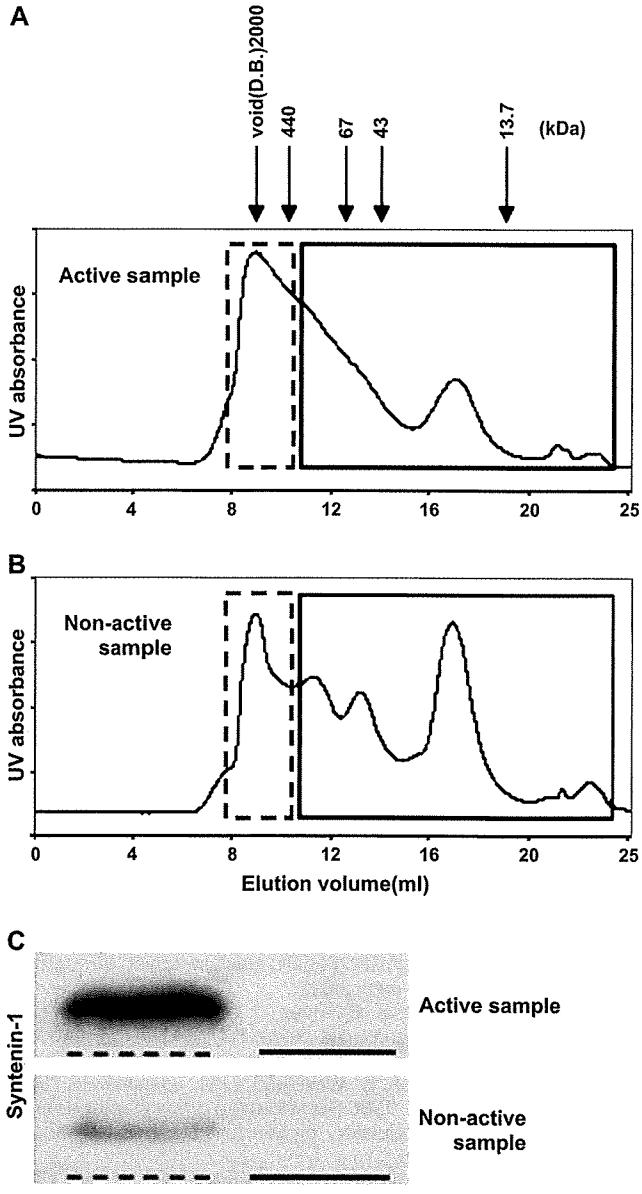

Fig. 7. Elution of syntenin-1 in the fractions with IgA-inducing activity of Superdex 200 10/300 GL column chromatography. (A) Superdex 200 10/300 GL column chromatography of the active colostrum. The dashed and solid squares show the fractions with and without IgA-inducing activity, respectively. (B) Superdex 200 10/300 GL column chromatography of the non-active colostrum. The squares show the corresponding fractions of the active colostrum (A). (C) Syntenin-1 (32 kDa) detected by western blot was found in the IgA-inducing activity fraction (≥440 kDa) and the corresponding fraction of the non-active sample, whereas no syntenin-1 band could be detected in the proteins eluted in the other fractions. The amount of syntenin-1 in the active sample was much higher than that in the non-active one. Molecular weight markers are the same as in Fig. 4. The figure is representative of three separate experiments.

(Table 1 and Fig. 5). Of the identified seven proteins, we focused on lactoferrin, lactadherin and syntenin-1. Bovine lactoferrin has been described to induce both mucosal and systemic immune response in mice where IgA and IgG secretion was enhanced in Peyer's patches and spleen from

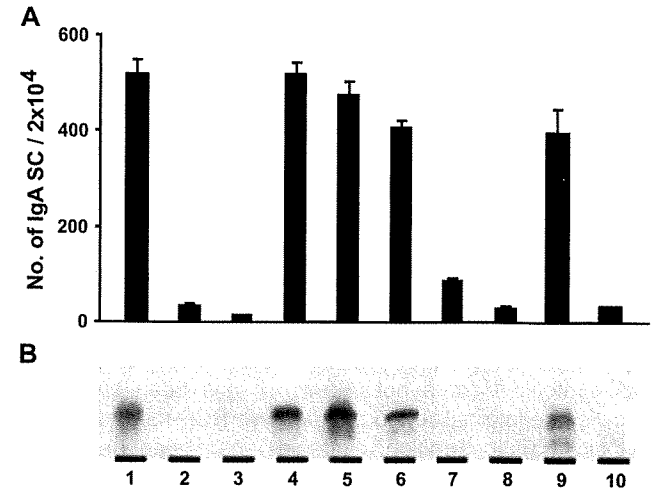

Fig. 8. Association of IgA-inducing activity with syntenin-1 in colostrum samples. Ten different colostrum samples were checked for IgA-inducing activity as described in the legend of Fig. 1. Syntenin-1 was examined by western blot analysis using 10 μl of each sample. Syntenin-1 was detectable only in samples with IgA-inducing activity but not in non-active samples. Values in the cultures are means ± SEMs (n = 3). Results are representative of three separate experiments.

lactoferrin-fed mice (28). Another study has shown that orally administrated lactoferrin restored humoral immune response in immunocompromised mice (29), and the addition of lactoferrin to the culture medium increased IgA secretion by B cells from mice administered lactoferrin orally (30). Lactadherin binds specifically to rotavirus and inhibits its replication (31) and works as an inhibitor of *E. coli* attachment to intestinal villi *in vitro* (32). Syntenin-1 is known as syndecan-binding protein. Syndecan is proteoglycan expressed on pre-B cells and plasma cells. Interestingly, syntenin-1 is implicated in IL-5 receptor signaling (33), and IL-5 has been described to have a role in IgA-inducing activity (34).

Among these three candidate proteins, we demonstrated that only sytenin-1 might have the ability to induce the generation of IgA SC from naive B cells (Fig. 6). Furthermore, western blot analysis disclosed that syntenin-1 was detectable in colostrum samples with the IgA-inducing activity but not in ones without IgA-inducing activity (Figs 7 and 8). These findings led us to the conclusion that syntenin-1 in human colostrum might be an important factor affecting IgA production. It was shown that IgA induction by colostrum itself was higher than that induced by GST-syntenin-1 fusion protein. In fact, 20 μg ml^{-1} of GST-syntenin-1, which concentration is almost equivalent to those of active colostrums, was less effective to the induction of IgA SC (Figs 6 and 8). So, it is possible that syntenin-1 is not the sole factor in colostrum responsible for IgA-inducing activity, and other factors in colostrum may be implicated with syntenin-1 in IgA induction. Another possibility is that syntenin-1 in human colostrum is in a bound form with other molecules because syntenin-1 (32 kDa) was eluted in a high molecular weight fraction (≥440 kDa). This binding may affect the activity of syntenin-1 in colostrum to be more active than recombinant GST-syntenin-1 in its produced form. Concerning a role of

syntenin-1 in the process of B cell differentiation, it might be involved in proliferation or survival of post-switched B cells rather than the IgA class switching of naive B cells by the following reasons. AID mRNA have already expressed in the control cells cultured only with DC and CD40L-transfected L cells and GST-synenin-1 did not increase the level of the message (Fig. 6D). On the other hand, the recombinant protein could increase the number of IgA-secreting B cells (Fig. 6C).

Concerning how syntenin-1 might act on naive B cells, it remains unclear whether syntenin-1 could transfer into the cells by endocytic transport. However, we found that syntenin-1 was recognized inside B cells by fluorescence microscopy after 24 h co-culture with cord blood B cells and GST-syntenin-1 (data not shown). It has been shown that B cells can internalize the antigen with BCR-mediated endocytosis (35). In addition, syntenin-1 has been detected in soluble fraction of colostrums and mature milk (36). Therefore, we speculate syntenin-1 is synthesized in mammary epithelial cells, secreted with breast milk and reaches the neonate mucosal immune cells and could be internalized in the cells. Syntenin-1 has also been detected in exosomal fractions of colostrums (37). As exosomes are small microvesicles released from cells and are possible to fuse with plasma membranes and endosomal membranes, syntenin-1 might be more easily released into cytoplasm through the fusion of these membranes *in vivo*.

In conclusion, we propose that syntenin-1 serves as one of IgA-inducing factors for B cells. However, the precise role of syntenin-1 in IgA induction awaits further studies, including animal experiments.

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Abbreviations

AID	activating-induced cytidine deaminase
BVA	biological variation analysis
cDNA	complementary DNA
CD40L	CD40 ligand
DC	dendritic cell
DTT	dithiothreitol
ELISPOT	Enzyme-linked immunosorbant spot
GFC	gel filtration chromatography
GST	glutathione S-transferase
IEF	iso-electric focusing
IPG	immobilized pH gradient
IPTG	isopropyl β-D-thiogalactoside
LC-MS/MS	liquid chromatography-mass spectrometry
MACS	magnetic-activated cell sorting
MBP	maltose-binding protein
mRNA	messenger RNA
MNC	mononuclear cells
MS	mass spectrometry
rIL-10	recombinant IL-10
RT	reverse transcription
SC	secreting cells
TGF	transforming growth factor
TNF	tumor necrosis factor

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Mutation of the *BTK* Gene and Clinical Feature of X-Linked Agammaglobulinemia in Mainland China

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Abstract
Introduction X-Linked agammaglobulinemia is a prototypical humoral immunodeficiency with the mutation of the Bruton's tyrosine kinase gene.
Methods We investigated the gene mutation and clinical features of 30 Chinese X-linked agammaglobulinemia (XLA) patients from 27 families. There were 26 mutations, including 11 novel and 15 recurrent mutations, distributing over the entire gene. The nucleotide and amino acid aberration, 1129C>T(H333Y) and 1196T>A(I355N), in SH2 have not been reported before. Five (I355N, W124R, R520X, I590F, G594E) of the 24 mutations not detected in the mothers receiving gene analysis were determined to be de novo. Two mutations occurred within intronic splice-site sequences (intron5(−2)A>G, intron17(−2)A>T).
Results and Discussion There are eight mutations in the PH domain, two mutations in the SH3 domain, three mutations in the SH2 domain, one mutation in the TH domain, and other 16 mutations in the TK domain. The mutations of protein domain is most common in TK (53%) domain and then in PH(8%) domain. Missense and nonsense mutations were found equal in 46% of the detected mutations. All of the patients are alive, but one died of liver cancer. Clinical features and serum Igs levels range variedly and were not correlated with genotypes. Our results demonstrated molecular genetic characteristics of XLA in mainland China.

Keywords Humoral immunodeficiency · X-linked agammaglobulinemia · mutation · Bruton's tyrosine kinase

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Introduction

X-linked agammaglobulinemia (XLA, MIM# 300300) is a prototypical humoral immunodeficiency disease. Bruton described the first case of XLA in a male patient with recurrent bacterial infection [1]. The increased susceptibility to infection in XLA is now known to be caused by a block in B lymphocyte differentiation, leading to profound hypogammaglobulinemia with few or no circulating B cells [1–3]. A gene was mapped that it is localized at Xq21.3-Xq22, and mutation was determined in patients with agammaglobulinemia, and the gene was called Bruton's tyrosine kinase (BTK) [4, 5]. BTK encompasses 37.5 kb containing 19 exons. It consists of five domains, each with a distinctive function: pleckstrin homology (PH), Tec homology (TH), Src homology 3 (SH3), SH2, and the kinase domain TK [5]. The genetic defect in XLA impairs early B cell development resulting in marked reduction of mature B cells in the blood. Studies from different countries and

ethnic groups have demonstrated that approximately 90% of males with presumed XLA bear mutations in BTK. In the last updated lists of online BTKbase, 1,155 entries have been compiled from 974 unrelated families with 602 unique molecular events, indicating that the vast majority of BTK mutations are private. The mutations are widely distributed over the gene, with only a few spared regions. This data would support a high spontaneous mutation rate for XLA. The BTK mutation has been already reported from many different regions and ethnic groups, but there is no report of large numbers BTK mutation of XLA from mainland China. In this study, we report for the first time the occurrence of BTK mutations in 30 Chinese male patients.

Methods

Patients

Most patients with XLA presented in this study were diagnosed and treated in the immunodeficiency clinic at the Children's Hospital of Fudan University, Shanghai, China, with a few patients referred for genetic counseling and molecular genetic analysis from other hospitals in China. XLA was diagnosed according to the recently updated criteria of a World Health Organization scientific group for primary immunodeficiency diseases [6]: low levels of circulating B cells (measured by levels of CD19-positive cells in blood samples), decreased or absent immunoglobulins in serum, and a typical clinical history with recurrent bacterial infection or a positive family history. Clinical characteristics, including the levels of immunoglobulins and B cell number, are described in Table I.

BTK Mutation Detection

The *BTK* mutation detection was performed as described previously [7]. Total RNA was extracted from PBMCs by using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA) and subjected to reverse transcriptase-polymerase chain reaction (PCR). The first complementary DNA (cDNA) strand was synthesized from 2 μg of total RNA using the SuperScript II preamplification system (Invitrogen Corp.). The *BTK* gene was amplified from cDNA with seven overlapping PCR primers. Genomic DNA was extracted by using DNAzol Reagent (Invitrogen Corp.) from PBMCs and subjected to PCR. The sequencing reaction was performed with a BigDye Terminator Cycle Sequencing Kit with ABI PRISM 3700 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Detected mutations were confirmed by sequencing from the opposite direction and sequencing genomic DNA encompassing exon–intron boundaries.

Results and Discussion

There have been reports for some Chinese cases of XLA from Hong Kong and Singapore and several cases from mainland China [8, 9]. We previously reported primary study results of this project in Chinese version, in that seven of XLA patient's mutations were described in brief but with limited clinical information [10]. We report presently the largest group of *BTK* mutations studied in China mainland, with a total of 26 mutations found in 30 patients with XLA (including the above seven cases detail). The mutations of *BTK*, including 11 novel mutations (42%) that did not appear in the *BTK*base or in the literature and 15 recurrent mutations (58%) that have been described previously (*BTK*base http://bioinf.uta.fi/BTKbase), are close to that of previous studies with 45% recurrent mutations in a group of 26 patients [11]. The wide spectrum in the type, location, and preponderance of unique *BTK* mutations in our patients with XLA mirrors previous reports (*BTK*base). In the noncoding region, two mutations occurred within intronic splice-site sequences. For these two splice-site mutations, deletion of exon 6 and exon 18 were detected, respectively, by sequencing cDNA using seven overlapping PCR primers; then, corresponding exons encompassing exon–intron boundaries were sequenced by using genomic DNA to find mutations(intron5(−2)A>G and intron17(−2)A>T). Intronic splice-site mutation can cause aberrant splicing in the coding region. Each splice-site mutation was a single nucleotide substitution observed within the splice junction of introns 5 (3′ end), 17 (3′ end). Other mutations were distributed at all different exons. There were seven mutations in the PH domain, one mutation in the TH domain, one mutation in the SH3 domain, three mutations in the SH2 domain, and 12 mutations in the TK domain. The mutations of protein domain is most common in TK (46%) and then in PH (27%). Our samples contained 14 mutations leading to a truncated *BTK* protein (54%). This is similar to the data reported to the *BTK*base, in which 55.4% of the mutations have been associated with truncation of the BTK protein. Missense and nonissense mutations were found to be equal in 46% of the detected mutations and distinctly different from the data in *BTK*base, and in previous reports, more missense than nonsense was identified. In contrast to premature termination mutations, missense mutations leading to amino acid substitution were not equally distributed over the *BTK* coding region. Missense mutations were found in the PH, TH, SH2, and TK domains, with a propensity for the PH and TK domains [12]. Our data showed that the missense mutations were mainly in the PH and TK domain but two in the SH2 domain. No disease-causing missense mutation has been described for the SH3 domain. Two cases from the same family with a nonsense-mutation-induced

Table I Clinical Characteristics of XLA Patients

Patient no.	Present age	Onset age	Age at diagnosis	Ig (g/L) levels at diagnosis			B cells (%)	Clinical presentation
				IgG	IgM	IgA		
p1	15y	3y	6y	3.00	0.21	0.31	2	Recurrent pneumonia, sinusitis, skin infection
p2	3y	1y	2.2y	2.80	0.30	0.11	0	Recurrent pneumonia, arthritis
p3	8y	9m	3y	2.70	0.50	0.38	1	Recurrent pneumonia
p4	12y	1y	7y	1.20	0.16	0.21	0	Recurrent pneumonia, meningitis
p5	5y	8m	5y	1.52	0.24	0.28	0	Recurrent pneumonia, otitis media, enteritis, sinusitis
p6	28y	8m	2y	1.50	0.09	0.08	0	Recurrent pneumonia, eczema, otitis media, bronchiectasis
p7	7.5y	1y	7y	0.70	0.07	0.21	0	Recurrent pneumonia, otitis media
p8	16y	2y	4y	0.35	0.10	0.05	0	Recurrent pneumonia, arthritis, encephalopathy (viral?, chronic meningoencephalitis), brain atrophy
p9	10y	2y	5.5y	1.90	0.07	0.04	0	Recurrent pneumonia, bronchiectasis
p10a	Died	3y	7y	0.20	0.30	0.49	0	Recurrent pneumonia, arthritis, liver cancer
p10b	3y	–	0	NE	NE	NE	0	
p11	16y	1y	7y	0.10	0.01	0.03	1	Recurrent pneumonia, arthritis
p12	11y	3y	10y	0.82	0.21	0.13	2	Pneumonia, otitis media
p13	9y	1y	6y	0.60	0.10	0.10	0	Otitis media, Recurrent pneumonia
p14	4y	2.5y	3.5y	2.39	0.06	0.07	0	Skin infection, pneumonia
p15	23y	2y	20y	1.38	0.16	0.24	0	Recurrent pneumonia, bronchiectasis
p16	12y	8m	10y	1.30	0.16	0.07	0	Recurrent pneumonia, enteritis, sinusitis, otitis media
p17	12y	3y	10y	0.40	0.12	0.14	0	Skin infection, recurrent pneumonia, purulent meningitis, deaf
p18	7y	4y	6.5y	0.81	0.10	0.07	0	Upper respiratory airway infection, pneumonia, otitis media
p19a	6y	6m	3.5y	2.10	0.16	0.07	0	Recurrent pneumonia
p19b	1.5y	–	0	NE	NE	NE	1	
p20	13y	2y	13y	4.15	0.58	0.54	0	Central nervous system infection, upper respiratory airway infection, enteritis
p21	11y	1y	9y	1.35	0.21	1.42	0	Recurrent pneumonia
p22	21y	4m	4y	2.90	0.16	0.08	0	Recurrent pneumonia, otitis media, enteritis, osteomyelitis, poliomyelitis, deaf
p23	25y	4m	9y	0.88	0.02	0.17	0	Recurrent pneumonia, meningitis, hepatitis C, bronchiectasis
p24	13y	9m	8y	0.55	0.06	0.07	1	Recurrent pneumonia, arthritis
p25	5y	6m	5y	0.50	0.07	0.13	0	Recurrent pneumonia
p26	9y	6m	8y	0.07	0.17	0.23	0	Recurrent enteritis, pneumonia, otitis media, arthritis
p27a	8y	1y	7y	1.56	0.23	0.09	0	Recurrent otitis media, skin infection, pneumonia, arthritis
p27b	3y	1y	2y	2.01	0.26	0.07	0	Oral infection, pneumonia, arthritis

y year(s), *m* months, *NE* not examined

termination in SH3 were detected in the present group. Two coding region mutations, H333Y and I355N, in SH2 have not been reported previously. Five (I355N, W124R, R520X, I590F, G594E) of 24 mutations were determined to be de novo because the corresponding carrier females did not show the mutation in peripheral blood lymphocytes. The mutations for individual patients are summarized in Table II.

The most frequently recurrent mutation sites in *BTK* are in arginine-coding CpG dinucleotides (e.g., R520 and R525). These sites contain the sequence purine-CpG-pyrimidine, which is the single most mutable tetranucleotide [11, 12]. It is notable that in this study one mutation in two unrelated patients, affecting exon 16 codon R525, the c.1706G>A change, is resulting in amino acid substitution R525Q. Several studies describe mutations at this codon [13–15], while the *BTK*base lists four mutations and 11 families with the R525Q mutations in this codon. Nineteen of the mutations lead to R520X in *BTK*base as is seen in patient 14. The defined "hot spot" suggests an important functional element that associates with the wild-type arginine in the TK domain. A second mutational array is located in the PH domain from L11 to T33 [16]. This second array contains a cluster of conserved positively charged amino acids probably involved in the phosphatidyl inositol-mediated membrane anchoring of the *BTK* protein [13, 17, 18]. Three families in our group have L11P, R13X, and R28C substitution, respectively, in this array.

We analyzed the data on disease course, B cell and immunoglobulin levels, and mutations in 30 patients from 27 families (Tables I and II). All the clinical presentation

Table II BTK Protein Expression and BTK Mutation Identified in the Present Study

Patient no.	Localization	Nucleotide aberration	Amino acid aberration	Protein domain	Pattern of mother
p1	Exon2	164T>C	L11P	PH	Heterozygous
p2	Exon2	169C>T	R13X	PH	Heterozygous
p3	Exon2	213C>T	R28C	PH	Heterozygous
p4	Exon5	472del TTCTCCCC	F114delX115	PH	Heterozygous
p5	Exon5	502T>C	W124R[a]	PH	Normal
p6	Exon5	503G>A	W124X	PH	Heterozygous
p7	Intron6	Intron5(−2)A>G[a]		PH	NE
p8	Exon6	546ins ACAGTGATCT	L138insX140[a]	PH	Heterozygous
p9	Exon8	721insTACATAG	I197insX207[a]	TH	Heterozygous
p10a	Exon8	832C>T	Q234X	SH3	Heterozygous
p10b	Exon8	832C>T	Q234X	SH3	Heterozygous
p11	Exon10	975G>A	W281X	SH2	Heterozygous
p12	Exon12	1129C>T	H333Y[a]	SH2	Heterozygous
p13	Exon12	1196T>A	I355N[a]	SH2	Normal
p14	Exon15	1690C>T	R520X	TK	Normal
p15	Exon16	1706G>A	R525Q	TK	Heterozygous
p16	Exon16	1706G>A	R525Q	TK	Heterozygous
p17	Exon16	1735insT	V535insX539[a]	TK	Heterozygous
p18	Exon16	1763G>A	R544K	TK	NE
p19a	Intron17	intron17(−2)A>T[a]		TK	Heterozygous
p19b	Intron17	intron17(−2)A>T[a]		TK	Heterozygous
p20	Exon17	1817G>T	R562L[a]	TK	Heterozygous
p21	Exon18	1896delGGA	W588delX588[a]	TK	Heterozygous
p22	Exon18	1900A>T	I590F	TK	Normal
p23	Exon18	1904A>C	Y591S	TK	Heterozygous
p24	Exon18	1913G>A	G594E	TK	Normal
p25	Exon18	1926T>G	Y598X[a]	TK	Heterozygous
p26	Exon18	1978C>T	L616F	TK	Heterozygous
p27a	Exon18	2025C>G	#Y631X	TK	Heterozygous
p27b	Exon18	2025C>G	#Y631X	TK	Heterozygous

Numbering of nucleotide and amino acid positions refers to cDNA sequence (GeneBank accession number NM_000061.1). Intron sequence information was obtained from the reference GeneBank sequence (accession number U78027.1)
NE not examined
[a] Novel mutation; TH Tec homology domain; SH3 Src homology; 3 domain SH2 Src homology 2 domain; TK kinase domain

was in the patients before diagnosis. Two of patients (p10b and p19b) were diagnosed after they were born because the family history was documented. One of the patients (p10a) died of liver cancer. It is well known that the high occurrence of neoplastic complications in primary immunodeficiency disease and thymoma, colorectal cancer, gastric carcinoma, hematological malignancies, and skin cancer have been reported [19, 20], but there have been no reports of liver cancer in XLA patient. The persistence of positive HBsAg in this patient may be one of the responsible factors of the liver cancer. Patient 8 suffered encephalopathy approximately 10 years after treatment with IVIG and then displayed brain atrophy. The clinical course of p8 was also with dermatomyositis, and he was suspected as to have enterovirus infection but without serum evidence. Encephalomyelitis and atrophic changes in the cerebral hemispheres were also reported by other authors [21]. Treatment of this case is still in challenge. As reported previously, there were no clear genotype/phenotype correlations established in patients with XLA [12, 22]. In our group, it is also difficult to find the prominent phenotype and genotype correlations. Patient 20 is one distinct patient; his IgG was above 4 g/L with slightly low IgM, IgA in serum, and absence of B cell. The clinical features were two episodes of mild and easy controlled central nerve infection and mild recurrent upper respiratory infection; otherwise, he was healthy. The mutation of patient 20 is R562L substitution in TK domain. Mild clinical presentation was mostly reported in adult diagnosed XLA groups. There appears to be no direct correlation between mutations in any specific domain with adult presentation of XLA. It has been suggested that these milder clinical phenotypes may be associated with *BTK* splice-site mutations, which produce lower levels of wild-type *BTK* transcripts [23, 24]; this still needs to be confirmed.

In conclusion, mutations found in our patients are heterogenous. The features of mutations of *BTK* were generally kept to that reported from other regions and ethnic groups. The predominant mutation data state that missense mutations were not absolutely numerous in comparison with nonsense in our patients. These are the first large numbers describing mutations in the *BTK* gene from mainland China.

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LETTER TO THE EDITOR

Pneumocystis jiroveci pneumonia as an atypical presentation of X-linked agammaglobulinemia

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X-linked agammaglobulinemia (XLA) is usually presented with clinical manifestations of bacterial respiratory and/or gastrointestinal infections below the age of 1 year, when the maternal IgG through placenta disappears from the circulation of the baby. Here, we describe an infant with XLA, who presented with interstitial pneumonia suggestive of *Pneumocystis jiroveci* (formerly *Pneumocystis carinii)* infection.

1 Patient report

A 3-month-old boy was admitted to Mie Hospital because of a long-standing cough, tachypnea and cyanosis. Physical examination showed a weight of 5.8 kg, temperature of 36.3°C, pulse of 170/min, respiration rate of 68/min, oxygen saturation on room air of 60%, respiratory retraction, and abnormal lung auscultation, but there was no lymphadenopathy. Chest radiology showed alveo-interstitial pneumonia (Fig. 1a), and chest computed tomography demonstrated diffuse ground-glass opacities (Fig. 1b). Laboratory tests showed a white blood count of 15,500/μl with 31.9% neutrophils and 54.4% lymphocytes, along with C-reactive protein of 0.02 mg/dl. The patient needed supplement oxygen, but did not require mechanical ventilation. He was first suspected of viral or *Chlamydia* pneumonia, and clarythromycin was administered but there was no improvement. Serum IgM against *Chlamydia trachomatis* and cytomegalovirus were negative. The patient's serum IgG, IgA and IgM levels were 108, 0 and 15 mg/dl, respectively, and the peripheral B cells were absent, suggesting an XLA despite the lack of a family history. With parental consent, the patient was diagnosed as having XLA based on a missense mutation (Arg28His) in the Bruton's tyrosine kinase (*BTK*) gene. The responses to phytohemagglutinin and concanavalin A are 56900 and 30900 cpm (control 141 cpm), respectively. Although T cell number and function were normal, he was suspected to have *Pneumocystis jiroveci* pneumonia. An elevated level of β-D glucan of >300 pg/ml (normal value <20) and KL-6 of 8750 U/ml (normal value, <500), suggested interstitial pneumonia caused by *Pneumocystis jiroveci*; however, polymerase chain reaction of sputum showed a negative result of *Pneumocystis jiroveci*. This may have been due to inadequate collection of sputum. He was treated with administration of sulfamethoxazole-trimethoprim (ST) and intravenous immunoglobulin (IVIG), with clinical improvement. The patient is currently well with IVIG replacement therapy and prophylactic administration of ST (Fig. 2).

2 Discussion

Interstitial pneumonia is caused by many organisms such as cytomegalovirus, adenovirus, fungus, and *Pneumocystis jiroveci*. An infant with interstitial pneumonia may be suspected of having an immunocompromised host with T cell deficiency, although there may have been *Pneumocystis jiroveci* pneumonia even in immunocompetent infants [1, 2]. XLA is a humoral immunodeficiency resulting from a block in early B cell development, and it is

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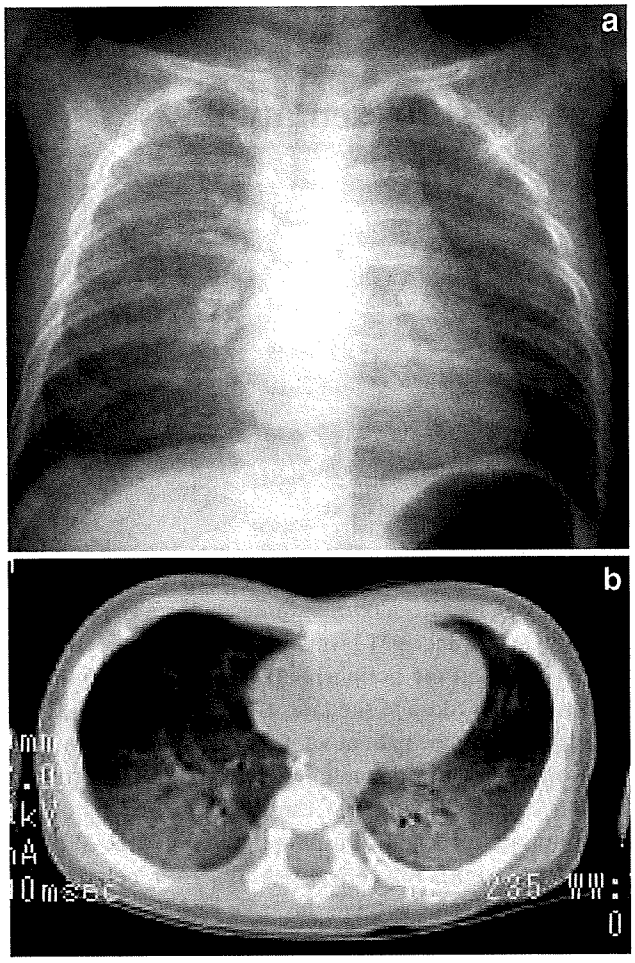

Fig. 1 Radiological findings in the patient. **a** Chest radiograph demonstrated bilateral perihilar opacities. **b** Computed tomography demonstrated diffuse ground-glass opacities in both lungs

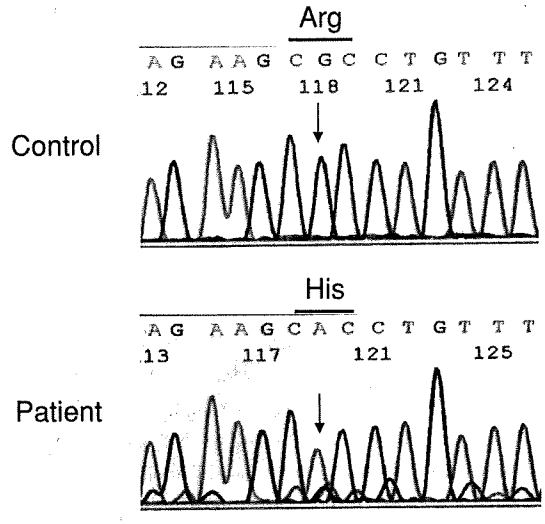

Fig. 2 *BTK* mutational analysis. DNA sequences of the exon 2 of the *BTK* gene in the patient and a control are shown. *Arrows* indicate position 215 in which the patient demonstrates a G to A mutation, indicating an amino acid substitution of Arg28His

clinically characterized by recurrent bacterial infections [3]. Although XLA patients sometimes demonstrate enteroviral infections, they usually show a normal response to viral and fungus infections because of normal T cell functions. However, 3 XLA patients with *Pneumocystis jiroveci* pneumonia have been reported [4–6]. One of these three was an adult receiving immunosuppressive therapy, but 2 patients were infants as in our case. It has recently been reported that Toll-like receptor signaling is impaired in XLA [7, 8] and that may be associated with the development of *Pneumocystis jiroveci* infection in XLA patients. Prophylactic administration of ST as well as IVIG replacement therapy would be recommended for the treatment of XLA patients.

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LETTER TO THE EDITOR

Early lineage switch in an infant acute lymphoblastic leukemia

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Childhood acute leukemia is usually associated with a good prognosis due to intensive chemotherapy [1]. However, infant leukemia still has a poor prognosis, which is associated with leukemic cell abnormalities involving the *MLL* (also known as *ALL1* or *HRX*) gene [2]. Lineage switches of the leukemic cell clones are rarely observed, especially in infant leukemia [3–9]. This phenomenon may cause poor prognosis for infant leukemia. This report presents a case of infant acute lymphoblastic leukemia (ALL) that demonstrated an early lineage switch to acute monocytic leukemia (AMoL).

The patient was a male infant born after a 37-week gestation and weighed 2.96 kg. He was delivered via a cesarean section because of fetal bradycardia. The family history was negative for cancer. He began vomiting frequently at 21 days of age and visited a family physician at 23 days of age. The gain in body weight was poor, and he presented with jaundice and marked hepatosplenomegaly. A laboratory examination showed a hemoglobin level of 5.2 g/dl, platelet count of 16,000/μl, white blood cell (WBC) count of 400,000/μl, aspartate amino transferase of 2,960 IU/l, alanine transferase of 745 IU/l and lactate dehydrogenase of 2,971 IU/l. He was diagnosed with acute leukemia, and admitted to the Toyama University Hospital. A physical examination at admission showed a body weight of 3,122 g (7 g gain per day from birth), a temperature of 37.3°C and a pulse of 150/min. His skin color was pale and icteric, and a bluish nodule was found on the right cubital fossa. Hepatosplenomegaly was detected, but no lymph node swelling was observed. The WBC count was 391,500/μl with 0.5% lymphocytes, 3.5% monocytes and 96% blastic cells (Fig. 1a). The blastic cells were negative for myeloperoxidase staining, and the immunophenotypic analysis disclosed that the cells were negative for CD10 and positive for CD19, CD22, CD34 and CD38. He was thus diagnosed with ALL of the pro-B phenotype. A cytogenetic analysis disclosed the karyotype of 46, XY, *t*(4;11)(q21;q23). Southern blotting revealed a rearrangement of the *MLL* gene, and a fluorescence in situ hybridization (FISH) analysis revealed split signals of the *MLL* gene. The patient underwent an exchange transfusion, resulting in the reduction of the WBC to 85,000/μl. The patient was treated with prednisolone for 7 days and the WBC count fell to 5,100/μl with 2.5% blastic cells. However, on day 8, the WBC count increased to 11,400/μl with 12.5% neutrophils, 6.5% lymphocytes, 70.5% monocytes, and 9% blastic cells (Fig. 1b), and atypical monocytes increased. An immunophenotype analysis disclosed that the cells were positive for CD20, CD13, CD14, CD15, CD33, CD41, CD61 and CD38. The patient was then diagnosed with AMoL. The peripheral blood smear at diagnosis was therefore reevaluated, and a small component of the monocytes showed an atypical morphology (Fig. 1c). The central nervous system was also involved.

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LETTER TO THE EDITOR

Three brothers of X-linked agammaglobulinemia: the relation between phenotype and neutropenia

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1 Introduction

X-linked agammaglobulinemia (XLA, i.e. Bruton's agammaglobulinemia [1]) is an X chromosomal recessive primary immunodeficiency syndrome, which is caused by insufficiency of antibody production. In 1993, *BTK* (Bruton's tyrosine kinase) gene, the critical gene of XLA, was discovered in Xq22 by Tsukada et al. [2] and Vetrie et al. [3]. We report a family case with XLA, and describe the difference of clinical outcome between the proband and his brothers.

2 Case report

The proband is a 2 years and 4-month-old male with anorexia as the chief complaint. His past medical history was not so severe, except that he had cold several times. He had two elder brothers, 7-year-old and 9-year-old, whose mother was a Phillipina, and father was a Japanese. Their past history was not remarkable.

His blood leukocytes was 5.1×10^9/L (neutrophil = 2.5×10^9/L), and all classes of serum immunoglobulin were decreased (IgG = 1.09, IgA = 0.17, IgM = 0.28 g/L) markedly. His bone marrow was normoplastic, and chromosomal analysis of blood sample (G-banding) showed 46XY. B cells in his blood lymphocytes were notably less (CD19+CD20+ = 0.08%) than normal. Analysis using flow cytometry of BTK-protein positive monocytes (established by Futatani et al. [4]) revealed that fluorescence intensities were equally decreased in three boys (the data of BTK-protein were reported in the previous report [5], Fig. 1). And a same missense mutation [5] (in exon 18, 1912G → C, Gly594Arg) was found by *BTK* gene analysis. Their mother was a carrier because this basepair was G/C heterotype.

Interestingly, his blood neutrophils began to decrease (min = 0.05×10^9/L) simultaneously from about 4 weeks after the first consultation. In addition, the proband had periodically six episodes of infection (pneumonia, tonsillitis, acute otitis media and bronchitis) in only 6 months. These episodes were cured by antibiotics alone, without intraveneous gammaglobulin replacement. However, these episodes had disappeared dramatically after administration of gammaglobulin (0.25 g/kg/dose per month). According to the serum IgG accumulated, the neutrophil count increased above the normal level. Elder brothers have been asymptomatic without administration of gammaglobulin.

3 Discussion

Figure 1 illustrates the family tree and their summaries. They had a same *BTK* gene mutation (1912G → C, Gly594Arg). Furthermore, their blood B cells and fluorescent intensities of BTK-protein decreased commonly. However, the frequency of infection episodes markedly differed between the proband and his brothers. Although total seven cases of XLA patients with Gly594Arg have ever been reported, we could not compare the phenotypes

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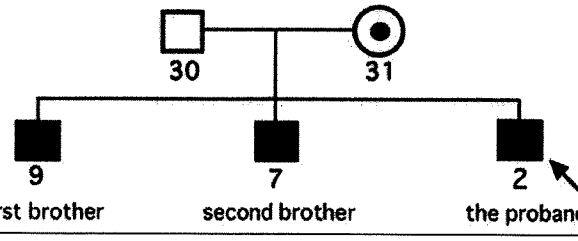

Fig. 1 The family tree and the summary of laboratory data and clinical episodes in three brothers

	first brother	second brother	the proband
Blood $CD19^+CD20^+$ lymphocytes	0.02% $0.2x10^6/L$	0.20% $1.6x10^6/L$	0.08% $0.2x10^6/L$
BTK protein positive monocytes (%)	8.9	5.1	7.2
BTK genotype	Gly594Arg	Gly594Arg	Gly594Arg
Serum IgG (g/L)	3.0	4.0	1.0
Episodes of bacterial infection	none	none	frequent (6 times / 6 months)
Blood neutrophil count ($x10^3/L$)	7.0	5.0	2.5 →0.05 →4.5

in our cases with those of other cases because they were published without clinical data. Generally in XLA patients, it has been recognized that the correlation between genotype and phenotype were not seen [6]. Kornfeld et al. [7] and Bykowsky et al. [8] have reported, respectively, a family with XLA whose same *BTK* mutation and discordant clinical outcome, as in our case. In elder brothers, IgG levels were relatively higher than that in the patient and neutrophil counts had kept above the normal level (their serum IgA and IgM were 0.26, 0.20 g/L in first brother and 0.15, 0.20 g/L in second brother, respectively). The proband only had neutropenia, and his neutrophil increased reversibly after replacement of gammaglobulin. These differences of clinical phenotypes and laboratory data may indicate that the neutropenia is associated with the frequency of bacterial infection in XLA.

In about 10–20% of XLA patients, episodes of neutropenia have been reported by Farrar et al. [9] (13/50 cases = 26%), Kanegane et al. [10] (16/87 cases = 18%), Lederman et al. [11] (10%) and Plo Rodriguez et al. [12] (4/37 cases = 10.8%). These authors have concluded that neutropenia is useful as a key to early diagnosis of XLA. Interestingly in those reports, no case with neutropenia was observed in any patients after receiving gammaglobulin.

The mechanism of neutropenia in XLA is still unknown. It is generally recognized that both neutrophil and gammaglobulin collaborate in bacterial infection. In XLA cases, the neutrophil life span may be shorter than that in intact cases, because gammaglobulin exhaust extremely and neutrophil has to fight against bacteria single-handedly. Kanegane et al. [10] have discussed, bacterial infections in some XLA cases might be caused by neutropenia why the mobilization activity of neutrophil were less than those in intact cases from bone marrow to peripheral blood.

On the other hand, it is possible that BTK deficiency is directly associated with neutrophil disfunction or neutropenia. Jefferies et al. [13] reported that BTK works as one of participants of Nuclear Factor kappa B activation by Toll-like receptor 4 in lipopolysaccharide stimulating model. Mangla et al. [14] concluded that BTK is likely to play a role at several stages on cell growth and development of myeloid lineages. However, these works cannot explain clearly the reason why genetically common cases complicate with or without neutropenia. Further investigations are needed to elucidate a mechanism of neutropenia in XLA patients.

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Detection of four genetic subgroup-specific antibodies to human metapneumovirus attachment (G) protein in human serum

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Human metapneumovirus (hMPV) strains are classified into two genetic groups, A and B, each of which is further divided in two genetic subgroups, A1, A2, B1 and B2. hMPV encodes two major surface glycoproteins, the fusion (F) and attachment (G) proteins, which may be immunogenic and protective antigens. Although the amino acid sequences of hMPV F protein are highly conserved, those of the G protein are highly variable with low amino acid identity between the two groups. To address the antigenic variation between the genetic subgroups, we developed an immunofluorescence assay (IFA) method using *Trichoplusia ni* (Tn5) insect cells infected with each recombinant baculovirus-expressed hMPV G (Bac-G) protein of the four genetic subgroups. The titre of each antibody to the four Bac-G proteins was measured by the IFA in 12 paired serum samples obtained from children infected with hMPV of each genetic subgroup. Although 11 of the 12 acute-phase serum samples in paired samples were negative for the antibody to any Bac-G proteins, all of the convalescent-phase serum samples in those paired samples were positive for the antibody to only one of the four Bac-G proteins of the infecting genotype of hMPV. Since the antibody response to hMPV G protein was transient and genetic subgroup-specific without cross-reactivity, four genetic subgroups on the basis of hMPV G protein could be identified as different serotypes. This assay may be useful for the study of immune responses of humans to different hMPV strains, especially for clarifying the risk of reinfection with hMPV.

INTRODUCTION

Human metapneumovirus (hMPV) has recently been recognized as an aetiologic agent of respiratory tract infections in children and adults (van den Hoogen *et al.*, 2001). hMPV and human respiratory syncytial virus (hRSV) are grouped in the same subfamily, *Pneumovirinae*, of the family *Paramyxoviridae*. Phylogenetic analysis of the nucleotide sequences indicated that there were two genetic groups, groups A and B, similar to the grouping of hRSV. Furthermore, each group could be subdivided into two subgroups, A1, A2, B1 and B2 (Bastien *et al.*, 2004; Biacchesi *et al.*, 2003; Ishiguro *et al.*, 2004; Ludewick *et al.*, 2005; Mackay *et al.*, 2004; Peret *et al.*, 2004; van den Hoogen *et al.*, 2004).

hMPV encodes two major surface glycoproteins, the fusion (F) and attachment (G) proteins, which may be immunogenic and protective antigens. The hMPV F protein has been shown to be a major antigenic determinant that mediates effective neutralization and protection against the two genetic groups of hMPV (Ishiguro *et al.*, 2005; Ma *et al.*, 2005; Skiadopoulos *et al.*, 2004, 2006). Since the amino acid sequences of hMPV F protein, as well as those of hRSV F protein, are highly conserved between the two groups, the hMPV F protein also plays a major role in the antigenic relatedness between the two groups of hMPV (Skiadopoulos *et al.*, 2004, 2006). Our previous studies indicated that the hMPV F protein was a major antigenic determinant that mediates extensive cross-lineage neutralization and protection (Ishiguro *et al.*, 2005; Ma *et al.*, 2005).

On the other hand, the hMPV G gene was shown to be highly variable, particularly in the extracellular domain, as a result of nucleotide substitutions and insertions and the use of alternative termination transcription codons (Biacchesi *et al.*, 2003; Peret *et al.*, 2004; van den Hoogen

A table showing the sequences of the primers used in this study is available with the online version of this paper.

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ORIGINAL ARTICLE

The syndrome of inappropriate secretion of antidiuretic hormone associated with SCT: clinical differences following SCT using cord blood and BM/peripheral blood

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Previously, we reported the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) as an underestimated complication associated with SCT. In the present report, we analyzed detailed data on a larger number of patients with SIADH following SCT and found different SIADH clinical features following cord blood SCT (CBSCT) and BMT/PBSCT. The median onset of SIADH following CBSCT and BMT/PBSCT was 19 and 46 days after SCT, respectively, and the median numbers of WBC at the onset of SIADH were 1.0 and 3.1 $\times 10^9$/l, respectively. Furthermore, severe symptoms such as seizures, somnolence and rigidity of limbs were observed only in patients with CBSCT (8/15 *vs* 0/10). These differences were statistically significant ($P<0.01$). Although the precise basis for SIADH following SCT still remains unknown, the different features of SIADH observed following CBSCT and BMT/PBSCT may provide important clues to the disease mechanism following SCT. Additionally, we confirmed our previous results that patients with SIADH showed a higher overall survival and event-free survival rates. However, we first suggested that they had some neurological disorders and that neurological sequelae such as developmental delay and seizures would consequently occur.

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Introduction

Hematopoietic SCT has been an approved treatment for hematological diseases (for example, leukemia, lymphoma and aplastic anemia), certain solid organ tumors, immunodeficiencies, metabolic diseases, among other disorders. However, SCT can cause many complications, such as GVHD, opportunistic infections and graft failure. Although the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) also seems to be one of these complications, there are only few case reports available on SIADH following SCT,[1,2] including our previous report.[3] Therefore, SIADH following SCT has had its importance underestimated.

In our previous report,[3] severe hyponatremia of less than 125 mmol/l was a complication in 27 (19.3%) of the 140 SCT patients, and 16 (56.9%) of them were diagnosed with SIADH. Moreover, multivariate analysis revealed that alternative donor and recipient below the age of 4 years were the only independent predictors of SIADH. However, the mechanism causing SIADH following SCT and the clinical characteristics are still poorly understood. Thus, in this report, we analyzed detailed data on a larger number of patients with SIADH following SCT.

Patients and methods

This study comprised 197 consecutive pediatric patients (126 boys and 71 girls) who received SCT for malignant (149 patients) or non-malignant disease (48 patients) at the Department of Pediatrics, Hokkaido University Hospital between February 1988 and March 2007. Of these patients, 164 received allogeneic transplantation and the remaining 33 patients received autologous transplantation. As prophylaxis for GVHD, almost all patients receiving BMT/PBSCT received CsA or tacrolimus (FK506) with MTX, whereas almost all patients undergoing cord blood SCT (CBSCT) received CsA with methylprednisolone. CsA and FK506 doses were appropriately adjusted to maintain trough levels of 150–200 ng/ml and 10–20 ng/ml, respectively. All patients received antifungal prophylaxis with amphotericin B or micafungin, antiherpes simplex virus prophylaxis with acyclovir and gut sterilization with polymixin B. Moreover, i.v. immunoglobulin and G-CSF were administered to all patients during the peritransplant

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period. Transplant characteristics of all patients are summarized in Table 1.

Serum sodium levels were measured in every patient by a routine automated analyzer every morning until 40 days after SCT and two to three times a week afterwards. We defined hyponatremia as a serum sodium level of less than 125 mmol/l on two consecutive days because serum sodium levels transiently between 125 and 135 mmol/l may not be clinically important. SIADH was diagnosed using the approach reported by Bartter and Schwartz.[4] Diagnosis requires the presence of all of the following criteria: (1) hyponatremia with hypotonicity of plasma; (2) urine osmolality in excess of plasma osmolality; (3) increased renal sodium excretion; (4) absence of edema or volume depletion and (5) normal renal and adrenal function.

A *t*-test or a χ^2 test was used to compare patients with CBSCT and BMT/PBSCT. Statistical analyses were performed using SPSS II for Windows (release 11.0.1J, SPSS Japan Inc.). A *P*-value of less than 0.05 was regarded as statistically significant.

Table 1 Transplant characteristics of all patients

	No. of patients
Disease	
Neoplastic	
ALL	62
First CR	30
Second CR	21
Third or later CR	11
AML	33
First CR	17
Second CR	11
Third or later CR	4
Not CR	1
Neuroblastoma	12
Non-Hodgkin's lymphoma	10
Myelodysplastic syndrome	8
Juvenile myelomonocytic leukemia	8
Rhabdomyosarcoma	7
CML	6
Yolk sac tumor	1
Hepatoblastoma	1
Primitive neuroectodermal tumor	1
Non-neoplastic	
Aplastic anemia	29
Wiskott–Aldrich syndrome	5
SCID	3
Kostmann syndrome	3
Hurler syndrome	2
Chronic granulomatous disease	2
Hunter syndrome	1
X-linked hyper IgM syndrome	1
Chronic active EBV infection	1
Hurler–Scheie syndrome	1
Donor	
Matched related	62
Mismatched related	16
Matched unrelated	48
Mismatched unrelated	38
Autologous	33
Source of stem cells	
BM	122
PB	25
BM + PB	4
Cord blood	46
Conditioning regimen	
TBI	93
BU	69
Melphalan (L-PAM)	57
CY	125
Etoposide (VP16)	93
Antithymocyte globulin	43
Acute GVHD (>2)	33
GVHD prophylaxis	
CsA	119
MTX	113
Tacrolimus (FK506)	33
Methylprednisolone	44

Results

Syndrome of inappropriate secretion of antidiuretic hormone developed in 25 (12.7%) of the 197 patients who received SCT, and a summary of the backgrounds of the patients with SIADH is provided in Table 2. They included 17 boys and 8 girls, ranging from 0 to 15 years old. Of them, 15 received CBSCT, 9 received BMT and 1 received PBSCT. No patient receiving autologous transplantation developed SIADH. Donors were HLA-mismatched unrelated (13 patients), HLA-matched unrelated (6), HLA-mismatched related (5) and HLA-matched related (1). In other words, 24 (96.0%) of the 25 patients with SIADH had received SCT from alternative donors. The symptoms of SIADH were nausea (15 patients), seizure (5), somnolence (2) and rigidity of limbs (1), but two patients exhibited no symptoms. The median of minimum serum sodium level was 120 mmol/l. The median onset of SIADH was 27 days after SCT and the median number of WBC at the onset of SIADH was 2.3×10^9/l.

Neurological sequelae such as developmental delay and seizure occurred in 5 (20.0%) of the 25 patients with SIADH, whereas in 2 (1.2%) of the 172 patients without SIADH ($P<0.01$). All patients with neurological sequelae had received allogeneic transplantation. In analysis limited to survivors, neurological sequelae occurred in 5 (23.8%) of the 21 survivors with SIADH, but they occurred in 2 (2.1%) of the 94 survivors without SIADH ($P<0.01$).

In the patients with SIADH, we compared the above-mentioned factors between patients after CBSCT and those after BMT/PBSCT (Table 3). Age, gender and minimum serum sodium level did not differ between them. However, severe symptoms such as seizure, somnolence and rigidity of limbs were observed in 8 (53.3%) of the 15 patients with CBSCT, but in none of the 10 patients with BMT/PBSCT ($P<0.01$). Additionally, SIADH developed earlier in patients receiving CBSCT (median onset, 19 days after SCT; range, 15–54 days) than in those receiving BMT/PBSCT (median onset, 46 days after SCT; range, 18–74 days), and the median numbers of WBC at the onset of SIADH were lower in patients after CBSCT (median, 1.0×10^9/l; range, $0.1–4.2 \times 10^9$/l) than in those after BMT/PBSCT (median, 3.1×10^9/l; range, $2.5–13.3 \times 10^9$/l) (Figure 1). These differences were statistically significant ($P<0.01$).