

Letter to the Editor

Quantification of κ -deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects

To the Editor:

X-linked agammaglobulinemia (XLA) is a primary immunodeficiency caused by severely decreased numbers of mature peripheral B lymphocytes as a result of a mutation in the *BTK* gene. Non-XLA is characterized by hypogammaglobulinemia with decreased B-cell counts (less than 2% of mature B cells) in the absence of the *BTK* gene mutation. Both XLA and non-XLA are caused by an early B-cell maturation defect.¹ In patients with XLA and non-XLA, recurrent infections appear between 3 and 18 months of age, whereas the mean age at diagnosis is 3 years.² This delayed diagnosis results in frequent hospitalization because of pneumonia, sepsis, meningitis, and other bacterial infections, which frequently require intravenous administration of antibiotics and can be fatal. Frequent pneumonia results in a high incidence of chronic lung diseases.³ Thus, early diagnosis and early treatment, including periodical intravenous immunoglobulin replacement therapy, is essential to improve the prognosis and the quality of life of patients with XLA and non-XLA.

In the process of B-cell maturation, immunoglobulin κ -deleting recombination excision circles (KRECs) are produced during κ -deleting recombination allelic exclusion and isotypic exclusion of the λ chain.⁴ Coding joint (cj) KRECs reside within the chromosome, whereas signal joint (sj) KRECs are excised from genomic DNA. cjKREC levels remain the same after B-cell division, whereas sjKREC levels decrease, because sjKRECs are not replicated during cell division.⁵ Because the B-cell maturation defects in XLA and non-XLA occur before κ -deleting recombination, KRECs are not supposed to be produced. Therefore, measurements of KRECs have the potential to be applied to the identification of these types of B-cell deficiencies in patients, which consist of around 20% of all B-cell defects.⁶ In addition, some types of combined immunodeficiencies show an arrest in B-cell maturation and can also be identified by this method. The success of newborn screening for T-cell deficiencies by measuring T-cell–receptor excision circles⁷ prompted us to develop a newborn screening method for XLA and non-XLA by measuring KRECs derived from neonatal Guthrie cards.

The study protocol was approved by the National Defense Medical College institutional review board, and written informed consent was obtained from the parents of normal controls, the affected children, and adult patients, in accordance with the Declaration of Helsinki.

First, we determined the sensitivity of detection levels of cjKRECs and sjKRECs in Guthrie cards using real-time quantitative PCR.⁵ Normal B cells from a healthy adult were isolated from peripheral blood (PB; mean purity, 88.5%). PB was also obtained from 1 patient with XLA (P20) whose B-cell number was 0.09 in 1 μ L whole blood and who was negative for sjKRECs ($<1.0 \times 10^2$ copies/ μ g DNA). Various numbers of normal B cells were serially added to 1 mL whole PB obtained from this patient with XLA. The B-cell-added XLA whole blood was then applied to filter papers, and 3 punches (3 mm in diameter) of dried blood spots were used for DNA extraction. At least 3 DNA samples containing the same B-cell concentrations (0.09–400 B cells/ μ L)

were used for the real-time quantitative PCR of cjKRECs and sjKRECs. The percentages of the positive samples ($>1.0 \times 10^2$ copies/ μ g DNA) of cjKRECs and sjKRECs increased constantly as the B-cell concentrations increased (Fig 1). None of the samples were positive for sjKRECs when the B-cell numbers were less than 20/ μ L, but cjKRECs were often positive. It has been reported that 90% of patients with XLA have less than 0.2% B cells in the PB at diagnosis.¹ Because peripheral lymphocyte numbers in neonates range from 1200 to 9800/ μ L,⁸ the absolute B-cell numbers of 90% of patients with XLA are estimated to be 2.4 to 19.6/ μ L at the time of blood collection for Guthrie cards, although exact B-cell numbers of XLA in neonatal periods are not known at this moment. Because neonates are known to have fewer B cells than infants,⁹ and we observed that B-cell numbers are constantly low in patients with XLA throughout infancy (Nakagawa, unpublished data, June 2010), which is consistent with the fact that BTK plays an essential role in B-cell maturation. It is likely that neonates with XLA also have severely decreased B cells. On the other hand, all samples obtained from 400 B cells/ μ L were positive for both cjKRECs and sjKRECs. We also observed that all healthy infants (1–11 months old; $n = 15$) were sjKREC-positive (Nakagawa, unpublished data, June 2010) and might have at least 600 B cells/ μ L whole blood.⁹ From these data, it is assumed that at least 90% of patients with XLA are sjKREC-negative, and healthy neonates are positive for sjKRECs on neonatal Guthrie cards.

Next, we measured cjKRECs and sjKRECs in dried blood spots in filter papers or Guthrie cards from 30 patients with XLA and 5 patients with non-XLA and from 133 neonates born at the National Defense Medical College Hospital during this study period (August 2008 to October 2009) and 138 healthy subjects of various ages (1 month to 35 years old) to investigate the validity of this method. The levels of B cells of the patients ranged from 0.0% to 1.1% of total lymphocytes and 0.0 to 35.78/ μ L. IgG levels were 10 to 462 mg/dL (see this article's Tables E1 and E2 in the Online Repository at www.jacionline.org). Patients with leaky phenotypes^{1,10} were included; 1 patient (P30) had more than 1% B cells and 34.22/ μ L total B cells, and 4 patients had more than 300 mg/dL serum IgG (P12, P30, P31, P33). All of the normal neonatal Guthrie cards were positive for both cjKRECs and sjKRECs ($7.2 \pm 0.7 \times 10^3$ and $4.8 \pm 0.6 \times 10^3$ copies/ μ g DNA, respectively). All healthy subjects of various ages were also positive for both cjKRECs and sjKRECs (Nakagawa, unpublished data, June 2010). In contrast, specimens from all 35 B-cell-deficient patients were sjKREC-negative ($<1.0 \times 10^2$ copies/ μ g DNA; Fig 2). All 5 patients with leaky phenotypes were also sjKREC-negative, which might be explained by the hypothesis that leaky B cells of patients with XLA are long-lived B cells that divided several times and have fewer sjKRECs than naive B cells. One patient (P27) was positive for cjKRECs, but other patients were negative for it. *RPPH1* (internal control) was detectable at the same level as in normal controls in all samples.

These results indicate that sjKRECs are undetectable in XLA and non-XLA and suggest that measurement of sjKRECs in neonatal Guthrie cards has the potential for the use of newborn mass screening to identify neonates with early B-cell maturation defects. Greater numbers of neonatal Guthrie cards should be examined to confirm this potential, and the data obtained from

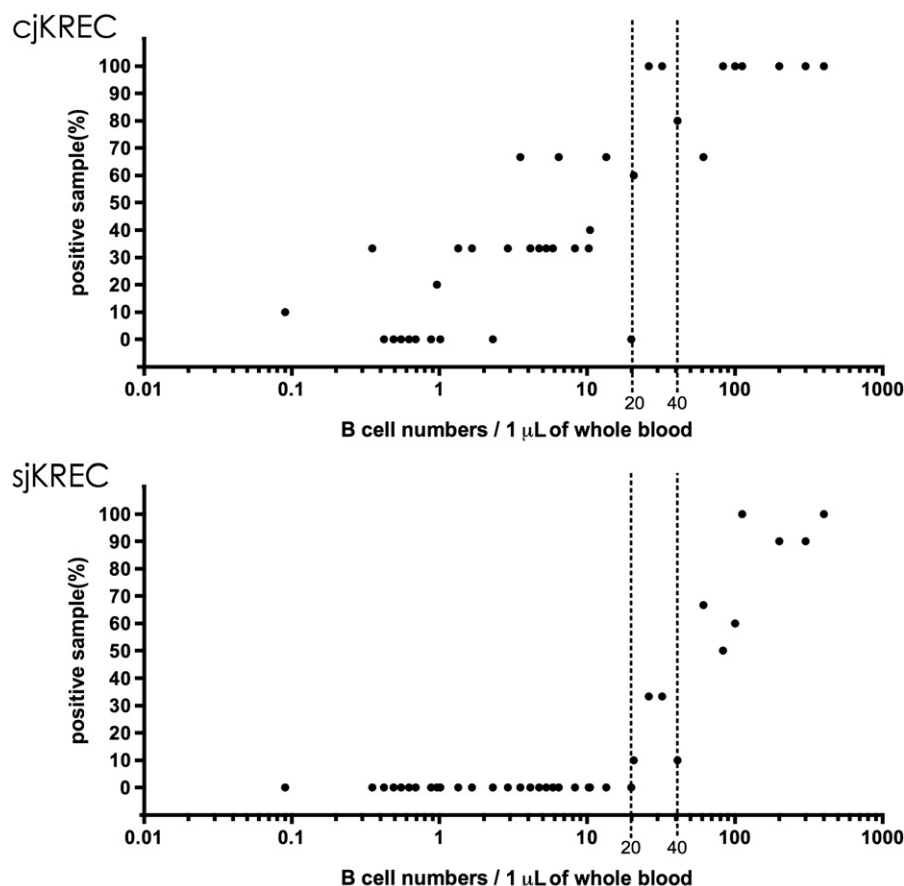


FIG 1. Sensitivity levels of cjKRECs and sjKRECs. Various numbers of purified normal B cells were serially added to whole PB from a patient with XLA (P20) to obtain B-cell-added XLA whole blood. cjKRECs and sjKRECs were measured in 3 to 10 samples of each concentration in triplicate. In all analyses, RNaseP (internal control) was positive ($2.3 \pm 0.2 \times 10^5$ copies/ μ g DNA). X-axis, B-cell numbers in 1 μ L whole blood from a patient with XLA. Y-axis, Percentages of the KREC-positive results in the tests.

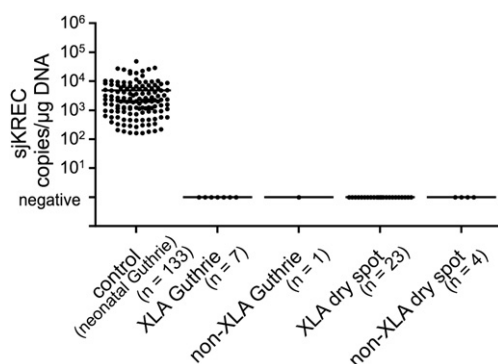


FIG 2. Copy numbers of sjKRECs measured in neonatal Guthrie cards or dried blood spots obtained from B-cell-deficient patients. On all samples from control, neonatal Guthrie cards (n = 133) were sjKREC-positive ($4.8 \pm 0.6 \times 10^3$ copies/ μ g DNA). B-cell-deficient patients were negative for sjKRECs in neonatal Guthrie cards (XLA, n = 7; non-XLA, n = 1) and dried blood spots (XLA, n = 23; non-XLA, n = 4).

dried blood spots on filter papers must be examined to prove that they truly reflect the data obtained from neonatal Guthrie cards. We should also examine whether screening can reduce the cost of treatment of the bacterial infections and chronic lung diseases in patients with XLA and non-XLA and increase the benefits for

these patients. An anticipated pilot study using a large cohort of newborns must address these problems. We also found that T-cell-receptor excision circles and sjKRECs can be measured simultaneously on the same plate. Thus, a pilot study of neonatal screening for both T-cell and B-cell deficiencies could be performed simultaneously.

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Noriko Nakagawa, MD^a
 Kohsuke Imai, MD, PhD^{a,b}
 Hirokazu Kanegane, MD, PhD^c
 Hiroki Sato, MS^b
 Masafumi Yamada, MD, PhD^d
 Kensuke Kondoh, MD, PhD^e
 Satoshi Okada, MD, PhD^f
 Masao Kobayashi, MD, PhD^g
 Kazunaga Agematsu, MD, PhD^g
 Hidetoshi Takada, MD, PhD^h
 Noriko Mitsuiki, MD^{i,j}
 Koichi Oshima, MD^{j,k}

Osamu Ohara, PhD^j
 Deepti Suri, MD^j
 Amit Rawat, MD^j
 Surjit Singh, MD^j
 Qiang Pan-Hammarström, MD, PhD^m
 Lennart Hammarström, MD, PhD^m
 Janine Reichenbach, MD, PhDⁿ
 Reinhard Seger, MD, PhDⁿ
 Tadashi Ariga, MD, PhD^d
 Toshiro Hara, MD, PhD^b
 Toshio Miyawaki, MD, PhD^c
 Shigeaki Nonoyama, MD, PhD^a

From ^athe Department of Pediatrics, National Defense Medical College, and ^bthe Department of Medical Informatics, National Defense Medical College Hospital, Saitama, Japan; ^cthe Department of Pediatrics, University of Toyama, Toyama, Japan; ^dthe Department of Pediatrics, Hokkaido University, Hokkaido, Japan; ^ethe Department of Pediatrics, St Marianna University School of Medicine, Kanagawa, Japan; ^fthe Department of Pediatrics, Hiroshima University, Hiroshima, Japan; ^gthe Department of Pediatrics, Shinshu University, Nagano, Japan; ^hthe Department of Pediatrics, Kyushu University, Fukuoka, Japan; ⁱthe Department of Pediatrics, Tokyo Medical and Dental University, Tokyo, Japan; ^jthe Department of Human Genome Technology, Kazusa DNA Research Institute, Chiba, Japan; ^kthe Department of Clinical Application, Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan; ^lthe Advanced Pediatric Centre Post Graduate Institute of Medical Education and Research, Chandigarh, India; ^mthe Division of Clinical Immunology, Department of Laboratory Medicine, Huddinge Hospital, Karolinska Institute, Stockholm, Sweden; and ⁿthe Department of Immunology/Hematology/BMT, University Children's Hospital Zurich, Zurich, Switzerland. E-mail: kimai@ndmc.ac.jp.

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TABLE E1. Characteristics of patients with XLA

Patient no.	Unique patient no.	Age (y)	Sex	Serum Ig (mg/dL)			CD19 ⁺		BTK mutation			Source	
				IgG	IgA	IgM	% Lymph	/μL	Genomic DNA	cDNA	Amino acid	Guthrie	Dry spot
P1	670	0	M	87	<6	10	0.21	12.99	29269G>T	1178-1G>T	Splice acceptor defect	x	
P2	718	0	M	215	<10	<10	0.07	7.04	11593_11594 insA	144_145insA	Arg49 frameshift	x	
P3	722	0	M	80	<1	1	<1.00	NA	25644C>T	763C>T	Arg255X	x	
P4	727	8	M	295	59	57	0.11	3.52	29269G>T	1178-1G>T	Splice acceptor defect		x
P5	732	34	M	1140*	<6	8	0.02	0.24	11631T>A	182T>A	Ile61Asn		x
P6	811	24	M	458*	0	13	0.50	5.32	23570T>G	426T>G	Tyr142X		x
P7	813	18	M	628*	109	6	0.60	6.87	23570T>G	426T>G	Tyr142X		x
P8	814	19	M	260	0	NA	0.20	3.01	16180C>T	344C>T	Ser115Phe		x
P9	815	13	M	600*	<10	<5	0.08	1.72	11590G>T	142-1G>T	Splice acceptor defect	x	
P10	816	11	M	12	0	5	0.00	0.00	150kb deletion of <i>BTK</i> , <i>TIMM8A</i> , <i>TAF7L</i> , <i>DRP2</i>				x
P11	817	10	M	10	2	24	0.80	35.78	36288C>T	1928C>T	Thr643Ile		x
P12	824	13	M	462	6	27	0.41	14.49	27518C>A	895-11C>A	Splice acceptor defect		x
P13	834	5	M	<237	<37	43	0.00	0.00	25715_26210del	776+57_839+73del	Exon 9 deletion		x
P14	838	21	M	<50	<5	7	0.00	0.00	31596G>C	1631+1G>C	Splice donor defect		x
P15	839	16	M	604*	<1	<2	0.04	0.66	31596G>C	1631+1G>C	Splice donor defect		x
P16	847	11	M	698*	26	11	0.08	1.86	25536delG	655delG	Val219 frameshift		x
P17	877	14	M	20	19	8	0.21	NA	32357T>C	1750+2T>C	Splice donor defect		x
P18	880	5	M	233	39	41	0.06	NA	10941-?_14592+?del	1-?_240+?del	Exon 1-3 deletion		x
P19	888	8	M	<212	<37	150	0.15	6.60	11023G>A	83G>A	Arg28His		x
P20	891	21	M	195	<6	37	0.02	0.09	32243C>G	1638C>G	Cys502Trp		x
P21	958	0	M	<50	<10	9	0.80	27.14	31544_31547 delGTTT	1580_1583del GTTT	Cys527 frameshift		x
P22	701	2	M	115	<2	4	0.09	1.99	16172C>A	336C>A	Tyr112X		x
P23	911	0	M	<10	<6	<4	0.00	0.00	29955A>C	1350-2A>C	Splice acceptor defect	x	
P24	937	0	M	60	<2	58	0.00	0.00	11022C>T	82C>T	Arg28Cys	x	
P25	938	0	M	<20	<4	<6	0.00	0.00	36269-?_36778+?del	1909-?_2418+?del	Exon 19 deletion	x	
P26	939	0	M	60	<2	22	0.00	0.00	11022C>T	82C>T	Arg28Cys	x	
P27	890	12	M	<237	<37	<20	0.03	NA	36261G>A	1909-8G>A	Splice acceptor defect		x
P28	944	6	M	12	<1	1	0.02	NA	36281C>T	1921C>T	Arg641Cys		x
P29	948	5	M	<237	<37	<20	0.01	0.70	36261G>A	1909-8G>A	Splice acceptor defect		x
P30	1053	5	M	386	5	113	1.10	34.22	32259A>C	1654A>C	Thr552Pro		x

Age, Age at analysis of KRECs; CD19⁺ % Lymph, CD19-positive cell percentage in lymphocytes; CD19⁺ /μL, CD19-positive cell number in 1 μL whole peripheral blood; M, male; NA, not available; Serum Ig, serum levels of immunoglobulins at diagnosis.

BTK mutation's reference sequences are NCBI NC_000023.9, NM_000061.2, and NP_000052.1.

*Trough level during intravenous immunoglobulin therapy.

TABLE E2. Characteristics of patients with non-XLA

Patient no.	Unique patient no.	Age (y)	Sex	Serum Ig (mg/dL)			CD19 ⁺		BTK mutation	Source	
				IgG	IgA	IgM	% Lymph	/μL		Guthrie	Dry spot
P31	596	4	F	386	<6	6	0.42	21.27	Normal		x
P32	719	0	F	<50	<5	<5	0.00	0.00	Normal	x	
P33	835	8	M	311	323	20	0.09	1.88	Normal		x
P34	915	0	M	<212	<37	<20	0.00	0.00	Normal		x
P35	947	0	M	<21	<37	<39	0.00	0.00	Normal		x

Age, Age at analysis of KREC; CD19⁺ % Lymph, CD19-positive cell percentage in lymphocytes; CD19⁺ /μL, CD19-positive cell number in 1 μL whole peripheral blood; F, female; M, male; Serum Ig, serum levels of immunoglobulins at diagnosis.