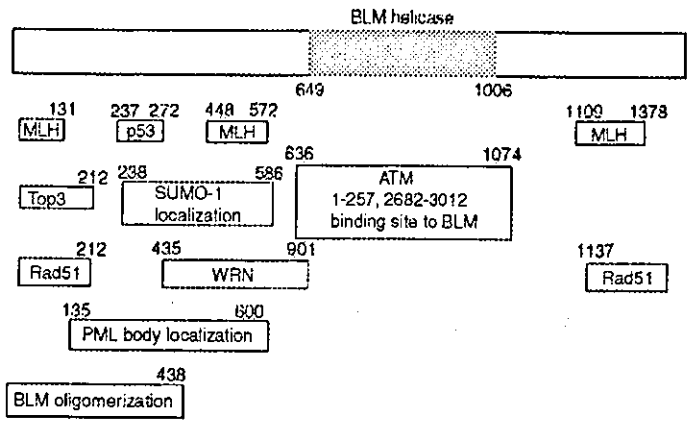

Figure 5. Proteins that interact with BLM. Regions of the protein where interactions with other proteins have been identified are shown.

mice are viable although by 20 months, nearly a third of them had developed cancer, mainly lymphomas and carcinomas. Luo then demonstrated that the increased rate of loss of heterozygosity resulting from mitotic recombination *in vivo* constitutes the underlying mechanism of tumor susceptibility in those mice.

Expert opinion & five-year view

Chromosome instability syndromes have a common feature: they are frequently associated with neoplasia. BS is considered as one of the chromosome instability syndromes, since the fibroblasts or lymphocytes of BS patients show excessive spontaneous chromosome instability. Several lines of reports indicate that BLM helicase is involved in the reinitiation of DNA replication at sites where replication forks have been arrested or collapsed. Evaluation of viable animals reveals an inverse correlation between the quantity of BLM and the level of chromosome instability and a similar genotypic relationship for tumor predisposition. This indicates that BLM is rate limiting for maintaining genomic instability and for the avoidance of tumors. These observations define a type of genetic instability that has significant implications for the evolution of cancer. The aim to elucidate the precise function of RecQ helicase in DNA repair and replication should not only to improve our understanding of the molecular basis for tumorigenesis but extend the range of potential therapeutic targets.

Key issues

- RecQ helicases are highly conserved from bacteria to humans. BLM, the causative gene for Bloom syndrome (BS), belongs to the RecQ helicase family.
- BS is uniquely associated with a predisposition to cancers of all types. BLM seems to maintain genomic stability by functioning at the interface between DNA replication and DNA repair.
- The BLM protein translocates into the nucleus. The distal arm of the bipartite basic residues in the C-terminus of the BLM protein is essential for targeting the nucleus.
- The combinational analysis of immunoblotting and immunohistochemistry is a useful approach for laboratory diagnosis of BS.
- BLM interacts with numerous binding partners. Ataxia telangiectasia mutated protein, the causative gene for Ataxia-telangiectasia, is one such associated protein.

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Systematic optimization of active protein expression using GFP as a folding reporter

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Received 1 April 2004, and in revised form 27 April 2004
Available online 10 June 2004

Abstract

Many recombinant proteins have been used as drugs; however, human proteins expressed using heterologous hosts are often insoluble. To obtain correctly folded active proteins, many optimizations of expression have been attempted but usually are found to be applicable only for specific targets. Interleukin-18 (IL-18) has a key role in many severe disorders including autoimmune diseases, and therapeutic approaches using IL-18 have been reported. However, production of IL-18 in *Escherichia coli* resulted in extensive inclusion body formation and previous conventional screenings of expression conditions could obtain only a condition with a low yield. To address the problem, we applied a folding reporter system using green fluorescent protein (GFP) for screening of the expression conditions for hIL-18. The established system efficiently screened many conditions, and optimized conditions for the expression of hIL-18 significantly enhanced the final yield of the active protein. Systematic screening using a GFP reporter system could be applied for the production of other proteins and in other organisms.

Production of recombinant proteins such as cytokines has been an important method for developing new therapeutic molecules. However, expression using heterologous target genes has production problems, especially the inactivation of the expressed proteins [1]. Recent advances in the proteomics require the availability of an efficient protein production system, but most of the target proteins cannot be properly produced, being an inactive aggregation [2]. To obtain correctly folded active proteins, many approaches such as expression condition optimization have been tried [3–5]. However, the methods reported have been relatively specific for their targets and are actually applicable only to specific or related proteins. Laborious and time-consuming screening steps for optimization of the expression conditions specific for the protein must be performed on an individual basis.

Interleukin-18 (IL-18) is functionally similar to IL-12 in terms of IFN-γ production; and the aberrant expression of IL-18 has been inferred to be associated with severe inflammatory conditions, such as autoimmune diseases, allergies, or neurological disorders [6–8]. Therapeutic approaches using recombinant IL-18 have been investigated for treatment of cancers, including in a clinical trial in humans [9–11]. Recently, we have successfully determined the 3D-structure of human IL-18 (hIL-18) and the structural basis of its receptor activation mechanisms using mutant proteins and receptor binding assays [12,13]. However, production of IL-18 in *Escherichia coli* resulted in extensive inclusion body formation and previous conventional screenings of expression conditions could obtain only a condition with a low yield ([12], Kato et al., unpublished data).

To address these problems, we have applied a folding reporter system using green fluorescent protein (GFP) for screening of the expression conditions of hIL-18 [14]. We constructed the expression vector encoding the hIL-18 with GFP protein in the C-terminus and evaluated the expression conditions by measuring fluorescence from the hIL-18-GFP fusion protein. The established system efficiently screened many conditions, and the optimized condition for the expression of hIL-18

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doi:10.1016/j.pep.2004.04.023

significantly enhanced the final yield of the active protein. This GFP reporter system could be applied to the production of other proteins and in other organisms.

Materials and methods

Construction of plasmid of GST-hIL-18wild-GFP fusion protein

mRNA was extracted from a blood sample obtained from a healthy volunteer, and cDNA was synthesized at 72 °C for 60 min using reverse transcriptase, MMLV, and oligo(dT) primers. The coding region for mature hIL-18wild (157 residues) was amplified by polymerase chain reaction (PCR). The coding region for GFP was also amplified from pEGFP-N2 vector (Clontech). Between the amplified fragments, the DNA fragment, 5′-GGATCC GCTGGCTCCGCTGCTGGTTCTGGCGAGTTC-3′, coding for amino acid linker GSAGSAAGSGEF, was placed [14]. The hIL-18wild-linker-GFP was cloned into a T-vector (Invitrogen). The primers were designed to insert the *Eco*RI and Factor Xa-cleavage sites immediately before the mature hIL-18wild sequence and the *Eco*RI site after the stop codon. The T-vector was digested with *Eco*RI and purified by electrophoresis. The purified product was then subcloned into the pGEX-4 T-1 vector (Pharmacia) and the DNA sequence of the clone was confirmed by bi-directional sequencing. The clone was named GST-hIL-18wild-GFP (Fig. 1).

Screening of induction temperatures

Escherichia coli BL21(DE3) (Novagen) was transformed by GST-hIL-18wild-GFP according to the manufacturer's instructions. The expression of the protein was performed as follows: each of the transformed colony was grown in 5 ml Luria–Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter of medium) with 100 μg/ml ampicillin and 2% glucose, and the cultures were shaken at 37 °C until an optical density at 600 nm (OD_{600}) = 0.4. *E. coli* cultures were transferred to each temperature (15, 25, and 37 °C) and continuously shaken until OD_{600} = 0.5. Protein production was induced by addition of isopropylthio-β-D-galactoside (IPTG) at a final concentration of 1 mM. After a 5 h induction of the protein, 1.5 ml of each culture was used as a sample for expression analysis by GFP. Fluorescences were measured using VersaFluor (Bio-Rad) (excitation, 490 nm; emission, 510 nm, averaging time, 4 s). For more precise examinations, induction temperatures from 23 to 37 °C were tested by the same procedures.

For SDS–PAGE analysis, 1.5 ml of each culture was centrifuged at 10,000 rpm for 2 min and the pellet was resuspended in 1.5 ml lysis buffer (50 mM Tris–HCl, pH 8.0, 400 mM KCl, 10 mM 2-mercaptoethanol (2-ME), and 1 mM EDTA) with 1 mM Pefabloc (Roche). The cells were completely lysed by sonication of 0.5-s active sonication with a 0.5-s interval on an ice-water bath for 10 min and then centrifuged for 10 min at 12,000 rpm, 4 °C. The pellet was then resuspended in 1.5 ml of lysis buffer; then 10 μl of the supernatant and the resuspended pellet were used for SDS–PAGE analysis.

Screening of concentrations of IPTG and induction periods

The *E. coli* culture incubated until OD_{600} = 0.4 at 37 °C was transferred into conditions at 26 °C and incubated until the cell density became OD_{600} = 0.5, which

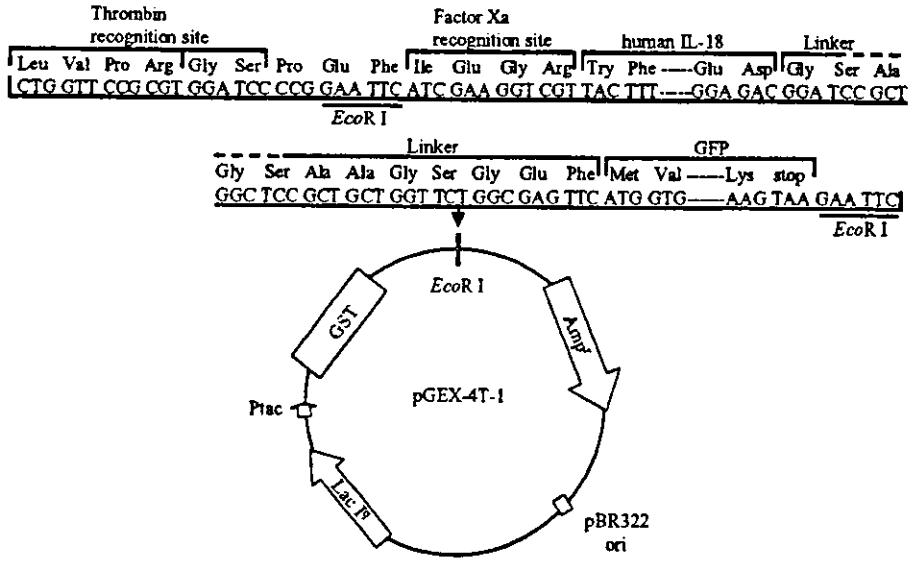

Fig. 1. Schematic representation of the expression vector for GST-hIL-18 fusion protein (pGEX-4T-Xa-hIL-18wild-GFP). The sequence encoding hIL-18wild and GFP was inserted into *Eco*RI site of pGEX-4T-1.

was usually after 5 h incubation. Then protein expression was induced by IPTG at final concentrations of 0.1, 0.5, 1, and 2 mM. OD_{600} and GFP expression of each culture was monitored for 30 h.

Large scale expression and purification

We used the expression vectors GST-hIL-18wild and GST-hIL-18opti for the examination to compare large scale production yields under different conditions. The vector, GST-hIL-18wild, had been constructed by subcloning wild-type hIL-18 into pGEX-4 T-1, and the vector, GST-hIL-18opti, contains the newly synthetic gene with optimized codons but without amino acid changes for expression in *E. coli* (BL21) [12].

Large scale expression and purification of the wild-type hIL-18 protein were carried out as described previously with minor modifications [12]. Briefly, BL21 was transformed by the vectors, GST-hIL-18wild or GST-hIL-18opti, and protein expression was performed as follows: the colony with the high expression level was cultivated overnight in 200 ml of the LB medium with 100 μg/ml ampicillin shaken at 160 rpm. The culture was transferred into 2 L of the LB medium with 100 μg/ml ampicillin. The culture was incubated at 37 °C until $OD_{600} = 0.45$ and then cooled to 26 °C. IPTG (final concentration 1 mM) was added to the medium when $OD_{600} = 0.5$. The culture was incubated at 26 °C with shaking at 160 rpm for 15 h. The culture was centrifuged at 5000*g* for 10 min and the *E. coli* pellet was stored at −80 °C until purification.

The bacterial cell pellet was resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 400 mM KCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA) with 1 mM Pefabloc, lysed by sonication, and then centrifuged. The clear lysate was applied onto a GST affinity column (Pharmacia) and the column was then washed by lysis buffer. The captured fusion protein was eluted with elution buffer (50 mM Tris–HCl, pH 8.0, and 10 mM glutathione). The protein-containing fractions were concentrated and cleaved by bovine Factor Xa (Funakoshi) at a ratio of 1% (w/w) at 4 °C. Mature hIL-18 protein was isolated using Sephacryl S-100 26/60 (Pharmacia). The fractions were then stored at 4 °C until further experiments. The concentration of the purified hIL-18 protein was estimated using the absorbance constant (6160) for hIL-18.

Biological activity assay

Human myelomonocytic KG-1 cells were grown in the culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mmol/L), penicillin (100 U/ml), and streptomycin (100 μg/ml). KG-1 cells (3.0×10^5 cells) were cultured in the presence of 0.1–50.0 ng/ml of recombinant hIL-18 for 24 h in a volume of 0.2 ml at 37 °C in a humidified atmosphere containing 5% CO_2. Culture supernatants in the microtest plates were spun to remove cells after the cultures and were stored frozen at −80 °C. The IFN-γ concentration was measured as previously described with minor modification by fluorometric microvolume assay technology using FMAT 8100 HTS system (ABI) [15].

Results

Screening of induction temperature

With expression at 37 °C, the fusion protein mostly precipitated as an inclusion body, while it was not observed in the pellet in expressions at 15 and 25 °C (Fig. 2A). Further, the expression level at 15 °C was lower than the level of expression at 25 °C. As examined on SDS-PAGE, the induction temperature at 25 °C showed the best yield. In addition, the fluorescence of the fusion protein at 25 °C was the highest among the three temperatures (Fig. 2B). To determine the optimized induction temperature, examinations measuring fluorescence were carried out at 15–37 °C. The fluorescence of *E. coli* culture expressing GST-hIL-18wild-GFP at 26 °C was the highest; at a level that was 4.3 times higher than that at 37 °C (Fig. 3).

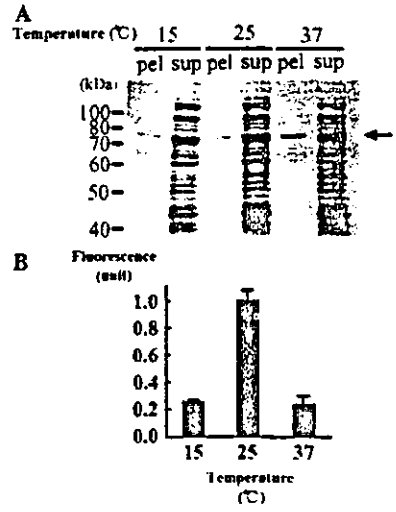

Fig. 2. Expression of GST-hIL-18wild-GFP. (A) SDS-PAGE (7.5% gel) of the expression of the GST-hIL-18wild-GFP fusion protein after 5 h of induction by IPTG (1 mM). The fusion protein migrated as a 71 kDa band. The band in the pellet at 37 °C indicated an extensive inclusion body formation. Mw, BenchMark Protein Ladder (Invitrogen); sup, supernatant of the cell lysate; pel, pellet of the lysate. Arrow indicates the GST-hIL-18wild-GFP protein. (B) Fluorescence of GFP at three different induction temperatures with 1 mM IPTG and 5 h of induction. The fluorescence was normalized by dividing by the highest fluorescence. Mean values of triplicate assays are shown with standard deviation. The fluorescence at 25 °C showed the highest in the three temperatures.

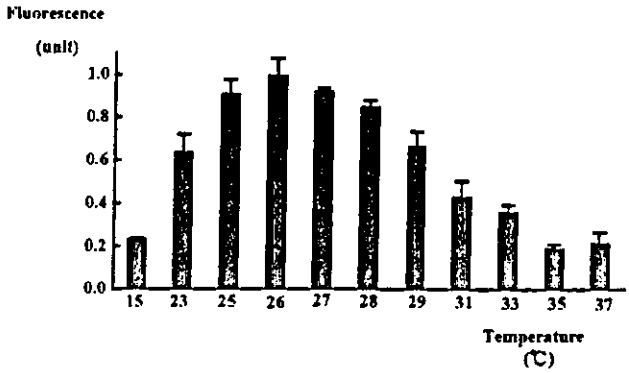

Fig. 3. Fluorescence at different induction temperatures with 1 mM IPTG and 5 h of induction. The fluorescence was normalized by dividing by the highest fluorescence. Mean values of triplicate assays are shown with standard deviation. The fluorescence at 26 °C showed the highest in the three temperatures.

Screening of IPTG concentration and induction period

The same approach could also be used to screen IPTG concentrations and induction periods (Fig. 4). Four IPTG concentrations were examined and fluorescence increased IPTG dose-dependently, but the fluorescence was saturated at 1 mM IPTG. The maximum intensity of fluorescence at 0.1 mM was obtained at 9 h, while the maximum intensity of fluorescence at 0.5, 1, and 2 mM was obtained at around 15 h. After that the fluorescence decreased gradually.

Large scale expression and purification

The target proteins, GST-hIL-18wild and GST-hIL-18opti, were expressed at 26 °C in *E. coli*. The culture medium of *E. coli* expressed GST-hIL-18wild showed $OD_{600} = 0.9$ after 15 h of induction, and 7.9 g of *E. coli* pellet was obtained from a 2-liter culture. On the other hand, culture medium expressed GST-hIL-18opti showed $OD_{600} > 3.0$ after 15 h induction and 13.0 g of *E. coli* pellet was obtained from a 2-liter culture. A GST affinity column was used to capture the GST fusion protein, which was concentrated and cleaved by Factor Xa. The cleavage was completed at 4 °C after about 16 h. Following gel filtration chromatography of the cleaved protein, highly purified hIL-18wild and hIL-18opti were obtained.

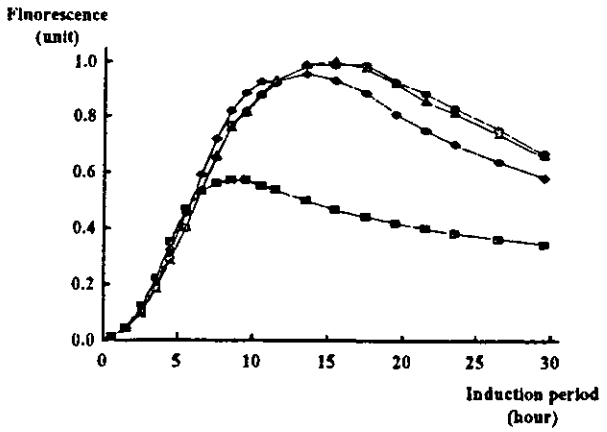

Fig. 4. Fluorescence at different concentrations of IPTG and induction periods. IPTG concentration: 0.1 mM (■), 0.5 mM (◆), 1 mM (●), and 2 mM (▲). Induction temperature was 26 °C. The fluorescence was normalized by dividing by the highest fluorescence at the optimal condition of 1 mM IPTG and 15 h. Mean values of triplicate assays are shown. The fluorescence induced with 1 and 2 mM IPTG showed the highest values at 15 h.

Actual fluorescence intensity by GST-IL-18-GFP fusion protein expressed in optimized conditions (11,191 at 26 °C, IPTG 1 mM, 15 h) showed a level about three times higher than that in the previous condition (4207 at 25 °C, IPTG 1 mM, 5 h). The yield of hIL-18wild protein was 3.0 mg from 2 L of the culture medium (1.5 mg/L; 0.38 mg/g wet weight cell), while the yield of the hIL-18opti protein was 23.1 mg from 2 L of the culture medium (11.5 mg/L; 1.78 mg/g wet weight cell) (Table 1). These yields obtained under the newly optimized conditions showed about three times higher levels than those previously used conditions indicating the correlation between fluorescence intensity and actual protein yield.

Biological activities of hIL-18 by different production methods

The biological activities of hIL-18 produced by different methods, commercially available hIL-18 (MBL),

Table 1
Comparison of final yields on large scale expression

Genes	Previous condition		Optimized condition	
	mg/L	mg/g cell	mg/L	mg/g cell
IL-18wild	0.5	0.21	1.5	0.38
IL-18opti	4.4	1.17	11.5	1.78

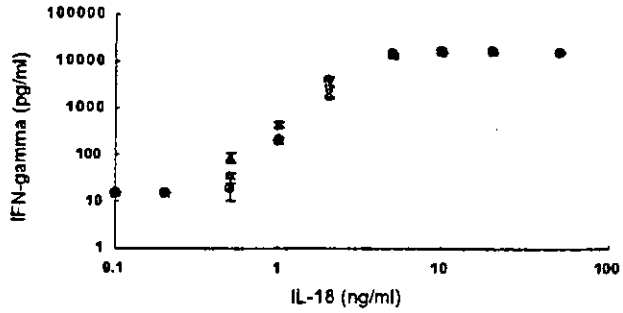

Fig. 5. IFN-γ induction by differently produced hIL-18s. Solid circle, commercially available hIL-18 (MBL); open circle, hIL-18wild produced by the previous protocol; and solid triangle, hIL-18opti produced by the new protocol. Mean values of triplicate IFN-γ induction assays are shown with standard deviation. IFN-γ production by different concentrations of the three IL-18 proteins showed no significant differences among them showing almost the same sigmoid curves. These findings indicate that the new expression conditions can produce high amounts of protein retaining the same biological activity as those made by conventional methods and conditions.

hIL-18wild or hIL-18opti, were estimated. IFN-γ production by different concentrations of the three IL-18 proteins showed no significant differences among them showing almost the same sigmoid curves (Fig. 5). These findings indicate that the new expression condition can produce high amount of protein retaining the same biological activity as those by the conventional methods. Conservation between the 3D-structures of the purified proteins using the hIL-18wild and hIL-18opti genes was previously confirmed by multidimensional-, multinuclear-, magnetic resonance techniques [12,13].

Discussion

Expression and purification of hIL-18 in *E. coli* have been described in two reports [7,16]; one a direct expression of mature IL-18 protein, which had methionine before the mature sequence; the other an expression of the GST-hIL-18 fusion protein, because direct expression of the mature hIL-18 polypeptide like the previous one by the authors did not yield any active protein [16]. The two studies used similar expression conditions (Ushio et al. 37 °C, 18 h, 1 mM IPTG; Liu et al. 37 °C, 3 h, 0.1 mM IPTG), but no findings concerning expression condition optimization were described. Neither were the final yields of the protein described, but the yields should have been quite low if in accord with our results.

Our previously reported method using a codon optimized synthetic gene could yield five times higher amounts of protein than that using a non-optimized wild-type gene [12]. The expression condition (25 °C, 5 h, 1 mM IPTG) used for the study was according to the results of a preliminary study; that showed that extensive inclusion body formation occurred and that the yield was very low at higher temperatures, such as 37 °C ([12], Kato et al., unpublished). However, this condition was not fully optimized and remained in a kind of empirical state.

To establish a systematic screening system for expression conditions, we have introduced GFP as the "folding reporter." The original usage of GFP as a folding reporter was described in an evaluation of mutagenesis to obtain a target protein as a soluble protein; but not to obtain the optimized expression conditions [14]. This strategy utilized the rationale that the chromophore formation of GFP depends on the correct folding of the protein. A "folding reporter" vector was constructed, in which the target protein was expressed as an N-terminal fusion protein before GFP. The fluorescence from *E. coli* cells expressing GFP was correlated to the correct folding of the upstream protein; and this system led them to achieve structural determination of a soluble mutant protein [17]. Recently, another application using GFP for screening of randomly cloned cDNAs has been reported to search for a soluble domain [18].

In our study, a systematic search of the expression conditions of temperature, inducer concentration, and induction period has been successfully performed (Figs. 3 and 4). Preliminary trials concerning condition optimization have been reported previously, but the actual effect for yields using these conditions remained unknown [19]. In contrast, we have here confirmed that the conditions obtained in small scale examinations could also be applied to large scale production with predicted yields of active protein using two different genes (Table 1). Moreover, this study also has indicated that the time required for screening can be significantly reduced. For example, large scale examination for one condition requires about 1 week in our system, and actual yields can be obtained only at the final steps. If we screen the conditions examined in this study using conventional methods, it would take more than 1 year; this reporter system requires only 1 week to carry out the same screening.

In conclusion, more efficient and cost-effective screening procedures could be performed using our GFP reporter system that should be applicable for the optimization of many expression conditions; not only of temperature, inducer, and periods, but also of other factors, such as host strains, additives, cell free systems in the production of many other proteins and using other organisms [5].

Acknowledgments

Part of this work was supported by Uehara Memorial Foundation and by The Naito Foundation.

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INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE 14: 439-442, 2004

Relatively common mutations of the Bloom syndrome gene in the Japanese population

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Received February 24, 2004; Accepted April 23, 2004

Abstract. Bloom syndrome (BS) is a rare autosomal recessive genetic disorder characterized by lupus-like erythematous facial telangiectasia, sun sensitivity, infertility, stunted growth and a high predisposition to various types of cancer. Chromosomal abnormalities are hallmarks of this disorder, and high frequencies of sister chromatid exchanges and quadriradial configurations in lymphocytes and fibroblasts are diagnostic features. BLM is the causative gene for BS. We investigated the mutation in the BLM gene in 4 Japanese BS kindreds. Taken together with previously documented mutations, 2 kindreds were homozygous for 631delCAA and 2 were compound heterozygous for 631delCAA. The silent mutation of A1055C (Thr to Thr) was detected in control Japanese individuals. The 6-bp deletion/7-bp insertion at position 2,281, which most Askenazi Jewish BS patients carry, was not detected in 200 Japanese alleles. These results suggest that 631delCAA is a relatively common mutation among the Japanese BS patients.

Introduction

Bloom syndrome (BS) is a rare autosomal recessive genetic disorder characterized by growth deficiency, abnormal faces, sun-sensitive telangiectatic erythema, immunodeficiency and a high predisposition to various types of cancer (1,2). The gene responsible for BS was identified to be BLM, which was shown to have 4437bp that encodes 1,417 amino acid peptides homlogous to RecQ helicases, a subfamily of DExH box-containing DNA and RNA helicases (3). It is assumed that the BLM protein plays an important role in the maintenance of genomic stability in somatic cells. In this BS, increased spontaneous sister chromatid exchanges have been observed, and it is considered to be the most malignancy-prone chromosomal disorder. The underlying mechanism may be caused by a deficiency in DNA damage repair.

German and Ellis have reported 14 unique mutations in 20/25 of the BS patients examined (4): Three of the mutations are putative missense substitutions, 6 are nonsense mutations, 2 are frameshift mutations, 2 are exon-skipping mutations and 1 is a gross deletion detected by Southern blot analysis. The mutation of the BLM gene that occurs at a relatively high frequency in Askenazi Jews, has been reported as blmAsh, which is a 6-bp deletion/7-bp insertion at nucleotide 2,281 of the open reading frame of the BLM gene that results in a frameshift and a stop codon at nucleotide 2,292 (5). Li *et al* (6) have reported that blmAsh mutation is present in 1/107 of this particular Askenazi Jewish population, or has a carrier frequency of 0.0093. In Hispanic BS patients, the expected carrier frequency of the blmAsh mutation was also reported (7). However, in the Japanese population such a common BLM mutation has not yet been reported. In this study, we describe relatively common mutations of the BLM gene in the Japanese population.

Materials and methods

BS patients. Case 1: a 26 year-old female was diagnosed as having BS based on clinical findings when she was 5 years old. Her younger sister was also diagnosed as having BS. She developed Burkitt's lymphoma at 13 years old, which was cured by chemotherapy. She developed breast cancer at 26 years old. She also developed diabetes mellitus.

Case 2: a 13 year-old female was diagnosed as having BS by the clinical findings and elevated sister chromatid exchanges. She developed Burkitt's lymphoma at 13 years old. She also developed diabetes mellitus.

Cases 3 and 4: Two sibilings, a 23 year-old man and a 30 year-old woman were identified, respectively, as 96 (HiOk)

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Key words: Bloom syndrome, BLM, Japanese population, 631delCAA, 1610insA

Table I. PCR primer sets for BLM genomic DNA amplification.

	Forward		Reverse
1S	5'-CCTCCCCTCAAAAAACATTG-3'	1A	5'-AATCAATGAATCTCTCAGCT-3'
2S	5'-AGTTTTGTAGAGTTGGGGGG-3'	2A	5'-AAGTATCAAAGTCATCCATA-3'
3S	5'- AAATCCCGGGATACTGCTCT-3'	3A	5'-TCATGACTATTCCCAATGGC-3'
4S	5'-TCGCTCATGCCCTGTTCTTT-3'	4A	5'-AACAATTTAAAGTATCCCAG-3'
5S	5'-ATATATTGTCTGATCAGTGG-3'	5A	5'-CACAGGTTCAAAACACAATC-3'
6S	5'-AGTCATGAGCCACCATGCCT-3'	6A	5'-AGCCTGAACATTTTGCCCTG-3'
7S	5'-TTTGGTATGAAAACTACAGA-3'	7A	5'-TTTCACAGCAGTGCTTGTGA-3'
8S	5'-AGTAGCAACTGGGCTGAAAC-3'	8A	5'-GCAATGATGATTTGCTATGG-3'
9S	5'-GCAGGGCAAGGGAAATGCTA-3'	9A	5'-ATGAAATACTGTGTAGGTGG-3'
10S	5'-TGCTCTGAAGACAGAACCTG-3'	10A	5'-AAAAAGGTTATCCAGAGGAC-3'
11S	5'-GGTTTGATATGTGACTAATA-3'	11A	5'-CCATTTGGGGTTTCTGGATG-3'
12S	5'-TTAAGTTGTGATGGAATTTG-3'	12A	5'-CTCGTTCACTCAGTGTGGGT-3'
13S	5'-TACTGAAGAATAAGGTAGTT-3'	13A	5'-GCGTTAAAGCGCCACAGGTG-3'
14S	5'-AATACCATCACACTGGGGGT-3'	14A	5'-GGAATGTTTAATATGCAATT-3'
15S	5'-CAGTATAAGAACACTACGGG-3'	15A	5'-TTGCATTCTACATGTGCATG-3'
16S	5'-ATGATAGAGCTTTTAGAAGC-3'	16A	5'-TACCACAATAGCAGGAGTAG-3'
17S	5'-AATATAGAATGCCATGTTGA-3'	17A	5'-GAGACCACCTTTTGCAATCT-3'
18S	5'-CTGGAAATGGGTTATGATGA-3'	18A	5'-TTCTATGTACTTGTAATAAA-3'
19S	5'-ATGAGTGTCTGTGCCAGGGA-3'	19A	5'-TTGGACAAAGACACTATACT-3'
20S	5'-CCCCAAAAATGCAATTAAGC-3'	20A	5'-ATTTATCACTTAAGGAGTTC-3'

and 97 (AsOk) in the BS Registry. Their clinical courses and mutation of BLM gene were previously reported (8-10).

Case 5: YoYa (BS registry 93) was described in reference 3. Informed consent for gene analysis was obtained from cases 1-4 or their parents.

Cell lines. EBV-transformed lymphoblasts established from BS patients (GM03403F, GM09960, GM04408B) were purchased from ATCC.

Amplification and electrophoresis of BLM gene. Peripheral blood monocytes (PBMCs) were separated using Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden). RNA was prepared from PBMCs and cDNA was synthesized with MMTV reverse transcriptase. Genomic DNA from PBMCs was prepared using a Sepa Gene kit (Sanko Jyunyaku, Tokyo, Japan). PCR primers for genomic DNA are listed in Table I. PCR consisted of 35 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The amplified DNA fragment was electrophoresed using 4% agarose gel or 20% acrylamide gel (11).

For the concise detection of 631delCAA mutation we used mismatch primers, which were introduced artificially into the HinfI site. The underlined nucleotide was a mismatched nucleotide. Sense, 5'-ACTTTGTAAGAGTAAGCACTGCTCAGGA AT-3'; anti-sense, 5'-AGTCATCCTTCTGTTCCTCAGTCA AATC-3'. Following PCR amplification, the PCR product was digested using HinfI. DNA was electrophoresed using 4% agarose gel or 20% acrylamide gel.

For the concise detection of the blmAsh mutation, the following primers were used. Sense, 5'-GGTTTGATATGTGA CTAATA-3'; anti-sense, 5'-CCATTTGGGGTTTCTGGATG-3'. Following PCR amplification, the PCR product was digested using BstNI. DNA was electrophoresed using 20% acrylamide gel.

Table II. Molecular basis of Bloom syndrome in the Japanese population.

	Zygosity of the mutation	mRNA sequence alteration	Predicted protein alteration
Case 1	heterozygous	631delCAA 1610insA	S186X 514-1-X
Case 2	homozygous	631delCAA	S186X
Case 3	homozygous	631delCAA	S186X
Case 4	homozygous	631delCAA	S186X
Case 5	homozygous	1610insA	514-1-X

Number of amino acids starting from the first in-frame ATG found in the BLM cDNA. An X indicates a stop codon. The amino acids that are changed appear before the codon number. The effect of a frameshift is shown by first indicating the number of the BLM amino acid residues that are incorporated, followed by the number of out-of-frame residues that are incorporated until a stop codon is reached. Cases 3 and 4 are siblings.

Sequencing of the BLM gene. The PCR fragment was subcloned into a T-vector (Novagen, Madison, WI, USA) and sequenced using a dye primer or a dye terminator method with an autosequencer (Applied Biosystems, Indianapolis, IN,

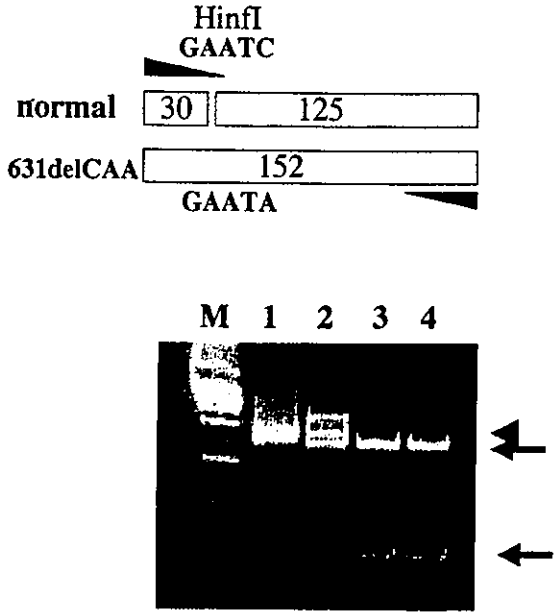

Figure 1. 631delCAA BLM gene mutation. The upper panel shows the strategy for the detection of the mutation. The triangle indicates the primer. A sense-strand mismatch primer which introduced a HinfI site *de novo* in the normal sequence was used. Arrows indicate HinfI-digested bands of a normal DNA. The arrowhead indicates a band corresponding to mutant DNA. (M), marker DNA; (1), case 4; (2), mother of case 3 and 4; (4), normal control.

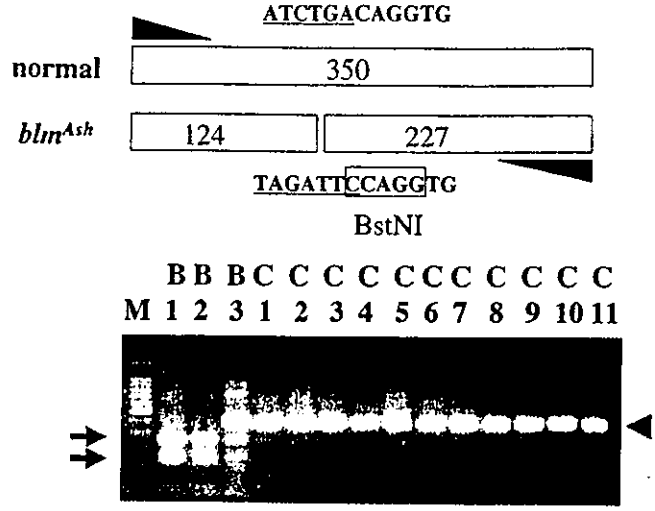

Figure 2. blmAsh gene mutation. The upper panel shows the strategy for the detection of the mutation. The triangle indicates the primer. The underlined sequence indicates 6-bp deletion/7-bp insertion. Arrows indicate BstNI digested bands of mutated DNA. The arrowhead indicates a band corresponding to a normal DNA. (M), marker DNA; (B1-3), EBV-transformed lymphoblasts established from BS patients (GM03403F, GM09960, GM04408B); (C1-11), normal control.

USA). For a dye primer, 5 independent colonies were picked and sequenced.

Results and Discussion

All 4 BS patients developed maligancy. Four out of 5 BS patients developed malignant lymphomas, such as Burkitt's lymphoma or B cell lymphoma at 10-20 years old. Diabetes mellitus was also observed in 4/5 BS patients.

For the analysis of BLM mutation, the PCR fragment from genomic DNA was sequenced. We identified 631delCAA and 1610insA mutations in 5 and 3 alleles, respectively, in a total of 8 mutant alleles in Japanese BS patients (Table II). For the concise detection of the 631delCAA mutation, we used mismatch primers which were introduced artificially into the HinfI site. The wild-type allele was digested into 125-bp and 30-bp fragments using HinfI (Fig. 1). This indicated that the parents had both normal and mutant alleles. Using this method, the 631delCAA mutation was analyzed, but was not detected in 200 normal alleles.

The silent mutation of A1055C (Thr to Thr) was detected in the control Japanese individuals. The 6-bp deletion/7-bp insertion at position 2281, designated as blmAsh, which most BS individuals of Askenazi Jewish carry, was not detected in 200 Japanese alleles, although EBV-transformed lymphoblasts established from BS patients (GM03403F and GM09960) exhibited the homozygous pattern of blmAsh, and those established from BS patient (04408B) exhibited the heterozygous pattern (Fig. 2). These results suggest that 631delCAA is a relatively common mutation in the Japanese population. 1610insA were also identified in 3 alleles out of a total 8 mutant alleles of Japanese BS patients. It may be possible to consider 1610insA as the common mutation in the BLM gene in Japanese BS patients, although the BLM gene analysis of more Japanese BS patients is necessary. Four out of 5 BS patients developed malignant tumors, particularly non-Hodgkin lymphoma between 10-20 years old. Consistent with previously reported cases, BS is one of the most malignant-prone disorders. Therefore, a definitive diagnosis of BS is essential for careful monitoring to follow up the BS patients with the treatment of malignant tumors.

BS is caused by the mutation of the BLM gene, which encodes 1417 amino acids. It is a laborious task to identify the BLM gene mutation. Moreover, it is sometimes difficult to distinguish between BS and other DNA instability syndromes, such as Fanconi anemia or ataxia-telangiectasia based on clinical manifestations. For BS diagnosis, it is vital to confirm the existence of DNA instability, particularly the elevated frequency of SCE. SCE is sensitive and specific for BS. Similarly, the striking cytogenetic feature of Fanconi anemia is the unusual sensitivity to DNA cross-linking agents such as mitomycin C. It is useful for the diagnosis of Fanconi anemia. The initial screening should be based on clinical symptoms and chromosome instability. However, we often treat patients with borderline features of BS.

Previously, we demonstrated that EBV-transformed lymphoblasts and PHA-stimulated PBMCs obtained from BS patients can be used in the diagnosis of BS by immunoblot and immunohistochemical analysis, based on the absence of the BLM protein (12). Because we could not detect the BLM protein consistently in freshly isolated PBMCs, it is possible that cells in the resting state expressed the BLM protein in much smaller amounts than cells in the proliferating state, such as EBV-transformed lymphoblasts or PHA-stimulated lymphoblasts.

The combination of immunoblotting and immunohistochemistry is a useful approach for laboratory diagnosis of BS. However, the detection of the mutation in the BLM gene

is required for the definitive diagnosis. The detection of the relatively common mutations in the BLM gene in the Japanese population and our concise detection method are useful for the screening of the BLM gene mutation.

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Clin Genet 2004: 66: 236–238
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CLINICAL GENETICS
doi: 10.1111/j.1399-0004.2004.00292.x

Short Report

Identification of somatic and germline mosaicism for a keratin 5 mutation in epidermolysis bullosa simplex in a family of which the proband was previously regarded as a sporadic case

Nagao-Watanabe M, Fukao T, Matsui E, Kaneko H, Inoue R, Kawamoto N, Kasahara K, Nagai M, Ichiki Y, Kitajima Y, Kondo N. Identification of somatic and germline mosaicism for a keratin 5 mutation in epidermolysis bullosa simplex in a family of which the proband was previously regarded as a sporadic case.
Clin Genet 2004: 66: 236–238. © Blackwell Munksgaard, 2004

Epidermolysis bullosa simplex (EBS) is an autosomal-dominant inherited blistering skin disease characterized by intraepidermal blistering due to mechanical stress-induced degeneration of basal keratinocytes. EBS is caused by mutations in either keratin 5 or keratin 14, the major keratins expressed in the basal layer of the epidermis. We experienced a unique EBS-affected family. The proband had a heterozygous 1649delG mutation in the keratin 5 gene and had been reported as a case of *de novo* mutation, because the mutations were not detected in the parents' DNA from blood samples. However, the proband's younger sister was revealed to have the same disease at birth and we found the same mutation in her. We reinvestigated the familial segregation of the 1649delG mutation and it was shown that the mother's DNA from hair bulb and buccal cell samples had the 1649delG mutation heterozygously, but her DNA from blood samples did not. A careful check on the mother's history disclosed that she had migratory circinate pigmentation in her skin in childhood, which means maternal somatic and germline mosaicism. The demonstration of somatic and gonadal mosaicism in the keratin 5 gene is important for accurate genetic counselling of families with sporadic cases of EBS.

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Key words: Epidermolysis bullosa simplex – keratin 5 – mosaicism – familial analysis

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Received 26 February 2004, revised and accepted for publication 20 April 2004

Epidermolysis bullosa simplex (EBS) is a group of cutaneous disorders characterized by blister formation caused by minor mechanical trauma. It has been traditionally divided into three broad categories – epidermolytic (simplex), junctional and dermolytic (dystrophic) – based on the level of blister formation (1). EBS is characterized by blister formation due to cytolysis of the epidermal basal cells and the majority of the cases are autosomal-dominant transmissions. Clinically, EBS is divided into three main subtypes, based on the severity, distribution and onset age of blistering. However, all the subtypes of EBS could be caused by mutations in either the keratin 5 (KRT5) gene or the keratin 14 (KRT14) gene (2). More than 20 missense mutations in KRT5 and KRT14 have been reported (3).

Germline mosaicism, with or without somatic mosaicism, has been confirmed by molecular mutation studies for many dominant disorders, such as osteogenesis imperfecta, Ehlers-Danlos syndrome and congenital contractural

arachnodactyly (4–6). The demonstration of gonadal and somatic mosaicism is important for accurate genetic counselling.

We previously reported that a Japanese EBS patient with migratory circinate erythema had a *de novo* 1649delG mutation in the V2 region of KRT5 (7). In this report, we describe a somatic mosaicism in the mother of this patient, which was not previously identified. This mosaicism was discovered, because the proband's younger sister was revealed to be affected with EBS at birth, upon reexamination of the familial segregation of the mutation.

Materials and methods

Informed consent for DNA analysis of the affected children and for familial analysis was obtained from their parents. Genomic DNA was extracted from peripheral blood lymphocytes, hair bulbs and buccal smears using an Isogen kit (Nippon Gene, Tokyo, Japan). Polymerase chain reaction (PCR) amplification of exon 9 of KRT5 was as described (7). Exon 9 of KRT5 was directly sequenced with the PCR-amplified fragment.

Case report

A 3-day-old newborn baby girl was transferred to the Department of Pediatrics, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu, Japan, because of blistering and erosion of both cutaneous lower extremities since birth. Physical examination revealed some variable-sized blisters and erosive areas on the bilateral knee. The lesions healed without scarring but with brown pigmentation. A clinical diagnosis of EBS was made, because her elder sister was previously diagnosed as having EBS and was recently reported as a novel type of EBS with migratory circinate erythema, which was caused by 1649delG of the KRT5 gene (7). In that report, the mutation was not detected in the parents' DNA and the mutation appeared to have occurred as a *de novo* event. Hence, the parents were told that the next baby would not have the same disorder. Because the younger sibling was revealed to have EBS, we reinvestigated the familial segregation of the mutation.

First, using the younger sister's blood mononuclear cells as a DNA source, we confirmed that she also had the same 1649delG mutation. As shown in (Fig. 1) (lane P), the younger sister had this mutation heterozygously. We resequenced the exon 9 fragments amplified from the parents' DNAs extracted from their

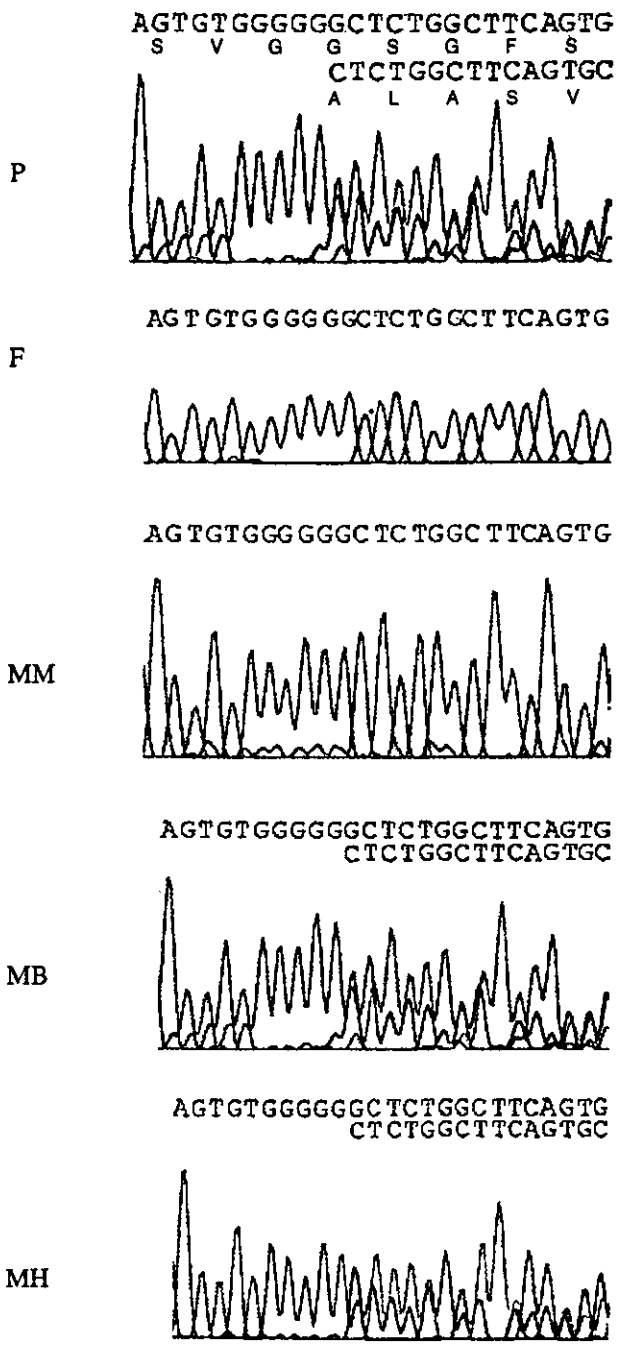

Fig. 1. The detection of 1649delG by direct sequencing of exon 9. Arrows indicate heterozygous 1649delG sites. P, patient; F, father; MM, mother (DNA from blood mononuclear cells); MB, mother (DNA from buccal smear samples); MH, mother (DNA from hair bulbs).

blood mononuclear cells. The mutation was not detected in their samples (Fig. 1, lanes F and MM). We then performed a careful check on the history of the parents. The mother remembered that she had migratory circinate pigmentation in her skin in childhood. Hence, we hypothesized that the mother is a mosaic of the mutation. We performed mutation analysis using DNAs from

her buccal smear samples and hair bulbs and showed that the mutant and normal alleles were identified in both of them (Fig. 1, lanes MB and MH).

Discussion

In this paper, we have described a somatic mosaicism in the mother of EBS patients. EBS is autosomal-dominant inherited blistering skin disease. Hence, if a proband's mutation is not detected in both the parents' DNA samples, it is regarded as a *de novo* mutation. Our results indicate that this simple interpretation is sometimes risky in autosomal-dominant disorders.

Mosaicism of a mutation in disorders with autosomal-dominant traits may not always give a clinical manifestation and sometimes gives few clinical manifestations. Such examples were observed in the cases of osteogenesis imperfecta (4), Ehlers-Danlos syndrome type IV (5) and contractural arachnodactyly (6). In the case of the contractural arachnodactyly, a mutation was detectable in DNA from the hair bulbs and buccal cells but not in white blood cell DNA. This is the same pattern as our case.

To our knowledge, this is the first case of somatic and germline mosaicism in EBS. The demonstration of somatic and germinal mosaicism in the KRT5 gene is important for accurate genetic counselling of families with sporadic cases of EBS. If a parent of the proband is affected, the risk to the sibs is 50%. When the parents are clinically unaffected, the risk to the sibs of a proband appears to be low but above the population risk because of reported (but rare) cases of somatic and germline mosaicism. In the latter case, DNA analysis using hair bulbs and blood mononuclear cells (DNA samples from two or more sources) from clinically 'unaffected' parents may be informative for genetic counselling of EBS, together with careful familial history taking.

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PEDIATRICS

Methylenetetrahydrofolate Reductase Polymorphism in Patients with Bronchial Asthma

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ABSTRACT

Objective: **Bronchial asthma is a chronic inflammatory condition of the respiratory tract. The C677T mutation in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene is reported to confer susceptibility to cardiovascular diseases and inflammatory conditions. We hypothesized that TT genotype of MTHFR may influence the development of bronchial asthma and thus examined the C677T polymorphism in our Japanese asthmatic patients.**

Design: **Clinical Investigation.**

Methods: **Genotypes for MTHFR were determined in 461 asthmatic patients (male/female ratio: 248/213) by the polymerase chain reaction and restriction fragment length polymorphism method and the results were compared with those obtained from 1430 healthy subjects (male/female ratio: 939/491).**

Results: **For the male population, the frequency of the TT genotype in asthmatic patients was significantly higher than in healthy subjects (16.9% vs. 11.0%, odds ratio = 1.65, 95% confidence interval: 1.12-2.44, P = 0.011). For the female population, the frequency of the TT genotype in atopic asthmatic patients was insignificantly higher than in non-atopic asthmatic patients (17.3% vs. 11.8%).**

Conclusion: **Our findings suggest that the TT genotype of MTHFR is a probable genetic risk factor for the development of bronchial asthma in Japanese males.**

KEY WORDS

bronchial asthma, homocysteine, methylenetetrahydrofolate reductase, oxidative stress, polymorphism

INTRODUCTION

Bronchial asthma is a condition characterized by episodic reversible airway obstruction, airway hyperresponsiveness and allergic inflammation of the airway. Multiple inflammatory cells, cytokines and other mediators participate in the pathogenesis of bronchial asthma[1]. Several lines of evidence suggest that oxidative stress contributes to airway inflammation and epithelial damage and to alterations in the immune system, and that individuals with lowered antioxidant capacity are at increased risk for developing asthma[2,3]. Therefore, genetic, environmental and dietary factors that diminish antioxidant defenses could potentially increase the vulnerability to oxidative stress and thus the risk for developing asthma. Accordingly, study of

Received on December 12, 2003 and accepted on March 18, 2004

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genetic polymorphism related to oxidative stress may be of help to our understanding of the complex pathophysiology of asthma, and could lead to the development of new and effective management protocols for this common disease.

There is general interest in hyperhomocysteinemia as a risk factor for vasculopathy, which is thought to exert its effects through oxidative damage[4,5]. Recently, a common C to T mutation at nucleotide position 677 (C677T) has been identified in the gene coding for 5,10-methylenetetrahydrofolate reductase (MTHFR), which is involved in the remethylation of homocysteine to methionine[6,7]. The C677T mutation causes a valine-for-alanine substitution, which decreases MTHFR activity and tends to be associated with elevated blood homocysteine levels. Other studies suggest that the TT genotype of MTHFR is significantly associated with coronary artery disease and ischemic stroke[8,9]. It has also been reported that the TT genotype may represent a genetic risk factor for the development and aggravation of inflammatory conditions such as inflammatory bowel disease, Kawasaki disease and chronic glomerulonephritis[10-13].

Based on the above considerations, we hypothesized that the TT genotype of MTHFR may influence the development of bronchial asthma and thus examined the C677T polymorphism in Japanese asthmatic patients. This is the first report to demonstrate the association between MTHFR C677T mutation and development of bronchial asthma.

METHODS

Subjects

We studied 461 Japanese patients with bronchial asthma who had been seen at the outpatient clinics of Fukui Medical University Hospital (Fukui), Tenri Hospital (Nara), Osaka University Hospital (Osaka), Hokkaido University Hospital (Hokkaido), Gifu University Hospital (Gifu) and their affiliated hospitals. They included 248 males and 213 females and their age ranged from 1 to 85 years (mean 33 years; median 28 years). The diagnosis of asthma was based on the criteria of the National Asthma Education and Prevention Program, Expert Panel Report II[14]. Atopy was defined by the presence of high levels of specific serum IgE (≥ 0.35 KU_A/l) to at least one aeroallergen (such as house dust mite) using the CAP radioallergosorbent test fluoro enzyme immunoassay system (Pharmacia Upjohn Diagnostics, Tokyo, Japan)[15]. As age-matched healthy controls, 1430 subjects (939 males and 491 females) were derived from studies conducted by our group (n = 339)[11] (unpublished results) and other groups (n = 1091)[8,16,17]. Informed consent was obtained from our subjects (461 patients and 339 healthy controls) and/or their parents before blood samples were collected. This study protocol was approved by the ethics committees of all the hospitals that participated in the present study.

Genetic analysis

Genomic DNA was extracted from peripheral-blood leukocytes. Identification of the C677T mutation in the MTHFR gene was performed by polymerase chain reaction (PCR) using primers described by our group[11]: 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' and 5'-AGGACGGTGCGGTGAGAGTG-3'(Toagosei, Tsukuba, Japan). PCR thermal cycling conditions were a 10-min denaturation period at 94℃ and 43 cycles of the following: 94℃ for 1 min, 55℃ for 30 s and 72℃ for 1 min. This was followed by a 10-min extension at 72℃ (Program Temp Control System PC-700, ASTEC, Fukuoka, Japan). The amplified products were digested with *Hinf*I (TaKaRa Biomedicals, Ohtsu, Japan) at 37℃ for 4 hours. The *Hinf*I-treated PCR fragments were electrophoresed in 5% polyacrylamide gel and visualized with ethidium bromide. The C to T mutation creates a *Hinf*I recognition site that leads to digestion of the 198-bp PCR product into 175- and 23-bp fragments. Heterozygote subjects show three fragments (198 bp, 175 bp and 23 bp) and a homozygous C to T mutation results in the production of two fragments of 175 bp and 23 bp.

Although we did not examine blood homocysteine levels in our subjects, data from another group on healthy Japanese individuals showed that the mean homocysteine level was significantly higher in subjects with the TT genotype (15.7 μM) than in those carrying the CC (11.6 μM) or CT (11.8 μM) genotype[18].

Statistical analysis

The analysis of genotype involved comparing the number of subjects carrying the TT genotype with the number of subjects carrying the other genotypes. Statistical analysis was also performed on the numbers of C677 alleles and T677 alleles. The differences were examined by the chi-squared test or Fisher's exact test where appropriate. Statistical significance was inferred when the P value was < 0.05.

RESULTS

The genotype frequencies of MTHFR in healthy subjects and asthmatic patients are listed in Table 1. The frequencies of the C677T polymorphism in healthy subjects were CC: CT: TT = 579 (40.5%): 681 (47.6%): 170 (11.9%). The frequency of the TT genotype was higher in both male and female asthmatic patients (16.9% vs. 11.0%, 16.0% vs. 13.6%, respectively) and the difference was significant for the male population (odds ratio = 1.65, 95% confidence interval: 1.12-2.44, P = 0.011). The allele frequency of the T mutation was significantly higher in male asthmatics than in male healthy controls (41.3% vs. 34.3%; odds ratio = 1.35, 95% confidence interval: 1.10-1.65, P = 0.004).

According to the criteria described in the METHODS section, 378 patients (82%) were classified as "atopic" asthmatics and 83 (18%) were "non-atopic" asthmatics. The distribution of the MTHFR C677T polymorphism was examined according to atopy (Table 2). For the female population, the frequencies of the TT genotype and the T677 alleles were higher in atopic asthmatics than those in non-atopic asthmatics (17.3% vs. 11.8%, 40.7% vs. 34.3%, respectively), but the differences were statistically insignificant.

Table 1. Genotype distribution of the MTHFR gene in healthy subjects and asthmatic patients

	MTHFR		
	CC	CT	TT
Healthy subjects			
Male (n = 939)	398 (42.4%)	438 (46.6%)	103 (11.0%)
Female (n = 491)	181 (36.9%)	243 (49.5%)	67 (13.6%)
Asthmatic patients			
Male (n = 248)	85 (34.3%)	121 (48.8%)	42 (16.9%)*
Female (n = 213)	80 (37.6%)	99 (46.5%)	34 (16.0%)

MTHFR, 5,10-methylenetetrahydrofolate reductase. Numbers in parentheses represent percentage of individuals.
*P = 0.011 vs. corresponding healthy subjects.

Table 2. Genotype distribution of the MTHFR gene in patients with atopic and non-atopic asthma

	MTHFR		
	CC	CT	TT
Atopic asthmatic patients			
Male (n = 216)	72 (33.3%)	107 (49.5%)	37 (17.1%)
Female (n = 162)	58 (35.8%)	76 (46.9%)	28 (17.3%)
Non-atopic asthmatic patients			
Male (n = 32)	13 (40.6%)	14 (43.8%)	5 (15.6%)
Female (n = 51)	22 (43.1%)	23 (45.1%)	6 (11.8%)

MTHFR, 5,10-methylenetetrahydrofolate reductase. Numbers in parentheses represent percentage of individuals.

DISCUSSION

Advances in asthma management are likely to depend on a better understanding of how genetic and environmental factors influence susceptibility to, and outcome in, this disease. The implication of the MTHFR C677T polymorphism in the pathogenesis of bronchial asthma is entirely novel. The MTHFR gene is located on chromosome 1 at 1p36.3. The complementary DNA sequence is 2.2 kilobases long and consists of 11 exons[6,7]. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate and carbon donor for the remethylation of homocysteine to methionine. In recent years, accumulating evidence suggests that mild to moderate hyperhomocysteinemia and its genetic promoter, MTHFR C677T mutation, may be associated with the development and progression of cardiovascular and inflammatory diseases[6-13].

Reactive oxygen species (ROS) are implicated in the cellular toxicity of hyperhomocysteinemia[4,5]. ROS, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), are generated during oxidation of the sulfhydryl group of homocysteine. Excessive O_2^- inactivates nitric oxide (NO) with the formation of the adduct peroxynitrite ($ONOO^-$). These oxidants are thought to account for the homocysteine-induced cytotoxicity. Furthermore, homocysteine both inhibits glutathione peroxidase activity *in vitro* and leads to a marked reduction in mRNA levels for the intracellular isoform; the inhibition of glutathione peroxidase is unique to homocysteine compared with other biologic thiols. Homocysteine significantly decreases the intracellular thiol redox state, as measured by the ratio [glutathione]/[glutathione disulfide], through these oxidative mechanisms.

The reported frequency of homozygotes for the mutation, having the TT genotype, varies in different populations from one geographic area to another (ranging from 5 to 20%)[6,7]. Our results showed that among Japanese healthy subjects the frequency of the TT genotype was 11.9% (170/1430), which was comparable to that in Caucasians.

The present study is the first to demonstrate the association between the TT genotype and development of bronchial asthma in male subjects. The mechanism(s) of the predisposition of the TT genotype to asthma remains speculative. The contribution of oxidative stress induced by this genotype is one possibility. Oxidative stress has been implicated in the initiation and worsening of asthma[2,3]. Increased release of ROS has been reported in exhaled condensates[19] and from circulating neutrophils and monocytes, bronchoalveolar lavage cells of patients with asthma[20]. ROS can cause cellular damage by oxidizing membrane lipids, proteins and nucleic acids. Furthermore, oxidative stress can activate the transcription factor, nuclear factor kappa-B (NF-κB), and previous studies reported high expression of this factor in airway cells of patients with asthma[21,22]. Increased NF-κB expression and DNA binding may underlie the increased expression of several inflammatory proteins in the asthmatic airway, including tumor necrosis factor-α, interleukin (IL)-1β; RANTES, eotaxin, macrophage chemotactic protein-1, macrophage inflammatory protein-1α; granulocyte-macrophage colony stimulating factor; inducible NO synthase, inducible cyclooxygenase; and intercellular adhesion molecule-1, vascular cell adhesion molecule-1[21-25]. It seems reasonable to consider that these changes create feed-forward amplifying loops that could form the basis for the development and progression of the chronic inflammatory process in asthma and that the MTHFR TT genotype enhances these pathological events especially in male subjects.

It is of note that more males than females develop asthma during childhood, probably because of narrower air-

ways, increased airway tone and possibly higher IgE in boys[14]. For the male asthmatic patients, the frequency of the TT genotype in children was insignificantly lower than in adults (patients aged < 16 years: 15.3% [20/131] vs. patients aged ≥ 16 years: 18.8% [22/117]). In addition, the frequency of the TT genotype in atopic asthmatics was almost comparable to that in non-atopic asthmatics (17.1% vs. 15.6%) (Table 2).

It needs to be taken into account that some proportion of the healthy controls reported by other groups (n = 1091)[8,16,17] may have allergic diatheses including bronchial asthma. The prevalence of asthma in Japanese children and adults is reported to be 3%[26]. If these individuals of the above healthy controls (3% of the total of 1091 subjects) were assumed to have the same distribution of the C677T polymorphism as the patient group, the frequency of the TT genotype would remain higher in both male and female asthmatics than that of the corresponding "non-asthmatic" healthy controls (16.9% vs. 10.8%, 16.0% vs. 13.6%, respectively). Under this condition, the difference would remain significant for the male population (odds ratio = 1.68, 95% confidence interval: 1.14-2.47, P = 0.007).

Atopy is a significant predisposing factor for the development of asthma[14]. Certain genetic and environmental factors drive the development of a Th2 lymphocyte-predominant immune response, which is associated with atopy and IgE-mediated inflammation. Th2 lymphocytes generate cytokines, including IL-4, IL-5 and IL-13, which play a primary role in B cell switching to IgE synthesis. In contrast, Th1 lymphocytes, which play a primary role in interferon-γ production, inhibit B-cell IgE synthesis. Th1- and Th2-type cytokines reciprocally regulate the Th1/Th2 immune responses. Recent *in vitro* studies have suggested that oxidative environments may act differentially on activated human Th cells by inhibiting Th1 cytokine production but promoting the expression of Th2 cytokines; thiol antioxidants such as N-acetyl-L-cysteine and glutathione decrease IL-4 production in human T cells and IgE production by B cells[27,28]. Moreover, glutathione depletion in antigen presenting cells inhibits Th1 cytokines and/or favors Th2 responses[29]. In studies using human B cell lines, Yanagihara *et al.*[30] showed that N-acetyl-L-cysteine regulates the IgE isotype switching by inhibiting the activation of NF-κB. It was expected that altered redox status induced by the TT genotype may drive the development of the Th2-predominant immune responses to environmental stimuli, which is associated with atopy and allergic inflammation in the airway. For the female population, the frequency of the TT genotype in atopic asthmatics was higher than in non-atopic asthmatics, although the difference was statistically insignificant. Since the number of non-atopic asthmatics was rather small, further studies using larger population samples may be required to confirm this contention.

In conclusion, our results suggest that the MTHFR TT genotype is a probable genetic risk factor for the development of bronchial asthma, possibly through the oxidative mechanism, in male Japanese subjects. Although further investigations are required to clarify the molecular mechanisms governing whether a Th1 or Th2-immune response predominates in the hyperhomocysteinemic state, the findings presented here should provide new insights into the pathophysiology of bronchial asthma. The homocysteine-lowering effects of folate and vitamins B_6 and B_{12} have been anticipated for individuals carrying the TT genotype[7]. The other relevant subject of exploration may be the investigation of the possible favorable effects of these vitamins on the development and worsening of asthma.

ACKNOWLEDGEMENTS

We thank Ms. Michiyo Toyooka for the excellent technical assistance. This study was supported by a Health Sciences Research Grant from the Ministry of Health, Labor and Welfare, Japan.

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