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Identification of polymorphisms in the promoter region of the human *NRF2* gene

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Abstract

Transcription factor Nrf2 regulates the basal and inducible expression of detoxifying and antioxidant genes. Recent studies using *nrf2*-null mice suggest that Nrf2 dysfunction might be involved in the pathogenesis of human diseases. To gain insight into the relationship between impairment in the *NRF2* gene and human diseases, we attempted to identify polymorphisms in the human *NRF2* gene. We determined the structure of the *NRF2* gene and found three single nucleotide polymorphisms and one triplet repeat polymorphism in its regulatory region. These results provide a molecular basis for the genetic analysis of the *NRF2* gene. The frequency of each polymorphism was examined in two groups of patients with systemic lupus erythematosus and chronic obstructive pulmonary disease. This study did not reveal a close connection between the risk of these diseases and the polymorphisms. However, available lines of evidence suggest the importance of examining the link between *NRF2* polymorphisms and other oxidative stress-related diseases.

Keywords: Human *NRF2* gene; Transcription; Initiator site; Promoter; Polymorphism; SNP; Japanese; 5′-RACE; RNase protection; Systemic lupus erythematosus; Chronic obstructive pulmonary diseases

Nrf2 belongs to the CNC family of transcription factors harboring a characteristic basic-leucine zipper (b-Zip) motif and is essential for the basal and inducible expression of a battery of detoxifying and antioxidative enzyme/protein genes [1–3]. Nrf2-deficient mice are susceptible to xenobiotic and oxidative stress owing to an impaired expression of their cytoprotective enzymes. Consequently, Nrf2-deficient mice display various pathological phenotypes, some of which are similar or related to human disorders [4–7]. For instance, systemic exposure to butylated hydroxytoluene and acetaminophen leads to acute pulmonary injury [4] and acute hepatotoxicity [5], respectively. Inhalation of diesel exhaust results in the accumulation of high levels of DNA adducts [6]. Mechanisms underlying cancer chemoprevention are also defective in Nrf2-deficient mice [7,8], with oltipraz and sulforaphane failing to exert their protective effects against xenobiotic-induced carcinogenesis, thus indicating the critical roles that Nrf2 plays in cancer chemoprevention.

Recent studies also unveiled that Nrf2 is an important regulator of oxidative stress-inducible genes, such

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as heme oxygenase-1 and peroxiredoxin 1 [9]. Susceptibility to hyperoxia turned out to be tightly linked to the *nrf2* locus [10]. A single nucleotide polymorphism was detected in the promoter region of the *nrf2* gene belonging to C57BL/6J mice, a strain sensitive to hyperoxic stress. Supporting this contention, *nrf2*-null mutant mice were found to be highly susceptible to hyperoxic lung injury [11]. Electron paramagnetic resonance (EPR) and spin probe kinetic analysis showed that the impaired defense mechanism against oxidative stress in the liver and kidney of *nrf2*-null mutant mice substantially decreases the ability to eliminate reactive oxygen species (ROS) [12]. It has been assumed that ROS play a prominent role in the pathogenesis of various human diseases including nephritis. The impaired elimination of ROS is exacerbated in aged female Nrf2-deficient animals [12]. In fact, elderly female *nrf2* -deficient mice often develop lupus-like autoimmune nephritis [13].

Available evidence indicates that the activation of Nrf2 is an important cue for the inducible expression of cytoprotective genes. Exposure to electrophilic reagents dissociates Nrf2 from its cytoplasmic repressor Keap1, resulting in stabilized Nrf2 which is free to translocate from the cytoplasm to the nucleus [14–18]. In some cell lineages, transcription of the *nrf2* gene is also facilitated in response to stress stimuli, implying the presence of a positive feedback system to attain a high level of Nrf2 expression [19].

These analyses using Nrf2-deficient animals led us to hypothesize that the dysregulation of Nrf2 activity may explain, at least in part, the pathogenesis of certain human diseases. To address this issue further, we attempted to search the human *NRF2* gene locus for polymorphisms. To this end, we determined the structure of the *NRF2* gene and identified its transcription initiation site using extension and protection methods. We then determined the nucleotide sequences of the gene from healthy volunteers, and found three SNPs and one triplet repeat polymorphism in the regulatory region. We could not find any polymorphisms in the coding exons. Thus, this study provides a solid molecular basis for the genetic analyses of the human *NRF2* gene. In our preliminary analysis, the frequencies of the promoter polymorphisms were examined in healthy and disease-prone Japanese populations, especially those with systemic lupus erythematosus (SLE) and chronic obstructive pulmonary disease (COPD). While we were unable to find a close relationship between the identified polymorphisms and SLE or COPD, we feel it is highly likely that a significant association between certain diseases and the polymorphisms identified in this study may become apparent if a more adequate population is selected.

Materials and methods

Oligonucleotides and human NRF2 plasmids. The oligonucleotides used for PCR and 5′-rapid amplification of the cDNA ends (RACE) are shown in Tables 1 and 2. The genomic PCR oligonucleotides were designed for the detection of polymorphisms so that the dye–primer sequence reaction could be followed directly; an 18 bp oligonucleotide (5′-tgtaaaacgacggccagt-3′) was attached to the 5′-ends of forward

Table 1
Sequence of oligonucleotides used for 5′-RACE, RPA, and promoter sequencing

Name	Sequence
5′-RACE primers	
E2/89-68	5′-TACTCTTTCCGTCGCTGACTGAA-3′
E2/64-35	5′-CAAATACTTCTCGACTTACTCCAAGATCTA-3′
Primers for making RPA probes	
Probe/A-F	5′-ccgcggatccCGGGCGGTAAAGTGAGATAA-3′
Probe/B-F	5′-ccgcggatccGGGATTTTCGGAAGCTCAG-3′
Exon 1-R1	5′-GAGCTGTGGACCGTGTGTT-3′
Exon 1-F1	5′-ATCATGATGGACTTGGAGCTG-3′
Probe/E2-R	5′-gccggaattCTGGTTTCTGACTGGATGTGC-3′
Exon 1-R2	5′-GCAGCTCCAAGTCCATCAT-3′
Primers for genotyping polymorphism	
hNrf2/P2-F	5′-tgtaaaacgacggccagtGCGTGGTGGCTGCGCTTT-3′
hNrf2/P2-R	5′-caggaaacagctatgaccGCCCGCGAGATAAAGAGTTG-3′
hNrf2/E1-F	5′-tgtaaaacgacggccagtCGTGTAGCCGATTACCGAGTGCC-3′
hNrf2/E1-R	5′-caggaaacagctatgaccCTCTGGCCAGACGTGGGGGAAG-3′
hN/E1g-F	5′-Cy5-TAGCCGATTACCGAGTGCCG-3′
hN/E1g-R	5′-GGCAGCTCCAAGTCCATCATG-3′

Upper-case letters represent the sequences derived from the human *NRF2* gene locus, while lower-case letters represent synthetic sequences. *Bam*HI and *Eco*RI restriction sites are underlined. Synthetic sequences attached to the 5′-ends of primers for genotyping were hybridized by the dye-primers used in an ABI PRISM BigDye Primer Cycle Sequencing Ready Reaction Kit for direct sequencing analysis. The 5′-end of hNE1g-F was modified with Cy5 for gene scan analysis for genotyping the triplet polymorphism.

Table 2
Sequence of oligonucleotides used for sequencing the coding region of the human *NRF2* gene

Name	Sequence
hNrf2/E2-F	5′-tgtaaaacgacggccagtACCATCAACAGTGGCATAATGTG-3′
hNrf2/E2-R	5′-caggaaacagctatgaccGCAAAGCTGGAACTCAAATCCAG-3′
hNrf2/E3-F	5′-tgtaaaacgacggccagtATTATTGAATATTTAGCTTGGC-3′
hNrf2/E3-R	5′-caggaaacagctatgaccGGAGATTCATTGACGGGACTTAC-3′
hNrf2/E4-F	5′-tgtaaaacgacggccagtTGTAGTGGTGCCTTAGAGCTTAC-3′
hNrf2/E4-R	5′-caggaaacagctatgaccAATAGCACCCTCCAATCCTTCC-3′
hNrf2/E5a-F	5′-tgtaaaacgacggccagtCTGAAGATAATGTGGGTAGGGAG-3′
hNrf2/E5a-R	5′-caggaaacagctatgaccCATTCTGTTTGACACTTCCAGGG-3′
hNrf2/E5b-F	5′-tgtaaaacgacggccagtTGATTCTGAAGTGGAAGAGCTAG-3′
hNrf2/E5b-R	5′-caggaaacagctatgaccCTAAATCTTGCTCTAGTTCTAC-3′
hNrf2/E5c-F	5′-tgtaaaacgacggccagtGTAAGAATAAAGTGGCTGCTCAG-3′
hNrf2/E5c-R	5′-caggaaacagctatgaccTCAACATACTGACACTCCAATGC-3′

Upper-case letters represent the sequences derived from the human *NRF2* gene locus, while lower-case letters represent synthetic sequences.

primers and an 18 bp oligonucleotide (5′-caggaaacagctatgacc-3′) was attached to the 5′-ends of reverse primers. One of the primers for gene scan (hN/E1g-F) was modified at the 5′-end with Cy5. The plasmid harboring the human NRF2 cDNA (pcDNAI/hNRF2) was a kind gift from Dr. Etsuro Ito, and the P1 phage containing the human *NRF2* gene was from Genome Systems.

Cell culture and RNA isolation. To isolate human total RNA, Jurkat, HeLa, and Hep3B cells were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. These cells were incubated at 37 °C in 5% CO_2 until sub-confluency. The cells were harvested in Isogen (Nippon Gene) to isolate total RNAs.

5′-RACE analysis. Poly(A)$^+$ RNA was purified from 250 μg total Jurkat cell RNA using Oligotex-MAG mRNA purification kit (TaKaRa), and 5′-RACE analysis was carried out with SMART RACE cDNA Amplification Kit (Clontech Laboratories). Two reverse primers corresponding to the second exon sequence of the *NRF2* gene were designed; E2/89-68 is a downstream primer and E2/64-35 is an upstream primer for nested PCR analysis. The 5′-RACE products were subcloned into pGEM-T Easy Vector (Promega) and sequenced with SP6 or T7 primers by an ABI PRISM 377 Genetic Analyzer (Applied Biosystems).

Ribonuclease protection assay. To generate the ribonuclease protection assay (RPA) probes, artificial NRF2 plasmids containing the promoter region followed by NRF2 cDNA were constructed by PCR. PCR primers were designed as shown in Fig. 1A. The promoter region was amplified with either Probe/A-F (Fig. 1A, a) and Exon 1-R1 (Fig. 1A, c) or Probe/B-F (Fig. 1A, b) and Exon 1-R1 (Fig. 1A, c) using the P1 phage harboring the *NRF2* gene locus as a template. The cDNA fragment containing exon 1 and exon 2 was amplified with Exon 1-F1 (Fig. 1A, d) and Probe/E2-R (Fig. 1A, e) using pcDNAI/hNRF2 as a template. *Bam*HI sites were added to the 5′-ends of the primers Probe/A-F and Probe/B-F, while an *Eco*RI site was added to 5′-end of the primer Probe/E2-R. The PCR products generated from the P1 phage and from the NRF2 cDNA were mixed and sub-cloned into the *Bam*HI/*Eco*RI sites of pBluescript II SK (+) (Stratagene). The hybrid hNRF2 plasmids were linearized by *Not*I digestion and used as templates for antisense RPA probes that were synthesized in the presence of [α-^{32}P]dCTP (Promega). Total RNA (30 μg) was used for hybridization with each probe, and RPA was performed in the reaction system of RPA III (Ambion). Yeast tRNA was used as a negative control. To prepare the size marker, a genomic fragment amplified with Probe/B-F and Exon 1-R2 was cloned into pGEM-T Easy Vector and the DNA sequencing reaction was performed with SP6 primer according to the Sanger method (BcaBEST Dideoxy Sequencing Kit, TaKaRa).

Human DNA isolation. DNA samples were obtained from three types of Japanese populations; 51 SLE patients, 87 COPD patients [20], and 81 healthy controls. SLE patients were diagnosed based on the criteria revised by American College of Rheumatology in 1997 [21,22]. Out of 51 SLE patients, 40 suffered from lupus nephritis as a complication and 47 were female. COPD patients were as described in a previous report [20]. Among 50 healthy controls, 25 were female and the rest were male. Genomic DNAs were isolated from whole blood using a QIAamp DNA Blood Midi Kit (Qiagen). The study protocol was approved by the Institutional Ethical Review Board of the University of Tsukuba.

Identification and genotyping of polymorphisms. Two and six sets of PCR primers were designed to amplify the promoter and the coding regions of the *NRF2* gene, respectively (Tables 1 and 2). Direct sequencing of the PCR products was performed to identify polymorphisms (ABI PRISM BigDye Primer Cycle Sequencing Ready Reaction Kit). The genotypes of SNPs were determined by directly sequencing PCR products amplified with hNrf2/P2-F and hNrf2/P2-R. Briefly, 500 ng of genomic DNA was used as a template, and PCR was performed using 200 nM of each primer, 200 nM of each deoxynucleotide triphosphate, 1× Ex *Taq* buffer, and 1 U Ex *Taq* polymerase (TaKaRa). PCR products were electrophoresed in a 2% agarose gel and purified using a QIAquick Gel Extraction kit (Qiagen). Sequence reactions were carried out using an ABI PRISM BigDye Primer Cycle Sequencing Ready Reaction Kit and genotyped by an ABI 3100 DNA Sequencer. Genotyping triplet repeat polymorphism was performed by Gene scan analysis using an ABI 310 Sequencer, determining the repetition number of the ccg triplet repeats. Cy5-modified hN/E1g-F and hN/E1g-R were used to amplify the promoter region containing the triplet repeat by PCR with 500 ng of genomic DNA, 200 nM of each primer, 400 nM of each deoxynucleotide triphosphate, 1× GC buffer II (TaKaRa), and 1.5 U Ex *Taq* polymerase (TaKaRa).

Statistical analysis of human samples. The genotypic and allelic frequencies between patients and controls were both analyzed by Fisher's exact test. The genotypic frequencies were calculated between a homozygous population of one allele and the other populations including a homozygous population of the other allele and a heterozygous population. Significance was accepted when the p value was less than 0.05.

Results and discussion

Structural analysis of the human NRF2 gene

In our initial analysis, we searched the human genome databases and found information on the *NRF2* gene structure including the location of each exon on the genome and the sequences of exon–intron boundaries

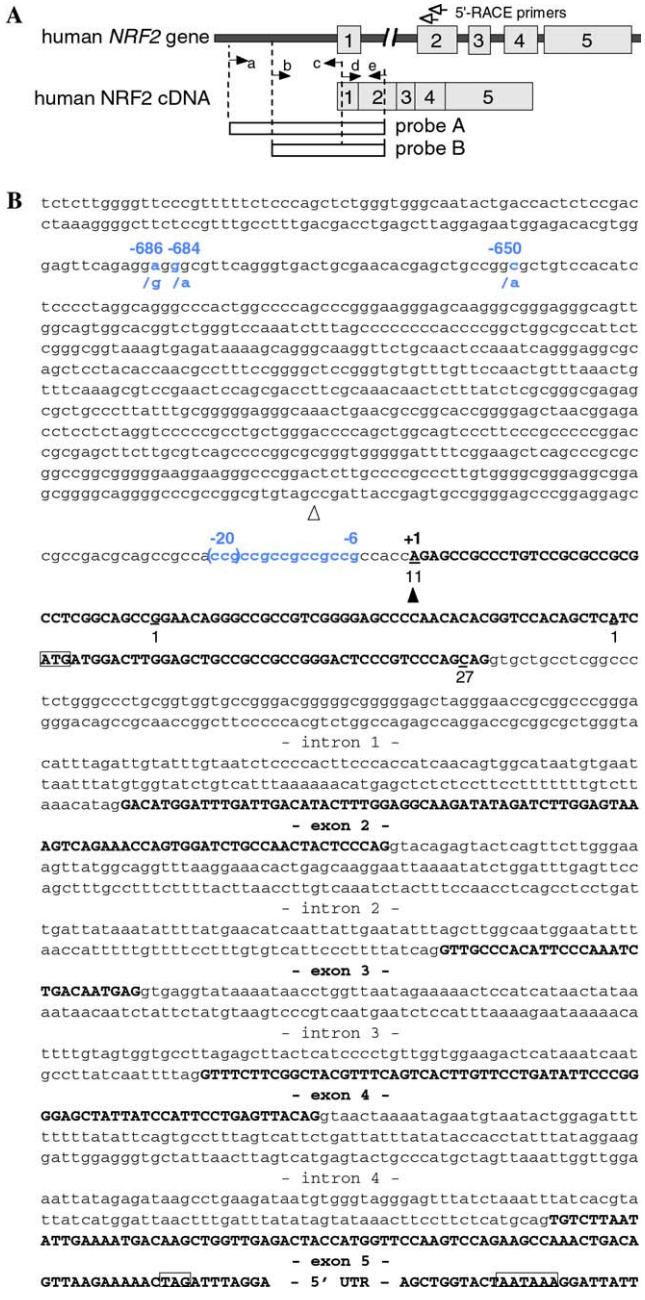

Fig. 1. Structure of the human *NRF2* gene and identification of the first exon. (A) Illustration of the human *NRF2* gene and NRF2 cDNA. Five exons were identified in the *NRF2* gene, and each is indicated by a numbered box, while NRF2 cDNA is depicted as an elongated box below. Two open arrows above exon 2 indicate the positions of the 5′-RACE primers. Five closed arrows (a–e) at the 5′ region of the *NRF2* gene and the NRF2 cDNA represent the primers for making RPA probes, which are indicated by two open bars at the bottom. (B) The sequences of the first exon and the promoter region followed by the exon-intron boundary sequences of the *NRF2* gene. The exon sequences are written in bold upper-case letters. The nucleotides at the 5′-ends of the 5′-RACE clones are underlined, and the numbers written beneath represent the obtained clone numbers. The major transcription initiation site determined by RPA is indicated by a closed arrowhead and numbered as +1, and the minor initiation site is indicated by an open arrowhead. The identified polymorphisms are highlighted in blue. The translation start codon, the stop codon, and poly(A) signal are boxed.

(Figs. 1A and B). However, we noticed that the database reports showed several different transcription start sites (BC011558, NM.006164, hCT1952818, S74017, hCT 12360, and BX649047) and, out of these six reports, four contained the same first exon harboring the translation initiation codon (BC011558, NM.006164, hCT1952818, and S74017), while the other two contained the same non-coding first exon (hCT12360 and BX649047).

In order to identify the major first exon of the *NRF2* gene, we performed 5′-RACE analysis using RNA isolated from human Jurkat cells. For this purpose, we synthesized two sequential reverse primers, E2/89-68 and E2/64-35, corresponding to the second exon sequence of the human *NRF 2* gene (Table 1 and Fig. 1A). The downstream primer (E2/89-68) was used for the first extension, while the upstream primer (E2/64-35) was used for the following nested PCR. The products of this 5′-RACE reaction were cloned, with 40 clones being analyzed further. To our surprise, all 40 clones basically shared the same exon sequence that is located approximately 30 kb upstream of the second exon. The first exon sequences of the four database reports (BC011558, NM.006164, hCT1952818, and S74017) coincided with the one identified in this analysis. The sequence of the first exon of the human *NRF2* gene shows only limited homology (70%) with that of the mouse *nrf2* gene, which is located 25 kb from the second exon.

Eleven out of 40 clones retained the longest fragment and shared the same 5′-end adenine nucleotide, while the remaining 27 clones contained shorter fragments (Fig. 1B). We tentatively assigned this 5′-end adenine as the transcription initiation site. This result indicates that a single exon was dominantly utilized for *NRF2* gene expression in Jurkat cells.

Verification of the transcription initiation site

We further asked where is the major transcription initiation site, since three different transcription start sites had been described for the first exon identified by 5′-RACE analysis (BC011558, NM.006164, hCT1952818, and S74017). The reason for these discrepancies is unclear at present, and it was important for us to verify our proposed transcription initiation site more precisely. Therefore, we performed RPA (ribonuclease protection assay) using RNA samples from Jurkat, HeLa, and Hep3B cells. The first exon and 5′-flanking sequences identified through the 5′-RACE analysis were linked to the second exon sequence to generate the RPA probes. Two probes were synthesized; probe A is 890 bp while probe B covers 556 bp (see Fig. 1A, probes A and B). RNase digestion and subsequent electrophoresis revealed several protected bands in the RNA from Hep3B cells (Fig. 2A). The most intense band was observed at 373 bp, and a minor band was observed at 443 bp. We found that both probes gave rise to protected bands of a similar size.

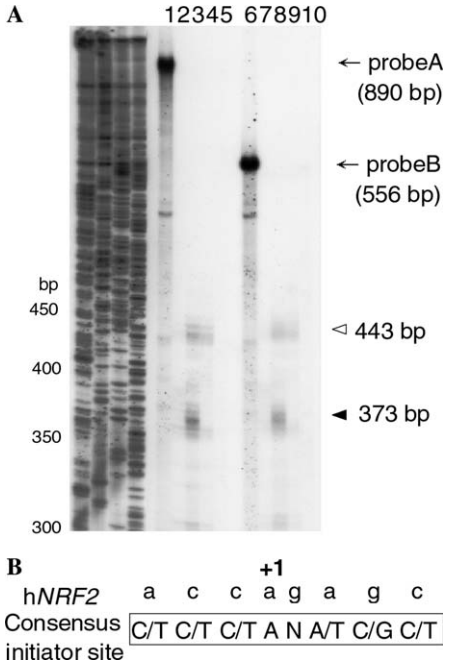

Fig. 2. Transcription initiation site of the human *NRF2* gene. (A) RPA for determining the 5′-end of the *NRF2* gene. Thirty micrograms of total RNA from HeLa (lanes 2 and 7), Hep3B (lanes 3 and 8), and Jurkat (lanes 4 and 9) cells and yeast tRNA as a control (lanes 5 and 10) was hybridized with ^{32}P-labeled probe A (lanes 2–5) or probe B (lanes 7–10), and digested with RNase A. The digested products were separated by electrophoresis with undigested probes (probe A in lane 1 and probe B in lane 6). The major and minor protected bands are indicated by closed and open arrowheads, respectively. DNA ladders applied in the four lanes on the left-hand side serve as size markers. (B) Comparison of the sequence surrounding the transcription initiation site of the *NRF2* gene and the consensus initiator site.

When we carried out the RPA experiment with RNAs derived from Jurkat and HeLa cells, the same pattern of protection was produced, but the bands were much fainter than those obtained with Hep3B cell-derived RNA. This observation implies that Nrf2 is expressed at a much lower level in Jurkat and HeLa cells than in Hep3B cells. The size of the major protected band matches that of the first nucleotide of the longest clones identified by 5′-RACE analysis (Fig. 1B). Furthermore, the nucleotide sequence surrounding the assigned transcription initiation site (Fig. 2B) corresponded well with the consensus initiator site sequence [23,24]. Overall, we conclude that the adenine nucleotide does indeed represent the transcription start site. Since the same initiation site was commonly used in three different cell lines, the single promoter is likely to specify transcription of the *NRF2* gene.

Polymorphisms identified in the promoter region of the NRF2 gene

In the hope of finding sequence polymorphisms in the coding, promoter, and upstream promoter regions of the *NRF2* gene, we sequenced approximately 1 kb of the *NRF2* promoter and regions upstream from the transcription initiation site in the P1 phage. Comparison of this sequence with the one registered in the NCBI human genome database (AC079305) revealed a single nucleotide difference. We then determined the sequences for the *NRF2* promoter and upstream regions using DNA samples from 12 healthy volunteers within the Japanese population. As a result, we found three single nucleotide polymorphisms (SNPs), including the one found in the above inspection. These SNPs were located at positions −686 (A/G), −684 (G/A), and −650 (C/A) (Fig. 3A). In addition to these SNPs, a triplet repeat polymorphism was found between −20 and −6 (four-time repetition versus five-time repetition of CCG; Fig. 3B).

We also determined the sequences for all the *NRF2* exons utilizing the 12 DNA samples from the volunteers. The primer sets used for this analysis are summarized in Table 2. However, we could not find any SNPs in the coding exons (data not shown), suggesting that the gene structure of *NRF2* is strictly conserved, and we therefore ceased our search for coding SNPs. Considering the high sensitivity of C57BL/6J strain mice to oxidative stress

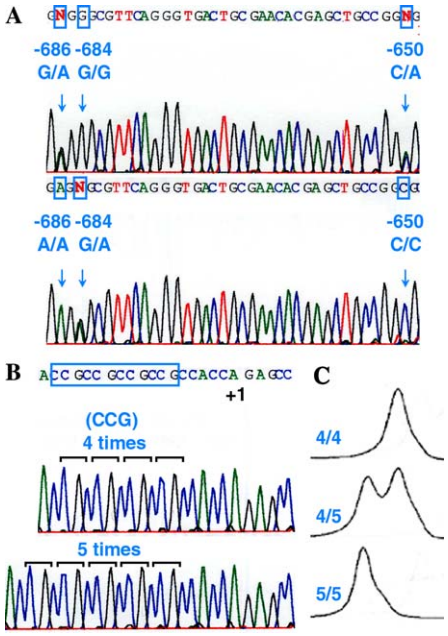

Fig. 3. Polymorphisms found in the promoter region of the human *NRF2* gene. (A) Representative electropherograms in two different patterns of the *NRF2* gene promoter region from position −685 to −649. The positions of the three SNPs, −686 G/A, −684 G/A, and −650 C/A, are indicated by arrows, and the polymorphic nucleotides are boxed in blue. (B) Representative electropherograms of homozygotes for 4- (top) or 5-time (bottom) triplet repeat polymorphisms. The transcription initiation site is numbered as +1. (C) Representative gene scan images of three different patterns of triplet repeat polymorphisms between positions −20 and −6. The homozygous pattern of 4-time repetition (top), the heterozygous pattern of 4- and 5-time repetitions (middle), and the homozygous pattern of 5-time repetition (bottom) are shown.

linked to the single nucleotide polymorphism (SNP) in the promoter region of *nrf2* gene [10], we decided to concentrate on the promoter region of the human *NRF2* gene for the search of the polymorphisms.

Study of polymorphism frequency in the Japanese population

The frequencies at which the promoter polymorphisms occurred in a healthy Japanese population were examined (Table 3, control). DNA samples were obtained from 81 healthy volunteers and genomic DNA was isolated from whole blood. SNPs were genotyped by direct sequencing (Fig. 3A), while the triplet-repeat polymorphism was determined by Gene Scan analysis (Fig. 3C). The results revealed that these polymorphisms commonly exist within the Japanese population, except for the one at position –684, for which allele A is quite rare compared to the other allele G. As for the triplet repeats, the four-time repeat is more frequent than the five-time repeat in the Japanese population.

Association of the promoter polymorphisms with human diseases

We performed a case-control study using these promoter polymorphisms in two human diseases, SLE and COPD. SLE was chosen because we previously found that female *nrf2*-deficient mice over 12 months of age often develop lupus-like autoimmune nephritis [13]. Furthermore, genome-wide linkage analysis of SLE patients pointed a genomic locus 2q31 as one of the candidate loci, which is very close to the human *NRF2* gene locus [25–27]. On the other hand, COPD was chosen as a representative of chronic inflammatory diseases. Recent analyses of *nrf2* -null mutant mice revealed that inflammation tends to be prolonged due to the reduced sensitivity of inflammatory cells to 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) [28,29]. 15d-PGJ_2 was found to activate Nrf2 and regulate the late stage of inflammation, suggesting that reduction in Nrf2 activity may be one of the exacerbating causes of persistent inflammation.

We therefore collected Japanese DNA samples from 81 control subjects, 51 SLE patients, and 87 COPD patients and the four polymorphisms were genotyped (Table 3). All genotype results of the polymorphisms in the control samples were in the Hardy–Weinberg equilibrium. Because the minor allele frequency of −684G > A SNP was low (less than 7% in all groups), we excluded this polymorphism from the case control analysis. *p* values of each case-control study were calculated using genotypes and allele frequencies of three polymorphisms (Table 3). Neither genotypic nor allelic

Table 3
Genotypic and allelic frequencies of human NRF2 promoter polymorphisms in SLE and COPD patients compared with healthy controls in the Japanese population

Position	Genotype allele	Control	SLE	*p* value	COPD	*p* value
−686	G/G	27	16	0.8149[a]	29	1.0000[a]
	G/A	39	28		46	
	A/A	15	7	0.4718[a]	12	0.4047[a]
	G	93	60	0.8205	104	0.5256
	A	69	42		70	
−684	G/G	75	44		82	
	G/A	5	7	ND	5	ND
	A/A	1	0		0	
	G	155	95	ND	169	ND
	A	7	7		5	
−650	C/C	35	27	0.2754[a]	49	0.0894[a]
	C/A	41	20		31	
	A/A	5	4	0.7109[a]	7	0.6376[a]
	C	111	74	0.4862	129	0.1978
	A	51	28		45	
−20 to −6 (ccg)*n*	*n* = 4/4	38	19	0.2753[a]	32	0.1832[a]
	n = 4/5	34	27		43	
	n = 5/5	9	5	0.8123[a]	12	0.5994[a]
	n = 4	110	65	0.4847	107	0.2198
	n = 5	52	37		67	

[a] Homozygous population of one allele was compared to the rest of the population including the homozygote of the other allele and the heterozygote.

frequencies were substantially different between the disease-prone and control populations (Table 3).

Concluding remarks

In order to generate a solid basis for the human genetic study of oxidative stress-related diseases, we analyzed the human *NRF2* gene structure in detail in this study. We also identified three SNPs and one triplet polymorphism within the promoter and upstream regions of the *NRF2* gene. Preliminary analyses of the link between these polymorphisms and human diseases were conducted. Although significant association was not observed in this study using a small number of patients with SLE or COPD, we still believe that Nrf2 dysfunction is one of the important prerequisites for the development of a certain group of diseases, especially those related to oxidative stress, considering the critical contribution of Nrf2 to the regulation of inflammation and elimination of ROS. Based on the connection between polymorphism of the promoter region and vulnerability to hyperoxic stress in C57BL/6J mice [10], and the absence of polymorphisms within the coding region of the human *NRF2* gene so far, we suppose that the polymorphisms of the promoter region would be indispensable tools for screening the *NRF2*-related human disorders.

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