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Detection of allelic imbalance in the gene expression of *hMSH2* or *RB1* in lymphocytes from pedigrees of hereditary, nonpolyposis, colorectal cancer and retinoblastoma by an RNA difference plot

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Abstract A number of phenotypes in hereditary disorders or common diseases are associated with specific genotypes. However, little is known about the molecular basis of phenotypic variation among individuals carrying the same mutation or polymorphism. Here, a highly quantitative approach was taken to examine a relative amount of mRNA from two polymorphic alleles with a coefficient of variation of less than 10% using an RNA difference plot (RDP). RDP analysis revealed that most genes examined were expressed in equal amount from the two alleles in normal lymphocytes. In contrast, the relative amounts of *hMSH2* or *RB1* mRNAs carrying

premature termination codons were significantly reduced compared with those of wild-type mRNAs in lymphocytes from carriers of hereditary, nonpolyposis, colorectal cancer and hereditary retinoblastoma. The balance of allelic expression of the *RB1* was also significantly impaired in a pedigree of retinoblastoma carrying a missense mutation in codon 661. The relative expression of the mutant to the wild-type *RB1* alleles among the carriers varied from 0.40 to 2.39. The analysis of the expression diversity of a disease-associated allele by RDP could provide a novel approach to elucidating the mechanisms underlying phenotypic variation among individuals carrying an identical mutation or polymorphism at a single locus.

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Introduction

Most autosomal genes are transcribed from both alleles, except for a group of genes regulated by genomic imprinting (Robertson and Jones 2000; Surani 2001). However, human individuals harbor variations in the nucleotide sequences in the genome, including single nucleotide polymorphisms (SNPs). Polymorphic changes in the gene promoter, enhancer, or other regions are expected to affect the amount or pattern of gene expression from two alleles through efficiency of transcription and the stability of mRNA (Relling and Derieux 2001; Mendell and Dietz 2001). Such a difference could possibly modify the phenotype of each individual simply through quantitative variation of the gene products or together with their additional qualitative difference. Quantitative analysis of allelic expression would therefore provide valuable information to the

understanding of the biological significance of DNA polymorphism and mutations.

Several assays have been developed so far for the quantitative measurement of allele-specific transcripts, including allele-specific ligation (Landegren et al. 1988), single nucleotide primer extension (Powell et al. 1993; Singer-Sam and Riggs 1993), the hot-stop PCR assay (Uejima et al. 2000), digital SNP (Zhou et al. 2001), and the fluorescent dideoxy terminator-based method (Yan et al. 2002a). Most of them, however, are either not sufficiently sensitive or require several complicated techniques. Single-strand conformation polymorphism (SSCP) analysis is a rapid and sensitive method to detect a single nucleotide substitution (Orita et al. 1989a,b). We have previously demonstrated that the PCR-SSCP analysis of a reverse-transcribed product can distinguish the mRNAs from two alleles if the mRNA carries a cSNP in its sequence (Murakami et al. 1991). Fluorescence-based SSCP using blunt-end DNA fragments was subsequently developed for the quantitative detection of two polymorphic DNA fragments (Makino et al. 1992; Sugano et al. 1996). Here, we present an RNA difference plot (RDP), a simple and highly quantitative method to detect a relative amount of mRNA from two polymorphic alleles based on PCR-SSCP with a coefficient of variation (CV) of less than 10%. We found a significant imbalance in the allelic expression of the disease-associated genes in the pedigrees of familial cancers.

Materials and methods

The analyses of human materials were approved by the ethics committee in the institute and carried out in accordance with institutional guidelines.

RDP analysis

Total cellular RNA was extracted using an SV Total RNA Isolation System (Promega, Madison, WI, USA) followed by treatment with RQ1 RNase-free DNase 1 (Promega) in the presence of RNasin (Promega) at 37°C for 1 h. The absence of genomic DNA in each sample was confirmed by PCR of the relevant gene using an aliquot of an RNA sample as a template. One microgram of total cellular RNA was annealed with random hexamers and transcribed with 200 μ of Super Script II reverse transcriptase (Gibco BRL, Rockville, MD, USA) in the presence of an RNase inhibitor (Gibco BRL) in a reaction mixture (20 μ l). An aliquot of the reaction mixture (2 μ l) was subjected to PCR to amplify the relevant fragments. For the analysis of genomic DNA, 50 ng of DNAs were used as the template for PCR. Mutations and SNPs analyzed and primers used are summarized in Table 1. The 5' ends of the reverse primers were labeled with a fluorescent dye—Texas Red. PCR was carried out using AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ, USA) in 10 μ l of reaction volume followed by treatment with 0.5 μ of Klenow fragments (Takara, Tokyo, Japan), as described previously (Sugano et al. 1996). For SSCP, an aliquot was diluted with 10 volumes of a loading solution containing 90% deionized formamide and 0.01% fuchsin (Sigma, St Louis, MO, USA), denatured at 90°C, and applied (2 μ l/lane) to a 0.5×MDE gel solution containing 1×TBE (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). Electrophoresis was carried out at 20°C, 30 W for 2 h using an SF5200 autosequencer (Hitachi Ltd, Tokyo, Japan). The signal intensity of the polymorphic or mutated fragments was analyzed

Table 1 Mutations and polymorphisms analyzed by RNA difference plot (RDP)

Gene	SNP ID	Exon/UTR	Nucleotide change	Length of fragments (bp)	Primer sequences
<i>TP53</i>	rs1042522	4	C > G	115	5'-AGCTCCCAGAATGCCAGAG-3' 5'-CTGGGAAGGGACAGAAGATG-3'
<i>hMSH2</i>	Germline mutation ^a	11	2 bp del AA	84	5'-TTCTTTAAATGAAGAGTATACCAAAAATAAA-3' 5'-GAAATATTGACAATTTCTTTAACAATGG-3'
<i>RB1</i>	Germline mutation ^b	20	T > C	117	5'-TGTATCGGCTAGCCTATCTC-3' 5'-CATACTCATTCTGCAGGGTG-3'
<i>RB1</i>	Germline mutation ^b	2	1 bp del T	110	5'-AGTTTGAAGAAACAGAAGAACCTGA-3' 5'-CACAGATGAAACTTTCTCCCAAG-3'
<i>RB1</i>	Germline mutation ^b	18	G > T	100	5'-TTGATCTTATTAAACAATCAAAGGAC-3' 5'-GCTGCAGTGTGATTATTCTGG-3'
<i>MYC</i>	IMS-JST005487	3	A > G	103	5'-CGGAACCTCTTTGTGCGTAAGG-3' 5'-TGCATTTGATCATGCATTTG-3'
<i>APC</i>	IMS-JST087688	3'UTR	G > A	105	5'-TCCTTAACAGGCAGGGATTC-3' 5'-CACTTTGCCATGCTCAGAAA-3'
<i>NF1</i>	IMS-JST005699	3	G > A	161	5'-TTTGCTTTTGTTCAGGAGA-3' 5'-CCAAAACCTAGGATCCATTATTCTTTC-3'
<i>BRCA1</i>	IMS-JST005850	1	C > T	145	5'-CAGTCAGGCACAGCAGAAAC-3' 5'-CCAGGTGCATTTGTAACTTCA-3'
<i>BRCA2</i>	rs180146	11	G > A	136	5'-ACACCTAGCCAAAAGGCAGA-3' 5'-TCTGGTTTTTCAGGCACTTCA-3'

^aNomura et al. (2000)

^bSugano et al. (2004)

using the software program Allele Links (Hitachi Ltd). All experiments were repeated nine times independently, and the expression ratio was calculated by dividing the RNA ratio by the DNA ratio.

Haplotype analysis

Polymorphic fragments were amplified by PCR using 50 ng of genomic DNA as a template and separated by SSCP to detect the polymorphism. The DNA sequence of the fragments was determined to confirm the genotype using the BigDye terminator cycle sequencing ready-kit (Perkin-Elmer) on an ABI 377 DNA auto-sequencer (Applied Biosystems, Foster City, CA, USA).

Results

Measurement of allele-specific expression by RDP analysis

To detect the relative amounts of each mRNA from two polymorphic alleles highly quantitatively, we amplified

the same fragments from the cDNA and genomic DNA flanking a cSNP using an identical set of primers. The absence of genomic DNA in each RNA sample was confirmed by PCR of the relevant gene using an aliquot of an RNA sample as a template (Fig. 1a). Amplified fragments were then subjected to fluorescence-labeled SSCP analysis (Makino et al. 1992; Sugano et al. 1996) using a DNA autosequencer, SF5200 (Hitachi Ltd.), which can control the temperature of the gel for electrophoresis.

We initially examined the allelic expression of the *TP53* gene in lymphocytes from a heterozygous individual for a cSNP at codon 72 within exon 4 (Buchman et al. 1988). The *TP53* gene is known to be expressed ubiquitously, including in peripheral blood lymphocytes. As shown in Fig. 1b, the relative ratio of the amplified genomic DNA fragments from alleles 1 and 2 (D1/D2) was 1.08 ± 0.08 [mean $\pm$ standard deviation (SD)] in nine independent experiments although the template DNAs from alleles 1 and 2 were identical in amount. This bias, therefore, appeared to be due to a difference in the efficiency of amplification between the two fragments caused by the cSNP. The ratio of cDNA from alleles 1 and 2 (R1/R2) was also determined as 1.08 ± 0.08 . The amplification bias between R1 and R2 was then adjusted by dividing R1/R2 by D1/D2, and the relative ratio of mRNA per allele [defined as the expression ratio, (ER)] was then calculated to be 1.0 ± 0.10 . This indicates that an identical amount of p53 mRNA from the two alleles was present in normal lymphocytes. These ratios were reproducibly obtained with a coefficient of variation of less than 10%. Furthermore, when lymphocyte DNAs from two homozygous individuals carrying alleles 1 and 2 were mixed together and used as templates, the ratio of the PCR products was essentially constant between 24 and 34 cycles of PCR (Fig. 1c) and correlated well with

Fig. 1 Validation of RNA difference plot (RDP) analysis. **a** Amplification of the *TP53* gene or its transcript using identical primers within exon 4. RTase indicates a reverse transcriptase. **b** PCR-single-strand conformation polymorphism (SSCP) analysis of the *TP53* gene and its transcript. Fragments of 115 bp carrying a cSNP in codon 72 (CCC/proline in allele 1 and CGC/arginine in allele 2) were examined in lymphocytes from a heterozygous individual. **c** Relative ratio of polymorphic DNA fragments of the *TP53* gene at various cycles of PCR. Lymphocyte DNAs from homozygous individuals carrying alleles 1 or 2 were mixed together at a ratio of 1:2 and used as templates. **d** Relative ratio of two polymorphic DNA fragments of the *TP53* gene amplified from the mixture of lymphocyte DNA from two homozygous individuals in various proportions

Fig. 2 Expression imbalance of the *hMSH2* gene in a pedigree of hereditary, nonpolyposis, colorectal cancer (HNPCC). **a** Pedigree of HNPCC. Filled symbols indicate individuals suffering from colorectal and several other cancers. Individuals I-1, II-1, and III-1 are carriers of a germline mutation of the *hMSH2* gene while II-2 is a noncarrier. Individual III-1 was 18 years old and had not developed any tumors by the time this study was conducted. The arrow indicates the site of the 2-bp deletion. **b** RNA difference plot (RDP) analysis of *hMSH2* in lymphocytes from carriers. Fragments of 84 bp containing a 2-bp deletion in codon 569 were analyzed: *mut* and *wt* indicate mutant and wild-type fragments, respectively. Average expression ratios (ERs) with standard deviations (SD) in nine independent experiments are indicated in the right-hand panel. **c** Genotype analysis around the *hMSH2* gene. Note that all SNPs in II-2, a noncarrier, are homozygous. Positions of six SNPs and a mutation examined are indicated on the basis of the sequence of AC079775 (gi:19848453). The expected haplotype is shown in a box

the ratio of the mixed template from 1:9 to 9:1 (Fig. 1d). In addition to its simple procedure, RDP is as sensitive as and more practical than other methods used for the detection of the relative amount of allelic expression. RDP analyses revealed that several autosomal genes, including *MYC*, *NF1*, *APC*, *BRCA1*, and *BRCA2*, were expressed in equal amounts from two alleles in normal lymphocytes (data not shown).

Imbalance in the allelic expression of the *hMSH2* or *RB1* genes carrying truncating mutations in a pedigree of hereditary, nonpolyposis, colorectal cancer and retinoblastoma

The amount of mRNA carrying a premature termination codon is often reduced compared with that of a wild-type mRNA (Frischmeyer and Dietz 1999). Therefore, we next examined RDP to quantify the amount of the allele-specific expression of the *hMSH2* gene in lymphocytes from three carriers of hereditary, nonpolyposis, colorectal cancer (HNPCC) harboring a frameshift mutation (I-1, II-1, and III-1 in Fig. 2a). The *hMSH2* gene is also shown to be expressed ubiquitously, including in peripheral blood lymphocytes. Individual

Fig. 3 Expression imbalance of the *RB1* gene in hereditary retinoblastoma patients. Lymphocytes from hereditary retinoblastoma patients were analyzed by RNA difference plot (RDP). **a** Fragments of 110 bp containing a 1-bp deletion in codon 76 or **b** fragments of 100 bp containing a non-sense mutation in codon 580 were analyzed. Average expression ratios (ERs) with standard deviation (SD) in three independent experiments are indicated in the right-hand panel

I-1 suffered from rectal, colon, uterine, ovarian, and stomach cancers after her forties, and individual II-1 suffered from colon and stomach cancers in his forties while individual III-1 was 18 years old and had not developed any tumors by the time this study was conducted. SSCP could distinguish the mutant fragment from the wild-type fragment when segments containing a 2-bp deletion in exon 11 were examined (Nomura et al. 2000). As shown in Fig. 2b, the expression ratios of a mutant to a wild-type *hMSH2* in carriers I-1, II-1, and III-1 were 0.60 ± 0.06 , 0.59 ± 0.06 , and 0.60 ± 0.06 , respectively, indicating a significant reduction in the

children. In contrast, a haplotype of the wild-type allele of the nonpenetrant father (I-1), which came from either of his parents, was distinct from those of the affected children (Fig. 4a). It is possible, therefore, that wild-type alleles with different haplotypes might have a different level of expression.

We then examined the total amount of RB1 mRNA in the lymphocytes by quantitative RT-PCR using G3PDH mRNA as an internal control and found that RB1 mRNA in the father's lymphocytes was significantly increased to 1.9-fold (SD is 0.25) relative to those in the children's lymphocytes (data not shown). These results suggest that the mutant RB1 mRNA is substantially increased in the father's cells but substantially decreased in the children's cells. Thus, modifier genes are more likely to be implicated in this expression imbalance than the difference in haplotypes of the wild-type alleles.

Discussion

RDP analysis provides highly quantitative information of allelic expression. The amplification of identical fragments from cDNA and genomic DNA followed by the adjustment of the amplification bias through PCR is the key issue to obtain accurate and reproducible results. Whereas we used PCR-SSCP analysis for the detection of cSNPs in the present study, polyacrylamide gel electrophoresis of the PCR products can also be used for RDP when the polymorphism is based on the length of the fragments, such as microsatellite polymorphism (data not shown). A pyrosequencing-based method would be an alternative approach to measure the relative amount of allelic expression by RDP analysis (Lavebratt et al. 2004).

Using RDP, a significant decrease, but not a complete loss, was detected in the relative expression of mRNAs carrying premature termination codons. An almost-identical rate of reduction in mutant hMSH2 mRNA observed in the carriers from three generations of an HNPCC pedigree suggests that the degradation mechanism of mRNA, known as non-sense-mediated RNA decay, is a strictly regulated system in our cells. Expression imbalance was also observed in mRNA carrying a missense mutation in a pedigree of hereditary retinoblastoma. In this case, the expression ratio was quite different between the father (I-1), an unaffected carrier, and the children (II-1, II-2), who developed bilateral retinoblastomas. In fact, individuals harboring an identical mutation or polymorphism often present considerable diversity in the phenotype. Such variations have been considered to be due to modifier genes or environmental factors. However, it is difficult to assess the effect of such modifiers in human individuals because there could be a number of modifiers, some of which might be implicated in the upstream or downstream cascades of the relevant gene product while others could be involved in the transcriptional regulation of the

relevant genes. We demonstrated in the present study that diversity in the amount of expression of the disease-associated allele could be a potential moderator of phenotypic variation among individuals.

The development of retinoblastoma requires the inactivation of the wild-type allele as a second hit. In this connection, it is noteworthy that a previous study reported that overexpression of the mutant RB protein in codon 661 could rescue the tumor suppressor activity in vitro (Whitaker et al. 1998). Overexpression of the mutant RB1 could then be possibly associated with a nonpenetrant phenotype of the father if the overexpression were to compensate for the partial defect of its activity in his possible tumor precursor cells in which the wild-type RB1 is inactivated. On the other hand, a reduced expression of the mutant RB1 in two children might be implicated in their severe phenotypes with bilateral retinoblastomas if the mutant RB1 had lost its function as a result of the reduction of expression in addition to the amino acid substitution in their tumor precursor cells lacking the wild-type RB1. To address this issue, analyses of mutant RB1 expression in primary retinoblastoma cells would be necessary. However, as no tumor had developed in the father and tumor samples were not available in the children, the pathogenic significance of this expression imbalance could not be elucidated further.

We finally examined the possibility that the wild-type alleles with different haplotypes might have a different level of expression. This model did not fit in with the 1.9-fold increase in total RB1 mRNA from the father's lymphocytes in this case. However, since a number of common diseases are shown to be associated with specific haplotypes, some haplotypes might be implicated in the phenotypic variation through modifying the level of allelic expression. In any case, allelic expression could constitute a novel and rather direct indicator to predict the phenotype of individuals in the pedigrees of hereditary disorders or in the carriers of polymorphisms associated with susceptibility to common diseases. Highly quantitative detection by RDP would be useful for studying the allelic expression in these cases.

Yan et al. (2002b), in a comprehensive study using a fluorescent dideoxy terminator-based method, demonstrated that allelic variation in human gene expression is relatively common among normal individuals and consistent with Mendelian inheritance. In fact, the majority of more than two million SNPs identified so far do not directly change the structures of the gene products, which supports the idea that the amounts or patterns of gene expression play a considerable role in the development of each phenotype. The balance of allelic expression might possibly be altered in a tissue-specific manner or through the developmental or aging processes of each individual. The analysis of an allele-specific expression by RDP in combination with haplotyping would cast a new light on the study of the phenotypical diversity of each individual, including the susceptibility to common diseases, in the post-genome era.

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