A Novel Single-Nucleotide Polymorphism in the 3′-Untranslated Region of the Human Dihydrofolate Reductase Gene with Enhanced Expression

Yoshinori Goto, Lijie Yue, Ayano Yokoi, Ryosei Nishimura, Takahiro Uehara, Shoichi Koizumi, and Yutaka Saikawa
Department of Pediatrics, Kanazawa University School of Medicine, Kanazawa, Ishikawa 920-8641, Japan

ABSTRACT
A novel single-nucleotide polymorphism (SNP), 829C→T in the 3′-untranslated region of the human dihydrofolate reductase (DHFR) gene transcript, was identified in the study population of 37 patients with childhood leukemias/lymphomas and 83 healthy Japanese children. Frequencies of the DHFR 829C/C, 829C/T, and 829T/T genotypes were 83.8, 10.8, and 5.4%, respectively, in the cases and 74.7, 19.3, and 6.0% in the controls, showing no significant difference in genotype frequencies between the cases and controls. When determined by real-time quantitative reverse transcription-PCR analysis, the highest expression of the DHFR transcript was demonstrated in the samples with a DHFR 829T/T polymorphism (P < 0.001). Direct association of the presence of the SNP with methotrexate-related adverse events in each patient was not demonstrated in this limited analysis. These data suggest that the novel DHFR 829 polymorphism is associated with a positive role in gene expression and provide evidence of a functional SNP in the 3′ regulatory region of the gene.

INTRODUCTION
Human genomic DNA contains a variety of known polymorphisms, such as single-nucleotide substitutions, insertion/deletions, and copy number variations in nucleotide repeat motifs. A single-nucleotide substitution is the most common form of polymorphism and is called a SNP. SNPs are not only important as markers for constructing dense genetic maps but also have potential as direct functional polymorphic variants involved in common and genetically complex diseases as well as drug response because (a) SNPs within the coding regions (cSNPs) of functional genes introduce biological variations directly into the gene products through the creation of missense substitutions or premature termination codon; (b) SNPs present in noncoding regions have effects on gene expression by affecting regulatory elements; and (c) some intrinsic SNPs activate cryptic splice sites, leading to alternative splicing.

Recently developed technologies using high-throughput genotyping methods have accelerated SNP discovery by identifying the specific location in a gene at a frequency of >1% across a population (1–5). It is necessary to build a comprehensive catalogue of SNPs in candidate genes in the human population to better understand the role of common genetic variants in common diseases. Recently, several studies characterizing SNPs on a large scale have been reported for this purpose (6–10). In addition, one goal of pharmacogenetics must be to exploit all of the relevant SNP variations to improve the diagnosis and treatment of disease. In the near term, concerning adverse drug reactions, analyses of SNPs in the genes for the enzymes involved in the metabolic pathways of chemotherapeutic drugs may directly provide gene-based information that predicts drug response for the treatment of cancer patients.

DHFR (EC 1.5.1.3) catalyzes the reduction of dihydrofolate to THF, which rejoins the pool of active folate cofactors (11, 12). MTX acts as a tight-binding inhibitor of DHFR and is the most widely used antimetabolite in pediatric malignancies, being especially effective in the treatment of ALL, non-Hodgkin’s lymphoma, the histocytoses, and osteosarcoma (13). MTX is also used in the treatment of rheumatoid arthritis, psoriasis, and autoimmune diseases, and for the prevention of graft-versus-host disease after transplantation. In actively proliferating tumor cells, inhibition of DHFR by MTX leads to accumulation of folates in the inactive dihydrofolate form, with a partial depletion of reduced folates. Similar to naturally occurring folates, which exist as polyglutamates within cells, intracellular MTX undergoes polyglutamation through the action of folypolyglutamyl synthetase. MTX polyglutamates retained in cells subsequently inhibit DHFR and also enhance their inhibitory effects on folate-requiring enzymes of thymidylate and purine synthesis, thus contributing to MTX cytotoxicity (11, 12). In both experimental and clinical settings, increased levels of DHFR and a decreased DHFR binding affinity for MTX are common findings in cells manifesting an MTX-resist-

Received 12/27/00; revised 3/27/01; accepted 4/10/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (09670793, 11470170, and 11877128).
2 To whom requests for reprints should be addressed, at Department of Pediatrics, Kanazawa University School of Medicine, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan. Phone: 81-76-265-2314; Fax 81-76-262-1866; E-mail: saikawa@ped.m.kanazawa-u.ac.jp.
3 The abbreviations used are: SNP, single-nucleotide polymorphism; cSNP, SNP within the coding region; DHFR, dihydrofolate reductase; THF, tetrahydrofolate; MTX, methotrexate; ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; nt, nucleotide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region; cSNP, SNP within the coding region; DHFR, dihydrofolate reductase; THF, tetrahydrofolate; MTX, methotrexate; ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; nt, nucleotide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region; importance as markers for constructing dense genetic maps but also have potential as direct functional polymorphic variants involved in common and genetically complex diseases as well as drug response because (a) SNPs within the coding regions (cSNPs) of functional genes introduce biological variations directly into the gene products through the creation of missense substitutions or premature termination codon; (b) SNPs present in noncoding regions have effects on gene expression by affecting regulatory elements; and (c) some intrinsic SNPs activate cryptic splice sites, leading to alternative splicing.

Recently developed technologies using high-throughput genotyping methods have accelerated SNP discovery by identifying the specific location in a gene at a frequency of >1% across a population (1–5). It is necessary to build a comprehensive catalogue of SNPs in candidate genes in the human population to better understand the role of common genetic variants in common diseases. Recently, several studies characterizing SNPs on a large scale have been reported for this purpose (6–10). In addition, one goal of pharmacogenetics must be to exploit all of the relevant SNP variations to improve the diagnosis and treatment of disease. In the near term, concerning adverse drug reactions, analyses of SNPs in the genes for the enzymes involved in the metabolic pathways of chemotherapeutic drugs may directly provide gene-based information that predicts drug response for the treatment of cancer patients.

DHFR (EC 1.5.1.3) catalyzes the reduction of dihydrofolate to THF, which rejoins the pool of active folate cofactors (11, 12). MTX acts as a tight-binding inhibitor of DHFR and is the most widely used antimetabolite in pediatric malignancies, being especially effective in the treatment of ALL, non-Hodgkin’s lymphoma, the histocytoses, and osteosarcoma (13). MTX is also used in the treatment of rheumatoid arthritis, psoriasis, and autoimmune diseases, and for the prevention of graft-versus-host disease after transplantation. In actively proliferating tumor cells, inhibition of DHFR by MTX leads to accumulation of folates in the inactive dihydrofolate form, with a partial depletion of reduced folates. Similar to naturally occurring folates, which exist as polyglutamates within cells, intracellular MTX undergoes polyglutamation through the action of folypolyglutamyl synthetase. MTX polyglutamates retained in cells subsequently inhibit DHFR and also enhance their inhibitory effects on folate-requiring enzymes of thymidylate and purine synthesis, thus contributing to MTX cytotoxicity (11, 12). In both experimental and clinical settings, increased levels of DHFR and a decreased DHFR binding affinity for MTX are common findings in cells manifesting an MTX-resist-

OR, odds ratio; CI, confidence interval; MTHFR, methylenetetrahydrofolate reductase.
MATERIALS AND METHODS

Study Population and Sample Collection. We studied 32 cases of acute leukemia (25 ALL and 7 AML) and 5 cases of lymphoma who were diagnosed between 1990 and 1997, as well as 83 non-malignancy-bearing children, with ages ranging from 0.75 to 15 years. Informed consent was obtained from the patients or the patients’ guardians. The diagnosis of leukemia was based on French-American-British morphological criteria and cytochemical assays. Immunophenotyping was performed using panels of monoclonal antibodies. Mononuclear cells from leukemic patients’ BM at diagnosis (>80% of leukemic blasts) or peripheral blood mononuclear cells from nonleukemic children were isolated by Ficoll-Hypaque density-gradient centrifugation and stored in liquid nitrogen. All patients were enrolled in the Children’s Cancer and Leukemia Study Group trial in Japan and were treated according to the risk-stratified protocols (15).

Reverse Transcription-PCR and DNA Sequencing. Total RNA was extracted from cryopreserved cell samples by a standard technique. The first-strand cDNA was generated with 2 μg of total RNA, random hexadeoxynucleotide primer (Takara, Tokyo, Japan), and RAV-2 reverse transcriptase (Takara). The oligonucleotide primers for the DHFR gene were 5’-CTGTCATGGTTGGTTCGC-3’ (sense), with coordinates 38/55, and 5’-ACCATAATCACCAGATTCTGT-3’ (antisense), with coordinates 962/979 [numbers correspond to the reference GenBank sequence (J100140)]. PCR amplification was performed in a DNA thermal cycler (GeneAmp PCR system 9600; Perkin-Elmer, Norwalk, CT) for 30 cycles (95°C for 1 min. and 72°C for 1 min.). The PCR primers for DHFR were designed as 5’-AAACTGCATCGTCGCTGTGTC-3’ (sense; nt 55–77) and 5’-ACCCATAATCACCAGATTCTGT-3’ (antisense; nt 183–204), and the primers for GAPDH were purchased from PE Biosystems.

Amplification reactions contained 5 μl of the first-strand cDNA reaction; 300 nM each PCR primer; 200 μM each of dATP, dCTP, and dGTP; 400 μM dUTP; 0.5 units of AmpErase uracil-N-glycosylase; 5 mM MgCl₂; and 1.25 units of AmpliTaq Gold DNA polymerase in a total volume of 50 μl. All components except the fluorogenic probes and amplification primers were supplied in a TaqMan PCR Core Reagent Kit (Perkin-Elmer). DNA amplifications were carried out in a 96-well reaction plate format in an ABI PRISM 7700 Sequence Detection System with a Sequence Detector V1.6.3 program (Perkin-Elmer Applied Biosystems). After a holding step at 50°C for 120 s to allow uracil-N-glycosylase digestion in the reaction, the thermal cycling was initiated with a first denaturation step of 10 min at 95°C, followed by 40 cycles of 95°C for 1 s and 60°C for 1 s.

To create a standard curve for relative quantification, a representative DNA sample without the SNP in the DHFR gene was chosen as a standard control, serially diluted in water (10⁰, 10⁻¹, and 10⁻²), and subjected to real-time quantitative PCR in triplicate. The dilution value (starting quantity) of the standard was plotted against the threshold cycle number (Ct) at which fluorescence first increased above background. The expression of the indicated gene in each sample was evaluated by this standard curve and shown as a value relative to the expression in the standard sample. These relative DHFR expression values were then corrected for the relative values obtained for GAPDH from the same DNA samples. Triplicate DHFR expression values were individually divided by the relative GAPDH values, and the mean was reported as DHFR/GAPDH normalized.

Statistical Analysis. All data are presented as the mean ± SD or as a percentage. The Mann-Whitney test was used for differences between the gene expression values and a specific genotype. ORs and 95% CIs were used for estimating the risk of association with genotypes. Fisher’s exact probability tests were used to compute the risk estimates. All statistical analyses were performed using StatView (version 4.5; Abacus Concepts, Inc., Berkeley, CA). P < 0.05 was considered significant.
RESULTS

Identification of SNPs. In each sample we analyzed an amplified DHFR cDNA fragment that contained a part of the 5'-UTR (nt 38–42), an entire coding region (nt 43–606), and a subsequent 373-bp-long portion of the 3'-UTR (nt 607–979). None of the 120 samples tested contained a cSNP within a coding region of the DHFR gene. Two SNPs were identified within the 3'-UTR. A 721T→A substitution was observed in all alleles in this Japanese population and found identical to a candidate SNP proposed from the Cancer Genome Anatomy Project-Genetic Annotation Index. This 721A→T SNP may represent a population-specific polymorphism. A novel 829C→T variant (designated as DHFR 829) was identified in 34 of a total of 240 alleles. The representative sequence traces and a PCR-RFLP assay for validation were shown in Fig. 1, A and B, respectively. Among the 37 patients with leukemia/lymphoma, the DHFR 829 polymorphic allele frequency was 10.8%, compared with 15.7% among the 83 control subjects. The frequencies of the DHFR 829C/C, 829C/T, and 829T/T genotypes were 83.8, 10.8, and 5.4% in the cases and 74.7, 19.3, and 6.0% in the controls, respectively (Table 1). Statistical analysis for estimation of the risk did not show any significant association between susceptibility to the disease and specific DHFR genotypes (ORs and 95% CIs for 829C/T and 829T/T relative to 829C/C were 0.50 and 0.15–1.62, and 0.80 and 0.15–4.36, respectively). Thus, the allele frequency of DHFR 829C→T was 14.2% in this Japanese population.

DHFR mRNA Expression with 829C→T SNP. The absence of SNPs in a coding region of the DHFR gene suggested that variation of the reductase activity attributable to missense substitutions was extremely rare in a human population. To investigate the role of 829C→T SNP in the 3'-UTR, we quantitated the DHFR mRNA present in the samples from nonleukemic children as well as from leukemia/lymphoma patients. Fifteen randomly selected samples (10 from nonleukemic children and 5 from leukemia/lymphoma patients) with 829C/C, 12 random samples (8 from nonleukemic children and 4 from leukemia/lymphoma patients) with 829C/T, and 10 (8 from nonleukemic children and 2 from leukemia/lymphoma patients) with 829T/T were analyzed for DHFR expression by real-time quantitative PCR analyses. The relative expression in the non-leukemic samples with C/C, C/T, and T/T was 0.22 ± 0.11.
with 829T/T was significantly higher (11-fold) than with prototype (Fig. 2). The relative expression in the leukemic samples with C/C, C/T, and T/T was 0.39 ± 0.20 (median, 0.28), 0.95 ± 0.45 (0.60), and 4.05, respectively. Two patients with 829T/T showed higher DHFR mRNA expression relative to the patients with 829C/C, although statistical significance was not obtained because of low subject numbers.

**DISCUSSION**

We demonstrated the enhanced expression of the DHFR transcripts with a novel SNP identified within the 3′-UTR of the gene. Consistent with the lack of reports of human diseases associated with DHFR polymorphisms, cSNPs introducing premature termination codons or missense substitutions were not found in this population, probably because of the critical role of this enzyme, making many mutations lethal (16). Three isoforms of DHFR mRNA molecules arising from 3′-UTR heterogeneity have been identified in human cell lines (17). The 3′-UTR of the human DHFR gene contains three polyadenylation sites that produce mRNAs 0.8, 1.0, and 3.8 kb in size; the latter two isoforms are the major transcripts in most human cells (17, 18). Because the DHFR 829 polymorphism site is located at nt 223 downstream from the stop codon and is between the first (nt 89) and second polyadenylation sites (nt 336; Ref. 17), the majority of DHFR mRNAs contain this SNP site.

At least four pseudogenes derived from processed RNA molecules have been identified and located on separate chromosomes (19). Despite highly homologous sequences, including the 3′ portion of the DHFR pseudogenes to the DHFR gene (19), a considerable number of single-nucleotide alterations existed around the SNP site, making it possible for us to discriminate between the functional DHFR transcripts and amplicons derived from the pseudogenes. The role of the 3′-UTR of mRNA is becoming clear and is seen to be as important as that of 5′-UTR in regulating gene expression. In addition to the key role of poly(A) tail, which protects the RNA chain from degradation by 3′-to-5′ exonucleases, resulting in enhancement of translation, there are a number of motif sequences within the 3′-UTR that regulate mRNA stability, translational efficiency, or both (20). Searching for the known regulatory UTR motifs, Day and Tuite (20) did not find any matches in the 3′-UTR sequences with or without the DHFR 829 polymorphism.

A comparison of the human and mouse DHFR 3′-UTR sequences revealed that only 100 nucleotides downstream from the terminator codon were conserved between the two species (18). These structural features of the 3′-UTR of the human DHFR gene suggest that the downstream regions are less important for post-transcriptional gene regulation and are not part of the DHFR ancestral gene. However, the following evidence supports the possible involvement of the DHFR 829 polymorphism in the regulatory region within the 3′-UTR. Goldsmith and Cowan (21) constructed several DHFR minigenes with deletions, in which the first two polyadenylation sites and the DHFR 829 site turned out to be eliminated from the 3′-UTR. The level of DHFR induction and RNA transcripts by these constructs was reduced by 50% relative to the wild type, indicating the presence of regulatory elements in this region. Furthermore, studies using a human DHFR minigene construct supported the regulatory role of DHFR UTRs in gene expression during MTX exposure (22). Recent studies on 3′-UTR-mediated regulation have focused on the interaction between trans-acting proteins and cis-acting mRNA sequences (20, 23). A specific RNA-protein interaction associated with the 3′-UTR regulatory elements would likely be involved in this enhancement, although the mechanism(s), e.g., increased mRNA stability, efficient translation, interaction of regulatory elements of 5′- and 3′-UTRs, or alteration of nucleo-cytoplasmic transport, of enhanced expression of DHFR mRNA associated with the DHFR 829 polymorphism need(s) to be clarified.

It would be anticipated that increased DHFR reduces MTX cytotoxicity in normal cells while conferring resistance in target cells. However, when we compared DHFR genotypes with MTX-related adverse events and patient outcomes, increased DHFR expression associated with the 829T/T genotype did not provide direct evidence of the clinical significance of the polymorphism (data not shown). This was possibly in part because MTX cytotoxicity is multifactorial. MTX-induced inhibition involves several folate-dependent enzymes in both de novo purine and thymidylate synthesis (12). The coincidence and grades of MTX-related adverse reactions depend on the extent of the status of DHFR as well as the enzymes involved in the metabolic pathways of folates and their differential expression between normal and malignant cells. Therefore, the expression of these enzymes, including thymidylate synthetase, glycaminide ribonucleotide and aminomimidazole carbboxamide ribonucleotide transformylases, folyopolyglutamyl synthetase, and folyopolyglutamate hydrolase, in both target and non-target cells is the critical determinant of drug response. A high-density oligonucleotide array (DNA chip)-based analysis could be applied for this purpose, but it is still experimental in clinical

![Figure 2](image_url)
practice. Profiling the combination of SNPs in particular reactions would help to circumvent unfavorable events and provide benefit for the treatment of patients. An additional, larger study with accumulation of clinical samples is required to find conclusive associations between the SNPs and drug-related adverse events.

A recent study by Skibola et al. (9) demonstrated that polymorphisms in the gene that encodes MTHFR, an enzyme that reduces methylene-THF to methyl-THF, decrease the risk of adult ALL. MTHFR is thought to have a protective role in leukemogenesis, with decreased MTHFR activity attributable to cSNPs consequently reducing the misincorporation of uracil into DNA, a process that is mediated by enhanced availability of methylene-THF in the DNA synthesis pathway (9). Theoretically, MTX polyglutamates inhibit de novo thymidylate synthesis, resulting in enhanced uracil nucleotide misincorporation as a result of an increase in the dUTP pool (12); therefore, enhanced DHFR activity may anticipate a preventable function similar to reduced MTHFR activity. However, our results showed no evidence of risk association (either predictive or protective) between DHFR genotype and the disease. This is attributable in part to the difference in the metabolic fate of folates affected by the two enzymes (9, 12). Notably, the same MTHFR polymorphism failed to show evidence of a protective effect against AML (9), indicating that cells may not be affected equally because of different biological features involved in the different leukemia types.

Efforts to analyze drug targets for polymorphisms related to drug treatment response are being made because gene-based information will make it possible to establish individual therapy as well as to improve the diagnosis of disease. Our data provide both evidence of the role of a genetic variant in the 3′-UTR in gene regulation and the framework to establish an additional therapeutic strategy based on genetically determined drug responsiveness in risk-stratified protocols for the treatment of childhood malignancies.

ACKNOWLEDGMENTS

We thank Dr. Bruce A. Chabner for critical review of the manuscript. We also thank M. Kitakata and T. Yonezawa for technical assistance.

REFERENCES

A Novel Single-Nucleotide Polymorphism in the 3′-Untranslated Region of the Human Dihydrofolate Reductase Gene with Enhanced Expression

Yoshinori Goto, Lijie Yue, Ayano Yokoi, et al.