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Deletion of Dinucleotide Repeat (Δ14 Allele) in the Methylthioadenosine Phosphorylase (MTAP) Promoter and the Allelotype of MTAP Promoter in the Japanese Population

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5′-Deoxy-5′-methylthioadenosine phosphorylase (MTAP) is an enzyme involved in purine and polyamine metabolism and is ubiquitously expressed in normal human tissues and cells. However, this enzyme has been found to be deficient in a variety of human cancers. Although the enzyme deficiency is known to be caused by MTAP gene deletion, human diffuse histiocytic lymphoma cell line DHL-9 without any detectable MTAP activity has been found to possess the intact MTAP gene. These lines of evidence suggested that promoter abnormality might cause the MTAP deficiency in DHL-9. Therefore, we analyzed the MTAP promoter region of DHL-9 and found the deletion of 14 bases in its sequence. We designated the allele lacking $(GT)_6GC$ as dinucleotide repeat deletion (Δ14 allele) and determined the effect of the Δ14 allele on the MTAP promoter activity by a luciferase reporter assay. We have also analyzed the distribution of the Δ14 allele and wild-type (WT) allele in the Japanese population by PCR assay. A reporter plasmid harboring the Δ14 allele exhibited luciferase activity comparable to that of a plasmid containing the WT allele. Forty-six (22%) out of 210 people were homozygous for WT allele in the MTAP promoter, whereas 43 (20.5%) were homozygous for Δ14 allele. The remaining 121 people (57.5%) possessed Δ14/WT alleles in the MTAP promoter region. These results indicated that the Δ14 allele has nothing to do with MTAP deficiency in DHL-9. The Δ14 allele is distributed among the general population irrespective of gender.

Key words: MTAP — DHL-9 — Promoter activity — Δ14 allele

MTAP is an enzyme abundantly expressed in all normal human cells and tissues including erythrocytes and hematopoietic stem cells.[1, 2] The gene for the enzyme maps to the short arm of human chromosome 9 at band p21, where the cyclin-dependent kinase inhibitor genes for p16 and p15 also reside.[3–6] MTAP catalyzes the irreversible phosphorolysis of MTA (a natural substrate of MTAP) to adenine and 5′-methylthio-D-ribose-1-phosphate, which are then recycled to adenine nucleotide and methionine, respectively, as an important salvage pathway of adenine and methionine in human cells.[7]

Previous studies have shown that MTAP is deficient in various human malignant cell lines[2] and primary tumors such as pancreatic carcinoma,[8] brain tumors,[9] leukemias,[10–12] melanoma[13] and non-small cell lung cancers.[14, 15] MTAP enzyme deficiency was found to be caused by the gene deletion in all cancer cell lines but DHL-9, which lacks the enzyme activity without gene deletion. Neither MTAP mRNA nor the product protein was detected by RT-PCR or immunoblotting, respectively. In this study, genomic DNAs extracted from 210 Japanese volunteers were analyzed to determine the allelotype of MTAP promoter. We found a 14 bp deletion in the MTAP promoter of DHL-9 and determined its effect on the expression of the MTAP gene *in vitro*.

MATERIALS AND METHODS

Cloning of human wild-type and DHL-9 MTAP promoters A phage library constructed from human placenta DNA (Stratagene, La Jolla, CA) was screened with human MTAP cDNA probe as described previously.[3] A phage clone containing wild-type (WT allele) undeleted MTAP promoter was obtained and sequenced. Based upon the sequence data of MTAP promoter, two sets of PCR primers were synthesized to amplify the 1.2 kb promoter region. Promoter DNA purified from DHL-9 (Δ14 allele) was analyzed by PCR using these sets of primers followed by DNA sequencing.

PCR analysis of genomic DNA Another set of primers

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Abbreviations: MTAP, 5′-deoxy-5′-methylthioadenosine phosphorylase; DHL-9, diffuse histiocytic lymphoma-9; MTA, 5′-deoxy-5′-methylthioadenosine; RT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered saline; SSM, slipped strand mispairing.

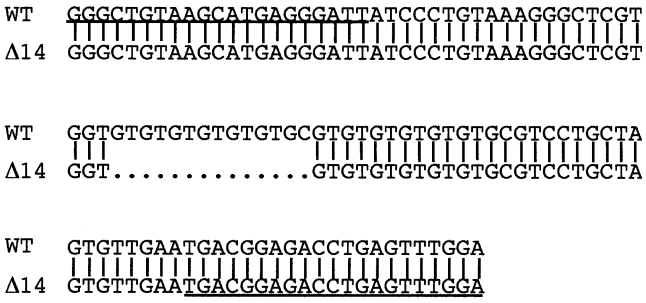

Fig. 1. MTAP promoter sequence harboring the WT allele and Δ14 allele. Underlined sequences were used as primers for PCR analysis.

was synthesized to diagnose Δ14 allele and WT allele as shown in Fig. 1. PCR reactions were carried out in a total volume of 25 μl reaction mixture containing 100 ng genomic DNA as a template, 1× PCR buffer (10 m*M* Tris-HCl, pH 8.0/50 m*M* KCl/1.5 m*M* $MgCl_2$), 250 μM of each of four deoxynucleoside triphosphates, 10 ng each of sense and antisense primers, and 1.25 units of Ex. *Taq* DNA polymerase (TaKaRa, Otsu, Shiga). The thermal cycling conditions consisted of an initial denaturation at 95°C for 7 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s with a final extension at 72°C for 7 min. The amplification products were resolved by 3% MetaPhor agarose gel electrophoresis to differentiate the 95 bp product of the Δ14 allele from the 109 bp product of the WT allele. PCR products amplified from DNA samples containing Δ14 allele (DHL-9) and WT allele (human placenta) were subcloned into pCR 2.1 (Invitrogen, San Diego, CA).

Purification of genomic DNA Lymphoblastoid cell lines established from members of a family (CEPH/UTAH PEDIGREE 1331) were obtained from Coriell Cell Repositories (Camden, NJ) for DNA extraction. Blood samples were collected from 210 Japanese volunteers, who had given their informed consent, and genomic DNA was purified by using the QIA amp system (Qiagen, Valencia, CA) according to the supplier's protocol. The volunteers were 40–60 years old and had visited hospital for a regular general health check-up.

Plasmid construction for transfection experiments The plasmids containing either the Δ14 allele or the WT allele were digested with *Pst*I restriction enzyme. *Pst*I fragments were purified with the gel extraction kit (Qiagen) and were subcloned into pGEM-3z vector (Promega, Madison, WI) linearized with *Pst*I. Then pGEM-3z vector containing the *Pst*I fragment of MTAP promoter was digested with *Kpn*I and *Hin*dIII restriction enzymes. Digested products were resolved by 1% agarose gel electrophoresis and *Kpn*I-*Hin*dIII fragments containing the MTAP promoter were purified. Purified MTAP promoter fragments (1.2 kb) were subcloned into the luciferase expression vector pGL2-Basic (Promega) linearized with *Kpn*I and *Hin*dIII. Each construct was verified by DNA sequence analyses. Plasmid DNA was purified with a Midi preparation kit (Qiagen).

Cell culture Human cervical carcinoma cell line HeLa S3 was grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 mg/ml) in a humidified incubator at 37°C under an atmosphere of 5% CO_2 and 95% air.

Transfection and luciferase reporter assay Transfection reactions were carried out in 12-well plates. HeLa cells were transferred into 12-well plates 24 h prior to the transfection procedure. HeLa cells were transfected at 60% to 70% confluence using Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. To obtain the optimum transfection efficiency, conditions for transfection were pre-optimized as recommended by the supplier's protocol. In order to normalize the transfection efficiency of the constructs, cells were cotransfected with pCMVβ vector which was designed to express β-galactosidase from cytomegalovirus promoter (Clontech, Palo Alto, CA). Transient transfection was carried out for 24 h along with the positive (pGL2-Promoter) and negative (pGL2-Basic) controls in each set of experiment.[16, 17] The cells were then washed with 1× PBS twice and harvested. Cell lysates were prepared using 1× Reporter lysis buffer (Promega). Luciferase activity was measured by the luciferase assay system using a "PicaGene" luminescence kit (Toyo Ink, Tokyo) on the same day that cell lysates were obtained. The β-galactosidase activity was determined using the β-galactosidase enzyme assay system (Promega). The total protein of cell lysates was estimated with the BCA protein assay kit (Pierce, Rockford, IL). Results were normalized to the total protein content and β-galactosidase activity. Three different independent experiments were carried out and mean values were taken for the graphic presentation. Statistical significance was determined by Student's *t* test. There was no significant difference in luciferase activity between the WT allele and Δ14 allele of the MTAP promoter.

RESULTS AND DISCUSSION

Sequence analysis of MTAP promoter in DHL-9 The MTAP promoter was PCR-amplified from DHL-9 and human placenta, followed by direct DNA sequencing. By comparison with the sequence of the cloned MTAP promoter, DHL-9 was found to have a 14 bp deletion from −924 to −939 in its promoter region. A new set of primers was synthesized as shown in Fig. 1 to amplify the 109 bp

product from the WT allele and the 95 bp product from Δ14 allele.

Analysis of Utah family 1331 Results on the Utah family showed that consecutive generations were also affected, indicating the genetic inheritance of the Δ14 allele in their offspring (Fig. 2). The pattern of transmission of the Δ14 allele follows the principles of inheritance. Eight (57%) out of 14 family members had the Δ14 allele in heterozygous form, with males and females affected almost equally. The MTAP gene has already been mapped on human chromosome 9p21. Therefore, genetic inheritance of Δ14 allele in consecutive generation is autosomal dominant.

Luciferase reporter assay Transfection results revealed that there was no significant difference of expression activity between the two constructs. Relative luciferase activity of the WT/WT allele and the Δ14/Δ14 allele of MTAP gene turned out to be almost equal (Fig. 3). This result suggested that the Δ14 allele is a normal DNA variation in which some bases within the MTAP promoter region are lacking without altering the expression of the gene as a whole *in vitro*. From this result, we can predict that the MTAP deficiency in DHL-9 is not due to the Δ14 allele.

The mechanism through which the Δ14 allele is generated is still unknown. Several years back, the SSM mechanism was first suggested by Efstratiadis *et al.*[18] and the same mechanism was proposed by Oron-Karni *et al.*[19] for the generation of short deletions. On the basis of the SSM mechanism, a single-stranded loop may be formed at the time of DNA replication, deleting 14 bases, followed by further elongation of the DNA strand.

Distribution of the Δ14 allele in the Japanese population In the process of screening the samples from Japanese volunteers, PCR products showed three distinct categories of amplified DNA bands in ethidium bromide-stained MetaPhor agarose gels (Fig. 4). One of them was a

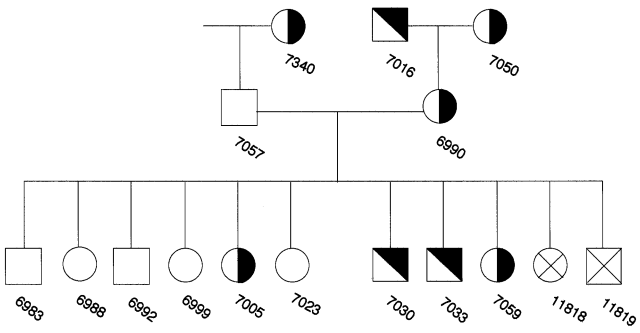

Fig. 2. Utah family analysis. Family pedigree showing the pattern of MTAP promoter sequences of the Δ14 allele and the WT allele in each member of the family. Each family member is given a separate number according to Coriell Cell Repositories. Male, square; female, circle; white, WT/WT; black and white, Δ14/WT; crossed, sample not available.

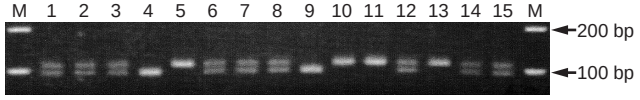

Fig. 4. Representative PCR assay of genomic DNA samples from Japanese volunteers. PCR amplification of genomic DNA was performed as described in "Materials and Methods" to determine the allelotype of the MTAP promoter. The bands corresponding to the WT allele (109 bp) and the Δ14 allele (95 bp) were distinctly visualized. Lanes: M, 100 bp DNA size marker; 1–3, 6–8, 12, 14 and 15, Δ14/WT allele; 4 and 9, Δ14/Δ14 allele; 5, 10, 11 and 13, WT/WT allele.

Table I. Distribution of Δ14 Allele in the Japanese Population

Allelotype	Frequency
Δ14/Δ14	20.5% (43/210)
WT/WT	22.0% (46/210)
Δ14/WT	57.5% (121/210)

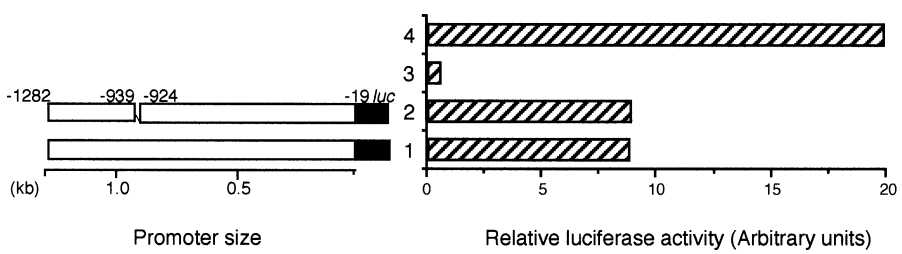

Fig. 3. Promoter activity of the MTAP gene. Relative luciferase activity of: 1, WT/WT allele promoter sequence; 2, Δ14/Δ14 allele promoter sequence; 3, pGL2 basic sequence (negative control); 4, pGL2 promoter sequence (positive control). No. 2 transfectant has deleted 14 bases at the position of (−924) to (−939) in the DHL-9 MTAP promoter sequence.

single band (95 bp) similar in size to the 100 bp ladder due to deletion of 14 bases from the MTAP promoter (Δ14/Δ14 allele). Another single band (109 bp) was bigger than the 100 bp marker (14 bases longer than the previous one), which was due the presence of undeleted MTAP promoter (WT/WT allele). In the third category, there were almost equally dense double amplified bands differing by 14 bases from each other. One was similar in size to the 95 bp band and the other was 109 bp in size. This is due to the presence of the heterozygous Δ14/WT allele in the MTAP promoter region. Forty-three (20.5%) Japanese out of 210 had a single band lacking 14 bases homozygously in the MTAP promoter region (Δ14/Δ14 allele). Forty-six (22%) had a single band without lacking 14 bases in the MTAP promoter (WT/WT allele). The rest (57.5%) had double bands indicating the presence of Δ14 allele in the MTAP promoter heterozygously. In aggregate, 50.45% Japanese had the WT allele and 49.55% Japanese had the Δ14 allele either homozygously or heterozygously in their MTAP promoter sequences. The ratio of Δ14/Δ14 allele:Δ14/WT allele:WT/WT allele among the Japanese population was approximately 1:2.8:1, whereas the simple ratio of Δ14 allele:WT allele was almost 1:1 (Table I). This study suggested that there might be a 50% chance of having the Δ14 allele among the Japanese population. This ratio was quite different from Utah family's result. For genotyping analyses of the Δ14 allele, we had collected blood samples from many people who had come to the hospital for their regular general health check-up. They were from different families and ethnic groups, whereas the Utah family members were few in number and from a single family. These differences may account for the genotyping analysis differences between the Japanese population and the Utah family.

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REFERENCES

1) Yu, J., Batova, A., Shao, L., Carrera, C. J. and Yu, A. L. Presence of methylthioadenosine phosphorylase (MTAP) in haematopoietic stem/progenitor cells: its therapeutic implications for MTAP (−) malignancies. *Clin. Cancer Res.*, **3**, 433−438 (1997).
2) Kamatani, N., Nelson-Rees, W. A. and Carson, D. A. Selective killing of human malignant cell lines deficient in methylthioadenosine phosphorylase, a purine metabolic enzyme. *Proc. Natl. Acad. Sci. USA*, **78**, 1219−1223 (1981).
3) Nobori, T., Takabayashi, K., Tran, P., Orvis, L., Batova, A., Yu, A. L. and Carson, D. A. Genomic cloning of methylthioadenosine phosphorylase: a purine metabolic enzyme deficient in multiple different cancers. *Proc. Natl. Acad. Sci. USA*, **93**, 6203−6208 (1996).
4) Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., III, Johnson, B. E. and Skolnick, M. H. A cell cycle regulator potentially involved in genesis of many tumor types. *Science*, **264**, 436−440 (1994).
5) Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K. and Carson, D. A. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature*, **368**, 753−756 (1994).
6) Carrera, C. J., Eddy, K. L., Shows, T. B. and Carson, D. A. Assignment of the gene for methylthioadenosine phosphorylase to human chromosome 9 by mouse-human somatic cell hybridization. *Proc. Natl. Acad. Sci. USA*, **81**, 2665−2668 (1984).
7) Kamatani, N. and Carson, D. A. Dependence of adenine production upon polyamine synthesis in cultured human lymphoblasts. *Biochim. Biophys. Acta*, **675**, 344−350 (1981).
8) Zhi-Hao, C., Zhang, H. and Savarese, T. M. Gene deletion selectivity: codeletion of the gene for $p16^{INK4}$, methylthioadenosine phophorylase, and the α- and β-interferons in human pancreatic cell carcinoma lines and its implications for chemotherapy. *Cancer Res.*, **56**, 1083−1090 (1996).
9) Olopade, O. I., Jenkins, R. B., Ransom, T. D., Malik, K., Pomykala, H., Nobori, T., Cowan, J. M., Rowley, J. D. and Diaz, M. O. Molecular analysis of deletions of the short arm of chromosome 9 in human gliomas. *Cancer Res.*, **52**, 2523−2529 (1992).
10) M'soka, T. J., Nishioka, J., Taga, A., Kato, K., Hawasaki, H., Yamada, Y., Yu, A., Komada, Y. and Nobori, T. Detection of methylthioadenosine phosphorylase (MTAP) and p16 gene deletion in T cell acute lymphoblastic leukemia by real time quantitative PCR assay. *Leukemia*, **14**, 935−940 (2000).
11) Batova, A., Diccianni, M. B., Nobori, T., Vu, T., Yu, J., Bridgeman, L. and Yu, A. L. Frequent deletion in the methylthioadenosine phosphorylase gene in T-cell acute lymphoblastic leukemia: strategies for enzyme targeted therapy. *Blood*, **88**, 3083−3090 (1996).
12) Hori, Y., Hori, H., Yamada, Y., Carrera, C. J., Tomonaga, M., Kamihira, S., Carson, D. S. and Nobori, T. The methylthioadenosine phosphorylase gene is frequently co-deleted with the $p16^{INK4a}$ gene in acute type adult T-cell leukemia. *Int. J. Cancer*, **75**, 51−56 (1998).
13) Fountain, J. W., Karayiorgou, M., Ernstoff, M. S., Kirkwood, J. M., Vlock, D. R., Titus-Ernstoff, L.,

Bouchard, B., Vijayasaradhi, S., Houghton, A. N., Lahti, J., Kidd, V. J., Housman, D. E. and Dracopoli, N. C. Homozygous deletions within human chromosome band 9p21 in melanoma. *Proc. Natl. Acad. Sci. USA*, **89**, 10557–10561 (1992).

14) Schmid, M., Malicki, D., Nobori, T., Rosenbach, M. D., Campbell, K., Carson, D. A. and Carrera, C. J. Homozygous deletion of methylthioadenosine phosphorylase (MTAP) are more frequent than $p16^{INK4A}$ (CDKN2) homozygous deletions in primary non-small cell lung cancers (NSCLC). *Oncogene*, **17**, 2669–2675 (1998).
15) Olopade, O. I., Buchhagen, D. L., Malik, K., Sherman, J., Nobori, T., Bader, S., Nau, M. M., Gazdar, A. F., Minna, J. D. and Diaz, M. O. Homozygous loss of the interferon genes defines the critical region on 9p that is deleted in lung cancers. *Cancer Res.*, **53**, 2410–2415 (1993).
16) Kaneko, S., Nishioka, J., Tanaka, M., Nakashima, K. and Nobori, T. Transcriptional regulation of the CDK inhibitor $p16^{INK4a}$ gene by a novel pRB-associated repressor, RBAR1. *Biochem. Mol. Biol. Int.*, **47**, 205–215 (1999).
17) Ohkubo, T., Tanaka, M. and Nakashima, K. Molecular cloning of the chicken prolactin gene and activation by pit-1 and cAMP-induced factor in GH3 cells. *Gen. Comp. Endocrinol.*, **119**, 208–216 (2000).
18) Efstratiadis, A., Posakony, J. W., Maniatis, T., Lawn, R. M., O'Connell, C., Spritz, R. A., DeRiel, J. K., Forget, B. G., Weissman, S. M., Slightom, J. L., Blechl, A. E., Smithies, O., Baralle, F. E., Shoulders, C. C. and Proudfoot, N. J. The structure and evolution of the human β-globin gene family. *Cell*, **21**, 653–668 (1980).
19) Oron-Karni, V., Filon, D., Rund, D. and Oppenheim, A. A novel mechanism generating short deletion/insertions following slippage is suggested by mutation in the human α_2-globin gene. *Hum. Mol. Genet.*, **6**, 881–885 (1997).