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Characterization and Prognostic Significance of Methylthioadenosine Phosphorylase Deficiency in Nasopharyngeal Carcinoma

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Abstract: Identification of cancer-associated genes by genomic profiling contributes to the elucidation of tumor development and progression. The *methylthioadenosine phosphorylase* (*MTAP*) gene, located at chromosome 9p21, plays a critical role in tumorigenicity and disease progression in a wide variety of cancers. However, the prognostic impact of MTAP in patients with nasopharyngeal carcinoma (NPC) remains obscured. Through data mining from published transcriptomic database, *MTAP* was first identified as a differentially downregulated gene in NPC. In this study, our aim was to evaluate the expression of MTAP in NPC and to clarify its prognostic significance.

MTAP immunohistochemistry was retrospectively performed and analyzed in biopsy specimens from 124 NPC patients who received standard treatment without distant metastasis at initial diagnosis. The immunoexpression status was correlated with the clinicopathological variables, disease-specific survival (DSS), distant metastasis-free survival (DMFS), and local recurrence-free survival (LRFS). Real-time quantitative polymerase chain reaction (PCR) was used to measure *MTAP* gene dosage. In some cases, we also performed methylation-specific PCR and pyrosequencing to assess the status of promoter methylation.

MTAP deficiency was significantly associated with advanced tumor stages ($P = 0.023$) and univariately predictive of adverse outcomes for DSS, DMFS, and LRFS. In the multivariate comparison, MTAP deficiency still remained prognostically independent to portend worse DSS ($P = 0.021$, hazard ratio = 1.870) and DMFS ($P = 0.009$, hazard ratio = 2.154), together with advanced AJCC stages III to IV. Homozygous deletion or promoter methylation of *MTAP* gene were identified to be significantly associated with MTAP protein deficiency ($P < 0.001$).

MTAP deficiency was correlated with an aggressive phenotype and independently predictive of worse DSS and DMFS, suggesting its role in disease progression and as an independent prognostic biomarker of NPC, which potentially offers new strategy of targeted treatment for patients lacking MTAP expression.

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Abbreviations: 6-TG = 6-thioguanine, aCGH = array comparative genomic hybridization, DMFS = distant metastasis-free survival, DSS = disease-specific survival, EBV = Epstein–Barr virus, LRFS = local recurrence-free survival, MMP = matrix metalloproteinase (MMP), MTA = methylthioadenosine, MTAP = methylthioadenosine phosphorylase, NPC = Nasopharyngeal carcinoma, ODC = ornithine decarboxylase, PCR = polymerase chain reaction, PRPP = 5-phosphoribosyl-1-pyrophosphate, RT = radiotherapy.

INTRODUCTION

Nasopharyngeal carcinoma (NPC), a common head and neck malignancy in southeastern Asia and Taiwan, is caused by a combination of factors, including Epstein–Barr virus (EBV) infection, environmental influence, and genetic susceptibility.[1] In endemic areas, NPC is strongly associated with EBV infection.[2] However, little is known about the molecular mechanism underlying NPC pathogenesis. Although advances in concurrent chemoradiotherapy have led to better locoregional control and overall survival, treatment options for advanced disease are still limited.[3] Therefore, it would be of great value to work out molecular mechanisms of this cancer and to search for potential prognostic biomarkers. New biomarkers can help to stratify the risk of disease progression and to develop novel therapeutic strategies for NPC patients in a personalized manner.

Mounting evidence has suggested that DNA losses of chromosome 9 are identified in a variety of cancers, particularly 9p21 that harbors several candidate or established tumor suppressor genes, such as *CDKN2A* (also known as $p16^{INK4A}$/$p14^{ARF}$), *CDKN2B* (also known as $p15^{INK4B}$), and *MTAP* (*methylthioadenosine phosphorylase*).[4–10] The *MTAP* gene, which lies about 100 kb telomeric to *CDKN2A*, is frequently co-deleted with the *CDKN2A* and *CDKN2B* genes in many different cancers.[6,7,10–12] *MTAP* encodes a key enzyme in the catabolism of methylthioadenosine (MTA), which is generated during the biosynthesis of polyamines. MTAP is expressed abundantly in a wide range of normal cells and tissues. In normal cells, MTAP cleaves MTA into adenine and

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5-methylthioribose-1-phosphate. The latter compound is further metabolized to methionine.[13] Adenine and methionine are further metabolized and hence salvaged.

Lack of MTAP expression, either due to homozygous deletion or promoter methylation, is found in numerous hematologic malignancies[4,12,14–19] and solid tumors, such as mesothelioma, pancreatic cancer, osteosarcoma, chondrosarcoma, soft tissue sarcoma, gliomas, gastrointestinal stromal tumor, endometrial cancer, esophageal carcinoma, chordoma, biliary tract cancer, malignant melanoma, nonsmall cell lung cancer, etc.[5,7,10,20–32] Information on MTAP-deficiency in primary NPC is lacking, and its association with various clinicopathological variables and survival has never been systematically studied. In this study, we first identified deletion of *MTAP* gene and corresponding downregulation of mRNA in NPC through data mining from array comparative genomic hybridization (aCGH) and gene expression profiling datasets. We further analyzed *MTAP* gene status, its promoter methylation and immunoexpression in a well-defined cohort of NPC tissues, trying to clarify its prognostic significance and correlation to different clinicopathological parameters.

MATERIALS AND METHODS

Analysis of Public Transcriptomic Datasets of NPC

To identify clinically relevant genes that are critical in the pathogenesis of NPC, we reappraised gene expression profiling datasets for NPC versus non-neoplastic nasopharyngeal tissues from the Gene Expression Omnibus, which contains transcriptome and copy number data (GSE34573) obtained using Affymetrix Human Genome U133 Plus 2.0 and Mapping 250K Nsp SNP Arrays, respectively. The raw CEL files obtained from Affymetrix Human Genome U133 Plus 2.0 and Mapping 250K Nsp SNP Array platforms were imported into Nexus Expression 3 (BioDiscovery, EI Segundo, CA) and Nexus 6 (BioDiscovery) to analyze all probe sets without preselection or filtering, respectively. For the analysis of expression profiling, supervised comparative analysis and functional profiling were performed to identify statistically significant genes that were differentially expressed, with special attention given to the nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process (GO:0006139). Those genes with $P < 0.01$ and $\log_2$-transformed expression fold change $>\pm 0.1$ were chosen for validation. For the purpose of exploring the copy number alteration of target genes, Mapping 250K Nsp SNP Arrays was also analyzed.

Patient Characteristics and Tumor Samples

In this study, there were 124 NPC patients who received biopsy between January 1993 and December 2002 as previous described.[33–37] Patients with distant metastasis were excluded. Adequate paraffin-embedded tissue blocks were obtained from BioBank of Chi-Mei Medical Center, which were approved by the Institutional Review Board of Chi-Mei Medical Center (IRB103-02-013). The slides of tumor tissues were reviewed by 2 pathologists (H-LH and C-FL) who did not know the patients' clinical information. The identification of histopathologic type of tumors was based on the current WHO classification. The 2 pathologists followed the principle of the 7th American Joint Committee on Cancer (AJCC) system to assess tumor staging.

Immunohistochemical Study

The paraffin-embedded blocks were sectioned at 3-mm thickness onto precoated slides, deparaffinized with xylene and then rehydrated with gradient ethanol. The slides were heated and incubated in a 10 mM citrate buffer (pH 6.0) for 7 min. After inactivating endogenous peroxidase by 3% H_2O_2, the slides were incubated with primary antibody targeting MTAP (MGC:31876, 1:100, Proteintech, Chicago, IL) for 1 h. Primary antibodies were detected by the use of ChemMate DAKO EnVision kit (DAKO, K5001, Glostrup, Denmark), washed with Tris buffered saline for 15 min and then incubated with a primary antiboarpinteria, CA. Two pathologists (H-LH and C-FL) evaluated the immunostainings of MTAP according to the previous published criteria.[6,20,25,38] No cytoplasmic staining or faint cytoplasmic staining in $<10\%$ of tumor cells was defined as MTAP deficiency. Moderate or strong cytoplasmic staining in $>10\%$ of tumor cells was defined as intact MTAP expression.

Conditions of Real-Time Quantitative PCR Used to Measure MTAP Gene Dosage

In order to isolate specific tumor cells of interest, we performed manual macrodissection in 14 selected tumor samples that contain 90% of tumor cells. The quantity of the DNA extracted was measured by an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). The relative fold change of the *MTAP* gene copy number between normal and tumor tissues was measured by a quantitative polymerase chain reaction (PCR) assay with the use of comparative threshold cycle (C_t) method. The TaqMan assays targeting *PIK3R1* (Hs06028467_cn) in 5q13.1 and the exon 8 of the *MTAP* gene (Hs02079487_cn) in 9p21.3 were purchased from Applied Biosystems. They were separately amplified using the ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). In each well of the PCR reaction contained a total volume of 20 μL, including 1 μL of 20× TaqMan assay probe, 20 ng of genomic DNA, and 10 μL of TaqMan Genotyping Master-Mix. The PCR conditions was started at an initial denaturation step of 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 s, and then extension at 60°C for 1 min. All reactions were conducted in duplicate with a negative control run in parallel.

To measure *MTAP* gene dosage, we conducted an assay using TaqMan real-time quantitative PCR, with *MTAP* as the test sequence and *PIK3R1* as the reference gene. To obtain gene dosages, the *PIK3R1* gene was regarded as a control with the gene copy number of 2, and the normalized ratio of *MTAP*/*PIK3R1* is expected to yield a value of 1 in individuals without deletion. Following our previous defined criteria for formalin-fixed samples, the *MTAP*/*PIK3R1* gene ratio <0.2 was considered as homozygous deletion.[25,39] Also, a calibration standard curve was plotted using input templates of 100, 20, 8, and 0.4 ng human genomic DNA (Clontech, Mountain View, CA, USA) to ensure the PCR efficiency and precision in the experiment

Methylation-Specific PCR to Detect MTAP Promoter Hypermethylation

It is known that promoter hypermethylation might be an alternative mechanism for *MTAP* gene silencing in those with intact or hemizygously deleted *MTAP* gene. We thus evaluated its promoter methylation for selected cases lacking

MTAP immunoexpression but showing no evidence of *MTAP* homozygous deletion by real-time quantitative PCR. The methylation-specific PCR was performed as previously described.[25] In brief, the first-run PCR was performed with primers specific for bisulfite-converted DNA (sense primer, 5′-ATTTTTGAGTTTTGGGTTAAGTTTATTT-3′; antisense primer, 5′-AAAAAACAACACTCCCTACTTAACC-3′), which did not discriminate between methylated (M) and unmethylated (U) sequences. PCR amplification was first run for 30 cycles at the annealing temperature of 59°C, in a reaction mixture of 15 μL containing 15 ng bisulfite-converted DNA, 0.1 μM of each primer, and 0.3 U HotStar Taq DNA polymerase (Qiagen, Gmb Hilden, Germany). The nested PCR for M and U promoters were both performed for 40 cycles at the annealing temperature of 61°C in a reaction mixture of 1 μL of 10-fold diluted, first PCR products, 0.1 μM of each primer, and 0.3 U HotStar Taq DNA polymerase (Qiagen) with the following primer sequences:

M, sense primer, 5′-GTTGTTTAAGAGAATTTTCGTG GTC-3′; antisense primer, 5′-ACTTAACCCAATATTAATAA CGTCG-3′.

U, sense primer, 5′-AGTTGTTTAAGAGAATTTTTGT GGTT-3′; antisense primer, 5′-CCCTACTTAACCCAATAT TAATAACATCA-3′.

Determination of MTAP Gene Promoter Methylation Using Pyrosequencing

Promoter methylation of *MATP* was quantified by pyrosequencing to crossly validate that of methylation-specific PCR. For this purpose, after bisulfate treatment and cleanup of DNA by using EpiTect 96 Bisulfite Kit (Qiagen), PCR amplification and sequencing were performed by using Hs_MTAP_01_PM PyroMark CpG assay (Qiagen, Product No. 978746) following our previous protocol.[40] Finally, PyroMark Q24 Software was used to quantity cytosine methylation. Methylation was called when the average methylation percentage of all CpG islands tested was higher than 4.56%, which was the mean +3 SD of the average methylation percentage of CpG islands from 10 nontumor nasopharyngeal mucosa, as described previously.[41]

Treatment and Follow-Up

A complete course of radiotherapy (RT) with a total dose > or =7000 cGy was performed in all 124 patients, following a uniform protocol of RT. In addition, those who with stage II to IV received cisplatin-based chemotherapy. The 124 patients were under regular follow-up after treatment until their last visit or death. The mean duration of follow-up was 59.6 months, ranging from 4 to 117 months.

Statistical Analysis

We used the SPSS 14 software package (SPSS, Inc., Chicago, IL) to perform statistical analysis. The relationships between MTAP expression and various clinicopathological parameters or *MTAP* gene dosage were assessed by the use of Chi-squared test. This study contained 3 endpoints, including disease-specific survival (DSS), distal metastasis-free survival (DMFS), and local recurrence-free survival (LRFS). We performed Kaplan–Meier method and log-rank test to analyze the survival data. Multivariate analysis was performed using Cox proportional hazards model. For all analyses, the statistical difference was considered significant if the P value was <0.05 under two-sided tests.

RESULTS

Identifying MTAP as a Significantly Differentially Downregulated Gene Associated With Nucleobase, Nucleoside, Nucleotide, and Nucleic Acid Metabolic Process in NPC

From the transcriptomic dataset of 16 NPCs and 4 nontumor nasopharyngeal samples deposited in the public domain, we focused on 151 probes covering 67 genes associated with nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process. The genes with $P < 0.01$ and $\log_2$-transformed expression fold change $>\pm 0.1$ are listed in Table 1 and Figure 1. Among these statistically significant genes, deficiency of *MTAP* appeared as the only candidate that can be directly linked to pharmaceutical targeting,[25,39] prompting us to characterize the protein expression level in NPC. Since the *MTAP* gene locus at 9p21.3 has been reported to be frequently deleted in various human malignancies,[5,7,10,20–32] we thus evaluated the copy number alterations of *MTAP* gene by analyzing GSE34573 dataset. Interestingly, *MTAP* deletion can be identified in 68.8% (11/16) of NPCs including 2 with homozygous and 9 with hemizygous deletion, suggesting a potential role of *MTAP* gene deletion in its protein deficiency in NPC (Fig. 2).

MTAP Deficiency and Its Association With Clinicopathological Factors in NPC

The tumor samples consisted of 5 keratinizing squamous cell carcinomas, 54 nonkeratinizing differentiated carcinomas, and 65 nonkeratinizing undifferentiated carcinomas. The study population included 29 women and 95 men. The age of patients ranged from 20 to 83 years (mean, 48.6). MTAP deficiency was identified in 37 (29.8%) cases, which was significantly associated with advanced tumor stage (AJCC stage III to IV, $P = 0.023$; Table 2). However, there was no association between MTAP deficiency and other clinicopathological factors. In cases with intact MTAP expression, the tumor cells retained moderate or strong cytoplasmic staining, although some nuclear staining was also seen (Fig. 3).

Prognostic Significance of MTAP Deficiency in NPC

All disease-specific mortality, local recurrence, and distal metastasis were significantly associated with T3 to T4 status ($P = 0.029$ for DSS; $P = 0.009$ for DMFS; $P = 0.018$ for LRFS), N2 to N3 status ($P < 0.001$ for DSS; $P = 0.013$ for DMFS; $P = 0.016$ for LRFS), and stage III to IV ($P = 0.002$ for DSS; $P = 0.007$ for DMFS; $P = 0.003$ for LRFS; Table 3). MTAP deficiency correlated with a significantly shorter DSS ($P = 0.002$), DMFS ($P < 0.001$), and LRFS ($P = 0.013$) in patients with NPC (Fig. 4). In multivariate analysis, MTAP deficiency and stage both independently portended inferior DSS (MTAP deficiency, $P = 0.021$, hazard ratio [HR] = 1.870) and worse DMFS (MTAP deficiency, $P = 0.009$, HR = 2.154), respectively (Table 4).

Correlation of MTAP Homozygous Deletion and Promoter Hypermethylation With Immunoexpression

As shown in Table S1, http://links.lww.com/MD/A553, the presence of either *MTAP* homozygous deletion or promoter hypermethylation had a strong correlation to the loss of protein expression ($P < 0.001$) in 14 selected NPC samples. In the

TABLE 1. Summary of Differentially Expressed Genes Associated With Nucleobase, Nucleoside, Nucleotide, and Nucleic Acid Metabolic Process (GO:0006139) in the Transcriptome of Nasopharyngeal Carcinoma

Probe	Comparison Log Ratio	Comparison *P* Value	Gene Symbol	Gene Name	Biological Process	Molecular Function
1553734_at	−1.253	<0.001	*AK7*	Adenylate kinase 7	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	ATP binding, adenylate kinase activity, kinase activity, nucleotide binding, nucleotide kinase activity, transferase activity
1569555_at	−1.289	<0.001	*GDA*	Guanine deaminase	Nervous system development; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	Guanine deaminase activity, hydrolase activity, zinc ion binding
202587_s_at	−1.490	<0.001	*AK1*	Adenylate kinase 1	ATP metabolic process, cell cycle arrest; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	ATP binding, adenylate kinase activity, kinase activity, nucleotide binding, nucleotide kinase activity, protein binding, transferase activity
211363_s_at	−0.613	<0.001	*MTAP*	Methylthioadenosine phosphorylase	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	S-methyl-5-thioadenosine phosphorylase activity; phosphorylase activity; transferase activity; transferase activity, transferring glycosyl groups; transferase activity, transferring pentosyl groups
216325_x_at	−0.478	0.004	*RTEL1*	Regulator of telomere elongation helicase 1	Anti-apoptosis, apoptosis; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	ATP binding, ATP-dependent DNA helicase activity, ATP-dependent helicase activity; DNA binding; helicase activity; hydrolase activity; hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides; nucleic acid binding; nucleotide binding; protein binding; receptor activity
217134_at	−0.448	0.007	*MTAP*	Methylthioadenosine phosphorylase	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	S-methyl-5-thioadenosine phosphorylase activity; phosphorylase activity; transferase activity; transferase activity, transferring glycosyl groups; transferase activity, transferring pentosyl groups
224209_s_at	−2.793	<0.001	*GDA*	Guanine deaminase	Nervous system development; nucleobase; nucleoside, nucleotide, and nucleic acid metabolic process	Guanine deaminase activity, hydrolase activity, zinc ion binding
230976_at	−1.217	<0.001	*C9orf98*	Chromosome 9 open reading frame 98	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	ATP binding, adenylate kinase activity, kinase activity, nucleotide binding, nucleotide kinase activity, transferase activity
200762_at	2.149	0.002	*DPYSL2*	Dihydropyrimidinase-like 2	Cell differentiation, multicellular organismal development, nervous system development; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process; signal transduction	Dihydropyrimidinase activity, hydrolase activity, protein binding
202338_at	1.735	0.007	*TK1*	Thymidine kinase 1; soluble	DNA replication; muscle development; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	ATP binding, kinase activity, nucleotide binding, protein binding, thymidine kinase activity, transferase activity
202589_at	3.986	<0.001	*TYMS*	Thymidylate synthetase	DNA repair; DNA replication; dTMP biosynthetic process; deoxyribonucleoside monophosphate biosynthetic process; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process; nucleotide biosynthetic process; phosphoinositide-mediated signaling	Methyltransferase activity, thymidylate synthase activity, transferase activity

Probe	Comparison Log Ratio	Comparison *P* Value	Gene Symbol	Gene Name	Biological Process	Molecular Function
202613_at	2.249	0.005	*CTPS*	CTP synthase	CTP biosynthetic process; glutamine metabolic process; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process; pyrimidine nucleotide biosynthetic process; response to drug	CTP synthase activity, catalytic activity, ligase activity, protein binding
203058_s_at	2.909	<0.001	*PAPSS2*	3′-phosphoadenosine 5′-phosphosulfate synthase 2	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process; skeletal development; sulfate assimilation	ATP binding; adenylylsulfate kinase activity; catalytic activity; kinase activity; nucleotide binding; nucleotidyltransferase activity; protein binding; sulfate adenylyltransferase (ATP) activity; transferase activity; transferase activity, transferring phosphorus-containing groups
203060_s_at	3.695	<0.001	*PAPSS2*	3′-phosphoadenosine 5′-phosphosulfate synthase 2	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process; skeletal development; sulfate assimilation	ATP binding; adenylylsulfate kinase activity; catalytic activity; kinase activity; nucleotide binding; nucleotidyltransferase activity; protein binding; sulfate adenylyltransferase (ATP) activity; transferase activity; transferase activity, transferring phosphorus-containing groups
203302_at	2.320	<0.001	*DCK*	Deoxycytidine kinase	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process; pyrimidine nucleotide metabolic process	ATP binding; deoxycytidine kinase activity; kinase activity; nucleotide binding; phosphotransferase activity, alcohol group as acceptor; transferase activity
204972_at	3.150	<0.001	*OAS2*	2′-5′-oligoadenylate synthetase 2; 69/71 kDa	RNA catabolic process; immune response; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	2′-5′-oligoadenylate synthetase activity, ATP binding, RNA binding, nucleotidyltransferase activity, transferase activity
208955_at	1.136	0.003	*DUT*	dUTP pyrophosphatase	DNA replication; dUTP metabolic process; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process; nucleotide metabolic process	dUTP diphosphatase activity, hydrolase activity, magnesium ion binding
209265_s_at	1.771	0.010	*METTL3*	Methyltransferase like 3	RNA methylation; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	RNA binding, mRNA (2′-O-methyladenosine-N6-)-methyltransferase activity, methyltransferase activity, transferase activity
212175_s_at	2.266	0.003	*AK2*	Adenylate kinase 2	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	ATP binding; adenylate kinase activity; kinase activity; nucleoside kinase activity; nucleotide binding; nucleotide kinase activity; phosphotransferase activity, phosphate group as acceptor; transferase activity
213653_at	1.861	0.002	*METTL3*	Methyltransferase like 3	RNA methylation; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	RNA binding, mRNA (2′-O-methyladenosine-N6-)-methyltransferase activity, methyltransferase activity, transferase activity
217870_s_at	1.955	0.001	*CMPK*	Cytidylate kinase	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process; pyrimidine nucleotide biosynthetic process; pyrimidine ribonucleotide biosynthetic process	ATP binding; cytidylate kinase activity; kinase activity; nucleotide binding; nucleotide kinase activity; phosphotransferase activity, phosphate group as acceptor; transferase activity; uridine kinase activity
218400_at	2.170	0.003	*OAS3*	2′-5′-oligoadenylate synthetase 3; 100 kDa	Immune response; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	ATP binding, RNA binding, nucleotidyltransferase activity, transferase activity

Probe	Comparison Log Ratio	Comparison *P* Value	Gene Symbol	Gene Name	Biological Process	Molecular Function
224655_at	1.859	0.005	*AK3*	Adenylate kinase 3	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	ATP binding; GTP binding; adenylate kinase activity; kinase activity; nucleoside triphosphate adenylate kinase activity; nucleotide binding; nucleotide kinase activity; phosphotransferase activity, phosphate group as acceptor; transferase activity
238736_at	1.866	<0.001	*REV3L*	REV3-like; catalytic subunit of DNA polymerase zeta (yeast)	DNA repair; DNA replication; DNA-dependent DNA replication; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process; response to DNA damage stimulus	DNA binding, DNA-directed DNA polymerase activity, metal ion binding, nucleic acid binding, nucleotide binding, nucleotidyltransferase activity, transferase activity, zeta DNA polymerase activity, zinc ion binding
242111_at	1.528	0.009	*METTL3*	Methyltransferase like 3	RNA methylation; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	RNA binding, mRNA (2′-O-methyladenosine-N6-)-methyltransferase activity, methyltransferase activity, transferase activity
216325_x_at	−1.253	<0.001	*AK7*	Adenylate kinase 7	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	ATP binding, adenylate kinase activity, kinase activity, nucleotide binding, nucleotide kinase activity, transferase activity
217134_at	−1.289	<0.001	*GDA*	Guanine deaminase	Nervous system development; nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	Guanine deaminase activity, hydrolase activity, zinc ion binding

FIGURE 1. Data mining on public transcriptomic datasets of nasopharyngeal carcinoma. By reappraising the published transcriptomic datasets of nasopharyngeal carcinoma (NPC) versus non-neoplastic nasopharyngeal tissue samples (GSE34573), *MTAP* is one of the significantly downregulated gene associated with nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process (GO:0006139) in clustering analysis. NPC (blue lines) and non-neoplastic (red lines) tissue specimens were indicated on top of the heatmap, and expression levels of upregulated and downregulated genes were expressed as a series of brightness of red and green colors, respectively, with those unaltered in mRNA expression being coded as black.

TABLE 2. Associations Between MTAP Expression With Other Important Clinicopathological Variables

		MTAP Exp		
Parameters	**Category**	**Intact**	**Deficient**	***P* Value**
Gender	Male	65	30	0.443
	Female	22	7	
Age, y	<60	67	31	0.397
	≥60	20	6	
Primary tumor (T)	T1–T2	59	21	0.239
	T3–T4	28	16	
Nodal status (N)	N0–N1	44	12	0.063
	N2–N3	43	25	
Stage	I–II	32	6	0.023*
	III–IV	55	31	
Histological grade	Keratinized	3	2	0.400
	Nonkeratinized	35	19	
	Undifferentiated	49	16	

MTAP = methylthioadenosine phosphorylase.
* Statistically significant.

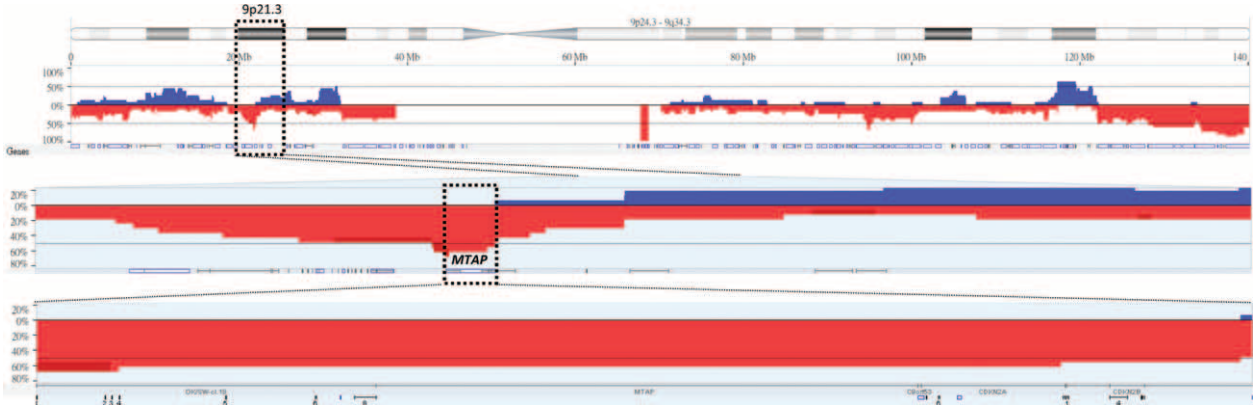

FIGURE 2. Analysis of copy number alterations of MTAP gene in public transcriptomic datasets of nasopharyngeal carcinoma. Reappraising copy number data in the same public domain dataset (GSE34573) further confirmed that chromosome region 9p21.3 is frequently (68.8%, 11/16) deleted (upper). Of this region, *MTAP* is located at the most frequently altered gene segment (middle), showing 68.8% (11/16) deletion and 12.5% (2/16) homozygous deletion (lower). MTAP = methylthioadenosine phosphorylase.

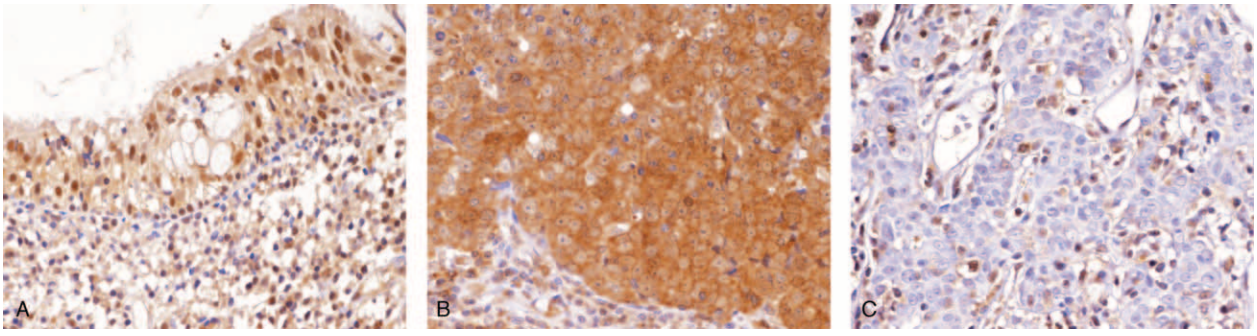

FIGURE 3. Immunohistochemical staining of MTAP in nasopharyngeal carcinoma. Non-neoplastic nasopharyngeal epithelial tissue (A) and representative low-stage NPC (B) show intact MTAP expression. While representative high-stage NPC (C) is deficient for MTAP expression. In addition, the expression of MTAP is also present either in the lymphocytes (A and C) or stromal cells (B) adjacent to the tumor or non-neoplastic mucosa. MTAP = methylthioadenosine phosphorylase, NPC = nasopharyngeal carcinoma.

TABLE 3. Univariate Log-Rank Analyses

			DSS		DMFS		LRFS	
Parameters	**Category**	**No. of Case**	**No. of Event**	***P* Value**	**No. of Event**	***P* Value**	**No. of Event**	***P* Value**
Gender	Male	95	45	0.787	38	0.613	30	0.324
	Female	29	14		11		7	
Age, y	<60	98	48	0.860	42	0.309	29	0.821
	≥60	26	11		7		8	
Primary tumor (T)	T1–T2	80	32	0.029*	25	0.009*	19	0.018*
	T3–T4	44	27		24		18	
Nodal status (N)	N0–N1	56	18	<0.001*	17	0.013*	12	0.016*
	N2–N3	68	41		32		25	
Stage	I–II	38	10	0.002*	9	0.007*	5	0.003*
	III–IV	86	49		40		32	
Histological grade	Keratinized/nonkeratinized	47	20	0.198	17	0.275	15	0.952
	Undifferentiated	77	39		32		22	
MTAP	Intact	87	34	0.002*	27	<0.001*	22	0.013*
	Deficient	37	25		22		15	

DMFS = distal metastasis-free survival, DSS = disease-specific survival, LRFS = local recurrence-free survival, MTAP = methylthioadenosine phosphorylase.

* Statistically significant.

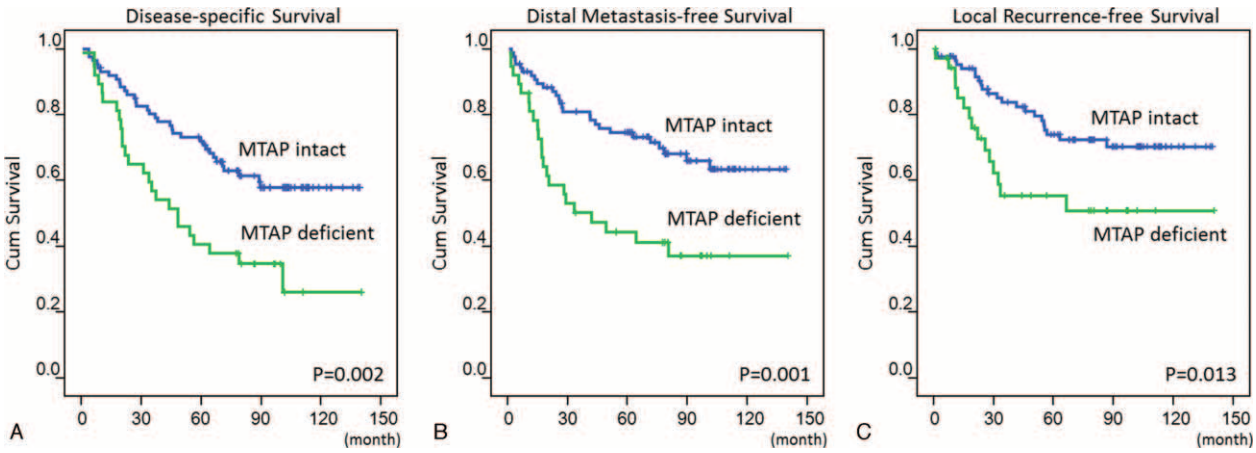

FIGURE 4. Survival analysis for assessing prognostic significance of MTAP deficiency in nasopharyngeal carcinoma. By using log-rank test, an MTAP deficiency is univariately predictive of inferior disease-specific survival (A), distant metastasis-free survival (B), and local recurrence-free survival (C), MTAP = methylthioadenosine phosphorylase.

14 selected NPC cases, there were 8 cases showing MTAP protein deficiency, including 4 cases with MTAP gene homozygous deletion characterized by *MTAP/PIK3R1* gene ratio < 0.2 and another 4 cases with promoter hypermethylation (Fig. 5).

DISCUSSION

The *MTAP* gene is located at human chromosomal locus 9p21. Homozygous deletions are not uncommon in several kinds of cancer associated with the loss of tumor suppressor genes as *CDKN2A* and/or *CDKN2B*.[6,7,10–12] Whether the loss of *MTAP* activity contributes to tumorigenesis remains debated owing to its frequent codeletion with adjacent tumor suppressor genes. However, mounting evidence has suggested that MTAP may play a role as an independent tumor suppressor in carcinogenesis. First, *MTAP* deletion may also occur without the loss of neighboring *CDKN2A* in gliomas and non-small cell lung cancers.[10,29] Second, reintroduction of MTAP into *MTAP*-deleted breast cancer cells (MCF-7) results in dramatic suppression of anchorage-independent growth in vitro and tumorigenicity in vivo.[11] Third, in hepatocellular carcinoma, downregulation of MTAP expression is mainly attributed to promoter hypermethylation, rather than genomic loss or mutation.[24] Moreover, MTAP deficiency is associated with increased tumorigenicity and induces progression of hepatocellular carcinoma via accumulation of 5′-deoxy-5′-methylthioadenosine (the MTAP substrate).[42] As for NPC, the role of MTAP expression is still unclear. In this study, we have first worked out the gene status and protein expression of *MTAP* at 9p21.3 in NPC and provided convincing evidence that MTAP protein deficiency was correlated with disease progression characterized by worse DSS and DMFS.

Through accumulation of the MTA (the MTAP substrate), MTAP depletion might increase the invasive and vasculogenic capability of melanoma cells by induction of matrix metalloproteinase (MMP) and angiogenic growth factor gene expression. In addition, MTA played a tumor-supporting role in melanoma via activating fibroblasts to construct an ideal microenvironment for tumor growth.[43] In hepatocellular carcinoma, Kirovski et al[42] also demonstrated similar findings that downregulation of MTAP resulted in MTA secretion from cancer cells, leading to induced expression of MMP and fibroblast growth factors. Furthermore, they found that low MTAP protein expression correlated with advanced tumor stages, which was compatible with our result in NPC. There was evidence suggesting that MTAP depletion may lead to upregulation of ornithine decarboxylase (ODC).[44] ODC is the rate-limiting enzyme in the production of polyamines having stimulatory effect on cell proliferation and therefore cancer growth.

TABLE 4. Multivariate Survival Analyses

		DSS			DMFS			LRFS		
Parameter	**Category**	**HR**	**95% CI**	***P* Value**	**HR**	**95% CI**	***P* Value**	**HR**	**95% CI**	***P* Value**
Stage	I–II	1	–	0.014*	1	–	0.041*	1		0.013*
	III–IV	2.389	1.189–4.802		2.170	1.033–4.556		3.370	1.289–8.811	
MTAP Exp.	Intact	1	–	0.021*	1	–	0.009*	1	–	0.095
	Deficient	1.870	1.099–3.180		2.154	1.207–3.842		1.770	0.906–3.459	

CI = confidence interval, DMFS = distal metastasis-free survival, DSS = disease-specific survival, HR = hazard ratio, LRFS = local recurrence-free survival, MTAP = methylthioadenosine phosphorylase.
* Statistically significant.

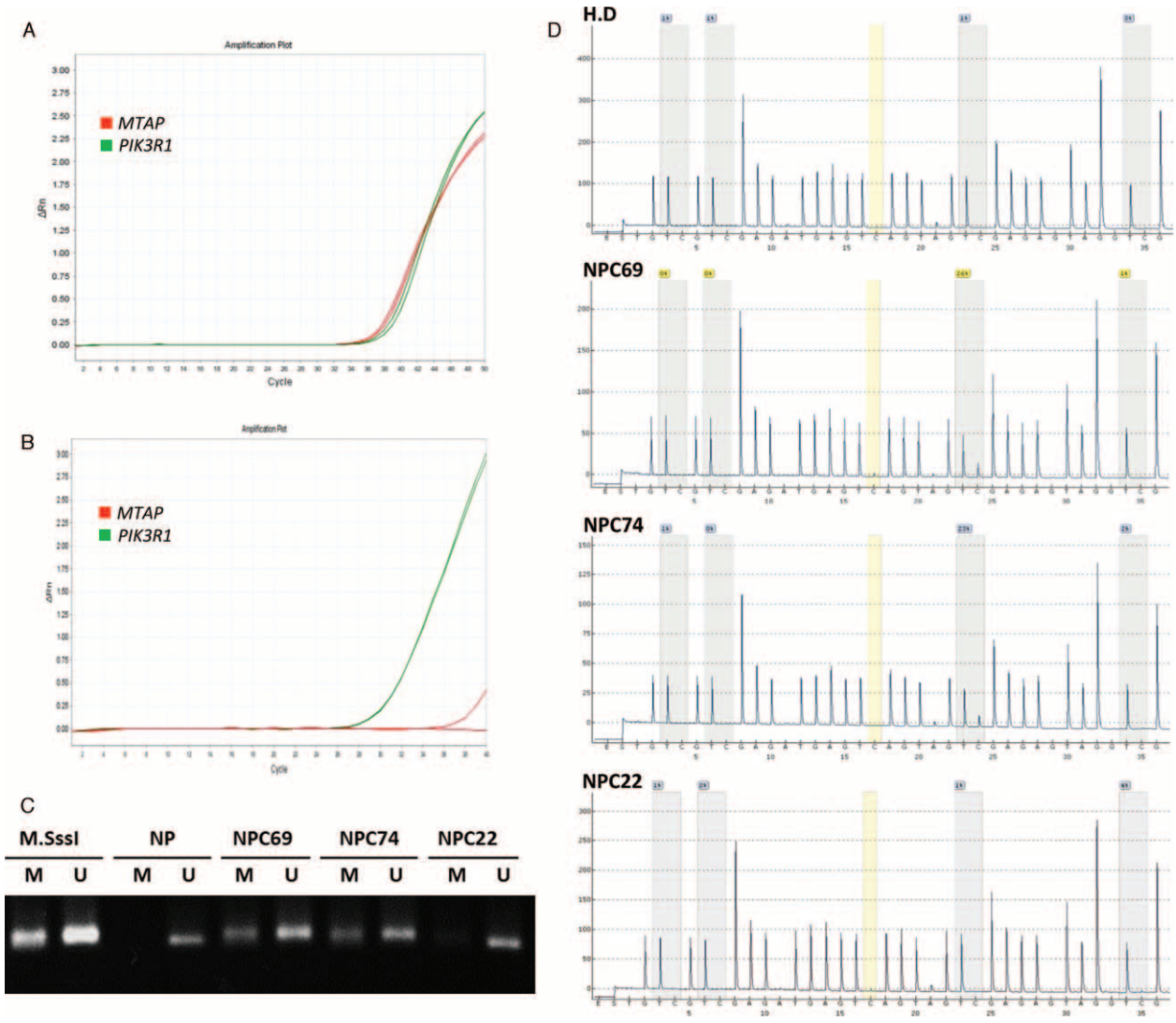

FIGURE 5. MTAP gene dosage and methylation-specific PCR for analysis of MTAP promoter methylation in nasopharyngeal carcinoma. Representative low-stage NPC with intact MTAP expression has a preserved *MTAP* gene, with a Ct value similar to that of *PIK3R1*, both <40 in a quantitative DNA-PCR (A). Representative high-stage and MTAP-deficient NPC has nondetectable *MTAP* gene, characterized by a Ct value no less than 40 (B). Methylation-specific PCR detects *MTAP* promoter methylation in MTAP protein-deficient NPCs without homozygous gene deletion (C). PCR products amplified from *MTAP* promoter with methylation-specific primers can be distinctly visualized in both NPC69 and NPC74, while the *MTAP* gene promoter of a representative MTAP-intact NPC, NPC22, is unmethylated. Pyrosequencing is performed to quantify the percentage of methylation in specific CpG sites of MTAP (D). The percentages of methylation in NPC69 and NPC74 are 23% and 26%, respectively. While in NPC22, the percentage is 4%, which is less than the cut-off value of methylation and regarded as unmethylated. M = methylation-specific primers, M.SssI = a healthy donor's blood DNA treated with M. SssI methyltransferase, MTAP = methylthioadenosine phosphorylase, NP = DNA from nontumor nasopharyngeal mucosal tissue without treatment of M.SssI methyltransferase, NPC = nasopharyngeal carcinoma, PCR = polymerase chain reaction, U = unmethylation-specific primers.

Introduction of MTAP into *MTAP*-deleted breast cancer cells (MCF-7) significantly reduced ODC activity, accompanied by reduction in polyamine levels. These findings indicated that other enzyme, in addition to MTAP, in the methionine salvage pathway may also involve in regulating tumor growth.

In normal cells, MTAP cleaves MTA, generated during polyamine biosynthesis, to adenine and 5-methylthioribose-1-phosphate. The latter compound is further metabolized to methionine and adenine is converted to AMP. In MTAP-deficient cells, MTA is unable to be catabolized to generate adenine and methionine. As a consequence, MTAP-deficient cells are more sensitive than MTAP-positive cells to inhibitors of de novo purine synthesis and to methionine deprivation, presenting a possible therapeutic target for cancer treatment.[13,45] The inhibitors of purine biosynthesis include methotrexate, 6-mercaptopurine, 6-thioguanine (6-TG), azaserine, and L-alanosine.[28,45,46] Both 6-mercaptopurine and 6-TG are rapidly converted to active nucleotide forms in MTAP-deficient cells. 6-TG can be incorporated into DNA, and cells without mismatch repair gene exhibits more resistance to 6-TG treatment.[47] In addition, MTA may act as a protective agent. MTA alone, which serves as an anti-inflammatory agent, has

been found to possess suppression effect on tumor growth in melanoma and colon cancer.[48,49] In recent years, a new strategy of treatment with selective killing of MTAP-deficient tumors has emerged. MTAP-deficient tumors are treated with MTA, followed by high dose of a purine or pyrimidine analog, such as 5-fluorouracil and 6-TG, which needs phosphoribosylation for conversion to its toxic nucleotide. In normal host cells, substantial adenine, generated from MTA, blocks the conversion of the analog to its toxic nucleotide via effective competition for phosphoribosylation by 5-phosphoribosyl-1-pyrophosphate (PRPP). In MTAP-deficient tumor cells, PRPP persists at adequate level due to lack of adenine. And the analog is converted to its toxic nucleotide, selectively killing the tumor cells.[50] Thus, it would be of great value to apply this strategy of targeted treatment to NPC in the future.

Finally, there is an interesting issue with regard to the immunolabeling for MTAP which needs to be further elucidated. Although MTAP is originally found to be a cytoplasmic protein, both previous studies and our current one disclose the possibility of its nuclear expression in some kinds of tumors or precancerous lesions, including gastroesophageal high-grade dysplastic lesions, mantle cell lymphomas, and NPCs.[17,30] However, little is known about the pathophysiologic process underlying this phenomenon. Further investigation is needed to confirm this expression pattern and to recognize its function as a nuclear protein.

In conclusion, we have found homozygous deletion or promoter hypermethylation of MTAP in a subset of NPCs either through published transcriptomic datasets or in our cases. MTAP deficiency was demonstrated in the immunohistochemical study. Furthermore, MTAP deficiency was associated with advanced tumor stage, as well as worse DSS and DMFS. This result implied that MTAP deficiency plays an important role in tumorigenesis and exhibits an aggressive behavior. It also acts as an independent factor to predict poor survival. This study sheds light on the potential application of target treatment for tumors lacking MTAP activity in NPC.

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