ANTICANCER RESEARCH *25*: 4455-4462 (2005)

Prognostic Significance of the Polymorphisms in Thymidylate Synthase and Methylenetetrahydrofolate Reductase Gene in Lung Cancer

AKIRA TAKEHARA, KAZUYUKI KAWAKAMI, NAOHIRO OHTA, KAEKO OYAMA, YASUHIKO OTA, MAKOTO ODA and GO WATANABE

Department of Surgery, Kanazawa University School of Medicine, Kanazawa 920-8641, Japan

Abstract. *Background: Thymidylate synthase (TS) and methylenetetrahydrofolate reductase (MTHFR) play important roles in folate metabolism. Previous studies have suggested that TS expression is a prognostic factor in non-small cell lung cancer (NSCLC). The TS gene has a variable number of tandem repeats (VNTR) and single nucleotide polymorphism (SNP) in the 5'-untranslated region, which are associated with TS expression. This association suggests that the TS polymorphism is a novel prognostic factor in NSCLC. In the present study, multiple genetic polymorphisms, TS VNTR, TS SNP and MTHFR C677T, were analyzed in NSCLC and compared with clinicopathological features and patients' prognoses. Materials and Methods: Genomic DNA was isolated from 294 surgically resected NSCLC tissues. The genotypes were determined by PCR and PCR-RFLP. The TS VNTR and SNP were combined, followed by functional stratification of H/H (3G/3G), H/L (2R/3G, 3G/3C) and L/L (2R/2R, 2R/3C, 3C/3C). Patients' prognoses were compared with TS and/or MTHFR genotype groups. TS was divided into the H- (H/H, H/L) and L-groups (L/L) according to functional stratification and MTHFR C677T was divided into C- (C/C) and T-groups (C/T, T/T). Results: TS VNTR, the SNP and the TS functional type, along with MTHFR C677T, showed no significant association with clinicopathological factors. There were no differences in prognosis between each genotype or functional group when the TS and MTHFR groups were considered separately. However, we found a unique association between prognosis and the TS functional group in stage I NSCLC, taking both TS and MTHFR groups into consideration. The patients in the TS L-group survived longer than those in the H-group when limited to stage I and MTHFR C-group ($p=0.086$). This relationship between the TS genotype group and prognosis was statistically significant in the subgroup of stage IB and MTHFR C-group ($p=0.030$). In contrast, the patients in the TS H-group survived longer than those in the L-group when limited to stage I and MTHFR T-group ($p=0.052$). Conclusion: The TS and MTHFR genotypes can be prognostic factors in NSCLC, where gene-gene interactions between the genotypes may occur. Further validation and investigation of the involvement of genotypes of folate metabolizing enzymes in the prognosis of NSCLC patients are required.*

Thymidylate synthase (TS) plays an important role in DNA synthesis through catalyzing the reductive methylation of dUMP by 5,10-methylenetetrahydrofolate (CH_2FH_4) to form dTMP and dihydrofolate (Figure 1). As it is a critical reaction for cell proliferation, the activity of TS is related to the biological features of cancer (1, 2). In addition, TS is a target enzyme of a variety of chemotherapeutic drugs, such as 5-FU, UFT and capecitabin (3, 4). TS expression in cancer tissue has been reported to be associated with the efficacy of chemotherapy and prognosis in many malignancies, including lung cancer. The high level of TS expression in lung cancer was shown to be associated with poor prognosis (5), which suggested the possibility of individualized therapy based on TS expression.

The TS gene has a unique tandem-repeat sequence in its 5'-untranslated region. The number of repeats was found to be polymorphic, where triple (3R) and double tandem repeats (2R) were mainly reported (6). This variable number of tandem repeats (VNTR) of the TS gene are associated with TS protein expression (7, 8) leading to the hypothesis that TS VNTR might be a novel prognostic and predictive factor for the clinical outcome of patients with

Correspondence to: Kazuyuki Kawakami, MD, Ph.D., Department of Surgery, Kanazawa University School of Medicine, 13-1 Takaramachi, Kanazawa 920-8641, Japan. Tel: +81-76-265-2355, Fax: +81-76-222-6833, e-mail: kawakami@med.kanazawa-u.ac.jp

Key Words: TS polymorphism, MTHFR polymorphism, prognosis, non-small cell lung cancer.

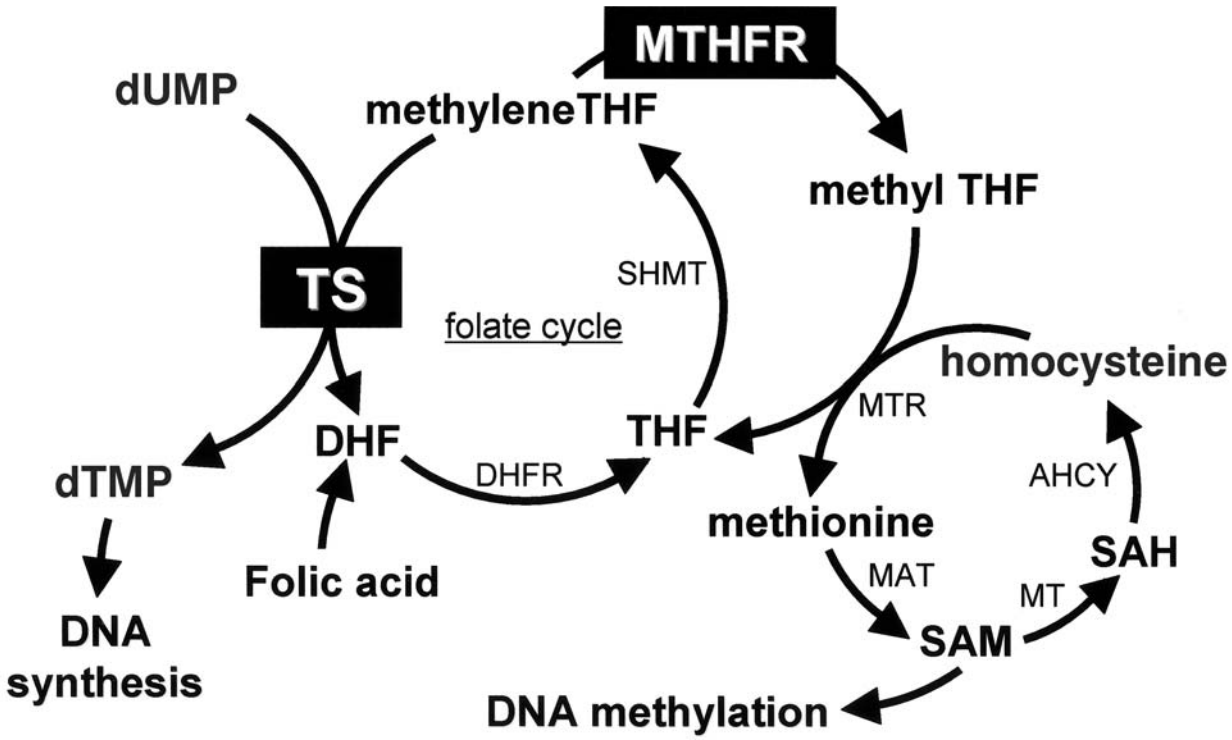

Figure 1. *The metabolic map of folate. Abbreviations: THF, tetrahydrofolate; DHF, dihydrofolate; dUMP, deoxyuridylate; dTMP, thymidylate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; DHFR, dihydrofolate reductase; SHMT, serine hydroxymethyltransferase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; MAT, methionine adenosyltransferase; MT, generic methyltransferase; AHCY, S-adenosylhomocysteine hydrolase.*

gastrointestinal cancer (9, 10). In addition to the VNTR, a novel G/C SNP within the 28-bp repeat component of TS VNTR was recently identified, which had a critical effect on TS expression (11, 12). Both the VNTR and the SNP were shown to be associated with TS expression, suggesting that the two polymorphisms should be considered in using TS genotype for prognostic prediction. There have been a number of studies of the relationship between TS polymorphism and the outcome of patients with colorectal cancer treated with 5-FU-based chemotherapy (10, 13-15). However, there has been only one previous study of the relevance of TS polymorphism with the clinical characteristics of lung cancer (16).

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism and is at the crossroad of DNA synthesis and methylation (Figure 1). The MTHFR has been suggested to interact with the TS in the metabolic pathway of folate because the enzymes compete for CH_2FH_4 (17). The MTHFR gene has a common polymorphism, C677T, which has been reported to affect enzymatic activity (18). Therefore, both TS and MTHFR polymorphism may be associated with characteristics of cancer on the basis of the suggested interaction between TS and MTHFR on folate metabolism, DNA synthesis and methylation.

In the present study, the VNTR and SNP of the TS gene together with MTHFR C677T polymorphism in NSCLC were investigated and their clinical significance evaluated.

Materials and Methods

Patients and DNA isolation. A total of 295 tumor samples were obtained by surgical resection from patients with NSCLC at the Department of Cardiothoracic and General Surgery, Kanazawa University Hospital, Japan. The patient population consisted of 197 males and 98 females, ranging in age from 13-83 years, with a mean age of 64.2. The resected tissues were fixed in formalin and embedded in paraffin followed by histological diagnosis with H&E staining. Tumor tissue was dissected manually from sections of the formalin-fixed paraffin-embedded tissue 10 μm thick. After deparaffinization using xylene and ethanol, genomic DNA was isolated using a QIAamp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Approval of this project was obtained from the Kanazawa University School of Medicine Ethics Committee.

Genotyping of the VNTR and SNP in the thymidylate synthase enhancer region. The TS genotypes of the VNTR and the SNP were determined by PCR and PCR-RFLP using the forward primer TS25 AGGCGCGCGGAAGGGGTCCT and reverse primer TS18 TCCGAGCCGGCCACAGGCAT as described previously (11), with a modification of the PCR conditions. PCR with the genomic

Table I. *TS genotypes of VNTR and SNP.*

	2R/2R	2R/3R		3R/3R			3R/5R
		2R/3G	2R/3C	3G/3G	3G/3C	3C/3C	
Gender							
Male	14	27	26	32	54	43	1
Female	6	13	9	18	34	18	0
Total	20	40	35	50	88	61	1

DNA template was performed in reaction mixtures containing 1x TaKaRa HS Taq buffer (TaKaRa, Japan), 200 μM deoxyribonucleoside triphosphates, 500 nM each primer, 0.5 unit of TaKaRa HS Taq DNA polymerase (TaKaRa) and 100 ng of genomic DNA. The cycling conditions were 1 cycle of 95˚C for 3 min, 35 cycles of 98˚C for 10 sec and 68˚C for 60 sec, with a final extension at 72˚C for 5 min. Aliquots of amplified fragments were separated on 3% agarose gels and the TS VNTR genotype was determined. Samples showing the 2R/3R or 3R/3R genotypes were analyzed further for G/C polymorphism by RFLP. *Hae*III digestion of the 3R fragment produced 66-, 37-, 28- and 10-bp bands for the 3G allele and 94-, 37- and 10-bp bands for the 3C allele after separation on 3% agarose gels. Accordingly, the TS genotype was classified into 2R/2R, 2R/3G, 2R/3C, 3G/3G, 3G/3C, or 3C/3C by comprehensive genotyping of the VNTR and SNP in the TS 5' untranslated region. Analyses were performed at least twice to confirm the genotype.

Genotyping of the MTHFR C677T polymorphism. The MTHFR genotype was examined using the PCR-RFLP method. PCR was performed in the same reaction mixture as used for TS genotyping except with the use of a forward primer, TGAAGGAGAAGGTGT CTGCGGGA and reverse primer, AGGACGGTGCGGTGAG AGTG. The cycling conditions were 1 cycle of 94˚C for 3 min, 40 cycles of 94˚C for 40 sec, 60˚C for 1 min and 72˚C for 40 sec, with a final extension at 72˚C for 5 min. The amplified DNA fragments were digested with *Hinf*I for at least 2 h at 37˚C, followed by electrophoresis on 3% agarose gels. The C allele produces a 198-bp band and the T allele produces bands of 175 and 23 bp. The MTHFR C677T genotypes were classified as C/C, C/T, or T/T.

Statistical analysis. The relationships between each genotype and clinicopathological variables were analyzed by either Chi-square analysis or ANOVA. The cumulative survival rate was obtained by the Kaplan-Meier method, and statistical significance was analyzed by the log-rank test.

Results

TS and MTHFR genotype in NSCLC. The TS genotypes of VNTR and SNP in the 295 NSCLC patients are summarized in Table I. There was no gender difference between the genotypes, which was observed in our previous study in colorectal cancer (11). We found the rare genotype 3R/5R in 1 specimen, which was excluded from further analysis. Analysis of the genotype for VNTR and/or SNP also showed no association between the genotype and clinicopathological features, including patient's age, histopathology and stage of the disease (data not shown). Then, the functional stratification of the TS genotype was employed for further analyses. The TS allele of 3G was considered a high expression (H) allele, while 2R and 3C were considered low expression (L) alleles according to the results of *in vitro* analyses (11, 12). Therefore, we assigned TS genotypes as H/H (3G/3G), H/L (2R/3G and 3G/3C), and L/L (2R/2R, 2R/3C and 3C/3C) from the functional viewpoint and investigated their clinicopathological significance (Table II). However, there were no significant relationships between TS functional groups and clinicopathological factors: *i.e.*, age, gender, histological type of the tumor, tumor size, T factor, N factor, M factor, pathological stage, preoperative serum CEA value, or Brinkmann index.

The genotypes of MTHFR C677T and clinicopathological features in the 294 NSCLC patients are summarized in Table III. The genotype frequency was similar to those observed in a previous study (19). There were no associations between the MTHFR C677T genotype and clinicopathological factors. There was no sequence disequilibrium between the TS and MTHFR genotypes (data not shown).

Prognostic significance of TS and MTHFR genotypes in stage I NSCLC. We investigated the associations of the TS and MTHFR genotypes with prognosis. The TS and the MTHFR genotypes were each stratified into two groups because the statistical power was insufficient by analysis with more than two genotype groups. The TS functional groups were classified into the H-group (H/H and H/L) and L-group (L/L). The MTHFR C677T genotypes were also stratified into two groups: the C-group(C/C) and the T-group (C/T and T/T).

There were no significant relationships between overall prognosis and the genotype groups when all patients were included in the analysis (Figure 2A and 2B). Thus, the

Table II. *TS functional groups and clinicopathological factors.*

	H/H	H/L	L/L	*p*-value
Total	50	130	114	
Gender				
Male	32	83	81	
Female	18	47	33	0.45
Age (yrs)	66.0±8.8	64.1±8.9	63.7±10.5	0.30
Histology				
Adeno	27	79	66	
Large	2	7	8	
Squamous	21	44	40	0.81
pT				
0	0	1	0	
1	17	53	42	
2	22	43	50	
3	4	15	13	
4	7	18	9	0.58
pN				
0	38	88	72	
1	3	11	8	
2	8	29	26	
3	1	2	8	0.29
pM				
0	50	124	113	
1	0	6	1	0.08
p-Stage				
0	0	1	0	
I	31	69	59	
II	4	14	11	
III	15	40	43	
IV	0	6	1	0.40

Table III. *MTHFR C677T and clinicopathological factors.*

	C/C	C/T	T/T	*p*-value
Total	154	115	25	
Gender				
Male	102	76	18	
Female	52	39	7	0.84
Age(yrs)	65.0±9.7	63.9±9.2	62.0±10.1	0.23
Histology				
Adeno	90	70	12	
Large	8	6	3	
Squamous	56	39	10	0.68
pT				
0	1	0	0	
1	64	39	9	
2	62	43	10	
3	12	16	4	
4	15	17	2	0.56
pN				
0	109	73	16	
1	9	11	2	
2	26	30	7	
3	10	1	0	0.07
pM				
0	151	111	25	
1	3	4	0	0.51
p-Stage				
0	1	0	0	
I	90	56	13	
II	14	11	4	
III	46	44	8	
IV	3	4	0	0.66

prognostic significance of the genotypes was further evaluated in sub-groups by the stage of disease. In this subgroup analysis, unique associations were found among patients' prognoses, TS genotype and MTHFR genotype in stage I NSCLC. Neither the TS genotype nor the MTHFR genotype showed a significant correlation with prognosis in stage I when the genotypes were analyzed separately (Figure 3A and 3B). However, the patients in the TS L-group survived longer than those in the H-group when limited to stage I and the MTHFR C-group (Figure 3C, $p=0.086$). The relationship between the TS genotype group and prognosis was statistically significant in the sub-group of stage IB and the MTHFR C-group ($p=0.030$). In contrast, the patients in the TS H-group survived longer than those in the L-group when limited to stage I and the MTHFR T-group (Figure 3D, $p=0.052$).

Discussion

The VNTR of the TS gene has been reported to be associated with intra-tumoral TS expression. The role of VNTR in TS expression suggests that the polymorphism may be a useful prognostic and predictive factor of 5-FU-based chemotherapy, which has attracted a great deal of clinical interest. The clinical implications of the TS VNTR have been investigated mainly in gastrointestinal cancer (10, 13-15), with only one previous report regarding TS genotype in NSCLC. Shintani *et al.* reported that the TS VNTR genotype was correlated with immunohistochemically-determined TS expression in NSCLC. They also confirmed that TSmRNA levels were significantly higher in cancer tissues with the 3R/3R genotype as compared to those with

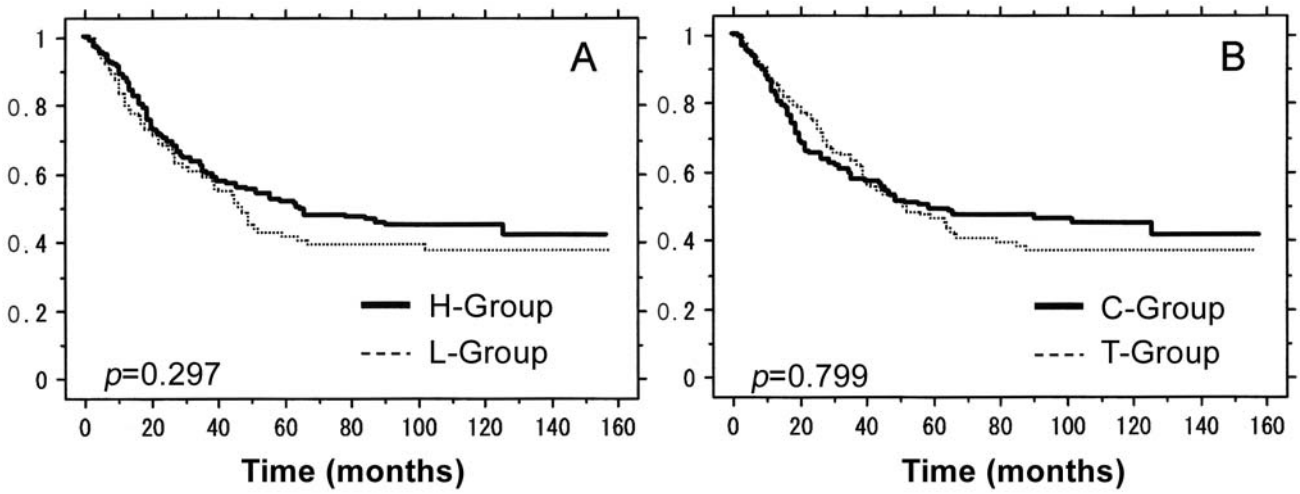

Figure 2. *A: Survival curves of all patients distributed into two groups according to TS functional classification. TS genotypes of 2R/2R, 2R/3C, or 3C/3C were classified as L-group and those of 2R/3G, 3G/3C, or 3G/3G as H-group. B: Survival curves of all patients distributed into two groups according to the genotype of MTHFR C677T. MTHFR C677T genotypes of T/T or C/T were classified as T-group and that of C/C as C-group.*

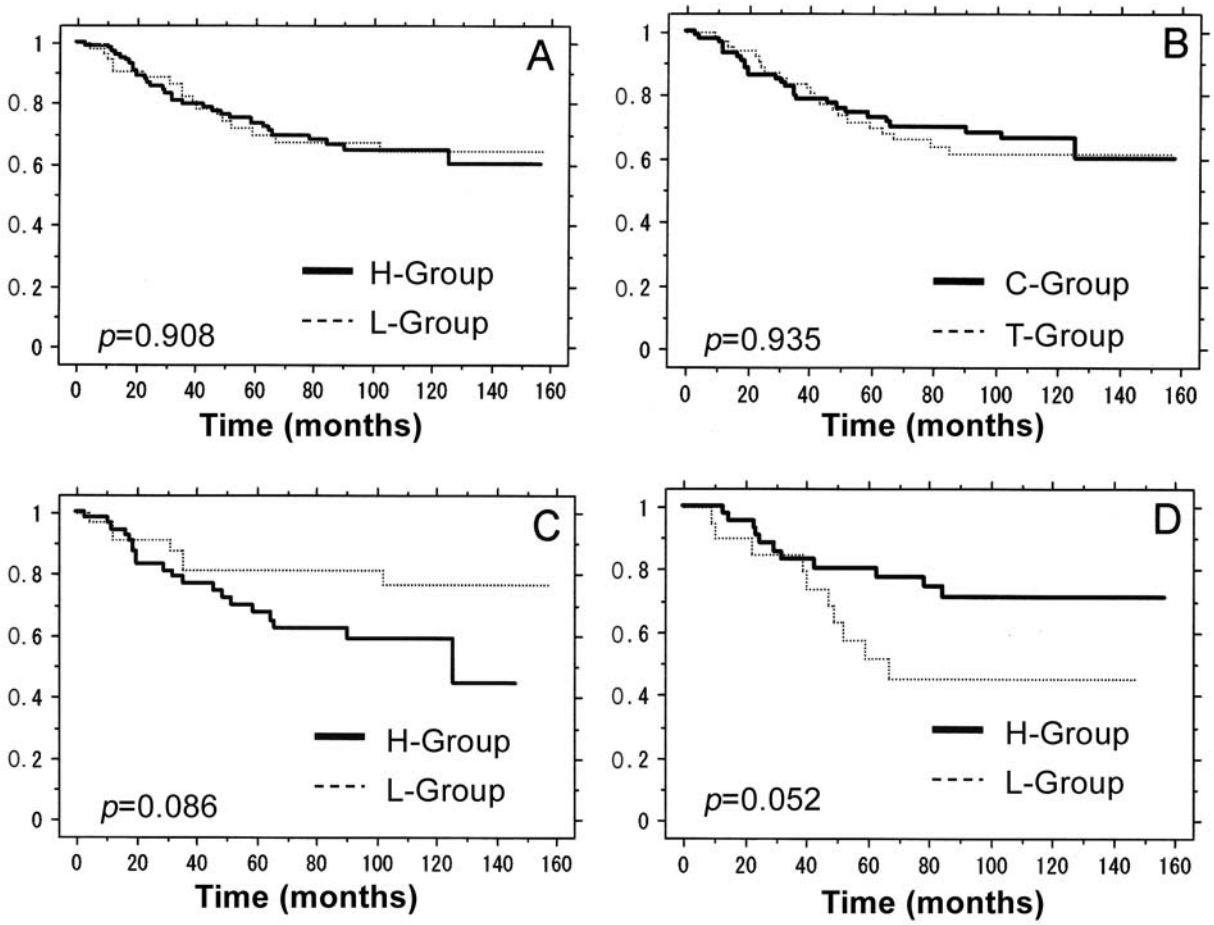

Figure 3. *Survival analysis of stage I patient. Neither the TS (A) nor the MTHFR genotype (B) correlated with patients' prognosis of stage I. C: The patients in the TS L-group survived longer than those in the H-group when limiting to stage I and the MTHFR C-group (p=0.086). D: The patients in the TS H-group survived longer than those in the L-group when limiting to stage I and the MTHFR T-group (p=0.052).*

the 2R genotype (16). They observed no relationship between the VNTR genotype and prognosis, suggesting that the single factor, *i.e.*, TS VNTR, may be insufficient as a prognostic factor. Therefore, we employed a multiple genotyping approach in the present study to investigate the newly identified TS SNP and well-characterized MTHFR C677T, in addition to TS VNTR.

To our knowledge, this is the first report of the investigation of multiple genetic polymorphisms of the TS VNTR, TS SNP and the MTHFR C677T in NSCLC. Although no relationships were found between the genotypes and clinicopathological features, we observed the unique association of TS functional type and MTHFR genotype with prognosis in stage I NSCLC. In this specific disease stage, the patients in the TS H-group survived longer than those in the L-group when limited to the MTHFR T-group, while on the contrary, the patients in the TS L-group survived longer than those in the H-group when limited to the MTHFR C-group. These results suggested that the MTHFR genotype might interfere with the effect of TS functional type on the tumor biology of NSCLC. As TS and MTHFR compete for CH_2FH_4 in folate metabolism, this gene-gene interaction can be attributed to folate status in NSCLC tissue. We reported that the MTHFR C677T genotype is associated with folate intermediates, CH_2FH_4 or tetrahydrofolate, in colorectal cancer tissue in both the Japanese (20) and Australian population (21). Therefore, the prognostic role of the TS functional type may be dependent on the FI level in NSCLC, assuming that the association between the MTHFR genotype and folate intermediates is consistent among different organs. Further studies, especially regarding folate status in NSCLC tissue, are required to validate the prognostic role of TS and to elucidate the implications of folate metabolism in the genotype-phenotype associations.

In the present study, we examined NSCLC of all stages to explore the clinical roles of the TS and MTHFR genotypes. However, the prognostic roles of these genotypes were observed only in stage I NSCLC and some association was more evident in stage Ib. NSCLC is a malignancy with poor prognosis, with a 5-year survival rate of 38 to 70% in stage Ib (22, 23). The relatively poor prognosis in stage Ib patients has led to a demand for prognostic prediction and selection of patients who require adjuvant therapy. The prognostic roles of TS and MTHFR in stage Ib meet this demand and warrant further investigation in this specific patient sub-group. In addition to the stage sub-group, studies of histologically homogeneous sub-groups may be required. Adenocarcinoma and squamous carcinoma of NSCLC are quite different tumor types (1). The role of folate metabolism in cancer biology has been demonstrated mainly in colorectal cancers, most of which are adenocarcinomas. Therefore, future studies of both stage Ib and adenocarcinoma should be performed. We observed no associations of the TS or MTHFR genotypes with clinicopathological features and prognosis in the patients with stage Ib and adenocarcinoma in the present study (data not shown), probably due to the small number of patients in this sub-group.

In conclusion, we investigated the relationships between the TS VNTR, TS SNP and MTHFR C677T in NSCLC, and found an association between the genotypes and prognosis of stage I patients. The TS and MTHFR genotypes are prognostic factors in NSCLC, where gene-gene interactions between the genotypes may be present. These results warrant further validation and investigation of the genotypes of folate-metabolizing enzymes with regard to prognosis in NSCLC patients.

References

1 Nakagawa T, Otake Y, Yanagihara K, Miyahara R, Ishikawa S, Fukushima M, Wada H and Tanaka F: Expression of thymidylate synthase is correlated with proliferative activity in non-small cell lung cancer (NSCLC). Lung Cancer *43*: 145-149, 2004.

2 Allegra C: Thymidylate synthase levels: prognostic, predictive, or both?, J Clin Oncol *20*: 1711-1713, 2002.

3 Danenberg PV: Thymidilate synthase – a target enzyme in cancer chemotherapy. Biochem Biophy Acta *473*: 73-92, 1977.

4 Danenberg PV, Malli H and Swenson S: Thymidylate synthase inhibitors. Semin Oncol *26*: 621-631, 1999.

5 Nakagawa T, Tanaka F, Otake Y, Yanagihara K, Miyahara R, Matsuoka K, Takata T, Yamada T, Fukushima M and Wada H: Prognostic value of thymidylate synthase expression in patients with p-stage I adenocarcinoma of the lung. Lung Cancer *35*: 165-170, 2002.

6 Horie N, Aiba H, Oguro K, Hojo H and Takeishi K: Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase. Cell Struct Funct *20*: 191-197, 1995.

7 Kawakami K, Omura K, Kanehira E and Watanabe Y: Polymorphic tandem repeats in the thymidylate synthase gene is associated with its protein expression in human gastrointestinal cancers. Anticancer Res *19*: 3249-3252, 1999.

8 Kawakami K, Salonga D, Park JM, Danenberg KD, Uetake H, Brabender J, Omura K, Watanabe G and Danenberg PV: Different lengths of a polymorphic repeat sequence in the thymidylate synthase gene affect translational efficiency but not its gene expression. Clin Cancer Res *7*: 4096-4101, 2001.

9 Ishida Y, Kawakami K, Tanaka Y, Kanehira E, Ohmura K and Watanabe G: Association of thymidylate synthase gene polymorphism with its mRNA and protein expression and with prognosis in gastric cancer. Anticancer Res *22*: 2805-2810, 2002.

10 Iacopetta B, Grieu F, Joseph D and Elsaleh H: A polymorphism in the enhancer region of the thymidylate synthase promoter influences the survival of colorectal cancer patients treated with 5-fluorouracil. Br J Cancer *85*: 827-830, 2001.

11 Kawakami K and Watanabe G: Identification and functional analysis of single nucleotide polymorphism in the tandem repeat sequence of thymidylate synthase gene. Cancer Res *63*: 6004-6007, 2003.

12 Mandola MV, Stoehlmacher J, Muller-Weeks S, Cesarone G, Yu MC, Lenz HJ and Ladner RD: A novel single nucleotide polymorphism within the 5'tandem repeat polymorphism of the thymidylate synthase gene abolishes USF-1 binding and alters transcriptional activity. Cancer Res *63*: 2898-2904, 2003.
13 Pullarkat ST, Stoehlmacher J, Ghaderi V, Xiong Y-P, Ingles SA, Sherrod A, Warren A, Tsao-Wei D, Groshen S and Lenz H-J: Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy. Pharmacogenomics J *1*: 65-70, 2001.
14 Villafranca E, Okruzhnov Y, Dominguez MA, Garcia-Foncillas J, Azinovic I, Martinez E, Illarramendi JJ, Arias F, Martinez Monge R, Salgado E, Angeletti S and Brugarolas A: Polymorphisms of the repeated sequences in the enhancer region of the thymidylate synthase gene promoter may predict downstaging after preoperative chemoradiation in rectal cancer. J Clin Oncol *19*: 1779-1786, 2001.
15 Etienne MC, Chazal M, Laurent-Puig P, Magne N, Rosty C, Formento JL, Francoual M, Formento P, Renee N, Chamorey E, Bourgeon A, Seitz JF, Delpero JR, Letoublon C, Pezet D and Milano G: Prognostic value of tumoral thymidylate synthase and p53 in metastatic colorectal cancer patients receiving fluorouracil-based chemotherapy: phenotypic and genotypic analyses. J Clin Oncol *20*: 2832-2843, 2002
16 Shintani Y, Ohta M, Hirabayashi H, Tanaka H, Iuchi K, Nakagawa K, Maeda H, Kido T, Miyoshi S and Matsuda H: New prognostic indicator for non-small-cell lung cancer, quantitation of thymidylate synthase by real-time reverse transcription polymerase chain reaction. Int J Cancer *104*: 790-795, 2003.
17 Trihn BN, Ong CN, Coetzee GA, Yu MC and Laird PW: Thymidylate synthase: a novel genetic determinant of plasma homocysteine and folate levels. Hum Genet *111*: 299-302, 2002.
18 Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJH, Heijer M, Kluijtmans LAJ, Heuvel LP and Rozen R: A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet *10*: 111-113, 1995.
19 Shen H, Spitz MR, Wang LE, Hong WK and Wei Q: Polymorphisms of methylene-tetrahydrofolate reductase and risk of lung cancer. Cancer Epidemiol Biomarkers Prev *10*: 397-401, 2001.
20 Kawakami K, Omura K, Kanehira E and Watanabe Y: Methylenetetrahydrofolate reductase polymorphism is associated with folate pool in gastrointestinal cancer tissue. Anticancer Res *21*: 285-290, 2001.
21 Kawakami K, Ruszkiewicz A, Bennett G, Moore J, Watanabe G and Iacopetta B: The folate pool in colorectal cancers is associated with DNA hypermethylation and with a polymorphism in methylenetetrahydrofolate reductase. Clin Cancer Res *9*: 5860-5865, 2003.
22 Mountain CF: Revisions in the international system for staging lung cancer. Chest *111*: 1710-1717, 1997.
23 Iwasaki A, Shirakusa T, Shiraishi T and Yamamoto S: Results of video-assisted thoracic surgery for stage I/II non-small cell lung cancer. Eur J Cardiothorac Surg *26*: 158-164, 2004.

Received March 9, 2005
Accepted June 29, 2005