20 Xio S, Li D, Vijg J, Sugarbaker DJ, Corson JM, Fletcher JA. Codeletion of p15 and p16 in primary malignant mesothelioma. *Oncogene* 1995; **11**: 511–15.
21 Sekido Y, Pass HI, Bader S *et al.* Neurofibromatosis type 2 (NF2) gene is somatically mutated in mesothelioma but not in lung cancer. *Cancer Res* 1995; **55**: 1227–31.
22 Bianchi AB, Mitsunaga SI, Cheng JQ *et al.* High frequency of inactivating mutations in the neurofibromatosis type 2 gene (NF2) in primary malignant mesotheliomas. *Proc Natl Acad Sci USA* 1995; **92**: 10854–8.
23 Murthy SS, Testa JR. Asbestos, chromosomal deletions, and tumor suppressor gene alterations in human malignant mesothlioma. *J Cell Physiol* 1999; **180**: 150–7.
24 Jaurand MC, Fleury-Feith J. Pathogenesis of malignant pleural mesothelioma. *Respirology* 2005; **10**: 2–8.
25 Metcalf RA, Welsh JA, Bennett WP *et al.* p53 and Kirsten-ras mutations in human mesothelioma cell lines. *Cancer Res* 1992; **52**: 2610–15.
26 Papp T, Schipper H, Pemsel H *et al.* Mutational analysis of N-ras, p53, 16INK4a, p14ARF and CDK4 genes in primary human malignant mesotheliomas. *Int J Oncol* 2001; **18**: 425–33.
27 Kumar K, Rahman Q, Schipper H, Matschegewski C, Schiffmann D, Papp T. Mutational analysis of 9 different tumour-associated genes in human malignant mesothelioma cell lines. *Oncol Rep* 2005; **14**: 743–50.
28 Usami N, Fukui T, Kondo M *et al.* Establishment and characterization of four malignant pleural mesothelioma cell lines from Japanese patients. *Cancer Sci* 2006; **97**: 387–94.
29 Kivipensas P, Bjorkqvist AM, Karhu R *et al.* Gains and losses of DNA sequences in malignant mesothelioma by comparative genomic hybridization. *Cancer Genet Cytogenet* 1996; **89**: 7–13.
30 Bjorkqvist AM, Tammilehto L, Anttila S, Mattson K, Knuutila S. Recurrent DNA copy number changes in 1q, 4q, 6q, 9p, 13q, 14q and 22q detected by comparative genomic hybridization in malignant mesothelioma. *Br J Cancer* 1997; **75**: 523–7.
31 Bjorkqvist AM, Tammilehto L, Nordling S *et al.* Comparison of DNA copy number changes in malignant mesothelioma, adenocarcinoma and large-cell anaplastic carcinoma of the lung. *Br J Cancer* 1998; **77**: 260–9.
32 Balsara BR, Bell DW, Sonoda G *et al.* Comparative genomic hybridization and loss of heterozygosity analyses identify a common region of deletion at 15q11.1-15 in human malignant mesothelioma. *Cancer Res* 1999; **59**: 450–4.
33 De Rienzo A, Testa JR. Recent advances in the molecular analysis of human malignant mesothelioma. *Clin Ther* 2000; **151**: 433–8.
34 Krismann M, Müller KM, Jaworska M, Johnen G. Molecular cytogenetic differences between histological subtypes of malignant mesotheliomas: DNA cytometry and comparative genomic hybridization of 90 cases. *J Pathol* 2002; **197**: 363–71.
35 Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
36 Tagawa H, Karnan S, Suzuki R *et al.* Genome-wide array-based CGH for mantle cell lymphoma: identification of homozygous deletions of the proapoptotic gene BIM. *Oncogene* 2005; **24**: 1348–58.
37 Pinkel D, Segraves R, Sudar D *et al.* High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. *Nat Genet* 1998; **20**: 207–11.
38 Ota A, Tagawa H, Karnan S *et al.* Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. *Cancer Res* 2004; **64**: 3087–95.
39 Karnan S, Tsuzuki S, Kiyoi H *et al.* Genomewide array-based comparative genomic hybridization analysis of acute promyelocytic leukemia. *Genes Chromosomes Cancer* 2006; **45**: 420–5.
40 Tagawa H, Tsuzuki S, Suzuki R *et al.* Genome-wide array-based comparative genomic hybridization of diffuse large B-cell lymphoma: comparison between CD5-positive and CD5-negative cases. *Cancer Res* 2004; **64**: 5948–55.
41 Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 2002; **30**: e36.
42 Heintz NH, Janssen YM, Mossman BT. Persistent induction of *c-fos* and *c-jun* expression by asbestos. *Proc Natl Acad Sci USA* 1993; **90**: 3299–303.
43 Shivapurkar N, Virmani AK, Wistuba II *et al.* Deletions of chromosome 4 at multiple sites are frequent in malignant mesothelioma and small cell lung carcinoma. *Clin Cancer Res* 1999; **5**: 17–23.
44 Bjorkqvist AM, Wolf M, Nordling S *et al.* Deletions at 14q in malignant mesothelioma detected by microsatellite marker analysis. *Br J Cancer* 1999; **81**: 1111–5.
45 Sandhu H, Dehnen W, Roller M, Abel J, Unfried K. mRNA expression patterns in different stages of asbestos-induced carcinogenesis in rats. *Carcinogenesis* 2000; **21**: 1023–9.
46 Knuuttila A, Jee KJ, Taskinen E, Wolff H, Knuutila S, Anttila S. Spindle cell tumours of the pleura: a clinical, histological and comparative genomic hybridization analysis of 14 cases. *Virchows Arch* 2006; **448**: 135–41.
47 Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. *Biochim Biophys Acta* 1991; **1072**: 129–57.
48 Lin DW, Coleman IM, Hawley S *et al.* Influence of surgical manipulation on prostate gene expression: implications for molecular correlates of treatment effects and disease prognosis. *J Clin Oncol* 2006; **24**: 3763–70.
49 Klabatsa A, Sheaff MT, Steele JP, Evans MT, Rudd RM, Fennell DA. Expression and prognostic significance of hypoxia-inducible factor 1α (HIF-1α) in malignant pleural mesothelioma (MPM). *Lung Cancer* 2006; **51**: 53–9.
50 Omasa T. Gene amplification and its application in cell and tissue engineering. *J Biosci Bioeng* 2002; **94**: 600–5.
51 Sasaki S, Kitagawa Y, Sekido Y *et al.* Molecular processes of chromosome 9p21 deletions in human cancers. *Oncogene* 2003; **22**: 3792–8.
52 Florl AR, Schulz WA. Peculiar structure and location of 9p21 homozygous deletion breakpoints in human cancer cells. *Genes Chromosomes Cancer* 2003; **23**: 141–8.
53 Raschke S, Balz V, Efferth T, Schulz WA, Florl AR. Homozygous deletions of CDKN2A caused by alternative mechanisms in various human cancer cell lines. *Genes Chromosomes Cancer* 2005; **42**: 58–67.
54 Suzuki M, Toyooka S, Shivapurkar N *et al.* Aberrant methylation profile of human malignant mesotheliomas and its relationship to SV40 infection. *Oncogene* 2005; **24**: 1302–8.
55 Shigemitsu K, Sekido Y, Usami N *et al.* Genetic alteration of the β-catenin gene (CTNNB1) in human lung cancer and malignant mesothelioma and identification of a new 3p21.3 homozygous deletion. *Oncogene* 2001; **20**: 4249–57.
56 Usami N, Sekido Y, Maeda O *et al.* Beta-catenin inhibits cell growth of a malignant mesothelioma cell line, NCI-H28, with a 3p21.3 homozygous deletion. *Oncogene* 2003; **22**: 7923–30.

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Genotype-Based Methods for Anticipating Gemcitabine-Related Severe Toxicities May Lead to False-Negative Results

TO THE EDITOR: In their recently published clinical study, Sugiyama et al[1] investigated the effects of cytidine deaminase (CDA) genetic polymorphisms on gemcitabine toxicities and altered pharmacokinetics. They conclude, from the observation of a single Japanese patient with the nonsynonimous mutation 208G > A (Ala70Thr) and displaying an abnormal gemcitabine pharmacokinetic profile resulting in subsequent neutropenia, that haplotype *3 harboring the 208 G more than A single nucleotide polymorphism (SNP) could be associated with the occurrence of severe toxicities after gemcitabine administration, and possibly, in combination with other chemotherapy regimens. Such a patient with severe toxicities was actually, repeatedly selected out of a group of five,[2] and then 256[1] carcinoma patients for whom linkage disequilibrium and haplotype analyses were performed in relation to CDA activities, gemcitabine pharmacokinetics analyses, and toxicity monitoring. Little correlation was evidenced among the various diplotype groups, the pharmacokinetic parameters of gemcitabine, and the occurrence of severe toxicities, other than the *3/*3 diplotype recorded in the single patient. Surprisingly, little impact was also reported between CDA activities and gemcitabine exposure levels, an observation contradictory to the pharmacokinetics of this drug,[3] and no data on a possible relationship between CDA phenotypic status and gemcitabine-related toxicities was reported. Finally, although Sugiyama et al claimed that plasma CDA activities correlated well with the CDA genotypes, it was not clear by their data whether the difference was statistically significant, apart from the homozygous *3 carrier.

At our institute, we have phenotyped CDA activity and performed genetic screening, including of the 208G > A mutation reported by Sugiyama et al, in 80 cancer patients (70 white, nine African, and one Asian patient) treated with gemcitabine alone or as part of combinational therapies with platinum derivatives or capecitabine. Four (5%) of 80 patients displayed severe, hematologic toxicities (eg, higher than grade 3 by the National Cancer Institute Common Toxicity Criteria), including a lethal one.[4] We found that all four of these patients with severe toxicities had markedly lower CDA activities (mean deficiency, −75%) than those recorded in the 76 patients showing good gemcitabine tolerance. This observation strongly suggests that CDA downregulation was a culprit for increased toxicities with gemcitabine, including, for the first time, in the toxic-death case we reported. Conversely to what was reported by Sugiyama et al, genotypic screening at our institute failed to identify genetic polymorphisms associated with the occurrence of toxicities, since for instance, none of our four toxic patients exhibited the 208G > A (Ala70Thr) mutation. This observation is fully consistent with other studies describing controversies regarding genotype-to-phenotype associations with CDA,[5,6] much likely due to the genetic and epigenetic regulations of the *CDA* gene that remain to be elucidated, and to the possible influence of ethnical origin in the relevance of particular single nucleotide polymorphisms.[7] Taken together, in total contradiction with the Sugiyama study, our own experience strongly suggests that genotypic approaches are probably insufficient to identify patients at risk of gemcitabine toxicity, with an elevated risk of precluding the right diagnostic. Conversely, phenotype-based methods seem to be a safer strategy for ensuring a better outcome in the handling of gemcitabine, a major drug used extensively in clinical oncology.

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REFERENCES

1. Sugiyama E, Kaniwa N, Kim SR, et al: Pharmacokinetics of gemcitabine in Japanese cancer patients: The impact of a cytidine deaminase polymorphism. J Clin Oncol 25:32-42, 2007
2. Yonemori K, Ueno H, Okusaka T, et al: Severe drug toxicity associated with a single-nucleotide polymorphism of the cytidine deaminase gene in a Japanese cancer patient treated with gemcitabine plus cisplatin. Clin Cancer Res 11:2620-2624, 2005
3. Abbruzzese JL, Grunewald R, Weeks RA, et al: A phase I clinical, plasma, and cellular pharmacology of gemcitabine. J Clin Oncol 9:491-498, 1991
4. Mercier C, Raynal C, Dahan L, et al: Toxic death case in a patient undergoing gemcitabine-based chemotherapy in relation with cytidine deaminase down regulation. Pharmacogenetics 17:841-844, 2007
5. Kirch HC, Schroder J, Hoppe H, et al: Recombinant gene products of two natural variants of the human cytidine deaminase gene confer different deamination rates of cytarabine in vitro. Exp Hematol 26:421-425, 1998
6. Schroder JK, Kirch C, Seeber S, et al: Structural and functional analysis of the cytidine deaminase gene in patients with acute myeloid leukaemia. Br J Haematol 103:1096-1103, 1998
7. Gilbert JA, Salavaggione OE, Ji Y, et al: Gemcitabine pharmacogenomics: Cytidine deaminase and deoxycytidylate deaminase gene resequencing and functional genomics. Clin Cancer Res 12:1794-1803, 2006

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IN REPLY: We appreciate the comments raised by Mercier et al and the opportunity to respond to them. We agree that the reduced intracellular CDA level is one of the major factors increasing gemcitabine-mediated toxicities. We also recognize that the genotyping based on *CDA* 208G>A (Ala70Thr) itself gives false-negative results with respect to the prediction of hematological toxicities (Table 7 in our article[1]), as is often the case with genotyping. Thus, phenotype-based methods are useful for identification of patients at a higher risk toward gemcitabine-mediated toxicities. However, as far as Japanese patients are concerned, the genetic method is fairly useful for predicting severe toxicities of gemcitabine because *CDA* 208G>A, a tagging SNP of haplotype *CDA*3*, is one of the factors that reduce CDA activity as clearly demonstrated by us.[1]

According to the letter by Mercier et al, four patients displayed severe hematologic toxicities (> grade 3) without any associations with *CDA* genotypes in their study. Their observations are quite reasonable from the following points: *CDA* 208G>A has not been detected in white people, and its allele frequency is relatively low in other populations (probably variable within African populations[2,3]; only nine Africans and one Asian were included in their study); all other genetic polymorphisms that we detected, including *CDA* 79A>C (**2*, Lys27Gln),[4,5] failed to show any significant associations with altered pharmacokinetics and toxicities of gemcitabine and plasma CDA activity.[1] Therefore, we consider that, in white people, no validated genotype is currently available for predicting gemcitabine toxicities.

Mercier et al pointed out that little correlation was evident among the various diplotype groups, the pharmacokinetic parameters of gemcitabine, and the occurrence of severe toxicities, other than the **3/*3* diplotype recorded in the single patient. However, as presented in our article,[1] significant differences were observed between **3/*1* and **1/*1* for pharmacokinetic parameters (our Fig 2), and the incidences of grade ≥ 3 or grade 4 neutropenia in the combined chemotherapies with fluorouracil or platinum-containing drugs were mostly higher in the non-**3/*3* patients than in the non-**3*/non-**3* patients (Table 7). Our Figures 3A (gemcitabine as a substrate) and 3B (cytidine as a substrate) show that when plasma CDA activities of the **3/*1* and **3/*2* patients were compared with those of the **1/*1* patients by Dunn's multiple comparison test, statistically significant differences were obtained ($P < .001$ and <0.05 for **3/*1* and **3/*2* groups, respectively, in Fig 3A; $P < .001$ for **3/*1* group in Fig 3B; P values were not provided in our report).[1]

In order to reply to the comments by Mercier et al, we re-evaluated the association between grade 4 neutropenia and gemcitabine area under the curve (AUC) or CDA activity (one patient with an extremely high level was excluded) either for the monotherapy or the combined therapy (fluorouracil, carboplatin, or cisplatin) group by the Mann-Whitney test. The median values of AUC were higher in the grade 4 group than in the grade ≤ 3 group (Δ, +9% for the monotherapy; Δ, +30% for the combined therapy), and the median values of plasma CDA levels were lower in the grade 4 group than in the grade ≤ 3 group (Δ, −29% for the monotherapy; Δ, −40% for the combined therapy). Both the increase in AUC and decrease in plasma CDA activity observed in the grade 4 group who received the combined therapies were mainly attributable to the **3*-bearing patients. Appropriate cutoff values could not be set for both AUC and plasma CDA activity to effectively screen grade 4 neutropenia since the median values of the two patient groups were not sufficiently different in our hands. Notably, these biomarkers successfully identified the patient who encountered life-threatening toxicities, because he had **3/*3* and showed extremely high AUC and low plasma CDA activity. As for the relationship between plasma CDA activities and AUC values (gemcitabine exposure levels), a moderate but statistically significant correlation was obtained ($r = -0.30$; $P = .0009$). It was reported that CDA released from damaged neutrophils diffuses into blood, and thus CDA activity in the blood is considered to be one of the markers of inflammatory diseases.[5] It must be noted that pretreatment neutrophil counts also showed a moderate correlation with CDA activity ($r = 0.37$; $P < .0001$; gemcitabine used as a substrate). Moreover, aging and sex influence on the pharmacokinetic parameters of gemcitabine.[1] Therefore, it is not surprising that very strong correlations were not obtained between plasma CDA activity and the pharmacokinetic parameters of gemcitabine.

Taken together, both predictive genotype (**3*) and phenotype markers, gemcitabine AUC and plasma CDA activity, could predict grade 4 neutropenia, but with some false-negative cases and with increased false-positive cases for AUC and plasma CDA. At least, *CDA* 208G>A is a useful marker to predict gemcitabine toxicities in Japanese and probably East Asians.

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REFERENCES

1. Sugiyama E, Kaniwa N, Kim S-R, et al: Pharmacokinetics of gemcitabine in Japanese cancer patients: The impact of a cytidine deaminase polymorphism. J Clin Oncol 25:32-42, 2007

2. Gilbert JA, Salavaggione OE, Ji Y, et al: Gemcitabine pharmacogenomics: Cytidine deaminase and deoxycytidylate deaminase gene resequencing and functional genomics. Clin Cancer Res 12:1794-1803, 2006

3. Fukunaga AK, Marsh S, Murry DJ, et al: Identification and analysis of single-nucleotide polymorphisms in the gemcitabine pharmacologic pathway. Pharmacogenomics J 4:307-314, 2004

4. Kirch HC, Schroder J, Hoppe H, et al: Recombinant gene products of two natural variants of the human cytidine deaminase gene confer different deamination rates of cytarabine in vitro. Exp Hematol 26:421-425, 1998

5. Thompson PW, Jones DD, Currey HLF: Cytidine deaminase activity as a measure of acute inflammation in rheumatoid arthritis. Annals Rheum Dis 45:9-14, 1986

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Hypofractionated Stereotactic Radiotherapy (HypoFXSRT) for Stage I Non-small Cell Lung Cancer: Updated Results of 257 Patients in a Japanese Multi-institutional Study

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Introduction: Hypofractionated stereotactic radiotherapy (HypoFXSRT) has recently been used for the treatment of small lung tumors. We retrospectively analyzed the treatment outcome of HypoFXSRT for stage I non-small cell lung cancer (NSCLC) treated in a Japanese multi-institutional study.

Methods: This is a retrospective study to review 257 patients with stage I NSCLC (median age, 74 years: 164 T1N0M0, 93 T2N0M0) were treated with HypoFXSRT alone at 14 institutions. Stereotactic three-dimensional treatment was performed using noncoplanar dynamic arcs or multiple static ports. A total dose of 18 to 75 Gy at the isocenter was administered in one to 22 fractions. The median calculated biological effective dose (BED) was 111 Gy (range, 57–180 Gy) based on $\alpha/\beta = 10$.

Results: During follow-up (median, 38 months), pulmonary complications of above grade 2 arose in 14 patients (5.4%). Local progression occurred in 36 patients (14.0%), and the local recurrence rate was 8.4% for a BED of 100 Gy or more compared with 42.9% for less than 100 Gy ($p < 0.001$). The 5-year overall survival rate of medically operable patients was 70.8% among those treated with a BED of 100 Gy or more compared with 30.2% among those treated with less than 100 Gy ($p < 0.05$).

Conclusions: Although this is a retrospective study, HypoFXSRT with a BED of less than 180 Gy was almost safe for stage I NSCLC, and the local control and overall survival rates in 5 years with a BED of 100 Gy or more were superior to the reported results for conventional radiotherapy. For all treatment methods and schedules, the local control and survival rates were better with a BED of 100 Gy or more compared with less than 100 Gy. HypoFXSRT is feasible for curative treatment of patients with stage I NSCLC.

Key Words: Stereotactic radiotherapy, Non-small cell lung cancer, Stage I, Hypofractionated.

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In Japan, due to the routine use of computed tomography (CT), detection of early-stage lung cancer is increasing. For patients with stage I (T1 or 2, N0, M0) non-small cell lung cancer (NSCLC), full lobar or greater surgical resection and regional lymphadenectomy is the standard treatment choice; the local control rates exceed 80% and the overall 5-year survival rates surpass 50%.[1] However, surgical resection is often not feasible or involves a high risk for lung cancer patients with tobacco-related pulmonary illnesses, severe cardiovascular disease, or other medical conditions. Moreover, a small proportion of the patients who are fit for surgery may refuse it for personal reasons.

Radiotherapy (RT) can offer a therapeutic alternative in these cases, but the outcome with conventional RT is unsatisfactory.[2] The reason for the poor survival with conventional RT is thought to be that the dose of conventional RT is too low to control the local tumor. To give a higher dose to the tumor without increasing the adverse effects, hypofractionated high-dose stereotactic RT (HypoFXSRT) has recently been used to treat small cell lung tumors, particularly in Japan.[3–6] Although the optimal treatment technique and

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schedule of HypoFXSRT for stage I NSCLC are unknown, the nationwide number of Japanese patients with stage I NSCLC who are treated with small-volume stereotactic RT (SRT) has increased rapidly.

Therefore, it is meaningful to investigate the results of SRT for stage I NSCLC from many institutions, even in a retrospective manner, despite the large differences in treatment protocols. Previously, we reported the result of a Japanese multi-institutional review of 300 patients with stage I NSCLC treated with SRT.[7] We concluded that SRT with a biological effective dose (BED) of less than 150 Gy is effective for the curative treatment of patients with stage I NSCLC and that the local control and survival rates are better with a BED of 100 Gy or more compared with less than 100 Gy.

The survival rates in selected medically operable patients with a BED of 100 Gy or more were promising and potentially comparable with those of surgery. These results for SRT were encouraging for stage I NSCLC patients; however, the 300 subjects in that report included 17 patients irradiated with comparatively small fractions (<4 Gy) and 26 patients irradiated in combination with conventional RT. This article presents the results for patients irradiated with HypoFXSRT alone in a multi-institutional study. In this study, we compared the reported results for surgery and conventional RT with those for HypoFXSRT.

PATIENTS AND METHODS

Eligibility Criteria

This was a retrospective study to review patients who were treated by HypoFXSRT for their stage I NSCLC in 14 different hospitals in Japan.

All the patients enrolled in this study satisfied the following eligibility criteria: identification of T1N0M0 or T2N0M0 primary lung cancer on chest and abdominal CT, bronchoscopy, bone scintigraphy, or brain magnetic resonance imaging; histological confirmation of NSCLC; performance status of 2 or less according to the World Health Organization (WHO) guidelines; and an inoperable tumor due to a poor medical condition or refusal to undergo surgery.

No restrictions were imposed concerning the locations of eligible tumors, irrespective of whether they were located adjacent to a major bronchus, blood vessel, chest wall, or the esophagus. Patients were informed of the concept, methodology, and rationale of this treatment, which was performed in accordance with the 1983 revision of the Declaration of Helsinki.

Patient Characteristics

The patient pretreatment characteristics are summarized in Table 1. From April 1995 to March 2004, a total of 257 patients with primary NSCLC was treated using high-dose HypoFXSRT in the following 14 institutions: Hokkaido University, Kyoto University, Cancer Institute Hospital, Tokyo Metropolitan Komagome Hospital, Kitasato University, Tohoku University, Hiroshima University, Tokyo Metropolitan Hiroo Hospital, Sapporo Medical University, Institute of Biomedical Research and Innovation, International Medical Center of Japan, Tenri Hospital, Kitami Red Cross Hospital, and University of Yamanashi. Of the 257 patients, 158 were considered medically inoperable mainly because of chronic pulmonary disease, advanced age, or other chronic illness. The remaining 99 patients were considered medically operable, but had refused surgery or had been advised to select HypoFXSRT by medical oncologists.

TABLE 1. Patient Pretreatment Characteristics

Total cases: 257
Age: 39–92 yr (median, 74)
Performance status: PS 0, 109; PS 1, 103; PS 2, 39; PS 3, 6
Pulmonary chronic disease: 168 positive, 89 negative
Histology: 111 squamous cell, 120 adenocarcinoma, 26 other
Stage: 164 IA, 93 IB
Tumor diameter: 7–58 mm (median, 28)
Medical operability: 158 inoperable, 99 operable

Treatment Methods

All the patients were irradiated using stereotactic techniques. For the purposes of this study, all the hypofractionated stereotactic techniques met five requirements: reproducibility of the isocenter of 5 mm or less, as confirmed for every fraction; slice thickness on CT of 3 mm or less for three-dimensional (3-D) treatment planning; irradiation with multiple noncoplanar static ports or dynamic arcs; dose per fraction size more than 4 Gy; and a total treatment period of fewer than 25 days. Details of the techniques and instruments used to achieve SRT in the 14 institutions were summarized in a previous report.[7] The clinical target volume (CTV) marginally exceeded the gross target volume (GTV) by 0 to 5 mm. The planning target volume (PTV) comprised the CTV, a 2- to 5-mm internal margin and a 0–5-mm safety margin. A high dose was concentrated on the tumor-bearing area, while sparing the surrounding normal lung tissues using SRT. The irradiation schedules also differed among the institutions. The number of fractions ranged between 1 and 14, with single doses of 4.4 to 35 Gy. A total dose of 30 to 84 Gy at the isocenter was administered with 6- or 4-MV x-rays within 20% heterogeneity in the PTV dose. No chemotherapy was administered before or during RT.

To compare the effects of various treatment protocols with different fraction sizes and total doses, the BED was used in a linear-quadratic model.[8] Here, the BED was defined as $nd(1 + d/\alpha/\beta)$, with gray units, where n is the fractionation number, d is the daily dose, and α/β is assumed to be 10 for tumors. The BED was not corrected with values for the tumor doubling time or treatment term. In this study, the BED was calculated at the isocenter. The median BED was 111.0 Gy (range, 57.6–180.0). The BED was 100 Gy or more in 215 patients and less than 100 Gy in 42 patients. The median BED for the less than 100 Gy and 100 Gy or more subgroups was 79.6 Gy (range, 57.6–98.6) and 117.0 Gy (range, 100.0–180.0), respectively.

Dose constraints were set for the spinal cord only. The BED limit for the spinal cord was 80 Gy (α/β was assumed to be 2 Gy for chronic spinal cord toxicity).

Evaluation

The objectives of this study were to retrospectively evaluate the toxicity, local control rate, and survival rate according to the BED. All patients underwent follow-up examinations by radiation oncologists. The first examination took place 4 weeks after treatment, and patients were subsequently seen every 1 to 3 months. Tumor response was evaluated using the Response Evaluation Criteria in Solid Tumors by CT.[9] Chest CT (slice thickness, 2–5 mm) was usually obtained every 3 months for the first year and repeated every 4 to 6 months thereafter. A complete response (CR) indicated that the tumor had disappeared completely or was replaced by fibrotic tissue. A partial response (PR) was defined as a 30% or more reduction in the maximum cross-sectional diameter. It was difficult to distinguish between residual tumor tissue and radiation fibrosis. Any suspicious confusing residual density after RT was considered evidence of a PR, so the actual CR rate might have been higher than that given here. Local recurrence was considered to have taken place only when enlargement of the local tumor continued for more than 6 months on follow-up CT. Two radiation oncologists interpreted the CT findings. The absence of local recurrence was defined as locally controlled disease. Lung, esophagus, bone marrow, and skin were evaluated using version 2 of the National Cancer Institute–Common Toxicity Criteria (NCI-CTC).

Statistical Analysis

The local recurrence rates in the two groups were compared with the χ^2 test. The BED among patient groups at each pulmonary toxicity grade was compared using the Kruskal-Wallis test. The cumulative local control and survival curves were calculated and drawn applying the Kaplan-Meier algorithms with day of treatment as the starting point. Subgroups were compared using log-rank statistics. Values of $p < 0.05$ were considered statistically significant. Statistical calculations were conducted using version 5.0 StatView software (SAS Institute, Cary, NC).

RESULTS

All the patients completed the treatment with no particular complaints. The median duration of follow-up for all patients was 38 months (range, 2–128).

Local Tumor Response

Of the 257 patients evaluated using CT, CR was achieved in 66 (25.7%) and PR in 157 (61.1%). The overall response rate (CR + PR) was 86.8%. The overall response rates for tumors with a BED of 100 Gy or more ($n = 215$) or less than 100 Gy ($n = 42$) were 87.5% and 86.7% in 3 years (?), respectively. A typical case of a T1 tumor after HypoFXSRT is shown in Figure 1.

Toxicity

Symptomatic radiation-induced pulmonary complications (NCI-CTC criteria grade >1) were noted in 28 patients (10.9%). Pulmonary fibrosis or emphysema before treatment was observed in 25 (89%) of the 28 patients with pulmonary complications above grade 1. Pulmonary complications of NCI-CTC criteria above grade 2 were noted in only 14

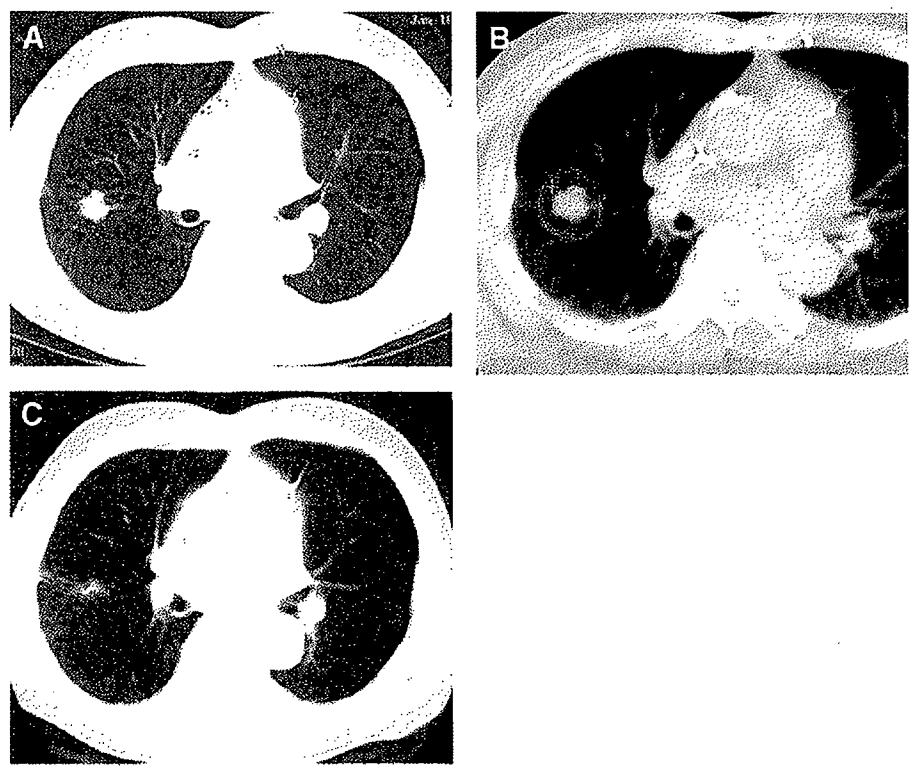

FIGURE 1. A typical example involving SRT for a 76-year-old man with T1N0 adenocarcinoma. He was treated with HypoFXSRT. (*A*) Before hypofractionated stereotactic radiotherapy (HypoFXSRT). (*B*) The calculated dose distribution. The isocenter dose was 75 Gy/10 fractions/5 days, and the tumor was fully enclosed with the 90% dose line. (C) Twelve months after HypoFXSRT, a scarred tumor is rated as a partial response.

TABLE 2. Recurrence Rate According to the BED and Stage

	Total cases	BED <100 Gy	BED ≥100 Gy	p	Stage IA	Stage IB	p
Local tumor	36/257 (14.0%)	18/42 (42.9%)	18/215 (8.4%)	<0.01	20/164 (12.2%)	16/93 (17.2%)	0.21
Regional nodal metastasis	29/257 (11.3%)	9/42 (21.4%)	20/215 (9.3%)	<0.05	17/164 (10.4%)	12/93 (12.9%)	0.54
Distant metastasis	51/257 (19.8%)	11/42 (26.2%)	40/215 (18.6%)	0.3	32/164 (19.5%)	19/93 (20.4%)	0.87

BED, biological effective dose.

patients (5.4%). The pulmonary symptoms resolved in most patients without steroid therapy, but six patients who had very poor respiratory function or severe pulmonary fibrosis before irradiation needed continuous oxygen. Chronic segmental bronchitis and wall thickening causing atelectasis in the peripheral lung was observed in one patient (0.4%). Transient grade 3 esophagitis was observed in two patients (0.8%) with tumors adjacent to the esophagus. Grade 3 or 4 dermatitis was observed in three patients (1.2%) with tumors adjacent to the chest wall. Rib fracture adjacent to the tumor was found in four patients (1.6%). No vascular, cardiac, or bone marrow complications had been encountered as of the last follow-up.

Recurrence

The recurrence rates of local, regional nodal, and distant lesions according to the BED and stage are listed in Table 2. The local recurrence rate was significantly lower for a BED of 100 Gy or more compared with a BED of less than 100 Gy (8.4 versus 42.9%, $p < 0.01$). For greater BED subgroups, the local recurrence rate was 11.8% for a BED of 120 Gy or more ($n = 93$) and 8.1% for a BED of 140 Gy or more ($n = 37$). The local recurrence rates for adenocarcinoma and squamous cell carcinoma were 13.3% (16/120) and 17.1% (19/111), respectively in 3 years. The cumulative local control rate curves according to BED subgroup are shown in Figure 2. The 5 (3? according to Table 2)-year local control rates of the BED of 100 Gy or more and less than 100 Gy subgroups were 84.2% (95% confidence interval [CI]: 77.7%–90.8%) and 36.5% (95% CI: 10.4%–62.6%), respectively. According to subgroup analysis, stage IB patients had a significantly higher rate of local recurrence than stage IA patients. The nodal and distant recurrence rates were almost identical in the stage IA and IB subgroups.

In the patients with regional nodal recurrence, nodal failures overlapped local failure in 3.1%, distant metastases in 3.9%, or both in 0.8% of the patients. Isolated local, nodal, and distant recurrences were observed in 8.6%, 5.1%, and 13.6% of the patients, respectively.

Survival

The overall 3- and 5-year survival rates for all patients were 56.8% (95% CI: 50.2%–63.5%) and 47.2% (95% CI: 38.7%–53.5%), respectively. The cause-specific 3- and 5-year survival rates were 76.9% (95% CI: 70.6%–83.2%) and 73.2% (95% CI: 66.1%–80.2%), respectively. The overall survival rates differed significantly according to medical operability, with intercurrent death in 36.8% of inoperable patients and 10.3% of operable patients. The overall 5-year survival rates of medically operable and inoperable patients (Figure 3) were 64.8% (95% CI: 53.6%–75.9%) and 35.0% (95% CI: 25.9%–44.1%), respectively. The overall survival rates according to the BED in all patients differed significantly between the BED of less than 100 Gy and 100 Gy or more subgroups. The overall 5-year survival rates of the BED 100 Gy or more and less than 100 Gy subgroups were 53.9% (95% CI: 46.0%–61.8%) and 19.7% (95% CI: 5.9%–33.4%), respectively. For the subgroup of medically operable patients with a BED of 100 Gy or more, the 3- and 5-year overall survival rates were 80.4% (95% CI: 71.0%–89.7%) and 70.8% (95% CI: 59.3%–82.2%), respectively (Figure 2). The overall 5-year survival rate according to stage in the operable patients irradiated with a BED of 100 Gy or more was 72.3%

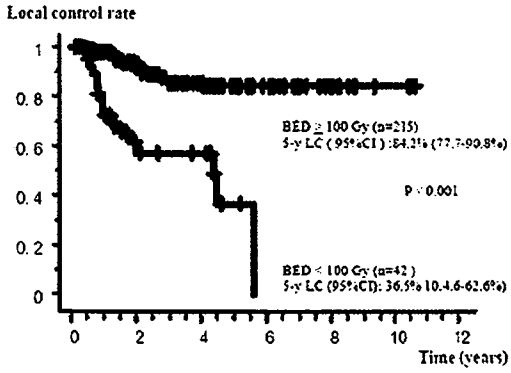

FIGURE 2. Cumulative local control rate according to the biological effective dose (BED). LC, local control rate; CI, confidence interval.

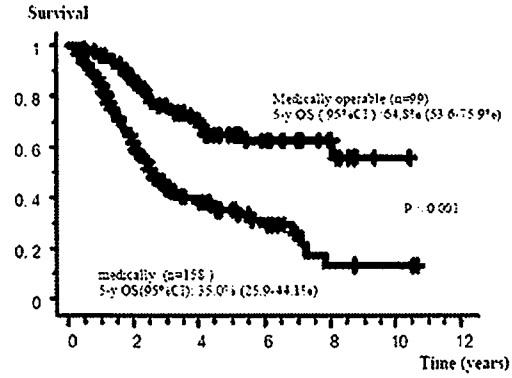

FIGURE 3. Overall survival rate according to medical operability. OS, overall survival rate; CI, confidence interval.

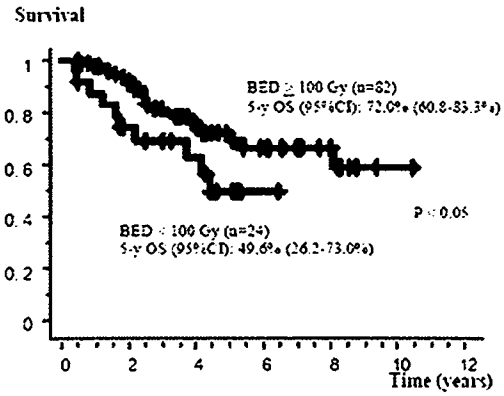

FIGURE 4. Overall survival rate in operable patients according to the biological effective dose (BED). OS, overall survival rate; CI, confidence interval.

(95% CI: 59.1%–85.6%) for stage IA and 65.9% (95% CI: 43.0%–88.9%) for stage IB patients (Figure 4).

Reproducibility of the Data Among Institutions

Table 3 compares the irradiation method and results for three major institutions enrolled in this study. These institutions used a BED of 100 Gy or more. The local control and 3-year survival rates were almost identical.

DISCUSSION

At present, surgery is the standard treatment for stage I NSCLC. RT is offered to patients who are unsuitable for surgery because of medical problems and to patients who refuse surgery. Most information on the results of RT for stage I NSCLC is based on retrospective studies of RT-treated inoperable NSCLC cases. Therefore, the role of RT for stage I NSCLC, as a curative modality, has not yet been established.

Qiao et al. summarized 18 papers on stage I NSCLC treated with conventional RT alone published between 1988 and 2000.[10] Local recurrence was the most common reason for treatment failure of stage I NSCLC with conventional RT, but the frequency of recurrence varied considerably according the report (between 6.4% and 70%). The 3-year recurrence rate was approximately 60%,[11–13] with a median time to relapse that ranged from 21 to 30 months.[12,14,15] Generally, smaller tumor size, low T stage, and increased dose had a favorable impact on local control, and increased local control was followed by increased survival.[14,16] However, the overall treatment results were disappointing. The median survival in these studies ranged from 18 to 33 months. The 3- and 5-year overall survival rates were 34 ± 9% and 21 ± 8% (mean ± 1 SE), respectively. The cause-specific survival rates at 3 and 5 years were 39 ± 10% and 25 ± 9% (mean ± 1 SE), respectively. Regarding treatment toxicity, severe (grade 3 or above) radiation esophagitis[14] and pneumonitis[11] occurred in 4.1% and 6.1% of the cases, respectively. Better local control may be achieved when the total dose is increased,[15,16] and a trend has been growing toward seeking better local control by increasing the BED[13–15] for a relatively limited span of doses (BED 59–76 Gy). Dose escalation has been the focus of developmental therapeutic strategies for inoperable stage I NSCLC to improve local control and survival.

Mehta et al.[17] provided a detailed theoretical analysis regarding the responses of NSCLC to RT and a rationale for dose escalation. They concluded that a greater BED irradiated during a short period must be given to gain local control of lung cancers. Giving a higher dose to the tumor without increasing the adverse effects was shown to be possible using the SRT technique; this is now feasible due to the technological progress that allows increasing the accuracy of localization to the tumor-bearing area using various imaging tools. SRT can also reduce the overall treatment time substantially, from several weeks for conventional RT to a few days, offering an important advantage to the patient.

After Uematsu et al.[18] reported a landmark study on SRT for stage I NSCLC using a CT-linac system, SRT has been actively investigated for stage I NSCLC in Japan and the United States. In the reports listed in Table 4,[3–6,19–21] the local control rates of primary lung cancer with SRT ranged from 87% to 97% when the BED exceeded 100 Gy. Uematsu et al.[3] showed excellent survival rates for medically operable patients, approximating those for full lobar surgical resection; however, they studied only a few patients, and it is not known whether the result is reproducible. Table 5 compares the results of Uematsu et al.[3] with the HypoFXSRT results presented here. These results suggest that the local control and survival rates of HypoFXSRT for stage I NSCLC are promising and reproducible when the BED exceeds 100 Gy.

In Japan, we consider a BED greater than 100 Gy to be a satisfactory dose for HypoFXSRT of stage I NSCLC, with a local control rate better than 85%, and a further dose escalation study is not necessary for tumors smaller than 4 cm in diameter. Conversely, in the United States, Timmerman et al.[22] concluded that 60 Gy in three fractions (BED = 180 Gy) is the proper dose, and they adopted this dose and fraction protocol for their prospective study. We need to observe the

TABLE 3. Comparison of the Irradiation Methods and Results for Three Major Institutions

Institution	No. of Patients	Total Isocenter Dose (Gy)	Single Isocenter Dose (Gy)	BED (Gy)	Median Follow-up (mo)	Local Failure, %	5-yr Overall Survival, %
Kyoto	42	48	12	106	40	3	64
Cancer Institute	30	50–62.5	10–12.5	100–141	25	4	77
Kitami	27	50–60	7.5–10	100–105	71	4	63

BED, biologically effective dose (α/β = 10) recalculated at the isocenter.

TABLE 4. Reports of Stereotactic Radiotherapy for Stage I Non-small Cell Lung Cancer

Author (ref.)	No. of Patients	Total Dose* (Gy)	Single Dose* (Gy)	BED† (Gy)	Median Follow-up (mo)	Local Progression, %	3-yr Overall Survival, %
Uematsu et al.[3]	50	72	7.2	124	60	6	66
Nagata et al.[4]	42	48	12	106	52	3	82
Fukumoto et al.[5]	17	48–60	6–7.5	99–137	24	6	NA
Onishi et al.[6]	28	72	7.2	124	24	8	75
Hof et al.[19]	10	19–26	19–26	55–94	15	20	NA
McGarry et al.[20]	47	75	25	263	15	13	NA
Wulf et al.[21]	12	26–57	19–26	94–165	11	5%	NA

BED, biologically effective dose; NA, not assessed.
*Stereotactic radiotherapy dose is calculated at the isocenter.
†BED (α/β = 10) recalculated at the isocenter

results of ongoing phase II studies on SRT for stage I NSCLC conducted in Japan (12 Gy × 4 = 48 Gy prescribed to the isocenter) and the United States (20 Gy × 3 = 60 Gy prescribed to cover 95% of the PTV).

The 5-year overall survival rate for medically operable patients with HypoFXSRT is encouraging (Table 6). Representative 5-year overall survival rates for clinical stage IA and IB with surgery range from 61% to 72% and 40% to 50%, respectively.[23–25] According to our data, the survival rate for SRT was not worse than that for large surgical series. Furthermore, concerning toxicity, approximately 3% of patients died as a result of surgery, and chronic morbidity occurs in 10% to 15% of patients after surgery.[26] HypoFXSRT is much less invasive than surgery, and it is postulated that SRT will become a major treatment choice for stage I NSCLC, at least for medically inoperable patients.

TABLE 5. Comparison of the Results between the Multi-institutional Study and the Uematsu et al. Study

	Uematsu et al.[3]	Multi-institutional
Total no. of cases	50	215
T1N0M0	24	141
T2N0M0	26	75
Follow-up duration, mo (median)	22–66 (36)	2–128 (38)
Local control, %	94	90
Regional lymph nodes metastases, %	4	7
Distant metastases, %	14	19
Grade ≥3 toxicity, %	0	3
3-yr overall survival rate, %	66	64
3-yr cause-specific survival rate, %	88	83
5-yr overall survival rate, %	55	55
5-yr cause-specific survival rate, %	81	77
3-yr overall survival rate in operable patients, %	86	82
5-yr overall survival rate in operable patients, %	77	72

TABLE 6. Comparison of 5-Year Overall Survival Rate between Stereotactic Radiotherapy and Surgery

Stage	Author			
	Mountain[23]*	Naruke et al.[24]*	Shirakusa and Koybayashi[25]*	Onishi†
IA	61%	71%	72%	72%
IB	40%	44%	50%	66%

*Surgery.
†HypoFXSRT presented here

Multi-institutional phase II studies of SRT for stage I NSCLC have been started in Japan (JCOG0403)[27] and the United States (RTOG0236).[28] However, it will take several years to obtain conclusive results, and an inevitable selection bias exists in comparing SRT with surgical series for patients in retrospective or phase II studies.

Although the differences in techniques and schedules of the institutions enrolled in this study may be large, it is meaningful that a safe, effective BED was suggested because the optimal dose-fraction schedule of SRT for stage I NSCLC is not known. Furthermore, this is the only report that gives the results of SRT for a large number of medically operable stage I NSCLC patients. Based on our excellent SRT results, it is arguable that a phase III study comparing surgery and SRT for medically operable patients is warranted. However, it is very difficult to perform a phase III study because most patients will opt for less invasive therapy such as SRT. We need much more experience and must study more patients with a longer follow-up duration to establish a safe, effective irradiation method that will instill both medical and social confidence in SRT for treatment of stage I NSCLC.

When we compare the results of conventional RT and surgery with those of HypoFXSRT, we conclude that HypoFXSRT has the following benefits for stage I NSCLC. First, HypoFXSRT is a safe and promising treatment modality. Second, the local control and survival rates are superior to those of conventional RT. Third, HypoFXSRT should be a standard of care for medically inoperable patients. Fourth, HypoFXSRT should be randomly compared with surgery for medically operable patients. Finally, we need additional experience with a longer follow-up duration to conclusively validate these points.

REFERENCES

1. Smythe WR. American College of Chest Physicians: treatment of stage I non-small cell lung carcinoma. *Chest* 2003;123:S181–S187.
2. Qiao X, Tullgren O, Lax I, et al. The role of radiotherapy in treatment of stage I non-small cell lung cancer. *Lung Cancer* 2003;41:1–11.
3. Uematsu M, Shioda A, Suda A, et al. Computed tomography-guided frameless stereotactic radiography for stage I non-small-cell lung cancer: 5-year experience. *Int J Radiat Oncol Biol Phys* 2001;51:666–670.
4. Nagata Y, Takayama K, Matsuo Y, et al. Clinical outcomes of a phase I/II study of 48 Gy of stereotactic body radiotherapy in 4 fractions for primary lung cancer using a stereotactic body frame. *Int J Radiat Oncol Biol Phys* 2005;63:1427–1431.
5. Fukumoto S, Shirato H, Shimizu S, et al. Small-volume image-guided radiotherapy using hypofractionated, coplanar, and noncoplanar multiple fields for patients with inoperable stage I nonsmall cell lung carcinomas. *Cancer* 2002;95:1546–1553.
6. Onishi H, Kuriyama K, Komuyama T, et al. Clinical outcomes of stereotactic radiotherapy for stage I non-small cell lung cancer using a novel irradiation technique: patient self-controlled breath-hold and beam switching using a combination of linear accelerator and CT scanner. *Lung Cancer* 2004;45:45–55.
7. Onishi H, Araki T, Shirato H, et al. Stereotactic hypofractionated high-dose irradiation for stage I nonsmall cell lung carcinoma. *Cancer* 2004;101:1623–1631.
8. Yaes RJ, Patel P, Maruyama Y. On using the linear-quadratic model in daily clinical practice. *Int J Radiat Oncol Biol Phys* 1991;20:1353–1362.
9. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 2000;92:205–216.
10. Qiao X, Tullgren O, Lax I, et al. The role of radiotherapy in treatment of stage I non-small cell lung cancer. *Lung Cancer* 2003;41:1–11.
11. Sibley GS. Radiotherapy for patients with medically inoperable stage I nonsmall cell lung carcinoma. *Cancer* 1998;82:433–438.
12. Cheung PC, Mackillop WJ, Dixon P, et al. Involved-field radiotherapy alone for early-stage non-small-cell lung cancer. *Int J Radiat Oncol Biol Phys* 2000;48:703–710.
13. Hayakawa K, Mitsuhashi N, Saito Y, et al. Limited field irradiation for medically inoperable patients with peripheral stage I non-small cell lung cancer. *Lung Cancer* 1999;26:137–142.
14. Jeremic B, Shibamoto Y, Acimovic YL, et al. Hyperfractionated radiotherapy alone for clinical stage I non-small cell lung cancer. *Int. J. Radiat Oncol Biol Phys* 1997;38:521–525.
15. Kaskowitz L, Graham MV, Emami B et al. Radiation therapy alone for stage I non-small cell lung cancer. *Int J Radiat Oncol Biol Phys* 1993;27:517–523.
16. Kupelian PA, Komaki R, Allen P. Prognostic factors in the treatment of node-negative non-small cell lung carcinoma with radiotherapy alone. *Int J Radiat Oncol Biol Phys* 1996;36:607–613.
17. Mehta M, Scrimger R, Mackie R, et al. A new approach to dose escalation in non-small cell lung cancer. *Int J Radiat Oncol Biol Phys* 2001;49:23–33.
18. Uematsu M, Shioda A, Tahara K, et al. Focal, high dose, and fractionated modified stereotactic radiation therapy for lung carcinoma patients: a preliminary experience. *Cancer* 1998;82:1062–1070.
19. Hof H, Herfarth KK, Munter M, et al. Stereotactic single-dose radiotherapy of stage I non-small cell lung cancer. *Int J Radiat Oncol Biol Phys* 2001;49:23–33.
20. McGarry RC, Papiez L, Williams M, et al. Stereotactic body radiotherapy of early-stage non-small cell lung carcinoma: phase I study. *Int J Radiat Oncol Biol Phys* 2005;63:1010–1015.
21. Wulf J, Hadinger U, Oppitz U, et al. Stereotactic radiotherapy for primary lung cancer and pulmonary metastases: a noninvasive treatment approach in medically inoperable patients. *Int J Radiat Oncol Biol Phys* 2004;60:186–96.
22. Timmerman R, Papiez L, McGarry R, et al. External stereotactic radioablation: results of a phase I study in medically inoperable stage I non-small cell lung cancer patients. *Chest* 2003;124:1946–1955.
23. Mountain CF. The international system for staging lung cancer. *Semin Surg Oncol* 2000;18:106–115.
24. Naruke T, Tsuchiya R, Kondo H, et al. Prognosis and survival after resection for bronchogenic carcinoma based on the 1997 TNM-staging classification: the Japanese experience. *Ann Thorac Surg* 2001;71:1759–1764.
25. Shirakusa T, Kobayashi K. Lung cancer in Japan: analysis of lung cancer registry for resected cases in 1994. *Jpn J Lung Cancer* 2002;42:555–562.
26. Deslauriers J, Ginsberg RJ, Dubois P, et al. Current operative morbidity associated with elective surgical resection for lung cancer. *Can J Surg* 1989;32:335–339.
27. http://www.clinicaltrials.gov/ct/show/NCT00238875.
28. http://www.rtog.org/members/protocols/0236/0236.pdf.

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Impact of one-carbon metabolism-related gene polymorphisms on risk of lung cancer in Japan: a case–control study

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There is substantial evidence that the decreased risk of lung cancer with high intake of vegetables and fruits is linked to folate as a specific nutrient. Functional polymorphisms in genes encoding one-carbon metabolism enzymes, methylenetetrahydrofolate reductase (*MTHFR* C677T and A1298C), methionine synthase (*MTR* A2756G), methionine synthase reductase (*MTRR* A66G) and thymidylate synthase, influence folate metabolism and thus might be suspected of impacting on lung cancer risk. We therefore conducted a case–control study with 515 lung cancer cases newly and histologically diagnosed and 1030 age- and sex-matched non-cancer controls to clarify associations with these five polymorphisms according to lung cancer subtype. Gene–environment interactions with smoking and drinking habit and folate consumption were also evaluated by logistic regression analysis. None of the polymorphisms showed any significant impact on lung cancer overall risk by genotype alone, but on histology-based analysis increase in *MTHFR* 677T and 1298C alleles was associated with reduced risk of squamous/small cell carcinoma ($P = 0.029$), especially among heavy smokers ($P = 0.035$), whereas the *MTHFR* 677TT genotype was linked to decreased risk for these subtypes among heavy drinkers (odds ratio = 0.17, 95% confidence interval: 0.03–0.98). In addition, we found interactions between the *MTRR* A66G polymorphism and smoking ($P = 0.015$) and the *MTHFR* A1298C polymorphism and alcohol consumption ($P = 0.025$) for risk of lung cancer overall. In conclusion, the results suggest that *MTHFR* polymorphisms contribute to risk of squamous/small cell carcinomas of the lung, along with possible interactions among folate metabolism-related polymorphisms and smoking/drinking habits. Further evaluation is warranted.

Introduction

Lung cancer, with its four major histological types (adenocarcinoma, squamous cell carcinoma, large cell carcinoma and small cell carcinoma), currently claims >55 000 lives annually in Japan and has become the leading cause of cancer death (1). Despite rapid advances in treatment over recent decades, the prognosis has not greatly improved. Therefore, efforts toward primary prevention in addition to early detection have come under the spotlight.

Abbreviations: CI, confidence interval; FFQ, food frequency questionnaire; 5,10-methylene THF, 5,10-methylenetetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; OR, odds ratio; PCR, polymerase chain reaction; 2R, two repeat; TS, thymidylate synthase; VNTR, variable number of tandem repeat.

Many epidemiological studies have provided evidence that high consumption of vegetables and fruits is associated with a reduced risk of lung cancer (2–4). Folate is one of the constituents found in vegetables and fruits, and dietary folate may be one of the micronutrients that provide protection against lung carcinogenesis (5–7).

Biological functions of folate within so-called 'one-carbon metabolism' are to facilitate *de novo* deoxynucleoside triphosphate synthesis and to provide methyl groups required for intracellular methylation reactions. Folate deficiency is thought to increase the risk of cancer through impaired DNA repair synthesis and disruption of DNA methylation that may lead to proto-oncogene activation (8–10).

Methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR) and thymidylate synthase (TS) play important and interrelated roles in folate metabolism (Figure 1). The MTHFR reduces 5,10-methylenetetrahydrofolate (5,10-methylene THF) to 5-methyl THF, the primary circulating form of folate (11). The TS catalyzes the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate using 5,10-methylene THF (12). The MTHFR product, 5-methyl THF, is the methyl group donor for the remethylation of homocysteine to methionine catalyzed by MTR (13). MTR activity is maintained by MTRR (14). Polymorphisms in the genes for *MTHFR* C677T and A1298C, *MTR* A2756G, *MTRR* A66G and *TS* 28 bp variable number of tandem repeat (VNTR) in the promoter region are known to have functional relevance (15). Thus, they might play roles in the etiology of lung cancer in combination with environmental factors such as folate consumption. Since information for this area of lung cancer is limited (16–22), we conducted the present case–control study, taking tobacco smoking, alcohol drinking and intake of folate into consideration.

Materials and methods

Subjects

The cases were 515 patients who were newly and histologically diagnosed as having lung cancer and not having any earlier history of cancer. Controls ($n = 1030$) were randomly selected and matched by age (±3 years) and sex to cases with a 1:2 case–control ratio from among the 2395 cancer-free individuals. All the subjects were recruited in the framework of the Hospital-based Epidemiologic Research Program at Aichi Cancer Center, as described elsewhere (23,24). In brief, information on lifestyle factors was collected using a self-administered questionnaire, checked by a trained interviewer, from all first-visit out-patients at Aichi Cancer Center Hospital aged 18–79 who were enrolled in Hospital-based Epidemiologic Research Program at Aichi Cancer Center between January 2001 and November 2005. Out-patients were also asked to provide blood samples. Each patient was asked about his or her lifestyle when healthy or before the current symptoms developed. Approximately 95% of eligible subjects complete the questionnaire and 60% provide blood samples. The data were loaded into a Hospital-based Epidemiologic Research Program at Aichi Cancer Center database and routinely linked with the hospital-based cancer registry system to update the data on cancer incidence. All participants gave written informed consent and the study was approved by Institutional Ethical Committee of Aichi Cancer Center.

Genotyping of MTHFR, MTR, MTRR and TS

DNA from each subject was extracted from the buffy coat fraction using BioRobot EZ1 and an EZ1 DNA Blood 350 ml Kit (Qiagen, Tokyo, Japan). The genotyping method was described in our previous reports with the polymerase chain reaction (PCR) TaqMan method using the GeneAmp PCR System 9700 or the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Briefly, for the *MTHFR* C677T (dbSNP ID: rs677) and A1298C (rs1801131), as well as *MTR* A2756G (rs1805087) and *MTRR* A66G (rs1801394) polymorphisms, extracted DNA was amplified with validated probes (assay IDs: C__11975651_10, C__850486_20, C__12005959_10 and C__3068176_10, respectively; Applied Biosystems). The *TS* VNTR polymorphism was defined by PCR using 5′-CGTGGCTCCTGCGTTTCC-3′ and 5′-GAGCCGGCCACAGGCAT-3′ primers. In our laboratory, quality of genotyping is routinely assessed statistically using the Hardy–Weinberg test.

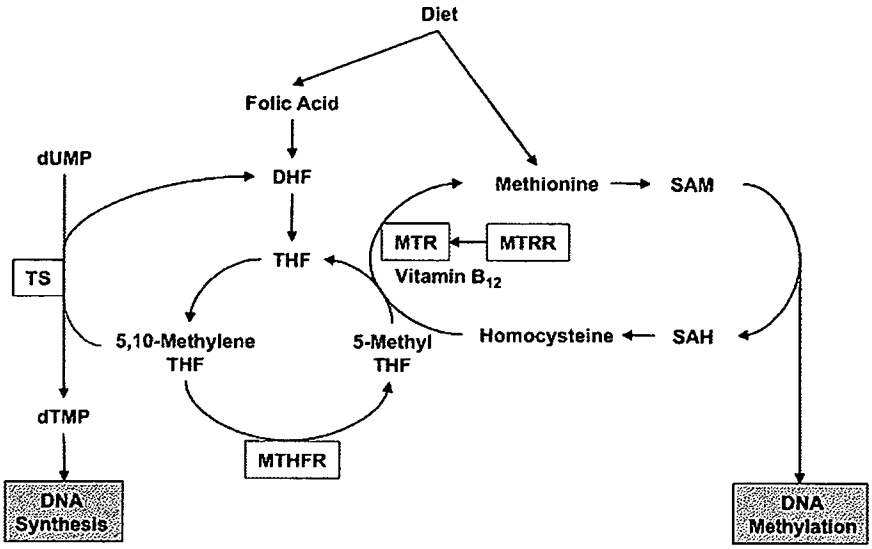

Fig. 1. Overview of folate metabolism. Enzymes with polymorphisms investigated in this study are boxed. THF, tetrahydrofolate; DHF, dihydrofolate; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; SAM, *S*-adenosylmethionine and SAH, *S*-adenosylhomocysteine.

When allelic distributions for controls depart from the Hardy–Weinberg frequency, genotyping is assessed using another method.

Intake assessment for folate and other nutrients

The consumption of folate and other nutrients was determined using a food frequency questionnaire (FFQ), described in detail elsewhere (25,26). Briefly, the FFQ consisted of 47 single food items with frequencies in the eight categories. We estimated the average daily intake of nutrients by multiplying the food intake (in grams) or serving size by the nutrient content per 100 g of food as listed in standard tables of food composition. Consumption of folate and other vitamins from supplements was not considered in total consumption because the questionnaire for multivitamins was not quantitative. Energy-adjusted intake of nutrients was calculated by the residual method (27). The FFQ was validated by referring to a 3-day weighed dietary record as a standard, which showed reproducibility and validity to be acceptable (28). The deattenuated correlation coefficients for energy-adjusted intakes of folate were 0.36 in men and 0.38 in women.

Consumption of tobacco and alcohol

Cumulative smoking dose was evaluated as pack-years, the product of the number of packs consumed per day and years of smoking. Smoking habit was entered for four categories of never, former and current smokers of <40 and ≥40 pack-years. Former smokers were defined as those who quit smoking at least 1 year before the survey. Consumption of each type of beverage (Japanese sake, beer, shochu, whiskey and wine) was determined by the average number of drinks per day, which was then converted into a Japanese sake (rice wine) equivalent. One drink equates to one 'go' (180 ml) of Japanese sake, which contains 23 g of ethanol, equivalent to one large bottle (633 ml) of beer, two shots (60 ml) of whiskey and two and a half glasses of wine (200 ml). One drink of 'shochu' (distilled spirit), which contains 25% ethanol, was rated as 108 ml. Total amount of alcohol consumption was estimated as the summarized amount of pure alcohol consumption (gram per drink) of Japanese sake, beer, shochu, whiskey and wine among current regular drinkers. Drinking habit was entered for four categories of never, former, current moderate and heavy drinkers. Heavy drinkers were defined as those currently drinking alcoholic beverages 5 days or more per week in a daily amount of 46 g (two Japanese drinks) or more, whereas moderate drinkers were defined as those currently consuming less frequently than 5 days/week, in lower amounts, or both. Former drinkers were defined as those who quit drinking at least 1 year before the survey. Former or current smokers and drinkers were categorized as 'smokers' and 'drinkers', respectively.

Statistical analysis

To assess the strength of the associations between polymorphic genes involved in folate metabolism and risk of lung cancer, odds ratio (ORs) with 95% confidence intervals (CIs) were estimated using age- and sex-matched conditional logistic models adjusted for potential confounders. For stratified and interaction analysis by smoking and drinking habit and folate intake, an unconditional logistic regression model was used because the matching was not retained after stratification by smoking and drinking habit and folate intake. Folate and other nutrient intakes were categorized into three groups as: first, second and third tertiles of dietary intake among controls. Potential confounders considered in the multivariate analyses were age, sex, smoking habit (never smokers, former smokers, current smokers of <40 or ≥40 pack-years), drinking habit (never drinkers, former drinkers, moderate drinkers or heavy drinkers), body mass index (<18.5, 18.5–24.9 or ≥25.0), total energy intake (as a continuous variable), dietary carotene intake (μg/day, tertiles), dietary vitamin C intake (mg/day, tertiles), dietary vitamin E intake (mg/day, tertiles), dietary folate intake (μg/day, tertiles), multivitamin use (at least once per week for 1 year or longer: yes or no) and referral pattern (patient's discretion, family recommendation, referral from other clinics, secondary screening after primary screening or others). Missing values for each covariate were treated as an additional category in the variable and were included in the logistic model.

For the histology-based analysis, we combined squamous cell carcinoma and small cell carcinoma, because tumors of these subtypes were small in number and both are consistently more related with smoking as compared with adenocarcinomas. Considering potential effects of two polymorphisms (*MTHFR* C677T and *MTHFR* A1298C) on lung risk, we evaluated associations with their combined genotypes. Trend of genotype was assessed by score test applying score for each genotype (0, homozygous for reference allele or combined reference genotypes; 1, heterozygote or one reference genotype and 2, homozygous non-reference allele or non-reference genotype).

Gene–environment interactions between smoking and drinking habit and folate intake and genotypes in each polymorphism were evaluated under the multiplicative assumption. Products of scores for genotype (0, homozygous; 1, heterozygote and 2, homozygous or 0, referent alleles and 1, non-referent alleles) and smoking habit (0, non-smoker and 1, smoker), drinking habit (0, non-drinker and 1, drinker), folate intake (0, tertile 1 and 1, tertile 2 + 3) or combined smoking–drinking habit (0, non-smoker and non-drinker; 1, smoker and non-drinker or drinker and non-smoker and 2, smoker/drinker) were included as interaction terms. Differences in categorized demographic variables between the cases and controls were tested by the Chi-squared test. Mean values for age and total energy intake were compared for cases and controls by the Student's *t*-test. Accordance with the Hardy–Weinberg equilibrium was checked for controls using the Chi-squared test and the exact *P*-value was used to assess any discrepancies between genotypes and allele frequencies. A *P*-value <0.05 was considered statistically significant. All analyses were performed using STATA version 9 (Stata Corp., College Station, TX).

Results

Data from 515 lung cancer cases, comprising 316 (61.4%) adenocarcinomas, 91 (17.7%) squamous cell carcinomas, 55 (10.7%) small

cell carcinomas, 40 (7.8%) large cell carcinomas and 13 (2.5%) others, and 1030 controls were available for analysis. Table I shows the distribution of cases and controls by background characteristics. Age and sex were appropriately matched. Smoking habits differed to a large extent between cases and controls. The proportion of 40 pack-years or more current smokers in cases was significantly higher than controls. Heavy drinkers in the cases were significantly higher than for the controls. Among cases, the proportion of lower body mass index was higher, consistent with previous study (29). Total energy intake did not differ between cases and controls. Significant lower intake of dietary carotene was found among the cases. For other nutrients lower proportions of the highest intake group among the cases also were found, including for folate, but these were not statistically significant. With regard to referral pattern, referral from other clinics was frequent, whereas patient discretion and secondary screening after primary screening were less common among the case group than the control group.

Table II shows genotype distributions for *MTHFR*, *MTR*, *MTRR* and *TS* and their ORs and 95% CIs for lung cancer risk according to histological subtypes. The genotype frequencies for all the polymorphisms were in accordance with the Hardy–Weinberg law in controls: *MTHFR* C677T (P = 0.17), *MTHFR* A1298C (P = 0.51), *MTR* A2756G (P = 0.17), *MTRR* A66G (P = 0.85) and *TS* VNTR (P = 0.51). On analysis of lung cancer overall, a slightly reduced risk was observed with the *MTHFR* 677TT genotype, but without statistical significance. The genotype frequencies for *TS* VNTR were quite varied; however, two repeat (2R) and three repeat alleles were dominant. The 2R/2R genotype showed decreased risk of lung cancer as compared with the non-2R homozygous, although again this was not significant. On subanalysis according to histological subtypes, the combination of *MTHFR* C677T and A1298C polymorphisms showed a significant decreased risk of squamous/small cell carcinoma among individuals with two or more *MTHFR* 677T and/or 1298C alleles (OR = 0.34, 95% CI: 0.13–0.92, trend P = 0.029), compared with those with *MTHFR* 677CC and 1298AA genotypes. In contrast, none of the polymorphisms showed any significant impact on adenocarcinoma risk.

To further evaluate the impact of *MTHFR* polymorphisms with regard to squamous/small cell carcinoma, we conducted stratified analysis by smoking and drinking habit (Table III). Among heavy drinkers, the *MTHFR* 677TT genotype conferred a significant decreased risk (OR = 0.17, 95% CI: 0.03–0.98, trend P = 0.041). A significant decreased risk among 40 pack-years or more current smokers was observed as number of *MTHFR* 677T or 1298C alleles increased (trend P = 0.035). No clear association was found for lung cancers overall or for adenocarcinomas in the stratified analysis (data not shown).

Table IV shows data for the combinations of gene and environmental factors with reference to lung cancer overall risk. The interaction with smoking was significant for the *MTRR* A66G genotype (P = 0.015). Among non-smokers, risk was reduced with increase in the number of *MTRR* G alleles, whereas a trend for increased risk was observed among smokers. A significant interaction between drinking habits and the *MTHFR* A1298C genotype was found (P = 0.025). These two interactions were especially noteworthy for adenocarcinomas when histology-based analyses were conducted (data not shown). We were not able to analyze the smoking interaction for squamous/small cell due to insufficient number of non-smokers in this category. No obvious interaction was found between folate intake and the polymorphisms.

Considering the possible effects of both tobacco smoking and alcohol drinking on folate, we further examined the impact of four-way combinations of these two factors, folate intake and the polymorphisms on lung cancer risk (Table V). The *MTRR* A66G genotype showed a significant interaction among the subjects with tertiles 2 or 3 of folate intake (P = 0.023). The risk with the *MTRR* 66GG was consistently decreased among non-smoker/non-drinker subjects with adequate folate intake (OR = 0.20, 95% CI: 0.04–0.91).

Table I. Characteristics of cases and controls

	Cases (n = 515), n(%)	Controls (n = 1030), n(%)	P-value
Age			
<50	53 (10.3)	108 (10.5)	
50–59	142 (27.6)	283 (27.5)	
60–69	193 (37.5)	389 (37.8)	
70–79	127 (24.7)	250 (24.3)	1.00
Mean age ± SD	61.9 ± 9.9	61.8 ± 9.8	0.87
Sex			
Male	381 (74.0)	762 (74.0)	
Female	134 (26.0)	268 (26.0)	1.00
Smoking status			
Never	129 (25.0)	401 (38.9)	
Former[a]	111 (21.6)	310 (30.1)	
Current (pack-years)			
0–39	71 (13.8)	149 (14.5)	
≥40	197 (38.3)	161 (15.6)	<0.01
Unknown	7 (1.4)	9 (0.9)	
Drinking status			
Never	196 (38.1)	378 (36.7)	
Former[a]	15 (2.9)	56 (5.4)	
Current			
Moderate[b]	192 (37.3)	454 (44.1)	
Heavy[c]	98 (19.0)	119 (11.6)	<0.01
Unknown	14 (2.7)	23 (2.2)	
BMI			
<18.5	38 (7.4)	55 (5.3)	
18.5–24.9	381 (74.0)	720 (69.9)	
≥25.0	94 (18.3)	249 (24.2)	0.03
Unknown	2 (0.4)	6 (0.6)	
Mean total energy ± SD, kcal/day	1670 ± 372	1677 ± 352	0.73
Carotene (μg/day)			
Tertile 1 (1331.2–2305.9)	200 (38.8)	341 (33.1)	
Tertile 2 (2306.0–3312.6)	149 (28.9)	341 (33.1)	
Tertile 3 (3312.7–12801.4)	158 (30.7)	341 (33.1)	0.04
Unknown	8 (1.6)	7 (0.7)	
Vitamin C (mg/day)			
Tertile 1 (26.8–74.5)	188 (36.5)	342 (33.2)	
Tertile 2 (74.6–102.0)	161 (31.3)	342 (33.2)	
Tertile 3 (102.1–364.5)	159 (30.7)	341 (33.1)	0.15
Unknown	7 (1.4)	5 (0.5)	
Vitamin E (total α-mg/day)			
Tertile 1 (1.5–4.8)	193 (37.5)	342 (33.2)	
Tertile 2 (4.9–6.3)	168 (32.6)	342 (33.2)	
Tertile 3 (6.4–17.1)	151 (29.3)	342 (33.2)	0.29
Unknown	3 (0.6)	4 (0.4)	
Folate intake (μg/day)			
Tertile 1 (139.5–274.5)	191 (37.1)	342 (33.2)	
Tertile 2 (274.6–354.9)	156 (30.3)	342 (33.2)	
Tertile 3 (355.0–1481.0)	162 (31.5)	341 (33.1)	0.18
Unknown	6 (1.2)	5 (0.5)	
Multivitamin use (at least once per week for 1 year or longer)			
Yes	111 (21.6)	253 (24.6)	
No	380 (73.8)	721 (70.0)	0.30
Unknown	24 (4.7)	56 (5.4)	
Referral pattern to our hospital			
Patient's discretion	52 (10.1)	306 (29.7)	
Family recommendation	86 (16.7)	195 (18.9)	
Referral from other clinics	287 (55.7)	300 (29.1)	
Secondary screening after primary screening	83 (16.1)	214 (20.8)	
Others	2 (0.4)	10 (1.0)	<0.01
Unknown	5 (1.0)	5 (0.5)	

SD: standard deviation, BMI: body mass index.
[a]Former smokers and drinkers were defined as subjects who had quit smoking and drinking at least 1 year previously.
[b]Moderate drinker means <46 g ethanol/drink and/or <5 days/week.
[c]Heavy drinker means ≥46 g ethanol/drink and ≥5 days/week.

Table II. *MTHFR*, *MTR*, *MTRR* and *TS* genotype distributions, and ORs for lung cancer according to histology

	All			Adenocarcinoma			Squamous + small cell carcinoma		
	Cases (n = 515), n (%)	Controls (n = 1030), n (%)	ORs[a] (95% CIs)	Cases (n = 316), n (%)	Controls (n = 632), n (%)	ORs[a] (95% CIs)	Cases (n = 146), n (%)	Controls (n = 292), n (%)	ORs[a] (95% CIs)
MTHFR (C677T)									
CC	182 (35.3)	379 (36.8)	1.00 (ref.)	109 (34.5)	237 (37.5)	1.00 (ref.)	54 (37.0)	103 (35.3)	1.00 (ref.)
CT	256 (49.7)	474 (46.0)	1.05 (0.81–1.37)	158 (50.0)	288 (45.6)	1.01 (0.72–1.41)	72 (49.3)	134 (45.9)	0.83 (0.43–1.58)
TT	77 (15.0)	177 (17.2)	0.75 (0.52–1.09)	49 (15.5)	107 (16.9)	0.85 (0.53–1.34)	20 (13.7)	55 (18.8)	0.44 (0.16–1.18)
P_{trend}[b]			0.260			0.567			0.129
MTHFR (A1298C)									
AA	341 (66.2)	652 (63.3)	1.00 (ref.)	210 (66.5)	416 (65.8)	1.00 (ref.)	94 (64.4)	175 (59.9)	1.00 (ref.)
AC	149 (28.9)	322 (31.3)	0.85 (0.65–1.13)	90 (28.5)	189 (29.9)	0.94 (0.67–1.33)	46 (31.5)	99 (33.9)	0.84 (0.42–1.68)
CC	22 (4.3)	45 (4.4)	1.01 (0.56–1.83)	14 (4.4)	22 (3.5)	1.46 (0.68–3.16)	5 (3.4)	14 (4.8)	0.40 (0.07–2.28)
UK[c]	3 (0.6)	11 (1.1)		2 (0.6)	5 (0.8)		1 (0.7)	4 (1.4)	
P_{trend}[b]			0.428			0.822			0.348
MTHFR C677T and A1298C combined Number of Variants									
0	76 (14.8)	174 (16.9)	1.00 (ref.)	43 (13.6)	118 (18.7)	1.00 (ref.)	23 (15.8)	41 (14.0)	1.00 (ref.)
1	273 (53.0)	471 (45.7)	1.19 (0.83–1.71)	171 (54.1)	293 (46.4)	1.46 (0.93–2.27)	76 (52.1)	130 (44.5)	0.52 (0.19–1.40)
≧2	163 (31.7)	374 (36.3)	0.84 (0.58–1.24)	100 (31.6)	216 (34.2)	1.10 (0.68–1.77)	46 (31.5)	117 (40.1)	0.34 (0.13–0.92)
UK[c]	3 (0.6)	11 (1.1)		2 (0.6)	5 (0.8)		1 (0.7)	4 (1.4)	
P_{trend}[b]			0.110			0.819			0.029
MTR (A2756G)									
AA	319 (61.9)	698 (67.8)	1.00 (ref.)	192 (60.8)	423 (66.9)	1.00 (ref.)	100 (68.5)	195 (66.8)	1.00 (ref.)
AG	175 (34.0)	291 (28.3)	1.23 (0.94–1.60)	109 (34.5)	184 (29.1)	1.26 (0.91–1.75)	42 (28.8)	84 (28.8)	0.80 (0.42–1.52)
GG	21 (4.1)	40 (3.9)	1.04 (0.55–2.00)	15 (4.7)	25 (4.0)	1.35 (0.62–2.91)	4 (2.7)	13 (4.5)	0.49 (0.07–3.38)
UK[c]	0 (0)	1 (0.1)							
P_{trend}[b]			0.227			0.146			0.364
MTRR (A66G)									
AA	235 (45.6)	484 (47.0)	1.00 (ref.)	148 (46.8)	294 (46.5)	1.00 (ref.)	63 (43.2)	136 (46.6)	1.00 (ref.)
AG	226 (43.9)	446 (43.3)	1.02 (0.79–1.31)	139 (44.0)	275 (43.5)	0.93 (068–1.28)	64 (43.8)	131 (44.9)	1.18 (0.60–2.31)
GG	54 (10.5)	100 (9.7)	0.96 (0.62–1.47)	29 (9.2)	63 (10.0)	0.91 (0.52–1.58)	19 (13.0)	25 (8.6)	1.11 (0.38–3.21)
P_{trend}[b]			0.939			0.638			0.718
TS VNTR									
Non-2R/non-2R	372 (72.2)	721 (70.0)	1.00 (ref.)	236 (74.7)	434 (68.7)	1.00 (ref.)	101 (69.2)	212 (72.6)	1.00 (ref.)
2R/non-2R	132 (25.6)	278 (27.0)	0.96 (0.73–1.27)	73 (23.1)	181 (28.6)	0.81 (0.57–1.13)	43 (29.5)	69 (23.6)	1.26 (0.61–2.59)
2R/2R	10 (1.9)	31 (3.0)	0.63 (0.29–1.39)	6 (1.9)	17 (2.7)	0.62 (0.22–1.73)	2 (1.4)	11 (3.8)	0.23 (0.03–1.62)
UK[c]	1 (0.2)	0 (0)		1 (0.3)	0 (0)				
P_{trend}[b]			0.394			0.137			0.653

[a]ORs were matched for age and sex and adjusted for smoking habit, drinking habit, body mass index, total energy intake, carotene intake, vitamin C intake, vitamin E intake, multivitamin use and referral pattern to our hospital.

[b]Trend of genotype was assessed by score test applying score for each genotypes (0, homozygous for reference allele or combined reference genotypes; 1, heterozygote or one reference genotype and 2, homozygous non-reference allele or non-reference genotype).

[c]UK denotes genotype unknown.

Table III. Stratification analysis by smoking and drinking habit for the *MTHFR* polymorphisms in squamous/small cell carcinoma

	Smoking status						Drinking status							
	Smokers		0–39 pack-years		40 ≥ pack-years		Non-drinkers		Drinkers		Moderate drinkers		Heavy drinkers	
	Cases/ controls	ORs[a] (95% CIs)	Cases/ controls	ORs[a] (95% CIs)	Cases/ controls	ORs[a] (95% CIs)	Cases/ controls	ORs[a] (95% CIs)	Cases/ controls	ORs[a] (95% CIs)	Cases/ controls	ORs[a] (95% CIs)	Cases/ controls	ORs[a] (95% CIs)
MTHFR (C677T)														
CC	53/229	1.00 (ref.)	7/65	1.00 (ref.)	35/58	1.00 (ref.)	15/143	1.00 (ref.)	39/236	1.00 (ref.)	22/168	1.00 (ref.)	14/38	1.00 (ref.)
CT	72/293	0.94 (0.61–1.45)	10/65	1.83 (0.40–8.47)	41/73	0.85 (0.43–1.67)	23/176	0.89 (0.36–2.21)	49/298	1.08 (0.63–1.84)	26/206	1.02 (0.48–2.16)	14/61	0.37 (0.09–1.63)
TT	20/107	0.81 (0.44–1.51)	4/19	5.63 (0.60–53.25)	11/30	0.50 (0.20–1.29)	3/59	0.52 (0.09–3.00)	17/118	0.90 (0.44–1.84)	8/80	0.85 (0.30–2.43)	5/20	0.17 (0.03–0.98)
P_{trend}[b]		0.528		0.151		0.179		0.519		0.871		0.817		0.041
MTHFR (A1298C)														
AA	93/406	1.00 (ref.)	15/106	1.00 (ref.)	57/97	1.00 (ref.)	29/232	1.00 (ref.)	65/420	1.00 (ref.)	36/293	1.00 (ref.)	18/74	1.00 (ref.)
AC	46/186	1.04 (0.68–1.61)	5/33	0.84 (0.15–4.63)	26/55	0.68 (0.35–1.33)	11/123	0.67 (0.26–1.77)	35/199	1.17 (0.69–2.01)	18/139	0.99 (0.46–2.11)	13/40	3.61 (0.87–14.96)
CC	5/30	0.80 (0.28–2.26)	0/8	NA[d]	4/9	0.84 (0.21–3.31)	1/20	0.30 (0.02–3.82)	4/25	1.05 (0.31–3.51)	2/17	1.03 (0.19–5.74)	2/5	1.05 (0.08–13.61)
UK[c]	1/7		1/2				0/3		1/8		0/5			
P_{trend}[b]		0.916		0.381		0.362		0.263		0.649		0.998		0.300
MTHFR C677T and A1298C combined														
Number of Variants														
0	22/105	1.00 (ref.)	3/37	1.00 (ref.)	15/22	1.00 (ref.)	6/64	1.00 (ref.)	17/110	1.00 (ref.)	13/81	1.00 (ref.)	4/15	1.00 (ref.)
1	76/295	1.08 (0.61–1.92)	11/72	0.63 (0.10–3.98)	47/73	0.97 (0.39–2.43)	28/169	1.02 (0.30–3.50)	48/302	1.13 (0.57–2.27)	22/211	0.57 (0.23–1.44)	17/58	0.93 (0.12–6.96)
≦2	46/222	0.88 (0.48–1.63)	6/38	2.40 (0.30–18.97)	25/66	0.44 (0.17–1.17)	7/142	036 (0.08–1.56)	39/232	1.15 (0.56–2.36)	21/157	0.82 (0.32–2.13)	12/46	0.30 (0.04–2.55)
UK[c]	1/7		1/2				0/3		1/8		0/5			
P_{trend}[b]		0.557		0.397		0.035		0.109		0.751		0.860		0.148

Data were not available in non-smokers because of absence of subjects in this category.

[a]ORs were adjusted for age, sex, smoking habit, drinking habit, body mass index, total energy intake, carotene intake, vitamin C intake, vitamin E intake, folate intake, multivitamin use and referral pattern to our hospital.

[b]Trend of genotype was assessed by score test applying score for each genotypes (0, homozygous for reference allele; 1, heterozygote and 2, homozygous non-reference allele).

[c]UK denotes genotype unknown.

[d]NA indicates not available because of absence of subjects in this category.

Table IV. Interaction between *MTHFR*, *MTR*, *MTRR* and *TS* polymorphisms and smoking and drinking habit and folate intake for lung cancer risk

	Smoking habit			Drinking habit			Folate intake		
	Non-smoker	Smoker	*P* interaction[b]	Non-drinker	Drinker	*P* interaction[b]	Tertile 1 (139.5–274.5 μg/day)	Tertile 2 + 3 (274.6–1481.0 μg/day)	*P* interaction[b]
	ORs[a] (95% CIs)	ORs[a] (95% CIs)		ORs[a] (95% CIs)	ORs[a] (95% CIs)		ORs[a] (95% CIs)	ORs[a] (95% CIs)	
MTHFR (C677T)									
CC	1.00 (ref.)	2.59 (1.61–4.17)		1.00 (ref.)	1.02 (0.67–1.55)		1.00 (ref.)	0.82 (1.34–0.82)	
CT	1.09 (0.69–1.73)	2.84 (1.79–4.52)		1.17 (0.77–1.78)	1.07 (0.72–1.60)		1.03 (1.58–1.03)	0.93 (1.50–0.93)	
TT	0.68 (0.36–1.29)	2.55 (1.48–4.40)	0.430	1.21 (0.68–2.14)	0.74 (0.45–1.23)	0.207	0.98 (1.82–0.98)	0.69 (1.22–0.69)	0.851
MTHFR (A1298C)									
AA	1.00 (ref.)	2.80 (1.88–4.18)		1.00 (ref.)	0.76 (0.56–1.04)		1.00 (ref.)	0.81 (1.24–0.81)	
AC	0.88 (0.55–1.39)	2.61 (1.67–4.07)		0.73 (0.48–1.12)	0.77 (0.53–1.11)		0.73 (1.14–0.73)	0.80 (1.27–0.80)	
CC	1.60 (0.61–4.19)	1.84 (0.85–3.96)	0.464	0.36 (0.12–1.04)	1.20 (0.58–2.47)	0.025	1.72 (4.44–1.72)	0.52 (1.18–0.52)	0.824
MTR (A2756G)									
AA	1.00 (ref.)	3.08 (2.07–4.57)		1.00 (ref.)	0.93 (0.68–1.28)		1.00 (ref.)	0.93 (1.40–0.93)	
AG	1.65 (1.05–2.59)	3.49 (2.26–5.40)		1.55 (1.03–2.33)	1.12 (0.77–1.61)		1.56 (2.40–1.56)	1.08 (1.70–1.08)	
GG	0.90 (0.27–2.94)	3.08 (1.45–6.53)	0.348	0.76 (0.27–2.14)	1.18 (0.54–2.56)	0.798	1.24 (3.45–1.24)	0.85 (1.92–0.85)	0.285
MTRR (A66G)									
AA	1.00 (ref.)	2.04 (1.31–3.16)		1.00 (ref.)	1.21 (0.83–1.76)		1.00 (ref.)	0.73 (1.15–0.73)	
AG	0.70 (0.45–1.09)	2.31 (1.49–3.58)		1.53 (1.02–2.27)	0.90 (0.61–1.32)		0.75 (1.14–0.75)	0.83 (1.31–0.83)	
GG	0.46 (0.20–1.09)	2.60 (1.49–4.56)	0.015	0.88 (0.45–1.73)	1.18 (0.67–2.06)	0.212	1.41 (2.82–1.41)	0.58 (1.09–0.58)	0.907
TS VNTR									
Non-2R/non-2R	1.00 (ref.)	2.62 (1.78–3.85)		1.00 (ref.)	0.93 (0.69–1.26)		1.00 (ref.)	0.88 (1.32–0.88)	
2R/non-2R	0.84 (0.51–1.37)	2.52 (1.64–3.88)		1.01 (0.65–1.55)	0.85 (0.58–1.24)		1.02 (1.60–1.02)	0.80 (1.28–0.80)	
2R/2R	0.60 (0.15–2.36)	1.70 (0.64–4.53)	0.668	0.82 (0.19–3.53)	0.44 (0.17–1.12)	0.550	0.66 (2.62–0.66)	0.48 (1.29–0.48)	0.673

[a]ORs were adjusted for age, sex, smoking habit, drinking habit, body mass index, total energy intake, carotene intake, vitamin C intake, vitamin E intake, folate intake, multivitamin use and referral pattern to our hospital.
[b]Interaction was modeled as a product of smoking habit (0, non-smoker and 1, smoker), drinking habit (0, non-drinker and 1, drinker), folate intake in score (0, tertile 1 and 1, textile 2 + 3) and genotype in score.

Discussion

The present study showed a significant impact of *MTHFR* C677T and *MTHFR* A1298C in combination for risk of the most smoking related subtypes of lung cancer, squamous and small cell carcinomas. Moreover, this effect was prominent among heavy smokers. The *MTHFR* 677TT genotype was inversely associated with squamous/small cell carcinoma risk among heavy drinkers. In combination analysis of smoking, drinking and folate consumption, several potential gene–environment interactions were suggested, between (i) the *MTRR* A66G polymorphism and smoking and (ii) the *MTHFR* A1298C polymorphism and alcohol consumption.

High dietary intake of folate has been found to decrease the risk of lung cancer in several epidemiological studies (5–7). Although our result for folate did not reach statistically significance, the observed trend was accordant with other studies. Two small-sized clinical trials found folate and vitamin B_{12} supplementation to reverse atypia among patients with bronchial squamous metaplasia, a precursor of squamous cell carcinoma of the lung (30,31). One might therefore hypothesize a protective effect of folate on lung cancer, but there are also epidemiological studies providing no support for this concept (32–35). Considering the fact that functional polymorphisms in folate-related genes may contribute to alteration of folate metabolism (15), it is biologically plausible to hypothesize that the polymorphisms or the gene–environment interactions rather than the folate intake alone have the impact on lung cancer risk.

Hitherto, only a few studies have investigated associations between one-carbon metabolism-related gene polymorphisms and lung cancer risk. The *MTHFR* 677TT genotype has been reported to decrease risk of lung cancer in female Caucasians (20), but the results were inconsistent in other case–control studies (17,19). The *MTHFR* 1298CC and *MTRR* 66AG or GG genotypes were associated with significantly increased risk (20,21), whereas *MTR* and *TS* enhancer region polymorphisms in the Caucasians studies demonstrated no link (21,22). Our results of overall analysis added evidence for a null association in this controversial issue. However, of note in this study was the fact that *MTHFR* 677T and/or *MTHFR* 1298C alleles were associated with reduced risk of squamous/small cell carcinomas, especially among heavy smokers and drinkers. It has been shown that subjects with the *MTHFR* 677TT and *MTHFR* 1298CC genotypes have a reduction in enzyme activity compared with the wild-type homozygous, 677CC and 1298AA genotypes (36–38). This would lead to high 5,10-methylene THF concentrations, which may provide more one-carbon groups for thymidylate synthesis, thereby enhancing DNA synthesis and repair ability. Thus, it is biologically reasonable that individuals harboring the *MTHFR* 677T and *MTHFR* 1298C alleles among heavy smokers and drinkers have lower risk of squamous/small cell carcinoma development, given that carcinogenesis is strongly related with the accumulation of DNA damage. To our knowledge, this is the first indication of protective effects of combinations of *MTHFR* polymorphisms for this histologic subtype. These data provide support for the hypothesis of links between one-carbon metabolism and tobacco and alcohol influence on squamous/small cell carcinoma carcinogenesis. Regarding other body sites, our previous study on esophagus cancers, which are almost all squamous cell carcinomas in Japan, demonstrated that the *MTHFR* 677TT had the protective effects among heavy drinkers, consistent with the present study (39).

One difficulty exists in distinguishing effects of smoking and drinking on lung cancer risk. In the present study, of 33 heavy drinkers in squamous/small cell carcinoma cases, 24 (72.7%) cases were heavy smokers, so we may not conclude an independent protective effect of *MTHFR* 677TT genotypes among heavy drinkers, although adjustment for smoking habits was performed. On the other hand, all cases with squamous/small cell carcinomas were smokers except one and 60% (85/142) in this subtype were heavy smokers (40 pack-years or more). Alcohol drinking as well as tobacco smoking is considered to induce DNA damage and resultant modification of nucleotides (40,41). In addition, high intake of alcohol can lead to folate depletion

Table V. Impact of combination of smoking and drinking habit by folate intake and the polymorphisms on lung cancer risk

	Folate intake							
	Tertile 1 (139.5–274.5 μg/day)				Tertile 2 + 3 (274.6–1481.0 μg/day)			
	Non-smoker/non-drinker	Smoker/non-drinker or non-smoker/drinker	Smoker/drinker[a]	*P* interaction[c]	Non-smoker/non-drinker	Smoker/non-drinker or non-smoker/drinker	Smoker/drinke[a]	*P* interaction[c]
	ORs[b] (95% CIs)	ORs[b] (95% CIs)	ORs[b] (95% CIs)		ORs[b] (95% CIs)	ORs[b] (95% CIs)	ORs[b] (95% CIs)	
MTHFR (C677T)								
CC	1.00 (ref.)	1.33 (0.44–3.99)	1.60 (0.58–4.41)		1.00 (ref.)	1.82 (0.90–3.67)	2.89 (1.43–5.84)	
CT + TT	0.61 (0.19–1.98)	1.66 (0.62–4.48)	1.55 (0.58–4.18)	0.763	1.54 (0.83–2.89)	1.48 (0.79–2.80)	2.93 (1.50–5.73)	0.389
MTHFR (A1298C)								
AA	1.00 (ref.)	3.18 (1.20–8.42)	2.78 (1.06–7.27)		1.00 (ref.)	0.97 (0.57–1.65)	1.70 (0.97–3.00)	
AC + CC	1.68 (0.52–5.48)	1.63 (0.55–4.81)	2.33 (0.84–6.47)	0.701	0.56 (0.29–1.08)	1.04 (0.56–1.91)	2.04 (1.10–3.76)	0.078
MTR (A2756G)								
AA	1.00 (ref.)	2.13 (0.90–5.04)	1.95 (0.83–4.62)		1.00 (ref.)	1.11 (0.64–1.92)	2.74 (1.53–4.89)	
AG + GG	1.63 (0.46–5.72)	3.00 (1.12–8.00)	3.28 (1.34–8.02)	0.826	1.38 (0.74–2.55)	2.03 (1.09–3.77)	2.16 (1.15–4.05)	0.069
MTRR (A66G)								
AA + AG	1.00 (ref.)	2.17 (1.00–4.73)	2.27 (1.05–4.91)		1.00 (ref.)	1.14 (0.71–1.82)	1.98 (1.18–3.32)	
GG	3.96 (0.49–32.23)	3.19 (1.02–10.03)	2.77 (0.88–8.78)	0.384	0.20 (0.04–0.91)	0.67 (0.24–1.88)	2.34 (1.12–4.90)	0.023
TS VNTR								
Non-2R/non-2R	1.00 (ref.)	1.75 (0.74–4.16)	2.14 (0.92–4.96)		1.00 (ref.)	0.95 (0.56–1.60)	2.04 (1.17–3.55)	
2R/non-2R + 2R/2R	0.87 (0.24–3.07)	2.38 (0.92–6.18)	1.48 (0.57–3.82)	0.367	0.58 (0.28–1.20)	1.30 (0.71–2.39)	1.65(0.90–3.05)	0.769

[a]Subjects who are both smoker and drinker.
[b]ORs were adjusted for age, sex, body mass index, total energy intake, carotene intake, vitamin C intake, vitamin E intake, multivitamin use and referral pattern to our hospital.
[c]Interaction was modeled as a product of smoking/drinking habit (0, non-smoker/non-drinker; 1, smoker/non-drinker or drinker/non-smoker and 2, smoker/drinker) and genotype in score.

(42). Therefore, it is within expectation that the *MTHFR* 677T allele, associated with high 5,10-methylene THF concentrations, may have the potential to protect against squamous/small cell carcinomas in tobacco consumers drinking large amounts of alcohol.

It was previously reported that lung cancer risk is higher with the *MTRR* 66AG/GG genotypes than the *MTRR* 66AA genotype among former smokers, but this did not extend to never and current smokers (21). Here, interaction between this gene and smoking habit was observed. Furthermore, the *MTRR* GG genotype exhibited a protective effect in low-risk subjects (non-smokers/non-drinkers with adequate folate intake). Several cytogenetic biomarker studies suggested that some polymorphisms involved in metabolic activation/deactivation or in DNA repair have been expected to be of special importance in modulating tobacco and alcohol carcinogen effects (43). A recent study reported a positive association with the modulating effect of the *MTRR* polymorphism on micronucleus frequency in peripheral blood lymphocytes, one of the cytogenetic markers (44), which is probably to increase by smoking (45) and drinking (46). The higher micronucleus frequency recorded in *MTRR* 66GG genotype with respect to AG or AA genotype is suggestive of a role of this polymorphism in modulation of chromosome stability, so that the findings may be consistent with our results. Further studies on the underlying mechanisms of the *MTRR* polymorphism thus appear warranted.

We found an interaction between the drinking habit and *MTHFR* A1298C polymorphisms for lung cancer risk, with decreased risk among non-drinkers. A Caucasian study showed that the *MTHFR* 1298CC genotype elevated risk among both drinkers and non-drinkers but only in women (20). The *MTHFR* A1298C is associated with decreased enzymatic activity (37,38) and would be expected to exert a similar effect to *MTHFR* C677T, with mutant alleles more protective among drinkers (27,39). There is no clear biological explanation for our results, and we cannot rule out the possibility that our observations for *MTHFR* A1298C were due to chance. Replication in a future study is needed.

Several potential limitations of the present study warrant consideration. First, internal validity of this hospital-based study is a potential threat to causal inference. We used non-cancer patients at our hospital as controls, given the likelihood that our cases arose within this population base, but individuals selected randomly from our control population were earlier shown to be similar to the general population in terms of the exposure of interest (47). Equivalence in the genotype distribution for the *MTHFR* C677T polymorphism between our controls and the general population has also been reported (48). To account for variation between cases and controls, we adjusted for referral pattern to our hospital. Second, as with other case–control studies, this study may suffer from recall bias. Although the questionnaires were completed before the diagnosis in our hospital, in some cases, patients referred from other institutions might have known the diagnosis. Third, we used a self-administered questionnaire to evaluate the nutrients intake, including folate. Data obtained from FFQ may not reflect intake as accurately as those from other methods, such as biological markers. We could not find any association with intake of vitamin C and E or folate for lung cancer risk, contrasting with our previous demonstration using the same population of protective effects of vegetables and fruits (4). The estimation of consumption by FFQ may be one possible explanation for this apparent anomaly. However, the reproducibility and validity of the FFQ were acceptable (28). We could not consider consumption of folate from supplements in total consumption, but the proportion of user with folate supplement is very low in Japan (0.1%) (49). Lastly, the limited number of cases, especially in subanalysis, is another factor and replication of our findings in a larger study is warranted.

In conclusion, we observed significant associations between *MTHFR* C677T and combined *MTHFR* C677T/A1298C polymorphisms and squamous/small cell carcinoma risk among heavy smokers and drinkers. Moreover, interactions between *MTRR* polymorphisms and smoking as well as the *MTHFR* A1298C polymorphism and alcohol consumption were also suggested. Our results thus support the hypothesis that folate metabolism-related gene polymorphisms may play a role in the genesis of lung cancer in combination with environmental factors. Replication in large epidemiological studies as well as studies of the mechanisms of the metabolisms is to be recommended.

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References

1. Kuroishi,T. *et al.* (2005) Cancer mortality in Japan. In Tajima,K., Kuroishi,T. and Oshima,A. (eds) *Gann Monograph on Cancer Research* Japan Scientific Societies Press, Tokyo, pp. 1–93.
2. Steinmetz,K.A. *et al.* (1991) Vegetables, fruit, and cancer. I. Epidemiology. *Cancer Causes Control*, **2**, 325–357.
3. Steinmetz,K.A. *et al.* (1991) Vegetables, fruit, and cancer. II. Mechanisms. *Cancer Causes Control*, **2**, 427–442.
4. Takezaki,T. *et al.* (2001) Dietary factors and lung cancer risk in Japanese: with special reference to fish consumption and adenocarcinomas. *Br. J. Cancer*, **84**, 1199–1206.
5. Bandera,E.V. *et al.* (1997) Diet and alcohol consumption and lung cancer risk in the New York State Cohort (United States). *Cancer Causes Control*, **8**, 828–840.
6. Voorrips,L.E. *et al.* (2000) A prospective cohort study on antioxidant and folate intake and male lung cancer risk. *Cancer Epidemiol. Biomarkers Prev.*, **9**, 357–365.
7. Shen,H. *et al.* (2003) Dietary folate intake and lung cancer risk in former smokers: a case-control analysis. *Cancer Epidemiol. Biomarkers Prev.*, **12**, 980–986.
8. Duthie,S.J. (1999) Folic acid deficiency and cancer: mechanisms of DNA instability. *Br. Med. Bull.*, **55**, 578–592.
9. Choi,S.W. *et al.* (2000) Folate and carcinogenesis: an integrated scheme. *J. Nutr.*, **130**, 129–132.
10. Wei,Q. *et al.* (2003) Association between low dietary folate intake and suboptimal cellular DNA repair capacity. *Cancer Epidemiol. Biomarkers Prev.*, **12**, 963–969.
11. Bailey,L.B. *et al.* (1999) Polymorphisms of methylenetetrahydrofolate reductase and other enzymes: metabolic significance, risks and impact on folate requirement. *J. Nutr.*, **129**, 919–922.
12. Radparvar,S. *et al.* (1988) Characteristics of thymidylate synthase purified from a human colon adenocarcinoma. *Arch. Biochem. Biophys.*, **260**, 342–350.
13. Leclerc,D. *et al.* (1996) Human methionine synthase: cDNA cloning and identification of mutations in patients of the cblG complementation group of folate/cobalamin disorders. *Hum. Mol. Genet.*, **5**, 1867–1874.
14. Leclerc,D. *et al.* (1998) Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. *Proc. Natl Acad. Sci. USA*, **95**, 3059–3064.
15. Sharp,L. *et al.* (2004) Polymorphisms in genes involved in folate metabolism and colorectal neoplasia: a HuGE review. *Am. J. Epidemiol.*, **159**, 423–443.
16. Shen,H. *et al.* (2001) Polymorphisms of methylene-tetrahydrofolate reductase and risk of lung cancer: a case-control study. *Cancer Epidemiol. Biomarkers Prev.*, **10**, 397–401.
17. Siemianowicz,K. *et al.* (2003) Methylenetetrahydrofolate reductase gene C677T and A1298C polymorphisms in patients with small cell and non-small cell lung cancer. *Oncol. Rep.*, **10**, 1341–1344.
18. Jeng,Y.L. *et al.* (2003) The methylenetetrahydrofolate reductase 677C–>T polymorphism and lung cancer risk in a Chinese population. *Anticancer Res.*, **23**, 5149–5152.
19. Shen,M. *et al.* (2005) Polymorphisms in folate metabolic genes and lung cancer risk in Xuan Wei, China. *Lung Cancer*, **49**, 299–309.
20. Shi,Q. *et al.* (2005) Sex differences in risk of lung cancer associated with methylene-tetrahydrofolate reductase polymorphisms. *Cancer Epidemiol. Biomarkers Prev.*, **14**, 1477–1484.
21. Shi,Q. *et al.* (2005) Polymorphisms of methionine synthase and methionine synthase reductase and risk of lung cancer: a case-control analysis. *Pharmacogenet. Genomics*, **15**, 547–555.
22. Shi,Q. *et al.* (2005) Case-control analysis of thymidylate synthase polymorphisms and risk of lung cancer. *Carcinogenesis*, **26**, 649–656.
23. Tajima,K. *et al.* (2000) A model of practical cancer prevention for outpatients visiting a hospital: the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC). *Asian Pac. J. Cancer Prev.*, **1**, 35–47.
24. Hamajima,N. *et al.* (2001) Gene-environment interactions and polymorphism studies of cancer risk in the Hospital-based Epidemiologic Research Program at Aichi Cancer Center II (HERPACC-II). *Asian Pac. J. Cancer Prev.*, **2**, 99–107.
25. Tokudome,S. *et al.* (1998) Development of data-based semi-quantitative food frequency questionnaire for dietary studies in middle-aged Japanese. *Jpn. J. Clin. Oncol.*, **28**, 679–687.
26. Tokudome,S. *et al.* (2004) Development of a data-based short food frequency questionnaire for assessing nutrient intake by middle-aged Japanese. *Asian Pac. J. Cancer Prev.*, **5**, 40–43.
27. Ma,J. *et al.* (1997) Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. *Cancer Res.*, **57**, 1098–1102.
28. Tokudome,Y. *et al.* (2005) Relative validity of a short food frequency questionnaire for assessing nutrient intake versus three-day weighed diet records in middle-aged Japanese. *J. Epidemiol.*, **15**, 135–145.
29. Kabat,G.C. *et al.* (1992) Body mass index and lung cancer risk. *Am. J. Epidemiol.*, **135**, 769–774.
30. Heimburger,D.C. *et al.* (1988) Improvement in bronchial squamous metaplasia in smokers treated with folate and vitamin B12. Report of a preliminary randomized, double-blind intervention trial. *JAMA*, **259**, 1525–1530.
31. Saito,M. *et al.* (1994) Chemoprevention effects on bronchial squamous metaplasia by folate and vitamin B12 in heavy smokers. *Chest*, **106**, 496–499.
32. Le Marchand,L. *et al.* (1989) Vegetable consumption and lung cancer risk: a population-based case-control study in Hawaii. *J. Natl Cancer Inst.*, **81**, 1158–1164.
33. Speizer,F.E. *et al.* (1999) Prospective study of smoking, antioxidant intake, and lung cancer in middle-aged women (USA). *Cancer Causes Control*, **10**, 475–482.
34. Hartman,T.J. *et al.* (2001) Association of the B-vitamins pyridoxal 5′-phosphate (B(6)), B(12), and folate with lung cancer risk in older men. *Am. J. Epidemiol.*, **153**, 688–694.
35. Jatoi,A. *et al.* (2001) Folate status among patients with non-small cell lung cancer: a case-control study. *J. Surg. Oncol.*, **77**, 247–252.
36. Frosst,P. *et al.* (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat. Genet.*, **10**, 111–113.
37. Weisberg,I. *et al.* (1998) A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Mol. Genet. Metab.*, **64**, 169–172.
38. van der Put,N.M. *et al.* (1998) A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *Am. J. Hum. Genet.*, **62**, 1044–1051.
39. Yang,C.X. *et al.* (2005) Gene-environment interactions between alcohol drinking and the MTHFR C677T polymorphism impact on esophageal cancer risk: results of a case-control study in Japan. *Carcinogenesis*, **26**, 1285–1290.
40. Church,D.F. *et al.* (1985) Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ. Health Perspect.*, **64**, 111–126.
41. Mufti,S.I. *et al.* (1993) Alcohol-associated generation of oxygen free radicals and tumor promotion. *Alcohol.*, **28**, 621–628.
42. Halsted,C.H. *et al.* (2002) Metabolic interactions of alcohol and folate. *J. Nutr.*, **132**, 2367S–2372S.
43. Norppa,H. (2004) Cytogenetic biomarkers and genetic polymorphisms. *Toxicol. Lett.*, **149**, 309–334.
44. Zijno,A. *et al.* (2003) Folate status, metabolic genotype, and biomarkers of genotoxicity in healthy subjects. *Carcinogenesis*, **24**, 1097–1103.
45. Bonassi,S. *et al.* (2003) Effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the Human MicroNucleus project. *Mutat. Res.*, **543**, 155–166.
46. Iarmarcovai,G. *et al.* (2007) Exposure to genotoxic agents, host factors, and lifestyle influence the number of centromeric signals in micronuclei: a pooled re-analysis. *Mutat. Res.*, **615**, 18–27.
47. Inoue,M. *et al.* (1997) Epidemiological features of first-visit outpatients in Japan: comparison with general population and variation by sex, age, and season. *J. Clin. Epidemiol.*, **50**, 69–77.
48. Yoshimura,K. *et al.* (2003) Allele frequencies of single nucleotide polymorphisms (SNPs) in 40 candidate genes for gene-environment studies on cancer: data from population-based Japanese random samples. *J. Hum. Genet.*, **48**, 654–658.
49. Imai,T. *et al.* (2006) Dietary supplement use by community-living population in Japan: data from the National Institute for Longevity Sciences Longitudinal Study of Aging (NILS-LSA). *J. Epidemiol.*, **16**, 249–260.

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Influence of histological type, smoking history and chemotherapy on survival after first-line therapy in patients with advanced non-small cell lung cancer

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The usual primary endpoint in clinical trials for first-line chemotherapy in advanced non-small cell lung cancer is overall survival. Second-line chemotherapy can also prolong overall survival. Non-smoking history has been associated with a treatment effect for epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) versus placebo for overall survival. We performed a retrospective analysis to identify prognostic factors for progression-free survival and overall survival in patients with advanced non-small cell lung cancer treated with first-line carboplatin/paclitaxel, and to examine the effect of second-line therapy on progression-free survival and overall survival. Ninety-eight patients (median age 61 years, 35 female, 74 adenocarcinoma, 68 smokers, 56 performance status 0) fulfilled our criteria, of which 75 patients (78%) received more than second-line therapy (docetaxel [54%] gefitinib [48%] erlotinib [4%]). For overall survival, smoking history and histology were significant prognostic factors. The 2-year overall survival rates were as follows: smokers, 17%; non-smokers, 52%, $P < 0.0001$; adenocarcinoma, 40%; other 15%, $P = 0.0017$. Multivariate analysis in patients who received second-line therapy showed treatment with EGFR-TKI was an independent predictor of overall survival. Smoking history and adenocarcinoma histology were prognostic factors for an improved outcome with carboplatin/paclitaxel in patients with non-small cell lung cancer. Our study results suggest that the use of EGFR-TKI after first-line treatment may be associated with an improvement in overall survival. (*Cancer Sci* 2007; 98: 226–230)

Lung cancer is the malignant tumor with the highest mortality rates in the world.[1] Approximately 80% of all lung cancer cases are non-small cell lung cancer (NSCLC) and patients with postoperative recurrence or advanced NSCLC may be treated with systemic chemotherapy. Platinum-based chemotherapy is widely used as first-line treatment. Various combination regimens are available – the Four-Arm Cooperative Study (FACS) conducted in Japan between October 2000 and June 2002 did not demonstrate any superiority of three experimental platinum-based regimens (cisplatin/gemcitabine, cisplatin/vinorelbine and carboplatin/paclitaxel) compared with the reference arm of cisplatin/irinotecan.[2,3] However, due to its good tolerability, ease of use and experience in Western countries, carboplatin/paclitaxel is currently the standard first-line chemotherapy for NSCLC in Japan.

Docetaxel has been widely used as second-line therapy for NSCLC in Japan. However, since its approval in July 2002, the use of gefitinib (IRESSA), an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), has been increasing each year. Erlotinib, another EGFR-TKI, which is approved in a number of Western markets has also been used in clinical registration trials in some Japanese medical institutions. Gefitinib was the first molecular targeted agent to be approved for the treatment of NSCLC in Japan. Two international cooperative Phase II studies (IRESSA Dose Evaluation in Advanced Lung Cancer Trial: IDEAL1 and 2) demonstrated efficacy (response rates, 12.0–18.9%) and favorable tolerability of gefitinib in the treatment of NSCLC after failure of platinum-based chemotherapy.[4,5] Furthermore, the results of subset analyses of IDEAL1 indicated that the patient characteristics of Japanese nationality, female gender and adenocarcinoma histology were associated with longer overall survival (OS).[4]

In a placebo-controlled Phase III study (BR21) erlotinib significantly prolonged OS compared with placebo in patients with previously treated NSCLC.[6] A similar Phase III study (IRESSA Survival Evaluation in Lung Cancer [ISEL]) of gefitinib in refractory, advanced NSCLC showed an improvement in survival compared with placebo in the overall study population, which did not reach statistical significance.[7] However, in a subset analysis, statistically significantly longer survival was demonstrated in patients of Asian origin and in patients who had never smoked.[7] With the availability of new second-line anticancer agents such as gefitinib and erlotinib, it is necessary to consider more fully the influence of second-line treatment on evaluation of OS following standard first-line treatment. Since the opening of our department in October 2002, carboplatin/paclitaxel has been used as the standard first-line therapy for NSCLC, while the use of gefitinib as second-line therapy is increasing each year. In this study we performed retrospective analyses of data from patients who had received carboplatin/paclitaxel, in order to identify prognostic variables affecting OS and progession-free survival (PFS), and also to determine the contribution of second-line and subsequent treatment to prolongation of OS.

Patients and Methods

Patients. This retrospective study recruited patients with NSCLC who had received chemotherapy at the Thoracic Oncology Division, Shizuoka Cancer Center, Japan, between October 2002 and September 2005. Patients met all of the following criteria:(1) clinical stage IIIB or IV;(2) patients were administered carboplatin area under the curve (AUC) 6 + paclitaxel 200 mg/m^2 as first-line chemotherapy; and(3) performance status (PS) 0 or 1.

Target patients were identified in our electronically controlled clinical database and the following information extracted from their data:(1) patient demographics at the start of first-line chemotherapy (age, gender, smoking history, histology, stage);(2) objective tumor response;(3) time to disease progression;(4) OS;

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and second-line and subsequent chemotherapy regimens.[5] The tumor response was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) using existing images and graded as complete response, partial response, stable disease, progressive disease or not evaluable.

Treatment. Patients received carboplatin and paclitaxel as first-line chemotherapy. Patients received paclitaxel 200 mg/m^2 as a 3-h intravenous infusion, followed by carboplatin AUC 6 (Calvert's setting) as a 1-h infusion on Day 1. Courses of treatment were repeated every 3 or 4 weeks for 4–6 cycles, until disease progression or severe toxicity. When a patient developed National Cancer Institute Common Toxicity Criteria (NCI-CTC) grade 3 non-hematological toxicity (except nausea and anorexia) after the start of treatment, the dose was reduced to carboplatin AUC 5 + paclitaxel 150 mg/m^2.

Statistical analysis. Kaplan-Meier plots were prepared for OS and PFS and median values were calculated. OS was measured from the first day of first-line treatment to the day of death or the day last seen alive (cut-off). PFS was measured from the first day of first-line treatment to the earliest observation of documented progressive disease, or the day of death if the patient died before observation of progressive disease. Univariate and multivariate analyses were performed for OS and PFS stratified by baseline factors. To identify factors influencing PFS and OS, multivariate analysis was performed with covariates including disease stage (IIIB versus IV), histology (adenocarcinoma versus other), smoking history (non-smoker versus smoker), gender (female versus male) and PS (0 versus 1). Multivariate analysis was performed by the stepwise regression method using a Cox proportional hazards model. To evaluate potential interaction between clinical variables such as smoking history or histology and EGFR-TKI treatment, patients who received second-line therapy were included in subsequent exploratory Cox analysis in which non-smokers and adnocarcinoma patients were divided by EGFR-TKI treatment, with smokers and nonadnocarcinoma patients set as references, respectively. Statistical analyses for this study were conducted using the Stat View software statistical tool.

Results

Patient characteristics. In total, 98 patients met the eligibility criteria and their demographic data are presented in Table 1. The majority of patients were male (64%), had a smoking history (69%), adenocarcinoma histology (76%), stage IV disease (70%) and PS 0 (57%). The median duration of first-line carboplatin/paclitaxel therapy was 3 cycles (range, 1–6 cycles). The median follow-up time was 24.8 months (range: 4.2–43.9). 57 patients died. 41 patients were still alive.

Table 1. Patient demographics (n = 98)

Gender *n* (%)	
Male	63 (64)
Female	35 (36)
Median (range) age, years	61 (34–78)
ECOG PS, *n* (%)	
0	56 (57)
1	42 (43)
Smoking history, *n* (%)	
Smoker	68 (69)
Non-smoker	30 (31)
Histology, *n* (%)	
Adenocarcinoma	74 (76)
Other	24 (24)
Stage, *n* (%)	
IIIB	29 (30)
IV	69 (70)

ECOG, European Cooperative Oncology Group; PS, performance status.

Table 2. Best overall objective response, *n* (%)

	Total population (n = 98)	By histology	
		Adenocarcinoma (n = 74)	Other (n = 24)
Partial response	20 (20)	15 (20)	5 (21)
Stable disease	53 (54)	42 (57)	11 (46)
Progressive disease	25 (26)	17 (23)	8 (33)

Efficacy. The overall response rate to first-line carboplatin/paclitaxel therapy was 20% (20/98), with outcomes similar in patients with adenocarcinoma and other histological subtypes (20% versus 21%, respectively) (Table 2). In the overall population, median PFS was 4.8 months and median OS 16.5 months, with a 1-year survival rate of 64%.

For PFS, only disease stage was a significant prognostic factor (Table 3). For OS, histology, smoking history and PS were significant prognostic factors (Table 3).

Multivariate analyses assessing the effects of histology and smoking history on PFS and OS were performed. No significant difference was observed for PFS between adenocarcinoma versus other histology (P = 0.40; Fig. 1) or non-smokers versus smokers (P = 0.22; Fig. 2). In contrast, OS differed significantly between adenocarcinoma versus other histology (P = 0.0017)

Table 3. Efficacy among patient subgroups: Cox regression analysis

Factor	Variable	PFS *P*-value HR (95% CI)	OS *P*-value HR (95% CI)
Histology	Adenocarcinoma versus other	0.2045 –	0.0020 0.410 (0.233–0.723)
Smoking	Non-smoker versus smoker	0.1351 –	<0.0001 0.222 (0.109–0.450)
Gender	Female versus male	0.2206 –	0.2691 –
PS	0 versus 1	0.9575 –	0.0109 0.499 (0.292–0.852)
Stage	IIIB versus IV	0.0074 0.536 (0.339–0.847)	0.2024 –

PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; NS, not significant; PS, performance status.