Promoter Hypermethylation Is the Predominant Mechanism in *hMLH1* and *hMSH2* Deregulation and Is a Poor Prognostic Factor in Nonsmoking Lung Cancer

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Abstract

Purpose and Experimental Design: The etiologic association and prognostic significance of mismatch repair gene/protein alterations have never been examined in nonsmoking lung cancer. Therefore, we investigated protein expression and promoter hypermethylation of *hMLH1* and *hMSH2* genes in the tumor specimens from 105 nonsmoking female non–small cell lung cancer (NSCLC) patients. Immunohistochemistry and restriction enzyme–based multiplex PCR were used to examine the protein expression and promoter hypermethylation, respectively. The occurrence of gene/protein alteration for each gene was compared with the patients' clinicopathologic variables as well as the overall survival and cancer-specific survival rates.

Results: Protein expression alteration and promoter hypermethylation were observed in 66% to 67% and 30% to 34% of tumor specimens for *hMLH1* and *hMSH2* genes, respectively. Loss of hMLH1 and hMSH2 protein expression was significantly associated with their promoter hypermethylation ($P < 0.0001$ and $P = 0.049$). The overall survival and cancer-specific survival rates were significantly lower in patients with promoter hypermethylation of *hMSH2* gene than in those without hypermethylation ($P = 0.038$ and $P = 0.004$). The poor prognosis was still especially significant in adenocarcinoma ($P = 0.035$ and $P = 0.061$) and early-stage NSCLC patients ($P = 0.067$ and $P = 0.041$).

Conclusion: Our data suggest that *hMLH1* is the major altered mismatch repair gene involved in nonsmoking NSCLC tumorigenesis and that promoter methylation is the predominant mechanism in *hMLH1* and *hMSH2* deregulation. In addition, promoter methylation of the *hMSH2* gene may be a potential prognostic factor in nonsmoking female lung cancer.

Lung cancer has became the leading cause of cancer death in many industrialized countries; in Taiwan, lung cancer clams >7,000 lives annually (1). In recent years, much attention has been focused on the rapidly increasing incidence of primary lung cancer in nonsmoking patients (2–4). Some gender differences exist in the distribution of lung cancer, including histologic type and exposure to tobacco (5–9). Although 80% of female lung cancer patients worldwide have smoked, <10% of Taiwanese women with lung cancer have ever smoked. The low smoking status and high incidence rate of adenocarcinoma constitute distinctive characteristics of Taiwanese female lung cancers. Ryberg et al. showed that susceptibility to DNA damage caused by environmental carcinogens such as polycyclic aromatic hydrocarbon–like compounds may be higher among women than among men. They concluded that women are at greater risk of tobacco and/or environmental-induced lung cancer (10). In the study of Takagi et al., distinct mutational spectrum of the *p53* gene in lung cancer tissues from nonsmoking Chinese women in Hong Kong was observed, suggesting that environmental and/or genetic factors might be involved in the development of lung cancer in women (11).

Molecular biological studies have shown that overt cancers carry multiple genetic and epigenetic alterations, indicating inactivation of tumor suppressor genes and activation of dominant oncogenes during the process of carcinogenesis and subsequent progression of cancers (12, 13). Alteration analysis in the genes controlling acquired somatic mutations, such as genes involved in the DNA repair system, may explain some of the observed significance in susceptibility to various conditions caused by environmental factors in nonsmoking female lung cancer. *hMLH1* and *hMSH2* are two of the genes known to be implicated in the DNA mismatch repair system. Inactivation of these two genes by promoter hypermethylation has been reported to be associated with some human cancers (14–18). Herman et al. suggested that DNA methylation associated with

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transcriptional silencing of *hMLH1* is the underlying cause of mismatch repair defects in most sporadic colorectal cancers (14). Xinarianos et al. have shown that 58.6% and 57.8% of lung cancer tumor specimens had reduced expression levels of the hMLH1 and hMSH2 proteins, respectively (19). However, the distribution of the gender related to the protein expression of the genes was not mentioned in the article. In addition, the clinical significance of the protein expression of *hMLH1* and *hMSH2* in nonsmoking lung cancers remains unclear. Recently, Brooks et al. reported that in patients with stage III non–small cell lung cancer (NSCLC), low protein expression of hMSH2 in the positive mediastinal nodal specimens was associated with poor treatment response and cancer death (20). In the previous report from our laboratory, we examined the protein expression and the status of promoter hypermethylation of *hMLH1* and *hMSH2* genes in 77 NSCLC tumors and found that protein expression of *hMLH1* and *hMSH2* in the tumor specimens from female patients with lung cancer was higher than in specimens from male patients, although only a few female tumor samples were examined (21). Following this finding, the present study collected more tumor specimens, focusing on nonsmoking female NSCLC patients, to investigate the protein expression of *hMLH1* and *hMSH2* and promoter hypermethylation of these two genes in these tumor tissues. The clinical association and prognostic significance of both genes in the nonsmoking female patients with lung cancer were also analyzed.

Materials and Methods

Study population and tumor samples. From 1996 to 1998, 105 nonsmoking female patients who had operations for NSCLC were enrolled in this study. The demographic data of these patients including the smoking habit, age, tumor type, and the stage of disease were collected by chart review. There was complete follow-up for all patients. The end of the follow-up period was defined as October 2004. Overall survival was calculated from the day of surgery to the date of death or the last follow-up. Cancer-specific survival was calculated from the day of surgery to the date of either lung cancer death or the last follow-up. The mean follow-up period for all patients was 32 months (range, 0.5-78 months). Of 105 patients, 42 (40%) patients died from lung cancer and had a median cancer-specific survival of 17 months (range, 0.5-44 months).

Analysis of protein expression:immunohistochemistry assay. Paraffin blocks of tumors were cut into 5-μm slices and then processed using protocols described in our previous publication (21). hMLH1 and hMSH2 protein expression was evaluated by the immunohistochemistry assay. The monoclonal antibodies used were G168-728 (1:250; PharMingen, San Diego, CA) for the hMLH1 protein and FE11 (1:50; Oncogene Science, Cambridge, MA) for the hMSH2 protein. The normal staining pattern for hMLH1 and hMSH2 is nuclear. Tumor cells that exhibit an absence of nuclear staining in the presence of nonneoplastic cells and infiltrating lymphocytes with nuclear staining are considered to have an abnormal pattern.

Restriction enzyme-based multiplex PCR methylation assay. The promoter methylation status of the *hMLH1* and *hMSH2* genes was investigated using the *Hpa*II/*Hha*I- and *Hha*I-based restriction enzyme–based PCR (RE-PCR) analysis, respectively. The *hMLH1* promoter region (−670 to −67) contains four *Hpa*II sites and two *Hha*I sites, whereas there are four *Hha*I sites for the *hMSH2* promoter region (−354 to −126). Genomic DNA (200 ng) was digested either with 10 units of the methylation-sensitive enzyme (*Hha*I and/or *Hpa*II, New England Biolabs, Beverly, MA) or placed in the appropriate buffer without enzyme for 10 hours at 37°C. The DNA was subjected to a second round of digestion by another 10 units of freshly added enzymes to improve specificity and completeness of digestion. The digested DNA was purified by phenol/chloroform extraction followed by ethanol precipitation. One hundred nanograms of the digested DNA were subjected to the multiplex PCR. The primer nucleotide sequences for the *hMLH1* promoter region and an internal control D3S3281 sequence, which contains no *Hpa*II and *Hha*I sites, were reported previously (21). The primer nucleotide sequences for the *hMSH2* gene were sense 5′-GTTTCCTTCTGATGTTACTCC-3′ and antisense 5′-CCGC-GCACGCTGGTG ACT-3′. Methylation was determined by the ratio of the normalized intensity (target gene/internal control) of the samples pretreated with digested (+) over the undigested (−) samples. DNA of 30 normal lung tissues from NSCLC patients was examined; the ratio was <0.2 for all samples examined. Therefore, a ratio of >0.2 was defined as aberrant methylation. DNA from normal lymphocytes as well as CL2 and CL1-5-F4 lung cancer cell line was included in each assay to serve as unmethylated and methylated controls, respectively, of the *hMLH1* and *hMSH2* genes.

Statistical analyses. The Pearson χ^2 test was used to compare the hMLH1 and hMSH2 alterations among cases and between various clinicopathologic variables. The difference on age distribution between patients with and without the alteration was analyzed by the independent sample t test. Type III censoring was done on subjects who were still alive at the end of the study. Censoring for the cancer-specific survival analysis was done at the end of the study on subjects who were still alive or who had died of some other cause. Survival curves were calculated by the Kaplan-Meier method, and comparison was done by the log-rank test. $P < 0.05$ was considered statistically significant.

Results

Genetic alterations of hMLH1 protein/gene and its correlation with clinicopathologic variables in nonsmoking female patients with lung cancer. Immunohistochemical staining for hMLH1 protein was done on 105 tumor samples. Staining within the nucleus of tumor cells was considered positive. Thirty-five lung tumors showed staining of moderate to strong intensity (Fig. 1A). The remaining 70 tumor cancer specimens showed a complete absence of nuclear staining of hMLH1 protein (66.7%; Fig. 1B and C). There was no significant correlation between the hMLH1 protein expression and age, smoking habit, tumor cell type, and tumor stage (Table 1). RE-PCR methylation assay indicated that 70 of these 105 tumor specimens showed promoter hypermethylation of *hMLH1* gene (66.7%; Fig. 2A). Analysis of the relationship between the expression of hMLH1 protein and its promoter hypermethylation was done. Aberrant hMLH1 protein expression was significantly associated with promoter hypermethylation of *hMLH1* gene ($p < 0.0001$; Table 1).

Genetic alterations of hMSH2 protein/gene and its correlation with clinicopathologic variables in female patients with lung cancer. Seventy-four tumor specimens showed moderate to strong staining of hMSH2 protein (Fig. 1D). The remaining 31 lung cancer specimen showed a complete absence of nuclear staining of hMSH2 protein (29.5%; Fig. 1E and F). There was also no significant correlation between the hMSH2 protein expression and patients' age, smoking habit, tumor cell type, and tumor stage (Table 2). Thirty-six of these 105 tumor specimen showed promoter hypermethylation of the *hMSH2* gene (34.3%; Fig. 2B). The tumor specimens of late-stage (stages III and IV) patients displayed more promoter hypermethylation of the *hMSH2* gene (45.5%) than those

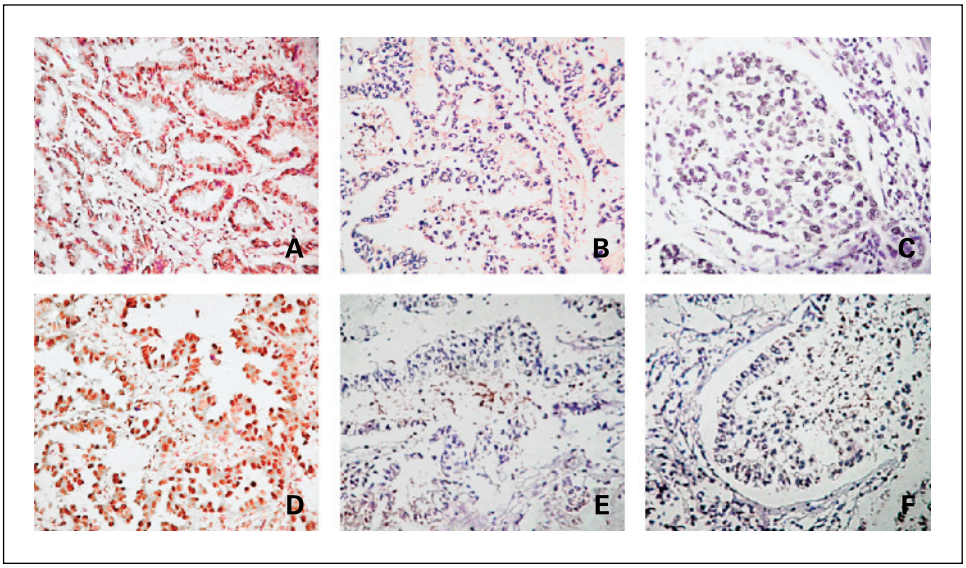

Fig. 1. Representative figures of the immunohistochemical analysis of hMLH1 (*A-C*) and hMSH2 (*D-F*) protein expression in paraffin sections of lung tumor specimens. hMLH1 and hMSH2 nuclear immunoreactivity was found in (*A*) and (*D*). *B*, negative hMLH1 expression in an adenocarcioma. *C*, negative hMLH1 expression in a squamous cell carcinoma. *E*, negative hMSH2 expression in an adenocarcinoma. *F*, negative hMSH2 expression in a squamous cell carcinoma. Original magnification, ×100.

early-stage patients (26.2%; $P = 0.041$). The aberrant hMSH2 protein expression was significantly associated with promoter hypermethylation of the *hMSH2* gene ($P = 0.049$; Table 2).

Correlation of hMLH1 and hMSH2 alterations with the prognosis of nonsmoking female non–small cell lung cancer patients. The relationship between survival and the alteration of *hMLH1* and *hMSH2* was analyzed (Fig. 3). The hMLH1 protein expression and DNA hypermethylation were not significantly associated with the prognosis of female patients with lung cancer. However, the overall survival and cancer-specific survival rates were significantly lower in patients with promoter hypermethylation of *hMSH2* gene than in those without promoter hypermethylation (Fig. 3A, $P = 0.038$; Fig. 3B, $P = 0.004$). In patients with early stage and without lymph node metastasis, the overall survival and cancer-specific survival were also significantly lower in patients with promoter hypermethylation of *hMSH2* than in patients without promoter hypermethylation (Fig. 3C and D, $P = 0.067$ and $P = 0.041$ for the early-stage patients; Fig. 3E and F, $P = 0.211$ and $P = 0.007$ for the patients without lymph node metastasis). In patients with adenocarcinoma of the lung but not with squamous carcinoma, the overall survival and cancer-specific survival were significantly low in patients with promoter hypermethylation of *hMSH2* (Fig. 3G and H, $P = 0.035$ and $P = 0.061$). The data indicated that for female patients, promoter hypermethylation of *hMSH2* was associated with a poorer prognosis.

Discussion

In this study, the significance of the DNA mismatch repair genes, *hMLH1* and *hMSH2*, in nonsmoking female NSCLC patients was investigated by analysis of protein expression and promoter methylation of the *hMLH1* and *hMSH2* genes in 105 tumor specimens. Protein expression alteration and promoter hypermethylation were observed in 66% to 67% and 30% to 34% of tumor specimens for the *hMLH1* and *hMSH2* genes, respectively. A significant concordance was observed between the alterations in protein and DNA hypermethylation of both genes, suggesting that promoter hypermethylation is the predominant mechanism in *hMLH1* and *hMSH2* deregulation. Note that promoter hypermethylation of *hMSH2* was significantly associated with poor prognosis in these nonsmoking female NSCLC patients, especially in the case of adenocarcinoma and early-stage patients. The registry data indicate that most of the female patients with lung cancer did not smoke and that adenocarcinoma was the predominant tumor cell type in

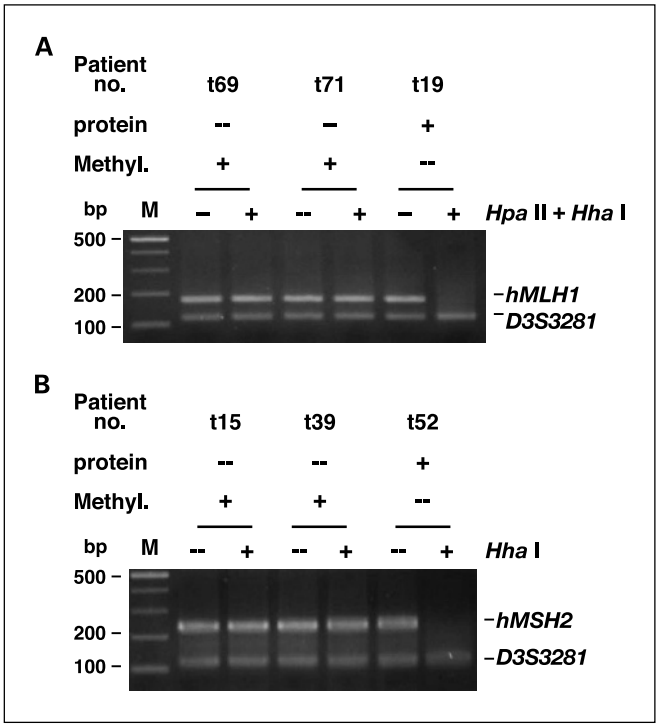

Fig. 2. Representative figures of RE-PCR methylation analysis of the *hMLH1* (*A*) and *hMSH2* genes (*B*). Restriction enzyme digest (+). Mock digest (−). Positions of the target *hMLH1* and *hMSH2* promoter regions and internal control are indicated. Two patients with promoter methylation and one patient without promoter methylation. Results of their protein expression status (*top*).

Table 1. Genetic alterations of *hMLH1* protein/gene in relation to the clinicopathologic variables of resected nonsmoking female lung cancer tumors

Characteristics	Protein expression				DNA hypermethylation			
	Total	+	−* (%)	*P*	Total	−	+* (%)	*P*
Overall	105	35	70 (66.7)		105	35	70 (66.7)	
Age (mean ± SD)		61.2 ± 11	59.4 ± 13	0.475		61.6 ± 11	59.4 ± 13	0.324
Age								
>60	57	22	35 (61.4)	0.213	57	23	34 (59.6)	0.096
≤60	48	13	35 (72.9)		48	12	36 (75.0)	
Tumor type†								
AD	65	22	43 (66.2)	0.457	65	22	43 (66.2)	0.919
SQ	17	4	13 (76.5)		17	6	11 (64.7)	
AS	2	0	2 (100.0)		2	1	1 (50.0)	
Other	21	9	12 (57.1)		21	6	15 (71.4)	
Tumor stage								
I + II	61	19	42 (68.9)	0.576	61	19	42 (68.9)	0.576
III + IV	44	16	28 (63.6)		44	16	28 (63.6)	
Protein expression								
+					35	21	14 (40.0)	0.00004
−					70	14	56 (80.0)	

Abbreviations: AD, adenocarcinoma; SQ, squamous carcinoma; AS, adenosquamous carcinoma.
*These groups represent patients with alteration in the *hMLH1* gene/protein.
†The comparison was made between SQ and AD.

all patients with NSCLC in Taiwan. The importance of identifying factors that increase the risk of lung adenocarcinomas in nonsmoking females has long been recognized. To our knowledge, comprehensive molecular and clinical analyses on the alterations of *hMLH1* and *hMSH2* in nonsmoking lung cancer patients to reveal the contribution of mismatch repair inactivation to NSCLC tumorigenesis have never been reported. This is also the first report of an association of *hMSH2* promoter

Table 2. Genetic alterations of *hMSH2* protein/gene in relation to the clinicopathologic variables of resected nonsmoking female lung cancer tumors

Characteristics	Protein expression				DNA hypermethylation			
	Total	+	−* (%)	*P*	Total	−	+* (%)	*P*
Overall	105	74	31 (29.5)		105	69	36 (34.3)	
Age (mean ± SD)		60.5 ± 12	58.9 ± 13	0.556		60.0 ± 12.4	60.1 ±12.0	0.969
Age								
>60	57	41	16 (28.1)	0.722	57	35	22 (38.6)	0.311
≤60	48	33	15 (31.3)		48	34	14 (29.2)	
Tumor type†								
AD	65	45	20 (30.8)	0.685	65	40	25 (38.5)	0.462
SQ	17	11	6 (35.3)		17	11	6 (35.3)	
AS	2	2	0 (0.0)		2	2	0 (0.0)	
Others	21	16	5 (23.8)		21	16	5 (23.8)	
Tumor stage								
I + II	61	44	17 (27.9)	0.662	61	45	16 (26.2)	0.041
III + IV	44	30	14 (31.8)		44	24	20 (45.5)	
Protein expression								
+					74	53	21 (28.4)	0.049
−					31	16	15 (48.4)	

Abbreviations: AD, adenocarcinoma; SQ, squamous carcinoma; AS, adenosquamous carcinoma.
*These groups represent patients with alteration in the *hMSH2* gene/protein.
†The comparison was made between SQ and AD.

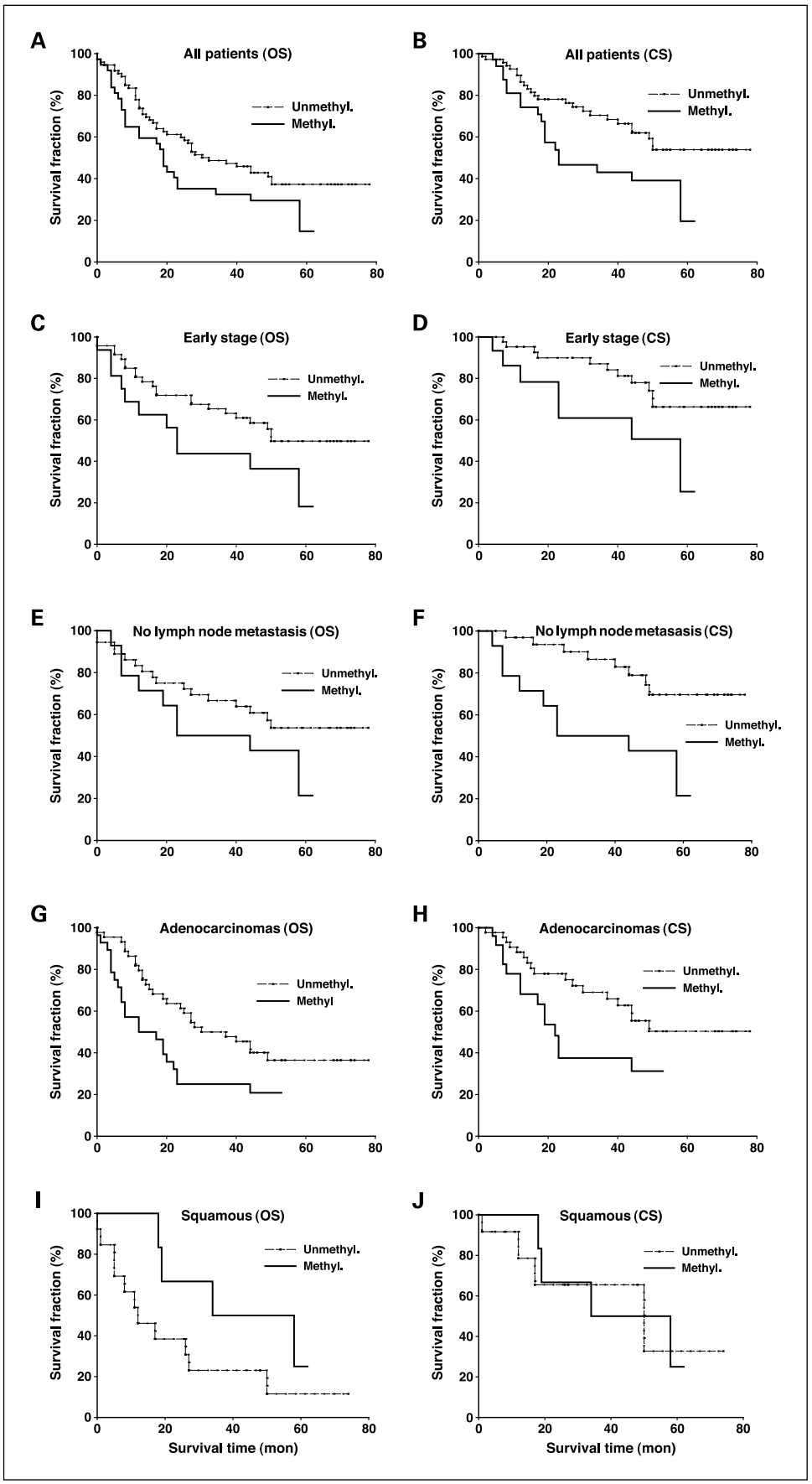

Fig. 3. Overall survival (*OS*) and cancer-specific survival (*CS*) of all nonsmoking female NSCLC patients (*A-B*), early-stage nonsmoking female patients (*C-D*), nonsmoking female patients without lymph node metastasis (*E-F*), nonsmoking female adenocarcinoma patients (*G-H*), and nonsmoking female squamous patients (*I-J*) according to the results of *hMSH2* promoter hypermethylation. *P* value for each analysis is also indicated.

hypermethylation with prognosis in nonsmoking female NSCLC patients.

The data on mismatch repair gene alterations in lung cancer is scarce. Xinarianos et al. previously reported the expression levels of hMLH1 and hMSH2 proteins in the tumor specimens from 59 males and 91 female patients with lung cancer in the United Kingdom and showed that 53% and 82% of adenocarcinoma specimens of both male and female had reduced expression levels of the hMLH1 and hMSH2 protein, respectively (19). Supporting the results of our study, Xinarianos et al. have also reported a high frequency of reduced expression levels of the hMLH1 protein. However, the expression level of hMSH2 protein in their study was apparently higher than in our study. In contrast to our study, which focused on nonsmoking female NSCLC, there were only six nonsmokers in their study. In addition, the promoter methylation status was not examined in their study. A study by Aubry et al. showed by immunohistochemistry assay that mismatch repair proteins hMLH1, hMSH2, and hMSH6 were not inactivated in 33 bronchioloalveolar carcinomas of the lung (22). This type of inconsistency also occurred in a U.S. study, which found no promoter methylation of the *hMLH1* gene in 20 NSCLC tumors by the methylation-specific PCR (MSP) assay (23). In the study of small cell lung cancer, Hansen et al. showed that low hMLH1 protein expression was not linked to promoter methylation in a panel of 21 small cell lung cancer cell lines using the MSP assay (24). The discrepancy may be due to the patients with different clinicopathologic variables examined in the contrasting studies. For example, there were more patients who were nonsmoking female adenocarcinoma lung cancer patients in our study compared with previous studies. Alternatively, the methylation regions examined and sensitivities of the assay used in various studies were different. It is also possible that geographic and/or ethnic factors account for frequent *hMLH1* and *hMSH2* alterations in Taiwanese NSCLC patients. In addition, environmental factor(s) other than cigarette smoke and/or endogenous factor(s) may be involved in the observed association of *hMLH1* and *hMSH2* alterations with nonsmoking female adenocarcinomas.

Some authors suggested that a relationship exists between the expression of hMLH1 and hMSH2 protein and drug resistance or the response to therapy including chemotherapy or radiotherapy for cancer patients (20, 25, 26). Strathdee et al. investigated the role of methylation of *hMLH1* in drug resistance in ovarian cancer cell lines and suggested that methylation of the *hMLH1* promoter may be a common mechanism for cisplatin resistance in ovarian cancer (25). In addition, Mackay et al. reported that reduction of hMLH1 protein expression in breast tumor samples after chemotherapy was strongly associated with poor disease-free survival (26). Note that Brooks et al. investigated the expression *hMSH2* in the positive mediastinal nodal specimens of 59 NSCLC patients with stage III diseases who were treated with chemotherapy and irradiation therapy and found that low expression of *hMSH2* was associated with poor overall survival (20). In our study, we also found that there was a relationship between the promoter hypermethylation of *hMSH2* gene and the poor prognosis of the nonsmoking female NSCLC patients. In the patients with early-stage lung cancer and without lymph node metastasis, in whom clinical oncologists need selection factors to decide whether adjuvant therapy is necessary, the patients with promoter hypermethylation of *hMSH2* had a poorer cancer-specific survival than those without DNA hypermethylation. To the best of our knowledge, this is the first study showing a relationship between the status of *hMSH2* promoter hypermethylation and the prognosis of patients with lung cancer. How the expression of *hMLH1* or *hMSH2* could influence the results of chemotherapy or radiotherapy is a question that warrants further analysis.

The inactivation of DNA mismatch repair gene has been mentioned as being associated with the genetic instability of some cancers. Loss of *hMLH1* expression was shown as one of the main causes of microsatellite instability in colorectal cancer (16, 18). Selective defect in some mismatch repair genes can cause genomic instability and activate the malignant transformation as well as the progression of gastric cancer, renal cell carcinoma, and endometrial carcinoma (15, 17, 27). In our previous article, we had indicated that aberrant methylation, which can result in the transcriptional silencing of the target gene, was frequently found in the *hMLH1* alterations and microsatellite instability in NSCLC tumors (28). A highly significant correlation was found between gene methylation and negative protein expression for mismatch repair *hMLH1* and *hMSH2* genes, suggesting that promoter hypermethylation is the predominant mechanism by which these two mismatch repair genes are silenced in NSCLC. This may eventually lead to genetic instability and poorer prognosis of lung cancer. However, Ward et al. (29) reported that poor prognostic effect of DNA methylation is lost in colorectal patients with microsatellite instability. It seems that methylated tumors, both unstable and stable, have distinct clinicopathologic features, which warrants further analysis in nonsmoking lung cancer. Mechanisms involving DNA damage signaling, promoter hypermethylation of mismatch repair genes, and the target drug-resistant genes after chemotherapy are also under investigation.

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