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Microsatellite instability and clinicopathological features in esophageal squamous cell cancer

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Abstract. Since multiple genetic alterations are involved in the molecular pathogenesis of esophageal squamous cell cancer (ESCC), the role of microsatellite instability (MSI) in its carcinogenesis is not well defined. The reported frequency of MSI in ESCC ranges from 2 to 66.7% but the majority of the results are derived from relatively small studies. Therefore, we carried out a precise MSI analysis on a large number of ESCC samples to clarify the significance of MSI in the ESCC tumorigenesis. The MSI status of the DNA extracted from 62 ESCC samples and 62 counterpart-normal esophageal epitheliums were studied with five NCI panel markers and ten microsatellite markers located in 17q24-25. Forty-four paraffin-embedded samples and 18 frozen samples from the ESCC patients who underwent surgery were studied. The MSI status was classified as MSS (microsatellite stable), MSI-L (low-level MSI; <30% of markers examined showed instability) and MSI-H (high-level MSI; >30% of markers reported instability). Among the 62 ESCC cases analyzed by the 15 microsatellite markers, 38 out of 62 cases (61.3%) showed MSS, 19 out of 62 cases (30.6%) showed MSI-L and 5 out of 62 cases (8.1%) showed MSI-H. Although the MSI status was not associated with the status of lymph node metastasis or a histological type of cancer, the depth of cancer invasion was significantly associated with the frequency of MSS status and the levels of MSI-L were inversely correlated with the depth of invasion (T1/T2 vs. T3/4; P=0.0007). However, MSI status was not associated with the prognosis of the ESCC patients. This is the first large scale MSI analysis of the ESCC in comparison with the clinicopathological features. Relatively high frequency of MSI-L was observed in ESCC and the frequency of MSI-L was inversely correlated with the depth of invasion.

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Abbreviations: ESCC, esophageal squamous cell cancer; MSI, microsatellite instability; MSS, microsatellite stable; MSI-L, low-level microsatellite instability; MSI-H, high-level microsatellite instability; HNPCC, hereditary non-polyposis colorectal cancer; MGMT, O^6-methylguanine DNA methyltransferase; MMR, mismatch repair

Key words: esophageal squamous cell cancer, microsatellite instability, depth of invasion, prognosis, early stage

Introduction

Esophageal cancer is a frequent fatal cancer throughout the world (1). Squamous cell carcinoma and adenocarcinoma are the two major pathological types in esophageal cancer. Despite the increased incidence of esophageal adenocarcinoma in North America and Europe, esophageal squamous cell cancer (ESCC) remains a common type of malignancy worldwide (1). Tobacco and alcohol consumption represent major environmental risk factors, however, the molecular events leading to the ESCC are largely unknown (2).

High-level microsatellite instability (MSI-H), which is defined as >30% of microsatellite markers showing instability in tumor DNA (6,7), has been described in hereditary non-polyposis colorectal cancer (HNPCC) and in 15 to 20% of sporadic colorectal cancers (3-5). In colorectal cancer, MSI-H correlates well with the proximal colon location, mucinous and poorly differentiated histology and better prognosis (6,7). In sporadic MSI-H colorectal cancer, the silencing of *hMLH1* through promoter hypermethylation is the main mechanism for its mismatch repair defect (8-10). Low-level microsatellite instability (MSI-L) is defined as <30% of markers showing instability (11,12). No clear clinical or pathologic differences were noted between MSI-L and microsatellite stable (MSS) tumors (12,13). Since MSI-L phenotype is not well defined, MSI-L has been frequently considered as MSS. We observed previously that MSI-L phenotype was frequently detected in

early colorectal cancers compared with advanced ones and we proposed that MSI-L phenotype is a separate category in colorectal cancer (14).

In ESCC, MSI has not been considered as a major event in its tumorigenesis. According to the relatively small studies, the frequency of MSI in ESCC is in a range of 2 to 66.7% (15-22). To clarify the true significance in pathogenesis of ESCC, we analyzed the precise MSI status of the disease in a large-scale study.

Materials and methods

Patients. Sixty-two patients with ESCC, who underwent a curative esophagectomy at the Okayama University Hospital (18 patients) between 1995 to 1998 and the Keiyuukai Sapporo Hospital (44 patients) in 1994, were collected after obtaining informed consent from all the patients. None of the patients received any preoperative chemotherapy or radiation. The patients included 58 men and 4 women ranging in age from 45-80 years (mean age 63.2 years). None of them had a hereditary background of ESCC.

DNA extraction. The genomic DNA from 18 patients from the Okayama University Hospital was obtained from the frozen tumor specimens and matched normal mucosal tissue specimens using phenol-chloroform extraction after proteinase K treatment. The genomic DNA was extracted from paraffin-embedded tissue derived from the 44 patients treated at the Keiyuukai Sapporo Hospital by a microdissection technique. DNA was also extracted from paraffin-embedded non-malignant stromal tissue on the same block by a microdissection technique.

Microsatellite analysis. The MSI status of the DNA extracted from ESCC and from counterpart normal tissue was determined by a panel of 15 markers using the fluorescence autosequencer (SQ-5500E, Hitachi Co., Tokyo, Japan). Five primers (*BAT25*, *BAT26*, *D2S123*, *D5S346* and *D17S250*) were the NCI recommended panel for MSI (12). Ten primers were located on 17q24-25, aimed at searching for the novel tumor suppressor gene. Seven primers (*D17S949*, *D17S1862*, *D17S1352*, *D17S709*, *D17S650*, *D17S785* and *D17S939*) were available through an Internet genome database and 3 primers (*17q25MS1*, *17q25MS2* and *17q25MS3*) were our designed markers (Table I). PCR was performed in 50 μl reaction mixtures comprising of 3 μl of the 5X DNA sample, 9 μl of Gene Releaser (Bio Ventures Inc. Murfreesboro, TN), 0.3 μM of each oligonucleotide primer pair (one end-labeled with Texas Red), 200 mM each dNTPs, 5 μl of 10X PCR buffer and 1.25 unit of Taq polymerase (Ampli-TaqGold, Perkin-Elmer, Foster City, CA). After denaturation by formaldehyde at 95˚C for 5 min, the amplified PCR products were electrophoresed on a 6% LongRanger-6.1 M urea gel on Autosequencer SQ-5500 and analyzed by FRAGRYS version 2 software (Hitachi Inc., Tokyo, Japan). MSI was classified as MSS, MSI-L; <30% of markers examined showed instability and MSI-H; >30% of markers showed instability.

Statistical analysis. The Chi-square and Wilcoxon/Kruskal-Wallis tests were performed using software JMP 5.0.1 J software (SAS Institute Inc., NC). $P<0.05$ was considered to be statistically significant.

Table I. Microsatellite markers used in this study.

Markers	Repeat	Product length (bp)	Locus
BAT25	mono-nucleotide	125	4q12
BAT26	mono-nucleotide	122	2p16
D5S346	di-nucleotide	138	5q21-5q22
D17S250	di-nucleotide	163	17q11.2-17q12
D2S123	di-nucleotide	140	2p16
D17S949	di-nucleotide	191	17q24
D17S1862	di-nucleotide	145	17q24
D17S1352	di-nucleotide	143	17q25
D17S709	di-nucleotide	106	17q25
D17S650	di-nucleotide	119	17q25
17q25MS3	di-nucleotide	152	17q25
17q25MS1	di-nucleotide	164	17q25
17q25MS2	di-nucleotide	120	17q25
D17S785	di-nucleotide	175	17q25
D17S939	di-nucleotide	122	17q25.3

Results and discussion

We collected 62 ESCC samples and analyzed the MSI status. Fig. 1A demonstrates a histological section of ESCC with submucosal invasion before and after microdissection. Microdissection of a cancer rich area was carefully performed in order to minimize the amount of contaminating normal tissue and stroma. Fifteen microsatellite markers were used to determine the MSI status (Fig. 1B). Interpretation of the microsatellite analysis was performed by two independent experts on fragment analysis. If agreement was not reached, re-analysis of the samples was undertaken. Among the 62 ESCC cases analyzed, 38 out of 62 cases (61.3%) showed MSS, 19 out of 62 cases (30.6%) showed MSI-L and 5 out of 62 cases (8.1%) showed MSI-H.

Association of MSI status with clinicopathological features in a total of 62 ESCCs are shown in Table II. Although the MSI status was not associated with the status of lymph node metastasis or a histological type of cancer, the depth of invasion was significantly related to the frequency of MSS status and MSI-L was inversely correlated with the depth of invasion ($P=0.0007$) (Fig. 2A-C). Although the depth of invasion is a factor involved in the altitude of stage, association of the stage level and MSI-L status reported the same trend observed in the depth of invasion, but was not remarkable (Fig. 2D). This was due to the fact that MSI-L cancers tend to reveal more lymph node metastasis within the same levels of stage. However, MSI status was not associated with the prognosis of the ESCC patients either in overall survival or disease-free survival (Fig. 3).

This is the first large scale MSI analysis of the ESCC and the results were compared with the clinicopathological features of the ESCC. In our study, the frequency of MSS, MSI-L and MSI-H in the ESCC were 61.3, 30.6, and 8.1%, respectively. The incidence of MSI-H in the ESCC patients was 8.1% (5 out

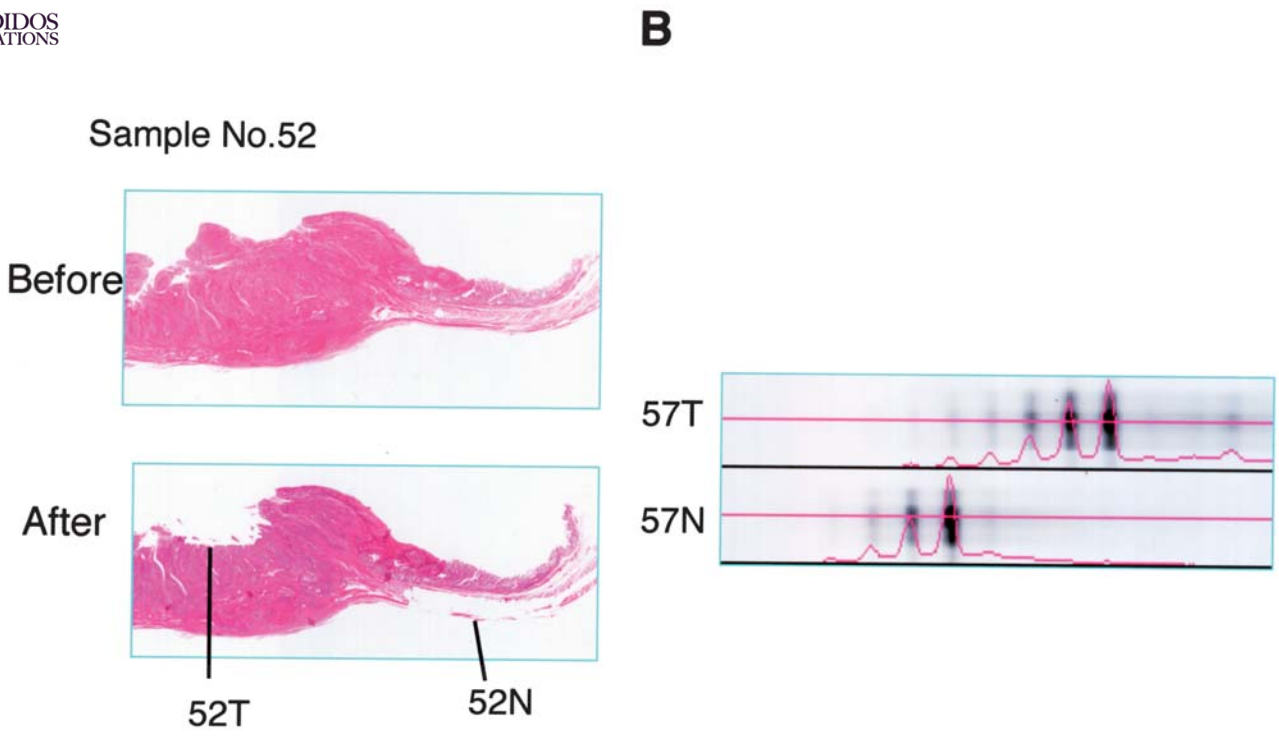

Figure 1. Microdissection of the paraffin-embedded esophageal samples and microsatellite instability analysis. (A) With the guide of H&E stained slides, cancer regions were micro-dissected from paraffin-embedded esophageal samples. Non-cancerous tissue from the counterpart adjacent regions was also captured for the analysis. (B) Representative example of the results of the fluorescent microsatellite analysis of the DNA extracted from the tumor in comparison with the counterpart normal tissue. The sample (T57) shows microsatellite instability of the D17S250 in comparison with the normal counterpart (N57).

Table II. Microsatellite status vs clinicopathological features in esophageal squamous cell carcinoma.

	Total (%)	MSS	MSI-L	MSI-H	P[a]
Gender					
Male	58 (93.5)	36 (62.1)	18 (31.0)	4 (6.9)	0.5619
Female	4 (6.5)	2 (50.0)	1 (25.0)	1 (25.0)	
Age	63.2±7.6	61.6±7.4	65.8±7.5	65.2±8.3	0.2489
Tumor stage					
Stage 0, I	11 (17.7)	5 (45.5)	6 (54.5)	0 (0)	0.0967
Stage II	29 (46.8)	17 (58.6)	9 (31.0)	3 (10.3)	
Stage III	22 (35.5)	16 (72.7)	4 (18.2)	2 (9.1)	
Pathological type					
Well	31 (53.4)	18 (58.1)	10 (32.3)	3 (9.7)	0.8017
Moderately	23 (39.7)	14 (60.9)	8 (34.8)	1 (4.3)	
Poorly	4 (6.9)	2 (50.0)	1 (25.0)	1 (25.0)	
Lymphnode metastasis					
n (-)	21 (33.9)	12 (57.1)	8 (38.1)	1 (4.8)	0.5723
n (+)	41 (66.1)	26 (68)	11 (58)	4 (80)	
Depth of tumor invasion					
T1, T2	24 (40)	11 (45.8)	13 (54.2)	0 (0)	0.0007
T3, T4	36 (60)	26 (72.2)	5 (13.9)	5 (13.9)	

MSS, microsatellite stable; MSI-H, high-frequent microsatellite instability; MSI-L, low-frequent microsatellite instability; well, well-differentiated adenocarcinoma; moderately, moderately differentiated adenocarcinoma; poorly, poorly differentiated adenocarcinoma; n (-), free of lymph node metastasis; n (+), positive for lymph node metastasis. [a]The Chi-square test was used to compare all variables for MSI status except mean age, for which the Wilcoxon/Kruskal-Wallis test was employed.

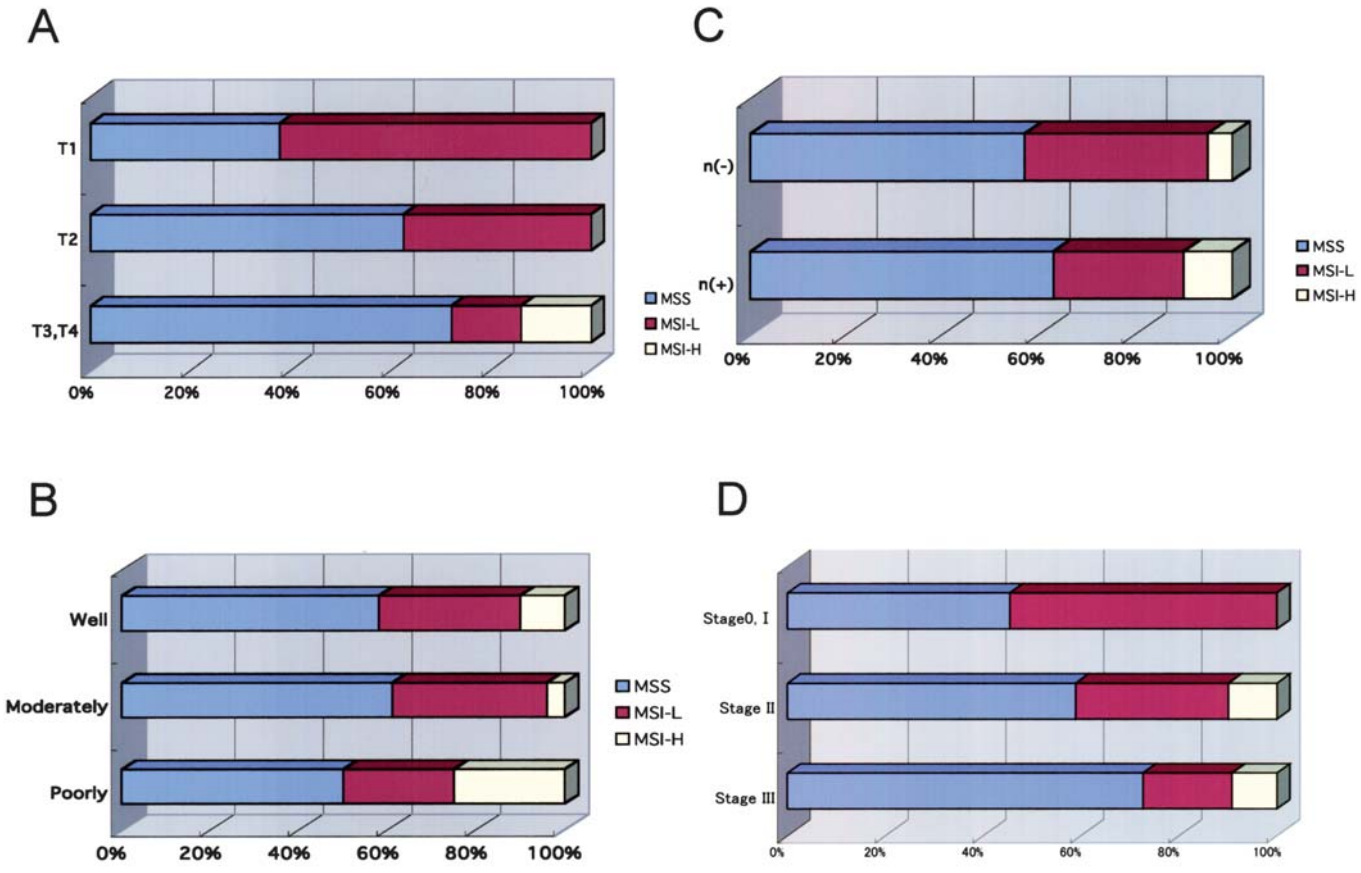

Figure 2. (A) Comparison of the frequency of MSS, MSI-L and MSI-H phenotype in differing depth of invasion of the esophageal squamous cell carcinoma (ESCC). The frequency of MSI-L in T1 ESCC was significantly higher than that in T2 and the same with T2 vs. T3, T4 ESCC. (B) Frequency of MSS, MSI-L and MSI-H phenotype between lymph node positive and negative ESCC. (C) Frequency of MSS, MSI-L and MSI-H phenotype between differing histology of the ESCC. No difference was observed between the histological types. (D) Comparison of the frequency of MSS, MSI-L and MSI-H phenotype at differing stages of the ESCC. Frequency of MSI-L is higher at stage 0 and I ESCC compared with stage II and III ESCC, but the difference was not statistically significant.

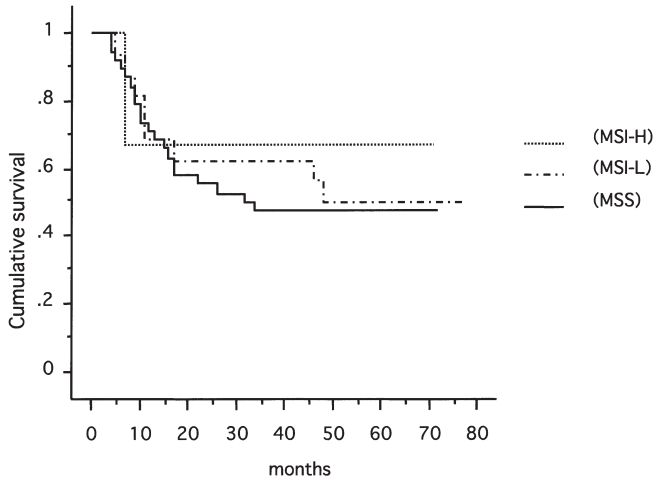

Figure 3. Differing overall survival of the ESCC patients by the MSI status.

of 62) and correspond with the previous studies (17,23,24). However, relatively high-levels of MSI-L status were observed in ESCC compared with the colorectal cancer or stomach cancers (14). The prevalence and the clinical significance of MSI-L in ESCC are poorly understood. In colorectal and stomach cancer, the MSI-L and MSS cancers are sometimes considered inseparable because of the lack of particular clinicopathological phenotype in MSI-L (12,13). In our study, MSI-L status was significantly associated with the depth of invasion into the esophageal wall similarly to our observations in colorectal cancer cases (14). However, there was no difference in stage between MSI-L and MSS ESCC. Therefore, MSI-L was not different from MSS in overall or disease-free survival.

It is also noteworthy that MSI-H was only observed in T3 and T4 ESCC. This suggests that a subset of the MSI-L cancer may develop to MSI-H cancer along with the cancer invading the wall of the esophagus. Since progression of the MSI-L ESCC may be slow compared with that of the MSS, more MSI-L cancers are identified in the earlier sages.

A possible biological event causing MSI-L phenotype has gradually been revealed. Promoter methylation of the *O*^{6}*-methylguanine DNA methyltransferase* (*MGMT*) followed by the loss of *MGMT* expression has been related to the MSI-L phenotype in colorectal cancer (25,26). MGMT is a DNA repair enzyme that rapidly repairs adducts at the *O*^{6}-position of guanine and acts with the mismatch repair (MMR) system. Almost 80% of the ESCC showed promoter methylation of *MGMT* in our study (data not published), thus, loss of function of MGMT may affect the observed high frequency of MSI-L in our series of ESCC. It is also possible that the MSI-L observed in ESCC may not be directly due to the mismatch repair deficiency (11,12,27), but rather be caused by other

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ms that may affect the MMR system. Oxidative
one of the probable mechanisms that may affect the MMR system (28-30). The reactive oxygen species produced in the environment of chronically inflamed esophageal epithelium may lead to DNA damage. Over-production of free radicals saturates the ability of cells to repair DNA damage prior to replication. The resulting imbalance in base excision-repair enzymes may cause MSI-L in chronic inflammation (31).

In conclusion, a relatively high frequency of MSI-L was observed in ESCC and the frequency of MSI-L was inversely associated with the depth of invasion. However, the presence of MSI-L did not correlate with other clinicopathological features such as tumor stage, degree of differentiation, or the presence of lymph node metastasis. There was no correlation between the presence of MSI and survival. A fraction of the MSI-L cancer cells observed at an early stage of ESCC might develop in the MSI-H cells at a later stage of cancer development.

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