

# Prognostic Value of Microsatellite Instability in Resectable Pancreatic Cancer

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## ABSTRACT

The significance of microsatellite instability (MSI) as a prognostic predictor for resectable pancreatic cancer patients was examined. Forty-six histologically confirmed pancreatic cancer patients who had undergone resection were studied. DNA was extracted from the paraffin block sections by means of the microdissection method. PCR was performed using eight microsatellite primer marker sets. The mixed PCR sample was analyzed by a genetic analyzer. The number of MSI-positive patients was eight (17.4%) as determined by assessment of microsatellite variations in three or more of the eight tested markers. Univariate analysis revealed that patients with MSI-positive tumors had significantly longer survival times than patients with MSI-negative tumors, although there were no significant differences in clinicopathological factors between the two groups (median survival term, 62 months *versus* 10 months, respectively;  $P = 0.011$ ). According to univariate survival analysis, patients with T<sub>3</sub>/T<sub>4</sub>, N<sub>1</sub>, or M<sub>1</sub> tumors, as classified by Union Internationale Contre le Cancer staging, had significantly shorter survival times than patients with less progressive tumors. Multivariate survival analysis indicated that MSI status had an independent predictive value (hazard ratio = 5.577;  $P = 0.007$ ). The tumor-infiltrating leukocyte intensity in MSI-positive tumors was significantly larger than that in MSI-negative tumors, suggesting that MSI-positive tumors may induce stronger antitumor immunity. In conclusion, a patient with MSI-positive pancreatic cancer may have a comparatively better prognosis after resection, possibly due to intensive immunoreaction to the tumor.

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## INTRODUCTION

Patients with pancreatic ductal cancer have extremely poor prognoses. The 5-year survival rate of patients who undergo a potentially curative operation is 5–20% (1). Various clinicopathological factors such as tumor size and lymph node metastasis have been examined as prognostic predictors in patients with resectable pancreatic cancer (2, 3); however, these traditional factors are still unsatisfactory for predicting the long-term survival of patients. Recently, various molecular biological factors, including p53 overexpression (4), decreased levels of transmembrane 4 superfamily genes (MRP-1/CD9 and KAI1/CD82; Ref. 5), *K-ras* mutation in plasma DNA (6), MMP-1<sup>2</sup> overexpression (7), and the increment of type IV collagenase genes (MMP-2 and MMP-9):E-cadherin gene ratio (8), have been shown to be associated with worse prognosis in pancreatic cancer patients.

MSI is caused by mutations or epigenetic changes of mismatch repair genes such as *MLH1*, *MSH2*, *MSH3*, *MSH6*, *PMS1*, and *PMS2*, which products mend DNA replication errors (9). It has been reported that the prognosis of patients with MSI-positive tumors was better than that of patients with MSI-negative tumors in colorectal cancer (10–12), gastric cancer (13–15), and cancer of the papilla of vater (16). Conversely, it has been demonstrated that the prognosis of patients with MSI-positive breast cancer (17, 18) and non-small cell lung cancer (19, 20) is worse than that of patients with MSI-negative tumors. However, to the best of our knowledge, few studies have been conducted on the association between MSI and prognosis in pancreatic cancer (21, 22). Therefore, using microsatellite markers, we examined the predictive value of MSI for the prognosis of patients with resectable pancreatic cancer.

## PATIENTS AND METHODS

**Patients.** Forty-six patients with histologically proven pancreatic cancer who had undergone resection of the primary tumor between 1989 and 2000 at the First Department of Surgery, Osaka City University Hospital were studied. According to UICC staging (23), lesions were classified as follows: 7 stage I lesions; 6 stage II lesions; 23 stage III lesions; 5 stage IVA lesions; and 5 stage IVB lesions. In terms of histological classification by UICC, the tumors studied here included 22 G1 (well-differentiated) tumors, 20 G2 (moderately differentiated) tumors, 2 G3 (poorly differentiated) tumors, and 2 G4 (undifferentiated) tumors. The median follow-up period was 12.5 months, ranging from 2.5 months to 130 months after surgery.

**DNA Extraction.** Specimens of formalin-fixed and paraffin-embedded tissues were prepared in 16- $\mu$ m-thick sections

<sup>2</sup> The abbreviations used are: MMP, matrix metalloproteinase; MSI, microsatellite instability; UICC, Union Internationale Contre le Cancer (International Union Against Cancer); TIL, tumor-infiltrating lymphocyte; MSI-H, high-frequency MSI; MSI-L, low-frequency MSI.

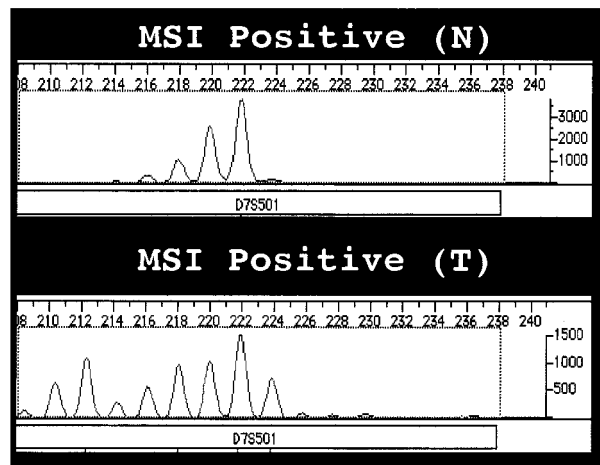


Fig. 1 Electropherogram using microsatellite markers for MSI analysis. The tumor sample (T) from the MSI-positive case shows far more numerous extra peaks as compared with the normal sample (N).

for each tumor. Based on microscopic observation of the H&E-stained section, tumor cells and corresponding nontumorous cells, *i.e.*, acinar cells or islet cells, were respectively collected by microdissection with a sterile razor blade. The excised samples were placed in microtubes, immersed in GeneReleaser (BioVentures, Inc., Murfreesboro, TN), and incubated at the appropriate temperature following the manufacturer's instructions, and then proteinase K (Life Technologies, Inc., Rockville, MD) was added, followed by incubation at 55°C for 1 week. A neoplastic cellularity ranging from 70–90% was obtained in all cases.

**MSI Analysis.** Primer sets for the eight microsatellite loci examined in this study were *D2S123*, *D3S1611*, *D5S346*, *D7S501*, *NM23*, *TP53-Penta*, *TP53-Dint*, and *D18S35* (Applied Biosystems, Foster City, CA). We used a 6- $\mu$ l aliquot of reaction mixture containing 0.4  $\mu$ l of DNA extracted by microdissection from a tumor or normal tissue, 4.5  $\mu$ l of each primer marker set, 0.06  $\mu$ l of AmpliTaq Gold (Applied Biosystems), and 1.04  $\mu$ l of distilled water. PCR conditions were as follows: 95°C for 10 min followed by 50 cycles of 96°C for 10 s, 55°C for 30 s, and 70°C for 3 min; with a final elongation at 70°C for 30 min. After amplification, all marker reaction products for each tissue samples were mixed. Then, 1  $\mu$ l of TAMRA (Applied Biosystems) and 12  $\mu$ l of deionized formamide were added to 2  $\mu$ l of the mixture. The mixed sample was heated at 95°C and immediately cooled on ice and then analyzed by the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The definition of MSI was estimated according to the manufacturer's manual: peak height of the relevant microsatellite loci for independent injections of each normal and each tumor sample was obtained. Microsatellite variation was determined when the electropherogram of informative microsatellite markers showed extra peaks in the tumor sample (Fig. 1). A tumor observed to show microsatellite variation at three or more of the eight loci was scored MSI positive. MSI status was confirmed in all cases in duplicate experiments using independently extracted DNA samples.

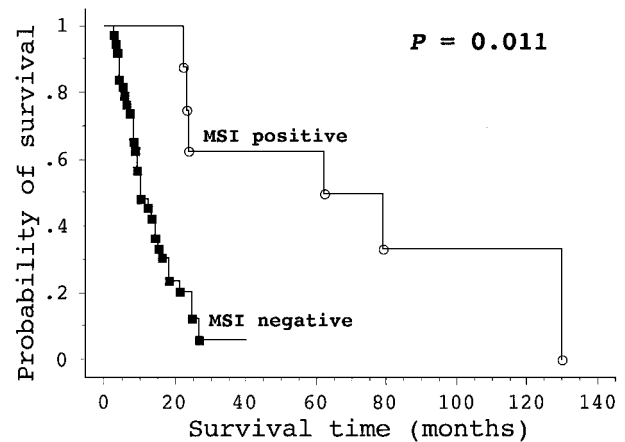


Fig. 2 Probability of survival for resectable pancreatic cancer patients in relation to the MSI status of their tumor. A statistically significant difference in survival rate was observed between the MSI-positive and MSI-negative groups.

**Evaluation of TIL.** The majority of TILs are T cells, although B cells, plasma cells, and natural killer cells are also present in cancer tissues. To detect T cells in tissues, we performed immunohistochemical staining using the avidin-biotin-peroxidase complex method. Formalin-fixed and paraffin-embedded tissues, which were used for MSI analysis, were prepared in 4- $\mu$ m-thick sections. The tissues were heated for 15 min at 121°C by autoclave in Target Retrieval Solution (DAKO, Carpinteria, CA). Slides were incubated for 30 min at room temperature with a 1:25 dilution of the antihuman CD3 mouse monoclonal antibody PC3/188A (DAKO). A Catalyzed Signal Amplification System (DAKO) was used to strengthen the avidin-biotin-peroxidase complex reaction. The number of CD3<sup>+</sup> lymphocytes and cancer cells in the 10 most infiltrated fields of  $\times 200$  magnification were counted, and the five highest values were averaged to obtain the final lymphocyte score/1000 cancer cells.

**Statistical Analysis.** The survival data were estimated by the Kaplan-Meier method and examined by log-rank test for the purpose of univariate survival analysis. The Cox proportional hazards model was used for the multivariate analysis of survival. The  $\chi^2$  test (Fisher's exact method) was used to compare the two independent groups. We used the unpaired *t* test to compare ages and CD3<sup>+</sup> lymphocyte scores between the two groups. All reported *P*s were two-sided, and *P*s < 0.05 were considered statistically significant.

## RESULTS

**Univariate Analysis to Assess Influence of MSI on Survival Rates in Pancreatic Cancer Patients.** MSI positivity was detected in 8 of 46 (17.4%) resectable pancreatic cancers. The median survival term of patients with MSI-positive tumors was significantly longer than that of patients with MSI-negative tumors (Fig. 2). There were no statistical differences in background between MSI-positive and MSI-negative groups (Table 1). Table 2 shows the predictive values of clinicopathological factors in relation to survival rates in patients with resectable

Table 1 Characteristics of patients with resectable pancreatic cancer stratified by MSI

Features	MSI		P <sup>a</sup>
	Positive	Negative	
Age (yrs)	68.3 ± 8.4	62.5 ± 11.0	0.170
Sex			
Male	7	26	0.409
Female	1	12	
Histology <sup>b</sup>			
G1	5	17	0.451
G2/G3/G4	3	21	
T factor <sup>c</sup>			
T <sub>1</sub> /T <sub>2</sub>	3	10	0.669
T <sub>3</sub> /T <sub>4</sub>	5	28	
N factor			
N <sub>0</sub>	4	10	0.223
N <sub>1a</sub> /N <sub>1b</sub>	4	28	
M factor			
M <sub>0</sub>	8	33	0.569
M <sub>1</sub>	1	5	

<sup>a</sup> P<sub>s</sub> were examined by Student's *t* test for age and  $\chi^2$  test (Fisher's exact method) for the other features.

<sup>b</sup> Histology was divided according to UICC classification (G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; and G4, undifferentiated).

<sup>c</sup> TNM classification by UICC staging.

pancreatic cancer as analyzed by univariate analysis. Patients with T<sub>3</sub>/T<sub>4</sub> tumors (extending directly into surrounding organs or adjacent large vessels), lymph node metastasis, or distant metastasis had significantly shorter survival than those whose tumors lacked these features. However, histological findings did not have an influence on prognosis when tumors were divided into G1 and G2–4 tumors according to UICC classification.

**Multivariate Analysis to Assess Influence of MSI on Survival.** The clinicopathological factors and MSI status of the tumors were analyzed using the Cox proportional hazards model. MSI negativity was an independent and strong indicator that correlated significantly with worse prognosis for patients with resectable pancreatic cancer (Table 3).

**TIL Intensity Stratified by MSI Status.** The mean of CD3<sup>+</sup> lymphocyte scores in MSI-positive tumors was significantly greater than that in MSI-negative tumors (mean ± SD, 114.6 ± 52.3 versus 37.9 ± 20.9, respectively; *P* < 0.0001).

**DISCUSSION**

There have been several reports concerning MSI in pancreatic cancer. The frequencies of MSI-positive pancreatic cancer vary from 3.7–67% (21, 24–26). Wilentz *et al.* (27) reported that medullary carcinoma of the pancreas, which they named for a histologically distinct entity from poorly differentiated carcinoma, showed 22% MSI positivity in 18 tumors, whereas non-medullary carcinomas showed no MSI in any of 77 tumors. The wide range of MSI incidence reported in pancreatic cancer may be due to differences in the numbers and locations of the loci tested and to discrepancies in methods of assessment. The criteria set out by the National Cancer Institute Workshop on MSI are recommended to be applied only to colorectal neoplasms (28). However, the definition of MSI-H, defined as microsatellite variation in  $\geq 30$ –40% of the markers tested, has

Table 2 Univariate analysis of prognostic indicators in resectable pancreatic cancer patients

Variable	No. of patients	Median survival (mo)	P (log-rank test)
Histology <sup>a</sup>			
G1	22	18	0.329
G2/G3/G4	24	10	
T factor <sup>b</sup>			
T <sub>1</sub> /T <sub>2</sub>	13	25	0.026
T <sub>3</sub> /T <sub>4</sub>	33	10	
N factor			
N <sub>0</sub>	14	22	0.047
N <sub>1a</sub> /N <sub>1b</sub>	32	10	
M factor			
M <sub>0</sub>	41	15	0.048
M <sub>1</sub>	5	4	
MSI			
Positive	8	62	0.011
Negative	38	10	

<sup>a</sup> Histology was divided according to UICC classification (G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; and G4, undifferentiated).

<sup>b</sup> TNM classification by UICC staging.

been recognized to be useful in assessment of other types of malignancy. On the other hand, there has been no supportive evidence that MSI-L, defined as microsatellite variation in <30–40% and >0% of the markers tested, is caused by defective DNA mismatch repair (22). We classified MSI status as MSI positive or MSI negative; MSI positivity was defined a microsatellite variation at three or more of eight loci. The MSI markers *BAT25*, *BAT26*, *D5S346*, *D2S123*, and *D17S250* are recommended for MSI analysis in colorectal cancer. However, MSI markers specific for other malignancies, including pancreatic cancer, have not yet been established. We selected these eight MSI markers based on their uniqueness as nonisotopic MSI markers and their commercial availability. In our study, 17.4% of pancreatic cancer specimens were MSI positive, and this percentage is consistent with the findings in recent reports of MSI in Japanese cases of pancreatic cancer. Ouyang *et al.* (29) reported MSI positivity in 9 of 60 (15%) pancreatic cancers, which was demonstrated by altered-size bands in two or more loci among five or more MSI markers. Yamamoto *et al.* (22) demonstrated MSI-H in 13% of 100 sporadic pancreatic cancers, with positivity determined according to the NCI workshop criteria.

To our knowledge, previous studies on MSI as a prognostic predictor of patients with resectable pancreatic cancer are very limited. Although they did not show statistically analyzed data for the prognosis, Goggins *et al.*, (21) suggested that patients with MSI-positive pancreatic cancer might have a comparatively better prognosis. More recently, Yamamoto *et al.* (22) reported that 7 patients with MSI-H tumors had a significantly longer survival than 33 patients with MSI-L or microsatellite stable tumors, based on findings of univariate analysis in stage II or III pancreatic cancer. Their results are consistent with those from our series of 46 resectable pancreatic cancer patients; we showed that MSI had predictive value comparable to that of traditional clinicopathological features, such as invasion of surrounding organs (T factor), lymph node metastasis (N factor),

Table 3 Multivariate analysis of prognostic indicators in resectable pancreatic cancer patients by the Cox proportional hazards model

Variable	Coefficient	SE	P	95% CI	Hazard ratio
Histology <sup>a</sup> (G2–G4)	0.201	0.352	0.567	0.613–2.439	1.223
T factor <sup>b</sup> (T <sub>3</sub> /T <sub>4</sub> )	0.853	0.461	0.064	0.950–5.789	2.346
N factor (N <sub>1a</sub> /N <sub>1b</sub> )	0.301	0.452	0.506	0.557–3.277	1.351
M factor (M <sub>1</sub> )	0.195	0.538	0.718	0.423–3.490	1.215
MSI negative	1.719	0.637	0.007	1.601–19.431	5.577

<sup>a</sup> Histology was divided according to UICC classification (G2, moderately differentiated; G3, poorly differentiated; and G4, undifferentiated).

<sup>b</sup> TNM classification by UICC staging.

and distant metastasis (M factor), as determined by univariate analysis for survival rates. The median survival term of patients with MSI-positive tumors was longer than that of patients with MSI-negative tumors, although no significant differences in clinicopathological background were found between the groups. Furthermore, we examined the independent prognostic predictive values of MSI status. Multivariate survival analysis showed that MSI negativity is an independent and powerful indicator of poor prognosis in resectable pancreatic cancer patients.

Thus far, it has been demonstrated in colorectal cancer that a number of unrepaired errors in genes including the HLA locus gene could lead to the appearance of new surface molecules, which trigger the T-cell-mediated immune response to improve survival (30–32). In this study, TIL intensity in the MSI-positive tumor group was significantly stronger than that in the MSI-negative tumor group. The mechanism by which MSI positivity contributes to longer survival in resectable pancreatic cancer patients remains unknown; it may be associated with an enhanced immunogenicity in pancreatic cancers that are deficient in DNA replication error repair.

Taking all of the above-mentioned results into consideration, we conclude that MSI positivity in resectable pancreatic cancer may be one of the most reliable predictors of a favorable prognosis.

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