Distal Colorectal Cancers with Microsatellite Instability (MSI) Display Distinct Gene Expression Profiles that Are Different from Proximal MSI Cancers

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Abstract

Promoter methylation of the mismatch repair gene plays a key role in sporadic microsatellite instability (MSI) colorectal cancers. However, promoter methylation often occurs in proximal colon cancers, and molecular phenotypes underlying MSI cancers in distal colon have not been fully clarified. Our goal was to clarify the difference between MSI and microsatellite stability (MSS) cancers and, furthermore, to determine distinct characteristics of proximal and distal MSI cancers. By DNA microarray analysis of 84 cancers (33 MSI and 51 MSS), we identified discriminating genes (177 probe sets), which predicted MSI status with a high accuracy rate (97.6%). These genes were related to phenotypic characteristics of MSI cancers. Next, we identified 24 probe sets that were differentially expressed in proximal and distal MSI cancers. These genes included promoter methylation-mediated genes, whose expression was significantly down-regulated in proximal MSI cancers. Among discriminating genes between MSI and MSS, nine methylation-mediated genes showed down-regulation in MSI cancers. Of these, 7 (77.8%) showed down-regulation in proximal MSI cancers. Furthermore, methylation-specific PCR confirmed that frequency of *hMLH1* promoter methylation was significantly higher in proximal MSI cancers (P = 0.0317). These results suggested that there is a difference between proximal and distal MSI cancers in methylation-mediated influence on gene silencing. In conclusion, using DNA microarray, we could distinguish MSI and MSS cancers. We also showed distinct characteristics of proximal and distal MSI cancers. The inactivation form of *hMLH*, per se, differed between proximal and distal MSI cancers. These results suggested that distal MSI cancers constitute a distinct subgroup of sporadic MSI cancers. (Cancer Res 2006; 66(20): 9804-8)

Introduction

Microsatellite instability (MSI) cancers have unique characteristics compared with microsatellite stability (MSS) cancers, such as proximal anatomic location and severe inflammatory cell infiltration (1, 2). We have also shown that MSI cancers show better prognosis compared with MSS cancers (3). In the carcinogenesis of MSI cancers, mononucleotide repeat is considered to be the target site, whereas the chromosomal instability pathway underlies MSS cancers (4). With respect to the disruption of mismatch repair system, promoter methylation plays a key role in most sporadic MSI cancers, whereas germ-line mutation is the most common cause in hereditary nonpolyposis colon cancer (HNPCC). Most of the promoter methylation of the genes is known to occur in proximal colon cancers (5). This accounts for why many sporadic MSI cancers show proximal predominance. However, some sporadic MSI cancers are located in the distal colon and the molecular phenotypes underlying distal MSI cancers have not been fully clarified.

Recent advances in DNA microarray have shown the potential use of expression profiles for molecular classification of cancer as well as disease outcome (6). We too have reported recently that gene expression profiles could be used for prediction of response to radiotherapy in rectal cancer (7). Using the same strategy, we conducted DNA microarray analysis to identify novel genes whose expressions differ significantly in MSI and MSS cancers. We then clarified the difference between proximal and distal MSI cancers in gene expression profiles. Several studies have examined expression profiles of MSI cancers using DNA microarray. However, these studies either examined MSI cancer cell lines, or the number of MSI cancer samples was comparatively small (8–13). Furthermore, no studies have highlighted the difference between proximal and distal MSI cancers with regard to gene expression profiles. To the best of our knowledge, the present study examined the largest number of MSI cancers and showed distinct expression signatures for MSI cancers. Furthermore, we showed for the first time a significant difference between proximal and distal MSI cancers in gene expression profiles. Our goal in the current study was to clarify the difference in gene expression between MSI and MSS cancers and, furthermore, to determine the distinct characteristics of proximal and distal MSI cancers in global molecular phenotypic data.

Materials and Methods

Patient Samples

Informed consent was obtained from patients for the collection of specimens, and the study protocol was approved by the local Ethics Committee. Eighty-four patients who had undergone surgical resection of colorectal cancer were studied. Familial adenomatous polyposis and HNPCC patients were excluded from the study (14). Specimens were taken

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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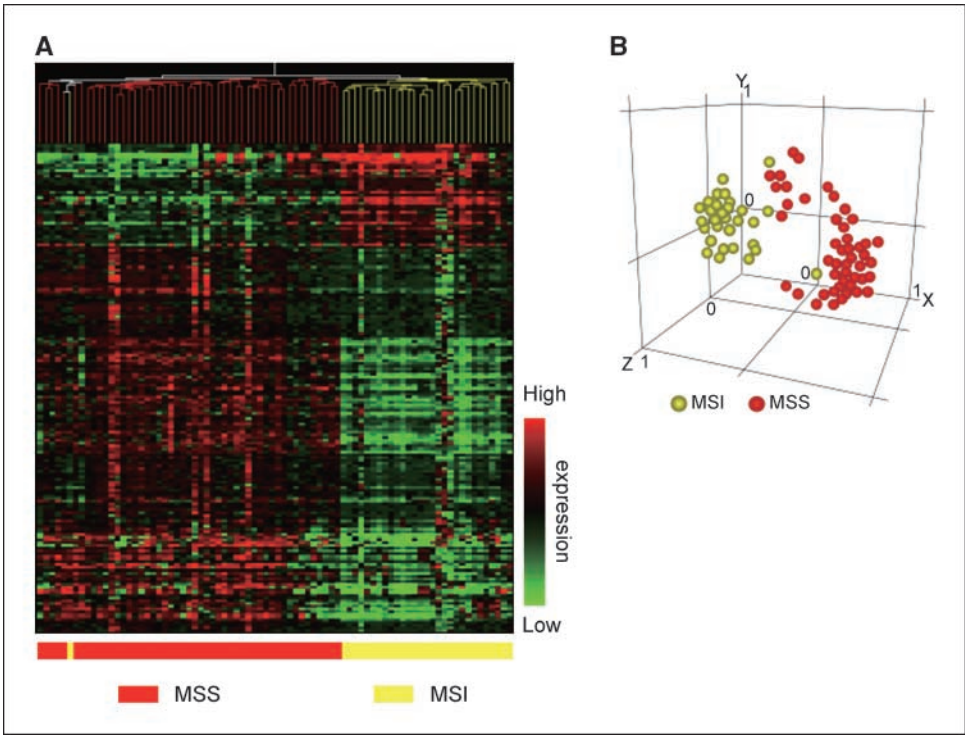

Figure 1. Supervised clustering and PCA of MSI and MSS cancers, using 177 probe sets. *A,* two-way hierarchical clustering was used to order samples (*columns*) and array targets (*rows*). *Red,* overexpression; *green,* underexpression. *Bottom, yellow,* MSI cancers; *red,* MSS cancers. Samples were classified correctly into MSI and MSS, except one MSI cancer. *B,* discriminating genes were used to generate a three-dimensional (from 177-dimensional plot) plot of the data. *X, Y,* and *Z axes,* first three principal components fitted to the MSI and MSS molecular profile data. The cumulative proportion of the variance captured by each principal component axis is (*a*) principal component axis, 48.02%; (*b*) principal component axis, 5.51%; and (*c*) principal component axis, 3.93%. PCA-based multidimensional scaling visualization separated MSI (*yellow*) and MSS (*red*) samples into linearly separable 177-gene expression data space.

from cancers and corresponding normal tissues in surgically resected specimens and were snap frozen in liquid nitrogen and stored at −80°C until use. Paralleled tumor specimens were formalin fixed and paraffin embedded for histologic examination. MSI tumor samples were classified as either proximal colon (ascending, transverse colon) or distal colon (descending, sigmoid colon and rectum).

DNA Isolation and Determination of MSI

DNA was extracted from paired tumor and normal tissue using frozen samples. Analysis of MSI was done using the following loci: BAT25, BAT26, D2S123, D5S346, and D17S250. MSI status was determined according to the criteria of the National Cancer Institute workshop as described previously (15, 16). Samples were classified as MSI high (MSI-H) when at least 40% of loci showed MSI.

RNA Isolation and Microarray Procedures

Total RNA was isolated from each of the frozen samples using RNeasy Mini kit (Qiagen, Chatswort, CA) for gene expression analysis. Gene expression profiles were determined using Affymetrix HGU133A and HGU133B GeneChip (Affymetrix, Santa Clara, CA) according to the manufacturer's recommendations as described previously (7). The entire microarray data set is available online under the data series accession number GSE4554.[6]

Analysis of MSI versus MSS Cancers

Microarray analysis. Expression analysis was carried out on GeneSpring software version 7.2 (Silicon Genetics, Redwood, California). Gene expression data, when classified as either flag-P or flag-M in >30% of all samples, were loaded into the software. All expression data on a chip were normalized to the 50th percentile of all values on that chip followed by normalization to the median expression level of that gene across all samples. To identify discriminating genes, the expression profiles of MSI and MSS cancers were compared, using Welch's *t* tests with Benjamini and Hochberg false discovery rate (FDR) and fold-change. Two-dimensional hierarchical clustering was then applied to the log-transformed data with average-linkage clustering with standard correlation as the similarity metric for the discriminating genes that we identified as differentially expressed in MSI and MSS cancers. Variation in multigene expression between MSI and MSS cancers was also compared by principal component analysis (PCA). Next, we carried out supervised class prediction using the k-nearest neighbor (KNN) method, support vector machine (SVM), and a leave-one-out cross-validation with the discriminating genes (17).

Gene functional category analysis of discriminating genes between MSI and MSS cancers. Gene Ontology categories were analyzed by the BioScript Library tool on GeneSpring version 7.2. Genes were classified according to their annotated role in biological processes, molecular function, and cellular components from Gene Ontology (The Gene Ontology Consortium). A hypergeometric *P* was used to measure statistical significance of the overlap (i.e., the likelihood that it is a coincidence that this many genes were in both the experimentally extracted gene list and the category).

Analysis of the Proximal and Distal MSI Cancers

Microarray analysis. Comparative gene expression analysis and gene functional category analysis of discriminating genes were carried out for proximal and distal MSI cancers, with the same method used in the analysis of MSI and MSS cancers as described above.

Methylation-specific PCR of hMLH1. After modifying DNA with sodium bisulfite, we did methylation-specific PCR (MSP) and determined the methylation status of *hMLH1* as described previously (Supplementary Fig. S1; ref. 16). Proximal and distal MSI cancers were compared for promoter methylation of *hMLH1*.

Results

Analysis of MSI and MSS Cancers

Gene expression profiling: MSI versus MSS cancers. Among 592 patients, MSI-H was found in 58 (9.8%) patients, MSI low (MSI-L) was found in 62 (10.5%) patients, and MSS was found in 472 (79.7%) patients. To identify molecular signatures of MSI cancers, we examined only MSI-H cancers and not MSI-L ones. Furthermore, because genetic alteration accumulates as the tumor

[6] http://www.ncbi.nlm.nih.gov/geo/info/linking.html.

Table 1. List of discriminating genes (24 probe sets) between proximal and distal MSI cancers

Probe ID	Fold change*	*P*	Gene symbol	Genbank	Gene name
227736_at	12.01	0.00906		AA553959	Homo sapiens clone DNA92219 RLLV1833 (UNQ1833)
213369_at	11.39	0.00906	*KIAA1775*	AI825832	MT-protocadherin
209994_s_at	9.307	0.00906	*ABCB1*	AF016535	ATP-binding cassette, subfamily B (MDR/TAP)
209993_at	8.661	0.00906	*ABCB1*	AF016535	ATP-binding cassette, subfamily B (MDR/TAP)
243951_at	5.361	0.00906	*ABCB1*	AA887211	ATP-binding cassette, subfamily B (MDR/TAP)
210115_at	5.313	0.000392	*RPL39L*	L05096	Ribosomal protein L39-like
217281_x_at	4.948	0.00906	*IGHG3*	AJ239383	Immunoglobulin heavy constant γ3 (G3m marker)
206211_at	4.912	0.00906	*SELE*	NM_000450	Selectin E (endothelial adhesion molecule 1)
221286_s_at	4.513	0.00906	*PACAP*	NM_016459	Proapoptotic caspase adaptor protein
230389_at	4.023	0.00906	*FNBP1*	BE046511	Formin binding protein 1
204871_at	3.733	0.00906	*MTERF*	NM_006980	Transcription termination factor, mitochondrial
235580_at	3.691	0.00906		AW272167	Homo sapiens cDNA clone IMAGE:2768440
226435_at	3.485	0.00906	*PAPLN*	AU145309	Papilin, proteoglycan-like sulfated glycoprotein
220023_at	3.167	0.00906	*APOB48R*	NM_018690	Apolipoprotein B48 receptor
222859_s_at	2.827	0.00906	*DAPP1*	AA150186	Dual adaptor of phosphotyrosine and 3-phosphoinositides
221205_at	2.806	0.00906		NM_018041	Homo sapiens hypothetical protein FLJ10254 (FLJ10254)
204797_s_at	2.508	0.00906	*EML1*	NM_004434	Echinoderm microtubule associated protein like 1
212288_at	2.179	0.00906	*FNBP1*	AB011126	Formin binding protein 1
207440_at	2.178	0.00906	*SLC35A2*	NM_005660	Solute carrier family 35 (UDP-galactose transporter)
207943_x_at	2.123	0.00906	*PLAGL1*	NM_006718	Pleiomorphic adenoma gene-like 1
238079_at	2.121	0.00906		AV713323	Homo sapiens, clone IMAGE:6200207, mRNA
229912_at	2.069	0.00906	*SDK1*	AL042166	Sidekick homologue 1 (chicken)
205159_at	2.026	0.00906	*CSF2RB*	AV756141	Colony-stimulating factor 2 receptor, β, low-affinity
225380_at	0.246	0.00906	*LOC91461*	BF528878	Hypothetical protein BC007901

*Fold change: distal MSI cancers/proximal MSI cancers.

shows progression, we compared expression profiles of MSI-H cancers with those of tumor stage–matched MSS cancers (Supplementary Table S1). After RNA extraction procedures, we could establish gene expression profiles using DNA microarray in 33 of 58 MSI-H and in 51 of 66 MSS cancers. Using class-comparison analysis, we identified a list of genes (177 probe sets) that were differentially expressed at significant levels ($P < 0.05$) in MSI and MSS cancers (Supplementary Table S2). Thirty-seven probe sets showed higher and 140 lower expression in MSI compared with MSS cancers. Mucin 5 (MUC5AC), apoptosis-related gene (CASP2), immunomodulatory genes (MSTP9 and MST1), and tumor suppressor genes (TFAP2A and CRIP1) showed over-expression in MSI cancers. On the other hand, insulin-like growth factor 2 (IGF2), βGlcNAc (B4GALT1), and WNT inhibitory factor 1 (WIF1) showed lower expression in MSI cancers. Using a hierarchical cluster analysis, the MSI status was correctly classified in 83 of 84 (98.9%) samples (Fig. 1*A*). We also used discriminating genes to generate a three-dimensional plot of the data. The three axes are the first three principal components fitted to the patient's molecular profile data. PCA-based multidimensional scaling visualization separated MSI and MSS cancers into a linearly separable gene expression data space (Fig. 1*B*).

Gene functional category analysis of discriminating genes between MSI and MSS cancers. Discriminating genes are associated with various functions, including translation regulator activity, chemokine activity, cell-cell signaling, apoptosis program, and others. When selected discriminating genes were compared with all genes whose expression profile could be evaluated, categories of cellular lipid metabolism (eight genes; $P = 0.00387$), cell-cell signaling (eight genes; $P = 0.00171$), apoptotic program (two genes; $P = 0.0245$), and translation initiation factor activity (three genes; $P = 0.0153$) showed a significantly higher proportion among selected genes.

Gene expression profiling: class prediction of MSI and MSS cancers. Using all samples, we carried out supervised class prediction by the KNN, SVM, and a leave-one-out cross-validation with the 177 probe sets. The accuracy of class prediction was 97.6% (82 of 84 correct calls) by KNN and 96.4% (81 of 84 correct calls) by SVM.

Analysis of Proximal and Distal MSI Cancers

Gene expression profiling: proximal MSI cancers versus distal MSI cancers. To identify molecular signatures of MSI cancers with respect to the tumor location, the gene expression profiles of proximal (22 lesions) and distal (9 lesions) MSI cancers were compared. Using class-comparison analysis, we identified 24 probe sets that were differentially expressed at significant levels ($P < 0.05$) between proximal and distal MSI cancers (Table 1). We did class prediction of proximal and distal MSI cancers by the KNN, SVM, and a leave-one-out cross-validation with the 24 probe sets. The accuracy of class prediction was 90.3% (28 of 31 correct calls) by KNN and 100.0% (31 of 31 correct calls) by SVM. Of 24 probe sets, 23 (95.8%) showed higher expression in distal MSI cancers. Some genes were related to human malignancies, including breast, gastric, and colon cancers (*EML, PLAGL1, ABCB1, KIAA1775, FNBP1, SELE*, and *PACAP*). A hierarchical cluster analysis using 24 probe sets confirmed the rather successful classification of proximal and distal MSI cancers (Fig. 2*A*). PCA analysis also separated proximal MSI cancers and distal MSI cancers (Fig. 2*B*). Most of the probe sets [(23 of 24) 95.8%] showed higher expression in distal MSI cancers than in proximal MSI cancers. Genes that showed lower expression in the proximal colon included *ABCB1*

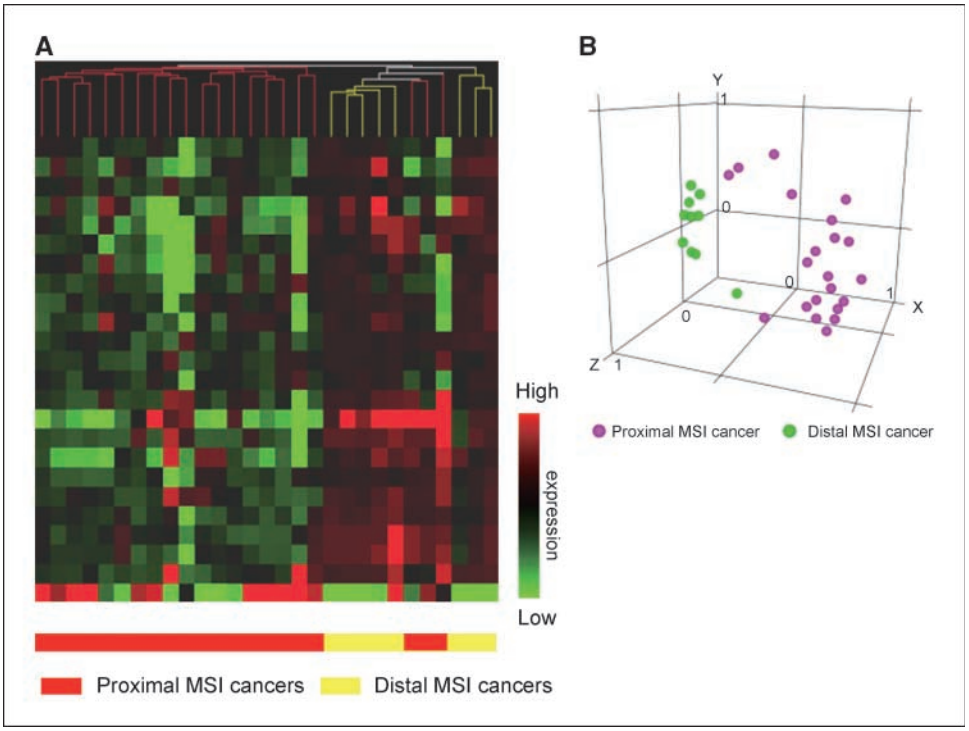

Figure 2. Supervised clustering and PCA of proximal and distal MSI cancers, using 24 probe sets. *A,* two-way hierarchical clustering was used to order samples (*columns*) and array targets (*rows*). *Red,* overexpression; *green,* underexpression. *Bottom, yellow,* MSI cancers; *red,* MSS cancers. Samples were classified into proximal and distal MSI cancers. *B,* discriminating genes were used to generate a three-dimensional (from 24-dimensional plot) plot of the data. *X, Y,* and *Z axes,* first three principal components fitted to the proximal and distal MSI cancer molecular profile data. The cumulative proportion of the variance captured by each principal component axis is (*a*) principal component axis, 41.1%; (*b*) principal component axis, 11.27%; and (*c*) principal component axis, 8.33%. PCA-based multidimensional scaling visualization separated proximal MSI (*purple*) and distal MSI (*green*) cancer samples into linearly separable 24-gene expression data space.

(fold change, 8.661; $P = 0.0091$) and *PLAGL1* (fold change, 2.123; $P = 0.00906$), which have been reported to be down-regulated in various cancers by their promoter methylation.

Comparison of expression level of methylation-related genes in proximal and distal MSI cancers. We next compared the expression level of methylation-related genes in proximal and distal MSI cancers. An on-line public database search using PubMed and keywords related to methylation for each gene targeted by a probe set revealed that 11 of 177 probe sets were targets of promoter methylation-mediated gene silencing, all of which showed lower expression in MSI cancers (Table 2). In 8 of 11 (72.7%) probe sets or 7 of 9 (77.8%) genes, distal MSI cancers showed higher expression than proximal MSI cancers.

Comparison of expression level and methylation status of *hMLH1* by MSP in proximal and distal MSI cancers. Distal MSI cancers showed significantly higher expression than proximal ones (fold change, 2.49; FDR $P = 0.00159$). We further compared promoter methylation of *hMLH1* by MSP. Distal MSI cancers showed a significantly lower rate of promoter methylation in hMLH1 than proximal MSI cancers [80.0% (16 of 20) versus 33.3% (3 of 9); $P = 0.0317$, Fisher's exact test].

Discussion

Using DNA microarray, we identified gene expression signatures that could distinguish MSI from MSS cancers. Furthermore, we showed that gene expression profiles differ significantly in proximal and distal MSI cancers. To date, the precise characteristics of distal MSI cancer have not been elucidated. Our study is the first to show the distinct characteristics of distal MSI cancers.

Table 2. List of promoter methylation-mediated genes (11 probe sets) and its expression between proximal and distal MSI cancers

Probe ID	Fold change MSI/MSS	Fold change Distal MSI/proximal MSI	Gene symbol	Genbank	Gene name
1555086_at	0.239	1.937	*STAT5B*	BC020868	Signal transducer and activator of transcription 5B
202410_x_at	0.236	2.348	*IGF2*	NM_000612	Insulin-like growth factor 2 (somatomedin A)
204712_at	0.207	0.695	*WIF1*	NM_007191	WNT inhibitory factor 1
205892_s_at	0.146	1.764	*FABP1*	NM_001443	Fatty acid binding protein 1, liver
206754_s_at	0.132	0.644	*CYP2B6*	NM_000767	Cytochrome *P*450, family 2, subfamily B, polypeptide 6
206755_at	0.247	0.879	*CYP2B6*	NM_000767	Cytochrome *P*450, family 2, subfamily B, polypeptide 6
218963_s_at	0.0649	1.336	*KRT23*	NM_015515	Keratin 23 (histone deacetylase inducible)
226400_at	0.227	1.262	*CDC42*	N92917	Cell division cycle 42 (GTP binding protein)
231693_at	0.229	1.239	*FABP1*	AV655991	Fatty acid binding protein 1, liver
234702_x_at	0.24	2.589	*CFTR*	S64699	Cystic fibrosis transmembrane conductance regulator, ATP-binding cassette (subfamily C, member 7)
244023_at	0.158	1.084	*SYK*	AW467357	Spleen tyrosine kinase

First, by comparing gene expression profiles in MSI and MSS cancers, we identified discriminating genes (177 probe sets). With this gene set, two-way hierarchical clustering correctly classified 84 colon cancers into MSI or MSS, except for one case, and PCA analysis also clearly separated MSI and MSS cancers. Furthermore, by KNN and SVM, MSI status could be predicted with an accuracy of 97.6% and 96.4%, respectively. Overexpressed discriminating genes were related to apoptosis (CASP2) or to previously reported phenotypic characteristics, such as intense immune response (MSTP9 and MST1) and increased mucin production (MUC5AC) unique to MSI cancers. On the other hand, down-regulated genes included *IGF2*, as has been described previously (18). These genes were considered to be highly associated determining the characteristics of MSI cancers.

We then identified genes (24 probe sets), whose expression differed significantly between MSI cancer in proximal and distal colon. Using these genes, two-way hierarchical clustering and PCA analysis distinguished proximal from distal MSI cancers. One of the top-ranked discriminating genes was *ABCB1*, whose expression is associated with outcome or drug sensitivity in human malignancies (19). *ABCB1* showed significantly higher expression in distal MSI cancers (fold change, 9.307; $P = 0.00906$). *PLAGL1*, candidate tumor suppressor gene, also showed higher expression in distal MSI cancers (fold change, 2.123; $P = 0.00906$). The expression levels of ABCB1 and PLAGL1 are down-regulated by promoter methylation (20). Although epigenetic changes have been reported to be important in the carcinogenesis of MSI cancers, these results suggest that there might be a difference between proximal and distal MSI cancers in methylation-mediated influence on gene silencing.

Therefore, we examined the difference between proximal and distal MSI cancers in expression level of other methylation-related genes. Among MSI discriminating genes (177 probe sets), we identified nine methylation-mediated genes, which showed lower expression in MSI cancers. Of these, 7 genes (77.8%) showed lower expression in proximal MSI cancers. We then focused on *hMLH1*. By microarray analysis, distal MSI cancers showed a significantly higher expression of *hMLH1* than proximal ones (fold change, 2.49; FDR $P = 0.0159$). We further showed that distal MSI cancers also showed a significantly lower frequency of promoter methylation in *hMLH1* than proximal MSI cancers ($P = 0.0317$). Although previous studies reported that the majority of sporadic MSI cancers show promoter methylation of *hMLH1*, we showed that there exists a significant difference between proximal and distal MSI cancers.

These results suggested that epigenetic pathways seem to have a smaller role in the carcinogenesis of distal MSI cancers compared with proximal MSI cancers. Using a different gene expression-based approach, Mori et al. (12) identified genes inactivated through promoter methylation in MSI cancers. They showed that expression of *RAB32* and *PTPRO* was significantly down-regulated due to promoter methylation in MSI cancers. They also showed that the frequency of promoter methylation of these genes was higher in the proximal colon, which agrees with our results. In the present study, we also showed that the frequency of promoter methylation of *hMLH1* and other methylation-mediated genes was higher in proximal MSI cancers.

In summary, using DNA microarray, we identified a significant difference between MSI and MSS cancers. Furthermore, we showed that proximal and distal MSI cancers show distinct expression profiles. The inactivation form of the *hMLH* gene, per se, differed in proximal and distal MSI cancers. These expression signatures may represent differences in the development of proximal and distal MSI cancers. Distal MSI cancers may constitute a distinct subgroup of sporadic MSI cancer. These signatures provide new insights into the role of genetic instability in cancer development and may suggest new strategies for diagnosis and therapeutic intervention.

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