[CANCER RESEARCH 54, 3853–3856, July 15, 1994]

Genetic Diagnosis Identifies Occult Lymph Node Metastases Undetectable by the Histopathological Method

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ABSTRACT

The mutant allele-specific amplification (MASA) method is capable of detecting one tumor cell containing genetic changes in a sample containing thousands of normal cells. To investigate whether MASA can be applied to sensitive detection of lymph node metastasis, we screened 22 colorectal cancers for K-*ras* and *p53* mutations and examined corresponding regional lymph node at the genetic level by the MASA method. Six of the primary tumors were found to certain K-*ras* mutations, and nine exhibited mutations of the *p53* gene. In seven of the 14 cases in which genetic alterations were identified (mutations in both genes were found in one tumor), we found discrepancies between the genetic and the histopathological diagnoses with respect to the presence or absence of cancer cells in lymph nodes, in that these patients were histologically diagnosed lymph node negative, hn(−) but genetically diagnosed lymph node positive, gn(+). Because disease recurs in 20–30% of cancer patients whose lymph nodes are histopathologically negative after surgery, genetic evaluation of lymph nodes for metastasis may become a useful prognostic indicator.

INTRODUCTION

Colorectal cancer is the third most common malignancy in the world, with an estimated 570,000 new cases in 1993. Although colorectal cancers diagnosed at an early stage can usually be cured by surgical or colonoscopic excision, individuals with advanced disease have a poor prognosis. Several prognostic factors have already been identified; for example, the presence of metastatic tumors in the regional lymph nodes of a patient who has undergone resection of a colorectal cancer is of major prognostic significance. On the basis of conventional histology, the mean 5-year survival of patients without lymph node metastasis is reported as 70–80%, whereas it is 30% for those with metastatic deposits (1, 2). Thus, even among patients without histologically detectable lymph node metastasis, 20–30% will die as a result of distant metastases or local recurrences. These figures show that routine histological techniques often fail to detect occult metastases and may result in falsely optimistic tumor staging.

In recent years, a number of alterations in genes such as *APC*, K-*ras*, *p53*, and *DCC* have been identified in colorectal cancers. Mutations in oncogenes other than K-*ras*, and losses of heterozygosity of various loci in tumors, have indicated a potential for many tumor-specific markers (3, 4). K-*ras* mutations occurring at codons 12, 13, or 61 are present in approximately 40–50% of colorectal cancers (5). Mutations in this oncogene can be observed at a relatively early stage of carcinogenesis in large adenomas as well as in carcinomas, and they can serve as clonal markers for the tumor cell populations (6, 7). Inactivation of the tumor suppressor gene *p53* is considered to play a role in malignant transformation of an adenoma cell to a cancer cell in the colon or rectum. In 75–80% of colorectal cancers, both *p53* alleles have been inactivated, usually one through partial or complete loss of chromosome 17p and the other through a point mutation (8). Mutations found in recurrent tumors may also serve to establish their clonal origin with respect to a primary tumor, when the mutation found in both tissues is identical (9).

We recently reported a highly sensitive method for detecting K-*ras* mutations in DNA samples isolated from sputum. By this technique, MASA[2] (10), one tumor cell containing genetic changes can be detected in a background of thousands of normal cells. Hence, we hypothesized that this method should allow genetic diagnosis of occult metastases whenever the molecular alteration in the primary tumor can be identified. To examine this possibility, we looked for mutations in K-*ras* (codons 12, 13, and 61) and *p53* (exons 5, 6, 7, and 8) in 22 cancer specimens and, when we found them, used MASA to analyze regional lymph nodes for the presence or absence of cells containing the same mutations.

MATERIALS AND METHODS

Materials and DNA Preparation. Tissue specimens from 22 colorectal cancers and corresponding lymph nodes were obtained from surgical resections performed in the Department of Surgery II, Kumamoto University Hospital, Kumamoto, and at the Cancer Institute Hospital, Tokyo, Japan. As shown in Table 1, no cases had any distant metastases. Lymph nodes were selected after operation, and each of them was divided into two specimens, one of which was fixed in 17% formaldehyde and submitted for histopathological diagnosis; each cancer and lymph node was examined conventionally after hematoxylin and eosin staining by an experienced histopathologist (A. Y.). The remaining half was immediately frozen in liquid nitrogen and stored at −80°C. DNA was extracted from the frozen tissue as described elsewhere (11).

Detection of Genetic Alterations of Primary Tumors. Codons 12, 13, and 61 of K-*ras* were examined by the MASA method described previously (10). The 3′-ends of 20-base pair oligonucleotides used as PCR primers corresponded to variants of the first or the second nucleotides of K-*ras* codons 12, 13, and 61. The third nucleotide at codon 61 was also examined by a similar method.

DNA sequences corresponding to exons 5, 6, 7, and 8 of *p53* were PCR amplified, and the PCR products were sequenced as described by Miyoshi *et al.* (12).

Detection of Genetic Alterations of Lymph Nodes. To look for corresponding genetic alterations in lymph nodes where mutations had been identified in primary tumors, we synthesized MASA primers with 3′-ends corresponding to each variant and amplified the respective lymph node DNA by PCR using these primers.

Polymerase Chain Reaction. PCR was performed with 35 cycles for 0.5 min at 95°C, 0.5 min at 54°C, and 0.5 min at 72°C, as described elsewhere using a GeneAmp PCR system 9600 (Perkin Elmer) (13). MASA was performed with 35 cycles for 0.5 min at 95°C, 1.5 min at 64 or 65°C, and 1.5 min at 70°C by the same method (13) except without dimethyl sulfoxide. All specimens were analyzed at least twice to confirm the results.

RESULTS

In each of the primary tumors, we looked for somatic mutations in selected regions of the K-*ras* and *p53* genes. We used MASA (10) to

Received 2/3/94; accepted 5/9/94.

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[2] The abbreviations used are: MASA, mutant allele-specific amplification; PCR, polymerase chain reaction; Asp, aspartic acid; Gly, glycine.

Table 1 *Clinicopathological summary of 22 patients examined for the mutation of K-ras and p53 genes in primary tumors of colorectal cancers*

Patient	Age (yr)	Sex	Location[a]	Histological type[b]	TNM[c]	Classification[d] (stage)
CRC 1	84	M	S	MD	T2N0M0	Ib
CRC 2	61	F	A	WD	T2N0M0	Ib
CRC 3	54	M	S	WD	T2N0M0	Ib
CRC 4	61	F	S	WD	T2N0M0	Ib
CRC 5	74	M	A	WD	T2N0M0	Ib
CRC 6	65	M	A	WD	T1N0M0	Ia
CRC 7	54	F	S	MD	T2N1M0	III
CRC 8	45	M	S	MD	T1N0M0	Ia
CRC 9	22	M	S	Mucinous	T3N4M0	IV
CRC 10	62	M	S	MD	T2N1M0	III
CRC 11	62	F	R	WD	T2N1M0	III
CRC 101	64	M	S	WD	T2N0M0	Ib
CRC 102	61	F	A	WD	T1N0M0	Ia
CRC 103	63	M	S	WD	T2N0M0	Ib
CRC 104	73	F	R	Mucinous	T2N1M0	III
CRC 105	63	M	A	WD	T2N1M0	III
CRC 106	71	M	R	WD	T2N1M0	III
CRC 107	66	M	S	WD	T2N0M0	Ib
CRC 108	64	M	T	WD	T1N0M0	Ia
CRC 109	70	F	S	WD	T2N0M0	Ib
CRC 110	66	M	D	WD	T2N1M0	III
CRC 111	63	F	A	WD	T2N0M0	Ib

[a] A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum.
[b] WD, well-differentiated adenocarcinoma; MD, moderately differentiated adenocarcinoma.
[c] T, tumor; N, node; M, metastasis.
[d] According to the classification by the International Union Against Cancer (17).

examine mutations at codons 12, 13, and 61 of K-*ras*. The K-*ras* mutations detected in six of the 22 primary tumors examined are summarized in Table 2. Exons 5, 6, 7, and 8 of the *p53* gene were examined by direct DNA sequencing; *p53* mutations were detected in nine of the primary tumors (Table 3). In total, 14 of 22 primary tumors were found to contain mutation(s) of the K-*ras* and/or *p53* genes (mutations in both genes were found only in case CRC6).

We synthesized PCR primers corresponding to each mutation and performed PCR for MASA using DNAs isolated from lymph nodes as templates, to look for the presence or absence of cells containing the same genetic alterations observed in the primary tumors. An example of the results is shown in Fig. 1. In the case illustrated, CRC1, in which the primary tumor cells contained a K-*ras* mutation of Gly (GGT) to Asp (GAT) at codon 13, histopathological diagnosis detected no metastases in any of seven regional lymph nodes examined. However, by the MASA method, the same mutation observed in the primary tumor was detected in five of the seven nodes (Fig.1, *lanes 2, 3, 4, 5,* and *7*). To further confirm this result and the sensitivity of the MASA method, we subcloned the PCR product that was amplified with primers flanking codon 13 and performed colony hybridization with the oligonucleotide corresponding to the Asp^{13} variant as described previously (10) (Fig. 2). Among nearly 10,000 subclones from PCR products amplified from lymph node DNA, only 10 colonies hybridized with the oligonucleotide corresponding to the Asp^{13} variant (Fig. 2*B*), although nearly half of the clones derived from the primary tumor carrying the Asp^{13} variant hybridized with it (Fig. 2*A*). DNA sequencing confirmed the same mutation in positive colonies

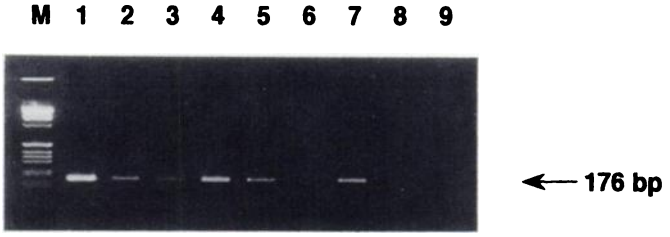

Fig. 1. Ethidium bromide staining of PCR products for detection of point mutations in the K-*ras* gene. PCR products (5 μl) were electrophoresed in a 2% agarose gel containing 0.5 μg/ml ethidium bromide. *Lane M,* size marker; *lane 1,* primary tumor CRC1; *lanes 2–8,* lymph nodes; *lane 9,* negative control (normal WBC).

Table 2 *Detection of k-ras gene mutations in primary tumors and their lymph nodes*

Patient	Age (yr)	Sex	Location[a]	Dukes' stage[b]	Mutation in primary tumor	No. of lymph node metastases (positive/examined) Genetic diagnosis	Histological diagnosis
CRC 1	84	M	S	B	Codon 13 GGC (Gly) → GAC (Asp)	5/7	0/7
CRC 6	65	M	A	A	Codon 12 GGT (Gly) → GAT (Asp)	0/3	0/3
CRC 10	62	M	S	C	Codon 12 GGT (Gly) → GAT (Asp)	8/9	1/9
CRC 102	61	F	A	A	Codon 13 GGC (Gly) → GAC (Asp)	1/15	0/15
CRC 106	71	M	R	C	Codon 13 GGC (Gly) → TGC (Cys)	5/18	2/18
CRC 111	63	F	A	B	Codon 12 GGT (Gly) → GAT (Asp)	9/16	0/16

[a] A, ascending colon; S, sigmoid colon; R, rectum.
[b] Dukes' stage: A, tumors not penetrating the bowel wall and without lymph node metastases; B, tumors penetrating the wall but still without lymph node metastases; C, tumors with lymph node metastases, regardless of the depth of penetration of cancer.

Table 3 *Detection of p53 gene mutations in primary tumors and their lymph nodes*

Patient	Age (yr)	Sex	Location[a]	Dukes' stage[b]	Mutation in primary tumor	No. of lymph node metastases (positive/examined) Genetic diagnosis	Histological diagnosis
CRC 4	61	F	S	B	Codon 213 CGA (Arg) → TGA (stop)	0/3	0/3
					Codon 258 GAA (Glu) → AAA (Lys)	0/3	0/3
CRC 5	74	M	A	B	Codon 270 TTT (Phe) → ATT (Ile)	3/3	0/3
CRC 6	65	M	A	A	Codon 213 CGA (Arg) → TGA (stop)	0/3	0/3
CRC 8	45	M	S	A	Codon 213 CGA (Arg) → TGA (stop)	7/8	0/8
CRC 11	62	F	R	C	Codon 282 CGG (Arg) → TGG (Trp)	9/9	1/9
CRC 101	64	M	S	B	Codon 282 CGG (Arg) → TGG (Trp)	4/22	0/22
CRC 107	66	M	S	A	Codon 245 GGC (Gly) → GAC (Asp)	0/6	0/6
CRC 109	70	F	S	A	Codon 248 CGG (Arg) → TGG (Trp)	2/8	0/8
CRC 110	66	M	D	C	Codon 282 CGG (Arg) → TGG (Trp)	5/18	2/18

[a] A, ascending colon; D, descending colon; S, sigmoid colon; R, rectum.
[b] Dukes' stage: A, tumors not penetrating the bowel wall and without lymph node metastases; B, tumors penetrating the wall but still without lymph node metastases; C, tumors with lymph node metastases, regardless of the depth of penetration of cancer.

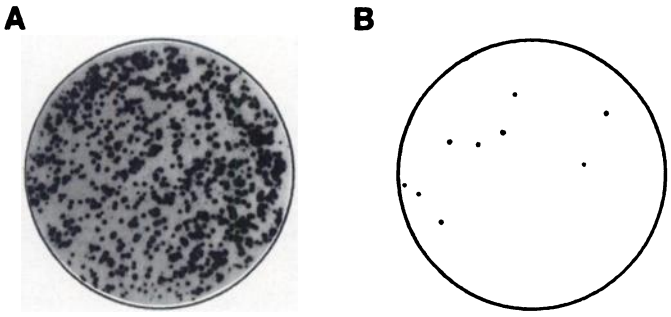

Fig. 2. Colony hybridization to detect the Asp^{13} variant in codon 13 of K-*ras*. PCR products (20 μl) were used for subcloning. Transformants were transferred to the membrane and hybridized with the oligonucleotide corresponding to the Asp^{13} variant. *A*, primary tumor (CRC1); *B*, lymph node.

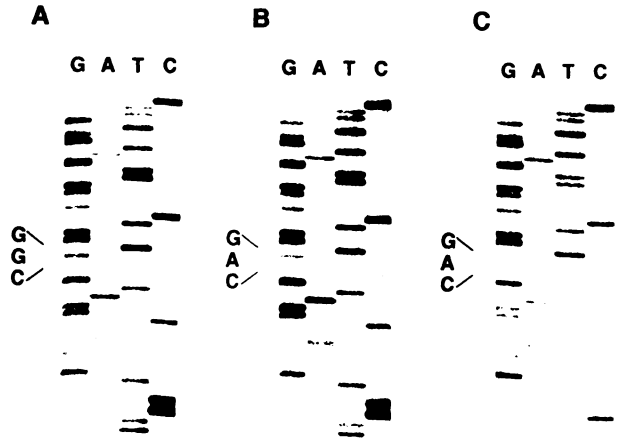

Fig. 3. DNA sequencing of PCR products. *A*, normal DNA obtained from a nonhybridized colony derived from primary tumor CRC1; *B*, primary tumor DNA obtained from a hybridized colony; *C*, lymph node DNA (obtained from a hybridized colony derived from a lymph node of patient CRC1).

obtained from the primary tumor and a corresponding lymph node (Fig. 3). These results indicated that the mutant allele was amplified sensitively and selectively by the MASA method and that tumor cells constituting 0.1% of the population in a background of normal cells could be detected.

Comparisons between genetic and histopathological diagnoses of lymph node metastasis are summarized in Tables 2 and 3. Among a total of 145 lymph nodes resected from 14 patients whose primary tumors had mutations in the K-*ras* and/or *p53* genes, examination by hematoxylin and eosin staining diagnosed only six as positive; 139 were diagnosed free of metastases. All six tissue samples with nodal metastases detected by hematoxylin and eosin staining were confirmed by DNA diagnosis. However, 52 of the 139 lymph nodes diagnosed as negative by hematoxylin and eosin staining were found to contain cells that had the same genetic alterations detected in the primary tumors. When reviewing the corresponding lymph nodes to examine multiple sections of each node, we detected no focus of tumor cells.

In summary, four cases were positive by both histological and genetic diagnoses, seven cases were negative by histology but positive by genetic diagnosis, and three cases were negative by both histological and genetic diagnoses.

DISCUSSION

Establishment of an easy method for detecting a small number of tumor cells, either as minimal residual diseases or as micrometastases, is clinically important. The detection of metastatic deposits may well be significantly improved by sampling a large number of lymph nodes from each case, examining multiple sections of each node, and using a panel of monoclonal antibodies including antibodies against tumor-associated antigens known to be expressed by colorectal cancers. However, these measures are not practical as routine histopathological services (14). Techniques such as morphological observation, immunohistochemistry, cell culture, or detection of tumor-specific molecular markers by Southern hybridization are limited in sensitivity; they will detect at best 1–10% of tumor cells in a normal background (15).

The fact that changes in K-*ras* or other genes are representative of the clonal expansion of malignant cells, and apparently remain stable as molecular markers throughout the course of the disease, renders possible the detection of oligocellular deposits of colorectal cancer cells (9). The MASA method (10) has proven to be simple, fast, sensitive, and specific for the diagnostic detection of single-point mutations in the K-*ras* and the *p53* genes in metastases of colorectal tumors to lymph nodes. Amplification of tumor-specific molecular markers by PCR, as this study has shown, is now able to offer a much more sensitive and rapid way of detecting the presence of one tumor cell among thousands of normal cells. This genetic method is more sensitive than conventional histology in detecting micrometastatic colorectal cancer cells.

In our study of 22 patients with colorectal cancer, the clinical status of seven patients, judged on the basis of conventional histopathological diagnosis, was changed by using genetic diagnosis of lymph node metastasis. The presence of cancer cells in lymph nodes could represent the first step forward lymphatic dissemination; moreover, cancer cells present in distant organs of these patients may be able to grow and proliferate to a level of clinical significance (16). Our results indicated that a small number of cancer cells had already spread into several lymph nodes that were diagnosed as negative histopathologically. Failure to detect this low-level migration might be one of the reasons why tumors recur in some patients with conventionally negative lymph nodes. Hence, detection of micrometastases in regional lymph nodes by the genetic method reported here might have an important impact on treatment of colorectal cancer and reduce the frequency of recurrence.

The results presented in this study suggest that carrying out genetic diagnosis in addition to conventional hematoxylin and eosin staining would permit more accurate diagnoses of lymph node metastasis. However, we are uncertain whether the tumor cells we detected in lymph nodes by this genetic method are associated with overt clinical metastasis. Hence, the presence of a positive lymph node by the MASA method may not necessarily indicate the likelihood of recurrent disease. Further studies involving a larger number of cases, and comparisons between genetic and histopathological diagnoses, should be carried out to confirm our results. Although the genetic method is limited presently to cases in which the specific genetic alterations in primary tumors can be identified, the approach could become applicable to a variety of tumor types if common mutations are discovered in other oncogenes or tumor suppressor genes.

ACKNOWLEDGMENTS

We acknowledge with thanks advice from Drs. Shigetoshi Ichii and Satoshi Takeda and technical assistance from Kiyoshi Noguchi. We are also grateful to physicians in the Department of Surgery II, Kumamoto University Medical School, and to physicians in the Department of Surgery, the Cancer Institute Hospital, Tokyo, for providing appropriate tissue samples.

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Cancer Res 1994;54:3853-3856.