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Detection of Gastric Cancer Micrometastases in Lymph Nodes by Amplification of Keratin 19 mRNA with Reverse Transcriptase-Polymerase Chain Reaction

Shinzaburo Noguchi,[1] Masahiro Hiratsuka, Hiroshi Furukawa, Tomohiko Aihara, Tsutomu Kasugai, Sumihito Tamura, Shingi Imaoka, Hiroki Koyama and Takeshi Iwanaga

Department of Surgery, Center for Adult Diseases, Osaka, 3-Nakamichi 1-Chome, Higashinari-ku, Osaka 537

A sensitive method for the detection of gastric cancer micrometastases in lymph nodes was developed. The method was based on amplification of keratin 19 mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR). Keratin 19 RT-PCR showed that keratin 19 mRNA was expressed in all 12 gastric cancers, but not in any of 20 normal control lymph nodes, indicating that keratin 19 mRNA is a good target of RT-PCR for the detection of gastric cancer micrometastases in lymph nodes. Serial dilution studies of RNA extracted from gastric cancers against RNA extracted from control lymph nodes demonstrated that the detection sensitivity of the keratin 19 RT-PCR method was one cancer cell in 10^3–10^5 lymph node cells. Detectability of lymph node metastases was compared between keratin 19 RT-PCR and conventional histological examination, using 100 lymph nodes obtained from 12 gastric cancer patients. Keratin 19 mRNA was detected in all of the seven lymph nodes which were histologically metastasis-positive. Of the 93 lymph nodes which were histologically metastasis-negative, 79 were found not to express keratin 19 mRNA but 14 were found to express keratin 19 mRNA, indicating that these lymph nodes contained micrometastases which could not be detected by histological examination. These results demonstrate that keratin 19 RT-PCR is a more sensitive method than histological examination for the detection of gastric micrometastases in lymph nodes.

Key words: Gastric cancer — Micrometastasis — Lymph node — Keratin 19 — RT-PCR

Multivariate analysis of various prognostic factors for gastric cancer has shown that lymph node status is the most important discriminant of patient outcome.[1,2] In spite of its prognostic importance, lymph node status is usually examined by a routine histological examination using only one hematoxylin and eosin (HE) section prepared from a representative cut surface of each lymph node. Since most parts of a lymph node are left unexamined, it is very likely that micrometastases are overlooked.

It was reported that a meticulous analysis with serial sectioning of lymph nodes resulted in disclosure of micrometastases in about 20% of the patients who had been diagnosed as lymph node metastasis-negative by routine histological examination.[3] This technique, however, is too time-consuming and cumbersome to be acceptable as a routine method. In order to overcome this drawback, new means based on polymerase chain reaction (PCR) have recently been developed to detect micrometastases in lymph nodes. Hayashi *et al.*[4] reported that the mutant allele-specific amplification (MASA) method was very useful in the detection of lymph node micrometastases from colon cancer and that patients with lymph node micrometastases disclosed by the MASA method showed poorer prognosis than those without them. They took advantage of mutant alleles of k-*ras* and *p53* genes because these genes are frequently mutated in colorectal cancers.[5,6] However, the frequency of mutation is less than 10% for k-*ras* and 40–50% for *p53* in gastric cancers.[5,6] Thus, the MASA method is not applicable to about a half of gastric cancer patients.

Another method recently developed for the detection of micrometastases in lymph nodes is reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of mRNA expressed in cancer cells, but not in lymph node cells. This method has been successfully applied to detection of micrometastases of breast cancer[7,8,9] and malignant melanoma[10] in lymph nodes. We[7,8] have been studying breast cancer micrometastases in axillary lymph nodes by RT-PCR targeted at keratin 19 mRNA or MUC1 mRNA and have shown that the RT-PCR-based method is sensitive enough to detect micrometastases which can not be detected by histological examination. Immunohistochemical study has demonstrated that keratin 19 protein is highly expressed in virtually all gastric cancers.[11] Thus, it is suggested that the keratin 19 mRNA RT-PCR method is applicable to detection of gastric cancer micrometastases in lymph nodes in almost all cases and seems to be superior to the MASA method, which is applicable to only about half of the cases. In the present study, we attempted to develop a sensitive method for detection of lymph node micro-

[1] Correspondence and reprint requests should be addressed to Shinzaburo Noguchi.

metastases of gastric cancer using keratin 19 mRNA RT-PCR.

MATERIALS AND METHODS

Patients Gastric cancer tissues and lymph nodes were obtained from 12 primary gastric cancer patients who underwent partial or total gastrectomy during the period from March 1995 to July 1995. All the gastric cancers were histologically proven to be adenocarcinoma. Special care was taken to prevent contamination of the lymph nodes by cancer cells from the main tumor and by normal epithelial cells in the stomach. Lymph nodes were separated from the surgical specimen immediately after its resection, and, then, samples were obtained from cancer tissue. Each lymph node was stripped carefully of adipose tissue and blood. Then, it was cut into halves with a scalpel. The scalpel was cleaned each time by rinsing it with saline before it was used for the bisection of a lymph node. One half of a lymph node was fixed in 10% buffered formalin and embedded in paraffin for histological examination. The HE section was prepared from the cut surface of the bisected lymph node. The other half and the cancer tissue were snap-frozen in liquid nitrogen and kept at −80°C until RNA extraction.

Lymph nodes of the hepato-duodenal ligament obtained from patients with cholelithiasis and axillary lymph nodes obtained from patients with benign phyllodes tumor were used as normal control lymph nodes, and they were also processed as described above.

RNA extraction Total cellular RNA was extracted from gastric cancers and lymph nodes by a guanidinium thiocyanate-phenol-chloroform method using an RNA isolation kit (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. The extracted RNA was finally resuspended in DEPC-treated water at a concentration of 0.5-2 $\mu g/\mu l$.

RT-PCR Oligonucleotide primers for keratin 19 mRNA and β-actin mRNA were designed as previously reported.[12, 13] Fragment sizes amplified from the keratin 19 mRNA and β-actin mRNA templates by RT-PCR were 460 bp and 154 bp, respectively; they were different from those amplified from the corresponding genomic DNA templates because the primers were selected from two different exons with at least one intervening intron. Primer sequences were as follows: 5′-AGGTGGATTCCGCTCCGGGCA and 5′-ATCTTCCTGTCCCTCGAGCA for keratin 19 mRNA and 5′-CACTGTGTTGGCGTACAGGT and 5′-TCATCACCATTGGCAATGAG for β-actin mRNA.

Total RNA (1 μg of RNA) was reverse-transcribed in a 20 μl reaction mixture containing 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 50 μM DTT, 5 m*M* $MgCl_2$, dNTPs (1 m*M* each), 2.5 μM oligo $(dT)_{20}$, 20 units of RNase inhibitor and 5 units of avian myeloblastosis virus reverse transcriptase (Takara Shuzo Co., Ltd., Kyoto) for 30 min at 42°C and the reaction was terminated by heating at 99°C for 5 min. From this cDNA solution, 2 μl was removed to be subsequently used for PCR amplification by adding each sample to 48 μl of a solution containing 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 1000 μM UTP, downstream and upstream primers (50 pmol each), 0.5 unit of uracil-*N*-glycosylase (Perkin-Elmer Cetus, Norwalk, CT) and 0.3 unit of Taq polymerase (Perkin-Elmer Cetus). Uracil-*N*-glycosylase was included to prevent contamination by the carry-over products. At first, samples were incubated at 37°C for 10 min, followed by 95°C for 10 min, to cleave the carry-over products, and then they were amplified in a Perkin-Elmer Cetus thermal cycler for 50 cycles (1 min, 94°C; 1 min, 60°C; 1 min, 72°C). After the amplification, the entire PCR reaction mixture (50 μl) was analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide. RT-PCR amplification of keratin 19 and β-actin were conducted in separate runs.

RESULTS

Specificity and sensitivity of keratin 19 RT-PCR Keratin 19 RT-PCR using total RNA from 12 primary gastric cancers demonstrated amplification of a 460 bp fragment in all cases. Five representative results are shown in Fig. 1. Twenty normal control lymph nodes were also examined for keratin 19 mRNA expression by RT-PCR, but keratin 19 fragment was not demonstrated in any of

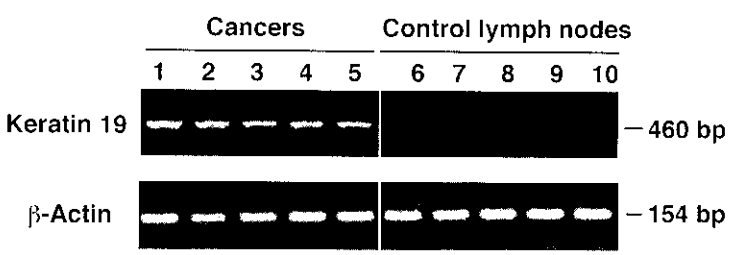

Fig. 1. RT-PCR amplification of keratin 19 mRNA and β-actin mRNA using total RNA extracted from gastric cancers (lanes 1–5) and normal control lymph nodes (lanes 6–10). Expression of mRNAs for keratin 19 and β-actin genes was examined by RT-PCR, and the results of agarose gel electrophoresis are shown. bp, base pairs.

them. Five representative results are shown in Fig. 1. RT-PCR amplification of β-actin mRNA, which was conducted to rule out RNA degradation, resulted in the appearance of a 154 bp fragment in every sample, guaranteeing the integrity of the total RNA used for RT-PCR.

Sensitivity was determined by performing serial dilutions of total RNA extracted from gastric cancers, and preparing mixtures with total RNA extracted from normal control lymph nodes to represent 10^{-3}–10^{-6} μg RNA of gastric cancer in 1 μg RNA of normal control lymph nodes. Three representative results are shown in Fig. 2. Keratin 19 mRNA was detected at a concentration of 10^{-5} μg (cancer 1), 10^{-4} μg (cancer 2), or 10^{-3} μg (cancer 3) cancer RNA in 1 μg control lymph node RNA. In all, such experiments were performed on five gastric cancers. Sensitivities of keratin 19 RT-PCR were 10^{-5} μg (n=1), 10^{-4} μg (n=2), or 10^{-3} μg (n=2) cancer RNA in 1 μg control lymph node RNA.

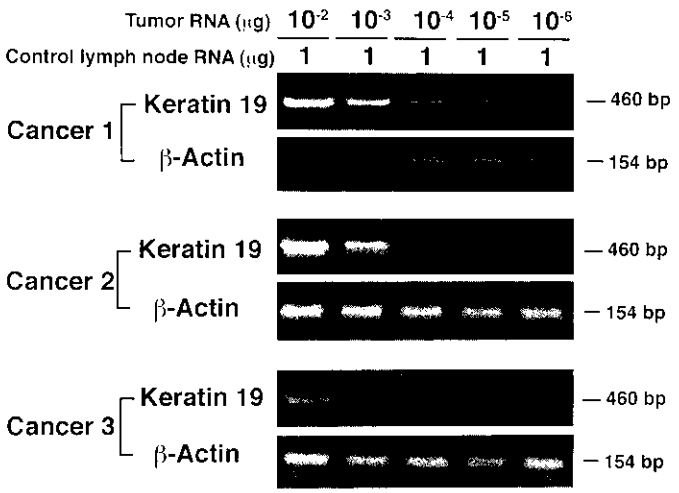

Fig. 2. Sensitivity of keratin 19 RT-PCR in detection of gastric cancer cells against lymph node cells. Detection sensitivity was determined by performing serial dilutions of total RNA from gastric cancers (cancers 1–3) and preparing mixtures with total RNA from normal control lymph nodes to represent 10^{-3}–10^{-6} μg RNA of gastric cancer/1 μg RNA of normal control lymph node. Keratin 19 mRNA and β-actin mRNA expression was studied with these RNA mixtures by RT-PCR. The results of agarose gel electrophoresis are shown. bp, base pairs.

Comparison of detectability of lymph node metastases between histological examination and keratin 19 RT-PCR The results of histological examination were compared with those of keratin 19 RT-PCR for the detection of micrometastases in 100 lymph nodes obtained from 12 patients (Table I). Keratin 19 mRNA was detected in all of the seven lymph nodes which were found to have metastases by histological examination. The extent of tumor involvement in each lymph node was estimated and ranged between 5 and 95% of the node. Of the 93 lymph nodes which were diagnosed to be devoid of metastases by histological examination, 79 were found not to express keratin 19 mRNA, but 14 were found to express keratin 19 mRNA, indicating that these 14 lymph nodes contained micrometastases which could not be detected by histological analysis.

Representative results of keratin 19 RT-PCR using total RNA extracted from histologically positive (n=3) and negative (n=7) nodes are shown in Fig. 3. Keratin 19 mRNA expression was observed in all three histologically positive lymph nodes (lanes 1–3) and in two histologically negative lymph nodes (lanes 4 and 7). Clinicopathological characteristics of the twelve patients as well as a summary of the keratin 19 RT-PCR analyses are shown in Table II.

Table I. Comparison between Histological Examination and Keratin 19 RT-PCR for Detection of Gastric Cancer Micrometastases in Lymph Nodes

Keratin 19 RT-PCR	Histological examination	
	Positive	Negative
Positive	7[a)]	14
Negative	0	79

a) Number of lymph nodes.

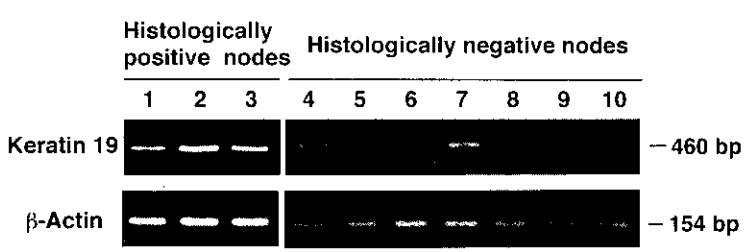

Fig. 3. Detection of gastric cancer micrometastases in lymph nodes by keratin 19 RT-PCR. Total RNA was extracted from each of 100 lymph nodes obtained from 12 patients, and it was analyzed by RT-PCR for keratin 19 mRNA and β-actin mRNA expression. Representative RT-PCR results of three histologically positive nodes (lanes 1–3) and seven histologically negative nodes (lanes 4–10) are shown. bp, base pairs.

Table II. Clinicopathological Characteristics of the Patients and Results of Keratin 19 RT-PCR

	Age (year)	Sex[a]	Size (cm)	Depth of invasion[b]	Histologic type[c]	Lymph node metastases[d]
1	85	M	8.7	se	pap	●●●◐◐◐◐○○
2	27	F	3.9	mp	por2	●●●◐◐○○○○○
3	68	M	3.9	ss	tub2	●◐◐◐◐○○○○○
4	52	F	3.8	ss	por2	◐◐◐○○○○○○○
5	66	F	13.0	se	por2	◐○○○○○
6	55	M	12.0	se	por2	○○○○○○○○○○
7	46	F	5.8	ss	sig	○○○○○○○○○○
8	63	M	8.0	sm	sig	○○○○○
9	47	F	3.8	sm	sig	○○○○○○○○○○
10	70	M	2.3	m	pap	○○○○○
11	74	F	1.0	m	tub1	○○○○○
12	46	M	0.6	m	sig	○○○○○○○○○○

a) F, female; M, male. *b, c*) Abbreviations according to the Japanese Research Society for Gastric Cancer.[20] *d*) ●, Both histology and keratin 19 RT-PCR positive lymph node; ◐, keratin 19 RT-PCR positive but histology negative lymph node; ○, both histology and keratin 19 RT-PCR negative lymph node.

DISCUSSION

Keratin 19 mRNA was detected by RT-PCR in all 12 gastric cancers, but not in any of 20 control lymph nodes obtained from patients with benign diseases. These results indicate that keratin 19 mRNA is a good target of RT-PCR for the detection of gastric cancer micrometastases in lymph nodes. Detection sensitivity studies using a serial dilution of RNA extracted from gastric cancer against RNA extracted from control lymph nodes has revealed that keratin 19 RT-PCR is sufficiently sensitive to detect one cancer cell in 10^3–10^5 lymph node cells, provided that the yield of total RNA from a lymph node cell is equal to that from a cancer cell. This sensitivity is comparable to that reported in the detection of breast cancer micrometastases in lymph nodes by keratin 19 RT-PCR.[8]

All of the seven histologically positive lymph nodes were keratin 19 RT-PCR positive and, in addition, 14 (15%) of the 93 histologically negative lymph nodes were also keratin 19 RT-PCR positive. These results demonstrate that keratin 19 RT-PCR is a more sensitive method than histological examination and it can detect micrometastases which can not be detected by histological examination. Very recently, Mori *et al.*[14] claimed in a preliminary report that lymph node micrometastases of gastric cancer can be detected with a sensitivity of one cancer cell in 10^4 lymphocytes through nested (two rounds) RT-PCR amplification of carcinoembryonic antigen (CEA) mRNA. We feel, however, that keratin 19 mRNA is a more suitable target of RT-PCR than CEA mRNA. The detection sensitivity of one-round keratin 19 RT-PCR presented in this paper is comparable to that of the nested CEA RT-PCR, probably due to a higher expression level of keratin 19 mRNA than CEA mRNA in gastric cancer cells. If the detection sensitivity is the same, one-round keratin 19 RT-PCR seems to be superior to the nested CEA RT-PCR because the latter method is time-consuming and more susceptible to contamination.

In order to conduct such a sensitive assay as RT-PCR with accuracy, special attention should be paid to the prevention of false-positive results. Contamination with carry-over products from previous PCR amplifications seems to be a major cause of the occurrence of false-positive results. Thus, we conducted PCR using dUTP instead of dTTP so that the amplicons contained dUTP. Before each PCR, the PCR reaction mixture was pretreated with uracil-*N*-glycosylase in order to cleave selectively the carry-over products containing dUTP but not cDNA templates containing dTTP. By incorporating this carry-over prevention system, we have been able to eliminate false-positive results attributable to contamination by carry-over products. Another important cause of false-positive results is contamination of lymph nodes by cancer cells and normal epithelial cells. Thus, special care was taken to prevent such a contamination when the lymph nodes were separated from the surgical specimens, as described in "Materials and Methods." We feel that the keratin 19 RT-PCR method is unlikely to be flawed by a false-positive problem if the above-mentioned precautions are taken.

In conclusion, we have shown in the present study that keratin 19 RT-PCR is a highly sensitive method to detect lymph node micrometastases which can not be identified by histological examination. The prognostic significance

of micrometastases remains controversial.[15, 16] Therefore, we are now prospectively studying this important issue by routinely examining lymph node micrometastases by keratin 19 RT-PCR. This method seems to be applicable to the detection of micrometastases of various cancers expressing keratin 19 mRNA, not only in the lymph nodes, but also in the peripheral blood as well as bone marrow, because keratin 19 mRNA is not expressed in these tissues.[17] Preliminary results[18] have suggested that this method is useful for the detection of breast cancer cells in the peripheral blood and bone marrow, although a conflicting result has been reported on lung cancer.[19]

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