Differential Expression of Motility-Related Protein-1 Gene in Gastric Cancer and Its Premalignant Lesions

Yao Xu
Jie Zheng
Wentian Liu
Jun Xing
Yanyun Li
Xin Geng
Weiming Zhang

Central Laboratory of Basic Medical Science, Tianjin Medical University. Tianjin 300070,China.

Correspondence to: Yao Xu
E-mail: xuyao@eyou.com
Tel: 86-22-23542775

OBJECTIVE To identify genes related to gastric cancer and to analyze their expression profiles in different gastric tissues.

METHODS The differentially expressed cDNA bands were assayed by fluorescent differential display from gastric cancer specimens, matched with normal gastric mucosa and premalignant lesions. The motility -related protein-1 (MRP-1/CD9) gene expression was studied by Northern blots and reverse transcription polymerase chain reaction (RT-PCR) in different kinds of gastric tissue

RESULTS A differentially expressed cDNA fragment showed lower expression in all gastric cancers compared to the normal gastric mucosa and premalignant lesions; and it was found to be homologous to the MRP-1/CD9 gene. Northern blot analysis confirmed the differential expression. RT-PCR analysis showed that the MRP-1/CD9 gene was expressed at a much lower rate in gastric cancers (0.31±0.18) compared to the matched normal gastric tissue (0.49±0.24) and premalignant lesions (0.47±0.18)(P<0.05). Furthermore, its expression in intestinal-type of gastric cancer (0.38±0.16) was higher than that expressed in a diffuse-type of gastric cancer (0.22±0.17)(P<0.05).

CONCLUSION The MRP-1/CD9 gene expression was down-regulated in gastric cancer and its expression may be related to the carcinogenic process and histological type of gastric cancer.

KEYWORDS: gastric cancer, mRNA differential display, motility -related protein-1/CD9 gene, Northern blot.

Gastric cancer is the second most common cause of cancer-related deaths in the world, being the first in China; it is a major cause of mortality. Knowledge of the pathogenesis of gastric cancer can play an important role for the early diagnosis and individualized treatment. Gastric carcinogenesis is a multistep process in which genetic alterations are important in its pathogenesis. It is generally recognized that the development of gastric cancer arises gradually from premalignant lesions, chronic atrophic gastritis with intestinal metaplasia and dysplasia. With the development of molecular biology techniques, some cDNA fragments which are closely related to the development of human gastric cancer have recently been identified . However, very little is currently known about the genes that may cause a predisposition to gastric cancer.

Fluorescent mRNA differential display (FDD) has been widely applied to identify cancer-related genes, a technique which is faster, safer and more cost-effective than the use of procedures that require

Received March 18, 2004; acceptecd May 26, 2004.

radioactivity. In this study, FDD was used to identify and characterize differentially expressed genes in gastric cancer specimens, matched with normal gastric mucosa and premalignant lesions. A cDNA, G1, that was formed at a very high level from normal gastric mucosa and premalignant lesions was produced at much lower levels from gastric cancers and was shown to be identical to the MRP1/CD9 gene.

MATERIALS AND METHODS

Specimens

Thirty gastric cancers and matched non-cancerous gastric mucosal tissues and 10 premalignant lesions were collected during gastroscopies (male: 28, female: 12; mean age: 59 ±7 years). The 30 gastric cancers included 18 intestinal type and 12 diffuse type. The 10 premalignant lesions included 8 moderate atrophic gastritis with intestinal dysplasia (4 also with metaplasia) and 2 severe atrophic gastritis with moderate dysplasia and metaplasia. Each specimen was bisected. One half was preserved in liquid nitrogen and the other half used to confirm the pathological diagnosis.

RNA preparation and differential display

Total RNA was extracted using a TRIzol reagent (Invitrogen Corp. Carlsbad, CA, USA), followed by treatment of the RNA with DNase I. Differential displays were carried out using a fluoroDD kit (Genomyx Corp. Foster city, CA, USA).

The set of primers used for generation of the MRP-1/CD9 cDNA fragment consisted of an anchored primer T7-AP6 and an arbitrary primer M13-ARP10. Approximately 1 μg of total RNA was reverse transcribed using 40 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp.) in the presence of 20 pmol of the T7-AP6 (5′-ACGACTCACTATAGGGCTTTTTTTTTTTCC-3′) primer mixture and 500 pmol of dNTP for 5 min at 25℃, followed by 10 min at 42℃and 60 min at 50℃. After heat inactivation of the reverse transcriptase at 70℃ for 15 min and subsequent cooling to 4℃, 3 μl of the samples were added to 7 μl of PCR labeling mixture containing 3.5 pmol of T7AP6, which was labeled with tetramethylrhodamine (TMR), 3.5 pmol of M13-ARP10 (5′-ACAATTTCACACAGGAGATCTCAGAC-3′), 500 pmol of dNTP and 0.5 unit of Taq DNA polymerase (Invitrogen Corp.). The cycling parameters were 94℃ for 2 min, then, 4 cycles as follows: 94℃ for 30 s, 50℃ for 30 s and 72℃ 2 min, then another 25 cycles of 94℃ for 30 s, 60℃ for 30 s and 72℃ for 2 min. Finally, the samples were heated to 72℃ for 7 min and then cooled to 4℃. The PCR products were analyzed on 5.6% HR-1000 clear denaturing gels (Genomyx Corp.). Duplicate samples were run for all the targeted differential display sample.Bands of interest were cut from the dried gel and reamplified using PCR with the same primers.

Cloning and sequencing

The cDNA fragments of interest were subcloned into pGEM-T Easy vector (Promega Corp. Madison, WI, USA) and confirmed by EcoR I (Invitrogen Corp.) digestion. Sequence analysis was performed with a CEQ8000 DNA sequencer (Beckman Coulter, Inc. Fullerton, CA, USA). All nucleotide sequence databases maintained by the National Center for Biotechnology Information were searched for homologous sequences using the basic local alignment search tool (BLAST).

Northern blot analysis

DIG Northern Starter Kit (Roche Diagnostic Corp. Ottweiler, Germany) was used. The procedure of hybridization was performed according to the manufacturer's protocol. An antisense cRNA probe labeled with digoxigenin was generated from a digested cDNA insert by means of in vitro transcription. A digoxigenin labeled sense cRNA probe was used as a negative control. The hybridization signals were visualized with chemiluminescence that was recorded on X-ray films.

Reverse transcription polymerase chain reaction (RT-PCR)

First-strand cDNAs were synthesized by Superscript II reverse transcriptase (Invitrogen Corp.) using oligo d(T) primers according to the suggested protocol. The sequences of primers used for reverse transcription of the MRP-1/CD9 and β-actin are as shown in Table1[1].

Table 1. The primer sequences and fragment size of MRP-1/CD9 and β-actin

Item	Fragment size(bp)	Primer sequences(5′-3′)
MRP-1/CD9	801	F:TGCATCTGTATCCAGCGCCA
		R:GCTCAGGGATGTAAGCTGACTμC
β-actin	274	F:CAAGAGATGGCCACGGCTGCT
		R:CCTTCTGCATCCTGTCGGCAA

The cycling parameters were 94℃ for 5 min, then 25 cycles at 94℃ for 30 s, 60℃ for 40 s and 72℃for 90 s.

Finally, the samples were heated to 72℃ for 7 min and then cooled to 4℃. The amplified DNA samples were electrophoresed on 1% agarose gels and visualized with ethidium bromide.

The signals of DNA bands were quantified by Cooled CCD Imaging Systems (AAB Corp. Fullerton, CA, USA). The relative intensities of MRP-1/CD9/β-actin were estimated by assessing the ratio of the MRP-1/CD9 band to the β-actin band.

RESULTS

Isolation of differentially expressed cDNA by FDD from gastric tissue

Direct comparisons of mRNA fingerprints from 3 sets of gastric cancer, premalignant lesions and normal gastric tissue samples were made (ages: 57-62, male). Most amplified cDNA bands were similar in size and intensity in the samples, suggesting that most of the genes expressed in this subpopulation of mRNAs were not uniquely associated with cancer. In contrast, a 650-bp band, named G1, exhibited higher levels of expression in premalignant lesions and normal gastric tissue compared to gastric cancer (Fig. 1). A BLASTN search for sequence homology performed in the GenBank revealed that the sequence of G1 was identical (100% homology) to that of the 3′end of human CD9 mRNA.

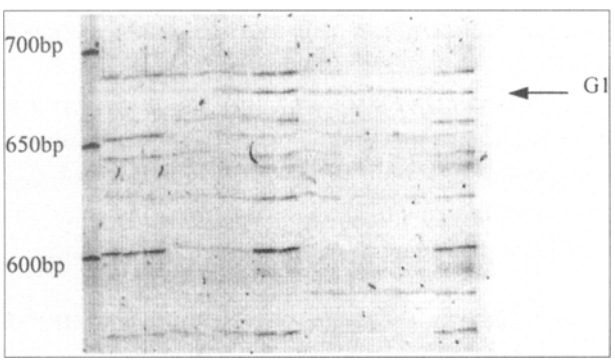

Fig.1. Differentially expressed genes in gastric cancer, matched normal gastric tissue and premalignant lesions. The differentially expressed cDNA fragment G1 (arrowed) was expressed less in gastric cancer; C: cancer; P: premalignant; N: normal; M: marker.

Northern blot analysis

Northern blot analysis showed that G1 was expressed less in gastric cancer compared to premalignant lesions and normal tissues (Fig.2). This result confirmed the differential expression initially observed by differential display after PCR amplification of the G1 mRNA gene product.

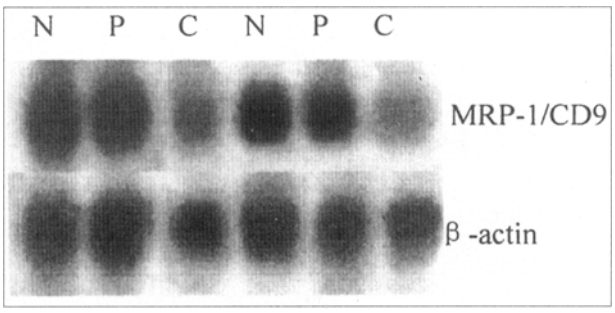

Fig.2. Northern blot analysis of MRP-1/CD9 gene expression in gastric mucosa tissues.C: cancer; P: premalignant; N: normal.

RT-PCR analysis

RNAs from 10 premalignant lesions, 30 gastric cancers and 30 normal gastric tissues were analyzed by RT-PCR with specific primers for each gene. β-actin was used as a control. RT-PCR analysis showed that the MRP-1/CD9 gene was expressed much less in gastric cancers (0.31±0.18) compared to the matched normal gastric tissue (0.49±0.24) and premalignant lesions (0.47±0.18) ($P<0.05$) (Fig.3, Table 2).

Table 2. The differential expression of MRP-1/CD9 gene in gastric cancer, normal gastric tissue and premalignant lesions

Group	n	$\bar{x}\pm s$
Gastric cancer	30	0.31±0.18*※
Normal gastric mucosa	30	0.49±0.24♦
Gastric premalignant lesions	10	0.47±0.18

Compared with normal gastric tissue:* $P<0.05$
Compared with premalignant lesions: ※ $P<0.05$
Compared with premalignant lesions: ♦ $P>0.05$

Of the 30 cases of gastric cancers, 18 cases were intestinal type, while the other 12 cases were diffuse type. MRP-1/CD9 showed higher expression in intestinal-type gastric cancer (0.38±0.16) compared to that in diffuse-type gastric cancer(0.22±0.17) ($P<0.05$) (Table 3). MRP-1/CD9 was missing in 5 gastric cancers, of which 4 were intestinal type and 1 for a diffuse type.

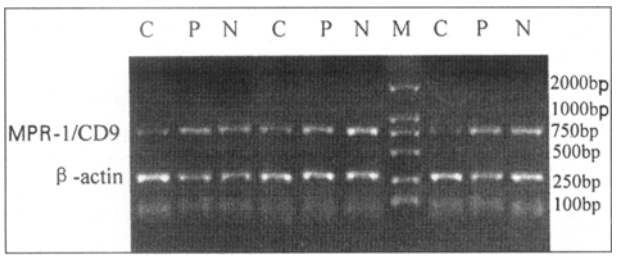

Fig.3. RT-PCR analysis of MRP-1/CD9 gene expression in gastric cancer, matched normal gastric tissue and premalignant lesion.C: cancer; P: premalignant; N: normal; M: DL2000marker.

Table 3. The expression of MRP-1/CD9 gene in intestinal and diffuse type gastric cancer

Group	n	$\bar{x}\pm s$
Intestinal	18	0.38±0.16*
Diffuse	12	0.22±0.17

Compared with diffuse type gastric cancer, *$P<0.05$.

DISCUSSION

Advances in molecular biology have revealed a consistent set of genetic alterations that may correspond to multi-step stomach cancer development. Aberrant expression and amplification of oncogenes such as ras, c-met, c-erb-2, APC, DCC, E-cadherin and survivin, etc.[2-8], are reported common events in the steps of carcinogenesis. However very little is currently known about genes that may cause a predisposition to gastric cancer. The primary aim of this study was to identify genes that were closely related to human gastric cancer by means of FDD.

FDD has been widely applied to identify cancer-related genes[9-12], some of which appear to be of clinical interest. If we can identify gastric cancer-related genes directly from the tissues it might reflect crucial genetic changes for carcinogenesis. Technical difficulties encountered while utilizing the differential RNA technique are related, in part, to the relatively low stringency of PCR amplification, which gives rise to false positive results, and problems of reproducibility. In our studies, duplicate samples were run for all the targeted differential display samples. Bands were considered of interest only when displayed in duplicate lanes and a differential display was used to identify a series of gastric-related genes. One of these cDNA fragments, G1, was found to be identical to MRP-1/CD9, a gene previously implicated in tumor cell motility and metastasis.

MRP-1/CD9 belongs to the transmenbrane-4 superfamily(TM4SF), which has at least 15 members or tetraspans. Members of the TM4SF bind with one another to form a cell surface-associated tetraspan network. The MRP-1/CD9 gene is located at human chromosome 12q13, encoding a Mr 24,000 protein that contains four hydrophobic domains and an extracellular domain. The hydrophobic domains allow the molecule to span four times within the cell membrane and the extracellular domain functions as a cell surface-anchoring receptor.

The biological function of MRP-1/CD9 in normal and cancer cells is complex. Some studies have implicated CD9 in cell proliferation and cell adhesion to the extracellular matrix proteins may be enhanced by MRP-1/CD9. Studies have shown that it regulates cell motility by suppressing cellular migration and also may have the ability to prevent formation of experimental metastasis.[13] It may regulate cell-matrix and/or cell-cell interactions. CD9-directed monoclonal antibodies induce platelet and tumor-cell aggregation, and furthermore, multiple cell membrane-associated proteins, including the β1 integrin subunit, and other members of the TM4SF and CD9 itself, are ligands for MRP-1/CD9[13].

Other investigators have demonstrated its inhibitory activity on tumor cell migration of lung cancer, oral squamous cell carcinoma and breast cancer, etc.[14-16], but little has been reported on the expression of MRP-1/CD9 in gastric cancer. In this study, MRP-1/CD9 showed lower expression in gastric cancers compared to normal gastric and premalignant lesions, a result confirmed by Northern blot analysis. RT-PCR analysis showed that the MRP-1/CD9 gene was expressed much less in gastric cancers compared to the matched normal gastric tissue and premalignant lesions. Its expression was slightly lower in premalignant lesions compared to normal tissues, but not to statistical significance. Furthermore, its expression in intestinal-type gastric cancer was higher compared to diffuse-type gastric cancer, the latter tended to be more malignant and metastasize earlier in its development. Our study shows that lower expression and lost of MRP-1/CD9 may be linked with the pathogenesis, development, metastatic tendency and poor prognosis of gastric cancer.

REFERENCES

1 Adachi M, Taki T, Konishi T, et al. Novel staging protocol for non-small-cell lung cancers according to MRP-1/CD9 and KAI1/CD82 gene expression. J Clin Oncol. 1998;16: 1397-1406.

2 Stadtlander CT, Waterbor JW. Molecular epidemiology, pathogenesis and prevention of gastric cancer. Carcinogenesis. 1999;20: 2195-2208.

3 Yasui W, Oue N, Kuniyasu H, et al. Molecular diagnosis of gastric cancer: present and future. Gastric Cancer. 2001;4: 113-121.

4 Hiyama T, Haruma K, KTadai Y, et al. K-ras mutation in helicobacter pylori-associated chronic gastritis in patients with and without gastric cancer. Int J Cancer. 2002;97: 562-566.

5 Nakajima M, Sawada H, Yamada Y, et al. The prognostic significance of amplification and overexpression of c-met and c-erbB-2 in human gastric carcinomas. Cancer.1999; 85:1894-1902.

6 Sato K, Tamura G, Tsuchiya T, et al. Frequent loss of expression without sequence mutations of the DCC gene in primary gastric cancer. Br J Cancer. 2001;85:199-203.

7 Cai J, Ikeguchi M, Tsujitani S, et al. Significant correlation between micrometastasis in the lymph nodes and reduced expression of E-cadherin in early gastric cancer. Gastric Cancer. 2001;4:66-74.

8 Yu J, Leung WK, Ebert MP, et al. Increased expression of survivin in gastric cancer patients and in first degree relatives. Br J Cancer. 2002; 87:91-97.

9 Chung JG, Yeh KT, Wu SL, et al. Novel transmembrane GTPase of non-small cell lung cancer identified by mRNA differential display. Cancer Res. 2001; 61: 8873-8879.

10 Yoshikawa Y, Mukai H, Hino F,et al. Isolation of two novel genes down-regulated in gastric cancer. Jpn J Cancer Res. 2000;91:459-463.

11 Ebert MP, Gunther T, Hoffmann J, et al. Expression of metallothionein II in intestinal metaplasia, dysplasia,and gastric cancer. Cancer Res. 2000:60:1995-2001.

12 Shiozaki K, Nakamori S, Tsujie M, et al. Human stomach-specific gene, CA11, is down-regulated in gastric cancer. Int J Oncol. 2001;19:701-707.

13 Miyake M, Koyama M, Seno M, et al. Identification of the motility -related protein (MRP -1), recognized by monoclonal antibody M31-15, which inhibits cell motility. J Exp Med.1991;174:1347-1354.

14 Funakoshi T, Tachibana I, Hoshida Y, et al. Expression of tetraspanins in human lung cancer cells: frequent downregulation of CD9 and its contribution to cell motility in small cell lung cancer. Oncogene. 2003; 22: 674-687.

15 Kusukawa J, Ryu F, Kameyama T, et al. Reduced expression of CD9 in oral squamous cell carcinoma: CD9 expression inversely related to high prevalence of lymph node metastasis. J Oral Pathol Med. 2001; 30: 73-79.

16 Huang CI, Kohno N, Ogawa E, et al. Correlation of reduction in MRP -1/CD9 and KAI1/CD82 expression with recurrences in breast cancer patients. Am J Pathol.1998;153: 973-983.