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• GASTRIC CANCER •

A novel gene, GCRG224, is differentially expressed in human gastric mucosa

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Abstract

AIM: To clone genes that may predispose us to human gastric cancer and to analyze it's expression in gastric tissues.

METHODS: Specimens of paired tumor, paratumor and normal gastric mucosa tissues collected from fifteen patients who suffered from stomach antrum adenocarcinoma were used for analysis. Seven out of the fifteen cases were first studied by fluorescent differential display reverse transcription polymerase chain reaction (DDTR-PCR) analysis. The differentially expressed bands of interest were cloned, analyzed by Northern blot, sequencing and RT-PCR. Through BLAST, the sequencing results were compared with GenBank database for homology analysis. *In situ* hybridization with DIG-labeled cRNA probes was used to analyze the expression of interesting cDNA bands in paraffin embedded paired normal gastric mucosa and cancer tissues isolated from 30 gastric adenocarcinoma patients.

RESULTS: DDRT-PCR showed that one of the interesting cDNA bands, which was named W2, expressed much higher in all seven tested tumor and paratumor samples than in their normal counterparts, it was sub-cloned into a pGEM-T Easy vector. Two subclones were subsequently obtained. One of the subclone, GCRG224, was studied further. The sequencing result showed that GCRG224 consisted of 1 159 base pairs and had one open reading frame (ORF). It located at human chromosome 11q14. No homologue was found in GenBank database with GCRG224-ORF. This nucleotide sequence data were submitted to GenBank with accession No. AF438406. RT-PCR showed that GCRG224 expressed higher in 11/15 gastric cancer tissues than in non-tumor tissues. However, the result of Northern blot analysis showed a higher GCRG224 expression in the non-tumor tissue than in the tumor one. Human multiple tissue Northern blot analysis revealed that GCRG224 also expressed in human normal colon tissue, and peripheral blood leukocyte. *In situ* hybridization analysis showed that only 5/30 adenocarcinoma, 3/18 dysplasia and 6/18 intestinal metaplasia showed higher GCRG224 expression level than the normal gastric glands. However, GCRG224 was over-expressed predominantly in 26/30 cases of normal mucosal epithelium.

CONCLUSION: A novel gene named GCRG224 was identified from human gastric mucosal tissue. It overexpressed in almost all gastric mucosal epithelium but only a small portion of cancer and precancerous leisions. The role of GCRG224 expression in gastric epithelium needs further study.

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INTRODUCTION

Gastric cancer is one of the most commonly diagnosed malignancies and remains an important cause of mortality world wide[1]. Considerable evidence supports the pivotal role of genetic factors in the pathogenesis of gastric cancer[2-7]. However, the mechanism of the process of multistage carcinogenesis is still unknown for gastric cancer. Abundant clinical and histopathogical data suggest that most, if not all, intestinal-type gastric cancer arise from precancerous leisions, which indicated intestinal-type gastric cancer to be an excellent model for studying the genetic alterations involved in the development of human neoplasms. It would be desirable, therefore, to screen directly from human intestinal-type gastric cancer and its precursor leisions the differentially expressed genes that are closely related to human gastric cancer.

In this study, differential display reverse transcription polymerase chain reaction (DDRT-PCR) analysis was used to identify and characterize differentially expressed genes in human gastric cancer tissues in comparison with their surrounding paratumor and nontumor counterparts. One novel cDNA with a complete cds was identified. We designated this gene as gastric cancer related gene 224 (GCRG224). Expression of GCRG224 in gastric tissues was analyzed using RT-PCR and *In situ* hybridization (ISH).

MATERIALS AND METHODS

Tissue acquisition

Fresh primary intestinal-type gastric adenocarcinoma, paratumor tissue which is 1.0 cm away from the tumor mass and their surrounding noncancerous stomach mucosal tissues obtained from 15 patients (11 male, 4 female, with average age 54±15 years) undergoing surgery were used for reverse transcription polymerase chain reaction (RT-PCR) analysis. 7 cases (4 male, 3 female, with average age 51±18 years) out of the 15 patients were used for differential display analyses. Paraffin embedded gastric adenocarcinoma and their corresponding normal gastric mucosal tissues obtained from 30 advanced gastric adenocarcinoma patients (21 male, 9 female, with average age 59±10 years) were used for ISH analysis. All tissues were histologically confirmed by pathologists.

RNA preparation and differential display

Total RNA was extracted from tissues using TRIzol reagent (Life Technologies, Inc., Rockville, Maryland). The fluorescent

DDRT-PCR procedure was performed essentially as described previously[8]. The primers used in the assay included 4 T_{12}MN primers and 20 arbitrary oligonucleotide primers (ARP-1 to ARP-20) (Genomyx Corporation, Foster City, CA).

Cloning and sequencing

The cDNA fragment of interest were subcloned into pGEM-T Easy vector (Promega Corporation, Madison, WI) and confirmed by EcoR I (Life Technologies, Bethesda, MD) digestion according to the manufacturer' s instruction. Sequence analysis was performed with CEQ™ 2000 DNA sequencer (Beckman Coulter, Fullerton, CA). The sequenced cDNA were analyzed via the BLAST program for homology matches in the GenBank database[9].

Northern blot analysis

Samples containing 20 μg of total nontumor and tumor RNAs were fractionated on an 1.2 % agarose gel containing 5.5 % formaldehyde and 1×MOPS and transferred to a nylon membrane. Fix the RNA to the membrane by baking at 80 ℃ for 2 hours. Human multiple tissue Northern (MTN) blot II membrane (Clontech Laboratories, Inc., Palo Alto, CA) was used for analysis. Anti-sense cRNA probe labeled with digoxigenin was generated from a digested cDNA insert using Dig Northern Starter Kit (Roche Diagnostic Corporation, Indianapolis, IN) by means of *in vitro* transcription. The membrane was prehybridized and then hybridized according to the manufacturer' s protocol. The results were detected using chemiluminescent detection.

RT-PCR

First-strand cDNAs were synthesized by SuperscriptII Rnase H^- reverse transcriptase (Life Technologies, Bethesda, MD) using oligo d(T) primers according to the protocal. PCR primers were designed using Primer Premier software (Premier biosoft international, Palo Alto, CA). The PCR primers used were: forward primer: 5' AAGGGTCACCTCTGTTCAAAGTG3' , reverse primer: 5' GCAGGGTTTATGGGCTCAATAG3' , Product length: 929 bp. G3PDH was used as internal control. Reactions were carried out in 10 μl solutions containing 100 ng cDNA, 0.2 μM of each primer, 50 μM of each dNTP, and 0.5 U of AmpliTaq DNA polymerase (Life Technologies, Bethesda, MD). The amplification conditions were (25 cycles): 1 min at 94 ℃, 40 sec at 56 ℃, 1 min at 72 ℃.

In situ hybridization

Specimens were fixed in 10 % neutral buffered formalin and embedded in paraffin wax. A series of 5 μm sections were cut for analysis. The antisense cRNA probe was prepared according to procedure described in Northern blot analysis section. ISH was performed as described previously[10,11]. Negative control (no probe applied) was performed. Slides were examined using an Olympus microscope. Images were captured using a cooled CCD camera (JVC, Japan). Cytoplasmic staining was noted. Any appreciable blue staining was considered positive, and graded as ± if very light blue were barely detectable, 1+ if light blue staining were seen diffusely throughout the cytoplasm, 2+ if easily seen fine staining were present throughout the cytoplasm, and 3+ when dark blue were observed. Generally, more than 100 cells (nontumor or tumor) were quantified in each measurement and at least one measurement was taken per slide. H&E slides were then reviewed to determine diagnosis and to map the location of the various histological patterns and correlate with the staining patterns observed in the ISH preparations.

Informed consents

The study protocol was approved by the Institutional Review Board of the hospital under the guidelines of the 1975 Declaration of Helsinki. Written informed consents were obtained from patients.

RESULTS

Isolation of differentially expressed cDNA by DDRT-PCR in gastric cancer

In order to run the assay efficiently, among 80 combinations of primer pairs (4 T_{12}MN primers *vs* 20 ARP primers), 4 pairs able to amplify more bands in one set of paired tissues were chosen and used to amplify cDNA from 7 sets of paired tissues. One primer pair (T_{12}GG *vs* ARP-3: 5' GACCATTGCA3') was found to amplify genes expressed differentially in 7 of 7 sets of the paired tissues. As shown in Figure 1, in comparison with results in the normal tissues, a cDNA fragment (arrowed) was found to be more abundant in the tumor and paratumor samples in all tested patients. The results of mRNA differential display were reproducible. This differentially expressed cDNA band was named as W2.

Sub-clone and sequence analysis

Two subclones, GCRG213 and GCRG224, were idnetified from the subcloning procedure. GCRG213 consisted of 1 194 base pairs, while GCRG224 consisted of 1 159 base pairs and had one open reading frame (ORF). The GCRG224-ORF consists of 35 amino acids with an estimated molecular weight of 3.8 kDa. BLAST analysis revealed that GCRG224 nucleotide sequence had 98 % homologue with Homo sapiens clone RP11-718B12, but the deduced amino acid sequence of GCRG224-ORF had no homology with any known peptide in the GenBank database. GCRG224 nucleotide sequence data was submitted to GenBank with accession No.AF438406. Further bioinformatic study in the GenBank database showed that GCRG224 is located at human chromosome 11q14.

RT-PCR and northern blot analysis

To confirm the expression pattern of GCRG224 in human gastric cancer, we performed RT-PCR analyses in 15 cases of paired gastric adenocarcinoma, paratumor and non-tumor

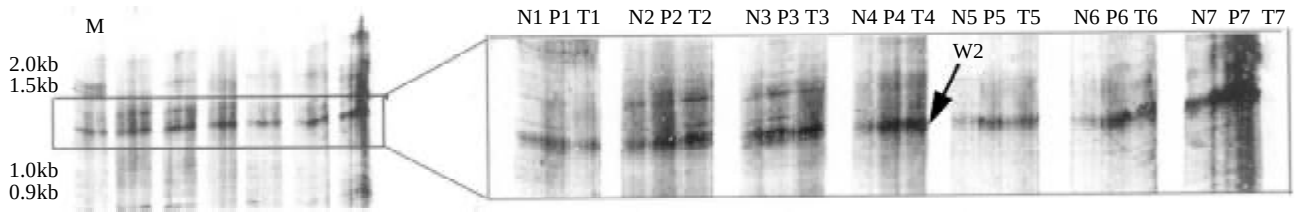

Figure 1 Differential analysis of expressed genes among human gastric cancer, paratumor and normal stomach mucosal tissues by means of fluorescent differential display. The differentially expressed cDNA fragment W2 (arrowed) showed higher expression in tumor and paratumor tissues than that in normal ones. N: normal; P: paratumor; T: tumor; 1-7: patient number.

tissues. GCRG224 expressed higher in 11/15 gastric cancer tissues than non-tumor tissues. However, the result of Northern blot analysis was contrary to that of DDRT-PCR, the non-tumor tissue showed higher GCRG224 expression level than the tumor did (Figure 2a). MTN results showed that GCRG224 also expressed in human normal colon tissues and peripheral blood leukocyte (Figure 2b).

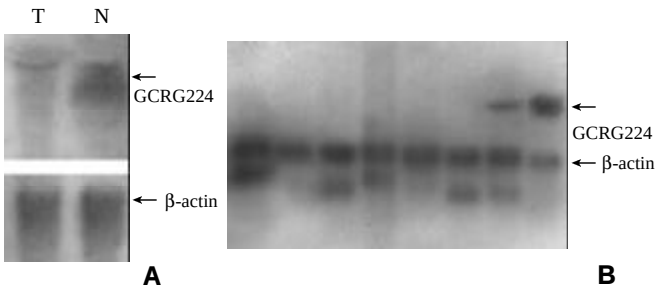

Figure 2 Northern blot analysis of GCRG224 expression in (a) human gastric cancer T/N pairs. T: tumor, N: non-tumor. (b) various adult human tissues. From left to right: spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocyte.

In situ hybridization

GCRG224 expression was analyzed at mRNA level. The hybridization signal that appears as blue is restricted to the cytoplasmic portion. All normal gastric glands showed ±-1+ staining. Only 5 out of 30 cases of adenocarcinoma had 2+ staining while the rest had ±staining. Of the 18 cases of intestinal metaplasia (IM) and dysplasia found in the paratumor site, 6/18 IM and 3/18 dysplasia had 2+ staining, respectively. Interestingly, in 26/30 cases the gastric mucosal epithelial cells were stained 2+-3+ (Table 1, Figure 3).

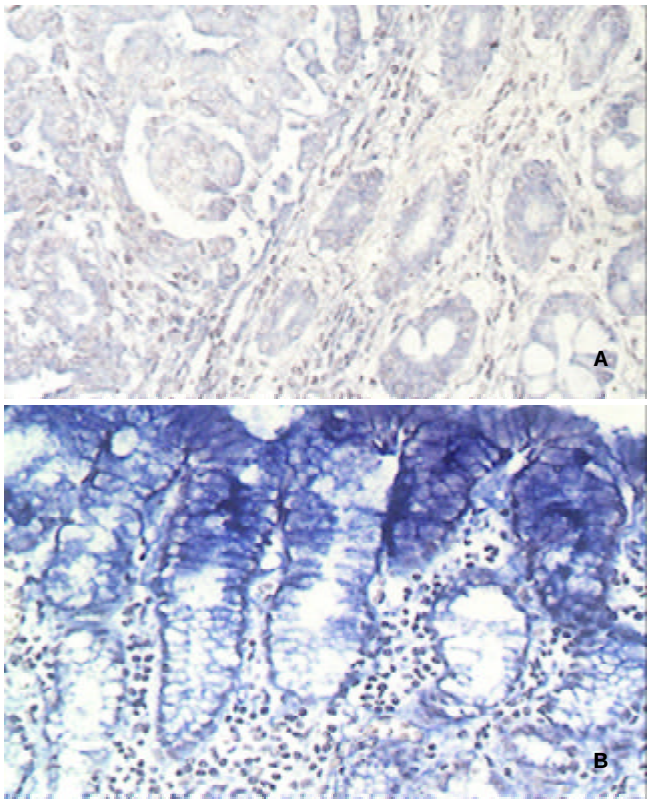

Figure 3 ISH analysis of GCRG224-ORF mRNA in formaldehyde-fixed, paraffin-embedded gastric tissues with a digoxigenin-labeled anti-sense probe, NBT/BCIP was used as alkaline phosphatase substrates, the expression appeared as cytoplasmic staining (blue precipitates). (a): Mild expression in tumor (left) and intestinal metaplasia glands (right) (200); (b): Strong expression in gastric mucosal epithelium cells, while the adjacent normal gastric glands showed little staining (200×).

Table 1 GCRG224 expression in different types of cells in gastric mucosa

	gastric mucosal epithelium	Gastric glands	IM	DYS	adenocarcinoma
±	0	19	8	13	25
1+	4	11	4	2	0
2+	15	0	6	3	4
3+	11	0	0	0	1
Total	30	30	18	18	30

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 c-met, c-myc, K-ras, c-erbB-2[12-16], etc., inactivation of tumor suppressor genes such as p53, p16, Rb, DCC, APC, PTEN[17-24], etc, DNA ploidy and microsatellite instability[25-28], abnormal transcript of genes related to metastasis like nm23, CD44, E-cadherin[29,30], etc., are reported common events in the steps of carcinogenesis. Newly found cancer related genes such as COX-2, survivin, metallothionein II and RUNX3, etc. were also expressed abnormally in gastric cancer[31-38]. Recognition of genetic factors had also improved the the treatment of cancer[39-41]. However, very little is currently known about genes that may predispose us to gastric cancer. The primary aim of this study was to identify genes that were closely related to human gastric cancer by means of DDRT-PCR.

DDRT-PCR has been widely applied to identify cancer-related genes[42-47], some of these genes look like to be of clinical value. It might reflect the true patterns of genetic changes in human gastric cancer if we identify gastric cancer-related genes directly from human gastric tissues.

One of the defects when using tissues for DDRT-PCR analysis is that some cDNA fragments amplified in the tumor tissues may actually originate from normal cells present in the tumor tissues, which may affect the results of the analyses. In order to decrease the chance of error in this study, we used 7 sets of tumor, paratumor and non-tumor tissues for the assay, displayed the results simultaneously and chose interesting bands showing distinctive patterns in all sets of specimens. One cDNA fragment which is up-regulated in gastric cancer tissues was identified. However, two subclones were identified from the subsequent subcloning procedure. Sequencing results revealed that these two subclones contained nucleotides of nearly the same size but of totally different sequences. This should be the result of PCR amplification in which arbitrary primers were used.

Because the deduced amino acid sequence of GCRG224-ORF had no homology with any known peptide in the GenBank database, further study was focused on this gene. The result of RT-PCR analysis was consistent with that of DDRT-PCR, but Northern blot analysis revealed an opposite result. Furthermore, ISH analysis also failed to see a general higher expression of GCRG224 in tumor cells. On the other hand, ISH showed an extensively strong expression of GCRG224 in gastric mucosal epithelial cells in almost all tested cases. This indicated GCRG224 might come mainly from normal gastric epithelium

other than tumor cells. Although the tissues applied for DDRT-PCR, Northern blot and RT-PCR in this study were examined by pathologists before their usage, they were actually a mixture of different types of cells, the exact percentage of tumor and non-tumor cells in each tissue could not be identified, this resulted in the contradiction we faced. It would be better, therefore, to separate different types of cells using techniques such as microdissection in tissues like gastric mucosa prior to the analyses[48-51].

Correlations between prognoses and the expression of genes such as p53, ras, myc, nm23, etc.were studied extensively in gastric cancer[15-17, 52-57]. However, the results turned out to be controversial. It is meaningful to find a biomarker that might predict the diagnosis or prognosis of gastric cancer. The consistent over-expression of GCRG224 in all tested tumor tissues in the DDRT-PCR and RT-PCR analyses prompted us to study further the expression pattern of GCRG224 in gastric mucosal tissues using ISH. However, GCRG224 did not show the expression pattern in gastric cancer and its precancerous leisions as RT-PCR revealed. Only around 20 percent tumor and precancerous leisions showed GCRG224 over-expression comparing with their normal gastric glands. Thus, GCRG224 may not serve as a potential marker for the diagnosis of gastric cancer.

A significant finding in this study is the extensive staining of GCRG224 in gastric epithelium. Up till now, the function of gastric mucosal cells is believed to be a barrier of the stomach as well as secretion of alkali and other ions such as Na^+, K^+ and Cl^-. It will be of great interest to study the role of GCRG224 expression in gastric mucosal epithelium.

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