A gene encoding an apurinic/apyrimidinic endonuclease-like protein is up-regulated in human gastric cancer

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INTRODUCTION

Gastric cancer is the second most common cause of cancer-related deaths in the world. It is widely accepted that genetic alterations play an important role in the pathogenesis of gastric cancer\[1,2\]. The expression of oncogenes such as c-met, c-myc, ras, c-erbB-2\[3-5\], the inactivation of tumor-suppressor genes, such as p53, p16, Rb, DCC, APC\[6-12\], and the abnormal transcription of genes related to metastasis, such as nm23, CD44, and E-cadherin\[12,13\], have been reported in patients with gastric adenocarcinoma. Recent data showed that cancer related genes such as COX-2\[14,15\], survivin\[16,17\], metallothionein II and RUNX3, etc.\[18-22\] were also expressed abnormally in gastric cancer. With the development of molecular biology techniques, such as cDNA array and differential display reverse transcription polymerase chain reaction (DDRT-PCR), some novel genes or cDNA fragments closely related to the development of human gastric cancer were identified recently\[23-32\]. However, the genetic factors in human gastric cancer and their mechanisms of carcinogenesis remain uncertain and deserve further study. We, therefore, used DDRT-PCR to screen the human intestinal-type gastric adenocarcinoma and its precursor lesions for the differential expression of gastric cancer related genes (GCRGs).

In our previous reports, we described a cDNA fragment which was upregulated in human gastric cancer tissues. Two subclones, GCRG213 and GCRG224, were subsequently identified. GCRG224 was overexpressed in almost all gastric mucosal epithelia but only in a small portion of gastric cancer and precancerous lesions\[33\]. In this study, we investigated the subclone, GCRG213 and found that it was a gene encoding an apurinic/apyrimidinic endonuclease1(APE)-like protein. GCRG213 was overexpressed in gastric cancer and in its precancerous lesions.

MATERIALS AND METHODS

Patients and tissue acquisition

Fresh primary intestinal-type gastric adenocarcinoma, para-tumor tissues and non-cancerous gastric mucosal tissues were collected from 7 patients (male: 4, female: 3; mean age: 51±18 years old) and their corresponding noncancerous tissues were collected for 32 cases of gastric adenocarcinoma, 15 cases were early gastric cancer while the other 17 cases were advanced carcinoma. The diagnosis of cancer was confirmed through histological findings.

Differential display, cloning and sequencing

Total RNA was extracted from tissues using TRizol reagent (Life Technologies, Inc., Rockville, Maryland) according to the instructions of the manufacturer. The fluorescent differential display was performed as previously described\[34\]. The primers used in the assay were T_{12}GG vs. ARP-8: 5’ TGGTAAAGGG3’
(Genomyx Corporation, Foster City, CA). The intensity of differentially expressed bands was quantified by Image Quant software (Molecular Dynamics, Sunnyvale, CA). The differentially expressed cDNA fragments were sub-cloned and sequenced as described previously[33, 34]. The sequenced cDNA was analyzed via the BLAST program for matches in the GenBank database[36, 37], and DNASIS software (Hitachi Software Engineering America Ltd., San Bruno, CA) was used for bioinformatic analysis.

**Northern blot analysis**

Dig Northern Starter Kit (Roche Diagnostic Corporation, Indianapolis, IN) was used. The procedure of hybridization was performed according to the manufacturer’s protocol. Anti-sense cRNA probe labeled with digoxigenin was generated from a digested cDNA insert by means of in vitro transcription. Digoxigenin labeled sense cRNA probe was used as a negative control. The hybridization signals were visualized with chemiluminescence which is recorded on X-ray films. The exposure time was 10 minutes.

**In situ hybridization**

All specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. A series of 5 µm thick sections were cut for analysis. In situ hybridization (ISH) was performed as previously described[36, 37] using anti-sense cRNA probe labeled with digoxigenin. Briefly, the slides were dried at 40°C overnight, dewaxed, rehydrated and pretreated with DEPC-treated PBS containing Triton X-100. The sections were then permeabilized with 20 µg/ml RNase-free proteinase K (Merck, Darmstadt, Germany) for 20 min, incubated at 37°C for at least 20 min with prehybridization buffer. Each section was overlaid with 30 µl of hybridization buffer containing 10 ng of digoxigenin-labeled cRNA probe and incubated at 42°C overnight. The positive signal for GCRG213 mRNA was finally detected by using NBT/BCIP as substrate. Sense cRNA probes were used as negative control. The presentation of blue staining in cytoplasm was considered positive. The positive staining of cytoplasm was semi-quantified as Grade ±: barely detectable light blue, Grade 1+: diffuse light blue, Grade 2+: blue staining, and Grade 3+: dark blue. More than 100 non-tumor or tumor cells were quantified in each measurement, and more than one measurement was required to confirm the diagnosis. Consequent slides with H&E staining were then reviewed to compare the histological patterns to the staining patterns 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**

**Identification of a human gastric cancer related gene**

One differentially expressed cDNA band named W2 was found to be more abundant in the tumor and paratumor samples in all tested patients. W2 was sub-cloned into a pGEM-T easy vector, and confirmed by EcoRI digestion. Two subclones, GCRG213 and GCRG224, were subsequently identified[33]. Sequencing results showed that GCRG213 consisted of 1094 base pairs with an open reading frame (ORF) which encoded 142 amino acids with an estimated molecular weight of 16.4 kDa (Figure 1). This nucleotide sequence data were submitted to GenBank with accession No. AY053451. BLASTN analysis revealed that GCRG213 shared 88% homology with human

| 3 GTA AAG GGA TCA ATA TGG CCA CAA GAA GAG CTA ACT ATC CTA CAA GAT TGA GCT ATT ACA AAT M Q E L T I L N I Y A P N T 20 |
| 63 AGA CGC CCA AGT TTC ATA AAG AGG GAG GGG GGA TTT AGA CTA ATT CAA ACT GAA GCA TCA AGG CAG T T A I G M D F N T Q L S I L E R S T R Q 40 |
| 123 ACA ATA ATG GGA GAC TTT AAC ACC CAA CTA ATA TTA GAA AGA TCA ACA AGG CAG T I T I L N D I Q D L N S A L Q T D P I D 80 |
| 183 AAG GTT AAG GAT ATC CAG GAC CGT AAC TCA GCT CTA CAA CAA ACA GAC CCA ATA GAC T H R T L H P K S T E C T T F F S A P H L 100 |
| 243 ATC CAG AGT ACT CTC CAC CCC AAA TCA ACA GAG TGT ACA TCT TTA GCA GCA CCA CAT CTC 81 |
| 303 ACT TAT TCT AAA TTT GGC CAC ATC ATA ATT GGA AGT AAA GCA CTC CTC ACC AAA TGT AAA AGA T Y S K F G H I I I G S K A L L T K C K R 120 |
| 363 ACA GAA ATC ACA GAC TGT CTC TCA GAC CAC ATG GGC ATC AAA TTA GCA AGT CTA G A I T E T T D C L S D H S A I K F E L R I 140 |
| 423 AAG AAC ATC TCT AAA AAT GTA AGT AGA AAA ATG AAA CGG AAA GTA AAT TAT TGA AAT TGG CTG CTC TCT ATG AAT 482 |
| 141 K L T Q N * 160 |
| 483 TAC TGG GTA AAT AAC AAA ATG AAG AGG AAT TTA GAA ACC AAT GAG AAC 542 |
| 543 GAA GAC ACA ATG TAC CAG CTA TCT GTC GAC ACA AAT TAA AAA GCA GTA TGT AGT CGG GAG AAATGT 602 |
| 603 ATA GCA ACA TAT GAC GAG AAG CAA GAG CAA TAC TAA TAT GAC CCC TTA ACATCA 662 |
| 663 CAA TTA AAA GTA CTA GAG AAG AAA AGG CAA ACA CAT TCA AAAGGCTTGACAGA AGG AAA AAG 722 |
| 723 TAA GCT GAC AGC ACT GGA GAC GAG AGC ACA AAA AAGGCTCCTAAA AAG CAATGA 782 |
| 783 ATC CAG CAG CTT GGG TGT TTA AGA GAT CAA CAA AAT TGA TAG ACT GCT AGC AAG ACT AAT 842 |
| 843 AAA GAG AAA AGA AGG AAT AAA ATG ATT GTA AAT GCA AAA TAA AAG GGA TAT CAC CAC 902 |
| 903 TGA GCC CAG GAA GAT AAA AAC TAC CAT GAG AGA ATA CTA AAA ACC CTA CAA AAA TAA 962 |
| 963 ACT TGA ACA TCT GGA ACA ATT ACA AAT GGA ATT CGG AAT ACA TAC CTT AGT CTC TGA ACT CTA AAT 1022 |
| 1023 CCA GGA AGA GGT TGA ATC TCT GCA TAG ACC AAT ACG AAG TCC TGA AGT TGA GGC AGT AAT 1082 |
| 1083 TAA TAT CTT ACC AAA AAA AAA AAA 1106 |

**Figure 1** Sequence of the human GCRG213 cDNA. The DNA sequence encoding the predicted start (ATG) and stop (TGA) codons is bolded and underlined. Three possible polyadenylation signals (AATAAA) are in italics. The protein encoded by the open reading frame is indicated in the single-letter code below the DNA sequence.
retrotransposable LINE-1 element LRE2. Through conserved
domain database search in GenBank, a putative conserved
domain, apurinic/apyrimidinic endonuclease1 (APE), was
detected in the deduced amino acid sequence of GCRG213-
ORF, it shared 61.0 % alignment with the C-terminal region
of APE conserved domain (Figure 2). Northern blot analysis
showed that GCRG213 was over-expressed in tumor tissues
than in their non-tumor counterparts (Figure 3).

Figure 2 NCBI conserved domain search results. A: GCRG213 ORF produced significant alignments with gnl|Pfam|pfam 01260
AP_endonucase1, AP endonuclease family 1; CD-length =250 residues, only 61.0 % aligned; Score=88.2 bits (217), Expect=3e-19. B:
Human APE protein produced significant alignments with gnl|Pfam|pfam01260 AP endonuclease family 1; CD-
length=250 residues, 100.0 % aligned. Score=161 bits (407), Expect=1e-40. GCRG213-ORF contained residues such as Y171, D210,
N212, D308 and H309 (arrowed) that are important for the endonuclease activity of APE.

Figure 4 In situ hybridization analysis of GCRG213 in formalin-fixed, paraffin-embedded 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) gastric adenocarcinoma invading into the muscle layer showed grade 2+ ~ 3+ staining, (B) gastric IM
epithelium showed grade 2+ ~ 3+ staining, (C) normal gastric glands showed grade ± ~ + staining, (D) negative control. Magnification was 10×10.
Figure 3 Northern blot analysis of GCRG213 in human gastric tissues. GCRG213 showed higher expression in tumor tissues than in non-tumorous ones. The expression of β-actin served as an internal control. N: non-tumorous tissue; T: tumor; 1-3: patient number.

**In situ hybridization**

GCRG213 expression was analyzed at the mRNA level using in situ hybridization. The hybridization signal appeared as a blue color in cytoplasm.

Both the early and advanced gastric cancer tissues were stained grade 2+ ~ 3+. Eight of the 17 cases of advanced adenocarcinoma showed an invasion of cancer into the muscle layer, with all eight cases showing grade 3+ staining in the invading tumor cells (Figure 4A). Dysplasia tissues at the para-tumor region were found in 22 patients. 15/22 dysplasia tissues showed grade 2+ ~ 3+ staining of GCRG213 expression while the rest showed grade 1+ staining. Nineteen patients had intestinal metaplasia (IM) epithelia at the para-tumor tissue. 9/19 IM showed grade 2+ ~ 3+ staining, the rest showed non-staining to grade 1+ staining (Figure 4B). All normal gastric glands showed grade ± ~ 1+ staining (Figure 4C).

**DISCUSSION**

In the present study, we used differential display to study the gene expression profile of human gastric cancer. One cDNA fragment, named by us as gastric cancer related gene 213 (GCRG213), was up-regulated in the gastric adenocarcinoma tissues of all 7 tested patients. Northern blot analysis confirmed the differential expression of GCRG213. As for the consistent up-regulation in gastric cancer tissues, further studies are necessary to confirm the role of GCRG213 and its expression pattern in tumors.

The nucleotide sequence of GCRG213 shared 88% homology with human retrotransposable LINE1 element LRE2. LINE-1 elements are very ancient, they constitute 20% or more of some mammalian genomes and presumably play a role in the evolution, structure, and function of mammalian genomes. LINE-1 elements contain regulatory signals and encode two proteins: one is an RNA-binding protein and the other is an APE-like enzyme which has both endonuclease and reverse transcriptase activities. BLASTP analysis in this study showed that the deduced amino acid sequence of GCRG213-ORF shared 84% homology with the S terminal of LINE1-ORF2 where the repair nuclease domain of APE exists.

We proposed that GCRG213-ORF be a new APE-like protein based on the following facts. First, APE conserved domain could be detected in GCRG213-ORF and the latter shared homology with the C terminal of human APE protein which is of particular relevance to the endonuclease function of APE. Furthermore, GCRG213-ORF also contains residues proposed to be important to the endonuclease activity of APE (Y171, D210, N212, D308 and H309). AP endonucleases have been divided into two families based on their amino acid sequence identity to either exonuclease III or Endo IV. Typically, the exonuclease III family of endonucleases accounts for approximately 95% of the repair activity in the organism. In mammals, the predominant AP endonuclease is APE (also called HAP1 or APEX), an enzyme that belongs to the ExoIII family. Human APE plays an important role in the base excision repair machinery of eukaryotic cells. The DNA repair activity of APE resides in the C-terminal region.

Many mechanisms such as hydrolysis of purine or pyrimidine, ionizing radiation, UV irradiation, and N-glycosylases may act on endogenous apurinic/apyrimidinic sites to modify DNA bases. Unrepaired apurinic/apyrimidinic sites result in mutations during DNA replication. Apart from its DNA repair function, APE exhibits as Redox-factor-1 which is important for the activation of transcription factors, such as activator protein 1, p53 and nuclear factor kappa B. APE also regulates the transactivation and pro-apoptotic functions of p53. Therefore, a role of APE in human tumorigenesis has been suggested.

APE protein is expressed in a wide range of human cells. Through immunohistochemistry detection, APE can be mainly localized in nucleus or cytoplasm or both depending on the cell type. Elevated expression of APE has been reported in a number of tumors such as prostate, ovarian, cervical, colorectal and germ cell tumors, malignant gliomas, whereas the cellular localization (nuclear/cytoplasmic ratio) differs in some neoplasia (colorectal carcinomas, epithelial ovarian cancers, primary breast carcinomas and thyroid carcinomas). In breast cancer, APE protein expression correlates with lymph node status and angiogenesis while in head-and-neck cancer, nuclear expression of APE is associated with its resistance to chemoradiotherapy and poor outcome. In this study, GCRG213 overexpressed in gastric intestinal metaplasia and dysplasia of the stomach as well as early and advanced gastric adenocarcinoma. The patterns of GCRG213 expression in cancerous tissue of the early gastric cancer did not differ significantly from those in the advanced gastric carcinoma. Thus, the GCRG213 expression appears “early” in the stage of gastric adenocarcinoma. This expression pattern is consistent with that of APE in cervical, prostate, colorectal cancer and their premalignant lesions reported.

There are two immediate implications of these findings of elevated GCRG213 expression in cancers. First, if the expression of GCRG213 could be modulated downward to, or below normal levels in the cancer cells, there may be an effect on the progression of the cancer or, the cells may become more sensitive to chemotherapeutic treatment. The latter presupposes that the increase in AP endonuclease activity result in increased DNA repair activity, protecting more cancer cells against base damage than normal cells.

In conclusion, a gene, GCRG213, overexpressed in tumors was identified in this study. Because of the similarity of the expression pattern in tumors between APE and GCRG213, as well as the 61% alignment between the amino acid sequences of GCRG213-ORF and APE conserved domain, it is likely that GCRG213-ORF is an important member of the APE family. A greater understanding of alterations in the function of GCRG213 in human cancers may explore its epidemiological and therapeutic significance.

**ACKNOWLEDGEMENTS**

The authors would like to thank Dr. Sien-Sing Yang, the Cathay General Hospital, Taipei, Taiwan and Timothy K Lee, Ph.D., FDA, U.S.A. for their comments.

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Edited by Zhao P