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Isolation of Two Novel Genes, Down-regulated in Gastric Cancer

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Using a differential display technique, we identified two genes that are down-regulated in human gastric cancer tissue as compared to normal gastric mucosa. The down-regulated expression of these genes in gastric cancer tissue was confirmed by northern blotting analysis and RT-PCR. One, CA11, was a novel gene expressed predominantly in the stomach and was depleted in all of the gastric cancer cell lines examined. The other gene, GC36, was homologous to the digestive tract-specific calpain gene, *nCL-4*. The expression of both *GC36* and *nCL-4* was suppressed or depleted in gastric cancer cell lines of differentiated and poorly differentiated types. This is the first report of genes, the expression of which is down-regulated with considerable frequency in gastric cancer.

Key words: Differential display — Down-regulated expression — Gastric cancer — Digestive tract-specific calpain *nCL-4*

Despite a decrease in incidence, gastric cancer is still one of the most common malignancies in the world. The genetic basis of this disease is not well understood. Amplification of c-*met*, c-*erbB-2* and K-*sam* genes, or point mutations of K-*ras*, *p53* and *APC* are known to occur in gastric cancer.[1,2] Recently, it was reported that germline mutation of E-cadherin caused a reduction in the amount of functional protein and led to a high rate of gastric cancer in a Maori family.[3] However, the frequencies of these genetic alterations are not high.

Differential display (DD)[4] is a rapid and effective method to identify differences in gene expression between related cells. Using DD, genes that are differentially expressed between normal gastric mucosa and gastric cancer tissue were investigated. The results are illustrated in Fig. 1. The bands of about 760 bp and 190 bp derived from normal tissue, the intensities of which were higher than those of the corresponding bands from the tumor tissue, were cut out from the polyacrylamide gel. We isolated the cDNA fragments of interest in the excised bands from the polyacrylamide gels with a rapid selection system using agarose gel electrophoresis containing a base-specific DNA ligand without cloning, as described previously.[5] We also cut out the corresponding regions showing low or no expression in the tumor lane. After reamplification of these fragments from the normal and tumor lanes, the cDNA fragments of interest were selected by side-by-side comparison between normal and tumor lanes on agarose gels containing the base-specific DNA ligand bisbenzimide-polyethylene glycol polymer.[6] The selected cDNA fragments were again purified by preparative electrophoresis on agarose gels containing phenyl neutral red-polyethylene glycol polymer. A cDNA library of mRNA extracted from normal gastric mucosa was constructed in the Uni-ZAP XR vector (Stratagene, La Jolla, CA) and screened using ^{32}P-labeled reamplified fragments as probes. We isolated two novel genes, *CA11* and *GC36*, showing tissue-specific expression and down-regulated expression in gastric cancer tissue.

The size of the largest clone of *CA11* isolated from the cDNA library was 738 bp, which was shorter than the DD fragment excised from the gel. We tried to isolate the 5′ end of the transcript using "Human Sure-RACE" a Multi-Tissue RACE panel (OriGene Technologies, Inc., Rockville, MD) consisting of 24 human RACE cDNAs. RACE products appeared from cDNAs derived from stomach, uterus and placenta (Fig. 2). We successfully isolated the longest RACE fragment (132 bp), although most of the fragments consisted of 79 bp. A cDNA clone deduced to have nearly the whole sequence was isolated by screening using a commercially available human stomach cDNA library (Takara Shuzo, Shiga) with the longest RACE product (132 bp) as a probe. Isolated cDNAs were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing FS Ready Reaction Kit in a 373A automated DNA sequencer (both from Perkin Elmer Applied Biosystems Division, Norwalk, CT). The 5′ end of *CA11* mRNA has some self-complementary sequences, i.e., the 8 nucleotides between residues 39 and 46 corresponding to the sequence between residues 48 and 55. Most of the cDNAs were thought to have lost the 5′ ends during synthesis of the first-strand cDNAs because of the strong secondary structure, and the fragments including the 5′ ends could be amplified from a few cDNAs having nearly the whole sequence in DD RT-PCR and RACE.

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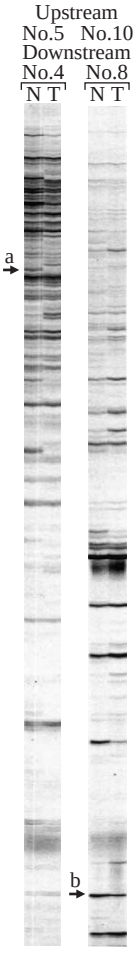

Fig. 1. Differential display using total RNAs derived from normal gastric mucosa (N) and poorly differentiated adenocarcinoma (T). DD was performed using a "Fluorescence Differential Display Kit" Rhodamine version (Takara Shuzo) according to the manufacturer's instructions. The upstream primer No. 5 (5′-CTTGATTGCC-3′) and downstream primer No. 4 (5′-rhodamine-labeled T_nCA-3′) to screen the *CA11* gene, and the upstream primer No. 10 (5′-GTTGCGATCC-3′) and downstream primer No. 8 (5′-rhodamine-labeled T_nGC-3′) for *GC36* were used, respectively. Arrows "a" and "b" indicate the bands derived from *CA11* and *GC36*, respectively.

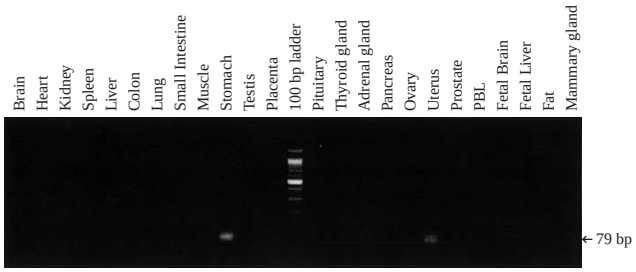

Fig. 2. 5′-RACE of *CA11* gene using "Human Sure-RACE" a Multi-Tissue RACE panel. This panel was used in combination with the *CA11*-specific outer primer, 5′-CTTTGTTTGGGTTGACTGAG-3′, and the *CA11*-specific inner primer, 5′-TTGTCTTCACGAAAGCAGTG-3′. The fragments from each tissue were cloned into the plasmid pT7Blue T-vector (Novagen, Madison, WI) using a TA cloning system. The sequence of the longest RACE product (132 bp) obtained from the placenta was identified.

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ATAACACCTAGTTTGAGTCAACCTGGTTAAGTACAAATATGAGAAGGCTTCTCATTCAGGTCC    63

ATGCTTGCCTACTCCTCTGTCCACTGCTTTCGTGAAGACAAGATGAAGTTCACAATTGTCTTT   126
M  L  A  Y  S  S  V  H  C  F  R  E  D  K  M  K  F  T  I  V  F

GCTGGACTTCTTGGAGTCTTTCTAGCTCCTGCCCTTGCTAACTATAATATCAACGTCAATGAT   189
A  G  L  L  G  V  F  L  A  P  A  L  A  N  Y  N  I  N  V  N  D

GACAACAACAATGCTGGAAGTGGGCAGCAGTCAGTGAGTGTCAACAATGAACACAATGTGGCC   252
D  N  N  N  A  G  S  G  Q  Q  S  V  S  V  N  N  E  H  N  V  A

AATGTTGACAATAACAACGGATGGGACTCCTGGAATTCCATCTGGGATTATGGAAATGGCTTT   315
N  V  D  N  N  N  G  W  D  S  W  N  S  I  W  D  Y  G  N  G  F

GCTGCAACCAGACTCTTTCAAAAGAAGACATGCATTGTGCACAAAATGAACAAGGAAGTCATG   378
A  A  T  R  L  F  Q  K  K  T  C  I  V  H  K  M  N  K  E  V  M

CCCTCCATTCAATCCCTTGATGCACTGGTCAAGGAAAAGAAGCTTCAGGGTAAGGGACCAGGA   441
P  S  I  Q  S  L  D  A  L  V  K  E  K  K  L  Q  G  K  G  P  G

GGACCACCTCCCAAGGGCCTGATGTACTCAGTCAACCCAAACAAAGTCGATGACCTGAGCAAG   504
G  P  P  P  K  G  L  M  Y  S  V  N  P  N  K  V  D  D  L  S  K

TTCGGAAAAAAACATTGCAAACATGTGTCGTGGGATTCCAACATACATGGCTGAGGAGATGCAA   567
F  G  K  N  I  A  N  M  C  R  G  I  P  T  Y  M  A  E  E  M  Q

GAGGCAAGCCTGTTTTTTTACTCAGGAACGTGCTACACGACCAGTGTACTATGGATTGTGGAC   630
E  A  S  L  F  F  Y  S  G  T  C  Y  T  T  S  V  L  W  I  V  D

ATTTCCTTCTGTGGAGACACGGTGGAGAACTAAACAATTTTTTAAAGCCACTATGGATTTAGT   693
I  S  F  C  G  D  T  V  E  N  *

CGTCTGAATATGCTGTGCAGAAAAAATATGGGCTCCAGTGGTTTTTACCATGTCATTCTGAAA   756

TTTTTCTCTACTAGTTATGTTTGATTTCTTTAAGTTTCAATAAAATCATTTAGCATTG        814
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Fig. 3. Nucleotide and deduced amino acid sequences of *CA11*. The deduced initial codon of the open reading frame is enclosed in a box. The nucleotide sequence of *CA11* appears in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB039886.

CA11 consisted of 814 bp and it contained the marker stSG31094, indicating that this gene is localized on human chromosome 2 (D2S292-D2S145), genetic instability of which has not been reported. This cDNA probably encodes a soluble protein of 199 amino acids, which contains a characteristic asparagine-rich sequence (Fig. 3). Homologous amino acid sequences to *CA11* were not found in the public database and there were no characteristic protein sequence motifs. By northern blotting analysis using Multiple Tissue Northern Blot (Clontech Co., Inc., Palo Alto, CA), mRNA of *CA11* (1−1.1 kb) was detected only in the stomach (Fig. 4A). Northern blotting analysis and RACE indicated that *CA11* is expressed predominantly in the stomach, and at low levels in the uterus and placenta. Northern blotting analysis using mRNA derived from a gastric cancer patient revealed very high expression of *CA11* in normal tissue, but not in cancer tissue. Also, various gastric cancer cell lines showed loss of *CA11* expression regardless of histological type (Fig. 4B).

Of the cDNA fragments showing down-regulated expression in cancer tissue on DD, *GC36* showed similar properties to those of *CA11*; tissue-specific expression and loss of expression in various gastric cancer cell lines. *GC36*

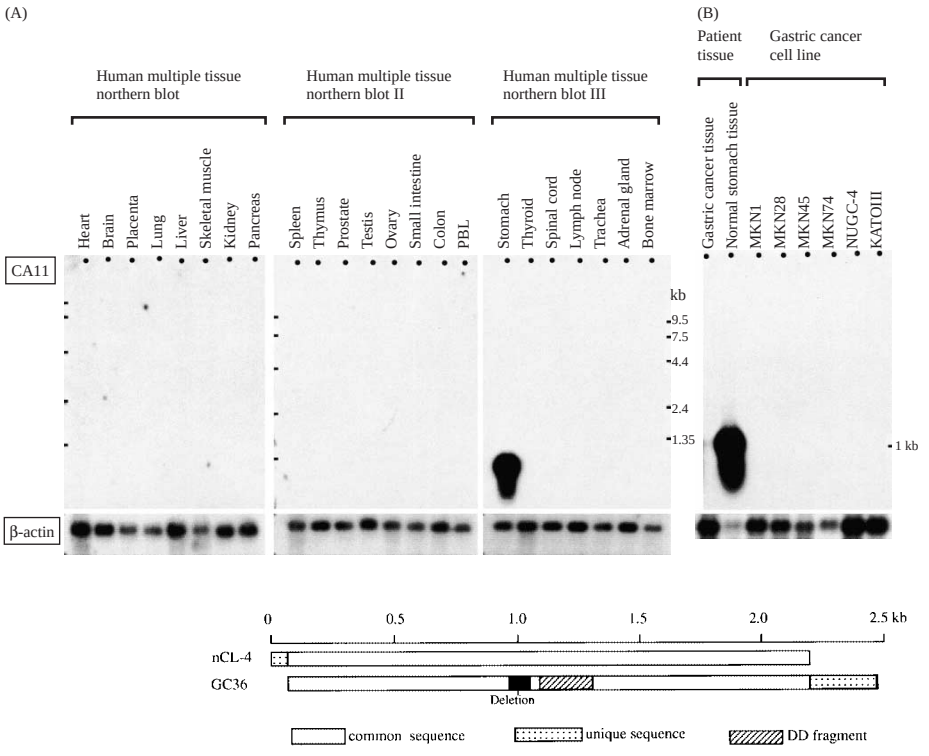

Fig. 4. Northern blotting analysis of *CA11* mRNA. (A) Human Multiple Tissue Northern ("MTN") Blot membranes were pre-hybridized for 30 min at 65°C with Rapid-hyb buffer (Amersham Life Science, Buckinghamshire, UK), and hybridization was carried out by adding ^{32}P-labeled *CA11* cDNA (738 bp) followed by incubation under the same conditions for 2 h. Membranes were washed once at 65°C with 2× SSC and 0.1% sodium dodecyl sulfate for 20 min, then twice with 0.1× SSC and 0.1% sodium dodecyl sulfate for 20 min before exposure to Kodak X-OMAT AR-5 film at −80°C. The RNA was qualified using a β-actin probe, which was supplied with the MTN Blot. (B) Aliquots of 2.5 μg of poly A$^+$ RNAs derived from a gastric cancer patient and from various gastric cancer cell lines were separated in a 1% agarose/6.2% (v/v) formamide gel. RNA was blotted onto a nylon membrane (Biodyne B, Pall Corporation, NY), which was then hybridized with the same probes as in (A). The histological types of the human gastric carcinoma cell lines we examined are adenosquamous carcinoma (MKN1), differentiated adenocarcinoma (MKN28 and MKN74), poorly differentiated adenocarcinoma (MKN45) and signet-ring cell carcinoma (KATOIII and NUGC-4).

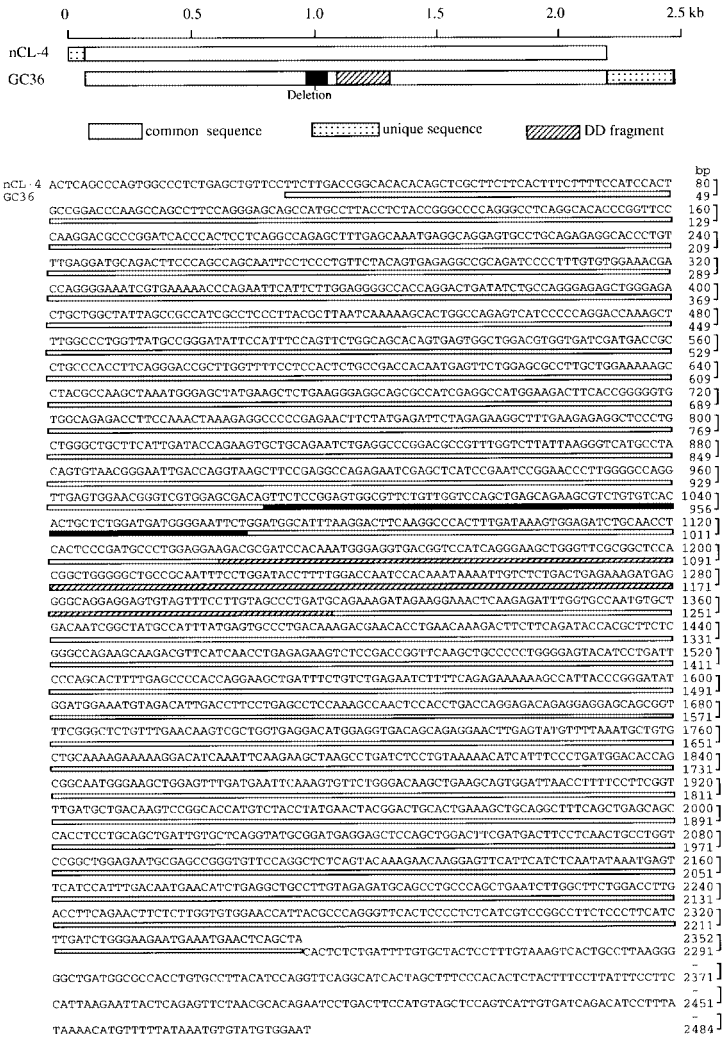

Fig. 5. Comparison of nucleotide sequences of *nCL-4* and *GC36*. *GC36* has been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB038463.

consisted of 2484 bp (Fig. 5) and showed a high degree of homology to the human digestive tract-specific calpain *nCL-4* cDNA.[7] The amino acid sequence of the protein encoded by *GC36* was identical to that of *nCL-4* except for one amino acid substitution (amino acid 292 coding serine in *nCL-4* to arginine) and deletion of the subsequent 26 amino acids (amino acids 293 to 318 in *nCL-4*). In the cDNA of *GC36*, 78 nucleotides were deleted between residues 987 and 1065 of the *nCL-4* cDNA and 241 bp were added at the 3′-terminal (Fig. 5). The fragment found by DD was not derived from the 3′-terminal of *GC36* cDNA, but from the middle region common to *nCL-4* and *GC36*. The clone derived from *nCL-4* was not found among the ten clones isolated from the cDNA library, even though the cDNA fragment common to *nCL-4* and *GC36* was used as a probe.

Calpain is an intracellular cytoplasmic non-lysosomal cysteine endopeptidase that requires calcium ions for its activity.[8] Calpain isoenzymes have been reported and they can be divided into two groups; one showing ubiquitous and constitutive expression, the other showing organ-specific expression. Similarly to *nCL-4*, the calpain isozymes *nCL-2* and *nCL-2′*, alternative splicing products, are predominantly expressed in the stomach.[9] We performed genomic library screening using a human BAC (I) library membrane (Genome Systems, St. Louis, MO) with the cDNA fragment from the common region as a probe. Two BAC clones showing positive signals were derived from the same locus and they were confirmed to have both the deleted region in *GC36* cDNA and the region unique to *GC36* by PCR (data not shown). So, *GC36* and *nCL-4* were thought to be generated by alternative splicing. The gene encoding *nCL-4* is localized on human chromosome 1, near the marker D1S225,[7] and *GC36* is probably also localized on this chromosome. LOH (loss of heterozygosity) on chromosome 1q occurs at high frequency in gastric cancer, and it has been suggested that there may be a tumor-suppressor gene on the normal chromosome 1.[2] Further studies are required to determine the systems involved in regulation of the expression of *GC36* and *nCL-4*.

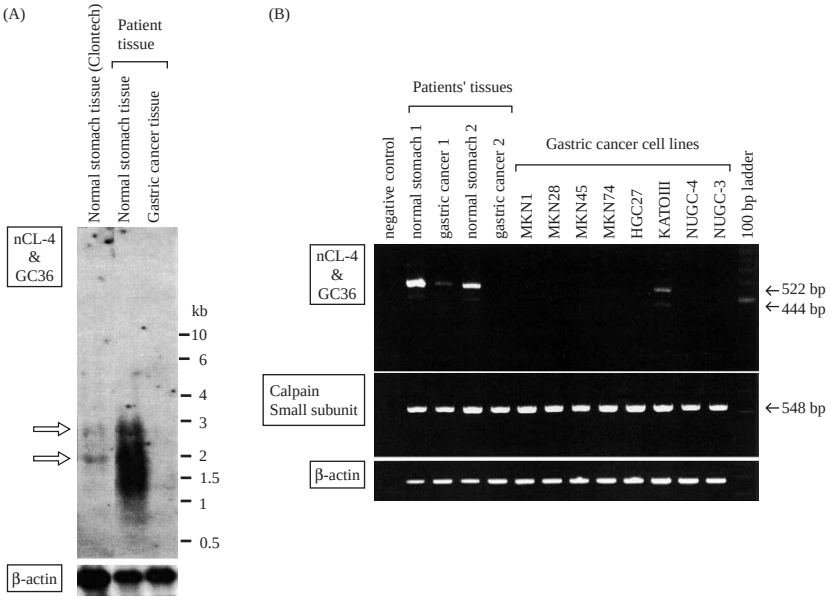

Fig. 6. Northern blotting analysis (A) and RT-PCR (B) showing the down-regulated expression of *GC36* and *nCL-4* in gastric cancers. (A) Stomach poly A^+ RNAs derived from a human victim of traumatic death (Clontech) and derived from a gastric cancer patient were blotted onto a nylon membrane. The *GC36* cDNA (503 bp), which was amplified by RT-PCR using the sense primer 5′-ATGAGGGGCAGGAGGAGTGT-3′ and antisense primer 5′-GTCCTTTTTCTTTTGCAGCA-3′, was used as a probe. (B) For semi-quantitative RT-PCR, total RNA treated with DNase I was reverse-transcribed using AMV reverse transcriptase, and cDNA was amplified by PCR using Ex Taq polymerase (Takara Shuzo). *GC36* and *nCL-4* were detected using 5′-CCCTGCTGGGCTGCTTCATT-3′ and 5′-CATCAGGGCTACAAGGAAA-3′ primers (amplicon: 444 bp for *GC36* and 522 bp for *nCL-4*). Primers for detection of the calpain small regulatory subunit (30K) were 5′-ACCGATCAGGGACCATTTGC-3′ and 5′-GCACAGGTACAGGGGAGAGG-3′ (amplicon: 548 bp). As a positive control, β-actin cDNA was amplified by PCR using primers 5′-CAAGAGATGGCCACGGCTGCT-3′ and 5′-TCCTTCTGCATCCTGTCGGCA-3′ (amplicon: 275 bp). The sequences of amplified fragments were confirmed by direct sequencing. The histological type of the human gastric carcinoma cell line HGC27 is poorly differentiated adenocarcinoma, and that of NUGC-3 is unknown.

On northern blotting analysis, two signals were mainly detected at about 2.5–2.7 kb and 2.0 kb in the lanes of mRNAs derived from the normal gastric tissues (Fig. 6A). There were no signals in the tumor lane of the gastric cancer patient (Fig. 6A) or in those of mRNAs derived from gastric cancer cell lines (data not shown). The decreases in *GC36* and *nCL-4* expression were confirmed by RT-PCR. To detect the expression of both *GC36* and *nCL-4* simultaneously by RT-PCR, we selected primers beyond the deleted region of *GC36* in comparison with *nCL-4*; fragments of 444 and 522 bp were amplified from *GC36* and *nCL-4*, respectively. The intensity of the band derived from *nCL-4* was higher than that from *GC36*, and the ratio of the former to the latter differed among individuals. RT-PCR analysis showed suppressed expression of both genes in gastric cancer tissues from patients and in all gastric cancer cell lines examined (Fig. 6B). *nCL-4* was reported to show activity only when coexpressed with a calpain small regulatory subunit (30K) and probably exists as a heterodimeric complex with this molecule.[10] The expression of 30K subunit in the cancer cell lines was also examined by RT-PCR, and all showed expression of this subunit (Fig. 6B).

Many substrates of the calpain isozymes have been reported, such as the transcription factors c-FOS and c-Jun, the tumor suppressor protein p53, protein kinase C, and the adhesion molecule integrin. The expression of calpain I mRNA was reported to increase with malignancy of renal tumor samples.[11] In this study, we found suppressed expression of tissue-specific calpain isozymes *GC36* and *nCL-4* in gastric cancer, and the patterns of expression of these genes differed from that of the constitutive calpain isozyme 30K subunit. These observations indicate that the mechanism of regulation of the expression and the physiological functions of the tissue-specific calpain isozymes *GC36* and *nCL-4* are different from those of the constitutive calpain isozymes during carcinogenesis and cancer progression.

In a preliminary study using clinical specimens, more than 60% of the patients with gastric cancer exhibited suppressed expression of both *CA11* and *GC36* mRNAs in the gastric tumor tissue (data not shown). Monitoring the expression of many genes simultaneously and in many different tissues is important. Microarrays that allow thousands of genes to be monitored may be useful for such studies. If the first-strand cDNAs synthesized with the mRNAs derived from different histological types of gastric cancers are used as probes, heterogeneous gene expression among the histological types of gastric cancers can be rapidly and extensively screened. Further investigations using various gastric cancer tissues of different histological types are now being carried out to study the feasibility of using these genes for molecular diagnosis of gastric cancer.

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