Cloning of novel LERGU mRNAs in GPR30 3′ untranslated region and detection of 2 bp-deletion polymorphism in gastric cancer

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The improved IGCR (In–Gel Competitive Reassociation) method was applied to the analysis of human gastric cancer genomic DNA to identify its alterations, and it appeared that the IGCR library contained a fragment of 3′-untranslated region (3′ UTR) of G-protein coupled receptor 30 (GPR30) mRNA. When we searched genomic DNA pairs of gastric cancer patients with this IGCR clone, we found the deletion polymorphism with or without 2 bp (Cytosine and Thymine; CT). We confirmed the existence of a novel mRNA in GPR30 3′UTR by northern blotting, cloned this novel mRNA and named it Leucine Rich Protein in GPR30 3′UTR (LERGU). The EST database search gave one alternative splicing form in this 3′ UTR, which was named as LERGU-1. A novel alternative splicing form of this mRNA was also identified from the stomach total RNA, which was named LERGU-2. The LERGU mRNA was also detected in eight gastric cancer cell lines, but GPR30 mRNA scarcely existed. Furthermore, we detected the 2 bp-deletion form in genomic DNAs and mRNAs derived from gastric cancers, but not in other type cancers. Since the 2 bp-deletion position on LERGU corresponds to its alternative splicing site, this deletion may produce a frame-shifted protein. Overall, our findings suggest that a mutation or disappearance of the normal LERGU protein may have a function in the development of gastric cancer. (*Cancer Sci* 2005; 96: 191–196)

Gastric cancer is the most frequent malignancy of the gastrointestinal tract in Japanese and certain South-east Asian populations, and one of the leading causes of cancer mortality in the world. Genetic aberrations associated with gastric cancers have been reported in the gene encoding E-cadherin, p53, the transforming growth factor-β receptor and so on, but it is believed that a much greater number of genetic changes could account for the phenomena.[1–4]

The original In-Gel Competitive Reassociation (IGCR) technique is one of the DNA subtractive hybridization techniques, which consists of the size separation of double strand DNA fragments by electrophoresis, the denaturation in gel with an alkaline buffer and the renaturation by exchanging the buffer.[5–11] This technique had achieved the sufficient enrichment of the DNA sequences only in the target to detect small aberrations between two DNA populations such as recombination junctions in extrachromosomal DNA,[9] restriction fragment length polymorphism in the genome,[10] and methylation sites in the genome.[6] We have improved the IGCR technique to overcome several disadvantages such as time-consuming and complex process with poor reproducibility. In the denaturing step, we employed a heat denature method instead of the alkaline denature protocol. In addition, to reduce the genomic complexity and prevent the amplification of undesirable polymerase chain reaction (PCR) bands, smaller fragments were cut off with DNA binding resins in the purification of Mse I cut fragment mixture, and a 'competitor' was introduced to the IGCR technique. Our improved IGCR method efficiently detected Epstein-Barr virus (EBV) genomic DNA fragments, which were contained only in a tester DNA of gastric cancer.[12]

G protein coupled receptor 30 (GPR30) is a seven transmembrane receptor, and its expression is up-regulated by progestin in the MCF7 breast cancer cell,[13] which is also called CMKRL2, CEPR, GPCR-Br, LyGPR, or FEG-1.[14–18] It is reported that the growth inhibition in MCF7 occurs through progestin after Erk-1 and Erk-2 are inactivated by GPR30.[19,20] Furthermore, GPR30 is necessary for the activation of EGFR-MAPK signaling by estrogen in breast cancer cells.[21,22]

In this paper, we report the identification of a novel RNA from GPR30 3′-untranslated region (3′UTR) of the gastric cancer patients' genomic DNA by our improved IGCR method and the detection of aberration of a novel mRNA only in the gastric cancer samples.

Materials and Methods

Human samples. Gastric cancer, leiomyoma in stomach and colon adenoma tissues were prepared from patients under their informed consent in the Second Department of Surgery, Gunma University Faculty of Medicine (Maebashi, Japan). The genomic DNA from non-cancer developmental twins was kindly gifted from Professor Ian W. Craig, Genetic and Developmental Psychiatry Center, Institute of Psychiatry, King's College London (London, UK) under informed consent.

Genomic DNA was extracted from the frozen sections of both the gastric cancer tissues and its adjacent normal tissues. The genomic DNA pair used in the improved IGCR method was obtained from a 57-year-old male patient who had undergone gastrectomy for T1N1M0 stage II disease. Histology of this gastric cancer was moderately differentiated tubular adenocarcinoma.

Human cDNA pairs from gastric cancer patients were purchased from BD Biosciences Clontech (Palo Alto, CA, USA), and human total RNA pairs from gastric cancer patients were obtained from Ambion Inc. (Austin, TX, USA) and Stratagene (La Jolla, CA, USA).

Human genomic DNA was purchased from Promega (Madison, WI, USA) except for gastric cancer samples, and human total RNA was purchased from BD Biosciences, Clontech, and Ambion Inc.

Total RNA was purified from cultured cells by Isogen (Nippon Gene Co., Ltd, Toyama, Japan) according to the manufacturer's

The nucleotide sequences reported in this paper have been submitted to the GeneBank™/EBI Data Bank with accession numbers AY265807-AY265809.

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protocols, and the contaminated DNA was digested with DNase I (Takara Shuzo Co., Ltd, Shiga, Japan). The mRNA was purified from cultured cells by a FastTrack 2.0 kit (Invitrogen Corp., Carlsbad, CA, USA) under the conditions instructed by the manufacturer. cDNAs were constructed from total RNA or mRNA by using Superscript II reverse transcriptase (Invitrogen Corp.) with 9mer random primers (Hokkaido System Science Co., Ltd, Sapporo, Japan) as described in the manufacturer's instruction.

Cell lines. Human gastric cancer cell lines H-111-TC (well differentiated tubular adenocarcinoma) St-4 (defuse-type, well differentiated tubular adenocarcinoma), MKN-7 (well differentiated tubular adenocarcinoma), SH-10-TC (poorly differentiated adenocarcinoma), Kato III (signet ring cell carcinoma), GCIY (poorly differentiated adenocarcinoma, scirrhous), and AZ521 (unspecified) were obtained from the Cell Bank, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Human gastric cancer cell line MKN-45 (poorly differentiated adenocarcinoma) was obtained from RIKEN Cell Bank (Tsukuba, Japan). Human embryonic kidney cell line (HEK) 293T was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Human breast cancer cell line MCF7 and colorectal carcinoma cell line KM12SM were obtained from Department of Tumor Biology, the University of Texas, M. D. Anderson Cancer Center (Houston, TX, USA). Human gastric cancer cell lines, breast cancer cell line MCF7, and colorectal carcinoma cell line KM12SM were cultured in RPMI1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS. HEK293T and HeLa cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen Corp.) supplemented with 10% FBS. Human colorectal adenocarcinoma cell line SW480 was cultured in Leibovitz's L-15 medium (Invitrogen Corp.) supplemented with 10% FBS. All cells were cultured at 37°C in humidified atmosphere of 95% air and 5% CO_2.

Improved IGCR. Improved IGCR was performed as described previously.[12] The normal genomic DNA was used as a tester, and the tumor genomic DNA was used as a driver as well as a competitor. Sequenced clones were analyzed by MKI in silico Analyzing System (Mitsui Knowledge Industry Co. Ltd, Tokyo, Japan) which is an automated blast search using several public databases.

Semi-real time PCR and real time PCR. Semi-real time PCR was performed by Ex Taq Polymerase (Takara Shuzo Co., Ltd) under the manufacturer's conditions. PCR steps were performed preheating at 96°C for 3 min, 30 cycles consisting of 30 s at 95°C and 1 min at 72°C followed by extension at 72°C for 15 min. The primers used in the semireal time PCR were 5′-GCCTGCCGCTGCAGGAAACATTTC-3′ and 5′-CTCGGCGGTGCGCTAGCTAGCTCAG-3′.

To quantify the GPR30 3′UTR fragment which was contained in the improved IGCR library, Ex Taq Polymerase R-PCR version (Takara Shuzo Co., Ltd) and i-Cycer iQ™ Real Time PCR Detection System (Bio-Rad Laboratories Inc.) were used. The primers for the semi-real time PCR experiments were amplified and used as the standard in the real time PCR. The concentration of the amplified standard PCR fragments was measured after the purification using QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany). The real time PCR step was performed under almost the same conditions for the semi-real time PCR except for the number of cycles; 40 cycles were carried out in the real-time PCR. The nucleic acid staining dye, SYBR Green I (Cambrex Corp., East Rutherford, NJ, USA) was employed for the quantification.

To quantify glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA as the control in the total and mRNA from cultured cells, Superscript II reverse transcriptase (Stratagene) and 9mer random primers were used. This reaction mixture was directly used as the template of real-time PCR. Real-time PCR was performed as described above. The primers employed were 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′.

Northern blotting. Northern blotting was carried out with 2 μg of mRNA of the cultured cells, whose amount was adjusted with the G3PDH mRNA content. To determine whether a novel mRNA is related to GPR30 mRNA or not, the specific probe was amplified using a DIG PCR labeling kit (Roche Diagnostics GmbH, Mannheim, Germany). Specific primers were described below;

Primer A: 5′-CGCTTCTTTCTAAAGATGGATTCACC-3′
Primer B: 5′-GCTTGTCCCTGAAGGTCTCCCCGAG-3′
Primer C: 5′-ATGTCTCTAAACTGCGGTCAGATGTGG-3′
Primer D: 5′-GGGAAGGTTTTTACATCATCACCGCAG-3′

A specific probe of the full-length GPR30 for DIG-labeling PCR was amplified by using Primer A and Primer D. The translated region of GPR30 was amplified with Primer A and Primer B, and its 3′UTR was done with Primer C and Primer D (Fig. 1). G3PDH probe was used as a control. Detection of northern blotting was performed using a DIG detection system (Roche Diagnostics GmbH).

To examine estrogen- and progestin-induced expression of GPR30 and LERGU, MCF7 cells were stimulated by 1 nM β-estradiol and 10 nM medroxyprogesterone (MPA), respectively.

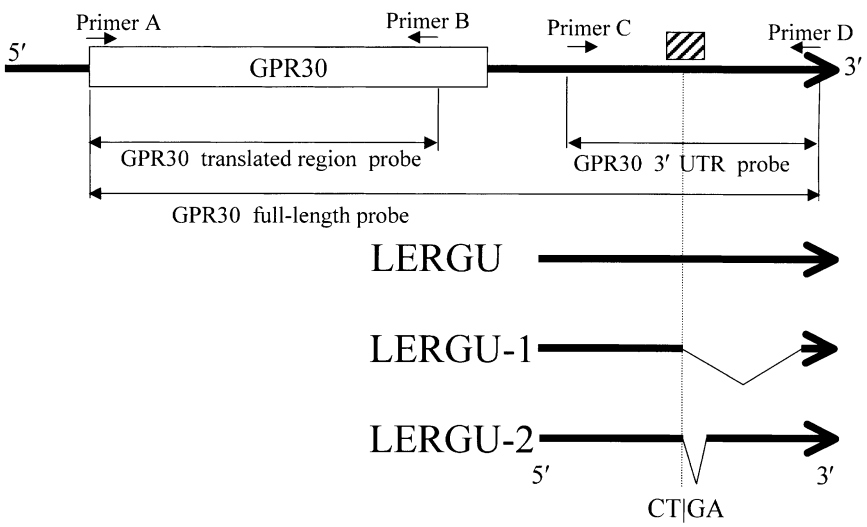

Fig. 1. The models of transcription patterns of the GPR30 region. The models of transcription patterns were displayed on the downstream of the GPR30 transcribed region. On the upper position, GPR30 mRNA exon 2 was shown. The primers for probes of northern blotting were displayed under the GPR30 mRNA. The probes used for northern blotting were shown by double-headed arrows under the GPR30 mRNA. The cloned fragment by improved IGCR is shown by a striped box. On the lower position, the transcription patterns of novel mRNA of LERGU, alternative splicing forms LERGU-1 and LERGU-2 are also demonstrated. The position of 2 bp-deletion polymorphism site is indicated by a dotted line, and alternative splicing junctions are also shown in LERGU-1 and LERGU-2.

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After a 24-h treatment cells were harvested and total RNA was prepared. A 25 μg aliquot of total RNA was analyzed by northern blotting using a GPR30 full length probe.

Cloning of novel mRNA. To clone a novel mRNA and alternative splicing form of this mRNA, we performed reverse transcription-PCR (RT-PCR). The cDNA transcribed from human normal total RNA (Ambion Inc.) was used as a template, and Primer C and Primer D described above were used for reverse transcription-PCR (RT-PCR). The PCR mixture was prepared using an Advantage-GC 2 PCR kit (BD Biosciences Clontech) as described in the manufacture's protocol. The amplified PCR mixture was directly sequenced, and the sequence data were compared with NCBI database (http://www.ncbi.nlm.nih.gov/). The candidate mutant fragments were cloned using pGEM-T Easy Vector System I (Promega).

Western blotting. A polyclonal antibody against LERGU from Rabbit was produced by SIGMA GENOSIS Japan K.K. (Hokkaido, Japan). The antigen was a peptide of LTPSTRKATRRGHCG, which was the C-terminal of the LEGRU protein. A GFP fusion protein of LERGU was expressed in HEK293T and used for the quality check of the polyclonal antibodies. Human gastric cancer H-111-TC and St-4 cells were harvested from subconfluent cultures and lyzed by RIPA buffer. A 40 μg aliquot of protein of the cell lysate was subjected to SDS polyacrylamide gel electrophoresis followed by western blot. In the western blot, rabbit anti-LERGU antibody was used for the first antibody reaction, and rabbit anti-LERGU antibody with preabsorption by the peptide antigen and mouse anti-human β-actin monoclonal antibody (Sigma-Aldrich) were used as controls. The secondary reaction was done with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology, Beverly, MA, USA) or horseradish peroxidase-conjugated anti-mouse IgG (Cell Signaling Technology). Positive bands were detected by using ECL plus Western Blotting Detection Reagents (Amersham Biosciences) and a Light Capture AE-6962 (ATTO Corp., Tokyo, Japan).

Fragment analysis for detection of 2 bp-deletion in LERGU Mrna. To investigate whether novel mRNAs derived from various genomic DNAs and RNAs have the 2 bp-deletion, we performed a fragment size analysis. PCR was carried out using an Ex Taq polymerase (Takara Shuzo Co., Ltd) under the manufacturer's conditions with 0.5 μM of R110-dCTP (Applied Biosystems, Foster City, CA, USA) or 0.5 μM of R6G-dCTP (Applied Biosystems) as a fluorescent dye. DNA templates used were genomic DNA and cDNA samples, which were transcribed from the total RNA. The primers used were 5′-GAATTGCTACAATCCCAAAGCGC-3′ and 5′-GCTTTAGGGTTCCTGTGTAACTG-3′.

PCR fragments were treated with calf intestine alkaline phosphatase (CIP), and then the CIP-treated PCR fragments and a GeneScan 400HD ROX size standard (Applied Biosystems) were mixed in formamide and analyzed by an ABI 310 Genetic Analyzer (Applied Biosystems). The analysis of fragment patterns was performed with GenScan 3.1 program (Applied Biosystems).

Results

Performance of improved IGCR and cloning of a novel mRNA named LERGU. The gastric cancer genomic DNA as a driver was subtracted from the normal stomach tissue genomic DNA as a tester by the improved IGCR method. The IGCR subtraction library was constructed and the obtained clones were sequenced, and then the database search was performed using MKI *in silico* Analyzing Systems. One clone out of 290 was identified as a GPR30 3′ UTR fragment (GPR30 mRNA: Accession No. NM_001505) which was mapped on 7p22.3.[23]

When the mutation search was carried out to check if the subtracted clone, the GPR30 3′UTR fragment (approximately 300 bp) would contain genomic aberration, we identified a 2 bp-deletion (Cytosine and Thymine; CT) in the genomic DNA pair of gastric cancer. The database search showed that the 2 bp-deleted GPR30 had been reported as 'FEG-1' (Accession No. AF015257) in GenBank. In contrast, the 2 bp-retentive form was submitted as 'similar to GPR30' (Accession No. BC011634) and 'GPCR-Br' (Accession No. U63917). This phenomenon, namely the existence of GPR30 with or without 2 bp (CT), was reported to be a deletion/insertion polymorphism in the NCBI website (refSNP ID: rs3840681; NCBI Assay ID: ss5027205) by Yusuke Nakamura, Laboratory of Molecular Medicine, The Institute of Medical Science, The University of Tokyo. The 2 bp-deleted GPR30 mRNA having approximately 3 kb was cloned from B-cell lymphoblasts and its expression in Burkitt lymphoma was confirmed.[14]

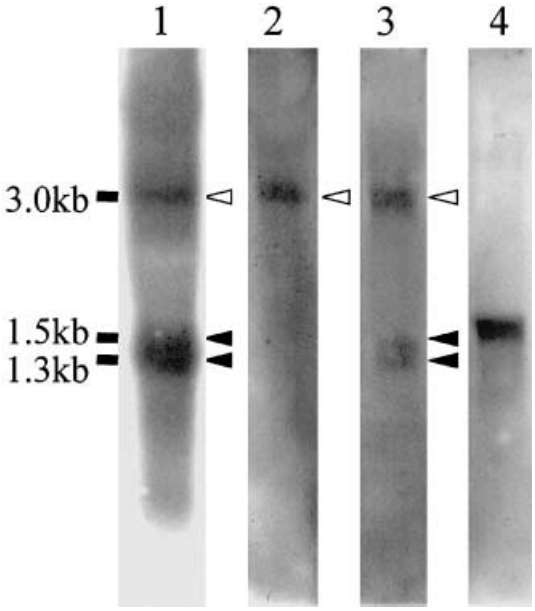

Fig. 2. Northern blotting by various GPR30 probes on MCF7 mRNA. Various probes in the GPR30 region shown in Fig. 1 were used to detect GPR30 mRNA and LERGU mRNAs in MCF7 mRNA. Open and closed arrowheads indicate GPR30 mRNA and LERGU mRNAs, respectively. Sizes are shown on the left. 1, GPR30 full-length probe; 2, GPR30 translated region probe; 3, GPR30 3′UTR probe; 4, G3PDH probe as a control.

However, the expressed sequence tag (EST) database search showed the existence of human ESTs in the 3′UTR region of GPR30; one is a 2 bp-retentive form and the other is its alternative splicing form (Fig. 1). The comparative analyzes of these two ESTs showed that the alternative splicing donor site used by them was not a general 5′ splice site consensus (AG|GT) but the 2 bp (CT|) mentioned above.

We carried out further analyzes of the EST fragment in 3′UTR of GPR30. To confirm the existence of a novel mRNA, but not GPR30 mRNA, northern blotting was performed with the mRNA prepared from MCF7 breast cancer cells, which were known to express GPR30 mRNA.[16,19,20] The probes used in the northern blotting were summarized in Fig. 1. A GPR30 full-length probe (2.1 kb) and a GPR30 3′UTR probe (800 bp) detected GPR30 mRNA having 3 kb and smaller ones having 1.5 and 1.3 kb, but a GPR30 translated-region probe (1.0 kb) hybridized with only the GPR30 mRNA (Fig. 2). When the cloning by RT-PCR was performed, another novel alternative splicing form was obtained in addition to two ESTs in 3′UTR region of GPR30 mentioned above (Fig. 1). Together with the results from the EST database search and the cloning, we named

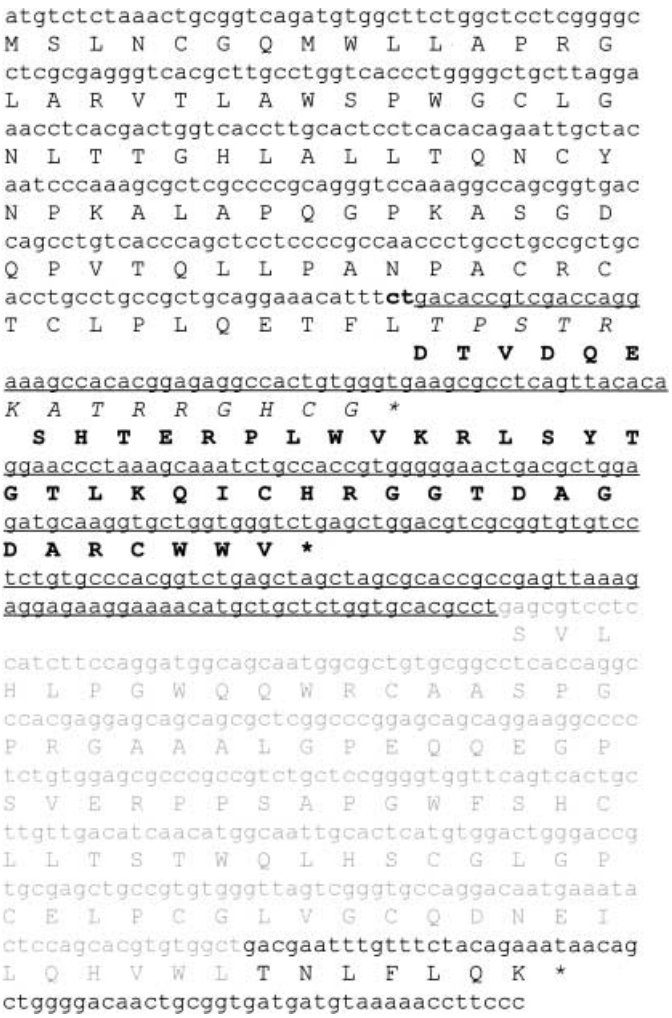

Fig. 3. Nucleotide and deduced amino acid sequences of the LERGU mRNAs. The 2 bp-deletion polymorphism was indicated in the nucleotide sequences in bold. The LERGU mRNA was deduced to the amino acid sequence shown under nucleotide sequence, and the downstream of 2 bp-deletion and the alternative splicing site were displayed in italic. The frame-shifted amino acid sequence of the 2 bp-deletion type mRNA was shown under the deduced amino acid sequence from the 2 bp-retention type mRNA written in bold. Alternative splicing form 1 was produced by splicing of the underlined sequence and the gray-colored sequence, and alternative splicing form 2 was produced by splicing of the underlined sequence.

a novel mRNA of a 2 bp-retentive form as 'LERGU' after Leucine Rich Protein in GPR30 3′UTR, an alternative splicing form from the EST database as 'LERGU-1' after LERGU alternative splicing form 1, and a novel alternative splicing form obtained by cloning as 'LERGU-2' after LERGU alternative splicing form 2. Figure 3 shows the nucleotide sequences and the deduced amino acid sequences of LERGU mRNAs. Although no homologies to any protein sequences were detected in a variety of database searches, the predicted protein was a leucine-rich protein. Two alternative splicing forms, LERGU-1 and LERGU-2, have the same consensus (CT|GA) at 5′ and 3′ sites as a donor and an acceptor, respectively.

We checked the expression of GPR30 mRNA and LERGU mRNA in the MCF7 breast cancer cell line and eight gastric cancer cell lines (Fig. 4a). Although the MCF7 cell line expressed both GPR30 mRNA and LERGU mRNA, gastric cancer cell lines expressed high levels of LERGU mRNA and a

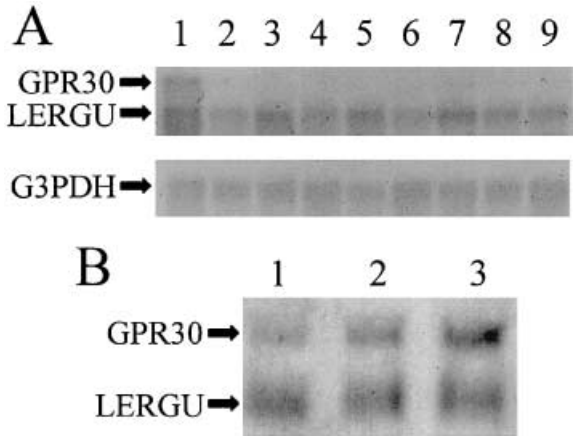

Fig. 4. Expression of GPR30 and LERGU mRNA in various human gastric cancer cell lines and breast carcinoma MCF7 cells stimulated with β-estradiol and MPA. (a) To determine the expression of GPR30 and LERGU mRNAs in gastric cancer cell lines, we performed northern blotting with a GPR30 full-length probe. 2 μg of mRNA which were normalized with G3PDH were loaded. 1, MCF7 mRNA as a positive control for GPR30; 2, MKN-45 mRNA; 3, H-111-TC mRNA; 4, St-4 mRNA; 5, MKN-7 mRNA; 6, SH-10-TC mRNA; 7, KatoIII mRNA; 8, GCIY mRNA; 9, AZ521 mRNA. Upper panel, GPR30 full-length probe; Lower panel, G3PDH probe as an internal control. (b) Expression of GPR30 mRNA and LERGU mRNA in MCF7 cells were analyzed to investigate their different responses to the stimulation by β-estradiol and MPA. 1, mRNA from control MCF7 cells; 2, mRNA from MCF7 cells stimulated by 1 nM β-estradiol; 3, mRNA from MCF7 cells stimulated by 10 nM MPA.

trace amount of GPR30 mRNA. Since it is known that GPR30 mRNA was up-regulated by MPA stimulation,[13,19,20] we examined whether LERGU mRNAs were also up-regulated or not by MPA stimulation (Fig. 4b). GPR30 mRNA was up-regulated by β-estradiol and MPA stimulation about 1.68- and 2.16-fold, respectively, but LERGU mRNA was not affected by β-estradiol or MPA stimulation (0.96- and 1.00-fold, respectively).

Furthermore, we confirmed the expression of LERGU protein by western blotting (Fig. 5). We detected a band having approximately 16.6 k daltons in H-111-TC cell lysate, though LERGU protein is composed of 99 amino acids with a calculated molecular mass of 10.6 k daltons.

Detection of 2 bp-deletion in the LERGU region. We recognized two forms of the LERGU region with or without 2 bp-deletion that had been reported as a deletion/insertion polymorphism in NCBI site. To know the physiological role of this 2 bp-deletion, we performed searching for a 2 bp-deletion by a fragment size analysis with various genomic DNAs and RNAs. We could detect 2 bp-deletion only in the gastric cancer samples, but not others as shown in Fig. 6. Furthermore, we investigated the frequency of 2 bp-deletion in the LERGU region in differentiated and undifferentiated gastric cancers. A 2 bp-deletion was detected in 8 out of 13 (61.5%) of differentiated gastric cancers, and 14 out of 27 (51.9%) of undifferentiated gastric cancers.

It is known that the allelic imbalance is an important event for cancer development. Loss of heterozygosity (LOH) was detected in normal allele and in 2 bp deletion allele at the LERGU locus in our gastric cancer samples (data not shown), but it had no correlation with gastric cancer development. It is also known that a somatic mutation is an important event for cancer development; however, in most cases, 2 bp-deletion was found in both of normal and gastric cancer genomic DNA. We found only one patient with a 2 bp-deletion in his gastric cancer genomic DNA, but not in his normal tissue DNA, by the fragment size analysis and the sequencing (Fig. 7).

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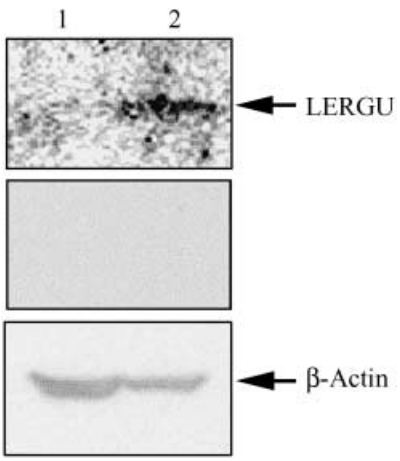

Fig. 5. LERGU protein detected in St-4 and H-111-TC cell lysates by western blotting. The normal LERGU protein was detected by western blotting using 40 μg protein of St-4 and H-111-TC cell lysates. The antibodies and protocols were described in Materials and Methods. 1, St4 cell lysate; 2, H-111-TC cell lysate. Upper panel, western blotting with the LERGU antibody; middle panel, western blotting with the LERGU antibody pre-absorbed by the antigen; lower panel, western blotting with β-actin antibody. Detected proteins were indicated on the right.

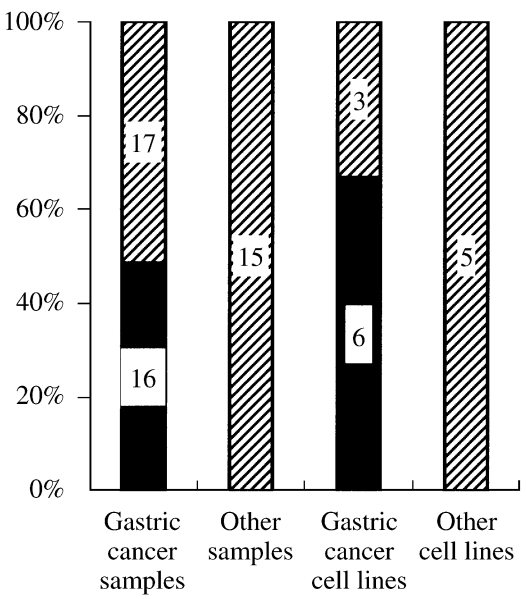

Fig. 6. The 2 bp-deletion in the LERGU region was detected only from gastric cancers. Gastric cancer samples (n = 33), other type cancer samples (n = 15, two samples of colon adenocarcinoma, one sample of leiomyoma, one sample of commercial control human genomic DNA, one sample of control human cDNA and 10 samples of twin genomic DNA) gastric cancer cell lines (n = 9) and other cancer cell lines (n = 5, MCF7, HEK293T, HeLa, SW480 and KM12SM) were subjected to the fragment analysis to detect 2 bp-deletion in the LERGU region. Solid bars indicate the samples which have both 2 bp-deletion and 2 bp-retention, striped bars indicate the samples which have only 2 bp-retention. The numbers of samples are indicated in the bars.

Discussion

The improved IGCR technique is one of the subtractive methods and a good technique for the gene hunting. This technique can

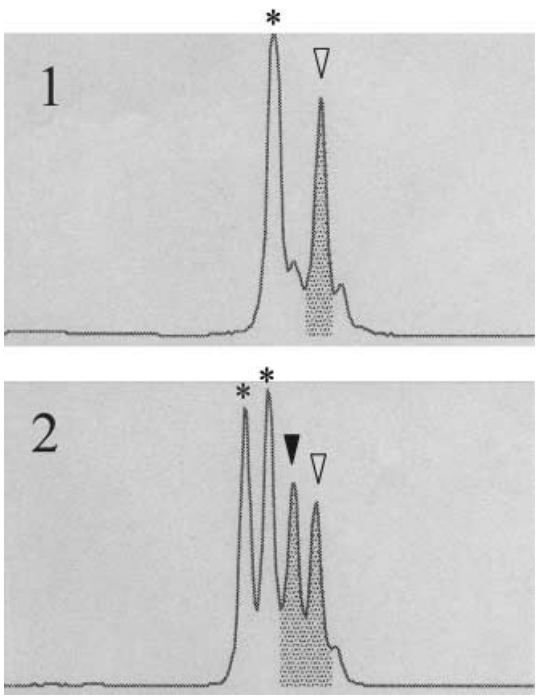

Fig. 7. The 2 bp-deletion in the LERGU region is detected in gastric cancers by fragment size analysis. 1, normal tissue genomic DNA; 2, gastric cancer genomic DNA. Open arrowheads indicate the fragment of LERGU region with 2 bp-retention, and a closed arrowhead indicates the fragment with 2 bp-deletion. Asterisks show the extra peaks which appeared as a set of peaks in the LERGU region, suggesting 4 bp repeat slipped peaks.

be applied to the genome-wide comparison easily. In fact, we detected EBV genomic DNA fragments when the genomic DNAs from the gastric cancer tissue and the adjacent normal tissue were used as a tester and a driver, respectively.[12] In this paper, we applied this tool to the gastric cancer genome analysis. In our improved IGCR results, only 7.2% (21 clones out of 290) of the repetitive sequence fragments were detected (data not shown). This result suggests that the non-specific amplifications of repetitive sequences were well suppressed. And the results of semi-real time PCR and real time PCR showed that-3′UTR of GPR30 mRNA increased about 62 000-fold by only two cycles of the improved IGCR (data not shown). Since EBV genomic DNA fragments were amplified 60 000–110 000-fold by two cycles of the improved IGCR, the results from the present study were satisfactory and showed a good improvement in our IGCR techniques.[12]

Regarding GPR30, there are some reports that the growth of MCF7 inhibited by progestin after both Erk-1 and Erk-2 were inactivated by GPR30,[19,20] and that GPR30 was necessary for the activation of EGFR-MAPK signaling by estrogen in breast cancer cells.[21,22]

We identified a novel mRNA named LERGU in the GPR30 3′UTR region (Fig. 2). Although MCF7 expresses both mRNAs, there has been no evidence for the relationship in expression of GPR30 and LERGU so far (Fig. 4). We speculate that their transcriptional regulations such as the promoter activity may be different because the expression of LERGU mRNA was not influenced by MPA stimulation (Fig. 4b), though it was reported that the expression of GPR30 mRNA was up-regulated by MPA in MCF7 cells.[13,19,20] This result suggests that LERGU mRNA expression may not be regulated by estrogen and progestin signals, differing from GPR30 mRNA expression.

We found that gastric cancer samples had a 2 bp-deletion of the LERGU region, but others had a 2 bp-retention of the LERGU region (Fig. 6). This evidence might correlate between the 2 bp-deletion of the LERGU region and the gastric cancer occurrence. In fact, there was an example of the gastric cancer patient in which one of the alleles showed the 2 bp-deletion of LERGU during the gastric cancer generation (Fig. 7). In other gastric cancer samples the 2 bp-deletion allele was found in both tumor and normal stomach tissues. Considering the existence of the patient in which LERGU deletion was found only in the gastric cancer tissue, there is a possibility that LERGU mutation may be one of the factors to generate gastric cancer. Furthermore, since the frequencies of the appearance of a 2 bp-deletion were almost the same between differentiated and undifferentiated gastric cancers, we also assume that the LERGU protein might work on the common step in cancer development, though it is believed that the genetic pathways of tumorigenesis are different between differentiated and undifferentiated gastric cancers.

Generally, the gain of chromosome occurs for oncogenes and its loss occurs for tumor suppressor genes, and both play important roles in cancer initiation and progression. It is true that we could not detect a close relationship between gastric tumorigenesis and the allelic imbalances at the LERGU region in our gastric cancer samples. However, our results suggest a close relation of two events to the gastric tumorigenesis; one is that the 2 bp-deletion in the LERGU mRNA may cause the frameshift and produce a mutated protein, and the other is that it may not be able to produce alternative splicing forms (LERGU-1, LERGU-2), considering that the 2 bp-deletion site may be a donor site of the alternative splicing. From this and the evidence that 2 bp-deletion alleles were found only in gastric cancer samples, we thought that 2 bp-deletion might not be simple polymorphism, and this deletion may be important to LERGU translation, but not to GPR30 translation. Furthermore, the normal LERGU protein was not detected in the lysate of St-4 cells although one normal allele in the LERGU region exists and the normal LERGU mRNA was expressed in St-4 cells (data not shown). This phenomenon may be explained by rapid degradation of the normal LERGU protein, suppression of translation or poor stability of the normal LERGU mRNA in St-4 cells. The LERGU protein with a molecular mass of 16.6 k daltons was detected in the lysate of well differentiated H-111-TC cells by western blotting. This protein has not yet been characterized for structure and functions and will be investigated from the point of view of carcinogenesis and differentiation. At present we speculate that the generation of mutant LERGU protein or the disappearance of normal LERGU protein might have a role in the development of gastric cancer.

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