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Original article

Cytochrome P450 aromatase gene (*CYP19*) expression in gastric cancer

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Abstract

Background. **The secretion of biologically active estrogen through the conversion of circulating precursor androgens has been suggested to play important roles in the pathophysiology of estrogen-dependent carcinomas. In the present study, we examined aromatase expression in gastric carcinoma.**

Methods. **Nineteen specimens of gastric carcinoma were obtained from Japanese patients at the Department of Surgery, Tottori University Hospital, Japan. Nontumoral tissues adjacent to the carcinoma were also available for analysis. The histological features of the gastric carcinomas were as follows: 8 intestinal-type and 11 diffuse-type adenocarcinomas. Tissue specimens removed at surgery were used for the preparation of RNA or for immunohistochemical analysis. Six cell lines derived from human gastric cancers were also used as a model system. Using conventional and real-time reverse transcription-polymerase chain reactions, aromatase mRNA expression and promoter usage were assayed. Immunohistochemical analysis was performed using an anti-aromatase antibody.**

Results. **We demonstrated the molecular basis of aromatase mRNA expression, which depended on three proximal promoters in tumoral and nontumoral tissues, for the first time. The tumoral tissues exhibited positive staining for anti-aromatase antibody. At the same time, positive staining was also observed in nontumoral mucosa, predominantly in the parietal cells.**

Conclusion. **We provide evidence suggesting a mechanism for the secretion of estrogen through the conversion of a precursor androgen in tumoral and nontumoral tissues in the stomach.**

Key words Aromatase · Estrogen secretion · Gastric carcinoma

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Introduction

Estrogen plays an important role not only in cell growth, differentiation, and apoptosis in various normal organs but also in benign and malignant tumors as well [1, 2]. Most estrogen has been believed to be synthesized depending on the expression of aromatase in the ovary and placenta in premenopausal women. However, the secretion of biologically active estrogen in situ through the conversion of circulating precursor androgens has been demonstrated to play important roles not only in normal extraovarian tissues [3–5] but also in estrogen-dependent carcinomas, including breast and ovarian cancers [6, 7].

Until the discovery of estrogen receptors (ERs) in human gastric cancer tissues, by Tokunaga et al. [8] in 1986, the stomach was not believed to be a direct target organ for estrogen. Since then, a number of investigators have demonstrated the expression of ERα and β mRNAs in both tumoral and nontumoral tissues from gastric cancer patients [9–11]. Of note, a pioneering study by Saitoh et al. [12] suggested the mechanism of estrogen production by aromatase in gastric carcinoma. Ueyama et al. [13] recently demonstrated aromatase expression and estrogen secretion in rat parietal cells of the gastric mucosa. Against the background of these observations, in the present study we further evaluated the ability of gastric carcinoma to secrete estrogen depending on the expression of aromatase.

Using a quantitative real-time polymerase chain reaction (PCR) and exon I-specific reverse transcription (RT)-PCR, we demonstrated the molecular basis of aromatase mRNA expression in gastric carcinoma for the first time. To support this observation, we submitted gastric tissue sections to immunohistochemical analysis, using an anti-aromatase monoclonal antibody.

Patients, materials, and methods

Patients and tissue preparation

Nineteen specimens of gastric carcinoma were obtained from Japanese patients at the Department of Surgery, Tottori University Hospital, Yonago, Japan. Nontumoral tissues adjacent to the carcinoma were also available for analysis. The histological features of the gastric carcinomas were as follows: 8 intestinal-type and 11 diffuse-type adenocarcinomas. Tissue specimens removed at surgery were immediately either stored in RNA*later* (Ambion, Tokyo, Japan) for the preparation of RNA (5 specimens) or submitted to formalin-fixation and paraffin-embedding for immunohistochemical analysis (14 specimens). Informed consent was obtained from all patients before surgery. Approval for the study was obtained from the Institutional Review Board of the Faculty of Medicine, Tottori University (approval number, 283).

Cell culture

Six cell lines derived from human gastric cancers were used throughout the experiments. MKN-1 was established from an adenosquamous carcinoma [14]. MKN-28 was established from a well-differentiated adenocarcinoma [14]. MKN-45 [14] and MKN-74 [15] were established from a poorly differentiated and a well-differentiated adenocarcinoma, respectively. KATO-III [15, 16] and HSC-39 [17] were established from a signet-ring cell carcinoma. COS-1 monkey kidney tumor cell was used in the analysis of exogenous aromatase. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) at 37℃ under 5% CO_2.

17β-Estradiol (E_2) secretion assay

The ability of gastric cancer cells to secrete E_2 was evaluated by quantifying the E_2 level in the culture medium. In brief, cells were plated at a density of 1×10^5 cells per well in a 12-well plate, and cultured in RPMI 1640 supplemented with 10% FCS for 24 h. Then the culture media were changed to FCS-free RPMI 1640 supplemented with or without 10^{-5} M testosterone. Eight hours after the media change, the E_2 concentration was assayed, using an enzyme immunoassay (EIA) kit (Amerlite P; Amersham Pharmacia, Tokyo, Japan).

Aromatase assay

Aromatase activity in vitro was determined by measuring the amount of tritiated water released upon the conversion of [1β-^{3}H] androstenedione to estrone, according to the method of Ackerman et al. [4], with minor modification. In brief, cells were scraped off the culture dishes or recovered by brief centrifugation with 0.1 M phosphate buffer (pH 7.6), and then subjected to sonication for 5 min at 4˚C, using a Bioruptor (Cosmo Bio, Tokyo, Japan). The resultant cell lysates were then subjected to centrifugation at 1000 rpm for 3 min at 4˚C, and the supernatant was used for the aromatase assay. Following the addition of [1β-^{3}H] androstenedione (5 nM, 936 GBq/mmol; NEN Life Science Products, Boston, MA, USA) and nicotinamide adenine dinucleotide phosphate, reduced (NADPH) buffer, the supernatant was incubated for 60 min at 37˚C. At the end of the incubation, the reaction mixture was extracted with chloroform and then treated with dextran-coated charcoal to remove the remaining [1β-^{3}H] androstenedione. After brief centrifugation, [^{3}H]-H_2O included in the supernatant was assayed in a liquid scintillation counter (LSC-6100; ALOKA, Tokyo, Japan). The protein concentration was determined by the method of Bradford [18].

Analysis of aromatase mRNA expression and alternative promoter usage

Total cellular RNA was prepared using the RNeasy kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. Using 2 μg of total RNA as template, single-stranded cDNA was prepared in 20 μl of reaction mixture, containing 1 mM each of dNTP, 50 units of murine leukemia reverse transcriptase (Applied Biosystems, Branchburg, NJ, USA), 20 units of RNase inhibitor (RNasin; Promega, Madison, WI, USA) and oligo-dT_{16} primer at 37˚C for 90 min. Then, 1 μl of the cDNA reaction mixture was subjected to PCR amplification in 50 μl of PCR mixture containing 0.25 units of ExTaq polymerase (Takara, Kyoto, Japan) and 50 pmol each of forward (AromF, 5′-GACTCTAAATTGC CCCCTCTGA-3′ in Exon II) and reverse (AromR, 5′-CATGCTGGAAATGATCTTTACCC-3′ in Exon X) primers, which were designed from the human placenta aromatase cDNA sequence (GenBank accession number, M22246) to amplify the sequence corresponding to the total open reading frame (ORF; 1517 bp). The conditions for PCR were 1 min at 94˚C, 1 min at 62˚C, and 1 min at 72˚C, for 38 cycles. At the end of the PCR cycles, 18 μl of the reaction mixture was removed and electrophoresed on a 1.5% agarose gel in Tris-borate-ethylenediaminetetraaceticacid (EDTA) buffer at a constant voltage of 100 V. At the end of the electrophoresis, the gel was removed and visualized by staining with ethidium bromide. As an internal control, β-tubulin mRNA expression was monitored. The primer sets to amplify a 577-bp sequence were: 5′-TGGATCT AGAACCTGGGACC-AT-3 (sense) and 5′-ACCATG TTGACTGCCAACTTGC-3′ (anti-sense). In the promoter assay, unique exon I primers (PII, I.1, I.3, I.4, I.5,

and I.6) and exon II primers were used, as described previously [19]. GenBank locations and accession numbers for the specific primers are: I.1, 708–727, M32245; I.3, 867–887,S85356; I.4, 512–531, D21240; I.5, 619–642, S71536; I.6, 161–181, S85356; PII, 965–984, S85356; exon II-f, 1030–1050, S85356; exon II-r, 1198–1179, S85356. The PCR condition was 1 min at 94˚C, 1 min at 54˚C, and 1 min at 72˚C, for 35 cycles. At the end of the PCR cycles, 10 µl of the reaction mixture was removed and electrophoresed as described above. Separated DNA fragments were then transferred to a nylon membrane (Hybond N+; GE Healthcare, Buckinghamshire, UK), and hybridized to ^{32}P-labeled aromatase exon II cDNA at 42˚C for 18 h in a solution containing 50% formamide, 5 × standard saline citrate (SSC), 10 × Denhardt's solution 50 mM sodium phosphate buffer (pH 6.5), salmon testis DNA (100 µg/ml), and 1% sodium dodecyl sulfate (SDS). At the end of the hybridization period, the nylon membrane was removed, washed according to the manufacturer's protocol, and exposed to BioMax film (Eastman Kodak, Rochester, NY, USA).

Quantitative real-time PCR

Total cellular RNAs and single-stranded cDNAs were prepared, as described above, from the tissue specimens and cell lines, and aromatase mRNA expression was assessed using real-time PCR. As an internal control, β-tubulin mRNA expression was assessed in parallel. In brief, 1 µl each of the cDNA samples (~50 ng) as indicated above was subjected to TaqMan real-time PCR in triplicate. The real-time PCR was performed using the Universal Probe Library system (Roche Diagnostics, Tokyo, Japan). The specific probes (aromatase #55 and β-tubulin #43) were selected on the Web site of the Universal Probe Library Assay Design Center (https://www.roche-applied science.com/sis/rtpcr/upl/adc.jsp). FastStart TaqMan Probe Master (Roche Diagnostics) was used for the reaction solution. Finally, the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Tokyo, Japan) was used for the real-time PCR.

Aromatase expression vector and the transient expression

Single-stranded cDNA was prepared from the total cellular RNA of MKN-74 cells. Using the cDNA as a template, a 1559-bp sequence encoding the total ORF of aromatase was amplified by PCR, as described above. The primer set used was AromF (in exon ll) and 5′-CCAGCCTTCTCTAGTGTTCCAG-3′ (in exon X). The amplified cDNA sequence was cloned into the *Hin*dIII/*Eco*RV sites of pcDNA3 (Invitrogen, Tokyo, Japan). COS-1 cells were transfected with either pcDNA3 or pcDNA3/aromatase, using FuGENE6 reagent (Roche Diagnostics). The transfection efficiency was more than 80%. Twenty four hours after the transfection, the cells were used for Western blot analysis.

Western blot analysis

Cells were scraped off the dishes and suspended in ice-cold 10% trichloroacetic acid for 10 min. Following centrifugation at 15000 *g* for 10 min at 4˚C, the resultant precipitates were solubilized into sample buffer (125 mM Tris-HCl [pH 6.8], 2.3% SDS, 10% glycerol, 20 µg/ml bromophenol blue, and 5% 2-mercaptoethanol), and then incubated in boiling water for 5 min. The protein sample (10 µg) was electrophoresed on a 10% SDS-polyacrylamide gel (150 V, for 80 min) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was pre-incubated with phosphate-buffered saline (PBS) containing 5% skim milk and 0.1% Tween 20, and then incubated with anti-aromatase mouse monoclonal antibody (H4,1: 250; Serotec, Oxford, UK) at 4˚C for 15 h. Then the membrane was incubated with anti-mouse IgG conjugated with horseradish peroxidase (1: 2000; MBL, Osaka, Japan) at room temperature for 1 h and subjected to a chemiluminescence assay (ECL, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Finally, the membrane was exposed to BioMax film (Eastman Kodak) at room temperature for appropriate time intervals.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were cut into serial tissue sections at a thickness of 4 µm. Following deparaffinization, the sections were immunostained using the streptavidin-biotin alkaline phosphatase complex method, according to the manufacturer's protocol (HISTOFINE SAB-AP (M) Immunohistochemical Staining Kit; Nichirei, Tokyo, Japan). In brief, the tissue section slides were incubated at room temperature for 20 min in blocking serum to eliminate nonspecific binding of the primary antibody. The slides were then serially incubated with an anti-cytochrome P450 aromatase monoclonal antibody (H4, 1: 50; Serotec) [20] overnight at 4˚C, followed by incubation with the secondary antibody. As a negative control, the sections were incubated in parallel without the primary antibody. At the end of the incubation with the secondary antibody, the sections were washed and then incubated with streptavidin-alkaline phosphatase reagent. The sections were finally visualized with new fuchsin and counterstained with hematoxylin.

Results

Aromatase expression in gastric cancer cell lines

As an initial step to verify aromatase expression in gastric cancer, we examined its expression in gastric cancer cell lines. Using RT-PCR, we demonstrated aromatase mRNA expression in all gastric cancer cell lines examined (Fig. 1A). The level of aromatase expression depends on the cell type, and here it was similar to or less than that in skin fibroblastic cells, which are known

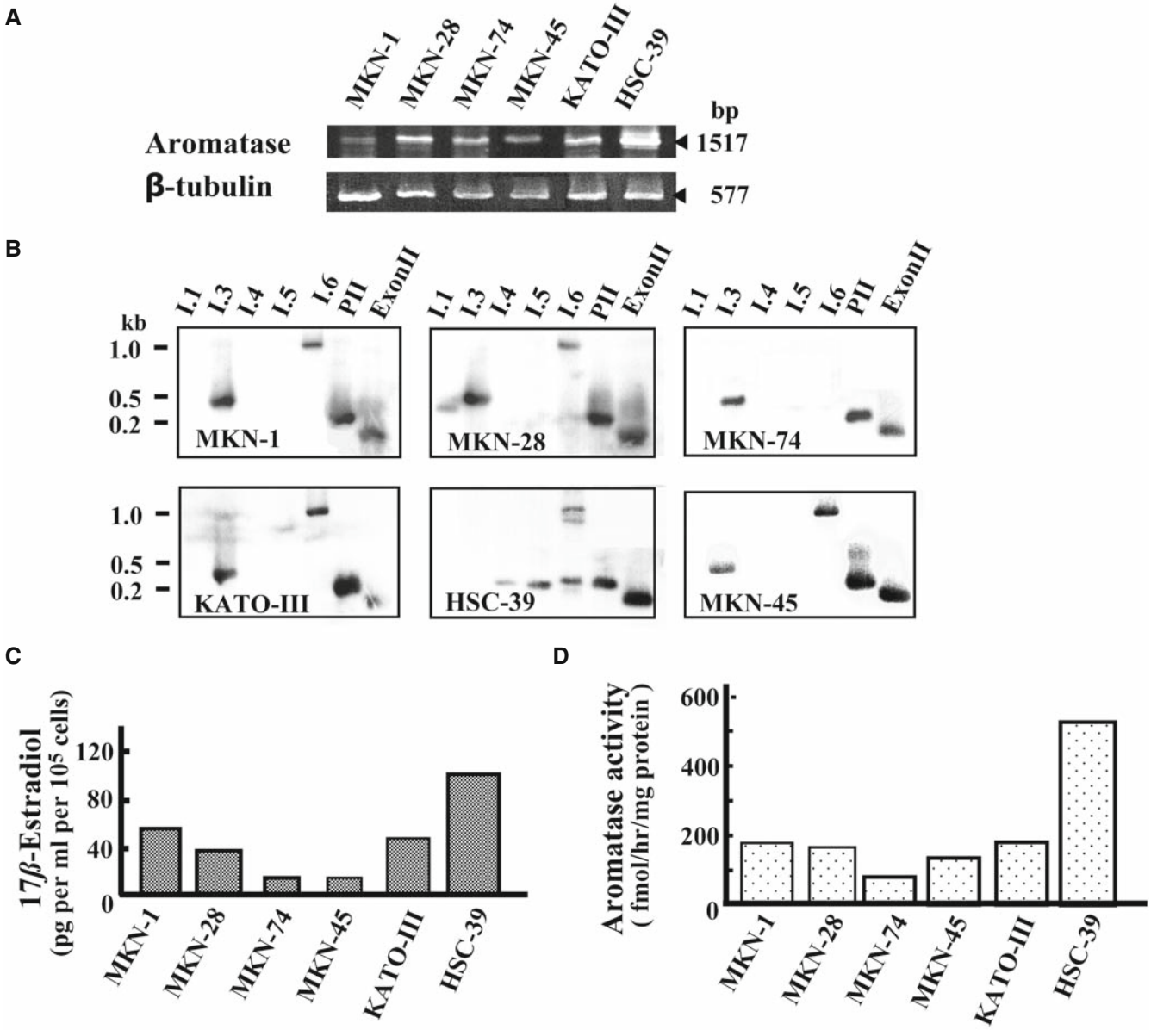

Fig. 1. **A** Aromatase mRNA expression in gastric cancer cell lines. Total cellular RNA and single-stranded cDNA were prepared from gastric cancer cells, and subjected to polymerase chain reaction (PCR). A sequence corresponding to the total open reading frame from exon II to exon X (1518 bp) was amplified and electrophoresed on a 1.5% agarose gel. As an internal control, β-tubulin mRNA was assayed in parallel. The amplified sequence was visualized using ethidium bromide staining under UV light. **B** Promoter involvement in aromatase mRNA transcription. Using the procedure described by Okubo et al. [19], unique exon I (PII, I.1, I.3, I.4, I.5, and I.6) and exon II primers were used for exon I-specific reverse transcription (RT)-PCR. **C** Estradiol production. Gastric cancer cells were incubated in fetal calf serum (FCS) (–) RPMI 1640 medium in the presence of 5×10^{-6} M testosterone. At the end of 8-h incubation, the culture medium was removed and submitted to estimation of the 17β-estradiol level, using an enzyme immunoassay, in duplicate. Values are means and are expressed as pg per ml per 10^5 cells. The intraassay variation was less than 10%. **D** Aromatase activity in vitro. The aromatase activity of the gastric cancer cell lines was determined by measuring the amount of [^{3}H]-H_2O released upon the conversion of [1β-^{3}H] androstenedione to estrone. The activity was expressed as fmol per h per mg protein

to express abundant aromatase. Among six promoters examined (Fig. 1B), PII, I.3, and I.6 were used in MKN-1, MKN-28, MKN-45 and KATO-III cells, while PII, I.4, l.5, and I.6 promoters were used in HSC-39 cells. In MKN-74 cells, two active promoters, PII and I.3, were recognized. None of these cells seemed to secrete E_2 in the culture media during an incubation period of up to 15 h (data not shown). However, in the presence of 5 μM testosterone in the culture, these cells produced E_2 even in a short incubation period of 8 h (Fig. 1C). Aromatase activity in vitro was demonstrated in all cell lines examined (Fig. 1D).

Aromatase expression in gastric cancer tissues

To demonstrate aromatase mRNA expression in gastric cancer tissue, tumoral tissues were randomly removed from five patients (three with intestinal-type and two with diffuse-type carcinoma) and subjected to RT-PCR analysis. Representative results from two patients are shown in Fig. 2A. A 1.5-kb sequence corresponding to the expected ORF of aromatase mRNA was amplified. Using quantitative real-time PCR analyses, aromatase mRNA expression was demonstrated in the five gastric cancer tissues examined (Fig. 2B). The expression level varied depending on the specimens, with an approximately tenfold difference. In these tumoral tissues, PII, I.3, and I.6 promoters were used (Fig. 2C). At the same time, nontumoral mucosa removed from the same patients was submitted to aromatase mRNA expression analysis in parallel. These tissues were shown to express aromatase mRNA, and the expression levels were similar to the levels in tumoral tissues (Fig. 2B). The predominant promoters

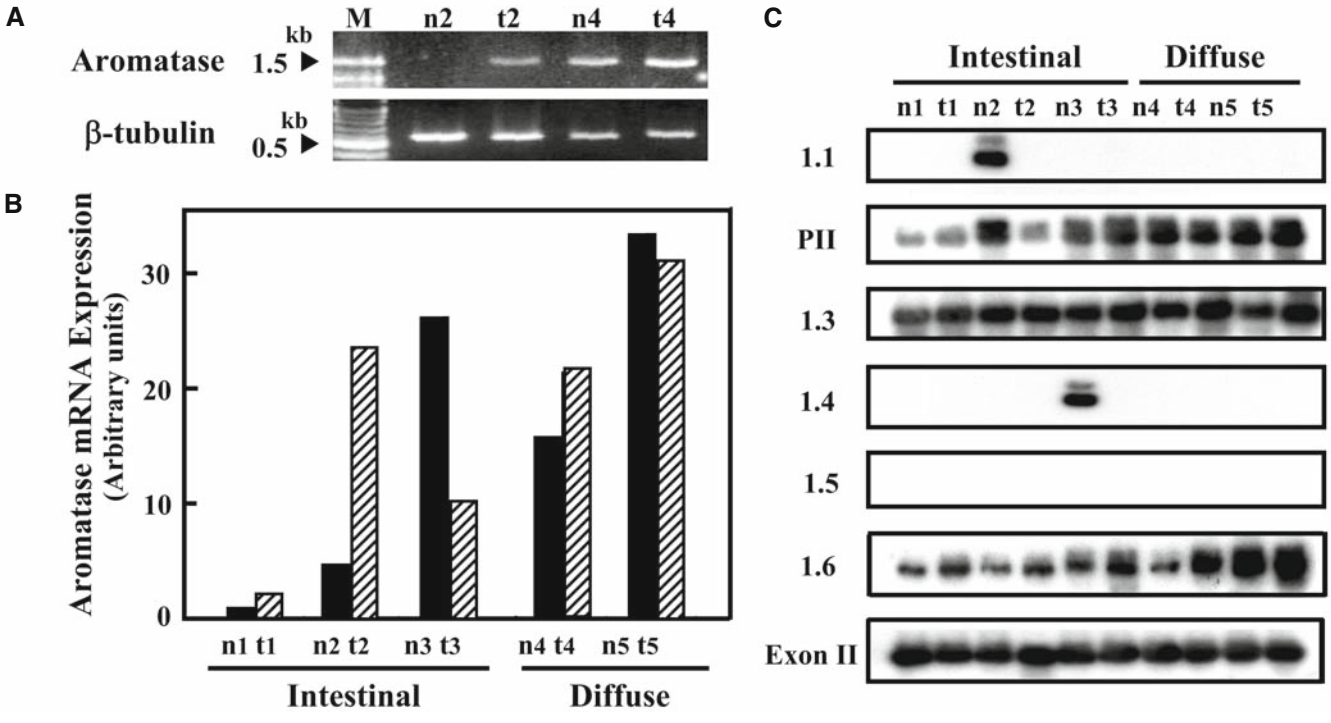

Fig. 2A–C. Aromatase mRNA expression in gastric cancer tissues. Gastric cancer tissues from five patients (three with intestinal-type and two with diffuse-type carcinoma) were removed for the preparation of total cellular RNA. Tumoral tissues (*t1*, *t2*, *t3*, *t4*, and *t5*) and nontumoral mucosa (*n1*, *n2*, *n3*, *n4*, and *n5*) are indicated, with the *numbers* shown corresponding to the patient number. **A** Aromatase mRNA expression. Representative results from two patients, patients 2 and 4. *As described in the legend to Fig. 1*, total cellular RNA and single-stranded cDNA were prepared from gastric cancer tissues (t2 and t4) and nontumoral mucosa (n2 and n4), and subjected to PCR. A 1518-bp sequence, corresponding to the total open reading frame, was amplified and separated on 1.5% agarose gel electrophoresis. As an internal control, β-tubulin mRNA (577 bp) was assayed in parallel. The amplified sequence was visualized using ethidium bromide staining under UV light. *M*, Molecular size marker. **B** Quantitative analysis of aromatase mRNA expression by real-time PCR. The single-stranded cDNAs used in the experiment whose results are shown in **A** were subjected to TaqMan real-time PCR in triplicate. Specific probes for aromatase (#55) and β-tubulin (#43) mRNAs were selected using the Universal ProbeLibrary System. The ABI PRISM 7900HT Sequence Detection System was used for the PCR. Relative aromatase mRNA expression was estimated using β-tubulin mRNA expression as an internal control. Using the lowest value in nontumoral mucosa (*n1*) as a unit, values are expressed as arbitrary units. **C** Promoter usage in aromatase mRNA transcription. Unique exon I (PII, 1.1, 1.3, 1.4, 1.5, and 1.6) and exon II primers were used for the exon I-specific RT-PCR [19]

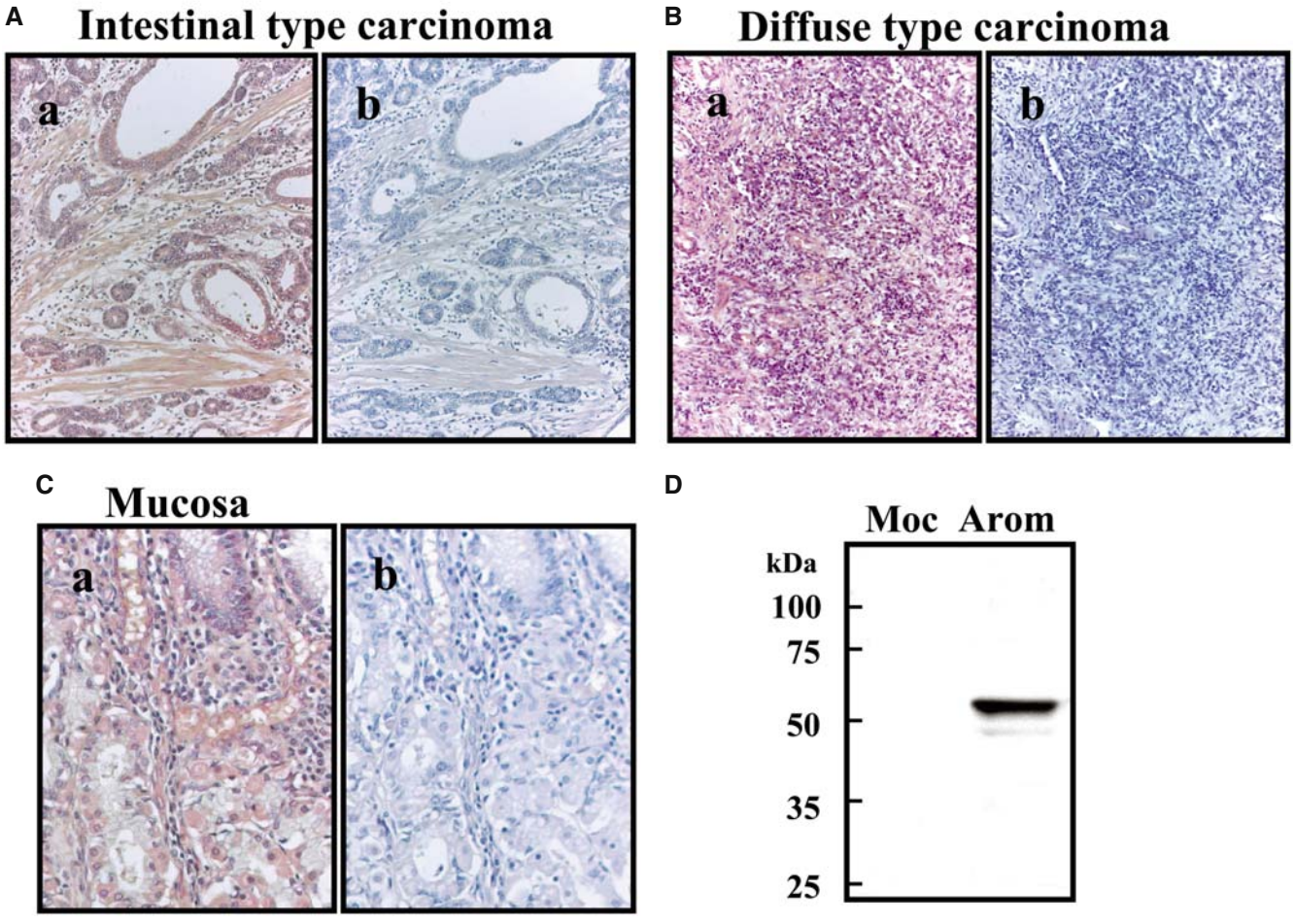

Fig. 3. A–C Expression of aromatase in paraffin-embedded specimens of gastric carcinoma and nontumoral gastric mucosa tissues. Sectioned tissues were stained with an anti-aromatase monoclonal antibody, H4. Strong cytoplasmic aromatase staining was observed in carcinoma cells in intestinal-type (**A**) and diffuse-type carcinoma (**B**), and in the parietal cells of nontumoral gastric mucosa (**C**) in the presence (*a*) or absence (*b*) of the anti-aromatase monoclonal antibody. **D** A single protein of 55 kDa, corresponding to aromatase, was specifically recognized by the monoclonal antibody H4 in cell lysates from COS-1 cells transfected with pcDNA3/aromatase (*lane Arom*). Cell lysates from COS-1 cells transfected with pcDNA3 are shown in *lane Moc*

used were identical to those used in the tumoral tissues (Fig. 2C).

Immunohistochemistry

Immunoreactivity for aromatase was examined in 14 gastric carcinoma specimens. Positive staining was observed in 4 of the 5 intestinal-type sections, and in 4 of the 9 diffuse-type sections. Representative staining for intestinal-type and diffuse-type carcinoma is shown in Fig. 3A and Fig. 3B, respectively. In the nontumoral mucosa, positive staining was observed predominantly in the parietal cells (Fig. 3C). The monoclonal antibody used above specifically recognized a single protein band of aromatase, which had been expressed in COS-1 cells (Fig. 3D).

Discussion

In the present study, we have demonstrated, for the first time, aromatase mRNA expression, which depended on three proximal promoters, in gastric carcinoma specimens (obtained from 5 patients; Fig. 2). Immunohistochemical analysis of gastric carcinoma specimens from 14 patients showed positive staining for aromatase (Fig. 3) in 8 specimens. It is important to note that the observation of positive staining for aromatase does not always indicate the exact evaluation of aromatase expression in tissue specimens. Gastric tissues expressing lower levels of aromatase mRNA sometimes exhibit less reactivity to the anti-aromatase monoclonal antibody used in the present study than gastric tissues expressing high levels of aromatase mRNA (data not shown).

Interestingly, aromatase mRNA expression was also demonstrated in nontumoral mucosa from the same patients (Fig. 2). Immunoreactive staining for aromatase in nontumoral mucosa was restricted predominantly to within the parietal cells (Fig. 3C). This finding is consistent with a previous observation in rat gastric mucosa [13]. The observation suggests that the stomach is an endocrine organ that secretes biologically active estrogen. Estrogen secreted in the stomach seems to be trapped in the portal vein and mostly metabolized in the liver [13]. At the same time, estrogen secreted in the stomach may influence hepatic function including the estrogen-dependent expression of ERα [13]. If the metabolic activity of liver is lost because of hepatic dysfunction, the estrogen may flow out. It is well recognized that gynecomastia occurs against a background of a relative increase in estrogenic activity caused through hepatic fibrosis and cirrhosis [21–23].

In our pilot experiments using gastric cancer cell lines (Fig. 1A), we found a mode of aromatase mRNA expression similar to that seen in gastric carcinoma. Although the expression levels in these cells seemed much lower than the levels in gastric tissues (data not shown), these cells produced a significant level of E_2 in culture media only in the presence of exogenous testosterone (Fig. 1C). Aromatase activity in vitro was also demonstrated (Fig. 1D). The lower aromatase mRNA expression in these cell lines may have been caused by an epigenetic disorder of the aromatase gene, as has been suggested to occur in endometriosis [24]. Likewise, epigenetic modification of aromatase gene expression may also occur in gastric carcinoma.

Promoters are well known to be involved in the molecular basis of the differential regulation of aromatase gene expression, in a tissue-specific manner. In normal breast adipose tissues, a marginal level of aromatase expression depends on the promoters I.3, I.4, and PII, while in breast cancer, I.3 and PII activities were markedly increased along with the upregulation of promoter I.7. The sum of promoter activities (PII, I.3, I.4, and I.7) markedly upregulated aromatase expression in breast cancer [7]. In gastric cancer, as well as in normal gastric mucosa, we found that the promoters, PII, I.3, and I.6 were involved in five patients with gastric cancer (Fig. 2C). At present, it is too early to conclude whether additional promoters are involved in gastric cancer. The question of most interest is whether or not aromatase mRNA expression is higher in tumoral tissues than in normal mucosa. Further study focusing on these issues may help to clarify the pathophysiological role of aromatase expression in gastric cancer.

Recently, a nongenomic action of ERs, without transcriptional effect, which mediates the anti-apoptotic effect of estrogens, has been suggested [25]. Interestingly, ER α and β forms have been demonstrated in gastric carcinoma [8–11], and gastric cancer cells exhibited an apoptosis-resistance phenotype [26, 27], suggesting the pathophysiological behavior of estrogen in gastric carcinoma.

In conclusion, our results provide evidence suggesting a mechanism for secreting biologically active estrogen in gastric carcinoma in vivo, through the conversion of circulating precursor androgens. Further understanding of the molecular basis and the role of estrogen secretion may provide insights into the diagnosis and treatment of gastric cancer.

References

1. Katzenellenbogen BS. Estrogen receptors: bioactivities and interactions with cell signaling pathways. Biol Reprod 1996;54:287–93.
2. Izawa M, Yeh J. Apoptosis in human reproductive tissues: emerging concepts. Reprod Med Rev 1997;6:23–36.
3. Lephart ED. A review of brain aromatase cytochrome P450. Brain Res Rev 1996;22:1–26.
4. Ackerman GE, Smith ME, Mendelson CR, MacDonald PC, Simpson ER. Aromatization of androstenedione by human adipose tissue stromal cells in monolayer culture. J Clin Endocrinol Metab 1981;53:412–7.
5. Harada N, Sasano H, Murakami H, Ohkuma T, Nagura H, Takagi Y. Localized expression of aromatase in human vascular tissues. Circ Res 1999;84:1285–91.
6. Mackay A, Urruticoechea A, Dixon JM, Dexter T, Fenwick K, Ashworth A, et al. Molecular response to aromatase inhibitor treatment in primary breast cancer. Breast Cancer Res 2007;9: R37.
7. Bulun SE, Chen D, Lu M, Zhao H, Chen Y, Demura M, et al. Aromatase excess in cancers of breast, endometrium and ovary. J Steroid Biochem Mol Biol 2007;106:81–96.
8. Tokunaga A, Nishi K, Matsukura N. Estrogen and progesterone receptors in gastric cancer. Cancer 1986;57:1376–9.
9. Matsuyama S, Ohkura Y, Eguchi H, Kobayashi Y, Akagi K, Uchida K, et al. Estrogen receptor β is expressed in human stomach adenocarcinoma. J Cancer Res Clin Oncol 2002;128: 319–24.
10. Zhao XH, Gu SZ, Liu SX, Pan BR. Expression of estrogen receptor and estrogen receptor messenger RNA in gastric carcinoma tissues. World J Gastroenterol 2003;9:665–9.
11. Wang M, Pan JY, Song GR, Chen HB, An LJ, Sx Q. Altered expression of estrogen receptor alpha and beta in advanced gastric adenocarcinoma: correlation with prothymosin alpha and clinicopathological parameters. Eur J Surg Oncol 2007;33:195–201.
12. Saitoh Y, Sasano H, Naganuma H, Ohtani H, Sasano N, Ohuchi A, et al. De novo expression of aromatase in gastric carcinoma, light and electron microscopic immunohistochemical and immunoblot study. Pathol Res Pract 1992;188:53–60.
13. Ueyama T, Shirasawa N, Numazawa M, Yamada K, Shelangousky M, Ito T, et al. Gastric parietal cells: potent endocrine role in secreting estrogen as a possible regulator of gastro-hepatic axis. Endocrinology 2002;143:3162–70.
14. Hojo H. Establishment of cultured cell lines of human stomach cancer origin and their morphological characteristics (in Japanese). Niigata Igakkai Zasshi (Niigata Med J) 1977;9:281–5.
15. Sekiguchi K, Sakakibara M, Fujii G. Establishment of cultured cell lines derived from a human gastric carcinoma Jpn J Exp Med 1978;48:61–8.

16. Owen-Schaub L-B, Radinsky R, Kruzel E, Berr K, Yonehara S. Anti-Fas antibody on nonhematopoietic tumors: levels of Fas/APO-1 and bcl-2 are not predictive of biological responsiveness. Cancer Res 1994;54:1580–6.
17. Yanagihara K, Seyama T, Tsumuraya M, Kamada N, Yokoro K. Establishment and characterization of human signet ring cell gastric carcinoma cell lines with amplification of the c-myc oncogene. Cancer Res 1991;51:381–6.
18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
19. Okubo T, Mok SC, Chen S. Regulation of aromatase expression in human ovarian surface epithelial cells. J Clin Endocrinol Metab 2000;85:4889–99.
20. Turner KJ, Macpherson S, Miller MR, McNeilly AS, Williams K, Cranfield M, et al. Development and validation of a new monoclonal antibody to mammalian aromatase. J Endocrinol 2002; 172:21–30.
21. Coen P, Kulin H, Ballantinee T, Zaino R, Frauenhoffer E, Boal D, et al. An aromatase-producing sex-cord tumor resulting in prepubertal gynecomastia. N Engl J Med 1991;324:317–22.
22. Braunstein GD. Aromatase and gynecomastia. Endocr Relat Cancer 1999;6:315–24.
23. Rosai J. Breast disease in males. In: Rosai J, editor. Surgical pathology, vol. 2. 2nd ed. New York: Mosby; 2004. p. 1837–9.
24. Izawa M, Harada T, Taniguchi F, Ohama Y, Takenaka Y, Terakawa N. An epigenetic disorder may cause aberrant expression of aromatase gene in endometriotic stromal cells. Fertil Steril 2007 (in press).
25. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, et al. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 2001;104:719–30.
26. Izawa M, Teramachi K. Down-regulation of protein kinase C activity by sorbitol rapidly induces apoptosis in human gastric cancer cell lines. Apoptosis 2001;6:353–8.
27. Izawa M, Mori M, Satoh T, Teramachi K, Sairenji T. Identification of an alternative form of caspase-9 in human gastric cancer cell lines: a role of a caspase-9 variant in apoptosis resistance. Apoptosis 1999;4:321–5.