Overexpression of Human X-box Binding Protein 1 (XBP-1) in Colorectal Adenomas and Adenocarcinomas

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Abstract. *Background: Human X-box binding protein 1 (XBP-1) is a transcription factor essential for hepatocyte growth, as well as for plasma cell differentiation. Recently, overexpression of XBP-1 has been reported in breast cancer including non-invasive carcinomas, and was suggested to play an important role in breast carcinogenesis. To investigate the involvement of XBP-1 in colorectal tumorigenecity, the expression of XBP-1 was examined in four colon cancer cell lines, six colorectal polyps and five colorectal carcinomas. Materials and Methods: The study population consisted of eleven patients who had undergone resection for colorectal cancer or adenoma from 2000 to 2002. Four colon cancer cell lines, DLD1, SW480, HCT15 and WiDr, were also analyzed for expression of XBP-1. Reverse transcription-polymerase chain reaction was performed using eleven primary colon tumors. XBP-1 expression was then investigated using an immunohistochemical method for archived paraffin-embedded sections. Results: The XBP-1 gene was overexpressed in four cases out of five primary colorectal carcinomas and in four cases out of six colorectal adenomas. Also all four cancer cell lines expressed XBP-1 mRNA. Immunohistochemical staining demonstrated that XBP-1 protein was strongly stained in the cytoplasms of cancer cells, whereas it was unreactive in the normal colon epithelial cells and stromal cells. Conclusion: These data indicate that increased expression of XBP-1 gene may play some role in human colon carcinogenesis through impairment of cell differentiation regulation.*

Human X-box binding protein 1 (XBP-1) is a transcription factor. The protein product was originally identified as a protein binding to the cis-acting X-box present in the promoter regions of human major histocompatibility complex class II genes (1), and also as a protein that binds to the tax-responsive element present in the long terminal repeat of human T cell leukemia virus type 1 (2). Normal XBP-1 function is essential for hepatocyte growth (3), as well as for plasma cell differentiation (4). XBP-1 was found to be indispensable for immunoglobulin secretion and development of plasma cells. Recent studies on XBP-1 have revealed that XBP-1 mRNA is induced by activating transcription factor 6 (ATF6) (5) and spliced by inositol-requiring 1 protein kinase (IRE1) (6) in response to stress upon the endoplasmic reticulum (ER) to produce a highly active transcription factor. Interestingly, the spliced form of XBP-1 is able to efficiently activate the unfolded protein response (UPR) (7).

A recent report has shown increased mRNA expression and highly-reactive immunohistochemical staining of XBP-1 in human primary breast carcinomas, as well as in breast cancer cell lines, but not in the corresponding non-cancer tissues, indicating involvement of XBP-1 in human breast carcinogenesis (8). Also, some lines of reports can indicate the relationship between XBP-1 and cancer (9-12). Additionally, IRE1-XBP1 branch of the UPR mediates cell survival and tumor growth. Inhibition of this pathway will be possible as a therapeutic strategy (13). In this study, XBP-1 expression in colorectal tumors, including adenomas and carcinomas, was investigated.

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Abbreviations: XBP-1: Human X-box binding protein 1, RT-PCR: reverse transcription-polymerase chain reaction, ER: endoplasmic reticulum, UPR: unfolded protein response, ATF6: activating transcription factor 6, IRE1: inositol-requiring 1 protein kinase, G3PDH: glyceraldehyde-3-phosphate dehydrogenase.

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Key Words: Human X-box binding protein 1 (XBP-1), colorectal adenoma, colorectal adenocarcinoma.

Table I. *Clinicopathological features of the patients.*

Case	Age	Gender	Histology	Location	Size (mm)
1	60	male	tubular adenoma severe atypia	A	20
2	65	male	tubular adenoma mild atypia	T	9
3	60	male	tubular adenoma moderately atypia	A	13
4	60	femle	tubular adenoma moderately atypia	Rs	9
5	60	male	tubular adenoma moderately atypia	S	10
6	61	male	tubularo-villous adenoma moderately atypia	Ra	12
7	70	male	well-differentiated adenocarcinoma	Rs	60
8	73	male	moderately-differentiated adenocarcinoma	T	30
9	45	male	moderately-differentiated adenocarcinoma	Rb	29
10	53	male	well-differentiated adenocarcinoma	Rb	82
11	77	female	well-differentiated adenocarcinoma	T	17

A, ascending; T, transverse; S, sigmoid; Rs, recto-sigmoid; Ra, upper rectum; Rb, lower rectum.

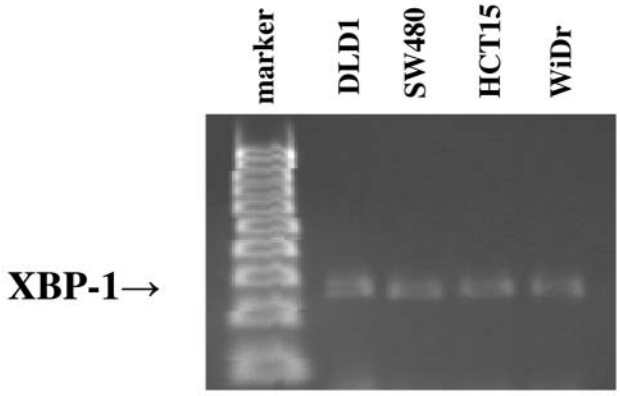

Figure 1. *XBP-1 gene expression by semi-quantitative RT-PCR analysis in colon cancer cell lines, DLD1, SW480, HCT15 and WiDr.*

Materials and Methods

Patients, cell lines and RNA isolation. Eleven patients who underwent surgery or colonoscopic polypectomy for colon cancer or adenoma at Tokyo Women's Medical University Medical Center East, between 2000 and 2002, were recruited to this study. Informed consent was obtained from each patient prior to the study, which was approved by the ethical committee of the hospital. None of the patients had undergone radiotherapy or chemotherapy prior to resection. Samples were immediately frozen at –80˚C. The four colon cancer cell lines, DLD1, SW480, HCT15 and WiDr, were cultured in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, as described previously (14). Total RNA was extracted from each specimen using TRIzol (Life Technologies, Inc., Rockville, MD, USA) according to the manufacturer's instructions.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Paired normal and tumor fresh-frozen tissues from these eleven patients with primary colon tumors were analyzed for abnormalities of XBP-1 mRNA levels by semiquantitative RT-PCR using the total RNAs extracted from the respective tissue source. Total RNA (5 μg) was treated with DNase I (Epicentre Technologies, Madison, WI, USA) and reverse-transcribed for single-stranded cDNA using an oligo (dT) 12-18 primer with Reverscript II reverse transcriptase (Wako Pure Chemical Industries Ltd., Osaka, Japan) (15). Each single stranded cDNA was diluted for subsequent PCR amplification by monitoring house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) using a quantitative control (8). Each PCR was carried out in a 30 μl volume of 1X PCR buffer for 2 min at 94˚C for initial denaturing, followed by 25-35 cycles of 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec, with a final extension step of 3.5 min at 72˚C in a GeneAmp PCR system 9600 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) (16). The sequences of the primers used for RT-PCR were as follows: XBP-1 forward (5'-TTTAGAAGAAGAGAACCAAA-3'), XBP-1 reverse (5'-CTGAAGAGTCAATACCGCCA-3'), that were designed to extend over two exons. Control RT-PCR was carried out using the G3PDH primers; G3PDH-forward, 5'-GGAAGGTGAAGGTCG GAGT-3'; G3PDH-reverse, 5'-TGGGTGGAATCATATTGGAA with some modifications (8, 17, 18). The PCR products were electrophoresed in 2.5% agarose gels.

Real-time RT-PCR. Real-time RT-PCR was performed using a qPCR™Core Kit for Sybr™ Green (Eurogenetec SA, Belgium) according to the manufacturer's instructions. Profile times for this kit on this system are as follows: 10 min at 95˚C for the initial step, 25-45 cycles of 95˚C for 15 sec, 60˚C for 60 sec and 25˚C forever for hold.

Immunohistochemistry of XBP-1 protein. The procedure for XBP-1 immunohistochemistry was performed, as previously reported (8). Briefly, after deparaffinization, the sections were incubated in 1 mM EDTA (pH8.0) at 100˚C for 20 min and sequentially treated with 0.3% H_2O_2 in methanol for 30 min and skim milk in PBS for 1 hr before incubation with diluted monoclonal XBP-1 antibody (Santa Cruz Biotechnology. Inc., CA, USA) for 2 h at room temperature. The sections were exposed to biotin-labeled goat anti-rabbit IgG for 30 min and immunoreactions were developed with an avidin-biotin complex (Vectastain ABC elite kit, Vector Laboratories, CA, USA) and the novel signal was amplified by an immunohistochemical method (CSA system, DAKO, CA, USA). The sites of peroxidase binding were demonstrated with diaminobenzidine.

Results

Overexpression of XBP-1 transcripts in colorectal cancers and adenomas. The clinicopathological features of the eleven patients with colorectal benign or malignant tumors examined in this study are summarized in Table I. Six cases were adenomas and five cases were adenocarcinomas. Representative results are shown in Figure 1 where

increased expression of XBP-1 is observed in four colon cancer cell lines (DLD1, SW480, HCT15 and WiDr). When XBP-1 mRNA expression in colorectal adenoma tissue was compared with normal counterpart tissue, it was found to be overexpressed in five out of six cases examined (Figure 2A). Densitometric quantification showed that the intensity of the bands in the tumor samples was increased about 3- to 10-fold in comparison to those in normal counterpart (data not shown). This difference was confirmed by the result of real-time RT-PCR (Figure 2B). A similar result (four out of five cases) was observed in the adenocarcinoma cases (Figure 3).

Immunohistochemical staining of XBP-1 in colorectal adenoma and adenocarcinoma. Figure 4 displays representative results for normal colon tissues (Figure 4A and Figure 4C) and for colon adenocarcinoma (Figure 2B) and for colon adenoma (Figure 2D). While the cytoplasm of the majority of colon adenoma and adenocarcinoma cells were strongly reactive for the XBP-1 antigen, the normal epithelial cells and stromal cells were unreactive. Therefore, the XBP-1 gene mRNA overexpression and XBP-1 protein overexpression were observed in most of the human colorectal tumors examined here.

Discussion

The XBP-1 gene was overexpressed in four cases out of five primary colorectal cancers, in four cases out of six adenomas and all four colon cancer cell lines expressed XBP-1 mRNA. The mRNA overexpression may be caused by mechanisms, such as gene amplification, transcriptional activation by hypomethylation of the promoter region and trans-activation by other cellular factors. Further detailed examination of the XBP-1 genome region may provide clues to elucidate such different mechanisms. The up-regulation of the XBP-1 gene in the primary colorectal tumors was further evidenced by detection of a high level of XBP-1 protein expression in the colorectal cancer cells by immunohistochemical staining procedures using anti-XBP-1 antibody.

XBP-1 was originally identified as a basic region-leucine zipper transcription factor, that binds to the X2 box in the promoter region of the major histocompatibility complex proteins, thereby regulating its expression (1, 19). XBP-1 was then found to be identical to the tax-responsive element binding protein 5 (2). A targeted disruption experiment in mice of the XBP-1 gene has demonstrated that it plays an essential role in the maintenance and growth of cardiac myocytes and hepatocytes (3, 20). XBP-1 was also shown to be critical for the development of the skeleton, tooth buds and exocrine glands, including the pancreas and salivary glands, especially in the stage of active branching, during mouse embryogenesis (21).

Additionally, XBP-1 is a critical transcriptional regulator of ER stress. Hypoxia favours tumors with an increased malignant phenotype and increases the metastatic potential of tumor cells. Tumor cells respond to hypoxia and ER stress through the activation of the UPR. The UPR is an adaptive response to increase cell survival during ER stress (22, 23). Many tumors contain hypoxic regions (<5 mm Hg oxygen) that could adversely affect therapy.

The presence of tumor hypoxia has been correlated with increased tumor recurrence rates in various tumor sites (24). In head and neck cancers, Gatenby *et al.* correlated poor response to radiation with increased hypoxia in these tumors (25). In prostate cancer, hypoxic tumors were associated with increased recurrences (26). Interestingly, even in cancer patients treated with surgery alone, an examination of cervical tumor specimens following radical tumor resection has shown that hypoxic tumors were more likely to metastasize compared to well-oxygenated tumors of similar clinical stage and size (27). Further examination of colorectal tumors, including adenomas, investigating changes related to ER stress, such as the hypoxic circumference, are warranted.

In summary, the present study suggests that up-regulation of the XBP-1 gene might play an important role in colon carcinogenesis by accelerating the cell growth.

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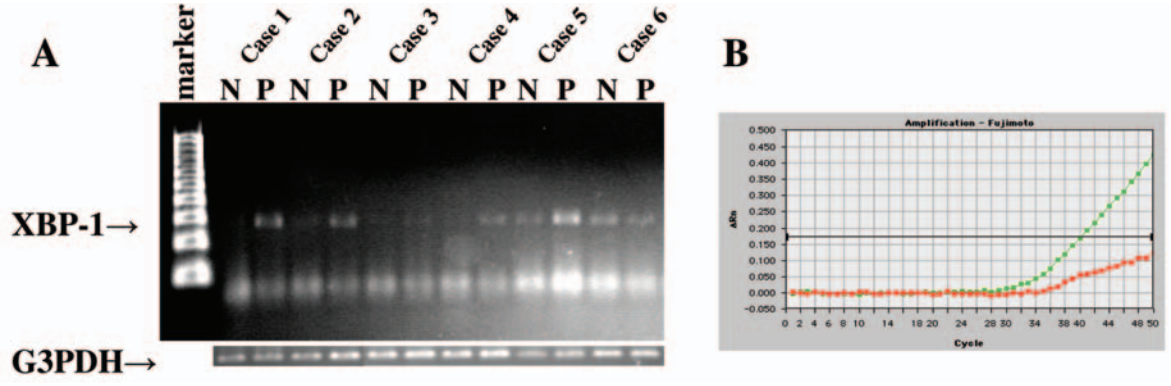

Figure 2. *XBP-1 gene expression by semi-quantitative RT-PCR analysis in colorectal polyps and corresponding normal epithelial tissues. N; normal epithelial tissue, P; colorectal polyp. Expression of G3PDH was used as a control (A). Representative real-time PCR was obtained from case 3 (B).*

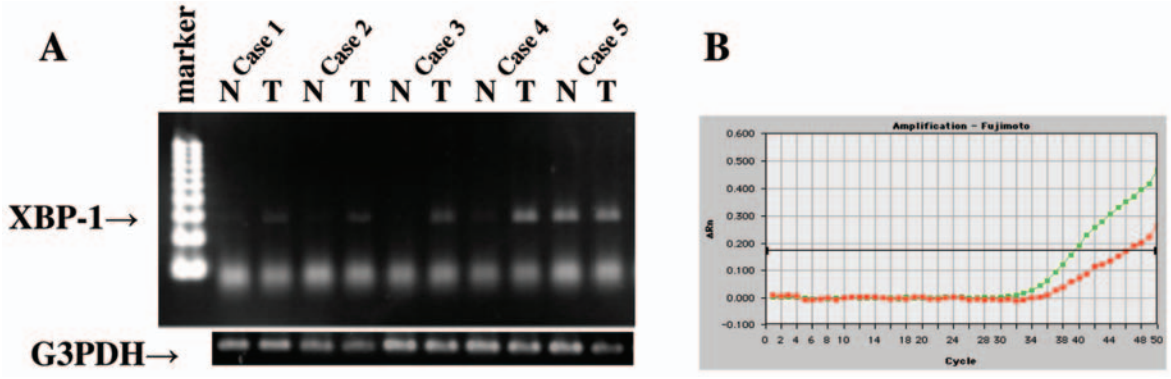

Figure 3. *XBP-1 gene expression by semi-quantitative RT-PCR analysis in colorectal carcinomas and corresponding normal epithelial tissues. N; normal epithelial tissue, T; colorectal cancer. Expression of G3PDH was used as a control (A). Representative real-time PCR was obtained from case 2 (B).*

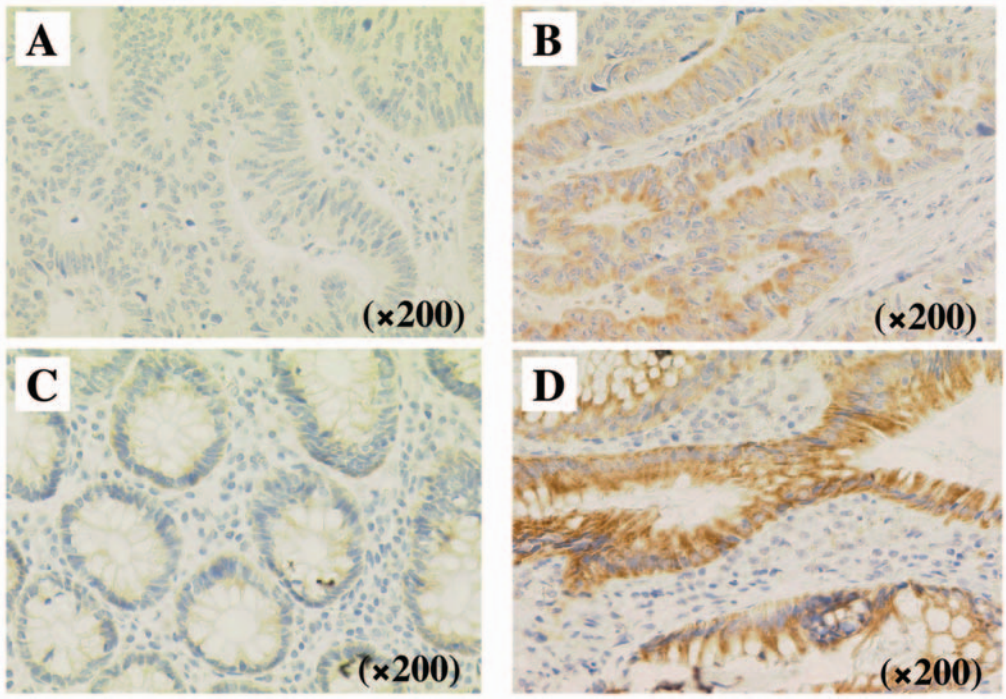

Figure 4. *Immunohistochemical staining using anti-XBP-1 antibody for colorectal tumors. (A) Normal epithelial tissue at magnitude x200, (B) colon adenocarcinoma at x200, (C) normal epithelial tissue at x200, (D) colon adenoma at x200.*

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Received September 26, 2006
Revised November 27, 2006
Accepted November 29, 2006