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Enhanced gene expression of transforming growth factor-α and c-*met* in rat urinary bladder cancer

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Abstract To investigate the roles of growth factors in bladder cancer, changes in the expression of messenger RNAs (mRNAs) for several growth factors and their receptors were examined during rat bladder carcinogenesis induced with *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN). Northern blot analysis showed that the contents of mRNAs for transforming growth factor-α (TGF-α) and c-*met*/hepatocyte growth factor (HGF) receptor increased with BBN treatment. Epidermal growth factor (EGF) receptor mRNA was hardly affected by the treatment; while mRNA for fibroblast growth factor (FGF) receptor 1 and transforming growth factor-β (TGF-β) type II receptor decreased with BBN treatment. A rat bladder tumor cell line, NBT-II, expressed both TGF-α and c-*met* mRNAs, and HGF showed apparent scattering and growth-stimulating effects on the cells. These results indicate the possibility that TGF-α produced by a bladder cancer, in addition to urinary EGF, plays a role in the development of bladder cancer, and that enhanced cell motility due to activation of the c-*met*/HGF receptor participates in the invasion and metastasis of the cancer cells.

Key words Bladder carcinoma · Gene expression · Growth factors · Growth factor receptors · Cell motility

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Introduction

A variety of polypeptide growth factors have been shown to play an essential role(s) in the growth and differentiation of virtually all cell types as positive or negative modulators. Aberrations in the expression of growth factors and their receptors are frequently associated with benign and malignant transformation. In the case of bladder cancer, epidermal growth factor (EGF) receptor has been studied most extensively, and more increased expression of the EGF receptor protein on malignant than on normal urothelium has been established [21, 24]. Although the production of EGF or transforming growth factor-α (TGF-α) in bladder cancer tissue has not been reported, there are several lines of evidence indicating that urinary EGF stimulates the growth of cancer cells in vivo [17, 22, 40]. In addition to the EGF receptor, recent studies have demonstrated the presence of several growth factors and their receptors in bladder cancer tissue and/or cell lines and increased concentrations of growth factors in the urine of bladder cancer patients. Included among them are basic fibroblast growth factor (bFGF) and its receptor [1, 25], acidic FGF (aFGF) [2, 5], TGF-α [11], insulin-like growth factor I receptor [13], and granulocyte-colony stimulating factor receptor [27]. The significance of these factors in the development and progression of bladder cancer in vivo, however, is not clear, as most of these studies were carried out using bladder cancer cell lines. It is important to know the changes in major growth factor systems during bladder cancer development in vivo to clarify the relative significance of and relationship between the growth factor systems in bladder cancer. However, there are difficulties in analyzing multiple factors, which exist in small quantities, at the protein level in the bladder. In the present study, we examined the changes in the contents of messenger RNAs (mRNAs) for a panel of growth factors and receptors during rat bladder

Table 1 Nucleotide sequences of oligonucleotide primers used for polymerase chain reaction (*PCR*) and related information (*F* forward primer, *R* reverse primer, *EGF* epidermal growth factor, *TGF* transforming growth factor, *bFGF* basic fibroblast growth factor, *KGF* keratinocyte growth factor, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

Proteins	Oligonucleotide primers (5′ → 3′)	Origins of target cDNAs[a]	Sizes of PCR products (bp)	Sizes of mRNAs (kp)	References
H4 histone	F: CCC TGC TGT TTT CAA ACA GGT CCG R: TCA GCC GCC GAA GCC ATA GAG CGT	Liver	414	0.5	36
EGF	F: CAA TTG GTG GTG GAT GCT GGC ATC R: CCC CGT GTG TTT GTT GGC TAT CCA	Submaxillary gland (mouse)	624	4.9	29
EGF receptor	F: TTG CTG ATT CAG GCT TGG CCT GAA R: GTG GCA CTG TAT GCA CTC AGA GTT	A431	465	10.0 6.0, 2.4	14, 34
TGF-α	F: GCT GCA GCG CCC TGC GCT CGG AAG R: CAT CGG CCA CCT GGC CAA ATT CCT	Brain	540	4.5	20
TGF-β1	F: GCC CTG GAT ACC AAC TAT TGC TTC R: GCT GCA CTT GCA GGA GCG CAC AAT	Liver	333	2.5	6
TGF-β type II receptor	F: TGG ACG CGC ATC GCC AGC ACG ATC R: GCT GAT GTC AGA GCG GTC GTC CTC	Liver	636	5.2	33
bFGF	F: ATG GCT GCC GGC AGC ATC ACT TCG R: TCA GCT CTT AGC AGA CAT TGG AAG	Brain	464	6.0	18, 30
FGF receptor 1	F: GGT CCA GAG AAC TTG CCG TAT GTC R: CTT CAT CTT GTA GAT GAT GAC GGA	Brain	297	4.3	35
KGF	F: GCG ATC AAC TCA AGG TCC AGT TCT R: AGC TGA TGC ATA GGT GTT GTA ATG	Lung	525	2.4	8, 38
GAPDH	F: GCC ATC AAC GAC CCC TTC ATT GAC R: ACG GAA GGC CAT GCC AGT GAG CTT	Liver	609	1.5	9

[a] DNA templates (first-strand cDNA) were prepared from 5 μg of total RNA isolated from various rat or mouse tissues and a human cell line (A431) using a first-strand cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden)
[b] Reported size of mRNAs for the corresponding proteins

carcinogenesis induced by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) to find candidates for a cancer-associated growth factor system.

Materials and methods

Animals

A total of 60 male Sprague-Dawley rats of 12 weeks of age (CLEA Japan, Tokyo, Japan) were divided into four groups. The rats were given drinking water containing 0.05% BBN (groups BBN-12 and BBN-20, 15 rats each) or without BBN (groups C-12 and C-20, 15 rats each) for 12 (BBN-12 and C-12) or 20 weeks (BBN-20 and C-20). The rats were fed at 20–25 °C *ad libitum* with Oriental MF solid diet (Oriental Yeast, Osaka, Japan). At the end of the treatment the rats were killed by cervical dislocation. The bladder tissue was immediately dissected out. A part (about one-quarter) of the tissue was subjected to histological examination, and the remaining tissue was frozen in liquid nitrogen for the preparation of total RNA. The frozen tissue was stored at −85 °C until use.

RNA isolation and northern blot analysis

Total RNA was extracted from the frozen bladder tissue specimens by the method of Chomczynski and Sacchi [4] using Isogen (Nippon Gene, Tokyo, Japan). After quantification, the total RNA preparation was subjected to northern blot analysis for H4 histone mRNA and poly(A)$^+$RNA isolation. Poly(A)$^+$RNA was isolated using a PolyATtract mRNA isolation system (Promega, Madison, USA).

Northern blot analysis was carried out as described previously [26]. Denatured total RNA or poly(A)$^+$RNA preparations were electrophoresed on 1% agarose, 2.2 M formaldehyde gels in 3-(*N*-morpholino) propansulfonic acid (MOPS) buffer, and then transferred onto nylon membranes. The RNAs were cross-linked to the nylon membranes by ultraviolet (light) irradiation. The blots were prehybridized in a solution of 5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.1% (w/v) *N*-lauroylsarcosine, 0.02% (w/v) sodium dodecyl sulfate (SDS), 0.5% (w/v) blocking reagent (Boehringer Mannheim, Mannheim, Germany) and 100 μg/ml denatured salmon sperm DNA for 3 h at 65 °C. After prehybridization, the blots were transferred to a fresh prehybridization solution containing 1 ng/ml of probes labeled with digoxigenin-11-2′-deoxy-uridine-5′-triphosphate (duTP; see below), and then incubated for 15 h at 65 °C. Following hybridization, the blots were washed with 2 × SSC/0.1% SDS at room temperature, and then with 0.1 × SSC/0.1% SDS at 65 °C. After treatment with buffer 1 (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5), containing 0.5% blocking reagent, for 30 min, the blots were incubated with 150 mU/ml of alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) in buffer 1 for 30 min. The blots were washed with buffer 1 containing 0.1% (w/v) Tween 20. The hybridized probes were detected by chemiluminescence using Lumi-Phos 530 (Lumigen, South Field, USA). Blots which hybridized to different probes were washed with 0.1% SDS for 30 min in a boiling-water bath.

Probes for northern blot analysis

All the probes used for northern blot analysis, except for those for hepatocyte growth factor (HGF) and c-*met*/HGF receptor, were

obtained by means of the polymerase chain reaction (PCR). The primers and the origin of target complementary DNAs (cDNAs) are summarized in Table 1. The cloned cDNA fragments of HGF [31] and c-*met* [23] inserted at the *Eco*RI and *Nde*I sites, respectively, of pBluescript were used as probes to detect HGF and c-*met* messages. All the probes were labeled with digoxigenin-11-dUTP by the method of Lanzillo [19].

Cell culture and HGF treatment of the cells

The NBT-II cell line established by Toyoshima et al. [32] was obtained from ICN Biomedicals (Costa Mesa, USA) and routinely grown on 100-mm culture dishes in minimum essential medium with Earle's salts, supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10% fetal bovine serum (FBS, standard medium). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were harvested by incubation with 0.25% (w/v) trypsin and 0.02% (w/v) EDTA in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} for 10 min at 37 °C, after which the cells were suspended in 5 ml/dish of the standard medium, and then centrifuged at $150 \times g$ for 5 min. The resulting cell pellet was resuspended in culture medium. To detect cell-scattering activity of HGF, the cells were plated at a density of 1×10^4 cells/35-mm dish and cultured in the standard medium for 24 h before adding samples. After a 48-h culture in the presence or the absence of HGF, the cells were fixed with 3.7% (w/v) formaldehyde in PBS and then stained with a Giemsa solution. To determine the growth-modulating effect of HGF, the cells were plated at a density of 3×10^4 cells/35-mm dish and cultured in the standard medium or the medium with a reduced FBS concentration (1%). Six hours after plating, by which time most cells had attached to the substratum, HGF was added to the culture medium. Cells were trypsinized and counted by a Coulter counter after 4 days of culture.

Results

Development of tumors and H4 histone

Histological observation showed that 20-week BBN treatment resulted in the development of noninvasive transitional cell carcinoma in all the animals examined. After 12-week BBN treatment, however, the appearance of papillary and nodular hyperplasia, and papillomas of the bladder epithelium was evident in most specimens, in accordance with previous studies [12, 41]. The steady state concentration of H4 histone mRNA is known to be coupled with DNA replication. The increase in the mRNA content in BBN-20 but not BBN-12 rat bladder (Fig. 1) is consistent with the histological changes observed in the present study and the changes in the proliferating cell population reported previously [12].

Growth factors and their receptors

TGF-α is the factor which shares the EGF receptor with EGF for signal transduction. An increase in the TGF-α mRNA content was evident in BBN-12 rat bladder and it was further increased after 20 weeks of BBN treatment (Fig. 2). EGF receptor mRNA is known to exist in multiple forms. We detected two or three kinds of EGF receptor mRNA species, ranging from 2.4 to 10 kb in size in rat bladder (Fig. 3). The contents of the EGF receptor mRNA species were not significantly affected by BBN treatment.

HGF, also known as scatter factor, was first identified as a potent mitogen for parenchymal hepatocytes, but now is known to regulate the growth and motility of various types of cells [28]. HGF mRNA was not detected in either normal or BBN-treated rat bladder specimens under the conditions used; while normal bladder expressed mRNA for c-*met*/HGF receptor, and the content of mRNA was increased in BBN-12 and -20 rat bladder (Fig. 4).

The changes in mRNAs for bFGF, TGF-β1, EGF, and keratinocyte growth factor (KGF) during bladder carcinogenesis were not unequivocally determined, as the contents of the mRNAs were very low (bFGF and TGF-β1) or below detectable levels (EGF and KGF) in

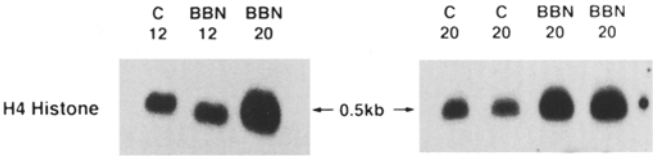

Fig. 1 Northern blot analysis of H4 histone mRNA expression in normal and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) treated rat bladder. Total RNA (20 μg) prepared from control and BBN-treated rat bladder was used. Blots were hybridized to a cDNA probe labeled with digoxigenin, and the hybridized probe was detected on X-ray film as to chemiluminescence, as described in *Materials and methods*. *C-12*, *C-20* control rats given drinking water without BBN for 12 and 20 weeks, respectively, *BBN-12*, *BBN-20* rats given drinking water containing 0.05% BBN for 12 and 20 weeks, respectively

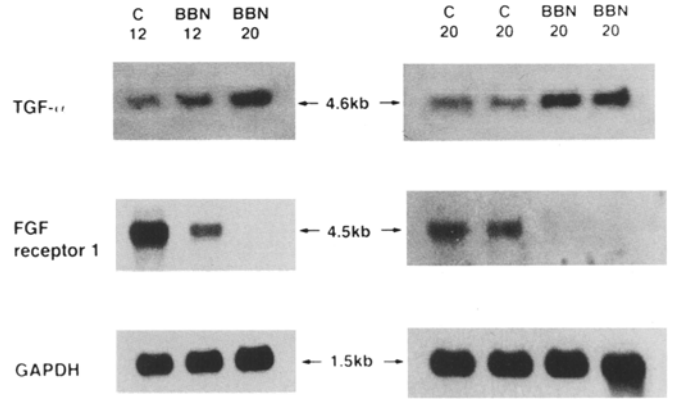

Fig. 2 Northern blot analysis of transforming growth factor-α (TGF-α) and fibroblast growth factor (FGF) receptor 1 mRNA expression in normal and BBN-treated rat bladder. Poly(A)$^+$RNA (1.5 μg) prepared from control and BBN-treated rat bladder was used. Blots were successively hybridized to cDNA probes for TGF-α, FGF receptor 1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Other conditions were as given in the legend to Fig. 1

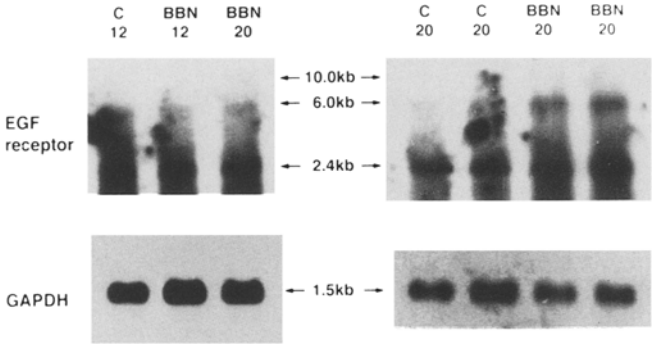

Fig. 3 Northern blot analysis of epidermal growth factor (EGF) receptor mRNA expression in normal and BBN-treated rat bladder. Poly(A)$^+$RNA (1.5 μg) prepared from control and BBN-treated rat bladder was used. Blots were successively hybridized to cDNA probes for EGF receptor and GAPDH. Other conditions were as given in the legend to Fig. 1

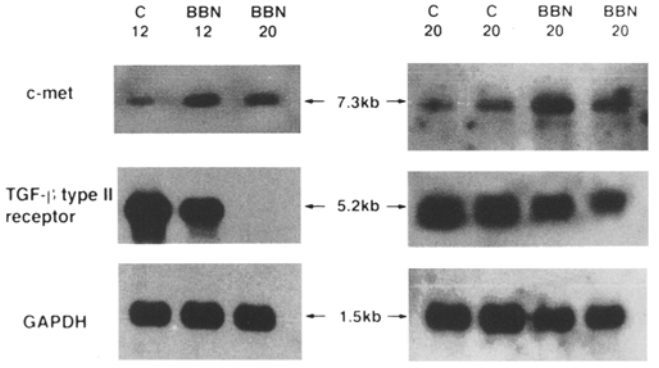

Fig. 4 Northern blot analysis of c-*met* and TGF-β type II receptor mRNA expression in normal and BBN-treated rat bladder. Poly(A)$^+$RNA (1.5 μg) prepared from control and BBN-treated rat bladder was used. Blots were successively hybridized to cDNA probes for c-*met*, TGF-β type II receptor, and GAPDH. Other conditions were as given in the legend to Fig. 1

both normal and tumor tissues. On the other hand, the expression levels of the mRNAs for FGF receptor 1 and TGF-β type II receptor were relatively high in normal tissues and decreased with BBN treatment (Figs. 2, 4). It is noteworthy that the two mRNA species almost completely disappeared in some BBN-20 rat bladder specimens.

Effect of HGF on a bladder cancer cell line

To investigate the function of c-*met*/HGF receptor in bladder tumor cell growth and motility, we examined the effect of HGF on a rat cell line, NBT-II, established from a bladder tumor induced with BBN. Northern blot analysis showed that NBT-II constitutively expressed c-*met* mRNA in addition to TGF-α and EGF receptor mRNAs (Fig. 5). HGF, when added to the culture medium, showed a strong scattering effect on

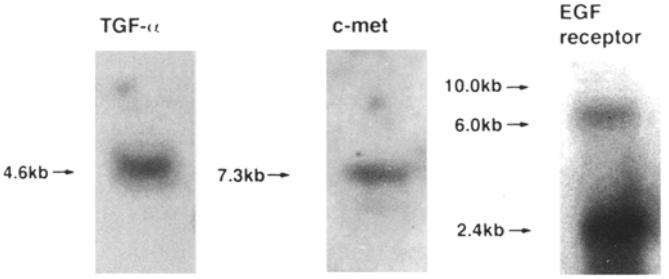

Fig. 5 Northern blot analysis of c-*met*, TGF-α, and EGF receptor mRNA expression in the NBT-II rat bladder tumor cell line. Poly(A)$^+$RNA (1.5 μg) prepared from NBT-II cells cultured in minimum essential medium supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10% fetal bovine serum was used. Other conditions were as given in the legend to Fig. 1

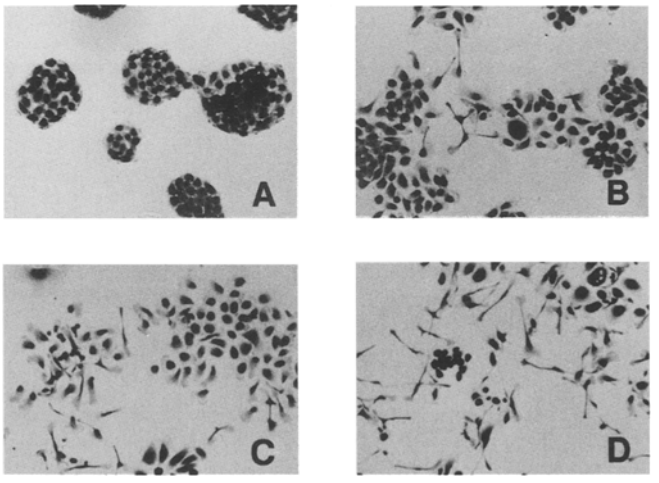

Fig. 6A–D Effect of hepatocyte growth factor (HGF) on the cell motility of NBT-II. NBT-II cells (1×10^4 cells/35-mm dish) were cultured in minimum essential medium supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10% fetal bovine serum for 24 h before adding samples. The cells were fixed with formaldehyde and then stained with a Giemsa solution 48 h after sample addition. **A** Control cells cultured in the absence of HGF; **B–D** Cells cultured in the presence of 1, 3, and 30 ng/ml of recombinant human HGF, respectively

NBT-II (Fig. 6) and slightly stimulated proliferation of the cells under the standard culture conditions (Fig. 7). These observations are consistent with the results recently reported by Bellusci et al. [3].

Discussion

Among various growth factors and their receptors, the EGF receptor has been studied most extensively in bladder cancer. As a result, most investigators, using clinical specimens, have found that malignant tissues contain more EGF receptor protein or mRNA than normal tissues, and there is a correlation between the EGF receptor concentration and the tumor grade

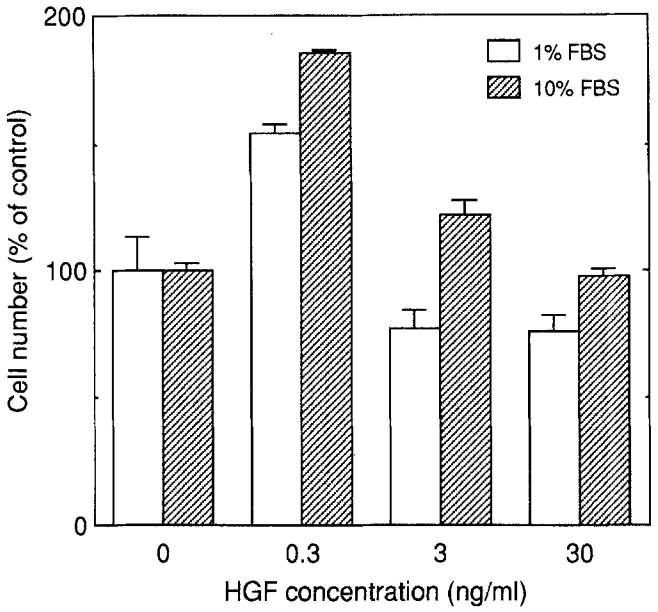

Fig. 7 Effect of HGF on the growth of NBT-II. NBT-II cells (3×10^4 cells/35-mm dish) were cultured in minimum essential medium supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 1% or 10% fetal bovine serum (*FBS*) for 6 h before adding HGF. The cells in triplicate dishes were trypsinized and then counted after 4 days of culture. The control values (1% FBS, $1.20 \pm 0.16 \times 10^5$ cells/dish; 10% FBS, $5.13 \pm 0.15 \times 10^5$ cells/dish) were taken as 100%. Values are means ± SEM

and/or invasive activity [21, 24, 37]. In addition, Messing reported a change in the EGF receptor distribution that made bladder epithelial cells become accessible to urinary EGF in premalignant and malignant urothelium [21]. Although we could not detect an increase in EGF receptor mRNA expression after BBN treatment, the expression of mRNAs for TGF-α and EGF receptor in rat bladder tumor suggests that tumor-derived TGF-α in addition to urinary EGF participates in growth stimulation of cancer cells via the EGF receptor in vivo.

HGF stimulated proliferation of NBT-II at a concentration of 0.3 ng/ml, but the growth-stimulating effect was diminished at higher concentrations, while the cell-scattering activity of HGF became apparent at concentrations higher than 1 ng/ml (Fig. 7). Geimer and Bade [10] have reported that the cell migration induced by EGF is associated with a transient inhibition of DNA synthesis. Thus it is likely that the cell scattering induced by HGF resulted in the suppression of cell growth. The cell-scattering activity of HGF is thought to be involved in several physiologic and pathologic events, including morphogenesis and cancer cell invasion and metastasis. In fact, Kuniyasu et al. [16] and Di Renzo et al. [7] reported that overexpression of c-*met*/HGF receptor mRNA or protein was correlated with tumor invasion and/or metastasis in human gastric and thyroid carcinomas, respectively. The present results indicate the possibility that activation of c-*met* also participates in bladder tumor growth, invasion, and metastasis. As HGF is known to act not only through an autocrine/paracrine mechanism but also through an endocrine mechanism [39], HGF synthesized in a distant organ(s) may activate c-*met* in bladder tumor tissue.

Although the idea that neoplastic transformation, in some instances, results from a loss of the negative growth regulatory influence of TGF-β may not be applicable to all tumors, it is possible that the decrease in TGF-β type II receptor mRNA in bladder tumors contributes to the proliferation of tumor cells. FGFs transduce signals through a family of transmembrane receptors, FGF receptors, and at least four mammalian FGF receptor genes, and their products are known [15]. As we analyzed the expression of only one FGF receptor, the apparent decrease in or disappearance of FGF receptor 1 mRNA in bladder tumors does not exclude the possibility that FGFs in tumor tissue and/or in urine stimulate the growth of bladder tumors in vivo.

In the present study, we examined changes in the gene expression of several growth factor-receptor systems during bladder carcinogenesis induced with BBN. As a result, the HGF-c-*met* system in addition to the EGF-TGF-α-EGF receptor system has appeared as a candidate for the growth factor system responsible for the development and progression of bladder cancer. The above-mentioned results were obtained by analyzing poly(A)$^+$RNA or total RNA fraction prepared from whole bladder tissue, which is composed of epithelial and stromal cells. The epithelium to stroma ratio may change during BBN treatment, and such a change may affect the interpretation of the results. Therefore, further studies are needed to clarify the role of these growth factor systems in bladder cancer by analyzing the content and distribution of the factors constituting the systems, especially TGF-α, EGF receptor, and c-*met*, at the protein level.

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