

A New Family of Heparin-binding Growth/Differentiation Factors: Increased Midkine Expression in Wilms' Tumor and Other Human Carcinomas¹

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ABSTRACT

Midkine (MK) and heparin-binding growth-associated molecule/pleiotrophin form a new family of heparin-binding growth/differentiation factors. We studied MK gene expression in human tumors. In normal human reference tissues, MK was highly expressed in the mucosal tissue of the small intestine, moderately in the thyroid, weakly in the tissues of the lung, colon, stomach, kidney, and spleen, and not at all in the liver. All of 6 surgically removed specimens of Wilms' tumor highly expressed MK. Also, a moderate to intense level of MK expression was noted in the majority of surgically removed hepatocellular carcinomas. The MK mRNA level was analyzed in a number of cultured and nude mice-transplanted lines of human tumors. In stomach, colon, pancreatic, lung, and esophageal carcinomas, a moderate to high level of MK expression was found in the majority of them. These results suggest an important role of MK in the development and/or biological behavior of tumors and raised a possibility to use MK as a diagnostic marker. Heparin-binding growth associated molecule/pleiotrophin mRNA was low or scarcely detectable in samples analyzed thus far except for significant levels of the expression that were observed in PA-1 teratocarcinoma cells and in some surgical specimens of Wilms' tumor.

INTRODUCTION

Growth factors or cytokines are involved in the regulation of a number of cellular processes, especially cell proliferation and differentiation (1-5). Many growth factors belong to distinct families or superfamilies such as that of fibroblast growth factor (6). A new family of heparin-binding growth/differentiation factors has recently been found (7-16); MK,³ the first to be discovered, is a product of a retinoic acid responsive gene, which is transiently activated during differentiation of teratocarcinoma stem cells (7). MK has a molecular mass of 13,000 and is rich in basic amino acids and cysteine (8).

HB-GAM (9), which is also called as PTN (10), heparin binding neurotrophic factor (11), and osteoblast specific factor 1 (12) have 50% sequence identity with MK. Both HB-GAM/PTN and MK have neurite extension activity in embryonic brain cells (14-16). HB-GAM/PTN also promotes the growth as well as the colonization of endothelial cells in soft agar (17, 18).

The modes of developmentally regulated expression of MK and HB-GAM/PTN are entirely different. MK is highly expressed during the midgestation period of mouse embryo genesis; in the adult, it is significantly expressed only in the kidney and uterus (19, 20). On the other hand, HB-GAM/PTN is highly expressed in the neonatal brain (14).

Growth factors and signal transducers triggered by them are often overexpressed in tumor cells, either in normal or aberrant forms, and the phenomenon is often considered to be a cause of tumorigenesis

(21-23). Thus, we investigated whether MK is overexpressed in human tumors. The MK mRNA level was examined in a large number of human tumor cells, specimens, and normal human tissues. In addition, HB-GAM/PTN mRNA level was also examined.

MATERIALS AND METHODS

Cells, Tumors, and Normal Tissues. The Wilms' tumor cell line G401 (24) and the renal cell carcinoma cell line VMRC-RCW were provided by the Japan Cancer Research Cell Bank. G401 cells were cultured in McCoy 5A medium containing 10% FCS and VMRC-RCW cells were in Dulbecco's modified minimum essential medium with 10% FCS. Other cell lines including NRC renal cell carcinoma (25), gastric cancer lines KATO III (26), MKN 28, MKN 45, and MKN 74 (27), lung cancer QG 56 (28), T-cell leukemia Molt 4 (29), and Burkitt's Lymphoma cell line Daudi (30) were provided by the Japan Immunoresearch Laboratories and were cultured in RPMI 1640 supplemented with 10% FCS. PA-1 human teratocarcinoma cells were cultured as described (31). Tumor specimens and the adjacent normal tissues were surgically removed from cancer patients. Various tumors were transplanted into nude mice and established as cell lines (32). The sigmoid colon carcinoma line COK18 and the rectal carcinoma line COK23 were established from lymph node metastases; all other lines were established from primary tumors. Harvested cells, tumors and the removed tissues were frozen in liquid nitrogen as soon as possible and stored at -70°C until use.

RNA Preparation and Northern Blots. Total cellular RNA was extracted from about 1 g of frozen tissues or cells using guanidine isothiocyanate/cesium chloride (33). Twenty or 15 µg of total cellular RNA was denatured with glyoxal, electrophoresed through an 1% agarose gel, and transferred to a Hybond nylon membrane (Amersham). Membranes were prehybridized and hybridized with a [³²P]-labeled probe prepared using a random oligonucleotide primer. Northern hybridization was performed overnight at 42°C in 50 mM Tris-HCl buffer, pH 8.0, containing 1 M NaCl, 10 mM EDTA, 0.1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, and 0.2% bovine serum albumin. Membranes were then washed twice with 2 × SSC (1 × SSC, 0.15 M NaCl containing 0.015 M sodium citrate) containing 0.1% SDS at room temperature for 10 min and twice with 0.2 × SSC containing 0.1% SDS at 56°C for 30 min. Membranes were exposed to X-ray film at -80°C with an intensifying screen for 2 days.

Southern Blot Analysis. Genomic DNA was isolated from G401, normal kidney, and renal cell carcinoma surgical specimens by the standard methods (33). Ten µg of genomic DNA were digested with restriction endonucleases for electrophoresis on a 0.8% agarose gel and then transferred to nitrocellulose filters. Hybridization and autoradiography were performed by the same methods as described for the RNA studies.

Probes. The cDNA probes used were as follows: a 487-base pair human MK cDNA fragment (nucleotide number 76-562) (13), a 590-base pair human HB-GAM/PTN cDNA fragment (nucleotide number 101-690) (12), and 3-kilobase human β-actin cDNA, a gift of Dr. T. Furukawa, Kagoshima University. The human HB-GAM/PTN cDNA was isolated from PA-1 teratocarcinoma cell cDNA by PCR (34) using the synthetic oligonucleotides 5'-AGAGAG-GACGTTTCCAATC-3' and 5'-GCAATAGTTAACTGATCCTG-3' as primers. The PCR conditions were as follows: 30 cycles of denaturation (93°C; 1 min); DNA reannealing (54°C; 2 min); and extension (72°C; 3 min). PCR products were subcloned into pUC18, and the DNA sequence was confirmed by dideoxy chain termination (35).

RESULTS

MK Expression. MK gene expression in human tumors and the corresponding normal tissues was studied by Northern blotting using

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³ Abbreviations: MK, midkine; HB-GAM, heparin-binding growth-associated molecule; PTN, pleiotrophin; HBNF, heparin-binding neurotrophic factor; FCS, fetal calf serum; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; cDNA, complementary DNA.

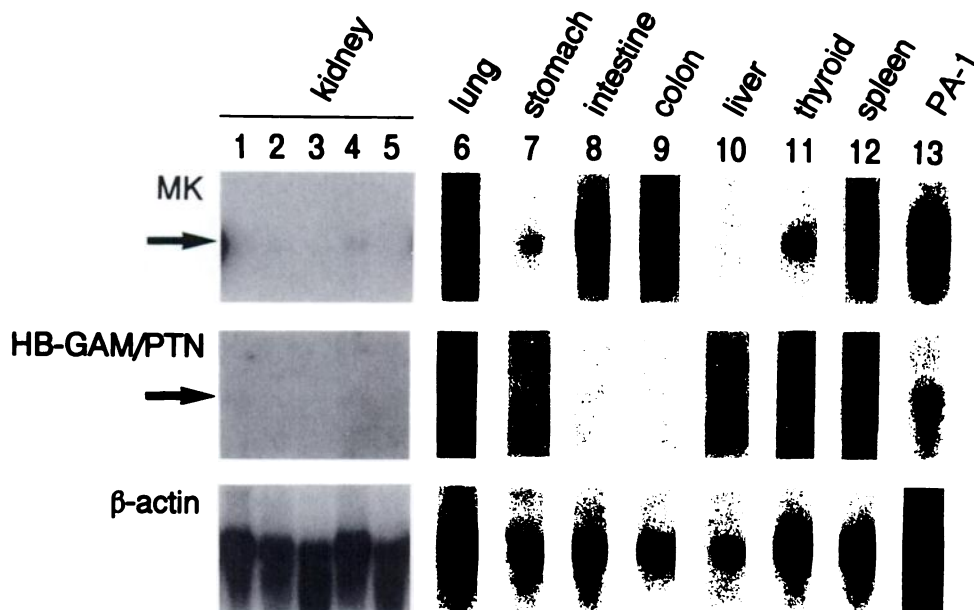


Fig. 1. MK and HB-GAM/PTN mRNA expression in normal human tissues and PA-1 ovarian teratocarcinoma. Northern blot hybridization was performed as described in "Materials and Methods." Upper lane, MK mRNA; arrow, about 1 kilobase MK mRNA. Middle lane, HB-GAM/PTN mRNA. Signals which are located in peripheral regions are due to those from adjacent lanes; arrow, about 1.4 kilobases HB-GAM/PTN mRNA. Lower lane, β -actin mRNA. Lanes 1-5, kidney; Lane 6, alveolus of the lung; Lane 7, mucosal tissue of the stomach; Lane 8, mucosal tissue of the intestine; Lane 9, mucosal tissue of the colon; Lane 10, liver; Lane 11, thyroid; Lane 12, spleen; Lane 13, PA-1 teratocarcinoma cells. Lanes 1-5 and 13 contain 20 μ g total RNA. Other lanes contain 15 μ g total RNA.

the β -actin gene expression as a reference. Since PA-1 teratocarcinoma cells highly express MK mRNA (13), PA-1 mRNA was included as a positive control.

In normal tissues, MK was weakly expressed in the kidney (Fig. 1, Lanes 1-5), lung alveoli (Lane 6), mucosal tissues of the stomach (Lane 7), colon (Lane 9), and spleen (Lane 12), moderately in the thyroid (Lane 11) and highly in the mucosal tissue of the small intestine (Lane 8). No MK mRNA was detected in the liver (Lane 10).

MK was highly expressed in all of 6 surgically removed specimens of Wilms' tumor (Fig. 2, Lanes 1-6). Lane 7 shows normal kidney tissue from the same source as the Wilms' tumor (Lane 6), and only weak MK expression was confirmed. An established cell line of Wilms' tumor G401 also highly expressed MK (Lane 8). MK mRNA was detected at high levels in one of two renal cell carcinoma cell lines (Lane 10), and weakly in a surgically removed specimen of renal cell carcinoma (Lane 11).

Southern blots showed that MK gene in Wilms' tumor cell line G401 was indistinguishable from that in surgically removed renal cell carcinoma and the adjacent normal tissue (Fig. 3). The intensity of the band was also similar. Therefore, gene amplification or a drastic structural change of MK gene is probably not the reason for the high MK expression levels in Wilms' tumor cells.

MK gene expression was studied in surgically resected specimens of other carcinomas (Fig. 4A) and in a number of human cancer lines, which were maintained either as cultures (Fig. 4B) or nude mice transplants (Fig. 4C). MK was weakly expressed in one of two surgical neuroblastoma specimens (Fig. 4A, Lane 1). In 4 surgical hepatocellular carcinomas specimens (Fig. 4A, Lanes 3-6), 3 expressed MK from moderate to intense levels. All of 9 colorectal adenocarcinoma lines expressed MK (Fig. 4C, Lanes 5-13); intensely to moderately in 8 lines and weakly in 1. Two lines of nude mice transplanted pancreatic adenocarcinoma highly expressed MK (Fig. 4C, Lanes 18 and 19). A nude mouse transplanted lung carcinoma (Fig. 4C, Lane 1) and its cultured cell line (Fig. 4B, Lane 4) highly expressed MK. Among 4 cultured gastric carcinoma cells (Fig. 4B, Lanes 1-4), 3 moderately expressed MK; in the nude mice transplanted lines (Fig. 4C, Lanes 2-4), intense expression was noted in 1 of 3. Two lines of esophageal carcinomas transplanted into nude mice moderately expressed MK (Fig. 4C, Lanes 15 and 16), but one weakly expressed it (Fig. 4C, Lane 14). Thus, among the cell and tumor lines so far examined, intense to moderate levels of MK were expressed in at least the majority of colon, stomach, pancreatic, lung, and esophageal car-

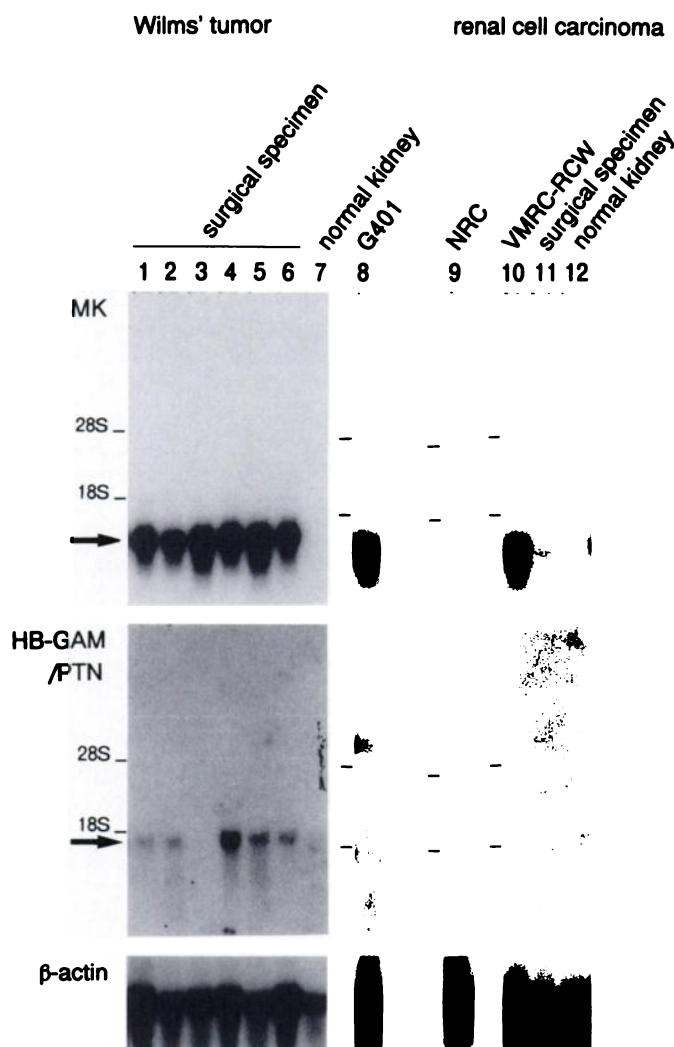


Fig. 2. MK and HB-GAM/PTN mRNA expression in Wilms' tumors, renal cell carcinomas, and the normal kidney. Upper lane, MK mRNA. The arrow indicates about 1 kilobase MK mRNA. Middle lane, HB-GAM/PTN mRNA; arrow, indicates about 1.4 kilobases HB-GAM/PTN mRNA. Lower lane, β -actin mRNA. Each lane contains 20 μ g total RNA. Lanes 1-6, Wilms' tumors from a surgical specimen. Lane 7, adjacent normal kidney of the Wilms' tumor Lane 6. Lane 8, G401 Wilms' tumor cell line. Lane 9, NRC renal cell carcinoma cell line. Lane 10, VMRC-RCM renal cell carcinoma cell line. Lane 11, renal cell carcinoma from a surgical specimen. Lane 12, Adjacent normal kidney of the renal cell carcinoma in Lane 11. 28S and 18S indicate the position of ribosomal RNAs.

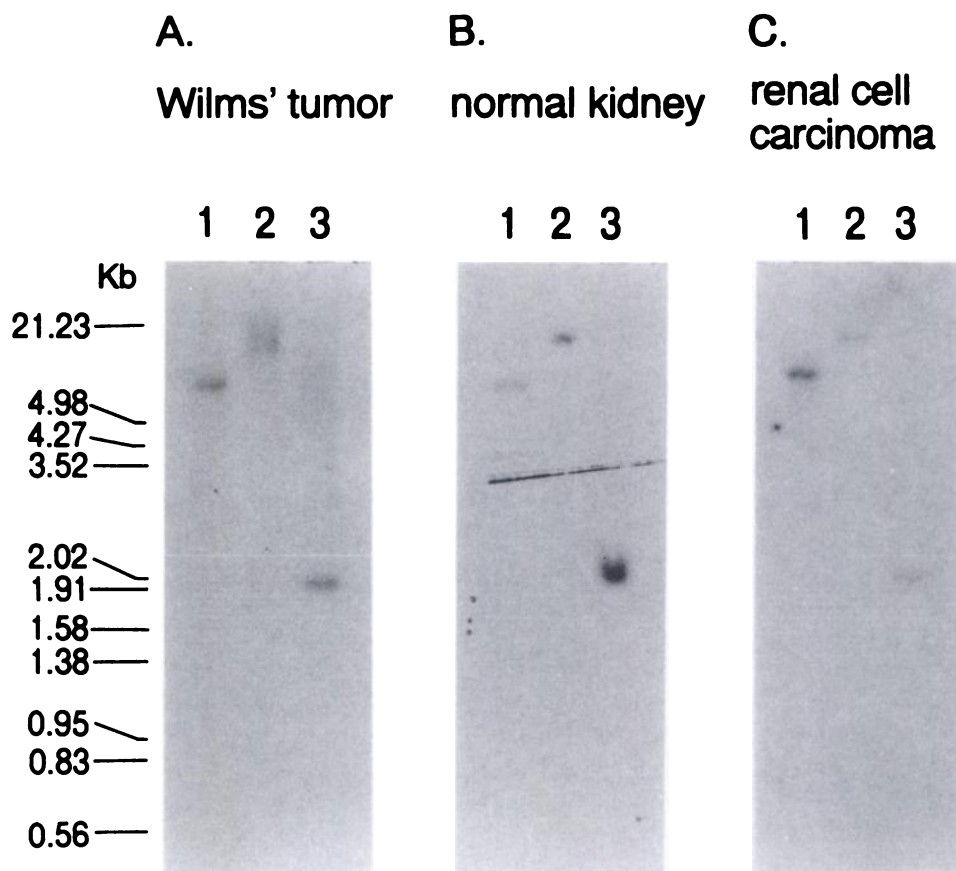


Fig. 3. Southern blots of genomic DNA in the Wilms' tumor cell line G401. (A), adjacent normal kidney (B), and renal cell carcinoma of surgical specimen (C). Lane 1, digested with XhoI; Lane 2, BglII; Lane 3, HindIII and XhoI. Molecular mass standards were λ DNA/HindIII \diamond EcoRI fragments (Nippon gene).

cinomas. MK expression was not observed in Molt4 T-cell leukemia and Daudi Burkitt's lymphoma cells (Fig. 4B, Lanes 6 and 7).

HB-GAM/PTN Expression. Using human HB-GAM/PTN cDNA as a probe, HB-GAM/PTN expression was studied in all samples that expressed MK. HB-GAM/PTN was not detected in normal tissues except for a weak expression in a specimen of the kidney (Fig. 1). In tumor specimens and lines, only PA-1 teratocarcinoma cells and one surgical specimen from a patient with a Wilms' tumor moderately expressed HB-GAM/PTN (Fig. 2). A lower level of HB-GAM/PTN was observed in 4 other surgically removed Wilms' tumors (Fig. 2). Among other tumor specimens and lines, HB-GAM/PTN was not detected (Fig. 4, A-C). Thus, it is likely that HB-GAM/PTN is not highly expressed in many human tumors as is MK.

DISCUSSION

By analyzing MK gene expression in human tumors, we arrived at the following conclusions. Firstly, MK gene expression was highly activated in all samples of Wilms' tumor thus far examined. Growth factors and their receptors have already been implicated in oncogenesis of this tumor. mRNA of platelet-derived growth factor and insulin-like growth factor II are increased in the tumor (36-39), and insulin-like growth factor II may promote tumor growth in an autocrine manner (40, 41). In addition to the high MK mRNA expression, G401 Wilms' tumor cells and PA-1 teratocarcinoma cells do secrete MK protein in the culture medium.⁴ Thus, it is possible that overexpression of MK is related to oncogenesis of Wilms' tumor as an autocrine manner. The Wilms' tumor locus gene at 11p13 (*WT1*) is a

transcription regulator of the Cys-His Zinc finger DNA binding protein (42, 43) the binding site sequences (CGC-CCCCGC) of which (43) are repeated twice in the 5' upstream sequence of the human MK gene antisense strand (nucleotides -157 ~ -149 and -904 ~ -896) (44). However, the product of the Wilms' tumor recessive oncogene is not always deleted in the tumor. Thus, altered expression of the Wilms' tumor recessive oncogene is not the sole reason, if any, for the increased expression of MK gene in the tumor. In any event, the intense expression of MK in all Wilms' tumor specimens implicates its potential diagnostic significance.

Secondly, at least in the majority of cases examined, moderate to high levels of MK gene were expressed in hepatocellular, stomach, lung, pancreatic, colon, and esophageal carcinomas. Whereas normal pancreatic tissue was not analyzed, MK gene expression level was much lower in other normal tissues. Intense to moderate expression of MK gene was also observed in some other tumors such as renal cell carcinoma. The widespread expression of MK gene in human tumors suggests an important role either in tumorigenesis or in the biological behavior of tumor cells. Furthermore, because of the broad expression, MK may be of value as a general diagnostic and/or follow-up marker of tumors.

In adult mice, a significant level of MK gene expression was observed only in the kidney and the uterus in 129/slc mice (19, 20). Using 8-week-old ICR mice, we confirmed that MK signal was absent in the lung, as well as in the mucosal tissues of the stomach, intestine, colon, and liver. A significant message was found in the kidney, and a trace amount was found in the brain.⁴ However, in the human, MK gene was expressed in a variety of organs, although at low levels except in the small intestine and thyroid. It is possible that the different levels of MK gene expression between the human and mouse

⁴ J.-i. Tsutsi *et al.*, unpublished results.

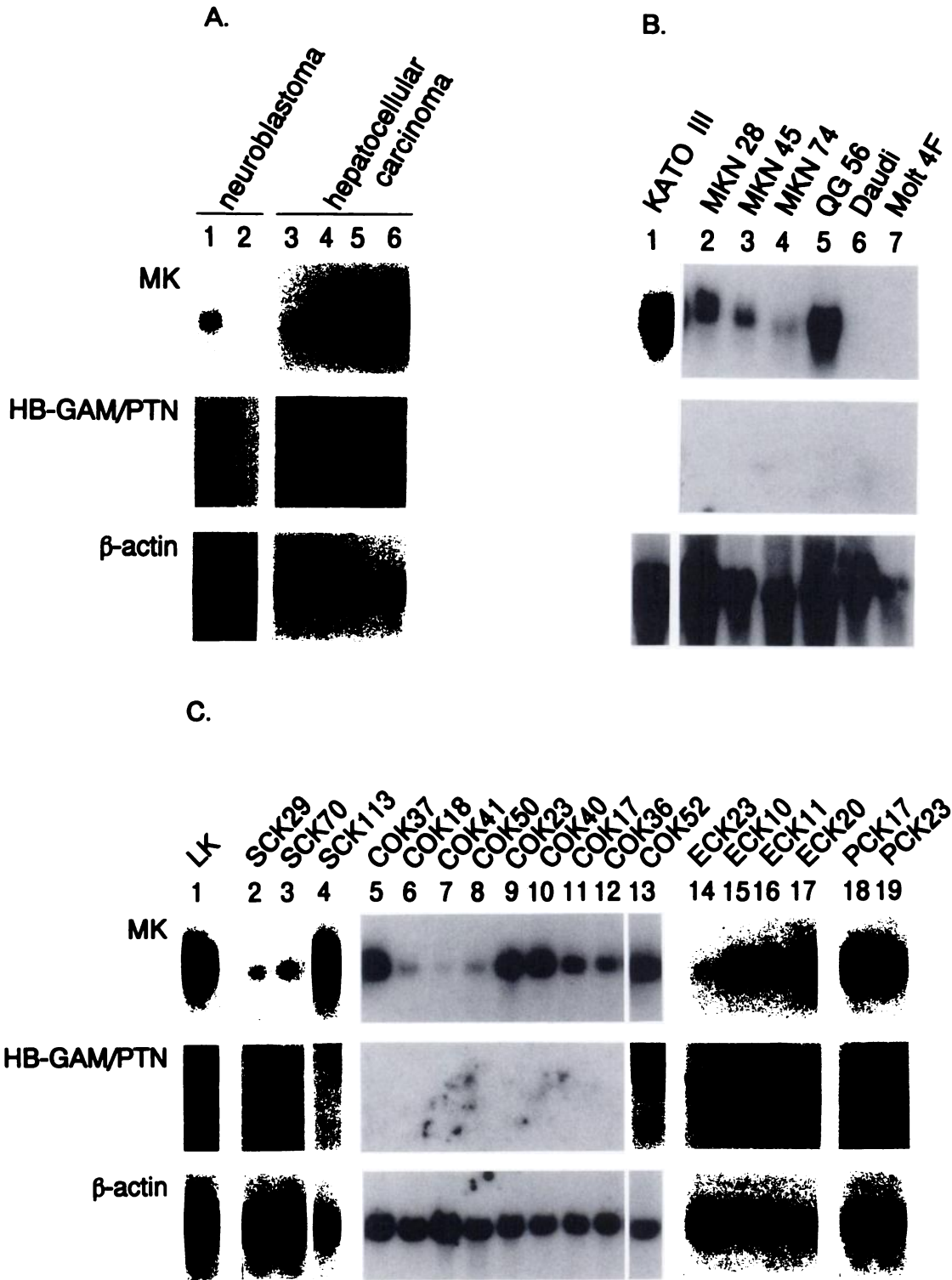


Fig. 4. MK and HB-GAM/PTN mRNA expression in surgically removed specimens (A), in cultured cell lines (B), and in nude mice-transplanted human carcinomas (C). A, Lanes 1 and 2, neuroblastomas from surgical specimens; 3–6, hepatocellular carcinomas from surgical specimens. B, Lane 1, KATO III; Lane 2, MKN28; Lane 3, MKN45; Lane 4, MKN74; Lane 5, QG56; Lane 6, Daudi; Lane 7, Molt 4F. C, Lane 1, lung adenocarcinoma LK; Lane 2–4, stomach adenocarcinomas (Lane 2, moderately differentiated carcinoma SCK29; Lane 3, well differentiated carcinoma SCK70; Lane 4, well differentiated carcinoma SCK113); Lane 5–13, colorectal adenocarcinoma (Lane 5, well differentiated colon carcinoma COK37, Lane 6, well differentiated sigmoid colon carcinoma COK18; Lane 7, well differentiated rectal carcinoma COK41; Lane 8, well and mucinous colon adenocarcinoma CO50; Lane 9, moderately differentiated rectal carcinoma COK23; Lane 10, well differentiated colon carcinoma COK40; Lane 11, moderately differentiated sigmoid colon carcinoma COK17; Lane 12, mucinous rectal carcinoma COK36; Lane 13, mucinous colon carcinoma COK52); Lane 14–17, esophageal carcinoma (Lane 14, well differentiated squamous carcinoma ECK23; Lane 15, poorly differentiated squamous carcinoma ECK10; Lane 16, small cell carcinoma ECK11; Lane 17, moderately differentiated squamous carcinoma ECK 20; Lane 18–19, pancreatic carcinoma (Lane 18, poorly differentiated adenocarcinoma PCK17; Lane 19, moderately differentiated tubular adenocarcinoma PCK23). Northern blot analysis was performed as described in Fig. 1.

are related to different physiological activities of the human and mouse tissues, since, for example, humans have a longer life span than mice.

Compared with MK, HB-GAM/PTN level was not high in the normal human tissues and tumors analyzed thus far. However, this does not mean that HB-GAM/PTN level is low in all human tumors.

For example, HB-GAM/PTN was intensely expressed in some other neuroblastomas.⁵ Furthermore, HB-GAM/PTN has been found in human breast carcinomas, and implicated as a tumor growth factor, which increased growth of endothelial cells in soft agar (17, 18).

In conclusion, gene expression of the new heparin-binding factors, MK and HB-GAM/PTN, is an useful parameter with which to characterize human tumors.

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⁵ A. Nakagawa, unpublished results.