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DISTINCT TUMOR SPECIFIC EXPRESSION OF TGFB4 (*ebaf*)*, A NOVEL HUMAN GENE OF THE TGF-β SUPERFAMILY

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1. ABSTRACT

We recently identified a novel gene of the TGF-β superfamily, endometrial bleeding associated factor, TGFB4 (*ebaf*), that, throughout the menstrual cycle, exhibited a defined expression in human endometrium. Here, we report on the expression of TGFB4 (*ebaf*) in normal and neoplastic human tissues. The expression of this gene was absent in a host of normal tissues including lung, stomach, small bowel, liver, kidney, breast, lymph node, spleen, ovary and fallopian tube. However, a weak expression of the 2.1 kb variant of the TGFB4 (*ebaf*) mRNA was observed in rectal, ovarian, and testicular tissues and the 2.1 and 2.5 kb TGFB4 (*ebaf*) mRNAs were observed in the pancreatic tissue. The expression of the mRNA of this gene was absent in sarcomas, Hodgkin's and non-Hodgkin's lymphomas, melanomas, squamous cell carcinomas, hepatocellular carcinomas, renal cell carcinomas, and adenocarcinomas of the breast, endometrium and lung. The expression of the TGFB4 (*ebaf*) mRNA was observed primarily in adenocarcinomas that exhibited a mucinous differentiation. This included colonic, duodenal, and ovarian adenocarcinomas. The expression of TGFB4 (*ebaf*) mRNA was absent in non-mucinous colonic, gastric and ovarian adenocarcinomas and adenocarcinomas of colon metastatic to the liver. However, some serous adenocarcinomas of the ovary also exhibited TGFB4 (*ebaf*) mRNA. The testicular tumors, seminomas and embryonal carcinomas, also expressed TGFB4 (*ebaf*) mRNA. These findings show that the TGFB4 (*ebaf*) mRNA has distinct tumor specific expression.

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2. INTRODUCTION

Some human genes such as β-actin and GAPDH mRNAs are present in virtually every cell (1-2). These genes, in view of their abundance, are considered part of the housekeeping gene repertoire. Some other genes are expressed in distinct tissues. For example, thyroglobulin or prostate specific antigen are, respectively, expressed in the thyroid and prostate (3-4). A different set of genes are expressed in cells of distinct lineages. For example, various isoforms of cytokeratin are expressed in the epithelial cells (5-7), vimentin is expressed in the mesenchymal and lymphoid cells (7-11), LCA in the lymphoid cells (12), desmin in the muscle cells (9-11), and neurofilament in the glial cells (9-11). Such a confined gene expression is shared by tumors derived from these tissues allowing the gene product to be used as a tumor marker. Among the most commonly used tumor markers is carcinoembryonic antigen (13). Its serum level is primarily used for the detection of gastrointestinal tumors. The serum level of the CA125 is used in the diagnosis of ovarian tumors and that of prostate specific antigen in the detection of the prostate cancers (4,13). We recently identified a novel member of the TGF-β superfamily, endometrial bleeding associated factor (*ebaf*), which was expressed in the endometrium (15). According to the guidelines of the Human Gene Nomenclature (http://www.gene.ucl.ac.uk/nomenclature/guidelines.html#Heading6),e*baf* has been designated as TGFB4. The expression of the TGFB4 (*ebaf*) mRNA in endometrium was confined to the late secretory and menstrual phases and was seen in

endometria with active bleeding (15). This gene was not expressed in endometrium during proliferative or early or mid-secretory phases of the menstrual cycle (15). *In situ* hybridization revealed that the gene was expressed primarily in the stroma and rarely in the endometrial glands. Endothelial cells failed to express the gene (15). The expression of the *lefty*, the mouse homolog of the TGFB4 (*ebaf*), also showed a narrow tissue-specific distribution. Meng *et al*, by *in situ* hybridization showed that the expression of *lefty* was found only in the mesenchymal cells of the mouse embryo on the left side of the body (16). The expression of *lefty* disappeared shortly after birth (16). These findings show that the expression of this gene is exquisitely controlled in the body, both in the mouse and in the human. Therefore, to gain an insight on the tissue distribution of the TGFB4 (*ebaf*) mRNA, in this study, we carried out Northern blot analysis on a panel of normal human tissues. In addition, we also examined the TGFB4 (*ebaf*) mRNA expression in tumors derived from epithelia, melanocytes and mesenchyme including adenocarcinomas, squamous cell carcinomas, melanomas, lymphomas and various types of sarcomas.

3. MATERIALS AND METHODS

3.1 Materials

A 1.1 kb cDNA fragment of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was obtained from Clontech (Palo Alto, CA). Deoxycytidine 5' tri-phosphate dCTP α-^{32}P (3000 Ci/mmol) was from Dupont NEN Research Products (Boston, MA). Prime-a-Gene labeling kit was from Promega (Madison, WI). RNA STAT-60™ was from Tell-Test, Inc (Friendwood, TX). Silane-coated, RNAse free, slides coated (Silane-Prep™) for *in situ* hybridization and the Kodak-OMAT films were obtained from Sigma Chemical Company (St Louis, MO). Nick columns were obtained from Pharmacia Biotech (Piscataway, NJ). Digoxygenin labeling kit (SP6/T7) and DIG nucleic acid detection kit were from Boehringer Mannheim Corporation (Indianapolis, IN). All other chemicals were from either Sigma Chemical Company or Fisher Scientific (Pittsburgh, PA).

3.2 Processing of tissues

Tissues were obtained from the tumor bank at the H. Lee Moffitt Cancer Center according to the rules and regulations of the institution. Tumor tissues and, when available, normal tissues surrounding the tumor were frozen in liquid nitrogen and maintained at -70°C until used. Samples of the normal tissues and tumors were embedded in paraffin, sectioned and stained by hematoxylin and eosin for light microscopic examination and for establishing the diagnosis and determination of the tumor type.

3.3 Isolation of RNA and Northern blotting

RNA was isolated by acid guanidinium thiocyanate-phenol-choloroform extraction method as described (17). Briefly, the tissues were homogenized in RNA STAT-60™. Each, 50-100 mg of tissue was homogenized in 1 ml of RNA STAT-60™ in a glass or Teflon Dounce homogenizer. Each homogenate was stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added for each ml of RNA STAT-60™ used. Each sample was covered and shaken vigorously for 15 seconds and allowed to stand at room temperature for 2-3 min. Following centrifugation at 12,000xg for 15 min at 4°C, each homogenate was separated into a lower phenol/chloroform phase and an upper aqueous phase. RNA in the upper aqueous phase was transferred to fresh tubes and mixed with isopropoanol to precipitate the total RNA. After centrifugation and drying, the precipitated RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by vigorous pipetting and by a gentle heating at 55°-60°C. The amount of RNA in each sample was determined spectrophotometrically. The quality of RNA was judged by the integrity of ribosomal. Northern blotting was done as described (18). Briefly, 20 µg of total RNA of each sample was denatured at 65°C in a RNA loading buffer, electrophoresed in 1% agarose containing 2.2 M formaldehyde gel, and blotted onto a Hybond nylon membrane using a positive pressure transfer apparatus (Posiblot, Stratagene, La Jolla, CA). The RNA was fixed to the membrane by UV crosslinking. Using the Prime-a-Gene kit, cDNA was labeled with [^{32}P] to a high specific activity, and purified by Nick columns. Membranes were prehybridized in 50% formamide, 10x Denhardt's solution, 4% saline sodium citrate (SSC), 0.05 M sodium pyrophosphate and 0.1 mg/ml of denatured Hering sperm DNA at 42°C for 2-4 hr and hybridized for 16 hr at 42°C with 10^6 cpm/ml of heat-denatured probe in the same buffer containing 10% dextran sulphate. Then, membranes were sequentially washed three times in 4x SSC, one time in 0.5x SSC and then one time in 0.1x SSC. All washes contained 0.1% sodium dodecyl sulphate (SDS), and were done at 65°C for 20 min each. The membranes were subjected to autoradiography at -70°C with intensifying screens. The same blot was stripped and reprobed for GAPDH. To reprobe a blot, the probe was stripped from the membrane in 75% formamide, 0.1x saline sodium phosphate ETDA (SSPE), and 0.2% SDS at 50°C for one hour.

3.4 In situ hybridization

Digoxigenin-labeled sense and anti-sense RNAs of TGFB4 (*ebaf*) were synthesized by *in vitro* transcription of the, full length cDNA, cloned into pBluescript^{R} SK^{-} using digoxygenin dUTP. After alkaline hydrolysis, the probes were subjected to agarose gel electrophoresis to determine the size of the digested RNA fragments. Dot blotting was performed on the RNA fragments to insure that they were labeled. *In situ* hybridization was performed as previously described (19-20) Briefly, frozen sections of endometria were mounted on silane-coated, RNAse-free, slides and fixed in 4% formalin in PBS for 15 min at 4°C. The tissue sections were rinsed in 2xSSC and then treated

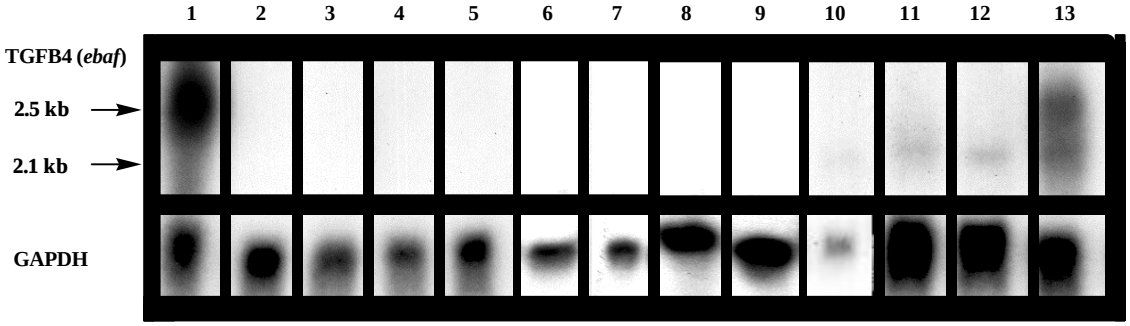

Fig 1. Northern blot analysis of TGFB4 (*ebaf*) mRNA in normal tissues. 20 µg total RNA from each tissue (lane 1: normal menstrual endometrium serving as the positive control) and other normal tissues (lane 2: spleen, lane 3: lymph node, lanes 4 and 5: stomach, lane 6: lung, lane 7: breast, lane 8: liver, lane 9 and 10: ovary, lane 11: rectum, lane 12: testis, lane 13: pancreas) was subjected to the Northern blot analysis using the entire placental-derived TGFB4 (*ebaf*) cDNA as the probe (upper panel). The integrity of RNA and equal loading was verified by staining the 18S and 28S ribosomal RNAs (not shown) and hybridization of the blots with a cDNA probe to GAPDH (lower panel). As shown, bands of TGFB4 (*ebaf*) mRNA in the size of 2.1 and 2.5 kilobase (kb) are detected in the endometrium. A weak 2.1 kb TGFB4 (*ebaf*) mRNA is detected in the ovary, rectum, and testis. In the pancreas, both the 2.1 and 2.5 kb TGFB4 (*ebaf*) mRNA are detected.

with proteinase K (1 µg/ml in 0.1 M Tris, 50 mM EDTA, 20 min, 37°C) and acetylated for 10 min in 0.1 triethanolamine (pH 8.0), 0.9% sodium chloride and 0.25% acetic anhydride. The slides were dipped once in 2x SSC and then were dehydrated in ascending series of ethyl alcohol and air dried. The slides were prehybridized for 1 hr at 37°C in 50% formamide, 1x Denhardt's solution and 500 µg/ml tRNA, 0.3 M sodium chloride, 10 mM Tris, 1 mM EDTA (pH 8), and 10% dextran sulfate. Then, sections were incubated at 55°C overnight in the same solution containing the appropriate concentration of the probe. The amounts of labeled probes needed were empirically determined first by a series of *in situ* hybridization experiments using various dilutions of the probes. Sense probe was used as the control. After hybridization, slides were washed three times for 10 min each at room temperature in 2x SSC, and the excess SSC was removed. The sections were then incubated with RNAse A (20 µg/ml) in 500 mM NaCl, 1 mM EDTA and 10 mM Tris HCl pH 8 at 37°C for 30 min to remove the non-hybridized RNA. The sections were washed three times at room temperature, for 15 min each, in 2x SSC, 1xSSC, and 0.5x SSC and a final wash in 0.1x SSC at 55°C for 45 min. Slides were washed in 100 mM Tris (pH 8), 150 mM sodium chloride for 10 min. Then, sections were blocked in 5% normal horse serum in the same buffer for 20 min at 37°C. Slides were incubated with alkaline phosphatase labeled, anti-digoxygenin antibody for 1 hr at 37°C, washed and developed in a mixture of Nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

4. RESULTS

We first examined the expression of the TGFB4 (*ebaf*) mRNA in normal tissues (Fig 1, Table 1). RNAs from several normal late secretory and menstrual endometrial tissues were used as control in these experiments (Fig 1-5). As reported previously (15), the prominent TGFB4 (*ebaf*) mRNA exclusively expressed in endometrium during the late secretory and menstrual phases was 2.5 kb (Fig 1). However, additional, smaller in size mRNAs were also noted in the menstrual endometria (Fig 3, Fig 5). These mRNAs were 2.1 and 1.5 kb in size (Fig 3, Fig 5). From a large number of tissues tested, the TGFB4 (*ebaf*) mRNA was expressed only in the ovary, rectum, testis and pancreas (Fig 1, Table 1). Both 2.5 and 2.1 kb TGFB4 (*ebaf*) mRNAs were expressed in the pancreas, whereas the TGFB4 (*ebaf*) mRNA, weakly expressed, in the rectum, in an ovary and testis, was 2.1 kb (Fig 1, Table 1). TGFB4 (*ebaf*) mRNA was not expressed in the breast, stomach, small bowel, colon, kidney, lung, fallopian tube, spleen and lymph node (Fig 1, Table 1).

We then examined the TGFB4 (*ebaf*) mRNA expression in the cancers derived from cells of different lineages. In eleven adenocarcinomas of colon, adjacent normal colonic tissues, non-involved by the tumor were available for the study. The RNAs from the neoplastic and surrounding normal tissues were both subjected to the Northern blot analysis for the detection of the TGFB4 (*ebaf*) mRNA (Fig 2, Table 2). Whereas the TGFB4 (*ebaf*) mRNA was not detected in the normal colon, the expression of the 2.1 kb TGFB4 (*ebaf*) mRNA was detected in seven of the eleven cases of adenocarcinomas of colon (Fig 2, Table 2). The histologic evaluation of the positive cases revealed these cases to have a mucinous differentiation. Similarly, an adenocarcinoma of the duodenum and three cases of mucinous adenocarcinomas of the ovary that also exhibited mucinous differentiation expressed the 2.1 kb TGFB4 (*ebaf*) mRNA (Fig 3, Table 2). The non-mucinous adenocarcinomas, whether primary in the

Table 1. Expression of TGFB4 (*ebaf*) mRNA in normal human tissues.

TISSUE	NUMBER OF TISSUES	NORTHERN BLOT FINDING
Breast	5	-
Stomach	3	-
Small Bowel	1	-
Colon	11	-
Rectum	2	2.1 kb
Liver	6	-
Pancreas	2	2.1 and 2.5 kb
Kidney	1	-
Lung	1	-
Fallopian Tube	1	-
Ovary	7	-
Ovary	1	2.1 kb
Testis	2	2.1 kb
Spleen	1	-
Lymph node	1	-

Table 2. Expression of TGFB4 (*ebaf*) mRNA in human tumors

TUMOR TYPE	NUMBER OF TUMORS	NORTHERN BLOT FINDING
Mucinous adenocarcinoma of colon*	7	2.1 kb
Mucinous adenocarcinoma of colon*	4	-
Mucinous adenocarcinoma of the duodenum	1	2.1 kb
Mucinous adenocarcinoma of the ovary	3	2.1 kb
Serous cystadenocarcinoma of ovary	2	2.1 kb
Serous cystadenocarcinoma of ovary	2	2.5 kb
Serous cystadenocarcinoma of ovary	5	-
Non-mucinous colonic adenocarcinoma metastatic to ovary	1	-
Non-mucinous adenocarcinoma of colon	7	-
Non-mucinous adenocarcinoma of uterine cervix	1	-
Non-mucinous adeoncarcinoma of the stomach	3	-
Endometrioid adenocarcinoma of ovary	1	-
Hepatocelluar carcinoma	3	-
Renal Cell Carcinoma	3	-
Liver metastasis; consistent with colonic primary*	6	-
Adenocarcinoma of lung	7	-
Adenocarcinoma of breast*	5	-
Adenocarcinoma of endometrium	3	-
SCC of the Larynx	1	-
SCC of the Lung	4	-
SCC of the Uterine cervix	1	-
Teratoma-embryonal cell carcinoma	1	2.5 kb
Germ cell tumor-embryonal cell carcinoma	1	2.5 kb
Seminoma	2	2.1 kb
Seminoma	1	-
Leiomyosarcoma, gastric	1	-
Leiomyosarcoma, colon	1	-
Leiomyosarcoma, pelvic	1	-
Chondrosarcoma, thoracic wall	1	-
Osteosarcoma, metastatic to the lung	3	-
Liposarcoma, retroperitoneum	1	-
Synovial sarcoma, metastatic to the chest wall	1	-
Synovial sarcoma, parotid	1	-
Synovial sarcoma, leg	1	-
Angiosarcoma, mediastinal	1	-
Lymphoma	1	-
Lymphoma, B cell type	1	-
Lymphoma, B cell, spleen	1	-
Lymphoma, T cell, groin	1	-
Lymphoma, T cell, angiocentric, hip	1	-
Hodgkin's disease, mixed cell type, lymph node	1	-
Melanoma	5	-

*: Normal tissues around the tumors were available for the Northern blot analysis and did not exhibit TGFB4 (*ebaf*) mRNA

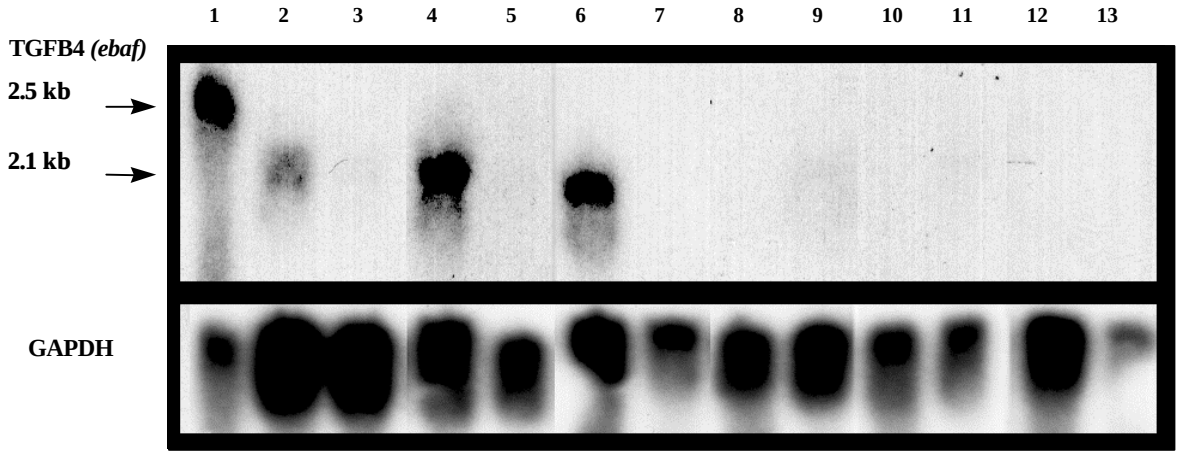

Figure 2. Northern blot analysis of TGFB4 (*ebaf*) mRNA in colonic adenocarcinomas. 20 μg total RNAs from a normal late secretory endometrium which served as the positive control (lane 1) as well as mucinous adenocarcinomas of colon (lanes 2, 4, 6), non-mucinous adenocarcinomas of colon (lanes 8, 10, and 12) and adjacent normal colon (lanes 3, 5, 7, 9, 11 and 13) were subjected to the Northern blot analysis using the entire placental-derived TGFB4 (*ebaf*) cDNA as the probe (upper panel). A 2.1 kb TGFB4 (*ebaf*) mRNA is detected in the colonic adenocarcinomas with mucinous differentiation and not those which did not exhibit a mucinous differentiation or the adjacent normal colonic tissues. The integrity of RNA and equal loading was verified by staining the 18S and 28S ribosomal RNAs (not shown) and hybridization of the blots with a cDNA probe to GAPDH (lower panel).

colon, metastatic to the liver, or the ovary, did not express TGFB4 (*ebaf*) mRNA (Table 2). In addition, in colonic adenocarcinomas metastatic to the liver, the adjacent liver, non-involved by the tumor, also did not show any evidence of TGFB4 (*ebaf*) mRNA expression (Table 1). In the ovary, besides the mucinous adenocarcinomas some serous adenocarcinomas (n=4/9) also expressed the TGFB4 (*ebaf*) mRNA (Table 2). In two cases, the TGFB4 (*ebaf*) mRNA was 2.1 and in two cases 2.5 kb (Fig 3, Table 2). On the other hand, other adenocarcinomas of ovary including those exhibiting endometrioid differentiation failed to express TGFB4 (*ebaf*) mRNA (Fig 3). To localize the cells that express the TGFB4 (*ebaf*) mRNA in the adenocarcinomas of colon and ovary, *in situ* hybridization was carried out on the positive cases. As a positive control, tissue sections from a late secretory endometrium, was used. As reported previously (15), the hybridization signal was noted primarily in the endometrial stroma and endometrial glands or the endothelium did not show presence of signal (Fig 4A). Only rarely, few glands close to the surface epithelium exhibited a positive hybridization signal (data not shown). Hybridization with the sense RNA did not produce any signal in the normal endometrium (Fig 4B). In tumor tissue sections hybridized with the anti-sense TGFB4 (*ebaf*) RNA, there was some minimal expression of TGFB4 (*ebaf*) mRNA in the tumor stroma. However, by far the most prominent expression of TGFB4 (*ebaf*) mRNA was noted in the neoplastic epithelial cells both in the colon (Fig 4C) and in the ovary (Fig 4E). The sense probe of the TGFB4 (*ebaf*) failed to show a hybridization signal in the same tissues (Fig 4D, 5F). In the adenocarinomas of other organs such as breast, lung, pancreas, cervix, stomach, liver, kidney and endometrium, TGFB4 (*ebaf*) mRNA was not detectable by the Northern blot analysis (Table 2).

Five cases of testicular cancer were examined for TGFB4 (*ebaf*) mRNA expression by the Northern blot analysis. Two cases that showed an embryonal carcinoma component exhibited the 2.5 kb TGFB4 (*ebaf*) mRNA. On the other hand, two out of three cases of seminomas expressed the 2.1 kb TGFB4 (*ebaf*) mRNA (Fig 5).

The Northern blot analysis of squamous cell carcinomas derived from larynx, lung and uterine cervix did not show any evidence of the TGFB4 (*ebaf*) mRNA expression (Table 2). Furthermore, the TGFB4 (*ebaf*) mRNA was not detectable in the non-epithelial tumors including sarcomas such as leiomyosarcoma, chondrosarcoma, osteosarcoma, liposarcoma, synovial sarcoma, as well as Hodgkin's and non-Hodgkin's lymphomas, or melanomas (Table 2).

5. DISCUSSION

In the present report, we defined the pattern of expression of TGFB4 (*ebaf*) mRNA in normal tissues. In addition to the endometrium, the 2.1 and 2.5 kb TGFB4 (*ebaf*) mRNAs were expressed in the pancreas and the expression of the 2.1 kb TGFB4 (*ebaf*) mRNA in the rectum and testis was weak and was rarely noted

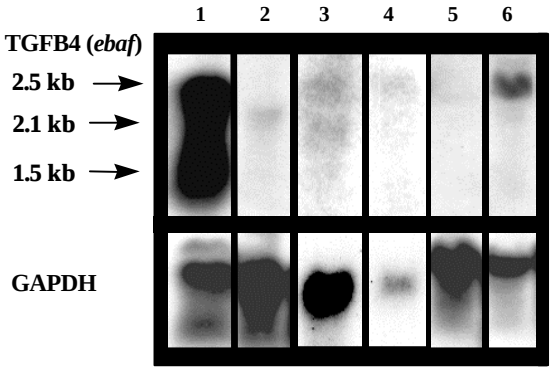

Figure 3. Northern blot analysis of TGFB4 (*ebaf*) mRNA in ovarian adenocarcinomas. 20 μg total RNA from a normal menstrual endometrium which served as the positive control (lane 1) as well as other tumors (lanes 2-4, serous cystadenocarcinomas; lane 5: endometrioid adenocarcinoma; lane 6, mucinous cystadenocarcinoma) was subjected to the Northern blot analysis using the entire placental-derived TGFB4 (*ebaf*) cDNA as the probe (upper panel). There is relatively more TGFB4 (*ebaf*) mRNAs in the menstrual endometrium than the tumor tissues leading to overexposure of the band of TGFB4 (*ebaf*) mRNAs in endometrium. The 2.5 kb TGFB4 (*ebaf*) mRNA is detected in serous (lanes 3 and 4) and mucinous cystadenocarcinomas (lane 6) of the ovary. The 2.1 kb mRNA is detected in serous cystadenocarcinoma (lane 2). The endometrioid adenocarcinoma of the ovary does not exhibit TGFB4 (*ebaf*) mRNA. The integrity of RNA and equal loading was verified by staining the 18S and 28S ribosomal RNAs (not shown) and hybridization of the blot with a cDNA probe to GAPDH (lower panel).

in the ovary. The weak to lack of expression of TGFB4 (*ebaf*) mRNA may be due to the proportionally low number of cells that express TGFB4 (*ebaf*) in these tissues. However, in contrast to the endometrium that primarily exhibits the 1.5 kb, 2.1 kb and 2.5 kb TGFB4 (*ebaf*) mRNAs, the major TGFB4 (*ebaf*) mRNA species expressed in these tissues was the 2.1 kb in size. Sequencing of the TGFB4 (*ebaf*) cDNAs obtained from normal endometria, revealed that the underlying basis for the difference in the sizes of TGFB4 (*ebaf*) mRNAs were attributable to the deletion of parts of the coding sequence of TGFB4 (*ebaf*) (unpublished data). Therefore, TGFB4 (*ebaf*) mRNA seems to exist as spliced variants. In addition, TGFB4 (*ebaf*) mRNA seems to have a distinct tissue distribution pattern. *In situ* hybridization revealed that in endometrium, TGFB4 (*ebaf*) mRNA expression was primarily confined to the endometrial stroma (15). Few, if any, endometrial glands expressed TGFB4 (*ebaf*) mRNA and the endometrial endothelial cells did not express TGFB4 (*ebaf*) mRNA (15). The expression of *lefty*, the mouse homolog of the TGFB4 (*ebaf*), was also limited to the mesenchymal cells in the mouse embryos (16). This expression was polarized and during embryogenesis was seen only on the left side of the body (16). Furthermore, the expression of *lefty* mRNA quickly disappeared postnatally. These findings show that TGFB4 (*ebaf*) mRNA has distinct tissue and cell specific expression in the embryo and in the adult tissues. It is conceivable that different splice variants of TGFB4 (*ebaf*) mRNA may exist in different cell types and lineages.

Northern blot analysis of tumors derived from different cell lineages confirmed the specific tissue distribution of the TGFB4 (*ebaf*) mRNA. TGFB4 (*ebaf*) mRNA was not expressed in tumors derived from lymphoid cells such as T and B cell lymphomas, and Hodgkin's disease. Tumors derived from endothelial cells, smooth muscle, bone, cartilage, synovium and melanocytes also did not express TGFB4 (*ebaf*) mRNA. The TGFB4 (*ebaf*) mRNA was expressed in adenocarcinomas of colon, ovary and testis. The type of the tumor in the colon and ovary that expressed the gene was derived from glandular structures of these tissues, namely adenocarcinomas. *In situ* hybridization showed that the TGFB4 (*ebaf*) mRNA was expressed primarily in the neoplastic glands rather than the tumor stroma. Histologic evaulation of adenocarcinomas expressing the TGFB4 (ebaf) mRNA showed that these tumors exhibit mucinous differentiation. In the testis, both the seminomas and tumors that contained an embryonal carcinoma component exhibited TGFB4 (*ebaf*) mRNA expression. However, the TGFB4 (*ebaf*) mRNA that was expressed in the seminomas was different in size from that expressed in the embryonal carcinomas. This difference may be attributable to the cell lineage of these tumors. Taken together, these findings show that TGFB4 (*ebaf*) mRNA is expressed in tumors of specific types and cell lineages. The difference in the expression of the 2.5 versus the 2.1 kb of TGFB4 (*ebaf*) mRNA may be dependent on the cell type that expresses the gene.

The predicted protein sequence of TGFB4 (ebaf) showed homology with and structural features of the members of TGF-β superfamily (15). The members of the TGF-β superfamily are synthesized as prepro-proteins which are cleaved at RXXR site to release the mature form of the protein. The predicted protein of TGFB4 (ebaf) exhibits two such RXXR sites which are located respectively at amino acid residues of 73-76 and 131-134 (15). The deduced amino acid sequence of lefty also contained two potential cleavage sites at amino acid residues of 74-77 and 132-135 (15). Therefore, TGFB4 (ebaf) gene products may be secreted by the tumor cells and may be released into the peripheral circulation. The confined expression of TGFB4 (ebaf) to tumors of distinct phenotype, therefore, will make detection of TGFB4 (*ebaf*) mRNA or its protein a useful tumor marker. Tumor markers in the blood include glycoproteins secreted by the solid

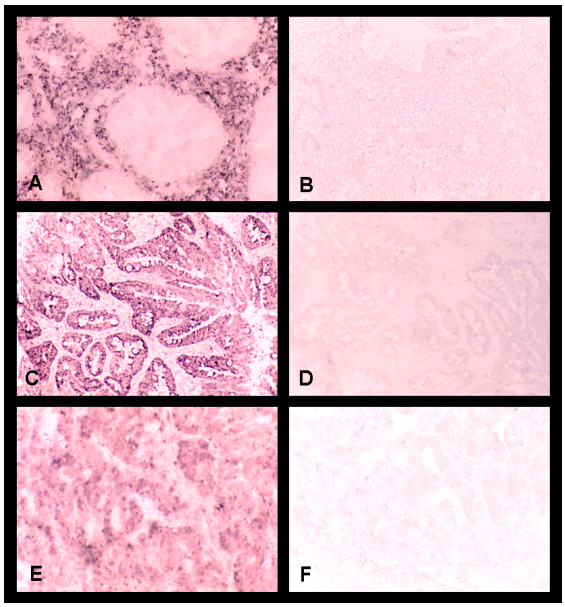

Fig 4. *In situ* hybridization of TGFB4 (*ebaf*) mRNA in adenocarcinomas of colon and ovary. Digoxigenin-labeled anti-sense (A, C, E) and sense RNAs (B, D, F) of TGFB4 (*ebaf*) were synthesized by *in vitro* transcription of the full length TGFB4 (*ebaf*) cDNA cloned into pBluescript^{R} SK(+/-). Sections of a late secretory endometrium were used as the positive control (A). Hybridization signal is seen in the endometrial stroma (A) and in the epithelial cells of a colonic adenocarcinoma with mucinous differentiation (C) and ovarian adenocarcinoma (E). The sense probe did not reveal any hybridization signal in the sections of the endometrium (B) or the same tumors (D, F).

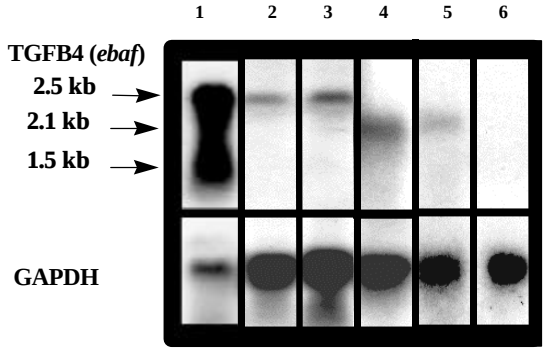

Fig 5. Northern blot analysis of TGFB4 (*ebaf*) mRNA in testicular cancers. 20 µg total RNA from a normal menstrual endometrium which served as the positive control (lane 1) and each tumor tissue (lane 2: teratoma-embryonal cell carcinoma, lane 3: mixed germ cell tumor containing embryonal carcinoma, lanes 4-6; seminoma) was subjected to the Northern blot analysis using the entire placental-derived TGFB4 (*ebaf*) cDNA as the probe (upper panel).). The blot was exposed for long duration to detect TGFB4 (*ebaf*) mRNA in the neoplastic tissues. This resulted in the overexposure of the TGFB4 (*ebaf*) mRNAs detected in the endometrium. The 2.5 kb TGFB4 (*ebaf*) mRNA is detected in the tumors containing embryonal carcinoma. The 2.1 kb mRNA is detected in two of three cases of seminoma. The integrity of RNA and equal loading was verified by staining the 18S and 28S ribosomal RNAs (not shown) and hybridization of the blots with a cDNA probe to GAPDH (lower panel).

tumors as well as those expressed on the cell surface. The most successful markers for the diagnosis of solid tissue cancers have been α-fetoprotein and prostate specific antigen (14). The serum level of markers such as CEA and a number of carbohydrate epitopes, e.g., CA 15.3, CA 19.9, CA 50, CA 242, and mucin epitopes, such as MCA, CA 125, and DU-PAN-2 are now being used to determine the prognosis of a variety of cancers and to monitor their response to therapy (13-14). If secreted, presence of TGFB4 (ebaf) in the peripheral circulation may be potentially used for screening, diagnosis, prognosis, and monitoring of the treatment, or the detection of relapse in tumors expressing the gene.

In summary, in the present report, we defined the expression of TGFB4 (*ebaf*) mRNA in normal and neoplastic tissues. In normal tissues, the expression of TGFB4 (*ebaf*) was limited to the endometrium, and only a weak expression of TGFB4 (*ebaf*) mRNA was detectable in the rectum, ovary and testis. In the pancreas, the TGFB4 (*ebaf*) mRNA was detectable. In the neoplastic tissues, the expression of the TGFB4 (*ebaf*) mRNA was restricted to tumors of colon, ovary and testis and tumors of lymphoid, melanocytic, and mesenchymal origins did not express the TGFB4 (*ebaf*) mRNA. These findings show that TGFB4 (*ebaf*) mRNA has a narrow and distinct tissue distribution in both normal and neoplastic tissues.

6. ACKNOWLEDGMENT

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