

# Glypican-3 Expression in Wilms Tumor and Hepatoblastoma

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**Background:** Glypican-3 (GPC3) is a heparan sulfate proteoglycan. When it is disrupted, it causes the X-linked gigantism-overgrowth Simpson-Golabi-Behmel syndrome. Its involvement in growth control is consistent with recent reports that it can bind to growth factors, possibly including insulin-like growth factor 2. Further, it has been hypothesized that it may function as a tumor suppressor gene in breast and ovarian carcinomas and mesotheliomas.

**Patients and Methods:** RNA and protein were extracted from Wilms tumor and hepatoblastoma tissue samples and GPC3 levels were measured in these extracts by Northern blotting, reverse transcription polymerase chain reaction, and immunoblotting.

**Results:** In contrast to published results with carcinomas, high levels of GPC3 expression were found in Wilms tumor and hepatoblastoma. Low or undetectable expressions of this gene were found in normal tissue surrounding the tumor.

**Conclusions:** Increased expression of GPC3 in Wilms tumor and hepatoblastoma suggests a growth-promoting or neutral activity for this gene product rather than a growth-suppressive effect.

**Key Words:** Glypican-3—Insulin-like growth factor 2—Wilms tumor—Hepatoblastoma.

Glypican-3 (GPC3) is a heparan sulfate matrix glycoprotein thought to bind tissue factor pathway inhibitor (1), fibroblast growth factor 2 (2), and insulin-like growth factor 2 (IGF2) (3). It has been suggested that GPC3 functions by limiting effective levels of IGF2 in embryonic tissues and that its absence may promote cell growth by increasing the amount of IGF2 available to cells. Glypican-3 is widely expressed in fetal tissues, including the placenta, but expression in adult tissues is limited to the lung, ovary, small intestine, and possibly testis (4).

Clinical overgrowth syndromes, including Beckwith-Wiedemann syndrome (BWS) (5) and the X-linked Simp-

son-Golabi-Behmel syndrome (SGBS) (3), are thought to reflect a disturbance of fundamental growth control mechanisms. Simpson-Golabi-Behmel syndrome is caused by loss-of-function mutation of GPC3, and patients with SGBS are believed to be at increased risk for malignancy (6,7). The true prevalence of tumors in SGBS is unknown; however, it is estimated to be 10% to 15% based on case reports (7). Tumors reported in SGBS include neuroblastoma, Wilms tumor, and hepatocellular carcinoma. Patients with BWS often have loss of imprinting of the *IGF2* gene, with resultant overexpression of IGF2 (8). Patients with BWS also have an increased incidence of hepatoblastoma and Wilms tumor (5,9), presumably because IGF2 is a critical growth factor for both tumors (10).

The possibility that GPC3 may have a tumor-suppressive function is supported by the observations that ovarian carcinomas often show loss of GPC3 activity and exogenous GPC3 expression suppresses the growth of ovarian cancer cells (11). Exogenous GPC3 expression also suppresses the growth of breast carcinoma cells and mesothelioma cells lacking endogenous GPC3, but not of NIH3T3 cells (12). Because IGF2 overexpression has been hypothesized to contribute to Wilms tumor and hepatoblastoma growth, the authors evaluated GPC3 expression levels in these tumors relative to adjacent normal tissues.

## METHODS

### Sample Acquisition and Preparation

Tumor samples were obtained from patients undergoing diagnostic surgical resection or biopsy of abdominal masses at the authors' institution. This study represents data from consecutive patients at the University of Maryland, with all patients included from 1997 to 1999. None of the University of Maryland patients had BWS or SGBS diagnosed. The authors also received random samples from Dr. Julie Ross and the Cooperative Human Tissue Network to enlarge the number of hepatoblastomas. Institutional Review Board-approved informed consent forms were signed by the parent or guardian. Tissues were frozen in liquid nitrogen as soon as possible after removal from the patient and a piece was sectioned, stained, and reviewed by a pathologist (C.C.S.) to confirm tumor or normal tissue type. The remaining tumor was then ground into a fine powder using liquid nitrogen in a mortar and pestle and stored at  $-80^{\circ}\text{C}$ . Rabbit antihuman GPC3 antibody was provided by D. Schlessinger.

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### Protein and RNA Extraction

Protein was isolated using lysis buffer and RNA was extracted using Trizol (Life Technologies, Grand Island, NY, U.S.A.), which were added to frozen powder without thawing. One complete Mini-Protease Inhibitor tablet (Boehringer Mannheim, Indianapolis, IN, U.S.A.) was added per 10 mL lysis buffer (50 mmol/L Hepes [pH 7.9], 100 mmol/L NaCl, 4 mmol/L NaPP, 10 mmol/L edetic acid, 10 mmol/L NaF, 1% Triton X-100). One hundred microliters of lysis buffer was added for each milligram of powdered tissue and tumbled overnight at 4°C. Samples were then spun at 14,000 rpm (Eppendorf benchtop centrifuge [Brinkman, Westbury, NY]) for 1 hour at 4°C to remove any insoluble debris, and the supernatant was analyzed. RNA was extracted using Trizol per the manufacturer's instructions.

### RNA Analysis

Northern gels were performed according to the Northern Max kit protocol (Ambion, Austin, TX, U.S.A.) and using ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA, U.S.A.). Blots were probed with an  $\alpha^{32}\text{P}$ -dCTP-labeled random-primed probe to a polymerase chain reaction-amplified fragment of GPC3 exons 1 through 3, as previously reported (13). Band density was determined using ImageQuant software on a STORM (Molecular Dynamics, Piscataway, NJ, U.S.A.) apparatus. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a GeneAmp kit per the manufacturer's instructions (Perkin Elmer, Branchburg, NJ, U.S.A.). Primers were located in exon 2, 5'-GCAAGTATGTCTCCCTAAGG-3', and exon 3B, 5'-AGGTCACGTCTTGCTCCTC-3', to yield a 430-base pair product. Thirty cycles of PCR (95°C for 1 min, 60°C for 1 min, 72°C for 1 min) were performed in a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer, Norwalk, CT, U.S.A.).

### Protein Analysis

Immunoblots were performed while undergoing nonreducing conditions. Protein lysate (100  $\mu\text{g}$ , as determined by BCA Protein Assay) was combined with nondenaturing loading dye (10% sodium dodecyl sulfate, 300 mmol/L Tris [pH 6.8], bromophenol blue 0.05%), separated using 6% Tris glycine gels, and transferred onto nitrocellulose paper (Schleicher & Schuell, Keene, NH, U.S.A.). Gels were stained with Bluestain Reagent (Pierce, Rockford, IL, U.S.A.) to ensure equal protein loading. Housekeeping proteins normally used for equal loading were too small for analysis on the 6% gel. Immunoblots were blocked with 5% milk TNE (10 mmol/L Tris, 50 mmol/L NaCl, 2.5 mmol/L edetic acid) for 1 hour at room temperature. Immunoblots were then probed with an affinity-purified rabbit antihuman GPC3 antibody 1 ng/ $\mu\text{L}$  in 2.5% milk TNET (TNE plus 0.05% Tween-20) overnight with rocking at 4°C, and then by secondary goat antirabbit (Amersham Life Science, Ar-

lington Heights, IL, U.S.A.), and detected using ECL Plus (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

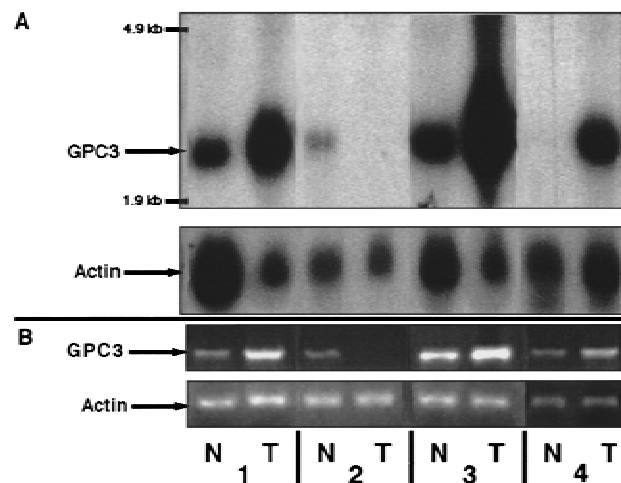
### Quantitation and Statistics

All RNA levels were quantified as described previously. Quantitation is reported to show relative levels, not absolute quantities. Reverse transcription polymerase chain reaction quantitation was determined by visualization and is meant to show relative levels. Each positive increment represents approximately a two-fold change in level and was reproducible to one positive increment by three masked observers. Relative immunoblot levels were determined by visualization and quantified in similar fashion to the RT-PCR.

## RESULTS

### Expression of Glypican-3 in Nephroblastoma

Increased IGF2 levels are believed to contribute to excess tumorigenesis in patients with BWS and possibly with SGBS compared with unaffected individuals (5,9). Because GPC3 is thought to alter IGF2 levels, GPC3 RNA levels were measured in three primary Wilms tumors with matching adjacent normal kidneys and one lung metastatic recurrence with matching adjacent normal lung. A single 2.5-kb band was observed on Northern blot (Fig. 1). Two of three primary Wilms tumors and one lung metastatic tumor showed increased GPC3 expression levels relative to the matched normal tissues. One additional tumor/normal pair was studied, as were four additional unpaired samples, including a primary Wilms tumor, two Wilms tumor lung metastases, and a renal cell carcinoma. All four of the ad-



**FIG. 1.** Glypican-3 RNA is overexpressed in Wilms tumor. Shown here is the expression of the GPC3 message from 30  $\mu\text{g}$  total RNA in paired normal (N) and tumor (T) by Northern blot (A) and RT-PCR (B). Total RNA was separated in agarose, and the transferred blot was probed with  $\alpha^{32}\text{P}$ -CTP-labeled fragment from exons 1 to 3. Glypican-3 is shown at the arrow, approximately 3.2 kb. Reverse transcription polymerase chain reaction using primers from exon 2 and 3B after agarose separation showed a 430-base pair fragment. Actin controls are shown below each figure. Tumor numbers correspond to Table 1.

**TABLE 1.** *GPC3 Expression in Wilms Tumor*

Sample (Patient number, age, sex, hist, stage)	Northern (pixel intensity)		RT-PCR		Immunoblot	
	Tumor	Normal	Tumor	Normal	Tumor	Normal
1. 5 y, F, FH, II	89	32	++++	++	84	12
2. 2 y, F, FH, II	4	14	0	+	182	17
3. 4 y, F, FH, II	206	53	++++	++	71	2
4. 6 y, F, lung met	57	7	+++	+	30	12
5. 7 y, M, FH, II	n.d.	n.d.	+++	0	60	15
6. 5 y, M, lung met	120	n.d.	+	n.d.	9	n.d.
7. 14 y, F, abdomen	33	n.d.	++	n.d.	40	n.d.
8. 5 y, M, FH, I	80	n.d.	++	n.d.	31	n.d.
9. 16 y, M, RCC, IV	5	n.d.	0	n.d.	10	n.d.

n.d., test not done due to inadequate or absent tissue; F, female; M, male; FH, favorable histology; RCC, renal cell carcinoma.

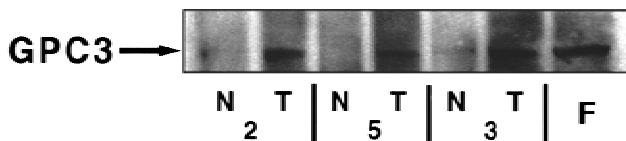
RT-PCR scores of 0, none seen, to 4+ were performed independently by three blinded observers are relative not absolute quantitative differences. Both Northern blot and immunoblot levels were determined densitometry.

ditional Wilms tumor samples had high levels of GPC3 message, whereas the renal cell carcinoma lacked expression, even by RT-PCR (Table 1). One primary tumor/normal pair (number 5) had partially degraded RNA that was adequate for RT-PCR but not for Northern analysis. Fetal kidney also expressed GPC3 message by Northern analysis at similar levels to the tumor tissues (data not shown). Overall, seven of eight Wilms tumors expressed high mRNA levels of GPC3, whereas only one of five normal kidney specimens expressed detectable GPC3.

The investigators assessed whether increased mRNA levels translated into higher protein levels. Nondenaturing conditions were used to measure GPC3 protein levels because denaturation reduced the recognition of the epitope as a result of GPC3 glycosylation. Expression of the expected 200-kD band was detected in tumor samples 2, 3, 5, and fetal kidney (Fig. 2). The polyacrylamide gel containing these samples was stained and showed equal loading (data not shown). The protein results are summarized in Table 1. Concordance was observed between RNA expression and protein levels in four of five paired samples; overall concordance between tumor RNA and protein was found in seven of nine samples.

#### Expression of Glypican-3 mRNA by Hepatoblastomas

Insulin-like growth factor 2 has also been implicated in the tumorigenesis of hepatoblastoma (10). Because GPC3



**FIG. 2.** Glypican-3 protein expression in Wilms tumor and fetal kidney. Shown here is the expression of the GPC3 protein in paired normal (N) and tumor (T) as well as fetal kidney (F) by immunoblot. Total protein extracts (100 µg) were separated while undergoing nondenaturing conditions on a 6% polyacrylamide gel, and the transferred blot was probed with a rabbit polyclonal antibody to GPC3. The 200-kD band is the expected size of the nondenatured GPC3. Tumor numbers correspond to Table 1.

has been suggested to alter IGF2 levels, seven paired samples of hepatoblastoma and adjoining normal liver were evaluated. Northern analysis and/or RT-PCR evaluation for GPC3 showed that six of seven hepatoblastomas expressed GPC3 in greater quantity than expressed in adjoining liver (Table 2). Fetal liver also expressed GPC3 (data not shown). As in Wilms tumors, the hepatoblastomas expressed GPC3, whereas the adjoining liver expressed little or no GPC3 message.

## DISCUSSION

Beckwith–Wiedemann syndrome predisposes patients to Wilms tumor and hepatoblastoma, and patients with the phenotypically similar SGBS lack GPC3. The investigators therefore predicted that Wilms tumor and hepatoblastoma would also have lost GPC3 expression, with an attendant release from growth control. Contrary to this expectation, the results indicate that both GPC3 mRNA and protein are present in Wilms tumor, based on direct assays. One sample pair, in patient 2, was discordant between RNA and protein expression; the authors cannot fully explain this, given a lack of additional patient material. The authors have also shown expression of GPC3 mRNA in hepatoblastoma

**TABLE 2.** *GPC3 expression in hepatoblastoma*

Sample (Patient number, age, sex)	Northern (pixel intensity)		RT-PCR	
	Tumor	Normal	Tumor	Normal
1. 10 mo, F	66	0	++++	+
2. 9 mo, M	70	29	++	+
3. 4.7 y, M	n.d.	n.d.	+	0
4. 2 y, M	n.d.	n.d.	++	0
5. 1.7 y, M	n.d.	n.d.	+	0
6. 6 y, M	n.d.	n.d.	0	0
7. 9 y, M	n.d.	n.d.	+	0

n.d., test not done due to inadequate or absent tissue; F, female; M, male.

Scores of 0, none seen, to 4+ were performed independently by three blinded observers.

samples. The levels of GPC3 were much higher in tumors than in the adjoining normal kidney and liver tissues in all but one patient. Fetal liver and kidney both also showed GPC3 expression.

These findings can be interpreted in several ways. First, because GPC3 was expressed in both fetal kidney and liver, and because Wilms tumor and hepatoblastoma are both derived from fetal cells, tumor-specific GPC3 expression suggests oncofetal continuation of gene expression, analogous to the expression of  $\alpha$ -fetoprotein in hepatoblastomas. Therefore, the pathway to tumorigenesis might travel in a direction that is not controlled by GPC3. Second, if GPC3 acts through the modulation of growth factor levels, the levels of interacting growth factors might be high enough to overcome GPC3 as a critical controlling element in these cells. In fact, IGF2, which has been suggested as a target of GPC3 control, is expressed at high levels in these tumors (10). Third, GPC3 may be involved in a different, growth-promoting pathway rather than a growth-suppressing pathway in these cells, a hypothesis supported by these data. Glypican-3 growth suppression has been hypothesized to be cell line-specific (12). If the tumor-suppressing activity of GPC3 were not tissue-specific, one would expect that Wilms tumors and hepatoblastomas, like ovarian and breast carcinomas, would have lost expression of the gene.

There is evidence that GPC3 sequesters IGF2, making it unavailable for tumor growth. Ovarian tumors were found to have loss of heterozygosity for GPC3 expression as a result of hypermethylation in 30% of the tumor cell lines evaluated (11). An ovarian tumor model showed that reexpression of GPC3 from the silenced allele resulted in growth suppression (11), perhaps from sequestration of IGF2. In mouse embryos, regional GPC3 expression correlates with IGF2 expression (4). Murine embryo GPC3 is highly expressed in the interdigital spaces of the extremities, where apoptosis is known to cause separation of the digits (4). Glypican-3 binding of IGF2 in normal development could thus negatively modulate this critical growth factor, and the consequential decrease in IGF2 levels could initiate apoptosis (12).

Additional studies are necessary to evaluate which of these hypotheses contributes to the role of GPC3 in em-

bryonal tumors. Studies should address whether expression of GPC3 is part of normal growth regulation. Primary fetal liver and kidney cells could be transfected with antisense constructs to reduce GPC3 expression. If GPC3 contributes to growth, cells with reduced GPC3 should arrest. To evaluate whether GPC3 contributes to IGF2 signaling, IGF type I receptor phosphorylation could be studied in cells with different levels of GPC3 protein.

Glypican-3 function, whether it suppresses, enhances, or has a neutral effect on growth, is probably dependent on additional protein interactions and overall ligand levels. Further evaluation of these findings will require a functional model of GPC3.

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