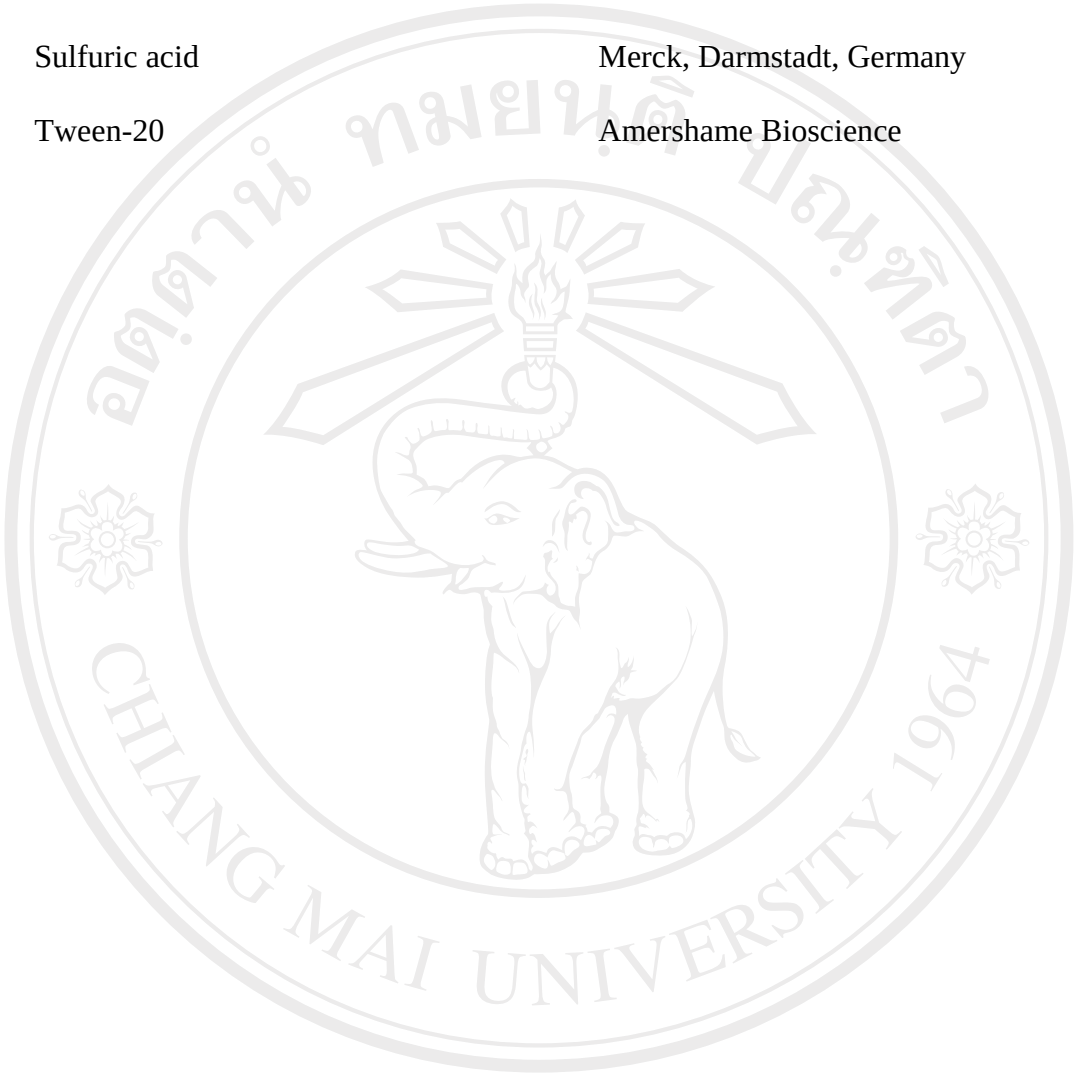
APPENDIX A

List of chemicals and materials used in the study

All chemicals and reagents used in this study are analytical grade and are listed as follows:

Chemicals	Source
95% tert-butly alcohol	Carlo Erba, Germany
96-well ELISA-plate (Nunc®, Maxisorb)	Nunc, Denmark
Anti-human glypican-3 antibody	R&D Systems
Bovine serum albumin	Sigma-Aldrich, St. Louis, MO, USA
Copper (II) sulfate	Carlo Erba, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO, USA
Hydrochloric acid	Lab scan, Ireland
Hydrogen peroxide	Merck, Darmstadt, Germany
N-acetylneuraminic acid	Sigma-Aldrich, Germany
OPD substrate	Sigma-Aldrich, St. Louis, MO, USA
Periodic acid	Fluka, Germany
Potassium chloride	Sigma-Aldrich, St. Louis, MO, USA
Recombinant human glypican-3	R&D Systems
Resorcinol	Fluka, Switzerland
Sodium bicarbonate	Merck, Darmstadt, Germany

Sodium chloride	Merck, Darmstadt, Germany
Sodium hydrogen phosphate (NaH_2PO_4)	Merck, Darmstadt, Germany
Sulfuric acid	Merck, Darmstadt, Germany
Tween-20	Amershame Bioscience



APPENDIX B

List of instrument used in the study

Instrument	Source
Analytical balance (HK160)	Mettler Instrument , Switzerland
ELISA plate reader (Titertek multiscan Mcc/340)	ICN, Flow, USA
Magnetic stirrer (MS101)	GEM
Microcentrifuge (Microcen13)	Herolab, Germany
Microplate shaker (MTSA)	Janke & Kunkel Gmblt& Co. KG, Germany
pH meter (SevenEasy)	Mettler Toledo, USA
Pipette	eppendorf
Refrigerator -20^{o}C	Sanyo
UV visible spectrophotometer (UV 1601)	Shimadzu, Japan
Vortex mixer (Vortex-Genie)	Scientific industry
Water bath (Imperial III)	Labline, USA

APPENDIX C

Reagent and buffers preparation

1. Stock 6 g% resorcinol reagent

Resorcinol	6.00 g
Copper (II) sulfate	1.00 mg
28% hydrochloric acid	60 ml
distilled water	40 ml

The solution was mixed thoroughly. This reagent was stored at -20 ^{o}C and should be warmed at room temperature before used.

2. Phosphate buffer saline (PBS)

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.44 g
Na_2PO_4	0.24 g

All reagents were dissolved in distilled water and made up volume to 1 L.

3. Citrate phosphate buffer

Citric acid monohydrate	10.30 g
$Na_2HPO_4 \cdot 3H_2O$	18.16 g

All reagents were dissolved in 900 ml of distilled water, adjusted pH to 5.0 and made up volume to 1 L. Stored reagent at 4^{o}C.

4. Tris Incubation buffer

BSA	1.0 g
Tween-20	1.0 ml
NaCl	8.77 g
Tris-HCl	1.21 g

All reagents were dissolved in 900 ml of distilled water, adjust pH to 7.4 and made up volume to 1 L. Stored at 4^{o}C.

5. Substrate solution

OPD	8 mg
Citrate phosphate buffer	12 ml
30% H_2O_2	5 µl

Prepare reagent fresh for 1 plate; keep in dark before use.

6. 0.1 M sodium hydrogen carbonate buffer pH 8.5

$NaHCO_3$	0.84 g

Dissolved in 80 ml of distilled water, adjust pH to 8.5 and made up volume to 100 ml

Anti-human Glypican 3 Antibody

ORDERING INFORMATION

Catalog Number: AF2119

Lot Number: UWW03

Size: 100 µg

Formulation: 0.2 µm filtered solution in PBS with 5% trehalose

Storage: -20° C

Reconstitution: sterile PBS

Specificity: human Glypican 3

Immunogen: NS0-derived rhGlypican 3

Ig Type: sheep IgG

Applications: Western blot
Flow cytometry
Immunohistochemistry
Direct ELISA

Preparation
Produced in sheep immunized with purified, NS0-derived, recombinant human Glypican 3 (rhGlypican 3). Human Glypican 3 specific IgG was purified by human Glypican 3 affinity chromatography.

Formulation
Lyophilized from a 0.2 µm filtered solution in phosphate-buffered saline (PBS) with 5% trehalose.

Reconstitution
Reconstitute with sterile PBS. If 0.5 mL of PBS is used, the antibody concentration will be 0.2 mg/mL.

Storage
Lyophilized samples are stable for twelve months from date of receipt when stored at -20° C to -70° C. Upon reconstitution, the antibody can be stored at 2° - 8° C for 1 month without detectable loss of activity. Reconstituted antibody can also be aliquotted and stored frozen at -20° C to -70° C **in a manual defrost freezer** for six months without detectable loss of activity. **Avoid repeated freeze-thaw cycles.**

Specificity
This antibody has been selected for its ability to recognize human Glypican 3 in the applications listed below. In direct ELISAs, this antibody shows less than 5% cross-reactivity with rhGlypican 2, rhGlypican 5 and rhGlypican 6.

Applications
Western blot - This antibody can be used at 0.1 - 0.2 µg/mL with the appropriate secondary reagents to detect human Glypican 3. The detection limit for rhGlypican 3 is approximately 5 ng/lane under non-reducing and reducing conditions.

Flow Cytometry - This antibody has been tested on human HepG2 cells for use in flow cytometry. Dilute this antibody to 50 µg/mL and add 10 µL of the diluted solution to 1 - 2.5 x 10^5 cells in a total reaction volume not exceeding 200 µL. The binding of unlabeled polyoclonal antibodies may be visualized by adding 10 µL of a 25 µg/mL stock solution of a secondary developing reagent such as goat anti-sheep IgG conjugated to a fluorochrome.

Immunohistochemistry - This antibody will detect Glypican 3 in cells and tissues. The working dilution is 2 - 5 µg/mL. For chromogenic detection of labeling, use R&D Systems' Cell and Tissue Staining Kits (CTS Series).

Direct ELISA - This antibody can be used at 0.5 - 1.0 µg/mL with the appropriate secondary reagents to detect human Glypican 3. The detection limit for rhGlypican 3 is approximately 2.0 ng/well.

Optimal dilutions should be determined by each laboratory for each application.

Recombinant Human Glypican 3

Catalog Number: 2119-GP

Specifications and Use

Source

Human CD33 signal peptide (Met 1 - Ala 16)	Human GPC 3 (Gln 25 - His 559)	HHHHHH

N C

- A DNA sequence encoding the mature human Glypican 3 (GPC 3) (Gln 25 - His 559) (Shen, T. *et al.*, 1997, Mamm. Genome **8**(1):72) was fused to the signal peptide of human CD33 and the N-terminus and to a six histidine tag at the C-terminus. The protein was expressed in a mouse myeloma cell line, NS0.

Molecular Mass

- Glypican 3 is subject to endoproteolytic processing by proprotein convertases (PC). By amino acid sequencing, three peptides (the first with a blocked N-terminus most likely starts with Gln 25, the second peptide starts with Ser 359 after a furin cleavage site, and the third peptide starts with Val 483) are present in the recombinant GPC 3 preparation. Peptides 2 and 3 are detected at a 1:1 ratio. All three peptides remained associated via disulfide bonds. Under non-reducing conditions in SDS-PAGE, the glycanated GPC 3 appears as a smear with an apparent molecular mass of 60 - 100 kDa.

Purity

- > 97%, as determined by SDS-PAGE and visualized by silver stain.

Endotoxin Level

- < 1.0 EU per 1 μg of the cytokine as determined by the LAL method.

Activity

- Measured by its ability to bind FGF-basic in a functional ELISA.
- Immobilized rhGPC-3 at 5 μg/mL (100 μL/well) will bind rhFGF-basic with a linear range of 0.16 - 10 ng/mL in a functional ELISA.

Formulation

- Lyophilized from a 0.2 μm filtered solution in PBS containing 50 μg of bovine serum albumin per 1 μg of cytokine.

Reconstitution

- It is recommended that sterile PBS containing at least 0.1% human serum albumin or bovine serum albumin be added to the vial to prepare a stock solution of no less than 10 μg/mL.

Storage

- Lyophilized samples are stable for up to twelve months from date of receipt at -20° C to -70° C.
- Upon reconstitution, this cytokine, in the presence of a carrier protein, can be stored under sterile conditions at 2° - 8° C for one month or at -20° C to -70° C **in a manual defrost freezer** for three months without detectable loss of activity.
- **Avoid repeated freeze-thaw cycles.**

Human Glypican 3

Glypicans (GPC) are a family of heparan sulfate proteoglycans that are attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor. Six members of this family have been identified in mammals (GPC1-GPC6). All glypican core proteins contain an N-terminal signal peptide, a large globular cysteine-rich domain (CRD) with 14 invariant cysteine residues, a stalk-like region containing the heparan sulfate attachment sites, and a C-terminal GPI attachment site. While glypican proteins do not share strong amino acid sequence identity (they range from 17 - 63%), the conserved cysteine residues in their CRDs suggests similarity in their three-dimensional structure. [1-2]

Mutations in GPC3 cause a rare disorder in humans, Simpson-Golabi-Behmel Syndrome, which is characterized by pre and postnatal overgrowth of multiple tissues and organs and an increased risk for developing embryonic tumors.[3] These features are also present in the mouse knock-out of GPC3 indicating that GPC3 regulates cell survival and inhibits cell proliferation during development.[4] Glypican 3 has been implicated in regulating many different signaling pathways including: IGF, FGF, BMP and Wnt. An endoproteolytic processing of GPC3 by proprotein convertases is required for the modulation of Wnt signaling.[5] Direct interaction with FGF-basic has been observed and is mediated by the heparan sulfate chains.[6]

References:
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R&D Systems, Inc.
1-800-343-7475

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CURRICULUM VITA

Name	Theerawut Chanmee
Sex	Male
Nationality	Thai
Marital status	Single
Date of birth	29 June, 1982
Birth place	Prachaubkhirikhan
Home address	136/1 Moo5, Tambol Tongmongkol, Bangsaphan district, Prachaubkhirikhan, 77230, Thailand

Education

1995-2000	High school at Bangsaphanvittaya, Prachaubkhirikhan, Thailand
2001-2004	B.Sc. (Medical Technology), Faculty of Associated Medical science, Chiang Mai University, Chiang Mai, Thailand.