Overexpression of Glycosylphosphatidylinositol (GPI) Transamidase Subunits Phosphatidylinositol Glycan Class T and/or GPI Anchor Attachment 1 Induces Tumorigenesis and Contributes to Invasion in Human Breast Cancer

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Abstract

Based on the oncogenic role of phosphatidylinositol glycan (PIG) class U in human tumors, we explored the role of two additional subunits of the glycosylphosphatidylinositol (GPI) transamidase complex in human breast cancer. We found that PIG class T (PIG-T) and GPI anchor attachment 1 (GPAA1) were overexpressed in breast cancer cell lines and primary tumors. Forced expression of PIG-T and GPAA1 transformed NIH3T3 cells *in vitro* and increased tumorigenicity and invasion of these cells *in vivo*. Suppression of PIG-T expression in breast cancer cell lines led to inhibition of anchorage-independent growth. Moreover, we found that PIG-T and GPAA1 expression levels positively correlated with paxillin phosphorylation in invasive breast cancer cell lines. Furthermore, suppression of PIG-T and GPAA1 expression led to a decrease in paxillin phosphorylation with a concomitant decrease in invasion ability. These results suggest that the GPI transamidase complex is composed of a group of proto-oncogenes that individually or as a group contribute to breast cancer growth. This aberrant growth is mediated, at least partially, by phosphorylation of paxillin, contributing to invasion and progression of breast cancer. (Cancer Res 2006; 66(20): 9829-36)

Introduction

Cancer is a disease caused by accumulation of multiple genetic and epigenetic alterations in two main categories of genes, tumor suppressor genes and oncogenes (1). Chromosomal translocation and gene mutations, which will lead to inactivation of tumor suppressor genes and activation of oncogenes, are common genetic alterations. Another common genetic alteration in cancer is manifested by genomic amplification and/or DNA copy number gain, leading to the overexpression of oncogenes (2–4). Examples include ERBB2 at the 17q12 amplicon (5), c-MYC at the 8q24 amplicon (6), CCND1 at the 11q13 amplicon (7, 8), ZNF217 and CYP24 at the 20q13.2 amplicon (9–11), and PPM1D and TBX2 at the 17q23 amplicon (12–15). Among these amplicons, 8q24, 20q11-13, and 17q23 are frequently altered in breast cancer, the most common cause of cancer death in women in the Western world (16, 17).

We recently found that phosphatidylinositol glycan (PIG) class U (PIG-U), a component of the glycosylphosphatidylinositol (GPI) transamidase complex located on chromosomal band 20q11, is amplified and overexpressed in bladder cancer cell lines and primary tumors and causes malignant transformation *in vitro* and *in vivo* (18). The GPI anchor is one of many anchors that mediate many membranous enzymes, receptors, differentiation antigens, and other biologically active proteins bound to the plasma membrane in unicellular and higher eukaryotes. It is essential for the expression of those proteins on the cell surface. In eukaryotic cells, both biosynthesis of GPI precursors and post-translational protein modifications with GPI proceed in the endoplasmic reticulum (ER). The COOH-terminal GPI attachment signal peptide is split from the protein, and the resulting new COOH terminus is then combined to GPI precursors. These processes are all mediated by the GPI transamidase complex. At present, there are five subunits identified in the GPI transamidase complex [PIG class T (PIG-T), GPI anchor attachment 1 (GPAA1), GPI8, PIG class S (PIG-S), and PIG-U; refs. 19–21]. Although PIG-U is an oncogene in human bladder cancer, the function of the other GPI transamidase subunits in human cancer has not been clarified. We found that, like PIG-U, both GPAA1 and PIG-T are located on chromosomal regions that harbor gain of copy number in breast cancer. GPAA1 is located on chromosomal region 8q24, and PIG-T is located on chromosomal region 20q13.12 (22). We thus hypothesized that individual subunits of the GPI transamidase complex might function either separately or as a functional group of oncogenes in breast cancer.

In the current study, we found overexpression and gain of copy number of PIG-T, GPAA1, and PIG-U in human breast cancer cell lines and primary tumors. We also cloned *PIG-T* and *GPAA1* and analyzed their tumorigenicity *in vitro* and *in vivo*. In addition, we discovered that overexpression of GPI transamidase subunits could induce tumor invasion possibly through activated paxillin.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Human breast cancer cell lines and tumors. The breast cancer cell lines examined, MDA-MB157, MDA-MB231, MDA-MB468, MCF7, and BT20, were kindly provided by Drs. Nancy E. Davidson and Scott E. Kern (Johns Hopkins University School of Medicine, Baltimore, MD) and Dr. Fergus J. Couch (Mayo Clinic, Rochester, MN), whereas others were obtained from the American Type Culture Collection (Manassas, VA). A total of 59 primary infiltrating ductal adenocarcinomas and 10 paired normal and tumor tissues were obtained from patients undergoing surgical treatment for breast cancer at Johns Hopkins. Institutional Review Board approval was obtained for this research (04-09-21-02e). Each tumor contained >70% tumor cells by H&E staining and was of high grade (Elston). Genomic DNA was prepared using the proteinase K method followed by extraction with phenol-chloroform and precipitated with ethanol. Total RNA was extracted using Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Quantitative real-time PCR. Specific primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA) to amplify *PIG-T, PIG-U*, and *GPAA1* genes and control β-actin (see Supplementary Data 1). The samples were run in triplicate using protocol described by Mambo et al. (23). Primers and probes to β-actin were run in parallel to standardize the input DNA. Standard curves were developed using serial dilutions of DNA extracted from MCF12A cells. Real-time PCR was run on the ABI 7900 Taqman (Applied Biosystems) according to the manufacturer's protocol. For measuring the expression level, we did the reverse transcription reactions with a cDNA synthesis kit (Invitrogen), and 1 μL of the total 20 μL was used for quantitative PCR with fluorogenic Taqman probes. The relative expression level was calculated as $2^{-(\text{cycle number of tested gene} - \text{cycle number of } \beta\text{-actin})}$.

Northern blotting. Total RNA (10 μg) was loaded per lane and transferred to a GeneScreen membrane (Schleicher and Schuell BioScience, Inc., Keene, NH). Normal epithelial cells, MCF10A, were used as control. The membranes were prehybridized in ExpressHyb hybridization buffer (Clontech Laboratories, Palo Alto, CA) at 60°C for 30 minutes, hybridized in 62°C for 2 hours, washed at 60°C, and exposed overnight.

Fluorescence *in situ* hybridization. Bronchioloalveolar carcinoma (BAC) clones RP11-714N16 and RP11-826A16 for GPAA1 and PIG-T were obtained from Research Genetics (Invitrogen), and the P1-3916 genomic clone for PIG-U was obtained from the National Center for Human Genome Research (Bethesda, MD). Both P1 and BAC clones were cultured as recommended, and the DNA was prepared according to standard protocols. For fluorescence *in situ* hybridization (FISH) analysis, the P1 and BAC probes were labeled by nick translation with SpectrumOrange-dUTP (Vysis, Inc., Downers Grove, IL). SpectrumGreen-dUTP-labeled chromosome 20 and chromosome 8 α-satellite probes were used as reference probes. Slide preparation and probe hybridization were according to standard FISH protocols (24). The results were evaluated under a Zeiss Axiophot microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Construction of the expression vectors and establishment of stable clones. Full-length *PIG-T* and *GPAA1* open reading frames were amplified by PCR with primers containing the 1× hemagglutinin (HA) sequence at the 3′-end and cloned into the pIRES-EGFP expression vector (Invitrogen). NIH3T3 cells were transfected with 1 μg of empty pIRES-EGFP vector or *PIG-T* and *GPAA1* expression vectors DNA using Fugene 6 (Roche Molecular Diagnostics, Branchburg, NJ) in six-well plates according to standard transfection protocols. Stable expression clones were selected with G418 (1 mg/mL) for 3 weeks. Selected clones were screened by Western blotting using the anti-HA monoclonal antibody (Sigma, St. Louis, MO).

Soft agar assay. Cells (5×10^3) from each stable clone or mock (empty vector) clones and polyclonal population cells were placed in 1 mL of 0.3% low-melting agarose in 10% fetal bovine serum (FBS)/DMEM and then overlaid onto 1 mL of 0.5% agarose/10% FBS/DMEM in each well of a six-well plate. The medium was changed every 3 days, and clones were grown for 2 weeks. Colony formation (2-3 mm) was counted and repeated in triplicate. Statistical analysis was done using the t test.

Tumor xenografts. Cells (5×10^6) were injected s.c. into athymic nude mice (CD-1-nuBR from Charles River, Wilmington, MA), and animals were killed 36 days after injection. Tumor size was measured each week, and tumor volume (V) was estimated from length (L) and width (W) using the following formula: $V = (\pi / 6) \times [(L + W) / 2]^3$. Animal experiments were conducted under protocol approved by the Central Animal Facility at the Johns Hopkins Medical Institute.

RNA interference constructs. RNA interference (RNAi) constructs were designed using the GeneSuppressor System. We designed two RNAi constructs for *GPAA1* and *PIG-U* and three RNAi constructs each for PIG-T (see Supplementary Data 2). For the negative control plasmid, a scrambled sequence is provided in the GeneSuppressor manual from Imgenex (San Diego, CA; Supplementary Data 2). Forward and reverse oligonucleotides (60 bp) were synthesized at Invitrogen. The oligonucleotides were then annealed and cloned into an IMG-8 (pSuppressorNeo) vector at the *Xho*I and *Bam*H sites. Correct constructs were picked after restriction digestion and subsequent sequencing. To check the effect of different RNAi constructs, we transfected 1 μg of different gene RNAi constructs or scrambled control RNAi construct into six-well dish plated with breast cancer cells. Expression was checked by Western blot analysis. Double-stranded small interfering RNA (siRNA) targeting human paxillin, control nontargeting siRNA1, and transfection reagent DharmaFECT 1 were purchased from Dharmacon (Lafayette, CO). Sequence targeted by siRNA was selected using Dharmacon Smartdesign: paxillin, GTGTGGAGCCTTC-TTTGGT.

Western blot. This was done according to standard protocols. The antibodies used included the following: paxillin (1:400; Cell Signaling, Danvers, MA), phosphorylated paxillin (1:400; Cell Signaling), matrix metalloproteinase 2 (MMP2; 1:400; Lab Vision, Fremont, CA), integrin-β_1 (1:500), E-cadherin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), fusin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), PIG-T and GPAA1 antiserum (1:400), Brk (1:400; Santa Cruz Biotechnology), and Flag M2 antibody (1:500; Sigma). The horseradish peroxidase–conjugated secondary antibodies (anti-mouse or anti-rabbit IgG; Amersham, Piscataway, NJ) were used at a 1:1,000 dilution.

Cell invasion assay. Cell invasion was done using a Matrigel invasion chamber according to the manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ). Breast cancer cells (1×10^5) were seeded in each well of a 24-well plate. The cells from a mixed culture of clones with different RNAi transfections were selected with G418 (1,000 μg/mL) for 20 days. For the paxillin experiment, cells were collected 96 hours after siRNA was transfected into the cells. Three independent experiments were done for each cell type.

Statistics. Statistical analysis was done using SigmaStat software. Student's t test was used to compare the difference in two groups, and the rank sum test was used when appropriate.

Results

Three GPI transamidase subunits are overexpressed in both human breast cancer cell lines and tumors. We first examined the expression pattern of the three GPI transamidase subunits, *PIG-T, GPAA1,* and *PIG-U,* in eight breast cancer cell lines by Northern hybridization. As shown in Fig. 1*A*, PIG-T was found to be overexpressed in six of eight (75%) cell lines. The highest level was seen in the BT20, MDA468, and MDA231 cells. *PIG-U* was found to be overexpressed in four of eight (50%) cell lines with the highest expression in MCF7, BT20, Hs.578t, and T47D cells. In addition, *GPAA1* was found to be overexpressed in two of eight (25%) breast cancer cell lines, MDA157 and MDA436. To confirm MCF10A cells as an appropriate control, we compared the expression levels of PIG-U, PIG-T, and GPAA1 in MCF10A, MCF12A, HMEC, and a normal breast tissue. We found the expression level of all three genes was almost identical in MCF10A and MCF12A but slightly less in HMEC and normal breast tissue (Supplementary Data 3).

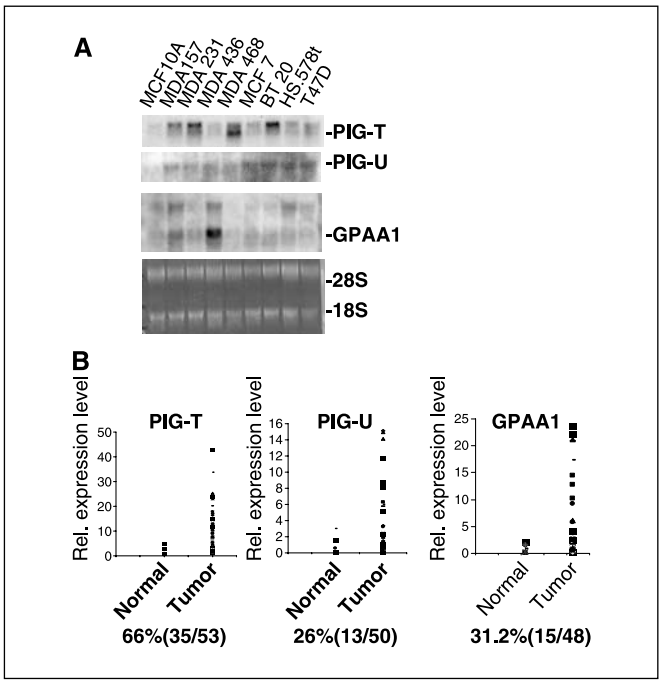

Figure 1. Overexpression of three GPI transamidase subunits in breast cancer. *A,* Northern blotting confirms overexpression of the three GPI transamidase subunits (>2-fold) in breast cancer cells. 28S and 18S RNA are loading controls. PIG-T was found to be overexpressed in six of eight (75%) cell lines. PIG-U was found to be overexpressed in four of eight (50 %) cell lines. GPAA1 was found to be overexpressed in two of eight (25%) breast cancer cell lines. *B,* overexpression of GPI transamidase subunits in 53 cases of primary breast cancer. *Numbers below graph,* number and percentage of cases where expression level is higher in tumor compared with normal tissue.

In addition, we investigated the expression pattern of these three GPI transamidase subunits in tissue from 53 cases of primary breast cancer, 4 organoids, and 4 cases of reduction mammoplasty (the latter two serving as a source of normal tissue) using quantitative real-time PCR. As can be seen in Fig. 1*B*, *PIG-T, PIG-U,* and *GPAA1* expression level is above the highest level of normal controls in 66%, 26%, and 31.2% of tumor samples, respectively, with the average expression level of PIG-T and PIG-U statistically higher than the normal control ($P < 0.01$).

Low-level amplification or gain of copy number of PIG-T, GPAA1, and PIG-U in breast cancer cells and tumors. To explore the relationship between gene copy number and overexpression in breast cancer, we did FISH with BAC RP11-826A16 (*PIG-T*), BAC RP11-714N16 (*GPAA1*), and P1-3916 (*PIG-U*) probes to determine gene copy number in eight breast cancer cell lines. As shown in Fig. 2*A* and Table 1, almost all the cell lines were hyperploid for chromosome 20 or 8 as determined by the chromosomal satellite probes, whereas normal control lymphocytes were confirmed to be diploid. We observed that three of eight (37.5%), four of eight (50%), and five of eight (62.5%) cell lines harbored more than four copies of *PIG-U, PIG-T,* and *GPAA1,* respectively. Similar copy number results were also obtained using quantitative real-time PCR (data not shown). In addition, we found excellent correlation between gene copy number and the expression level for each gene. For example, for *PIG-T,* the highest level of overexpression was seen in the BT20, MDA468, and MDA231 cells that also harbored the highest genomic copy number. Similar correlations were also found for *PIG-U* and *GPAA1* (Table 1).

To determine the gene copy number of *PIG-T, GPAA1,* and *PIG-U* in sporadic breast tumors, we used quantitative real-time PCR to examine copy number in 69 cases of primary breast cancer, including 10 cases with matched normal epithelial breast tissue. We found that, in the paired cases, for all three genes, the tumors generally showed a higher copy number than the normal controls (Fig. 2*B*). For all 69 cases of primary breast cancer, we found that, for PIG-T, 9 of 69 (13%) tumor cases had more than five copies of the gene. For *PIG-U,* 29 of 69 (42%) tumor cases harbored more than five copies, whereas for *GPAA1,* 8 of 69 (11.6%) tumor cases showed more than five copies (Fig. 2*C*). Furthermore, we found that coamplification of all three genes in these tumors was rare (1 of 69). Only four tumors exhibited coamplification of *PIG-U* and *GPAA1,* three cases of tumors harbored *PIG-U* and *PIG-T* coamplification, and one case showed *PIG-T* and *GPAA1* coamplification (Supplementary Data 4). Taken together, these results show that gain of copy number or low-level amplification of these three subunits is a frequent genetic event, whereas coamplification of any of these genes is rare in human breast cancer.

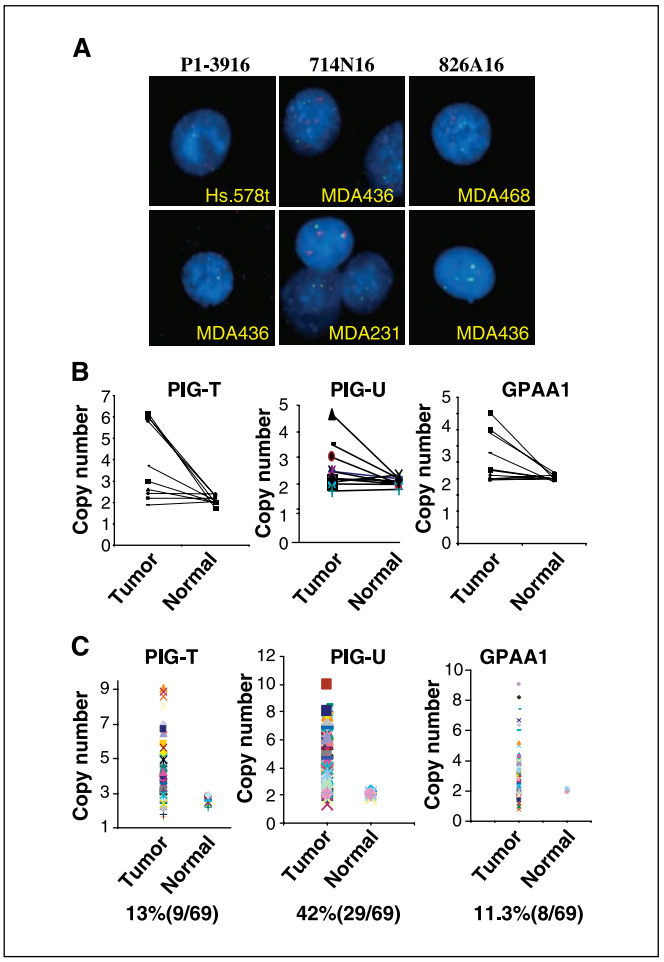

Figure 2. Gain of copy number of the GPI transamidase subunits PIG-T, PIG-U, and GPAA1 in breast cancer. *A,* representative images of FISH analyses. The α-satellite centromere probe (*green*) and BAC probes for the specific genes (*red*) were hybridized. *Top,* amplification and/or gain of gene copy number in selected breast cancer cell lines; *bottom,* no gain of gene copy. *B,* gain of three GPI transamidase subunits copy number in 10 cases of paired normal and tumor breast tissues. *C,* gain of copy number of three GPI transamidase subunits in 69 cases of primary breast tumor compared with 10 normal breast tissues. The cutoff for gain of copy number is more than five copies. *Numbers below graph,* percentage of cases containing gain of copy number.

PIG-T and GPAA1 contribute to cell transformation *in vitro* and induce tumorigenicity *in vivo*. We have previously reported that PIG-U can transform cells *in vitro* and induce tumorigenicity *in vivo*. To ascertain whether PIG-T and GPAA1 possess oncogenic potential, the full-length cDNA of both *PIG-T* and *GPAA1* was cloned into the pIRES-EGFP vector and expressed in NIH3T3 cells. Western blotting and immunofluorescence assays were done to confirm expression from the constructs and to confirm their localization in the ER (Supplementary Data 5A and B). We then established NIH3T3 clones that stably expressed either the gene or the empty vector. Clones from each gene were verified by Western blotting and immunofluorescence for expression; PIG-T clone 5 and GPAA1 clone 6 showed higher gene expression than the other clones tested (Supplementary Data 5C and D). Three independent clones for each transfected gene and one vector control clone were sequentially analyzed for growth rate in culture and for anchor-independent growth capacity. No significant differences in cell growth rate were seen in a 5-day period (data not shown). We then tested the same three clones and polyclonal populations (to rule out clonal variability) for both *PIG-T* and *GPAA1* genes in soft agar assays and found a significant increase in clone numbers compared with the controls ($P < 0.001$). For example, the PIG-T clone 5 showed a 10-fold increase in cloning efficiency compared with the mock controls (Fig. 3*A*; Supplementary Data 6). These data suggest that overexpression of the GPI transamidase subunits contributes to cell transformation manifested by an increase in cell anchorage-independent growth.

We next used RNAi to abrogate expression of *PIG-T* in breast cancer cells. The GeneSuppressor System constructs along with the negative control constructs were transiently transfected into breast cancer cells BT20. We identified a significant decrease in the expression level of *PIG-T* in two of three RNAi constructs transfected into BT20 cells (Fig. 3*B*). We then plated the same number of BT20 in a soft agar assay after transfection of *PIG-T* and control RNAi constructs. We found that the clone number was markedly decreased in cells where the expression level was inhibited by the RNAi constructs (Fig. 3*B*). Similar results were observed for *PIG-U* in MCF7 cells (Supplementary Data 7). This observation further indicates that GPI transamidase subunits contribute to anchorage independence in breast cancer cells.

To determine whether overexpression of PIG-T and GPAA1 can induce tumorigenicity *in vivo*, we injected a total of 5×10^6 cells from each stable clone into both flanks of nude mice. For each clone, five injections were done on three mice. Tumors were observed for all four clones overexpressing PIG-T and GPAA1 but not for the mock clones and parental cells. We observed a good correlation between latency, growth speed, and size of the tumors with the expression level of the genes tested. The clones with highest expression, PIG-T clone 5 and GPAA1 clone 6, showed a shorter latency, faster growth, and larger tumors than other clones (Fig. 3*C* and *D*).

Overexpression of GPI transamidase subunits can contribute to tumor invasion through phosphorylated paxillin. To further evaluate the biological effect of overexpression of GPI transamidase subunits in tumor progression, we first examined tissue sections of lung, lymph nodes, and tumor from the nude mice with tumors. We observed a marked increase in tumor invasion in lung, the adjacent lipid tissue and lymph nodes in nude mice with the highest overexpressing clones (clone 5 of PIG-T, clone 6 of GPAA1, and clone 1 of PIG-U). Representative images are shown in Fig. 4. These data indicate that overexpression of the GPI transamidase subunits not only promotes tumor initiation but might also contribute to tumor invasion and metastasis.

To better understand the molecular mechanism underlying the role of GPI transamidase subunits in tumor initiation and invasion, we tested the endogenous expression pattern of several well-known cell invasion–related proteins, including MMP2, paxillin, fusin, integrin-β, E-cadherin, and Brk, in two invasive cell lines, MDA231 (which overexpresses PIG-T) and MDA436 (which overexpresses GPAA1). PIG-U is not overexpressed in these invasive cells. We found that the expression level of phosphorylated paxillin, E-cadherin, integrin-β_1, but not fusin, MMP2, and Brk, correlated with the protein expression status of both PIG-T and GPAA1 in these two breast cancer cells (Fig. 5*A*). We then did immunofluorescence analysis to determine whether PIG-T and GPAA1 colocalize with paxillin in breast cancer cells. As shown in Supplementary Data 8A, PIG-T and paxillin cytoplasmic expression strongly overlapped in MDA231 cells, whereas cytoplasmic expression of GPAA1 and paxillin strongly overlapped in MDA436 cells. A physical interaction between PIG-T or GPAA1 and paxillin was confirmed by immunoprecipitation (Supplementary Data 8B).

Table 1. Copy number and expression pattern of three GPI transamidase subunits in breast cancer cell lines

Cell lines	PIG-U			PIG-T			GPAA1		
	Satellite	P1-3916	Expression	Satellite	826A16	Expression	Satellite	714N16	Expression
MDA157	3	ND	−	3	4-5	+	2-4	3-6	+
MDA231	2	4	−	2	4-5	+	2	2-4	−
MDA436	2-4	2-4	−	2	2	−	2-4	4-8	+
MDA468	3-5	3-5	−	4	5-6	+	3	3-5	−
MCF7	3-4	4	+	3-4	4	−	3-4	4	−
BT20	3	5	+	3	5	+	3-4	3-4	−
Hs.578t	2-3	4-6	+	2	3-4	−	3	4-5	−
T47D	4	5	+	4	4	−	4	4-5	−
Lymphocytes	2	2	ND	2	2	ND	2	2	ND

Abbreviations: ND, not determined; −, no expression; +, overexpression.

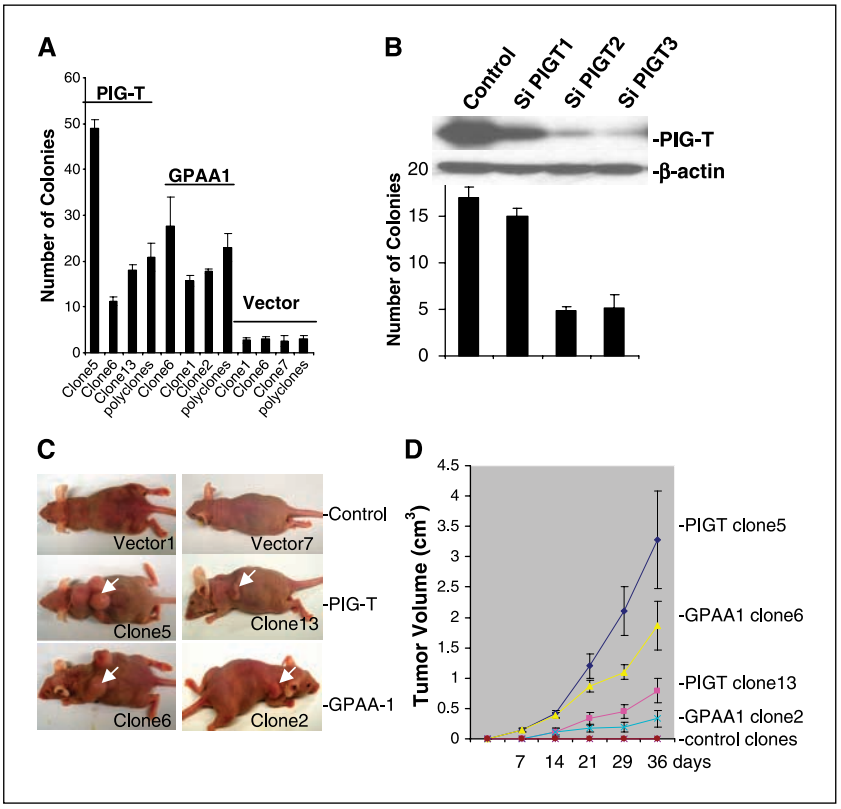

Figure 3. PIG-T and GPAA1 increase tumorigenicity *in vitro* and *in vivo*. *A,* soft agar assay comparing numbers of colonies among different cell clones. *Numbers,* average of total colonies (>3 mm in diameter) in 10 fields at ×200 magnification. *Columns,* mean of three independent experiments; *bars,* SD. Statistical analysis was done by the *t* test. $P < 0.001$. *B,* RNAi decreases expression of PIG-T in BT20. Expression levels correlated with a decrease in colony formation activity. *Top,* protein levels by Western blotting; *bottom,* colony formation assay. Colonies were counted as described in (*A*). *Columns,* mean of three independent experiments; *bars,* SD. *C* and *D,* tumorigenesis in nude mice injected with NIH3T3 cell clones stably expressing PIG-T and GPAA1. No tumors grew in mice implanted with vector-only controls. *D,* tumor size was measured every week and is on a log scale. *Points,* mean weights of five tumors per clone; *bars,* SD.

A specific PIG-T and GPAA1 band was seen only after precipitation of protein lysates with paxillin antibody but not with control anti-Flag antibody.

To further explore the biological significance of these correlations, we next did invasion studies using breast cancer cells. Initially, using the BD Matrigel invasion chamber, we confirmed a more invasive phenotype for MDA231 and MDA436 cells compared with MDA157 and MCF7 cells, which is identical to previous reports (Supplementary Data 9; ref. 25). We next used RNAi to suppress the expression of PIG-T and GPAA1 in the breast cancer cells. We observed a decrease in the level of phosphorylated paxillin along with a decrease in expression levels of PIG-T and GPAA1 in the breast cancer cell lines MDA231 and MDA436 (Fig. 5*B*). In addition, we did not observe marked expression level changes of other tested proteins, including paxillin, MMP2, Brk, integrin-β_1, and fusin (E-cadherin, MMP2, and integrin-β_1 remained undetectable before and after siRNA treatment). We next did an invasion study with these breast cancer cells. We found that the decrease in PIG-T expression by siRNA treatment led to a 32.3% decrease of the invasion ability in MDA231 cells ($P = 0.003$). Likewise, decreased GPAA1 expression resulted in a 29.3% decrease of the invasion ability in MDA436 cells ($P = 0.016$; Fig. 5*C*). To further test whether paxillin is the mediator of GPI transamidase role in cell invasion, we use siRNA to knock down paxillin expression in MDA231 cells, which overexpress PIG-T. We observed that the expression levels of both phosphorylated paxillin and total paxillin were reduced 96 hours after transfection of paxillin siRNA (Fig. 5*D*). We further observed a significant decrease in invasion of MDA231 cells (79.6%; $P = 0.0001$) concomitant with the decrease in expression level of phosphorylated paxillin (Fig. 5*D*).

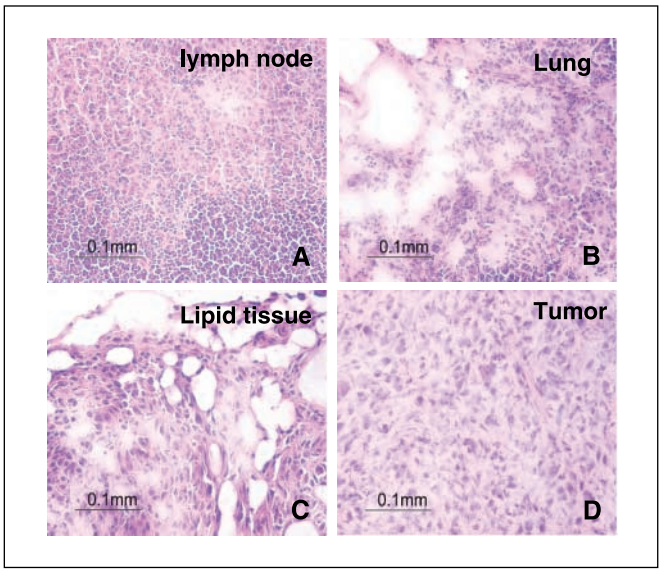

Figure 4. GPI transamidase subunit overexpression induces tumor invasion. H&E staining shows tumor invasion in lung, lipid tissue, and lymph nodes in the nude mice with tumor. The tissue type is described in the images. Magnification bars are in 0.1 mm increments.

Discussion

We have shown amplification and overexpression of three components of the GPI transamidase complex in primary breast cancer and cell lines. PIG-T, GPAA1, and PIG-U are now added to a group of oncogenes in breast cancer that includes HER-2, CCND2, and AKT2. The GPI transamidase complex contains five subunits: PIG-T, GPAA1, GPI8, PIG-S, and PIG-U. Each subunit of this

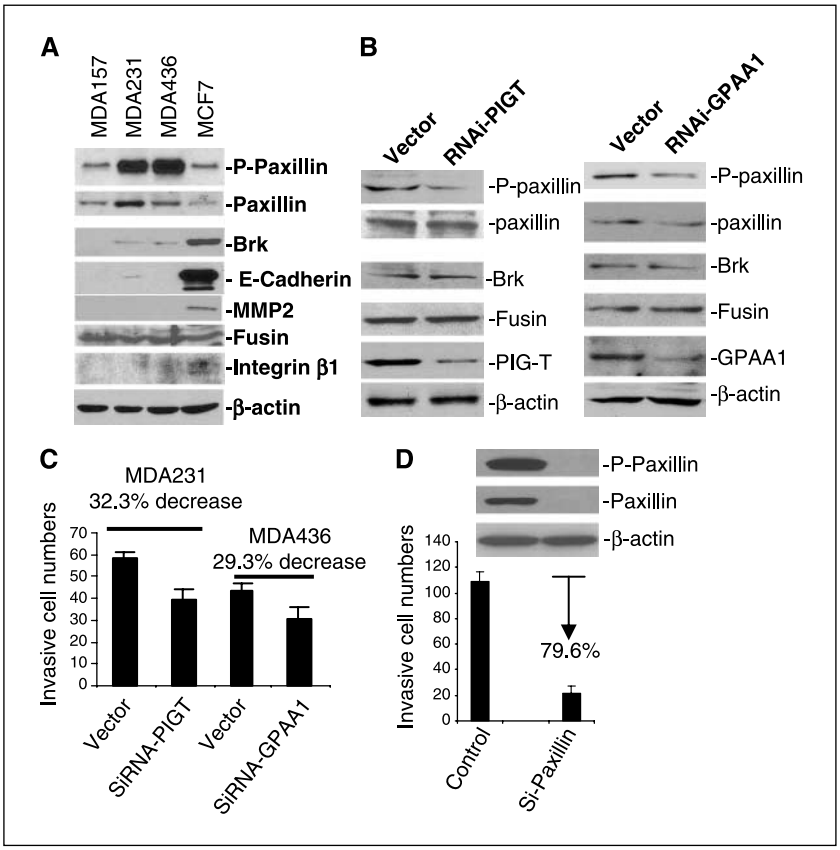

Figure 5. GPI transamidase subunits contribute to tumor invasion through phosphorylated paxillin. *A,* Western blot analysis shows the expression pattern of phosphorylated paxillin (*P-Paxillin*), paxillin, Brk, MMP2, E-cadherin, integrin-β, and fusin in PIG-T- and GPAA1-overexpressing cell lines MDA231 and MDA436. β-Actin was used as a protein loading control. *B,* effect of PIG-T (*left*) and GPAA1 (*right*) siRNA on protein expression. RNA knockdown of PIG-T or GPAA1 in cell lines MDA231 and MDA436 results in a reduction of phosphorylated paxillin. No marked changes were observed in expression of total paxillin, Brk, and fusin. Integrin-β_1, E-cadherin, and MMP2 remained undetectable before and after siRNA treatment. *C,* cell invasion assay using BD Matrigel invasion chambers shows that siRNA suppression of PIG-T and GPAA1 expression leads to a decrease in invasion ability of MDA231 (32.3%; $P = 0.003$) and MDA436 (29.3%; $P = 0.016$) cells. *Columns,* mean of three independent experiments; *bars,* SD. Statistical analysis was done using a *t* test. *D,* biological effect of suppression of paxillin in invasive breast cancer cell MDA231. *Top,* Western blotting shows that paxillin siRNA specifically knocked down the expression level of both total and phosphorylated paxillin. β-Actin was used as the protein loading control. *Bottom,* cell invasion assay using BD Matrigel invasion chambers shows that siRNA suppression of paxillin expression leads to a decrease in invasion ability of MDA231 cells (79.6%; $P = 0.0001$). *Columns,* mean of three independent experiments; *bars,* SD. Statistical analysis was done using a *t* test.

complex is critical for maintaining the complex and is essential for both cleavage of GPI anchoring protein and the attachment of the split protein to GPI precursors. As reported previously, the targets of GPI attachments are diverse and these proteins have different cellular functions, such as cell adhesion, signal transduction, nutrient uptake, and regulation of complement activity (26). Therefore, the overexpression of individual GPI transamidase subunits might lead to an increased number of specific GPI anchoring proteins attaching to the cell membrane, further activating downstream signaling pathways through adaptors, such as paxillin. It would be interesting to investigate whether the overexpression of a single transamidase subunit will influence the biological activity of the GPI transamidase complex. This kind of study will allow us to establish whether the oncogenic property of GPI transamidase subunits depends on their biological function in human cells.

In our current study, we found that coexpression and coamplification of all three GPI transamidase subunits in breast cancer cell lines and primary tumors is rare. Genetic alterations of different components in a known oncogenic pathway are not uncommon. For example, inactivation of adenomatous polyposis coli and axin-1 or point mutation activation of β-catenin is frequently seen in colorectal cancer (27–33). Epidermal growth factor receptor (EGFR) mutations and ras mutations inversely segregate in primary non–small cell lung cancers (NSCLC; ref. 34). Thus, our study extends these observations by showing that different components in a specific protein complex with similar biological functions can individually function as activating oncogenes in cancer.

In addition to breast and bladder cancers, we also observed overexpression and gene copy number changes of the GPI transamidase subunits in several different kinds of human cancers, including head and neck, ovarian, colon, and lung cancers (data not shown). One recent study also reported that *GPAA1* was one of the seven overexpressed genes in both hepatitis B virus–positive and hepatitis C virus–positive hepatocellular carcinoma (HCC; ref. 35). During the revision of this article, another group reported that increased expression of GPAA1 was associated with gene amplification in HCC and increased GPAA1 expression was also significantly associated with poor cellular differentiation and poor prognosis of HCC (36). These data, together with ours, strongly suggest that activation of the GPI transamidase complex is a molecular mechanism underlying the progression of different human cancers. It is thus worthy to investigate the amplification or overexpression pattern of GPI transamidase subunits in cancers with different pathologic characteristics and evaluate them as potential prognostic or diagnostic molecular markers in human cancer. In addition to the three subunits of the GPI transamidase complex studied here, there are two other subunits, PIG-S and GPI8, residing on chromosomal region 17p13.2 and 1p31.1, respectively, regions frequently harboring loss in various human cancers (22). Thus, further exploration of these targets and the biological effects of all GPI transamidase complex subunits will aid us in understanding the role of this complex in human cancer.

In addition to the ability of GPI transamidase subunits to transform cells, our data suggest that these subunits might also play a role in tumor invasion. We screened the expression pattern of several invasive related proteins, including MMP2, paxillin, fusin, integrin-β, E-cadherin, and Brk. We found that phosphorylated paxillin (and several other proteins) positively correlated with the expression level of both PIG-T and GPAA1. But suppression of

PIG-T and GPAA1 expression led to a marked decrease only in the expression level of phosphorylated paxillin (and not the other proteins) and a concomitant decrease in invasive ability of highly metastatic breast cancer cell lines. When we specifically knocked down paxillin in the invasive MDA231 cell line, which overexpresses PIG-T, we observed a significant decrease in the expression level of phosphorylated paxillin and a concomitant decrease of cell invasion ability. Moreover, we also found that both PIG-T and GPAA1 colocalize and interact with paxillin intracellularly in invasive breast cancer cell lines MDA231 and MDA436, respectively. Paxillin is a multidomain adaptor found at the interface between the plasma membrane and actin cytoskeleton, which provides a platform for the integration and processing of adhesion-related and growth factor–related signals (37–39). In response to various physiologic stimuli, paxillin is activated by Brk-mediated phosphorylation and in turn promotes cell migration and invasion and has also been linked to tumor metastasis (40–42). In our study, the effects of inhibition on MDA231 cell invasion for PIG-T and paxillin are different. This might be due to the efficiency of the different siRNAs. si-PIG-T only partially inhibited the expression of PIG-T resulting in partial inhibition of paxillin, whereas si-paxillin almost totally inhibits the expression of paxillin in MDA231 cells. However, we cannot exclude the possibility that there are other mechanisms contributing to the phosphorylation of paxillin in metastatic breast cancer cells. Taken together, our data suggest that paxillin is one of the molecules through which the GPI transamidase subunits mediate the invasion of breast cancer cells.

Isolation and identification of oncogenes, whose overexpression is driven by gain of copy number or gene amplification, has facilitated our understanding of cancer development and contributed to cancer therapy. For example, treatment of HER-2-positive breast cancers with specific monoclonal antibody, Herceptin, has been successful (43). Cetuximab (IMC-C225, Erbitux), a monoclonal antibody targeted to EGFR, has shown antitumor activity in a wide variety of tumor types, including colon cancer (44, 45). Gefitinib (ZD1839, Iressa) and erlotinib (Tarceva) are small molecular EGFR inhibitors that have been intensively studied in advanced NSCLC and colon cancer patients (46–49). Our current study not only implicates GPI transamidase subunits as novel oncogenes but also adds credence to the notion that the GPI anchoring process is a new and common pathway inducing tumor formation and contributing to tumor invasion. Further study is needed to clarify the feasibility of using GPI transamidase subunits as potential therapeutic targets in breast cancer.

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Overexpression of Glycosylphosphatidylinositol (GPI) Transamidase Subunits Phosphatidylinositol Glycan Class T and/or GPI Anchor Attachment 1 Induces Tumorigenesis and Contributes to Invasion in Human Breast Cancer

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