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Research Paper

Frequent genomic copy number gain and overexpression of GATA-6 in pancreatic carcinoma

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Multiple genetic alterations are well recognized as contributing to pancreatic carcinogenesis, although the finding of recurrent copy number changes indicates additional targets remain to be found. The objective of this study was to identify novel targets of genetic alteration that contribute to pancreatic cancer development or progression. We used Representational Oligonucleotide Microarray Analysis (ROMA) to identify copy number changes in pancreatic cancer xenografts, and validated these findings using FISH, quantitative PCR, Western blotting and immunohistochemical labeling. With this approach, we identified a 0.36-Mb amplification at 18q11.2 containing two known genes, *GATA-6* and *cTAGE1*. Using a cutoff value of 3.0 fold compared to haploid controls, copy number gain or amplification was confirmed in 4 of 42 (9.5%) pancreatic carcinomas analyzed. Combined genetic and transcriptional analyses showed consistent overexpression of GATA-6 in all carcinomas with 18q11.2 gain, as well as in the majority of pancreatic cancers examined (17 of 30 cancers, 56.7%) that did not have gain of this region. By contrast, overexpression of cTAGE1 was rare in these same cancers suggesting *GATA-6* is the true target of this copy number increase. GATA-6 mRNA overexpression corresponded to robust nuclear protein expression in cancer cell lines and resected tissues consistent with its role as a transcription factor. Intense nuclear labeling was significantly increased in PanIN-3 lesions and infiltrating carcinomas compared to normal duct epithelium ($p < 0.000001$ and $p < 0.003$, respectively). Forced overexpression of GATA6 in MiaPaca2 cells resulted in increased proliferation and growth in soft-agar. Gain and overexpression of the development-related transcription factor GATA-6 may play an important and hitherto unrecognized role in pancreatic carcinogenesis.

Introduction

Multiple genetic alterations are well recognized as contributing to pancreatic carcinogenesis.[1,2] These alterations are largely represented by inactivating mutations or deletions of critical tumor suppressor genes, activating mutations in oncogenes, or epigenetic modifications. In most instances, the alterations disrupt regulatory checkpoints in the cell cycle, the response to DNA damage or cellular stress, and signaling pathways related to growth and development. However, with the application of global analysis methods to human cancers, the finding of recurrent allelic losses or copy number gains of specific loci indicates additional genetic targets remain to be found.[3-7] For example, novel candidate oncogenes include *SMURF1* on 7q21, *FGFR1* on 8p12, *BIRC2* and *BIRC3* on 11q22 and *PAK4* on 19q13 while novel candidate tumor suppressor genes include *TUSC3* on 8p22 and *FEZ1* on 8p23.[4-8]

The objective of this study was to identify novel targets of genetic alteration that contribute to pancreatic cancer development or progression. Towards this goal, we used the high resolution method Representational Oligonucleotide Microarray Analysis (ROMA) to identify novel copy number changes of significance in pancreatic cancer.[9] A major advantage of ROMA is its high resolution (~4 Mb) compared to other copy number technologies due to its ability to reduce genomic complexity with a corresponding increase in hybridization efficiency and signal to noise ratio. With this approach, we identified an amplification not recognized by other copy number methods on chromosome 18q11.2 that contains only two known genes, *GATA-6* and *cTAGE1*, and have confirmed the target of this amplification is *GATA-6*.

Results

Identification of *GATA-6* as a target of amplification. Representational Oligonucleotide Microarray Analysis (ROMA) was used to characterize the copy number alterations in 16 low-passage pancreatic cancer xenografts from four patients.[9,10] Matched samples were used to account for the prominent genomic variability characteristic of human pancreatic cancers[16] and to better identify copy number alterations consistently present in all samples from the same patient. Despite the prominent genomic instability present within each xenograft, consistent deletions and amplifications were identified among two or more samples from each individual by ROMA (Suppl. Fig. 1). However, no copy number changes were identified that were common to any two or more patients' samples. The copy number alterations found are summarized in Supplemental Table 1.

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Review of the copy number changes identified by ROMA revealed many that were previously reported in human pancreatic cancer. These included a homozygous deletion of 18q21.1 that encompassed *SMAD4*, amplification of 8q24 that included *c-MYC,* and a homozygous deletion of 9p21 that included *p16/CDKN2A*.[6,17,18] However, a consistent 0.36 Mb amplification of 18q11.2 was found in all three xenografts derived from one patient representing the primary carcinoma (13A Xenograft, 13B Xenograft) and a matched lung metastasis (13D Xenograft). This amplification was predicted to span from 19.6 to 19.9 Mb on chromosome 18q (chr18: April 2003 assembly). Examination of the RefSeq database in the human genome assembly revealed only two genes, *GATA-6* and *cTAGE1/2*, were localized in this amplified region. Review of more recent genome assemblies indicate this region has been re-assigned to bps 17,945,134-18,307,805 on chromosome 18, although *GATA-6* and *cTAGE1* remain the only two genes assigned to this region.

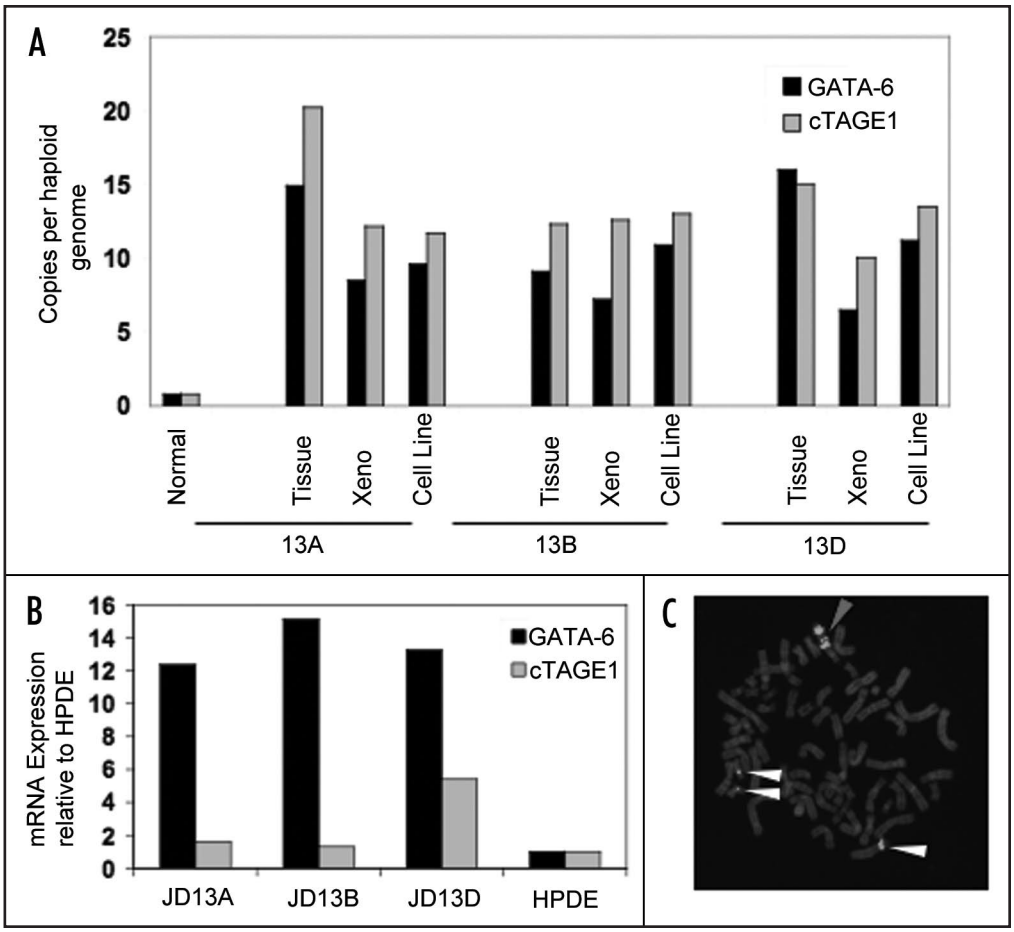

Figure 1. Expression of GATA-6 and cTAGE1 in cancer samples from patient A13. (A) Copy number analysis of the GATA-6 and cTAGE1, the only two genes within the 18q11.2 amplification in the neoplastic tissues, xenografts and cell lines of the primary carcinoma and matched lung metastasis from patient A13. All values in cancer and normal DNA samples were normalized to that of flanking genomic DNA from within 2–3 Mb upstream and downstream of the 18q11.2 amplicon determined from the same template DNA. Increased copy number for both genes is evident in both samples of the primary carcinoma (13A and 13B) and lung metastasis (13D). (B) Relative mRNA expression of GATA-6 and cTAGE1 in the immortalized normal cell line HPDE and in cell lines derived from the primary carcinoma (JD13A, JD13B) and lung metastasis (JD13D) of patient 13. All three cells derived from patient A13 show >12-fold overexpression of GATA-6 mRNA relative to that of HPDE. By contrast, cTAGE1 expression in the primary cancer cell lines is approximately the same as in HPDE, while up to 5-fold overexpression is seen in the metastatic cell line JD13D. (C) Validation of GATA-6 gene amplification in the cell line JD13A by fluorescent in situ hybridization (FISH) analysis using a probe corresponding to GATA-6 located within the minimal amplicon of 18q11.2. The grey arrow highlights the homogeneous staining region (HSR) whereas the white arrows indicate single copies of GATA-6.

Identification and validation of GATA-6 as a target of amplification. To validate this amplification we first performed quantitative real-time PCR for evaluation of the DNA copy number within the 0.36 Mb amplicon and normalized these values to flanking genomic regions on 18q (primer sequences available in Suppl. Table 2). Increased DNA copy number for both *GATA-6* and *cTAGE1* was confirmed in the three xenografts analyzed by ROMA, in microdissected samples of the matched original tumor tissues and in the low-passage cell lines generated from these xenograft tissues but not in the corresponding normal tissue from this patient (Fig. 1A). Consistently increased DNA copy number for both genes was also observed in all other primary and metastatic cancer tissues available from the same patient (data not shown). The predicted copy numbers were higher in both the microdissected tissues and cell lines than in the xenograft enriched tissues, most likely due to the presence of contaminating mouse DNA. However, the possibility of copy number loss with passaging ex vivo might have also contributed to the lower copy numbers in the cell lines JD13A and JD13D compared to their corresponding original tumor tissues.

To gain insight into the gene targeted by this amplification, we examined the mRNA levels of these two genes in parallel by quantitative real-time PCR in the three low passage cell lines. Consistent overexpression of GATA-6 mRNA was found in all three cell lines relative to the normal diploid cell line HPDE (Fig. 1B). By contrast, overexpression of cTAGE1 was detected in only one of these same three samples (JD13D) despite its co-amplification with GATA-6, and the level of overexpression was less than that for GATA-6 in this same cell line. This finding suggested to us that *GATA-6* is the potential gene targeted by this amplification. To confirm that *GATA-6* is amplified in this cancer we performed FISH using a bacterial artificial chromosome probe specific to GATA-6 (Fig. 1C). This probe revealed a homogeneously staining region, confirming amplification of the *GATA-6* gene in this cell line and consistent with the findings by ROMA and quantitative PCR.

Expression of GATA-6 and cTAGE1 in pancreatic cancer. A logical question was whether the amplification of 18q11.2 and

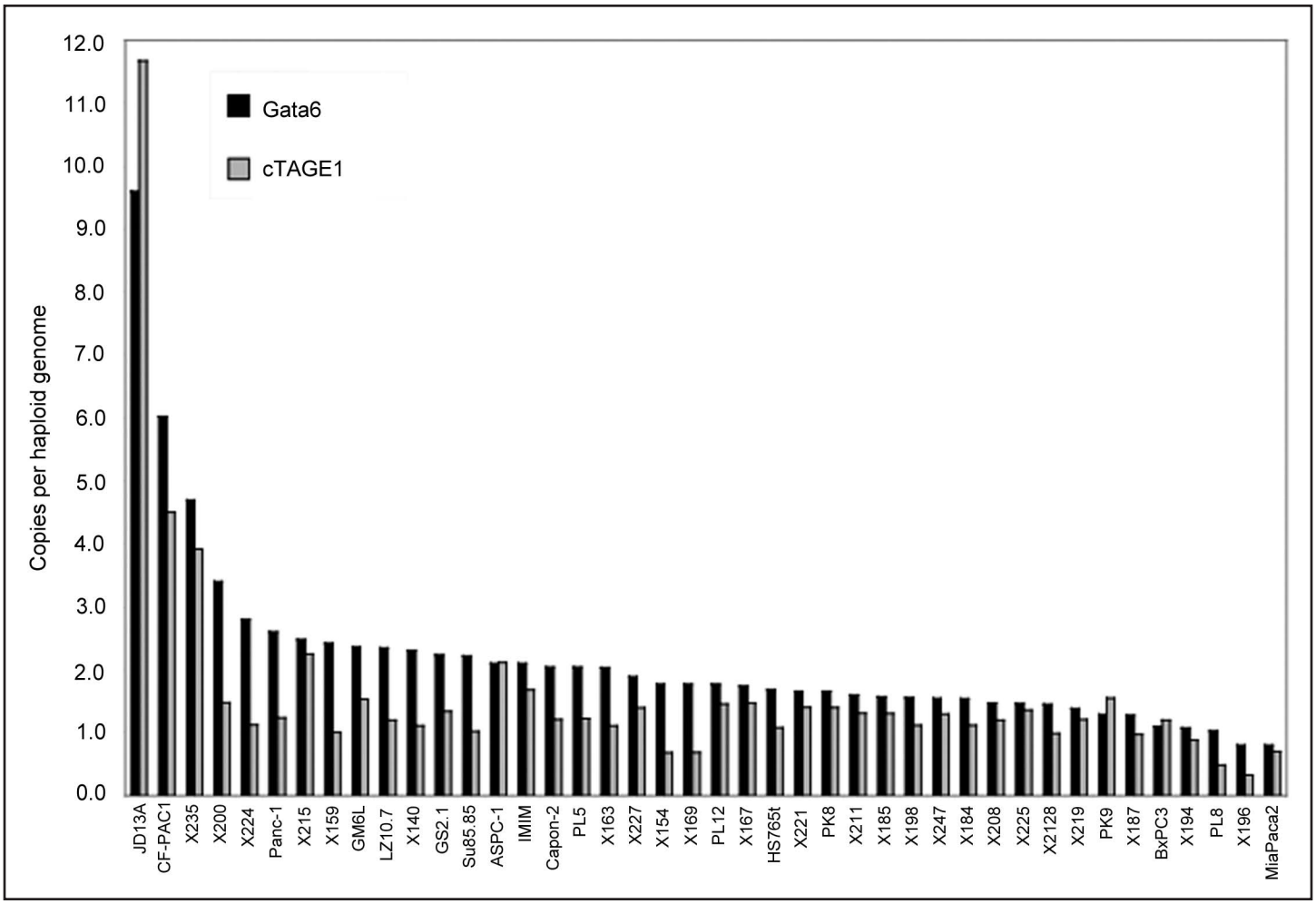

Figure 2. Frequency of copy number gain of GATA-6 and cTAGE1 in pancreatic cancer. Copy number values per haploid genome of GATA-6 and cTAGE1 in pancreatic cancer cell lines and first passage xenografts were quantified by real-time PCR. The relative cut-off value for copy number gain was set at 3.0. Using this criterion, 4 of 42 pancreatic cancers showed increased copy number of GATA-6. Three of these same cancers also contained copy number gain of cTAGE1.

overexpression of GATA-6 were specific for this one carcinoma or represents a more general feature of human pancreatic cancers. To determine the frequency of *GATA-6* copy number gain we screened 38 pancreatic cancers by quantitative PCR (Fig. 2). *GATA-6* copy number gain (defined as three or more copies per haploid genome) was found in an additional three pancreatic cancers at levels of ~6 copies (CFPAC1), ~5 copies (Xenograft 235) and ~3 copies (Xenograft 200) per haploid genome. When these findings were combined with data of the four cancers screened by ROMA, *GATA-6* gain was present in 4 of 42 (9.5%) pancreatic cancers, one of which corresponded to a true amplification. We also evaluated DNA copy numbers of *cTAGE1*, which indicated gain in three of these same 42 cancers (7%), all of which also contained *GATA-6* copy number gain (Fig. 2).

We next examined the mRNA expression of GATA-6 and cTAGE1 in 30 of these same pancreatic cancers using quantitative real-time PCR. Overexpression of GATA-6 mRNA (>5.0 fold compared to normal pancreatic duct cell line HPDE) was demonstrated in 17 of 30 (56.7%) pancreatic cancers. All three samples with copy number gain also showed mRNA overexpression of GATA-6 (Fig. 3). Moreover, in the 14 cancers with mRNA overexpression >5 fold but without genomic amplification, levels of overexpression often exceeded that of the samples with amplification. By contrast, overexpression of cTAGE1 was only found in 3 of these 30 cancers samples examined (10%), which was significantly lower than the frequency of GATA-6 overexpression ($p < 0.0001$). Surprisingly, two of the three cancers with cTAGE1 overexpression were those without genomic amplification and with the lowest levels of expression of GATA-6 (Xenograft 185 and cell line PL8). Taken together, these data lend further support to our hypothesis that *GATA-6*, and not *cTAGE1*, is the target of copy number gain or amplification within the 0.36 Mb region identified by ROMA. Given the finding of *GATA-6* genomic amplification in pancreatic cancer, we next sought to determine if *GATA-6* is additionally targeted by intragenic mutation. The entire coding region of GATA-6 was sequenced in 25 pancreatic cancers but no mutations were found.

Characterization of GATA-6 protein expression. To confirm that GATA-6 copy number gain and/or mRNA overexpression results in translation to protein, we performed Western blotting and immunohistochemical labeling using a GATA-6 specific antibody raised against an epitope common to both the long and short forms of human GATA-6. Shown in Figure 4A, both the long and

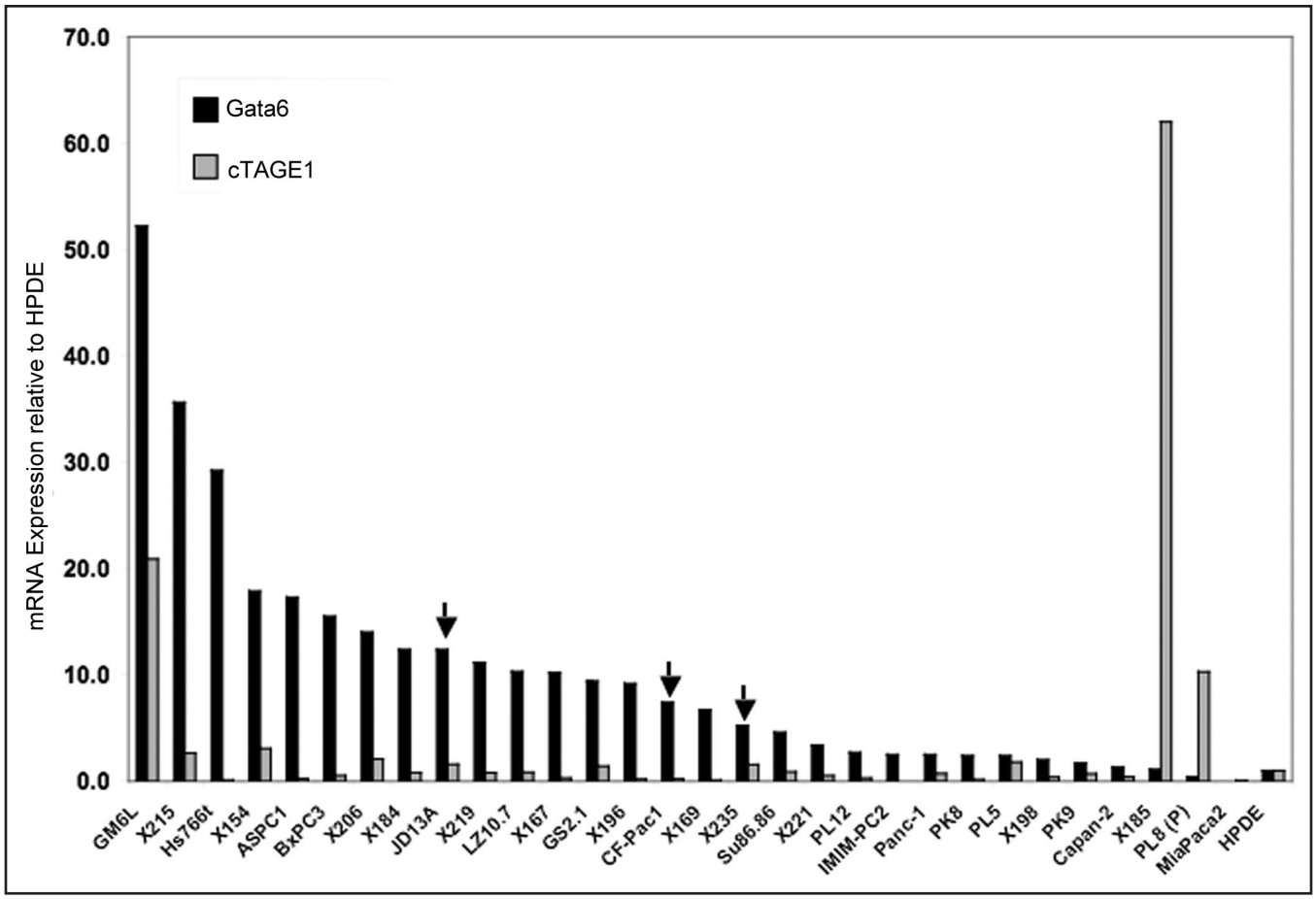

Figure 3. Expression of GATA-6 and cTAGE1 in pancreatic cancer. Relative mRNA expression of GATA-6 and cTAGE1 in pancreatic cancer cell lines and first passage xenografts using quantitative real-time PCR. All values are normalized to the immortalized normal cell line HPDE for which GATA-6 and cTAGE1 are both arbitrarily assigned a value of 1.0. Relative values of GATA-6 expression of 5.0 fold or more are seen in 17 of 30 pancreatic cancers analyzed, whereas only three of these same cancers showed cTAGE1 overexpression. Arrows indicate those cell lines with copy number gain at 18q11.2.

short forms of GATA-6 are detected in cancer cell lines, although increased expression of GATA-6 is generally associated with increased amounts of the long form of GATA-6 protein. Protein expression of GATA-6 was also highly correlated with mRNA expression levels (Fig. 4B). Immunolabeling for GATA-6 revealed that overexpression of GATA-6, whether by copy number gain, amplification or mRNA overexpression, corresponded to strong nuclear labeling in formalin-fixed cell pellets created from these same cell lines consistent with the known function of GATA-6 as a transcription factor (Fig. 4C).

Immunolabeling for GATA-6 protein expression in pancreatic cancer tissues. To determine the frequency of GATA-6 protein overexpression in pancreatic cancer tissues, we performed immunolabeling for GATA-6 protein in 14 normal ducts, 8 samples of pancreatic intraepithelial neoplasia-1a (PanIN-1a), 13 samples of PanIN-1b, 18 samples of PanIN-2, 13 samples of PanIN-3, and 193 samples of infiltrating pancreatic cancer from resection specimens (Table 1). Immunolabeling was evaluated by calculation of the Histology (H) score for each case with H = Intensity (scale 0–3 with three most positive) x% Positive Cells. GATA-6 labeling was detected in the majority of samples of normal duct epithelium, seen as scattered cells with positive nuclei (~30% of cells) (Fig. 5A). This is consistent with the known role of GATA-6 in differentiation of progenitor cells into ductal epithelium.[19] Among samples of PanIN1 or 2, GATA-6 labeling was also present with an equal frequency and distribution. However, while labeling of early stage PanINs was not significantly different from normal duct epithelium (or from each other), a striking difference emerged when comparing normal epithelium to samples of PanIN3 ($p < 0.000001$) (Fig. 5B) or infiltrating cancer ($p < 0.003$) (Fig. 5C and D). In these stages of disease, GATA-6 nuclear labeling was of greater intensity and was present in the majority of cells, often 80% or greater. No difference was found in comparing PanIN3 labeling to infiltrating carcinoma, suggesting the upregulation of GATA-6 occurs late in carcinogenesis but prior to the development of infiltrating carcinoma. Among the infiltrating cancers specifically, 133 of 193 cancers (69%) showed an H score ≥100, and 54 of these (28%) showed an H score ≥200, indicating GATA-6 is also overexpressed at a significant frequency in resection specimens.

To assess possible functional consequences of GATA-6 overexpression in pancreatic cancer, we stably expressed the long form of GATA-6 in MiaPaca2, a cell line in which GATA-6 expression was absent (Fig. 3), to generate MiaPaca2-hG6. Forced expression of GATA-6 in MiaPaca2 was verified by RT-PCR and Western blotting (Suppl. Fig. 2). MiaPaca2-hG6 cells exhibited a statistically significant increase in cell growth in culture ($p < 0.01$) and in soft agar ($p = 0.01$) compared with control transfected MiaPaca2 cells

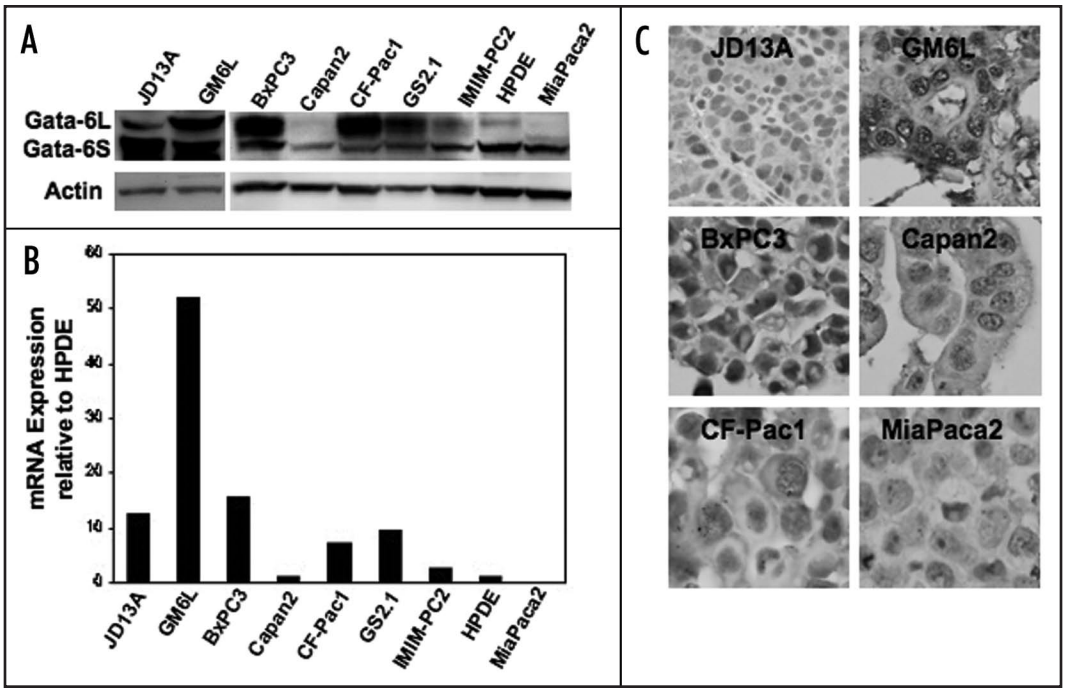

Figure 4. Correlation of GATA-6 mRNA and protein expression in pancreatic cancer. (A) Detection of GATA-6 protein in selected pancreatic cancer cells by Western blot. Actin levels were also determined on the same blot as a loading control. The mRNA expression of GATA-6 in these same cell lines is also shown below the Western blot (B), indicating a good correlation among mRNA and protein expression. (C) Immunohistochemical labeling for GATA-6 protein in representative pancreatic cancer cell lines with high (JD13A, GM6L, BxPC3, CFPAC1) and low (Capan2, MiaPaca2) expression. Protein expression corresponds to nuclear localization of GATA-6. Cytoplasmic labeling is also seen in CFPAC1.

Table 1 **Summary of GATA-6 immunolabeling in normal, PanIN and cancer samples**

Total samples	Normal ducts N = 14	PanIN1A N = 8	PanIN1b N = 13	PanIN2 N = 18	PanIN3 N = 13	Infiltrating carcinoma N = 193
H Score						
Mean ± S.D.	76.8 ± 35.3	71.3 ± 56.7	98.5 ± 48.1	101.1 ± 40.1	114.2 ± 98.5	142.9 ± 85.0
Number >100	7 (50%)	3 (38%)	10 (77%)	13 (72%)	6 (46%)	133 (69%)
Number >200	0	0	0	0	5 (38%)	54 (28%)
p values	-	NS	NS	NS	$p < 0.000001^a$	$p < 0.003^a$

[a]Two-sided Student's t-test versus normal duct epithelium.

(MiaPaca2-pcDNA3.1) (Fig. 6A and B). To determine if GATA-6 is oncogenic, we also created stable transfectants of GATA-6 and control vectors using the hTERT-immortalized but non-tumorigenic normal cell line HPNE. Similar to MiaPaca2-hG6 cells, forced GATA-6 expression caused increased growth of HPNE-hG6 in vitro (Fig. 6C). However, GATA-6 expression in HPNE did not result in increased colony formation in soft-agar nor did it permit xenograft formation in nude mice (data not shown). Thus, GATA-6 may contribute to tumorigenicity of pancreatic cancer but does not appear to be oncogenic by itself when overexpressed in association with hTERT immortalization.

Discussion

The data presented provide compelling evidence for a role of GATA-6 in pancreatic cancer based on our identification of increased copy number of this gene by the high-resolution method ROMA, and validation of this finding by qPCR, Western Blotting and immunohistochemistry. Using FISH, we also demonstrate that GATA-6 undergoes true amplification, seen in a homogeneous staining region in a cancer cell line. Given the frequency of GATA-6 overexpression and copy number gain or amplification, it is curious that GATA-6 has not been identified in other genomic studies of pancreatic cancer. Chromosome copy number studies in pancreatic cancer have extensively utilized array-based comparative genomic hybridization (aCGH)[3-7,20,21] yet almost all of these studies failed to identify the 18q11.2 amplification even although CFPAC1 cell was included in almost all. This may be due to the low resolution sensitivity (0.1 to 1.0 Mb) of aCGH compared with ROMA (4 Kb), although other contributing factors include whether the BAC and/or cDNAs utilized in these studies provided coverage of 18q11.2, the local clone density, or the filtering criteria in data analysis of each study. To our knowledge, the only other study that has identified gain of

18q11.2 in pancreatic cancer was reported by Heidenblad et al.[7] in which a large genomic region (~25 Mb) covering 18q11.1-18q12.3 was identified. However, that study focused on a more prominent region (about 7 Mb) within distal 18q12. In addition, since some studies only focused on genes within genomic amplifications, it is not surprising that GATA-6 overexpression was not identified.[3,6,8]

Despite frequent reports of copy number gain, relatively few amplified genes have been demonstrated to be involved in pancreatic carcinogenesis. These well-characterized genes include *c-ERBB-2* (27% by FISH), *Cyclin D1* (25% by Southern blot), *MYB* (10% by Southern blot), *AKT2* (20% by Southern blot), *C-MYC* (>30% by FISH), *AURKA* (83% by CGH) and *AIB1* (66.7% by FISH).[22-27] Thus, *GATA-6* has a relatively low frequency of copy number gain (9.5%) in pancreatic cancer. Our findings do not rule out the possibility that additional genes located qter to *GATA-6* are targeted by copy number gain or amplification.[7] Thus, *GATA-6* gain or amplification may simply reflect variation among the spectrum of amplified genes on this chromosome as well as the molecular complexity of pancreatic cancer. For example, in addition to gain and amplification, deletion of 18q11.2 in pancreatic cancer has also been described.[3]

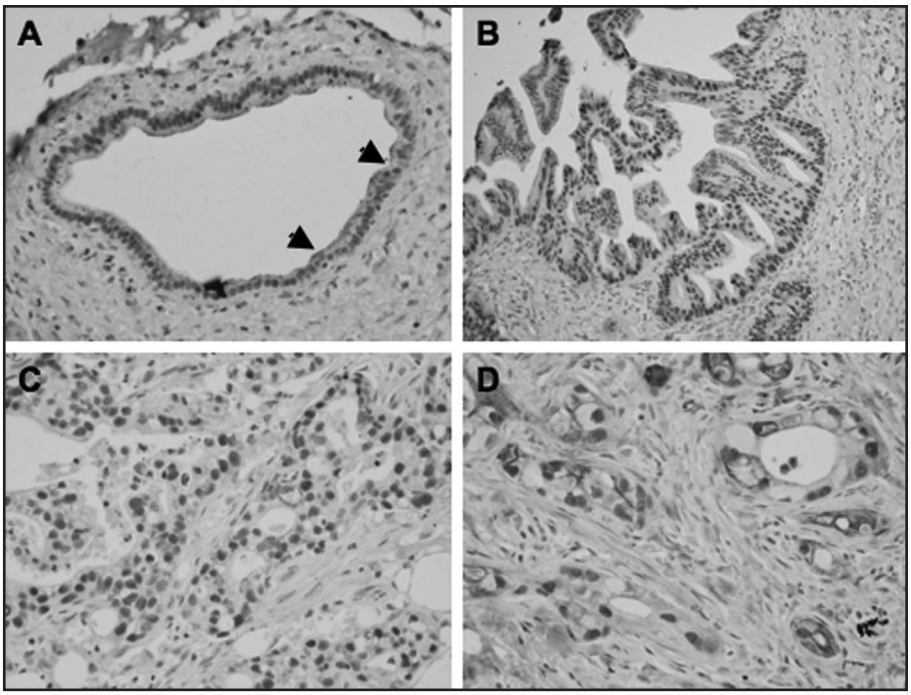

Figure 5. Immunohistochemical labeling for GATA-6 protein. Shown are GATA-6 labeling patterns in normal pancreatic duct epithelium (A, 400x), in PanIN 3 (B, 200x), and in two different pancreatic cancers (C and D, 400x). Unlike normal epithelium in which scattered positive nuclei are seen (indicated by arrows), the higher grade lesions show a marked increase in nuclear labeling and intensity.

Despite a relative low rate of amplification, GATA-6 is overexpressed in the majority of pancreatic cancers analyzed indicating that more than one mechanism contributes to its overexpression. In this respect GATA-6 is quite similar as ERRB2 in which elevated expression is frequently observed in pancreatic cancers in the absence of gene amplification.[28] As gene expression studies of pancreatic cancer indicate that numerous genes are deregulated during carcinogenesis,[29-31] copy number-independent mechanisms may play a more important role in gene de-regulation in pancreatic cancer. For example, in pancreatic cancers many overexpressed genes identified by expression profiling are associated with hypomethylation.[32] Thus, it will be worthwhile to characterize the mechanisms underlying GATA-6 upregulation in pancreatic cancer.

At present, it is unclear through which mechanism GATA-6 may exert a pro-oncogenic function. GATA-6, together with other five zinc-finger transcriptional regulators, is well known for its indispensable role in the development and differentiation of numerous endodermal and mesodermal derived organs.[19,33] GATA-6 also specifically directs pancreatic development by initiating cell type specification and differentiation.[34] Recent studies, however, have revealed that GATA-6 may also contribute oncogenic signals in cancer as shown by the ability to induce anomalous dedifferentiation of ovarian tumor cells and to downregulate NSAID-mediated 15-LOX-1 expression that contributes to the escape of apoptosis in colon cancer.[35,36] Recently, GATA-6 was shown to participate in tissue-specific immune responses in both *C. elegans* and Drosophila, and to protect human lung epithelial cells from infection, suggesting post-development GATA-6 is a modulator of the innate immune response.[37,38] In accordance, GATA-6 may represent a key regulator of some unrevealed regulatory machinery in pancreatic cancer development. As such, GATA-6 provides a new marker for the assessment of clinical outcome and as a potential therapeutic intervention for human pancreatic cancer.

Methods

Human pancreatic cancer specimens, xenografted tissue and cell lines. Xenograft enriched samples of matched primary infiltrating and metastatic pancreatic cancer were generated as previously described from patients in the Johns Hopkins Gastrointestinal Cancer Rapid Medical Donation Program (GICRMDP).[10] Additional xenograft enriched samples of pancreatic cancer were generously provided by Dr. Anirban Maitra at Johns Hopkins Medical Institutions. Fourteen established pancreatic cell lines were obtained from the ATCC (Manassas VA) and an additional 10 low passage pancreatic cancer cell lines were created in our own laboratory.[11] All cancer cell lines were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum. HPDE was maintained in Keratinocyte-SFM supplemented with EGF and BPE (Invitrogen) and HPNE was cultured in a mixture of M3 Base/DMEM (1:3) (Incell) with 5% fetal bovine serum and 10 ng/mL EGF. Samples of paraffin-embedded pancreatic cancer tissue from resection specimens were obtained from the Department of Surgical Pathology at The Johns Hopkins Hospital. The collection and use of all tissue samples for this project was approved by the Johns Hopkins Institutional Review Board.

DNA and RNA preparation. Genomic DNA was prepared using a DNAeasy Tissue Kit (Qiagen, Valencia CA). DNA was dissolved in low TE buffer and stored at -20°C. Total RNA was extracted using

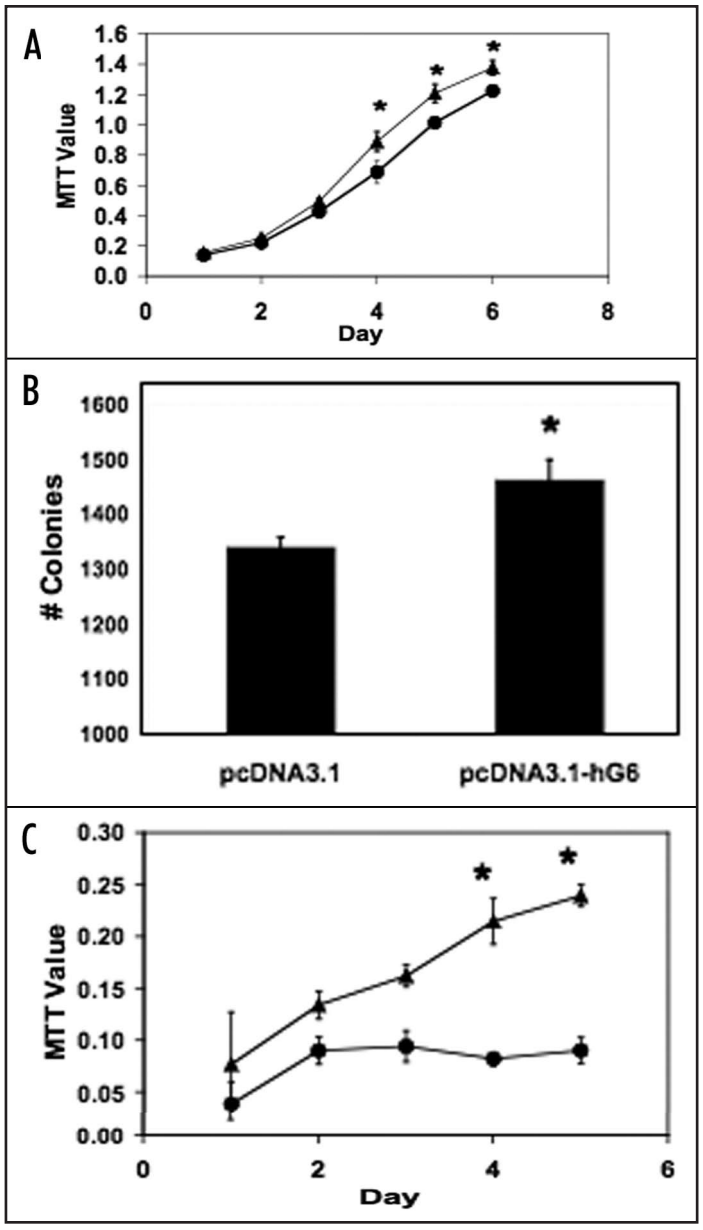

Figure 6. Oncogenic assays for GATA-6. (A) MiaPaca2 cells were stably transfected with control empty (pcDNA3.1, black circle) or GATA-6 expression vectors (pcDNA3.1-hG6, black triangle) as described in Materials and Methods, and proliferative activity determined by MTT assay. A significant increase in proliferation is noted in GATA-6 expressing MiaPaca2 cells compared to control transfected cells (p = 0.001, Day four; p = 0.00002, Day five, p = 0.0005, Day six). (B) Colony formation in soft agar of control and GATA-6 transfected MiaPaca2 cells. A significant increase in colony formation is noted in GATA-6 expressing MiaPaca2 cells compared to controls (p = 0.00009). (C) HPNE cells were stably transfected with the control empty vector (pcDNA3.1, black circle) or GATA-6 expression vectors (pcDNA3.1-hG6, black triangle), and proliferative activity determined by MTT assay. Similar to that found for MiaPaca2 cells, a significant increase in proliferation is noted in association with GATA-6 expression (p = 0.0001). All values shown represent the mean and standard error of three independent experiments.

RNAeasy Mini Kits (Qiagen), quantified using NanoDrop (NanoDrop Technologies, Wilmington, Delaware) and stored at -80°C.

ROMA. Sample preparation, *Bgl*II representations, and arrays were described in detail previously.[9] Briefly, the arrays are based on representational techniques and thus all oligonucleotides map to *Bgl*II fragments that are within the representation size range of 200–1000 bp. The array was originally designed to the June 2002 (NCBI Build 30) and the coordinates were updated to the April 2003 build for this study (NCBI Build 33). They have since been updated to May 2004 (NCBI Build 35). There are roughly 84,000 features on the array and these are scattered across the genome resulting in an average resolution of 30 kb. For each sample 2 ug of DNA were labeled and hybridized to microarrays and arrays were scanned with an Axon GenePix 4000B scanner set at a pixel size of 5 μm. GenePix Pro 4.0 software was used to quantify the intensity for the arrays. Array data were imported into S-PLUS for further analysis. Measured intensities without background subtraction were used to calculate ratios. Data were normalized using an intensity-based lowest curve fitting algorithm. Segmentation has been described previously.[12] Briefly, the ratios are arranged in genome order and are divided into blocks of 100 data points with arbitrary boundaries, and the boundaries of the segments are iteratively moved by minimizing the variance.

Flourescent in situ hybridization (FISH). FISH was performed as described in detail by Fox et al.,[13] using bacterial artificial chromosome clones CTD-2376C8 containing the genomic sequences of the 18q11.2 amplicon at 0.11 Mb (Invitrogen, Carlsbad, CA).

Quantitative real-time PCR. Quantitative real-time PCR was performed to determine genomic DNA copy numbers and gene expression levels using protocols recommended by the manufacturer (Invitrogen). Primers were specifically designed to exclude cross-reaction with potential mouse tissue in xenografts and tested for performance in quantitative real-time PCR. Only those primer pairs showing specific and robust PCR products without detectable primer dimers were selected for analysis. PCR reactions were performed using an ABI 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). For copy number evaluations, primers both within the 0.36 amplicon and flanking regions within 2–3 Mb upstream and downstream were used. For mRNA expression, the results were expressed as the relative fold change ($2^{\Delta\Delta Ct}$) compared to the normal cell line HPDE using beta-glucuronidase as an internal control. All PCR primers are listed in the Supplemental Table 2.

Western blot analysis. Protein lysates were separated by SDS/PAGE using 4–12% gels (Invitrogen) and subsequently transferred to a 0.45-μm nitrocellulose membrane (Invitrogen). The membrane was blocked in wash solution (0.1% Tween 20 in PBS) containing 5% nonfat dry milk. Rabbit anti-human Gata-6 antibody (Sc-9055 Santa Cruz Biotechnology) raised against amino acids 358–449 of human Gata-6 protein and rabbit TrueBlot (eBioScience) were used as the primary and secondary antibodies respectively, followed by Lumingen PS-3 as detecting reagent (Amersham). Protein was visualized using a Bio-Rad Quantity One Imaging Device (Bio-Rad).

Immunohistochemistry. Cell pellets and cancer tissues were formalin-fixed for 24 hours and embedded in paraffin, and 5 μm sections cut from each block. Immunolabeling was performed following standard methods described in detail in our prior publications[14] using a 1:50 dilution of primary antibody (rabbit anti-human Gata-6, sc-9055, Santa Cruz Biotechnology) and overnight incubation at room temperature. Immunolabeling was detected using the CSA II kit (DAKO) following the manufacturers' protocol and slides were counterstained with hematoxylin. A negative control was used in each run in which the antibody was replaced by an equal volume

of phosphate-buffered saline. For resected cancer tissues, immunohistochemical labeling was scored on an intensity scale of 0–3, with zero corresponding to no labeling of neoplastic epithelial cells, one to weak labeling of neoplastic epithelium (labeling best seen at 10X objective or greater), two to unequivocal labeling of epithelial cells, and three to intense labeling. The percentage of labeled neoplastic epithelial cells was scored from zero (complete absence) to 100% (all cells labeling). The labeling intensity and labeling percentage were used to generate a Histology Score (H-Score) ranging from 0 to 300, with H-score = Intensity of immunolabel (range 0 to 3) X percentage of reactive cancer cells. H scores of ≥50 were considered positive.

Establishment of stable GATA-6 expression clones. The GATA-6 expression vector hG6-MALT was generously provided by Dr. Clement Ho.[15] This vector expresses the long form of human GATA-6 using the pcDNA3.1 vector (Invitrogen). MiaPaca2 and HPNE cells were grown in 6-well plates to 80–90% confluence and transfected with empty pcDNA3.1 vector or the pcDNA3.1-hG6 GATA-6 expression vectors using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Stable expression clones were selected with 700 ug/mL geneticin for two weeks and clones were screened by RT-PCR and Western blotting using anti-GATA-6 antibody.

Cell proliferation and colony formation assays. Cells (2×10^3) were seeded in 96-well plates and the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) assay was performed after 24, 48, 72 and 96 hours based on the absorbance at 590 nm using an ELISA reader 1420 Multilabel Counter (PerkinElmer, Shelton CA). For anchorage-independent growth assays, each well of 6-well plates was plated with a basal layer of 2 ml of 4% agarose containing 75% culture medium. After solidification, 2 ml of 4% agarose containing 5,000 cells and 87.5% culture medium were added to the wells. Each well was finally covered with 1 ml of culture medium with geneticin at 250 ug/mL, and the plates were incubated at 37°C. Fourteen days after seeding, cells were stained with 0.05% crystal violet (Sigma, St. Louis, MO) containing 10% buffered formalin (Sigma) and counted using a Bio-Rad Quantity One Imaging Device (Bio-Rad laboratories, Hercules CA). Data were expressed as the mean ± 1 standard deviation from triplicates.

Statistical analysis. Statistical significance of data was determined by using a Student's t-test for parametric distributions, or a Chi-squared test for frequency distributions. p values ≤0.05 were considered statistically significant.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/FuCBT7-10-Sup.pdf

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