Identification of Candidate Biomarkers and Cancer Genes AHNAK2 and EPPK1 in Pancreatic Cancer

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NCWG and KD conceived and designed the experiments. Authors AS and LP performed the experiments. Authors NCWG, AS and LP analyzed the data. Authors NCWG and KD contributed reagents/materials/analysis tools. Author NCWG wrote the paper. All authors read and approved the final manuscript.

Short Research Article

ABSTRACT

Aims: The lack of specific symptoms at early tumor stages, together with a high biological aggressiveness of the tumor contribute to the high mortality rate for pancreatic cancer (PC). Improved screening for earlier diagnosis, through the detection of diagnostic and prognostic biomarkers provides the best hope of increasing the rate of curatively resectable carcinomas. The aim of this study is to provide new targets for use as biomarkers in PC.
Study Design: In a previous study, we identified novel candidate cancer genes and biomarkers that were significantly upregulated in PC, through a meta-analysis of large number of microarray datasets, using bioinformatics methods. In this study, we analyzed the expression of these genes in a panel of pancreatic cancer cell lines by quantitative Reverse Transcription-PCR (qRT-PCR).
Place and Duration of Study: Department of Chemistry and Biochemistry and Department of Biology, University of Northern Iowa, USA, between June 2014 and Dec 2015.
Methodology: We analyzed the expression of three genes, AHNAK2, EPPK1 and IGHG3 in a panel of seven standard PC cell lines, AsPC-1, BxPC-3, Capan-2, CFPAC-1, HPAF-II, PANC-1,

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and SW 1990 by Relative Quantification. qRT-PCR experiments were conducted in triplicate, and each experiment was replicated twice using different passages.

Results: AHNAK2 was significantly upregulated in all PC cell lines tested, with P values < 0.005 except for PANC-1 (P < 0.05). EPPK1 too was significantly upregulated (P < 0.05) in six of seven PC cell lines tested. While IGHG3 was nominally upregulated in all PC cell lines, upregulation was significant (P < 0.05) in only four PC cell lines.

Conclusion: Our results confirm that AHNAK2 and EPPK1 are novel candidates for use as biomarkers in pancreatic cancer. IGHG3 does not appear to be a suitable candidate, due to its low levels of expression in both PC and control cell lines.

ABBREVIATIONS

PC: *Pancreatic Cancer*
PCR: *Polymerase Chain Reaction*
qRT-PCR: *Quantitative reverse transcriptase-PCR*
SEM: *Standard error of the mean*

1. INTRODUCTION

Pancreatic cancer (PC) is a highly lethal cancer with poor diagnosis and dismal prognosis, with a 5-year survival rate of less than 5% [1]. It is projected to be the second leading cause of cancer death in Western societies within a decade [2]. In nearly 95% of PC patients there is neither an associated family history nor specific symptoms at the early stages of PC, when the disease can be effectively treated by surgical resection [3]. Early detection is projected to increase survival by 30–40% [4]. PC can also display a high biological aggressiveness, and a high resistance to current therapeutics. There are two fundamental ways by which the high mortality of PC can be reduced. One is to develop biomarkers for the early detection of PC and the second is to identify new targets in PC, against whom therapeutics can be developed. Our efforts are aimed at contributing to both these objectives.

Improved screening for earlier diagnosis, through diagnostic and prognostic biomarkers, provides the best hope of increasing the rate of curative, resectable carcinomas. For example, recent data has suggested that the time frame from the initiation of the pancreatic tumor to the development of metastasis could be a decade or more [5,6], suggesting a broad window of opportunity for early detection of PC. Though many serum markers have been reported to be elevated in patients with pancreatic cancer, so far, most of these biomarkers have not been implemented into clinical routine due to low sensitivity or specificity, with the exception of CA19-9 [7]. CA19‑9 can discriminate between patients with pancreatic cancer and healthy individuals with a sensitivity of 80.3% and a specificity of 80.2% [8]. However, it is estimated that an assay for early diagnosis would have to perform with a minimum sensitivity of 88% and a specificity of 85% [9] to significantly impact patient survival rates and reduce healthcare expenditure. As such, CA19-9 is used clinically to monitor PC response to therapy, but its utility for screening and risk-assessment is limited. Against this rather bleak landscape pertaining to biomarkers in PC, recent investigations [10,11] on glypican 1 (GPC1) circulating exosomes show promise as potential biomarkers in PC - if these results can be replicated in a larger study, with a greater representation of patients with early-stage disease. One drawback in the use of exosomes in a population-screening context is that it is a time consuming and a labor-intensive process with the need for specialized equipment [12].

The approach we previously took to discovering new leads for biomarkers and therapeutic targets in PC is through a meta-analysis of microarray data on PC [13]. In this analysis, sophisticated statistical techniques were used to combine results from many microarray studies to increase statistical power and generalizability compared to a single study [14]. This strategy also addressed some of the issues of biological and technical variations, which can have a significant effect on microarray measurements [15]. Using this methodology, we obtained a ranked list of genes that were upregulated in PC compared with normal pancreatic tissue [13].

Most genes in this list had well-established associations with pancreatic and other cancers. Some well-known examples included MUC4 [16], CEACAM5/6 [17], S100P [18], CLDN18 [19], KRT19 (CK19) [20] and COLA1/2

[21]. Importantly, our analysis was also able to uncover three novel candidate cancer genes, AHNAK2, EPPK1 and IGHG3 among the top 25 upregulated genes in the list, which had no previously known association with pancreatic cancer. Upregulation of AHNAK2 in PC microarray data has subsequently been confirmed [22].

Here, we have determined the expression level of AHNAK2, EPPK1 and IGHG3 in a panel of seven pancreatic cancer cell lines, and confirm the status of AHNAK2 and EPPK1 as candidate cancer genes and biomarkers in PC.

2. MATERIALS AND METHODS

2.1 Cell Culture and Culture Conditions

Seven human pancreatic cancer cell lines and one immortalized non-tumorigenic pancreatic epithelial cell line (HPDE-6) were chosen for this study. The PC cell lines AsPC-1, BxPC-3, Capan-2, CFPAC-1, HPAF-II, PANC-1, and SW 1990 were obtained from the American Tissue Culture Collection (Manassas, VA). Fresh cultures reconstituted from frozen stocks were maintained at 37℃ in a humidified atmosphere of 5% CO_2 in air except for SW 1990, which were maintained at 37℃ without CO_2. Cell culture media was as recommended by vendor: AsPC-1 and BxPC-3 in RPMI 1640 (ATCC 30-2001); Capan-2 in McCoy's 5A medium (ATCC-30-2007); CFPAC-1 in Iscove's Modified Dulbecco's Medium (ATCC-30-2005); HPAF-II in Eagle's Minimum Essential Medium (ATCC-30-2003); PANC-1 in Dulbecco's Modified Eagle's Medium (ATCC-30-2002); SW 1990 in Leibovitz's L-15 Medium, (ATCC-30-2008). The media was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The cell line HPDE-6 was obtained from Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada) and maintained in keratinocyte serum-free medium supplemented with 0.2 ng/mL epidermal growth factor and 30 µg/mL bovine pituitary extract (Invitrogen).

2.2 Quantitative Reverse Transcription-PCR (qRT-PCR)

The mRNA levels of AHNAK2, EPPK1, IGHG3 and the endogenous gene, GAPDH, were analyzed by qRT-PCR for the seven PC cell lines and control described in section 2.1 above. Total RNA isolation was performed using Qiashredder and RNEasy Mini kit (Qiagen). RNA quality and quantity were measured by a GE Nanovue spectrophotometer. One microgram of total RNA was used for reverse transcriptase (RT) reactions (20 µL total volume) to synthesize cDNA, and was carried out using the iScript cDNA Synthesis kit (Bio-Rad). Quantitative PCR was performed in 15 µL reactions with 200 nM of each primer, iQ SYBR Green Supermix (Biorad) and 1 µL of cDNA template using the ABI PRISM 7300 Real-time PCR system (Applied Biosystems, USA). Melting curves were performed to ensure that only a single product was amplified. Each experiment was performed in triplicate, and repeated twice using different passages. The relative expression of mRNA was evaluated by the delta delta Ct method. Data shown represent the mean ± SEM (n = 3). Specific primers were synthesized by Integrated DNA Technologies (Iowa City, IA) and the primer sequences are given in Table 1. The efficiency of each primer pair was determined to be within 5% of the value for GAPDH.

Table 1. Primers used in qRT-PCR analysis

Gene	Primers
AHNKA2	F: 5' GATGTGCGACTGCTTCCAC 3'
	R: 5' CAGCCTCAGTCGTGTATTCG 3'
EPPK1	F: 5' GTACGAGCGGTTGGAGCAT 3'
	R: 5' TGTTTGCTTTTGGGTCGATGA 3'
IGHG3	F: 5' AGGACTCTACTCCCTCAGCA 3'
	R: 5' GGCATGTGTGAGTTGTGTCA 3'
GAPDH	F: 5' CCATGTTCGTCATGGGTGTG 3'
	R: 5' CAGGGGTGCTAAGCAGTTGG 3'

3. RESULTS AND DISCUSSION

3.1 AHNAK2

3.1.1 Introduction

The AHNAK family of scaffold PDZ proteins include two large proteins (600–700 kD), AHNAK (desmoyokin) and AHNAK2 [23]. AHNAK has been associated with several muscular diseases, including cardiomyopathy and limb-girdle muscular dystrophy, and this effect is believed to be mediated through its association with the b-subunit of cardiac Ca(v) calcium channel [24]. AHNAK & AHNAK2 have also been shown to be components of the costameric network, associated with linking of the extracellular matrix to the cytoplasmic microfilament system [25]. Recently, AHNAK2 has been linked to two molecules that promote carcinogenesis: AHNAK2 has been associated with the export of

the growth factor FGF1 [26]. Also, Olsen et al. [27] have shown that AHNAK2 is a target of SMYD2, a protein lysine monomethyltransferase, whose overexpression is associated with malignant outcome in gastric cancer [28] and esophageal squamous cell carcinoma [29].

3.1.2 Expression in pancreatic cancer cell lines

AHNAK2 was significantly upregulated in all seven PC cell lines tested (Fig. 1), with P values < 0.005 except for PANC-1 (P < 0.05). The level of expression was over 10-fold in about 40% of PC cell lines. These results strongly support our prediction [13] that AHNAK2 is a candidate biomarker and cancer gene in PC. One possible role of AHNAK2 in PC could be through its association with the costameric network. In muscle cells, costameres mediate the lateral transmission of force from the sarcomeres to the extracellular matrix [25]. Thus, it is possible that AHNAK2 could impact PC through its effect on cell motility. For example, experiments on metastatic human tumor cell lines [30] have shown that knockdown of a related protein, AHNAK, inhibited cell migration. Another possible way in which AHNAK2 could impact PC is through its role as a critical component of the stress-induced FGF1 secretion pathway [26]. FGF1 is an important regulator of tissue repair, angiongenesis and inflammation, and secretion of FGF1 can be stimulated by hypoxia [31], a condition associated with growing tumors.

3.2 EPPK1

3.2.1 Introduction

Epiplakin belongs to the plakin family of giant proteins that are associated with the cytoskeleton. Plakins connect the microfilament, microtubule (MT), and intermediate filament (IF) systems with each other and with junctional complexes in organelle and plasma membranes, thereby contributing to cell shape and polarity. They modulate fundamental biological processes such as cell adhesion, migration, and signaling pathways [32]. Epiplakin is rather an unusual plakin in that it consists solely of plakin repeats organized into 13 plakin repeat domains (PRD's) and does not contain a plakin domain characteristic of other plakins. There is evidence to suggest that Epiplakin associates with keratin networks in migrating keratinocytes during wound healing [33].

3.2.2 Expression in pancreatic cancer cell lines

EPPK1 too was significantly upregulated in all but one of the PC cell lines tested (Fig. 2). Among cell lines overexpressing EPPK1, the level of expression is at least 2-fold for all except for CFPAC-1. Again, these results are consistent with our prediction [13] that EPPK1 is a candidate biomarker and cancer gene in PC. EPPK1 has recently been associated with Intrahepatic cholangiocarcinoma (ICC) as a putative driver gene [34], and thus it holds promise as a therapeutic target in PC as well. It is intriguing that PANC-1 is the only cell line in which EPPK1 was not upregulated. The reason for this exception is not entirely clear, beyond the well-documented fact that multiple combinations of mutations can give rise to the development of PC [35]. For example, PC has been recently classified into four different molecular subtypes [36]. A specific phenotypic difference between PANC-1 and BxPC-3 (in which EPPK1 is highly expressed) is that PANC-1 cells have a 5-fold greater motility [37]. This may be significant since there is evidence that plakins, of which EPPK1 is a member, can modulate cell migration activity [32].

3.3 IGHG3

3.3.1 Introduction

IGHG3 (Immunoglobulin heavy constant c-3) is an antigen binding protein belonging to the major immunoglobulin class in body secretions. High blood Immunoglobulins have been reported in patients in several cancers [38]. While elevation of IGHG3 has been reported in the pancreatic juice of smokers [39], IGHG3 has not previously implicated in pancreatic cancer.

3.3.2 Expression in pancreatic cancer cell lines

The status of IGHG3 as a putative cancer gene and biomarker is more nuanced; IGHG3 is nominally upregulated in all seven PC cell lines tested (Fig. 3), with > 10-fold overexpression in two cell lines (BxPC-3 and SW 1990). However, in 40% of cell lines tested, the overexpression is not significant. The main reason appears to be the low level of expression of IGHG3 in both PC and control cell lines (as evidenced by high threshold cycle (Ct) values in qPCR), which makes it difficult to detect the expression of this gene with high precision.
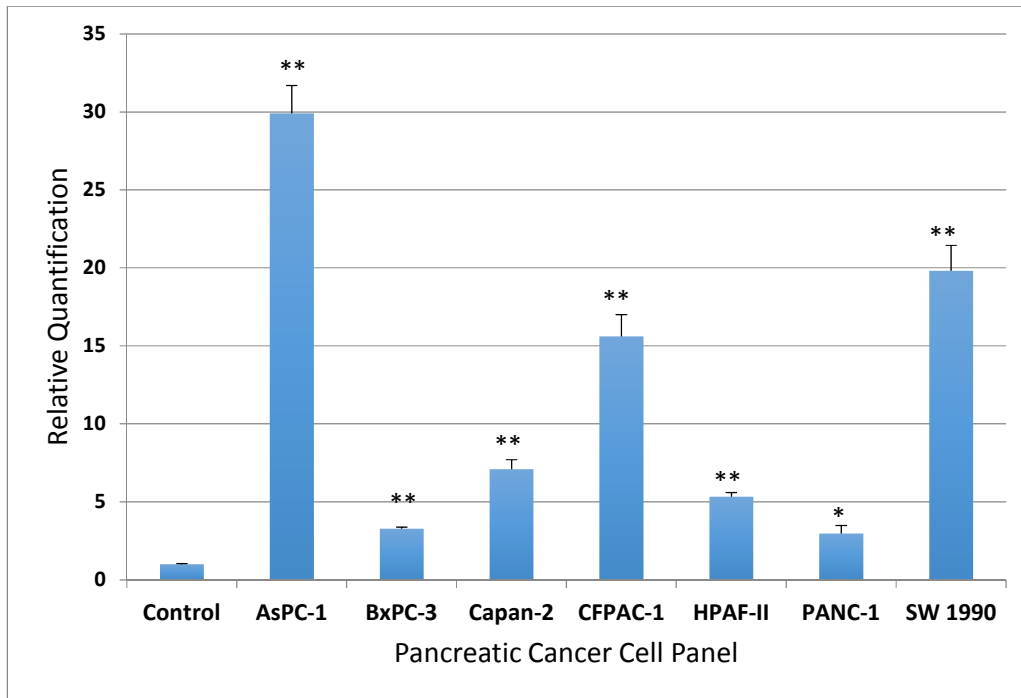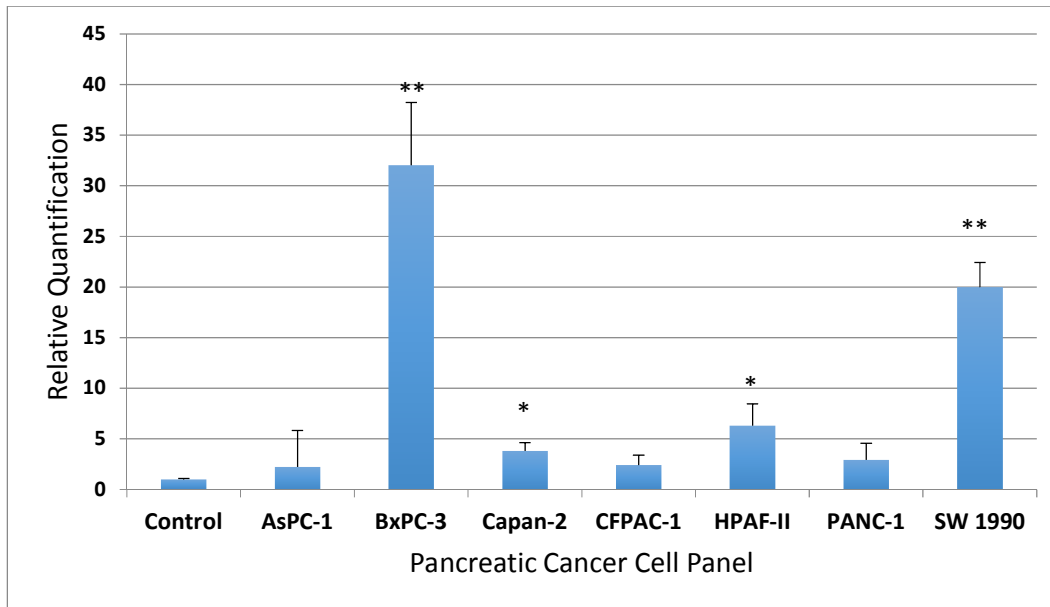
Fig. 1. Relative quantification of the expression of AHNAK2 in a panel of PC cell lines by qRT-PCR

*Significant from Control, * P < 0.05; ** P < 0.005 (unpaired t-tests)*
Mean ± S.E.M = Mean values ± Standard error of means (n=3)
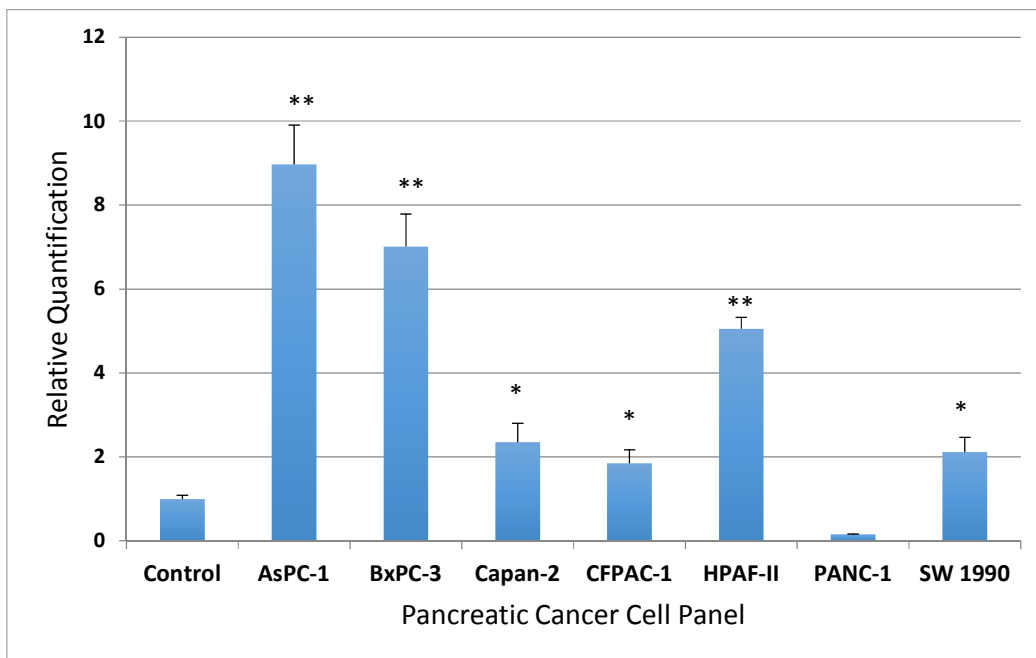
Fig. 2. Relative quantification of the expression of EPPK1 in a panel of PC cell lines by qRT-PCR

*Significant from Control, * P < 0.05; ** P < 0.005 (unpaired t-tests)*
Mean ± S.E.M = Mean values ± Standard error of means (n=3)

Fig. 3. Relative quantification of the expression of IGHG3 in a panel of PC cell lines by qRT-PCR
*Significant from Control, * P < 0.05; ** P < 0.005 (unpaired t-tests)*
Mean ± S.E.M = Mean values ± Standard error of means (n=3)

4. CONCLUSION

The main objective of this paper was to evaluate the expression of three genes AHNAK2, EPPK1 and IGHG3 in a panel of standard PC cell lines, to assess their potential as biomarkers in PC. A qRT-PCR analysis revealed that AHNAK2 is upregulated in all seven standard PC cell lines tested (Fig. 1), with > 10-fold overexpression in 40% of cell lines (3 of 7). These results provide strong evidence for the status of AHNAK2 as a candidate biomarker for PC. Further support comes from Bhasin et al. [22], who have included AHNAK2 in a 5-gene classifier for PC through an analysis of PC microarray data performed subsequent to our analysis [13]. They also performed a qRT-PCR assay of 22 microdissected paired retrospective FFPE patient samples containing PC and matched non-tumor tissue samples, and showed that AHNAK2 was upregulated in all but one sample, with an average fold change of 5. The second gene, EPPK1, too was significantly upregulated in most PC cell lines tested by us. EPPK1 has been previously shown to be upregulated in pancreatic intraepithelial neoplasia (PanIN) [40], a precursor lesion of PC. We expect further studies to validate the usefulness of these candidates for earlier diagnosis in clinical practice. Interestingly, EPPK1 has also been suggested as a potential plasma biomarker in cervical squamous cell carcinoma [41]. Our results are inconclusive regarding the status of the third gene, IGHG3, as a biomarker for PC. While IGHG3 was overexpressed in all PC cell lines, the results were not significant in nearly half the cell lines tested.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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