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Research paper

Microarray-based gene expression profiling reveals genes and pathways involved in the oncogenic function of REG3A on pancreatic cancer cells

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ABSTRACT

We previously reported that regenerating islet-derived protein 3 alpha (REG3A) exacerbates pancreatic malignancies. The mechanism of this effect has not been clearly elucidated. Here we first identified key differentially expressed genes (DEGs) and signal pathways in the pancreatic cancer cell line SW1990, compared to two control cell lines, by microarray analysis. We then identified key genes and pathways regulated by REG3A or the cytokine IL6 in SW1990 cells. Afterwards, these DEGs induced by REG3A or IL6 were subjected to KEGG pathway enrichment analysis and GO function analysis by the DAVID online tool. Ultimately, we constructed protein–protein interaction networks among the DEGs by Cytoscape. Among the three pancreatic cell lines, SW1990 exhibited highly deterioration with the activation of genes and pathways related to proliferation, survival, angiogenesis, and invasion. As a result, 50 DEGs enriched in 11 pathways were identified in REG3A-treated SW1990 cells, and 28 DEGs enriched in 9 pathways were detected in IL6-treated cells. Overall, results of microarray analysis followed by qRT-PCR and Western blotting suggest that REG3A regulates pancreatic cell growth by increasing the expression of at least 8 genes: *JAK1*, *STAT3*, *IL10*, *FOXM1*, *KRAS*, *MYC*, *CyclinD1*, and *c-fos*; and activation of at least 4 signal pathways: TGFβ, PDGF, angiogenesis and RAS. Similar results were obtained with IL6 treatment. Regulation network analysis confirmed the cell growth related DEGs, and further uncovered three transcription factor families with immune functions regulated by REG3A.

1. Introduction

Pancreatic cancer, one of the most lethal and intractable human malignancies, has a reported five year survival rate of less than 5% (Hidalgo, 2010). It was estimated that the number of patients with pancreatic cancer in 2015 would increase to 48,960 in the United States, and of those, 40,560 would die (Siegel et al., 2015). Nevertheless, this lethal disease is still difficult to treat effectively, in part because its pathogenesis has not been thoroughly characterized.

One widely-held hypothesis is that infaust changes of certain genes are implicated in the etiology of pancreatic cancer. Specifically, alterations of Kirsten rat sarcoma viral oncogene homolog (KRAS),

Abbreviations: AP, Acute pancreatitis; *BACH1*, BTB and CNC homology 1, basic leucine zipper transcription factor 1; *BACH2*, BTB and CNC homology 1, basic leucine zipper transcription factor 2; *CARD9*, Caspase recruitment domain family, member 9; *CDK4*, Cyclin-dependent kinase 4; *CDKN2A*, Cyclin-dependent kinase inhibitor 2A; *c-fos*, FBJ murine osteosarcoma viral oncogene homolog; *c-Jun*, Jun proto-oncogene; *CLDN2*, Claudin 2; DAVID, The Database for Annotation, Visualization, and Integrated Discovery; DEGs, Differentially expressed genes; *DOK3*, Docking protein 3; *EGF*, Epidermal growth factor; *FOXM1*, Forkhead box M1; *FOXO3*, Forkhead box O3; *GATA1*, GATA binding protein 1 (globin transcription factor 1); *GATA2*, GATA binding protein 2 (globin transcription factor 2); GO, Gene Ontology; *GRB2*, Growth factor receptor-bound protein 2; *IL10*, Interleukin-10; *IL6*, Interleukin-6; *JAK1*, Janus kinase 1; *JAK2*, Janus kinase 2; KEGG, Kyoto Encyclopedia of Genes and Genomes; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *MAPK1*, Mitogen activated kinase-like protein 1; *MAPK3*, Mitogen activated kinase-like protein 3; *MAPK4*, Mitogen activated kinase-like protein 4; *MAPK9*, Mitogen activated kinase-like protein 9; *MAPK14*, Mitogen activated kinase-like protein 14; *MRAS*, Muscle RAS oncogene homolog; *MYC*, V-myc avian myelocytomatosis viral oncogene homolog; *NF-κB*, Nuclear factor kappa B; PAP, Pancreatitis-associated protein; *PAX4*, Paired box 4; PDAC, Pancreatic ductal adenocarcinoma; *PDGF*, Platelet derived growth factor; *PID1*, Phosphotyrosine interaction domain containing 1; *PIK3CA*, Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; *PPP2CB*, Protein phosphatase 2, catalytic subunit, beta isozyme; *RALB*, V-ral simian leukemia viral oncogene homolog B; *RAPGEF6*, Rap guanine nucleotide exchange factor (GEF) 6; *RASAL2*, RAS protein activator like 2; *REG3A*, Regenerating islet-derived protein 3 alpha; RT-PCR, Real-time polymerase chain reaction; *SHC1*, Src homology 2 domain containing transforming protein 1; *SMAD4*, SMAD family member 4; *SOCS3*, Suppressor of cytokine signaling 3; *SPRY1*, Sprouty homolog 1, antagonist of FGF signaling (*Drosophila*); *STAT3*, Signal transducer and activator of transcription 3; *STAT5A*, Signal transducer and activator of transcription 5A; *TGFβ*, Transforming growth factor β; *TIMP1*, Tissue inhibitor of metalloproteinase 1; *TIMP2*, Tissue inhibitor of metalloproteinase 2; *TP53*, Tumor protein p53; *TWIST1*, Twist family bHLH transcription factor 1; *Tyk2*, Tyrosine kinase 2; *VEGF*, Vascular endothelial growth factor.

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cyclin-dependent kinase inhibitor 2A (p16/CDKN2A), tumor protein p53 (TP53) and SMAD family member 4 (SMAD4) are all detected in more than 50% of pancreatic cancer patients (Sheng et al., 2014). The excessive activation of KRAS in particular is an essential step in the development of many cancers (Yu et al., 2010). KRAS exerts its carcinogenic effects through the activation of mitogen activated kinase-like protein (MAPK) and PI3 kinase signaling pathway (Ji et al., 2007). In approximately 80% of pancreatic ductal adenocarcinoma (PDAC) cases, there is a loss-of-function of p16/CDKN2A, a tumor suppressor gene involved in cell cycle regulation (Heilmann et al., 2014). Mutations in TP53 are also prevalent. TP53 codes tumor protein p53 to inhibit cancer formation through several pathways involved in cell cycle arrest and apoptotic death (Mohamadkhani et al., 2013). SMAD4 codes proteins which are intracellular mediators of transforming growth factor β (TGFβ). Previous studies indicate that the absence of SMAD4 is necessary for the progression of pancreatic cancer (Legendre et al., 2014).

REG3A (regenerating islet-derived protein 3 alpha, also known as pancreatitis-associated protein or PAP) is a C-type lectin protein secreted by acini during acute pancreatitis. REG3A is a member of a family of REG proteins. It exerts its tissue protection function at least in part through activating nuclear factor kappa B (NF-κB) signaling pathways in acute pancreatitis (AP) (Okochi et al., 2014). However, overexpression of REG3 in pancreas may also promote cell proliferation of islet tumor cells in mice and acinar epithelial cells in rats (Folch-Puy et al., 2006; Cui et al., 2009). In addition, REG3 stimulates cell growth by activating Akt kinase and up-regulating cyclinD1/cyclin-dependent kinase 4 (CDK4) signaling pathway (Cui et al., 2009). Our previous studies have indicated that REG3 is involved in the regeneration or growth of islet β cell (Hou et al., 2011). We reported that REG3A overexpression, in synergy with suppressor of cytokine signaling 3 (SOCS3) methylation, promoted cell proliferation in pancreatic cancer as well as during inflammation-linked pancreatic carcinogenesis (Wang et al., 2014). Further, we found that REG3A could constitute a REG3A-janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) positive feedback loop, and that REG3A shared the canonical inflammation-related carcinogenic JAK2/STAT3 pathway with the inflammatory cytokine interleukin 6 (IL6) (Liu et al., 2015). IL6 is secreted by T cells and macrophages to stimulate immune response. The cytokine can also promote the progression of pancreatic intraepithelial neoplasia and development of pancreatic cancer by activating STAT3/SOCS3 signaling pathways (Lesina et al., 2011) and by stimulating the secretion of multiple Th2 cytokines (Feurino et al., 2007).

mRNA expression array analysis is an efficient tool for high throughput analysis and for the screening of differentially expressed genes involved in carcinogenesis, including the pathogenesis of pancreatic cancers (Xie et al., 2015). Here, we applied this technique to identify and compare abnormalities in gene expression during tumor initiation in the high malignant pancreatic cancer cell line SW1990 (Zhou et al., 2015), with noncancerous HPDE6c7, an immortalized but not transformed pancreatic ductal epithelial cell line, and PANC-1, a less malignant pancreatic ductal cancer cell line, as controls. The genetic background of the primary pancreatic cancer cell line PANC-1 is mutant KRAS, mutant TP53, wild type (wt) SMAD4, and homozygous deletion of p16. The genetic background of the metastatic pancreatic cancer cell line SW1990 is mutant KRAS, wt TP53, not determined (ND) SMAD4, and ND p16. The noncancerous cell line HPDE6c7 is near normal genotype and phenotype (Ouyang et al., 2000; Deer et al., 2010). We focused on, but did not restrict our analysis to, the expressions of KRAS, p16/CDKN2A, TP53, and SMAD4, genes that have been identified to be mutation in these cells (Oshima et al., 2013). Our previous studies have revealed that among these five pancreatic cancer cells AsPC-1, BXPC-3, Mia Paca-2, PANC-1 and SW1990, and the normal control cell HPDE6c7, REG3A expressed highest in SW1990, secondly in PANC-1, and nearly not expressed in HPDE6c7. Besides, after incubation with 50 ng/mL REG3A protein for 24 h, SW1990 possessed higher cell viability, more colony numbers than PANC-1. Scarcely any studies locate the receptor of REG3A, and our previous research speculated that EGFR might function as the receptor of REG3A (Liu et al., 2015). In addition, IL6 receptor gp130 expressed equally in pancreatic cancer cell lines as well as noncancerous pancreatic cell HPDE6c7 (Goumas et al., 2015). On the basis of those findings, we then investigated the mechanism of action of the growth factor REG3A in inducing inflammation-linked pancreatic cancer development in SW1990 (Wang et al., 2014).

In this study, we approached this problem by: 1) comparing genetic profiles of the SW1990 cancer cell line with those of two control cell lines; and 2) identifying genes and pathways in SW1990 cells that are activated or induced by treatment with REG3A or IL6. Based on comparisons of results obtained by microarray, KEGG pathway enrichment, and GO function analyses, we confirmed the involvement of eight key genes, as well as four specific signal pathways, in the induction of tumorigenesis by REG3A in the SW1990 cell model of pancreatic cancer.

Table 1
The primers of human genes used in qRT-PCR.

Gene	Primer sequence (5′ → 3′)
IL10	F: ATG CCC CAA GCT GAG AAC CAA GAC CCA
	R: TCT CAA GGG GCT GGG TCA GCT ATC CCA
FOXM1	F: AAC CGC TAC TTG ACA TTGG
	R: GCA GTG GCT TCA TCT TTCC
JAK1	F: CTG GTA TGC TCC AAA TCG
	R: CAT CCC TAG ACA CTC GTT CT
CyclinD1	F: TGC ACA GTG TCA CGA ACA GA
	R: ACC TCG GAG AAG GCT AAA CA
c-fos	F: TGT CAA CGC GCA GGA CTT CT
	R: CCT TCT CCT TCA GCA GGT TG
MYC	F: CAA ACC TCC TCA CAG CCC ACT
	R: TGA CAC TGT CCA ACT TGA CCC
KRAS	F:GAG GCC TGC TGA AAA TGA CTG
	R:ATT ACT ACT TGC TTC CTG TAGG
STAT3	F:GTG TAT GCG TCG GCT TCA
	R:GAC TCT GCG GGT CCT GTT

2. Methods and materials

2.1. Cell lines and reagents

Human pancreatic cancer cell lines SW1990 and PANC-1 were obtained from American Type Culture Collection (ATCC, USA). The noncancerous cell line HPDE6c7, purchased from GuangZhou Jennio Biotech Co., Ltd. was used as a normal pancreas cell line. The cell lines were cultivated in RPMI 1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) and 100 units/mL penicillin/streptomycin (Invitrogen, USA) at 37 °C under a 5% CO_2 environment. HPDE6c7 cells and SW1990 cells were incubated with 100 ng/mL IL6 protein (SAB, USA) or 50 ng/mL REG3A protein (Sino Biological Inc., China), respectively, for 24 h with the control group disposed with the same volume of phosphate buffered saline (PBS) (Wang et al., 2014). Afterwards, total cellular RNA was extracted with TRIzol reagent (Life Technologies, USA) in accordance with the manufacturer's instructions. RNA quality was evaluated by ultraviolet spectrophotometer, Agilent 2200 Bioanalyzer (Agilent, USA), and agarose gel electrophoresis (A260/A280 ≥ 1.8, A260/A230 ≥ 1, RIN value ≥ 7). Synthetic antisense RNA was generated in the condition of Amino Allyl message Amp II Kit 9 (Life Technologies, USA), subsequently hybridized to RiboArray™ Custom Array 1 × 2 K (RiboBio, China). Cells were treated identically for the microarray and real-time PCR studies described below.

2.2. Microarray data and screening of differentially expression genes

Our microarray analysis targeted 444 genes which have been implicated in pancreatic cancer on a genome-wide scale. Data with the coefficient of variation [CV = SD (standard deviation)/mean