ORIGINAL ARTICLE

The Gamma-Aminobutyric Acid A Receptor π Subunit Is Overexpressed in Pancreatic Adenocarcinomas

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ABSTRACT

Context The identification of genes involved in tumorigenesis is essential for the development of new treatment strategies or diagnostic approaches for pancreatic cancer.

Objective To identify genes overexpressed in pancreatic cancer we employed differential display, a PCR-based method of differential expression cloning. Using this method, we identified a PCR product that was consistently overexpressed in pancreatic tumors relative to normal pancreatic tissues.

Setting Five pancreatic ductal adenocarcinomas and 5 bulk pancreatic ducts isolated from independent pancreatic specimens without malignancies were analyzed by differential display. A panel of 12 pancreatic tumors at various stages of differentiation and a set of 6 pancreatic ducts without malignancies were then used to verify expression by RT-PCR.

Results Sequence analysis of a cDNA detected by differential display revealed that it was a portion of the recently cloned gamma-aminobutyric acid A receptor π subunit. RT-PCR analysis of a panel of RNAs prepared from pancreatic ducts isolated from organs without malignancies and pancreatic tumors confirmed that that the gamma-butyric acid A receptor π subunit was significantly overexpressed in pancreatic carcinomas. Analysis of 12 pancreatic tumors revealed that the π subunit was overexpressed in 10 of the tumors (83%). The expression varied among the tumors, however, overexpression was observed in tumors of each histopathological grade. In contrast, none of the normal pancreatic tissues analyzed displayed high levels of expression.

Conclusions The expression of the $GABA_A$ receptor π subunit may thus play an important role in the pathogenesis of pancreatic cancer.

INTRODUCTION

Pancreatic cancer is the fifth most common cause of cancer death. Conventional therapeutic approaches have not had much impact on the course of this aggressive neoplasm. The dismal overall 5-year survival associated with pancreatic cancer is 3% and is largely a result of diagnosis late in the course of the disease. Currently, the only curative intervention is surgery, however only 15-20% of patients present with resectable lesions. A better understanding of the pathogenesis of pancreatic cancer and more effective screening techniques are required to increase the proportion of patients presenting with early resectable disease and to improve current survival rates [1].

Studies have begun to uncover the molecular alterations that are most prevalent in

pancreatic cancer. Among these, activating mutations in K-*ras* and inactivating mutations in the tumor suppressors *p53*, *p16*, and *DPC4* are the most common alterations [2]. Other important events in pancreatic tumorigenesis include changes in expression of receptor tyrosine kinases including the epidermal growth factor receptor and *HER2/neu* oncoprotein. Both are overexpressed in pancreatic cancer and are capable of transducing extracellular growth stimulatory signals [3].

Pancreatic cancers are resistant to both chemo- and radiation therapy. The exact mechanisms of resistance remain poorly understood. Early studies suggested a role for P-glycoprotein, the product of the multidrug resistance gene (*MDR-1*), but later studies could not show upregulation of P-glycoprotein in pancreatic cancer cell lines. However, another transmembrane ATP-dependent transporter, multidrug resistance associated protein (MRP) is upregulated in these cell lines [3].

The identification of genes involved in tumorigenesis is essential for the development of new treatment strategies or diagnostic approaches. Thus, we tried to identify genes differentially expressed between pancreatic cancer and pancreatic tissues without malignancies. To investigate those genes overexpressed in pancreatic cancer we employed differential display, a PCR-based method of differential expression cloning. Using this method, we found that the gamma-aminobutyric acid (GABA) A receptor π subunit was overexpressed in pancreatic tumors.

GABA is the major inhibitory neurotransmitter in the brain and is essential for the overall balance between neuronal excitation and inhibition. GABA influences neurons via a large number of receptor subtypes which are grouped on the basis of their pharmacology under three major classes of receptors: $GABA_A$, $GABA_B$, and $GABA_C$ receptors. $GABA_A$ and $GABA_C$ are ligand-gated ion channels, while $GABA_B$ are G-protein coupled receptors. $GABA_A$ receptors are GABA-gated chloride ion channels that cause inhibition of neuronal firing [4].

In mammals, there are 14 known subtypes of $GABA_A$ receptor subunits that are thought to assemble in different pentameric complexes. In addition to their location on central neurons and astroglia, functional $GABA_A$ receptors have been detected on peripheral neurons and non-neuronal cells. The non-neuronal cells include endocrine cells of the pituitary pars intermedia, adrenal medulla, islets of Langerhans, placenta, and smooth muscle cells of the urinary bladder and uterus. The precise function of $GABA_A$ receptors in non-neuronal cells is unclear. In endocrine cells they have been implicated in regulation of hormone secretion and in the uterus their function appears to be related directly to tissue contractility. The receptor π subunit was identified recently by searching a database of expressed sequence tags (ESTs) with a peptide consensus sequence of known $GABA_A$ receptor family members. The receptor π subunit cDNAs were expressed as recombinant proteins and shown to assemble with known $GABA_A$ receptor subunits and confer unique ligand binding properties to the resulting recombinant receptor [5]. Here we describe the identification and subsequent characterization of the expression of the π subunit of the $GABA_A$ receptor family in pancreatic cancer.

METHODS

Human Tissue Samples

Tissue samples from patients with adenocarcinoma of the pancreas were provided by the Cooperative Human Tissue Network (CHTN) which is funded by that National Cancer Institute. For RT-PCR analysis of $GABA_A$ receptor π subunit expression in pancreatic tumors of various histopathological grades, 12 tumors were used and included 1 well-differentiated, 4 moderately, 4 moderately-to-poorly, and 3 poorly differentiated tumors. A set of 6 pancreatic ducts without evidence of

malignancy from organ donors without suitable recipients were obtained from the Department of Surgery, University of Arkansas for Medical Sciences (UAMS).

RNA Isolation

RNA from tissue samples less than 250 mg was isolated by selective absorption on to silica gel-based membranes using commercially available spin columns (Qiagen, Valencia, CA, USA). For samples greater than 250 mg, such as donor pancreas, RNA was isolated using standard guanidine methods for total RNA preparation [6]. Total RNA (50 µg) was treated with 10 units of DNase I, phenol extracted, and ethanol precipitated. The treated RNAs were suspended with diethyl pyrocarbonate-treated water (1.0 µg/µL, final concentration) and stored at -80°C until use.

Differential Display

Messenger RNA expression in 5 pancreatic ductal adenocarcinomas (3 poorly and 2 moderately-to-poorly differentiated) and bulk pancreatic ducts isolated from 5 independent pancreatic specimens without malignancies was analyzed by differential display. Differential display was performed essentially as described by Liang and Pardee [7] using RNAimage Kits (GenHunter, Nashville, TN, USA). Complementary DNA fragments detected by differential display in at least 3 out of the 5 tumors analyzed were isolated, reamplified, and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). DNA sequences of the cloned PCR products obtained in this manner were used to search GenBank databases (*e.g.,* non-redundant and EST) to determine if they represent known or novel gene products.

Relative Quantitative PCR

The relative expression level of GABA$_A$ receptor π subunit was examined by quantitative PCR essentially as described by Tanimoto *et al*. [8]. Sequence-specific primers used to amplify the GABA$_A$ receptor π subunit were 5′-CGTCGAGGTCGGCAGAAGT-3′ (sense) and 5′-GCGGGCATCCAGAGTGAAG-3′ (antisense) which amplifies a sequence that corresponds to nucleotides 237-487 of the π subunit mRNA sequence (accession # NM_014211). Primers for beta-actin, 5′-GCATGGGTCAGAAGGAT-3′ (forward) and 5′-CCAATGGTGATGACCTG-3′ (reverse), were included as an internal control. First-strand cDNAs were synthesized from 2 µg of DNase I-treated total RNA from pancreatic ducts and adenocarcinomas by Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) using oligo (dT) and random primers. Two microliters of each cDNA product (about 50 ng) were amplified in a mixture containing 5 pmol of GABA$_A$ receptor π subunit-specific primers, 2.5 pmol of beta-actin-specific primers, 200 µM dNTPs, 5 µCi [alpha-^{32}P] dCTP, and 1 unit *Taq* DNA polymerase with reaction buffer in a final volume of 25 µL. The PCR amplification was carried out for 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. The linearity of the PCR reaction for 35 cycles of PCR was confirmed with the two sets of primers. Reaction products were separated on 1.5% agarose gels containing ethidium bromide and the level of amplification was determined using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). The relative expression was measured as a ratio of GABA$_A$ receptor π subunit expression to beta-actin expression.

STATISTICS

The overexpression cutoff value was defined as the mean value for pancreatic duct expression +2 standard deviations (SD). An unpaired t test was used for the comparison of the mean values of normal pancreatic ducts with tumors (Prism software, GraphPad Software, San Diego, CA, USA). A significant difference in the relative mean expression is defined using as a two-tailed P
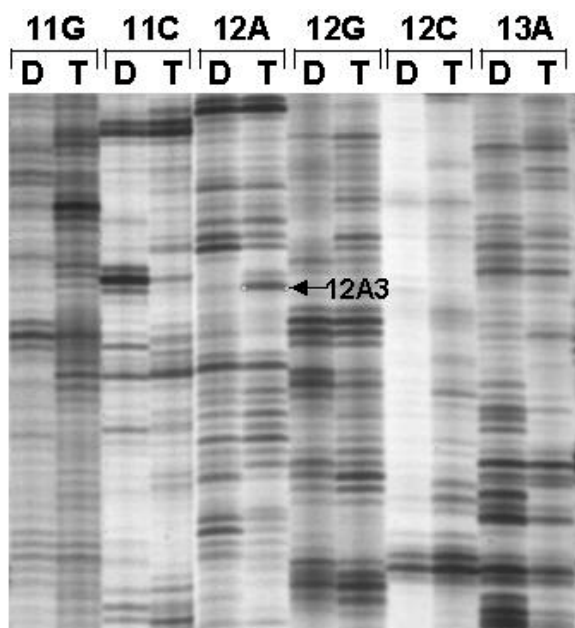
Figure 1. Portion of a representative differential display gel comparing pancreatic duct (D) and tumor (T) cDNAs amplified with arbitrary primers (11, 12, 13) and anchored oligo (dT) primers (A, G, C) as described in Methods. Tumor cDNA 12A3 (arrow) was cloned and identified as GABA$_A$ receptor π subunit.

value less than 0.05. Expression in each RNA sample was measured 3 to 5 times.

ETHICS

All research using human tissues was reviewed and approved by the UAMS Human Research Advisory Committee.

RESULTS

Differential Display of Pancreatic Tissues

A cDNA fragment, 12A3, was identified by differential display that was consistently overexpressed in pancreatic tumors compared to its expression in pancreatic ducts from normal organs (Figure 1). The cDNA fragment was reamplified with the primers used in differential display and the resulting 250-bp PCR product was cloned and sequenced. GenBank database searches revealed that the clone corresponded to a portion of the GABA$_A$ receptor π subunit.

RT-PCR Analysis of GABA$_A$ Receptor π Subunit Expression in Pancreatic Tissues

To verify the overexpression of the GABA$_A$ receptor π subunit observed in the differential display profile, the expression level of the receptor π subunit was measured in RNA prepared from pancreatic ducts isolated from organs without malignancies and pancreatic adenocarcinomas. Gene-specific primers were designed from the GABA$_A$ receptor π subunit cDNA sequence and used in a RT-PCR assay to quantify its expression relative to beta-actin. A comparison of π subunit expression in pancreatic adenocarcinomas with pancreatic ducts from donor organs indicated that the GABA$_A$ receptor π subunit is expressed at significantly higher levels in the pancreatic tumors (Figure 2).

To assess the correlation of GABA$_A$ receptor π subunit expression in pancreatic tumors with progressing stages of tumor differentiation, RT-PCR analysis was performed on RNA isolated from tumors with various histopathological grades (Figures 2 and 3). Using a cutoff level for overexpression of the mean expression in nontumor pancreatic duct +2SD, 10 of 12 (83%) adenocarcinoma cases were above the cutoff value. The expression of the GABA$_A$ receptor π subunit varied among the tumors, however, overexpression was observed in tumors at each stage of differentiation. In contrast, none of the normal tissues displayed high levels of expression. The expression
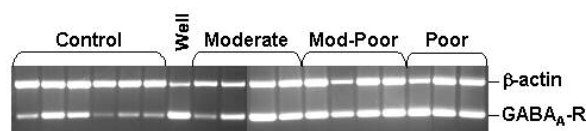

Figure 2. RT-PCR analysis for the mRNA expression of GABA$_A$ receptor π subunit and beta-actin in pancreatic ducts without malignancies (Control) and pancreatic adenocarcinomas of various histopathological grades (Well, Moderate, Mod-Poor, Poor). RT-PCR products were electrophoretically separated on agarose gels and stained with ethidium bromide as described in Methods.
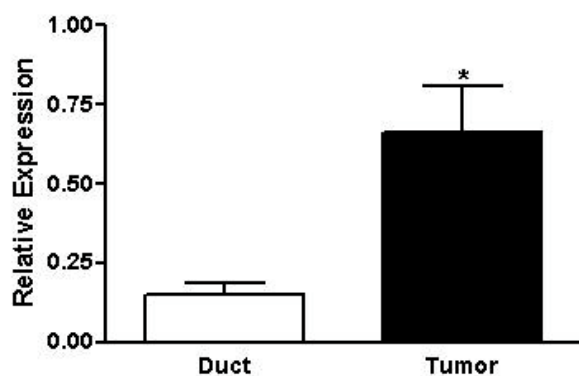
Figure 3. Quantitation of $GABA_A$ receptor π subunit expression relative to beta-actin. Expression of the receptor π subunit mRNA was measured by RT-PCR in pancreatic ducts without malignancies (Duct, n=6) and pancreatic adenocarcinomas (Tumor, n=12) and defined relative to beta-actin mRNA expression in each tissue. $GABA_A$ receptor π subunit mRNA expression levels were significantly elevated in tumors compared to normal pancreatic ducts (*P=0.026).

level of $GABA_A$ receptor π subunit was highest in the well-differentiated tumor, however the significance of this observation is tempered by the analysis of only a single tumor at this stage of differentiation. Seventy-five percent of the moderately and moderately-to-poorly differentiated adeno-carcinomas overexpressed the π subunit as did all three of the poorly (G3) differentiated tumors that were analyzed.

DISCUSSION

The $GABA_A$ receptor family is considered to be the most complicated of the GABA receptor families due to the large number of receptor subtypes and the variety of ligands that interact with specific sites on the receptors. No selective agonist of the $GABA_A$ receptors has been identified that does not also have significant agonist action on the ligand-gated ion channels of the $GABA_C$ receptors. There are at least 11 proposed structurally specific sites on $GABA_A$ receptors that can be bound by oleamide, thyroid hormones, peptide hormones (*e.g.,* somatostatin-14), amyloid-beta protein, and even insulin which promotes the rapid translocation of $GABA_A$ receptors from the intracellular compartment to the plasma

membrane in transfected human embryonic kidney cells. Other chemicals acting on $GABA_A$ receptors include nitric oxide, flavonoids, terpenoids, and many therapeutic agents [4].

Transepithelial solute transport and bicarbonate secretion are major functions of pancreatic duct cells and both functions are thought to involve the presence of chloride channels in the apical membrane of the cell. In the pancreatic adenocarcinoma cell line Capan-1, a high density of chloride-selective channels was identified. These cells express vasoactive intestinal peptide receptors associated with adenylyl cyclase that may be involved in the secretion of ions. There is also a high basal level of cAMP in Capan-1 cells that may account for the existence of ion transport in the absence of hormone stimulation [9]. In pancreatic tumor tissues, we identified a subunit of the $GABA_A$ receptor that may associate with other $GABA_A$ receptor subunits to form a functional chloride channel. $GABA_A$ receptor subunits were identified in the carcinoma cell line P19. Neurons derived from the embryonal carcinoma cell line P19 were found to express mRNAs for alpha, beta and gamma_2 subunits and to possess GABA receptor-activated chloride currents [10]. The association of malignancy with elevated diamine oxidase (DAO), an enzyme producing GABA, is well documented. Elevated urine GABA levels were observed in ovarian cancer patients providing evidence for the association of a GABA producing enzyme in malignant tissues and the possibility of functional GABA-activated chloride channels in these tissues [11].

Chloride channels regulated by different mechanisms have been associated with several types of cancer. The multidrug resistant gene (*MDR-1*) which produces the P-glycoprotein, a drug efflux pump, is often considered in relation to ion channels overexpressed in malignant tissues. The P-glycoprotein and the multidrug resistance associated protein (MRP) have been implicated in cancers that are highly resistant to chemotherapy such as pancreatic

carcinoma. Other anion channels have also been associated with cancer; in particular, the volume-sensitive chloride currents that are stimulated by cell swelling. In multidrug-resistant lung cancer cells chloride channels that do not require ATP and are not associated with *MDR-1* gene expression were observed [12]. Volume-sensitive chloride channels are also expressed in colon adenocarcinoma, transformed tracheal epithelium, and cervical cancer among others [13, 14].

In many epithelial cells, chloride channels are essential for the transport of salt and water across the membrane bilayer. Three distinct chloride currents, regulated by cAMP, Ca^{2+}, and cell volume have been demonstrated in airway epithelial cells and in the T84 colonic carcinoma cell line [14]. In cervical cancer, cell swelling was shown to activate ATP-dependent Cl^- currents but not in normal cervix suggesting that activation of volume-activated chloride currents is associated with malignant transformation of human cervical squamous epithelium. Volume-sensitive chloride channels have been reported to be important for regulation of cellular volume during mitosis and osmotic challenge, activation of the transport of amino acids and other organic substrates, and also are related to the cytoskeleton or motility of cells. From these studies it was suggested that activation of Cl^- channels might confer on the cells a selective advantage for continuous growth and survival [14].

Volume-regulated anion channels (VRAC) have been linked with cell cycle progression in cervical cancer. During cell cycle progression, cells undergo a significant increase in size (especially at the G1/S transition) that perturbs cell volume homeostasis and is counterbalanced by regulatory volume decrease. Several studies suggest that differential expression of K^+ channels and concomitant changes in membrane potential are critical for cell cycle checkpoints. Indeed, when the VRAC was investigated in cervical cancer, arrest of cell growth in G_0/G_1 phase was accompanied by a marked decrease of VRAC current density and that activity recovered upon re-entry into the cell cycle [15].

These studies implicate a role for anion channels in carcinogenesis. In the present study, we have observed overexpression of the $GABA_A$ receptor π subunit in pancreatic tumors. These findings are consistent with the original identification of the π subunit from a pancreatic carcinoma cDNA library [5]. The π subunit was overexpressed in tumors from all histopathological grades analyzed, including well to poorly differentiated tumors. This suggests that increased transcription of the π subunit gene is an early and sustained event in the tumorigenic process. Expression of this anion channel in tumor tissues indicates it may have a role in pancreatic carcinogenesis by as yet undiscovered mechanisms. Further studies will have to be performed to determine if other $GABA_A$ subunits are expressed which can interact with the π subunit, associate, and form a functional chloride channel. In addition, confirmation of increased levels of protein expression in these tumors must await the development of specific antisera directed against this GABA receptor subunit for immunohistochemical analysis of tumor tissues. This study, however, has provided a new avenue for study of pancreatic carcinogenesis by determining another mechanism by which pancreatic tumor cells may enhance proliferation and growth in this aggressive disease.

Arkansas for Medical Sciences; VRAC: volume-regulated anion channels

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