The overexpression of cytochrome c oxidase subunit 6C activated by Kras mutation is related to energy metabolism in pancreatic cancer

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Background: Kras mutation is frequently detected in pancreatic cancers and leads to altered energy metabolite. Here we investigated molecule markers related with Kras mutation, which could be used as developing new target for Kras mutant driven cancer.

Methods: A knockin BxPC-3/Kras<sup>G12D</sup> cell line was constructed by CRISPR/Cas9 system. Proliferation and metabolite characterization in BxPC-3/Kras<sup>G12D</sup> was compared with wild type BxPC-3 by using colony formation assay and mitochondrial dyes. The differential genes were screened using mitochondrial metabolite-related genes PCR array. The expression of COX6C was confirmed by real time polymerase chain reaction (RT-PCR) and western blot. COX6C expression in 30 pairs of tissue microarray of pancreatic carcinoma and matched adjacent tissues was analyzed by immunohistochemistry. ATP production stimulated by metabolites substrate assay was carried out to investigate whether the decreased COX6C by siRNA transfection affected the metabolite characterization of pancreatic cancer cells.

Results: The BxPC-3/Kras<sup>G12D</sup> displayed faster proliferation, increased mitochondrial mass and ATP, elevated mitochondrial membrane potential than BxPC-3. Using mitochondrial metabolite-related genes array, we identified COX6C was an up-regulated gene driven by Kras<sup>G12D</sup>. There was a notable difference of COX enzyme activity between BxPC-3 and BxPC-3/Kras<sup>G12D</sup>. The overexpression of COX6C was found in pancreatic cancer tissues. Using COX6C siRNA-mediated knockdown, the cell viability, COX enzyme activity and ATP production of BxPC-3/Kras<sup>G12D</sup> cells significantly decreased.

Conclusions: These results suggested that activation of Kras<sup>G12D</sup> in pancreatic cancer cells increased the COX activity and ATP production of mitochondrial via up-regulation of COX6C. This regulatory subunit of COX may have utility as a Ras effector target for development of anti-pancreatic cancer therapeutics.

Keywords: Kras; COX enzyme; mitochondrial; energy metabolite; pancreatic cancer

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Introduction

RAS proteins are molecular switches that control cell growth and proliferation. Ras mutations are found in approximately 20–30% of all human cancers including most commonly three types of mutations, Hras, Nras and Kras. Kras is the most frequently isoform detected mutation occurring in about 90% of pancreatic cancers, about 40% colon rectal cancer and lung cancer (1,2). Kras mutations are characterized by single base missense mutations, 98% of which are found at residues G12, G13 or Q61 (the predominant substitution is G12D) (3). Ras mutations activate downstream signaling can promote tumorigenesis. Moreover, Kras mutation cancers are resistant to current therapies and often accompanied by poor prognosis and low survival rate. Kras mutations not only promote aerobic glycolysis and glutamine metabolism reprogramming to provide energy (4,5), but also facilitate other branched metabolism pathways including autophagy and macropinocytosis (6,7). Since no directly targeted therapy currently exists for cancers with Kras mutations (8,9), targeting the enzymes involved in metabolic pathways may provide an alternative therapeutic approach for Kras mutation cancer.

In this study, we constructed a BxPC-3/Kras\textsuperscript{G12D} cell line by using CRISPR/Cas9 system. By searching differential genes correlated with bioenergy metabolite in a pair of cells between BxPC-3/Kras\textsuperscript{G12D} and wild type BxPC-3, we identified an up-regulated gene COX6C which encodes a subunits of the protein involved in respiration chain in mitochondrial inner membrane. We further characterized the function of COX6C in BxPC-3/Kras\textsuperscript{G12D} cells in the context of mitochondrial function and cell viability. Our finding indicates COX6C might be as a potential new protein driven by Kras\textsuperscript{G12D} mutant in pancreatic cancer.

Methods

Cell culture

The human pancreatic cancer BxPC-3 cell line (provided by the Cell Resource Center, Institute of Basic Medical Sciences, China) was cultured in RPMI-1640 medium (Hyclone; Thermo Fisher Scientific, Hudson, NH, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco; Life Technologies, Carlsbad, CA, USA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) under humidified conditions with 5% (v/v) CO\textsubscript{2} at 37 °C.

Establishment of stable Kras\textsuperscript{G12D} site mutation BxPC-3 cell line by CRISPR/Cas9

PCS-sgRNA (CRISPR/Cas9 vector) and TV-Kras\textsuperscript{G12D} (Targeting vector) were designed and constructed by Biocytogen Co. Ltd (Beijing, China). BxPC-3 was transfected TV-Kras\textsuperscript{G12D} and PCS-sgRNA by using the Neon transfection system (Life Technologies, Grand Island, NY). Transfected cells were selected in selective medium containing 200 ng/mL puromycin antibiotic for 3–5 days. Multiple monoclones were picked and cultured individually in separate wells.

Genotyping PCR

The puromycin-resistant clones were screened for homologous recombination by genomic PCR primers. The correct colony was detected Kras mutations by sequencing. The PCR fragments were directly sequenced or cloned into the pMD18-T (Takara, Japan) vector and then sequenced to identify Kras mutations.

Clonogenic Assay

Five hundred cells were seeded into each well of 6-well plate in triplicates, and colonies were stained 7–10 days later with 0.2% crystal violet in 80% methanol.

Anchorage-Independent Growth Assay

Ten thousand cells per well of 6-well plate were seeded in medium containing 1.6% Methyl cellulose (Sigma, M0512) with 10% fetal calf serum and plated over a layer of 0.9% agar-coated six-well plates. Standard medium (1 mL) was added to the top of the gelled matrix. After 15 days of culture, colonies were counted in five random three-dimensional fields per well.

Flow cytometry analysis of mitochondria mass and transmembrane potential

Cells were stained with 200 nM Mito Tracker Green (Invitrogen, Carlsbad, CA, USA) for 30 min to measure the mitochondrial mass and with 1 μM rhodamine-123 (30 min) to evaluate the mitochondrial transmembrane potential (MMP). Cells were washed with ice-cold phosphate-buffered saline (PBS) and kept on ice in the dark for immediate detection with a flow cytometry (FACS Aria II, BD Biosciences, CA, USA).
**Immunofluorescence and confocal microscopy**

Cells were cultured on sterilized glass slide covers until 70–80% confluence and incubated with 200 nM Mito Tracker Green for 30 min and with 5 μg/mL Hoechst 33342 for 15 min. Then the cells were washed with PBS fixed with 3.7% paraformaldehyde. The slide covers were mounted on glass slides. Images were taken by LSM 710 confocal microscope (Carl Zeiss AG, Oberkochen, German).

**Isolation of functional mitochondrial**

The crude mitochondrial fractions were obtained using differential centrifugation from cultured cells as described previously with some modification (10,11). Cells were suspended in 1 mL IBC buffer (10 mM Tris-MOPS, 200 mM Sucrose, pH=7.4). The suspension was homogenized 30-gauge needle of syringe. Cells were lysed by forceful passage of the homogenate with 5–10 strokes. Lysis was monitored microscopically. After cell disruption, an aliquot of the lysate was examined to ensure that 80% of the cells were lysed. The homogenate was centrifuged at 600 g for 10 min at 4 °C. The supernatant was centrifuged at 7,000 g for 10 min at 4 °C. The pellet was collected and washed with IBC buffer once. The mitochondrial suspension is ready to be used for functional analysis.

**Spectrofluorometry measurements of isolated mitochondrial mass**

The cells were stained by 200 nM Mito Tracker Green for 30 min and mitochondrial was isolated. The experimental work was performed by exciting Mito Tracker Green at 480 nm and detecting the peak of fluorescence emission at 515 nm.

**Western blot and qPCR**

Active Ras protein was measured using RAS active detection kit (Cell Signaling Technology, Danvers, MA, USA) according to instruction. For regular western blot, protein extracts were prepared with the ice-cold high efficiency RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). Twenty micrograms of each total protein were applied on 5–15% SDS-PAGE according to the molecular weight of detected proteins, then subjected to electrophoretic analysis and blotting. All primary antibodies (Cell Signaling Technology) were incubated overnight at 4 °C; and then incubated with peroxidase-coupled antibody at RT for 1 h which was used for detecting the primary antibody binding. Protein bands were visualized with an enhanced chemiluminescence kit (Merck Millipore).

For qPCR, cells were collected and total RNA was isolated with RNAiso Plus (Takara). qPCR was performed with SYBR Green (Takara). For COX6C mRNA expression, the primer sequences were: forward 5’-ctttgtataagtttcgtgtgg-3’ and reverse 5’-attcatgtgtcatagttcagg-3’.

**siRNA transfection**

The COX6C specific siRNA (RiboBio, Guangzhou, China) was transfected with Lipofectamine RNAiMAX (Life Technologies) in six-well plates. The siRNA sequence against COX6C was sense 5’-GGACCACAUUAGGAAGGUUTT-3’ and anti-sense 5’-AACCUUCCUAAUGGUCCAG-3’. The negative control siRNA was used as non-targeting control for all siRNA experiment. Following 48 h transfection, the cells subject to be detected by Western Blot or isolated mitochondrial.

**ATP quantification**

The detection of ATP from the isolated mitochondrial was determined based on luciferin/luciferase method with bioluminescent ATP determination assay kit (Molecular Probes, Bethesda, MD, USA) according to the manufacturer’s protocol.

**Gene array analysis**

The human mitochondrial energy metabolism PCR array (Qiagen, #PAHS-008Z, Hilden, Germany) was used, which includes 84 key genes involved in mitochondrial respiration. Briefly, total RNA was extracted from cells and cDNA was mixed with the SYBR Green qPCR Master Mix and loaded into a 96-well plate for qPCR analyses performed on an ABI7500 Fast PCR machine (Applied Biosystems, Foster City, CA, USA) following the instructions provided by the PCR array kit. qPCR data was analyzed using online software (Qiagen).

**Tissue microarray and immunohistochemistry staining**

The tissue microarray chips containing a total of 30 pairs of pancreatic carcinoma and matched adjacent tissues were
provided by Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). Briefly, the main experimental procedure is as follows: sections were proceeded as dewaxing, microwave antigen retrieval, endogenous peroxidase blocking, and then incubated with the COX6C antibody (TA506178, 1:100, OriGene, Rockville, MD, USA) overnight, and the second antibody for 0.5 h, respectively. Finally, the specimens were determined with TCA decontamination and hematoxylin staining.

**COX enzyme assays**

Isolated mitochondrial fraction was resuspended in 20 volumes of ice-cold resuspended buffer (25 mM potassium phosphate, 5 mM MgCl₂, 1 mM EDTA, 0.6 mM lauryl maltoside) with protease inhibitors. Samples were freeze-thawed three times. Protein concentration was determined according BCA kit. Homogenates were diluted 10× assay buffer and added to 96-well plate containing assay buffer and reduced 0.05 mM cytochrome c. Absorbance at 550 nm was followed for 30 min in a spectrophotometer (Beckman, Brea, CA, USA).

**Statistical analysis**

Results were expressed as mean ± SD. Comparisons were made between different treatments using unpaired Student’s t-test.

**Results**

**Fast proliferation characterization of BxPC-3/Kras⁶¹²D cells**

We generated a transgenic cell line which was induced Kras⁶¹²D by taking advantage of CRISPR/Cas9 genome modification in Kras wild type cells (BxPC-3). In this new cell line-BxPC-3/Kras⁶¹²D, G12D (12th amino acid glycine in Kras gene replaced with aspartate) was introduced to exon 1 locus. The endogenous Kras gene and therefore was expressed under the control of Kras promoter. By inserting Kras⁶¹²D into downstream of starting codon in BxPC-3 cells Kras locus, we expect Kras⁶¹²D to be expressed under the endogenous Kras transcriptional control. Briefly, a pair of vectors including sgRNA vector and knockin Kras⁶¹²D vector were mixed and electo-transfected into BxPC-3 cell. Anti-neomycin colonies were expanded. Subsequently genotyping PCR screening was to confirm the homologous recombination and include Kras⁶¹²D mutation site. The correct colonies were established and named as BxPC-3/Kras⁶¹²D cells. Activated Kras expression of BxPC-3/Kras⁶¹²D cells was detected by the GTP-bound GTPase pull down assay. In Figure 1A, activated Kras expression in BxPC-3/Kras⁶¹²D cells was higher than wild type BxPC-3. This indicated the Kras activity in BxPC-3/Kras⁶¹²D cells was increased by Kras mutation. BxPC-3/Kras⁶¹²D cells exhibit enhanced proliferative properties compared with wild type BxPC-3 by using colony formation assay (Figure 1B). Next, the anchorage-independent growth both of cells were examined using the soft agar assay. We observed that the activation of Kras⁶¹²D expression enabled an elevated anchorage independent growth capability of BxPC-3/Kras⁶¹²D cells compared with BxPC-3 (Figure 1C).

**Mitochondrial characterizations of BxPc-3/Kras⁶¹²D cells**

The fluorescence microscopy was used to observe the mitochondrial morphology. The wild type BxPC-3 cells staining with Mito Tracker Green were shown network connection. However, BxPC-3/Kras⁶¹²D cells were seen more disconnected (more dot-shaped) mitochondrial (Figure 2A). Western blot analysis showed that Drp-1 was up-regulated and Mfn-2 was down-regulated in BxPC-3/Kras⁶¹²D cells compared with the wild type BxPC-3 (Figure 2B). We further characterized mitochondrial mass in BxPC-3/Kras⁶¹²D cells. Fluorescence intensity of cells staining Mito Tracker Green was detected by flow cytometry. The mitochondrial mass in BxPC-3/Kras⁶¹²D cells was moderately increased (Figure 2C). The isolated mitochondrial labeled Mito Tracker Green was detected by spectrofluorometer. The fluorescence intensity of mitochondrial in BxPC-3/Kras⁶¹²D cells was much higher than that in wild type BxPC-3 (Figure 2D). Western blot analysis showed that overexpression of Tom22, a core component of the mitochondria outer membrane protein translocation pore, was up-regulated in BxPC-3/Kras⁶¹²D cells (Figure 2B). All these data showed that the mitochondrial mass was increased after Kras⁶¹²D activation. Mitochondrial membrane potential of cells staining with Rhodamine 123 was estimated by flow cytometry. BxPC-3/Kras⁶¹²D cells showed higher mitochondrial membrane potential than wild type BxPC-3 cells (Figure 2E). ATP production was estimated by isolated mitochondrial from both of the cells. ATP content showed similar basal levels in both cells, whereas which was increased after adding TCA metabolites. This indicated the isolated mitochondrial from the cells kept intact functional. The increased ATP content was average around 2 times after adding TCA.
COX6C overexpression activated by Kras mutation

Figure 1 Proliferation of BxPC-3/Kras\textsuperscript{G12D} cells. (A) RAS activity detection in wild type BxPC-3 cells and BxPC-3/Kras\textsuperscript{G12D} cells; (B) quantification of colony formation between wild type BxPC-3 cells and BxPC-3/Kras\textsuperscript{G12D} cells; (C) quantification of the anchorage-independent growth. Cells were suspended in methylcellulose to evaluate anchorage-independent growth potential over 15 days. All Data was with average of five random fields per dish from two cells. Data shown was mean ± SD. *, P<0.05 **, P<0.01 vs. wild type BxPC-3 cells.

cycle intermediates include citrate (Cit), \(\alpha\)-ketoglutarate (AKG), succinate (Suc), malate (Mal) and pyruvate (Pyr) compared with basal levels in wild type BxPC-3 cells (Figure 2F). However, ATP of isolated mitochondrial in BxPC-3/Kras\textsuperscript{G12D} cells was remarkably increased when AKG and Cit treatment. 4.5 folds were increased treated with AKG and 3.7 folds were increased treated with Cit.

Expression and activities of COX6C in BxPC-3/Kras\textsuperscript{G12D} cells

We employed the human mitochondrial energy metabolism PCR array to screen the differential energy metabolism related genes from the pairs of cells. Figure 3A is the scatter plot which compared the normalized expression of each gene on the array between two group cells (BxPC-3/Kras\textsuperscript{G12D} cells vs. wild type BxPC-3 cells). The central line indicated unchanged gene expression and the dotted lines indicated the 2-fold regulation threshold. Based on gene selection criteria (P<0.05 and fold change ≥ 2), there were 4 of 84 genes showed up-regulated expression and 4 genes showed down-regulated expression in BxPC-3/Kras\textsuperscript{G12D} cells compared with wild type BxPC-3 (Figure 3B). Gene expression profiles differed maximum between two cells was COX6C gene. The relative expression ratio of the mRNA of COX6C gene in both cells was analyzed by RT-PCR. The mRNA expression of COX6C in BxPC-3/Kras\textsuperscript{G12D} cells (18.2-fold elevation) was higher than in wild type BxPC-3 cells. We then detected the activities of cytochrome c oxidase. As shown in Figure 3D, mean enzymatic activity was increased by 35% in BxPC-3/Kras\textsuperscript{G12D} cells compared with wild type BxPC-3.

The expression level of COX6C via tissue microarray and immunohistochemistry

On the basis of the above gene microarray, we tested expression of COX6C through tissue microarray of pancreatic carcinoma and matched adjacent tissues. The scanning pattern image of COX6C was presented in Figure 4A. The evaluation standards were presented in Figure 4B. According to the evaluation standards, the classification of samples was shown in Figure 4C. Weak cytoplasmic COX6C IHC staining were shown in adjacent tissue group, and moderate to strong staining cytoplasmic COX6C IHC staining were shown in tumor group. COX6C immunoreactivity was significantly more intense and diffuse in tumor tissue compared with matched adjacent tissue. As shown in Figure 4D, the representative results between tumors and their matched adjacent tissues denoted significant difference. Evidently, it suggested that significant COX6C overexpression was identified in pancreatic cancer tissue compared with matched adjacent tissue.

Effect of COX6C siRNA on ATP production in BxPC-3/Kras\textsuperscript{G12D} cells

To investigate whether silencing COX6C expression could affect the metabolite characterization of cells, BxPC-3/Kras\textsuperscript{G12D} cells were transfected with COX6C siRNA for 48 h. As shown in Figure 5A, COX6C siRNA transfection...
Figure 2 Mitochondrial characterization of BxPC-3/Kras\textsuperscript{G12D} cells. (A) Mitochondrial morphology of BxPC-3/Kras\textsuperscript{G12D} cells. Mitochondria and nuclear were stained with Mito Tracker Green and Hochest 33342 and then fixed by 4% paraformaldehyde and photographed by confocal microscopy. Scale bar as 10 μm; (B) protein expression of BxPC-3/Kras\textsuperscript{G12D} cells and BxPC-3 cells. Protein expression was detected by immunoblotting with specific antibody. GAPDH was used as a loading control; (C) alteration of mitochondrial mass in BxPC-3/Kras\textsuperscript{G12D} cells. Cells were staining with Mito Tracker Green and fluorescent intensity of Mito Tracker Green were detected by flow cytometry; (D) Fluorescent spectrometer detection of isolated mitochondrial from both of cells labeled by Mito Tracker Green; (E) flow cytometry analysis of the change of mitochondrial transmembrane potential in BxPC-3/Kras\textsuperscript{G12D} cells and BxPC-3 cells staining rhodamine 123; (F) alterations of production of ATP in isolated mitochondrial from BxPC-3/Kras\textsuperscript{G12D} cells and BxPC-3 cells by incubated with substrate. Mitochondrial was isolated from 10×10\textsuperscript{7} cells and incubated with 10 mM substrate for 15 min at 37 °C. The ATP was measured by bioluminescent assay. Relative increased ATP abundance was expressed as normalized the Bioluminescence (AU) with substrate to bioluminescence without substrate. Error bar indicate SD (n=3). Data was shown as mean± SD from triplicate experiments. **, P<0.01 vs. wild type BxPC-3 cells. Cit, Citrate; AKG, a-ketoglutarate; Mal, malate; Suc, succinate; Pyr, pyruvate.
COX6C overexpression activated by \textit{Kras} mutation

decreased significantly COX6C levels compared with the negative siRNA control. Cells viability was inhibited by COX6C siRNA compared with the negative siRNA control. (Figure 5B). Enzyme activity of COX decreased significantly in COX 6C siRNA transfected BxPC-3/\textit{Kras}^{G12D} cells (Figure 5C). We further investigated whether silencing COX6C expression could affect the ATP production of mitochondrial. Both of cells were transfected with COX6C siRNA for 48 h, mitochondria were isolated and incubated with 10 mM different substrates for 15 min at 37 °C in dark 96-well plates. The amount of ATP generated from mitochondria was quantified using ATP determination kit. As shown in Figure 5D, there are no statistical significance in endogenous mitochondria BxPC-3/\textit{Kras}^{G12D} cells compared with wild type BxPC-3 cells (ATP generation from residual endogenous substrates, no exogenous substrate added). However, when treated with Cit or AKG, mitochondria isolated from BxPC-3/\textit{Kras}^{G12D} cells produced a significant amount of ATP increase. When transfected with COX6C siRNA, there was a significant decrease of ATP production

\textbf{Figure 3} COX6C was up-regulated expression in \textit{Kras}^{G12D} driven cells. (A) The scatter plot compared the normalized expression of every gene on the array between the BxPC-3/\textit{Kras}^{G12D} vs. wild type BxPC-3. The central line indicated unchanged gene expression. The dotted lines indicated the fold regulation threshold (±2). Data points beyond the dotted lines in the upper left and lower right sections meet the selected fold regulation threshold (left); (B) fold change of changed genes in BxPC-3/\textit{Kras}^{G12D} compared with wild type BxPC-3; (C) protein expression of COX6C was measured in both cells by western blot analysis. Equally amount of whole cell lysate was loading. GAPDH was used as a loading control; (D) cytochrome c oxidase (COX) activity in BxPC-3/\textit{Kras}^{G12D} and with wild type BxPC-3. Data was mean ± SD of COX enzymatic activity measured by following the oxidation of ferrocytochrome c at 550-540 nm in mitochondrial lysate. *, P<0.05 vs. wild type BxPC-3 cells.
in BxPC-3/Kras<sup>G12D</sup> cells and slightly decrease in wild type BxPC-3 cells.

**Discussion**

There are newer reviews on the contribution of Ras mutations and altered Ras signaling in cancers, and in pancreatic cancer in particular (12). There are still no specific therapies for directly targeting mutant RAS protein currently available in clinics (13). Targeting Ras downstream pathway is currently strategy. Kras mutation has been linked to many alterations of metabolism-related characteristics.
These metabolic adaptations including the Warburg effect have been recognized as one of the hallmarks of cancer (14). Taken together, the alteration of metabolic adaptations of Ras-mutant cancer cell may provide novel therapeutic opportunities.

Normally, different Kras mutation cells lines are independently derived cell lines that differ in many phenotypic and genetic properties. In this study, by taking advantage of the CRISPR/Cas9 system, we generated a KrasG12D knockin cell line. Compared with wild type BxPC-3, BxPC-3/KrasG12D maintained maximum genetic similarity besides the specific Kras mutation site, which allowed us to explore the precise alteration driven by Kras mutation.

It is known mitochondria is the highly dynamic organelle that constantly changes shape and structure in response to different metabolic demands of the cells. BxPC-3/KrasG12D cells led to rapid mitochondrial division, and expression of Drp1 compared with the wild type BxPC-3, which indicates KrasG12D activation leads to going through more mitochondrial division.

We searched the differential genes between the wild type BxPC-3 and BxPC-3/KrasG12D cells by screening human mitochondrial energy metabolism PCR array. In all the tested 84 key genes involved in mitochondrial respiration, the COX6C is the highest overexpressed gene driven in BxPC-3/KrasG12D cells. COX, as one of the electron-driven proton pumps of oxidative phosphorylation, plays a critical role in energy metabolism (15,16). COX is encoded by two genomes: 10 nuclear-encoded subunits and three subunits encoded by mitochondrial DNA (17,18). Alteration a single enzyme complex (COX) is commensurate with tumor-altered metabolism (19,20). Varieties of COX subunits have
been connected with cancer. The expression of cytochrome c oxidase subunit is overexpressed in colon cancer (21,22). The COX5A play a role in invasion of lung cancer (23). Functional effects of altered cytochrome c oxidase are related to prostate cancer (24,25).

In our study, COX6C is found not only the highest up-expression gene among all the 84 detected mitochondrial respiration related genes, but also the only up-expression gene among all the 14 of COX genes. COX6C as one of the subunits of COX holoenzyme is encoded by a nuclear gene. COX6C protein involves in the respiration chain in the mitochondrial inner member. Enhanced expressions of COX6C in prostate carcinoma cancer have been described (26). However, limited literature has been found to report its biology function with cancer. Here, we first provided evidence that COX6C was up-regulated expression in both mRNA and protein level in \( \text{Kras}^{G12D} \) driven cells. Similar result of linkage COX and Ras mutation has also been reported that activation of HRas (V12) increases COX activity and mitochondrial respiration in part via up-regulation of COX Vb (20). The high expression of COX6C in pancreatic cancer tissue compared with that adjacent normal tissue provided strong evidence allowing us to determine its value as a marker related to energy metabolite. COX6C knockdown significantly decreased ATP production treated with Cit (60% decrease) and with AKG (40% decrease) in isolated mitochondrial from BxPC-3/\( \text{Kras}^{G12D} \) cells. Although the expression levels of COX subunits differ between two cells, the assays for COX activity we employed reflect differences in COX holoenzyme levels between two cell lines. The other subunits involved in COX activity detected in our PCR array, 3 of them are down-regulated and other genes weren’t affected in BxPC-3/\( \text{Kras}^{G12D} \) cells. These findings suggested that expression of COX6C could play a crucial role in elevated COX activity and ATP production in BxPC-3/\( \text{Kras}^{G12D} \) cells.

Our studies carry out that activation of \( \text{Kras}^{G12D} \) in pancreatic cancer cells increases the COX activity and ATP production of mitochondrial via up-regulation of COX6C. These results indicate this regulatory subunit COX6C may have utility as a Ras effector target for development of anti-pancreatic cancer therapeutics, especially against tumors in which the RasG12D driven singling pathway is activated.

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**Footnote**

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

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