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DOMINANT-NEGATIVE CONSTRUCTS OF HUMAN 6-PHOSPHOFRUCTO-2-KINASE/ FRUCTOSE-2,6-BISPHOSPHATASE-3 AND -4: EFFECT ON THE EXPRESSION OF ENDOGENOUS 6-PHOSPHOFRUCTO-2-KINASE/FRUCTOSE-2,6-BISPHOSPHATASE mRNA

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Expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB), a key regulatory enzyme of glycolysis, is significantly increased in different malignant tumors provides a potential mechanism of enhanced glycolysis and cancer cell proliferation. We created dominant-negative constructs of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 and -4 (dnPFKFB-3 and dnPFKFB-4) cDNA for suppression of strongly enhanced expression endogenous PFKFB-3 and PFKFB-4. We introduce point mutation in ATP-binding domain of 6-phosphofructo-2-kinase part of PFKFB-3 as well as PFKFB-4 cDNA for suppression of 6-phosphofructo-2-kinase activity in the products of dnPFKFB-3 and dnPFKFB-4 expression. Cancer cells were stable transfected with these dominant-negative constructs for suppression of endogenous PFKFB-3 and PFKFB-4 expression and cell proliferation. We have shown that PFKFB-3 expression in pancreatic cancer cell line Panc1, stable transfected by dnPFKFB-3, was significantly reduced in normal as well as in hypoxic conditions. Pancreatic cancer cells proliferation, stable transfected by dnPFKFB-3 or dnPFKFB-4, was also reduced. Results of this investigation demonstrate possibility to apply the dominant-negative constructs of PFKFB-3 and PFKFB-4 for suppression of glycolysis and tumor cells proliferation via reduction of endogenous PFKFB expression.

Key words: PFKFB-4 mRNA, PFKFB-3 mRNA, dominant-negative constructs, cancer cells.

The homodimeric bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase [6-phosphofructo-2-kinase (EC 2.7.1.105); fructose-2,6-bisphosphatase (EC 3.1.3.46)] has both kinase and bisphosphatase activities and catalyses the synthesis and degradation of fructose-2,6-bisphosphate, a signal molecule that control glycolysis [1–4]. Moreover, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) is a key enzyme in the regulation of glycolysis as well as gluconeogenesis in normal and different pathological conditions, in particular in malignant tumors [4–10]. X-ray crystallography shown that 6-phosphofructo-2-kinase domain has the same fold as adenylate kinase [11]. The fructose-2,6-bisphosphatase domain of the enzyme subunit bears sequence, mechanistic and structural similarity to the histidine phosphatase family of enzymes, including

the acid phosphatases and phosphoglycerate mutases [5, 11]. Therefore, fructose-2,6-bisphosphate plays a unique role in the control of glucose homeostasis by allowing the liver to switch from glycolysis to gluconeogenesis [4, 5, 7]. There are four 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoenzymes (PFKFB-1, PFKFB-2, PFKFB-3 and PFKFB-4) in mammals, each coded by a different gene (*pfkfb*1, *pfkfb*2, *pfkfb*3 and *pfkfb*4). Moreover, these genes express several isoforms of each isoenzyme with different kinetic and regulatory properties [4, 5, 12, 13]. It was shown that different tissues as well as cancer cell lines express more than one isoform [13–16]. This multiple expression of PFKFB isozymes with different properties suggests that each isoenzyme can play a specific role in the regulation of glycolysis in some physiologic and different pathophysiological conditions.

Overexpression of PFKFB isozymes as well as enhanced glycolysis is observed in most malignant tumors and in different cancer cell lines and significantly increases in hypoxic conditions [13–19]. The metabolism within a solid tumor is significantly different from that of the surrounding normal tissue. Tumors growing under conditions of normal oxygen tension show elevated glycolytic rates, produce high levels of lactate and pyruvate (the Warburg effect) that correlate with the increased expression of glycolytic enzymes and glucose transporters via HIF-1 dependent mechanism [1–4, 20–25]. In tumor over 50% of the cellular energy is produced by glycolysis with the remainder being generated at the mitochondria. The reliance of tumor cells on glycolysis for energy production causes them to consume more glucose because of the low efficiency of glycolysis in generating ATP [23]. Thus, glycolysis is essential for tumor survival and spread. Moreover, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase has a key role for the neoplastic transformation and provide rationale for the development of agents that selectively inhibit the PFKFB-3 enzyme as antineoplastic agents. Moreover, hypoxia is a common feature of many cancers and has been linked to malignant transformation, metastasis, and treatment resistance [23–27]. Most of tumors are subjected to hypoxic conditions due to the abnormal vasculature that supply them with oxygen and nutrients. Transcription complex HIF-1 is overexpressed in a variety of tumors and its expression appears to correlate with poor prognosis and responses to chemo- or radiotherapy. The most PFKFB isozymes are contribute to *de novo* nucleic acid synthesis in tumor cells, are uniformly increased in the malignant tissues and provide a potential mechanism to explain the apparent coupling of enhanced glycolysis and cell proliferation [6, 7, 17–19, 28, 29].

Previously, we have shown that the *pfkfb*4 gene is overexpressed in several cancer cell lines and is highly induced by hypoxia via HIF-1 dependent mechanism [14].

The *pfkfb*3 gene encoded 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isozyme ubiquitously expressed in different organs and tumor cells [8, 13–15, 17–19]. Several alternative splice variants of PFKFB-3 mRNA with variable C-terminus were identified in human brain and different rat and mouse tissues [30–35]. There were isolated eight isoforms of PFKFB-3 mRNA from brain and some other tissues. The cDNA sequences encoding the 5′-untranslated region, the amino-terminal domain, and the catalytic core domain were identical among all these isoforms. However, heterogeneity of the carboxyl-terminus was found by sequence analysis.

Many genes whose expression is regulated by hypoxia are overexpressed in malignant tissues and cell lines and contain HIF-1 binding site (hypoxia-responsible element, HRE) [14, 16, 36–38]. HRE was recently identified in *pfkfb*3 and *pfkfb*4 genes [14, 39–41]. Transcription factor HIF is central in coordinating many of the transcriptional adaptations to hypoxia and a necessary mediator of the hypoxic effect as well as Pasteur effect in mammalian cells and Warburg effect in tumors [29, 37, 42, 43]. Thus, targeting PFKFB enzymes, either directly or through inhibition of HIF-1, appears as a promising approach for the treatment of certain tumors.

Despite importance of PFKFB-3 and PFKFB-4 in the regulation of glycolysis, the dominant-negative constructs of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 and -4 cDNA were not been created and used yet for suppression of glycolysis. In this work we have created dnPFKFB-3 and dnPFKFB-4 constructs and studied the expression of endogenous PFKFB-3 and PFKFB-4 mRNA in pancreatic cancer cells stable transfected with these constructs.

Materials and methods

Cell lines. Human pancreatic cancer cell lines Pank1 and PSN-1 were obtained from the American Type Culture Collection (ATCC,

USA) and grown according to the supplier's protocols. The cells were incubated at 37 °C before harvesting under normoxic (21% oxygen and 5% carbon dioxide) or hypoxic conditions, which were modulated by dimethyloxalylglycine (1 mM during 6 hours) [15].

RNA isolation. Total RNA was extracted using Trizol reagent according to the manufacturer's protocols (Invitrogen, USA). RNA pellet was washed with 75% ethanol, dissolved in nuclease-free water and used for reverse transcription.

Synthesis and cloning of PFKFB-3 and PFKFB-4 cDNA. The human PFKFB-3 and PFKFB-4 cDNA was synthesized by RT-PCR using total RNA from human pancreatic cancer cell lines Pank1 and oligo(dT). For first-strand cDNA synthesis was used Sensiscript RT Kit (QIAGEN, Germany). Human PFKFB-3 PCR amplification was performed with the following oligonucleotides: 5′-GATGCCGTTGGAACTGACGC-3′ (forward primer) and 3′-CTGAGGCAGACGTGTCGGTT-5′ (reverse primer) using HotStarTaq Master Mix Kit (QIAGEN). These oligonucleotides correspond to nucleotide sequences 329–348 and 1889–1908 of human PFKFB-3 mRNA (GenBank accession number NM_004566). The amplification of human PFKFB-4 was performed with the following oligonucleotides: 5′-GATGGCGTCCCCACGGGAATTG -3′ (forward primer) and 3′-GCTCACCAGTGACCATGTTC-5′ (reverse primer) using HotStarTaq Master Mix Kit (QIAGEN). These oligonucleotides correspond to nucleotide sequences 17–38 and 1416–11435 of human PFKFB-3 mRNA (GenBank accession number NM_004566). The PFKFB-3 and PFKFB-4 cDNA were cloned into pCRII-TOPO (Invitrogen, USA) vector as described previously [14] and used for creation of dominant-negative constructs (dnPFKFB-3 and dnPFKFB-4). These constructs were verified by sequencing the insert in the plasmid. Sequence analysis was performed using ABI Prism (Model 3100, version 3.7). The dnPFKFB-3 and dnPFKFB-4 cDNA were recloned into pcDNA3.1 (Invitrogen, USA) and used for transfection assays.

The expression of PFKFB-3 mRNA in Panc1 cells was examined by ribonuclease protection assays as described previously [15, 46], but probes corresponds to 3′-region (3193–3540; GenBank accession number NM_004566). The 18S rRNA antisense probe was used to ensure equal loading of the sample of total RNA. The expression of PFKFB-3 and PFKFB-4 mRNA in Pst-1 cells was examined by real time RCR analysis assays. Quantitative PCR was performed on «Stratagene Mx 3000P cycler», using SYBRGreen Mix as described previously [47]. Reaction was performed in triplicate. Analysis of quantitative PCR was performed using special computer program «Differential expression calculator» and statistic analysis — in Excel program.

Results and Discussion

We synthesized the human PFKFB-3 and PFKFB-4 cDNA using total RNA from human pancreatic cancer cell line Panc1. Both cDNA were amplified and cloned into pCRII-TOPO vector. For inactivation of 6-phosphofructo-2-kinase we changed four nucleotide residues in domain «A» using special primers. As shown in Fig. 1 and 2, this nucleotide replacement in

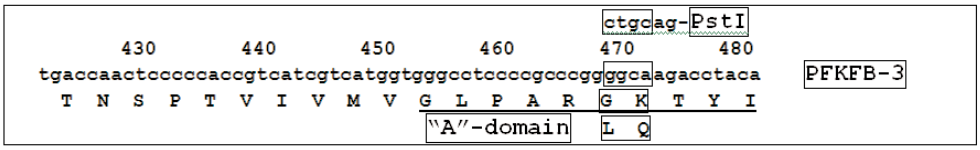

Fig. 1. **Fragment of 6-phosphofructo-2-kinase part of human PFKFB-3 cDNA (GenBank accession number NM_004566), containing domain «A» (underlined). Four nucleotide residues (GGCA) into domain «A» were replaced by CTGC. These changes create new restriction site (PstI) and replace two amino acids residues: G and K**

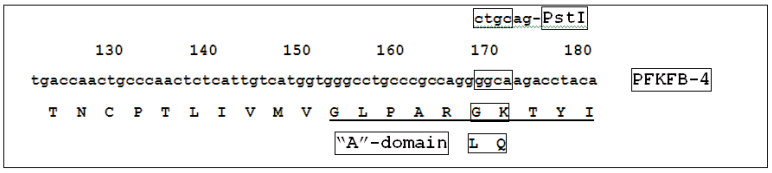

Fig. 2. **Fragment of 6-phosphofructo-2-kinase part of human PFKFB-4 cDNA (GenBank accession number NM_004567), containing domain «A» (underlined). Four nucleotide residues (GGCA) into domain «A» were replaced by CTGC. These changes create new restriction site (PstI) and replace two amino acids residues: G and K**

PFKFB-3 and PFKFB-4 creates new restriction site (PstI) and changes two amino acids residues: G to L and K to Q. These changes should suppress the 6-phosphofructo-2-kinase activity because K residue is crucial for keeping kinase activity. These modified PFKFB-3 and PFKFB-4 cDNA represent dominant-negative variants in respect to 6-phosphofructo-2-kinase activity.

We recloned modified human PFKFB-3 and PFKFB-4 cDNA into eukaryotic expression vector pcDNA3.1(+) using EcoRI and XbaI restriction nuclease sites. Structure of dominant-negative construct of PFKFB-3 (dnPFKFB-3) shown on Fig. 3. Same structure has dnPFKFB-4. Both dominant-negative constructs were used for transfection experiments. We received clone of Panc1 cells stable transfected by dnPFKFB-3.

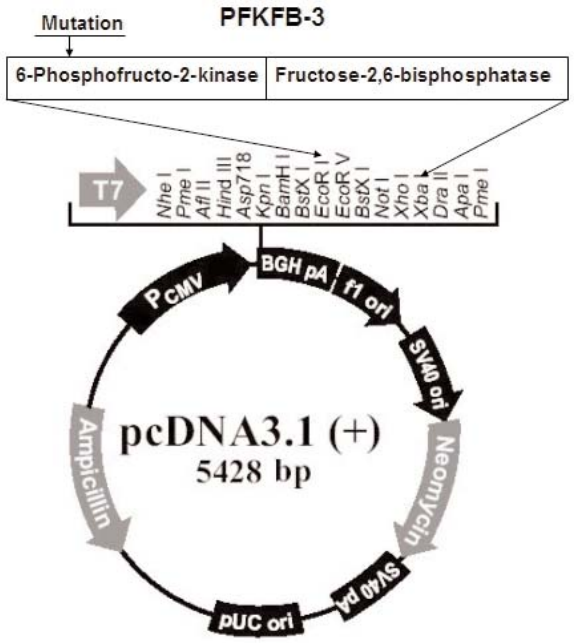

Fig. 3. **Dominant-negative plasmid construction of human PFKFB-3 cDNA, which contains PFKFB-3 cDNA with modified 6-phosphofructo-2-kinase domain «A» into pcDNA3.1(+) vector**

The effect of PFKFB-3 dominant-negative construct on the expression of endogenous PFKFB-3 mRNA is shown in Fig. 4. For this analysis was used 3′-terminus of PFKFB-3 mRNA because dnPFKFB-3 construct does not contains this region of PFKFB-3. Pancreatic cancer cells, stable transfected by dnPFKFB-3, have significantly lower expression of endogenous PFKFB-3 mRNA as in normoxic and hypoxic conditions, which were modulated by dimethyloxalylglycine (Fig. 4 and 5).

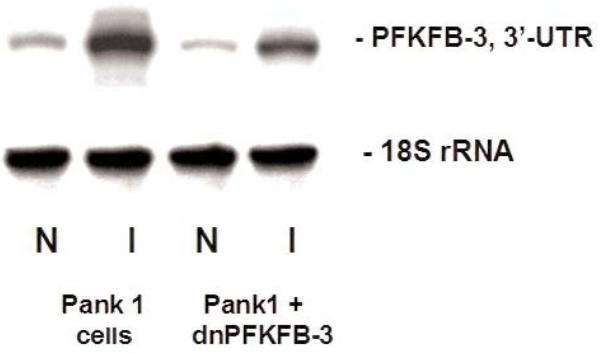

Fig. 4. **Endogenous PFKFB-3 mRNA expression in pancreatic carcinoma cell line Panc1 stable transfected by pcDNA3.1(+) vector (Panc1 cells) and stable transfected by dnPFKFB-3 (Panc1 + dnPFKFB-3) in normoxic (N) and hypoxic conditions (I), which were modulated by dimethyloxalylglycine (1 mM during 6 hours). The 18S rRNA antisense probe was used to ensure equal loading of the sample of total RNA**

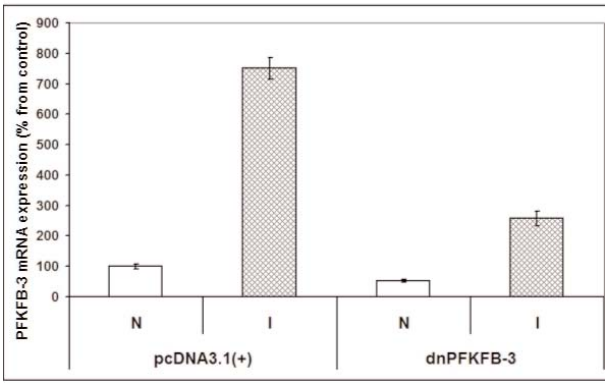

Fig. 5. **Quantification of endogenous PFKFB-3 mRNA expression in pancreatic carcinoma cell line Panc1 stable transfected by pcDNA3.1(+) vector or dnPFKFB-3 in normoxic (N) and hypoxic (I) conditions; $n = 5$**

PFKFB-3 and PFKFB-4 mRNA expression was also measured in other pancreatic carcinoma cell line PSN-1 stable transfected by pcDNA3.1(+) vector, dnPFKFB-3 or dnPFKFB-4. Results of these experiments are shown on Fig. 6. PSN-1 cells transfected with dnPFKFB-3 shown lower level of endogenous PFKFB-3 mRNA expression. Moreover, the expression of PFKFB-4 mRNA in these cells also decreased. It is possible that there is some interaction between different PFKFB genes in molecular mechanisms of PFKFB suppression by dominant-negative constructs. Same effect has dnPFKFB-4 on the of endogenous PFKFB-4 and PFKFB-3 mRNA.

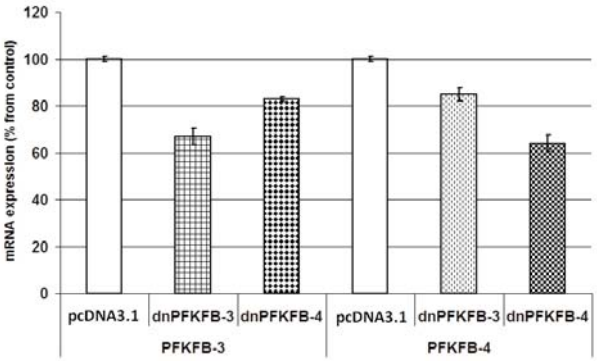

Fig. 6. **Endogenous PFKFB-3 and PFKFB-4 mRNA expression in pancreatic carcinoma cell line PSN-1 stable transfected with pcDNA3.1(+) vector, dnPFKFB-3 and dnPFKFB-4**

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ДОМІНАНТНЕГАТИВНІ КОНСТРУКЦІЇ 6-ФОСФОФРУКТО-2-КІНАЗИ/ФРУКТОЗО-2,6-БІСФОСФАТАЗИ-3 ТА -4 ЛЮДИНИ: ВПЛИВ НА ЕКСПРЕСІЮ ЕНДОГЕННИХ мРНК 6-ФОСФОФРУКТО-2-КІНАЗИ/ ФРУКТОЗО-2,6-БІСФОСФАТАЗ

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Експресія 6-фосфофрукто-2-кінази/фруктозо-2,6-бісфосфатази (PFKFB), ключового регуляторного ензиму гліколізу, різко зростає в різних злоякісних пухлинах, що розкриває можливий механізм посиленого гліколізу в ракових клітинах та їх проліферації. Ми створили домінантнегативні конструкції кДНК 6-фосфофрукто-2-кінази/фруктозо-2,6-бісфосфатази-3 та -4 (dnPFKFB-3 та dnPFKFB-4) для пригнічення посиленої експресії ендогенних PFKFB-3 та PFKFB-4. Для цього вводили точкові мутації в АТР-зв'язувальний домен 6-фосфофрукто-2-кінази як PFKFB-3, так і PFKFB-4 кДНК для пригнічення 6-фосфофрукто-2-кіназної активності у продуктах експресії цих конструкцій. Проводили трансфекцію ракових клітин цими домінантнегативними конструкціями для пригнічення експресії ендогенних PFKFB-3 і PFKFB-4 та проліферації клітин. Встановлено, що експресія PFKFB-3 в клітинах карциноми підшлункової залози лінії Panc1, стабільно трансфекованих dnPFKFB-3, знижується як у нормальних умовах, так і за гіпоксії. У клітинах з посиленою експресією dnPFKFB-4 спостерігали пригнічення ендогенних як PFKFB-4, так і PFKFB-3. Проліферація клітин карциноми підшлункової залози, стабільно трансфекованих як dnPFKFB-3, так і dnPFKFB-4, знижується. Результати цих досліджень показують можливість використання домінантнегативних конструкцій PFKFB-3 та PFKFB-4 для зменшення інтенсивності гліколізу та проліферації ракових клітин шляхом зниження експресії ендогенних PFKFB.

Ключові слова: PFKFB-4 мРНК, PFKFB-3 мРНК, домінантнегативні конструкції, ракові клітини.

ДОМИНАНТНЕГАТИВНЫЕ КОНСТРУКЦИИ 6-ФОСФОФРУКТО-2-КИНАЗЫ/ФРУКТОЗО-2,6-БИСФОСФАТАЗЫ-3 И -4 ЧЕЛОВЕКА: ВЛИЯНИЕ НА ЭКСПРЕССИЮ ЭНДОГЕННЫХ мРНК 6-ФОСФОФРУКТО-2-КИНАЗЫ/ФРУКТОЗО-2,6-БИСФОСФАТАЗ

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Экспрессия 6-фосфофрукто-2-киназы/фруктозо-2,6-бисфосфатазы (PFKFB), ключевого регуляторного энзима гликолиза, резко возрастает в различных злокачественных опухолях, что раскрывает возможный механизм усиленного гликолиза в раковых клетках и их пролиферации. Мы создали доминантнегативные конструкции кДНК 6-фосфофрукто-2-киназы/фруктозо-2,6-бисфосфатазы-3 и -4 (dnPFKFB-3 и dnPFKFB-4) для угнетения усиленной экспрессии эндогенных PFKFB-3 и PFKFB-4. Для этого вводили точечные мутации в АТР-связывающий домен 6-фосфофрукто-2-киназы как PFKFB-3, так и PFKFB-4 для угнетения 6-фосфофрукто-2-киназной активности в продуктах экспрессии этих конструкций. Проводили трансфекцию раковых клеток этими доминантнегативными конструкциями для угнетения экспрессии эндогенных PFKFB-3, а также пролиферации клеток. Установлено, что экспрессия PFKFB-3 в клетках карциномы поджелудочной железы линии Panc1, стабильно трансфецированных dnPFKFB-3, снижается как в нормальных условиях, так и при гипоксии. В клетках с усиленной экспрессией dnPFKFB-4 наблюдали угнетение эндогенных как PFKFB-4, так и PFKFB-3. Пролиферация клеток карциномы поджелудочной железы, стабильно трансфецированных как dnPFKFB-3, так и dnPFKFB-4, снижается. Результаты этих исследований показывают возможность использования доминантнегативных конструкций PFKFB-3 и PFKFB-4 для уменьшения интенсивности гликолиза и пролиферации раковых клеток путем снижения экспрессии эндогенных PFKFB.

Ключевые слова: PFKFB-3 и PFKFB-4 мРНК, доминант-негативные конструкции, раковые клетки.