УДК 577.112.7:616-006.04:616.24

6-PHOSPHOFRUCTO-2-KINASE/FRUCTOSE-2,6-BISPHOSPHATASE GENE FAMILY OVEREXPRESSION IN HUMAN LUNG TUMOR

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Вивчали експресію різних форм 6-фосфофруктo-2-кінази/фруктозо-2,6-бісфосфатази (ФФКФБ) в злоякісних пухлинах легень людини. Виявлена посилена експресія ФФКФБ-1, -2, -3 and -4 мРНК в злоякісних пухлинах порівняно з нормальною тканиною легень, взятою від тих самих пацієнтів. Методом імуноблотінгу показано значне збільшення рівня ФФКФБ-4 і -3. Встановлено, що експресія ФФКФБ-2, -3 та -4 мРНК в A_{549}-клітинах карциноми легень також збільшена порівняно з нормальною тканиною легень і різко посилюється в умовах гіпоксії. Результати дослідження свідчать про посилену експресію ізоферментів ФФКФБ і можливу роль їх в ефекті Варбурга.

Ключові слова: мРНК ФФКФБ-1-4, білок ФФКФБ-4, злоякісні пухлини, легені, A_{549} клітини карциноми легень.

Tumor cells, growing under conditions of normal oxygen tension, show elevated glycolytic rates, produce high levels of lactate and pyruvate (the Warburg effect), which correlates with an increased expression of glycolytic enzymes and glucose transporters via a hypoxia-inducible factor (HIF) dependent mechanism [1–3]. Hypoxia is a common feature of many cancers and has been linked to malignant transformation, metastasis, and treatment resistance [4]. Hypoxia is one of the most potent inducers of gene expression especially genes involved in glycolysis for maintaining cellular energy [5,6]. This change from aerobic respiration to glycolysis is essential for cell survival in hypoxic conditions. A key regulator of glycolytic flux is fructose-2,6-bisphosphate, an allosteric activator of 6-phosphofructo-1-kinase [7,8]. Steady-state levels of fructose-2,6-bisphosphate are maintained by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase [PFKFB; 6-phosphofructo-2-kinase (EC 2.7.1.105); fructose-2,6-bisphosphatase (EC 3.1.3.46)], which has both kinase and phosphatase activities. There are four different genes coding different isozymes 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB-1, -2, -3 and -4), which differ not only in their tissue distribution, but also in their kinetic and regulatory properties [9,10]. Importantly, tissue-specific isoforms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase are not completely exclusive and several tissues express more than one isoforms [11–14]. Since, the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase catalyses the synthesis and degradation of fructose-2,6-bisphosphate, it controls glycolysis and plays a significant role in the Warburg effect [14–16]. This multiple expression of isozymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase with different kinetic and regulatory properties suggests that each isozyme plays a key role in the regulation of glycolysis in different physiologic or pathophysiologic conditions.

Recently, we have shown that in vivo hypoxia leads to a significant mRNA increase of four isozymes (PFKFB-1, -2, -3 and -4) in an organ-specific manner [12]. The PFKFB-4 gene is expressed in several cancer cell lines and is highly induced by hypoxia by HIF-1 dependent mechanism [13]. The PFKFB-3 gene encoded 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isozyme ubiquitously expressed in different organs and tumor cells [14,15,17]. The inducible isozyme of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (iPFKFB-3 or iPFK-2), which contributes to de novo nucleic acid synthesis in tumor cells, is uniformly increased in the malignant tissues and provides a potential mechanism to explain the apparent coupling of enhanced glycolysis and cell proliferation [15–18]. 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 [14,17,19–22]. Transcription factor HIF

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is a central one 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 [3,22–26].

Despite its importance in the regulation of glycolysis and gluconeogenesis, the expression of different forms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB-1, -2, -3 and -4) in the lung malignant tumors has not been characterized. We report herein that the expression of PFKFB-1, PFKFB-2, PFKFB-3 and PFKFB-4 uniformly increased in the lung malignant tumors as compared to corresponding control tissues and that all isozymes of PFKFB possibly play a significant role in the Warburg effect.

Materials and Methods

Frozen human breast solid malignant tumors (from 20 patients) and non-malignant tissue counterparts from the same patients were obtained from the National Cancer Center Hospital East (Kashiwa, Japan). A_{549} lung adenocarcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and grown according to manufacturer protocols. For hypoxic stimulation, the cultures were exposed in 1% oxygen, 5% carbon dioxide and 94% nitrogen for six hours.

Total RNA was extracted from normal and tumor tissue as well as from the A_{549} cells using Trizol reagent according to manufacturer protocol (Invitrogen, Carlsbad, CA, USA). RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water.

The plasmids for synthesis of human PFKFB-1, 2, -3 and -4, glucose transporter-1 (Glut1), vascular endothelial growth factor (VEGF) and 18 S rRNA probe for ribonuclease protection assays were described previously [12–14,27]. The synthesis of radiolabeled probes and ribonuclease protection assay was carried out as described previously [13]. The expression of mRNA was quantified using Fujix BAS 2000 Bio-Image Analyzer (Fuji Photo Film Co.). Intensity of each mRNA band was normalized for 18 S ribosomal RNA levels. PFKFB-1 mRNA expression in the lung cancers and normal tissues counterparts were examined using RT-PCR. PCR fragments were separated in agarose gel and stained with ethidium bromide.

Western blot analysis was carried out as described previously [13]. For detection of PFKFB-4 a rabbit polyclonal anti-PFKFB-4 antibody was used with a dilution of 1:10,000 [13]. For detection of PFKFB-3 we used a goat polyclonal anti-PFKFB-3 [PFK-2br/pl (N-11); sc-100890; a dilution of 1:1,000] antibody from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Horseradish peroxidase-conjugated anti-rabbit, anti-goat or anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a secondary antibody with a dilution of 1 : 5,000. The β-actin was used to ensure equal loading of the sample. The results are expressed as mean ± standard error of the mean (SEM) of three or more independent experiments. Comparison of two means was performed by the use of unpaired Student's *t* test. Statistical significance was assumed at a value of $p < 0.05$.

Results and Discussion

Using ribonuclease protection assays, we examined the expression of four different isozymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in the human lung cancers and corresponding normal tissues counterparts from 20 patients. As shown in Fig. 1*A*, there is a significant increase in the expression of PFKFB-2, PFKFB-3 and PFKFB-4 mRNA in the lung cancers as compared to corresponding normal tissue counterparts. It is interested to note, that the expression of different isoforms of PFKFB in normal lung tissue was variable, being intense for PFKFB-3 and PFKFB-2. We have also studied the expression of the transcript levels of PFKFB-2, -3 and -4 in A_{549} lung carcinoma cell line to compare with normal lung tissue. As shown in Fig. 1, *A*, the expression of PFKFB-2, PFKFB-3 and PFKFB-4 mRNA in the A_{549} lung carcinoma cells is increased as compared with normal lung tissue, especially for PFKFB-4 and PFKFB-2. Moreover, hypoxia strongly induces the expression of PFKFB-3 and particularly PFKFB-4 mRNA in the A_{549} lung carcinoma cells. Overexpression of PFKFB-2, PFKFB-3 and PFKFB-4 mRNA in the lung cancers was correlated with enhanced expression of HIF-dependent genes Glut1 and VEGF (Fig. 1, *A*). Quantification of the expression of PFKFB2, PFKFB-3, PFKFB-4, Glut1, and VEGF mRNA is shown in Fig. 1, *B*. The intensity of each mRNA band was normalized to that of 18S rRNA level. Thus, the transcript levels in the lung tumors were significantly increased for PFKFB-2 (+178%; $P < 0.01$), PFKFB-3 (+274%; $P < 0.01$), PFKFB-4 (+603%; $P < 0.01$), Glut1 (+583%; $P < 0.05$) and VEGF (+146%; $P < 0.05$). Of interest, the expression of PFKFB-4 and Glut1 mRNA in the human lung cancers is much higher as compared with VEGF and other members of PFKFB gene family. However, the expression of PFKFB-1 mRNA in the lung cancers as well as in normal tissues counterparts was much weaker (Fig. 2, *A*) as compared to other isoforms of PFKFB (Fig. 1, *A*). There is an increase in the expression of PFKFB-1 mRNA in lung tumors as compared to corresponding normal tissue counterparts but a signal was not clear. Consequently, we also examined PFKFB-1 mRNA expression in the lung cancers and normal tissues counterparts by

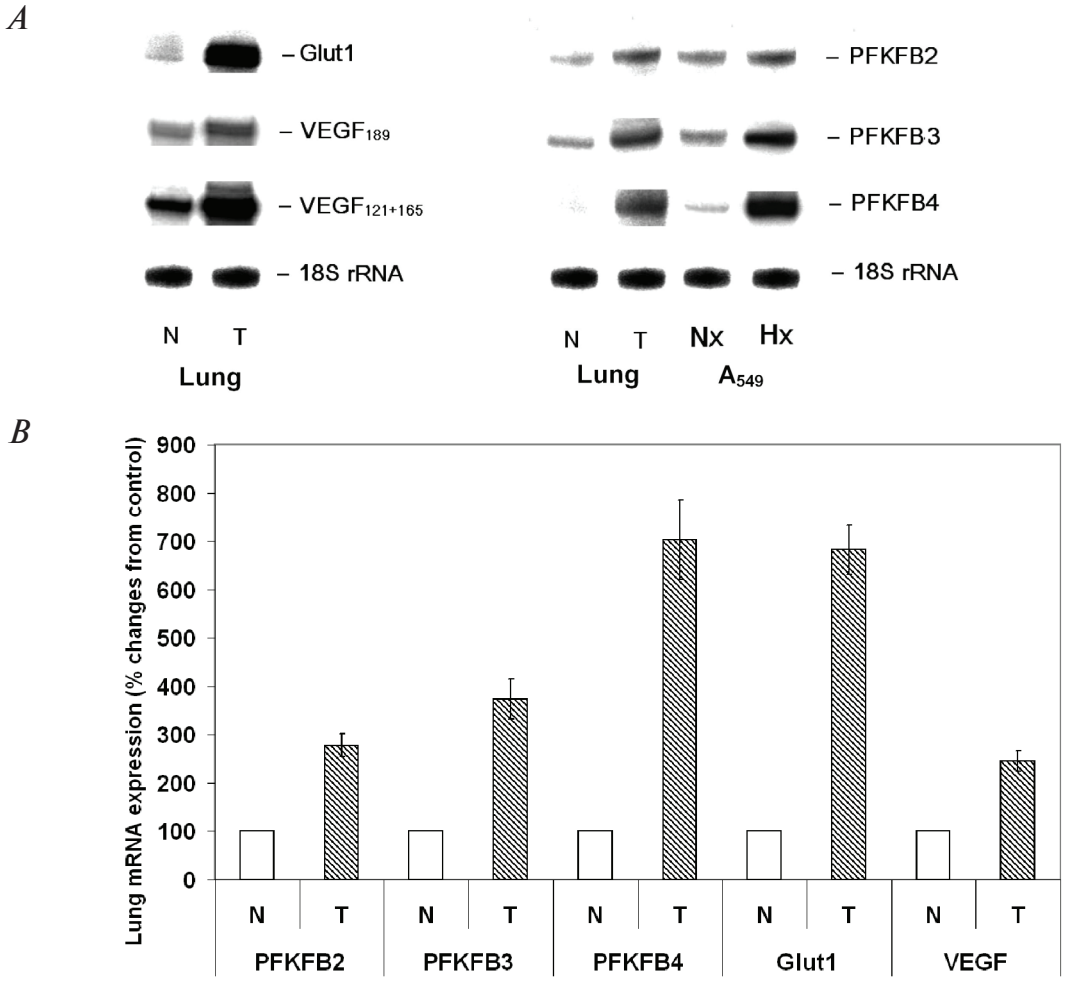

Fig. 1. A – Representative polyacrylamide gel autoradiograph employed in a typical ribonuclease protection assay of PFKFB-2, PFKFB-3, PFKFB-4, Glut1, and VEGF mRNA from the human lung malignant tumors (T) and control (normal) tissues counterparts (N) from the same patients as well as PFKFB2-4 in A_{549} lung carcinoma cells exposures under normoxic (Nx) and hypoxic (Hx) conditions. Ribonuclease protection assay of these mRNA was described previously (12-14). The 18 S rRNA was used to standardize total RNA quantity. B – Quantification of PFKFB2, PFKFB3, PFKFB4, Glut1 and VEGF mRNA levels in the human lung cancers and normal tissues counterparts from the same patients. The intensities of different mRNA bands were determined using Fujix BAS 2000 Bio-Image Analyzer (Fuji Photo Film Co., Japan) and normalized to 18 S rRNA. We have analyzed the lung tumors and corresponding control (normal) tissues from 20 patients. Bar heights are mean values ± standard errors of the mean.

RT-PCR. As shown in Fig. 2, *B*, RT-PCR analysis clearly demonstrated an increase in the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-1 in lung tumors as compared to normal tissue counterparts.

Using Western blot analysis, we examined the PFKFB-4, PFKFB-3 and inducible PFKFB-3 (iP-FKFB-3) protein levels in the lung cancer and normal tissues counterparts. As shown in Fig. 3, the protein levels of PFKFB-4 as well as PFKFB-3 and iPFKFB-3 isozymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase were significantly higher in lung cancers as compared to corresponding normal tissue counterparts.

Importantly, this study clearly demonstrated that tissue-specific isoforms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is not completely exclusive and that the lung normal and malignant tissues express all four isoforms of this enzyme. It has been previously reported that many mouse organs and human cell cultures express more than one isoform of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase [12–14]. This multiple expression of the isozymes of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase with different kinetic and regulatory properties in lung cancers is, possibly,

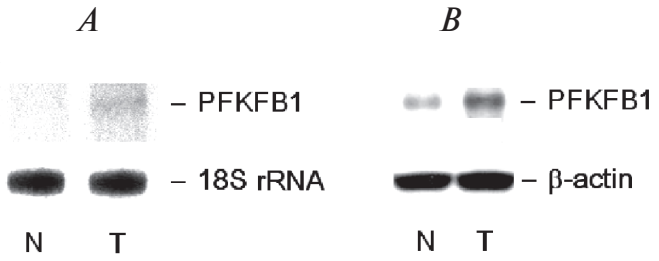

Fig. 2. A – Representative polyacrylamide gel autoradiograph employed in a typical ribonuclease protection assay of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-1 mRNA from the human lung malignant tumors (T) and control (normal) tissues counterparts (N) from the same patients. The 18 S rRNA was used to standardize total RNA quantity. B – Representative agarose gel employed in a typical RT-PCR analysis of PFKFB-1 mRNA expression in the human lung cancers (T) and control (normal) tissues counterparts (N) from the same patients. First-strand cDNA was synthesized using Sensiscript RT Kit (QIAGEN, Germany). PCR amplification was performed with the following oligonucleotides primers: 5'-GCCACCTGTCCTACATCAAG-3' (forward) and 3'-GAGATTGATGCGGGTGTCTG-5' (reverse) using HotStarTaq Master Mix Kit (QIAGEN, Germany). The amplified DNA fragments were separated on a 1.5% agarose gel and that visualized by ethidium bromide staining. The β-actin was used to standardize total RNA quantity.

important in the adaptation and survival of tumor cells in their hypoxic microenvironment.

This study provides evidence that PFKFB-4 gene is highly expressed gene of the PFKFB gene family in the lung cancer, strongly responds to hypoxia, and possibly has a dominant role in the Warburg effect in this malignant tissue. There are very low constitutive levels of PFKFB-4 mRNA expression in the normal human lung tissue as compared to the expression of other 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isozymes, but strongly enhanced in the lung cancer and A_{549} lung carcinoma cells under conditions of normal oxygen tension as compared to normal tissues and significantly induced under hypoxia. Thus, PFKFB-4 should be considered a highly hypoxia-sensitive enzyme in the lungs. The precise molecular mechanisms, whereby PFKFB family enzymes are overexpressed in the lung tumors as well as a role of protein kinase enzymes in the regulation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase activity, await further study.

Thus, the major finding reported here is that several isozymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase with different kinetics and regulatory properties are highly expressed in cancers from human lungs as compared with normal tissue counterparts via a hypoxia-inducible factor dependent mechanism, suggesting that all four isozymes may contribute to the high glycolytic rate observed in tumors (the Warburg effect). PFKFB-4 should be considered a highly hypoxia-sensitive and tumor-specific enzyme in the lungs. Overexpression of PFKFB-1, PFKFB-2, PFKFB-3 and PFKFB-4

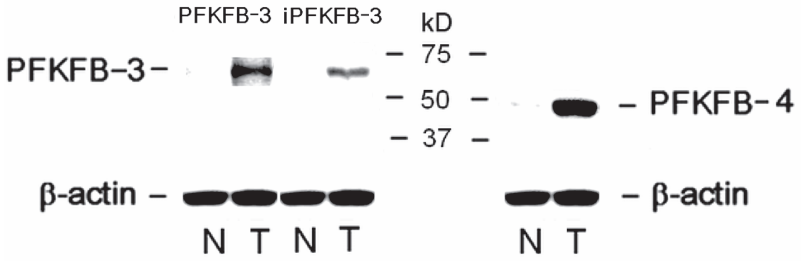

Fig. 3. Representative Western blot analysis of PFKFB-4, PFKFB-3 and inducible PFKFB-3 (iPFK-2) protein levels in the human lung malignant tumors (T) and control (normal) tissues counterparts (N) from the same patients. For detection of PFKFB-3 was used goat polyclonal anti-PFKFB-3 [PFK-2br/pl (N-11); sc-100890] antibody from Santa Cruz Biotechnology, Santa Cruz, CA, USA. For Western blotting of inducible PFKFB-3 was used rabbit polyclonal anti-iPFK-2 antibody [17]. Rabbit polyclonal anti-PFKFB4 antibody was used for detection of PFKFB-4 protein. An antibody against human PFKFB4 was generated by immunization of rabbits with a synthetic peptide from (MASPRELTQNPLKK-Cys). The bands were visualized by enhanced chemiluminescence's reagents. The β-actin was used to ensure equal loading of the sample.

mRNA in the lung cancers was correlated with enhanced expression of HIF-dependent genes Glut1 and VEGF. Moreover, the isozymes of PFKFB gene family may find clinical utility as a novel targets for the development of antineoplastic agents.

6-PHOSPHOFRUCTO-2-KINASE/ FRUCTOSE-2,6-BISPHOSPHATASE GENE FAMILY OVEREXPRESSION IN THE LUNG TUMOR

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Summary

The 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) is a family of bifunctional enzymes which is responsible for maintaining the cellular levels of fructose-2,6-bisphosphate, a powerful allosteric activator of glycolysis. Here we report the overexpression of PFKFB-1, -2, -3 and -4 mRNA in the human lung cancers when compared with corresponding normal tissues counterparts as well as PFKFB-4 and -3 protein levels. The lung carcinoma cell line A_{549}, under conditions of normal oxygen tension, has also shown increased transcript levels of PFKFB-2, -3 and -4 when compared to normal tissues. Moreover, hypoxia highly induced the expression of PFKFB-2, PFKFB-3 and especially PFKFB-4 isozymes are highly induced in the lung carcinoma cells. Thus, our results clearly demonstrated overexpression of PFKFB gene family isozymes in the lung cancers and they possible role in the Warburg effect.

Key words: PFKFB-1-4 mRNA; lung, PFKFB-4 protein, human cancers, A_{549} lung carcinoma cells.

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Received 06.06.2005