Expression of vascular endothelial growth factor (VEGF), glucose transporter Glut1 and two members of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase family (PFKFB-3 and PFKFB-4) in lung, colon and gastric cancers was studied. We have shown that expression of VEGF and glucose transporter Glut1 mRNA significantly increased in lung and colon cancers and much less – in gastric cancers comparing with corresponding normal tissues counterparts. However, expression of mRNA of glucose transporter Glut1 was increased in all tested cancers more distinctly as compared with mRNA for VEGF. Moreover, we have shown that PFKFB-3 and -4 mRNA were overexpressed in different cancers, but much more marked changes were observed in the lung and colon. PFKFB-4 protein level was also increased in different cancers being more clearly induced in the lung cancer. However, normal tissue counterparts from colon and stomach have much higher levels of PFKFB-4 protein, as compared to the lung. Thus, our data demonstrated that VEGF, glucose transporter Glut1, PFKFB-3 and PFKFB-4, known HIF-dependent genes, are overexpressed in malignant tumors from lung, colon and stomach and thus can participate in regulation of tumor growth.

Key words: VEGF, Glut1, PFKFB, mRNA expression, human cancers.

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INTRODUCTION

The metabolism within a solid tumor significantly differs from that in the surrounding normal tissues. Tumors growing under conditions of normal oxygen tension show elevated glycolytic rates, produce high levels of lactate and pyruvate (Warburg effect) that correlate with an increased expression of glycolytic enzymes and glucose transporters via HIF-1 dependent mechanism [5–7, 11–13, 22]. Over 50% of the cellular energy is produced in
tumor by glycolysis with the remainder being generated in mitochondria. The reliance of tumor cells on glycolysis for energy production induces them on consuming more glucose because of low efficiency of glycolysis in generating ATP [7]. Glucose transporter Glut1 is the most important glucose transporter which is significantly increased in tumors and provides a potential mechanism of enhanced glycolysis and cell proliferation.

Vascular endothelial growth factor (VEGF) is one of the most intimately involved in pathological angiogenesis taking place in tumors [8]. VEGF expression is increased in tumors and strongly induced by hypoxia via HIF-1 dependent mechanism [8]. The biological effects of VEGF are mediated by two receptor tyrosine kinases, VEGFR-1 and VEGFR-2, which differ considerably in signaling pathways. New vessels’ growth and maturation are highly complex and coordinated processes, requiring a sequential activation of a series of receptors by numerous ligands, and VEGF signaling represents a critical rate-limiting step in physiological and pathological angiogenesis [8].

The key regulator of glycolysis is 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)], that belongs to a family of bifunctional enzymes which possess kinase and bisphosphatase activities [17, 18]. The PFKFB controls the level of fructose-2,6-bisphosphate in the cell, because one part of this enzyme possess kinase activity and induces its synthesis and is responsible for dephosphorylation of the fructose-2,6-bisphosphate. Fructose-2,6-bisphosphate is a powerful allosteric activator of 6-phosphofructo-1-kinase and key regulator of glycolytic flux [16–18]. Four dissimilar genes located in different chromosomes coding diverse isozymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB-1, -2, -3 and -4) in the mammalian cells [17, 18]. These isoenzymes differ not only in their tissue distribution, but also in their kinetic and regulatory characteristics [17]. Importantly, tissue-specific variants of PFKFB are not completely exclusive and many cancer cells, as well as normal cells, express more than one isoform PFKFB in cell-specific manner [14, 15]. This multiple expression suggests that each variant of this enzyme can play a certain role in different physiological or pathological conditions.

Recently, we have shown that two isoenzymes of PFKFB (PFKFB-3 and PFKFB-4) are highly induced by hypoxia in several cancer cell lines by HIF-1-dependent mechanism [13, 15]. These regulatory isoenzymes contribute to de novo nucleic acid synthesis in tumor cells and provide a potential mechanism to explain apparent coupling of enhanced glycolysis and cell proliferation. The PFKFB-3 gene encoded 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoenzyme ubiquitously expressed in different organs and tumor cells [1, 13–15, 17, 18]. PFKFB-3, as well as PFKFB-4 and PFKFB-2, possess high kinase/phosphatase ratio and maintain high fructose-2,6-bisphosphate levels which, in turn, regulate glycolysis. We have also shown that in vivo hypoxia leads to a significant increase in mRNA coding for all four isoenzymes PFKFB in cell-specific manner [14]. There are data that inducible isozyme of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 uniformly increased in the malignant tissues when compared with corresponding normal tissues [1, 2]. Overexpression of inducible PFKFB-3 protein in situ was especially high in colon, prostate, breast, ovary and thyroid cancers. It was also shown that in breast carcinoma, aggressive infiltrating carcinoma cells were markedly positive for inducible PFKFB-3 protein and adjacent normal epithelium within the same specimen displayed only weak staining. Neoplastic cells express higher inducible PFKFB-3 than adjacent epithelial cells [1].

Hypoxia is an important component of many pathophysiological processes including tumor formation and growth [10–12, 21]. It is known that during hypoxia, cells shift to a preliminary glycolytic metabolic mode for their energetic needs. This change from aerobic
respiration to glycolysis is essential for maintaining cell energy and survival in the hypoxic conditions. The regulation of gene expression by hypoxia appears to be linked to a common mechanism which includes activation of the transcriptional complex termed hypoxia-inducible factor-1 (HIF-1) that binds specific enhancer elements in hypoxia-responsive genes [19–21, 24, 25]. Hydroxylation of specific prolyl and asparaginyl residues in alpha subunits of HIF by a series of non-haeme iron-dependent dioxygenases has been defined as a novel mechanism of protein modification that transduces oxygen-sensing signals [20, 23]. Hypoxia is one of the most potent inducers of gene expression, especially genes involved in glycolysis. Many genes whose expression is regulated by hypoxia, contain HIF-1 binding site (hypoxia-responsive element/enhancer) and are overexpressed in malignant tissues and cell lines [4, 14, 15, 25]. This is also manifested in the HIF-dependent up-regulation of many glycolytic genes [13, 14, 19]. Transcription factor HIF-1 is an obligatory mediator of the hypoxic effect as well as Warburg effect in the mammalian cells. Hypoxia-inducible factor is a key transcription factor in coordinating many transcriptional adaptation to hypoxia in normal and cancer cells, as well as up-regulates a series of genes involved in regulation of tumor glycolysis and growth [5, 7, 15].

Despite its importance in regulating glycolysis and tumor growth, the expression of Glut1, VEGF and PFKFB isoforms in different malignant tumors has not been well characterized yet. We demonstrated here that Glut1, VEGF, PFKFB-3 and PFKFB-4 mRNA is are uniformly increased in lung, colon and gastric malignant tumors when compared with corresponding normal tissues.

**MATERIALS AND METHODS**

**Tissue Samples.** Frozen human lung, colon and stomach tumor tissues and their normal counterparts were obtained from the National Cancer Center Hospital East, Kashiwa, Japan.

**RNA Isolation.** Total RNA was extracted from different tumor tissues and normal tissue counterparts by using Trizol reagent according to the manufacturer protocol (Invitrogen, USA). RNA pellets were washed with 75% ethanol and dissolved in RNase-free water.

**Plasmid construction.** The plasmids for human PFKFB-3, Glut1, VEGF genes and mouse 18S ribosomal RNA probe for ribonuclease protection assays were performed as described [13, 15]. The 18S ribosomal RNA antisense probe was used to evaluate total RNA.

**Ribonuclease protection assays.** Synthesis of radiolabeled probes for ribonuclease protection assay was carried out according to BD Biosciences protocol using T7 RNA polymerase (BD Biosciences Pharmingen, San Diego, CA, USA) and [α^{32}P]-UTP (Amersham Biosciences). Solutions of total RNA were dried under vacuum and dissolved in 25 μl of 80% formamide hybridization buffer containing radiolabeled probes. Samples were preincubated for 5 min at 85°C and then kept for 16 hours at 45°C. The extracted, protected probe fragments were run in 6% polyacrylamide sequencing gel in 1x TRIS-borate-EDTA buffer for 2 hours at 50 mA. The gel was then dried and expression of mRNA was determined using Fujix BAS 2000 Bio-Image Analyzer (Fuji Photo Film Co.). Intensity of each mRNA band was normalized for 18S ribosomal RNA amount.

**Reverse transcription and real time PCR Analysis.** The expression of PFKFB-3 mRNA was examined by polymerase chain reaction (PCR) of complementary DNA (cDNA). Total RNA isolated from normal and tumor tissues was utilised as template for cDNA synthesis using SuperScript II Reverse Transcriptase („Invitrogen”, USA) and oligo(dT). Reverse transcription was performed according to the manufacturer protocol by 0.4 mg of total RNA in total volume 20 ml.
For amplification of VEGF cDNA, forward (5'-AACATGAACTTTCTGCTGTC-3') and reverse (3'-CATCACCATGCAGATTATCCGG-5') primers were used. The nucleotide sequences of these primers correspond to sequences 1,028–1,048 and 1,334–1,355 of human VEGF165 cDNA (GenBank accession number NM_001025368).

The amplification of Glut1 cDNA was performed using forward primer (5'-TTGCTAACAACACTGGAGTC-3') and reverse primer (3'-CAATGCTGATGATGACCTGC -5'). These oligonucleotides correspond sequences 256–275 and 463–483 of human Glut1 cDNA (GenBank accession number NM_006516).

For amplification of PFKFB-3 cDNA real time RCR analysis was performed using two oligonucleotides primers: forward – 5'-CTCATGAGACGCAATAGTGTC-3' and reverse – 3'-GAAACACTGAGGCACGTGT-5'. The nucleotide sequences of these primers correspond sequences 1,695–1,715 and 1,883–1,903 of human PFKFB-3 cDNA (GenBank accession number NM_004566). Two other primers were used for real time RCR analysis of PFKFB-4 cDNA expression: forward – 5'-ATCTGCCACAGGTGATG-3' and reverse – 3'-GTGACCATGTTCATCCACTGT-5'. The nucleotide sequences of these primers correspond to sequences 1182–1202 and 1424–1444 of human PFKFB-4 cDNA (GenBank accession number NM_004567).

The amplification of β-actin cDNA was performed using primers: forward – 5'-CGTACATGACAATGATGTC-3' and reverse – 5'-GTGTTGGCGTACAGGTCTTT-3'. The expression of β-actin mRNA was used as control of analyzed RNA amount. The primers were received from „Sigma“ (USA).

Quantitative PCR (qPCR) was performed in „Stratagene Mx 3000P cycler“ using SYBR Green Mix. The reaction was performed in triplicate. Quantitative PCR analysis was performed using special computer program „Differential expression calculator“ and statistical analysis – in Excel program. The amplified DNA fragments were separated in 1.5% agarose gel and visualized by ethidium bromide staining.

Western Blotting. Cytoplasmic extracts were prepared using buffers A, as previously described [15]. Proteins were resolved using electrophoresis in sodium dodecyl sulfate-10% polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Immobilon-P Transfer Membrane; Millipore, USA) by semi-dry blotting system. Excess sites on the membrane were saturated by 5% nonfat dried milk in T-PBS (PBS, containing 0.1% Tween-20). The membrane was incubated for 16 h at 4°C with primary antibody. For detection of PFKFB-4 protein rabbit polyclonal anti-PFKFB-4 (1:10,000 dilution) antibody as described was used [15]. For detection of PFKFB-3 protein rabbit polyclonal anti-PFKFB-3 (1:10,000 dilution) antibody from Dr. T. Atsumi was used [1]. Horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, USA) was used as secondary antibody at 1:5,000 titer. The bands were visualized by enhanced chemiluminescence reagents (Amersham Biosciences). β-Actin was used for normalization of loading of analyzed proteins.

RESULTS

Expression of VEGF mRNA in lung, colon and gastric cancers

We examined VEGF mRNA expression in lung, colon and gastric cancers and normal tissues counterparts by using ribonuclease protection assays. As shown in Fig. 1, there is a significant increase in the expression of VEGF mRNA isoforms in lung tumors as compared with corresponding normal tissue counterparts from the same patients. It should be noted that very low level of VEGF mRNA expression has place in normal lung tissue samples, as compared with malignant tumors, being more variable in cancer tissues. Moreover, induction of VEGF mRNA expression in the lung tumors is more vari-
able, as compared with VEGF mRNA encoding shorter variants of VEGF protein. We also analyzed the VEGF mRNA expression by using real time PCR in lung and other malignant tumors. As shown in Fig. 2, there is a significant increase (230%) in VEGF mRNA expression not only in lung tumors, but also in colon cancers, as compared with corresponding normal tissue counterparts. Moreover, the VEGF mRNA expression in the gastric tumors is also increased by 60% but not too strong as compared with lung or colon cancers.

Expression of Glut1 mRNA in lung, colon and gastric cancers

We also examined Glut1 mRNA expression in lung cancers and normal tissues counterparts by using ribonuclease protection assay and real time PCR. As shown in Fig. 3, there is a significant increase in the expression of Glut1 mRNA in lung tumors, as compared with corresponding normal tissue counterparts from the same patients. Similar levels of Glut1 mRNA expression were observed in normal tissue lung samples obtained from different patients, as compared with malignant tumors, being more variable in cancer tissues.

Results of real time PCR analysis of Glut1 mRNA expression in lung and other tumors are presented in Fig. 2. There is a significant increase (+631%) of Glut1 mRNA
expression not only in lung tumors but also in colon tumors (+724%), as compared with corresponding normal tissue counterparts. An increase in Glut1 mRNA expression in gastric tumors is not so distinct, as compared with lung or colon tumors (only +146%).

Expression of PFKFB-3 and PFKFB-4 mRNA in lung, colon and gastric cancers

The results of ribonuclease protection assay analysis of PFKFB-3 mRNA expression in seven different lung tumors and normal tissues counterparts are presented in Fig. 4. There is a significant increase in the expression of PFKFB-3 mRNA in all analyzed lung tumors, as compared with corresponding normal tissue counterparts from the same patients. Similar high levels of PFKFB-3 mRNA expression were observed in the malignant lung tumor samples obtained from different patients, as compared with normal tissue, being more variable in the normal tissues. As shown in Fig. 4, there are two bands of PFKFB-3 mRNA protected fragments: the upper band of PFKFB-3 representing the main variant of PFKFB-3 and the lower band of PFKFB-3 representing alternative splice variant of PFKFB-3.

We also analyzed the PFKFB-3 and PFKFB-4 mRNA expression by using real time PCR in lung, as well as in colon and gastric tumors. As shown in Fig. 5, there is a significant increase of PFKFB-3 and PFKFB-4 mRNA expression in lung tumors – +257% and +613%, respectively, as compared with corresponding normal tissue counterparts. The PFKFB-3 and PFKFB-4 mRNA expression in colon tumors was also increased (+199% and +250%, respectively), as compared with corresponding normal tissue counterparts. However, an increasing of PFKFB-3, as well as PFKFB-4 mRNA expression, was not too strong in gastric malignant tumors (+74 and 58%, respectively), as compared to lung or colon tumors.

Expression of PFKFB-3 and PFKFB-4 protein in normal and tumor tissues

We used Western blot analysis for examination of PFKFB-3 and PFKFB-4 protein expression in lung, colon and stomach tumors and normal tissue counterparts. As shown in Fig. 6, lung tumors overexpress both tested isoforms of 6-phosphofructo-2-kinase/fruc-
tose-2,6-bisphosphatase, as compared with corresponding normal tissue counterparts, whereas a smaller increase of expression was observed in colon and stomach. The expression of different isoforms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in normal colon and stomach tissue was much higher, comparing with lung tissue.

DISCUSSION

The major finding reported in this paper is that VEGF, Glut1 and two isozymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase possessing different kinetics and regulatory properties are overexpressed in solid tumors of different organs, especially in lung tumors, as compared with normal tissues. Overexpression of VEGF and Glut1 is very important for tumor growth since the reliance of tumor cells on glycolysis for energy production causes was a reason for consuming more glucose for generating ATP because the efficiency of glycolysis in generating ATP is very low [7]. Our results clearly demonstrated overexpression of glucose transporter Glut1, the most important glucose transporter in different tumors that provides a potential mechanism of enhanced glycolysis and cell proliferation. We found that vascular endothelial growth factor expression increased significantly in lung and colon cancers that caused pathological angiogenesis associated with tumors [8]. Data exist that expression of Glut1 and VEGF, as well as PFKFB, is increased by HIF-dependent mechanism [8, 13, 15]. Overexpression of different isoforms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in tumor tissues was observed, but breast and lung

Fig 5. Real time PCR analysis of PFKFB-3 and PFKFB-4 mRNA expression in different tumors (T) and corresponding normal tissue counterparts (C). Amplification of PFKFB-3 and PFKFB-4 cDNA was carried out using specific forward and reverse primers. Values of PFKFB-3 and PFKFB-4 mRNA expression were normalized to β-actin mRNA expression. VEGF and Glut1 mRNA expression was examined in lung, colon and gastric tumors from seven patients, as well as in normal tissues counterparts from the same patients as control.

Fig 6. Results of representative Western-blot analysis of PFKFB-3 and PFKFB-4 protein level in lung, colon and stomach tumors (T) and control (normal) tissues counterparts (N) from the same patients. β-actin was used to ensure equal loading of protein samples.
normal tissues have very low expression of these isozymes. The colon and stomach normal tissues have much higher levels of different isoforms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and weak increasing of these isozymes in tumors, as compared with lung tissue. Moreover, the expression levels of different isoforms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in tumor tissues of lung, colon and stomach were similar. These data are consistent with our previous results. Recently, we have shown that the expression of all four genes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB-1-4) are responsive to hypoxia in vivo, however regulation of the expression of these PFKFB isozymes following hypoxic treatment is different and can occur in organ-specific and possibly cell-specific manner [14]. Moreover, we observed different basal and hypoxia-inducible levels of PFKFB-4 mRNA and protein in different breast cancer cell lines. These data clearly demonstrated absence of positive correlation between PFKFB mRNA and protein levels in different organs and cell lines. It should be noted that tissue-specific isoforms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase are not completely exclusive and cancer cells can express 3–4 various isoforms. This variable expression suggests that each isozyme can play a key role at different physiologic or pathologic conditions.

Our results clearly demonstrated tumor-specific overexpression of PFKFB-3 and PFKFB-4, especially in lung tumors. Cancer cells maintain high aerobic glycolytic rates under conditions of normal oxygen tension and produce high levels of lactate and pyruvate that correlated with increased expression of glycolytic enzymes and glucose transporters, usually via HIF-1 dependent mechanism [2, 9, 12]. Preferential reliance on glycolysis is correlated with a disease progression in some types of cancers.

In summary, our results show that VEGF, Glut1, PFKFB-3 and PFKFB-4 genes are strongly overexpressed in the malignant tumors of lung, colon and stomach, and thus, can participate in Warburg effect. Moreover, this study demonstrated tumor-specific overexpression of PFKFB-4, especially in lung cancers. PFKFB-4, as well as PFKFB-3, might find clinical application, as a novel target of the antineoplastic drugs.
мРНК PFKFB-3 та PFKFB-4 значно збільшений у різних злоякісних пухлинах, але ці зміни яскравіше виражені у злоякісних пухлинах легень та прямої кишки порівняно з пухлинами шлунку. Максимальне посилення експресії протеїну PFKFB-4 було виявлено у злоякісних пухлинах легень, хоча в нормальній тканині прямої кишки та шлунку спостерігали досить високий рівень експресії даного протеїну, порівняно з легенями. Таким чином, отримані нами результати свідчать про посилену експресію VEGF, переносника глюкози Glut1, PFKFB-3 та PFKFB-4, добре відомих HIF-залежних генів, у злоякісних пухлинах легень, прямої кишки і шлунку та про їхню можливу участь у рості пухлин.

ЭКСПРЕССИЯ VEGF, Glut1 И 6-ФОСФОФРУКТО-2-КИНАЗЫ/ФРУКТОЗО-2,6-БИСФОСФАТАЗЫ-3 И -4 В ЗЛОКАЧЕСТВЕННЫХ ОПУХОЛЯХ ЛЮДЕЙ ИЗ ЛЕГКИХ, ПРЯМОЙ КИШКИ И ЖЕЛУДКА

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Исследовали экспрессию эндотелиального фактора роста сосудов (VEGF), переносника глюкозы Glut1 и двух членов семейства 6-фосфофрукто-2-киназ/фруктозо-2,6-бисфосфатаз (PFKFB-3 и PFKFB-4) в злокачественных опухолях легких, прямої кишки и желудка. Экспрессия мРНК VEGF и переносчика глюкозы Glut1 была значительно повышенной в злокачественных опухолях легких и прямої кишки и в меньшей степени — в опухолях желудка, если сравнивать с нормальной тканью тех же органов. Установлено, что экспрессия мРНК переносчика глюкозы Glut1 была повышенной во всех исследованных злокачественных опухолях в большей мере по сравнению с экспрессией VEGF. Мы также показали, что уровень мРНК PFKFB-3 и PFKFB-4 был значительно увеличенным в разных злокачественных опухолях, но эти изменения были ярче выражены в злокачественных опухолях легких и прямої кишки по сравнению с опухолями желудка. Максимальное увеличение экспрессии протеина PFKFB-4 было выявлено в злокачественных опухолях легких, хотя в нормальной ткани прямої кишки и желудка наблюдали достаточно высокий уровень экспрессии данного протеина, по сравнению с легкими. Таким образом, полученные нами результаты свидетельствуют об усиленной экспрессии VEGF, переносчика глюкозы Glut1, PFKFB-3 и PFKFB-4, хорошо известных HIF-зависимых генов, в злокачественных опухолях легких, прямої кишки и желудка и указывают на их возможное участие в регуляции роста опухолей.


