ORIGINAL ARTICLE

Bioinformatics analysis and expression study of fumarate hydratase in lung cancer

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Abstract

Background: As its etiology and pathogenesis is obscure, illustrating the molecular mechanism of lung cancer has become a serious and urgent task. Studies have shown that fumarate hydratase (FH) is a tumor suppressor related to tumorigenesis, development, and invasion. Our aim was to analyze the biological information of FH, and detect the messenger ribonucleic acid (mRNA) and protein expression of FH in lung cancer cells to explore its role in tumorigenesis and in the development of lung cancer.

Method: We analyzed the biological characteristics of FH, then utilized reverse transcription-polymerase chain reaction (RT-PCR) to study FH mRNA expression in A549 and 16 human bronchial epithelial (HBE) cell lines. The protein expression of FH was detected in 57 cases of human lung cancer tissues and 19 cases of normal lung tissues by immunohistochemistry.

Results: 1. Bioinformatic analysis: FH mainly exist in the mitochondria; the common structural elements of FH are mainly α -helix, random coil, β -turn, and extended strand; there are five possible transmembrane domains in the entire polypeptide chain; FH is a hydrophilic and soluble protein. 2. RT-PCR result: FH mRNA expression was downregulated in A549 cells compared with 16HBE cells. 3. Immunohistochemistry: FH protein expression was significantly lower in lung cancer cells than in normal lung tissues ($P < 0.05$), but was not correlated with the patients' age, gender, tumor size, pathological type, or lymph node, distant, or tumor node metastasis stage.

Conclusion: FH was under-expressed in lung cancer, suggesting that it may be an indicator of tumorigenesis and could be a potential target for therapies against lung cancer in the future.

Introduction

Lung cancer is currently the most common malignancy, as well as the leading cause of cancer death in the world.¹ Mortality related to this malignant disease has risen by 465% during the last 30 years in the People's Republic of China.² It has long been acknowledged that the aggressive nature of lung cancer is closely related to mutations of oncogenes, as well as tumor suppressor genes and abnormalities in several growth factors and their receptors.^{3–5} But as multiple molecular alterations may be involved in the development of lung cancer,⁶ the specific etiology and pathogenesis of this disease is still obscure. Conventional treatments are not efficient enough to improve the prognosis of lung cancer. Therefore,

there is a severe and urgent need to discover novel cancer-specific molecular targets and signaling pathways to develop new therapeutic strategies against this devastating malignancy.

In our prophase research on mitochondria proteomics, we had screened and identified a series of different proteins including fumarate hydratase (FH).⁷ FH is an enzyme responsible for converting fumarate to malate in the tricarboxylic acid (TCA) cycle. Genetic analysis of tumor samples indicates that FH acts as a tumor suppressor gene.^{8,9} The deactivation of or missing FH leads to an increase in intracellular fumaric acid, which is the competitive inhibitor of hydroxypyruvate of hypoxia-inducible factor (HIF), thus, HIF cannot be hydroxylated and eliminated by

proteasome.^{10–14} HIF may regulate the expression of multiple genes, such as vascular endothelial growth factor (VEGF), erythropoietin (EPO), platelet derived growth factor (PDGF), epidermal growth factor receptor (EGFR), glucose transporter protein 1 (Glut-1), and transforming growth factor- α (TGF- α).^{13,15,16} These gene products play an important role in maintaining the energy metabolism of tumor cells, promoting tumor angiogenesis, invasion, and metastasis. The survival of tumor cells depends on sugar anaerobic glycolysis, while the dysfunction of FH causes false hypoxia, which could strengthen the angiogenesis and glycolysis.¹⁷ Vanharanta *et al.*¹⁸ confirmed this in a study of myoma of the uterus by applying gene expression microarray. Previous research has shown that germline mutations in FH predisposed individuals to multiple cutaneous leiomyomas, uterine leiomyomas, and, in some families, renal cell cancer.¹⁹ FH mutations have also been associated with uterine fibroids without skin leiomyomas,²⁰ cerebral cavernomas,²¹ Leydig cell tumors,²² and ovarian mucinous cystadenoma.²³

However, until now there have been no reports studying whether FH is involved in the development of lung cancer. In the present study, we analyzed the biological characteristics of FH. Using reverse transcription-polymerase chain reaction (RT-PCR), this study first quantified the messenger ribonucleic acid (mRNA) expression level of FH in lung adenocarcinoma cell lines A549 and 16 normal human bronchial epithelial cell lines (HBE). Moreover, we utilized immunohistochemistry to detect FH protein expression in lung cancer tissues and examined the relationship with clinicopathologic factors. This study may provide important references for the possible role of FH in human lung cancer progression.

Materials and methods

Bioinformatics analysis

The biological characteristics of FH were analyzed using National Center for Biotechnology Information, ProtParam, Self-optimized Prediction Method with Alignment (SOPMA), TMpred, SMART, ProtScale, SOSUI, PSORT II, and Swiss-MODEL Repository, among others. Biological characteristics include physicochemical properties, homology, secondary structure, transmembrane domain, functional domain, subcellular localization, three dimensional structure, phosphorylation sites, and functions. The gene and protein interaction networks of FH were established based on the platforms of GeneMANIA and STRING9.0.

Human cell lines and growth conditions

Human lung adenocarcinoma cells A549 were obtained from the central laboratory of Xi'an Jiaotong University and maintained in RPMI 1640 supplemented with 10% fetal bovine

serum and pen/strep (100 U/mL penicillin and 100 U/mL streptomycin). The cells were grown until confluent at 37°C in an incubator with a humidified atmosphere of 5% CO₂. The tumor cell library of the Chinese Academy of Medical Sciences kindly provided 16HBE.

Patients and specimens

Human lung cancer samples were obtained from 57 patients with primary lung cancer who underwent surgery at the Department of Chest Surgery of the Second Affiliated Hospital of Xi'an Jiaotong University, between June 2006 and June 2011, without preoperative chemotherapy and/or radiation therapy. All resection samples were confirmed to be lung cancer by clinical pathology. Nineteen cases of normal lung tissues were benign lung lesions or at least 5 cm far away from the cancer. The lung cancer samples comprised 32 adenocarcinomas, 19 squamous carcinomas, two adenosquamous carcinomas, one carcinoid, one small cell lung cancer, one pulmonary metastasis tumor from the esophagus, and one pulmonary metastasis tumor from the cervix. The average age was 57.72 ± 9.70 years (range: 21–75 years) and the sample set included 30 men and 27 women. All of the patients were staged based on the 2009 Union for International Cancer Control (UICC) staging system. Of the 57 patients, 31 (54.4%) had lymph node metastasis, four (7.0%) had distant metastasis, 26 (45.6%) had T1–T2 stage, and 31 (54.4%) had T3–T4 stage. Patients' characteristics, such as gender, age, pathological pattern, lymph node invasion, and UICC stage were obtained from medical records. The Human Research Committee of the University approved the study, and all samples were taken with informed consent of the donors. Fresh tumor specimens and normal lung tissues were collected in the operating room, snap frozen in liquid nitrogen, and stored at –80°C until analysis.

Primary reagent

RPMI 1640 and fetal bovine serum were purchased from Hyclone Laboratories (Logan, UT, USA). RNAfast200 and RevertAi First Strand cDNA Synthesis Kit were separately obtained from Feijie Biotechnology (Shanghai, China) and Fermentas (Hanover, MD, USA). Rabbit polyclonal antibody against FH was purchased from ProteinTech (Chicago, IL, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cells with RNAfast200 according to manufacturer's instructions. A total of 1 μ g RNA was used for RT, and cDNA was generated and used as the

template for RT-PCR analysis. RT-PCR was performed using the RevertAid First Strand cDNA Synthesis Kit. PCR conditions for all of the reactions were as follows: 94°C for three minutes, 94°C for 30 seconds, 52°C for 30 seconds, 72°C for one minute (30 cycles) and 72°C for five minutes; β -actin: 94°C for three minutes, 94°C for 30 seconds, 58°C for 30 seconds and 72°C for one minute (30 cycles), and 72°C for five minutes. Gel images were collected using conventional electrophoresis on the PCR product. Using Gel-Pro software and under the reference of β -actin, the mRNA expression levels of FH in cell lines A549 and 16HBE were evaluated. Each experiment was performed in triplicate.

Immunohistochemistry

Immunostaining was carried out according to standard streptavidin-peroxidase (SP) methods. Briefly, the tissue specimens were fixed in neutral buffered formalin and then embedded in paraffin wax. Four μ m-thick tissue sections were dewaxed, rehydrated, heat-induced antigen retrieved, and then blocked with normal goat serum for 15 minutes. The slides were respectively incubated with rabbit polyclonal antibody against FH at 4°C overnight, rinsed with phosphate buffered saline (PBS), and incubated with horseradish peroxidase labeled secondary antibody. The localization of FH was revealed using 3,3'-diaminobenzidine (DAB) as the chromogen. The negative control was performed by replacing the primary antibody with PBS. The immunostaining level of FH was assessed using a semiquantitative staining index method and evaluated as the percentage of positive cells and staining intensity. The percentage of positive cells was assessed quantitatively and scored as: 0 for staining of <5% of total cells counted; 1 for staining of 5–24%; 2 for staining of 25–50%; and 3 for staining of >50%. Intensity was graded as follows: 0, no signal; 1, weak; 2, moderate; and 3, strong staining. Multiplying the percentage of positive cells and staining intensity for each sample generated a staining index ranging from 0 to 6. The total staining score was graded as negative (–, score 0), weak (+, score 1–2), moderate (++, score 3–4), or strong (+++, score 5–6). Negative expression was defined as “–”, and “+”, “++” were defined as positive. All slides were examined and scored independently by two pathologists blinded to patient data.

Statistical analysis

For statistical analysis, the Statistical Package for the Social Sciences 19.0 (SPSS) was performed. The associations between immunohistochemical staining and the clinicopathological factors were examined using the χ^2 test wherein a *P*-value less of than 0.05 was considered statistically significant.

Results

The biological characteristics of fumarate hydratase (FH)

The FH gene is located in chromosome 1q42.1 and the whole length includes 22 229 bp. The mRNA length of the FH gene is 1877 bp; the initiation codon of the gene is ATG, and the termination codon is TGA. The open reading frame is from 64 bp to 1596 bp. FH mRNA contains 10 exons and 9 introns; and the human, wren, gibbon, and chimpanzee species share a high degree of genetic homology. The FH protein is composed of 510 amino acids; the relative molecular mass is about 54636.9; the theoretical isoelectric point is 8.85; and the half-life is 30 hours. The common structural elements of FH are mainly α -helix, random coil, β -turn and extended strand (Fig 1). There are five possible transmembrane domains in the entire polypeptide chain. FH is a hydrophilic and soluble protein and there are two peptidase activity regions in FH protein: one is lyase and the other is fumarate hydratase. Analysis of its subcellular localization shows that a 78.3% opportunity may exist in the mitochondria, 8.7% may be located in the cytoplasm, 8.7% in the nucleus, and 4.3% in the cytomembrane (Fig 2). Figure 3 shows the three-dimensional structure of FH protein which is predicted by Swiss-MODEL Repository software; using GeneMANIA and STRING9.0 software and imposing restrictions on the search conditions within 20 genes, the Network diagram of FH gene and protein interaction are achieved and constructed (Fig 4).

Messenger ribonucleic acid (mRNA) level of FH in cell lines A549 and 16 human bronchial epithelial (HBE)

Figure 5 shows the electrophoresis results of the mRNA level of FH in cell lines A549 and 16HBE. As evident by the naked eye, the stripe corresponding to cell lines A549 is darker than that of 16HBE, which reveals that the mRNA level of FH in A549 is down-regulated. Using Gel-Pro software and under the reference of β -actin, the relative expression level of FH in 16HBE and A549 were measured for 0.19 ± 0.02 and 0.08 ± 0.01 , respectively. The difference between the two is about 2.4 times.

FH protein expression in tissue specimens

Immunohistochemistry staining of human lung cancer and normal lung tissue specimens showed that FH was generally expressed in the cell cytoplasm, appearing as brown granules (Fig 6). The expression of FH in normal lung tissues is 57.9% (11/19), while in the 57 cases of lung cancer, only 13 cases showed positive expression with a positive rate of only 22.8%. Compared with normal samples, the expression level of FH in

SOPMA :

Alpha helix	(Hh) :	283 is	55.49%
3 ₁₀ helix	(Gg) :	0 is	0.00%
Pi helix	(Ii) :	0 is	0.00%
Beta bridge	(Bb) :	0 is	0.00%
Extended strand	(Ee) :	34 is	6.67%
Beta turn	(Tt) :	24 is	4.71%
Bend region	(Ss) :	0 is	0.00%
Random coil	(Cc) :	169 is	33.14%
Ambiguous states (?)	:	0 is	0.00%
Other states	:	0 is	0.00%

Figure 1 The secondary structure prediction result of fumarate hydratase (FH) by SOPMA.

adenocarcinoma was uniformly and significantly decreased ($P = 0.019$). Similarly, applying the χ^2 test, the P -value between normal tissues and squamous carcinomas is calculated as 0.020. Thus, it can be seen that FH expression is absent or decreased in lung cancer.

The correlation between the clinicopathological characteristics and FH protein expression is shown in Table 1. The

expression of FH in lung cancer was uncorrelated with lymph node metastasis ($P = 0.147$). Similarly, it was not related to patients' age, gender, tumor size, pathological type, distant, or tumor node metastasis (TNM) stage. Adenosquamous carcinoma, carcinoid, small cell lung cancer, and metastatic carcinoma samples were removed during the analysis because there were too few cases.

Figure 2 The analysis of the subcellular localization of fumarate hydratase (FH).

Figure 3 The prediction of the tertiary structure of fumarate hydratase (FH).

Figure 4 Network diagram of the fumarate hydratase (FH) (a) gene and (b) protein interaction.

Figure 5 Electrophoresis of fumarate hydratase (FH) gene polymerase chain reaction products in cell lines A549 and 16 human bronchial epithelial (HBE) (M is the DNA marker).

Discussion

Bioinformatics is a new discipline combining computer technique and applied mathematics, and is one of the core areas of life science and natural science analysis. In the presented work, we used bioinformatics to analyze the biological characteristics of FH. A homogeneity analysis of FH showed a high homology in humans and other species, suggesting that it is conservative and plays an important role in vivo. FH mainly exists in the mitochondria, conforming to the function of participating in the Krebs cycle. Most of the genes and proteins interacting with FH participate in the Krebs cycle and the respiratory chain process to provide energy for cellular activities. We can use this data to analyze the biological process and signal transduction pathways, which FH may participate in. Thus, the bioinformatics approach offers opportunity and convenience in understanding the structure and function of FH more deeply.

Figure 6 The expression of fumarate hydratase (FH) in (a) normal lung tissue, (b) adenocarcinoma, and (c) squamous carcinoma (immunohistochemistry, $\times 400$).

Table 1 Correlation between clinicopathological characteristics and fumarate hydratase (FH) expression

Variables	Patients [n(%)]	Positive	Negative	Positive rate (%)	P
Age					
≥65	31	7	24	22.6	0.888
65	20	5	15	25.0	
Gender					
Male	27	8	19	29.6	0.275
Female	24	4	20	16.7	
Tumor size (cm)					
≥3.5	26	6	20	23.1	0.920
3.5	25	6	19	24.0	
Histology					
Adenocarcinoma	32	8	24	25.0	0.748
Squamous carcinoma	19	4	15	21.1	
Lymph node metastasis					
Node-positive	29	9	20	31.0	0.147
Node-negative	22	3	19	13.6	
Distant metastasis					
Yes	4	2	2	50.0	0.493
No	47	10	37	21.3	
Tumor Node Metastasis stage					
I-II	24	3	21	12.5	0.080
III-IV	27	9	18	33.3	

FH is one of the key enzymes in the TCA cycle. Previous studies have reported that FH acts as a tumor suppressor gene. For example, germline mutations of FH will lead to the occurrence and development of renal cell carcinoma and leiomyoma.^{25–27} However, the specific mechanism has not been studied in detail. Researchers have not found other genetic modification factors, apart from the FH gene.²⁸ They now believe that FH may participate in tumorigenesis through false hypoxia, oxidative stress, apoptotic resistance, and so on.^{29–32} However, further research is needed to clarify the exact pathogenesis.

In this study, we provided quantification of the FH mRNA level between lung adenocarcinoma cells and normal human bronchial epithelial cells by RT-PCR. Our study is the first to report that the expression of FH in lung adenocarcinoma cells was significantly lower than in normal cells. As the expression of mRNA may not truly reflect the level of protein, we used immunohistochemistry to detect the level of protein in order to provide an independent confirmation to this conclusion. The results showed that the protein level of FH varied with the level of the corresponding FH mRNA in lung cancer and normal tissues, but was not related to patients' age, gender, tumor size, pathological type, lymph node or distant metastasis, or TNM stage. These findings suggest that FH presents low expression in lung cancer, and its deficiency could play an important role in early-onset lung cancer, contributing to lung cancer formation and progression.

In past research, FH has been shown as a tumor suppressor gene, which promotes differentiation and apoptosis and

inhibits proliferation in a variety of tumors. This study suggested that FH is associated with human lung cancer and could be considered as a potentially valuable indicator in patients with lung cancer. This information might be useful to clinicians in providing individualized therapy for lung cancer patients with optimal benefit.

Conclusion

In summary, our data showed for the first time that the expression of FH decreased in human lung cancer, and the FH level was not correlated to patients' age, gender, tumor size, pathological type, lymph node or distant metastasis, or TNM stage. FH might serve as a novel marker in early-onset human lung cancer. However, as this is only a first study, further experiments are required to confirm our conclusion. According to our experience, we would expand the sample size and establish cell excessive expression vectors in order to study the specific role of FH in lung cancer.

Disclosure

No authors report any conflict of interest.

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