Full Length Research Paper

Expression and significance of glyceraldehyde-3-phosphate dehydrogenase in patients with osteosarcoma

Dan Fei[1], Hejia Zhang[1], Zhongli Gao[2], Dan Jiao[1], Guoqing Sui[1] and Kaijun Zhao[1*]

[1]Department of Ultrasonographic, China-Japan Union Hospital of Jilin University, Chang Chun130033, China.
[2]China-Japan Union Hospital of Jilin University, Chang Chun130033, China.

To observe the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in patients with osteosarcoma and its relationship with surgical stage, 60 patients with osteosarcoma, including 20 cases in Stage I, 17 cases in Stage II and 23 cases in Stage III were sequentially enrolled in this study. The expression of GAPDH in osteosarcoma and osteochondroma tissues on mRNA and protein levels were measured by fluorescent quantitative polymerase chain reaction (qPCR) and immunohistochemical stain, respectively. It was found that GAPDH was significantly up-regulated in osteosarcoma in comparison to the healthy control on mRNA and protein levels ($P < 0.05$). The positive rate of GAPDH osteosarcomas in Stage III were significantly higher than those in Stages I and II ($P < 0.05$). These data demonstrated that the expression of GAPDH correlates with the clinical stage of osteosarcomas, which implied that over-expressions of GAPDH play an important role in the development of osteosarcoma.

Key words: Osteosarcoma, GAPDH, qPCR, immunohistochemical stain.

INTRODUCTION

Osteosarcoma is an aggressive sarcoma of the bone characterized by a high level of chromosomal instability and very complex karyotypes (Man et al., 2004; Selvarajah et al., 2008). Osteosarcoma is also the most common primary malignancy of bone, comprising 2.4% of all malignancies in pediatric patients and approximately 20% of all bone cancers. The main incidence is during the second decade of life and the tumors frequently localize to the distal femur and proximal tibia (Longhi et al., 2006; Chou and Gorlick, 2006). The 5-year survival rate is between 50 and 65% (Stiller et al., 2001). Current standard treatment is to use neoadjuvant chemotherapy followed by surgical resection. The cause of death is usually due to pulmonary metastases since local disease can be surgically treated (Kim et al., 2004; Kager et al.,

2003). Despite extensive research effort, the molecular genetics events and mechanistic pathways involved in osteosarcoma remain to be elucidated (Clark et al., 2008). Thus, it is crucial and important to study molecular mechanism of osteosarcoma for control and treatment osteosarcoma disease and for development effective drug.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a classical glycolytic enzyme for energy production in the cytosol and functions as a homotetramer (Alexander et al., 1988; Sabath et al., 1990). GAPDH contributes to various biological processes related to glycolysis, nuclear tRNA export, DNA replication and repair, neuronal apoptosis, endocytosis, exocytosis and cytoskeletal organization (Harada et al., 2007; Cale et al., 1997; Sirover, 2011). It has been commonly regarded as a constitutive housekeeping gene and widely used as a reference gene/protein for the quantifications of level changes of genes/proteins in cells and tissues based on

*Corresponding author. E-mail: kaijun618@yahoo.cn.

the conventional fact that GAPDH level was usually unaffected under the experimental or physiological conditions (Rubporn et al., 2009; Jung et al., 2007).

Whereas, as a multifunctional gene/protein, the use of GAPDH as a reference gene/protein has been questioned and challenged. GAPDH mRNA levels were highly differentially regulated in a variety of types of cells under certain metabolic conditions. GAPDH might play a role in cancer pathogenesis and translocate to nucleus in response to several anti-cancer agents (Kim et al., 1999). The accumulated results indicated that GAPDH was differentially expressed in prostate, breast, lung, and cervical carcinomas under certain conditions (Nguewa et al., 2008; Lau et al., 2000; Gong et al., 1996; Chang et al., 2011; Revillion et al., 2000; Yamagata et al., 1998; Rienzo et al., 2012). However, the alteration of GAPDH gene expression has little report in osteosarcoma. Thus, in this study, we evaluated the GAPDH expression change on protein and transcript level in osteosarcoma tissue of patients with osteosarcoma by fluorescent quantitative polymerase chain reaction (qPCR) and immunohistochemical stain, respectively. We then associated their expression with clinicopathological features of the patients.

MATERIALS AND METHODS

Patients and their tissue samples

Sixty (60) patients (24 males and 36 females, aging 5 to 60 years, mean ± SD = 40.5 ± 0.6 years) with primary osteosarcoma were from Department of Orthopaedics, China-Japan Union Hospital of Jilin University, ChangChun, P.R. China from April, 2011 to August, 2011. All patients recruited in this study underwent systemic neoadjuvant chemotherapy (methotrexate, adriamycin, cisplatin, isofosfamide).

Closed biopsies of all patients were performed by fine-needle aspiration or trephine for diagnosis and then surgical treatment. The biopsy samples with bone tissue were decalcified before chemotherapy. Non-cancerous adjacent tissues were available in 30 of 60 patients. The pathological diagnosis was performed by the same group of two senior pathologists experienced in osteosarcoma diagnosis. The classification of osteosarcoma of patients was according to the 6th edition of the tumor-node-metastasis classification of the International Union against Cancer (UICC) (Shaha et al., 2007), including 20 case in Stage I, 17 cases in Stage II and 23 cases in Stage III. The study was approved by the Research Ethics Committee of Ministry of Public Health of China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

RNA preparation and quantitative RT-PCR

Total RNA was isolated from frozen bone tissue using tissue RNA extraction kit (Tiangen, China) according to the manufacturer's protocol and as described in the online supplement. The quality and quantity of the RNA was verified by the presence of two discrete electropherogram peaks corresponding to the 28S and 18S rRNA at a ratio approaching 2:1.Using mRNA as template, single-stranded cDNAs were generated by Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's

directions. Real-time quantitative PCR experiments were conducted by ABI Prism 7900 sequence-detection system (Applied Biosystems, Foster City, USA) and SYBR Green PCR Master Mix according to the manufacturer's protocol. The qPCR conditions are as follows: an initial 95°C for 10 min and followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. A dissociation curve was established after each PCR in order to verify amplification specificity.

Immunohistochemistry analysis

We carried out immunohistochemical detection of GAPDH using the avidin-biotin complex method. The specimens were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. The paraffin-embedded tissues were cut at 5 mm and then deparaffinized with xylene and rehydrated for further H&E or peroxidase (DAB) immunohistochemistry staining employing DAKO EnVision System (Dako Diagnostics, Zug, Switzerland). Following a brief proteolytic digestion and a peroxidase blocking, the tissue slides were divided into two groups and incubated with different primary antibodies rabbit antihuman GAPDH polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100) against GAPDH proteins, respectively, overnight at 4°C. After washing, peroxidase labeled polymer and substrate-chromogen were then employed in order to visualize the staining of the interested proteins. The quality (number, intensity and pattern) of every staining procedure for GAPDH has been comparatively evaluated using consecutive control sections and the immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological data and clinical outcomes of the patients.

The number of positive-staining cells in ten representative microscopic fields was counted and the percentage of positive cells was calculated. Given the homogenicity of the staining of the target proteins, tumor specimens were scored in a semi-quantitative manner based on the percentage of tumor cells that showed immunoreactivity. The staining results of GAPDH were classified into negative (0, staining of <15% of cells) or positive (1, staining of >15% of cells) (Salzer-Kuntschik et al., 1983).

Statistics analysis

To calculate the statistical differences between the control and osteosarcoma, the statistical package SPSS13.0 (SPSS Incorporated, USA) was used for all analysis. Student's t-test was used to determine the significance of differences among the groups. All values were expressed as mean ± SD. In general, p-values less than 0.05 were considered statistically significant.

RESULTS

In this study, we analyzed the expression of levels in 60 cases of osteosarcoma and adjacent normal bone tissues, including 20 cases in Stage I, 17 cases in Stage II and 23 cases in Stage III. We found that GAPDH were significantly over-expressed in osteosarcoma compared to the adjacent normal bone tissue on mRNA level (p < 0.05) (Figure 1A), which showed that GAPDH mRNA expression increased in patients with osteosarcoma.

The expression of GAPDH was detected in 44/60 (73.33%) of patients with osteosarcoma and in 10/30 (33.33%) of non-cancerous adjacent tissues (Table 2).
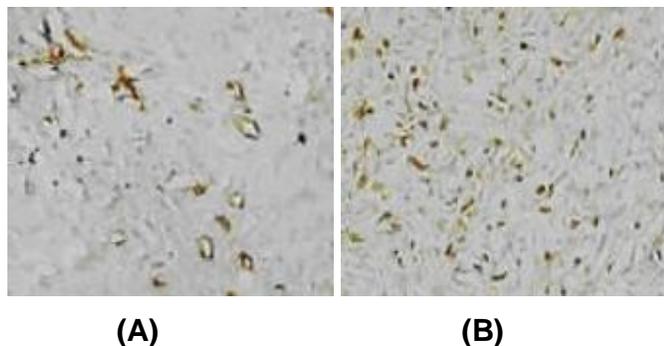
(A)   **(B)**

Figure 1. GAPDH expression in osteosarcoma and the adjacent normal bone tissue. (A), Normal bone tissue; (B), osteosarcoma (Stage III).

Table 1. Expression level of GAPDH mRNA in osteosarcoma and the adjacent normal bone tissue.

Group	n	$\overline{x}$ ± s (copy/µg RNA)
Osteosarcoma	60	217865.35 ± 48321.15[A]
Normal	30	97658.48 ± 28434.78[B]

Different letter represent the significant difference at $p < 0.05$.

Table 2. The GAPDH expression and staging of osteosarcoma,

Staging	n	Positive GAPDH (%)
Normal	30	6 (20.00)
Stage I	20	9 (45.00)[A]
Stage II	17	13 (76.47)[B]
Stage III	23	22 (95.74)[C]

Different letter represent the significant difference at $p < 0.05$.

Their signals concentrated primarily within the cytoplasm and membrane of the tumor cells (Figure 1B). These results showed that the expression of GAPDH levels in osteosarcoma was significantly higher than those of adjacent non-cancerous tissue on protein level ($p < 0.05$) (Table 1). Furthermore, GAPDH proteins was significantly over-expressed in ostesarcoma patients with surgical Stage III as compared to those with lower surgical Stages (I - II), which showed that the expression of GAPDH was correlated with the clinical stage osteosarcomas .

DISCUSSION

In the present study, we show a correlation between GAPDH gene expression and pathologic stage for patients with osteosarcomas. Average GAPDH levels for Stage III osteosarcomas specimens were significantly higher than those for Stage (I - II), specimens on protein.

Previous studies have shown that GAPDH expression is increased in other human tumors and tumor-derived cell lines from colon, kidney, liver, lung and pancreas (Nguewa et al., 2008; Lau et al., 2000; Gong et al., 1996; Chang et al., 2011; Revillion et al., 2000; Yamagata et al., 1998), which complies with our results. Because of the variability of expression of GAPDH in osteosarcomas, GAPDH expression should not be used as housekeeping genes in analyses on mRNA level and protein level in osteosarcomas studies.

The elevation of GAPDH mRNA level or protein level might accelerate the malignant cell proliferation, since enhanced expression of GAPDH was accompanied by an increase in the levels of enolase and glucose transporter and an accelerated rate of glucose transport has long been known to accompany cellular transformation (Gong et al., 1996). The up-regulation of GAPDH in tumor could contribute to augmented rate of glycolysis in tumor cells and/or maintenance of a transformed phenotype (Vila et al., 2000). Thus, we infer that GAPDH may have a role in osteosarcomas progression by some its function, which will depend on further study in future by a series of molecular method.

In conclusion, our data indicate that GAPDH expression was significantly up-regulated in patients with osteosarcomas, which implied that GAPDH may play an important role in osteosarcoma.

REFERENCES

Alexander MC, Lomanto M, Nassir N, Ramaika C (1988). Insulin stimulates glyceraldehyde-3-phosphate dehydrogenase gene expression through cis-acting DNA sequences. Proc .Nat. Acad. Sci. 85:5092-5096.
Cale JM, Millican DS, Itoh H (1997).Pregnancy Induces an Increase in the Expression of Glyceraldehyde-3-Phosphate Dehydrogenase in Uterine Artery Endothelial Cells. Reprod. Sci. 4(6):284-292.
Chang YT, Tseng HC, Huang CC, Chen YP, Chiang HC, Chou FP (2011). Relative down regulation of apoptosis and autophagy genes in colorectal cancer. Eur. J. Clin. Investig. 41(1):84-92.
Chou AJ, Gorlick R (2006). Chemotherapy resistance in osteosarcoma: current challenges and future directions. Expert. Rev. Anticancer. Ther. 6:1075e85.
Clark JC, Dass CR, Choong PF (2008). A review of clinical and molecular prognostic factors in osteosarcoma. J. Cancer. Res. Clin. Oncol .134:281-297.
Gong Y, Cui L, Minuk GY (1996). Comparison of glyceraldehyde-3-phosphate dehydrogenase and 28s-ribosomal RNA gene expression in human hepatocellular carcinoma. Hepatology 23(4):734-737.
Harada N, Yasunaga R, Higashimura Y (2007). Glyceraldehyde-3-phosphate Dehydrogenase Enhances Transcriptional Activity of Androgen Receptor in Prostate Cancer Cells J. Biol. Chem. 282:22651-22661.
Kager L, Zoubek A, Bielack SS (2003).Primary metastatic osteosarcoma: presentation and outcome of patients treated on

neoadjuvant Cooperative Osteosarcoma Study Group protocols. J. Clin. Oncol. 21:2011-2018.

Kim SJ, Choi JA, Lee SH (2004). Imaging findings of extrapulmonary metastases of osteosarcoma. Clin. Imaging 28:291-300.

Kim JW, Kim TE, Kim YK (1999) Antisense oligodeoxynucleotide of glyceraldehyde-3-phosphate dehydrogenase gene inhibits cell proliferation and induces apoptosis in human cervical carcinoma cell lines. Antisense Nucl. Acid Drug 9:507-513.

Lau WY, Lai PB, Leung MF (2000). Differential gene expression of hepatocellular carcionma using cDNA microarray analysis. Oncol. Res. 12(2): 59-69.

Longhi A, Errani C, De Paolis M (2006). Primary bone osteosarcoma in the pediatric age: state of the art. Cancer. Treat. Rev. 32:423-436.

Man TK, Lu XY, Jaeweon K (2004). Genome-wide array comparative genomic hybridization analysis reveals distinct amplifications in osteosarcoma. BMC Cancer 4:45.

Nguewa PA, Agorreta J, Blanco D(2008). Identification of importin 8 (IPO8) as the most accurate reference gene for the clinicopathological analysis of lung specimens. BMC Mol. Biol. 17(9):103.

Jung M, Ramankulov A, Roigas J (2007) In search of suitable reference genes for gene expression studies of human renal cell carcinoma by real-time PCR. BMC Mol. Biol. 8:47

Revillion F, Pawlowski V, Hornez L (2000). Glyceraldehyde-3-phosphate dehydrogenase gene expression in human breast cancer. Eur. J. Cancer 36(8):1038-1042.

Rienzo M, Schiano C, Casamassimi A, Grimaldi V, Infante T, Napoli C (2012). Identification of valid reference housekeeping genes for gene expression analysis in tumor neovascularization studies. Clin. Transl. Oncol. 25:671-679.

Rubporn A, Srisomsap C, Subhasitanont P (2009) Comparative proteomic analysis of lung cancer cell line and lung fibroblast cell line. Cancer. Genom. Proteom. 6:229-237.

Sabath DE, Broome HE, Prystowsky MB (1990). Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2- induced transcript in a cloned T-helper lymphocyte. Gene 91:185-191.

Salzer-Kuntschik M, Delling G, Beron G, Sigmund R (1983). Morphological grades of regression in osteosarcoma after polychemotherapy- study COSS 80. J. Cancer Res. Clin. Oncol. 106(Suppl):21-24.

Selvarajah S, Yoshimoto M, Ludkovski O (2008). Genomic signatures of chromosomal instability and osteosarcoma progression detected by high resolution array CGH and interphase FISH. Cytogenet. Genome. Res. 122:51-55.

Sirover MA (2011).On the functional diversity of glyceraldehyde-3-phosphate dehydrogenase: biochemical mechanisms and regulatory control. Biochim. Biophys. Acta. 1810:741-751.

Stiller CA, Craft AW, Corazziari I (2001). Survival of children with bone sarcoma in Europe since 1978: results from the EUROCARE study. Eur. J. Cancer .37:760-766.

Vila MR, Nicolas A, Morote M (2000). Increased glyceraldehyde-3-phosphate dehydrogenase expression in renal cell carcinoma identified by RNA based, arbitrarily primed polymerase chain reaction. Cancer 89:152-164.

Yamagata M, Mori M, Begum NA (1998). Glyceraldehyde-3-phosphate dehydrogenase mRNA expression in hepatocellular carcinoma. Int. J. Oncol. 12(3):677-683.