© IJHG 2021 Int J Hum Genet, 21(1): 23-29 (2021)
PRINT: ISSN 0972-3757 ONLINE: ISSN 2456-6330 DOI: 10.31901/24566330/21.01.772

Association of MMP-9 and PCNA Protein Expression in Osteosarcoma are Associated with Clinical Stage, Metastasis, and Prognosis

Xiaohu Wang[1] and Bo Zhao[2*]

[1]Department of Orthopaedics, Hanchuan People's Hospital, Hanchuan, PR China
E-mail: [1]<jnbv8956@126.com>, [2]<hcd89569@126.com>

KEYWORDS Biomarkers. Clinical Stage. Matrix Metallopeptidase 9. Proliferating Cell Nuclear Antigen. Osteosarcoma. Prognosis

ABSTRACT Present work studies the expressions of MMP-9 and PCNA in osteosarcoma patients and its correlation with clinical stage. 53 specimens of osteosarcoma were surgically removed and 28 osteochondroma tissues were used for immunohistochemical staining to detect the expression of proliferating cell nuclear antigen (PCNA) as well as matrix metalloproteinase-9 (MMP-9). The expression rates of MMP-9, as well as PCNA, were 75.5 percent and 86.8 percent in osteosarcoma, while were 10.7 percent and 7.5 percent in osteochondroma, respectively, which was statistically significant ($P<0.05$). The expression levels of MMP-9, PCNA, and the total survival time were connected with the Enneking stage. It shows an upward trend for the expression levels of PCNA and MMP-9 in osteosarcoma tissues. The expression levels of MMP-9 and PCNA are related to the clinical stage, metastasis, and prognosis.

INTRODUCTION

Osteosarcoma is a primary tumor that occurs in male adolescents. As a highly malignant tumor, osteosarcoma often results in poor prognosis due to its early metastasis, strong invasiveness, and rapid growth (Benini et al. 2015; Cong et al. 2016). According to data surveys, worldwide, the incidence of osteosarcoma is as high as 4/105 per year (Yaheng et al. 2014). Several markers were identified in correlation to the prognosis of osteosarcoma, and numerous studies have proved that Matrix metallopeptidase 9 (MMP-9), as well as proliferating cell nuclear antigen (PCNA), are widely expressed in malignant tumor tissues such as gastric cancer and colorectal cancer (Yonemura et al. 2015; Cong et al. 2016). MMP-9 protein is mostly expressed in the cytoplasm, and PCNA protein is mainly expressed in the nucleus. It is suggested that MMP-9 and PCNA are tumor factors, but there are few studies on the association between MMP-9 and PCNA and osteosarcoma. Although, numerous studies have already tested the prognostic role of proliferating cell nuclear antigen (PCNA) expression in patients with osteosarcoma with no consistent conclusion. Hence the researchers found that these results are contradictory and cannot be reliable for their significance in expression for the prognosis of osteosarcoma (Tang et al. 2017; Zhang et al. 2016). The hypothesis of this study was that expression of MMP-9 and PCNA protein might be associated with a different stage of metastasis, and prognosis of osteosarcoma. Briefly, the researchers compared the expression levels of PCNA and MMP-9 performing in osteosarcoma as well as osteochondroma, respectively, and the clinical features and prognosis of osteosarcoma patients with different expression levels to explore the relevance between the expressions of PCNA and MMP-9 in patients with osteosarcoma as well as its clinical stage as well as prognosis.

*Address for correspondence:
Bo Zhao,
Department of Orthopaedics,
Hanchuan people's Hospital,
Hanchuan City, Hubei Province, China
E-mail: hcd89569@126.com

Objectives

The present study aimed to evaluate the expressions of MMP-9 and PCNA in osteosarcoma patients and their correlation with the clinical stage.

Experimental

Clinical Data

Approved by the ethics committee, 53 patients with osteosarcoma diagnosed by patho-

logical biopsy from August 2011 to June 2013 were selected. The specimens of osteosarcoma surgically removed were collected into the study group, including 36 males and 17 females, who aged 12-34 years old, were staged by the international Enneking staging system, including 16 cases in phase IIA, 10 cases in phase IIB, and 27 cases in phase III. Another 28 patients with osteochondroma were selected as the control group which was diagnosed as osteochondroma using x-ray results, including 19 males and 9 females, aged 10-33 years old. Osteosarcoma is a malignant (cancerous) tumor of bone, while osteochondroma occurs in bones near joints usually a benign tumor. Inclusion criteria selected patients with not yet accepting radiotherapy and chemotherapy before the study; being followed up regularly; voluntarily having signed informed consent. Patients combined with severe cardiovascular and cerebrovascular diseases; severe mental disorders; and metabolic diseases such as diabetes without complete data, were excluded.

Main Reagents

Xylene (Shanghai Ziqi Biotechnology Co., Ltd.), sodium citrate solution (Shanghai Harling Biotechnology Co., Ltd.), phosphate buffer (Shanghai Yuanmu Biotechnology Co., Ltd.), 3% hydrogen peroxide solution (Shanghai source leaf) Biotechnology Co., Ltd., mouse anti-human MMP-9 polyclonal antibody (Wuhan Elitet Biotechnology Co., Ltd.), rabbit anti-human PCNA polyclonal antibody (Wuhan Elitet Biotechnology Co., Ltd.), sheep anti- Mouse IgG (Shanghai Xinfan Biotechnology Co., Ltd.), goat anti-rabbit IgG (Shanghai Xinfan Biotechnology Co., Ltd.), SP kit (Beijing Bole Life Science Development Co., Ltd.), DAB chromogenic kit (Shanghai Yanhui Biotechnology Co., Ltd.).

METHODOLOGY

The researchers employed Immunohistochemical staining to explore the relative expressions of MMP-9 and PCNA in the two groups. The paraffin-embedded tissue was treated with a tissue microtome, and the tissue was cut into 4 ìm thick serial sections. Following the kit instructions, the researchers employed xylene, sodium citrate solution, phosphate buffer, 3 percent hydrogen peroxide solution, and rabbit serum in turn to dewax, antigen repair, rinse, catalase block, and block the tissue sections. Mouse anti-human MMP-9 polyclonal antibody, as well as the rabbit anti-human PCNA polyclonal antibody of the first antibody, were added dropwise for incubating overnight, respectively, also, of the second antibody, goat anti-rabbit IgG and goat anti-mouse IgG were both added dropwise to incubate for 15 min, and horseradish peroxidation was added dropwise. The horseradish catalase-labeled streptavidin was added and was let to react for 15 min. The tissue sections were stained and counterstained with DAB coloring solution and hematoxylin, followed by dehydration, transparency, and sealing, with an observation with a microscope.

Determination of Results

The process was observed and analyzed by two pathologists with qualifications, respectively. Positive rate score of the tumor cell : zero stands for $\leq$ 5%, 1 for 6% -25%, 2 for 26% -50%, 3 for 51 %-75%, 4 for 76%-100%; staining intensity score: 0 for tumor cells without colors, 1 for light yellow, 2 for brown, and 3 for tan. The two types of scores were added together, and the final score of 0-4 was the low expression and >4 was the high expression.

Observation Indicators

The expression situations of MMP-9 and PCNA in osteochondroma as well as osteosarcoma, and the pathological parameters and prognosis of osteosarcoma patients with different expression levels of MMP-9 and PCNA.

Follow-up

Follow-up was performed by telephone and hospital review. The first year after the study, the patients were followed up after 1, 3, 6, and 12 months, and then once a year followed up to 48 months after the study or until death. Routine consultations, physical examinations, chest radiographs, and primary X-rays were performed at each follow-up.

Statistical Processing

All the data of the experiment were investigated taking advantage of the SPSS20.0 statisti-

cal software, among which a t-test was applied to the measurement data, χ^2 test was employed for the count data. Kaplan-Meier method and Log-rank method were utilized to investigate the association between pathological parameters and patient prognosis, and the survival curve was drawn. The test standard α=0.05, and when P<0.05, with statistical significance.

RESULTS

Expression Situation of MMP-9 and PCNA in Osteosarcoma and Normal Tissues

The high expression rates of MMP-9 protein in osteosarcoma and osteochondroma were 75.5 percent (40/53) and 10.7 percent (3/28), respectively, while the PCNA protein was 86.8 percent (46/53) and 7.1 percent (2/28), respectively. Briefly, the high expression rates of MMP-9 and PCNA in osteosarcoma were prominently higher than that in osteochondroma, with statistical significance (Table 1, Fig. 1). The expression of MMP-9 and PCNA was highly significantly different between the study and control group with χ^2 Value higher than 30 in both cases and p-value lower than 0.001.

The Relationship between the Expression Level of MMP-9 and Clinicopathological Features in Osteosarcoma

The high expression rate of MMP-9 protein (Fig. 1) in osteosarcoma tissues with clinical stage

Table 1: Comparison of expressions of PCNA and MMP-9 in osteosarcoma as well as osteochondroma

Group	*MMP-9*		*PCNA*	
	High expression	*Low expression*	*High expression*	*Low expression*
Study group (n=53)	40	13	46	7
Control group (n=28)	3	25	2	26
χ^2	30.849		48.143	
P	<0.001		<0.001	

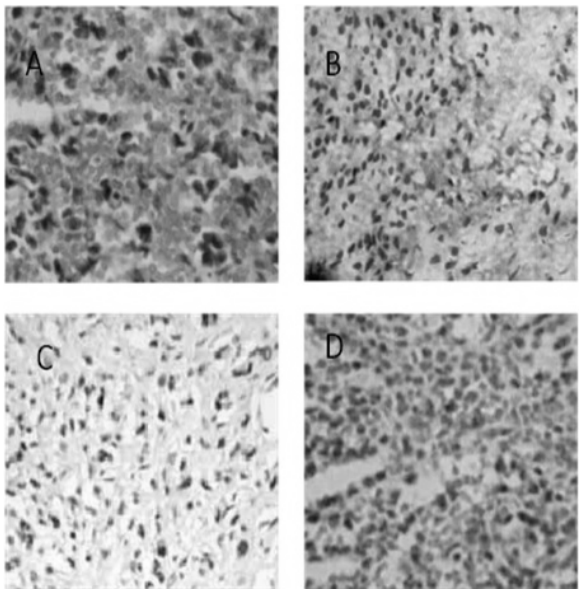

Fig. 1. Expression of MMP-9 and PCNA in osteosarcoma and osteochondroma (×400). A: Expression of MMP-9 in osteosarcoma; B: Expression of MMP-9 in osteochondroma; C: Expression of PCNA in osteosarcoma; D: Expression of PCNA in osteochondroma

IIB/III was 97.3 percent (36/37), which was prominently higher than 25 percent (4/16) of IIA period, demonstrating the statistical significance (P<0.001); the high expression rate of MMP-9 protein in osteosarcoma tissues in patients with metastasis was 93.8 percent (30/32), which was higher than 47.6 percent (10/21) of the absence of metastasis. The difference was statistically significant (P<0.001); Also, it existed no diversity in the expression level of MMP-9 protein in osteosarcoma in patients with different genders, ages, and histologies (Table 2) (P>0.05).

Table 2: Association between the expression level of MMP-9 and the characteristics of various clinical cases in osteosarcoma

Pathological features	*MMP-9*			
	High expression	*Low expression*	χ^2	*P*
Gender			2.203	0.137
Male	25	11		
Female	15	2		
Age			2.198	0.138
>14	29	12		
≤14	11	1		
Clinical Stage			31.538	<0.001
Phase IIA	4	12		
Phase IIB/III	36	1		
Histological Type			5.799	0.122
Osteoblast type	21	4		
Chondrocyte type	8	2		
Fibroblast type	4	5		
Hybrid	7	2		
Ability to Transfer			14.576	<0.001
Yes	30	2		
No	10	11		

The Association between the Expression Level of PCNA and Clinicopathological Features in Osteosarcoma

The high expression rate of PCNA protein in osteosarcoma tissues with clinical stage IIB/III was 97.3 percent (36/37), which was prominently higher than 62.5 percent (10/16) in IIA period, showing statistical significance (P< 0.05); The high expression rate of PCNA protein in osteosarcoma in patients with metastasis was 96.9 percent (31/32), which was remarkably higher than that of non-existing metastasis (71.4%, 15/21). The difference was statistically significant (P<0.05); and, it existed no diversity in the expression of PCNA protein in osteosarcoma of patients with different genders, ages, and histological types (Table 3) (P>0.05).

Table 3: Association between the expression level of PCNA and characteristics of various clinical cases in osteosarcoma

Pathological features	*MMP-9*			
	High expression	*Low expression*	χ^2	*P*
Gender			1.172	0.279
Male	30	6		
Female	16	1		
Age			0.322	0.571
>14	35	6		
≤ 14	11	1		
Clinical Stage			11.799	<0.001
Phase IIA	10	6		
Phase IIB/III	36	1		
Histological Type			1.977	0.577
Osteoblast type	21	4		
Chondrocyte type	8	2		
Fibroblast type	9	0		
Hybrid	8	1		
Ability to Transfer			7.162	0.007
Yes	31	1		
No	15	6		

Analysis of Factors Related to the Survival Rate of Patients with Osteosarcoma

The survival time of patients with osteosarcoma with different clinical-stage, metastasis, MMP-9, and PCNA expression levels showed statistical significance (P<0.05), while the difference of survival time between patients with different ages, gender, and the histological type was on the contrary (P<0.05). The ability to transfer the cells has p-value lower than 0.001 and hence it was significantly associated with the survival rate of patients (Table 4, Figs. 2 and 3).

Table 4: Single-factor analysis of survival rate

Pathological parameters	*Univariate analysis*	
	Log-Rank	*P*
Age	0.749	0.387
Gender	1.062	0.303
Clinical stage	11.367	0.001
Histological type	4.173	0.224
Transfer	28.068	<0.001
MMP-9	11.609	0.001
PCNA	08.467	0.004

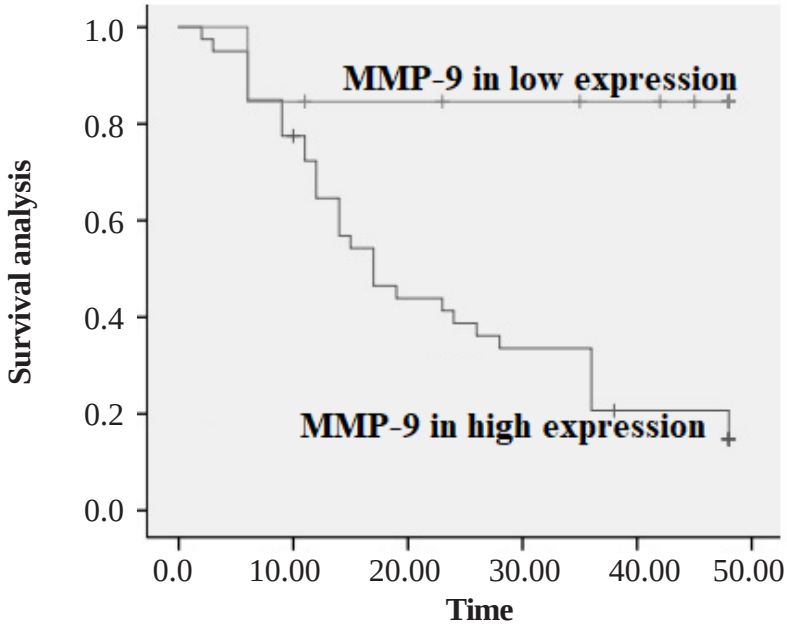

Fig. 2. Contrast of survival curves of MMP-9 between in high and low expression groups in osteosarcoma

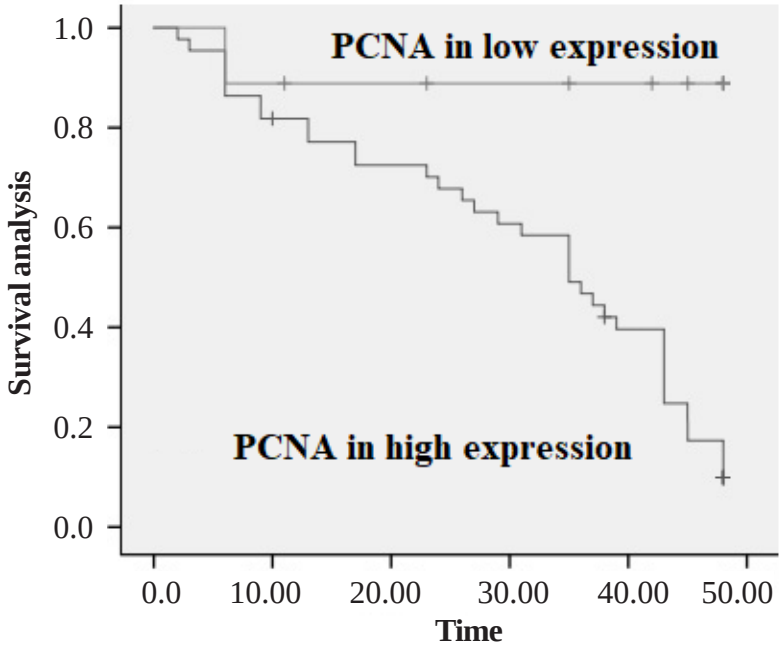

Fig. 3. Contrast of survival curves of PCNA high and low expression groups in osteosarcoma

DISCUSSION

Osteosarcoma, often occurring in adolescents, is regarded as a malignant tumor. As the most common bone malignant tumor, osteosarcoma cells can directly produce bone-like tissue and bone tissue through the cartilage stage, and thus osteosarcoma grows rapidly (Benini et al. 2015). Besides, osteosarcoma is prone to early metastasis, and the lung is its most commonly affected organ. Bhattasali et al. (2015) found that 20 percent of patients with osteosarcoma had lung metastases at the first hospitalization. Based on the above pathological features, patients with osteosarcoma often have a poor prognosis, and the mortality and disability rates remain high.

Matrix metalloproteinase-9 (MMP-9) is a zinc ion-dependent extracellular proteolytic enzyme mainly expressed in the cytoplasm. As a member of the matrix metalloproteinase family, it serves a vital function in tumor invasion (Fan et al. 2015). Xueliang et al. (2016) discovered MMP-9 showed a high expression trend in colorectal cancer tissues. It revealed that the high expression rate of MMP-9 protein in osteosarcoma tissue was remarkably higher than in osteochondroma tissue, which corresponds with the results of Xueliang et al. (2016). Furthermore, patients with osteosarcoma with different clinical stages and metastases have significant differences in the expression levels of MMP-9 in cancer tissues. Also, patients with high MMP-9 expression have a worse prognosis. This indicates that the expression level of MMP-9 in osteosarcoma patients is correlated with clinical stage, metastasis, and prognosis. Analysis of the reason suggests that osteosarcoma cells express abundant extracellular matrix metalloproteinase-inducing molecules and induce the synthesis of MMP-9 protein (Lin et al. 2014). As a gelatinase, MMP-9 can degrade the extracellular matrix (ECM), reduce the tightness between tissue structure and the adhesion ability of tumor cells. Meanwhile, MMP-9 can also induce tumor cells to secrete angiogenic factors and promote tumor angiogenesis (Candido et al. 2016; Ho et al. 2016). Therefore, MMP-9 protein can encourage tumor growth, metastasis, and invasion.

Proliferating cell nuclear antigen (PCNA), in essence, is a protein diffusely expressed in proliferating cells in vivo and serves a vital function in the process of DNA replication in eukaryotic cells (Paradiso et al. 2015; Zhoushuai et al. 2014). Jupeng et al. (2015) found that PCNA is important for the growth of gastric cancer. The expression level of PCNA in gastric cancer tissues is prominently higher than that in normal ones. This study found that PCNA showed a high expression trend in osteosarcoma, which corresponds with the results of Jupeng et al. (2015). Additionally, in patients with osteosarcoma with different clinical stages and metastases, the expression level of PCNA in cancer tissues is significantly different, and patients with higher expression of PCNA had a worse prognosis. This conveys that the expression level of PCNA in osteosarcoma patients is connected with clinical stage, metastasis, and prognosis. To analyze the reason, PCNA may be served as an accessory protein of DNA polymerase to engage in the regulation of DNA synthesis during eukaryotic cell proliferation, whose expression level in the nucleus varies periodically with cell proliferation (Zhoushuai et al. 2014; Yang et al. 2014).

CONCLUSION

PCNA features in the tumor cell proliferation, which can be used to evaluate tumor cell proliferation status. In general, the expression levels of MMP-9 and PCNA protein in osteosarcoma tissues show an upward trend, and the expression levels of MMP-9 and PCNA are associated with clinical stage, metastasis, and prognosis.

RECOMMENDATIONS

For patients with osteosarcoma with different clinical stages, the expression level of MMM-9 and PCNA varies significantly and patients with higher expression of PCNA showed no improvement, hence the study recommends that the expression level of PCNA be connected with clinical stage, metastasis, and prognosis.

REFERENCES

Benini S, Baldini N, Manara MC et al. 2015. Redundancy of autocrine loops in human osteosarcoma cells. *Int J Cancer,* 80(4): 581-588.

Bhattasali O, Vo AT, Roth M et al. 2015. Variability in the reported management of pulmonary metastases in osteosarcoma. *Can Med,* 4(4): 523-531.

Candido S, Abrams SL, Steelman LS et al. 2016. Roles of NGAL and MMP-9 in the tumor microenvironment and sensitivity to targeted therapy. *Biochimicaet Biophysica Acta (BBA) - Mol Cell Res*, 1863(3): 438-448.

Cong M, Li J, Jing R et al. 2016. Long non-coding RNA tumor suppressor candidate 7 functions as a tumor suppressor and inhibits proliferation in osteosarcoma. *Tumour Biol*, 37(7): 9441-9450.

Fan SH, Wang YY, Lu J et al. 2015. CERS2 suppresses tumor cell invasion and is associated with decreased V-ATPase and MMP-2/MMP-9 activities in breast cancer. *J Cel Biochem*, 116(4): 502-513.

Ho HY, Lin CW, Chien MH et al. 2016. Melatonin suppresses TPA induced metastasis by downregulating matrix metalloproteinase 9 expression through JNK/SP 1 signaling in nasopharyngeal carcinoma. *J Pineal Res*, 61(4): 479-492.

Jupeng Yang, Daguang Wang, Yagang Li et al. 2015. Expression of β-catenin and proliferating cell nuclear antigen in gastric cancer and its relationship with prognosis. *Chinese J Gen Sur*, 30(8): 635-638.

Lin Kang, Wei Du, Bin Wang et al. 2014. Preliminary study on the degradation pathway of extracellular matrix metalloproteinase inducer. *J Neuroanat*, 30(5): 541-545.

Liu Gang, Ying-hua Guan, Xiao-qiang Ren et al. 2012. Correlation of both MMP-9 and uPA expression with prognosis of ureter transitional cell carcinoma. *Chinese J Clin Exp Pathol*, 10: 1.

Paradiso A, Rabinovich M, Vallejo C et al. 2015. p53 and PCNA expression in advanced colorectal cancer: Response to chemotherapy and long term prognosis. *International J Cancer*, 69(6): 437-441.

Tang Y, Yang C, Guo Z et al. 2017. P16 protein expression as a useful predictive biomarker for neoadjuvant chemotherapy response in patients with high-grade osteosarcoma: A systematic meta-analysis under guideline of PRISMA. *Medicine*, 96: e6714.

Xueliang Wu, Jun Xue, Likun Wang et al. 2016. Expression of DNA-binding differentiation inhibitory protein 1 and matrix metalloproteinase-9 in colorectal cancer and its correlation with microvessel density. *J Chinese Aca Med Sci*, 38(6): 696-701.

Yang Qingyuan, Chao Ou, Mei Liu et al. 2014. NRAGE promotes cell proliferation by stabilizing PCNA in an ubiquitin-proteasome pathway in esophageal carcinomas. *Carcinogen*, 35(7): 1643.

Yaheng Zhao, Helin Feng, Lihua Zheng et al. 2014. Progress in the pathogenesis of osteosarcoma. *Can Res*, 41(3): 283-286.

Yonemura Y, Kimura H, Fushida S et al. 2015. Analysis of proliferative activity using anti proliferating cell nuclear antigen antibody in gastric cancer tissue specimens obtained by endoscopic biopsy. *Cancer*, 71(8): 2448-2453.

Zhang Q, Liu F, Wang B et al. 2016. HER-2 expression in biopsy and surgical specimen on prognosis of osteosarcoma: A systematic review and meta-analysis of 16 studies. *Med*, 95: e3661.

Zhoushuai Qin, Chuanlin Zhang, Wei Xiao 2014. DNA damage tolerance mechanism regulated by PCNA post-translational modification. *Life Science*, 26(11): 1143-1156.

Paper received for publication in November, 2020
Paper accepte for publication in February, 2021