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Original Article

Expression and significance of matrix metalloprotein 9 (MMP-9) mRNA in ascites

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Abstract: This study aims to discuss the expression of matrix metalloprotein 9 (MMP-9) mRNA in the ascites and its value for the differential diagnosis of nature of ascites. 34 patients with the malignant ascites and 15 patients with the benign ascites were selected as the subjects. The real-time fluorescent quantitative polymerase chain reaction (PCR) was employed to detect the expression of MMP-9 mRNA in the ascites. The difference in the expression of MMP-9 mRNA in the benigh ascites and malignant ascites and the expression of MMP-9 mRNA under the different causes in the ascites were compared. The expression of MMP-9 mRNA in 31 patients with the malignant ascites and 15 patients with the benign ascites was 14.67 (9.19, 38.08) and 0.90 (0.71, 2.26) respectively. The expression of MMP-9 mRNA in the malignant ascites was significantly higher than that in the benign ascites, with statistical significance ($P<0.05$). There was no significant difference in the expression of MMP-9 mRNA in the malignant ascites under different primary diseases ($P>0.05$), as well as under liver cirrhosis and tuberculous peritonitis ($P>0.05$). The expression of MMP-9 mRNA in the malignant ascites is significantly higher than that in the benign ascites, which is of critical significance for the differential diagnosis of nature of ascites. In the clinical practice, the detection of expression of MMP-9 mRNA in the ascites can specify the nature of ascites in the early stage.

Keywords: Ascites, MMP-9 mRNA, fluorescent quantitative PCR, tumor

Introduction

The ascites is one of the common clinical symptoms, which can be divided into benign ascites and malignant ascites. The differential diagnosis of benign and malignant ascites is always a clinical problem. The treatment and prognosis of ascites because of different causes are totally different. The palliative treatment after definite diagnosis can significantly improve the quality of life for patients and thus it will be of critical importance to specify the nature of ascites. Presently, the indicators for differential diagnosis of ascites include the ascitic cytology, ascitic tumor markers, ascitic biochemical markers and imaging [1, 2]. But these indicators are still less than ideal for the differential diagnosis of benign and malignant ascites. The ascitic cytology is the gold standard for the diagnosis of malignant ascites, with the high specificity, but according to the foreign research, its highest sensitivity was only 50%-60% [3]. Besides, it requires many times of drawing the ascites. In case of abundant ascites, there are few exfoliated tumor cells per unit volume, resulting in the lower positive rate and misdiagnosis and missed diagnosis. The study on the effective method to rapidly diagnose the nature of ascites is critically important for the clinical diagnosis and treatment. The research indicated that increased expression of matrix metalloprotein 9 (MMP-9) in tumor was related to the invasion and metastasis of tumor [4-6]. In addition to the solid tumor, the expression of MMP-9 was also increased in the pleural effusion, which played a key role in the formation of pleural effusion [7]. But there have been few researches on the expression of MMP-9 in the ascites. Because of the increased expression of MMP-9 in the tumor cells and its involvement in the invasion and metastasis of tumor, we presumed that the cancer cells might be exfoliated in the ascites during the peritoneal metastases of advanced tumor. The expression of MMP-9 in

Table 1. General data of patients with malignant ascites and benign ascites

Item		Malignant asctes (n=34)	Benign ascites (n=15)	*T*	*P*
Age (years)		55±15.96	52.9±9.43	0.391	0.698
Gene	Male	15 (44.12%)	12 (80%)	1.929	0.193
	Female	19 (55.88%)	3 (30%)		

Quantitative data between the two groups were compared using *t* test (*P*>0.05).

the exfoliated cells of malignant ascites should be higher than that in the benign ascites. Accordingly, in the clinical practice, it may be possible to detect the expression of MMP-9 in the exfoliated cells of ascites to diagnose the malignant ascites.

Materials and methods

General data

49 patients with the ascites treated and diagnosed in the Fifth Affiliated Hospital of Sun Yat-sen University from June 2014 to February 2015 were collected prospectively. According to results of the medical history, clinical manifestations, B-type ultrasound scan and CT, patients were divided into the benign ascites group and malignant ascites group, with 34 cases of malignant ascites and 15 cases of benign ascites. Patients who were with unclear diagnosis or whose ascites that could not be decided whether it was caused by the tumor or non-neoplastic diseases were excluded. This study had been approved by the Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University. All patients had understood all aspects of the informed consent and signed the consent.

Sampling

After signing the informed consent, patients were given the abdominal puncture and drainage or diagnostic abdominal paracentesis. The ascites were collected by 50 ml after the operation and then centrifuged at 3500 rpm and 4°C for 15 min. The supernatant was removed and the precipitate was stored at -80°C for the extraction of total RNA.

Design and synthesis of primers

GAPDH was chosen as the reference gene. Primer Premier 5.0 was employed to design the primer sequence of gene by referring to the mRNA sequence of human MMP-9 and GAPDH in NCBI. The primer sequence of MMP-9 was 5'-AGTCCACCCTTGTGCTCTTC-3' (forward primer), 5'-ACTCTCCACGCATCTCTGC-3' (reverse primer), with the primer size of 117 bp. The primer sequence of GADPH was 5'-GTGGGGCGCCAGGCACCAG-3' (forward primer), 5'-CTCCTATGTCACGCACATTC-3' (reverse primer), with the primer size of 138 bp.

Extraction of total RNA from the ascites

The precipitate of ascites was collected and dissolved. Then 1 ml Trizol (TaKaLa, Dalian) was added per 5×10^6-1×10^7 cells for the cell lysis. The total RNA was extracted from the ascites according to the instruction manual of Trizol. The UV spectrophotometer was used to detect the concentration and purity of total RNA.

Reverse transcription

500 ng total RNA was collected and then PrimeScript™ RT reagent Kit (TaKaLa, Dalian) was employed to transcript into cDNA, with the reaction conditions of 37°C for 15 min and 85°C for 5 s.

PCR amplification

cDNA was chosen as the template. SYBR PremixEx Taq™ reagent kit (TakaLa, Dalian) was used for the real-time fluorescent quantitative PCR, with the reaction system of 25 μl and reaction conditions of 95°C for 30 s, 95°C for 5 s and 60°C for 30 s, 40 cycles. The relative expression of MMP-9 was expressed by $2^{-\Delta\Delta Ct}$, with 3 repeats for each gene of each sample.

Statistical analysis

SPSS19.0 was employed for the statistical analysis. When the data met the normal distribution, the "mean ± standard deviation" was selected for the statistical description. The t test was used for the comparison between two groups, while the analysis of variance for the comparison among groups. When the data did not meet the normal distribution (non-normal distribution), the "median (interquartile range (P25, P75))" was selected for the statistical description. The Mann-Whitney rank sum test was used for the comparison between two groups, while Kruskal-Wallisa rank sum test for the comparison among groups. *P*<0.05 indicated the significant difference.

Table 2. Relationship between expression of MMP-9 mRNA in ascites and gender andage

	Number	Median (interquartile range)	*P* value
Malignant ascites			
Age (years)			
<50	11	13.71 (9.01, 36.81)	0.24
≥50	20	11.33 (8.50, 42.58)	
Gene			
Male	15	13.70 (9.01, 50.11)	0.53
Female	16	15.64 (9.23, 27.68)	
Benign ascites			
Age (years)			
<50	6	2.21 (1.18, 2.83)	0.08
≥50	9	0.80 (0.77, 1.14)	
Gene			
Male	12	1.14 (0.72, 2.32)	0.92
Female	3	0.89 (0.83, 2.01)	

Quantitative data between the two or three groups were compared using Mann-Whitney U and Kruskal-Wallis test (*P*>0.05).

Table 3. Expression of MMP-9 mRNA in ascites

Group	Number	Median (interquartile range)	*P* Value
Malignant ascites	31	14.67 (9.19, 38.08)	0.000
Benign ascites	15	0.90 (0.71, 2.26)	

Quantitative data between the two groups were compared using Mann-Whitney U test (*P*<0.05).

Results

General data of patients

There were 34 patients with the malignant ascites in this study, including 15 males and 19 females, with the age range of 33-94 year-old and average age of 55±15.96 year-old; 15 patients with the benign ascites, including 12 males and 3 females, with the age range of 39-65 year-old and average age of 52.9±9.43 year-old. The statistical analysis indicated that there was no significant difference in the age and gender between the malignant ascites group and benign ascites group (*P*>0.05, **Table 1**).

There were 3 cases with the malignant ascites that had no amplification of MMP-9. Patients were divided into different groups according to the gender and age and then the difference in the expression of MMP-9 mRNA in the ascites was compared. The results showed that there was no significant difference in the expression of MMP-9 mRNA in the benign ascites and malignant ascites at the gender and age composition (*P*>0.05, **Table 2**).

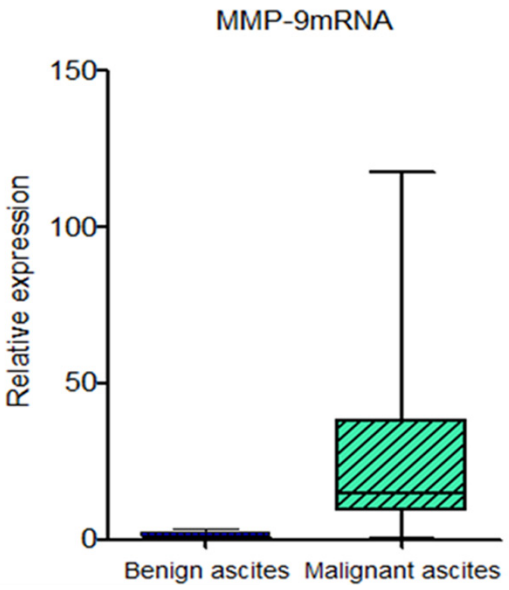

Figure 1. Expression of MMP-9 mRNA in ascites.

Expression of MMP-9 mRNA in ascites

The relative expression of MMP-9 mRNA in 31 patients with malignant ascites was 14.67 (9.19, 38.08), which was significantly higher than 0.90 (0.71, 2.26) of patients with benign ascites, with the statistical difference (*P*<0.05) (**Tables 1**-**3**; **Figure 1**). It indicated that MMP-9 mRNA was of significance in the differential diagnosis of benign and malignant ascites (**Table 3**; **Figure 1**).

The malignant ascites was divided into 7 groups according to the primary tumor, including 12 cases with the liver cancer, 6 cases with the ovarian cancer, 2 cases with the gastric cancer, 3 cases with the endometrial cancer, 2 cases with the pancreatic cancer, 4 cases with the peritoneal primary cancer and 2 cases with the intestinal cancer. The relative expression of ascitic MMP-9 mRNA was different in the different primary tumors. The results of this study showed that the relative expression of MMP-9 mRNA in the malignant ascites was highest in the intestinal cancer, followed by the pancreatic cancer and ovarian cancer. But there were no significant difference of MMP-9 mRNA expression level in the malignant ascites of different primary tumors (*P*>0.05, **Table 4**).

Table 4. Expression of MMP-9 mRNA in malignant ascites of different primary tumors

Group	Number	Median (interquartile range)	*P* Value
Liver cancer	12	9.84 (4.87, 33.04)	
Ovarian cancer	6	25.67 (11.33, 61.57)	
Gastric cancer	2	33.51 (30.21, 36.81)	0.169
Endometrial cancer	3	69.98 (22.42, 117.54)	
Pancreatic cancer	2	40.40 (29.69, 51.11)	
Peritoneal primary cancer	4	16.46 (10.18, 22.74)	
Intestinal cancer	2	4.89 (0.44, 9.33)	

Table 5. Expression of MMP-9 mRNA in cirrhosis and tuberculous peritonotis

Group	Number	Median (interquartile range)	*P* value
Cirrhosis	11	0.80 (0.67, 2.32)	0.64
Tuberculous peritonotis	4	1.29 (1.03, 1.33)	

Quantitative data between the two groups were compared using Mann-Whitney U test ($P>0.05$).

There were only 2 diseases of cirrhosis and tuberculous peritonitis in the benign ascites, including 11 cases with the cirrhosis and 4 cases with the tuberculous peritonitis. The expression of MMP-9 mRNA in the cirrhosis and tuberculous peritonitis was 0.80 (0.67, 2.32) and 1.29 (1.03, 1.33) respectively, with no significant difference ($P>0.05$, **Table 5**).

Discussion

MMP-9 is also named as the gelatinase B, as the enzyme with the largest molecular weight in the family of matrix metalloproteinases, which can degrade the extracellular matrix [8, 9]. In addition to the degradation of extracellular matrix, the activated MMP-9 can also degrade type IV, V, VII and X collagens, laminin, proteoglycan and core proteins, directly destroy the barrier of basement membrane and play the key role in the infiltration and invasion of tumor into the surrounding tissues and its distant metastasis [10, 11]. More and more researches had reported that the increased expression of MMP-9 in the tumor and its involvement in the occurrence and development of tumor were related to the invasion and metastasis of tumor cells [12, 13]. During the metastasis and invasion of tumor cells, the first step was the binding of tumor cells and surface receptor of basement membrane. Afterwards, it would secrete the enzymes such as MMP to degrade the basement membrane and extracellular matrix. Finally, the tumor cells could spread around along the defect parts of basement membrane [8, 14]. The current knowledge about the relationship between MMP-9 and tumor has not been limited to the matrix degradation and tumor invasion, but all aspects of the biological behavior of tumor, such as the early formation of tumor, angiogenesis of tumor, metastasis, invasion and activity of tumor, regulation of tumor immune surveillance and promotion of growth of metastatic cells [6]. MMP-9 is mainly distributed in the tumor cells and some interstitial cells around the tumor [15]. The malignant ascites is an obvious clinical manifestation for the invasion and metastasis of malignant tumor cells into the peritoneal cavity. Thus during the metastasis of tumor cells into the peritoneal cavity, the tumor cells or interstitial cells around the tumor can be released into the ascites. In this way, MMP-9 can be detected in the malignant ascites. MMPs have been widely reported as key factors in the druge delivery systems [16, 17]. According to one-stage clinical research on the perfusion of matrix metalloproteinase inhibitors in 23 patients with the malignant ascites in 1998 (BB94), MMP inhibitors could inhibit the formation of malignant ascites [18], which indicated the close relationship between MMP-9 and the formation of ascites.

Fluorescent Quantitative PCR is also named as qPCR for short. To be specific, the fluorescent molecule is added in the PCR amplification system, while the proportion of fluorescent signal is used to reflect the increase of DNA amount. It can solve the problem of uncertainty of traditional PCR on the quantitative template at the end point, with the advantages of high efficiency, accuracy and rapidity. It has been widely applied in the fields of biological medicine and molecular diagnosis, as some kind of reliable method to detect the micro-RNA in different cells, tissues and organs. Besides, it can also be used for the detection of quantitative expression of gene and load of bacterium and virus and mutation [19]. Because of the small sample size required by the study, it only needs small amount of RNA to detect the expression level of genes in the sample. The sample in this study was ascites with the convenient sampling

and limited paints for the patients, which was easily accepted by the patients and also suitable for the clinical application.

Accordingly, fluorescent quantitative PCR was employed in this study to detect the expression of MMP-9 mRNA in the ascites. 31 of 34 cases with the malignant ascites had the amplification of MMP-9 mRNA, while 15 cases with the benign ascites all had the amplification of MMP-9 mRNA. There was no significant difference in the expression of MMP-9 mRNA in the benign and malignant ascites between the ages and gender composition, which indicated that the expression of MMP-9 mRNA in the ascites was irrelative of the age and gender. The relative expression of MMP-9 mRNA in the malignant ascites of 31 cases was 14.67 (9.19, 38.08), while the one in the benign ascites was 0.90 (0.71, 2.26), namely the relative expression of MMP-9 mRNA in the malignant ascites was 14.67 (9.19, 38.08) times higher than that in the benign ascites. The expression of MMP-9 mRNA in the malignant ascites was significantly higher than that in the benign ascites, with the statistical difference, which indicated that the detection of the expression of MMP-9 mRNA in the ascites could be used for the differential diagnosis of benign and malignant ascites. Zhang et al. [20] used the ELISA method to detect that the expression of MMP-9 in the malignant ascites was higher than that in the cirrhosis ascites and tuberculous ascites. Shen [21] adopted the ELISA method to detect the expression of MMP-9 in the ascites of 23 patients with the benign ovarian lesions and 50 patients with the malignant ovarian tumor, which indicated that the expression of MMP-9 in the malignant ascites was significantly higher than that in the benign one. The expression of MMP-9 in the lymph node metastasis group was higher than that in the group without the lymph node metastasis and the one in the group with the ascites volume larger than 1000 ml was higher than that in the group with the ascites amount less than or equal to 1000 ml, which indicated that the detection of expression of MMP-9 in the ascites could become the marker for the differential diagnosis of benign and malignant ovarian lesions. The findings of above two researches were similar with the ones of this study, which all indicated that the expression of MMP-9 in the malignant ascites was higher than that in the benign ascites. Sun et al. [22] adopted the gelatin zymography assay to detect the expression of MMP-9 in the ascites of 67 patients and the results showed that there was no expression of MMP-9 in the liver cirrhosis and tuberculous peritonitis, but had the high expression in the malignant ascites. But the results of this study showed the amplification of MMP-9 in the ascites of patients with the liver cirrhosis and tuberculous peritonitis, namely the expression of MMP-9 in the liver cirrhosis and tuberculous peritonitis, which was different from the findings of SUN and might be related to the different experimental methods, sample size and research objects. Based on such above analysis, the results of this study indicated that MMP-9 played the certain role in the formation of malignant ascites and thus in the clinical practice, it could detect the expression of MMP-9 mRNA in the ascites to identify the benign and malignant ascites, which was of critical significance for the differential diagnosis of the nature of ascites and could also provide the theoretical foundation for the study on the relationship between MMP-9 and the formation of ascites.

Is it possible to detect the expression of MMP-9 mRNA in the ascites to differentiate the primary diseases with the unclear ascites for the further examination and treatment plan? After analyzing the expression of MMP-9 in the ascites with the different causes, the results showed that the relative expression of MMP-9 mRNA was different in the different primary tumors, with the highest relative expression in the intestinal cancer, followed by the pancreatic cancer and ovarian cancer. But there was no significant difference in the relative expression of MMP-9 mRNA in the malignant ascites of different primary tumors. For the benign ascites, there was no significant difference in the expression of MMP-9 mRNA between the liver cirrhosis and tuberculous peritonitis. Therefore, this study indicated that the detection of expression of MMP-9 in the ascites could only be used for the differential diagnosis of benign and malignant ascites, but not for the differential diagnosis of origin of primary tumor or the differential diagnosis of cirrhosis ascites and tuberculous ascites. But there were limitations in this study, namely the small sample size, which should be increased in the further study.

To sum up, the conclusion could be drawn that in the clinical practice, it could detect the expression of MMP-9 mRNA in the ascites for

the differential diagnosis of benign and malignant ascites, but there was no evidence to prove that the detection of expression of MMP-9 mRNA in the ascites could differentiate the origin of primary tumor or the ascites of liver cirrhosis and tuberculous peritonitis. The differential diagnosis of benign and malignant ascities is of critical importance for the treatment and prognosis. It's hoped that there will be a new vision of future studies in such field.

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Disclosure of conflict of interest

None.

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References

[1] Kaleta EJ, Tolan NV, Ness KA, O'Kane D and Algeciras-Schimnich A. CEA, AFP and CA 19-9 analysis in peritoneal fluid to differentiate causes of ascites formation. Clin Biochem 2013; 46: 814-818.

[2] Lu J, Li XF, Kong LX, Ma L, Liao SH and Jiang CY. Expression and significance of cyclooxygenase-2 mRNA in benign and malignant ascites. World J Gastroenterol 2013; 19: 6883-6887.

[3] Sangisetty SL and Miner TJ. Malignant ascites: A review of prognostic factors, pathophysiology and therapeutic measures. World J Gastrointest Surg 2012; 4: 87-95.

[4] Ławicki S, Zajkowska M, Głażewska EK, Będkowska GE and Szmitkowski M. Plasma levels and diagnostic utility of VEGF, MMP-9, and TIMP-1 in the diagnosis of patients with breast cancer. Onco Targets Ther 2016; 9: 911-919.

[5] Chen Y, Wang W, Li Z, Zhou J, Xiao L, Liu H and Liu B. Expression and significance of MTDH and MMP-9 in laryngeal squamous cell carcinoma. Lin Chung Er Bi Yan Hou Jing Wai Ke Za Zhi 2016; 30: 60-63.

[6] Farina AR and Mackay AR. Gelatinase B/MMP-9 in Tumour pathogenesis and progression. Cancers (Basel) 2014; 6: 240-296.

[7] Apostolidou E, Paraskeva E, Gourgoulianis K, Molyvdas PA and Hatzoglou C. Matrix metalloproteinases 2 and 9 increase permeability of sheep pleura in vitro. BMC Physiol 2012; 12: 2.

[8] Kessenbrock K, Plaks V and Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 2010; 141: 52-67.

[9] Nagase H, Visse R and Murphy G. Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 2006; 69: 562-573.

[10] Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM and Shafie S. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 1980; 284: 67-68.

[11] Candido S, Abrams SL, Steelman LS, Lertpiriyapong K, Fitzgerald TL, Martelli AM, Cocco L, Montalto G, Cervello M, Polesel J, Libra M and McCubrey JA. Roles of NGAL and MMP-9 in the tumor microenvironment and sensitivity to targeted therapy. Biochim Biophys Acta 2016; 1863: 438-448.

[12] Oguić R, Mozetič V, Cini Tešar E, Fučkar Čupić D, Mustać E and Dorđević G. Matrix metalloproteinases 2 and 9 immunoexpression in prostate carcinoma at the positive margin of radical prostatectomy specimens. Patholog Res Int 2014; 2014: 262195.

[13] Verma S, Kesh K, Ganguly N, Jana S and Swarnakar S. Matrix metalloproteinases and gastrointestinal cancers: Impacts of dietary antioxidants. World J Biol Chem 2014; 5: 355-376.

[14] Gialeli C, Theocharis AD and Karamanos NK. Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. FEBS J 2011; 278: 16-27.

[15] Mehner C, Hockla A, Miller E, Ran S, Radisky DC and Radisky ES. Tumor cell-produced matrix metalloproteinase 9 (MMP-9) drives malignant progression and metastasis of basal-like triple negative breast cancer. Oncotarget 2014; 5: 2736-2749.

[16] Zhang X, Wang X, Zhang W, Ren X, Sha X and Fang X. Matrix metalloproteinases-2/9-sensitive peptide-conjugated polymer micelles for site-specific release of drugs and enhancing tumor accumulation: preparation and in vitro and in vivo evaluation. Int J Nanomedicine 2016; 11: 1643-1661.

[17] Roy R, Yang J and Moses MA. Matrix metalloproteinases as novel biomarkers and potential therapeutic targets in human cancer. J Clin Oncol 2009; 27: 5287-5297.

[18] Beattie GJ and Smyth JF. Phase I study of intraperitoneal metalloproteinase inhibitor BB94 in patients with malignant ascites. Clin Cancer Res 1998; 4: 1899-1902.

[19] Yu S, Kielt M, Stegner AL, Kibiryeva N, Bittel DC and Cooley LD. Quantitative real-time poly-

merase chain reaction for the verification of genomic imbalances detected by microarray-based comparative genomic hybridization. Genet Test Mol Biomarkers 2009; 13: 751-760.

[20] Zhang CL. An explore of MMP-2, MMP-9, VEGF and Survivin in the diagnosis and significance of malignant ascites. Guide of China Medicine 2012; 10: 21-22.

[21] Shen SN. A study on expression of matrix metalloproteinases in the ascites and tissues of ovarian cancer. Tianjin Medical University 2006; 47.

[22] Sun XM, Dong WG, Yu BP, Luo HS and Yu JP. Detection of type IV collagenase activity in malignant ascites. World J Gastroenterol 2003; 9: 2592-2595.