Mashhad University of Medical Sciences (MUMS)

Reviews in Clinical Medicine

Clinical Research Development Center Ghaem Hospital

Evaluation of cyclooxygenase 2- gene expression in benign and malignant ascites

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ARTICLE INFO

Article type
Original article

Article history
Received: 16 Jun 2020
Revised: 29 Jun 2020
Accepted: 30 Jun 2020

Keywords
Ascites
Cyclooxygenase-2
Malignancy

ABSTRACT

Introduction: This study aimed to evaluate the mRNA expression of cyclooxygenase-2 (Cox-2) in ascites caused by various diseases. Moreover, it was attempted to investigate its usefulness in the differential diagnosis between malignant and benign ascites.

Methods: A total of 52 ascitic fluid samples were collected from cirrhotic patients referred to Ghaem Hospital affiliated to Mashhad University of Medical Sciences, Mashad, Iran. Subsequently, the samples were divided into two experimental groups, namely benign ascites (n=26) and malignant ascites (n=26). Reverse transcriptase-polymerase chain reaction (RT-PCR) was utilized to determine the presence of Cox-2 mRNA in samples.

Results: According to the results, the mean age of the patients was 56.94±12.04 years (age range: 30-80 years), and the majority of the patients were male (1.88 to 1). There was no significant difference between the two groups in terms of age and gender. The Cox-2 mRNA was detected in 4 (15.4%) and 15 (57.7%) patients from the benign and malignant groups, respectively (P=0.003). Moreover, the sensitivity, specificity, as well as positive and negative predictive values of Cox2 to differentiate malignant from benign ascites were estimated at 57.7%, 84.6%, 78.9%, and 66.7%, respectively.

Conclusion: The Cox2 mRNA expression assessed by RT-PCR could be a useful method in differential diagnosis and screening of malignant ascites.

Please cite this paper as:
Jabini R, Mirbolouk A, Farzanefar MR. Evaluation of cyclooxygenase -2 gene expression in benign and malignant ascites. Rev Clin Med. 2020;7(2):78-82

Introduction

Ascites is an abnormal accumulation of fluid in the peritoneal cavity which is common in people with cirrhosis, malignant neoplasia, cardiac insufficiency, tuberculous peritonitis, and nephrotic syndrome (1, 2). The diagnosis and management of malignant ascites is an important problem in clinical practice, and a new noninvasive method can improve diagnostic accuracy. Although cytological detection of tumor markers, such as AFP, CEA, CA125, and CA19-9 in the diagnosis of malignant and benign ascites has become a gold standard, its sensitivity is low (3), and it increases the diagnostic yield of malignancy from 40% to 60% cases (4, 5).

Despite the use of laparoscopy in the diagnosis of ascites, its role is markedly decreased since its

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procedure is too invasive for the patients and is not available at all facilities (2, 6). Cyclooxygenase (Cox) is known as the rate-limiting enzyme in prostaglandin synthesis (1). There are two cox isoforms, namely Cox-1 and Cox-2. The Cox-2 mRNA is not detectable in many normal human tissues, and it is induced as an intermediate-early gene in cells, such as monocytes, lymphocytes, macrophages, and endothelial cells (7, 8). Moreover, it is involved in pathophysiological phenomena, including inflammation and cancer development, and has been detected in various tumor tissues (9). Therefore, the increased gene expression of Cox-2 may have prognostic value in diagnosing cancer in its early stages. This study hypothesized a relationship between malignancy and Cox-2 mRNA detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in ascitic fluid.

Methods

Samples and General data

A total of 52 ascitic fluid samples were collected from ascitic patients over 18 years of age admitted to the Ghaem Hospital affiliated to Mashhad University of Medical Sciences, Mashhad, Iran, who were candidates for paracentesis through 2015. All subjects underwent ultrasound, computed tomography (CTScan), and pathology examination. Regarding patient medical history and physical examination, the cases were divided into two groups of 26. The malignant ascites group consisted of 16 and 10 male and female patients, respectively, with HBV (n=14), HCV (n=7),cryptogenic cirrhosis (n=2), Wilson disease (n=1), autoimmune hepatitis (n=1), and cardiac cirrhosis (n=1).

On the other hand, the benign ascites included 18 and 8 male and female patients, respectively, suffering from colon cancer (n=10), ovarian (n=3) and lung cancer (n=3), cholangio adenocarcinoma (n=2), stomach adenocarcinoma(n=2), breast cancer (n=2), metastatic adenocarcinoma (n=1), squamous cell carcinoma (n=1) (SCC), melanoma (n=1), and pancreatic cancer (n=1) according to disease type (Table 1 and Table 2).

Table 1. Patients' demographic characteristics (n=52).

Characteristics	Benign ascites	Malignant ascites
Gender		
Female	8	10
Male	18	16
(Age (year		
Mean±SD	54±11.8	59.8±11.6
Range	30-75	41-80

Centrifugation of samples was performed at 1500 r/min for 15 min immediately upon collection, and the supernatant was removed. The pellet was kept at -80ºC in a freezer for total RNA isolation. The study protocol was approved by the Human Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran. Moreover, written informed consent was obtained from the participants.

Table 2. Distribution of different cases according to the etiology of benign and malignant ascites (n=52).

Diseases	Number of (%) cases	Number of (%) cases
Benign ascites		
HBV	(53.8) 14	
HCV	(26.9) 7	
Wilson disease	(3.8) 1	
Cryptogenic cirrhosis	(7.6) 2	
Autoimmune hepatitis	(3.8) 1	
Cardiac cirrhosis	(3.8) 1	
Malignant ascites		
Ovarian cancer		(11.5) 3
Colon cancer		(38.4) 10
Metastatic Adenocarcinoma		(3.8) 1
Cholangiocarcinoma		(7.6) 2
SCC		(3.8) 1
Melanoma		(3.8) 1
Pancreatic cancer		(3.8) 1
Lung cancer		(11.5) 3
Stomach adenocarcinoma		(7.6) 2
Breast cancer		(7.6) 2
Total number	(100) 26	(100) 26

HBV: Hepatitis B virus; HCV: Hepatitis C virus; SCC: Squamous Cell Carcinoma.

Reverse Transcription-Polymerase Chain Reaction Primers

The RT-PCR was performed for Cox-2 using the following primers (Roobin Teb Gostar): forward 5'- TTCAAATGAGATTGTGGGAAAATTGCT-3' and reverse 5'- AGATCATCTCTGCCTGAGTATCTT-3' yielding a 305 bp amplicon. The forward and reverse primers for GAPDH which were amplified as reference genes were 5'- TGGGTGTGAACCATGAGAAG-3' and 5'- GCTAAGCATTGGTGGTGC-3', respectively, yielding an amplicon size of 80 bp (1).

Total RNA extraction

Total RNA was extracted from the samples using YTA Total RNA Extraction Mini Kit (No, YT9065). Moreover, nanodrop was used to measure RNA concentrations and purity.

Reverse transcription

After total RNA quantitation, reverse transcrip-

tion was performed using a cDNA synthesis kit (No, 6130, Takara).

Polymerase Chain Reaction Amplification

The reverse transcription products were employed as a template in PCR with primers for Cox-2 and GAPDH. The PCRs were in two replicates of each sample in 48-well plates in a step one RT-PCR System (Applied Biosystem, USA). The mRNA expression of Cox-2 was normalized to GAPDH. The RT conditions were 95ºC for 2 min followed by 40 cycles of 95ºC for 10 s, 53ºC for 10 s, and 72ºC for 30 s with data acquisition after each cycle (1).

Statistical analysis

The data were analyzed in SPSS software (version 16), and the Kolmogorov-Smirnov was utilized to evaluate the normal distribution of the data. Furthermore, the relationship between the quantitative variables was investigated using T-student or Mann- Whitney tests. A p-value less than 0.05 was considered statistically significant.

Results

In the current study, the mean age of the patients (n=52) was 56.94±12.04 years (age range: 30-80 years). The majority of the ascitic patients were male (n=34) with a male/female ratio of 1.88 to 1. The participants were divided into two groups of benign ascites (18 and 8 males and females, respectively) and malignant ascites (16 and 10 males and females, respectively) with the same number of samples (n=26) (Table 1). Hepatic cirrhosis and colon cancer were the most common causes of malignant ascites in the benign (14/26) and ascites (10/26) groups, respectively (Table2). The positive rate of Cox-2 mRNA in the malignant group was estimated at 57.7% (15/26), which was significantly higher than that in the benign group (15.4%, 4/26) (Tables 3 and 4).

Table 3. Positive rate of Cox-2 among different disease types in benign and malignant groups

Disease	Total no. of cases	Cases with Cox-2 mRNA presence (%)
Benign ascites (n=26)		
HBV	14	4(15.3)
Malignant ascites (n=26)		
Ovarian cancer	3	3(11.5)
Colon cancer	10	6(23)
Cholangiocarcinoma	2	1(3.8)
Pancreatic cancer	1	1(3.8)
Lung cancer	3	2(7.69)
Breast cancer	2	2(7.69)

Table 4. mRNA expression of Cox-2, sensitivity, specificity, PPV, and NPV in benign and malignant ascites samples (n=52).

	RT-PCR							
	(+)	(-)	Positive (%)rate of cox-2	P value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Benign ascites	4	15	15.4	0.002	57.7	84.6	78.9	79.8
Malignant ascites	15	11	57.7					

PPV: Positive Predictive Value; NPV: Negative Predictive Value; RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction.

Discussion

Many diseases can cause intra-abdominal fluid production, including cirrhosis, congestive heart failure, nephrosis, pancreatitis, peritonitis, primary malignancy, or hepatic metastases. Malignant ascites is a sign of poor prognosis and indicates the presence of advanced cancer (10, 11). Differentiation between benign and malignant ascites is not possible by physical examination or imaging alone. Biochemical and cytological examination of ascitic fluid (including tumor markers) are the other diagnostic tests (12)used widely for the diagnosis of malignant ascites. However, according to studies, cytology is diagnostic in only 50%-60% of the cases with malignant ascites (10, 13). Considering the low sensitivity and specificity, they are still less than ideal (14) and should be regarded as complementary methods for the diagnosis of malignant ascites (3). Prostaglandin-endoperoxide synthase or Cox, is an important rate-limiting enzyme in prostaglandin biosynthesis, which metabolizes arachidonic acid to prostaglandin (9). The Cox-2 is an enzyme that is expressed in areas of inflammation (15). There are several studies of altered Cox-2 expression in human cancers (16), such as breast cancer (17), pancreatic cancer (18), squamous cell carcinoma of the head and neck (19), non-small cell lung cancer (20), and colorectal cancer (21, 22) Considering the presence of Cox-2 in pre-malignant cells and promotion of healthy cells to malignant phenotype (23), Cox-2 mRNA gene expression was assessed in ascitic fluid sam-

ples in order to identify a molecular marker for diagnosis of malignant ascites. It has been demonstrated that regular use of Cox inhibitors, such as aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs), is associated with a 40%-50% decreased risk of colorectal cancer (24). It has also been reported that the administration of celecoxib resulted in a 25% reduction in the number of palpable tumors in mammary rats (25). In 1999, Hla et al. generated transgenic mice overexpressing Cox-2 gene in mammary epithelial cells resulted in the development of mammary tumors showing that Cox-2 was sufficient to induce mammary tumorigenesis (26). In recent years, many studies have been conducted on Cox-2 expression in different stages of cancer progression, and most of them show that tissue cancer expression of Cox-2 is associated with higher stage of cancer (22, 27-29). It is of utmost importance to find a molecular parameter as an indicator in diagnosis of malignancy in clinical decision making. Furthermore, the diagnosis of malignant and benign ascites is a significant clinical issue (2). In this study, ascitic fluid samples were collected from 52 hospitalized patients with benign (n=26) and malignant ascites (n=26) before treatment.

As revealed in a similar study conducted by Jing Lu et al. (9), the results of this study showed that Cox-2 mRNA could be a valuable indicator in distinguishing benign ascites from malignant ascites (Table 4, P=0.002). Jing Lu et al. revealed that the most common cause of benign ascites was the cirrhosis of the liver, and malignant ascitic samples were most commonly found with ovarian cancer (9). In our study, hepatitis B cirrhosis was the most common disease among the benign samples (14 out of 25), whereas colon cancer was the most common one in the malignant group (10 out of 25).

Similar to some other studies (30, 31), the results of the present study indicated the number of males were more than females with statistical significance. Gue Gl et al. (2003) examined Cox-2 expression in ovarian cancer samples. The results of this study showed that the expression level of Cox-2 was significantly higher in cancerous and adjacent tissues than in healthy and distant tissues (32).

Increased expression of Cox-2 is an early event in carcinogenesis. Therefore, the combination of non-steroidal anti-inflammatory drugs and Cox-2 selective inhibitors with chemotherapeutic drugs could enhance the effectiveness of therapy (33). Evaluation of Cox-2 expression status before chemotherapy may be useful in identifying patients that have a lower probability of response to chemotherapy (34). Li et al. reported that the rate of Cox-2 mRNA in rectal cancer samples was significantly higher than that in colorectal adenomas (35). It has also been demonstrated that in rectal cancer, high Cox-2 expression is significantly associated with poor survival (36). However, Parbhu et al. found no statistically significant association between Cox-2 gene expression and survival in colon cancer samples (15). In our study, it was difficult to collect samples, and small sample size was the main limitation of our study. Therefore, according to the results of Table 4, it is suggested that further studies be conducted on more samples.

Conclusion

Our study showed that although the evaluation of Cox-2 gene expression in ascites fluid is not a fully accurate method to differentiate malignant from benign ascites, considering the acceptable sensitivity and specificity, it could be a useful technique in early diagnosis of malignant ascites.

Acknowledgements

The authors are thankful for the financial support of the Mashhad University of Medical Sciences, Mashhad, Iran.

Conflict of Interest

The authors declare no conflict of interest.

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