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The Diagnostic Potential of Telomerase Level in Malignant Ascites

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ABSTRACT

Background: The differentiation of the non-malignant ascites (NMA), and malignancy-related ascites (MA) in early phases, with subsequent appropriate management remains a considerable clinical challenge.

Objective: This study aimed to investigate the possible diagnostic value of the level of telomerase enzyme in ascitic fluid in discrimination between MA and NMA.

Patients and Methods: The study included 117 patients, divided into two groups: 45 (38.5%) in group I with NMA as a control group and 72 (61.5%) in group II with MA. Group II was subdivided into group IIa including patients with primary hepatocellular carcinoma, and IIb that included patients with other non-hepatic malignancies. All patients were subjected to complete history taking, cytological examination, and biochemical analysis of the level of telomerase enzyme in ascitic fluid in comparison to cytological examination of ascetic fluid and the serum level of some specific traditional tumor markers (CA19.9, CEA, CA125, and alpha fetoprotein).

Results: In the malignant group, telomerase level was higher than cut off value (1.2 ng/ml) in (34/40) 85.0% of group IIa, and (25/32) 78.1% in group IIb, while it was high in (14/45) 31.1% of control group. Cytological examination was positive in (6/40) 15.0 % of group IIa and (12/32) 37.5% of group IIb. Moreover, in group IIa, telomerase at cut off level of ≥ 5.25 ng/ml and serum alpha-fetoprotein (AFP) at level of ≥ 210.8 ng/ml demonstrated a sensitivity of 88.4%, 77.1%, and a specificity of 67.7%, 77.3%, respectively.

Conclusion: The level of telomerase enzyme could be a useful tool in evaluating the diagnostic performance of cytological examination especially if used in combination with another more specific tumor marker and may provide a better differentiation between MA and NMA.

Keywords: Malignant ascites, Telomerase level, Hepatocellular carcinoma, Tumor markers, Cytological examination.

INTRODUCTION

Telomeres are specialized DNA structures located at the end of chromosomes. They are essential for stabilizing chromosomes by protecting them from end-to-end fusion and DNA degradation [(1)]**.** Telomerase is a ribonucleoprotein complex containing a telomerase RNA component (TR), and a catalytic protein with telomere-specific reverse transcriptase activity (TERT). TERT which synthesizes de novo telomere sequences by using TR as a template, is the rate-limiting component of the telomerase complex [(2)].

It adds DNA sequence repeats ("TTAGGG" in all vertebrates) to the 3' end of DNA strands in the telomere regions at the ends of eukaryotic chromosomes that contain non-coding DNA, hindering the loss and damage of important DNA from chromosomal ends whenever the chromosome is copied. Critical shortening of telomeres occurs during progressive cell division leading to dysfunctional telomeres, provoke DNA damage responses that result in cellular senescence. Telomerase is suggested to play an important role in development and survival of tumors, as it has a detectable activity in large majority (~90%) of cancer cells, at which telomere length is maintained by telomerase enzyme. This is mostly achieved through upregulation of human TERT gene (hTERT) that encodes telomerase [(3-5)].

Ascites is pathological accumulation of fluid in the peritoneal cavity that is caused by a wide variety of benign and malignant causes [(6)]**.** Ascites can be classified by the underlying pathophysiological mechanism that includes portal hypertension, peritoneal diseases, and hypoalbuminemia, in addition to miscellaneous disorders. In the Western world, liver cirrhosis is the most common cause of ascites (75%), followed by malignancy (10%), heart failure (3%), tuberculosis (2%), and pancreatitis (1%) [(7)]. Malignant ascites is an initial diagnostic feature in about 50% of patients with cancer in organs of pelvic and abdominal cavities that is associated with significant morbidity [(8)]. This could be found in many malignancies as adrenal, bladder, cervical, colorectal, endometrial, hepatobiliary, gastric, lymphoma, melanoma, mesothelioma, neuroendocrine, ovarian, pancreatic, and renal tumors [(9, 10)], mostly it is a poor prognostic sign.

The differentiation between malignancy-related ascites (MA) and non-malignant ascites (NMA) is important in therapeutic and prognostic terms, considering that optimum management should be able to maximize patient's comfort and quality of life [(11)]. Management options for malignant ascites include, diuretic therapy, therapeutic paracentesis, and peritoneo-venous shunts. Oncological interventions could be helpful in ovarian carcinoma, lymphoma, as well as hormonal therapy in hormone-sensitive malignancies [(6)].

Cytological analysis is considered a well-established golden diagnostic standard of malignancy in ascites [(12, 13)]. Clearly, positive cytology is highly indicative for peritoneal carcinomatosis. However, the sensitivity of cytology is variable, ranging between 60-83%, and it could be as high as 97% if three samples from separate paracentesis are analyzed [(14)]. But, many

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crucial factors should be considered as the avoidance of long time gap between sampling and cytology processing, as well as obtaining the largest possible amount of ascitic fluid at least 50 ml, but even 1000 ml may be needed if the first test was negative to confirm the finding. This may explain the relatively low sensitivity of cytology in the ascetic fluid of patients with hepatocellular carcinoma, it could be as low as (~27%) [15, 16].

Various laboratory parameters have been evaluated in ascitic fluid, such as total protein, lactate dehydrogenase (LDH), fibronectin, cholesterol, amylase, triglycerides, serum-ascites albumin gradient (SAAG), but none of them are satisfactory as a single diagnostic test. Likewise, several tumor markers such as carcinoembryonic antigen (CEA), cancer antigen 125 (CA125), and carbohydrate antigen19-9 (CA 19-9) have also been evaluated, but there was persistent discrepancies among these tests, with insufficient clinical performance [17 -19]. Therefore, a more reliable novel biomarker with higher sensitivity and specificity is needed to discriminate between MA and NMA, and for early diagnosis, determination of prognosis, and the development of more convenient therapeutic protocols[20].

We aimed in the current study to assess the diagnostic value of the level of telomerase enzyme in comparison with cytological examination of the ascitic fluid, and the correlation between its level and those of four well studied serum tumor markers (alpha fetoprotein, CEA, CA19.9, CA125) in the diagnosis of malignant ascites.

PATIENTS AND METHODS

The present study included 117 patients, 87 (74.4%) males, and 30 (25.6%) females with different causes of ascites. They were admitted into Ain Shams University Hospital, Cairo, Egypt between January 2018 and January 2020. The range of age of enrolled patients was 41 - 60 years with a mean of 50.5 ± 9.8. The subjects were divided into 2 groups based on the cause of ascites: group I, included 45 patients with non-malignant ascites (NMA). Group II, contained 72 patients with malignancy-related ascites (MA).

Ethical approval:

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Institutional and national) and with the Helsinki Declaration of 1975. Informed consent was obtained from every patient for being included in the study after approval of The Ethical Committee of Ain-Shams University.

All patients were subjected to the following: history taking: age, sex, onset of symptoms, course and possible complications, history of weight loss or prolonged fever, clinical data related to the complaint and suspected etiology.

Routine analysis of complete blood count, liver and renal function tests, abdominal ultrasound, and computed tomography (abdomen, chest, pelvis, bone scan and PET scan) were done for patients with malignant ascites on individual basis. The staging of malignant cases was described based on the criteria of the International Union of Cancer Control (UICC), TNM staging system [21]. Patients had either tumor limited to its primary origin (T1-4, N0, M0), lymph node involvement (T1-4, N1, M0), or distant metastasis (T1-4, N0-1, M1).

Their results were analyzed and compared, a panel of tumor markers has been measured in blood including: AFP, CEA, CA125 and CA19-9 with the values 10 ng/ml, 5 ng/ml, 35 U/ml and 37 U/ml respectively, which were adopted as upper limits of normal in healthy subjects. In addition to their planned work up for management, the included patients had undergone diagnostic paracentesis then the ascitic fluid sample was taken to be subjected to cytological examination and test for telomerase level by ELISA according to the instructions of the manufacturer.

Inclusion criteria: patients with ascites due to liver disease, gastrointestinal tumors or gynecological tumors.

Exclusion criteria: patients < 18 years, patients with ascites of unknown etiology, malignant ascites with previous management (systemic or local chemotherapy or radiotherapy), and patients who refused to sign the consent.

Collection and handling of ascitic fluid:

At least 50 ml of ascitic fluid was collected under strict sterile condition and divided into 2 samples; one sample sent for cytological examination and the other was stored in -80˚C for total proteins, and telomerase assay.

Cytological examination:

5 ml fluid was taken and fluid was centrifuged at 2500 rpm for 15 minutes and a minimum of four thin smears were prepared from the sediment and were immediately fixed in 95 % alcohol and stained with H & E stain. Other stains like Giemsa stain was used whenever required [22]. Liver cirrhosis have shown benign cytology, while malignant cytology was detected most commonly in patients with hepatocellular cancer.

Principles of Telomerase (TE) test by ELISA method

This experiment use double-sandwich Elisa technique and the kit was provided by (MyBioSource, Inc. San Diego, CA, USA). The pre-coated antibody is human TE monoclonal antibody and the detecting antibody is polyclonal antibody with biotin labeled. Samples and biotin labeling antibody were added into ELISA plate wells and washed out with PBS. Then Avidin-peroxidase conjugates were added to ELISA wells in order; TMB substrate was used for coloring after thorough washing out of reactant by PBS. TMB

turns into blue in presence of hydrogen peroxide by peroxidase catalytic activity, and finally an acid stop solution was added to terminate the reaction. This resulted in converting the endpoint color to yellow. The intensity of the color was measured at 450 nm using a microtitration plate reader (Stat Fax_ 2100, Awareness Technology, Inc., FL 34990, USA). TE level (ng/ml) was calculated from a standard calibration curve.

Statistical Methods

Data were analyzed using statistical package for social sciences (SPSS version 24). Qualitative data were expressed as number of cases and their percent of expression. Chi square test was used to compare categorical data. Quantitative data were expressed as Mean ± S.D. Comparative analyses were done using Student t, and ANOVA . Correlations were done using Pearson's correlation coefficient. Receiver operating characteristic (ROC) curve analysis was done to estimate the predictive performance of telomerase in ascitic fluid as a marker of malignant ascites in hepatocellular carcinoma. Difference were considered significant for $P \leq 0.05$.

RESULTS

In the current study 117 patients with different causes of ascites were enrolled, the patients were divided into 2 groups according to the etiology of ascites. Group I: 45 patients with ascites of benign origin (non-malignant liver cirrhosis). Group II: it was subdivided into IIa: 40 patients with ascites of malignant liver origin (hepatocellular carcinoma) and IIb: 32 patients with ascites of malignant origin other than hepatocellular carcinoma (HCC) (10 patients with ovarian carcinoma, 9 patients with gastric carcinoma, and 13 patients with colon cancer). Patients in both groups were age- and gender-matched with no significant difference in their mean age. Male sex was predominant in group I and II (Table 1).

Table (1): Demographic characteristics of the studied patients

		Group I (NMA) n= 45 (100%)	Group II (MA) n= 72 (100%)	P value
Age (years) (mean ± SD)		51.4 ± 9.4	52.31 ± 8.9	>0.05
Gender	Male	35 (77.8%)	52 (72.2%)	>0.05
	Female	10 (22.2%)	20 (27.8%)	

NMA: non-malignant ascites: Group I, MA malignant ascites: group II

Table (2) showed that the differences between laboratory data were statistically not significant between the studied groups except for median of AST and ALT. Both were significantly higher in patients with HCC compared to each of the other 2 groups. Serum albumin was below normal value in all studied groups with no significant difference.

Table (2): Comparison of routine laboratory data in-between NMA and MA

	Benign Ascites Group I n=45	HCC Group IIa n= 40	Malignant Ascites (Other Tumors) Group IIb n= 32	P value
White blood cells count cell/ml (mean ± SD)	4.72 ± 0.7	6.5 ± 1.9	5.99 ± 1.6	0.09
Hemoglobin g/dl (mean ± SD)	11.1 ± 1.44	10.4 ± 1.8	10.0 ± 1.8	0.06
Albumin g/dl (mean ± SD)	2.6 ± 0.47	2.42 ± 0.67	2.48 ± 0.51	0.74
Platelet mcl (mean ± SD)	147.0 ± 35.8	102.0 ± 17.3	164.0 ± 30.1	0.15
AST IU/l (mean ± SD)	49.0 ± 4.8	149.0 ± 35.7	63.0 ± 14.9	<0.01*
ALT IU/l (mean ± SD)	46.0 ± 6.1	126.0 ± 40.8	30.0 ± 5.6	<0.01*
Total Bilirubin mg/dl (mean ± SD)	2.0 ± 0.81	2.6 ± 0.72	1.9 ± 0.41	0.51
Creatinine mg/dl (mean ± SD)	1.3 ± 0.61	1.3 ± 0.58	1.2 ± 0.39	0.86
INR (mean ± SD)	1.7 ± 0.1	1.8 ± 0.16	1.4 ± 0.17	0.054

*Statistically significant at p value < 0.05.

Patients in group IIa (HCC) had tumor limited to the liver in 40%, spread to local lymph nodes (LNs) in 20% and had distant metastasis in 40% of patients. In group IIb, patients had tumor limited to the primary origin in 25% of patients, LNs in 12.5% and had distant metastasis in 62.5% (Table 3).

Table (3): Clinico-pathological staging of included malignant cases

Tumor spread	IIa n= 40 (100%)	IIb n= 32 (100%)
Locally spread (T1-4, N0, M0)	16 (40.0%)	8 (25.0%)
Lymph nodes +ve (T1-4, N1, M0)	8 (20.0%)	4 (12.5%)
Metastasis +ve (T1-4, N0-1, M1)	16(40.0%)	20(62.5%)

Results are expressed as number and % of patients

Malignant cells were detected by cytological examination in 6 (15%) of HCC patients (IIa) and in 15 (37.5%) of patients with other causes of malignant ascites (IIb). There was a significant difference in frequency of detection of malignant cells in ascitic fluid between both groups (Table 4).

Table (4): Results of cytological examination of malignant cases

Malignant cells in ascitic fluid	IIa n= 40	IIb n= 32	P value
Positive	6 (15.0%)	12 (37.5%)	< 0.05
Negative	34 (85.0%)	20 (62.5%)	

Results are expressed as number and % of patients,
*Statistically significant at p value < 0.05

The telomerase enzyme in ascitic fluid was significantly positive in cases of group II as compared to group I (Table 5).

Table (5): Results of telomerase enzyme of ascitic fluid positivity evaluation in malignant group (II) as compared to control group (I)

Telomerase detection in ascitic fluid	I n= 45	II n= 72	P value
Positive	14 (31.1%)	59 (81.9)	< 0.01
Negative	31 (68.9%)	13 (18.1%)	

* Cases with level ≥ 1.2 ng/mL are considered Positive,
*Statistically significant at p value < 0.05

Moreover, the positivity of telomerase enzymes in ascitic fluid was significantly higher than cytological positivity in both subgroups IIa, and IIb (Table 6).

Table (6): The comparison of positive telomerase in ascetic fluid and cytological examination in malignant cases

Groups	Positive Telomerase	Positive Cytology	P Value
IIa n= 40	34 (85.0%)	6 (15.0%)	< 0.001
IIb n=32	25 (78.1%)	12 (37.5%)	< 0.001

*Statistically significant at p value < 0.05

The mean level of telomerase was high in group IIa and IIb as compared to group I with significant difference. The mean level of AFP was significantly different between all studied groups (Table 7).

Table (7): The levels of telomerase enzyme in ascitic fluid and alpha fetoprotein in the blood

	Group I n= 45 (mean ± SD)	Group IIa n= 40 (mean ± SD)	Group IIb n= 32 (mean ± SD)	P Value
Telomerase (ng/mL)	0.7 ± 0.12	5.5 ±1.3	4.5 ± 1.01	<0.001
AFP (ng/mL)	24.8 ±5.1	515 ±16.8	158 ±35.9	<0.001

*Statistically significant at p value < 0.05

Table (8) demonstrated the significant positive correlation between telomerase level and serum levels of AFP, CA19.9, and CA125.

Table (8): The correlation between serum telomerase level and serum tumor markers in malignant subgroups

	Telomerase	
	Correlation Coefficient (r)	P Value
AFP	0.828	<0.01*
CEA	0.236	>0.05
CA19.9	0.860	<0.01*
CA125	0.880	<0.01*

*Statistically significant at p value < 0.05

In The diagnostic performance of telomerase and AFP as markers of malignant ascites in group IIa; telomerase in ascitic fluid at cut off value ≥ 1.2 had sensitivity of 86.4% and specificity of 73.3% in diagnosis of malignant ascites. Serum AFP at cut off value ≥ 61 had sensitivity of 80.3% and specificity of 63.2% (Figure 1).

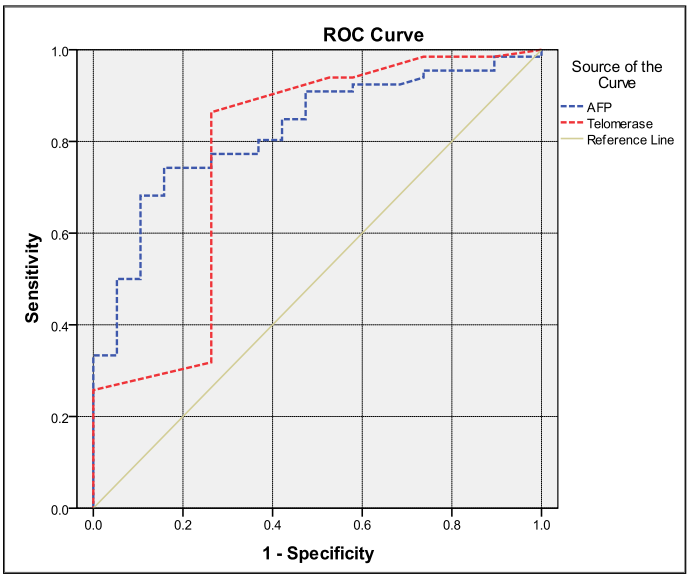

Figure (1): ROC curve of AFP and telomerase in diagnosis of malignant ascites due to hepatocellular carcinoma.

Telomerase in ascitic fluid at cut off value ≥ 5.25 had sensitivity of 88.4% and specificity of 67.7%. While, serum AFP at cut off value ≥ 210.8 had sensitivity of 77.1% and specificity of 77.3% in diagnosis of HCC (Figure 2).

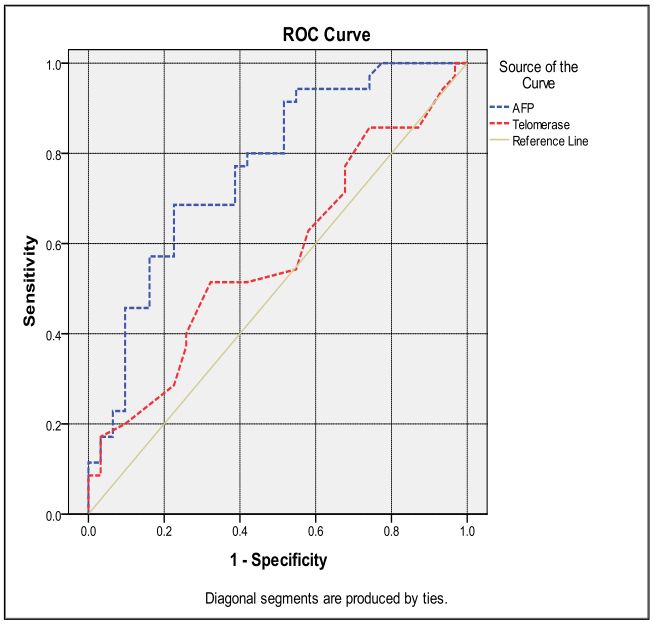

Figure (2): ROC curve of AFP and telomerase in diagnosis of malignant ascites due to hepatocellular carcinoma.

DISCUSSION

The diagnostic analytic markers in ascitic fluid have been proposed for the initial differential diagnosis, as well as for predicting prognosis in certain disorders, most of the discoveries are aiming to simplify, accelerate or reduce the costs of those processes. Pathological examination from tissue biopsy or body fluids is the confirmatory diagnostic test of most forms of cancer. Cytological evaluation of ascitic fluid has high diagnostic specificity but low sensitivity in differentiation of MA and NMA. The low sensitivity could be explained by inadequate sample collection, or low number of tumor cells shed into the specimen, or error in differentiation between neoplastic and atypical inflammatory cells [(23)].

In the present study, positive telomerase in ascitic fluid level was significantly increased in MA (81.9%) group IIa and IIb as compared to 31.1% in NMA (group –I). This goes in a way with the work of **Rahamtalla *et al.*** [(24)] who studied telomerase activity in the blood and postulated that, telomerase activity was significantly elevated in 70% of patients with HCC and only in 18% of cirrhotic patients.

As regards ascetic fluid cytology, malignant cells were detected in 15% of patients of group-IIa and in 37.5% of patients of group IIb. Comparing the rate of detection of malignant cells to that of telomerase in malignant ascites, telomerase activity had significantly higher rate of detection than cytological examination (85% in group-IIa and 78.1% in group-IIb), suggesting better sensitivity of telomerase measurement**. Li *et al.*** [(25)] have detected telomerase activity in 52% of gastrointestinal carcinoma, in 25% of patients with HCC and in only 4% of patients with liver cirrhosis. They reported that cytology was positive in 58% of gastrointestinal carcinoma and 2.5% of HCC patients. **Zhao *et al.*** [(26)] have found telomerase activity in 41.7% of patients compared to 25% of positive cytology, both studies recommended the use of both methods for better diagnosis of MA. The lower rate of telomerase detection in their study compared to our work may be due to the difference in telomerase measurement method, and tumor heterogeneity. In addition, **Tangkijvanich *et al.*** [(27)] detected telomerase activity in patients with peritoneal carcinomatosis and HCC (81.3% and 66.7% respectively), while cytology was positive in 56.3% and 11.1% respectively.

Several studies have addressed the diagnostic value of tumor markers in ascitic fluid including AFP, des-gamma-carboxy prothrombin, CEA, CA 19-9 and CA125. Increased concentrations have been associated with the underlying malignancies, but their levels may be elevated in other non-malignant medical conditions, such as gastritis, diverticulitis, cirrhosis and pancreatitis [(28)].

In the current study, both levels telomerase in ascitic fluid and serum AFP were significantly lower in the group of NMA as compared to the group of MA. On the other hand no significant difference was detected in the level of telomerase between subgroups IIa and IIb of MA. This could be referred to the non-specificity of telomerase, which is considered as marker of malignancy in general. In contrast, AFP was significantly higher in group IIa compared to group IIb. This finding could be explained by the well-known high specificity of AFP with lower degree of sensitivity as a tumor marker for diagnosis of HCC [(29)]**.** A positive correlation was noticed between the levels

of telomerase in ascetic fluid and the tumor markers in the serum as AFP in HCC with **r** value 0.83, CA125 in ovarian cancer with **r** value 0.88, and with CA 19.9 in colon cancer with **r** value 0.86. A relatively high sensitivity could be of value in the work up for diagnosis of ascites of unknown origin to support exclusion of malignancy.

Result of the present study showed that telomerase in ascitic fluid at concentration ≥ 1.2 ng/ml had sensitivity of 86.4% and specificity of 73.3% in diagnosis of MA in HCC patients. While, at concentration of ≥ 5.25 ng/ml it had sensitivity of 88.4% and specificity of 67.7%. In contrast on the other hand, AFP at serum level ≥ 210.8 ng/ml had sensitivity of 77.1% and specificity of 77.3%. Emphasizing that AFP had higher specificity in detection of HCC than telomerase. **Hady *et al.*** [30] found a positive correlation between AFP, PIVKA II (protein induced vitamin -K absence II), serum telomerase and pathological grades of the tumor in patients with HCC. They reported that the highest sensitivity and specificity was for telomerase being 88.2% and 79.6% respectively compared to 80.5% & 69.3% for PIVKA II and 72.6% & 61.5% for AFP. Similar study by **Musaev *et al.*** [31] has suggested that the determination of telomerase activity and the level CA 19-9 in the contents of the mucinous cystic liver neoplasms is a valuable method, which makes it possible to carry out a preoperative differential diagnosis.

Symptomatic malignant ascites is a significant problem in the palliative care setting and associated with a progressive deterioration in the quality of life as well as poor prognosis. The quality of life and survival of these patients may be improved by better understanding of the pathophysiology of malignant ascites, better diagnostic evaluation and the use of multimodality therapy [32- 35]. Level of telomerase can be used in screening for malignancy in patients with ascites of unknown etiology with high sensitivity (86.4%) especially when combined with conventional cytology it might help increase the sensitivity with subsequent use of the suitable guidelines in management. Moreover, showing higher sensitivity but less specificity compared to serum AFP, telomerase can be combined with the later to improve its sensitivity as tumor marker in the screening and follow up of HCC.

There are some limitations in the present study as the small sample size, broad inclusion criteria, and some degree of heterogeneity in patients with different types of primary tumors that should be considered in future work.

CONCLUSION

The level of telomerase enzyme in ascitic fluid can represent a beneficial adjunct in order to improve the diagnostic performance of cytology and tumor markers. Thus, it acts as a distinguishing guide for patients with malignant and benign ascites, to select those who need further invasive procedure and/or palliative management plan that could give maximum possible improvement in the quality of life of cancer patients.

Declarations:

Consent for publication: Not applicable.

Availability of data and material: Please contact author for data requests.

Competing interests: The authors declare no competing conflict of interest.

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