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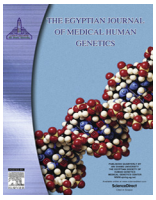

Original article

Metadherin mRNA expression in hepatocellular carcinoma

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ABSTRACT

Background: Metadherin (MTDH) has been known as an essential oncogene in carcinogenesis and tumor spread in several malignancies, via its effect on pathways of signal transduction.
Objective: We aimed to evaluate the role of serum MTDH mRNA expression in the diagnosis of hepatocellular carcinoma (HCC) and to compare its expression levels with serum levels of Alpha-fetoprotein (AFP).
Subjects & methods: A total of 150 subjects (90 HCC patients & 60 healthy volunteers) were enrolled in the current study. Serum MTDH mRNA relative expression was analyzed by Real Time PCR technique.
Results: There was a significant statistical increase of serum MTDH mRNA expression in HCC group when compared to controls ($P < 0.05$). MTDH mRNA expression was significantly associated with clinicopathological data, advanced tumor stage and poor histological differentiation in HCC patients ($p < 0.05$). There was direct positive correlation between MTDH mRNA expression and serum AFP levels in HCC group ($r = 0.445$), P value = <0.05. ROC curve was used to verify the accuracy of MTDH mRNA expression and compare it with accuracy of serum AFP in HCC diagnosis; MTDH mRNA expression had higher accuracy (92%), sensitivity (91%) and specificity (93%) than AFP.
Conclusion: MTDH mRNA is up-regulated in serum of HCC patients; MTDH may be considered as non-invasive biomarker for HCC diagnosis and it could replace serum AFP in HCC diagnosis as it had higher accuracy.

1. Introduction

Hepatocellular carcinoma (HCC) is considered as the 5thmost common cancer and the 3rdmost abundant cause of death from cancer worldwide [1]. In Egypt HCC is one of the most dangerous health problems. Egypt is the 6thlargest country in the Middle East and Arab world, the 3rdin Africa and the 15thin the world regarding population size, having around 90 million inhabitants. According to a study done by [2], they stated almost 2 times increase in HCC among chronic liver disease patients over a decade [3]. The results of the national population-based cancer registry program revealed that most common cancer sites in Egypt were liver (23.8%), breast (15.4%), and bladder (6.9%). Liver cancer is a serious if not the most serious cancer problem in Egypt [4].

As the diagnosis of HCC is usually late, the percent of morbidity and mortality are nearly the same. Early diagnosis can be useful to decrease mortality rate [5]. Serum Alpha-fetoprotein (AFP) is an approved marker for diagnosis and monitoring of HCC, but its specificity and sensitivity are not satisfactory [6]. Therefore, identification of a new marker that contribute to the detection and progression of HCC would be extremely useful for diagnosis, prognosis and act as a target for therapy [1]. Metadherin (MTDH) is a new oncogene known as Astrocyte Elevated Gene-1 (AEG-1) and also identified as lysine-rich carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-1-coisolated (LYRIC) protein. Metadherin mRNA has increased expression in 90% of HCC patients and markedly mediates aggressive disease behavior [1]. Metadherin firstly identified in 2002, at fetal astrocytes of persons exposed to HIV-1 and then known as a vital oncogene mediating carcinogen-

Abbreviations: AEG-1, Astrocyte Elevated Gene-1; AFP, Alpha-fetoprotein; AJCC, American Joint Committee on Cancer; AUC, area under the curve; cDNA, complementary DNA; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; ELISA, Enzyme Linked Immunosorbent Assay; LYRIC, lysine-rich carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-1-coisolated; HCC, hepatocellular carcinoma; HIV, human immunodeficiency virus; MTDH, Metadherin; mRNA, messenger ribonucleic acid; NF-κB, nuclear factor kappa beta; NPV, negative predictive value; PPV, positive predictive value.

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esis, progress, invasion, and cancer metastasis [7]. MTDH might be a vital molecule in relation of cancer, innate immunity and inflammation [8]. Its gene product is known as metadherin or metastasis adhesion protein. It is a basic membrane protein composed of 582 amino acids, its molecular weight is 64 kDa, and has isoelectric point of 9.33 [9]. Human MTDH gene is located on long arm of chromosome 8; the gene spans 12 exons and 11 introns. MTDH is a transmembrane protein of single pass regulating many signaling pathways strictly related to cancer, as nuclear factor-kappa B, Wnt/β-catenin, MAPK/ERK, PI3K/AKT, and AP-1 [19]. NF-κB signal pathway significantly accounts for MTDH mediated oncogenic effects [10]. Although MTDH does not interact with DNA directly, it binds with NF-κB and also with transcription coactivator-cAMP response element-binding protein (CREB), also MTDH functions as a bridge between NF-κB and CBP attaching them to each other and to the basal machinery of transcription [11]. MTDH is considered as an anti-apoptosis gene in glioma cells and could be used as a target of microRNA-136 in anticancer treatment [12].

2. Aim of the study and search strategy

The aim of our study is to detect alternations of Metadherin gene expression in sera of HCC patients using a case control study, we present evidence with its role as a marker for diagnosis of HCC and correlation of its relative expression level with both of serum AFP levels and clinico-pathological data such as tumor stage, histological grade and vascular invasion.

3. Subjects and methods

3.1. Subjects

This study was fulfilled by cooperation between Molecular Biology and Medical Biochemistry & Internal Medicine Departments, Faculty of Medicine and Hepatology Department at National Liver Institute, Menoufia University. This study was carried out during the period from August 2014 to October 2016). A total number of 150 subjects were enrolled in the present study: 90 previously diagnosed HCC patients and 60 healthy volunteers, age and gender matched as the control group. For HCC patients tumor staging was done according to the American Joint Committee on Cancer (AJCC) (8th edition) [13]. All studied individuals were subjected to complete history taking, full clinical examination and laboratory investigations; serum AFP levels were assessed using Enzyme Linked Immunosorbent Assay (ELISA) technique. Expression profile of metadherin in serum was analyzed by Real-Time Polymerase Chain Reaction. Before collection of blood samples, written consent approved by the Committee of Ethics and Human Rights in Research at Menoufia University was obtained from all studied cases and controls. The work has been carried out in accordance with the code of ethics of The World Medical Association (Declaration of Helsinki) for experiments in humans.

3.2. Sampling and primary test

4 ml of venous blood were withdrawn from every subject by sterile vein-puncture and transferred into a plain tube, left to clot at 37 °C, centrifuged for 20 min at 4000 r.p.m. The clear fresh supernatant serum was separated from the clot and, then divided into 2 plain tubes; 2 ml were used immediately for total RNA extraction step, the other tube containing 2 ml was kept frozen at −80 °C until determination of serum AFP levels by ELISA. Alpha fetoprotein levels were assessed using Leinco Technologies kit, USA according to manufacturer's instruction [14].

3.3. Metadherin mRNA serum expression by Real Time PCR

Total RNA was extracted from fresh serum samples using miRNeasy kit, QIAGEN, USA. The yield and purity of RNA were measured by Nano-Drop instrument (Thermo Scientific, USA). RNA extract was stored at −80 °C. SensiFASTcDNA synthesis Kit, Bioline, Germany was used for reverse transcription step and production of complementary DNA (cDNA). Each reaction was carried out on ice with a total volume of 20 μl, containing 1 μl of reverse transcriptase enzyme, 4 μl of reverse transcriptase Buffer, 10 μl of template RNA and 5 μl of nuclease free water. Incubation was done using 2720 thermal cycler, Applied Bio systems (Singapore) for one cycle as follows: 10 min at 42 °C then, 5 min at 95 °C to inactivate reverse transcriptase enzyme and finally for 5 min at 4 °C. cDNA produced was stored at −20 °C till real-time PCR step. Real-time PCR was performed using SensiFAST™ SYBR Lo-ROX Kit, USA. Total volume of 20 μl was applied, in the form of 10 μl of SYBR green Master Mix; 1 μl of Nuclease-free water, 6 μl of template cDNA and 1.5 μl of each primer (forward & reverse). The following primers (Midland, Texas) were used; for Metadherin gene: forward primer sequence was: 5′-AAGAGGAAAACTGAG-CCATCTG-3′ and reverse primer sequence was: 5 CGGCTAACATCCCACTGA-TAAT-3′ and for GAPDH gene: forward primer sequence was: 5 GAAGGTGAAGGTCGGAGTC3 and reverse primer sequence was: 5 GAAGATGGTGATGGGATTTC 3. The PCR condition for Metadherin gene amplification consisted of three phases: initial activation phase at 95 °C for 5 min followed by 45 cycles at 95 °C for 20 s; 60 °C for 30 s; 72 °C for 1 min; and a final extension phase at 72 °C for 10 min. Finally, fluorescence detection and data analysis were done using 7500 ABI PRISM (Applied Biosystems, USA) v.2.0.1. The relative quantification (RQ) of Metadherin gene expression was calculated using comparative $^{\Delta\Delta}$Ct method [15]. As in Figs. 1 and 2, the amount of the Metadherin gene is normalized to an endogenous housekeeping gene (GAPDH) and relative to a control.

3.4. Statistical analysis

Results were collected, tabulated and statistically analyzed by IBM personal computer and statistical package (SPSS version 23, Inc., Chicago, Illinois, USA). Data were expressed into two phases: I-Descriptive: Mean value and Standard Deviation [SD]: for quantitative data, frequency and percentage for qualitative date. II- Analytic: Chi-square test [χ^2]: was used to study association between two qualitative variables. Student *t*-test used for comparison between two groups having quantitative variables. *U* test (Mann Whitney) comparison between patients and control for continuous variables not normally distributed. F test (one way ANOVA): for comparison of more than two independent quantitative variables normally distributed. Pearson's correlation coefficient (r) was used to assess the relationship between MTDH mRNA expression and serum AFP levels. ROC-curve is Receiver operating characteristic Curve analysis. Sensitivity: Probability that the test results will be positive when the disease is present, true positive rate, expressed as percentage. Specificity: Probability that the test results will be negative when the disease is absent, true negative rate, expressed as percentage. PPV: positive predictive value: probability that the disease is present when the test is positive. NPV: negative predictive value: probability that the disease is present when the test is negative. Accuracy: the ratio of the true positive and true negative on all patients. P value < 0.05 was considered statistically significant.

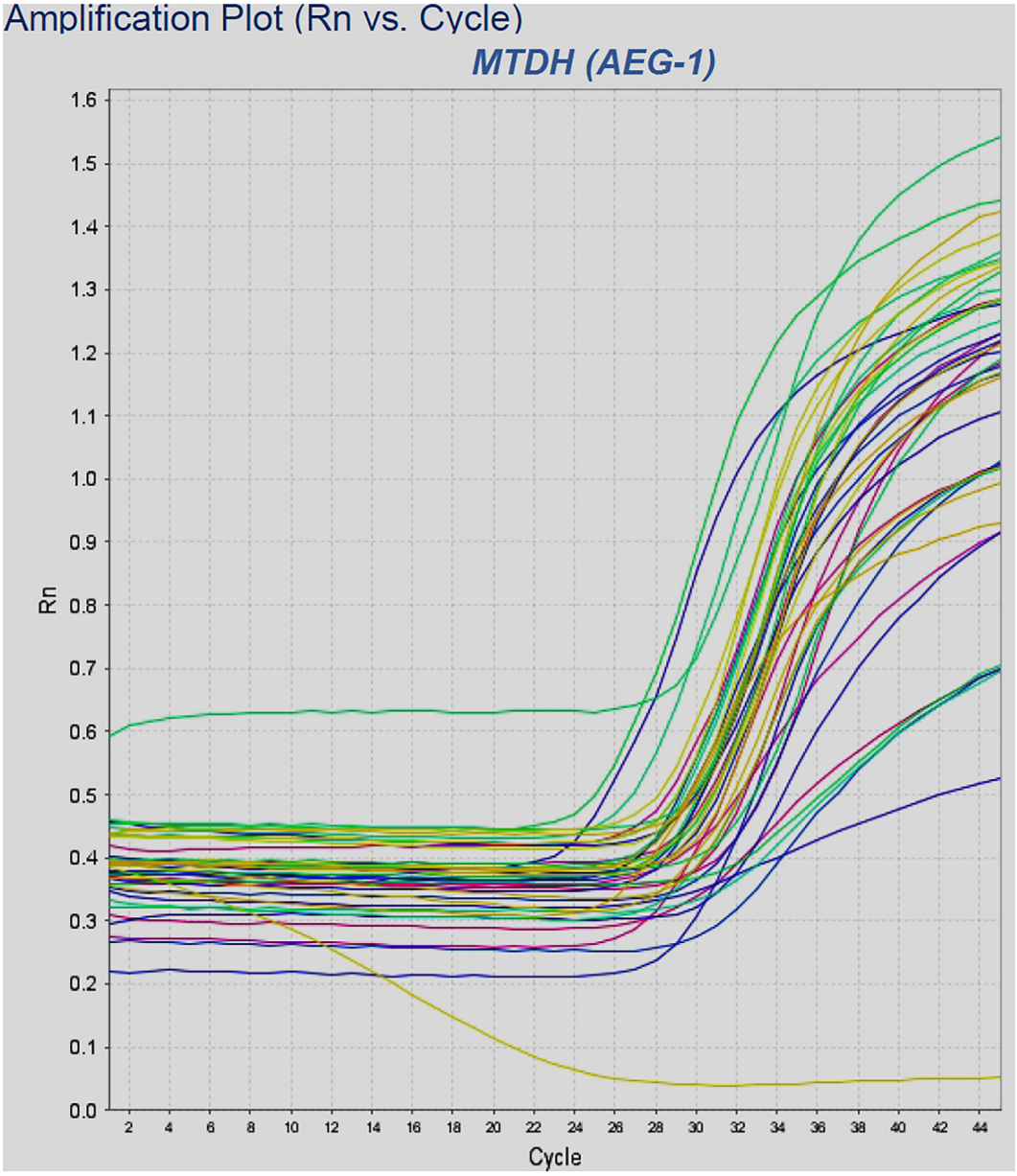

Fig. 1. Amplification plot of MTDH expression (normalized fluorescence signal (Rn) plotted versus cycle number.

4. Results

The results of the present study showed that, there were no significant statistical differences between the two studied groups regarding age and gender, indicating matching between two studied groups ($P > 0.05$). There was a significant statistical difference between group I & II as regards smoking and serum AFP levels being significantly increased in HCC group than in controls ($P < 0.05$) as shown in Table 1. Also, there was a significant statistical increase of MTDH mRNA relative expression level (RQ) in group I when compared to the control group ($P < 0.05$) as demonstrated in Table 1 and Fig. 3a (see Table 2).

Regarding, relationship between MTDH mRNA relative expression levels and both of demographic &established clinicopathological parameters in HCC, our study revealed that MTDH mRNA levels were significantly associated with HCC of advanced T (Tumor size & number) classification (being increased with increased tumor size, number and presence of vascular invasion) ($p < 0.05$), advanced N (Nodal, that describes regional lymph nodes that are involved) classification ($p < 0.05$) and M (describes distant metastasis) classification ($p < 0.05$). Furthermore, its level was significantly increased with advanced AJCC stage and poor histological differentiation ($p < 0.05$). On the other hand there was no significant association as regards age and gender. There was direct positive correlation between MTDH mRNA expression levels and serum AFP levels in patients with HCC($r = 0.445$), P value = <0.05 as in Fig. 3b (see Table 3.).

ROC curves were used to verify the diagnostic accuracy of MTDH mRNA expression and serum AFP for HCC. For serum AFP: a cutoff value of 13.75 ng/ml gives a sensitivity of 75%, a specificity of 73.3%, PPV of 80.9%, NPV of 66.7%, accuracy of 74.7% and the area under the curve was 0.83 as shown in Fig. 4a.

While, for MTDH mRNA expression: a cutoff value of 3.5gives a sensitivity of 91%, a specificity of 93%, PPV of 95%, NPV of 87.5%, accuracy of 92% and the area under the curve of 0.89 as shown in Fig. 4b.

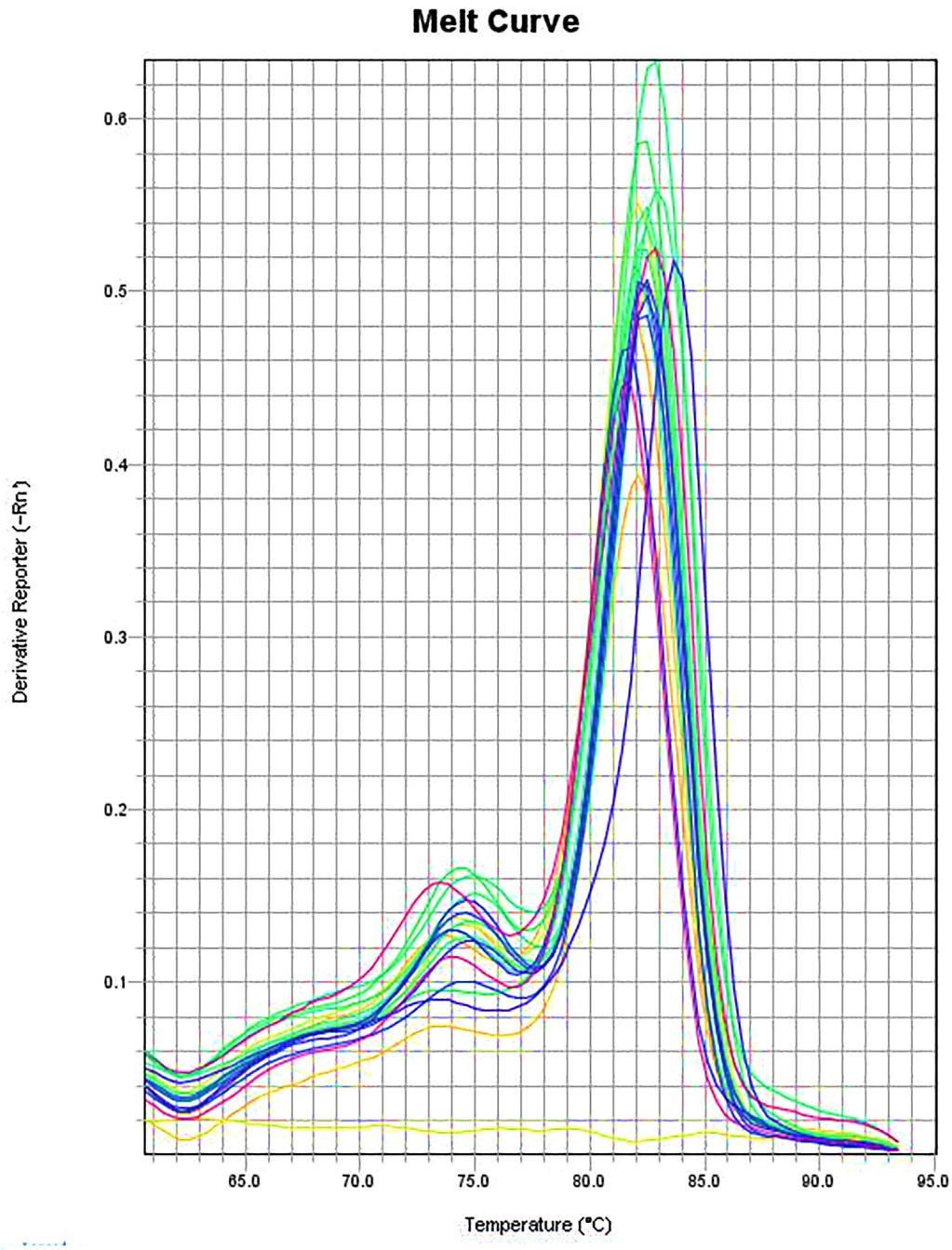

Fig. 2. Melting curve identifying specificity of chosen primers.

5. Discussion

HCC is caused by bilharziasis, hepatitis B or C viral infections, alcoholism, smoking, exposure to toxins such as aflatoxin, and cirrhosis [16]. The most common features of HCC are rapid rate of growth, early vascular invasion, poor histological differentiation and multiple drug resistance, so early diagnosis and treatment are mandatory for better management [17]. It is necessary to identify effective biomarkers for diagnosis of HCC. The gold standards for HCC diagnosis are; serum AFP, imaging techniques then pathological verification. However, the accuracy of serum AFP is not satisfactory [18].The aim of our study is to quantitate Metadherin mRNA expression in HCC patients and to evaluate its role in HCC. The results of this study showed that there was a significant statistical increase regarding smoking distribution in HCC group than controls; these results come in line with [19] who stated that cigarette smoking increases the risk of development of and mortality from HCC. Also a study from Egypt done by [20] demonstrated that tobacco ingredients metabolized in the liver had carcinogenic effect causing HCC. The present study showed that there was a significant statistical increase of serum levels of AFP in HCC group when compared to controls, these results are in agreement with Behne and Copur, [21] who reported that, the first test for detection and monitoring of patients with HCC was serum AFP which has been the standard marker for HCC for several years. The current study demonstrated that, there was a significant statistical increase of MTDH mRNA relative expression level in group I when compared to controls; these results are in accordance to Wang

Table 1
Demographic & laboratory data and smoking distribution.

	HCC patients N = 90	Control N = 60	Test of sig.	P value
Age (years)	53.4 ± 6.3	52.9 ± 7.2	*T* test	0.665
			0.434	
Gender			0.041#	0.84
Female	39(43.3%)	27(45%)		
Male	51(56.75)	33(55%)		
Smoking			19.9#	<0.05*
−ve	39(43.3%)	48(80%)		
+ve	51(56.7%)	12(20%)		
			U test	<0.05*
AFP (ng/ml)	355.7 ± 255.7	8.7 ± 5	11.6	
MTDH mRNA relative expression level	7.5 ± 2.8	1.4 ± 0.9	*U* test 16.4	<0.05*

#: χ^2 = Chi square test, *U* test: Mann Whitney test *: Statistically significant at p value ≤ 0.05.
HCC: hepatocellular carcinoma, AFP: Alpha-fetoprotein, MTDH: Metadherin and mRNA: messenger ribonucleic acid.

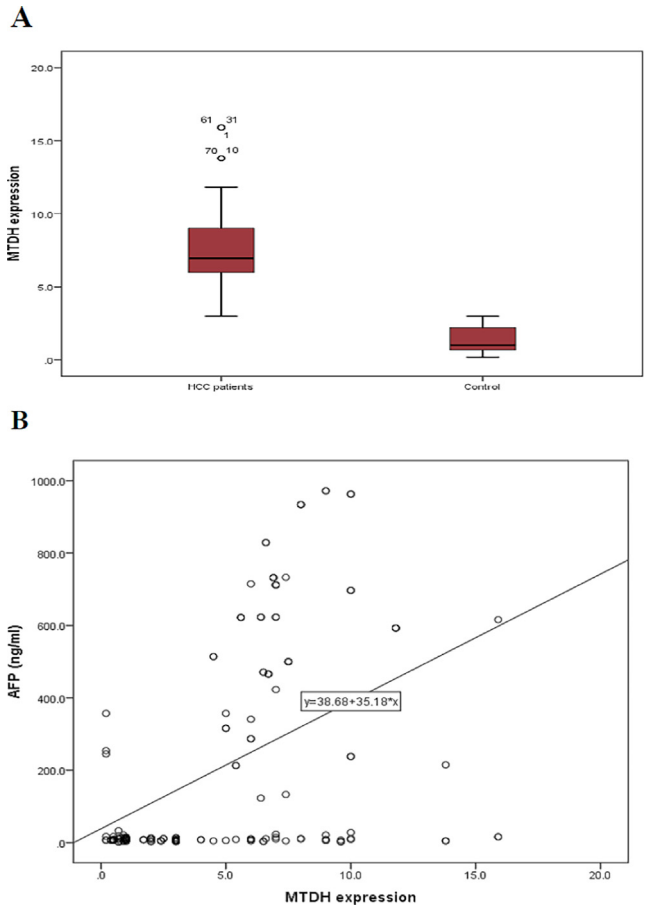

Fig. 3. a. Expression level of MTDH mRNA in studied groups, b. Correlation between MTDH mRNA relative expression &serum AFP levels in HCC patients (r = 0.823), P value = <0.05.

Table 2
Correlation between MTDH mRNA expression and demographic & clinicopathological parameters in patients with HCC.

Parameters		*T* test	P value
MTDH mRNA relative expression level			
		F test	
Age		0.008	0.992
Gender	6.01 ± 4.2	1.2	0.21
Female (n = 39)	6.8 ± 3.5		
Male(n = 51)			
Tumor size			
>5 cm (n = 27)	11 ± 2.3	13.3	<0.05*
<5 cm (n = 63)	6.1 ± 1.2		
Tumor number			
1 (n = 63)	6.1 ± 1.2	13.3	<0.05*
>1 (n = 27)	11 ± 2.3		
Vascular invasion			
+ve (n = 54)	9.1 ± 2.6	8.3	<0.05*
−ve (n = 36)	5.3 ± 1.03		
Nodal classification			
N0(n = 72)	6.5 ± 1.6	12	<0.05*
N1 (n = 18)	11.9 ± 2.3		
Distant metastasis			
M0 (n = 81)	6.9 ± 1.8	10.8	<0.05*
M1 (n = 9)	13.8 ± 1.8		
AJCC staging			
1 (n = 36)	5.3 ± 1	F test	0.05*
2 (n = 27)	7.1 ± 0.4		
3 (n = 9)	9.2 ± 0.3	118.03	
4 (n = 18)	11.9 ± 2.3		
Grading			
G1 (n = 39)	0.9 ± 0.1	F test	<0.05*
G2 (n = 33)	1.9 ± 0.3	80.3	
G3 (n = 18)	2.3 ± 0.5		

F test: one way ANOVA,*: Statistically significant at p value ≤ 0.05, MTDH: Metadherin and mRNA: messenger ribonucleic acid and AJCC: American Joint Committee on Cancer.

et al. [22], who reported that MTDH overexpression is associated with carcinoma progression, vascular invasion, angiogenesis, and metastasis.

Our study revealed that, MTDH mRNA levels were significantly associated with large tumor size, tumor number and presence of vascular invasion, advanced AJCC stage and poor histological differentiation. On the other hand there were no significant association as regards age and gender; these results come in line with Hu et al. [23], & Meng et al. [24], & Wang et al. [1], & He et al. [25], who confirmed that MTDH overexpression was correlated with clinicopathologic characters including cell proliferation through inhibition of cancer cell apoptosis & vascular invasion & distant metastasis & resistance to chemotherapy and hence poor prognosis.

Wang et al. [1], had linked MTDH with tumor development and poor prognosis in HCC. Also clinical study by Yoo et al. [26], had linked MTDH with tumor development and poor prognosis in several cancer types, such as breast, prostatic, esophageal and colorectal cancer.

MTDH is a chief mediator of cancer and a crucial part of oncogenic signaling pathways as it regulates different signaling path-

Table 3
Validity of serum AFP &MTDH mRNA expression for differentiation between of HCC patients and controls.

	MTDH mRNA expression	Serum AFP
AUC	0.89	0.83
P value	<0.05	<0.05
Cutoff	3.5	13.75
Sensitivity	91%	75%
Specificity	93%	73.3%
PPV	95%	80.9%
NPV	87.5%	66.7%
Accuracy	92	74.7%

MTDH: Metadherin and mRNA: messenger ribonucleic acid, AFP: Alpha-fetoprotein, AUC: area under the curve, PPV: positive predictive value and NPV: negative predictive value.

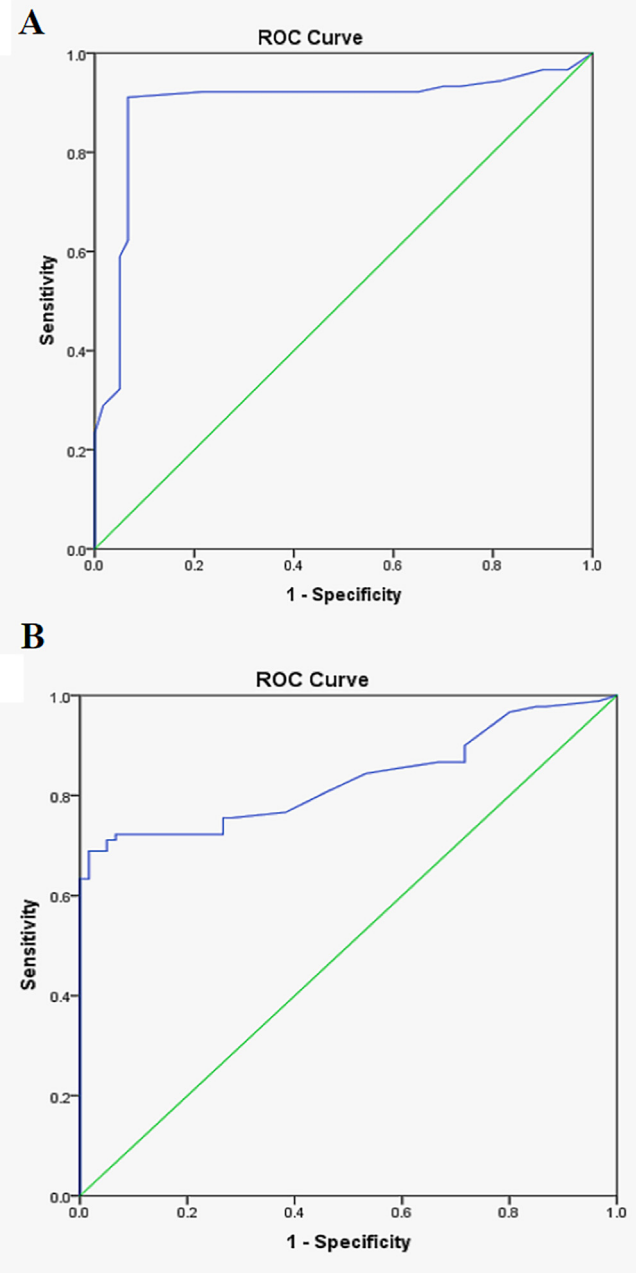

Fig. 4. a. ROC curve of serum AFP levels for diagnosis of HCC, b. MTDH mRNA expression level for diagnosis of HCC.

ways that are strictly related to cancer, such as nuclear factor-kappa B, Wnt/β-catenin, MAPK/ERK, PI3K/Akt, and AP-1 [27].

The current study showed direct positive correlation between MTDH mRNA expression and serum AFP levels in patients with HCC. In an attempt from our study to compare the validity of MTDH mRNA expression and serum AFP as diagnostic markers for HCC, ROC curve analysis revealed that, MTDH mRNA expression gives a higher sensitivity, specificity, PPV, NPV and accuracy than that of serum AFP. Paul et al. [28], stated that AFP cannot be considered to be efficient diagnostic tool for HCC, AFP may be elevated in serum from patients with chronic disease. AFP is not useful for screening in patients suffering from cirrhosis or hepatitis C therefore elevated AFP in these patients may not be indicative, or be only suggestive for HCC. Similarly, Kim et al. [29], reported that, AFP is considered a valuable marker only for monitoring of HCC patients after treatment and to detect tumor recurrence. Wang et al. [1], showed that, MTDH mRNA may be an accurate serum marker to distinguish HCC patients from non-cancer patients.

An experimental study done by [26] revealed that, after MTDH gene knocking in HCC mice by siRNA, an obviously enhanced efficacy of anti-cancer drugs such as 5-fluorouracil and docetaxel was observed which indicates that MTDH had a potential therapeutic effect in malignancies.

Wang et al. [24], stated that, MTDH is an essential marker for prediction of cancer survival and delivers information to improve chemotherapy.

6. Conclusion

Serum MTDH is up-regulated in of HCC patients than controls; MTDH may be considered as a sensitive and specific non-invasive biomarker for HCC diagnosis. It is superior to serum AFP as it had higher accuracy. So, MTDH could replace AFP in HCC diagnosis. However, we recommend further studies to evaluate role of MTDH in HCC management and cancer therapy.

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Conflict of interest

The authors declare no conflict of interest.

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