Diagnostic Value of hTERT mRNA and in Combination With AFP, AFP-L3%, Des-γ-carboxyprothrombin for Screening of Hepatocellular Carcinoma in Liver Cirrhosis Patients HBV or HCV-Related

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ABSTRACT: Diagnosis of hepatocellular carcinoma (HCC) in early-stage, to give an effective treatment option and improve quality of life for cancer patients, is an important medical mission globally. Combination of AFP with some biomarkers may be more supportive in both diagnosis and screening of HCC, but the range value of these markers can be applied as daily markers were unclearly. In some studies, human telomerase reverse transcriptase (hTERT mRNA) was reported as an advantage marker to diagnose cancer. The present study identified serum of 340 patients that were infected chronic hepatitis B virus or hepatitis C virus and divided in 2 groups including Hepatocellular carcinoma (HCC) and liver cirrhosis (LC) to measure their values of hTERT mRNA, AFP, AFP-L3%, and DCP, as well as combination of them. As a result, the concentration of hTERT mRNA, AFP, AFP-L3%, and DCP in HCC groups were significantly higher than that in LC group ($P < .01$). For detecting HCC, hTERT mRNA had sensitivity of 88% and specificity of 96% (at the cutoff value of 31.5 copies/mL), AFP sensitivity of 73% and specificity of 92% (at the cutoff value of 5.1 ng/mL), AFP-L3% sensitivity of 69% and specificity of 90% (at the cutoff value of 1.05%), DCP sensitivity of 82% and specificity of 92% (at the cutoff value of 29.01 mAU/mL). The largest area under the curve (AUC) of combination hTERT mRNA with DCP was 0.932 (sensitivity of 98.2% and specificity of 88.2%). New combination of DCP with hTERT mRNA gave a useful choice for screening of HCC in chronic HBV or HCV patients associated liver cirrhosis.

KEYWORDS: hTERT mRNA, AFP, AFP-L3%, Des-γ-carboxyprothrombin, hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of liver cancer which is estimated with 905 677 new cases and the third cause leading cancer-related death (death rate about 8.3%) worldwide, according to cancer statistics of the GLOBOCAN 2020. In the same year, approximately 26 418 (14.5%) new cases of HCC are reported in Vietnam. HCC progresses quietly without any specific symptom in pre-clinical stage, so most patients are diagnosed in advanced stage of HCC who spend expensive cost of cancer treatment and healthcare, but have a high rate of postoperative recurrence and metastasis and has the 5-year survival rate lower than 9%.[1]

Vietnam was one of the developing countries in the epidemiological area accounting for about 80% of HCC cases and had epidemiological characteristics of HCC such as infection with hepatitis virus (HBV) or hepatitis C virus (HCV), alcohol use, diabetes, or cirrhosis in the same high prevalence rate. Disease burden and low effectiveness of treatment HCC were the facts that happened commonly here. Most Vietnamese HCC patients had 1 or more comorbidities that were risk factor of HCC. However, due to their low-socioeconomic and lack of knowledge about this disease, only when their health got worse and worse, they accepted to visit the hospital for treatment. When advanced disease, all therapeutic options were limited extremely because of bad prognosis.[2]

To improve survival rate of HCC patients, clinically diagnostic of HCC at early-stage plays a significant role to screen and choose an optimal therapy for high-risk patients. Regularly, from the findings of abdominal ultrasonography and symptoms of liver cirrhosis, patients are diagnosed of HCC by combination of methods. Non-invasive tests identification biomarkers in serum such as Alpha fetoprotein (AFP), lens culinary agglutinin-reactive fraction of AFP (AFP-L3%), and Des-gramma-carboxy prothrombin (DCP) known as PIVKA II (protein introduced by vitamin K absence or antagonists II) are used widely. However, these tests of sensitivity and specificity are controversial and limited. For example, AFP levels may be not increased or normal in approximately 40% HCC patients, especially small tumor size, and increased in pregnancy, severe hepatitis or cirrhosis.[3] In addition, PIVKA-II test has higher sensitivity and specificity than AFP[3] but its levels are also overexpressed in coagulation dysfunction, liver cirrhosis, or several non-tumor factors.[4] AFP-L3 have been

proven as potential biomarker for diagnosing discrimination of HCC with AFP level-normal.[5] In levels of AFP <20 ng/mL, Hie-Won suggested that combination of AFP+AFP-L3+DCP had sensitivity of 75% in chronic hepatitis patients with or without HCC. The present meta-analysis demonstrated that these biomarkers should be combined to perform the overall sensitivity and specificity better than[6] just as 88% and 79%[3] or 92.2 and 81.7,[7] respectively. Notably, almost HCC patients had liver cirrhosis and approximately 70% of those were related to HBV or HCV infection.[8,9] Besides, level of each biomarker may be different from patients with or without liver cirrhosis.[10]

Using correct diagnosis methods in detection HCC had important implication for patients that had background disease from liver cirrhosis because HCC was a serious type cancer, complex progress, short life-time of patient HCC, and over-necessary diagnosis should be avoided. There were no-evidence clearly and lack of specific guidance on early detection HCC in patients at risk, especially in Vietnam. Human telomerase reverse transcriptase (hTERT) mRNA in serum was reported that was similar to process of HCC, tumor size and number.[11] The sensitivity/specificity of combinations of hTERT mRNA with AFP was 96.0%/87.2%.[11] From this result, hTERT was predicted a potential biomarker for diagnosis and monitored the treatment course.[11,12] Based on limitation of the previous studies, we hoped finding an appropriate diagnosis from identifying value of hTERT alone and in combination with AFP, AFP-L3, DCP to assess sensitivity and specificity of biomarkers in detecting early-stage HCC for high-risk Vietnamese patients that had cirrhosis liver HBV or HCV-related.

Materials and Methods

Ethics statement

This study was approved by the University of Medicine and Pharmacy HCM City Ethics Review Committee (approval number #2018-295). Clinical databases and serum of patients were collected after written informed consent from participants. Results of study will support medical doctors to select the best regimen for each patient.

Study design and collect sample

During the period from April 2018 to December 2019 at University of Medical Center—Ho Chi Minh City University of Medicine and Pharmacy, 1900 chronic hepatitis patients with HBsAg and/ or HBV-DNA positive over 6 months recently and 550 patients with anti HCV + HCV-RNA or HCV Core-Ag positive were retrospectively enrolled from medical databases of hospital. Patients suffering from liver cirrhosis (LC) or in ultrasound were "liver cirrhosis" to be collected in 2 groups. Based on diagnosis and treatment guidelines of HCC from Vietnamese Ministry of Health and the Barcelona Clinic Liver Cancer (BCLC) system,[13] patients had 1 of 3 diagnostic results with HCC: (1) histopathology, (2) computed tomography (CT scan) with contrast, or (3) magnetic resonance imaging (MRI), were recruited in HCC group. Non-HCC and liver cirrhosis patients were participated in LC group. For the current study, we excluded participants such as metastatic liver cancer, pregnancy, co-infection HIV, or received any HCC treatments (surgical resection, radiotherapy, chemotherapy) before. A total of 340 patients with 170 HCC cases and 170 cirrhosis cases were enrolled finally. After writing informed consent, each patient was collected 3 mL blood into serum tube to determine 4 markers. All blood samples were obtained from patients at the time of admission

Identified of serum hTERT mRNA, AFP, AFP-L3, DCP

hTERT mRNA was evaluated by Real-time PCR method using SYBR Green. Serum AFP+AFP-L3+DCP were assessed by μTASWako® i30 a clinical automated immunoanalyzer system using a micro-chip capillary electrophoresis (Liquid-phase binding assay). Concentrations of AFP and AFP-L3% were calculated on the basis of the peak region of the fluorescence intensity of complex 1 (part AFP-L3) and complex 2 (part AFP-L1). The AFP concentration is the total concentration of AFP-L1, AFP-L3 and AFP-L3% determined by the following formula: (AFP-L3 concentration)/(AFP-L1 + AFP-L3 concentration) × 100. These tests require accuracy and all patient samples are only performed when the internal examination results are satisfactory. Epidemiological information was collected including age, gender, Child-Pugh score and BCLC stage, tumor size, number of tumors, location of tumor in HCC group.

Statistical analysis

SPSS software version 20.0 was used for statistical analysis. All differences were statistically significant at the 95% confidence interval (CI) with *P*-values were 2-sided and <.05. Categorical variables include Child-Pugh grade, BCLC stage, tumor size, number of tumors, location of tumor, were presented as case number and percentages. For normally distributed continuous variables, data was showed as mean ± standard derivation (std), or median (interquartile range, lower quartile – upper quartile) for its abnormally. Receiver operation characteristic (ROC) curve analysis and the area under the curve (AUC) were used to evaluate the sensitivity, specific for each cut-off value and to compare the diagnostic values of AFP, AFP-L3%, DCP, and hTERT mRNA. Using *t*-test and chi-square test to compare differences in groups. For continuous variables or quantitative variables (mean/median), Mann-Whitney *U* test, Kruskal-Wallis, or ANOVA tests was applied to identified difference of each biomarker and to predict the best diagnostic performance of them.

Table 1. Clinical features of patients in 2 groups.

	HCC (N = 170)	LC (N = 170)	*P* VALUE
Age (mean ± std, y)	59.8 ± 11.3	46.5 ± 13.8	<.001*
Gender (male/female)	136/34	90/80	<.001*
Child-Pugh grade			
A	152 (89.4)	136 (80.0)	.032
B	17 (10.0)	32 (18.8)	
C	1 (0.6)	2 (1.2)	
BCLC stage			
0 & A	92 (54.1)	170	
B, C, D	78 (45.9)	0	
Tumor size, n (%)			
<2 cm	44 (25.9)	0	
2-5 cm	70 (41.2)	0	
>5 cm	56 (32.9)	0	
Number of tumors			
1	100 (58.8)	0	
⩾2	70 (41.2)	0	
Location of tumor			
The right lobe	111 (65.3)	0	
The left lobe	21 (12.4)	0	
Both of lobes	38 (22.4)	0	
AFP (ng/mL)	33 (5.60-347.95)	1.95 (1.30-2.90)	<.001*
AFP-L3 (%)	7.5 (0.5-39.8)	0.5 (0.5-05)	<.001*
DCP (mAU/mL)	206.94 (42-3790)	16 (12.75-20)	<.001*
hTERT mRNA (copies/mL)	1610 (498.5-3927.5)	0.0 (0.0-0.0)	<.001*

Data were expressed as mean ± std or median (interquartile range, lower quartile – upper quartile) and numbers (n) with percentage (%). The age variable was measured by *T*-test. The binary variable (gender) was identified by Chi-squared test. The continuous variables (levels of AFP, AFP-L3, DCP, hTERT) with abnormal distribution were analyzed by Mann-Whitney *U* test.
**P*-value < .05.

Results

Clinical features

Clinical characteristics of both the HCC and LC groups were shown in Table 1. The mean age of HCC patients was 59.8 (±11.3) years, significantly higher than of the LC patients was 46.5 (±13.8) years ($P < .001$). In addition, the male/ female ratios were 4.0 and 1.12 among HCC group and LC group respectively. This difference was statistical significance ($P < .001$). To BCLC system, there are 5 stages to assess liver function. Stage 0 & A were the early-stage of HCC in which patients should be diagnosed and follow-up HCC. More haft of HCC patients had 1 tumor in liver and the majority location of tumor was the right lobe (65.3%).

The present study did not detect hTERT mRNA in serum of liver cirrhosis patients but it showed a median of 1610 (range 498.5-3927.5) copies/mL of HCC patients. Median level of AFP, AFP-L3% and DCP in HCC patients were 33 (5.60-347.95) ng/mL, 7.5 (0.5-39.8)%, and 206.94 (42-3790) mAU/mL, higher than those in LC patients were 1.95 (1.30-2.90) ng/mL, 0.5 (0.5-05)%, and 16 (12.75-20) mAU/mL. All these differences were statistical significance ($P < .001$, Table 1).

Table 2. Correlation between concentration of hTERT mRNA, AFP, AFP-L3%, DCP with tumor size.

	TUMOR SIZE			P^A	P^B	P^C	P^D
	<2 CM	2-5 CM	>5 CM				
AFP, ng/mL	8.7 (3.8; 58.5)	183.6 (8.4; 9647.5)	34.8 (8.8; 290.6)	.05	.005*	.027*	.101
AFP-L3, %	2.4 (0.5; 7.7)	16.7 (0.7; 76.1)	9.2 (0.9; 50.3)	.001*	.001*	.221	<.001*
DCP, mAU/mL	31.5 (20.9; 70.7)	3747.7 (750.4; 35422.5)	178.0 (60.5; 1866.0)	.291	<.001*	<.001*	.003*
hTERT, copies/mL	1374.0 (504.3; 2617.5)	1795.0 (550.8; 6826.8)	1876.5 (386.3; 4900.0)	.405	.405	.676	.044*

Data were showed as median (lower quartile; upper quartile).
[a]Tumor size group <2 cm with 2 to 5 cm.
[b]Tumor size group <2 cm with >5 cm.
[c]Tumor size group 2 to 5 cm with >5 cm.
[d]Tumor size with each biomarker.
**P*-value < .05, using Kruskal-Wallis test to calculate difference of each biomarker.

Table 3. Diagnostic values of AFP, AFP-L3, DCP and hTERT mRNA alone in detection HCC.

MARKERS	AUC	CUT-OFF	SENSITIVITY (%)	SPECIFIC (%)
AFP	0.910	5.1 (ng/mL)	0.73	0.92
AFP-L3	0.814	1.05 (%)	0.69	0.90
DCP	0.925	20.91 (mAU/mL)	0.91	0.76
hTERT mRNA	0.942	31.50 (copies/mL)	0.88	0.96

Correlation between concentration of AFP, AFP-L3, DCP and hTERT mRNA with tumor marker

As shown in Table 2, the median levels of hTERT mRNA were more and more significantly increasing in the development of tumor size, and expressed the highest concentration compared with other markers ($P < .05$). However, difference median levels of hTERT mRNA among tumor size group were not statistically significant. Median concentration of AFP, AFP-L3, or DCP was increasing between tumor size <2 cm with 2 and 5 cm, but decreasing between tumor size 2 and 5 cm with >5 cm. Median levels of AFP, AFP-L3, and DCP in tumor size group <2 cm were much lower significantly than in 5 cm. There was statistically significant between the median level of AFP-L3 in tumor size group <2 cm with 2 to 5 cm ($P < .05$). Otherwise, the median level of AFP and DCP were significant changes in tumor size group <2 and 5 cm.

Diagnostic values of AFP, AFP-L3, DCP, and hTERT mRNA alone

To assess the risks for development of HCC progress in patients with chronic liver and HBV/ HCV-related, the study applied ROC analysis AUC in calculation levels of AFP, AFP-L3, DCP, and hTERT mRNA individually. As shown in Table 3 and Figure 1, DCP marker exhibited the best sensitivity of 91%, but the worst specific of 76%, at cut-off point of 20.91 mAU/mL and AUC = 0.925. In 4 serological markers, the performances of hTERT mRNA gave desirable diagnostic values with sensitivity of 0.88, specific of 0.96 at cut-off point of 31.5 copies/mL and the highest AUC value was 0.942. In the present study, AFP and AFP-L3 showed lower sensitivity of 0.73 and 0.69, respectively, although their specific had the better values.

Diagnostic values of AFP, AFP-L3, DCP, and hTERT mRNA in combination

The results showed that when combining the 2 markers DCP + hTERT mRNA, the AUC area was the highest at cut-off point of 0.932 ($P < .05$) with sensitivity of 98.2% and specificity of 88.2% higher than that of the 3 individual markers AFP, AFP-L3, and DCP but lower than that of the hTERT mRNA marker alone (see Table 4 and Figure 2). When combining 3 markers AFP+DCP+hTERT mRNA, the AUC area was 0.921, the highest diagnosis value with sensitivity of 100% and specificity of 84.1% than other combinations, but lower than of the hTERT mRNA marker used alone. When all 4 markers were combined, the AUC was only 0.503 although it increased the sensitivity but decreased the specificity as non-diagnostic value.

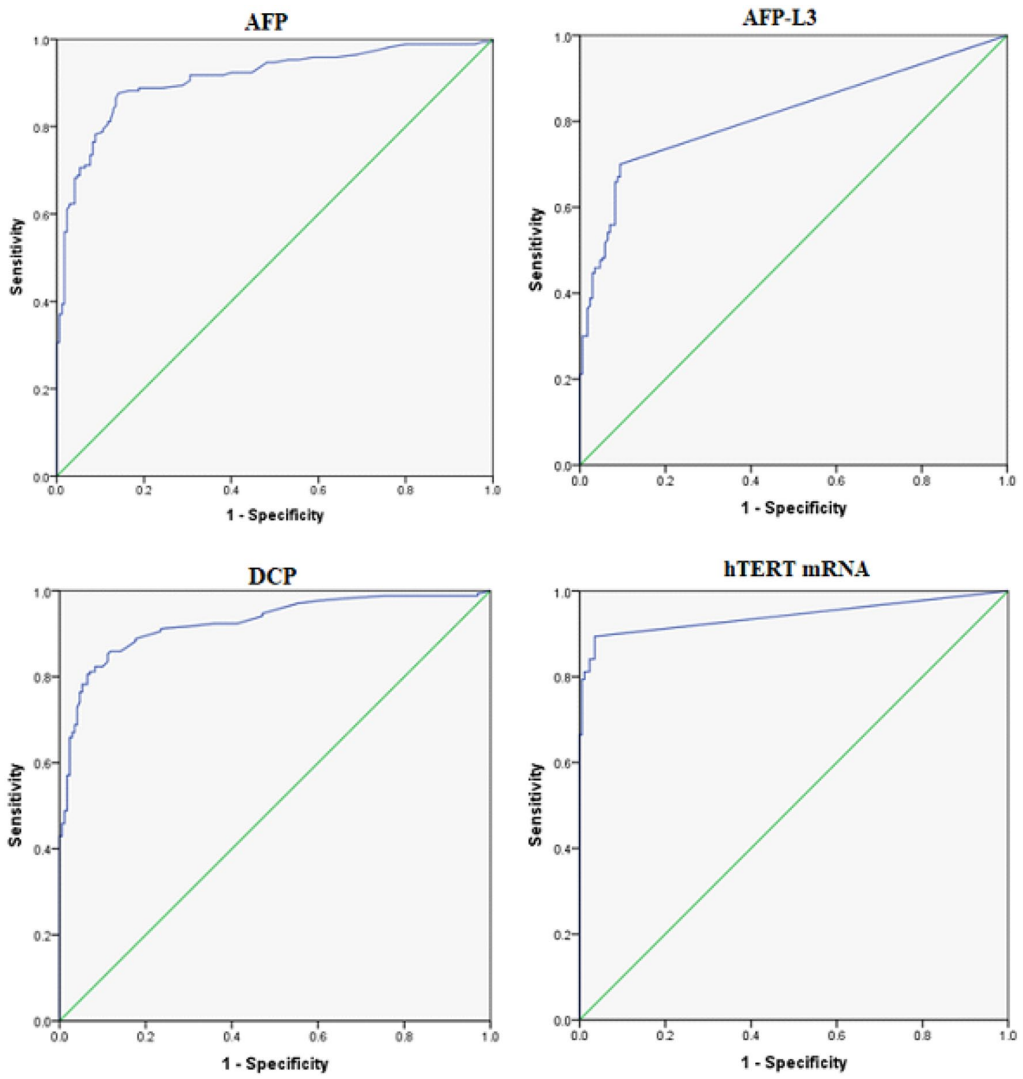

Figure 1. Receiver operating characteristic (ROC) curves for AFP, AFP-L3, DCP, and hTERT mRNA in detection HCC for liver cirrhosis patients HBV or HCV-related.

Table 4. Diagnostic values of AFP, AFP-L3, DCP, and hTERT mRNA in combination.

	AUC	SENSITIVITY (%)	SPECIFIC (%)
AFP+AFP-L3	0.853	81.2	89.4
AFP+DCP	0.900	92.4	87.6
AFP-L3+DCP	0.891	91.2	87.0
AFP+AFP-L3+DCP	0.897	93.5	85.8
hTERT+AFP	0.926	97.6	87.6
hTERT+AFP-L3	0.915	95.9	87.0
hTERT+DCP	0.932	98.2	88.2
hTERT+AFP+ DCP	0.921	100.0	84.1
hTERT+AFP+AFP-L3	0.918	97.6	85.8
hTERT+AFP-L3+ DCP	0.915	99.4	83.5
hTERT+AFP+AFP-L3+DCP	0.503	99.4	1.2

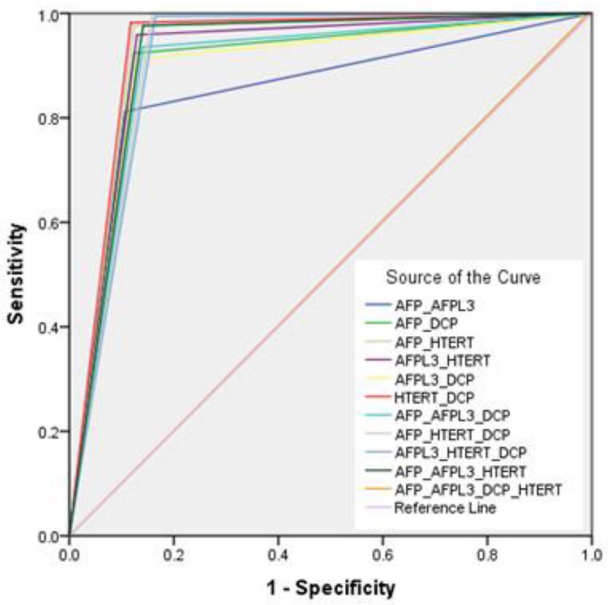

Figure 2. Analysis ROC combination of hTERT mRNA, AFP, AFP-L3, and DCP to distinguish between hepatocellular carcinoma patients and liver cirrhosis patients.

Discussion

Contemporary medicine has made remarkable advances in diagnosis and treatment HCC. However, finding an effective screening test about sensitive, easily available, cheap, timely for early HCC diagnosis is still the challenge. Some biomarkers such as AFP, AFP-L3, DCP, or the combination of these markers have been applied commonly to HCC surveillance in Asia and Western regions. Unfortunately, it is difficult to be reached international consensus due to their limitation of sensitivity and specificity.[14] Based on recent technological advancements in medicine, a few studies have suggested that peripheral blood telomerase activity may be used as a potentially molecular marker in diagnosis, prognosis and treatment response assessment of HCC.[11,15,16] Telomerase enzyme is a complex of 2 major components in which, human telomerase reverse transcriptase (hTERT) plays a catalytic subunit of its expression. Chen et al[17] proved that there was expression of hTERTmRNA in tissue (94%) and in serum (the sensitivity/specificity about 40%/100%) of breast cancer patients, but undetectable in benign disease or healthy persons. hTERT mRNA was absent in the healthy liver but low levels of its activity may be observed in conditions of physiological activation, chronic liver diseases or injury.[18,19] Actually, hTERTmRNA was demonstrated to be significantly associated with HCC include tumor size/number/grade and presence of intrahepatic metastasis.[11,12,20] Furthermore, with respect to the diagnosis of HCC (with different causes), hTERTmRNA showed sensitivity of 90.2% and specificity of 85.4%, higher than AFP, AFP-L3, and DCP,[11] and even be applied to detect HCC in patients with low AFP levels.[21,22]

In the present study, it was noteworthy that the hTERT mRNA serum level only occurred in HCC group, significant difference with LC group ($P < .001$). At cut-off point of 31.50 copies/mL and AUC = 0.941, hTERTmRNA had the best sensitivity (88%) and specificity (96%). From report of El-Mazny et al,[21] the role of hTERTmRNA in screening HCC was similar to our results. Even so, hTERTmRNA was found both in HCC patients, LC patients and healthy persons with 200.03 ± 35.3, 84.3 ± 27.3, and 42.8 ± 15.2 copies/mL, respectively. At the cut-off point of 144 copies/mL, hTERTmRNA had sensitivity of 77.14% and specificity of 100%, lower than the present study. This difference could be explained by the technical sensitivity of the Real-time PCR kit that was used from 2 studies.

HCC has been known as a complex disease with many causes in which cirrhosis was the main etiologies. In addition, HBV and HCV infection are defined to association with HCC,[23] mechanisms of HCC development and liver cirrhosis, which caused leading to HCC.[24] Hence, it is necessary to detect early-stage HCC for high-risk patients, especially in patients with chronic HBV/HCV infection and liver cirrhosis. Previous studies have selected participants include patients with different etiologies of liver diseases (HCC, cirrhosis, or benign liver disease) and healthy persons. The current study enrolled mainly patients with chronic HBV/HCV infection and liver cirrhosis but not included control group (healthy individuals) that are reported to have little or be absent of hTERTmRNA.[11,15] All HCC patients in our study had chronic HBV/HCV infection and liver cirrhosis for a long time before detected with HCC. Therefore, these patients reflected the natural history of HCC progression. We mainly focused on the analysis of hTERTmRNA with serum AFP, AFP-L3, and DCP to identify the most effective markers to complement the imaging technology for detection HCC at an early stage, of small tumors particularly. The results showed that concentration of AFP, AFP-L3, and DCP were increased much in HCC group than in LC group (significant difference with $P < .05$). However, the ROC curve analysis of each marker was limit because of low sensitivity. As shown above, serological markers were affected by some potential factors.[3,4,25] Caviglia et al[26] and Park et al[27] demonstrated combined application of AFP, AFP-L3, DCP gave a better overall specificity diagnostic performance. Interestingly, when adding hTERTmRNA in analysis of other biomarkers had alterations in AUC, and sensitivity/specific. Combining of DCP and hTERTmRNA, the ROC curve and the AUC area were the largest at 0.932, with sensitivity of 98.2% and specificity of 88.2%, and higher than that of the 3 individual markers AFP, AFP-L3 and DCP but lower than that of the hTERT mRNA marker used alone. When all 4 markers were combined, the AUC was only 0.503 which is similar to study of Park et al[27] noted that when AFP, AFP-L3, and DCP were combined, the AUC was 0.684 smaller than when using AFP or DCP alone. Clearly, combination of AFP, AFP-L3%, DCP, and hTERT mRNA markers showed improvement of HCC diagnostic better than using individual marker.

There was correlation between AFP-L3, DCP, and hTERT mRNA with tumor size ($P < .05$). According to the study of Yamamoto et al,[28] there was statistically significant difference in level of AFP ($P < .05$) and DCP ($P < .001$) as tumor size increased, while AFP-L3 of changes was not significant. Regarding the quantitative results of hTERT mRNA, our study illustrated a significant change when the tumor size increased ($P < .05$). This result was consistent with the study of El-Mazny et al[21] ($P < .001$) and the study of Miura et al[11] ($P < .033$). These findings indicate that hTERT mRNA could be a biomarker for better diagnostic efficacy. Besides, HCC often recurs repeatedly, so using serum hTERTmRNA gives useful choice to access recurrence or therapeutic effect.[11]

There was non-evidence of levels hTERT mRNA, AFP, AFP-L3 and DCP be affected by age and gender. However, the results showed that the mean age of HCC patients was 59.8 ± 11.3(years), significantly higher than in LC patients, consistent with the previous studies and the natural progression of the disease from hepatitis, cirrhosis to HCC about 10 to 15 years.[29] On the other hand, the proportion of men was also more dominant than women and difference significantly between HCC group and LC group. This result was the same with other studies which were reported that male to female ratio incidence in males was significantly higher and averages between 2:1 and >4:1.[30] Sex hormones, drink much alcohol, or diabetes may be cause for the difference in the male/female ratio in HCC patients.

In brief, the advantages of study gave medicine evidences about diagnostic value of new biomarker namely hTERT mRNA combined with AFP, AFP-L3, and DCP for screening HCC in liver cirrhosis patients HBV/HCV-related. However, hTERT mRNA test was a new method to be applied uncommonly in the world and in Vietnam. Thus, using hTERT mRNA as a daily test in diagnosis early-stage HCC should be needed to validate by more studies with larger sample size and multicenter.

Conclusion

This was the first study showed that new combination of hTERTmRNA with DCP or AFP to gave a useful choice for HCC detection in Vietnamese chronic HBV or HCV associated liver cirrhosis patients. Besides, the result also demonstrated that hTERTmRNA was a vital biomarker and only expressed in HCC patients. In screening of HCC, clinicians can consider to add hTERTmRNA in combination with both AFP and DCP for the highest sensitivity, but its specificity is lower than in other combinations.

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Author Contributions

Bang-Suong Thi Nguyen and Hoang-Bac Nguyen contributed to planning, conduct and writing of the manuscript; Xuan-Thao Thi Le, Huu-Huy Nguyen contributed to design and collect sample; Thanh Thanh Vo, Ngan Trung Nguyen, and Thien Minh Do-Nguyen analysis of the results; Minh-Khoi Le and Cong-Minh Truong Nguyen edit language.

Data Availability

All databases of the study were stored security at University Medical Center, Ho Chi Minh City Medicine and Pharmacy University. For a reasonable request in the results of this article, please contact our corresponding author.

Ethics Approval Statement

Our study was approved by the University of Medicine and Pharmacy HCM City Ethics Review Committee (approval number #2015-61).

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