Clinical significance of combined circulating TERT promoter mutations and miR-122 expression for screening HBV-related hepatocellular carcinoma

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Telomerase reverse-transcriptase (TERT) gene promoter mutations in circulating cell-free DNA (cfDNA) as well as the levels of circulating microRNA-122 (miR-122) have been reported as potential noninvasive biomarkers for several. This study evaluates the diagnostic performance of potent biomarker-based panels composing of serological AFP, miR-122 and circulating TERT promoter mutations for screening HBV-related HCC. TERT promoter mutations (C228T and C250T) and miR-122 expression were assessed in the plasma samples from 249 patients with HBV-related liver diseases by nested PCR and qRT-PCR assays, respectively. The diagnostic values of TERT promoter mutations, miR-122 expression and biomarker-based panels were assessed by computation of the area under the curve (AUC). Nested-PCR assays were optimized to detect C228T and C250T mutations in TERT promoter with detection limit of 1%. The common hotspot C228T was observed in 22 HCC cases. The triple combinatory panel (AFP@TERT@miR-122) acquired the best diagnostic value to distinguish HCC from CHB (AUC = 0.98), LC (AUC = 0.88) or non-HCC (LC + CHB, AUC = 0.94) compared to the performance of double combinations or single biomarkers, respectively. Notably, among patients with AFP levels ≤20 ng/μl, the double combination panel (TERT@miR-122) retains satisfactory diagnostic performance in discriminating HCC from the others (HCC vs. CHB, AUC = 0.96; HCC vs. LC, AUC = 0.88, HCC vs. non-HCC, AUC = 0.94). The triple combination panel AFP@TERT@miR-122 shows a better diagnostic performance for screening HCC in HBV patients, regardless of AFP levels. The newly established panels can be a potential application in clinical practice in Vietnamese setting.

The prevalence of the hepatitis B virus (HBV) infection and the incidence and mortality of HBV-related hepatocellular carcinoma (HCC) represent a serious health problem in most Asian countries including Vietnam1. The high mortality rate of HCC is due to the lack of suitable tools for early detection. So far, ultrasound and AFP (alpha-fetoprotein) are recommended for surveillance and early screening of HCC in high-risk groups2,3. However, roughly 30% of HCC patients had normal AFP levels at diagnosis even with advanced disease or high levels of AFP (>200 ng/ml) were also found in several non-HCC patients4. In addition, using ultrasound for HCC screening generates a poor sensitivity of 47%5 and the accuracy of ultrasound is highly dependent on the expertise and tumor size. The diagnostic performance of other serum protein biomarkers for routine surveillance of HCC is unsatisfactory6. Therefore, the implementation of potential biomarkers with better diagnostic performance and/...
and chronic hepatitis (CHB, n = 98) were allocated into three groups including hepatocellular carcinoma (HCC, n = 96), liver cirrhosis (LC, n = 55) and chronic hepatitis (CHB, n = 98) based on the clinical manifestations, biochemical and liver function tests, imaging modalities (abdominal ultrasound, MRI or CT scanner). Histopathological analyses were mandatory for the diagnostic confirmation in case of HCC. All patients were negative for antibodies against HCV and HIV.

Materials and Methods

All methods used in this study were in accordance with the relevant guidelines and regulations and were approved by the institutional review board and an independent Ethics Committee of the 108 Military Central Hospital, Hanoi, Vietnam.

Patients and sampling. Two hundred forty-nine patients with HBV-related liver diseases were recruited for the study. These patients were treated in the 108 Military Central Hospital between 2016 and 2019. Patients were allocated into three groups including hepatocellular carcinoma (HCC, n = 96), liver cirrhosis (LC, n = 55) and chronic hepatitis (CHB, n = 98) based on the clinical manifestations, biochemical and liver function tests, imaging modalities (abdominal ultrasound, MRI or CT scanner). Histopathological analyses were mandatory for the diagnostic confirmation in case of HCC. All patients were negative for antibodies against HCV and HIV.

Blood samples were obtained from all patients. Plasma was immediately separated from blood cells and were stored at −80 °C until use.

Biochemical and serological tests. The levels of albumin, globulin, total and direct bilirubin, alanine transaminase (ALT), aspartate aminotransferase (AST) were measured on an auto-analyzer (Hitachi Automatic Analyzer, Tokyo, Japan). ALT and AST levels were assessed for both the patients and HCs. Markers for HBV infection (HBsAg, anti-HBc-IgM, anti-HBcIgG, HBeAg, and anti-HBe) were assessed by commercial immunoassay kits (General Biologicals Corp, Taipei, Taiwan and DiaSorin, Saluggia, Italy). ALT and AST levels were measured using a commercial ELISA kit (General Biologicals Corp., Taipei, Taiwan) as previously described29.

Circulating cell-free DNA extraction. Peripheral blood samples were spun at 1,600 × g for 10 min at 4 °C. The plasma portion was re-centrifuged at 16,000 × g for 10 min at 4 °C to obtain cell-free plasma and then were stored at −70 °C until further analyses. DNA was extracted from 4 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (QIAGEN, Hilden, Germany) and following the manufacturer’s recommended protocol. The extracted circulating cell-free DNA (cfDNA) was then reconstituted in 100 μl of 25 mM Tris-HCl pH 8.0 containing 0.5 mM EDTA and stored at −20 °C for further analyses.

Nestested PCR assays for the identification of TERT promoter mutations. The promoter region of TERT gene (Accession number AH007699.2) was amplified in Eppendorf thermocycler using 5 μl of reconstituted cfDNA as template with the outer primer pairs: Tr-Tert-seq-F: 5’-GCA GCG CTG CCT GCC CTT CAC CTT CCA-3’ and Tr-Tert-seq-R: 5’-GTC CTG CCC CTT GCT GAA ACT CG-3’ plus 0.2 unit of Promega Gold Tag DNA Polymerase (M3001), 8% DMSO and 1.25 M Betaine to generate an amplicon of 163 bp. The PCR condition was set with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 sec and 62 °C (annealing) for 45 sec and elongation at 72 °C for 30 sec. Afterwards, the outer amplicon products were then diluted 1000 times; 5 μl of diluted outer PCR product was used as a template for the nested PCRs.
For identification of TERT C228T and C250T mutations, two allele-specific PCRs with individual forward primers: Tr-ARMS-TERT-MT(C228T)-F2: 5’-CCC CGG CCC AGC CC GT-3’ and Tr-ARMS-C250T-MT-F: 5’-CGC CCC GTC CCG ACC CC GTC-3’ (the underlined letters are mismatched nucleotide to further eliminate the unspecific pairing of forward primers to wild-type templates) coupling to a single reverse primer (Tr-Tert-seq-R: 5’- GCA GCG CTG CCT GAA ACT CG-3’) were used. Thermal cycling condition was the same as outer PCR to generate 93 bp amplicon (for C228T mutation) and 116 bp amplicon (for C250T mutation), respectively. The reaction mixtures were then electrophoresed against 2% agarose to visualize the amplicon products (Suppl. Figure 1).

miRNA extraction and cDNA synthesis. Total RNA, including miRNA fractions, was isolated from 200 µl plasma with TRIzol reagent and reconstituted in 50 µl water treated with diethylpyrocarbonate (DEPC). The quality of total RNA preparations was assessed by NanoDrop spectrometer (NanoDrop Technologies, Wilmington, USA) at 260 and 280 nm (A260/280). Approximately 200 ng of total RNA were used for reverse transcription (RT) by RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific Inc, Singapore) following the manufacturer’s instruction. The specific primer used for miR-122 cDNA synthesis (miR-122 accession number: MIMAT0000421) was designed according to stem-loop theory as described previously (sequence: 5’-GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA ACC AAA CA-3’).

### Table 1

| Characteristics                  | CHB (n = 98) | LC (n = 55) | HCC (n = 96) | P value |
|----------------------------------|-------------|-------------|--------------|---------|
| Age (years)                      | 42 [21–85]  | 57 [27–79]  | 55 [23–92]  | <0.05*  |
| Male (%)                         | 81          | 78          | 91           | NS      |
| AST (IU/L)                       | 96 [35–293] | 69 [50–134] | 69 [48–123] | NS      |
| ALT (IU/L)                       | 117 [36–453]| 46 [29–85]  | 44 [30–84]  | <0.0001*|
| Total bilirubin (mg/dl)          | 16 [12–61]  | 31 [22–58]  | 15 [11–20]  | <0.0001*|
| Direct bilirubin (mg/dl)         | 5 [3–50]    | 15 [8–33]   | 4 [3–6]     | <0.0001*|
| Albumin (g/L)                    | 41 [38–43]  | 32 [28–38]  | 39 [35–42]  | <0.0001*|
| Prothrombin (% of standard)      | 95 [81–108] | 65 [53–79]  | 95 [85–106]| <0.0014*|
| WBC (x10^9/ml)                   | 7.0 [5.6–8.6]| 5.0 [4.3–6.7]| 7.0 [5.7–8.7]| <0.0001*|
| RBC (x10^6/ml)                   | 5.0 [4.6–5.3]| 4.0 [3.4–4.2]| 5.0 [4.3–5.2]| <0.0001*|
| PLT (x10^9/ml)                    | 191 [153–237]| 88 [64–113]  | 194 [142–238]| <0.0001*|
| HBV-DNA - log10(copies/ml)       | 6.0 [4.4–8.4]| 5.2 [3.9–7.8]| 5.0 [4.2–6.6]| NS      |
| AFP (IU/L)                       | 2.0 [1.5–9.3]| 11.0 [4.0–25.8]| 126.0 [12.9–315.2]| <0.0001*|

Table 1. Characteristics HBV patients segregated according to clinical presentation. CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; RBC, red blood cells; WBC, white blood cells; PLT, platelets; AST and ALT, aspartate and alanine amino transferase; AFP, alpha-fetoprotein; IU, international unit; NR, normal range. Values given are medians with 25–75% percentiles. P values were calculated by chi-squared (α) or Kruskal-Wallis test (β).
Quantification of miRNA by real-time PCR. After reverse transcription, cDNA was reconstituted in 100 µl of 25 mM Tris-HCl pH 8.0. The real-time PCR (qPCR) reaction mixtures consisted of 10 µl of 2xSybr-Green I master mix (Applied Biosystems, Foster City, CA, USA), 5 µl of cDNA preparation, 5 pmol of miRNA universal reverse primer 5’-GTG CAG GGT CCG AGG T-3’ and 5 pmol of miR-122-specific forward primer (5’-GGT GTG GAG TGT GAC AAT GG-3’). The qPCR reaction was performed using Stratagene M3000p device (Stratagene, San Diego, CA, USA) with a pre-incubation step at 50 °C for 15 min, initial denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 sec and 60 °C for 60 sec. The RT-PCR reactions were finalised by amplicon melting dissociation. The cycle of threshold (Ct) values was recorded and analysed according to the comparative Ct method\textsuperscript{31}, in which the Ct value of miRNA-16 was used as normalisation factor as recommended previously\textsuperscript{29,32}.

Statistical analysis. All statistical analyses were performed using R v3.2.2 (https://www.r-project.org/). Values were presented as either mean with standard deviation (SD), median with 25–75% percentiles, or numbers with percentages where appropriate. Chi-squared tests were used to test the significant differences of categorical variables between groups. Kruskal-Wallis or Mann Whitney Wilcoxon tests were used to compare non-parametric data of quantitative variables between different groups where appropriate. Logistic regression models were built to establish the potent biomarkers-based panels (AFP, miR-122 expression and circulating \textit{TERT} promoter mutations) to predict HCC from individuals with chronic hepatitis B who did not have cancer at the time of the study. Receiver operating characteristic (ROC) curves were generated and the diagnostic value of different panels was assessed by computation of the area under the ROC curve (AUC). The level of significance was set at a two-sided \( P \) value of <0.05.

Results
Clinical characteristics of the patients. The baseline characteristics of the enrolled 249 HBV-infected patients are shown in Table 1. Most patients were male (84%). The median age of patients was higher in patients with later phase of liver disease progression. The levels of ALT were higher in patients with CHB compared to other subgroups (\( P < 0.0001 \)). Total and direct bilirubin levels were significantly lower in patients with HCC.
compared to other subgroups ($P < 0.0001$). Albumin and prothrombin levels and blood cell counts were significantly lower in patients with LC compared to the other patient groups ($P < 0.0001$). AFP levels were significantly higher in HCC patients compared to CHB and LC groups ($P < 0.0001$) (Table 1).

**Expression of miR-122 in HBV patients.** We assessed and compared the relative expression of miR-122 in different HBV patient subgroups. The results show that miR-122 relative expression was significantly higher in HCC patients compared to non-HCC patients (CHB + LC) ($P < 0.0001$). Relative expression of miR-122 was highest in HCC patients followed by LC and CHB patients indicating that miR-122 expression is increased according to the development of HBV-related liver diseases (Fig. 1).

**Circulating TERT promoter mutations in HBV related liver diseases.** In order to determine the concentration of spiked TERT C228T and TERT C250T mutant alleles at which the nested PCR assays can detect, the genomic DNA of HCC cell lines HUH733 (C228T carrier) and Mahlavu (C250 carrier) were mixed against genomic DNA myeloid leukaemia cell line HL-60 (unknown TERT promoter mutation) to make serial dilutions of 50%, 25%, 10%, 1%, 0.5%, 0.1% and 0% of either TERT C228T or TERT C250T mutations. All the dilutions were formulated to 20 ng/μl and we used 5 μl (equal to 100 ng) as a template for nested PCRs. The nested PCR for the identifications of TERT C228T and TERT C250T acquired detection limits of 1% and 0.5% respectively (Supl. Figure 1). We then applied these allele-specific nested PCR assays onto the plasma samples collected from 249 patients with HBV-related liver diseases (96 HCC, 55 LC and 98 CHB). TERT C228T mutation was detected from 22 HCC plasma samples. However, we did not detect any TERT promoter mutations in CHB and LC subgroups and the C250T mutation was also not identified in our current study cohort.

**Diagnostic performance of the biomarkers and combined panels in differentiating HCC.** We built logistic regression models to assess the diagnostic performance of the study biomarkers and different biomarkers-combinations (namely panels) for screening HCC. Single biomarkers: miR-122 expression, AFP levels and TERT C228T mutation) showed a poor or moderate diagnostic performance in differentiating HCC from LC (AUC = 0.73, 0.75, and 0.61, respectively), HCC from CHB (AUC = 0.88, 0.84, and 0.61, respectively) and

![Figure 3. Diagnostic performance of the models with combination of the biomarkers (double or triple combination) in differentiating HCC from other groups: HCC vs. CHB; HCC vs. LC; HCC vs. non-HCC.](https://example.com/figure3.png)
HCC from non-HCC (AUC = 0.82, 0.81, and 0.61, respectively) (Fig. 2). Double combination of the biomarkers miR-122@AFP, AFP@TERT and TERT@miR-122 gained better diagnostic performance in differentiating HCC from LC (AUC = 0.83, 0.82, and 0.79, respectively), HCC from CHB (AUC = 0.96, 0.89 and 0.89, respectively) and HCC from non-HCC (AUC = 0.90, 0.87 and 0.86, respectively) (Fig. 3). Triple combination of the biomarkers (TERT@miR-122@AFP panel) showed the highest diagnostic performance in differentiating HCC from other forms of chronic liver diseases (HCC from LC, AUC = 0.88; HCC from CHB, AUC = 0.98; HCC from non-HCC or CHB + LC, AUC = 0.94) (Fig. 4). We also analyzed HCC diagnostic performance of single biomarkers and combined panels in HBV patients who had low AFP levels (≤20 ng/μl), the TERT@Mir122 panel still sustained its diagnostic accuracy in distinguishing HCC from the other groups [CHB patients, AUC = 0.96; LC patients AUC = 0.88, patients with non-HCC, AUC = 0.94] (Fig. 4).

**Discussion**

The implementation of effective and reliable strategies to interrogate the risk populations such as HBV carriers to screen and detect HCC at early stage could improve the survival of suffered patients. Recently, a number of genetic lesions involving in key etiological pathways of HCC has been identified in patients' liver tumor tissues. Somatic mutations of TERT in the promoter region and circulating miR-122 have reported as potential noninvasive biomarkers for neoplastic diseases, including liver cancer. In this study, we developed a PCR assay that could detect TERT promoter mutations from circulating DNA with high sensitivity and established the diagnostic panels with combinatory use of TERT promoter mutations, miR-122 expression and AFP levels for identifying HCC among chronic HBV-infected patients. The established models with the triple combination could provide a better diagnostic performance in the prediction of HCC occurrence among high-risk populations.

We used TERT promoter mutations and miR-122 in the combination with AFP in the predictive models based on the evidence on clinical relevance of these biomarkers in the association with liver cancer. MicroRNA (miRNAs) have been suggested as potential genetic biomarkers to predict tumor load, early disease recurrence...
and therapeutic response in various cancer entities, including HCC. Among several important miRNAs, miR-122 is one of the most crucial microRNAs, which have been reported in liver cancer. However, so far, only little is known about the values of miR-122 in diagnosis, treatment and prognosis of HCC even though miRNAs can be isolated from blood samples and easily integrated into standard follow up procedures. TERT promoter mutations are the most frequent somatic genetic alterations in HCC and TERT is the first recurrent gene somatically mutated in preneoplastic lesions in hepatocytes. However, it is not clear how frequent these somatic lesions are detected in the blood of HCC patients. In fact, more than 90% of the region covering 1000 bp around TERT gene transcription start site is dominated by GC content, which makes a challenge for amplification of TERT promoter fragment especially from clinical samples containing a little amount of DNA. Therefore, the method for identification of TERT promoter mutations directly from peripheral blood, as well as the prevalence of circulating TERT promoter mutations in HCC has yet been described. With the application of amplification refractory mutation system, our optimized nested PCR assays for identifying TERT promoter mutations C228T (−124G ≥ A) and C250T (−146G ≥ A) could detect TERT promoter mutation at a limit of 0.1–1%. Therefore, for the first time in this study, the prevalence of TERT gene promoter mutations circulating in blood was reported.

In our study cohort, TERT promoter mutations C228T (−124G ≥ A) and C250T (−146G ≥ A) were not detected in CHB and LC samples, whereas C228T (−124G ≥ A) mutation was detected in 23% of HCC samples. This prevalence is lower than that detected in liver tumor tissues as described previously. Due to the low prevalence of C228T (−124G ≥ A) mutation in HCC plasma samples in our study compared to tumor tissues, the single use of circulating C228T (−124G ≥ A) mutation acquires unsatisfactory diagnostics performance in assisting diagnosis of HCC. In addition, the diagnostic performance of either miR-122 expression or AFP levels alone did not gain enough accuracy, especially in discriminating HCC from LC. Nevertheless, the combinatorial use of C228T (−124G ≥ A) mutation with two biomarkers miR-122 expression and AFP levels acquired a good diagnostic performance in distinguishing HCC from LC (AUC = 0.88) or from the group without HCC (LC + CHB, AUC = 0.94). Importantly, patients who have AFP levels lower than 20 ng/ml, the miR-122/TERT panel also provides accuracy in discriminating HCC from the other groups (CHB, AUC = 0.96; LC, AUC = 0.88, CHB + LC, AUC = 0.94). This result indicates that TERT promoter mutations could be an additional genetic marker for diagnostics of HCC in the circumstances of risk group and/or other biomarkers lacking accurate diagnostic performance.

There are some limitations remaining in our current study. Firstly, our assay could not be used for quantitatively monitoring the circulating TERT promoter mutations, therefore, it is impossible at the moment to use TERT promoter mutations for a treatment-follow-up purpose. Secondly, our study cohort was not big enough that allows us to investigate the association of TERT promoter mutations with liver function parameter and the progression of HCC. In conclusion, the combinatorial use of TERT promoter mutation, miR-122 expression, together with serological AFP levels, is a potential biomarker assist the diagnostic establishment of HCC in HBV patients, particularly in HBV-related LC patients with normal AFP levels.

**Ethics approval and consent to participate.** The study was approved by the institutional review board and an Independent Ethics Committee of the 108 Military Central Hospital, Hanoi, Vietnam. Informed written consent was obtained from all study patients.

**Data availability**
Data and supporting materials associated with this study will be shared upon request.

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**Author contributions**

N.T.T. and L.H.S., designed and supervised the studies. P.Q.T., M.T.B. conducted the experiments. N.X.H., M.H.B. evaluated the clinical data and provided the clinical samples, N.T.T., N.X.H. L.H.S., analysed the data and wrote the manuscript, N.L.T., H.V.T. revise the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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