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Diagnostic Accuracy of Non-Invasive Liquid Biopsy Approach for Diagnosis of Hepatocellular Carcinoma Keeping Histopathology as Gold Standard

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ABSTRACT

Early noninvasive identification of hepatocellular carcinoma could significantly lower its high global fatality rate. A possible non-invasive method for liquid tumor biopsy is provided by the quantitative measurement of cell-free circulating DNA in plasma or serum. To evaluate the diagnostic accuracy of non-invasive liquid biopsy test for screening and early detection of hepatocellular carcinoma in high-risk individuals taking histopathology as gold standard. Free circulating DNA in plasma was amplified using a real-time polymerase chain reaction (PCR) technology. Nanodrop 2000 spectrophotometry was used to measure the amount of free circulating DNA, and gel electrophoresis was used to quantify the amount of DNA in blood. The amplification status of hTERT and β-Globulin gene as markers of hepatocellular carcinoma were estimated. Hepatocellular carcinoma patients had a concentration of free circulating DNA (61.6 ng/ul), which was around six times higher than the value seen in healthy people (10.3 ng/ul). The 95% CI for the area under the ROC curve was 0.890-0.967, with a P-value of less than 0.01. Considerable variation in the copy numbers of hTERT gene and β-Globulin gene were seen in patients with hepatocellular carcinoma and patients without hepatocellular carcinoma ($P<0.01$). The hTERT gene had maximum sensitivity of 90% but at expense of specificity. Whereas, β-Globulin gene had highest specificity of 74.9%. The amplification of cell free DNA as well as estimation of hTERT gene quantification status seems to higher in patients than healthy individuals. That's why liquid biopsy can be helpful in early diagnosis of HCC.

***Keywords*:** Histopathology, polymerase chain reaction, Hepatocellular carcinoma, biomarkers, diagnostic, Cell-free DNA, β-Globulin, hTERT.

INTRODUCTION

Cancer is the second greatest cause of death worldwide, accounting for one out of every six fatalities. Despite being the sixth most prevalent type of cancer globally, hepatocellular carcinoma (HCC) is the third most common cause of cancer-related fatalities and has one of the highest mortality-to-incidence ratios (Grewal et al., 2012). More than 90% of liver malignancies are HCC, which also causes an estimated 782,000 new cases of cancer and over 746,000 deaths annually. HCC often develops in cirrhotic livers, and there are notable regional and racial differences in the disease's incidence. Chronic viral hepatitis infection (hepatitis B or C) or exposure to toxins like alcohol or aflatoxin are closely associated with HCC (Serag and Hashem, 2011). Alpha 1-antitrypsin deficiency and hemochromatosis are two

conditions that significantly raise the chance of developing HCC. Additionally, metabolic disorders are becoming more often acknowledged as HCC risk factors. The majority of HCCs diagnosed in Sub-Saharan Africa and South-East Asia are caused by chronic hepatitis B virus (HBV) infection, which is widespread in these regions (Bardelli et al., 2017). Pakistan's cancer care system has ominous restrictions that negatively affect patient outcomes. The incidence of hepatobiliary malignancies has been steadily rising. According to the findings of a trustworthy hospital-based registry in Pakistan, hepatobiliary malignancies account for 10.7% of all cancers and are the most prevalent cancer in adult males (Kelley et al., 2015). In Pakistan, the age-standardized rate of HCC is 2.8 for females and 7.6 for males per 100,000 people year. Hepatitis B (hep-B) and hepatitis C (hep-C) patients provide the data on HCC, and we don't know the natural history of non-hep-B/hep-C HCC in our group (Kowalik et al., 2017). In contrast, hep-B continues to be the most common cause in many other Asian Pacific nations. Hereditary conditions include hemochromatosis and α-1-antitrypsin deficiency, exposure to aflatoxin B1 through diet, drinking, and male gender are further recognized risk factors for HCC. Depending on the cause, location or ethnic group, and stage of cirrhosis, patients with cirrhosis have a 5-year cumulative risk for HCC that varies from 5 to 30% (Bettegowda et al., 2014). In patients with HCC, early diagnosis through screening programs has been shown to improve survival. Serum Alpha fetoprotein is the most often utilized blood-based biomarker (AFP). is constrained by its low sensitivity and specificity, even when screening high-risk patients. Although patients with chronic liver disease may also exhibit high serum AFP levels, a significant percentage of HCC patients do not. In fact, AFP is not advised as the only diagnostic marker at this time (Wang et al., 2017). Numerous research over the last few decades have shown the powerful efficacy of circulating cancer byproducts detection, such as circulating tumor cells (CTC) and cell free nucleic acid (cfNA), which is known as "liquid biopsy." A standard liquid biopsy can be performed without any clinical issues because it is a noninvasive procedure. Unlike tissue samples, which only provide physicians with a confined view, it enables us to do repeated samplings that provide a complex portrait of the disease across time (Allen, 2024). It is significantly quicker and less expensive than traditional biopsy testing for early disease diagnosis, and it has a higher chance of capturing the entire genetic complexity of both primary and metastatic lesions in patients with advanced disease. In this area, research is still in its very early stages. Prior to the introduction of these highly promising cfDNA biomarkers into clinical practice, standardized and reliable protocols must be developed (Karachaliou et al., 2015). The diagnostic accuracy of a panel of cfDNA markers in detecting Hepatocellular carcinoma using a highly proficient real time PCR based methodology. Histopathology is Gold standard for diagnosis of hepatocellular carcinoma which is highly invasive, time consuming and costly test in contrast with economical liquid biopsy is non- Invasive, fast and cheaper test if its diagnostic accuracy will be promising it can be a better candidate substitute for hepatocellular carcinoma diagnosis.

MATERIALS AND METHODS

Quantitative Real time PCR for *ß-Globulin*

Primer Designing

The primers used to amplify β-Globulin were already reported by Hui and co- workers (Hui *et al.*, 2012). These were further rechecked by using bioinformatics tools ‘Primer blast’.

Table 3: Characteristics of Forward and Reverse primers for β-Globulin

Primers	Sequence (5'->3')	Length	Tm (°C)	GC %	Productsize (bp)
Forward	GAATTCCGATCTTAACAGGCCAGAAATGC	29	62.6	44.8	
Reverse	AGATCTCCACCAGACAGAAGGACCAGAGT	29	65.4	51.7	72

β-Globulin Primer Optimization Conditions by Gradient PCR

For amplification of β-Globulin the PCR were standardized by using following recipe. The mixture for PCR was prepared by using 1.5 mM $MgCl_2$, 5 μl of fcDNA solution, 1X PCR buffer, 1 μM forward primer, 1 μM reverse primer, 200 μM dNTPs, and 5 units of Hot start Taq polymerase enzyme and distilled water was used to make up the volume 25 μl. iCycler (Bio-Rad) was used for optimization of β-Globulin. PCR tubes containing reaction mixture were placed in it. The first denaturation stage of the reaction took place at 95°C for five minutes. 50 cycles of denaturation at 95°C for 30 seconds, annealing at various temperatures (48°C, 48.6°C, 49.5°C, 50.8°C, 52.5°C, and 53.8°C) for 30 seconds, and extension at 72°C were performed after the denaturation phase. The last elongation phase was carried out for five minutes at 72°C. For 30 seconds, the ideal annealing temperature was 63°C.

Quantitative Real time PCR for β-Globulin

Quantitative Fluorogenic The CFX96 Real-time Detection System (Applied Bio Systems) was used to perform real-time PCR. 10 µl of TOPreal™ qPCR 2X PreMIX, 0.25 pmol of forward primer, 0.25 qPCR 2X PreMIX, 0.25

pmol of forward primer, 0.25 pmol of reverse primer, and 3 µl of sterile water are needed for a 20 µl PCR reaction mixture. Each real-time PCR experiment used 5 µl of fcDNA solution. A denaturation step of 95°C for 10 minutes was used to start the thermal cycling process. 50 cycles of 95°C for 20 seconds and 60°C for 1 minute came next. CFX Manager software was used to analyze the data from 50 amplification cycles.

Table 4: Components of Real Time PCR Reaction Mixture for β-Globulin

Components (stock concentration)	Volume (final concentration)
TOPreal™ qPCR PreMIX (2X)	10 μl (1X)
Forward primer (5 pmol)	1 μl (0.25 pmol)
Reverse primer (5 pmol)	1 μl (0.25 pmol)
Template (fcDNA)	5μl
Water	3 μl
Total	20 μl

The 96-well plate was used for the real-time PCR amplifications. Samples from patients, controls, and a water blank served as a negative control on each plate. The calibration curve was constructed using suitable serial dilutions of Control Human Genomic DNA. The unknown amount of DNA in the test sample was ascertained using this standard amplification curve of known DNA quantity.

Specificity Determination of β-Globulin qPCR Assay

Melt curve analysis was used to check specificity of qPCR assay along with analysis of qPCR products on agarose gel.

Melt Curve Analysis

In real time PCR protocol melt curve analysis was inserted after amplification cycles completion. For melt curve analysis temperature conditions was 63.0°C to 95.0°C with an increment of 0.5°C for 5 second (Figure 6). CFX manager software was used to analyze melt peak and curve. For indication of single specific target amplification single sharp peak was checked.

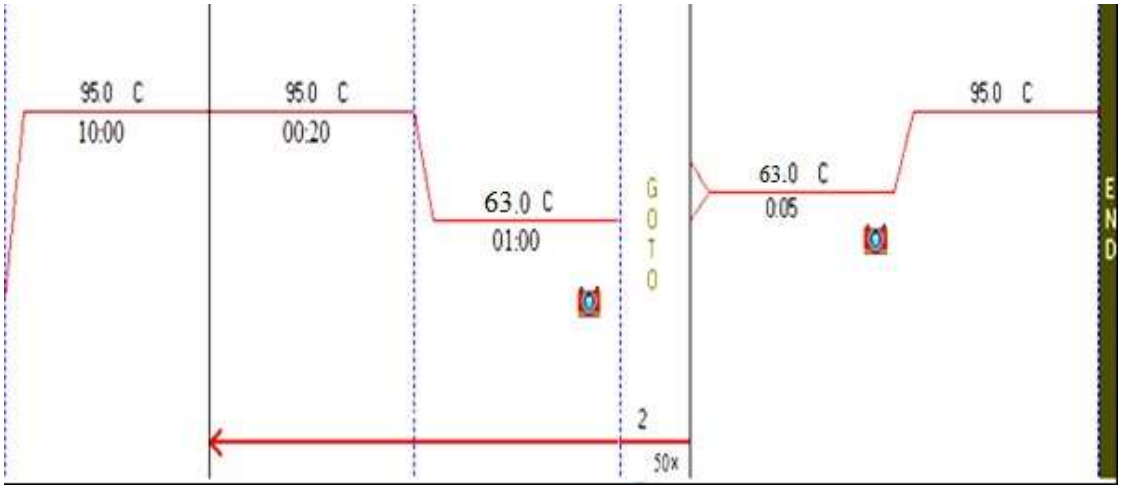

Fig. 1: Cyclic conditions of Real time PCR & Melt Curve Analysis for β-Globulin.

Agarose Gel Electrophoresis of β-Globulin Real Time PCR Products

Agarose gel electrophoresis was used to check amplified PCR product. 5 μl of PCR products including negative and positive control along with patient's samples was run on 2% agarose gel. GeneRuler™50 bp ladder was used to check single β-Globulin band.

Convential PCR for β-Globulin

Convential PCR analysis of β-Globulin was also done to check β-Globulin status in control and patient samples. For amplification of β-Globulin the PCR was performed by using following recipe. The mixture for PCR was prepared by using 1.5 mM $MgCl_2$, 5 μl of fcDNA solution, 1X PCR buffer, 1 μM forward primer, 1 μM reverse primer, 200

μM dNTPs, and 5 units of Taq polymerase enzyme and distilled water was used to make up the volume 25 μl. iCycler (Bio-Rad) was used for amplification of β-Globulin. PCR tubes containing reaction mixture were placed in it. The first denaturation stage of the reaction took place at 95°C for five minutes. 50 cycles of denaturation at 95 °C for 30 seconds, annealing at various temperatures (63 °C for 30 seconds), and extension at 72 °C were performed after the denaturation stage. The last elongation phase was carried out for five minutes at 72°C. Agarose Gel Electrophoresis of β-Globulin Conventional PCR Products. PCR products were than analyzed on 2% agarose gel using GeneRuler™50 bp ladder tocheck presence of amplified product

Traits of Study Population

From hepatitis clinic and Jinnah Hospital Lahore, 137 suspected individuals presentingthe clinical signs and symptoms were initially selected. Blood samples were taken after consent from both the suspects and patients having hepatitis and undergoing surgical liver tissue biopsy. Their Histopathology reports were also collected for comparative analysis of the proposed diagnostic accuracy of the assay with the gold standard. The collected blood was immediately transported, stored and were processed. All samples have been amassed from Jinnah Hospital Lahore.

Table 4: Demographics and Histopathological presentation of patients

Participants (N=137)			Traits
HistopathologyResults	Gender		Age (Years)
	Male	Female	Range Mean ± S.D
Liver cancer positivepatients (n=50)	42	08	(30-60) 44 ± 21.2
Liver cancer negativepatients (n=87)	66	21	(22-59) 42 ± 26.1

Histopathological and Clinicopathological findings

Among the total 137 participants, there were 50 confirmed HCC patients while 87 cases were found to be negative (Non-cancerous cases) diagnosed in histopathology section, Pathology Department, Allama Iqbal Medical College, Lahore. Prior to initiating anticancer therapy, blood samples from cancer patients were taken. Only patients with primary tumors were included in thestudy population; patients with cancer recurrence or those coming for follow-up were omitted. It is reflected in the Figure 2 that the prevalence of HCC is high in middle aged males as compared to female patients.

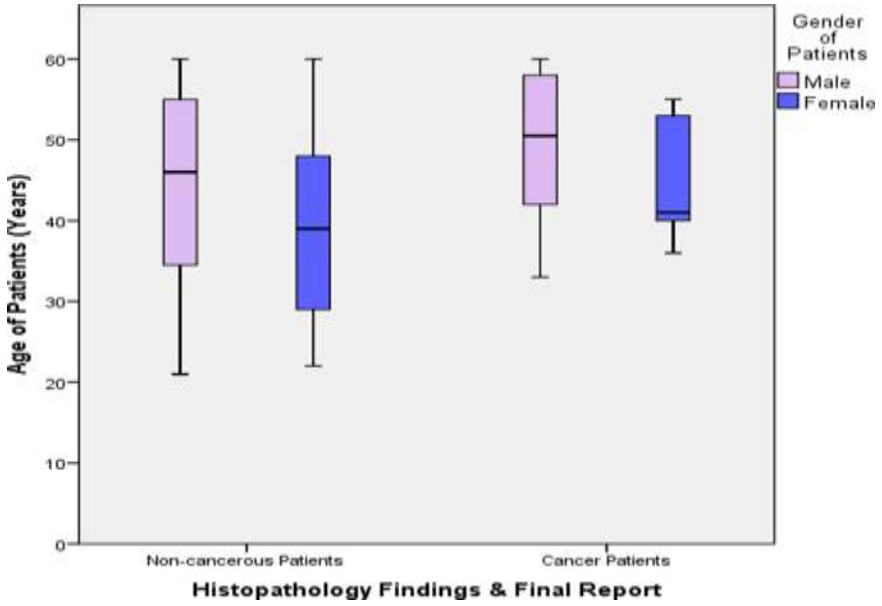

Fig. 02: Box plots of HCC patients and non-cancerous patients categorized with respect to their ageand sex.

Quantification of cfDNA by DNA spectrophotometer

The DNA spectrophotometer (NanoDrop 2000) was used to measure the concentration of circulating cell-free DNA in plasma at 260 nm. Patients with hepatic cancer had higher quantitiesof cfDNA than those without the disease indicated in the Table 10, 11 &12. Figure 8 and Table 12 reflected the hepatic cancer patient had six-fold higher mean

cfDNA concentration (61.6 ng/uL) compared to hepatic cancer negative individuals (10.3 ng/uL). Hepatic cancer patients had high cfDNA concentration values, while only a small percentage of cancer patients had low cfDNA concentration levels up to 5.43 ng/uL.

Table 5: Statistics of cfDNA in HCC patients and healthy individuals

	cfDNA (ng/µl)			
	Mean	S.D	Min	Max
HCC Patients	61.6	22.1	18.346	111.321
Healthy Individuals	10.3	2.2	5.4	14.7

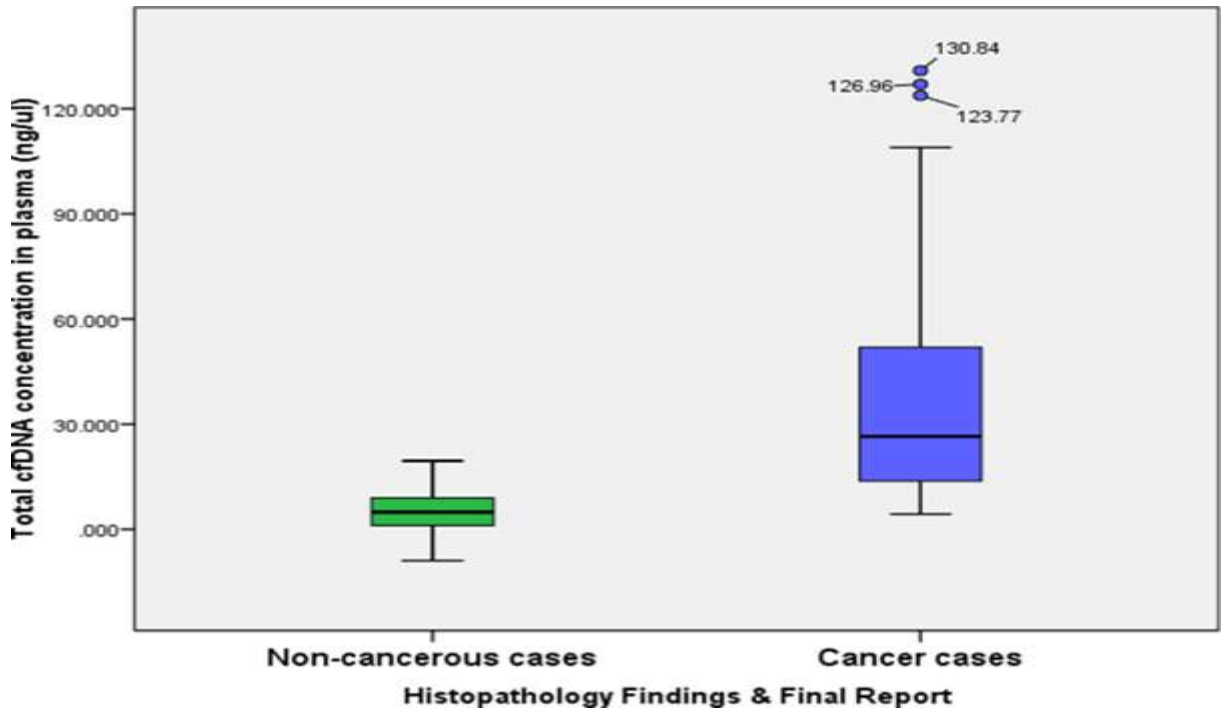

Fig. 03: Box plot of total cfDNA concentrations in plasma of Hepatocellular carcinoma(HCC) cases and non-cancerous cases by DNA spectrophotometer (NanoDrop 2000).

Table 6: Diagnostic performance of cfDNA quantification at cutoff-value 5.435 ng/uL

Marker	AUC	Sensitivity %	Specificity %	Positive predictivevalue %	Negative predictive value %	Accuracy	95%CI	P value
cfDNA	0.898	90.0	70.8	65.9	91.9	72.03	0.860-0.967	0.001

The receiver operating characteristic (ROC) curve was constructed by graphing the sensitivity against the 1-specificity. The AUC quantifies an assay's ability to discriminate cancer patients from non-cancerous individuals. The diagnostic power grows as the area approaches unity. The AUC was determined to be 0.898 (95 percent confidence interval [CI]: 0.890-0.967; P.001; Figure 9). Youden's index (YI) was used to select cut-off values by using the formula (YI = Sensitivity% + Specificity% −100) whereas, the cut-off point for having an acceptable Youden's index (YI) is 50 and above.

Optimization of PCR conditions for hTERT

While the Tm of the forward and reverse primers was 54.9◦C and 56.3◦C, respectively, gradient PCR was used to optimize the hTERT primers, with an annealing temperature range of 50◦C to 55◦C. Samples were examined on a 2% agarose gel after 45 cycles of amplification.

Figure 6 and Figure 7 depicted the graphical representation of ct (cycle threshold) values of GAPDH and β-Globulin after real time PCR amplification. The early graphs indicated the sample had low ct value and higher gene load and late amplification represented higher ct value and low gene load. These results validate all PCR process as these are housekeeping genes.

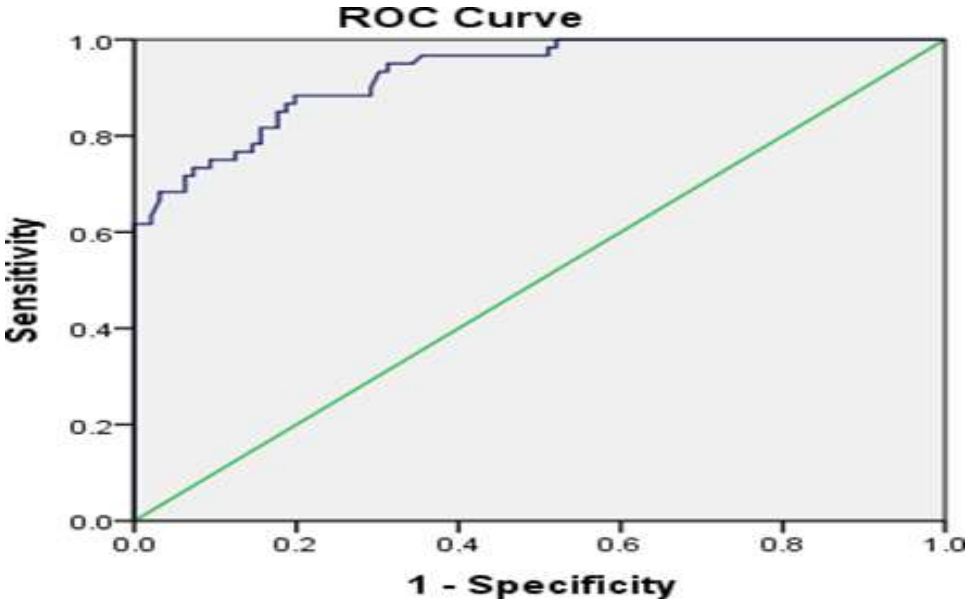

Fig. 04: Receiver operating characteristics (ROC) curve for the diagnosis of hepatic cancer using plasma-free circulating DNA cfDNA) concentrations.

Fig. 5: Analysis of amplified PCR fabricated from hTERT gene at 50°C. Lane A, 50 bp GeneRuler DNA ladder of Thermo Fisher Scientific InvitrogenTM GeneRuler™50 bp ladder and lane 1st amplified PCR merchandise of hTERT gene had been run.

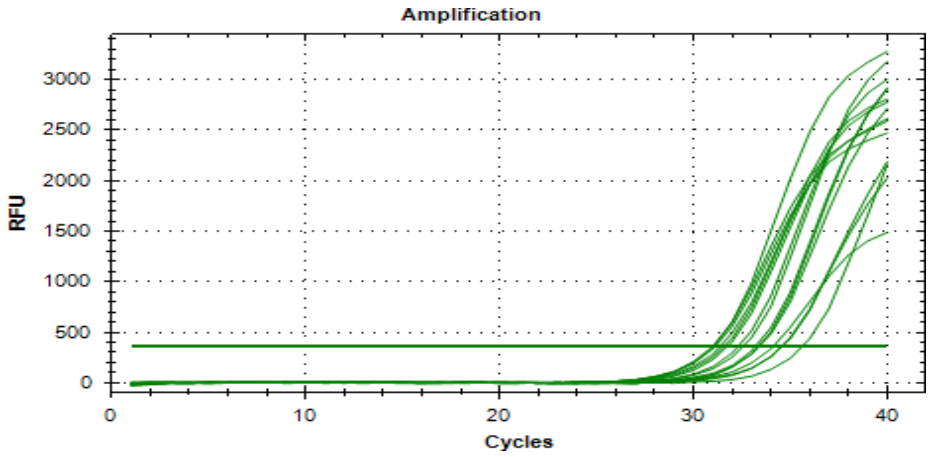

Fig. 6: Reatime PCR Amplification Graph of GAPDH.

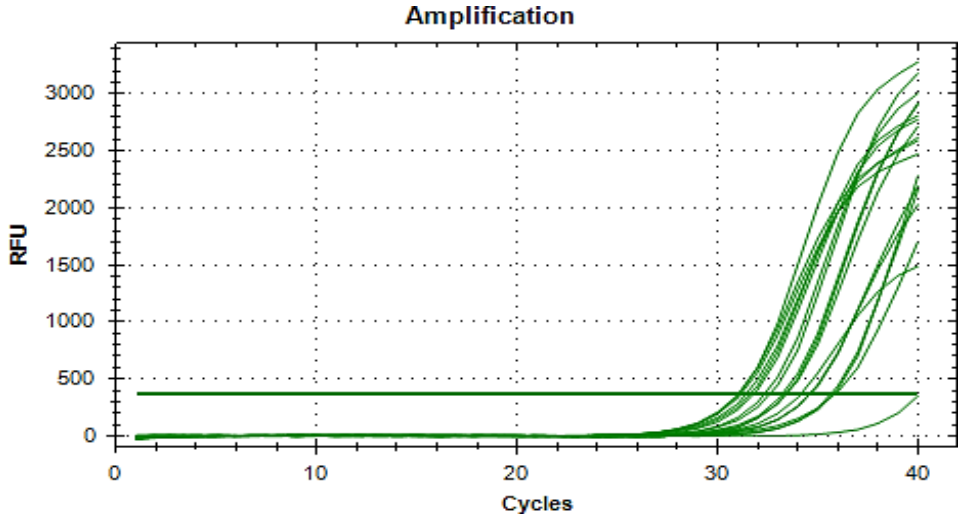

Fig.7: Real time PCR Amplification Graph of β-Globulin

Table 7: Mean ct values of hTERT and β-Globulin in HCC patients and healthy individuals

	hTERT	β-Globulin	GAPDH
	Mean ± S.D	Mean ± S. D	Mean ± S.D
HCC Patients	28.7 ± 1.41	29.1 ± 1.38	18.0 ± 0.7
Healthy Individuals	31.0 ± 1.25	31.6 ± 0.76	17.6 ± 0.8

Table 7 indicates the mean ct (cycle threshold) values of hTERT. It showed that ct values of HCC patient were lesser than ct value of healthy individual. It means the ct value is inversely proportional to gene expression, GAPDH and β-Globulin is used as internal control to monitor and validate all procedure.

DISCUSSION

Hepatocellular carcinoma is the most often diagnosed cancer, reported highest mortality rates globally in both sexes. The highest mortality rate and low survival rate of the malignancy is primarily due to its late diagnosis as this deadly cancer is asymptomatic at the initial stages (Hartke et al., 2017). Approximately, 75% of the hepatocellular carcinoma cases are diagnosed at metastasized stages followed by reduced prognosis. Survival of these patients strongly relies on early detection methods. The findings demonstrated that ctDNAs may be found in 50% of patients with localized malignancies and in over 75% of patients with advanced illnesses, such as hepatocellular carcinoma. According to Jabbour et al. (2015), there were no instances when CTCs were found but ctDNAs were not present. Conversely, ctDNAs were found in a large number of cases (13 out of 16 cases; 81.25%). Remarkably, hepatocellular carcinoma patients accounted for five of these cases, suggesting that ctDNA detection is probably more sensitive than CTC detection in hepatic malignancy (Charlotte et al., 2018). The diagnosis of hepatocellular carcinoma is typically verified by surgical or fine needle biopsy, which is not only invasive but also not required in the majority of benign tumor cases. Therefore, the creation of non-invasive and more practical biomarkers that enable earlier identification of hepatocellular carcinoma has received a lot of attention and research efforts (Candita et al., 2023). The circulating extracellular vehicles (EVs) in peripheral blood, nipple aspirate fluid (NAF), sweat, urine, tears, circulating carcinoma antigens (CAs), circulating tumor cells (CTCs), circulating cell-free tumor nucleic acids (DNA or RNA), circulating microRNAs (miRNAs), and volatile organic compounds (VOCs) in exhaled breath are among the non-invasive body fluid-based tests that have been reported to have the potential to complement current clinical approaches to the early detection of hepatocellular carcinoma.

Blood tests are not only easy to use and non-invasive (or minimally invasive) as compared to imaging and biopsy methods for cancer screening, but they are also widely accepted, easily repeatable, and reasonably priced. In addition to releasing living cells or dead cell debris into the bloodstream, cancer cells frequently create particular proteins, nucleic acids, or other cellular vesicles. Examining whether such substances are present in the blood could offer a way to identify cancer (Wald et al., 2017).

Better results are frequently obtained when hepatocellular carcinoma is detected early. Females with early-stage hepatic tumors had a significantly greater relative survival rate than those with advanced hepatic cancers, per Cancer Australia's National Cancer Control Indicators. At one, three, and five years after diagnosis, the survival rate for early-stage (stage 1) hepatic cancer stayed at 100%. Nevertheless, the survival rate for stage 4 metastatic hepatic carcinoma dropped to 69% after one year, 47% at three years, and 32% at five years after diagnosis. Consequently, early identification of hepatic cancer is essential for lowering the death rate (Vengateswaran et al., 2024). Due to intricate nature of Hepatic cancer, early diagnosis and therapeutic treatment can meet many challenges. As conventional fine

and core tissue biopsy which is still considered the gold standard for the diagnosis of Hepatic tumor are highly invasive techniques, so research on novel diagnostic approaches like liquid biopsy which is a non-invasive, rapid and cost-effective technique of cancer detection is the need of the hour. Early diagnosis and screening, prognostic prediction, early relapse detection, serial sampling, and effective longitudinal monitoring of disease progression and treatment response are just a few of the ways that liquid biopsy can enhance the management of hepatic cancer. In this regard, scientific study is still in its very early stages (Chartampilas et al., 2022). There is need to develop robust molecular procedures for bringing these promising ct DNA biomarkers into clinical practice. By using highly sensitive and specific biomarkers, we could investigate the diagnostic accuracy of blood based cfDNA/ctDNA molecular marker act as specimen of liquid biopsy for screening andearly detection of hepatic cancer (Nowicki et al., 2017). This is the need of time to change the gold standard technique of diagnosing cancer with the modern advance molecular techniques. Histopathological diagnostic technique proved to be highly invasive, expensive, and full of complications becauseit not only requires surgical intervention for proper tissue biopsy but it also needs radiological intervention and other imaging techniques (Ronot et al., 2023). On the other hand, real time rapid molecular techniques are noninvasive, cost efficient and least complicated diagnostic technique that has potential to detect hepatic cancer at an early stage with high diagnostic accuracy along with high sensitivity and specificity (Park et al., 2021). Undoubtedly, liquid biopsy will greatly help to increase the survival rate of Hepatic cancer patients and decrease the mortality rate consequently by this non-invasive,cost efficient and rapid diagnostic technique (Xu-Welliver et al., 2017).

CONCLUSIONS

In conclusion, all biomarkers used in the current study were based on blood so the contaminationrisks were lower to a greater extent and the technique can be regarded as non-invasive. The real time quantification of hTERT and cfDNA in blood / plasma samples presents significant clinical utility in the early diagnosis of hepatic carcinoma. The present study revealed that these biomarkers can act as liquid biopsy of the tumor and may possibly be promising blood-based biomarkers in diagnosing primary stage hepatic cancer. In future, studies comprising large number of hepatic cancers affected individuals are obligatory to further confirm the clinical usefulness and efficacy of plasma cfDNA and somatic gene mutation biomarkers.

Recommendations

It is recommended that prospective studies with long follow-ups should be conducted with large number of carcinomas affected individual to further validate and standardize the present technique for sample collection, processing and analysis. This will confirm the clinical usefulness and efficacy of serum cfDNA and somatic gene mutation biomarkers for early diagnosis of cancers.

Limitations

Despite the fact that liquid biopsy technique is non-invasive, rapid and cost effective in comparison to gold standard; however liquid biopsy approach of cancer diagnosis still lacks appreciation and implementation. It needs extensive research to identify some highly sensitive and specific biomarkers for its global recognition and acceptance.

DECLARATION

Acknowledgement: Not applicable

Conflict of Interest: The authors declare no conflict of interest.

Data Availability: All data is given in the manuscript

Ethics Statement

Not applicable

Author's Contribution

Muhammad Abdullah; conceptualized, Data curation, Original Draft Writing, Saif Ullah; Methodology, Data analysis, Writing, Muhammad Shahzad Taaj; Data Collection, Data analysis, Muhammad Ahsan; Writing and Review, Qadeer Liaquat; Data Analysis, Interpreted Results, Aneeqa Javed; Writing, Reviewing, Editing, Zoha Ali; Review, Editing, Writing, Ayisha Hafeez; Review, Editing, Writing.

Generative AI Statements

The authors confirm that no generative-AI tools (including DeepSeek) were used in the writing or preparation of this manuscript.

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