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RESEARCH ARTICLE

Identification of Non-Invasive Biomarkers for Early Detection of Hepatocellular Carcinoma in Pakistani Patients: A Correlation Study

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ABSTRACT

Hepatocellular carcinoma (HCC) is a type of liver cancer that is often detected at an advanced stage, resulting in high mortality rates. Current diagnostic procedures for HCC have low efficacy, highlighting the need for non-invasive, early detection methods. Several molecular markers have been identified for HCC occurrence, including Alpha fetoprotein (AFP), hepatitis C virus (HCV) viral load, and methylation of tumour suppressor genes (RASSF1A). The aim of this study is to analyse these molecular markers and their correlation for the non-invasive, early detection of HCC. The study included three groups: a control group (healthy individuals), chronic HCV patients, and HCC diagnosed patients. Patient histories were recorded, including liver function tests, age, gender, and viral load (for HCV patients). Methylation was detected using Methylation Specific PCR. The study analysed fifty blood samples. Correlations were found between AFP and viral load, as well as between liver enzymes and AFP. Methylation was detected in HCC patients, but not in the other two groups. The study found that 20% of HCC (hepatocellular carcinoma) patients had methylation, which is a lower percentage than previous studies that reported 50% methylation in blood samples from HCC patients. The study also found that there are direct relationships between AFP (alpha-fetoprotein) and viral load, as well as between liver function enzymes, which supports previous research. Patients with HCV (hepatitis C virus) who have high viral load and AFP should receive antiviral therapy and regular monitoring. This study highlights the potential for non-invasive methods to detect HCC and emphasizes the importance of early detection and treatment to improve patient outcomes.

Keywords: Hepatocellular carcinoma (HCC), Non-invasive biomarkers, Alpha-fetoprotein (AFP), RASSF1A methylation.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a prevalent cancer, with an estimated global incidence of 500,000 to 1 million cases annually (Stella et al., 2022). The majority of cases occur in individuals with cirrhosis and chronic hepatitis B or C infections, which account for 80% of cases worldwide (Nardone et al., 2023). HCC is most common in Asia and sub-Saharan Africa and developing countries, where there is a higher prevalence of hepatitis B and C infections (Mitchell et al., 2023). In Pakistan, there has been an increase in HCC cases due to the rising exposure to risk factors in the local population (Mitchell et al., 2023). HCV infection significantly increases the risk of developing HCC, with infected patients being 17 times more susceptible than non-infected individuals (Mitchell et al., 2023). Several factors, including male sex, older age, HBV coinfection, heavy alcohol intake, diabetes, and HCV infection from a transfusion-related source, increase susceptibility to HCC (Anzola, 2004). Alpha fetoprotein is a useful marker for detecting HCC, with

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over 70% of HCC patients exhibiting elevated levels (Anzola, 2004). The commonly used cut-off value to distinguish HCC patients from healthy adults is a serum concentration of 20ng/Ml (Yao et al., 2016). The hypermethylation of RASSF1A is a mechanism that leads to uninterrupted cell growth and division, a hallmark of cancer cells (Oršolić & Jazvinšćak Jembrek, 2022). This process results in the transcriptional silencing and loss of the RASSF1A gene product. Detection of the hypermethylation of RASSF1A in serum DNA could be a valuable biomarker for early-stage diagnosis in populations at high risk of HCC, as it occurs at a moderate to high frequency in a wide range of tumor types but is rare in normal tissue (Fan et al., 2023).

MATERIALS AND METHODS

The study involved the use of 50 blood samples, which were obtained from three groups of individuals: HCC patients (20), HCV positive patients (20), and healthy individuals (Ouvrard et al., 2022). The patients' clinical history, including their age, gender, viral load, and liver functional enzymes, was recorded. To extract DNA from the plasma, a kit from Thermos-scientific was used in accordance with the kit protocol.

After extraction, the DNA was confirmed using Nano-drop and agarose gel electrophoresis. Methylation was detected through methylation specific PCR, which involved treating the DNA with bisulphite using a Bisulphite kit from Thermo-scientific. The resulting PCR product was then confirmed using agarose gel electrophoresis. The primers used during the methylation specific PCR were able to amplify the targeted site and produce the expected product.

To calculate the alpha fetoprotein level, an ELISA kit was used. The results were then correlated to enable early diagnosis of HCC. This study provides valuable insights into the use of various techniques for detecting methylation and alpha fetoprotein levels in blood samples, which could prove useful for the early detection of liver cancer

Statistical Analysis:

The statistical analysis of the data was performed using Microsoft Excel 2023

RESULTS

Performed agarose gel electrophoresis to confirm the presence of extracted DNA. The results, shown in Fig. 1, indicate that the DNA had a high molecular weight, causing it to remain at the top of the gel during the analysis.

To detect DNA methylation, the MSP technique uses two sets of primers specific to either the methylated or unmethylated version of the target DNA sequence. The primers used in this line are specific to the promoter region of the RASSF1A gene and designed to amplify either the methylated or unmethylated version of the DNA, depending on the primer set used.

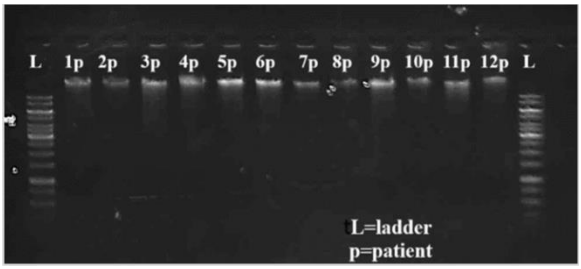

Fig. 1: Extracted DNA.

By comparing the amplification results obtained with each primer set, it is possible to determine the methylation status of the RASSF1A promoter region (Aibel et al., 2023). If the methylated primer set amplifies the target sequence, it indicates the presence of DNA methylation at that site, whereas the unmethylated primer set amplification indicates the absence of DNA methylation at that site (Rand et al., 2002) (Ting et al., 2005). The primers used are given below.

Methylated Primer

PRIMERS	SEQUENCE	TM	PRODUCT SIZE
FORWARD (F)	5' GTGTTAACGCGTTGCGTATC 3'	60.4°C	93BP
REVERSE (R)	5' AACCCCGCGAACTAAAAACGA 3'	60.6°C	

UN-Methylated Primer

Primers	*Sequence*	*Tm*	*Product size*
Forward (F)	*5' TTTGGTTGGAGTGTGTTAATGTG 3'*	*59.2°C*	*105bp*
Reverse (R)	*5' CAAACCCCACAAACTAAAAACAA*	*57.4C*	

Methylation specific PCR has been performed by using above primers and their thermo-cycler condition are given in Table 1.

Table 1: Thermo-cycler conditions for MS PCR.

Condition	*Temperature*	*Time*
Initial denaturation	*950C*	*4:00 mints*
Final denaturation	*96 oC*	*0:30 seconds*
Annealing	*60 oC*	*0:40 seconds*
Initial extension	*72 oC*	*0:30 seconds*
Final extension	*72 oC*	*5:00 mints*

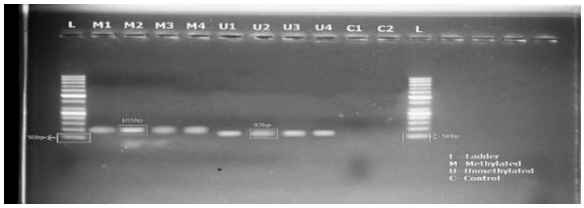

Fig. 2: confirmation of PCR product. The size and presence of the PCR product have been validated through agarose gel electrophoresis, as depicted in Fig. 2. To ensure accuracy, we employed a 50-base pair ladder and two controls, C1 and C2. C1 consisted of untreated DNA, while C2 served as a PCR control without a template. Our results showed that the methylated region produced a product size of 105 base pairs, while the un-methylated region generated a product size of 93 base pairs. Methylation detected in four HCC patients (20%) but no methylation has been observed in normal and HCV group.

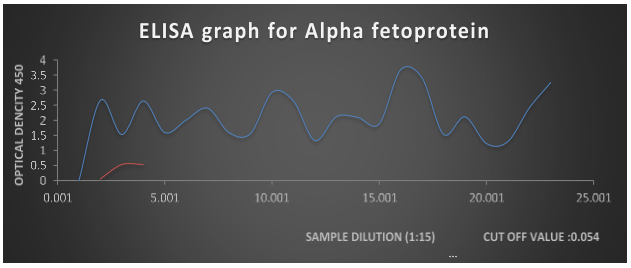

Fig. 3: Graphical representation of ELISAThe passage describes the use of the Enzyme-Linked Immunosorbent Assay (ELISA) test to measure Alpha-fetoprotein levels in patients with Hepatitis C Virus (HCV) infection. The test measures the optical density of the samples at 450 nm wavelength. A cut-off value of 0.054 is used to distinguish between positive and negative samples. Samples with values below the cut-off are considered negative, while those with values above the cut-off are considered positive for the presence of Alpha-fetoprotein.

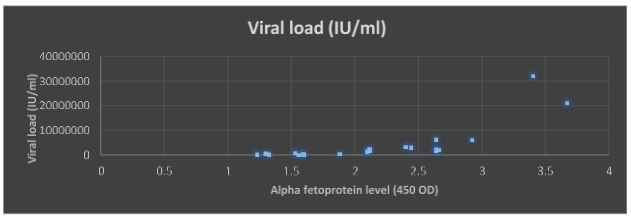

Fig. 4: Direct relationship between the levels of Alpha fetoprotein and HCV viral load. Specifically, as the level of Alpha fetoprotein increases, so does the level of HCV viral load.

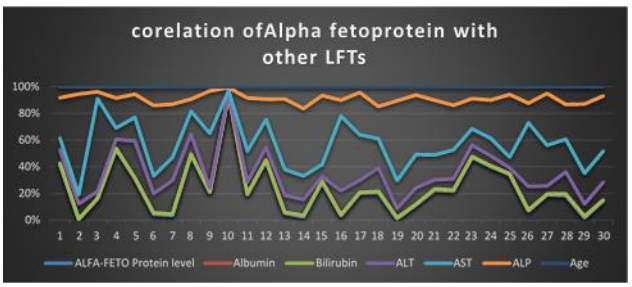

Fig. 5: The relationship between Alpha-fetoprotein levels and liver function tests. A graph is used to represent the number of patients on the X-axis and their Alpha-fetoprotein concentration on the Y-axis. When the Alpha-fetoprotein levels are elevated, the liver function tests show disturbance. The graph shows that almost all patients have increasing Alpha-fetoprotein levels, which correspond with the disturbed liver function tests

DISCUSSION

Numerous studies have been conducted on the non-invasive detection of hepatocellular carcinoma (HCC) (Mariam et al., 2022). In our research, we aim to develop a panel of molecular markers for the early detection of HCC, which includes HCV viral load, alpha fetoprotein, and methylation of the RASSF1A gene. HCV viral load has been reported as a major factor in disease progression (Stella et al., 2022), while alpha fetoprotein and RASSF1A methylation are both potential non-invasive molecular markers (Nomeir et al., 2022). We found aberrant methylation in approximately 20% of our HCC patients, but no methylation was detected in chronic hepatitis C or control groups (healthy individuals). Methylation in various genes, including RASSF1A genes, could be induced by chronic HCV infection, potentially acting as an epi-mutagen and playing a role in hepatic carcinogenesis (Nishida et al., 2008). Earlier studies have observed hyper methylation of the RASSF1A gene in tumor tissue samples to be 96%, while in blood, it is below 50% (Aibel et al., 2023). We also conducted an ELISA for alpha fetoprotein and correlated it with other variables such as age, gender, and liver function tests. Alpha fetoprotein is directly related to HCV infection (viral load), increasing with the viral load, and liver function tests are also disturbed with the elevation of alpha fetoprotein. Previous studies have observed the same elevation and relationship, with patients with elevated HCV infection having abnormal values of alpha fetoprotein and liver functional enzymes (Nomeir et al., 2022). While methylation has been detected in HCC patients, no significant correlation has been observed between alpha fetoprotein and RASSF1A promoter methylation. In earlier studies, the same correspondence has been perceived between them (Nishida et al., 2008).

The suggested diagnostic approach is attractive as it does not require invasive methods and utilizes ELISA and PCR-based molecular assays. This detection approach will be more cost-effective than the recently used radiographic techniques, allowing for regular examination of high-risk patients before the onset of symptoms. However, some limitations must be addressed, including the need for detailed gene methylation studies to examine RASSF1A methylated with high incidence in hepatocellular carcinomas associated with many etiologic factors. Identifying other tumor suppressor genes that are frequently methylated regarding that disease would be valuable epigenetic biomarkers for early diagnosis (Alshammari et al., 2022). It will also be important to establish high sensitivity and specificity for each marker. Ultimately, our study provides an excellent starting point for additional studies aimed at non-invasive detection of HCC in the Pakistani population.

REFERENCES

Aibel, C., Coll De Peña, A., & Tripathi, A. (2023). An optimized CoBRA method for the microfluidic electrophoresis detection of breast cancer associated RASSF1 methylation. *BioTech*, *12*(1), 7.

Alshammari, E., Zhang, Y., Sobota, J., & Yang, Z. (2022). Aberrant DNA methylation of tumor suppressor genes and oncogenes as cancer biomarkers. *Genomic and Epigenomic Biomarkers of Toxicology and Disease: Clinical and Therapeutic Actions*, 251-271.

Anzola, M. (2004). Hepatocellular carcinoma: role of hepatitis B and hepatitis C viruses proteins in

hepatocarcinogenesis. *Journal of Viral Hepatitis, 11*(5), 383-393.

Fan, Z., Zhou, P., Jin, B., Li, G., Feng, L., Zhuang, C., & Wang, S. (2023). Recent therapeutics in hepatocellular carcinoma. *American Journal of Cancer Research, 13*(1), 261.

Mariam, A., Miller-Atkins, G., Moro, A., Rodarte, A. I., Siddiqi, S., Acevedo-Moreno, L.-A., & Rotroff, D. M. (2022). Salivary miRNAs as non-invasive biomarkers of hepatocellular carcinoma: a pilot study. *PeerJ, 10*, e12715.

Mitchell, T., Nayagam, J. S., Dusheiko, G., & Agarwal, K. (2023). Health inequalities in the management of chronic hepatitis B virus infection in patients from sub-Saharan Africa in high-income countries. *JHEP Reports, 5*(2), 100623.

Nardone, A., Nerlander, L., Duffell, E., Valenciano, M., Buti, M., Marcos-Fosch, C., & Filip, P. V. (2023). A pilot sentinel surveillance system to monitor treatment and treatment outcomes of chronic hepatitis B and C infections in clinical centres in three European countries, 2019. *Eurosurveillance, 28*(6), 2200184.

Nishida, N., Nagasaka, T., Nishimura, T., Ikai, I., Boland, C. R., & Goel, A. (2008). Aberrant methylation of multiple tumor suppressor genes in aging liver, chronic hepatitis, and hepatocellular carcinoma. *Hepatology, 47*(3), 908-918.

Nomeir, H., El-Sheredy, H. G., Nomeir, A., Mostafa, N., & El-Hamshary, S. (2022). Diagnostic performance of RASSF1A and CDKN2A gene methylation versus a-fetoprotein in hepatocellular carcinoma. *Clinical and Experimental Hepatology, 8*(3), 243-252.

Oršolić, N., & Jazvinšćak Jembrek, M. (2022). Molecular and cellular mechanisms of propolis and its polyphenolic compounds against cancer. *International Journal of Molecular Sciences, 23*(18), 10479.

Ouvrard, J., Muniz, L., Nicolas, E., & Trouche, D. (2022). Small interfering RNAs targeting a chromatin-associated RNA induce its transcriptional silencing in human cells. *Molecular and Cellular Biology, 42*(12), e00271-00222.

Rand, K., Qu, W., Ho, T., Clark, S. J., & Molloy, P. (2002). Conversion-specific detection of DNA methylation using real-time polymerase chain reaction (ConLight-MSP) to avoid false positives. *Methods, 27*(2), 114-120.

Stella, L., Santopaolo, F., Gasbarrini, A., Pompili, M., & Ponziani, F. R. (2022). Viral hepatitis and hepatocellular carcinoma: From molecular pathways to the role of clinical surveillance and antiviral treatment. *World Journal of Gastroenterology, 28*(21), 2251.

Ting, A. H., Schuebel, K. E., Herman, J. G., & Baylin, S. B. (2005). Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. *Nature Genetics, 37*(8), 906-910.

Yao, M., Zhao, J., & Lu, F. (2016). Alpha-fetoprotein still is a valuable diagnostic and prognosis predicting biomarker in hepatitis B virus infection-related hepatocellular carcinoma. *Oncotarget, 7*(4), 3702.