RESEARCH ARTICLE

Determine the frequency distribution of hepatocellular carcinoma (HCC) and evaluation of viral persistence in the development of HCC among Pakistani patients

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Abstract

Objective: To determine the frequency distribution of hepatocellular carcinoma, and to evaluate viral persistence and its association with viral titres.

Method: The retrospective study was conducted at the Lahore Hepatobiliary/Liver Transplantation Unit, Shaikh Zayed Hospital, Lahore, Pakistan, and comprised data between December 2010 and September 2019 related to patients with liver complications. For viral persistence evaluation, immunohistochemistry analysis was performed on explanted liver tissues/liver resections from patients who also had hepatitis B and C, assessing hepatitis C virus non-structural protein 5B and hepatitis B Virus X protein expression using qualitative immunohistochemistry scoring. Data was analysed using R version 3.3.5.

Results: Of the total 1,384 patients admitted, hepatocellular carcinoma was noted in 256(18.5%) cases, with age and gender being significantly different between positive and negative patients ($p<0.05$). Further, 13/16 (81%) of hepatocellular carcinoma patients with hepatitis C had immunohistochemistry score 1), followed by 2/16 (13%) with score 3, and 1/16 (6%) score 2 with respect to hepatitis C virus non-structural protein 5B protein expression. In hepatocellular carcinoma patients with hepatitis B, 4/5(40%) had score 1),4/5 (40%) had score 2, and 1/5(20%) had score 3 with respect to hepatitis B Virus X protein expression.

Conclusion: Hepatocellular carcinoma was found to be the most frequently reported liver-associated disease, predominantly affecting males. Viral protein detection in viral-induced hepatocellular carcinoma samples underscored the persistence of the virus in hepatocytes.

Keywords: Hepatitis, Hepatocellular carcinoma, NS5B, HBX, Liver neoplasm, Hepacivirus. (JPMA 75: 1409; 2025)

DOI: https://doi.org/10.47391/JPMA.22562

Introduction

Hepatocellular carcinoma (HCC) is responsible for over 80% cases among all the cancers primarily originating from the liver.[1] The prevalence of HCC varies geographically, and currently HCC is the fifth most common cancer representing almost 6% of all newly diagnosed cancers globally.[1,2] It is on the rise in Pakistan as well. Asia and Africa have the highest incidence rates of HCC3. HCC is the most common cause of death in cirrhotic patients, mainly due to viral hepatitis with hepatitis C virus (HCV) or hepatitis B virus (HBV) infection.[3,4] In the absence of a national cancer registry and the lack of screening programmes, the prevalence of HBV, HCV and HCC are only estimates of the true extent of the problem.[5] Globally, it ranks as the 6th most prevalent cancer among frequently occurring malignancies and constitutes one of the primary causes of mortality, claiming approximately 800,000 lives annually. Particularly prevalent in developing and low-income regions, such as East Asia and Sub-Saharan Africa, HCC accounts for 80-85% of cases in these areas.[6] Chronic viral hepatitis stands as the leading factor contributing to HCC development, with approximately 80% of cases attributed to this aetiology. Among viral-associated HCC cases, approximately 25% are HBV-related and 58% are linked to HCV.[7]

Among all the HCV proteins, the non-structural 5B (NS5B) protein holds significant importance, serving pivotal roles in HCC development and regulation of HCV replication via ribonucleic acid (RNA)-dependent RNA polymerase (RdRp) activity. Consequently, it emerges as a prime target for therapeutic interventions aimed at combatting HCV. NS5B's involvement in ubiquitination of tumour suppressors, such as retinoblastoma (Rb) and Novel Ras effector-1 (NORE-1A) /Ras association domain family member 5 (RASSF5) further underscores its importance in HCC progression. More than half of virus-related HCC cases are caused by HBV, demonstrating the significance of HBV in the disease's pathophysiology. Host, viral and environmental variables can all play a part in the intricate multistep process that leads to the formation and progression of HBV-related

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Submission complete: 24-11-2024 **1st Revision received:** 21-01-2025

Acceptance: 31-05-2025 **Last Revision received:** 27-05-2025

HCC.[8]

Similarly, in HBV, the regulatory hepatitis B Virus X (HBX) protein plays a pivotal role. Predominantly located in the cytoplasm of hepatic cells but also present to a lesser extent in the nucleus and cytosolic mitochondria, HBX regulates protein degradation, signal transduction, apoptotic pathways, and cell cycle progression within the host. Mounting evidence underscores HBX's indispensable role in HCC development both in vitro and in vivo, mediated through mechanisms encompassing transcriptional activity, covalently closed circular deoxyribonucleic acid (cccDNA) regulation, inhibition of innate immunity effectors, and degradation of host factors with antiviral properties.

The current study was planned to determine the frequency distribution of HCC, and to evaluate viral persistence and its association with viral titers.

Materials and Methods

The retrospective study was conducted at the Lahore Hepatobiliary/Liver Transplantation Unit, Shaikh Zayed Hospital (SZH), Lahore, Pakistan, and comprised data between December 2010 and September 2019 related to patients with liver complications. Approval was obtained from the ethics review committee (ERC) of the Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Science and Technology (NUST), Islamabad, Pakistan, and the SZH ERC.

The demographic profiles of all patients were recorded. The most commonly diagnosed liver cancers and their other liver associated diseases were divided along age, gender, disease profile and surgical interventions. HCC liver tissues were collected, and those with lack of information about HCC aetiology were excluded. HCC samples with associated HBV and HCV were used as the cases, while non-viral HCC samples were used as controls (Table 1). For viral persistence evaluation, sections of the liver tissue were used to extract viral nucleic acid, which was then measured using quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Figure 1). Using immunohistochemistry (IHC), the liver tissue sections were stained for NS5B and HBX assessment.

Table-1: Inclusion and exclusion criteria.

Inclusion Criteria	Exclusion Criteria	Control Group
All confirmed HCC patients (moderate, poor and well differentiated) patients, positive for HCV, HBV HCC were included.	All HCC patients with less tissue and incomplete data record were excluded from IHC experiments	HCV +ve chronic hepatitis liver tissue is used as positive control. Non-viral HCC tissue, Gall bladder, cholecystitis, Cholangiocarcinoma, Cirrhosis liver tissues are used as negative control.

HBV: Hepatitis B virus, HCV: Hepatitis C virus. HCC: Hepatocellular carcinoma.

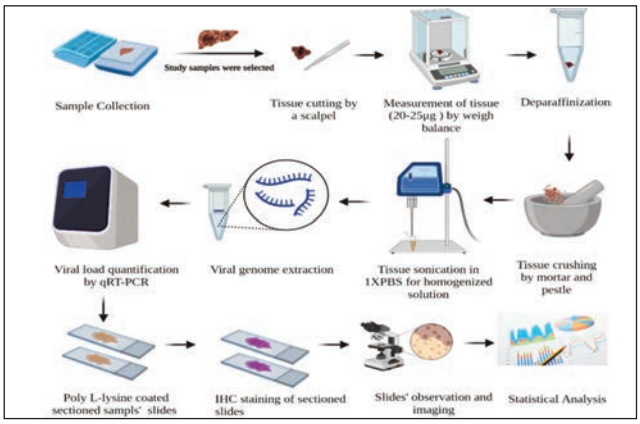

Figure-1: Methodology of research work.

Approximately, 20-25µg of liver tissue was cut from a formalin-fixed paraffin embedded (FFPE) tissue block using a scalpel. Tissue was weighed and was then shifted to an Eppendorf tube.

Viral nucleic acid was extracted from the FFPE blocks using the relevant kit (Innu PREP Virus DNA/RNA Kit; Catalogue # 845-KS-4800050, United States), and qRT-PCR was performed (Qiagen's Artus HCV RG RT PCR kit; Catalogue # 4518363, USA) for viral-induced liver tissue samples. For qRT-PCR, temperature was set at 50°C for reverse transcription of RNA into complementary DNA (cDNA) for 15-30 minutes. Then the temperature was set for initial activation of hot start enzyme at 95°C for 10-15 minutes. The temperatures for denaturation, annealing and elongation steps of amplification of cDNA were set at 95° for 30 seconds, 50° for 60 seconds and 72° for 30 seconds. The fluorescence detection range was also adjusted according to the fluorescence intensities in the PCR tubes. Temperature was set at 50°C to match the annealing temperature of amplification. Dyes used 'FAM/Sybr' (dyes) and 'ROX' (Passive dye) used in qPCR. After adjustments, all the values were calibrated, and PCR was run. Viral titre values >34IU/ml were detectable, and those <34 IU/ml were undetectable.

For IHC, a microtome (HS2205, Leedo company) was used to cut 4um thin sections that were fixed on poly L-lysine coated slides. IHC[9] was performed on tissue sections in duplicates. The sections were dried overnight at room temperature. After dewaxing and rehydration, the sections were pretreated in citrate buffer with potential of hydrogen (pH) 6.0, using microwave at 130°C for 30 minutes, followed by blocking of endogenous peroxidase. Nonspecific protein binding was blocked through incubation with 10% normal bovine serum for 20 minutes at room temperature. To block the nonspecific binding of antibodies, the samples were treated with hydrogen peroxide. The sections were incubated with primary monoclonal antibodies (anti-NS5B

of HCV; Catalogue # MA1-7343, USA), anti-HBX of HBC; Catalogue # MA1-081, Invitrogen, USA). All the primary antibodies were diluted to 1% BSA/PBS (Bovine Serum Albumin/Phosphate buffer Saline) and applied to sample which was left to incubate at room temperature. Secondary polyclonal horseradish peroxidase (HRP)-labelled antibody (Catalogue # A16160, Invitrogen, USA) was diluted with 1% BSA/PBS (Bovine serum albumin/ phosphate buffer saline) and was applied to the samples. The samples were stored in a covered dark tray, undisturbed for 1 hour at room temperature. To visualise the antigens (stained proteins), microscopy was used. HRP Label, 3, 3′-diaminobenzidine (DAB) substrate were used (Abcam DAB Staining Kit, Catalogue # ab64238, Shanghai, China) which imparted an intense brown colour to HRP-labelled proteins. Non-viral HCC tissues were used as negative experiment control. Internal negative control tissues were incubated with 1X PBS instead of primary antibody. The scoring was done on 0-3 scale, staining intensity for NS5B and HBX protein. Though IHC is semi-quantitative technique, it provides valuable information concerning the localisation and distribution of HCV proteins in histological sections of liver tissues.[9]

Each slide was assessed independently in a light microscope using Image J software. Positive staining was defined as brown colour stain observed in the hepatocytes, which was measured by intensity stain under 100X microscopy. NS5B and HBX protein expression was scored using qualitative IHC grade 0-3; 1=low, 2=moderate; and 3=high.[10]

Data was analysed using R version 3.3.5. Data was presented as frequencies and percentages. The groups were compared using Fisher's exact probability method. For IHC data, one-way analysis of variance (ANOVA) and Spearman Rank Order Correlation was used on Graph Pad Prism version 9.0, SPSS (version 27.0.1). P<0.05 was considered significant.

Results

Of the total 1,384 patients admitted, HCC was noted in 256(18.5%) cases, with age and gender being significantly different between positive and negative patients (p<0.05) (Tables 2-3, Figure 2). There was a preponderance of males aged >40 years in HCC cases (Figures 3). The trend of HCC, HBV and HCV prevalence across the study period was noted (Figures 4-5). Of the 70 patient samples available, 40(57%) were discarded because of missing data about HCC aetiology. Of the remaining 30(43%) samples, 18(60%) were HCV-positive, 5(16.6%) were HBV-positive, and 6(20%) had non-viral HCC (Figure 6).

Histological and IHC staining of NS5B protein in HCV-associated HCC liver tissues showed expression in brown colour and were counterstained with haematoxylin, while HCV-associated HCC liver tissue section with 1X PBS

Table-2: The distribution of top three liver-associated diseases by gender.

Gender characteristics	Total (n)	Top three liver associated malignancies			
		HCC n (%)	Obstructive jaundice n (%)	Cholangiocarcinoma n (%)	Others (other than liver disease
All gender groups	1384	256* (18.01)	68 (4.91)	53 (3.83)	1007
Males	868	176 (20.27)	40 (4.61)	31 (3.57)	621
Females	516	59 (11.43)	20 (3.88)	14 (2.71)	423

HCC: hepatocellular carcinoma; * Complete data was unavailable for 21 patients.

Table-3: HCC cases by gender and age.

Variables	Liver-related Complications [n (%)]		Total n (%)	OR (95% CI)	*p-value
	HCC	Non-HCC			
Gender**					
Male	176(20.27)	692 (79.72)	868 (100)	1.89	0.00
Female	59 (11.43)	457 (88.57)	516 (100)	(1.3974-2.5779)	
Age (years)					
≤ 40	18 (3.79)	457 (96.21)	475 (100)	8.58	0.00
>40	229 (25.19)	680 (74.80)	909 (100)	(5.238-14.064)	

*p<0.05; ** Complete data was not available for 21 patients; HCC: Hepatocellular carcinoma, OR: Odds ratio, CI: Confidence interval.

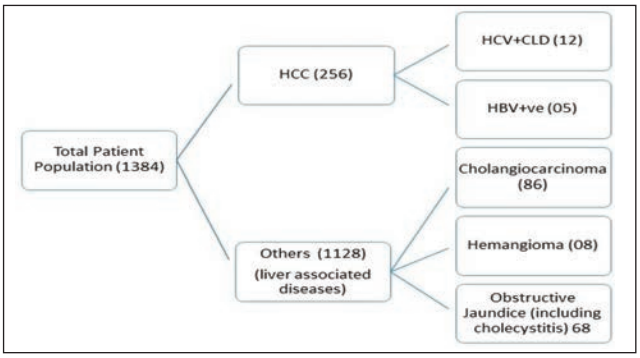

Figure-2: Flow diagram showing the stratification of total patient population (Epidemiological Data).
HBV: Hepatitis B virus, HCV: Hepatitis C virus. HCC: Hepatocellular carcinoma.

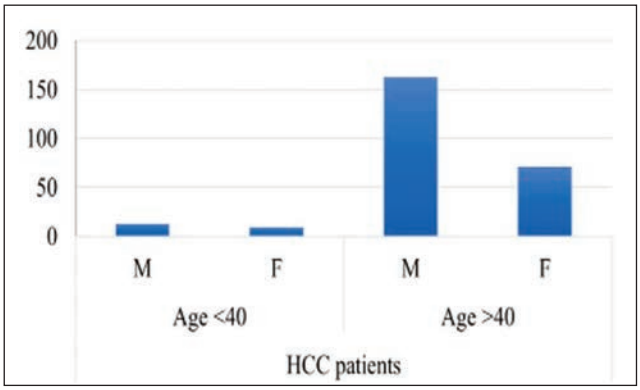

Figure-3: Preponderance of males aged >40 years in HCC cases.

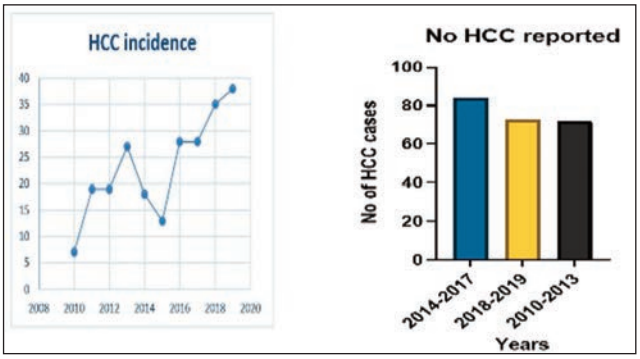

Figure-4: (a) Year-wise distribution of HCC cases; and the (b) increasing trend of HCC cases.
HCC: Hepatocellular carcinoma.

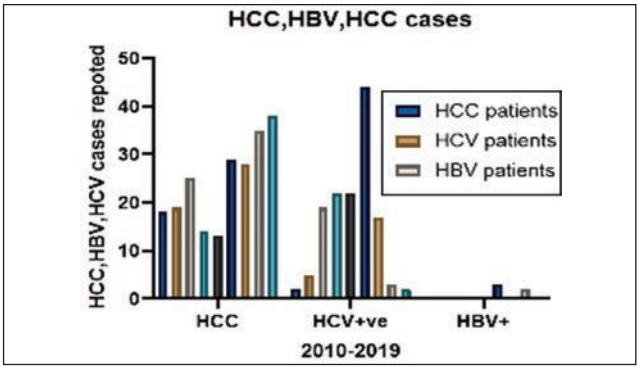

Figure-5: Distribution of HCC, HCV and HBV cases.

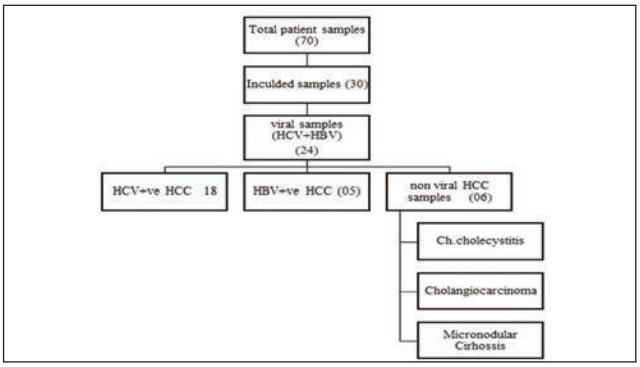

Figure-6 Out of 70 collected samples that were HCC-positive, 40 were excluded because of lack of data about HCC aetiology. The remaining 30 were further processed for the IHC. Out of them, 6 were positive for HCC. Those with non-viral aetiology were used as the control group.
HBV: Hepatitis B virus, HCV: Hepatitis C virus. HCC: Hepatocellular carcinoma.

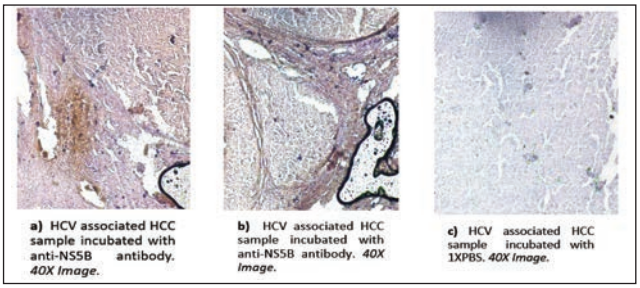

Figure-7: IHC staining of HCV-NS5B protein in HCV-associated HCC liver tissues.
IHC: Immunohistochemistry, HCV: Hepatitis C virus. HCC: Hepatocellular carcinoma, NS5B: Nonstructural protein 5B.

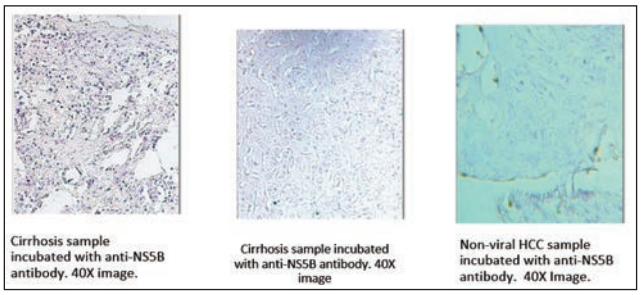

Figure-8: IHC staining of HCV-NS5B protein in cirrhotic and non-viral HCC liver tissues.
IHC: Immunohistochemistry, HCV: Hepatitis C virus, NS5b: Nonstructural protein 5B, HCC: Hepatocellular carcinoma.

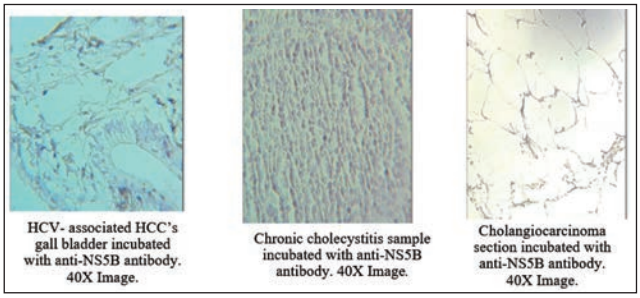

Figure-9: IHC staining of HCV-NS5B protein in cirrhotic, chronic cholecystitis and cholangiocarcinoma samples.
IHC: Immunohistochemistry, HCV: Hepatitis C virus. NS5B: Nonstructural protein 5B.

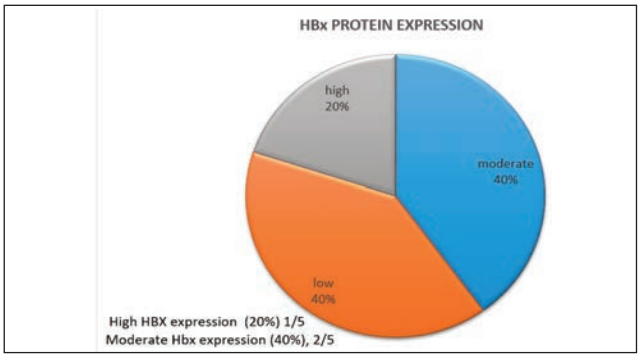

Figure-10: Total samples of HBV-HCC cases.
HBV: Hepatitis B virus, HCC: Hepatocellular carcinoma.

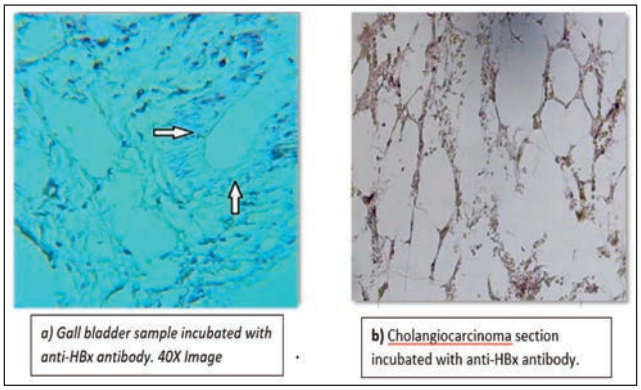

Figure-11: (a) Immunohistochemical (IHC) staining of hepatitis B Virus X (HBX) protein in gall bladder sample and liver tissue of cholangiocarcinoma and (b) gall bladder samples stained with anti-HBX antibody. The liver tissue of cholangiocarcinoma incubated with anti-HBX antibody showed no HBX expression in the form of brown colour and was only stained with purple-coloured haematoxylin stain.

showed no brown staining and worked as internal control (Figure 7).

The haematoxylin-counterstained cirrhotic liver tissue sections stained with anti-NS5B antibody did not exhibit

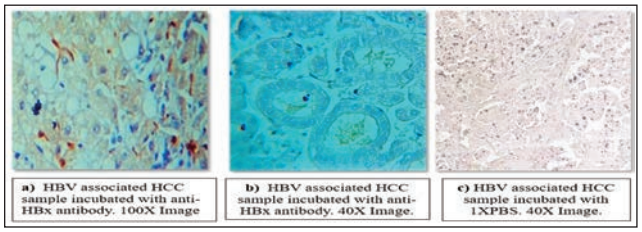

Figure-12: IHC staining of HBV-HBX protein in HBV-associated HCC liver tissues.

IHC: Immunohistochemistry, HBV: Hepatitis B virus, HBX: Hepatitis B X protein, HCC: Hepatocellular carcinoma.

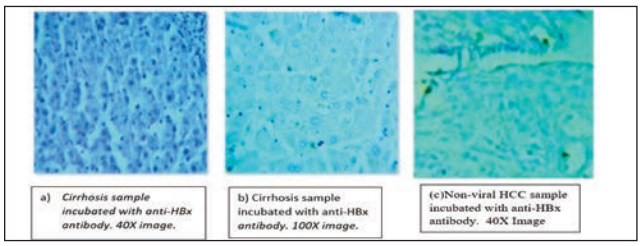

Figure-13: IHC staining of HBV-HBX protein in non-viral HCC and cirrhotic liver tissues.

IHC: Immunohistochemistry, HBV: Hepatitis B virus, HBX: Hepatitis B X protein.

a) HBV associated HCC sample incubated with anti-HBx antibody. 100X Image.

b) HBV associated HCC sample incubated with anti-HBx antibody. 40X Image.

c) HBV associated HCC sample incubated with 1XPBS. 40X Image.

Table-4: Demographic profile of viral-induced HCC patients including viral load (titer IU/ml).

Serial No.	Age (year)	Gender	Viral load (IU/ml)	IHC Score: Null (0)	Low (01)	Moderate (02)	High (03)
HCV Associated Samples							
1.	66	M	Undetectable	-	01	-	-
2.	70	M	Undetectable	-	01	-	-
3.	49	M	4.8×10^4	-	-	-	03
4.	66	M	Undetectable	-	01	-	-
5.	55	M	Undetectable	-	01	-	-
6.	52	M	1.3×10^3	-	01	-	-
7.	60	M	Undetectable	-	01	-	-
8.	55	M	Undetectable	-	01	-	-
9.	70	F	1.4×10^3	-	01	-	-
10.	63	M	Undetectable	-	01	-	-
11.	50	F	2.1×10^4	-	-	02	-
12.	72	M	Undetectable	-	01	-	-
13.	65	M	Undetectable	-	01	-	-
14.	63	M	1.7×10^3	-	01	-	-
15.	42	M	1.5×10^3	-	01	-	-
16.	55	M	5.1×10^4	-	-	-	03
HBV Associated Samples							
17.	45	M	Undetectable	-	01	-	-
18.	44	M	Undetectable	-	-	02	-
19.	65	M	2.8×10^4	-	-	-	03
20.	42	F	Undetectable	-	01	-	-
21.	56	M	Undetectable	-	-	02	-

HBV: Hepatitis B virus, HCV: Hepatitis C virus, HCC: Hepatocellular carcinoma, IHC: Immunohistochemistry.

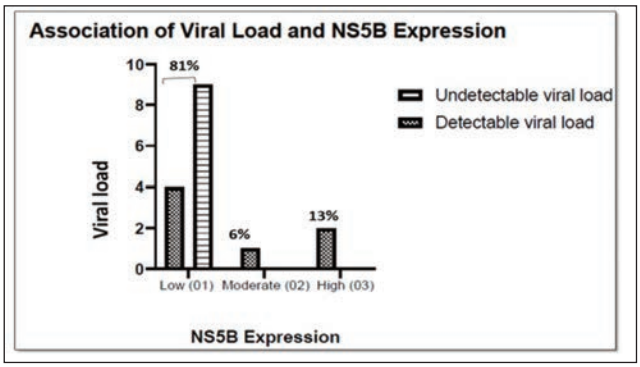

Figure-14: Association of viral load and HCV-NS5B expression.

IHC: Immunohistochemistry, HCV: Hepatitis C virus, NS5B: Nonstructural protein 5B.

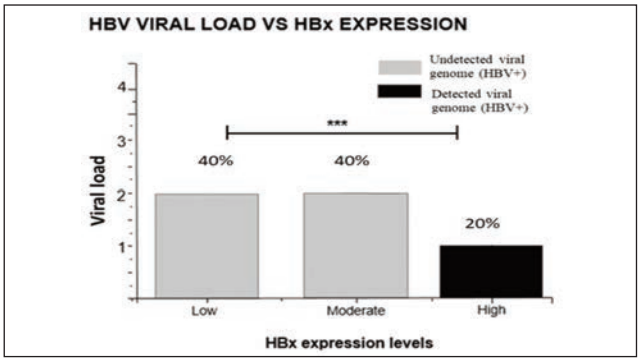

Figure-15: In hepatocellular carcinoma (HCC) patients with hepatitis B virus (HBV), 40% had low score 1), 40% had moderate score 2, and 20% had high score 3 with respect to hepatitis B Virus X protein (HBX) expression ($p<0.005$).

NS5B expression, and anti-NS5B antibody-incubated non-viral HCC liver tissue sections did not exhibit NS5B expression and were taken as negative control (Figure 8).

The sections of gall bladder tissue incubated with anti-NS5B antibody did not express NS5B, and the same was the case with chronic cholecystitis and cholangiocarcinoma tissues incubated with anti-NS5B antibody (Figure 9).

Among the HBV-associated HCC liver tissue samples, HBX expression was low in 2/5 (40%), moderate in 2/5(40%) in high in 1/5(20%) (Figure 10). Cirrhotic as well as non-viral HCC tissues were stained, and the control samples, cholangiocarcinoma and gall bladder tissues showed no HBX expression (Figure 11).

The HBX protein on HCC liver tissues stained with anti-HBx antibody was visualised under a microscope, and the brown region showed HBX expression in the tissue, while the purple region in the vicinity of the hepatocytes showed no expression (Figure 12).

Cirrhotic liver tissue sections incubated with anti-HBX antibody showed no HBX expression in the

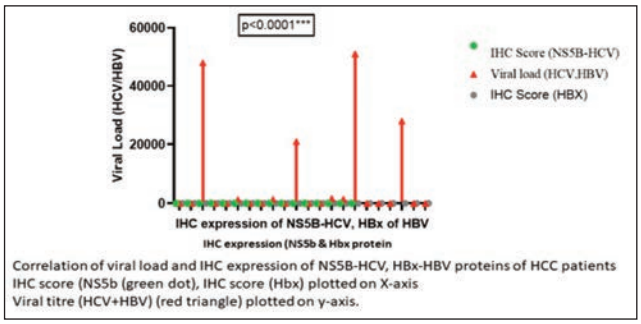

Figure-16: Correlation of viral load with HCV-NS5B and HBV-HBx.
HCV: Hepatitis C virus, HBV: Hepatitis B virus, HBX: Hepatitis B X protein, NS5b: Nonstructural protein 5B.

form of brown colour, and was only stained with purple-coloured haematoxylin, and the same was the case with non-viral HCC liver tissue section (Figure 13).

HCV-associated HCC and chronic hepatitis were significantly correlated with viral loads (Table 4, Figure 14).

A significant correlation between the HBV viral load and HBX expression in the hepatocytes of HCC tissues was found (r=0.972, p<0.01) (Figure 15).

IHC expression of NS5B-HCV and HBX-HBV proteins was significantly associated with viral load (p<0.0001) (Figure 16).

Discussion

HCC stands as the predominant form of liver-originating cancers, accounting for over 75% of cases[11] globally.[6] Ranking 6th among the most prevalent cancers worldwide, HCC imposes a substantial burden on public health systems.[2] Notably, it ranks among the top four cancer types contributing to mortality rates.[6] Many factors contribute to poor treatment outcomes of HCV-infected Pakistani patients receiving treatments. Pakistan faces disturbing limitations in cancer care that have an adverse impact on patient outcomes.[12,13] A steady increase in the incidence of hepatobiliary cancers has been observed. Based on the results of a reliable hospital-based registry in Pakistan, hepatobiliary cancers are the most common malignancy in adult males and represent 10.7% of all cancers.[12,14] Globally, majority of the patients, approximately 25% to 70%, are usually diagnosed at late stages of HCC, after which treatments are quite less effective. Chemotherapeutic treatment with drugs, like sorafenib, does not increase the survival duration in patients to a greater extent, showing a median survival of 2-3 months only. Late diagnosis of patients leaves no definite treatment options, and only symptomatic relief in the form of palliative care is provided in most cases.[12] If the individuals are diagnosed at early infectious stages, HCV-associated HCC treatments' costs are unbearable for people in Pakistan. In alignment with these epidemiological trends, the current study revealed that HCC accounted for 24/30 (80%) of all liver disease cases examined (first objective: IHC analysis). However, upon investigating the underlying reasons, various factors contributed to HCC development, and the key parameter found was viral persistence.

HCV is a significant public health concern, responsible for an estimated 3-4 million new infections annually, with an existing burden of approximately 200 million infected individuals worldwide.[2] In Pakistan, HCV-associated infections are alarmingly prevalent, affecting around 6% of the population, equating to nearly 10 million people, and making it one of the countries most burdened globally. HBV infections also pose a substantial health challenge in Pakistan, affecting approximately 3% of the population, or up to 4.55 million individuals.[15] However, the incidence rate of HBV infections is relatively lower than that of HCV, largely attributed to a national-level immunisation programme targetting HBV. Despite these efforts, HBV infections persist in various regions of Pakistan, driven by socioeconomic factors and inadequate hygiene practices.

HCV has multiple genotypes and the most common HCV genotype in Pakistan is type 3a.[16] The same is the case with HBV and its genotypes. The most common genotypes in Asia are B and C, but a study[17] showed that genotype D was more common, and that HBV infection was a serious health concern in Pakistan.

The current findings also indicate that HCV is the predominant aetiological agent among hepatitis viruses, accounting for 59% of all associated cases in the sample cohort compared to 19% attributed to HBV and 22% non-viral cases. This observation aligns with existing literature, which underscores HCV as a leading cause of HCC in the Asian region, contributing to approximately 60% of the cases.

Moreover, the current study revealed a notable gender disparity in HCC incidence, with males being predominantly affected. Specifically, among HCV-HCC and HBV-HCC patients, 14/16 (87%) were males, while only 2/16 (12%) were females. This gender discrepancy is consistent with global trends demonstrating a higher incidence of HCC among males compared to females.[18]

The current study validated previous findings suggesting that in Pakistan, HCC among males is primarily attributed to HCV infection[19] underscoring the significant role of HCV in hepatocarcinogenesis within this population.

The current findings revealed a notable age-dependent incidence pattern of HCC, with a high prevalence observed among individuals aged >40 years. Specifically, among HCV-HCC and HBV-HCC patients in the current cohort, 75% (15/20) patients were aged >40 years, while 25% (5/20) were aged <40 years old. This age distribution aligns with

existing literature[20] indicating that the prevalent age for HCC in Asia is typically >40 years. Similarly, studies in Pakistan have shown that individuals aged 50-70 years are predominantly affected by HCV, encompassing both males and females.[21]

Notably, the observed age distribution in the current study is comparatively lower than in countries where the prevalent age for HCC is 60 years and beyond.[20] Furthermore, the current study reflected a trend consistent with previous studies indicating that HBV-associated HCC patients tend to be younger compared to those with other aetiological factors contributing to HCC.[20] Specifically, the current findings demonstrated that 40% of HBV samples were aged >40 years, while 60% of HBV-associated HCC samples were aged <40 years old.

Detection of HCV-RNA and HBV-DNA in blood, known as the 'viral load,' has been crucial indicator of viral persistence and treatment efficacy.[15] Post-treatment monitoring of individuals undergoing therapy for HBV and HCV typically involves the quantification of HBV-DNA and HCV-RNA in blood or serum to assess the viral load. Undetectable levels of HCV-RNA and HBV-DNA in blood are conventionally considered indicative of viral clearance and restored health.[15] However, it is important to note that in HCV-treated individuals, the absence of detectable HCV-RNA in blood does not necessarily indicate the eradication of the virus, as persistent HCV infection in hepatocytes may still exist, with viral particles maintaining a quiescent state despite their absence in blood or serum,[22] Consequently, relying solely on blood-based quantification methods may yield false-negative results due to potential interference from sources outside the liver. To mitigate this limitation, quantification of viral load should ideally be performed directly in liver tissues or in conjunction with blood-based assessments to ensure greater accuracy and reliability of results.[22] The current study used IHC of liver tissues for semi-quantification of NS5B and HBX protein.

Viral load quantification revealed detectable viral loads in the liver tissues of the HCV-associated HCC, chronic hepatitis tissues, and, notably, these levels were higher than those observed in detectable HCV-HCC tissues. This observation aligns with previous research, indicating elevated HCV-RNA levels in the stages of HCV-related liver diseases preceding HCC.[23] Similarly, HBV-associated HCC tissues, akin to HCV-associated samples, exhibited a high percentage 4/5 (80%) of undetectable HBV-DNA compared to a minority 1/5 (20%) with detectable HBV-DNA. Numerous studies have verified that an advancement in the histological grade of HCC is linked to a decrease in HBV-DNA loads.[24,25] Consistently, the current study demonstrated a similar pattern, wherein an increase in the histological grade of HBV-HCC correlated with undetectable viral loads. IHC was employed for the semi-quantitative analysis of two key proteins, NS5B of HCV and HBX of HBV, in the liver tissues of the collected samples. NS5B, an RdRp enzyme, holds pivotal significance in HCV replication within the host. The unavailability of an effective HCV vaccine is largely attributed to the formation of HCV quasispecies by the NS5B protein owing to its error-prone replication mechanism. However, specific treatments targeting NS5B, such as antiviral ribavirin (RBV) and direct-acting antivirals (DAAs), like sofosbuvir, have been developed.

On the other hand, HBX is a multifunctional protein integral to the occurrence and perpetuation of HBV infection both in vitro and in vivo. Its mechanisms include transcriptional activity, regulation of cccDNA, degradation of host factors with antiviral properties, and inhibition of effectors of innate immunity.[26] Additionally, the HBX protein plays a crucial role in various cellular processes, such as cell cycle progression, signal transduction, protein degradation, and apoptotic pathways. Dysregulation in these normal pathways mediated by HBX significantly impacts the prognosis of HCC.[27]

The key parameters responsible for developing HCC in the current study was viral persistence. The degree of HBX and NS5B expression was taken into account when manually grading liver tissue sections for the purpose of analysing protein expression. The current findings are noteworthy because they show that HBx and NS5B proteins were consistently expressed in liver tissues, even in cases where HBX-DNA and HCV-RNA were not detectable. The correlation between viral loads and protein expression levels in the current study aligned with previous studies, indicating a strong correlation between viral load and IHC results in HCC liver tissues.[28] Specifically, a significant correlation was observed between HBV viral load and HBX protein expression ($p<0.001$).

Nevertheless, IHC is a semi-quantitative technique and old, the liver tissue samples can be further evaluated with Western Blot and SDS-PAGE analysis. Increasing sample size for both study objectives would enable generalize the actual prevalence of HCC in Asian region especially Pakistan.

Conclusion

Pathogenicity, progression of liver diseases, response to treatment, and viral replication are different among HBV and HCV mutants. Understanding the complex relationship between viral genome variations and host signalling pathway alteration is likely to improve understanding of the molecular pathogenesis of virus-induced HCC. From epidemiological result, the most frequently reported liver-associated disease was HCC. There is a dire need to

implement proper surveillance for identifying HCC cases at an early stage. However, it is also inferred that virus still persists in the liver tissues of HCC patients. HCV's NS5B and HBV's HBX protein expression was also found evident through immunostained liver tissue irrespective of the virus status. Better antiviral therapeutic options need to be explored, targetting the virus persistence.

Acknowledgment: We are grateful to Dr Hassam Ahmed and Dr Shahla Jaleel for facilitating the study.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: The National University of Science and Technology (NUST), Islamabad, Pakistan.

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Author Contribution:

SR: Concept, methodology, software, visualization and investigation.
MK & ER: Data curation and writing-original draft preparation.
FA: Co-supervision and final approval.
SM: Supervision, review and final approval.