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Detection of hepatitis through proteases and protease inhibitors genes expression and identification of HCV untypable genotype in Abbottabad, Pakistan

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Summary. – Liver cancer is the 5th most common cancer caused mainly due to the late detection of hepatitis. Therefore, the early detection of hepatitis through genetic markers boosts effective and remedial management. In addition, to determine the occurrence of hepatitis C virus (HCV), genotyping is indispensable as majority of hepatitis cases remain undiagnosed. The current study was designed to find the gene expression of proteases and proteases inhibitors in different hepatitis patients and to determine HCV genotypes mainly focusing on untypeable genotypes of HCV in Abbottabad, Pakistan. PCR was conducted to find the expression of proteases and protease inhibitors genes in hepatitis patients and healthy individuals. HCV genotyping was done by PCR based method and untypeable genotypes were sequenced and verified using online tools. Controlled individuals showed normal expression of cystatin C and leptin, low expression of cathepsin B while high expression of other studied genes including cathepsin D and G, TPP1 and serpin B1 could be seen. Hepatitis A patients showed high expression of leptin while other genes showed low expression. Hepatitis B patients revealed considerable variations in the cathepsin and cystatin C gene expression. Therefore, low cystatin C (high cathepsin B) and/or high cystatin C (low cathepsin B) levels can be regarded as a potential marker for hepatitis B. Hepatitis C infected patients showed high gene expression of cystatin C and leptin, so they could be useful markers for the diagnostics and prediction of the severity of HCV infections. While genotyping findings showed that about 45% of total PCR positive samples (110) were found to be of 3a genotype followed by 3b in 18%, 1a in 13.6% and 1b in 10%. About 9% of infections turned out to be mixed infections, whereas only 4.5% were untraceable by our genotyping system. Sequencing of untypeable genotypes and applying online tools revealed that the described untypeable genotypes of HCV were in fact variants of 3a genotype. Furthermore, full length characterization of these variants could help to classify them into types and subtypes.

Keywords: hepatitis; genotyping; genes expression; proteases and protease inhibitors; ML; NJ

Introduction

Viral hepatitis refers to inflammation of liver after specific hepatitis virus infection followed by direct persistent inflammation, cirrhosis and hepatic carcinoma (El-Saeed *et al*., 2017; Ringehan *et al*., 2017). Globally, liver cancer is the fifth most common cancer with average rate of 7.9% of all the cancers (Ashraf and Ahmad, 2015; Wong *et al*., 2019). Regulating inflammation is the foremost task of the liver by secreting special proteins which control both systemic and local inflammatory responses (Zampino *et al*., 2013). In acute infection, danger signals detection generates inflammatory response, while in chronic infection it is either induced by extreme alcohol use, metabolic

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Abbreviations: cDNA = complementary deoxyribonucleic acid; HCV = hepatitis C virus; RT-PCR = reverse transcriptase polymerase chain reaction; RNA = ribonucleic acid

factors or viral hepatitis. The chronic liver disease leads to hepatocellular carcinoma and liver cancer (Szabo *et al.*, 2007). In Pakistan, hepatitis A is common among children (50–60%) while prevalence of hepatitis B is 4% and that of hepatitis C is around 5% (Bosan *et al.*, 2010; Umer and Iqbal, 2016). Hepatitis C infection is a foremost reason of fibrosis, cirrhosis, hepatocellular carcinoma and end stage liver disease (ESD) (Ali *et al.*, 2011). About 85% of acute infections turn out to chronic infection, within which 20% leads to cirrhosis which ultimately develops hepatocellular carcinoma and ESD (Kanwal and Mahmood, 2014). In Pakistan, HCV infection occurrence varies amongst the provinces, specifically 1.1% frequency was reported in KPK, prevalence rate in Baluchistan was 1.5% following Sindh and Punjab with 5% and 6.7%, respectively (Umer and Bilal, 2012).

Genotypes are a group of species which have nucleotide dissimilarity of 31–34%, while in each genotype, there are closely related subtypes which have distinction of 20–23%. Whereas individual viruses comprised of composite genetic variants, known as quasi species with nucleotide dissimilarity of 1–9% (Walewski *et al.*, 2002). Overall, there are six foremost genotypes of HCV with more than hundred subtypes (Safi *et al.*, 2010). Development of novel variants arise frequently with time due to deficiency of modification refinement capability of RNA polymerase (Lindenbach *et al.*, 2005). PCR based genotyping involving specific primers often meets with failure due to sequence mutations. The precise recognition of HCV genotypes has taxonomic applications along with solid links with implementation of appropriate treatment procedures i.e., epidemiological studies, therapy duration, therapy outcome and response rate (Liew, 2004; Rauch *et al.*, 2010).

Previous findings highlighted the significance of definite host genetic markers in influencing the treatment outcome of HCV infection. They also provide a framework for clinically appropriate pharmaco-genomic approach for initialing hepatitis treatment (McHutchison, 2011).

Cathepsins are a collection of catalytically non-conserved protease enzymes with numerous members ubiquitously present in human body. Cathepsins A and G are serine proteases, cathepsins D and E are aspartate proteases, and the rest are lysosomal cysteine proteases including human isoforms B, C, F, H, K, L, O, S, V, X and W2 (Siklos *et al.*, 2015). Literature has revealed that apart from their identified role in protein turnover, cathepsins can also control numerous processes like cell growth, antigen presentation, neo-vascularization of endothelial progenitor cells, tissue homeostasis, liver damage and fibrogenesis. Consistent with these functions, cathepsin D (CtsD) has been involved in cancer development including invasion and metastasis. In experimental fibrogenesis, CtsD gene has been observed to be activated with high levels of CtsD in patients with cirrhosis and hepatocellular carcinoma (Moles *et al.*, 2009).

Cystatins are endogenous, competitive and reversible inhibitors of lysosomal cysteine proteinases, produced by all nucleated cells. The serum cystatin C concentrations significantly increased with liver disease progression making it a potential genetic marker for hepatic-fibrosis (Finney *et al.*, 2000; Magister and Kos, 2013). Serpin B1 is a cytoplasmic serine protease inhibitor which distinctively inhibits the activity of cathepsin G by means of suicide inhibition (Remold-O'Donnell *et al.*, 1992; Gong *et al.*, 2011). Previous studies have identified the involvement of serpin B1 in the regulation of hepatocellular carcinoma metastasis (Cui *et al.*, 2014). Leptin, the peptide product of the obesity gene, has been implicated in hepatic fibrogenesis but its role has not been well illuminated (Treeprasertsuk and Huntrup, 2006; Myers *et al.*, 2007). According to literature, in patients with chronic HCV related steatosis, leptin levels were concomitant with impaired hepatic function which accelerates fibrosis progression. Meaning, that serum leptin might be a predictive indicator of chronic HCV infection with steatosis (Ellidokuz *et al.*, 2003). Tripeptidyl peptidase 1 is associated with sedolis in family of serine proteases that is activated and auto-proteolyzed upon acidification. Researchers identified that hepatocellular carcinomas, prostate cancers as well as endometrial and renal cancers displayed moderate to strong granular cytoplasmic positivity while the remaining cancers were weakly stained or negative.

Current study was designed to characterize and compare the above-mentioned genes in hepatitis patients and controlled individuals in Pakistani population for the first time. The results might be helpful in disease diagnostics and eradication of HCV infection. Moreover, in Khyber Pakhtunkhwa (KP), HCV infection is dominant including its untypable genotype (Attaullah *et al.*, 2001; Ali *et al.*, 2011). Frequency of untypable genotype in study population encouraged us to analyze these genotypes employing PCR based genotyping.

Materials and Methods

Population samples. The study comprised of 100 samples from hepatitis patients collected from different hospitals and health care units of Abbottabad, KP. In addition, there were 50 healthy individuals who served as a control for the levels of the seven candidate genes. A total of 150 Immunochromatography technique (ICT) and ELISA positive samples for HCV genotyping were collected from several medical centres of KP. Serum was isolated and kept at -20°C for further studies.

RNA isolation and gene expression study. About 2 ml of venous blood was taken from each infected and control subject

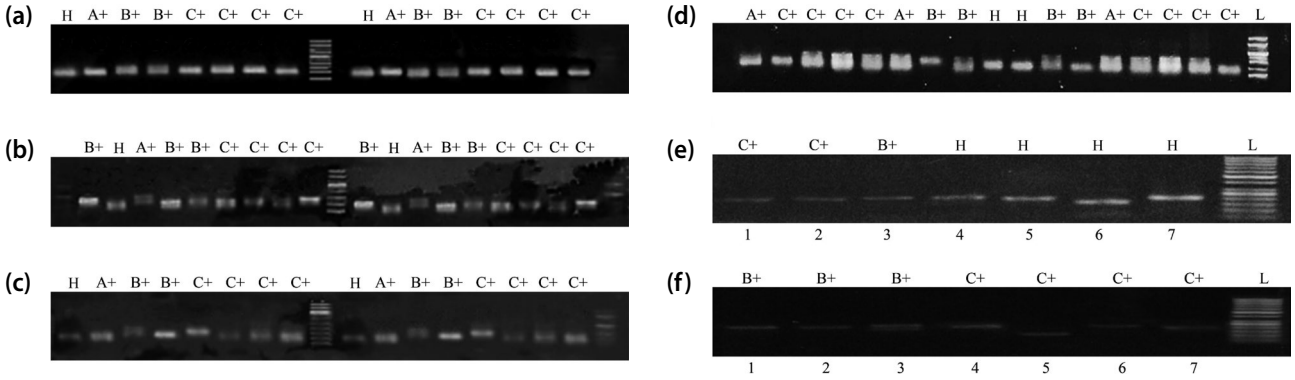

Fig. 1

Proteases and proteases inhibitors gene expression

Beta actin **(a)**, cystatin C **(b)**, cathepsin B **(c)**, leptin gene **(d)**, cathepsin D (lane 4), cathepsin G (lane 7), TPP1 gene (lane 6) and serpin B1 (lanes 1-3,5) **(e),** cathepsin D (lanes 3, 4), cathepsin G (lanes 1, 6), TPP1 gene (lane 5) and serpin B1 (lanes 2, 7) **(f)** genes expression through PCR in healthy (H) individuals and hepatitis patients (A+, B+, C+) visualized on 1% agarose gel electrophoresis.

in EDTA to study the gene expression. Initially, ICT and ELISA positive samples were processed further by RNA extraction using Trizol reagent (Invitrogen, USA) followed by cDNA synthesis using TOPscript™ cDNA synthesis kit (Enzynomics, South Korea). Polymerase chain reaction was carried out to analyse the expression of proteases and protease inhibitors genes i.e., cathepsin B, cathepsin D, cathepsin G, cystatin C, Serpin B1, Tpp1 and leptin by type specific primers (Hass *et al.*, 2016). Beta actin was used as control gene in all the procedures (Lin and Redies, 2012; Lévy *et al.*, 2017).

Quantification of genes. The quantification of the genes was done using *GelAnalyzer-19.1* software (www.gelanalyzer.com) in which the band intensity is defined by three attributes: start index, end index, and peak index.

HCV RNA detection and qualitative PCR. Sera samples were investigated for HCV RNA presence. Briefly, total RNA was isolated from 150 µl of sera samples according to the protocols provided by RNA extraction kit (Gentra, Puregene, USA). Qualitative detection of HCV RNA was carried out by PCR based assay previously mentioned by Idrees using primers that target HCV 5'NCR region (Idrees, 2001).

Genotyping. HCV genotyping was performed according to the PCR based genotyping method of Ohno *et al.* (1997) and Gul *et al.* (2016). Briefly, RT-PCR was done to reverse transcribe RNA to cDNA (Spitz *et al.*, 2019). Two rounds of polymerase chain reaction were conducted. In first round, synthesized cDNA was used to amplify 470 bp targeting 5' NCR and core region. In next round, type-specific primers were employed to detect genotypes 1, 2 and 3 with their subtypes 'a' and 'b' respectively. The amplified PCR product was visualized on a 2% agarose gel and 50 bp DNA ladder (Invitrogen, Corp, USA) according to the protocol described by Jamil *et al.* (2020).

qRT-PCR. The untypeable genotypes were quantified in real time RT-PCR employing sequence specific primers and probes of ATRUS HCV RT-PCR kit (Qiagen, Netherland). The diagnostic sensitivity of above-mentioned kit was approximately 80 copies/ml.

Type specific PCR and sequencing. HCV untypeable genotype was verified by amplifying 216 bp region of *Core* gene using specific primers (forward primer 5' ATGTGCACACTTCCTAAACCC 3' and reverse primer 5'-CGATATCGCTTCGACGCGCCT-3'). PCR products were purified by ethanol precipitation and sent for sequencing by Big Dye method (Applied Biosystem, USA).

Phylogenetic analysis. Phylogenetic analysis was carried out using *Core* gene sequences of HCV untypeable genotype. "Oxford subtyping tool" (http://www.bioafrica.net/rega.../html/subtypinghcv.html) and "COMET" online genotyping program (http://comet.retrovirology.lu/hcv/) were employed for confirmation of genotypes. The subject nucleotide sequences were translated using HCV and HIV online databanks. (http://www.hiv.lanl.gov/content/sequence/TRANSLATE/translate.html). BLASTn and BLASTp were carried out to find identical sequences already submitted at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Then phylogenetic analysis was performed to discover the evolutionary relationship of query genes with already existing genes. Evolutionary tree was constructed with 1000 bootstrap replicates using Maximum Likelihood (ML) algorithms and Neighbor Joining (NJ) algorithms in Mega 6 software (Tamura *et al.*, 2013).

Results

Gene expression and quantification

Expression analysis of proteases and protease inhibitors genes in healthy and hepatitis infected individuals

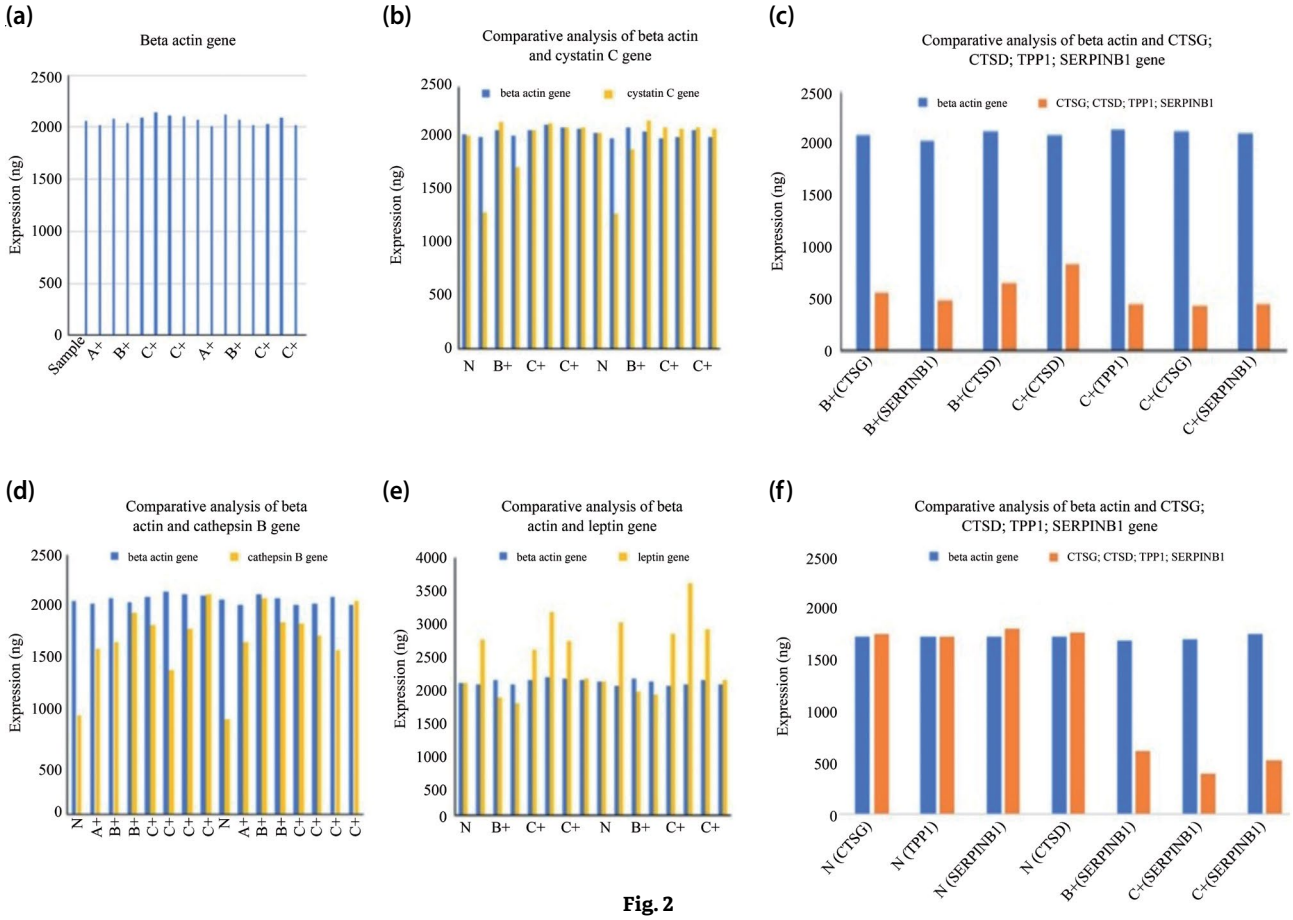

Fig. 2

Proteases and proteases inhibitors gene expression quantification

Beta actin **(a),** cystatin C **(b),** cathepsin B **(c)**, leptin gene **(d)**, cathepsin D, cathepsin G, TPP1 gene and serpin B1 **(e, f)** genes quantification by Gel analyzer and their comparison with beta actin.

was carried out to discover their role in early detection and eradication of disease.

In this study, beta actin was used as a reference gene. We observed same bands intensity in both control and hepatitis patients which was confirmed by Gel analyzer (Fig. 1a, 2a). Substantial difference in cystatin C gene expression was discovered between healthy subjects and patients with hepatitis. In comparison with healthy individuals where gene expression was normal, hepatitis A patients showed lower gene expression. While in hepatitis B the cases varied significantly. Overall cystatin C expression was moderate high with some exceptions with high or low expression. In case of hepatitis C patients, the gene expression was moderate high to high as compared to healthy subjects. Band intensities of cystatin C were compared with beta actin bands (Fig. 1b, 2b).

In contrast to cystatin C, cathepsin B gene showed inverse expression in some cases. In healthy subjects, cathepsin B gene expression was low. Hepatitis A patients showed low to normal gene expression while in hepatitis B the expression varied significantly with moderate low to moderate high expression in infected individuals. In case of hepatitis C patients, the gene expression was similar to healthy subjects (Fig. 1c, 2c).

In comparison with healthy individuals where leptin gene expression was normal, hepatitis A patients showed high gene expression. In hepatitis B patients, it was observed to be moderate low while hepatitis C patients showed high gene expression (Fig. 1d, 2d).

The expression of cathepsin G and TPP1 genes showed same patterns. Their expression was moderate to high in healthy individuals while in hepatitis B and C patients low expression was observed. Serpin B1 gene expression was observed to be low in hepatitis patients while high in healthy individuals. In case of hepatitis C, the level was very low and in some cases negligble while in case of hepatitis B the expression level was low due to its suicide mode of action (Fig. 1e, 1f, 2e, 2f).

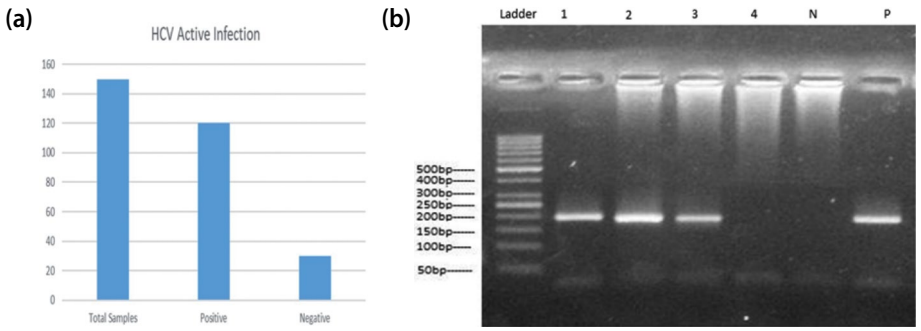

Fig. 3

Hepatitis C infection
Frequency of active hepatitis C infection **(a)** and PCR amplification of HCV 170 bp product **(b)**.

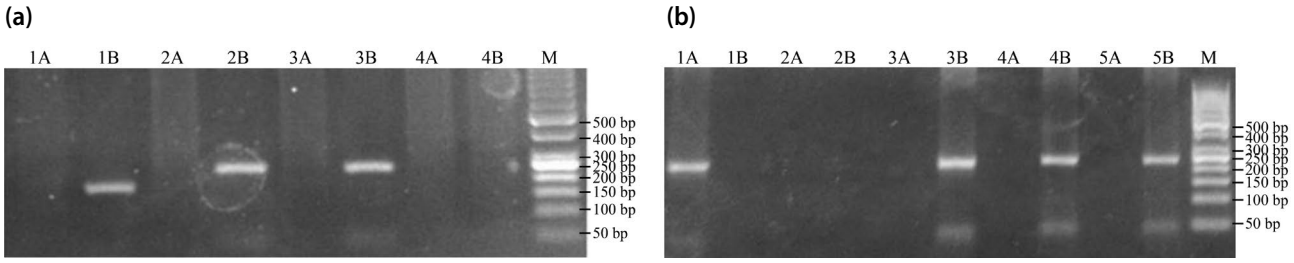

Fig. 4

Genotyping of HCV
Gel pictures of genotyping, **(a)** Lane M showing DNA ladder of 50 bp; sample 1 having 2a genotype (190 bp), samples 2 and 3 infected with 3a genotype (230 bp) and sample 4 with untypeable genotype. **(b)** Lane M showing DNA ladder of 50 bp; sample 1 having 1b genotype (200 bp), samples 3, 4 and 5 infected with 3a genotype (230 bp) and sample 2 with untypeable genotype.

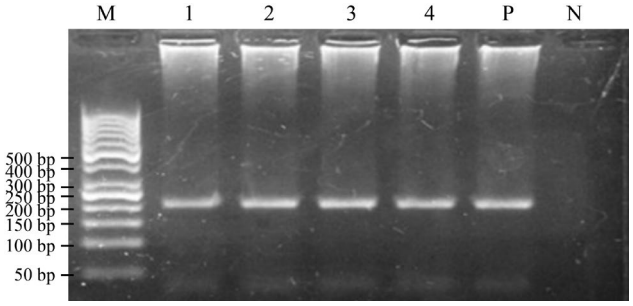

Fig. 5

PCR amplification of *Core* gene (220 bp)
Lane M showing DNA ladder of 50 bp; Lane 1-3 representing HCV positive samples with 220 bp product of *Core* gene. PC showing positive control while NC representing negative control.

HCV genotyping and phylogenetic analysis

Out of 150 ELISA positive samples, 120 (80%) samples were actively infected while 30 (20%) samples were detected as negative. Samples from actively infected subjects were visualized on agarose gel (Fig. 3a, b). Out of 120 PCR positive samples, 110 (91%) were found to show greater viral load and were analyzed in further study. After qualitative and quantitative analysis of HCV infected samples, genotyping by type specific PCR was carried out. Products amplified by PCR with type specific primers showed different bands, however untypeable genotypes could not be detected by these primers. Meaning that some new genotypes may be present in Pakistan or there might be some variation in primer binding sites of isolates that make them undetectable by designed specific primers.

Table 1. Viral load of untypeable genotype of HCV by qRT-PCR

Patient ID	Viral load
A	36615 copies/ml
B	1710 copies/ml
C	64315 copies/ml
D	52821 copies/ml
E	111253 copies/ml

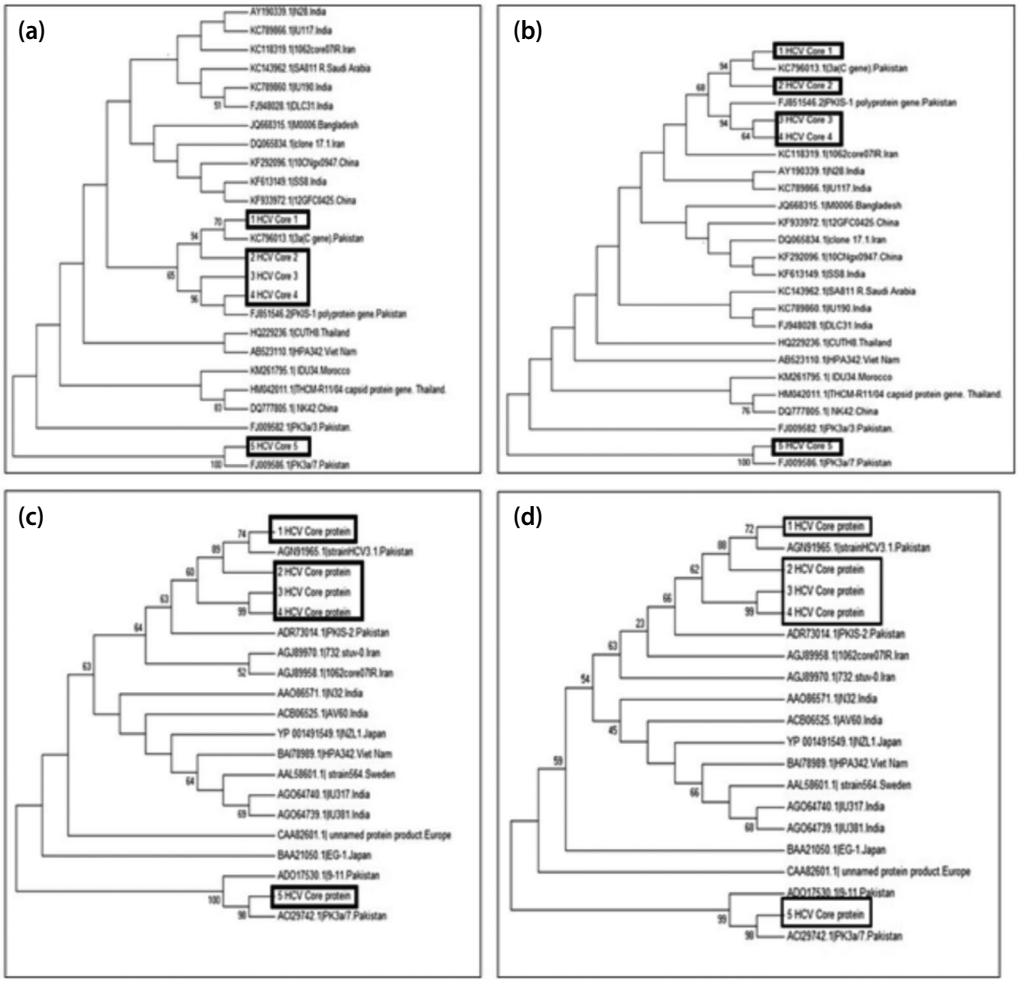

Fig. 6

Evolutionary relationship of *Core* genes

Evolutionary relationship of *Core* genes using the Maximum Likelihood method based on the Tamura-Nei model **(a)** (Tamura *et al.*, 1993); using the NJ method **(b)** (Saitou *et al.*, 1992). Phylogenetic analysis of deduced amino acid sequences using the ML method that was based on the JTT matrix-based model **(c)** (Jones *et al.*, 1992); using the Neighbor-Joining method **(d)**. Evolutionary analyses were carried out in MEGA 6 software.

About 45% (50) of total PCR positive samples were found to be 3a genotype followed by 3b in 18% (20), 1a in 13.6% (15) and 1b in 10% (10). Nine percent (10) of infections turned out to be mixed infections. Whereas only 4.5% (5) were untraceable by our genotyping system (Fig. 4a,b). These untypeable genotypes showed high copies/ml of viral load on qRT-PCR, confirming HCV infection (Table 1).

Core genes of HCV untypable genotypes were amplified (Fig. 5) and sequenced. Online programs were used to assign genotypes to these sequences from individual isolates which showed that these genotypes were actually variants of 3a genotype, not detected by current genotyping system. After BLAST analysis, phylogenetic analysis employing ML method verified the occurrence of our subject gene sequences in three diverse assemblies. Our tested sequences grouped together with genes with accession no. KC796013, FJ851546 and FJ009586 from Pakistan with strong bootstrap values of 94, 96 and 100 correspondingly validating the association. Our isolates exhibited homology with isolates from Iran, India, and China.

Nucleotides based tree constructed by NJ method validated the results of ML method by showing relationship of our tested sequences with same Pakistani isolates with strong bootstrap values of 94 and 100. By measuring of character and distance, it was shown that query isolates were like that of isolates from Pakistan (Fig. 6 a,b). For verification, we have performed phylogenetic analysis based

Table 2. Gene expression of beta-actin, cystatin C, cathepsin B, cathepsin D, cathepsin G, leptin TPP1 and serpin B1 in control and hepatitis patients

Genes	Expression in healthy and hepatitis patients			
	Control	**Hepatitis A+**	**Hepatitis B+**	**Hepatitis C+**
beta-actin	normal	normal	normal	normal
cystatin C-1	normal	low	low in some patients moderate high in some patients	moderate high to high
cathepsin B	low	low to normal	moderate high in some patients moderate low in other patients	close to normal
cathepsin D	moderate to high	NA	low	low
cathepsin G	moderate to high	NA	low	low
leptin	normal	high	moderate low	high
TPP1	moderate to high	NA	low	low
serpin B1	high	low	low	low

on deduced amino acids of query *Core* genes. Evolutionary tree showed the connection of the deduced amino acids of *Core* gene with those of reference sequences retrieved from NCBI. Evolutionary analysis of deduced amino acids with NCBI retrieved sequences using Maximum Likelihood method showed the relationship of query sequences with Pakistani isolates having strong bootstrap values of 74, 99 and 98 (KC796013, FJ851546 and FJ009586). NJ method confirmed their correspondence with Pakistani isolates of 3a genotype as shown in trees based on nucleotides and amino acids (Fig. 6 c,d).

Discussion

Liver diseases have been a significant health concern since severe hepatic diseases could lead to persistent inflammation, fibrosis, cirrhosis and even liver cancer (Wang *et al.*, 2014). In hepatitis A, B and C patients of Abbottabad, we analyzed cystatin C, cathepsin B, cathepsin D, cathepsin G, Tpp1, serpin B1 and leptin gene expression. We also analyzed the correlation of proteinases with protease inhibitors. Earlier study discovered that cystatin C, which is a specific inhibitor for proteolytic enzymes, was enhanced directly with the hepatic disease severity. Mean serum concentrations of cystatin C in patients with liver diseases has been observed to be higher as compared to the healthy controls (Yap *et al.*, 2017). This suggests that cystatin C can be a relevant potential monitoring marker for progression of liver fibrosis and liver function (Ahmed *et al.*, 2006). It has been suggested that cystatin C plays a regulatory and defensive role against exogenous cysteine proteinases present in body fluids (Vincents *et al.*, 2008). The over expression of cystatin C may have the role in inhibition of human cysteine proteases that are over expressed in inflammatory conditions and if uncontrolled can cause severe tissue degeneration leading to liver fibrosis. In addition, these cysteine proteases are involved in the disease-related tissue remodeling by cleavage of membrane and extracellular matrix proteins to make infected cells suitable for viral propagation (Korolenko *et al.*, 2008). Furthermore, cystatin C secretion is linked directly to the TGF-α, thus emphasizing a potentially crucial role of cysteine proteases in progression of liver fibrosis.

Likewise, a previous study showed that the serum cystatin C levels gradually increased along the initial and intermediary stages of liver fibrosis in chronic HCV infection, which demonstrate that serum cystatin C level could even predict the degree of liver fibrosis and inflammation in animal chronic liver disease models (Ladero *et al.*, 2012). Thus, cystatin C can be an efficient monitoring marker for the diagnosis and prediction of disease severity in HCV infections; same was revealed by the work of Takeuchi *et al.* (2001). Overexpression of cystatin C may have the inhibitory effect on viral cysteine proteases (NS2/3) as suggested by Behairy *et al.* in a study conducted in 2012, revealing that serum cystatin C levels correlated negatively with the viral load and did not fluctuate with the severity of infection. This would be of greatest importance assuming a potential role of cystatin C as an inhibitor of viral replication, because its antiviral functions have already been reported in different studies done with virus-infected cell (Behairy *et al.*, 2012).

In comparison with healthy individuals, the hepatitis A patients showed low cystatin C gene expression. However, relative gene expression of cathepsin B in this case was low to normal. This means that cathepsin B might perform normal/controlled proteolysis in the liver and was not affected by cystatin C. This might be due to that HAV infection typically does not cause cytopathic effect,

cellular RNA degradation, or protein synthesis shut-off, and also inhibits apoptosis induced by dsRNA (Shi*et al.*, 2016). Therefore, neither cystatin C nor cathepsin B can be used as a potential marker for hepatitis A.

Hepatitis B patients showed considerable variations in the cystatin C gene expression. We found moderate high cystatin C gene expression in some patients while in others we found moderate low cystatin C gene expression or no expression at all. In first case of moderate high gene expression, the serum concentration of cystatin C protein was also moderate high, and it effectively inhibited the cathepsin B gene expression representing the chronic stage. In comparison with healthy group, level of cystatin C was found to be substantially increased continuously with the liver disease progression (El-Saeid *et al.*, 2012). In second case of moderate low cystatin gene expression with relatively moderate high gene expression of cathepsin B, it showed that the acute stage progressed towards chronic stage. So, low cystatin C (high cathepsin B) and high cystatin C (low cathepsin B), both can be used as a potential marker for hepatitis B. The third case of cystatin gene expression absence, together with considerably high cathepsin B expression does not give any information about the stage of infection.

In comparison with healthy individuals, we found moderate high cystatin C gene expression in hepatitis C patients. Cathepsin B has been inhibited by cystatin C with close to normal expression in hepatitis C patients. There was no significant difference in cystatin C concentrations between hepatitis B (1st case) and hepatitis C virus infected patients so, it also clearly defined the chronic stage of hepatitis C.

By comparing liver cirrhosis and HCC, HBV and HCV infected patients, we found higher expression of cystatin in liver cirrhosis and HCC than in HBV and HCV infected patients (Takeuchi *et al.*, 2001; Chen *et al.*, 2005). In chronic hepatitis patients and liver cirrhosis or fibrosis patients' serum leptin concentrations were observed to be higher, but results were very conflicting. However, substantial correlation has been observed between leptin level and histopathological inflammation grades (Piche *et al.*, 2004).

Upon analyzing the gene expression, we found some interesting relationship between leptin expression and the acute stage of hepatitis. Hepatitis A patients showed high leptin gene expression. This better defines the acute stage at peak and can be used as potential marker for hepatitis A. The same gene expression has been observed in hepatitis C patients. So, leptin can be used as a potential marker for both hepatitis A and C (acute). In case of hepatitis B, the gene expression was moderate low.

The difference between production and degradation of extracellular matrix has been renowned to be one foremost source of liver fibrosis, cirrhosis and hepatoma (Zhang *et al.*, 2016). At first, this inequity results typically due to the inflammation of the liver or viral infection caused by hepatitis A, B or C virus. By gene expression, acute and chronic phases of the hepatitis infection particularly the acute hepatitis A infection might be detected. We reported altered results in different hepatitis patients for cathepsin D, cathepsin G, serpin B1 and TPP1 gene expression. In contrast with healthy persons, the hepatitis B patients showed low level of cathepsin D, cathepsin G, serpin B1 and TPP1 gene expression.

HCV genotyping is important for treatment of HCV because dosage, medication type and therapy duration depend on virus type (Zein, 2000). There is a number of assays that detect genotypes, like PCR-RFLPs, type-specific PCR and serologic genotyping, (Ohno *et al.*, 1997; Idrees *et al.*, 2009). Literature revealed that PCR based genotyping system is used extensively in majority of investigations because of its economical nature. It was claimed that these methods can identify numerous genotypes proficiently, but they all have plusses and several restrictions as well. However, it was assumed that these assays were supposed to be inappropriate for typing of HCV isolates due to the emergence of mixed infections and rising reports of untypeable genotypes (Pol *et al.*, 2013).

HCV has RNA genome with modifications frequency of 10^{12} virions/day due to deficient capability of RNA polymerase proof reading (Lauring *et al.*, 2010). Thus, as a consequence of these mutations, new HCV variants might originate eventually. Type specific primers based genotyping system to detect HCV isolates is unproductive. In present study, a percentage of untypable genotype (4.5%) was described and corresponded with formerly identified records (Attaullah *et al.*, 2001; Ali *et al.*, 2011). Presence of untypeable isolates in Pakistan discovered the existence of certain novel or alternative genotypes. Hepatitis C infection rate is continuously increasing and has become an economic burden due to poor health care facilities. It has been well documented that disease progression and therapy response vary according to the type of virus so it's utmost to have knowledge about HCV type. In Pakistan, usually genotyping is not done earlier to recommend medication and therapeutic measures are solely based on detection of virus. Current investigation showed that HCV genotype 3a has highest frequency followed by 2a and untypeable genotypes. It has been previously investigated that 2a and 3a genotypes of HCV are most susceptible to interferon-ribavirin therapy (Mejer, *et al.*, 2018).

There are some rare cases when HCV types are not detected by PCR based genotyping systems. We attempted to find out the frequency of these untypable genotypes in Pakistani population and to create awareness about the importance of genotyping in combination with qualitative detection of infection. In Pakistan, untypable

genotypes have previously been studied. In our study, PCR based genotyping system could not determine considerable number of HCV genotypes in chronic HCV patients. By using bioinformatics tools, amino acid sequences were obtained from query nucleotides. BLAST algorithms were employed to search for identical sequences which confirmed high similarity scores of query *Core* region of HCV 3a genotype with reference sequences. Phylogenetic analysis of collected sequences was carried out using NJ and ML algorithms (Lole *et al.*, 2003; Idrees *et al.*, 2009). Evolutionary analysis discovered reported genotypes as variants of HCV 3a genotype which grouped with Pakistani isolates. Even though they were closely linked and progressed together but again were distinct.

Maximum Likelihood algorithm is character based and guesstimates the evolutionary relatedness of sequences based on their informative sites, while Neighbor Joining method describes the likeness as measures of distance employing numerical values. It is suggested to construct more than one tree for reliable results of each dataset and core of both trees must be same. In recent study, both the methods (ML and NJ) verified the relationship of reported sequences with those of reference sequences from NCBI by displaying almost matching outcomes.

As nationwide information indicated, present study suggested that HCV genotype 3a was the foremost genotype. Occurrence of other genotypes 1a and 2a was in line with already existing studies (Attaullah *et al.*, 2001; Ali *et al.*, 2011). Prevalence of HCV genotype 3a is a good hope for therapeutic measures because it necessitates brief treatment duration, less side effects and cost than others (Qazi *et al.*, 2006). The incidence of 3a genotype in Pakistan and the bordering countries like India, Iran, Bangladesh and China provided evidence about the transmission routes of HCV variants (Asif *et al.*, 2009; Hussain *et al.*, 2013; De Sabato *et al.*, 2018).

This study suggests that appropriate diagnostics and therapeutic approaches should be carried out in order to minimize the rate of infection. Genotyping must be carried out along with quantitative or qualitative PCR in case of untypeable genotype infection. Sequencing of the untypeable genotypes is required to fully understand it, not only for academic explanations but also for future therapeutic regimes. Every system must be updated after duration of 6–10 years.

Conclusion

It was concluded that proteases and protease inhibitors can be implied as diagnostic markers for disease progression and some of them could be used for disease control in future. Low cystatin C (high cathepsin B) and/or high cystatin C (low cathepsin B) levels can be regarded as potential markers for hepatitis B. While in hepatitis C, high cystatin C expression inhibits cathepsin B activity which better defines the chronic stage and can be used as a potential marker. Cystatin C can be a relevant potential monitoring marker for progression of liver function while leptin can be used as potential marker in case of acute stages of hepatitis A and C only. Moreover, genotyping is imperative to decide therapeutic measures against HCV. In case of untypable genotype infection, therapeutic strategies should not be solely based on type detection, quantitative or qualitative detection of virus must be carried out before genotyping, as current genotyping system does not detect virus completely. In addition, query genotypes might be the new HCV types, or it might be the limitation of our system, so full length characterization of untypeable genotypes is required. Furthermore, using bioinformatics tools, more therapeutic genes in different species can be identified and used for infection suppression.

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