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Research Article

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Identification of Ankyrin Repeat, RNASE Domain of RNASE L Gene Mutation in Hepatocellular Carcinoma and its Association with HCV Viral Load

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Abstract

Background: *Hepatocellular Carcinoma (HCC) is the most common primary malignant tumour of the liver. This is multiple risk factors are associated with its pathogenesis. Chronic liver infection via Hepatitis is by far most common cause of HCC worldwide. It is a viral infection by Hepatitis C virus (HCV), Hepatitis B virus (HBV) and Hepatitis D virus (HD). Pathogenesis of HCC is a combination of multiple genetic factors. Certain gene mutations are directly involved in the development of carcinoma; one that stands out most is RNASE-L. We assume that mutation of this gene also co-relates with viral load of hepatitis which in turn increases the severity of HCC.*

Methodology: *This study was conducted in Dow University of Health Sciences, department of Molecular Pathology DDRRL and National Institute of Liver & GI Diseases (NILGID), we investigated 80 whole blood samples, from which we extracted DNA and then PCR performed. The amplified PCR products were sent to Macrogen, Korea for analysis and sequencing of RNASE-L gene mutation followed by sequence analysis via BioEdit and MEGA 7 software. After which HCV viral load was correlated with those sequences.*

Result: *The categories of our sample were; 1) HCV positive patients, 2) HCV positive patients with HCC and 3) HCC diagnosed patients. Out of the 80 blood samples, 5% are of HCC, 50% are of HCC along with HCV, and the remaining blood samples are of HCV positive individuals. Out of which 57.5% are males and 42.5% are females. Sequence analysis revealed, only one sample to have SNV G>an in RNASE domain, and none of the samples have SNV in ARD.*

Conclusion: *In this study we tried analyse the genetic aspect of HCC, so that it can help us therapeutically. Our health care system is in dire need of some immediate reforms that are necessary to control the prevalence of HCC.*

Keywords: RNASEL, HCC, HCV, ARD, RNASE

Introduction

Hepatocellular carcinoma (HCC) is the 5th most common malignancy and the second most leading cause of cancer related deaths worldwide [1, 2]. Each year the death ratio is about 662,000 people across the globe. According to the WHO about 564,000 cases of HCC are identified each year. In Pakistan prevalence of HCC is about 3.7% to 16% of all malignant tumours [3, 4]. There are multiple risk factors that are involved in the pathogenesis of HCC via liver cirrhosis [2, 4-7]. However, 80% of cases of HCC are due to chronic liver infections, caused by HCV, HBV and HDV [2]. After the primary viral infection the immune response is via cellular immunity specifically through type 1 interferon, which is a cytokine produced by T-cells and activated by NK cells. Interferon up-regulates 2-5' OLIGOADENYLATE SYNTHETASE, which then induces apoptosis in the infected cells [8-12].

RNASE-L/ 2-5`OAS pathway is the first antiviral response that was discovered in the 1970s [11]. In the presence of a viral infection, interferon induces the transcription and modification of Oligoadenylate Synthetase (OAS) by binding with the cell surface receptors. Double stranded RNA (dsRNA) of viruses interact with OAS which leads to the production of 2-5`OAS. This dimerization eventually causes the activation of RNASE-L [13].

RNASE-L is an endo-ribonuclease protein which is usually present in an inactive state and is activated during viral infections [14]. RNASE-L blocks the activity of different types of viruses through a variety of mechanisms; depending upon the extent of ribonuclease activity and the specificity of RNA substrates [15, 16]. RNASE L consists of three Domains: N –terminal Ankyrin (ANK) repeat domain, C (catalytic) terminal Ribonuclease domain (RNASE), and proteins kinase (PK) homology domain [17]. ANK repeats 2 and 4 of RNASE-L are important as they interact with the nucleic acid and 2-5OAS. This binding of 2-5A within the Ankyrin-region activates RNASE-L which causes degradation and cleavage of viral nucleic acid leading to cell death [18]. RNASE and PK domains

also have kinase and endoribonuclease activity and are involved in the unfolded protein response [13, 19, 20].

It has recently been discovered that RNASE-L along with antiviral properties also allows suppression of tumours due to its pro-apoptotic and anti-proliferative activities. It seems to have a significant impact on the pathogenesis and metastasis of prostate cancer; as mutations in the RNASE-L gene are associated with reduced enzyme activity, which increases the risk of development of prostate cancer and also leads to an increase in the expression of inflammatory biomarkers [21-23]. It has also been reported that a mutation in the RNASE L gene is associated with a higher viral load of XMRV virus in prostate cancer in this study was find out of the four missense varients found, R462Q showed an association with HPC. According to Madsen ET. Al an increased risk of head and neck, cervical and breast cancer is seen with RNASE-L germline mutation. In this study to find out that the genotype frequencies of only one of the 15 mutations, uncharacterized 5′UTR mutation rs3738579 differed significantly between cancer patients, the viral infection is implicated in close to 100% of all uterine cervix cancer cases, we hypothesized that if a defective RNase L is responsible for the lack of ability to clear a viral infection [24, 25].

In accordance to these discoveries; we believe that mutations in the RNASE-L gene are associated with an increase in the viral load of HCV, which in turn increases the incidence of HCC. Therefore, in this study we investigated the mutations in RNASE-L ANK domain (2, 5A) and RNASE domain in patients with/without HCC and correlated it with the viral load of HCV. We also investigated RNASE-L mutations in individuals suffering from HCC, but were negative for HCV infection.

Material and Methods

The study was conducted at Dow University of Health Sciences, Molecular Pathology Department DDRRL and National Institute of Liver & GI Diseases (NILGID). The blood samples were collected from 3 groups of patients. HCV positive patients, patients with HCV and associated HCC and patients diagnosed with HCC alone. Informed consent was taken, and the details regarding the research were given to all the patients included in the study. The research was conducted after receiving approval from IRB (Institutional Review Board) and BASR (Board of Advance Studies &Research). For this research 80 samples were selected through subjective sampling from Dow University of Health Sciences Karachi, Pakistan. Blood Samples for patients with HCC were obtained from the National Institute of Liver and Gastrointestinal Disease (NILGID), Dow Hospital Karachi and the HCV samples were obtained from Dow Diagnostic Reference and Research Laboratory (DDRRL) DUHS, Ojha Campus. Out of the 80 samples, 4 were of individuals diagnosed with HCC, 40 patients had HCC along with HCV and 36 samples were of patients infected with HCV only. The blood samples were collected in the EDTA tube, they were then centrifuged and the buffy coat was removed for the extraction of DNA.

• HCV RNA Extractions from Plasma Specimens:

The RNAviral extraction kit, QIAamp Viral RNA Mini Kit (Germany, QIAGEN) was follow-up according to the company's instructions given for the extraction of RNA from the samples. Purified RNA was eluted from the spin column QIA amp in solution form, concentrated in AE buffer and was stored at -20°C. We used 15µl of the extracted RNA for HCV viral load detection by using the commercially available kit artus® RT-PCR (Qiagen, Germany) HCV RG Kit, according to the manufacturer's instructions.

• RNA Quantification:

The purified sample of RNA (1µl) after diluting with deionised 100µl water was measured using spectrophotometer at wavelengths of 260nm and 280nm in cuvette quartz.

• Complimentary DNA Synthesis:

M-MuLV RT enzyme was used to make the c-DNA. The following measures were taken to make the c-DNA: The reaction mix was prepared by adding 1µl random hexamer, 10µl of purified RNA, 1µl of dNTP mix (10mm each) and 2.5µl of nuclease water in the PCR tube. The tube was then placed at 65°C for 5 minutes then on an ice block for an extra few minutes. 5.5µl of RTmix (5X RT Buffer 4µl, Ribo Lock RNase Inhibitor 0.5µl (20U), Maxima Reverse Transcriptase 1µl (200U) was added to the PCR tube and incubated at 50°C for 30 minutes. Deactivation of RT enzyme was carried out by incubating it at 85°C for 5 minutes and the product was stored at -70°C.

• Real Time for HCV Viral load:

The quantitative Real Time PCR was done for the quantification of HCV viral load by using the commercially available Artus HCV RG RT-PCR Kit (Qiagen, Germany), according to the as per protocol.

• Genotyping of HCV:

HCV genotyping was performed by using a commercially available Abbott Real-time HCV Genotype II Kit (Abbott, USA) on an automated nucleic acid extractor and Real Time-PCR system, m2000 Sp (Abbott, USA), according to the protocol

• Mutation of ARD and RNASE Domain of RNASE L Gene by PCR:

Followed by the amplification of ARD and RNASE domain of RNASE L gene, PCR was performed in order to ascertain beta-globin gene amplification by using already published primers. PCR reaction volume (25 µl) was prepared by adding five microliters of eluted DNA sample, 1X PCR DreamTaq PCR Master Mix (Thermo Scientific, USA) (2X) was used as per protocol. The total reaction volume came down to 25µl.The thermal cycling for PCR reaction mixture and temperature profiles was performed under the following conditions: 35 cycles with three cycle loop in each initial temperature at 95 ◦C for 10 mint, 95 ◦C for 60 sec, 56 ◦C for 60 sec, 72 ◦C for 30sec, and 72 ◦C for 6 mint, the program lasted 1 mint at 60 ◦C.

• Agarose Gel Electrophoresis of PCR products:

All the amplified products of PCR reaction were analysed using gel-electrophoresis. A stock solution of 2-3µl was made using 0.2-0.5µg/mL ethidium bromide. This solution was then added to 100ml of agarose for the preparation of 2% agarose gel. 2% Agarose gel showing 302bp product obtained by PCR amplification of a region in ARD Domain of RNASE L gene, 02, 03, are amplified samples of ARD, while 4,5 are amplified products of RNASE domain of RNASE L gene, The PCR amplified product size was determined with 50bp plus ladder DNA. Agarose gel was kept running at 120 Volt for 30 minutes and the results were photographed after they were visualised under UV light.

• Ethical Consideration:

This research was presented in the Scientific Committee (Ref. No. DUHS/SC-O/2018/-) for approval. After acceptance from

Institutional Review Board (IRB) (IRB-658/DUHS/Approval/18/) on 07th APRIL 2018, and Board of Advanced Studies and Research (BASR), further research was continued. Information of all the participants involved has been kept confidential until it is required for publication and scientific research purposes.

- **Statistical Analysis:**

Complete information of all the patients was gathered which included their baseline demographics such as their names, age, and marital status, number of children and different gastroenterological complications. These factors were considered to analyse their association with RNASE-L gene HCC. Data analysis was completed by performing it on all varieties of variables, so we can rule out percentages, frequencies and their graphical representation. This statistical analysis was performed on SPSS 17.

Results

Table 1: Over all distribution of Samples

GENDER (n=80)	Total no (%)
Male	46 (57.5%)
Female	34 (42.5%)
Age(n=80)	
Group1(10-25yrs)	5 (6.25%)
Group2(26-41yrs)	23(28.7%)
Group3(42-57yrs)	23(28.7%)
Group4(58-73yrs)	29(36.25%)
HCV Genotype 3(n=76)	76(100%)
HCC	4(5%)
HCC WITH HCV	40(50%)

Demographic Profile: Age and gender

The distribution of age and gender of 80 patients of this study showed 5 (6.2%) were among Age group 10-25, 23 (28.7 %) were among age group 26-41, 23 (28.7%) were among age group 42-57 and 29 (36.4%) were among age group 58-73. While 46 (57.6%) were males and 34 (42.4%) were females.

HCV Viral Load Quantification

Quantification of HCV viral load in 36 HCV and 40 HCC with HCV positive samples showed that 4 (5.2%) have viral load among 180-22400IU/ml, 36 (47.3%) have viral load 22500-44720IU/ml, 30 (39.4%) have viral load 44820-66940IU/ml and 6 (7.8%) have viral load 66950-89,160IU/ml. While the median HCV viral load was 280,419IU/ml.

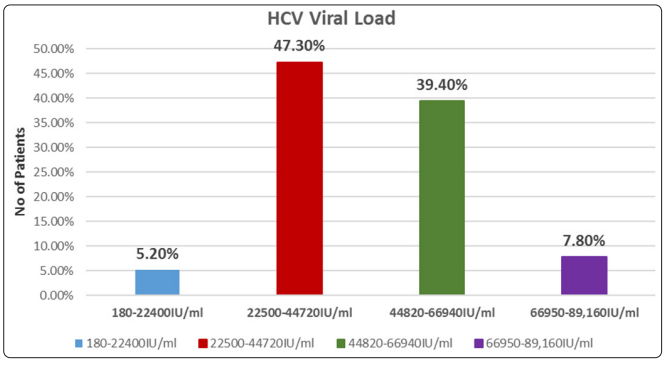

Figure: Represents the percentage of patients showing different ranges of the HCV viral load

HCV Genotype Analyses

Real Time PCR analyses by using a commercially available kit for HCV Genotyping revealed that all of the 76 HCV positive samples including the 40 HCC and 36 HCC with HCV positive samples have found to be infected with Genotype 3.

Amplification of ARD and RNASE Domain

For the mutation analysis, ARD and RNASE domain of the RNASE L Gene were amplified by PCR. The 59 successfully amplified products were sent for sequencing. The successful amplification of specific 302 bp band of ARD and 302 bp band of RNASE domain of RNASE L gene.

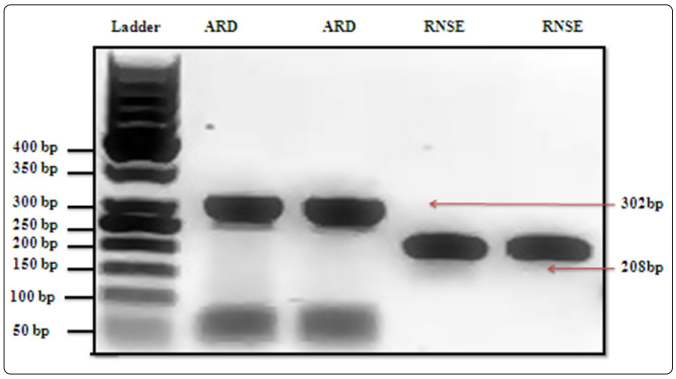

Figure: PCR-amplification of RNASE L gene

2% Agarose gel showing 302bp product obtained by PCR amplification of a region in ARD Domain of RNASE *L* gene, 02, 03, are amplified samples of ARD, while 4, 5 are amplified products of RNASE domain of RNASE L gene, and LD is 50 base pairs DNA ladder.

Mutation Analyses of ARD and RNASE domain of RNASE L gene

Out of the 59 samples that have been sequenced for the ARD & RNase domain of RNASEL gene, only one sample (1.69%) have SNV G>A in RNASE Domain which is found to be a synonymous mutation. However, none of the samples showed any SNV in ARD domain. The details of the SNV observed are as follows:

S.No	Sample ID	Genomic Mutation	A.A Change
1	60RNASE	NG_009024.2: g.15656**G>A** **(Heterozygous)**	None

Discussion

HCC is a multi-factorial disease; this is because it exhibits a complicated intracellular pathway in its pathophysiology by viral infectivity and genetic variants. Pakistan is located in Southern Asia, being a developing country it has a narrow resource index towards health and sanitariness. In Pakistan 193.2 million of the population is composed of the younger generation. This is a worrisome situation because number for high risk individuals for viral infection increase by the day. The scarcity of resources in dealing with these health hazards is a huge contributing factor for this predicament. Topographical location, socio-economic status and unawareness about health risks, all play a huge part in this phenomenon. Besides infectious diseases, cancers are also the major cause of mortality in

Pakistan, in which HCC is the second most common. Unfortunately in Pakistan cancers are not registered nationwide, which makes it difficult in dealing with them on a larger scale. Only a small number of provincial data is available via, cancer registry of Punjab, the cancer registry of Karachi and the Dow University of Health Science Hospital Cancer registry. Very few studies have been conducted regarding risk factors and progression of HCC, which concluded that Hepatitis C and Hepatitis B are the most significant risk factors in progression of HCC in Pakistan.

Our study is limited because we have small sample size of 80 patients and we are only targeting high-risk individuals who are on the verge of developing HCC i.e. purposive sampling. This is clearly not the representation of the population of Pakistan. But due to lack of resources and financial constraints we had to settle with these limitations.

For future prospects, it is essential to study the exact mutation in infected subjects. Models of the structures are to be additionally evaluated for drug targets which can be therapeutic in recovery of patients. Hepatocellular carcinoma is a disease of concern in Pakistan; most of the cases remain undiagnosed and ultimately lead to death. Global incidence of HCC is also increasing and many studies are showing different genomic mutations responsible for the disease. Mutation in RNASE-L gene like any other genetic mutations, affects our innate immune response to infection. Infection of HCV is most common cause of cirrhosis and HCC in Pakistan which is similar to other countries such as India, Japan, European countries and also in North America. Therefore Pakistani population is at higher risk for HCV associated cirrhosis and ultimately leading to HCC [26, 27]. Different types of gene mutations are also involved HCC pathogenesis. RNASE L was also proposed as a candidate gene for this region by Carpten et al. in 2002 [23]. Moreover, Urisman et al. reported the presence of XMRV in prostate cancer tissues and found an association between XMRV and the R462Q mutant of the RNASE L gene [28, 29]. To investigate whether XMRV is present in tissues obtained from prostate cancer and the RNASE L R462Q variants are associated with the risk of prostate cancer [29, 30].

HPV, Epstein Barr virus (EBV) and sequences from mouse mammary tumor virus (MMTV) have been detected in breast tumors and believed to both major risk factor associated RNASE L mutations for uterine cervix cancer and for a subset of head and neck squamous cell carcinomas (HNSCC). As defective RNASE L would be unable to clear out the viral infection that ultimately leads to carcinoma [25]. However in this study ARD domain which is crucial for RNASE L activation as well as RNASE domain which degrades the cellular RNA thus involved in viral clearance from the cell has been sequenced to determine the effect of mutations on HCV viral loads and HCC progression because RNASE L have antiviral activity if there is a mutation in RNASE L gene so viral load will be high. However, in this study we have only identifed one mutation g.15656G>An in RNASE domain of RNASE L in HCC patient who is also positive for HCV with 683, 208 IU/ml viral load. None of the SNVs have been observed in ARD domain. According to best of our knowledge this is a first study of its kind on HCC. Therefore, it is important to conduct more research on RNASE L mutation analysis in HCC and its association with HCV viral load. It is necessary for all Healthcare institutes to promote general public awareness regarding HCV infections and HCC. Pakistan is lacking national level cancer registry due to which it is difficult generate a population-based data and develop strategies to decrease mortality rate. Globally mortality ratio of Hepatocellular carcinoma is increasing day by day, mainly this is due to late diagnosis and hence the treatment. The Medical and Research communities of Pakistan should focus on prevention and treatment of HCC. This would be mainly via public awareness and educating general population about all the risk factors and their exposures. There is a dire need of programs that focus on screening of HCC prior to the development of the disease. Hopefully using such measures we can avoid this potential national crisis [31-35].

Conclusion

We have observed only single SNV (g.15656G>A) in RNase domain of RNASEL gene in one sample. None of the samples had any SNV in ARD domain. Majority of the patients were males and belonged to age group 58-73 years age. The mean age of the patients was 50.86 ±14.84 years. All the HCV infected individuals had HCV genotype 3 and had viral loads mean range 837404.21 ±1302318.

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