

Research article

**Mutational analysis of MDM2 gene in hepatocellular carcinoma**Hafiz Muzaffar Ali<sup>1</sup>, Shahzad Bhatti<sup>1</sup>, Muhammad Naeem Iqbal<sup>2,3\*</sup>, Shahzad Ali<sup>4</sup>, Aftab Ahmad<sup>5</sup>, Muhammad Irfan<sup>5</sup>, Ali Muhammad<sup>5</sup><sup>1</sup>Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore 54000, Pakistan<sup>2</sup>The School of Life Sciences, Fujian Agricultural and Forestry University, Fuzhou 350002, P R China<sup>3</sup>Department of Microbiology, <sup>4</sup>Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Lahore 54000, Pakistan<sup>4</sup>National Academy of Young Scientists (NAYS), University of the Punjab, Lahore 54000, Pakistan<sup>5</sup>Department of Zoology, PMAS Arid Agriculture University, Rawalpindi 46000, Pakistan**Abstract**

Hepatocellular carcinoma (HCC) represents the fifth most prevalent cancer worldwide, and accounts for the top three causes of death in the Asia-Pacific region. The risk factors associated with HCC include age, sex, alcohol, diet, and infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV). The incidence rate of HCC is plausibly linked to geographic area and geo-economic conditions and it is possible that gene polymorphism may be associated with the risk of HCC. In this research, the PCR technique was used for the diagnosis of point mutation in the MDM2 gene. The point mutation at MDM2-SNP309 from T to G was detected which may be responsible for HCC in patients with HCV. In this study, five cases showed the mutational changes in the allele T/G. The frequency of the mutated alleles was 10% of the mutations. So it is concluded that the mutation is responsible for HCC and the frequency of a mutated T/G allele is more liable to cause the disease.

**Keywords:** Hepatocellular carcinoma, cancer, polymorphism, mutation.

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**Introduction**

Murine double minute 2 (MDM2) is responsible for the ubiquitination and degradation of p53 [1] that functions as an E3 ubiquitin ligase. Ubiquitination is a complex series of events involving E1, E2 and E3 proteins [2]. The E1 enzyme binds and activates ubiquitin and then E2 conjugating enzyme accepts the activated ubiquitin from E1 and transfers it to the E3 enzyme, a ligase that covalently bonds the ubiquitin to the substrate. The MDM2 ubiquitinates p53 at several lysine residues [3]. The p53 and MDM2 proteins form an auto regulatory feedback loop in which p53 positively regulates MDM2 levels and MDM2 negatively regulates p53 levels and activity [4]. Furthermore, as an oncogene, MDM2 plays a regulatory role in its interaction with other tumor-related genes that are important for cell-cycle control. It also contributes to carcinogenesis independently of p53 through interaction with transcriptional factors of the E2F family [5], inhibition of the Rb growth regulatory function [6] and inhibition of G0/G1-S-phase transition in normal cells. The MDM2 can also ubiquitinate itself and induce its own degradation [7]. The human MDM2 gene located on chromosome 12q13-14 with genomic size of 34 kb comprises two promoters, constitutive promoter and p53-responsive intronic promoter [8].

It is considered that 170 million people at least are infected with hepatitis C virus (HCV) worldwide, which often leads to the dreadful sequels of cirrhosis

and hepatocellular carcinoma [9]. The risk of hepatocellular carcinoma development increases with the severity of inflammation and liver fibrosis [10]. Disease progression in HCV infections has known to accelerate due to the presence of several factors, such as alcohol intake, older age at time of infection, male gender, and co-infection with the hepatitis B virus or HIV [11]. Host genetic factors in addition to these factors had also been reported to affect the outcome of HCV infection [12]. In a large population of HCV patients it has been found out that three single nucleotide polymorphisms (SNP) in three genes (SCYB14, GFRA1, and CRHR2) were significantly associated with hepatocellular carcinoma development in Japanese patients with chronic HCV infection [13]. The association of both the germ-line and somatic inactivating mutations of the p53 gene with increased tumor development is well known [14]. Gene polymorphisms at critical nodes of the p53 pathways have also been reported to be associated with the development of cancers [15].

Hepatocellular carcinoma (HCC) is most prevalent fifth worldwide cancer and accounts for the top three causes of death in the Asia-Pacific region [16]. The risk factors associated with HCC include age, sex, alcohol, diet, and infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) [17]. MDM2 and p53 form central nodes in the p53 signal transduction network, many inputs and outputs that connect almost every function of the pathway [18].

The MDM2 protein level in a cell or organism seems to have a large effect upon the p53 response and cancer formation [19]. A failure to arrest division after DNA damage results in the propagation of many mutations and a cancer prone phenotype.

The objective of the present study was the determination of mutation T to G in hepatocellular carcinoma. This mutation in the MDM2 gene at 309 in intron 1 of P1 has been evaluated in different types of cancers including hepatocellular carcinoma. As mutated GG allele is more common in hepatocellular carcinoma than normal TT.

## Materials and Methods

### Sample collection

The blood samples of 14 patients were collected from Sheikh Zaid Hospital, Lahore, Pakistan. Blood samples were taken in EDTA containing vacutainer tubes and stored at -20°C until nucleic acid extraction.

### DNA extraction

Blood obtained was subjected to DNA extraction to get the genomic DNA using the phenol/chloroform method [20]. Blood samples (750 µl) were mixed with 2.5 ml Tris EDTA (lysis buffer) and centrifuged at 3000 rpm for 15 minutes. The supernatant was removed and the process of centrifugation was repeated by adding the same quantity of lysis buffer each time until the pellet of sediments became clear (pink in color). The supernatant was removed and 0.75 ml of TNE buffer (Tris-NaCl-EDTA buffer), 20 µl of 10% SDS and 15 µl of proteinase K were added. The solution was incubated at 70 °C for 2-3 hours and then placed on ice. Later, 1 ml of saturated 6M NaCl was added, shaken vigorously and placed for 10-15 minutes at room temperature. The solution was centrifuged at 3000 rpm for 15 minutes and the supernatant was transferred to another tube. Equal volume of chilled isopropanol was added and after 10 minutes, centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded and DNA was washed with 70% ethanol followed by centrifuged at 3000 rpm for 10 minutes. The pellets were dried at 37 °C and dissolved in 1.5 ml of distilled water.

### PCR amplification

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was performed to determine the genotype of the T/G polymorphism of MDM2 gene, as described previously [21]. The 450 bp fragment encompassing the T to G polymorphic site in the intronic p53-response promoter of MDM2 region was amplified

using specific primers 5'-CGGGAGTTCAGGGTAAAGGT-3' and 5'-AGCAAGTCGGTGCTTACCTG-3'.

The amplification was performed in Gene Amp PCR System 9700 thermocycler (Applied Biosystems, Singapore) with 100 ng of genomic DNA, 25 pmol of each primer, 200 mM total dNTP, 1.5 mM MgCl<sub>2</sub>, 10× PCR buffer and 2.5 U Taq DNA polymerase (Promega, Madison, WI, USA). The following cycling conditions were used: 95 °C for 5 min, followed by 30 cycles of 94 °C for 60 s, 55 °C for 60 s and 72 °C for 60 s, with a final extension at 72 °C for 5 min.

### Gel electrophoresis

The quality of DNA and PCR products were analyzed by 1.5% agarose gel electrophoresis containing 0.5 mg/ml ethidium bromide and visualized under UV illumination.

### DNA sequencing

The PCR products were directly sequenced using the ABI BigDye Terminator v 3.1 Sequencing Standard Kit (Applied Biosystems, Foster City, CA) and run on an ABI 3130 Genetic Analyzer. The sequence data were analyzed by comparison to the consensus sequence of the MDM2 gene using ABI SeqScape v2.5 software [22]. Any changes in the hyper-variable region were detected by sequencing both sense and antisense strands.

## Results and Discussion

To check the quality of extracted DNA, samples were randomly subjected to gel electrophoresis. The illumination of the bands of the sample DNA indicated its quality. The PCR amplification was done through specific primers for MDM2 gene and compared with 1kb ladder. The amplicon of the normal MDM2 gene sequence was 450 bp (Fig. 1).

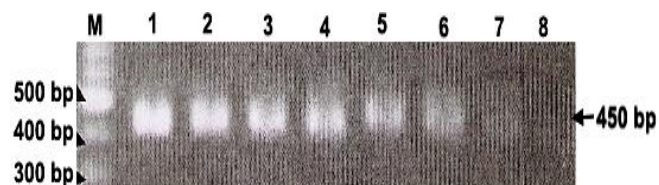


Fig. 1: MDM2 gene amplification. M = 1kb ladder; 1 to 6 = PCR amplified DNA from the samples.

All samples were subjected to sequence analysis and sense and antisense strands were analyzed for T to G mutation as shown in Fig. 2. Studies have shown that G allele increased the binding affinity of

*Sp1* to the promoter of MDM2, resulting in increased MDM2 expression and abolished TP53 DNA damage response [23]. Considering the role of MDM2 in cancer formation, one might expect that individuals who carry the G allele have boosted expression of MDM2 and over a lifetime, are at higher risk for developing cancer.

In this study, five out of 14 cases showed the mutational changes for the allele T/G. The frequency of the mutated alleles was 10% for the mutations. The MDM2 is a key negative regulator of the TP53 pathway that targets TP53 for degradation and over expression. The amplification of MDM2 has been frequently observed in many human cancer types [24]. Various studies showed a 2-fold increase in the MDM2 protein for cell lines with the heterozygous (TG) genotype and a 4-fold increase for cell lines with the homozygous variant (GG) genotype. It was shown that the MDM2-transgenic mice, which produced an average of fourfold more MDM2 in various tissues relative to non-transgenic mice, developed spontaneous tumors throughout their lifetime [25]. The p53 pathway plays a key role in maintaining genomic integrity and preventing against oncogenic transformation of cells, and the loss of TP53 function by gene mutations is a common genetic alteration found in human cancer [26]. The importance of the MDM2/p53 interaction has been demonstrated convincingly by *in vivo* experiments. The loss of MDM2 serves as a critical indicator of p53 activity [27].

The MDM2 TT genotype increases risk was unexpected but associations had found increased risk with the GG genotype that are consistent with the impact of this genotype on MDM2 RNA and protein

levels, and hence, on inhibition of p53. Some results showed a 4.5-fold higher risk in those with the GG, genotypes relative to TT. It has been shown that attenuated p53 pathway resulting from germ-line polymorphisms is associated with increased risk of carcinogenesis [28].

A few studies have investigated the association between the MDM2-SNP 309T>G polymorphism and the cancer risk but the results were inconsistent [29]. For instance, Leu et al have found that the MDM2 SNP309 genotypes were not associated with HCC susceptibility in Taiwanese population [30]. The MDM2 polymorphism might affect cancer risk, as MDM2 has been shown to interact with several key tumor suppressors, including Rb [6], p53 family protein, p73 [31], the growth suppressor p14/ p19, p53 [8] and consistent in several ways with previous studies. The putative effect of MDM2-SNP309 is to enhance that transcription of the MDM2 gene, and to affect the p53 regulatory pathway for tumor development. The SNP309 polymorphism, in the intronic promoter of MDM2 gene, is associated with a significantly increased risk of HCC in a Turkish population. This finding is consistent with previous studies that the 309G allele is the risk allele for HCC [29], and suggests that the MDM2 309 T>G polymorphism may be used as a marker for genetic susceptibility to HCC. In HCC patients, the ratio of men and women was 3.46 and 84.5% of patients also had hepatitis. The average age at diagnosis for HBV- and HCV- infected HCC patients was 59.53 years. Akkiz et al reported 89.9% HCC cases in males and the average age of the patients was 57.42 years in Turkish population [32].

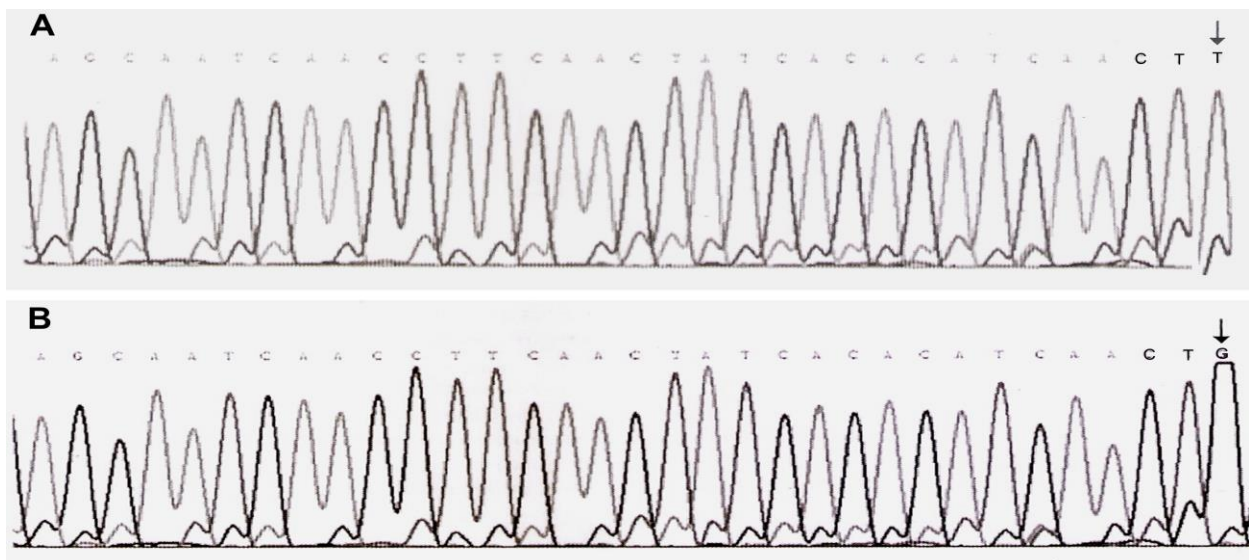


Fig. 2: MDM2 gene sequence showing normal CTT (A) and mutated CTG (B).

## Conclusions

In Pakistan, the average age of presentation of hepatocellular carcinoma is less as compared to western countries; in addition, males are more affected as compared to females. Chronic hepatitis C and B are found to be the major known factors. Patients with chronic hepatitis C and B should undergo vigorous HCC surveillance to detect early, potentially respectable HCC. Recently, it has been reported that a single nucleotide polymorphism (SNP) in the promoter region of MDM2 oncogene, MDM2SNP309, is associated with the risk of HCC in Japanese and Korean patients infected with hepatitis C and hepatitis B virus, respectively. Our results showed a weak interaction of HCC and MDM2-SNP309 mutation from T to G. There was no report of significant mutation in patients of HCC.

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