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Research Article

Insinuations of p53 and MDM2 in Renal Cell Carcinoma progression

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Abstract

Aim: In response to DNA damage, p53 comes into play for rescue and Mouse Double Minute 2 homologue (MDM2) negatively regulates p53. Single Nucleotide Polymorphisms (SNPs) in these genes showed strong association with cancers. Henceforth, the present study was aimed to estimate the gene frequencies of p53 variant (Arg72Pro), MDM2 (T309G) and their expression analysis in RCC from Indian population.

Methods: Genotyping of p53 variant (Arg72Pro) and MDM2 (T309G) in 50 RCC cases and 50 age-sex matched controls was carried out and confirmed by DNA sequencing. Their expression in renal tumor tissue and adjacent normal tissue was analyzed by RT PCR.

Results: A higher proportion of patients had Pro/Pro genotype (26%) and Arg/Arg genotype (18%) in comparison to 14% and 4% in controls respectively which was significantly higher (*p* value: 0.013) when the whole genomic distribution was considered. However, the distribution of MDM2 genotypes (TT, TG, GG) was comparable in patients to that of controls. In tumor tissue, p53 and MDM2 expression, was significantly higher (3 fold increase; *p* value: 0.001) as compared to adjacent normal parenchyma. However, no association was observed in their expression with the tumor stages and grades.

Conclusion: A significant relation of p53 polymorphism with RCC, and the augmented expression of p53, MDM2 genes suggest that they may be involved in the pathogenesis of RCC.

Keywords: MDM2 (T309G) polymorphism; p53 (Arg72Pro) polymorphism; Renal cell carcinoma

Introduction

Renal Cell Carcinoma (RCC) is the most common type of kidney tumors, which comprises ~90% of them [1]. Annually, the incidence of Kidney cancer is ~60,000 cases and the mortality rate is ~13,000 deaths in the United States [2]. Notwithstanding the higher incidence rate of kidney cancer in Europe, Australia, North America, the incidence is lower in Africa, India and China [3]. According to Heidelberg classification, RCC is categorized into many types like clear cell RCC, chromophobe RCC, papillary RCC, collecting duct carcinoma and unclassified RCC [4]. It presents with few symptoms but it can have diverse paraneoplastic manifestations. Inspite of advances in diagnosis, about 20-30% of the cases are identified with distant metastasis [1]. Median survival rate for the distant metastatic disease is about ~13 months [5]. Surgical excision is the standard treatment at early stages. RCC shows relative resistance to radiation and chemotherapy, but shows occasional durable responses to IL-2 based immunotherapy. The relapse rate during follow up is 20% [6]. TNM staging & Fuhrman grading are used as prognostic indicators.

Etiologic studies have demonstrated the association of Von Hippel-Lindau tumor suppressor gene with familial and sporadic RCC [7]. The tumor suppressor gene p53 is present on chromosome 17*p*13.1. In severe DNA damage, it activates DNA repair proteins, arrests cell cycle, initiates apoptosis, thus, having an anticancer role. When there is DNA damage, up regulation of p53 causes cell cycle arrest specifically at G1 to S phase *via p*21 (CDKN1A, waf1) which is as an inhibitor of cyclin dependent kinase [8]. The MDM2 oncogene, the negative regulator of p53, was first identified in a mouse 3T3-DM cell line [9]. The MDM2 oncogene encodes a 90-kDa nuclear phosphoprotein. MDM2 binds p53, and causes inhibition of its transactivation activity. It is also an E3 ubiquitin protein ligase which makes N-terminal TAD of p53 susceptible for proteasomal degradation. Upregulation of MDM2 is related to increased tumor growth and resistance to treatment [10]. Recent findings in the genetic changes associated with RCC have become the targets for therapy.

p53 variant (Arg72Pro), where arginine is replaced by proline led to altered protein structure which downregulates the cellular apoptosis mechanism [11]. Conversely, it increases the cellular proliferation and shows a poor inflammatory response [12]. Thus, this p53 variants showed involvement in tumor cell expansion and apoptosis, which may encourage testing this hypothesis in Renal Cell Carcinoma. p53 is inactivated by the overexpression of MDM2 oncogenes. A polymorphism in the 309th nucleotide of MDM2, where T base is replaced by G base has been noted (rs2279744). This polymorphism is present in an intronic promoter region, which shows increased affinity to stimulatory protein (Sp1) [13]. There is an increased RCC risk in subjects with SNP309 GG genotype of MDM2, which is associated with increased expression of MDM2 [14]. However there

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Table 1: Genotyping, primer sequences, length of PCR products.

Gene	Primer sequences [16,17]	Genotyping	Product size (bp)
p53 (Arg72Pro)	FP 5′-TTGCCGTCCCAAGCAATGGATGA-3′	RFLP	199
	RP 5′-TCTGGGAAGGGACAGAAGATGAC-3′		
MDM2 (SNP309)	FP 5′-CGCGGGAGTTCAGGGTAAAG-3′	RFLP	158
	RP 5′-CTGAGTCAACCTGCCCACTG-3′		

Table 2: Characteristics of RCC cases and controls.

Characteristics	RCC patients, n (%)	Controls, n (%)
Age years		
Mean	53.1	47.14
Range	23-78	24-80
Gender		
Males	36(72)	33(66)
Females	14(28)	17(34)
Grade		
1	13(26)	
2	21(42)	
3	5(10)	
4	2(4)	
Other types (not graded)	9(18)	
Stage		
1	25(50)	
2	3(6)	
3	10(20)	
4	11(22)	
Recurrent	1(2)	
Pathological types		
Clear cell	39(78)	
Chromophobe	6(12)	
Papillary	2(4)	
Clear cell s/o Translocation	3(6)	

Total no. of RCC patients=50, Total no. of age and sex matched controls=50
Tumor stage: Earlier stages 1, 2; Late stages 3, 4, recurrent
Tumor grades: Lower grades 1, 2; Higher grades 3, 4.

is no information on these polymorphisms of p53, MDM2 and their gene expression in RCC from Indian population. In view of the above findings, this study was conducted to estimate the frequency of p53 variant (Arg72Pro) and MDM2 (SNP309) genotypes. Additionally, the expression studies of p53 and MDM2 genes were also carried out to ascertain any association with the pathogenesis of RCC.

Materials and Methods

Samples

Total 50 cases (36 males, 14 females) of histopathologically proven RCC, who were treated by surgery under Urology department of Nehru Hospital, at PGIMER, Chandigarh and 50 age, sex matched controls were enrolled to take part in the present study after taking a proper informed consent. Grading and staging were done using Fuhrman grading and TNM classification respectively. WHO criteria was used for classifying them. Patients with any other nephropathy like diabetic nephropathy, chronic glomerular nephritis, drug induced nephropathy were excluded from the study. The samples were obtained from the renal tumor tissue and the adjacent normal renal parenchyma, separately after nephrectomy, from the patients. Peripheral blood was also collected for SNP analysis from these subjects.

Genotyping

Genomic DNA from the blood was isolated by Daly method [15] which was then quantified on spectrophotometer by taking OD at 260nm and the quality was checked by 2% agarose gel electrophoresis. Specific sites of exon 4 of p53 gene and intronic promoter region of MDM2 gene from genomic DNA were amplified using their respective primer pairs [16,17] (Table 1). PCR was done in 25 ml mixture which contained 1X Taq Buffer, 200 μM dNTPs mixture, 25 pmol of reverse and forward primers, 200 ng genomic DNA and 1 unit of Taq polymerase. Amplification was performed in thermal cycler (Agilent Technologies Sure Cycler 8800). The PCR amplification programme used was: a hot start (95°C, 5 minutes) and an additional 40 cycles (denaturation: 94°C for 30 seconds, annealing: 68°C for p53/62°C for MDM2 for 30 seconds, and extension: 72°C for 15 seconds), followed by an incubation at 72°C for 5 minutes. 10 μl of PCR products each of p53 and MDM2 was digested with BstUI, MspA1I restriction enzymes respectively and was checked on 8% polyacrylamide gel. Automated DNA sequencing was done with forward and reverse primers at DNA sequence ABI Prism (Model 3130, Perkin Elmer, USA) to confirm the genotypes detected by RFLP. For sequencing reaction, PCR product was used as template together with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, USA).

Gene expression study

RNA was obtained from the tissue using trizol method. It was then quantified on spectrophotometer by taking OD at 260nm wavelength and the quality was checked by 1% agarose gel electrophoresis. Complementary DNA was synthesized from the isolated RNA using the biorads cDNA synthesis kit. Amplification of the cDNA was done using RT-PCR with their primers, p53: 5'-ACCTATGGAAACTACTTCCTG-3'(FP), 5'-ACCATTGTTCAATATCGTCC-3'(RP) [product size - 99 bp], MDM2: 5'-CCTTAGCTGACTATTGGAAATG-3'(FP), 5'-TGTTGAGTTTTCCAGTTTGG-3'(RP) [product size - 161 bp]. The condition for RT-PCR was initial denaturation (94°C, 2min), fourty cycles of denaturation (95°C, 30 sec), annealing (58°C, 35 sec) followed by final extension (72°C, 5min). 18S RNA was used for normalization. The 2^-DDCT method is used to analyze the relative changes in gene expression from RT-PCR experiments. The fold change in gene expression was calculated using the formula: Fold change = 2^-DDCT = 2^-[(CT gene of interest - CT internal control) sample A - (CT gene of interest -CT internal control) sample B].

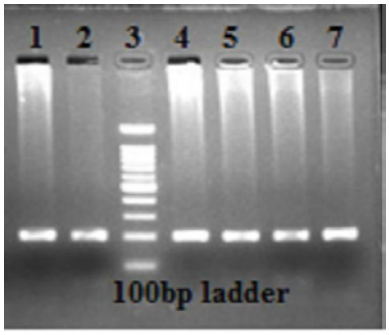

Figure 1: p53 PCR agarose gel showing the 100 bp ladder on the third lane, and the amplified products of p53 which are of 199 bp product size on the other lanes.

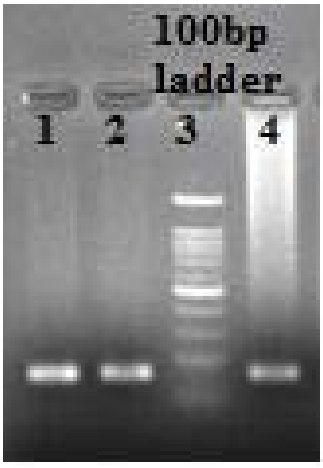

Figure 2: MDM2 PCR agarose gel showing the 100 bp ladder on the third lane, and the amplified PCR products which are of 158 bp product size on the other lanes.

Statistical analysis

Statistical analysis of the obtained results was done by using SPSS program (version 20.0). Comparison between the cases and controls was done by using two tailed T test and Pearson Chi-square test. The gene expression in normal and tumor renal tissues was compared by one sample T- test. *P* value of ≤ 0.05 was taken as significant.

Results

Characteristics of RCC cases and controls

The Table 2 shows the mean age, sex distribution in patients and controls; frequency of tumor grades, staging, pathological types of RCC in patients. Our study included 50 patients with RCC and 50 controls. The mean age of RCC patients was 53.1 years, having a range of 23-78 years. The mean age of controls was 47.14, which had a range of 24-80 years. In RCC patients, majority were males (72%) and the number of males was significantly higher than the females. In RCC cases, 28 cases (56%) cases fall in earlier stages (stage 1 and 2), 34 cases (68%) fall in lower grades (grade 1 and 2). Majority of the RCC

Table 3: Restriction enzymes and their digestion products to detect SNPs of p53, MDM2

Genes	Restriction enzymes	Fragment sizes
p53 (Arg72Pro)	BstUI	199 bp (Pro/Pro)
		199,113,86 bp (Arg/Pro)
		113,86 bp (Arg/Arg)
MDM2 (SNP309)	MspA1I	158 bp (TT)
		158,112,46 bp (TG)
		112,46 bp (GG)

Table 4A: p53 gene genotypes and renal cancer risk.

Genotypes	RCC patients, n (%)	Controls, n (%)	Statistical analysis
p53 codon 72			
Arg/Arg	9(18)	2(4)	Chi-square test
Arg/Pro	28(56)	41(82)	Cases *Vs* controls *p* value = 0.013
Pro/Pro	13(26)	7(14)	

Total no. of RCC patients = 50, Total no. of age and sex matched controls = 50 Statistical significance between RCC patients and controls were done by Chi-square test. *p*-value of ≤ 0.05 was considered as significant.

Table 4B: MDM2 gene genotypes and renal cancer risk.

Genotypes	Cases, n (%),	Controls, n (%),	Statistical analysis
MDM2 (SNP 309)			
TT	11(22)	7(14)	Chi-square test
TG	28(56)	35(70)	Cases *Vs.* controls *p* value = 0.343
GG	11(22)	8(16)	

Total no. of RCC patients = 50, Total no. of age and sex matched controls = 50 Statistical significance between RCC patients and controls were done by Chi-square test. *p*-value of ≤ 0.05 was considered as significant.

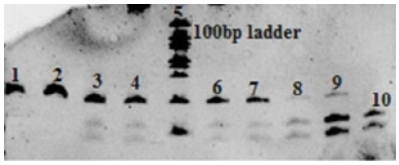

Figure 3: Native PAGE for visualizing the restriction enzyme BstUI digested products of p53. The lane 1,2 show 199 bp band of p53 (Pro/Pro), which was not cut by the restriction enzyme. The lane 3,4,6-9 show 199, 113, 86 bp bands of p53 (Arg/Pro; Hz), which was partially digested by the restriction enzyme. The fifth lane shows the 100bp ladder. The last lane shows 113, 86 bp bands of p53 (Arg/Arg), which was completely digested by the restriction enzyme.

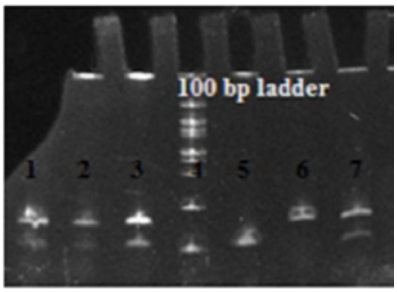

Figure 4: Native PAGE for visualizing the restriction enzyme MspA1I digested products of MDM2. The lanes 1, 2, 3 and 7 show 158, 112 bp bands of MDM2 (TG; Hz), which was partially digested by the restriction enzyme. The fourth lane shows the 100bp ladder. The fifth lane shows 112 bp band of MDM2 (GG), which was completely digested by the restriction enzyme. The sixth lane shows shows 158 bp band of MDM2 (TT), which was not digested by the restriction enzyme.

cases were in stage 1 [25 cases (50%)] and of grade 2 [21 cases (42%)].

Polymorphism analysis

After PCR amplification of p53 codon 72 and MDM2 (SNP309) in DNA, the quality was checked by resolving PCR products on 2% agarose gel for the presence of the sharp bands of 199bp, 158bp size respectively (Figure 1,2). 8% Native PAGE was performed to check for different alleles of p53 and MDM2. They were checked by the presence of specific restriction enzyme sites (Table 3) with the bands of different sizes (p53: 199bp, 113bp, 86bp and MDM2: 158bp, 112bp, 46bp) according to specific genotypes (Figure 3,4). The genotypes of p53 and MDM2 were confirmed by sequencing of the PCR products with the presence of specific peaks for the different bases (Figure 5,6).

The Table 4A shows the distribution of genotypes of p53 variant (Arg72Pro) between cases and the controls. p53 variants of 72, *viz.* Arg/Arg, Arg/Pro, Pro/Pro were 9, 28, 13 in number in RCC cases when compared to 2, 41, 7 in controls respectively. A significant rise in the Pro/Pro genotype and Arg/Arg genotype was seen in patients in comparison to controls (p value: 0.013) when the whole genomic distribution of p53 codon 72 was considered (Figure 7). A borderline significance was noted for proline genotype (p 0.054) between the groups of Pro/Pro and Arg/Pro genotypes. Though higher proportion of cases had p53 Arg/Arg genotype (18%) and Pro/Pro genotype (26%) when compared to controls, the difference was not significant statistically among them. The distribution of MDM2 gene polymorphism (TT, TG, GG) was not significantly different between RCC cases and controls (Table 4B, Figure 8).

Gene expression analysis

Further, Real Time PCR was conducted to elucidate any effect of these polymorphisms on p53 and MDM2 gene expression in RCC pathogenesis. RT-PCR analysis demonstrated that the expression of p53 and MDM2 genes at transcription level was significantly higher (3 fold increase; p value: 0.001) in RCC tissues as compared to adjacent normal renal parenchyma (Figure 9,10). The frequency of p53 expression was significant with Pro/Pro genotype in tumor tissue as compared to adjacent normal renal parenchyma (p value: 0.05; Figure 9). Strikingly, no significant difference of p53 expression was found between the subjects of Pro/Pro genotype and Arg/Arg genotype. Notably, a significant difference was found in p53 expression in RCC stages without distant metastasis when compared with its expression in the distant metastasis stage (p value: 0.003). However, there was no association of p53 and MDM2 expression with tumor stage (earlier stages 1, 2 *Vs.* late stages 3, 4 recurrent) and grades (lower grades 1, 2 *Vs.* higher grades 3, 4).

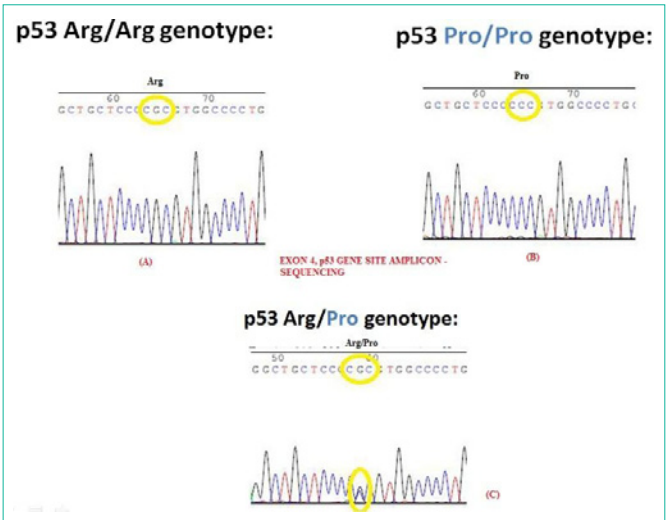

Figure 5: (A). The figure shows the sequencing of the p53 PCR product of Arg/Arg genotype. The yellow circle highlights the p53 variant codon 72, showing CGC codon coding for arginine. (B). The Figure shows the sequencing of the p53 PCR product of Pro/Pro genotype. The yellow circle highlights the p53 variant codon 72, showing CCC codon coding for proline. (C). The Figure above shows the sequencing of the p53 PCR product of Arg/Pro genotype. The upper yellow circle highlights the p53 variant codon 72, showing CGC codon. The lower circle highlights both C and G peaks.

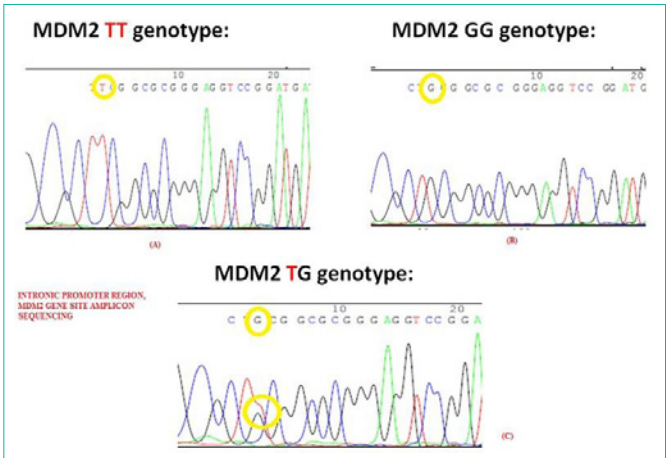

Figure 6: (A).The Figure shows the sequencing of the MDM2 PCR product of TT genotype. The yellow circle highlights the MDM2 309th nucleotide, showing T base. (B). The Figure shows the sequencing of the MDM2 PCR product of GG genotype. The yellow circle highlights the MDM2 309th nucleotide, showing G base. (C). The Figure shows the sequencing of the MDM2 PCR product of TG genotype. The upper yellow circle highlights MDM2 309th nucleotide, showing G base. The lower circle highlights both T and G peaks.

Discussion

p53, the gatekeeper of the genome, is a critical factor of the signalling complex networks in reaction to genetic stress. It is the most frequent gene to get mutated in cancers of individuals. SNPs which are located in the p53 signalling complex gene loci, affect cancer risk and the clinical outcome [18]. In many varieties of cancers, SNPs at codon 72 of p53 had been thoroughly investigated [19]. p53 is negatively regulated by MDM2 and its expression is raised in varieties of cancers that continues to hold wild type p53. The upregulated MDM2 will cause more degradation of p53 [20]. As there is an important role of these polymorphisms of p53 (Arg72Pro) and MDM2 (SNP309) in cancer risk, biological and clinical effects, we studied the association of these SNPs with the risk of RCC in 50 patients. In RCC patients, majority were males and this may be due to the increased prevalence of risk factors of smoking and alcohol among males. Majority of the RCC cases were in stage 1 and of grade 2 and this may be due to advances in diagnosis. Huang *et al.* [21], identified that pro/pro genotype of p53 and GG carriers of MDM2 (SNP 309) were associated with the development of RCC in comparison with the other genotypes of those genes. Hirata *et al.* [14], showed a notable rise in GG carriers of MDM2 (SNP309) in cases with RCC

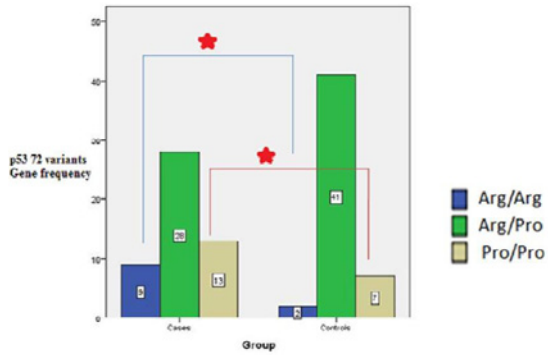

Figure 7: p53 genotypes and renal cancer risk. A significant rise in the Pro/Pro genotype and Arg/Arg genotype was seen in patients compared to controls (*p* value = 0.013) when the whole genomic distribution of p53 codon 72 was considered.

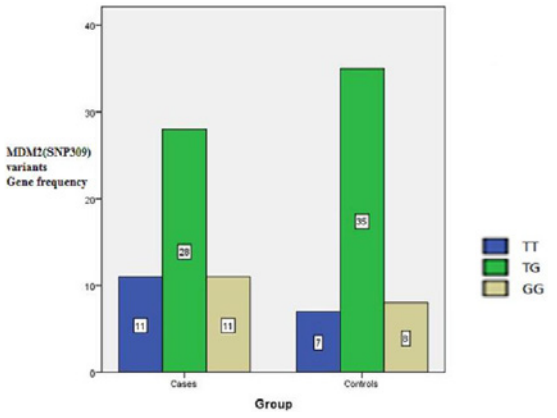

Figure 8: MDM2 genotypes and renal cancer risk. The number of cases of MDM2 genotypes (TT, TG, GG) were 11, 28, 11 when compared to 7, 35, 8 in controls respectively. The distribution of MDM2 gene polymorphism was not significantly different as compared to controls (*p* value = 0.343).

when compared to controls as well as significantly expressed GG genotype in cases to that of controls. In this study, when the whole genotypic distribution of p53 was considered, Pro/Pro genotype and Arg/Arg genotype showed association with the development of renal tumor appreciably. However, the distribution of MDM2 gene polymorphism showed no significant difference as compared to controls. In other types of cancers like liver cancer, p53 and MDM2 were found to play an important role. Yang *et al.* [22], showed that p53 Pro/Pro genotype, MDM2 GG genotype in combination has increased risk for developing Hepatocellular carcinoma. In this study, Pro/Pro genotype of p53, GG genotype of MDM2 in combination has no increased risk for developing RCC when matched with controls.

In tissue samples, the expression of p53 and MDM2 at transcription level was significantly higher in renal tumor as compared to normal renal parenchyma. A significant expression was also observed with Pro/Pro genotype of p53 in tumor tissue as compared to adjacent normal renal parenchyma. Noon *et al.* [23], reported that increased p53 expression, but not p53 mutation, is associated with the reduced overall survival/more rapid disease progression in RCC. They also noticed that over expression of MDM2 is related with reduced disease-free survival. They showed that p53

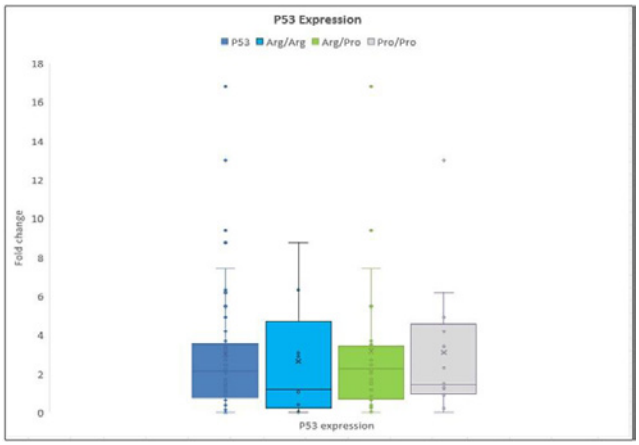

Figure 9: The box plot depicts the p53 expression profile on the whole and according to different genotypes. In tumor tissue samples, p53 expression at transcription level was significantly higher (3 fold increase; *p* value = 0.001) as compared to adjacent normal renal parenchyma. A significant expression was also observed with Pro/Pro genotype in tumor tissue as compared to adjacent normal renal parenchyma (*p* value = 0.05) which showed 3.11 fold increase.

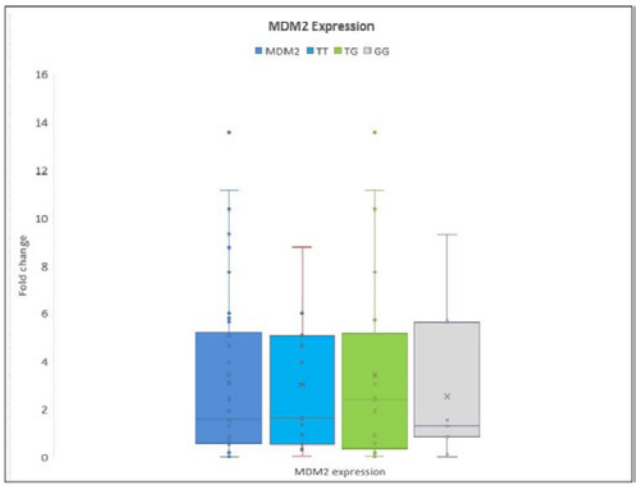

Figure 10: The box plot depicts the MDM2 expression profile on the whole and according to different genotypes. In tumor tissue samples, MDM2 expression at transcription level was significantly higher (3 fold increase; *p* value = 0.001) as compared to adjacent normal renal parenchyma. GG genotype of MDM2 showed 2.54 fold increase of expression, which has not attained statistically significant value (*p* value = 0.1).

overexpression is closely related to MDM2 overexpression. Erdem *et al.* [24], showed AEG-1 and p53 overexpression is related with stages and tumor grades and also associated with the disease progression in RCC cases. Moch *et al.* [25], showed that only p53 was found to predict the disease prognosis in RCC and not the MDM-2 gene. TP53 overexpression in cases was found to have worse prognosis in comparison with MDM-2. However, both p53 and MDM2 were over expressed in RCC renal tissues in this study. Further, attempts were made to elucidate any association of the expression of p53, MDM2 with the tumor stages and tumor grades. Notably, no association was found between them. Haitel *et al.* [26], revealed that MDM2/p53 co-overexpression, nuclear grade, and preoperative presence of distant metastasis are independent predictors of poor survival in clear

cell RCC. p53 and MDM2 co-overexpression in the present study, is consistent with the above study. Laird *et al.* [27], found that over expression of genes like Ki67, p53, SLUG and SNAIL in the cases of distant metastasis in comparison with the other stages of RCC. Notably, a critical difference was found in expression of p53 in stages without metastasis when compared with its expression in the distant metastasis stage. This might be due to the anti-cancer role of p53, which gets lost in advanced stages.

Javid *et al.* [28] study, showed more than 6-fold increased expression of MDM2 and about 7-fold decreased in p53 expression levels in cases with non-small cell lung cancer compared to healthy controls. It was concluded that the increased MDM2 expression causes decreased expression of p53 at messenger RNA level. In contrast, in this study, both p53 and MDM2 expression was significantly higher (3 fold) in renal tumor in comparison to normal renal parenchyma.

Conclusion

In conclusion, in Indian population, this is the special report to reveal significant relation between p53 gene polymorphism and RCC risk. Augmented expression of p53 and MDM2 in tumor tissue, put forward for consideration of the involvement of these genes in the pathogenesis of RCC.

Acknowledgment

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