Results

Objective 1: To assess *p53* and *MDM2* gene polymorphisms, *p53* mutations and HPV infections in oral cancer patients

1.1. Genetic polymorphisms in *p53* and *MDM2* genes and oral cancer risk

Genotyping for the detection of *p53* and *MDM2* polymorphisms was carried out in the present study and their influence on oral cancer susceptibility was determined.

1.1.1. Distribution of genotypes and allele frequencies among subjects

The genotype and allele frequency distribution of 16 bp duplication in intron 3, Arg72Pro in exon 4 and G>A transition in intron 6 of *p53* and T>G transition in the promoter region of *MDM2* are provided in table 4.1. Controls and cases showed good fit to HWE for intron 3 and 6 polymorphisms of *p53* and also for *MDM2* polymorphism. In case of exon 4 polymorphism, controls showed good fit to HWE. No significant difference in the distribution of *p53* and *MDM2* genotypes between cases and controls was observed.

Table 4.1: Genotype and allelic frequency distribution of various polymorphisms of *p53* and *MDM2* among cases and controls

Polymorphism	Group	Genotypes			Pearson's χ^2	AF 1:2	HWE χ^2
		1/1 No. (%)	1/2 No. (%)	2/2 No. (%)			
16 bp duplication (*p53*;Intron 3) rs17878362	Controls	145 (68.4)	61 (28.8)	6 (2.8)	2.055 p=0.358	0.83:0.17	0.034 p=0.983
	Cases	169 71.3)	57 (24.1)	11 (4.6)		0.83:0.17	4.258 p=0.119
Arg72Pro (*p53*;Exon 4) rs1042522	Controls	46 (21.7)	102 (48.1)	64 (30.2)	3.091 p= 0.213	0.46:0.54	0.209 p=0.901
	Cases	63 (26.6)	95 (40.1)	79 (33.3)		0.47:0.53	8.975 p=0.011
G>A (*p53*;Intron 6) rs1625895	Controls	6 (4.5)	58 (29.1)	148 (66.4)	0.705 p= 0.703	0.17:0.83	0.081 p=0.961
	Cases	8 (3.4)	57 (24.1)	172 (72.6)		0.15:0.85	1.570 p=0.456
T>G (*MDM2*;SNP309) rs2279744	Controls	57 (26.9)	107 (50.5)	48 (22.6)	0.425 p=0.808	0.48:0.52	0.028 p=0.986
	Cases	70 (29.7)	115 (48.7)	51 (21.6)		0.46:0.54	0.086 p=0.958

1= A1 at intron 3, Proline at exon 4, A at intron 6, G at *MDM2*; 2= A2 at intron 3, Arginine at exon 4, G at intron 6, T at *MDM2*; A1 represents absence of 16 bp duplication allele at intron 3 loci; AF=Allele Frequency

1.1.2. Risk estimation of oral cancer associated with *p53* and *MDM2* genotypes

Higher OR were observed with the presence of 16 bp duplication allele in intron 3 after adjustment for confounding variables, like, age, sex and tobacco use (OR=2.08,

95%CI=0.55-7.90, table 4.2). It was observed that Arg72Pro in exon 4, G>A transition in intron 6 of *p53* and T>G transition in the promoter region of *MDM2* did not show any association with oral cancer risk even after adjusting for variables, like, age, sex and tobacco habits.

Table 4.2: Risk estimates of *p53* and *MDM2* genotypes in oral cancer patients

Genotype	Controls No. (%)	Cases No. (%)	OR (95% CI) *p* value	Adjusted OR (95% CI) *p* value
16 bp duplication (*p53*; Intron 3)				
A1/A1	145 (68.4)	169 (71.3)	1.0 (Referent)	
A1/A2	61 (28.8)	57 (24.1)	0.80 (0.52 - 1.22) *p*= 0.307	0.81 (0.45 - 1.41) *p*= 0.450
A2/A2	6 (2.8)	11 (4.6)	1.57 (0.57 - 4.36) *p*= 0.384	**2.08 (0.55 - 7.90)** *p*= 0.285
A1/A2 + A2/A2	67 (31.6)	68 (28.7)	0.87 (0.58 - 1.30) *p*= 0.502	0.90 (0.52 - 1.54) *p*= 0.698
Arg72Pro (*p53*; Exon 4)				
Arg/Arg	64 (30.2)	79 (33.3)	1.0 (Referent)	
Arg/Pro	102 (48.1)	95 (40.1)	0.76 (0.49 - 1.16) *p*= 0.201	0.82 (0.45 - 1.48) *p*= 0.504
Pro/Pro	46 (21.7)	63 (26.6)	1.11 (0.67 - 1.84) *p*= 0.686	1.40 (0.72 - 2.73) *p*= 0.323
Arg/Pro + Pro/Pro	148 (69.8)	158 (66.7)	0.87 (0.58 - 1.29) *p*= 0.475	1.01 (0.59 - 1.72) *p*= 0.985
G>A (*p53*; Intron 6)				
G/G	148 (66.4)	172 (72.6)	1.0 (Referent)	
A/G	58 (29.1)	57 (24.1)	0.85 (0.55 - 1.30) *p*= 0.441	0.87 (0.49 - 1.52) *p*= 0.618
A/A	6 (4.5)	8 (3.4)	1.15 (0.39 - 3.38) *p*= 0.803	0.99 (0.21 - 4.74) *p*= 0.990
A/G + A/A	64 (33.6)	65 (27.5)	0.87 (0.58 - 1.32) *p*= 0.519	0.88 (0.51 - 1.51) *p*= 0.876
Total	212	237		
T>G (*MDM2*; SNP309)				
G/G	57 (26.9)	70 (29.7)	1.0 (Referent)	
G/T	107 (50.5)	115 (48.7)	0.88 (0.57 - 1.36) *p*= 0.55	0.95 (0.42 - 1.58) *p*= 0.551
T/T	48 (22.6)	51 (21.6)	0.87 (0.51 - 1.47) *p*= 0.59	0.82 (0.55 - 1.63) *p*= 0.839
G/T + T/T	155 (73.1)	166 (70.3)	0.76 (0.50 - 1.15) *p*= 0.193	0.90 (0.54 - 1.51) *p*= 0.697
Total	212	236		

1.1.3.Determination of genotype combinations and risk of oral cancer

Present study also determined the genotype distribution of all the three polymorphisms of the *p53* gene in combination and risk associated with these

combined genotypes (Table 4.3). It was observed that heterozygous genotypes at intron 3 and exon 4 in combination with G/G genotype at intron 6 exhibited protective effect from oral cancer development (OR=0.13; 95%CI: 0.02-1.13; *p*=0.065).

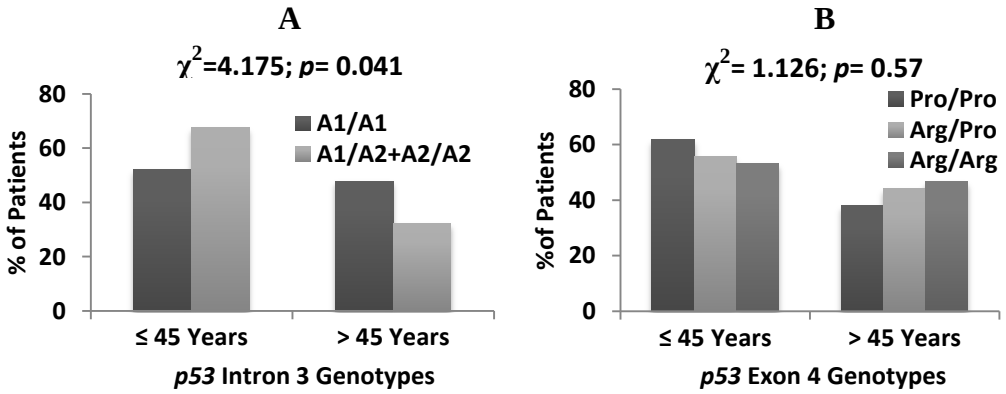
Table 4.3: Distribution of combined genotypes of *p53* among subjects and risk estimation

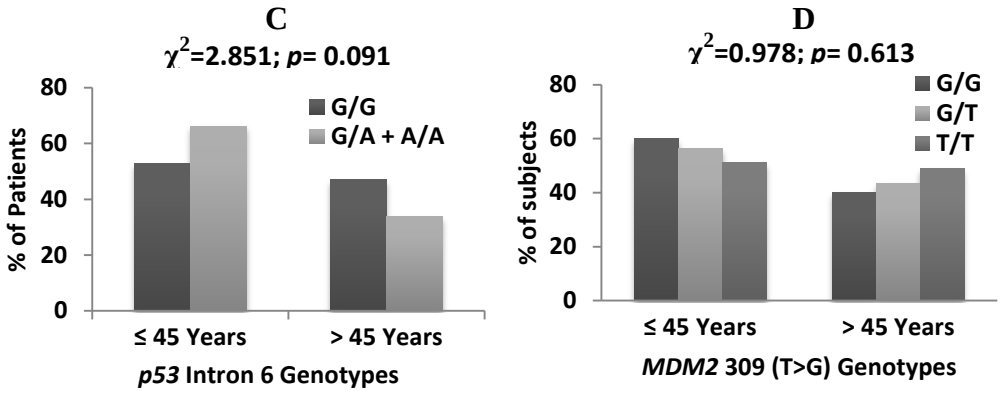
16 bp duplication+Arg72Pro+G>A	Controls No.	Cases No.	OR (95%CI)	*p* value
A1/A1+Arg/Arg+G/G	62	78	1.0 (Referent)	
A1/A1+Pro/Pro+G/G	22	27	0.98 (0.51 - 1.88)	0.941
A1/A2+Pro/Pro+G/G	4	1	3.18 (0.35 – 29.17)	0.306
A1/A2+Pro/Pro+G/A	15	22	1.17 (0.56 - 2.24)	0.683
A2/A2+Pro/Pro+A/A	6	8	1.06 (0.35 - 3.22)	0.918
A1/A1+Arg/Pro+G/G	56	62	0.88 (0.54 - 1.44)	0.611
A1/A2+Arg/Pro+G/A	37	29	0.62 (0.35 - 1.12)	0.116
A1/A2+Arg/Pro+G/G	6	1	**0.13 (0.02 - 1.13)**	**0.065**
(A1/A2+A2/A2)+(Arg/Pro+Pro/Pro) +(G/A + A/A)	58	62	0.85 (0.52 - 1.39)	0.514

1.1.4. Distribution of *p53* and *MDM2* genotypes according to the age of onset

The patients were categorized according to the age groups. Median age (45 years) of the cohort was considered as the cut-off. Distribution of *p53* intron 3, exon 4 and intron 6 as well as *MDM2* genotypes according to the age of disease onset are presented in Figure 4.1A, 4.1B, 4.1C and 4.1D, respectively. It was observed that 16 bp duplication and A allele at intron 3 and 6 were associated with early age of disease onset (*p*=0.041 and *p*=0.091, respectively). Whereas, more number of patients having Pro/Pro genotype (61.9%) for *p53* and G/G genotype (60%) for *MDM2* had early age of onset.

Figure 4.1: Distribution of *p53* genotypes according to the age of onset





1.1.5. Estimation of haplotype frequency and risk of oral cancer

Pair-wise haplotype frequencies for all possible combinations of the three *p53* polymorphisms were also estimated. The frequencies of pair-wise haplotypes did not differ significantly between controls and cases (Table 4.4). LD analysis was also performed to examine the linkage among the three loci (intron 3-exon 4-intron 6). There was significant LD between these three loci in both the groups (Table 4.4). The LD analysis also revealed that the intron 3 and exon 4 polymorphisms and intron 3 and 6 polymorphisms were in strong LD association, whereas exon 4 and intron 6 polymorphisms were in low LD association.

Table 4.4: Frequencies of pair-wise haplotypes and linkage disequilibrium analysis among controls and cases

Groups	Pair-wise haplotype frequencies				Pearson's χ^2	D	*p* value	D'	r^2
	1-1	1-2	2-1	2-2					
16 bp duplication-Arg72Pro									
Controls	0.293	0.535	0.165	0.007	0.406	0.0862	< 0.0001	0.94	0.21
Cases	0.308	0.525	0.158	0.009	*p*= 0.939	0.0802	< 0.0001	0.88	0.18
Arg72Pro-G>A									
Controls	0.161	0.296	0.004	0.539	0.483	0.0856	< 0.0001	0.22	0.21
Cases	0.151	0.316	0.003	0.530	*p*=0.923	0.0791	< 0.0001	0.20	0.20
16 bp duplication-G>A									
Controls	0.012	0.816	0.153	0.019	1.761	0.1246	< 0.0001	0.88	0.78
Cases	0.004	0.829	0.150	0.017	*p*=0.623	0.1243	< 0.0001	0.99	0.86

1= A1 at intron 3, Proline at exon 4, A at intron 6; 2= A2 at intron 3, Arginine at exon 4, G at intron 6
D=linkage disequilibrium (LD), p value through Pearson's chi square test to test LD, D'=1 is called Complete LD; r^2 =1 is called Perfect LD.

The extended *p53* haplotypes (3 loci haplotypes) in controls and cases are reported in the order of intron 3-exon 4-intron 6 (Table 4.5). For haplotype analysis, absence of 16 bp duplication at intron 3, Pro at exon 4 and A at intron 6 is defined as allele 1 at each of the three loci, respectively. The predominant haplotype consisted of the highly

prevalent alleles (1-2-2). It was followed by 1-1-2 and 2-1-1, respectively. 1-1-1 haplotype was more frequent in controls compared to the cases (Table 4.6).

Table 4.5: Frequencies of extended haplotypes among controls and cases

Groups	Extended Haplotype frequencies							Pearson's χ^2
	1-2-2	1-1-2	1-1-1	2-2-2	2-1-2	2-1-1	2-2-1	
Controls	0.535	0.281	0.012	0.004	0.015	0.150	0.003	2.596
Cases	0.525	0.303	0.004	0.004	0.013	0.146	0.004	p= 0.858

1= A1 at intron 3, Proline at exon 4, A at intron 6; 2= A2 at intron 3, Arginine at exon 4, G at intron 6

Table 4.6: Distribution of haplotypes and associated risk of oral cancer among controls and cases

Haplotypes	Controls (n=424)	Cases (n=474)	OR (95%CI)	*p* value
1-2-2	228 (53.8)	250 (52.7)	1.0 (Referent)	
1-1-2	118 (27.8)	143 (30.2)	1.11 (0.82 - 1.50)	0.517
1-1-1	5 (1.2)	2 (0.4)	0.37 (0.07 - 1.90)	0.231
2-2-2	1 (0.3)	2 (0.4)	1.82 (0.16 - 20.25)	0.625
2-1-2	7 (1.7)	6 (1.3)	0.78 (0.26 - 2.36)	0.662
2-1-1	64 (15.1)	70 (14.8)	1.00 (0.68 - 1.46)	0.989
2-2-1	1 (0.3)	1 (0.2)	0.91 (0.06 - 14.67)	0.948

n = no of chromosomes; 1= A1 at intron 3, Proline at exon 4, A at intron 6; 2= A2 at intron 3, Arginine at exon 4, G at intron 6

1.2. Gene-environment interaction and risk of oral cancer development

Interaction between *p53* genotypes and tobacco exposure (16 bp duplication in intron 3, Arg72Pro in exon 4 and G>A transition in intron 6) are presented in table 4.7, 4.8 and 4.9, respectively. It was observed that A2/A2 genotype at intron 3, Pro/Pro genotype at exon 4 and A/A genotype at intron 6 increased the risk of oral cancer in conjunction with any type of tobacco habit (OR=13.62, 95%CI=3.48-53.3, p=0.0002, OR=7.19, 95%CI=3.0.1-16.88, p<0.0001 and OR=9.82, 95%CI=2.4-40.14, p=0.0015, respectively).

Table 4.7: Gene-environment interaction (*p53* intron 3 genotypes and tobacco exposure)

Genotypes	Controls No.	Cases No.	OR (95% CI)	*p* value
A1/A1*NHT	78	21	1.0 (Referent)	
A1/A1*WHT	67	148	8.20 (4.68 - 14.39)	<0.0001
A1/A1*Chewers	55	89	6.01 (3.34 - 10.82)	<0.0001
A1/A2*NHT	29	4	0.51 (0.16 - 1.62)	0.255
A1/A2*WHT	32	53	6.15 (3.21 - 11.80)	<0.0001
A1/A2*Chewers	24	30	4.64 (2.26 - 9.55)	<0.0001
A2/A2*NHT	3	0	-	-
A2/A2*WHT	3	11	**13.62 (3.48 - 53.3)**	0.0002
A2/A2*Chewers	3	7	8.67 (2.06 - 36.43)	0.0032

NHT: No habit of tobacco, WHT: With habit of tobacco

Table 4.8: Gene-environment interaction (*p53* exon 4 genotypes and tobacco exposure)

Genotypes	Controls No.	Cases No.	OR (95% CI)	*p* value
Arg/Arg *NHT	31	10	1.0 (Referent)	
Arg/Arg *WHT	33	69	6.48 (2.84 - 14.79)	<0.0001
Arg/Arg *Chewers	26	40	4.77 (2.00 - 11.35)	0.0004
Arg/Pro*NHT	58	10	0.53 (0.20 - 1.42)	0.210
Arg/Pro*WHT	44	85	6.00 (2.69 - 13.33)	<0.0001
Arg/Pro *Chewers	37	47	3.94 (1.71 - 9.06)	0.0013
Pro/Pro*NHT	21	5	0.74 (0.22 - 2.47)	0.622
Pro/Pro*WHT	25	58	**7.19 (3.01 - 16.88)**	<0.0001
Pro/Pro *Chewers	19	39	6.36 (2.59 - 15.64)	0.0001

NHT: No habit of tobacco, WHT: With habit of tobacco

Table 4.9: Gene-environment interaction (*p53* intron 6 genotypes and tobacco exposure)

Genotypes	Controls No.	Cases No.	OR (95% CI)	*p* value
G/G*NHT	81	22	1.0 (Referent)	
G/G*WHT	67	150	8.24 (4.75 - 14.32)	<0.0001
G/G*Chewers	54	88	6.00 (3.36 - 10.72)	<0.0001
G/A*NHT	26	3	0.42 (0.12 - 1.54)	0.192
G/A*WHT	32	54	6.21 (3.27 - 11.81)	<0.0001
G/A*Chewers	25	34	5.01 (2.49 - 10.07)	<0.0001
A/A*NHT	3	0	-	-
A/A*WHT	3	8	**9.82 (2.40 - 40.14)**	0.0015
A/A*Chewers	3	4	4.91 (1.02 - 23.58)	0.047

NHT: No habit of tobacco, WHT: With habit of tobacco

G/T genotype of *MDM2* SNP309 exhibited increased oral cancer risk in conjunction with any type of tobacco habit (OR=10.4, 95%CI=3.99-27.01, $p<0.0001$, table 4.10).

Table 4.10: Gene-environment interaction (*MDM2* SNP309 genotypes and tobacco exposure)

Genotypes	Controls No.	Cases No.	OR (95% CI)	*p* value
G/G*NHT	26	6	1.0 (Referent)	
G/G*WHT	31	61	8.50 (3.18 - 22.89)	<0.0001
G/G*Chewers	26	36	6.00 (2.16 - 16.66)	0.0006
G/T*NHT	64	12	0.81 (0.28 - 2.39)	0.71
G/T*WHT	43	103	**10.40 (3.99 - 27.01)**	<0.0001
G/T *Chewers	37	61	7.10 (2.69 - 18.98)	0.0001
T/T*NHT	20	7	1.52 (0.44 - 5.22)	0.51
T/T*WHT	28	44	6.81 (2.49 - 18.63)	0.0002
T/T*Chewers	19	28	6.40 (2.21 - 18.47)	0.0006

NHT: No habit of tobacco, WHT: With habit of tobacco

1.3. Association of *p53* and *MDM2* polymorphisms with clinico-pathological features, recurrence and survival of oral cancer patients

In the present study, we also analyzed association of *p53* and *MDM2* genotypes with different clinico-pathological parameters, like, tumor differentiation, size, stage, lymph node metastasis, invasion as well as disease recurrence and survival of the patients.

1.3.1. Association of *p53* and *MDM2* genotypes with clinico-pathological parameters of oral cancer patients

Higher OR for moderate differentiation was observed in patients having variant genotypes at all the three loci of *p53* (Table 4.11).

Table 4.11: Distribution of *p53* genotypes in oral cancer cases by differentiation

Genotypes	Well No. (%)	Moderate No. (%)	OR (95% CI)	*p* value
Intron 3 (16 bp Duplication)				
A1/A1	61 (74.4)	90 (69.8)	1.0 (Referent)	
A1/A2	20 (24.4)	32 (24.1)	1.08 (0.57 - 2.07)	0.806
A2/A2	1 (1.2)	7 (5.4)	**4.74 (0.57 - 39.5)**	0.150
A1/A2 + A2/A2	21 (25.6)	39 (29.5)	1.26 (0.68 - 2.35)	0.469
Total	82	129		
Exon 4 (Arg72Pro)				
Arg/Arg	30 (36.6)	42 (32.6)	1.0 (Referent)	
Arg/Pro	37 (45.1)	51 (39.5)	0.98 (0.52 - 1.85)	0.962
Pro/Pro	15 (18.3)	36 (27.9)	**1.71 (0.80 - 3.68)**	0.166
Arg/Pro + Pro/Pro	52 (63.4)	87 (67.4)	1.20 (0.67 - 2.14)	0.548
Total	82	129		
Intron 6 (G>A)				
G/G	60 (73.2)	92(71.3)	1.0 (Referent)	
A/G	21 (25.6)	32 (24.8)	0.99 (0.52 - 1.88)	0.985
A/A	1 (1.2)	5 (3.9)	**3.26 (0.37 - 28.60)**	0.286
A/G + A/A	22 (26.8)	37 (28.7)	1.10 (0.59 – 2.04)	0.770
Total	82	129		

Likewise, higher OR for advanced stage was seen in patients having A2 allele, Pro allele, A allele at all the three loci, respectively (Table 4.12). *MDM2* SNP309 (T>G) genotypes did not show association with any clinico-pathological parameters.

Table 4.12: Distribution of *p53* genotypes in oral cancer cases by stage of the disease

Genotypes	Early No. (%)	Advanced No. (%)	OR (95% CI)	*p* value
Intron 3 (16 bp Duplication)				
A1/A1	63 (79.7)	104 (68.4)	1.0 (Referent)	
A1/A2	14 (17.7)	41 (27.0)	1.77 (0.90 - 3.51)	0.100
A2/A2	2 (2.5)	7 (4.6)	2.12 (0.43 - 10.52)	0.358
A1/A2 + A2/A2	16 (20.3)	48 (29)	**1.82 (0.95 - 3.45)**	**0.070**
Total	79	152		

Exon 4 (Arg72Pro)				
Arg/Arg	31 (39.2)	47 (30.9)	1.0 (Referent)	
Arg/Pro	31 (39.2)	62 (40.8)	1.31 (0.71 - 2.47)	0.386
Pro/Pro	17 (21.5)	43 (28.3)	1.67 (0.81 - 3.43)	0.165
Arg/Pro + Pro/Pro	48 (60.8)	105 (69.1)	**1.44 (0.82 - 2.54)**	0.206
Total	79	152		
Intron 6 (G>A)				
G/G	62 (78.5)	108 (71.1)	1.0 (Referent)	
A/G	15 (19.0)	40 (26.3)	1.53 (0.78 - 2.99)	0.213
A/A	2 (2.5)	4 (2.6)	1.15 (0.20 - 6.45)	0.875
A/G + A/A	17 (21.5)	44 (28.9)	**1.49 (0.78 - 2.82)**	0.226
Total	79	152		

1.3.2. Association of *p53* and *MDM2* genotypes with disease recurrence

No significant association of *p53* genotypes with recurrence of the disease was observed. However, G/T genotype as well as T allele (G/T+T/T) of *MDM2* marginally associated with recurrence of the disease (OR=1.88, 95%CI=0.93-3.79, p=0.080; OR=2.10, 95%CI=0.92-3.50, p=0.087, respectively, table 4.13).

Table 4.13: Distribution of *MDM2* genotypes in oral cancer cases by recurrence

Genotype	Non-Recurrent No. (%)	Recurrent No. (%)	OR (95% CI)	*p* value
T>G (*MDM2*; SNP309)				
G/G	38 (31.7)	16 (20.5)	1.0 (Referent)	
G/T	57 (47.5)	45 (57.7)	**1.88 (0.93 - 3.79)**	**0.080**
T/T	25 (20.8)	17 (21.8)	1.62 (0.69 - 3.77)	0.269
G/T + T/T	82 (68.3)	62 (79.5)	**2.10 (0.92 - 3.50)**	**0.087**
Total	120	78		

As *MDM2* SNP309 (T>G) showed marginal association with recurrence, we further analyzed association of *MDM2* SNP309 (T>G) genotypes with recurrence of the disease according to various clinico-pathological parameters. It was observed that G/T genotype as well as T allele (G/T+T/T) of *MDM2* exhibited significant association with recurrence of the disease in patients having moderately differentiated, large size, advanced stage, lymph node positive and invasive tumors (table 4.14). Further, T/T genotype of *MDM2* was also significantly associated with recurrence of the disease in patients having advanced stage and lymph node positive tumors (table 4.14). However, *MDM2* SNP309 (T>G) genotypes were not associated with recurrence in early disease.

Table 4.14: Association of *MDM2* SNP309 (T>G) genotypes with disease recurrence according to various clinico-pathological parameters

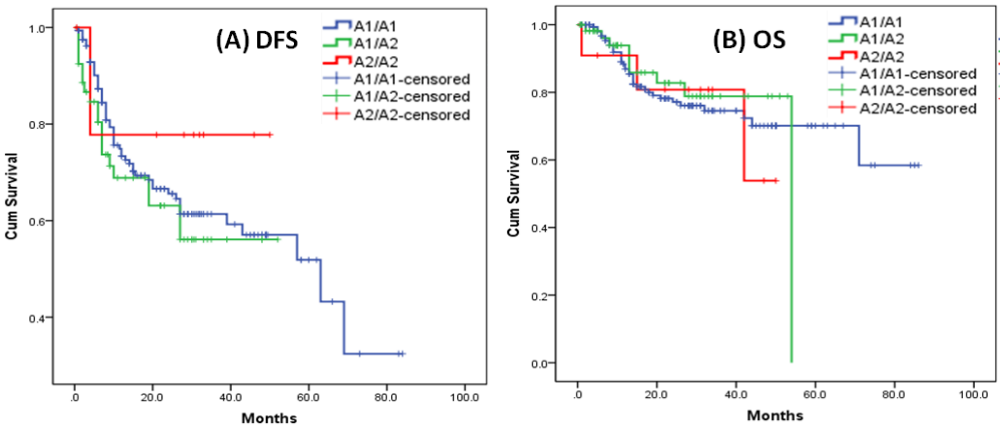
Genotype	Non-Recurrent No. (%)	Recurrent No. (%)	OR (95% CI)	*p* value
Differentiation (Moderate)				
G/G	20 (35.1)	9 (17.6)	1.0 (Referent)	
G/T	24 (42.1)	31 (60.8)	2.87 (1.11 – 7.42)	**0.03**
T/T	13 (22.8)	11 (21.6)	1.88 (0.61 – 5.79)	0.271
G/T + T/T	37 (64.9)	42 (82.4)	2.52 (1.02 – 6.22)	**0.045**
Total	57	51		
Tumor size (≥ 4 cm)				
G/G	17 (35.4)	6 (16.2)	1.0 (Referent)	
G/T	23 (47.9)	24 (64.9)	2.96 (1.00 – 8.81)	**0.052**
T/T	8 (16.7)	7 (18.9)	2.48 (0.63 – 9.80)	0.196
G/T + T/T	31 (64.6)	31 (83.8)	2.83 (1.00 – 8.14)	**0.053**
Total	48	37		
Disease stage (Advanced)				
G/G	25 (37.8)	9 (15.2)	1.0 (Referent)	
G/T	30 (44.1)	37 (62.7)	3.43 (1.40 – 8.40)	**0.007**
T/T	13 (19.1)	13 (22.0)	2.78 (0.94 – 8.20)	0.064
G/T + T/T	43 (63.2)	50 (84.7)	3.23 (1.36 – 7.66)	**0.008**
Total	68	59		
Lymph node metastasis (LN(+))				
G/G	15 (41.7)	7 (14.6)	1.0 (Referent)	
G/T	15 (41.7)	30 (62.5)	4.30 (1.44 – 12.78)	**0.009**
T/T	6 (16.7)	11 (22.9)	3.90 (1.03 – 15.00)	**0.045**
G/T + T/T	21 (58.3)	41 (85.4)	4.20 (1.48 – 11.84)	**0.007**
Total	36	48		
Invasion (Invasive)				
G/G	25 (34.7)	8 (16.3)	1.0 (Referent)	
G/T	32 (44.4)	29 (59.2)	2.83 (1.11 – 7.30)	**0.030**
T/T	15 (20.8)	12 (24.5)	2.50 (0.80 – 7.51)	0.103
G/T + T/T	47 (65.3)	41 (83.7)	2.73 (1.11 – 6.70)	**0.029**
Total	72	49		

1.3.3. Association of *p53* and *MDM2* genotypes with survival of oral cancer patients

It was observed that DFS and OS were higher in patients having A1/A1 genotype compared to patients having A1/A2 and A2/A2 genotypes at *p53* intron 3 locus (Figure 4.2). For exon 4 genotypes, DFS was lower in patients having Pro/Pro genotype (44.5 months) compared to patients having Arg/Pro genotype (55.7 months) (Figure 4.3), whereas, OS was higher in patients having Arg/Arg (64.3 months) and Arg/Pro genotypes (63.7 months) compared to patients having Pro/Pro genotype (54.6 months) (Figure 4.3). For intron 6, patients having G/G genotype had higher DFS and OS compared to patients having G/A and A/A genotypes (Figure 4.4). For *MDM2*

SNP309, DFS and OS were higher in patients having G/G genotype compared to patients having G/T and T/T genotypes (Figure 4.5).

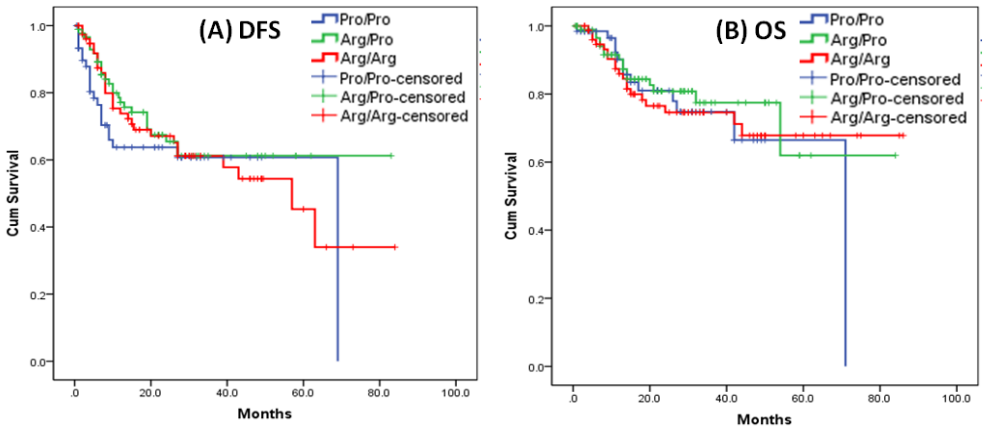
Figure 4.2: Disease free and overall survival of oral cancer patients according to *p53* intron 3 genotypes



Genotype	Disease Free Survival		Overall Survival	
	Mean survival (Months)	*p* value	Mean survival (Months)	*p* value
A1/A1	49.1	*0.429	64.0	*0.738
A1/A2	33.6	#0.412	45.6	#0.821
A2/A2	39.8	@0.312	39.9	@0.559

*A1/A1 vs. A1/A2;
#A1/A1 vs. A2/A2;
@A1/A2 vs. A2/A2

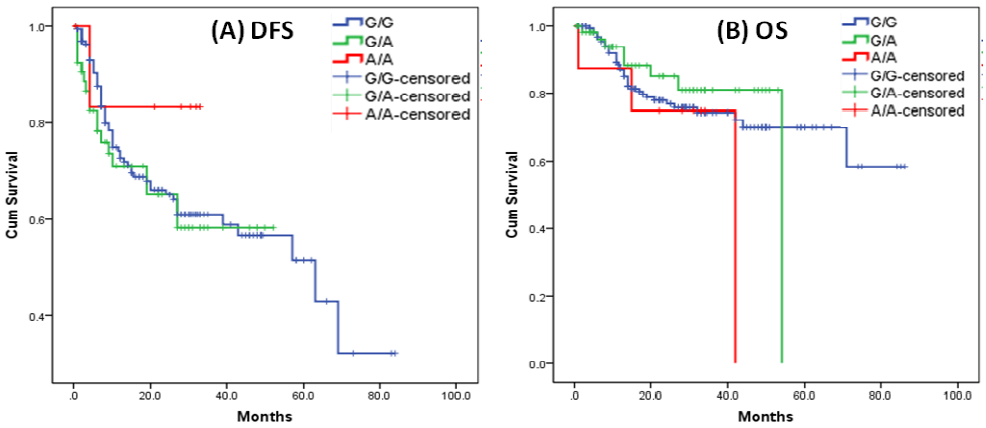
Figure 4.3: Disease free and overall survival of oral cancer patients according to *p53* exon 4 genotypes



Genotype	Disease Free Survival		Overall Survival	
	Mean survival (Months)	*p* value	Mean survival (Months)	*p* value
Arg/Arg	48.1	*0.619	64.3	*0.535
Arg/Pro	55.7	#0.507	63.7	#0.919
Pro/Pro	44.5	@0.301	54.6	@0.542

*Arg/Arg vs. Arg/Pro;
#Arg/Arg vs. Pro/Pro;
@Arg/Pro vs. Pro/pro

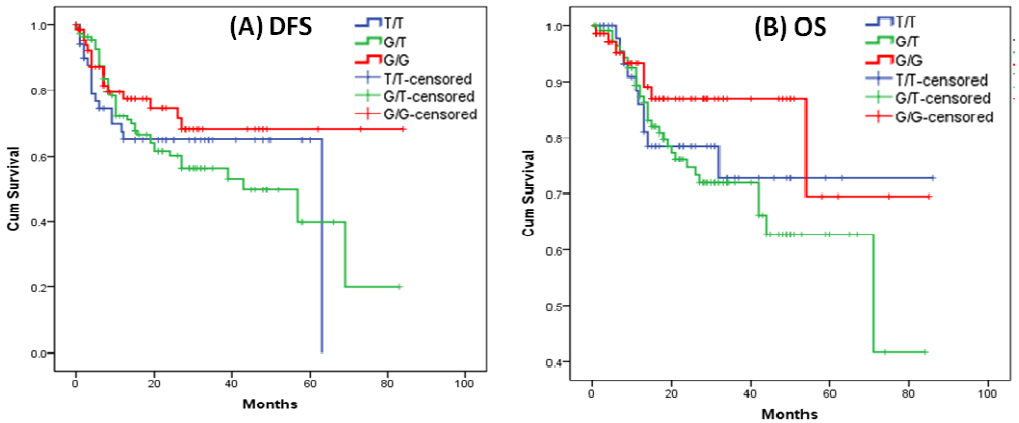
Figure 4.4: Disease free and overall survival of oral cancer patients according to *p53* intron 6 genotypes



Genotype	Disease Free Survival		Overall Survival	
	Mean Survival (Months)	*p* value	Mean Survival (Months)	*p* value
G/G	48.8	*0.622	63.9	*0.535
G/A	34.5	#0.361	46.5	#0.430
A/A	28.2	@0.312	33.5	@0.185

*G/G vs. G/A;
#G/G vs. A/A;
@G/A vs. A/A

Figure 4.5: Disease free and overall survival of oral cancer patients according to *MDM2* SNP309 genotypes



Genotype	Disease Free Survival		Overall Survival	
	Mean Survival (Months)	*p* value	Mean Survival (Months)	*p* value
G/G	60.6	*0.148	63.9	*0.535
G/T	43.6	#0.270	46.5	#0.430
T/T	42.7	@0.866	33.5	@0.185

*G/G vs. G/T;
#G/G vs. T/T;
@G/T vs. T/T

1.4. Gene-gene interaction (*p53* and *MDM2* polymorphisms) and risk of oral cancer

It was observed that Arg/Arg genotype of *p53* exon 4 in combination with T/T genotype of *MDM2* SNP309 showed marginal protective effect from oral cancer development (OR=0.38; 95%CI=0.14-1.07; p=0.067, table 4.15). However, no interaction was observed between *p53* intron 3 (16bp duplication), intron 6 (G>A) and *MDM2* SNP309 (T>G) polymorphisms.

Table 4.15: Gene-gene interaction between *p53* exon 4 (Arg72Pro) and *MDM2* SNP309 (T>G) polymorphisms and risk of oral cancer

Genotypes	Controls No.	Cases No.	OR (95% CI)	*p* value
Arg/Arg+G/G	14	23	1.0 (Referent)	
Arg/Pro +G/G	26	24	0.56 (0.23 – 1.34)	0.192
Pro/Pro+G/G	17	23	0.82 (0.33 – 2.05)	0.677
Arg/Arg +G/T	34	45	0.81 (0.36 – 1.79)	0.596
Arg/Pro +G/T	55	46	0.51 (0.24 – 1.10)	0.086
Pro/Pro +G/T	18	24	0.81 (0.33 – 2.00)	0.650
Arg/Arg +T/T	16	10	**0.38 (0.14 – 1.07)**	**0.067**
Arg/Pro +T/T	21	25	0.72 (0.30 – 1.75)	0.474
Pro/Pro +T/T	11	16	0.89 (0.32 – 2.44)	0.814

1.5. Association of gene-gene interaction (*p53* and *MDM2* polymorphisms) with clinico-pathological features, recurrence and survival of oral cancer patients

Results of earlier gene-gene interaction analysis suggested that interaction was present between *p53* Arg72Pro and *MDM2* SNP309 (T>G) polymorphisms (Table 4.15). Thus, the present study also evaluated the effect of this gene-gene interaction on oral cancer progression.

1.5.1. Gene-gene interaction between *p53* Arg72Pro and *MDM2* SNP309 (T>G) polymorphisms and their association with various clinico-pathological parameters

It was observed that interaction between *p53* Arg72Pro and *MDM2* SNP309 (T>G) polymorphisms could significantly affect the stage of oral cancer progression (Table 4.16). Patients having Pro/Pro genotype of *p53* with G/G and G/T genotypes of *MDM2* in combination had significantly high risk to have advanced stage (OR=4.16, 95% CI=1.13-15.30, p=0.032; OR=3.68, 95% CI=1.06-12.77, p=0.040, respectively, table 4.16). Further, patients having Arg/Arg genotype of *p53* with G/T genotype of *MDM2* in combination had also high risk to have advanced stage (OR=2.79, 95% CI=0.98-7.88, p=0.054, table 4.16). The effect of this gene-gene interaction on tumor differentiation, size, lymph node involvement and invasion was also analyzed; however, the results were not significant.

Table 4.16: Gene-gene interaction between *p53* Arg72Pro and *MDM2* SNP309 (T>G) genotypes and their association with tumor stage

Genotypes	Early No.	Advanced No.	OR (95% CI)	*p* value
Arg/Arg+G/G	13	10	1.0 (Referent)	
Arg/Pro +G/G	7	17	3.16 (0.95 -10.55)	0.062
Pro/Pro+G/G	5	16	**4.16 (1.13 – 15.3)**	**0.032**
Arg/Arg +G/T	14	30	**2.79 (0.98 – 7.88)**	**0.054**
Arg/Pro +G/T	16	28	2.28 (0.81 – 6.36)	0.117
Pro/Pro +G/T	6	17	**3.68 (1.06 – 12.77)**	**0.040**
Arg/Arg +T/T	4	6	1.95 (0.43 – 8.83)	0.386
Arg/Pro +T/T	8	17	2.76 (0.85 – 8.97)	0.091
Pro/Pro +T/T	6	10	2.17 (0.59 – 7.99)	0.246

1.5.2. Gene-gene interaction between *p53* Arg72Pro and *MDM2* SNP309 (T>G) polymorphisms and its association with disease recurrence

It was observed that Arg/Arg as well as Pro/Pro genotypes of *p53* in combination with G/T genotype of *MDM2* showed higher OR for oral cancer recurrence (OR=2.00, 95%CI=0.67-6.00; OR=1.81, 95%CI=0.52-6.33, respectively, table 4.17).

Table 4.17: Gene-gene interaction between *p53* Arg72Pro and *MDM2* SNP309 (T>G) genotypes and their association with disease recurrence

Genotypes	Non-Recurrent No.	Recurrent No.	OR (95% CI)	*p* value
Arg/Arg+G/G	14	7	1.0 (Referent)	
Arg/Pro +G/G	13	5	0.77 (0.19 - 3.04)	0.708
Pro/Pro+G/G	11	4	0.73 (0.17 - 3.13)	0.669
Arg/Arg +G/T	20	20	**2.00 (0.67 - 6.00)**	0.216
Arg/Pro +G/T	26	15	1.15 (0.38 - 3.49)	0.800
Pro/Pro +G/T	11	10	**1.81 (0.52 - 6.33)**	0.348
Arg/Arg +T/T	4	2	1.00 (0.15 - 6.85)	1.000
Arg/Pro +T/T	13	8	1.23 (0.35 - 4.36)	0.748
Pro/Pro +T/T	8	7	1.75 (0.45 - 6.82)	0.420

The present study also evaluated association of interaction between *p53* Arg72Pro and *MDM2* SNP309 (T>G) genotypes with recurrence of the disease in patients having advanced stage tumors.

Results suggested that patients with advanced stage of the disease and having Arg/Arg and Pro/Pro genotypes of *p53* in combination with G/T genotype of *MDM2* had significantly high risk to have recurrence (OR=6.61, 95%CI=1.13-38.69, p=0.036; OR=7.00, 95%CI=1.04-46.95, p=0.045, respectively, table 4.18).

Table 4.18: Gene-gene interaction between *p53* Arg72Pro and *MDM2* SNP309 (T>G) genotypes and their association with disease recurrence in advanced stage tumors

Genotypes	Non-Recurrent No.	Recurrent No.	OR (95% CI)	*p* value
Arg/Arg+G/G	7	2	1.0 (Referent)	
Arg/Arg +G/T	9	17	**6.61 (1.13 – 38.69)**	**0.036**
Arg/Arg +T/T	3	1	1.17 (0.07 – 18.35)	0.912
Arg/Pro +G/G	10	4	1.40 (0.19 – 9.87)	0.736
Arg/Pro +G/T	16	6	1.31 (0.21 – 8.18)	0.771
Arg/Pro +T/T	5	5	3.50 (0.47 – 25.90)	0.220
Pro/Pro+G/G	8	3	1.31 (0.17 – 10.26)	0.796
Pro/Pro +G/T	5	10	**7.00 (1.04 – 46.95)**	**0.045**
Pro/Pro +T/T	5	4	2.80 (0.36 – 21.73)	0.325

1.5.3. Gene-gene interaction between *p53* Arg72Pro and *MDM2* SNP309 (T>G) and its association with DFS and OS

Association of *MDM2* SNP309 (T>G) genotypes with DFS and OS was also evaluated according to *p53* exon 4 genotypes. It was observed that patients having Arg/Arg genotype had higher DFS compared to patients having Pro/Pro genotype at *p53* exon 4 locus in combination with T/T genotype at *MDM2* locus (Table 4.19). DFS was higher in patients with T/T genotype compared to patients with G/T genotype at *MDM2* locus in combination with Arg/Arg genotype at *p53* exon 4 locus. Also, OS was higher in patients with G/G genotype compared to patients with G/T genotype at *MDM2* locus in combination with Arg/Arg genotype at *p53* exon 4 locus (Table 4.19).

Table 4.19: Disease free and overall survival of oral cancer patients according to and *p53* Arg72Pro and *MDM2* SNP309 (T>G) genotypes in combination

	Disease Free Survival		Overall survival	
Genotypes	Mean survival (Months)	*p* value	Mean survival (Months)	*p* value
Arg/Arg+G/G	61.0		74.4	
Arg/Arg +G/T	35.1		48.3	
Arg/Arg +T/T	55.8		76.4	
Arg/Pro +G/G	46.9	*0.081	55.4	
Arg/Pro +G/T	54.2	$^{\#}$0.084	68.3	$^{@}$0.077
Arg/Pro +T/T	38.9		42.2	
Pro/Pro+G/G	22.1		24.6	
Pro/Pro +G/T	43.5		55.2	
Pro/Pro +T/T	23.3		36.0	

*=Arg/Arg+T/T vs. Pro/Pro+ T/T, #= Arg/Arg+T/T vs. Arg/Arg+ G/T,@= Arg/Arg+G/G vs. Arg/Arg+ G/T

1.6. *p53* gene mutations (exon 4-9) frequencies

Total 92 tissues (46 paired tissue samples) were studied for *p53* mutations by PCR-SSCP followed by DNA sequencing for exon 4 to 9. Sequencing data were analyzed using mutation surveyor software (version 4.0.9) by taking NM_000546 as a reference sequence.

The results revealed a total of 51 mutations (adjacent normal and malignant tissues) in a total of 24/46 (52.2%) oral cancer patients. Out of these 24, 11 patients had *p53* mutations only in malignant tissues, 6 patients had *p53* mutations only in adjacent normal tissues and 7 patients had *p53* mutations in both malignant as well as adjacent normal tissues. Mutations were maximally clustered in exon 4 (39.1%) followed by exon 5 (8.7%) (Table 4.20). Interestingly, we could not find any mutation in exon 6. We also found multiple *p53* mutations (two or more) in 15 cases. Gene sequencing data documented that out of the total mutations, 49 were point mutations (49/51; 96.07%) and 2 were frameshift mutations (2/49; 4.08%). Among the point mutations, majority were missense mutations (40/49; 81.6%) and 5/49 (10.2%) were silent mutations and 3/49 (6.1%) were splice-site mutations and 1/49 (2.04%) was nonsense mutation. Majority of these point mutations were transitions (40/49; 96.07%) followed by transversions (9/49; 4.08%). The most prevalent type of *p53* mutation observed in our study were C>T transition followed by T>C and then G>A transition; and G>T followed by G>C transversion (Table 4.20). Also, of these 49 point mutations, 5 occurred in CpG sequence (Table 4.21).

1.6.1. Novel mutations observed in oral cancer patients

Interestingly, 3 novel mutations which have still not been reported in IARC *p53* mutation database in exons 4 and 9 were also observed in four patients (out of these three novel mutations one is recurring). Among the novel mutations, one was frameshift mutation observed in exon 4 (c.326_delT) (sample no: 9M-14) (Figure 4.6A) resulting into truncating *p53* protein. Second was a missense mutation resulting into G>T transversion at codon 117 in exon 4 (sample no: 9M-1 and S-50) (Figure 4.6B). This transversion results into a non-functional *p53* protein. The third one was a silent mutation resulting into G>A transition at codon319 in exon 9 (sample no: 10M-43) (Figure 4.6C). These patients have noteworthy case histories as follows.

Case 1: Patient having c.326_delT mutation in exon 4 was a 33 year old male having both tobacco chewing and smoking habits. He was presented with advanced stage

tumor of lip with lymph node involvement and infiltration in skin and muscles. Patient underwent surgery followed by radiotherapy. Patient had 6 months DFS. Later the patient was lost to follow-up. This patient also had splice-site mutation in intron 8 (c.919+1G>T, Table 4.20).

Case 2: Two patients had G>T transversion at codon117 in exon 4. One patient was 46 year old male having exclusive tobacco chewing habit. This patient had early stage tumor involving multiple sites and underwent surgery, had 6 months DFS and then loss to follow-up. He also had multiple mutations in exon 4 (c.328 C>T and c.325T>A, Table 4.20).

Case 3: Another patient was 54 year old male having tobacco chewing habit. This patient had advanced stage tumor of tongue with lymph node involvement. Patient underwent surgery followed by radiotherapy. Patient had 4 months DFS and 8 months OS. This patient had multiple mutations in exon 5 (c.524 G>C and c.527G>T, Table 4.21) and exon 7 (c.678C>T and c.679T>C, Table 4.20).

Case 4: One patient had G>A transition at codon 319 in exon 9 (silent mutation). The patient is 42 year old male having tobacco chewing habit. This patient had advanced stage tumor of lip with lymph node involvement and infiltration in skin. The patient underwent surgery followed by radiotherapy and had no recurrence. Patient has 50 months of OS, still reports for follow-up and he has a good general condition. Also, this patient had only single mutation which is silent.

In addition, we also identified several noteworthy mutations at codons 90, 91, 109, 110, 116, 117, 168, 176, 179, 226, 227, 282 and 329;other than the codons considered as mutation hot spots (www: iarc.fr/*p53*). Besides several recurring mutation sites were detected in exon 4 (TCC to CCC at codon 90 in 9 cases resulting in a serine to proline substitution in a protein; codon 116 in 14 cases resulting in a serine to phenylalanine substitution in a protein) in our population (Table 4.20).

Table 4.20: Characteristics of the patients with *p53* mutations in oral cancer

Sample No.	Age/sex	Habit	Site	TNM	Differentiation	Exon	Tissue	Codon	Base Change	Codon alteration	Amino acid change	Mutation type	*p53* in surgical margin	Outcome (Months)	Recurrence (Months)
9M1	46/M	EC	Multiple	pT2N0M0	Moderate	4 4 4	M M N	110 109 117	c.328C>T c.325T>A c.349G>T	CGT>TGT TTC>ATC GGG>TGG	p.R110C p.F109I p.G117W	Missense Missense Missense	+	LTF (21)	Recurrence (6)
9M8	53/M	EC	Alveolus	pT4N0M0	Well	4 4	N N	90 116	c.268T>C c.347C>T	TCC>CCC TCT>TTT	p.S90P p.S116F	Missense Missense	+	LTF (25)	NAD (23)
9M9	45/M	EC	BM	cT4N1M0	Moderate	4 4	M M	90 116	c.268T>C c.347C>T	TCC>CCC TCT>TTT	p.S90P p.S116F	Missense Missense		LTF (1)	LTF (1)
9M14	33/M	MixCS	Lip	pT4N2M0	Well	4 I-8	M N	0	c.326delT c.919+1G>T	TTC>? -	p.F>? p.?	FS Splice	+	LTF (10)	Recurrence (6)
9M18	42/F	NHT	Alveolus	pT4N2M0	Moderate	4 I-8	M M	116 0	c.347C>T c.920-1G>C	TCT>TTT -	p.S116F p.?	Missense Splice		LTF (3)	Recurrence (2)
9M20	32/M	EC	Tongue	pT4N0M0	Well	8	M	282	c.844C>T	CGG>TGG	p.R282W	Missense		LTF (23)	NAD (22)
9M22	62/F	NHT	BM	pT3N2M0	Well	I-8	M	0	c.919+1G>T	-	p.?	Splice		Dead (11)	Recurrence (10)
10M43	42/M	EC	Lip	pT4N2M0	Well	9	N	319	c.957G>A	AAG>AAA	p.K319K	Silent	+	Alive (50)	NAD (49)
10M44	55/M	EC	Central Arch	pT2N1M0	Moderate	8	M	301	c.902_903ins1	CCA>?	p.P>?	FS		Dead (9)	Recurrence (5)
10M50	50/M	EC	BM	pT2N0M0	Well	4 4	N N	90 116	c.268T>C c.347C>T	TCC>CCC TCT>TTT	p.S90P p.S116F	Missense Missense	+	Alive (42)	NAD (41)
10M52	60/M	EC	Multiple	pT4N0M0	Moderate	4 4	N N	90 116	c.268T>C c.347C>T	TCC>CCC TCT>TTT	p.S90P p.S116F	Missense Missense	+	LTF (5)	LTF (4)
10M55	43/M	EC	Tongue	pT4N0M0	Moderate	4	M	116	c.347C>T	TCT>TTT	p.S116F	Missense		LTF (29)	NAD (28)
10M59	55/F	EC	BM	pT4N2M0	Moderate	5 4	M N,M	175 116	c.524G>A c.347C>T	CGC>CAC TCT>TTT	p.R175H p.S116F	Missense Missense	+	LTF (10)	Nodal Recurrence (6)
10M63	60/M	ES	Lip	pT2N0M0	Moderate	4 4	M M	116 90	c.347C>T c.268T>C	TCT>TTT TCC>CCC	p.S116F p.S90P	Missense Missense		Alive (36)	LTF (35)

10M68	73/M	EC	Alveolus	pT4N0M0	Moderate	4 4	N,M N,M	116 90	c.347C>T c.268T>C	TCT>TTT TCC>CCC	p.S116F p.S90P	Missense Missense	+	Alive (46)	NAD (46)
10M74	45/F	NHT	Tongue	pT4N1M0	Moderate	7 7 7	M M M	245 226 227	c.733G>A c.678C>T c.679T>C	GGC>AGC GGC>GGT TCT>CCT	p.G245S p.G226G p.S227P	Missense Silent Missense		LTF (1)	NAD (1)
S-2	55/M	ES	Multiple	pT2N0M0	Well	4	N,M	116	c.347C>T	TCT>TTT	p.S116F	Missense	+	Dead (6)	Recurrence (3)
S-5	32/M	EC	BM	pT2N1M0	Moderate	4	M	91	c.273G>A	TGG>TGA	p.W91*	Nonsense		Alive (63)	LTF (60)
S-6	67/M	EC	BM	pT2N2M0	Moderate	4 4	M M	116 90	c.347C>T c.268T>C	TCT>TTT TCC>CCC	p.S116F p.S90P	Missense Missense		LTF (4)	LTF (3)
S-39	31/M	MixCS	Multiple	pT2N1M0	-	5 5 4 4	M M M M	179 175 116 90	c.535C>T C.524G>C c.347C>T c.268T>C	CAT>TAT CGC>CCC TCT>TTT TCC>CCC	p.H179Y p.R175P p.S116F p.S90P	Missense Missense Missense Missense		Dead (13)	Recurrence (7)
S-50	54/M	EC	Tongue	pT2N2bM0	Well	5 5 4 7 7	M M N,M M M	175 176 117 226 227	C.524G>C C.527G>T c.349G>T c.678C>T c.679T>C	CGC>CCC TGC>TTC GGG>TGG GGC>GGT TCT>CCT	p.R175P p.C176F p.G117W p.G226G p.S227P	Missense Missense Missense Silent Missense	+	Dead (8)	Recurrence (4)
S-54	28/M	MixCS	Alveolus	pT2N1M0	Moderate	5 4	N,M N	168 116	c.504C>T c.347C>T	CAC>CAT TCT>TTT	p.H168H p.S116F	Silent Missense	+	LTF (4)	Recurrence (2)
S-58	34/M	EC	Multiple	pT4N0M0	-	4	N	116	c.347C>T	TCT>TTT	p.S116F	Missense	+	LTF (44)	Recurrence (4)
S-64	28/M	MixCS	Tongue	pT1N1M0	Moderate	4	N	90	c.268T>C	TCC>CCC	p.S90P	Missense	+	LTF (22)	NAD (22)

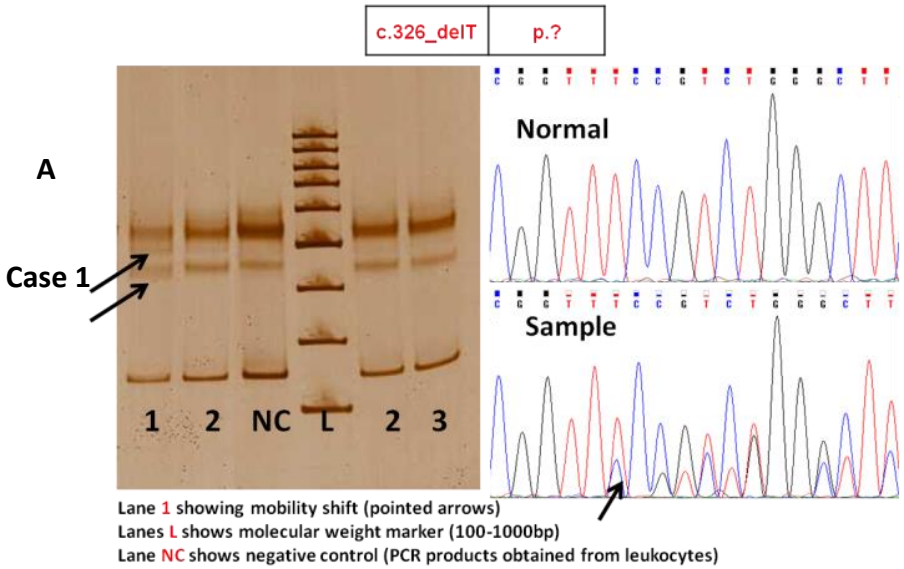
EC: Exclusive chewers; NHT: No habit of tobacco; ES: Exclusive smokers; MixCS (chewer and smoker); BM: Buccal mucosa; Multiple: involving more than one site; NAD: Not any disease; LTF: Lost to follow up; FS: Frame shift; N: adjacent normal tissue, M: Malignant tissue

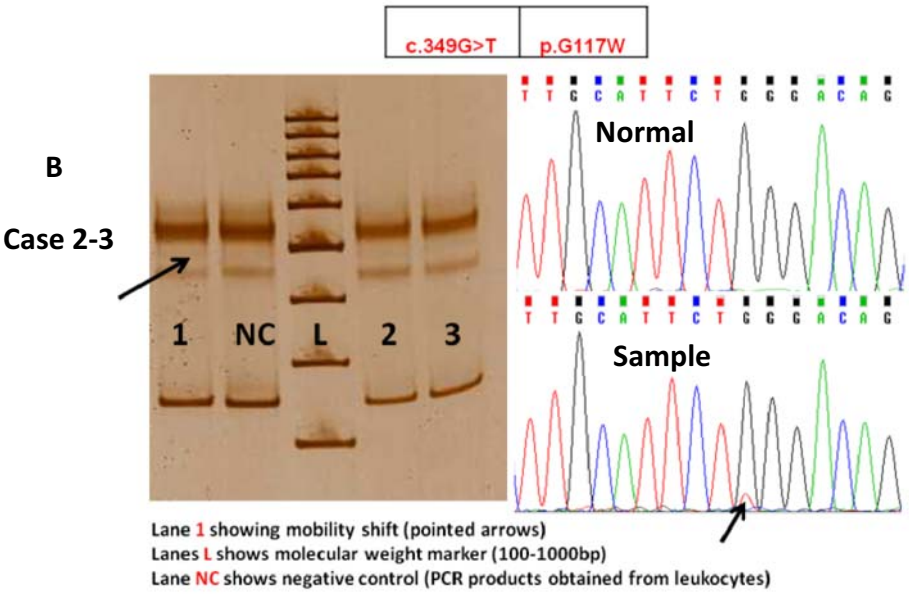
Table 4.21: Types of mutations observed in the *p53* gene (exon 4-9)

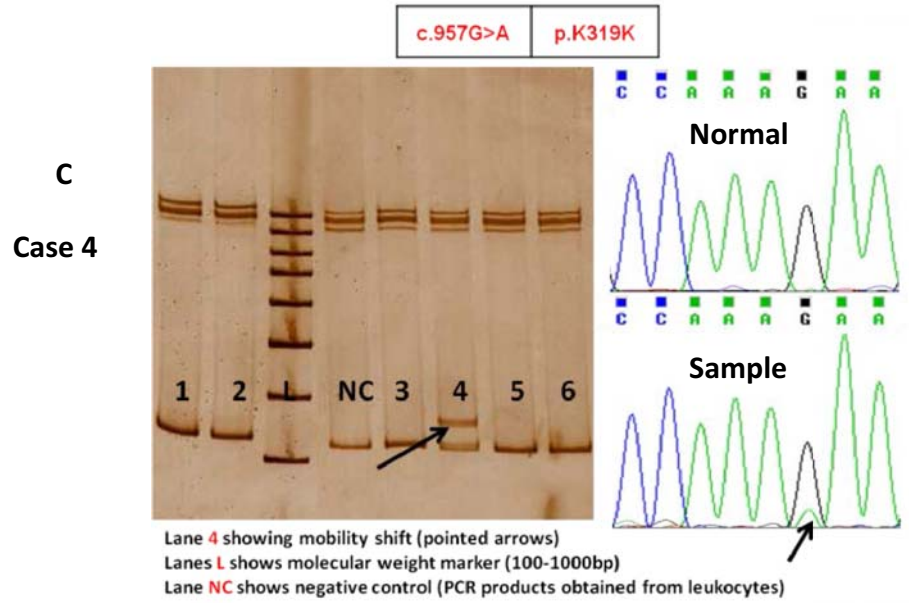
No of Samples	Tissues	c.DNA Description	Protein Description	Exons	Splice site	CpG site	Effect	Mutation classification	Transcriptional activation
9	N:4, M:4, N&M:1	c.268T>C	p.S90P	4	no	no	missense	neutral	functional
1	M	c.273G>A	p.W91*	4	no	no	nonsense	NA	NA
1	M	c.325T>A	p.F109I	4	no	no	missense	deleterious	non-functional
1	M	c.328C>T	p.R110C	4	no	yes	missense	deleterious	non-functional
14	N:5, M:6,N&M:3	c.347C>T	p.S116F	4	no	no	missense	deleterious	functional
1	**M**	**c.326_delT**	**p.?**	**4**	**NA**	**NA**	**FS**	**NA**	**NA**
2	**N:1, N&M:1**	**c.349G>T**	**p.G117W**	**4**	**no**	**no**	**missense**	**deleterious**	**non-functional**
2	M	c.524G>C	p.R175P	5	no	yes	missense	deleterious	non-functional
1	M	c.527G>T	p.C176F	5	no	no	missense	deleterious	partially functional
1	N&M	c.504C>T	p.H168H	5	no	no	silent	NA	NA
1	M	c.524G>A	p.R175H	5	no	yes	missense	deleterious	non-functional
1	M	c.535C>T	p.H179Y	5	no	no	missense	deleterious	partially functional
2	M	c.678C>T	p.G226G	7	no	no	silent	NA	NA
2	M	c.679T>C	p.S227P	7	no	no	missense	deleterious	partially functional
1	M	c.733G>A	p.G245S	7	no	yes	missense	deleterious	non-functional
1	M	c.844C>T	p.R282W	8	no	yes	missense	deleterious	non-functional
1	M	c.897_898ins1	p.?	8	no	no	FS	NA	NA
1	**N**	**c.957G>A**	**p.K319K**	**9**	**no**	**no**	**silent**	**NA**	**NA**
2	N:1, M:1	c.919+1G>T	p.?	I8	Consensus SD	no	splice	NA	NA
1	M	c.920-1G>C	p.?	I8	Consensus SA	no	splice	NA	NA

FS: Frame shift, NA: Not available, N: adjacent normal tissue, M: Malignant tissue, SD: splice donor site, SA: splice acceptor site

Figure 4.6: Pattern of PCR- SSCP gels and DNA sequencing pattern for novel *p53* mutations







1.6.2. Association of *p53* mutation status with age of onset and tobacco habits

We studied a relationship between age and *p53* mutation status. For this, the patients were categorized according to the age groups. Median age (45 years) of the cohort was considered as the cut-off. Out of the total (46) oral cancer patients, 25 (54.3%) patients were below 45 years of age while 21 (45.7%) patients were above 45 years of age. However, no relation was observed (χ^2=0.104; p=0.74). Likewise, we grouped the patients according to tobacco habits. The cohort had 40 cases with tobacco habits (exclusive tobacco chewers n:26; exclusive tobacco smokers n:4 and mixed habits n: 10) and 6 cases with no tobacco habits. χ^2 analysis revealed no relation between tobacco habits and *p53* mutations. However, tobacco chewers were at higher risk of acquiring *p53*mutations (OR=1.42; 95% CI=0.24-8.26; p=0.69). C to T transition was the most

predominant mutation observed and associated with tobacco chewing (12/17). Of these 12 patients, 8 also had additional T>C transition.

1.7. Association of *p53* mutations with clinico-pathological features, recurrence and survival of oral cancer patients

1.7.1. Association of *p53* mutation status with clinico-pathological parameters and recurrence

We also explored the relationship between frequency of *p53* mutations and clinico-pathological features, including tumor size, degree of differentiation, lymph node involvement, stage and mode of invasion. It was observed that the frequency of *p53* mutations was higher in moderately differentiated (58.3%), in advanced stage tumor (55.6%) and also in tumors having lymph node involvement (66.7%). Moreover, tumors with *p53* mutations had high risk to develop lymph node metastasis (OR=3.0, 95%CI=0.9-10.1, p=0.075) (Table 4.22).

Table 4.22: Association of *p53* mutation with clinico-pathological parameters of oral cancer patients

	No. (%)	**No. (%)**	**χ^2 *p* value**	**OR (95%CI)**	***p* value**
Tumor differentiation					
	Well	**Moderate**			
Wild *p53*	9 (52.9%)	10 (41.7%)	0.155	1.0 (Referent)	0.477
Mutant *p53*	8 (47.1%)	14 (58.3%)		**1.6 (0.5 - 5.5)**	
Total	17	24			
Disease stage					
	Early	**Advanced**			
Wild *p53*	6 (60.0%)	16 (44.4%)	0.610	1.0 (Referent)	0.388
Mutant *p53*	4 (40.0%)	20 (55.6%)		**1.9 (0.5 - 7.8)**	
Total	10	36			
Lymph node (LN) metastasis					
	LN (-)	**LN (+)**			
Wild *p53*	15 (60.0%)	7 (33.3%)	0.130	1.0 (Referent)	**0.075**
Mutant *p53*	10 (40.0%)	14 (66.7%)		**3.0 (0.9 - 10.1)**	
Total	25	21			

Further, we also evaluated relationship of *p53* mutation with recurrence of disease by stratifying the patients according to various clinico-pathological variables (Table 4.23). It was observed that presence of *p53* mutations was higher in recurrent well differentiated (66.7%), small (85.7%), lymph node positive (50%), localized (85.7%) and early stage tumors (100%). Interestingly, small tumors with *p53* mutations had significantly high risk to have recurrence compared to small tumors with wild-type *p53* gene (OR=15.0, 95%CI=1.03-218.3, p=0.047) (Table 4.23). However, presence of *p53* mutations did not associate with recurrence in advanced disease.

Table 4.23: Association of *p53* mutation with recurrence of the disease

	Non-recurrent No. (%)	Recurrent No. (%)	χ^2 *p* value	OR (95%CI)	*p* value
Differentiation (Well)					
Wild *p53*	7 (63.6)	2 (33.3)	0.492	1.0 (Referent)	0.241
Mutant *p53*	4 (36.4)	4 (66.7)		3.5(0.4 - 28.5)	
Tumor Size (<4 cm)					
Wild *p53*	5 (71.4)	1 (14.3)	0.105	1.0 (Referent)	
Mutant *p53*	2 (28.6)	6 (85.7)		**15.0(1.03 - 218.3)**	**0.047**
Lymph node metastasis (-) (LN(-))					
Wild *p53*	11 (68.8)	3 (50.0)	0.130	1.0 (Referent)	0.420
Mutant *p53*	05 (31.2)	3 (50.0)		2.2 (0.3 - 15.0)	
Invasion (Localized)					
Wild *p53*	3 (100)	1 (14.3)	**0.067**	NA	
Mutant *p53*	0(0)	6 (85.7)			
Disease Stage (Early)					
Wild *p53*	5 (83.3)	0 (0)	0.206	NA	
Mutant *p53*	1 (16.7)	2 (100)			

Moreover, it was also observed that all five patients harboring truncating mutations had lymph node involvement and they had advanced disease. Out of these five patients, 4 patients had recurrence and 1 patient was loss to follow up. Additionally, total 13 patients had *p53* gene mutations in adjacent normal tissues also (6 had mutations only in adjacent normal and 7 had mutations both in adjacent normal and malignant tissues). Of the 6 cases having mutations only in adjacent normal tissues, only one had recurrence (Sample no: S-58, table 4.20). Of the 7 cases with mutations in both adjacent and malignant tissues, 6 developed recurrence. Of these, three were the cases where we identified novel mutations and interestingly in two cases these were present in adjacent normal tissues (9M-1 and S-50, table 4.20).

1.7.2. Association of *p53* mutation status with disease free and overall survival of oral cancer patients

Present study also evaluated association of *p53* mutation status of tumors with DFS and OS of oral cancer patients. In the present study, all patients were followed-up for 5 years. It was observed that 20 (43.5%) patients had loco-regional recurrence, 20 (43.5%) patients had no recurrence and 6 (13.0%) patients were lost to follow-up. Further, based on the information available in IARC database, *p53* mutations were also classified as deleterious, truncating, transcriptionally active and non-active type (Table 4.21).

DFS and OS were low in patients having mutant *p53* as compared to those having wild-type *p53* (Figure 4.7). Further, DFS and OS were low in patients having deleterious,

missense, truncating, transcriptionally active and transcriptionally non-active mutations as compared to wild-type *p53* gene. This was significant for comparison made between truncating and transcriptionally non-active mutations versus wild-type *p53* gene (Figure 4.8 and 4.9).

Figure 4.7: Disease free and overall survival of oral cancer patients according to *p53* mutation status

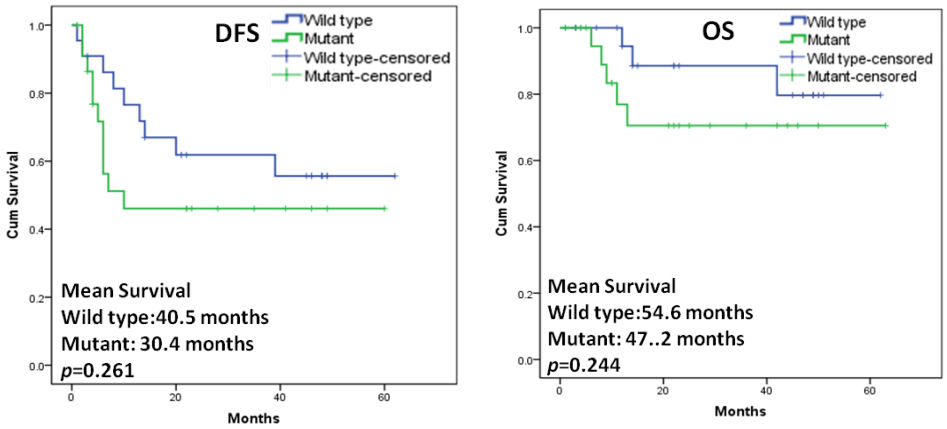


Figure 4.8: Disease free and overall survival of oral cancer patients according to *p53* mutations status (Truncating mutations vs. Wild type)

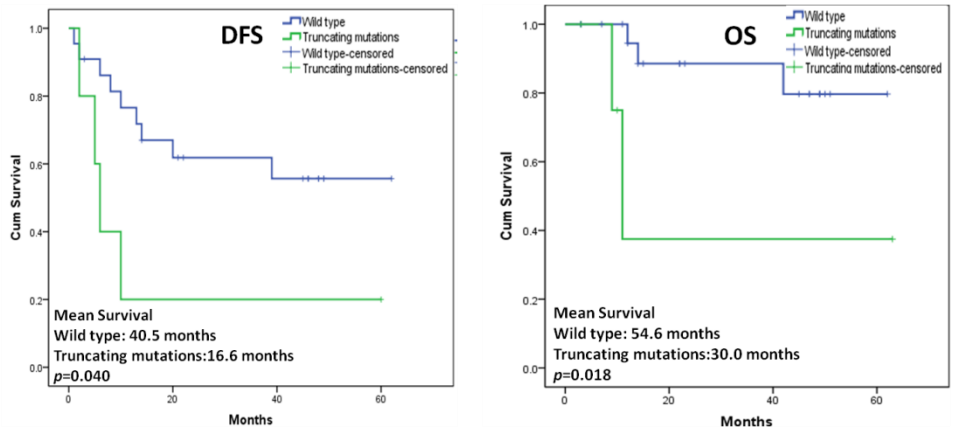
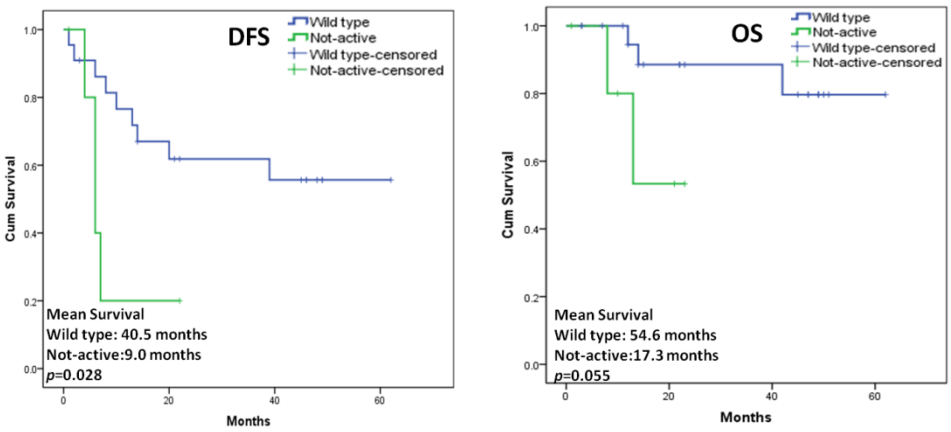


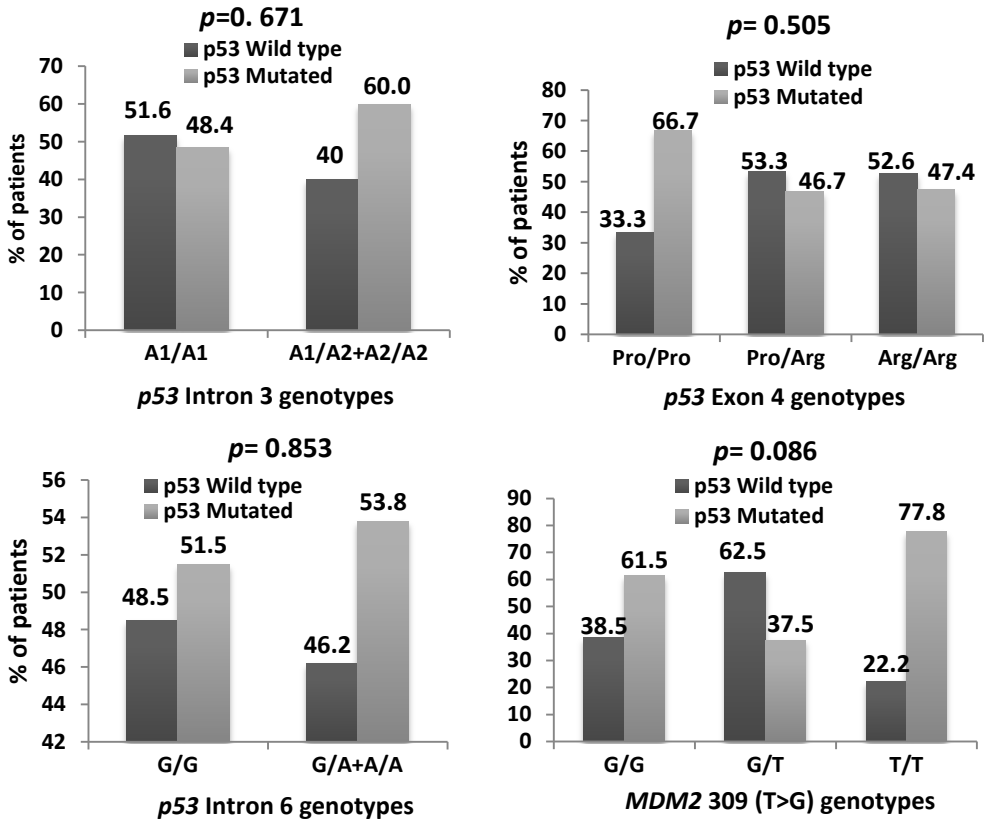
Figure 4.9: Disease free and overall survival of oral cancer patients according to *p53* mutations status (Transcriptionally not-active vs. Wild type)



1.8. Association of *p53* mutations with *p53* and *MDM2* polymorphisms

It was observed that patient harboring A1/A2+A2/A2 and G/A+A/A genotypes at intron 3 and intron 6 loci, respectively had higher number of *p53* mutations (60% and 53.8%, respectively, Figure 4.10). Also, frequency of *p53* mutations was also higher in patients having Pro/Pro genotype (66.7%) and T/T genotype (77.8%) at *p53* exon 4 and *MDM2* SNP309 loci (Figure 4.10).

Figure 4.10: Association of *p53* mutations frequency with *p53* and *MDM2* genotypes



Association between *p53*, *MDM2* polymorphisms and *p53* mutation status and its effect on oral cancer progression was also evaluated. It was observed that more number of patients having A2 allele in combination with mutant *p53* were in advanced stage, had lymph node involvement and recurrence (Table 4.24).

Table 4.24: Interaction between *p53* intron 3 genotypes and *p53* mutation status and their association with various clinico-pathological parameters and recurrence in oral cancer patients

***p53* intron 3 Genotypes* *p53* Mutation**	**No. (%)**	**No. (%)**	**OR (95% CI)**	***p* value**
Disease Stage				
	Early	**Advanced**		
A1/A1*wild	3 (30.0)	13 (36.1)	1.0 (Referent)	
A1/A1*mutant	4 (40.0)	11 (30.6)	0.63 (0.12 - 3.47)	0.600
A1/A2 + A2/A2*wild	3 (30.0)	3 (8.3)	0.23 (0.03 - 1.76)	0.158

A1/A2 + A2/A2*mutant	0 (00.0)	9 (25.0)	-	-
Total	10	36		
Lymph node (LN) metastasis				
	LN (-)	**LN(+)**		
A1/A1*wild	9 (36.0)	7 (33.3)	1.0 (Referent)	
A1/A1*mutant	8 (32.0)	7 (33.3)	1.13 (0.27 - 4.64)	0.871
A1/A2 + A2/A2*wild	6 (24.0)	0 (00)		
A1/A2 + A2/A2*mutant	2 (8.0)	7 (33.3)	**4.50 (0.70 – 28.80)**	0.112
Total	25	21		
Disease recurrence				
	Non-recurrent	**Recurrent**		
A1/A1*wild	7 (35.0)	8 (40.0)	1.0 (Referent)	
A1/A1*mutant	6 (30.0)	5 (25.0)	0.73 (0.15-3.47)	0.692
A1/A2 + A2/A2*wild	5 (25.0)	1 (5.0)	0.18 (0.02 – 1.88)	0.150
A1/A2 + A2/A2*mutant	2 (10.0)	6 (30.0)	2.63 (0.39-17.46)	0.318
Total	20	20		

More number of patients having Arg/Arg genotype in combination with mutant *p53* were in advanced stage and had lymph node involvement. Patients having proline allele in combination with wild *p53* had lower risk to have recurrence (OR=0.17, 95%CI=0.02-1.12, *p*=0.065, Table 4.25).

Table 4.25: Interaction between *p53* exon 4 genotypes and *p53* mutation status and their association with various clinico-pathological parameters and recurrence in oral cancer patients

***p53* exon 4 Genotypes* *p53* Mutation**	**No. (%)**	**No. (%)**	**OR (95% CI)**	***p* value**
Disease stage				
	Early	**Advanced**		
Arg/Arg*wild	2 (20.0)	8 (22.2)	1.0 (Referent)	
Arg/Arg*mutant	1 (10.0)	8 (22.2)	2.00 (0.15 – 26.74)	0.600
Arg/Pro+Pro/Pro*wild	4 (40.0)	8 (22.2)	0.50 (0.07 – 3.55)	0.488
Arg/Pro+Pro/Pro *mutant	3 (30.0)	12 (33.3)	1.00 (0.13 – 7.39)	1.000
Total	10	36		
Lymph node (LN) metastasis				
	LN (-)	**LN(+)**		
Arg/Arg*wild	5 (20.0)	5 (23.8)	1.0 (Referent)	
Arg/Arg*mutant	3 (12.0)	6 (28.6)	2.00 (0.31-12.84)	0.465
Arg/Pro+Pro/Pro*wild	10 (40.0)	2 (9.5)	0.20 (0.03-1.42)	0.108
Arg/Pro+Pro/Pro *mutant	7 (28.0)	8 (38.1)	1.14 (0.23-5.67)	0.870
Total	25	21		
Disease recurrence				
	Non-recurrent	**Recurrent**		
Arg/Arg*wild	3 (15.0)	6 (30.0)	1.0 (Referent)	
Arg/Arg*mutant	3 (15.0)	3 (15.0)	0.50 (0.06 – 4.15)	0.521
Arg/Pro+Pro/Pro*wild	9 (45.0)	3 (15.0)	**0.17 (0.02 – 1.12)**	**0.065**
Arg/Pro+Pro/Pro *mutant	5 (25.0)	8 (40.0)	0.80 (0.13 – 4.75)	0.806
Arg/Arg*wild	20	20		

Also, patients having A allele in combination with mutant *p53* had high frequency to have advanced stage, lymph node involvement and recurrence (Table 4.26).

Table 4.26: Interaction between *p53* intron 6 genotypes and *p53* mutation status and their association with various clinico-pathological parameters and recurrence in oral cancer patients

***p53* intron 6 Genotypes* *p53* Mutation**	**No. (%)**	**No. (%)**	**OR (95% CI)**	***p* value**
Disease stage				
	Early	**Advanced**		
G/G*wild	3 (30.0)	13 (36.1)	1.0 (Referent)	
G/G*mutant	4 (40.0)	13 (36.1)	0.75 (0.14 – 4.04)	0.738
G/A+A/A*wild	3 (30.0)	3 (8.3)	0.23 (0.03 – 1.76)	0.158
G/A+A/A*mutant	0 (00.0)	7 (19.4)		
Total	10	36		
Lymph node (LN) metastasis				
	LN (-)	**LN(+)**		
G/G*wild	9 (36.0)	7 (33.3)	1.0 (Referent)	
G/G*mutant	8 (32.0)	9 (42.9)	1.44 (0.37 – 5.70)	0.598
G/A+A/A*wild	6 (24.0)	0 (00)		
G/A+A/A*mutant	2 (8.0)	5 (23.8)	3.21 (0.47 – 21.80)	0.232
Total	25	21		
Disease recurrence				
	Non-recurrent	**Recurrent**		
G/G*wild	7 (35.0)	8 (40.0)		
G/G*mutant	6 (30.0)	7 (35.0)	1.02 (0.23 – 4.53)	0.978
G/A+A/A*wild	5 (25.0)	1 (5.0)	0.18 (0.02 – 1.88)	0.150
G/A+A/A*mutant	2 (10.0)	4 (20.0)	1.75 (0.24 – 12.64)	0.579
Total	20	20		

Patients having G/G genotype in combination with mutant *p53* had higher frequency to have moderately differentiated tumors and lymph node involvement. It was also observed that patients having G/G genotype in combination with mutant *p53* had high risk to progress towards advanced stage (Table 4.27). Also, patients having T allele in combination with mutant *p53* had higher frequency to have moderate differentiation, advanced stage, lymph node involvement as well as recurrence (Table 4.27).

Table 4.27: Interaction between *MDM2* SNP309 (T>G) genotypes and *p53* mutation status and their association with various clinico-pathological parameters and recurrence of oral cancer patients

***MDM2* Genotypes* *p53* Mutation**	**No. (%)**	**No. (%)**	**OR (95% CI)**	***p* value**
Tumor differentiation				
	Well	**Moderate**		
G/G*wild	3 (17.6)	2 (8.3)	1.0 (Referent)	
G/G*mutant	3 (17.6)	4 (16.7)	2.0 (0.19 - 20.61)	0.560
G/T + T/T *wild	6 (35.3)	8 (33.3)	2.0 (0.25 - 15.99)	0.513

G/T + T/T *mutant	5 (29.4)	10 (41.7)	3.0 (0.37 - 24.17)	0.302
Total	17	24		
Disease stage				
	Early	**Advanced**		
G/G*wild	2 (20.0)	3 (8.3)	1.0 (Referent)	
G/G*mutant	1 (10.0)	7 (19.4)	4.67 (0.3 – 73.4)	0.273
G/T + T/T *wild	4 (40.0)	13 (36.1)	2.17 (0.3 – 17.9)	0.473
G/T + T/T *mutant	3 (30.0)	13 (36.1)	2.89 (0.32– 25.7)	0.341
Total	10	36		
Lymph node (LN) metastasis				
	LN (-)	**LN(+)**		
G/G*wild	5 (20.0)	0 (0.0)	NA	
G/G*mutant	3 (12.0)	5 (23.8)		
G/T + T/T *wild	10 (40.0)	7 (33.3)		
G/T + T/T *mutant	7 (28.0)	9 (42.9)		
Total	25	21		
Disease recurrence				
	Non-recurrent	**Recurrent**		
G/G*wild	4 (20.0)	0 (0.0)	NA	
G/G*mutant	4 (20.0)	3 (15.0)		
G/T + T/T *wild	8 (40.0)	9 (45.0)		
G/T + T/T *mutant	4 (20.0)	8 (40.0)		
Total	20	20		

Further, present study also evaluated DFS and OS associated with *p53* and *MDM2* genotypes and mutation status of tumors. It was observed that DFS was low in patients having mutant *p53* (10.4 months) compared to patients having wild type *p53* (40.2 months) in combination with A1/A2+A2/A2 genotypes in intron 3 (p=0.075, Table 4.28). However, there is no difference in DFS in patients having A1/A1 genotype with respect to *p53* mutation status. Further, DFS was also low in patients having A1/A2+A2/A2 genotypes (10.4 months) compared to patients having A1/A1 genotype (38.1) in combination with mutant *p53* (Table 4.28). However, there is no difference in DFS in patients having wild *p53* with respect to intron 3 genotypes.

It was observed that DFS was low in patients having mutant *p53* (18.6 months) compared to patients having wild type *p53* (48.6 months) in combination with Arg/Pro+Pro/Pro genotypes in exon 4 (p=0.076, Table 4.28). However, DFS was higher in patients having mutant *p53* (38.1 months) compared to patients having wild type *p53* (27.3 months) in combination with Arg/Arg genotype in exon 4 (Table 4.28). Further, DFS was also low in patients having Arg/Pro+Pro/Pro genotypes (18.6 months) compared to patients having Arg/Arg genotype (38.1 months) in combination with

mutant *p53* (Table 4.28). However, DFS was higher in patients having Arg/Pro+Pro/Pro genotypes (48.6 months) compared to patients having Arg/Arg genotype (27.3 months) in combination with wild *p53* (Table 4.28). OS was low in patients having mutant *p53* (37.2 months) compared to patients having wild *p53* (57.2 months) in combination with Arg/Pro+Pro/Pro genotypes (Table 4.28). However, there is no difference in OS in patients having Arg/Arg genotype with respect to mutant *p53*. Further, OS was low in patients having Arg/Arg genotype (42.8 months) compared to patients having Arg/Pro+Arg/Arg genotypes (57.2 months) in combination with wild *p53* gene (Table 4.28). However, there is no difference in OS in patients having mutant *p53* with respect to exon 4 genotypes.

Further, DFS was also low in patients having mutant *p53* (12.3 months) compared to patients having wild type *p53* (40.2 months) in combination with G/A+A/A genotypes in intron 6 (Table 4.28). However, there is no difference in DFS in patients having G/G genotype with respect to *p53* mutation status. Further, DFS also low in patients having G/A+A/A genotypes (12.3 months) compared to patients having G/G genotype (33.5 months) in combination with mutant *p53* (Table 4.28). However, there is no difference in DFS in patients having wild *p53* with respect to intron 6 genotypes.

For intron 3 and intron 6, OS was not calculated as there was no death in patients having wild type *p53* with A1/A2+A2/A2 genotypes in intron 3 and A/G+G/G genotypes at intron 6. Similarly, there was no recurrence as well as death in patients having wild type *p53* with *MDM2* G/G genotype.

Table 4.28: Disease free and overall survival of oral cancer patients according to *p53* genotypes and mutations status

Genotypes	**Disease Free Survival**				**Overall Survival**			
	Mean Survival	*p* value	Mean Survival	*p* value	Mean Survival	*p* value	Mean Survival	*p* value
	A1/A1		**A1/A1 + A1/A2**		**A1/A1**		**A1/A1 + A1/A2**	
Wild *p53*	36.0	0.810	40.2	**0.075**				
Mutant *p53*	38.1		10.4					
	Wild *p53*		**Mutant *p53***		**Wild *p53***		**Mutant *p53***	
A1/A1	36.0	0.220	38.1	0.104				
A1/A1 + A1/A2	40.2		10.4					
	Arg/Arg		**Arg/Pro+Pro/Pro**		**Arg/Arg**		**Arg/Pro+Pro/Pro**	
Wild *p53*	27.3	0.594	48.6	**0.076**	42.8	0.325	57.2	0.471
Mutant *p53*	38.1		18.6		39.7		37.2	
	Wild *p53*		**Mutant *p53***		**Wild *p53***		**Mutant *p53***	
Arg/Arg	27.3	0.125	38.1	0.350	42.8	0.358	39.7	0.279
Arg/Pro+Pro/Pro	48.6		18.6		57.2		37.2	

	G/G		A/G + A/A		G/G		A/G + A/A	
Wild *p53*	36.0	0.782	40.2	0.159				
Mutant *p53*	33.5		12.3					
	Wild *p53*		**Mutant *p53***		**Wild *p53***		**Mutant *p53***	
G/G	36.0	0.220	33.5	0.415				
A/G + A/A	40.2		12.3					

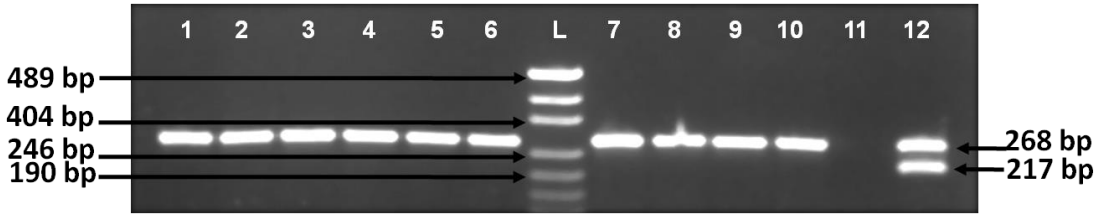
1.9. HPV status of the oral cancer patients and its association with *p53*

Total of 97 cases with oral cancer were screened for HPV 16 and 18 infections using highly sensitive TS-PCR based assay. Oral cancer cohort screened for HPV prevalence involved 84 (86.6%) males with age range 28 to 75 years (mean 46.8 years) and 13 (13.4%) females with age range 22 to 62 years (mean 46.3 years). Patients were also categorized according to age group. Median age (46 years) of the cohort was considered as cut-off. Out of 97 oral cancer patients, 48 (49.5%) patients were below 46 years of age while 49 (50.5%) patients were above 46 years of age. Majority of patients had buccal mucosa as a primary site (40.2%) followed by tongue (20.6%).

1.9.1.HR-HPV type 16 and 18 infections did not present in oral cancers patients

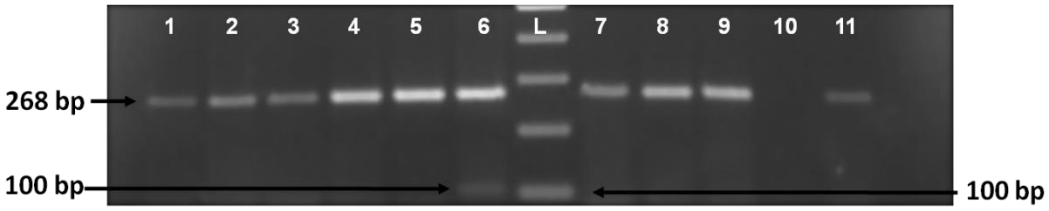
We have screened all 97 oral carcinoma tissues for HPV 16 and HPV 18 infection by TS-PCR method. Figures 4.11 and 4.12 are representative figures for HPV 16 and HPV 18 genotyping, respectively. Positive HPV 16 and 18 infections were interpreted by the presence of 217 bp and 100 bp bands, respectively along with 268 bp band of β-globin gene (Figures 4.11 and 4.12). Presence of only one band of β-globin gene (268 bp) indicated absence of HPV 16 and 18 infections. Interestingly, we did not find any of the oral cancer patients with infection of HPV 16 and 18. These results suggest that the prevalence of HPV 16 and 18 associated oral cancers is absent or rare in this population. Thus, present study was unable to analyze association of HPV infection with *p53* mutations.

Figure 4.11: Representative pattern for the HPV16 genotyping by PCR



Lane 1-10 show band of β globin gene (268 bp) from oral carcinoma tissues
Lane 12 = Positive control, show bands of HPV16 gene (217 bp) and β globin gene (268 bp) from cervical carcinoma tissues
Lane 11 = Negative control
Lane L = PUC19/MspI digest DNA ladder

Figure 4.12: Representative pattern for the HPV18 genotyping by PCR



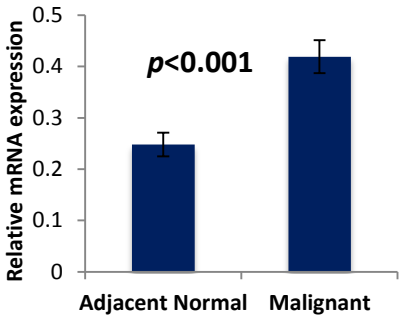
Lane 1, 2, 3, 4, 5, 7, 8, 9, 11 show band of β globin gene (268 bp) from oral carcinoma tissues.
Lane 6 = Positive control, show bands of HPV18 gene (100 bp) and β globin gene (268 bp) from cervical carcinoma tissue
Lane 10 = Negative control
Lane L = 100bp DNA ladder

2. To investigate the expression levels of genes involved in major hallmarks of cancer i.e. immortalization (*hTERT*), angiogenesis (*VEGFA*, *VEGFC* and *VEGFD*) invasion and metastasis (*MMP2* and *MMP9*) in oral cancer patients

2.1. *hTERT* mRNA levels in oral cancer patients

Expression of *hTERT* was analyzed by semi-quantitative RT-PCR from adjacent normal and malignant tissues. Transcript levels of *hTERT* were significantly higher in malignant tissues as compared to the adjacent normal tissues ($p<0.001$) (Figure 4.13).

Figure 4.13: Transcript levels of *hTERT* in adjacent normal and malignant oral carcinoma tissues

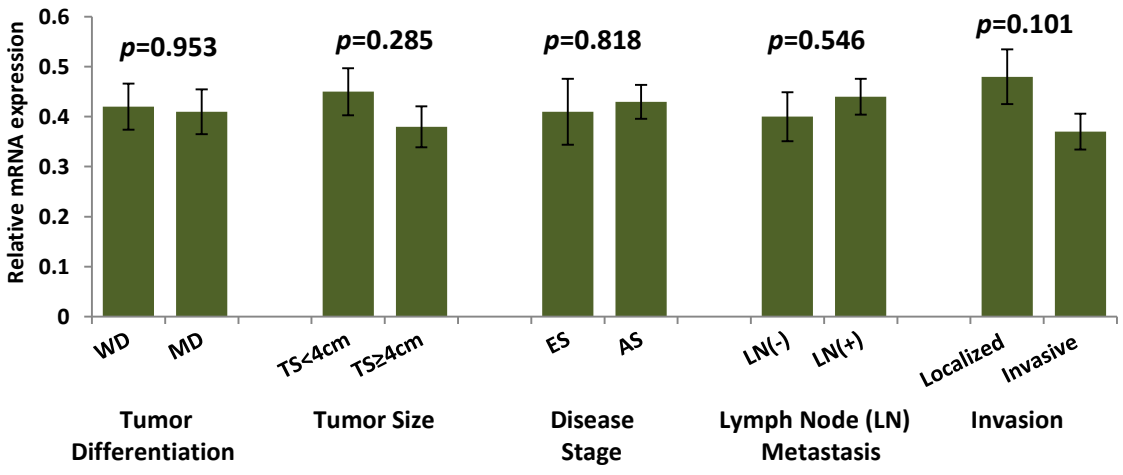


2.2. Association of mRNA levels of *hTERT* with clinico-pathological features, recurrence and survival of oral cancer patients

2.2.1. Association of *hTERT* mRNA levels with clinico-pathological parameters

The present study also analyzed association of *hTERT* transcript levels with various clinico-pathological parameters. No significant association of *hTERT* transcript levels with clinico-pathological parameters was observed (Figure 4.14).

Figure 4.14: Transcript levels of *hTERT* according to various clinico-pathological parameters



WD: Well Differentiated; MD: Moderately Differentiated; TS<4cm: Tumor Size<4cm; TS≥4cm: Tumor Size≥4cm; ES: Early Stage; AS: Advanced Stage; LN(-): Lymph node Metastasis (-); LN(+):Lymph node Metastasis (+)

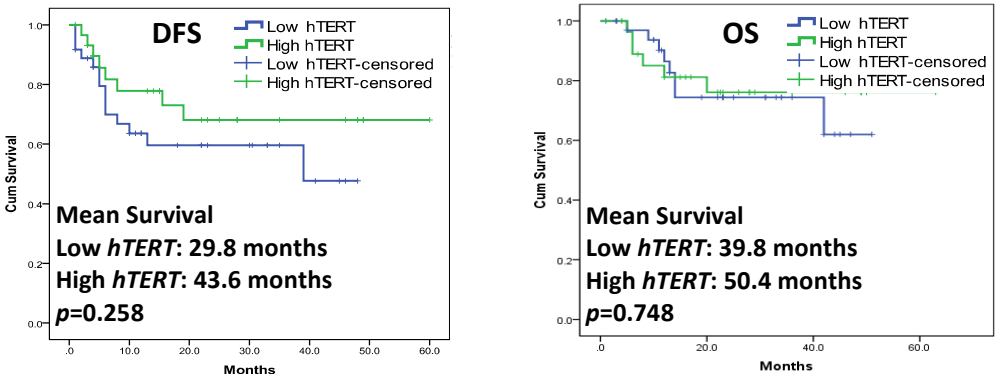
2.2.2. Association of *hTERT* mRNA levels with recurrence of the disease

Present study also analyzed association of *hTERT* mRNA expression with recurrence of the disease. It was observed that *hTERT* mRNA expression did not significantly associate with recurrence of the disease even after stratifying tumors according to different clinico-pathological parameters.

2.2.3. Association of *hTERT* mRNA levels with survival

The present study also analyzed association of DFS and OS of oral cancer patients with transcript levels of *hTERT*. It was observed that *hTERT* transcript levels did not significantly associate with DFS and OS (Figure 4.15).

Figure 4.15: Association of *hTERT* mRNA levels with disease free as well as overall survival of oral cancer patients



2.3. Expression of *VEGFA* isoforms in adjacent normal and malignant oral carcinoma tissues

All forms of *VEGFA* were expressed in malignant as well as adjacent normal tissues. A total of 67/68 (98.5%) adjacent normal tissues and 68/68 (100%) malignant tissues expressed *VEGF121*; 66/68 (97.1%) adjacent normal tissues and 64/68 (94.1%) malignant tisuues expressed *VEGF165*; 49/68 (72.1%) adjacent normal tissues and 55/68 (80.9%) malignant tissues expressed *VEGF183*; 58/68 (85.3%) adjacent normal tissues and 64/68 (94.1%) malignant tissues expressed *VEGF189*; whereas 54/68 (79.4%) adjacent normal tissues and 31/68 (45.6%) malignant tissues expressed *VEGF206* isoforms.

2.3.1. Transcript levels of *VEGFA* isoforms in adjacent normal and malignant oral carcinoma tissues

Semiquantitative RT-PCR analysis was performed and levels were normalized to *β-actin*. The results revealed that transcript levels of *VEGF183* and *VEGF165* were significantly lower in malignant tissues as compared to the adjacent normal tissues (p=0.001 for both *VEGF183* and *VEGF165*, Figure 4.16). When correlation was performed between all

all isoforms, it was observed that *VEGF189* exhibited significant positive correlation with *VEGF183* ($p<0.001$) and *VEGF165* ($p<0.001$). While *VEGF165* also exhibited significant positive correlation with *VEGF121* ($p=0.001$, table 4.29). Only a small number of malignant tissues showed *VEGF206* expression, hence it was omitted from further analysis.

Figure 4.16: Transcript levels of *VEGFA* isoforms in adjacent normal and malignant oral carcinoma tissues

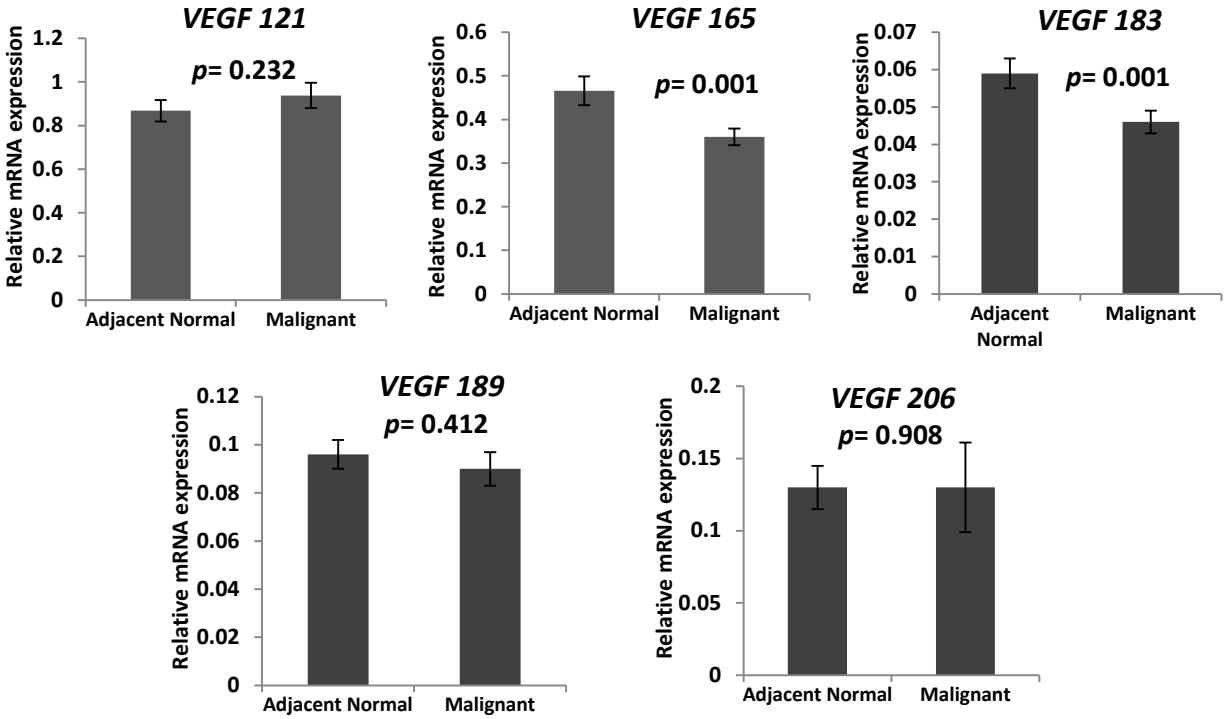


Table 4.29: Correlation of *VEGFA* isoforms in oral carcinoma tissues

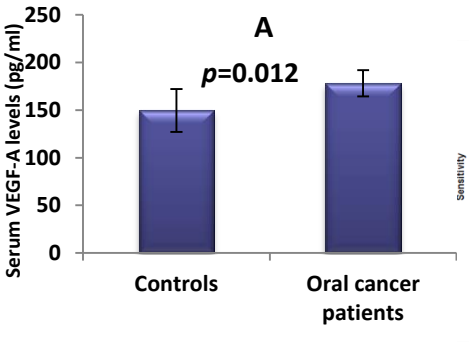
	VEGF 206	VEGF 189	VEGF 183	VEGF 165	VEGF 121
VEGF 206	1.000	-0.097	-0.067	0.070	0.049
VEGF 189	-0.097	1.000	**0.838****	**0.520****	-0.027
VEGF 183	-0.067	**0.838****	1.000	0.068	-0.107
VEGF 165	0.070	**0.520****	0.068	1.000	**0.422****
VEGF 121	0.049	-0.027	-0.107	**0.422****	1.000

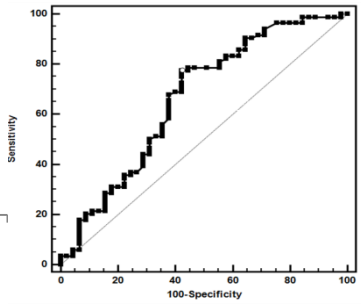
**$p<0.001$

2.4. Serum VEGF-A levels in controls and oral cancer cases

Mean serum VEGF-A levels were significantly higher in oral cancer patients [165.3 ± 11.9 (range: 14 – 504 pg/ml)] as compared to controls [114.4 ± 15.67 (range: 10 – 410 pg/ml)] ($p=0.012$, Figure 4.17A). When the serum VEGF-A values for cancer cases versus controls were plotted for ROC curve, a statistically significant ($p= 0.002$) AUC (0.663) with 73.5 pg/ml as a cut off value (sensitivity: 77.4% with 95%CI=67.0-85.8 and specificity: 57.8% with 95%CI=42.2-72.3) was obtained (Figure 4.17B).

Figure 4.17: Serum VEGF-A levels in oral cancer patients and controls and ROC curve analysis





B

AUC	0.663
95% CI	0.57 - 0.74
p value	0.002
Cut off level	>73.5
Sensitivity 95% CI	77.4 (67.0 – 85.8)
Specificity 95% CI	57.8 (42.2 – 72.3)

2.4.1. Correlation of *VEGFA* isoforms in oral carcinoma tissues with serum VEGF-A levels

The present study also correlated circulatory VEGF-A levels with *VEGFA* transcripts levels. Among all the VEGF isoforms, *VEGF165* and *VEGF121* are diffusible forms, hence we correlated serum VEGF-A levels with *VEGF165* and *VEGF121* transcript levels. However, we did not observe a significant correlation of circulatory VEGF-A with transcript levels of *VEGF121* (r=0.041, *p*=0.790) and *VEGF165* (r=0.122, *p*=0.454).

2.5. Association of mRNA as well as protein levels of *VEGFA* isoforms with clinico-pathological features, recurrence and survival of oral cancer patients

2.5.1. Association of *VEGFA* isoforms with different *clinico-pathological* parameters

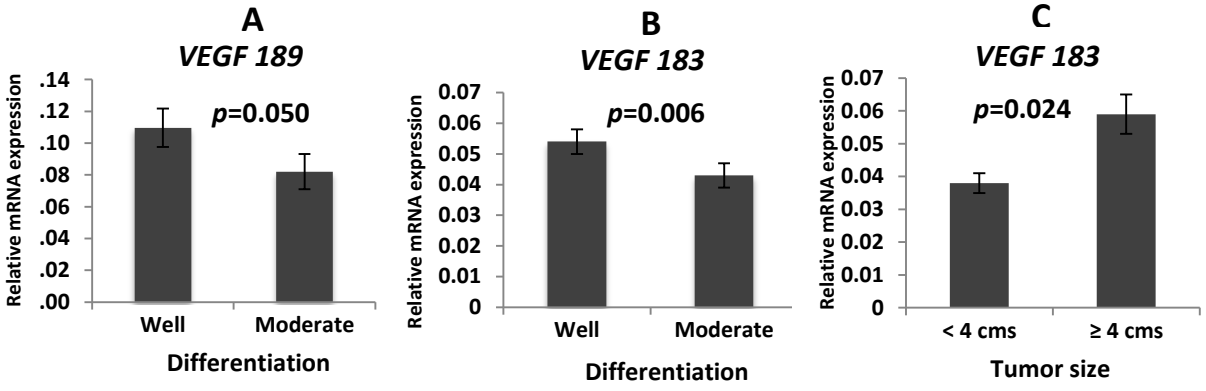
Multivariate analysis of *VEGFA* isoforms with different clinico-pathological parameters suggests that *VEGF183* was significantly associated with tumor differentiation and tumor size (F=4.09, *p*=0.050 and F=8.59, *p*=0.006, respectively). *VEGF189* was significantly associated with tumor differentiation (F=5.50, *p*=0.024) (Table 4.30). Pairwise comparison revealed that *VEGF183* and 189 were significantly down regulated in moderately differentiated tumors in comparison to well differentiated tumors (Figure 4.18A and 4.18B). *VEGF183* was significantly higher in large tumors (≥4cm in size) as compared to small tumors (<4 cm in size) (Figure 4.18C).

Table 4.30: Multivariate analysis of *VEGFA* isoforms with different clinico-pathological parameters

Biomarkers	Tumor Differentiation		Tumor size		LN Mets		Invasion		Disease stage	
	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* value	F	*p* Value
VEGF 189	4.087	**0.050**	1.455	0.235	1.233	0.275	0.034	0.854	0.042	0.838
VEGF 183	5.503	**0.024**	8.592	**0.006**	0.006	0.938	0.746	0.393	1.205	0.279
VEGF 165	1.602	0.213	0.000	0.993	0.520	0.475	2.052	0.160	0.216	0.645
VEGF 121	0.144	0.706	0.730	0.398	2.720	0.107	0.077	0.783	2.222	0.144

LN Mets: Lymph node metastasis

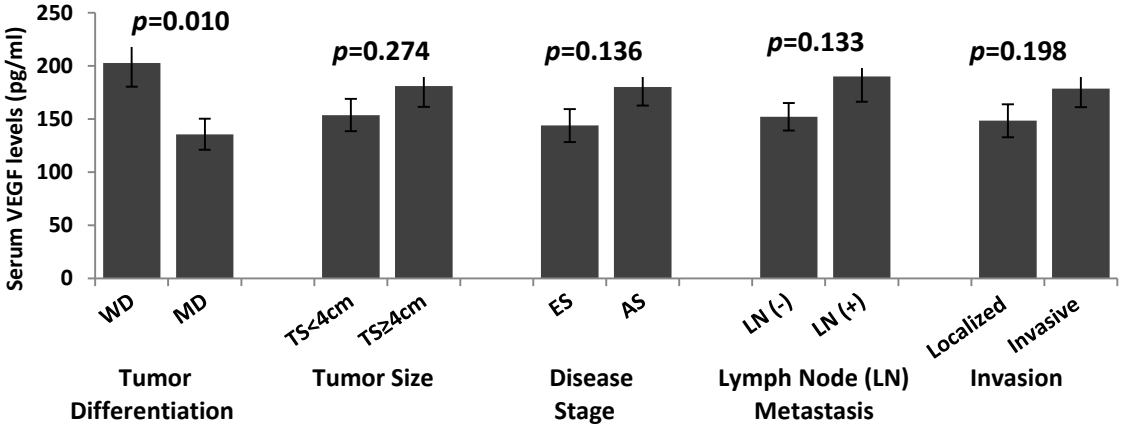
Figure 4.18: Association of *VEGFA* isoforms with differentiation and tumor size



2.5.2. Association of serum VEGF-A levels with clinico-pathological parameters

When the association between serum VEGF-A levels with various clinico-pathological parameters was analyzed, it was observed that serum VEGF-A levels were significantly decreased in patients having tumors with moderate differentiation as compared to the patients having tumors with well differentiation (p=0.010) (Figure 4.19).

Figure 4.19: Association of serum VEGF-A levels with clinico-pathological parameters



WD: Well Differentiated; MD: Moderately Differentiated; TS<4cm: Tumor Size<4cm; TS≥4cm: Tumor Size≥4cm; ES: Early Stage; AS: Advanced Stage; LN(-): Lymph node Metastasis (-); LN(+):Lymph node Metastasis (+)

2.5.3. Association of *VEGFA* isoforms with recurrence of the disease

VEGFA isoforms were did not significantly associate with recurrence of the disease. However, a significant association was observed between *VEGF165*, differentiation, and recurrence (F=4.54, p=0.041); tumor size and recurrence (F=5.47, p=0.025); stage and recurrence (F=8.15, p=0.007) (Table 4.31).

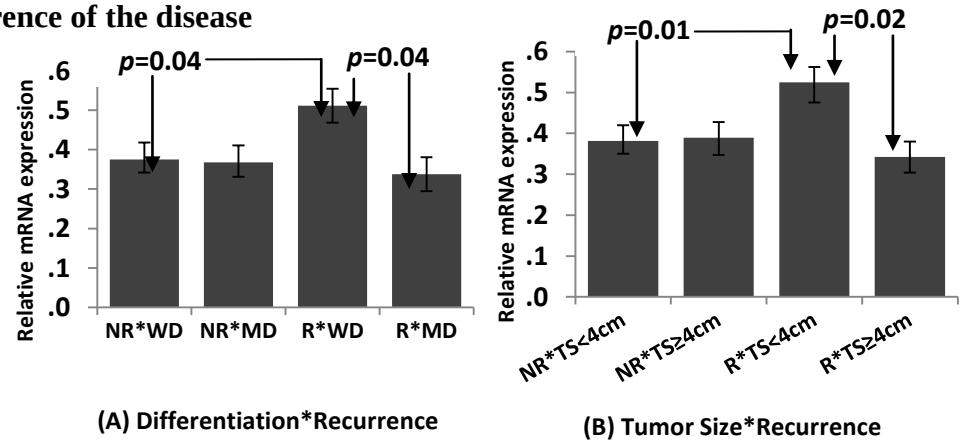
Table 4.31: Association of *VEGFA* isoforms with recurrence of the disease according to different clinico-pathological parameters

Biomarkers	Tumor Differentiation* Recurrence		Tumor size* Recurrence		LN Mets* Recurrence		Invasion* Recurrence		Disease stage* Recurrence	
	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* Value
VEGF 189	0.731	0.399	0.233	0.632	2.132	0.153	0.016	0.901	0.001	0.971
VEGF 183	0.569	0.456	0.007	0.932	1.808	0.187	0.327	0.571	0.009	0.925
VEGF 165	4.537	**0.041**	5.468	**0.025**	0.000	0.993	2.074	0.159	8.149	**0.007**
VEGF 121	3.855	0.058	0.000	0.990	0.094	0.761	0.443	0.510	1.682	0.203

LN Mets: Lymph node metastasis

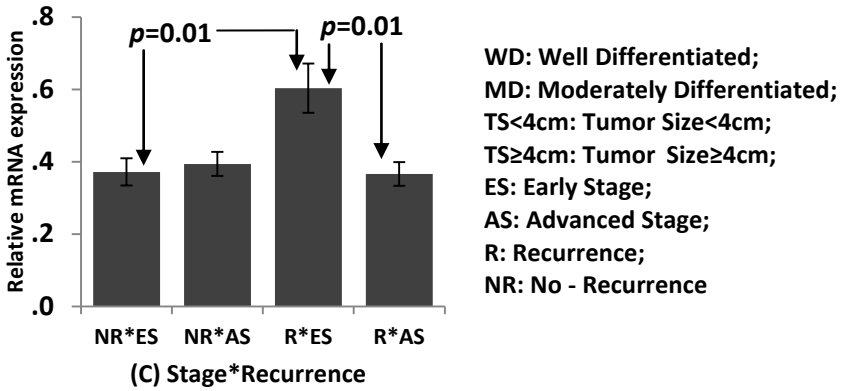
Further, results revealed that transcript levels of *VEGF165* were significantly elevated in recurrent well differentiated tumors as compared to the non-recurrent well differentiated tumors (p=0.04). In recurrent tumors, *VEGF165* was significantly decreased in moderately differentiated tumors compared to well differentiated tumors (p=0.04). No difference was found between well and moderately differentiated non- recurrent tumors (Figure 4.20A). Association of *VEGFA* isoforms with tumor size and recurrence suggests that transcript levels of *VEGF165* were significantly elevated in recurrent small tumors as compared to the non-recurrent small tumors (p=0.01). However, in recurrent tumors, levels of *VEGF165* transcript were significantly down regulated in large tumors compared to small tumors (p=0.02). No difference was found between large and small non- recurrent tumors (Figure 4.20B). Association of *VEGFA* isoforms with stage and recurrence suggest that transcript levels of *VEGF165* were significantly elevated in recurrent early stage tumors compared to non-recurrent early stage tumors (p=0.01). However, *VEGF165* transcript levels were significantly down regulated in advanced stage compared to an early stage in recurrent tumors (p=0.01). No difference was observed between early stage and advanced stage in non- recurrent tumors (Figure 4.20C).

Figure 4.20: Association of *VEGF165* with differentiation, tumor size, stage and recurrence of the disease



(A) Differentiation*Recurrence

(B) Tumor Size*Recurrence



Above results suggest that *VEGF165* isoforms plays a definitive role in recurrence of the disease in early stage. Therefore, we also evaluated the predictive value of *VEGF165* for recurrence in early stage by logistic regression. Mean *VEGF165* transcript levels of primary tumors were set as cut-off and tumors were categorized into having low and high *VEGF165* transcript levels. It was observed that well differentiated tumors having high levels of *VEGF165* had high risk to develop recurrence (OR=8.00, 95%CI=0.75-85.32, p=0.085, table 4.32). Moreover, small and early stage tumors having high levels of *VEGF165* levels had also high risk to develop recurrence (OR=2.73, 95%CI=0.44-16.75 and OR=2.25, 95%CI=0.29-17.76 for small and early stage tumors, respectively, table 4.32).

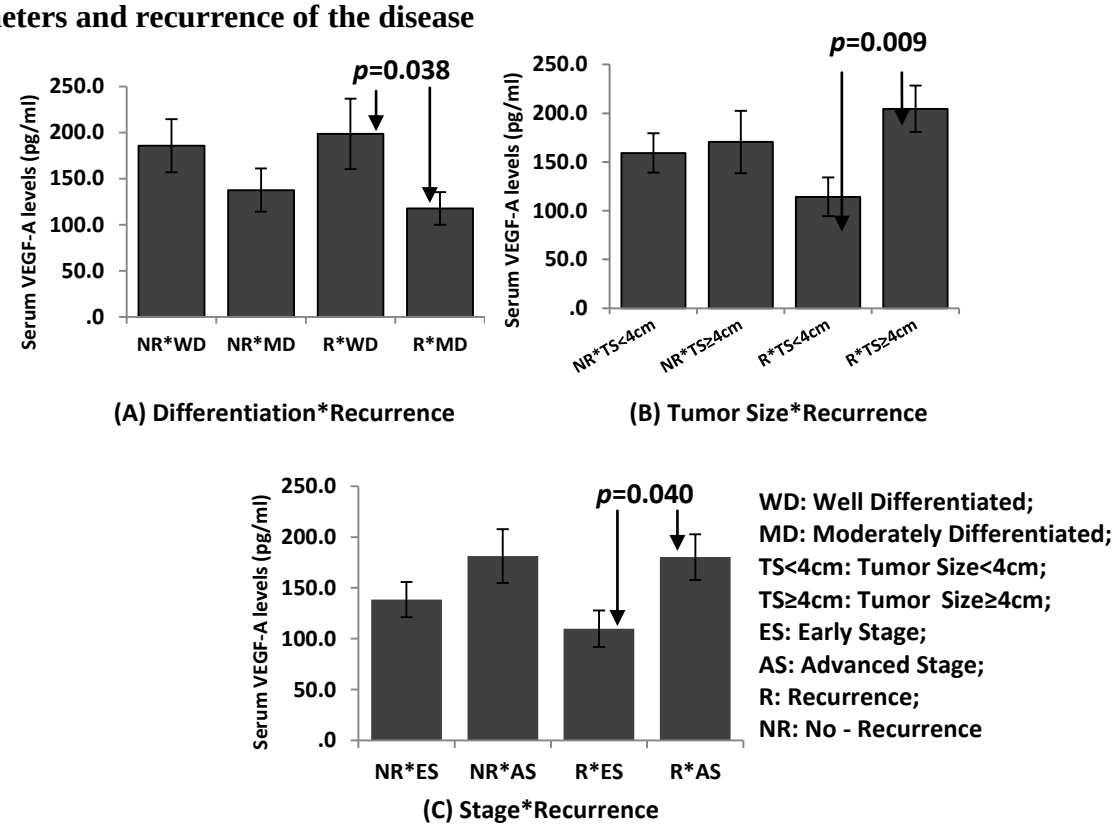
Table 4.32: Predictive values of *VEGF165* for recurrence in early disease

***VEGF165* levels**	**Non-Recurrence No. (%)**	**Recurrence No. (%)**	**OR (95%CI)**	***p* Value**
Tumor Differentiation (Well)				
Low	8 (57.1)	1 (14.3)	8.00 (0.75-85.32)	0.085
High	6 (42.9)	6 (85.7)		
Tumor – size (<4 cm)				
Low	10 (47.6)	2 (25.0)	2.73 (0.44-16.75)	0.279
High	11 (52.4)	6 (75.0)		
Disease stage (Early)				
Low	9 (60.0)	2 (40.0)	2.25 (0.29-17.76)	0.442
High	6 (40.0)	3 (60.0)		

2.5.4. Association of serum VEGF-A with recurrence of the disease

Serum VEGF-A levels did not associate with recurrence of the disease. However, association of serum VEGF-A levels with recurrence of the disease according to various clinico-pathological parameters suggested that serum VEGF-A levels were significantly higher in recurrent tumors having moderate differentiation (p=0.038) (Figure 4.21A), larger size of tumors (p=0.009) (Figure 4.21B) and advanced stage (p=0.040) (Figure 4.21C).

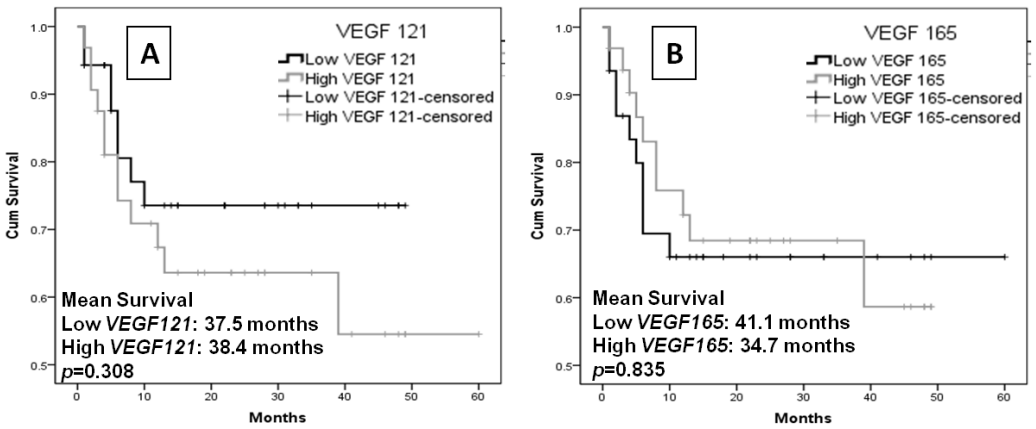
Figure 4.21: Analysis of serum VEGF-A with different clinico-pathological parameters and recurrence of the disease



2.5.5. Disease free and overall survival analysis of oral cancer patients according to mRNA and protein levels of *VEGFA*

The present study also evaluated DFS and OS associated with transcript levels of *VEGFA* isoforms and serum VEGF levels. It was observed that patients having low *VEGF165*, *VEGF183* and *VEGF189* transcript levels had higher DFS and OS as compared to the patients having high *VEGF165*, *VEGF183* and *VEGF189* transcript levels (Figure 4.22 and 4.23).

Figure 4.22: Association of disease free survival of oral cancer patients with transcript levels of *VEGFA* isoforms



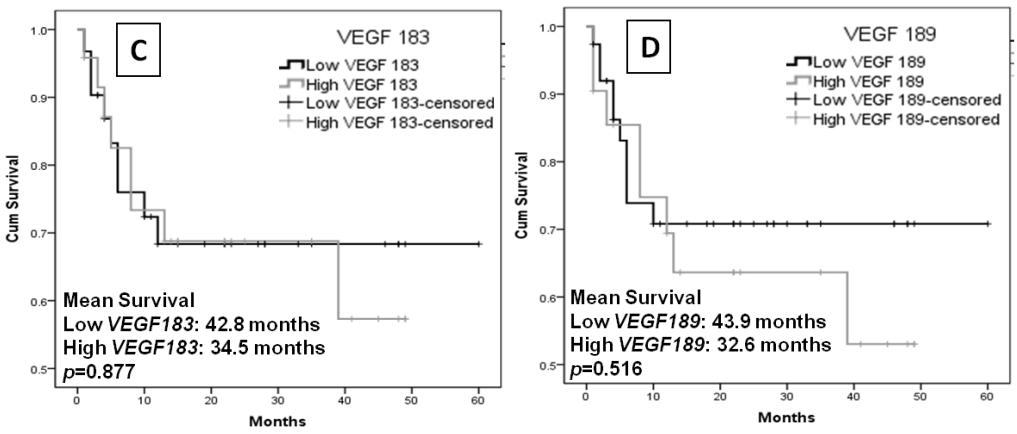
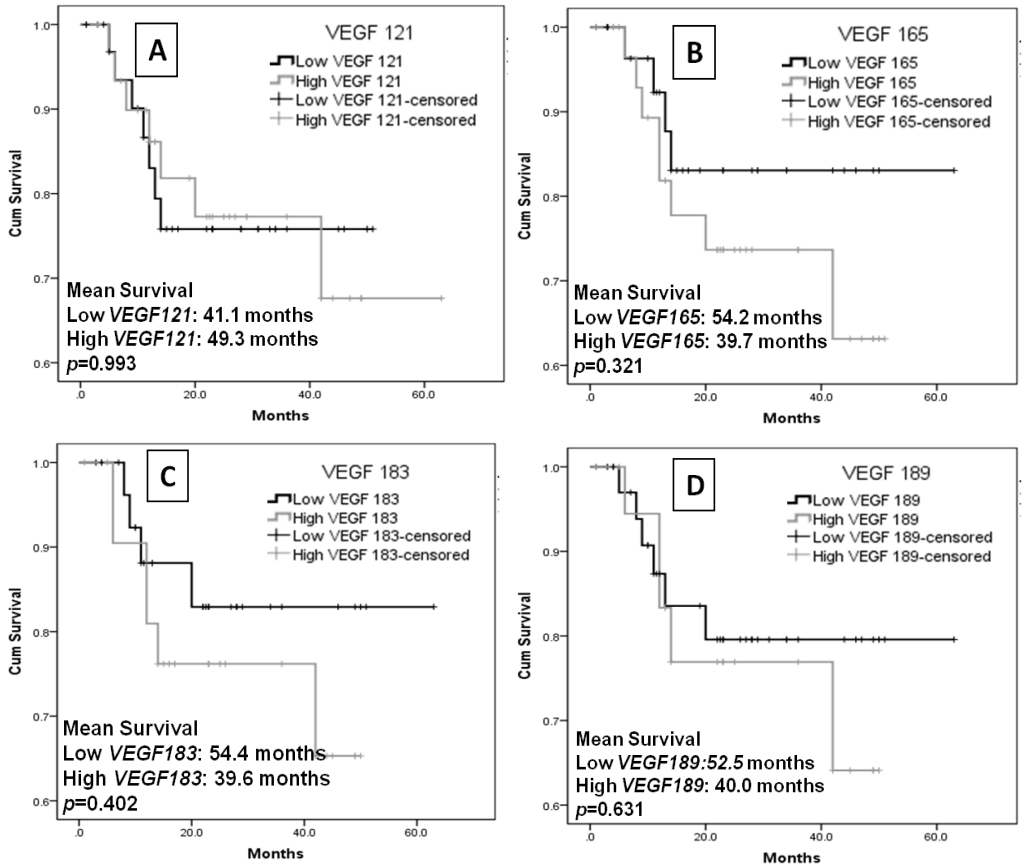


Figure 4.23: Association of overall survival of oral cancer patients with transcript levels of *VEGFA* isoforms

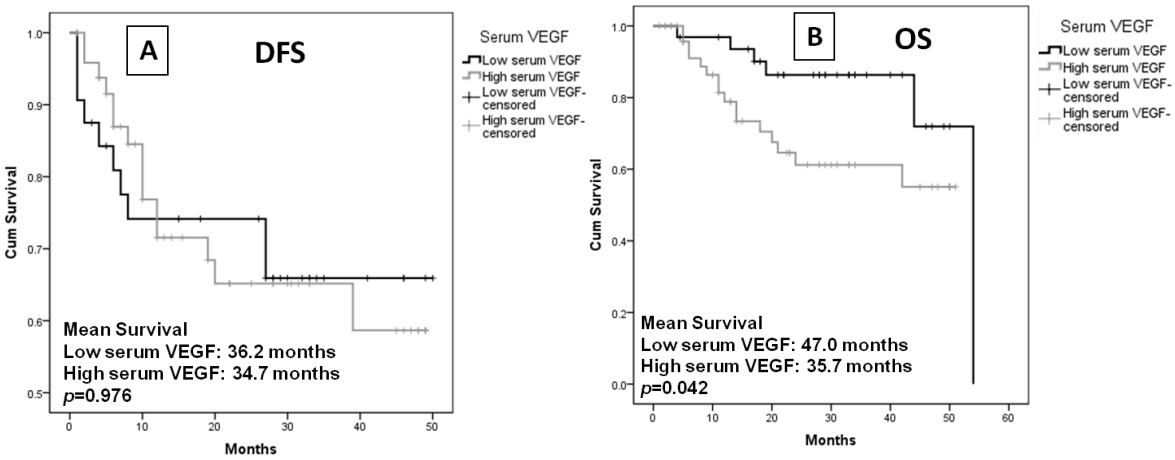


Further, multivariate cox proportional hazard model suggest that among all *VEGFA* isoforms, patients having high levels of *VEGF165* had worse prognosis (HR:5.46, 95%CI: 0.61 - 48.96) (Table 4.33). It was also observed that patients having low serum VEGF-A levels had significantly higher OS compared to patients having high serum VEGF-A levels (Figure 4.24B). Univariate cox proportional hazard ratio analysis suggest that the estimated risk of death was 2.7 fold higher in the patients having high serum VEGF-A levels (HR: 2.7, 95%CI: 1.0 - 7.4, p=0.052).

Table 4.33: Multivariate cox proportional regression analyses of *VEGFA* isoforms

VEGFA isoforms	HR ratio	*p* value
VEGF121	1.53 (0.38 - 6.12)	0.549
VEGF165	5.46 (0.61 - 48.96)	0.130
VEGF183	1.35(0.21 - 8.66)	0.754
VEGF189	0.71 (0.11 - 4.80)	0.728

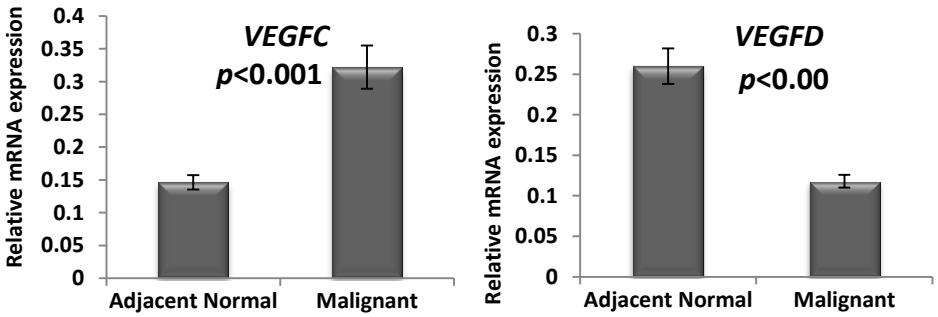
Figure 4.24: Association of disease free and overall survival of oral cancer patients with serum VEGF-A levels



2.6. mRNA levels of *VEGFC* and *VEGFD* in oral cancer patients

Expression of *VEGFC* and *VEGFD* were analyzed by semi-quantitative RT-PCR. Transcript levels of *VEGFC* were significantly elevated in malignant tissues as compared to the adjacent normal tissues ($p<0.001$) (Figure 4.25). However, transcript levels of *VEGFD* were significantly down regulated in malignant tissues as compared to the adjacent normal tissues ($p<0.00$) (Figure 4.25).

Figure 4.25: Transcript levels of *VEGFC* and *VEGFD* in adjacent normal and malignant oral carcinoma tissues

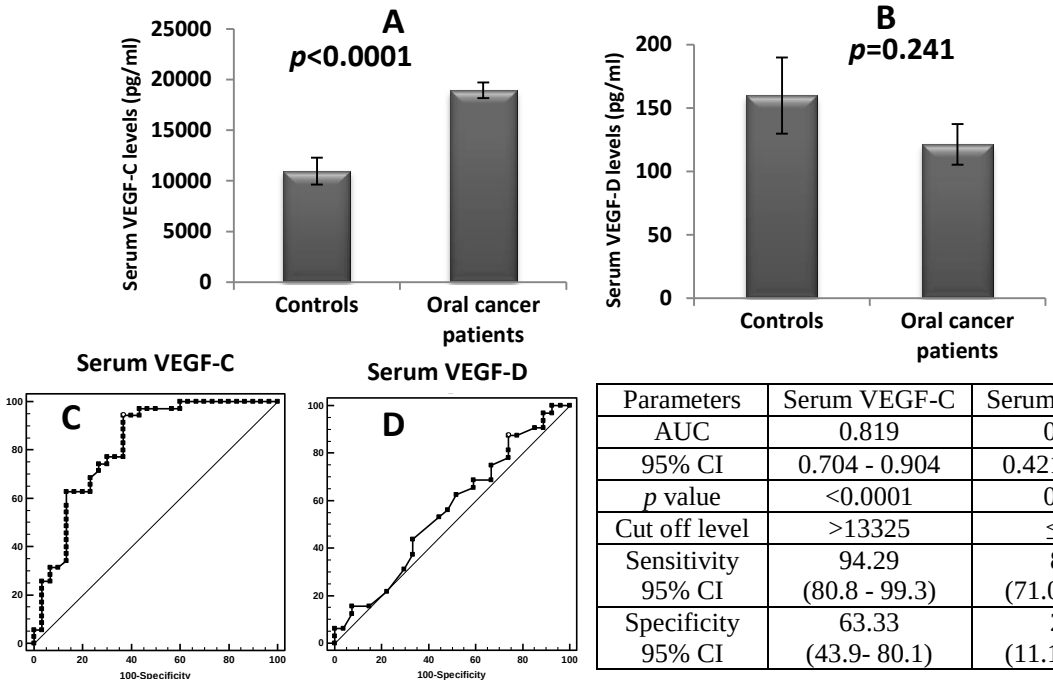


2.7. Serum VEGF-C and VEGF-D levels in controls and oral cancer cases

Mean serum VEGF-C levels were significantly higher in oral cancer patients [18934.3 ± 780.8 (range: 8425 - 31475 pg/ml)] as compared to the controls [10953.3 ± 1325.0

(range: 925 - 26225 pg/ml)] (p<0.0001) (Figure 4.26A). However, mean VEGF-D levels were lower in oral cancer patients [121.3 ± 16.0 (range: 5 - 430 pg/ml)] as compared to controls [159.8 ± 30.0 (range: 25 – 695 pg/ml)] (Figure 4.26B). ROC curve analysis revealed that serum VEGF-C levels could significantly discriminate between oral cancer patients and controls (AUC=0.819, p<0.0001) with 13325 pg/ml as a cut off value (sensitivity: 94.3% with 95%CI = 80.8 - 99.3 and specificity: 63.3% with 95%CI = 43.9 - 80.1) (Figure 4.26C). However, serum VEGF-D levels could not discriminate between oral cancer patients and controls (Figure 4.26D).

Figure 4.26: Serum VEGF-C and VEGF-D levels in oral cancer patients and controls and ROC curve analysis



Parameters	Serum VEGF-C	Serum VEGF-D
AUC	0.819	0.557
95% CI	0.704 - 0.904	0.421 - 0.686
p value	<0.0001	0.455
Cut off level	>13325	≤185
Sensitivity 95% CI	94.29 (80.8 - 99.3)	87.5 (71.0 – 96.5)
Specificity 95% CI	63.33 (43.9- 80.1)	25.9 (11.1 – 46.3)

2.7.1. Correlation of *VEGFC* and *VEGFD* in oral carcinoma tissues with serum VEGF-C and VEGF-D levels

The present study also correlated circulatory VEGF-C and VEGF-D levels with *VEGFC* and *VEGFD* transcripts levels in tissues, respectively. However, we did not observe a significant correlation of circulatory VEGF-C with transcript levels of *VEGFC* (r=0.056, p=0.764) and circulatory VEGF-D with transcript levels of *VEGFD* (r=0.123, p=0.558).

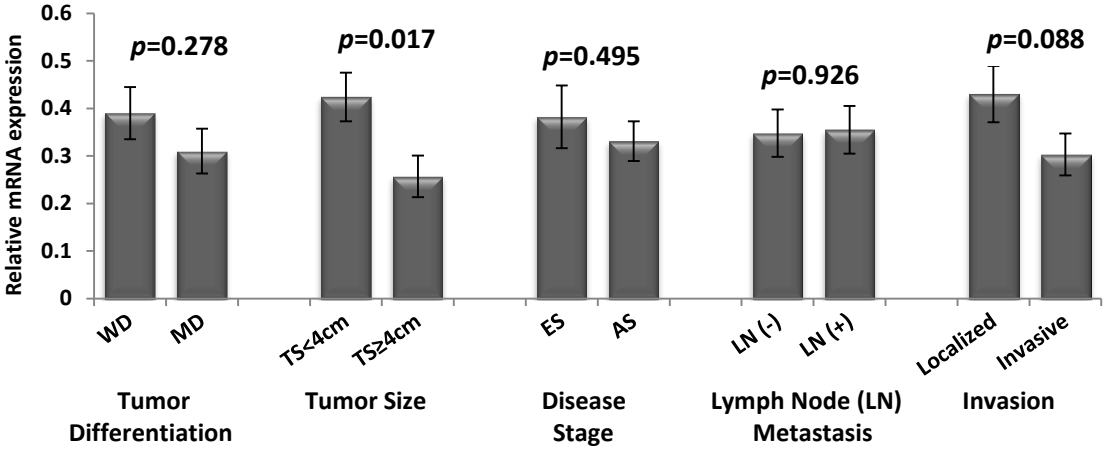
2.8. Association of mRNA as well as protein levels of *VEGFC* and *VEGFD* with clinico-pathological features, recurrence and survival of oral cancer patients

2.8.1. Association of *VEGFC* and *VEGFD* transcript levels with clinico-pathological parameters

The present study evaluated association of *VEGFC* and *VEGFD* with clinico-pathological characteristics of oral cancer patients (Figure 4.27 and 4.28, respectively). It

was observed that transcript levels of *VEGFC* were significantly higher in small tumors compared to large tumors (p=0.017, Figure 4.27).

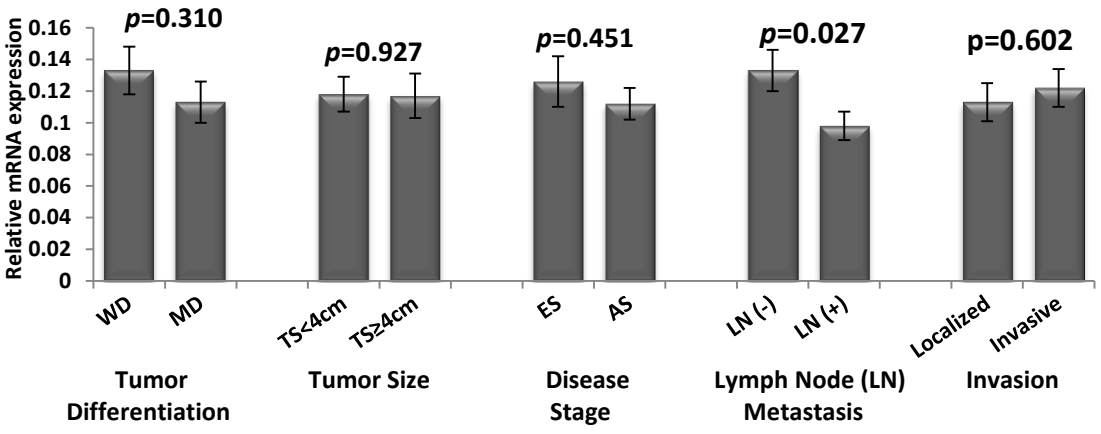
Figure 4.27: Association of *VEGFC* with different clinico-pathological parameters of oral cancer patients



WD: Well Differentiated; MD: Moderately Differentiated; TS<4cm: Tumor Size<4cm; TS≥4cm: Tumor Size≥4cm; ES: Early Stage; AS: Advanced Stage; LN(-): Lymph node Metastasis (-); LN(+):Lymph node Metastasis (+)

Further, transcript levels of *VEGFD* were significantly higher in tumors without lymph node involvement compared to tumors with lymph node involvement. (p=0.027, Figure 4.28).

Figure 4.28: Association of *VEGFD* with different clinico-pathological parameters of oral cancer patients



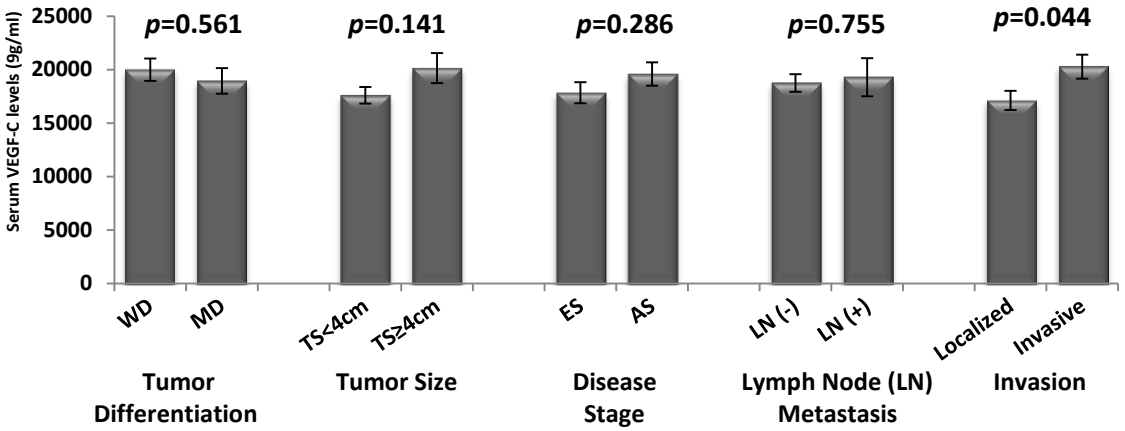
WD: Well Differentiated; MD: Moderately Differentiated; TS<4cm: Tumor Size<4cm; TS≥4cm: Tumor Size≥4cm; ES: Early Stage; AS: Advanced Stage; LN(-): Lymph node Metastasis (-); LN(+):Lymph node Metastasis (+)

2.8.2.Association of serum VEGF-C and VEGF-D levels with clinico-pathological parameters

Association of serum VEGF-C and VEGF-D levels with various clinico-pathological parameters was also analyzed. Results revealed that serum VEGF-C levels were significantly higher in patients having invasive tumors as compared to patients having

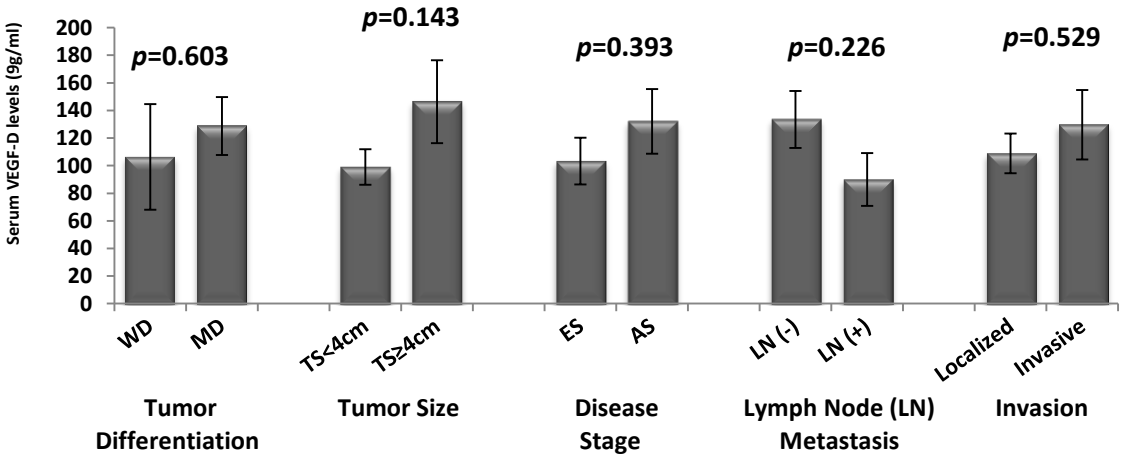
localized tumors (p=0.044) (Figure 4.29). Serum VEGF-D levels were lower in patients having lymph node involvement (Figure 4.30).

Figure 4.29: Association of serum VEGF-C levels with clinico-pathological parameters



WD: Well Differentiated; MD: Moderately Differentiated; TS<4cm: Tumor Size<4cm; TS≥4cm: Tumor Size≥4cm; ES: Early Stage; AS: Advanced Stage; LN(-): Lymph node Metastasis (-); LN(+):Lymph node Metastasis (+)

Figure 4.30: Association of serum VEGF-D levels with clinico-pathological parameters



WD: Well Differentiated; MD: Moderately Differentiated; TS<4cm: Tumor Size<4cm; TS≥4cm: Tumor Size≥4cm; ES: Early Stage; AS: Advanced Stage; LN(-): Lymph node Metastasis (-); LN(+):Lymph node Metastasis (+)

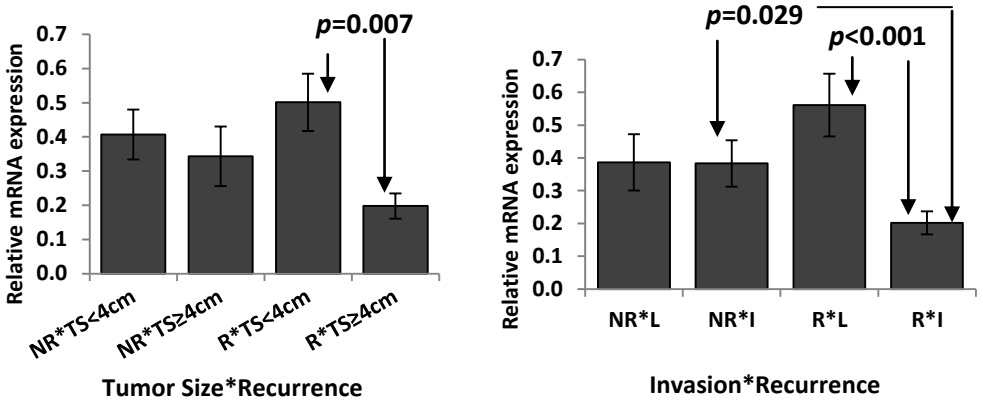
2.8.3. Association of *VEGFC* and *VEGFD* transcript levels with recurrence of the disease

VEGFC and *VEGFD* was not associated significantly with recurrence of the disease. Further, association of *VEGFC* and *VEGFD* with recurrence of the disease was analyzed by stratifying tumors according to different clinico-pathological parameters. It was observed that transcript levels of *VEGFC* were significantly higher in small and localized tumors compared to large and invasive tumors that had recurrence (p=0.007 and p=0.001, respectively, Figure 4.31). Moreover, *VEGFC* levels were significantly higher

in non-recurrent invasive tumors as compared to recurrent invasive tumors (p=0.029, Figure 4.31). However, there is no difference in *VEGFC* levels in non-recurrent tumors having different types differentiation, size and invasive potential (Figure 4.31).

However, for VEGF D transcript levels, there was no significant association with recurrence of the disease when analyzed by stratifying tumors according to various clinico-pathological parameters.

Figure 4.31: Association of *VEGFC* with recurrence of the disease according to different clinico-pathological parameters



TS<4cm: Tumor Size<4cm; TS≥4cm: Tumor Size≥4cm; I: Invasive tumors; L: Localized tumors; R: Recurrence; NR: No - Recurrence

2.8.4. Disease free and overall survival analysis of oral cancer patients according to *VEGFC* and *VEGFD* transcript levels

The present study also evaluated association of DFS and OS with transcript levels of *VEGC* and *VEGFD* (Figure 4.32 and Figure 4.33, respectively). Results revealed that *VEGFC* and *VEGFD* transcript levels did not significantly associate with DFS as well as OS.

Figure 4.32: Association of *VEGFC* transcript levels with disease free and overall survival of oral cancer patients

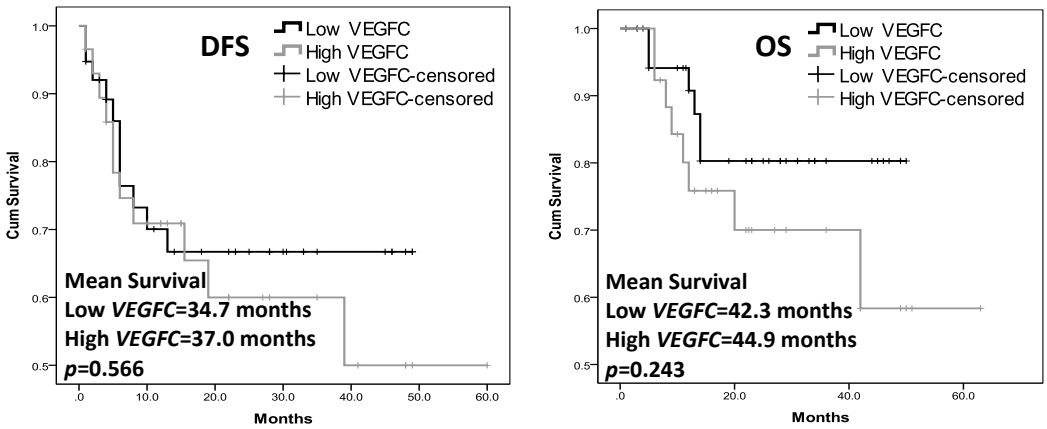
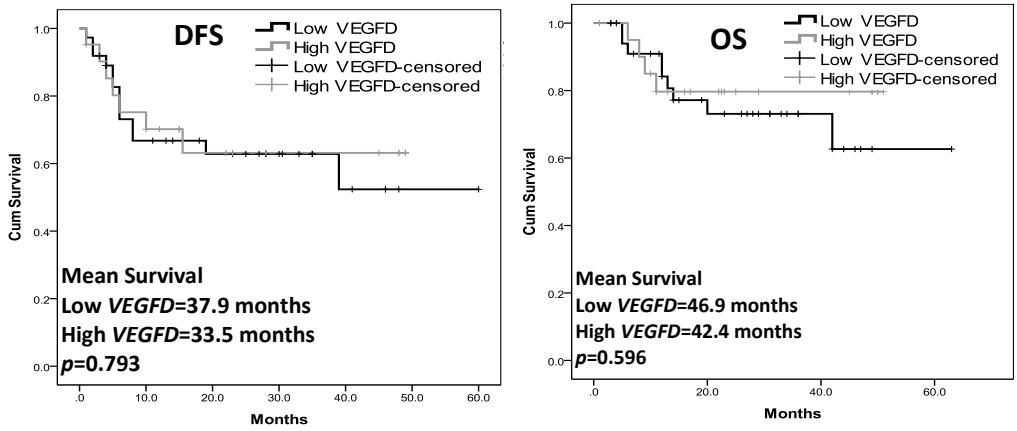


Figure 4.33: Association of *VEGFD* transcript levels with disease free and overall survival of oral cancer patients

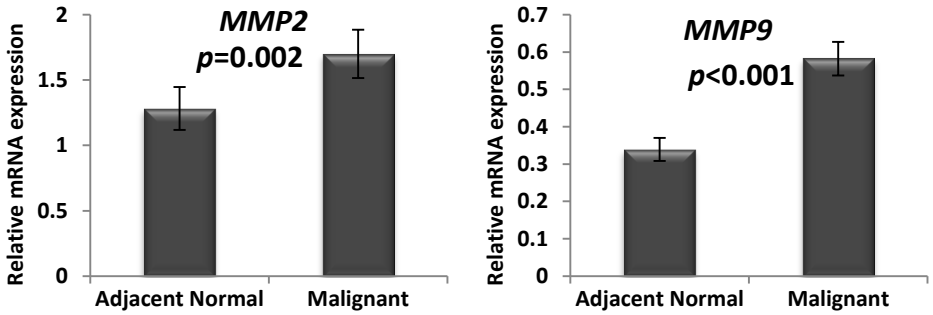


Association of serum VEGF-C and VEGF-D levels with recurrence of the disease as well as DFS and OS were not evaluated due to small sample size.

2.9. mRNA levels of *MMP2* and *MMP9* in oral cancer patients

Transcript levels of *MMP2* and *MMP9* were significantly elevated in malignant tissues compared to the adjacent normal tissues (p=0.002 and p<0.001, respectively, Figure 4.34). Significant positive correlation was observed between *MMP2* and *MMP9* (p=0.001).

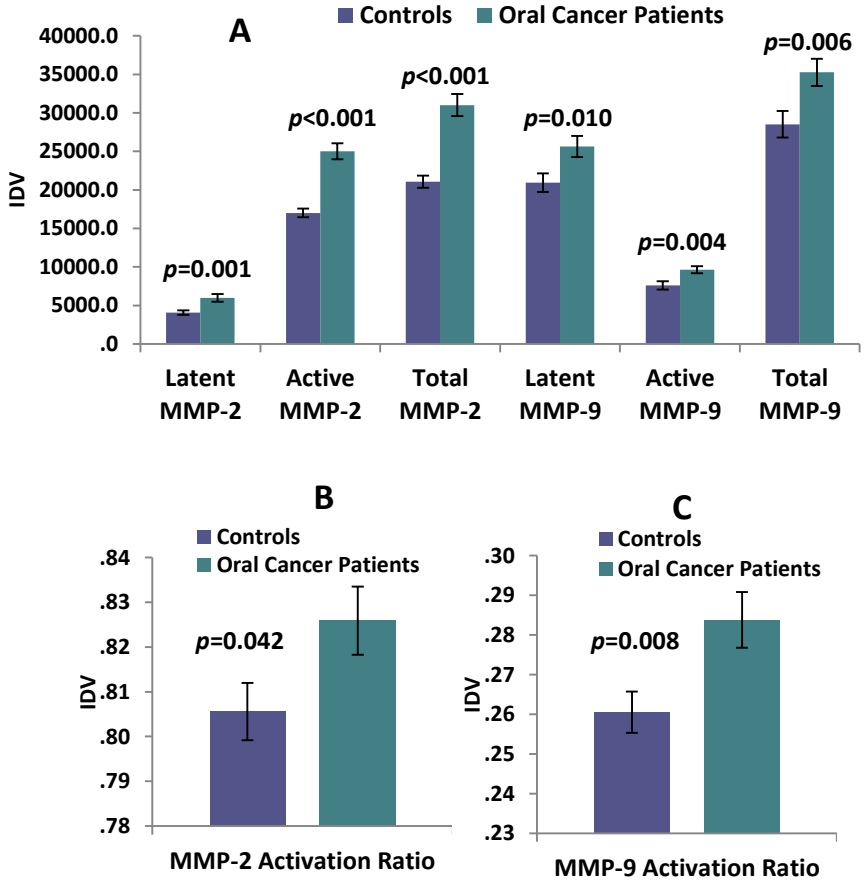
Figure 4.34: Transcript levels of *MMP2* and *MMP9* in adjacent normal and malignant oral carcinoma tissues



2.10. Comparison of MMP-2 and MMP-9 protein levels between controls and oral cancer patients

The levels of latent, active and total forms of MMP-2 and MMP-9 exhibited a significant increase in oral cancer patients as compared to the controls (p<0.05, Figure 4.35A). Activation ratio of MMP-2 and MMP-9 were also significantly higher in oral cancer patients compared to the controls (p=0.042 and p=0.008, respectively, Figure 4.35B).

Figure 4.35: Comparison of latent, active, total MMP-2 and MMP-9 protein levels in controls and oral cancer patients



2.10.1. Correlation among different forms of MMPs and their activation ratio

The different forms of MMP-2 and MMP-9 were significantly inter-correlated ($p<0.001$, table 4.34). Activation ratio of MMP-2 exhibited negative correlation with latent MMP-2, total MMP-2 and latent MMP-9 ($p<0.001$, $p<0.001$ and $p=0.053$, respectively, table 4.34). Further, activation ratio of MMP-9 showed significant negative correlation with latent MMP-2, latent MMP-9, total MMP-2 and total MMP-9 ($p<0.001$, $p<0.001$, $p=0.050$ and $p=0.029$, respectively, table 4.34). In addition, activation ratio of MMP-9 exhibited significant positive correlation with active MMP-9 ($p=0.001$, table 4.34). Moreover, significant positive correlation was observed between activation ratio of MMP-2 and MMP-9 ($p<0.001$, table 4.34).

Table 4.34: Correlation among different forms of MMPs and their activation ratio

	Latent MMP-2	Active MMP-2	Latent MMP-9	Active MMP-9	Total MMP-2	Total MMP-9	MMP-2 Activation ratio	MMP-9 Activation ratio
Latent MMP-2	1	**0.656**	**.568**	**.349**	**.833**	**.532**	**-.636**	**-.253**
Active MMP-2	.656	1	**.713**	**.620**	**.964**	**.716**	-.052	-.063
Latent MMP-9	.568	.713	1	**.812**	**.723**	**.988**	**-.133**$^{@}$	**-.268**
Active MMP-9	.349	.620	.812	1	**.578**	**.891**	.101	**.222***
Total MMP-2	.833	.964	.723	.578	1	**.712**	**-.262**	**-.135**$^{@}$
Total MMP-9	.532	.716	.988	.891	.712	1	-.077	**-.150***
MMP-2 Activation ratio	-.636	-.052	.133	.101	-.262	-.077	1	**.412**
MMP-9 Activation ratio	-.253	-.063	-.268	.222	-.135	-.150	.412	1

*:p<0.05 and @:p=0.05

2.10.2. Correlation between transcript and protein levels of MMPs

Levels of latent and total MMP-2 exhibited significant positive correlation with *MMP2* transcript levels (p<0.001 and p=0.036, respectively). Levels of latent, active and total forms of MMP-9 were positively correlated with *MMP9* transcript levels (Table 4.35). Further, *MMP9* transcript levels were also positively correlated with latent and total MMP-2 (p=0.021 and p=0.060, respectively, table 4.35). Activation ratio of MMP-2 showed significant negative correlation with *MMP2* and *MMP9* transcript levels (p<0.001 and p=0.011, respectively, table 4.35). Moreover, activation ratio of MMP-9 was also negatively correlated with transcript levels of *MMP2* (p=0.062, table 4.35).

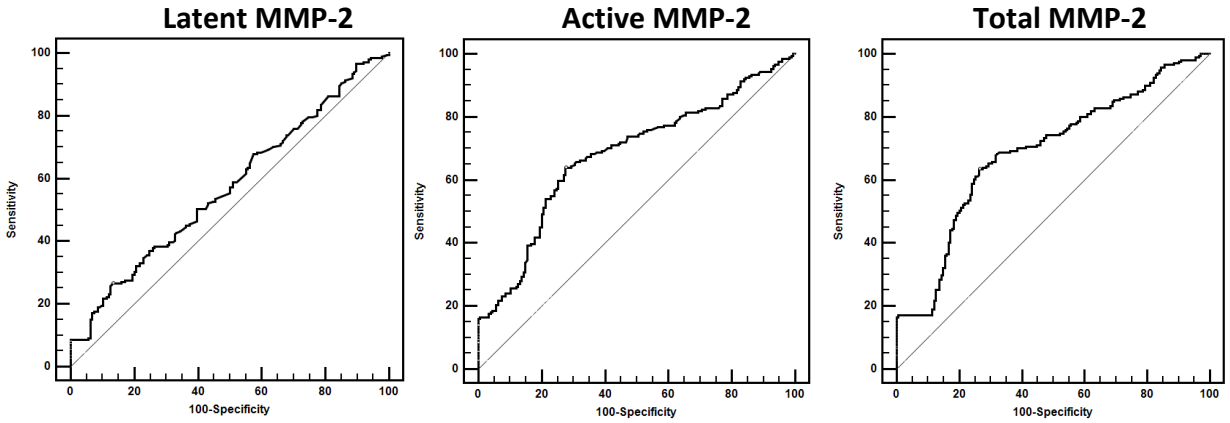
Table 4.35: Correlation between transcript and protein levels of MMPs

		Latent MMP-2	Active MMP-2	Latent MMP-9	Active MMP-9	Total MMP-2	Total MMP-9	MMP-2 Activation ratio	MMP-9 Activation ratio
MMP2	**r**	**.464***	.156	.179	.077	**.257**	.159	**-.604***	**-.229**
	p Value	.000	.207	.147	.536	.036	.200	.000	.062
MMP9	**r**	**.282**	.194	**.215**	**.232**	**.231**	**.226**	**-.308**	.061
	p Value	.021	.115	.081	.059	.060	.066	.011	.624

2.10.3. ROC curve analysis of MMPs protein levels

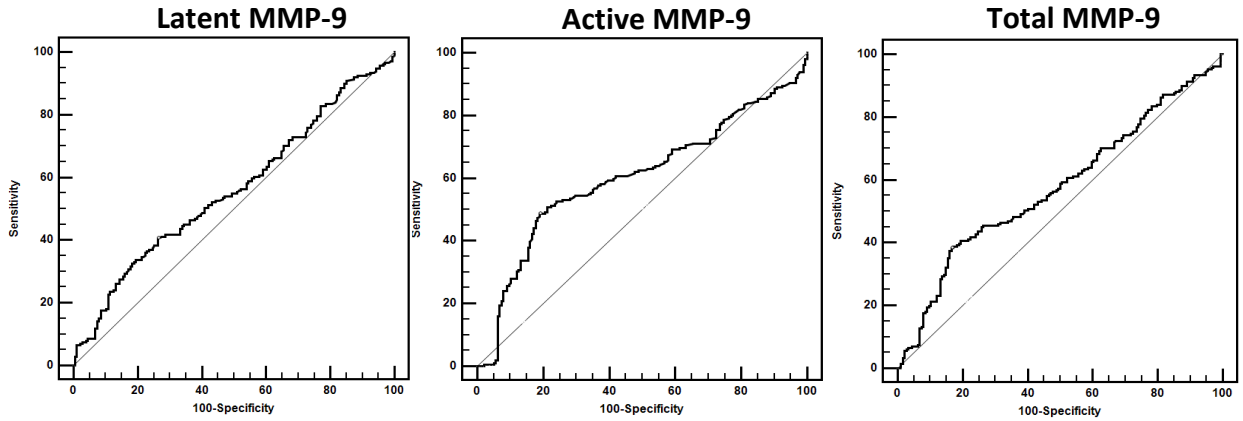
The present study also performed ROC curve analysis to evaluate efficacy of latent, active, total MMP-2 and MMP-9 as well as their activation ratio. It was observed that all forms of MMP-2 and MMP-9 and their activation ratio could significantly discriminate between oral cancer patients and controls (p<0.05, Figure 4.36, 4.37 and 4.38).

Figure 4.36: ROC curve analysis of latent, active and total MMP-2 levels



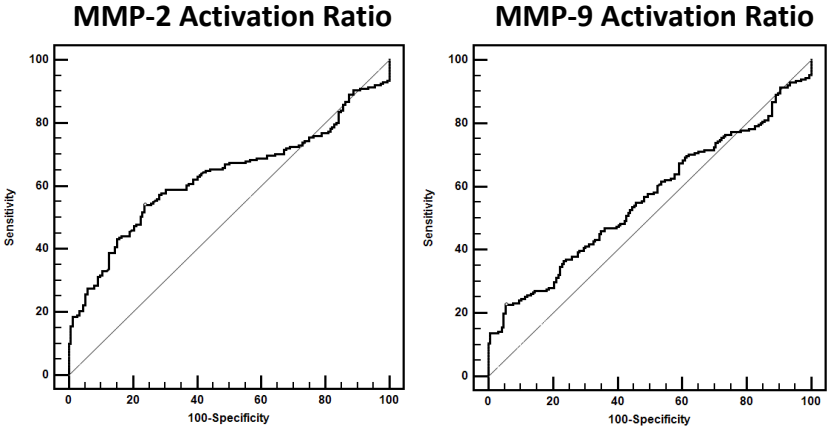
Parameters	Latent MMP-2	Active MMP-2	Total MMP-2
AUC	0.570	0.679	0.688
95% CI	0.519 - 0.620	0.630 - 0.725	0.639 - 0.734
p value	0.016	<0.0001	<0.0001
Cut off level	>6160	>17640	>20460
Sensitivity 95% CI	26.54 (20.7-33.0)	63.98 (57.1 - 70.5)	63.51 (56.6 - 70.0)
Specificity 95% CI	86.78 (80.8 - 91.4)	72.41 (65.1 - 78.9)	73.56 (66.4 - 79.9)

Figure 4.37: ROC curve analysis of latent active and total MMP-9 levels



Parameters	Latent MMP-9	Active MMP-9	Total MMP-9
AUC	0.560	0.603	0.578
95% CI	0.509 - 0.611	0.552 - 0.652	0.526 - 0.627
p value	0.039	0.0004	0.008
Cut off level	>23520	>8272	>37248
Sensitivity 95% CI	40.76 (34.1 - 47.7)	48.82 (41.9 - 55.8)	38.39 (31.8 - 45.3)
Specificity 95% CI	73.56 (66.4 - 79.9)	81.03 (74.4 - 86.6)	83.33 (76.9 - 88.5)

Figure 4.38: ROC curve analysis of activation ratio of MMP-2 and MMP-9



Parameters	MMP-2 Activation Ratio	MMP-9 Activation Ratio
AUC	0.628	0.561
95% CI	0.576 - 0.678	0.509 - 0.612
p value	<0.0001	0.038
Cut off level	>0.8656	>0.3678
Sensitivity 95% CI	54.03 (47.1 - 60.9)	22.75 (17.3 - 29.0)
Specificity 95% CI	76.32 (68.7 - 82.8)	94.58 (90.0 - 97.5)

2.11. Association of mRNA as well as protein levels of *MMP2* and *MMP9* with clinico-pathological features, recurrence and survival of oral cancer patients

2.11.1. Association of *MMP2* and *MMP9* transcript levels with clinico-pathological parameters

Multivariate analysis of *MMP2* and *MMP9* transcript levels with different clinico-pathological parameters suggested that *MMP2* and *MMP9* transcript levels did not associate with any clinico-pathological parameters (Table 4.36).

Table 4.36: Association of *MMP2* and *MMP9* transcript levels with clinico-pathological parameters

Biomarkers	Tumor Differentiation		Tumor size		LN Mets		Invasion		Disease stage	
	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* Value
MMP2	0.895	0.349	1.633	0.207	0.116	0.734	1.418	0.239	0.452	0.504
MMP9	0.653	0.423	1.974	0.166	0.399	0.531	0.633	0.430	0.033	0.857

LN Mets: Lymph node metastasis

2.11.2. Association of MMP-2 and MMP-9 protein levels with clinico-pathological parameters

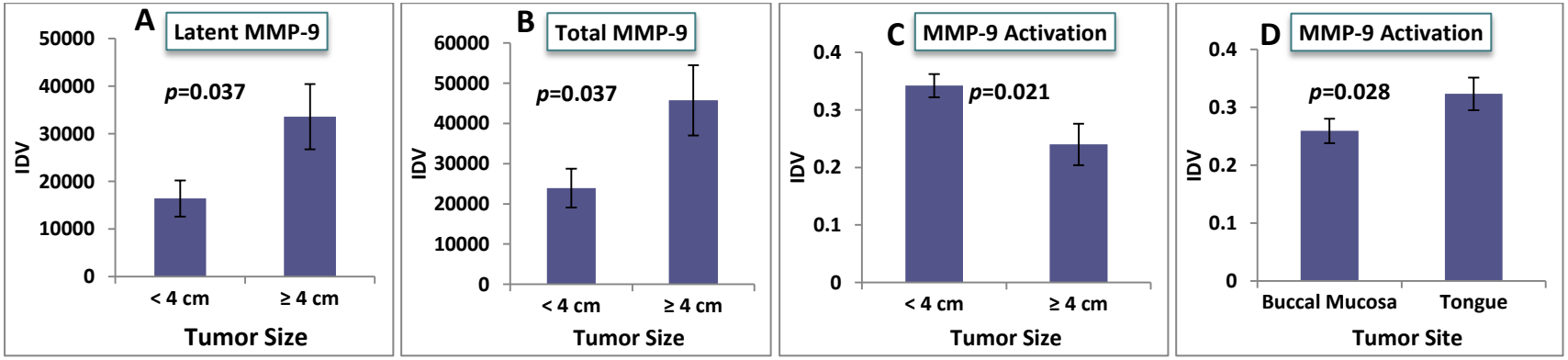
It was observed that levels of latent and total MMP-9 were significantly associated with tumor size (F=4.542, *p*=0.037 and F=4.516, *p*=0.037, respectively, table 4.37). Activation ratio of MMP-9 was also significantly associated with tumor size and primary site of tumor (F=5.630, *p*=0.021 and F=5.052, *p*=0.028, respectively, table 4.37). Pair-wise comparison revealed that latent and total MMP-9 were significantly higher in larger tumors in comparison to smaller tumors (Figure 4.39A and 4.39B). However, activation ratio of MMP-9 was significantly higher in small tumors (<4 cm) as compared to large tumors (≥4 cm) (Figure 4.39C). Also, tongue carcinoma patients exhibited significantly higher MMP-9 activation ratio compared to buccal carcinoma patients (Figure 4.39D). However, for different forms of MMP-2, no association was observed.

Table 4.37: Association of MMP-2 and MMP-9 protein levels with clinico-pathological parameters (Multivariate analysis)

Biomarkers	Tumor Differentiation		Tumor Size		LN Mets		Invasion		Disease stage		Tumors grade		Tumor site	
	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* Value
Latent MMP-2	0.120	0.730	1.999	0.162	0.970	0.328	0.393	0.533	0.115	0.735	0.062	0.804	0.085	0.771
Active MMP-2	0.017	0.895	0.816	0.370	1.880	0.175	0.000	0.982	0.442	0.509	0.015	0.901	0.220	0.640
Total MMP-2	0.001	0.976	1.331	0.253	1.780	0.187	0.058	0.810	0.357	0.552	0.000	1.000	0.194	0.661
Latent MMP-9	0.138	0.711	**4.542**	**0.037**	0.013	0.910	0.147	0.703	0.075	0.785	0.252	0.617	0.849	0.360
Active MMP-9	0.009	0.927	3.090	0.083	0.048	0.828	0.345	0.559	0.524	0.472	0.919	0.341	0.041	0.841
Total MMP-9	0.071	0.790	**4.516**	**0.037**	0.021	0.885	0.204	0.653	0.162	0.689	0.412	0.523	0.448	0.505
Activation Ratio MMP-2	0.718	0.400	2.001	0.162	0.496	0.484	1.938	0.169	0.092	0.762	0.028	0.867	0.069	0.793
Activation Ratio MMP-9	0.315	0.576	**5.630**	**0.021**	1.012	0.318	0.087	0.769	1.940	0.168	0.798	0.375	**5.052**	**0.028**

LN Mets: Lymph node metastasis

Figure 4.39: Association of latent, total MMP-9 levels and its activation ratio with tumor size and site of tumor



2.11.3. Association of *MMP2* and *MMP9* transcript levels with recurrence of the disease

MMP2 and *MMP9* mRNA levels did not associate with recurrence of the disease even after stratifying tumors according to various clinico-pathological parameters (Table 4.38).

Table 4.38: Association of *MMP2* and *MMP9* transcript levels with different clinico-pathological parameters and recurrence of disease

Biomarkers	Tumor Differentiation* Recurrence		Tumor size* Recurrence		LN Mets* Recurrence		Invasion* Recurrence		Disease stage* Recurrence	
	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* Value
MMP2	0.842	0.364	0.019	0.890	0.123	0.727	0.004	0.951	0.029	0.865
MMP9	1.581	0.215	0.096	0.758	0.652	0.423	0.342	0.561	1.588	0.213

LN Mets: Lymph node metastasis

2.11.4. Association of MMP-2 and MMP-9 protein levels with recurrence of the disease according to different clinico-pathological parameters

Further, latent, active and total MMP-2 and MMP-9 levels as well as their activation ratio was not associated with recurrence of the disease even after stratifying tumors according to various clinico-pathological parameters (Table 4.39).

Table 4.39: Association of MMP-2 and MMP-9 protein levels with different clinico-pathological parameters and recurrence of the disease

		Latent MMP-2	Active MMP-2	Latent MMP-9	Active MMP-9	Total MMP-2	Total MMP-9	MMP-2 Activation ratio	MMP-9 Activation ratio
Differentiation *Recurrence	F	0.114	0.401	0.182	0.282	0.204	0.223	0.456	0.772
	p Value	0.892	0.670	0.834	0.755	0.816	0.800	0.634	0.464
Tumor Size *Recurrence	F	0.055	0.374	0.007	0.627	0.280	0.023	0.080	2.079
	p Value	0.814	0.541	0.935	0.430	0.598	0.879	0.778	0.151
LN Mets *Recurrence	F	1.640	0.542	1.753	3.421	0.992	2.319	0.022	1.024
	p Value	0.202	0.462	0.187	0.066	0.321	0.130	0.881	0.313
Invasion *Recurrence	F	0.388	1.660	0.162	0.011	1.332	0.116	0.097	0.924
	p Value	0.534	0.199	0.688	0.915	0.250	0.734	0.755	0.338
Stage *Recurrence	F	0.043	0.027	0.702	0.654	0.002	0.750	0.278	0.058
	p Value	0.836	0.869	0.403	0.420	0.965	0.388	0.598	0.810
Grade *Recurrence	F	0.007	0.104	0.136	0.461	0.069	0.221	0.214	0.167
	p Value	0.934	0.748	0.713	0.499	0.794	0.639	0.644	0.683
Site *Recurrence	F	0.834	0.077	0.003	0.007	0.027	0.004	0.600	0.419
	p Value	0.363	0.782	0.954	0.932	0.870	0.947	0.440	0.519

LN Mets: Lymph node metastasis

2.11.5. Disease free and overall survival analysis of oral cancer patients according to *MMP2* and *MMP9* mRNA levels

The present study also evaluated association of DFS and OS of patients with *MMP2* and *MMP9* transcript levels. Results revealed that DFS as well as OS did not differ in patients with respect to *MMP2* and *MMP9* mRNA levels (Figure 4.40 and 4.41, respectively).

Figure 4.40: Association of *MMP2* transcript levels with disease free and overall survival of oral cancer patients

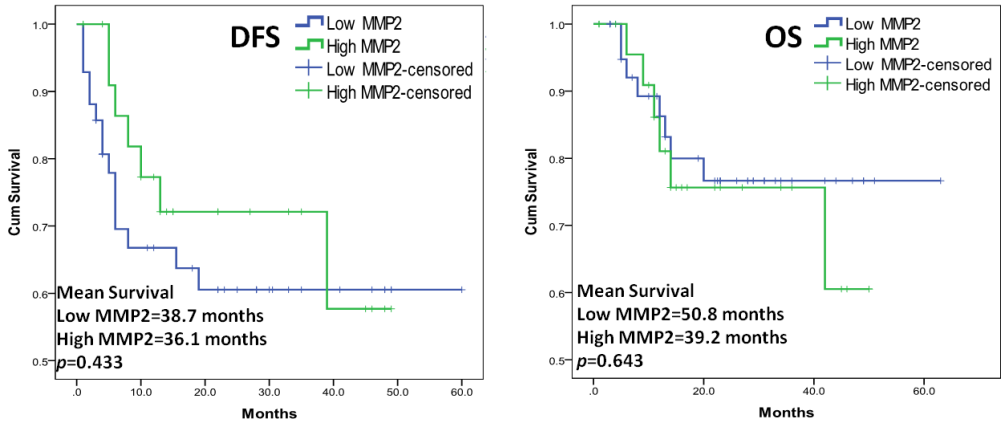
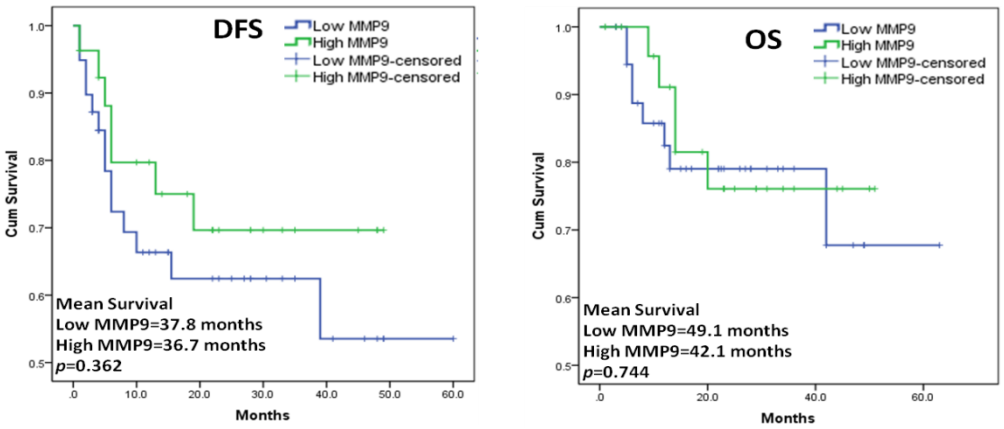


Figure 4.41: Association of *MMP9* transcript levels with disease free and overall survival of oral cancer patients



2.11.6. Disease free and overall survival analysis of oral cancer patients according to MMP-2 and MMP-9 protein levels

DFS and OS analysis of oral cancer patients according to latent, active, total MMP-2 and MMP-9 levels and their activation ratio suggested that there was no significant difference in DFS and OS of oral cancer patients according to latent, active, total MMP-2 and MMP-9 levels and their activation ratio (Figure 4.42 (A,B) and 4.43 (A,B)).

Figure 4.42A: Disease free survival of oral cancer patients according to MMP-2 protein levels

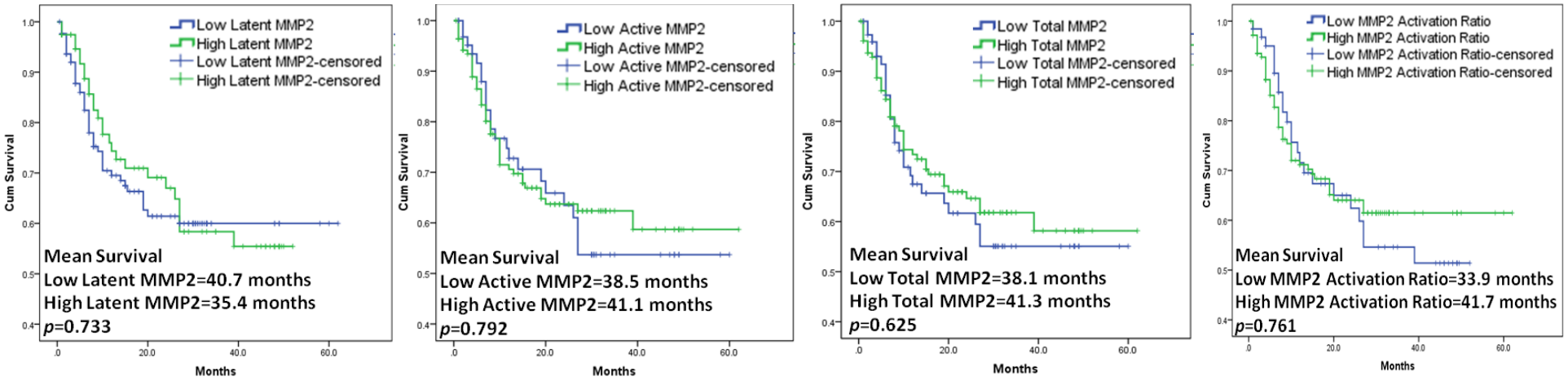


Figure 4.42B: Overall survival of oral cancer patients according to MMP-2 protein levels

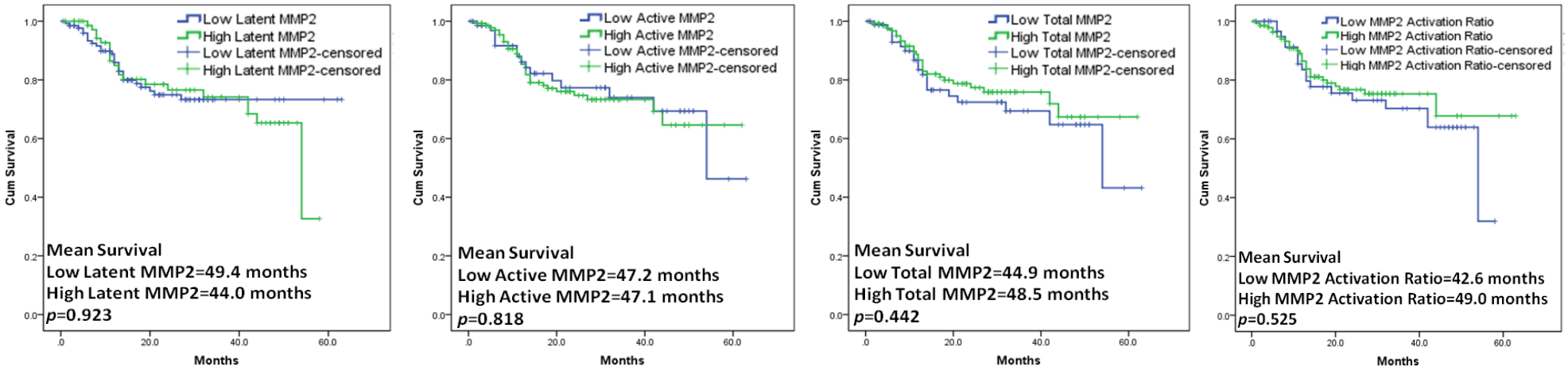


Figure 4.43A: Disease free survival of oral cancer patients according to MMP-9 protein levels

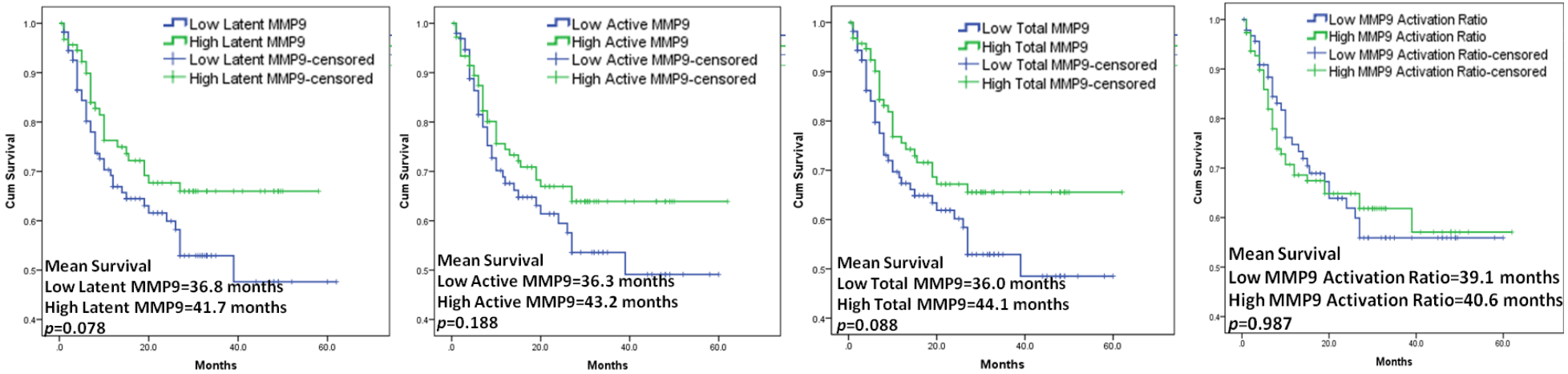
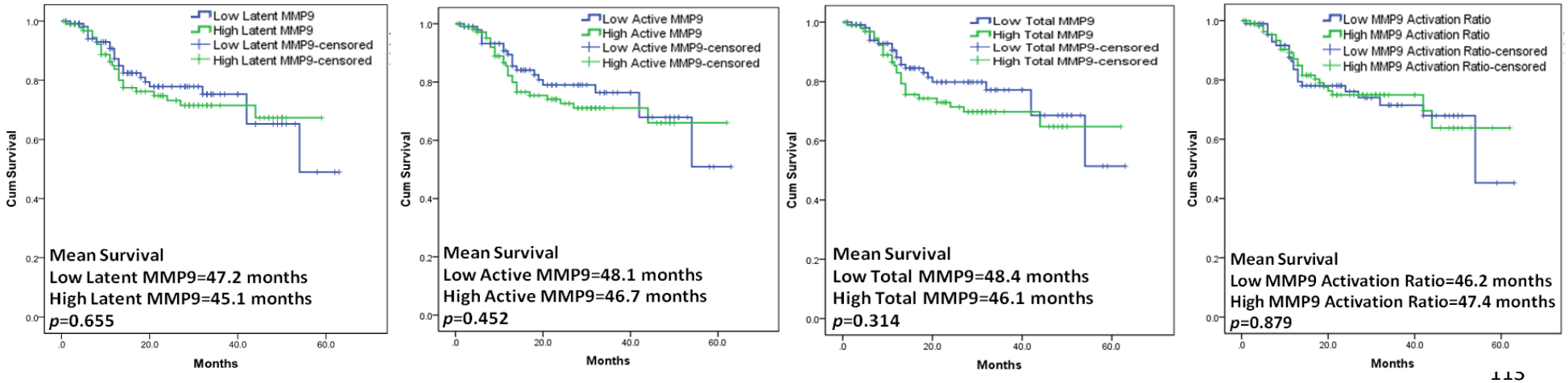


Figure 4.43B: Overall survival of oral cancer patients according to MMP-9 protein levels



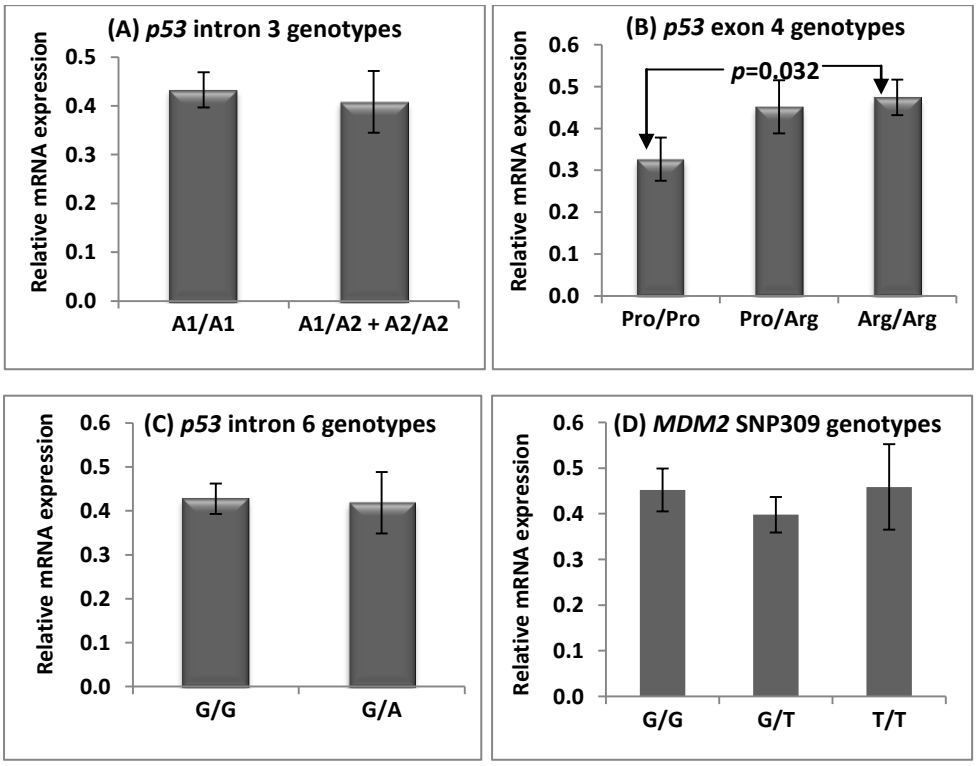
3. Objective 3: To evaluate the correlation between *p53*, *MDM2* polymorphisms, *p53* mutations, *hTERT*, *VEGFA*, *VEGFC*, *VEGFD*, *MMP2*, *MMP9* and their role in molecular pathogenesis of oral cancer

3.1. Correlation of *hTERT* transcript levels with *p53* and *MDM2* polymorphisms and *p53* mutations

3.1.1. Correlation of *hTERT* transcript levels with *p53* and *MDM2* genotypes

The present study evaluated correlation between *p53* genotypes and transcript levels of *hTERT*. It was observed that the transcript levels of *hTERT* were significantly higher in homozygous oral cancer patients harboring Arg allele compared to homozygous patients harboring Pro allele (p=0.032, Figure 4.44B). For *MDM2* SNP309 (T>G) polymorphism, no correlation among the types of genotypes and *hTERT* transcript levels was observed.

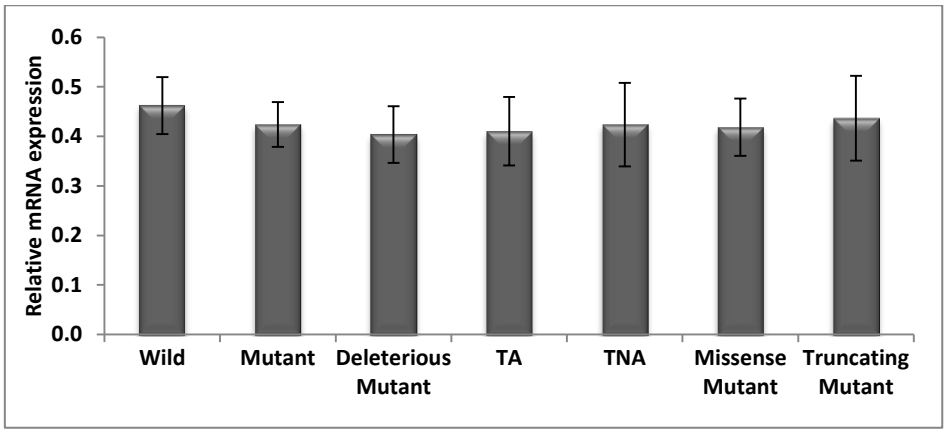
Figure 4.44: **Correlation of *hTERT* transcript levels with *p53* and *MDM2* genotypes**



3.1.2. Correlation of *hTERT* transcript levels with types of *p53* mutations

Figure 4.45 represents mRNA levels of *hTERT* according to types of *p53* mutations. However, there was no significant difference in mRNA levels of *hTERT* according to *p53* mutations status.

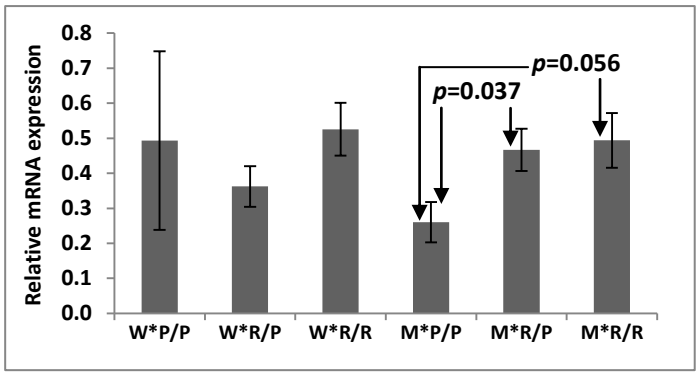
Figure 4.45: **Correlation of *hTERT* transcript levels with types of *p53* mutations**



TA=Transcriptionally active: TNA=Trancriptionally not-active

Further, we also analyzed *hTERT* transcript levels according to *p53* mutations and *p53* polymorphisms in combination. Interestingly, in combination with mutant *p53*, it was observed that *hTERT* levels was significantly higher in heterozygous patients (p=0.037) as well as homozygous individuals harboring Arg allele (p=0.056) compared to homozygous patients harboring Pro allele (Figure 4.46). No significant association of *hTERT* levels with *MDM2* genotypes and *p53* mutation in combination was observed.

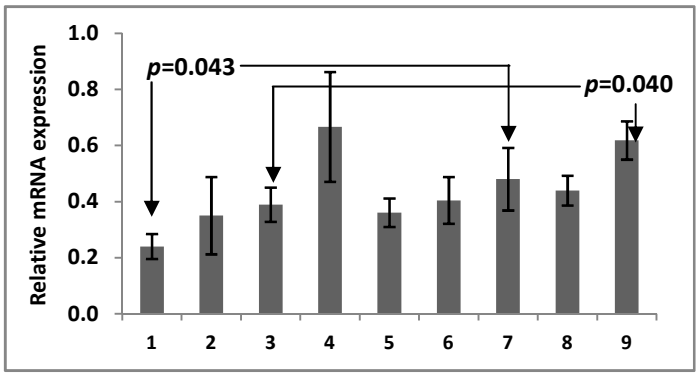
Figure 4.46: **Correlation of *hTERT* transcript levels with *p53* exon 4 genotypes and *p53* mutations in combination**



P/P=Pro/Pro,
R/P=Arg/Pro,
R/R=Arg/Arg,
W=Wild type *p53*
M=Mutant *p53*

hTERT levels were significantly higher in patients with Arg/Arg genotype compared to patients with Pro/Pro genotype at *p53* exon 4 locus in combination with T/T genotype at *MDM2* locus (p=0.043, Figure 4.47). *hTERT* levels were also significantly higher in patients with Arg/Arg genotype compared to patients with Pro/Pro genotype at *p53* exon 4 locus in combination with G/G genotype at *MDM2* locus (p=0.040, Figure 4.47).

Figure 4.47: **Correlation of *hTERT* transcript levels with *p53* exon 4 and *MDM2* SNP309 genotypes in combination**



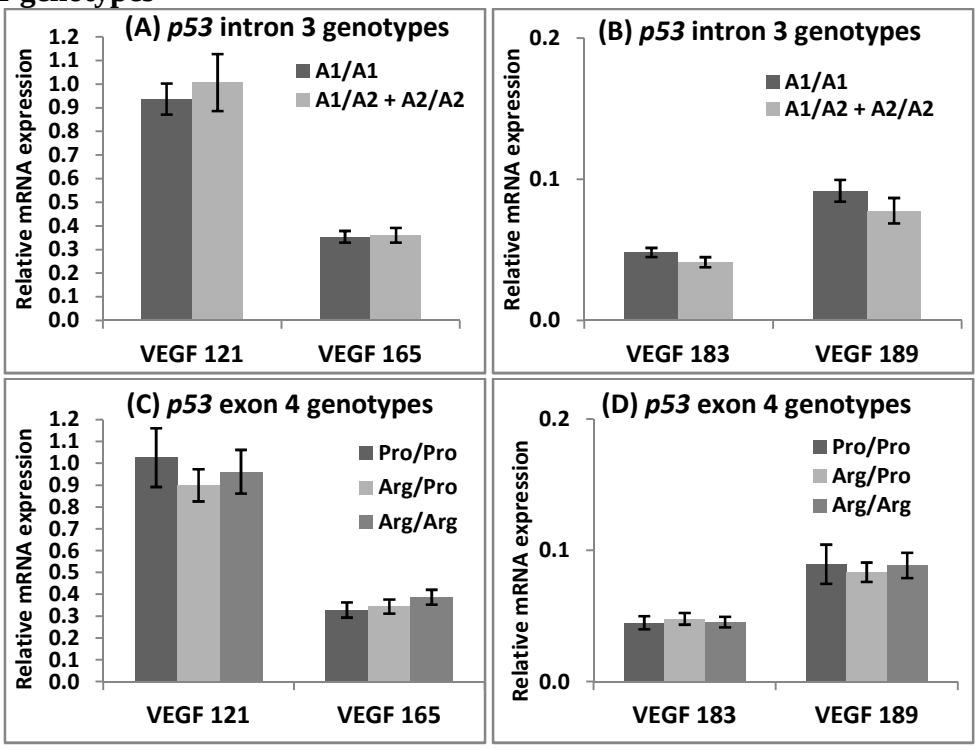
1=Pro/Pro+T/T,
2=Pro/Pro+G/T,
3=Pro/Pro+G/G,
4=Arg/Pro+T/T,
5=Arg/Pro+G/T,
6=Arg/Pro+G/G,
7=Arg/Arg+T/T,
8=Arg/Arg+G/T,
9=Arg/Arg+G/G

3.2. Correlation of transcript as well as protein levels of *VEGFA*, *VEGFC* and *VEGFD* with *p53* and *MDM2* polymorphisms and *p53* mutations

3.2.1. Correlation of *VEGFA*, *VEGFC* and *VEGFD* transcript as well as protein levels with *p53* and *MDM2* genotypes

It was observed that *VEGFA* isoforms and *VEGFC* transcript levels did not show any association with *p53* and *MDM2* genotypes (Figure 4.48 and 4.49). However, *VEGFD* transcript levels were significantly higher in homozygous patients (A1/A1) for *p53* intron 3 genotypes compared to patients harbouring A2 allele (A1/A2+A2/A2) (p=0.048, Figure 4.49A). Further, *VEGFD* transcript levels did not show association with *MDM2* genotypes (Figure 4.49).

Figure 4.48: Correlation of transcript levels of *VEGFA* isoforms with *p53* and *MDM2* genotypes



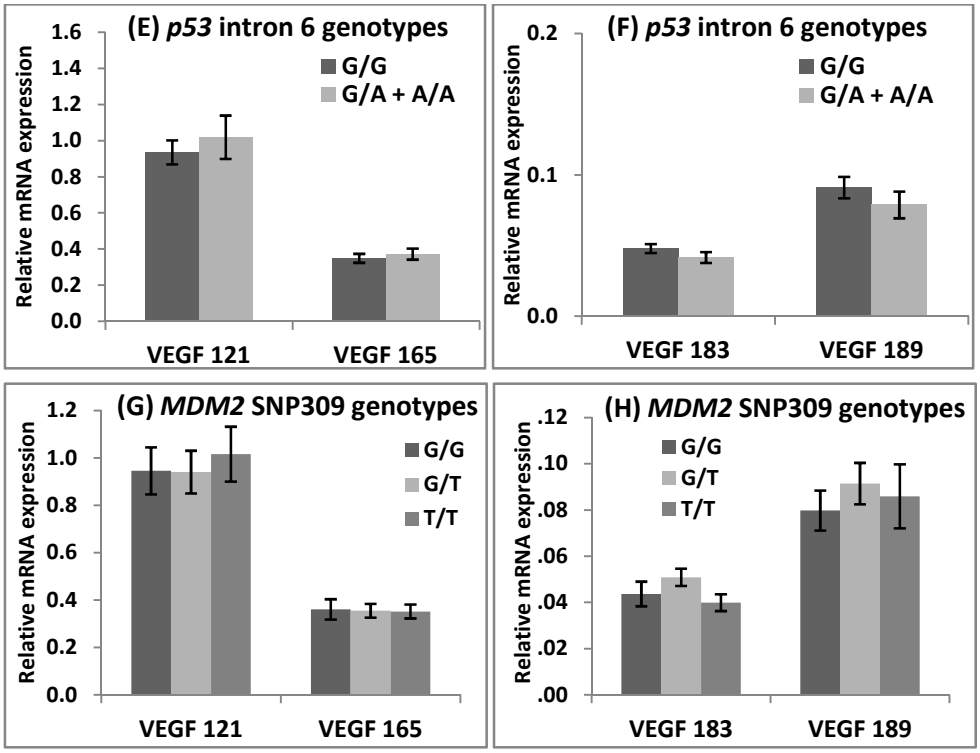
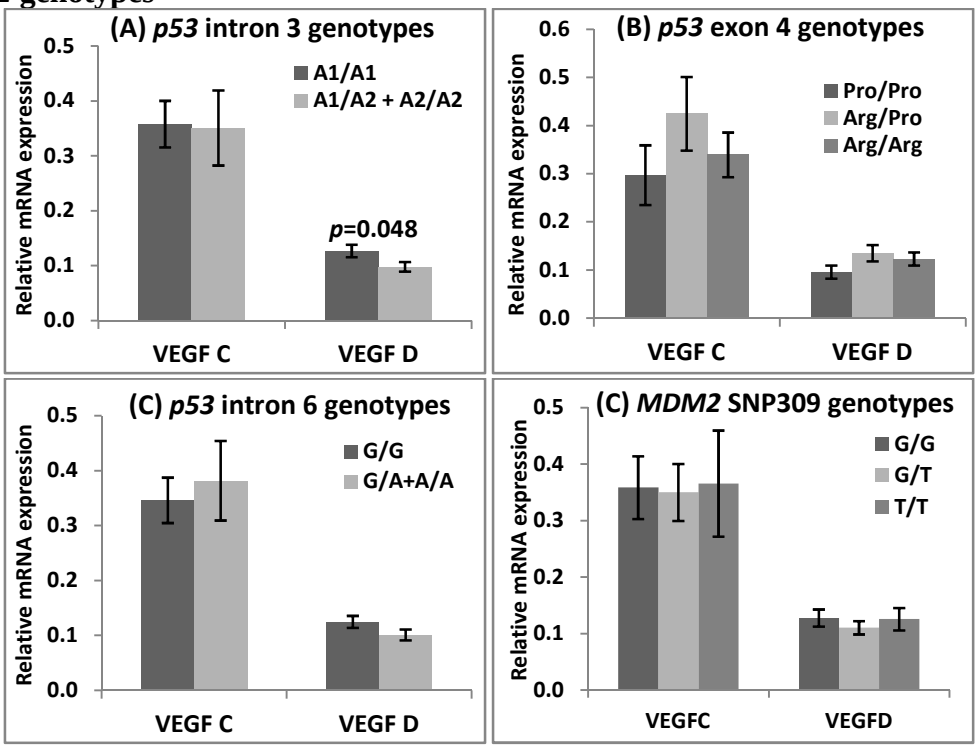


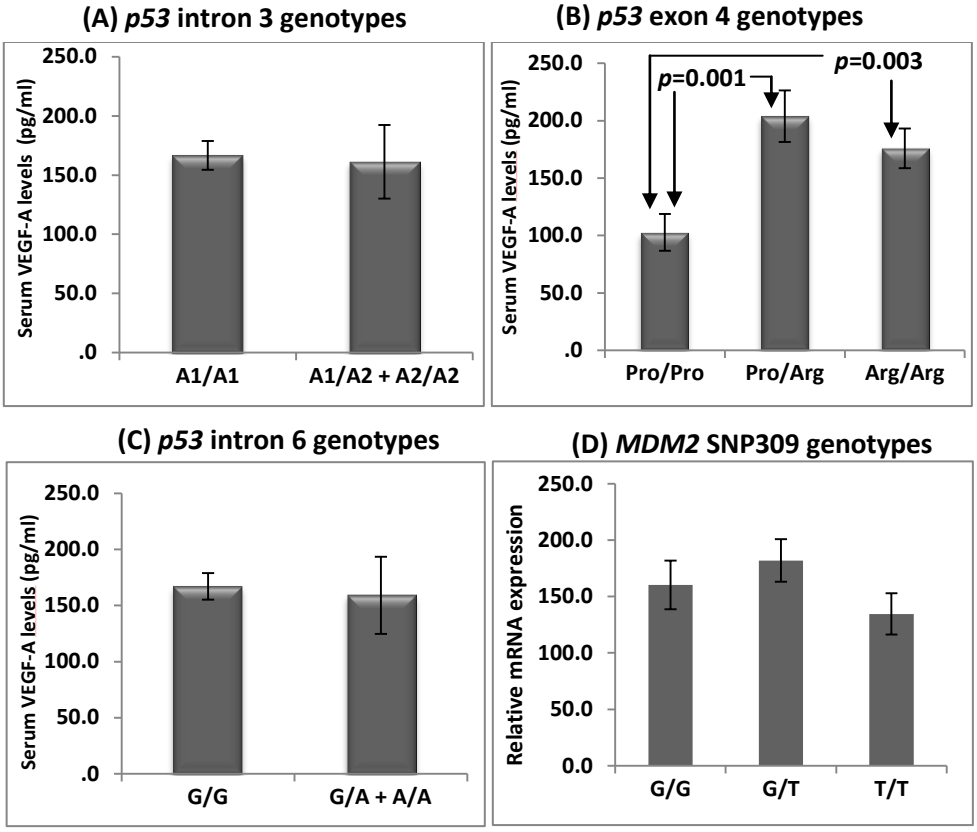
Figure 4.49: Correlation of *VEGFC* and *VEGFD* transcript levels with *p53* and *MDM2* genotypes



Analysis of circulatory levels of VEGF-A were significantly higher in heterozygous patients (Pro/Arg) and homozygous patients (Arg/Arg) compared to homozygous patients harbouring Pro allele at exon 4 (p=0.001 and p=0.003, respectively, Figure 4.50B). Correlation of serum VEGF-A levels with *MDM2* SNP309 (T>G) genotypes

revealed that serum VEGF-A levels were higher in oral cancer patients having G/T genotype compared to patients having T/T as well as G/G genotype (Figure 4.50D).

Figure 4.50: Correlation of serum VEGF-A levels with *p53* and *MDM2* genotypes



Serum VEGF-C and VEGF-D levels were also analyzed in association with *p53* and *MDM2* genotypes. It was observed that serum VEGF-C and serum VEGF-D levels were also higher in oral cancer patients harboring A2 allele (A1/A2+A2/A2) and A allele (G/A+A/A) compared to oral cancer patients having A1/A1 genotype at intron 3 locus and G/G genotype at intron 6 locus, respectively (Figure 4.51A and 4.51C, 4.52A and 4.52C, respectively). Serum VEGF-C levels were higher in heterozygous individuals (Pro/Arg) and homozygous individuals (Arg/Arg) compared to homozygous individuals harboring Pro allele at exon 4 in oral cancer patients (Figure 4.51B). However, in case of serum VEGF-D, their levels were higher in heterozygous individuals (Pro/Arg) and homozygous individuals (Pro/Pro) compared to homozygous individuals harboring Arg allele at exon 4 in oral cancer patients (Figure 4.52B). Serum VEGF-C levels did not show association with *MDM2* SNP309 genotypes (Figure 4.51D). Serum VEGF-D levels were higher in oral cancer patients having G/G genotypes compared to patients having G/T as well as T/T genotypes (Figure 4.52D).

Figure 4.51: Correlation of serum VEGF-C levels with *p53* and *MDM2* genotypes

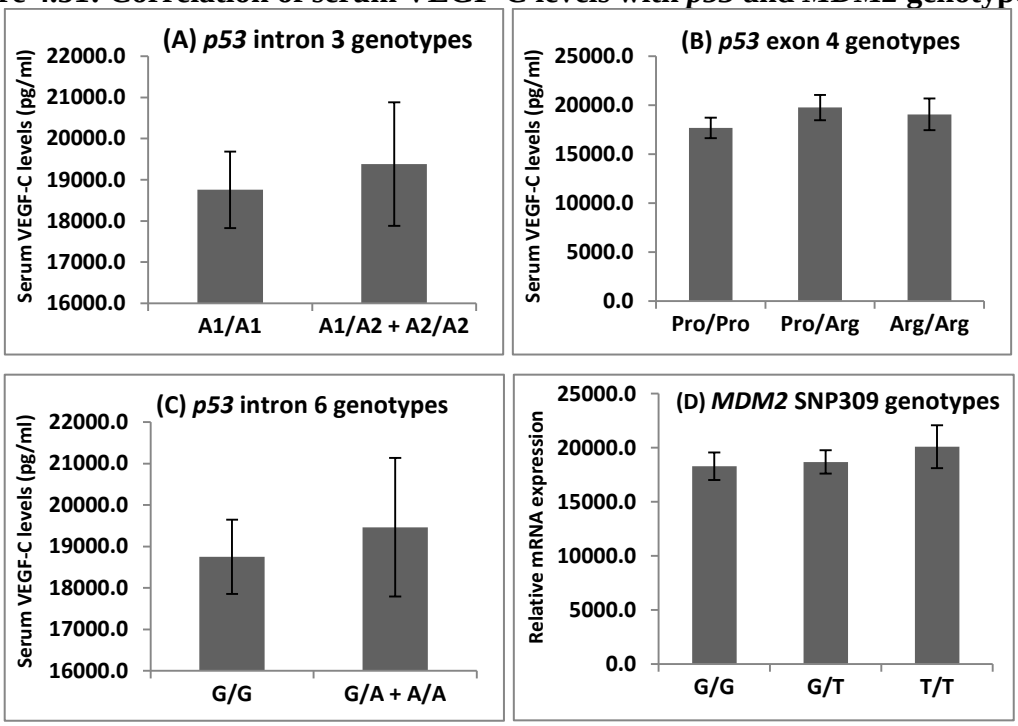
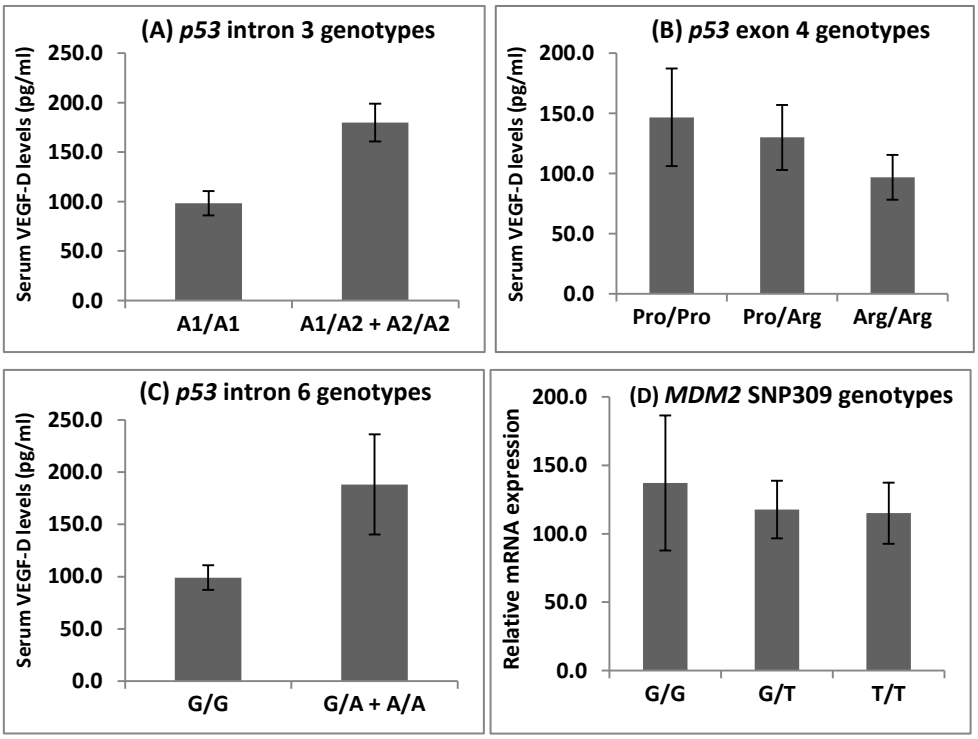


Figure 4.52: Correlation of serum VEGF-D levels with *p53* and *MDM2* genotypes



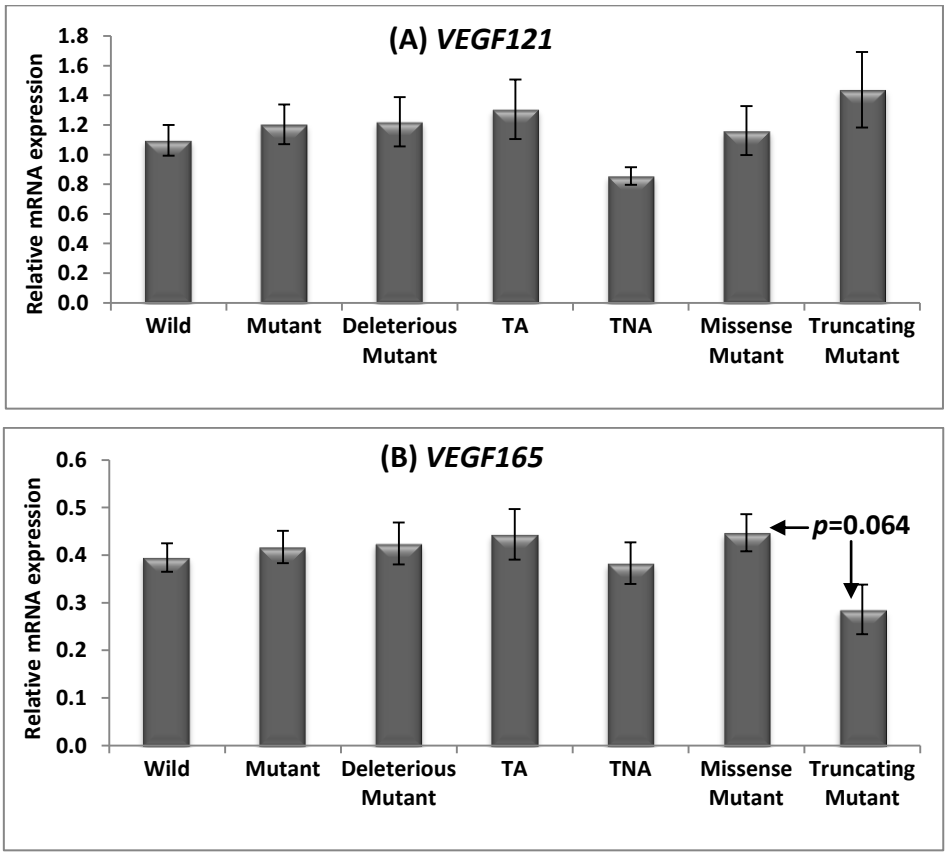
3.2.2. Correlation of *VEGFA, VEGFC* and *VEGFD* transcript as well as protein levels with the types of *p53* mutations

Figure 4.53 represents correlation between *VEGFA* isoforms and types of *p53* mutations. *VEGF165*, *VEGF183* and *VEGF189* decreased in tumors having truncating mutations compared to tumors having missense type of *p53* mutations and this decrease was significant for *VEGF189* (p=0.064 (Figure 4.53B), p=0.065 (Figure 4.53C) and p=0.029

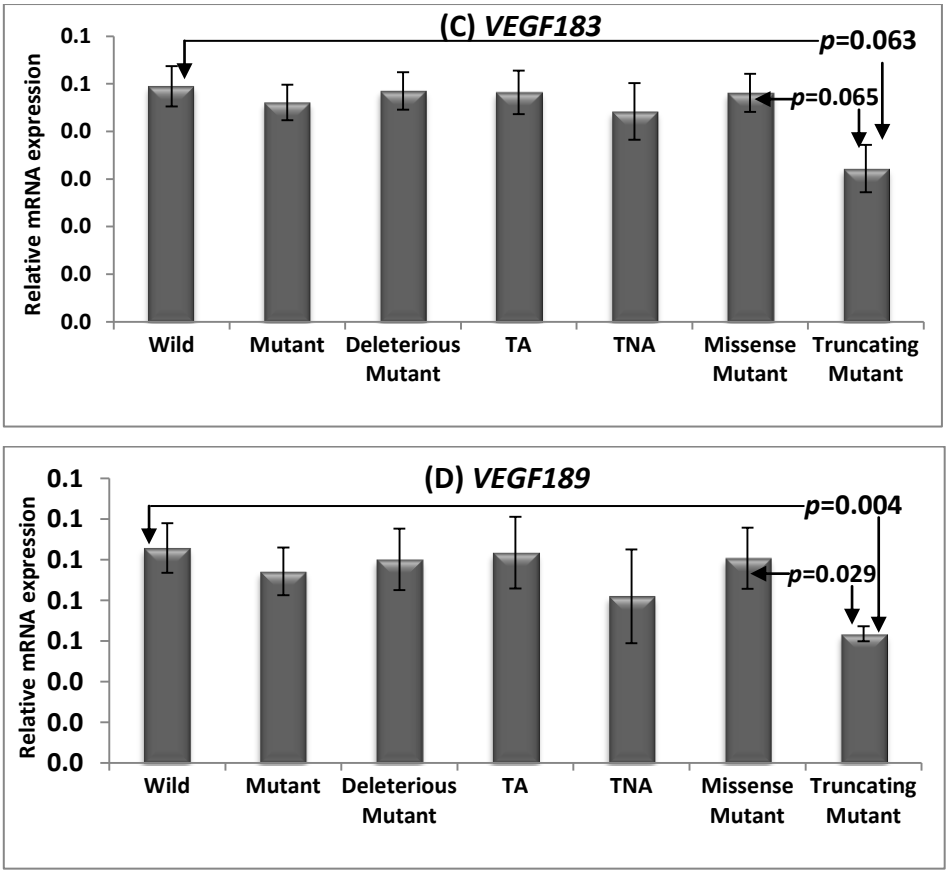
(Figure 4.53D) respectively). Moreover, *VEGF183* was decreased in tumors having mutant *p53* (truncated) compared to tumors having wild *p53* (p=0.063 Figure 4.53C). It was observed *VEGF189* was significantly decreased in tumors having mutant *p53* (truncated) compared to tumors having wild *p53* (p=0.004 Figure 4.53D). In contrast to *VEGF165*, *VEGF183* and *VEGF189*, levels of *VEGF121* were increased in tumors having truncating type of *p53* mutations compared to tumors having missense (Figure 4.53A).

For *VEGFC*, the mRNA levels were significantly higher in tumors having transcriptionally not-active *p53* mutations than tumors with transcriptionally active *p53* mutations (p=0.010, Figure 4.54A). *VEGFC* mRNA levels were also higher in tumors having transcriptionally not-active *p53* mutations compared to tumors having wild type *p53*. However, *VEGFD* mRNA levels decreased in tumors having truncating type of *p53* mutations compared to tumors having missense type (p=0.016, Figure 4.54B).

Figure 4.53: Correlation of transcript levels of *VEGFA* isoforms with type of *p53* mutations

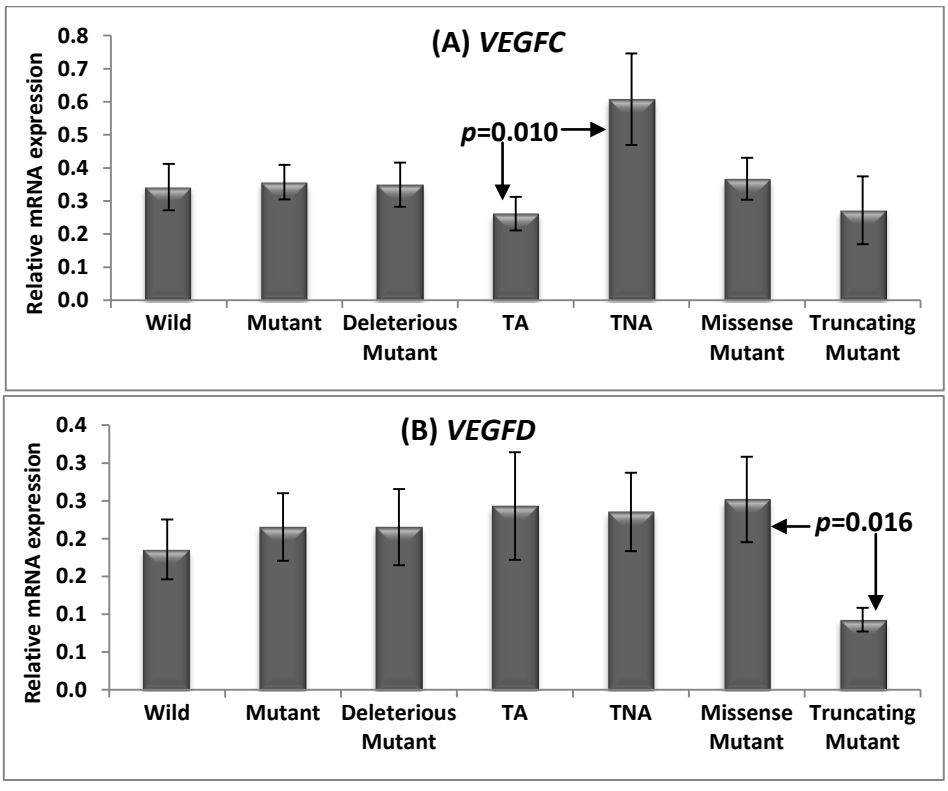


TA=Transcriptionally active: TNA=Trancriptionally not-active



TA=Transcriptionally active: TNA=Trancriptionally not-active

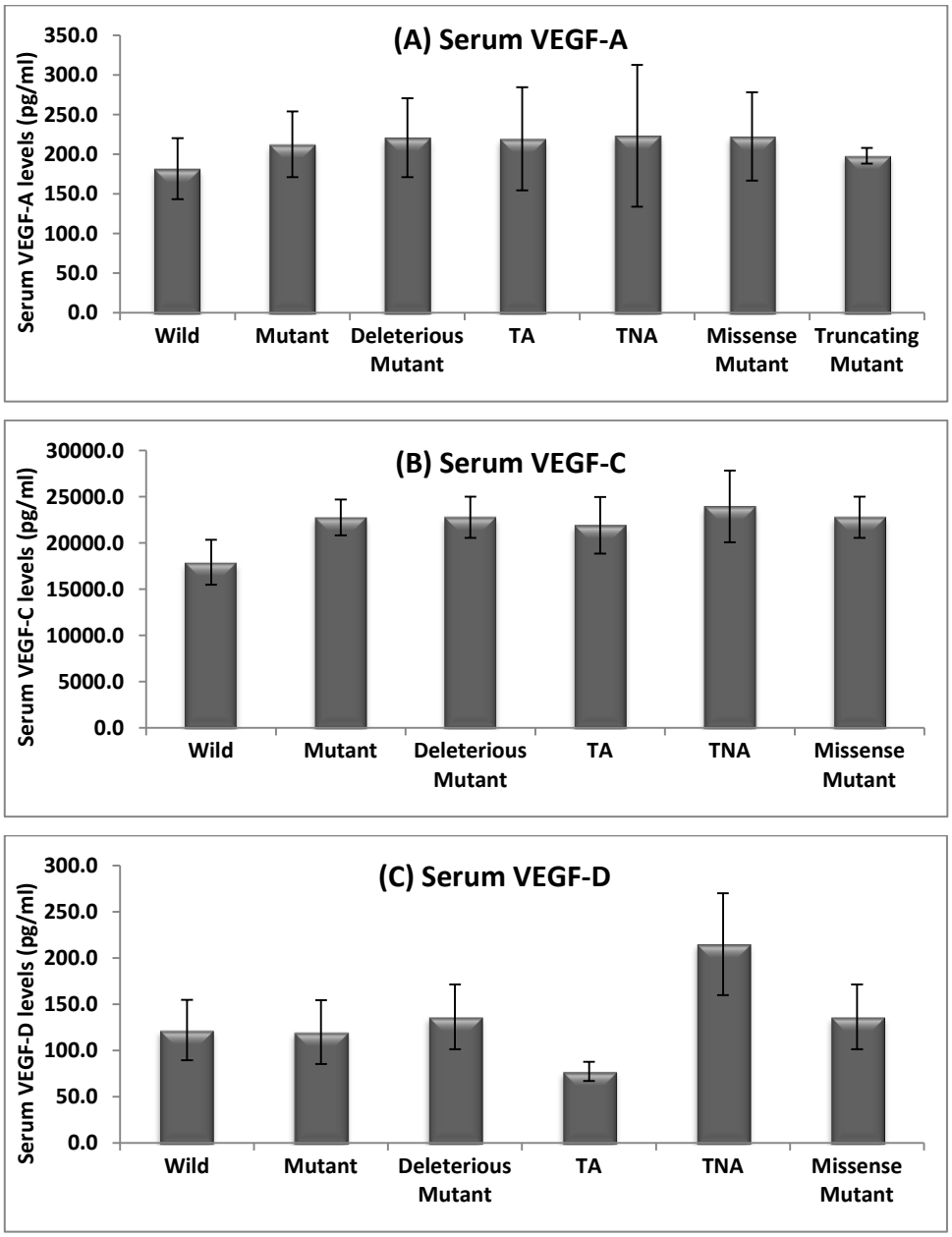
Figure 4.54: Correlation of *VEGFC* and *VEGFD* transcript levels with the types of *p53* mutations



TA=Transcriptionally active: TNA=Trancriptionally not-active

Association of serum VEGF-A, VEGF-C and VEGF-D levels with *p53* mutation status was also analyzed. There was no significant difference in the serum levels of VEGF-A and VEGF-C according to types of *p53* mutations (Figure 4.55A and 4.55B). However, levels of serum VEGF-C were higher in patients having mutant *p53* irrespective of mutation types compared to patients having wild type *p53*. Serum VEGF-D levels were higher in patients having transcriptionally not active mutations in *p53* compared to patients having transcriptionally active, missense, deleterious mutations in *p53* or wild type *p53* (Figure 4.55C).

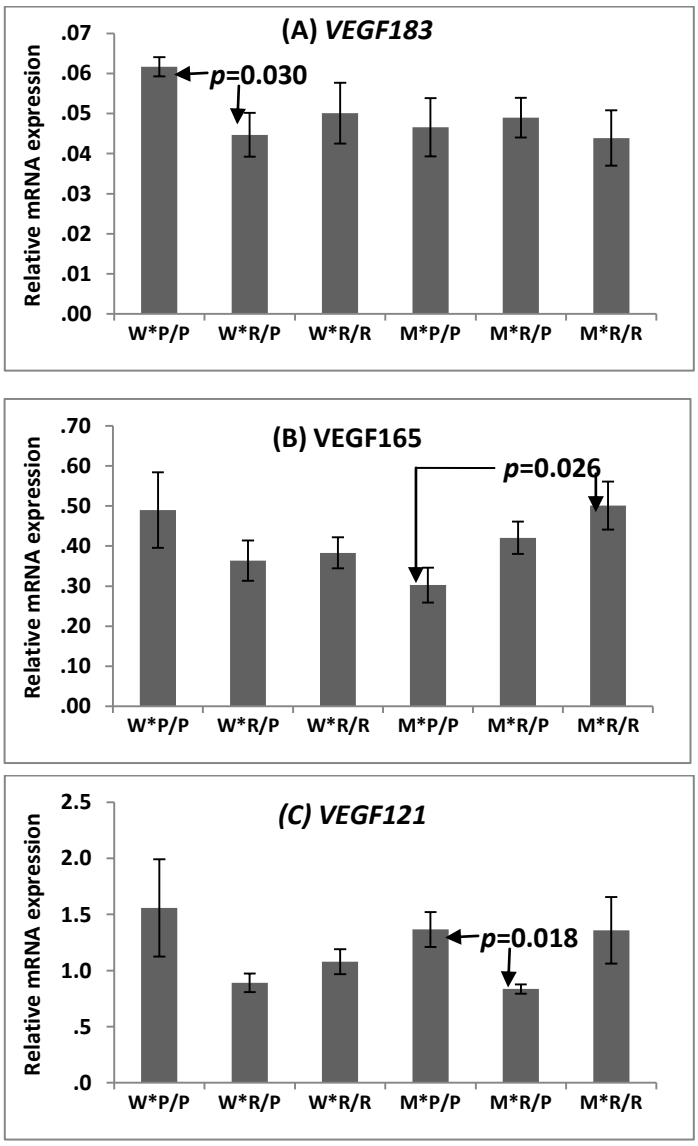
Figure 4.55: Correlation of serum VEGF-A, VEGF-C and VEGF-D levels with the types of *p53* mutations



TA=Transcriptionally active: TNA=Trancriptionally not-active

Further, association of *VEGFA* isoforms, *VEGFC* and *VEGFD* transcript as well as protein levels with *p53*, *MDM2* genotypes and mutations in combination was also analyzed. It was observed that in combination with wild type *p53*, *VEGF183* transcript levels was significantly higher in patients with Pro/Pro genotype compared to patients with Arg/Pro genotype at *p53* exon 4 locus (p=0.030, Figure 4.56A). *VEGF165* transcript levels was significantly higher in patients with Arg/Arg genotype compared to patients with Pro/Pro genotype at *p53* exon 4 in combination with mutant *p53* (p=0.028, Figure 4.56B). *VEGF121* transcript levels was also significantly higher in patients with Pro/Pro genotype compared to patients with Arg/Pro genotype at *p53* exon 4 locus in combination with mutant *p53* (p=0.018, Figure 4.56C).

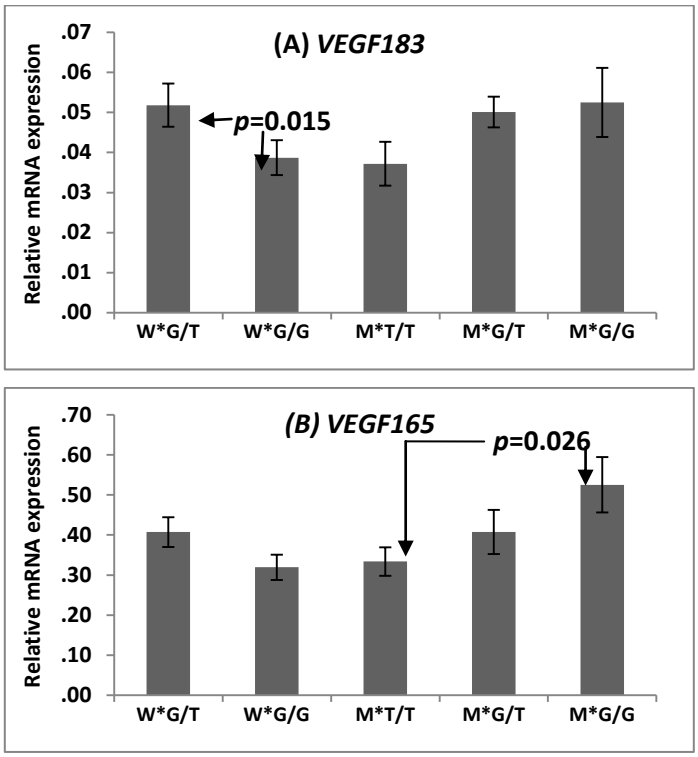
Figure 4.56: Correlation of transcript levels of *VEGFA* isoforms with *p53* exon 4 genotypes and mutations in combination



P/P=Pro/Pro; R/P=Arg/Pro; R/R=Arg/Arg; W=Wild type *p53*; M=Mutant *p53*

VEGF183 levels were also significantly higher in patients with G/T genotype compared to patients with G/G genotype at *MDM2* locus in combination with wild type *p53* (p=0.015, Figure 4.57A). In combination with mutant *p53*, *VEGF165* transcript levels was significantly higher in patients with G/G genotype compared to patients with T/T genotype at *MDM2* locus (p=0.026, Figure 4.57B).

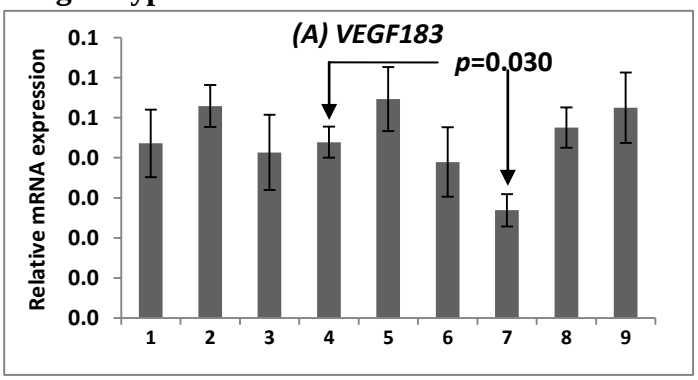
Figure 4.57: Correlation of transcript levels of *VEGFA* isoforms with *MDM2* SNP309 genotypes and mutations in combination



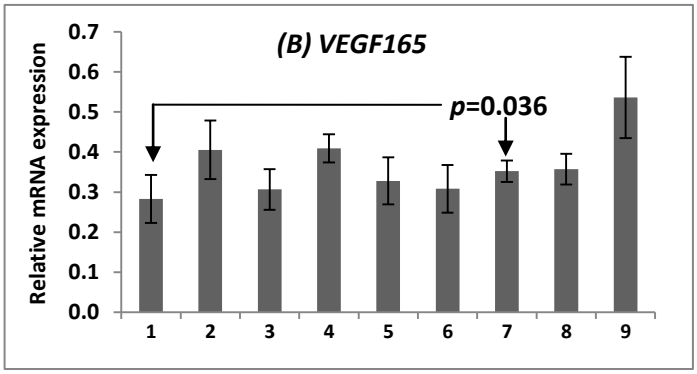
W=Wild type *p53*,
M=Mutant *p53*

VEGF183 transcript levels were significantly higher in patients with Arg/Pro genotype compared to Arg/Arg genotype of *p53* exon 4 in combination with T/T genotype of *MDM2* (p=0.030, Figure 4.58A). *VEGF165* transcript levels were significantly higher in patients with Arg/Arg genotype compared to Pro/Pro genotype of *p53* exon 4 in combination with T/T genotype of *MDM2* (p=0.036, Figure 4.58B).

Figure 4.58: Correlation of transcript levels of *VEGFA* isoforms with *p53* exon 4 and *MDM2* SNP309 genotypes in combination



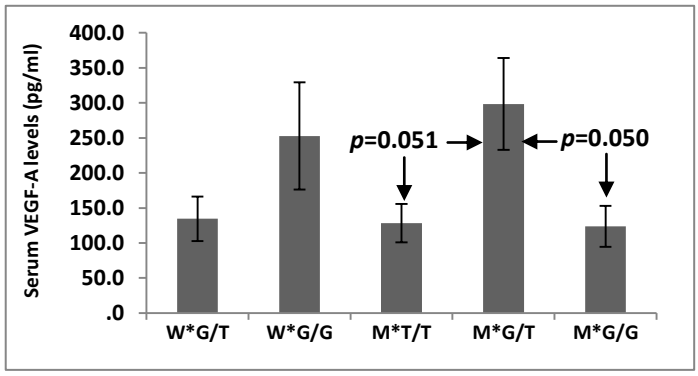
1=Pro/Pro+T/T,
2=Pro/Pro+G/T,
3=Pro/Pro+G/G,
4=Arg/Pro+T/T,
5=Arg/Pro+G/T,
6=Arg/Pro+G/G,
7=Arg/Arg+T/T,
8=Arg/Arg+G/T,
9=Arg/Arg+G/G



1=Pro/Pro+T/T,
2=Pro/Pro+G/T,
3=Pro/Pro+G/G,
4=Arg/Pro+T/T,
5=Arg/Pro+G/T,
6=Arg/Pro+G/G,
7=Arg/Arg+T/T,
8=Arg/Arg+G/T,
9=Arg/Arg+G/G

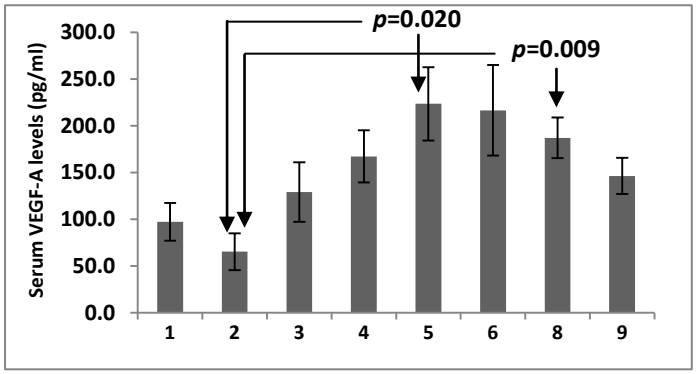
Serum VEGF-A levels were significantly higher in patients with G/T genotype compared to patients with G/G as well as T/T genotype in combination with mutant *p53* (p=0.051 and p=0.050, respectively, Figure 4.59). Serum VEGF-A levels were also significantly higher in patients with Arg/Pro and Arg/Arg genotypes compared to patients with Pro/Pro genotype at *p53* exon 4 locus in combination with G/T genotype at *MDM2* locus (p=0.002 and p=0.009, respectively, Figure 4.60). Levels of serum VEGF-C and D did not alter according to presence of *p53*, *MDM2* polymorphisms and *p53* mutations simultaneously.

Figure 4.59: Correlation of serum VEGF-A levels with *MDM2* SNP 309 genotypes and *p53* mutations in combination



W=Wild type *p53*,
M=Mutant *p53*

Figure 4.60: Correlation of serum VEGF-A levels with *p53* exon 4 and *MDM2* SNP309 genotypes in combination



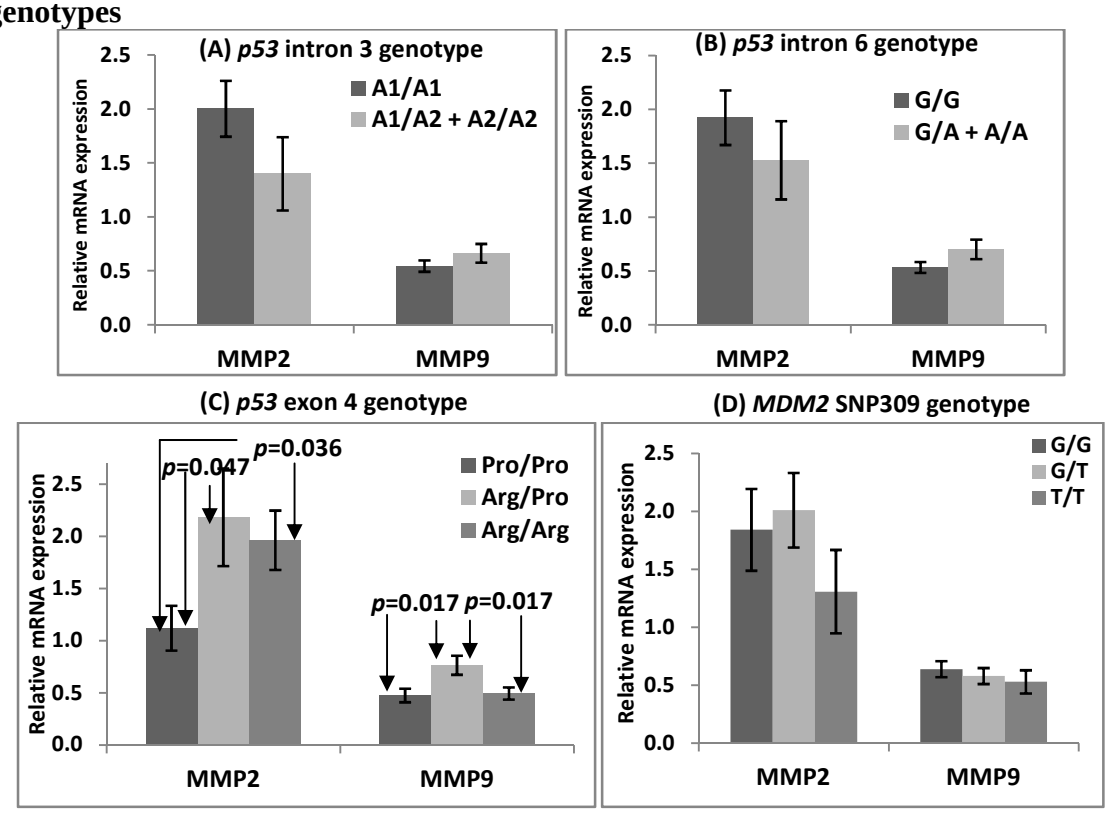
1=Pro/Pro+T/T,
2=Pro/Pro+G/T,
3=Pro/Pro+G/G,
4=Arg/Pro+T/T,
5=Arg/Pro+G/T,
6=Arg/Pro+G/G,
7=Arg/Arg+T/T,
8=Arg/Arg+G/T,
9=Arg/Arg+G/G

3.3. Correlation of *MMP2* and *MMP9* transcript as well as protein levels with *p53* and *MDM2* polymorphisms and *p53* mutations

3.3.1. Correlation of *MMP2* and *MMP9* transcript as well as protein levels with *p53* and *MDM2* genotypes

When correlation between *MMP2* and *MMP9* mRNA levels and *p53* genotypes was analyzed, it was observed that heterozygous patients (Arg/Pro) and homozygous patients (Arg/Arg) for *p53* exon 4 had significantly elevated transcript levels of *MMP2* as compared to homozygous patients (Pro/Pro) (p=0.047 and p=0.036, respectively, Figure 4.61C). Also, heterozygous patients (Arg/Pro) had significantly higher transcript levels of *MMP9* compared to homozygous patients harboring Arg or Pro allele (p=0.017 and p=0.017, respectively, Figure 4.61C). At intron 3 and intron 6 locus, the levels of *MMP9* mRNA were higher in patients having A1/A2+A2/A2 and G/A+A/A genotypes compared to patients having A1/A1 and G/G genotypes, respectively (Figure 4.61A and 4.61B, respectively). However, levels of *MMP2* mRNA were higher in patients having A1/A1 and G/G genotypes compared to patients having A1/A2+A2/A2 and G/A+A/A genotypes at intron 3 and intron 6, respectively (Figure 4.61A and Figure 4.61B, respectively). Transcript levels of *MMP2* and *MMP9* were also higher in oral cancer patients having G/T and G/G genotypes compared to the patients having T/T genotype (Figure 4.61D).

Figure 4.61: Correlation of *MMP2* and *MMP9* transcript levels with *p53* and *MDM2* genotypes



Further, correlation between protein levels of latent, active and total MMP-2 and MMP-9 levels and *p53* genotypes was also analyzed. Levels of latent, active and total MMP-9 were lower in oral cancer patients having A1/A2+A2/A2 genotypes as compared to oral cancer patients having A1/A1 genotype at intron 3 locus (Figure 4.62B).

Latent and total MMP-2 were significantly higher in oral cancer patients homozygous for Arg allele compared to oral cancer patients homozygous for Pro allele (p=0.026 and p=0.047, respectively). Active MMP-2 was also higher in oral cancer patients homozygous for Arg allele as compared to oral cancer patients homozygous for Pro allele (p=0.083). Also, active MMP-2 was higher in oral cancer patients homozygous for Arg allele as compared to heterozygous (Arg/Pro) oral cancer patients (p=0.074) (Figure 4.63A). Latent, active and total MMP-9 levels were also higher in oral cancer patients having Arg/Arg genotype compared to oral cancer patients with Pro/Pro (Figure 4.63B).

Levels of latent, active and total MMP-9 were lower in oral cancer patients having G/A+A/A genotypes compared to oral cancer patients having G/G genotype (Figure 4.64B).

Correlation of latent, active and total MMP-2 and MMP-9 with combined *p53* genotypes was also analyzed. It was observed that levels of latent and total MMP-2 were higher in patients having Arg/Arg genotype compared to patients having Arg/Pro and Pro/Pro genotypes with common intron 3 (A1/A1) and intron 6 (G/G) genotypes and this was significant for latent MMP-2 (p=0.015 and p=0.043, respectively, Figure 4.65). However, no such observation was found for latent, active and total MMP-9 levels with respect to combined *p53* genotypes in oral cancer patients (Figure 4.66).

Figure 4.62: Correlation of latent, active and total MMP-2 and MMP-9 protein levels with *p53* intron 3 genotypes

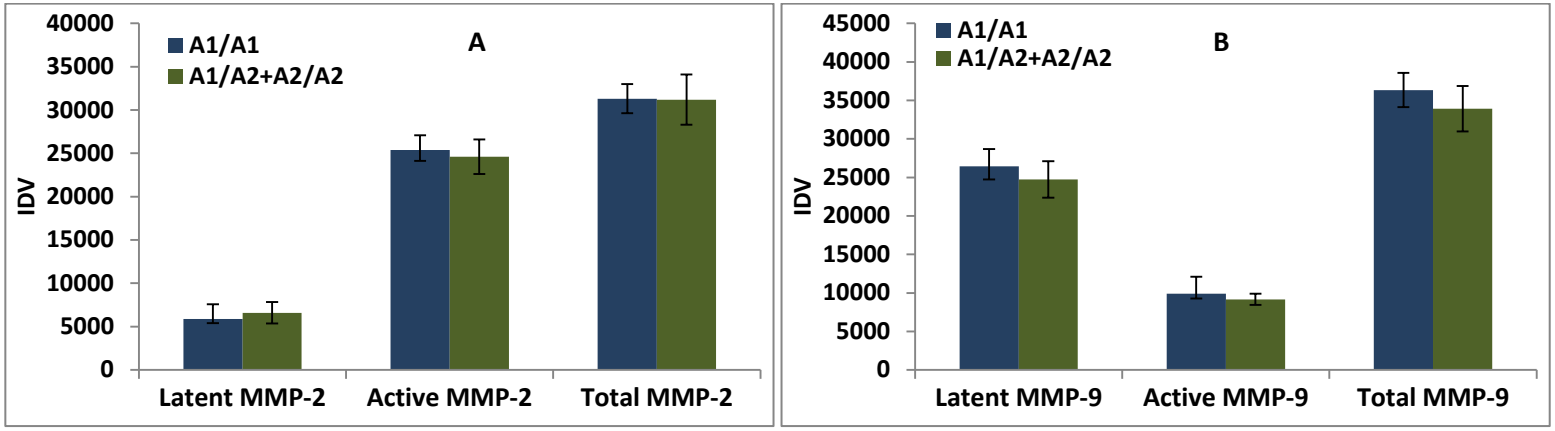


Figure 4.63: Correlation of latent, active and total MMP-2 and MMP-9 protein levels with *p53* exon 4 genotypes

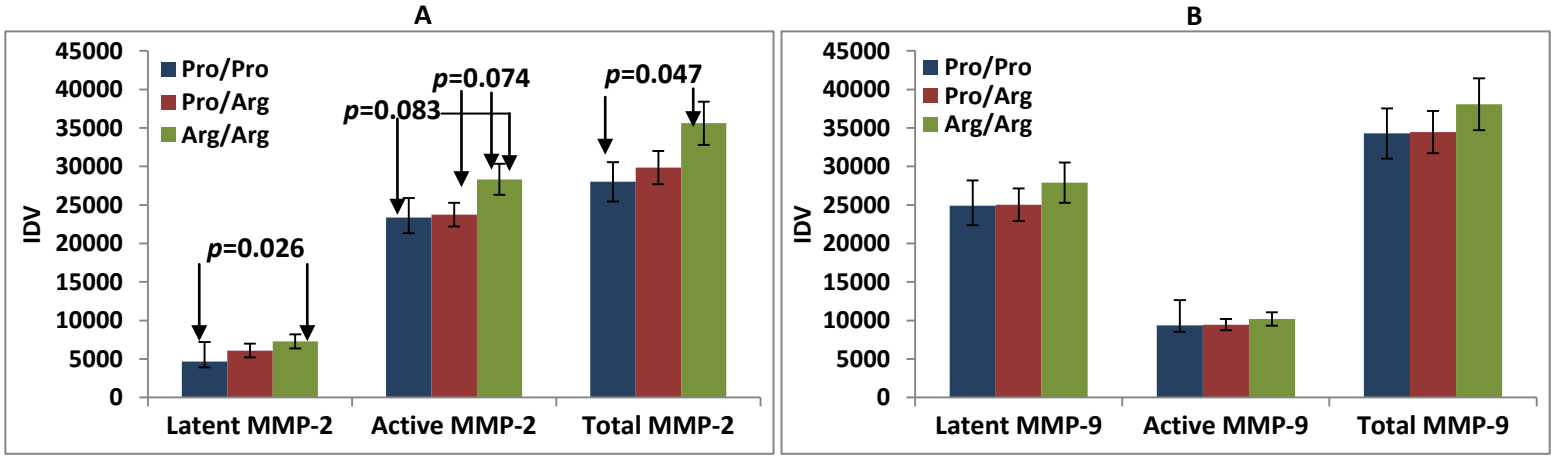


Figure 4.64: Correlation of latent, active and total MMP-2 and MMP-9 protein levels with *p53* intron 6 genotypes

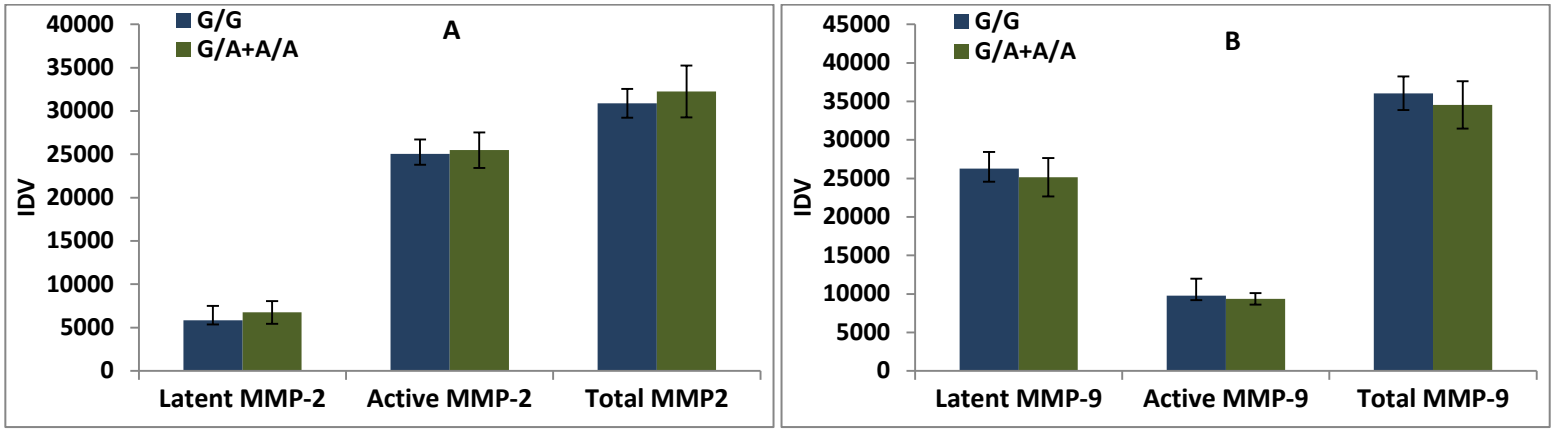
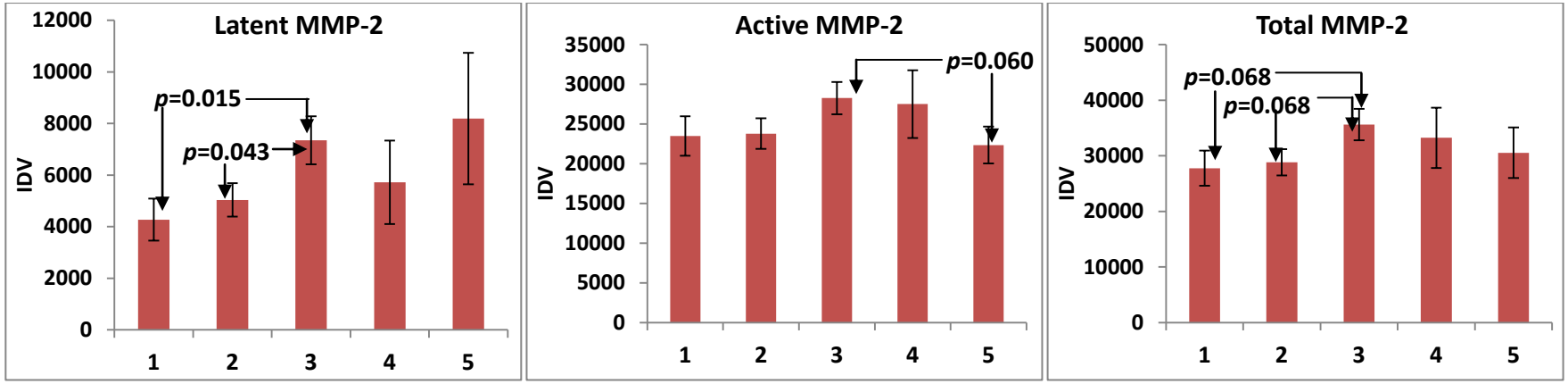
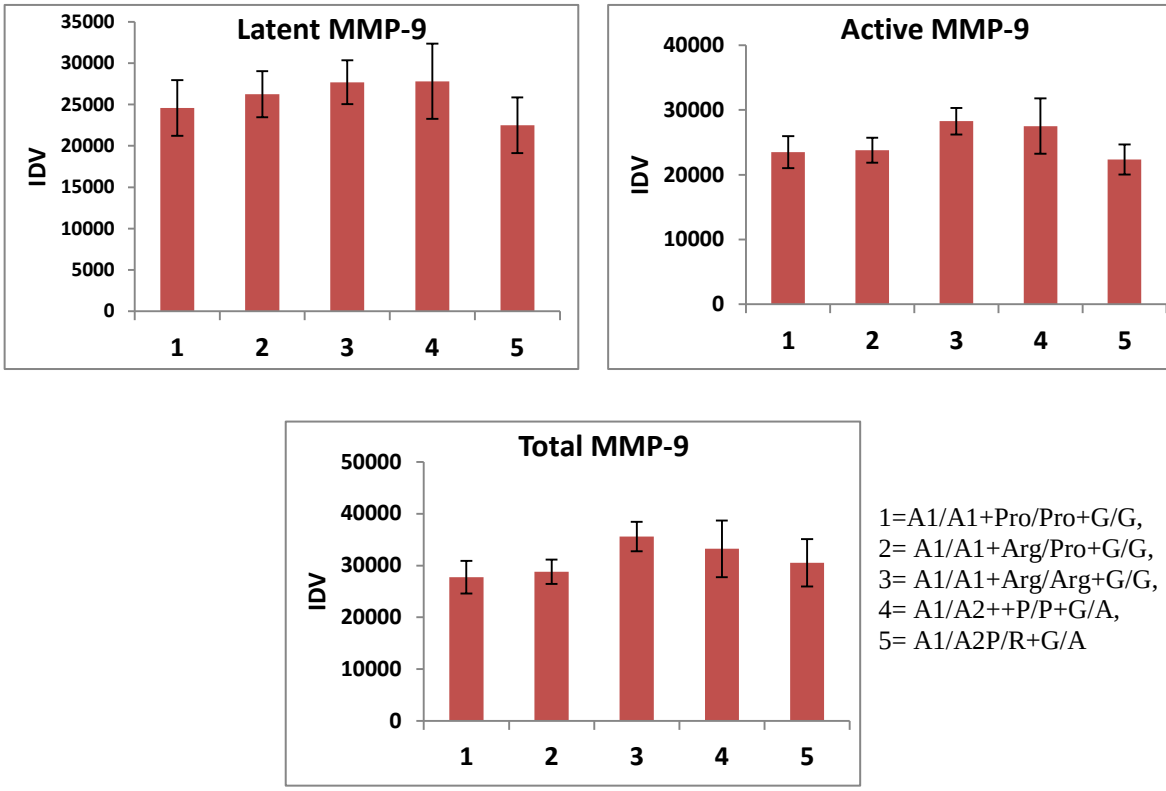


Figure 4.65: Correlation of latent, active and total MMP-2 protein levels with *p53* combined genotypes



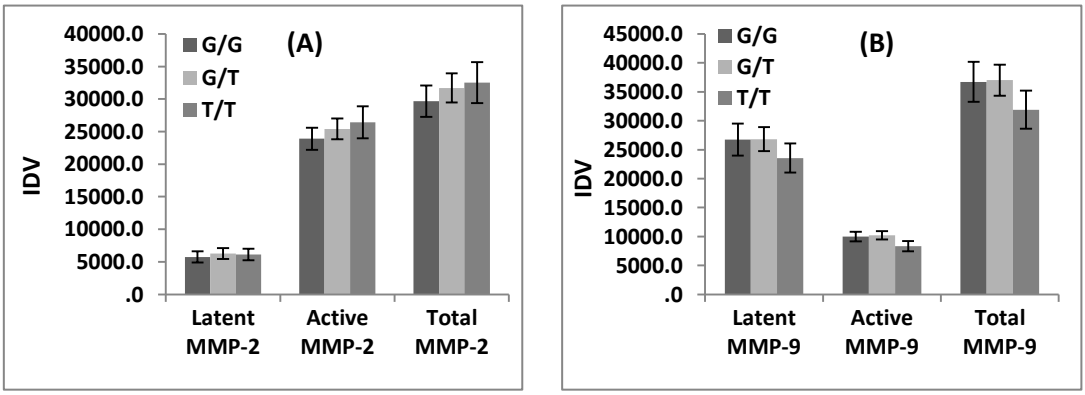
1=A1/A1+Pro/Pro+G/G, 2= A1/A1+Arg/Pro+G/G, 3= A1/A1+Arg/Arg+G/G, 4= A1/A2+P/P+G/A, 5= A1/A2+P/R+G/A

Figure 4.66: Correlation of latent, active and total MMP-9 protein levels with *p53* combined genotypes



Further, correlation of protein levels of MMP-2 and MMP-9 with *MDM2* SNP309 (*T>G*) genotypes revealed that levels of all the different forms of MMP-2 were higher in oral cancer patients having G/T and T/T genotypes compared to patients having G/G genotype (Figure 4.67A). Levels of all the different forms of MMP-9 were higher in oral cancer patients having G/G and G/T genotypes compared to patients having T/T genotypes (Figure 4.67B).

Figure 4.67: Correlation of latent, active and total MMP-2 and MMP-9 protein levels with *MDM2* SNP309 genotypes

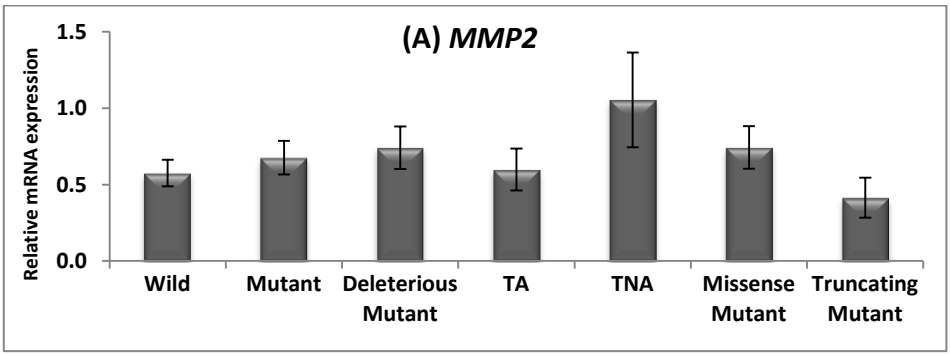


3.3.2. Correlation of *MMP2* and *MMP9* transcript as well as protein levels with the types of *p53* mutations

Correlation of transcript levels of *MMP2* and *MMP9* with types of *p53* mutations revealed that *MMP2* and *MMP9* mRNA levels were upregulated in tumors having transcriptionaly not active *p53* mutations compared to tumors having wild type *p53* (Figure 4.68A and 4.68B, respectively). This was significant for *MMP9* (p=0.050, Figure 4.68B). Levels of *MMP2* and *MMP9* mRNA were also found to be upregulated in tumors having transcriptionaly not active *p53* mutations compared to tumors having trancriptionally active *p53* mutations (Figure 4.68A and 4.68B, respectively). Moreover, *MMP2* and *MMP9* mRNA levels were lower in tumors having truncating *p53* mutations compared to tumors having missense *p53* mutations as well as wild type *p53* (Figure 4.68A and 4.68B, respectively).

Correlation of levels of latent, active and total MMP-2 and MMP-9 with types of *p53* mutations revealed that latent MMP-2 levels were higher in patients having transcriptionaly not active *p53* mutations compared to patients having transcriptionaly active *p53* mutations and also compared to patients having wild type *p53* (Figure 4.69A). There was no difference in the levels of active and total MMP-2 with respect to types of *p53* mutations (Figure 4.69B and 4.69C). Further, levels of latent, active and total MMP-9 were found to be higher in patients having wild type *p53* compared to patients having any type of mutant *p53* (Figure 4.70A, 4.70B and 4.70C).

Figure 4.68: Correlation of *MMP2* and *MMP9* transcript levels with the types of *p53* mutations



TA=Transcriptionally active: TNA=Trancriptionally not-active

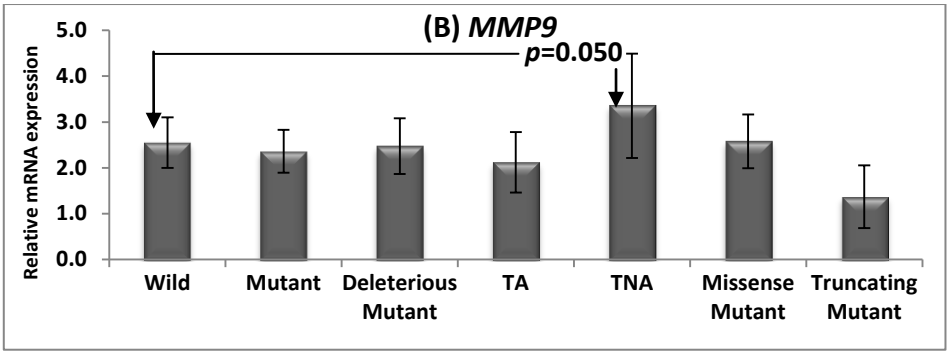
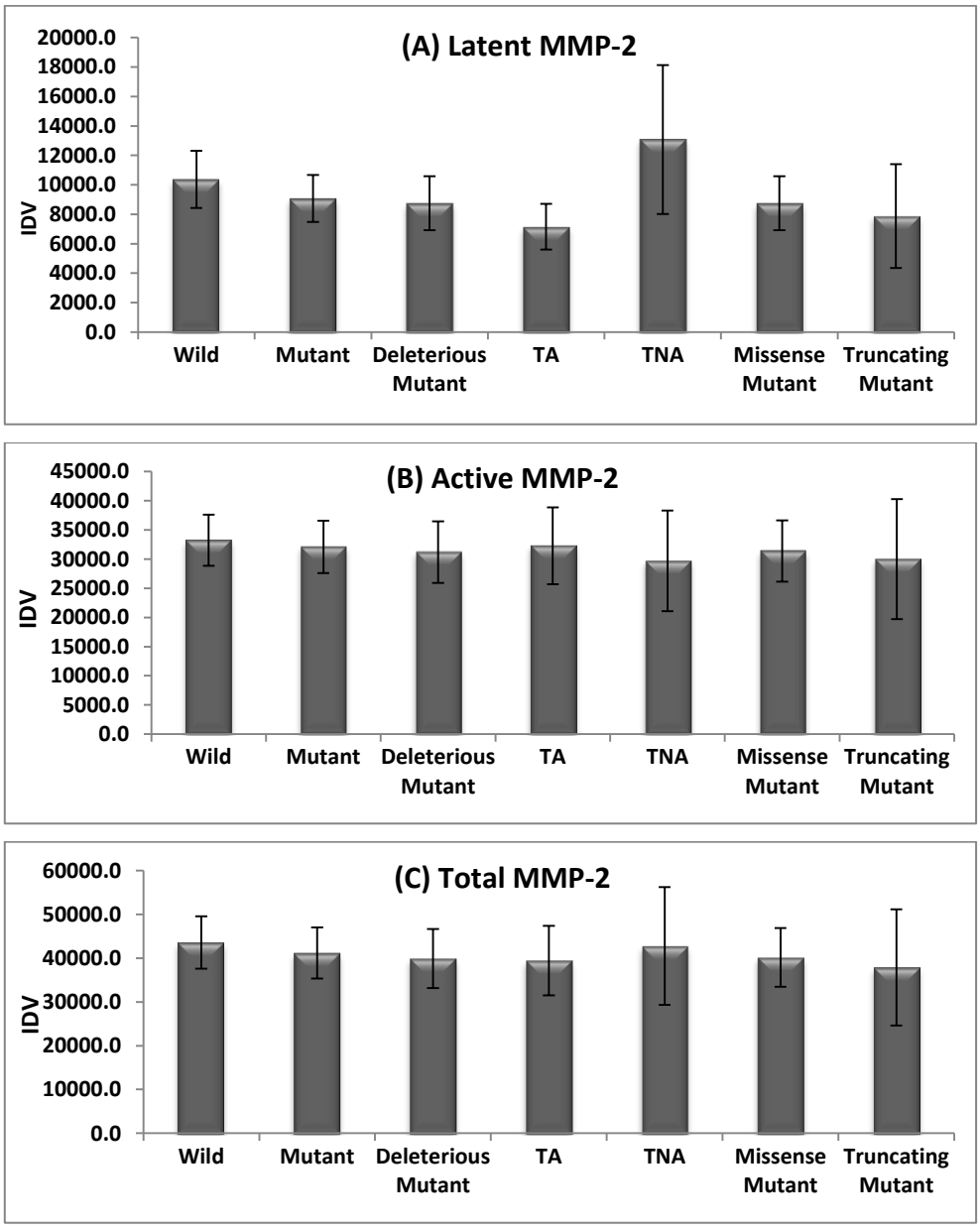
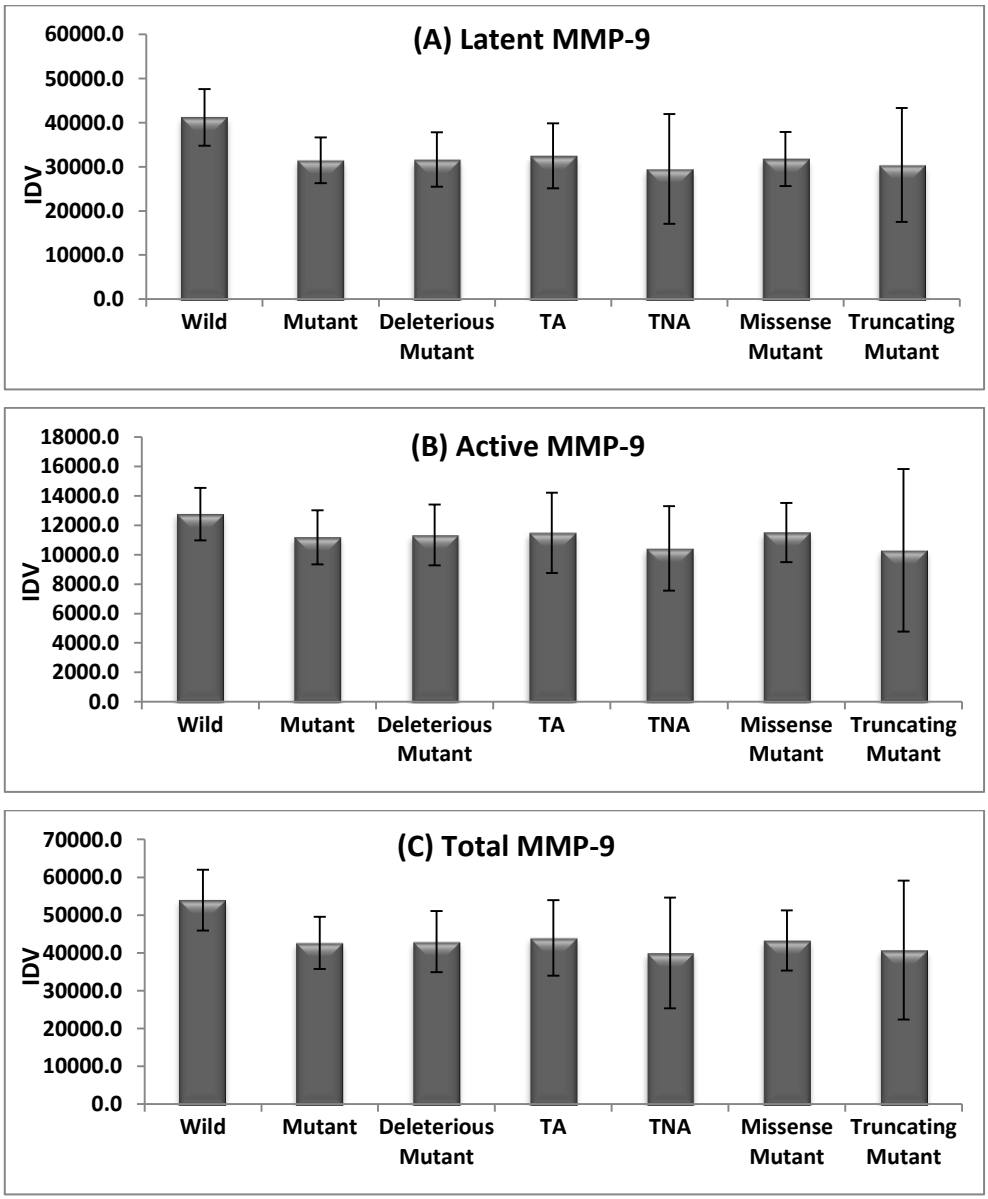


Figure 4.69: Correlation of latent, active, total MMP-2 protein levels with the types of *p53* mutations



TA=Transcriptionally active: TNA=Trancriptionally not-active

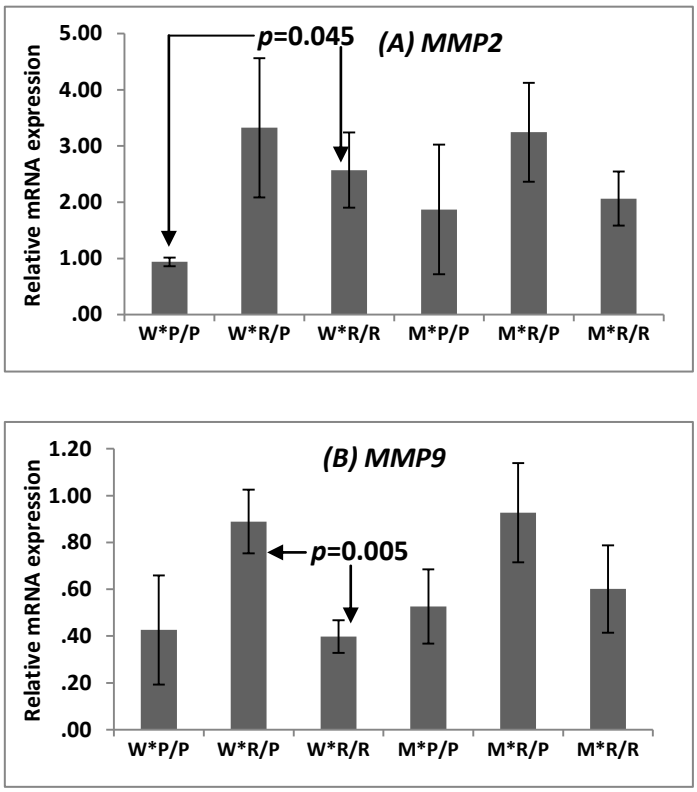
Figure 4.70: Correlation of latent, active, total MMP-9 protein levels with types of *p53* mutations



TA=Transcriptionally active: TNA=Trancriptionally not-active

Further, the present study also evaluated transcript as well as protein levels of *MMP2* and *MMP9* with *p53* and *MDM2* genotypes and *p53* mutations in combination. It was observed that *MMP2* transcript levels were significantly higher in patients with Arg/Arg genotype compared to Pro/Pro genotype at *p53* exon 4 locus in combination with wild type *p53* (p=0.045, Figure 4.71A). Similarly in combination with wild type *p53*, *MMP9* transcript levels were significantly higher in patients with Arg/Pro genotype compared to patients with Arg/Arg genotype at *p53* exon 4 locus (p=0.005, Figure 4.71B).

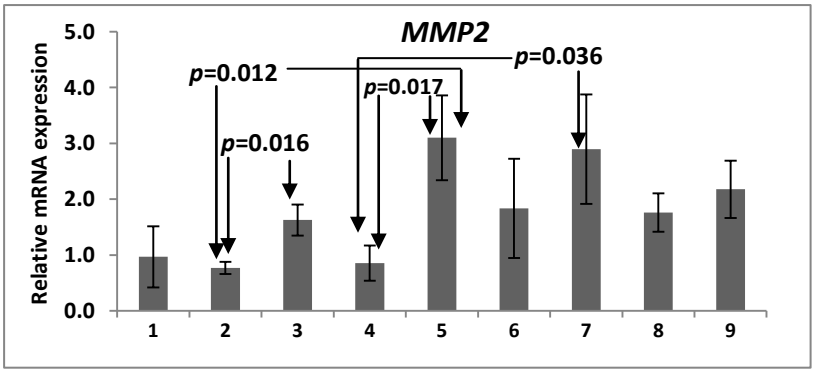
Figure 4.71: Correlation of *MMP2* and *MMP9* transcript levels with *p53* exon 4 genotypes and *p53* mutations in combination



P/P=Pro/Pro,
R/P=Arg/Pro,
R/R=Arg/Arg,
W=Wild type *p53*
M=Mutant *p53*

MDM2 polymorphisms and *p53* mutations in combination did not alter *MMP2* and *MMP9* transcript levels. *MMP2* transcript levels were significantly higher in patients with Arg/Arg genotype compared to patients with Arg/Pro genotype at exon 4 locus in combination with T/T genotype at *MDM2* locus (p=0.036, Figure 4.72). *MMP2* transcript levels were significantly higher in patients with Arg/Pro genotype compared to patients with Pro/Pro genotype at exon 4 locus in combination with G/T genotype at *MDM2* locus (p=0.012, Figure 4.72). *MMP2* transcript levels were significantly higher in patients with G/G genotype compared to patients with G/T genotype at *MDM2* locus in combination with Pro/Pro genotype at *p53* exon 4 locus (p=0.016, Figure 4.72). *MMP2* transcript levels were significantly higher in patients with G/T genotype compared to patients with T/T genotype at *MDM2* locus in combination with Arg/Pro genotype at *p53* exon 4 locus (p=0.017, Figure 4.72).

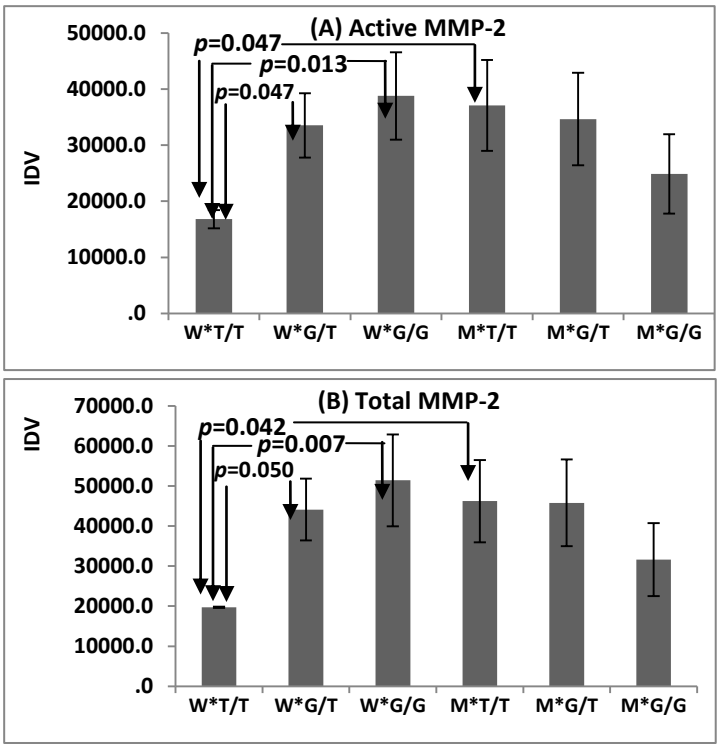
Figure 4.72: Correlation of *MMP2* transcript levels with *p53* exon 4 and *MDM2* SNP309 genotypes in combination



1=Pro/Pro+T/T,
2=Pro/Pro+G/T,
3=Pro/Pro+G/G,
4=Arg/Pro+T/T,
5=Arg/Pro+G/T,
6=Arg/Pro+G/G,
7=Arg/Arg+T/T,
8=Arg/Arg+G/T,
9=Arg/Arg+G/G

There was no significant association of MMP-2 and MMP-9 protein levels and *p53* genotypes and mutation combination. Active and total MMP-2 levels were significantly higher in patients with mutant *p53* compared to patients with wild type *p53* in combination with T/T genotype at *MDM2* locus (p=0.047 and p=0.042, respectively, Figure 4.73A and 4.73B). However, in combination with wild type *p53*, active and total MMP-2 were significantly higher in patients with G/T as well as G/G genotypes compared to patients with T/T genotype at *MDM2* locus (p=0.013, p=0.007 (G/T vs. T/T) and p=0.047, p=0.050 (G/G vs. T/T) , Figure 4.73A and Figure 4.73B).

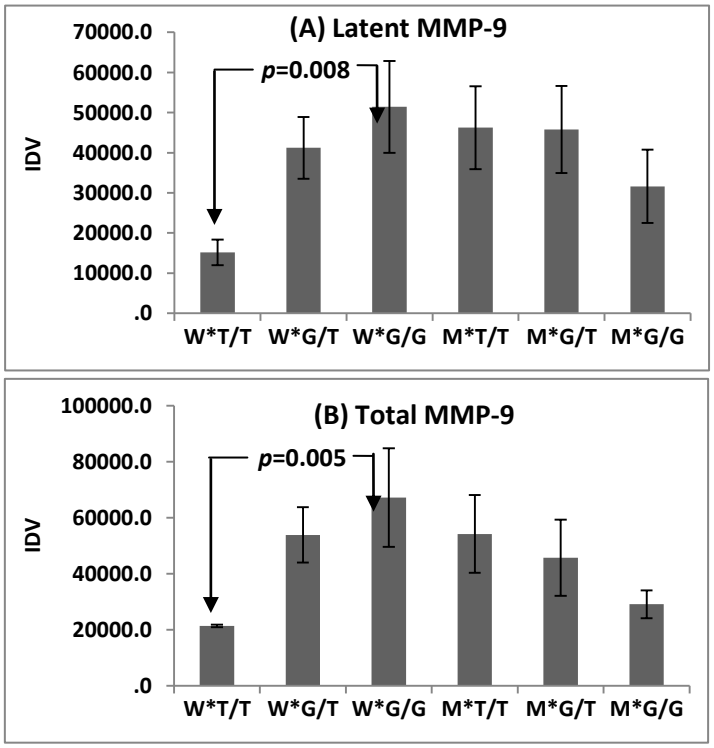
Figure 4.73: Correlation of MMP-2 protein levels with *MDM2* SNP309 genotypes and *p53* mutations in combination



W=Wild type *p53*
M=Mutant *p53*

Latent and total MMP-9 were also significantly higher in patients with G/G genotype as compared to patients with T/T genotype at *MDM2* locus (p=0.008 and p= 0.005) in combination with wild type *p53* (Figure 4.74A and B).

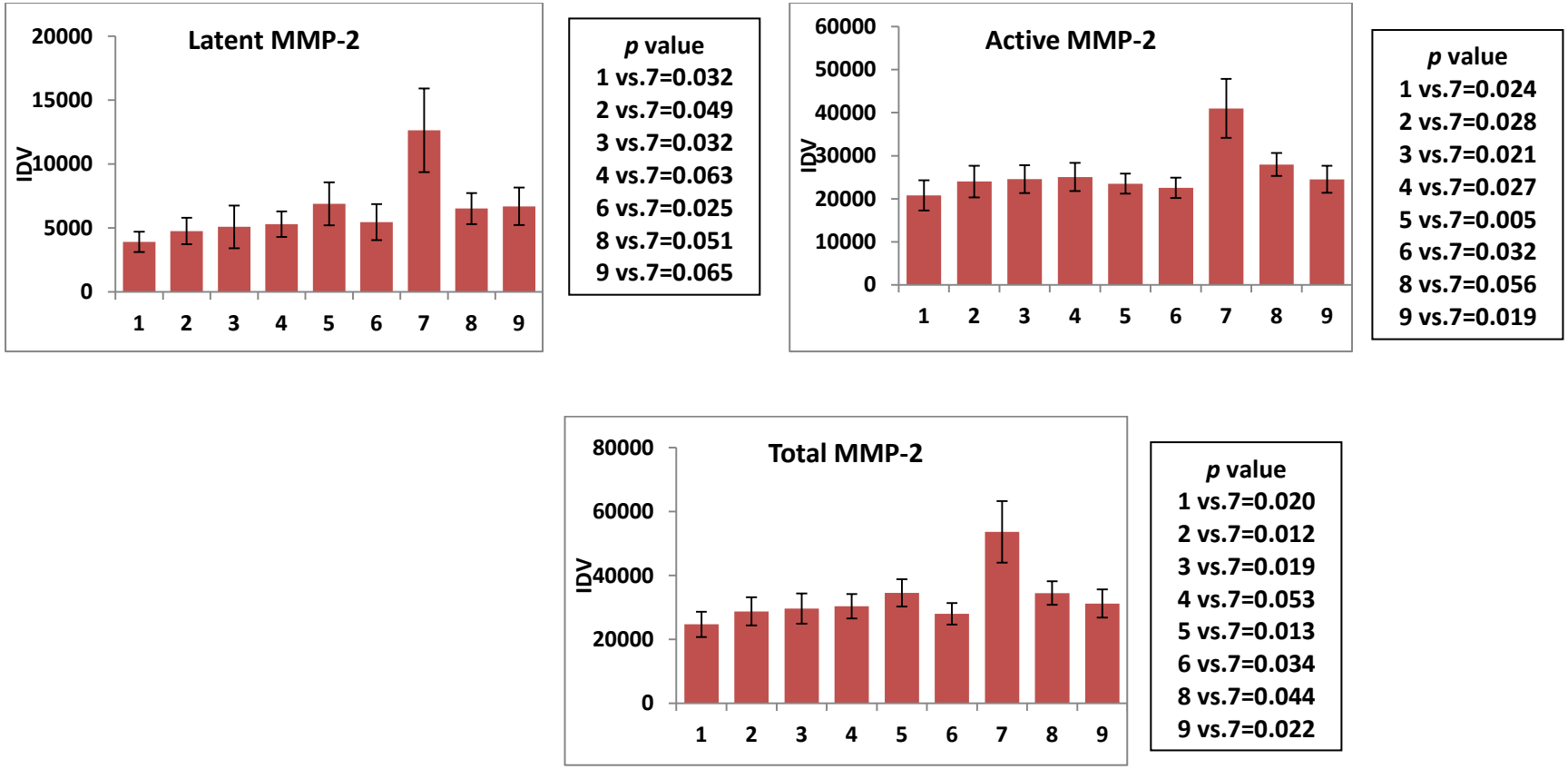
Figure 4.74: Correlation of MMP-9 protein levels with *MDM2* SNP309 genotypes and *p53* mutations in combination



W=Wild type *p53*
M=Mutant *p53*

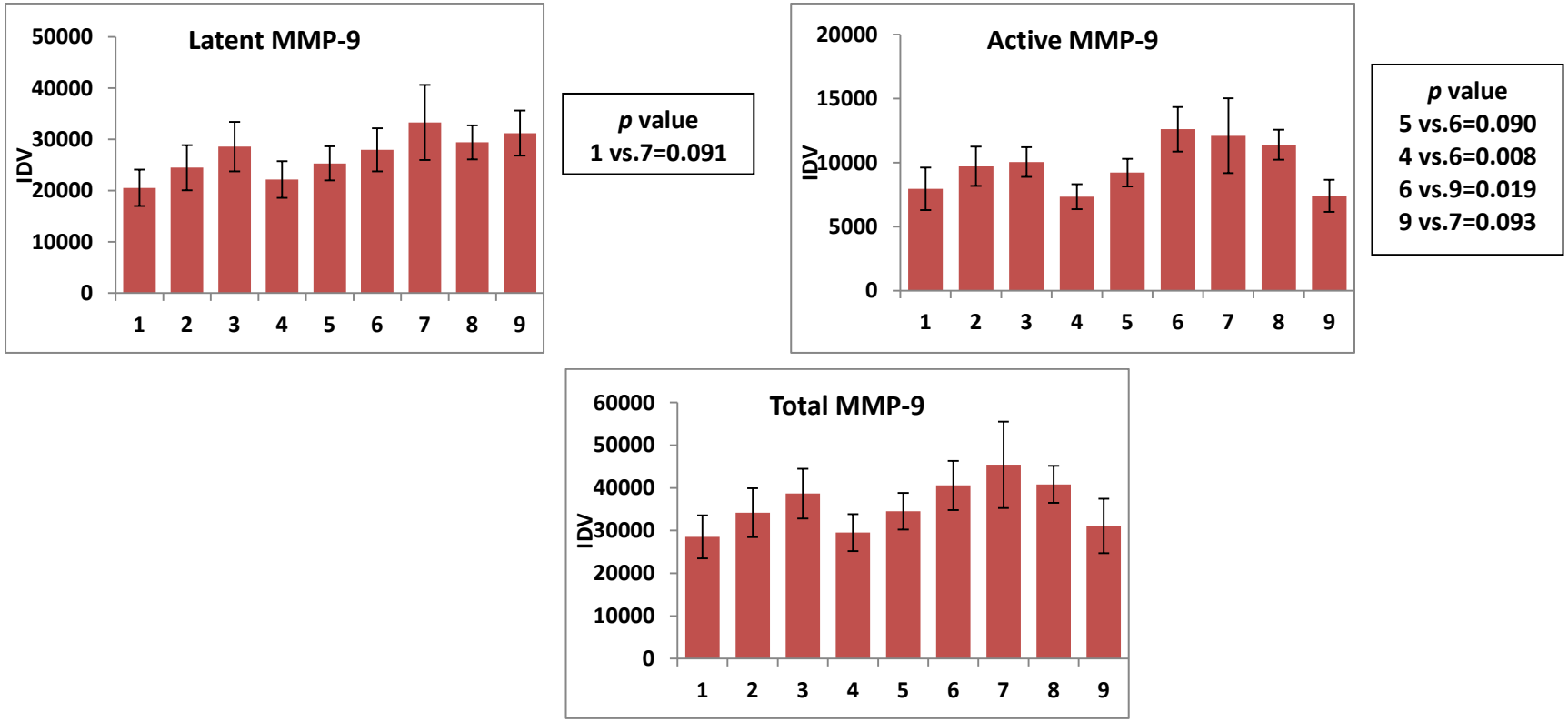
Interestingly, all forms of MMP-2 were significantly higher in patients having Arg/Arg genotype of *p53* exon 4 polymorphism and T/T genotype of *MDM2* SNP309 (T>G) polymorphism in combination as compared to the patients having any other genotypes of these two polymorphisms in combination (Figure 4.75). All forms of MMP-9 were higher in oral cancer patients as we move from T/T, G/T to G/G genotypes of *MDM2* SNP309 (T>G) polymorphism in combination with Pro/Pro as well as Arg/Pro genotypes of *p53* exon 4 polymorphism (Figure 4.76). In contrast, active and total MMP-9 levels were lower in oral cancer patients as we move from T/T, G/T to G/G genotypes of *MDM2* SNP309 (T>G) polymorphism in combination with Arg/Arg genotype of *p53* exon 4 polymorphism (Figure 4.76).

Figure 4.75: Correlation of latent, active and total MMP-2 protein levels with *p53* exon 4 and *MDM2* SNP309 combined genotypes in oral cancer patients



1=Pro/Pro+T/T, 2=Pro/Pro+G/T, 3=Pro/Pro+G/G, 4=Arg/Pro+T/T, 5=Arg/Pro+G/T, 6=Arg/Pro+G/G, 7=Arg/Arg+T/T, 8=Arg/Arg+G/T, 9=Arg/Arg+G/G

Figure 4.76: Correlation of latent, active and total MMP-9 protein levels with *p53* exon 4 and *MDM2* SNP309 combined genotypes in oral cancer patients



1=Pro/Pro+T/T, 2=Pro/Pro+G/T, 3=Pro/Pro+G/G, 4=Arg/Pro+T/T, 5=Arg/Pro+G/T, 6=Arg/Pro+G/G, 7=Arg/Arg+T/T, 8=Arg/Arg+G/T, 9=Arg/Arg+G/G

3.4. Correlation between *hTERT*, VEGF, MMPs in oral cancer patients

The statistical analysis documented that the transcript levels of *VEGF189* showed significant positive correlation with *MMP2* transcript levels (p=0.024, table 4.40). *VEGF183* also exhibited positive correlation with *MMP2* (p=0.093) and a negative correlation was seen with *MMP9* transcript levels (p=0.085, table 4.40). *MMP2* and *MMP9* transcript levels were also positively correlated with transcript levels of *VEGFD* (p=0.058 and p=0.026, respectively, table 4.40). *hTERT* transcript levels showed significant positive correlation with *VEGF121* transcript levels (p=0.051, table 4.40). Further, transcript levels of *MMP9* showed significant negative correlation with *hTERT* and *VEGF121* (p=0.029 and p=0.011, respectively, table 4.40).

Table 4.40: Correlation between *VEGFA* isoforms, *VEGFC, VEGFD, MMP2, MMP9, hTERT* mRNA levels in malignant tissues

		hTERT	***MMP2***	***MMP9***	***VEGFC***	***VEGFD***
VEGF 189	r	-.039	**.293**	-.165	.070	.090
	p value	.772	.024	.213	.603	.537
VEGF 183	r	-.040	**.229**	**-.234**	-.003	-.025
	p value	.778	.093	.085	.984	.868
VEGF 165	r	.190	.166	-.041	.124	.118
	p value	.143	.194	.752	.338	.398
VEGF 121	r	**.244**	-.127	**-.310**	.010	.017
	p value	.051	.306	.011	.933	.898
hTERT	r	1	-.077	**-.269**	.159	-.041
	p value		.540	.029	.206	.762
MMP2	r	-.077	1	**.379**	.074	**.248**
	p value	.540		.001	.554	.060
MMP9	r	-.269	.379	1	-.009	**.292**
	p value	.029	.001		.939	.026
VEGFC	r	.159	.074	-.009	1	**.255**
	p value	.206	.554	.939		.053
VEGFD	r	-.041	.249	.289	.255	1
	p value	.762	.058	.026	.053	

It was observed that latent and total MMP-2 exhibited significant positive correlation with serum VEGF-C (p=0.014 and p=0.046, respectively, table 4.41) and VEGF-D (p<0.0001 and p=0.016, respectively, table 4.41). Active MMP-2 was also positively correlated with serum VEGF-C and VEGF-D levels. Further latent MMP-9 was also positively correlated with serum VEGF-D levels (p=0.071). Serum VEGF-C and VEGF-D levels negatively correlated with activation ratio of MMP-2 (p=0.066 and p<0.0001, respectively) (Table 4.41).

Table 4.41: Correlation between VEGF-A VEGF-C, VEGF-D, MMP-2 and MMP-9 protein levels

		Serum VEGF-A	Serum VEGF-C	Serum VEGF-D
Latent MMP-2	r	-.099	0.413	0.645
	p value	.375	**0.014**	**<0.0001**
Active MMP-2	r	-.160	0.288	0.289
	p value	.150	**0.093**	0.109
Latent MMP-9	r	-.120	0.254	0.323
	p value	.283	0.141	**0.071**
Active MMP-9	r	-.090	0.169	0.154
	p value	.420	0.331	0.400
Total MMP-2	r	-.148	0.340	0.423
	p value	.183	**0.046**	**0.016**
Total MMP-9	r	-.117	0.243	0.293
	p value	.294	0.159	0.103
MMP-2 Activation Ratio	r	-.071	-0.314	-0.604
	p value	.529	**0.066**	**<0.0001**
MMP-9 Activation Ratio	r	.096	-0.114	-0.260
	p value	.392	0.515	0.151
Serum VEGF-A	r		0.190	-0.094
	p value		0.298	0.627
Serum VEGF-C	r	0.190		0.148
	p value	0.298		0.462
Serum VEGF-D	r	-0.094	0.148	
	p value	0.627	0.462	

VEGF189 transcript levels exhibited significant positive correlation with latent MMP-2 (p=0.006), latent MMP-9 (p=0.018) and total MMP-2 (p=0.009) (table 4.42). However, transcript levels of *VEGF189* showed significant negative correlation with activation ratio of MMP-2 (p=0.041) (table 4.42). In addition, *VEGF165* transcript levels exhibited positive correlation with active MMP-2 and total MMP-2 (p=0.018 and p=0.084, respectively) and negative correlation with activation ratio of MMP-2 (p=0.013) (table 4.42). *VEGF121* was also negatively correlated with activation ratio of MMP-9 (p=0.052) (table 4.42).

VEGFC transcript levels were negatively correlated with active MMP-9 levels (table 4.42). Moreover, transcript levels of *VEGFD* were exhibited significant positive correlation with latent MMP-2 (p=0.017), latent MMP-9 (p=0.035) and total MMP-2 (p=0.021) (table 4.42). Transcript levels of *VEGFD* were negatively correlated with activation ratio of MMP-2 (p=0.069, table 4.42). Serum VEGF-A was positively correlated with transcript levels of *MMP9* (p=0.054, table 4.42).

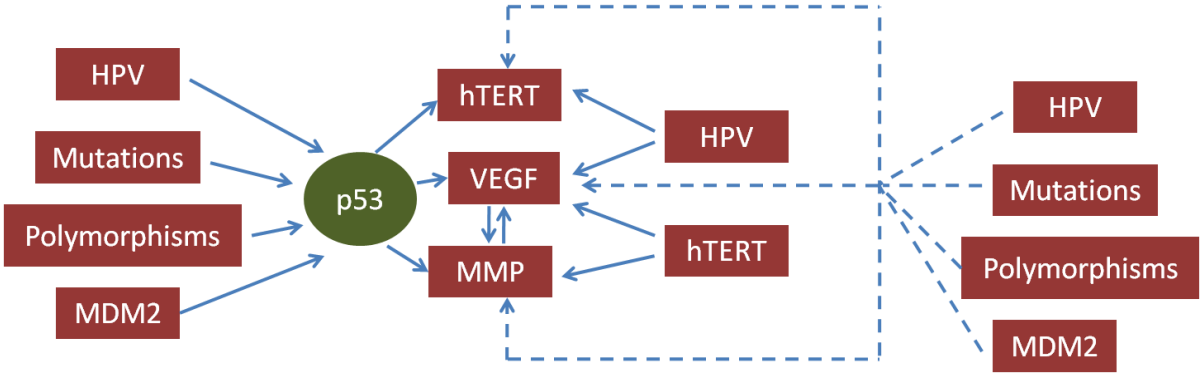
Serum VEGF-C levels exhibited significant positive correlation with *MMP9* transcript levels (p=0.005) and negative correlation with *hTERT* transcript levels (p=0.091). Serum VEGF-C levels were also positively correlated with transcript levels of *VEGF165* (p=0.084). Serum VEGF-D levels exhibited significant positive correlation with *MMP2* transcript levels (p=0.023) (table 4.42).

The hypothetical and observed correlation between *p53* polymorphisms, *MDM2* polymorphism, *p53* mutations, hTERT, VEGFA, VEGFC, VEGFD, MMP2 and MMP9 in the present study is summarized in figure 4.77 and 4.78, respectively.

Table 4.42: Correlation between VEGFA isoforms, VEGFC, VEGFD, MMP2 and MMP9 mRNA and protein levels

		VEGF206	*VEGF189*	*VEGF183*	*VEGF165*	*VEGF121*	*VEGFC*	*VEGFD*	*MMP2*	*MMP9*	*hTERT*
Latent MMP-2	r	-.110	**.358**	.120	**.300**	.019	-.066	**.314**	**.464**	**.282**	-.051
	p value	.556	.006	.389	.018	.879	.597	.017	.000	.021	.688
Active MMP-2	r	.003	**.309**	-.007	.174	-.048	-.092	**.277**	.156	.194	-.005
	p value	.986	.018	.960	.177	.703	.464	.035	.207	.115	.966
Latent MMP-9	r	.132	.189	.028	.074	-.071	-.183	.178	.179	**.215**	-.049
	p value	.479	.154	.843	.565	.569	.141	.181	.147	.081	.701
Active MMP-9	r	-.058	.076	-.090	-.005	-.165	**-.249**	.143	.077	**.232**	-.185
	p value	.758	.570	.516	.970	.186	.044	.285	.536	.059	.140
Total MMP-2	r	-.026	**.342**	.030	**.221**	-.030	-.089	**.302**	**.257**	**.231**	-.019
	p value	.888	.009	.830	.084	.808	.477	.021	.036	.060	.878
Total MMP-9	r	.082	.166	-.001	.057	-.097	**-.205**	.175	.159	**.226**	-.085
	p value	.662	.212	.994	.662	.438	.099	.189	.200	.066	.503
MMP-2 Activation Ratio	r	.202	**-.269**	-.136	**-.313**	-.055	.036	**-.240**	**-.604**	**-.308**	.094
	p value	.276	.041	.326	.013	.661	.772	.069	.000	.011	.456
MMP-9 Activation Ratio	r	-.201	-.173	-.174	-.074	**-.240**	.108	.000	**-.229**	.061	-.114
	p value	.279	.194	.209	.569	.052	.390	.999	.062	.624	.364
Serum VEGF-A	r	0.339	-0.168	-0.186	0.122	0.041	.203	.059	-.010	**.292**	.140
	p value	0.183	0.334	0.291	0.454	0.790	.185	.721	.949	.054	.376
Serum VEGF-C	r	-0.110	0.104	-0.047	**0.326**	-0.009	0.056	0.296	0.201	**0.488**	**-0.350**
	p value	0.763	0.630	0.835	0.084	0.962	0.764	0.134	0.278	0.005	0.058
Serum VEGF-D	r	-0.094	0.036	-0.122	0.206	0.149	0.163	0.123	**0.435**	0.102	-0.055
	p value	0.772	0.878	0.608	0.322	0.457	0.418	0.558	0.023	0.614	0.791

Figure 4.77: Hypothetical correlation between the molecular signatures in oral cancer



Dashed lines suggest hypothetical correlation

Figure 4.78: Observed correlation between the molecular signatures in oral cancer