

Epilogue

Summary

Study of alterations in p53 responses using comprehensive approaches and their effect on molecular signatures involved in major hallmarks of cancer have great potential to help in screening and early detection of recurrence which ultimately leads to better management of the dreaded disease. With this central idea, we investigated *p53* and *MDM2* polymorphisms and their effect on the risk of oral cancer along with gene-gene and gene-environment interactions, frequency of *p53* mutations, HPV16/18 infections, expression of *hTERT*, *VEGFA*, *VEGFC*, *VEGFD*, *MMP2* and *MMP9* in oral cancer patients. Further, the present study also analyzed the association of all these molecular signatures with clinico-pathological parameters, recurrence of the disease as well as survival of oral cancer patients.

The major findings of the present study were as follows:

***p53*, *MDM2* polymorphisms, *p53* mutations and HPV infections**

- Higher OR for oral cancer development was observed for the presence of 16 bp duplication allele at intron 3 locus of *p53*. Heterozygous genotypes at intron 3 and exon 4 in combination with G/G genotype at intron 6 of *p53* exhibited protective effect towards oral cancer development. Haplotype analysis 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. 16 bp duplication allele at *p53* intron 3 locus was also significantly associated with early age of onset.
- Gene-environment interaction analysis revealed that homozygous individuals harboring 16 bp duplication, Pro and A allele at *p53* intron 3, exon 4, intron 6, loci, respectively and heterozygous individuals for *MDM2* SNP309 polymorphism with any type of tobacco habits were at significant risk for development of oral cancer.
- 16 bp duplication, Pro and A allele at *p53* intron 3, exon 4 and intron 6 exhibited higher OR for moderate differentiation and advanced stage. G/T and G/T+T/T genotypes of *MDM2* were significantly associated with recurrence in patients with advanced disease. DFS and OS were also higher in patients having A1/A1, Arg/Arg, G/G genotype at intron 3, exon 4, intron 6 loci of *p53*, respectively and G/G genotype at *MDM2* SNP309 locus.

- Gene-gene interactions between *p53* exon 4 and *MDM2* polymorphisms influence the risk of oral cancer as well as the stage of oral cancer progression. Also, these interactions further significantly increased the risk of recurrence in patients with advanced disease.
- Apart from a very high frequency of *p53* mutations, *p53* sequencing data revealed distinct mutation spots and novel mutations.
- Frequency of *p53* mutations was higher in patients with advanced disease. The presence of *p53* mutations was also higher in patients with early disease who developed recurrence later on. Interestingly, small tumors with *p53* mutations had significantly higher risk to have recurrence compared to small tumors with wild-type *p53* gene. More number of patients having truncating type of *p53* mutations and mutations in both malignant and adjacent normal tissues had recurrence. Also, patients with truncating and transcriptionally non-active *p53* mutations had poor DFS as well as OS.
- Frequency of *p53* mutations was higher in patients having A1/A2+A2/A2, G/A+A/A, Pro/Pro genotype at *p53* intron 3, exon 4, intron 6 loci and T/T genotype at *MDM2* locus. More number of patients having A2 allele, Pro allele, A allele at *p53* intron 3, exon 4, intron 6 loci in combination with mutant *p53* were in advanced stage, had lymph node metastasis and recurrence. Individuals with A2 and Pro allele at *p53* intron 3 and exon 4 with mutant *p53* had lower DFS. Interaction between *MDM2* polymorphism and *p53* mutations also influences differentiation, stage, lymph node involvement as well as recurrence of the disease.
- HPV 16 and 18 infections were absent in oral cancer patients in this cohort from Gujarat, West India.

Expression of *hTERT*, *VEGFA* isoforms, *VEGFC*, *VEGFD*, *MMP2* and *MMP9*

- *hTERT* expression levels were significantly higher in malignant tissues as compared to the adjacent normal tissues.
- *VEGF183* and *VEGF165* were significantly elevated in adjacent normal tissues as compared to malignant tissues. A significant positive correlation was observed between *VEGF189*, *VEGF183*, *VEGF165* and *VEGF121*. Mean serum VEGF-A levels were significantly elevated in oral cancer patients as compared to the controls and also could significantly discriminate between controls and patients.

- Both *VEGF183* and *VEGF189* were significantly decreased in moderately differentiated tumors whereas *VEGF183* significantly increased in large size tumors. Serum VEGF-A levels were also significantly decreased in patients with moderate differentiation. *VEGF165* levels were significantly elevated in recurrent well differentiated, small and early stage tumors as compared to non-recurrent tumors with same clinico-pathological features. Logistic regression analysis also revealed that well differentiated, early stage and small tumors with higher levels of *VEGF165* had high risk to develop recurrence. Serum VEGF-A levels were significantly higher in recurrent tumors with moderate differentiation, large size and advanced stage. Patients with high levels of *VEGF165* and serum VEGF-A levels had worse prognosis.
- *VEGFC* mRNA levels were significantly elevated in malignant tissues, however, *VEGFD* mRNA levels were significantly downregulated in malignant tissues as compared to adjacent normal tissues. Serum VEGF-C levels were significantly higher in oral cancer patients whereas VEGF-D levels were lower in oral cancer patients. Serum VEGF-C levels could significantly discriminate oral cancer patients from controls. Further, *VEGFC* and *VEGFD* transcript levels were significantly upregulated in small tumors and tumors without lymph node involvement, respectively. However, serum VEGF-C was higher in cases with invasive tumors. Transcript levels of *VEGFC* were significantly higher in recurrent small and localized tumors as compared to recurrent large and invasive tumors. Moreover, *VEGFC* levels were significantly higher in non-recurrent invasive tumors as compared to recurrent invasive tumors.
- *MMP2* and *MMP9* transcript levels were significantly elevated in malignant tissues as compared to the adjacent normal tissues. Significant positive correlation was observed between *MMP2* and *MMP9* transcript levels. Various forms of MMP-2 and MMP-9 protein levels were significantly elevated in patients as compared to the controls and could significantly discriminate between oral cancer patients and controls. The different forms of MMP-2 and MMP-9 were significantly inter-correlated. Plasma levels of latent and total MMP-2 exhibited significant positive correlation with *MMP2* transcript levels whereas all the forms of plasma MMP-9 exhibited positive correlation with *MMP9* transcript levels. Further, latent and total MMP-9 were significantly elevated in large size tumors

whereas activation ratio of MMP-9 was significantly high in small tumors and in tongue carcinoma patients.

Correlation between *p53*, *MDM2* polymorphisms, *p53* mutations, expression of *hTERT*, *VEGFA* isoforms, *VEGFC*, *VEGFD*, *MMP2* and *MMP9*

- In patients with Arg/Arg genotype at exon 4 of *p53*, *hTERT* transcript levels were significantly higher as compared to patients with Pro/Pro genotype. *hTERT* transcript levels were significantly higher in cases with Arg/Arg and Arg/Pro genotype as compared to cases with Pro/Pro genotype at *p53* exon 4 locus in combination with mutant *p53*. Significantly higher *hTERT* levels were also observed in patients with Arg/Arg genotype as compared to patients with Pro/Pro genotype at *p53* exon 4 locus with G/G and T/T genotypes at *MDM2* locus.
- Further, in cases with Arg allele at *p53* exon 4 locus, serum VEGF-A levels were significantly higher as compared to cases having Pro/Pro genotype. *VEGFD* transcript levels were significantly higher in patients having A1 allele as compared to patients having A2 allele at *p53* intron 3 locus. *VEGF189* transcript levels were significantly decreased in tumors having truncating mutations as compared to tumors with missense mutations and wild type *p53*. *VEGFC* transcript levels were significantly higher in tumors having transcriptionally not active mutations as compared to tumors having transcriptionally active *p53* mutations. *VEGFD* transcript levels were significantly low in tumors having truncation/nonsense mutations as compared to patients having missense mutations.
- In combination with wild type *p53*, expression of *VEGF183* transcript was higher in patients having Pro/Pro or G/T genotypes as compared to patients having Arg/Pro or G/G genotypes at *p53* exon 4 and *MDM2* locus, respectively. However, in combination with mutant *p53*, *VEGF165* transcript levels were higher in patients with Arg/Arg or G/G genotypes as compared to patients having Pro/Pro or T/T genotypes at *p53* exon 4 and *MDM2* locus, respectively. *VEGF121* transcript levels were also higher in patients having Pro/Pro genotype as compared to patients having Arg/Pro genotype at *p53* exon 4 locus in combination with mutant *p53*.
- In combination with T/T genotype at *MDM2* locus, *VEGF183* transcript levels were higher in patients having Arg/Pro genotype as compared to patients having

Arg/Arg genotype at *p53* exon 4 locus. However, *VEGF165* transcript levels were higher in patients having Arg/Arg genotype as compared to patients having Pro/Pro genotype at *p53* exon 4 locus in combination with T/T genotype at *MDM2* locus.

- In combination with mutant *p53*, serum VEGF-A levels were higher in patients with G/T genotype as compared to patients with T/T as well as G/G genotypes of *MDM2* polymorphism. Serum VEGF-A levels were also higher in patients with Arg/Arg and Arg/Pro genotypes as compared to patients having Pro/Pro genotype at *p53* exon 4 locus in combination with G/T genotype at *MDM2* locus.
- Patients having Arg allele at *p53* exon 4 locus had significantly higher *MMP2* transcript levels as compared to patients having Pro/Pro genotype. While transcript levels of *MMP9* was significantly higher in patients with Arg/Pro genotype as compared to homozygous patients with Arg and Pro allele at *p53* exon 4 locus. Protein levels of latent and total MMP-2 were also significantly higher in patients with Arg/Arg genotype as compared to patients with Pro/Pro genotype at *p53* exon 4 locus even with same genotypes at *p53* intron 3 and 6 loci. *MMP9* transcript levels were significantly higher in tumors having transcriptionally not active mutations as compared to tumors having wild type *p53*.
- In combination with wild type *p53*, *MMP2* transcript levels were significantly higher in patients having Arg/Arg genotype as compared to patients having Pro/Pro genotype at *p53* exon 4 locus. Transcript levels of *MMP9* were also higher in patients having Arg/Pro genotype as compared to patients with Arg/Arg genotype at *p53* exon 4 locus in combination with wild type *p53*.
- In combination with G/T genotype at *MDM2* locus, *MMP2* transcript levels were significantly higher in patients with Arg/Pro genotype as compared to patients having Pro/Pro genotype at *p53* exon 4 locus. *MMP2* transcript levels were also significantly higher in patients with Arg/Arg genotype as compared to patients with Arg/Pro genotype at *p53* exon 4 in combination with T/T genotype at *MDM2* locus. Further, *MMP2* transcript levels were also significantly higher in patients with G/G genotype as compared to patients with G/T genotype at *MDM2* locus in combination with Pro/Pro genotype at *p53* exon 4 locus. *MMP2* transcript levels were also significantly higher in patients with G/T genotype as compared to

patients with T/T genotype at *MDM2* locus in combination with Arg/Pro genotype at *p53* exon 4 locus.

- In combination with T/T genotype at *MDM2* locus, levels of active and total MMP-2 were significantly higher in patients with mutant *p53* as compared to wild type *p53*. Levels of active and total MMP-2 were also significantly higher in patients with G/G and G/T genotypes as compared to patients having T/T genotype at *MDM2* locus in combination with wild type *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 in combination with wild type *p53*.
- All forms of MMP-2 were significantly higher in patients having Arg/Arg and T/T genotype at *p53* exon 4 and *MDM2* locus, respectively than in patients with any other genotypes of these two polymorphisms in combination.
- There was significant inter-correlation exists between hTERT, VEGFA isoforms, VEGFC, VEGFD, MMP2 and MMP9 as hypothesized.

Conclusions

- The results suggested that tobacco use in combination with genetic factors play an important role in the etiopathogenesis of oral cancer. Polymorphisms in the *p53* and *MDM2* genes as well as interactions between them also influence oral cancer risk, progression as well as treatment outcome. The results of *p53* mutation spectrum strongly suggest its usefulness for predicting loco-regional recurrence in oral cancer patients at an early stage. Detection of *p53* mutations in apparently normal adjacent mucosa along with mutations with tumor tissues could further aid in the prognostication of oral cancer patients. HPV 16 and 18 infections play negligible role in oral carcinogenesis in this region. These observations support the hypothesis that the incorporation of information of inherited genetic polymorphisms of the *p53* pathway along with the somatic mutations in *p53* is very essential to improve our understanding of oral cancer pathogenesis.
- Further, specific gene signatures i.e. *hTERT*, *VEGFs*, *MMPs* contributing in major hallmarks of cancer also play a significant role in oral cancer progression. The study also revealed that *VEGF165* has the potential to be an important prognostic factor in oral cancer. Preoperative assessment of *VEGF165* might be useful to understand aggressive malignant potential of tumors. Polymorphisms in *p53* and *MDM2* as well as *p53* mutations also influence the major genes involved in

various hallmarks of cancer and hence further contribute to aggressive behavior of oral cancer.

- Comprehensively, these results support the notion that *p53*, a key tumor suppressor is a molecular node at the crossroads of an extensive and complex network of various cellular pathways. Ultimately, this comprehensive analysis might aid in the prevention, early detection, follow-up strategies and genetic counseling. This can also be helpful for better management. The use of these molecular events as an adjunct to standard clinico-pathological criteria may eventually help in prognostication and effective management of oral cancers.
- Moreover, these striking observations of the present study might be helpful to improve *p53*-targeted therapy as well as identification of newer effective drug targets for oral cancer patients.