Discussion

It has become evident that p53, a multi-faceted "guardian of the genome" is a molecular node at the crossroads of an extensive and complex network of various cellular processes (Bai and Zhu, 2006). Deregulation of p53 has enormous influence on carcinogenesis. The molecular events affecting normal functioning of p53 in oral cancer (somatic mutations in *p53*, germ-line polymorphisms in *p53,* polymorphic variants of *MDM2* and/or degradation of p53 by E6 protein of HR-HPV) might have association with aggressive behavior of oral cancer. Further, many p53 family transcriptional targets have been identified as having the capacity to modulate various cellular processes suggesting that mutant *p53* also plays a key role in malfunction of almost all hallmarks of cancer (Freed-Pastor and Prives, 2012). Hence, it is biologically plausible that alterations in *p53* responses other than *p53* mutations also influence the genes involved in major hallmarks of cancer. Therefore, in the present study, we made a comprehensive analysis of the mechanisms by which normal function of p53 is affected. Also, the study analyzed as to how the altered p53 affects the expression of other genes involved in various hallmarks of cancer i.e. immortalization (*hTERT),* angiogenesis *(VEGFs)* and invasion and metastasis *(MMPs*). Further, the present study also evaluated effect of all these molecular alterations on oral cancer progression and outcome individually as well as in comprehensive manner.

1. *p53* and *MDM2* gene polymorphisms, *p53* mutations and HPV infections in oral cancer patients

1.1. *p53* and *MDM2* polymorphisms in oral cancer patients

In the present study, we evaluated three polymorphisms of the *p53* gene i.e. 16 bp duplication in intron 3, Arg72Pro in exon 4 and G>A transition in intron 6 and *MDM2* SNP309 (T>G) in order to predict the oral cancer risk associated with these polymorphisms in the West Indian population. Also their association with oral cancer progression and outcome was also evaluated. To the best of our knowledge, this type of study has so far not been carried out from West Indian population.

1.1.1. Allelic frequency of p53 and MDM2 polymorphisms

An allele frequency of Arg72Pro polymorphism has been reported to vary with respect to ethnicity and latitude (Nagpal *et al.*, 2002). The allele frequency of proline at codon 72 varies from 0.12 to 0.69 worldwide (Francisco *et al.*, 2011) whereas for the Indian population, it ranges from 0.42 to 0.72 (Mitra *et al.*, 2003; Mittal *et al.*,

2011). In the studied population, the frequency of proline was 0.46. Further, A2 and A allele frequencies at intron 3 and intron 6 were 0.17. The frequencies of these alleles range from 0.10 to 0.23 for A2 allele of intron 3 and 0.19 to 0.32 for A allele of intron 6 among different populations (Mitra *et al.*, 2005; Hrstka *et al.*, 2009; Hu *et al.*, 2010).

For *MDM2*, an allele frequency of *MDM2* G allele in the studied population was 0.52. From India, the only study on the association of *MDM2* polymorphism with oral cancer risk has reported the frequency of G allele as 0.54 in the North Indian population (Misra *et al.*, 2009). The frequency of G allele is also variable between different races and ethnic groups (Yu *et al.*, 2011). The Chinese population had high frequency of G allele (>0.50) whereas the Caucasian had lowest frequency (<0.40) (Al-Hadyan *et al.*, 2012). However, in the present study, the controls as well as the cases belonged to same ethnicity and were from the same geographic location i.e. West India.

1.1.2. Risk of oral cancer associated with p53 and MDM2 polymorphisms

There are only few reports which have assessed the role of these *p53* polymorphisms in oral cancer from India. Among them, most of the reports are on Arg72Pro polymorphism of the *p53* gene (Tandle *et al.*, 2001; Nagpal *et al.*, 2002; Katiyar *et al.*, 2003; Mitra *et al.*,2005; Chakrobarty *et al.*, 2014). There is only one study from eastern region of India, which has explored the role of all the three polymorphisms of *p53* in oral cancer (Mitra *et al.*, 2005). Majority of the studies from India did not find any association between Arg72Pro polymorphism and oral cancer risk (Tandle *et al.*, 2001; Nagpal *et al.*, 2002; Katiyar *et al.*, 2003; Mitra *et al.*,2005; Chakrobarty *et al.*, 2014). However, there are studies from different region of India suggested that Pro/Pro genotype might be a risk for oral cancer (Addala *et al.*, 2012b; Adduri *et al.*, 2014). However, a recent meta-analysis by Mandal *et al.* (2014) suggested that Arg72Pro polymorphism may not be an independent risk factor for cancer in Indian population. Studies from other populations also showed conflicting results for the association of Arg72Pro polymorphism with oral cancer risk (Kuroda *et al.*, 2007; Saini *et al.*, 2011; Jing *et al.*, 2012; Sina *et al.*, 2014). Francisco *et al.* (2011) suggested that ethnicity, allelic frequency, histological and anatomical sites may modulate the penetrance of Arg72Pro polymorphism in cancer susceptibility. However, it was suggested that this polymorphism did not associate with oral cancer

risk even after stratifying by ethnicity (Zhuo *et al.*, 2009; Jiang *et al.*, 2013). In accordance with these studies, our results also could not find any oral cancer risk associated with Arg72Pro polymorphism suggesting that this polymorphism did not play a significant role in oral cancer susceptibility in West Indian population.

There are evidences that the Arg72Pro polymorphism had a profound effect on the primary structure of p53 protein and its biochemical and biological activities (Ozeki *et al.*, 2011). It has been shown that the Pro-72 form of p53 has increased transcriptional trans-activation capacity, induces a higher level of G1 arrest and senescence compared to the Arg-72 form (Frank *et al.*, 2011). In contrast, both Pro-72 and Arg-72 form of p53 are capable of inducing equal levels of apoptosis but with different kinetics (Thomas *et al.*, 1999). Dumont *et al.* (2003) observed that the Arg-72 form has a much stronger capacity to induce apoptosis than the Pro-72 form of *p53* in tumor cells but not in normal cells. Cell-line based studies suggest that the Arg-72 has superior pro-apoptotic function in human tumor cell-lines. Recently, studies on mouse model indicate that the Arg-72 variant induces increased apoptosis in mouse embryo fibroblast (MEF) and in the small intestines of mice along with decreased apoptosis in the thymus compared to Pro-72 (Zhu *et al.*, 2010; Azzam *et al.*, 2011). Thus, there is tissue specific influence of Arg72Pro polymorphism on apoptosis. Such tissue specific function of this polymorphism may explain as to why most of the epidemiological studies remain inconclusive.

Very few studies have reported the association between two intronic polymorphisms of *p53* and oral cancer risk. Galli *et al.* (2009) have reported that intron 3 polymorphism was associated with increased oral cancer risk, while intron 6 polymorphism was associated with reduced oral cancer risk in the Italian population. It is also suggested that association of 16 bp duplication allele with cancer risk varies according to population and tumor type (Sagne *et al.*, 2013). A study from Eastern India has suggested that A allele at intron 6 was found to be protective for oral cancer development however, no association between intron 3 polymorphism and oral cancer risk (Mitra *et al.*, 2005). Our study is the first study from West India which has analyzed association of these intronic polymorphisms with oral cancer risk. However, our results revealed a higher OR for the presence of 16 bp duplication allele at intron 3 locus and no risk for intron 6 polymorphism of the *p53* gene. From the above discussion including our results, it can be suggested that association of intronic

polymorphisms of *p53* with oral cancer risk is contradictory. Literature suggests that the intronic polymorphisms may affect the function of wild type p53 protein and hence cancer risk (Avigad *et al.*, 1997; Lehman *et al.*, 2000). However, various studies exploring the functional role of these intronic polymorphisms remain indecisive (Wang-Gohrke *et al.*, 1999; Gemignani *et al.* 2004; Wu *et al.*, 2002; Hu *et al.* 2008). Recently, it was suggested that the presence of the intron 3 16bp duplication allele could impact on *p53* regulatory activity through the modulation of *p53* mRNA transcript patterns and subsequent isoform expression (Marcel *et al.*, 2011; Sagne *et al.*, 2013). However, the functional role of intron 6 G>A polymorphism is still unclear.

When genotypes of three polymorphisms were assessed in combination for the association with oral cancer risk, we found that Arg/Pro genotypes in combination with A1/A2 and G/G genotypes were protected from oral cancer development. More interestingly, Wu *et al.* (2002) have observed that proline at exon 4 in conjugation with intron 3 and 6 variant alleles exert a protective effect rather than a detrimental effect for lung and colorectal cancers though they found significant risk of cancer associated with these variants. There are no reports on the association of these three genotypes combinations and oral cancer risk from India till date. Interestingly, our results suggest that these three polymorphisms play vital role in combination to modulate the oral cancer susceptibility in the studied population.

For *MDM2*, there is only one study from India which has suggested that there is no association of *MDM2* (T>G) polymorphism with oral cancer risk (Misra *et al.*, 2009). Moreover, most of the studies on different population suggested no association of *MDM2* polymorphism with oral cancer risk (Tu *et al.*, 2008; Huang *et al.*, 2009; Hamid *et al.*, 2009; Misra *et al.*, 2009). Similarly, no risk association of *MDM2* SNP309 (T>G) polymorphism with oral cancer was observed in the studied population even after adjusting with cofounders like age, sex and habits. On the contrary, study on non-hispanic white patients suggested that G allele of *MDM2* was significantly associated with decreased oral cancer risk (Chen *et al.*, 2010). However, it is important to mention that this study also included cases of oropharynx. Most interestingly, a recent meta-analysis also suggested that G allele of *MDM2* might be a protective factor for head and neck squamous cell carcinoma (HNSCC) in Caucasians, in contrast no such relationship was found in Asian population (Liu *et al.*, 2011). Wo

et al. (2011) have suggested that *MDM2* SNP309 G allele was associated with increased risk for most types of cancers whereas significantly decreased risk was found in prostate cancer. Also, G allele was associated with decreased risk for prostate cancer in Caucasian population whereas no association was observed in Asian population (Yang *et al.*, 2012). Thus, overall this suggests that the association of *MDM2* polymorphism with cancer risk might be influenced by ethnicity and tumor types. Other reason may be explained by recently discovered second promoter polymorphism in *MDM2* gene i.e. 285G>C located on 24 bps upstream from SNP309. The C-variant of SNP285 is located on the SNP309G allele forming a distinct SNP285C/309G haplotype. Knappskog and Lønning (2011a) had confirmed that SNP285C significantly reduced Sp1-binding to the *MDM2* promoter. Importantly, the combined SNP285C/SNP309G haplotype had a reduced affinity towards Sp1 as compared to the SNP285G/309T haplotype (Knappskog *et al.*, 2011b). It was also observed that the presence of SNP285C was also associated with reduced risk of the various malignancies among carriers of the SNP309G-allele. Notably, SNP285C was found at a similar frequency in different Western populations (Dutch, British, Norwegian) but was absent from Asians (Chinese) (Knappskog and Lønning, 2011c). The finding that the SNP285C/309G haplotype accounted for about 12% of all SNP309G alleles among Caucasians may be of importance explaining the potential difference regarding the effect of SNP309 status on cancer risk among Caucasians versus Asians (Hu *et al.*, 2007; Economopoulos and Sergentanis, 2010). Our study as well as all other studies carried out on Asian population also observed no association of *MDM2* polymorphisms with oral cancer risk (Tu *et al.*, 2008; Huang *et al.*, 2009; Hamid *et al.*, 2009; Misra *et al.*, 2009) suggesting that this polymorphism do not play significant role in oral cancer susceptibility among Asians.

1.1.3. p53 haplotype analysis and oral cancer risk

Haplotype structure of a population is indicative of its evolutionary history and different haplotypes are associated with cancer in different ethnic population. There are many studies which showed positive association with one or more haplotypes constructed from these three polymorphisms with cancer risk. 1-2-2 haplotype was more common in Caucasian population and 2-1-1 haplotype was associated with breast cancer risk in Caucasian population (Weston *et al.*, 1998; Wu *et al.*, 2002). Whereas, haplotype 1-2-2 was found more frequent in breast cancer patients in

Pakistani ethnic groups (Khaliq *et al.*, 2000). They also observed that 1-1-2 haplotype was most common in the Makrani, Punjabis, and Sindhis. Significant differences in haplotype distribution among three Indian caste populations were also observed (Mitra *et al.*, 2003). Thus, distributions of haplotype frequencies also tend to differ due to differences in the ethnicity among Indians. There is only one study from eastern India which has performed haplotype analysis in oral cancer (Mitra *et al.*, 2005). They have observed that 1-2-1 haplotype was present and 2-2-2 and 2-2-1 haplotypes were absent in Eastern Indian population. In the present study, all pair-wise haplotypes showed significant linkage disequilibrium. 1-2-1 haplotype was completely absent in the studied population whereas in the population from Eastern India, 1-2-1 haplotype was present (Mitra *et al.*, 2005). They also found that individuals who were carriers of haplotype 1-2-2 were at risk of developing oral cancer that was also observed to be more prevalent in the studied population.

1.1.4. Risk of oral cancer associated with p53 and MDM2 polymorphisms, age at disease onset and tobacco habits

16 bp duplication allele in intron 3 was found to be significantly associated with early age of disease onset in the present study. There are no earlier studies regarding the association between *p53* intron 3 polymorphisms and age at disease onset in oral cancer patients. However, 16 bp duplication allele was associated with early age of disease onset in breast cancer patients (Costa *et al.*, 2008; Faghani *et al.*, 2011). Further, we also observed that frequency of Pro allele in exon 4 and A allele in intron 6 was also higher in younger patients. Addala *et al.* (2012b) also reported that frequency of Arg was higher in oral cancer patients with age range of 45-65 years. Proline allele was also associated with early age of disease onset in other malignancies (Shen *et al.*, 2002; Lang *et al.*, 2009; Rogler *et al.*, 2011; Shi *et al.*, 2013). However, no association between *p53* exon 4 polymorphism and age of disease onset in head and neck cancer was also reported (Mojtahedi *et al.*, 2010). It has also been reported that the manifestation of functional role of *p53* polymorphisms is tissue and age specific, thus effect of these polymorphisms on *p53* controlled process may vary between cell types and age groups (Bonafe *et al.*, 2004; Salvioli *et al.*, 2005; Azzam *et al.*, 2011). Results of the present study also support this notion.

In the present study, 60% patients having G/G genotype at *MDM2* SNP309 (T>G) locus had early age of disease onset. Most of studies on various cancers supported this

observation (Nakashima *et al.*, 2008; Yu *et al.*, 2011; Liu *et al.*, 2011). Huang *et al.* (2009) found that the *MDM2* SNP309 G-allele was associated with earlier age of oral cancer onset in a Taiwanese population. In contrast, Hamid *et al.* (2009) found that the G allele is associated with a delayed onset of oral squamous cell carcinoma (OSCC), particularly in women. Interestingly, this association between genotype and mean age of diagnosis was not observed in men. Moreover, they also suggested that *MDM2* SNP309 may modulate disease onset in a gender-specific manner. It is likely that primarily female-specific hormones, such as estrogen, could allow for the SNP309 G-allele to accelerate tumor formation in women (Yu *et al.*, 2011). In the present study, such gender specific analysis was not possible due to small number of female subjects. On the contrary, Tu *et al.* (2008) have reported no association between *MDM2* polymorphism and age at disease onset in oral cancer patients. Thus, above discussed studies including our study suggest that association of *MDM2* polymorphism with age of oral cancer onset remains contradictory. Further, well designed studies are recommended to rule out this gender-specific association of this polymorphism with age of the oral cancer onset from India.

In this cohort of study, tobacco habituates were significantly higher in cases compared to controls and they were at significant risk to develop oral cancer. The gene-environment interaction analysis revealed that the interaction of A2/A2 genotype of intron 3, Pro/Pro genotype of exon 4 and A/A genotype of intron 6 of *p53* gene and G/T genotype of *MDM2* gene with tobacco habits further significantly increased the risk of oral cancer. However, the only study by Mitra *et al.* (2005) from India failed to observe any association between Arg72Pro polymorphism and oral cancer development in tobacco users. They have suggested that combination of A/A and A/G genotypes at intron 6 locus showed protective effect towards oral cancer development but at a low smoking dose. However, it is important to mention here that in our study, most of the subjects were tobacco chewers. For *MDM2*, Misra *et al.* (2009) have also observed that G/T genotype was associated with increased oral cancer risk in mix tobacco habituates. Results of the present study for *MDM2* polymorphism was also in agreement with this observation.

It has been suggested that a simultaneous account of *p53* and *MDM2* polymorphisms and their tissue and age specific effects along with ethnic specific genetic background and environmental exposure may reveal how *p53* and *MDM2* germ line variations

modify cancer risk (Denisov *et al.*, 2012). Our results also supported this notion as effect of *p53* and *MDM2* polymorphism on oral cancer risk was greatly affected by age at diagnosis and tobacco habits.

1.1.5. Association of p53 and MDM2 polymorphisms with clinico-pathological parameters

Studies on such kind of association are scant in the literature and mainly involved malignancies other than the oral cancer. Recently, Addala *et al.* (2012b) reported that frequency of Pro/Pro genotype was higher in advanced stage oral cancer patients. They have only analyzed the role of *p53* (Arg/Pro) genotypes on oral cancer progression. Our results revealed that the frequency of variant genotypes of *p53* intron 3, exon 4 and intron 6 genotypes were found to be higher in moderately differentiated and advanced stage tumors. Also, studies have found that Pro/Pro genotypes were associated with poor differentiation, advanced stage of the disease and lymph-node involvement in various malignancies (Mojtahedi *et al.*, 2010; Pandith *et al.*, 2010; Shi *et al.*, 2013). *p53* intron 3 and intron 6 variant genotypes were also associated with lymph-node metastasis in breast cancer patients (Costa *et al.*, 2008; Hrstka *et al.*, 2009). No association was also observed between *p53* Arg72Pro polymorphism and clinico-pathological features in cervical cancers (Jiang *et al.*, 2010). Overall, studies regarding the association of these polymorphisms with cancer progression are more numerous than studies showing no association. Thus, it can be suggested that, though, these *p53* polymorphisms do not play significant role in oral cancer susceptibility but might play role in oral cancer progression.

1.1.6. Association of p53 and MDM2 polymorphisms with recurrence and survival

There is dearth of studies regarding the association of these polymorphisms with recurrence and survival of oral cancer patients. In our study, the referent genotypes of all these polymorphisms were found to be associated with higher DFS and OS compared to heterozygotes as well as variant genotypes. One study from Japan has observed that the oral cancer patients with the Pro/Pro genotype had a poorer prognosis than those with Arg/Pro genotype (Kuroda *et al.*, 2007). In contrast, Arg/Arg genotype was associated with poor OS and DFS of irradiated oral cancer patients in Taiwan (Tu *et al.*, 2008). There are no reports regarding *p53* intronic polymorphisms and oral cancer prognosis in the literature. For *MDM2*, G/T and G/T+T/T genotype of *MDM2* marginally increased the risk of recurrence in the

present study. Most importantly, this effect was significant in cases with advanced stage tumors. Patients having G/G genotype had better DFS compared to patients having G/T as well as T/T genotype. Whereas, OS was favorable in patients having G/G as well as T/T genotypes compared to patients having G/T genotypes. On the contrary, Tu *et al.* (2008) reported that *MDM2* G/G genotype was associated with poor OS in advanced oral cancers. However, our results of survival analysis are in accordance with our previous findings of oral cancer outcome associated with *MDM2* polymorphism. Moreover, G/G genotype was found to be associated with better OS in bladder cancer patients (Sanchez-Carbayo *et al.*, 2007; Shinohara *et al.*, 2009).

1.1.7. *Gene-gene interaction between p53 and MDM2 polymorphisms, oral cancer risk and progression*

The vast majority of epidemiological studies showing association between genotypes and susceptibility are largely based on the effects of single genes. Generally, the effect of a SNP might be less compared to the genetic effect of combinations of functionally relevant SNPs that may additively or synergistically contribute to the increased cancer risk. These interactions might determine the functional outcomes over the independent effects of any single susceptibility gene or its genetic polymorphism. The biological events associated with cancer risk that are modestly affected by a SNP may be more greatly affected by a SNP in combination with additional SNPs (Moore, 2003; Goodman *et al.*, 2006). Early studies on crucial role of MDM2 in the control of p53 functions recommend that polymorphisms in the *MDM2* gene should be responsible for probable alteration in p53 functions, hence this inspired us to further investigate a possible synergistic role of SNPs in *MDM2* and *p53* in oral cancer development (Whibley *et al.*, 2009; Post *et al.*, 2010; Yu *et al.*, 2011). In the present study, interaction between all the three polymorphisms of *p53* and *MDM2* SNP309 (T>G) polymorphisms suggested no significant interaction between *p53* intron 3 and intron 6 polymorphisms and *MDM2* SNP309 (T>G) polymorphism. However, interaction between *p53* Arg72Pro and *MDM2* T>G suggested that individuals harboring Arg/Arg genotype in combination with T/T genotype were marginally protected from oral cancer development as compared to the individuals harboring Arg/Arg and G/G genotypes in combination in the present study. This observation of the present study was similar to the results observed by Wan *et al.* (2011). They also observed that Pro/Pro, Arg/Pro, Arg/Arg in combination with G/G genotypes increased the risk of cancer more largely with reference to

combination of Arg/Arg and T/T genotypes. However, as discussed earlier, both these two polymorphisms might influence cancer risk in tissue specific manner. Thus, comparison of results with other tumor types in not feasible. Further, cancer risk associated with *p53* and *MDM2* polymorphisms also varies according to ethnicity of the studied population (Francisco *et al.*, 2011; Liu *et al.*, 2011; Yang *et al.*, 2012). However, there is no data on the gene-gene interactions between *p53* and *MDM2* polymorphisms and the risk of oral cancers among the Indians. Further, we have observed that interactions between these two polymorphisms also affect the stage of oral cancer progression. Pro/Pro genotype of *p53* in combination with G/G as well as G/T genotypes of *MDM2* significantly increased the risk of having advanced stage of oral cancer. G/T genotype of *MDM2* in combination with Arg/Arg also increased the risk of having advanced stage. Thus, it can be suggested that gene-gene interactions between *p53* exon 4 (Arg72Pro) and *MDM2* SNP309 (T>G) polymorphisms might influence the progression of oral cancer.

Further, the present study observed that gene-gene interaction between *p53* exon 4 and *MDM2* (T>G) polymorphisms also modulate the risk of recurrence. G/T genotype of *MDM2* in combination with Arg/Arg as well as Pro/Pro genotypes of *p53* exon 4 exhibited high OR (OR=2.00, 95%CI=0.67-6.00; OR=1.81, 95%CI=0.52-6.33, respectively) for recurrence in oral cancer patients. Further, T/T genotype in combination with Pro/Pro genotype also exhibited high OR for recurrence in oral cancer patients. Also, G/T genotype of *MDM2* in combination with Arg/Arg as well as Pro/Pro genotype of *p53* exon 4 polymorphism significantly increased the risk of having recurrence in patients with advanced stage tumors. Overall, the analysis suggested that the G/T genotype of *MDM2* individually or in combination with Arg/Arg and/or Pro/Pro genotypes of *p53* exon 4 polymorphism is a poor prognosticator for oral cancer patients. The OS and DFS associated with *MDM2* polymorphism also varied according to presence of *p53* exon 4 genotypes in the present study.

1.2. p53 mutations in oral cancer patients

1.2.1. Frequency and types of p53 mutations

In our study, the *p53* mutations occurred in 52.2% (24/46) of cases which is clearly higher than earlier reported from different regions of India (17-21%) (Munirajan *et al.*, 1996; Heinzel *et al.*, 1996; Saranath *et al.*, 1999; Ralhan *et al.*, 2001). On the

contrary, no mutations have been reported for Orissa, the eastern part of the country (Patnaik *et al.*, 1999). In our study, the mutations were clustered pre-dominantly in exon 4 followed by exon 5; whereas a study on north Indian population (Ralhan *et al.*, 2001) found maximum mutations in exon 5. The authors have suggested that exon 5 might be one of the specific targets for betel quid ingredients (Ralhan *et al.*, 2001). It also needs to be highlighted that they have covered exon 5-9 for mutation analysis. The possible explanations in the discrepancy of mutation rate might be the: (i) ethnic and geographic factors, (ii) partial exon analysis which also affects the value of *p53* mutations (Chang *et al.,* 2005). Complicating this further is the fact that *p53* mutations vary in the frequency with which they occur in specific tumors suggesting that environmental mutagens leave their mark on *p53* in a tumor and tissue selective manner (Freed-Pastor and Prives, 2012). There is a wide regional variation in betel- and tobacco consuming habits in different regions of India (Sherin *et al.*, 2008). Our region has maximum consumption of smokeless tobacco but in form of gutkha and pan masala (Joshi *et al.*, 2010).

The vast majority of cancer associated mutations reported in *p53* are missense mutations, single base-pair substitutions that result in the translation of a different amino acid in that position in the context of the full length protein (Freed-Pastor and Prives, 2012). In the present study also, missense mutations were observed maximally though we also found two frameshift mutations. The most prevalent type of point mutations found in our study were C>T transitions followed by T>C transitions. Interestingly in our series, we had 15 cases that had multiple mutations. The pattern of transitions and transversions observed in the present study is quite different from the pattern of *p53* mutations observed in oral cavity tumors as reported by the other studies (Hsieh *et al.*, 2001). The type of transition and transversion observed depends primarily on the type of tobacco exposure (Saranath *et al.*, 1999). Like in Southern part of the country, G>A transitions have been reported predominantly, which is mainly attributed to exposure of benzo(a)pyrene, a major carcinogen of tobacco smoke (Munirajan *et al.*, 1996). Also, a study from the Northern part of the country by Ralhan *et al.* (2001) found transversions mutations in *p53* gene which were different from the *p53* mutations reported in studies from the other parts of the country. The different type of base pair changes and the multiple mutations observed in our oral cancer patients suggest DNA damage by several different carcinogens

which are present in smokeless tobacco (gutkha and pan masala). In our study, a trend of a higher frequency of *p53* mutations remains among chewers. Of the 24 patients harboring *p53* mutations, 21 were tobacco chewers. However, in literature, studies showing positive association between *p53* mutation and tobacco smoke are more numerous (Brennan *et al.*, 1995; Liloglou *et al.*, 1997; Koch *et al.*, 1999; Ko *et al.*,2001) than studies with no association (Obata *et al.*, 2000; Chaves *et al.*, 2004; Poeta *et al.*, 2007) though these studies have mainly covered head and neck cancers.

1.2.2. Novel p53 mutations:comparison with IARC database

Our sequencing data described the occurrence of three novel mutations in four patients (one is recurring) when compared with IARC *p53* mutation database (www-*p53*.iarc.fr) which is the major strength of the present study. One was frameshift deletion in exon 4. Another was a recurring missense mutation at codon 117 of exon 4 and a third one was a silent mutation at codon 319 in exon 9. Of these four cases, three developed recurrence and all these three cases were having multiple mutations. One case which was not having recurrence had single silent mutation. Most of the *p53* mutations described in the IARC database affect exons 5-8 which encodes for residues 130-286 (www-*p53*.iarc.fr). We found a high frequency of recurring mutation sites in codon 90 and codon 116 in exon 4 in the studied population. These have not been reported previously for oral cancer, though they have been reported for other malignancies like, stomach and nasal cancer (www-*p53*.iarc.fr). According to the IARC database, high frequency of recurring mutation sites observed in the studied population in codon 90 and codon 116 in exon 4 are missense mutations leading to serine to proline and serine to phenylalanine substitution in protein, respectively. The present investigation also found *p53* mutations at codons which are not considered as mutation "hot-spots" by IARC database. It needs to be mentioned that none of the studies from Southern, Eastern and Western part of the country have reported mutations in these codons (Heinzel *et al.*, 1996; Munirajan *et al.*, 1996; Patnaik *et al.*, 1999). This suggests that these codons might be important to inactivate wild-type *p53* or substitutions at these codons might offer distinct activities to the neomorphic protein (Freed-Pastor and Prives, 2012). We also found mutations in codons considered as mutation "hot-spots" (codons 175, 245, 282). But their number was quite low. Though, *p53* mutations at particular "hot-spots" have been indicated in tobacco-chewing associated OSCC from India (Saranath *et al.*, 1999).

1.2.3. Association of p53 mutations with clinico-pathological parameters

We also examined the association of *p53* mutations with clinical parameters. It was observed that the frequency of *p53* mutations was higher in moderately differentiated and advanced stage tumors. The results of OR analysis also revealed that the patients having mutations in *p53* gene were at higher risk of developing lymph-node metastasis. Similar results have been obtained by Peltonen *et al.* (2010). The authors have suggested that the *p53* mutations were not associated with clinico-pathological parameters such as histological grade and stage of the disease but the frequency of node metastasis was higher in patients with *p53* mutations (83%) than those patients with a wild-type *p53* (50%) in tumors. On the contrary, Erber *et al.* (1998) have reported that the occurrence of lymph node metastasis was significantly higher in patients harboring *p53* mutations than patients with a wild-type *p53*. The study by Yamazaki *et al.* (2003) also did not find any association of *p53* mutations and any of the clinico-pathological parameters. However, they found that tumors containing specific *p53* mutations were significantly associated with loco-regional failure, lymph-node metastasis and distant metastasis. For the present study, the *p53* mutation results were also analyzed taking into consideration the clinico-pathological features and recurrence simultaneously. Results showed that loco-regional recurrence was higher in cases with well differentiated, small, localized and early stage tumors having *p53* mutations. Most interestingly small tumors with *p53* mutations were at a significant risk of developing recurrence. Thus, the present study suggests that evaluation of oral cancer patients for the presence of *p53* mutations would be helpful to predict aggressive potential of tumors in early stage.

1.2.4. p53 mutations in adjacent normal oral tissues

We also observed a higher percentage (13/24; 52.2%) of cases having mutations in the adjacent normal tissues. We had 6 cases that had mutations only in adjacent normal tissues whereas 7 cases had mutations both in adjacent normal and malignant tissues. There has been considerable discussion in the literature about the concept of mucosal fields based on the accumulating evidence that the extent of spread of altered cells is much greater than previously analyzed (Partridge *et al.*, 2000; Braakhuis *et al.*, 2002; Huang *et al.*, 2007). Studies have established conclusively that large areas of the oral mucosa may harbor the genetic mutations associated with tumors and the concept of a field-cancerization aptly describes the location of these aberrations throughout the

superficial tissues (Huang *et al.*, 2007). Mutations in adjacent normal mucosa are believed to increase the risk of local recurrence (Thode *et al.*, 2010). Though, the rate of local recurrence associated with mucosal margins is low (Partridge *et al.*, 2000; van Houten *et al.*, 2002; van Houten *et al.*, 2004). Study by Braakhuis *et al.* (2003) has shown identical genetic mutations in the tumor suppressor gene *p53* in tumors and the tumor-free margin in 25% of patients with oral cancer. In the present study, of the total 7 cases having mutation both in adjacent normal and malignant tissues, 6 developed recurrence and of the 6 cases having mutations only in adjacent normal tissues, one developed recurrence. It needs to be mentioned that these mutations found in adjacent normal and malignant cases were not always identical. This suggests that molecular analysis of adjacent normal and malignant tissues together for *p53* mutations is more useful in terms of predicting risk of recurrence.

1.2.5. Association of p53 mutations with survival of the disease

Our results of survival rate analysis demonstrated lower DFS and OS in patients with *p53* mutations in comparison to patients with wild- type *p53* gene. Data regarding the association of *p53* mutation with survival of oral cancer patients remains contradictory (Tsuji *et al.*, 1995; Sommer and Olofsson *et al.*, 1997; Ostwald *et al.*, 2000; Yamazaki *et al.*, 2003; Siegelmann-Danieli *et al.*, 2005; Kozomara *et al.*, 2005; Huang *et al.*, 2009; Ogmundsdóttir *et al.*, 2009). Most of the studies on role of *p53* on survival have compared only tumors with or without *p53* mutations. We have further categorized the mutations and studied their effect on DFS and OS. We found significant low OS and DFS in patients harboring truncating and transcriptionally non-active mutations in comparison to patients harboring wild- type *p53* gene. Recently, it was suggested that a truncating mutations remained a significant prognosticator while a missense mutation did not influence prognosis of oral cancer patients (Lindenbergh-van der Plas *et al.*, 2011). The results highlight the importance of particular type of *p53* mutations in the prognostication of oral cancer.

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

The present study analyzed association of frequency of *p53* mutations with *p53* and *MDM2* germline genotypes. It was observed that patients harboring 16 bp duplication allele, Pro/Pro genotype and A allele at intron 3, exon 4 and intron 6 of *p53*, respectively had higher frequency of *p53* mutations. In contrast to this, a study by Hsieh *et al.* (2005) have observed that OSCC patients with the Arg allele had a

significantly higher frequency of *p53* mutations than those with Pro/Pro genotype among patients with common alleles of intron 3 and intron 6. Further, 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 in the present study. In the literature, this type of association study has not been reported previously. For *MDM2*, we observed increased frequency of *p53* mutations in patients with T/T genotypes. Further, patients having G/G genotype of *MDM2* as well as mutant *p53* had high risk to have advanced stage tumors. In addition, patients having G/T+T/T genotype as well as mutant *p53* developed lymph-node metastasis and recurrence more frequently. However, results could not achieve statistical significance. This might be due to small number of patients samples analyzed for mutations in *p53*. In accordance with previous studies (Agarwal *et al.*, 1999; Huang *et al.*, 2009), current study suggests that interaction of *p53, MDM2* polymorphisms and *p53* mutations influences oral cancer progression.

Further, we have also observed that DFS was low in patients having variant allele of *p53* intron 3 and intron 6 polymorphisms and mutant *p53* in combination. For exon 4 polymorphism, DFS was low in patients harboring Proline allele and mutant *p53* in combination. However, in contrast to that, both DFS and OS were high in patients harboring Proline allele and wild *p53* in combination. Various studies have confirmed that Arg72Pro polymorphism can affect the levels of apoptosis both in the context of wild type *p53* and mutant *p53*. It was suggested that wild type *p53* in combination with Arg allele mediates the *p53* dependent apoptotic response more efficiently. Interestingly, with mutant *p53* protein, Pro allele could be associated with higher levels of apoptosis (Vazquez *et al.*, 2008). However, this type of analysis has not been carried out in oral cancer patients previously.

Thus, oral cancer progression might be influenced by the presence of *p53, MDM2*, polymorphisms as well as mutations in combination. Hence, our study highlighted the importance of comprehensive analysis of alterations in *p53* responses. In the era of personalized medicine, it will be important to not only differentiate between wild type and mutant *p53* tumors, but it may also prove beneficial to delineate the particular inherited genotypes of *p53, MDM2* as well as type of mutation that a patients' tumor has. Assessing status of *p53* responses might be beneficial in early detection and

monitoring of tumor relapse which further aids in the prediction of effective therapeutic regimens for oral cancer management.

1.3. HPV 16 and HPV 18 infections in oral cancers

HPV associated oral cancers are on a rise in India (Shukla *et al.*, 2009; Kulkarni *et al.*, 2011). This has resulted into the demand of specific prevention programs including screening and vaccination across the country. HPV has gained much interest recently because of its acceptance as important risk factor for cervical cancer. However, oral HPV infections have not been studied to the degree as those of the genital tract. Oncogenic HPVs are associated with oral malignancies but their prevalence varies widely in different studies (Shukla *et al.*, 2009; Kumaraswamy and Vidhya, 2011). The insights from recent studies on HPV infection and oral cancer have also raised certain unanswerable questions as to why: (i) there are large differences in the reported prevalence rates of HPV infected tumors, even when the results are stratified for tumor sites and assays with a comparable performance and (ii) there are large regional and time trend variations in prevalence rates (Leemans *et al.*, 2011).

The present study was carried out keeping into consideration that: (i) the oral cancers are the leading malignancies in India. Also, the incidence of oral cancer is increasing in our region, especially in young adults and (ii) no such study has been carried out in the studied population. WHO has accepted that the knowledge of baseline epidemiology of the disease should be known which is of sufficient importance to justify prioritizing the intervention in form of screening and vaccination (Mattheij *et al.*, 2012). We have chosen specifically HPV 16 and 18 because of their strongest association with oral cancer as published extensively in the literature (Campisi *et al.*, 2007).

Emerging literature on prevalence of HR-HPV 16 and 18 from India have suggested that the prevalence of HR-HPV type 16 and 18 infection in oral cancer varies widely across the different geographical regions of India. Prevalence of HPV 16 infection varies from 6% to 45.8% whereas HPV 18 infection varies from 0% to 54.2% in oral cancers (Balaram *et al.*, 1995; D'Costa *et al.*, 1998; Saranath *et al.*, 1999; Nagpal *et al.*, 2002; Katiyar *et al.*, 2003; Koppikar *et al.*, 2005; Mishra *et al.*, 2006; Gheit *et al.*, 2009; Kulkarni *et al.*, 2011). HPV positive oral cancers are highest from Southern India (Balaram *et al.*, 1995; Kulkarni *et al.*, 2011) while in the Western part of the

country (Mumbai), there is low incidence of HPV positive oral cancers (D'Costa *et al.*, 1998; Saranath *et al.*, 1999; Koppikar *et al.*, 2005). Our results of prevalence of HPV 16 infection in oral cancer in West Indian population are entirely different from other reports from different geographical regions of India. However, the results of the present study on prevalence of HPV 18 infection in oral cancer are in concordance with reports from North, West and Central India (D'Costa *et al.*, 1998; Saranath *et al.*, 1999; Mishra *et al.*, 2006; Gheit *et al.*, 2009). The results suggested unequivocally that HPV 18 does not play any role in oral carcinogenesis.

As documented, the discrepancies in the current results of prevalence of HPV infection in oral cancer may be due to: (i) life-style differences, which play an important role in HPV infections in oral cancer as the infection is mainly transmitted through sexual behaviour of the population (Heck *et al.*, 2010), (ii) the mobile nature of oral cavity with constant salivary secretion having cleaning ability may possibly be responsible for the lower detection rate of HPV (Chen *et al.*, 2012) and (iii) accuracy of distinction between cancer at oral and oropharyngeal site (Kumaraswamy and Vidhya, 2011). It is also very essential to mention that India is a vast country having enormous genetic and cultural diversity with diverse groups of ethnicity and life-style differences (Majumder, 2001).

2. The expression levels *hTERT, VEGFA, VEGFC, VEGFD, MMP2* and *MMP9* in oral cancer patients

2.1. hTERT expression in oral cancer

The activity of telomerase could be regulated by the extent of *hTERT* transcription which is one of the major hallmarks of cancer progression. Thus, the present study investigated *hTERT* mRNA expression in oral cancer tissues. It was observed that *hTERT* mRNA expression was significantly higher in malignant tissues as compared to adjacent normal oral tissues. Various studies evaluated hTERT protein and mRNA expression in oral cancer tissues (Chen *et al.*, 2007; Pannone *et al.*, 2007; Freier *et al.*, 2007; Palani *et al.*, 2011; Abrahao *et al.*, 2011). In most of the studies, it was observed that hTERT expression was higher in oral carcinoma tissues and suggested that hTERT expression was frequent and early event in oral carcinogenesis (Luzar *et al.*, 2004; Chen *et al.*, 2007; Pannone *et al.*, 2007; Palani *et al.*, 2011) which was in agreement with our observation. Further, we did not observe any association between *hTERT* mRNA expression and clinico-pathological parameters. Similar results were

also observed by various investigators (Lee *et al.*, 2001; Pannone *et al.*, 2007; Abrahao *et al.* 2011). On the contrary, Chen *et al.* (2007) observed that high hTERT expression was associated with larger tumor size and advanced stage of tumors. However, Falchetti *et al.* (2000) suggested that at lower stage, many solid tumors, most probably as a consequence of a critical size increase and insufficient vascularization, become necrotic in their central region and are associated to a marked down regulation of *hTERT* gene expression.

We have also analyzed association of *hTERT* mRNA expression with recurrence of disease as well as survival of the patients. We did not observe any significant association of *hTERT* mRNA expression with recurrence of disease and survival even after stratifying tumors according to various clinico-pathological parameters. However, it has been suggested by Chen *et al.* (2007) that nuclear staining of hTERT was associated with high risk of recurrence. They have also suggested that high hTERT expression was associated with poor OS. Pannone *et al.* (2007) suggested that stage I oral cancer patient shaving high *hTERT* mRNA as well as protein expression had worst OS. However, we did not observe any prognostic value associated with *hTERT* expression.

2.2. VEGFA isoforms expression in oral cancers

VEGFA is not only a critical angiogenic factor but also a tumor growth factor which acts in autocrine manner (Mărgăritescu *et al.*, 2009). *VEGFA* isoforms are different molecular entities having different biological activites (Shintani *et al.*, 2004). Moreover, Woolard *et al.* (2009) suggested that *VEGFA* isoforms play a pivotal role in progression and clinical outcome of a variety of cancers. Present study evaluated mRNA levels of all *VEGFA* isoforms in malignant and adjacent normal tissues and serum VEGF-A levels in oral cancer patients simultaneously. It was observed that *VEGF165* and *VEGF183* were significantly elevated in adjacent normal than malignant tissues. Studies regarding transcript levels of *VEGFA* isoforms in oral cancer are very scant in the literature. Moreover, most of the studies on VEGF-A in oral carcinoma have reported IHC to study VEGF-A expression and have depicted contradictory results (Johnstone and Logan, 2006; Mărgăritescu *et al.*, 2009, 2010). Nayak *et al.* (2012) have studied *VEGFA* mRNA levels in oral cancer patients. However, they have analyzed total *VEGFA* mRNA levels. They have suggested that *VEGFA* mRNA levels were 53 fold higher in oral carcinoma tissues as compared to

the normal tissues. However, they obtained normal tissues from healthy individuals which might differ in physiology from oral cancer patients. Further, O' charoenrat *et al.* (2001a) have also analyzed *VEGFA* isoforms in head and neck cancers. The authors have reported that all *VEGFA* isoforms (121, 165, 189 and 206) were significantly elevated in tumor tissues as compared to normal epithelium. However, they obtained tumor tissues from advancing edge of the tumor and also included cases of larynx, oropharynx and hypopharynx. IHC based study by Tae *et al.* (2000) suggested that VEGF-A expression was higher in normal tissues as compared to the malignant head and neck tissues. Moreover, Gandolfo *et al.* (2011) observed increased expression of VEGF-A in non tumoral epithelial borders of oral carcinoma tissues and concluded that epithelial VEGF-A expression could be an additional aid to evaluate malignant potential of oral lesions.

In the present study, serum VEGF-A levels were significantly higher in oral cancer patients as compared to controls. Further, serum VEGF-A levels did not show correlation with tissue transcript levels of *VEGFA* isoforms. Moreover, serum VEGF-A levels showed wide variations both in oral cancer patients (14 to 504 pg/ml) and healthy individuals (10 to 410 pg/ml). Nayak *et al.* (2012) has reported that circulating VEGF-A may serve as surrogate marker for tissue expression of VEGF-A. However, they were unable to find correlation between serum VEGF-A and mRNA expression of *VEGFA* in tissues. Friedrich *et al.* (2010) and Shang *et al.* (2007) have also reported wide range of circulating VEGF-A in serum of oral cancer patients. It was suggested that this wide range of serum VEGF-A are possibly due to the different cellular sources like inflammatory cells (Friedrich *et al.*, 2010).

Further, *VEGF183* and *VEGF189* were significantly downregulated in moderately differentiated tumors as compared to well differentiated tumors. *VEGF183* was significantly elevated in large tumors as compared to small tumors. O' charoenrat *et al.* (2001a) have suggested that *VEGF121* and *VEGF165* play a dominant role in nodal metastasis. To the best of our knowledge, this is the first study which has analyzed association of *VEGFA* isoforms with clinico-pathological parameters in oral cancer. It was also reported that the pattern and biological activity of VEGFA isoforms expression may vary depending upon tumor types (O-charoenrat *et al.*, 2001a). It was also observed that serum VEGF-A levels were significantly higher in well differentiated tumors as compared to moderately differentiated tumors.

Regarding, the association of serum VEGF-A levels with clinico-pathological parameters in oral cancer, data show discrepancy in the literature (Shang *et al.*, 2007; Friedrich *et al.*, 2010). However, various IHC based studies suggested that tissue VEGF-A expression was decreased in moderately differentiated tumors (Shintani *et al.*, 2004; Li *et al.*, 2005; Johnstone and Logan, 2006; Mărgăritescu *et al.*, 2009).

As loco-regional recurrence is very common in oral cancer, present study attempted to analyze the role of *VEGFA* isoforms in loco-regional recurrence. We observed that *VEGF165* was significantly higher in recurrent well differentiated tumors, recurrent small tumors and recurrent early stage tumors as compared to their counter parts. Results of the present study suggested that *VEGF165* may play significant role in development of recurrence in early stage of oral cancer patients. It was also observed that *VEGF165* acts as potent autocrine survival factor for cancer cells (Woolard *et al.*, 2009) additional to its angiogenic properties. Thus, association of *VEGF165* with aggressive behavior of oral cancer might be due to cumulative effect of these functions. However, here it is important to emphasize that this is the first study that has analyzed the role of *VEGFA* isoforms in loco-regional recurrence in oral cancer patients. Further, larger sample size including more number of early stage patients with follow-up study might give more conclusive results.

Serum VEGF-A levels were significantly higher in recurrent well differentiated, large and advanced stage tumors as compared to recurrent moderately differentiated, small and early stage tumors. It was observed that molecular and phenotypic expression of *VEGFA* showed opposite results, when stratified according to recurrent potential of tumors. Thus, it can be suggested that the mRNA expression of *VEGFA* might play an important role in recurrence of early stage tumors and the protein expression in circulatory system might play an important role in recurrence of advanced stage tumors.

Survival analysis suggested that patients having higher levels of *VEGF165*, *VEGF183* and *VEGF189* have shorter DFS and OS. Also, patients having higher levels of *VEGF165* had 5 fold higher risk of death. This may be due to its function as survival factor in addition to angiogenesis (Woolard *et al.*, 2009). The higher serum VEGF-A levels were significantly associated with shorter OS and worst prognosis. There are various IHC based reports suggesting that VEGF-A expression in tissue was significantly associated with worst prognosis (Johnstone and Logan, 2006;

Mărgăritescu *et al.*, 2009; Cheng *et al.*, 2011). Also, it has been reported that circulating VEGF-A may serve as surrogate marker for tissue expression of VEGF-A (Nayak *et al.*, 2012).

Overall, the results suggest that *VEGFA* isoforms play a significant role in oral cancer progression. The study also revealed that *VEGF165* and serum VEGF-A has the potential to be important prognostic factors in oral cancer.

2.3. VEGFC and VEGFD mRNA as well as protein expression in oral cancer

Lymph node metastasis which occurs very early in this malignancy is touted as a major clinical problem and is responsible for a majority of cancer related deaths (Roomi *et al.*, 2009). Ability to identify the presence of metastatic potential of a tumor at an early stage would condition the therapeutic strategy (Cortesina and Martone, 2006). VEGFC and VEGFD are major molecules playing role in lymphangiogenesis. Thus, we also evaluated VEGFC and VEGFD mRNA and protein levels in oral cancer patients. We have observed that *VEGFC* mRNA levels were significantly higher in malignant tissues as compared to adjacent normal oral tissues. Similarly, circulatory protein levels of VEGF-C were also significantly higher in oral cancer patients as compared to the controls. However, mRNA levels of *VEGFD* were significantly lower in malignant tissues as compared to adjacent normal tissues. Serum VEGF-D levels were also lower in oral cancer patients as compared to the controls. However, difference was not statistically significant. ROC curve analysis also suggested that serum VEGF-C levels could significantly discriminate oral cancer patients from controls, while, serum VEGF-D could not. There are studies suggesting that expression of *VEGFC* mRNA as well as protein expression was significantly higher in oral carcinoma than in normal oral tissues (Wen *et al.*, 2001; Yu *et al.*, 2002). However, there is absence of studies regarding serum VEGF-C levels in oral cancer patients. The role of VEGFD is contradictory in oral cancer as well in lymph-angiogenesis (O-charoenrat *et al.*, 2001a). Also, there are no studies on serum VEGF-D levels in oral cancer patients.

Surprisingly mRNA levels of *VEGFC* were significantly higher in small tumors as compared to large tumors. In addition, transcript levels of *VEGFC* were higher in localized tumors as compared to invasive tumors. In contrast, circulatory serum VEGF-C levels were significantly higher in invasive tumors compared to localized

tumors. This difference in tissue transcript levels and circulatory levels need to be explored. Previous studies indicate that VEGF-C expression by tumor cells correlates significantly with lymph-node metastasis in oral cancer (Mărgăritescu *et al.*, 2009; Sugira *et al.*, 2009; Kono *et al.*, 2013). However, there are reports which did not support this observation (Miyahara *et al.* 2007; Warburton *et al.* 2007; Faustino *et al.* 2008; Oliveira *et al.* 2011). Thus, it can be suggested that association of VEGF-C with the lymph-node involvement by IHC method yields contradictory results. In contrast, *VEGFD* transcript levels were significantly lower in tumors with lymph-node metastasis in the present study. High *VEGFC*/low *VEGFD* mRNA levels were correlated with lymph node metastasis in lung adenocarcinomas (Niki *et al.*, 2000). O-charoenrat *et al.* (2001a) suggested that VEGFD exerts an antagonistic effect relative to VEGFA or VEGFC and could have a role later in the angiogenesis such as in stabilization of the newly formed blood or lymphatic vessels. Further, circulatory serum VEGF-D did not associate with oral cancer progression. However, decreasing trend was observed in patients with lymph-node positive tumors. Hence, it might be possible that lower levels of VEGFD might antagonize the effect of VEGFC in lymph-node positive tumors of oral cancer patients in the present study. The data suggested that VEGFC and VEGFD play an important role in early stage of oral carcinogenesis.

2.4. MMP2 and MMP9 mRNA as well as protein expression in oral cancer

Numerous studies in the literature suggest that there is link of MMPs with aggressive malignant progression (Ruokolainen *et al.*, 2006; Hong *et al.*, 2006; Zhou *et al.*, 2010; Barros *et al.*, 2011). However, attempts to correlate gelatinase expression with clinical outcome for patients with oral cancer have been inconclusive and the predictive value of the MMPs in invasion and metastasis of oral cancer has been controversial. This may be partly because of the different methodologies like, IHC, substrate zymography, ELISA and RT-PCR used to detect MMP expression and partly because of the heterogeneity of oral cancer as well as the contradictory facts related to the role of MMPs in oral tumorigenesis (Vilen *et al.*, 2013). We quantified MMP2 and MMP9 mRNA and protein expression using substrate zymography and highly sensitive RT-PCR assays taking into the account that MMPs are synthesized in tissues and released into the blood stream and regulation of MMP activity takes place at various levels i.e. transcription, translation and enzyme activity.

Results of the present study demonstrated that the expression of *MMP2* and *MMP9* mRNA was significantly higher in oral carcinoma tissues as compared to adjacent normal tissues. These results are similar with the study from O-charoenrat *et al.* (2001b) and also with study from our laboratory (Singh *et al.*, 2010). We also compared the mRNA levels of *MMP2* and *MMP9* with clinico-pathological variable. We did not observe significant differences in the mRNA expression of *MMP2* and *MMP9* with clinico-pathological parameters including lymph node metastasis, stage and localization of tumor. Previous study (Singh *et al.*, 2010) from our laboratory also did not observe any significant association of *MMP2* and *MMP9* transcript levels with clinico-pathological parameters of oral cancer patients.

The results of the current study for zymographic analysis demonstrated significantly elevated levels of various forms of MMP-2 and MMP-9 in patients with oral cancer cases as compared to controls. The results for oral cancer cases are in agreement with the results from previous studies (Kuropkat *et al.*, 2002; Ranuncolo *et al.*, 2002). Our results of circulating levels of MMPs are reflecting the direct tissue situation. Also, significantly increased levels of latent, active and total forms of MMP-2 and MMP-9 strengthen results of our studies in oral cancer and breast cancer (Patel *et al.*, 2007; Shah *et al.*, 2009; Singh *et al.*, 2010). Moreover, ROC curve analysis suggested that all forms of MMP-2 and MMP-9 as well as their activation ratio significantly discriminated between oral cancer patients and healthy individuals. The relationship of plasma MMP-2 and MMP-9 with clinico-pathological variables was also analyzed. The results suggested that the levels of active and total forms of MMP-9 were increased in cases of large size tumors compared to small size tumors. In addition, activation ratio of MMP-9 was decreased in case of large size tumors compared to small size tumors. This may be due to lower activation rate from latent to active MMP-9. However, MMP-9 failed to show any association with lymph-node involvement. On the other hand, activation ratio of MMP-9 was increased in tongue carcinoma patients compared to buccal carcinoma patients. This indicates that MMP-9 play a significant role in oral cancer progression. These data are in agreement with a study by Mohtasham *et al.* (2013) which suggested that MMP-9 is the most reliable one for invasive grading in oral cancer. The present study failed to find association of MMP-2 with clinico-pathological parameters. This can be due to complexity of the metastatic process which involves multiple MMPs. Moreover, it was also observed

that *MMP2* transcript levels were significantly associated with latent MMP-2 and negatively associated with its activation ratio. However, *MMP9* transcript levels significantly associated with latent MMP-2 levels and tend to be positively associated with latent, active and total MMP-9. However, it is significantly negatively associated with MMP-2 activation ratio. Thus, it can be concluded that MMP2 and MMP9 were inter-correlated in oral cancer patients and involved in regulation of each other.

3. The correlation between *p53, MDM2* polymorphisms, *p53* mutations, *hTERT, VEGFA, VEGFC, VEGFD, MMP2, MMP9*

3.1. Association of hTERT expression with p53 gene status and MDM2 polymorphism

In this study, we observed that *hTERT* mRNA expression was significantly associated with *p53* exon 4 (Arg72Pro) polymorphism. *hTERT* mRNA levels were significantly higher in oral cancer patients having Arg/Arg genotype as compared to the oral cancer patients having Pro/Pro genotype. However, *hTERT* expression did not show association with *p53* intron 3, 6 and *MDM2* polymorphism as well as *p53* mutation status individually. A study by Roos *et al.* (1998) has suggested that telomerase activity was not associated with frequency of *p53* gene mutations in breast cancer, however, was significantly associated with *p53* protein accumulation. Tang *et al.* (2006) has also suggested positive correlation between *hTERT* mRNA expression and *p53* protein expression in breast cancer. Positive association of telomerase activation or *hTERT* mRNA levels with *p53* overexpression was observed in various malignancies (Dai *et al.*, 2001; Wisman *et al.*, 2003; Boldrini *et al.*, 2004). However, in oral cancer, a study by Wu *et al.* (2005) suggested no significant correlation between *hTERT* mRNA expression and *p53* expression. It is important to mention that there is no data in the literature regarding the association of *hTERT* expression with *p53* and *MDM2* polymorphisms. When combination analysis of *p53* polymorphisms, mutations and *hTERT* expression was done, it was observed that *hTERT* expression was significantly increased in cases with Arg allele as compared to cases with Pro/Pro genotype with *p53* mutations. *hTERT* expression was also higher in patients with Arg/Arg genotype as compared to Pro/Pro genotype in combination with G/G or T/T genotypes for *MDM2* polymorphisms. Thus, it can be suggested that association of *hTERT* mRNA expression with *p53* mutations may be influenced by *p53* (Arg72Pro) and *MDM2* polymorphism. Overall, these results indicate that *p53* exon 4 (Arg72Pro) play an important role individually as well as in combination with *MDM2*

polymorphisms and *p53* gene mutations in the process of immortalization in oral cancer through regulation of *hTERT*.

3.2. Association of VEGF A expression with p53 gene status and MDM2 polymorphism

In the present study, it was observed that *VEGFA* isoforms did not show any association with *p53* genotypes. However, serum VEGF-A levels were significantly higher in heterozygous cases (Pro/Arg) and homozygous cases (Arg/Arg) as compared to homozygous cases harboring Pro allele at *p53* exon 4 locus. Transcript levels of *VEGFA* isoforms as well as serum VEGF-A levels did not show significant association with *MDM2* genotypes. Various recent studies suggested that *p53* mutations up-regulates VEGFA (Cho *et al.*, 2007; Khromova *et al.*, 2009; Yoshioka *et al.*, 2012). A study by Maeda *et al.* (1998) suggested that there was no association between VEGF-A positivity and *p53* mutations in oral cancer. Similarly, in the present study, we did not observe any significant difference in *VEGFA* isoforms levels between patients with wild and mutant *p53*. However, we observed that *VEGFA* isoforms (*VEGF189*, *VEGF183*, *VEGF165*) levels were lower in patients with truncating type of *p53* mutations when compared to wild type *p53*. The discrepancy between these results might be explained by differences in the methods used to assess *p53* mutation and VEGFA expression in cancer tissues, the antibodies used, and the patient populations. In several studies, VEGFA expression was assessed by IHC, which is frequently influenced by tissue preparation and antibodies used (Yuan *et al.*, 2002). Moreover, presence of various VEGFA isoforms in the tissues might influence the association of VEGFA expression and *p53* mutations. Further, *VEGF189* was significantly down regulated in tumors harboring truncating type of *p53* mutations as compared to the tumors harboring missense type of mutations in *p53* and wild type *p53* gene. However, there is no data regarding the association of *VEGFA* isoforms expression with *p53* mutations and also with *p53* and *MDM2* polymorphisms in the literature.

In addition, it was observed that *VEGF165* and *VEGF183* were significantly altered in the presence of specific combination of *p53* polymorphism and mutations, *MDM2* polymorphism and *p53* mutations, *p53* and *MDM2* polymorphisms in the present study. More specifically, *VEGF165* was significantly higher in cases with Arg/Arg and G/G genotypes as compared to Pro/Pro and T/T genotypes at *p53* exon 4 locus

and *MDM2* locus, respectively in combination with mutant *p53*. *VEGF165* was also higher in cases with Arg/Arg genotype as compared to cases with Pro/Pro genotype in combination with T/T genotype at *MDM2* locus. Thus, in cases with Arg/Arg genotype, *VEGF165* might be higher if there is mutant *p53* or T/T genotype at *MDM2* locus present. Further, *VEGF165* was also higher in cases with G/G genotype at *MDM2* locus if mutant *p53* is also there in combination. For *VEGF183*, it was significantly higher in cases with Pro/Pro and G/T genotype as compared to Arg/Pro and G/G genotype at *p53* exon 4 locus and *MDM2* locus, respectively in combination with wild type *p53*. It was also higher in cases with Arg/Pro genotype as compared to cases with Arg/Arg genotype in combination with T/T genotype at *MDM2* locus. These results suggested no clear pattern for the association of *VEGF183* expression with the presence of *p53*, *MDM2* polymorphisms and *p53* mutation in combination. However, the results do suggest that in case with wild type *p53*, expression of *VEGF183* is altered according to presence of *p53* and *MDM2* polymorphisms. Further, its' expression tends to be higher in cases with Pro allele in combination with wild type *p53* or T/T genotype at *MDM2* locus. For *VEGF121*, in cases with mutant *p53*, it was also significantly higher in patients with Pro/Pro genotypes as compared to patients with Arg/Pro genotypes at *p53* exon 4 locus. Serum VEGF-A levels were also higher in patients with G/T genotype as compared to patients with both homozygous genotypes at *MDM2* locus with mutant *p53*. Further, interestingly, in combination with this G/T genotype at *MDM2* locus, serum VEGF-A levels were significantly higher in patients with Arg allele as compared to patients with Pro/Pro genotype. Thus, previously published conflicting results of association between VEGFA expression and *p53* mutations might be explained by presence of *p53* and *MDM2* polymorphisms in the population. It was reported that MDM2 plays important role in regulation of VEGFA expression (Narasimhan *et al.*, 2007; 2008). It was also reported that VEGFA expression is regulated through p53/MDM2 pathway (Zietz *et al.*, 1998). On the contrary, recent studies suggest that MDM2 regulates VEGFA expression in a *p53* independent way (Narasimhan *et al.*, 2007; Carroll and Ashcroft *et al.*, 2008; Zhou *et al.*, 2011; Rathinavelu *et al.*, 2012; Xiong *et al.*, 2014, Muthumani *et al.*, 2014). However, in the present study, we observed that MDM2 might regulate *VEGFA* expression through p53 dependent manner. The overall results indicate that *p53* exon 4 (Arg72Pro) polymorphism, *p53* mutations and *MDM2*

polymorphism play an important role in the process of angiogenesis through affecting VEGFA levels in oral cancer.

3.3. Association of VEGFC and VEGFD expression with p53 gene status and MDM2 polymorphism

VEGFD transcript levels were higher in homozygous cases (A1/A1) for *p53* intron 3 genotypes as compared to the cases harboring A2 allele (A1/A2+A2/A2). *VEGFC* transcript levels were significantly higher in tumors harboring transcriptionally not active *p53* mutations as compared to tumors harboring transcriptionally active *p53* mutations. Whereas, *VEGFD* transcript levels were significantly lower in tumors having truncating type of *p53* mutations as compared to tumors having missense type of *p53* mutations. Serum VEGF-C and VEGF-D did not show significant association with *p53* genotypes as well as mutations. Both transcript as well as protein levels did not significantly associate with *MDM2* polymorphisms. Further, the presence of specific combination of *p53* polymorphism and mutations, *MDM2* polymorphism and *p53* mutations, *p53* and *MDM2* polymorphisms also did not alter the transcript as well as protein levels of VEGFC and VEGFD levels. There is dearth of data on how *p53* affect the VEGFC and VEGFD expression. However, the results suggest that *p53* polymorphisms and mutations might play some unexplored role in the regulation of VEGFC and VEGFD expression and thus contribute to lymphangiogenesis in oral carcinogenesis.

3.4. Association of MMP2 and MMP9 expression with p53 gene status and MDM2 polymorphism

We have observed that heterozygous cases (Arg/Pro) and homozygous cases (Arg/Arg) for *p53* exon 4 had significantly elevated transcript levels of *MMP2* as compared to homozygous cases (Pro/Pro). Also, levels of latent, active and total MMP-2 were higher in oral cancer patients homozygous for Arg allele compared to oral cancer patients homozygous for Pro allele. Correlation of latent, active and total MMP-2 with combined *p53* genotypes was also analyzed. It was observed that levels of latent MMP-2 were significantly higher in patients having Arg/Arg genotypes compared to patients having Arg/Pro and Pro/Pro genotypes with same genotypes at intron 3 and intron 6 loci. These results suggest that presence of Arg allele results into MMP2 over expression. Correlation of transcript and protein levels of MMP2 with types of *p53* mutations as well as *MDM2* polymorphisms revealed no significant observation.

Further, the present study also evaluated transcript as well as protein levels of MMP2 according to various combinations like *p53* polymorphisms and mutations, *MDM2* polymorphism and *p53* mutations, *p53* and *MDM2* polymorphisms. It was observed that *MMP2* transcript levels were significantly higher in patients with Arg/Arg genotypes as compared to Pro/Pro genotypes at *p53* exon 4 locus in combination with wild type *p53*. These results further suggest that presence of Arg allele results in to higher *MMP2* transcript levels in cases with wild type *p53*. *MMP2* transcript levels were also significantly higher in patients with Arg/Arg genotypes compared to patients with Arg/Pro genotypes at exon 4 locus in combination with T/T genotypes at *MDM2* locus. Further, it was also higher in Arg/Pro genotypes as compared to patients with Pro/Pro genotypes at exon 4 locus in combination with G/T genotypes at *MDM2* locus. Additionally, it was significantly higher in patients with G/G genotypes compared to patients with G/T genotypes at *MDM2* locus in combination with Pro/Pro genotypes at *p53* exon 4 locus. It was also significantly higher in patients with G/T genotypes compared to patients with T/T genotypes at *MDM2* locus in combination with Arg/Pro genotypes at *p53* exon 4 locus. This combination analysis do not reveal clear pattern of association however, it might be possible that presence of Arg and T allele at *p53* exon 4 and *MDM2*, respectively together results into higher *MMP2* transcript levels. Also, presence of Pro and G allele at *p53* exon 4 and *MDM2*, respectively together might results into higher *MMP2* transcript levels. Further, levels of active and total MMP-2 were significantly higher in cases with G allele as compared to cases with T/T genotype at *MDM2* locus in combination with wild type *p53*. In contrast, in combination with T/T genotype at *MDM2* locus, levels of active and total MMP-2 were higher in cases with mutant *p53* as compared to cases with wild type *p53*. Thus, presence of *p53* mutations also alters the association of *MDM2* polymorphisms and MMP-2 protein levels. Further, the present study also evaluated protein levels of MMP-2 with combined genotypes of *p53* exon 4 and *MDM2* polymorphisms. Most interestingly, all forms of MMP-2 were significantly higher in patients having Arg/Arg genotypes of *p53* exon 4 polymorphism and T/T genotypes of *MDM2* polymorphism in combination compared to patients having any other genotypes of these two polymorphisms in combination. The overall results suggest that presence of Arg allele might results in to over-expression of MMP2 and this association was further altered according to presence of *p53* mutations and *MDM2* polymorphism.

Further, heterozygous individuals for *p53* Arg72Pro polymorphism had significantly higher transcript levels of *MMP9* compared to homozygous individuals harboring Arg and Pro allele. Moreover, there was no significant difference in latent, active and total MMP-9 levels with respect to individual as well as combined *p53* genotypes in oral cancer cases. Further, transcript as well as protein levels of MMP9 were not significantly associated with *MDM2* polymorphisms in the present study. Also, *MMP9* mRNA levels were significantly upregulated in tumors having transcriptionaly not active *p53* mutations as compared to tumors having wild type *p53*. Franchi *et al.* (2002) reported that *p53* mutation results into MMP-9 over-expression and not the MMP-2 in head and neck carcinoma. However, they did not analyze different types of *p53* mutations. Recently, Wang *et al.* (2013) also suggested that loss of *p53* results in to increased MMP2 and MMP9 expression and hence invasion and metastasis of prostate cancer.

p53 exon 4 genotypes and mutation combination analysis suggests that *MMP9* transcript levels were higher in patients with Arg/Pro genotypes as compared to patients with Arg/Arg genotypes at *p53* exon 4 locus in combination with wild type *p53*. Further, *MDM2* genotypes and *p53* mutation in combination suggest that latent and total MMP-9 were higher in patients with G/G genotype as compared to patients with T/T genotypes at *MDM2* locus in combination with wild type *p53*. In addition, 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* polymorphism in combination with Pro/Pro as well as Arg/Pro genotypes of *p53* exon 4 polymorphism. In contrast, active and total MMP-9 were lower in oral cancer patients as we move from T/T, G/T to G/G genotypes of *MDM2* polymorphism in combination with Arg/Arg genotypes of *p53* exon 4 polymorphism. Recently, Chen *et al.* (2013) suggested that *MDM2* promotes invasion and metastasis in invasive ductal breast carcinoma by inducing MMP9.

Overall, the results suggest that *MMP9* transcript levels were higher in patients with Arg/Pro genotype as compared to patients with Arg/Arg genotype, more specifically in combination with wild type *p53*. Further, *MDM2* polymorphism was also found to be associated with altered protein levels of MMP-9 in combination with wild type *p53*.

Above results suggests that *p53*, *MDM2* polymorphisms and *p53* mutations affect transcript as well as protein levels of MMP2 and MMP9 and hence contributes to

invasion and metastasis of oral cancer cell. However, there are lack of evidences that have analyzed effect of *p53*, *MDM2* polymorphisms and *p53* mutations on MMP2 and MMP9 levels in oral carcinogenesis.

3.5. Correlation between hTERT, VEGF, MMPs in oral cancer patients

In the present study, *hTERT* transcript levels showed a significant positive correlation with *VEGF121* transcript. Previous reports have suggested that *hTERT* mRNA expression was significantly associated with *VEGF165* and *VEGF189* expression in breast cancer and could explain the poor prognosis reported in breast tumors with high levels of *hTERT* (Kirkpatrick *et al.*, 2004). *hTERT* transcript levels also showed significant negative correlation with *MMP9* transcript levels. It is also reported that there was no association with *hTERT* and *VEGFC* expression in breast cancer (Mansfield *et al.*, 2007). However, in the present study *hTERT* was negatively correlated with serum VEGF-C. These results of correlation of *hTERT* with *VEGF121*, *MMP9* and *VEGFD* support the notion that *hTERT* affects the transcription levels of these molecules in a manner independent of its telomerase activity (Zhou *et al.*, 2014).

The previous studies have suggested that there is a significant interplay between MMP2, MMP9 and VEGFA (Hoeben *et al.*, 2004; Hollborn *et al.*, 2007; Ebrahem *et al.*, 2010; Ugarte-Berzal *et al.*, 2010). In the present study, we have also observed that transcript levels of *VEGF189* showed significantly positive correlation with *MMP2* transcript levels. *VEGF183* also exhibited positive correlation with *MMP2* but a negative correlation was seen with *MMP9* transcript levels. Further, transcript levels of *MMP9* showed significant negative correlation with *VEGF121*. *VEGF189* transcript levels exhibited significant positive correlation with latent MMP-2, latent MMP-9 and total MMP-2. However, transcript levels of *VEGF189* showed significant negative correlation with activation ratio of MMP-2. In addition, *VEGF165* transcript levels exhibited positive correlation with active MMP-2 and total MMP-2 and negative correlation with activation ratio of MMP-2. *VEGF121* was also negatively correlated with activation ratio of MMP-9. Serum VEGF-A was positively correlated with transcript levels of *MMP9*. Thus, both MMP2 and MMP9 show association with *VEGFA* isoforms but the interaction between these molecules is highly complex in oral carcinogenesis. Lee *et al.* (2005) have suggested that VEGF-A bioavailability is regulated by MMPs. They have also suggested that matrix bound VEGF-A and not

bound VEGF-A provide different signaling outcome. Also, there is no study in the literature regarding the association of VEGFA isoforms with MMP2 and MMP9. Moreover, it was also observed that *p53* status also responsible for alterations in VEGF-MMP-2/9 pathway (Hu *et al.*, 2012; He *et al.*, 2013). Thus, it might be possible that presence different VEGFA isoforms and alterations in *p53* might influence the correlation between VEGFA, MMP2 and MMP9 expression.

VEGFC transcript levels were negatively correlated with active MMP-9 levels. In contrast, serum VEGF-C levels exhibited significant positive correlation with *MMP9* transcript levels. Serum VEGF-C levels negatively correlated with activation ratio of MMP-2. However, it is important to mention that there is no correlation between transcript levels of *VEGFC* with circulatory serum VEGF-C levels. This might be one of the reasons for this discrepancy. Serum VEGF-C levels were also positively correlated with transcript levels of *VEGF165*. Transcript levels of *VEGFD* exhibited significant positive correlation with *MMP2* and *MMP9* transcript as well as latent MMP-2, latent MMP-9 and total MMP-2. However, transcript as well as protein levels of VEGFD were negatively correlated with activation ratio of MMP-2. Serum VEGF-D levels exhibited significant positive correlation with *MMP2* transcript levels as well as latent and total MMP-2. Serum VEGF-D levels exhibited significant positive correlation with latent MMP-9. There is dearth of data in the literature regarding the correlation between VEGFC, VEGFD, MMP2 and MMP9 in oral cancer. However, it can be suggested that correlation between these molecules might be responsible for invasion and metastasis in oral cancer. Further, hTERT, VEGFA, VEGFC, VEGFD, MMP2, MMP9 show a complex interplay between them; both at transcript and protein levels. Possibly this complex interplay play a significant role in aggressive behavior associated with this malignancy.

Overall interpretation of the present correlation study is depicted in figure 5.1. It is revealed that *p53*, a key tumor suppressor is master regulator of various signaling pathways involved in major hallmarks of cancer. The results of this correlation analysis between molecules suggests significant interaction and these molecules together through complex interplay affect various hallmarks of cancer including cell cycle regulation, evasion of apoptosis, immortalization, angiogenesis, invasion and metastasis.

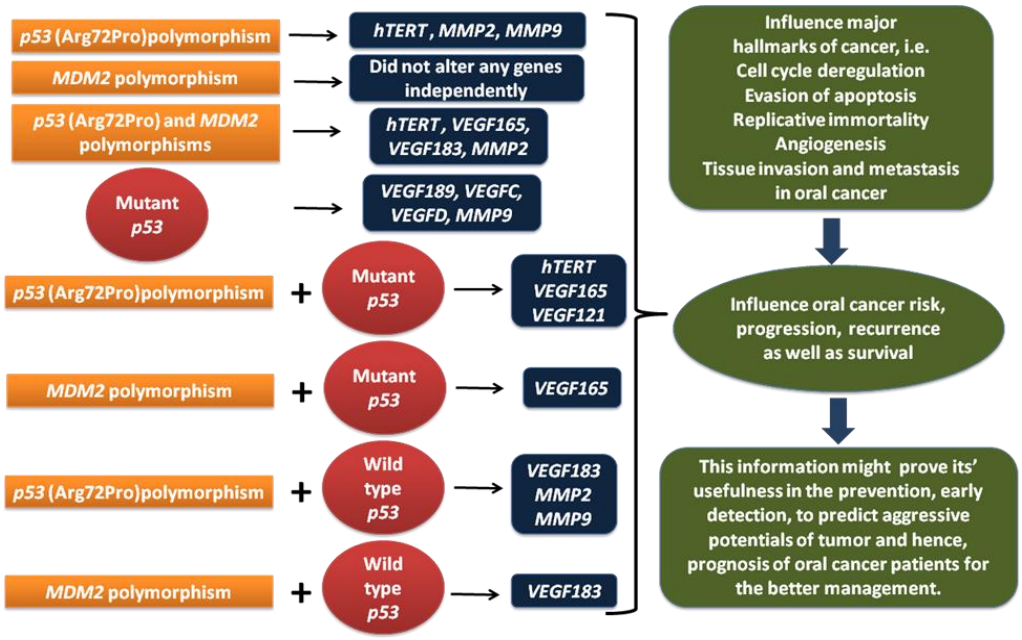

Figure 5.1: Clinical relevance of observations of the present investigation

Together, this investigation suggests that inherited genotypes of *p53* and *MDM2* as well as somatic *p53* mutations influence the progression as well as outcome of oral cancer. Genes involved in major hallmarks of cancer i.e. *hTERT*, *VEGFA*, *VEGFC*, *VEGFD*, *MMP2*, *MMP9* also play a significant role in oral cancer development. Most importantly, alterations in *p53* responses (*p53* and *MDM2* polymorphisms, *p53* mutations) modulate the expression of these genes. Thus, the data revealed that oral cancer is a multi-factorial disease involving multiple molecular changes including *p53* as a key molecule. Ultimately, this information might prove its usefulness in the prevention, early detection of oral cancer, to predict aggressive potentials of tumors and hence prognosis of oral cancer patients for the better management of oral cancer.

Most importantly, our results suggest that oral cancer patients could have a different *p53* gene status that also revealed the complexity of p53 response pathway. This information might be helpful to provide guidance to personalize the precise therapeutic strategy to the mechanism by which the *p53* pathway has been disrupted. Also, this information might be useful to improve personalized targeted therapy for oral cancer patients as well as identification of newer effective drug targets for oral cancer patients.