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.,
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
Pakistan 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 pairwise 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
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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
Discussion

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 (Muniraj 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; Koppickar 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
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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.
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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 transcriptionally 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.