# *Introduction*

## 1. Oral cancer incidence and mortality

India has highest prevalence of oral cancer in the world and accounts for nearly one-third of all oral cancer cases reported worldwide (77,003/1,98,975) (Ferlay *et al.*, 2013). Hence, oral cancer is also the leading malignancy in India (Krishna Rao *et al.*, 2013). Oral cancer remains the most common cancer amongst the male population. However, it is the third most common cancer after cervical and breast cancer amongst women in India (Khan, 2012). Moreover, 52,067 deaths were registered due to oral cancer from India in 2012 (Ferlay *et al.*, 2013), which documented it as a major health burden. Oral cancer in particular will continue to be a major problem. In figure 2.1, crude incidence projections by Globocan demonstrate that oral cancer crude incidence will increase in India by 2020 and 2030 in both sexes. Variability in the age-adjusted incidence rates of oral cancer in different regions of India has increased over the years (Coelho, 2012).

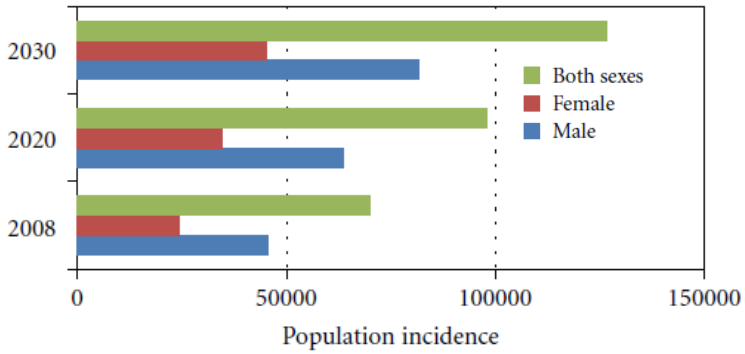


**Figure 2.1: Crude incidence projections for lip/oral cavity cancer (2008 to 2030) (Coelho, 2012)**

Oral cancer is highly prevalent in Gujarat, Western India (Ferlay *et al.*, 2010) where the rate of oral cancer is also increasing in the younger age groups (Patel *et al.*, 2009). The HBCR of the Gujarat Cancer & Research Institute (GCRI), Ahmedabad have also shown that oral cancer is the leading malignancy among all cancers diagnosed and treated at the Institute.

## 2. Oral cancer characteristics

***Age:*** Oral cancer is considered to be a disease that occurs mainly in the elderly and most of the cases of oral cancer occur between 50 and 70 years of age (Bhurgri *et al.*, 2006). Recently, there has been a shift towards younger age at diagnosis (Krishna Rao

*et al.*, 2013). About 17% of the younger patients are below 40 years of age (Halboub *et al.*, 2011).

***Sex:*** Considering all the age groups, men are more affected than women. However, a few reports from one of the Indian registries show male to female ratio of 1 (Krishna Rao *et al.*, 2013). Taking into account only young adults, men are predominantly affected (Krishna Rao *et al.*, 2013).

***Site:*** There is also a considerable variation in the distribution of cancer in intra oral sites. It is reported that buccal mucosa is the commonest site for oral cancer in India followed by anterior two thirds of tongue, the lower gum, the lip, the hard palate, the floor of the mouth and the upper gum (Byakodi *et al.*, 2012). However, Addala *et al.* (2012a) suggested that the tongue is the leading site not only among oral cancers followed by the buccal mucosa and gingiva in India. It was also reported that young adults of 45 years or below suffer more from tongue cancers (Ibayashi *et al.*, 2011). Differences in site preference were seen on comparing older (≥ 40 yrs) and younger adults (< 40 yrs). Older people have tendency to develop cancer of buccal mucosa, whereas younger people have tendency to develop tongue followed by buccal mucosa and other sites (Krishna Rao *et al.*, 2013).

***Recurrence:*** Recurrence is an important aspect of treatment outcome. It indicates a poor prognosis. Recurrence can be local, regional, locoregional or second primaries. According to an institutional study from India, the average disease free survival (DFS) time is around 35 months (Priya *et al.*, 2012). Recurrence rate vary between 25-40% after a follow-up period of 2-4 years (Priya *et al.*, 2012; Wang *et al.*, 2012; Hakeem *et al.*, 2013). The clinico-pathological factors that influence the treatment outcome and likelihood of recurrence are use of tobacco, advanced stage including local regional nodal involvement, positive resection margin and thickness of tumor (Krishna Rao *et al.*, 2013).

***Mortality and survival:*** Even with the technological advancement in treatment modalities and diagnosis, the 5 year survival rate has been low for oral cancer. Patients with advanced stage of the disease have a higher mortality rate than those at the initial stages. The probability of survival in the presence of nodal involvement reduces from 80% for stage I to less than 20% for stage IV after 5 years following diagnosis (Ma'aita, 2000; Kruaysawat *et al.*, 2010). Subsequent to diagnosis, the

treatment provided based on the stage at diagnosis could influence the survival. However, survival following surgical treatment depends on the pre-operative characteristics of tumor including positive nodes, thickness of the tumors, poorly differentiated tumors etc.

## 3. Etiology

***Tobacco use:*** Tobacco being an independent risk factor, the relative risk of occurrence of oral cancer in tobacco users is 11 times that of people who never used tobacco. It is used in smoking as well as smokeless forms (Madani *et al.*, 2010).

Though, cigarette smoking is seen in all Asian countries, bidi smoking is common in countries like India. Smoking bidi is the major risk factor contributing to a considerable number of oral cancer cases in India. Contrary to this, in a Karunagappally cohort (India), bidi smoking did not show any risk for oral cancer in tobacco chewers but an elevated risk was observed with bidi smoking when analysis was restricted to non-tobacco chewing smokers (Jayalekshmi *et al.*, 2011). It appears that tobacco chewing has a stronger effect than bidi smoking, masking the effect of bidi in the chewers.

Smokeless tobacco is used in a variety of forms and it is wide spread among both men and women. Most commonly used forms are, panmasala and gutkha which are blends of tobacco, areca nut, lime and catechu and this combination of ingredients is strongly genotoxic and carcinogenic (Nair *et al.*, 2004). It is observed that smokeless tobacco users are at greater risk than those smoking tobacco (Muwonge *et al.*, 2008; Madani *et al.*, 2010). Oral cancer is largely associated with gutkha followed by tobacco flakes (Madani *et al.*, 2010). Gutkha and panmasala are termed as "polyingredient oral dip products" and are more carcinogenic because of other carcinogenic compounds present (Madani *et al.*, 2012). A greater incidence of oral cancer is observed in Asian societies where consumption of smokeless tobacco is high. In a study conducted in Kerala, tobacco chewing is found to be a greater risk for gum and mouth cancer but slightly less for tongue (Krishna Rao *et al.*, 2013). This could be due to direct contact of tobacco and other carcinogenic compounds with oral mucosa for a considerably longer time.

Prevalence studies of tobacco use in India have shown wide variations between urban and rural areas, regions, age, gender, education, and other socio-demographic

variables across the country. In Gujarat, tobacco chewing is the most frequent form of using tobacco by adolescents than smoking (Makwana *et al.*, 2007). In addition, Joshi *et al.*, (2010) suggested that every 4 out of 10 residents in Gujarat were found to be exposed to chewing tobacco with mawa-masala and gutkha, being the predominant forms.

***Alcohol consumption:*** Drinking alcohol is another important risk factor for oral cancer. Alcohol use may be under reported in communities where alcohol consumption is restricted like Gujarat. However, alcohol has a synergistic effect with tobacco chewing and smoking (Gangane *et al.*, 2007; Subapriya *et al.*, 2007; Muwonge *et al.*, 2008; Lin *et al.*, 2011).

***Diet:*** Diet is influenced by the culture of a population. The complexity of the diet makes it difficult to assess the role of dietary components in cancer development. There are no well designed epidemiological studies from Asian countries regarding this aspect. A few studies have been able to show some relation with oral cancer. The protective effect of fruits and vegetables on oral cancer is unequivocal. Studies from our laboratory also suggested association between deficiency of important nutrients in diet and oral cancer risk (Raval *et al.*, 2001; 2002). It was also reported that risk of oral cancer for non-vegetarians is greater than vegetarians by 85% (Subapriya *et al.*, 2007). This could be attributed to reduced exposure to polycyclic aromatic hydrocarbons (PAHs) among vegetarians. However, the oral cancer risk attributable to dietary PAH exposure is still unclear.

***Biological agents:*** Role of oncogenic viruses in human cancer is an emerging area of research. Viruses are capable of hijacking host cellular apparatus and modifying DNA and the chromosomal structures and inducing proliferative changes in the cells (Ram *et al.*, 2011). HPV and herpes simplex virus (HSV) have been established in recent years as causative agents of oral cancer (Campisi *et al.*, 2007; Chocolatewala and Chaturvedi, 2009; Kumaraswamy and Vidhya, 2011; Ram *et al.*, 2011). Epstein-barr virus (EBV), human herpesvirus-8 (HHV-8) and human immunodeficiency virus (HIV) have also been reported as risk factors of oral cancer in different studies (Ram *et al*., 2011).

***Genetic susceptibility:*** Although considerably larger numbers of individuals use tobacco, a small percentage of users develop cancer. Conversely, not all oral cancer

patients are tobacco habitués (Ram *et al.*, 2011). These observations suggest the importance of inherent genetic factors in oral cancer. Genetic susceptibility to oral cancer is significant especially in young patients and based on inherited difference in the efficiencies of metabolizing carcinogens, DNA repair and cell cycle control alone or in combination (Scully, 2011). Single nucleotide polymorphism (SNP) leads to phenotypic effect by several mechanisms including enhanced or reduced transcription, altered post transcriptional activity or change in protein structure (da Silva *et al.*, 2011). Candidate genes have been evaluated DNA repair, cell cycle, xenobiotic metabolism and growth factor pathways (Hopkins *et al.*, 2008). However, identification of genetic variants in humans that modify cancer risk has been complicated by a number of factors including genetic heterogeneity in the population, the multiplicity of genes involved and variation in environmental exposure. As a result, many studies are underpowered, produce conflicting results, and are difficult to replicate in independent studies (Houlston and Peto, 2004). Epidemiological evidence suggests that exposure profiles and inherited susceptibility to the action of carcinogenic and mutagenic stimuli are both important in the development of cancer (Wolf, 1990).

## 4. Molecular pathogenesis of oral cancer

Oral carcinogenesis like any other cancer is a progressive disease and normal epithelium passes through stages starting from dysplasia to finally transforming into invasive phenotypes (Ram *et al.*, 2011). The development of oral cancer is a multistep process modulated by genetic predisposition and environmental influences, such as, tobacco, alcohol and viral infection. All of these factors can lead to a wide range of genetic and molecular alterations (Markopoulos, 2012). The alterations mostly affect two large groups of genes: oncogenes and tumor suppressor genes which can be either overexpressed or inactivated. From these alterations, tumor cells acquire autonomous self-sufficient growth and evade growth inhibitor signals and lead to uncontrolled tumor growth. Tumor cells also escape apoptosis and then replicate infinitely through the immortalization process by telomere lengthening (Choi and Myers, 2008). As cancer cells grow, new blood vessels formation is critical. During oral carcinogenesis, tumor cells are able to create a blood supply by stimulating endothelial cell proliferation and new blood vessel formation. The subsequent progression of oral cancer includes tissue invasion and metastasis. Invasion to adjacent normal tissue

requires the degradation of basement membrane by proteolytic enzymes. Thus, oral cancer develops from complex process enabled by occurrence of key hallmark events like sustaining proliferative signaling, evading growth suppressors, resisting apoptotic cell death, enabling replicative immortality, inducing angiogenesis, activating invasion, metastasis (Chandra *et al.*, 2013). Although, all histopathological types of carcinomas are seen in oral cavity, the most common form of oral cancer is squamous cell carcinoma.

## 5. Importance of molecular signatures

As documented above, multiple factors are involved in oral carcinogenesis. Many investigators have evaluated a range of molecular markers involved in the oral carcinogenesis, including molecules involved in the cell cycle regulation, DNA repair, apoptosis, angiogenesis and extracellular matrix (ECM) degradation (Campo-Trapero *et al.*, 2008; Choi and Myers, 2008; Pérez-Sayáns *et al.*, 2009; Ram *et al.*, 2011; da Silva *et al.*, 2011). However, the existing data about the role of molecular markers in oral cancer development still remains unclear and inconclusive.

Moreover, oral cancer is associated with high incidence of loco-regional recurrence, post surgery and radiotherapy, which account for the majority of treatment failures. The rates of oral cancer recurrence vary from 18 to 76% for patients who underwent standard treatment, and it is considered the major cause of poor survival (da Silva *et al.*, 2012). Though, clinical tumor-node-metastasis (TNM) classification is the major determinants for the prognosis and outcome of oral cancer, it does not provide any information on the biological characteristics and aggressive nature of the tumor (Brocić *et al.*, 2009). Paucity of early diagnostic and prognostic markers is one of the contributory factors for higher mortality rates. Therefore, it is essential to stamp newer molecular biomarkers for earlier detection of aggressive potential of tumors which can help to identify the high risk patients likely to develop recurrence and hence might further aid in better treatment management. It is also important to emphasize here that oral cancer progression is a multi-factorial and multistep process impacting nearly all hallmarks of cancer. Hence, multiple marker evaluation may logically be required to estimate the changes related to early, intermediate and late end points like prognosis and treatment outcome.

## 6. p53, the guardian of genome, is a key player

*p53*, a tumor suppressor gene has come up to the forefront of cancer research because it is commonly mutated in human cancer. Curiosity in the *p53* has generated a large amount of information regarding the complexity of its function and regulation in carcinogenesis (Vousden and Prives, 2009; Liu *et al.* 2014; Muller and Vousden, 2014). The *p53* gene is located on the short arm of chromosome 17 with 11 exons of which 10 are coding. The p53 protein is comprised of 393 amino acids and divided into five domains with each domain having a different structure and function (Figure 2.2). It contains five evolutionary conserved domains, of which four are located in the DNA-binding core.

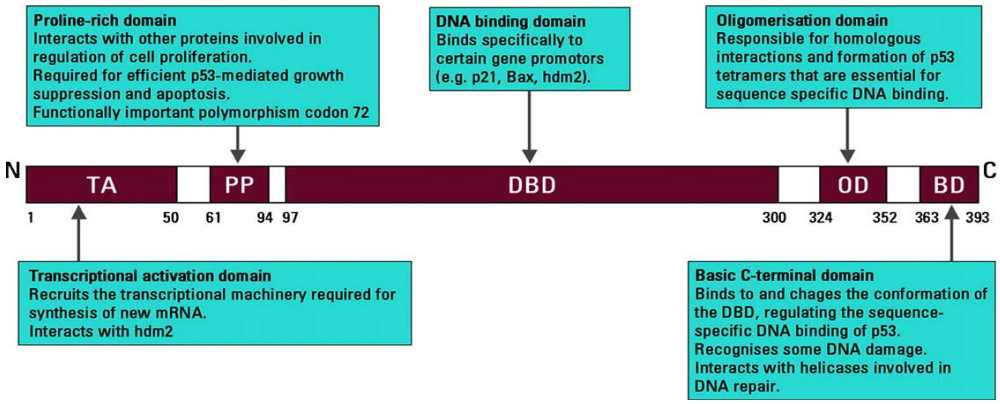


**Figure 2.2: Structure of p53 protein (Partridge *et al.*, 2007)**

The most important role of p53 is to integrate signals emerging from a wide range of cellular stresses including stresses due to DNA damage, hypoxia, oncogene overexpression, environmental carcinogen exposure (Whibley *et al.*, 2009). p53 induces adaptive and protective cellular responses by triggering transcription of specific genes. p53 prevents erroneous cell proliferation, by inducing growth arrest at the stage of cell cycle followed by DNA damage or apoptosis, if the damage is not repairable (Figure 2.3). Thus, it preserves the integrity of the genome (Partridge *et al.*, 2007).

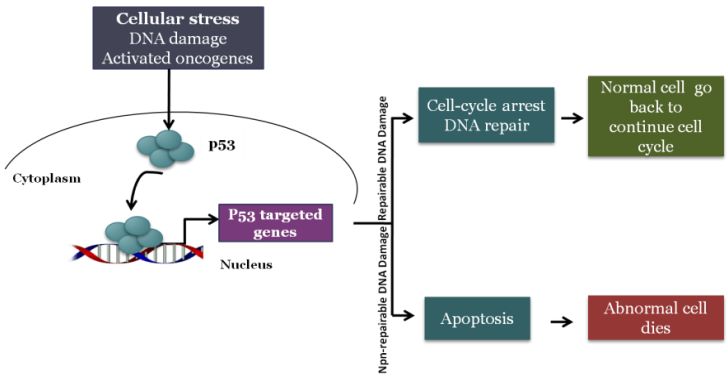


**Figure 2.3: Tumor suppressor gene *p53*; the guardian of the genome**

Somatic mutations in *p53* with the aim of absence or dysfunction in p53 protein is one of the most universal mechanisms by which the p53 pathway is damaged during carcinogenesis. These mutations are mainly single base substitutions that result in amino acid substitution (Whibley *et al.*, 2009). However, in the tumors, where wild-type *p53* is intact, there might be alterations in the regulation of the p53 pathway. Thus, understanding why p53 is unable to perform its role as a tumor suppressor in these wild-type tumors is very crucial. Unlike somatic mutations, the majority of polymorphisms in the *p53* gene are expected to be associated with cancer risk by compromising the normal activities of p53 (Whibley *et al.*, 2009). The levels of p53 in normal cell are tightly controlled. Under normal conditions, MDM2, a key negative regulator of p53 binds to its transactivation domain and promotes ubiquitin-dependent proteasomal degradation of p53 (Bond *et al.*, 2005). Since p53 transcriptionally activates *MDM2*, the expression levels of p53 and MDM2 are balanced through a feedback negative loop. This balance is altered in stresses such as, DNA damage which leads to increased p53 levels (Tang *et al.*, 2008). The crucial role of MDM2 in the control of p53 functions recommends that polymorphisms in the *MDM2* gene should be responsible for probable alteration in p53 functions. Moreover, degradation of p53 by E6 protein of HR-HPV is also one of the mechanism by which normal function of p53 is altered in HPV associated oral carcinogenesis (Campisi *et al.*, 2007). Together, all of the above mentioned mechanisms lead to alterations in p53 responses in cancer cell (Figure 2.4).

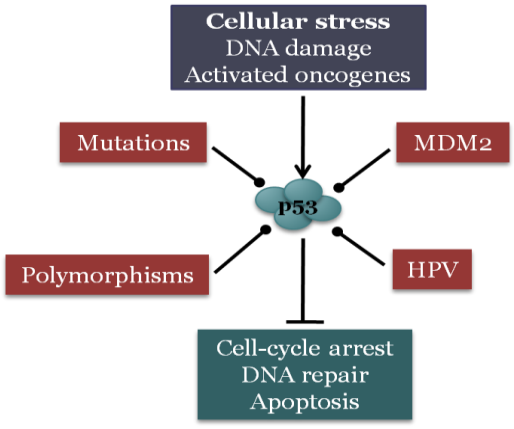


**Figure 2.4: Mechanisms that leads to alterations in p53 responses**

Thus, exploring mechanisms by which p53 responses is affected in the comprehensive way might aid in the identification of tumor characteristics, prognosis and thus in the development of a new approach to treat the cancer.

## *6.1. p53 polymorphisms in oral cancer*

Several sequence variations are present in the *p53* gene. Most of these variations are intronic and have no cancer related biological consequences (Whibley *et al.*, 2009). However, two intronic polymorphisms in the *p53* gene; 16 bp duplication in intron 3 (rs17878362) and G>A transition in intron 6 (rs1625895) have been suggested to affect the levels of *p53* gene expression as well as its functions (Pietsch *et al.*, 2006).

Introns were originally believed to be non functional because they do not code for proteins, still they have been implicated in regulation of gene expression and DNA – protein interactions (Wu *et al.*, 2002). Thus, sequence variations in introns may affect function of proteins and hence cancer risk. However, various studies exploring the functional role of these intronic polymorphisms remain indecisive. In cell culture analysis, these two intronic polymorphisms did not seem to be enough to impair p53 functions during the neoplastic transformation but required an additional coding region mutation (Wang-Gohrke *et al.*, 1999). They have also reported that no splicing errors have been linked to these polymorphisms. Wu *et al.* (2002) have reported low apoptotic index and less repair capacity of lymphoblastoid cell lines that harbor variant alleles of intron 3 and intron 6 polymorphisms. In contrast, Hu *et al.* (2008) observed that variant alleles of intron 3 and intron 6 have significantly higher levels of Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) induced apoptotic index compared to its homozygous wild-type genotypes. Gemignani *et al.* (2004) have also found that 16 bp duplication in intron 3 reduced the *p53* mRNA levels. However, intron 3 and exon 4 polymorphisms are in strong linkage disequilibrium (LD). Thus, it remains to be determined that whether intron 3 polymorphism alone influence mRNA stability or need the presence of the exon 4 polymorphism. Hence, the functional role of these two intronic polymorphisms of *p53* in cancer risk remains uncertain. Only one study has reported the association between these two intronic polymorphisms and oral cancer risk. The authors have suggested that intron 3 polymorphism was associated with increased oral cancer risk whereas intron 6 polymorphism was associated with reduced oral cancer risk (Galli *et al.*, 2009).

Of the 19 exonic polymorphisms, 11 polymorphisms in *p53* are non-synonymous, resulting in an amino-acid change and only four of these have been validated (Whibley *et al.*, 2009). Out of these, there are sufficient molecular evidences for two

polymorphisms (Pro47Ser and Arg72Pro) suggesting their role in functional change of the p53 protein (Whibley *et al.*, 2009). The rest two polymorphisms (V217M and G360A) have not been associated with an altered cancer risk till date (Whibley *et al.*, 2009). The codon 47 polymorphism results in proline to serine substitution is rare whereas arginine to proline substitution in codon 72 (rs1042522) is common (Felley-Bosco *et al.*, 1993; Beckman *et al.*, 1994). It was reported that Arg to Pro polymorphism at codon 72 in exon 4 affects the structure of p53 protein and its biochemical and biological activities (Ozeki *et al.*, 2011). It has also been reported that this polymorphism affects various important functions of p53 such as cell cycle regulation, apoptosis, transcriptional trans-activation capacity and senescence (Figure 2.5) (Thomas *et al.*, 1999; Bergamaschi *et al.*, 2003; Dumont *et al.*, 2003; Pim and Banks, 2004; Frank *et al.*, 2011). The relationship between the polymorphism in codon 72 and oral cancer has been studied but the results are inconsistent (Zhuo *et al.*, 2009).

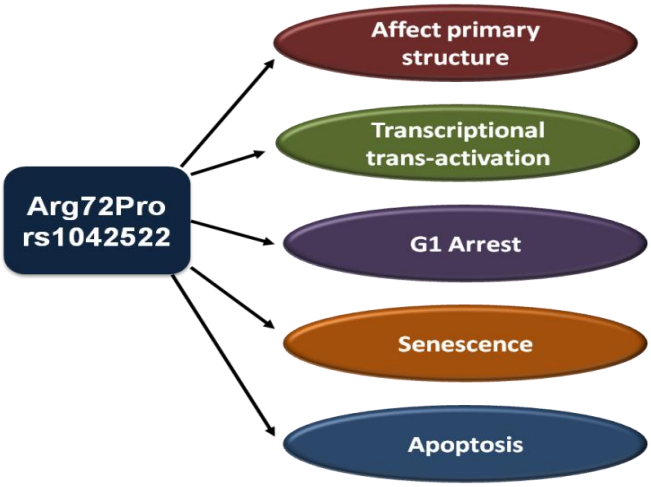


**Figure 2.5: *p53* Arg72Pro polymorphism and its' influence on p53 functions**

It has been speculated that both ethnic back-ground and lifestyle differences contribute to the differences in predisposing genetic factors to cancer development (Chakrabarti *et al.*, 2001). This fact is very crucial for country like, India where wide variations in ethnicity and life style exist. There is a single study from east region of India which analyzed association between these three polymorphisms and oral cancer till date (Mitra *et al.*, 2005). It has also been reported that the frequencies of the polymorphisms tend to differ among Indians and this is mainly due to differences in ethnicity (Mitra *et al.*, 2003). Therefore, the present study aimed to analyze whether the polymorphisms in exon 4, intron 3 and intron 6 of the *p53* gene are associated

with differential oral cancer risk in the population from West India where incidence of oral cancer is dramatically high.

## *6.2. p53 mutations in oral cancer*

Approximately 40% of all human cancers are found to have somatic mutations in the *p53* gene (www-p53.iarc.fr). The frequency of *p53* mutations varies according to the tumor type (Muller and Vousden, 2014). The frequency of *p53* mutations have also been observed to vary between groups which differ in ethnicity or nationality for some cancer types (Chitra *et al.*, 2010). This may be due to specific carcinogen exposure or inherited features in those populations. Wide variations have been reported between different studies of the same tumor type, probably reflecting methodological and geographic differences (Royds and Iacopetta, 2006).

Cancer-associated mutations in *p53* are primarily mis-sense substitutions, non-randomly distributed along the molecule, particularly in the central DNA binding domain (www-p53.iarc.fr). These single amino acid changes affect transcriptional activity of p53 to various degrees. However, all mutations do not have an equal deleterious effect on p53 function, and some mis-sense mutants may even acquire new functions (Martin *et al.*, 2002; Kato *et al.*, 2003; Petitjean *et al.*, 2007; Oren and Rotter, 2010). Different mutations have different effect on the function of the p53 protein. Evidences also have suggested that mutant p53 not only loses its tumor suppressive function, but also has dominant negative effect on the remaining wild-type allele (Hong *et al.*, 2014). Moreover, mutant p53 has also ability to transcriptionally activate genes involved in increased proliferation, inhibition of apoptosis, limitless replication, angiogenesis, invasion and metastasis etc. (Katara and Singh, 2008; Freed-Pastor and Prives, 2012; Liu *et al.*, 2014; Powell *et al.*, 2014). Thus, mutant p53 promotes tumorigenesis by influencing many different aspects of oncogenic processes affecting almost all the ‘‘hallmarks of cancer’’ (Figure 2.6). However, how these different forms of mutant p53 have an impact upon tumorigenesis still remains unclear.

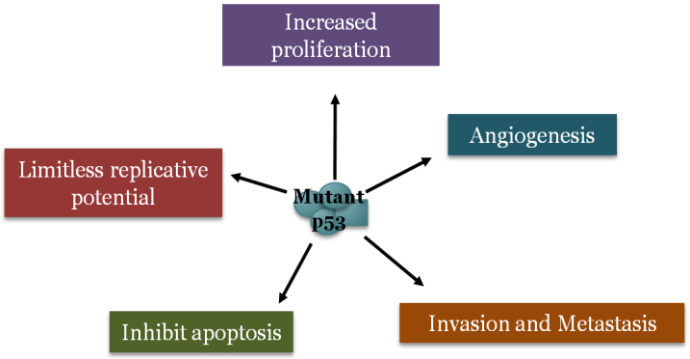


**Figure 2.6: Mutant p53 contributes to tumorigenesis**

In head and neck tumors, the prevalence of *p53* mutation varies from 30% to 70%, depending on the methodologies used to assess *p53* mutations, types of the tumor material and heterogeneity of the tumor sites examined (Chitra *et al.*, 2010). The other reason for these wide variations could be different levels of exposure to risk factors like, tobacco and alcohol in the studied populations as mentioned earlier. Studies from various regions of India have shown that the frequency of *p53* mutation in oral cancer ranges from 17 to 21%, which is lower than the western reports (Heinzel *et al.*, 1996; Saranath *et al.*, 1999; Ralhan *et al.*, 2001). In contrast, no *p53* mutations were reported in the study from Orissa, the Eastern region of India (Patnaik *et al.*, 1999). Moreover, accumulation of p53 protein has also been observed in histologically normal epithelium adjacent to oral tumors (Bilde *et al.*, 2009). Though, the role of these mutations in adjacent normal tissues in prognosis is unknown. Therefore, present study aimed to analyze *p53* gene mutation spectrum and role of *p53* gene mutation in initiation, tumor progression and prognosis. Present study also aimed to find role of *p53* mutations in histologically normal epithelium adjacent to malignant tumor in recurrence of the disease.

## *6.3. MDM2 polymorphism*

*MDM2* is a proto-oncogene that encodes an E3 ubiquitin ligase which regulates the activity of p53 by binding to its transactivation domain, inhibits its transcriptional activity, shuttles p53 out of the nucleus to the cytoplasm of the cell and promotes its ubiquitin-dependent proteasomal degradation (Singh *et al.*, 2008). Importantly, MDM2 forms a negative feedback negative loop in regulating p53 activity, in which p53 induces transcription of *MDM2*, and, in turn, the MDM2 protein inhibits p53 activity (Figure 2.7) (Yu *et al.*, 2011)

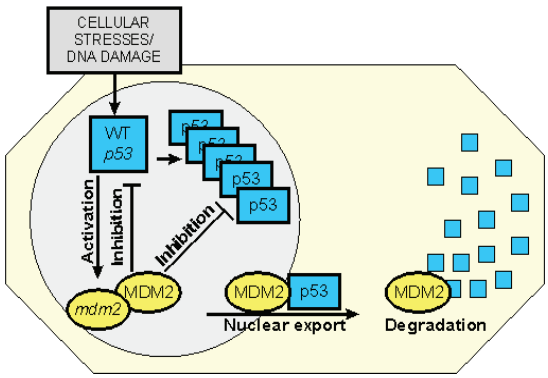


**Figure 2.7: Feedback negative loop between p53 and MDM2**

Because, MDM2 is a key negative regulator of p53 activity, over-expression of MDM2 inhibits the p53 responses, thus contributing to human carcinogenesis (Lozano and Zambetti, 2005). It has been reported that *MDM2* transgenic mice expressing higher levels of MDM2 in various tissues, develop spontaneous tumors compared to non-transgenic mice (Jones *et al.*, 1998; Lozano and Zambetti, 2005). Thus, over-expression of *MDM2* has an effect on tumor susceptibility in mice. Amplification of the *MDM2* gene and over-expression of MDM2 by unknown mechanisms have been observed in a subset of human tumors, some of which retain wild type *p53* (Yu *et al.*, 2011). These data suggest that increased levels of MDM2 may represents an alternative mechanisms by which tumor cells escape from the tumor suppressive activities of p53 (Valentin-Vega *et al.*, 2007). In fact, reports suggest that over-expression of MDM2 commonly occurs in oral cancer (Shwe *et al.*, 2001; Lim *et al.*, 2005). Recently, novel *MDM2* splice variants were identified in oral cancer at a high frequency and were found to be significantly associated with oral cancer development (Sam *et al.*, 2012). Taken together, MDM2 may play a significant role in oral carcinogenesis. Therefore, it is biologically plausible that functional genetic variants in the *MDM2* gene may have an effect on cancer development in the general population.

It has been reported that one intronic polymorphism (T>G in the Sp1-binding site within the intronic promoter region, rs2279744) of *MDM2* may increase the affinity of the Sp1 transcriptional factor, which results in higher *MDM2* mRNA and hence protein levels (Post *et al.*, 2010). In turn, this alteration in MDM2 levels disturb the p53/MDM2 ratio and affects the oscillating levels of p53 and MDM2 in response to various stresses like DNA damaging agents. Thus, the presence of this polymorphism may attenuate the p53 responses, hence increasing cancer susceptibility (Bond *et al.*, 2004; Whibley *et al.*, 2009; Grochola *et al.*, 2010). This polymorphism has been

associated with an increased cancer risk in some human tumors that express wild type *p53* (Bond *et al.*, 2006; Dharel *et al.*, 2006; Yarden *et al.*, 2008; Grochola *et al.*, 2010). However, a significant number of reports have failed to confirm such a notion (Krekac *et al.*, 2008; Khan *et al.*, 2008). As oral cancer is closely linked to carcinogenic exposure through tobacco chewing and smoking, a compromised DNA damage surveillance in these patients through *MDM2* over-expression and attenuation of p53 responses may lead an increased risk to oral cancer in individuals who practice these habits. There are very few studies which have investigated the association of this *MDM2* polymorphism with oral cancer risk. Two risk association studies highlighted that *MDM2* polymorphism did not alter the risk of oral cancer, but may influence the age of disease onset (Hamid *et al.*, 2009; Huang *et al.*, 2009). Therefore, present study aimed to assess *MDM2* gene polymorphisms alone as well as in combination with *p53* polymorphisms and mutations in oral cancer patients as a risk modifier.

## *6.4. HPV infection*

HPV are non-enveloped, small DNA viruses with circular and double-stranded DNA genome of approximately 8,000 base pairs (bp) that can infect epithelial cells. Currently, nearly 150 different HPV types are recognized, of which 120 HPV types are fully sequenced (Rautava and Syrja¨nen, 2012). The coding sequences have been classified as early (E) containing the early genes E1, E2, E4, E5, E6 and E7, and late (L) containing the late genes encoding the major (L1) and minor (L2) capsid proteins. The genomic organization is presented in figure 2.8. It contains promoter and enhancer DNA sequences critical to regulate viral replication and transcription by both viral and cellular genes. During early stages of the viral life cycle, early transcripts are initiated by a promoter referred to as either p97 or p105 in the HPV16/31 or HPV18 subtypes, respectively. The p97 promoter is located just upstream of the E6 open reading frame (ORF) (Miller *et al.*, 2012). The major promoter of late genes is located further downstream and varies slightly depending on the virus subtype, but it is generally referred to as p742 (Miller *et al.*, 2012). All of these play role in viral replication, transcription and carcinogenesis (Mannarini *et al.*, 2009). The early genes E1–E7 play a role in regulating, promoting and supporting viral DNA transcription and replication. The late genes, L1 and L2, are transcribed only in productively infected cells and encode the major and minor capsid proteins

required for assembly of progeny virions and eventual accumulation and release into the environment (Ragin *et al.*, 2007).

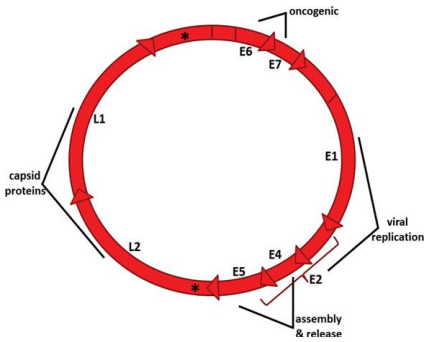


**Figure 2.8: Genomic organization of HPV (Miller *et al.*, 2012)**
HR-HPV genomes contain two viral promoters (*) encoding early (E) and late (L) genes.

In cancer cells, HPV can be found in episomal (extra-chromosomal) form, integrated, or in mixed forms of both. Integrant-derived transcripts are more stable than those derived from episomal viral DNA, and HPV integration has been associated with a selective growth advantage for the affected cells (Ruutu *et al.*, 2002). The cellular abnormalities induced by HPV reflect complex interactions between viral and cellular proteins. HPV can utilize three different strategies commonly used by higher organisms, including: (1) transcription; (2) RNA splicing and finally, (3) post-translational modification of the proteins (Rautava and Syrja¨nen, 2012).

Based on the oncogenic potentials, they are classified as Low-Risk (LR) and High Risk (HR). HR-HPVs are mainly associated with potentially malignant lesions e. g. HPV 16 and 18, whereas LR-HPVs e.g. HPV 6 and 11 are more commonly associated with benign diseases (Campisi *et al.*, 2007). The molecular mechanisms by which, HPVs disrupt key cellular elements responsible for cell cycle regulation and apoptosis have been identified. The two oncoproteins, E6 and E7 coded by the HR-HPVs are considered oncogenic due to their transforming and growth stimulating properties. E6 and E7 proteins of HR-HPVs can bind to several cellular targets leading to interference with transcription, chromatin remodeling, cytokine signaling, protein degradation, cell polarity and apoptosis, leading to genomic instability (Rautava and Syrja¨nen, 2012). The E6 protein is a major transforming protein. E6 protein binds to cellular ubiquitin ligase E6AP resulting in p53 degradation and blocking of p21 (Rautava and Syrja¨nen, 2012). E6 protein also has ability to activate the telomerase reverse-transcriptase (*hTERT)* gene which is essential for telomerase activity (Ragin *et al.*, 2007; Rautava and Syrja¨nen, 2012). Moreover, HPV E7 also plays a role in the

maintenance of telomerase activity in stable cell lines and augments acute, E6-induced *hTERT* promoter activity (Liu *et al.*, 2008). Thus, cells that contain damaged DNA enter aberrantly into mitosis, sustain proliferative ability and may eventually contribute to the propagation of structural chromosomal abnormalities in HR-HPV associated cancer (Figure 2.9) (Korzeniewski *et al.*, 2011). Moreover, HPV16 E6 oncoprotein also induce vascular endothelial growth factor-A (*VEGFA*) expression through the same promoter region as that utilized by p53 (Loureiro and D'Amore, 2005).

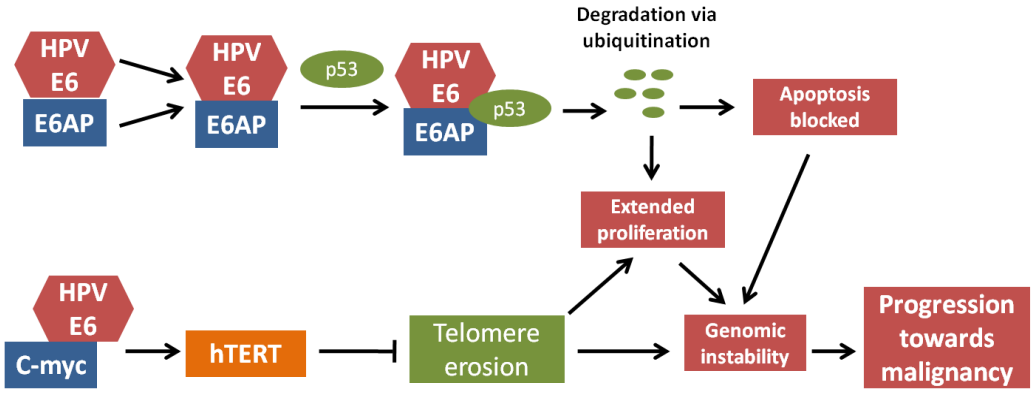


**Figure 2.9: Schematic presentation of progression towards malignancy by means of HPV E6 oncoprotein (modified from Rautava and Syrja¨nen, 2012)**

The participation of HPV in oral carcinogenesis was first proposed by Syrjanen *et al.* in 1983. There is growing evidence that HPV contributes to increased risk of oral cancer, along with alcohol and tobacco use (Campisi *et al.*, 2007; Chocolatewala and Chaturvedi, 2009; Kumaraswamy and Vidhya, 2011; Smith *et al.*, 2012). The International Agency for Research on Cancer (IARC) has declared that there is ample evidence that HPV 16 is acting as a biological carcinogen to humans and it is causally associated with oral cancer (IARC Working Group, 2012). Recent studies suggest that HPV, especially type 16 and 18 may be responsible for a small subgroup of oral cancers (Mannarini *et al.*, 2009). Moreover, HPV positive oral cancer patients have different molecular properties and are considered as a distinct clinico-pathological entity with different outcome and response to treatment (Chen *et al.*, 2012). The prognosis for patients with oral cancer is largely determined by the stage at presentation (Leemans *et al.*, 2011). Recently, Gogilashvili *et al.* (2012) have suggested that HPV status has a significant role as a confounding factor for prognostication of oral cancer. It has been suggested that HPV positive oral cancer patients have significantly improved outcome compared to HPV negative counterparts

(Zhao *et al.* 2009; Śnietura *et al.*, 2010; Elango *et al.*, 2011; Chen *et al.*, 2012; Laco *et al.*, 2012; Klozar *et al.*, 2013). The reason for the improved survival is unclear. However, better outcomes seem to be attributed to the ability of HPV positive cancer cells to induce apoptotic cell death in response to DNA damage as they contain wild type p53. Another reason appears to be attributed to the absence of carcinogen induced early genetic changes in the epithelium and the development of multifocal tumors (Mannarini *et al.*, 2009). However, it has been recently shown that among HPV positive oral cancer, the worst outcome is related to smoking (Ragin *et al.*, 2007; Sinha *et al.*, 2012). Thus, these carcinogens might potentiate the transformation effect of HPV. Prevalence of HPV in oral cancer has been reported from different geographic regions of India. However, it needs to be emphasized that incidence of HPV associated oral cancer widely varies across the different regions of India (Shukla *et al.*, 2009). Hence, it is important to investigate its prevalence and distribution in oral cancers in various parts of India. Such studies on the prevalence of HR-HPV type 16 and 18 infections in oral cancer have not been carried out from Gujarat, West India. Thus, present study aimed to evaluate the HR-HPV type 16 and 18 genotypes in oral cancers for the first time from Gujarat, West India using PCR based assay and its association with *p53* polymorphisms, *p53* mutations and *hTERT* expression.

## 7. Human telomerase reverse transcriptase

Human telomeres in somatic cells undergo progressive shortening with each replication cycle. This shortening acts as a signal for replicative senescence and probably functions as a tumor suppressor mechanism (Feldser and Greider, 2007, Deng and Chang, 2007). Activation of telomerase can bypass this replicative senescence and could lead to immortalization (Artandi and DePinho, 2010). Telomerase possess an RNA-dependent DNA polymerase activity that is specialized in the synthesis and maintenance of the telomeric ends of linear chromosomes, protecting them from degradation and end-to-end fusion and hence playing an important role in cell survival (Pannone *et al.*, 2007). The activity of telomerase is repressed in many human somatic tissues, whereas the enzyme is activated during tumor progression in most human cancers (Li *et al.*, 1999). The human telomerase is a complex RNA-protein heterotrimeric enzyme. The three main component of this enzyme are: 1) hTR: human telomerase RNA that acts as template in the process of repeat addition; 2) TP1: telomerase associated protein 1 of unknown function and 3)

hTERT: human telomerase reverse transcriptase, the catalytic subunit of the complex (Pannone *et al.*, 2007).

The telomere dependent senescence is mediated by *p53* tumor suppressor gene. Telomeres shorten progressively with cell division due to the end-replication problem in settings of insufficient telomerase. Critical telomere shortening compromises the telomere cap and results in a DNA damage response that activates the p53 tumor suppressor protein. This activation of p53 induces replicative senescence or mortality 1 (M1) phase and strongly suppresses tumor formation (Artandi and DePinho, 2010). However, inactivation of p53 prevents the p53-mediated growth arrest signal, allowing the cells to continue to divide until mortality 2 (M2) phase, when telomeres become so shortened that chromosomal end–end fusions occur. The resulting dicentric chromosomes threaten cell viability either by blocking mitosis per se or via a fusion breakage bridge cycle that leads to the loss of essential chromosomal material (Newbold, 2002). However, some cells that activate telomerase continue to proliferate and when the fused chromosomes attach to opposite spindle poles, chromosome breakage occurs. These broken ends serve as potent catalysts for translocations, focal amplifications and focal deletions. These alterations lead to drive development of carcinomas (Artandi and DePinho, 2010). Reactivation of telomerase, to acquire the capacity to proliferate indefinitely is an important characteristic of cancerous cells. This observation was put forward as telomere-telomerase hypothesis (Figure 2.10).

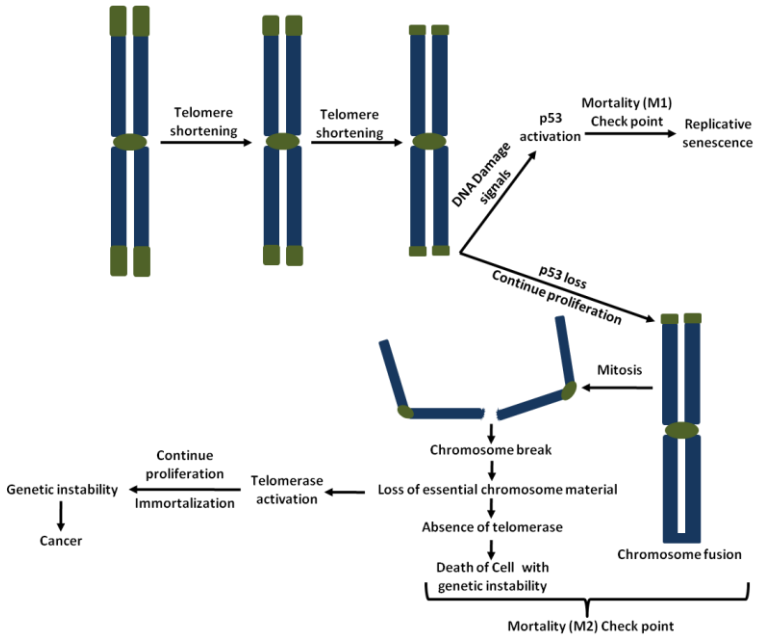


**Figure 2.10: Telomere telomerase hypothesis (modified from Artandi and DePinho, 2010)**

Of the telomerase subunits, hTERT is concomitantly expressed with the activation of telomerase during cellular immortalization and tumor progression. It has been observed that introduction of *TERT* into telomere negative cells leads to expression of telomerase activity, elongate telomeres and extends cellular lifespan (Cao *et al.*, 2002). The ectopic expression of hTERT along with activated oncogenes results in tumorigenesis (Hahn *et al.*, 1999; Qi *et al.*, 2011). hTERT activity is repressed in normal somatic tissues, but elevated in most human tumors (Rahman *et al.*, 2005). Moreover, it was also suggested that hTERT maintains cell survival and proliferation via both telomerase enzymatic activity dependent telomerase lengthening and enzymatic activity independent intra-molecular interactions involving p53 (Cao *et al.*, 2002; Rahman *et al.*, 2005; Lai *et al.*, 2007; Jin *et al.*, 2010). It was also observed that the expression of hTERT affects a variety of other genes. In a recent study, 284 genes were found to be either positively or negatively affected by changes in hTERT, suggesting that hTERT may have extra-telomeric effects. Interestingly, most of the genes affected were involved in cell-cycle regulation, signaling, metabolism, differentiation and apoptosis (Perrault *et al.*, 2005).

There is an overall agreement on the critical role played by hTERT in the mechanism of immortalization during the process of oral carcinogenesis both *invitro* and *invivo* (Lee *et al.*, 2001; Kim *et al.*, 2001; Fujimoto *et al.*, 2001; Luzar *et al.*, 2004; Kumar *et al.*, 2005; Pannone *et al.*, 2007; Freier *et al.*, 2007; Chen *et al.*, 2007). In addition, the recent increase in understanding the structure and function of telomeres and telomerase has led to the possible use of them as diagnostic and prognostic markers in several types of neoplasms, including, oral cancer. However, data regarding the association of hTERT expression levels with clinico-pathological characteristics were contradictory (Pannone *et al.*, 2007).

A number of transcription factors, tumor suppressors, cell cycle inhibitors, cell fate determining molecules, hormone receptors and viral proteins have been implicated in the control of *hTERT* expression (Figure 2.11) (Ducrest *et al.*, 2002; Janknecht, 2004). Kanaya *et al.* (2000) showed that the *hTERT* gene has two p53 binding motifs upstream of the 5′ core promoter region. Further, over expression of p53 and its subsequent binding to these two motifs with the help of transcription factor Sp1 leads to the repression of the *hTERT* promoter (Figure 2.11) (Janknecht, 2004; Lai *et al.*, 2007).

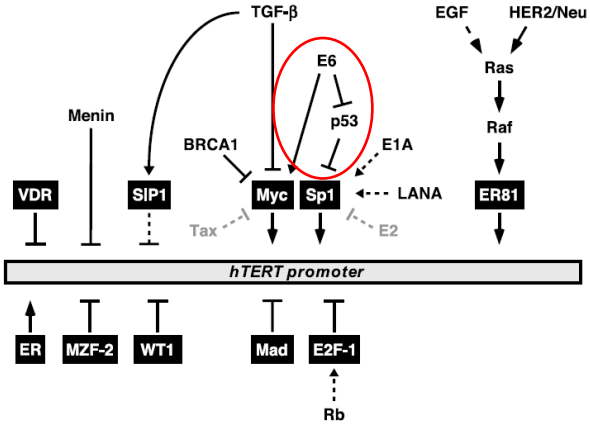


**Figure 2.11: A number of transcription factors that regulate *hTERT* expression (Janknecht, 2004)** (*hTERT* promoter binding proteins are boxed. Dashed arrows indicate hypothetical regulatory pathways.)

Transcription of *hTERT* was shown to be down regulated following the induction of *p53* (Shats *et al.*, 2004; Toh *et al.*, 2005; Yan *et al.*, 2006; Cukusić *et al.*, 2008). Thus, it may be suggested that *p53* polymorphisms as well as mutations may affect the levels of *hTERT* expression. Moreover, *hTERT* expression might be influenced indirectly by polymorphic variant of *MDM2* through down regulation of p53. Further, HPV E6 oncoprotein also has ability to activate *hTERT* expression (Ragin *et al.*, 2007; Rautava and Syrja¨nen, 2012). Moreover, HPV E7 also plays a role in the maintenance of telomerase activity in stable cell lines and augments acute, E6-induced *hTERT* promoter activity (Liu *et al.*, 2008). Interestingly, it has been reported that hTERT regulates *VEGFA* expression at transcriptional levels and matrix metalloproteinases (*MMPs)* family gene expression via NF-κB-dependent transcription, in a manner independent of its telomerase activity (Zhou *et al.*, 2014). Thus, present investigation also intended to evaluate *hTERT* mRNA expression in association with *p53* polymorphisms, *p53* mutations, *MDM2* polymorphism, HPV infection, *VEGF* and *MMPs* expression.

## 8. Vascular endothelial growth factor

Angiogenesis play an important role in tumor growth as solid tumors do not grow beyond 3 mm without new vessel formation (Yavrouian and Sinha, 2012). Thus, angiogenesis is a major hallmark for tumor growth. Angiogenesis is subject to a complex control system with proangiogenic and antiangiogenic factors. Angiogenesis is tightly controlled by a physiological balance between the proangiogenic and antiangiogenic factors called “angiogenic balance”. A switch to the angiogenic phenotype depends on a local change in the balance between proangiogenic and

antiangiogenic factors. One of the most important proangiogenic factors is VEGF also known as VEGF-A (Johnstone and Logan, 2006; Nayak *et al.*, 2012).

VEGF-A is a 34 to 42 kDa, dimeric, disulfide-bound glycoprotein. Various isoforms of VEGF-A are generated as a result of alternative splicing from a single gene of *VEGFA* known as *VEGF121*, *VEGF165*, *VEGF183*, *VEGF189* and *VEGF206*. The aminoacids encoded by exons 1 to 5 and 8 are conserved in all isoforms, whereas alternative splicing occurs in exon 6 and exon 7 (Figure 2.12). Exon 6 and 7 encode two distinct heparin binding domains. The presence or absence of these domain influences solubility and receptor binding. The heparin binding domain encoded by exon 6 determines binding to the ECM and therefore isoforms containing this domain (VEGF189 and VEGF206) are bound tightly to cell surface heparin containing proteoglycans, in the ECM, whereas those lacking the domain are diffusible. VEGF165 contains only one heparin binding region encoded by exon 7, is moderately diffusible and VEGF121 which lacks the domain encoded by both exon 6 and 7 is highly diffusible. Thus, the different VEGF-A isoforms differ in their heparin binding ability as well as in their binding capacity to receptors VEGF-R1 and VEGF-R2 (Stimpfl *et al.*, 2002).

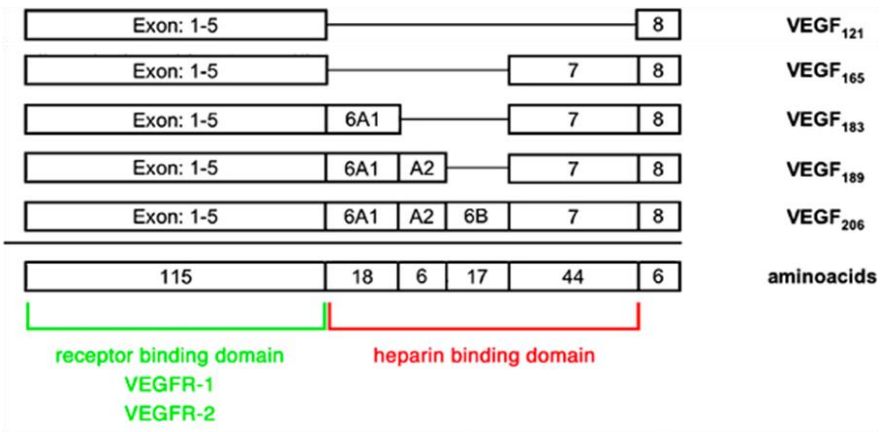


**Figure 2.12: Alternative splicing results in the generation of several isoforms of VEGF-A (modified from Eming and Krieg, 2006**)

The affinity of the VEGF-A isoforms variants to bind heparin has an overall effect on isoform spatial distribution and their bioavailability; these variations then determine whether blood-vessel growth is organized and directed or disordered (Woolard *et al.*, 2009). It has been suggested that VEGF-A isoforms perform unique functions and act in a co-ordinated fashion to optimize the formation and development of new vessels networks (Johnstone and Logan, 2006). It has also been suggested that various isoforms do not demonstrate equivalent function (Zacchigna *et al.*, 2008). VEGF121

is a freely diffusible from the cells and promotes the proliferation of vascular endothelial cells, macromolecular extravasation and angiogenesis (Woolard *et al.*, 2009). Moreover, this isoform showed much lower receptor binding affinity than that of VEGF165 (Tas *et al.*, 2006). VEGF165 is a potent enhancer of vascular permeability and predominant regulator of both physiological and pathological angiogenesis (Woolard *et al.*, 2009). VEGF183 is secreted from endothelial cells at low concentrations and predominantly cell associated and does not exhibit mitogenic or proliferative capabilities unless released by heparin or cleaved by plasmin (Woolard *et al.*, 2009). VEGF189 is almost entirely sequestered in the ECM making this a reservoir of VEGF-A that can be mobilized via proteolysis (Mărgăritescu *et al.*, 2009). VEGF189 induces endothelial cell proliferation and migration in vitro to a similar extent to that observed with VEGF165 (Herve *et al.*, 2005); and plays a role in cell adhesion and survival (Hutchings *et al.*, 2003). VEGF206 is tightly bound to extracellular heparin containing proteoglycan. This splice variant is poorly exported from the cell and does not demonstrate mitogenic properties (Woolard *et al.*, 2009). It has also been reported that changes in the expression pattern of these isoforms in cancer may influence other genes involved in malignant transformation and pro-angiogenic cascade in particular (Biselli-chicote *et al.*, 2012). Further, Woolard *et al.* (2009) suggested that VEGF-A isoforms play a pivotal role in progression and clinical outcome associated with a variety of cancers.

Regarding the role VEGF-A in oral cancer, data from literature are incongruous (Johnstone and Logan, 2006; Mărgăritescu *et al.*, 2009; 2010). Most of studies evaluating role of VEGF-A in oral cancer have used immunohistochemistry (IHC) as a technique. It is suggested that the immunostaining patterns of VEGF-A in the tumoral tissues were heterogeneous from case to case and also within the same case but in different regions (Mărgăritescu *et al.*, 2009). Moreover, studies regarding the role of various VEGF-A isoforms in oral cancer are scant in the literature. It is also suggested that previously published conflicting results may be due to the presence of various VEGF-A isoforms in the tissues and also due to the cross reactivity of the antibodies used (Johnstone and Logan, 2006). Also, association of VEGF-A isoforms with clinico-pathological characteristics, disease progression and outcome of oral cancer patients has not been studied till date. Thus, determination of VEGF-A

isoforms profiles within oral tissues is also necessary to improve our understanding regarding the role of VEGF-A in oral cancer.

In addition, lymphangiogenesis, the growth of new lymphatic vessels, is believed to underlie lymph node metastasis. The extent of regional lymph node metastasis is an important indicator of tumor aggressiveness and is a prognostic factor for several kinds of carcinoma. Most of oral cancers undergo lymphatic metastasis to the regional lymph node (Sugiura *et al.*, 2009). The patient prognosis depends on the presence of lymph node metastasis. However, little is known about the molecular mechanisms underlying lymph node metastasis in oral cancer. VEGF-A have also lymphangiogenic effects that may be linked to the recruitment of inflammatory cells and secrete lymphangiogenic factor. However, the most potent factor implicated in lymphangiogenesis is VEGF-C and VEGF-D (Mărgăritescu *et al.*, 2009).

VEGF-C and VEGF-D represent a novel subgroup of the VEGF family because of their unique sharing of the most closely related VEGF homology domain, a long N and C terminal extension and the same receptors, VEGF-R2 and VEGF-R3. VEGF-C and VEGF-D are first produced by pre-pro-polypeptides and stepwise, proteolytic processing increases their affinities for VEGF-R3 which is expressed exclusively in lymphatic endothelial cell (Sugiura *et al.*, 2009). The fully processed mature forms also bind to and activate VEGF-R2. Therefore, both VEGF-C and VEGF-D can also exert angiogenic activity via VEGF-R2 (He *et al.*, 2004).

Because of the close similarity between VEGF-C and VEGF-D, it has been postulated that VEGF-C and VEGF-D could contribute collaboratively in cancer cell. They might be involved in modifying the permeability of lymphatic vessels and involved in the formation of nodal metastasis (O'charoenrat *et al.*, 2001). The role of VEGF-C and VEGF-D in oral cancer remains largely unknown and its association with lymph node involvement remains contradictory (Shintani *et al.*, 2004; He *et al.*, 2004; Sagiura *et al.*, 2009; Mărgăritescu *et al.*, 2009). Thus, evaluation of VEGF-C and VEGF-D mRNA as well as protein expression is essential to study potential of oral cancer cells to metastasize in lymph node. Also, evaluation of VEGF-A isoforms, VEGF-C and VEGF-D simultaneously may provide insights into oral carcinogenesis. Hence, the present investigation aimed to study contribution of VEGF-A isoforms, VEGF-C and VEGF-D in oral cancer.

It is not surprising that VEGF-A is regulated at multiple levels from transcriptional to post-translational. Transcriptional regulation accounts for much of the up and down regulation of VEGF-A in tumors. Transcription regulation of VEGF-A occurs via both the core promoter and through enhancers or repressors outside of the core promoter (Loureiro and D'Amore, 2005). The *VEGFA* promoter is predicted to be controlled by a Sp1 site that is 50 base pairs upstream of the transcription start site. Interestingly, the p53, an important inhibitor of angiogenesis promotes the expression of inhibitors of angiogenesis such as thrombospondin, and inhibits the expression of *VEGFA* but not *VEGFC* (Hoeben *et al.,* 2004). In addition, active tumor growth is accompanied by hypoxia. This hypoxia conditions led to the up regulation of both p53 and hypoxia-inducible factors-1α (HIF-1α). HIF-1α binds to and stabilizes p53. This association initiates apoptosis and turn off the angiogenic switch. The loss of p53 expression or attenuated p53 responses enhances the heterodimerization of HIF-1α with HIF-1β and upregulates the expression of *VEGFA* in tumor cells (Hoeben *et al.*, 2004). Moreover, it is accepted that wild type p53 indirectly repress *VEGFA* expression by interaction and inhibition of transcription factors such as Sp1 and E2F (Pal *et al.*, 2001; Qin *et al.*, 2006). In addition to the indirect repressive role of p53 on *VEGFA* promoter activity, it seems that p53 is also required for *VEGFA* induction during the initial phases of hypoxia. A highly conserved unconventional but functional p53-binding site adjacent to the main HIF-1α binding site was identified within the *VEGFA* promoter and shown to be essential for this p53-mediated *VEGFA* induction. p53 rapidly induces *VEGFA* transcription upon hypoxia exposure by binding, in an HIF-1α-dependent manner, to a highly conserved and functional p53-binding site within the *VEGFA* promoter. However, p53 indirectly downregulates *VEGFA* expression in a p21-dependent manner during sustained hypoxia (Figure 2.13) (Farhang Ghahremani *et al.*, 2013). Recently, mutant p53 has been implicated in the transcriptional regulation of *VEGFA* (Hong *et al.*, 2014; Liu *et al.*, 2014). Thus, it may be suggested that *p53* mutations as well as *p53* polymorphisms may affect the levels of *VEGFA* expression. Moreover, *VEGFA* expression might be influenced indirectly by polymorphic variant of *MDM2* through down regulation of p53. Furthermore, HPV16 E6 oncoprotein also induce *VEGFA* expression through the same promoter region as that utilized by p53. However, this E6 dependent *VEGFA* activation was shown to be p53 independent (Loureiro and D'Amore, 2005). It was also reported that MMPs upregulate *VEGFA* (Loureiro and D'Amore, 2005). Most

importantly, MMP-9 induces an upregulation of *VEGFA* (Hoeben *et al.*, 2004). As mentioned earlier, hTERT was also found to regulate *VEGFA* expression at transcriptional levels (Zhou *et al.*, 2014).

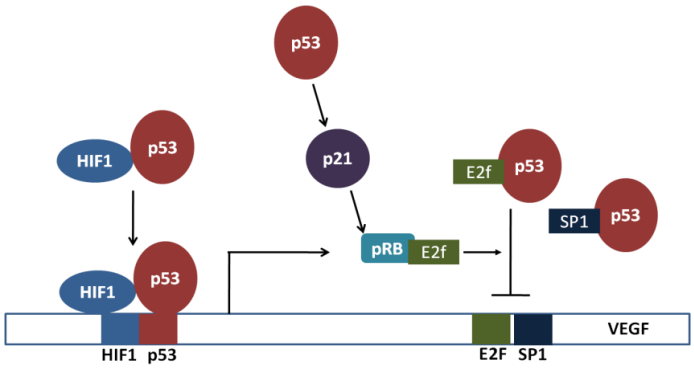


**Figure 2.13: Schematic representation of VEGF promoter regulation by p53 (modifired from Farhang Ghahremani *et al.*, 2013)**

*VEGFA* expression can also be regulated post-transcriptionally. As mentioned previously, varying amounts of each *VEGFA* isoform mRNA can be generated during pre-mRNA splicing, so that cells can express certain subsets or all *VEGFA* isoforms. It is not known if the various upstream effectors of *VEGFA* transcription also modulate the ratio of *VEGFA* isoforms expressed by tumor cells, but this information could be important since the *VEGFA* isoforms have differential effects on tumor growth (Loureiro and D'Amore, 2005).

Further, the regulation of VEGF-A in the extracellular environment has been implicated in the angiogenic switch (Bergers *et al.*, 2000). Studies have indicated two modes for the regulation of VEGF-A interaction with ECM. First is alternative splicing however, the role of multiple VEGF-A isoforms, in the interaction of with the ECM, has remained elusive. Second is proteolytic processing depending on microenvironmental levels of specific MMPs (Lee *et al.*, 2005). It was observed that VEGF-A bioavailability is regulated extracellularly by MMPs through intra-molecular processing. Specifically, a subset of MMPs can cleave matrix-bound isoforms of VEGF-A, releasing soluble fragments. Thus, MMP activity could have an impact on VEGF-A regulation and vascular patterning through the direct alteration in the status of bound versus soluble VEGF-A (Lee *et al.*, 2005). Fang *et al.* (2000) reported that the suppression of MMP-2 alone inhibits the transition from the pre-vascular to the vascular stage during tumor development. They showed that MMP-2 is required to switch to the angiogenic phenotype during the development of chondrosarcoma and during the progression of plasma cell tumors. Bergers *et al.* (2000) showed that

MMP-9 is a functional component of the angiogenic switch during multistage pancreatic angiogenesis. It was also suggested that MMP-9 was regulated by VEGF-A at the transcriptional level in B chronic lymphocytic leukemia (Ugarte-Berzal *et al.*, 2010). Recently, Hollborn *et al.* (2007) suggested positive feedback regulation between MMP-9 and VEGF-A in retinal pigment epithelial (RPE) cells. Regarding the regulation of VEGF-C and VEFG-D, there is no much data in the literature. Thus, evaluation of VEGF-A, VEGF-C, VEGF-D mRNA as well as protein levels in association with *p53* polymorphisms, *p53* mutations, *MDM2* polymorphisms, HPV infection, hTERT and MMPs expression was also one of the objective of the present investigation.

## 9. Matrix metalloproteinases

Tumor invasion and the process of metastasis formation are characteristics for malignant neoplasia. The metastatic spread of tumors continues to be the main barrier to successful treatment of malignant tumors. Thus, better understanding of the cellular and molecular processes leading to invasion and metastasis is of paramount importance. Specific sequential steps are necessary for the formation of metastasis and they include genetic alterations that induce the invasive metastatic phenotype during tumor progression, breakdown of cell-cell and cell-matrix adhesion (tumor cell detachment from the primary tumor and basement membrane invasion), tumor cell migration, modulation of ECM and proteolysis and angiogenesis (Cortesina and Martone, 2006). Degradation of the ECM is the one of the important hallmark of cancer progression that allows cancer cells to invade the surrounding tissue. Interactions involving tumor cells and the ECM strongly influence tumor development, affecting cell proliferation and survival as well as ability to migrate beyond the original location into other tissues to form metastases (Pereira *et al.*, 2005).

MMPs are zinc-dependent endopeptidases that efficiently degrade the components of the ECM and basement membranes, hence, contributing to the formation of a microenvironment that promotes tumor growth and subsequently metastasis. They selectively degrade various components of the ECM and release growth factors and cytokines that reside in the ECM (Roy *et al.*, 2009; Hatfield *et al.*, 2010; Vilen *et al.*, 2013). The MMPs are also capable of activating various latent growth factor, cytokines and chemokines and cleaving cell surface proteins (Kessenbrock *et al.*,

2010; Rodriguez *et al.*, 2010; Klein and Bischoff, 2011). Most of them are involved in promoting aspects of tumor growth such as cell proliferation, adhesion, migration, differentiation, angiogenesis, apoptosis and host defense evasion (Figure 2.14) (Hadler-Olsen *et al.*, 2013). Indeed, recent studies have revealed that MMPs are important during the early stages of tumor development even before metastasis occurs.

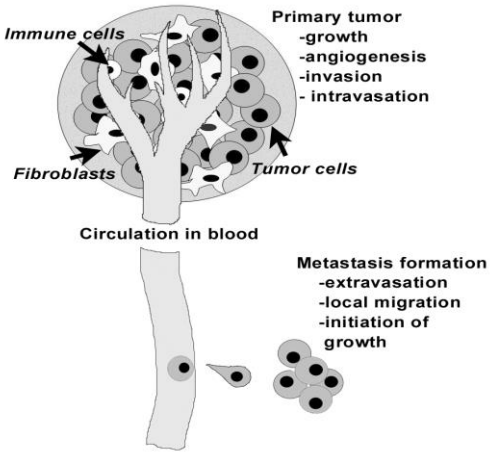


**Figure 2.14: Schematic representation of the steps in tumor progression**
MMPs have been implicated to play a role in most of these steps

Expression of various MMPs has been found to be up regulated in virtually every types of human cancer. Of the various MMPs thought to be involved in cancer, attention has focused on the gelatinases because they are overexpressed in a variety of malignant tumors (Patel *et al.*, 2005; 2007; Roy *et al.*, 2009; Shah *et al.*, 2009; Klein and Bischoff, 2011). It was also observed that knockout of either MMP-2 or MMP-9 is found to decrease tumorigenesis, supporting their role as cancer-promoting enzymes (Hadler-Olsen *et al.*, 2013).

The MMP-2 and -9 also known as gelatinases A and B or type IV collagenases are members of the MMP family. MMP-2 and MMP-9 are highly similar enzymes in many respects, but significant differences exist in the regulation of expression, glycosylation, proenzyme activation and substrate selectivity. MMP-2 is a 72-kDa nonglycosylated protein, whereas the 92-kDa MMP-9 contains two N-glycosylated sites in the prodomain and the catalytic domain (Kotra *et al.*, 2002) and a number of O-linked glycans (Rudd *et al.*, 1999; Mattu *et al.*, 2000). Gelatinase substrates include a wide variety of proteins including ECM proteins, proteinases, proteinase inhibitors, blood clotting factors, chemotactic molecules, latent growth factors and growth factor binding proteins, cell surface receptors, adhesion molecules and even intracellular

substrates (Bjorklund and Koivunen, 2005). The substrate specificities of MMP-2 and MMP-9 are similar but not identical. The most notable difference is the ability of MMP-2 to degrade native type I collagen (Bjorklund and Koivunen, 2005). Some substrates show over 200-fold selectivity towards MMP-2 (Kridel *et al.*, 2001; Chen *et al.*, 2002).

As gelatinases are capable of degrading a broad spectrum of substrates, their synthesis and activity are kept under tight control at various levels including transcription, activation, inhibition, complex formation and localization (Hadler-Olsen *et al.*, 2013). The gelatinases are secreted as proenzymes, and they need to be activated for full catalytic activity. The activation of the gelatinases occurs on the cell surface or in the extracellular milieu (Sato *et al.*, 1996; Yana and Weiss, 2000; Zucker *et al.*, 2003). MMP-2 is synthesized and secreted as an inactive proenzyme. It is reported that after secretion, proMMP-2 binds to its specific inhibitor, tissue inhibitor of metalloproteinases-2 (TIMP-2). This proenzyme is stable in normal physiology conditions. The main activation route of MMP-2 on the cell surface is by the formation of a molecular complex containing MMP-2, membrane type 1-matrix metalloproteinase (MT1-MMP) and TIMP-2 (Visse and Nagase, 2003). The N-terminal domain of TIMP-2 binds to and inhibits MT1-MMP, whereas the C-terminal domain of the same TIMP-2 molecule binds the hemopexin-like domain of MMP-2 forming a ternary complex. An adjacent TIMP-free MT1-MMP subsequently cleaves the MMP-2 to the intermediate 64-kDa form. This intermediate form is then processed to the fully mature 62-kDa form through cleavage active MMP-2 molecule (Visse and Nagase, 2003). Although TIMP-2 is normally required for the MMP-2 activation, higher TIMP-2 levels lead to inhibition of MMP-2 activation. On the other hand, soluble MT1-MMP activates MMP-2 with a high efficiency in the absence of TIMP-2 (Pei and Weiss, 1996). MMP-2 can also activate itself by auto-activation leading to the formation of multiple smaller activation products. MMP-2 activity has little regulation at the transcription level and most of the regulation occurs at the post-transcriptional level and during enzyme activation and inactivation (Overall and López-Otín, 2002). MMP-9 is also synthesized as a precursor, bound to TIMP-1 (Moll *et al.*, 1990). However, in cell cytosol, the enzyme can be stored in either a latent or an active form, which is in contrast to MMP-2 which can be stored only in a latent form (Nguyen *et al.*, 2001). The activation of proMMP-9 is a complex process,

which is regulated by interactions with TIMP and other MMPs. Numerous enzyme, leukocyte elastase, tissue kallikrein, collagenase I, stromelysin and trypsin now been suggested to be capable of proMMP-9 activation. Interestingly, MMP-2 has been also suggested to be capable of activating proMMP-9 (Kolkenbrock *et al.*, 1996; Bu and Pourmotabbed, 1996; Sorsa *et al.*, 1997; Ferry *et al.*, 1997). It can also be activated by plasmin dependent pathway involving MMP-3 (Rollin *et al.*, 2007). More complexity comes from the finding that the proMMP-9 displays significant catalytic activity in the presence of the propeptide (Bannikov *et al.*, 2002). Substrate binding appears thus to be sufficient to trigger the cysteine switch and provides one possible solution to the dilemma that active MMP-9 is often not found in the tissues despite the catalytic activity of MMP-9 (Bjorklund and Koivunen, 2005). MMP-9 is also highly regulated at the transcriptional level in contrast to MMP-2 (Martin *et al.*, 2001; Sternlicht and Werb, 2001). Growth factors, cytokines, cell-cell and cell-ECM adhesion molecules are the most important agents that are known to control the induction of MMP-9. Both gelatinases can be activated via free radical-generating 2- and 4- hydroxyestradiols (Paquette *et al.*, 2003).

It has been reported that MMP is regulated by VEGF at transcriptional levels in B chronic lymphocytic leukemia (Ugarte-Berzal *et al.*, 2010). hTERT also regulates *MMP* family gene expression via NF-κB-dependent transcription (Zhou *et al.*, 2014). It is also observed that p53 regulates the expression of *MMPs*, specifically, *MMP2*, However, the regulation of *MMPs* by p53 is complex, as it upregulates *MMP2* but downregulates *MMP9* (Katara and Singh, 2008; Powell *et al.* 2014). Thus, it can be hypothesized that attenuated p53 reponses by *p53* polymorphisms, *p53* mutations, *MDM2* polymorphisms, HPV infections as well as VEGF, hTERT might be implicated in altered expression of *MMPs*. This may be one of the potent reasons why most of the studies regarding MMPs remain indecisive. Hence, present investigation also proposed to analyze association of MMP-2 and MMP-9 mRNA and protein levels with *p53* polymorphisms, *p53* mutations, *MDM2* polymorphisms, HPV infections, hTERT and VEGF levels.

Overall, it is very crucial to identify the molecular signatures which can aid in identification of susceptible individuals for oral cancer development, help in earlier detection of aggressive potential of tumors and also assist to categorize the high risk patients likely to develop recurrence and hence might further aid in better treatment

management. As documented above, comprehensive analysis of alterations in p53 responses might facilitate in both identification of susceptible individuals and recurrence potential of tumors, early at diagnosis. Also, evaluation of alterations in the genes involved in major hallmarks in context with alterations in p53 throw some light on molecular pathogenesis of oral cancer in relevance to p53.