Introduction
(Review of literature)
ORAL CANCER
NATURAL HISTORY.

Incidence and prevalence

The term 'Oral cancer' is used to describe any malignancy that arises from the oral cavity comprising of lip, tongue, buccal mucosa and oropharynx. (Metha and Hamner, 1993; Smith, et.al, 1990). Oral cancer regrettably still poses a major health problem in many parts of the world. It is also a leading cause of death among the 10 most common cancers with highest rates being reported in Indian and French population (Smith, et.al, 1990). In India, at least 56,000 new cases are thought to occur each year, resulting in about 100,000 individuals suffering from the disease at any given time (La Vecchia, et.al 1997). Moreover, the age adjusted rates of 24.2/105 for males and 11.2/105 for females in the Thiruvananthapuram district of Kerala State are probably among the highest reported rates in the world (Sankaranarayanan, et.al, 1992). Hospital tumor registry data shows an average of 1000 new cases per year reporting to Regional Cancer Centre, Thiruvananthapuram. Cancer of the oral cavity constitutes 16.5% of all cancers and ranks first among the leading sites of cancer in male populations. In females, cancer of the oral cavity constitutes 10.5% of all cancers and ranks third among the leading sites of cancer, with breast cancer continues to rank first. (Hospital Tumor Registry Regional Cancer Centre, Annual Report, 1995).
Globally, cancer of the oral cavity ranks fourth among all cancers in men accounting for 233,000 new cases annually. In females, it ranks 6th among all cancers and accounts for 107,000 new cases. Among the developing countries India ranks first in oral cancer. Oral cancer is prevalent in both males and females over the age of 35 (Johnson, 1991)

Most oral cancer patients have a history of long term tobacco use such as cigarette smoking or chewing with or without alcohol consumption. Oral cancer accounts for 13.7% of all tobacco related cancers and ranks first among tobacco related cancers. Most patients with oral cancer include subjects with low socio-economic status (casual laborers, fisherman, wayside vendors, drivers etc). These subjects have the habit of chewing tobacco with betel or pan, smoking beedi or cigarettes while at work. A study report shows that labour class people in Kerala chew tobacco containing betel quids at frequencies of almost 17± 9 times per day. (Stich, et.al., 1988). Consumption of alcohol, common among this population is also an additive factor.

**Etiology**

Etiological factors considered as risk factors in oral cancer include those with chemical and biological origins. Evidence accumulated over the past 200 years indicates a link between oral cancer and tobacco use. Oral cancer is more prevalent among populations with tobacco habits. Consumption of alcohol often in combination with tobacco is also known to increase the risk for oral cancer. The
carcinogenic effect of tobacco and alcohol appear to be synergistic rather than additive. Polycyclic aromatic hydrocarbons are regarded as proximate-carcinogens present in tobacco smoke activated to ultimate carcinogens by microsomal enzymes. Chewing of tobacco results in release of N'-nitrosonomicotine, which is nitrosylated by bacterial flora to active nicotine. (Johnson, 1991)

The biological causative agents include Human Papillomavirus, Herpes Simplex Virus, syphilis, candidiasis, adenovirus etc. However the precise role of these agents in oral carcinogenesis remains to be understood. Host factors include, low socio economic status as a major risk factor. Poor nutritional status in the population associated with impaired antioxidant and immune defense system increases the risk potential if exposed to carcinogens. In addition to the above-described risk factors, these patients report to hospital only after they develop cancer often at advanced stages and perhaps it is still rare to see a pre-malignant being reported. (Paterson, et.al 1996)
DIAGNOSIS OF ORAL CANCER

Histopathological staging of oral cancer

It is unlikely that oral squamous cell cancer arises from normal surface epithelium. The surface epithelial cells undergo gradual changes from clinically undetectable pre-malignant lesion to clinically identifiable pre-malignant lesion. These pre-malignant stages are often reversible and are readily curable. Symptoms of pre-malignant conditions can be identified by screening alone; however most often these remain unnoticed. Patients report only after the disease advances to an irreversible malignant lesion.

Oral pre-cancerous lesion has been defined by an International Working Group as 'morphologically altered tissue', which in cancer is more likely to occur than in its apparently normal counterpart. (Axell, et.al 1991) There are two major clinically visible pre-malignant lesions namely leukoplakia and erythroplakia. Leukoplakia, appears as white plaque, 5mm or more in width, which cannot be attributed to any other disease. Erythroplakia appears as a red plaque. The dysplastic changes may or may not be appear in these stages. It is however universally accepted that squamous cell carcinoma can develop from these pre-malignant lesions. However this concept may not be applicable in developed countries such as UK or US with low prevalence of oral cancer, since there are no reports available with clinically described pre-malignant lesions. (Johnson, 1991)

Dysplastic lesions are more likely to undergo malignant change since chances of malignant transformation increase with increase in the severity of dysplasia. The
main morphological features of dysplasia are hyperchromatism and loss of polarity of basal cells. Increased nuclear to cytoplasmic ratio is often characteristic of dysplastic lesions. Carcinoma in situ has the highest risk among the histologically identifiable pre-cancerous lesions, showing marked epithelial dysplasia involving full thickness of the epithelium. Since carcinoma in situ is a stage that appears briefly and quickly progresses on to invasive lesion, it is not generally reported (Smith, et.al 1990).

High-risk squamous cell carcinomas are those that are associated with short survival time. This is usually due to early recurrence of neoplasm after the treatment. Despite identical staging, prediction of prognosis based on histopathology alone has not proven useful in oral cancer. Within the individual oral cancer there is often considerable variation in histological features in different parts. Histopathology reports based on structural criteria is suggestive of malignancy rather than the functional activity of the neoplastic cells (Johnson, 1991). Broder's classification based on degree of differentiation is the most commonly followed pathological grading system. Percentage of differentiated cells are used to grade the tumor as well differentiated, moderately differentiated, poorly differentiated and undifferentiated tumors (Broder, 1941 and Pindborg, et.al., 1997). Further modification of this grading was done by including additional information such as structural cohesiveness of cells, tendency to keratinize, nuclear aberrations, and number of mitoses above the basal layer (Anneroth, et.al 1987). Tumor host relationship such as invasiveness and inflammatory responses were also included.
To date no prospective study adopting this approach has been described, since variations in an individual sample is more common and the interpretation becomes very subjective and therefore unreliable.

Representative samples showing various histopathological grades are shown in Plate 1.

Clinical staging

The clinical staging of oral cancer follows International Union Against Cancer (UICC) guidelines. It has 3 parameters, "T" - the extent of primary tumor, "N" - the condition of regional lymph nodes and "M" - the presence or absence of distant metastasis. This is known as TNM staging.

T stages

TX Primary tumor cannot be assessed
TO No evidence of primary tumor
TIS Carcinoma in situ
T1 Tumor 2 cm or less
T2 Tumor 2-4 cm in the greatest dimension
T3 Tumor greater than 4 cm in the greatest dimension
T4 Tumor invading adjacent structures in lip and oral cavity

Photographs showing various T2 stage tumor and leukoplakia shown in plate 2.

N Stages

NX Lymph node cannot be assessed
NO No regional lymph node metastasis
N1 Metastasis in single ipsilateral lymph node, 3 cm or less in the greatest dimension
N2 Metastasis in single ipsilateral / contralateral / bilateral lymph nodes 3-6 cm in the greatest dimension
N3 Metastasis in lymph nodes with greater than 6 cm in the greatest dimension

M Stages
Presence of distant metastasis cannot be assessed
No distal metastasis
Distant metastasis

(Hermanek and Sobin., 1992))

Based on survival analysis clinical staging is a more reliable prognostic indicator than that of histopathological grading. Survival analysis shows no significant correlation with histopathological grading. Based on TNM staging, T4N0 and T4N3 when analyzed for survival shows 28% difference in complete response to treatment and 30% difference in survival percentage. In general, higher T status and N status often show poor prognosis when compared with T1 or N0 status. Thus it is clear that reporting without staging would lead to a loss of prognostically important data and therefore TNM staging is widely used for deciding options for treatment. (Ensley, et.al., 1987)

In summary, it may be possible to identify clinical or histopathological features that may statistically correlate with treatment response or survival in a large population study. However, they do not have the ability to predict such outcome for an individual patient. In particular, they cannot predict response or resistance prior to treatment. (Stafford, 1989)
Histopathological grades

1. Normal
2. Mild dysplasia
3. Moderate dysplasia
4. Well differentiated carcinoma
5. Well differentiated carcinoma
6. Moderately differentiated carcinoma
7. Poorly differentiated carcinoma
   (Magnification 25X)

Plate 1
A. Leukoplakia and tumor in tongue.

B. Tumor Stage T1 (Tongue).

C. Tumor stage T2 (Tongue).

D. Tumor stage T4 (Tongue).

D. Tumor stage T2 (Lip).

E. Tumor stage T4 (Buccal mucosa).

Tumor Grades (Clinical staging)
Plate 2
MANAGEMENT OF ORAL CANCER

Cancers of oral cavity were treated surgically or by radiotherapy long before understanding the tumor biology or radiobiology. Most centers practice radiotherapy as the primary mode of management of oral cancer. If it fails then salvage excision with or without supplementary chemotherapy may be used. However, advanced stages fail to attain cure rate with surgery or radiotherapy alone. Such failures can be overcome with combined treatment involving chemotherapy, radiotherapy and surgery. Most often treatment plans are made based on some management policy designed with current state of knowledge. However these are still arbitrary, because sometimes high-grade tumors responds well to pre-operative chemotherapy, while certain low-grade tumors often do not respond for any treatment. Complications such as disease recurrence or residual disease do occur despite giving a post-operative radio or chemotherapy. Thus it is obvious that in addition to tumor staging, analysis of biological behavior might have better prospects in designing individual treatment strategies.


A tumor 1cm in diameter, at the threshold of clinical detectability, contains $10^9$ cells and, if there has been no loss of clonogenic cells from the tumor, is at 30th or 40th or so generations. By the time the tumor is clinically detected and diagnosed, it is at the end of its rapid growth and a large proportion of cells become dormant rather than dividing. As both radiotherapy and chemotherapy act primarily against actively
dividing cells, this kinetic change with increased size of the tumor has important implications for treatment. (Stafford, 1989)

**Tumor kinetics**

Tumor cell cycle does not have constant timing just as that of normal cells. The rate of tumor doubling time is affected by relative cell loss by mechanisms such as exfoliation, metastasis, necrosis etc., Squamous cell carcinoma of head and neck have very high cell loss and this is important while assessing response to therapy. In order to eradicate a tumor permanently it is necessary to destroy the reproductive integrity of every last clonogenic cell. Thus reproductive viability of tumor cells determines the extent of tumor response to treatment. Unfortunately, there is no means of assessing this in a biopsy of a tumor. A biopsy after treatment may show a mixture of cells ranging from cells which are totally necrotic, through cells with apparently normal looking cytoplasm but pyknotic nuclei, to cells which look normal. The cells looking normal may or may not be reproductively viable. For slow growing tumors, cells may persist long time after treatment, yet the tumor might be permanently controlled. (Jacobs, 1987)

The major limiting factor in anti-cancer treatment is the tolerance of the normal tissues. For radiotherapy, the normal tissue of relevance and those adjacent to tumors. For cytotoxic drugs, the dose limiting normal tissues are largely the actively proliferating cells of bone marrow, epithelium of skin, mucosa and gonadal tissues. Considering all these factors various modalities of treatments are planned.
Clinicians mostly rely on TNM staging in deciding their treatment as to single or combined mode of treatment, which includes surgery, radiation and chemotherapy (Munro, 1989)

Chemotherapy

One of the major problems in using cytotoxic drugs for chemotherapy in the treatment of human cancer is drug resistance. Drug resistance may be acquired by increased deactivation of the drug or decreased uptake or increased pumping out of the drug. The cells may find alternate substrate if metabolic inhibitors are used. Drug resistance is a major problem while treating squamous cell cancer of the head and neck. The common drugs used for the treatment of oral cancers are methotrexate, 5-fluorouracil, bleomycin and cis-platinum.

**Methotrexate** is an antimitabolite, acting primarily by inhibiting the enzyme dihydrofolate reductase, which is needed for conversion of dihydrofolate to tetrahydrofolate required for the biosynthesis of thymidine. Folinic acid is used to rescue the normal cells from the toxic effects of methotrexate. Toxic effects of methotrexate are nausea, vomiting, myelosuppression, mucositis, renal damage and hepatic fibrosis. Methotrexate is more useful in palliative management of the oral cancer. A dose of 100mg is given intravenously followed by 15 mg folinic acid after 24 hours. This schedule is repeated every 21 days provided the renal functions and blood counts are normal.
5-Fluorouracil is an S-phase specific antimetabolite and it disrupts thymidine synthesis by blocking thymidylate synthase. A dose of 500mg/24 hours can be infused daily for 3 to 6 months without any complications. The half-life of 5-FU is only 10 minutes in the plasma. Therefore, 5-FU is used usually in combination with other drugs (cis-platinum) or along with radiation and is a value in treating advanced stages of the disease.

Bleomycin is a versatile drug, which acts by causing single or double strand breaks in the DNA. It is active in G2 and M phase of the cell cycle. A dose less than 150mg is normally given during combination treatment, to avoid adverse effects such as pulmonary fibrosis and pigmentation. Unlike other drugs given as bolus infusion, bleomycin is given as 24-hour infusion.

Cis-platinum: The mode of action of cis-platinum is believed to act by alkylating DNA, and is usually given as 1-6 hour intravenous infusion. Nephrotoxicity is a common side effect that can be overcome by hydrating the patients with saline or dextrose during the treatment. Modified form of cis platinum such as carboplatin are less nephrotoxic but expensive. Cis-platinum sensitizes the cells to cytotoxic actions of radiation and also interferes with DNA damage repair. Cis-platinum is synchronously used along with radiotherapy.
Radiotherapy

Radiotherapy is based on the use of ionized ration to treat disease. Macromolecular damage is a common effect of radiation, production of reactive chemical species such as free radicals that attacks DNA and interferes with replication. The value of linear energy transfer (LET) indicates density of ionization. Single strand and double strand breaks are common during radiation treatment. Such effects depend on oxygenated status. The responses by hypoxic cells are very low.

Radiation dose is defined by amount of energy absorbed by a unit mass of tissue as a result of radiation and is usually expressed as Gray. Analysis shows that lower LET is more efficient and sub-lethal to normal cells. However it is sparsely ionizing and therefore biological effects depend on concentration of oxygen in the irradiated tissues. Tumor cells are generally hypoxic and therefore high LET radiation is used for treatment. Fractionation of dosage allows the normal cells to repair the damage. Since repair processes in tumor cells may be less efficient than that of normal cells. In the hyper-fractionated regimen, increased frequency of exposure to radiation allows tumor destruction more effectively as it does not facilitate tumor reoxygenation. Clinical trials using hypoxic cell sensitizers show better response to treatment with low LET radiation. Conventional practice of radiotherapy involves external beam radiotherapy or interstitial implantation (Brachytherapy).
External beam therapy

External beam therapy using X-ray is classified according to the energy of the beam employed. The electron volt (eV) is the unit used to define the beam energy of the beam of radiation. Megavoltage radiation (above 2MV) spares skin, penetrates tissue well and no excess energy deposition in bone occurs. Electron beam therapy is used for superficial tumors. For neck nodes, external beam therapy is useful particularly after radical neck dissection. Production of megavoltage for clinical use utilizes either a linear accelerator or isotopes. Linear accelerator produces electrons and smashes them into a tungsten alloy target, resulting in the production of X-rays that are appropriately collimated and focused on the target. Caesium 137 or Cobalt 60 is used as radioactive sources of electron beam. The beam of radiation applied to the target tissue after marking the area of the tumor and immobilizing the head of the patient.

Radiation dose, time and fractionation.

The units of dose of radiation are based on energy deposited in the tissue by radiation. The Gray is the international SI unit, represented as Gy (1Gy=100rad). Clinically radiotherapy is given as radical or palliative radiotherapy. Palliative doses are intended to relieve symptoms without producing side effects while radical doses aim to eradicate the tumor and tolerate toxicity to normal cells. Palliative treatment is usually given as large doses in a short duration (for example 2500 cGy in 5 fractions over a week or 3000 cGy in 10 fractions over 2 weeks). There are two main types of schedule used for radical treatment: shorter 3-4 week course, and the
longer 6-7 week course. The total dose is usually prescribed on the basis of the
tumor volume. Radical schedules can vary from 5000-5750 cGy in 16-20 fractions
over 3-4 week shorter schedule or 6000-7000 cGy in 30-35 fractions over 6-7
weeks (longer schedule).

**Interstitial implantation (Brachytherapy)**

Interstitial techniques for radiotherapy imply putting radioactive sources within the
tumor volume rather than irradiating from external source. The advantage of
interstitial implantation is that it allows much higher dose of radiation to be given to
the tumor relative to the normal tissue. Doses may be as high as 10000 cGy. The
disadvantage of implantation is the use of anesthetics at the time of implantation,
exposure to radiation by health personnel, visitors and other patients. Iridium192
pins are commonly used for this purpose. A combination of external beam and
implantation can exploit the advantages of both techniques.

**Treatment Planning.**

<table>
<thead>
<tr>
<th>Tumor stage and Size</th>
<th>Treatment plan</th>
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<tbody>
<tr>
<td>T1 N0</td>
<td>Radiotherapy or surgery to primary.</td>
</tr>
<tr>
<td>N+ &lt;2cm</td>
<td>Radiotherapy or surgery to primary and neck.</td>
</tr>
<tr>
<td>&gt;2cm</td>
<td>Surgery to primary and neck.</td>
</tr>
<tr>
<td>T2 N0</td>
<td>Radiotherapy or surgery to primary and neck.</td>
</tr>
<tr>
<td>N+ &lt;2cm</td>
<td>Radiotherapy or surgery to primary and neck.</td>
</tr>
<tr>
<td>&gt;2cm</td>
<td>Surgery to primary and neck.</td>
</tr>
<tr>
<td>T3 N0</td>
<td>Superficial tumors: Radiotherapy or surgery to primary and neck</td>
</tr>
<tr>
<td>N+ &lt;2cm</td>
<td>Deeply infiltrating tumors: Surgery to primary and neck with post-operative radiotherapy to primary site.</td>
</tr>
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<td>Tumor stage and Size</td>
<td>Treatment plan</td>
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<tr>
<td>&gt;2cm</td>
<td>Superficial tumors: Surgery to primary and neck. Deeply infiltrating tumors: Surgery to primary and neck with post-operative radiotherapy to primary site and to neck if indicated.</td>
</tr>
<tr>
<td>T4 N0</td>
<td>Surgery to primary and neck with planned postoperative radiotherapy to primary site, and to neck if indicated.</td>
</tr>
<tr>
<td>N+ &lt;2cm</td>
<td></td>
</tr>
<tr>
<td>&gt;2cm</td>
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(Stafford, 1989)

The progress in developing combination therapy depends on following objectives: production of high clinical response rates, complete response that can be translated to histologically negative resected specimen. Based on current modalities the overall response rate observed clinically is about 75-90%, while a complete response is seen only in 40-60% cases. Recurrence or disease relapse remains as major issue in about 40% of the treated cases (Jacobs, et.al, 1987)

Currently stage of the disease is used for assessing the prognosis and clinical outcome for patients with oral cancer. The biological characteristics of these tumors are often variable resulting in divergent clinical disease courses despite similar or identical staging. Tumor pathology and histological differentiation may also often be subjective and are unreliable predictors of disease course (Clayman, et.al, 1996). Identification of biological factors contributing to the clinical aggressiveness of oral cancer can provide a basis for the identification of low and high-risk groups as well as help predict tumor response to therapy. Therefore understanding the molecular
aspects of oncogenic transformation as well as response to treatment by the tumors is necessary for such evaluations.
MOLECULAR BASIS OF ONCOGENIC TRANSFORMATION IN ORAL CANCER

Oral cavity is exposed to various chemical and biological agents capable of inducing cancer. Tobacco chewing and smoking has found to be linked to oral carcinogenesis. Tobacco contains potent carcinogens such as benzopyrenes and nitroso amines capable of causing mutations in the DNA. The effect of tobacco is not only dose and time dependent but also acts synergistically with alcohol intake to multiply the risk. Tobacco chewing in the form of betel quid consisting of betel leaf, areca nut with calcium and other spices and additives is a common habit seen in our populations. The tumors are in general enophytic and may be deeply penetrating. Oral cancer associated with tobacco chewing, however develops at the site of application of the bolus and is thus common in buccal sulcus and buccal mucosa (Metha, 1993). Food products contain additives, toxins, harmful products like peroxides produced during cooking all of which interact directly with cells of the oral cavity. The oral cavity is also exposed to pathogenic organisms such as papillomaviruses, bacterial and fungal agents. These agents independently or in concerted action play a potential role in oral carcinogenesis. (Johnson, 1991)

Chromosomal aberrations are observed during premalignant transformation changes. Alterations in chromosomes 3p, 5q, 8p, 9p, 18q are common in oral cancers and populations showing such alterations are at increased risk of developing cancer (Bockmuhl, et.al., 1998). Chromosomal alterations lead to genomic instability and mutations disrupting tumor suppressor genes resulting in transformation (Paterson, et.al 1998) According to Knudson, a single hit is not
sufficient for transformation and loss of more than one gene 'multi-hit' leads to malignant transformation. These multiple hits are the result of mutagens damaging potential tumor suppressor genes such as p53 and retinoblastoma gene or activation of oncogenic genes such as ras, bcl-2, myc etc. The damage caused are fixed in the genes resulting in reprogramming the growth control mechanisms by specific activation of proliferation associated genes (myc, ras, cyclin D1, EGF, EGFR etc.,) and inactivation of tumor suppressor genes (Paterson, 1996). Cells acquiring malignant potential shows increased proliferation rates and develop as a tumor, which further depends on angiogenesis for its continued growth and proliferation as well as for invasion and metastasis to distant sites.

The most common defect widely reported is the mutation in p53 gene (frequency being 82% in most of all cancers). (Sidransky, 1996). p53 mutation has been detected in 47% of oral lesions. The ras gene is the next common gene, mutated in 35% of oral cancers. Amplification of myc, ras and EGFR is also common and variations are observed among different ethnic populations (Carter, 1992).

The p53 tumor suppressor protein.

The p53 protein was first reported by two independent investigators Linzer, et.al., and Lane. et.al., (1979). Thereafter for past two decades p53 occupies a singular position among the tumor suppressor genes, inactivated with startlingly high frequency in most types of human cancers. Its sole essential function in normal cells seems to be to prevent them from developing tumors (Harris et.al., 1999 and
p53 is a 53 kilodalton phosphoprotein, the product of a 20 kilo base tumor suppressor gene located on the short arm of human chromosome 17. Wild type p53 protein blocks the division of genetically damaged cells either by suppressing growth and subsequently promoting DNA repair or forcing the cells to undergo apoptosis for permanent elimination of the damaged cells (Vogelstein, et al., 1988). p53 is also involved in cellular differentiation and development. (Almog et al., 1997). Cells carrying DNA damage are potentially neoplastic and since p53 functions to eliminate such cells it is well regarded as tumor suppressor or "guardian of the genome". (Sidransky, et al., 1996)

In the event of DNA damage, p53 causes cell cycle arrest by induction of cyclin kinase inhibitor p21 [also known as WAF1 (Wild type p53 Activated Fragment 1)]. p21 binds to cyclin kinases to block the phosphorylating events required for progression of the cell cycle (Figure 1). p53 protein is also involved in the regulation of gene expression, inducing pro-apoptotic genes and repressing the expression of antiapoptotic genes (Levine, 1977). The most interesting discovery is the direct role of p53 protein in triggering apoptosis by induction of a group of red-ox related genes and the products involved in formation of reactive oxygen species resulting in oxidative degradation of the mitochondria culminating in cell death (Polyak et al., 1997 and Wyllie, 1997). During this process bcl-2 protein is pulled out from mitochondrial membrane and bax protein gets inserted in mitochondrial membrane leading to the central co-ordinating event in apoptosis, the change in permeability transition pores located in mitochondrial membrane. This results in the collapse of
Figure 1. Functional pathways of p53 protein in blocking cell cycle

Drugs, Radiation

Transcription

Cyclin / cdk

Rb

E2F

p21

GADD 45

Cell cycle arrest

Cyclin / cdk

Rb

E2F

PCNA
mitochondrial membrane potential. This phenomenon appears to be a significant event in almost all forms of apoptosis (Marchetti, et al. 1996).

p53 structural details and mutational spectrum.

More than a thousand mutations have been detected in the p53 gene in various human tumors. Majority of such mutations are located between 100 – 300 amino acid residues, the region containing DNA binding core domain. Therefore it is obvious that mutations in these regions would affect the DNA binding ability and therefore the function of the protein. The regions shown as A-E are conserved regions among various species (Cho et al., 1994).

Figure 2: Mutation spectra in the coding regions of the p53 gene.

There are 6 ‘hotspots’ where the mutation frequency is high and as well as affect the function of protein drastically. These are Arg 248 (9.6%), Arg 273 (8.8%), Arg 175 (6.1%), Gly (6%), Arg 249 (5.6%) and Arg 282 (4%) of all the mutations.
detected in p53. Minor ‘hot spots’ are Arg 280 (2.1%), Cys 176 (1.5%), His 179 (1.9%), Cys 238 (1.8%) and Cyc 242 (1.4%). All these amino acids are involved either in DNA–protein interaction or maintain protein conformation. Therefore the consequences of mutations in these spots would lead to loss of important functions needed for tumor suppressing activity. It thus leads to perturbations in cell cycle, DNA repair and induction of apoptosis, eventually leading to malignant transformation. (Cho, et.al 1994)
APOPTOSIS. ( Programmed Cell Death )

Historical perspectives of apoptosis

Developmental biologists over decades has observed this phenomenon and referred to it as cellular senescence, anergy or programmed cell death. John Kerr (1972) and Andrew Willie (1981) characterized apoptosis and distinguished it from other modes of cell death like necrosis. The typical patterns of apoptotic behavior led them to define apoptosis as a fundamental physiological process to have the critical role in maintaining tissue homeostasis and started a new era in cancer biology. It has been now studied extensively and numerous genes regulating apoptosis and their derangement in cancer have been identified. Further prospective role of apoptosis as the primary mode of cell death in cancer treatment resulted in opening newer insights in cancer therapy. Apoptosis has now become indispensable in cancer pathology and management.

SIGNIFICANCE OF APOPTOSIS IN CANCER

In cancer biology, evaluation of growth control mechanisms has shown that cells often fail to succumb to death programme and gain immortality. Apoptosis is a distinct form of cell death that can result from activation of a genetically regulated cell suicide program or from cell injury induced by various stimuli including radiation and chemotherapy (Hannun, 1997).

The apoptotic failure is genetically determined or acquired during the development of a cancer. Possibilities of a defective programmed cell death that occurs during
malignant alterations stems from the changes in observed in tumor associated genes involved in the regulation of apoptosis (Decaudin, et al., 1997). Normal tissue homeostasis depends on balance between cell division and cell death. In cancer the cell division proceeds unopposed by cell death since the regulation of cell death and cell division are mediated by common group of interrelated factors. The presence of excess growth promoters of cell division would enhance proliferation while undermining cell death mechanisms Similarly inactivation of cell death promoters would cell division to go unchecked (Collins, et al, 1994).

**Morphological features of apoptosis.**

Programmed cell death or apoptosis, is a complex network of biochemical pathways with fine regulatory mechanism controlling death events in cell. It is now widely recognized that apoptosis is an essential and fundamental biological phenomenon occurring in various biological processes including growth, differentiation, tissue remodeling and immunological development (Bowen, 1993). Cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation are characteristic changes observed during the execution phases of apoptosis (Wyllie 1992). (Apoptotic manifestations shown in Plate 3). Apoptotic cells loose contact with neighbours, and lose specialized surface elements such as microvilli and intercellular junctions (Bowen, 1993). These changes appear to be similar but may not be common in all forms of apoptosis. The similarity is more likely due to sharing of final effectors involved in the death programme. The final effectors
1. Normal cell.  
2. Membrane blebbing.  
3. Chromatin condensation.  
4. Apoptotic fragmentation.

A. Morphological changes observed during drug induced apoptosis in Erlich's ascites carcinoma cells.

B. Apoptotic events observed in human oral tissue showing chromatin condensation, margination and fragmentation. Such fragments are rarely observed in intact tissues as fragments rapidly cleared by neighbouring cells.
altering morphology can in turn induce secondary triggers to ensure successful execution of the death programme.

Figure 3. Diagram showing apoptotic events.
Radiation and chemotherapy induces apoptosis

Various treatment approaches in cancer attempt to induce apoptosis (Dixon et al., 1997). Apoptotic resistance might lead to failure of response to treatment by tumor cells (Reed, 1999). Therefore it is essential to know about the detailed mechanisms of apoptosis as how the tumor responds during treatment and if this occurs then the option, for alternate means apoptotic pathways and exploit them for treatment.

Apoptotic machinery and apoptotic mechanisms.

Apoptosis is a process triggered when there is a threat to cellular integrity. This process is active during development and continues throughout life and is involved in the elimination of superfluous or damaged cells to maintain tissue morphology and size. Net loss of cells by apoptosis per year on an average is equal to our weight (Reed, 1999). The mere dependence on extracellular survival signals by the cells suggests that this death by default mechanism might ensure that cell survives only when and where it is required (Collins, et al., 1994). All nucleated cells except blastomeres depends on survival signals to defend against execution of the death programme. It is now established that apoptotic machinery is constitutively expressed and the survival signals suppress the activation cascade (Weil, et al., 1996). The sequence of events involved apoptotic commitment appears to begin from cytoplasm and gradually spread to plasma membrane and nucleus. The earliest event known to occur at the onset of apoptosis is loss of mitochondrial membrane potential, leakage of mitochondrial contents leading to decrease in
intracellular pH, vacuolation and dilation of cytoplasmic organelles giving a transient blistered appearance to cytoplasm (Bowen, 1993).

**Actions of apoptotic enzymes results in manifestation of characteristic features of apoptosis**

Proteases, transglutaminases and nucleases are the major enzymes involved in execution of apoptosis. Signals arising from drug treatment, radiation, cytokines, hormonal treatment, withdrawal of survival signals and death signals from mitochondria enters final common pathway to convert a group of inactive proteases, known as procaspases to active caspases (Collins, et.al 1994). Caspases are so called since they have cysteine in their active site and have substrate specificity by cleaving residues next to aspartic acid. These proteases are present in large in numbers, preformed and stored as (30 kD - 50 kD) proenzymes, which upon activation causes specific and irreversible proteolysis. The proteolytic action further amplifies the activation of caspases by autocatalysis. The functions of caspases are regulated by feedback mechanisms, and also by co-existing inhibitors of caspases. Mitochondrial release of cytochrome C serves as important signal to activate caspases (Thornberry and Lazebnik, 1998).

Radiation, drug and cytokine act through specific receptors. Some of the well-characterized death receptors are CD95/Fas/Apo1or Tumor Necrosis Factor Receptor family TNFR1/p55/CD20a, DR3/Apo3, DR4 and DR5/Apo2/Killer. The distributions of these receptors vary among different cell types. These receptors
interact with a cytoplasmic homolog known as ‘death effector domains’ (DED), which has direct control over the apoptotic machinery. DED proteins also respond to cytotoxic drugs regardless of the requirement of any receptors (Ashkenazi and Dixit, 1998). Signal transducers also respond to death receptors by releasing ceramide through spingomyelinase pathway resulting in activation of Stress activated Protein Kinase C / J N K, which in down stream activates the caspases. Ceramide is also a potent apoptogenic factor capable of disrupting the electron transport chain of mitochondria (Zanke, et.al 1998).

Activated caspases induce DNA fragmentation, chromatin condensation, blocking of synthesis and repair mechanism leading to the disruption of nuclear architecture (De Murica, et.al., 1997, Ding 1994, Rao, et.al 1996). Gross alterations in cytoskeletal proteins occur as the result of action of proteases producing characteristic morphological features visualized in the apoptotic cells (Kondo, et.al 1997, Brancolini, et.al., 1997 and Mills, et.al., 1998). Interleukin 1 beta converting enzyme (ICE protease) and Apoptosis Inducing Factor affects the function of aminophospholipid translocase and influence lipid scramblase activity leading to loss of membrane asymmetry and exposes phosphatidyl serine in the outer leaflet of the plasma membrane resulting in membrane blebbing (Naito, et.al, 1997). The presence of phosphatidyl serine in the outer leaflet serves as a signal to promote the clearance of apoptotic cells. Lamins, the structural proteins present in the nucleus are also modified by the proteases (Rao, et.al., 1996).
DNA Fragmentation Factor activated by caspases causes chromatin condensation and DNA fragmentation (Liu, et al., 1998). Cleavage of repair enzyme Poly ADP Ribose Polymerase (PARP) by protease results in loss of catalytic domains. The DNA binding domain of PARP binds to broken ends of DNA and remains attached without any function and blocks the access by other DNA repair enzymes (Smulson, et al., 1998). DNA fragmentation pattern appears as large fragments and subsequently in many but not in all forms of apoptosis into oligonucleosomal sized fragments of 180 base pair and multiples of 180 base pair in different forms of apoptosis (He, et al., 1998). The nuclear fragments are found enclosed within membrane bound apoptotic bodies dispersed at late stages of apoptosis. These apoptotic bodies are rapidly cleared by adjacent cells and degraded within secondary lysosomes. The concerted damaging events drives the cells to 'a point of no return' forcing the cells to accept death.

Mitochondria, which is the cross road for several metabolic pathways is where various regulatory signals converge plays central role in apoptosis. Mitochondria also plays vital role in regulating cell division. Cancer cell expressing antiapoptotic proteins such as bcl-2 or cytokine response modifier A (crmA) are mostly targeted to protect mitochondria and is a wise attempt to prevent damages to mitochondria, which would otherwise results in irreparable and irreversible damage to cell. Maintenance of mitochondrial membrane integrity appears to be essential to escape or delay apoptosis (Zamzami, et al., 1996 and Green, et al., 1998). Mitochondria is essential for continued supply of energy to repair damaged targets.
and under extreme conditions of failure to repair should possibly induce stress on mitochondria resulting induction of cell death.

The mitochondrial regulation of apoptosis is gaining sharp attention since the discovery of the role of p53 and bcl-2 group of proteins. In this aspect and pathways independent to p53 also appear to converge on mitochondria (Heedt, et.al., 1998). It could be therefore be assumed that hurdles in signaling mitochondrial death is more likely to contribute apoptosis resistance in cells.

The genetic regulation of apoptosis

Mitochondrial death appears to be the more perfect and dynamic attempt to induce efficient apoptosis. Results from findings such as the ability of mitochondria to transfer apoptosis from apoptotic cells to a cell free system (Liu, et.al., 1996) and the ability of mitochondria to induce apoptosis in the non nucleated cells substantiates that mitochondria is central to apoptosis (Zamzami, et.al., 1996). In nucleated cells, mitochondrial death precedes nuclear or cellular death. The possible mechanism being the release of apoptogenic factors, dissipation of metabolic fuels causing gross alterations in the intracellular cellular pH (Zanke, et.al., 1998), release of dATP the universal currency for energy in large excess into cytosol to meet the energy demands of cell death machinery aiding destruction to the last (Eguchi, et al., 1997). The role of p53 protein and bcl-2 group of proteins in regulating mitochondria mediated apoptosis is beginning to be understood. The role of other signals from different pathways converging on mitochondria is currently
under investigation. Some of the recent developments in these aspects are discussed in following sections.

**Role of p53 and Bcl-2 group of proteins**

The wild type p53 protein induces the expression of apoptosis promoting proteins such as bax and also represses the expression of apoptosis inhibitors such as the bcl-2 protein. In response to DNA damage p53 up-regulates apoptosis associated receptors such as DR 5. (Wu et.al., 1997). Inactivation or mutation of the p53 gene may therefore result in over expression of bcl-2 and down regulation of bax leading to defective apoptosis. Loss of control over the cell cycle and cell death can also lead to increased tumor proliferation rates. The analysis of p53 under these aspects would possibly provide clues regarding apoptotic status of a cell. It is therefore imperative to understand as to how these proteins carry out such tasks in a cell.

**Bcl-2 family proteins:** A group of structurally related genes identified as Bcl-2 family codes for protein with contrasting functions. There are anti-apoptotic proteins like Bcl-2, Bcl-XL, Mcl-1 etc., and also pro-apoptotic proteins like Bax, Bad, Bid etc.,. The functions of Bcl-2 group proteins are regulated by phosphorylation mechanisms and they also interact with each other to regulate apoptosis besides direct involvement in apoptosis (Jacobson 1997). The important anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins are discussed below.
The bcl-2 proto-oncogene is identified as the site of reciprocal translocation human chromosome 18 in follicular lymphoma (Ngan et al., 1988). The gene encodes for membrane protein found distributed in outer mitochondrial membrane, endoplasmic reticulam and nuclear membrane (He, et al., 1997). Bcl-2 is widely expressed during embryonic development, but in adults is confined to developing immature cells, resting B cells and stem cells of the immune system. Bcl-2 confers resistance to death in these cells. In malignant cells over-expression of Bcl-2 has been found to block apoptosis, thus interfering with treatment (Yang, et al., 1997).

The main anti-apoptotic function of bcl-2 protein is to protect the mitochondrial membrane integrity by blocking the alterations in permeability transition pores and blocking the release of apoptotic mediators like Apoptosis Inducing Factor (AIF) protease, cytochrome C, dATP and calcium ions. Other possible associated changes are the discharge of acidic metabolic intermediates decreasing intracellular pH favoring the action of acidic endonuclease. Cytochrome C initiates the caspase cascade by activating caspase 9. AIF has been found to exist in sufficient quantities and does not require de novo synthesis at the onset of apoptosis. AIF activates the preformed nuclear DNases to induce DNA fragmentation. Both these actions involved in apoptosis do not require de novo protein synthesis, which is in contrast to earlier reports on the requirement of de novo protein synthesis being considered essential for executing the death programme (Susin, et al., 1996). The protein synthesis inhibitor cycloheximide and transcription inhibitor actinomycin D are found to induce apoptosis. Thus it is clear
that cell death proteins are constitutively expressed and their functions remain suppressed by survival signals (Weil, et.al., 1996). It has been well established that all nucleated mammalian cells except blastomeres depend on survival signals from other cells to protect themselves from being executed by an internal and ever existing cell death programme (Weil, et.al., 1996). The role of bcl-2 protein localized on endoplasmic recticulum has been found to prevent the efflux of calcium ions even if the extracellular calcium levels are depleted. Endoplasmic recticulum is a major organelle involved in the synthesis of protein, packing and sorting. Dilation of endoplasmic recticulum has been observed at the onset of apoptosis associated with the release of calcium ions. Bcl-2 protein plays no role in protecting the nucleus at the onset of apoptosis (He, et.al., 1996). Thus the major target for bcl-2 function appears to protect mitochondria.

Bax belongs to bcl-2 family of protein, but is pro-apoptotic in function. The expression of bax protein is inducible by p53 protein. Though bcl-2 and bax protein forms heterodimers, it now appears that they function in an independent manner (Knudson, et.al., 1997). This finding is further substantiated by discovery of movement of bax from cytosol to mitochondria at the onset of apoptosis. Bcl-2 protein found to be localized in outer membrane of mitochondria, membranes of endoplasmic recticulum and nucleus. Localization of bax protein analyzed by tagging the bax protein with Green Fluorescence Protein (GFP) shows that bax is found dissolved in cytoplasm. The levels showed no marked changes with increase in co-expression of bcl-2 or bcl-XL protein. Upon induction of apoptosis, Bax-GFP
showed a dramatic movement to a punctate distribution that is partially localized to mitochondria. Bax protein, similar to that of perforins forms channels facilitating the release of mitochondrial contents (Marzo, et.al., 1998). The movement of bax from cytosol to mitochondria occurs prior to cell shrinkage or nuclear condensation. Bax has been found to associate with transition pore complex and found to co-operate adenine nucleotide transporter to trigger cell death (Wolter, et.al., 1997).

Other regulators of apoptosis

Mitochondrial membrane potential disruption is found to be an obligatory step in early (pre-nuclear) apoptosis (Wyllie, 1992). In p53 independent apoptotic pathways too the status of mitochondrial membrane is critical (Heedt, et.al., 1998). Apoptosis inducing agents like doxorubicin, etoposide, and ceramide in the presence of mitochondrial membrane stabilizing agents like, N-benzyloxy carbonyl-val-ala-asp fluormethyl ketone (Z-VAD.fmk) and bonkrekic acid fails to induce apoptosis. Ceramide is a second messenger in sphigomylinase-mediated pathway of radiation, fas mediated and TNFR mediated pathways of apoptosis (Friedman, et.al., 1996). Cytokine response modifier A (crmA) over expression prevents to disruption of mitochondrial membrane protein; this essentially leads to apoptosis resistance in fas mediated apoptotic cascade.
Tumor Angiogenesis

Angiogenesis or neovascularisation, the process involved in the growth of new blood vessels is an important requirement for several biological processes, including embryonic development, wound healing and chronic inflammation (Lingen, 1999). Angiogenesis is a prerequisite for successful establishment, growth and dissemination of tumors. It was first demonstrated by Folkman (1985) and his group showed that angiogenesis is a part of tumor evolution consisting of two distinct phases: prevascular and vascular phase. Tumor cells remain dormant in a prevascular phase and they induce angiogenic mediators thereby switching over to a relatively vascular (angiogenic) phenotype (Folkman, 1992). (Events during tumor angiogenesis shown in Figure 4).

Figure 4. Tumor neovascularisation.
This leads to rapid expansion of the tumor cells gaining adequate nutrients, increased propensity for distant metastasis (Folkman, 1992). Tumors possess the ability to induce proliferation of proximal capillaries, which arise from small venules in response to angiogenic stimulus imparted by the tumor. Initially, local dissolution of the basement membrane occurs, possibly caused by proteinases synthesized and released by endothelial cells. This is followed by migration of endothelial cells towards the source of angiogenic factors, as sprouts and they align to form a lumen. The angiogenic factors identified to be secreted by the tumors are Vascular Endothelial Growth Factor, Transforming Growth Factor Beta, Fibroblast Growth Factors etc., (Brown, et.al., 1998)

Recent studies on several different tumors by Holash and his group (1999) challenge the prevailing view that malignancies and metastases generally are initiated as avascular masses that only belatedly induce vascular support. Instead, they report that malignant cells rapidly co-opt existing host vessels to form an initially well-vascularized tumor mass. Paradoxically, the co-opted vasculature does not undergo angiogenesis to support the growing tumor, but instead regresses (perhaps as part of a normal host defense mechanism) via a process that involves disruption of basement membrane and endothelial cell apoptosis. This vessel regression in turn results in necrosis within the central part of the tumor. However, robust angiogenesis is initiated at the tumor margin, rescuing the surviving tumor and supporting further growth. Angiopoietin-2 (the
natural antagonist for the angiogenic Tie2 receptor) and vascular endothelial growth factor (VEGF) strongly implicates these factors in the above processes.

The tumor suppressor gene p53, which controls the progression of cell cycle and apoptosis has been found to inhibit angiogenesis as well. Angiogenic inhibition by p53 is mediated by inhibition of the expression of basic FGF and or down regulating thrombospondin 1 expression (TSP-1). TSP-1 is required for cell adhesion, motility and has been found to inhibit tumor growth and metastasis. Expression of bFGF is also regulated by TSP-1. The link between p53 mutation and angiogenesis favours proliferation potential shifting tissue kinetics towards tumor progression (Dameron, et.al 1994).
Tumor proliferation compartment

Proliferation compartment refers to the group of cells undergoing cell division, which maintains steady state equilibrium with cells dying at the given time. Oncogenes and growth factors are involved in the regulation of cell cycle. In cancer cells these genes are mutated leading to loss of control over the brakes of cell cycle thereby favours the function of promoters of cell cycle (Jiang, et.al., 1993). Mutations also lead to over-expression of such positive regulators of the cell cycle.

The Cell cycle and Cyclins.
Cell cycle consists of sequence of events during the cell division and classified as distinct phases described below.

**G1** (Gap 1) First growth phase passes through restriction point R approximately 2 hours before the end of G1.

**S** DNA synthesis and chromosomal doubling.

**G2** Second growth phase.

**M** Mitotic phase, sub-divided as follows.

- **Prophase** Chromatin condensation, disruption of nucleoli, centrioles constructed and dissolution of lamina.
- **Metaphase** Completion of spindle and chromosomes attached to kinetochore.
- **Anaphase** Separation of chromatids.
- **Telophase** Chromosome uncoils, reappearance of nuclear envelope, cleavage furrow appears at the equator, constricts to give two daughter cells.

The timing and the chronological orders of events in cell cycle is determined by host of proteins known as 'cyclins,' without which cell division cannot take place. The cyclins were first described by Ewans et al. (1983) as 50-60 kD proteins with varying concentrations at various phases of the cell cycle. The cyclins and their role in cell cycle are described below.

**Cyclin A** is an S phase protein, coincides with DNA synthesis.

**Cyclin B** (1-3) appears between G2/M phases and is essential for mitosis.

**Cyclin C** appears in mid G1 phase.

**Cyclin D** (D1-D3), group of nuclear proteins appears in mid G1 phase with an half-life of 38 minutes and becomes cytoplasmic at the end of S phase.
The other cyclins discovered so far include cyclin E, F, G and H. The functions of these proteins are not clearly understood.

Alteration in the levels of cell cycle proteins such as amplification or over-expression leads to increased proliferation as observed in tumors. Cyclin D known as BCL-1 was first identified in the break points on chromosome 11q13 in B lymphocyte tumors with translocation t(11:14). Amplification and over-expression of cyclin D1 is frequent among 25-48% of head and neck cancers. (Bartkova, et.al, 1995). Increased levels of cyclin D1 result in accelerated G1 phase, without DNA repair or completion of replication. The cyclin D1 pathway of induction of proliferation is shown below in Figure 5.

Figure 5. Induction of Proliferation mediated by cyclin D1.
Cyclin D1 exists as a complex consisting of cell division kinases, retinoblastoma protein and E2F transcription factor. Activation of cyclin D1 results in phosphorylation of rb protein and release of E2F protein, which in turn induce gene expression. In cancer over-expression of cyclin D1 is found to be associated with tumor progression and a major link between oncogenes and cyclins was established from the studies on cyclin D1. (Jiang et.al., 1993). The induction of G1 by cyclin D1 can be blocked by wild type p53 protein, therefore in order to analyze the extent of proliferation in the absence of wild type p53 protein, cyclin D1 was analyzed in this study.
p53, apoptosis and angiogenesis.

Thus from this detailed discussion it is evident that p53 play a central role a tumor suppressor by arresting proliferation or enhancing apoptosis and blocking growth of new blood vessels creating an unfavorable environment for the tumor growth, multiplication and its spread. Considering this background and rationale, the present study examines p53 as the mediator of treatment, along with apoptosis and angiogenesis as a function of treatment response. The ratio of proliferation and apoptosis determines the tumor progression and the proliferative potential depends on angiogenesis enhancing progression during its natural course. On the other hand, angiogenesis offers advantage during treatment by increasing the access of drugs or production of free radicals thus aiding tumor destruction by inducing apoptosis. The dual role of angiogenesis influencing apoptosis and other factors needs to be analyzed in order to understand the functional relations both during tumor progression as well as response to treatment. The tri-functional roles of wild type and mutant p53 protein summarized below. Figure 6.