Chapter-I

Introduction & Review of literature
1. Introduction

The term 'cancer' represents a range of different type of malignant diseases; all of them exhibit uncontrolled cell division, tissue invasion and spreading as common characteristics. Cancer has been considered to be very dreadful disease since the time when man started to think over therapeutics and is a major public health concern due to its high incidence and life-threatening nature.

Cancer continues to be a global killer, even though enormous amount of research and rapid developments seen during the past decade. Epidemiological data so far shows a significant decrease in the death rate due to heart and other diseases however cancer related mortality rate has remained unaffected since 1950. According to World Health Organization (W.H.O., 2004), cancer is the leading cause of death among fatal diseases, next to cardiovascular diseases, in the industrialized countries and second fatal disease in developing word. In 2008, about 12.7 million cancer cases and 7.6 million cancer deaths have occurred throughout the globe (Jemal et al., 2011). Global human population is expected to reach up to 7.5 billion by 2020. Out of these, about 15 million new cancer cases will be diagnosed, and approximately 12 million cancer patients will die (Bray and Moller, 2006). Although, overall incidence rate of cancer in developing countries is half to that of the developed world, but it is increasing rapidly as a result of population aging and growth as well as an adoption of cancer-associated lifestyle choices. In the past decade 19 % increase in the rate of cancer incidence has been observed, most of which was associated to cases in developing countries (Stewart and Kleihues, 2003). It is expected that by the 2020 about 70% of all the new cases of cancer diagnosed, will be from developing world (Jones, 2003). A wide range of risk factors have been associated with the cancer development (Fig.1). In general, major cancer risk factors includes, smoking and chewing of tobacco, eating habits, lifestyle, living or working environments and infectious agents. Epidemiological studies have shown that 70 to 90 % of human
cancers are usually related to environmental factors and life style (Doll and Peto, 1981; Doll 1998; Higginson and Muir, 1977). A carcinogen can be the physical, chemical or biological agent.

Fig. 1. Major risk factors for cancer development

2. The Skin

Skin is the largest organ in the human body and primarily acts as a protective barrier between the body and the external environment. It serves as the first source of protection against physical, biological, chemical and mechanical damage, such as harmful radiation, abrasions, wounds, microbial infection and dehydration. The human skin is both a physical and biochemical barrier to the absorption and penetration of potentially damage-causing compounds of the environment.

2.1 Structure

The skin is divided into two main layers, the outer epidermis and the inner dermis separated by a basement membrane (Fig. 2).

Epidermis

The epidermis is the outermost layer of the skin and provides most of the skin’s barrier functions. It consists of five layers, the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and the stratum basale. Specific cell types, such as keratinocytes, melanocytes (melanin producing cells), Langerhans cells (antigen detecting,
processing and presenting cells), and Merkel cells (neuroendocrine cells) are present in epidermis.

**Dermis**

It is found below the epidermis and is composed of a tough, supportive cell matrix. The dermis is attached to the epidermal stratum basale by a basement membrane and consists of mainly extracellular matrix of collagen and elastin fibres. Cells, such as fibroblasts, mast cells, lymphocytes and macrophages are present in the dermis. Nerves, glands, blood vessels and lymph vessels are situated in the lower dermis region. The hypodermis situated below the dermis consists of adipose tissue and contains adipocyte cells, which produce fatty acids and triglycerides. The hypodermis is an important part for thermoregulation, energy store and a buffer against mechanical injury.

![Structure of skin](image)

**Fig.2. Structure of skin**

**2.2 Skin cancer**

Skin cancer (including melanoma, basal and squamous cell carcinoma) is the most common type of human cancer and its incidence is increasing at an astonishing rate. Skin tumors in humans represent about 30% of all new cancers reported annually (Fguard et
al., 2003). About 3.5 million skin cancer cases are diagnosed each year (Rogers et al., 2010; Stewart and Kleinhues, 2003). Furthermore, it is estimated that one in five Americans will develop skin cancer in their life time (Robinson, 2005). The American Cancer Society estimated that 76,250 men and women will be diagnosed with melanoma and 12,190 men and women will die (9,180 from melanoma and 3,010 from other non-epithelial skin cancers) in the United States in 2012 (American Cancer Society, 2012). The World Health Organization estimates that as many as 65,161 people a year worldwide die from too much sun exposure, mostly from malignant skin cancer.

In white populations, epidemiological studies have revealed a correlation between an increase of skin cancer incidence and exposure to UV radiation. In fact, this increase was predominantly recorded in Caucasians living near the equator. Recent data assess the incidence of non melanoma skin cancer in the U.S. at about 232/100,000, whereas, in Queensland (Australia) numbers as high as 2398/100,000 males and 1908/100,000 females have been reported (Schaart et al., 2003). After Australia and New Zealand, Denmark has the highest incidence of melanoma skin cancer (Krarup et al., 2011).

Skin cancer places a huge burden on health care services due to the high incidence and cost of treatment. It is a major public health problem in many countries, as considerable amounts of money and resources are wasted on a disease, which is preventable by employing simple personal sun protection strategies. For example, it was estimated in 2002 that in England, the total costs contributed by skin cancer was over £190 million and approximately a third of a dermatologist’s and a plastic surgeon’s work load is accounted for by just skin cancer care alone (Hiom, 2006). In the United States of America (USA), non-melanoma skin cancer is reported to be a substantial burden on the health care system and it is estimated that melanoma treatment will cost approximately $5 billion annually by 2010 (Housman et al., 2003). Skin cancer is Australia’s most costly
cancer and results in over $300 million in expenses for the healthcare system every year (International Union Against Cancer, 2006).

2.2.1 Types of skin cancer

Melanoma and non-melanoma are the two major types of skin cancer. The non-melanoma includes basal cell carcinoma (BCC) and squamous cell carcinoma (SCC).

(i) Basal cell carcinoma (BCC)

BCCs are slow growing, locally invasive malignant epidermal skin tumors. It is the most common type of skin cancer worldwide and accounts for more than 70% of all non-melanoma skin cancers (Fig. 3a) (Boi et al., 2003). Usually looks like a raised, smooth, pearly bump on the sun exposed areas of skin, such as the face, head and neck. Metastasis is relatively rare, only occurring in 0.0028–0.5% of cases and rarely cause death (Vu and Laub, 2011).

(ii) Squamous cell carcinoma (SCC)

The second most common skin cancer worldwide, accounting for 22% of all non-melanoma skin cancers is SCC (Fig. 3b) (Boi et al., 2003). This type of skin cancer develops from keratinocytes in the epidermis and usually occurs on the head, neck and hands. It is commonly a red, scaling, thickened patch on sun exposed skin. It is locally invasive and has the potential to metastasize to other body organs and cause death.
(iii) Melanoma

Cutaneous malignant melanoma (Fig. 3 c) accounts for approximately 9% of all skin cancers (Boi et al., 2003) and is the third most common cancer worldwide (Mqoqi et al., 2003). It is the most rapidly increasing cancer in white populations. It is the sixth most common fatal malignancy in the United States, responsible for 4% of all cancer deaths and 6 of every 7 skin cancer-related deaths (Losina et al., 2007). Melanoma arises from melanocytes that invade the dermis, and can develop from precursor lesions and directly from the skin. Melanoma is an aggressive cancer that metastasizes quickly to organs, such as the brain, lung, liver, intestine and other skin sites with increased mortality risk. Generally occur on sun-exposed areas like head and neck, but also on less exposed areas like the trunk and legs.

Other types of skin cancer include cutaneous T-cell lymphoma, Kaposi carcinoma, Merkel cell carcinoma and sebaceous carcinoma.

2.2.2 Skin cancer risk factors

According to American Cancer Society, the risk factor for human skin cancer includes ultraviolet radiation, types of exposure, sunbeds, skin type, hair and eye color, sunscreen, vitamin D, environmental pollutants, chronic skin injury or inflammation, occupational exposure to coal tar, pitch, creosite, a past history of basal cell or squamous cell skin cancers and arsenic compounds (de Gruijl et al., 2003; Berwick et al., 2005; Leiter and Garbe, 2008). Established risk factors for skin cancer include exposure to solar ultraviolet radiation, white race, and advancing age (Armstrong and Kricker, 2001; Cho et al., 2005). Immunosuppression also increases the risk of certain skin cancers. The risk in solid organ transplant recipients and people with human immunodeficiency virus (HIV) infection is extremely high for Kaposi’s sarcoma (KS), to some extent elevated for cutaneous non-Hodgkin’s lymphoma (NHL), Merkel cell carcinoma, appendageal skin carcinomas and melanoma (Lanoy et al., 2010).
3. Carcinogenesis

The concept of multi-stage carcinogenesis was first proposed by Berenblum and Schubik in 1948 and supported by later studies. Development of cancer is a complex and multi-step process viz., initiation, promotion and progression (Fig.4). Development of cancer is usually accompanied by changes in the central genomic information coded in the DNA leading to activation of dominantly acting oncogenes and inactivation of tumor-suppressor genes implicated in regulatory circuits governing cell proliferation, differentiation and homeostasis.

Initiation phase of cancer development involves modification of genes controlling cellular homeostasis. It essentially requires irreversible changes in appropriate target somatic cells. This is considered to be the first step in carcinogenesis, where the cellular genome undergoes mutations, creating the potential for neoplastic development, which predisposes the affected cell and its progeny to subsequent neoplastic transformation.

![Diagram of carcinogenesis](image)

Fig.4. An overview of carcinogenesis

Promotion is the event in which the initiated cells become expanded into different phenotypic outgrowths like papillomas, nodules, or polyps, which may develop into cancer. It is a long-term, 15 to 30 years process and requires continuous exposure to a promoter or promoters. During promotion, proliferation and clonal expansion of initiated cells occurs and finally benign mass of abnormal cells called tumor develops.
Conversion of benign papillomas into malignant carcinomas is known as tumor progression. Progression is another important step in which further genetic and epigenetic changes occur and lead to development of characteristic phenotypes, such as invasiveness metastasis and angiogenesis.

3.1 Chemically induced two-stage mouse skin carcinogenesis-A complex and multi-step process

So far, different types of animal models of cancer have been developed to understand the cellular and molecular alterations associated with the process of carcinogenesis. Chemically induced two-stage mouse skin carcinogenesis represents one of the well-established *in vivo* models for study the sequential and step wise development of tumors. Furthermore, this model can be use for novel cancer prevention strategies and therapeutic concepts for human epithelial neoplasia. This is the most thoroughly studied mouse cancer model and has been used to test a large number of hypothesis in almost all fields of cancer biology and genetics, including DNA repair, signaling, immunology and cell biology (Kemp, 2005; DiGiovanni, 1992).

This is a well described and proven model for studying the evolution of tumors (e.g., papillomas, keratoacanthomas and squamous cell carcinomas) in mouse skin (Abel et al., 2009; Kemp, 2005; Perez-Losada and Balmain, 2003; Quintanilla et al., 1986). It involves treatment of the dorsal mouse skin with an initiating dose of DMBA (7, 12-dimethylbenz[a]anthracene) as a single application typically, followed by multiple applications of tumor promoter TPA (12-O-tetra-decanoylphorbol-13-acetate) (Abel et al., 2009; Roe et al., 1972) over several months (Fig.5). This results to the formation of hyperplasic epidermis, stroma and outgrowths of pre-malignant papillomas. With continued TPA application, these lesions enlarge and a small percentage of papillomas transform into malignant invasive squamous cell carcinomas.
Two-stage skin carcinogenesis protocols have been designed to study skin tumors in mice, such as tumor incidence, latency, multiplicity and progression. It has several advantages like:

(i) Initiation and promotion stages can be distinctly separated both operationally and mechanistically.

(ii) Tumor development can be easily examined visually throughout the study.

(iii) Efficacy of chemopreventive agents can be assessed easily. By using genetically engineered mouse models (GEMMs), role of different genes and signal transduction pathways can be elucidated.

(iv) We can understand the molecular mechanisms involved in all the three stages of carcinogenesis through the evaluation of specific short-term markers.

(v) This model serves as a useful model of human cancers of epithelial origin (Kemp, 2005; Abel et al., 2009).

Fig. 5. Chemically induced two-stage skin carcinogenesis. Source, Abel et al., 2009.
3.1.1 Initiation

The initiation stage is essentially an irreversible step in which genetic changes occur in genes, controlling differentiation (Boutwell, 1982). Initiation can be done by the topical application of a single sub-carcinogenic dose of DMBA. Initiation only requires a small amount of time and single dose of carcinogens and may result from mutation, translocation, and/or amplification of target cells (Mukhtar et al., 1989; Sun, 1990). Development of neoplasm may be the result of chemical or biological insults to normal cells through multistep processes that involves genomic changes. It starts when normal cells are exposed to carcinogenic substances and their DNA undergoes damage that remains unrepaired or misread. In chemical carcinogenesis, initiation involves the uptake of a given carcinogen, which is subsequently distributed to organs for metabolism. Metabolic activation leads to reactive (electrophilic) species, which can bind to DNA.

A large number of chemicals have been identified as initiating agents for carcinogenesis in animal model systems. The initiating agents for murine skin include polynuclear aromatic hydrocarbons (PAHs), nitrosamines, nitrosamide, aromatic amines and various alkylating agents (Slaga, 1985; Boutwell, 1964; Yuspa, 1986). Humans are exposed to some of these agents through, polluted air, cigarette smoke, automobile exhaust, drugs, diet and other air-borne pollutants (Mukhtar et al., 1989). Some of Polycyclic aromatic hydrocarbons (PAHs) are the common environmental pollutants and known known to have carcinogenic and mutagenic effects (Singer and Grunberger, 1983). The PAHs themselves are relatively inert, biologically and essentially act as pre-carcinogens that must first undergo metabolic activation by the cytochrome P-450 dependent enzymes to their biologically active ultimate carcinogenic metabolites and manifest their mutagenic and carcinogenic effects (Conney, 1982). Cytochrome P-450 is the major enzyme system responsible for metabolism of carcinogen to their DNA binding metabolites, which is considered essential for tumor initiation (Das et al., 1986; 1987). The DNA binding can
then cause coding errors at the time of replication leading to mutation. The somatic mutation in a damaged cell can be reproduced during mitosis to produce clones of mutated cells.

It has been well known for long time that skin tumors (e.g., papillomas, Keratoacanthomas, and squamous cell carcinomas) can be induced by the sequential application of a sub threshold dose of a carcinogen followed by repetitive treatment with a non-carcinogenic tumor promoter (Slaga and Nesnow, 1985). Electrophilic reactants formed spontaneously or after metabolic activation interact covalently with and damage critical macromolecules especially epidermal DNA, to initiate skin carcinogenesis (Mukhtar et al., 1989).

3.1.2 Promotion

During promotion, proliferation and clonal expansion of initiated cells occurs and finally benign mass (papillomas, nodules, or polyps) of abnormal cells called a tumor develops (Yuspa, 2000). In this stage two important events take place one is that clonal expansion of the initiated cells and second is the genomic instability to these multiplying cells by progressive DNA damage (Slaga, 1985; Weinstein, et al., 1984; Diamond, 1987; Rundhaug, 2010). Genomic instability is responsible for the development of all the requisites needed to become invasive neoplastic cells. Tumor promoters can be defined as compounds which have very weak or no carcinogenic activity when tested alone but clearly enhance tumor growth when applied again and again following a low or suboptimal dose of a carcinogen (initiator) (Slaga, 1983 ). Promoters include various agents, such as drugs, plant products and hormones that do not directly interact with host cellular DNA but somehow influence the expression of genetic information encoded in the cellular DNA (Slaga, 1983; Pelling and Slaga, 1985; Rundhaug, 2010). Multiple molecular mechanisms are involved in the promotion of skin carcinogenesis. Induction of constant cell proliferation and hyperplasia is one of the prime features of
this stage. Activation of growth signaling pathways via direct or indirect (like chronic inflammation and oxidative stress) means, is required for the clonal expansion of initiated cells with DNA mutations to form tumors (Rundhaug, 2010). The growth stimulating properties of the tumor promoters is thought to be mediated via oxidative stress and inflammation, an environment, which is not favourable for normal cells (Yoshimura, 2006; Nishigori et al., 2004; Balkwill, 2005). Promoters can also induce the synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, alter cell morphology and induce epigenetic effects that promote carcinogenesis by altering gene expression (Rundhaug, 2010). Thus, tumor promoters usually interact at the cell’s surface with specific receptors or other cell components that elicit several processes/responses, including enhanced DNA synthesis, increased production of eicosanoids, cytokines, growth factors, over production of free radicals and alterations in cell surface properties leading to changes in cell adhesion and cell-cell communication.

TPA is the most potent and most widely used distinguished skin tumor promoting agent to understand the biochemical, cellular and molecular alterations associated with promotion stage as it activate a series of protein kinase C (PKC) iso-enzymes and induce a pleiotropic tissue response encompassing a strong inflammatory reaction. Topical application of TPA on the mouse skin is one of the well recognized models for the induction of oxidative stress, ROS production, cutaneous inflammation and hyperplasia (Nakadate, 1989; Ha et al., 2006; Riehl et al., 2010; Clark et al., 1985). It is believed that TPA acts indirectly through pro-oxidant generation which ultimately leads to genomic instability. It also causes a decrease in the activity of various antioxidant and phase II detoxifying enzymes while it increases the activity of xanthine oxidase, peroxidation of lipids and generation of hydrogen peroxide in the skin tissue.

The topical application of phorbol ester TPA to mouse skin or its treatment in certain epidermal cells result in biochemical alterations, inflammation, changes in cellular
functions and histological alterations (Rundhaug, 2010; Perchellet and Perchellet, 1988; Ha et al., 2006). Histological alterations, which include induction of dark basal keratinocytes and dermal infiltration of polymorphonuclear leukocytes, in addition to inflammation and hyperplasia are believed to be important events in the process of TPA induced skin tumor promotion (Ha et al., 2006; Mukhtar et al., 1989). The induction of inflammation in skin induced by TPA is believed to be governed by number of cellular and molecular inflammatory mediators, such as infiltration of leucocytes, production of proinflammatory cytokines like TNF-α, IL-6, IL1-β, elevation of cycloxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) activity and activation of the ubiquitous redox sensitive transcription factor nuclear factor-kappa B (NF-kB) (Marks and Furstenberger, 1995). The most important biochemical response to the brief exposure to TPA is induction of ODC activity which catalyzes the first step in biosynthesis of polyamines (Slaga, 1984). High levels of polyamines enhance cell proliferation, reduce apoptosis, induce angiogenesis and the expression of genes involved in invasion and metastasis (Gerner and Meyskens, 2004). Such an assumption is strongly supported by the studies showing that inhibitors of these metabolic pathways inhibit the tumor promotion (Gerner and Meyskens, 2004).

PKC is a receptor of phorbol ester, and tumor promotion by these agents is mediated by their binding with PKC receptors (Blumberg, 1988; Droms and Malkinson, 1991). TPA activates PKC in vivo in the presence of calcium and phospholipid, forming a part of the signal transducing system. Several lines of evidences have clearly established that activation of phospholipase C by external stimuli generates two separate arms of secondary messengers, diacylglycerol and inositol triphosphate (Perchellet et al., 1990; Blumberg, 1988). Diacylglycerol is endogenous activators of protein kinase C. TPA binds to protein kinase C at the same site where diacylglycerol binds to it. The activation of protein kinase C, phosphorylates various growth factor receptors which then receive
unscheduled signals leading to the enhanced synthesis of DNA along with the amplified expression of oncogenes.

Tumor promotion in mouse skin can be divided into at least two stages; stage I (conversion) and phase II (propagation). Stage I is characterized by the histopathological manifestation like induction of dark basal keratinocytes, synthesis of prostaglandins and activation of growth factors like TGFα (Slaga et al., 1982). The agents capable of producing such effects are known as stage I promoters. In the stage I of tumor promotion, a phorbol ester (TPA) binds to membrane receptor and one to four applications of TPA are sufficient to trigger the stage I. Stage II involves biochemical changes like induction of ODC activity, activation of PKC, elevation in polyamines, enhanced DNA synthesis and sustained cellular proliferation (Slaga, 1983; Slaga et al., 1982). The agents capable of producing such effects are known as stage II promoters.

TPA is a complete tumor promoter. The best example of stage II promoter is mezerein which is as potent as TPA in inducing ODC activity and enhancing DNA synthesis in mouse epidermis (Slaga, 1983). Other evidence for the existence of two-stage tumor promotion comes from the inhibition studies where anti-inflammatory steroid fluocinolone acetonide and the protease inhibitor, tosyl phenylalanine chloromethyl ketone, have been shown to inhibit stage I tumor promotion, while retinoic acid inhibits stage II tumor promotion (Mukhtar et al., 1989). The major purposes of studying the modification of carcinogenesis are to identify tumor promoters that should be eliminated from our environment, to find inhibitors or anti-promoters that benefit us in the chemoprevention of cancer and establish the mechanisms of promoting or inhibitory action.

3.1.3 Progression

Progression is an accelerated process in which the tumour exhibits characteristic phenotypes like invasiveness, metastasis and angiogenesis (Hanahan and Weinberg, 2000). During progression, there is further exposure to mutagens, which produce
mutations in genes, such as tumour suppressors and oncogenes (Bowden et al., 1995). In the skin benign papillomas may progress to malignant SCC. Tumor progression, therefore, is proposed to involve a number of genetic alterations. The benign and malignant phenotypes can be induced sequentially, when normal epidermal cells are transfected first with the v-ras and then with the v-fos oncogene (Greenhalgh et al., 1990). Ha-ras and Ha-fos therefore, may cooperate in converting papilloma-derived keratinocytes to malignant tumors (DiGiovanni, 1992; Greenhalgh et al., 1990). In addition loss of a tumor suppressor gene located on the distal part of chromosomes, could be important in skin tumor progression (Greenhalg et al., 1990). The gradual increase in malignancy causes disappearance or decrease in the organelles and metabolic function necessary for normal cellular functions. The malignant conversion stage of tumor progression occurs spontaneously (Mukhtar et al., 1989), but may be enhanced by treatment of papilloma-bearing mice with genotoxic agents and free radical generating compounds. For example, it has been shown that after promotion of SENCAR mice with TPA for 10 to 12 weeks to produce large number of papillomas, repeated treatment of the papilloma bearing mice with genotoxic agents like 4-nitroquinoline N-oxide or urethane substantially increased the rate of conversion of papillomas to carcinomas (Sun, 1990). These studies suggested that the papillomas with the highest spontaneous rate of malignant conversion are also most sensitive to induction of malignant conversion by treatment with chemicals.

3.2 Oncogene and tumor suppressor gene in multistage carcinogenesis

The majority of genetic changes found in cancer fall into two categories: gain-of-function mutations in proto-oncogenes, which stimulate cell growth, division, and survival; and loss-of function mutations in tumor suppressor genes that normally help to prevent unrestrained cellular growth and promote DNA repair and cell cycle checkpoint activation (Lee and Muller, 2010).
Oncogenes are genes that all normal cells contain and in their natural states are known as proto-oncogene (Klein and Klein, 1984; Balmain, 1985). Protooncogenes are activated by point mutations, chromosomal translocation, amplification, or retroviral insertion that may ultimately result in tumor growth or tumorigenesis (Klein and Klein, 1984). More than 50 different oncogenes have been found to have transforming capabilities (Balmain, 1985). Some common examples of oncogenes implicated in cancer include ras, myc, fos, and src. The oncogene that has been demonstrated in many chemically induced skin tumors is mutated Harvey ras gene (H-ras) (Abel, et al., 2009). This gene is a member of a multigene family, present in the genome of all multicellular organisms (Balmain et al., 1984). The activation of H-ras in skin appears to be mediated by point mutation in the cellular oncogene. Majority of skin tumours produced following DMBA initiation possess a h-ras mutation with amino acid change CAA to CTA in codon 61, over 90% (Balmain et al., 1984; Quintanilla et al., 1986). DMBA can induce different types of mutations, eg. k-ras (it’s mutagenic action is random) it appears that promotion specifically by TPA selects for outgrowth of h-ras bearing papillomas. For instance, when the DMBA treated skin is subjected to a different method of tumour promotion, like constitutive over expression of ornithine decarboxylase (Megosh et al., 1998), less than 50% showed typical codon 61 h-ras mutation, instead a variety of k-ras mutations were observed in the majority (Megosh et al., 1998).

The expression of recessive mutations (i.e. tumor suppressor genes) has been shown to be involved in carcinogenesis in both humans and animals. These genes probably play a role in carcinogenesis when their function is inactivated by point mutations, deletions or chromosomal loss (monosomy). Ruggeri et al., 1991 have reported that alterations in the putative tumor suppressor gene, p53, occur in 25 50% of murine SCCs induced by the two-stage initiation-promotion protocol. These changes in p53 may be relatively late events in the progression of SCCs to more malignant and/or poorly differentiated
phenotypes (Ruggeri et al., 1991) although another study found similar alterations in p53 in well differentiated SCCs (Burns et al., 1991).
It has been found that in tumors promoted by TPA, there is an associated over expression of adhesion molecules, keratins, growth factors, cyclins and cyclin-dependent kinases (Owens et al, 1999). The prolonged duration of promotion is conducive for the acquisition of additional genetic mutations, commonly found hits include, p53 mutation (Burns et al, 1991) and trisomy of chromosome 7 (h-ras is found on chromosome 7) (Bianchi et al, 1990). These additional hits appear to be crucial in promoting transformation from benign papilloma into malignant carcinoma (Perez-Losada and Balmain, 2003).
Hence, mouse skin model of multistage carcinogenesis is ideally suited for studying the stepwise evolution of genetic events associated with tumor progression due to the ease of generating large numbers of tumors at defined time points during the overall carcinogenesis process.

3.3 Inflammation and carcinogenesis

Inflammation is the one of the pivotal functions of the innate immune system that protects against injury caused by wounding, chemical irritation/damage, or infection. In another way it can also be defined as inflammation is a component of the host response to either internal or external stimuli. This process is mediated through the cascade of cytokines and chemokines that attract innate immune cells, such as dendritic cells (DCs), natural killer (NK) cells, macrophages, neutrophils, basophils, eosinophils and mast cells, to form first line of defence against tissue damage/ to infiltrate at the site of disrupted and damaged tissue. During acute inflammation, innate immune cells form the first line of immune defence and regulate activation of adaptive immune responses. These responses can be pyrogenic, as indicated by fever. When acute inflammation is manifested for a short period of time, it has a therapeutic consequence.
By contrast, when inflammation becomes chronic or lasts too long, all these roles can be reversed-adaptive immune responses can cause ongoing and excessive activation of innate immune cells which is harmful and may lead to disease (De Luca and Iaquinto, 2004). Now it has been clearly observed that if the inflammation persists for a long period of time, the condition of chronic inflammation develops which is associated with most of the chronic human diseases including cancer (Mantovani et al., 2008).

Because of the destructive nature, acute inflammation is normally a self-limiting process and tightly regulated by a large number of pro- and anti-inflammatory mediators. Usually, removal of the eliciting inflammatory stimulus, e.g. a bacterial infection, leads to quick resolution of the inflammatory process and a subsequent healing phase (Winsauer and de, 2007). However, if the eliciting stimulus cannot be removed, e.g. in case of a chronic infection or autoimmune disease, sustained release of pro-inflammatory mediators lead to chronic inflammation, endothelial hyper-activation, induction of angiogenesis, and tissue damage.

Connection between inflammation and cancer is not new. In 19th century, it was the German physician Rudolf Virchow, who noticed the presence of leukocytes in neoplastic tissue and suggested that the “lymph reticular infiltrate” reflected the origin of cancer at the site of chronic inflammation (Balkwill and Mantovani, 2001).

The functional relationship between inflammation and cancer is based on Virchow’s hypothesis that origin of the cancer at the site of chronic inflammation is based on his assumption that some classes of irritants cause both tissue injuries and inflammation which leads to enhance cell proliferation (Balkwill and Mantovani, 2001). Continuous cell proliferation, an environment rich in inflammatory cells, growth factors, activated stroma and DNA-damage-promoting agents, certainly potentiates and/or promotes neoplastic risk. In case of tissue injury associated with wounding, cell proliferation enhanced while the tissue regeneration, proliferation and inflammation subside after the assaulting agent is removed or the repair completed. In contrast, proliferating cells that sustain DNA
damage and/or mutagenic assault (for example, initiated cells) continue to proliferate in microenvironments rich in inflammatory cells and growth/survival factors that support their growth (Dvorak, 1986).

The condition of chronic inflammation has been associated with most of the chronic human diseases including cancer, cardiovascular disease, diabetes, obesity, pulmonary disease, and neurologic disease (Aggarwal et al., 2006). Epidemiological findings have shown that chronic inflammation predisposes individuals to different types of cancer and about 15% of all global cancers are initiated by chronic inflammation (Balkwill and Mantovani, 2001). The 15-20% of all deaths from cancer related deaths can be attributed to inflammation (Mantovani et al., 2008). Further, it is also well known that chronic inflammation associated with different steps involved in carcinogenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (Coussens and Werb, 2002; Mantovani 2005).

Chronic inflammation plays significant roles in the development of cancer. On the basis of various evidences it is now confirm that chronic inflammation act as an important trigger and play key role at initiation, promotion and progression stages of cancer development (Fig.6).

Cellular and molecular mechanisms involved in inflammation induced cancer development includes, over production of free radicals causes damage to important cellular components (e.g., DNA, proteins and lipids), which can directly or indirectly contribute to malignant cell transformation, induction of genomic instability, alterations in epigenetic events and subsequent inappropriate gene expression, enhanced proliferation of initiated cells, resistance to apoptosis, aggressive tumor neovascularization, invasion through tumor-associated basement membrane and metastasis.
3.3.1 Pathways connecting inflammation and cancer

There are two, an intrinsic and an extrinsic, pathways that link inflammation and cancer (Fig. 7). The intrinsic pathway is activated by genetic a modification that causes inflammation and cellular transformation.

These modifications incorporate the activation of different types of oncogene by mutation, chromosomal rearrangement or amplification and the inactivation of tumor suppressor genes. Transformation of cells by above means induce production of various types of inflammatory mediators that lead to construction of an inflammatory milieu. This type of inflammatory conditions is also observed in tumors which are epidemiologically unrelated to inflammatory conditions (Mantovani et al., 2008). In the extrinsic pathway inflammatory or infectious conditions promote cancer development.
Selected chronic inflammatory conditions affecting organs such as liver, pancreas, stomach, colon, and prostate are associated with increased risk of cancer (Mantovani et al 2008).

Fig. 7. Pathways connecting inflammation to cancer. Source, Mantovani et al., 2008.

3.3.2 Cellular and Molecular links between inflammation and cancer

Inflammatory responses, to counteract the negative/harmful effects of various types of stimuli are mediated by different cellular and molecular players. It involves activation of various cellular inflammatory components, such as lymphocytes, neutrophils, basophils, eosinophils, macrophages and mast cells. These cells migrate from circulation to the site of tissue damage and get activated (Bennet and Brown, 2003).
Aberrant production of different types of cellular inflammatory mediators like macrophages, neutrophils, eosinophils, dendritic cells, mast cells, and lymphocytes are known to play important roles in tumor development. Most of these inflammatory cells are capable of producing cytokines, cytotoxic mediators including reactive oxygen/nitrogen species, proteases, MMPs and membrane-perforating agents, and soluble mediators of cell killing (Coussens and Werb, 2002).

Role of macrophages (tumor associated macrophages) in the tumorigenesis is well elucidated. They are known to play important role in tumor development by different ways. They can suppress the anti-cancerous action of different agents (Elgert et al., 1998) and help in the angiogenesis by releasing angiogenic factors like vascular endothelial growth factor (VEGF) and endothelin-2 (Pollard, 2004). Macrophages also help in the tumor cell invasion and metastasis through modulating the function of matrix metalloproteinases (Pollard, 2004). In addition, they may induce production of proinflammatory cytokines, reactive moieties and also helps in cell migration and proliferation (Leek and Harris, 2002).

Mast cells are another important type of cellular inflammatory mediator involved in cancer development. As they play key role in the angiogenesis, invasion and metastasis by generating angiogenic growth factors, such as VEGF and basic fibroblast growth factor, specific angiogenic regulators histamine and heparin, MMP-9, and mast cell–specific proteases MCP-4 and MCP-6. Neutrophils are well known for their critical roles in neoplasm formation like angiogenesis, metastasis and induction of genetic instability in tumor cells (Lu et al., 2006).

Now, it can be said that inflammatory component of a developing cancer includes different leukocytes, such as neutrophils, dendritic cells, macrophages, eosinophils and mast cells as well as lymphocytes. All these cells are capable of producing various groups of cytokines, cytotoxic mediators including free radicals (ROS/RNS), proteases, MMPs and membrane-perforating agents and soluble mediators of cell killing including TNF-α.
Studies of genetically modified mice, experiment of inflammatory cells adoptive transfer and analysis of human tumors have highlighted some of the molecular pathways that link inflammation and cancers. Cytokines, COX-2, inducible nitric oxide synthase (iNOS), nitric oxide (NO) intermediates and the transcription factors NF-κB, represent the major molecular players linking inflammation and cancers (Kundu and Surh 2008; Mantovani et al. 2008).

Cytokines

Cytokines are the low molecular weight (8–60 kDa) regulatory proteins secreted by leucocytes and other cells. They act across short distance in autocrine or paracrine manner. These molecules play pivotal roles in most of the important biological process, like cellular immunity, inflammation, proliferation, fibrosis, repair and angiogenesis. Their roles in different types of human diseases have been extensively studied in past decades. The pleiotropic (ability of one cytokine to act on different cell types) and redundant (multiple cytokines having the same functional effects and cytokines often affect the action and synthesis of other cytokines) actions of cytokines include numerous effects on immune cells and modulation of inflammation (Thomson and Lotze, 2003). It has been recognized that a variety of cytokines induces reactive toxic moieties like ROS/RNS production in different types of cells.

Tumor necrosis factor-α (TNF-α)

TNF-α is one of the important proinflammatory cytokine and a powerful activator of immune responses of the innate immune system including cytokine production and activation, immune-cell proliferation, expression of adhesion molecules and induction of inflammatory processes. Originally, it was identified as a factor inducing tumors necrosis but shortly it was found that TNF-α have pro-tumoral functions. It is produced by immune cells, like macrophages, monocytes, NK cells, B- and T-cell and non-immune cells like fibroblasts, smooth muscle and tumor cells. Most of the biological effects of
TNF-α are mediated through its binding to TNFR1 that leads to recruitment of an adaptor protein that activate multiple signaling pathways (Hehlgans and Pfeffer, 2005; Varfolomeev and Ashkenazi, 2004). TNF-α has been shown to regulate cell death, proliferation, differentiation and survival (Tracey and Cerami, 1994). Unregulated production of TNF-α is associated with various chronic inflammatory diseases including cancer.

Over expression of TNF-α is detected in various human cancers, such as breast, prostate, colorectal, bladder, lymphoma and leukemia (Kundu and Surh, 2008). One of the findings shows that mice deficient in TNF-α are resistant to skin tumor development thus provide genetic evidence linking TNF-α mediated inflammation and cancer (Balkwill, 2009; Moore et al., 1999). Pharmacological inhibition of TNF-α by pentoxifylline inhibits chemically induced papilloma formation in mouse skin (Robertson et al., 1996). TNF-α also orchestrate inflammatory responses along with other proinflammatory cytokines, chemokines and growth factors at the site of damage. TNF-α builds tumor microenvironment and promote tumor growth by the induction of tumor promoting cytokines, release of MMPs and pro-angiogenic factors (Coussens and Werb, 2002). It is an important inducer of NF-kB, the master regulator of inflammation. It can cause DNA damage by inducing the production of toxic moieties (Yan et al., 2006).

**Interleukin-6 (IL-6)**

IL-6 is another pleiotropic cytokine play important role in several biological activities such as regulation of immune reactivity, inflammation, oncogenesis and hematopoiesis. It promotes carcinogenesis and shows many functions in synergy with IL-1α, β and TNF-α (Heinrich et al., 1990). IL-6 produced by monocytes, macrophages, endothelial cells and fibroblasts in response to inflammatory stimulus. It play vital role in inflammatory pathways and promote cancer initiation and progression (Rose-John and Schooltink,
2007). IL-6 regulates cell cycle progression and inhibition of apoptosis (Lin and Karin, 2007). IL-6 is also one of the effectors of activated NF-kB signal.

Elevated level of IL-6 has been observed in both serum and biopsy of cancer patients that suggests a key role of this cytokine in cancer-related inflammation (Chung and Chang 2003; Kai et al., 2005). Studies on the genetically modified animals reveal the importance of IL-6 in cancer development. Induction of IL-6 by ras oncogene is critical for tumor growth in DMBA-TPA-induced two-stage skin carcinogenesis (Ancrile et al., 2007). Number and onset of skin tumors was less in IL-6 deficient mouse as compared to those in wild type, in two-stage skin carcinogenesis and the same results were found in case of murine plasmacytoma development (Lattanzio et al., 1997).

**Interleukin-1β (IL-1β)**

IL-1β is one of the important pleiotropic proinflammatory cytokines produce by monocytes, macrophages, dendritic cells and play different roles in both physiological and pathological states. It is produce in response to different stimuli and has many effects in common same like TNF-α and IL-6. Like other cytokines its role is protective in case of acute immune response or at low concentration while at high concentration or in case of chronic inflammation it contribute significantly in inflammation associated cancer development, like it promotes tumor proliferation, angiogenesis and metastases by its autocrine and paracrine effects (Lewis, 2006; Apte and Voronov, 2002). Studies on the different animal models of cancer revealed that IL-1β deficient mice are resistant to metastasis associated with melanoma, mammary and prostate cancer (Giavazzi et al., 1990).

**Cyclooxygenase-2 (COX-2)**

Cyclooxygenases, also known as prostaglandin H synthase (PGHS), are the rate-limiting enzymes in arachidonic acid metabolism that catalyze the conversion of arachidonic acid (AA) into the unstable pros glandins (PGs). PGs regulate different pathophysiological
processes, such as inflammatory reaction, haemostasis and thrombosis, gastrointestinal cytoprotection and ulceration, as well as in renal hemodynamics. Three isoenzymes of COX (COX-1, COX-2 and COX-3) with different functions have been identified; COX-1 is constitutively expressed in most tissues and control normal physiological processes. It exhibits housekeeping functions. COX-3 is a splice variant of COX-1. Mostly expressed in the cerebral cortex and in heart tissue and associated with temperature regulation (Ghosh et al., 2010; Ayoub et al., 2004).

Cox-2 is an inducible form of cyclooxygenase. Expression of COX-2 generally absent in normal cells. Its expression rapidly elevated in response to various stimuli like, proinflammatory cytokines, mitogenes, endotoxins, UV, ROS and phorbol ester (Surh and Kundu, 2007; Vane et al., 1998). Cox-2 plays central roles in the inflammation associated cancer development and act as interface between inflammation and cancer. Unregulated expression of COX-2 is implicated in all the three steps of carcinogenesis i.e., initiation promotion and progression. Over expression of COX-2 causes/ help in cellular proliferation of transformed cells, inhibit apoptosis inducing mediators, activate endothelial growth factor for angiogenesis and also alleviate metastasis and invasion (Ghosh et al., 2010; Dannenberg et al., 2001; Surh et al., 2001). Deregulated expression of COX-2 also causes oxidative stress and genetic instabilities (chromosomal breaks, fusion and tetraploidy).

Multiple lines of evidence indicate that genetically modified animals to over express COX-2 are more prone to develop malignancy in different organs, such as skin, mammary glands and intestine. Further, it was also found that COX-2 knockout animals are resistant or less prone to malignancies of different organs including skin. Use of selective COX-2 inhibitors or its functional inactivation and or use of genetically modified animals provide substantial evidence about the role of COX-2 in tumorigenesis (Kundu and Surh, 2008).
Inducible nitric oxide synthase (iNOS) and nitric oxide (NO)

NOS and NO are the other important inflammatory mediators. Endothelial NOS (e-NOS), neuronal NOS (nNOS) and inducible NOS (iNOS) are the three isoforms of NOS. Expression of eNOS and nNOS is constitutive while the expression of iNOS is inducible. NOS enzymes are responsible for the endogenous synthesis of NO from the amino acid L-arginine (Moncada et al., 1991). Low level of NO is helpful for various cellular physiological processes and in host defense by the virtue of its antimicrobial effects. In response to various stimuli such as LPS, proinflammatory cytokines, phorbol esters and sun exposure induction of iNOS in macrophages and other types of cell increases therefore concentration of NO is also increases (Yoshida et al., 2000; Surh et al., 2001). At high level, NO may be toxic, mutagenic, and or carcinogenic to the host cells. In case of chronic infection/damage or chronic inflammatory diseases and different types of cancer, expression of iNOS and the level of NO elevates (Chen and Stoner, 2004; Jaiswal et al., 2001). NO play important roles in the inflammation associated cancer development as it help in angiogenesis, metastasis and cell proliferation, causes DNA damage and induces suppression of both antitumor defence and DNA repair enzymes (Jaiswal et al., 2001; Hussain et al., 2004). Moreover, role of iNOS and NO in tumorigenesis was confirm from the previous study which shows that use of pharmacological inhibitor of iNOS suppressed DMBA/TPA induced carcinogenesis (Chun et al., 2004).

Nuclear factor-kappaB (NF-kB)

Nuclear factor-kB, one of the important families of transcription factors, critically associated in the regulation of various biological processes, such as cell proliferation, apoptosis, immunity, inflammation and development. In mammals five members of NF-kB, viz., p65 (RelA), p50, p52, c-Rel and RelB, are known. In inactive state or in un-stimulated cells NF-kB present in the cytoplasm as homo or heterodimeric complexes
along with its cytosolic repressor inhibitory protein IkB. Upon cell stimulation, proteolytic degradation of IkB by IkB kinase (IKK) results in nuclear translocation/activation of NF-kB (Hayden and Ghosh, 2008). A range of stimuli such as, hypoxia, growth factors, ionizing radiation, infectious agents, inflammatory cytokines, oxidative stress and phorbol ester causes activation of IKK thereby releasing active form of NF-kB, which is now free to enter the nucleus and induce transcription of genes to provide defence (Pahl, 1999).

It is responsible for the transcriptional induction of inflammatory (proinflammatory cytokins, chemokines, COX-2, iNOS and MMP), anti-apoptotic (cIAP1, cIAP2, XIAP, Bcl-2, Bcl-3 and Bcl-XL0) pro-angiogenic (VEGF and angioipoetin), cell proliferation regulatory genes and invasive factors (Karin, 2006; Chen et al., 2001). Aberrant and constitutive activation of NF-kB play pivotal roles in the development of almost all human diseases including malignancies due to its ubiquitous presence and multiple functions. Implication of NF-kB in the most of the hallmarks of cancer biology such as growth factor independent proliferation, inhibition of apoptosis, limitless replicative potential and tissue invasion and metastasis, provides direct support about it roles in cancer development (Naugler and Karin, 2008). NF-kB has been recognized as a key molecular bridge between inflammation and cancer and also as a prime endogenous tumor promoter (Pikarsky et al., 2004). Induction of various inflammatory mediators, for example proinflammatory cytokines, chemokines, iNOS, COX-2, matrix MMPs and a number of adhesion factors are generally mediated by DNA binding of NF-kB (Tak and Firestein, 2001). Thus, unregulated NF-kB activation may lead to the over expression of the above mentioned proteins thereby creating a tumorigenic environment (Karin, 2006).

Several lines of evidence from different studies revealed that NF-kB play central role in the development of neoplasm (Pahl, 1999; Pikarsky et al., 2004; Greten et al., 2004). It
has been observed that deregulated activation of NF-kB plays potential role in the development of various skin pathological conditions including skin carcinogenesis (Bell et al., 2003).

In 2000, Hanahan and Weinberg suggested six basic characteristic of cancer cell biology ‘hallmarks of cancer’ which were acquired during the multi-stage cancer development. The hallmarks include self-sufficiency in growth signals, insensitivity to anti-growth signals, able to evade apoptotic signals, limitless replicative potential, sustained angiogenesis and ability to invade tissues and metastasis (Hanahan and Weinberg, 2000).

Fig. 8. Inflammation as the seventh hallmark of cancer. Source, Colotta et al., 2009.

Further, on the basis of the evidences obtained from the large number of preclinical and clinical observations, now it is well established that there is a strong connection between inflammation and cancer and in 2009 Colotta et al., suggested that cancer related inflammation should be the ‘seventh hallmark’ in Hanahan and Weinberg’s model (Fig. 8).
3.4 Free radicals, oxidative stress and carcinogenesis

A free radical is any chemical species capable of independent existence that contains one or more unpaired electrons (Wilcox et al., 2004). The presence of unpaired electrons makes free radicals highly reactive and may also trigger a chain of reactions each capable of generating new radicals. Free radicals play key roles in the cellular signaling. They have been found to stimulate a number of signal transduction pathways that are important in maintaining cellular homeostasis.

Over production of the free radicals, such as highly reactive oxygen and nitrogen species causes damage to the major cellular building macromolecules, such as lipids, proteins and nucleic acids, hence most of the pathological events are linked with excess of free radicals production (Wilcox et al., 2004; Halliwell, 2007). Oxidative stress results when there is an imbalance between biochemical processes leading to production of reactive oxygen species (ROS) and the antioxidant defence mechanism of cell or tissue (Sies, 1991). This imbalance leads to damage of important biomolecules and cells. Generally, the condition of oxidative stress occurs due to one of the following three reasons: 1) increased oxidants or ROS, 2) decreased anti-oxidants, 3) failure to repair oxidative damage induced by ROS.

**Reactive oxygen species**

ROS is a more inclusive term that describes both radical and non-radical oxidants which may be oxygen, halide or nitrogen centered. ROS derived from oxygen are the superoxide anion (\(O_2^-\)), the perhydroxyl radical (protonated superoxide, HOO\(^-\)) the hydroxyl radical (HO), Lipid peroxy radical (LOO\(^-\)), nitric oxide radical (NO\(^-\)). The one electron reduction of oxygen results in formation of \(O_2^-\) (also known as the superoxide radical), whereas the two-electron reduction product of oxygen, when fully protonated, forms hydrogen peroxide (H\(_2\)O\(_2\)). Superoxide radical directly reacts with various types of biological molecules and also damages cell membrane (Klaunig and Kamendulis, 2004).
Reactive nitrogen species

NO is synthesized from the amino acid L-Arginine by a family of enzymes termed nitric oxide synthase. Generation of NO and O$_2^-$ favors the production of a toxic reaction product, peroxynitrite anion (ONOO$^-$). Once near or inside a cell, ONOO$^-$ can damage or deplete various vital components e.g., DNA by strand scission, lipids by peroxidation.

Sources of ROS and RNS

ROS can be produced from both endogenous as well as exogenous source (Fig. 9). Endogenous sources include; mitochondria, peroxisomes, activated inflammatory cells, activated phagocytes, xanthine oxidase, transition metals-mediated reactions and arachidonate pathways. Neutrophils, eosinophils, and macrophages are the other endogenous sources and major contributor to the cellular ROS production (Klaunig and Kamendulis, 2004). Mitochondrial respiratory chain is primarily responsible for the ROS production. During the mitochondrial process of reducing oxygen for production of water, several short-lived intermediates are formed, including superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO$^*$), and organic peroxides (Fridovich, 1978). RNS including NO also generated in mitochondria under hypoxic condition (Poyton et al., 2009).

ROS can be also produced by the exogenous sources, such as environmental agents (Radiations), pharmaceuticals and industrial chemicals (Pesticides). Environmental agents like nongenotoxic carcinogens can directly generate reactive oxygen species in cells (Rice-Evans and Burdon, 1993). Exposure to xenobiotic of varied structures and activities result in oxidative stress and associated damages. Chlorinated compounds, radiation, metal ions, barbiturates, phorbol esters, and some peroxisome proliferating compounds are also important exogenous sources of ROS/RNS production (Klaunig and Kamendulis, 2004).
Oxidative stress and carcinogenesis

In the last two decades, there has been a considerable amount of interest in the role of ROS in various pathological conditions, such as malignancies, diabetes and arthritis (Apel and Hirt, 2004; Behrend et al. 2003; Witz, 1991).

Low level of ROS and RNS play vital roles inside the cells, such as cellular defense against infectious agents, transcriptional expression of antioxidant enzymes, regulation of cellular redox homeostasis, expression of various genes, signal transduction, cell growth and differentiation (Dröge, 2002; Evans and Halliwell, 2001). For example, super oxide and hypochlorous acid (HOCL) produce by phagocytic cells and leukocytes to eliminate potentially pathogenic microorganisms (Bahorun et al., 2006). NO involve in the regulation of various cellular and physiological processes, such as vascular system,
nervous system, cell adhesion, platelet aggregation, thrombosis and inflammatory response (Bahorun et al., 2006).

Over production of ROS cause oxidative stress, a condition which ultimately leads to the damage of major cellular macromolecules like DNA, lipid and proteins that may result in somatic mutations and neoplastic transformation. Further, oxidative stress play important role in various cellular pathophysiological events, including apoptosis, cytotoxicity, inflammation, differentiation, proliferation and angiogenesis (Kryston et al., 2011; Ames and Shigenaga, 1992; Witz, 1991). Free radicals play important roles in the inflammation induced cancer development (Halliwell, 2007; Seril et al., 2003; Cerutti and Trump, 1991) as the activated immune cells and cytokines are the prime producer of ROS. Proinflammatory cytokine like tumor necrosis factor-α (TNF-α) is one of the important mediators of inflammation and a well known producer of ROS (Yan et al., 2006).

Now, it is well known that over production of ROS play important role in all the three stages of cancer development via inducing DNA damage or increasing DNA mutations, genomic instability, cellular proliferation differentiation and angiogenesis (Fang et al., 2009; Khandrika et al., 2009, Visconti and Grieco, 2009; Witz, 1991).

Over production of ROS and RNS play important role in the inflammation associated cancer development. Reactive oxygen species generated during the inflammatory process damages DNA and cause mutations, which contribute towards the neoplasm development (Fig.10). Inflammatory cells, such as neutrophils, macrophages, mast cells and eosinophils are the important endogenous ROS producer and contribute significantly to the cellular oxidative stress load (Reuter et al., 2010; Forman and Torres, 2002). They produce ROS and RNS in order to destroy microbial invaders and foreign particles during the oxidative respiratory burst by the enzyme nicotine adenine
dinucleotide phosphate reduced (NADPH) oxidase (Bartsch and Nair, 2006; Carreras et al., 1994).

Myeloperoxidase (MPO) is important pro-oxidative and proinflammatory enzyme present in polymorphonuclear neutrophils and macrophages that catalyze the conversion of chloride and hydrogen peroxide to hypochlorous acid and other reactive oxygen species to kill foreign microbial invaders. Hypochlorous acid is a highly reactive oxidant and in the presence of $O_2^{-}\cdot$, it produces $OH\cdot$ radical. MPO also produces free Cl$_2$, which act as a potent halogenating agent. Over expression of MPO result in excessive production of free radicals and DNA damage which may lead to the genesis of various chronic human diseases including cancer (Reynolds et al., 1997).

Overall, various preclinical and clinical data suggest that there is a close relationship between oxidative stress, inflammation and carcinogenesis. Extensive research during the past 2 decades has revealed that sustained oxidative stress/over production of ROS can

Fig.10. Proposed mechanisms for the role of inflammatory oxidative stress induced DNA damage. Source, Kawanishi and Hiraku, 2006.
lead to chronic inflammation, a phenomenon which is critically associated with carcinogenesis. Various transcription factors, such as NF-κB, AP-1, p53 and Nrf2 have been activated by oxidative stress. Further, activation of transcription factors lead to expression of genes for various growth factors, inflammatory mediators and cell cycle regulatory molecules (Reuter et al., 2010).

**Cellular antioxidant defense system**

The cell is able to handle and survive the continuous production of ROS because of the existence of a delicate balance between the various pro-oxidants and the antioxidant defence system. In the human body, this balance exists and is in slight favour towards oxidants to allow the body to complete its metabolic duties. If the generation of ROS overwhelms the available antioxidant defences, oxidative stress can arise. The antioxidant rationale is based on the assumption that antioxidants can limit the deleterious effects caused by oxidative stress thereby slowing the incidence of disease and the process of ageing.

The skin is chronically exposed to both endogenous and environmental pro-oxidants and to protect against this overload of oxidant species, it has a well-organized system of both enzymatic and non-enzymatic antioxidants (Briganti and Picardo, 2003). Antioxidants can be exogenous (natural or synthetic) or endogenous (enzymatic and non-enzymatic) substances or compounds that ease the burden of free radicals (ROS or RNS) through various mechanisms (Halliwell and Gutteridge, 1995) (Fig.11 and 12).

Both enzymatic and non-enzymatic antioxidants exist in the skin to protect it from oxidative stress or free radical induced damage via acting as electron donor to unstable free radicals. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are considered the primary antioxidant enzymes, since they are involved in the direct elimination of active oxygen species. Glutathione S-transferase (GST), glutathione reductase (GR), and glucose 6-phosphate dehydrogenase (G6PD) are secondary...
antioxidant enzymes which help in the detoxification of reactive oxygen species by decreasing peroxide level or by maintaining a steady supply of metabolic intermediates like glutathione (GSH) and nicotamide adenine dinucleotide phosphate (NADPH) for the primary antioxidant enzymes (Tabrez and Ahmad, 2011; Valko et al., 2006; Sun, 1990; Cerutti et al., 1994). The non enzymatic antioxidants consist of sulfhydryl compounds, such as GSH and thiol, NADPH, ascorbate, tocopherol, β-carotene, bilirubin, selenium, and urate, is to afford protection against the adverse effect of oxidant or reactive metabolites of pro-carcinogens (Matough et al., 2012; Clarkson and Thompson, 2000; Prior and Cao, 1999).

**Glutathione**

Reduced glutathione (GSH), the most abundant low-molecular-weight tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) in mammalian cells, is a major antioxidant that provides reducing equivalents to the GPx-catalyzed reduction of hydrogen peroxide and lipid hydroperoxides to water and the respective alcohol (Young and Woodside, 2001). GSH is synthesized in cytosol by consecutive reactions of two enzymes: γ-glutamylcysteine (γGlu-Cys) synthetase and GSH synthetase.

GSH synthesis can be limited by the ATP availability (Shan et al., 1989). In nucleus, GSH maintains the redox state of critical protein sulfhydryls that are necessary for DNA repair and expression.

Inside the cells GSH is present in two, reduced (GSH) and oxidized (GSSG) forms. Oxidized glutathione is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress (Rossi et al., 2006).

Oxidized glutathione can be reduced to GSH by the NADPH-dependent glutathione reductase as well as via the thioredoxin/glutaredoxin systems. GSH has a major intracellular antioxidant activity being involved in detoxification of peroxides and electrophilic toxins as a substrate for glutathione peroxidase and glutathione s-
transferase. Glutathione can also work independently of GPx by directly scavenging radicals such as hydroxyl radicals and singlet oxygen (Afaq and Mukhtar, 2001).

Glutathione peroxidase (GPx) and glutathione reductase (GR)

Glutathione peroxidase catalyze the oxidation of glutathione at the expense of a hydroperoxide, which might be hydrogen peroxide or another species such as a lipid hydroperoxides (Arthur, 2000)

$$\text{ROOH} + 2\text{GSH} \rightarrow \text{GSSG} + \text{H}_2\text{O} + \text{ROH}$$

Other peroxides, including lipid hydroperoxides, can also act as substrates for these enzymes, and therefore play a role in repairing damage resulting from lipid peroxidation. Glutathione peroxidases require selenium at the active site, and deficiency might occur in case of severe selenium deficiency. Glutathione peroxidase is widely distributed in almost all tissues. The activity of the enzyme is dependent on the constant availability of reduced glutathione.
The product of the oxidation of GSH is glutathione disulfide or reduced glutathione (GSSG). GSH is regenerated from GSSG within the cells in a reaction catalyzed by glutathione reductase (GR). The ratio of reduced to oxidize glutathione is usually kept very high as a result of the activity of GR:

\[ \text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \]

This enzyme regenerates GSH by transferring reducing equivalent from NADPH to GSSG. NADPH regeneration during GSH redox cycling in cells depends on NADPH–regenerating enzymes like G6PD. G6PD is a cytoplasmic enzyme that affects the production of reduced form of cytosolic nicotine adenosine dinucleotide phosphate coenzyme (NADPH) by controlling the step from glucose-6-phosphate to 6-phosphogluconate in the pentose phosphate pathway (Pinna et al., 2008; Kletzien et al., 1994).

**Glutathione-S-transferase (GST)**

Glutathione S-transferases, another important group of antioxidant enzyme, are thought to play vital role in the detoxification of potential alkylating agents including pharmacologically active compounds and ROS. These enzymes are present throughout the animal and plant kingdom and have been detected in almost all human tissues. They protect cells from cytotoxic and carcinogenic agents by conjugating reactive chemical species to reduced glutathione, thereby neutralizing their electrophilic sites and rendering the products more water-soluble (Coles and Kadlubar, 2003; Marshall et al., 2000).

**Superoxide dismutase (SOD)**

This enzyme catalyzes the dismutation of the superoxide radical anions into \( \text{H}_2\text{O}_2 \), which is converted to water and oxygen. Based on the metal ion requirements and the anatomical distribution, three types of superoxide dismutase exist in cells: Cu, ZnSOD (SOD1), MnSOD (SOD2) and extracellular SOD (SOD3).
MnSOD constitutes one of the major cellular defense mechanisms against the adverse effects of toxic agents that cause oxidative stress. In addition to being essential for survival, increased expression of MnSOD has been shown to protect against numerous agents and conditions that cause oxidative stress and/or cell death. It also functions as a tumor suppressor gene. It plays important role in the chemically induced skin cancer development and has been demonstrated to reduce tumorigenesis (Oberley, 2005). SOD not only suppresses cell proliferation, but also affects inflammation. One of the recent findings revealed that over expression of MnSOD reduced tumor incidence and multiplicity. Further, SOD deficiency increases TPA-induced oxidative stress markers in mouse skin (St Clair et al., 2005; Zhao et al 2001; 2002).

**Catalase**

The defense of skin cells against peroxide mediated oxidative damage is essential for maintaining cutaneous functioning. Catalase and the glutathione system both are important for cellular detoxification of $\text{H}_2\text{O}_2$ (Ames et al., 1993; Davies, 2000). Under physiological conditions, catalase accepts only $\text{H}_2\text{O}_2$ as substrate but not organic hydroperoxides (Aebi et al., 1984). The haem-containing enzyme converts $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and $\text{O}_2$.

Catalase was the first antioxidant enzyme to be characterized that catalyzes the two stage conversion of hydrogen peroxide to water and oxygen:

\[
\text{Catalase–Fe (III) + H}_2\text{O}_2 \rightarrow \text{compound I}
\]

\[
\text{Compound I + H}_2\text{O}_2 \rightarrow \text{catalase–Fe (III)} + 2\text{H}_2\text{O} + \text{O}_2
\]

Catalase is largely located within cells peroxisomes, which also contain most of the enzymes capable of generating hydrogen peroxide. The amount of catalase in cytoplasm and other sub cellular compartments remains unclear, because peroxisomes are easily ruptured during the manipulation of cells. Catalase is a diffusion-controlled enzyme and
therefore is especially effective when the clearance of high concentration of \( \text{H}_2\text{O}_2 \) is required (Young and Woodside, 2001).

**Lipid peroxidation (LPO)**

Lipid peroxidation is one of the major outcomes of free radical-mediated injury that directly damages biological membranes and generates a variety of highly reactive products including malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), which in turn causes DNA damage together with deregulation of cell homeostasis (Negre-Salvayre et al., 2008; Bartsch and Nair 2004) (Fig.12). Lipid radicals oxidizes proteins and react with DNA to form DNA adducts such as ethano and propano-DNA adducts and MDA deoxyguanine adducts (Negre-Salvayre et al., 2008; Williams et al., 2006). Malondialdehyde is a mutagen and also a carcinogen (Niedernhofer et al., 2003).

![Fig.12. Mechanism of lipid peroxidation and antioxidant defense system](image-url)

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Lipid peroxidation has been defined as the oxidative deterioration of polyunsaturated lipids i.e. those lipids containing more than two carbon-carbon double covalent bonds. This process proceeds by free radical chain reaction mechanism that consists of three major steps: initiation, propagation, and termination. Several experimental evidences indicate that extensive lipid peroxidation results in loss of membrane integrity, impairment of the function of membrane-transport proteins and ion channels, disruption of cellular ion homeostasis and eventual rupture leading to release of cell organelle contents such as lysosomal hydrolytic enzymes (Fong et al., 1973; Mattson, 1998).

4. Chemoprevention

The fact that about 7 million people die from various types of cancer every year, making this disease responsible for 12.5% of deaths worldwide, raises an overwhelming demand to develop new, more potent and effective therapeutic agents (Coseri, 2009). Despite massive effort to defeat cancer over the last few decades, the outcome of conventional strategies to combat cancer appears inadequate as the incidence and mortality of cancer is not reducing globally. Use of natural products appears to be a good promising approach to reduce cancer load. In the present era, importance has been given to the natural products for the treatment of various degenerative diseases due to their antioxidant as well as anti-inflammatory potential. Drug development from natural products is a rapidly emerging and highly promising strategy to identify novel anticancer agents. In recent years, novel combination treatments with conventional cancer therapies and natural chemopreventive agents have received much attention in cancer research. Moreover most of the plant based chemopreventive agents are considered to be safe. The term chemoprevention was coined by Michael Sporn, a pioneer in cancer prevention research, in the mid-1970s. Chemoprevention, a rapidly growing area of preventive oncology, can be defined as prevention of cancer by administration of one or more
naturally occurring or synthetic compounds to block/reverse or suppress and or delay the process of carcinogenesis (Surh, 2003). Chemoprevention therefore, is the means of cancer control in which the occurrence of this disease as consequence of exposure to carcinogenic agents can be slowed, blocked or reversed by the administration of one or more naturally occurring and synthetic compounds individually or in combination therapy has emerged as a promising and pragmatic medical approach to reduce cancer risk (Sarkar and Li, 2007; Morse and Stoner, 1993; Bertram et al., 1987). Epidemiological findings also revealed that use of natural compounds is a well promising approach for the chemoprevention and management of human cancers (Nakachi et al. 1996; Kroon and Williamson, 2005).

![Diagram of some of the natural chemopreventive agents](image)

Fig. 13. Some of the natural chemopreventive agents
It is well documented that plant derived foods contain chemopreventive agents, including several micronutrients, like vitamins and minerals, and a variety of bioactive compounds, such as organosulfur compounds, polyphenols, and isoflavones carotenoids, indoles and sterols (Fig. 13).

Many natural compounds, particularly plant products and dietary constituents, have been found to exhibit cancer chemopreventive activities (Zhao et al., 2010; Kelloff et al., 2000). Experimental studies and clinical trials have demonstrated the beneficial effects of chemopreventive agents, including soy isoflavone, curcumin, epigallocatechin-3-gallate (EGCG), non-steroidal anti-inflammatory drugs (NSAIDs), resveratrol, indole-3-carbinol (I3C), and 3,3’-diindolyl-methane (DIM) in cancer prevention and treatment (Sarkar and Li, 2007).

Evidence from epidemiologic and in vivo studies have shown that natural products, like fruits, vegetables, spices, tea, and medicinal herbs rich in antioxidant and anti-inflammatory phytochemicals suppress different types of experimentally induced carcinogenesis (Ulbricht and Chao, 2010; Mehta et al., 2010; Wattenberg, 1993). Various antioxidants including plant polyphenols inhibit skin tumor initiation, promotion and progression in experimental animals (Afaq and Katiyar, 2011; George et al., 2011; Liu et al., 2009; Stoner and Mukhtar, 1995). The incidence of skin cancer may possibly be reduced by a combination of a healthy lifestyle, a diet rich in naturally occurring chemopreventive compounds and the use of sunscreens and specially formulated health care products containing botanical antioxidant compounds (Velasco et al., 2008; Afaq et al., 2002; Katiyar, 2002).

**Types of chemopreventive agents**

According to the Wattenberg, chemopreventive agents can be divided in two main categories;

(a) Blocking agent  (b) Suppressing agent (Wattenberg, 1985) (Fig.14)
(a) Blocking agent

These are typically those compounds that can inhibit initiation either by inhibiting the formation of carcinogens from precursor molecules or reactive metabolites from the parent carcinogens, or by preventing interaction of the ultimate electrophilic/carcinogenic species with critical cellular target molecules, such as DNA, RNA, and proteins (Surh, 2003; Wattenberg, 1985). Diverse classes of compounds, such as chemicals that inhibit metabolic activation of carcinogens or enhance their detoxification; antioxidants that scavenge free radicals; and chemicals that trap ultimate electrophilic carcinogens, act as blocking agents.

Fig. 14. Mechanisms of action of chemopreventive agents

Since cytochrome P450 (CYP) play important role in the metabolic activation of carcinogens that lead to formation of highly electrophilic intermediates. Blocking agents can inhibit the conversion of carcinogen by inhibiting the CYP. Diallyl sulfide, a naturally occurring constituent of Allium vegetables, inhibits cytochrome CYP enzymes (Hong et al., 1991) and tumorigenesis in a number of animal models (Tadi, et al 1991; Wargovich,
et al, 1987). Curcumin also inhibits aflatoxin induced carcinogenesis by modulating the function of CYP (Firozi et al., 1996). Previous *in vivo* and *in vitro* observations show that curcumin inhibits the metabolic activation of different carcinogen by inhibiting CYP (Duvoix et al., 2005). Resveratrol is another natural chemopreventive agent known to inhibit the carcinogenesis by inhibiting the CYP (Delmas et al., 2006). Egallic acid also inhibits the metabolic activation of procarcinogen to carcinogen by suppressing CYP (Wood et al., 1982).

Induction of detoxification enzymes by naturally occurring or synthetic agents represents a promising strategy for cancer chemoprevention (Wilkinson and Clapper, 1997). Detoxification enzymes protect cells from a wide variety of carcinogens and endogenous toxins (Wilkinson and Clapper, 1997). Induction of phase-2 detoxifying or antioxidant genes represents an important cellular defence in response to oxidative and electrophilic insults. Phytochemicals are promising cancer blocking agents that could prevent the occurrence of DNA mutation caused by carcinogens. While some of them directly react with carcinogens, many of them elicit their chemopreventive effects indirectly through the modulation of phase II metabolizing enzymes existing in the tissues where carcinogens/procarcinogens are metabolized (Johnson, 2007; Kensler, 1997). Decreased mutagenic risk could result from the induction of several phase II detoxifying or antioxidant enzymes, leading to the excretion or inactivation of the carcinogen (Gopalakrishnan and Tony Kong, 2008). Plant based natural products have been reported to reduce or inhibit the cancer risk by the induction of the detoxifying enzymes, such as GST, quinone reductase (QR), SOD, Catalase, and GPx and GR (Zhao et al., 2010; Lee and Surh, 2005; Wood et al., 1982).

**(b) Suppressing agent**

These agents act in either promotion or progression stage of the carcinogenic process to inhibit malignant expression of initiated cells. Because carcinogenesis is a multistep
process that often progresses slowly in the early stages and, hence, there is great potential for suppressing its development. Suppressing agents can be classified as compounds that inhibit polyamine metabolism; induce terminal cell differentiation; modulate signal transduction; modulate hormonal/growth factor activity; inhibit oncogene activity; promote intercellular communication; restore immune response; induce apoptosis; correct DNA methylation imbalances; inhibit basement membrane degradation; and inhibit arachidonic acid metabolism (Hail et al., 2008; Stoner et al., 1997). They reduce the consequences of altered gene expression by reducing proliferation of initiated cells or restoring apoptosis to normal levels, thus preventing the accumulation of damaged cells (Surh, 2003; Manson et al., 2000; Wattenberg, 1985). Generally, the chemopreventive activity of suppressing agents is attributed to their influence on cell proliferation, differentiation, senescence, and/or apoptosis and act by following ways:

- Scavenge reactive oxygen species
- Alter gene expression
- Decrease inflammation
- Suppress proliferation
- Induce differentiation
- Encourage apoptosis
- Enhance immunity
- Discourage angiogenesis

**Molecular targets of chemopreventive agents**

Now, it is well known that natural chemopreventive compounds offer great potential in the fight against different chronic diseases including cancer by inhibiting the process of carcinogenesis through modulation of various cell signaling pathways (Karikas, 2011; Khan et al., 2007; Manson, 2003).
Natural products can interfere with all the three steps of cancer development to prevent carcinogenesis. The ability of natural products to modulate cell signal transduction pathways, through the activation/suppression of multiple redox-sensitive transcription factors, such as NF-kB and Nuclear factor-E2-related factor-2 (Nrf-2), provides molecular basis for chemopreventive agents (Surh and Na, 2008; Surh et al., 2005; Chen et al., 2005) (Fig.15).

Effectiveness of chemopreventive agents reflects their ability to counteract certain upstream signals that leads to genotoxic damage, redox imbalances and other forms of cellular stress. Targeting malfunctioning molecules along the disrupted signal transduction pathway in cancer represents a rational strategy in chemoprevention. NF-kB provides mechanistic links between inflammation and cancer. Components of the cell signaling network, especially those which converge on the ubiquitous eukaryotic redox-sensitive transcription factor NF-kB, have been implicated in pathogenesis of many inflammation-associated disorders.

![Fig. 15. Molecular targets of chemopreventive agents](image)

Modulation of cellular signaling involved in chronic inflammatory response by anti-inflammatory agents hence represents an important strategy in molecular target-based...
chemoprevention and cytoprotection (Surh et al., 2005; Surh, 2003). Thus cell signaling cascades and their components have become important targets of chemoprevention, phenolic phytochemicals and plant extracts seem to be promising in this endeavour (Neergheen et al., 2009). Many natural chemopreventive agents have been shown to suppress constitutive NF-κB activation in malignant cells or NF-κB activation induced by the external tumor promoter phorbol 12-myristate-13-acetate (PMA) or TNF-α (Shu et al., 2010; Surh, 2003).

Nrf2 is another important redox-sensitive transcription factor play vital role in the transcriptional up-regulation of antioxidant and phase II enzymes. It regulates the cytoprotective genes expression through a common DNA regulatory element called the antioxidant-response element (ARE). Therefore, the activation of Nrf2–ARE signaling pathway has been considered as an effective strategy for cancer chemoprevention. Natural chemopreventive agents induce antioxidant response element (ARE)-mediated gene expression of cytoprotective enzymes by increasing the levels of Nrf2 protein (Zhao et al., 2010; Surh, 2003; Surh and Na, 2008).