Introduction

Cancer is potentially a deadly disorder which is mainly caused by environmental factors that mutate the genes encoding cell-regulatory proteins. The consequent aberrant behaviour of cells that leads to increased abnormal cell mass destroys the surrounding normal tissues and can spread to important organs bringing about dispersed sickness, commonly an indication of imminent death of the patient. Alterations primarily occur in three main classes of genes such as proto oncogenes, tumour suppressor genes and DNA repair genes. These events collectively contribute to the development of cancer genotype and phenotype that resists the natural and inherent death mechanisms embedded in cells, coupled with the dysregulation of cell proliferation. There is an increasing evidence to suggest that cancer is also driven by ‘epigenetic changes’ like DNA methylation and altered patterns of histone modifications, leading to alterations in chromatin condensation status, thereby regulating expression of certain set of specific genes (Bayil and Ohm, 2006).

The fundamental defect in cancer is the loss of the ability to control the growth and division of cells. This overgrowth is often referred to as a tumour. Some tumours are not cancerous and are termed ‘benign’; those tumours that are cancerous are termed ‘malignant’. Uncontrolled growth of cells causes the tissue to look abnormal. If malignant cells are contained within the original tissue, the cancer is called in situ, whereas if they invade neighbouring tissue the cancer is said to be invasive. One property of most malignant tumours is their ability to undergo metastasis—the spread of cancerous cells to other parts of the body. Several factors may cause a cell to become cancerous, e.g. environmental agents in the food we eat, chemical agents, radiation and viruses. These agents are called carcinogens and cause mutations in the genetic make-up of the cell (Tortora and Grabowski, 2004).
Multistage carcinogenesis

The process of conversion of a normal cell to the malignant state is called carcinogenesis, and the agents that induce it are called carcinogens. Carcinogenesis is a complicated, multi stage process; essentially, a small population of abnormal cells are generated which then increases in abnormality as a result of a series of mutations and variations in the patterns of gene expression.

Initiation

A cellular phenomenon characterized by genetic changes that are irreversible, **initiation** involves alterations in one or more genes that are more likely to be either oncogenes or tumour suppressor genes. Initiation requires first, the binding of the carcinogen or its reaction products with DNA and second, cell proliferation.

Promotion

**Promotion** is the process whereby an initiated tissue or organ develops focal proliferations, hyperplasia, dysplasia and benign tumour, one or more of which may act as precursors for subsequent steps in the carcinogenic process. An essential feature of promotion is the creation of a mitogenic environment and is considered to be the result of non-mutagenic agents which stimulate growth. Promotion on the other hand, is considered as reversible and generally a slow process influencing the proliferation of initiated cells and requires the presence of continuous stimulation, which may enhance the possibility for the acquisition of additional genetic alterations (Moolgavkar and Luebeck, 2003).

Progression

Tumour **progression** is a term widely used with reference to phenotypic changes occurring in already formed neoplastic lesion. Thus, progression is the natural tendency of tumours to become more aggressive or
malignant with time. Overall mechanisms involve the acquisition of genetic instability by the preneoplastic cell; the occurrence of spontaneous and/or induced mutations create a heterogeneous cell population in the tumour and the selection of the more aggressive neoplastic subpopulation. The more aggressive clones are those that have a selective advantage such as increased proliferation potential, deficient terminal differentiation and blocked apoptotic mechanisms (Sugimura, 1992).

![Multistep Carcinogenesis](http://www.bvsde.paho.org)

**Figure 1:** Multistep carcinogenesis (adapted from http://www.bvsde.paho.org)

**Colorectal cancer (CRC) incidence and prevalence**

CRC is the most common type of gastrointestinal cancer which can be derived from either inherited or somatic genetic alterations that develop over the course of a lifetime. CRC is the third most common type of cancer in males and the second in females with the highest rates in America, Australia, Newzealand and Western Europe. Estimates made by the American Cancer Society for the year 2014 reveal 96,830 new cases of colon cancer, 40,000 new cases of rectal cancer and 50,310 deaths from CRC (American Cancer Society 2014). In Asia the incidence of CRC is increasing and research shows that this type of tumour is the third most common malignant disease in both men and women. The rate of CRC incidence was low in India when compared
to western countries but is presently increasing; out of 3.5 million cancer cases, 35,000 suffer from CRC in India, due to the migration of rural population to the cities, increase in life expectancy and changes in lifestyle (Shrikhande et al., 2007). Although the prevalence of some cancers has declined, that of colon cancer has increased, and the rise is thought to reflect socioeconomic affluence. Inheritance contributes about 5-10% to the causation of this disease, whereas dietary and environmental factors have a large influence. Knowledge that low-fiber, high-calorie diets contribute to the development of CRC has led to the use of systematic dietary interventions and chemotherapy for reducing the prevalence of CRC (Krzyzanowska et al., 2010).

**Types of cancer in the colon and rectum**

There are many different types of cancer which can start in the colon or rectum.

**Adenocarcinoma:** More than 95% of CRCs are a type of cancer known as adenocarcinoma which begins in the cells that form glands and secrete mucus to lubricate inside the colon and rectum.

**Carcinoid tumours:** These tumours are initiated from hormone secreting cells in the intestine.

**Gastrointestinal stromal tumours (GISTs):** These tumours start from specialized cells in colon wall known as interstitial cells of Cajal. Some tumours are benign; others are malignant. These tumours can be found anywhere in the GI tract, but it is unusual in the colon.

**Lymphomas:** These are cancers of immune cells that typically originate in lymph nodes, but they may also originate in the colon, rectum, or other organs.

**Sarcomas:** These tumours can originate in blood vessels also in muscle and connective tissue in the wall of the colon and rectum. Colon or rectum sarcomas are rare (Gorbach and Goldin, 2012).
Risk factors for CRC

Dietary factors

Diet is definitely the most important exogenous factor identified up to now in the aetiology of colon cancer. It has been estimated that 70% of CRC’s could be prevented by nutritional intervention; various promoting and protective factors have been identified in prospective and case-control studies.

High alcohol consumption probably increases the risk of colon cancer. A majority of epidemiological studies on alcohol intake have reported either an increased risk or no association between alcohol intake and colon cancer. The effect generally seems to be related to total ethanol intake, regardless of the type of drink.

Several epidemiological studies have examined meat intake and the risk of colon cancer. The mechanisms by which red meat and processed meat may increase the risk of colon cancer include the facilitating effect of fat on bile acid production, and the formation of carcinogens when meat is cooked or processed.

Processed meats may contribute to the production of nitrosamines. The evidence shows that red meat probably and processed meat possibly increases the risk of colon cancer (Saldanha and Tollefsbol, 2012).

Obesity, greater adult height, frequent eating, and diets high in sugar, total and saturated fat and eggs, all possibly increase the risk.

Physical activity has consistently been associated with decreased risk of colon cancer. Evidences show that physical activity especially when lifelong decreases the risk of colon cancer (Burstein, 1993).

Non dietary factors

Smoking has consistently been associated with adenomatous polyps. One study has suggested that smoking early in life is likely to increase risk of colon cancer. Approximately 20% of the large bowel cancers in men appear to be attributable to smoking (Slattery et al., 1990).

Age increases the risk of developing CRC. This disease is more common in people who are above 50.
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Hereditary factors

Genetic vulnerability to colon cancer has been attributed to either the polyposis or nonpolyposis syndromes. The main polyposis syndrome is familial adenomatous polyposis (FAP) which is associated with a mutation or loss of FAP (also called the adenomatous polyposis coli (APC)) gene (Thliveris et al., 1991).

Hereditary nonpolyposis CRC (often referred to as HNPCC) syndrome is associated with germline mutations in six DNA mismatch repair genes. The prevalence of CRC associated with the HNPCC syndrome is very low as estimated by the Modena Cancer registry: slightly more than 7% of the total CRC prevalence (Jemal et al., 2010).

Signs and Symptoms of colon cancer

Early CRC does not cause any symptoms, that is why screening is very important. Most of the CRC begins as a polyp, a small growth in the colon wall. Once a polyp grows, it can bleed or obstruct the intestine. The following symptoms are most common in the multi stage CRC (Zepp and Li, 2012).

- Blood in the stool after having a bowel movement
- Bleeding from the rectum
- Dark-coloured stools
- A change in the pattern of the stool
- New onset of constipation or diarrhoea
- Cramping pain in the lower abdomen
- Unprecedented weight loss

Screening test for colon cancer

- **Fecal occult blood test (FOBT):** This test detects occult (hidden) blood. This test should be performed every 1-2 years in people between the age of 50 and 80 years.

- **Sigmoidoscopy:** Sigmoidoscope is a thin flexible tube with a light to examine the inside of the colon for polyps, tumours and other abnormalities.
• **Barium enema:** Liquid containing barium is introduced into the rectum through anus. This procedure may be effectual in detecting large polyps and also cancers. Recommended interval is every 3-5 years.

• **Colonoscopy:** Colonoscopy is similar to sigmoidoscopy, except that the entire colon is examined. This procedure is the most efficient method of examining the colon. It is commonly recommended to be performed every 10 years.

**Genetic alterations in the growth of colon cancer**

The stepwise progression from normal to dysplastic epithelium and to carcinoma is earmarked by specific genetic alterations at known oncogenes and tumour suppressor genes (figure 2). Loss of functional mutations at the adenomatous polyposis coli (APC) tumour suppressor gene on chromosome 5q21 occurs in over 80% of colon adenocarcinoma (Smith et al., 1993; Kinzler and Vogelstein, 1996), thus representing the earliest and rate limiting genetic event in colorectal tumour initiation (Powell et al., 1992). Indeed, APC mutations were present already at the preneoplastic stage and they are related with the degree of dysplasia of those early lesions (Jen et al., 1994; Smith et al., 1994). The inactivation of both alleles of the APC gene can be detected in most of the intestinal tumours at early stages of development (Powell et al., 1992; Miyoshi et al., 1992), in agreement with Knudson’s two hit hypothesis. In a situation in which a germline mutation was inherited or occurred spontaneously as in the familial adenomatus polyposis (FAP) syndrome, the rate of initiation of colonic polyps was dramatically increased with the development of thousands of colorectal adenomas and the inevitable progression of some of these into carcinoma, unless the intestine is not surgically resected (Kinzler and Vogelstein, 1996).

K-ras mutations are found in at least 50% of colorectal adenomas larger than 1 cm and in carcinomas but are infrequent in adenomas smaller than 1 cm in size (Vogelstein et al., 1988), indicating a role in adenoma progression rather than initiation. K-ras mutations affect only specific codons (12-13, 59, 61) relevant for the endogenous guanine triphosphatase activity, leading to the constitutive activation of the Ras/Raf/MEK/ERK signal transduction.
pathway. Activation of this pathway results in the transduction of signals from
the surface receptors to the nucleus for the transcriptional activation of target
genesis involved in cell proliferation and inhibition of apoptosis like cyclin
dependent kinases, cyclins and Bcl-2 (Kim and Lance, 1997) resulting in
malignant transformation. Atleast, 50% of large adenomas and 75% of
carcinomas show loss of heterozygosity at chromosome 18q (Vogelstein et al.,
1988; Vogelstein et al., 1989; Jen et al., 1994). The first candidate tumour
suppressor gene in this chromosomal interval, the "deleted in colorectal
cancer" gene (DCC), has now been identified as a component of a receptor
complex that mediates axon guidance in neurons (Keino-Masu et al., 1996).
However DCC mutations are rarely found in colorectal cancers (Cho et al.,
1994). Other tumour suppressor genes have been subsequently identified in this
region and, among others, two intracellular mediators of the TGF-β signal
transduction pathway SMAD2 and SMAD4. When SMAD2 or SMAD4 are
mutated, TGF- β signal is not transduced into the nucleus of the cell. TGFBR2
mutations are also frequently found to affect TGF-β signalling in colorectal
cancer, mainly among microsatellite instable (MSI) tumours but also in
approximately 55% of microsatellite stable (MMS) tumours (Grady et al., 1999).

Figure 2: The stepwise genetic alterations that lead to colorectal cancer
(Adapted from Powell et al., 1992)
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Management of colorectal cancer

Chemotherapy uses powerful drugs to kill cancer cells, control their growth, or relieve pain symptoms. Chemotherapy may involve one drug, or a combination of two or more drugs, depending on the type of cancer and its rate of progression. However, side effects can occur in other tissues that are dividing rapidly. Chemotherapy can be used in combination with other treatments such as surgery or radiation, to make sure all cancer cells have been eliminated. 5-fluorouracil (5FU), Capecitabine, Oxaliplatin, Irinotecan are currently used chemotherapeutic drugs to treat CRC but these drugs cause severe side effects.

Radiation

Ionizing radiation (IR) is used for the treatment of cancer and it produces ions as it passes through a tissue and the ions thus produced can cause biological damage (Jagetia, 2007). The principal types are:

- **Photons** (x-rays and gamma rays), which are most widely used and
  - **Particle radiation** (electrons, protons, neutrons, alpha and beta particles)

Some types of IR have more energy than others. In general, higher the energy, more deeply, the radiation can penetrate into the tissues.

Mechanism of ionizing radiation

Radiation is termed “ionizing” when it possesses the capacity to accelerate electrons in atomic orbits of matter. When this matter happens to be the double helix molecule of deoxyribonucleic acid (DNA) that forms the genetic material of our chromosomes, IR becomes a mechanism for mutagenesis. Mutations in the genome of somatic body cells form a point of initiation of carcinogenesis. Mutations can be of three broad kinds which include point mutations, dimerisations and direct physical changes.
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Cellular damage induced by IR is implicated in both carcinogenesis and also in cancer therapy. Advances in radiation therapy aims to decrease the dosage of radiation and thus the consequences; however, the development of radioresistance by cancer cells and toxicity of radiation to normal tissues till date is a major concern. The development of radioresistance involves several mechanisms. The present strategy of combining standard cytotoxic chemotherapeutic agents and radiation effectively leads to unwanted side effects. These chemotherapeutic agents improve the radiation efficacy by killing the cancer cells and preventing the normal cells from damage caused by the direct and bystander effects of radiation. Chemopreventive phytochemicals are generally non-toxic agents that exert both cancer preventive and therapeutic properties, and could be beneficial for this kind of approach (Nambiar et al., 2011).

Targeted effects of radiation

Chromosomal aberrations

Chromosomal aberrations can be detected during post-irradiation mitosis and are best scored during metaphase. The first reported evidence suggested that X-rays induced chromosomal aberrations in Drosophila (Muller, 1927). This study was again correlated by Painter and Muller, (1929) cytological studies. Sax, (1941) later modified and suggested ‘breakage first’ hypothesis on the X-ray of origin induced chromosome aberrations, followed by Revell, (1955) who proposed the substitute exchange hypothesis. In essence, Sax, (1941) proposed that the damaged regions of separate chromosomes come into contact after complete breaks have been induced, and the ends move about and eventually combine to form exchanges.

Micronuclei

A number of biomarkers have been assessed to test the environmental genotoxicity and the micronuclei (MN) test has served as one of the most popular approaches as an index of cytogenetic damage for more than 30 years
(Schmid, 1975; Heddle et al., 1991). MN arise from chromosome fragments or whole chromosomes that lag at cell division due to the lack of centromere, damage in centromere region, or defect in cytokinesis (Fenech, 2000). Thus MN are small, secondary structures of chromatin, surrounded by membranes, located in the cytoplasm which have no detectable link to the cell nucleus (Heddle et al., 1991). The most preferred method for measuring micronuclei in cultured cells is cytokinesis block micronucleus (CBMN) assay. Cytokinesis is inhibited by cytochalasin-B and it is recognized by the appearance of binucleated cells. The measurement of micronuclei frequency is widely used in both radio and chemo-toxicity testing.

**DNA damage**

DNA is known to be the prime target of IR-induced damage in the cell. It provokes an array of changes ranging from mutations, base lesions, cross linking, single and double strand breaks. Single strand breaks (SSBs) are more easily repaired by the cell, hence less likely to be mutagenic or lethal, whereas double strand breaks (DSBs) are more difficult for cells to repair and are more likely to result in mutagenesis, hence DSBs represent mostly the lethal cellular event (Neijenhuis, et al., 2009). Comet assay is a neutral pH assay to quantify double-stranded DNA breakages in single cells (Singh, 1988). The comet assay, also called the alkaline single cell gel electrophoresis assay, has been used in many *in vitro* applications to assess DNA damage in individualized mammalian cells. Comet assay sensitively detects DNA single and double strand breaks induced by chemical and plant bioactive compounds. Alkaline comet assay was recognized to be a very sensitive test for the detection of DNA damage but it must be carried out under carefully controlled experimental conditions. It is a highly sensitive method, and requires only a limited number of individualized cells (Sasaki et al., 1997).

Apoptosis, a programmed mode of cell death under hereditary control that may come about because of growth factor or hormonal controls, DNA damage and the aberrant expression of genes (Miller, 1999). As aberrant
apoptotic regulation is frequently seen in malignant cells, the mechanisms of apoptotic response that was elicited by chemotherapeutic drugs and IR is of particular interest for the design of new novel strategies for the treatment of cancer (Martin and Green, 1994). Apoptosis is mainly characterized by distinct changes of morphological, e.g., chromatin condensation at the nuclear periphery, cell shrinkage and membrane blebbing (Falcieri et al., 1994; Gomez-Angelats et al., 2000). These changes are accompanied by a number of biochemical alterations, where activation of caspses plays a central role. Another prominent feature of apoptotic cells is fragmentation of DNA progressing from high molecular weight (300–700 kbp) fragments (Bicknell et al., 1994) to fragments of size around 50 kbp (Zhivotovsky et al., 1994). At late stages of apoptosis, fragmentation of DNA in intranucleosomal regions may also occur, giving rise to a characteristic “ladder” of fragments as revealed by electrophoresis in agarose gels (Wyllie, 1980) (DNA fragmentation assay).

Currently, several morphological staining methods are available to detect apoptosis in vitro and one such includes ethidium bromide/acridine orange (EB/AO) dual staining method (Cohen, 1992; Coligan et al., 1995). Acridine orange (AO) permeates all cells and makes the nuclei appear green, whereas ethidium bromide (EB) is taken up by the cells only when the integrity of cytoplasmic membrane is lost, making the nucleus stain red. Thus live cells have a normal green nucleus; early apoptotic cells have bright green nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange chromatin; cells that have died from direct necrosis have a structurally normal orange nucleus (Renvoize et al., 1998).

**Damage to lipids**

The biological membranes which are mainly formed by the lipids are also affected adversely by IR exposure. Free radicals generated are responsible for the maximum extent of damage sustained by the membranes. Oxidative damage to the membrane is generally mediated by the degradation of phospholipids, which are the major constituents of the plasma membrane.
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This process is mainly brought about by the activation of the enzyme sphingomyelinase by IR, which converts sphingomyelins in membrane to ceramide (Otori et al., 1995).

Damage to proteins

IR damages the protein component of the cellular machinery, either by direct impact on protein chains, causing cross linking or by amino acid conversions or indirectly by ROS-induced redox reactions. Some of the most common chemical changes in proteins induced by IR, includes oxidation, carbonylation, cleavage and cross linking. One of the first reports of ROS-mediated protein damage was given by Garrison, by exposing solutions of amino acids and peptides to IR (Garrison, 1987).

Figure 3: Radiation-induced biological effects in cells (Adapted from Nambiar et al., 2011).

IR-induced bystander effects

Cells exposed to IR show apoptosis, DNA damage, chromosomal aberrations and for a long time it was commonly accepted that these above mentioned effects resulted from cell structure ionization and the action of

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reactive oxygen species (ROS) formed by water radiolysis, and these biological effects were attributed to irreparable or misrepaired DNA damage in cells directly hit by radiation. In the last two decades an increasing number of studies have described phenomena termed “bystander effects” i.e. the induction of damage in neighbouring non hit cells by signals released from directly-irradiated cells (Prise and O’Sullivan, 2009). Bystander effects include reduced clonogenic survival, increased sister chromatid exchange (Narayanan et al., 1997), formation of micronuclei and apoptosis and altered gene expression and levels of RNA transcripts (Rzeszowska-Wolny et al., 2009).

Figure 4: Bystander effect induced by radiation (adapted from http://www.photobiology.info/Widel.html)

Radiotherapy

Radiation therapy or radiotherapy is one of the major treatment options for cancer management and according to the best available evidence (Delaney et al., 2005), about 52% of cancer patients should receive radiotherapy at least once in the due course of their treatment. Radiation
therapy uses powerful energy sources such as X-rays to kill cancer cells that might remain after chemotherapy, shrinks the larger tumours before an operation hence it can be removed easily, or to relieve symptoms of colon cancer and rectal cancer. One of the major limitations of radiotherapy is that, solid tumours cells become oxygen deficient. Hypoxia is a condition in which solid tumours can outgrow their blood supply causing a low-oxygen state. Oxygen is a strong radiosensitizer which increases the effectiveness of a given radiation dose by synthesizing DNA-damaging free radicals. Hypoxic cell radiosensitizers such as metronidazole, misonidazole and tirapazamine have several side effects and lack target specificity, so research is focussed to enhance the effectiveness of the radiosensitizer, with lesser side effects and protective to normal cells (radioprotectors) (Steel and Peckham, 1979). The result will be decreased radiation toxicity coupled with a suitable anti-tumour effect.

Goals of radiotherapy

Despite a good therapeutic index, radiotherapy can cause tissue injury to normal tissues in long-term cancer survivors (Meister, 2005). The main health concerns during exposure to radiation are poorly understood, although the risk of carcinogenesis and degenerative diseases has been reported (White and Averner, 2001). Thus, a major challenge to radiation therapy is to increase the tolerance of normal cells by preventing their transformation to malignant cells, thereby improving the quality of patient’s life (Weichselbaum, 2005). Previous report has shown that the cellular damage caused by IR is predominantly mediated through free radicals and the resultant ROS (Weiss and Landauer, 2003).

Types of radiotherapy

Teletherapy or External-beam radiotherapy

The most frequent form of radiotherapy is teletherapy or external beam radiotherapy. The patient sits on a couch and an external radiation source is focussed at a particular area of the body.
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Linear Accelerator Unit (LINAC)

The linear accelerator (LINAC) (Figure 5), a device that uses electromagnetic waves of high frequency through a linear tube that accelerate the charged particles i.e. electrons to high energies (Haimson and Karzmark, 1963).

Figure 5: Linear accelerator (LINAC) [http://www.medical.siemens.com]

LINACS have various applications which include X-rays generation for medicinal purposes, injector for higher-energy accelerators, investigating the subatomic particle properties. The LINAC design depends on the type of the particle which is being accelerated.

Internal radiotherapy or Brachytherapy

Brachytherapy (internal radiation therapy) is delivered by placing radiation source(s) inside or behind the part of the body requiring treatment. Brachytherapy is generally used as an effective treatment for skin, breast, prostate and cervical cancer and also used in the treatment of other tumours which occurs in many other sites of body (Polgar and Major, 2009).

Unsealed source radiotherapy or Systemic radioisotope therapy

The term “unsealed source radiotherapy” relates to the use of soluble forms of radioactive substances which are administered to the body either by
injection or ingestion. Strontium-89 is taken up preferentially by the skeleton and may be used in palliation of bone metastases from, for example, carcinoma of the prostate (Robinson, 2007).

**Radioprotectors and Radiosensitizers**

Radioprotectors and radiosensitizers are chemicals that modify cells in response to radiation. Radioprotectors protect normal cells (non-cancerous) from the damage caused during radiotherapy and radiosensitizer makes the cancer cells more sensitive to radiotherapy. Since exposure to radiation during radiotherapy or accidental exposure to radiation can produce significant unwanted side effects, it is important to ameliorate such effects by the use of radioprotectors. However, only few modifiers are approved by the food and drug administration for clinical use. They are **amifostine, a protector, tirapazamine, a hypoxic cell cytotoxin** and 5-fluouracil, postoperative radiotherapeutic. These drugs cause several toxic effects which include nausea, fatigue, blood count depression, decreased parathyroid hormone level, decrease in systolic blood pressure, etc., (Caffo, 2001). Hence, the use of efficient antioxidants as radiation modifiers is becoming an important strategy in the field of radiation therapy (Glimelius et al., 2008).

**Preneoplastic lesions**

**Aberrant crypt foci (ACF)** are the initial lesions in colon cancer development that can be microscopically identified after methylene blue staining on the whole mount surface of colonic mucosa. They are identified in carcinogen treated rodents (Bird and Good, 2000) and also in humans at high risk of developing colon cancer (familial history or personal) (Pretlow and Pretlow, 2005). A number of studies in humans and rodents as well as molecular analysis has revealed that ACF are lesions that are valuable intermediate biomarkers in the development of colon carcinogenesis (Cheng and Lai, 2003). ACF are characterized by 1) crypt components that are larger than normal crypts, 2) pericryptal spaces are expanded, 3) crypts stain
darker than adjacent crypts and 4) crypt orifices differ in configuration from circular openings of normal crypts. ACF has been used in assessing and identifying the preventive or promotional role of pharmacological and natural compounds including environmental and dietary factors, in the development of colon carcinogenesis (Corpet and Pierre, 2005).

ACF have been extensively used as a biomarker for screening compounds for their chemopreventive and therapeutic activities (Bird, 1987). Total number of aberrant crypts can be considered to be an appropriate biomarker only at a very early stage of carcinogenesis, while in following weeks, higher ACF crypt multiplicities (more than 4 crypts) are considered a more specific biomarker than the total number of ACF, while in more advanced stages of colon cancer development, ACF may not be considered as a consistent intermediate colon biomarker. It is also important to reveal that ACF are not distributed equally among the proximal, middle, or distal colon. Since ACF are considered precursors for cancer, interventions and therapeutics are targeted to alter this stage or earlier to either halt disease progression, reverse it or prevent ACF formation (Stopera and Bird, 1993).

Ochiai et al., (2005) defined ACF with nuclear atypia as “dysplastic ACF”. ACFs that retain methylene blue staining even after methanol decolourisation are recognised as dysplastic ACF (DACF) and are characterized by increased basophilia, nuclear atypia, loss of mucin and atypical mitosis (Kinzler and Vogelstein, 1996). It is implied that dysplastic type ACF already have neoplastic potential in rodents and humans and a certain percentage of such lesions in the large intestine of rodents or humans could be micro-adenomas or small adenomas (Mori et al., 2005). It was common in familial adenomatous polyposis (FAP) patients, and also occurred in sporadic patients at a low frequency (Losi et al., 1996). Sporadic ACF have characteristics similar to those of dysplastic ACF in FAP patients with less frequent APC mutations and more frequent methylation. Hence DACF could be considered as a potential preneoplastic lesion rather than ACF.

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β-Catenin is a multifunctional protein; it is a critical component of cell-cell adhesion junctions and participates in wingless Wnt cell-cell signalling pathways. **β-catenin accumulated crypts (BCAC)** were identified in rodent’s colon as a new type of precancerous lesion. β-catenin has been shown to have an influence in almost all stages of colorectal carcinogenesis via the adenoma and adenocarcinoma sequence (Hirose et al., 2003; Yamada et al., 2003). BCAC develop when β-catenin protein accumulates in large volumes in the cytoplasm and/or the nuclei as a result of suppressed phosphorylation of β-catenin protein due to an abnormality in the Wnt signalling pathway (Yamada et al., 2000). Mutations in the β-catenin gene or accumulation of β-catenin are the first process in rat colon cancer development, crypts with increased expression of β-catenin have been proposed as a relevant colon cancer biomarker than ACF (Mori et al., 2004).

Thus ACF, DACF and BCAC are now frequently used as surrogate endpoint biomarkers which clarify resemblances and differences among early appearing preneoplastic lesions and provide a more rational basis for their use in DMH induced experimental model for the identification of numerous chemopreventive agents against colon carcinogenesis.

**Reactive oxygen species (ROS)**

Oxygen derived radicals represent the most important category of radical species that were generated in living systems. Molecular oxygen (dioxygen) has unique electronic configuration and is itself a radical. Superoxide anion radical (O$_2^-$) is formed by the addition of one electron to dioxygen (Miller et al., 1990). Superoxide anion, arising either through metabolic processes or following oxygen “activation” by physical irradiation, is considered the “primary” ROS and further interacts with other molecules to generate “secondary” ROS, either directly or indirectly through enzymes or metal-catalyzed processes (Valko et al., 2005).
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The hydroxyl radical (•OH) is the neutral form of hydroxide ion. With high reactivity these hydroxyl radicals, are very dangerous radicals with a very short period in vivo half-life of approximately 10^{-9} sec (Pastor et al., 2000). Additional reactive radicals derived from oxygen that can be formed in living systems are peroxyl radicals (ROO•). The simplest peroxyl radical is HO2•, which is the protonated form and is usually termed either perhydroxyl radical or hydroperoxyl radical.

Reactive nitrogen species (RNS)

Most abundant reactive radical is nitric oxide (NO•) that acts as an important signalling molecule in a large variety of diverse physiological processes which include defence mechanisms, blood pressure regulation, immune regulation, smooth muscle relaxation and neurotransmission (Bergendi et al., 1999). Overproduction of RNS is called nitrosative stress (Ridnour et al., 2004). This may occur when RNS generation in a system exceeds the system’s capacity to counterbalance and get rid of them. Nitrosative stress may perhaps leads to nitrosylation reactions which may modify protein structure and hence normal function gets inhibited.

![Diagram of Oxidative Stress]

Figure 6: Sources of ROS and the general mechanisms by which oxidative stress can alter cellular function (adapted from Peter et al., 2009).
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Lipid peroxidation

Lipid peroxidation (LPO) has been associated with several types of diseases including cancer (Kurimura et al., 1968). LPO can be defined as the oxidative degradation of lipids containing a number of carbon-carbon double bonds. Lipid hydroperoxides are intermediates of non-radicals that are derived from unsaturated fatty acids, glycolipids, phospholipids, cholesterol esters and cholesterol itself. They are formed in enzymic or non enzymic reactions with involvement of activated chemical species, and are called as reactive oxygen species (ROS), which in turn are toxic to the body. These ROS include superoxide radical, hydroxyl radical, alkoxy radical, hydrogen peroxide and lipid oxyl or peroxyl radical.

Oxidative stress is known to be associated with the pathogenesis of inflammation-related CRC (Seril et al., 2003). Biomembranes especially that of the erythrocytes are vulnerable targets for oxidative stress due to their high intracellular concentration of iron and the presence of numerous double bonds in the membrane-bound polyunsaturated fatty acids. Highly reactive hydroxyl radicals are generated through the interaction of iron and ROS and result in molecular damage.

Antioxidant defence system

An antioxidant is defined as any substance when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of those substrates. Antioxidants can inhibit LPO through competitive binding of oxygen, retarding the propagation step by destroying or binding with free radicals, inhibiting catalysts or stabilizing hydroperoxides (Halliwell and Gutteridge, 1999b). Co-operative and interactive antioxidants synergistically rely on sequential degradation of free radicals and peroxides and also on the mutual protection of enzymes. This antioxidant network plays a vital role in the regulation of protein expressions and/or activity at transcriptional and post-translational levels which also induces metabolic deviations (Rahman, 2007). The intracellular antioxidants include low molecular weight scavengers of oxidizing enzymes and species, which degrade various radicals especially O$_2^-$ and H$_2$O$_2$. 

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Antioxidant enzymes

**Superoxide dismutase (SOD)** is a primary antioxidant enzyme, which catalyzes the dismutation of $\cdot O_2^{-}$ to the less-reactive species $H_2O_2$ and $O_2$. The cellular SOD is represented by a group of metalloenzymes with various prosthetic groups. The three forms of SOD are mitochondrial Mn-SOD, cytosolic Cu-Zn-SOD and extracellular Cu-SOD.

**Catalase (CAT)** is a tetrameric haemprotein, which is located in peroxisomes and very efficiently promotes the conversion of $H_2O_2$ to water and molecular oxygen. CAT has the highest turnover rates: one molecule of CAT can convert approximately 6 million molecules of $H_2O_2$ to water and oxygen in a single minute (Halliwell and Gutteridge, 1999a; Valko et al., 2006).

**Glutathione peroxidase (GPx)** is a $H_2O_2$ degrading enzyme, which is widely distributed in animal tissues. It can also catalyze reduced glutathione (GSH)-dependent reduction of fatty acid hydroperoxides and cholesterol 7α-hydroperoxide but cannot act upon esterified fatty acid peroxides in lipoproteins or membranes. The phospholipid hydroperoxide, glutathione peroxidase is a member of GPx family which can act on peroxidised fatty acid residues within membranes and lipoproteins and reduce them to alcohols (Halliwell and Gutteridge, 1999a).

**Glutathine reductase (GR)** is an intracellular flavoenzyme, which recycles GSSG with the expense of NADPH, and helps to sustain high levels of GSH as well as GSH/GSSG ratio. Due to the central role of GSH in the scavenging and removal of deleterious ROS, GR also plays an important role in the antioxidant defence mechanisms of the cell (Dreher and Junod, 1996).

**Non enzymatic antioxidants**

The nonenzymatic antioxidants are low molecular weight substances, which include vitamin C, vitamin E, natural flavonoids, melatonin (a hormonal product of the pineal gland) and other compounds. Some antioxidants act in an hydrophilic environment, others in a hydrophobic environment, and some act in both the environments of the cell (Valko et al., 2006). The intracellular redox buffering capacity is supported primarily by GSH.
Reduced glutathione (GSH) is a tripeptide, which is the major non-enzymatic regulator of intracellular redox homeostasis. GSH is highly abundant in cytosol (1-11 mM), mitochondria (5-11 mM), nuclei (3-15 mM). The protective role of GSH originates from multifactorial mechanisms that involve detoxification, modulation of cellular redox state, the subsequent redox sensitive cell signalling pathways and interaction with pro and antiapoptotic signals (Masella et al., 2005).

Vitamin C (ascorbic acid) is a powerful and an important antioxidant that works in aqueous environments of the body, which include lungs and lens of the eye. Its primary antioxidant partners are vitamin E (α-tocopherol), carotenoids and also acts along with other antioxidant enzymes. Vitamin C co-operates with vitamin E to regenerate α-tocopherol from α-tocopherol radicals in membranes and lipoproteins (McCall and Frei, 1999; Upritchard et al., 2000).

Vitamin E (α-tocopherol) is a fat-soluble vitamin that exists in eight different forms. The most active form of vitamin E in humans is α-tocopherol, and it is a major powerful membrane bound antioxidant employed in the cell (Burton and Ingold, 1989). Vitamin E functions as a chain breaking antioxidant that prevents the propagation of free radical reactions. It quenches the lipid peroxides and protects the cell structure from free radical attack (Upritchard et al., 2000).

Xenobiotic metabolic enzymes

The identification plants which help in detoxification has increased rapidly during recent years. Detoxication enzyme systems were identified in agronomically important plants like wheat, maize and oat and also in various plant cell cultures. The intake of antimutagens and anticarcinogens present in food components may lead to a decreased risk of cancer onset (Negishi et al., 1989). On entering the food chain, environmental contaminants may be subjected to metabolism by xenobiotic metabolizing enzymes. Drugs and other commonly used organic chemicals are all xenobiotics, and are
metabolized by the same enzymes as drugs. The xenobiotic metabolism is often classified into phase I and phase II reactions. The cytochrome P450 and cytochrome P450 isoenzyme systems are considered as most important in the phase I biotransformation of both endogenous and exogenous compounds. It consists of different enzymes with specific but overlapping substrate specificity.

Important phase II enzymes include UDP glucuronyl transferases (UDP-GT), sulphatases, acetyltransferases, DT-diaphorase and glutathione S-transferases (GST). Phase II enzymes generally reduce the bioactivity of the xenobiotic, increasing its hydrophilicity and thus promoting its excretion. Both phase I and phase II enzymes can be induced by exogenous compounds. With respect to contaminants, two main types of inducers can be distinguished. Exposure to planar molecules, like polycyclic aromatic hydrocarbons, result in the increased activity of the CYP1A subfamily (Boon et al., 1992). Xenobiotic metabolizing enzymes also play an important role in the balance of endogenous compounds. Most carcinogens and xenobiotics are primarily metabolized by the microsomal cytochrome P450 system, localized in liver more abundantly than in other organs such as lung, kidney or intestine (Connely and Bridges, 1982).

**Bacterial transforming enzymes of the colonic microflora**

Many foreign compounds are detoxified by glucuronide formation in the liver before entering the intestine via the bile. Because of its wide substrate specificity, the bacterial enzymes have the ability to hydrolyse many glucuronides and may thus liberate carcinogenic aglycones in the intestinal lumen. Several other bacterial enzymes have also been implicated in the carcinogenic process, releasing carcinogens in the intestinal tract.

A relationship between a western-style diet (fats, low vegetable intake and red meat) and the changes in gut microbiota composition has been observed in human and animal studies. This was linked to the increased activities of faecal bacterial enzymes, as well as modification of sulphidogenesis and metabolism of biliary acid with an impact on the development of procarcinogenic conditions (Keefe et al., 2009).
**β-glucuronidase** exists in almost in all tissues and is involved in the stepwise degradation of glucosamine-glucuronides (Kyle et al., 1992). β-glucuronidase has been considered as a key enzyme for the activation of DMH metabolites to carcinogens in the colonic lumen (Reddy et al., 1974). Bacterial β-glucuronidase a hydrolase that catalyzes the cleavage of terminal glucuronic acid, is believed to be largely responsible for the hydrolysis of glucuronide conjugates in the gut and thus plays an important role in the generation of toxic and carcinogenic substances (Chipman, 1982). Bacterial **β-glucosidase** hydrolyses plant glycosides to release aglycones, many of which are mutagenic, although some also have anticarcinogenic activity (Rowland, 1995).

**Mucins** (highly glycosylated macromolecules) form the initial barrier between the epithelial cells and gut contents, shielding them from direct contact with their components and commensal bacteria. Changes in the amount and/or the mucus composition which can lead to inflammatory responses (Linden et al., 2008). The epithelial layer, which is covered by glycocalyx, forms a major barrier between the host and the environment.

**Nitroreductases** are widely distributed among bacteria, but nitroreductase-like proteins are also found in archaea and eukaryotic organisms. In addition, most bacteria contain several types of nitroreductases. Thus, four different nitroreductases have been isolated from the gut microbial community organism *Bacteroides fragilis* (Kinouchi and Ohnishi, 1983). Nitroreductases, first described in the bacteria were able to reduce chloramphenicol and p-nitrobenzoic acid to their respective amines (Villanueva, 1964). Nitroreductase participates in the conversion of aromatic compounds such as dinitrotoluene, nitrobenzenes and nitropyrenes to amines which often exhibit toxic, mutagenic or carcinogenic activities (Facchini and Griffiths, 1981). Genes coding for nitroreductase-like proteins are present in most bacterial genomes, and are also found in archaea and eukaryotic organisms (Marques de Oliveira et al., 2007).
Sulphatases are categorized under four headings by Fraomagot, according to the nature of the organic moiety: phenylsulphatases, glucosulphatases, myrosulphatases and chondrosulphatases. Fecal sulphatase activity should also be considered in the desulphation of conjugated toxins and in the degradation of sulphated mucins. Changes in the expression of sulphated molecules such as mucins and other glycoconjugates have been demonstrated in the transformed colonic epithelial cells.

Cell proliferation

Increased cell proliferation has been proposed to be a biomarker of increased susceptibility to gastrointestinal cancer (Lipkin, 1988; Lippamnn et al., 1990). Colonic epithelial cell proliferation changes are considered to be indicators of increased risk of colon cancer (Bostick et al., 1997).

Nucleolar organizer regions (NOR) are loops of DNA that contain ribosomal RNA genes. These genes are transcribed by RNA polymerase I and ultimately result in direct ribosomal formation and protein synthesis. NOR-associated acidic proteins are related to sites of rRNA transcription during interphase of the cell cycle (Roussel et al., 1994). Agyrophillic nucleolar organizing region (AgNORs) dots in a nucleus reflect the status of cell activation and therefore are useful as an index of cell proliferation (Bostick et al., 1997). The amount of AgNORs represent a cell kinetic parameter used in tumour pathology for prognostic purposes. AgNOR counts have been reported as an useful, complementary diagnostic tool for several neoplasias including breast carcinoma, lymph node metastasis, pancreatic carcinoma and colon cancer (Cohen and Ellwein, 1990).

Proliferating cell nuclear antigen (PCNA) is a 36 kDa nuclear protein which functions as an auxiliary protein for DNA polymerase δ and is an absolute requirement for DNA synthesis (Fairman, 1990; Hall and Levison, 1990). It is a stable cell-cycle regulated nuclear protein that is expressed differentially during the cell cycle and whose rate of
synthesis is correlated directly with the proliferative rate of cells. The levels of PCNA increase in the nucleus during the late G1-phase, immediately before the onset of DNA synthesis, become maximal during the S-phase, decline during the G2-phase and reach a low level in the M-phase and quiescent cells. Moreover, during the cell cycle, two populations of cells with differing PCNA can be distinguished. Abnormal cellular proliferation is one of the crucial mechanisms in carcinogenesis (Jia and Han, 2000).

**Cyclin D1** protooncogene is an important regulator of G1 to S phase progression in many different cell types. Together with its binding partners cyclin dependent kinases 4 and 6 (CDK4 and CDK6), cyclin D1 forms active complexes that promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein (RB) (Kato et al., 1993; Lundberg and Weinberg, 1998; Weinberg, 1995). The cyclin D1 protein has been shown to be unstable with a short half life (~24 min) (Diehl et al., 1998) and is degraded mainly via the 26S proteasome in an ubiquitin-dependent manner. Cyclin D1 is important for the development and progression of several cancers including those of the breast, oesophagus, bladder and lung (Hall and Peters, 1996). Overexpression of cyclin D1 has also been linked to the development of endocrine resistance in breast cancer cells (Hodges et al., 2003; Hui et al., 2002; Kenny et al., 1999). Cyclin D1 overexpression is a common event in cancer but does not occur solely as a consequence of gene amplification.

**Apoptotic response and cancer**

Apoptosis was first at the beginning described through its morphological traits, together with cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Kerr et al., 1972; Wyllie et al., 1980). Apoptosis (programmed cell death) is a genetically regulated form of cell death and is essential for the balance between proliferation, growth arrest, and cell death thereby ensuring normal development of multicellular organisms and maintenance of tissue homeostasis. Apoptosis
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plays a vital role in cancer prevention. In the event that a cell is not able to undergo apoptosis due to mutation or biochemical restraint, it keeps on dividing and progresses into a tumour. Apoptosis is an important phenomenon in cytotoxicity induced by anticancer drugs. The execution of apoptosis, or programmed cell death, is associated with characteristic morphological and biochemical changes mediated by a series of gene regulations and cell signalling pathways (Evan and Vousden, 2001). Stimulation of apoptosis by natural components provide a protective mechanism against cancer by eliminating genetically damaged cells before they can transform to tumour cells. Apoptosis is mediated via an intrinsic pathway in which activation takes place inside the cell mitochondria or via an extrinsic pathway in which activation occurs from outside the cell by means of cell surface receptors [APO1/CD95 and the tumour necrosis factor (TNF) receptor family]. In the intrinsic pathway, cytochrome c is a released from the mitochondria into the cytosol, where it binds and activates an adaptor protein called apoptotic-protease-activating factor1 (APAF1). This protein binds and aggregates procaspace -9 molecules, which leads to the cleavage of these molecules and the triggering of the caspase cascade (Albers et al., 2002).

The p53 protein, also called tumour protein 53, is one of the best known tumour suppressor proteins encoded by the tumour suppressor gene TP53 located at the short arm of chromosome 17 (17p13.1). It is named after its molecular weight, i.e., 53 kDa (Levine et al., 1991). p53 is a transcription factor placed at the nexus of a number of pathways that mediate apoptosis response to a wide range of cellular stress. These include DNA damage, hypoxia and nutrient deprivation, cell survival and proliferation (Hofseth et al., 2003; Lowe et al., 2004). Activation of p53 results in the activation of oncogenes, hypoxia and DNA damage, resulting in growth arrest and/or apoptosis by stimulating the expression of various p53 target genes such as p21, Bax, Puma, Mxa, Apaf-1 and Fas (Vousden and Lu, 2002) or by repressing the expression of antia apoptotic

Combined therapeutic efficacy of CVC and X-radiation
proteins e.g. Bcl-2, Bcl-xl (Wu et al., 2000; Hoffman et al., 2002; Vogelstein et al., 2002). The varied functions of p53 including control of the cell cycle, DNA repair and programmed cell death have earned it the name, “guardian of the genome”. Defects in the p53 tumour suppressor gene have been linked to more than 50% of human cancers.

**CDX-2** is an intestine-specific transcription factor, caudal-type homeobox, which is expressed early in intestinal development and involved in the regulation of differentiation and proliferation of intestinal epithelial cells (Beck, 2004). Both CDX-2 and CDX-1, is of particular concern as intestine is the only organ containing obvious gene product levels. This restricted gene expression pattern is unusual for homeobox genes. CDX-2 domain activation by phosphorylation can modulate its different spatial expression patterns and functions in intestinal epithelium.

The **Bcl-2** family of proteins play a critical role in controlling death via the intrinsic, or mitochondrial, programmed cell death pathway. Bcl-2, the namesake of the family, was identified at the breakpoint of the t(14;18) translocation common to follicular lymphoma (Tsujimoto et al., 1984; Cleary and Sklar, 1985; Bakhshi et al., 1985).

Bcl-2 proteins largely interact at the mitochondria, the nexus of events that irreversibly commit a cell to programmed cell death via the intrinsic pathway. Some Bcl-2 proteins are localized to the mitochondria even during normal cellular conditions while many have other subcellular locations. Bcl-2 itself is found not only in the mitochondria, but also in the endoplasmic reticulum where it is implicated in calcium homeostasis (Bassik et al., 2004). Many Bcl-2 family members have identified roles outside the control of apoptosis, and it is likely that Bcl-2 family members are important in other aspects of cellular homeostasis.

**Bax** is the death promoter that is inactivated in certain types of colon cancer in hematopoietic malignancies (Meijerink et al., 1998). Upon apoptotic stimuli bax translocated from cytoplasm to mitochondria, which induces
apoptosis in two ways either by binding to Bcl-2 family antiapoptotic proteins or by disrupting the function of mitochondrial membrane barrier by ion-permeable pore formation in mitochondrial membranes.

**Cytochrome C** is an essential component of the mitochondrial respiratory chain. It is a soluble protein, localized in the intermembrane space, and is loosely attached to the surface of the inner mitochondrial membrane. Cytochrome C plays an important role in initiating apoptosis (Arnoult et al., 2002). Once released into the cytosol, Cytochrome C binds to apoptotic protease activating factor-1 (Apaf-1), leading to an unmasking of its caspase requirement domain and the subsequent binding and autoproteolytic activation of procaspase-9. The complex of procaspase-9, cytochrome C and Apaf-1 are known as the apoptosome.

**Cysteine aspartic proteases**, called *caspases* upon activation through the intrinsic and/or extrinsic pathway destroys essential proteins leading to controlled cell death. There are two tiers of caspase activation during apoptosis. Initiator caspases (caspases-4, -8, -9, -10, -12) are activated through the apoptosis-signalling pathway and activate the effector caspases (caspases-3, -6, -7, -14) which in an expanding cascade, carryout apoptosis (Thornberry and Lazebnik, 1998; Lavrik et al., 2005). Upon activation by an initiator caspase cleaving certain cellular substrate to cause demolition of the cell (Ho and Hankins, 2005). Apoptosis proceeds through caspase activation cascades, known as the extrinsic and intrinsic pathways. The extrinsic pathway-induced apoptosis is mediated by receptors (FADD), which activate initiator caspase-8 or 10 signalling that leads to activation of executioner caspases such as caspase-3, 6, 7 and 9. Steps in the intrinsic pathway, which is induced by stress, radiation and chemotherapeutic drugs, include cytochrome C release from mitochondria, caspase-9 activation and then activation of effectors caspases, particularly caspase-3 (Green and Reed 1998).
Inflammation and cancer

The link between inflammation and cancers, rather than a recent concern, was noticed 150 years ago (Balkwill and Mantovani, 2001). Inflammation is a physiologic process in response to tissue damage resulting from microbial pathogen infection, chemical irritation, and/or wounding (Philip et al., 2004). At the very early stage of inflammation, neutrophils are the first cells to migrate to the inflammatory site under the regulation of molecules produced by rapidly responding macrophages and mast cells petitioned in tissues (Okada, 2002; Nathan, 2002). Cytokines are the membrane-bound molecules that play a regulatory role in the growth, differentiation, and activation of immune cells (Dranoff, 2004). Cytokine signalling could contribute to the progression of tumours in two aspects: the stimulation of cell growth and differentiation and the inhibition of apoptosis of altered cells at the inflammatory site (Pollard, 2004).
Cyclooxygenase (COX)-2 is an inducible enzyme, whose expression is upregulated by NF-κB mediated tumourigenesis. COX-2, the inducible isoform of prostaglandin H synthase, has been implicated in the growth and progression of a variety of human cancers. Epidemiologic studies recently have shown 40–50% mortality reduction from colorectal cancer in individuals who consume nonsteroidal anti-inflammatory drugs on a regular basis compared with those not consuming these agents. These drugs having their ability to inhibit COX, a key enzyme in the synthesis of prostaglandins from arachidonic acid. Enhanced COX-2 expression has been found in colon cancer tissues from subjects with clinically diagnosed colorectal cancer (Sano et al., 1995; Tsuji et al., 1997, 1998). Cyclooxygenase regulates colon carcinoma-induced angiogenesis by two mechanisms: COX-2 can modulate the production of angiogenic factors by colon cancer cells, while COX-1 regulates angiogenesis in endothelial cells (Einspahr et al., 2003). COX-2 expression in human tumours can be induced by various growth factors, cytokines, oncogenes and other factors.

Interleukin-6 (IL-6) is a pleiotropic inflammatory cytokine. First discovered as a B-cell growth factor, it is synthesized by many cell types, including T-cells, macrophages and stromal cells, in response to stimulator from tumour necrosis factor-alpha (TNF-α) and IL-1 (Ardestani et al., 1999). IL-6 is a pleiotropic immune regulatory cytokine that plays an important role in tumour progression, metastasis and angiogenesis (Rose-John and Neurath, 2004). IL-6 can antagonize p53 function, and therefore favour target-cell survival in foci of chronic inflammation (Hudson et al., 1999). Komoda et al., (1998) found an approximately eightfold increase of IL-6 protein in cancerous lesions when compared to normal colonic mucosa.

Mast cells play a vital role in acute inflammation due to their newly synthesized and stored inflammatory mediators release after activation. Mast cells releases various factors which are well known to develop angiogenic phenotypes which includes histamine, heparin, heparanase, metallo and serine proteinase and diverse polypeptide growth factors such as vascular
endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Coussens et al., 1999). Hence, mast cells provide direct mitogens for endothelial cells, epithelial cells, fibroblasts and diverse enzymatic activities which are involved in remodeling of extra cellular matrix.

**Angiogenesis**

Angiogenesis is the process of formation of new blood vessels from preexisting blood vessels, is critical for furnishing the nutrients and oxygen necessary for tumor growth and metastasis. It is well known that angiogenesis is primordial for solid tumour growth and dissemination (Folkman, 1990).

**Vascular endothelial growth factor (VEGF)** and its receptors play a pivotal role in normal and pathologic angiogenesis. Activation of the VEGF/VEGF-receptor axis triggers multiple signalling networks that result in endothelial cell- survival, mitogenesis, migration, and differentiation, and vascular permeability and mobilization of endothelial progenitor cells (EPCs) from the bone marrow into the peripheral circulation. In addition, VEGF mediates vessel permeability, and has been associated with malignant effusions (Zebrowski et al., 1999). The permeability induced by VEGF leads to deposition of proteins in the interstitium that facilitate angiogenesis. Overexpression of VEGF has been associated with tumour progression and poor prognosis in several tumour systems, including colorectal carcinoma, (Dvorak, 2002) gastric carcinoma, pancreatic carcinoma, breast cancer, prostate cancer, lung cancer, and melanoma. VEGF and its receptors are regulators of angiogenesis, emphasizes its importance in the genesis and maintenance of vasculature necessary for the progression and metastasis of malignant disease. VEGF 165 is the predominant isoform and commonly overexpressed in a variety of human solid tumours.

**Hypoxia-inducible factor 1 (HIF-1)** is a heterodimeric protein consisting of an oxygen regulated HIF-1α and a constitutively expressed HIF-1β subunit. HIF-1α is rapidly degraded under normoxia, whereas hypoxia leads to stabilization and accumulation of HIF-1α (figure 9). However, under
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certain normoxic conditions, HIF-1α expression can be increased; for example, mutations in the von Hippel-Lindau (VHL) protein stabilize HIF-1α protein and PI3K/AKT/mTOR activity stimulates translation of HIF-1α mRNA (Rade makers et al., 2008). Moreover, reactive oxygen and nitrogen species inhibit proteasomal degradation of HIF-1α (Semenza, 2010). After stabilization of HIF-1α, the heterodimeric protein activates transcription of numerous genes involved in proliferation, angiogenesis, pH regulation and glycolytic tumour metabolism. Mutations in VHL are associated with renal cancer and cerebellar haemangioblastomas (Maxwell et al., 1999). HIF activity is deregulated in many human solid tumours, especially those that are highly hypoxic. Hypoxic tumour cells are generally resistant to radiotherapy and most conventional chemotherapeutic agents, depicting them highly aggressive and metastatic. Overexpression of HIF-1α, the regulatory subunit of HIF, is associated with increased vascular density, treatment failure, severity of tumour grade and a poor prognostic outcome with conventional therapies. Increased levels of HIF-1α are related with increased proliferation and increased expression pattern of VEGF. HIF-1α is overexpressed in renal, colon, gastric, breast, lung, skin, ovarian, prostate, and pancreatic carcinomas associated with tumour cell proliferation (Zhong et al., 1999).

Figure 8: Schematic representation of the HIF-1 pathway (adapted from Meijer et al., 2012)
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NF-κB in cancer

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a collective term referring to dimeric transcription factors of the Rel family. The nuclear factor κB (NF-κB) family of eukaryotic transcription factors influence a number of important cellular and organismal processes, including cellular growth control, apoptosis, immune and inflammatory responses, and cellular stress responses (Loop and Pahl, 2003). The mammalian NF-κB family includes five related proteins (p50, p52, p65, c-Rel, and RelB) that form various combinations of homodimers and heterodimers to control the activity of numerous genes. In many cell types, NF-κB is located in the cytoplasm in a latent, inactive form primarily bound to the inhibitor protein IκBα. NF-κB can be activated by a multi-component signal transduction pathway. Namely, in response to a variety of inducers (e.g., cytokines, growth factors), an IκB kinase (IKK) is rapidly activated that then phosphorylates two serine residues (Ser32 and Ser36) in IκBα (Hayden and Ghosh, 2004). Phosphorylated IκBα then undergoes polyubiquitination and is subsequently proteolytically degraded by the 26S proteasome (Deng and Chen, 2003). The freed NF-κB complex can then enter the nucleus to regulate target gene expression. Several reports have shown that NF-κB is constitutively active and present in the nucleus in a variety of human tumour cell types and cell lines (Gilmore et al., 2002). Moreover, inhibition of this chronic NF-κB activity can, in many cases, slow the growth of these tumour cell lines or induce cell death. For example, the blocking of NF-κB signalling by arsenic-mediated inhibition of IKK sensitizes Hodgkin/Reed-Sternberg cell lines to apoptosis (Mathas et al., 2003).

Role of Nrf2 in cancer

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a member of the basic leucine zipper family which plays an essential role in the antioxidant responsive element mediated expression of phase II detoxifying enzymes and stress inducible genes (Zhang and Gordon, 2004). It is present as an
inactive complex in the cytoplasm with the inhibitory protein, Keap-1 (Kelch-like ECH-associated protein-1). Once Nrf2 is translocated to the nucleus, it interacts with a small Maf protein forming a heterodimer that binds to the antioxidant response element (ARE) present in the enhancer/promoter area of genes that encoding many detoxifying and antioxidants enzymes. Through the phosphorylation of Nrf2 it is assumed that dissociation of this Nrf2-Keap1 complex by different upstream kinases like PKC, MAPK and P13K. Activation of protein kinases upon stimulation such as P13K, PKC, JNK, p38, MAPK and ERK induce Nrf2 phosphorylation which facilitates the dissociation of Nfr2 from its repressor Keap1 and subsequent translocation to nucleus and interaction with the coactivator CBP/p300 (Surh and Na, 2007). Alternatively, prooxidants/electrophiles may directly interact with cysteine residues present in Keap-1 thereby stimulating Nrf2 dissociation.

Nrf2, has the ability to control many different aspects of cellular protection, including DNA repair (Aoki et al., 2007), haem metabolism (Ishii et al., 2000), quinine reduction (Itoh et al., 1997), efflux transport (Maher et al., 2007) and glutathione synthesis (Sekhar et al., 2002). In fact, the regulation of these genes is typically ascribed as the main function of Nrf2, and this regulation was clearly beneficial from an evolutionary standpoint.

**TGF-β/Smad pathway**

Transforming growth factor-betas (TGF-βs), the cytokines expressed in colon which play an important role in tumour suppressing and tumour promoting during colorectal carcinogenesis (Ewen et al., 1993). TGF-β signalling pathway involves Smad-2 and Smad-3 activation by type I receptor and Smad-2/3/4 heteromeric complexes formation which enters the nucleus transcriptional regulation. Most human CRCs are resistant to the tumour suppressor effects of TGF-β and a subset of human CRCs have mutations in Smad-2 and Smad-4 (Li et al., 2005). Emerging studies on TGF-β signalling pathway have shed light on the role of TGF-β in carcinogenesis. DPC4 (Smad-4/ALK3) and Smad-2 are the genes that encode small proteins related to the
Drosophila mad (mothers against decapentaplegic). These proteins are essential intracellular components of the TGF-β pathway and act as tumour suppressors. Alterations of the TGF-β pathway are also a frequent finding in CRC either by inactivation of Smad-4 or by mutations of the TGF-β type II receptor (Hahn et al., 1996). TGF-β signalling pathway can behave both as a tumour suppressor and as a tumour promoter pathway (Derynck et al., 2001; Siegel et al., 2003). Whatever the scenario, TGF-β gains an unique position in the process of carcinogenesis and dietary agent targeting TGF-β would be beneficial in the field of cancer prevention.

**MAPK pathway**

The mitogen activated protein kinases (MAPKs) are a family of signal transduction proteins that convert extracellular signals, such as stresses and growth factors, to the activation of intracellular pathways of MAPKs have been highly conserved from yeast to multicellular organisms. In mammals, at least four subfamilies of MAPKs have been described including the extracellular signal regulated kinase (ERKs), the c-jun-NH2-terminal kinases (JNKs), the p38 isoforms (p38) (Pearson et al., 2001; Wagner and Nebreda, 2009). MAPKs are mainly modulated by upstream MAPK kinases (MKKs) and MAPK phosphatases that modify the phosphorylation of the MAPK threonine and tyrosine (T-X-Y) motif. Activated MAPKs catalyze the phosphorylation on specific serine and threonine residues of target substrates and trigger a wide range of cellular responses, such as proliferation, differentiation, and apoptosis. In consistence with their important roles in cellular processes, MAPKs have been found to play key physiological roles such as regulating embryonic development and maintaining tissue homeostasis. On the other hand, extracellular stresses can induce MAPK activation in pathological conditions. For example activation of MAPK activation is critical in regulating inflammation associated cancer development (Wagner and Nebreda, 2009). Moreover, MAPKs have been considered promising targets for therapies, and
great efforts are therefore being made to explore new functions and mechanisms regulated by MAPKs in disease conditions. Previous report quoted that the expression of NF-κB, p38, MAPK and JNK are high and active in the stroma of human colonic adenomatous polyps (Hardwick et al., 2001).

**DNA repairing genes**

Repairing of DNA plays a crucial role in protection of normal individuals from radiation effects, including cancer. Genes controlling the process of DNA repair encode enzymes which catalyzes DNA damage from cellular responses. Repair function loss or changes in repair processes control has severe on cells and individuals. More than 130 genes are known to be involved in the repair of different types of DNA damage and the disruption of the transcription of these genes accounts for the lethal effects of DNA damage. **Human 8-oxoguanine glycosylase (hOGG1)** is a DNA glycosylase enzyme involved in base excision repair (BER). hOGG1, the enzyme responsible for the 8-oxoguanine excision, a mutagenic base byproduct occurs as a outcome of exposure to ROS. It is a bifunctional DNA glycosylase and apurinic/apyrimidinic (AP) lyase activities. hOGG1 recognizes both 8-oxoguanine and 8-oxoadenine and removes these oxidized bases from double stranded DNA, initiating the base lesion repair. **X-ray repair cross-complimenting protein 1 (XRCC1)** is a protein that is encoded by the XRCC1 gene in humans. Its major function is involvement in DNA repair where it forms complexes with DNA ligase III. It plays a vital role in processing of DNA during recombination and meiogenesis in germ cells. XRCC1 is overexpressed in non-small cell lung carcinoma (NSCLC) (Kang et al., 2010) and at an even increased level in NSCLC metastatic lymph nodes (Kang et al., 2009). Several polymorphisms in DNA repair genes have been reported to be associated with cancer risk (Ishikawa et al., 2005). Mutations in DNA repair genes could be either responsible for the occurrence of tumours or could arise due to random accumulation of mutations during cycling of cancer cells. The presence of incorrect DNA repair in tumour cells predisposes them to accumulate even more genetic alterations.
Experimental models in colon cancer research

In vitro

Human blood lymphocytes

Human blood lymphocytes represent a cellular population which is predominantly in a DNA pre-synthetic stage of the cell cycle (i.e. the G0 phase). Moreover, the lymphocyte concentrations in the peripheral blood are also variable. Nowell, (1960) was the first person to show that phytohaemagglutinin (PHA) stimulates peripheral ‘human leukocytes’ to undergo in vitro mitoses, while Carstairs, (1962) showed that ‘small lymphocytes’ are the target cells for initiation of mitogens by PHA. This makes lymphocytes ideal targets for looking for induced aberration (Fabry and Coton, 1985). Therefore studies using peripheral blood lymphocytes as a model system to identify in vitro cytogenetic and biochemical alterations could be of immense significance in persons exposed to radiation accidentally and therapeutically.

Cell lines

The term cell line has been coined to describe cells that proliferate continuously. Cell culture has been performed since the beginning of the 20th century (Harrison, 1907; Sanford et al., 1948). The development of mortal and immortal cell lines has been a significant benefit to the study of human disease. Although in those days, it represented a relatively primitive methodology in biomedical research, cell culture nevertheless generated some very impressive results. A crude definition of cell culture could be a method to perform experiments on cell populations without being encumbered by the whole organism (Gruenert, 1987; Hopfer et al., 1996). The second major improvement in cell culture technology was cryopreservation and the subsequent recoverability of the stored cells. This in vitro experimental system, using cell line based research is the best method for therapeutic drug production and animal welfare.
HT-29 cells produce secretory component of carcinoembryonic antigen (CEA) and Immunoglobulin A (IgA). The HT-29 cell line has been designated heterotransplantable that forms well-distinguished grade I tumours. The structure of HT-29 cells which includes microfilaments, mitochondria, microvilli, lipid droplets, rough and smooth endoplasmic reticulum with free ribosomes, limited primary lysosomes and many secondary lysosomes.

SW480 cells are the least differentiated and most quickly proliferating, which make them fundamentally the same to carcinogenic colon cells in the human body. The more distinguished cells like Caco-2 cells, are actually more representative of small intestinal cells than colon cells (Izuishi et al., 2000; Meunier et al., 1995). This cell line is positive for several oncogene mutations including myc, ras, fos, and p53 which makes it genetically similar to tumours found in the human colon. The SW480 cell line is also poorly differentiated, which is typical of cancer cells, and this characteristic makes it an appropriate model to study colon cancer (Brattain et al., 1999).

**In vivo**

**Animal models**

The study of colon carcinogenesis in rodents has long history, dating back to almost 80 years (Krebs, 1928). Recently identification of genetic alterations in polyps and tumours of colon has permitted the creation of hypothesis for the molecular origin of colon carcinogenesis. Suitable animal models are crucial to the testing of molecular postulates and also in the development of markers and colon cancer treatment. Mouse models are essential tools for the preclinical testing of novel therapeutic options *in vivo* (Pories et al., 1993).

1, 2-dimethylhydrazine (DMH) and its metabolite, azoxymethane (AOM), are procarcinogens form DNA-reactive products through metabolic activation. The mutagenic effects of DMH, AOM and other alkylating agents occur initially through methylation of guanine in DNA at the N-7 position. The alkylated guanines are modified so as to pair with thymidine rather than
cytosine. They donate a proton to cytosine and lead to the modification of both bases. During subsequent replication, mispairing of guanine to thymine and cytosine to adenine occurs, leading to mutations of DNA. DMH and AOM injected at the doses of 10 to 20 mg/kg body weight produce colonic adenomas and adenocarcinomas in rats, mice, and hamsters (Thurnherr et al., 1973). The latency period is approximately six months. Even a single injection of 10 mg/kg DMH or AOM produces colon cancers in rats after a latency period of 15 to 20 months (Pozharisski et al., 1975). Metabolism of these compounds involve multiple xenobiotic metabolising enzymes, which proceed through several hydroxylation and N-oxidation steps, including the methyldiazonium (MAM) formation following AOM hydroxylation. The reactive metabolite, MAM, readily yields a methyldiazonium ion, which can alkylate macromolecules in the liver and colon (Fiala et al., 1984).

MAM, a substrate of nicotinamide adenine dinucleotide (NAD) dependent dehydrogenase is present in the liver and colon, suggesting that the active metabolite of MAM might be the corresponding aldehyde (Zedde et al., 1979). The metabolites of CYP2E1 are transported to the colon through the bloodstream. Alkylation of DNA is extensively seen in the colonocytes, but some metabolites are present in the liver. There are probably two mechanisms of initiation of carcinogenesis. The main pathway involves hepatic conversion of DMH to AOM and azoxymethanol which subsequently undergoes glucuronic acid conjugation and biliary excretion (Fiala et al., 1977). The glucuronides undergo bacterial hydrolysis and metabolism to the active carcinogen in the colonic lumen (Weisburger, 1971).
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Figure 9: Diagrammatic representation of DMH metabolism

The etiology of CRC is very complex, and both genetic and environmental factors are thought to be involved in this process. Among environmental factors, and dietary habits play an important role. High consumption of meat and fat, together with low consumption of fruits, vegetables, vitamins, and fibers, have been suggested to increase the risk of CRC (Health Canada, 2005; Johnson and Mukhtar, 2007). Many epidemiological studies have demonstrated a positive relationship between dietary fat intake and CRC. Experimental studies have shown that a high-fat diet rich in omega-6 polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) promote colon carcinogenesis, particularly in the post initiation or promotional phases and/or both (West et al., 1989; Lin et al., 2004; Oba et al., 2006; Theodoratou et al., 2007). Peanut oil was used as a tumour promoter in our study. Peanut oil is rich in omega-6 fatty acids that are metabolized into precursors of inflammatory molecules such as eicosanoids and prostaglandins that can stimulate tumour growth and metastasis.
Chemotherapy

Chemotherapy is different from radiation therapy and surgery in that the drugs act against cancer, circulating in blood to body parts where the cancer cells get killed or eliminated at locations great distance from the area of the original cancer occurrence. Accordingly chemotherapy is considered as a systemic treatment in cancer. Many people who are diagnosed with cancer receive chemotherapy, respond well and, this approach helps in effective treatment enabling them to get full productive lives (Cragg et al., 1997).

Medicinal plants and cancer research

For the past many decades, there are many studies to evaluate the chemotherapeutic and chemopreventive role of substances present in natural products. Medicinal plants are frequently used by traditional healers to treat a variety of ailments and symptoms including diabetes and cancer. Over 80% of the world’s population rely upon such habitual plant-based medicine systems to provide them primary healthcare according to the World Health Organization (WHO) (Calixto, 2005). Dietary intake of phytochemicals has been associated with decreased risk of cancer and significant survivability of cancer patients. Over 60% of anticancer drugs available in the market are of natural origin (Ho et al., 2002). Protection against IR induced damage has realistic applications in radiotherapy treatment of cancer and also in decreasing the risk to exposed individuals. Many natural compounds have been investigated in the past for the efficacy to reduce the adverse effects of ionizing radiation (Weiss and Landauer, 2003; Gutteridge and Halliwell, 2000). However, the inherent toxicity of some of the synthetic agents at their radioprotective concentrations warranted further search for safer and effective compounds (Landauer et al., 1992; Monig et al., 1990; Uma Devi, 1998). So, within these restraints, the strategy to evaluate the radioprotective/radiosensitizing ability of physiologically acceptable and non toxic compounds seems to be promising and warrants investigation. The use of naturally occurring compounds as radiation modifier has become an important strategy in the field of radiotherapy. In human diet naturally occurring compounds are commonly non toxic within certain doses.
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Carvacrol

Carvacrol (2-methyl-5-(1-methylethyl-phenol; CVC), used in this study, is a monoterpenoic phenol which is present in many essential oils of the family Labiatae including *Origanum, Satureja, Thymbra, Thymus*, and *Coridothymus* species, which have widespread use in folk medicine and aromatherapy (Lemos et al., 1990; Krimer et al., 1995). CVC has been reported to be a constituent in the essential oils not only of *Labiatae* but also of other families such as Chenopodiaceae, Plantaginaceae, Umbelliferae, Verbenaceae, etc. CVC-rich oils reported earlier from *Labiatae* are as follows: *Lavandula multifida* (47-86%), *Coridothymus capitatus* (86%), *Origanum syriacum* (83%), *Origanum dictamnus* (59-82%), *Origanum onites* (51-81%), *Thymus eugii* (75%), *Origanum majorana* (48-74%). Non-Labiatae oils in which carvacrol is the major constituent are the following: *Anabasis setifera* (Chenopodiaceae) (85%), *Carum carvi* (Umbelliferae) (60%), *Lippia graveolens* (Verbenaceae) (25%), *Lippia sidoides* (Verbenaceae) (25%), *Plantago asiatica* (Plantaginaceae) (18%). It is categorized as Generally Recognized as Safe (GRAS) by United states Food and Drug Administration. It is used as a flavoring agent in sweets, beverages, chewing gum, condiment relish, frozen dairy, gelatin pudding, nonalcoholic beverages, and soft candy (Ultee et al., 1999; De Vincenzi et al., 2004).

![Chemical structure of carvacrol (CVC)](image)

Figure 10: Chemical structure of carvacrol (CVC)
**Biological properties of CVC**

**Pharmacokinetics of CVC**

CVC appears to be absorbed slowly from the rabbit’s intestine following 1.5 g oral administration with 30% remaining in the GI tract and about 25% of the dose is being excreted in the urine 22 h post administration (Opdyke, 1979). The amount of CVC in blood, urine, feces and tissues of rats were measured at 2–24 h subsequent administration with CVC by gavage, resulted in carvacrol distribution to the intestine, stomach, urine with small amounts in muscle, liver, lungs (Schroder and Vollmer, 1932). Results from a study examining the metabolism of the CVC in male Wistar rats by gas chromatographic-mass spectrometric methods showed that the urinary excretion of metabolites was rapid with only very small amounts being excreted after 24 h and no metabolites detected in the 48-h to 72-h post treatment sample (Austgulen et al., 1987).

**Toxicity and safety**

In rats it has been reported that the median lethal dose (LD$_{50}$) of CVC is 810 mg/kg of b.w. when administered orally (Hagan et al., 1967). In mice the lethal dose of CVC has been found to be 80 and 73.3 mg/kg b.w. when treated intraperitoneally or intravenously (Andersen, 2006). The LD$_{50}$ in rabbits following dermal application has been estimated at 2700 mg/kg (McOmie et al., 1949). The LD$_{50}$ following subcutaneous administration of CVC in mice has been estimated at 680 mg/kg (Andersen, 2006). In dogs, the lethal dose of intravenously administered CVC was 0.31 g/kg (Coujolle and Franck, 1944; Andersen, 2006).