**Table 4.** Continued

| Quartile | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 3 | | | | | 4 | | | | | $P_{trend}$ |
| Range | No. of Cases | No. of Controls | Odds Ratio | 95% Confidence Interval | Range | No. of Cases | No. of Controls | Odds Ratio | 95% Confidence Interval | |
| | | | | | | | | | | 0.71[b] |
| 92.6–<119 | | | | | ≥119 | | | | | |
| 68.2–<88.2 | | | | | ≥88.2 | | | | | |
| | 47 | 56 | | | | 22 | 20 | | | |
| | | | 0.88 | 0.54, 1.43 | | | | 1.08 | 0.54, 2.17 | 0.89 |
| | 105 | 86 | | | | 176 | 123 | | | |
| | | | 1.41 | 0.94, 2.10 | | | | 1.53 | 1.06, 2.20 | 0.70 |
| | | | | | | | | | | 0.39[b] |
| 3,274–<4,145 | | | | | ≥4,145 | | | | | |
| 1,880–<2,575 | | | | | ≥2,575 | | | | | |
| | 43 | 55 | | | | 27 | 15 | | | |
| | | | 0.90 | 0.54, 1.48 | | | | 2.05 | 1.01, 4.17 | 0.24 |
| | 93 | 87 | | | | 209 | 128 | | | |
| | | | 1.35 | 0.89, 2.03 | | | | 1.95 | 1.36, 2.79 | 0.01 |

[a] Men and women were combined according to sex-specific quartiles of each measurement of visceral adiposity.
[b] Values are $P_{interaction}$ instead of $P_{trend}$.
[c] Body mass index was dichotomized on sex-specific median values for controls (23.5 and 21.8 kg/m$^2$ for men and women, respectively).
[d] Adjusted for cigarette smoking, alcohol drinking, physical activity, height, total energy intake, family history of colorectal cancer, and nonsteroidal antiinflammatory drug use, as well as sex and matching variables.

was associated with the prevalence of colorectal adenoma independently of body mass index. Further adjustment for body mass index on a continuous scale did not essentially change these results (data not shown).

We then examined whether body mass index modified the association of visceral fat area and visceral fat volume with colorectal adenoma (Table 4). In this analysis, men and women were combined according to sex-specific quartiles of each measurement of visceral adiposity and stratified by body mass index based on sex-specific median values for controls. Compared with men and women with a lower body mass index as well as in the lowest quartile of visceral adiposity, those with a higher body mass index as well as in the highest quartile had a significantly higher prevalence of colorectal adenoma, with adjusted odds ratios of 1.53 (95% CI: 1.06, 2.20) and 1.95 (95% CI: 1.36, 2.79) for the visceral fat area and visceral fat volume, respectively. In addition, the adjusted odds ratio was also statistically significant among those with a lower body mass index but in the highest quartile of visceral fat volume (odds ratio = 2.05, 95% CI: 1.01, 4.17). On the other hand, when comparison was made among those with a higher body mass index, adjusted odds ratios of colorectal adenoma for the highest compared with the lowest quartile of visceral fat area and visceral fat volume were 1.33 (95% CI: 0.60, 2.92) and 2.69 (95% CI: 1.11, 6.55), respectively (data not shown). Importantly, statistical evaluation of interaction between visceral adiposity and body mass index revealed that body mass index was unlikely to modify the association of visceral fat area or visceral fat volume with colorectal adenoma, either ($P_{interaction}$ = 0.71 and 0.39 for visceral fat area and visceral fat volume, respectively). When the above analysis was conducted for men and women separately, results were essentially the same.

## DISCUSSION

In this study, we directly quantified the degree of visceral adiposity among middle-aged and elderly Japanese men and women and observed that, although visceral fat volume was highly correlated with body mass index, an increase in the visceral fat volume was associated with a higher prevalence of colorectal adenoma independently of body mass index in both sexes. Conversely, body mass index was unlikely to modify the association between visceral fat volume and colorectal adenoma.

To our knowledge, this is the first study to examine the association of visceral fat volume with any type of colorectal neoplasm. In contrast, 3 epidemiologic studies have investigated the association between visceral fat area and

colorectal adenoma (12–14); of these, 2 demonstrated a statistically significant positive association, albeit with relatively small study sizes ($n \leq 200$) (12, 14), whereas the third found no association in a larger, but still small, population ($n = 458$) (13). Although this apparent inconsistency may simply reflect sparse data from a limited number of studies or chance due to the small number of subjects, another likely contributor is that all 3 studies combined men and women, with different male/female ratios. As shown in the present study, however, the degree of visceral adiposity differs considerably between men and women. Moreover, the influence of visceral adiposity on risk may differ by sex. Further studies with a sufficient number of men and women to enable sex-specific analyses are needed.

Several mechanisms that implicate visceral adiposity in colorectal carcinogenesis have been hypothesized. One well-known hypothesis is that visceral adiposity may be associated with factors that promote the growth of colorectal adenomas, thereby increasing the risk of colorectal cancer. Visceral adiposity is, in fact, a strong determinant of insulin resistance and subsequent hyperinsulinemia (2), while insulin is an important growth factor for colonic mucosal cells and colonic carcinoma cells in vitro (23) and may have the potential to mediate the association between visceral adiposity and colorectal neoplasms.

Computed tomography allows the accurate quantification of visceral fat and is presently the optimum technique in this regard (11). However, there are several disadvantages associated with the use of this technique as an assessment tool of visceral adiposity in practical settings, including health hazards of exposure to ionizing radiation. On the other hand, although the waist/hip ratio and waist circumference provide inexact measurements of visceral adiposity, they are not only safe but also cheap and relatively easy to perform (11). These anthropometric measurements therefore remain useful for the general classification of large numbers of people by visceral adiposity.

Among the strengths of the present study, the provision of total colonoscopy to all study subjects would have decreased the likelihood of misclassification between cases and controls. Moreover, the number of subjects was by far larger than in previous colorectal neoplasm studies that evaluated visceral adiposity by using computed tomography (12–14). Finally, case-control studies of colorectal adenoma have a methodological advantage over those of colorectal cancer, in that bias related to changes in body composition caused by the cancer itself is avoided.

A major limitation of this study is that colorectal adenomas were identified by magnifying colonoscopy with indigo carmine dye spraying, which may have resulted in some misclassification among adenoma cases. Given that pit-pattern classification based on magnifying chromoendoscopy differentiates colorectal lesions with approximately 90% accuracy (24), however, and that our institution has reported accuracy in differential diagnosis of ≥95% (25), the influence of this misclassification on the present results is likely to have been minimal. A second limitation is the relatively small body size of the study population. For male and female controls, the median body mass index was 23.5 and 21.8 kg/m$^2$, respectively, and the prevalence of overweight and obesity was 28% and 13%, respectively. Our findings may not therefore be directly applicable to severely obese populations, often found in North American and European countries, where more than half of adults are overweight or obese (1). Further studies in populations with larger body sizes are thus required. Third, because of their lower prevalence of colorectal adenoma, sample sizes for women were relatively small, and in some cases significant results could not be obtained because of limited statistical power. Nonetheless, statistical evaluation of interaction between visceral adiposity and sex revealed that, despite an obvious sex difference in the degree of visceral adiposity, its positive association with colorectal adenoma was likely to be similar between sexes. Finally, the present study was based on not incident but prevalent cases, meaning that the odds ratios of colorectal adenoma presented in this study did not necessarily indicate the risk of "developing" colorectal adenoma. Rather, they represent the risk of "having" colorectal adenoma at a point in time, and they should therefore be interpreted with caution.

In summary, with an optimal technique for the direct quantification of visceral fat, our present study corroborates previous findings obtained by using inexact surrogate markers of visceral adiposity, namely, waist/hip ratio and waist circumference. Our findings add to accumulating evidence that visceral adiposity exerts an important influence on the pathogenesis of colorectal neoplasms. The mechanisms of this potential association between visceral adiposity and colorectal carcinogenesis warrant further investigation.

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# Toxicity

## Definition

Refers to unwanted biological activity.

►ADMET Screen

# Toxicity Testing

►Preclinical Testing

# Toxicokinetics

## Definition

Refers to study of the fate of administered drug and metabolites in the animals used in toxicity studies. The term usually refers to those studies of drug disposition that form part of toxicity study rather than specialist studies of adsorption, distribution, metabolism and excretion conducted as separate studies.

►Preclinical Testing

# Toxicological Carcinogenesis

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## Synonyms

Chemical carcinogenesis; Experimental carcinogenesis

## Definition

In the broadest possible sense, ►carcinogenesis is a process of generation of benign and malignant neoplasm. Agents such as viruses, radiation, and chemicals are able to induce ►cancer in humans and experimental animals. However, the importance of chemicals as a cause of cancer has long been recognized in basic and clinical studies, and is emphasized by the epidemic of ►tobacco-related lung cancer in the twentieth century. Carcinogenesis may be considered as a form of toxicity in which cells achieve a different steady state from the normal and do not respond normally to homeostatic mechanisms. Carcinogenesis induced by chemicals is called "toxicological (chemical) carcinogenesis." Basic and clinical research in the field of toxicological carcinogenesis has led to many major advances, ranging from the fields of epidemiology and international human studies to laboratory research on mechanisms involved in the complex processes that are associated with the initiation and development of malignant disease (cancer).

Many chemical carcinogens have been identified, and their effects documented in experiments in which animals exposed to the agents at the maximum tolerated dose develop neoplasm. Toxicological carcinogenesis and ►human cancer epidemiology studies have clearly identified specific chemicals that can act as human carcinogens in both occupational and environmental settings. The main groups of relevance to human disease include ►polycyclic aromatic hydrocarbons, aromatic amines, nitrosamines, ►alkylating agents, and heterocyclic amines. Cancer resulting from exposure to chemicals in the environment has taken on new importance. Knowledge about the mechanisms and natural history of cancer development from toxicological carcinogenesis as well as epidemiology of human cancer is critical in the control and prevention of human neoplastic disease.

## Characteristics

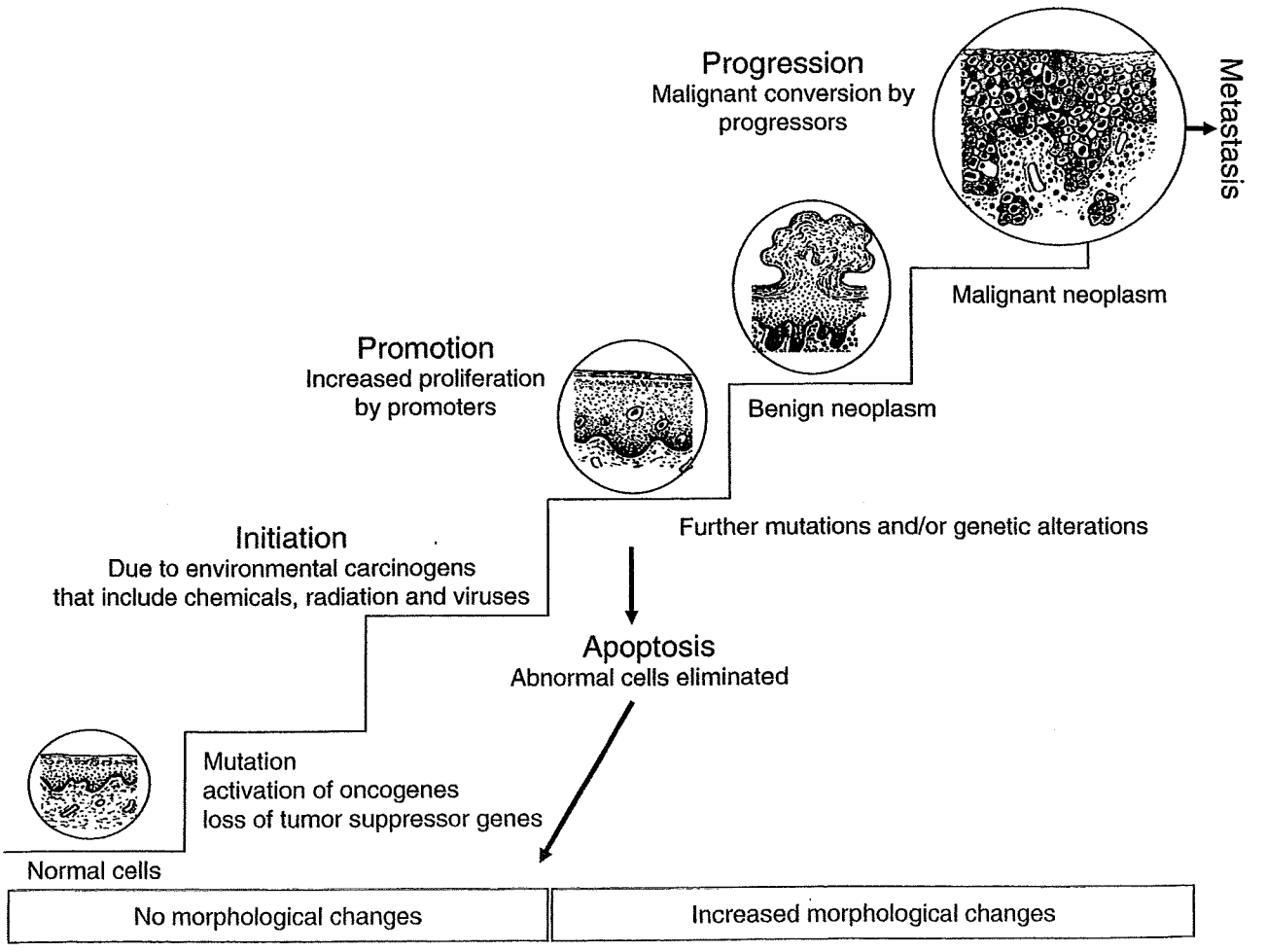
Mutagens are agents that can permanently alter the genetic constitution of a cell. The most widely used screening test, the Ames test, uses the appearance of mutants in a culture of bacteria of the *Salmonella* species. Approximately 90% of known carcinogens are mutagenic in this system. Moreover, most, but not all, mutagens are carcinogenic. This close correlation between carcinogenicity and mutagenicity presumably occurs because both reflect ►DNA damage. The *in vitro* mutagenicity assay is a valuable tool in screening for the carcinogenic potential of chemicals. Cultured human cells are also being increasingly used for assays of mutagenicity.

Chemical carcinogens may cause development of neoplasm either directly or indirectly. They can be grouped into two main classes according to the mechanism by which they stimulate development of neoplasm: (i) ►Genotoxic carcinogen causes direct damage to DNA by forming chemical: ►DNA adducts. The abnormal areas of DNA are prone to damage in replication and some adducts are resistant to normal ►DNA repair mechanisms. (ii) ►Non-genotoxic carcinogen is a carcinogen for which there is no evidence of direct interaction with cellular DNA. This type of carcinogen can be divided into two subgroups. ►Mitogenic carcinogen binds to receptors on or in cells and stimulates cell division without causing

direct DNA damage. In experimental skin carcinogenesis such agents have been shown to bind to and activate ►protein kinase C, causing sustained epidermal hyperplasia. ►Cytotoxic carcinogen produces tissue damage and leads to hyperplasia with cycles of tissue regeneration and damage. In some cases it is believed that cytokines generated in response to tissue damage act as mitogenic factors. Chemical carcinogens can be further divided into two groups: (i) ►Direct-acting carcinogen: the agent is capable of directly causing neoplasia. (ii) ►Procarcinogen: the agent requires conversion to an active carcinogen. This conversion takes place through normal metabolic pathways. In procarcinogens the ►cytochrome P450 (CYP) monooxygenase system plays an important role in conversion in many instances. ►Detoxification reactions also occur, the accumulation of carcinogen being determined by a balance between: (i) dose of procarcinogen; rate of detoxification and elimination; and (ii) rate of conversion to the active form.

Three stages have been defined in toxicological carcinogenesis (Fig. 1). Studies of toxicological carcinogenesis among experimental animals have shed light on the individual stages in the progression of normal cells to cancer. From these studies, one can define three stages (►multistep development) of toxicological carcinogenesis:

1. *Initiation* is the first stage and likely represents mutations in a single cell. The nature of the initial changes in cells is still uncertain. In experimental toxicological carcinogenesis in skin, the Harvey ►*ras* gene has been identified as being frequently mutated. This gene is involved in epidermal proliferation and when it becomes abnormal epidermal cells are less responsive to signals that normally cause terminal differentiation. Only relatively few genes have been identified as being mutated in other animal models of toxicological carcinogenesis.
2. *Promotion* follows initiation and is characterized by clonal expansion of the initiated cell. Induction of cell proliferation takes place at this stage. The altered cells do not exhibit autonomous growth, but remain dependent on the continued presence of the promoting stimulus, including an exogenous chemical or physical agent or an endogenous mechanism, such as hormonal stimulation. In this phase of carcinogenesis a promoting agent brings about increased cell proliferation. Promotion is initially reversible if the promoting agent is withdrawn.




**Toxicological Carcinogenesis. Figure 1** Toxicological carcinogenesis as a multi-step process.

3. *Progression* is the third stage, in which growth becomes autonomous and is independent of the carcinogen or promoter. At this stage, additional genomic changes presumably endow cells with a relative growth advantage that, in turn, results in their further clonal expansion. Cancer is the end result of the entire sequence and is established when the cells acquire the capacity to invade and metastasize. If there is persistent cell proliferation, initiated cells acquire secondary genetic abnormalities in oncogenes, which first lead to dysregulation and eventually to autonomous cell growth. The ultimate end-point of progression is development of an invasive neoplasm.

The various tests that have been applied to identifying agents with carcinogenic potential may be classified into several general areas on the basis of the time involved in the assay: short, medium, and long. These include short-term tests for mutagenicity (e.g., the Ames test), gene mutation assays *in vivo* (e.g., The LacZ mouse, the LacI mouse, the LacI rat), assay for chromosomal alterations (e.g., ▶micronucleus assay, sister chromatid exchange), measurement of primary DNA damage *in vitro* and *in vivo*, and chronic bioassays for carcinogenicity (e.g., chronic 2-year bioassay, medium-term bioassays-Ito model, multi-stage models of neoplastic development, transgenic and knockout mice as models of carcinogenesis).

### History

It is widely recognized that exposure to chemicals in the workplace and the environment can contribute to human cancer risk. This was first indicated in 1775 by Dr. Pott, who attributed scrotal skin cancers to prolonged exposure to soot in London chimney sweeps. In 1914, Dr. Boveri first hypothesized that cancer was a genetic disease, prior to the discovery of the genetic material. In 1915, Dr. Yamagiwa and co-workers successfully induced skin cancer in rabbits by painting their ears continuously with benzene solutions of tar. In the 1930s Dr. Kenneway and co-workers demonstrated that pure chemicals isolated from coal tar could also produce tumors in animals. In the 1950s there were parallel discoveries of the structure of the DNA double helix and its establishment as the hereditary material and mutagenic potential of ionizing radiation and certain chemical carcinogens in humans and experimental systems, and extensive investigations into the relationship between chemically induced mutations and human cancer. The 1980s saw the elucidation of the first oncogenes that appeared to be responsible for the initiation of cancer as first predicted by Dr. Boveri. This era also saw the development of the Ames *Salmonella* bacterial mutagenesis assay (the Ames test) and similar genetic toxicology assays. These developments firmly established the basic paradigm for the field of toxicological carcinogenesis: chemicals capable of induction of mutations are presumed to be carcinogens. It was predicted that any chemical or physical agent that can covalently damage DNA could also cause mutations through its DNA-damaging mechanism, and hence can be a carcinogen. The data that followed in the 1990s appeared to strongly support this central assumption, as numerous chemicals that were initially tested for DNA damage or mutations were also carcinogens in experimental animals. Since then, our understanding of the molecular basis of cancer has improved substantially. In addition, investigations into the molecular basis of toxicological carcinogenesis, as well as more extensive human cancer epidemiology studies using modem molecular tools, have greatly expanded our knowledge in this area.

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## Toxicological Pathologist

### Definition

Is usually a veterinary or medical graduate with experience in the pathological changes that can be induced by chemicals agents including drugs.

▶Preclinical Testing

## Toxicology

### Definition

Study of the nature, effects, and detection of poisons in living organisms. The basic assumption of toxicology is

# Colorectal Carcinogenesis and Suppression of Tumor Development by Inhibition of Enzymes and Molecular Targets

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**Abstract:** Colorectal cancer (CRC) is the fourth most common cancer in the world. If detected at an early stage, treatment often might lead to cure. Of course prevention is better than cure. Epidemiological studies reveal that having a healthy diet often protects from CRC development. An important consideration in evaluating new drugs and devices is determining whether a product can effectively treat a targeted disease. There are a number of agents making their way into clinical trials by estimating their effects on biomarkers' expression. Also, some are awaiting the preclinical efficacy and safety results to enter into clinical trials. Oncologic researchers are facing challenges in modifying trial design and defining the right control population, validating biomarker assays from the biological and analytical perspective. However, the results are disappointing from many of the large clinical trials. To avoid these disappointments, selection of biomarkers and its target agents needs to be evaluated in appropriate animal models for their efficacies as well as toxicities. This review focuses on the few of the potential molecular targets and their biomarkers in CRC development.

## INTRODUCTION

Neoplasm (or tumor) is a disease group that is characterized by excessive and uncontrolled growth and spread of structurally and biologically abnormal differentiated cells that can be originated from any tissues of the body. Benign neoplasms show a close morphological resemblance to their tissue of origin, grow in a slow expansile fashion, and form circumscribed and (usually) encapsulated masses. They may stop growing and regress. Benign tumors do not infiltrate through local tissues and do not metastasis. They are rarely fatal. In contrast, malignant neoplasms including cancer resemble their parent tissues less closely and are composed of increasingly abnormal cells in terms of their cellular/structural form and function. Well-differentiated examples still retain recognizable features of their tissue of origin but these characteristics are progressively lost in moderately- and poorly-differentiated malignancies: undifferentiated or anaplastic tumors are composed of cells which resemble no known normal tissue. Most malignant tumors grow rapidly, spread progressively through adjacent tissues and metastasis to distant sites. Tumors are conventionally classified according to the anatomical site of the primary tumor and its microscopical appearance, rather than by cause. Cancer that is epithelial origin is caused by both external and internal factors. These causal factors may act together or in sequence to initiate and or promote cancer. In spite of knowing more than ever about the genetic and cellular events that can accelerate or inhibit cancer induction, cancer is still the number one health concern in the world, especially western and westernized countries.

Globally, colorectal cancer (CRC) is the fourth most common cancer in men and the third most common cancer in women. CRC is more prevalent in North America, Argentina, Australia, New Zealand and parts of Europe, Japan, and Israel, and for this reason is commonly regarded as a western lifestyle disease. However, although incidence and mortality are higher in western lifestyle countries, global incidence is rising and the majority of the world's cases of CRC occur outside of countries in which traditional western lifestyles are dominant. CRC is the third most common disease in the USA, prevalent in both men and women. In Japan, there are 65795 new cases of colon cancer and 34342 new cases of rectal cancer, in 2001. In the USA, as per the statistics of the National Cancer Institute of 2007, there are 112 340 new cases of colon cancer, 41 420 new cases of rectal cancer and 52 180 deaths from both cancers combined. In the USA, average-risk patients account for approximately 75% of CRC and include persons older than 50 years with no other known risk factor; moderate-risk patients account for 15%-20% of CRC and include those with a positive family history of colorectal adenomatous polyps or cancer; and high-risk patients account for 5%-15% of CRC and include those with familial adenomatous polyposis (FAP), hereditary nonpolyposis colorectal cancer (HNPCC) or long-standing inflammatory bowel disease (IBD) [1]. Thus, the majority of CRC are non-hereditary and sporadic, which makes early detection important. There is good evidence demonstrating reduced morbidity and mortality associated with early detection of invasive lesions and precursor adenomatous polyps. However, most CRC in the world is diagnosed at an advanced stage. Therefore, attention has most focused on screening for targets to aim through cancer chemoprevention to reduce the number of CRC patients.

Chemoprevention strategies can be benefited from observations linking the identification of intermediate and surrogate biomarkers or various nutrients and/or drugs with specific molecular targets involving cancer development. Currently, genetic information about cancer, molecular signaling and metabolic pathways has been translated into certain therapy that targets specific molecules for prevention. Developing new technologies to provide knowledge of the functions of non-coding RNA [2], such as small interfering RNA (siRNA), microRNA (miRNA) and piwi interacting RNA (piRNA), can form a basis for the development of novel chemopreventive agents, which result in intervention at dif-

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ferent steps during the multistage carcinogenesis process: preventing the initial mutation; blocking promotion to premalignant tumors; stopping progression from the premalignant state to in situ carcinomas; or preventing invasion or metastasis. As the early stages of tumor promotion and progression are rate limiting, successful targeting of molecular events during these stages can have great impact on outcomes.

## ENZYMATIC AND MOLECULAR BIOMARKERS IN CRC

Most sporadic CRCs (adenoma-carcinoma sequence) develops through multistep and multigenetic alteration (Fig. **1**). Recent advances in cancer pathogenesis help us in untangling the valid enzymatic and molecular biomarkers that are involved in colon tumorigenesis. Identification/discovery of these biomarkers ranges from exposure assessment, risk assessment and management to clinical trials. This is helpful for us to develop and discover novel therapeutic interventions, preventive strategies and chemopreventive agents. Along with these, there is also a need to develop and validate molecular biomarkers reflective of exposure and risk from etiological factors [3-7]. There are high-risk people who are most susceptible to chronic disease, including cancer. Hence, methods that identify such high-risk individuals will greatly facilitate the implementation of a spectrum of targeted prevention techniques directed to reducing individual risk. Currently developed technologies, -omics (genomics and proteomics) and miRNAs, are guiding us through the development of agents which are also able to target the biomarkers. A link between miRNAs function and cancer pathogenesis is supported by studies investigating the expression of miRNAs in clinical samples. miRNAs are emerging as a new class of genes involved in cancer [2, 8]. Knowledge about micro RNA's and its specific functions offers opportunities to target genetic or epigenetic changes that influence cancer risk. Indeed a more detailed understanding of micro RNA (miRNA) functions is required to identify and manipulate the molecular targets for cancer prevention. However, specific molecular processes have been targeted for therapeutic intervention, including growth factor receptors, proliferation signaling, cell cycling, apoptosis, angiogenesis, the immune system, etc. Most cancers are characterized by alternations in certain signaling pathways, and identification of the aberrant pathway in cancer patients allows for targeted therapy to such specific pathway.

Surrogate endpoint markers provide opportunities to understand the cancer development and also to evaluate efficacy of agents' intervention. All the biomarkers will not achieve the status of surrogate endpoints, only a subset of biomarkers may achieve surrogate endpoint status. Based on epidemiologic, therapeutic, pathophysiologic, clinical, and cost benefit, adenomas are considered as surrogate endpoints in CRC, since removal of adenomatous polyps has been shown to reduce the risk of development of CRC. However, intervention using adenomas as endpoint may not be fruitful, because colonic polyps often take several years to develop and become adenocarcinomas. Much interest is currently shown on research in the use of surrogate endpoints biomarkers that are altered early in colonic carcinogenesis, prior to polyp (adenoma) formation, to predict the clinical effectiveness of chemopreventive agents or drugs, since it takes 10-20 years for a normal cryptal cell to undergo molecular changes and to be clinically detected as a neoplasm. Rather, aberrant crypt foci (ACF, Fig. **2a**) can be used as endpoint in CRC development, because aberrant crypts are postulated to be the earliest identifiable potential precursors of CRC in rodents and human. Analysis of ACF may facilitate the study of the early pathological and molecular changes that precede adenoma to CRC [9, 10]. Therefore, ACF may eventually evolve into polyps and, subsequently, CRC in the case of adenoma-carcinoma sequence. Hence, it provides a simple and economical tool for preliminary screening of potential

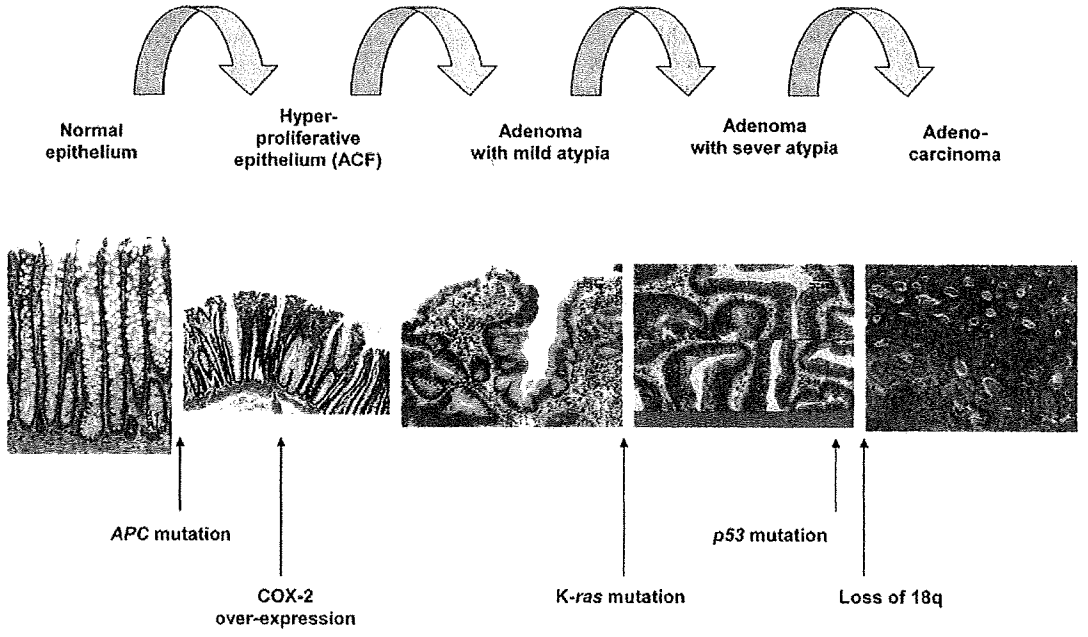


**Fig. (1).** Adenoma-carcinoma sequence.

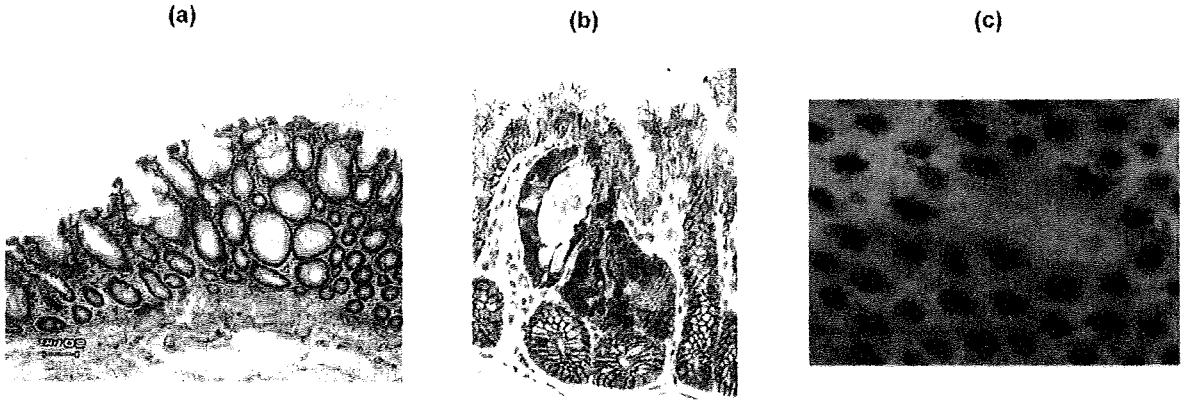


**Fig. (2).** Putative precursor lesions for CRC. (**a**) histopathology of ACF; (**b**) BCAC; and (**c**) MDF.

chemopreventive agents, and it allows a quantitative assessment of the mechanisms of colon carcinogenesis. Beta-catenin-accumulated crypts (BCAC, Fig. **2b**) and mucin-depleted foci (MDF, Fig. **2c**) are also early lesions that develop CRC [11], but detection of these lesions from human samples is not easy by routine laboratory techniques.

The most important studied molecular markers/targets involved in signal pathways are polyamine (ornithine decarboxylase), cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), 3-hydroxy-3-methylglutatyl coenzyme A (HMG-CoA) reductase, retinoid X receptor (RXR)-α, estrogen receptor (ER)-β, β-catenin, 5-lipoxygenase (5-LOX), signal transducers and activators of transcription (STAT)3, nuclear factor kappaB (NF-kB), and heme oxygenase (HO)-1 (Table **1**). These can be also considered as biomarkers and/or surrogate endpoint markers in colorectal carcinogenesis, and also enzymatic and molecular targets for colorectal malignancies. Also, non-protein-coding RNAs that include miRNA, siRNA, and piRNA and are recently highlighted in their role in carcinogenesis and are shortly reviewed in this review article.

## POLYAMINES

The polyamines, putrescine, spermidine, and spermine, are naturally occurring polycationic alkylamines that are absolutely required for eukaryotic cell growth. Importantly, the polyamine metabolic pathway (Fig. **3**), as well as the requirement of polyamines for cell growth, is frequently dysregulated in cancer cells, thus providing a unique set of targets for therapy and chemoprevention. Ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis (Fig. **3**), is frequently up-regulated in preneoplastic cells, and is implicated as an oncogene in multiple neoplasm types [12, 13]. Several model systems have demonstrated that inhibition of ODC's enzymatic activity and down-regulation of its expression are rational strategies for both chemoprevention and chemotherapy [14]. Specific inhibitors of ODC, most notably 2-difluoromethylornithine (DFMO), have been used experimentally to validate polyamine metabolism as an anti-neoplastic strategy [15]. However, multiple biochemical and clinical limitations to these ODC-targeting strategies minimize their value as therapeutic tools. Included among these limitations are poor bioavailability of the inhibitor, and

**Table 1. Different Expression Pattern of Several Biomarkers of Colon Carcinogenesis in Rodents and Human**

| | Lesions | Expression of Biomarkers | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | β-Catenin | COX-2 | iNOS | HMG-CoA Reductase | RXR-α | ER-β | 5-LOX | STAT-3 | NF-κB |
| Rodents' colon | Normal/non-lesional crypts | 1+ | - | - | 1+ | 4+ | 1+ | +/- | 1+ | 1+ |
| | ACF | 2+ | +/- | +/- | 1+ | 2+ | 2+ | 2+ | - | 2+ |
| | AD | 3+ | 3+ | 2+ | 3+ | 1+ | 3+ | 3+ | 3+ | 3+ |
| | ADC | 4+ | 4+ | 4+ | 4+ | - | 4+ | 4+ | 4+ | 4+ |
| Human colon | Normal/non-lesional crypts | 1+ | - | - | 1+ | 4+ | 1+ | +/- | 1+ | ? |
| | ACF | 2+ | ? | ? | 1+ | ? | - | ? | - | ? |
| | AD | 3+ | 3+ | 2+ | 2+ | 1+ | 3+ | 3+ | 3+ | ? |
| | ADC | 4+ | 4+ | - | 3+ | - | 4+ | 4+ | 4+ | ? |

(?), not yet determined; (-), negative or no over-expression; (+/-), very weak over-expression; (1+), weak over-expression; (2+), strong over-expression; (3+), very strong over-expression, and (4+), intensive over-expression.

COX, cyclooxygenase; iNOS, inducible nitric oxide synthase; HMG-CoA, 3-hydroxy-3-methylglutatyl coenzyme A; RXR, retinoid X receptor; ER, estrogen receptor, LOX, 5-lipoxygenase; STAT, signal transducers and activators of transcription; NF-kB, nuclear factor-kappaB; ACF, aberrant crypt foci; AD, adenoma; and ADC, adenocarcinoma.

the compensatory up-regulation of polyamine metabolism and transport that allow tumor cells to escape the growth inhibitory effects of blockers specifically targeting ODC. As a strategy to overcome the limitations of direct enzyme inhibition, several groups have pursued the design of polyamine analogues that specifically target the dysregulated polyamine metabolism observed in tumors [16]. These analogues have been developed specifically to target the specific polyamine transporter, thus competing with circulating natural polyamines. Additionally, most of the analogues examined thus far maintain the regulatory function of the natural polyamines, but are unable to functionally substitute for them in promoting growth. Specifically, individual analogues have demonstrated the ability to down-regulate each of the biosynthetic enzymes without causing compensatory increases in parallel systems or increases in polyamine uptake. Additionally, specific analogues produce tumor specific up-regulation of the rate-limiting enzymes in polyamine catabolism.

The initial studies on polyamines revealed increased amounts of one or more of the polyamines in the serum or urine of patients with certain malignancies, such as adenocarcinomas, leukemia, lymphoma, and melanoma [17]. Polyamines were, therefore, proposed as biochemical markers of neoplasia, with elevated concentrations of polyamines in physiological fluids being diagnostic of malignant disease. Disappointingly, raised amounts of polyamines were not restricted to malignant conditions, and high concentrations were also found in body fluids of patients with other diseases, including cystic fibrosis and psoriasis, and during pregnancy. The focus then switched from measuring polyamines as a diagnostic tool to using polyamine content as a means of monitoring therapeutic efficacy. It had been shown in a number of cases that patients in remission had urinary polyamine outputs similar to those found in normal individuals and that this remained within the normal range while the patient remained in remission [18, 19]. More importantly, urinary polyamine output was found to increase when patients suffered a tumor recurrence. Although this finding still holds true, disappointingly, little use is made of this fact in clinical practice. There is, however, a clear link between increased polyamine content and cancer, and thus it is reasonable to suggest that strategies aimed at depletion of polyamine content will have anti-proliferative activity [20]. The relationship between polyamines and CRC development is well studied [21] and inhibitors of polyamine biosythesis are postulated to be good chemopreventors in CRC [22]. Recently several analogues [19] and antizyme inhibitors [23] of polyamine biosynthesis have been developed to combat against CRC [22].

## COX-2

COX is the rate-limiting enzyme responsible for converting arachidonic acid (AA) to prostaglandin (PG) $H_2$, which is the precursor module of a number of proinflammatory cytokines including prostaglandins, prostacyclins and thromboxanes [24] (Fig. **4**). At least two isoforms of COX (Table **2**) have been identified which are labelled COX-1 and COX-2 [25, 26]. COX-1 is constitutively expressed and is thought to be involved in 'house-keeping' roles in tissue homeostasis. In contrast, COX-2 is an inducible isoform which has been

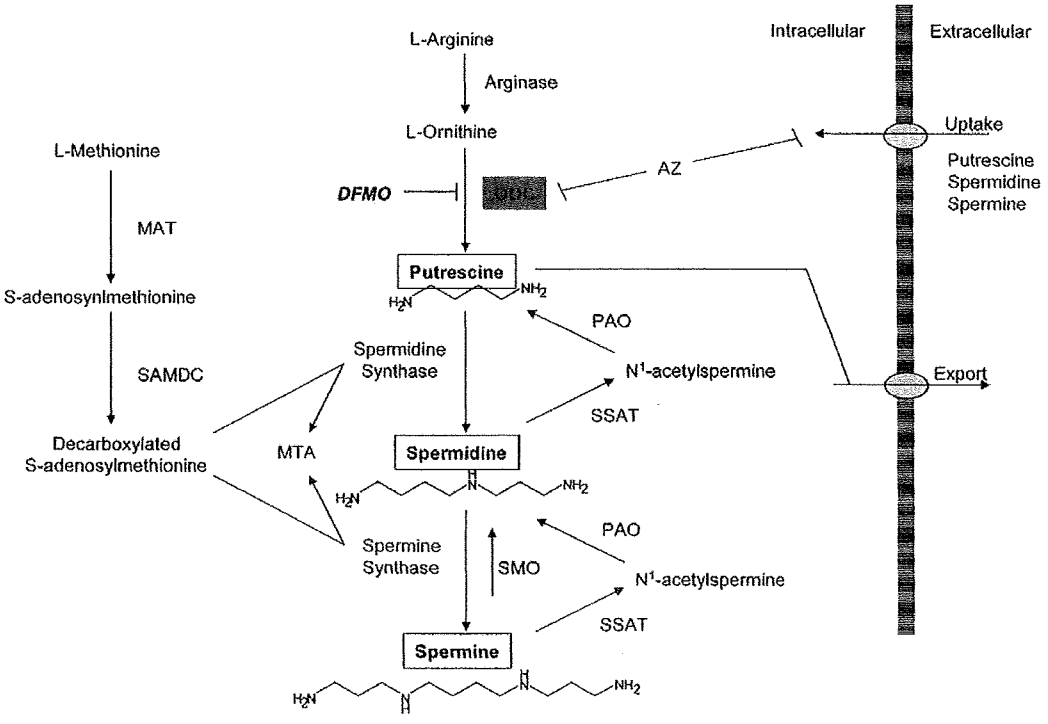


**Fig. (3).** Mammalian polyamine metabolic pathway. MAT, methionine adenosyltransferase; SAMDC, S-adenosylmethionine decarboxylase; ODC, irnithine decarboxylase; DFMO, α-difluoromethylornithine; MTA, 5'-methylthioadenosine; SSAT, spermidine/spermine acetyltransferase; PAO, polyamine oxidase; SMO, spermine oxidase; AZ, antizyme.

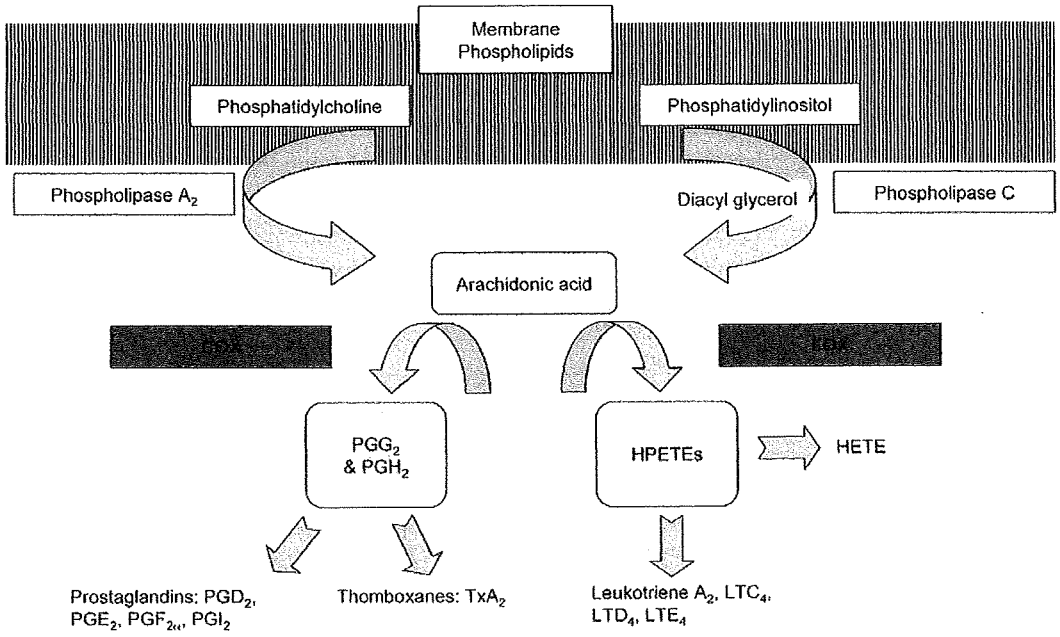


**Fig. (4).** COX and LOX pathways -1.

**Table 2. Some Features of Cyclooxygenases**

| | COX-1 | COX-2 |
|---|---|---|
| Molecular weight | 72 kDa | 72 or 74 kDa |
| Expression | Constitutive | Inducible |
| Expressional control | - | +: LPS, IL-1, TNF-α<br>-: Glucocorticoids |
| Expressing cells | Ubiquitous | Ubiquitous (macrophages, smooth muscle cells, fibroblasts, epithelial cells, etc) |
| Cellular localization | Endoplasmic reticulum | Endoplasmic reticulum, nuclear envelope |
| Cofactors | Heme, glycosylation required for optimal activity | |
| $[Ca^{2+}]$-activated | Yes | Yes |
| Post-translational modifications | Homodimerisation, N-glycosylation | Homodimerisation, N-glycosylation (72 kDa species 3 groups, 74 kDa species 4 groups) |
| Substrate specificity | Narrow (AA, dihomo-γ-linoleate) | Wide due to larger catalytic cleft (AA, dihomo-γ-linoleate, α-linolenate, eicosapentaenoic acid) |

AA, arachidonic acid; IL-1, interleukin-1; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor alpha; $[Ca^{2+}]_i$, intracellular calcium.

shown to have a key role in pathological processes, such as inflammation and carcinogenesis [27]. The first reported evidence of COX-2 upregulation in gastrointestinal malignancies was in 1994 [28], and since then the role of COX-2 in a wide range of malignancies has been reported.

COX-1 and COX-2 catalyze the conversion of AA to eicosanoids, namely PGs and thromboxanes *via* endoperoxides. COX-1 isoform synthesizes PGs that are required for normal physiologic function like gastrointestinal cytoprotection and platelet activity. While COX-2 is not detectable in most normal tissues, it is induced at sites of inflammation by cytokines, growth factors, tumor promoters and other factors. Both isoforms are also responsible for the synthesis of prostaglandin (PG) $E_2$. There is some evidence for a correlation between increased levels of $PGE_2$ and tumorigenesis [29]. PGs, especially $PGE_2$, are important in the pathogenesis of cancer secondary to the effects on mitogenesis, cellular adhesion, immune surveillance and apoptosis. PGE2 exerts its actions by binding to one (or a combination) of its four subtypes of receptor ($EP_1$, $EP_2$, $EP_3$ and $EP_4$) (Table **3**). Malignancies have been found to over-express PGs, when compared with adjacent normal tissues [30]. COX-2 is known to be a key mediator/player in the development of CRC. COX-1 and COX-2 differently expressed and regulated in different tissues. COX-1 is constitutively expressed in the colon, but COX-2 is inducible and markedly up-regulated in CRC [28, 31] (Table **1**). COX-2 over-expression is also found to be associated with chronic inflammations. In several animal carcinogenesis models and also human cases, the inhibition

**Table 3. Prostaglandin Receptors, Signal Transduction and Tissue Distribution**

| Receptor Type | Subtype | Second Messenger | Tissue Distribution |
|---|---|---|---|
| DP | | cAMP ↑ | Brain, meninges, intestine |
| EP | $EP_1$ | $Ca^{2+}$ | Kidney, lung, intestine |
| | $EP_2$ | cAMP ↑ | Uterus |
| | $EP_3$ | cAMP ↑/↓/PI *) | Kidney tubuli, brain, smooth muscle, intestine, enteric ganglia |
| | $EP_4$ | cAMP ↑ | Kidney glomeruli, intestine |
| FP | | PI | Corpus luteum, kidney, heart, lung, intestine |
| IP | | cAMP ↑/PI | Platelets, smooth muscle |
| TP | TPα | PI/cAMP ↓ | Lung, kidney, heart, thymus, spleen |
| | TPβ | PI/ cAMP ↑ | Lung, kidney, heart, thymus, spleen |

↑/↓, increase/decrease; cAMP, cyclic adenosine-3',5'-monophosphate; DP, prostaglandin $D_2$ receptor; EP, prostaglandin $E_2$ receptor; FP, prostaglandin $F_2$ receptor; IP, prostaglandin $I_2$ receptor; TP, thromboxane receptor; PI, phosphoinositol cascade. * ) Isoform (e.g. EP3 A-D) specific action.

of PG formation by blocking COX-2 results in protection against many types of epithelial malignancies, including breast, colon, esophageal, lung, skin and head and neck cancers. Several mechanisms have been proposed to explain the important role of COX-2 in tumorigenesis [32]. Increased COX-2 gene expression has been shown in human CRC and in chemically induced rodents' colonic tumors [33, 34]. COX-2 is also known to modulate tumor angiogenesis [35]. Recent observations of COX-2 expression with colonic tumor samples from patients explain a progressive overexpression of COX-2 during stepwise sequence from adenoma to carcinoma [36]. Although in depth detailed study is necessary to improve the efficacy of drugs to target COX-2, suppression of COX-2 is now a crucial target for control of CRC with chronic/persistent inflammation due to various evidences of its oncogene actions.

Epidemiological and rodent studies have documented a protective effect of non-steroidal anti-inflammatory drugs (NSAIDs) in preventing CRC. NSAIDs showed inhibition of COX-2 activity and $PGE_2$ synthesis both *in vitro* and *in vivo* [37, 38]. Recent findings have suggested that NSAIDs, COX-2 specific inhibitors in particular, are effective chemopreventive agents in CRC [39]. The concern over gastric toxicity associated with NSAID (aspirin) use led to efforts to develop COX-2 specific inhibitors (COXibs) [40, 41]. Although COXibs are found to be better drugs, several large randomized controlled trials provided unequivocal evidence of the cardiotoxicity of COXibs, which led to withdraw certain COXibs from US market [42, 43], but a selective COX-2 inhibitor, celecoxib, is used to treat FAP. Celecoxib is approved as an adjunctive (secondary) treatment among patients with FAP. Celecoxib is found to be effective in combination with docosahexaenoic acid (DHA) in HCA-7 (human colon cancer cell line) [44]. Different clinical trials (phase I/II/III) are being carried out using celecoxib individually and also in combination with other agents/drugs (curcumin, docetaxel, prednisolone, zoledronate, eflornithine) in CRC patients with different case histories to study its efficacy, reduce its toxicity and for better outcome. However, there is compelling evidence for the cancer-chemopreventive potential of aspirin and other NSAIDs [36, 44, 45]. A recent report on use of a classical NSAID aspirin in men and women with differential expressions of COX-2 showed varied responses on CRC. Regular use of aspirin appeared to reduce risk in those patients with high levels of COX-2 expression, but there was no effect shown on patients with very low or no COX-2 expression [36]. The differences between CRC that expresses high or low levels of COX-2 have to be investigated, and this understanding can form the basis for clinical implications. These findings led us to identify who is at risk of COX-2-expressing colon cancers and for new drugs that can provide benefits for colon cancer without the risks associated with the drug. Nitric oxide (NO) releasing NSAIDs (NO-NSAIDs) are other promising alternatives to aspirin in chemoprevention of colon carcinogenesis, which possess the beneficial effects of parent compound (aspirin) and at the same time are devoid of side effects [46-48]. NO-aspirin and NO-indomethacin are experimented against the development of colon adenocarcinoma in animal colon carcinogenesis models. These agents suppress colonic adenocarcinomas with or without invasion [49]. Various mechanistic pathways are suggested for the beneficial effects of NO-NSAIDs [46]. Functional studies of PG-related polymorphisms, including biochemical studies that evaluate the response to NSAIDs and COXibs [50], will provide information on possible subgroups of individuals who might be more susceptible to the toxic effects of either COXibs or standard NSAIDs. NO-NSAIDs and COXibs need to be further evaluated for their eventual use for human treatments.

## LOX

LOX consists of four enzymes, 5-LOX, 8-LOX, 12-LOX and 15-LOX, whose nomenclature is dependent on their activity to insert oxygen at carbon 5, 8, 12 and 15, respectively, of the AA molecule [51]. The products of this conversion are the hydroperoxyeicoasatetraenoic acids (HPETEs) (Fig. **5**). These acids are further metabolized by 5-LOX to form leukotriene (LT)$A_4$. $LTA_4$ is subsequently converted to 5(S)-hydroxy-6-trans8,11,14-cis-eicosatetranoic acid (5-HETE) or the LTs, $LTA_2$, $LTC_4$, $LTD_4$ and $LTE_4$. The latter three LTs were previously known as the, slow-reacting substance of anaphylaxis.

Evidence is emerging that the LOXs play an important role in carcinogenesis. Blockade of 5-LOX has been shown to inhibit lung and prostate carcinogenesis [52, 53]. 12-LOX

has been found to be over-expressed in a number of tumors including melanomas, prostate cancer and epidermal cancers [54], and to relate to the metastatic potential of prostate cancer cells [55], whilst blockade of 12-LOX results in an increase in the apoptotic index of sarcoma cells [56]. The exact role of 15-LOX in carcinogenesis is unclear as yet, although limited data suggest that 15-LOX inhibition induces apoptosis in colon cancer cells [57]. 8-LOX is a relatively new discovery and the function of 8-LOX in carcinogenesis has not been fully evaluated.

LOX is one of the two important enzymes that metabolize polyunsaturated fatty acids and affect carcinogenesis. Cell membrane phospholipids are converted to AA, which serves as a substrate that gives rise, in turn, to two powerful and potentially damaging classes of inflammation mediators, known as eicosanoids: the PGs and LTs. AA release and production of eicosanoids are prerequisites for inflammation. The inflammatory PGs and LTs are formed by the action of COX-2 and 5-LOX enzymes, respectively [58]. This forms the crux of dual inflammatory pathways: COX-2 and 5-LOX. There is clear evidence of COX pathway generating inflammatory PGs and its role in colon carcinogenesis. However, research has largely ignored the potentially damaging effects of 5-LOX, which forms the second branch of the dual AA inflammation pathways. Most emphasis was given to block COX-2 activity, ignoring the effects of 5-LOX, which may actually increase the 5-LOX levels, worsening the inflammation. This may be due to shifting of AA toward synthesis of LTs through the 5-LOX pathway, when COX-2 is blocked. COX-2 inhibition alone was ineffective in slowing the progression of CRC. From animal models and *in vitro* studies, it is evident that expression of 5-LOX appears to be occasionally up-regulated during neoplastic transformation [59]. Expression of 5-LOX is characterized in early stage of colon carcinogenesis and is up-regulation of 5-LOX in colonic adenomatous polyps and CRC [60, 61]. These observations suggest that 5-LOX plays a role in CRC development and may be an early target for chemoprevention of CRC.

LTs are involved in cell survival signals [62]. 5-LOX is involved in exerting both anti- and pro-inflammatory activities. The other bioactive agents, which need to be studied further to understand the network of anti- and pro-inflammatory mediators include lipoxins and resolvins. Biosynthesis of LOX-derived eicosanoids, lipoxins, occurs through different pathways: one of the pathways involves 5-LOX in the formation of metabolically active lipoxins, such as $LTA_4$, $LTB_4$, or 15(R)-HETE for 15-epi-LTs (aspirin triggered LTs) ($LTA_4/LTB_4$). These lipoxins promote the resolution of inflammation and also development of tumors. Biologically active metabolites of the 5-LOX cascade are LTB, and the so-called cysteinyl LTs ($LTC_4$, $LTD_4$, and $LTE_4$), which are pro-inflammatory mediators. Cysteinyl leukotrienes (CysLT) are important pro-inflammatory mediators, which exert effects on several cellular functions, including smooth muscle contraction, bronchial mucus production and chemotaxis [63]. These products may contribute to the development of CRC and several other cancers [64, 65]. Lipoxins are high affinity antagonists to the cystienyl leukotriene receptor 1 (CysLT1), to which several LTs ($LTC_4$, $LTD_4$ and $LTE_4$) mediate their smooth muscle contraction and eosinophil chemotactic effects. Also, the modulation of CysLT1 mediated inflammatory processes *in vivo* by anti-inflammatory lipids [66]. 5-LOX is involved in E series resolvins (anti-inflammatory), suggesting its protective role in addition to its LTs production. A recent report [67] of exacerbated inflammatory responses in 5-LOX deficient mice confirmed these findings. Since the findings are contradictory, we need a further insight into actual underlying mechanisms and the networks involved during inflammation. 5-

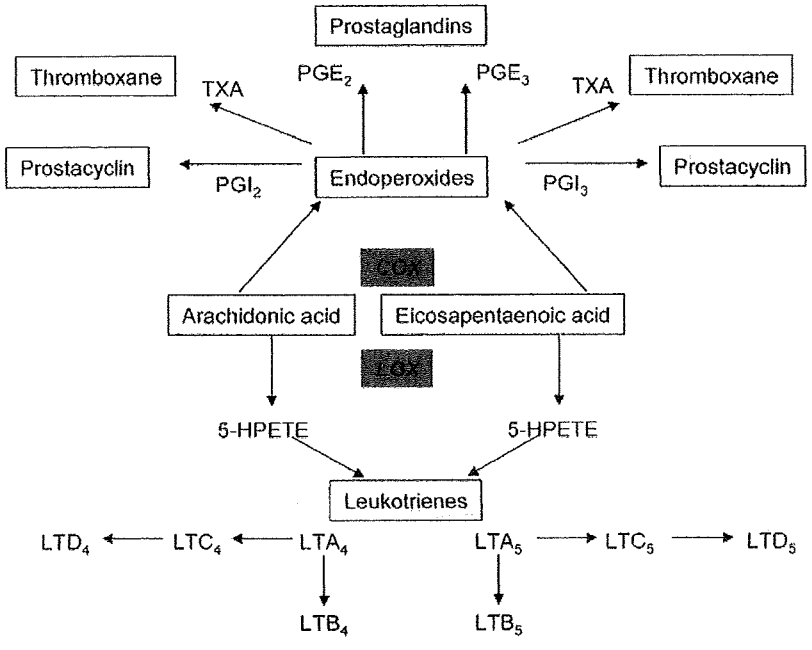


**Fig. (5).** COX and LOX pathways -2. Biosynthetic pathways of prostaglandins and leukotrienes from arachidonic acid and eicosapentaenoic acid.

LOX protective mediation is through lipoxins and resolvins. As 5-LOX is not the only pathway for the production of these anti-inflammatory components, use of 5-LOX inhibitors is beneficial. Thus, as a therapeutic approach, inhibitors of 5-LOX may come without many side effects. Each of these pro-inflammatory mediators activates specific signaling mechanisms. However, the potential role of lipoxins and resolvins in preventing chronic inflammation needs to be investigated. The use of experimental animal models in colon carcinogenesis will help to underpin the relative importance of lipoxins and resolvins in colon tumorigenesis. Data available on lipoxins and resolvins as promoting the resolution of inflammation urge the need to develop agonists of lipoxins and resolvins. Nevertheless, drugs able to block the LOX pathway, 5-LOX inhibitors or LTs receptor antagonists appear to be an insufficient single therapeutic approach of inflammation, although they are included among the effective therapies of asthma. It is likely that these results reinforce a growing body of research that dual inflammatory pathway inhibition may be needed to fully realize the promise of anti-inflammatory therapy [68].

Recent studies have shown that COX-2 is important in renal physiology. This means that selective COX-2 inhibitors, while undoubtedly safer than NSAIDs in terms of gastric toxicity, are not devoid of renal toxicity, in addition to their now clearly established adverse effects on coronary heart disease risk. Both the gastric and renal toxicities induced by traditional NSAIDs and selective COX-2 inhibitors seem to be related to inhibition of PGs, but not LT synthesis. Maintaining the correct balance between PGs and LTs is essential for continuing good health, but both classes of mediators also play an important role in the pathogenesis of several diseases. Recently, a new class of anti-inflammatory drugs, the LOX/COX inhibitors, has been developed as a means of simultaneously inhibiting the synthesis of PGs, thromboxanes and LTs. Inhibition of LTs synthesis increases anti-inflammatory efficacy, particularly in rheumatic diseases, while reducing the risk of gastric damage. The class of dual 5-LOX/COX inhibitors, such as indometacin, dexamethasone, ER-34122, BW 755C, diclofenac, tepoxalin and licofelone has emerged as an effective and well tolerated therapy that could offer safety advantages over COX inhibition alone [69]. These dual inhibitors are tested and are effective in pre-clinical animal models and clinical trials [70, 71]. Tepoxalin is better than indomethacin in having no gastro-intestinal toxicity [72]. Based on various observations, tepoxalin have future prospects as preventive and therapeutic drug in colon carcinogenesis. A novel dual 5-LOX/COX inhibitor, licofelone, is in phase III clinical development that effectively inhibits the synthesis of cysteinyl LTs [73, 74].

## iNOS

NO (Fig. **6**) is an important mediator in inflammation and carcinogenesis [75, 76]. Nitric oxide is a free radical and is produced from the conversion of L-arginine and molecular oxygen to L-citrulline by the enzyme nitric oxide synthase (NOS) [76] (Fig. **7**). There are three isoforms of the NOS

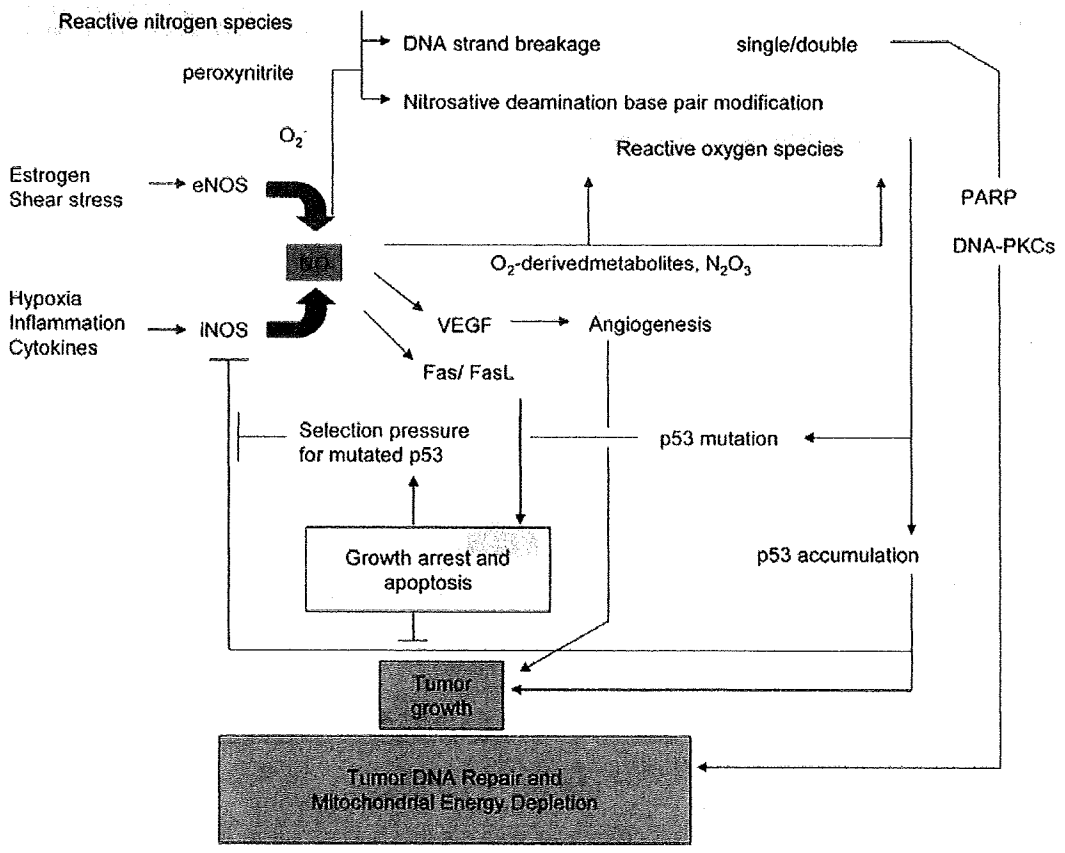


**Fig. (6).** NO involves in tumorigenesis. A representation of the dual action of nitric oxide on tumour growth.

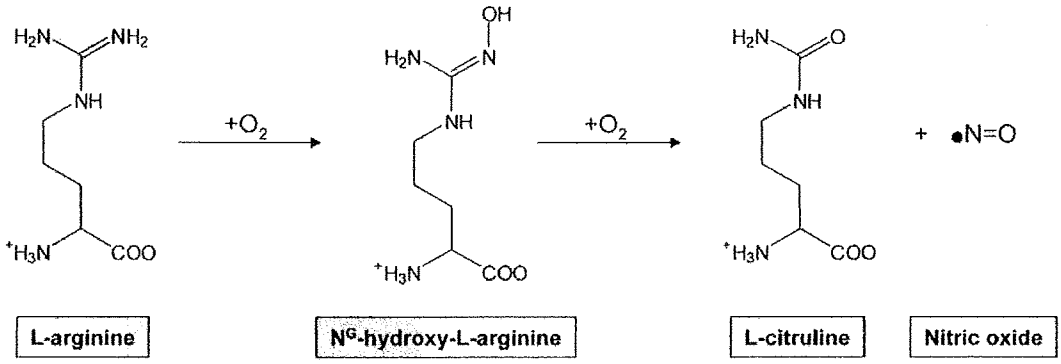


**Fig. (7).** The reaction catalyzed by NOS.

**Table 4. Features of Nitric Oxide Synthases**

| | nNOS, NOS-I | eNOS, NOS-III | iNOS, NOS-II |
|---|---|---|---|
| Molecular weight | 160 kDa | 130 kDa | 130 kDa |
| Expression | Constitutive | Constitutive | Inducible |
| Expressional control | | | +: LPS, IL-1, TNF-α<br>-: glucocorticoids |
| Expressing cells | Neurons | Endothelial cells | Macrophages, lymphocytes, epithelial cells, smooth muscle cells etc. |
| NO production | Picomoles | Picomoles | Nano- to micromoles |
| Cellular localization | Cytosolic | Membrane associated | Cytosolic |
| Cofactors | Calmodulin, tetrahydrobiopterin, NADPH, FAD, FMN, heme | | |
| $[Ca^{2+}]_i$ -activated | + | + | - |
| Post-translational modifications | Homodimerisation | Homodimerisation, Myristoylation | Homodimerisation |

FAD, flavine-adenine dinucleotide; FMN, flavine mononucleotide; LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor alpha; NOS, nitric oxide synthase; NADPH, reduced form of nicotine adenine dinucleotide phosphate; $[Ca^{2+}]_i$, intracellular calcium.

enzyme (Table **4**). Endothelial NOS and neuronal NOS are expressed constitutively and produce low amounts of NO, involved in house-keeping roles such as neurotransmission and maintaining vascular tone. The third isoenzyme, iNOS, is expressed by certain types of cancer cells and by activated macrophages [77].

The exact role of iNOS on carcinogenesis in unclear. Nitric oxide (NO) regulates prostaglandin production, activates the COX enzymes [78, 79] and regulates expression of the proinflammatory interleukin (IL)-8 [80], and by these mechanisms may promote cancer cell growth (Fig. **8**). However, high levels of NO have been demonstrated to have a pro-apoptotic role on tumor cells [81, 82] and induces G1 arrest [83], whereas low levels of iNOS and NO may enhance tumor progression and metastasis [84].

Intestinal inflammation is almost invariably accompanied by intestinal dysfunction, which is a major clinical complication. NO may be a key component in this process. NO that is produced by three isoforms of NOS, namely neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS is highly reactive. Under normal physiological conditions, endogenous NO is produced by the constitutive NOS isoforms, eNOS and nNOS. These two are important for peristalsis (by nNOS) and maintaining mucosal blood flow (by eNOS) depend on Ca. Independent on Ca, iNOS is expressed in many cells, extravascular resident leucocytes (macrophages), intravascular and/or infiltrating leucocytes (neutrophils and monocytes), endothelium, and parenchymal cells, including intestinal epithelium after exposure to various inflammatory stimuli, such as lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α, or IL-1β. iNOS produces large amounts of NO for a limited period of time and is an element of the innate immunity. Excessive and prolonged NO production has been suggested to cause intestinal dysfunction in certain diseases, including inflammatory bowel disease (IBD) and sepsis. For inflammatory reaction that leads to injury, the role of NO is controversial, with evidence for pro-inflammatory as well as anti-inflammatory effects [85]. NO and its metabolites have a role in cytotoxicity and genotoxicity [86, 87]. Effect of NO on tumor progression is dependent on the type of tissues/cells and activity of NOSs. Production of NO and expression of iNOS has often been detected in several established human tumor cell lines [88-91]. However, considering the physiological role of NO, inhibition of NO generation could have harmful effects.

Chronic inflammation and continuous exposure to NO produced by iNOS leads to neoplastic transformation, which is a key step in carcinogenesis. NO produced by iNOS is able to initiate and/or promote carcinogenesis [92, 93]. Mice with mutations in both adenomatous polyposis coli (*Apc*) and

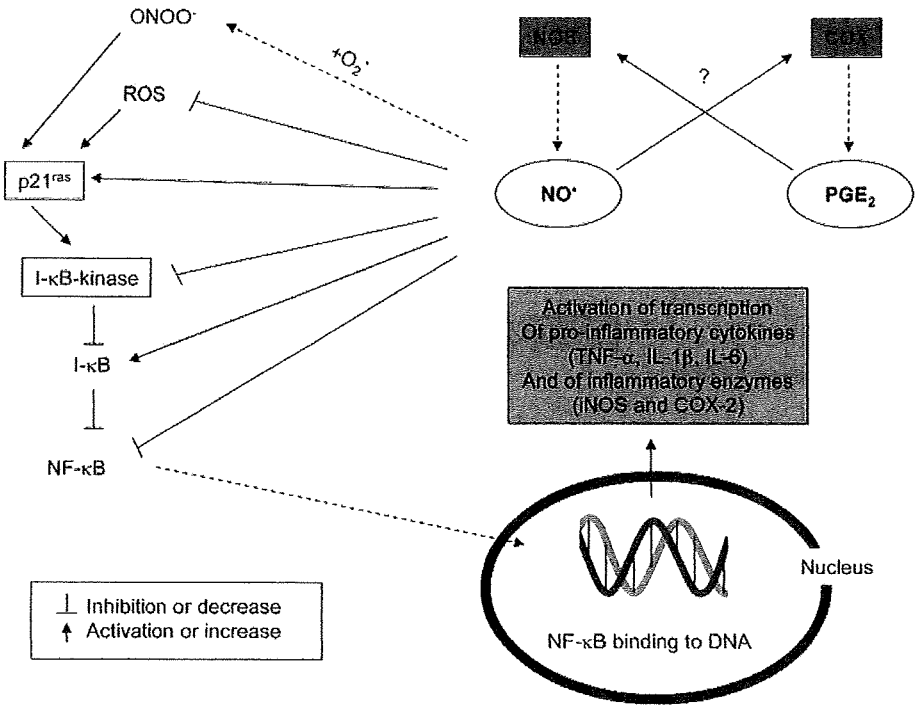


**Fig. (8).** Biphasic regulation of inflammation by NO. Biphasic regulation of inflammation by nitric oxide (Connelly *et al.*, 2001). COX-2, cyclooxygenase-2; I-κB, inhibitor of NF-κB; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor κB; NO, nitric oxide; $ONOO^-$, peroxynitrite anion; p21ras, a G-protein; $PGE_2$, prostaglandin $E_2$; ROS, reactive oxygen species; TNF-α; tumor necrosis factor α.

*iNos* have fewer adenomatous polyps in the small and large bowels, when compared with mice with the mutation in *Apc* alone [94]. $iNos^{-/-}$ mice lowered gastric carcinogenesis induced by *Helicobacter pylori* [95]. These findings suggest iNOS as a target for tumor chemoprevention in digestive tract, including colon. Indeed, certain iNOS inhibitors reduced azoxymethane (AOM)-induced rat colon carcinogenesis and tumorigenesis in *Apc*-mutant mice [96]. Also, our recent study revealed that inhibition of iNOS result in suppression of inflammation-related colon carcinogenesis [97-99] in the mouse model [100], in which inflammation and iNOS expression are involved in carcinogenesis [101-106]. In contrast, there are conflicting results regarding different NOSs in carcinogenesis: both increasing and decreasing NO signaling as a potential strategy for cancer prevention [107, 108]. Further studies are needed to know what approach can be applied to balance the activities of various NOSs and target the iNOS. Currently, both treatment strategies to increasing NO signaling and decreasing NO signaling are being tested. Whereas, pre-clinical studies indicate that NO down-regulation might be of value in cancer chemoprevention. Pre-clinical studies in colon carcinogenesis models are encouraging in prevention of tumorigenesis with iNOS selective inhibitors. These studies are in agreement with genetic studies of iNOS knockout mice, but conflicting results in some $iNos^{-/-}$ mouse studies have also been known [94, 95, 109, 110]. Because of these contradictory findings, more detailed evaluation in pre-clinical models will be required prior to the clinical evaluation of this strategy, keeping in mind the multiple physiological roles of NO. However, NO promotes tumorigenesis, when associated with chronic inflammation and angiogenesis. NO interaction between COX-2 signaling and NO signaling is well documented [111], and this observation can be utilized in aiming cancer prevention. A combination of COX-2 inhibitor and iNOS inhibitor exerts a better chemopreventive effect against colon carcinogenesis [96]. Combinations of antiangiogenic agents and NOS inhibitors might also be more effective [112]. Exploitation of combination treatments which target multiple targets will be beneficial, although there are many gaps to be filled in to efficiently target iNOS.

## NF-κB

NF-κB comprises of a family of transcription factors which activate the expression of a wide array of genes involved in tumorigenesis, metastasis, differentiation, embryonic development, apoptosis and inflammation [113]. The family comprises of NF-κB1, NF-κB2, Rel A, c-Rel and Rel B. These proteins consist of hetero- or homodimers and share a 300-residue long Rel domain. In normal conditions NF-κB interacts with the protein Iκ-Bα, which covers the signal sequence responsible for DNA binding and nuclear translocation, on the NF-κB subunit [114]. This interaction results in the sequestration of NF-κB in the cytoplasm of cells. Stimulation can occur *via* a wide number of different pathogenic stimuli and results in phosphorylation of Iκ-Bα followed by degradation by the 26S proteasome. This uncovers the nuclear localization signal, enabling nuclear translocation of the NF-κB complex with activation of a number of different genes, ultimately leading to increased expression of pro-inflammatory and pro-oncogenic messenger mole-

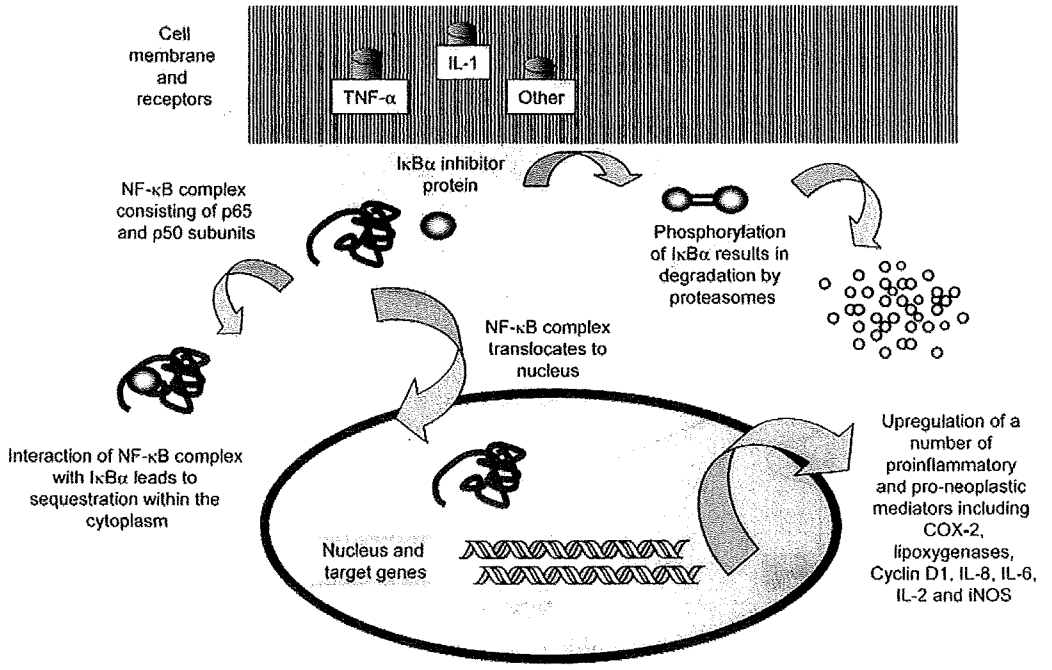


**Fig. (9).** NFκB increases expression of pro-inflammatory and pro-oncogenic messenger molecules.

cules, such as iNOS, COX-2, cyclin D1, and IL-8 [115-117] (Fig. **9**). An alternate mechanism, by which NF-κB promotes cancer growth, may involve activation of the mitogenic cyclin D1 gene [116, 118] which has been shown to be overexpressed in human colonic cancer tissue and to have an inverse correlation with patient survival [117, 119-121]. In addition to promoting cell survival and growth, NF-κB has been implicated in increasing the angiogenic potential of pancreatic cancer cells *via* increased expression of the proangiogenic factors, vascular endothelial growth factor (VEGF) and IL-8 [117, 120, 121]. IL-8 is produced by cells in response to hypoxia and can directly stimulate tumor cell growth [122] as well as encouraging angiogenesis [123]. Hence, NF-κB may be implicated in influencing the aggressiveness and metastatic potential of colonic cancer cells.

NF-κB activation may contribute to the characteristic resistance of colonic tumor cells to the apoptotic effect of chemotherapeutic agents [124, 125]; hence, inhibiting the activation of NF-κB could be a useful adjunct in the medical management of the disease. *In vitro* work has shown that blocking NF-κB with the natural inhibitor IκBα suppresses tumorigenesis and increases the effect of chemotherapeutic agents such as gemcitabine, etoposide and doxorubicin [124, 126, 127]. Alternatively, direct blockers of NF-κB, such as the NEMO-binding domain (NBD) peptide [128], have been used. Other methods of targeting the NF-κB pathway include the proteasome inhibitor, MG132, which prevents proteasome-dependent degradation of the NF-κB inhibitor protein, IκBα [124], and the protein LDCOI which blocks upstream activation of the NF-κB cascade [129].

The formation of reactive oxygen species (ROSs), comprise one of the many pathogenic stimuli which activate NF-κB and this has led to attempts to use anti-oxidants in colon cancer cell lines [130]. Polyphenols are a huge and poorly defined class of substances which range from simple phenolic acids to highly polymerised condensed tannins. Polyphenols comprise a major constituent of human food and have been shown to exhibit a number of poly-mechanistic anti-cancer activity predominately through their anti-oxidant and anti-inflammatory effects. The polyphenols quercetin and resveratrol have shown potential to inhibit NF-κB activation in colon cancer cell lines [131]. There is limited evidence that polyphenols may, at least in part, exert this effect by inhibiting phosphorylation of Iκ-Bα [132]. The possibility that changes in diet, to include a higher intake of polyphenols, may reduce pancreatic cancer risk is an attractive and simple idea, but further work is needed.

## HO-1

The role of HO-1 in cancer stems from the demonstration that HO-1 is a potent regulator of cell growth (Fig. **10**) and angiogenesis. CO signaling has been established in the promotion of angiogenesis in human microvessel endothelial cells, presumably by increasing the levels of HO-derived CO [133]. In addition, HO-1 has been shown to accelerate tumor angiogenesis in certain human cancers [134]. Mice lacking Nrf2 are more susceptible to dextran sulfate sodium-induced colitis, suggesting that Nrf2 plays an important role in protecting intestinal integrity through regulation of proinflammatory cytokines and the induction of phase II detoxifying enzymes [135]. Constitutive over-expression of Nrf2-dependent HO-1 confers resistance to apoptosis induction by epigallocatechin 3-gallate; therefore, its inactivation may be a target for overcoming the resistance to chemoprevention and chemotherapy [136].

The up-regulation of phase II detoxifying and stress-responsive genes is believed to play an important role in

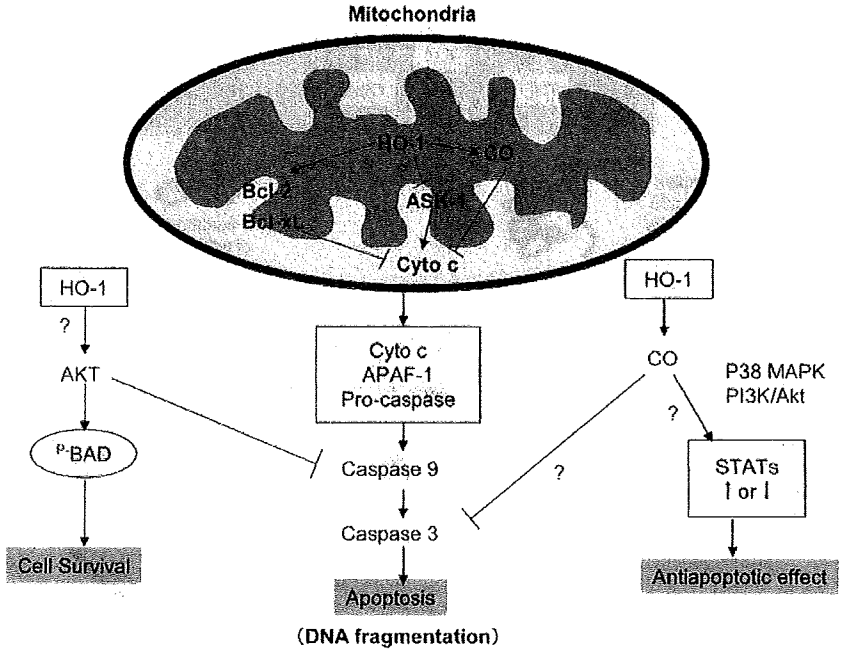


**Fig. (10).** HO-1 in cell growth.

cancer prevention, and many natural agents have been shown to be potent inducers of these genes. The antioxidant-responsive elements found in these genes can be bound by the transcription factor Nrf2 and are responsive to activation by chemopreventive agents and by oxidative stress. Nuclear Nrf2 activates antioxidant-responsive elements and induces the expression of stress-responsive genes, including HO-1 [108]. Nrf2 and HO-1, regulated by Nrf2, were not expressed in skin tumors from mice of either genotype whereas HO-1 expression in Nrf2-/- mice was much higher than that in Nrf2-/- mice in non-tumorous skin samples [137]. These results demonstrate that $Nrf2^{-/-}$ mice are more susceptible to skin tumorigenesis and that the chemopreventive effects of sulforaphane are mediated, at least in part, through Nrf2. HO-1 over-expression increased the viability, proliferation and angiogenic potential of melanoma cells, augmented metastasis, and decreased survival in tumor-bearing mice, suggesting that the induction of HO-1 may be detrimental in the treatment of melanoma [138].

A relationship between malignancy and perturbations in HO exists in cancerous tissues. Angiogenesis is necessary for the continued growth, invasion, and metastasis of tumors, and several studies have shown that HO-1 plays an important role in angiogenesis [133]. As such, HO-1 can be considered a target for anti-tumor therapy because the growth of most tumors depends on the activity of this enzyme. Inhibition of HO activity by ZnPP significantly reduces tumor growth in a rat model [139, 140]. Increased levels of human HO-1 protein accelerated tumor growth, stimulated the early stages of angiogenesis, and increased the occurrence of lung metastasis of pancreatic cancer in a mouse model [134]. Conversely, the inhibition of HO activity, was shown to be beneficial in controlling angiogenesis and both the growth and the spread of tumors [141]. The findings suggest that HO-1 is an attractive target for chemotherapeutic intervention.

The effects of HO-1 induction on cell survival were examined in a human colon cancer cell line, Caco-2 [142]. HO-1 induction resulted in resistance to apoptosis, activation of Akt, reduction in p21 (Cip/WAF1) levels, and modification of the Bcl-2/Bax ratio toward survival. Their study shows the anti-apoptotic effect of HO-1 in colon cancer cells, which was mediated through the formation of bilirubin and biliverdin. An anti-apoptotic role for HO-1 in these cells and provides a mechanism by which increased levels of HO-1 may promote tumor resistance to stress in conditions of limited nutrient supply. Thus, HO-1 may be an ideal target to control cancer growth; in fact, enhancement of the chemotherapeutic response of tumor cells was achieved by an HO inhibitor, ZnPP, *in vivo* and *in vitro* [139].

## REACTIVE OXYGEN SPECIES (ROSs) AND DNA DAMAGE

ROSs are highly reactive oxygen metabolites which include the superoxide radical $O_2^-$, hydrogen peroxide $H_2O_2$ and hydroxyl radical OH- [143]. ROS are produced as part of the inflammatory, by phagocytes, as well as being produced continually as part of normal cellular metabolism. ROS can cause lipid peroxidation, leading to mutagens, such as the carbonyl compound malondialdehyde (MDA) or, alternatively, they can directly damage DNA bases (Fig. **11**). A huge variation of oxidised DNA adducts can be formed, examples include 3-(2-deoxybeta-dierythropentafuranosyl) pyr[1,2-alpha]-purin-10(3H)one ($M_1G$) and 8-oxodeoxyguanosine (8-OH-dg) [144-146]. $M_1G$ is formed from malondialdehyde (MDA) which is a by-product of lipid peroxidation and prostaglandin synthesis. MDA reacts with DNA to form up to six different adducts, of which $M_1G$ is the predominant species and the most carcinogenic molecule [146]. 8-Oxo-deoxyguanosine (8-OH-dg) is formed by the

reaction of oxygen radical or singlet oxygen with DNA. In DNA it induces G-T tranversions, which have oncogenic potential [147]. DNA adducts can also be formed *via* the MDA pathway from the manufacture of prostaglandins, catalysed by the enzyme COX-2 [146] and by the influence of iNOS which produces ROS.

In addition to their role in damaging DNA, evidence is emerging that ROS can directly stimulate cancer growth. Fibroblasts transfected with the viral ras oncogene have increased superoxide production, and the generated superoxide may act as a second messenger molecule to promote cell proliferation [148]. Although traditionally ROS were thought to promote cell death, more recent data suggests that ROS may inhibit apoptosis in colorectal and pancreatic cancer cells [149, 150]. Up to 104 DNA lesions can occur each day from different contributory sources including oxidative damage [151]. Cellular defence against this damage comprises of three mechanisms. The first mechanism is from enzymatic inactivation of superoxide by superoxide dismutase, and inactivation of hydrogen peroxide with catalysis. The second line of defence is hydrolysis of oxidised bases and thirdly, complex DNA repair mechanisms to, including base excision repair (BER), transcription-coupled repair (TCR), global genome repair (GGR) and mismatch repair (MMR) [152] (Fig. **11**). Disturbances in the balance between DNA damage, and the protective prevention and repair mechanisms can occur through heavy sustained oxidative damage, such as in chronic inflammation, or by inherited or acquired defects in the intricate defence systems.

## HMG-CoA REDUCTASE

HMG-CoA reductase is the key regulated step in cholesterol synthesis (Fig. **12**) and represents the sole major drug target for contemporary cholesterol-lowering drugs. There are conflicting reports in the literature on the association between serum cholesterol level and CRC. The geographical incidence of CRC correlates well with high fat diets [153]. Patients with CRC have high levels of fecal bile acids and cholesterol. Results from animal studies also support the significance of fat intake in CRC development [154]. Fecal bile acid and cholesterol metabolites are considered to act as promoters, co-carcinogens or carcinogens in colon carcinogenesis. Cholesterol is an obligatory precursor of the bile acid. HMG-CoA reductase is a polytopic and transmembrane protein that catalyzes a key step in the mevalonate pathways [155], which is involved in the synthesis of sterols, isoprenoids and other lipids. These end products are important for many different cellular functions. Acetyl-CoA (citric acid cycle) is converted to acetoacetyl-CoA. Acetyl-CoA condenses with acetoacetyl-CoA to form HMG-CoA. HMG-CoA is reduced to mevalonate by NADPH. This reaction occurs in the cytosol. It is the committed step in cholesterol synthesis, which is why the enzyme (HMG-CoA reductase) catalyzing the reaction is a target of the HMG-CoA reductase inhibitors, statins. Blockade of rate limiting step by statins results in reduced levels of mavalonate and its down stream molecules, such as Ras, nuclear lamins, and small guanosine triphosphate (GTP)-binding proteins (members of the Rab, Rac, and Rho families), having significant influences on cellular functions. HMG-CoA reductase is observed to be overexpressed in colon cancer cell lines, which makes it a potential molecular target for prevention/treatment of CRC [156]. Statins also exert immunomodulatory and anti-inflammatory effects that are also beneficial for cancer inhibition [157].

HMG-CoA reductase inhibitors, such as lovastatin, atorvastatin, pravastatin, simvastatin, and pitavastatin have been in use for the past 15 years for their efficacy in reducing cardiovascular diseases. These studies suggest merits of statins use in chemoprevention of chronic diseases. Whereas, major randomized controlled trials demonstrated no association between the use of HMG-CoA reductase inhibitors and

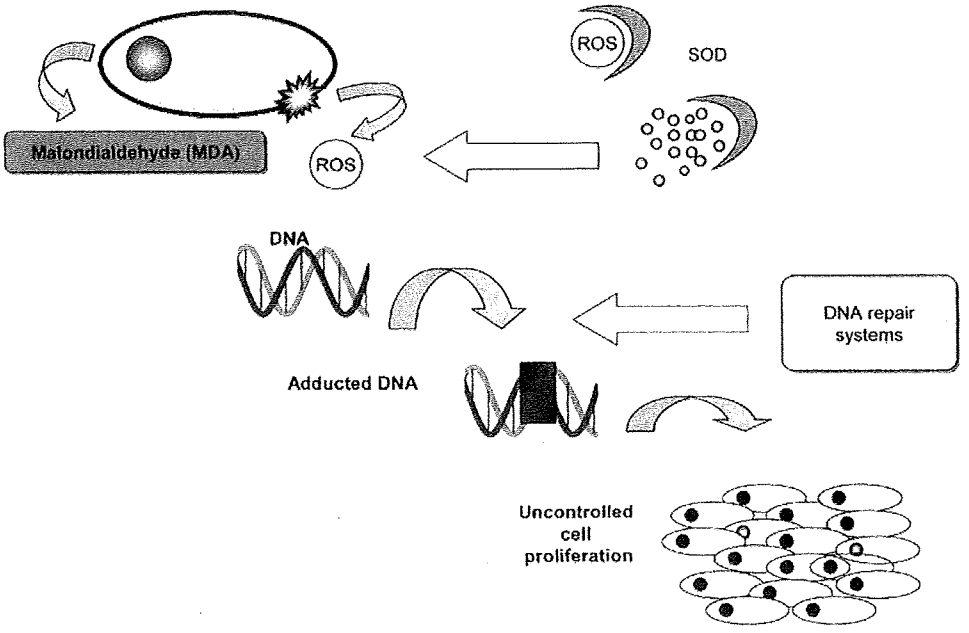


**Fig. (11).** ROS leads to mutagens.