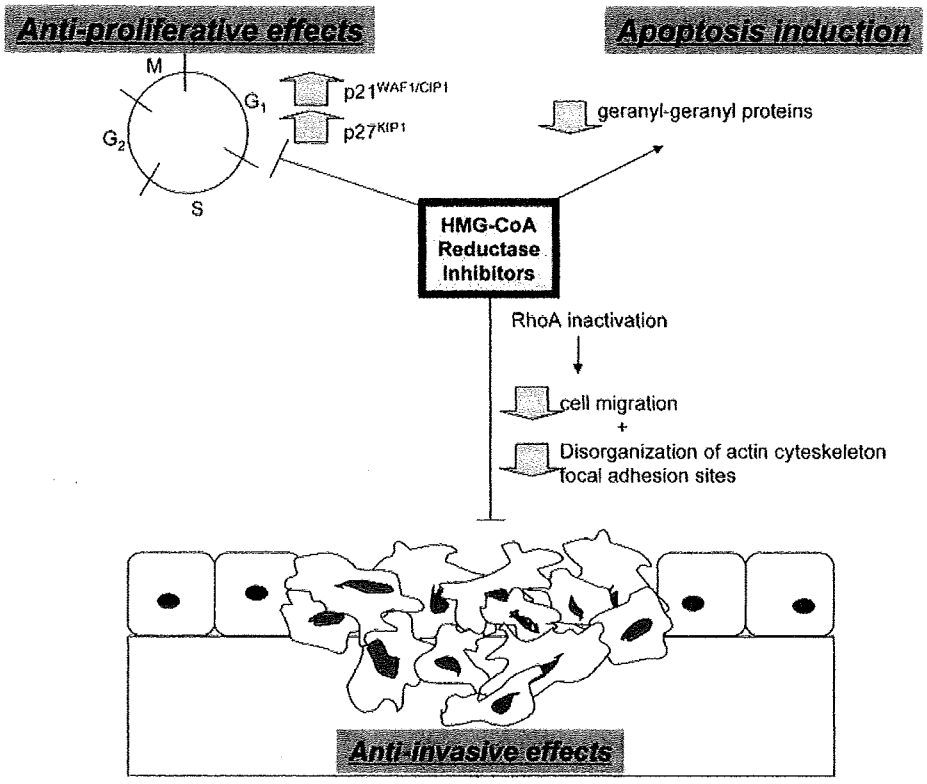

Fig. (12). HMG-CoA reductase and proliferation.

the risk of fatal and non-fatal colon cancers [158]. A recent large study, the Molecular Epidemiology of Colorectal Cancer (MECC) study, which included almost 4,000 people, showed that statin use for a period of more than 5 years was associated with a 47% reduction in the risk of CRC [159]. Eight small prospective studies found no or weak statistically significant association between statin use and the risk of CRC and significantly lowered risk with dose levels of specific statins [160-164]. Although these controversies exist on statins effect on CRC, many pre-clinical findings support the positive effects of statins in reducing CRC [165-168]. Our recent study also revealed that pitavatatin effectively inhibits colitis-related colon tumorigenesis in mice [169]. Anti-proliferative effects of HMG-CoA inhibitors on different cancer cell lines are through two cyclin dependent kinases and Rho small GTPases, geranylgeranylated by geranylgeranyl pyrophosphate (GGPP) [170, 171]. Statins in combination with cytotoxic drugs showed positive results in inhibition of colon carcinogenesis in pre-clinical assays [168, 172]. Since HMG-CoA reductase inhibitors exhibit diverse effects on various aspects of carcinogenesis *in vitro* and *in vivo*, they deserve further investigation in chemoprevention and therapeutic clinical trials. Clinical trials are under way to assess whether these actions will translate into significant clinical benefit.

RXR-α

Retinoids comprise a family of polyisoprenoid lipids consisting of vitamin A and its derivatives. Vitamin A and its natural and synthetic analogs exert their multiple functions through the RAR and RXR receptors (Fig. **13**). RXRs have important cross interactions between RXR and other 20 nuclear receptors. RXR forms heterodimers with RAR, VDR, TR, etc. through which they transmit the hormonal signals by interacting with co-activators and co-repressors to regulate genes [173, 174]. There are three retinoid X receptors (RXR-α, RXR-β, RXR-γ), and RXR-α, being a member of the nuclear receptor super family which, regulates development, organ physiology and cell proliferation. These receptors are altered distinctly in different tissues during carcinogenesis and provide evidence of unique functions of each receptor in different tissues. The expression patterns of these receptors in carcinogenesis give prognostic information, as these receptors have a prominent role in actions of gene expressions, when interacted with a ligand. Among the RAR and RXR receptor subtypes, RXR-α mRNA was observed to be expressed at the highest levels in gastric mucosa. Since RXR-α interacts with other receptors, such as Vit. D and peroxisome Peroxisome proliferator-activated receptor (PPAR)γ, it may also play a physiological role in the colon. This can be supported by the investigations of Kane *et al.* that among all the three receptors (RXR-α, RXR-β, RXR-γ) tested, a significant decrease in RXRα expression was found in human CRC, when compared with non-neoplastic tissue. Genetic studies with $RXR\alpha^{+/-}$ mice reported a significantly enhanced susceptibility to 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, when compared with $RXR\alpha^{+/+}$ mice [175]. RXR has a role in decreasing colonic inflammation in mouse models [175]. In addition, RXR-α induces β-catenin proteasomal degradation, replacing the function of APC and shows an interaction of

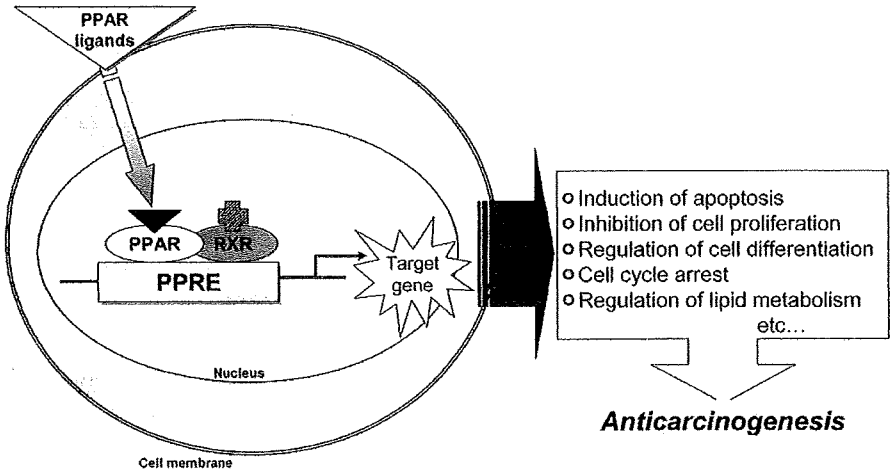

Fig. (13). RXR and PPAR pathway and transcriptional regulation of target genes.

RXR-α with β-catenin [176]. RXR-α may thus exert chemopreventive or chemotherapeutic effects on CRC *via* the regulation of β-catenin [176]. Dysregulation of β-catenin is a common outcome of mutations observed in CRC, resulting in β-catenin induced activation of oncogenes [176]. RXR agonists enhance interaction between RXRα and β-catenin, resulting in more efficient β-catenin degradation and subsequent anti-proliferative effects [98]. RXR-α acts as a carrier for nucleo-cytoplasmic translocation of orphan receptors [177]. Taken together, RXR-α is considered to be an attractive molecular target in CRC and RXR agonists might have a beneficial role as a therapy in CRC.

Rexinoids are ligands and agents that selectively bind to RXRs [178, 179]. They are found effective both in prevention and treatment of certain types of cancer. Most notable rexinoids are bexarotene (LGD1069), LG100268, VTP 194204 studied. Bexarotene is the first synthetic RXR-selective agonist to enter for clinical trials for cancer therapy. This agonist has minimum binding to RARs, when compared to other rexinoids and is effective in preventing ER^- and ER^+ mammary cancers [180]. Notably, bexarotene is well tolerated without any classical signs of traditional retinoid toxicities. Although it is tested for breast cancer, there are no studies on colon carcinogenesis using this agonist. LG100268 and VTP 194204 are potent selective RXR agonists with no biologically relevant binding to RARs, and are more effective than bexarotene [181]. However, they need to be tested in animal models of colon carcinogenesis, before being taken for clinical studies. RXR-specific agonists AGN195362, AGN195456, AGN195741, AGN196060, and AGN196459 were tested with CRC cell lines and induced growth inhibition through β-catenin degradation in *APC* independent manner [176]. These findings *in vitro* assay suggest the role of RXRs in ligand mediated protein degradations, but it needs to be validated in animal carcinogenesis models. Rather, induction or elevation of RXR-α level through specific ligands can be an efficient method in treating CRC. Chemopreventive potential of β-ionone, an end ring analog of β-carotene, which is naturally present in various vegetables, against chemically-induced ACF formation in rat colon has been recently reported [182]. The compound induces apoptosis and RXR-α expression. Identification of various natural and synthetic drugs selective to RXR-α can be beneficial in prevention/treatment of colon carcinogenesis.

ER-β

Estrogens (estradiol, estriol and estrone) are the steroid hormones which exert their actions through the estrogen receptors ER-β and ER-α (Fig. **14**), which have distinct expression patterns in human tissues [183]. ER is also attached to the cell membrane and is involved in signaling by forming complexes with G proteins, striatin, receptor tyrosine kinases, e.g., EGFR and IGF-1, and non-receptor tyrosine kinases and Cav-1 [184]. Several epidemiologic studies have suggested that CRCs are influenced by steroid hormones [185-187]. Studies on estrogen replacement therapy explain the protective effect on CRC risk, although mostly protective effects were provided by the progesterone rather than estrogen [188, 189]. Recent re-analysis of world wide data on the relationship between hormone replacement therapy and breast cancer showed that the risk of this cancer may be increased in women who are taking estrogen [190]. ERs are observed in colonic mucosa and CRC, and ER-β is the predominate receptor expressed in colonic tissue [191, 192].

The presence of five different isoforms of ER-β is suggested in the colon mucosa and CRC. Estradiol treatment significantly enhances the growth of CRC cells injected in mice, and the resulting tumors were notably larger in female than in male mice [193]. Since most of the actions of estrogens exert through the respective receptors [194, 195], ER might be important in regulating CRC risk associated with these hormones [196]. The changes in expression patterns of ER-β, rather than ER-α, suggest that ER-β gene is more important than ER-α in the etiology of CRC [197-199]. ER-α is certainly associated with more differentiated tumors in breast and other cancers, while the involvement or ER-β is controversial. ER-β protein level is lower in CRC than normal colonic mucosa, and loss of ER-β may have protective role in CRC development. However, certain investigations suggest that increase in ER-β level in colon tumorigenesis is associated with advanced stages and differentiation of CRC

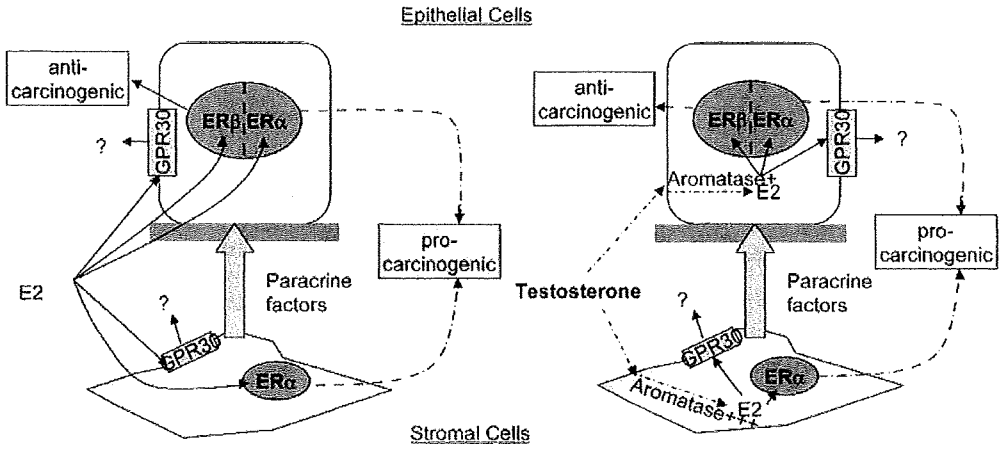

Fig. (14). Estrogens (E2) and estrogen receptors (ERs) in nuclear transcription. Systemic and local effects of estrogen in stromal-epithelial interactions. (**A**) Effects of systemic estrogens. Circulating E2 enters target tissues and can either bind to ER-α or ER-β of stromal or epithelial cells. E2 binding to ER-α in the stroma and epithelium is likely to be involved in the promotion of carcinogenesis. Stromal ER-α-signaling stimulates the secretion of putative paracrine factors to stimulate carcinoma formation. Unlike ER-α, ER-β when occupied by ligand slow epithelial growth and hence may be anti-carcinogenic. (**B**) Effects of local estrogens. Testosterone can be converted to E2 *via* aromatase and thus increase local concentration of E2, which may enhance estrogenic effects.

[200]. ER-$\beta^{-/-}$ knockout mice examinations indicated a role of ER-β in the organization and architectural maintenance of the colon, but it could not prove that an exact lack of ER-β is causing hyper-proliferation [201]. Therefore, ambiguity on the protective role of ER-β still exits.

Considering the differential functions of ERs, a class of drugs termed "selective estrogen receptor modulator (SERM)" has been developed. The concept of SERM is based on the ability to selectively activate or block one type of ER or to promote ER interactions with different proteins, such as transcriptional co-activator or co-repressor proteins. In addition, the different ERs combinations respond differently to various antagonists, and some agents exert partially agonistic and antagonistic effects, depending on the tissue [202]. One such anti-estrogens or SERMs, which is used to inhibit the growth of CRC and is widely used is anti-estrogen tamoxifene, which has shown good application in CRC cells and is also efficient in inhibiting liver metastasis of CRC in animal models [203]. However, the effect of tamoxifene on CRC in different observational and clinical studies were not encouraging either, there was no effect or increase of CRC risk [204, 205]. Because of side effects of tamoxifene, another SERM, raloxifene, is tested for its efficacy in cancers and prevention of osteoporosis. Raloxifene is safe when used as a preventative chemotherapy for women judged to have a high risk of developing breast cancer or who have breast cancer [206]. The accumulated results from three studies (MORE, CORE and RUTH) suggest that raloxifene use is safe and does not appear to increase the risk of CRC [207]. Raloxifene has biological effects on ERα-positive CRC cell lines [208]. Although other SERM agents, droloxifene, idofene and toremifene are known, but they still need to be experimented to be considered. Another anti-estrogen, ICI 182,780 (Faslodex), which acts as a complete antagonist, also promotes degradation of ER. ER-β isoforms predominate in the colonic mucosa, and each isoform needs to be evaluated for its ligand dependent and independent effects on cell growth, development or death [209]. In this context, we need investigations of estrogen effects by determining the ER-β expression in CRC and surrounding normal mucosa in patients (men and women) below 40 years.

β-CATENIN

β-catenin is a subunit of the cadherin protein complex. β-catenin is found at the plasma membrane in association with cadherins, in association with the tumor suppressor promoter APC and microtubules, in the cytoplasm and in the nucleus (Fig. **15**). Therefore, it cannot be presumed that it works only in the nucleus to "signal" a response. It is a multi-functional protein involved in cell adhesion, signaling and many more [210]. β-catenin regulates pre-mRNA splicing [211]. There are a number of reports pertaining to the functional interactions between nuclear receptors and the canonical, Wnt/β-catenin signaling pathway cascade [212]. β-catenin interacts with various nuclear receptors, and genetic interactions between ER and β-catenin may promote growth and tumorigenesis in eye of drosophila [213]. This significance remains to be experimented in other systems to give a better idea of the functions and interactions of β-catenin with nuclear receptors. The deregulation of β-catenin leads to various types of cancer, particularly CRC [214]. Normal function of APC in combination with glycogen synthase kinase (GSK) 3β and axin regulates free cytoplasmic β-catenin levels by binding to and targeting β-catenin for degradation by ubiquitination-dependent proteolysis [215-217]. This regulates the availability of free β-catenin for binding with the TCF-LEF family of transcription factors in the nucleus [218]. Mutations in *APC* or β-catenin result in the failure of β-catenin to be degraded, and they are retained in the cytoplasm of cells, which is often observed in CRC [214]. Subsequently, there will be an increase in β-catenin-TCF complex formation, causing

alterations in gene transcription (myc, cyclinD1, c-jun, Tcf-1, Lef-1, conductin/axing MMP7), leading to carcinogenesis [219, 220]. Somatic mutations in genes in the β-catenin pathway are found in >80% of CRC, either FAP or sporadic, and aberrant β-catenin activity plays an early and causative role in CRC development. This may occur by mutations in the APC protein, axin or in the β-catenin itself, which leads to dysregulation of β-catenin turnover and activation of genes involved in carcinogenesis [218]. Since these mutations are exclusively observed in CRC, β-catenin is considered to be a potential molecular target in colon carcinogenesis [219, 220]. Expression of nuclear β-catenin in macroscopic tumors is further upregulated in comparison with that in microadenomas, suggesting that activation of β-catenin/Tcf transcription plays a role not only in the initiation stage but also in the promotion stage of colon carcinogenesis in $Apc^{Min/+}$ mice [221]. A study with folic acid indicates that certain dietary supplements can enhance the tumor formations through β-catenin accumulations [222], suggesting that folate can act as a tumor inhibitor only under particular settings with a specific genetic status of the disease. A recent findings from a clinical study also support the data that folic acid did not show any effect in preventing CRC, rather enhanced CRC [223].

PPARγ and RXR-α interact with and stabilize β-catenin transcription complex in CRC cells. The interaction of ligands, such as NSAIDs with PPARγ, may induce the conformational change of the receptor, leading inhibition of transactivation function of β-catenin, finally blocking Wnt/β-catenin signaling [224]. The interaction of RXR receptors with β-catenin is reported to induce degradation of β-catenin in CRC cell lines [176]. This may suggest a significant value to the development and selective use of rexinoids and NSAIDs-like drugs as cancer chemopreventive and/or chemotherapeutic agents. A number of agents that inhibit the β-catenin pathway and show selective toxicity toward cancer cells have been identified. Currently, there are over 20 antisense oligonucleotides tested in clinical trials [225, 226]. Diverse β-catenin antagonists have also been developed [224, 227, 228]. The most advanced agents, the PRLX 8025 agentseries, are currently in pre-clinical efficacy testing in animal models of colon carcinogenesis [228, 229]. The development of small molecules which selectively target β-catenin interaction that transmits the cell proliferative signal of β-catenin, while leaving alone the other activities of β-catenin required for normal cell growth, may be a viable option for treatment and prevention. The development of drugs that selectively target the nuclear entry or exit of regulatory proteins altered in cancer may be another option for treatment and/or prevention.

STAT-3

The STAT family of cytoplasmic proteins translocate to the nucleus to stimulate gene expression (Fig. **16**). Stat (signal transduction and translation) proteins, normal constituents of cells activated by tyrosine kinases, have diverse biological functions [230]. Any aberrations in stat signaling are predicted to have a wide variety of consequences. Stat 1, 3 and 5 are observed to be more strongly associated with human cancers out of the known stat family members. Although dysregulated activity of STAT factors 1, 3 and 5 has been implicated in various cancers, not many studies have addressed these in the context of CRC. STAT3, which normally resides in the cytoplasm, becomes activated in response to a range of growth-factor signalling pathways through tyrosine phosphorylation. This leads to its dimerization and translocation to the nucleus. Stat3, which has been identified as an oncogene, not only regulates cell proliferation and survival but also has been implicated in cell migration. However, no target genes have been implicated in its effects on cell migration. Ng *et al.* [231] identified SCLIP, a member of a family of tubulin-binding proteins that includes stathmin (which destabilizes microtubules), in a yeast two-hybrid screen for Stat3-binding proteins. Thus, Stat3 appears to regulate microtubule dynamics and cell migration through interactions with stathmin in the cell cytoplasm independent of its ability to translocate to the nucleus and activate gene transcription.

Experimental and clinical data revealed the oncogenic potential of STAT3 through over expression and constitutive activation in a variety of human malignancies, including leukemia, melanoma, and carcinomas of head and neck carcinoma, breast, prostate, ovarian and colorectum [232-235].

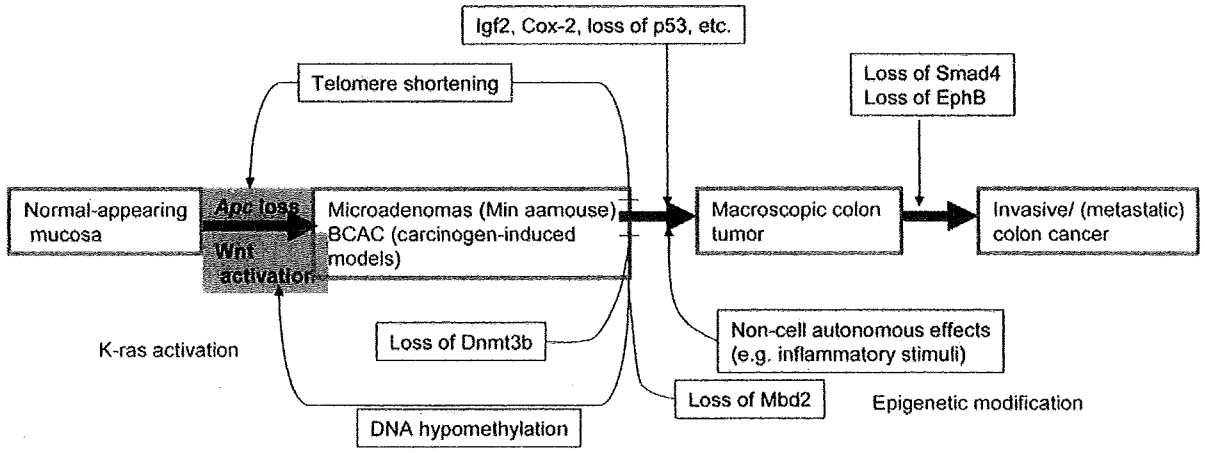

Fig. (15). Genetic alterations in multistage colon carcinogenesis. A model for multistage colon carcinogenesis in mice and its possible modifiers.

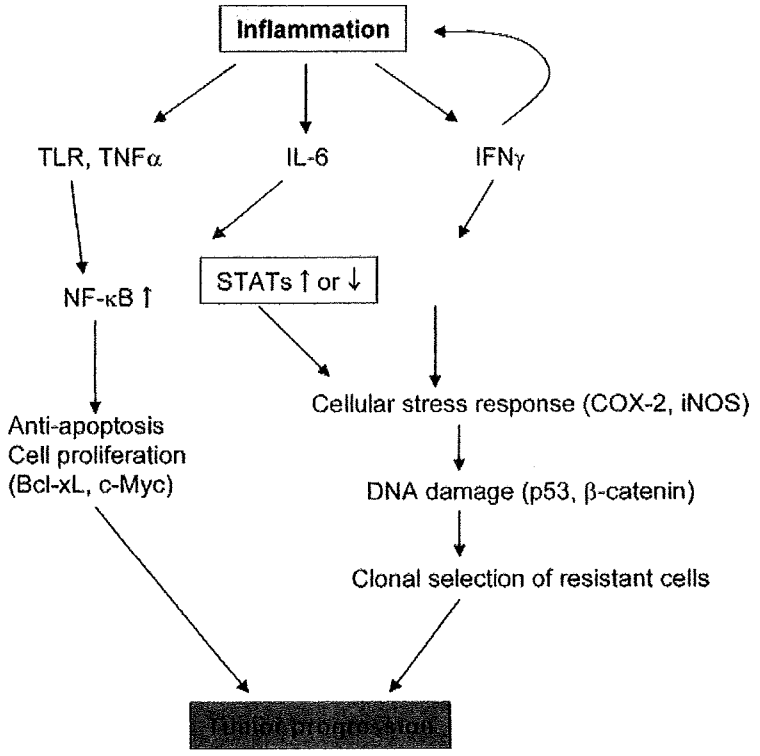

Fig. (16). STATs in tumorigenesis.

STAT3 is activated by cytokine and growth factor receptors, several viral or cellular oncogenes, such as *src, fps*, polyoma virus middle T-antigen and *sis* [235]. Cell transformation by aberrant STAT3 activity involves up-regulation of genes promoting cell cycle progression (cyclin Dl and c-myc) and/or preventing apoptosis (bcl-xL, mcl-1, and survivin) and cell migration [236, 237]. These findings suggest that STAT3 is a suitable target for disease intervention. STAT3 elicits permanent changes in the genetic constitution of the cell required for the initiation and maintenance of tumorigenesis. STAT3 also plays a vital role in tumor angiogenesis by acting as a direct transcriptional activator of vascular endothelial growth factor (VEGF) [238]. STAT3 is activated by modulation of both tyrosine phosphorylation and lysine acetylation to stimulate cancer cell growth and metabolism. These findings also suggest better targeting of STAT3. One can target upstream (JAK, SRC, EGFR) and downstream targets responsible for activation of STAT3. The results of STAT3 disruption in mouse fibroblasts demonstrate that deletion of STAT3 is not deleterious for normal functioning of cells [230]. This observation clears the fears of drastic effects to be normally associated by blocking STAT3 functioning. While the molecular mechanism of oncogenesis by STAT3 is yet to be defined in colon carcinogenesis, STAT3 protein is involved in mediating cytokine signaling, which in turn, has a vital role in inflammation of cells. Inflammatory cells have powerful effects during the early step of colon carcinogenesis and promote tumor development, facilitate genomic instability and promote angiogenesis. Thus, one might assume that STAT3 is playing a vital role in colon carcinogenesis [239]. Genetic approaches and also small molecule inhibitors, including anti-tumor cytokines might be better approaches for inhibiting activated STAT3. Efficacy studies in animal models should be carried out to screen various naturally occurring or synthetic anti-inflammatory molecules, which have a promise in inhibition of activated STAT-3 and further prevention of colon carcinogenesis [240].

NON-PROTEIN-CODING RNAS: miRNA, siRNA, AND piRNA

RNA silencing is a generic term for a group of pathways that utilize short RNA molecules to achieve sequence-specific gene repression, often post-transcriptionally. Recently, not only cancer but also bioscience research has surfed on the new wave of RNA knowledge. Most of those RNAs are non-protein-coding RNAs and are connected to cell development and differentiation, and thereby with cancer differentiation and treatment [2]. The 2nd Microsymposium on Small RNAs at Vienna highlighted .three major themes emerged: (i) characterization of the RNA silencing machineries and identification of their components, with particular emphasis on the miRNA pathway, (ii) prediction and characterization of miRNA targets, and (iii) characterization of piRNAs, one of the latest additions to the growing family of small RNAs.

1. miRNAs-Emerging New Science as a Target for Multiple Diseases

miRNAs are short non-coding RNA molecules that regulate expression of genes by repressing translation or by cleaving RNA transcripts. These play a crucial role in cell

differentiation/development, proliferation and apoptosis [241]. Therefore, they are believed to play an important role in cancer development and can become potential therapeutic targets. Each miRNA can target hundreds of transcripts directly or indirectly [241, 242], whereas more than one miRNA can converge on a single mRNA target [243, 244]. Recent studies indicated that miRNAs are deregulated in various cancers including CRC [245-247]. The expression profiles of miRNAs can be used for the classification, diagnosis and prognosis of malignancies [248]. miRNA expression is frequently disregulated in cancer cells, and specific miRNAs regulate both cell-cycle progression and apoptosis [249]. miRNAs are important components of the p53 transcriptional network in HCT116 colon adenocarcinomas cell line. The observation suggest an important role for miR-34a in mediating p53 tumor suppressor function [250]. Mutations in miRNAs are observed in breast cancer tissue and the role of mutations in miRNAs is still to be elucidated [248]. A number of molecular studies have shown that colon carcinogenesis results from an accumulation of epigenetic and genetic alterations, suggesting that alternative genetic events may occur during colorectal tumorigenesis. A few reports described that CRC is associated with altered expression of miRNAs [245, 251]. In addition, the expression level of miR-31 was correlated with the stage of CRC. MAPK transduction proteins, such as MAP3K and MAP4K4, are possible targets for miR-145 [252]. Because up to hundreds of target genes may be affected by a single miRNA, as predicted by bioinformatics approaches and thus, a given miRNA may target several hundreds of miRNAs that would include transcripts of oncogenic or anti-oncogenic genes. We need a study to identify specifically the altered miRNAs related to specific pathways and to target them to restore the normal functioning of the colonic cryptal cells. Also, we should further understand the biological and functional mechanisms of miRNA to know how miRNA contributes to carcinogenesis. Recently interesting findings that abrogation of miR-34a function could contribute to aberrant cell proliferation, leading to colon cancer development have been described [253]. Targeting miRNAs thus could provide an important diagnostic for prevention/ therapeutic strategy for human CRC in the future.

2. siRNAs

siRNAs are tiny bits of genetic material that can prevent the translation of genes into proteins, including specific proteins involved in biochemical reactions that promote cancer and other chronic diseases. siRNA interference is considered as an invaluable tool in biological research, and could also become a powerful therapeutic modality, because of its broad applicability, specificity and high efficiency [254]. There are numerous reports on use of siRNA in silencing a gene of interest in many different diseases, including cancer [255]. siRNA approach is effective against TGFβ1 and reduced its expression in HCT-116, a human CRC cell line [256]. Treatment with a COX-2 siRNA in CRC cells result in a significant knockdown of COX2 at the protein level of 57%, as compared to a non-silencing siRNA control [257]. Down-regulation of endogenous levels of COX-2 can be achieved in CRC by siRNA. This strategy should prove to be a valuable tool in revealing the specific function of COX-2 in colon tumorigenesis [257]. RNA-i based technologies have gained popularity by addressing the ability to knockdown several genes at the same time, but this can be a disadvantage if considered a specific knockdown. siRNA was used to obtain defined combinations of pro- and anti-apoptotic gene expression in CRC cells of varying p53 status [258]. siRNA is also used in elucidating functional role of lysophosphatidic acid (LPA) and LPA receptors in CRC cells [259]. The observation indictae Bcl-2 and LPA receptors accessibility for siRNA silencing, making specific gene targeting a very possible approach. siRNAs targeted against different oncogenesis-related pathways in colon carcinogenesis are tested in mice successfully, but there are many hurdles needed to be cleared before it can be used for the treatment. There are possible toxicities induced by partial inhibition of homologous genes/function of endogenous siRNAs. If we can overcome the problems, this strategy can become an attractive therapeutic model for colon carcinogenesis.

3. piRNAs

piRNAs are a new class of small non-coding RNAs that possess unique long length of nucleotides [260-262], which differ from other miRNAs and siRNAs. These are named as piRNAs due to their association with Piwi proteins, which belong to Argonaute proteins. These are found to be present in sperm-producing cells in mammals. The piRNAs are present during the initiation of meiosis of sperms and observed to disappear by the time sperm matures. Therefore, these may regulate germ cell maturations. piRNAs also have a possible role as a type of immune system against transposons. While the role of piRNAs in cancer development has not extensively been elucidated, hiwi gene (a human member of the piwi family) is known to cause germ cell malignancy [263]. Additionally, expression of hiwi is associated with proliferating human gastric cancer cells [264]. Such correlations may raise the questions of whether these piRNAs are related to expression of piwi proteins in relation to cancer development. piRNAs have not yet been studied in colon carcinogenesis, but the research is occurring and will hopefully give a lot of information for understanding the role of piRNAs in colon tumorigenesis that is useful for CRC chemoprevention and treatment..

CONCLUSIONS

Our goal of cancer prevention is the inhibition or retardation of carcinogenic step by intervening different and/or whole pathways in carcinogenesis, resulting in the reduction of cancer incidence. In this context, alterations of different enzymes and molecular events during colorectal carcinogenesis are good targets for chemoprevention and/or chemotherapy against this epithelial malignancy, as described. In fact, recent studies have shifted to multi-targeted strategies from mono-targeted strategies for the 'War against Cancer'. Since most epithelial malignancies are caused by dysregulation of about 500 different genes and alterations of several specific enzymes, agents targeting multiple gene products and enzymes are attractive for prevention or even treatment of malignant neoplasms at the late stage of carcinogenesis. Therefore, promising agents for prevention of cancer are

those interact with a wide variety of proteins, including inflammatory cytokines and enzymes, transcription factors, and gene products that that are involved proliferation and angiogenesis, and modify their expression and activity. In contrast, agents affecting mono- or a few targets are considerable at the early stage of carcinogenesis or in the specific conditions that are convenient for cancer development. The later include hyper-proliferation state and/or chronic inflammation. Additionally, the non-coding RNA may be used for treating CRC, although such a new technology in requires scientific proof that these strategies have true value.

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Review Article

Preclinical Assays for Identifying Cancer Chemopreventive Phytochemicals

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Dietary factors influence carcinogenesis in a variety of tissues. The consumption of fruits and vegetables is associated with a decreased risk of several types of epithelial malignancies. In addition, there are interrelationships between diet, environmental factors, and genetics that can affect cancer risk. Potential chemopreventive agents against cancer development can be found among nutritive and/or nonnutritive compounds in inedible and edible plants. To identify potential cancer chemopreventive agents, scientists are evaluating hundreds of phytochemicals for the prevention of cancer. This short review article describes in vitro and in vivo assays reported to identify potential cancer preventive compounds from plants.

1. Introduction

Cancer mortality rates in the developed countries have increased throughout this century. It is already the leading cause of death in some Western countries [1, 2]. In Japan, the progressive introduction of Western dietary habits, especially increased fat intake and reduced carbohydrate and dietary fiber intake, has increased the incidence of colon cancer and related deaths [3]. Great advances have been made in the pharmacologic-based treatment of malignant epithelial neoplasms (cancers). In addition, there is a marked increase in the understanding of cell and molecular mechanisms underlying carcinogenic processes. However, therapy for advanced neoplastic disease remains limited. This may be due to the fact that advanced neoplasms contain a large number of genetic and molecular alterations that contribute to the maintenance of their neoplastic progression.

The chemopreventive approach against cancer development is highly attractive. Although highly attractive from a theoretical point of view, practical limitations may exist with respect to developing novel and effective chemopreventive agents. Most importantly, practical clinical endpoints are not definite. The efficacy of cancer chemoprevention can be determined by comparing the incidence of nondeveloped disease in a treated group to that of a control group. Such a clinical trial is labor intensive, very costly, and time-consuming. In addition, practically such trials cannot be conducted for the rapidly increasing number of chemopreventive agents being identified. These considerations suggest the use of certain intermediate biomarkers as indicators of clinical efficacy. Validated intermediate biomarkers thus may allow relatively small chemopreventive trials to be conducted within a short-term period. However, there are no universal approaches to determine intermediate biomarkers and their method of validation has still not been proven.

Some herbal and botanical products are likely to possess cancer preventive activities [4–7]. Many cancer patients use complementary and alternative medicines, including phytochemicals in addition to, or following the failure of standard cancer therapy [8]. The term phytochemical applies to any plant-based substance, but in the field of nutrition and cancer this term is usually applied to nutritive and nonnutritive chemicals that occur naturally in fruits and vegetables. A diet rich in fruits and vegetables has long been suggested to correlate with reduced risk of certain epithelial malignancies, including cancers in the lung, colon, prostate, oral cavity, and breast [4–7, 9–12]. Also, the cancer prevention potential of Mediterranean diets based mainly on

Table 1: Proposed mechanisms of phytochemicals for cancer prevention.

Food sources	Chemicals	Modification of carcinogen metabolism	Antioxidant and/or anti-inflammatory properties	Modification of cancer cell biology	Induction of differentiation	Antiangiogenesis	Apoptosis induction
Rosemary	Carnosol	+	+	+			+
	Rosmarinic acid	+	+	+		+	+
	Ursolic acid	+	+	+		+	+
Carrots	Carotenoids	+	+	+	+	+	+
Tumeric	Curcumin	+	+	+	+	+	+
Garlic	Diallyl sulfide	+	+	+	+	+	+
Gingko	Gingkolides	+	+	+			
Crucifers	Isothiocyanates	+	+	+	+	+	+
	Sulforaphane	+	+	+	+	+	+
Citrus	Limonene	+	+	+	+	+	+
Mint	Menthol	+	+	+	+		+
Cherries	Perillyl alcohol	+	+	+	+	+	+
Onion	Quercetin	+	+	+	+	+	+
Grape seed	Resveratrol	+	+	+	+	+	+
Milk thistle	Silymarin	+	+	+	+	+	+
Soybean	Isoflavones	+	+	+	+	+	+
Tea	Catechins	+	+	+	+	+	+
Olive	Oleuropein	+	+	+	+	+	+

olive tree products is known [13]. The major component of the leaves and unprocessed olive drupes of Olea europaea is oleuropein and the majority of polyphenols found in olive oil or table olives are derived from its hydrolysis. Oleuropein is a novel, naturally occurring antioxidant compound, which may possibly be used to prevent cancer [14–16] and cardiotoxicity induced by doxorubicin [17]. Searching for medicinal benefits from edible or inedible plants is not a new idea since numerous modern medicines have plant origins. Given that the ingestion of some plant foods results in reduced risk for cancer, researchers are delving into the identification of phytochemicals with cancer preventive ability in studies in vitro (cell culture), in vivo (model animals) and those in humans [18]. Phytochemicals can be roughly classified into four groups based on their mechanisms of chemopreventive action, as shown in Table 1. Preclinical studies focus on the identification of possible cancer preventive agents, short-term pharmacology, and assessment of toxicity. Agents that are within tolerable safety limits in humans then moved clinical trials to test their efficacy.

This review briefly summarizes the in vitro and in vivo assays used to discover possible new chemopreventive agents possessing novel mechanisms of action. Surely there are many more assay systems currently used in different laboratories to find candidate cancer chemopreventive agents and determine their efficacy. Because of limited space, only limited assays that might be useful for carcinogenesis and chemoprevention studies are herein introduced.

2. Anti-Inflammatory, Antioxidative, Antiangiogenic, and/or Apoptosis-Inducing Compounds in the Prevention of Cancer Development

One of the most exciting areas of cancer chemoprevention is the effect of anti-inflammatory compounds against CRC. Almost a dozen case-control and cohort studies have concluded that the daily or alternate day consumption of aspirin over extended periods results in halving one's risk for colorectal cancer (CRC) [19]. Several prospective studies are underway to specifically assess whether the daily consumption of aspirin affects the recurrence rates of colorectal adenoma in high-risk subjects for CRC. These studies strongly suggest that eicosanoids, especially prostaglandins (PGs), are involved in colorectal oncogenesis [20]. Two genes encode the two major forms of cyclooxygenase (COX) that is responsible for the metabolic conversion of dietary arachidonate to PGs, thromboxanes, and leukotrienes. The constitutive form, COX-1, is present in different types of cells. COX-2, the isoform inducible by growth factors and tumor promoters, is rarely found in the normal intestinal epithelial cells but is overexpressed in colorectal neoplasms [21–23]. Since COX-1 is a housekeeping gene, drugs for COX-1 inhibition cause side effects, such as ulceration. The kidney function, platelet aggregation, and the ulceration in stomach are strongly affected by the overuse or overdose of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) [24]. These limit the wide-scale application of NSAIDs in the general

public for the chemoprevention of CRC. Although NSAIDs are excellent chemopreventive agents in animal models for CRC, dietary aspirin does not inhibit azoxymethane (AOM)-induced colon carcinogenesis in rats that received a nonhigh fat diet. Because of side effects of NSAIDs that affect both COX-1 and COX-2 expressions, specific COX-2 inhibitors (-coxibs) were introduced to obtain cancer chemopreventive ability without side effects [25, 26]. They are indeed effective for CRC development in animal studies [27]. However, certain COX-2 inhibitors have been reported to increase the risk of ischemic heart diseases [28].

Many phytochemicals and/or botanicals are routinely tested for anti-inflammatory properties in the hope of finding new sources of medicines for the treatment of chronic inflammation and for the cancer chemoprevention. The plant world is apparently rich in sources of COX and/or inducible nitric oxide (iNOS) inhibitors that are involved in inflammation-related carcinogenesis [29]. Several of these have been found to inhibit cancer development in certain animal models. Curcumin [30] is able to inhibit the COX activity [31] in the skin [32] and suppresses skin cancer development in mice [33], tongue [34] and CRC in rats [35]. Tea [36, 37], given to human volunteers in a clinical trial, could reduce PGE_2 activity in the rectal mucosa [38]. Resveratrol [39] is a potent inhibitor of COX [40] and is an inhibitor in the mouse skin and rat mammary carcinogenesis models [41]. Silymarin [42] a COX and iNOS inhibitor [43] was also found to inhibit skin [44, 45], colon [46], tongue [47], and prostatic [45, 48] cancer development. However, the cancer preventive ability of carnosol and ginger substances, both COX inhibitors [49, 50], has not fully been investigated [51, 52]. Numerous phytochemicals thus remain to be tested in animal models for the most common cancers such as colon, mammary, prostate, and lung. Nobiletin [53–56], zerumbone [57, 58], and garcinol [59–61] are potent anti-inflammatory compounds present in plants that inhibit carcinogenesis in different tissues. A recent study demonstrated the chemopreventive ability of zerumbone, which has been shown to possess anti-inflammatory effects in mouse lung and colon carcinogenesis [62]. These phytochemicals [63], for example, curcumin [31, 64], resveratrol [65, 66], silymarin [67], from plants have multifunctional effects, including COX-2 inhibition and anti-inflammatory function, on a variety of events that involved carcinogenesis [68–70].

Most phytochemicals with chemopreventive ability [71] have direct antioxidant activity but many can also induce oxidative stress within cells when applied at high doses [11, 72]. For example, a simple phenolic acid protocatechuic acid is a potential cancer chemopreventive agent in a variety of tissues [73], but it enhances tumor promotion and oxidative stress in female ICR mouse skin via enhancing a promoter-induced inflammatory responses and promotion by affecting tyrosinase-dependent oxidative metabolism of protocatechuic acid [74]. Therefore, care should be paid for applying strong antioxidants to clinical use as a chemopreventive agent. However, recently anti-inflammatory, antioxidative chemopreventive phytochemicals targeting signal transduction mediated NF-2 related factor-2 (Nrf2), nuclear factor-kappaB (NF-κB), and activator protein (AP)-1 that are redox-sensitive factors have been highlighted [72]. We recently have demonstrated that such a compound and melatonin effectively suppress inflammation-associated colon tumorigenesis [62, 75].

Angiogenesis the formation of new blood vessels from existing vasculature has been associated with neoplasms. Angiogenesis is critical to the transition of premalignant lesions in a hyperproliferative state to the malignant phenotype, which leads to tumor growth and metastasis. The intensity of angiogenesis as assessed by counting of microvessels in neoplastic tissue acts as a prognostic factor for many solid tumors, including CRC [76, 77]. Similarly, expression of angiogenic growth factors is associated with prognosis of a variety of cancers [76, 77]. Angiogenesis enables tumors to grow larger than 1-2 mm in diameter, invade surrounding tissue, and metastasise. Angiogenesis is already targeted by chemopreventive agents at various stages of drug development or in clinical practice [76, 77]. The biomarker measured in chemoprevention must have the potential for modification by therapeutic interventional agents. In this regard, angiogenesis is a particularly attractive biomarker. There are in vivo and in vitro assays using human endothelial cell neoplasms [78], umbilical vein endothelial cells [79] and for screening potential anti-angiogenic effects of candidate chemicals. Potentially nontoxic anti-angiogenic dietary compounds include green tea polyphenols, genistein, curcumin, resveratrol, linoleic acid, hesperidin, naringenin, and allyl disulfide [80].

The defect in apoptosis mechanism is recognized as an important cause of carcinogenesis [5, 6]. A dysregulation of proliferation alone is not sufficient for cancer development as suppression of apoptotic signaling is also required. Cancer cells acquire resistance to apoptosis by overexpression of anti-apoptotic proteins and/or by the downregulation or mutation of pro-apoptotic proteins. Various studies indicate that dietary constituents, particularly phytochemicals, can modulate the complex multistage process of carcinogenesis by several mechanism(s), including apoptosis-inducing effects [81]. They include (−)-epigallocatechin gallate, curcumin, genistein, indole-3-carbinol, resveratrol, isothiocyanates, luteolin, lycopene, caffeic acid, apigenin, sylymarin, gingerol, and capsaicin [81].

3. Mechanistic Screening Assays for Detecting Potential Chemopreventive Compounds

Potential chemopreventive agents are systematically screened in a battery of short-term assays which determine the inhibition or induction of biochemical and molecular processes involved in carcinogenic processes. As summarized in Table 2, these mechanistic-based assays are roughly divided into three major categories: (i) antimutagenesis assays which evaluate carcinogenesis blocking activities, (ii) antiproliferative and antiprogression screening assays, and (iii) assays assessing antioxidant and anti-inflammatory mechanisms.

In addition to these assays, studies are in progress to establish assays utilizing DNA microarray and proteomics to

Table 2: Various assays for chemopreventive mechanisms.

Categories	Assays	Culture cells or enzymes	Measurements (effects)
Antimutagenesis	B(*a*)P-DNA adduct formation	Bronchial cells (human)	DNA damage (inhibition)
	NAD(P)H:quinone reductase	Liver cells (human)	Detoxification (induction)
	GSH *S*-transferase	Liver cells (human)	Detoxification (induction)
	GSH synthesis & GSSG reduction	Liver cells (rat)	Detoxification (induction)
Antiproliferation	TPA-induced ODC	Tracheal epithelial cells (rat)	Proliferative activity (inhibition)
	Normal epithelial cell proliferation	Primary keratinocytes (human)	Proliferative activity (inhibition)
	Poly(ADP-ribose)polymerase	Primary fibroblasts (human)	DNA damage (inhibition)
	Calmodulin regulated phosphodieaaterase	Leukemia cells (HL60)	Signal transduction regulation (inhibition)
	TPA-induced tyrosine kinase	Leukemia cells (HL60)	Signal transduction regulation (inhibition)
	EGFR	A431 (human) and 3T3 (mouse) cells	Signal transduction regulation (inhibition)
	ras farnesylation	Brain farnesyl transferase (rat)	Signal transduction regulation (inhibition)
	HMG-CoA reductase	Liver HMG-CoA reductase (rat)	Signal transduction regulation (inhibition)
	Steroid aromatase	PMSG-stimulated ovarian aromatase (rat)	Estrogenic activity (inhibition)
	Estrogen receptor	Breast cancer cells (MCF-7)	Estrogenic activity (inhibition)
	5α-reductase	Prostate 5α-reductase (rat)	Androgenic activity (inhibition)
	Cell differentiation	Leukemia cells (HL60)	Differentiation (induction)
	DNA fragmentation	Leukemia cells (HL60) or histiocytic lymphoma cells (U937 cells)	Apoptosis (induction)
Antioxidant/Anti-inflammation	AA metabolism	Macrophages/keratinocytes (human)	Anti-inflammatory activity (AA metabolism inhibition)
	TPA-induced active oxygen	Leukemia cells (HL60)	Active oxygen (inhibition)
	COX-2	Placental COX-2 (sheep)	Anti-inflammatory activity (COX-2 inhibition)
	5-LOX	RBL-1 cells (rat)	Anti-inflammatory activity (5-LOX inihibition)

AA, arachidonic acid; B(*a*)P, benzo[*a*]pyrene; COX, cyclooxygenase; EGFR, epidermal growth factor receptor; GSH, glutathione; GSSG, oxidized glutathione; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LOX, lipoxygenase; PMSG, pregnant mares' serum gonadotropin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

facilitate the discovery of new chemopreventive agents and novel molecular mechanisms of action [82, 83]. Such new emerging technologies allow the screening and monitoring of the expression levels of thousands of genes simultaneously [84]. More importantly, the technology to make customized gene chips with specific genes is also possible. In addition to monitoring the alterations in gene expression patterns in tissues undergoing carcinogenesis, these chips can be utilized to evaluate subjects at risk, such as those carrying specific germline mutations and genetic polymorphisms [85].

4. In Vitro Efficacy Model Systems

Besides the mechanistic assays mentioned above, the systematic evaluation of cancer preventive agents includes screening compounds in short-term in vitro screens which aim to select agents for subsequent whole animal testing with insight into potential mechanisms of action. Use of primary cultured cells without aneuploidy is ideal because they possess relatively intact drug metabolizing systems and normal gene numbers. Epithelial cells used are rat

tracheal epithelial cells, human lung cells, hyperplastic alveolar nodules in mouse mammary gland organ cultures, JB6 epidermal cells, and human foreskin epithelial cells. In each assay, the substances are tested over a wide range of concentrations to determine EC_{50} values. The assays include (i) a rat tracheal epithelial cell (RTE) assay which measures the ability of candidate chemopreventive agents to block the benzo[*a*] pyrene (B[*a*]P)-induced transformation of primary RTE cells [86]; (ii) an anchorage independence assay which is an effective method for detecting compounds that block carcinogenesis in the postinitiation stages and evaluates inhibition of anchorage independence in human lung tumor (A427) cells [87]; (iii) a mouse mammary gland organ cultures (MMOCs) assay which assesses the inhibitory activity of test chemicals on the development of carcinogen-induced hyperplastic alveolar nodules (HANs) in MMOC [88]. This assay is similar in appearance to the alveolar nodules produced in mouse mammary glands in vivo [89]; (iv) an in vitro assay for antipromoters or antiprogressors which is designed to identify chemopreventive agents effective in the promotion or progressive stages of carcinogenesis in JB6 epidermal cells [90]; and (v) a human foreskin epithelial assay which determines the inhibitory potential of chemopreventive agents in blocking cell growth stimulation induced by the carcinogen propane sulfone [91].

5. In Vivo Short-Term Screening Assays

This type of short-term assays identifies agents that might block or arrest carcinogenesis in the early stages. Two experimental models, which reflect major cancers in humans, are being used. They include the rat and mouse colorectal aberrant crypt foci (ACF) assay [11, 12, 92, 93] and a rat model of breast ductal carcinoma in situ model (DCIS) [94].

5.1. ACF Assay. The ACF assay is a short-term model which can identify agents that may be effective in preventing CRC [11, 12, 92, 93]. ACF, which were first described by Bird [95], are putative preneoplastic lesions consisting of aggregates of single and multiple crypt cells that exhibit hyperplasia and/or dysplasia and are thought to be the earliest detectable lesions of CRC [96–99]. Two different protocols have been developed: one which identifies compounds that inhibit initiation and a second treatment schedule which evaluates potential chemopreventive agents during the postinitiation phase of colorectal carcinogenesis. Details of these regimens have been described previously [11, 12, 100]. In the former (the initiation protocol), rats are given a test agent in the diet one week prior to the administration of a colonic carcinogen, such as AOM and continuing throughout the five-week study period. In the latter regimen (the postinitiation protocol), rats are first treated with AOM, followed four weeks later by a test agent, which is given for additional weeks. Animals are sacrificed and the ACF frequency is determined by microscopic evaluation.

5.2. DCIS Assay. The DCIS assay provides both toxicity and efficacy data for identifying candidate chemopreventive agents prior to testing in common mammary carcinogenesis models. Therefore, the induction of mammary tumorigenesis is initiated in weanling female SD rats by the intraperitoneal (*i.p.*) injection of the carcinogen *N*-methyl-*N*-nitrosourea (MNU) [94]. In general, test agents are administered in the diet, starting one week after the carcinogen administration, and thereafter are continued until the termination of the study (45–50 days later). Mammary tissue specimens are excised and processed for histopathological analysis. The efficacy is estimated as the percent reduction in the number of DCIS lesions in comparison to controls that receive a carcinogen alone.

6. Animal Efficacy Assays

The use of animal efficacy models to establish organ specificity and to generate dose-response, toxicity, and other pharmacological data is a crucial component of the determinant process for chemoprevention agents. These assays with thetoxicity tests are used for decisions regarding recommendations for clinical use. Numerous animal models are used to study inhibition of chemical carcinogenesis in rodents. Important criteria considered in selecting an in vivo model for screening cancer chemoprevention agents include (i) short study duration and induction of carcinogenesis; (ii) target-specific experimental model evidenced by the induction of cancer in the target tissues comparable in such factors as histological type and hormone dependence to those found in humans; (iii) evaluation of in vitro mechanistic activities, efficacy profiles, and relevant published data prior to the selection of models for a given possible chemopreventive agent. Typically, test agents are administered in the diet unless problems with stability are encountered. During the course of chemoprevention studies a maximum tolerated dose (MTD), defined as the highest dose level that does not cause $\geq 10\%$ reduction or gain in body weight over a six-week period, is determined. The treatment schedules include the administration of test agents either before, concurrently, or following exposure to the carcinogen. Efficacy is based upon the percent inhibition of tumor incidence and/or multiplicity, or increased tumor latency in comparison to carcinogen-treated controls. Representative carcinogenesis models are listed in Table 3.

6.1. Head and Neck Carcinogenesis Models. Several well-established models of oral and respiratory tract cancer have been developed. In the hamster buccal pouch model [101], the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) is topically applied over a 12-week period, thus resulting in buccal pouch squamous cell carcinomas [102]. Rats [4] and mice [103] develop tongue cancers when exposed to 4-nitroquinoline 1-oxide (4-NQO) [104]. In rats, tongue squamous cell carcinomas and dysplasia can be induced by 4-NQO in drinking water (20 ppm) for 8 weeks [4]. Tongue dysplasia occurs during 4-NQO treatment and the incidence of tongue squamous cell carcinoma is over 50% at 32 weeks after the exposure. In this model, test chemicals can be orally administered either before, during,

Table 3: Preclinical animal models for identifying chemoprevention efficacy.

Target tissues	Species and carcinogens	Induced tumors
Oral cavity: tongue or buccal pouch	Hamster (buccal pouch): DMBA* Mouse (tongue): 4-NQO Rat (tongue): 4-NQO	SCC, PAP
Colon	Mouse: AOM, DMH, MAM acetate Rat: AOM, DMH, MAM acetate, MNU	ADC, AD, ACF
Esophagus	Rat: Nitrosaminea (MNAM, NMBA), 4-NQO Mouse: 4-NQO	SCC, PAP
Forestomach	Mouse: B(*a*)P	SCC, PAP
Liver	Mouse: various Rat: 2-AAF, DEN, DMN, 3′-Me-DAB	HCC, AD
Lung	Mouse: B(*a*)P, DMBA, NNK, Urethane, 4-NQO Hamster: DEN, MNU (trachea)	SCC, ADC, AD
Breast	Mouse: DMBA Rat: DMBA, MNU	ADC, AD, fibroadenoma
Pancrease	Hamster (duct cell)): BOP Rat (acinar cell): azaserine	ADC, AD, acinar cell carcinoma
Skin	Mouse: UV radiation, B(*a*)P/TPA, DMBA, DMBA/TPA, MC	SCC, PAP
Glandular stomach	Rat: MNNG, MNU	ADC
Urinary bladder	Mouse: OH-BBN Rat: MNU, OH-BBN	TCC

2-AAF, 2-acetylaminofluorene; ACF, aberrant crypt foci; AD, adenoma; ADC, adenocarcinoma; AOM, azoxymethane; B(*a*)P, benzo[*a*]pyrene; BOP, *N*-bis(2-oxopropyl)nitrosamine; DEN, diethylnitrosamine; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMH, 1,2-dimethylhydrazine; DMN, dimethylnitrosamine; HCC, hepatocellular carcinoma; MAM acetate, methylazoxymethanol acetate; MC, 3-methylcholanthrenel; 3′-Me-DAB, 3′-methyl-4-dimethylaminoazobenzene; MNAN, *N*-methyl-*N*-amylnitrosamine; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosourea; NMBA, *N*-nitrosomethylbenzylamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 4-NQO, 4-nitroquinoline 1-oxide; OH-BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; PAP, squamous cell papilloma; SCC, squamous cell carcinoma; TCC, transitional cell carcinoma; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

or following 4-NQO exposure [4]. For the induction of lung adenosquamous cell carcinoma or squamous cell carcinoma of hamsters, MNU is administered over a 15-week period, thus resulting in approximately 40–50% of the treated animals after 6 months [105]. Starting one week prior to MNU exposure, a test compound is administered over 180 days. In the second model, hamsters are given the carcinogen diethylnitrosamine (DEN), subcutaneously, twice per week over a 20-week period, resulting in the formation of lung adenocarcinomas in about 50% and tracheal tumors in over 90%, of treated animals [106]. As in the MNU model, test agents are given prior to carcinogen exposure. The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) can induce lung tumors in A/J mice and a large number of compounds are now available that inhibit the mouse lung tumorigenesis induced by NNK [107]. Another lung cancer bioassay is the strain A mouse model [108]. Strain A mice spontaneously develop lung tumors as early as 3-4 weeks, with lung tumor incidences approaching 100% at 24 months of age. A/J mice are also utilized for studies on colon tumorigenesis because of their susceptibility to colonic carcinogens [109].

6.2. Colorectal Carcinogenesis Models. Potential inhibitors of colorectal carcinogenesis can be assessed utilizing models in both rats and mice [11, 12, 110, 111]. According to established protocols, 1,2-dimethylhydrazine (DMH), AOM, or methylazoxymethanol (MAM) acetate is administered intraperitoneally or subcutaneously, thus resulting in colorectal adenocarcinoma development within 32–40 weeks in either species. DMH is first activated to form AOM and then is metabolized by the liver to form MAM, the ultimate carcinogen, which is excreted via glucuronide conjugation. In the AOM induction model, a single or multiple (up to 3 times) subcutaneous dose of AOM in male F344 rats results in the occurrence of colorectal adenocarcinoma and adenoma in approximately 70% of treated animals by 40 weeks. Again, the test agents can be orally administered either before, during, or following carcinogen treatment [112]. In comparison to rats, mice should receive multiple exposures of a colonic carcinogen to induce colonic tumors and tumor development needs long-term period. A mouse model recently established for colorectal carcinogenesis [113], in which different colonic carcinogens are followed by a colitis-inducing agent, dextran sodium sulfate (DSS),

is quite useful to identify potential chemopreventive agents within a short-term period [68, 114].

6.3. Mammary Carcinogenesis Models. Chemopreventive efficacy against mammary gland carcinogenesis is routinely assessed by either the MNU- or DMBA-induced models [115]. Both protocols utilize female SD rats and require that the carcinogen is given as a single dose at 50 days of age. In some instances, the carcinogen is administered to older animals (180 days) which is more representative of the human target population. Tumor incidences at 120 days after carcinogen treatment are similar, ranging from 80–100% in the DMBA protocol and 75–95% in the MNU model. However, the histological types of tumors induced by the two carcinogens are different. DMBA-induced mammary tumors are predominantly adenoma and fibroadenoma, with some adenocarcinoma, whereas MNU-induced mammary tumors are invasive adenocarcinomas. The chemopreventive activity of the test agents is determined by the percent reduction in tumor incidence or percent increase in tumor latency relative to controls treated with the carcinogen alone. These models produce hormonally responsive tumors. In addition to these chemically-induced mammary carcinogenesis models, several genetically engineered animals have been introduced to investigate breast carcinogenesis and evaluate the efficacy of candidate chemopreventive agents. They include a COX-2 overexpressing mouse model [116], a *Ras*-driven mouse mammary tumorigenesis model [117], and a HER-2/neu transgenic mouse model [118]. In addition, mammary stem cell models can be used for studying how the hormonally regulated paracrine interactions influence stem cells and the stem cell niche during mammary carcinogenesis [119]. Another interesting model system for understanding normal human breast development or tumorigenesis is the orthotopic xenograft model that has the potential to improve the understanding of crosstalk between tissue stroma and the epithelium as well as factors involved in breast stem cell biology tumor initiation and progression [120].

6.4. Urinary Bladder Carcinogenesis Models. Urinary bladder neoplasms are typically induced by the carcinogen *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (OH-BBN) which can induce invasive transitional cell carcinomas morphologically similar to those found in humans [121, 122]. This carcinogen is given either intragastrically or in drinking water over an 8-week period to 50-day old BDF mice (C57BL/6 × DBS/2-F_1) or F344 rats, thus resulting in a 40–50% incidence of bladder tumor incidence at 180 days after OH-BBN treatment. Treatment schedules for a test agent administration are as described above [123].

6.5. Skin Carcinogenesis Models. Agents effective in inhibiting skin carcinogenesis are identified in a two-stage skin carcinogenesis model utilizing DMBA and TPA, which are applied topically to the back skin of SENCAR or CD-1 mice [124, 125]. Both strains of mice are highly susceptible to skin tumor induction. Skin papillomas appear as early as 6 weeks postcarcinogen treatment, eventually progressing to squamous cell carcinomas by 18 weeks [126]. Test agents are generally administered in the diet or in some experiments are topically applied according to several predefined treatment regimens. As to melanoma chmoprevention study, we should read an elegant review for new perspectives of this research area [127]. In addition to in vitro screening assay [128], several genetically altered animal (mouse) models of melanoma [129–132], including hepatocyte growth factor/scatter factor (HGF/SF) transgenic mice [133–136], have been introduced for prevention [137] and biology [138] of this neoplasm. Also, a three-dimensional skin reconstruction model [139] is useful for determining the therapeutic efficacy of selected chemicals or drugs in cultured melanoma cells [140]. While epidemiological studies suggest sunlight as an etiologic agent for the pathogenesis of melanoma, recent experimental investigations by the group Meyskens, Jr. indicated that elevation of reactive oxygen species follows from melanin serving as a redox generator [127] and this may be involved in the etiology and pathogenesis of cutaneous melanoma. Such findings will help to establish novel preventive and therapeutic approaches to this malignancy.

7. Transgenic and Gene-Knockout Animal Models

Animal models that mimic the specific characteristics of human carcinogenesis may prove to be a valuable resource in both evaluating chemopreventive efficacy and identifying appropriate biomarkers for measuring the chemopreventive activity. Transgenic and gene knockout mice that carry well-characterized genetic lesions predisposing them to carcinogenesis are appropriate models for chemoprevention testing (Table 4). Some of the best developed models include the multiple intestinal neoplasia (*Min*) mouse [141] and other strains possessing lesions in the *Apc* gene [142]. The *Min* mouse carries an *Apc* mutation similar to that found in human familial adenomatous polyposis (FAP) patients. These mice are predisposed to develop predominantly small intestinal adenomas, but a few in the large intestine. By manipulating two or more carcinogenesis-associated genes, such as modifier genes, in a single animal, closer approximations of human carcinogenesis may be possible. Numerous colonic tumors develop in the large bowel in *Min* mice at 3 weeks after one-week-exposure of DSS [143], thus suggesting the importance of gene-environmental interaction in cancer development [68]. It might be feasible to knock out *p53* in an animal that already carries another tumor suppressor defect such as *Apc* or p16. Recently, new transgenic animal models for mammary [144], tongue [145, 146], pancreas [147], and gall bladder [148] cancers have been reported. These models might be useful for discovering possible novel cancer chemopreventive agents.

8. Combination Treatment

One strategy for improving the efficacy and lessening toxicity is using combinations of agents [149, 150]. Synergistic or additive effects may be observed when two agents with