



## A Review on Chemical Models of Colorectal Cancer: Criteria with Mechanism of Carcinogenesis

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### ABSTRACT

Colorectal cancer (CRC) is a third most malignant cancer globally with significant mortality and morbidity. Incidence of CRC is increasing in developing countries due to their forwarding life styles to westernization like smoking, alcohol consumption, obesity, poor physical activities and consumption of red and processed meat. Animal models provide opportunity to study multi-stage mechanism of tumorigenesis. There are some models like chemical, inoculated and genetic cause of cancer in mouse which are likely to recapitulate the pathophysiology of human colorectal cancer to evaluate anticancer potential of drugs against colorectal cancer. But still, none of them exactly mimic the human pathophysiology. Hence, it is very essential to choose specific model which is reproducible, economic, rapid induction time as well as similar mechanism of cancer initiation, development and progression. In the present article, we dealt chemical mouse models with mechanism of development of CRC and some methodology followed using the same models.

**Keywords:** Colorectal cancer, Chemical models, Azoxymethane, Dimethylhydrazine.

### INTRODUCTION

Cancer is a disease of cells where abnormal cells undergo uncontrolled proliferation. When this type of cells appeared in colon or rectum is called colorectal cancer (CRC). CRC initially develops as polyps, abnormal non-cancerous tissue on colon inner lining. After some decades few polyps especially adenomatous polyps (adenoma) grow slowly and become cancerous. Less than 10% of adenoma capable of invading into other organ. Adenoma start proliferating from glandular cells and lubricate the colon by producing mucus. The cancer arised from inner lining of colon is called as adenocarcinoma. The formed cancer then grown to the colon wall that can be carried in blood vessel and lymph nodes and further invades to other part of organ. Black or dark stool, rectal bleeding, urgency in bowel movement when bowel is empty, blood in toilet and stool, narrow stool than usually, discomfort in lower abdomen, passing excessive amount of stool and constipation or diarrhoea, weight loss, anaemia, abdominal pain, bloating, loss of appetite etc.,<sup>1</sup>

CRC is the third most diagnosed cancer in both men (after prostate and lung cancer) and women (after breast and lung cancer) in United State. In every year, one millions of people diagnosed colorectal cancer globally. American cancer society estimated that 1,01,420 colon cancer and 40,180 new cases of rectal cancer expected and estimated 51,020 cases of death caused by CRC in 2019. Incidence of CRC is increasing in developing countries due to their forwarding life styles to westernization like smoking, alcohol consumption, obesity, poor physical activities and consumption of red and processed meat.<sup>2</sup>

It is a major cause of morbidity and mortality throughout the world. Hence to overcome this issue there is a great need of developing anticancer drugs with fewer side effects using suitable *in-vitro*, *in-silico* and animal models. Especially animal models help researcher to understand cellular and molecular mechanism of cancer initiation, development and progression. And also help to determine role of biomarkers and cancer-related genes. Reduction of human CRC is directly related with identification of carcinogens in workplace, diet and environment through carcinogenesis in animal models. Chemicals induced CRC is widely accepted based on its reproducibility, less expensive, easily tested on animals with different genetics, comparatively rapid tumour induction and exactly mimicking the pathogenesis of initiation and progression of cancer in human.

The following carcinogens are used to induce CRC.

- i) Azoxymethane (AOM)
- ii) 1.2 Dimethylhydrazine (DMH)
- iii) Methylazoxymethanol (MAM) acetate
- iv) Heterocyclic amines (HCA) e.g. 2-amino-3,3-dimethylimidazo [4,5-f] quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)
- v) Aromatic amines (AA) e.g. 3,2'- dimethyl-4-aminobiphenyl (DMAB)
- vi) AlkylNitrosamide compounds e.g. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and methylNitrosourea (MNU)



### i) Azoxy methane

AOM is a procarcinogen requires cytochrome enzymes CYP2E1 to convert into MAM. Then MAM breakdown into formaldehyde and highly reactive species, methyl diazonium ion, leads to G→A transition by forming O<sup>6</sup> and N<sup>7</sup> methylguanine (MG) adduct in DNA in the colon and liver causes activation of several signalling pathways like TGF-β, β-catenin and K-ras for tumorigenesis. Inactivation or inhibition of CYP2E1 in mice was significantly inhibited CRC formation by reducing polyps formation and O<sup>6</sup>-MG in colon.<sup>3</sup> K-ras protein activated by transversion mutation of A:T from G:C by AOM. Later this protein causes activation of signalling pathways like MAPK and PI3K/Akt. These pathways responsible for cell growth and proliferation. PI3K/Akt phosphorylate the NF-κB and Bcl-xl to increase cell survival and decrease apoptosis by blocking the p53 and forkhead/Fas-ligand.<sup>4,5,6</sup> The cell proliferation regulated by deactivating GSK3 and by promoting myc and cyclin D1.<sup>7</sup> β-Catenin is an oncogenic protein and it has a role in cell adhesion. Mutation of N-terminal of β-catenin at codons 33 and 41 that were target for phosphorylation of GSK3β and regulate the cyclin D expression.<sup>8</sup> Mutated β-Catenin can't be degrade and can't form complex. Thus causes accumulation of free β-catenin then binds to TCF/LEF to activate cell proliferation and other gene transcription.<sup>9,10</sup> TGF induces apoptosis via several signalling pathways, however defects in TGFβ initiate tumorigenesis in 20-30% of colon cancer patients. Animals treated with AOM, decreased the TGF-β active form to develop carcinogenesis.<sup>11</sup>

AOM/ Dextran Sodium Sulphate (DSS) induced CRC model is outstanding model now as it recapitulate development of CRC from polyps-adenoma-carcinoma as in human CRC.<sup>12</sup> It's based on DNA damage associated with repeated cycles of colitis recapitulating the human cancer. DSS is a long chain polymer containing glucose and sulphur and supply of 5-10% of DSS solution as drinking water leads to acute and chronic signs of colitis. Effect of DSS on colon depends on concentration and number of DSS cycles. Risk factor of cancer is depends on extent and duration of colitis. Use of two chemicals AOM and DSS reduces induction time and produce accuracy in CRC model. Because of its stronger potency and greater stability during administration it's advantageous over DMH.<sup>13</sup> DNA hypomethylation occurred in animal treated with AOM was reported to human colorectal cancer. Gene specificity indicates the DNA methylation pattern like methylated Zik1 and Gja9 in cancer whereas, in tumour and in normal colon found methylated Cdkn2a/p16, Igfbp3, Mgmt, Id4 and Cxcr4. However unmethylated p19Arf, Tslc1, Hltf and Mlh1 were found in both AOM tumor and normal colon mucosa.<sup>14</sup> Mutation in K-ras, β-catenin, TGF-β, Src/PI3k/Akt and p53 plays major role in carcinogenesis, and TGF-βR2 and adiponectin were more prone to AOM in knockout mice.<sup>15</sup>

### ii) Dimethylhydrazine

DMH and its metabolites like AOM and MAM acetate are widely accepted agents to induce CRC. DMH is metabolically activated as AOM later as MAM then as Methyl diazonium ions. This series of activation takes place in liver and also in colon epithelial cells with or without presence colon bacteria. When activation of DMH takes place in liver, the active metabolite MAM excreted into bile and transported to colon epithelial via blood circulation.<sup>16</sup> Reactive metabolites forms DNA adducts with guanine and thymidine to induce mutation in many genes sequence to cause cell proliferation and growth and to inhibit the apoptosis. Development of cancer initiated from discrete microscopic mucosal lesions like Adenocarcinoma foci (ACF) to malignant tumor. ACF considered as pro-hallmarker of CRC, this was assessed by studying short-term (4 weeks) and medium-term (30 weeks) assay in DMH (40 mg/kg) administered rats twice a week. Most of ACF were found in middle and distal colon and they were increased with time. Continuous progression of multiple crypt in ACF was noticed from 4<sup>th</sup> to 30<sup>th</sup> week. And well differentiated adenocarcinoma were found in the 30 weeks of assay.<sup>17</sup> Trauma caused by implantation during operation, cancer recurrence and residual tumor in colon are considered as promoting factor for CRC. It was demonstrated in promotion of cancer in DMH induced tumor by partial colectomy found that carcinogenic rate increased 87.5% in partial colectomy with DMH group compared to DMH only treated group (58.8%).<sup>18</sup> On comparison AOM, DMH produced more efficient neoplasia, dysplasia and colon cancer and this method was also found 50 times cheaper than AOM.<sup>19</sup>

Increase in incidence of CRC is due to westernization like intake of food with low fruits, vegetable and fibres, decreased physical activities etc., The epidemiological data showed possible association of Diabetic Mellitus-2 (DM-2) with CRC. Increased duration of insulin treatment or insulin resistance have a higher risk of cancer. Where, transformation and proliferation of colorectal epithelial cell, cell apoptosis inhibition and cell cycle influence caused by immoderate release of insulin and insulin like growth hormone in serum.<sup>20,21</sup> The association of DM-2 with CRC was also investigated by administering low dose of STZ and DMH in rats model. The final result suggested that increased hexokinase and phosphokinase activity in colon was higher in intratumor than in peritumor. Whereas, the activity of phosphodehydrogenase decreased in both tumor, and increased number of ACF and foci containing different crypt were seen in diabetic associated cancer in rats.<sup>22</sup> Increased O<sup>6</sup>-MG and O<sup>4</sup>-Methylthymidine (MT) adducts formation associated with cancer development. Depletion of these two adducts affected by acetylguanine transferase. Treatment with O<sup>6</sup>-alkylguanine transferase inhibitors, O<sup>6</sup>-Benzylguanine causes depletion of alkylguanine transferase (AGT) that leads to removal of protumorigenics, O<sup>6</sup> MG and O<sup>4</sup>-MT lesions following DMH treatment.<sup>23</sup>



*Apc* is a tumor suppressor gene. The mutation in *Apc* responsible for majority of sporadic and familial adenomatous polyposis.<sup>24</sup> The function of this gene is responsible for regulation of  $\beta$ -catenin in concert with GSK-3 $\beta$  and other protein. This mutation was not withstanding in DMH/AOM induced CRC but mutation was predominant in different region of *Apc* seen in humans.<sup>25</sup> Mutation in tumor suppressor gene, p53 responsible for human CRC. However there is no report for the change in coding region (exon 5-8) from DMH/AOM induced CRC. This was confirmed by direct sequencing analysis, single strand conformation polymorphism and immunohistochemical analysis.<sup>26</sup> Wnt signalling plays major role in cell differentiation, cell proliferation and cell fate decision. Changes in Wnt signalling leads to development of ACF. Proliferation of *Apc*-depleted cells exhibited by activating mammalian target rapamycin complex-1 (mTORC1) pathways. So use of rapamycin, mTORC1 blocker blocks the tumour growth activity of Wnt signalling, however intestinal homeostasis regulation of Wnt signalling was unaffected.<sup>27</sup>

### iii) Methylazoxymethanol acetate

As MAM is a metabolic product of AOM, mechanism of CRC follows DNA Adduct formation as discussed earlier. Single injection of MAM is able to produce tumor in colon that had similar feature to human tumor and incidence of cancer by this model was significant in colon compared to small intestine.<sup>28</sup> The organ specific effect of MAM acted by alcohol dehydrogenase and NAD<sup>+</sup> dependent dehydrogenase. The acute and chronic effect of MAM carcinogenicity depends on NAD<sup>+</sup> dependent dehydrogenase activity in colon and liver, where the NAD<sup>+</sup> changed to its reduced form by MAM. However jejunum and ileum were resistant to tumor development due to the presence of little NAD<sup>+</sup>. Sensitivity of MAM to inhibit DNA synthesis is not correlated with the level of deacetylase activity in various segment of colon. The biological effect of carcinogenesis exerted in the absence of biliary transport and this was proved in animal study by cannulating the biliary duct.<sup>29</sup>

Expression of TNF- $\alpha$  and IL-1 $\alpha$  in colon act as growth factor in rats treated with MAM acetate and hydroxyanthroquinone (HAQ) to accelerate colon cancer when compared to rats treated only with HAQ and control.<sup>30</sup> The correlation between the tumor induction and level of methylated DNA were assessed by measuring the 7-MG in DNA isolated from descending colon and liver of rats treated with MAM. The 7-MG level was found more in liver compared to descending colon of rat. This data concluded that there is no relationship between the level of 7-MG and tumor induction.<sup>31</sup> MAM acetate causes no mutation in *Apc* in the entire coding region and intron flanking coding of exons, not in K-ras and p53 genes.<sup>32</sup> MAM absorbed through the distal region of colon and exhibiting its carcinogenic activity in order of large intestine, kidney and liver but CRC from MNNG showed by its direct contact on the large intestinal

mucosa.<sup>33</sup> Effect of X-irradiation before or after treatment of ACI/N male rats with MAM showed synergistic action to intestinal tumorigenesis but decreased tumor development in female rats.<sup>34</sup>

### iv) Heterocyclic amines

These are carcinogens produced by cooked meats like beef, fish and pork using high temperature methods like pan frying, grilling directly over an open flame. Hence increased risk of colon cancer associated with increased dietary HCA.<sup>35</sup> The microsatellite instability and mismatch repairs are associated with hereditary colon cancer and sporadic colon cancer. Possible environmental influence on microsatellite instability in colon cancer increases with increase in dose and duration of smoking as well as dietary heterocyclic aromatic amines.<sup>36</sup> The mutagenic activity of smoke condensate obtained from broiling fish was studied using *salmonella typhimurium TA100 and TA98*. Smoke condensate of some species were required metabolic activation to induce mutagenicity. Some condensate of charcoal broiling of beefsteak was found less mutagenic compared to that of fish. The mutagenic compounds also present in charred surface of broiled fish and meat, and exhibited mutagenicity after metabolic activation.<sup>37</sup> Synthesized HCA were tested in animals where it produced colon, breast and prostate cancers in rodents and only hepatoma in monkeys. Alteration of genes like *Apc*, beta-carotene and Ha-ras are responsible for cancer induction and lesion formation. Generally, HCA oxidised to hydroxylamine derivatives by CYP450 and further leads to formation of esters by acetyltransferase and sulfotransferase. This eventually form DNA adduct at guanine bases to develop cancer.<sup>38</sup>

Association of meat consumption and colon cancer is based on the presence of mutagenic HCA and Polycyclic Aromatic Hydrocarbons (PAHs) and this was showed in many cases. Case control study was conducted to analyse data in colon cancer cases and control cases from validated meat preparation questionnaire. Study revealed that statistically significant association of red meat derived compounds like 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (DiMeIQx) and its mutagenic activity.<sup>39</sup> The risk of mammary adenocarcinoma was increased in the second generation of rats in two generation exposure experiment (transplacental and trans-breast milk). This was inhibited by co-administration of chlorophyllin or 1-o-hexyl-2,3,5-trimethylhydroquinone (synthetic antioxidant).<sup>40</sup>

Mice Cyp1a was replaced by human CYP1A to metabolise PhIP by N(2)-hydroxylation thus activate Wnt signalling and Ctnnb1 mutations by exome sequencing in PhIP/DSS as driving force to develop CRC.<sup>41</sup> HCA alone was failed to induce colon cancer in C57BL but in combination with DSS adenocarcinoma experimentally.<sup>42</sup> PhIP is having complete carcinogenic, mutagenic and estrogenic activity. In exposed animals PhIP undergo N-hydroxylation in liver in the presence of CYP1A2 to N-hydroxy-PhIP. Later this



metabolite undergo esterification by N-acetyltransferase and Sulfotransferase to give N-acetoxy PhIP and Sulfonyloxy PhIP respectively. Either of these metabolites generates arylnitrenium ion to react with nucleophilic group of DNA to form adducts mainly dG-C8-PhIP adducts. More, the N-hydroxylated PhIP undergo detoxification by generating glucuronate conjugate in liver secreted into bile later by blood circulation taken by colon and deconjugated by gut flora. This deconjugated PhIP takes up by colon epithelial cell to activate further until adduct formation takes place.<sup>43</sup> IQ is also a genotoxic agent induce tumors in liver and colon. Role of NADPH oxidase (Nox) was investigated in PhIP model. It was revealed the overexpression of Nox1, Nox4, NFkB-50 and NFkB65 after 1 year treatment with PhIP.<sup>44</sup>

Activation of Wnt-catenin signalling pathways caused by mutation of  $\beta$ -catenin or *Apc* gene. But mutation in K-ras and p53 genes were rarely seen. The study on environmental carcinogens shows that DSS acts as tumor promoting agent in PhIP initiated CRC within a short period of time. Where  $\beta$ -catenin, COX-2 and iNOX expressions in colon epithelial were identified by immunohistochemically.<sup>45</sup> Single injection of PhIP or IQ (50 mg/kg) in C57BL/6J-Min/+ Mice which has heterozygous nonsense *Apc*(Min) mutation was found to increase 3 to 4 folds of tumor in small intestine, but increased colonic tumor was observed only in male rats. This tumor was induced by causing mutation of LOH and truncation in inactivated wild-type *Apc* gene.<sup>46</sup>

#### v) Aromatic amines

These are the class of carcinogen showed attention towards risk of human cancer. They found in cooked meats, fish, poultry, tobacco smoke condensate and diesel exhaust. Study of AA, their metabolites and DNA adduct in biological fluids and in tissue served as biomarkers in epidemiological study. Hence, provide evident on the role of these genotoxicants in the etiology of human cancer.<sup>47</sup> Mortality and incidence of prostate cancer and other types of tumors found to be increased in DMAB treated F344 rats when compared to ACI, Lewis, CD and Wistar rat strains. F344 rat strain identified as the most appropriate model for DMAB caused prostate carcinogenesis.<sup>48</sup> Main tumor sites was correlated well by excretion route of major metabolite of DMAB. DMAB was able to induce tumor in both urinary bladder and in intestine of the hamster. However, in contrast, the same could able to induce only colon cancer in rats. The presence of major metabolite, N-hydroxy-N-glucuronide conjugation of DMAB in hamster bile and urine, and in rat bile but not in urine provides evidence in confirmation of cancer site. Other metabolites like sulfates and glucuronides of C-hydroxylated DMAB and C-hydroxylated-N-acetyl DMAB metabolites were also identified partially.<sup>49</sup> Dose dependent reduction of DMAB formed DNA adduct was found with celecoxib treated animals. This report was supported for further investigation of anticancer activity of celecoxib in CRC.<sup>50</sup>

The effect of microbial flora and dietary fat was studied in DMAB induced colon and breast cancer in germfree and conventional F344 rats. The lower incidence of mammary and colon tumor was found in germfree than conventional F344 rats<sup>51</sup> and dietary fat had no effect in small intestinal tumor in germfree as well as in conventional rats but incidence and multiplicity was higher in conventional rats with DMAB and fat compared to germfree.<sup>52</sup> Colon tumor enhancement was observed in rats treated with both DMAB and PhIP but that was failed to enhance prostate cancer. This is due to DMAB which is not influenced by PhIP-adduct formation.<sup>53</sup> 2-Nitrosoamino-3-methylimidazo[4,5-f]quinolone (N-NO-IQ) and 2-Nitrosoamino-3,8-dimethylimidazo[4,5-f]quinoxaline (N-NO-MeIQx) are nitrosation of IQ and MeIQ under inflammatory conditions to form DNA adduct. These DNA adducts along with the adducts of Sulfates and Glucuronidase of C-hydroxylated DMAB, and C-hydroxylated metabolite of IQ and MeIQx causes genotoxicity headed CRC by further inflammatory mediators.<sup>54</sup> They were elicited mutagenicity in E-coli strain which expresses recombinant N-acetyltransferase (NAT), but not expressed neither in CYP450 1A2 and NADPH reductase nor in NAT negative control.<sup>55</sup>

Heterocyclic and aromatic amines undergoes metabolic activation by enzymes NAT1 and NAT2 to cause cancer. Humans are susceptible to cancer on exposure to aromatic and heterocyclic carcinogens along with high frequency of NAT1 and NAT2 polymorphism.<sup>56</sup> Irrespective of fatty acids, increased high fat intake promote the uncontrolled cell proliferation in prostate and beef tallow found to accelerate the prostate and intestinal carcinogenesis.<sup>57</sup>

#### vi) Alkyl nitrosamide compounds

MNU and MNNG are direct carcinogens that can induce cancer without metabolic activation. MNU was supplied to F344 and C3H rats with DMSO for 16-70 weeks. The study was found that 4.5% gastric adenocarcinoma, 90% survival rate, two induced adenocarcinoma, one squamous cell carcinoma and one sarcoma in rats.<sup>58</sup> Incidence of CRC increased in rats exposed to MNU and MAM acetate along with 20% dietary fat. And there was no significant difference in incidence of cancer in DMH treated and in 20% fat fed animals.<sup>59</sup>

Cellular senescence induced by MNNG in HCT-116 cells. Cellular senescence is a state in which cells ceases its normal growth and functions. The senescence like cell arrest at G0/G1 phase was associated with decreased level of *Apc*, p53, mRNA, microtubular organization and telomeric DNA.<sup>60</sup> MNNG possess synergic effect with *H. pylori* to cause gastric cancer formation. Tumorigenic property of MNNG related with oncogenic Ras activation and also through Ras-MAPK pathway. This pathway decreases the expression of E-cadherin to dissociate  $\beta$ -catenin from E-cadherin.<sup>61</sup> Gastric carcinogenesis development is associated with high salt intake and COX-2 over expression, with this knowledge the study was





performed by supplying of MNU (240ppm) in drinking water for 10 weeks and later with 10% NaCl for 10 weeks. After 50 weeks, study was revealed that increased tumor frequency was observed with high salt intake in transgenic COX-2 mice by causing induction of PG-E2, chronic inflammation, inflammatory cytokines and disruption of cell kinetic.<sup>62</sup> MNNG regulates epithelial-mesenchymal transition and cell proliferation in GES-1 cells and MC cells by activating CCL2/CCR2 signalling pathway to initiate cancer development.<sup>63</sup>

**Table 1:** Different chemical models used to induce CRC

MODELS	METHODOLOGY	REMARKS
AOM/DSS model	12 mg/kg i.p of AOM was administered and observed for 48 hours. DSS 1 <sup>st</sup> cycle was given (3% DSS solution for 3-5 days, DSS recovery period for 3-8days) then continue with 2 <sup>nd</sup> and 3 <sup>rd</sup> cycle.	Fast and efficient analysis of genetic and environmental modulators of colitis-associated cancer without the requirement of time-intensive genetic crossings. <sup>13</sup>
	10 mg/Kg i.p of AOM was administered. one week later, DSS cycle was started (DSS solution 2.5% 1 week, recovery 2 weeks) and continued with cycle 2 and 3.	Efficient model with increased ACF and other features of neoplasia. <sup>64</sup>
DMH model	Single oral dose of DMH (35mg/kg), examined for CRC after 1.5 years.	78.6% tumor development in treated rats showed 1-3 tumor formation in each rats. <sup>65</sup>
MAM acetate Model	Daily 1mg of MAM in 0.5 ml of water was infused rectally in rats for 7days to 26 days.	Tumor varies from polyps, polypoid, protuberant tumor with ulceration and other organ tumor depends on duration of exposures. <sup>33</sup>

## CONCLUSION

CRC caused by combined effects of hereditary, environmental factors and life styles. Human exposed to carcinogens in day-to-day life and those are genotoxic mutagens. Development of CRC can be prevented as colon cancer life span is about 10-15 years. There is a great need for preventative to overcome by fatal CRC disease. Chemical models for cancer induction have been developed over year to evaluate anticancer drugs. AOM/DSS induced model reduced the experimental period, feasible and mimic the pathology of inflammation induced CRC compared to other models. The modification of carcinogen model in genetically knockout mice (cancer susceptible) found highly reproducible and efficient model to study chemopreventive agents.

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