Review Article

Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents

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Studies of colon carcinogenesis in animal models are very useful to elucidate mechanisms and provide pointers to potential prevention approaches in the human situation. In the rat colon carcinogenesis model induced by azoxymethane (AOM), we have documented frequent mutations of specific genes. K-*ras* mutations at codon 12 were found to be frequent in hyperplastic aberrant crypt foci (ACF) and large adenocarcinomas. In addition, mutations of the *β-catenin* gene in its GSK-3β phosphorylation consensus motif could also be identified in many adenomas and adenocarcinomas, and altered cellular localization of β-catenin protein was observed in all of the dysplastic ACF, adenomas and adenocarcinomas examined, indicating that activation of Wnt signaling by accumulation of β-catenin is a major mechanism in the AOM-induced colon carcinogenesis model. Frequent gene mutations of *β-catenin* and altered cellular localization of the protein are also features of AOM-induced colon tumors in mice. Expression of enzymes associated with inflammation, such as inducible nitric oxide synthase (iNOS) and the inducible type of cyclooxygenase (COX), COX-2, is increased in AOM-induced rat colon carcinogenesis, and overproduction of nitric oxide (NO) and prostaglandins is considered to be involved in colon tumor development. We have demonstrated that increased expression of iNOS is an early and important event occurring in step with β-catenin alteration in rat colon carcinogenesis. Activation of K-ras was also found to be involved in up-regulation of iNOS in the presence of inflammatory stimuli. In addition, expression levels of prostaglandin E_2 (PGE_2) receptors may be altered in colon cancers. For example, the EP_1 and EP_2 subtypes have been shown to be up-regulated and EP_3 down-regulated in AOM-induced colon cancers in rats and mice. EP_1 and EP_4 appear to be involved in ACF formation, while alteration in EP_2 and EP_3 is considered to contribute to later steps in colon carcinogenesis. Increased expression of some other gene products, such as the targets of Wnt/β-catenin signaling, have also been reported. The further accumulation of data with this chemically-induced animal colon carcinogenesis model should provide useful information for understanding colorectal neoplasia in man. (Cancer Sci 2004; 95: 475–480)

In recent years, colorectal cancer has increasingly become a major cause of cancer mortality in Japan. Therefore, elucidation of the mechanisms of colon carcinogenesis and the search for chemopreventive agents are important and urgent tasks. Screening of colon cancer preventive agents has been carried out using several *in vivo* animal models, the majority using azoxymethane (AOM), a very potent carcinogen which induces colorectal cancers at high incidence in rats and mice. In relatively short-term experiments, aberrant crypt foci (ACF) induced by treatment with AOM in rats and mice can be used as biomarkers, since the formation and growth of these putative preneoplastic lesions are thought to be useful indices of the effects of carcinogens and agents promoting or preventing carcinogenesis in the colon.[1, 2] Recently, other pre-neoplastic lesions such as β-catenin-accumulated crypts and mucin-depleted foci have also been reported as specific biomarkers for colon carcinogenesis.[3–5] Compounds which appear to be effective in the short-term must then be further examined in long-term experiments focusing on AOM-induced colon cancer development. In order to identify novel prevention approaches, it is very important to take into account the mechanisms underlying colon carcinogenesis in this animal model, and this is the rationale for examining mutations in different genes and changes in expression of proteins. Understanding the relationship of such alterations to each step of colon carcinogenesis should help to elucidate the mechanisms of colon carcinogenesis, not only in rodents, but also in humans.

1. Gene mutations in colon carcinogenesis

Colon carcinogenesis is known to be a multistep process involving multiple genetic alterations. Findings for K-*ras*, *APC*, *DCC* and *p53* in tumors are summarized in Table 1. In human lesions, these genes are frequently mutated or deleted.[6–13] K-*ras* and *APC* gene mutations are involved in relative early stages of colon carcinogenesis, while alterations of *DCC* and *p53* are involved in the late stages.[13] K-*ras* mutations are frequent from the ACF stage, while *APC* mutations are frequent from the adenoma stage. Most ACF are hyperplastic and positive for K-*ras* mutations, but about 5% of ACF are dysplastic and harbor *APC* mutations.[9] K-*ras* is an oncogene which encodes an intracellular signaling molecule. Oncogenic mutations in Ras result in constitutive activation of Ras and its downstream signaling pathways, such as the Raf/MEK/MAPK and PI3K/Akt/PKB pathways. The other three are tumor suppressor genes. *DCC* encodes a protein that has homology to cell adhesion molecules, and p53 protein is a transcription factor which regulates the cell cycle and apoptosis. The *APC* gene has been identified as responsible for the inherited colon cancer syndrome adenomatous polyposis coli, the APC protein forming a complex with β-catenin and stimulating its degradation.[14, 15] Mutations in the GSK-3β phosphorylation

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Abbreviations: AOM, azoxymethane; iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; NO, nitric oxide; ACF, aberrant crypt foci; PGE_2, prostaglandin E_2; MNU, methylnitrosourea; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; SSCP, single strand conformation polymorphism; IL-1β, interleukin-1β; LPS, lipopolysaccharide.

consensus motif of the *β-catenin* gene, as well as *APC* mutations, cause stabilization of β-catenin in the cytoplasm, and induce constitutive transcriptional activation with Tcf-4, a member of the Tcf family of DNA binding proteins.[16, 17] In fact, Sparks *et al*. found mutations of the *β-catenin* gene in half of human colon tumors possessing an intact *APC* gene.[18] In APC-deficient tumors or cell lines, *β-catenin* mutations are generally not detected. It can be concluded that most colon tumors feature change in the APC/ β-catenin/Tcf pathway.

Table 1 also shows gene alterations that occur during chemical carcinogenesis in rat colon. In AOM-induced lesions, K-*ras* mutations are frequent, as seen in human cancers, but *Apc* and *p53* mutations are only rarely observed.[19, 20] This difference can not be explained by species differences, because *p53* mutations have been detected in methylnitrosourea (MNU)-induced rat colon tumors and *Apc* mutations have been found in 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced tumors.[21, 22] Furthermore, K-*ras* mutation does not appear to be a feature of PhIP-induced colon tumors.[23] The K-*ras* gene is activated by point mutations at codon 12 or 13, while most observed mutations in the *APC* gene in human colon tumors are due to frame-shift deletions or nonsense mutations that produce truncated products. It has also been reported that in the rat, PhIP causes specific one-base deletions of GGGA to GGA in the *Apc* gene.[22] AOM causes point mutations of G:C to A:T transversion type in the K-*ras* gene,[24, 25] which may derive from O^6-methyl-deoxyguanine adducts.[26, 27] Thus, the difference may be carcinogen-specific rather than species-specific. In order to ascertain whether the β-catenin/Tcf pathway is involved in AOM-induced rat colon carcinogenesis, we have focused on β-catenin in AOM-induced rat colon tumors, and we found frequent mutations and altered cellular localization.[28, 29] *β-Catenin* mutations have also been found in PhIP-induced rat colon tumors without *Apc* mutations.[30] These results indicate that Wnt/Apc/β-catenin signaling indeed plays important roles in chemically-induced rat colon carcinogenesis, as it does human cancers.

1.1 β-Catenin

Male F344 rats were treated with AOM to induce colon tumors, and adenocarcinomas obtained after 36 weeks were examined by PCR-single strand conformation polymorphism (SSCP) analysis and direct sequencing for *β-catenin* mutations in the GSK-3β phosphorylation consensus motif. Immunohistochemical staining and immunoblotting were also performed to examine the cellular localization and protein level, with an additional focus on small adenomas and ACF to investigate alteration of the β-catenin pathway in different steps of colon carcinogenesis.[29]

Fig. 1 shows the DNA sequence around the GSK-3β phosphorylation consensus motif of rat β-catenin. The *β-catenin* mutations found in our study were located in codons 33 and 41, which are encode serine and threonine residues important for GSK-3β phosphorylation, and in codons 32 and 34, encoding

Table 1. Gene alterations in colon cancers

Species	Carcinogen	Lesion	Mutation frequency (%)				
			APC	*β-Catenin*	*K-ras*	*DCC*	*p53*
Human		Adenocarcinoma	40–80	15	40–60	40–70	50–80
		Adenoma	40–65	—	0–40	—	—
		ACF	<5	—	10–95	—	—
Rat	AOM	Adenocarcinoma	8	75	30–60	—	0
		ACF	0	—	20–40	—	0
	PhIP	Adenocarcinoma	13	50	0	—	0
	MNU	Adenocarcinoma	—	—	18	—	27
Mouse	AOM	Adenocarcinoma	—	100	0–10	—	0

References: 6–13, 18–25, 28, 30, 32).
—: No data.

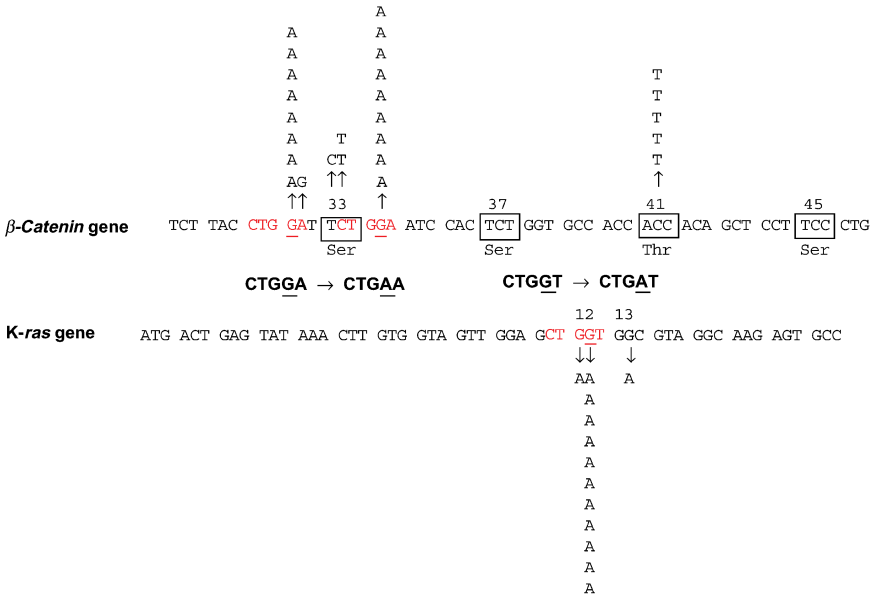

Fig. 1. Mutations of the *β-catenin* and *K-ras* genes in rat colon lesions induced by AOM. The serine residues encoded by codons 33, 37 and 45 and the threonine residue encoded by codon 41 of the *β-catenin* gene are GSK-3β phosphorylation sites. Mutational hot spots are the second G of CTGGA/T sequences, indicated in red.

Table 2. Alterations in AOM-induced colonic lesions in rats[1)]

Gene or protein	Alteration	Frequency (%) in				
		Hyperplastic ACF	Dysplastic ACF	Adenoma	Small adenocarcinoma[2)]	Large adenocarcinoma[3)]
K-ras	Mutation	70	0	0	8	43
β-Catenin	Mutation	0	66	33	75	79
β-Catenin	Cytoplasmic & nuclear translocation	0	100	100	100	100
iNOS	Increased expression					
	in epithelial cells	0	100	100	92	100
	in stromal cells	–	–	–	–	±
COX-2	Increased expression					
	in epithelial cells	0	0	17	42	79
	in stromal cells	+	+	++	++	+++

1) Reference: 29).
2) Small adenocarcinomas: 1–5 mm.
3) Large adenocarcinomas: >5 mm.

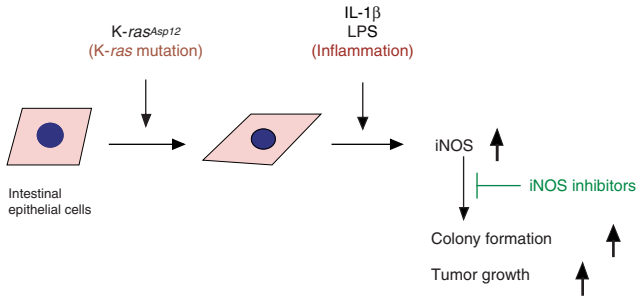

Fig. 2. K-*ras* codon 12 mutations can elevate iNOS expression mediated by IL-1β and LPS. Rat intestinal epithelial cells (IEC-6) were transfected with K-*ras*^{Asp12} mutant cDNA. In the presence of IL-1β or LPS stimuli, iNOS expression is markedly enhanced, and anchorage-independent growth is elevated. Note suppression of the *in vivo* growth of IEC-6/K-*ras*^{Asp12} cells by NOS inhibitors.

residues adjacent to the serine residue encoded by codon 33, and presumably affecting its phosphorylation. Frequent mutations in codons 32 and 34 are CTGGA to CTGAA. The common K- and H-*ras* mutations in codon 12 that have been observed frequently in AOM-induced rat colon tumors are also CTGGT to CTGAT, and CTGGA to CTGAA, respectively.[24, 25, 29, 31)] Thus, the second G in CTGGA or CTGGT sequences may be a hot spot for AOM-induced mutations. As shown in Table 2, mutations in the *β-catenin* gene were found to be frequent from the step of dysplastic ACF.[29)]

In normal colon epithelium, β-catenin exists mainly as a component of the cadherin-mediated cell-cell adhesion system and is immunohistochemically stained at the cellular membrane. In contrast, pronounced cytoplasmic and nuclear staining of β-catenin was seen in all AOM-induced colon adenocarcinomas examined. As summarized in Table 2, alteration of the cellular localization of β-catenin was observed in all dysplastic ACF, adenomas and adenocarcinomas examined, but not in any hyperplastic ACF.[29)] These results indicate the importance of dysplastic ACF as a precursor of colon cancer.

Analysis of mutations in the *β-catenin* gene and altered cellular localization in mouse colon carcinomas induced by AOM yielded similar results to those found in the rat. A hot-spot in the mouse *β-catenin* gene was found in codon 34 at the second G of the CTGGA sequence. Other mutations were identified in codons 33, 41 and 37, but not codon 32. In addition to the nuclear staining of β-catenin with a scattered heterogeneous pattern, which is common to the rat, mouse-distinctive homogeneous or focal heterogeneous nuclear staining was evident.[32)] Furthermore, reduced expression of Apc protein in AOM-induced mouse tumors has been reported.[33)] The results show that β-catenin alterations are early events in AOM-induced colon tumorigenesis, and may play important roles in causing dysplastic changes.

1.2 K-ras

Using the same DNA samples employed for the mutation analysis of the *β-catenin* gene, rat K-*ras* gene mutations were analyzed. Fig. 1 shows mutations detected in exon 1. All were G:C to A:T transitions, and the most frequent was CTGGT to CTGAT at the second base of codon 12. As shown in Table 2, in the AOM-induced rat colon carcinogenesis model, K-*ras* activating mutations at codon 12 were very frequently observed in ACF and tumors, especially in large tumors, as in human cancers, indicating that activation of K-ras may be involved in promotion of cell proliferation.[29)] On the other hand, K-*ras* mutations in mouse colon carcinomas induced by AOM proved rare.[32)] It has also been reported that K-*ras* mutations are not detected in mouse colon tumors induced by 1,2-dimethylhydrazine, a precursor of AOM.[34)] These findings suggest that activation of the K-*ras* gene is not essential for colon cancer development.

2. Altered gene expression in colon carcinogenesis

In human colorectal cancers, the expression of enzymes associated with inflammation, such as inducible nitric oxide synthase (iNOS) and inducible-type cyclooxygenase, COX-2, have been reported to be elevated,[35, 36)] and their reaction products, nitric oxide (NO) and prostaglandin E_2 (PGE_2), could contribute to colon tumorigenesis. However, the relation between their expression and gene alteration remains to be clarified .

In our studies of the expression of iNOS and COX-2 in AOM-induced rat colorectal cancers by immunoblotting and immunohistochemical staining, the levels of both proteins were found to be markedly elevated.[29, 37)]

2.1 iNOS

In normal colon mucosa, iNOS expression is hardly detectable in epithelial or stromal cells. As summarized in Table 2, increased expression of iNOS in epithelial cells is very frequently observed in dysplastic ACF, adenomas and adenocarcinomas, but not in hyperplastic ACF. Thus, iNOS expression is detected in almost all lesions in which β-catenin alterations are observed, indicating a possible direct or indirect causal relationship. However, iNOS expression within tumors was not homogeneous, in contrast to the β-catenin alteration.[29)] Positive

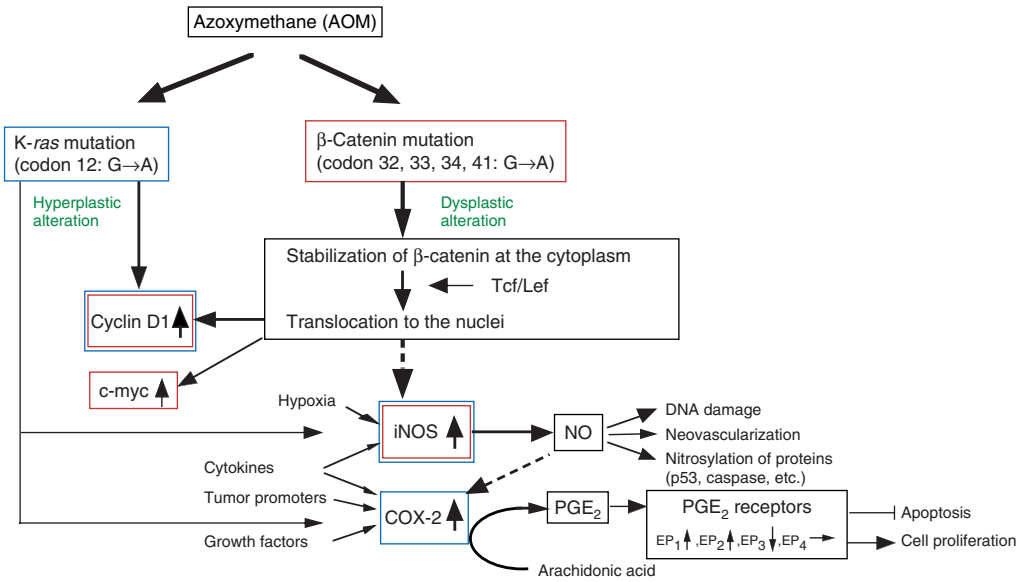

Fig. 3. Gene mutations and altered expression of proteins in rat colon carcinogenesis induced by AOM.

staining for iNOS was clearly observed in the carcinoma epithelial cells,[37] predominantly at the luminal surfaces of carcinoma cells forming glandular patterns, but not in moderately differentiated adenocarcinoma cells not forming clear glandular patterns, implying the involvement of factors other than alteration of β-catenin alone. Since some colon cancer cell lines are known to express iNOS on cytokine treatment,[38] cytokine receptors and/or subcellular components present in well-differentiated cells might be contributing to the iNOS expression. The results therefore suggest that increased expression of iNOS could be related to the altered localization of β-catenin in the presence of other factors.

Furthermore, we examined the relation between K-*ras* and increased expression of iNOS in a cell culture system (Fig. 2). When IEC-6 rat intestinal epithelial cells were transfected with K-*ras* mutant cDNA, iNOS expression mediated by interleukin-1β (IL-1β) or lipopolysaccharide (LPS) was markedly elevated, whereas transfection of the control vector or wild-type K-*ras* cDNA did not result in enhanced iNOS expression.[39] These results suggest that K-*ras* with an activating mutation can elevate iNOS expression in the presence of inflammatory stimuli, and thereby presumably promote tumorigenesis.

2.2 COX-2 and prostanoid receptors

As summarized in Table 2, COX-2 expression in stromal cells is a feature of normal colon mucosa and ACF, but is clearly increased in the stromal elements of adenomas and adenocarcinomas. On the other hand, COX-2 expression in epithelial cells is negative in normal mucosa and ACF, slight in adenomas, but frequent in adenocarcinomas, being located in the cytoplasm and nuclear membranes of carcinoma cells forming glandular patterns. COX-2 expression in both stromal and epithelial cells is particularly high in large carcinomas, suggesting that this protein enhances tumor growth.[29] COX-2 overexpression has been demonstrated to render tumor cells resistant to apoptosis and to stimulate neovascularization, thus conferring a survival advantage.[40–42]

Some evidence of an involvement of the Wnt/Apc/β-catenin/Tcf pathway in COX-2 expression has been presented.[43–45] However, our results indicate that β-catenin alteration is not in itself sufficient for induction of COX-2. It has also been reported that K-*ras* mutations and/or activation increased expression of COX-2, but β-catenin mutations did not, in AOM-induced rat colon tumors.[46] In our studies, a clear relationship between K-*ras* mutations and COX-2 expression was not evident, but both were frequent in relatively large adenocarcinomas. Like iNOS, preferential expression of COX-2 could be demonstrated in well-differentiated carcinoma cells forming glandular patterns,[29] and it has been reported that NO enhances activity and expression of COX-2 in several cell lines.[47–50] A causal relationship between the two is therefore conceivable.

In colon cancers, PGE_2 synthesis is generally elevated,[51, 52] and it is very likely that this would enhance carcinogenesis. Indeed, administration of PGE_2 enhanced colon carcinogenesis in rats treated with AOM through induction of cell proliferation and reduction of apoptosis.[53] Prostanoids exert their biological actions through binding to their specific membrane receptors and there are four PGE_2 receptor subtypes, EP_{1-4}. Using prostaglandin E receptor subtype-knockout mice, the roles of these receptors in colon carcinogenesis have been investigated in our laboratory.[54–56] Deficiencies of PGE_2 receptors EP_1 and EP_4 caused a decrease in ACF formation in the colons of mice treated with AOM.[54, 55] Deficiencies of EP_2 and EP_3 did not affect AOM-induced ACF formation,[55] but Sonoshita *et al*. reported that double knockout of *Apc* and *EP*$_2$ genes decreased intestinal polyp development.[57] On the other hand, deficiency of EP_3 enhanced colon tumor formation induced by AOM.[56] These observations suggest that EP_1 and EP_4 are promotive in early steps of colon carcinogenesis, and EP_2 and EP_3 play promotive and suppressive roles, respectively, in later steps. RT-PCR analysis of mRNA expression of PGE_2 receptors demonstrated up-regulation of EP_1 and EP_2 and down-regulation of EP_3 in AOM-induced rat and mouse colon cancers. EP_4 mRNA was constantly expressed in normal mucosa and tumors.[56]

2.3 Others

Bissonnette *et al*. reported that mutations in either K-*ras* or β-catenin increase expression of cyclin D1 in AOM-induced rat colon tumors.[46] It is well known that *cyclin D1* is a target gene of β-catenin/Tcf signaling and its increased expression has been observed in human colon cancers.[58, 59] Another target of β-catenin/Tcf signaling is c-myc mRNA expression, which has also been reported to be increased in the early stage of colon carcinogenesis in rats treated with AOM.[60, 61]

Conclusion

From the above observations, we have constructed a tentative schema for AOM-induced colon carcinogenesis, as shown in Fig. 3. AOM induces G-to-A transitions in the K-*ras* and/or *β-catenin* gene. K-*ras* mutations at codon 12 may contribute to induce hyperplastic changes, while *β-catenin* mutations seem to be involved in generation of dysplastic lesions. Mutated K-ras activates the MAPK and PI3K pathways, and then up-regulates cyclin D1 and COX-2, also enhancing iNOS expression in the presence of inflammatory stimuli. *β-Catenin* mutations stabilize β-catenin protein in the cytoplasm and activate transcription of the targets of β-catenin/Tcf signaling, such as cyclin D1 and c-myc. β-Catenin alteration may also be involved in increased expression of iNOS. NO produced by iNOS causes DNA damage and neovascularization, while activating COX-2. Overexpression of COX-2 produces excess prostaglandins, and causes an increase in cell proliferation and decrease of apoptosis, to some extent mediated by PGE_2 receptor subtypes EP_{1-4}.

The data obtained in these studies point to particular mechanisms of colon carcinogenesis and indicate that further investigations of cross-talk between the Wnt/β-catenin/Tcf pathway and the K-ras/MAPK pathway in colon carcinogenesis are warranted.

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