Review

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Genetic Alterations and Microenvironment that Drive Malignant Progression of Colorectal Cancer: Lessons from Mouse and Organoid Models

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Comprehensive genome analyses have identified frequently mutated genes in human colorectal cancers (CRC). These include *APC*, *KRAS*, *SMAD4*, *TP53*, and *FBXW7*. The biological functions of the respective gene products in cell proliferation and homeostasis have been intensively examined by in vitro experiments. However, how each gene mutation or combinations of specific mutations drive malignant progression of CRC in vivo has not been fully understood. Based on the genomic information, we generated mouse models that carry multiple mutations of CRC driver genes in various combinations, and we performed comprehensive histological analyses to link genetic alteration(s) and tumor phenotypes, including liver metastasis. In this review article, we summarize the phenotypes of the respective genetic models carrying major driver mutations and discuss a possible mechanism of mutations underlying malignant progression.

Key Words Transgenic mice, Organoid, Colorectal cancer, Mutations, Elastic modulus

INTRODUCTION

It has been proposed that the accumulation of genetic alterations causes the development and malignant progression of colorectal cancer (CRC) as a well-known concept of multistep tumorigenesis [1]. According to this concept, *APC* mutation induces adenoma development followed by additional mutations in *KRAS*, *SMAD4*, and *TP53*, resulting in gradual malignant progression of the primary cancer. A wide range of genome analyses have confirmed that these driver genes are frequently mutated in human CRC [2]. Moreover, transposon mutagenesis screening in mice also demonstrated that insertional mutations in these candidate genes were enriched in mouse intestinal tumors, indicating possible advantages of these gene mutations in CRC development [3]. Based on these genetic results, we generated mouse models that carry mutations in *Apc*, *Kras*, *Tgfbr2*, *Trp53*, and *Fbxw7* in various combinations by crossing, and examined tumor phenotypes of mice. Most mutations were introduced in intestinal epithelial cells conditionally using villin-CreER mice. Furthermore, we established tumor-derived organoids from these models and performed organoid transplantation to other mice to investigate metastatic ability.

MAIN SUBJECTS

Wnt signaling activation

Wnt signaling plays an important role in the regulation of stemness of tissue stem cells [4]. Wnt signaling via Frizzled receptors induces dissociation of the destruction complex, which consists of APC, AXIN and β-catenin, resulting in stabilization and activation of β-catenin, and the induction of the expression of Wnt-target. Thus, mutations in *APC* or *CTNNB1* encoding β-catenin constitutively activate the Wnt signaling pathway, which causes the development of adenoma. $Apc^{\Delta 716}$ mice carry an *Apc* gene truncation mutation at codon 716, which is a model for human familial adenomatous polyposis [5]. Somatic loss of wild-type *Apc* by loss of heterozygosity (LOH) directly causes the initiation of adenoma development by forming a unique out-pocketing structure in the

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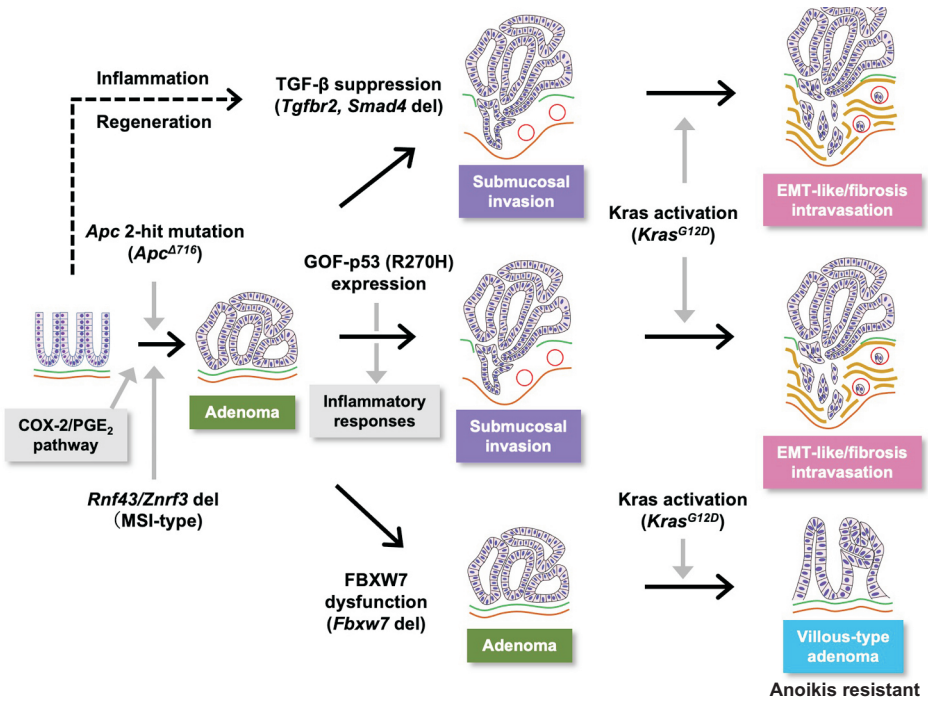

Figure 1. Intestinal tumor phenotypes linked to specific combinations of driver mutations, *Apc*, *Tgfbr2*, *Smad4*, *Trp53*, and *Fbxw7*. Typical morphology of the intestinal tumor tissue is drawn for each genotype. Green lines, basal membranes; thin brown lines, smooth muscle layers; thick brown lines, fibrotic niche; red circles, capillary vessels. Note that intravasations were found in triple mutant tumors. PGE_2, prostaglandin E_2; EMT, epithelial-mesenchymal transition; MSI, microsatellite instability; GOF, gain-of-function.

crypt (Fig. 1) [6]. Notably, the expression of COX-2 is induced in the stromal cells of adenoma, and genetic and pharmacological studies indicated that the COX-2/prostaglandin E_2 (PGE_2) pathway is required for the promotion of benign tumor development (Fig. 1) [7,8]. A recent analysis indicates that mesenchymal cell-derived PGE_2 supports the stemness of intestinal stem and tumor cells through the EP4 receptor [9]. Thus, the targeting of EP4 signaling is a possible strategy for the prevention of CRC.

Oncogenic Wnt activation is also regulated at the receptor level. RNF43 is a ubiquitin ligase that targets the Frizzled receptor. Thus, *RNF43* mutation stabilizes Frizzled receptors, resulting in increased Wnt signaling activation in a ligand-dependent manner. Notably, *RNF43* mutations are frequently found in CRC via the serrated adenoma pathway, which is often associated with microsatellite instability-type tumors [10-12]. A mouse model study confirmed that simultaneous disruption of *Rnf43* and its homolog *Znrf3*, encoding E3 ligases for Frizzled, resulted in the expansion of stem/progenitor cell population (Fig. 1) [13]. Of interest, Wnt activation levels are significantly lower in the serrated pathway of CRC in comparison to the *APC* mutant conventional pathway of CRC [12]. It is possible that the degree of Wnt activation that is induced by *RNF43* mutation is not sufficient for polyposis through the conventional pathway.

Suppression of TGF-β signaling

TGF-β binds the TGF-β type II receptor (TGFβRII), leading to the association and phosphorylation of TGFβRI, which further phosphorylates Smad2 or Smad3. Phosphorylated Smad2/3 forms a complex with Smad4, resulting in the expression of TGF-β target genes. TGF-β signaling induces the differentiation of intestinal epithelial cells, and thus plays a role as a tumor suppressor. When the mouse intestinal mucosa is damaged by irradiation, the remaining stem cells actively proliferate to regenerate. Notably, in *Tgfbr2*$^{-/-}$ mice, remaining stem cells showed significant proliferation after irradiation; however, they never differentiated and thus the villus-crypt structures were not reconstructed [14]. TGF-β signaling is therefore essential for the differentiation of regenerating intestinal stem cells. Importantly, compound mutation of *Apc*$^{\Delta 716}$ and *Tgfbr2*$^{-/-}$ or *Smad4*$^{-/-}$ caused submucosal invasion of intestinal tumors (Fig. 1) [14,15]. Interestingly, we found that the regenerating colonic epithelial cells of *Tgfbr2*$^{-/-}$ mice continuously proliferated and invaded into the submucosa when the colonic mucosa was inflamed. It is therefore possible that the cooperation of TGF-β suppression and the inflammatory microenvironment sufficiently promotes the development of invasive adenocarcinoma (Fig. 1).

Gain-of-function p53 expression

TP53 is a gene that is frequently mutated in CRC [2], and p53 protein has many tumor suppressor functions, including cell cycle arrest, DNA repair and senescence [16]. Notably, approximately 75% of p53 mutations in cancer are missense-type, which results in amino acid substitution. A mouse genetic study indicated that the expression of missense mutant p53 caused tumor development in the lung and intestine, while *Trp53* null mutation induced lymphoma and sarcoma [17], indicating that missense mutant p53 acquires a novel

oncogenic role by a gain-of-function (GOF) mechanism. We crossed *Apc*$^{\Delta 716}$ and *Trp53*$^{+/R270H}$ mice that expressed mutant p53 R270H (corresponding to human R273H), to generate heterozygous compound mutant mice [18]. Importantly, the expression of mutant p53 R270H induced the submucosal invasion of intestinal tumors (Fig. 1). An immunohistochemical analysis showed that p53 is stabilized and distributed to the nuclei/cytoplasm of *Trp53* heterozygous mouse tumor cells; however, it clearly accumulated in the nuclei of the *Trp53* homozygous mouse tumor cells. These results suggest that wild-type p53 interferes with the nuclear transportation of mutant p53 [18,19].

A gene expression analysis of established organoid cells indicated that the expression of mutant p53 R270H resulted in the significant upregulation of innate immunity and inflammatory pathway, including TNF, NFκB, TLR4, IRF7, and IFNG [18]. These results suggest that GOF p53 mutation in cancer cells contributes to the generation of an inflammatory microenvironment, which may support tumor cell survival and proliferation (Fig. 1).

Kras activation mutation

KRAS point mutation causes constitutive activation of the epidermal growth factor receptor signaling pathway, which is believed to play a central role in tumorigenesis. However, we did not find submucosal invasion of intestinal tumors of *Apc*$^{\Delta 716}$ and *Kras*^{G12D} compound mutant mice [20]. On the other hand, the number of colonic tumors in *Apc*$^{\Delta 716}$ *Kras*^{G12D} mice significantly increased in comparison to simple *Apc*$^{\Delta 716}$ mice. Although the underlying mechanism has not yet been elucidated, it is possible that activated Kras supports the survival and proliferation of initiated tumor cells by inducing the expression of COX-2.

Interestingly, *Kras*^{G12D} mutation in *Apc*$^{\Delta 716}$ *Tgfbr2*$^{-/-}$ or *Apc*$^{\Delta 716}$ *Trp53*^{R270H} mice, namely triple mutations, induced advanced phenotypes of submucosal tumors, such as loss of epithelial morphology and cell cluster formation with occasional solitary cells (defined as epithelial-mesenchymal transition-like morphology), accompanied by a fibrotic microenvironment (Fig. 1) [20]. Moreover, the invasion of tumor cells into lymph and capillary vessels was also found in submucosal tumors (Fig. 1). Accordingly, it is possible that submucosal invasion is a prerequisite for the *Kras* mutation-induced malignant progression of CRC.

Fbxw7 dysfunction mutations

FBXW7 is a gene that is frequently mutated in CRC tissues [2]; however, the mechanism through which FBXW7 dysfunction contributes to tumorigenesis is not yet fully understood. Like simple *Apc*$^{\Delta 716}$ mice, *Apc*$^{\Delta 716}$ *Fbxw7*$^{-/-}$ compound mice developed only adenomas. Interestingly, *Apc*$^{\Delta 716}$ *Fbxw7*$^{-/-}$ *Kras*^{G12D} triple mutant mice showed intestinal tumors with a distinct histology (i.e., villous-type adenomas), while other mice without *Fbxw7* mutation developed glandular-type tumors (Fig. 1) [20]. At present, we do not know whether such morphological changes are linked to malignant progression.

In the in vitro study, we found that the disruption of *Fbxw7* resulted in resistance to anoikis. When intestinal tumor-derived organoids were dissociated to single cells by enzyme treatment, only tumor cells that lost wild-type *Trp53* through the LOH survived and proliferated. Thus, wild-type p53 induces apoptosis or cell death of tumor cells after the loss of epithelial adhesion, a phenotypic change known as anoikis. However, if organoid cells lost *Fbxw7*, single dissociated cells survived and proliferated, even when they carry wild-type *Trp53* [19]. Furthermore, it has been shown that the expression of mutant p53 as well as knockdown of *Fbxw7* resulted in enhanced tumorigenicity through the expression of c-Myc in a glioblastoma model [21]. It is therefore possible that there are functional links between wild-type or mutant p53 and FBXW7 in apoptosis induction and oncogenicity, respectively; however, this remains to be investigated.

Combination of driver mutations for liver metastasis

We further generated mice carrying four or five driver mutations in their intestinal tumor cells, and established intestinal tumor-derived organoids from all mice [20]. To examine the metastatic ability of the tumor cells, we transplanted established organoids to the mouse spleen and examined liver metastasis via the portal vein. Notably, tumor organoid cells that carried triple mutations, *Apc*$^{\Delta 716}$ (A), *Kras*^{G12D} (K), *Tgfbr2*$^{-/-}$ (T) in their genotype (i.e., AKT, AKTP, AKTF, AKTPF organoid cells) developed multiple liver metastases (P, *Trp53*^{R270H}; and F, *Fbxw7*$^{-/-}$) [20]. Multiplicity and the incidence of liver metastasis were highest in AKTPF cells and the second highest in AKTP cells. Another type of triple-mutant organoid cell, AKP, also developed liver tumors to a lesser extent. These results indicate that a combination of AKT mutations is a possible minimum requirement for the acquisition of efficient metastatic ability.

Importantly, when AKTP cells with *Trp53*$^{+/R270H}$ (heterozygous mutation) were transplanted to the spleen, AKTP cells with *Trp53*$^{R270H/LOH}$ that lost wild-type *Trp53* were enriched in liver tumor lesions [22]. These results indicate that p53 LOH is an important genetic event for promotion of the metastasis process (Fig. 2A). Consistently, TP53 LOH is frequently found in addition to TP53 GOF mutation in human CRC [2]. Furthermore, we found that the combination of *Trp53* GOF/LOH mutation promotes the survival and clonal expansion of single dissociated organoid cells with the activation of stem cell regulation, growth factor signaling, and the inflammatory pathway [22].

Fibrotic niche generation and polyclonal metastasis

Histological analyses indicate that hepatic stellate cells (HSCs) are activated and proliferate inside or outside of the liv-

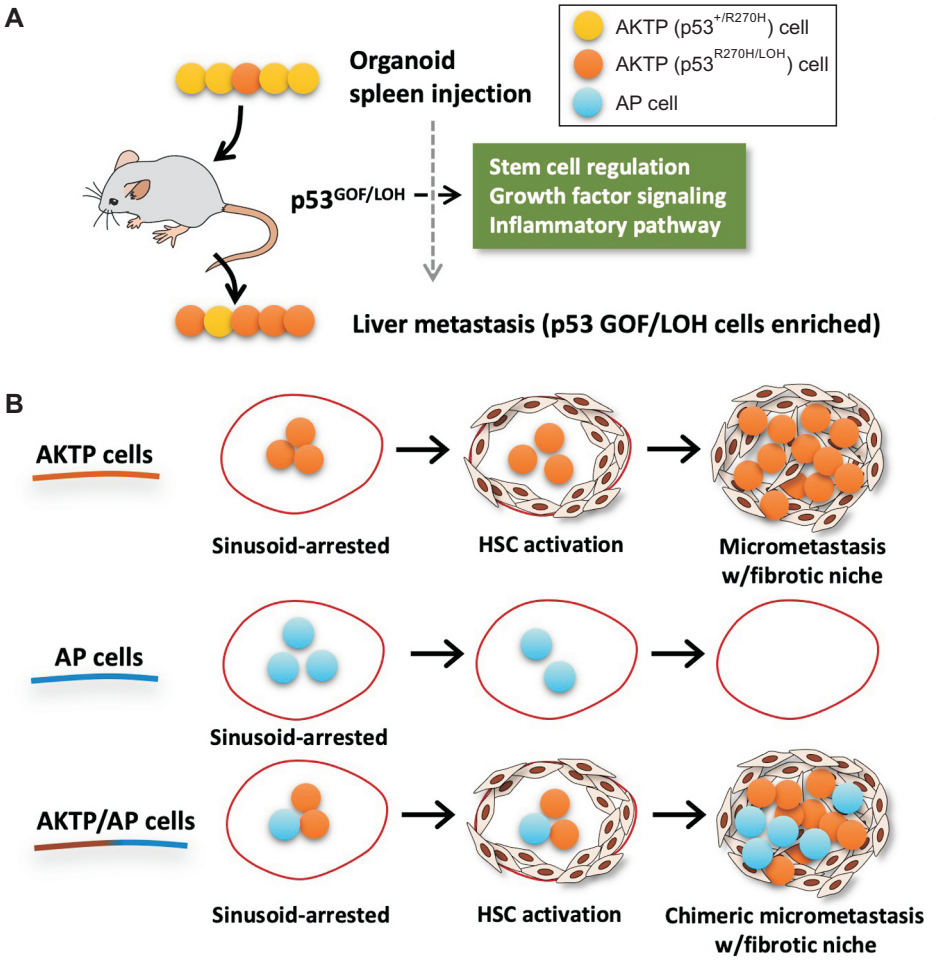

Figure 2. Schematic drawings of mechanisms of metastasis identified by organoid transplantation experiments. (A) When AKTP ($p53^{+/R270H}$) cells are transplanted to the spleen, AKTP ($p53^{R270/LOH}$) cells are enriched in metastatic foci in the liver. Loss of wild-type p53 by LOH causes the activation of stemness, growth factor signaling and the inflammatory pathway, which may contribute to metastasis. (B) AKTP cells arrested in the sinusoid (brown circle) activate HSCs, leading to the development of micrometastasis in the liver with generation of the fibrotic niche (top). AP cells do not activate HSCs and metastatic foci are not formed (middle). When AKTP and AP cells are co-disseminated, both AKTP and AP cells survive and form chimeric metastasis with the fibrotic niche (bottom). HSCs, hepatic stellate cells; GOF, gain-of-function. Modified from the article of Kok et al. (Nat Commun 2021;12:863) (Fig. 7) [23].

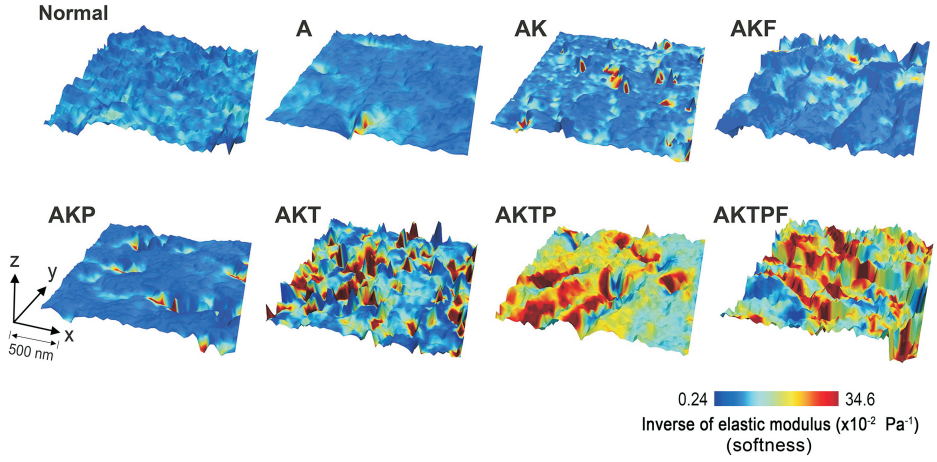

Figure 3. Representative 3D images of the cell surface of the organoid cells of the indicated genotype. Cells were examined by SICM. In each image, the cell surface topography (in nano-scale) and softness (inverse of elastic modulus; in color scale) are shown. SICM, scanning ion conductance microscopy. Modified from the article of Wang et al. (Biomaterials 2021;280:121256) (Fig. 3) [26].

er sinusoid, where metastatic AKTP cells are arrested, which leads to the generation of a fibrotic niche that surrounds CRC cells [23]. It has been established that TGF-β signaling is responsible for HSC activation and liver fibrosis. Notably, the disruption of host TGF-β signaling by *Tgfbr2* knockout significantly suppressed liver metastasis of AKTP cells [23], suggesting a role of the fibrotic niche in the development of metastatic lesions (Fig. 2B, top). On the other hand, this fibrotic reaction was not induced when non-metastatic organoids, such as AP cells, were arrested inside liver vessels, and

tumor cells are cleared (Fig. 2B, middle). Importantly, however, when non-metastatic AP cells were co-transplanted with AKTP cells, a fibrotic niche was generated, which surrounded chimeric cell clusters inside and outside the liver vessels, and non-metastatic AP cells survived and proliferated to form chimeric tumor lesions with AKTP cells (Fig. 2B, bottom) [23]. These findings support a novel concept of metastasis: polyclonal metastasis [24]. In this metastasis model, cancer cell clusters that have genetic and phenotypic heterogeneity break off from the primary site, move and disseminate to distant organs as circulating tumor cell-clusters, and survive and proliferate to form metastatic lesions, keeping the heterogeneity of the primary site. Accordingly, it is possible that targeting of the fibrotic niche will be an effective strategy against liver metastasis of CRC.

Physical properties of the organoid cell surface

Genetic alterations induce distinct gene expression profiles, leading to the acquisition of malignant phenotypes, such as metastatic ability. Mouse and organoid model studies have successfully linked genotypes and phenotypes. To further understand how genetic alterations cause the acquisition of malignant phenotypes, such as increased migration and invasiveness, we performed a nano-scale topography and mechanical property analysis using scanning ion conductance microscopy (SICM). An SICM analysis can create live images that simultaneously show topography and stiffness by contact-free scanning of the cell surface with a nanopipette that measures the ion migration through the pipette tip [25]. Notably, we observed a distinct topographic feature—a microridge structure—on the surface of AKT-common and AKF organoid cells, which actively fluctuate (Fig. 3) [26]. Moreover, cell surface stiffness was found to be significantly low (softer) in AKT-common highly metastatic tumor organoid cells. Therefore, SICM analyses indicate that fluctuating structures and softer surface properties are characteristic to metastatic cells (Fig. 3). Such information about the physical properties of CRC cells will be helpful for understanding the relationships among genetic alteration, expression profiles, and malignant phenotypes. We also believe that SICM can be used for evaluating the metastatic ability of CRC cells.

CONCLUSIONS AND PERSPECTIVES

Comprehensive genome analyses revealed frequently mutated genes in CRC as possible driver genes. Based on the genomic information, mouse genetics and organoid transplantation experiments demonstrated the role of each driver mutation and specific combinations of mutations in the promotion of malignant progression, including metastasis. Mouse and organoid model studies also showed the tumor-induced generation of microenvironment that promotes the survival and proliferation of cancer cells. These results will be helpful to understand the biological mechanism of malignant progression of CRC and the future development of clinical strategies for the treatment of malignant CRC.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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