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 extensively 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. ApcΔ716 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
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 E2 (PGE2) pathway is required for the promotion of benign tumor development (Fig. 1) [7,8]. A recent analysis indicates that mesenchymal cell-derived PGE2 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 knockout 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 ApcΔ716 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 knockout 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
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 ApcΔ716 mice, ApcΔ716 Fbxw7Δ/– compound mice developed only adenomas. Interestingly, ApcΔ716 Fbxw7Δ/– Trp53Δ/Δ 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 spleen and examined liver metastasis via the portal vein. Notably, tumor organoid cells that carried triple mutations, ApcΔ716 (A), Trp53Δ/Δ (K), Tgfr2Δ/– (T) in their genotype (i.e., AKT, AKTP, AKTF, AKTPF organoid cells) developed multiple liver metastases (P, Trp53Δ/Δ, and F, Fbxw7Δ/–) [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 Trp53Δ/ΔR270H (heterozygous mutation) were transplanted to the spleen, AKTP cells with Trp53Δ/ΔR270H/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-
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

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^{R270H/L0H}) 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].
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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**REFERENCES**

1. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: molecular basis of colorectal cancer. N Engl J Med 2009;361: 2449-60.
2. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012; 487:330-7.
3. Takeda H, Wei Z, Koso H, Rust AG, Yew CC, Mann MB, et al. Transposon mutagenesis identifies genes and evolutionary forces driving gastrointestinal tract tumor progression. Nat Genet 2015;47:142-50.
4. Nusse R, Clevers H. Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. Cell 2017;169:985-99.
5. Oshima M, Oshima H, Kitagawa K, Kobayashi M, Itakura C, Taketo M. Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. Proc Natl Acad Sci USA 1995;92:4482-6.
6. Oshima H, Oshima M, Kobayashi M, Tsutsumi M, Taketo MM. Morphological and molecular processes of polyp formation in Apc(delta716) knockout mice. Cancer Res 1997;57:1644-9.
7. Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, et al. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996;87:803-9.
8. Oshima M, Murai N, Kargman S, Arguello M, Luk P, Kwong E, et al. Chemoprevention of intestinal polyposis in the ApCdelta716 mouse by rofecoxib, a specific cyclooxygenase-2 inhibitor. Cancer Res 2001;61:1733-40.

9. Roulis M, Kaklamanos A, Schermthanner M, Bleiecki P, Zhao J, Kaffe E, et al. Paracrine orchestration of intestinal tumorigenesis by a mesenchymal niche. Nature 2020;580:524-9.

10. Yan HHN, Lai JCW, Ho SL, Leung WL, Lee JFY, et al. RNF43 germline and somatic mutation in serrated neoplasia pathway and its association with BRAF mutation. Gut 2017;66:1645-56.

11. Lannagan TRM, Lee YK, Wang T, Roper J, Bettington ML, Fennell L, et al. Genetic editing of colonic organoids provides a molecularly distinct and orthotopic preclinical model of serrated carcinogenesis. Gut 2019;68:684-92.

12. Yamamoto D, Oshima H, Wang D, Takeda H, Kita K, Lei X, et al. Characterization of RNF43 frameshift mutations that drive Wnt ligand- and R-spondin-dependent colon cancer [published online ahead of print January 17, 2022]. J Pathol. doi: 10.1002/path.5868.

13. Koo BK, van Es JH, van den Born M, Clevers H. Porcupine inhibitor suppresses paracrine Wnt-driven growth of Rnf43;Znrf3-mutant neoplasia. Proc Natl Acad Sci USA 2015;112:7548-50.

14. Oshima H, Nakayama M, Han TS, Naik K, Ju X, Maeda Y, et al. Suppressing TGFβ signaling in regenerating epithelia in an inflammatory microenvironment is sufficient to cause invasive intestinal cancer. Cancer Res 2015;75:766-76.

15. Kitamura T, Kometani K, Hashida H, Matsunaga A, Miyoshi H, Hosogi H, et al. SMAD4-deficient intestinal tumors recruit CCR1+ myeloid cells that promote invasion. Nat Genet 2007;39:467-75.

16. Vousden KH, Lu X. Live or let die: the cell’s response to p53. Cell 2000;111:594-604.

17. Olive KP, Tuveson DA, Ruhe ZC, Yin B, Willis NA, Bronson RT, et al. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell 2004;119:847-60.

18. Nakayama M, Sakai E, Echizen K, Yamada Y, Oshima H, Han TS, et al. Intestinal cancer progression by mutant p53 through the acquisition of invasiveness associated with complex glandular formation. Oncogene 2017;36:5885-96.

19. Nakayama M, Oshima M. Mutant p53 in colon cancer. J Mol Cell Biol 2019;11:267-76.

20. Sakai E, Nakayama M, Oshima H, Kouyama Y, Niida A, Fuji S, et al. Combined mutation of ApC, Kras, and Tgfbr2 effectively drives metastasis of intestinal cancer. Cancer Res 2018;78:1334-46.

21. Kim HS, Woolard K, Lai C, Bauer PO, Maric D, Song H, et al. Gliomagenesis arising from Pten- and InK4a/Arf-deficient neural progenitor cells is mediated by the p53-Fbxw7/Cdc4 pathway, which controls o-Myc. Cancer Res 2012;72:6065-75.

22. Nakayama M, Hong CP, Oshima H, Sakai E, Kim SJ, Oshima M. Loss of wild-type p53 promotes mutant p53-driven metastasis through acquisition of survival and tumor-initiating properties. Nat Commun 2020;11:2333.

23. Kok SY, Oshima H, Takahashi K, Nakayama M, Murakami K, Ueda HR, et al. Malignant subclone drives metastasis of genetically and phenotypically heterogenous cell clusters through fibrotic niche generation. Nat Commun 2021;12:863.

24. Cheung KJ, Ewald AJ. A collective route to metastasis: seeding by tumor cell clusters. Science 2016;352:167-9.

25. Watanabe S, Kitazawa S, Sun L, Kodera N, Ando T. Development of high-speed ion conductance microscopy. Rev Sci Instrum 2019;90:123704.

26. Wang D, Sun L, Okuda S, Yamamoto D, Nakayama M, Oshima H, et al. Nano-scale physical properties characteristic to metastatic intestinal cancer cells identified by high-speed scanning ion conductance microscope. Biomaterials 2022;280:121256.