The Role of Tn and STn Antigens in Progression and Metastasis of Colon Cancer

**Abstract:** Colorectal cancer (CRC) is a leading cause of cancer deaths in the Western world. To understand its initiation, progression, and metastasis, many different animal models for CRC have been established. Among them, mice with a germ line mutation in the gene encoding Adenomatous Polyposis Coli (APC) (APCmin−/−) which were originally obtained in an ENU mutagenesis screen, is widely accepted in the field because the gene with most predominant mutation in human CRC is APC. APC−/− mice can develop multiple intestinal neoplasia (min), yet without distal organ metastasis. Importantly, emerging evidence shows that tumor associated carbohydrate antigens, Tn and sialylTn (STn) appear at an early stage of colon carcinogenesis, and are associated with progression and metastasis. However, the molecular mechanisms underlying the role of Tn/STn antigens in CRC tumorigenesis and progression are unknown. We recently identified a novel X-linked gene Cosmc (C1GalT1C1 on human Xq24 and mouse Xe3), which encodes a molecular chaperone Cosmc regulating mucin O-glycosylation. Cosmc is required for folding of active core 1 β3-galactosyltransferase (T-synthase, C1GalT1 on 7p21.3), the key enzyme that converts Tn antigen to normal core 1 O-glycans on glycoproteins in all animal cells. Many studies from ours and others showed that Cosmc mutations lead to loss of T-synthase activity and resultant expression of Tn/STn antigens in human carcinoma cells and in other diseases. Remarkably, in our ongoing study, intestinal epithelial cell (IEC)-specific Cosmc-knockout (IEC-Cosmc-KO) mice spontaneously developed invasive CRC. The molecular mechanisms underlying the colorectal carcinogenesis in these Cosmc-KO mice are through complex pathways and require a comprehensive study. **Objective:** To investigate whether Tn/STn antigens promote CRC formation, progression and ultimate metastasis to distal organs in the IEC-Cosmcnull:APCmin−/− CRC animal model. **Methods:** using gene targeted strategy to generate IEC-Cosmcnull in APCmin−/− mice (IEC-Cosmcnull:APCmin−/+ ) and pathologically examine their primary CRC and metastatic tumors. **Results:** IEC-Cosmcnull:APCmin−/− mice could not be generated due to the short life span of APCmin−/− mice. We had successfully taken an alternative approach to generate IEC-APC:Cosmc-DKO mouse line by using APCem1/+ line. In the project period, 22 IEC-APC:Cosmc DKO mice (13 male and 9 female) and similar numbers of control mouse groups (APC-KO, Cosmc-KO and WT) in total were generated. Among them, the DKO male and female mice grew and developed normally as other controls in their first 2 months, then slower growth with smaller body size, stress signs, and mortality gradually occurred around 3-month age, while other controls IEC-APC-KO, IEC-Cosmc-KO and WT appeared normal. Four male DKO mice (IEC-APCem1/+;Cosmcem1/−;Cre−/−) (30%) died and a majority (8 of 9) of living male DKO mice at 3~4 moths age, and a fraction of (2 of 9) female DKO mice (IEC-APCem1/−;Cosmcem1/−;Cre−/−) had to be sacrificed due to their illness. DKO mice had significant lower survival rate (*P<0.01) compared to all other control groups. Importantly, multiple gross tumors in the small intestine were observed in both APC-KO and APC:Cosmc-DKO mice, but the colorectal tumors were seen in all DKO mice and only in 15% of APC-KO mice. Consistent with our early findings, thickened rectum was observed in Cosmc-KO male mice, but no tumor was found in Cosmc−/− female mice. Furthermore, the enlarged inguinal lymph nodes were only found in the DKO mice. These results clearly indicate that the Cosmc deletion promotes the colorectal tumorigenesis in APC-mutant mice. Unexpectedly, DKO mice suffered from severe anemia, likely due to an impaired iron homeostasis because of dysfunctional iron-pump ferroportin on duodenum epithelia which could be possibly O-glycosylated and degraded in DKO mice. Anemia of DKO mice might partially contribute to their slower growth phenotype. HE staining of GI tract tissue sections confirmed the adenomas and adenocarcinomas in the DKO, and adenomas in APC-KOs and Cosmc-KO male mice. The significant progression and metastasis of CRC were not observed thus far from these young mice, since it may require a longer time to develop. Nevertheless, these preliminary results suggest that the expression of Tn/STn antigens significantly promoted the colorectal carcinogenesis and perhaps progression in APC-mutant mice. More detailed
molecular analyses are underway, and the project will continue investigating the role of Tn/STn in CRC progression and metastasis.

(b) Objectives: 1. To generate APC:Cosmc-double knockout (DKO), APC-KO (A-KO), and Cosmc-KO (C-KO) mice;
2. To monitor and examine the development of colorectal tumors in the DKO, A-KO and C-KO animals;
3. To assess the malignancy of the colorectal tumors and possible metastatic tumors in those mice by pathology and molecular.

(c) Methods used:
1. Generating experimental animals using targeted gene-deletion technology: the breeding strategy is shown below:

![Breeding Strategy to generate Experimental Animals](image)

2. Assessing colorectal tumor development;
3. Analyzing the tumors including metastatic tumors by a pathological approach;
4. Analyzing Tn/STn Expression and tumor pathways by IHC: immunohistochemistry analysis on the Tn/STn antigen expression, Wnt/APC/β-Catenin pathway, TGFβ, and other pathways using specific antibodies against important players in these pathways.

(d) Results: At beginning of the project, the APC\textsuperscript{min/+} mice were used as an APC-mutant model as proposed in our original proposal. Yet, after ~4 months’ breeding, the double mutants, IEC.Cosmc\textsuperscript{null}.APC\textsuperscript{min/+} mice could not be generated due to the short life span (~3 months) and low productivity of APC\textsuperscript{min/+} mouse line. An alternative strategy (Figure) was immediately taken by replacing APC\textsuperscript{min/+} mouse line with APC-floxed (targeted mutation-1, APC\textsuperscript{em1}) mice, and worked well. In this period of research time, 22 of APC:Cosmc-DKO in total: 13 APC\textsuperscript{em1/+}.Cosmc Fy/Cr+/- (male) and 9 APC\textsuperscript{em1/+}.Cosmc Fy/Cr+/- (female), and similar number of control male and female (APC-KO, Cosmc-KO and WT) animals were generated; they grew normally at young ages (<2-months). Here are the major observations:
1. Gross Phenotypes: The male DKO mice started to show phenotypes when they grew older than 2-months: slower growth or even body weight loss, curled back, and unthrilled; 4 of them died at ~3 months age; while APC-KO, Cosmc-KO and WT controls developed normally. Eight of nine DKO male mice at age of 3–4 moths were sacrificed because of their illness, while all other control animals appeared to exhibit normal growth with the exception of one Cosmc-KO mouse which developed rectal prolapse and had to be terminated. The female DKO mice also had similar but minor phenotypes than the male DKO mice; 6 of 9
female DKO lost weight after 3-months age, 3 of them had to be sacrificed at 4-months’ age due to illness, and DKO mice had a significantly lower survival rate (*P<0.01);  
2. Tumors: multiple gross tumors grew in intestines in all DKO mice: 4~37 tumors with 0.1-0.3mm in size in small intestines and 2~6 tumors with 0.1~0.5 mm in size in colorectum; enlarged brachial and inguinal lymph nodes were found in 6 of those 18 DKOs, but there was no obvious tumor mass in distant organs such as liver and lung although they appeared pale. For 16 of APC-KO mice, 4~27 tumors were observed in their small intestines, but only 3 of them (16 total) had 1~2 gross tumors with sizes of 0.1-0.2 mm in colorectum. The thickened rectum was also observed in 9 of 10 male Cosmc-KO male mice;  
3. Histology: H&E staining confirmed multiple adenoma and some carcinomas developed in both small intestines and colorectum from DKO mice, but only adenomas were observed in small intestines (all APC-KOs), and in colon (3 of 16 APC-KOs); epithelial morphology changes also appeared in Cosmc-KO mice, mainly in rectum, consistent with our previous study;  
4. Anemia: unexpectedly, all DKO mice suffered from severe anemia based on analysis of packed blood cell volume (PCV: 20-30% in DKO versus 50% in control), while APC-KO and Cosmc-KO mice had a 10~15% decrease in PCV. Surprisingly, Feroportin (FPN), an important iron pump glycoprotein expressed on GI-tract epithelial cells (mainly in duodenum epithelial cells) appeared to be largely degraded in the DKO mice, suggesting that the dysfunction of FPN might result in an impaired iron homeostasis in the DKO animal. For the female DKO mice, because of the mosaicism of the Cosmc-deletion due to random X-chromosome inactivation, the phenotype was milder than the male DKO mice, and the DKO females survived longer.  
(e) Discussion: While the Tn and STn antigens are highly expressed in human colorectal carcinoma, which is mostly associated with gene mutations in the tumor suppressor APC, the role of Tn/STn in the development, progression and metastasis in conjunction with APC-mutation has not been investigated. In our study, for the first time, the expression of Tn/STn antigens was systematically investigated in an animal model with APC mutation, which is the most relevant and key question in the colorectal tumor glyobiology. Our preliminary results showed that the young IEC-APC:Cosmc-DKO mice developed more and larger colorectal tumors compared to either APC-KO or Cosmc-KO mice, strongly indicating that Tn/STn antigens did play a significant promoting role in colorectal carcinogenesis. In addition, unexpectedly, the DKO mice were also found to suffer from severe anemia possibly due to an impaired iron homeostasis since the iron-pump Feroportin on duodenum epithelia was significantly degraded, which might also contribute to the slower growth phenotype. While further molecular analyses are underway, this preliminary data is exciting and promising. Although the metastatic tumors were not observed in distal organs in these young mice (<4.5 months), they will be investigated in elder animals in a continued project. The preliminary study supported by the Mizutani Foundation has laid a foundation for this exciting project, which will ultimately answer the important question regarding the role of most common tumor antigens, Tn/STn in malignancy of colorectal carcinoma. This comprehensive study will have an important impact not only on our understanding of relevant tumor glyco-biology, but also on development of novel therapeutics in metastatic colon cancer in future. In addition, this work also leads to a new exciting project on the role of O-glycosylation in iron homeostasis, which is totally novel.  
(f) List of publications: There is no publication yet.  
(g) Name (signed) & Date:  

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