eGastroenterology

Contribution of colibactin-producing *Escherichia coli* to colonic carcinogenesis

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To cite: Ishikawa H, Aoki R, Mutoh M, *et al*. Contribution of colibactin-producing *Escherichia coli* to colonic carcinogenesis. *eGastroenterology* 2025;**3**:e100177. doi:10.1136/egastro-2024-100177

► Prepublication history and additional supplemental material for this paper are available online. To view these files, please visit the journal online (https://doi.org/10.1136/egastro-2024-100177).

Received 08 January 2025
Accepted 04 June 2025

Linked

► https://doi.org/10.1136/egastro-2023-100016
► https://doi.org/10.1136/egastro-2023-100001
► https://doi.org/10.1136/egastro-2023-100007
► https://doi.org/10.1136/egastro-2024-100086

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Familial adenomatous polyposis (FAP) is an autosomal dominant disorder characterised by multiple colorectal adenomas caused by a germline pathological variant of *APC*.[1] The development of colorectal cancer (CRC) has interindividual differences. Recently, colibactin-producing *E. coli* (pks+*E. coli*) has attracted attention as a factor affecting CRC development. Currently, pks+*E. coli* is detected in 67% of patients with sporadic CRC and in 21% of healthy individuals.[2] It has been reported that pks+*E. coli* injects colibactin, a genetic toxin, into colonic mucosal epithelial cells, resulting in DNA damage, G2/M arrest[3] and promotion of colon carcinogenesis in animal experiments.[2]

One study has reported an association between FAP and pks+*E. coli*.[4] In this study, banked frozen mucosal samples were obtained from 25 patients with FAP (two polyps and two normal tissues per patient) and 23 healthy individuals (mucosal samples from surgical resections or one biopsy of the ascending and descending colon). Subsequently, mucosal tissue was cultured for the presence of pks+*E. coli*, and mucosal tissue from patients with FAP was found to be significantly associated with pks+*E. coli* (68%) compared with healthy mucosa (22%). However, the percentage of pks+*E. coli* detected in the colonic mucosa unaffected by surgery or antibiotic therapy in patients with FAP is unknown. In addition, the association between colorectal adenocarcinoma and pks+*E. coli* is not well known, because previous studies on FAP have been limited to adenomas. On the other hand, we have been performing intensive endoscopic polypectomy,[5] which has advantages in assessing colonic polyps/mucosa that are unaffected by surgery or antibiotic therapy in patients with FAP. Therefore, we decided to clarify the contribution of pks+*E. coli* to colorectal carcinogenesis in patients with FAP.

Samples were collected between January 2018 and August 2019 (online supplemental file 2). 75 patients with FAP were included in this study (online supplemental table 1 and figure 1). The analysis failed to detect a statistically significant association between sex, age, alcohol consumption and the positive rate of pks+*E. coli* in polyp tissues (online supplemental table 1). Furthermore, pks+*E. coli* was not detected in the polyp tissues of patients with a history of colorectal surgery. Notably, the positive rate of pks+*E. coli* in the polyp tissues of smokers was significantly higher (p=0.004) (online supplemental table 1).

In patients with FAP, there was no observed statistical association of the positive rate of pks+*E. coli* in polyp tissues with the density of colonic polyps and a history of CRC (online supplemental table 2). When only analysing patients who had not undergone colon surgery, there was a significant difference in the rate of pks+*E. coli* between patients with and without a history of CRC (risk ratio=3.250, 95% CI: 1.336 to 7.907, p=0.041).

Among 10 patients with FAP who had not undergone colorectal surgery and had a previous adenoma canceration (all non-smokers), the adenomas positive for pks+*E. coli* had slightly higher nuclear atypia of tumour cells and more intraepithelial inflammatory cell infiltration (figure 1, online supplemental table 3). Compared with pks+*E. coli*-negative polyps, γ-H2AX was significantly upregulated in positive polyps, IL-6 showed an upward trend and there was no significant change in IL-1β (figure 2).

This study showed that the percentage of pks+*E. coli* detected in colorectal polyp tissues of patients with FAP was unaffected by surgery or antibiotic administration. Although previous studies on FAP have been conducted on surgically obtained colorectal specimens, especially colorectal adenomas, we addressed

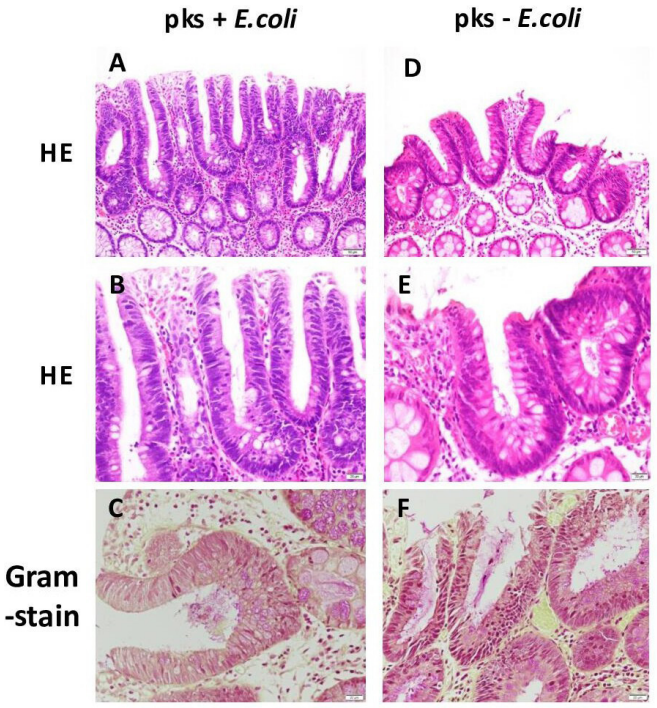

Figure 1 Representative histopathology of the polyps. A polyp lesion with colibactin-producing *E. coli* (pks+) is shown in panels A–C. (A) HE staining of the polyp lesion, which shows high-grade tubular adenoma. (B) High magnification of A. (C) A few bacteria with gram-positive and negative stains are seen in the crypts and cryptal lumen. A polyp lesion without colibactin-producing *E. coli* (pks–) is shown in D–F. (D) HE staining of the polyp lesion, which shows low-grade tubular adenoma. (E) High magnification of D. (F) Bacteria with gram-positive and negative stains are observed in the crypts and cryptal lumen. Figure permission is sought for the image.

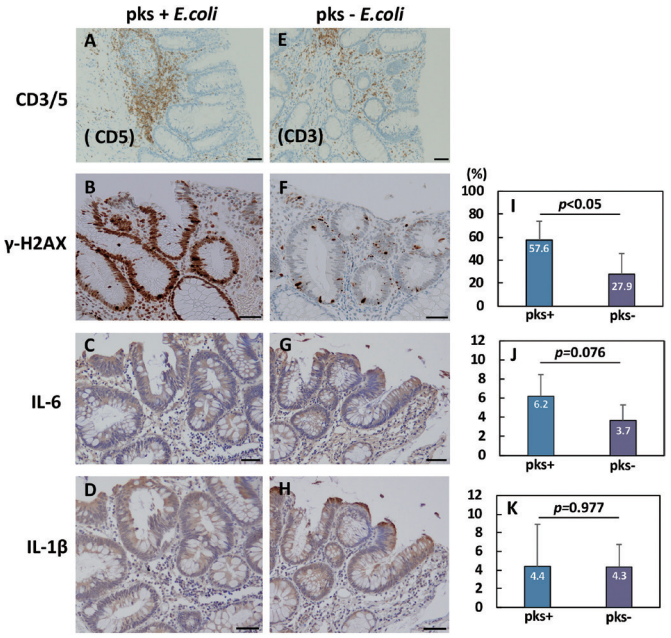

Figure 2 Representative immunohistopathology of the polyps. Polyp lesions with colibactin-producing *E. coli* (pks+, A–D) and without colibactin-producing *E. coli* (pks–, E–H) are shown. (A) Lymphocytes showing CD3-positive reaction infiltrating the mucosal lamina propria. (E) CD5-positive lymphocytes infiltrating the mucosal lamina propria. (B and F) γ-H2AX immunostaining. (I) The positive rate (%) of nuclei to the total number of adenoma cell nuclei is shown. (C and G) IL-6 immunostaining. (J) The immunoreactivity score (IRS) value was evaluated by IRS = (staining percentage score) × (staining intensity score) and shown. (D and H) IL-1ß immunostaining. (K) IRS values are shown. Bars, 50 µm. Figure permission is sought for the image.

the association between colorectal adenocarcinoma and pks+*E. coli* in association with a history of CRC.

Dense polyposis increases the risk of CRC development.[1] However, in this study, we did not find any association between the degree of polyp density and pks+*E. coli* infection. This is in line with a previous study that reported no statistically significant associations regardless of the tumour site or number of colorectal adenoma lesions.[6]

We have previously reported that smoking, a lifestyle factor, promotes polyp formation in patients with FAP.[7] Moreover, numerous reports have demonstrated that smokers have a high incidence of colorectal adenomas. In contrast, no association has been reported between pks+*E. coli* detected in the stool and the incidence of colorectal adenomas.[8] In this study, smokers showed a high prevalence of pks+*E. coli*. Therefore, here, there was a possibility that polyp development might be enhanced in pks+*E. coli*-positive FAP by smoking, but there was no such bias.

Notably, pks+*E. coli*-positive tissues showed intraepithelial inflammatory cell infiltration, suggesting that inflammation and immunity may be involved in colibactin-related carcinogenesis. We investigated the mechanism by which colibactin exposure induces inflammation in polyps of patients with FAP. Since it has been reported that colibactin-exposed cells induce senescence, the production of senescence-associated secretory phenotypes (SASPs) such as IL-6 may enhance inflammation. Therefore, we used tissue sections to confirm the status of SASPs and found a trend towards IL-6 induction and increased γ-H2AX expression. This finding suggests that senescence is involved in the induction of inflammation following colibactin exposure.

The strengths of our study are that (1) a large number of patients with FAP who did not undergo colorectal surgery. Since the standard of care is total colorectal resection at approximately 20 years of age, there are only a few patients over 20 years of age with FAP whose colon is preserved.[1] Due to intensive downstaging polypectomy, this study included many valuable patients with FAP whose colon was preserved. This made it possible to examine 79 non-surgical cases with an average age of 40 years, even though FAP is a rare disease. In addition, because surgery is known to drastically alter the intestinal microbiota,[9] it is necessary to examine non-surgical patients to determine the effect of pks+*E. coli* on colorectal carcinogenesis; (2) detailed clinical data were collected to address

these factors as potential confounders; and (3) efforts were made to evaluate colorectal polyp density; (4) when assessing colibactin positivity, blinding of the histological analysis added weight to the conclusions, counteracting the limited number of samples reviewed.

The limitations of this study are that (1) the sample size was small, limiting statistical interpretation; (2) only selected lifestyle factors were assessed, excluding key exposures like diet; (3) potential bias in polyp selection may exist, as not all polyps could be analysed; (4) post-surgical changes in gut microbiota and their impact on pks+*E. coli* were not evaluated; and (5) we did not have genetic diagnostic information due to the barriers in Japanese legislation. To reveal the longitudinal effect of pks+*E. coli*, it would be helpful to identify the mutational signature of pks+, that is, SPS88, or the recurrent variant in *APC* c.835–8 A>G. In summary, no definitive association can be established based on our current data set, and our findings should be interpreted as hypothesis-generating only. We also recognise the importance of validating these preliminary findings through larger, multicentre studies or consortia in the future to confirm whether such an association or causation truly exists.

In conclusion, endoscopically collected colonic polyp samples can be used to determine the presence of pks+*E. coli* in patients with FAP. Our results implied an association with CRC, but not with polyp density, a factor associated with the severity of FAP, and implied that colibactin may not be involved in the development and growth of adenomas. The pks+*E. coli* infection can be influenced by lifestyle factors, such as smoking. In the absence of previous colorectal surgery, a higher proportion of patients with FAP with a prior CRC diagnosis are carriers of colibactin-producing *E. coli*, which may point towards a potential role of these bacteria in carcinogenesis in this setting.

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Acknowledgements We thank Ms Eri Okuda (Department of Molecular-Targeting Prevention, Kyoto Prefectural University of Medicine) for their help with the administrative work of entering data and maintaining contact with the subjects.

Contributors HI and MM had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Moreover, HI, MM and KWat contributed to the concept, design and drafting of the manuscript. MM and KWat obtained funding. HI, NM, SM, TH and NY were responsible for acquisition, analysis or interpretation of data. SI, RA and TT performed the histopathological analysis. KWak reviewed and supervised this work. All authors approved the final submitted version.

Funding This study was funded by the Japan Society for the Promotion of Science (JSPS) (22H05119, 22H05121, 22H00362, 22H04979, 22K19158), SECOM Science (guarantor: KWat) and Technology Foundation and the Japan Agency for Medical Research and Development, AMED (24ck0106806h0002) (guarantor: MM).

Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involves human participants and was approved by the Ethical Review Committee of the University of Shizuoka (Ethics Approval Number: 29-27). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

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