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Induction of Aberrant Crypt Foci and Flat-type Adenocarcinoma in the Colons of Dogs by *N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine and Their Sequential Changes

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Sequential endoscopic observation of dog colons was performed during colon carcinogenesis. Two beagle dogs were given suppositories containing *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) every day for five months. In month 3, aberrant crypt foci (ACF), a putative preneoplastic lesion, were found in the colons of both dogs, but not in an untreated dog. The frequency of ACF increased until month 10, and then decreased. In month 9, very small lesions, less than 1 mm in diameter, which were similar to human early flat tumors, were first noticed. One of these lesions grew to about 7 mm in size without a change in its shape for 10 months. There were more than ten flat-type tumors in the two dogs, but such lesions were not found in the untreated dog. By biopsy, two of the lesions were proved to be well-differentiated adenocarcinomas histologically. Four polypoid lesions were found in one of the carcinogen-treated dogs. Thus, flat-type adenocarcinomas were induced in the dog colon by ENNG, and their development was followed by magnifying endoscopy.

Key words: Aberrant crypt foci — Flat-type adenocarcinoma — Dog — Colon carcinogenesis — Endoscopy

Adenocarcinoma of the colorectum is accepted as a representative example of multistep carcinogenesis, on the basis of histopathological and genetic studies.[1,2] However, the adenoma-carcinoma sequence theory was established mainly by studying the progression of adenoma in patients with familial adenomatosis colorectum. What proportion of sporadic colorectal cancers follow this process of tumor progression is still controversial. Hereditary non-polyposis colorectal cancer (HNPCC) is a representative disease for which a distinct genetic pathway of progression of colorectal cancer is documented.[3,4] The colorectal tumors in HNPCC patients harbor defects in DNA repair genes. Another important point is that completely flat or slightly depressed colorectal cancers have been found without a remnant of a polypoid lesion.[5] Kudo *et al.* classified the morphological types of the flat-type tumors.[6] Genetic alterations in this unique class of colorectal cancer revealed that flat-type colorectal tumors are distinct from ordinary polypoid lesions.[7,8] These flat-type tumors have been found to progress into invasive and metastasizing carcinomas more readily than adenomatous polyps.[9–11]

In recent years, minute colorectal lesions, aberrant crypt foci (ACF), have received much attention.[12–14] ACF were first identified microscopically in methylene blue-stained whole-mount preparations of colonic mucosa from carcinogen-treated mice and rats.[15,16] They were also found in the colons of patients with colorectal cancer, show K-*ras* activation at very high frequency and are diverse in their histology.[17–19] Although it is not established yet whether ACF is a precursor of clinical neoplastic lesions, ACF is a putative precursor of colorectal cancer.[12,19]

Follow-up study of colorectal tumors is very important to know what is the precursor of invasive cancer and what is the fate of preneoplastic lesions. Colorectal carcinogenesis models are well established in rats and mice, and sequential studies have been conducted to answer the above questions. However, direct follow-up study of the carcinogenesis process in these small animals is not easy. Pioneering work has been done in dogs by endoscopic follow-up,[20] but information from this study is limited. In the present study, we adapted this dog colorectal carcinogenesis model and used magnifying endoscopic examination to examine the very earliest steps in colorectal carcinogenesis.

MATERIALS AND METHODS

The carcinogen was administered to the dogs by the method of Kamano *et al.* with some modifications.[20] A suppository with a diameter of 1 cm and a length of 4 cm was prepared with a vehicle of Witepsol H15 containing 50 mg of *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG, Sigma Chemical Co., St. Louis, MO). Suppositories without the carcinogen were used as a vehicle control. The suppositories were stored at 4°C until use.

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Two 6-month-old beagle dogs weighing 10 kg were given one suppository containing ENNG about 10 cm from the anus every day for 5 months. As a control, one dog was given a suppository without the carcinogen in the same way. The animals were fed chow (DS; Oriental Yeast Co., Ltd., Tokyo), and given tap water *ad libitum*.

Endoscopic observation of the colon was carried out at 2- or 3-week intervals. During the endoscopic experiments, the animals were anesthetized by subcutaneous injection of ketamine chloride (Sankyo Co., Ltd., Tokyo). Atropine sulfate was additionally injected subcutaneously. The dog colon was observed with a magnifying videoscope (CF 200Z, Olympus Optical Co., Ltd., Tokyo). The pit pattern of the luminar surface of the colon was visualized by spraying the mucosa with 0.02% methylene blue solution. Careful observation was made mainly on the colonic surface between 5 and 20 cm from the anus, where a tattoo was made. Several lesions in the colons were tattooed for sequential survey.

RESULTS

Induction of aberrant crypt foci in colons by ENNG treatment At the beginning of carcinogen treatment, redness and slight erosion were observed but no definitive ulcer was detected in the 2 ENNG-treated dogs, designated dogs No. 1 and No. 2. Since melena became worse in both dogs, the ENNG administration was terminated after 5 months. Numerous small nodules, about 2–3 mm in diameter, were seen within 15 cm from the anus 2 weeks after the start of carcinogen treatment, and were present throughout the carcinogen administration. When several nodules were biopsied, histological examination revealed that those were hypertrophic lymphatic follicles (data not shown).

We first identified ACF in the two ENNG-treated dog colons in month 3. They consisted of single enlarged crypts in a focus (Fig. 1). In month 7, ACF containing multiple crypts were found in both ENNG-treated dogs,

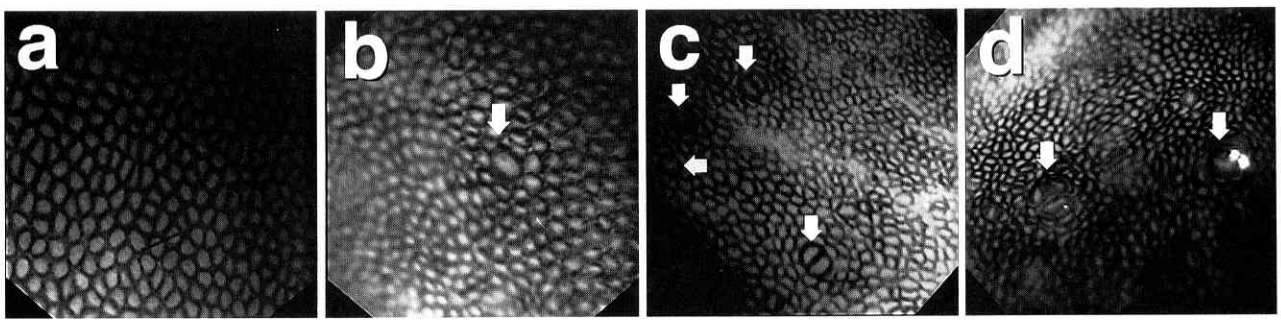

Fig. 1. Endoscopic features of aberrant crypt foci in the colons of ENNG-treated dogs. The mucosa of dog colons was stained with 0.02% methylene blue and then observed with a magnifying endoscope. Arrows indicate ACF. a, Mucosa of a control dog; mucosa of an ENNG-treated dog in months 3 (b), 7 (c), and 10 (d).

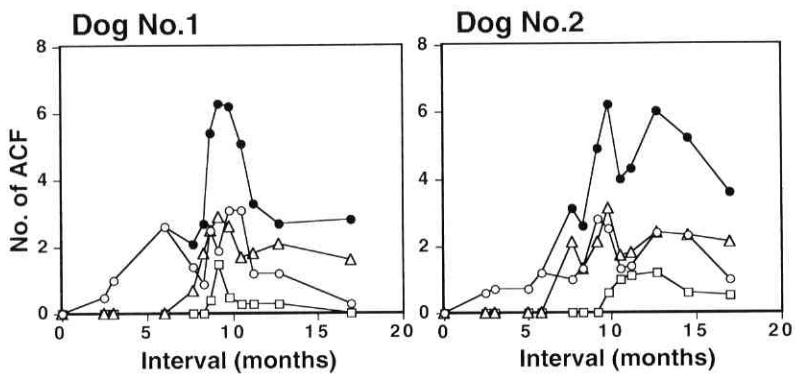

Fig. 2. Frequency of aberrant crypt foci in the colons of ENNG-treated dogs. The number of ACF in the mucosa of ENNG-treated dog colons was calculated by counting more than 10,000 crypts of methylene blue-stained mucosa in video prints obtained with a magnifying endoscope. Closed circles indicate number of total ACF. Open circles, triangles and squares indicate numbers of ACF consisting of one, two and three or more crypts, respectively.

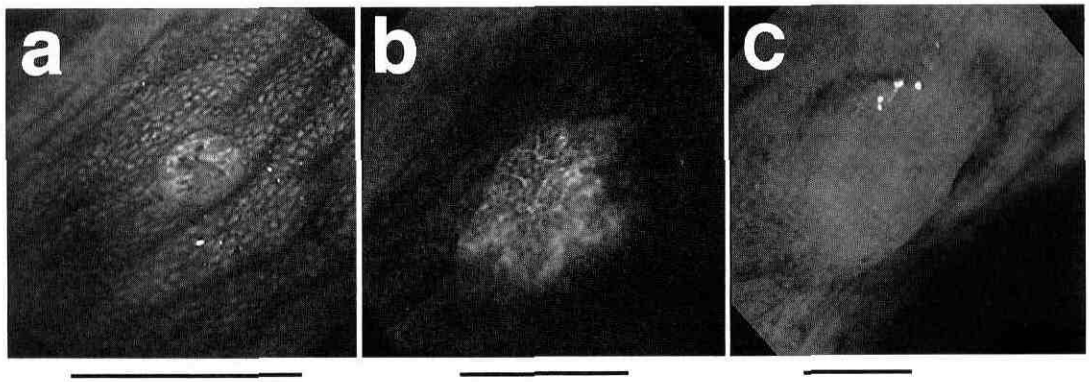

Fig. 3. Endoscopic features of minute flat tumors in the colons of ENNG-treated dogs. Black bars indicate 1 mm. a, Lesion 1F in month 9; b, lesion 1B in month 13; c, lesion 2A in month 18.

but not in the untreated dog. The morphology of the ACF was somewhat different from that in rats or mice.[15, 16] The dog ACF were not elevated above the adjacent mucosa. There was no ACF containing more than six aberrant crypts. The frequency of ACF was determined by counting the ACF in video prints of the colonic mucosa, and defined as the number of ACF per 10,000 normal crypts. The frequency of ACF reached the maximum in month 10, and then decreased (Fig. 2). Time-dependent changes in the frequency of ACF with multiple aberrant crypts were similar to those in total ACF. Irregularity in the size of the individual pits was found in about half the ACF containing three or more crypts (Fig. 1d).

Induction of flat-type tumors in the ENNG-treated dog colons We first found another type of minute lesion less than 1 mm in diameter, corresponding to 20 crypts, in month 9 (Fig. 3). We have identified and followed up six and nine lesions in dogs No. 1 and No. 2, respectively, by endoscopic examination up to now, month 30. Endoscopic characteristics of these lesions are summarized in Table I.

In dog No. 1, lesions 1A and 1F were first identified in months 10 and 9, respectively (Figs. 3 and 4). They were similar to slightly flat-elevated tumors with a central depression (IIa+IIc) in human colon. Lesion 1B was found in month 9. It was slightly depressed with a peripheral elevation (IIc+IIa) (Fig. 3). Lesion 1C showed a slightly flat-elevated shape (IIa). Lesions 1D and 1E were flat and not elevated (IIb). All the lesions had irregular pit patterns of cryptic openings, which were very similar to the oval or gyrus-like pits of adenomas and adenocarcinomas in human colons.[21] Therefore, they were thought to be lesions related to flat and/or depressed adenomas and adenocarcinomas of human colon.

Table I. Flat Tumors and Sessile Polyps in the Colons of ENNG-treated Dogs

Dog No.	Lesion designation	Endoscopic morphology[a]	First definition (month)	Histology[b]
1	1A	IIa+IIc	10	Carcinoma
1	1B	IIc+IIa	9	NT[c]
1	1C	IIa	16	NT
1	1D	IIb	22	NT
1	1E	IIb	25	NT
1	1F	IIa+IIc	9	NT
2	2A	IIc+IIa	18	NT
2	2B	IIa	13	Carcinoma
2	2C	IIa	19	NT
2	2D	Is	18	NT
2	2E	Is	15	Hyperplasia
2	2F	Is	15	NT
2	2G	IIa	19	NT
2	2H	Is	19	NT
2	2I	IIa	26	NT

a) Endoscopic morphology of minute lesions was classified according to Kudo *et al.*[6] IIa, flat elevated lesion; IIb, flat and not elevated lesion; IIa+IIc, elevated lesion with a central depression; IIc+IIa, depressed lesions with a peripheral elevation; Is, sessile polyp.
b) Histology was determined from hematoxylin-eosin (H & E)-stained sections of the lesions by one pathologist, one of the authors (T. H.).
c) Not tested.

Lesion 1A was endoscopically followed up until month 20. The shape of the lesion changed from a sloping flat elevation to a clear flat elevation with a central depression during 10 months, and the diameter increased from 1 to 7 mm (Fig. 4). In month 20, lesion 1A was examined histologically by biopsy and diagnosed as well-differen-

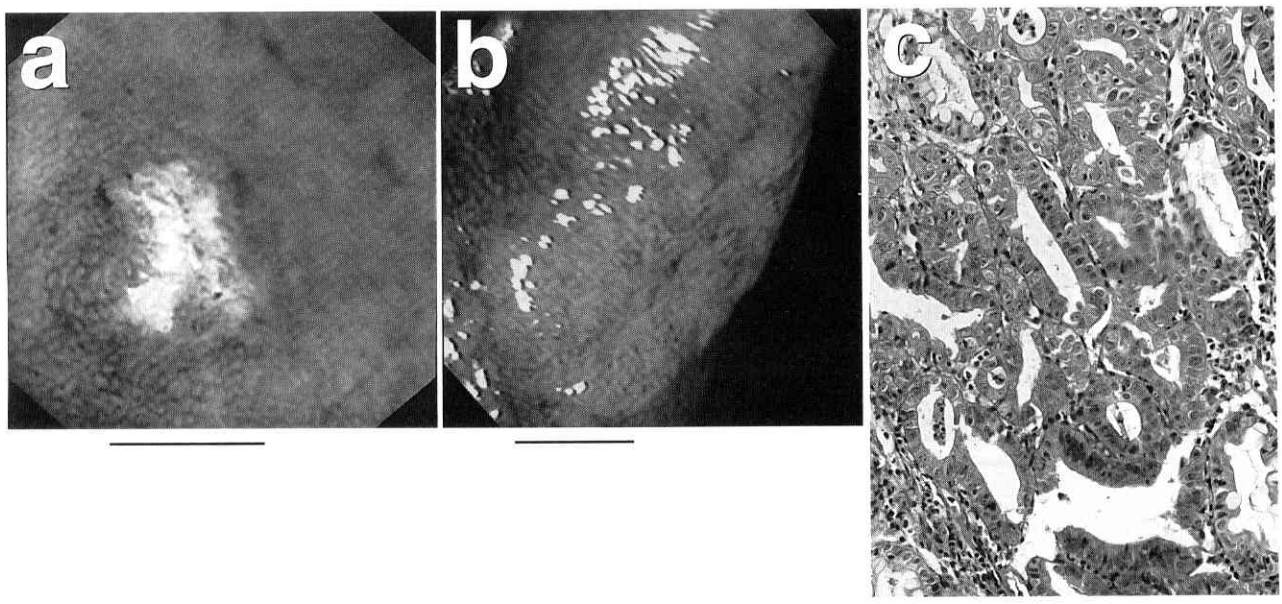

Fig. 4. A flat depressed adenocarcinoma in the colon of an ENNG-treated dog. Endoscopic features of lesion 1A in months 10 (a) and 20 (b) in dog No. 1. c, Well-differentiated adenocarcinoma in an H&E-stained section of lesion 1A. Black bars indicate 1 mm.

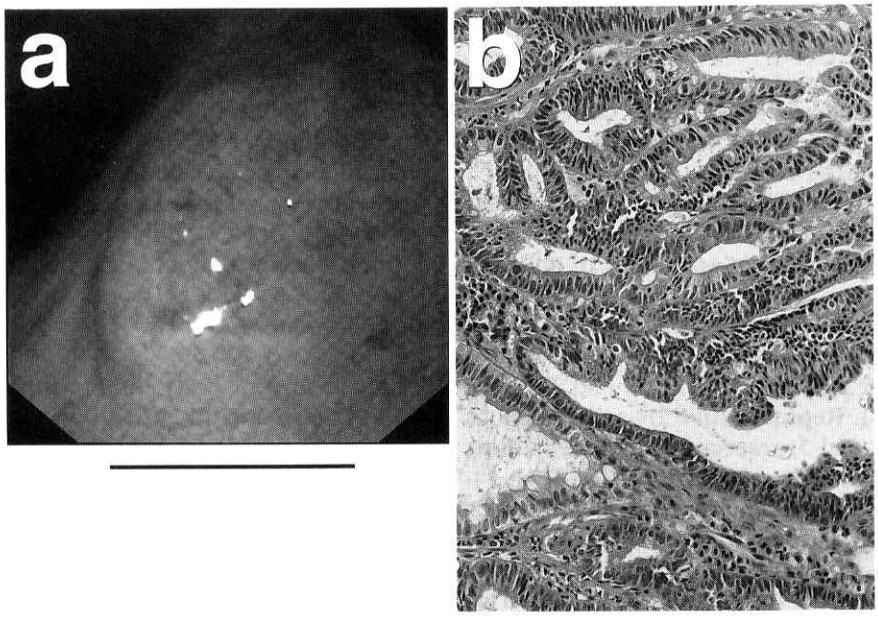

Fig. 5. A flat adenocarcinoma in the colon of an ENNG-treated dog. a, Endoscopic features of lesion 2B in month 13 in dog No. 2. b, Well-differentiated adenocarcinoma with adenomatous components in an H&E-stained section of lesion 2B. Black bar indicates 1 mm.

tiated adenocarcinoma (Fig. 4c). We tried to remove this lesion by mucosal resection endoscopically before the biopsy, but the submucosa could not be expanded by saline injection. Therefore we assumed that the carcinoma of lesion 1A had invaded the submucosal layer, though this was not confirmed histologically. Although

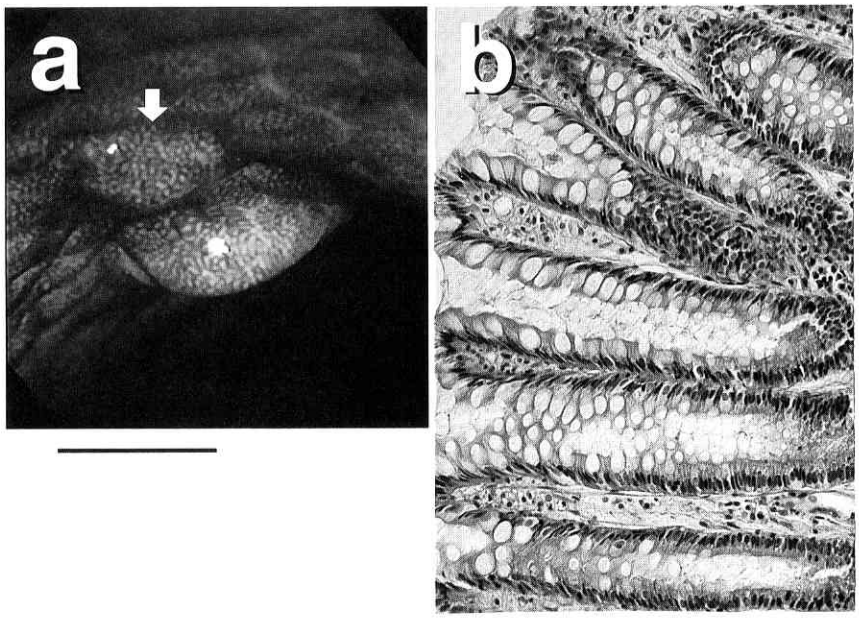

Fig. 6. A polypoid lesion in the colon of an ENNG-treated dog. a, Endoscopic features of lesions 2E (arrow) and 2F in month 13 in dog No. 2. b, Hyperplasia of glandular epithelium in an H&E-stained section of lesion 2E. Black bar indicates 1 mm.

not all these flat-type lesions in dog No. 1 could be observed every time endoscopically, it was clear that the shapes of lesions 1B, 1E and 1F at least did not change up to month 30. The diameter of lesion 1B increased from 1 to 3 mm during 21 months. These findings proved that at least one minute flat-depressed tumor developed into an invasive adenocarcinoma without an episode of polypoid growth.

In dog No. 2, nine minute lesions were found. Lesion 2A showed a slight depression with a peripheral elevation (IIc+IIa). The other lesions (2B, 2C, 2G and 2I) were slightly elevated (IIa). Representative 2A and 2B lesions are shown in Figs. 2 and 5, respectively. These flat-type lesions were first identified in months 13 to 26 (Table I). In addition to the flat-type tumors, four sessile polyps (2D, 2E, 2F and 2H) were found. They were first identified in month 13 or 15, and did not change in morphology by month 30 except for lesion 2E. This lesion was first found as a sessile polyp in month 15. Typical endoscopic features of 2E and 2F lesions are shown in Fig. 6.

Lesions 2B and 2E were resected by biopsy in month 20. Pathological examination revealed that lesions 2B and 2E were well-differentiated adenocarcinoma with adenomatous components and hyperplasia of the glandular epithelium, respectively (Figs. 5 and 6). The pit pattern of lesion 2E as well as other polyps was round, whereas the flat-elevated lesions displayed distorted pit patterns. The round and distorted patterns were similar to those found in hyperplasia and tumors, respectively, in human colons.[21] These findings indicate that the classifications of the pit pattern and elevation/depression of the mucosa in human colons are applicable to endoscopic examination of tumors in the dog colon.

DISCUSSION

In the present study, ACF were induced in the colons of ENNG-treated dogs, and most of them were smaller than six crypts/focus. Why could we not detect relatively large ACF consisting of more than six crypts in the dog colons? In the human colon, it is not rare to detect ACF consisting of more than 50 crypts. However, large ACF are also very rare in rats and mice treated with carcinogens.[15, 16] We examined the dog colon for more than two years in this study. Therefore, we cannot attribute the rarity of large ACF to the shortness of the observational period. The histological architecture of ACF might be more unstable in dogs and probably in mice and rats. This instability might facilitate faster progression of ACF. Some intrinsic factors in dog colon as well as rat and mouse colon might be involved.

The incidence of dog ACF decreased after month 10, and no other type of lesion with similar incidence

appeared. Probably, most of the ACF disappeared with time. Although we could not obtain direct evidence in the present study, it is most unlikely that a very small part of the ACF in the dog developed into minute flat tumors or unknown neoplasias for the following reasons. First, in about 50% of ACF containing three or more crypts, luminar openings of the crypts were irregular in size. Such irregularity is not common in small ACF of humans, and is considered to be one of the superficial characteristics of colon tumors.[21] Second, after the incidence of ACF reached the maximum, flat-type tumors appeared in the ENNG-treated dog colons.

In humans, flat-type tumors are found more frequently in Japan than in North America and Western Europe.[22–24] But Fujii *et al.* found that the incidence of these tumors in English Caucasians was similar to that in Japanese (unpublished results). Thus, differences in diet and methods of endoscopic examinations between these countries are possible explanations for the difference in incidence.[25] In dog No. 2, about 40% of the minute lesions were sessile polyps, while no polyp was found in dog No. 1. This finding showed that the type of lesions induced differed between dogs, even though the same carcinogen was administered. Therefore, although the number of dogs used in this study was limited, the results might indicate involvement of genetic factors and the metabolic state of the individuals in the induction of flat-type tumors.

The incidence of flat tumors was low in the rats treated with colon carcinogens, including dimethyl hydrazine.[26] Kamano *et al.* also reported that one of three adenocarcinomas in an ENNG-treated dog was a flat depressed adenocarcinoma (IIa+IIc).[20] It is not known whether induction of flat-type tumors depended on the type of carcinogen, the mode of carcinogen administration or the species of the animal.

Most advanced adenocarcinomas in human colons are deeply ulcerated. It is widely accepted that most of the advanced adenocarcinomas originated from malignant polyps. However, several investigators have speculated that not all the colorectal cancers developed from polyps, and flat-type tumors are one of the candidates for the precursor of ulcerative carcinomas, because remnants of adenomas are sometimes absent in invasive adenocarcinomas.[9–11] There is no reported study in which flat-type tumors were followed up until they developed into ulcerative advanced cancers in human colons. To establish whether flat-type tumors really develop into advanced cancers, it is desirable to have an animal model suitable for endoscopic examination of the colon. In the present study, we showed that ENNG-treated dogs may be a good model for investigating the progress of flat-type tumors in the colon. Further follow-up of the flat-type tumors should allow us to determine whether they progress into ulcerative advanced carcinomas.

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