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Title: A mutation spectrum that includes GNAS, KRAS, and TP53 may be shared by mucinous neoplasms of the appendix

Article Type: Original Article

Keywords: appendiceal mucinous neoplasm; TP53; GNAS; KRAS; mismatch repair gene; mutation

Corresponding Author: Dr. Tsuyoshi Saito, M.D., Ph.D.

Corresponding Author's Institution: Juntendo Univ.

First Author: Kieko Hara

Order of Authors: Kieko Hara; Tsuyoshi Saito, M.D., Ph.D.; Takuo Hayashi; Alkam Yimit; Michiko Takahashi; Keiko Mitani; Makoto Takahashi; Takashi Yao

Abstract: Appendiceal mucinous tumors (AMTs) are classified as low-grade appendiceal mucinous neoplasms (LAMNs) or mucinous adenocarcinomas (MACs), although their carcinogenesis is not well understood. As somatic activating mutations of GNAS are considered to be characteristic of LAMNs while TP53 mutations have been shown to be specific to MACs, MACs are unlikely to result from transformation of LAMNs. However, emerging evidence also shows the presence of GNAS mutations in MACs. We examined 16 AMTs (11 LAMNs and 5 MACs) for genetic alterations of GNAS, KRAS, BRAF, TP53, CTNNB1, and TERT promoter in order to elucidate the possibility of a shared genetic background in the two tumor types. Extensive histological examination revealed the presence of a low-grade component in all cases of MAC. GNAS mutations were detected in two LAMNs and in one MAC, although the GNAS mutation in this MAC was a nonsense mutation (Q227X) expected not to be activating mutation. TP53 mutations were detected in three LAMNs; they were frequently detected in MACs. KRAS mutations were detected in three LAMNs and three MACs, and CTNNB1 mutations were detected in two LAMNs. KRAS mutation and activating mutation of GNAS occurred exclusively in AMTs. BRAF and TERT mutations were not detected. Overexpression of p53 was observed in only two MACs, and p53 immunostaining clearly discriminated the high-grade lesion from a low-grade component in one. These findings suggest that p53 overexpression plays an important role in the carcinogenesis of AMTs and that, in addition to mutations of GNAS, KRAS and TP53 alterations might be shared by AMTs, thus providing evidence for the possible progression of LAMNs to MAC.

Pathology - Research and Practice,
Editor-in-Chief,

Dear Editor-in-Chief,

April 27th, 2015

Thank you very much for your e-mail on 4/23/2015 regarding our manuscript, entitled "A mutation spectrum that includes *GNAS, KRAS,* and *TP53* may be shared by mucinous neoplasms of the appendix "(PRP-D-15-00116).

We have revised our manuscript to address the concerns cited by reviewers #1 and #2. The purpose of this letter is to elaborate these concerns in a point-by-point manner and indicate how the manuscript has been revised to accommodate these issues.

Reviewers' Comments to Author:

Reviewer: 1

Comments to the Author
In the manuscript "A mutation spectrum that includes GNAS, KRAS, and TP53 may be shared by mucinous neoplasms of the appendix", the authors studied extensively 16 appendiceal mucinous tumors (AMT), including 11 low-grade appendiceal mucinous neoplasm (LAMNs) and 5 mucinous adenocarcinomas (MACs), with immunohistochemistry of p53 and beta-catenin and with mutation analysis of GNAS, KRAS, BRAF, CTNNB1, TP53, and hTERT. The work covered broad range of genetic alteration, and the paper is well organized. However, there are several concerns in the manuscript.

Major:
1. The authors suggest that GNAS mutation might be shared in LAMN and MAC, providing evidence of possible progression of LAMN to MAC. However, GNAS mutation was detected only one of five MAC, and the mutation was nonsense mutation which should not be regarded as activating mutation as the authors described in the discussion section. Thus, I think mutation and activation of GNAS would have little role in MAC, at least from the results of this study.

Response) We completely agree with this comment. We summarize and simplified the comments about this non-activating GNAS mutation in the "Discussion" section, to emphasize the exclusiveness of GNAS and KRAS mutation, as reviewer #1 advised us in Major comment# 2, below.

2. Because GNAS mutation in Case #12 is not thought as an activating mutation, activating mutation of GNAS and mutation of KRAS is mutually exclusive in AMT, which is an interesting point of the study. The authors should emphasize the exclusiveness in the result and discussion section.

Response) According to the reviewer's suggestion, we emphasize the exclusiveness of *GNAS* and *KRAS* mutation in "Result" and "Discussion" section.

3. Some TP53 mutation induces not overexpression but complete expression loss of p53, which is also thought to cause functional loss of p53. The authors should clarify if there was any case with 0% positivity of p53 in tumor cells.

Response) According to the reviewer's suggestion, we re-evaluated p53 immunohistochemistry to calculate p53 labeling index (LI). At least one thousand tumor cells were evaluated to calculate p53 LI. As for the cases containing less than 1000 cells in the representing section, all tumor cells in the representative section was evaluated. Complete expression loss of p53 (LI: 0%) was observed in 55% (6/11) for LAMNs and 40% (2/5) for MACs. Some *TP53* mutations observed in this study were considered to cause functional loss. We added this information in the "Material & Methods", "Result" section, and some comments in the "Discussion" section.

4. KRAS mutation was shared by LAMN and MAC. I think the authors should describe the significance of KRAS mutation found in the present study in the discussion section.

Response) According to the reviewer's suggestion, we added some description of the mutations in the "Discussion" section.

5. Mucinous adenocarcinoma of the colon is well-known for loss of expression of mismatch repair genes (MLH1, MSH2, PMS2, and MSH6) and microsatellite instability. Although I know the low-prevalence of loss of mismatch repair protein in AMT (e.g. Am J Surg Pathol 2013 Aug; 37:1192-200), I recommend the immunohistochemistry of those mismatch repair proteins in order to confirm the low-frequency of microsatellite instability in AMT.

Response) According to the reviewer's suggestion, we added already obtained data of MLH1 and MSH2 immunohistochemistry. In all of 16 cases, MLH1 and MSH2 expression were preserved, and we considered none of our series showed microsatellite instability. We added this information in the "Material & Methods", "Result" section, and some comments in the "Discussion" section. We also added the literature that reviewer kindly mentioned (Am J Surg Pathol 2013 Aug; 37:1192-200) as a reference.

6. Is the study approved by institutional review board? The authors should clarify the ethical issues in the "Sample preparation" section.

Response) Yes, the study is approved by institutional review board. We added this information in the "Material & Methods" section.

Minor:
1. On page 8 line 3, "The clinicopathological features of 16 the AMTs…" should be "The clinicopathological features of the 16 AMTs…"
Response) We appreciate the kindness of the reviewer. We corrected this typo.

2. On footnote of Table 3, the description "N.I.: data not informative…m: Methylated" should be removed. There is no abbreviation "N.I." or information of methylation in the Table 3.
Response) We appreciate the kindness of the reviewer. We removed these descriptions.

Reviewer: 2

Comments to the Author
This article focused on appendiceal mucinous tumors (AMTs) and gene mutation. AMTs are classified into low-grade appendiceal mucinous neoplasms (LAMNs) and mucinous adenocarcinomas (MACs), and it is unclear whether MACs arise from LAMNs or not. Authors investigated gene mutation and immunohistochemistry to clarify their carcinogenesis. It may be interesting that all five cases of MAC had a low-grade component from the view point of relationship between LAMNs and MACs. But several points should be revised.

1. In this study, histological difference between LAMNs and MACs must be significant point, but the difference was descriptive. Some representative histological photos about MACs feature, e.g. destructive invasion, high-grade cytological atypia, or complex epithelial proliferation, should be demonstrated.
Response) According to the reviewer's suggestion, we additionally demonstrate some representative histological photos about MACs feature, high-grade cytological atypia and complex epithelial proliferation in new Figure 1B, destructive invasion in new Figure 1C, respectively.

2. In Figure 2A, magnification was too low to understand details. High magnification photos should be inset. In Figure 2CD, magnification should be raised and the collision or transitional sites between LAMNs and MACs should be shown.
Response) According to the reviewer's suggestion, we additionally demonstrate some high magnification photos of low-grade component and high-grade component of case #15 in new Figure 1A and 1B. Unfortunately, we could not show the p53 stain of collision or transitional sites between LAMNs and MACs, because we have already run out of the

histological section for the sake of other immunostains and DNA analysis. Alternatively, we added some higher magnification photos of high-grade component and low-grade component of p53 stain in new Figures 2D and F.

3. There is gender difference and female cases were predominant. It might be natural that ovarian metastases were found, but the evidence should be demonstrated that the primary organ was appendix, but not ovary.
Response) As the reviewer#2 suggests, there is female predominance in our series. This series contained 3 cases with synchronous ovarian lesions, and all of them were presented as pseudomyxioma peritonei (PMP). Since the several histological and molecular genetic studies show the appendix as a primary origin of PMP (Ronnett BM et al. Human Pathol 1995; 26:509-524.; Mukherjee A et al. Eur J Gynaecol Oncol. 2004;25:411-4), we believe these 3 cases with ovarian lesions are also of appendiceal origin. For the remaining 13 cases, the appendiceal origin is confirmed clinically and histologically. We additionally mentioned about this information in the "Result" section and also added related references at the end of the literature.

4. In table 3, unrelated description was found at the bottom.
Response) We appreciate the kindness of the reviewer. We removed these unrelated descriptions.

5. Proportion of this article is imbalance, especially discussion is too diffuse. These should be summarized more briefly.
Response) According to the reviewer's suggestion, we summarized and shortened the "Discussion" section.

The comments offered by reviewers #1 and #2 have helped us to formulate what we believe a strong paper. We appreciate these thoughtful comments and hope that our responses and, in particular, our revisions will allow this paper to achieve a sufficient priority for publication in "**Pathology, Research and Practice**".

Thank you very much for your generous consideration.

Sincerely,

Tsuyoshi Saito, M.D., Ph.D.
Corresponding author,
Department of Human Pathology,

Juntendo University, School of Medicine.
Tokyo, Japan

A mutation spectrum that includes *GNAS, KRAS and TP53* may be shared by mucinous neoplasms of the appendix

Kieko Hara[1], Tsuyoshi Saito[1], Takuo Hayashi[1], Alkam Yimit[1], Michiko Takahashi[1], Keiko Mitani[1], Makoto Takahashi[2], Takashi Yao[1]

[1] Department of Human Pathology, Juntendo University School of Medicine

[2] Department of Coloproctological Surgery, Juntendo University School of Medicine

Correspondence to: Tsuyoshi Saito, MD, PhD

Department of Human Pathology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo, Japan

E-mail: tysaitou@juntendo.ac.jp

Tel: +81-3-3813-3111

Fax: +81-3-3813-3428

Keywords: appendiceal mucinous neoplasm; *TP53*; *GNAS*; *KRAS*; mismatch repair gene; mutation

Running title: Genetic alterations in appendiceal mucinous neoplasms

Abstract

Appendiceal mucinous tumors (AMTs) are classified as low-grade appendiceal mucinous neoplasms (LAMNs) or mucinous adenocarcinomas (MACs), although their carcinogenesis is not well understood. As somatic activating mutations of *GNAS* are considered to be characteristic of LAMNs while *TP53* mutations have been shown to be specific to MACs, MACs are unlikely to result from transformation of LAMNs. However, emerging evidence also shows the presence of *GNAS* mutations in MACs. We examined 16 AMTs (11 LAMNs and 5 MACs) for genetic alterations of *GNAS, KRAS, BRAF, TP53, CTNNB1,* and *TERT* promoter in order to elucidate the possibility of a shared genetic background in the two tumor types. Extensive histological examination revealed the presence of a low-grade component in all cases of MAC. *GNAS* mutations were detected in two LAMNs and in one MAC, although the *GNAS* mutation in this MAC was a nonsense mutation (Q227X) expected not to be activating mutation. *TP53* mutations were detected in three LAMNs; they were frequently detected in MACs. *KRAS* mutations were detected in three LAMNs and three MACs, and *CTNNB1* mutations were detected in two LAMNs. *KRAS* mutation and activating mutation of *GNAS* occurred exclusively in AMTs. *BRAF* and *TERT* mutations were not detected. Overexpression of p53 was observed in only two MACs, and p53 immunostaining clearly discriminated the high-grade lesion from a low-grade component in one. These findings suggest that p53 overexpression plays an important role in the carcinogenesis of AMTs and that, in addition to mutations of *GNAS*, *KRAS* and *TP53* alterations might be shared by AMTs, thus providing evidence for the possible progression of LAMNs to MAC.

Introduction

The classification and diagnosis of appendiceal mucinous tumors (AMTs) has been controversial for several decades. The latest classification of AMTs by the World Health Organization (WHO) is based on the classification of Misdraji et al., which was devised by reviewing 107 AMTs and classifying them as low-grade appendiceal mucinous neoplasms (LAMNs) or mucinous adenocarcinomas (MACs) according to their architectural and cytological features [1]. LAMNs are characterized by the replacement of normal appendiceal mucosa with a villiform, undulating, or flat mucinous epithelial proliferation [2]. Frequently, the wall of the appendix becomes increasingly fibrotic and hyalinized, and the appendix may become transformed into a fibrotic cyst that is lined by attenuated neoplastic mucinous epithelium [2]. Rarely, the neoplastic epithelium directly abuts the hyalinized cyst wall [2]. In contrast, MACs demonstrate destructive invasion of the appendiceal wall, high-grade cytological atypia, or complex epithelial proliferation [1]. However, there may be difficulties in differentiating LAMNs from MACs, because structural and cytological atypia of tumors sometimes varies within a single lesion. In practice, we frequently encounter MACs containing high-grade atypia together with residual luminal mucinous tumor that resembles LAMNs.

The molecular basis for the development of AMTs remains unclear. It has been shown that *KRAS* is frequently mutated in the majority of LAMNs and MACs [3,4], whereas *GNAS* mutation is only observed in LAMNs [5]. Overexpression of p53 is reported to be rare in appendiceal tumors [4,6], although *KRAS* mutation and p53 overexpression can be seen in half of pseudomyxoma peritonei (PMP) cases of appendiceal origin [7]. Furthermore, microsatellite instability is rare in appendiceal

carcinoma, and hypermethylation is not a mechanism for genetic instability in these tumors [8] although some hyperplastic polyps and sessile serrated adenomas of the appendix show decreased expression of MLH1 and *BRAF* mutation is more common in serrated polyps [9].The Wnt signaling pathway that involves β-catenin plays a critical role in colorectal carcinogenesis, and approximately 90% of cases of colorectal carcinoma express nuclear β-catenin. On the other hand, the impact of the activation of the Wnt signaling pathway on the tumorigenesis of AMTs has not been adequately elucidated, although it has been shown that MACs show a lower percentage (12%) of nuclear β-catenin expression [10]. Mutations in the *TERT* promoter were recently identified in several tumors [11,12], and were reported to be associated with tumor aggressiveness and poor patient survival [13,14]. *TERT* promoter mutations were reported to be very rare in gastrointestinal stromal tumors [15], and Killela et al. did not find mutations of the *TERT* promoter in an analysis of 22 cases of colorectal adenocarcinoma [16]. However, there has so far been no report of *TERT* promoter mutations in AMTs.

In this study, we examined genetic alterations of *GNAS, KRAS, BRAF, TP53, CTNNB1,* and *TERT* promoter in LAMNs and MACs, in order to better understand the underlying molecular pathogenesis/carcinogenesis of AMTs.

Materials and Methods

Sample preparation

Sixteen cases of AMTs that were surgically resected at Juntendo University Hospital, Tokyo, Japan were collated from the pathological files of the Department of Pathology. The clinicopathological records of the 16 patients were reviewed. All tumor samples were obtained from their primary sites. This study was approved by the Ethics Committee of Juntendo University School of Medicine.

Pathological evaluation

Hematoxylin-and-eosin (H&E)-stained slides were reviewed by four pathologists (KH, TS, TH, and TY). The AMTs were divided into two groups based upon their architectural complexity and degree of cytological atypia. Tumors that demonstrated low-grade cytological atypia (nucleomegaly, nuclear stratification, rare mitotic figures, and single-cell necrosis) and minimal architectural complexity (villiform, flat epithelial proliferation, and small papillary excrescences) were classified as LAMNs (Fig. 1A). Tumors were classified as MACs if they demonstrated any of the following: destructive invasion of the appendiceal wall, high-grade cytological atypia (extensive full-thickness nuclear stratification, vesicular nuclei, marked nuclear membrane irregularities, prominent nucleoli, and brisk mitotic activity), or complex epithelial proliferation (complex papillary fronds and cribriform glandular spaces) (Figs. 1B,C). This classification was proposed by Misdraji et al. in 2003 [1], and later adapted to the WHO classification [17]. In addition, we exhaustively evaluated all slides of each MAC in order to ascertain whether it contained lesions resembling LAMNs (a low-grade component). Representative sections that included both tumor tissue and non-tumor tissue were

selected for further analysis.

Immunohistochemical analysis

Immunohistochemical staining was performed on 4-μm-thick formalin-fixed, paraffin-embedded tissue sections using antibodies directed against the following human proteins and at the dilutions indicated: MLH-1 (1:50; G168-15, Diagnostic Biosystems, Pleasanton, CA, USA), MSH2 (1:50; PC57 Rabbit polyclonal, Calbiochem, La Jolla, CA, USA), p53 (1:50; PAb1801, Leica Biosystems, Nussloch, Germany), and β-catenin (1:200; 14/beta-Catenin, BD Transduction Laboratories, Franklin Lakes, NJ, USA). Antigen was retrieved by autoclaving slides, in Tris-EDTA buffer (pH 9.0) for hMLH-1 and MSH2 or in citrate buffer (pH 6.0) for p53 and β-catenin, and detected following incubation with the primary antibody overnight at 4°C using an EnVision™ kit (Dako, Grostrup, Denmark).

The immunohistochemical expressions of MLH1 and MSH2 were considered as being preserved if there was nuclear staining of neoplastic cells. The complete absence of nuclear staining of neoplastic cells despite internal control positivity (stromal cells, lymphocytes, and non-neoplastic crypt epithelium, if present) was regarded as complete loss of expression. The immunohistochemical expression of β-catenin was evaluated with respect to membranous and/or nuclear expression in epithelial cells. Membranous staining without nuclear expression was considered normal. Nuclear β-catenin accumulation in >5% of tumor cells was considered to represent positive nuclear staining. Staining of p53 was interpreted as either positive (moderate to strong staining) or negative (negative, weak staining). At least one thousand tumor cells were evaluated to calculate p53 labeling index (LI). As for the cases containing less than 1000 cells in the

representing section, all tumor cells in the representative section was evaluated.

Polymerase chain reaction and mutational analysis

Genomic DNA of each case was extracted from tumor-derived and non-tumor-derived tissue that was manually microdissected from 10-μm-thick unstained sections. *GNAS, KRAS, BRAF, TP53, CTNNB1,* and *TERT* promoter mutations were examined using PCR followed by direct sequencing. Primer sequences used in this study are listed in Table 1. As for MAC cases found to have genetic alterations, each component of high-grade and low-grade areas was microdissected using laser-capture microdissection system (LMD6500, Leica microsystems, Tokyo, Japan) for further PCR examination to determine from which component the genetic alterations were derived.

Results

Clinicopathological and histological analyses

The clinicopathological features of the 16 AMTs are summarized in Table 2; they were from two male and 14 female patients, with a mean age of 58 years and ranging from 36–86 years. There was a female predominance in this series, although we confirmed that bilateral ovaries were intact in all but three cases by intraoperative and histological findings. The remaining three cases were PMP cases with synchronous mucinous neoplasms of both appendix and ovary at the initial surgery, and were considered as appendiceal origin [18,19]. A histological re-evaluation revealed that the 16 AMTs comprised 11 LAMNs and 5 MACs. In this study, none of the 5 MACs contained areas with goblet-like mucinous cells/signet ring cells. In addition, the extensive review of all available slides revealed that all cases of MAC contained a focal area of a low-grade component. Low-grade components were observed at the edge of the tumor or the surface of the crypts. The bottom crypts showing high-grade atypia smoothly differentiated into the surface crypts with minimal atypia. In one case (Case#15), glands with high-grade atypia were clearly demarcated from low-grade components within the tumor (Figs. 1A,B,D).

The mean age of patients with MAC was 56.8 years, and it was 57.9 years for patients with LAMNs. There was no apparent difference between the two types of tumor with regard to tumor size (MAC: mean diameter = 56.6 mm; LAMN: mean diameter = 60.7 mm). Lymphovascular invasion was not observed in any of the cases. The tumors were confined within the appendix in 62.5% of cases. Lymph-node metastasis was observed in one out of eight informative cases, which was patient with

MAC, although an exploration of regional lymph nodes was not performed in eight of the 16 cases. Four patients (three with LAMNs and one with MAC) developed distant metastases (of the ovary and/or omentum). Tumor cells were cytologically positive in the ascites of one LAMN patient. Four cases of LAMN and one case of MAC had developed PMP. One patient with LAMNs relapsed soon after surgery. One patient with MAC died of the disease 20 months after surgery.

Immunohistochemistry of MLH1, MSH2, β-catenin and p53

All of the 16 AMTs showed preserved expression of MLH1 and MSH2. Nuclear expression of β-catenin was observed in 62.5% (10/16) of cases, being 73% (8/11) for LAMNs and 40% (2/5) for MACs. p53 LI was higher in MAC (mean: 22%) compered to LAMN (mean: 0.9%). High p53 LI over 10% was observed in two cases of MAC and immunohistochemical staining of p53 discriminated high-grade area from low-grade areas in a case of MAC (Figs. 2A-F). Complete expression loss of p53 (LI: 0%) was observed in 55% (6/11) for LAMNs and 40% (2/5) for MACs.

Genetic alterations

GNAS mutations were identified in three cases (two LAMNs and one MAC); the two LAMNs carried mutations of codon 201 (Fig. 3A), whereas a MAC had a nonsense mutation at codon 227 (Q227X; Fig. 3B) expected not to be activating one. *KRAS* mutations were identified in six cases, including three LAMNs (Fig. 4A); one MAC case with *KRAS* mutation also harbored a *GNAS* mutation expected to be non-activating one. Thus, the activating mutation of *GNAS* and mutation of *KRAS* was mutually exclusive in AMTs. *CTNNB1* mutations were found in two cases of LAMN and both cases showed nuclear expression of β-catenin (Fig. 4B). Mutations in *TP53* were detected in three of the 11 LAMNs

and three of the five MACs (Fig. 4C). Two MACs (cases #13 and 15) harbored multiple point mutations in *TP53*. In these cases, one of the multiple genetic alterations (G245D in Case#13 and R249G in Case#15) was observed only in each high-grade component (Fig. 4C). *BRAF* or *TERT* promoter mutations were not detected.

Discussion

Somatic activating mutations of *GNAS* have been reported in several neoplasms, such as pituitary adenomas [20], fibrous dysplasia [21], intramuscular myxomas [22], and villous adenomas of the colon and rectum [23]. Furthermore, *GNAS* mutations have been reported in low-grade malignancies, including appendiceal tumors, and intraductal papillary neoplasms of the pancreas [24] and bile duct [25], and thus have been considered as characteristic of low-grade tumors. Nishikawa et al. found activating *GNAS* mutations in 50% of LAMNs but in none of the three examined cases of MAC [5]. A higher incidence of activating *GNAS* mutations in LAMNs and low-grade mucinous carcinomatosis peritonei has also been reported [26]. From the standpoint of cancer genetics, MAC is unlikely to be transformed from LAMN, considering the above-mentioned quite different frequency of *GNAS* mutation in LAMN versus MAC [5]. However, the number of MAC cases that have been examined for *GNAS* mutation to date is too small to enable the frequency of *GNAS* mutation in this tumor to be concluded [5]. A recent study demonstrated that 35% of LAMNs harbored *GNAS* mutations, whereas 37% of high-grade MACs without a signet ring cell component carried *GNAS* mutations [27]. Another recent report also described a case of high-grade appendiceal mucinous neoplasm where the tumor contained *GNAS* mutations within a low-grade component [26]. From an extensive review of cases in this study, we found that all five cases of MAC had a low-grade component. Although the frequency was relatively low compared to that in previous studies [5,26], we found *GNAS* mutations in two out of 11 cases of LAMN. Furthermore, *GNAS* mutation was found in a case of MAC (Case#12). A *GNAS* mutation observed in this case of MAC

where there was simultaneous *KRAS* mutation was not of the hotspot-type observed at codon 201, but a nonsense mutation at codon 227, thereby expected not to be an activating mutation. Moreover, *TP53* mutations were found in three out of the 11 LAMNs examined here, while frequent *TP53* mutations were previously identified in MAC [28]. Out of five MACs, 2 cases (Case#13 and 15) harbored multiple *TP53* mutations. Among them, G245D in Case#13 and R249G in Case#15 were proven to be derived from the high-grade component of each tumor by the LCM based sequence analysis. Codons 245 and 249 in *TP53* are both known as common mutation spot in several tumors, including colorectal cancer, but this is the first report in appendiceal tumor [29]. Though there is no report that codon 245 and 249 mutants are active [29], these two cases (#13 and 15) showed high p53 LI of 14% and 95%, respectively. Furthermore, in Case#15, p53 immunohistochemistry clearly discriminated between high-grade and low-grade components. These findings suggest the possible involvement of p53 during the progression of LAMN to MAC. Collectively, these findings are the first evidence that confirmed co-existence of genetically distinct low-grade and high-grade components within AMTs and possible transformations of MAC from LAMN within a single tumor, different from those that occurred during the recurrent process of the tumor [30].

In this study, nuclear β-catenin expression was observed in 63% of all AMTs (73% of LAMNs and 40% of MACs); the incidence in MACs is rather higher than a previously reported value of 12% [10]. A higher incidence (60%) of loss of β-catenin expression in the cytoplasmic membrane has also been reported in MAC, compared to approximately 10% of LAMNs that showed loss of β-catenin expression [31]. In this study, two cases of *CTNNB1* mutation were observed in LAMNs that showed

nuclear β-catenin expression. Interestingly, neither of these cases harbored a *GNAS* mutation, suggesting the possibility that activation of the Wnt signaling pathway contributes in part to the tumorigenesis of LAMNs. Regarding the latter point, we have recently reported that *GNAS* mutations occur as an alternative mechanism of Wnt pathway activation in a small subset of gastric adenocarcinomas of fundic gland type that show frequent activation of the Wnt signaling pathway [32]. Crosstalk between G-protein and Wnt signaling pathways might also occur in AMTs, especially LAMNs, since we observed a lower frequency of *GNAS* mutation in LAMNs compared to a previous report [5].

It has been shown that the p53 LI in appendiceal adenoma/carcinoma is lower than that in colorectal adenoma/carcinoma [6]. It was unanticipated that *TP53* mutations were frequently observed in AMT cases in this study (in three of 11 LAMNs and three of five MACs). Generally, *TP53* mutations alter the conformation of p53, leading to a more stable protein that accumulates in the nuclei of tumor cells; the accumulation of p53 can be detected by immunohistochemistry. However, various false-positive and false-negative associations between *TP53* mutation and immunohistochemical status have been reported [33]. Our series contains 8 cases with complete loss of p53 expression, in three of which cases harbored p53 mutations. Since nonneoplastic appendiceal tissues show sporadic nuclear immunopositivity of p53 protein in low frequency (about 2%) [34], these cases with complete loss of p53 expression may represent loss of p53 function caused by *TP53* mutations with possible deletion of another allele which is not identified in our PCR analysis. Furthermore, it has been shown that some p53 mutant variants have no effect on cell

proliferation or cell viability [35], and do not influence the transcriptional activity of p21/WAF1, MDM2, or Bax promoters [36]. We suggest that the *p53* mutations we detected in p53-negative cases do not induce conformational changes that inhibit protein degradation, resulting in false-negative findings by immunohistochemistry. In this study, overexpression of p53 associated with *p53* mutation was observed in two cases of MAC, and one of the two patients with MAC died of the disease. These findings are consistent with the previously reported observation that the level of p53 overexpression increases according to the tumor grade in AMTs [7] and that p53 overexpression in MACs predicts adverse clinical outcomes [31]. Overexpression of p53 was observed in 44.3% of patients with PMP, and the incidence of p53 overexpression was significantly higher in high-grade PMP [7]. In this study, we encountered five cases of PMP comprising of four cases of LAMN and one case of MAC. However, only one case of LAMN harbored a *TP53* mutation, and none of these cases showed p53 overexpression.

High-level microsatellite instability (MSI-high) is found in approximately 15% of all colorectal adenocarcinomas and in at least 20% of right-sided bowel cancers [8]. In contrast, MSI-high in appendiceal adenocarcinoma is reported to be rare with the prevalence of MSI being approximately 3% [8]. Of our series of 16 AMTs, there was no case with MLH1 and MSH2 protein loss, consistent with the previous report [8].

A next-generation sequencing study recently identified the mutational spectra of AMTs [28]. Among 15 LAMNs, *KRAS* and *GNAS* mutations were each found in eight cases, and six LAMNs contained both *KRAS* and *GNAS* mutations [28]. In addition, *TP53* and *SMAD4* were identified as the

most frequently mutated genes in MACs [28]. These findings are in part consistent with—in part contradictory to—our results. An interesting finding in our study is that *KRAS* mutation was shared but *GNAS* and *KRAS* mutations exclusively occurred in AMTs, although the frequencies of mutation in these genes were quite lower than the previously reported values [5,28]. Further large-series studies are necessary in order to elucidate the true mutation spectra in AMTs, especially MAC.

In conclusion, our histological and genetic study shows that LAMNs and MACs might share a mutation spectrum that includes *KRAS* and *TP53*, and that some MACs can arise via transformation of LAMNs, in addition to their de novo occurrence.

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Conflict of Interest

The authors declare that there are no conflicts of interest to disclose.

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Figure Legends

Figure 1 Histological and immunohistochemical feature of appendiceal mucinous tumor.

(A) Low-grade mucinous neoplasm (LAMN) composed of mucinous epithelium with minimal atypia (x400).

(B) Mucinous adenocarcinoma (MAC) shows high-grade cytological atypia and complex epithelial proliferation (x400).

(C) Destructive invasion of mucinous adenocarcinoma. Irregular glands invade from the lumen (asterisk) into the appendiceal wall.

(D) Histology of mucinous adenocarcinoma (MAC) with a low-grade component (Case#15). Tumorous glands with a complex structure and high nucleo/cytoplasmic ratio (left side) are shown, accompanied by a low-grade component that resembles low-grade appendiceal mucinous neoplasms (LAMNs; right side); the two components are clearly demarcated. Figure 1A and 1B represent high power magnification of low-grade component and high-grade component of Case#15, respectively.

Figure 2. Differential p53 expression in a case of MAC with a low-grade component (Case#15).

(A) Low-power view shows coexistence of mucinous adenocarcinoma (right) and low-grade component (left). (B) Low-power view of p53 immunohistochemistry clearly demarcated the high-grade and low-grade components. (C, D) Tumor cells have high-grade atypia (C) and show diffuse and strong expression of p53 (D). (E, F) In contrast, the low-grade component (E) does not express p53 (F). (original magnification: A, B: x40, C-F: x400)

Figure 3: Genetic alterations in appendiceal mucinous tumors.

GNAS mutation in a case of low-grade appendiceal mucinous neoplasm (LAMN) (A) and in a case of MAC (B).

Figure 4: Genetic alterations in appendiceal mucinous tumors.

KRAS mutation in a case of LAMN (A), *CTNNB1* mutation in a case of LAMN (B). DNA from corresponding non-tumorous tissue showed the wild-type sequence in each case. *TP53* mutation in a case of MAC (C). Note that mutation is seen only in high-grade component.

Table 1

Table 1: Sequences of the PCR primers and anticipated product size

Gene	Forward primer	Reverse primer	Product size (bp)
GNAS exon 8	ACTGTTTCGGTTGGCTTTGGTGA	AGGGACTGGGGTGAATGTCAAGA	189
GNAS exon 9	GACATTCACCCCAGTCCCTCTGG	GAACAGCCAAGCCCACAGCA	136
KRAS	AGGCCTGCTGAAAATGACTG	GGTCCTGCACCAGTAATATGCA	164
BRAF	TGCTTGCTCTGATAGGAAAATG	CTGATGGGACCCACTCCAT	143
CTNNB1	CCAATCTACTAATGCTAATACTG	CTGCATTCTGACTTTCAGTAAGG	310
p53 exon 5	CTCTTCCTACAGTACTCCCCTGC	GCCCCAGCTGCTCACCATCGCTA	211
p53 exon 6	GATTGCTCTTAGGTCTGGCCCCTC	GGCCACTGACAACCACCCTTAACC	182
p53 exon 7	GCTTGCCACAGGTCTCCCCAAG	TGGCAAGTGGCTCCTGAC	188
p53 exon 8	TGGTAATCTACTGGGACGGA	GCTTAGTGCTCCCTGGGGGC	134
p53 exon 9	GCCTCTTTCCTAGCACTGCCCAAC	CCCAAGACTTAGTACCTGAAGGGTG	102
hTERT promoter	AGTGGATTCGCGGGCACAGA	CAGCGCTGCCTGAAACTC	235

Table 2

Table 2: Clinicopathological feature of 16 appendiceal mucinous tumors

Case #	Path Diag.	Age	Sex	Tumor size (mm)	T	N	M	Disseminated / metastasis site	Pseudo -myxoma peritonei	ly	v	Stage	Disease free survival	Relapse	Follow up months	Prognosis
1	LAMNs	49	F	60	2	c0	c0	-	-	0	0	I	8	-	8	NED
2	LAMNs	61	F	30	is	0	c0	-	-	0	0	0	70	-	70	NED
3	LAMNs	36	F	41	is	c0	c0	-	-	0	0	0	59	-	59	NED
4	LAMNs	57	F	57	4a	c0	1b	Lt.ovary, omentum	+	0	0	IVC	48	-	48	AWD
5	LAMNs	61	F	25	4a	c0	1b	Both ovaries, omentum	+	0	0	IVC	14	+	17	AWD
6	LAMNs	73	M	95	3	c0	c0	-	-	0	0	IIA	25	-	25	NED
7	LAMNs	86	F	115	3	c0	c0	-	-	0	0	IIA	24	-	24	NED
8	LAMNs	47	F	70	4a	c0	c0	-	-	0	0	IIB	29	-	29	NED
9	LAMNs	46	F	30	4a	c0	1b	Rt.ovary, omemtum	+	0	0	IVC	24	+	25	AWD
10	LAMNs	44	F	90	is	0	c0	-	-	0	0	0	11	-	11	NED
11	LAMNs	77	F	55	4a	0	c1a	Ascites	+	0	0	IVA	1	-	1	AWD
12	MAC	60	F	95	3	0	c0	-	-	0	0	IIA	127	-	127	NED
13	MAC	43	F	60	4a	0	c0	-	-	0	0	IIB	4	+	20	DOD
14	MAC	48	M	38	4a	0	1a	Omentum	+	0	0	IVB	30	-	30	AWD
15	MAC	54	F	40	4a	0	c0	-	-	0	0	IIB	8	-	8	NED
16	MAC	79	F	50	4b	1	c0	-	-	0	0	IIIB	11	-	11	NED

F: Female, M: Male, NED: No evidence of disease, DOD: Dead of disease, AWD: Alive with disease
cN0: lymph nodes are not pathologically examined, but clinically no evidence of lymph node metastasis.
cM0: Clinically no evidence of metastasis.

Table 3

Table 3: Results of Immunohistochemistry and genetic analysis in 16 appendiceal mucinous tumors

		IHC				Mutation analysis					
Case #	Path Diag.	MLH1 expression	MSH2 expression	p53 LI (%)	β-catenin nuclear expression	*GNAS*	*KRAS*	*BRAF*	*CTNNB1*	*TP53*	*hTERT*
1	LAMNs	+	+	0	-					C141R	
2	LAMNs	+	+	0	-		Q25X				
3	LAMNs	+	+	0	+	R201C				D184G	
4	LAMNs	+	+	0	+						
5	LAMNs	+	+	0.5	-					P250S	
6	LAMNs	+	+	2.2	+				T40A		
7	LAMNs	+	+	2	+		D30Y		T40A		
8	LAMNs	+	+	0	+						
9	LAMNs	+	+	0	+		G12S				
10	LAMNs	+	+	6.8	+	R201H					
11	LAMNs	+	+	0.5	+						
12	MAC	+	+	0	-	Q227X	G15D			D206G	
13	MAC	+	+	14	-		G12D			A161V, E171K **G245D**, A275V	
14	MAC	+	+	0	+						
15	MAC	+	+	95	+		G12V			**R249G**, S260T	
16	MAC	+	+	1	-						

High-grade area specific mutations are shown in bold.

Figure 1

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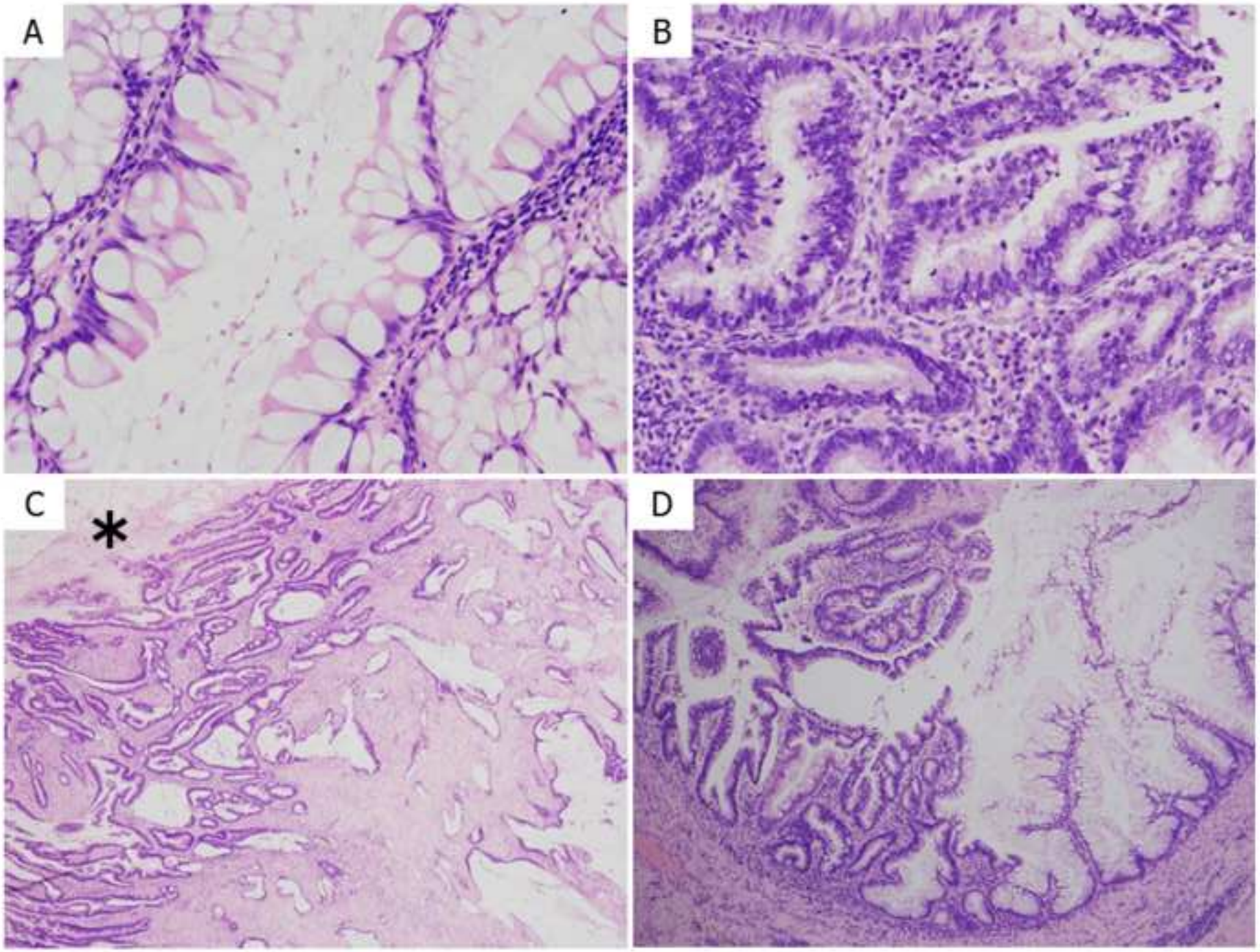

Figure 2

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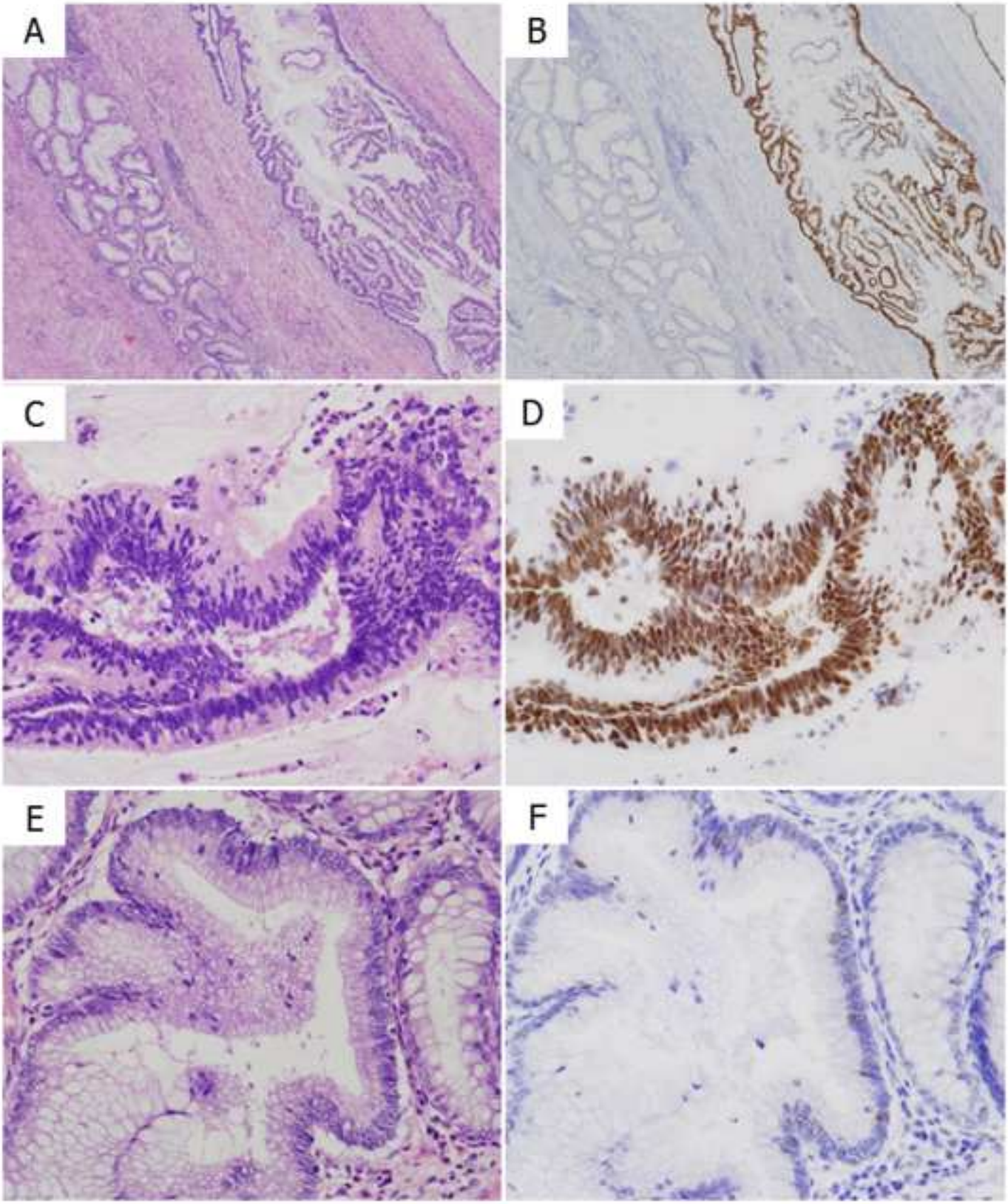

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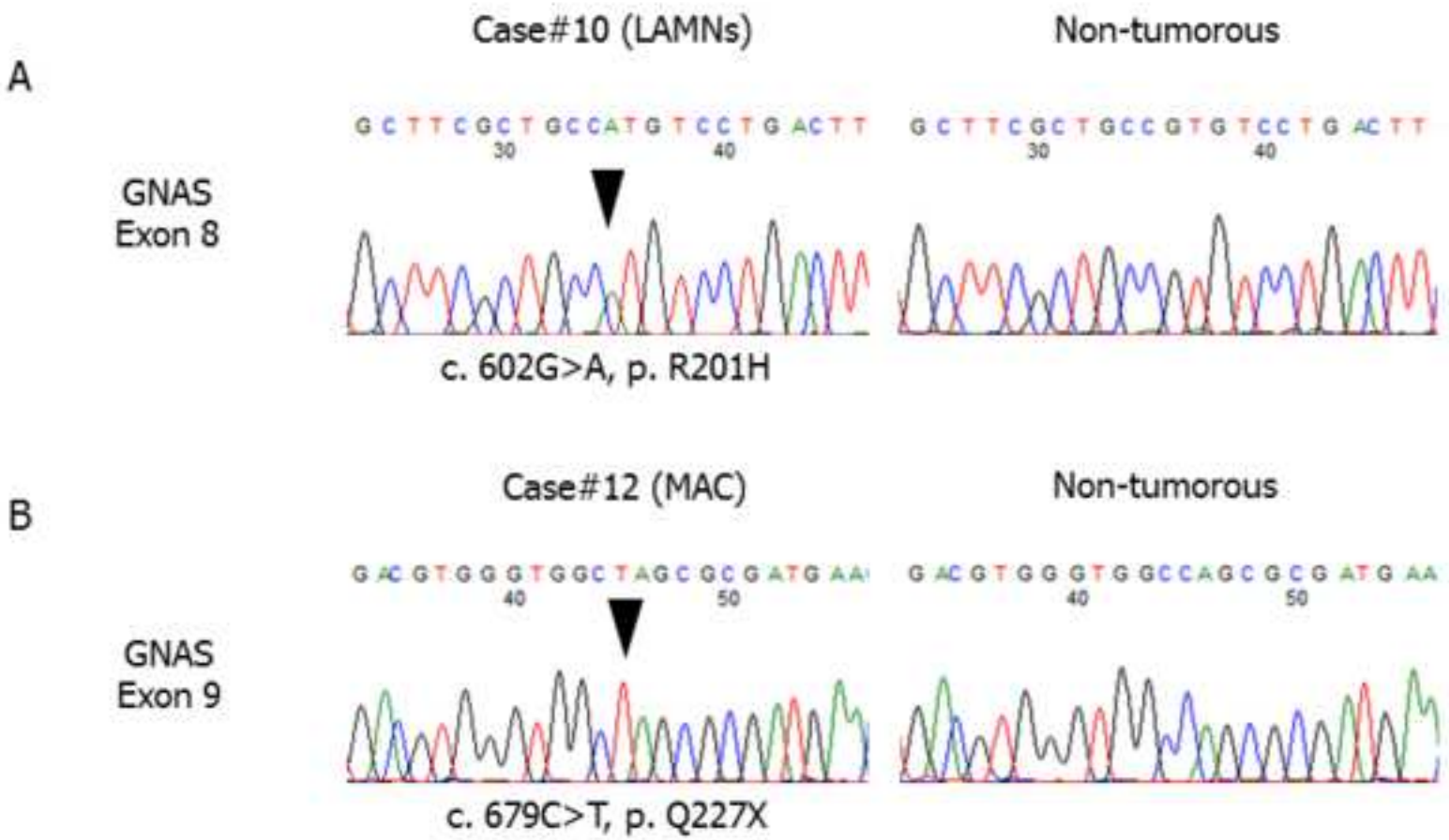

Figure 4

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