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Clinical Cancer Research

Molecular Profiling of Appendiceal Adenocarcinoma and Comparison with Right-sided and Left-sided Colorectal Cancer

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Abstract

Purpose: The natural history and prognosis of appendiceal adenocarcinomas differ from those of adenocarcinomas arising in other large bowel sites. We aimed to compare the molecular profiles exhibited by appendiceal adenocarcinomas and colorectal cancers, or between the histopathologic subtypes of appendiceal adenocarcinoma.

Experimental Design: A total of 183 samples from appendiceal adenocarcinoma [46 adenocarcinoma, not otherwise specified (NOS), 66 pseudomyxoma peritonei (PMP), 44 mucinous adenocarcinoma (MU), and 27 signet ring cell carcinoma (SR)], 994 from right-sided colorectal cancer (R-CRC), and 1,080 from left-sided CRC (L-CRC) were analyzed by next-generation sequencing (NGS) and IHC markers. Microsatellite instability (MSI) and tumor mutational burden (TMB) were tested by NGS, and programmed death ligand 1 (PD-L1) by IHC.

Results: We observed high mutation rates in appendiceal adenocarcinoma samples for *KRAS* (55%), *TP53* (40%), *GNAS* (31%), *SMAD4* (16%), and *APC* (10%). Appendiceal adenocarcinoma exhibited higher mutation rates in *KRAS* and *GNAS*, and lower mutation rates in *TP53*, *APC*, and *PIK3CA* (6%) than colorectal cancers. PMP exhibited much higher mutation rates in *KRAS* (74%) and *GNAS* (63%), and much lower mutation rates in *TP53* (23%), *APC* (2%), and *PIK3CA* (2%) than NOS. Alterations associated with immune checkpoint inhibitor response (MSI-high, TMB-high, PD-L1 expression) showed similar frequency in appendiceal adenocarcinoma compared with L-CRC, but not R-CRC, and those of NOS were higher than other subtypes of appendiceal adenocarcinoma and L-CRC.

Conclusions: Molecular profiling of appendiceal adenocarcinoma revealed different molecular characteristics than noted in R-CRC and L-CRC, and molecular heterogeneity among the histopathologic subtypes of appendiceal adenocarcinoma. Our findings may be critical to developing an individualized approach to appendiceal adenocarcinoma treatment.

Introduction

The natural history and prognosis of appendiceal adenocarcinomas differs from those of adenocarcinomas arising in other large bowel sites (1, 2). Compared with colorectal adenocarcinoma, appendiceal adenocarcinoma more commonly are associated with peritoneal dissemination and increased mucin production, and are distinguished by the diagnostic classification as pseudomyxoma peritonei (PMP; refs. 3, 4). Histologic variants of appendiceal epithelial neoplasia include low- and high-grade mucinous neoplasms, goblet cell tumors, neuroendocrine neoplasms, adenoma, and adenocarcinomas (common colonic type, mucinous type, and signet ring cell carcinoma; refs. 2, 5). The primary treatment for these neoplasms is surgical resection (6). Also in PMP, cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) are associated with favorable outcome when conducted in specialized centers of excellence (6–8). However, both patient selection and the expertise of the treating team are critical to best outcomes. Furthermore, in patients with metastatic appendiceal adenocarcinoma or recurrent PMP, the most effective chemotherapy and molecular targeted therapy is controversial. At present, patients with appendiceal adenocarcinoma typically receive therapies approved for colorectal cancer (3, 9, 10), although their efficacy, especially in low-grade tumors, suggests lower vulnerability of these slow growing tumors to conventional therapies as compared with colorectal cancers (11, 12).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Compared with colorectal cancer, appendiceal adenocarcinoma is more associated with peritoneal dissemination and increased mucin production. However, patients with appendiceal adenocarcinoma typically receive therapies approved for colorectal cancer in spite of lower vulnerability. The molecular profiling of appendiceal adenocarcinoma and comparing it with those of right-sided colorectal cancer (R-CRC) and left-sided colorectal cancer (L-CRC) are needed for a personalized treatment strategy. We found that appendiceal adenocarcinoma had higher mutation rates in *GNAS* and *KRAS*, and lower mutation rates in *TP53*, *APC*, and *PIK3CA* than colorectal cancer, and gene mutation rates differed among the histopathologic subtypes. Although the immune profile results (microsatellite instability, tumor mutational burden, and programmed death-ligand 1 expression status) of appendiceal adenocarcinoma were similar to those of L-CRC (not to those of R-CRC), not otherwise specified appendiceal adenocarcinoma had higher overexpression of the markers than did other subtypes of appendiceal adenocarcinoma and L-CRC. This molecular profiling may support developing a personalized treatment strategy in patients with appendiceal adenocarcinoma.

Molecular profiling has been used effectively to identify novel treatment options for malignant diseases. In colorectal cancer, genetic profiling, such as *RAS* and *BRAF* alterations, has suggested phenotypic clustering with profiles that are prognostic as well as predictive of different susceptibilities to molecularly targeted therapeutics (13). In addition, assessment of the presence or absence of microsatellite instability (MSI), tumor mutational burden (TMB), and programmed death ligand 1 (PD-L1) expression can be predictive of the likelihood that an individual patient will see a tumor response to treatment with immune checkpoint inhibitors (14). With molecular profiling, differences in the genetic and immune characteristics between the right-sided (R-CRC) and left-sided CRC (L-CRC) have been reported and have developed a personalized treatment strategy in colorectal cancer (15). On the contrary, studies on molecular profiling of appendiceal adenocarcinoma have been handicapped because of the rarity of appendiceal adenocarcinoma and assay failures in available PMP samples because of low cellularity (2), and these analyses have provided limited genetic data (16, 17). Although the prognosis varies according to the histopathologic subtypes of appendiceal adenocarcinoma (18), studies are lacking that correlate molecular profiles with appendiceal adenocarcinoma subtypes (19). Identification of molecular alterations of appendiceal adenocarcinoma is critical for the development and selection of more effective therapeutic strategies.

We performed molecular profiling of appendiceal adenocarcinoma and compared it with those of R-CRC and L-CRC, using integrated data within a total of 183 samples from appendiceal adenocarcinoma, 994 from R-CRC, and 1,080 from L-CRC. Our analysis demonstrates that histopathologic and molecular classification of appendiceal adenocarcinoma could be a key step toward personalized treatment strategies of appendiceal adenocarcinoma.

Materials and Methods

Tumor samples

Figure 1 summarizes the workflow of this study. Consecutive appendiceal cancer ($N = 224$) and colorectal cancer ($N = 4,600$) cases submitted to a commercial CLIA-certified laboratory from April, 2015 to January, 2018 were retrospectively analyzed for their molecular alterations. Formalin-fixed paraffin-embedded (FFPE) samples were sent for analysis from treating physicians around the world. The tissue diagnoses were submitted on the basis of pathologic assessment of physicians who requested

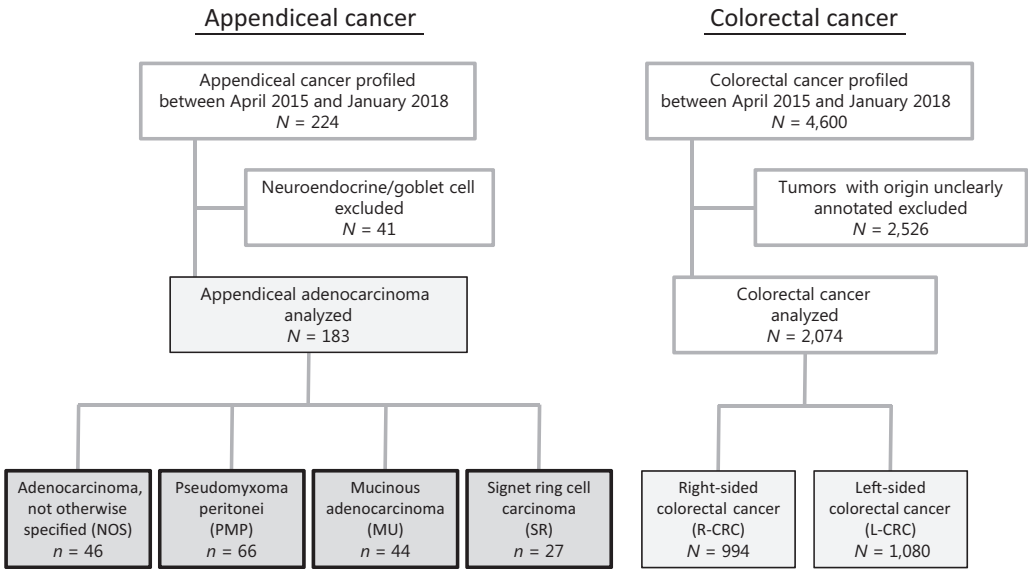

Figure 1.
CONSORT diagram. Flowchart showing the inclusion/exclusion criteria in this study.

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the assays and were further verified by a board-certified oncological pathologist at the Caris laboratory. A total of 183 appendiceal adenocarcinomas were analyzed and 41 tumors of neuroendocrine/goblet histology were excluded from their analysis. Included in the appendiceal adenocarcinoma cohort were 66 PMP, 44 mucinous adenocarcinoma (MU), and 27 signet ring cell carcinoma (SR). Forty-six tumors were determined to be adenocarcinoma, not otherwise specified (NOS) as no additional detailed histologic features were noted (Supplementary Fig. S1). R-CRC defined as tumors arising from the cecum to the hepatic flexure and transverse colon ($N = 994$) and L-CRC defined as those arising from the splenic flexure to the rectosigmoidal colon ($N = 1,080$) were analyzed; while tumors with origin unclearly annotated ($N = 2,526$) were excluded. Samples taken from original tumor sites were considered primary tumors and samples taken from organs other than the primary were considered metastases. Tissues were profiled by next-generation sequencing (NGS) and IHC analysis using Caris Molecular Intelligence. Human subjects were anonymized prior to analysis. This study was conducted in accordance with guidelines of the Declaration of Helsinki, Belmont report, and U.S. Common rule. In keeping with 45 CFR 46.101(b)(4), this study was performed utilizing retrospective, deidentified clinical data. Therefore this study is considered IRB-exempt and no patient consent was necessary from the subject.

NGS

NGS was performed on genomic DNA isolated from FFPE tumor samples using the NextSeq platform (Illumina, Inc.). A custom-designed SureSelect XT assay was used to enrich 592 whole-gene targets (Agilent Technologies). All variants were detected with >99% confidence based on allele frequency and amplicon coverage, with an average sequencing depth of coverage of 750 and an analytic sensitivity of 5%. Prior to molecular testing, tumor enrichment was achieved by harvesting targeted tissue using manual microdissection techniques. Genetic variants identified were interpreted by board-certified molecular geneticists and categorized as "pathogenic," "presumed pathogenic," "variant of unknown significance," "presumed benign," or "benign," according to the American College of Medical Genetics and Genomics (ACMG) standards. When assessing mutation frequencies of individual genes, "pathogenic," and "presumed pathogenic" were counted as mutations, whereas "benign", "presumed benign" variants, and "variants of unknown significance" were excluded.

MSI

MSI was examined using over 7,000 target microsatellite loci and compared with the reference genome hg19 from the University of California, Santa Cruz (UCSC) Genome Browser database, and the status was defined as MSI-high (MSI-H) or MSI-low/microsatellite stable (MSS). The number of microsatellite loci that were altered by somatic insertion or deletion were counted for each sample. Only insertions or deletions that increased or decreased the number of repeats were considered. Genomic variants in the microsatellite loci were detected using the same depth and frequency criteria as used for mutation detection. MSI-NGS results were compared with results from over 2,000 matching clinical cases analyzed with traditional PCR-based methods. The threshold to determine MSI by NGS was determined to be 46 or more loci with insertions or deletions to generate a sensitivity of >95% and specificity of >90%.

TMB

TMB was measured by counting all nonsynonymous missense mutations found per tumor that had not been described previously as germline alterations [592 genes and 1.4 megabases (MB) sequenced/tumor]. The threshold to define TMB-high (TMB-H) was greater than or equal to 17 mutations/MB and was established by comparing TMB with MSI by fragment analysis in colorectal cancer cases, based on reports of TMB having high concordance with MSI-H in colorectal cancer.

IHC analysis

IHC analysis was performed on full slides of FFPE tumor specimens using automated staining techniques (Benchmark XT, Ventana, and Autostainer Link 48, Dako). The primary antibody and details of evaluation for analysis are shown in Supplementary Table S1. Staining was scored for intensity (0 = no staining; 1+ = weak staining; 2+ = moderate staining; 3+ = strong staining) and staining percentage (0%–100%). Results were categorized as positive or negative by defined thresholds specific to each marker, based on published clinical literature.

Statistical analysis

All statistical analyses were performed with SPSS v23 (IBM SPSS Statistics), and all tests were two-sided at a significant level of 0.05. The comparison of age was analyzed using Student *t* test and that of molecular profile between groups were analyzed using Fisher exact test. Cases with missing information in any of the categorical data were not included in the analysis.

Use of standardized official symbols

In this study, HUGO (Human Genome Organisation)-approved official symbols for genes and gene products were used: all of which are described at www.genenames.org.

Results

Patient and tumor characteristics

Baseline patient and tumor characteristics in regard to age, gender, and location of tumor sampling are shown in Supplementary Table S2. Patients with appendiceal adenocarcinoma were significantly younger than patients with R-CRC ($P < 0.001$) and were more likely to be female than patients with L-CRC ($P = 0.011$). In addition, more appendiceal adenocarcinoma samples were collected from metastatic sites than R-CRC ($P < 0.001$) and L-CRC ($P < 0.001$) samples. Between histopathologic subtypes of appendiceal adenocarcinoma, there is no significant difference with regard to age, gender, and location of tumor sampling.

Common gene mutations in appendiceal adenocarcinoma, and comparison with R-CRC and L-CRC

The observed patterns of common gene mutations were totally different between appendiceal adenocarcinoma, R-CRC, and L-CRC. (Table 1) The most prevalent mutations seen in appendiceal adenocarcinoma were *KRAS* (55%), *TP53* (40%), *GNAS* (31%), *SMAD4* (16%), *APC* (10%), *ARID1A* (8%), *RNF43* (7%), *PIK3CA* (6%), and *BRAF* (5%). Compared with both R-and L-CRCs, appendiceal adenocarcinoma had significantly higher mutation rates in *GNAS* (31% vs. 2% vs. 1%) and *SMAD4* (16% vs. 11% vs. 10%), and lower mutation rates in *TP53* (40% vs. 66% vs. 75%), *APC* (10% vs. 70% vs. 83%), *PIK3CA*

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Table 1. Frequency of gene mutations in appendiceal adenocarcinoma, R-CRC, and L-CRC

	Appendiceal adenocarcinoma	R-CRC	L-CRC			
	N = 183	*N* = 994	*N* = 1,080	*P*[a]	*P*[a]	*P*[a]
Gene	%	%	%	AA vs. R-CRC	AA vs. L-CRC	R-CRC vs. L-CRC
TP53	40	66[b]	75[b]	<0.001	<0.001	<0.001
GNAS	31	2[b]	1[b]	<0.001	<0.001	0.049
SMAD4	16	11[b]	10[b]	0.042	0.029	
APC	10	70[b]	83[b]	<0.001	<0.001	<0.001
PIK3CA	6	22[b]	17[b]	<0.001	<0.001	0.008
FBXW7	3	11[b]	9[b]	0.002	0.015	
NRAS	1	3[b]	5[b]	0.048	0.004	
AMER1	0	9[b]	2[b]	<0.001	0.024	<0.001
ARID1A	8	26[b]	19	0.006		0.034
BRAF	5	17[b]	5	<0.001		<0.001
ATM	2	7[b]	5	0.012		0.041
KMT2D	2	7[b]	2	0.006		<0.001
PTEN	1	8[b]	3	<0.001		<0.001
MSH6	1	5[b]	2	0.016		<0.001
HNF1A	1	5[b]	1	0.024		<0.001
PTCH1	1	5[b]	1	0.009		<0.001
CTNNB1	0	4[b]	1	0.004		0.001
KRAS	55	56	43[b]		0.004	<0.001
RNF43	7	11	2[b]		0.004	<0.001

[a]*P* value was based on Fisher exact test. Blanks are $P > 0.05$.
[b]$P < 0.05$ compared with appendiceal adenocarcinoma.

(6% vs. 22% vs. 17%), *FBXW7* (3% vs. 11% vs. 9%), *NRAS* (1% vs. 3% vs. 5%), and *AMER1* (0% vs. 9% vs. 2%). In addition, compared with R-CRC, appendiceal adenocarcinoma had significantly lower mutation rates in *ARID1A* (8% vs. 26%), *BRAF* (5% vs. 17%), *ATM* (2% vs. 7%), *KMT2D* (2% vs. 7%), *PTEN* (1% vs. 8%), *MSH6* (1% vs. 5%), *HNF1A* (1% vs. 5%), *PTCH1* (1% vs. 5%), and *CTNNB1* (0% vs. 4%); and compared with L-CRC, appendiceal adenocarcinoma had significantly higher mutation rates in *KRAS* (55% vs. 43%) and *RNF43* (7% vs. 2%). Moreover, the mutation rates in *TP53*, *GNAS*, *APC*, *PIK3CA*, and *AMER1* had significant differences between appendiceal adenocarcinoma, R-CRC, and L-CRC. *BRAF* mutation rate was the highest in R-CRC (appendiceal adenocarcinoma, R-CRC, L-CRC: 5%, 17%, 5%), and *KRAS* mutation rate was the lowest in L-CRC (appendiceal adenocarcinoma, R-CRC, L-CRC: 55%, 56%, 43%).

Common gene mutations in the histopathologic subtypes of appendiceal adenocarcinoma

We further evaluated the pattern of common gene mutations in the histopathologic subtypes of appendiceal adenocarcinoma (NOS, PMP, MU, and SR; Table 2). Compared with NOS, PMP exhibited much higher mutation rates in *KRAS* (74% vs. 44%) and *GNAS* (63% vs. 7%), and much lower mutation rates in *TP53* (23% vs. 51%), *APC* (2% vs. 22%), and *PIK3CA* (2% vs. 15%); MU exhibited higher mutation rate in *KRAS* (64% vs. 44%) and *GNAS* (25% vs. 7%); and SR exhibited lower mutation rate in *KRAS* (15% vs. 44%), *GNAS* (4% vs. 7%), *TP53* (33% vs. 51%), *APC* (0% vs. 22%), and *PIK3CA* (0% vs. 15%). Notably, *BRAF* mutations were identified in NOS (7%), MU (9%), and SR (7%), but not identified in PMP.

Immune profiling of appendiceal adenocarcinoma, comparison with R-CRC and L-CRC, and differences among the histopathologic subtypes of appendiceal adenocarcinoma

The immune profile of appendiceal adenocarcinoma was similar to that of L-CRC but not that of R-CRC (Fig. 2). MSI-H, TMB-H, and PD-L1 high expression rates were much lower in appendiceal adenocarcinoma (MSI-H, TMB-H, PD-L1 high: 2.2%, 2.2%, 2.8%) than in R-CRC (MSI-H, TMB-H, PD-L1 high: 14.5%, 14.9%, 6.7%), but similar to L-CRC (MSI-H, TMB-H, PD-L1 high: 3.4%, 4.6%, 2.7%). The positive ratio of any of immune checkpoint inhibitor markers (MSI-H, TMB-H, PD-L1 high) is much higher in R-CRC (19.8%) than in appendiceal adenocarcinoma (5.1%) and in L-CRC (6.7%). Notably, some differences were

Table 2. Frequency of gene mutations in the histopathologic subtypes of appendiceal adenocarcinoma

	NOS	PMP	MU	SR			
	n = 46	*n* = 66	*n* = 44	*n* = 27	*P*[a]	*P*[a]	*P*[a]
Gene	%	%	%	%	NOS vs. PMP	NOS vs. MU	NOS vs. SR
KRAS	44	74[b]	64	15[b]	0.002		0.019
GNAS	7	63[b]	25[b]	4	<0.001	0.020	
TP53	51	23[b]	57	33	0.004		
SMAD4	15	15	20	11			
RNF43	9	6	7	4			
APC	22	2[b]	16	0[b]	<0.001		0.011
PIK3CA	15	2[b]	7	0[b]	0.009		0.042
BRAF	7	0	9	7			
ARID1A	11	0	15	11			

[a]*P* was based on Fisher exact test. Blanks are $P > 0.05$.
[b]$P < 0.05$ compared with NOS.

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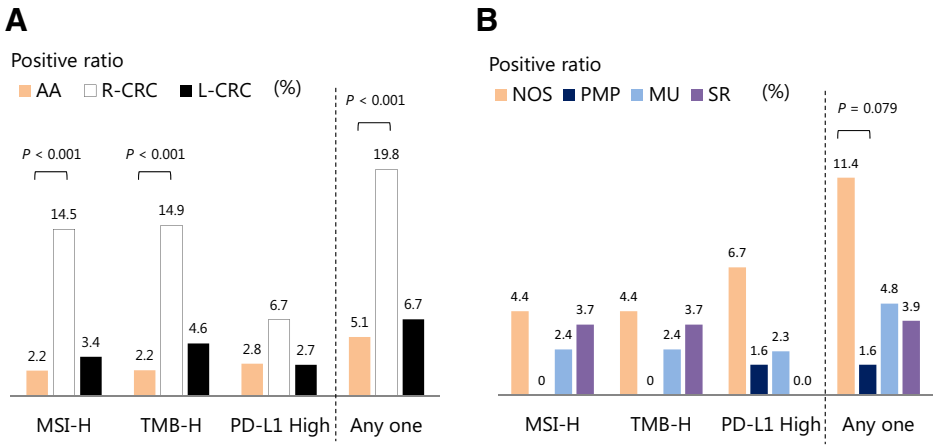

Figure 2.
Immune profiling (the status of MSI, TMB, and PD-L1 expression) of appendiceal adenocarcinoma, R-CRC and L-CRC, and the histopathologic subtypes of appendiceal adenocarcinoma. **A,** The characteristics of immune profiles in appendiceal adenocarcinoma, R-CRC, and L-CRC. **B,** The characteristics of immune profiles in the histopathologic subtypes of appendiceal adenocarcinoma.

observed between the histopathologic subtypes of appendiceal adenocarcinoma: PMP had no MSI-H or TMB-H case; and the positive ratio of any one marker (MSI-H, TMB-H, PD-L1 high) was higher in NOS (11.4%) than in PMP (1.6%), in MU (4.8%), and in SR (3.9%).

Protein expression of chemotherapeutic sensitivity markers in appendiceal adenocarcinoma, comparison with R-CRC and L-CRC, and differences among the histopathologic subtypes of appendiceal adenocarcinoma

As shown in Table 3, expression status of protein markers for chemotherapeutic sensitivity varied between appendiceal adenocarcinoma, R-CRC, and L-CRC, and between the histopathologic subtypes of appendiceal adenocarcinoma. The positive ratios of ERCC1, TOPO1, PTEN, and MGMT were higher in appendiceal adenocarcinoma than in R-CRC and L-CRC. Furthermore, thymidylate synthase (TS) was overexpressed most frequently in R-CRC. Between the histopathologic subtypes of appendiceal adenocarcinoma, although the protein expressions were similar in NOS, MU, and SR, those expressions were significantly different between NOS and PMP: ERCC1, TOPO1, PTEN, and MGMT were overexpressed in PMP, and TS was suppressed in PMP.

Comparison of molecular characteristics between the locations of tumor sampling

Finally, we analyzed the association of molecular characteristics with the location of tumor sampling (primary or metastatic sites; Supplementary Table S3) In appendiceal adenocarcinoma, the molecular characteristics had no difference between the locations of tumor sampling. On the other hand, in both R-CRC and L-CRC, the positive ratios of *ARID1A* mutation and MSI-H were significantly lower in metastatic sites.

Discussion

To the best of our knowledge, we performed so far the largest study to determine molecular profiling of appendiceal adenocarcinoma and to compare it with R-CRC and L-CRC, using integrated data within a total of 183 samples from appendiceal adenocarcinoma, 994 from R-CRC, and 1,080 from L-CRC. We found that appendiceal adenocarcinoma had higher mutation rates in *GNAS* and *KRAS*, and lower mutation rates in *TP53*, *APC*, and *PIK3CA* than R-CRC and L-CRC; gene mutation rates differed between the histopathologic subtypes of appendiceal adenocarcinoma; and although the immune profile results (MSI, TMB, and PD-L1 expression status) of appendiceal adenocarcinoma were

Table 3. Frequency of protein expressions in appendiceal adenocarcinoma, R-CRC, L-CRC, and the histopathologic subtypes of appendiceal adenocarcinoma

	Appendiceal adenocarcinoma	**R-CRC**	**L-CRC**		**P^a**	**P^a**	**P^a**
	***N* = 183**	***N* = 994**	***N* = 1,080**		**Appendiceal**	**Appendiceal**	**R-CRC**
Protein	**%**	**%**	**%**		**adenocarcinoma vs. R-CRC**	**adenocarcinoma vs. L-CRC**	**vs. L-CRC**
TS	19	31[b]	18		<0.001		<0.001
ERCC1	32	16[b]	15[b]		<0.001	<0.001	
TOPO1	65	52[b]	53[b]		0.001	0.003	
PTEN	88	65[b]	66[b]		<0.001	<0.001	
MGMT	69	52[b]	56		0.047		
	NOS	PMP	MU	SR			
	n = 46	*n* = 66	*n* = 44	*n* = 27	P^a	*P* value[a]	P^a
Protein	%	%	%	%	NOS vs. PMP	NOS vs. MU	NOS vs. SR
TS	26	8[b]	23	27	0.015		
ERCC1	22	44[b]	27	27	0.034		
TOPO1	53	76[b]	70	52	0.022		
PTEN	83	98[b]	88	76	0.010		
MGMT	50	95[b]	60	38	0.006		

[a]*P* value was based on Fisher exact test. Blanks are $P > 0.05$.
[b]$P < 0.05$ compared with appendiceal adenocarcinoma or NOS.

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similar to those of L-CRC (not to those of R-CRC), those of NOS had higher likelihood of overexpression than did other subtypes of appendiceal adenocarcinoma and L-CRC. Our results support developing a personalized treatment strategy in patients with appendiceal adenocarcinoma that is tailored to the individual's histopathologic subtypes.

The molecular feature of appendiceal adenocarcinoma is different from colorectal cancer. As in the past reports (16, 20–22), our current study showed that appendiceal adenocarcinoma had higher *GNAS* and *KRAS* mutation rates in comparison with colorectal cancer. The mutations of *GNAS*, a member of the G-protein family, cause constitutive activation of the protein kinase A (PKA) pathway via elevated levels of cyclic AMP (cAMP), and affected the MAPK and Wnt signaling pathways (20). *GNAS* mutation is common in benign tumors such as villous adenomas of the stomach (23) and colon (24), and intraductal papillary mucinous neoplasm (IPMN) of the pancreas (25, 26) and bile duct (27). Although the cAMP-PKA pathway can stimulate cell proliferation via MAPK or Wnt signaling, exogenous *GNAS* mutation in colorectal cancer (28) and pancreatic ductal adenocarcinoma (29) did not promote cell proliferation but increased expressions of MUC2 and MUC5AC (which induce mucin production). In addition, Taki and colleagues demonstrated that transgenic mice with pancreas-specific *GNAS* and *KRAS* mutations developed a cystic pancreatic tumor (30). However, intraperitoneal injection of colorectal cancer cells with exogenous *GNAS* mutation in mice did not produce PMP but resulted in the formation of solid tumors (28). These data suggest that both *GNAS* and *KRAS* may contribute to the oncogenesis of PMP. Furthermore, the mutation rates in *GNAS* and *KRAS* increased from NOS to MU and to PMP in our data, implying a functional interaction of these two oncogenes on mucin production. Moreover, several studies showed that *GNAS* and *KRAS* mutations were independent from pathologic grade, which is related to PMP activity (31–35). In support of these findings, both *GNAS* and *KRAS* mutations were not reported to be prognostic in patients with PMP (32, 36). *GNAS* and *KRAS* mutations might be a genetic feature of PMP but not be easy therapeutic targets.

Our data for the first time demonstrated that all the subtypes of appendiceal adenocarcinoma had much lower *TP53*, *APC*, and *PIK3CA* mutation rates in comparison with colorectal cancer, and the mutation rates were further much lower in PMP and SR than in NOS and MU. In colorectal cancer, *TP53*, *APC*, and *PIK3CA* are key driver genes for "adenoma-to-carcinoma sequence," and the mutation rates are high in any stage or tumor status (37, 38). Our findings suggest that carcinogenic mechanisms of appendiceal adenocarcinoma may be different from that of colorectal cancer. Wilson and colleagues showed that *GNAS* mutation cooperated with inactivation of *APC* leading to colorectal tumorigenesis, but not carcinogenesis (39). In addition, Noguchi and colleagues demonstrated that *TP53*, *PIK3CA*, and *AKT1* mutations were detected in peritoneal mucinous adenocarcinoma but not in PMP (21). Although *GNAS* mutation might contribute to APC-driven tumorigenesis, mutations in TP53 and/or genes related to the PI3K–AKT pathway may be necessary for malignant transformation in PMP. On the other hand, SR exhibited lower mutation rates not only in *TP53*, *APC*, and *PIK3CA*, but also in *KRAS* and *GNAS*. In addition, the mutation rate of *BRAF* was much lower (7%) compared with SR of colorectal cancer (about 40%; refs. 40, 41), suggesting that SR might differ from both other histopathologic subtypes of appendiceal adenocarcinoma and SR of colorectal cancer in both development and treatment strategies.

Recently, immune checkpoint–based therapy has demonstrated better survival and tolerance in subsets of patients with both solid and hematologic malignancies. Studies further showed benefit in immune checkpoint blockade in patients whose tumors are with MSI-H, TMB-H, and PD-L1 high expression (14). There is a need for molecular markers that can identify patients who are likely to benefit from immune checkpoint inhibitors. Therefore, overall immune profiling is an important emerging biomarker for cancer treatment. The appendix originated from the cecum has many lymphoid clusters, and regulates IgA production in the large bowel sites (42), suggesting that appendiceal adenocarcinoma may be subject to lymphocytic regulation more than R-CRC and L-CRC. However, interestingly, our study showed that immune profile of appendiceal adenocarcinoma was similar to L-CRC but not R-CRC. In addition, NOS had a higher positive likelihood of expressing any of the evaluated immune checkpoint inhibitor markers (11.4%) than other subtypes of appendiceal adenocarcinoma (PMP: 1.6%, MU: 4.8%, SR: 3.9%) and L-CRC (6.7%). Although the carcinogenic mechanism of NOS is different from that of R-CRC and L-CRC, the immune characteristics might be closer to R-CRC. Further experimental studies are necessary to better understand this mechanism.

The efficacy of systematic chemotherapy and molecular targeted therapy for appendiceal adenocarcinoma has not been well studied so far. Although a phase II trial in unresectable PMP suggested capecitabine combined with mitomycin C, the combination of fluorouracil (5-FU) and oxaliplatin/irinotecan is commonly used for the treatment of metastatic appendiceal adenocarcinoma and PMP despite the paucity of efficacy data (3, 43). Our data showed that the positive ratios of ERCC1 and TOPO1 were higher in appendiceal adenocarcinoma, especially in PMP, than in R-CRC and L-CRC, and that of TS was lower in appendiceal adenocarcinoma, especially in PMP. ERCC1, a protein of the nucleotide excision repair complex, is essential for repairing platinum–DNA adducts and is involved in drug resistance to oxaliplatin (44); TOPO1, a molecular target of SN38, is a plausible positive predictive marker for irinotecan (45); and TS, a rate-limiting enzyme in the synthesis of pyrimidine nucleotides, is required for DNA synthesis and the activity is a negative predictive marker for 5-FU (44, 46). Thus, compared with colorectal cancers, combination therapy with 5-FU and irinotecan (due to lower TS and higher ERCC1 and TOPO1) may be more effective for appendiceal adenocarcinoma, especially for PMP treatment. However, the predictive protein expressions for chemosensitivities are still controversial even in colorectal cancer. As increased mucin production is responsible for major complications and fatal outcome in patients with PMP, GNAS-related pathways (cAMP–PKA, MAPK, and Wnt signaling pathways) might be a potential therapeutic target: PKA inhibitor (28), BIM-46174 (inhibitor of heterotrimeric G-protein complex; ref. 47), and a MEK-inhibitor (48) were reported to cause mucin production reduction in tumors with GNAS mutation. Biomarker studies according to the histopathologic and molecular subtypes are needed to determine individualized treatment strategies for appendiceal adenocarcinoma.

We also compared the molecular profiles of samples from primary and metastatic tumors to identify features that are associated with distant metastasis in appendiceal adenocarcinoma. In

consensus with the data from past reports (38), a high level of genomic concordance was detected. However, *ARID1A* mutation and MSI-H were specifically enriched in primary tumors compared with distant metastases in both R-CRC and L-CRC, suggesting their potential protective effects. Recently, *ARID1A*, a subunit of the chromatin-remodeling complex SWI/SNF, mutation was reported to contribute to impaired mismatch repair and mutator phenotype in cancers (49). ARID1A could be a promising target for novel treatment strategies for immune checkpoint–based therapy.

Limitations in our study need to be mentioned. First, the retrospective study design could not exclude selection bias. Second, due to the loss of clinical data, such as precise TNM stage, treatment, and patient outcome, the direct effect of our findings in clinical perspectives is unclear, as well as the protective effect of *ARID1A* mutation and MSI-H for patients with colorectal cancer. However, using the biggest dataset (183 samples from appendiceal adenocarcinoma, 994 from R-CRC, and 1,080 from L-CRC), our results may support the past findings and compare molecular profiles between appendiceal adenocarcinoma, R-CRC, and L-CRC, and also between minor histopathologic subtypes of appendiceal adenocarcinoma. In addition, our dataset included immune profile and protein expressions, which could lead to selection of treatment strategies. Further large-scale prospective studies with detailed clinical data may be warranted to validate our findings and provide us more information for treatment strategies of appendiceal adenocarcinoma.

In conclusion, molecular profiling of appendiceal adenocarcinoma revealed different characteristics from R-CRC and L-CRC, and heterogeneity between the histopathologic subtypes of appendiceal adenocarcinoma. Our data suggest that these molecular differences should be recognized in treating the patients with appendiceal adenocarcinoma. Upon validation with clinical features, our findings may provide novel insight to develop individualized approach for appendiceal adenocarcinoma treatment.

Disclosure of Potential Conflicts of Interest

P.A. Philip reports receiving speakers bureau honoraria from Merck, Celgene, Halozyme, Ipsen, Bristol-Myers Squibb, and Bayer, and is a consultant/advisory board member for Halozyme, Caris, and Merck. A. Seeber is a consultant/advisory board member for Caris Life Sciences. A.F. Shields is a consultant/advisory board member for and reports receiving commercial research grants from Caris. J.L. Marshall is an employee of and has ownership interests (including patents) at Caris; reports receiving speakers bureau honoraria from Genentech, Amgen, Celgene, Tailho, and Bayer; and is a consultant/advisory board member for Caris SAB. H. Baba reports receiving speakers bureau honoraria from Eli Lilly Japan K.K. W.M. Korn has ownership interests (including patents) at Caris Life Sciences and is a consultant/advisory board member for Merck and Lilly. H.-J. Lenz reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Merck Serono, Bayer, and Genentech. No potential conflicts of interest were disclosed by the other authors.

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References

1. Compton C, Fenoglio-Preiser CM, Pettigrew N, Fielding LP. American Joint Committee on Cancer Prognostic Factors Consensus Conference: Colorectal Working Group. Cancer 2000;88:1739–57.
2. Turaga KK, Pappas SG, Gamblin T. Importance of histologic subtype in the staging of appendiceal tumors. Ann Surg Oncol 2012;19:1379–85.
3. Pietrantonio F, Maggi C, Fanetti G, Iacovelli R, Di Bartolomeo M, Ricchini F, et al. FOLFOX-4 chemotherapy for patients with unresectable or relapsed peritoneal pseudomyxoma. Oncologist 2014;19:845–50.
4. Carr NJ, Finch J, Ilesley IC, Chandrakumaran K, Mohamed F, Mirnezami A, et al. Pathology and prognosis in pseudomyxoma peritonei: a review of 274 cases. J Clin Pathol 2012;65:919–23.
5. Carr NJ, Bibeau F, Bradley RF, Dartigues P, Feakins RM, Geisinger KR, et al. The histopathological classification, diagnosis and differential diagnosis of mucinous appendiceal neoplasms, appendiceal adenocarcinomas and pseudomyxoma peritonei. Histopathology 2017;71:847–58.
6. Chua TC, Moran BJ, Sugarbaker PH, Levine EA, Glehen O, Gilly FN, et al. Early- and long-term outcome data of patients with pseudomyxoma peritonei from appendiceal origin treated by a strategy of cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. J Clin Oncol 2012;30:2449–56.
7. Sugarbaker PH. New standard of care for appendiceal epithelial neoplasms and pseudomyxoma peritonei syndrome? Lancet Oncol 2006;7:69–76.
8. Bijelic L, Kumar AS, Stuart OA, Sugarbaker PH. Systemic Chemotherapy prior to cytoreductive surgery and HIPEC for carcinomatosis from appendix cancer: impact on perioperative outcomes and short-term survival. Gastroenterol Res Pract 2012;2012:163284.
9. Sugarbaker PH, Bijelic L, Chang D, Yoo D. Neoadjuvant FOLFOX chemotherapy in 34 consecutive patients with mucinous peritoneal carcinomatosis of appendiceal origin. J Surg Oncol 2010;102:576–81.
10. Shapiro JF, Chase JL, Wolff RA, Lambert LA, Mansfield PF, Overman MJ, et al. Modern systemic chemotherapy in surgically unresectable neoplasms of appendiceal origin: a single-institution experience. Cancer 2010;116:316–22.
11. Pietrantonio F, Berenato R, Maggi C, Caporale M, Milione M, Perrone F, et al. GNAS mutations as prognostic biomarker in patients with relapsed peritoneal pseudomyxoma receiving metronomic capecitabine and bevacizumab: a clinical and translational study. J Transl Med 2016;14:125.
12. Farquharson AL, Pranesh N, Witham G, Swindell R, Taylor MB, Renehan AG, et al. A phase II study evaluating the use of concurrent mitomycin C and capecitabine in patients with advanced unresectable pseudomyxoma peritonei. Br J Cancer 2008;99:591–6.

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13. Tosi F, Magni E, Amatu A, Mauri G, Bencardino K, Truini M, et al. Effect of KRAS and BRAF mutations on survival of metastatic colorectal cancer after liver resection: a systematic review and meta-analysis. Clin Colorectal Cancer 2017;16:e153–e63.
14. Gibney GT, Weiner LM, Atkins MB. Predictive biomarkers for checkpoint inhibitor-based immunotherapy. Lancet Oncol 2016;17:e542–e51.
15. Stintzing S, Tejpar S, Gibbs P, Thiebach L, Lenz HJ. Understanding the role of primary tumour localisation in colorectal cancer treatment and outcomes. Eur J Cancer 2017;84:69–80.
16. Liu X, Mody K, de Abreu FB, Pipas JM, Peterson JD, Gallagher TL, et al. Molecular profiling of appendiceal epithelial tumors using massively parallel sequencing to identify somatic mutations. Clin Chem 2014;60:1004–11.
17. Raghav KP, Shetty AV, Kazmi SM, Zhang N, Morris J, Taggart M, et al. Impact of molecular alterations and targeted therapy in appendiceal adenocarcinomas. Oncologist 2013;18:1270–7.
18. Overman MJ, Fournier K, Hu CY, Eng C, Taggart M, Royal R, et al. Improving the AJCC/TNM staging for adenocarcinomas of the appendix: the prognostic impact of histological grade. Ann Surg 2013;257:1072–8.
19. Levine EA, Votanopoulos KI, Qasem SA, Philip J, Cummins KA, Chou JW, et al. Prognostic molecular subtypes of low-grade cancer of the appendix. J Am Coll Surg 2016;222:493–503.
20. Alakus H, Babicky ML, Ghosh P, Yost S, Jepsen K, Dai Y, et al. Genome-wide mutational landscape of mucinous carcinomatosis peritonei of appendiceal origin. Genome Med 2014;6:43.
21. Noguchi R, Yano H, Gohda Y, Suda R, Igari T, Ohta Y, et al. Molecular profiles of high-grade and low-grade pseudomyxoma peritonei. Cancer Med 2015;4:1809–16.
22. Ang CS-P, Shen JP, Hardy-Abeloos CJ, Huang JK, Ross JS, Miller VA, et al. Genomic landscape of appendiceal neoplasms. JCO Precis Oncol 2018;2:1–18.
23. Matsubara A, Sekine S, Kushima R, Ogawa R, Taniguchi H, Tsuda H, et al. Frequent GNAS and KRAS mutations in pyloric gland adenoma of the stomach and duodenum. J Pathol 2013;229:579–87.
24. Yamada M, Sekine S, Ogawa R, Taniguchi H, Kushima R, Tsuda H, et al. Frequent activating GNAS mutations in villous adenoma of the colorectum. J Pathol 2012;228:113–8.
25. Furukawa T, Kuboki Y, Tanji E, Yoshida S, Hatori T, Yamamoto M, et al. Whole-exome sequencing uncovers frequent GNAS mutations in intraductal papillary mucinous neoplasms of the pancreas. Sci Rep 2011;1:161.
26. Wu J, Matthaei H, Maitra A, Dal Molin M, Wood LD, Eshleman JR, et al. Recurrent GNAS mutations define an unexpected pathway for pancreatic cyst development. Sci Transl Med 2011;3:92ra66.
27. Tsai JH, Yuan RH, Chen YL, Liau JY, Jeng YM. GNAS is frequently mutated in a specific subgroup of intraductal papillary neoplasms of the bile duct. Am J Surg Pathol 2013;37:1862–70.
28. Nishikawa G, Sekine S, Ogawa R, Matsubara A, Mori T, Taniguchi H, et al. Frequent GNAS mutations in low-grade appendiceal mucinous neoplasms. Br J Cancer 2013;108:951–8.
29. Komatsu H, Tanji E, Sakata N, Aoki T, Motoi F, Naitoh T, et al. A GNAS mutation found in pancreatic intraductal papillary mucinous neoplasms induces drastic alterations of gene expression profiles with upregulation of mucin genes. PLoS One 2014;9:e87875.
30. Taki K, Ohmuraya M, Tanji E, Komatsu H, Hashimoto D, Semba K, et al. GNAS(R201H) and Kras(G12D) cooperate to promote murine pancreatic tumorigenesis recapitulating human intraductal papillary mucinous neoplasm. Oncogene 2016;35:2407–12.
31. Bazan V, Migliavacca M, Zanna I, Tubiolo C, Grassi N, Latteri MA, et al. Specific codon 13 K-ras mutations are predictive of clinical outcome in colorectal cancer patients, whereas codon 12 K-ras mutations are associated with mucinous histotype. Ann Oncol 2002;13:1438–46.
32. Singhi AD, Davison JM, Choudry HA, Pingpank JF, Ahrendt SA, Holtzman MP, et al. GNAS is frequently mutated in both low-grade and high-grade disseminated appendiceal mucinous neoplasms but does not affect survival. Hum Pathol 2014;45:1737–43.
33. Hosoda W, Sasaki E, Murakami Y, Yamao K, Shimizu Y, Yatabe Y. GNAS mutation is a frequent event in pancreatic intraductal papillary mucinous neoplasms and associated adenocarcinomas. Virchows Arch 2015;466:665–74.
34. Nummela P, Saarinen L, Thiel A, Jarvinen P, Lehtonen R, Lepisto A, et al. Genomic profile of pseudomyxoma peritonei analyzed using next-generation sequencing and immunohistochemistry. Int J Cancer 2015;136:E282–9.
35. Pietrantonio F, Perrone F, Mennitto A, Gleeson EM, Milione M, Tamborini E, et al. Toward the molecular dissection of peritoneal pseudomyxoma. Ann Oncol 2016;27:2097–103.
36. Austin F, Mavanur A, Sathaiah M, Steel J, Lenzner D, Ramalingam L, et al. Aggressive management of peritoneal carcinomatosis from mucinous appendiceal neoplasms. Ann Surg Oncol 2012;19:1386–93.
37. Hadac JN, Leystra AA, Paul Olson TJ, Maher ME, Payne SN, Yueh AE, et al. Colon tumors with the simultaneous induction of driver mutations in APC, KRAS, and PIK3CA still progress through the adenoma-to-carcinoma sequence. Cancer Prev Res 2015;8:952–61.
38. Yaeger R, Chatila WK, Lipsyc MD, Hechtman JF, Cercek A, Sanchez-Vega F, et al. Clinical sequencing defines the genomic landscape of metastatic colorectal cancer. Cancer Cell 2018;33:125–36.
39. Wilson CH, McIntyre RE, Arends MJ, Adams DJ. The activating mutation R201C in GNAS promotes intestinal tumourigenesis in Apc(Min/+) mice through activation of Wnt and ERK1/2 MAPK pathways. Oncogene 2010;29:4567–75.
40. Alvi MA, Loughrey MB, Dunne P, McQuaid S, Turkington R, Fuchs MA, et al. Molecular profiling of signet ring cell colorectal cancer provides a strong rationale for genomic targeted and immune checkpoint inhibitor therapies. Br J Cancer 2017;117:203–9.
41. Yalcin S, Onguru O. BRAF mutation in colorectal carcinomas with signet ring cell component. Cancer Biol Med 2017;14:287–92.
42. Masahata K, Umemoto E, Kayama H, Kotani M, Nakamura S, Kurakawa T, et al. Generation of colonic IgA-secreting cells in the caecal patch. Nat Commun 2014;5:3704.
43. Tejani MA, ter Veer A, Milne D, Ottesen R, Bekaii-Saab T, Benson AB III, et al. Systemic therapy for advanced appendiceal adenocarcinoma: an analysis from the NCCN Oncology Outcomes Database for colorectal cancer. J Natl Compr Canc Netw 2014;12:1123–30.
44. Shirota Y, Stoehlmacher J, Brabender J, Xiong YP, Uetake H, Danenberg KD, et al. ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy. J Clin Oncol 2001;19:4298–304.
45. Braun MS, Richman SD, Quirke P, Daly C, Adlard JW, Elliott F, et al. Predictive biomarkers of chemotherapy efficacy in colorectal cancer: results from the UK MRC FOCUS trial. J Clin Oncol 2008;26:2690–8.
46. Bendardaf R, Lamlum H, Elzagheid A, Ristamaki R, Pyrhonen S. Thymidylate synthase expression levels: a prognostic and predictive role in advanced colorectal cancer. Oncol Rep 2005;14:657–62.
47. Prevost GP, Lonchampt MO, Holbeck S, Attoub S, Zaharevitz D, Alley M, et al. Anticancer activity of BIM-46174, a new inhibitor of the heterotrimeric Galpha/Gbetagamma protein complex. Cancer Res 2006;66:9227–34.
48. Ang C, Stollman A, Zhu H, Sarpel U, Scarborough B, Sahni G, et al. Clinical benefit from trametinib in a patient with appendiceal adenocarcinoma with a GNAS R201H mutation. Case Rep Oncol 2017;10:548–52.
49. Shen J, Ju Z, Zhao W, Wang L, Peng Y, Ge Z, et al. ARID1A deficiency promotes mutability and potentiates therapeutic antitumor immunity unleashed by immune checkpoint blockade. Nat Med 2018;24:556–62.

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Molecular Profiling of Appendiceal Adenocarcinoma and Comparison with Right-sided and Left-sided Colorectal Cancer

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