Molecular Profiling of Appendiceal Epithelial Tumors Using Massively Parallel Sequencing to Identify Somatic Mutations


BACKGROUND: Some epithelial neoplasms of the appendix, including low-grade appendiceal mucinous neoplasm and adenocarcinoma, can result in pseudomyxoma peritonei (PMP). Little is known about the mutational spectra of these tumor types and whether mutations may be of clinical significance with respect to therapeutic selection. In this study, we identified somatic mutations using the Ion Torrent AmpliSeq Cancer Hotspot Panel v2.

METHODS: Specimens consisted of 3 nonneoplastic retention cysts/mucocele, 15 low-grade mucinous neoplasms (LAMNs), 8 low-grade/well-differentiated mucinous adenocarcinomas with pseudomyxoma peritonei, and 12 adenocarcinomas with/without goblet cell/signet ring cell features. Barcoded libraries were prepared from up to 10 ng of extracted DNA and multiplexed on single 318 chips for sequencing. Data analysis was performed using Golden Helix SVS. Variants that remained after the analysis pipeline were individually interrogated using the Integrative Genomics Viewer.

RESULTS: A single Janus kinase 3 (JAK3) mutation was detected in the mucocele group. Eight mutations were identified in the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) and GNAS complex locus (GNAS) genes among LAMN samples. Additional gene mutations were identified in the AKT1 (v-akt murine thymoma viral oncogene homolog 1), APC (adenomatous polyposis coli), JAK3, MET (met proto-oncogene), phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3CA), RB1 (retinoblastoma 1), STK11 (serine/threonine kinase 11), and tumor protein p53 (TP53) genes. Among the PMPs, 6 mutations were detected in the KRAS gene and also in the GNAS, TP53, and RB1 genes. Appendiceal cancers showed mutations in the APC, ATM (ataxia telangiectasia mutated), KRAS, IDH1 [isocitrate dehydrogenase 1 (NADP+)], NRAS [neuroblastoma RAS viral (v-ras) oncogene homolog], PIK3CA, SMAD4 (SMAD family member 4), and TP53 genes.

CONCLUSIONS: Our results suggest molecular heterogeneity among epithelial tumors of the appendix. Next generation sequencing efforts have identified mutational spectra in several subtypes of these tumors that may suggest a phenotypic heterogeneity showing mutations that are relevant for targeted therapies.

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Although cancer of the appendix is rare, representing <1% of gastrointestinal tumors, it affects approximately 2000 individuals annually in the US (1). Diagnostically these tumors can be challenging due to the heterogeneity of the diverse histologic types. Carcinoids historically have been the most common histologic type of appendiceal tumors identified, but the distribution of histology has changed with time, with adenocarcinomas representing 64% of appendiceal tumors and carcinoids representing 11% in one recent series (2). Adenocarcinomas (intestinal, colonic, and signet ring types), lymphomas, and mixed histology, are all examples of additional tumor types known to arise from the appendix.

The most frequent presenting symptoms of these tumors are those mimicking appendicitis, but they may also present with general abdominal pain, ascites, or peritoneal spread and pseudomyxoma peritonei.

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Carcinoids may occasionally present with symptoms of carcinoid syndrome (flushing, tachycardia, and diarrhea). In fact, cancers of the appendix are commonly noted incidentally upon appendectomy. In some instances, surgery alone is sufficient in the management of these tumors. However, for large carcinoids, adenocarcinomas, or lymphomas, for example, more extensive surgery and/or chemotherapy are required. There is currently no standard treatment for these tumors, and which chemotherapy or targeted agent therapy is best used for these tumors remains a subject of debate. At present, adenocarcinomas of the appendix are frequently treated similarly to colon cancer.

Lack of standardized management strategies for these tumors has contributed to high mortality rates. The identification of biomarkers is necessary for the development and selection of more appropriate therapeutic regimens contributing to more personalized medicine. In this era of personalized medicine, in which therapeutic strategies can be based on genomic profiling of tumor cells, we sought to identify genomic profiles of various types of appendiceal tumors. In the current study we used massively parallel sequencing techniques to identify somatic mutations in the hotspots of 50 known human cancer genes to identify molecular profiles which may be clinically actionable with respect to currently available targeted therapies.

**Materials and Methods**

**SAMPLES**

All samples were obtained from the Department of Pathology archives at the Dartmouth Hitchcock Medical Center (2003–2013). Formalin-fixed, paraffin-embedded (FFPE) specimens consisted of four groups: 3 nonneoplastic retention cysts/mucocele, 15 low-grade appendiceal mucinous neoplasms (LAMNs), 8 low-grade/well-differentiated mucinous adenocarcinomas with PMP, and 12 adenocarcinomas with goblet cell/signet ring cell features (Fig. 1). The specimen types included FFPE surgical resections and FFPE cy-

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8 Nonstandard abbreviations: PMP, pseudomyxoma peritonei; LAMN, low-grade appendiceal mucinous neoplasm; FFPE, formalin-fixed, paraffin-embedded; EGFR, epidermal growth factor receptor; CRC, colorectal cancer.
tology samples (cell blocks) of peritoneal fluid. All tissues were deidentified in compliance with the Committee for the Protection of Human Subjects. All samples were independently reviewed by two surgical pathologists who also determined the percentage of tumor cell content in each sample.

**DNA EXTRACTION**

FFPE tissue samples with more than 10% tumor content were macrodissected from 8 unstained tissue sections of 5 microns each. Macrodisssection enriched for tumor cell content to >50%. Genomic DNA was extracted using the Gentra Puregene kit (Qiagen) according to the manufacturer’s recommendations. Extracted DNA samples were quantified using the Quant-iTTM PicoGreen® dsDNA assay kit (Invitrogen) before library preparation for sequencing.

**AmpliSeq™ CANCER HOTSPOT PANEL v2**

Libraries were generated using Life Technology’s Ion AmpliSeq Cancer Hotspot Panel v2 as previously described (3). This panel consists of 207 amplicons covering over 20 000 bases of 50 genes with known cancer associations. Approximately 10 ng of genomic DNA from each sample was used to prepare barcoded libraries using IonXpress barcoded adapters (Life Technologies). Libraries were combined to a final concentration of 100 pmol/L using the Ion Library Quantification Kit (Life Technologies), and emulsion PCR was performed using the Ion Torrent OneTouch™ 2 System. Samples were sequenced on the Ion Torrent Personal Genome Machine (PGMTM) using Ion 318™ chips.

**DATA ANALYSIS**

Sequencing reads were first aligned to Human Genome version 19 (hg19) using Torrent Suite 3.4.2. The first step in the data analysis pipeline was to run the Variant Caller Plugin 4.0 provided by Ion Torrent using the high stringency thresholds for somatic variant detection. Variant annotation and prediction of functional significance was performed with the aid of Golden Helix’s SVS software version 7.7.8. Remaining variants were assessed using the Broad’s Integrated Genome Viewer (IGV 2.2). Read depth and uniformity of coverage across individual amplicons was assessed using the Coverage Analysis Plugin 4.0 provided by Ion Torrent.

**Results**

**HISTOLOGIC DISTRIBUTION OF CASES**

Nonneoplastic appendiceal lesions, namely retention cysts/mucoceles, showed a dilated cystic appendix filled with mucinous material. Two out of 3 of the cysts were lined by benign columnar epithelium, while the other was without an epithelial lining, possibly a result of extensive epithelial denudation (Table 1).

All patients with LAMNs also had dilated cystic appendices. Five out of 15 LAMNs showed gross evidence of rupture with acellular mucin on the serosal surface. Microscopically, the cyst walls were lined by a low-grade adenomatous mucinous epithelium (Table 1).

Low-grade/well-differentiated mucinous adenocarcinoma with PMP showed appendiceal rupture with mucin extravasation into the abdomen. Six of them revealed cellular mucin, while the other 2 presented with acellular mucin (Table 1).

Of the appendiceal adenocarcinomas, 11/12 cases showed features of adenocarcinoma and admixed single and clusters of goblet- like mucinous cells/signet ring cells. The other case had adenocarcinoma with mucin production and serrated features, moderately differentiated morphology, and no overt goblet cell features (Table 2).

**MUTATION PROFILING USING NEXT GENERATION SEQUENCING**

A total of 38 samples were sequenced for the AmpliSeq Hotspot Cancer Panel v2 and only 9 did not show any
mutations in the 50 genes tested. Among the 3 mucinous cell samples, 2 were wild type and the other had a mutation in the Janus kinase 3 (JAK3) gene (c.2164G>A, p.V722I) (Fig. 2A).

Of the 15 LAMNs, 80% (n = 12) had at least 1 hotspot mutation identified and 20% (n = 3) were wild type (Fig. 2B). The most common somatic mutations identified were in the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) and GNAS complex locus (GNAS) genes. There were 6 KRAS mutations mapped to codon 12 (c.35G>T, p.G12V and c.35G>A, p.G12D), 1 to codon 13 (c.38G>A, p.G13D), and 1 to codon 61 (c.183A>C, p.Q61H). Mutations in the GNAS gene were identified in 8 samples, 2 with c.2531G>A, p.R844H and 6 with c.2530C>T, p.R844C. Six out of 8 LAMNs contained both KRAS and GNAS mutations. Additional mutations were identified in the v-akt murine thymoma viral oncogene homolog 1 (AKT1) (n = 1), adenomatous polyposis coli (APC) (n = 5), JAK3 (n = 1), met proto-oncogene (MET) (n = 2), phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3CA) (n = 1), retinoblastoma 1 (RB1) (n = 2), and serine/threonine kinase 11 (STK11) (n = 1) genes. Samples with mutations in the MET and AKT genes did not show additional mutations. Three of the 4 APC mutated cases also had mutations in both KRAS and GNAS genes. Two samples had the same mutations in KRAS, GNAS, RB1, and APC genes. These samples had a total of 8 and 5 variants each.

Two out of 8 (25%) low-grade/well-differentiated mucinous adenocarcinoma with PMP samples were wild type and 6/8 (75%) had 2 variants identified (Fig. 2C). All of the mutated samples had a mutation in the KRAS gene mapped to codon 12 (c.35G>A, p.G12D; c.35G>T, p.G12V; c.34G>T, p.G12C). In addition, mutations were identified in the tumor protein p53 (TP53) (n = 2), GNAS (n = 1), and RB1 (n = 1) genes. Two tumor samples had the same mutations in the KRAS and GNAS genes. Two other tumor samples had mutations in the KRAS and TP53 genes; however, the mutations were not similar. And one sample had a mutation in the KRAS and RB1 genes.

Nine out of 11 adenocarcinoma cases with goblet cell features (82%) had at least 1 hotspot mutation, and 2 (18%) were wild type (Fig. 2D). Five mutations were identified in the TP53 and SMAD family member 4 (SMAD4) genes. Additional mutations identified in this cohort were mapped to the following genes: APC (n = 3), PIK3CA (n = 2), ataxia telangiectasia mutated (ATM) (n = 2), and neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) (n = 1). APC and ATM mutations were similar among samples. The 1 adenocarcinoma case without goblet cell features showed 2 unique mutations in the KRAS (c.37G>A, p.G13R) and isocitrate dehydrogenase 1 (NADP+) (IDH1) (c.394C>A, p.R132S) genes.

The genomic profiles across the 4 sample types identified mutations within 15 different genes. Comparatively, only 7 of them (KRAS, GNAS, TP53, RB1, APC, JAK3, and PIK3CA) were mutated in more than 1 group (Fig. 3). Mutations identified in the KRAS, GNAS, and APC genes were similar among samples and groups (Fig. 4). With KRAS, a total of 7 samples (LAMNs and low-grade/well-differentiated mucinous adenocarcinoma with PMP) had a G12D mutation and 2 samples had a G12V mutation. For GNAS, the most common mutations were R844C and R844H, identified in 7 and 4 samples, respectively. Of the 9 samples with a mutation in the APC gene, 7 had the P1415L and 2 had the G1299Q mutation.

**Discussion**

Appendiceal carcinomas are quite rare, with an age-adjusted incidence of 0.12 cases per 1 million people per year. Although most discussions about appendiceal cancers have revolved around carcinoid tumors historically, incidences of the noncarcinoid subtypes have been found to be higher as a whole, with rates per 1 million people per year of 1.3 for mucinous adenocarcinoma, 0.95 for adenocarcinoma, 0.5 for adenocarcinoid (goblet cell), and 0.15 for signet ring cell tumors (4). In an analysis of over 2500 cases from 1973 to 2001, it was noted that carcinoid and goblet cell tumors more often presented with localized disease, whereas mucinous and signet ring cell types presented more frequently with larger tumors (>2cm) and distant dis-

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8 Human genes: JAK3, Janus kinase 3; KRAS, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; GNAS, GNAS complex locus; AKT1, v-akt murine thymoma viral oncogene homolog 1; APC, adenomatous polyposis coli; MET, met proto-oncogene; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase; RB1, retinoblastoma 1; STK11, serine/threonine kinase 11; TP53, tumor protein p53; SMAD4, SMAD family member 4; ATM, ataxia telangiectasia mutated; IDH1, isocitrate dehydrogenase 1 (NADP+); N-RAS, neuroblastoma RAS viral (v-ras) oncogene homolog.
Tumors of the appendix tend to result in increased morbidity and mortality due to late or incidental detection. Mortality varies by histologic subtype, with 5-year survival rates, despite aggressive surgical resections, being 83% for carcinoid, 76% for goblet cell, 46% for mucinous, 42% for adenocarcinoma, and 18% for signet ring cell.

Traditional systemic therapies have been extrapolated from treatment of colorectal cancer (CRC) and, although data are limited, there have been several reports noting benefit with FOLFOX [folinic acid (leucovorin), 5-FU (fluorouracil), and oxaliplatin] therapy. Molecular targeting agents, as used and currently under investigation in treatment of colorectal adenocarcinoma, also might be worth evaluating as a treatment option for appendiceal adenocarcinoma, as similar molecular aberrations have been noted in colonic adenocarcinoma (5–9).

We have previously validated the AmpliSeq Cancer Hotspot Panel for use in clinical assessment of human cancers, including non–small cell lung cancer, melanoma, glioma, and colon cancer (3). In this study, we extended the application of this cancer gene panel to tumors of the appendix, for which there is little molecular profiling reported. The goal was to potentially identify clinically actionable mutations with respect to targeted therapy. We found that KRAS and GNAS mutations were the most common alterations identified in patients with LAMNs and low-grade/well-differentiated mucinous adenocarcinoma with PMP.

KRAS is a protooncogene that encodes proteins involved in cell growth, division, motility, differentiation, and apoptosis. Somatic mutations have been linked to the development of several types of cancer, but in CRC, they are detected in 35%–45% of the patients. The most common mutations result in amino
acid substitutions at codons 12 and 13, leading to the activation of the MAPK (mitogen-activated protein kinase) pathway. Although these mutations are not prognostic, they are well established biomarkers associated with a lack of response to anti–epidermal growth factor receptor (anti-EGFR) monoclonal antibodies, such as cetuximab and panitumumab. According to the US Food and Drug Administration, only patients negative for KRAS mutation (or wild type) should receive cetuximab and panitumumab. Therefore, patients diagnosed with CRC are screened for KRAS mutations. According to Patel and Karapetis, the discovery of these mutations as a biomarker allowed for personalized EGFR-targeted therapy for CRC patients (10). Among the appendiceal adenocarcinomas with goblet cell features, this study shows that this group does not have KRAS mutations, in agreement with previous findings (11).

KRAS mutations have been reported previously in appendiceal tumor studies that queried fewer genes. For example, Cuatrecasas and colleagues (12) demonstrated that 5 of 6 appendiceal tumors with PMP had a KRAS mutation in codon 12. In 2002, Bazan and colleagues reported that KRAS mutation in codon 12 might be involved in the regulation of mucins (13). Zauber and colleagues demonstrated that 100% of low-grade mucinous tumors had a KRAS mutation either in codon 12 or 13, with G12D and G12V being the most common (42% each) (14). These authors also studied 42 normal appendices without any neoplasms and all of them were wild type for the KRAS gene. Recently, a group of researchers identified 58% of patients with PMP as positive for KRAS mutation (15). In that study, 89% of the mutations were in codon 12 and 11% in codon 13. In 2013, Nishikawa and colleagues identified GNAS mutations as common in LAMNs and suggested that mutant GNAS might play a direct role in mucin production (16).

Among the appendiceal adenocarcinoma cases with goblet cell features, we found no samples with KRAS mutations. This observation confirms previous findings that these tumors lack KRAS mutations (11). Our study also demonstrated that this group has mutations in the TP53 and SMAD4 genes. Rammani and colleagues showed that 25% of the appendiceal goblet cell carcinomas had a TP53 mutation (17). According to Stancu and colleagues, goblet cell carcinoids did not show mutations in SMAD4 (18). However, a study with CRC samples performed in 2013 by Fleming and colleagues demonstrated that 9% of sporadic CRCs
had an SMAD4 mutation (19). The authors demonstrated that one of the mutations identified (c.1082G>A, p.R361H) was a pathogenic mutation. None of the appendiceal adenocarcinomas with goblet cell features demonstrated KRAS or GNAS mutations. This may indicate that a different molecular pathway of carcinogenesis exists in this group of tumors.

GNAS is a member of the G-protein family, which modulates signals from transmembrane receptors to the cAMP pathway. Mutations in GNAS may cause the activation of adenylyl cyclase and an increase of cAMP levels. If this pathway is dysregulated, it can increase cell proliferation and contribute to the development and/or progression of cancer. It has also been suggested that a mutated GNAS gene is responsible for mucin production in LAMNs.

Conclusion

The next generation sequencing molecular profiling data presented in this study have identified mutation spectra in several subtypes of epithelial tumors of the appendix, suggesting substantial intertumor genomic heterogeneity which may contribute to phenotypic heterogeneity and which warrants further study. These findings contribute to a better understanding of the molecular pathogenesis of these tumor types, whereas previous studies have focused on single or small numbers of genes. This is the first study making use of a multigene panel using next generation sequencing technology in appendiceal cancers and may support the off-label use of targeted therapies.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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