ORIGINAL ARTICLE

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Comprehensive Cancer Panel Sequencing Defines Genetic Diversity and Changes in the Mutational Characteristics of Pancreatic Cancer Patients Receiving Neoadjuvant Treatment

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Background/Aims: Pancreatic ductal adenocarcinoma (PDA) is associated with an extremely poor prognosis. This study assessed the genetic diversity among patients with PDA and compared their mutational profiles before and after treatment. **Methods:** Tumors and matched blood samples were obtained from 22 PDA patients treated with neoadjuvant chemoradiation therapy. The somatic mutations were analyzed with comprehensive cancer gene panel (CCP). In addition, the biopsy samples obtained at diagnosis and the surgically resected samples after treatment were compared for seven patients. The CCP provided formalin-fixed paraffin-embedded sample-compatible multiplexed target selection for 409 genes implicated in cancer. **Results:** Assessments of the *MLH1*, *MLH3*, *MSH2*, and *PMS2* genes showed that the four patients with the highest relative burdens of mutations harbored somatic mutations in at least three of these genes. Genes in the histone-lysine N-methyltransferase 2 (KMT2) family, such as *KMT2D*, *KMT2A*, and *KMT2C*, were frequently mutated in tumor samples. Survival was worse in patients with *ARID1A* gene mutations than those without *ARID1A* gene mutations. Mutation patterns were compared between tissue samples before and after neoadjuvant treatment in seven patients who underwent surgical resection. The allelic fraction of mutations in *KRAS* codon 12 was lower in the surgically resected samples than in the endoscopic ultrasonography-guided fine needle aspiration biopsy samples of six patients. The number of mutant alleles of the histone lysine methyltransferase gene *WHSC1* also decreased after treatment. **Conclusions:** These results indicate that tumor tissue from PDA patients is genetically diverse and suggest that *ARID1A* mutations may be a potential prognostic marker for PDA. **(Gut Liver 2019;13:683-689)**

Key Words: Pancreatic neoplasms; ARID1A; Histone-lysine N-methyltransferase

INTRODUCTION

Pancreatic cancer is the fifth most common cancer among Korean males, with a steadily increasing incidence rate in recent decades in Korea.[1,2] At present, the crude annual incidence of pancreatic cancer in Korea is approximately 10.9 per 100,000 persons. Pancreatic ductal adenocarcinoma (PDA) is the predominant histologic type, accounting for about 85% of pancreatic cancers. Risk factors for pancreatic cancer include chronic pancreatitis, heavy smoking, and obesity, but there is no effective screening tool for early diagnosis.[3]

Pancreatic cancer is also one of the top five causes of cancer deaths worldwide, as well as having the lowest 5-year survival rate among solid tumors. In Korea, the 5-year survival rate of both men and women with pancreatic cancer between 2010 and 2014 was only 10.1%.[1] Current treatments for PDA include a combination of gemcitabine and nab-paclitaxel, or a combination of fluorouracil, irinotecan, oxaliplatin, and leucovorin (FOLFIRINOX).[4,5] Although several clinical trials have shown that these treatments improve survival among patients with PDA, the effects of treatment vary. Genetic features of PDA may help identify targets for treatment, as well as genetic markers associated with patient prognosis, thereby providing clinical benefits for patients with PDA.

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Activating mutations of the *KRAS* gene are frequently detected in PDAs, with these mutations considered a genetic factor associated with poor prognosis.[6,7] Genetic studies have reported the inactivation of tumor suppressor genes, such as *TP53*, *CDKN2A*, and *SMAD4*, in more than 50% of PDAs.[8,9] In addition, SWI/SNF-mediated chromatin remodeling, including alterations in the *ARID1A*, *KDM6A* and *SMARCA2* genes, has emerged as the basis for additional pathways affected by somatic mutations in PDA.[10,11]

Genome analyses of PDAs have revealed considerable genetic diversity and a complex mutational landscape, making it difficult to determine genetic features associated with patient prognosis. However, the clinical value of serial monitoring of mutation profiles in PDA is unknown. Mutation patterns affected by treatment may help identify treatment targets. Genetic characteristics defined by a comprehensive cancer gene panel (CCP) have been used to predict clinical benefits with an accuracy similar to that of whole exome sequencing.[12-14] This study assessed genetic diversity among patients with PDA and compared the mutational profiles before and after treatment.

MATERIALS AND METHODS

1. Patients and collection of clinical data

Tumor samples were obtained from surgically resected blocks from 14 patients who underwent preoperative chemoradiotherapy with gemcitabine and eight patients who received induction chemotherapy and chemoradiotherapy. All of these patients had been enrolled in two phase II clinical trials at the National Cancer Center of Korea (ClinicalTrials.gov numbers: NCT01333124 and NCT01593475, respectively). These trials were started in 2012, and their protocols were approved by the Institutional Review Board of the National Cancer Center of Korea (IRB numbers: NCCCTC-10-500 and NCCCTC-10-567, respectively). Written informed consents were obtained.

Fourteen patients with resectable pancreatic cancer received gemcitabine 400 mg/m^2 as an intravenous 30-minute infusion on days 1, 8, 15, 22, and 29, along with radiotherapy. Eight patients with locally advanced pancreatic cancer received induction chemotherapy, consisting of gemcitabine (1,000 mg/m^2) and cisplatin (25 mg/m^2) as intravenous infusions on days 1, 8 and 15 of each 28-day treatment cycle.[15] Within 3 weeks of completing two cycles of induction chemotherapy, patients were treated with gemcitabine alone (300 mg/m^2) as a 30-minute intravenous infusion once weekly during radiation therapy. Four to six weeks after the end date of chemo-radiotherapy, patients underwent preoperative evaluation, including computed tomography, positron emission tomography, and measurement of serum carbohydrate antigen 19-9 (CA19-9). If resection was deemed feasible, surgery was performed 1 to 2 weeks later. Demographic and clinical characteristics, including age, sex, cancer stage, were evaluated and analyzed.

Survival was estimated using the Kaplan-Meier method, with patients alive at the time of follow-up censored. Survival curves were constructed using Prism 5 (GraphPad software, La Jolla, CA, USA). Between-group differences in survival were assessed with the log-rank (Mantel-Cox) test.

2. Comprehensive cancer panel

Genomic DNA of patients was extracted from blood samples using QIAamp blood DNA mini kits (Qiagen, Valencia, CA, USA) and from formalin-fixed paraffin embedded (FFPE) endoscopic ultrasound (EUS)-guided biopsy samples, and surgically resected tissue using QIAamp DNA FFPE tissue kits (Qiagen). To prevent sequencing artifacts, DNA samples were treated with uracil-DNA glycosylase prior to amplification.[16,17] Targeted panel sequencing was performed with the Ion AmpliSeq Comprehensive

Table 1. Demographic and Clinical Characteristics of Patients with Pancreatic Ductal Adenocarcinoma

Characteristic	Patient
Total patients	22
EUS specimens	7
Surgically resected specimens	22
Sex	
Male	12 (54.5)
Female	10 (45.5)
Age at cancer diagnosis, yr	67 (51–76)
Smoking history	
Yes	12
No	10
Pack-years for smokers	38 (10–98)
Tumor size, mL	2.7 (0.6–5.8)
Tumor location	
Head	15
Body and tail	7
Endolymphatic tumor emboli	
Absent	14
Present	8
Blood vessel invasion	
Absent	13
Present	9
Pretreatment CA19-9, U/mL	82 (0–3,334)
Adjuvant chemotherapy	
Yes	12
No	10
Recurrence	20
Death	18
Follow-up time, mo	20 (5–59)

Data are presented as number (%) or median (range).
EUS, Endoscopic ultrasound; CA19-9, carbohydrate antigen 19-9.

Cancer Panel covering 409 genes (Ion Torrent, Life Technologies, Carlsbad, CA, USA). Libraries were prepared for sequencing according to the manufacturer's instructions, and the quality of the libraries was determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed using the platform Nextseq 500 System platform, with 2×151 bp paired end sequencing runs (Illumina Inc., San Diego, CA, USA).

Next-generation sequencing data were generated from 22 tumor samples, seven biopsy samples, and 22 blood samples from the 22 patients with PDA. The cancer panel covering 1.3 Mbp from 409 cancer-related genes produced about 16.4 M reads per sample. Reads with low quality were excluded from further analyses. The targeted average coverage was greater than 1200× for tumor samples and 500× for germline DNA.

3. Analysis of somatic mutations

Sequencing reads for the 409 target genes were processed using CASAVA base calling software version 1.8.2 (Illumina, Hayward, CA, USA). The read length was 150 bp, with insert sizes of 125 to 175 bp. Sequence index, quality score from FASTQ and individual Phred scores were assessed for sequencing quality. Sequences were analyzed with Genome Analysis Toolkit version 3.3 (Broad Institute, Cambridge, MA, USA)[18] after mapping with BWA tools.[19,20] Somatic mutations were designated by MuTect and Strelka after comparing sequences in tumor tissues with their corresponding blood samples.[21,22] Variants were annotated with SNPEff 4.1 (GRCh 37.75). The effects of variants were predicted with Polyphen2 and SIFT scores.[23,24] Allelic fractions of somatic mutations were compared in sequences obtained from EUS-guided biopsy and surgically resected tumor samples. Mutations were visualized using OncoPrint and MutationMapper by cBioPortal.[25,26]

RESULTS

1. Clinical characteristics of PDA patients and treatment outcomes

The demographic features of the 22 patients with PDA are summarized in Table 1. These 22 patients included 12 men and 10 women, of median age 67 years. Median follow-up time was 20 months (range, 5–59 months). Smoking history and tumor size were not significant predictors of poor prognosis, whereas lymphatic invasion, as determined by endolymphatic tumor emboli, was significantly associated with poor median survival (13 months vs 26 months, p=0.0037) (Supplementary Fig. 1).

2. Somatic mutation pattern and related survival characteristics of 22 PDA patients

Following the analysis of somatic mutations in these 22 patients, nonsense, missense, frameshift and splice site mutations were selected for further analyses because of the effects of these mutations on their encoded proteins. Four patients had a high mutation burden as shown in Fig. 1, averaging 1,545

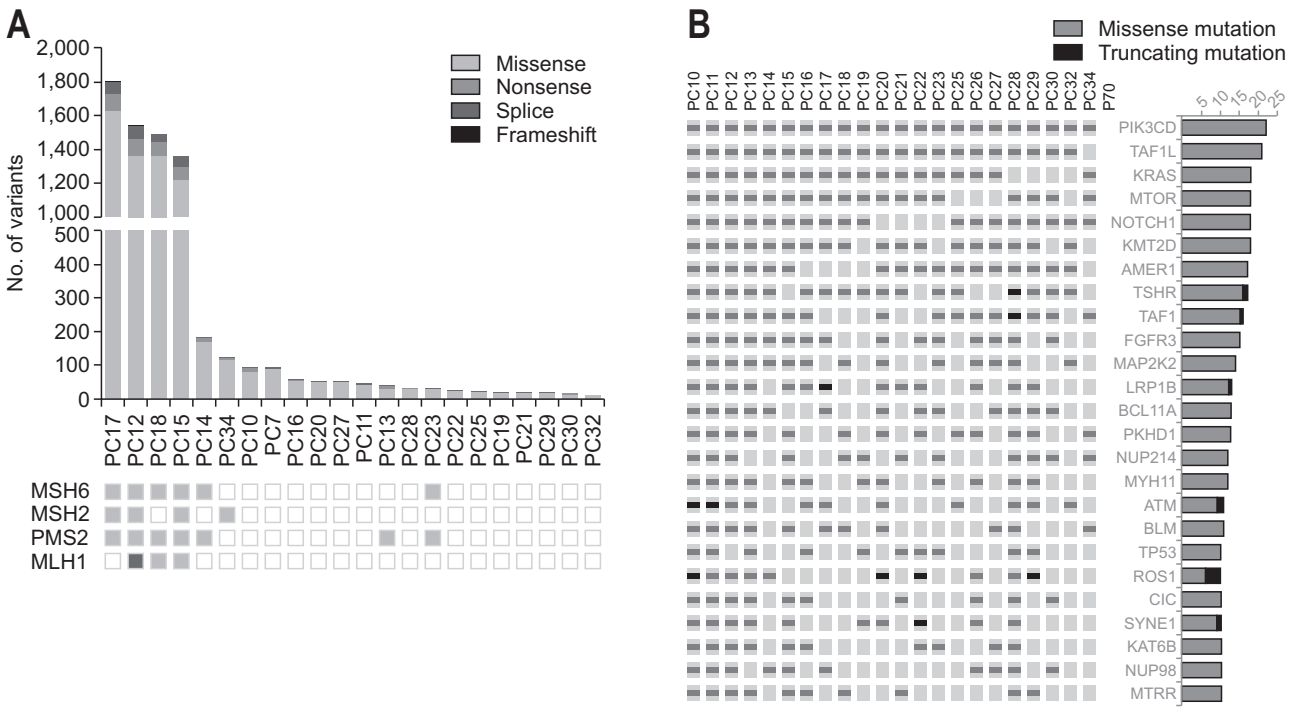

Fig. 1. Overall mutations from the cancer gene panel data of pancreatic ductal adenocarcinoma (PDA) patients. (A) Total number of variants in each of the 22 patients. Patients classified as hypermutated were enriched for mutations in mismatch repair genes. (B) Genes frequently altered in patients with PDA. The bar at the right shows the number of patients harboring alterations in each gene.

nonsynonymous mutations per patient, whereas the other 18 patients had a mutation burden of 50.7 nonsynonymous mutations per patient. To determine whether high mutation burden was associated with mismatch repair genes, mutations in the *MSH2*, *MSH6*, *MLH1*, *MLH3*, *POLE*, *EXO1*, and *PMS2* genes were examined. Assessments of the *MLH1*, *MLH3*, *MSH2*, and *PMS2* genes showed that the four patients with the highest relative burden of mutations harbored somatic mutations in at least three of these genes (Fig. 1). However, high mutational load was not associated with overall survival (Supplementary Fig. 2).

KRAS mutations, frequently observed in PDA, were detected in 18 of the 22 tumors (82%), with 17 having mutations in codon 12 and one in codon 61. *PIK3CD*, *TAF1L*, and *MTOR* were altered in most patients, whereas *TP53* was mutated in 12 patients. Genes altered in more than 10 patients and their major mutations are also shown in Fig. 1. We also tested the effect of somatic mutations in *TP53*, *KRAS*, and *ARID1A* on clinical outcome. Survival curves indicated that median survival was shorter in patients harboring *ARID1A* mutations than wild type (14 months vs 23.5 months, p=0.05) (Fig. 2). In contrast, *TP53* and *KRAS* mutations were not associated with survival (Supplementary Fig. 2).

Highly mutated genes showing high frequency and multiple mutation sites are shown in Table 2. Gene set enrichment analyses showed that these highly mutated genes included those involved in chromatin modification, such as *KMT2D*, *TAF1*, *ATM*, and *WHSC1*. Members of the histone-lysine N-methyltransferase 2 (KMT2) gene family, especially, *KMT2D*, *KMT2A*, and *KMT2C*, which are highly mutated in patients with mixed-lineage leukemia (MLL), were also highly mutated in our patients with PDA (Supplementary Fig. 3).

3. Genetic diversity before /after treatment in seven patients

The somatic mutation patterns were compared in biopsy samples obtained at diagnosis of seven patients and in their surgically resected samples after treatment. Of these, three had resectable tumors at initial diagnosis. The remaining four had locally advanced cancer. Somatic mutations with a reduced allelic fraction (<1.5-fold) after compared to before treatment in more than three patients are shown in Table 3. The allelic fraction of *KRAS* codon 12 mutations was lower in surgically resected specimens than in EUS samples of six patients, and the allelic fraction of the codon 1020 mutation P1020A in the histone lysine methyltransferase gene *WHSC1* was lower after than before treatment in four patients.

DISCUSSION

Pancreatic cancer is one of the most common causes of cancer deaths worldwide, with a very poor 5-year survival rate. Due to asymptomatic progress and a lack of effective screening markers, most patients are diagnosed at an advanced stage. Current chemotherapy regimens for patients with advanced pancreatic cancer frequently include gemcitabine, but treatment responses vary widely among patients, due to the molecular genetic diversity of individual tumors.[27,28]

This study analyzed the somatic mutations of 22 Korean patients with PDA using CCP. All patients received neoadjuvant treatment inside clinical trial. Four of these patients showed a hypermutated pattern, with more than 1,000 mutations in mismatch repair genes, a finding consistent with the mismatch repair deficiency and "mutator" phenotype of PDA.[29] Mutation burden was unrelated to smoking history or overall survival in

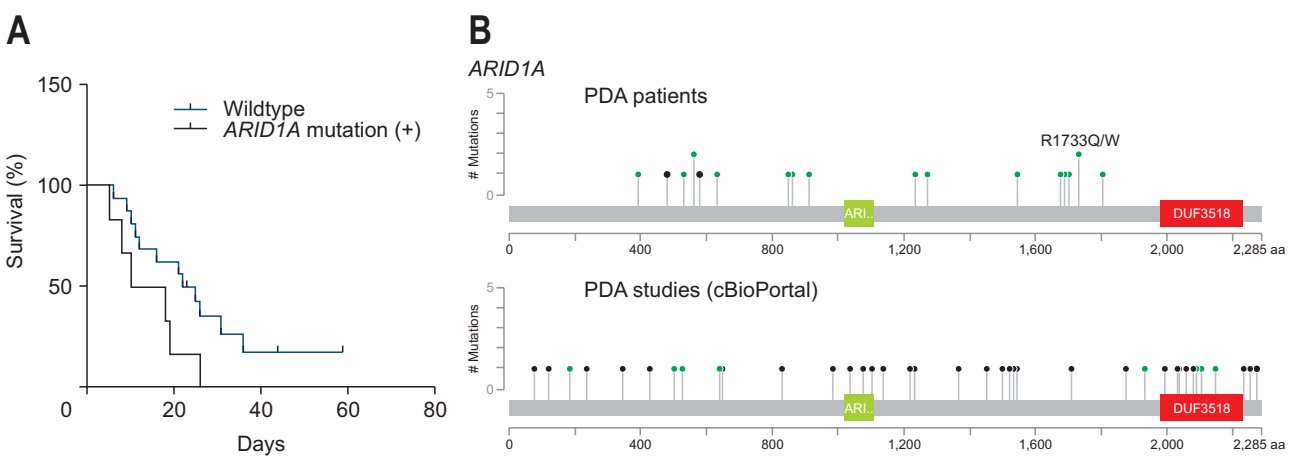

Fig. 2. *ARID1A* gene mutations and their association with disease outcome. (A) Kaplan-Meier analysis of overall survival in patients with and without *ARID1A* mutations. Median survival was shorter in patients harboring *ARID1A* mutations than in those with wildtype *ARID1A* (14 months vs 23.5 months, p=0.05). (B) Comparison of *ARID1A* gene mutations detected in our patients and those in pancreatic ductal adenocarcinoma (PDA) patients listed in cBioPortal. Gray indicates missense mutations, and black indicates truncating mutations, including nonsense and frameshift mutations.

Table 2. Genes Highly Mutated in Patients with Pancreatic Ductal Adenocarcinoma

GSEA	Gene	Description	Total score*	Frequent mutant type
Chromatin modification	KMT2D	Lysine (K)-specific methyltransferase 2D	131	L449Q
	KMT2A	Lysine (K)-specific methyltransferase 2A	81	
	TRRAP	Transformation/transcription domain-associated protein	78	
	KMT2C	Lysine (K)-specific methyltransferase 2C	68	
	TAF1L	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 210kDa-like	66	R1243Q
	TAF1	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250kDa	65	
	KDM5C	Lysine (K)-specific demethylase 5C	47	
	KAT6B	K(lysine) acetyltransferase 6B	46	
	ATM	ATM serine/threonine kinase	45	Q14R
	EP400	E1A binding protein p400	43	
	TET2	Tet methylcytosine dioxygenase 2	43	
	EP300	E1A binding protein p300	42	
	WHSC1	Wolf-Hirschhorn syndrome candidate 1	40	
Protein kinase activity	MTOR	Mechanistic target of rapamycin (serine/threonine kinase)	70	T600I
	IGF2R	Insulin-like growth factor 2 receptor	58	
	ROS1	ROS proto-oncogene 1, receptor tyrosine kinase	44	W729*
Cell cycle	DST	Dystonin	71	I761M
	USP9X	Ubiquitin specific peptidase 9, X-linked	55	R882C
	NUP98	Nucleoporin 98kDa	46	
	AKAP9	A kinase (PRKA) anchor protein (yotiao) 9	45	
	NOTCH2	Notch 2	42	
	APC	Adenomatous polyposis coli	41	
	NUP214	Nucleoporin 214kDa	41	
	SYNE1	Spectrin repeat containing, nuclear envelope 1	152	L1632P
	RNF213	Ring finger protein 213	104	
	PKHD1	Polycystic kidney and hepatic disease 1	74	
	LRP1B	Low density lipoprotein receptor-related protein 1B	63	I321S
	FN1	Fibronectin 1	61	
	PTPRT	Protein tyrosine phosphatase, receptor type, T	57	L1214P
	CSMD3	CUB and Sushi multiple domains 3	55	
	UBR5	Ubiquitin protein ligase E3 component n-recognin 5	55	
	MYH11	Myosin, heavy chain 11, smooth muscle	49	T1546M

*Total score included total number of mutations located in the same gene in all patients.
GSEA, gene set enrichment analysis.

this patient cohort. Because sample size is not large enough to ensure an adequate power to detect statistical significance, we cannot draw any firm conclusion.

Mutation profiling of patients with PDA has reported recurrent mutations in *KRAS*, *TP53*, *SMAD4* and *ARID1A*, all of which have been associated with patient prognosis.[10,29] Our results showed that mutations in *ARID1A* tended to be associated with poor prognosis, whereas mutations in *KRAS* and *TP53* were not. Mutations in *ARID1A* present before treatment decreased or disappeared after treatment in two patients. These results provide further evidence suggesting that *ARID1A* mutations may be a prognostic marker in patients with PDA.

We also found that some frequently mutated genes were involved in chromatin modification. Members of the histone lysine methyltransferase KMT2 (MLL) family of genes, including *KMT2D*, *KMT2C*, and *KMT2A*, showed a high frequency of alterations, including nonsynonymous and nonsense mutations. Interestingly, mutations in *MLL*, *MLL2*, and *MLL3* were closely associated with survival outcomes in patients with PDA,[30] suggesting that *MLL* mutation status was an independent prognos-

Table 3. Somatic Mutations with Decreased Allelic Fractions after Treatment in at Least 3 Patients

Gene	Description	Protein change	Patient
KRAS	Kirsten rat sarcoma viral oncogene homolog	G12*	6
WHSC1	Wolf-Hirschhorn syndrome candidate 1	P1020A	4
CDK6	Cyclin-dependent kinase 6	p.N284H	3
DDB2	Damage-specific DNA binding protein 2, 48kDa	p.W54L	3
EP300	E1A binding protein p300	p.G98A	3
ERCC3	Excision repair cross-complementation group 3	p.V193L	3
FBXW7	F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase	p.A105S	3
FLT3	Fms-related tyrosine kinase 3	p.R655G	3
KAT6A	K(lysine) acetyltransferase 6A	p.M1389L	3
KAT6B	K(lysine) acetyltransferase 6B	p.Q1513E	3
KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)	p.C246S	3
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	p.E258Q	3
PSIP1	PC4 and SFRS1 interacting protein 1	p.A168G	3
TET2	Tet methylcytosine dioxygenase 2	p.M1789I	3
XPA	Xeroderma pigmentosum, complementation group A	p.L226W	3
ZNF521	Zinc finger protein 521	p.D25E	3

*G12 indicates various mutant types on codon 12 of the *KRAS* gene including G12D, G12V and G12S.

tic factor associated with survival. Our study could not confirm these results, as only three of 22 patients lacked mutations in the *KTM2* genes.

Our comparison of somatic mutation patterns in EUS-guided biopsy specimens collected at initial diagnosis and surgical specimens collected after concurrent chemo-radiotherapy showed a reduction in the allelic fraction of mutations in *KRAS* and *WHSC1* after treatment. Decreased *KRAS* mutant fraction after treatment suggests the favorable response of neoadjuvant therapy. Furthermore, we demonstrated the feasibility of EUS-guided biopsy samples for mutation profiling that can also be compared with the resected tissues. However, this finding requires confirmation in future studies comparing paired samples from a larger nubmer of patients. Indeed, the primary limitation of our study was the small number of PDA patients. Moreover, clinical follow-up was relatively short. Further study and validation will be needed to determine the utility of the detected alterations in PDAs. Nevertheless, our study could determine that PDAs are genetically complex and that *ARID1A* mutations may be prognostic of survival.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

Study concept and design: K.A.Y., S.M.W., S.J.P. Data acquisition: K.A.Y., S.M.W., S.J.P. Data analysis and interpretation: K.A.Y., S.M.W., M.K.L., S.S.H., T.H.K., W.J.L., S.J.P. Drafting of the manuscript: K.A.Y., S.M.W. Statistical analysis: K.A.Y., M.K.L. Obtained funding: K.A.Y., S.J.P. Critical revision of the manuscript for important intellectual content: S.M.W., Y.H.K., S.Y.K., S.S.H., T.H.K., W.J.L., S.J.P. Administrative, technical, or material support: Y.H.K., S.Y.K., M.K.L., S.S.H., T.H.K., W.J.L., S.J.P. Study supervision: Y.H.K., S.Y.K., S.S.H., T.H.K., W.J.L., S.J.P.

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