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BRAF Rearrangements and *BRAF* V600E Mutations Are Seen in a Subset of Pancreatic Carcinomas With Acinar Differentiation

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• ***Context.***—**Comprehensive genomic profiling has demonstrated that approximately 20% of pancreatic carcinomas with acinar differentiation harbor potentially targetable *BRAF* fusions that activate the MAPK pathway.**

Objectives.—**To validate the above finding by *BRAF* break-apart fluorescence in situ hybridization (FISH) in a large series of pure acinar cell carcinomas (ACCs), evaluate tumors for the presence of *BRAF* V600E mutations, and compare clinicopathologic features of tumors with *BRAF* rearrangements with those without.**

Design.—**Thirty cases of pure ACC and 6 cases of mixed acinar-neuroendocrine carcinoma (ACC-NEC) were retrieved. A break-apart FISH probe was used to detect *BRAF* rearrangements. Immunohistochemistry for *BRAF* V600E was performed.**

Results.—***BRAF* rearrangements by FISH were found in 6 of 36 cases (17%), 5 of which were pure ACC and 1 was a mixed ACC-NEC. Follow-up was available in 29 of 36 (81%). The median survival was 22 months for *BRAF*-rearranged cases and 16 months for *BRAF*-intact cases; the 2-year overall survival was 50% for *BRAF*-rearranged cases and 35% for *BRAF*-intact cases. No significant clinicopathologic differences were identified in cases with *BRAF* rearrangement compared with those without *BRAF* rearrangement. *BRAF* V600E mutation was identified in 2 of 34 cases (6%), both of which were pure ACC and were *BRAF*-intact by FISH.**

Conclusions.—**This study supports the finding that *BRAF* rearrangements are present in approximately 20% of cases and identified *BRAF* V600E mutations in approximately 5% of cases. These cases may benefit from targeted therapy.**

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Pancreatic acinar cell carcinoma (ACC) is a rare, aggressive malignant epithelial pancreatic neoplasm that portends a grave 5-year survival of approximately 40% in resected cases and 20% in unresectable cases.[1,2] ACC comprises approximately 1% to 2% of all adult pancreatic neoplasms and 15% of all pediatric pancreatic neoplasms and is characterized by exocrine enzyme production, which can be demonstrated by immunohistochemical expression of trypsin, chymotrypsin, amylase, lipase, and carboxyl ester hydrolase (BCL10 antibody, clone 331.3).[3] Up to one third may be focally positive for neuroendocrine markers such as chromogranin A and synaptophysin. ACC are twice as frequent in males as in females and present over a wide age range. There is a slight predilection for the head of the pancreas, and presenting symptoms are usually referable to a mass, including abdominal pain, diminished appetite, weight loss, nausea, vomiting, and less commonly, jaundice. This is not surprising, because the tumors are generally large, averaging 10 cm. Macroscopically, ACC appears soft, fleshy, often multinodular, and relatively well circumscribed. Up to 10% of patients may develop lipase hypersecretion syndrome, which manifests as a constellation of fat necrosis, polyarthralgias, and peripheral eosinophilia.[3,4] Current standard management includes surgical resection and palliative radiotherapy and chemotherapy.[5]

While driver mutations underlying pancreatic ductal carcinoma have been well-established, including *KRAS*, *TP53*, *CDKN2A*, and *SMAD4*, these mutations are infrequent in pancreatic ACC, and the molecular pathophysiology of pancreatic tumors with acinar differentiation has only

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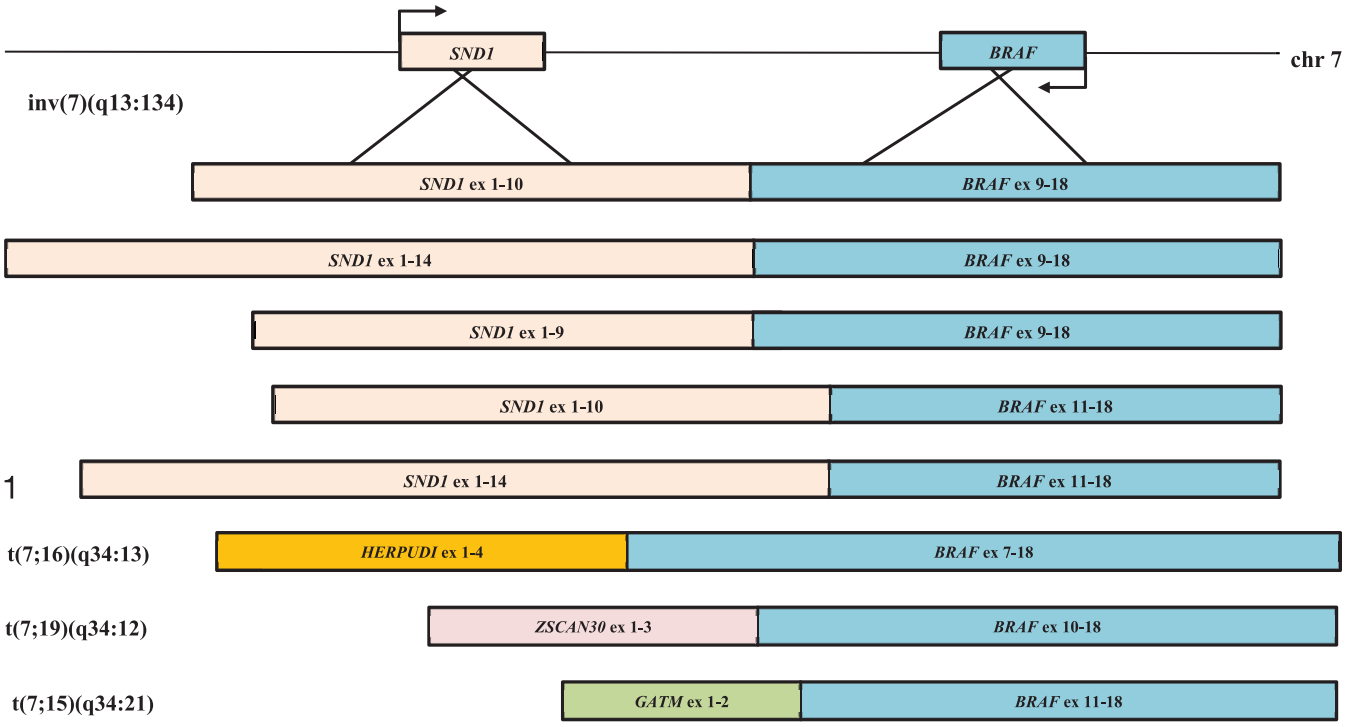

Figure 1. *All variant* SND1-BRAF *fusion genes resulting from inversions of chromosome 7 (chr 7) fusing the thermonuclease domain of* SND1 *with the serine/threonine kinase domain of* BRAF. *All of these variants are able to be detected by the fluorescence in situ hybridization probe developed in this study. Abbreviations: ex, exons; inv, inversion.*

Figure 2. *Additional fusions formed by translocations with* BRAF *and genes from other chromosomes are able to be detected by this fluorescence in situ hybridization probe, as well. Abbreviations: ex, exons; t, translocation.*

recently been examined.[6,7] In a comprehensive genomic profiling study, approximately 20% of pancreatic ACC cases were shown to harbor recurrent *BRAF* and *RAF1* (*CRAF*) rearrangements.[8] A study by Prall et al[9] confirmed the presence of *RAF1* rearrangements in 5 of 30 pancreatic tumors with acinar differentiation, indicating that the MAPK pathway has a recurrent role in such tumors. In addition, these findings suggest that ACC bearing these alterations may be amenable to *BRAF* and/or MEK inhibition.[9] While survival is improved with surgical management, only a minority of patients respond sustainably to chemotherapy and radiotherapy.[5] This underscores the value of identifying novel recurrent targets for inhibition.

To this end, this study sought to validate the findings of recurrent *BRAF* gene rearrangements with the development and utilization of a custom developed break-apart fluorescence in situ hybridization (FISH) probe, compare clinicopathologic characteristics of ACCs with *BRAF* rearrangements to those without *BRAF* rearrangements and evaluate for *BRAF* V600E mutations in these tumors.

METHODS

Cases

Thirty cases of pure ACC and 6 cases of mixed ACC-NEC were identified between 1985 and 2016 in adult patients, retrieved from the authors' institutions. The histologic sections of all cases underwent review by 7 submitting pathologists. The cases comprised 25 partial or total pancreatectomy specimens and 11 biopsy specimens (including primary and metastatic biopsy specimens). A representative tissue block or unstained formalin-fixed, paraffin-embedded tissue sections were selected in each case for ancillary immunohistochemical studies and FISH. Clinical data were extracted from the patients' medical records.

Immunohistochemistry was performed using commercially available antibodies on a Ventana Benchmark XT using standard laboratory protocols: trypsin (Biodesign, polyclonal), BCL10 (Santa Cruz, clone 331.1) chromogranin A (Ventana, clone LK2H10), synaptophysin (Leica (Novocastra), clone 27G12**)**, keratin 7 (Dako, clone OV-TL 12/30), and *BRAF* V600E (Spring, clone VE1). Cases that were positive for *BRAF* V600E by immunohistochemistry were subjected to a targeted *BRAF* V600E polymerase chain reaction assay using a previously published method.[10]

Mixed ACC-NEC were defined as neoplasms with at least 30% identifiable acinar and neuroendocrine components.

Interphase Break-Apart FISH

A break-apart FISH probe was designed to detect *BRAF* rearrangements, including all fusions previously described by comprehensive genomic profiling (Figures 1 and 2),[8] and was tested on all cases.

BRAF rearrangement was analyzed with a break-apart FISH probe set. Human bacterial artificial chromosomes flanking the *BRAF* gene region were identified using the University of California Santa Cruz February 2009 Assembly hg19. The 3′*BRAF* clones (RP11-577C22, RP11-96I22 and RP4-592P3) were labeled by nick translation with Spectrum Orange dUTP (Abbott Molecular/Vysis Products), and the 5′*BRAF* clones (CTD-2023L14, CTD-2655E10 and RP11-145N8) were labeled with SpectrumGreen dUTP (Abbott Molecular/Vysis Products). Labeled clones were combined to create a dual-color fusion break-apart probe set. The break-apart probe set was applied to individual slides, hybridized, and washed according to the Partially Automated Tissue Reduced Pepsin FISH

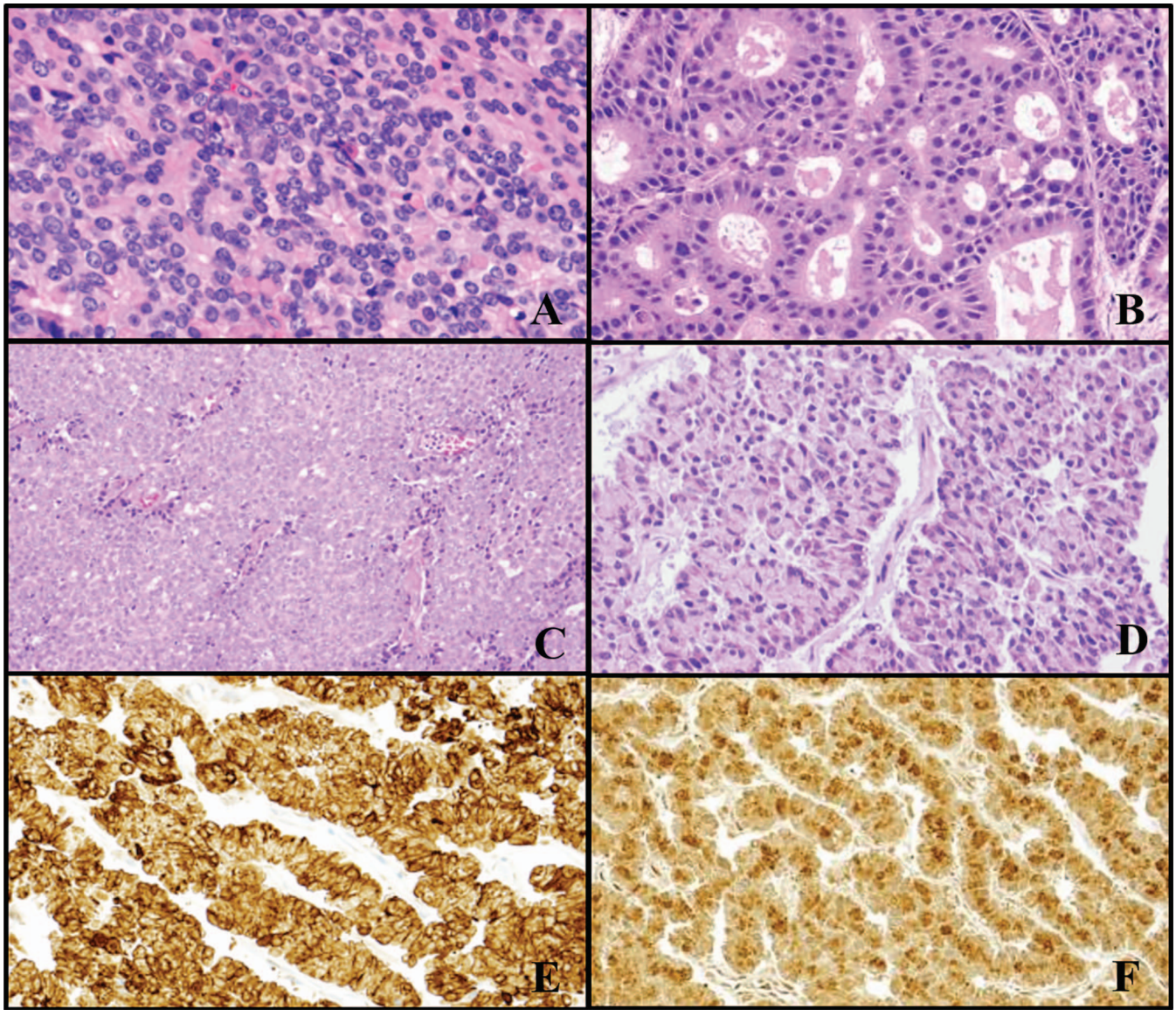

Figure 3. *Representative architectural patterns and immunohistochemical profile of pancreatic acinar cell carcinoma in our cohort. A, The classic acinar pattern demonstrates round-to-oval neoplastic cells with characteristic prominent nucleoli and abundant eosinophilic, granular cytoplasm. B, The "glandular" pattern depicts more dilated acinar formations. C, The solid pattern shows diffuse sheet-like growth. D, The trabecular pattern displays anastomosing islands of neoplastic cells with basally palisaded nuclei. E and F, The neoplastic cells demonstrate strong and diffuse immunohistochemical expression of keratin 7 and trypsin (hematoxylin-eosin, original magnification ×40 [A through D]; keratin 7, original magnification ×40 [E]; trypsin, original magnification ×40 [F]).*

protocol. Slide processing was done as previously reported by authors from our group.[11]

The slides were analyzed by 2 technologists using standard fluorescence microscopy methods. Each technologist independently scored 50 qualifying tumor nuclei (including both acinar and neuroendocrine tumor cells in mixed cases) for each sample, and the results were reviewed by 2 other authors. A positive *BRAF* rearrangement constituted at least 10% break-apart nuclei.

Statistical Analysis

Data were summarized as frequencies and percentages and medians or means and ranges (as appropriate). Comparisons between the *BRAF* FISH-positive and FISH-negative cases were performed using Fisher exact tests for categoric variables and with Wilcoxon rank-sum tests for continuous or ordinal variables. Median survival (along with the 95% CI) and the estimated 2-year survival was summarized with the Kaplan-Meier method. All analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina).

RESULTS

The cases in this cohort showed a male predilection (26 of 36; 72%). The mean age at diagnosis was 64 years (SD, 12.9). The median maximum tumor dimension in the *BRAF*-rearranged cohort was 4.5 cm (range, 3–7 cm), and the median maximum tumor dimension in the *BRAF*-intact cohort was 4.8 cm (range, 1.6–29 cm). The majority of cases displayed a mixture of architectural patterns, including acinar, trabecular, glandular, and solid (Figure 3, A through F). Keratin 7 was positive in 23 of 28 cases (82%) and focally positive in 1 of 28 (4%) for which sufficient material was available for immunohistochemistry.

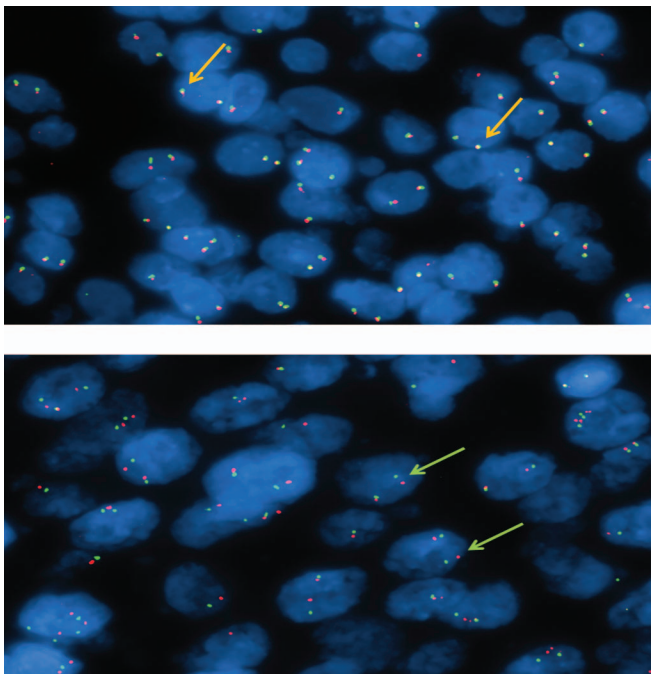

Figure 4. *Panels demonstrating intact and rearranged* BRAF *fluorescence in situ hybridization results. A, A normal result with 2 intact copies of the* BRAF *locus in each nucleus, as depicted by 2 yellow signals or 2 closely positioned red and green signals (orange arrows highlight intact signals). B,* BRAF *rearrangement with separation of the red and green signals (green arrows highlight split signals).*

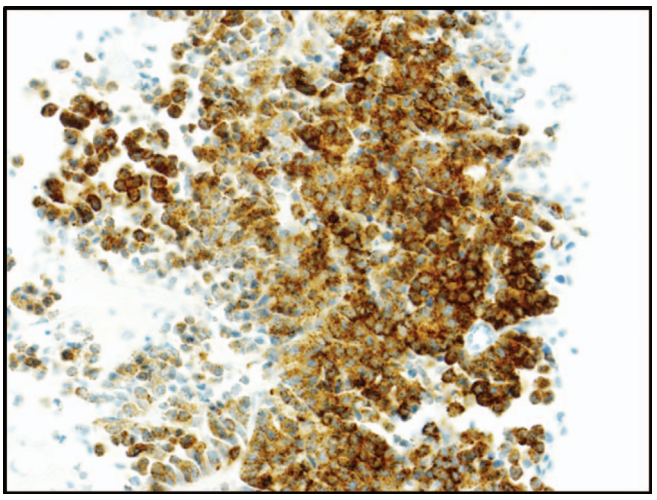

Figure 5. BRAF *V600E mutation by immunohistochemistry. This was identified in 2 cases, 1 was confirmed by polymerase chain reaction–based fragment analysis. Both cases were negative for* BRAF *rearrangement by fluorescence in situ hybridization and were cases of pure acinar cell carcinoma (original magnification ×40).*

BRAF Rearrangements

BRAF rearrangements were identified in 6 of 36 cases (17%), 5 of which were pure ACC and 1 of which was a mixed ACC-NEC (Figure 4, A and B). All of our cases demonstrated at least 60% of tumor nuclei showing break-apart signals (range, 66%–98%). The Table shows clinicopathologic comparisons between cases with *BRAF* rear-

Clinicopathologic Characteristics of Carcinomas With Acinar Differentiation With and Without FISH-Detected *BRAF* Gene Rearrangements[a]

Clinicopathologic Feature	*BRAF* Rearranged (n = 6)	*BRAF* Intact (n = 30)	*P* Value
Mean age (range)	71 (63–78)	63 (37–88)	.14
Sex (M:F)	1:1	3.3:1	.32
Tumor location	Head: 3 Body: 1 Body/tail: 1 Tail: 1	Head: 8 Head/body: 2 Neck/body: 1 Body: 1 Body/tail: 3 Tail: 9 Metastasis/primary location in pancreas unknown: 6	
Median maximum tumor size (range)	4.5 cm (3–7) (6 cases)	4.2 cm (1.6–29) (28 cases)	.93
Lymph node metastasis[b]	3/6	7/21	.64
Distant metastasis[b]	0/6	12/26	.06
Necrosis	4/6	14/27	.66
Lymphovascular invasion	6/6	15/27	.06
Perineural invasion	2/6	6/27	.62
Median mitoses/10 hpf (range)	2.5 (0–16) (6 cases)	9.5 (0–88) (28 cases)	.20
Keratin 7 expression	5/5	18/23	.64
Available follow-up (No. of deaths[c])	4 (3)	25 (19)	
Median survival, mo	22 (95% CI: 9.0–NA)	16 (95% CI: 9.0–26.0)	.64
2-year overall survival	50.0% (95% CI: 1.0%–99.0%)	35.4% (95% CI: 15.8%–55.0%)	

Abbreviation: NA, not available (data too sparse to estimate upper end of CI).

[a] Data were collected in available cases; some were consultation cases in which material was returned, and therefore data were unable to be obtained. Chromogranin and synaptophysin-positive cases constituted ≥30% positively staining cells; focally positive cases constituted 10%–29% positively staining cells. Trypsin and CK7-positive cases constituted ≥10% positively staining cells; focally positive cases constituted 5%–9% positively staining cells.

[b] At diagnosis.

[c] All deaths were due to disease.

rangement and those without. Cases designated as "*BRAF*-rearranged" refer to cases showing rearrangement by FISH, all of which were negative for *BRAF* V600E by immunohistochemistry.

Follow-up data were available in 29 of 36 of cases (81%). The median survival was 22 months for *BRAF*-rearranged cases and 16 months for *BRAF*-intact cases; the 2-year overall survival was 50% for *BRAF*-rearranged cases and 35% for *BRAF*-intact cases (Table).

BRAF V600E

Two cases were strongly and diffusely positive for *BRAF* V600E by immunohistochemistry (Figure 5) and negative for *BRAF* rearrangement by FISH. Both *BRAF* V600E mutant cases were pure ACC. Confirmatory polymerase chain reaction was successful in 1 case, and was positive for *BRAF* V600E. The other case failed sequencing due to poor-quality DNA, and there was no residual material from the small biopsy specimen. Both *BRAF* V600E-positive cases had metastatic disease at presentation, with the survival intervals measuring 8 and 22 months.

DISCUSSION

Our study validates the finding that *BRAF* rearrangements are seen in approximately 10% to 20% of pancreatic carcinomas with acinar differentiation, corroborating findings from both prior comprehensive genomic profiling studies and studies of *BRAF* rearrangements by FISH.[6,12,13] We furthermore identified *BRAF* V600E mutations that did not co-occur in the cases with *BRAF* rearrangements. Others have identified *BRAF* V600 alterations as part of whole-exome sequencing.[14,15] and genome-wide studies.[7] However, *BRAF* fusions were not detected in the whole-exome sequencing studies. Although the finding of *BRAF* fusions and *BRAF* point mutations is not new, there are only few studies on this biomarker with slightly different designs. Further, our study analyzed a relatively large number of cases and compares the clinicopathologic features between *BRAF*-rearranged tumors and *BRAF*-intact tumors. In our cohort, no statistically significant clinicopathologic difference was identified between *BRAF*-rearranged and *BRAF*-intact tumors.

This is the third study specifically examining *BRAF* rearrangements in pancreatic neoplasms with acinar differentiation. The first study included 44 pancreatic carcinomas with acinar differentiation, including 16 pure ACCs, 14 mixed ACC-NEC, and other mixed variants. Twenty percent of the tumors demonstrated recurrent *BRAF* gene rearrangements.[8] The most prevalent fusion genes were *SND1-BRAF* (50%), followed by *HERPUD1-BRAF* (18%), which may be amenable to MEK inhibition.[8,13] The same group then developed a *BRAF* break-apart FISH assay.[13] The second study by Prall et al[9] examined 25 pure ACCs, 2 mixed ACC-NEC, 1 mixed ACC-NET, and 2 pancreatoblastomas and identified *RAF1* rearrangements in 18.5% of cases and *BRAF* rearrangements in 2 of 28 cases (7%) (exclusive of pancreatoblastoma), with 1 *BRAF*-rearranged case also harboring *RAF1* rearrangement.

Our study provides a likely therapeutically targetable finding that adds to the data published in the prior studies.[8,9,13,14,16,17] *BRAF* V600E is a targetable alteration in several malignancies, including malignant melanoma,[18] hairy cell leukemia,[19] and colorectal carcinoma.[20] In cancer, *BRAF* alterations are targetable by *BRAF* inhibitors, but also may be targeted by MEK inhibition or combined *BRAF* and MEK inhibition, as these effectors are all part of the MAPK pathway.[20,21] This raises the possibility that *BRAF* inhibition or combination therapy may be effective in acinar cell carcinoma; this merits further study.

The frequency of *BRAF* rearrangements in our study is similar to that identified by Chmielecki et al[8] (20%) and somewhat higher than that identified by Prall et al[9] (7%). The low sample sizes of *BRAF*-rearranged cases likely accounts for these differences. These studies did not report clinicopathologic correlations between *BRAF*-rearranged tumors and those without *BRAF* rearrangements, and so comparison in this regard is not possible. Considering the findings of our study along with those of Chmielecki et al[8] and Prall et al,[9] pathogenic alterations involving *BRAF* or another member of the MAPK pathway underlie a significant subset of cases of ACC and suggests that these cases should be screened for pathogenic alterations in this pathway.

In this study, we developed and tested an efficient and economic FISH probe that can identify potentially targetable gene rearrangements. Currently, transcriptome sequencing for *BRAF* genetic rearrangements is not widely available and, in most institutions, has not been optimized for the most common biospecimen, formalin-fixed, paraffin-embedded tissue. As such, this FISH probe may be a useful clinical test to determine patients who may benefit from therapy. One advantage of this FISH assay is that it is informative on very small biopsy specimens and can be performed on a single unstained slide, unlike many sophisticated molecular assays. Next-generation sequencing provides the opportunity for a single assay to detect both *BRAF* gene rearrangements and point mutations, creating efficiency and analytic sensitivity in a cost-effective way. This is because next-generation sequencing can be designed to detect not only point mutations, such as polymerase chain reaction–based assays, but can also, using bioinformatics tools, recognize sequence changes that correspond to large structural rearrangements, such as fusions, or in RNA-based next generation sequencing, directly identify the fusion transcripts.

This study design does have a limitation based on use of FISH as the sole methodology. We note that false positives may occur with FISH, such that FISH signals may be disrupted by large genomic changes and not indicate an in-frame functional fusion gene. Therefore, *BRAF* rearrangements identified by this method do not necessarily indicate the presence of an in-frame fusion oncogene. Nonetheless, the frequency of *BRAF* rearrangements identified by our methodology accords well with the previously published frequencies of *BRAF* fusions in other studies.[8,9] This suggests that despite this study design limitation, our findings were valid. Our study was also limited by the lack of additional formalin-fixed, paraffin-embedded tissue to molecularly confirm the second *BRAF* V600E immunohistochemistry positive case. The strong and diffuse expression of the immunostain similar to that seen in the other case (and as shown in Figure 5), which was molecularly confirmed, suggests that this finding likely corresponds to the molecular event.

BRAF fusion genes and *BRAF* activating point mutations are alternate ways to achieve activation of the MAPK pathway. Therefore, it is not surprising that we identified cases positive for *BRAF* V600E by immunohistochemistry. This pattern of MAPK pathway activation by either *BRAF* fusion genes or *BRAF* point mutations has been previously identified in thyroid tumors, melanomas, and other tumors as described by Ross et al.[17]

In conclusion, in this series of pancreatic carcinomas with acinar differentiation, which includes the largest series of pure ACCs evaluated to date for *BRAF* rearrangements by FISH, we confirmed that *BRAF* rearrangements are present in approximately 20% of these tumors. We discovered that a subset of cases of ACC harbor *BRAF* V600E mutations, mutually exclusive of *BRAF* rearrangements. This study adds to the literature that suggests cases of pancreatic ACC should be examined for MAPK abnormalities and studies of therapeutic sensitivity to *BRAF* and MAPK pathway inhibition are timely.

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