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Sequencing of 279 cancer genes in ampullary carcinoma reveals trends relating to histologic subtypes and frequent amplification and overexpression of *ERBB2* (*HER2*)

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The biological relevance of histological subtyping of ampullary carcinoma into intestinal *vs* pancreaticobiliary types remains to be determined. In an effort to molecularly profile these subtypes of ampullary carcinomas, we conducted a two-phase study. In the discovery phase, we identified 18 pancreatobiliary-type ampullary carcinomas and 14 intestinal-type ampullary carcinomas using stringent pathologic criteria and performed next-generation sequencing targeting 279 cancer-associated genes on these tumors. Although the results showed overlapping of genomic alterations between the two subtypes, trends including more frequent *KRAS* alterations in pancreatobiliary-type ampullary carcinoma (61 *vs* 29%) and more frequent mutations in *APC* in intestinal-type ampullary carcinoma (43 *vs* 17%) were observed. Of the entire cohort of 32 tumors, the most frequently mutated gene was *TP53* ($n = 17$); the most frequently amplified gene was *ERBB2* ($n = 5$); and the most frequently deleted gene was *CDKN2A* ($n = 6$). In the second phase of the study, we aimed at validating our observation on *ERBB2* and assessed *ERBB2* amplification and protein overexpression in a series of 100 ampullary carcinomas. We found that (1) gene amplification and immunohistochemical overexpression of ERBB2 occurred in 13% of all ampullary carcinomas, therefore providing a potential target for anti-HER2 therapy in these tumors; (2) amplification and immunohistochemical expression correlated in all cases, thus indicating that immunohistochemistry could be used to screen tumors; and (3) none of the 14 *ERBB2*-amplified tumors harbored any downstream driver mutations in *KRAS*/*NRAS*, whereas 56% of the cases negative for *ERBB2* amplification did, an observation clinically pertinent as downstream mutations may cause primary resistance to inhibition of EGFR family members.
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Ampullary adenocarcinoma is a rare and heterogeneous malignancy occurring in 0.7 per 10 000 males and 0.4 per 10 000 females in the United States annually.[1] Prognosis is generally dismal, with 5-year survival ranging from 4% in patients with distant metastases to 45% in stage 1 patients (SEER data).[1] Because it forms at the junction of intestinal-type duodenal and pancreatobiliary-type ductal epithelium, ampullary carcinoma can have heterogeneous differentiation reflecting either or both of these types.[2,3] It has recently been shown that subtyping based on morphology, immunohistochemistry, and mRNA levels affects prognosis: patients with intestinal-type ampullary adenocarcinoma have a longer median overall survival of 70 months in comparison with the pancreatobiliary-type ampullary adenocarcinoma group, which has a median overall survival of 28 months.[4–6]

Recently, some oncologists have started treating ampullary carcinoma based on histologic subtype, using gemcitabine-based regimen for pancreatobiliary type and fluorouracil-based regimen for intestinal type. However, ~12% of cases have mixed intestinal and pancreatobiliary differentiation[2] and cannot be subtyped definitively into one category.

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Whether or not histologic subtype and cases with mixed differentiation have specific genetic signatures, and the influence of those signatures on prognosis and treatment response, remains to be investigated. In this study, we aimed to assess (1) whether histologic subtype correlated with differences in the somatic mutational and copy number profiles of 279 cancer-related genes; and (2) the targetable alterations that occur most frequently in ampullary carcinoma and their clinicopathologic and molecular correlates.

Materials and methods

Case Selection

After approval from our institutional review board, a discovery set and validation set were selected as follows. For the discovery set, unambiguous examples of 14 intestinal-type ampullary carcinomas and 18 pancreatobiliary-type ampullary carcinomas with matched normal tissues were selected for next-generation sequencing. Determination of intestinal *vs* pancreatobiliary subtype was performed on the basis of morphology and immunohistochemistry including expression of CDX2, CK7, CK20, MUC1, and MUC2.[2] Tissue microarrays were constructed to validate the *ERBB2* amplification finding discovered in the next-generation sequencing cases. For the validation set, all available institutional resection specimens from 1985 to 2013 were included. In total, 42 intestinal-type ampullary carcinomas, 44 pancreatobiliary-type ampullary carcinomas, 19 mixed intestinal and pancreatobiliary ampullary carcinomas, and one poorly differentiated ampullary carcinoma were studied. All cases tested by next-generation sequencing were also included in the tissue microarrays for correlation between methodologies, provided that adequate material was available.

Mutation Analysis

After macrodissection, genomic DNA was extracted from formalin-fixed paraffin-embedded tissue with the DNeasy Tissue KIT (Qiagen, Valencia, CA, USA). Next-generation sequencing of the discovery set was performed with the clinically validated next-generation sequencing assay, Integrated Mutation Profiling of Actionable Cancer Targets (IMPACT). This assay is a customized hybrid capture-based deep sequencing assay that evaluates 279 cancer-associated genes, listed in Supplementary Table 1. Detectable alterations include single-nucleotide variants, indels, and somatic copy number gains and losses. In brief, DNA was subjected to shearing, followed by library preparation. Matched normal tissue was processed in the same manner and samples were pooled together and sequenced on an Illumina HiSeq 2500. Burrows–Wheeler Aligner[7] was used to align 100-bp paired end sequence reads to reference human genome. Single-nucleotide variants were detected using MuTect,[8] whereas small indels were identified using SomaticIndelDetector. Germline variants were filtered out based on the matched germline DNA.

Manual review was performed using Integrated-GenomicsViewer for all candidate mutations.[9] Technical details of this assay are further described elsewhere.[10]

KRAS, *BRAF*, *NRAS*, and *PIK3CA* mutation testing was performed on all *ERBB2*-amplified cases discovered from the tissue microarrays that did not undergo IMPACT testing. The methodology used for this further testing was the MassARRAY system (Sequenom) with primers as previously described[11,12] at the following hotspots in duplicate for *KRAS*: c.34, 35, 37, 38, 181, 182, 183, 351, and 437; *NRAS* c. 34, 35, 37, 38, 181, 182, and 183; *BRAF* c. 1781, 1798 and 1799; and PIK3CA c. 1624, 1633, and 3140.

ERBB2 Analysis with Whole Sections and Tissue Microarrays

All cases that underwent IMPACT analysis from the discovery set were immunohistochemically stained for ERBB2 expression on whole sections. In addition, three 0.6-mm-diameter cores from separate areas of all available ampullary carcinoma cases were used to construct tissue microarrays. Duplicate tissue microarrays were constructed to account for core dropout. Immunohistochemistry was performed using PATHWAY anti- HER-2/neu (4B5) Rabbit Monoclonal Primary Antibody (Ventana Medical Systems; Tucson, AZ, USA). Immunohistochemical analysis paralleled that used for gastric carcinoma.[13] Basolateral and/or complete membranous staining were scored, whereas cytoplasmic and nuclear staining, as well as staining of nontumor elements, were not included. A cluster of at least five positive tumor cells was required, and immunohistochemical staining intensity was interpreted using magnification necessary to assess staining: 3+ intensity was visible at $\times 40$ original magnification, 2+ intensity was visible at $\times 100$–$\times 200$ original magnification, 1+ intensity was visible at $\times 400$ original magnification, and 0 intensity was used in the absence of staining.

Chromogenic *in situ* hybridization was performed using INFORM HER2 Dual ISH DNA Probe Cocktail Assay (Ventana Medical Systems). Twenty tumor cells per core were counted. An *ERBB2*: CEP17 ratio of >2 in one core was considered amplified, as were clusters of *ERBB2* signals that could not be counted individually owing to overlap of many signals.

Results

IMPACT Findings

Among 32 cases tested by IMPACT, mean target coverage of normal and tumor DNA was $327\times$ and

412×, respectively. Aside from one hypermutated case with 225 mutations, which was negative for *ERBB2* amplification, the number of mutations per tumor ranged from 1 to 16, with an average of five mutations per ampullary carcinoma case, excluding the single hypermutator case. Copy number alterations ranged from 0 to 6, with an average of one copy number alteration per tumor.

Histologic Subtype Trends for Common Mutations Seen in Colorectal and Pancreatic Carcinoma

Trends between molecular alterations and phenotype included more frequent *KRAS* alterations in pancreatobiliary-type ampullary carcinoma than intestinal-type ampullary carcinoma (61 *vs* 29%) and more frequent mutations in *APC* in intestinal-type ampullary carcinoma than pancreatobiliary-type ampullary carcinoma (43 *vs* 17%). *CDKN2A* was more frequently deleted in intestinal-type ampullary carcinoma than in pancreatobiliary-type ampullary carcinoma (30 *vs* 6%). However, these trends did not reach statistical significance ($P > 0.05$).

Approximately 13% of Ampullary Carcinomas Exhibit *ERBB2* Amplification, without Predilection for Subtype

Combining data from IMPACT and chromogenic *in situ* hybridization, 14 of 106 cases (13%) showed *ERBB2* amplification. The clinicopathologic features of all ampullary carcinoma tested by IMPACT or chromogenic *in situ* hybridization/immunohistochemistry for *ERBB2* are summarized in Table 1. No significant differences in age, sex, subtype, node status, peri-neural, or lymphovascular invasion were identified between *ERBB2*-amplified and nonamplified ampullary carcinoma. Interestingly, several cases had missense mutations in *ERBB2*. All *ERBB2* missense mutations occurred in *KRAS*/*BRAF* wild type. Two cases with *ERBB2* mutations occurred in *ERBB2*-amplified ampullary carcinoma (Table 2), whereas other *ERBB2* mutations in three nonamplified ampullary carcinoma included *ERBB2* p. R678Q, R103L, and R784C. *ERBB2* missense mutation did not correlate with histologic subtype.

ERBB2 Amplification Frequently Co-occurs with *TP53* Mutation but Not *KRAS*, *NRAS*, or *BRAF* Mutation

The most frequent gains (including five cases of *ERBB2* amplification), losses, and point mutations and indels in the discovery set are summarized in Figure 1. The molecular profiles of the ampullary carcinoma cases with *ERBB2* amplification are summarized in Table 2. None of the 14 *ERBB2*-amplified cases had *KRAS* or *NRAS* c. 12, 13, 61, or 117, whereas 15 of 27 (56%) of cases without *ERBB2* amplification were positive for a *KRAS* or *NRAS* c. 12, 13, 61, and 117 mutations. However, one *ERBB2*-

Table 1 Clinicopathologic features of *ERBB2*-amplified and nonamplified ampullary carcinoma

	ERBB2 amplified	*ERBB2 non-amplified*
Age (median, range)	62, 37–83	66, 35–87
Male: female	10:4	48:44
Presence of adenoma	5/14 (36%)	31/92 (34%)
Intestinal differentiation	4/14 (29%)	38/92 (41%)
Pancreaticobiliary differentiation	6/14 (43%)	38/92 (41%)
Mixed differentiation	4/14 (29%)	15/92 (16%)
Poor differentiation	0	1/92 (1%)
Nodal metastasis	10/14 (71%)	52/92 (57%)
Lymphovascular invasion	9/14 (64%)	50/92 (53%)
Peri-neural invasion	7/14 (50%)	38/92 (41%)
IHC score: 0	0/14	61/92 (66%)
IHC score: 1+	0/14	17/92 (18%)
IHC score: 2+	6/14 (46%)	14/92 (15%)
IHC score: 3+	7/14 (54%)	0/92
**KRAS*/*NRAS* mutation	0/14	15/27 (56%)
BRAF mutation	0/14	0/27
PIK3CA mutation	1/14 (7%)	2/27 (7%)

Abbreviation: IHC, immunohistochemical.
*$P = 0.0004$.

Table 2 Additional molecular alterations of *ERBB2*-amplified ampullary carcinoma on IMPACT

Case	*Molecular alteration, allele frequency/fold change*
1	*TP53* splice site, 65%
	TEK R1072G, 37%
	FLT3 A814T, 29%
	RNF43 S268*, 27%
	TOP1 E764A, 26%
	TSHR D487A, 27%
	ARID2 G262R, 20%
	RICTOR S1542C, 19%
	PALB2 S328C, 11%
	BAP1 E9D, 15%
	RICTOR H379D, 6%
	ERBB2 D639V, 5%
	***ERBB2* amplification, 8.7**
	CCNE1 amplification, 9.8
	CDK12 amplification, 6.1
	MYC amplification, 3.2
2	*TP53* splice site, 35%
	***ERBB2* amplification, 10**
3	*TP53* V157F, 12%
	***ERBB2* amplification, 2.5**
	GRIN2A amplification, 2.8
4	*TP53* R196*, 32%
	SMAD4 N129D, 30%
	PTEN C136R, 6.2%
	***ERBB2* amplification, 5.3**
	CDK12 amplification, 4.9
	RARA amplification, 3.6
5	*ERBB2* L313I, 95%
	TP53 W146*, 37%
	TGFBR2 K260fs, 31%
	PBRM1 I279fs, 29%
	TET1 R1694H, 25%
	***ERBB2* amplification, 8.1**
	CDK12 amplification, 7.4

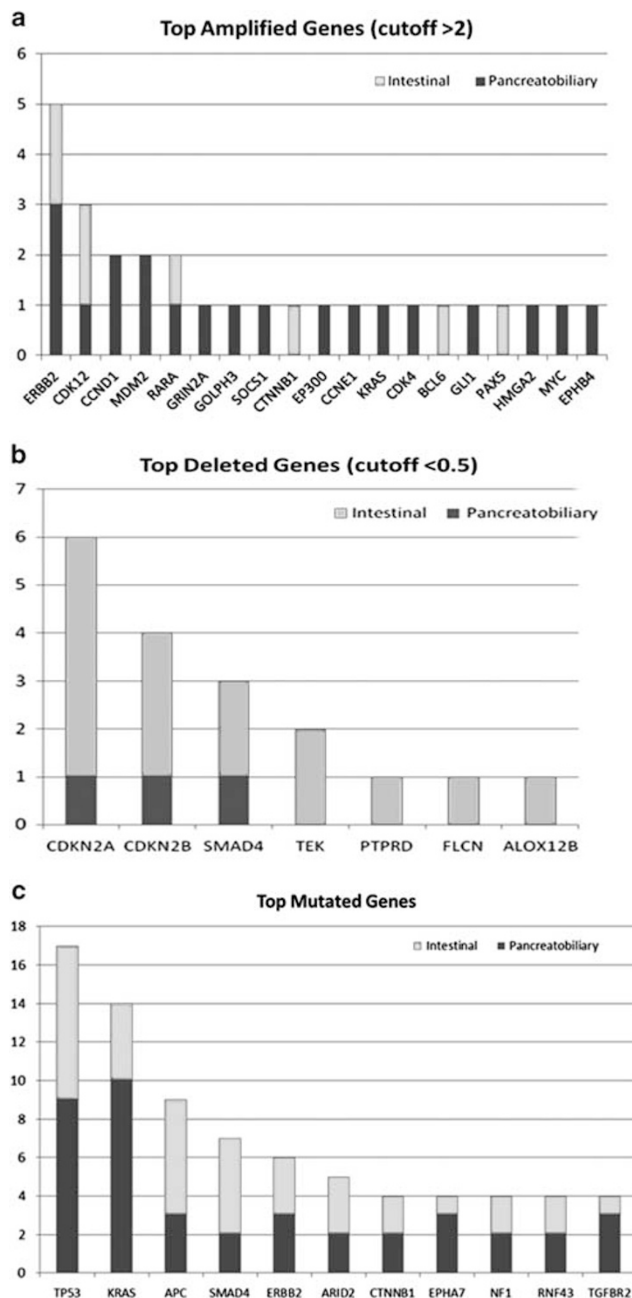

Figure 1 Most frequent genetic alterations detected via IMPACT. (**a**) *ERBB2* and neighboring gene *CDK12* were the most frequently amplified genes, whereas (**b**) *CDKN2A* was the most frequently deleted. (**c**) *TP53* was the most frequent of the missense/indel mutations followed by *KRAS* and *APC*, which each respectively occurred more frequently in pancreatobiliary and intestinal-type ampullary carcinoma.

amplified case (detected via chromogenic *in situ* hybridization) was positive for a *PIK3CA* p. H1047R (c. 3140A>G) mutation on Sequenom analysis. Interestingly, all five cases with *ERBB2* amplification on IMPACT had concomitant *TP53* mutations, whereas only 44% of ampullary carcinoma cases without *ERBB2* amplification had *TP53* mutations.

ERBB2 Immunohistochemistry Results Demonstrate High Sensitivity for Amplification by Chromogenic *In Situ* Hybridization and IMPACT

All five cases that were amplified on IMPACT displayed strong, diffuse immunohistochemical positivity on whole sections. Among the 29 cases that were negative for *ERBB2* amplification on IMPACT, 6 cases displayed heterogeneity on whole sections including 4 cases ranging from 0 to 2+ in intensity, 1 case that ranged from 1+ to 2+ in intensity, and 1 case that ranged from 0 to 1+ in intensity.

Among the 100 cases available for chromogenic *in situ* hybridization analysis, 13 were positive for *ERBB2* amplification, and the correlation between immunohistochemistry intensity and chromogenic *in situ* hybridization result is shown in Table 1. Immunohistochemical staining detected all 13 cases with *ERBB2* amplification, yielding a sensitivity of 100%. Furthermore, lack of 3+ immunohistochemical intensity predicted lack of *ERBB2* amplification in all 87 cases without *ERBB2* amplification, yielding a specificity of 100% (Figure 2). There were no discrepancies between IMPACT and chromogenic *in situ* hybridization on cases tested by both methods, including 4 *ERBB2*-amplified cases and 22 *ERBB2* nonamplified cases. Immunohistochemical heterogeneity between cores occurred in two amplified cases, yet on each of these two cases chromogenic *in situ* hybridization showed consistent homogeneous results between cores (Figure 2).

Discussion

In this study, we have found that the incidences of mutations frequently implicated in colorectal and pancreatic carcinogenesis, in particular mutations in *APC* and *KRAS*, correlate with the specific histologic subtype of ampullary carcinoma. Although neither *APC* nor *KRAS* mutations were 100% specific for pancreatobiliary-type or intestinal-type ampullary carcinoma, respectively, it is noted that *APC* mutations do occur, though rarely, in pancreatic carcinoma. The differences identified in the genetic signatures of subtypes of ampullary carcinoma may help assess prognosis, as well as treatment regimen, and may help assist with classification when the histologic subtype is mixed. In addition, but not surprisingly, a number of other alterations in tumor suppressors and oncogenes occurred in both groups, including *ERBB2* amplification. This is the first systematic investigation of *ERBB2* amplification and overexpression in ampullary carcinoma and their molecular correlations. In addition to identifying the most frequently mutated oncogenes and tumor suppressors, we have shown that *ERBB2* amplification is a relatively frequent event in ampullary carcinoma with specific molecular correlates and can be reliably identified using routine immunohistochemistry.

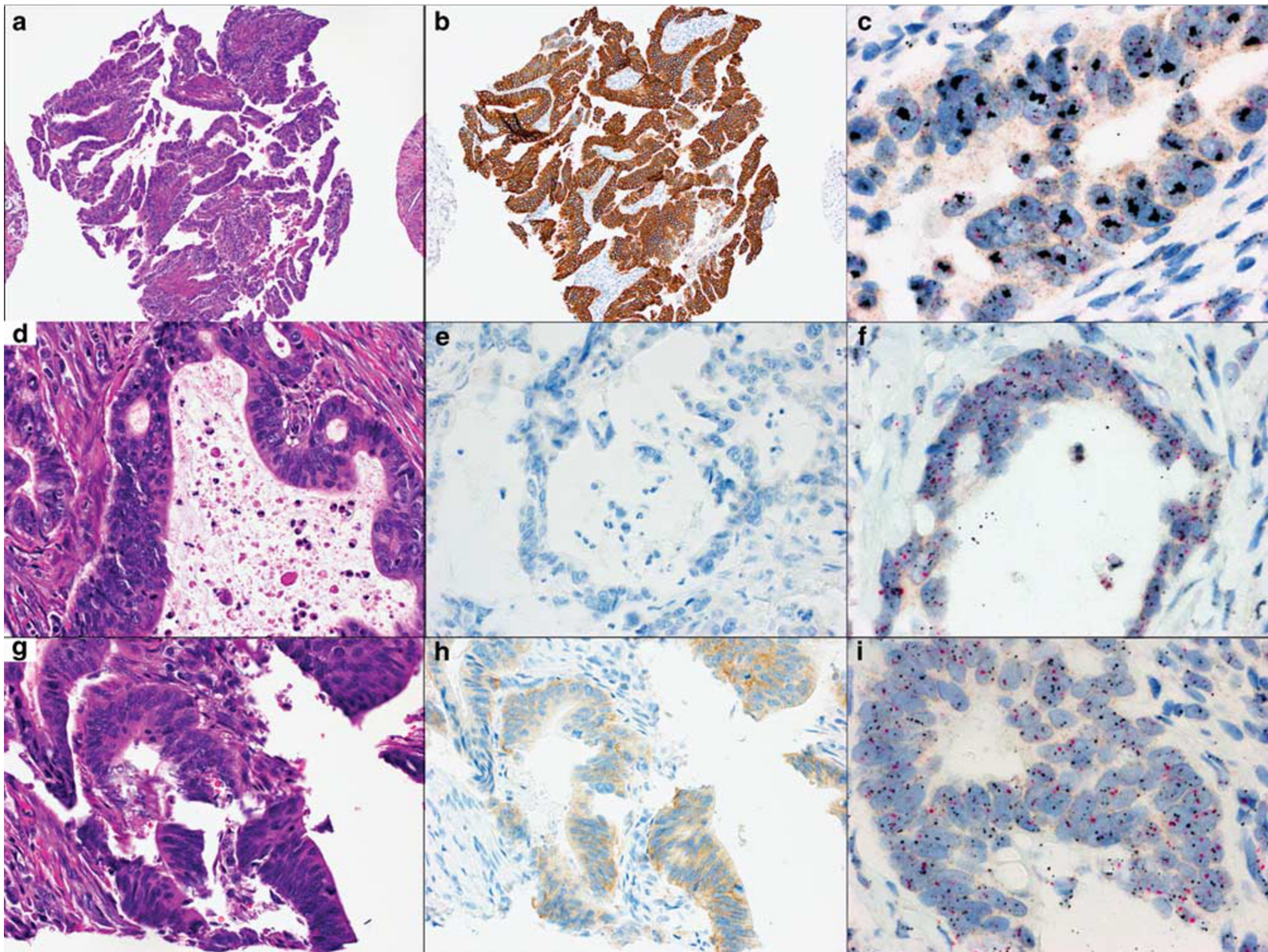

Figure 2 ERBB2 immunohistochemistry and chromogenic *in situ* hybridization correlation. (**a**) A pancreatobiliary-type ampullary carcinoma showing (**b**) strong membranous staining (3+) visible on low power and (**c**) clustering of numerous *ERBB2* signals per cell (black dots) on chromogenic *in situ* hybridization, consistent with amplification. (**d–h**) In this case, (**d**) a more poorly differentiated area had (**e**) no immunohistochemical reactivity, yet (**f**) it was amplified on chromogenic *in situ* hybridization, whereas (**g**) a more well differentiated area with intestinal differentiation had (**h**) 2+ immunohistochemical reactivity and (**i**) similar amplification on CISH (**a**, **d**, **g**, **h** and **e**), ×100 original magnification; **b**, **e**, **h**: 4B5 immunohistochemistry, ×100 original magnification; C, F, I: *ERBB2* CISH, ×1000 original magnification; both: Ventana Medical Systems; D and G: H&E, ×400 original magnification;**e** and **h**: 4B5 immunohistochemistry, ×400 original magnification).

Our results correlate well with those of previous studies that have demonstrated *ERBB2* amplification/overexpression in 0–23% of cases.[14–16] In addition to previous studies, we provide data correlating *ERBB2 in situ* hybridization and immunohistochemical results in ampullary carcinoma. We have found that the immunohistochemical scoring criteria used for gastric carcinoma work well for ampullary carcinoma for predicting gene amplification. Further, the molecular profiles of *ERBB2*-amplified ampullary carcinoma included frequent *TP53* co-mutation and a paucity of *KRAS* or *NRAS* mutations. Thus, *ERBB2*-amplified ampullary carcinomas have a molecular profile that is more similar to gastric than colorectal or pancreatobiliary carcinoma: provisional TCGA data show that pancreatobiliary carcinomas have a paucity of *ERBB2* amplification, and colorectal carcinomas do not share the positive association between *TP53* mutation and *ERBB2* amplification or the negative correlation between *KRAS*/*BRAF* mutation and *ERBB2* amplification.[17] The fact that *ERBB2*-amplified ampullary carcinoma did not harbor downstream mutations in *KRAS* or *BRAF* may be important therapeutically, as the latter may cause primary resistance to inhibition of EGFR family members.[18] With a similar molecular profile to *ERBB2*-amplified gastric carcinoma, it follows that *ERBB2*-amplified ampullary carcinoma might also derive benefit from similar targeted therapy. The significance of the *ERBB2* mutations detected in this study is yet to be determined. None of these mutations occurred at hot spots; however, two mutations occurred in cases positive for *ERBB2* amplification: the *ERBB2* p. L313I extracellular domain mutation occurred in 95% of reads, suggesting that the mutation was amplified, although the *ERBB2* p. D639V mutation occurred in only 5% of reads, suggesting that

it occurred in a nonamplified subclone. The other *ERBB2* mutations in three nonamplified cases (p. R678Q, R103L, and R784C) have not been described in the Cosmic database or other reports.

Intratumoral heterogeneity of *ERBB2* amplification is relatively common in gastric carcinoma, yet not as common in breast carcinoma. We identified intratumoral heterogeneity in 2/14 (13%) of cases based on immunohistochemistry, yet not by chromogenic in situ hybridization. It is important to note that all three cores per tumor were derived from the same paraffin block, and whether the *ERBB2* status of synchronous or metachronous metastases will correlate well with the *ERBB2* status of the primary ampullary carcinoma remains to be seen. One possible cause of the discordant immunohistochemistry results in these two cases is less than optimal tissue fixation. Ampullary carcinoma would be especially prone to fixation issues, as these resections typically include pancreatic tissue with high levels of digestive enzymes. Another possible reason would be epigenetic changes altering protein expression, as both cases were phenotypically heterogeneous.

Limitations to this study include the relatively small numbers of histologically typical intestinal-type and pancreatobiliary-type ampullary carcinoma, as well as relatively small numbers of *ERBB2*-amplified ampullary carcinoma, which precluded meaningful survival and treatment response comparisons. Our methodology was limited to DNA-level alterations without specific assessment of possible epigenetic mechanisms. It has recently been shown that differences in phenotypic differentiation of ampullary carcinoma are reflected in RNA profiling,[4] perhaps suggesting that epigenetic factors may additionally contribute to phenotypic differentiation. Regarding assessment of *ERBB2* amplification, specific fixation requirements were not required for inclusion in this study; whether stricter fixation requirements would alter immunohistochemical patterns in ampullary carcinoma remains to be seen.

In summary, certain molecular trends may be associated with intestinal or pancreatobiliary histologic subtype in ampullary carcinoma, and these molecular trends may be important for therapeutic decision-making and prognosis. *ERBB2* amplification occurs in ~13% of ampullary carcinoma, is virtually mutually exclusive with downstream mutations in *KRAS*/*NRAS*/*BRAF*, and is present regardless of subtype or nodal status. *ERBB2* amplification can be reliably screened for using immunohistochemistry with the scoring criteria developed for gastric carcinoma, and patients with *ERBB2*-amplified ampullary carcinoma may be candidates for targeted therapy.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol).