

Unraveling Neuroendocrine Gallbladder Cancer: Comprehensive Clinicopathologic and Molecular Characterization

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PURPOSE Neuroendocrine carcinomas and mixed neuroendocrine non-neuroendocrine neoplasms of the gallbladder (NE GBC) are rare and highly aggressive entities. The cell of origin of NE GBC has been a matter of controversy. Here, we performed a comparative histopathologic and molecular analysis of NE GBC cases and, if present, associated precancerous lesions.

PATIENTS AND METHODS We selected cases diagnosed between 2000 and 2019 in the Netherlands. Precursors and carcinomas were immunohistochemically compared and analyzed for mutations, gene amplifications, microsatellite instability, and tumor mutational burden using a next-generation sequencing panel containing 523 cancer-related genes. In addition, presence of fusion genes was analyzed using a panel of 55 genes.

RESULTS Sixty percent of neuroendocrine cases (6/10) presented with a precursor lesion, either intracholecystic papillary neoplasm (n = 3) or biliary intraepithelial neoplasia (n = 3). Immunohistochemically, neuroendocrine components were different from the epithelial precursor lesions. Molecular profiling, however, revealed *TP53* mutations shared between different components in five of six cases, indicating a clonal relation. Furthermore, 40% of cases (4/10) harbored at least one potentially actionable alteration. This included (likely) pathogenic mutations in *RAD54L*, *ATM*, and *BRCA2*; amplifications of *ERBB2* and *MDM2*; and a gene fusion involving *FGFR3-TACC3*. All cases were microsatellite-stable and had a tumor mutational burden of < 10 mutations/Mb.

CONCLUSION Our data provide insight into the development of NE GBC and suggest a common origin of precancerous epithelial lesions and invasive neuroendocrine components, favoring the hypothesis of lineage transformation. Moreover, nearly half of the NE GBCs carried at least one potentially actionable molecular alteration, highlighting the importance of molecular testing in this highly lethal cancer.

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INTRODUCTION

Neuroendocrine neoplasms (NENs) can arise from almost any anatomical site including, but not limited to, the lung, the prostate, and throughout the GI and hepatobiliary tract. Two major categories of NENs have been described: well-differentiated neuroendocrine tumors (NETs) and poorly differentiated neuroendocrine carcinomas (NECs), the latter being high grade by definition.¹ Morphologically, NECs are classified as either pure small-cell or large-cell NEC, and they might also be present together with an adenocarcinoma (AC) component. They are classified as mixed neuroendocrine non-neuroendocrine neoplasms (MiNENs) when both the neuroendocrine component (generally NEC) and the non-neuroendocrine component (generally AC) comprise at least 30% of the neoplasm.² Regardless of the anatomical site, NECs and MiNENs are highly aggressive tumors and carry a dismal prognosis.

NECs can either arise de novo or during progression of an epithelial neoplasm, for example, because of a therapy resistance mechanism.³ The molecular features of NECs have been best described in lung, pancreas, and prostate, but the cell of origin of NECs has been a matter of controversy, specifically in tumors with a mixed phenotype (ie, MiNENs). Two hypotheses have been postulated: either NEC cells develop from preexisting neuroendocrine cells and grow together with an AC component or AC cells become NEC cells through lineage transformation and thus have a common origin. Accumulating evidence favors the latter hypothesis, starting from the observation of amphicrine cells with both endocrine and exocrine features⁴ to more recent molecular studies supporting the multistep progression of MiNENs from a shared precursor lesion.⁵ The rare occurrence of NECs and MiNENs in organs lacking neuroendocrine cells such as the gallbladder may support the latter hypothesis.⁶

ASSOCIATED CONTENT

Appendix

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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CONTEXT

Key Objective

Neuroendocrine gallbladder cancer (NE GBC) has a dismal prognosis. The origin and molecular targetable alterations of NE GBC are poorly understood. To our knowledge, this study provides the first comprehensive clinicopathologic and molecular analysis of 10 (mixed) NE GBC cases, a very rare subtype of GBC, identified using a combination of two nationwide registries.

Knowledge Generated

We provide evidence for a shared origin between neuroendocrine and non-neuroendocrine components in mixed NE GBC. Furthermore, broad molecular testing in these patients adds useful information for treatment decisions as in a substantial number of patients potentially actionable alterations were identified.

Relevance

The clinical need for effective treatment options in NE GBC is high. The findings of this study advocate for molecular testing in NE GBC cases, which will allow patients to be included in basket trials.

Neuroendocrine cancers of the gallbladder (NE GBC) account for 0.5% of all NECs and 5% of all primary GB cancers.⁷ Literature on NE GBC is restricted to case reports and small case series, and its molecular pathogenesis remains poorly understood. Four NE GBC cases have been described in which the neuroendocrine and non-neuroendocrine components were associated with an intracholecystic papillary neoplasm (ICPN),⁸⁻¹¹ but only one case was supported with limited molecular data.⁸

Here, we performed a comparative histopathologic and molecular analysis of 10 NE GBC cases, including three arising in the context of ICPN and three in the context of biliary intraepithelial neoplasia (BillIN), and we provide an overview of potentially targetable alterations in this rare entity.

PATIENTS AND METHODS

Patient Selection

By combining databases of the Netherlands Cancer Registry (k171236) and the nationwide network and registry of histopathology and cytopathology in the Netherlands (PALGA, LZV2017-87), we anonymously selected consecutive patients with a high-grade NE GBC between 2000 and 2019.

By retrospective review of the pathology report and medical history, information on sex, age at time of diagnosis, location of the GBC, and relevant comorbidities was collected. This study was approved by the Radboud University Medical Center medical ethics committee (2018-4126).

Histopathologic Review

Formalin-fixed paraffin-embedded (FFPE) blocks were selected on the basis of pathology reports and histopathologic review. Cases were reviewed by a pathologist (R.S.v.d.P.), on the basis of 4- μ m hematoxylin and eosin-stained sections of available tissue blocks. Histologic typing and grading, including description of a precursor

lesion (either BillIN or ICPN), was performed using the WHO histologic classification of tumors of the gallbladder (fifth edition).² T classification was performed using the American Joint Committee on Cancer tumor-node-metastasis classification system (eighth edition).¹² Additionally, presence of vascular, lymphatic, and perineural invasion was assessed.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 4- μ m whole-slide FFPE sections semiautomated using the Lab Vision Autostainer (Immunologic, Duiven, the Netherlands) for MUC2, MUC5AC, MUC6, and TTF-1 essentially as described before¹³ or fully automated using Tissue-Tek Genie (Sakura Finetek, Torrance, CA) for chromogranin A, CD56, CK7, CK20, Ki-67, EMA (MUC1), p53, and synaptophysin with ready-to-use reagents. Details of antibodies are listed in the Data Supplement.

Immuno-expression was examined by two gastroenterology-dedicated pathologists (R.S.v.d.P. and I.D.N.), and intensity and % positive cells were scored semiquantitatively. Discrepancies in scoring were reevaluated until agreement.

Nucleic Acid Extraction

For molecular analyses, DNA and RNA were isolated from FFPE-derived tumor tissue and the precancerous lesion if available. DNA was isolated, quantified, and precipitated manually as described before.¹⁴ After precipitation, the final concentration was determined using the Qubit High Sensitivity Kit (Thermo Fisher Scientific, Waltham, MA).

RNA was isolated using the ReliaPrep FFPE Total RNA Miniprep System (Promega, Madison, WI) according to the manufacturer's protocol, omitting the DNase treatment step. RNA concentrations were measured with the Qubit RNA Broad Range Kit (Thermo Fisher); samples were diluted to a concentration of 30 ng/ μ l and measured again with the Qubit RNA High Sensitivity Kit (Thermo Fisher).

Subsequently, 60 ng of either DNA or RNA was used as input for the library preparation.

Library Preparation

DNA and RNA library preparations were performed separately using the hybrid capture-based TruSight Oncology (TSO) 500 DNA and RNA Library Preparation Kits, respectively (Illumina, San Diego, CA) according to the manufacturer's protocol essentially as described before.¹⁵ The TSO500 DNA assay targets 523 cancer-related genes for assessment of single- and multiple-nucleotide variants, gene amplifications, tumor mutational burden (TMB), and microsatellite instability (MSI), whereas the RNA kit targets 55 genes for fusion gene analyses (Data Supplement). The use of unique molecular identifiers allows sensitive analysis of unique DNA molecules sequenced at every position. In short, DNA samples were fragmented, and RNA samples were used for first- and second-strand cDNA synthesis. Next, samples underwent end-repair and A-tailing, followed by unique molecular identifier ligation and barcoding. Two target capture and purification steps, allowing for maximal target enrichment, were followed by polymerase chain reaction amplification and purification. Libraries were quantified and normalized for uniform library representation.

Sequencing and Data Analysis

Sequencing was performed on a NextSeq 500 system (Illumina), with 10 DNA libraries on a NextSeq high-output cassette or 16 RNA libraries on a NextSeq mid-output cassette. The raw sequencing data were processed and analyzed by the TruSight Oncology 500 Local App version 1.3 and 2.0 (Illumina), which produces a report with quality parameters, TMB and MSI values, gene fusions and splice variants, and a variant call file with single- and multiple-nucleotide variants. Presence of gene amplifications was determined as previously described on the basis of median coverage normalization.^{15,16} A relative coverage of ≥ 3 was considered as gene amplification. The number of gene copies in the tumor cells was estimated on the basis of the relative coverage corrected for the percentage of neoplastic cells in the sample.

Variant annotation was performed as described before via an in-house-developed pipeline.¹⁷ Variants were filtered by exclusion of (1) variants outside exons and splice site regions ($-8/+8$) except those in the *TERT* promoter region, (2) synonymous variants, unless present in a splice site region, (3) variants present with a frequency of $> 0.1\%$ in the ExAC (version 0.2) database, (4) variants with a variant allele frequency (VAF) of $< 5\%$, and (5) variants with < 5 variant reads. Remaining variants were manually inspected and curated and classified into five classes on the basis of their level of evidence for pathogenicity, largely on the basis of American College of Medical Genetics/Association for Molecular Pathology guidelines^{18,19}: class 1, not pathogenic; class 2, unlikely pathogenic; class 3, possibly

pathogenic; class 4, likely pathogenic; and class 5, pathogenic. Interpretation of pathogenicity for variants in tumor suppressor genes (TSG) was based on three prediction tools (sorting intolerant from tolerant (SIFT), PolyPhen-2, and Align-Grantham Variation Grantham Deviation (Align-GVGD)) and for both TSG and oncogenes (O) on various knowledgebases (ClinVar, OncoKB, JAX CKB, MyCancer Genome, and COSMIC) (references 20-25, all accessed July 17, 2020). For interpretation of variants in *TP53*, the *TP53*-IARC database was used (reference 25, accessed July 17, 2020). Potential effects on splicing were evaluated on the basis of the majority vote of SpliceSiteFinder-like, MaxEntScan, NNSPLICE, and GeneSplicer (all available from Alamut Visual version 2.13 [SOPHiA GENETICS, Lausanne, Switzerland]). Details on classes and their interpretation are provided in [Figure A1](#), online only.

Variant-specific level 1, 2, 3, or 4 evidence for actionability across all tumor types was derived from OncoKB.²¹ Level 1 and 2 biomarkers are defined as FDA-recognized or standard care biomarkers, respectively, that are predictive of response to FDA-approved drugs in specific tumor types. For level 3A biomarkers, there is compelling clinical evidence of drug response in a specific indication, and level 3B alterations are predictive of response to an FDA-approved or investigational drug in another indication. Level 4 alterations comprise alterations for which there is compelling biological evidence that predicts response to a drug. Since there are no biomarkers specifically available for NE GBC, we listed all alterations that are possibly targetable in any tumor type according to OncoKB (reference 21, accessed July 17, 2020).

RESULTS

Patient and Tumor Characteristics

Between 2000 and 2019, 10 consecutive patients with a high-grade NE GBC were identified using the Netherlands Cancer Registry and PALGA ([Table 1](#)). All but one case were female (90%), and the median age at diagnosis was 67.5 years (range, 42-79 years). None of the cases had a history of another malignancy, suggesting that, at least clinically, these were primary NE GBCs. Half of the cases presented with cholelithiasis. Three patients received adjuvant systemic therapy. Data on the type of adjuvant treatment or other treatments in the advanced setting were not available. All but one patient died during a median follow-up of 10.6 months after diagnosis (range, 4.3-29.4 months); one case with a small-cell NEC was still alive 62.3 months after diagnosis.

All cases presented with muscle-invasive NE GBC (pT2 or higher), five cases (50%) had lymph node metastases (pN1/2), and two cases (20%) presented with distant metastasis (pM1) to liver, peritoneum, and skin ([Table 2](#)) at time of diagnosis. Seven cases (70%) were pure high-grade NET or NEC, whereas three tumors (30%) showed both AC and NEC features and were classified as MiNENs. Five

TABLE 1. Patient Characteristics

Case	Age at Diagnosis	Sex	Vital Status	Follow-Up in Months After Diagnosis	Adjuvant Systemic Therapy ^a	Cholecystitis/Cholelithiasis
1	71	F	Deceased	10.7	No	Cholelithiasis
2	67	F	Alive	62.3	Yes	Cholelithiasis
3	55	F	Deceased	4.3	No	Cholelithiasis
4	42	F	Deceased	29.4	Yes	Absent
5	68	F	Deceased	7.3	No	Absent
6	77	F	Deceased	14.3	No	Chronic cholecystitis
7	74	F	Deceased	10.6	No	Cholelithiasis
8	66	F	Deceased	5.3	No	Absent
9	79	F	Deceased	8.3	No	Cholelithiasis
10	61	M	Deceased	13.1	Yes	Absent

^aThree patients received adjuvant systemic therapy according to data from the Netherlands Cancer Registry. Data on type of systemic therapy are not available.

tumors (50%) were large-cell NEC, four cases (40%) were small-cell NEC, and one case (10%) was a NET grade 3, hereafter collectively referred to as NE GBC. Sixty percent of cases showed a precancerous lesion, either ICPN (three cases) or BillIN (three cases). In 90% of cases, at least one tumor component (either NEC or, if present, AC) showed angioinvasion; lymphatic invasion and perineural invasion

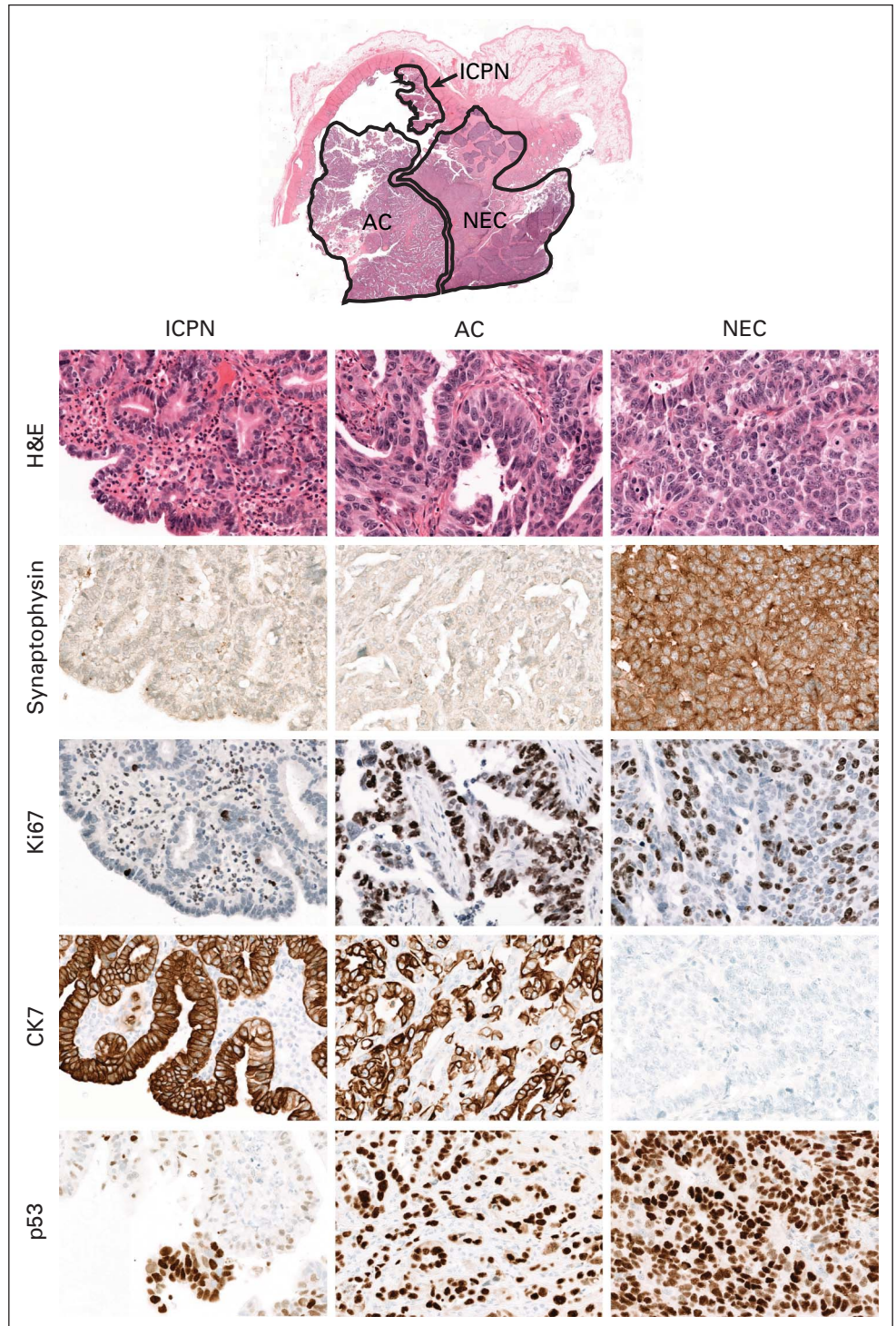
were observed in 80% and 30% of cases, respectively. Neuroendocrine expression with synaptophysin, chromogranin A, and CD56 confirmed neuroendocrine histology and was absent in the precancerous lesions (Table 2 and Fig 1). The three ICPNs expressed epithelial markers; all cases were CK7-positive and two of three were also partly CK20-positive, whereas corresponding NEC

TABLE 2. Tumor Characteristics

Case	Diagnosis	pTNM (M Location)	Angioinvasion/Lymphatic Invasion/Perineural Invasion	Component	Chromogranin A/Synaptophysin/CD56 (All %)	Ki67 (%)	TTF1	CK7	CK20
1	ICPN (mixed type) with NEC (SC)	T2aNx	+/-/-	ICPN	0/0/0	70	-	+	+
				NEC	100/100/100	70	-	-	+
2	BillIN with NEC (SC)	T3Nx	+/-/-	BillIN	0/0/0	20	-	+	+
				NEC	10/70/70	70	±	±	-
3	NEC (SC)	T3N2M1 (liver)	+/+/+	NEC	60/100/60	50	-	F	F
4	ICPN (intestinal type) with NEC (LC)	T3N1	+/+/-	ICPN	10/0/0	20	-	+	+
				NEC	0/100/100	20	-	-	-
5	ICPN (biliary type) with MiNEN (LC)	T2aN1	+/+/-	ICPN	0/0/0	2	-	+	-
				AC	0/0/0	30	-	+	-
				NEC	100/100/100	30	-	-	-
6	NET G3	T2aN1M1 (peritoneum and skin)	+/+/-	NET G3	5/95/0	30	-	-	-
7	BillIN with NEC (SC)	T3N2	+/+/-	BillIN/AC	0/0/0	80	-	+	±
				NEC	20/80/100	80	-	-	-
8	BillIN with MiNEN (LC)	T3Nx	+/+/+	BillIN	0/0/0	40	-	+	-
				AC	0/0/0	60	-	+	±
				NEC	90/100/100	20	-	+	-
9	NEC (LC)	T2Nx	+/+/-	NEC	20/50/0	20	-	-	F
10	NEC (LC)	T3NxM1 (peritoneum)	-/+/+	NEC	30/100/100	80	-	±	+

Abbreviations: +, > 50% positive tumor cells; ±, < 50% positive tumor cells; -, negative; AC, adenocarcinoma; BillIN, biliary intraepithelial neoplasia; F, focally positive tumor cells; ICPN, intracholecystic papillary neoplasm; LC, large cell; MiNEN, mixed neuroendocrine non-neuroendocrine neoplasm; n.a., not applicable; NEC, neuroendocrine carcinoma; NET G3, neuroendocrine tumor grade 3; SC, small cell; TNM, tumor-node-metastasis.

FIG 1. Immunohistochemical staining of case 5. Neuroendocrine marker expression with synaptophysin was positive in the NEC and negative in the ICPN and AC (chromogranin A and CD56 were comparable; [Table 2](#)), whereas epithelial marker expression (CK 7) was positive in the ICPN and AC and negative in the NEC (CK20-negative in all three; [Table 2](#)). The Ki-67 index was 30% for both the AC and NEC. Both the AC and NEC showed overexpression of p53, whereas the ICPN component showed predominantly a wild-type p53 expression with only a small region of overexpression. Lower panel: all magnifications 400x. AC, adenocarcinoma; H&E, hematoxylin and eosin; ICPN, intracholecystic papillary neoplasm; NEC, neuroendocrine carcinoma.



components were negative. Lineage subtyping of the ICPNs was aided by IHC expression profiles of MUC1, MUC2, MUC5AC, and MUC6 (Data Supplement and [Fig A2](#)). One ICPN was of biliary type, one was of intestinal type, and one showed a mixed biliary and intestinal phenotype. TTF-1 immuno-expression was absent in all cases supporting primary NE GBC. Since *TP53* is frequently altered in NECs of other anatomical origin, case

5 served as an exemplary case for which p53 IHC was performed next to a molecular screening, which was done for all cases. Both the AC and NEC components showed aberrant p53 expression, whereas the ICPN component showed predominantly a normal p53 expression with only a small region with overexpression ([Fig 1](#)). This reflects the presence of the missense mutation p.R273H.

Molecular Characteristics

For each case, DNA and RNA were isolated from the precursor lesion, the NEC component and, if available, the AC component. For case 7 and 8, the BillIN lesion was not sufficient in size for DNA and RNA isolation, and these were not included in molecular analyses. Quality control parameters of DNA and RNA libraries including reference values are listed in the Data Supplement. For case 1, both the NEC and ICPN components did not meet quality control for MSI, TMB, and CNV assessment.

All likely pathogenic and pathogenic single- and multiple-nucleotide variants (classes 4 and 5) were considered potentially clinically relevant and are listed in Figure 2 and the Data Supplement. The majority of cases had a mutation in *TP53* (70%), followed by *CTNNB1* (40%), *RB1* (30%), and *ATM* (20%). A variety of other genes harbored potentially clinically relevant mutations in individual cases. Mutations in *TP53* were shared among the different precursor, AC, and NEC components in all but one case (case 8), where two different mutations were observed in the AC versus the NEC. Interestingly, the ICPN of case 5 did share the same *TP53* mutation with the invasive tumor components, albeit with a low VAF (8.7%), reflecting the limited region with p53 overexpression (Fig 1). Three cases (30%) harbored potentially actionable variants (level 1) in *ATM*, *BRCA2*, and *RAD54L*, albeit with a low VAF for the *ATM* variant of case 4.

In six cases, gene amplifications were observed in a variety of genes including potentially actionable amplifications of *ERBB2* (level 1) and *MDM2* (level 3A), the latter being in a case without a *TP53* mutation (Fig 2). Whereas the majority of mutations were shared among different precancerous, AC, and NEC components, for gene amplifications this was not observed, except for a *CNNE1* amplification that was shared between the AC and NEC, but not the ICPN, of case 5.

A fusion involving *FGFR3* and *TACC3* genes was observed in all three components of case 5 (Fig 2). The fusion joins exons 1-17 of *FGFR3* to exons 10-16 of *TACC3* (Data Supplement and Fig A3). This fusion is considered targetable with erdafitinib in bladder cancer (level 1 evidence).

With respect to biomarkers predictive for response to immunotherapy, MSI and TMB, it was observed that all cases were microsatellite-stable and that their median TMB was 3.1 mutations/Mb (range, 0.8-6.3 mutations/Mb) (Data Supplement).

Collectively, whereas different precancerous, AC, and NEC components showed a heterogeneous immunophenotype, with the epithelial precancerous lesion closely resembling the AC component, molecular alterations were mostly shared between the individual components, including the NEC.

DISCUSSION

We have provided a comprehensive histopathologic and molecular analysis of 10 NE GBC cases and their associated precancerous lesions, a very rare subgroup of GBC that has not been studied to this extent before.

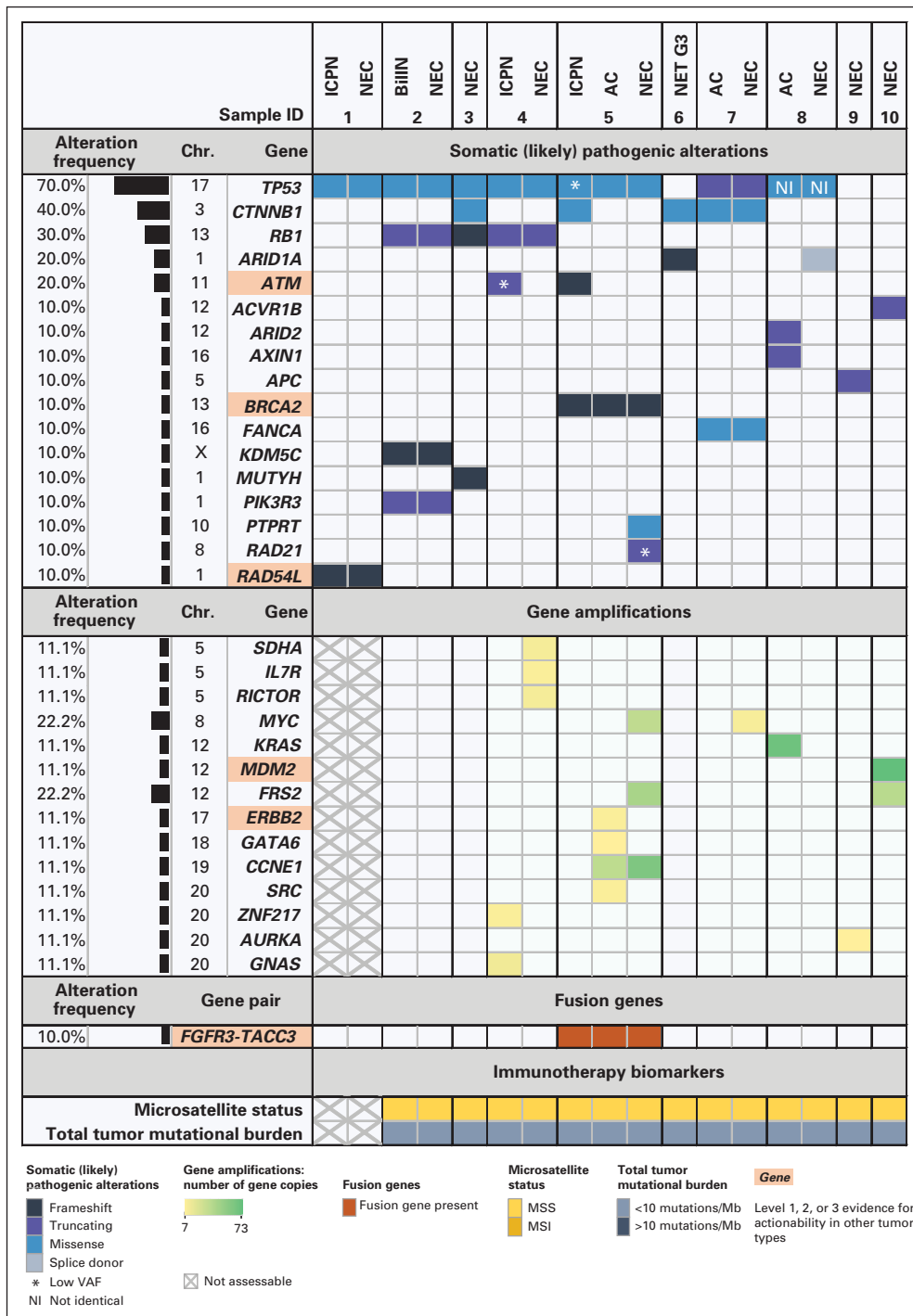
We observed that the majority of NE GBCs in our series were associated with an epithelial precancerous lesion: three with a BillIN and three with an ICPN. The presence of an ICPN together with a NE GBC (including MiNEN) has been observed before.⁸⁻¹¹ In only one of these case reports, a small next-generation sequencing panel was used and results suggested relatedness of both lesions on the basis of a shared *TP53* mutation.⁸ We observed that (immuno) phenotypically the NE components were different from their epithelial counterparts (precancerous lesion or AC). Despite these phenotypical differences, the shared *TP53* mutation in the different components in five of six cases strongly suggests a common origin for neuroendocrine and epithelial components of NE GBC. In addition, 50% of the cases also had cholelithiasis, which is a well-known risk factor for pure gallbladder AC,²⁶ and may further reinforce the concept of a common origin. Other studies of digestive system NECs also support the lineage transformation hypothesis with molecular analyses.^{5,27,28}

Nearly all cases had multiple pathogenic or likely pathogenic mutations in a variety of genes with *TP53*, *CTNNB1*, *RB1*, and *ATM* being the most frequently involved. Our findings are largely in line with observations in lung, prostate, and pancreatic NECs, where *TP53* and *RB1* are altered in roughly 80% of cases.³ Interestingly, also pure gallbladder AC is molecularly highly heterogeneous with *TP53* as the most frequently mutated gene (47.1%-59% of cases).²⁹ However, large-scale molecular data remain scarce and are mainly derived from endemic regions, which could hamper the extrapolation of findings to non-endemic regions.

Gene amplifications (n = 6 cases) were observed in a subset of cases and were in all but one case not shared between the different components. This suggests that, in contrast to mutations that are shared between the precursor and invasive components, oncogenic amplifications typically occur as a later event in carcinogenesis, which has been observed before in esophageal AC.³⁰ In case 5, a *CCNE1* amplification was shared between the AC and NEC, but not the ICPN. Possibly this amplification was present in a small-cell population such as the *TP53* mutation and could therefore not be detected in the ICPN.

Given the short survival time after diagnosis of NE GBC, the clinical need for innovative systemic treatment options is high. As there are currently no predictive biomarkers for targeted treatment options available for NE GBC specifically, we evaluated the presence of genetic aberrations that serve as predictive markers in other tumor types. Nearly half of the cases (40%) proved to have at least one

FIG 2. Somatic alterations in neuroendocrine gallbladder cancer. Potentially clinically relevant mutations (class 4 and class 5), gene amplifications, fusion genes, microsatellite status, and total tumor mutational burden, identified by TSO500. Left: frequency of alterations. Top: cases and individually sequenced components per case. Note that case 1 did not meet quality control for assessment of gene amplifications, TMB and MSI, and that potentially clinically relevant mutations could have been missed. BillIN, biliary intraepithelial neoplasia; ICPN, intracholecystic papillary neoplasm; MSS, microsatellite stable; MSI, microsatellite instability; NEC, neuroendocrine carcinoma; NET G3, neuroendocrine tumor grade 3; TMB, tumor mutational burden.



alteration that has level 1, 2, or 3 evidence for actionability in other tumor types, suggesting that molecular testing in these patients adds useful information for treatment decisions. For example, one NE GBC expressed an *FGFR3-TACC3* fusion transcript. A patient with GBC with a similar fusion showed stable disease after 30 weeks of treatment with an oral FGFR inhibitor in a phase I trial.³¹ This underscores the clinical importance to explore molecular testing in all NE GBC cases. Given the rarity and molecular

heterogeneity of this entity, future research should be directed toward the inclusion of patients with NE GBC in basket trials rather than the exploration of therapeutic options in preclinical disease models such as cell lines or mouse models which, to the best of our knowledge, do not exist for NE GBC.

The current study significantly contributes to existing literature by describing the clinicopathologic and molecular characteristics of a substantial number of rare subtype GBC

cases. Although we identified potential therapeutic targets in a substantial number of patients, the efficacy of these therapies remains unknown because of the retrospective nature of the study. Another limitation of this study is the limited information on used therapeutic regimens, such as the type and outcome of adjuvant systemic therapy received by three of the patients, because of the retrospective anonymized nature of this study.

In conclusion, we provide a comprehensive histopathologic and molecular overview of a very rare and understudied tumor type. We gained biological insight into the multistep development from an epithelial precursor lesion to invasive NEC/MiNEN and demonstrated that a substantial number of NE GBC cases carry at least one potentially actionable genetic alteration, expanding the treatment possibilities for this patient group.

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DISCLAIMER

The views expressed in the submitted article are of the authors' own and not an official position of the institution or funder.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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APPENDIX

Tumor suppressor genes				
In silico prediction tools - SIFT - PolyPhen-2 - Align-GVGD		TP53 variants - TP53-IARC database	Knowledgebases - ClinVar - OncoKB - JAX CKB - MyCancer Genome - COSMIC	Splice variants - SpliceSiteFinder-like - MaxEntScan - NNSPLICE - GeneSplicer
Class 1 Not pathogenic	Class 2 Unlikely pathogenic	Class 3 Possibly pathogenic	Class 4 Likely pathogenic	Class 5 Pathogenic
Validated single-nucleotide polymorphism (SNP) with frequency of >1% in dbSNP database	Neutral variant (no effect on amino acid) and no effect on splicing. Listed in dbSNP database, but frequency <1%. TP53-IARC: - TA-class functional	Prediction benign: -SIFT: tolerated -PolyPhen-2: benign -Align-GVGD: ≤C25 Prediction unsure: -SIFT: tolerated -PolyPhen-2: possibly damaging -Align-GVGD: C15 TP53-IARC: - TA-class partially functional	Prediction damaging: -SIFT: deleterious -PolyPhen-2: probably damaging -Align-GVGD: ≥C35 Small in-frame deletionst for which conserved amino acids are lost Splice variants for which all four tools predict >20% difference in wild type versus mutant	Truncating variants for which nonsense mediated decay is expected or for which conserved protein domains are completely lost Known pathogenic variants in (clinical) knowledgebases TP53-IARC: - TA-class nonfunctional

Oncogenes		
Knowledgebases - ClinVar - OncoKB - JAX CKB - MyCancer Genome - COSMIC		
Class 1 Not pathogenic	Class 2/Class 3/Class 4 Unlikely pathogenic/Possibly pathogenic/Likely pathogenic	Class 5 Pathogenic
Validated single nucleotide polymorphism (SNP) with frequency of >1% in dbSNP database	The variant is known somatic in COSMIC The variant is known transforming in literature The variant type is similar to known hotspot (same amino acid is affected or variant in nearby amino acid) The variant affects amino acids or conserved domains that are important for gene regulation ↓ Four times no: Class 2 Yes/no: Class 3/4 Four times yes: Class 4	Known pathogenic hotspot variants in (clinical) knowledgebases

FIG A1. Variant interpretation.

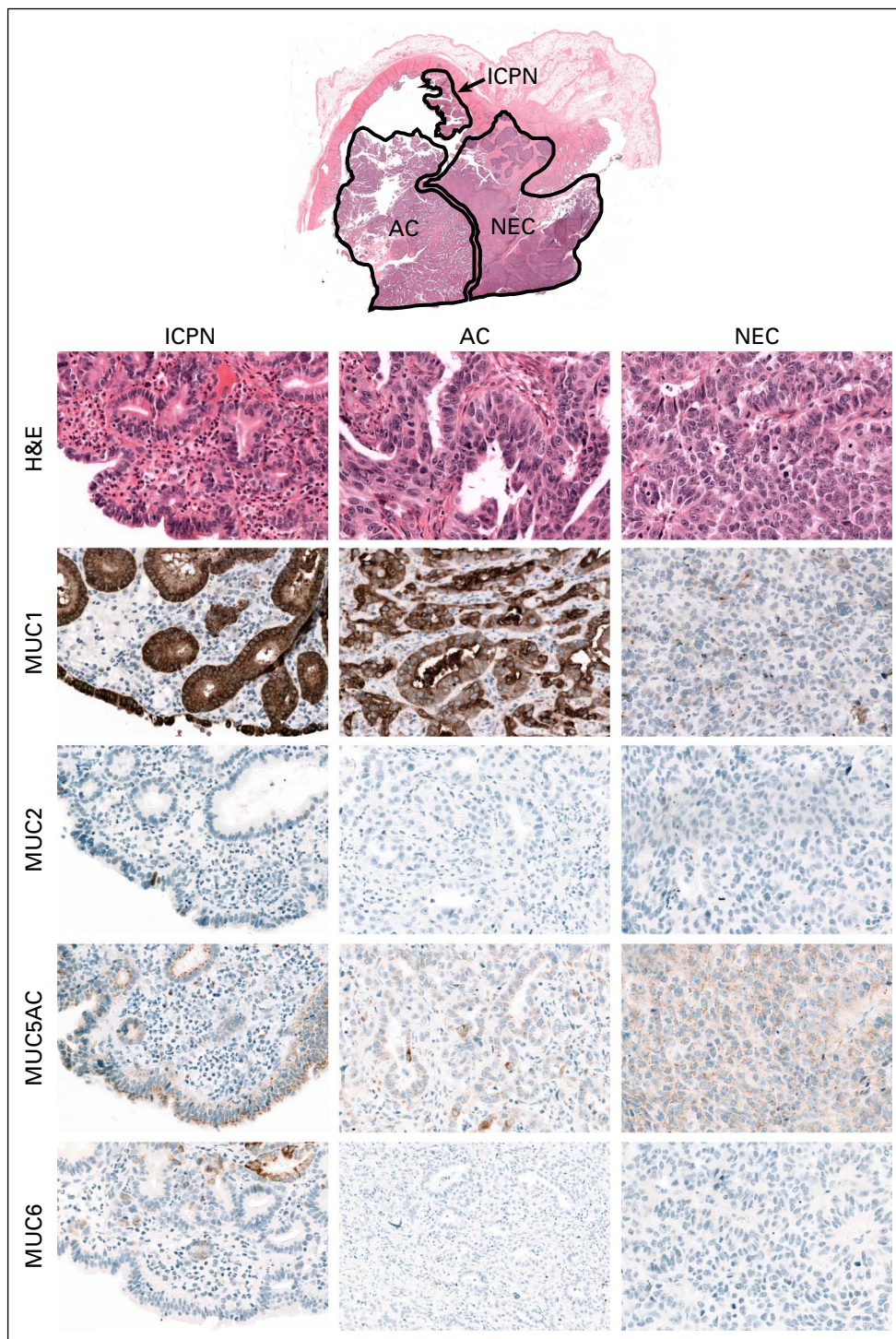


FIG A2. Immunohistochemical staining of case 5. Lower panel: magnifications 400x. AC, adenocarcinoma; H&E, hematoxylin and eosin; ICPN, intracholecystic papillary neoplasm (predominantly biliary subtype); NEC, neuroendocrine carcinoma.

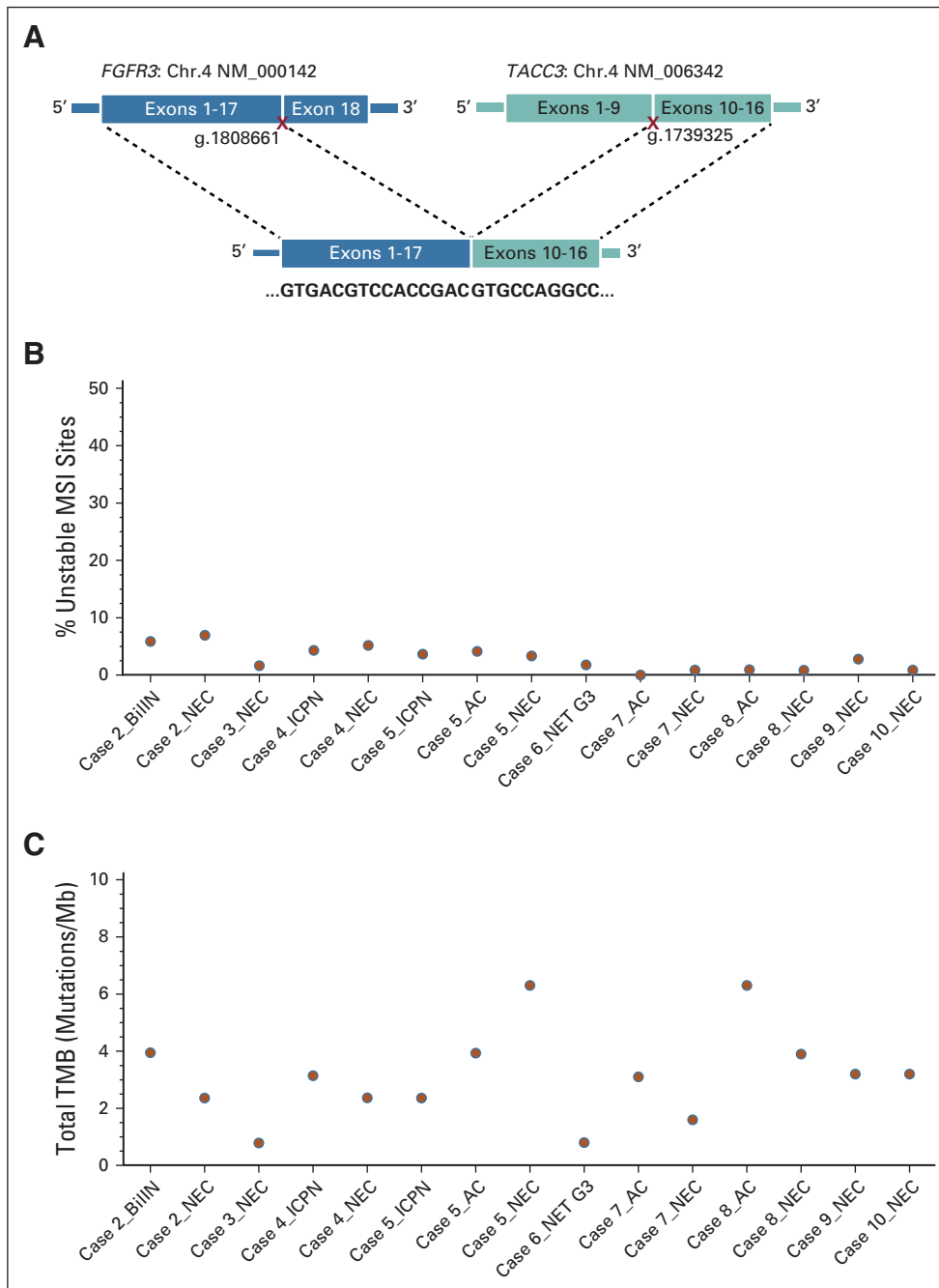


FIG A3. Somatic alterations in NE GBC. (A) *FGFR3-TACC3* fusion detected in all three components of case 5. (B) Microsatellite status. All cases were MSS (with < 15% unstable sites). (C) Total TMB. Case 1 was excluded for microsatellite instability and TMB analysis. AC, adenocarcinoma; BiLiN, biliary intraepithelial neoplasia; MSS, microsatellite stable; NEC, neuroendocrine carcinoma; NE GBC, neuroendocrine gallbladder cancer; TMB, tumor mutational burden.