Original Article

KRAS, NRAS and BRAF analysis of ampullary adenocarcinoma classified using CK7, CK20, MUC1 and MUC2

Marwa Ferchichi[1,2], Raja Jouini[2], Imen Ayari[1,2], Wafa Koubaa[2], Aschraf Chadli-Debbiche[2], Ehsen BenBrahim[2]

[1]University of Sciences, Farhat Hached Campus, Tunis El Manar, Tunis, Tunisia; [2]Pathology Department, Habib Thameur Hospital, Tunis, Tunisia

Contributions: (I) Conception and design: R Jouini, M Ferchichi; (II) Administrative support: W Koubaa; (III) Provision of study materials or patients: M Ferchichi, R Jouini, I Ayari; (IV) Collection and assembly of data: M Ferchichi, R Jouini; (V) Data analysis and interpretation: M Ferchichi, R Jouini, E BenBrahim; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Marwa Ferchichi, PhD. 8 Ali Ben Ayed Street, Montfleury, Tunis 1008, Tunisia. Email: marwaferchichi88@gmail.com.

Background: Ampullary carcinomas are rare and dominated by adenocarcinomas. They account for only 0.5% of all gastrointestinal malignancies. Ampullary adenocarcinoma (AAC) with pancreaticobiliary (PB) histology has a worse outcome than that with intestinal (IT) histology. The mixed subtype contains the two epitheliums. This subclassification remains a challenge for pathologists and induces a reasonable level of disagreement. Genetic features of these subtypes are unclear. In this study, we aimed to reclassify AAC cases then to evaluate differences in prognostic, pathological and molecular parameters including mutational status of three oncogenes between these subtypes.

Methods: AACs from 21 Tunisian patients were used in this study. Reclassification was made based on histology and immunohistochemistry (IHC) using CK7, CK20, MUC1 and MUC2. Mutational analysis included the pyrosequencing of KRAS, NRAS and BRAF.

Results: Fifteen cases were PB subtype, 2 cases were IT subtype and 4 cases were mixed subtype. CK20 and MUC2 were associated with N stage, MUC1 and histomolecular subtype with T stage. Nine cases were mutated and 12 were wild-type. Eight cases were KRAS mutated (5 G12D and 3 G12V). Only 1 case was NRAS mutated (G12D). No BRAF mutation was found. Genetic alterations didn't influence prognostic factors.

Conclusions: We validate the prognostic utility of AAC histomolecular classification.

Keywords: Ampullary adenocarcinoma (AAC); cytokeratin (CK); apomucin (MUC); RAS; RAF

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Introduction

Ampullary carcinomas are rare and dominated by adenocarcinomas. They account for only 0.5% of all gastrointestinal malignancies (1,2) and induce 36% of all pancreaticoduodenectomies (3). Although having a better prognosis than pancreatic ductal adenocarcinoma, ampullary adenocarcinoma (AAC) remains a deadly disease, killing 60% of affected patients (4). AAC with pancreaticobiliary (PB) histology has a worse outcome than this with intestinal (IT) histology (2). The IT type originates from the IT epithelium overlying the ampulla and the PB type originates from the epithelium of the distal common bile duct, distal pancreatic duct, or common ampullary duct. In addition, mixed type (M) contains both epitheliums (5). This subclassification remains a challenge for pathologists and induces a reasonable level of disagreement. Immunohistochemistry (IHC) using cytokeratin (CK) and apomucin (MUC) could facilitate subtyping. In fact, PB type expresses CK7 and MUC1, while IT type expresses CK20 and MUC2 and M type expresses all these markers (6). Genetic features of these subtypes remain unclear. In this study, we aimed to evaluate differences in prognostic, pathological and molecular parameters including mutational

status of three oncogenes KRAS, NRAS and BRAF, between these subtypes in AAC from 21 Tunisian patients; subtyped on HE and IHC using CK7, CK20, MUC1 and MUC2.

Methods

Cases selection

This retrospective study was approved by institutional ethics committee of Habib Thameur Hospital of Tunis (HTHEC-2017-03). Clinical, epidemiological and prognosis analysis; including the following parameters: age, sex, tumor size, TNM stage, differentiation, vascular emboli and perineural invasion, was determined referring to patients' files. Formalin fixed paraffin embedded (FFPE) tissues, resected in the period 2000–2016, were obtained from the archival tissue collection of pathology department. All specimens were fixed in 10% buffered formalin. Cases were revised by two pathologists to subclassify tumors.

MUC and CK IHC

IHC labeling was carried out in an automated Leica Bond Max (Leica Microsystems, Germany) through the following mouse monoclonal antibodies (anti-CK7: NCL-L-CK7-560, clone RN7, 1:100; anti-CK20: mouse NCL-L-CK20-561, clone PW31, 1:100; anti-MUC1: NCL-MUC-1, clone Ma695, 1:100; and anti-MUC2: NCL-MUC-2, clone Ccp58, 1:100; Novocastra, Leica Biosystems, Newcastle, Upon, UK). Briefly, 3 µm thick sections were prepared from each bloc and were dried overnight at 60 ℃. First, tissues were deparaffinized using xylene and pre-treated with the Epitope Retrieval Solution (EDTA-buffer, pH =8.8) in the rate of 98 ℃ for 20 min. After washing steps, peroxidase blocking was carried out for 10 min using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems). Tissues were again washed and then incubated with primary antibody for 30 min at 25 ℃. Subsequently, tissues were incubated with polymer for 10 min and developed with DAB-Chromogen for 10 min. IHC reaction was considered to be positive regardless the number of cells stained.

Histomolecular classification

Pancreatobiliary subtype

Most PB subtype have flat or micropapillary pattern, often complex with cribriform areas. The tumor cells are cubic without pseudostratification. Nuclei are enlarged and vesicular with dispersed chromatin. They possess varying degrees of mucosecretion with morphology similar to pancreatic ductal adenocarcinoma. In IHC, tumor cells express MUC1 and CK7.

IT subtype

Superimposed to digestive tract segments, the architecture of the lesion was tubulous, tubulo-villous or villous. Neoplastic cells are cylindrical with pseudostratified and elongated nuclei showing varying degrees of atypia. In IHC, tumor cells express CK20 and MUC2.

Mixed subtype

This subtype contains >10% of the two epitheliums, and express CK7, CK20, MUC1 and MUC2.

Molecular analysis

Molecular analysis was performed as described in (7). Briefly, the most representative blocks were selected. For each sample, 10 sections were used for DNA extraction. Genomic DNA extraction was performed according to Kit (QIAamp® DNA FFPE Tissue QIAGEN, Hilden, Germany) manufacturer's handbook. Nine hotspot sites of KRAS, NRAS and BRAF were analyzed in this order—KRAS: codons 12 and 13, codons 59 and 61, codon 117 and codon 146, NRAS: codons 12 and 13, codons 59 and 61, codon 117 and codon 146 and BRAF: codon 600. After each pyrosequencing, the mutated samples were excluded and only wild type samples could be amplified for following sequencing. PCR was performed in 30 µL final volume with 2 U of Taq polymerase (500 U Taq polymerase, Agilent Technologies, Wilmington, USA), 1X PCR buffer, 0.1 mM dNTPs, 30 pM of each primer and 50 ng of genomic DNA. A wild-type genomic DNA (QIAGEN) was used as a negative control. Real time sequencing was performed using PyroMark Q24 pyrosequencing instrument and software according to the manufacturer's instructions (www.pyrosequencing.com). Detailed information about methods and lists of primers' sequences, sequences to analyze and dispensation orders are described in our previous study (7), and are listed in the following articles (8,9).

Statistical analysis

Data were processed using SPSS 20.0 statistical software (SPSS, Inc., USA). Patients' characteristics were analyzed

using descriptive statistics. Qualitative and quantitative variables were analyzed, as appropriate, using non-parametric tests, with a significant P value less than 0.05.

Results

Clinicopathological characteristics

Thirteen patients were women and 8 were men. Mean age at operation was 60 years (35–75 years). Mean tumor size was 2.2 cm (0.5–7.5 cm). Resection margins were positive in one case. Tumor was well differentiated [13], moderately differentiated [7] and poorly differentiated [1]. T1 stage was observed in 4 cases, T2 in 12 cases and T3 stage in 5 cases with N1 stage in 5 cases. Perineural invasion and vascular emboli were present in 2 and 4 cases, respectively. Associated lesions were PanIN in 10 cases. Perineural invasion was statistically associated with vascular emboli and differentiation degree (P=0.029 and 0.033, respectively). T stage was associated with tumor size and perineural invasion (P=0.049 and 0.056, respectively). Sex and N stage were statistically associated (P=0.047). CK7, CK20, MUC1 and MUC2 were positive in 17, 8, 18 and 7 cases, respectively. MUC1 influenced T stage (P=0.033) while N stage was statistically associated with MUC2 and CK20 (P=0.025 and 0.047, respectively). These data are shown in *Table 1*.

Histomolecular classification

Based on HE and IHC evaluation, three subtypes were obtained:

- Fifteen cases MUC1+/CK7+ were classified as PB (*Figure 1*);
- Two cases MUC2+/CK20+ were classified as IT (data not shown);
- Four cases MUC1+/MUC2+/CK7+/CK20+ were classified as M (*Figure 2*).

Genetic characteristics

Nine cases were mutated and 12 were wild-type. Eight cases were KRAS mutated (5 G12D and 3 G12V). Only one case was NRAS mutated (G12D). No BRAF mutation was found. Seven of the 15 PB cases and 2 of the 4 M cases were mutated. The two IT subtypes were wild-type. Mutations classes' distribution through histomolecular subtypes and their influence on prognostic factors are shown in *Table 1*.

Histomolecular subtypes characteristics

Different characteristics of histomolecular subtypes are shown in *Table 2*. Briefly, 4/4 M and 1/15 PB subtype were N1, while 2/2 IT subtype, were N0. This difference was significant (P=0.001). Histomolecular subtype was correlated too with associated lesions (P=0.027). The only R1 case was PB subtype. The two IT subtype cases were wild-type, while 2/4 of M subtype and 7/15 of PB subtype were mutated.

Discussion

In this study, we found a statistically significant difference between histological subtypes of AAC in N stage and precursor lesions. Histologically, the IT type evolves through adenoma-carcinoma sequences and the PB type arises from precursor large-duct PanIN (5). Concerning our cases, 15/21 were PB, 2 were IT and 4 were M; which is comparable to other studies' findings. Indeed, the overall prevalence of the IT type ranges from 26% to 74%, the PB type from 22% to 72% and the M from 7% to 39% (4,6,10,11-24).

We found that IHC parameters (MUC1, CK7 and CK20) influence significantly T and N stage. In other studies, MUC1 and MUC2 expression was significantly associated with tumor differentiation, lymphatic and perineural invasion, tumor stage and survival (5,17).

Histomolecular subtypes are thought to be one of the most important prognostic factors for AAC. Patients with PB type have a worse overall survival than those with IT type (2,5,17-19,22). In addition, histomolecular subtypes are significantly associated with pathological grade, lymphatic and perineural invasion, tumor stage, CK7, CK20, MUC1 and MUC2 expression (4,5,11,13).

More recent studies have investigated additional markers such as DNA mutations to identify prognostically distinct AAC subtypes. Analysis revealed 19,143 genome-wide somatic point mutations, of which 30 maps within known annotated coding sequences. The most notable alteration is an activating KRAS mutation at codon 12 (G12V) (25). KRAS mutations were detected in 8/21 of our cases as other studies which found them in 23% to 47% of cases (4,5,11,12,19,26,27). Also, we found mutations in NRAS in one case. BRAF wasn't mutated. Seven of the 15 PB and 2 of the 4 M were mutated. The 2 IT subtypes were wild-type. This prevalence is also in line with observations

Table 1 Influence of clinicopathological and histomolecular parameters on prognostic factors

Characteristic	T stage				N stage			Differentiation			
	T1 (n=4)	T2 (n=12)	T3 (n=5)	P	N0 (n=16)	N1 (n=5)	P	Well differentiated (n=13)	Moderately differentiated (n=7)	Poorly differentiated (n=1)	P
Age (years)											
≤60	1	6	2	0.838	7	2	1	5	3	1	0.796
>60	3	6	3		9	3		8	4	0	
Sex											
Male	1	6	1	0.484	4	4	0.047	4	4	0	0.469
Female	3	6	4		12	1		9	3	1	
Perineural invasion											
Present	0	0	2	0.056	2	0	1	0	1	1	0.033
Absent	4	12	3		14	5		13	6	0	
Vascular emboli											
Present	0	2	2	0.449	2	2	0.228	2	1	1	0.272
Absent	4	10	3		14	3		11	6	0	
Resection limits											
R0	4	11	5	1	15	5	1	12	7	1	1
R1	0	1	0		1	0		1	0	0	
MUC1											
Positive	2	12	4	0.033	13	5	0.549	11	6	1	1
Negative	2	0	1		3	0		2	1	0	
MUC2											
Positive	1	5	1	0.83	3	4	0.025	4	3	0	0.768
Negative	3	7	4		13	1		9	4	1	
CK7											
Positive	2	11	4	0.109	12	5	0.532	10	6	1	1
Negative	2	1	1		4	0		3	1	0	
CK20											
Positive	2	5	1	0.698	4	4	0.047	5	3	0	1
Negative	2	7	4		12	1		8	4	1	
Mutational status											
Wild-type	3	6	3	0.838	10	2	0.611	7	4	1	1
Mutated	1	6	2		6	3		6	3	0	
Mutational subtypes											
KRAS-G12D	0	4	1	0.608	3	2	0.608	3	2	0	1
KRAS-G12V	0	2	0		2	1		2	1	0	
NRAS-G12D	1	0	0		1	0		1	0	0	

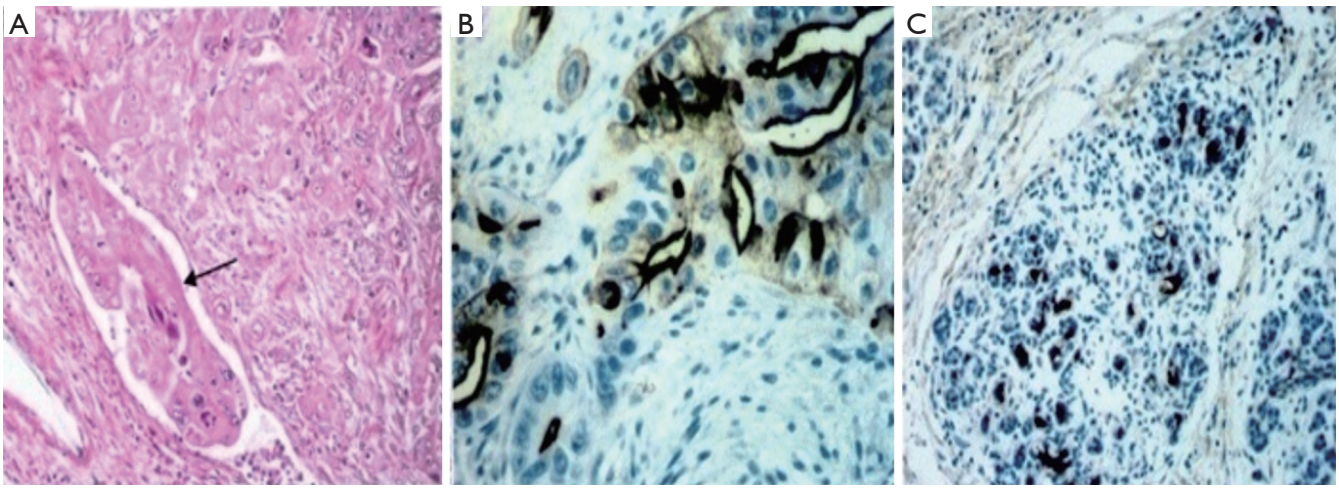

Figure 1 Pancreatobiliary subtype(hematoxylin-eosin staining) (A) classified by histology. This case expressed typically MUC1 in IHC (B) like pancreatic acini used as control (C) (magnification: A, ×200; B, ×400; C, ×200). The arrow shows a vascular emboli.

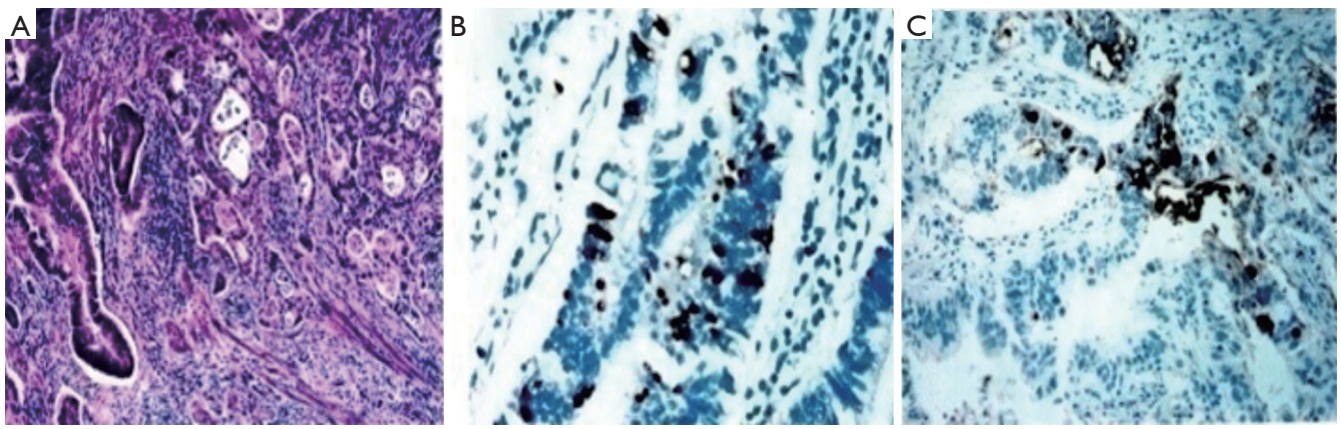

Figure 2 Mixed subtype (A) (hematoxylin-eosin stain) expressed MUC1 (B) and MUC2 (C) in IHC (magnification: A, ×200; B, ×400; C, ×200).

made by others. KRAS is mutated in 36% to 61% of PB subtype, in 5% to 52% of IT subtype and in 7% to 45% of M subtype. No mutation is detected in NRAS and BRAF is mutated in 0% to 9% of PB subtype, in 0% to 1% of IT subtype and in 0% to 1% of M subtype (4,5,14,19,28).

Studies analyzing the prognostic value of KRAS mutations in AAC reported conflicting results. In fact, KRAS mutations especially KRAS G12D are associated with shorter survival in some series (4,12,29,30). Interestingly, patients with mutations other than KRAS G12D do not appear to be different from those with KRAS wild-type (12). While, other groups don't show any influence of KRAS or BRAF status on survival (18,19,30).

Only few studies have described the prognosis and characteristics of M subtype. M subtype was reported to have an intermediate prognosis between the PB and IT phenotypes (17). In the other side, others indicated that the pathological characteristics of the M subtype is similar to those of the PB subtype, and prognosis of the M subtype is poor in comparison to that of the IT phenotype (5). In our series, we couldn't give more clarifications because of the limited number of M subtypes.

Our study has several limitations. First, our series was small. Then, 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 AAC are reflected in RNA profiling (15). Future trials should be designed in view of

Table 2 Influence of clinicopathological and molecular parameters on histomolecular subtypes

Characteristic	Histomolecular subtype			P
	Pancreatobiliary (n=15)	Intestinal (n=2)	Mixed (n=4)	
Age (years)				
≤60	8	0	1	0.489
>60	7	2	3	
Sex				
Male	4	1	3	0.071
Female	11	1	1	
Differentiation (#)				
Well differentiated	10	1	2	0.852
Moderately differentiated	4	1	2	
Poorly differentiated	1	0	0	
Perineural invasion				
Present	2	0	0	1
Absent	13	2	4	
Vascular emboli				
Present	2	0	2	0.175
Absent	13	2	2	
T stage				
T1	3	8	4	0.178
T2	8	0	1	
T3	4	4	0	
N stage				
N0	14	2	0	0.001
N1	1	0	4	
Resection limits				
R0	14	2	4	1
R1	1	0	0	
Mutational status				
Wild-type	8	2	2	0.621
Mutated	7	0	2	
Mutational subtypes				
KRAS-G12D	4	0	1	1
KRAS-G12A	2	0	1	
NRAS-G12D	1	0	0	
Tumor size				
≤2	7	0	3	0.327
>2	8	2	1	
Associated lesions				
PanIN	6	0	4	0.027
Others	9	2	0	

our increased understanding of the different anatomic and histomolecular profiled subtypes of these cancers.

In conclusion, we validate the prognostic utility of the histomolecular classification of AAC. This combination of HE and IHC classification should be incorporated into our clinical practice. We hypothesize that these tumors necessitate highly patient- and tumor-individualized therapy. Future efforts to understand the biological bases of these subgroups are needed.

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None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical statement: The study was approved by the Habib Thameur Hospital Ethics Committee HTHEC (No. HTHEC-2017-03). As this was a retrospective study, informed consent is not required.

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