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ORIGINAL ARTICLE

# Immunohistochemical Predictors for Intestinal and Pancreatobiliary Types of Adenocarcinoma of The Ampulla of Vater

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## Abstract

**Objectives** To investigate immunohistochemical predictors for intestinal and pancreatobiliary types of adenocarcinoma of ampulla of Vater and identify clinicopathological characteristics associated with the histological types and patient survival.

**Methods** Immunohistochemical markers included MUC1, MUC2, MUC5AC, CDX2, CK7, and CK20. The data were analyzed by univariate and multivariate methods. The two-step cluster method was used to determine the best immunohistochemical markers to discriminate the intestinal from the pancreatobiliary type.

**Results** This study identified 9 (33.3%) intestinal and 21 (66.7%) pancreatobiliary tumors. CK7 and CDX2 achieved the highest value (= 1) as predictor markers, while CK20, MUC1, and MUC2 showed degrees of importance equal to 0.77, 0.71, and 0.68, respectively. MUC5AC did not reach 0.50 of importance. In the univariate analysis, lymph node involvement, staging (TNM), and angiolymphatic and perineural invasions were associated with histological types. The independent clinicopathological variable in the multivariate model to predict the histological type was angiolymphatic invasion ($p = 0.005$), OR = 17 (95% CI 2.33 to 123.83). The final model showed positive nodes (N1) associated with shorter survival (HR = 9.5; $p = 0.006$). Overall survival at 12, 36, and 60 months was 88.5, 67.0, and 47.6%, respectively.

**Conclusions** CDX2 and CK7 were the immunohistochemical markers that best discriminated the intestinal from the pancreatobiliary type. Lymph node involvement had a high impact on survival and proved to be more frequent in the pancreatobiliary type.

**Keywords** Ampulla of Vater · Adenocarcinoma · Immunohistochemistry · Survival

## Introduction

Ampulla of Vater tumors (AVT) are rare neoplasms. These correspond to 0.5% of all gastrointestinal tract tumors and 6 to 20% of all periampullary tumors.[1] In 1994, Kimura et al.[2] were the first to describe the two histological types of AVT: the intestinal type (IT) and the pancreatobiliary type (PBT). In 2000, Albores-Saavedra et al.[3] defined the morphological characteristics of these tumors. Prevalence of the PBT is higher than IT.[1–5]

The intestinal-type epithelium covers the major duodenal papilla and the pancreatobiliary type lines the ampulla of Vater, the pancreatic duct, and the distal common bile duct.[6,7] Tumors of the IT, usually, originate from a precursor adenomatous lesion, following the adenoma-dysplasia-adenocarcinoma sequence.[8,9] Tumors of the PBT seem to be associated more frequently with neoplastic intraepithelial ductal precursor lesions, but they can also be developed from polypoid lesions with pancreatobiliary phenotypes.[10,11]

The correct classification of ampullary tumors remains a challenge for pathologists, and the best markers are still controversial.[1,6,10–17] In this context, the present study seeks to produce an immunohistochemical panel, including the cell markers MUC1, MUC2, MUC5AC, CDX2, CK7, and CK20, in an attempt to identify those with more accuracy in the

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discrimination of intestinal and pancreatobiliary histological types and associated clinical-pathological characteristics in patients with AVT submitted to surgical treatment with curative intent.

## Material and Method

This is a longitudinal study carried out in a single tertiary care institution, in which 33 patients were studied between 2005 and 2014. Informed consent was obtained from each recruited patient prior to surgery. This research was approved by the Research Ethics Committee of the Federal University of Minas Gerais (UFMG), logged under protocol number CAAE - 23377113.1.0000.5149.

Thirty patients with AVT, who had undergone complete tumor resection (R0) with curative intent were included in this study. Patients with distal cholangiocarcinoma, adenocarcinoma of the pancreas head, adenocarcinoma of the duodenum, and tumors not classified as adenocarcinomas, as well as patients submitted to biopsies and palliative surgical procedures, were excluded from this study. This study also excluded three patients whose paraffin blocks from their tumors were not located in the archives of the Department of Pathological Anatomy.

The following clinical data were collected from patient medical records: age, sex, smoking, alcohol intake, comorbidities, gallstones, Ca 19.9, family history of cancer, type of resection, and TNM stage. Tumor size, surgical resection margins, lymph node involvement, pTNM staging, and angiolymphatic and perineural invasions were obtained from anatomopathological reports. Tumors were classified according to the nomenclature set forth by the World Health Organization (WHO, 2010), while the staging was performed according to the TNM staging (AJCC/UICC), 7th edition, 2010.[18]

All surgical specimens were initially fixed in 10% formaldehyde for 24 h. The samples were automatically processed with subsequent inclusion in paraffin. The paraffin blocks were cut by microtomy, obtaining slices with a thickness of 4 μm, which were mounted on glass slides and subsequently stained by the hematoxylin-eosin (H&E) method.

The H&E-stained slides served as a parameter to select the paraffin block most suitable for immunohistochemistry that in which a representative area of the tumor was chosen. From the chosen paraffin block, new cuts of 4-μm thickness were obtained (*not tissue microarray*). For immunohistochemistry, the sections were deposited onto adhesive-coated glass slides.

Immunohistochemical staining was performed according to manufacturer instructions. The antibodies used and their respective dilutions are shown in Table 1. Briefly, the slides were initially left in an oven at 60 °C for 12 h. These were then deparaffinized in xylol and rehydrated in successive alcohol baths, followed by Epitope retrieval in a citrate buffer at pH 6.0 in a vegetable steamer (Cuisinart® Turbo Conection Steamer) for 30 min. The endogenous peroxidase was blocked using 3% hydrogen peroxide for 15 min and proteins for 10 min. Next, the specimens were incubated overnight with the primary antibody. After removing the antibody, the complement was placed on the slide, and the advanced HRP polymer was applied for 30 min. Staining was viewed using a 3,3′-diaminobenzidine substrate-chromogen (DAB) solution, followed by counterstaining with hematoxylin. For the expression of the anbibodies, an internal control was used as the positive labeling of the proteins in the bile duct and adjacent tissues. PBS was substituted for the primary antibody in negative controls.

**Table 1** Antibodies used in immunohistochemical reactions

| Monoclonal antibody | Clone | Brand | Dilution |
|---|---|---|---|
| Anti MUC 1 (rabbit) | EP1024Y | Abcam® | 1:200 |
| Anti MUC 2 (rabbit) | EPR6145 | Abcam® | 1:200 |
| Anti MUC 5 AC (Rat) | 45 M1 | Abcam® | 1:100 |
| Anti CDX2 (rabbit) | EPR2764Y | Abcam® | 1:200 |
| Anti CK7 (rabbit) | EPR11619Y | Abcam® | 1:200 |
| Anti CK20 (rabbit) | EPR1622Y | Abcam® | 1:200 |

Immunohistochemical analysis was performed by two blinded pathologists who were unaware of the patients' clinical data. If there was a disagreement, the final decision was made by a third senior pathologist. In the analysis of the mucins (MUC1, MUC2, MUC5AC) and the cytokeratins (CK7 and CK20), the expression was considered cytoplasmic, whereas in the analysis of the CDX2 transcription factor, the expression was nuclear. The evaluation conducted in this study was semi-quantitative and the tumors that showed positivity for the immunohistochemical markers in an area greater than or equal to 10% were considered positive.

Statistical analyses were performed using the Stata® software for MAC (Macintosh®), version 12, and the IBM SPSS Statistics, version 22 (SPSS Inc. Chicago, IL). The immunohistochemical predictors for tumor classification were obtained by the multivariate two-step cluster method. The recognized clusters were identified according to previous publications regarding immunohistochemical studies in AVT. Student's *t*, Fisher's, or chi-square tests were applied in order to verify the association of clinical-pathological variables and the subtypes of AVT. The magnitude of the associations was obtained by the odds ratio (OR). Based on the results of the univariate analysis, variables with $p$ values of less than 0.30 ($p < 0.30$) were selected as candidates for the multivariate model. In the multivariate analysis, the exact logistic regression model was used for the sequential deletion of the variables with a $p$ value of greater than 0.10 ($p > 0.10$). Survival curves were analyzed by the Kaplan-Meier method, and comparisons between groups were performed using the Logrank test. A multivariate Cox regression model was generated to

quantify the relationships between one or more factors of interest in survival. The obtained estimator was the hazard ratio (HR). The Schoenfeld residue analysis was performed to estimate the fit of the model. Survival at 12, 24, 36, and 60 months was obtained. Deaths that occurred within 30 days of the postoperative period were excluded from the survival analysis. The level of significance considered in all analyses was 5% ($p < 0.05$).

## Results

Thirty patients with AVT were studied, of which 10 were men and 20 were women. The mean age of the patients was 61.7 years (ranging from 30 to 79 years, SD 11.9). The median age was 62.5 years, IIQ = (54; 72). Smoking and alcohol consumption were absent in most patients, reaching only 30 and 10% of the cases, respectively. Three female patients had a previous history of malignant neoplasia (colon, breast, and brain).

Table 2 shows the immunohistochemical characteristics of AVT. The two-step cluster method classified the 30 carcinomas of the ampulla of Vater into two groups. One group with 21 cases (cluster 1—pancreatobiliary type) and another group with 9 cases (cluster 2—intestinal type). Table 3 shows the distribution of the tumors in each cluster. The degrees of importance of the CK7 and CDX2 markers, as predictors, were equal to 1, the highest possible value. The cytokeratin, CK20, and mucins—MUC1 and MUC2—presented degrees of importance equal to 0.77, 0.71, and 0.68, respectively. MUC5AC did not reach 0.50 of importance as a predictor. Figures 1 and 2 illustrate the histological types according to the expression of each immunomarker.

The association between the histological types of the resected tumors and the clinicopathological variables is shown in Table 4. The variables that were significantly associated with the histological types in the univariate analysis were lymph node (N) involvement, staging (TNM), angiolymphatic invasion, and perineural invasion. The independent variable that remained in the multivariate model to predict the histological type was angiolymphatic invasion ($p = 0.005$), OR = 17 (95% CI 2.33 to 123.83).

The overall survival of the patients was 32.3 months, with a median survival of 27.5 months (IIQ = 18; 48). The percentages of survivors at 12, 24, 36, and 60 months of follow-up were 87.8, 75.2, 69.9, and 60.5%, respectively. In the univariate statistical analysis, the prognostic factors associated to overall patient survival rate were age ($p < 0.05$), histological type ($p < 0.10$), TNM staging, lymph node involvement, angiolymphatic, and perineural invasions ($p < 0.05$), CK7 ($p < 0.10$), and CDX2 ($p < 0.10$). In the multivariate analysis, only neoplastic lymph node involvement (N1) had a significant impact on the patient survival rate. The hazard ratio was HR = 9.03 ($p = 0.009$).

**Table 2** Immunohistochemical characteristics of the 30 patients with adenocarcinoma of ampulla of Vater

| Variables | Total *n* (%) | *p* value |
|---|---|---|
| MUC1 | | |
| Negative | 6 (20.0) | 0.001 |
| Positive | 24 (80.0) | |
| MUC2 | | |
| Negative | 18 (60.0) | 0.361 |
| Positive | 12 (40.0) | |
| MUC5AC | | |
| Negative | 15 (50.0) | 0.999 |
| Positive | 15 (50.0) | |
| CDX2 | | |
| Negative | 20 (66.7) | 0.099 |
| Positive | 10 (33.3) | |
| CK7 | | |
| Negative | 10 (33.3) | 0.099 |
| Positive | 20 (66.7) | |
| CK20 | | |
| Negative | 18 (60.0) | 0.362 |
| Positive | 12 (40.0) | |

Exact binomial tests were performed to achieve an equality of proportions in each category

## Discussion

The mean of 62 years of age in the present study is consistent with those found in other studies in the literature[1], as was the mean age of patients with intestinal tumors (72 years) compared to those of the pancreatobiliary type (62 years). An increase in the incidence of AVT tumors has also been observed with advancing age. By contrast, unlike other reports[1,11,19–21], females were the majority in this study, representing 66.7% of the sample. Albores-Saavedra et al.[1] found a predominance of males, reporting no difference in prevalence among blacks and caucasians in the USA. Due to the high miscegenation rate in the Brazilian population, this parameter was not evaluated in this sample.

In the present study, the multivariate analysis method, called the "two-step cluster analysis", was used to determine which markers could best discriminate the histological types. This method recognized two different groups. The identification of cluster 1 (pancreatobiliary) and cluster 2 (intestinal) was based on published studies regarding the immunohistochemical classification of AVT. The pancreatobiliary type was responsible for 70% of the cases, and this result was in agreement with most reports in the literature[12,16,22–26]. However, this was the opposite of what was found in other publications[8,9,20,27]. The immunomarkers that were most important in the conformation of the two clusters were CK7 and

**Table 3** Classification of histological subtypes of adenocarcinoma of ampulla of Vater, using the two-step cluster statistical method according to immunohistochemical results

| Positive immunomarkers | Frequency (%) | Classification | |
|---|---|---|---|
| | | Cluster | Subtype |
| MUC2; CDX2, CK20 | 6 (23.3) | 2 | Intestinal |
| MUC1; CK7 | 5 (16.7) | 1 | Pancreatobiliary |
| MUC1; CK7; CK20 | 1 (3.3) | 1 | Pancreatobiliary |
| MUC1; CDX2 | 1 (3.3) | 1 | Pancreatobiliary |
| MUC1; MUC5AC; CK7 | 1 (3.3) | 1 | Pancreatobiliary |
| MUC1; MUC5AC; CK7 | 7 (21.3) | 1 | Pancreatobiliary |
| MUC1; MUC5AC; CK7; CK20 | 2 (6.7) | 1 | Pancreatobiliary |
| MUC1; MUC2; CDX2; CK20 | 2 (6.7) | 2 | Intestinal |
| MUC1; MUC2; MUC5AC; CK7 | 4 (10.0) | 1 | Pancreatobiliary |
| MUC1; MUC2; MUC5AC; CDX2; CK20 | 1 (3.3) | 2 | Intestinal |
| Total | 30 (100.0) | | |

CDX2. De Paiva Haddad et al.[6] described MUC1 and CK7 as being highly related to the pancreatobiliary subtype, and MUC2, CDX2, and CK20 as being highly related to the intestinal subtype. Our results were in agreement with those authors, except for the expression of mucins, which were not the most important in the present study.

Kawabata et al.[12] identified the immunophenotype CK20 +, MUC1 in 100% of cases, and CK20-, MUC1 + in 94% of

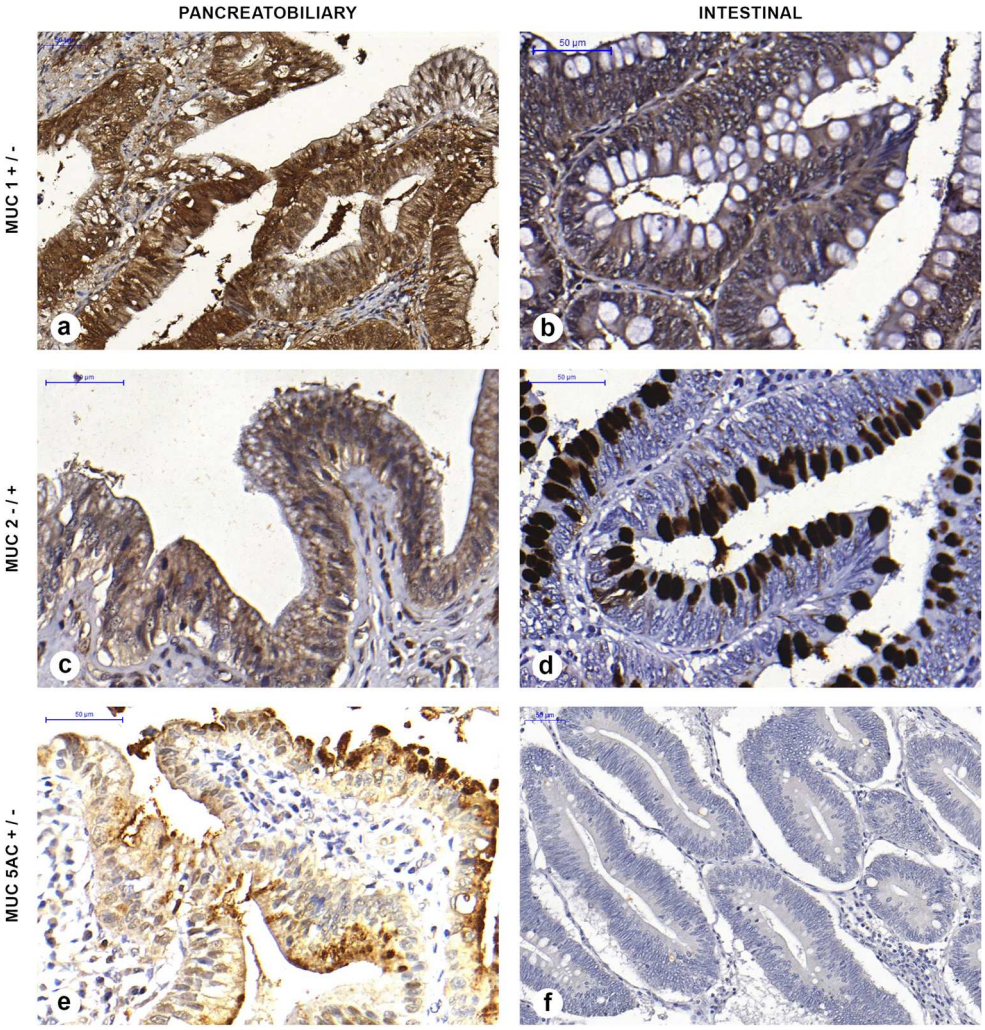


**Fig. 1** Immunohistochemical expression panel in pancreatobiliary and intestinal types of Ampulla of Vater adenocarcinoma: MUC 1 (**a**, **b**), MUC 2 (**c**, **d**), and MUC 5AC (**e**, **f**)

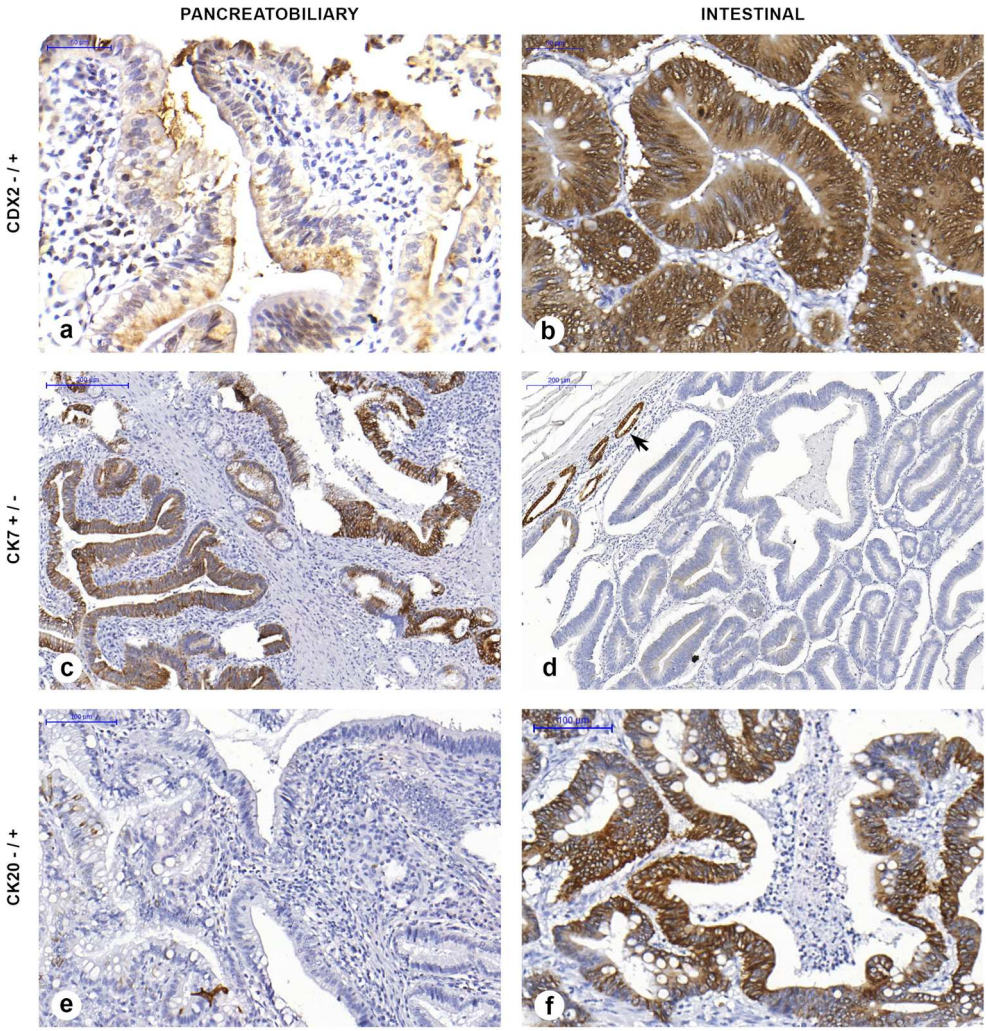


**Fig. 2** Immunohistochemical expression panel in pancreatobiliary and intestinal types of Ampulla of Vater adenocarcinoma: CDX2 (**a**, **b**), CK7 (**c**, **d**), and CK20 (**e**, **f**). Arrow shows internal positive control for CK7 expression in biliary tract (**d**)

pancreatobiliary type cases. Sessa et al.[18] studied MUC1, MUC2, MUC5AC, MUC6, and CDX2 in the subclass of 53 ampullary tumors. In this previous study, CDX2 was related to intestinal-type tumors, while MUC1 and MUC5AC were related to pancreatobiliary tumors. The present study's results were also in agreement with that reported by Chu et al.[28], who tested several immunomarkers. These authors found that CDX2 showed a sensitivity of 100% and a specificity of 83.3% for the intestinal type, while CK7 presented a sensitivity of 83.3% and a specificity of 81.8% for the pancreatobiliary type.

The advantage of the present study by using the complete histological cut, instead of the tissue array, was to allow an extensive area of the tumor to be examined, in addition to allowing the internal control of the reactions by positive or negative marking of adjacent tissues. In addition, it was noticed that, in some tumors, heterogeneous markings were found in different areas. Ohike et al.[10] described the mixed aspects of tumors that originate from precursor polypoid lesions, defined as intra-ampullary papillary-tubular neoplasm (IAPN). Reid et al.[29] showed that 40% of ampullary carcinomas are in fact mixed/hybrid by morphology. According to these authors, the results of immunohistochemical studies that utilize tissue microarrays prepared from a limited amount of tumor cells ought to be evaluated with caution, and may have to be avoided if a more "complete" picture of the tumor is intended. This fact can explain the difference between our results and the other publications that performed studies using the tissue microarray method. However, it was not the object of our study to explore the specific mixed characteristics of these tumors.

In the univariate analysis of the present study, the factors that negatively impacted the survival rate were immunophenotype, tumor T staging, lymph node involvement, and perineural and angiolymphatic invasions. Several publications corroborate these findings.[1,2,9,19,24,30,31]. An age of less than 40 years was also a poor prognostic factor in our patients. The youngest patient in our series was a 30-year-old woman who had a pancreatobiliary tumor, T4N1M0 (stage III), who survived only 6 months. Showalter et al.[31] and Colussi et al.[32] reported age as a factor of poor prognosis in the multivariate analysis.

**Table 4** Association between the intestinal and pancreatobiliary types of tumors and the clinicopathological variables in patients with adenocarcinoma of ampulla of Vater

| Variables | Subtypes | | Total | *p* values |
|---|---|---|---|---|
| | Intestinal 9 (30.0%) | Pancreatobiliary 21 (70.0%) | 30 (100.0%) | |
| Age (years) | | | | |
| Mean (SD) | 68.5 (8.7) | 58.5 (11.9) | 61.5 (11.8) | 0.031*a |
| Median (IIQ) | 72 (64; 75) | 62 (52; 66) | 62 (54; 72) | 0.046*b |
| < 50 | 0 (0.0) | 5 (100.0) | 5 (100.0) | |
| 50 to 59 | 2 (33.3) | 4 (66.7) | 6 (100.0) | 0.022* |
| 60 to 69 | 1 (10.0) | 9 (90.0) | 10 (100.0) | |
| ≥ 70 | 6 (66.7) | 3 (33.3) | 9 (100.0) | |
| Sex | | | | |
| Male | 4 (40.0) | 6 (60.0) | 10 (100.0) | 0.331 |
| Female | 5 (25.0) | 15 (75.0) | 20 (100.0) | |
| Smoking | | | | |
| No | 6 (28.6) | 15 (71.4) | 21 (100.0) | 0.999 |
| Yes | 3 (33.3) | 6 (66.7) | 9 (100.0) | |
| Alcohol | | | | |
| No | 9 (33.3) | 18 (66.7) | 27 (100.0) | 0.534 |
| Yes | 0 (0.0) | 3 (100.0) | 3 (100.0) | |
| Comorbidities | | | | |
| No | 3 (27.3) | 8 (72.7) | 11 (100.0) | 0.999 |
| Yes | 6 (31.6) | 13 (68.4) | 19 (100.0) | |
| Family cancer | | | | |
| Negative | 5 (30.5) | 8 (61.5) | 13 (100.0) | 0.673 |
| Positive | 3 (23.1) | 10 (76.9) | 13 (100.0) | |
| Gallstones | | | | |
| No | 6 (31.6) | 13 (68.4) | 19 (100.0) | 0.999 |
| Yes | 3 (27.3) | 8 (72.7) | 11 (100.0) | |
| CA19.9 | | | | |
| <37 | 3 (30.0) | 7 (70.0) | 10 (100.0) | 0.999 |
| ≥ 37 | 4 (36.4) | 7 (63.6) | 11 (100.0) | |
| T (TNM) | | | | |
| 1 | 4 (66.6) | 2 (33.3) | 6 (100.0) | |
| 2 | 4 (28.6) | 10 (71.4) | 14 (100.0) | 0,191 |
| 3 | 1 (14.3) | 6 (85.7) | 7 (100.0) | |
| 4 | 0 (0.0) | 3 (100.0) | 3 (100.0) | |
| N (TNM) | | | | |
| Negative | 9 (42.9) | 12 (57.1) | 21 (100.0) | 0.029* |
| Positive | 0 (0.0) | 9 (100.0) | 9 (100.0) | |
| TNM stage | | | | |
| I | 8 (53.3 | 7 (46.7) | 15 (100.0) | |
| II | 1(8.3) | 11 (91.7) | 12 (100.0) | 0.022* |
| III | 0 (0.0) | 3 (100.0) | 3 (100.0) | |
| Angiolymphatic invasion | | | | |
| No | 8 (61.54) | 5 (38.46) | 13 (100.00) | 0.002* |
| Yes | 1 (5.88) | 16 (94.12) | 17 (100.00) | |
| Perineural invasion | | | | |
| No | 8 (44.44) | 10 (55.56) | 18 (100.00) | 0.049* |
| Yes | 1 (8.33) | 11 (91.67) | 12 (100.00) | |

[a] Student's *t* test

[b] Test of proportions (Fisher's exact, based on the median cut-off point); Fisher's exact test was used for all other variables

*$p < 0.05$

However, inversely to what we found, in these studies, the risk increased in older patients (> 75 years). The only independent prognostic factor with statistical significance in our study was lymph node involvement (HR = 9.03, $p = 0.009$), which was present only in patients with pancreatobiliary tumors.

The distinction between PBT and IT ampullary carcinoma has significant implications for clinical management. The histopathological diagnosis could guide oncologists for chemotherapy treatment. PBT type mimics ductal adenocarcinoma of the pancreatic head and should be treated adjuvantly like pancreatic cancer, while IT mimics duodenal or colorectal cancer and might be treated in accordance with those entities. Therefore, gemcitabine-based treatment regimens could be used for PBT tumors and fluorouracil-based for IT tumors.[20,29] A substantial survival benefit was demonstrated for the PBT subgroup of patients receiving adjuvant gemcitabine chemotherapy compared with those PBT patients who did not (32 vs 13 months; $P = .0130$).[20]

A limiting factor of this study was the non-assessment of adjuvant chemotherapy due to the lack of well-established protocols. This may have influenced patient survival. However, it is known that the role of this treatment is not yet well defined in the literature.[33]

In conclusion, the present study found that, in the adenocarcinoma of the ampulla of Vater, the CDX2 was strongly related to the intestinal histological type and CK7 to the pancreatobiliary type. Therefore, the diagnosis regarding the histological classification could exclude the attainment of an immunohistochemical panel involving several markers, making the process more feasible. Because angiolymphatic and perineural invasions were more associated with the pancreatobiliary type, these pathological characteristics may aid in the prediction of this histological type. The only independent factor that had a negative impact on survival was lymph node involvement.

**Author Contributions** João Paulo Lemos da Silveira Santos and João Bernardo Sancio Rocha Rodrigues: collecting data, drafting, and final approval of the work.

Carla Jorge Machado: statistical analysis, drafting, and final approval of the work.

Eduardo Paulino Junior and Paula Teixeira Vidigal: interpretation and immunohistochemical analysis, drafting and final approval of the work.

Vivian Resende: conception and design, drafting and final approval of the work.

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## Compliance with Ethical Standards

This research was approved by the Research Ethics Committee of the Federal University of Minas Gerais (UFMG), logged under protocol number CAAE 23377113.1.0000.5149.

**Conflict of Interest** The authors declare that there is no conflict of interest.

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