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The Significance of Tumor Budding and Immunohistochemical Axl Expression in Gallbladder Adenocarcinomas

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Background: Tumor budding is a histopathological finding that is accepted as an indicator of epithelial-mesenchymal transformation in many solid tumors. Axl is a Receptor Tyrosine Kinase (RTK) family member and contributes to epithelial-mesenchymal transformation. It has been reported that its overexpression in various solid cancer cells is associated with a poor prognosis. It is claimed that Axl RTK may be the targeted molecule in treating some cancers due to its location in the cell membrane.

Aims: To investigate the relationship between immunohistochemical (IHC) Axl expression with tumor budding on the histopathological level and their prognostic significance in patients with gallbladder carcinoma. Thus, it is aimed to contribute to the emergence of a molecular option for targeted, personalized therapy in these patients.

Study Design: A retrospective cross-sectional study

Methods: Thirty-eight gallbladder cancer patients who underwent surgery between 2000 and 2017 were included in the study. The expressions of Axl RTK in tumor tissues were evaluated by the IHC method. Demographic data (age, sex) of patients, histopathological features (size, growth pattern), tumor differentiation, pathological T staging, lymphovascular invasion, perineural and serosal invasion, surgical margin, tumor infiltrated lymphocyte, and tumor budding were examined. The tumor budding of the tumor was made according to the International Tumor Budding Consensus Conference and was classified as low (0-4 buds), intermediate (5-9 buds), high (≥ 10 buds). The relationship between clinical pathologic features, the survival rate, and Axl expression was analyzed with Person's chi-square, Cox regression tests, and the Kaplan-Meier method.

Results: Tumor budding was determined as low in 12, intermediate in 10, and high in 16 cases. The increased degree of tumor budding was associated with focal-diffuse Axl expression ($p = 0.018$), infiltrative growth patterns ($p = 0.031$), poor differentiation ($p = 0.006$), advanced pathological stage ($p = 0.002$), and serosal ($p = 0.040$), perineural ($p = 0.008$), and lymphovascular invasion ($p < 0.0001$). Overall survival time was shorter in patients with intermediate to high tumor budding compared with those with low tumor budding ($p = 0.011$).

Conclusion: Axl expression appears to be associated with tumor budding capacity, which may be a poor prognostic criterion for patients with gallbladder cancer. It may be a good target to prevent tumor budding to reduce tumor invasion and metastasis.

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INTRODUCTION

Gallbladder cancer (GBC) ranks sixth among gastrointestinal cancers, and it is the most common malignant disease of the biliary tract with a poorly understood etiology[1-3]. Since the disease is often diagnosed at an advanced stage, GBC has a high mortality rate[3,4]. The probable reason for this is that it spreads to the other organs via lymph, perineural, and blood vessels or directly to the liver at an early stage[5]. The histopathological subtype, tumor size, differentiation, perineural and lymphovascular invasion, regional lymph node metastases, and surgical margins are known as the main prognostic features for today[1,4,6,7]. Mesenchymal-like cancer cells are known to play a decisive role in chemotherapy resistance and the metastatic stage of malignant neoplasms[8,9]. Tumor budding (TB) is the histopathological reflection of epithelial-mesenchymal transition and represents mesenchymal-like cancer cells. TB, the counterpart to these cells in the histological examination, has recently been an intriguing topic in explaining tumor features, and many studies have been carried out on its prognostic significance in various types of cancer[10-14]. A consensus-based on the histopathological criteria at the H&E level in colorectal cancers was established at the International TB Consensus Conference (ITBCC) in 2016[10]. However, there is no recognized TB system for gallbladder carcinomas, and unlike colon carcinomas, it is not one of the mandatory criteria that must be specified when reporting gallbladder carcinomas.

Only about 15% of GBC patients are diagnosed at an early stage and have the option of curative treatment through surgery alone[7]. Currently, treatment protocols have almost no effect on the average survival rate of this disease[1,3,4,7]. The primarily surgical treatment approach and protocols to improve the prognosis of patients with GBC remain controversial[7]. New, effective, and targeted therapy protocols are urgently needed to treat this disease[3-5].

Axl, a receptor tyrosine kinase (RTK), is a member of the TAM receptor family (TYRO3, AXL, MER TKs)[15-18]. Axl RTK is structurally similar to other RTK family members. It consists of two immunoglobulin-(Ig)-like domains and two transmembrane fibronectin III domains[15,19-21]. It is assumed that Axl RTK is responsible for cell plasticity, chemoresistance, immunosuppression, and the potential for metastasis[9,19,22,23]. Axl RTK, which has been activated by dimerization, is linked to many cellular functions after binding to its specific ligand-protein GAS6 (growth-arrest-specific protein 6); However, its critical role in cancer cells is not yet currently known. It has been shown that the pathways (PI3K, MAPK, STAT, and NF-KB) associated with cell proliferation, invasion, epithelial-mesenchymal transition, drug resistance were activated in different cell types[8,9,18,19,22-24]. Our study focused on only the pathways that play a role of Axl RTK in epithelial-mesenchymal transition and migration. Because histopathologically, TB is considered to represent epithelial-mesenchymal transition. There is molecular crosstalk between Axl and TGF-beta via Smad3 phosphorylation in the epithelial-mesenchymal transition pathway[25]. PI3K and P38 are some of the Axl downstream molecules and promote cell migration and epithelial-mesenchymal transition in GBM[26]. The migration and epithelial-mesenchymal transition pathways in which Axl RTK is involved are shown schematically in Figure 1[25,26]. The RTKs play an essential role in signal conversion both in normal and in malignant cells, and it is obvious that they could be a target molecule for individual therapy because they are located on the cell membrane[16,18,27-31]. The increased expression of Axl has been reported in brain, lung, breast, and pancreatic cancers have been associated with poor prognosis[22,23,29,30,32,33]. There is one study on the role of Axl RTK and it is prognostic importance in the GBC[5].

A few studies that have been carried out to date on TB in gallbladder cancers. The relationship between TB and other histopathological features has not been adequately studied. There is also no study linking TB to Axl RTK.

Our study aimed to investigate the prognostic significance of TB and the immunohistochemical (IHC) expression of Axl RTK its relationship to other histopathological features.

MATERIALS AND METHODS

Clinicopathological Data

Thirty eight patients who underwent cholecystectomy in our hospital between 2010 and 2017 and were diagnosed with gallbladder adenocarcinoma as histopathological were included in our retrospective cross-sectional study. All patients were followed up to June 2021. Gallbladder adenocarcinomas with neuroendocrine differentiation, adeno-squamous carcinomas, and other rare tumors were excluded from the study. Those who did not have access to overall survival information and those who were not eligible for

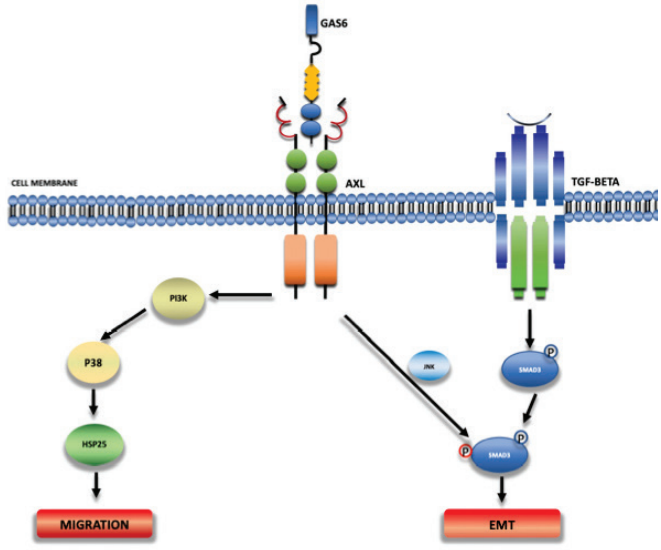

FIG. 1. The migration and epithelial-mesenchymal transformation pathways controlled by the Axl RTK; The migration and epithelial-mesenchymal transformation pathways controlled by the Axl RTK; Dimerization and activation of Axl RTK by binding of Gas 6 molecule provides migration via PI3K pathway, and it induces epithelial-mesenchymal transformation by attaching a phosphate group to different regions of Smad3 and via PI3K-AKT pathways[21,25,26]. Abbreviations: JNK: c-Jun N-terminal kinase, SMAD: mothers against decapentaplegic homolog

evaluating paraffin blocks were also excluded from the study. Three independent pathologists re-evaluated Hematoxylin-Eosin-stained sections from these cases. Pathological findings were recorded in a standard format: tumor size as numeric, tumor growth pattern; papillary-polypoid and infiltrative, histopathological diagnosis with a grade of differentiation (poor-moderate-good), neural and lymphovascular invasion; present-absent, serosal invasion; present-absent, TB; low, intermediate, high, tumor-infiltrated lymphocyte (TIL); absent, weak, moderate, dense. The presence of microscopic tumor at the resection margin was considered positive for the resection margin; resection margin 0 = no residual disease, resection margin 1 = microscopic residual disease. Tumor differentiation, pT, and pathological stage were classified using the tumor classification of tumors of the gastrointestinal system of the World Health Organization, 2019. The TB of the tumor was made according to the ITBCC[10]. Firstly, 10 individual fields were scanned at medium power (10x objective) of the Olympus CX40 microscope to identify the "hotspot" at the invasive front of tumor. Secondly, TB in the hotspot area was counted for the 20x objective lens of microscope. The TB was accepted as single cell or clusters of up to four cells. The TB of the tumor was classified as low (0-4 buds), moderate (5-9 buds), high (≥ 10 buds) (Figure 2/ a1, a2, b1, b2, c1, c2). All GBC slides were morphologically examined for the intensity of TIL. We examined all the tumor tissues, the tumor stroma, and the tumor microenvironment at the same time and modified the Zang et al. reported scoring system. The lymphocytic response was divided into four categories:(0) no infiltrating lymphocytes; (1) slight increase in infiltrating lymphocytes in the tumor tissue or stroma; (2) modest increase in infiltrating lymphocytes interwoven with tumor tissue; (3) strong intensity of infiltrating lymphocytes (Figure 3C, blue arrow) incorporated into tumor tissue and presence of the lymphoid aggregates[34]. The clinical and the macroscopic features of the tumors were recorded from our clinic's file system.

Survival Data

The prognostic information was acquired from the archive records of the Local Cancer Monitoring and Follow-up Center. Overall survival time (OS) time was calculated as the interval between the date of cholecystectomy and the date of death or between the date of resection and the last observation for the last follow-up of surviving patients. The patient follow-up data was completed in June 2021. The data were considered according to the last follow-up of the living patient.

Immunohistochemistry

Four micron-thick sections were obtained for IHC examination by choosing one of the blocks that had gone through the routine process and best reflected the characteristics of each patient tumor. Axl (C89E7) Rabbit Monoclonal Ab #8661 Cell Signaling, 1:75 dilution, Catalog number: 8661S) primary antibody was used for IHC. The Axl antibody was incubated at 37 °C for 32 minutes. The positive control was a cell paraffine bloc of Hep40 cell line known to possess high-level Axl protein expression. IHC, tumoral cytoplasmic, and membranous staining was considered positive. At least ten microscopic high-power fields were evaluated for each tumor, and the staining scoring of the tumor cells was determined. A mean percentage of positive staining tumor cells was determined,

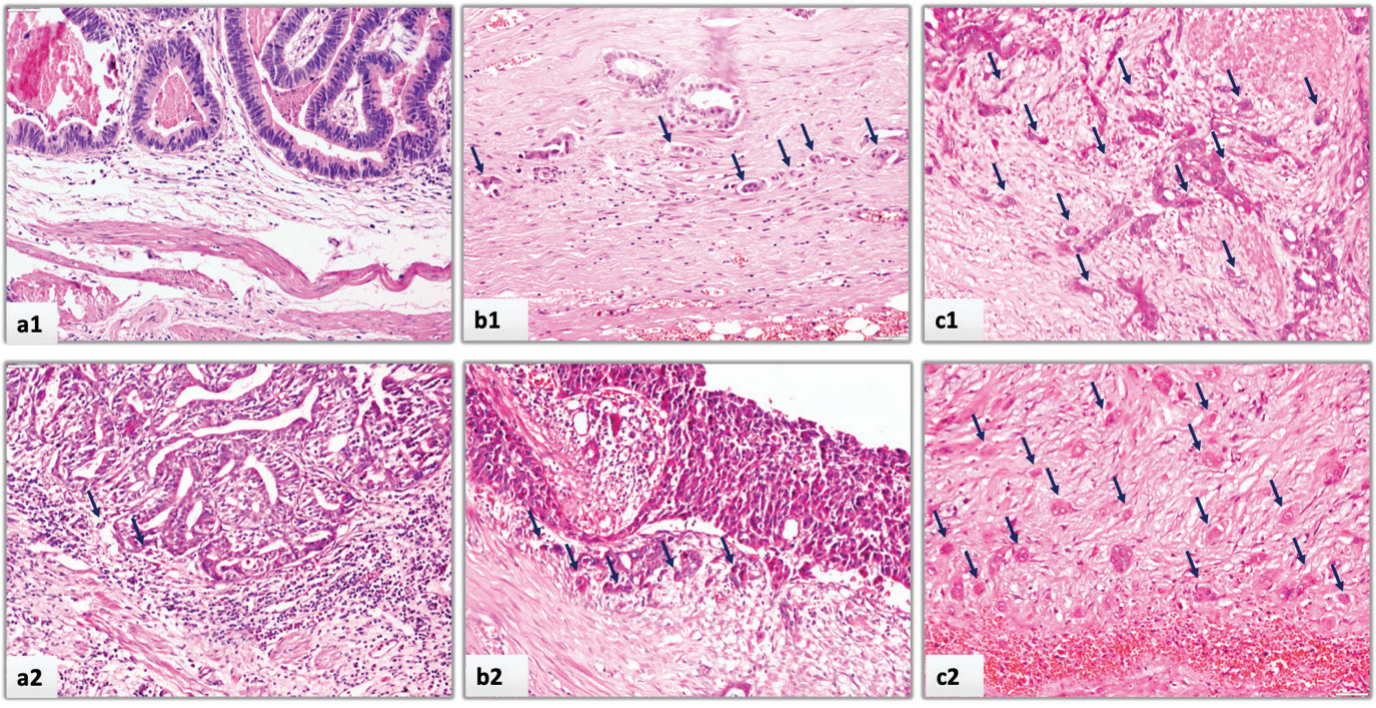

FIG. 2. Examples of tumor budding (TB) scoring of some gallbladder cancer cases; **a1, a2:** Two different H&E staining examples of low TB cases (x200), **b1, b2:** Two different examples of intermediate TB cases (x200), **c1, c2:** Two different examples of high TB cases (x200)

and a level of staging score was divided into three categories: negative (< 1%), focal positivity (2-10% staining), and common positivity (11-100% staining).

The whole staining of weak, moderate, or strong intensity was accepted, and only the extent was included in the scoring. Three pathologists evaluated the score of Axl expression without knowledge of the corresponding clinical data, and the mean value was considered.

Statistical Analysis

We performed statistical analyses with the IBM SPSS Statistics V21.0., the correlation between the Axl expressions and clinicopathological parameters was analyzed with the Pearson's chi-square test. Fisher's exact test was used to compare the categorical variables, and survival was analyzed univariately using the Kaplan-Meier method with a log-rank test for sub-groups comparison and multivariate Cox Regression (Forward Stepwise: Conditional) analysis was used to choose best model and to determine the parameter that is the best indicator. Logistic regression analysis was used to determine best model and parameter(s) between budding tumor groups. It was taken into consideration to be statistically significant p-value <0.05 in all statistical analyses.

Ethics and Disclosure of Potential Conflicts of Interest

All procedures performed in our study have been approved by the National Research Ethics Committee (reference number: 02, date: September 17th, 2020) by the Declaration of Helsinki in 1964 and its subsequent amendments. All authors have announced that no conflict of interest could affect the content of papers and participate in the research and article preparation.

RESULTS

Patients' Characteristics

There had been 12 men (31.6%) and 26 women (68.4%). The confidence level of our work, which was carried out considering the cumulative risk value[2], was over 99.99%. The mean age was 67.36 ± 11.48 years, and the median age was 68.00 (range, 43 ± 88 years). Average survival time was 29,605 ± 6,377 month (95% confidence interval:17,107-42,103). 12 month estimated survival rate: 68.4%, 24 months estimated survival rate: 36.8%, 36 months estimated survival rate: 5.3%. As the age increases, the average survival time decreases (p = 0.028). The average tumor size was 3.58 ± 2.19 cm (range, 0.30-9.50 cm), median tumor size was 3.05 cm. The relationship of tumor size with OS time could not be determined (p = 0.373).

Tumor Budding

TB was determined as low in 12 cases, intermediate in 10 cases, and high in 16 cases. In statistical analyses, high TB was strongly belonged with infiltrative growth pattern (p = 0.031), poor differentiation (p = 0.006), advanced pT stage (pT2-3) (p = 0.002), presence of serosal invasion (p = 0.040), presence of perineural invasion (p = 0.008), the presence of lymphovascular

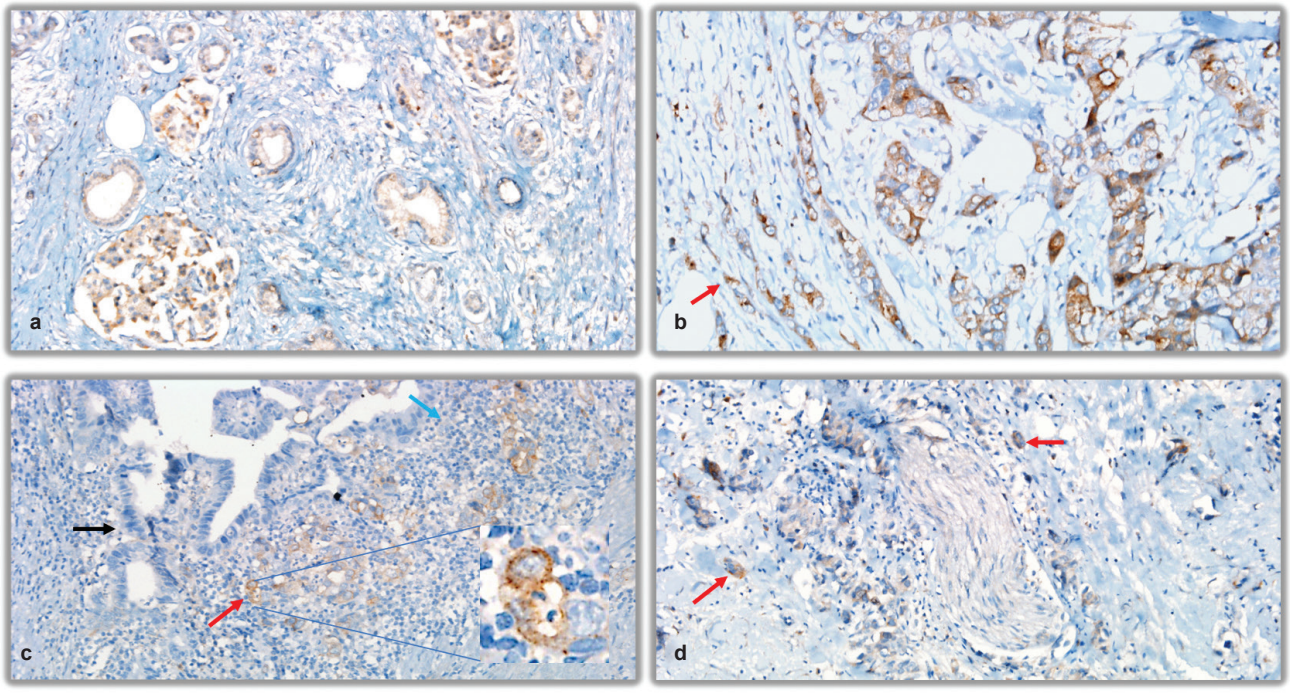

FIG. 3. Examples of immunohistochemical staining with AXL RTK in gallbladder cancer cases; Cytoplasmic and membranous staining was accepted positive. **a:** Positive staining in tumor tissue (x100), **b:** Positive staining in tumor budding area (red arrow) (x200), **c:** Negative staining in normal glandular epithelium (black arrow), positive staining in tumor (red arrow). Note intense tumor infiltrating lymphocytes (blue arrow) (x100), **d:** Positive staining in adenocarcinoma cell groups around perineural invasion area (red arrows) (x200)

invasion ($p < 0.0001$) and common Axl expression ($p = 0.018$). The average survival time of GB cancer patients with low TB cases was 58,750 ± 13,162 months, with moderate TB cases of 22,300 ± 12,664 months, with high TB cases being 12,313 ± 4,261 months. There was a relationship between the high TB scoring and poor prognosis ($p = 0.011$). The statistical correlation rates between TB, clinicopathological parameters and, Axl staining are shown in Table 1.

Immunohistochemical Axl Expression

Axl staining was negative in 6 patients, was focally positive in 19 patients, and diffuse positive in 13 patients. Axl expression was not associated with any histopathological features except tumor budding. The mean survival time was 16,333 ± 9,701 months in patients with Axl-negative tumors, 32,053 ± 9,660 months in patients with positive focal tumors, and 32,154 ± 11,651 months in those with diffuse positive tumors. Our Axl IHC staining samples in tumor tissue and TB areas are shown in figure 3. We determined no relationship between the presence of Axl expression and OS time ($p = 0.670$).

Correlation between Tumor Budding and Immunohistochemical Axl Expression and the Cox Regression Analysis of Their Relationship

The distribution between TB degree and Axl IHC staining intensity is getting increase positively in the especially high-grade TB group (Figure 4, a). It has been shown that the intensity of Axl IHC staining increases as the degree of TB increases, and these patients in particular have shorter survival times (Table 1, Figure 4, b, c, d).

TABLE 1. The relationship of Tumor Budding with Clinicopathological Features and Immunohistochemical Axl Expression

	TB-Low (%)	TB-Intermediate (%)	TB-High (%)	
Sex				
Female	10 (38.5)	4 (15.4)	12 (46.2)	0.071
Male	2 (16.7)	6 (50.0)	4 (33.3)	
Growth pattern				
Polypoid	5 (71.4)	0 (0)	2 (28.6)	0.031
Ulcero-infiltrative	7 (22.6)	10 (32.3)	14 (45.2)	
Histological grade				
Well-differentiated	6 (66.7)	2 (22.2)	1 (11.1)	
Moderate-differentiated	6 (37.5)	5 (31.3)	5 (31.3)	0.006
Poor-differentiated	0 (0)	3 (23.1)	10 (76.9)	
Primary tumor (pT)				
pT1	7 (87.5)	1 (12.5)	0 (0)	
pT2	0 (0)	1 (20.0)	4 (80.0)	0.002
≥ pT3	5 (20.0)	8 (32.0)	12 (48.0)	
Surgical margin				
Negative	10 (41.7)	5 (20.8)	9 (37.5)	0.205
Positive	2 (14.3)	5 (35.7)	7 (50.0)	
Serosal invasion				
Negative	7 (58.3)	1 (8.3)	4 (33.3)	0.040
Positive	5 (19.2)	9 (34.6)	12 (46.2)	
Perineural invasion				
Absent	9 (60.0)	3 (20.0)	3 (20.0)	0.008
Present	3 (13.0)	7 (30.4)	13 (56.5)	
Lymphovascular invasion				
Absent	11 (61.1)	5 (27.8)	2 (11.1)	<0.0001
Present	1 (5.0)	5 (25.0)	14 (70.0)	
Axl expression				
Negative	2 (33.3)	0 (0)	4 (66.7)	
Focal positive	8 (42.1)	8 (42.1)	3 (15.8)	0.018
Diffuse positive	2 (15.4)	2 (15.4)	9 (69.2)	

TB, Tumor budding

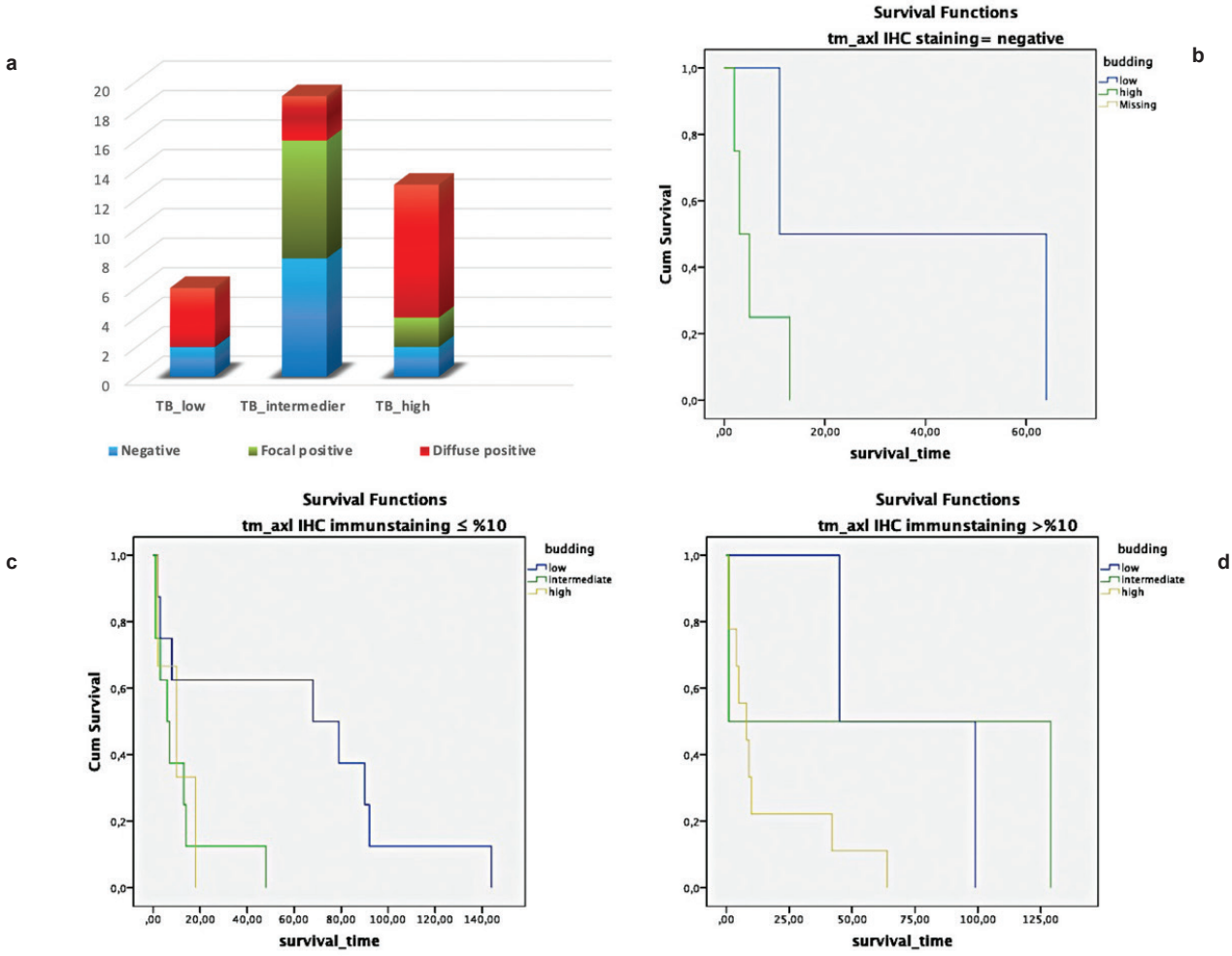

FIG. 4. The relationship of Tumor Budding with immunohistochemical Axl expression and the Cox Regression Analysis of their relationship: **a:** The histogram showing the distribution between tumor budding degree and axl immunohistochemical staining intensity, **b:** The Kaplan-Meier plot demonstrates the relationship between tumor budding level and negative Axl immunohistochemical staining, **c:** The Kaplan-Meier plot demonstrates the relationship between tumor budding level and focal positivity (2-10% staining) of Axl immunohistochemical staining. **d:** The Kaplan-Meier plot demonstrates the relationship between tumor budding level and common positivity (11-100% staining) of Axl immunohistochemical staining

Correlation Between Histopathological Features and Overall Survival Time

The infiltrative growth pattern ($p = 0.004$), poor differentiation ($p = 0.007$), advanced pT ($p < 0.0001$), RM1 ($p = 0.001$), dense TIL ($p = 0.021$), presence of perineural invasion ($p = 0.003$), the presence of lymphovascular invasion ($p = 0.003$), the presence of serosal invasion ($p < 0.0001$), high TB ($p = 0.011$) were associated with a short OS time. The distribution of the number of cases according to histopathological features, IHC staining Axl scoring protein, and their relationship with overall survival time was shown in Table 2.

Gallbladder Adenocarcinoma Tumor Budding Multivariate Cox and Logistic Regression Statistical Analysis Results

Differentiating Low-Moderate Tumor Budding: As a result of the multivariate Cox regression analysis performed among the TB Low-Intermediate groups, only the serosal invasion parameter came to the fore ($p = 0.002$). It is parallel with the model and result obtained in the logistic regression analysis (Figure 5, a).

Differentiating Low-High Tumor-Budding: In the multivariate Cox regression analysis (multivariate modeling) performed between the TB Low-High groups, serosal invasion ($p = 0.001$) and lymphovascular invasion ($p = 0.004$) were found to be effective in differentiating the low and high groups from each other in terms of survival time (Figure 5, b, c). It was determined that these two parameters contributed to determining the difference in survival time between TB Low and High groups, and these findings are also compatible with the model and result obtained in Logistic regression analysis.

Differentiating Intermediate-High Tumor-Budding: According to the multivariate Cox regression analysis performed between TB Intermediate and High groups, only the pT parameter came to the fore ($p = 0.026$) (Figure 5, d). It has been determined that the

TABLE 2. The Number of Cases According to Histopathological Features and Their Relationship with Overall Survival Time

	Number of the cases (%)	Overall survival	Log Rank (Mantel-Cox) *p* value
Sex			
Female	26 (68.4)	33.038 ± 8.460	0.435
Male	12 (31.6)	22.167 ± 8.561	
Growth pattern			
Papillary-polypoid	7 (18.4)	78.286 ± 17.050	0.004
Ulcerative-infiltrative	31 (81.6)	18.613 ± 5.146	
Histological grade			
Well-differentiated	9 (23.7)	57.889 ± 15.746	
Moderate-differentiated	16 (42.1)	30.688 ± 10.219	0.007
Poor-differentiated	13 (34.2)	8.692 ± 2.945	
Primary tumor (pT)			
pT1	8 (21.0)	87.375 ± 15.241	
pT2	5 (13.2)	37.000 ± 9.518	<0.0001
≥ pT3	25 (65.8)	9.640 ± 3.003	
Resection margin			
Negative	24 (63.2)	43.250 ± 8.996	0.001
Positive	14 (36.8)	6.214 ± 1.415	
Tumor infiltrated lymphocyte (TIL)			
Weak	11 (28.9)	15.000 ± 7.632	
Moderate	16 (42.1)	17.563 ± 7.123	0.021
Dense	11 (29.0)	61.727 ± 14.323	
Perineural invasion			
Absent	15 (39.5)	52.400 ± 12.945	0.003
Present	23 (60.5)	14.739 ± 4.241	
Lymphovascular invasion			
Absent	18 (47.4)	50.389 ± 11.339	0.003
Present	20 (52.6)	10.900 ± 2.836	
Serosal invasion			
Absent	12 (31.6)	69.667 ± 12.967	<0.0001
Present	26 (68.4)	11.115 ± 3.241	
Tumor budding			
Low	12 (31.6)	58.750 ± 13.162	
Intermediate	10 (26.3)	22.300 ± 12.664	0.011
High	16 (42.1)	12.313 ± 4.261	
Axl expression			
Negative	6 (15.8)	16.333 ± 9.701	
Focal positive	19 (50.0)	32.053 ± 9.660	0.670
Diffuse positive	13 (34.2)	32.154 ± 11.651	

pT grade is effective in differentiating TB intermediate and high levels. However, in paired analyzes to understand with which level of pT this effect is; The coexistence of pT2 and pT3 is influential in determining these two budding groups in terms of survival time with 17-fold in the direction of TB high (Figure 5, e). In the logistic regression analysis, it could not be obtained result similarly.

DISCUSSION

GBC is usually in the advanced stage when it is detected, and this situation contributes to the poor prognosis and shortens the OS time associated with this disease. Practical clinical diagnostic tools for early diagnosis do not yet exist, sometimes discovered

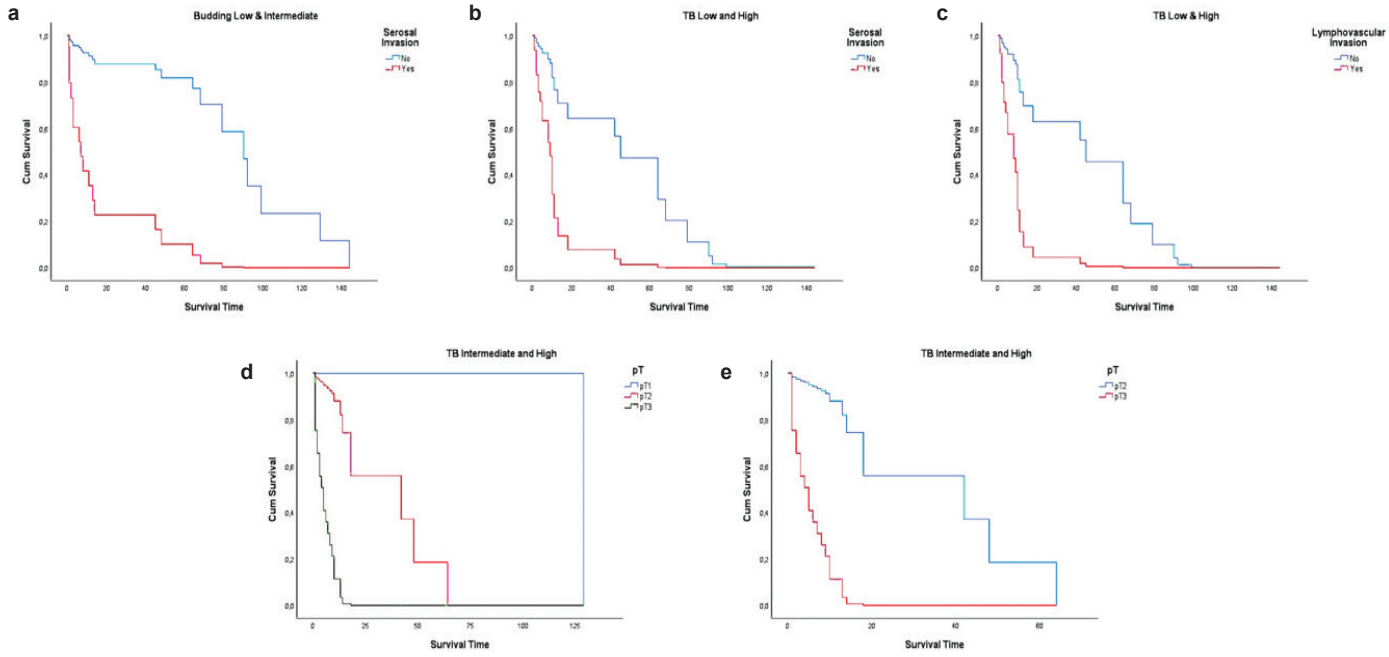

FIG. 5. Gallbladder Adenocarcinoma Tumor budding Cox and Logistic Regression statistical analysis results; The TB of the tumor was classified as low (0-4 buds), moderate (5-9 buds), high (≥10 buds) according to the ITBCC [10]. **a, b:** The presence of serosal invasion independently distinguishes low-grade tumor budding from intermediate- and high-grade tumor budding. **c:** The presence of lymphovascular invasion independently distinguishes low-grade tumor budding from high-grade tumor budding. **d, e:** pT grading was found to be an independent predictor of overall survival among the intermediate and high-grade tumor budding groups, particularly between the pT2 and pT3 groups

accidentally after cholecystectomy. The light of the literature shows that GBC's prognostic characteristics have been almost the same for many years[1,3,4,7,35]. Tumor size, pT staging, lymph node metastasis, perineural invasion, histological grading, RM, and liver invasion play decisive roles in the prognosis of GBC patients [1,4,6,7,35]. The present study found that the relationship between poor tumor differentiation, advanced pT, positive resection margin, the presence of perineural invasion, lymphovascular invasion, and serosal invasion with a short OS time is in agreement with the literature[1,3,6,7]. If we first reassess the prognostic features of the group again in the light of the literature, it would be appropriate to talk about resection margin, which is one of the accepted prognostic features, and then TIL and TB as the current features. The anatomical structure of the operation area and the thin wall of the gall bladder may not allow the preservation of surgical margins, unlike other solid tumors. Therefore, resection margin positivity is a common histopathological finding in GBC since they cannot be operated curatively. The ratio of resection margin 1 patients is 36.8% and is compatible with the literature[1,6,7,35]. The statistically significant distinction in the short overall survival time in this group of patients underlines the need for a complete resection during the operation.

In our series, dense infiltration of TIL was observed in approximately one-third of the cases, and the survival time of this group of patients was found to be significantly longer than that of the other patients. There are few studies with TIL in GBC[36]. The intensity of TILs were evaluated in our study, the positive prognostic effect of dense TIL was demonstrated (Table 2), suggesting that gallbladder cancers could also benefit from immunologically targeted therapies. Extensive studies are needed to determine the molecules that can be targeted for treatment in the host immune response against the tumor.

Wistuba reported that the budding of the tumor was first described in 1954 by Imai[3]. TB is a histopathological manifestation of epithelial-mesenchymal transition[37], and its prognostic significance in colorectal cancer was first published in 1993[38]. Since then, it has been an exciting topic to explain the properties of cancer, and in the last ten years, in particular, many studies have been carried out on its prognostic significance in various types of cancer[10,11,13,14]. As recommended by the ITBCC, it has been entered into some guidelines used in pathological practice today and widely used in daily routine in colorectal cancers worldwide. This histopathological finding, interpreted as an indicator of epithelial-mesenchymal transition at the H&E level, has not yet taken its place in assessing gallbladder cancer. Only two studies about the relationship between TB in the gallbladder and prognosis are reported in the literature[39,40], and our study is among the pioneering studies. TB and dedifferentiation in GBC

are potential prognostic factors, especially in T2 stage lesions[39,40] (Figure 5, d, e). Our results about both parameters are similar to those in the literature. Both of them have a negative prognostic effect on survival has been demonstrated. Since TB is considered to be a histopathological finding of epithelial-mesenchymal transition[37] and increased expression of Axl RTK has been reported to play a role in the epithelial-mesenchymal transition pathway, it is also a poor prognostic marker[8,9,18-20,22,24,28-30], our study not only examined the effect of TB on overall survival but also investigated their relationship with each other. The relationship between Axl protein expression and TB has not been reported in any previous study. Investigating this relationship is an innovative approach to assessing TB and was for the first time examined in our study.

In the absence of effective screening and laboratory tests for GBC, we have no choice today but to talk about what to do in advanced disease. Today, clinical oncological treatment options in GBC are limited. Because systemic chemotherapy protocols cannot increase overall survival in GBC cases, targeted molecular mechanisms are among the issues that need urgent investigation. There are some potential biomarkers for GBC. Among these, HER2[41,42] and EGFR[42,43] have been prominent molecules. With the importance of epithelial-mesenchymal transition in recent years and the understanding of the role of Axl RTK in epithelial-mesenchymal transition signaling pathways, both the prognostic importance of Axl protein expression and its potential as a treatment molecule option make it attractive[8,9,18-20,22,24,28-30]. We found a short overall survival time in GBC patients with intermediate-high TB scores in our study group, and we determined that Axl RTK protein expression was increased. Investigating the Axl-TB relationship may be an essential approach to determining targets for TB treatments and thus prevent disease progression. This specific relationship has not been studied in any cancer. Our study detected that a high level of Axl RTK expression is associated with high-grade TB (Figure 4); therefore, we believe that therapies that target the Axl protein can prevent the progression of TB and so clinical progression in GBC patients.

Our study detected that the histopathological features, which are well known to date, retain their effective place in predicting the prognosis of gallbladder cancer. In addition, in the current histological evaluation of cancers, we have found that TB, which is increasingly important, is an essential prognostic parameter for gallbladder cancers. Moreover, high expression of Axl RTK, one of the epithelial-mesenchymal transition-related molecules, was observed in tumors with high budding potential. RTKs have generally been accepted to be precise targets for the development of monoclonal antibodies and small-molecule kinase inhibitors. A promising direction of our result is the possibility that Axl RTK located in cell membrane might become a relevant therapeutic target in GBC, a disease where conventional therapies have had minimal impact on improving prognosis. These results have led us to believe that Axl RTK is a target molecule that could break the almost immutable fate of gallbladder cancers by inhibiting TB and its spreading of the tumor.

As a result of our study, it was concluded that TB scoring as a prognostic marker must be included in the routine practice in the evaluation of GBC. *Axl* gene expression should be evaluated together while the TB score is evaluated. Moreover, the detection of Axl gene expression may be a predictive marker for GBC patients' personalized molecular treatment options.

In conclusion, it can be predicted that our study will contribute to the literature as it investigates the effect of TB on survival of GBC and its relationship with the expression of Axl RTK, a molecule associated with epithelial-mesenchymal transition, and presents a new perspective.

Ethics Committee Approval: All procedures performed in our study have been approved by the National Research Ethics Committee (reference number: 02, date: September 17th, 2020) by the Declaration of Helsinki in 1964 and its subsequent amendments.

Data Sharing Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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