

CD24 Expression is an Independent Prognostic Marker in Cholangiocarcinoma

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Abstract CD24 has been described as an adverse prognostic marker in several malignancies. This study evaluates CD24 expression in cholangiocarcinoma and correlates the findings with clinicopathologic data and patient survival. Between 1996 and 2002, 22 consecutive patients with cholangiocarcinoma were treated at our institution. Demographic data, SEER stage, pathologic data, treatment, expression of CD24, mitogen-activated protein kinase (MAPK), phosphorylated MAPK, and survival were analyzed. The majority of the tumors demonstrated CD24 (81.8%) and p-MAPK (87%) expression. A negative association was noted between the expression of CD24 and p-MAPK. Median survival for patients with low expression of CD24 was 36 months and high expression was 8 months. Median survival for patients who received chemotherapy with low CD24 expression was 163 months, and for seven patients with high CD24 expression, it was 17 months ($p=0.04$). With the addition of radiation therapy, median survival for patients with low expression of CD24 was 52 months and high expression was 17 months ($p=0.08$). On multivariate analysis, the use of chemotherapy ($p=0.0014$, hazard ratio 0.069) and the CD24 overexpression ($p=0.02$, hazard ratio 7.528) were predictive of survival. CD24 is commonly expressed in cholangiocarcinoma, and overexpression is predictive of poor survival and possibly of lack of response to chemotherapy and radiation therapy. These findings may improve selection of patients for the appropriate treatment modality and the development of CD24-targeted therapy.

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Introduction

Malignancies of the biliary tract are uncommon in the Western world. Two-thirds arise in the gallbladder and the remainder in the biliary tree or periaampullary region. Cholangiocarcinoma or bile duct cancer is a rare but lethal malignancy with an incidence of 1–2 cases per 100,000 patients in the United States.¹ Clinicopathologic factors predictive of survival include curative resection, tumor stage and grade, serum bilirubin level <10 mg/dl, low CA19-9 level, hepatitis viral infection,^{2–4} lymphovascular or portal vein invasion,⁵ intrahepatic satellite lesions, inraductal papillary component, tumor angiogenesis,⁶ and DNA

ploidy.⁷ Reports of molecular markers predictive of survival in cholangiocarcinoma include cluster of differentiation CD24,⁸ MMP-2, TIMP-2,⁹ cholinesterase level,¹⁰ MUC-4,¹¹ cyclin D1,¹² VEGF-C,¹³ p27,¹⁴ p53, and p73.¹⁵

Recently, CD24 has been described in a wide variety of malignancies and shown to be a prognostic marker in several solid tumors including colorectal, stomach, lung, prostate, ovarian, and breast.^{16–21} CD24 is a small, heavily glycosylated, mucin-like, cell-surface protein expressed in developing cells including pre-B cells, keratinocytes, and renal tubular epithelium.^{22–24} It functions as an alternative ligand of P-selectin, an adhesion receptor expressed on activated endothelial cells and platelets which can enhance the metastatic potential of CD24-expressing tumor cells.^{25–28} CD24 has apoptotic activity, and its cross-linking induces the sustained activation of p38 MAPK (mitogen-activated protein kinases)—the magnitude of which may determine the survival or death of pre-B cells.²⁹ An improved understanding of the molecular pathways involved in the pathogenesis and progression of cholangiocarcinoma will contribute to the development of targeted therapy.

This study correlates CD24 and MAPK expression with patient survival in cholangiocarcinoma with the objective of identifying a subset of patients who may benefit from targeted molecular therapy.

Patients and Methods

Clinical Data

After obtaining approval of the Institutional Review Board, a review of the tumor registry at Roswell Park Cancer Institute identified 31 consecutive patients with histologically proven cholangiocarcinoma between 1996 and 2002. Twenty-two patients had adequate tissue for further histopathologic studies and constitute the basis of this study. Medical records of these patients were reviewed for demographic data including age; gender; surveillance, epidemiology, and end results (SEER) stage at presentation; treatment; and survival from the time of diagnosis.

Immunohistochemical Staining

For most of the patients, diagnosis was established by examination of conventional hematoxylin and eosin (H&E)-stained slides and, in the remainder diagnosis, was confirmed with ancillary techniques including immunohistochemistry and special histochemistry with mucin and PAS stains. Uniform tissue fixation techniques were used for all patients. For each patient, a representative block containing adequate neoplastic and nonneoplastic tissue was selected. Five-micrometer tissue sections from these blocks were placed

on charged slides and dried in a 60°C oven for 1 h. Upon return to room temperature, the slides were deparaffinized in three changes of xylene and rehydrated using graded alcohols. Endogenous peroxidase was quenched with 3% aqueous H₂O₂ for 15 min and washed with phosphate buffered saline with 0.05% Tween-20 (PBS/T). CD24 primary antibody was obtained from BD Biosciences (clone ML5) and used with the recommended incubation time and antigen retrieval procedures. The primary antibody used for MAPK was obtained from Cell Signaling and for phosphorylated/activated MAPK from Santa Cruz. After a PBS/T wash, 0.03% casein (in PBS/T) was used as a block for 30 min followed by the application of the primary antibody to the slides for an hour or overnight. Another PBS/T wash was followed by exposure to the biotinylated secondary antibody for 30 min. A third PBS/T wash was followed by exposure to the streptavidin–peroxidase complex for 30 min. A PBS/T wash was followed by the application of the chromogen DAB (DAKO, Carpinteria CA, USA) for 5 min. The slides were then counterstained with hematoxylin, rinsed with water, dehydrated, and cleared, and a coverslip was placed. The use of biomarkers, related controls, and interpretation of results using the HistoScore system for quantification of results have been described previously by our group.³⁰ HistoScore was defined as the product of the percentage of positive cells and the intensity of stain. The grade of positive staining depended upon the intensity of staining (0: no staining, 1: weak, 2: moderate, and 3: strong staining) and the percentage of cells stained. The final score was calculated as a sum of each stain intensity multiplied by the percentage of stained cells in the area of interest. For example: if tumor showed 50% weak, 30% moderate, and 20% strong staining, the score assigned was $(50 \times 1) + (30 \times 2) + (20 \times 3) = 170$.

Histological Grading

The cholangiocarcinoma tissue specimens were also stained by routine H&E stains. The specimens were graded based on the degree of tumor differentiation using the World Health Organization (WHO) system.

Statistical Analysis

Association between biomarker expressions in tumor tissue was investigated using the Kendall's tau. Biomarker expressions were classified as high and low based on whether their scores were above or below the median value, and survival between low and high expressions was compared using the log-rank test. The Kaplan–Meier method was also used to estimate the survival curves and median survival. The Cox's proportional hazards survival analysis was used in the multivariate analysis of survival

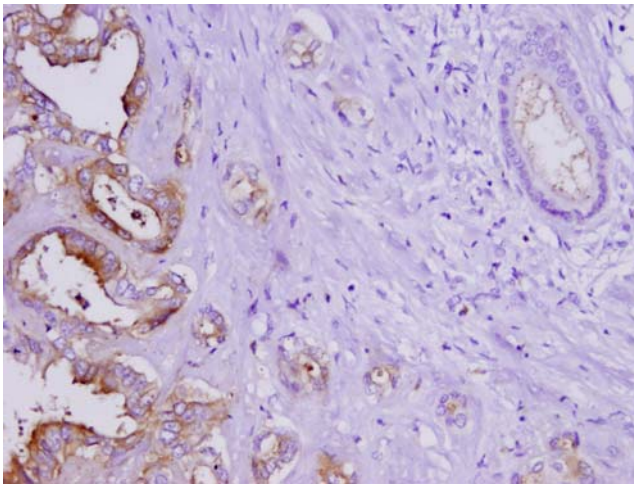


Figure 1 Moderately differentiated cholangiocarcinoma with over-expression of CD24. Normal bile duct (*right upper corner*) demonstrated weak apical staining, whereas neoplastic cells had strong apical and cytoplasmic staining (20 \times).

data to explain the effect of biomarker expressions together with other diagnostic parameters. Patient demographics including age, tumor grade, SEER stage, and treatment received were considered as possible parameters for explanatory variables in the model. All statistical tests were two-sided with statistical significance level at 5%.

Results

Patient Characteristics

Of the 22 patients included in the study, 7 were males and 15 females. The median age was 66.5 years (range: 35–77). SEER staging was local in 1 (4.6%), regional in 14 (63.6%), and distant in 7 (31.8%) patients. Differentiation of the tumor was classified as grade 1 in 3 (13.6%), grade 2 in 9 (40.9%), and grade 3 in 10 (45.5%) patients according to the WHO classification. Treatment for cholangiocarcinoma included surgery only ($n=8$), surgery and chemotherapy ($n=5$), surgery and radiation ($n=1$), chemotherapy only ($n=2$), and all three treatment modalities ($n=5$), and one patient did not receive any treatment.

Immunohistochemical Staining

Normal bile duct staining was used to set the score intensity. Most of the bile ducts were negative. Occasionally, they demonstrated weak focal and incomplete staining as seen in Fig. 1. The cholangiocarcinoma cells were scored as 1+ when they demonstrated weak expression of CD24, 2+ for moderate expression, and 3+ for strong expression of CD24. Figure 1 depicts cholangiocarcinoma positive for

CD24 expression adjacent to normal biliary epithelium. The majority of the tumors demonstrated CD24 (81.8%) and p-MAPK (87%) expression. Immunohistochemical staining for these proteins was higher in malignant tissue in comparison to normal biliary epithelium. The pattern of staining was usually a combination of cytoplasmic and apical, and few specimens demonstrated the apical pattern only.

Relationship Between Biomarkers

A negative association was suggested between the expression of CD24 and phosphorylated/activated p-MAPK (Kendall's $\tau=-0.32408$, $p=0.0501$).

Survival

Median survival was 36 months for nine patients with low expression of CD24 and 8 months for 13 patients with high expression of CD24 as shown in Fig. 2. The median survival for five patients who received chemotherapy with low CD24 expression was 163 months, and for seven patients with high CD24 expression, it was 17 months (Fig. 3, $p=0.04$). Median survival for four patients treated with radiation in the presence of low CD24 expression was 52 months, and it was 17 months for two patients with overexpression of CD24 (Fig. 4, $p=0.08$). Overexpression of CD24 continued to affect survival adversely despite the overall improvement noted with the addition of radiation therapy. Multivariate analysis using the Cox's proportional hazards survival analysis demonstrated that overexpression of CD24 ($p=0.02$, hazard ratio 7.528) and use of chemo-

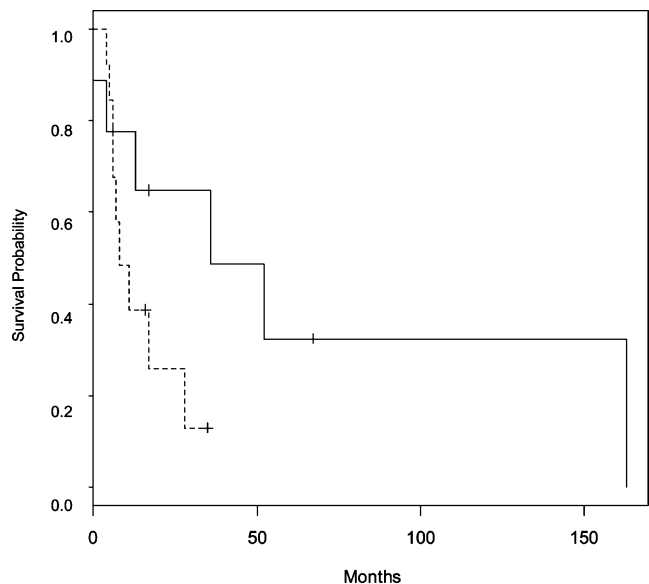
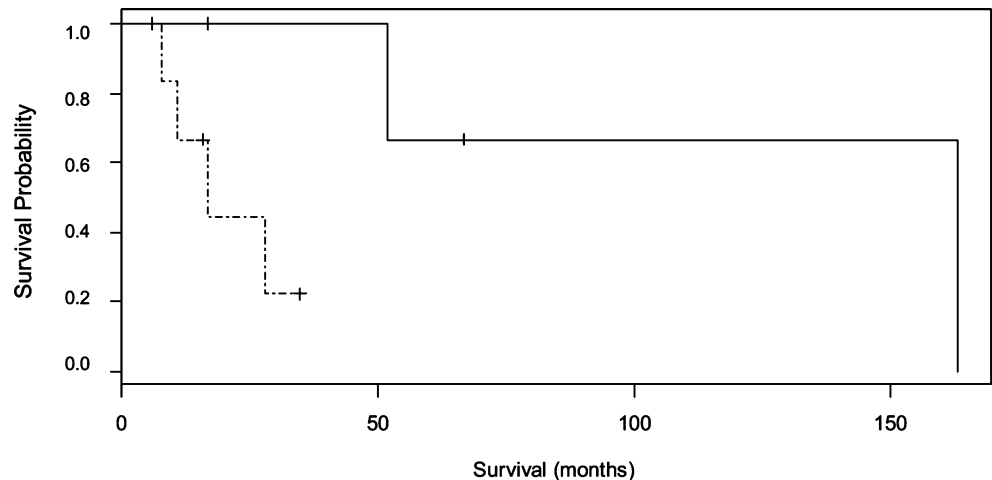


Figure 2 Kaplan–Meier survival curve for patients of cholangiocarcinoma with low and high levels of CD24 expression ($n=22$). $p=0.02$. Low CD24 (—), high CD24 (- - - - -).

Figure 3 Kaplan–Meier survival curve for patients who received chemotherapy with low and high levels of CD24 expression ($n=12$). $p=0.04$. Low CD24 (—), high CD24 (-----).



therapy ($p=0.0014$, hazard ratio 0.069) were predictive of survival (Table 1). There was no significant association noted between survival and patient's age, sex, SEER stage, grade of the tumor, surgery, radiation therapy, or expression of MAPK.

Discussion

The physiologic function of CD24 is incompletely understood but it has been shown to increase tumor proliferation, cell adhesion, motility, invasion, and apoptosis.^{22–24,31} Selectins are cell adhesion molecules involved in the rolling adhesion of leukocytes to endothelial cells and platelets under the shear forces of circulation, and P-selectin expressed by thrombin-activated platelets and endothelial cells is a major ligand for CD24 on carcinoma cells.^{26,27} This suggests that CD24-expressing tumor cells can disseminate more readily due to their capacity to form thrombi with activated platelets or to adhere to endothelial cells. Friederichs et al.²⁸ have demonstrated that the

carbohydrate sialylLex abundantly expressed on human cancers is essential for CD24-mediated rolling of tumor cells on P-selectin, and in its absence, human adenocarcinoma cells failed to arrest and colonize the lungs. CD24, a metastasis-associated protein, has been recently identified as a downstream target of Ral signaling.³² Ral GTPases are important mediators of transformation, tumorigenesis, and cancer progression. Microarray by immunohistochemistry of a human bladder cancer identified CD24 as a novel Ral-regulated target and a prognostic biological marker.

In this study, 81.8% of patients with cholangiocarcinoma expressed CD24. Median survival for patients with overexpression of CD24 was significantly shorter, and the addition of chemotherapy improved survival. A negative association was noted between the expression of CD24 and p-MAPK. The use of chemotherapy in patients with low expression of CD24 was associated with a median survival of 163 months compared to 17 months in patients with a high CD24 expression ($p=0.04$). The use of radiation therapy in patients with low expression of CD24 was also associated with an improved survival than with over-

Figure 4 Kaplan–Meier survival curve for patients who received radiation therapy with low and high levels of CD24 expression ($n=6$). $p=0.08$. Low CD24 (—), high CD24 (-----).

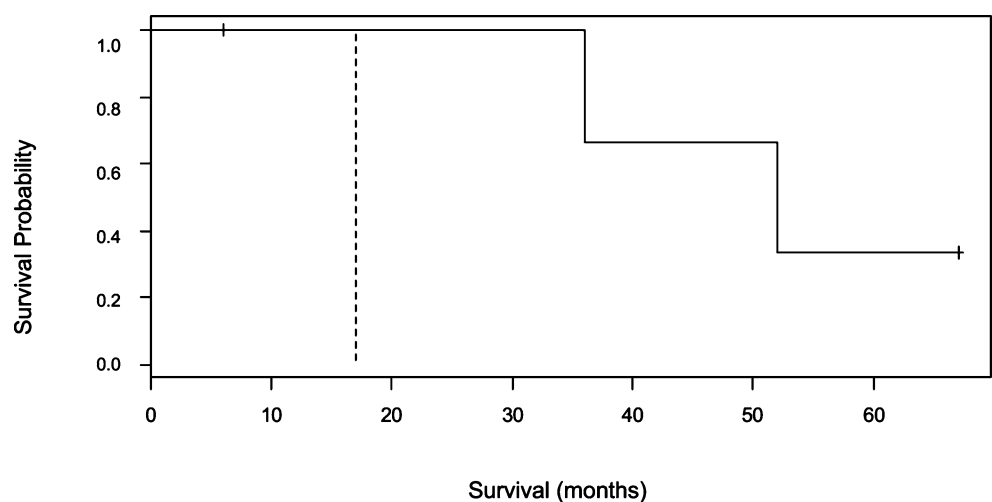


Table 1 Prognostic Variables for Survival in 22 Patients with Cholangiocarcinoma

Variable	<i>n</i>	Median survival (months)	Survival (<i>p</i> value)	Multivariate analysis (<i>p</i> value)
Age				
<68	11	17 (8–36)	0.60	
>68	11	11 (5–52)		
Gender				
Male	7	12 (8–163)	0.52	
Female	15	17 (6–52)		
Grade				
1, 2	12	15 (6–52)	0.99	
3	10	11 (8–*)		
SEER stage				
1, 2	15	11 (6–52)	0.48	
3	7	28 (6–163)		
Chemotherapy				
No	10	6 (4–13)	0.0005	0.0014
Yes	12	52 (17–163)		
Radiation				
No	16	8 (6–28)	0.12	
Yes	6	44 (17–*)		
Surgery				
No	3	8 (0–28)	0.17	
Yes	19	17 (7–52)		
MAPK				
Low	10	17 (8–36)	0.68	
High	11	28 (5–163)		
P-MAPK				
Low	10	13 (8–52)	0.34	
High	11	36 (4–163)		
CD24				
<120	9	36 (13–163)	0.10	0.02
>120	13	8 (6–28)		

*The estimate was not provided because the upper limit of the survival curve had not reached a 50% failure rate.

CD denotes cluster of differentiation.

p-MAPK denotes phosphorylated form of mitogen-activated protein kinase.

expression of CD24 although the data did not attain statistical significance possibly due to the small number of patients in this series.

It has been reported by Taguchi et al.²⁹ that the cross-linking of CD24 induces apoptosis in Burkitt's lymphoma enhanced by a B-cell antigen receptor (BCR)-mediated signal. They observed that simultaneous cross-linking of pre-BCR clearly inhibited CD24-mediated apoptosis in pre-B cells. CD24 cross-linking also induces the sustained activation of p38 MAPK, and whether pre-B cells survive or die may be determined by the magnitude of MAPK activation. Consistent with these observations, the present study suggests an inverse association between CD24 and

p-MAPK, and eventual cellular proliferation or apoptosis might be a consequence of the dominant effect in a complex interplay of opposing influences.³³

Our data indicate that high expression of CD24 remains an adverse prognosticator despite the use of additional therapy. Chemotherapy and radiation were noted to provide maximal survival benefit to low expressors of CD24 although the data for the use of radiation was statistically insignificant probably due to the small number of patients in this study. Furthermore, correlation between CD24 expression and radiation sensitivity has been noted to vary with the cell type as in human small cell lung cancer, and radiation doses required to induce apoptosis of CD24-negative human ALL (acute lymphoblastic leukemia) cells were higher than those required for CD24-positive cells, suggesting that lack of CD24 surface antigen expression is associated with intrinsic radiation resistance.^{34,35}

Hypoxia is a characteristic feature of tumor cells due to the sustained proliferation which progressively results in an acidic, nutrient-deprived, and hypoxic tumor microenvironment. Tumor oxygenation has been identified as an independent prognostic variable for locoregional control and overall survival following definitive irradiation for squamous cell carcinoma of the head, neck, and uterine cervix.^{36,37} Recent reports have indicated decreased efficacy of chemotherapy under hypoxic conditions in several tumor types including pancreatic cancer and testicular tumors.^{38,39} Because treatment failure was a consequence of hypoxia, the authors recommend novel treatment strategies aimed at improving tumor oxygenation or enhancing the treatment sensitivity of hypoxic tumor cells. Aimed at identifying potential oxygen-dependent markers in vascular endothelial cells for therapeutic intervention in tumor angiogenesis, Scheurer et al.⁴⁰ performed a broad-range transcriptomic analysis of selected extracellular matrix protein gene expression levels in human umbilical cord vein endothelial cells exposed in vitro to hypoxia for different time periods. They noted several genes transcriptionally upregulated including CD24 at late times of exposure to hypoxia, indicating that it was a useful marker of hypoxic activation in vascular endothelial cells. In the present series, low expressors of CD24 demonstrated greater survival benefit from chemotherapy and radiation than the high expressors, suggesting that its expression may be a marker for tumor hypoxia and response to therapy. This finding that shows that patients with low expression of CD24 may benefit from chemotherapy or radiation is of importance because it has been previously reported that adjuvant or palliative radiation had no effect on survival in patients with cholangiocarcinoma.⁴¹ However, the small number of patients in the present series limits interpretation of data suggesting that CD24 overexpression may be predictive of lack of response to radiation or chemotherapy.

CD24 has been shown to be a prognostic marker for shortened survival and disease progression in several malignancies including colorectal, stomach, lung, prostate, ovarian, and breast cancers.^{16–21} Weichert et al. report that in colorectal cancer, only the subset of patients with exceptionally strong cytoplasmic CD24 staining comprising 10% of their study group demonstrated a markedly shortened mean survival of 31.5 months compared to 67.5 months for the remaining patients.¹⁶ They also reported that cytoplasmic CD24 staining pattern is prognostically more significant than the membranous pattern—the biological significance of which was unclear. Su et al. noted a 51% expression of CD24 by immunohistochemistry in intrahepatic cholangiocarcinoma as compared to the 81.8% in the present series. They reported CD24 expression and tumor stage as independently predictive of survival on multivariate analysis and suggested membrane-bound CD24 protein as a potential target for immunotherapy.⁸

In conclusion, overexpression of the molecular marker CD24 in cholangiocarcinoma is predictive of poor survival. CD24 overexpressors demonstrated a lack of response to chemotherapy and possibly radiation therapy although these observations were limited by the small sample size. Additional properties of tumor proliferation, invasion, metastasis, and apoptosis make CD24 a potent target for specifically directed molecular therapy and its overexpression a potential criterion in the selection of patients for the appropriate conventional treatment modality.

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