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Clinical and Prognostic Significance of Neoplastic Spindle Cells in Gallbladder Cancer

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Abstract. *Background/Aim: Neoplastic spindle cells (NSCs) are believed to play a role in cancer invasion and metastasis, as well as in poor prognosis. The clinicopathological characteristics and prognostic relevance of NSCs was investigated in gallbladder cancer. Materials and Methods: Specimens were obtained from 62 patients with gallbladder cancer who underwent surgery. The emergence of NSCs and their correlation with clinicopathological factors, prognosis, and EMT markers was evaluated. Results: The NSC grade correlated with tumor size, preoperative CA19-9, surgical margin, the degree of differentiation, the depth of invasion, lymph node metastasis, lymphatic invasion, vascular invasion, and perineural invasion. Multivariate analysis of overall survival showed that NSCs were an independent prognostic factor. A correlation between NSCs and EMT was also suggested. Conclusion: NSCs are an independent prognostic factor for patients with postoperative gallbladder cancer, which also suggests a correlation between NSCs and EMT.*

Gallbladder cancer is considered to be one of the most malignant forms of cancer for which chemotherapy and radiation therapy have little effect, and radical surgery remains the most effective treatment. The prognosis for patients with advanced gallbladder cancer is very poor, with a 5-year survival rate of 6-20% (1). The recurrence rate in these patients is quite high, and many patients develop distal metastases or experience local recurrence within 12 months of radical resection. In a recent histopathological study of gallbladder cancer, it was reported that the prognostic factors for patients with postoperative gallbladder cancer included the depth of invasion (T), lymph node metastasis (N), the degree of tumor differentiation, the extent of surgical margin, lymphatic invasion (ly), and perineural invasion (pn) (2-8).

Neoplastic spindle cells (NSCs) are spindle-shaped cancer cells with no tubular structure (9). There have been reports suggesting that in conditions, such as pancreatic cancer, nasopharyngeal carcinoma, and vulvar squamous cell carcinoma, NSCs are associated with cancer invasion and metastasis and are factors for poor prognosis (10-12). It has also been suggested that changes in cell morphology, whereby epithelial cancer cells become spindle-shaped, may be a result of the cancer cells undergoing epithelial–mesenchymal transition (EMT), a process by which epithelial cells lose their polarity and cell–cell adhesion and gain migratory and invasive properties (10). It has been reported that when EMT is induced in epithelial cells, a decrease in E-cadherin expression and an increase in vimentin expression are observed (13, 14). Although it is conceivable that NSCs are found in highly malignant cancerous growth, to our knowledge, there are few reports on the characteristics of NSCs in gallbladder cancer, except for a few studies on the association between gallbladder cancer and EMT (15-18).

In this study, we investigated the clinicopathological characteristics of NSCs and their correlation with postoperative survival of patients in whom gallbladder cancer was treated using surgical resection.

Materials and Methods

Patient background and method of tissue collection. In total, 62 patients who were diagnosed with gallbladder cancer and underwent surgical resection (but not R2 resection) between 2006 and 2016 at the Kurume University Hospital, Fukuoka, Japan, were chosen for this study.

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Resected specimens were fixed with 10% neutral buffered formalin. The specimens were then cut into 5-μm thick slices, stained with hematoxylin and eosin (H&E), and observed under a microscope. Histopathological findings were evaluated in accordance with the TNM Classification of Malignant Tumours (8th Edition) of the Union for International Cancer Control (UICC) (19), by two pathologists who had no knowledge of the clinical characteristics of the patients involved. In cases the evaluations of the two pathologists were different, a reevaluation was conducted until a consensus could be reached. We collected the clinical data and information from follow-up consultations using medical records and the Cancer Database. This study was conducted with the approval of the Kurume University Ethics Review Board (Approval No. 17326).

Evaluation of NSCs. We defined spindle-shaped cancer cells or cancer cells with oval nuclei and no tubular structure as NSCs. Typical NSCs are shown in Figure 1. Using the H&E stained specimens, the sites that showed the most prominent transition into NSCs in or surrounding the tumor were evaluated. The specimens were assigned one of three grades as follows according to the proportion of NSCs: Grade 0, NSCs <10%; Grade 1, 10%-50% NSCs; and Grade 2, NSCs >50%.

Assessment of E-cadherin and vimentin. Formalin-fixed, paraffin-embedded sections of 5 μm thickness were mounted on glass slides and incubated with anti-rabbit monoclonal antibody against vimentin (Clone V9, dilution: 1:9, DAKO, Santa Clara, CA, USA) and E-cadherin (clone NCH-38 dilution: 1:100, DAKO), and assessments of these two proteins were conducted using the same fully automated Bond-III system (Leica Microsystems) with onboard heat-induced antigen retrieval solution 1 for 10 min at 99˚C for vimentin and with epitope retrieval solution 2 for 30 min at room temperature for E-cadherin. This automated system used a polymer refine detection kit with a horseradish peroxidase-polymer as a secondary antibody and 3,3-diaminobenzidine. The tissues were incubated with the secondary antibody for 30 min at room temperature.

To assess the levels of E-cadherin and vimentin, we studied the specimens that had shown high proportion of NSCs after H&E staining. The results of the E-cadherin assessment were considered positive when 10% or more of the cancer cell membranes were stained, whereas the results of vimentin assessments were considered positive when 10% or more of the cancer cell cytoplasm was stained, as reported by Kim *et al*. (20).

Statistical analyses. The correlation between NSC grade and clinicopathological factors, namely NSCs occurrence and E-cadherin/vimentin expression was analyzed using Fisher's exact test. Using the Cox proportional hazards model, univariate and multivariate analyses were conducted to identify the negative prognostic factors. Overall survival time refers to the period between the date of surgery and the last follow-up date or date of death. The Kaplan–Meier method was used to calculate the cumulative survival rate. The log-rank test was used to compare the survival rate between the groups having two different levels of NSCs detected. $p<0.05$ was set as the level of significance. The data were analyzed using JMP v. 13 (SAS Institute, Tokyo, Japan).

Table I. *Clinicopathological characteristics of patients with gallbladder cancer (N=62).*

Characteristics	Patients	%
Age (years)		
Range	41-86	
Median	71	
Gender		
Male	28	45.2%
Female	34	54.8%
Tumor size (mm)		
Median	29	
Range	10-90	
CEA (ng/ml)		
Median	2.4	
Range	0.5-50.5	
CA19-9 (ng/ml)		
Median	17.8	
Range	0.8-315.8	
Surgical margin		
R0	54	87.1%
R1	8	12.9%
Complication≥CD IIIa		
Absent	55	88.7%
Present	7	11.2%
Differentiation		
Pap	28	45.2%
Well	20	32.3%
Mode	6	9.7%
Poor	4	6.5%
Other	4	6.5%
T category		
Tis	9	14.5%
T1	8	12.9%
T2	32	51.6%
T3	12	19.4%
T4	1	1.6%
N category		
N0	51	82.3%
N1	10	16.1%
N2	0	0%
Nx	1	1.6%
Lymphatic invasion		
–	33	53.2%
+	29	46.8%
Vascular invasion		
–	38	61.3%
+	24	38.7%
Perineural invasion		
–	44	71.0%
+	18	29.0%
Stage		
Stage 0	9	14.5%
Stage I	7	11.3%
Stage II	25	40.3%
Stage III	19	30.6%
Stage IV	1	1.6%

CD: Clavien-Dindo Classification; pap: papillary adenocarcinoma; well: well differentiated tubular adenocarcinoma; mode: moderately differentiated tubular adenocarcinoma; poor: poorly differentiated adenocarcinoma.

Table II. *Correlation between clinicopathological characteristics and NSC grades in patients with gallbladder cancer.*

Characteristics	Total	NSCs0	NSCs1	NSCs2	*p*-Value*
Age (mean, 71)					
≤70	27	14	6	7	0.21
>70	35	26	4	5	
Gender					
Male	28	19	5	4	0.75
Female	34	21	5	8	
Tumor size (mm)					
≤30	33	26	4	3	0.013
>30	26	11	6	9	
CEA (ng/ml)					
≤5	50	35	7	8	0.078
>5	11	4	3	4	
CA19-9 (ng/ml)					
≤37	48	34	7	11	0.030
>37	13	5	5	1	
Complication ≥CD IIIa					
absent	55	37	7	11	0.11
present	7	3	3	1	
Surgical margin					
R0	54	39	6	9	0.0017
R1	8	1	4	3	
Differentiation					
Pap, well	48	38	5	5	<0.001
Mode, poor, other	14	2	5	7	
T category					
Tis, T1, T2	49	40	4	5	<0.001
T3, T4	13	0	6	7	
N category					
N0	51	36	9	6	0.028
N1	10	4	1	5	
Lymphatic invasion					
–	33	29	2	2	<0.001
+	29	11	8	10	
Vascular invasion					
–	38	32	3	3	<0.001
+	24	8	7	9	
Perineural invasion					
–	44	37	5	2	<0.001
+	18	3	5	10	

NSCs: Neoplastic spindle cells; CD: Clavien-Dindo Classification; pap: papillary adenocarcinoma; well: well differentiated tubular adenocarcinoma; mode: moderately differentiated tubular adenocarcinoma; poor: poorly differentiated adenocarcinoma. *Using the Fisher's exact test.

Results

The clinicopathological factors of 62 patients with gallbladder cancer. The median age was 71 years, and 55% of the patients were female. The median tumor size was 29 mm (range=10-90 mm), median preoperative carcinoembryonic antigen (CEA) was 2.4 ng/ml (range=0.5-50.5 ng/ml), and the median preoperative cancer antigen (CA) 19-9 was 17.8 ng/ml (range=0.8-315.8 ng/ml). Among all patients, 8 (13%) underwent R1 resection. The Clavien–Dindo Grade IIIa classification or above for surgical complications (21) was observed in seven patients. Most patients were diagnosed with papillary adenocarcinoma or well-differentiated adeno-carcinoma, and "three patients with adenosquamous carcinoma and 1 with squamous cell carcinoma. There were nine patients with an invasion depth of Tis, 8 with T1, 32 with T2, 12 with T3, and 1 with T4. Further, ly was positive in 29 patients (46.8%), vascular invasion (v) was positive in 24 patients (38.7%), pn was positive in 18 patients (29.0%), and N1 was observed in 10 patients (16.1%). With regard to the UICC TNM classifications, there were nine patients with Stage 0, seven with Stage I, 25 with Stage II, 19 with Stage III, and one with Stage IV (Table I).

Table III. *Univariate and multivariate analyses of the overall survival of patients after surgery.*

		Univariate Analysis*			Multivariate Analysis*		
Characteristics	Patients total	*p*-Value	HR	95%CI	*p*-Value	HR	95%CI
Age (years)							
≤70	27	0.34					
>70	35						
Gender							
Male	28	0.71					
Female	34						
Tumor size (mm)							
≤30	33	0.15					
>30	26						
CEA (ng/ml)							
≤5	50	0.11					
>5	11						
CA19-9 (ng/ml)							
≤37	48	0.82					
>37	13						
Complication≥CD IIIa							
Absent	55	0.68					
Present	7						
Surgical margin							
R0	54	0.002	7.08	2.26-18.65	0.007	8.73	1.88-38.64
R1	8						
Differentiation							
Pap, well	48	0.093					
Mode, poor, other	14						
T category							
Tis, T1, T2	49	0.045	2.69	1.02-6.38	0.20		
T3, T4	13						
N category							
N0	51	0.004	4.56	1.68-11.39	0.68		
N1	10						
Lymphatic invasion							
–	33	0.007	3.19	1.36-8.31	0.078		
+	29						
Vascular invasion							
–	38	0.033	2.48	1.08-5.75	0.21		
+	24						
Perinural invasion							
–	44	<0.001	5.16	2.22-12.12	0.034	4.87	1.13-19.51
+	18						
NSCs							
NSCs low (grade 0, grade 1)	50	<0.001	10.35	3.93-28.13	0.005	6.65	1.74-30.43
NSCs high (grade 2)	12						

NSCs: Neoplastic spindle cells; CD: Clavien-Dindo Classification; pap: papillary adenocarcinoma; well: well differentiated tubular adenocarcinoma; mode: moderately differentiated tubular adenocarcinoma; poor: poorly differentiated adenocarcinoma; HR: hazard ratio; CI: confidence interval. *Using the Cox proportional hazards model.

Correlation between NSC grade and clinicopathological factors. Totally, 40 patients (64.5%) had NSC Grade 0, 10 (16.1%) Grade 1, and 12 (19.3%) Grade 2. These grades showed a statistically significant correlation with tumor size (p=0.013), preoperative CA19-9 (p=0.030), surgical margin (p=0.0017), differentiation (p<0.001), T category (p<0.001), N category (p=0.028), ly (p<0.001), v (p<0.001), and pn (p<0.001) (Table II).

Survival rate analysis based on NSCs and clinico-pathological factors. The patients were divided into two groups according to NSC grade as follows: the low NSCs group (Grades 0 and 1) and the high NSCs group (Grade 2). Univariate analysis of overall survival after surgical resection showed that the prognostic factors with a significant difference were surgical margin, T category, N category, ly, v, pn, and NSCs. Multivariate analyses of these factors using

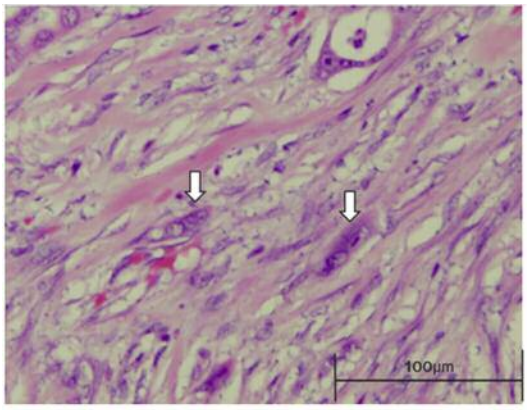

Figure 1. *Photomicrograph of NSCs. Spindle-shaped cancer cells or cancer cells with oval nuclei and no tubular structure (White arrow). NSCs: Neoplastic spindle cells.*

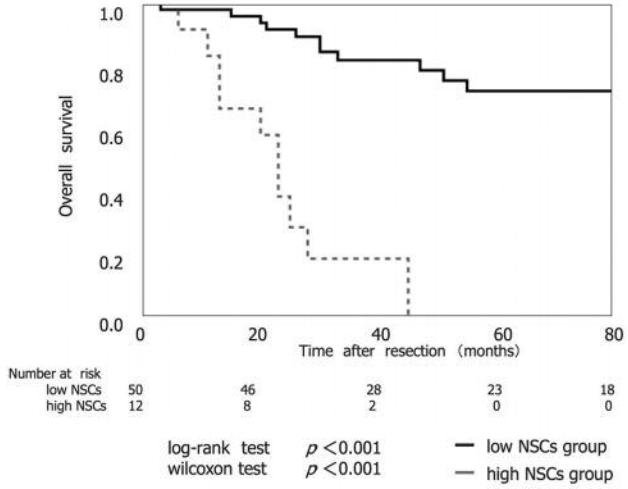

Figure 2. *The survival rate between the low NSCs and the high NSCs groups using the Kaplan–Meier method. The 5-year survival rate of the low NSCs group was 72.2% and that of the high NSCs group was 0%. The difference in survival rates between the two groups was significant (log-rank test, p<0.001). NSCs: Neoplastic spindle cells.*

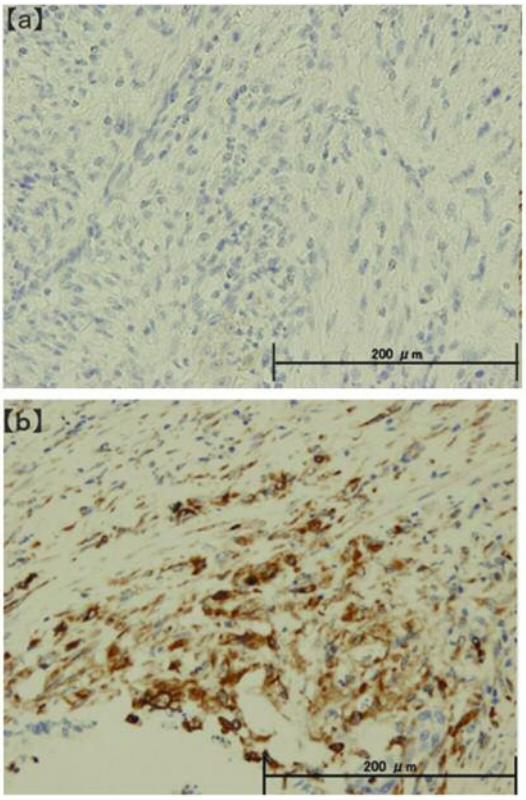

Figure 3. *Immunostaining of NSCs. (a) E-cadherin immunostaining was negative, (b) vimentin immunostaining was positive (×200). Evaluation of E-cadherin/vimentin expression in gallbladder cancer cells. NSCs: Neoplastic spindle cells.*

them as covariates showed that the independent prognostic factors after surgery for patients with gallbladder cancer were surgical margin [hazard ratio (HR), 8.73; 95% confidence interval (CI)=1.88–38.64; p=0.007], pn (HR=4.87; 95%CI=1.13-19.51; p=0.034), and NSCs (HR=6.65; 95%CI=1.74-30.43; p=0.005) (Table III).

Comparison of survival rate between low and high NSCs groups. We compared the survival rates between the low NSCs and high NSCs groups using the Kaplan–Meier method. The 5-year survival rate of the low NSCs group was 72.2% and that of the high NSCs group was 0%. The difference in survival rates between the two groups was significant (log-rank test, p<0.001) (Figure 2).

Correlation between NSC grade and E-cadherin/vimentin expression. The E-cadherin-negative group (Figure 3a) comprised 0 patients with NSC Grade 0, 1 with Grade 1, and 9 with Grade 2, whereas the vimentin-positive group (Figure 3b) comprised 0 patients with Grade 0, 3 with Grade 1, and 12 with Grade 2. NSC grade and E-cadherin expression negatively correlated with each other, whereas NSC grade and vimentin expression positively correlated with each other; both correlations were statistically significant (p<0.001) (Table IV).

Discussion

We conducted a clinicopathological evaluation of NSCs found in specimens taken from patients with gallbladder cancer who underwent surgical resection, and observed that

Table IV. *Correlation between NSC grades and E-cadherin/vimentin expression.*

	Total	NSCs0	NSCs1	NSCs2	*p*-Value*
All patients	62	40	10	12	
E-cadherin					
Negative	10	0	1	9	$p<0.001$
Positive	52	40	9	3	
Vimentin					
Negative	47	40	7	0	$p<0.001$
Positive	15	0	3	12	

NSCs: Neoplastic spindle cells. *Using the Fisher's exact test.

the NSC grades significantly correlated with tumor size, preoperative CA19-9, the extent of surgical margin, the degree of differentiation, the depth of invasion (T), lymph node metastasis (N), lymphatic invasion (ly), vascular invasion (v), and perineural invasion (pn). We also analyzed the overall survival with respect to NSCs and 13 other factors that are considered to be major prognostic factors for gallbladder cancer, such as T category and N category. Among those factors, the high NSCs grade had a strong impact on overall survival with an HR of 6.65, which was the highest following R0 resection revealing that NSCs are an independent prognostic factor that affects the overall survival after surgery in patients with gallbladder cancer. The 5-year survival rate was 72.2% in the low NSCs group and 0% in the high NSCs group, which suggests an extremely poor prognosis for those with high NSCs grade. It has been reported that there is a correlation between NSCs and tumor size, TNM stage, N, histological grade, invasion to the pancreatic nerve plexus, duodenal invasion, invasion to retroperitoneal tissue, ly, and venous invasion in patients with pancreatic cancer, and that NSCs are an independent prognostic factor for disease-free and overall survival (12). It has also been reported that NSCs show a correlation with the stage and lymph node metastasis of vulvar squamous cell carcinoma, which suggests that they are independent prognostic factor (11). In light of the above, it is suggested that there is a possibility that NSCs have a strong influence on gallbladder cancer invasion and proliferation and are one of the major prognostic factors for patients with this cancer who undergo surgical resection.

The expression of molecular markers that accompany EMT in relation to NSCs in patients with gallbladder cancer was also evaluated. During the EMT process, embryonic epithelial cells lose their polarity and cell–cell adhesion and gain migratory and invasive properties to become mesenchymal stem cells. Cancer cells are known to induce EMT (22) and, when induced, the adhesion between epithelial cells is weakened, the cytoskeleton is restructured, and the morphology changes from flat and scale-like into spindle-shaped cells (23-25). Molecules that are known to be related to EMT include E-cadherin, which is one of the epithelial markers involved in cell–cell adhesion, and vimentin, which is a mesenchymal cell marker involved in cytoskeletal interactions (13, 26). Increased expression of E-cadherin and decreased expression of vimentin is observed in cancer cells undergoing EMT resulting in an acceleration of migration and invasion capabilities of the cancer cells (27). This study also showed that NSCs in tumors negatively correlated with E-cadherin and positively correlated with vimentin. This suggests the possibility that in gallbladder cancer an EMT mechanism operates that has a negative impact on the biological malignancy. In patients with nasopharyngeal carcinoma, a significant decrease in E-cadherin expression and an increase in vimentin expression have been observed, along with an increased expression of snail and slug, which are transcription factors that play a central role in EMT (10). In association with gallbladder cancer, it has been reported that vimentin showed correlation with gallbladder cancer metastasis and had a strong influence on cancer progression (28). Given these findings, we suggest that there is a strong correlation between NSCs and EMT in gallbladder cancer. Moreover, the evaluation of NSCs is useful in formulating a prognosis after surgery for patients with gallbladder cancer, and may be used to determine the course of postoperative adjuvant therapy.

There were some problems associated with evaluating NSCs using H&E staining, one of which was that it is difficult to determine the three-dimensional structure of specimens when they are prepared on slides, and at times cancer cells that have transitioned into NSCs appear as ordinary cancer cells because of the way in which the specimens have been sliced. A second problem was that there is the possibility that cancer tissues contain cells at various stages of EMT, which means that some cells have not yet completed their morphological changes into NSCs. Although the evaluation of NSCs using H&E staining appears to be an important step in considering a prognosis, the development of more accurate methods to evaluate these cells is required.

In conclusion, NSCs influence the invasion and proliferation of gallbladder cancer cells and are an independent prognostic

factor for patients with gallbladder cancer who undergo surgical resection. Additionally, there is a possibility that NSCs are cancer cells in which EMT has been induced. We suggest that NSCs can be included in the evaluation criteria used for the prognosis of patients with highly malignant gallbladder cancer.

Conflicts of Interest

The Authors declare no conflicts of interest associated with this manuscript.

Authors' Contributions

Ryuta Midorikawa designed the study, and wrote the initial draft of the manuscript. Ryuta Midorikawa and Toru Hisaka contributed to analysis and interpretation of data, and assisted in the preparation of the manuscript. All other authors contributed to data collection and interpretation, and critically reviewed the manuscript. All authors approved the final version of the manuscript, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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