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RESEARCH

Squamous Cell/Adenosquamous Carcinomas and Adenocarcinomas of the Gallbladder: An Immunohistochemistry Study of Prognostic Markers

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Abstract Gallbladder cancers (GBCs) are highly aggressive and lethal diseases. However, the key molecular mechanisms responsible for the progression and prognosis of GBCs have not been identified. No biological markers for effectively identifying GBC subtypes have been reported. In this study the expression of keratin 19 (KRT19) and human achaete-scute homolog 1 (hASH1) proteins in 46 squamous cell/adenosquamous carcinomas (SC/ASC) and 80 adenocarcinomas (AC) were examined using immunohistochemistry. Negative KRT19 or positive hASH1 expression were significantly associated with lymph node metastasis, invasion and TNM stage of SC/ASC patients. In contrast, positive KRT19 and hASH1 expression were significantly associated with large tumor size, lymph metastasis, invasion, and TNM stage in AC patients. Univariate Kaplan–Meier analysis showed that loss of KRT19 or elevated hASH1 expression significantly correlated with decreased survival in SC/ASC patients. In contrast, positive KRT19 and hASH1 expression correlated with a shorter survival time in AC patients. Multivariate Cox regression analysis showed that negative KRT19 expression or positive hASH1 expression was an independent poor-prognostic predictor in SC/ASC, but positive KRT19 and hASH1 expression were poor-prognostic factors in AC patients. Our study suggested that hASH1 can be used to determine the malignancy of SC/ASC and AC tumors and is associated with poor prognosis. In contrast, KRT19 is a protective factor in AC patients but a sign of malignancy in SC/ASC patients.

Keywords Gallbladder cancer · Adenocarcinoma · Squamous cell carcinoma · Adenosquamous carcinoma · KRT19 · hASH1 · Prognosis · Metastasis

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Introduction

Gallbladder cancers (GBCs) are uncommon but highly lethal diseases [1, 2]. While the majority of GBCs are adenocarcinomas (>98 %) [3], other histopathologic subtypes, such as squamous cell/adenosquamous carcinoma (SC/ASC), are rarely identified [2, 4, 5]. Previous studies suggested that squamous carcinoma proliferates at a higher rate than adenocarcinoma, but squamous tumors are less frequently present with lymph node metastasis [6, 7]. However, the molecular events involved in proliferation and metastasis have not been identified. Overall, the clinicopathological characteristics of

SC/ASC have not been well documented because most reports on SC/ASC are individual case reports or analyses of small case series [8]. It is therefore important to document the clinicopathological and biological characteristics of SC/ASC using a larger sample size.

Keratins (KRTs) are intermediate filament proteins responsible for the structural integrity of epithelial cells and are subdivided into cytokeratins and hair keratins [9]. KRT19 belongs to the type 1 group of cytokeratins with normal expression in ductal epithelium and in the mucosa of the gastrointestinal tract (GIT) [10]. KRT19 immunohistochemistry was therefore used to confirm epithelial immunophenotype in undifferentiated appearing tumors or establish biliary, pancreatic, or renal ductular origin [11]. A previous study has shown that KRT19 is expressed in squamous carcinomas of the head and neck, and it is also expressed in more than 50 % of renal cell carcinomas and tumors arising from stratified squamous epithelium [12]. Recently, the predictive role of KRT19 in the prognosis of epithelial tumor has been investigated in papillary thyroid carcinomas, hepatocellular carcinomas, colorectal adenocarcinoma, and pancreatic neuroendocrine (NE) tumors [13, 14]. However, KRT19 was found to be relatively insensitive in comparison with Ki-67 as an independent marker of poor prognosis [11]. The expression of KRT19 in AC and SC/ASC and its implications in the invasion, metastasis, and prognosis of GBC have not been reported.

Human achaete-scute homolog 1 (*hASH1*) gene encodes a member of the basic helix-loop-helix (bHLH) family of transcription factors. hASH1 protein activates transcription by dimerizing with other bHLH proteins [15]. This protein plays a critical role in the development of central and autonomic nervous systems and in tissues of the so-called diffuse NE system [16]. Recent studies demonstrated that hASH1 expression is a cardinal feature of the NE tumors of the diffuse endocrine system, foregut and midgut [17, 18]. For example, hASH1 has been implicated to impart neuroendocrine behaviors to various NE-tumors such as small cell lung cell cancer (SCLC), gastrointestinal NE carcinoma (NEC), medullary thyroid cancer (MTC), and prostate small cell carcinoma where it is highly expressed [16–19]. High levels of hASH1 expression seen in these tumors correlate with poor prognosis. Therefore, hASH1 appears to be a useful marker for various NE-tumors. However, a recent study also detected hASH1 expression in non-NE lung cancer [20]. In addition, hASH1 was suggested to propagate a stem cell microenvironment in bronchiolization of the alveoli [21]. This suggests that hASH1 might also be a critical molecule involved in the pathology of some cancers. Primary NE tumors of the gallbladder are rarely identified during routine pathological examinations. However, the proof of hASH1 expression and its possible role in identifying the neuroendocrine differentiation characteristics in gallbladder tumors have not been reported.

In this study, the expression of KRT19 and hASH1 in surgically resected specimens, including AC and SC/ASC, was examined using immunohistochemistry. The correlations of KRT19 and hASH1 expression with clinicopathological characteristics and prognosis of AC and SC/ASC were comparatively evaluated.

Materials and Methods

Case Selection

A total of 46 squamous cell/adenosquamous carcinomas (SC/ASCs) of gallbladder cancers (1,060) from patients that underwent surgical resection or biopsy were collected from January 1995 to December 2009. The percentage of SC/ASCs in various gallbladder cancers is 4.34 % (46/1,060 GBCs). 80 adenocarcinomas (ACs) were collected from January 2005 to December 2009. AC and SC/ASC were diagnosed based on morphological criteria, immunohistochemical staining, and clinical findings. The TNM classification of malignant tumors 7th edition, published by the International union against cancer (IUAC) was used for TNM staging of gallbladder cancer. SC was diagnosed when most malignant cells are squamous cells with less than 10 % of the total being adenocarcinoma cells. ASC was diagnosed when the tumor contains both squamous cells and adenocarcinoma cells, but the tumor must contain at least 10 % adenocarcinoma or squamous cell carcinoma cells. However, no neuroendocrine elements were identified in these AC and SC/ASC tumors using routine pathological examinations. Among the 46 SC/ASC patients, 27 patients were female and 19 were male (F/M=1.42) with an age variation of 35 to 82 (55.8±9.6) years. Among the 80 AC patients, 54 patients were female and 26 patients were male (F/M=2.08) with an age variation of 33 to 80 (53.8±9.9) years. Survival information of all 46 SC/ASC and 80 AC patients was obtained through letters and phone calls. This study was pre-approved by The Ethics Committee for Human Research, Central South University.

Immunohistochemistry Staining

Rabbit anti-KRT19 and rabbit anti-hASH1 antibodies were purchased from Abgent Company (California, USA). Staining was conducted with the peroxidase-based EnVision™ Detection kit (Dako Laboratories, CA, USA) by following the user manual. Briefly, 4 μM sections were cut from routinely paraffin-embedded tissues. The sections were then deparaffinized and incubated with 3 % H_2O_2 for 15 min. After being soaked with phosphate buffered saline (PBS) for 3× 5 min, the sections were incubated with rabbit anti-KRT19 (1:100 dilution) or rabbit anti-hASH1 (1:100 dilution) antibody for 1 h at room temperature. After rinsing sections with

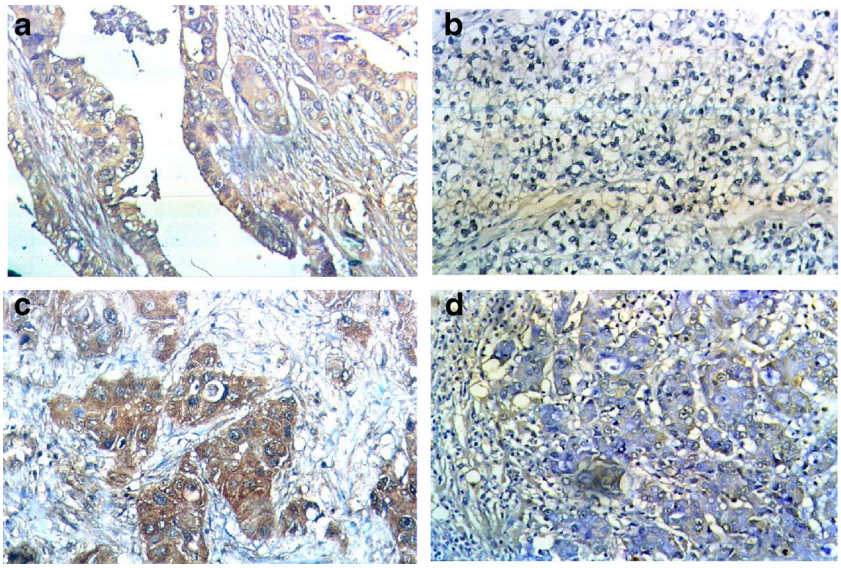

Fig. 1 KRT19 and hASH1 expression in SC/ASC. EnVision immunohistochemistry, original magnification ×200. KRT19 and hASH1 positive reaction was mainly localized in the cytoplasm. **a** Positive KRT19 expression in well differentiated SC/ASC. **b** Negative KRT19 expression in poorly differentiated SC/ASC. **c** Positive hASH1 expression in moderately differentiated SC/ASC. **d** Negative hASH1 expression in well differentiated SC/ASC

PBS for 3 times, HRP-conjugated second antibody was added for 30 min. The substrate DAB was added followed by hematoxylin counter-staining. The positive control was positive sections provided by Beijing Zhongshan Biotechnology Company (Beijing, China) while the negative control was designed by replacing the primary antibody with 5 % fetal bovine serum. The percentage of positive cells was calculated from 500 cells in 10 random fields. Cases with positive cells ≥25 % were considered positive, while cases with positive cells <25 % were considered negative [22].

Statistical Analysis

Data was analyzed using the statistical package for the Social Sciences Version 14.0 (SPSS 14.0). The inter-relationship of KRT19 or hASH1 expression with histological or clinical factors was analyzed using $\chi 2$ or Fisher's exact test. Kaplan–Meier and time series test (log-rank test) were used for univariate survival analysis. Cox proportional hazards model was used for multivariate analysis and to determine the 95 % confidence interval.

Results

Comparison of Clinicopathological Characteristics and KRT19 and hASH1 Expression Between SC/ASC and AC

The percentage of SC/ASC (46) in GBCs (1,060) is 4.34 %. The percentage of cases with age older than 45 years, tumor mass >3 cm, and well- or moderately-

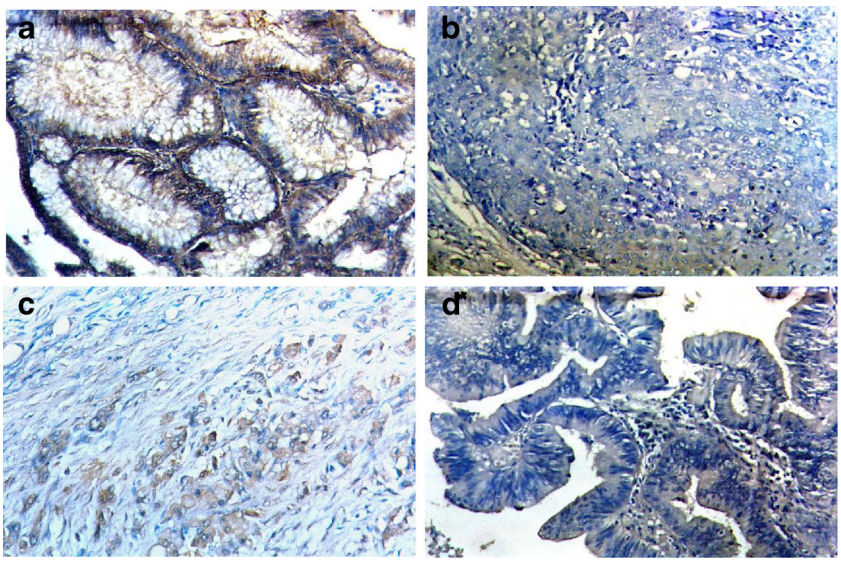

Fig. 2 KRT19 and hASH1 expression in AC. EnVision immunohistochemistry, original magnification ×200. KRT19 and hASH1 positive reaction was mainly localized in the cytoplasm. **a** Positive KRT19 expression in well differentiated AC. **b** Negative KRT19 expression in poorly differentiated AC. **c** Positive hASH1 expression in poorly differentiated AC. **d** Negative hASH1 expression in well differentiated AC

differentiated tumors was significantly higher in SC/ASCs than in ACs ($p < 0.05$). No significant differences in other clinicopathological characteristics as well as the percentage of positive KRT19 and hASH1 expression were observed between SC/ASC and AC patients. EnVision immunohistochemistry revealed that KRT19 and hASH1 positive reaction was mainly localized in the cytoplasm of SC/ASC (Fig. 1) and AC (Fig. 2). Among the 46 SC/ASC tumors, 23 tumors showed positive KRT19 expression (50 %) while 18 tumors showed positive hASH1 expression (39.1 %). Among the 80 AC tumors, 45 tumors showed positive KRT19 expression (56.3 %), while 31 tumors showed positive hASH1 expression (38.8 %).

The Association of KRT19 and hASH1 Expression with Clinicopathological Characteristics of Patients with SC/ASC and AC

As shown in Table 1, the percentage of negative KRT19 and positive hASH1 expression was significantly higher in SC/ASC with high TNM stage, invasion and lymph node metastasis compared to the cases with low TNM stage, no invasion, and no lymph metastasis ($p < 0.05$ or $p < 0.01$). Negative KRT19 expression is significantly associated with large tumor size ($p < 0.05$). Both KRT19 and hASH1 expression only showed a tendency to be associated with differentiation in SC/ASC. In AC tumors, the percentage of positive KRT19

Table 1 The association of KRT19 and hASH1 expression with the clinicopathological characteristics of SC/ASC and AC

Clinicopathological characteristics	Total no:	KRT19			hASH1		
		Pos no: (%)	χ^2	*P*	Pos no: (%)	χ^2	*P*
SC/ASC differentiation							
Well	16	11 (68.8)	5.083	0.079	6 (37.5)	5.940	0.051
Moderately	24	11 (45.8)			7 (29.2)		
Poorly	6	1 (16.7)			5 (83.3)		
Tumor mass size							
≤3 cm	20	14 (70.0)	5.662	0.017	5 (25.0)	2.966	0.085
>3 cm	26	9 (34.6)			13 (50.0)		
TNM stage							
I + II	12	10 (83.3)	9.905	0.007	2 (16.7)	6.403	0.046
III	20	10 (50.0)			7 (35.0)		
IV	14	3 (21.4)			9 (64.3)		
Lymph metastasis							
No	17	12 (70.6)	4.572	0.033	3 (17.6)	5.225	0.021
Yes	29	11 (37.9)			15 (51.7)		
Invasion							
No	16	12 (75.0)	6.133	0.015	3 (18.8)	4.278	0.041
Yes	30	11 (36.7)			15 (50.0)		
AC differentiation							
Well	27	11 (40.7)	6.823	0.033	6 (22.2)	5.336	0.066
Moderately	25	13 (52.0)			8 (32.0)		
Poorly	28	21 (75.0)			17 (60.7)		
Tumor mass size							
≤3 cm	50	22 (44.0)	8.130	0.004	13 (26.0)	9.132	0.003
>3 cm	30	23 (76.7)			18 (60.0)		
TNM stage							
I + II	21	7 (33.3)	12.092	0.002	4 (19.0)	10.660	0.006
III	38	20 (52.6)			13 (34.2)		
IV	21	18 (85.7)			14 (66.7)		
Lymph metastasis							
No	30	11 (36.7)	7.480	0.006	7 (23.3)	4.807	0.028
Yes	50	34 (68.0)			24 (48.0)		
Invasion							
No	31	12 (38.7)	6.327	0.012	7 (22.6)	6.071	0.018
Yes	49	33 (67.3)			24 (49.0)		

and hASH1 expression was significantly higher in cases with large tumor mass size, high TNM stage, lymph node metastasis, and invasion compared to cases with small tumor mass size, low TNM stage, no lymph node metastasis, and no invasion ($p<0.05$ or $p<0.01$). KRT19 expression significantly correlated with poor differentiation, but hASH1 only showed a tendency to be associated with poor differentiation in AC.

The Correlation of KRT19 or hASH1 Expression with Survival in Patients with SC/ASC and AC

Survival information was collected by phone calls and letters over a period of 2 years. Among the 80 AC patients, 57 patients survived <1 year and 23 patients survived ≥1 year (9 cases survived >2 years) with an average survival time of 10.34±0.63 months. Among the 46 SC/ASCs patients, 33 patients survived <1 year and 13 patients survived ≥1 year (4 cases survived >2 years) with an average survival time of 10.07±0.78 months. There was no significant difference in survival time between SC/ASC and AC patients.

The Kaplan–Meier survival analysis of SC/ASC patients revealed that the average survival time of KRT19 negative and hASH1 positive patients was significantly shorter than patients having positive KRT19 ($p=0.001$) and negative hASH1 expression ($p=0.01$) (Table 2, Fig. 3). The differentiation, tumor size, TNM stage, lymph node metastasis, and invasion were also significantly associated with average survival time in SC/ASC and AC patients ($p<0.001$) (Table 2). The Kaplan–Meier survival analysis of AC patients revealed that KRT19 and hASH1 positive patients survived significantly shorter than patients having negative KRT19 ($p<0.001$) and hASH1 expression ($p<0.001$) (Table 2, Fig. 4).

Cox multivariate analysis of SC/ASC patients' survival showed that the differentiation, tumor size (≥3 cm), TNM stage, invasion as well as KRT19-negative and hASH1-positive expression negatively correlated with overall survival, suggesting that they are independent risk factors of SC/ASCs (Table 3). Cox multivariate analysis of AC patients revealed that KRT19-positive and hASH1-positive expression negatively correlated with overall survival in SC/ASCs patients (Table 3).

Table 2 Relationship between KRT19 and hASH1 expression, clinicopathological characteristics and average survival of SC/ASC and AC patients

C.P characteristics	Samples (n)	Average survival (month)	Chi-square	P value
SC/ASC differentiation				
Well	16	13.81 (5–24)	19.125	0.000
Moderately	24	8.92 (4–18)		
Poorly	6	5.83 (4–9)		
Tumor mass size				
≤3 cm	20	14.35 (7–24)	31.337	0.000
>3 cm	26	7.04 (4–11)		
TNM stage				
I + II	12	17.00 (9–24)	51.139	0.000
III	20	9.20 (7–15)		
IV	14	5.86 (4–8)		
Lymph metastasis				
No	17	14.24 (4–24)	16.219	0.000
Yes	29	7.86 (4–15)		
Invasion				
No	16	15.75 (9–24)	32.271	0.000
Yes	30	7.27 (4–12)		
KRT19				
−	23	7.78 (4–24)	11.927	0.001
+	23	12.65 (6–24)		
hASH1				
−	28	11.68 (6–24)	6.611	0.010
+	18	7.94 (4–24)		
AC differentiation				
Well	27	15.07 (5–24)	32.501	0.000
Moderately	25	10.60 (4–24)		
Poorly	28	6.68 (3–14)		
Tumor mass size				
≤3 cm	50	13.70 (6–24)	68.283	0.000
>3 cm	30	5.80 (3–10)		
TNM stage				
I + II	21	18.96 (5–24)	105.825	0.000
III	38	9.29 (6–15)		
IV	21	5.14 (3–7)		
Lymph metastasis				
No	30	16.27 (4–24)	42.372	0.000
Yes	50	7.42 (3–14)		
Invasion				
No	31	16.68 (7–24)	55.535	0.000
Yes	49	6.98 (3–11)		
KRT19				
−	35	13.54 (4–24)	12.675	0.000
+	45	8.56 (3–24)		
hASH1				
−	49	13.10 (5–24)	15.830	0.000
+	31	7.00 (3–24)		

Discussion

Squamous cell/adenosquamous carcinoma (SC/ASC) is a rare subtype of GBC and our current understanding on its clinicopathological characteristics is mainly based on the reports of individual cases or small case series. The present study with a relative large simple size may provide a clearer picture on the clinicopathological natures of SC/ASC. Previous studies proposed that SC/ASC is more aggressive than ordinary AC. Previous observations also revealed that SC/ASC occurs predominantly in females (F/M=3.8) [4], and squamous

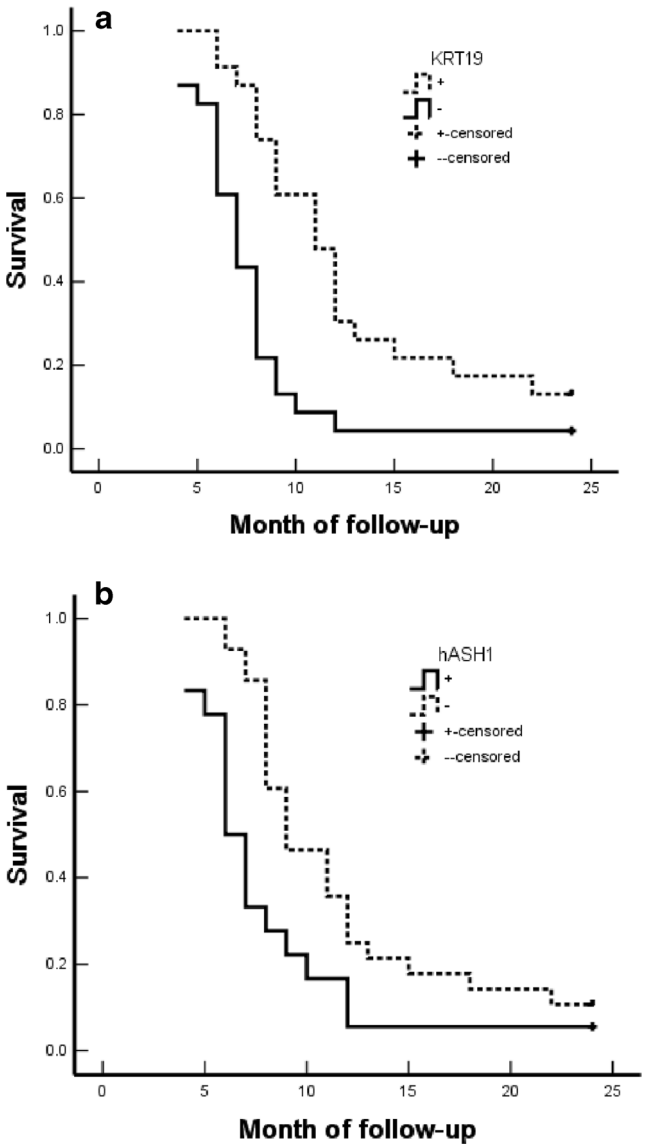

Fig. 3 KRT19 and hASH1 expression and survival in patients with SC/ASC of gallbladder. **a** Kaplan–Meier plots of overall survival in patients with SC/ASC and with KRT19 positive and negative expression. **b** Kaplan–Meier plots of overall survival in patients with SC/ASC and with hASH1 positive and negative expression

carcinomas proliferate at a higher rate than ACs but are less frequently presented with lymph node metastasis [6, 7]. In this study we demonstrated that SC/ASC has similar clinical and pathological characteristics to AC tumors. SC/ASC occurs with no significant difference between females and males (F/M=1.4). There are no differences in invasion and lymph node metastasis occurrence between AC and SC/ASC although more SC/ASC patients had large tumor size. There was no significant difference in differentiation, TNM stage, and survival time between SC/ASC and AC patients. These observations suggest that the clinicopathological presentations of SC/ASC did not seem to be significantly different from ordinary AC.

At present, the histopathologic typing of GBCs is mainly dependent on the histological characteristic of tumor cells, which is not very effective for undifferentiated tumors. However, biomarkers for accurately distinguishing AC from SC/ASC have not been identified. KRT19 immunohistochemistry was previously used to confirm epithelial immunophenotype in undifferentiated appearing tumors or to establish biliary, pancreatic, or renal ductular origin [11]. KRT19 was also proposed as a useful marker in tumors arising from stratified squamous epithelium. In contrast, hASH1 expression is thought to be a cardinal feature of the NE tumors of the diffuse endocrine system of the foregut and midgut [17, 18]. hASH1 is also highly expressed in epithelial malignancies with

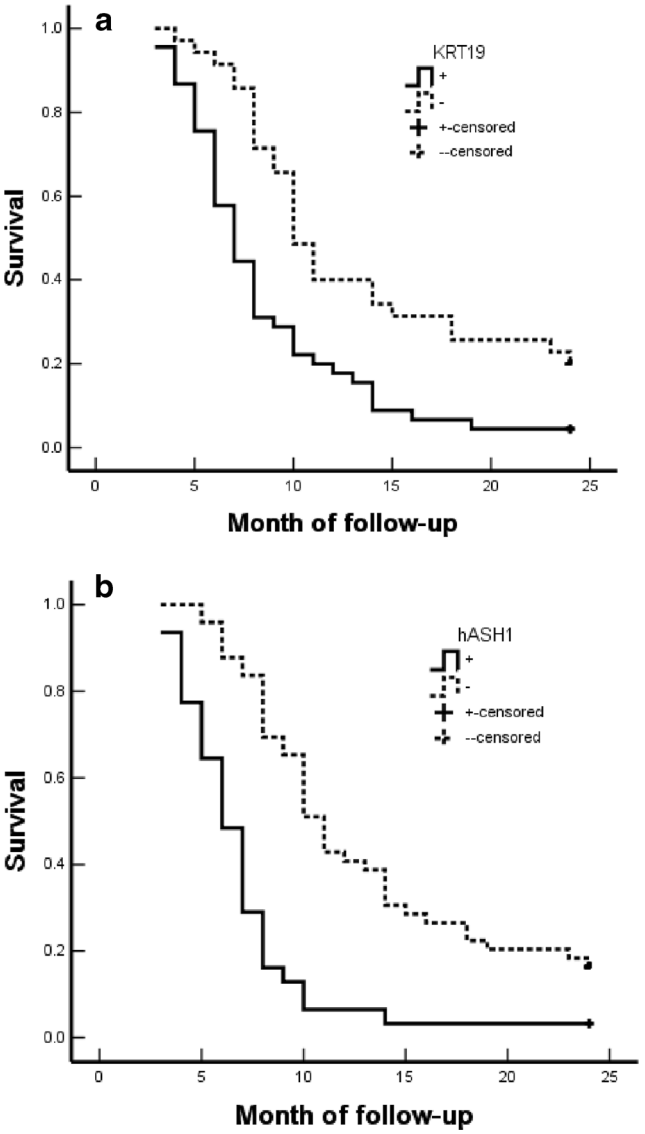

Fig. 4 KRT19 and hASH1 expression and survival in patients with AC of gallbladder. **a** Kaplan–Meier plots of overall survival in patients with AC and with KRT19 positive and negative expression. **b** Kaplan–Meier plots of overall survival in patients with AC and with hASH1 positive and negative expression

Table 3 Multivariate Cox regression analysis of survival rate in SC/ASC and AC patients

Groups	Factors	RC	SE	Wald	*P*	RR	95 % CI	
							Lower	Upper
SC/ASC								
Differentiation	Well /moderately /poorly	.892	.361	6.105	.013	2.440	1.203	4.951
Tumor mass size	≤3 cm/>3 cm	2.335	.765	9.316	.002	10.329	2.306	46.266
TNM stage	I + II/III/IV	1.610	.478	11.345	.001	5.003	1.960	12.767
Lymph metastasis	No/yes	1.457	.620	5.523	.019	4.293	1.274	14.472
Invasion	No/yes	2.525	.770	10.753	.001	12.491	2.762	56.498
KRT19	±	−.985	.426	5.346	.021	.373	.162	.861
hASH1	±	.924	.429	4.639	.031	2.519	1.087	5.841
AC								
Differentiation	Well /moderately /poorly	1.162	.508	5.232	.022	3.196	1.181	8.651
Tumor mass size	≤3 cm/>3 cm	1.096	.440	6.205	.013	2.992	1.263	7.088
TNM stage	I + II/III/IV	1.305	.403	10.486	.001	3.688	1.674	8.124
Lymph metastasis	No/yes	1.425	.498	8.188	.004	4.158	1.567	11.035
Invasion	No/yes	1.086	.503	4.661	.031	2.962	1.105	7.940
KRT19	±	.769	.321	5.739	.017	2.158	1.150	4.048
hASH1	±	.872	.327	7.111	.008	2.392	1.260	4.540

RC regression coefficients; *SE* standard error; *RR* relative risk

neuroendocrine differentiation characteristics [16]. In this study positive KRT19 expression was only detected in 50 % of SC/ASC tumors and 56.3 % of AC tumors. The low percentage of positive KRT19 expression in AC and SC/ASC as well as the lack of a difference in the percentage of positive KRT19 expression between AC and SC/ASC tumors suggest that KRT19 is not a specific marker to distinguish between these two tumor subtypes. In contrast, positive hASH1 expression was observed in 39.1 % of SC/ASC tumors and 38.8 % of AC tumors. The percentage is too high in both AC and SC/ASC tumors because they are not classic neuroendocrine tumors. Therefore, hASH1 could not be used as a specific marker for identifying neuroendocrine differentiation characteristics in gallbladder tumors.

Recently, hASH1 has been found to be significantly correlated with tumor progression, metastasis, invasion, and prognosis in epithelial tumors with neuroendocrine differentiation characteristics [16]. The predictive roles of hASH1 in GBCs have not been established. In this study, positive hASH1 expression was significantly associated with high TNM stage, invasion and lymph node metastasis in both AC and SC/ASC tumors. The average survival time of hASH1 positive patients was significantly shorter than patients having negative hASH1 expression in SC/ASC patients, and hASH1 positive expression was significantly associated with poor prognosis in AC patients. Therefore, hASH1 appears to be a useful marker for fast progression of SC/ASC and AC tumors and poor prognosis of SC/ASC and AC patients. Our findings show that hASH1 levels can be used to indicate which patients should receive more aggressive therapy in an attempt to reverse poor prognosis of SC/ASC and AC patients.

A notable finding in this study is that KRT19 is a protective factor in SC/ASC tumors, but a malignant factor in AC tumors. Negative KRT19 expression was significantly associated with high TNM stage, invasion, and lymph node metastasis in SC/ASC tumors and poor prognosis in SC/ASC patients. The role of KRT19 in AC tumor is the opposite. Positive KRT19 expression correlated with poor prognosis in AC patients. KRT19 was previously identified as a predictive marker of poor prognosis in several epithelial tumors, such as papillary thyroid carcinomas, hepatocellular carcinomas, colorectal adenocarcinoma, and pancreatic neuroendocrine tumors. However, no divergent role of KRT19 in different subtypes of a tumor was previously reported. KRT19 belongs to the type 1 group of cytokeratins, which are intermediate filament proteins responsible for the structural integrity of epithelial cells [9]. At present, the function of KRT19 as an intermediate filament protein cannot explain its divergent role in AC and SC/ASC. However, our finding indicates a biological difference between the rare SC/ASC subtype and ordinary AC subtype. This may suggest that different molecular mechanisms are involved in the tumorigenesis, progression, and prognosis of different subtypes of GBC.

Our study demonstrated that high hASH1 expression is associated with the aggressive characteristics and poor

prognosis of AC and SC/ASC. KRT19 is a protective factor in SC/ASC tumors but a malignant factor in AC tumors. Either KRT19 or hASH1 could be used as a prognostic marker in AC and SC/ASC.

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Conflict of Interest All authors declared no conflict of interest.

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