ORIGINAL ARTICLE

Concurrent Analysis of Human Equilibrative Nucleoside Transporter 1 and Ribonucleotide Reductase Subunit 1 Expression Increases Predictive Value for Prognosis in Cholangiocarcinoma Patients Treated with Adjuvant Gemcitabine-based Chemotherapy

Short title: hENT1 and RRM1 in cholangiocarcinoma

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ABSTRACT

Background: The aim of this study was to investigate the predictive and prognostic value of intratumoral human equilibrative nucleoside transporter 1 (hENT1) and ribonucleotide reductase subunit 1 (RRM1) expression in advanced cholangiocarcinoma patients treated with adjuvant gemcitabine-based chemotherapy (AGC).

Methods: Intratumoral hENT1 and RRM1 expression levels were investigated immunohistochemically in 127 patients with advanced cholangiocarcinoma who underwent surgical resection (68 with AGC and 59 without AGC). The impacts of hENT1 and RRM1 expression on survival were evaluated.

Results: High intratumoral hENT1 and RRM1 expression levels were observed in 86 (68%) and 67 (53%) patients, respectively. In multivariate analysis of 68 patients who received AGC, high hENT1 ($P = 0.044$) and low RRM1 expression ($P = 0.009$) were independently associated with prolonged disease-free survival (DFS), while low RRM1 expression ($P = 0.024$) was independently associated with prolonged overall survival (OS). Moreover, concurrent high hENT1 and low RRM1 expression was a powerful independent predictor of prolonged DFS ($P < 0.001$) and OS ($P = 0.001$) when the combined classification of hENT1 and RRM1 was introduced.

Conclusions: Concurrent analysis of hENT1 and RRM1 expression may increase the predictive value of these biomarkers for survival of advanced cholangiocarcinoma patients treated with AGC.

Keywords: hENT1, RRM1, cholangiocarcinoma, gemcitabine, adjuvant chemotherapy.

Cholangiocarcinoma, including intra- and extrahepatic cholangiocarcinoma, is a relatively uncommon disease in the United States, accounting for 4,410 deaths in 2012 (Siegel *et al*, 2013). However, this disease is the sixth leading cause of cancer-related deaths in Japan, with more than 18,000 deaths reported in 2012 (National Cancer Center, Japan, 2014). While surgical resection is the only curative treatment for cholangiocarcinoma, the 5-year overall survival (OS) rates of patients with resected cholangiocarcinoma are still 18%–40%, even in high-volume centers (Murakami *et al*, 2011; DeOliveira *et al*, 2007; Nagino *et al*, 2013). Therefore, several peri-operative therapeutic modalities, including adjuvant chemotherapy, have recently been proposed in order to improve the prognosis of patients with cholangiocarcinoma.

Since 2002, postoperative adjuvant gemcitabine-based chemotherapy (AGC) has been administered to patients with advanced cholangiocarcinoma (International Union Against Cancer [UICC] stage II–IV) in our institution, and we have previously reported a survival benefit associated with this therapy (Murakami *et al*, 2009, 2011, 2012). However, the efficacy of AGC varies among individuals, and the resulting survival rates are still unsatisfactory. To maximize the therapeutic benefit of adjuvant chemotherapy, identification of biomarkers that have predictive and prognostic value is important. Several clinical studies have revealed the predictive significance of intratumoral human equilibrative nucleoside transporter 1 (hENT1) for survival in pancreatic cancer patients treated with gemcitabine (Spratlin *et al*, 2004; Giovannetti *et al*, 2006; Farrell *et al*, 2009; Maréchal *et al*, 2012; Wei *et al*, 2013; Greenhalf *et al*, 2014). Moreover, our recent report demonstrated that hENT1 also predicts the survival of cholangiocarcinoma patients treated with AGC (Kobayashi *et al*, 2012). Thus, hENT1 has been recognized as a relevant predictive biomarker for response to gemcitabine.

In addition, researchers have recently also become interested in identifying other candidates for predictive biomarkers related to gemcitabine sensitivity. In particular, the expression of ribonucleotide reductase subunit M1 (RRM1), which is involved in the production of

deoxyribonucleotides for DNA synthesis, has been reported to be associated with gemcitabine resistance in several cancers (Jordheim *et al*, 2011; Gong *et al*, 2012; Akita *et al*, 2009; Ohtaka *et al*, 2008). Additionally, we have recently demonstrated that combined analysis of hENT1 and RRM1 expression was a more powerful predictor than analysis of either target alone in pancreatic cancer (Nakagawa *et al*, 2013). However, very few reports have revealed the predictive significance of RRM1 expression for gemcitabine resistance in cholangiocarcinoma. In addition, the predictive value of combined analysis of hENT1 and RRM1 expression in cholangiocarcinoma is still unclear. The aim of this study was to investigate the predictive and prognostic values of intratumoral hENT1 and RRM1 expression in advanced cholangiocarcinoma patients treated with AGC after surgical resection.

MATERIAL AND METHODS

Study Design. Patients with advanced cholangiocarcinoma (UICC stages II, IIA, IIB, III, IIIA, IIIB, IV, IVA, and IVB) who underwent surgical resection with curative intent (R0 or R1 resection) at the Department of Surgery, Hiroshima University Hospital, Hiroshima, Japan between April 1989 and August 2012 were enrolled in this study. All patients had a confirmed pathological diagnosis. Patients who experienced postoperative mortality were excluded from this study. Formalin-fixed, paraffin-embedded tumor tissues from the resected specimens were collected from eligible patients, and immunohistochemical staining for detection of intratumoral hENT1 and RRM1 was performed. The influences of clinicopathological factors and hENT1 and RRM1 expression on survival were evaluated by univariate and multivariate analyses. Written informed consent was obtained from all patients for surgical treatment and pathological examinations, as required by institutional guidelines.

Surgical Procedures and Pathological Assessment. Most patients with intrahepatic or perihilar cholangiocarcinoma underwent major hepatectomy, and all surgical procedures for perihilar

cholangiocarcinoma included caudate lobectomy. Patients with distal cholangiocarcinoma usually underwent pancreatoduodenectomy with or without pylorus preservation. Dissection of the regional lymph nodes was performed for all patients. All resected specimens were examined histologically by specialized pathologists; each tumor was classified as well-differentiated, moderately differentiated, or poorly differentiated adenocarcinoma according to the predominant histology. Residual tumor (R factor) was considered R1 if histological infiltrating carcinoma was present at the proximal or distal bile duct transaction line, the hepatic transaction line, or the dissected peripancreatic soft-tissue margins. All patients with R2 resections were excluded from this study. Tumor stage, lymph node metastasis, and final stage were classified based on the 7th edition of the UICC tumor-node-metastasis (TMN) classification (Sobin *et al*, 2010).

Adjuvant Gemcitabine-based Chemotherapy. The AGC regimen used in this study, which included 2 treatment options, has been reported previously (Murakami *et al*, 2009, 2011, 2012). First, intravenous chemotherapy consisted of gemcitabine 700 mg/m^2 administered biweekly. Second, intravenous and oral chemotherapy consisted of intravenous gemcitabine 700 mg/m^2 on day 1 and oral S-1 50 mg/m^2 for 7 consecutive days. These regimens were repeated every 14 days for 10 cycles. None of the patients received radiation therapy during this study period. Patients who had to switch to other chemotherapies before the 10 cycles were completed because of recurrent disease were considered to have received AGC in our group classification. Patients who received gemcitabine-based chemotherapy because of recurrent disease after completion of AGC were also included in this group.

Immunohistochemistry for hENT1 and RRM1. Polyclonal rabbit antibodies against human hENT1 (Abnova Co., Taipei, Taiwan) and against human RRM1 (Abcam, Cambridge, UK) were used to evaluate hENT1 and RRM1 expression, respectively. Following antigen retrieval by

autoclaving (100°C for 10 min in Dako Target Retrieval Solution High pH 1× for hENT1 or 121°C, 10 min in 0.01 M citrate buffer for RRM1), sections were immersed in methanol containing 3% hydrogen peroxide for 15 min, incubated in protein blocking solution (Dako, Carpinteria, CA) for 10 min, and incubated with anti-hENT1 antibodies (1:200 dilution) overnight at 4°C or anti-RRM1 antibodies (1:150 dilution) for 60 min at room temperature. Samples were then incubated in labeled streptavidin-biotin polymer (Envision Plus, Dako) at room temperature for 60 min as a secondary antibody and immersed for 10 min in 0.01% 3,3-diaminobenzidine solution in 50 mM Tris-HCl buffer with 10 mM hydrogen peroxide as a substrate. Sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. Negative controls were provided by omitting the primary antibodies.

Two observers (H.S. and N.K.), blinded to clinical characteristics and outcomes, assessed and scored the expression of hENT1 and RRM1. In cases of disagreement, consensus was reached by joint review. Because hENT1 is strongly expressed in cell membranes of lymphocytes (Spratlin *et al*; 2004; Farrell *et al*, 2009), and RRM1 is strongly expressed in plasma and stromal cells (Akita *et al*, 2009; Ohtaka *et al*, 2008), these were used as internal positive controls. The intensities of hENT1 and RRM1 staining were scored as follows: grade 0, not stained; grade 1, weakly stained compared with the internal positive control; grade 2, stained as strongly as the internal positive control; and grade 3, strongly stained compared with the internal positive control. For evaluation of intratumoral hENT1 and RRM1 expression, if grade 2 or 3 staining was observed in more than 50% of tumor cells, the sample was considered to have high expression, and if grade 0 or 1 staining was observed in more than 50% of tumor cells, the sample was considered to have low expression (Figure 1). This cutoff value was determined on the basis of a previous report (Santini *et al*, 2010).

Survival. Disease status was regularly assessed every 3 months by blood tests and computed tomography. If a patient had died, the survival time after surgery and cause of death were recorded.

For surviving patients (as of July 4, 2013), postsurgical time and recurrence status were recorded. The failure event for OS was defined as death of any cause, while that for disease-free survival (DFS) was defined as disease recurrence, diagnosed based on imaging findings, or death of any cause. Survival time was measured from the date of operation to the date of the failure event or last follow-up evaluation.

Statistical Analysis. Categorical clinicopathological variables were compared with chi-square test and Fisher's exact test, as appropriate. Survival endpoints were estimated using the Kaplan-Meier method and compared by univariate log-rank (Mantel-Cox) test. The Cox proportional hazards model was applied to the multivariate survival analysis for factors found to be significant on univariate analysis. The UICC stage was excluded in the multivariate analysis, even though it was significant by univariate analysis, because of its confounding with UICC pT factor and lymph node metastasis. *P*-values of less than 0.05 were considered statistically significant. Statistical analyses were performed by JMP software, version 10.2 (SAS Institute, Cary, NC, USA).

RESULTS

Patient Demographics and Pathological Assessment. A total of 132 consecutive patients with UICC stage II–IV cholangiocarcinoma underwent surgical resection (R0 or R1 resection) at our institution between April 1989 and August 2012. Of these 132 patients, 5 (3.7%) were excluded from this study because of operative deaths. In total, 127 cholangiocarcinoma patients were eligible for this study. This case series included 105 (83%) patients previously reported in our retrospective analysis of hENT1 expression in cholangiocarcinoma (Kobayashi *et al*,2012). Demographics and clinicopathological factors of enrolled patients are summarized in Table 1. The median age of these 127 patients was 69 years (range: 37–85 years). Lymph node metastasis was found in 65 (51%) patients, including 12 (9%) with para-aortic lymph node involvement. Finally, 32 (25%), 17 (13%),

24 (19%), 2 (2%), 7 (6%), 20 (16%), 6 (5%), 13 (10%), and 6 (5%) patients were diagnosed with stage II, IIA, IIB, III, IIIA, IIIB, IV, IVA, and IVB disease, respectively. All 12 patients with stage IV or IVB disease had para-aortic lymph node metastases detectable only by postoperative histological examination, but not by pre-operative imaging examinations.

Delivery of AGC. Of the 127 patients, 68 (54%) received postoperative AGC, and 59 (46%) did not. In the 68 patients who received AGC, 60 (88%) patients received adjuvant gemcitabine plus S-1 chemotherapy, and 8 (12%) patients received gemcitabine alone. Sixty-one (90%) patients received 10 cycles of AGC, while the remaining 7 (10%) patients had to switch to other chemotherapies at 7 or 8 cycles of AGC because of recurrent disease. The median total dose of gemcitabine administered to the 68 patients was 17,000 mg (range: 7,000–44,000 mg). No treatment-related deaths were reported in this case series. In the 59 patients without AGC, 55 (93%) patients received only surgical treatment, and 4 (7%) patients received adjuvant oral UFT chemotherapy.

Clinicopathological Factors and Intratumoral hENT1 and RRM1 Expression. Potential correlations of hENT1 and RRM1 expression levels with each clinicopathological factor are shown in Table 1. High intratumoral hENT1 and RRM1 expression levels were observed in 86 (68%) and 67 (53%) patients, respectively. Significant differences in hENT1 expression were found among samples with varying states of pathological differentiation ($P = 0.009$), and poorly differentiated adenocarcinoma samples were more likely to exhibit low RRM1 expression ($P = 0.017$). Additionally, the distribution of RRM1 expression was significantly different depending on the tumor location ($P = 0.020$), pathological differentiation ($P = 0.017$), and UICC stage ($P = 0.009$). Other clinicopathological factors did not correlate with hENT1 or RRM1 expression.

Univariate Survival Analysis for Patients with or without AGC. The median follow-up time

after surgery was 81 months (range: 9–294 months) for all 127 patients. The 5-year DFS and OS rates for these patients were 26% and 33%, respectively. The results of univariate DFS and OS analyses for patients with or without AGC are shown in Table 2. In 68 patients who received AGC, pathological differentiation ($P = 0.003$), UICC stage ($P = 0.042$), hENT1 expression ($P = 0.005$), and RRM1 expression ($P = 0.015$) were significantly associated with DFS, and pathological differentiation ($P = 0.011$), lymph node metastasis ($P = 0.009$), UICC stage ($P = 0.012$), hENT1 expression ($P = 0.036$), and RRM1 expression ($P = 0.035$) were also significantly associated with OS. In the 59 patients who did not receive AGC, residual tumor ($P < 0.001$) and lymph node metastasis ($P = 0.037$) were significantly associated with DFS, and residual tumor ($P < 0.001$), pathological differentiation ($P = 0.049$), lymph node metastasis ($P = 0.007$), and UICC stage ($P = 0.017$) were significantly associated with OS. However, both hENT1 and RRM1 expression were not significantly correlated with DFS (hENT1: $P = 0.796$, RRM1: $P = 0.642$) or OS (hENT1: $P = 0.913$, RRM1: $P = 0.883$) (Figure 2) (Figure 3).

Each of the 68 patients who received AGC was classified into 4 groups based on hENT1 and RRM1 expression levels as follows: high hENT1/low RRM1 expression (n = 20), high hENT1/high RRM1 expression (n = 25), low hENT1/low RRM1 expression (n = 13), and low hENT1/high RRM1 expression (n = 10), which was significantly associated with both DFS ($P = 0.003$) and OS ($P = 0.015$) by univariate analysis (Figure 4). Moreover, patients with high hENT1/low RRM1 expression experienced significantly longer DFS and OS than those with high hENT1/high RRM1 expression (DFS: $P = 0.001$, OS: $P = 0.006$), low hENT1/low RRM1 expression (DFS: $P < 0.001$, OS: $P = 0.002$), and low hENT1/high RRM1 expression (DFS: $P < 0.001$, OS: $P = 0.003$). Based on these findings, we further categorized these 68 patients who received AGC into the high hENT1/low RRM1 expression (n = 20) group and low hENT1 and/or high RRM1 group (n = 48) for comparative purposes. This combined classification was significantly associated with both DFS ($P < 0.001$) and OS ($P = 0.001$) by univariate analysis.

On the other hand, each of the 59 patients who did not received AGC was also classified into 4 groups: high hENT1/low RRM1 expression (n = 12), high hENT1/high RRM1 expression (n = 29), low hENT1/low RRM1 expression (n = 15), and low hENT1/high RRM1 expression (n = 3), which was not significantly associated with both DFS ($P = 0.778$) and OS ($P = 0.994$) by univariate analysis.

Multivariate Survival Analysis for Patients who Received AGC. Multivariate analysis including separated hENT1 and RRM1 expression for 68 patients who received AGC identified well differentiated (HR, 0.37; 95% CI, 0.17–0.76; $P = 0.007$), high hENT1 expression (HR, 0.49; 95% CI, 0.24–0.98; $P = 0.044$), and low RRM1 expression (HR, 0.41; 95% CI, 0.21–0.80; $P = 0.009$) as independent factors for prolonged DFS and well differentiated (HR, 0.45; 95% CI, 0.19–0.98; $P = 0.045$), absence of lymph node metastasis (HR, 0.39; 95% CI, 0.18–0.81; $P = 0.011$), and low RRM1 expression (HR, 0.43; 95% CI, 0.20–0.89; $P = 0.024$) as independent prognostic factors for prolonged OS (Table 3, Model 1).

Furthermore, multivariate analysis including combined hENT1 and RRM1 classification was performed among the 68 patients who received AGC. Well differentiated (HR, 0.38; 95% CI, 0.17–0.77; $P = 0.007$) and high hENT1/low RRM1 expression (HR, 0.22; 95% CI, 0.08–0.51; $P < 0.001$) were identified as independent factors of prolonged DFS, and absence of lymph node metastasis (HR, 0.39; 95% CI, 0.18–0.81; $P = 0.012$) and high hENT1/low RRM1 expression (HR, 0.22; 95% CI, 0.07–0.60; $P = 0.001$) were identified as independent factors for prolonged OS (Table 3, Model 2).

DISCUSSION

Since some clinical studies, including one randomized controlled trial, have revealed that gemcitabine-based chemotherapy provides a survival advantage for patients with unresectable biliary cancer (Valle *et al*, 2010; Okusaka *et al*, 2010), gemcitabine has also been recognized as a key

anticancer drug in adjuvant chemotherapy for resectable cholangiocarcinoma. Indeed, our previous studies have revealed that use of AGC was independently associated with prolonged survival (Murakami *et al*, 2009, 2011, 2012). Based on these findings, we believe that AGC can provide a survival benefit for patients with resectable cholangiocarcinoma and identification of biomarkers that could predict the clinical outcome of AGC may contribute to further optimization of adjuvant chemotherapy for cholangiocarcinoma. The current study and our previous study (Kobayashi *et al*, 2012) has revealed the predictive significance of hENT1 in advanced cholangiocarcinoma patients who received AGC in the adjuvant setting. These results suggested that hENT1 expression could be used as a predictive marker for the efficacy of AGC. In contrast to hENT1, however, reports investigating the predictive and prognostic values of intratumoral RRM1 expression with immunochemical staining in biliary cancer are extremely rare. To the best of our knowledge, this is the first clinical report concurrently investigating hENT1 and RRM1 expression in cholangiocarcinoma patients treated with AGC.

In this study, 68% and 53% of patients with cholangiocarcinoma had high intratumoral hENT1 and RRM1 expression, respectively. One analysis by Fisher *et al* (2013) of data from 63 patients who underwent surgical resection for biliary malignancies found that 81% of patients exhibited high RRM1 expression, which was slightly higher than that observed in the current study. Moreover, the current study revealed significant correlations between RRM1 expression and some clinicopathological factors, though no previous reports including a sufficient number of patients have previously demonstrated these correlations. Therefore, further, larger-scale studies on RRM1 expression in cholangiocarcinoma are needed.

RRM1 is the large subunit of human ribonucleotide reductase. In cellular replication, ribonucleotide reductase catalyzes the production of deoxynucleotide triphosphates, which are necessary for DNA synthesis. Gemcitabine is currently the most potent and most widely used ribonucleotide reductase inhibitor, and some clinical studies on gastrointestinal and other cancers

treated with gemcitabine have demonstrated the significant correlation between increased RRM1 expression and gemcitabine resistance (Jordheim *et al*, 2011; Gong *et al*, 2012; Akita *et al*, 2009; Ohtaka *et al*, 2008; Nakagawa *et al*, 2013). However, only a few studies on cholangiocarcinoma, which generally included only a small number of patients, have revealed the predictive significance of RRM1 expression in the palliative setting. The analysis by Ohtaka *et al* (2008) of data from 12 patients with recurrent biliary carcinoma treated with gemcitabine alone found a trend towards a better response rate in patients with low RRM1 expression compared to those with high RRM1 expression. The analysis by Nakamura *et al* (2010) of data from 10 patients with advanced biliary carcinoma demonstrated significant associations of low RRM1 expression with gemcitabine sensitivity and improved OS. In contrast, no previous study in the adjuvant setting has evaluated the impact of RRM1 expression on the efficacy and/or prognosis of cholangiocarcinoma patients treated with gemcitabine. The current study revealed that high RRM1 expression was independently associated with poor DFS and OS in patients treated with AGC, but not in those who did not receive AGC. These results suggested that RRM1 expression could be a relevant predictive marker of survival in cholangiocarcinoma patients treated with AGC. On the other hand, some basic studies have demonstrated that increased expression of RRM1 decreases the formation of metastasis and inhibits the development of carcinogen-induced lung tumors (Fan *et al*, 1997; Gautam *et al*, 2003, 2006). Indeed, a significant correlation between high RRM1 expression and improved outcomes has been reported in a few studies of lung cancer in patients who underwent surgery alone (Bepler *et al*, 2004; Zheng *et al*, 2007). However, no significant difference was found in the current study between RRM1 expression and survival of cholangiocarcinoma patients who did not receive AGC. The possible causes of this discrepancy are differences in cancer type and/or the small number of patients in this study. In addition, patients who did not receive AGC were selected without randomization and their postoperative courses were slightly different (a few patients received adjuvant oral UFT chemotherapy). Therefore, further basic and clinical studies on role of RRM1 in the growth and

proliferation of cholangiocarcinoma cells are needed.

Since both hENT1 and RRM1 were associated with survival in patients treated with AGC, the combined hENT1 and RRM1 classification was introduced in this study to reinforce the predictive values of these targets. The current results demonstrated that patients with high hENT1 and low RRM1 experienced longer DFS and OS compared with the other 3 groups. Additionally, these patients had dramatically reduced HRs compared to analyses of separated hENT1 and RRM1 expression. Based on these findings, the combined hENT1 and RRM1 classification enabled us to increase the predictive value of these targets for prognosis in cholangiocarcinoma patients treated with AGC compared with either factor alone and may contribute to the optimization of adjuvant chemotherapy for resected cholangiocarcinoma.

This study has some inherent limitations due to the small number of patients analyzed and the study's retrospective nature. First, patients who did not receive AGC (the control group in the current study) were selected without randomization. Second, oral fluoropyrimidines were administered to some of patients treated with and without gemcitabine in the current study. However, we believe this supplement had no effect on the results of this study because prior studies have revealed that hENT1 and RRM1 work as a predictive marker of gemcitabine but not fluoropyrimidine (Farrell et al, 2009; Fujita et al, 2010; Greenhalf et al, 2014). Third, some other biomarkers, including deoxycytidine kinase, 5'-nucleotidase, cytidine deaminase, and ribonucleotide reductases subunit 2, have been reported to be associated with response to gemcitabine in pancreatic cancer (Giovannetti *et al*, 2006; Fujita *et al*, 2010; Maréchal *et al*, 2012). The role of these candidates as potential predictive markers in cholangiocarcinoma is still unclear. Further prospective, large-scale, randomized studies are needed to overcome these limitations.

In conclusion, both intratumoral hENT1 and RRM1 expression levels were closely associated with the survival of patients with advanced cholangiocarcinoma treated with AGC after surgical resection. Additionally, combined analysis of hENT1 and RRM1 expression was more useful for

prediction of AGC efficacy than either factor alone. These findings warrant further investigations to establish appropriate postoperative treatments for resectable cholangiocarcinoma, which can be optimized based on hENT1 and RRM1 expression levels.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

Figure 1. Immunohistochemical analysis of human equilibrative nucleoside transporter 1 (hENT1) and ribonucleotide reductase subunit 1 (RRM1) expression in cholangiocarcinoma. These photomicrographs reveal (A) high hENT1 expression, (B) low hENT1 expression, (C) high RRM1 expression, (D) low RRM1 expression (original magnification, 200×; bar = 50 μm). Positive internal controls is established by staining of lymphocytes and stromal cells (arrows).

Figure 2. Disease-free survival (DFS) and overall survival (OS) curves stratified by intratumoral hENT1 expression. AGC(+) indicates subgroups of patients who received adjuvant gemcitabine-based chemotherapy; AGC(-) indicates subgroups of patients who did not receive adjuvant gemcitabine-based chemotherapy. (A) DFS curves in AGC(+) patients ($P = 0.005$). (B) DFS curves in AGC(-) patients ($P = 0.796$). (C) OS curves in AGC(+) patients ($P = 0.036$). (D) OS curves in AGC(-) patients ($P = 0.913$).

Figure 3. DFS and OS curves stratified by intratumoral RRM1 expression. AGC(+) indicates subgroups of patients who received adjuvant gemcitabine-based chemotherapy; AGC(-) indicates subgroups of patients who did not receive adjuvant gemcitabine-based chemotherapy. (A) DFS curves in AGC(+) patients ($P = 0.015$). (B) DFS curves in AGC(-) patients ($P = 0.642$). (C) OS curves in AGC(+) patients ($P = 0.035$). (D) OS curves in AGC(-) patients ($P = 0.883$).

Figure 4. DFS and OS curves stratified by combined analysis of intratumoral hENT1 and RRM1 expression. AGC(+) indicates subgroups of patients who received adjuvant gemcitabine-based chemotherapy; AGC(-) indicates subgroups of patients who did not receive adjuvant gemcitabine-based chemotherapy. (A) DFS curves in AGC(+) patients ($P = 0.003$). (B) DFS curves in AGC(-) patients ($P = 0.778$). (C) OS curves in AGC(+) patients ($P = 0.015$). (D) OS curves in AGC(-) patients ($P = 0.994$).

Figure.1

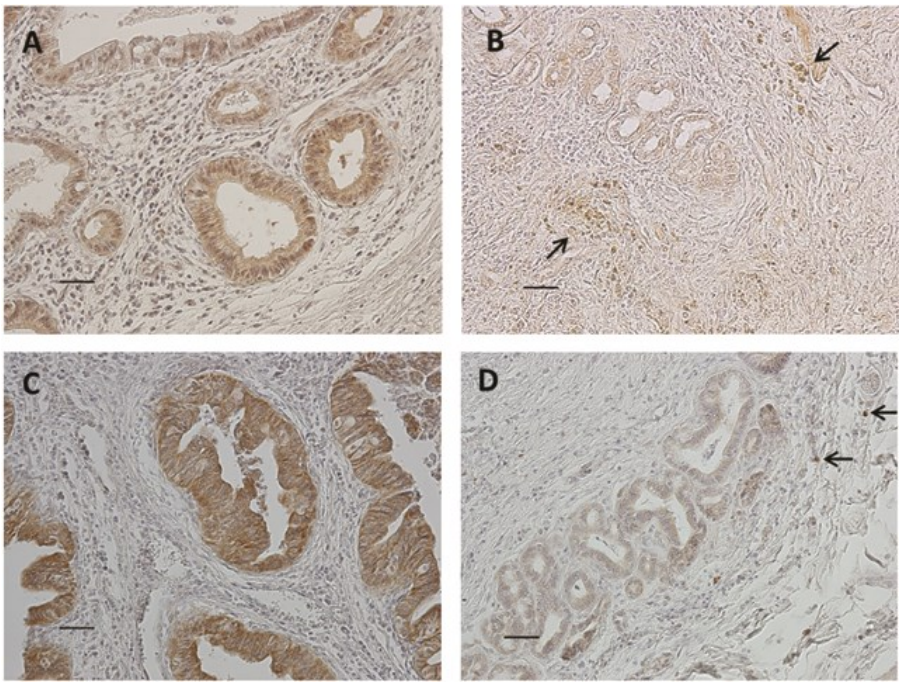
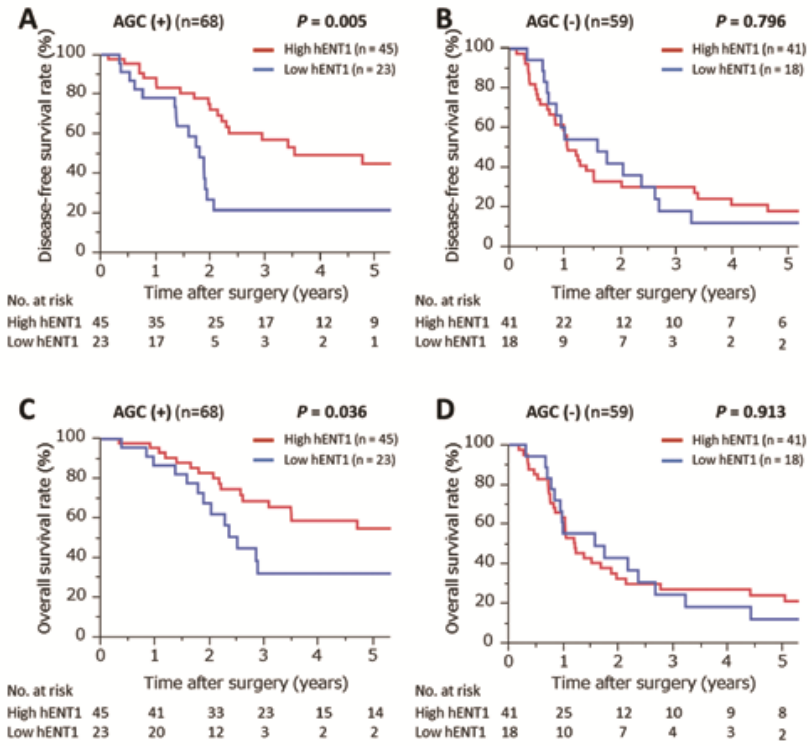


Figure.2



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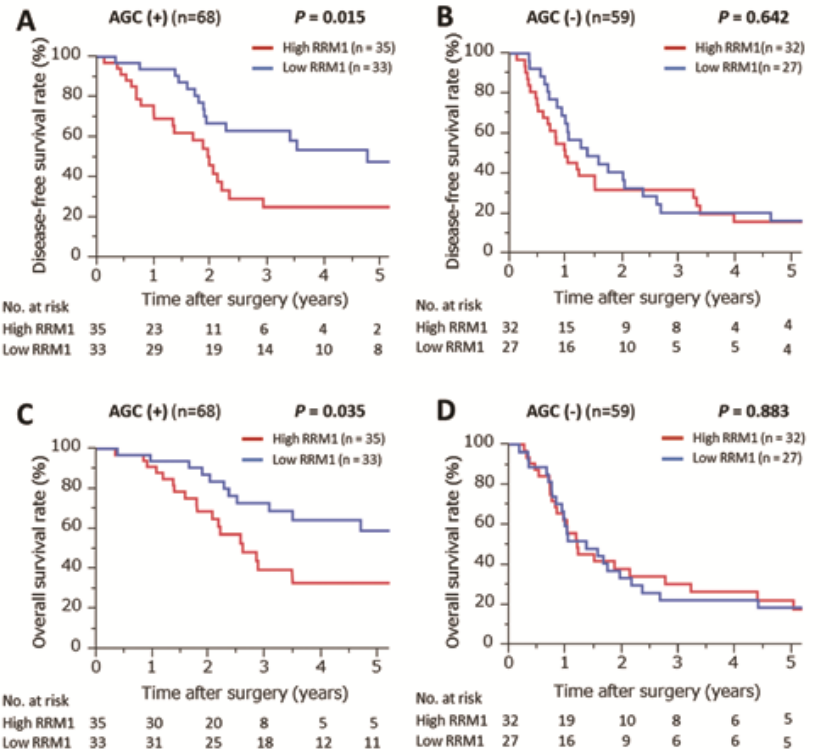

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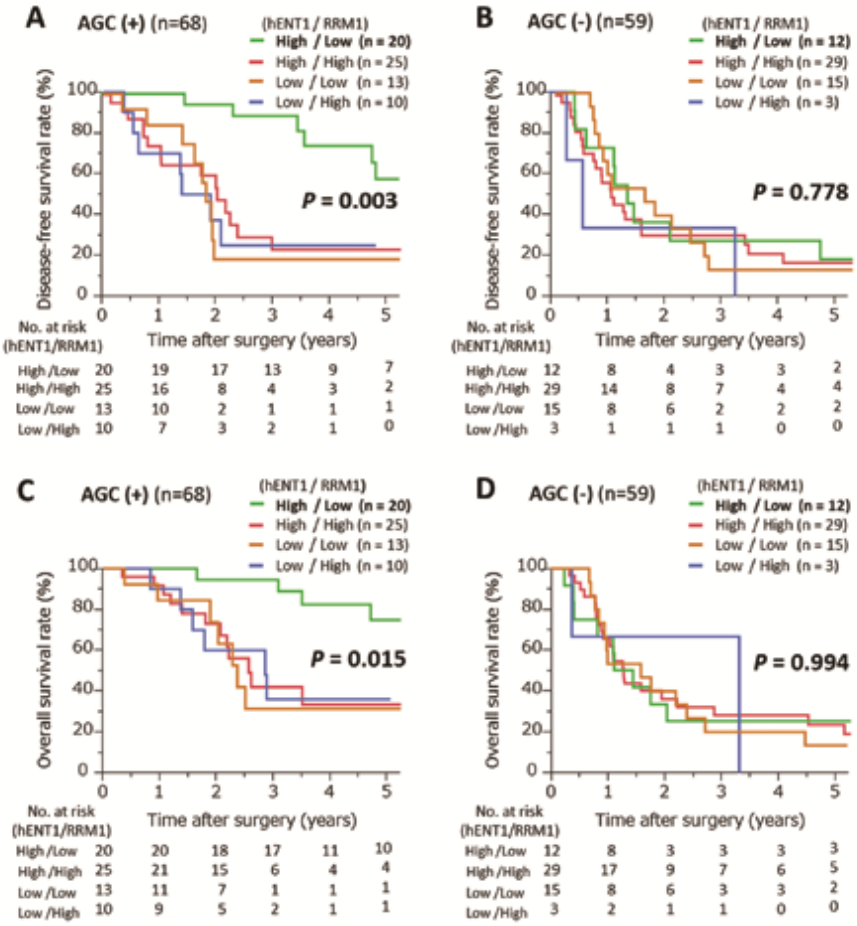

Table 1. Comparison of clinicopathological factors based on intratumoral hENT1 and RRM1 expression for all patients (*n* = 127)

	Total No. of Patients (%)	No. of Patients (%)			No. of Patients (%)		
		High hENT1	Low hENT1		High RRM1	Low RRM1	
		***n* = 86 (68)**	***n* = 41 (32)**	***P*-value**	***n* = 67 (53)**	***n* = 60 (47)**	***P*-value**
Ages(yrs)							
< 70	66 (52)	48 (56)	18 (44)	0.209	38 (57)	28 (47)	0.258
≥ 70	61 (48)	38 (44)	23 (56)		29 (43)	32 (53)	
Gender							
Male	85 (67)	56 (65)	29 (71)	0.529	43 (64)	42 (70)	0.486
Female	42 (33)	30 (35)	12 (29)		24 (46)	18 (30)	
Tumor location							
Intrahepatic	20 (16)	13 (15)	7 (17)	0.251	14 (21)	6 (10)	0.02
Perihilar	60 (47)	37 (43)	23 (56)		24 (36)	36 (60)	
Distal	47 (37)	36 (42)	11 (27)		29 (43)	18 (30)	
AGC							
Yes	68 (54)	45 (52)	23 (56)	0.69	35 (52)	33 (55)	0.755
No	59 (46)	41 (48)	18 (44)		32 (48)	27 (45)	
Residual tumor							
R0	95 (75)	64 (74)	31 (76)	0.885	54 (81)	41 (68)	0.112
R1	32 (25)	22 (26)	10 (24)		13 (19)	19 (32)	
Pathological differentiation							
Well	55 (43)	45 (52)	10 (24)	0.009	33 (49)	22 (37)	0.017
Moderately	49 (39)	29 (34)	20 (49)		28 (42)	21 (35)	
Poorly	23 (18)	12 (14)	11 (27)		6 (9)	17 (28)	
Lymph node metastasis							
Present	65 (51)	47 (55)	18 (44)	0.257	36 (54)	29 (48)	0.544
Absent	62 (49)	39 (45)	23 (56)		31 (46)	31 (52)	
UICC pT factor							
T1	3 (2)	2 (2)	1 (2)	0.345	3 (4)	0 (0)	0.126
T2,2a,2b	62 (49)	38 (44)	24 (59)		32 (48)	30 (50)	
T3	59 (46)	43 (50)	16 (39)		29 (43)	30 (50)	
T4	3 (2)	3 (4)	0 (0)		3 (4)	0 (0)	
UICC stage							
II, IIA, IIB	73 (57)	49 (57)	24(58)	0.544	40 (60)	33 (55)	0.009
III, IIIA, IIIB	29 (23)	18 (21)	11(27)		9 (13)	20 (33)	
IV, IVA, IVB	25 (20)	19 (22)	6(15)		18 (27)	7 (12)	

Abbreviations: hENT1=human equilibrative nucleoside transporter 1; RRM1=ribonucleotide reductase subunit 1; AGC=adjuvant gemcitabine-based chemotherapy.

Table 2. Univariate DFS and OS analysis of prognostic factors in patients with cholangiocarcinoma who received AGC (*n* = 68) and those who did not (*n* = 59)

	AGC(+) (*n* = 68)					**AGC(-) (*n* = 59)**				
		DFS		**OS**			**DFS**		**OS**	
	N	**5-y Survival (%)**	***P*-value**	**5-y Survival (%)**	***P*-value**	***N***	**5-y Survival (%)**	***P*-value**	**5-y Survival (%)**	***P*-value**
Age (yrs)										
< 70	35	38	0.941	61	0.225	31	18	0.834	26	0.472
≥ 70	33	36		30		28	14		15	
Gender										
Male	42	40	0.518	53	0.566	43	17	0.483	20	0.56
Female	26	19		35		16	13		21	
Tumor location										
Intrahepatic	12	20	0.129	56	0.479	8	25	0.56	13	0.841
Perihilar	36	43		49		24	17		27	
Distal	20	33		39		27	13		16	
Residual tumor										
R0	53	42	0.077	50	0.11	42	23	**< 0.001**	29	**<0.001**
R1	15	16		36		17	0		0	
Pathological differentiation										
Well	28	54	**0.003**	62	**0.011**	27	27	0.187	34	**0.049**
Moderate, poor	40	20		32		32	4		7	
Lymph node metastasis										
Present	31	34	0.057	31	**0.009**	34	9	**0.037**	10	**0.007**
Absent	37	40		60		25	26		35	
UICC pT factor										
T1,2,2a,2b	34	43	0.216	56	0.126	31	21	0.092	27	0.167
T3,4	34	28		35		28	10		12	
UICC stage										
II, A, B	35	43	**0.042**	66	**0.012**	38	21	0.22	27	**0.017**
III,A,B,IV,A,B	33	30		25		21	8		10	
hENT1 expression										
High	45	45	**0.005**	55	**0.036**	41	18	0.796	24	0.913
Low	23	21		32		18	12		12	
RRM1 expression										
High	35	25	**0.015**	33	**0.035**	32	16	0.642	22	0.883
Low	33	47		59		27	16		19	
Combined hENT1 and RRM1 Classification										
High hENT1 / Low RRM1	20	58	**0.003**	75	**0.015**	12	18	0.778	25	0.994
High hENT1 / HighRRM1	25	24		34		29	18		23	
Low hENT1 / Low RRM1	13	19		32		15	13		13	
Low hENT1 / High RRM1	10	25		36		3	0		0	
High hENT1 / Low RRM1	20	58	**< 0.001**	75	**0.001**					
The other 3 expression combimatstions	48	22		32						

Abbreviations: DFS=disease-free survival; OS=overall survival; AGC=adjuvant gemcitabine-based chemotherapy; hENT1=human equilibrative nucleoside transporter 1; RRM1=ribonucleotide reductase subunit 1; The other 3 expression combinations=high hENT1 / high RRM1 expression or low hENT1 / low RRM1 expression or low hENT1 / high RRM1 expression.

Table 3. Multivariate DFS and OS analysis of prognostic factors in patients who received AGC (*n* = 68)

	DFS			**OS**		
	HR	**95%CI**	***P*-value**	**HR**	**95%CI**	***P*-value**
Model 1 – multivariate analysis including separated hENT1 and RRM1 expression						
Pathological differentiation						
Well	**0.37**	**0.17–0.76**	**0.007**	**0.45**	**0.19–0.98**	**0.045**
Moderate, poor	1			1		
Lymph node metastasis						
Present				1	**0.18–0.81**	**0.011**
Absent				**0.39**		
hENT1 expression						
High	**0.49**	**0.24–0.98**	**0.044**	0.55	0.26–1.21	0.135
Low	1			1		
RRM1 expression						
High	1	**0.21–080**	**0.009**	1	**0.20–0.89**	**0.024**
Low	**0.41**			**0.43**		
Model 2 – multivariate analysis including combined hENT1 and RRM1 classification						
Pathological differentiation						
Well	**0.38**	**0.17–0.77**	**0.007**	0.49	0.21–1.05	0.066
Moderate, poor	1			1		
Lymph node metastasis						
Present				1	**0.18–0.81**	**0.012**
Absent				**0.39**		
Combined hENT1 and RRM1 classification						
High hENT1 / Low RRM1	**0.22**	**0.08–0.51**	**<0.001**	**0.22**	**0.07–0.60**	**0.001**
The other 3 expression combinations	1			1		

Abbreviations: DFS=disease-free survival; OS=overall survival; AGC=adjuvant gemcitabine-based chemotherapy; hENT1=human equilibrative nucleoside transporter 1; RRM1=ribonucleotide reductase subunit 1; HR=hazard ratio; CI=confidence interval; The other 3 groups=high hENT1 / high RRM1 expression or low hENT1 / low RRM1 expression or low hENT1 / high RRM1 expression.