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Expression of Cyclooxygenase-2 and Embryonic Lethal Abnormal Vision-Like Protein HuR in Gallbladder Carcinoma

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Background : Cyclooxygenase-2 (COX-2) is an enzyme that promotes proliferation of tumor cells. HuR is a member of the family of embryonic lethal abnormal vision-like proteins. Recent studies show that cytoplasmic HuR stabilizes the mRNA of COX-2 and regulates the expression of COX-2. Moreover, cytoplasmic HuR expression is associated with a poorer prognosis for patients with some cancers. The aim of this study was to investigate the expression patterns of and the relationship between COX-2 and HuR in gallbladder carcinoma. **Methods :** We analyzed COX-2 and HuR expression by immunohistochemical staining of 108 gallbladder carcinomas. **Results :** COX-2 expression and nuclear and cytoplasmic HuR expression were seen in, respectively, 61 (56.5%), 77 (71.3%), and 4 (3.7%) cases. COX-2 and nuclear HuR were simultaneously expressed in 44 of the 108 samples without any quantitative association between the levels of each. COX-2 expression correlated with tumor stage, differentiation (based on histology), lymph node metastasis, perineural invasion, and survival. Nuclear and cytological expression of HuR did not correlate with any clinical parameters. **Conclusions :** COX-2 expression but not HuR may play an important role in the prognosis of patients with gallbladder carcinoma.

Key Words : Gallbladder neoplasms; Cyclooxygenase 2; ELAV-like protein 1

Cyclooxygenase-2 (COX-2) is a well-characterized enzyme that controls the activity of many inflammatory mediators, including prostaglandins.[1,2] Increased expression of COX-2 is associated with promotion of tumor growth and metastasis, which leads to poorer prognoses for patients with various malignancies, including gastric cancer, colon cancer, breast cancer and chondrosarcoma.[3-10]

HuR is a member of the family of embryonic lethal abnormal vision (ELAV)-like proteins, a family that contains four members; (Hel-N1/HuB, HuC, HuD, and HuR). ELAV proteins are involved in the post-transcriptional regulation of mRNA turnover and mRNA stability,[11] which are important processes for the regulation of eukaryotic gene expression.[12,13] One of the best characterized cis-acting elements in mRNA turnover is the adenylate/uridylate-rich element (ARE) located in the 3′ untranslated region of many unstable transcripts, such as those for certain proto-oncogenes and cytokines.[13] There are several known proteins that can bind to AREs and control mRNA stability.[14] HuR is an ARE-binding factor related to a *Drosophila* ELAV protein, and HuR interacts with several types of mRNAs.[15-19] HuR is ubiquitously expressed, in contrast to other family members, which are mainly expressed in terminally differentiated neuronal tissue.[19] HuR is predominantly localized in cell nuclei, but can shuttle between nucleus and cytoplasm, and shows different activities in each location.[13] Some studies have shown that the cytoplasmic localization of HuR is important for its mRNA-stabilizing function.[20] COX-2 mRNA contains an ARE in the 3′ untranslated region that provides a binding site for HuR, which increases mRNA half life.[21] A correlation between COX-2 expression and cytoplasmic HuR expression was recently discovered in several types of malignant tumors, including breast, colon and ovarian cancers.[21-24]

Gallbladder carcinoma is a type of malignant tumor that occurs in the digestive tract. Although improvements in the treatment of gallbladder cancer have occurred, the prognosis for gallbladder carcinoma is poor and the five-year survival rate is lower than

that of the other common digestive tract tumors such as colon cancer or gastric carcinoma.

No immunohistochemical studies of COX-2 and HuR expression in gallbladder carcinoma have been reported. To evaluate possible associations between COX-2 and HuR expression in gallbladder carcinoma, we investigated the immunohistochemical expression and cellular distribution of HuR and COX-2 expression and compared the patterns of HuR expression in gallbladder carcinoma with that of other tumors.

MATERIALS AND METHODS

Patients and samples

We collected 108 gallbladder carcinomas from 1982 to 2008. Of these, 106 were from Kyung Hee University Hospital and two were from Kyung Hee East-West Neo Medical Center. The gallbladder carcinoma cases used in this study were all obtained from cholecystectomy specimens. Forty-eight patients died during the follow up period. We reviewed all the hematoxylin-eosin stained slides and representative slides were used to evaluate the immunohistochemical staining of each case. Research protocols for the use of human tissue were approved by and conducted in accordance with the policies of the Institutional Review Board at Kyung Hee Medical Hospital.

Immunohistochemistry

For immunohistochemical staining, all paraffin-embedded samples were sliced into sections of 4 μm thickness. For HuR immunohistochemical staining, we used a mouse anti-HuR antibody (1 : 300, Zymed, San Francisco, CA, USA) and a bond polymer intensity detection kit (DS9588, Vision Biosystem, Mount Waverley, VIC, Australia). For COX-2 immunohistochemical staining, we used a COX-2 monoclonal antibody (1 : 400, Dako, Carpenteria, CA, USA) and a polymer kit (Novocastra, Mount Waverley, VIC, Australia). We used a bond-max immunohistochemistry auto-stainer (Vision Biosystem) for the HuR and COX-2 immunohistochemical staining. As a positive

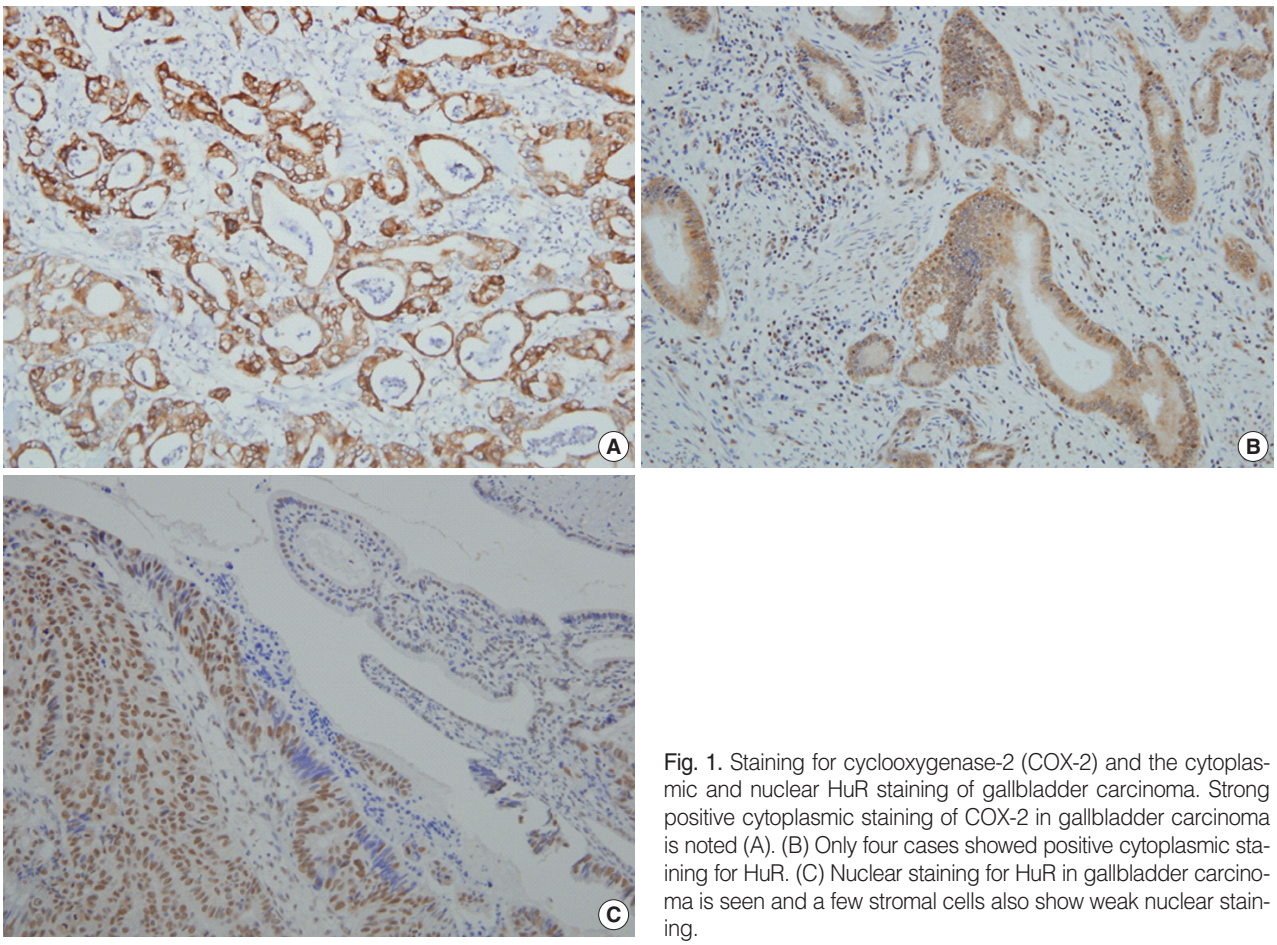

Fig. 1. Staining for cyclooxygenase-2 (COX-2) and the cytoplasmic and nuclear HuR staining of gallbladder carcinoma. Strong positive cytoplasmic staining of COX-2 in gallbladder carcinoma is noted (A). (B) Only four cases showed positive cytoplasmic staining for HuR. (C) Nuclear staining for HuR in gallbladder carcinoma is seen and a few stromal cells also show weak nuclear staining.

control, we evaluated a human colon carcinoma that's known to overexpress COX-2, and in each section, we used lymphocytes that showed HuR nuclear staining as internal positive controls. We evaluated the percentage of staining-positive cells and the intensity of staining. The percentage of positive cells was scored as follows: 0 (0% positive cells), 1 (< 10% positive cells), 2 (10-50% positive cells), and 3 (50-80% positive cells), and 4 (≥ 80% positive cells). The intensity of staining was scored as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). For the immunoreactivity score, we established a 0 to 12 point system by multiplying the percentage of positive cells by the intensity of the staining score. We classified as negative cases manifesting a immunoreactive scoresoof 0 to 5, and as positive expression cases manifesting scores of 6 to 12.

We used the SPSS ver. 15.0 (SPSS Inc., Chicago, IL, USA) for statistical evaluation. The relationships between variables were determined and a p-value < 0.05 was considered statistically significant.

RESULTS

The study cohort contained 52 males and 54 females with a mean age of 61 years (range, 27 to 80 years), clinical information was not available for two cases. Of the 108 cases, COX-2 expression was seen in 61 (56.5%), and nuclear HuR expression in 77 (71.3%) (Fig. 1). Some normal gallbladder mucosa showed weak COX-2 staining, but strong positive staining was not found. We independently investigated nuclear and cytoplasmic expression of HuR. Cytoplasmic HuR expression was observed in only four cases (Fig. 1). There was a significant correlation between COX-2 expression and tumor differentiation (p = 0.000), T stage (p = 0.001), lymph node metastasis (p = 0.010), tumor, node and metastasis (TNM) stage (p = 0.000) and perineural invasion (p = 0.022) (Table 1). Age and gender did not correlate with COX-2 expression. Nuclear and cytoplasmic HuR expression did not correlate with any clinicopathological parameters, including age, gender, differentiation, T stage, lymph node metastasis, TNM stage and perineural inva-

Table 1. Statistical analysis of the clinical parameters and the COX-2 expression

	COX-2 expression		
	Negative	Positive	p-value
Gender			
Male	23	29	0.716
Female	22	32	
Age			
≤ 61	22	25	0.413
> 61	23	36	
Differentiation			
Well	32	19	0.000[a]
Moderate	11	33	
Poor	3	9	
T stage			
1-2	37	30	0.001[a]
3-4	9	31	
Lymph node metastasis			
Absent	18	13	0.010[b]
Present	4	16	
TNM stage			
1	34	25	0.000[a]
2	12	32	
3	0	4	
Perineural invasion			
Absent	45	51	0.022[b]
Present	1	10	

Two cases did not have clinical information such as age or gender.
Pearson chi-square test.
[a]Linear-by-linear test; [b]Fisher's exact test.
COX-2, cyclooxygenase-2; TNM, tumor, node and metastasis.

Table 2. Statistical analysis of the clinical parameters with the nuclear and cytoplasmic expressions of HuR

	Nucelar HuR expression			Cytoplasmic HuR expression		
	Negative	Positive	p-value	Negative	Positive	p-value
Gender						
Male	12	40	0.241	51	1	0.618[b]
Female	18	36		51	3	
Age						
≤ 61	17	30	0.108	46	1	0.628[b]
> 61	13	46		56	3	
Differentiation						
Well	15	36	0.826[a]	49	2	0.731[a]
Moderate	13	31		43	1	
Poor	3	9		11	1	
T stage						
1-2	19	48	0.857[a]	65	2	0.597[a]
3-4	12	28		38	2	
Lymph node metastasis						
Absent	7	24	0.068[b]	31	0	0.149[b]
Present	10	10		18	2	
TNM stage						
1	14	45	0.275[a]	57	2	0.067[a]
2	16	28		44	0	
3	1	3		2	2	
Perineural invasion						
Absent	27	69	0.726[b]	92	4	1.000[b]
Present	4	7		11	0	

Pearson chi-square test.
[a]Linear-by-linear test; [b]Fisher's exact test.
TNM, tumor, node and metastasis.

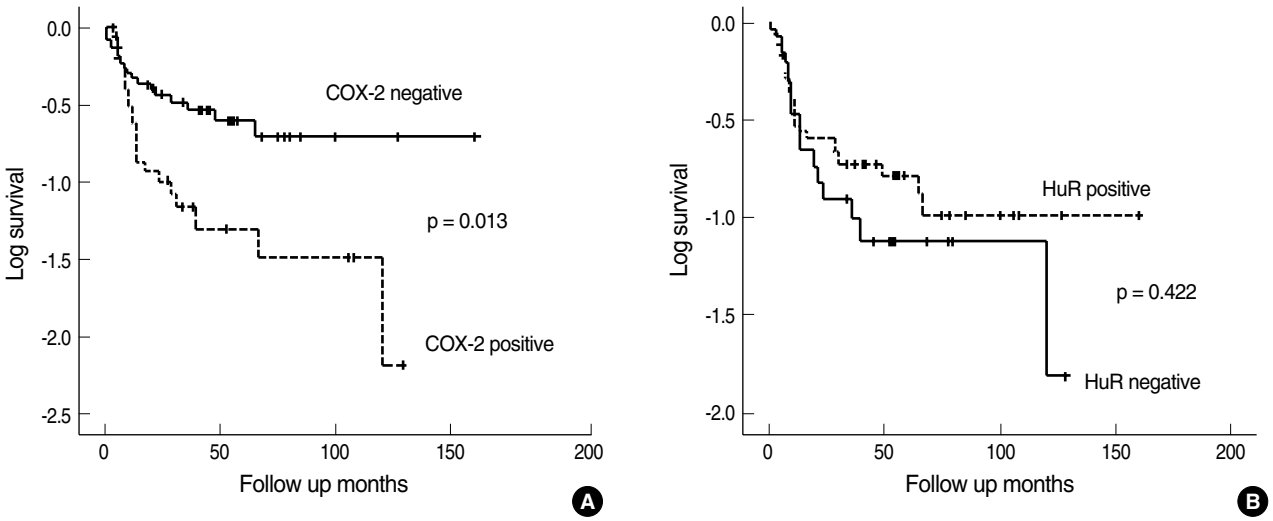

Fig. 2. Kaplan-Meier test for the cyclooxygenase-2 (COX-2) and nuclear HuR expressions. (A) The expression of COX-2 shows statistical correlation with survival (p = 0.013). (B) However, positive HuR staining shows no statistical correlation with survival (p = 0.422).

Table 3. Statistical analysis of the COX-2 expression and the nuclear and cytoplasmic expressions of HuR

	Nuclear HuR expression			Cytoplasmic HuR expression		
	Negative	Positive	p-value	Negative	Positive	p-value
COX-2 expression						
Negative	14	33	0.828	46	1	0.631[a]
Positive	17	44		58	3	

[a]Fisher's exact test.
COX-2, cyclooxygenase-2.

sion (Table 2). Co-expression of COX-2 and nuclear HuR was observed in 44 cases, and co-expressions of COX-2 and cytoplasmic HuR was observed in three cases. However, no significant correlation occurred between positive COX-2 expression and positive nuclear or cytoplasmic HuR expression (p = 0.828 and p = 0.631, respectively) (Table 3). COX-2 expression showed a statistically significant association with survival (p = 0.013, Kaplan-Meier test), but not with a nuclear or cytoplasmic HuR expression (p = 0.422 and p = 0.250, respectively, Kaplan-Meier test) (Fig. 2). In addition, COX-2 positive expression emerged as an adverse prognostic factor by Cox regression test (harzard ratio, 2.894; 95% confidence interval, 1.085 to 7.718; p = 0.034) (Table 4).

Table 4. Cox multivariate analysis for overall survival

Prognostic factor	Overall survival		
	HR	95% CI	p-value
Gender			
Male vs Female	1.572	0.544-4.540	0.403
Age	0.561	0.220-1.430	0.226
Differentiation			
Moderate vs Well	1.721	0.436-6.785	0.438
Poor vs Moderate	3.949	0.589-26.502	0.157
T stage			
3-4 vs 1-2	7.096	2.609-19.304	0.000
Lymph node metastasis			
Present vs Absent	0.856	0.232-3.167	0.816
TNM stage			
2 vs 1	4.369	0.974-19.594	0.054
3 vs 2	2.545	0.814-193.243	0.070
Perineural invasion			
Present vs Absent	1.031	0.264-4.026	0.965
COX-2			
Positive vs Negative	2.894	1.085-7.718	0.034
HuR nuclear staining			
Positive vs Negative	1.645	0.498-5.440	0.414
HuR cytoplasmic staining			
Positive vs Negative	0.280	0.036-2.179	0.224

HR, harzard ratio; CI, confidende interval; TNM, tumor, node and metastasis; COX-2, cyclooxygenase-2.

DISCUSSION

Increased expression of COX-2 is thought to be associated with poor outcomes for patients with several human malignancies, including gastric, colon, and breast carcinoma and chondrosarcoma.[3-10] In gallbladder carcinoma, COX-2 overexpression and increased COX-2 mRNA are regarded as adverse prognostic factors that increase angiogenesis, and mitogenesis and decrease apoptosis.[25-29] A correlation between COX-2 and HuR expression in several other malignant tumors has recently been

suggested, and cytoplasmic HuR expression has been proposed as an adverse prognostic factor.[21-24] In this study, we did not observe any correlation between COX-2 and HuR expression, regardless of the location of the HuR. Moreover, cytoplasmic HuR expression was observed in only four cases of gallbladder carcinoma. These finding suggest that shuttling of HuR from nuclear to cytoplasmic site is not easy in gallbladder carcinoma. However, there were no significant correlations between nuclear HuR expression and any clinicopathological parameters such as age, the tumor stage or survival.

Zhi *et al.*[25] reported that COX-2 expression, as measured by immunohistochemical staining, is associated with clinical stage ($p = 0.01$) and lymph node metastasis ($p = 0.01$) in gallbladder carcinomas. In this study, the COX-2 expression rate was consistent with the findings of previous studies, and COX-2 expression showed a significance correlation with clinicopathological parameters that influence prognosis directly or indirectly. These observations are in agreement with and support previous studies that showed that COX-2 expression may play a role in the poor prognosis of patients with gallbladder cancer. However, our results are not consistent with those of the studies on colon, ovary or breast cancer that showed that cytoplasmic HuR expression is associated with a COX-2 expression.[21-24] We have previously reported a correlation between the COX-2 and cytoplasmic HuR expression in Korean breast cancer patients,[30] and several studies have suggested that acytoplasmic HuR expression is associated with mRNA stabilization, but we did not observe any association of these two variables in gallbladder carcinoma.[13-18] HuR is known to be predominantly localize to the nucleus, but it shuttles between nucleus and cytoplasm, and the cytoplasmic location affects mRNA stabilization. Because our nuclear expression rate of HuR was similar to rates of previous studies, but our cytoplasmic HuR expression rate was much lower, we propose that the shuttling ability of HuR in gallbladder carcinoma is too weak to influence COX-2 mRNA stability. If our hypothesis is correct, further studies on gallbladder carcinoma may help to determine the HuR shuttling mechanism. To the best of our knowledge, this is the first study that has investigated the relationship between HuR and COX-2 expression in gallbladder carcinoma. Although the results were unexpected, we observed that a COX-2 expression is associated with a poor prognosis for gallbladder cancer patients. In conclusion, our study does not support the hypothesis that cytoplasmic HuR expression is important for COX-2 expression. However, COX-2 expression is associated with a poor prognosis for patients with gallbladder carcinoma. Further studies will be necessary to obtain more information on the shuttling of nuclear HuR and the influence of COX-2 in gall bladder carcinoma.

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