World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i12.1912

World J Gastroenterol 2013 March 28; 19(12): 1912-1918
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Expression and clinical significance of CD73 and hypoxia-inducible factor-1α in gastric carcinoma

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Supported by National Natural Science Foundation of China, No. 81071806
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Received: July 15, 2012 Revised: August 15, 2012
Accepted: September 28, 2012
Published online: March 28, 2013

Abstract

AIM: To investigate the expression of CD73 and hypoxia-inducible factor-1α (HIF-1α) in human gastric carcinoma, and explore their clinical significance and prognostic value.

METHODS: CD73 and HIF-1α expressions were detected by immunohistochemistry in consecutive sections of tissue samples from 68 gastric carcinoma patients. The peritumor tissues 2 cm away from the tumor were obtained and served as controls. The presence of CD73 and HIF-1α was analyzed by immunohistochemistry using the Envision technique.

RESULTS: CD73 and HIF-1α expressions in gastric carcinoma were significantly higher than those in gastric mucosal tissues as control ($P < 0.001$) and showed a close correlation (Spearman $r = 0.390$, $P = 0.001$). Overexpression of CD73 was positively correlated with differentiation of tumor ($P = 0.000$), histopathology ($P = 0.041$), depth of invasion ($P < 0.001$), nodal status ($P = 0.003$), metastasis ($P = 0.013$), and the American Joint Committee on Cancer (AJCC) stage ($P < 0.001$). High expression of HIF-1α was positively correlated with tumor diameter ($P = 0.031$), depth of invasion ($P = 0.022$), and AJCC stage ($P = 0.035$). The overall survival rate was low in the patients with high expression of CD73 ($P < 0.001$). Moreover, CD73+/HIF-1α+ patients had the worst prognosis ($P < 0.001$). CD73 expression was proven to be an independent predictor for patients with gastric carcinoma by both multivariate Cox regression analysis ($P = 0.021$) and receiver operating characteristic curves ($P = 0.001$).

CONCLUSION: CD73 expression correlates closely with HIF-1α expression in gastric carcinoma. CD73 could be an independent prognostic indicator for gastric carcinoma.

Key words: CD73; Hypoxia-inducible factor-1α; Gastric carcinoma; Immunohistochemistry; Prognosis

Lu XX, Chen YT, Feng B, Mao XB, Yu B, Chu XY. Expression and clinical significance of CD73 and hypoxia-inducible factor-1α in gastric carcinoma. *World J Gastroenterol* 2013; 19(12): 1912-1918 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i12/1912.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i12.1912

INTRODUCTION

Gastric carcinoma has been the fourth most common cancer in the world since the latter half of the 20th century[1,2]. In spite of the recent advances in diagnostic techniques

for early detection and the improvement in surgical treatment, gastric carcinoma remains the second leading cause of cancer-related deaths[3]. In China, Japan and Korea, the incidence of gastric carcinoma now has reached up to 80 new cases per 100 000 population annually[4]. Changes observed in expression of tumor specific biomarkers in gastric carcinomas may be helpful to understand the transformation of histological heterogeneity and the underlying molecular mechanisms. Searching for specific biomarkers which determine the biological nature and behavior of gastric carcinoma would be of utmost importance to optimize individualized therapy.

Ecto-5'-nucleotidase/CD73 is a homodimer linked to the plasma membrane through a glycosylphosphatidylinositol lipid anchor, which was found in most tissues[5]. It is a part of extracellular ATP metabolism, which dephosphorylates AMP into adenosine rapidly after CD39 catalyzes ATP, ADP and AMP[6]. Recent studies have demonstrated that CD73 could participate in a variety of physiological responses including ischemic preconditioning, platelet function, hypoxia, vascular leak and tissue injury[7,8]. CD73 was up-regulated in various human cancers, including those of lung, colon, breast, pancreas and ovary[9,10]. Importantly, the high expression of CD73 was correlated with tumor neovascularization, invasiveness, metastasis, as well as shorter patient survival[9-13]. These results suggested that CD73 might play a significant role in controlling tumor progression.

Oxygen is only able to diffuse 100-180 μm from a capillary to cells, which makes hypoxia a common feature of rapidly growing solid tumors[14]. Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric basic helix-loop-helix transcription factor composed of HIF-1α and HIF-1β subunits; and HIF-1α determines HIF-1 activity[15]. It is found that HIF-1α is wildly expressed in various types of carcinomas, such as those of brain, breast, lung and colon[16-18]. These results revealed that HIF-1α was correlated with tumor progression, aggressive behavior, and patient prognosis. As is known, pathophysiologic conditions of hypoxia can cause adenine nucleotide metabolic changes. A recent study found that CD73 is transcriptionally regulated by ambient hypoxia and is one of the mechanisms involved HIF-1[19].

The purpose of this study was to ascertain the correlation between CD73 and HIF-1α expressions and their clinicopathological significance in gastric carcinoma, including patient survival. We hypothesized that CD73 expression would be correlated with clinicopathological factors and HIF-1α expression, and the combination of the two molecules would predict recurrence and overall survival.

MATERIALS AND METHODS

Patients

Samples of gastric carcinoma were collected from the resected stomach of 68 patients who were diagnosed histologically as gastric carcinoma and underwent gastrectomy at the Nanjing General Hospital of Nanjing Military Command. None of the patients had previously received radiotherapy, chemotherapy or other medical interventions before surgery. Among them, 43 (63%) patients were male and 25 (37%) were female, with a mean age of 49.86 years. All patients were followed up from the date of surgery until either the date of death or December 2011. For analysis of patient survival, the patients who were lost to follow-up or those who died from causes other than gastric carcinoma were regarded as censored data.

This study was approved by the Institutional Review Board, and informed consent was obtained from each patient.

Immunohistochemistry

The peritumor tissues 2 cm away from the tumor were collected and served as healthy controls. Tumor specimens and healthy control gastric mucosal tissues, which were fixed in 10% buffered formalin and embedded in paraffin, were cut into 4-μm sections and placed on polylysine-coated slides. The staining was conducted by the avidin-biotin-peroxidase complex method. Each paraffin section was deparaffinized and rehydrated though graded alcohols, followed by antigen retrieval with epitope retrieval solution (10 mmol citrate buffer, pH 6.0) in a pre-heated water bath at 98 ℃ for 10 min. Endogenous peroxidase was blocked using 3% hydrogen peroxide. Subsequently, sections were incubated with the primary mouse monoclonal CD73 antibody (1:100, ab71322 Abcam) and mouse monoclonal HIF-1α antibody (1:100, MAB1935 R and D) overnight at 4 ℃, and then were stained with secondary antibody for 30 min. The sections were finally counterstained with haematoxylin (Zymed Laboratories Inc, San Francisco, CA, United States). Negative control was performed by replacing the primary antibody with a normal murine immunoglobulin G. Known immunostaining-positive sections were used as positive controls.

Evaluation of immunohistochemical analysis

We used semi-quantitative method. Five different perspectives were randomly selected under ordinary optical microscope at a magnification of 400. The percentage of positive cells was scored 0 for staining of < 1%, 1 for staining of 2%-25%, 2 for staining of 26%-50%, 3 for staining of 51%-75%, and 4 for staining > 75% of the cells examined. Staining intensity was calculated, no coloring, slightly yellow, brown yellow and tan stains were marked as 0, 1, 2 and 3. Finally, we calculated the product of staining intensity and positive cell percentage: ≤ 5 was defined as negative and ≥ 6 as positive. Two pathologists blinded to the clinical details reviewed the pathological films and staining points.

Statistical analysis

Categorical data were analyzed using the χ^2 or nonparametric test, while measurement data were evaluated with Student's *t* or one-way analysis of variance test. Correlation coefficient between expression of CD73 and HIF-1α was estimated by the Spearman correlation method.

Table 1 Correlation of CD73 and hypoxia-inducible factor-1α expression with clinicopathological characteristics of gastric carcinoma

Clinicopathological data	CD73 expression High	CD73 expression Low	*P* value	HIF-1α expression High	HIF-1α expression Low	*P* value
Gender			0.144			0.136
Male	18	25		21	22	
Female	13	12		15	10	
Age (yr)			0.157			0.107
< 49.82	10	15		11	14	
⩾ 49.82	21	22		25	18	
Tumor diameter (cm)			0.127			0.031
⩽ 5	7	17		9	15	
5-10	10	5		8	7	
> 10	14	15		19	10	
Differentiation			0.000			0.445
Well	1	4		4	1	
Moderate	6	22		13	15	
Poor	24	11		19	16	
Histopathology			0.041			0.168
Tubular adenocarcinoma	3	16		7	12	
Poorly differentiated adenocarcinoma	21	11		21	11	
Signet-ring cell carcinoma	3	3		2	4	
Mucinous adenocarcinoma	4	7		6	5	
Borrmann type			0.140			0.430
Ⅰ	5	19		9	16	
Ⅱ	9	12		9	12	
Ⅲ	15	13		17	11	
Ⅳ	2	2		1	3	
Depth of invasion			0.000			0.036
T1-T2	1	21		8	14	
T3-T4	30	16		28	18	
Nodal status			0.003			0.113
N0	4	17		9	12	
N1/N2	27	20		27	20	
Metastasis			0.013			0.192
M0	20	33		27	26	
M1	11	4		9	6	
AJCC stage			0.000			0.035
Ⅰ / Ⅱ	2	24		10	16	
Ⅲ / Ⅳ	27	15		26	16	

HIF-1α: Hypoxia-inducible factor-1α; AJCC: American Joint Committee on Cancer.

The Kaplan-Meier method was used to estimate the overall survival and the log-rank test was used to analyze the differences between the curves. Multivariate Cox proportional hazard regression model and receiver operating characteristic (ROC) curve analysis were established to assess the prognostic values of protein expression. All statistical analysis were performed using the SPSS software version 16.0 (SPSS, Chicago, IL, United States) and $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

The demographic and clinicopathological variables of the 68 patients are shown in Table 1. The ages of the patients ranged from 24 to 59 years with the mean age 49.82 years. Based on the American Joint Committee on Cancer (AJCC) classification, there were 26 stage Ⅰ / Ⅱ patients and 42 stage Ⅲ / Ⅳ patients. The patients were followed up for a period of 1-84 mo. Two were lost to follow-up and 37 patients died during the follow-up.

Immunohistochemical analysis of CD73 and HIF-1α expression

CD73 and HIF-1α were detected on consecutive sections and were found to be mainly expressed in the cytoplasm of gastric carcinoma (Figure 1). We repeated the experiment twice to exclude false positive results. Immunohistochemical analysis showed that in gastric carcinoma, 31 (45.6%) of 68 samples were CD73-positive and 36 (52.9%) of 68 were HIF-1α-positive, while in healthy control gastric mucosa, 8 (11.8%) of 68 were CD73-positive and 12 (17.6%) of 68 were HIF-1α-positive. CD73 ($P < 0.001$) and HIF-1α ($P < 0.001$) expression was significantly higher than in healthy controls. CD73 expression was concordant with HIF-1α expression in 69.1% (47 of 68) of gastric carcinoma cases (Spearman $r = 0.390$, $P = 0.001$). CD73 and HIF-1α were found double-positive in 23 cases, double-negative in 24 cases, and either CD73-positive or HIF-1α-positive only in 21 cases. The Spearman's rank correlation method was used to estimate the expression correlation coefficient ($r = 0.390$, $P = 0.001$), indicating a close correlation between CD73 and HIF-1α expression in gastric carcinomas.

Correlation of CD73 and HIF-1α expression with clinicopathological variables

Chi-squared test was used to investigate the correlation of CD73 and HIF-1α expression with clinicopathological variables. Statistically, CD73 overexpression was significantly correlated with tumor differentiation ($P = 0.000$), histopathology ($P = 0.041$), depth of invasion ($P = 0.000$), nodal status ($P = 0.003$), metastasis ($P = 0.013$), and AJCC stage ($P = 0.000$) (Table 1). In contrast, there was no correlation between the expression of CD73 and age, gender, tumor size, or Borrmann type ($P > 0.05$, Table 1). In addition, HIF-1α expression was significantly correlated with tumor size ($P = 0.031$), depth of invasion ($P = 0.036$) and AJCC stage ($P = 0.035$) (Table 1), but not with age, gender, tumor differentiation, histopathology, depth of invasion, nodal status, metastasis, or Borrmann type ($P > 0.05$, Table 1).

Survival analysis

Further Kaplan-Meier analysis demonstrated that high expression of CD73 (log-rank, $P < 0.001$) had a statistically significant correlation with a poor overall survival (Figure 2). But there was no significant correlation between overexpression of HIF-1α and survival time (log-rank, $P = 0.103$). Moreover, we classified the patients into four groups stratified according to CD73/HIF-1α expression, and a significant difference was observed

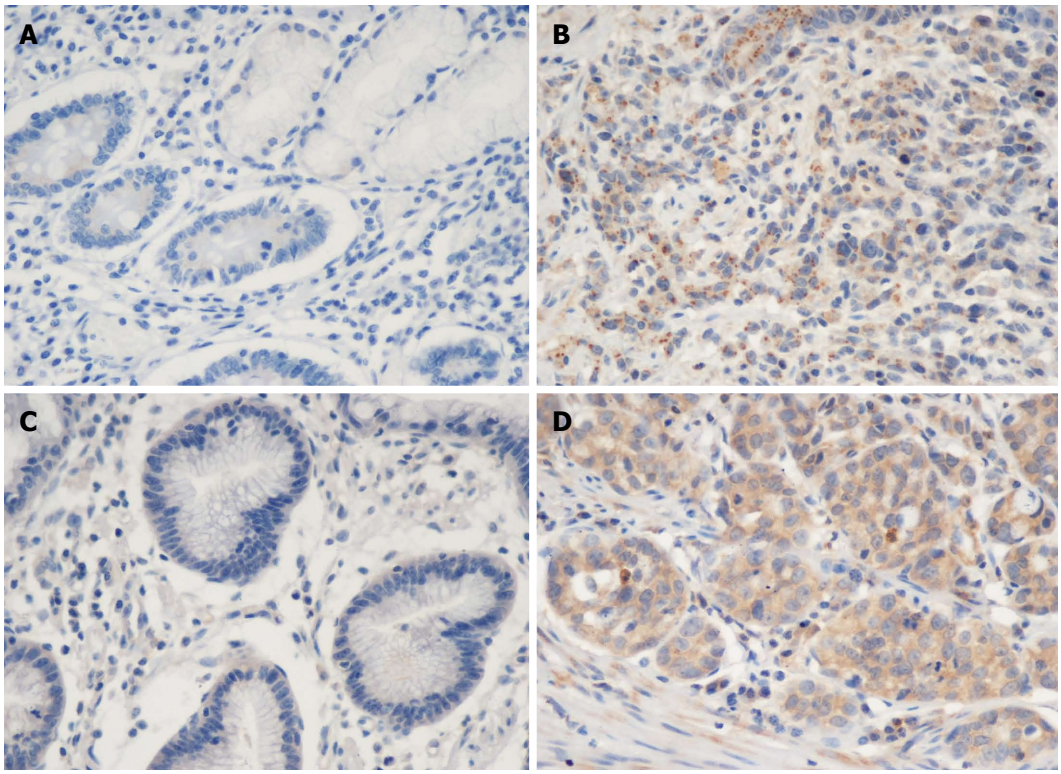

Figure 1 Expression of CD73 and hypoxia-inducible factor-1α in gastric carcinoma (immunohistochemical stain, ×400). A, C: Negative staining for CD73 (A) and hypoxia-inducible factor-1α (HIF-1α) (C) in healthy control gastric mucosa; B, D: Positive staining for CD73 (B) and HIF-1α (D) in gastric carcinoma.

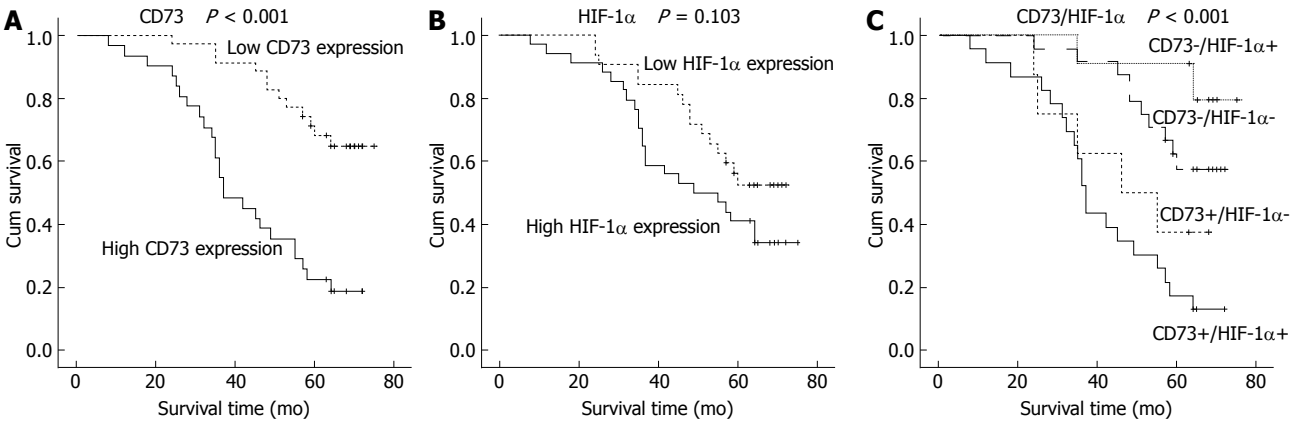

Figure 2 Kaplan-Meier curves for postoperative survival. A: The median survival time of patients with positive CD73 was shorter than that of patients with negative CD73 (log-rank test: $P < 0.001$); B: Hypoxia-inducible factor-1α (HIF-1α) expression had no correlation with the survival time of patients (log-rank test: $P = 0.103$); C: There was a significant difference among groups stratified according to CD73/HIF-1α expression ($P < 0.001$). Patients with CD73+/HIF-1α+ had the worst prognosis.

among the groups (log-rank, $P < 0.001$). The patients with CD73+/HIF-1α+ carcinomas had the worst prognosis. The independent effects of all the significant factors were tested using the Cox proportional hazards model. The exploratory multivariate analyses demonstrated that CD73 [$P = 0.021$, hazard ratio (HR) = 0.385, 95%CI: 0.171-0.865] and AJCC stage ($P = 0.035$, HR = 1.585, 95%CI: 1.032-2.433) were independent prognostic factors, while HIF-1α and others were not independent predictors. ROC curve analysis was also performed to further evaluate the prognostic value of CD73 and HIF-1α expression, which revealed that CD73 expression was encouragingly useful in predicting the overall survival of gastric carcinoma patients (area under the curve = 0.850, $P < 0.001$, Figure 3).

DISCUSSION

Gastric carcinoma is diagnosed with a high frequency throughout the world. In spite of improved diagnostic techniques and therapeutic methods, gastric carcinoma remains a major public health problem. Recently, some investigations showed that biomarkers might be promising predicting factors, and some of them were found

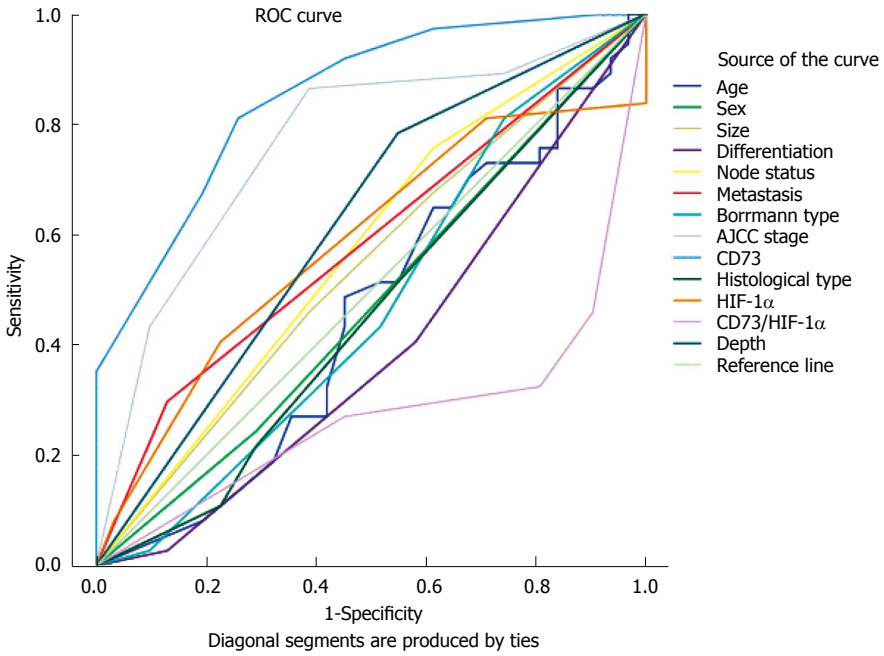

Figure 3 Receiver operating characteristic curves of clinicopathological variables, CD73 expression and hypoxia-inducible factor-1α expression based on outcomes of gastric carcinoma patients. CD73 expression (AUC = 0.850; $P < 0.001$), hypoxia-inducible factor-1α (HIF-1α) (AUC = 0.582; $P = 0.247$), AJCC stage (AUC = 0.765; $P < 0.001$), CD73/HIF-1α (AUC = 0.275; $P = 0.001$), Borrmann type (AUC = 0.472; $P = 0.689$), metastasis (AUC = 0.584; $P = 0.235$), nodal status (AUC = 0.572; $P = 0.310$), differentiation (AUC = 0.394; $P = 0.135$), histopathology (AUC = 0.459; $P = 0.559$), tumor diameter (AUC = 0.541; $P = 0.559$), gender (AUC = 0.476; $P = 0.740$), and age (AUC = 0.456; $P = 0.534$). ROC: Receiver operating characteristic; AJCC: American Joint Committee on Cancer; AUC: Area under the curve.

even superior to the AJCC staging system[20,21]. Increasingly more researches have been conducted to discover the specific biomarkers although some of the results remained conflicting and inconsistent.

CD73, known as a purine salvage enzyme, might play a regulatory role in the immune system response[22,23]. Jin *et al*[22] found that the adenosine generated by tumor-derived CD73 could inhibit both the activation phase and effector phase of the antitumor T cell response and promote T cell apoptosis. Besides, some studies have indicated that CD73 could promote invasion, migration and adhesion of cancer cells[24]. Moreover, the tumor-inhibiting effects of CD73 using siRNA or anti-CD73 antibody could restore efficacy of adoptive T cell therapy, leading to a long-term tumor-free survival[22,25,26]. Host-derived CD73 was also observed in recent years, which provided evidence that CD73 knockdown could significantly delay tumor growth by regulating host immune system[27-29].

The prognostic significance of CD73 has been studied in several cancers such as papillary thyroid carcinoma and breast cancer[9-13]. It was suggested that high expression of CD73 in papillary thyroid carcinomas could be a useful indicator in the differential diagnosis of thyroid tumors. Moreover, strong expression of CD73 was found to be associated with invasiveness, metastasis, and shorter clinical survival in breast cancer. However, few studies have investigated the correlation between CD73 and gastric carcinoma.

CD73 expression of tumor cells may be induced by the selective pressure of the host immune system. Among other influencing factors in tumor microenvironment, hypoxia is the one which has been clearly defined[19]. Hypoxia could induce upregulation of CD73 expression in brain microvessel endothelial cells, which will be reversed by reoxygenation of a short duration[30]. Synnestvedt *et al*[19] reported that hypoxia induced CD73 mRNA, increased protein expression levels and enhanced the CD73 activity in intestinal epithelial cells (T84 cells) and this involved direct binding of HIF-1 to the *Nt5e* gene.

In tumor cells, adaptations to hypoxia are regulated by the activation of specific genes through HIF. And the transcription factor HIF-1α which determines HIF activity is regarded as a hypoxia marker[31]. Overexpression of HIF-1α has been observed in various cancers, such as brain, bladder, lung, breast, esophagus, pancreas, colon, ovary, kidney, and prostate[16-18,32-35]. Furthermore, it was reported that HIF-1α overexpression was significantly correlated with highly aggressive disease, resistance to radiotherapy and chemotherapy, and poor prognosis in various carcinomas[36,37]. Dellas *et al*[38] found that high expression of HIF-1α was associated with tumor progression and metastasis in advanced cervical cancer. Lu *et al*[37] reported that elevated HIF-1α expression was significantly correlated with poor prognosis of rectal adenocarcinoma patients.

In this study, we investigated the relationship between CD73, HIF-1α, clinicalpathological significance, and clinical prognosis in gastric carcinoma. We found that the expression of CD73 was significantly higher in gastric carcinoma than that in normal gastric mucosa, indicating the important role of CD73 in carcinogenesis. Furthermore, CD73 expression was closely correlated with differentiation, histopathology, depth of invasion, nodal status, metastasis, and AJCC stage, but not associated with age, gender, tumor diameter, or Borrmann type. Overexpression of HIF-1α was found to be associated with tumor size, depth of invasion, and AJCC stage. The Spearman's rank correlation analyses indicated a close correlation between CD73 and HIF-1α expressions in gastric carcinoma.

Our data also demonstrated that the overall survival curves in the CD73-negative group were significantly higher than in the CD73-positive group. However, there was no significant correlation between HIF-1α overexpression and the poor overall survival. We classified the

patients into four groups stratified according to CD73/HIF-1α expression, and a significant difference was observed among the groups. The patients with CD73+/HIF-1α+ carcinomas had the worst prognosis. Multivariate analyses showed that only CD73 expression was a prognostic factor independent of certain well-established clinicopathologcial parameters.

In conclusion, CD73 was correlated with the clinicopathological features in gastric carcinoma. High expression of CD73 was an indicator of poor clinical prognosis in patients with gastric carcinoma. Moreover, immunoreactivity of the combined CD73 and HIF-1α could be a useful prognostic marker of gastric carcinoma.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Xiao-Jun Zhou and Xing-Hua Zhang for their technical assistance.

COMMENTS

Background

Gastric carcinoma has been the fourth most common cancer in the world since the latter half of the 20th century. Changes observed in expression of tumor specific biomarkers in gastric carcinomas may be helpful to understand the transformation of histological heterogeneity and the underlying molecular mechanisms. Searching for specific biomarkers which determine the biological nature and behavior of gastric carcinoma would be of utmost importance to optimize individualized therapy.

Research frontiers

Overexpression of CD73 has been observed in various cancers. However, few studies have investigated the correlation between CD73 and gastric carcinoma. Previous studies indicated that hypoxia could induce up-regulation of CD73 expression in different cells, but the correlation between CD73 expression and hypoxia-inducible factor-1α (HIF-1α) expression has not been observed. In this study, the authors demonstrate that CD73 expression is up-regulated in gastric carcinoma and shows close correlation with HIF-1α expression.

Innovations and breakthroughs

In this paper, data firstly shows that there are close correlation between the two biomarkers in gastric carcinoma and the combination of CD73 and HIF-1α could be a useful marker of the prognosis of gastric carcinoma. Moreover, high expression of CD73 was found to be an indicator of poor clinical prognosis in patients with gastric carcinoma.

Applications

Examination of CD73 and HIF-1α expression by immunohistochemistry (IHC) analysis could be used as an additional effective way to identify patients at high risk of tumor progression, thus to optimize individual treatment of patients with gastric carcinoma.

Terminology

Ecto-5'-nucleotidase/CD73 is a homodimer linked to the plasma membrane through a glycosylphosphatidylinositol lipid anchor, which was found in most tissues. It is a part of extracellular ATP metabolism, which dephosphorylates AMP into adenosine rapidly after CD39 catalyzes ATP, ADP and AMP; HIF-1 is a heterodimeric basic helix-loop-helix transcription factor composed of HIF-1α and HIF-1β subunits; and HIF-1α determines HIF-1 activity

Peer review

This manuscript investigate the expression of CD73 and HIF-1α in human gastric carcinoma by IHC. The results showed that CD73 and HIF-1α were higher expressions in gastric carcinoma than that of control and showed close correlation. They concluded that the combination of the two molecules and CD73 expression in gastric cancer tissue is associated with prognosis. The results are interesting and may represent an additional effective way to identify patients at high risk of tumor progression.

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