High Lactate Dehydrogenase 5 Expression Correlates with High Tumoral and Stromal Vascular Endothelial Growth Factor Expression in Gastric Cancer

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Key Words

Disease-free survival · Gastric cancer · Lactate dehydrogenase 5 · Overall survival · Vascular endothelial growth factor

Abstract

Background: Lactate dehydrogenase 5 (LDH5) is a major lactate dehydrogenase isoenzyme catalyzing the transformation of pyruvate to lactate to provide anaerobic energy. Vascular endothelial growth factor (VEGF) is expressed in both tumor and stromal cells in gastric cancer. Our aim was to study the prognostic effect of LDH5, and tumoral and stromal expression of the angiogenic factor VEGF in gastric cancer, and the intercorrelation of tissue expression of both factors. **Methods:** Tissue microarray analysis of 382 consecutive gastric cancer resection specimens was used for immunohistochemistry of LDH5 and VEGF, and expression of LDH5, tumoral VEGF, and stromal VEGF was categorized into low and high groups. **Results:** High expression was observed for LDH5 in 57.9% (219/378), tumoral VEGF in 35.7% (136/381), and stromal VEGF in 58.5% (223/381) of the specimens. Regarding high expression of LDH5 and VEGF, significant associations with intestinal type, advanced gastric cancer, lymph node metastasis, higher TNM stage, and upper-third location were noted. Positive intercorrelations occurred among the expression of LDH5 and VEGF. Results of survival analyses revealed a significant association of high expres-

sion of LDH5 and VEGF with lower survival (overall and disease-free survival). Five-year survival rates were significantly lower in tumors with high LDH5 and tumoral VEGF expression in diffuse- or mixed-type cancers and high expression of stromal VEGF in intestinal-type cancer. **Conclusion:** The results of our study showed that high LDH5 and VEGF expression in both tumor and stroma was a prognostic factor for patients with gastric cancers, especially diffuse- or mixed-type cancers. Therefore, LDH5 expression may play a role in the regulation of tumoral and stromal VEGF expression in gastric cancer. Our results suggest the potential use of LDH5 expression as a biomarker for response to VEGF-targeted therapy.

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Introduction

Gastric cancer is still the fourth most common malignancy in the world and the most common malignancy in Korea [1]. Surgery is the only possible curative treatment, and results of gastrectomy with respect to survival, morbidity, and mortality have improved through the years [2]. Despite these improvements, up to 80% of patients resected with curative intent develop locoregional recurrences [3]. Various regimens of adjuvant chemotherapy, either with or without radiotherapy, have been administered to prevent disease relapse and to improve the survival rate [4–6].

Hypoxia frequently develops in various solid tumors and provides a microenvironment with resistance to chemoradiotherapy, resulting in a poor prognosis. Glycolysis is a major source for the production of ATP from glucose, which is transformed to pyruvate while NAD⁺ (nicotinamide adenine dinucleotide) is reduced to NADH. The fate of the pyruvate molecules produced is dependent on the presence of oxygen. Adequate oxygenation allows for conversion of pyruvate to ATP, water and carbon dioxide, while lack of oxygen or biochemical defects relevant to the electron transport system or the citric acid cycle restrict ATP production to glycolysis. Such an oxidation occurs during the reversible transformation of pyruvate to lactate, a reaction catalyzed by lactate dehydrogenase (LDH) [5, 7].

Aerobic glycolysis has been shown to be under the direct control of hypoxia-inducible factors (HIF-1 α and 2 α) in the transcription of a variety of genes involved in angiogenesis, glycolysis, and apoptosis, including vascular endothelial growth factor (VEGF) and LDHA [8].

LDH is involved in the reversible transformation of pyruvate, the end product of glycolysis, to lactate. The LDH5 isoenzyme, which is composed of 4 M subunits encoded by the LDHA gene, is the most efficient in catalyzing the conversion of pyruvate to lactate. LDH is formed by the arrangement of up to 4 copies of two different subunits: subunit LDHH is encoded by the LDHB gene and is ubiquitously expressed in healthy tissues, whereas subunit LDHM is encoded by the HIF-1 target gene LDHA and is therefore induced by hypoxia. Increased expression of LDH5 is one of the hallmarks of cancer cells. Elevated expression of LDH5 is an unfavorable prognosticator in many human tumors, including non-small cell lung cancer, endometrial cancer, and colorectal cancer [7, 9–11].

VEGF is the most commonly expressed cytokine of oxygen-regulated proteins. VEGF-A is the most important proangiogenic factor for the formation and maturation of novel tumor blood vessels. In tumor development, the angiogenic switch is associated with the onset of expression and secretion of angiogenic factors by tumor cells. Secretion of growth factors by tumor cells leads to a complex interplay with subsequent stromal secretion of growth factors and activation of endothelial cell receptors [12]. Angiogenic markers are important in the interaction between different stromal and tumor cells [13]. VEGF also increases capillary leakiness and was first identified as a vascular permeability factor [14].

Most studies addressing the significance of VEGF in angiogenesis have focused on tumor cell expression. As

there is growing recognition that the tumor stroma plays a critical role in tumorigenesis [15], a complex interplay between tumor and stromal cells (e.g. macrophages, fibroblasts, lymphocytes, granulocytes and plasma cells) has been described in angiogenesis [14, 16].

In this study, we graded VEGF expression in tumor and stroma of gastric cancer separately in order to explore how their expression correlates with LDH5 expression in gastric cancer tissue. We assessed the prognostic significance of LDH5 and VEGF expression in gastric cancer patients treated with curative resection and adjuvant chemotherapy.

Materials and Methods

Patients and Clinical Samples

The study cohort included 404 consecutive patients who underwent curative gastrectomy for primary gastric cancer between January 1, 2004, and December 31, 2004, at the Seoul National University Hospital. The cohort consisted of 297 men and 107 women with a mean age of 58.1 years (range: 28–86 years). Information on tumor location was obtained for 403 cases, the upper third in 73, middle third in 113, lower third in 209, and the entire stomach in 8 cases. The histological type was classified as intestinal in 174, diffuse in 158, and mixed in 72 cases based on the Lauren classification. There were 128 early and 276 advanced gastric cancers. Lymph node metastases were present in 190 cases and absent in 214 cases. One hundred ninety-nine patients were diagnosed as stage I, 87 as stage II, 82 as stage III, and 36 as stage IV (table 1). Adjuvant chemotherapy following surgery was performed in 228 patients: 23 patients in stage I, 87 in stage II, 82 in stage III, and 36 in stage IV. The mean follow-up period was 56.3 months (range: 2–99 months). Local recurrence was noted in 96 (23.8%), distant metastasis in 8 (2.0%), and gastric cancer-related death in 91 of 404 patients (22.5%).

This study was approved by the Institutional Review Board of the Seoul National University Hospital (H-1010-065-336). Patient survival data, including dates and causes of death, were obtained from the Korean Central Cancer Registry, Ministry of Health and Welfare, Republic of Korea. Standard histopathological examination included the assessment of cancer type and pathological tumor stage according to the criteria established by the 6th edition of the *AJCC Staging Manual* [17].

Immunohistochemistry

For tissue microarray analysis, three core tissue specimens (diameter: 2 mm) were obtained from individual paraffin-embedded representative gastric cancer tissue samples (donor blocks) and arranged in three sets of new recipient paraffin blocks (tissue microarray blocks) using a trephine apparatus (Superbiochips Laboratories, Seoul, Republic of Korea). Each tissue microarray block contained up to 60 cores. From the tissue microarray blocks, sections of 4- μ m thickness were deparaffinized and rehydrated in graded alcohol. Antigen retrieval was achieved by pressure cooking in 0.01 mol/l citrate buffer for 5 min. The sheep polyclonal antibody 9002 (Abcam, Cambridge, UK) raised against human LDH5, purified from human placenta [11, 18, 19], and VEGF (1:200, sc-7269; Santa Cruz, Calif., USA) were used for immunohistochemistry.

Table 1. Clinicopathologic profiles of 404 primary gastric cancer (GC) patients analyzed in this study

	n	%
Gender		
Total	404	100
Male	297	73.5
Female	107	26.5
Age		
<60 years	201	49.8
≥60 years	203	50.2
Tumor location		
Upper	73	18.1
Middle	113	28
Lower	209	51.7
Entire	8	2
Not recorded	1	0.2
Lauren classification		
Intestinal	174	43.1
Diffuse	158	39.1
Mixed	72	17.8
Tumor invasion		
Early GC	128	31.7
Advanced GC	276	68.3
N stage		
N0	190	47
N1	214	53
TNM stage		
I	199	49.3
II	87	21.5
III	82	20.3
IV	36	8.9

Table 2. LDH5 grading system based on the intensity and extent of cytoplasmic and nuclear staining

	Score	n (%)
Complete absence of LDH5 reactivity	low (negative)	8 (2.1)
Weak cytoplasmic reactivity (regardless of the extent)	low	110 (29.1)
Strong cytoplasmic reactivity in <50% of cells	low	15 (4.0)
Nuclear expression in sporadic cells (<10%)	low	26 (6.9)
Strong cytoplasmic expression in >50% of cells	high	146 (38.6)
Nuclear expression in >10% of cells	high	73 (19.3)
Total		378 (100.0)

The percentage of cancer cells with cytoplasmic and nuclear LDH5 expression was assessed separately and then combined in a grading system proposed by Giatromanolaki et al. [11, 20] and Koukourakis et al. [18] (table 2). The gastric adenocarcinomas in the series were divided into tumors with high and low LDH5 expression.

Cytoplasmic positivity for VEGF was separately evaluated in tumor and stromal cells. Immunoreactivity was scored as the per-

centage of positive to total tumor cells for tumoral VEGF expression and as the percentage of positive to total stromal cells for stromal VEGF expression. Percentage scores were categorized using a four-category scoring system: 0 = no reactivity; 1 = 0–25%; 2 = 26–50%; 3 = 51–75%, and 4 = 76–100%. Scores of 3 and 4 were grouped as high VEGF expression and scores of 0–2 as low VEGF expression.

Statistical Analyses

The SPSS 15.0 package was applied for statistical analyses. The χ^2 test was used to determine correlations between each protein and clinicopathologic variables, and intercorrelations in protein expression. Survival curves for overall (OS) and disease-free survival (DFS) were plotted using the Kaplan-Meier method, and the log-rank test was employed to determine statistically significant differences between life tables. Five-year survival rates were obtained with the life table method. All p values are two sided and values of $p < 0.05$ were considered statistically significant.

Results

Immunohistochemical Expression of LDH5

Normal gastric mucosa showed weak or moderate nuclear staining of epithelial cells in antral and fundic glands. The frequency of each grade of LDH5 expression is shown in table 2. LDH5 expression was increased in 57.9% (219/378 patients) of the gastric adenocarcinomas studied. Immunohistochemical expression showed a nuclear and/or cytoplasmic pattern in gastric cancer cells (fig. 1a–c).

Immunohistochemical Expression of VEGF

In normal gastric mucosa, no or weak cytoplasmic expression was observed in both epithelial and stromal cells. VEGF expression was increased in the cytoplasm of tumoral cells in 35.7% (136/381 patients) and in stromal cells in 58.5% (223/381 patients). Immunohistochemical expression showed a cytoplasmic pattern in both tumoral and stromal cells (fig. 1a–c).

Association of LDH5 and VEGF Expression with Histopathological Variables

High LDH5 expression was associated with upper tumor location, intestinal type, advanced gastric cancer, lymph node metastasis, and higher TNM stage; high tumoral VEGF expression showed an association with older age (≥60 years), upper tumor location, intestinal type, advanced gastric cancer, lymph node metastasis, and higher TNM stage. Increased stromal VEGF expression was associated with older age (≥60 years), intestinal type, advanced gastric cancer, lymph node metastasis, and higher TNM stage (table 3).

Fig. 1. Immunohistochemical staining of LDH5 and VEGF in tissue sections of human gastric cancer ($\times 200$). **a** Low LDH5, low stromal VEGF expression. **b** High LDH5, high epithelial and stromal VEGF expression. **c** High LDH5, high stromal VEGF.

Table 3. Clinicopathologic correlation of LDH5 and VEGF expression in gastric cancer (GC)

		LDH5					Tumoral VEGF					Stromal VEGF				
		low		high		p	low		high		p	low		high		p
		n	%	n	%		n	%	n	%		n	%	n	%	
Total patients		159	42.1	219	57.9		245	64.3	136	35.7		158	41.5	223	58.5	
Gender	Male	113	40.8	164	59.2	0.408	175	62.7	104	37.3	0.287	108	38.7	171	61.3	0.071
	Female	46	45.5	55	54.5		70	68.6	32	31.4		50	49	52	51	
Age	<60 years	84	45.2	102	54.8	0.23	137	72.1	53	27.9	0.002	98	51.6	92	48.4	<0.001
	≥ 60 years	75	39.1	117	60.9		108	56.5	83	43.5		60	31.4	131	68.6	
Lauren	Intestinal	57	34.3	109	65.7	0.007	90	53.9	77	46.1	<0.001	59	35.3	108	64.7	0.032
	Diffuse/mixed	102	48.1	110	51.9		155	72.4	59	27.6		99	46.3	115	53.7	
Invasion	Advanced GC	97	36.7	167	63.3	0.001	154	58.1	111	41.9	<0.001	85	32.1	180	67.9	<0.001
	Early GC	62	54.4	52	45.6		91	78.4	25	21.6		73	62.9	43	37.1	
N stage	N0	83	48.5	88	51.5	0.02	126	72	49	28	0.004	96	54.9	79	45.1	<0.001
	N1	76	36.7	131	63.3		119	57.8	87	42.2		62	30.1	144	69.9	
TNM stage	I	89	49.4	91	50.6	0.03	130	71	53	29	0.053	100	54.6	83	45.4	<0.001
	II	26	31.3	57	68.7		50	59.5	34	40.5		32	38.1	52	61.9	
Location	III	32	40	48	60		48	59.3	33	40.7		20	24.7	61	75.3	
	IV	12	34.3	23	65.7		17	51.5	16	48.5		6	18.2	27	81.8	
	Upper	22	31	49	69	0.046	40	57.1	30	42.9	0.014	26	37.1	44	62.9	0.082
	Middle	45	45.5	54	54.5		79	74.5	27	25.5		55	51.9	51	48.1	
	Lower	91	45.7	108	54.3		123	62.4	74	37.6		75	38.1	122	61.9	
	Entire	1	12.5	7	87.5		2	28.6	5	71.4		2	28.6	5	71.4	

Correlation of LDH5 Expression with Tumoral and Stromal VEGF Expression in Gastric Cancers

A correlation study of protein expression levels was available in 360 cases for LDH5 versus tumoral and stromal VEGF, and in 381 cases for tumoral versus stromal

VEGF, respectively. Significant positive correlations were noted for all the comparative pairs mentioned ($p < 0.05$; table 4).

Among 381 cases available for tumoral and stromal VEGF expression, 148 (38.8%) cases showed high expres-

Table 4. Association of LDH5 and tumoral and stromal VEGF expression in gastric cancer

		LDH5					Tumoral VEGF				
		low		high		p	low		high		p
		n	%	n	%		n	%	n	%	
Tumoral VEGF	low	108	30	119	33.1	0.001					
	high	39	10.8	94	26.1						
Stromal VEGF	low	74	20.6	72	20	0.002	148	38.8	10	2.6	<0.001
	high	73	20.3	141	39.2		97	25.5	126	33.1	

sion in both, 10 (2.6%) showed high expression only in tumor cells, 97 (25.5%) showed high expression only in stromal cells, and 126 (33.1%) showed low expression in both.

Survival Analyses by LDH5 and VEGF Expression

The patients with high LDH5 expression had a significantly shorter DFS compared with those with low LDH5 expression. No significant association was noted between LDH5 expression and OS, while a significant association was found between high tumoral VEGF expression and poor DFS. High stromal VEGF expression showed an association with both poor OS and poor DFS (fig. 2).

The cases were classified into intestinal and diffuse or mixed types and 5-year survival rates were analyzed in two histological subgroups. High LDH5 expression and tumoral VEGF expression were associated with lower 5-year survival rates in patients with diffuse or mixed type ($p = 0.007$ and $p = 0.027$, respectively). In contrast, high stromal VEGF expression was associated with lower 5-year survival rates in patients with intestinal type ($p = 0.005$; table 5).

Discussion

VEGFs, apparently the most important angiogenic factors, are prime regulators of both physiological and pathological angiogenesis in malignancy [21]. Of particular importance, tissue oxygen tension tightly regulates VEGF levels. Exposure to hypoxia induces rapid and reversible expression of VEGF through both increased mRNA transcription and stabilization. Thus, hypoxic up-regulation of VEGF provides a compensatory mechanism by which tissues can increase their oxygenation via the induction of blood vessel growth. Normoxia downregulates VEGF production and leads to the regression of certain newly formed blood vessels.

Both the LDHA and VEGF genes are transcriptionally regulated by HIFs, therefore, serum LDH is assumed to serve as a predictive marker of response to the VEGF receptor (VEGFR) inhibitor combination with chemotherapy [22]. In our study, high LDH5 expression in gastric cancer tissue was correlated with both tumoral and stromal VEGF expression. The Lauren classification for gastric cancer has been used for a long time as consistent categories based on differentiation [23]. The results of our study revealed that expression of LDH5 and VEGF was more frequently increased in the intestinal type, and high expression of LDH5 and tumoral VEGF showed a significant correlation with poor prognosis in diffuse- or mixed-type gastric cancer, while stromal VEGF expression was correlated with outcome, regardless of the histologic type. The reason for this phenomenon is not clear; tumoral cell differentiation might be linked to lactate production and the regulation of angiogenesis.

In a meta-analysis of tissue VEGF expression as a prognosticator in gastric cancer, there are 26 eligible studies: 20 positive and 6 negative results. VEGF binds to VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), which is mainly expressed in vascular endothelial cells. VEGF-A, usually simply referred to as VEGF, is believed to play a major role in tumor growth and metastasis [24].

In the 1920s, Otto Warburg reported that in tumor cells of ascites rates of glucose consumption and lactate production were increased despite the availability of sufficient oxygen for the complete oxidation of glucose. The Warburg effect is regarded as a metabolic hallmark of aggressive tumors; however, the phenotype has also been observed in nontransformed cells during rapid proliferation [25]. It is widely believed that most tumor cells derive the major portion of metabolic energy from glycolysis rather than mitochondrial oxidation [26]. Reduction of pyruvate to lactate also allows glycolytic cells to maintain pyruvate levels low enough to avoid cell death [26, 27].

Fig. 2. Kaplan-Meier analysis of OS and DFS of gastric cancer patients according to LDH5 (**a, b**), tumoral VEGF (**c, d**), and stromal VEGF expression (**e, f**). Dotted line = Low expression; solid line = high expression.

This reversible reaction is catalyzed by LDH. Lactate produced by tumor cells may also affect tumor progression and metastasis [28].

There are 5 component isoenzymes as a result of the 5 different combinations produced by 2 polypeptide chains encoded by separate genes (M and H). LDH1 is composed of 4 H subunits, and LDH5 of 4 M subunits. As the number of M over H chains increases, the LDH isoenzyme becomes more efficient in catalyzing the conversion of pyruvate to lactate (LDH5), while an increase of H over M chains (LDH1) favors the conversion of pyruvate to acetyl-CoA that enters into the citric acid (Krebs) cycle. The prevailing type of LDH is dependent upon the metabolic demands of a particular tissue [29].

VEGF is a major inducer of angiogenesis and vessel permeability [30]. Expression of VEGF in tumor cells is

Table 5. Five-year survival rates according to LDH5, and tumoral and stromal VEGF expression in histological subgroups

Histological type/category	Expression	5-year survival, %	p
<i>Intestinal type</i>			
LDH5	low	75.6	0.585
	high	80.4	
Tumoral VEGF	low	79.5	0.619
	high	78.2	
Stromal VEGF	low	91.1	0.005
	high	72.0	
<i>Diffuse or mixed type</i>			
LDH5	low	80.2	0.007
	high	63.6	
Tumoral VEGF	low	79.0	0.027
	high	63.9	
Stromal VEGF	low	79.0	0.085
	high	69.2	

thought to be largely attributable to the hyperpermeability of tumor vessels, and increased permeability results in leakage of several plasma proteins, including fibrinogen and other clotting proteins. This can lead to the deposition of fibrin in the extravascular space, which subsequently retards the clearance of edema fluid and transforms the normally anti-angiogenic stroma of normal tissue into a proangiogenic environment. VEGF ligands expressed by tumor cells or host stromal cells stimulate VEGFR-1, VEGFR-2, or VEGFR-3 expressed by endothelial, lymphendothelial, or hematopoietic cells. Stimulation of VEGFR-1- and VEGFR-2-positive endothelial cells by VEGF activates proliferation, migration, survival, and vascular permeability [31].

There are limitations of both heterogeneity of tissue microarray-based immunohistochemistry and heterogeneity of gastric cancer itself in our results [32, 33]. However, the results of our study of stromal VEGF showed a strong correlation between tumoral and stromal VEGF expression in gastric cancer tissue and the prognostic effect showed a similar pattern according to the expression

level. In contrast, in non-small cell lung cancers, an inverse prognostic impact of angiogenic marker expression in tumor cells versus stromal cells was reported [34]. A significant association of LDH5 and VEGF expression was reported in endometrial cancer [11], and a direct correlation of LDH5 with VEGF was noted in non-small cell lung cancer [29]. In colon cancer, no association of LDH5 with either VEGF expression or vascular density was observed [9, 18], but the association with phosphorylated kinase domain receptor was significant [9].

Results of our retrospective study showed that high expression of LDH5, and expression of both tumoral and stromal VEGF in gastric cancer are indicators of tumor aggressiveness and a poor outcome, particularly in diffuse- or mixed-type cancers.

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