

Basic Study

Inverse correlation between CD8⁺ inflammatory cells and E-cadherin expression in gallbladder cancer: Tissue microarray and imaging analysis

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Abstract

AIM

To investigate the association between the tumor cells' expression of E-cadherin and the numbers of several types of inflammatory cells infiltrating into the invasive portion of gallbladder cancer (GBC).

METHODS

We analyzed 50 GBC cases for which a sufficient amount of tumor tissues for tissue microarray (TMA) had been saved. Three tissue cores (3.0 mm) of invasive lesion from each case were used for the TMA. The 4- μ m cut sections on slides were immunostained using primary antibodies including E-cadherin for cancer cells, leukocyte common antigen for leukocyte, myeloperoxidase for neutrophils, CD3 for T cells, CD4 for helper T cells, CD8 for killer T cells, CD20 for B cells and CD68 for macrophages. The immunostained slides were digitally analyzed by imaging analysis software.

RESULTS

A significant inverse correlation between the number of infiltrating CD8⁺ cells at invasive areas and the expression of E-cadherin by cancer cells was observed ($P = 0.0001$), although the degree of this correlation was relatively weak ($R = 0.32$). The number of CD8⁺ cells and the cancer cells' E-cadherin expression were also significantly correlated with tumor differentiation (well-differentiated vs poorly differentiated) ($P = 0.0467$ and $P = 0.0294$, respectively). Inverse correlation of T-stage

and the number of CD8⁺ cell infiltration was observed with statistical significance in comparison of T2 and T3 cases ($P = 0.0324$).

CONCLUSION

Our findings indicate an inverse correlation of CD8⁺ T cell infiltration and cancer cells' E-cadherin expression at invasive areas of GBC. Further analyses are essential to test these findings.

Key words: E-cadherin; Inflammation; CD8; Gallbladder cancer; Tissue microarray

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Core tip: We analyzed the association between the expression of E-cadherin in tumor cells and the type and amount of inflammatory cells infiltrating into the invasive portion of gallbladder cancer, using tissue microarray and imaging analyses. The results indicated an inverse correlation of CD8⁺ T cell infiltration and E-cadherin expression by cancer cells at invasive areas of gallbladder cancer. Further analyses are essential to test these results.

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INTRODUCTION

Gallbladder cancer (GBC) often shows the dedifferentiation or clustering of tumor cells (tumor budding) at the areas of infiltration, and a high degree of inflammatory response usually accompanies these findings^[1]. Dedifferentiation is often associated with increased aggressiveness of a tumor. Although genetic heterogeneity, genome instability and epithelial-mesenchymal transition are considered mechanisms of dedifferentiation, the mechanism of dedifferentiation has not been established^[2,3]. The concept of tumor budding was first established in colorectal cancer and was considered to be associated with the initial phase of tumor invasion and then with metastatic activity and prognostic outcome in various types of solid tumors^[1,4,5], but the mechanism underlying tumor budding has not been clearly demonstrated.

Cell-cell adhesion participates in histogenesis and the maintenance of cell polarity and tissue structure. It has long been known that the mutual adhesiveness of cancer cells is significantly weaker than that of the corresponding normal cells^[6,7]. Reduced cell-cell adhesiveness allows cancer cells to destruct of the histological structure of tumor tissue, resulting in the dedifferentiation of the tumor. E-cadherin is a well-known cell adhesion molecule

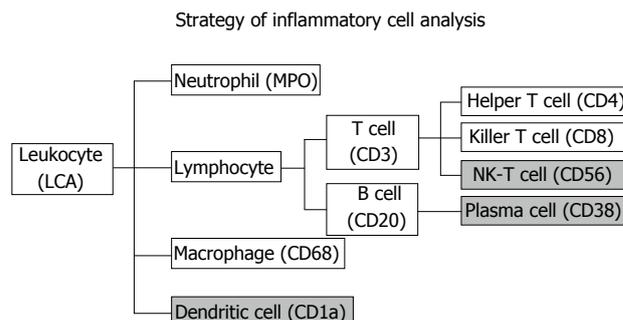


Figure 1 The inflammatory cell analysis strategy. Gray filled shows items which was not able to analyze by Tissue studio (because of positive stain of tumor cells). LCA: Leukocyte common antigen; MPO: Myeloperoxidase.

composed of a series of components, each comprised of approximately 110 amino acid residues. E-cadherin forms a complex with three cytoplasmic proteins (alpha-, beta- and gamma-catenins), and the E-cadherin-catenin complex plays a key role in cellular adhesion; the loss of this function has been associated with tumor dedifferentiation and metastasis^[8].

The microenvironment of cancer changes dramatically in relation to invasion into the stroma due to the changes of blood flow, metabolism and the direct interaction of inflammatory cells. We speculated that the type or amount of inflammatory cells that infiltrate into invasive areas may play some roles in the loss of E-cadherin function, and that tumor-infiltrating inflammatory cells thus contribute to the dedifferentiation of GBC. The purpose of this study was to determine the association between the expression of E-cadherin in tumor cells and the type and amount of inflammatory cells infiltrating into the invasive portion of GBC.

MATERIALS AND METHODS

Patients and tissue microarray preparation

We selected a total of 50 GBC cases for which a sufficient amount of formalin-fixed, paraffin-embedded tumor tissues for tissue microarray (TMA) had been saved from resection of the primary lesion at Saga University Hospital between 1994 and December 2010. Three tissue cores (3.0 mm) of the invasive lesion from each case were used for the TMA. Written informed consent for the use of resected tissue and clinical information was obtained from all patients, and the study protocol was approved by the Ethics Committee of the Faculty of Medicine at Saga University (approval No. 27-19). Clinical and histopathological staging were based on the TNM Classification of Malignant Tumors by the International Union against Cancer (7th edition)^[9].

The inflammatory cell analysis strategy and immunohistochemical stains

The inflammatory cell analysis strategy is summarized in Figure 1. The labels that were initially considered for inflammatory cells were as follows: Leukocyte common antigen (LCA) for leukocytes, myeloperoxidase (MPO) for

Table 1 The primary antibodies used for immunohistochemistry

Antibody	Dilution	Clone	Manufacturer
LCA	Prediluted	PD7/26, 2B11	Nichirei Biosciences, Tokyo
MPO	1:200	Polyclonal	DakoCytomation, Glostrup, Denmark
CD3	Prediluted	PS1	Nichirei Biosciences
CD4	Prediluted	4B12	Nichirei Biosciences
CD8	1:50	C8/144B	DakoCytomation
CD20	1:50	L26	DakoCytomation

LCA: Leukocyte common antigen; MPO: Myeloperoxidase.

neutrophils, CD3 for T cells, CD4 for helper T cells, CD8 for killer T cells, CD56 for natural killer T cells, CD20 for B cells, CD38 for plasma cells, CD68 for macrophages and CD1a for dendritic cells. Among them, CD56, CD38 and CD1a were excluded from the analysis because the imaging analysis software could not accurately detect inflammatory cells due to positive staining of the tumor cells. The primary antibodies used are summarized in Table 1. The 4- μ m cut sections on slides were heated in EDTA (pH 9.0) by a microwave for antigen retrieval. The Envision+[®] System (Dako Cytomation, Glostrup, Denmark) was used as the secondary antibody. The slides were visualized by diaminobenzidine tetrahydrochloride, and nuclei were counterstained with hematoxylin. An Autostainer plus[®] automatic stainer (Dako Cytomation) was used for staining.

Imaging analysis

Immunostained tissue microarray slides were digitally scanned by the NanoZoomer 2.0-HT digital slide scanner (Hamamatsu Photonics, Hamamatsu, Japan) with 20 \times magnification for the imaging analysis. The numbers of positively stained inflammatory cells were automatically counted by the imaging analysis software, Tissue Studio (Definiens, München, Germany) (Figure 2). The membranous expression of E-cadherin was assessed by the histological score that was automatically calculated by Tissue Studio (Figure 3). Tissue Studio digitally evaluated the membranous expression of E-cadherin and categorized the expression into three grades (high, medium and low), and the histological score was calculated by the following formula: $1 \times \%Low + 2 \times \%Medium + 3 \times \%High$.

Statistical analysis

We used JMP ver. 12 software (SAS Institute, Cary, NC, United States) for all statistical analyses. The comparisons of two groups were performed using a simple linear regression analysis, and each pair was analyzed using Student's *t*-test as appropriate. Probability values < 0.05 were considered significant.

RESULTS

The population of inflammatory cells at the invasive sites of GBC

The numbers of inflammatory cells per TMA core are

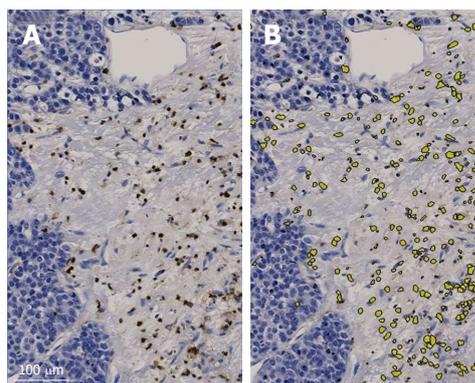


Figure 2 Images of CD8⁺ lymphocytes. A: Original image of CD8-immunostained slide of an invasive area; B: The software Tissue Studio detects the positive cells and counts them automatically.

summarized in Table 2. Many acute inflammatory cells such as MPO⁺ neutrophils and CD68⁺ macrophages (average numbers/core were 2692.39 and 2452.99, respectively) were infiltrated into invasive sites of the GBCs. Regarding the composition of infiltrating lymphocytes, CD8⁺ killer T cells showed the largest number, followed by CD4⁺ helper T cells; CD20⁺ B cells were the least in number (avg. nos./core were 919.44, 583.01 and 467.95). All of the types of inflammatory cells showed a large standard deviation, indicating that the number and composition of inflammatory cells were quite different in each case.

Correlation between E-cadherin expression and the type of inflammatory cells

The results of our single linear regression analysis between E-cadherin expression and the type of inflammatory cells are summarized in Table 3. The immunohistochemical markers of inflammatory cells that were shown to be significantly correlated with the expression of E-cadherin were LCA ($P = 0.0482$), CD3 ($P = 0.011$) and CD8 ($P = 0.0001$). The linear regression models of these factors are demonstrated in Figure 4. An inverse correlation between the number of CD8⁺ cells and the expression of E-cadherin was observed, but the degree of this correlation was relatively weak ($R = 0.32$).

Clinicopathological data and correlations with pathologically assessed differentiation

The clinicopathological data, number of CD8⁺ cells (means of analyzable cores in each case) and E-cadherin histological score (means of analyzable cores in each case) of the 50 GBC cases are presented in Table 4. The numbers of cases in which the pathologically assessed differentiation of invasive front was observed were as follows. G1 (well-differentiated), 16 cases (32.0%); G2 (moderately differentiated), 19 cases (38.0%); G3 (poorly differentiated), 15 cases (30.0%). The median \pm SD of CD8⁺ cells was 580.0 ± 1154.5 , and the E-cadherin histological score was 114.3 ± 37.8 .

Figure 5 illustrates the correlations of pathologically assessed differentiation with the number of infiltrating

Table 2 The numbers of inflammatory cells per tissue microarray core

	Maximum positive cell counts/core	Minimum positive cell counts/core	Average/core	Standard deviation/core	No. of analyzable cores
LCA	13962	73	3108.27	2734.79	136
MPO	10911	388	2692.39	1830.47	131
CD3	14702	74	1967.06	2058.45	130
CD4	6583	0	583.01	934.52	134
CD8	5572	10	919.44	1034.35	131
CD20	7097	4	467.95	834.74	133
CD68	11355	97	2452.99	2043.92	135

LCA: Leukocyte common antigen; MPO: Myeloperoxidase.

Table 3 Single linear regression analysis of the relationship between E-cadherin expression and the type of inflammatory cells

Cells	Regression equation	Adjusted R ²	R	Standard error	P-value
LCA	EHS = 117.67879 - 0.0029223 × LCA	0.0216	0.15	46.5793	0.0482
CD20	EHS = 112.37344 - 0.0083497 × CD20	0.0153	0.12	45.8466	0.083
CD3	EHS = 119.86794 - 0.0050479 × CD3	0.0421	0.21	45.7107	0.011
CD4	EHS = 109.63264 - 0.0003939 × CD4	< 0.0001	n/a	47.1913	0.9285
CD8	EHS = 121.37735 - 0.0146326 × CD8	0.1011	0.32	43.6707	0.0001
MPO	EHS = 104.23773 + 0.0012811 × MPO	-0.00523	n/a	46.9647	0.5701
CD68	EHS = 113.83072 - 0.0023317 × CD68	0.0029	0.05	46.8666	0.2412

EHS: E-cadherin histological score; n/a: Not available; LCA: Leukocyte common antigen; MPO: Myeloperoxidase.

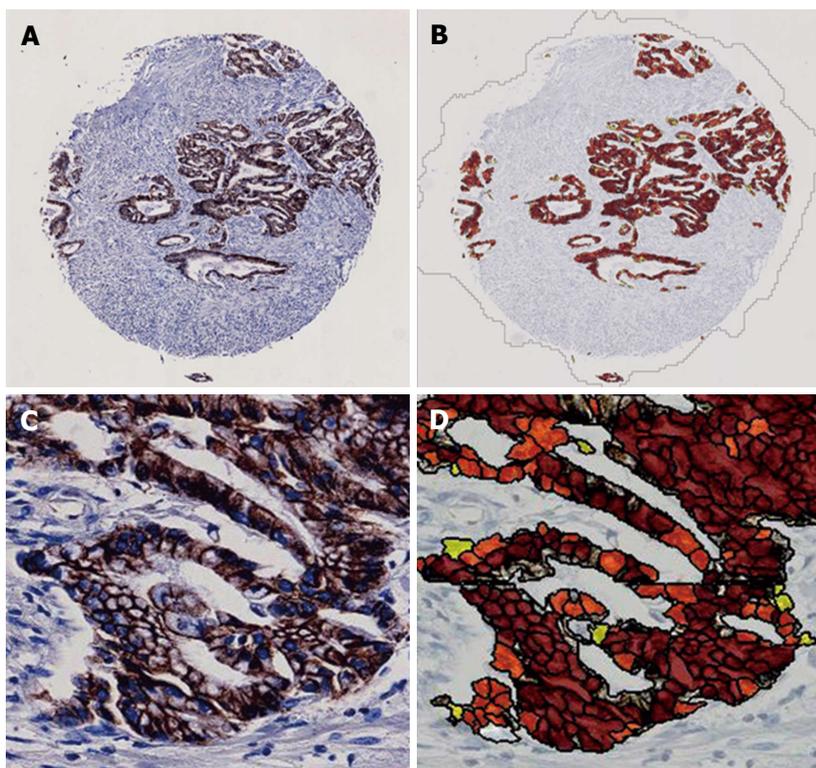


Figure 3 Images of E-cadherin expression in cancer cells. A: Low-magnification image of an E-cadherin-immunostained TMA core; B: Low-magnification image of E-cadherin-immunostained TMA core analyzed by Tissue Studio; C: High-magnification (× 400) image of an E-cadherin-immunostained TMA core; D: High-magnification (× 400) image of an E-cadherin-immunostained TMA core analyzed by Tissue Studio. The membranous expression of E-cadherin was assigned to three categories of the degree of expression: High (brown), medium (orange) and low (yellow). The histological score was calculated by the following formula: 1 × %Low + 2 × %Medium + 3 × %High. TMA: Tissue microarray.

CD8⁺ cells in each case or with the E-cadherin histological score in each case, with each pair analyzed by Student's *t*-test. A significant difference was observed in the comparison

of G1 and G3 cases according to the number of CD8⁺ cells (*P* = 0.0467) and according to the E-cadherin histological score (*P* = 0.0294).

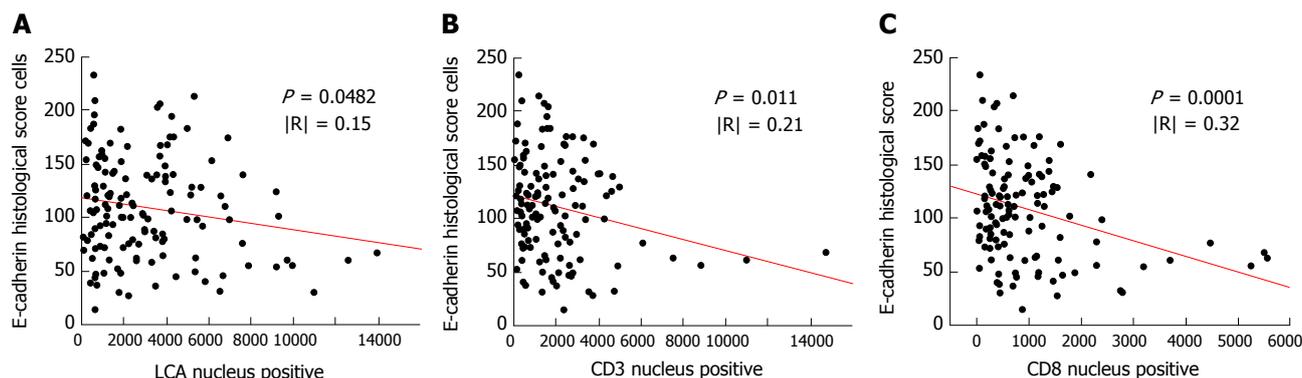


Figure 4 The single linear regression models of the relationship between E-cadherin expression (histological score) and the numbers of cells. The cells were positively immunostained by LCA (A), CD3 (B) and CD8 (C) antibodies.

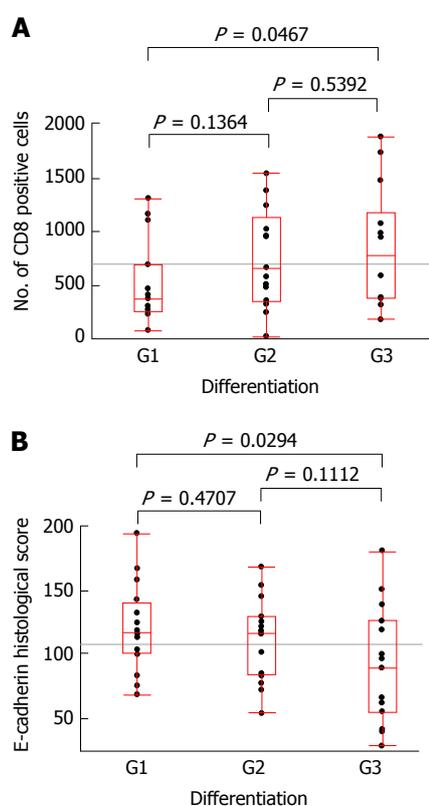


Figure 5 The box-and-whisker plots regarding pathologically assessed differentiation and the number of CD8⁺ cells (A) and the E-cadherin histological score (B). Significant differences were observed in the comparison of G1 and G3 cases according to the number of CD8⁺ cells ($P = 0.0467$) and according to the E-cadherin histological score ($P = 0.0294$), respectively.

Relationship between TMN-stage and E-cadherin expression or CD8⁺ cell infiltration

No distant metastasis (M1) case was involved in this study. The mean \pm SD of number of infiltrating CD8⁺ cells with T2, T3 and T4 tumors were 1489.05 ± 1607.22 , 747.33 ± 719.87 and 506.92 ± 336.37 , respectively. The mean \pm SD of E-cadherin histological score with T2, T3 and T4 tumors were 101.69 ± 37.90 , 108.27 ± 37.04 and 133.75 ± 42.30 , respectively. Figure 6 illustrates the correlations of T-stage with the E-cadherin histological score in each case or with the number of infiltrating CD8⁺

Table 4 Clinicopathologic features of the patients with gallbladder cancer ($n = 50$) n (%)

Age	Mean \pm SD	68.7 \pm 8.4
Gender	Male	15 (30.0)
	Female	35 (70.0)
Differentiation of invasive front	G1	16 (32.0)
	G2	19 (38.0)
	G3	15 (30.0)
T-stage	T2	18 (36.0)
	T3	28 (56.0)
	T4	4 (8.0)
	N	N0
	N1	33 (66.0)
No. of CD8-positive cells (means of analyzable cores in each case)	Median \pm SD	580.0 \pm 1154.5
E-cadherin histological score	Median \pm SD	114.3 \pm 37.8

cells in each case. A significant difference was observed in the comparison of T2 and T3 cases according to the number of CD8⁺ cells ($P = 0.0324$).

The mean \pm SD of number of infiltrating CD8⁺ cells of N0 and N1 cases was 1109.10 ± 1618.16 and 936.39 ± 849.25 , respectively. The mean \pm SD of E-cadherin histological score of N0 and N1 cases was 117.73 ± 9.11 and 102.90 ± 6.54 , respectively. Figure 7 illustrates the N-stage with the E-cadherin histological score in each case or with the number of infiltrating CD8⁺ cells in each case. No significant difference was observed in each comparison.

DISCUSSION

This study analyzed the association between the expression of E-cadherin at tumor cells and inflammatory cells infiltrating into the invasive area of GBC, using the TMA technique and imaging analysis software. Our observation that relatively large numbers of acute inflammatory cells such as neutrophils and macrophages had infiltrated into invasive areas seems reasonable, since the gallbladder frequently develops acute inflammation manifested as acute cholecystitis. Although neutrophils have the potential for cell damage/destruction by the degranulation of their arsenal of cytotoxic chemicals

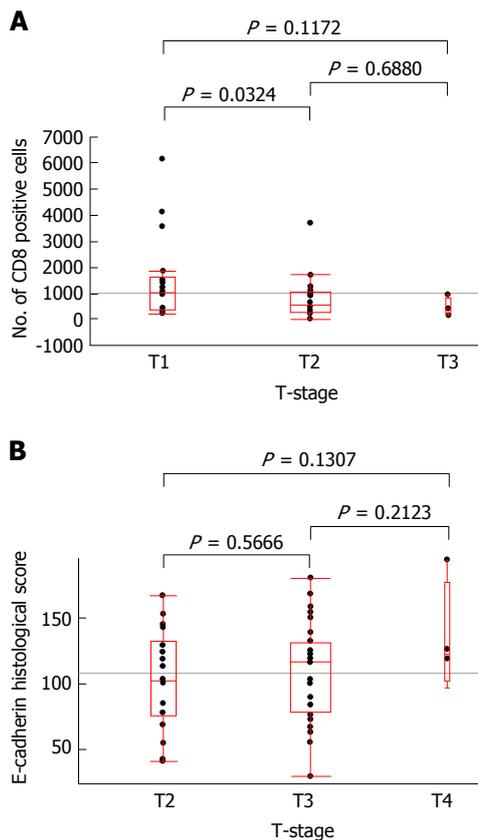


Figure 6 The box-and-whisker plots regarding T-stage and the number of CD8⁺ cells (A) and the E-cadherin histological score (B). A significant difference was observed in the comparison of T2 and T3 cases according to the number of CD8⁺ cells ($P = 0.0324$).

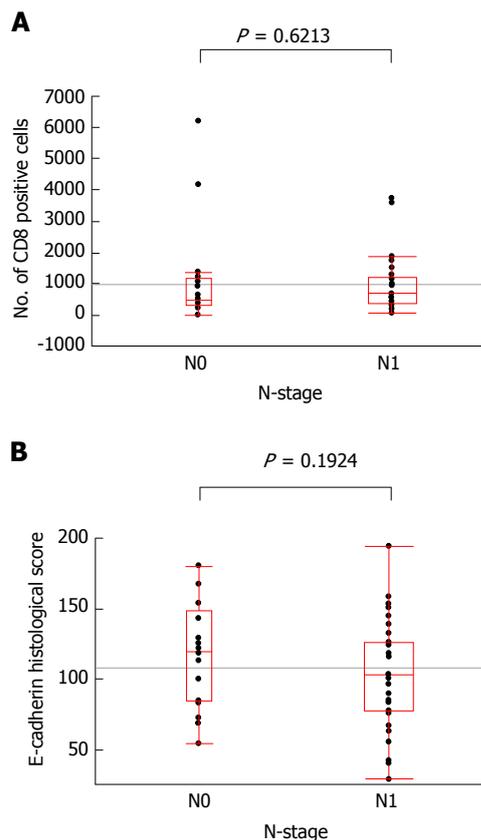


Figure 7 The box-and-whisker plots regarding N-stage and the number of CD8⁺ cells (A) and the E-cadherin histological score (B). No significant difference was observed in each comparison.

and enzymes^[10], we observed no association between neutrophil infiltration and the E-cadherin expression of the tumor cells in this study.

Among the analyzed inflammatory cells, significant associations with E-cadherin expression by tumor cells were observed in the infiltration of leukocytes (LCA), T cells (CD3) and CD8⁺ T cells. In light of the degree of statistical correlation and theoretical considerations, we interpret our present findings as showing that CD8⁺ T cells are involved in the loss or attenuation of E-cadherin expression in GBC.

Generally, CD8⁺ T cells have been considered manifestations of host immune reactions against cancer cells, and favorable prognostic impacts of CD8⁺ T cells have been found in a wide variety of solid cancer tissues^[11-18]. Regarding GBC, the prognostic impact of CD8⁺ T cell has been less investigated, and it remains controversial^[19,20]. Herein we investigated the correlation between the invasion of CD8⁺ T cells or E-cadherin expression of tumor cells and the prognosis of GBC cases (disease free survival, overall survival and disease specific survival), and no significant relationship was found (data not shown).

It is interesting finding that the number of infiltrating CD8⁺ cells were significantly fewer in T3 cases than that of T2 cases. Although it is not statistically significant, the number of infiltrating CD8⁺ cells were fewer in T4 cases than that of T3 cases. Similar results, namely

inverse correlation of T-stage and the density of CD8⁺ cell infiltration has been reported in colorectal cancer^[21]. It is hypothesized that progressive decrease of CD8⁺ cell densities along with tumor invasion could indicate a progressive immune escape and the magnitude of the immune reaction at the early stage of the disease could be a major determinant for controlling the evolution of colorectal cancer^[21].

Same as other sites of cancer, a reduced expression of E-cadherin by cancer cells compared to non-tumorous epithelium has been reported in GBC^[22]. The major finding of the present study is the inverse correlation of CD8⁺ T-cell infiltration and E-cadherin expression by cancer cells. This result might be interpreted in the following two ways. The first interpretation is that the expression of E-cadherin by tumor cells is initially reduced by unknown causes, and then the tumor invaded stroma and CD8⁺ T-cell infiltration is an immune response to the invading tumor. If this is so, it gives rise to the hypothesis that the more reduced the E-cadherin expression of the tumor is, the greater the amount of CD8⁺ T cells attracted will be.

A second interpretation of the results of the present study is that infiltrating CD8⁺ T cells affects the reduced expression of E-cadherin. The mechanisms of the inactivation of the E-cadherin are: (1) a consequence of genetic alterations of E-cadherin; (2) genetic alterations of beta-catenin; and (3) a loss of the interaction of beta-catenin and E-cadherin due to the tyrosine phosphorylation

of beta-catenin^[8]. If CD8⁺ T cells reduce E-cadherin expression, the focus of investigation should be to determine whether CD8⁺ T cells affect any or all of the above-described mechanisms for the inactivation of E-cadherin.

Either way, our results do not adequately establish the relationship between CD8⁺ T cell and E-cadherin expression. It also remains in doubt whether the results of a TMA analysis truly reflect the status of the entire tumor. In addition, the microenvironment of a tumor is not composed of only inflammatory cells but also extracellular matrix and surrounding blood vessels; a degree of hypoxia and tumor-associated fibroblasts also contribute to the tumor microenvironment and interact with each other in a complex manner. We found no study in the English literature that provides evidence that the loss of E-cadherin expression promotes CD8⁺ T-cell infiltration, or evidence that CD8⁺ T cells reduce the E-cadherin expression in cancer cells, which would support our results. Therefore, a prudent validation by different research methods is essential to establish the correlation between CD8⁺ T cells and E-cadherin expression in cancer cells.

In conclusion, the results of the present study indicate an inverse correlation of CD8⁺ T-cell infiltration and E-cadherin expression by cancer cells at invasive areas of GBC. However, our data are not enough to establish the above interaction, and further experimental and clinicopathological analyses are needed to test our results.

COMMENTS

Background

Gallbladder cancer (GBC) often shows the dedifferentiation or clustering of tumor cells at the areas of infiltration. The author speculated that the type or amount of inflammatory cells that infiltrate into invasive areas of GBC may play some roles in the loss of E-cadherin function, and that tumor-infiltrating inflammatory cells thus contribute to the dedifferentiation of GBC.

Research frontiers

This study quantitatively assessed the type and amount of inflammatory cells infiltrating into invasive front of GBC using tissue microarray technique and latest imaging analysis software.

Innovations and breakthroughs

The results of present study indicate an inverse correlation of CD8⁺ T cell infiltration and cancer cells' E-cadherin expression at invasive areas of GBC. No previous study in the English literature provides evidence that the loss of E-cadherin expression promotes CD8⁺ T cell infiltration, or evidence that CD8⁺ T cells reduce the E-cadherin expression in cancer cells. Their results also indicate the inverse correlation of T-stage and the number of CD8⁺ T cell infiltration.

Applications

The goal of the study is to clarify the correlation of CD8⁺ T cell infiltration and E-cadherin expression of cancer cells. These results would be applicable to clarify the mechanism of dedifferentiation and metastasis of cancer. However, further analyses are essential for conclusive result for the correlation of CD8⁺ T cell infiltration and E-cadherin expression of cancer cells.

Terminology

E-cadherin is a well-known cell adhesion molecule and the E-cadherin-catenin complex plays a key role in cellular adhesion; the loss of this function has been associated with tumor dedifferentiation and metastasis. A CD8⁺ T cell was known

as cytotoxic T cell or killer T cell that kills cancer cells, cells that are infected (particularly with viruses), or cells that are damaged in other ways.

Peer-review

This study investigates the association between the expression of E-cadherin and the numbers of several types of inflammatory cells infiltrating into the invasive portion of GBC.

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