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Cluster of Differentiation 44 Expression in Gastrointestinal Malignancies: A Study from South India

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Abstract

Introduction Cancer stem cell markers are now being tried in various cancers as prognostic markers including GI cancer but these kinds of studies are sparse in Indian population.

Materials and Methods This study conducted over a period 50 months. Hematoxylin and eosin-stained slides were screened for grading of the tumor, extent of invasion of tumor, confirmation of metastasis, and staging was done. Immunohistochemical expression of CD44 was graded on the basis of percentage of tumor cells positive for staining. Statistical analysis was done and results were tabulated.

Results: A total of 40 cases of GI cancer were studied. Ascending colon (37.5%) was the common site involved, 37 cases (92.5%) showed invasion beyond the muscularis externa. Most tumors were poorly differentiated (37.5%). Also, 50% of lymph nodes showed tumor deposits. The majority of the cases were in stage II (40%). There was a significant correlation between histopathological type of differentiation with lymph node metastasis and staging of tumor, lymph node metastasis also had significant association with staging.

Grade 2, CD 44 expression was most common followed by Grade 3. Significant association was observed between histopathological differentiations of tumor with CD44 expression. Tumors that are invading beyond muscularis externa and lymph node-positive cases showed moderate to high CD44 expression.

Conclusion CD44 expression was significantly noted in poorly differentiated tumors. Increased expression was also noted in cases of tumors invading beyond muscularis externa and lymph node metastasis. Combination of CSC markers will increase the sensitivity and specificity and predict better overall survival in GI tumors.

Keywords

- gastro intestinal cancer
- gastric cancer
- colorectal cancer
- staging
- immunohisto-chemistry
- CD 44
- cancer stem cells

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Introduction

Gastrointestinal (GI) cancer is ranked third most common cancer occurring worldwide.[1] In India, stomach cancer is the most common (9%), followed by colon and rectum (5.8%), esophagus (4.3%), liver (3.5%), biliary tract and gall bladder accounted for 3.1%.[2] The risk factor for gastric cancer is *Helicobacter pylori* infection. *H. pylori* is known to cause a range of gastric lesions such as chronic gastritis, gastric atrophy, intestinal metaplasia, dysplasia, and finally carcinoma.[1] Colorectal cancer (CRC) is in increasing trends in India. This rise in CRC can be attributed to westernized dietary lifestyle, increased life expectancy, smoking, physical inactivity, and other risk factors.[3] India is now in the stage of transition between developing and developed country.[4]

Histopathological differentiation, staging, lymph node metastasis, hematogenous/perineural invasion, depth of invasion beyond muscularis propria are established prognostic factors for GI cancer.[5] Among these, TNM staging is now the basis for therapy as well as for determining the outcome. However it is not precise; hence, the hunt for reliable biomarker which can predict prognosis is essential.[6]

Cancer stem cells (CSC), which are the subpopulation of tumor cells, are slowly proliferating and responsible for tumor initiation, progression, and metastasis. These cells are resistant to chemotherapy and radiotherapy and are responsible for the recurrence of tumor.[6,7] CD44, CD133, epithelial cell adhesion molecule (EpCAM, CD326) and aldehyde dehydrogenase 1 (ALDH1) are the currently available CSC markers.[6] These markers are now being identified in various cancers as prognostic markers in western countries but data on Indian population is very sparse. As CD44 expression is associated with stem cell ness and poor prognostic factor, studies are being evaluated for the targeted therapy using nanoparticles of salinomycin and paclitaxel coated with monoclonal Ab or hyaluronic acid.[8] Hence, this study is being undertaken to study the immune expression of CD44 among GI malignancy.

AIMS and Objective

1. To study the expression of CD44 among gastrointestinal cancer by immunohistochemistry.
2. To correlate the CD44 expression with various clinicopathological parameters.

Materials and Methods

This is a retrospective study conducted in the Department of Pathology, Tertiary Care Centre of south India. This study included surgically resected specimens of gastrointestinal malignancy received in the Department of Pathology, for a period of 50 months from January 2016 to February 2020. Guided biopsy was excluded from the study as the tumor yield is very less. Demographic profile of the patients such as age and gender was retrieved from pathology request forms. Site and dimension of the tumors were noted down from the gross notes. Hematoxylin and eosin (H&E)-stained slides were retrieved from the archives of pathology. If the H&E slide is not available, paraffin blocks were retrieved and 5 micron thickness sections were cut and stained by H&E.

On microscopic examination, each case was re-assessed by two pathologists independently. Grading of the tumor, extent of invasion of tumor, confirmation of metastasis to lymph nodes were noted. Staging was performed using the criteria of American Joint Committee on Cancer (AJCC).[9]

Immunohistochemical Procedure

Immunohistochemistry was carried out on all 50 cases simultaneously. Thin tissue sections of 3 to 4 µ were taken on silane-coated slides. The slides were incubated at 58 to 60 degree in an incubator. Deparafinization was done in xylene; 2 changes, 10 minutes each. Hydration was done in running tap water and then changed to distilled water for 5 minutes. Antigen retrieval was done with EDTA buffer using a pressure cooker. Slides were brought to room temperature and washed with distilled water. Slides were then treated with endogenous peroxidase block for 20 minutes. Further, slides were washed in Tris buffer solution (TBS), 2 changes 5 minutes each. Slides were treated with power block for 20 minutes. Primary antibody anti-CD44 (Biogenix) was applied and left for 1.5 hours then washed with TBS buffer, 2 changes 5 minutes each. Enhancer was applied and incubated for 20 minutes. Next, it was washed with TBS; 2 changes, 5 minutes each. Secondary antibody was applied for 30 minutes and then washed with TBS, 2 changes 5 minutes each. Diaminebenzidine chromogen was applied for 5 to 10 min and then it was washed with buffer to stop chromogen reaction. Counter staining was done with hematoxylin for 2 minutes and then finally washed with distilled water 2 changes. Dehydrated with alcohol and xylene and mounted with DPX.[10]

Immunohistochemical slides were graded by the two pathologists separately. Also, the IHC slides were scored twice by the same two pathologists to decrease intra-observer variability in a blinded fashion. In unmatched cases, slides were evaluated again by third pathologist and the average of the three were taken as the final score. As there are no standard criteria available in the literature to grade CD44 expression and most authors consider $>10\%$ as positivity is considered as overexpression. We graded into grade 0, grade 1, grade 2, and grade 3 using the following cutoff.

$0 = 0\%$ of tumor cells (negative, grade 0)
$1 \leq 10\%$ of tumor cells (positive) (grade I)
$2 = 11\%$-39% of tumor cells (positive) (grade II)
$3 \geq 40\%$ of tumor cells (positive) (grade III)

Statistical Analysis

All characteristics were summarized descriptively. For continuous variables, the summary statistics of mean $\pm$ standard deviation (SD) were used. For categorical data, the number and percentage were used in the data summaries and diagrammatic presentation. Chi-square (χ^2) test was used for the association between two categorical variables.

If the p-value was < 0.05, then the results were considered to be statistically significant; otherwise they were considered as not statistically significant. Data were analyzed using the SPSS software v.23.0. and Microsoft office 2007.

The study was cleared from the ethics committee bearing number No532/L/11/12/Ethics/ ESICMC&PGIMSR/ESTT Vol-IV dated 1.9.2022.

Results

During the study period, 40 cases of gastrointestinal carcinoma were available. Age of the patient ranged from 30 to 83 years with a median of 53.8 years. Also, 54.5% of the cases were in the range of 40 to 60 years, whereas 36.4% were above the age of 60 years. Males outnumbered the females (M:F = 1.5:1).

Out of 40 cases, 15 (37.5%) were in the ascending colon, 13 (32.5%) were in stomach, 9 (22.5%) were in rectosigmoid, and 3 (7.5%) were in the transverse colon. Also, 40% of the tumor had tumor size in the range of 4 to 6 cm, whereas 17.5% had tumor size more than 6 cm (►**Fig 1A**). Also, 37 cases (92.5%) showed invasion beyond the muscularis externa, while in 3 (7.5%) cases, tumor was confined to mucosa and submucosa. At microscopy, 37.5% of tumors were poorly differentiated, 35% were well-differentiated, and 27.5% were moderately differentiated. Lymph nodes were retrieved only in 30 cases, out of which 15 cases (50%) showed tumor deposits. Then, 40% of cases were in stage II, 32.5% cases were in stage III, 20% were in stage I, 7.5% were in stage IV at the time of diagnosis.

On statistical analysis, an association was noted between histopathological type of differentiation with lymph node metastasis (p-value 0.003) and histopathological type of differentiation with staging of tumor (p-value 0.020). Even the lymph node metastasis also had significant association with staging (p-value < 0.00001) (►**Table 1**).

Semi quantitative analysis of CD 44 immunohistochemistry:

Out of 40 cases of GI malignancy, 19 (47.5%) cases showed moderate expression of CD44 (grade 2), 11 cases (27.5%) showed high expression of CD44 (grade 3), 8 cases (20%) showed low expression of CD44 (grade1), and in 2 cases, the tumor score was negative.

There was a significant association between histopathological differentiation of tumor with CD44 expression (p-value = 0.0008). Also, 81.8% of poorly differentiated tumors showed grade 3 (>40%) expression, 38.9% of moderately differentiated tumors showed grade 2 (>10% and <40%) expression, and 55.6% of well differentiated tumors showed Grade 1 (<10%) expression. No significant correlation was observed between CD 44 expression and age (p-value: 0.972); gender (p-value: 0.093); site of the tumor (p-value: 0.867); size of the tumor (p-value: 0.241); invasion (p-value: 0.900); lymph node metastasis (p-value: 0.972) and staging (p-value: 0.561).

In the present study, it was also observed that tumors invading beyond muscularis propria and tumors with lymph node positivity showed more CD44 expression though statistical correlation was not present, reason may be due to the small sample size. Of the 37 cases that showed invasion beyond muscularis propria, 27 cases showed moderate and high CD44 expression (11 and 16 cases, respectively). Of the 15-lymph node-positive cases 14 cases showed moderate and high CD44 expression (9 and 5 cases, respectively) ►**Fig 1 B–L**.

It is evident from the present study that, poorly differentiated tumors have high expression of CD44. And tumors invading the muscularis propria and lymph node shows moderate to high expression of CD44. ►**Table 2** shows the correlation of CD44 expression with various clinicopathological parameters.

Discussion

GI cancer is a global problem, more so in the developed world than developing world. In 2016, among both the sexes, stomach cancer ranked first in incidence accounting to 9% while colorectal cancer constituted 5.8%.[1] Even though incidence among the Indian population is at the lower limit of the global range, it is showing an increasing trend due to globalization and adaptation of the western life style and food habits.[3] The disease affects equally among both the sex and commonly found after 50 years of age.[11]

GI epithelium is replaced once in every 5 days. The multipotent stem cells that are observed at the base of crypt are responsible for the renewal of the epithelium, these cells are multipotent and can differentiate into epithelial cells, goblet cells, and neuroendocrine cells.[12] Now it is an accepted theory that cancer arises from stem cells.[13]

Cancer stem cells play a crucial role in the pathogenesis of cancer from its initiation, progression, metastasis, resistant to treatment, and recurrence. Identification of CSC in the tumor and their percentage of expression are the key prognostic factors of the tumor and also for the initiation of targeted therapy.[14] CD133, CD44, Ep CAM, and ALDH1 are the potential CSC markers that can predict the prognosis and has been investigated in various cancers.[6]

CD133 was the first marker for the identification of CSC. It was first identified on hematopoietic stem cells in 1997. CD133 stains the normal stem cells, embryonic stem cells, circulating primordial endothelial cells. This marker is used to isolate the CSC in tumors of colon, brain, prostate, liver, pancreases, and lungs.[4]

Another still common and widely used CSC marker is CD44, which is a class I trans membrane protein and is a specific receptor for hyaluronic acid. It was first discovered on lymphocytes in 1982. It is also an extracellular adhesion molecule, which is responsible for regulating cell adhesion, proliferation, motility, migration differentiation and angiogenesis.[4]

By mRNA splicing, many variants of CD44 has been found, namely CD44v2, CD44 v3, CD44 v5, CD44 v6, CD44v, and CD44 s. CD 44 plays a role in many signaling pathways such as MAPK, Wnt, and P13/Akt. CD44s is the smallest of all CD44 molecule and is responsible for epithelial to mesenchymal transition. CD44 is overexpressed in many solid cancer, breast cancer, colon, gastric and bone tumor. It is now the established prognostic marker for hepatocellular cancer, colonic cancer, gastric cancer, and pancreatic cancer.[14]

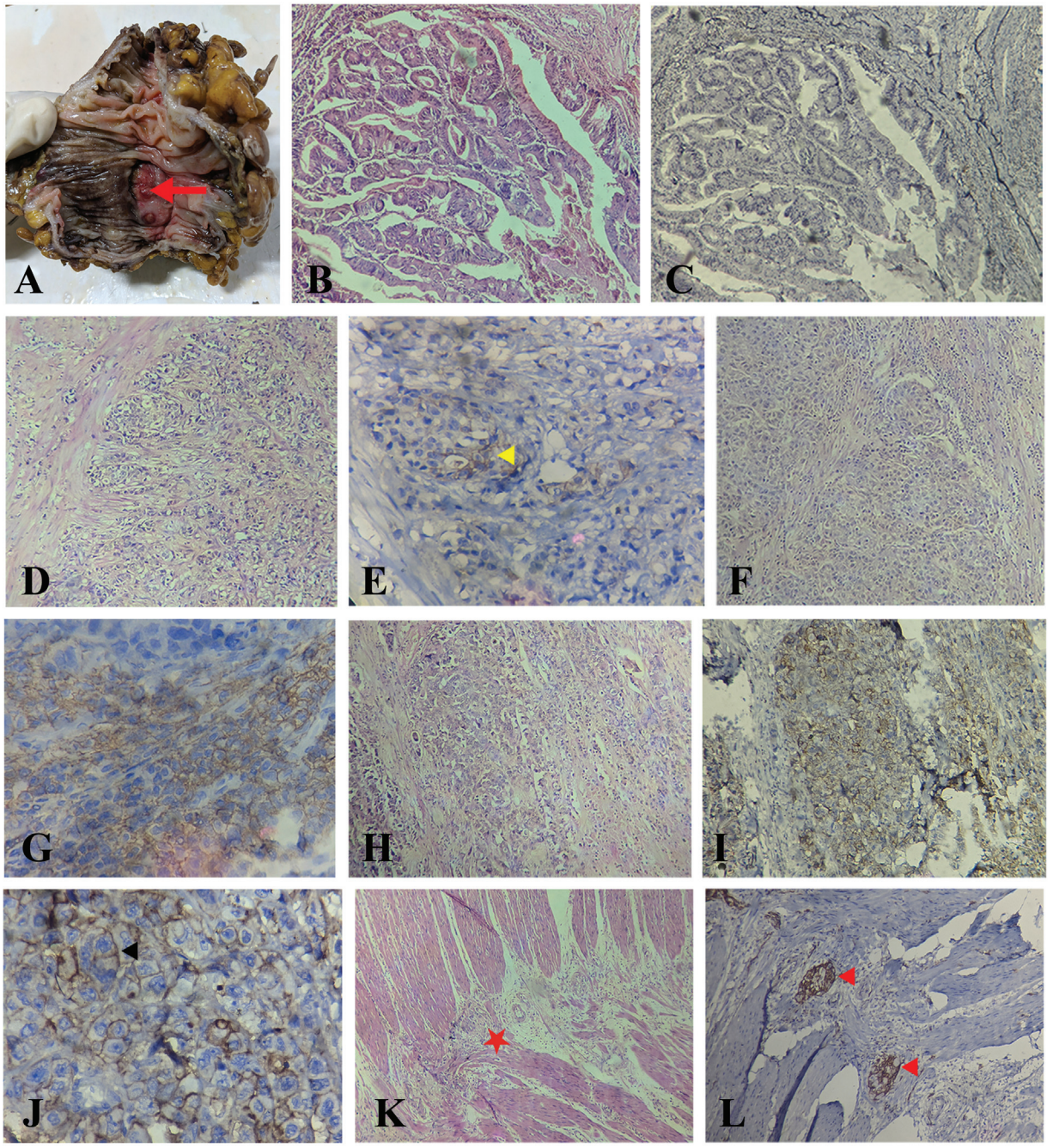

Fig. 1 **A**: Gross photograph of colon showing exophytic fleshy tumor. **B**: Microphotograph showing well differentiated adenocarcinoma (H&E, X20) same section in figure **C** stained negative on CD 44 staining (anti CD 44, X20). **D**: Moderately differentiated adenocarcinoma showing tumour nests. (H&E, X20), same section in figure **E** showing grade 1 staining. (anti CD44, X20). **F**: Poorly differentiated adenocarcinoma showing nests and single tumor cells (H&E, X20), same section in figure **G** showing grade 2 staining. (anti CD44, X40). **H**: Microphotograph of poorly differentiated adenocarcinoma, showing infiltration to deeper tissue (H&E, X20), same section scored grade 3 on anti CD44 staining (►**Fig B–L**) (anti CD44, X20). High power of the same showing grade 3 membrane staining (anti CD44, X40). **K**, **L**: Microphotograph showing deeper invasion of tumor tissue (K) (H&E, X20), which is highlighted by CD44 staining (anti CD44, X20).

Like CD44, Ep CAM is also an adhesion molecule identified as CSC in 2004. It is involved in proliferation, migration, and mitogenic signal transduction. It is expressed in all adenocarcinoma, a few squamous cell carcinomas, retinoblastomas, and hepatocellular carcinomas.[6] Aldehyde dehydrogenase 1 (ALDH1) is another CSC marker mainly in pediatric solid tumor and many adult tumours.[7]

In the present study, CD44 expression is found to have strong association with histopathological differentiation with significant *p*-value. Statistical correlation was not significant between CD44 expression and other clinicopathologic parameters such as age, sex, site, tumor size, lymph node metastasis, and staging. Chaitra et al[4] studied 26 cases of colorectal cancer, observed a positive correlation between

Table 1 Significant correlation between clinicopathological parameters

Lymph node metastasis	Histological type differentiation			Total	Chi square (2 df)	*p*-Value
	Poorly differentiated	Well-differentiated	Moderately differentiated			
Negative	2	10	3	15	11.7	0.003*
Positive	7	1	7	15		
Total	9	11	10	30		
Stage						
I	1	2	5	8	15	0.020*
II	7	1	8	16		
III	6	7	0	13		
IV	1	1	1	3		
Total	15	11	14	40		
Stage	**Lymph node metastasis** Positive	Negative				
I	0	8		8	15	0.020[a]
II	0	6		6		
III	12	1		13		
IV	3	0		3		
Total	15	15		30		

[a]Statistically significant.

CD44 with staging (*p*-value 0.003) and no significant association was observed between age, sex, site, and grade with CD44. Addition of another CSC marker CD133 showed a significant correlation with stage (*p*-value: 0.002), histopathological differentiation (*p*-value: 0.037), lymph node involvement (*p*-value: 0.001), hence authors concluded that a combination of CSC markers will better predict patient overall survival and increase the sensitivity, specificity, positive predictive value, and negative predictive value.

Wang et al[6] conducted a meta-analysis of CD44 expression in CRC, they studied 48 publications and concluded that CD44 expression has a significant correlation with lymph node metastasis (*p*-value: 0.004), differentiation (*p*-value: 0.05), distance metastasis (*p*-value: 0.044) and tumor size (*p*-value: 0.056) and no association was observed between location and staging.

In another meta-analysis study by Wang et al,[15] on gastric cancer and CD44 expression, authors observed a positive correlation between CD44 expression with stage (*p*-value: 0.02), tumor size (*p*-value: 0.01), lymph node metastasis (*p*-value: 0.004), lymphocytic invasion (*p*-value: 0.02), venous invasion (*p*-value: 0.001), but not with sex, differentiation of tumor and tumor type. It is evident from the observation of our study and review of literature that a good sample size is essential for correlating CD44 expression and clinicopathological parameters.

CD44 expression analysis can be carried out by RT-PCR, immunohistochemistry, and ELISA. ELISA detects the CD44 in the blood but blood level of CD44 is also influenced by immune system. RT-PCR detects the gene expression. Hence IHC is cheap, easily available, reliable and sensitive method for study of expression of CD44.[3]

Identification of CSC is not only of prognostic value but also for therapeutic importance. As these cells are resistance to conventional chemotherapy, they will cause therapeutic failure and recurrence. Now drugs such as pacitaxel, gemcitabine derivatives, salinomycin, 8-hydroxyquinoline, si RNA and others are now being targeted via nanoparticle aganist CSC in various cancers such as breast, colon, ovaries, solid tumors, hematological malignancy, and others.[8] Hence, in near future, the identification of CSC subpopulation will be the important factor in deciding the management of cancer.[16,17]

The limitation of the present study is less sample size. A multicentric study involving more sample size and also combination of CSC markers is required to document the role of cancer stem cell markers in GI carcinomas.

Conclusion

From the observation of the study, we conclude that CD44 overexpression was significantly correlated with poor differentiation. Increased expression was noted with tumors invading beyond muscularis externa and lymph node metastasis. It is also evident from review of literature that CD44 expression is having significant association with metastasis, staging, lymph node involvement. CD44 is also a prognostic marker and targeted therapy can be used to increase overall survival. A combination of CSC markers will increase the sensitivity and specificity and predict better overall survival in GI tumors.

Funding
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Table 2 CD44 expression with various clinicopathological parameters

Parameters	Number of cases (%)	CD44 grading								χ^2 value	*p*-Value
		Negative		<10%		11%-39%		≥40%			
		N	%	*N*	%	*N*	%	*N*	%		
Age (y)											
<40	3 (7.5)	0	0.0	1	11.1	1	5.6	1	9.1	1.30	0.972
40-60	25 (62.5)	1	50.0	6	66.7	12	66.7	6	54.5		
>60	12 (30)	1	50.0	2	22.2	5	27.8	4	36.4		
Sex											
Male	24 (60)	0	0.0	4	44.4	14	77.8	6	54.5	6.41	0.093
Female	16 (40)	2	100.0	5	55.6	4	22.2	5	45.5		
Site											
Ascending colon	15 (37.5)	1	50.0	4	44.4	8	44.4	2	18.2	4.61	0.867
Rectosigmoid	9 (22.5)	1	50.0	1	11.1	4	22.2	3	27.3		
Stomach	13 (32.5)	0	0.0	3	33.3	5	27.8	5	45.5		
Transverse colon	3 (7.5)	0	0.0	1	11.1	1	5.6	1	9.1		
Diameter											
0-2	5 (12.5)	1	50.0	1	11.1	2	11.1	1	9.1	11.53	0.241
2-4	12 (30)	0	0.0	1	11.1	5	27.8	6	54.5		
4-6	16 (40)	0	0.0	6	66.7	8	44.4	2	18.2		
>6	7 (17.5)	1	50.0	1	11.1	3	16.7	2	18.2		
Invasion of tumor (T)											
Serosa, muscularis propria and adjacent structure	37 (92.5)	2	100.0	8	88.9	16	88.9	11	100.0	0.59	0.900
Mucosa and submucosa	3 (7.5)	0	0.0	1	11.1	2	11.1	0	0.0		
Lymph node metastasis											
Negative	15 (50)	1	100.0	4	80.0	9	50.0	1	16.7	4.96	0.174
Positive	15 (50)	0	0.0	1	20.0	9	50.0	5	83.3		
Stage											
I	8 (20)	1	50.0	3	33.3	3	16.7	1	9.1	7.74	0.561
II	16 (40)	1	50.0	5	55.6	5	27.8	5	45.5		
III	13 (32.5)	0	0.0	1	11.1	8	44.4	4	36.4		
IV	3 (7.5)	0	0.0	0	0.0	2	11.1	1	9.1		
Histological type differentiation											
Poorly differentiated	15 (37.5)	0	0.0	2	22.2	4	22.2	9	81.8	20.45	0.008*
Moderately differentiated	11 (27.5)	0	0.0	2	22.2	7	38.9	2	18.2		
Well-differentiated	14 (35)	2	100.0	5	55.6	7	38.9	0	0.0		
Total	40	2	100.0	9	100.0	18	100.0	11	100.0		

*Statistically significant.

Conflict of Interest
None declared.

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