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ORIGINAL ARTICLE

Expression of clusterin in colorectal carcinoma in relation to clinicopathological criteria

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Abstract

Background/Aim: Colorectal carcinoma (CRC) carries a high incidence of morbidity and mortality. Prognosis is related to nodal metastasis and stage. Clusterin is a widely distributed glycoprotein with not yet fully understood functions. Clusterin may be overexpressed in some tumours or under expressed in other tumours. The aim behind this study is to examine the relation of clusterin cytoplasmic immunostaining to tumour characteristics, disease relapse, and survival in CRC. *Materials and Method*s: Paraffin blocks of 133 CRCs were retrieved from the Department of Pathology, King Abdulaziz University, Jeddah, Saudi Arabia. Immunostaining was done using antibody to clusterin. Staining expression in 10% of malignant cells was used as a cut-off to determine low immunostaining and high immunostaining. Statistical tests were used to evaluate the association of clusterin immunostaining with clinicopathological parameters. *Results:* Immunohistochemical results showed clusterin low immunostaining in CRC and nodal metastases. No association was found between clusterin immunostaining and tumour grade, age, tumour invasiveness, distant metastases, vascular invasion, nodal metastases, relapse, and survival. *Conclusion:* Our study showed low clusterin immunostaining in CRC with lack of association with prognostic indicators in CRC. These results raise the controversy of understanding the role of clusterin in CRC. Further molecular studies are required to explore more about possible mechanisms of clusterin association with tumorigenicity, apoptosis, tumour growth progression, local and vascular invasion, and metastasis of CRC.

Keywords: clusterin, colorectal carcinoma, immunohistochemistry, lymph node status

INTRODUCTION

Colorectal carcinoma (CRC) is associated with significant mortality and morbidity worldwide.[1] CRC prognosis is determined by distant tumour metastasis and local invasiveness. Metastasis is associated with poor treatment outcomes. Accordingly, markers of metastasis are essential to improve treatment outcomes.[2,3] CRC is most common cancer in Saudi men and represents 5.3% of all tumours. In women, CRC is the third most common cancer and represents 4% of all tumours.[4]

Clusterin is a cryptic glycoprotein with a wide distribution within different tissues. Although discovered about twenty years ago, its functions are still not fully understood.[5,6] Clusterin expression has been related to cellular response to stress,[7] cell damage recovery,[8,9] senescence,[10] tumorigenesis and apoptosis.[5] Clusterin is overexpressed in some tumours,[11] where it may inhibit apoptosis during tumour cell transformation and metastasis. However, there have been reports of diminised expression in other tumours, where it may have a pro-apoptotic

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function.[12] Abnormal clusterin expression has been shown in different cancers such as prostate and breast cancer.[13] In CRC, clusterin has been studied used as a marker in diagnosis.[14,15]

Several studies have examined the prognostic significance of clusterin in human cancer with conflicting results. The objective of this study is to analyse the immunostaining of clusterin in primary CRC and nodal metastasis and its relation to clinicopathological parameters and prognosis.

MATERIALS AND METHODS

Patients

Paraffin wax blocks of 133 primary CRC and their corresponding 56 regional nodal lymph node metastases were used in this study. Patients' data and pathological materials were obtained from the Department of Pathology at King Abdulaziz University, Jeddah, Saudi Arabia. Clinical and pathological data of patients are shown in Table 1. The Research Committee of the Biomedical

TABLE 1: Clinicopathological parameters of cases

Parameter		Number (%)
Age	< 60 years	67 (50.4%)
	≥ 60 years	66 (49.6%)
Sex	Male	68 (51.1%)
	Female	65 (48.9%)
Grade	Well-differentiated	32 (24.1%)
	Moderately-differentiated	85 (63.9%)
	Poorly-differentiated	16 (12%)
Tumour location	Right colon	34 (25.6%)
	Left colon	87 (65.4%)
	Rectum	12 (9%)
Tumour size	< 5cm	52 (39.1%)
	≥ 5cm	81 (40.9%)
Primary tumour stage	T1	2 (1.5%)
	T2	20 (15%)
	T3	102 (76.7%)
	T4	9 (6.8%)
Lymphovascular invasion	Negative	126 (85%)
	Positive	7 (15%)
Margin status	Free	126 (85%)
	Involved	7 (15%)
Nodal metastasis	Negative	71 (53.4%)
	Positive	59* (44.4%)
	Not available	3 (2.2%)
Distant metastasis	Negative	96 (72.2%)
	Positive	37 (27.8%)
Survival	Died of disease	31 (23.3%)
	Alive	91 (68.4%)
	Data not available	11 (8.3%)
Relapse	Negative	86 (64.7%)
	Positive	47 (35.3%)

T1: Tumour invades submucosa; T2: Tumour invades muscularis propria; T3: Tumour invades through the muscularis propria into the subserosa or into non-peritonealised pericolic or perirectal tissues; T4: Tumour directly invades other organs or structures, and/or perforates visceral peritoneum

*Only 56 of the 59 lymph nodes were available for TMA construction

Ethics Unit, Faculty of Medicine, King Abdulaziz University approved the study.

Tissue microarray

The construction and design of tissue microarrays were done as previously described.[16] Haematoxylin and eosin-stained sections of CRC and nodal metastasis were rechecked by a pathologist (WG). Areas of viable tumour tissues were identified on the slides. Areas of prominent stroma, autolysis, or necrosis were avoided. Paraffin blocks belonging to the chosen slides were examined for validity to perform TMA. Two donor blocks from each tumour and nodal metastasis were used. Two tissue cores with a 1.5 mm diameter were taken from donor blocks and inserted into a recipient paraffin block. Construction was done using an automated tissue microarrayer (TMA Master 1.14 SP3 from 3D Histech Ltd. Budapest, Hungary).

Clusterin immunostaining

Tumour and metastasis TMA paraffin blocks were cut at 4 μm thickness. Tissue sections were mounted on positive charged slides. Immunostaining was performed in an automated immunostainer (BenchMark XT, Ventana® Medical Systems Inc., Tucson, AZ, USA). Sections were deparaffinised in xylene and rehydrated. Pre-treatment was done using CC1 (prediluted cell conditioning solution) for 60 min. Anti-human Ventana® anti-clusterin mouse monoclonal primary antibody was used and incubated at 37°C for 16 minutes with tissue sections. The detection kit used was Ventana® I-view DAB. Slides were washed, counterstained with Mayer's haematoxylin, and mounted. Appropriate positive (normal breast tissue) and negative controls were used.

Interpretation of clusterin immunostaining

Sections were examined under light microscopy and the percentage of tumour cells exhibiting clusterin cytoplasmic immunostaining was noted independently without knowledge of the clinical and histopathological parameters of patients. A cut off percentage of 10% was used as previously described.[17] Based on staining percentage, the tumours were categorised as (1) low immunostaining [<10% of malignant cells are positive] and (2) high immunostaining [≥10 % of malignant cells were positive].

Statistical analysis

Statistical tests were done with SPSS® Release 21. Statistical significance was 2-sided and determined at p value ≤ 0.05. The difference between two variables was tested using the Mann Whitney test, while the association between three or more groups of patients was done by the Kruskal Wallis test. The variance along one variable was revealed by non-parametric chi-square. To test clusterin immunostaining as a predictor of lymph node metastasis, blood metastasis, resection margin positivity and lymphovascular invasion, logistic regression analysis was used. Disease free survival and overall survival probabilities were tested by Kaplan-Meier procedure.

RESULTS

Clusterin immunostaining

In primary tumours, low immunostaining was detected in 105 (78.9%) while 28 (21.1%) showed high immunostaining ($p < 0.001$) (Fig. 1 A & B). A higher percentage (87.5%; 49/56) of nodal metastasis showed low clusterin immunostaining ($p < 0.001$) (Fig. 1 C & D). However, no statistically significant difference between clusterin immunostaining in primary CRC and nodal metastasis could be found ($p = 0.688$).

The relationship between clusterin immunostaining and clinicopathological features of CRC

Clusterin immunostaining showed no statistically significant association with tumour differentiation, sex, age, tumour invasiveness, stage, lymphovascular invasion, and positive resection margins, regional lymph node metastasis, or distant metastasis (Table 2).

Clusterin immunostaining and patient outcome

In regression analysis, low clusterin immunostaining of the primary tumour could not independently predict resection margins, lymphovascular invasion, relapse, nodal metastasis, and distant metastasis (Table 3). Also, clusterin immunostaining in the primary tumour showed no relation to disease free survival Log Rank (Mantel-Cox) = 0.039, $p = 0.844$, and overall survival Log Rank (Mantel-Cox) = 0.726, $p = 0.394$ (Figs. 2 and 3).

DISCUSSION

New molecular biomarkers of metastasis are needed to improve therapeutic interventions and outcome in CRC. The introduction of new

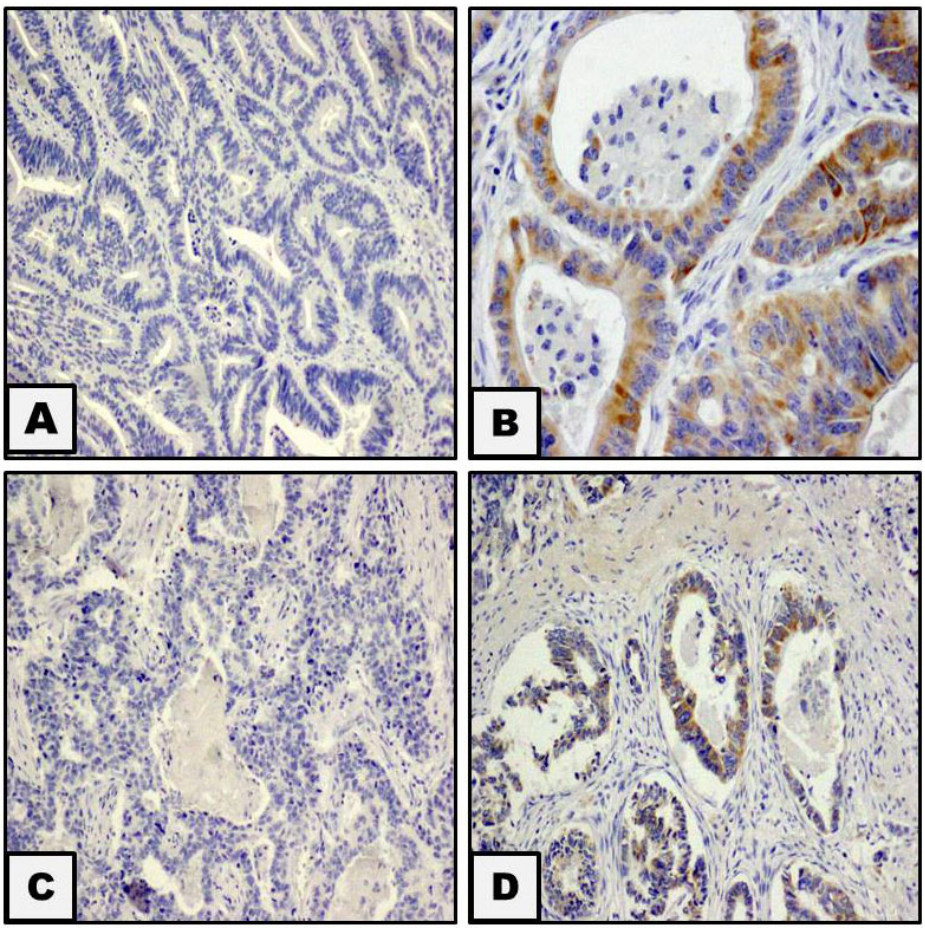

FIG. 1: Immunostaining of clusterin in colorectal carcinoma. Immunohistochemical staining was done with anti-clusterin. A: a moderately-differentiated CRC showing negative labelling for clusterin (100x). B: Positive cytoplasmic labelling for clusterin in a moderately-differentiated colorectal carcinoma (200x). C: Negative labelling for clusterin in a metastatic CRC in lymph node (200x). D: Positive cytoplasmic labelling for clusterin in a metastatic CRC in lymph node (200x)

TABLE 2: Clusterin immunostaining in relation to clinicopathological parameters

	***p* value**
Age	0.639✪
Sex	0.067✪
Grade	0.269*
Tumour location	0.827*
Tumour size	0.185✪
Depth of invasion (pT)	0.185*
Lymphovascular invasion	0.901✪
Margin status	0.148✪
Nodal metastasis	0.913✪
Distant metastasis	0.567✪
Relapse	0.624✪

*Kruskal-Wallis Test; ✪Mann-Whitney test

TABLE 3: Binary logistic regression analysis for clusterin immunostaining

Variable	exp(B)	95% CI for exp(B)	*p* value
Surgical resection margins	0.950	0.234 - 3.857	0.943
Lymphovascular invasion	0.544	0.145 - 2.043	0.367
Nodal metastasis	0.730	0.302 - 1.762	0.483
Distant metastasis	1.136	0.193 - 6.700	0.888
Relapse	0.833	0.165 - 4.219	0.826

markers for detection and diagnosis of CRC is a major goal of recent research. Clusterin is present in two isoforms: the nuclear form (nCLU) is located in the nucleus and secreted form (sCLU) presents in the cytoplasm. Loss of Ku80 and the cytoplasmic relocalisation of Ku70 clusterin isoforms are related to cell death inhibition and cancer progression. The debated role of clusterin in CRC lately was attributed to the differential expression of clusterin forms displaying antagonistic functions (nCLU and sCLU) reflecting the "tumour suppressor" and the "tumour promoting" roles of this protein in cancer.[18] Previous studies demonstrated nuclear expression of clusterin in normal mucosa where it serves as cell cycle regulator and apoptosis inducer.[19-21] The nuclear localisation of clusterin in normal colonic mucosa is consistent with the involvement of its proapoptotic nuclear form in cell cycle progression regulation and in induction of cell death.[22] When the proapoptotic nuclear form disappears and the prosurvival secreted/cytoplasmic isoform is overexpressed, the normal cell is triggered to transform to neoplastic phenotype. In addition, the acquisition of aggressiveness, the loss of the proapoptotic clusterin is associated with the loss of DNA repair activity of Ku70/80.[22] The incidence of increased circulating clusterin in patients' sera before the confirmation of tissue diagnosis raises the suggestion of using clusterin as marker of increased risk to CRC, which may be used in screening and preventive measures.[23]

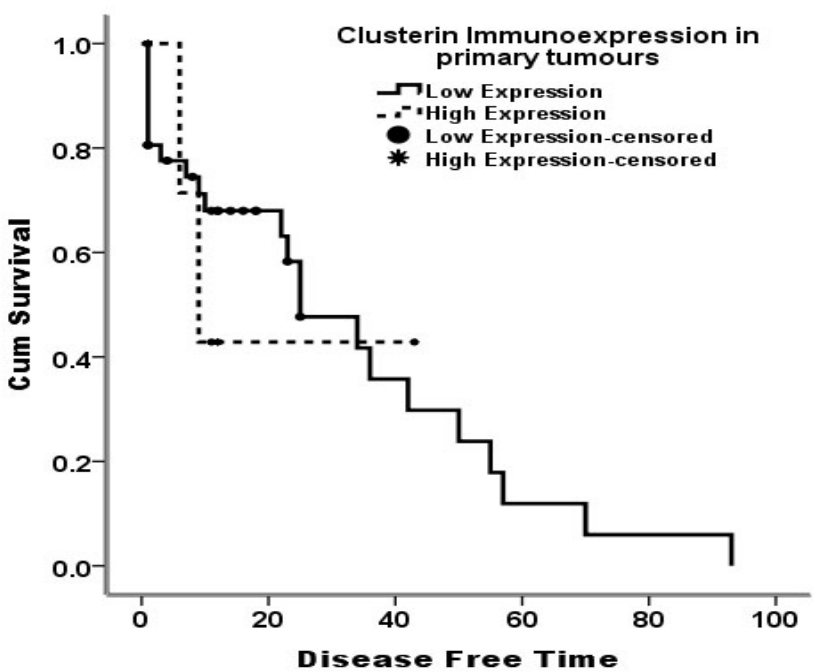

FIG. 2: Disease-free survival curve (Kaplan Meier) associated with clusterin immunostaining: low clusterin immunostaining; and high clusterin immunostaining (log-rank = 0.039, $p = 0.844$)

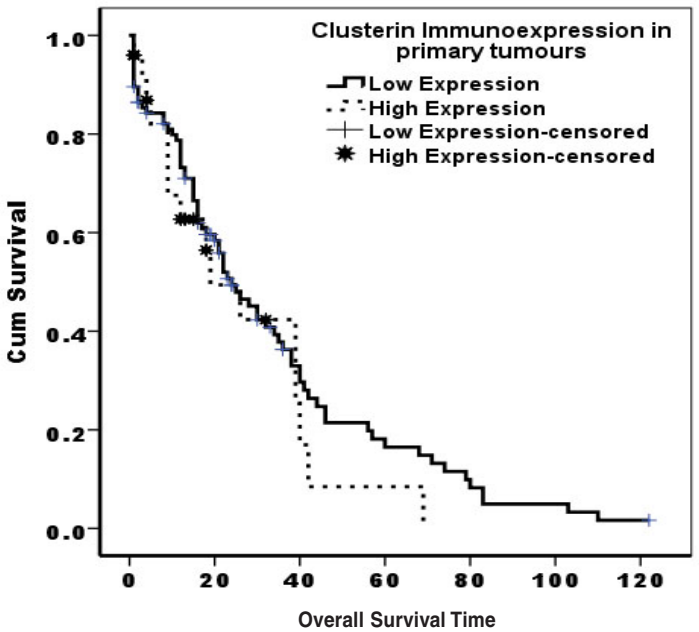

FIG. 3: Overall survival curve (Kaplan Meier) associated with clusterin immunostaining: low clusterin immunostaining; and high clusterin immunostaining (log-rank = 0.726, $p = 0.394$)

Cytoplasmic clusterin was overexpressed in certain tumours,[11] where it is assumed to have anti-apoptotic activity during cellular transformation and metastasis. On the other hand, clusterin is downregulated in other tumours[12] where it may play a pro-apoptotic role. Clusterin expression was increased in cervical cancer compared to normal cervical tissues. High clusterin expression predicts survival in metastatic uterine cervix cancers.[24,25] Clusterin immunostaining and nodal metastasis can be used to predict survival in pancreatic carcinoma.[26] Also, clusterin expression is significantly associated with the biological behaviour of ovarian carcinoma.[27]

In the current study, our findings indicate prevalence of low cytoplasmic clusterin immunostaining in this subset of CRC and associated nodal metastasis. In addition, cytoplasmic clusterin was not found to correlate to any of the tumour characteristics or patient outcome. Previous studies showed conflicting results on whether clusterin is upregulated[28,29] or downregulated[17,30,31]. Cytoplasmic clusterin correlated with tumour progression and metastatic potential, survival, and advanced stage in some studies.[22,28,29,32] The conflict in these results may arise from the material used (*in vivo* versus *in vitro*), number of patients included, methods used to detect clusterin cytoplasmic expression, and the method of scoring of immunostaining for clusterin. Further studies should focus on standardisation of the method of immunostaining and the scoring. Most studies mentioned above used only the intensity of immunostaining of clusterin to report the expression. In our study, we used the extent (%) of malignant cells expressing clusterin. Results based on intensity of immunostaining should be viewed critically because intensity of staining are affected by variations in fixation and tissue processing especially for specimens stored for a period of several years.[16]

Clusterin activity is double-faced; firstly, clusterin correlates with cell survival mediated by cytoplasmic retention of clusterin by calcium-dependent cellular mechanism. Secondly, clusterin translocation to the nucleus is a proapoptotic mechanism in calcium-deprived cells.[33]

Externally stimulated apoptosis is a major pitfall in chemotherapy. Recently, several molecular mechanisms have shown how malignant cells overcome apoptosis. Clusterin was recognised as having proapoptotic and/or antiapoptotic activity, which is related to calcium homeostasis.[33] Clusterin was reported to translocate from cytosol to nucleus at low intracellular Ca2þ levels, which correlates with apoptosis in colorectal cancer cells.[34] Tumour

cells undergoing apoptosis expressed low levels of clusterin.[31] High expression of cytoplasmic clusterin in CRC was inversely correlated with tumour apoptotic index.[29] The overexpression of clusterin was associated with adenomatous polyposis coli gene expression in colon cells.[30] Tumour progression to poorly differentiation metastasis is associated with cytoplasmic clusterin translocation. Thus, there is controversy about the exact role of clusterin in tumours. This may be related to the shift between clusterin isoforms and their production.[22]

The proper understanding of the functions of clusterin isoforms and the underlying molecular mechanisms involved in the regulation of clusterin activity may provide a prospective mechanism for overcoming malignant cell apoptosis resistance. This can subsequently help to avoid large doses of chemotherapy drugs, and lower the complications of chemotherapy.

The immunoexpression of clusterin in normal colonic mucosa is controversial as some studies have reported that clusterin immunostaining was intense in tumour tissues compared to normal tissues[17,31] while other studies have reported down regulation of clusterin CRC relative to paired normal mucosa[30]. A limitation of the current study is the lack of examination of clusterin immunostaining in normal colonic mucosa, as such material was not available to us. This would help to explore more about the pattern of clusterin staining in the non-neoplastic colon.

Conclusion

Contrary to some other studies, our study indicates that clusterin shows low immunostaining in CRC and is not related to prognostic indicators outcome and survival in CRC. This raises the controversy of the understanding of clusterin role in CRC. A molecular approach to the possible role of clusterin in CRC is required to clarify its role in tumourigenity (initiation, apoptosis, and cell growth progression). Also, its role in nodal and distant metastasis needs attention.

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