Decreased Immunoexpression of Standard Form of CD44 Is an Independent Favourable Predictor of Nodal Metastasis in Colorectal Carcinoma

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Abstract. *Background: CD44 is a transmembrane glycoprotein and is associated with cell–matrix and cell–cell interactions. CD44 expression was shown to be relevant to tumour progression in various types of human cancer. The objective of this study was to investigate the relationship between the expression levels of the standard form of CD44 (CD44s), and clinicopathological characteristics in a subset of colorectal carcinomas (CRC). Patients and Methods: A total of 96 cases of CRC were retrieved from the archives at the Department of Pathology at King Abdulaziz University, Jeddah, KSA. Immunohistochemistry was performed using antibodies to CD44s. A cut-off of <10% of positive neoplastic cells was used to define low expression, 10-50% to define moderate expression and >50% to define extensive expression. Statistical tests were used to determine the association of CD44s with clinicopathological characteristics in a subset of colorectal carcinomas and survival. Results: Immunostaining results showed that there was no association between C44 immunoexpression and age of patients, tumour grade, depth of invasion, vascular invasion, recurrence and survival. CD44s immunolabelling was found to have an association with nodal metastasis and to be an independent predictor of nodal metastasis. Conclusion: Loss of CD44s immunolabelling in CRC is an independent favourable predictor of regional lymph node metastasis. On the other hand, CD44s loss has no significant association with disease recurrence or survival. Extensive in vivo and in vitro molecular studies are required to elucidate the possible mechanistic association of CD44s with tumour initiation, progression, invasion and metastasis in primary CRC, and in nodal and distant metastases.*

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Key Words: CRC, CD44s, IHC, lymph node status.

Colorectal carcinoma (CRC) is a common type of cancer with a considerably poor prognosis and mortality all over the world. The disease outcome is determined by the extent of local invasion and tumour metastasis. Development of metastases heralds the failure of treatment, which will be subsequently palliative. Hence, the detection of molecular markers of metastasis is essential in order to improve treatment protocols (1, 2). In Saudi males, CRC is the most common malignancy. According to the Saudi Arabian National Cancer Registry in 2005, CRC represented 5.3% of all tumours. In females, CRC represented 4% of all tumours and was the third most common type of cancer. (3)

CD44, a transmembrane glycoprotein, is associated with cell–matrix and cell–cell interactions (4). It is encoded by a gene in which alternative splicing of exons forms a transcript that encodes a wide range of proteins, including a ubiquitous multifunctional standard form CD44s (5). CD44s is abundant and expressed in a wide range of epithelial and non-epithelial tissues, except for microglia. The colon and rectum exhibit strong CD44s labelling and milder expression of CD44 variants (6-8). CD44 exhibited structural and functional changes during malignant transformation (9). CD44 is expressed on malignant cells, and on cancer stem cells (10). In addition, CD44 has been shown to be linked to metastasis in some malignant tumours (11). There is accumulating evidence in the literature correlating CD44 expression with tumour growth, proliferation, and metastasis. It is also gaining interest as a molecular marker of metastasis.

The aim of the current study was to examine the expression of CD44s in a subset of primary CRC and determine its relation to tumour differentiation, invasion, metastasis, recurrence and disease-free survival.

Patients and Methods

The study included paraffin wax blocks of tumour from 96 cases of CRC (in the period from 2003 to 2009). Cases were retrieved from the archives of the Department of Pathology at King Abdulaziz University, Jeddah, Saudi Arabia. Clinicopathological characteristics of patients are listed in Table I. The study was approved by the Research Committee of the Biomedical Ethics Unit, Faculty of Medicine, King Abdulaziz University. Disease-free survival (DFS) and disease-specific survival (DSS) were calculated as the time from diagnosis to the appearance of recurrent disease (or date last seen disease-free), and time from diagnosis to death (due to disease) or to the date last seen alive, respectively. In calculating the DSS, the patients who died of other or unknown causes were censored.

CD44s immunostaining. Paraffin blocks of tumours were cut at 4 μm, and mounted on positive charged slides (Leica Microsystems Plus Slides, Menzel, Braunschweig, Germany). Sections were deparaffinised in xylene and rehydrated in graded alcohol. Slides were immersed in H_2O_2 (0.3%) for 12 minutes to block the endogenous peroxidase activity. Slides were then pre-treated in microwave oven in 10 mM citrate buffer (pH 6) for three cycles of 5 minutes each. Antihuman mouse monoclonal antibody to CD44s (clone DF 1485, Isotype: IgG1, kappa, from Dakocytomation Denmark) was used at a dilution of 1:25. Avidin biotin detection kit was used and immunostaining was carried out in an automated immunostainer (BenchMark XT, Ventana® Medical systems Inc., Tucson, AZ, USA) according to the manufacturer's instructions. Subsequently, slides were washed, counterstained with Mayer's haematoxylin, and mounted. Negative control (by substitution of primary antibody with Tris-buffered saline) and positive control slides (squamous cell carcinoma previously shown to be positive for CD44s) were included.

Interpretation of CD44s immunostaining. In order to evaluate CD44s immunostaining, malignant tumour cells showing membranous staining were regarded as being positive cells. All available tumour cells in each section were counted at microscope magnification of ×200. Positive cells were then counted. The mean values of positivity were calculated and expressed as a percentage of the total number of tumour cells. The percentage of membranous CD44s immunostaining was reported for all cases. For categorisation, cases were considered to have low CD44s immunoexpression when <10% of malignant cells were positive, moderate CD44s immunoexpression when 10-49% of malignant cells were positive, and high CD44s immunoexpression was reported when tumour cells exhibited CD44s labelling in more than 50% of cells (12).

Statistical analysis. Data is presented as the mean±standard error of the mean (SEM). Differences between two groups of cases for one variable were tested by using the Mann-Whitney test. To test association between the three groups of cases for one independent variable the Kruskal – Wallis test was used. Multivariate logistic regression analysis was used to predict lymph node metastasis and recurrence in relation to immunoexpression of CD44s. Beta coefficient and 95% confidence intervals (CI) were denoted for each analysis. The Kaplan-Meier procedure was used to calculate the survival probabilities and the log-rank test was used to compare the difference between survivals. The end-point for patients was death from tumour (disease-specific). Statistical procedures were performed using SPSS® Release 16.0. Statistical significance was determined at p-value of ≤0.05 and tests were two-sided.

Table I. *Clinicopathological parameters of cases*.

Parameter		Number (%)
Gender	Male	46/96 (47.9%)
	Female	50/96 (52.1%)
Tumour grade	Well differentiated	24/96 (25%)
	Moderately differentiated	67/96 (69.8%)
	Poorly differentiated	5/96 (5.2%)
Age	<60 years	46/96 (47.9%)
	≥60 years	50/96 (52.1%)
Tumour location	Right colon	31/96 (32.3%)
	Left colon	26/96 (27.1%)
	Rectum	39/96 (40.6%)
Tumour size	<5 cm	28/96 (29.2%)
	≥5 cm	24/96 (25%)
	Not applicable	44/96 (45.8%)
Primary tumour	T1	0/96 (0%)
	T2	12/96 (12.5%)
	T3	10/96 (10.4%)
	T4	30/96 (31.1%)
	Not applicable	44/96 (45.8%)
Nodal metastasis	Positive	30/96 (31.2%)
	Negative	24/96 (25%)
	Not applicable	42/96 (43.8%)
Lymphovascular invasion	Positive	7/96 (7.3%)
	Negative	45/96 (46.9%)
	Not applicable	44/96 (45.8%)
Margin status	Involved	6/96 (6.2%)
	Free	46/96 (47.9%)
	Not applicable	44/96 (45.8%)
Status at end point	Died of disease	23/96 (24%)
	Alive	73/96 (76%)
Recurrence	Recurrence	31/96 (32.3%)
	No recurrence	34/96 (35.4%)
	Not available	31/96 (32.3%)

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.

Results

CD44s immunoexpression. CD44s immunoexpression was observed in the colonic glands and crypts within the brush border in some cases and in many lymphocytes and stroma (Figure 1A). In tumour cells, positive membranous CD44s immunoexpression was observed (Figures 1B-D). There was a higher incidence of cases with low CD44s immunoexpression [66 cases (68.8%)], than moderate [28 (29.1%)] and high [2 (2.1%)] CD44s immunoexpression (p<0.001) (Table II).

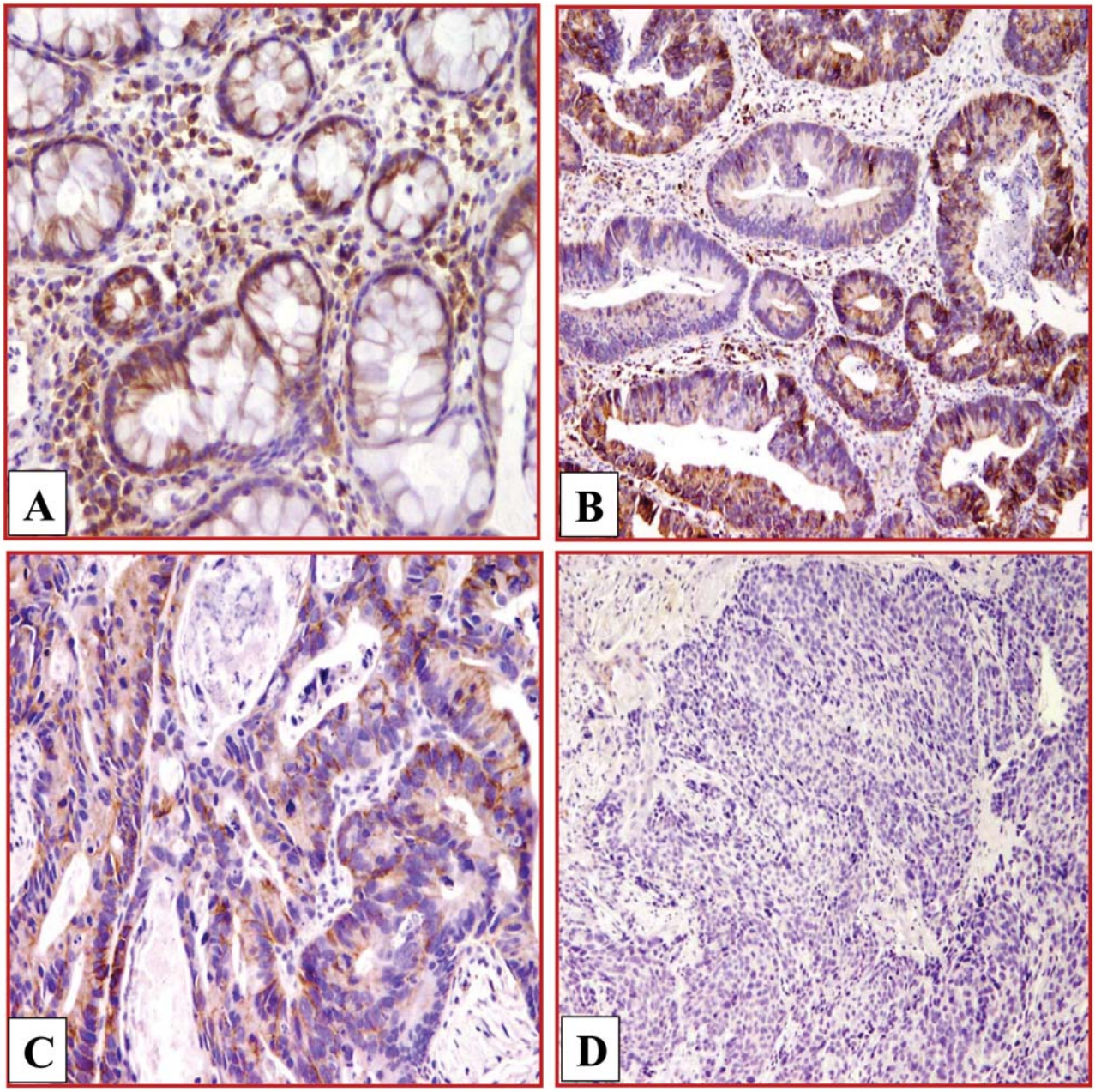

Figure 1. *CD44s immunoexpression in colorectal carcinoma using immunohistochemical labelling with anti-CD44s antibody with diaminobenzidine used as the chromogen and haematoxylin as counterstain. A: Membranous labelling of CD44s in the colonic crypts in sides and brush borders. CD44s is also expressed in stromal cells and lymphocytes (×200). B: Membranous labelling of CD44s in a well differentiated colorectal carcinoma. Staining does not involve all glands (×200). C: Membranous labelling of CD44s in a moderately differentiated colorectal carcinoma. Staining involves more glandular structures (×200). D: A poorly differentiated colorectal carcinoma showing no CD44s labelling (×200).*

Relation of CD44s expression to clinicopathological parameters. The CD44s expression in relation to different clinicopathological parameters is presented in Table III. Although the mean of CD44s expression in well-differentiated and moderately-differentiated tumours was higher than in poorly differentiated tumour, there was no statistically significant difference (p=0.318). On the other hand, tumour from females had a higher CD44s immunoexpression than that from males (p=0.001). There was no statistically significant difference in CD44s immunoexpression between different age

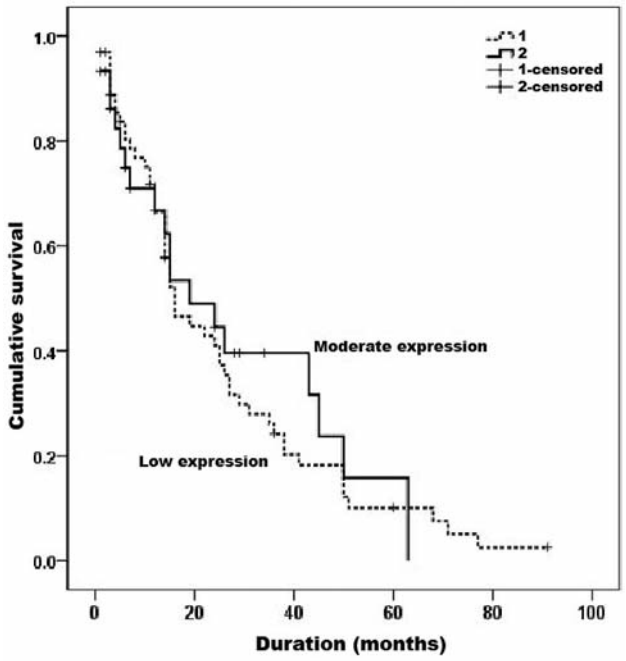

Figure 2. *Disease-free survival curve (Kaplan Meier) according to CD44s immunostaining 1: low CD44s immunoexpression; 2: moderate CD44s immunoexpression (log-rank=0.363, p=0.834).*

groups, tumour location, depth of invasion, lymphovascular invasion, recurrence, survival status or margin status. As regards nodal metastasis, there is higher immunoexpression of CD44s in cases with nodal metastasis than non-metastatic cases (p=0.05).

Multivariate logistic regression analysis showed that CD44s expression was an independent predictor of lymph node metastasis (beta coefficient=0.287, confidence interval=0.020-0.559, p-value=0.049). However, CD44s was not proven to be an independent predictor of recurrence: (beta coefficient=0.205, confidence interval=0.09-1.675, p-value=0.099). Kaplan–Meier survival analyses showed that CD44s immunoexpression in CRC had no significant association with more favourable disease-free survival (log-rank=0.363, p=834) (Figure 2).

Discussion

Although 70% of CRC cases undergo curative surgery, 50% of surgically cured patients will have advanced local recurrence or metastases at some point (1). CRC molecular pathogenesis is known to involve transformation of normal colonic epithelium adenoma and then carcinoma. This transformation occurs through a series of molecular alterations leading to abnormal cell differentiation, proliferation, motility, and apoptosis. Understanding the molecular pathology underlying CRC needs a continuous effort to discover more prognostic molecules to predict disease outcome and improve the therapeutic intervention and clinical outcomes.

Table II. *Category of CD44s immunoexpression of 96 colorectal cancer cases.*

Expression	Number (%)	Mean (SEM)	p-Value*
Low	66 (68.8%)	1.03 (0.229)	<0.001
Moderate	28 (29.1%)	21.785 (1.799)	
High	2 (2.1%)	55.5 (2.5)	

SEM: Standard error of the mean; *Kruskal Wallis test.

CD44 is a cell surface transmembrane glycoprotein which interacts with a major ligand, hyaluronate, together with other extracellular molecules. CD44 is associated with cell–matrix and cell–cell interactions (4). Several isoforms of CD44 exist resulting from alternative splicing, with considerable heterogeneous CD44 expression in different tissues (13). Alteration in CD44 expression has been reported in several types of malignancy in association with invasion and metastasis (2, 11, 14, 15). CD44 up-regulation has been shown to be an earlier marker of neoplastic transformation of colonic epithelium (16). On the other hand, CD44 down-regulation in the metastatic phase of CRC has been demonstrated (17). CD44s expression and variants are over expressed in colonic adenomas and CRC, and this correlated with poor prognosis (18). The expression of CD44s and variants has been strongly linked with tumour progression in CRC by several studies (12, 19-28). This is supported by the notion that CD44 is expressed normally in the lower two-thirds of colonic crypts where proliferation is maximum and is absent from the upper third where apoptosis occurs (29). In our study, CD44s was expressed at low level in about 70% of cases while it was moderate in 28%. Down-regulation of CD44s was defined previously (30) as being expression of less than 50%. This means that there is down-regulation of CD44s in our study. CD44s has been shown to be higher in normal colonic epithelium than CRC (27). In our subset of cases, only invading tumours are represented (T2, T3, and T4). This indicates that CD44s down regulation has a role in early tumorigenesis rather than in advanced tumours. This notion has been reported by Choi *et al.* (31), who reported CD44s down-regulation in CRC associated with liver metastasis and they also suggested metastasis to be related to up-regulation of CD44 variants. Wong *et al.* (32), also demonstrated that expression of CD44 variants reduces cell proliferation and increases metastatic potential *in vitro*. This finding has been contradicted by Bendardaf *et al.* (33), who found up-regulation of CD44s in primary CRC compared to

Table III. *Distribution of CD44s immunoexpression in relation to clinicopathological parameters.*

		Number (%)	Mean (SEM)	*p*-Value
Grade (n=96)	Well differentiated	13/24 (54.2%)	9.12 (2.832)	0.318*
	Moderately differentiated	35/67 (52.2%)	8.43 (1.575)	
	Poorly differentiated	1/5 (20%)	1.0 (1.0)	
Sex (n=96)	Male	15/46 (32.6%)	5.46 (1.703)	0.001#
	Female	34/50 (68%)	10.76 (1.921)	
Age (n 96)	>60 years	23/46 (50%)	6.98 (1.665)	0.796#
	≥60 years	26/50 (52%)	9.36 (2.005)	
Tumour location (n=96)	Right colon	14/31 (45.2%)	6.84 (2.214)	0.589*
	Left colon	14/26 (53.8%)	7.46 (2.401)	
	Rectum	21/39 (53.8%)	9.82 (2.210)	
Tumour size (n=52)	<5 cm	12/28 (42.8%)	5.14 (1.722)	0.205#
	≥5 cm	13/24 (54.2%)	7.2 (1.938)	
Depth of invasion (pT) (n=52)	T2	5/12 (41.7%)	4.17 (2.562)	0.665*
	T3	4/10 (40%)	4 (1.926)	
	T4	17/30 (56.7%)	7.60 (1.862)	
Nodal metastasis (n=54)	Positive	19/30 (63.3%)	8.80 (2.201)	0.05#
	Negative	9/24 (37.5%)	4.29 (1.774)	
Lymphovascular invasion (n=52)	Positive	2/7 (28.6%)	8.71 (5.626)	0.618#
	Negative	25/45 (55.5%)	5.71 (1.228)	
Margin status (n=52)	Involved	4/6 (66.6%)	9.33 (3.783)	0.278#
	Free	23/46 (50%)	5.70 (1.468)	
Status at end point (n=96)	Died of disease	14/23 (60.8%)	13.35 (3.715)	0.145#
	Alive	35/73 (47.9%)	6.6 (1.229)	
Recurrence (n=65)	Recurrence	17/31 (54.8%)	9.74 (2.317)	0.199#
	No recurrence	16/34 (47%)	5.29 (1.654)	

*Kruskal Wallis test; #Mann Whitney test. SEM: Standard error of the mean; 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.

their metastases. Alternatively, it has been reported that there was no apparent association with tumour progression. In CRC, CD44 immunoexpression was associated with areas of proliferation, while in non-proliferative colonic epithelium, CD44 immunoexpression was very low or absent. (25). Knockdown of CD44 strongly prevented clonal formation and inhibited tumourigenicity and cell proliferation, and stimulated apoptosis (34, 35). Several studies have demonstrated the association between CD44s over expression and invasion of tumour (1, 2, 12, 17, 36).

In the current study, there was no statistically significant relation between CD44s immunoexpression and clinicopathological parameters such as age, tumour size, tumour location, tumour grade, depth of invasion, lymphovascular invasion, margins, or recurrence. Most literatures reported similar results (2, 20, 27). Some findings are different from ours. Kunimura *et al*. (2) found significant association with depth of tumour invasion. This is because they studied only the deep invading portion of tumour. We averaged the CD44s immunoexpression from the entire tumour. The average of immunoexpression may be a reliable reflection of the state of CD44s, avoiding the effect of tumour heterogeneity.

The present study showed a statistically significant association between CD44s expression and nodal metastasis. In multivariate logistic regression analysis, we found that loss of CD44s expression is a favourable independent predictor of nodal status. Previous reports have shown association of CD44s with nodal metastasis (1, 2, 17). On the other hand, Sokmen *et al*. (20) showed that CD44 was not a predictor of metastasis.

The results from our study have shown no association between CD44s expression in primary CRC and recurrence and CD44s cannot be considered as an independent predictor for recurrence. Some other studies have shown the same (20, 37). Analysis of disease-free survival in our study revealed no association between CD44s expression and survival. Many previous reports found CD44s to be an independent predictor of survival in CRC and high levels of CD44s were associated with poor survival (1, 12, 37-40). In one study it was demonstrated that CD44 cannot be considered as a prognostic predictor of survival (20). Some studies that found association with survival used CRC cases of advanced stage or CD44s expression in certain areas of the tumour. This may explain why their results are different

from ours. We used the overall expression of CD44s in primary tumours only.

Due to inconsistent findings regarding the importance of CD44s immunoexpression as a prognostic molecular marker in CRC, further studies are required with standardization of methodologies and larger study samples with long clinical follow-up periods to establish the prognostic significance of CD44s. The inconsistency of results from various studies correlating CD44 immunoexpression in relation to clinicopathological parameters may be related to technical factors, such as the type of antibody, differences in immunostaining method, tissue blocks, or immunostaining scoring method and cut-off for negative/positive or low/high.

A limitation of our study was a loss of follow-up in some cases. A number of patients also had a very short survival time. In some cases, a colonoscopic biopsy was carried out without further resection specimens. This limited the availability of some pathological data that helps proper analysis.

Conclusion

In conclusion, we have shown that loss of CD44s immunolabelling in CRC is an independent favourable predictor of regional lymph node metastasis. On the other hand, CD44s loss has no significant association with disease recurrence or survival. The results from our studies need a more confirmative large-scale study on the immuno-localization of CD44s in CRC and its relation to nodal metastasis and survival. Moreover, extensive molecular studies, both *in vivo* and *in vitro*, are required to elucidate the possible mechanistic association of CD44s with tumour initiation, progression, invasion and metastasis in primary CRC, and in nodal and distant metastases.

Acknowledgements

This work was supported by the Scientific Chair for Colorectal Cancer, King Abdulaziz University, Jeddah, Saudi Arabia.

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Received April 23, 2012
Revised June 19, 2012
Accepted June 20, 2012