Disease-Free Survival of Colorectal Cancer Patients in Relation to CDw75 Antigen Expression

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Key Words

CDw75 · $\alpha(2,6)$ -Sialylation · Colorectal cancer · Prognostic value

Abstract

Objective: CDw75 is an $\alpha(2,6)$ -sialylated antigen associated with a poor prognosis in gastric cancer. In the present study, we examined if CDw75 expression in colorectal cancer (CRC) predicts tumour recurrence. Besides, we evaluated CDw75 expression in different colorectal tissue specimens to clarify their role in tumour development and progression. **Methods:** We analyzed CDw75 expression in 34 specimens of healthy disease-free colorectal mucosa, 19 specimens of inflammatory colorectal mucosa, 73 colorectal adenomas, 35 specimens of healthy tissue and 101 specimens of tumoural tissue from CRC patients. **Results:** None of the healthy disease-free and inflammatory colorectal mucosa specimens showed the presence of the epitope. CDw75 was expressed in 26% of the colorectal adenomas. In healthy and tumoural tissue from CRC patients, CDw75 was detected in 22.9% and 82.2% of the specimens, respectively. CDw75 expression in tumoural tissue was correlated with growth pattern ($p = 0.044$), Dukes stage ($p = 0.002$), TNM stage ($p = 0.020$) and distant metastasis ($p = 0.005$). Survival analysis showed that CDw75 expression is not associated with tumour recurrence.

Conclusion: CDw75 expression in CRC is not a prognostic factor for predicting disease-free survival. Nevertheless, CDw75 expression may be a good marker of tumour progression and of the malignant potential of CRC.

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Introduction

Compositional and structural alterations in cell surface glycoconjugates are commonly occur during malignant transformation [1]. One of the most frequent changes observed in tumour cell surface glycoconjugates is the increase in sialic acid linked to carbohydrate antenna residues. It is known that the sialylation of membrane components influences the metastatic potential of tumour cells [2]. Sialic acid residues are mainly found at terminal positions of glycoprotein and glycolipid oligosaccharide chains. They may be α -2,3- and/or α -2,6-linked to galactose (Gal), $\alpha(2,6)$ -linked to N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc), as well as $\alpha(2,8)$ -linked to other sialic acids in a specific post-transcriptional modification of N chains known as polysialic acid.

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The synthesis of these different sialyl linkages is catalyzed by different members of the sialyltransferase family, a class of glycosyltransferases [3].

CDw75 is a cell surface antigen synthesized by the β -galactosyl $\alpha(2,6)$ -sialyltransferase (ST6Gal I) and expressed by the majority of B cells. The enzyme transfers a sialic acid residue from CMP-NeuAc to a Gal $\beta(1,4)$ GlcNAc (lactosamine) sequence located in the terminal chains of cell surface and serum N-glycoproteins [4]. ST6Gal II is another enzyme with $\alpha(2,6)$ -sialyltransferase activity, the main acceptor of which is a free lactosamine sequence [5, 6]. Several studies have reported a significant increase in ST6Gal I activity in colorectal tumours compared with healthy colorectal mucosa [7–10]. Moreover, high activity levels of ST6Gal I in metastasizing colorectal tumours were found to be related to a poor prognosis [8, 11].

CDw75 antigen is a ligand for CD22 (siglec-2), an inhibitor coreceptor in B lymphocytes that controls the signalling threshold of B cell receptors [12]. It has been widely studied in blood cells neoplasias [13–16] and in solid tumours, such as benign breast lesions and carcinomas, as well as in gastro-intestinal carcinomas [17–21]. Several studies have reported increased CDw75 expression in gastric and colorectal tumours compared with healthy gastric and colorectal mucosa [19–21]. Moreover, CDw75 expression in gastric carcinomas has been associated with tumour progression and a worse prognosis [19, 21]. Regarding colorectal cancer (CRC), although CDw75 expression has been correlated with the malignant potential [20], the prognostic value of this antigen has not been described.

In a recent study, our group reported an increase in both CDw75 expression and ST6Gal I activity in CRC, as well as a possible implication of CDw75 in CRC development [22]. In the present study, we examined whether the expression of CDw75 antigen in CRC is associated with tumour recurrence. Besides, in an attempt to clarify the role of CDw75 in the development and progression of CRC, we analyzed its expression profile in different colorectal specimens that show the progressive malignant transformation of colorectal tissue, from healthy disease-free mucosa to adenocarcinoma.

Materials and Methods

Patients

CRC tissue specimens were obtained from 101 patients with CRC, diagnosed and treated between 2000 and 2004 at the University Hospital Complex (CHOU, Ourense, Spain). Tissue specimens of healthy mucosa (at a distance at least 10 cm from the pri-

mary carcinoma) were obtained from 35 of the above-mentioned CRC patients. Tumour staging was carried out by means of the TNM classification system described by the International Union against Cancer (UICC) [23]. In addition, 73 colorectal adenomas, 19 biopsies of colorectal inflammatory mucosa and 34 biopsies of healthy disease-free colorectal mucosa, obtained from CRC-free individuals, were included. Patients who underwent primary colorectal tumour resection were followed for at least 5 years or until disease recurrence or death. Patients who died within 30 days after surgery or with distant metastasis at surgical resection were excluded from the follow-up. All procedures involving human samples were performed according to the clinical practices of the 'Xunta de Galicia' and followed the tenets of the Helsinki Declaration.

Immunohistochemical Analysis

All fresh specimens were fixed in formalin (10% v/v) and embedded in paraffin. Sections (3 μ m) from selected paraffin-embedded tissue blocks were deparaffinized in xylene, hydrated in a graded ethanol series, and heated for 15 min in 0.1 M citric acid buffer (pH 6.0) in a microwave oven to unmask the epitopes. Endogenous peroxidase activity was blocked by using a commercial solution (Peroxidase block, EnVision™ Detection System; Dako, Calif., USA) for 15 min. To avoid non-specific binding, the tissue slides were incubated for 20 min with 2% bovine serum (Sigma-Aldrich, St. Louis, Mo., USA). Afterwards, the sections were incubated with the primary antibody against CDw75 (dilution 1/25, clone LN-1; Neomarkers, Fremont, Calif., USA) in a moist chamber for 30 min. After rinsing with PBS, the incubation with the secondary antibody bound to peroxidase (goat anti-mouse labelled polymer, HRP, EnVision™ Detection System) was carried out for 30 min in a moist chamber. The immunostaining was then visualized with 3,3'-diaminobenzidine reagent (EnVision™ Detection System). Finally, the sections were counterstained with haematoxylin, dehydrated through a graded ethanol series and xylene, and mounted in Dpex mounting medium. Negative controls were performed by replacing the primary antibody with PBS.

Evaluation of CDw75 Staining

The immunohistochemical staining, detected as a brown colour precipitate, was evaluated independently by two pathologists from CHOU, who reached a final result by consensus. The staining scores were classified semiquantitatively as follows: 0 = no staining, 1 = weak staining intensity, 2 = moderate staining intensity and 3 = strong staining intensity.

Statistical Analysis

Statistical analysis was performed using the PASW Statistics 18 program. Univariate analysis for categorical data was conducted by means of the χ^2 test or Fisher's exact probability test. For continuous data, we employed Wilcoxon's test and the Mann-Whitney U test. Disease-free survival rates were calculated by the Kaplan-Meier method [24] and differences between the survival curves were analyzed by the log-rank test [25]. Multivariate Cox regression analysis [26], using the forward stepwise data selection method, was carried out to determine the factors with independent prognostic value. The results were considered significant when $p < 0.05$.

Fig. 1. Immunohistochemical expression of CDw75. Sections from a healthy disease-free colorectal mucosa (**a**) and an inflammatory colorectal mucosa (**b**) showing no CDw75 expression. Sections from a colorectal adenoma specimen (**c**), a colorectal healthy specimen (**d**) and a colorectal tumour specimen (**e, f**) with positive CDw75 staining, evidenced by the presence of a brown precipitate (color refers to the online version). Scale bars = 25 μ m.

Results

Expression of CDw75 Antigen in Colorectal Specimens

No positive CDw75 antigen expression was found in healthy disease-free and inflammatory mucosa from CRC-free patients (fig. 1a, b). However, CDw75 expression was visualized as a brown staining located on the cytoplasm and the plasma membrane of adenomas, and in healthy and tumoural tissues from CRC patients (fig. 1c–f). Besides, both healthy and tumoural specimens from CRC patients showed consistently immunoreactive germinal center cells.

Of 101 CRC specimens analyzed (table 1), 83 showed positive expression for CDw75 (82.2%). Among these specimens, 27 (26.7%) were found to express CDw75 weakly, whereas 42 (41.6%) and 14 (13.9%) showed moderate and strong expression, respectively. In healthy tissue specimens from CRC patients ($n = 35$), CDw75 expression was found in 8 cases (22.9%), with weak staining in 7 (20%) specimens and moderate staining in 1 (2.9%) specimen. Likewise, we observed CDw75 antigen expression in 19 adenomas of a total of 73 analyzed (26.0%), the expression being weak in 16 (21.9%) and moderate in 3 (4.1%) (table 1).

The statistical analysis revealed significant differences between CDw75 expression in tumoural and healthy tis-

Table 1. Immunoreactivity of CDw75 antigen in the colorectal specimens analyzed

	CDw75 staining score			
	no expression	weak expression	moderate expression	strong expression
Healthy disease-free mucosa (n = 34)	34 (100%)	0	0	0
Inflammatory mucosa (n = 19)	19 (100%)	0	0	0
Adenomas (n = 73)	54 (74%)	16 (21.9%)	3 (4.1%)	0
Healthy tissue CRC (n = 35)	27 (77.1%)	7 (20.0%)	1 (2.9%)	0
Tumoural tissue CRC (n = 101)	18 (17.8%)	27 (26.7%)	42 (41.6%)	14 (13.9%)

Table 2. CDw75 antigen expression in colorectal specimens

Colorectal specimen	n	Score range	Score mean $\pm$ SD	T vs. DFM	T vs. I	T vs. A	T vs. H
Disease-free mucosa	34	0	0	$p < 0.001^a$	$p < 0.001^a$	$p < 0.001^a$	$p < 0.001^b$
Inflammatory mucosa	19	0	0				
Adenomas	73	0–1	0.26 ± 0.442				
Healthy tissue (CRC)	35	0–2	0.26 ± 0.505				
Tumoural tissue (CRC)	101	0–3	1.51 ± 0.945				

The score is the intensity of CDw75 expression evaluated according to the following gradation: 0 = without staining, 1 = weak staining intensity, 2 = moderate staining intensity and 3 = strong staining intensity. The intensity of the expression is indicated as the mean $\pm$ SD. ^a $p < 0.001$ based on the Mann-Whitney U test. ^b $p < 0.001$ based on the Wilcoxon test. SD = Standard deviation; DFM = healthy disease-free mucosa; I = inflammatory mucosa; A = adenomas; H = healthy tissue from CRC; T = tumoural tissue from CRC.

sues from CRC patients ($p < 0.001$, according to Wilcoxon's test), as well as between tumoural tissue and colorectal adenomas, inflammatory and healthy disease-free mucosa ($p < 0.001$, according to the Mann-Whitney U test) (table 2).

Relationship between CDw75 Expression in CRC and Clinicopathological Features

In order to elucidate whether CDw75 expression in CRC tumoural tissue was correlated to clinicopathological features, a correlation analysis was carried out. The χ^2 test did not show any correlation with patient's age or sex, tumour location, size, tumour differentiation, depth of invasion and lymph node metastasis. However, statistically significant correlations were found between CDw75 expression and growth pattern ($p = 0.044$), Dukes stage ($p = 0.002$), TNM stage ($p = 0.020$), and distant metastasis ($p = 0.005$) (table 3).

Further analyses were carried out (1) comparing the cases with negative expression (score 0) with those that showed positive expression (scores 1–3) and (2) compar-

ing the cases with low expression (score 0 or 1) with those with high expression (score 2 or 3). In both analyses, no significant correlations were found between CDw75 staining groups and clinicopathological features (data not shown).

A correlation analysis of CDw75 expression in colorectal adenomas with clinicopathological features was also performed, but no associations were found (data not shown).

Relationship between CDw75 Expression and Tumour Recurrence

To study the influence of CDw75 expression on the prognosis of CRC patients, a 5-year survival analysis was carried out. The analysis was performed on 78 of the 101 patients initially included in the study. During the post-surgery follow-up, recurrences were detected in 22 of the 78 patients (21.8%): local recurrences in 6 cases and distant metachronous metastases in 16 cases.

The results obtained according to the Kaplan-Meier method and the log-rank test indicated no difference in

Table 3. Relationship between patients' clinicopathological features and CDw75 antigen expression in colorectal tumour specimens

Clinicopathological features	CDw75 expression ¹				p value
	0	1	2	3	
Sex					
Female	6 (15.0)	12 (30.0)	17 (42.5)	5 (12.5)	0.882
Male	12 (19.7)	15 (24.5)	25 (41.0)	9 (14.8)	
Age					
<70 years	6 (17.6)	10 (29.4)	12 (35.4)	6 (17.6)	0.932
70–76 years	7 (20.0)	8 (22.8)	15 (42.9)	5 (14.3)	
>76 years	5 (15.6)	9 (28.1)	15 (46.9)	3 (9.4)	
Tumour location					
Colon, proximal	5 (25.0)	2 (10.0)	10 (50.0)	3 (15.0)	0.410
Colon, distal	5 (18.5)	8 (29.6)	9 (33.4)	5 (18.5)	
Rectum	8 (16.0)	17 (34.0)	21 (42.0)	4 (8.0)	
Tumour size					
<4.1 cm	11 (23.9)	14 (30.4)	16 (34.8)	5 (10.9)	0.639
4.1–5.5 cm	2 (8.7)	7 (30.4)	10 (43.5)	4 (17.4)	
>5.5 cm	4 (14.3)	6 (21.4)	13 (46.4)	5 (17.9)	
Growth pattern					
Polypoid	10 (23.8)	13 (31.0)	17 (40.5)	2 (4.7)	0.044 ^a
Non-polypoid	6 (10.9)	12 (21.8)	25 (45.5)	12 (21.8)	
Tumour differentiation					
Well differentiated	0 (0)	1 (25.0)	2 (50.0)	1 (25.0)	0.070
Moderately differentiated	9 (11.7)	24 (31.2)	34 (44.1)	10 (13.0)	
Poorly differentiated	0 (0)	0 (0)	1 (25.0)	3 (75.0)	
Dukes stage					
A	4 (30.8)	5 (38.5)	3 (23.1)	1 (7.6)	0.002 ^b
B	9 (23.1)	8 (20.5)	14 (35.9)	8 (20.5)	
C	5 (12.8)	12 (30.8)	22 (56.4)	0 (0)	
D	0 (0)	2 (20.0)	3 (30.0)	5 (50.0)	
pTNM classification					
pT (primary tumour extent)					0.376
pT1	1 (50.0)	0 (0)	1 (50.0)	0 (0)	
pT2	4 (28.6)	6 (42.9)	3 (21.4)	1 (7.1)	
pT3	11 (15.5)	19 (26.8)	32 (45.1)	9 (12.6)	
pT4	2 (14.3)	2 (14.3)	6 (42.8)	4 (28.6)	
pN (lymph node metastasis)					0.059
pN0	13 (23.2)	13 (23.2)	19 (33.9)	11 (19.7)	
pN1/N2	5 (11.1)	14 (31.1)	23 (51.1)	3 (6.7)	
pM (distant metastasis)					0.005 ^a
pM0	18 (19.8)	25 (27.5)	39 (42.9)	9 (9.8)	
pM1	0 (0)	2 (20.0)	3 (30.0)	5 (50.0)	
TNM stage					
I	4 (30.8)	5 (38.5)	3 (23.1)	1 (7.6)	0.020 ^b
IIa	9 (25.0)	8 (22.2)	12 (33.4)	7 (19.4)	
IIb	0 (0)	0 (0)	2 (66.7)	1 (33.3)	
IIIa	1 (50.0)	1 (50.0)	0 (0)	0 (0)	
IIIb	2 (9.5)	6 (28.6)	13 (61.9)	0 (0)	
IIIc	2 (12.5)	5 (31.3)	9 (56.2)	0 (0)	
IV	0 (0)	2 (20.0)	3 (30.0)	5 (50.0)	

Figures in parentheses are percentages. ^a $p < 0.05$, based on Fisher's exact probability test or ^b on the χ^2 test. ¹ According to the staining results scored from no expression (0) to weak (1), moderate (2) or strong (3) expression.

Table 4. Univariate analysis of factors affecting disease-free survival

Clinicopathological features	n	Survival rate, %	p value
Sex			
Female	34	75.5	0.364
Male	44	66.0	
Age			
<70 years	24	70.1	0.888
70–76 years	31	72.6	
>76 years	23	66.1	
Tumour location			
Colon, proximal	17	70.6	0.731
Colon, distal	17	63.1	
Rectum	42	75.3	
Tumour size			
<4.1 cm	38	72.6	0.959
4.1–5.5 cm	15	73.3	
>5.5 cm	22	69.9	
Growth pattern			
Polypoid	34	72.1	0.878
Non-polypoid	42	67.6	
Dukes stage			
A	12	83.3	0.077
B	34	80.3	
C	32	56.1	
pTNM classification			
pT (primary tumour extent)			
pT1	2	100	0.209
pT2	13	84.6	
pT3	54	70.0	
pT4	9	44.4	
pN (lymph node metastasis)			
pN0	46	81.0	0.024 ^a
pN1/N2	32	56.1	
TNM stage			
I	12	83.3	0.177
IIa	32	83.0	
IIb	2	50.0	
IIIa	2	100	
IIIb	17	52.3	
IIIc	13	53.8	
CDw75 expression			
Negative (0)	15	65.5	0.398
Weak (1)	23	68.3	
Moderate (2)	32	67.6	
Strong (3)	8	100	0.424
Negative (0)	15	65.5	
Positive (1, 2, 3)	63	71.4	
Low (0, 1)	38	66.9	0.381
High (2, 3)	40	73.2	

^a p < 0.05 based on the log-rank test.

disease-free survival rate between the groups of patients established according to the degree of CDw75 staining (table 4; fig. 2a–c). However, univariate analysis revealed that patient prognosis was influenced by lymph node metastasis ($p = 0.024$) (table 4). Finally, multivariate Cox regression analysis showed that lymph node metastasis was the only variable with independent prognostic value for predicting disease-free survival of CRC patients ($p = 0.018$, table 5).

Discussion

CDw75 epitope is an $\alpha(2,6)$ -sialylated antigen synthesized by the ST6Gal I enzyme. This antigen has been little studied in solid organ tumours. Nevertheless, several studies have revealed an increase in ST6Gal I activity in colorectal carcinomas, and an association between high enzyme levels and CRC progression [7–11].

In a previous study, we reported enhanced CDw75 expression in CRC specimens compared with healthy colorectal specimens [22]. The main objective of this work was to study the influence of CDw75 expression in tumour recurrences in patients with CRC. In addition, we analyzed CDw75 expression in a wide collection of colorectal adenomas, and in healthy and tumoural tissue specimens from CRC patients with the purpose to elucidate their implication in the development and progression of CRC. To obtain a complete picture of the malignant transformation process of colorectal tissue, we also included specimens from both disease-free individuals and from patients with inflammatory bowel disease.

Immunohistochemical analysis showed no expression of CDw75 antigen in healthy disease-free and inflammatory colorectal mucosa. However, we observed positive CDw75 staining in 22.9% of healthy tissue specimens from CRC patients. The expression detected in this tissue could be related to the tumour process since we have not observed expression of CDw75 in colorectal mucosa from disease-free patients. Concerning the colorectal adenomas, we detected expression of CDw75 antigen in 26% of the specimens, similar to our previous observations [22] in a study in which a smaller number of adenomas were analyzed. The majority of the adenomas showed weak CDw75 expression and the correlation analysis between the expression and the clinicopathological features did not reveal any association. These results suggest that the antigen is not implicated in the early stages of CRC development through the classical adenoma-carcinoma pathway.

Fig. 2. Disease-free survival of CRC patients in relation to immunohistochemical CDw75 antigen expression. The staining results were scored as no expression (0), weak (1), moderate (2) or strong (3) expression (a), no expression (0) and positive expression (1, 2 or 3) (b) or low expression (0 or 1) and high expression (2 or 3) (c).

Although CDw75 expression in colorectal adenomas has not been analyzed previously, different studies have reported the expression of $\alpha(2,6)$ -sialylated oligosaccharides in colorectal adenomas using SNA lectin [27, 28]. Nevertheless, the specificity of the SNA lectin differs from that of the anti-CDw75 monoclonal antibody. This lectin recognizes oligosaccharide chains bearing a galactose residue sialylated in the $\alpha(2,6)$ -position (sequence present in the CDw75 antigen) as well as in the $\alpha(2,6)$ -sialylated GalNAc residue. This last structure, known as the sialyl-Tn antigen, is mainly expressed in tumours in various organs although it has also been detected in healthy colorectal tissue [29, 30].

Table 5. Multivariate analysis of predictive factors for disease-free survival

Clinicopathological features	Relative risk	95% CI	p value
pN (lymph node metastasis)			
pN0 (absent) vs. pN1/N2 (present)	3.230	1.226–8.510	0.018 ^a
CI = Confidence interval.			
^a p < 0.05 based on Cox regression analysis.			

Regarding the tumoural specimens analyzed, 82.2% of the cases expressed CDw75 antigen, in accordance with our previous results [22]. However, Elpek et al. [20] detected the antigen in 51% of CRC specimens studied. The overexpression of CDw75 antigen in tumoural tissue compared with healthy tissue from CRC patients and in colorectal adenomas was corroborated statistically. Moreover, the absence of CDw75 expression in specimens of inflammatory bowel disease indicates that the enhanced antigen expression is specific of the tumour stage rather than being a consequence of the tumour-associated inflammation.

The correlation analysis carried out to study the relationship between antigen expression and clinicopathological features revealed a statistically significant association between CDw75 expression and tumour aggressiveness. We observed an increase in moderate or strong CDw75 expression in advanced tumour stages according to Dukes and TNM classifications, in non-polypoid tumours (tumours that expand progressively infiltrating the intestinal wall) as well as in cases with distant metastasis. Similar results have been reported regarding a statistically significant association between the expression of CDw75 in CRC and different characteristics of tumour aggressiveness, such as the degree of tumour infiltration into the intestinal wall, tumour stage, lymph node infiltration and distant metastasis [20]. Taking into account these results, we hypothesize that CDw75 antigen could be implicated in local tumour progression through the intestinal wall. In relation to this, it has been found that the increased cell surface $\alpha(2,6)$ -sialylation affects the adhesion of breast carcinoma cells, reducing their homotypic aggregation [31]. Similarly, recent studies have revealed the enhancement of $\beta 1$ -integrin $\alpha(2,6)$ -sialylation associated with progression of colorectal and mammary tumours [32, 33].

As far as we know, the relationship between CDw75 expression in CRC and patient survival has not been reported previously since the only report focusing on the expression of CDw75 in CRC did not analyze its prognostic value [20]. However, it has been reported that the strong SNA lectin reactivity in CRC is an independent prognostic factor that predicts overall survival [34], but as previously discussed, SNA lectin recognizes both CDw75 and sialyl-Tn antigens. In this work, the correlation analysis showed an association between high CDw75 expression and distant metastasis. This finding could indicate a positive correlation between CDw75 expression and tumour recurrence. However, the survival analysis did not reveal an association between antigen expression

and disease-free survival. The fact that CDw75 antigen was expressed in the majority of the carcinomas analyzed, limits its usefulness as a prognostic marker. Previous studies reporting an association between CDw75 expression and overall survival have demonstrated that CDw75 antigen expression in gastric cancer is associated with a poor prognosis [19, 21]. Nevertheless, we consider that disease-free survival is the parameter that better represents the patient's post-surgical evolution, since many aged CRC patients die of non-cancerous processes. Thus, an association between CDw75 expression in gastric or colorectal adenocarcinoma and tumour recurrence has not been reported to date.

In conclusion, we have demonstrated that CDw75 expression in CRC is not a prognostic factor for predicting disease-free survival. However, we observed an increase in CDw75 expression paralleling the progressive malignant transformation of healthy disease-free colorectal mucosa to adenocarcinomatous mucosa. Furthermore, the high expression levels of CDw75 antigen were associated with advanced tumour stages and distant metastasis. Therefore, our results suggest that the high CDw75 expression may be a marker of progression and of the malignant potential of CRC. Nevertheless, further studies are needed to elucidate the mechanisms leading to CDw75 antigen overexpression in CRC and the role of the antigen in tumour progression.

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