CD44v6 and Nm23-H1 protein expression related to clinico pathological parameters in colorectal cancer

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Abstract

Objective: *Nm23-H1 and CD44v6 expression has been shown to be correlated with the metastatic potential of colorectal cancer (CRC) in some studies but not in others. The present study was undertaken to evaluate immunohistochemically the expression of these markers and to correlate them with clinicopathological variables.*
Matherials and Methods: *Archival tissues of 41 non metastatic colorectal cancers were histopathologically evaluated and stained with monoclonal antibodies versus Nm23-H1 and CD44v6.*
Results: *Expression of Nm23-H1 was detected in 73% (n=30) of all CRC, and CD44v6 in 37% (n=15) of all CRC. CD44v6 was found to be statistically associated with tumour grading differentiation (p<0.03), but no correlation emerged between Nm23-H1 and CD44v6 and Dukes stage, site, peritumoral lymphocytic infiltration, venous infiltrating, perineural infiltrating, tumour budding, pushing and infiltrating tumour growth.*
Conclusion: *Even if the results are not statistically significant, the authors noticed that the expression of Nm23-H1 was correlated with those histopathological parameters that indicate local disease progression and metastases.*
Key words: Immunohistochemistry, prognostic variables, metastasis.

Riassunto

ESPRESSIONE DELLE PROTEINE CD44v6 E Nm23-H1 CORRELATA CON I PARAMETRI CLINICO-PATOLOGICI DEL CANCRO COLORETTALE

Obiettivi: *In alcuni studi è stata dimostrata l'esistenza di correlazione fra l'espressione di Nm23-H1 e CD44v6 ed il potenziale metastatico del cancro colo-rettale. Il presente studio ha lo scopo di valutare, utilizzando metodiche di immunoistochimica, l'espressione di questi markers e di correlarli con le varianti clinico-patologiche.*
Materiali e Metodi: *sono stati rivalutati rivalutati da un punto di vista istopatologico 41 vetrini ottenuti da pezzi in paraffina di 41 carcinomi colorettali non metastatici. Tali vetrini sono stati cimentati con anticorpi monoclonali verso Nm23-H1 e CD44v6.*

Introduction

The Nm23 (non metastatic clone n. 23) gene was first identified by Steeg in hybridization experiments in murine melanoma cell lines, with an inverse relationship between metastatic potential and Nm23 RNA and/or protein levels (1). This implies that Nm23 is a potential metastasis suppressor gene and could function in the invasion and migration steps of the metastatic pathways. Thus far, two human Nm23 genes, Nm23-H1 and Nm23-H2, have been cloned; they are 88% homologous to each other and encode two polypeptide subunits of a nucleoside diphosphate (NDP) kinase (2). Nm23 expression has been reported to be associated with advanced stages, high metastatic potential and poor prognosis in several different tumours, including breast, hepatocellular, ovarian and gastric carcinoma and melanoma (3-5). In other tumours, such as neuroblastoma and pancreatic carcinoma, the opposite results have been demonstrated (3). The role of Nm23-H1 in colorectal carcinoma (CRC) is still controversial. In fact, allelic deletion or mutation of the Nm23-H1 gene appears to be associated with distant metastasis in some studies, but not in others.
CD44, identified in 1982, is a transmembrane molecule found in different cell types. It is encoded by one gene located on the short arm of chromosome 11 (6). CD44

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Risultati: *l'espressione dell'Nm23 è stata rilevata nel 73% (n=30) di tutti i CRC, mentre l'espressione del CD44v6 è stata rilevata nel 37% (n=15) dei pezzi valutati. E' stata riscontrata un'associazione statisticamente significativa fra il CD44v6 ed il grading delle neoplasie (p<0,03), mentre nessuna correlazione è emersa fra Nm23-H1 e CD44v6 con la stadiazione secondo Dukes, la sede, l'infiltrato linfocitario peritumorale, l'infiltrazione venosa, l'invasione perineurale, il budding tumorale e la crescita espansiva o infiltrante della neoplasia stessa.*
Conclusioni: *Sebbene i risultati non siano statisticamente significativi, gli Autori ritengono che l'espressione dell'Nm23-H1 sia correlata con quelle varianti istopatologiche che indicano una progressione locale della malattia e metastasi.*
Parole chiave: Immunoistochimica, varianti prognostiche, metastasi.

is described as a family of transmembrane glycoproteins involved in cell to cell and cell to matrix interaction. This molecule is a receptor for hyaluronate, an important component of the extracellular matrix and a substratum for cell adhesion (8). CD44 is expressed in different variant forms owing to a differential splicing of various exons between exons 5 and 16 (8, 9). Variant forms of CD44 have been found to be expressed in inflammatory conditions such as ulcerative colitis and in neoplasms, including adenomas and carcinomas (9, 11) as well as in other human malignancies (10, 13).
In the present study CD44v6 and Nm23H1 protein expression was evaluated in non metastatic colorectal cancer related to clinicopathological parameters.

Material and methods

Clinical samples

For this study, a total of 41 cases of resected colorectal cancer were selected from the Clinical Surgery III Department of the University of Rome "La Sapienza" data base. Formalin-fixed paraffin embedded archival specimens were considered; the cases were selected after a review of hematoxylin and eosin stained slides. Fourteen cases of them were females (34%) and twenty-seven males (68%); mean age 65.5 (range 46-80 years). The tumours were located in the right colon (n=9), left colon (n=15) and rectum (n=17). All cases were selected without metastasis at surgery intervention. All patients underwent curative resection: twenty anterior resection, nine left hemicolectomy, eleven right hemicolectomy, one Miles operation. According to the International TNM staging system, two (5%) of the lesions were T1, fourteen (34%) were T2, twenty-five (61%) were T3, whereas according to the Dukes' parameters, sixteen (39%) were Dukes' A and twenty-five (61%) were Dukes' B. Histologically there were thirty-five (85%) adenocarcinomas and six were mucinous adenocarcinomas. With regard to diffusion, no metastases and lymphnode involvement were found. In all cases an accurate histopathological evolution was accomplished, including lymphocytic peritumoral infiltrating, venous infiltrating, perineural infiltrating, tumour budding, pushing growth and infiltrating growth. The presence of tumour budding, defined as small clusters of undifferentiated cancer cells ahead of the invasive front of the lesion, was then sought (11) (Tab. I). Median follow up was 73 months (range 1-154 months). At follow up eleven (27%) had died of the disease, four (10%) from other diseases, twenty-four (58%) were still alive and disease free and two (5%) were alive but presented metastases (Tab. II).

Immunohistochemistry

Nm23-H1

The anti Nm23-NDPK protein is a murine monoclonal antibody purified from human erythrocytes and purchased from Novocastra Diagnostic. Formalin fixed, paraffin embedded tissue sections, cut to 5 µ, were dewaxed in xylene, hydrated through graded ethanol and washed in PBS (pH 7.4). Endogenous peroxidase activities were quenched in 0.3% (v/v) hydrogen peroxidase in absolute methanol for 15 minutes. The primary monoclonal antibody was used at a dilution of 1:125 and incubated for 30 minutes at room temperature. Immunoperoxidase staining was performed using a streptavidin biotin system kit for primary mouse antibodies (Zymed, San Francisco, CA, USA), according to the manufacturer's specifications. The avidin biotine peroxidase reaction was performed with chromogen 3-3' diamonobenzidine supplemented with hydrogen peroxidase (Zymed, San Francisco, CA, USA)) and nuclei were counterstained with hematoxylin. In the experiment a case of breast carcinoma with high Nm23 protein expression was included as a positive control. The primary antibody was omitted for the negative control.

CD44v6

The anti CD44 protein is a murine monoclonal antibody raised against an epitope encoded by exon v6 of human variant CD44 (R & D System, Inc. Minneapolis, MN, USA). Formalin fixed, paraffin embedded tissue sections, cut to 5 µ, were dewaxed in xylene, hydrated through graded ethanols and washed in PBS (pH 7.4). Endogenous peroxidase activities were quenched in 0.3% (v/v) hydrogen peroxidase in absolute methanol for 15 minutes. The primary monoclonal antibody was used at the dilution of 1:100 and incubated for 60 minutes at room temperature. Immunoperoxidase staining was performed using a streptavidin biotin system kit for primary mouse antibodies (Zymed, San Francisco, CA, USA), according to the manufacturer's specifications. The avidin biotin peroxidase reaction was performed with chromogen 3-3'

Tab. I – CORRELATION BETWEEN NM23-H1 AND CD44V6 IMMUNOSTAINING IN NON METASTATIC COLORECTAL CANCER AND CLINICOPATHOLOGICAL PARAMETERS

	Tot.	*Nm23 Neg.*	*Nm23 Pos.*	*P value*	*Cd44 Neg.*	*Cd44 Pos.*	*P value*
	n. (%)	n. (%)	n. (%)		n. (%)	n. (%)	
	41 (100)	11 (27)	30 (73)		26 (63)	15 (37)	
HISTOLOGY:							
Adenocarcinoma	35 (85)	7 (20)	28 (80)		23 (66)	12 (34)	
Mucinous Adenocarcinoma	6 (15)	4 (67)	2 (33)	<.035°	3 (50)	3 (50)	N.S.
DUKES:							
A	16 (39)	3 (19)	13 (81)		11 (69)	5 (31)	
B	25 (61)	8 (32)	17 (68)	N.S.	15 (60)	10 (40)	N.S.
GRADING:							
G1	4 (10)	1 (25)	3 (75)		4 (100)	0	
G2	23 (56)	6 (26)	17 (74)		16 (70)	7 (30)	
G3	12 (29)	4 (33)	8 (67)		4 (33)	8 (67)	
G4	2 (5)	0	2 (100)	N.S.	2 (100)	0	<.036*
STAGE:							
pT1	2 (5)	0	2 (100)		1 (50)	1 (50)	
pT2	14 (34)	1 (7)	13 (93)		11 (79)	3 (21)	
pT3	25 (61)	3 (12)	22 (88)	N.S.	15 (60)	10 (40)	N.S.
Lymphocitic Peritumoral Infiltrating	27 (66)	7 (26)	20 (74)	N.S.	17 (63)	10 (37)	N.S.
Venous Infiltrating	2 (5)	0	2 (100)	N.S.	1 (50)	1 (50)	N.S.
Perineural Infiltrating	1 (2)	0	1 (100)	N.S.	1 (100)	0	N.S.
Tumour Budding	19 (46)	7 (37)	12 (63)	N.S.	12 (63)	7 (37)	N.S.
Pushing	12 (29)	3 (25)	9 (75)	N.S.	8 (67)	4 (33)	N.S.
Infiltrating	29 (71)	8 (28)	21 (72)	N.S.	18 (62)	11 (38)	N.S.

*Pearson chi-squares; °Fisher's exact test; Neg.= 0,+1 intensity and <10% staining cells; Pos.= +2, +3 intensity and 10% staining cells; N.S.= no significant.

Tab. II – RELATION BETWEEN Nm23H1 AND CD44V6 EXPRESSION AND SURVIVAL

	Tot.	*Nm23 Neg.*	*Nm23 Pos.*	*p value*	*Cd44 Neg.*	*Cd44 Pos.*	*p value*
	n. (%)	*n. (%)*	*n. (%)*		*n. (%)*	*n. (%)*	
FOLLOW UP:							
Disease Free Survival	24	8	16		14	10	
	(59)	(33)	(67)		(58)	(42)	
Survival With Disease	2	1	1	N.S.	2	0	N.S.
	(5)	(50)	(50)		(100)		
Dead For Other Disease	4	2	2	2	2		
	(10)	(50)	(50)	(50)	(50)		
Dead Of Disease	11	2	9		7	4	
	(27)	(18)	(82)		(64)	(36)	

*Pearson chi-squares; °Fisher's exact test; Neg.= 0,+1 intensity and <10% staining cells; Pos.= +2, +3 intensity and 10% staining cells; N.S.= no significant.

diaminobenzidine supplemented with hydrogen peroxide (Zymed, San Francisco, CA, USA) and nuclei were counterstained with hematoxylin. A breast cancer case was utilized for positive control. The primary antibody was omitted for the negative control.

Interpretation of immunoreactivity

The degree of expression of these markers was estimated by semiquantitative evaluation and classified in two group: positive (+2, +3 intensity and >10% staining cells) and negative (0,+1 intensity and <10% staining cells).
Slides were scored independently by two pathologists; the discordant cases were reviewed and reassigned scores based on consensus of opinion.

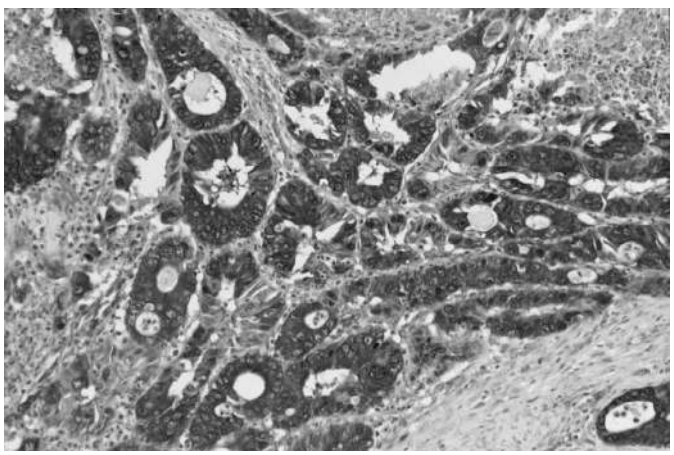

Fig. 1: Small enlargement; overall view of filtering adenocarcinoma: overexpression of the Nm23H1.

Statistical analysis

The statistical correlation among Nm23, CD44v6 and clinicopathological features were analyzed by Pearson chi-square and Fisher's exact test (SPSS version 7.5.2, Chicago, IL;1996). The statistical significance was set at $P < 0.05$.

Results

Nm23-H1 Immunohistochemical staining

Nm23-H1 protein accumulation was cytoplasmatic. Normal mucosa showed low level of immunostaining prevalently in the cytoplasm of the interstitial epithelial cells of the crypts. Nm23H1 positivity was observed in 30 cases while negativity was detected in 11 cases of the samples (Fig. 1-2).

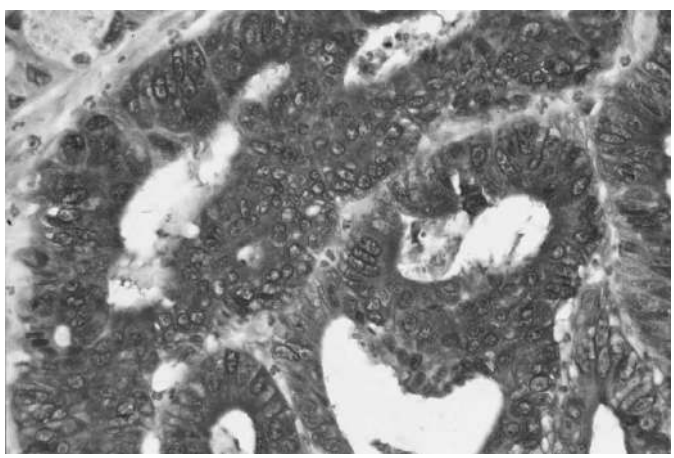

Fig. 2: A detail of Fig. 1 that shows the cytoplasmatic expression of the Nm23H1.

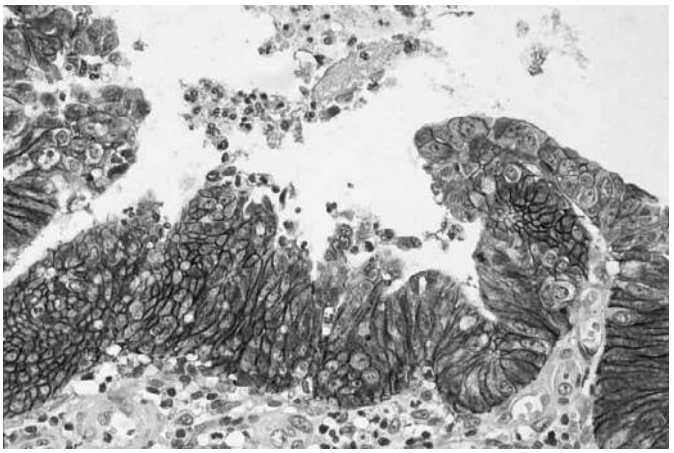

Fig. 3: Cytoplasmatic expression of CD44v6 close to the cytoplasmatic membrane, in adenocarcinoma with papillary aspect.

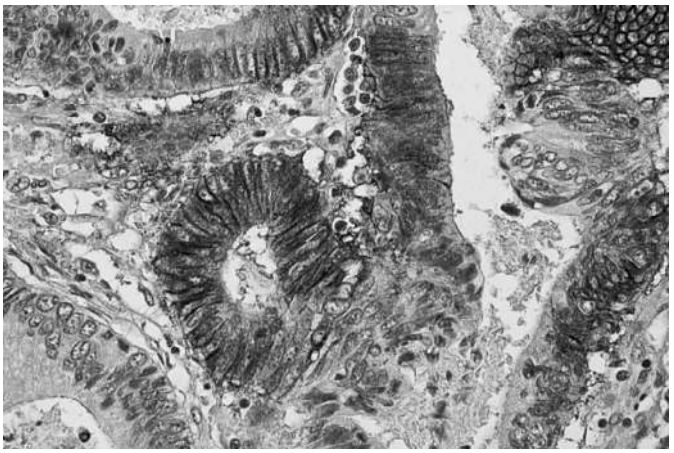

Fig. 4: Discontinuous cytoplasmatic expression of CD44v6 in the neoplastic cells.

CD44v6 Immunohistochemical staining

The expression of CD44v6 was not detected in normal colonic mucosa. CD44v6 immunostaining was prevalently cytoplasmatic and was observed in 15 cases of tumour samples with strong expression. Negative CD44v6 was detected in 26 cases (Fig. 3-4).

Expression of CD44v6 and Nm23-H1 related to clinico-pathological parameters

The correlation between CD44v6 expression and clinicopathological features is summarized in Table I. CD44v6 protein expression was not found to be statistically related to the tumoral Dukes stage, TNM stage, site, lymphocytic peritumoral infiltrating, venous infiltrating, perineural infiltrating, tumour budding, pushing and infiltrating tumour growth. However CD44v6 was found to be statistically associated with tumoral grading differentiation ($p<0.03$). In fact it seems that CD44v6 positivity is directly related to grading.
No correlation was found between Nm23-H1 and Dukes stage, grading, site, lymphocytic peritumoral infiltrating, venous infiltrating, perineural infiltrating, tumour budding, pushing and tumour infiltrating.

Discussion

In recent years numerous studies have been made to identify a biological marker, the expression of the aggressiveness of the tumour, which is statistically correlated with prognosis. Thus for to select group of patients with poor prognosis, to include in specific follow up and therapeutic programs.
The aim of this study is to evaluate the expression of two immunohistochemical markers in non metastatic colorectal cancer and to correlate them with some histopathological parameters and prognosis. The markers selected for the study, Nm23-H1 and CD44v6, are those implicated in local disease progression and metastases, as reported by several studies.
The Nm23 gene is thought to be a potential metastasis-suppressor gene because its mRNA and protein levels are greatly reduced in cell lines and tumours of high metastatic potential compared with those of low metastatic potential (12, 13). It seems that the Nm23/NDP kinase proteins are expressed on the cell surface and are important in the formation of the basement membrane. These proteins may alter the microenviroment of the basement membrane, thus modulating cellular responsiveness (12, 14, 15).
The place of Nm23 in CRC is controversial. Most studies have reported that reduced expression of Nm23, at both protein and mRNA levels, is associated with advanced tumor stage and liver metastasis (3). Moreover, several investigations showed that only the expression of Nm23-H1, but not Nm23-H2, was correlated with more advanced tumour stage (16). Cheah in 1998 reported one of the biggest series when studying the Nm23-H1 protein, showing Nm23-H1 expression to be inversely correlated with tumour staging (3). Nevertheless this result was not sufficient to predict the 5-year survival rate of CRC patients nor to predict whether an early stage CRC would progress to a more invasive tumour (3). The study thus concluded that although Nm23-H1 may be involved in suppressing metastasis, it was apparently not a major factor in metastasis suppression and was not an independent prognostic indicator in CRC (3, 17-20).
Nevertheless, in other studies Nm23H1proved to be elevated in most of the colorectal cancers examined (6). Zeng showed that, in a large series of CRC patients, Nm23-H1 RNA was significantly increased in all stages of primary CRC relative to adjacent normal mucosa. In particular high Nm23-H1 RNA levels were significantly correlated with locally advanced lesions (T2-T4) and

with large tumours than small tumours. These results suggest that in CRC the Nm23-H1 gene may play a more important role in local disease progression than in metastasis suppression (17). Subsequent works reported that overexpression of Nm23-H1 RNA and/or protein was correlated with metastasis potential and poor prognosis. Berney demonstrated in fact that patients with tumours that did not express Nm23 had an estimated 75% probability of being disease-free at 5 years; this percentage dropped to approximately 25% in those overexpressing this protein (20).

Differences in the relationship between Nm23 expression and disease progression in different tumours suggest possible tissue-specific functions. The overexpression of Nm23-H1 observed in metastatic CRC seems to be inconsistent with Nm23-H1 being a colorectal metastasis suppressor. One hypothesis is that mutations in the Nm23 gene produce a protein, functionally distinct from the wild type, that facilitates growth and metastasis (17-20).

To confirm the controversial role of Nm23 in CRC, Tabuchi recently reported a study that demonstrated no correlation between Nm23-H1 expression and prognostic variables including lymphnode and liver metastases, vascular invasion and histological stage, dCEA level, proliferative activity of cancer cells represented by AgNOR score or with the prognosis of advanced colorectal cancer patients (21).

In the study presented, the expression of Nm23-H1 does not appear to be associated with histopathological variables or with prognosis. However, even if the results are not statistically significant the authors observed that the abnormal expression of Nm23-H1 is correlated with the presence of venous infiltrating, perineural infiltrating, tumour budding, infiltrating growth of the tumour.

In the colon, CD44 is normally weakly expressed as the standard form (CD44s) in a few cells at the bases of crypt (22). Variant forms of CD44 have been found to be expressed in inflammatory conditions such as ulcerative colitis and in adenomas and carcinomas (7, 9). Many studies suggest an involvement of CD44v6 in the spread of metastatic tumours and so research has been focused primarily on this splice variant (23). Specifically, Herrlich reported that the increased expression of CD44v6 protein was a marker of metastases in human colon cancer (24). Other authors described an association between CD44v6 expression and both Dukes C and D stages in colorectal cancer and adverse prognosis (25, 26, 27). Furthermore CD44v6 was also reported to predict poor prognosis regardless of the Dukes stage (28).

On the other hand, some studies demonstrated that the expression of the CD44 variant epitopes did not correlate with either tumour progression or metastasis to the liver from colorectal carcinoma, and that CD44 expression was either lost in large invading tumours or decreasing during the formation of liver metastases from colorectal carcinoma (24, 29, 30). It seems that this loss may induce a defective binding of the tumours cells to the extracellular matrix, thus increasing their mobility and metastatic potential (24). It is possible that these differences in the reported results are due to different staining techniques, in particular reflecting the use of diverse antibodies, and perhaps due to different study populations (23, 30).

Other studies have shown CD44v6 expression in benign adenomatous polyps and have suggested that CD44v6 gene expression is an early event in colorectal carcinogenesis (23). Increasing CD44 expression has been associated with increasing dysplasia in adenomatous polyps (23). These results might be useful to identify patients at higher risk of residual carcinoma, local recurrence or lymphnode metastases (23).

In this study the authors demonstrated a correlation between grading of the tumour and the expression of the CD44v6; therefore it seems that the expression of CD44v6 may indicate a more aggressive tumour.

Nevertheless the authors consider that Nm23-H1 and CD44v6 expression should be subjected to further study in order to understand their prognostic significance.

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