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ORIGINAL ARTICLE

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Evaluation of the prognostic significance of nm23/NDP kinase and cathepsin D in anal carcinomas

An immunohistochemical study

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Abstract Reduced expression of nm23/NDP kinase and increased expression of cathepsin D seem to be correlated with a high metastatic potential for a variety of malignancies. Nm23/NDP kinase and cathepsin D have been correlated with several clinical variables, including survival in 96 patients with squamous cell carcinoma of the anal canal. Immunohistochemical methods were used on paraffin-embedded biopsies. Seventy-six (79%) anal carcinomas were nm23/NDP kinase positive, whereas 35 (36%) and 28 (29%) of the cases were cathepsin D positive in tumour cells and stromal cells, respectively. We have found no indication that the extent of cathepsin D staining has any prognostic significance. The overall survival of patients with tumours positive for nm23/NDP kinase in the cytoplasm was significantly shorter than that of patients with anal carcinomas negative for nm23/NDP kinase.

Key words Nm23/NDP kinase · Cathepsin D · Immunohistochemistry · Anal carcinoma

Introduction

Cancer invasion and metastasis are the result of a cascade of sequential steps involving multiple host–tumour interactions [21]. The mechanisms involved in cancer cell metastasis are not well understood, but the two gene products nm23 and cathepsin D have been reported to be associated with tumour metastasis [10, 13, 30, 34, 35].

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The nm23 gene, encoding nucleoside diphosphate (NDP) kinase, has been suggested to represent a new class of metastasis-associated genes [31]. In human tumours a strong association has been observed between reduced expression of nm23 gene and acquisition of vigorous metastatic behaviour in melanomas [10], breast carcinomas [4, 34], hepatocellular carcinomas [23], gastric carcinomas [20] and colorectal carcinomas [2, 36]. Other studies have failed to reveal any such association [9, 12, 14, 15, 28, 29, 37, 38].

Cathepsin D is an acidic lysosomal protease and may affect the invasive and metastatic potential of tumours by its ability to degrade the extracellular matrix [5, 27]. Abnormal high levels of cathepsin D are found in cancer cells and this overexpression is associated with an unfavourable prognosis in breast carcinomas in several [19, 26, 30, 35], but not all [1, 17], studies performed.

The role of nm23 and cathepsin D in the metastatic process of anal carcinomas has not yet been evaluated. In the present study, we examined the immunohistochemical expression of nm23/NDP kinase and cathepsin D in a comparatively large series of patients with squamous cell carcinoma of the anal canal and compared it with clinical and histopathological data.

Materials and methods

Patients

A total of 113 patients were admitted to the Norwegian Radium Hospital for squamous cell carcinoma of the anal canal during 1983–1991. "Basaloid" and "cloacogenic" tumours were included, as these represent variants of squamous cell carcinomas. All patients were examined clinically including digital ano-rectal examination, proctoscopy, CT or MR scans of the pelvic, ultrasonography of the liver, chest radiographs and blood tests. All patients received a standard treatment (combined radiochemotherapy) according to a protocol. All tumours were checked by histopathological examination. Sufficient material for immunohistochemical analyses of nm23/NDP kinase and cathepsin D was available for 96 tumours. Of these 96 patients, 73 (76%) were women and 23 (24%) were men. The mean age of the patients was 66.0 years (range 37–91). The tumours were staged according to the

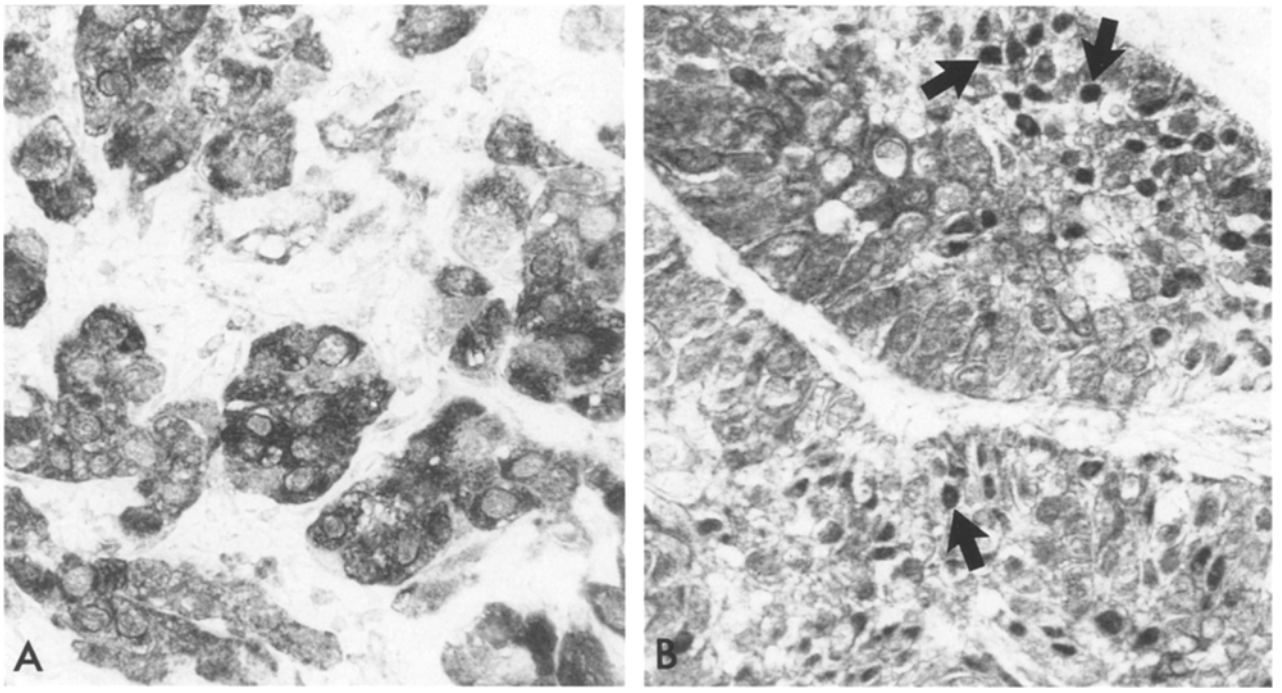

Fig. 1A, B Immunohistochemical staining for nm23/NDP kinase. Tumour with **A** strong cytoplasmic staining and **B** cytoplasmic and nuclear staining (*arrows*). ×400

TNM/sytem (UICC). There were 75 patients with primary tumours, 9 with T1, 22 with T2, 23 with T3 and 21 patients with T4 tumours. Twenty-one patients had a local recurrence following previous surgery. A total of 9 patients were lost during follow-up. The remaining 87 patients were followed up at regular intervals for 5 years after treatment.

Immunohistochemistry

Sections for immunohistochemistry were stained with the avidin–biotin-peroxidase complex (ABC) method [16]. Deparaffinized sections were treated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min to block endogenous peroxidase. To unmask the epitopes of cathepsin D, we microwaved the sections twice for 5 min in 10 mM citrate buffer pH 6.0 [6] in a household microwave oven (NF-4084, Electrolux) at the maximum power (800 W). The sections were then incubated for 20 min with normal serum from the species in which the secondary antibody was made. This was done to eliminate nonspecific staining. Excess normal serum was blotted from the slides before incubation with polyclonal nm23-H1/NDP kinase A antibody (Boehringer Mannheim, Mannheim, Germany) diluted 1:50 (1 µg IgG/ml) and polyclonal cathepsin D antiserum (Zymed Laboratories, San Francisco, Calif.) diluted 1:50 for 18–22 h at 4°C. The sections were then incubated with 1:200 dilution of biotin-labelled secondary antibody for 30 minutes and ABC (10 µg/ml of avidin and 2.4 µg/ml of biotin-labelled peroxidase) for 60 min (Vector, Burlingame, Calif.). Tissues were stained for 5 minutes with 0.05% 3′3-diaminobenzidine tetrahydrochloride (DAB) freshly prepared in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.6, containing 0.01% H_2O_2 and then counterstained with haemotoxylin, dehydrated, and mounted in Diatex. All the dilutions of normal sera, antibodies, biotin-labelled secondary antibodies and ABC were made with phosphate-buffered saline, pH 7.4, containing 5% bovine serum albumin. All series included positive controls. Negative controls included substitution of primary polyclonal antiserum/antibody with normal rabbit serum/antibody. All controls gave satisfactory results.

The immunostaining results for nm23/NDP kinase and cathepsin D were scored semi-quantitative according to the intensity of staining and proportion of cells stained. To score positive, more than 10% of the tumour cells had to show moderate to strong staining.

Statistical analysis

Statistical analyses were performed with the BMDP-PC programme package (Pearson Chi-square test) using a level of significance of 0.05.

Results

The 96 anal carcinomas included 76 (79%) that were nm23/NDP kinase positive. All positive tumours exhibited cytoplasmic staining (Fig. 1A). In addition 23 of the positive cases showed nuclear staining (Fig. 1B). Seventeen specimens of normal anal squamous epithelium adjacent to a tumour were negative for nm23/NDP kinase.

The cathepsin D content was evaluated as positive in 48 (50%) of the 96 anal carcinomas. Thirty-five (36%) of these cases exhibited cytoplasmic staining in tumour cells (Fig. 2), whereas in 28 (29%) of these cases stromal cells were immunoreactive. In 15 of the positive cases both tumour and stromal cells expressed cathepsin D. The expression of cathepsin D in carcinoma cells and stromal cells was statistically significantly associated (P=0.03). Weak cytoplasmic cathepsin D staining was observed in 17 specimens of normal anal squamous epithelium adjacent to a tumour and was evaluated as negative.

An inverse relationship was seen between nm23/NDP kinase and cathepsin D expression (P=0.03). Thirty-three (34%) of the tumours were positive for both nm23/NDP

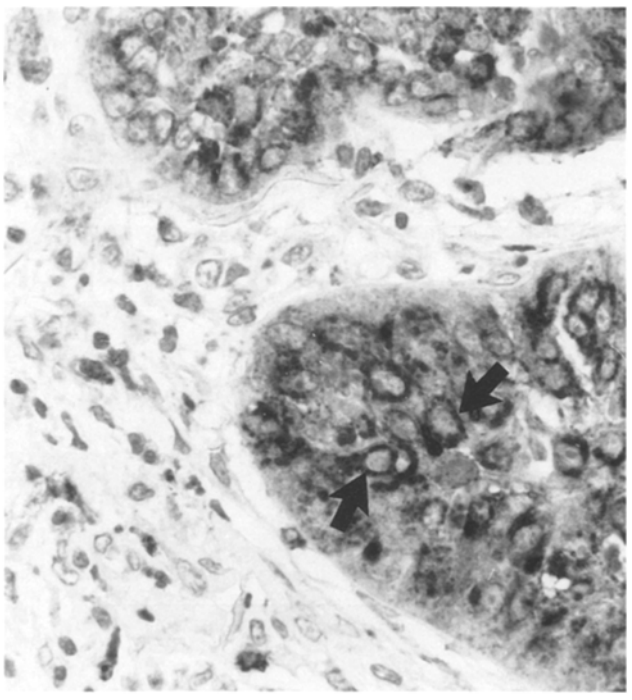

Fig. 2 Immunohistochemical staining for cathepsin D. Case with strong cytoplasmic staining in tumour cells (*arrows*). ×400

Table 1 Histopathological/clinical diagnosis compared with nm23/NDP kinase and cathepsin D expression

	Total no. of cases	nm23/NDP kinase positive (%)	Cathepsin D positive (%)[a]
Tumour stage			
T_1	9	9 (100)	2 (22)
T_2	22	19 (86)	4 (18)
T_3	23	14 (61)	11 (48)
T_4	21	19 (90)	10 (48)
R	21	15 (71)	8 (38)
Nodal involvement			
Negative	71	57 (80)	25 (35)
Positive	25	19 (76)	10 (40)
Differentiation			
High/moderate	51	40 (78)	17 (33)
Poor	45	36 (80)	18 (40)

[a] Cases with cathepsin D expression in tumour cells

kinase and cathepsin D or negative for both, whereas 63 (66%) of the tumours were positive for nm23/NDP kinase and negative for cathepsin D or negative for nm23/NDP kinase and positive for cathepsin D.

The extent of nm23/NDP kinase and cathepsin D staining showed no significant correlation with tumour stage, lymph node metastases or grade of differentiation (Table 1). Patients with tumours with cytoplasmic staining for nm23/NDP kinase had a poorer survival than those with a negative tumour (P=0.03). However, no significant relationship was found between nuclear nm23/NDP kinase staining and overall survival ($P > 0.10$). There was no correlation between tumour and/or stromal staining for cathepsin D and overall patient survival ($P > 0.10$).

Discussion

In malignant melanoma, breast carcinoma, hepatocellular carcinoma, gastric carcinoma, colorectal carcinoma and cervical adenocarcinoma, decreased nm23/NDP kinase expression was shown to correlate with a vigorous metastatic potential [18, 20, 31, 34], the presence of distant metastases [2, 36], early appearance of metastases [10] and poor prognosis [20, 22, 34]. These reports are in accordance with the hypothesis that nm23 functions as a tumour metastasis-suppressor gene. To our knowledge, no corresponding studies on anal carcinomas have been published. Our results are in contrast to these findings; we found poorer survival in patients with nm23/NDP kinase-positive anal carcinomas. Similar results have been reported in neuroblastoma [12], lung carcinoma [9] and thyroid carcinoma [38], with an increased expression of nm23/NDP kinase associated with an advanced stage of disease. Furthermore, others did not find any significant correlation between the extent of nm23/NDP kinase staining and lymph node metastases, distant metastases or survival rate in pulmonary adenocarcinoma [14], colorectal carcinoma [37] and medullary thyroid caracinoma [15]. These contradictory results seem to indicate that the correlation of nm23/NDP kinase expression with the metastatic process is dependent on the tissue type. The varying results may also be due to the identification of nm23/NDP kinase protein in some earlier studies [1, 14, 15, 28, 34, 36, 37] and in this work, whereas others have relied on nm23 mRNA detection [4, 9, 10, 18, 20, 37, 38]. Furthermore, the nm23/NDP kinase antibodies used may recognize different epitopes. The polyclonal nm23/NDP kinase used in the present study was raised against nm23-H1/NDP kinase A. However, it is known that nm23-H1/NDP kinase A has 88% identity to nm23-H2/NDP kinase B [11], and cross-reaction with nm23-H2/NDP kinase B cannot be excluded. In other studies, a monoclonal nm23 antibody identifying only nm23-H1/NDP kinase A has been used [34, 36].

Previously, immunohistochemical analyses of nm23/NDP kinase have focused solely on cytoplasmic staining. In the study of Bertheau et al. [3], malignant thyroid tumours expressing nm23/NDP kinase in the nuclei were associated with longer disease-free survival. In contrast to this finding we did not observe any significant relationship between nuclear nm23/NDP kinase staining and prognosis. However, we have demonstrated for the first time that increased cytoplasmic staining for nm23/NDP kinase in anal carcinomas is correlated with a poorer survival.

Recently, it has been demonstrated that a differentiation-inhibiting factor in a mouse myeloid leukaemia cell line is the murine homologue of nm23-H2 [24]. Furthermore, in lung carcinomas increased expression of both

nm23-H1 and nm23-H2 mRNA was correlated with poorly differentiated tumours [9]. Thus, nm23/NDP kinase may act as an inhibitor of differentiation in mouse myeloid leukaemia cells and lung tumours. In a previous report, nm23/NDP kinase has been shown to act as a transcriptional factor for *c-myc* expression [25]. Increased *c-myc* expression has commonly been noted in anal carcinomas [8], and cell lines overexpressing *c-myc* have a markedly higher resistance to differentiation than do parental lines lacking the overexpression of *c-myc* [7]. Therefore, it may be possible that elevated expression of nm23/NDP kinase up-regulates *c-myc* and inhibits the cellular differentiation. However, this concept was not supported in our series of anal carcinomas; we did not observe any relationship between nm23/NDP kinase expression and degree of cellular differentiation.

Cathepsin D expression has been identified as a poor prognostic factor in breast carcinomas [17, 30, 32]. With the cytosolic assay used in these reports, the cellular origin of the enzyme cannot be assessed. In this and in other studies, immunohistochemical methods have been used that demonstrate cathepsin D expression in both cancer cells and stromal cells [1, 19, 33]. In the present study there was no significant correlation between the extent of cathepsin D staining in tumour and/or stromal cells and tumour stage, lymph node metastases, grade of differentiation or overall survival in patients with anal carcinomas. This is in agreement with the immunohistochemical results obtained in breast carcinomas [1]. In contrast with these findings, the elevated extent of cathepsin D staining in tumour cells [19] and stromal cells [33] has been shown to be associated with a poor prognosis in patients with breast carcinomas.

The specimens used in the present study have been collected from 14 different pathology laboratories, and the fixation procedures may vary. Such variation has been claimed to influence immunohistochemical results. However, this is unlikely since recent work in our institution (unpublished findings) has identified similar immunostaining for nm23/NDP kinase and cathepsin D in tissue fixed in 10% acid formalin and in 10% buffered formalin.

In summary, we have found that the extent of cathepsin D staining has no clinical significance in anal carcinomas, while increased cytoplasmic staining for nm23/NDP kinase is correlated with poorer survival for patients with squamous cell carcinoma of the anal canal.

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