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Immunohistochemical detection of nm23/NDP kinase and cathepsin D in medullary carcinomas of the thyroid gland

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Abstract Reduced expression of nm23/NDP kinase and increased expression of cathepsin D seem to be correlated with the high metastatic potential in a variety of malignancies. The expression of nm23/NDP kinase and that of cathepsin D have been evaluated by means of an immunohistochemical technique in paraffin-embedded tissues from 44 primary medullary carcinomas of the thyroid gland (MCT) and from the corresponding lymph node metastases in 32 of these cases. In addition, lymph node metastases from 4 cases were studied. We found that 36 of 44 (82%) primary and 26 of 36 (72%) lymph node metastatic MCT were nm23/NDP kinase positive, whereas 14 of the 44 (32%) primary and 17 of the 36 (47%) lymph node metastatic MCT were cathepsin D positive. We found no indication that the nm23/NDP kinase level has any prognostic significance in MCT. The cathepsin D level is close to being prognostically significant in this study, and we cannot exclude the possibility that it could be of prognostic value. However, it seems to be quite weak, and therefore of little use in a clinical situation.

Key words Immunohistochemistry · nm23/NDP kinase · Cathepsin D · Medullary thyroid carcinoma

Introduction

Tumour metastasis is the major cause of death among cancer patients [31] and an understanding of the processes that are involved in metastasis and their regulation is crucial to the development of new strategies for treatment and prevention of the disease. The metastatic process comprises a number of definable stages, each of which is likely to entail the activation and/or repression of specific genes or gene products. Two such gene products, nm23 and cathepsin D, have been reported to be associated with tumour metastasis [11, 13, 29, 32, 34].

The former nm23, was first identified as a gene expressed in reduced amounts in highly metastatic rodent tumour cells [30]. The nm23 gene product is known to be identical to nucleoside diphosphate (NDP) kinase [26, 33]. In human tumours a strong association has been observed in some studies between reduced expression of nm23/NDP kinase gene and the acquisition of agressive metastatic behaviour in melanomas [11], hepatocellular carcinomas [23], breast carcinomas [4, 6, 13, 15, 32], colorectal carcinomas [3], gastric carcinomas [24], and papillary carcinomas of the thyroid gland [1]. However, other studies have not revealed such an association [9, 14, 19, 20, 27, 28, 36].

Proteases, such as cathepsin D, have proteolytic properties and may facilitate tumour invasion and metastasis by the ability to degrade the extracellular matrix [7]. A high cytosolic concentration of cathepsin D is associated with an unfavourable prognosis in breast carcinomas in several [18, 25, 29, 34] but not all [2, 17] studies performed.

The role of nm23 and cathepsin D in the metastatic process of medullary carcinomas of the thyroid gland (MCT) has not yet been evaluated. Since new prognostic markers for this tumour group are urgently needed, because of the unpredictable clinical course, we examined the immunohistochemical expression of nm23/NDP kinase and cathepsin D and compared the expression of each with clinical and pathological findings. Furthermore, we planned to address the issue of changes in nm23/NDP kinase and cathepsin D expression with tumour progression, since we had tumour samples available from 32 primary tumours and the corresponding lymph node metastases.

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Materials and methods

Patient material

Formalin-fixed and paraffin-embedded tissue from 48 patients with MCT diagnosed in the period from 1961 to 1992 were available for immunohistochemical study. The cases included 44 primary tumours, and in 32 of these the corresponding lymph node metastases were available. In addition, lymph node metastases from 4 other cases were studied. Clinical information was available for 45 patients, viz the 41 with primary tumours and the 4 for whom lymph node metastases alone were studied. The mean age of the 30 women and 15 men at diagnosis was 47 years (range 14–81 years). Eight (18%) of the patients had familial MCT, whereas 37 (82%) had sporadic MCT. Thirty-eight (84%) of the patients had lymph node metastases, and distant metastases were detected in 23 (51%) of the patients during the course of their disease. The observation period was from 2 to 33 years after primary diagnosis. Twenty-three of 43 (53%) patients died of disseminated disease within the follow-up period.

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 in 10 mM citrate buffer pH 6.0 [8]. 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 a 1:200 dilution of biotin-labelled secondary antibody for 30 min 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 min 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 haematoxylin, 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 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 heavy staining.

Statistical analysis

To test correlations between nm23/NDP kinase or cathepsin D levels in 45 MCT (41 primary and 4 lymph node metastatic tumours) and the clinical outcome, the Fisher exact probability test was used on the 2×2 contingency tables. When the changes in these levels from the primary tumours to the lymph node metastases were examined, McNemar significance tests were performed.

The difference in survival rates in the various prognostic groups were examined by log-rank tests in univariate analysis, and by Cox backward regression analysis in the multivariate case. *P*-values ≤0.05 (two-tailed) were considered statistically significant.

Table 1 Comparison of nm23/NDP kinase and cathepsin D immunoreactivity in primary and lymph node metastatic MCT (*Pr* primary tumour, *LN* lymph node metastatic tumour, + tumours positive; – tumours negative)

Antibody	Total no.	Immunoreactivity			
		Pr+ LN+	Pr– LN–	Pr+ LN–	Pr– LN+
nm23/NDP kinase	32	21 (66%)	5 (16%)	4 (12%)	2 (6%)
Cathepsin D	32	12 (38%)	18 (56%)	0 (0%)	2 (6%)

Results

In normal follicular epithelial cells adjacent to tumours a strong cytoplasmic staining for nm23/NDP kinase was present in 31 cases. In 7 of these cases nuclear staining was also identified. We observed that 36 of the 44 (82%) primary tumours and 26 of the 36 (72%) lymph node metastases were nm23/NDP kinase positive. All positive tumours exhibited cytoplasmic staining, while 4 primary and 3 metastatic tumours also showed nuclear staining. The nm23/NDP kinase immunoreactivity was compared in 32 primary tumours and corresponding lymph node metastases (Table 1). In 18% of the cases primary and lymph node metastatic tumours had different staining patterns (Fig. 1a, b), whereas in 82% of the cases both primary and corresponding lymph node metastases were either nm23/NDP kinase positive or nm23/NDP kinase negative.

Weak cytoplasmic cathepsin D staining observed in 31 normal thyroid tissues from sites adjacent to tumours was evaluated as negative. The cathepsin D content was evaluated as positive in 14 of the 44 (32%) primary MCT and 17 of the 36 (47%) lymph node metastases. The cytoplasmic staining was predominantly in tumour cells only. However, in one case both tumour and stromal cells were positive, whereas in another case only stromal cells were immunoreactive. Table 1 shows a comparison of cathepsin D immunoreactivity in primary tumours and the corresponding lymph node metastases. Of the 32 cases examined, 94% had an identical reaction pattern (Fig. 2a, b), while in 6% of the cases primary tumours were negative and lymph node metastatic lesions were cathepsin D positive (Fig. 2c, d).

No significant relationship between nm23/NDP kinase and cathepsin D expression could be demonstrated. In 27% of the cases positive staining for both nm23/NDP kinase and cathepsin D was observed; in 46% of the cases for nm23/NDP kinase only; in 10% of the cases for cathepsin D only; and in 17% for neither.

The levels of nm23/NDP kinase and cathepsin D showed no significant correlation with lymph node metastases or distant metastases ($P>0.10$ for each of these correlations).

In the univariate analysis of the subgroups for age, sex, nm23/NDP kinase or cathepsin D only age emerges

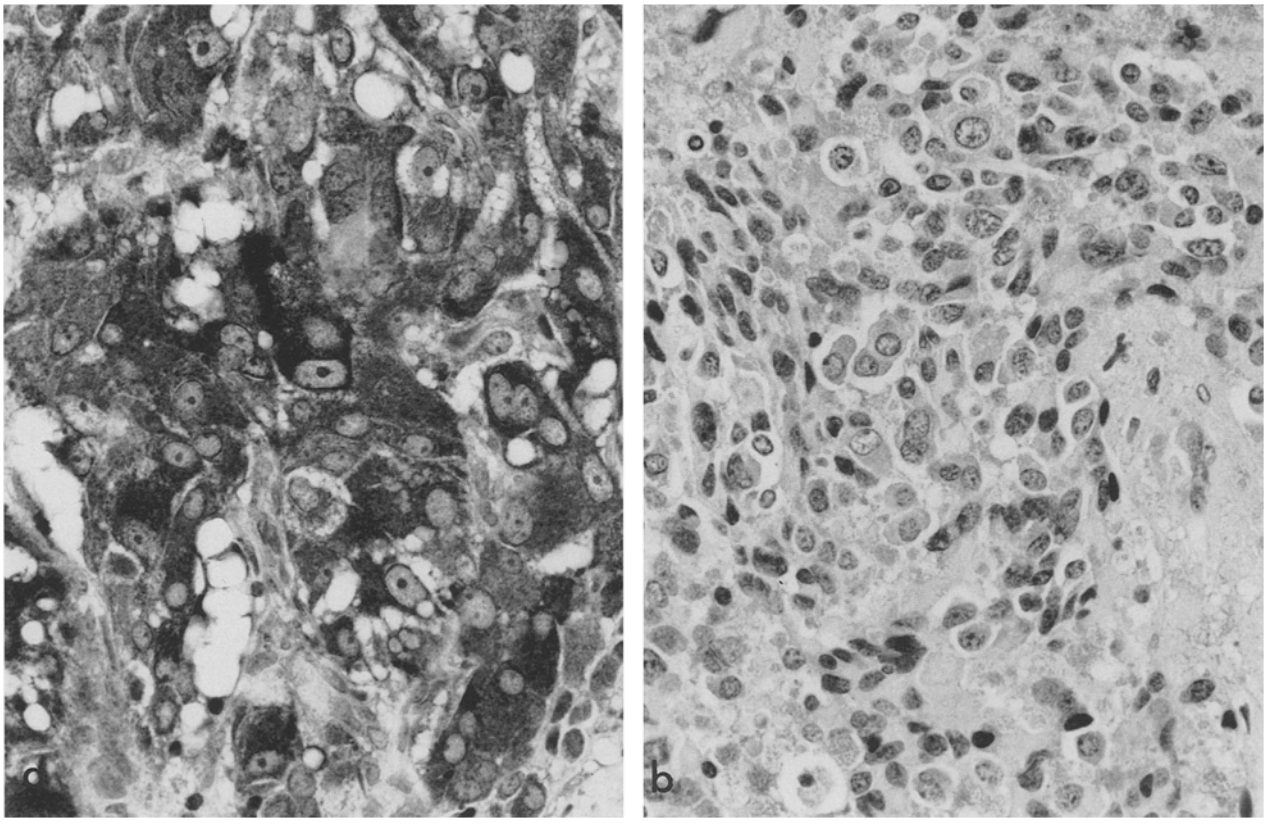

Fig. 1a, b Immunohistochemical staining for nm23/NDP kinase in a primary tumour and the corresponding lymph node metastases. **a** Primary tumour with heavy cytoplasmic staining; **b** corresponding nm23/NDP kinase-negative lymph node metastatic tumour. ×400

as a significant variable ($P<0.01$). The multivariate analysis shows that sex also has a significant prognostic value ($P<0.01$), which is hidden in the univariate analysis because of a large selection bias (mean age 50 years for women and 38 years for men). The prognostic significance of sex has little consequence for the survival analysis with regard to marker levels, since women and men are distributed proportionately over the nm23/NDP kinase and cathepsin D subgroups. However, there is some age selection bias in these subgroups. For nm23/NDP kinase this does not result in any substantial improvement in prognostic significance, while the age correction implied in the multivariate analysis brings cathepsin D up to an almost significant P-level ($P=0.07$). However, the numbers are small, and a much larger number of specimens would be needed to confirm the trends observed here.

Discussion

MCT is an uncommon tumour, constituting 5–10% of all thyroid carcinomas. A subgroup of the patients affected has aggressive disease with progression to distant metastases and eventual death. To investigate a role of nm23 and cathepsin D in the metastatic process of MCT, we evaluated the levels of nm23/NDP kinase and cathepsin D in a series of MCT. We did not find any significant correlation between the levels of nm23/NDP kinase and lymph node metastases, distant metastases or survival rate. Similar results have been reported in at least three different types of human cancer, including pulmonary adenocarcinomas [14], breast carcinomas [28] and papillary thyroid carcinomas [10, 20]. However, studies on melanomas [11], hepatocellular carcinomas [23], colorectal carcinomas [3], breast carcinomas [4, 13], gastric carcinomas [24] and papillary thyroid carcinomas [1] revealed an association between nm23/NDP kinase expression and the aggressiveness of the disease. These contradictory results may 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 [3, 4, 14, 23, 28] and in our own present work, whereas other groups have relied on nm23 mRNA detection [1, 10, 11, 13]. Furthermore, the nm23/NDP kinase antibodies used may have recognized 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 with nm23-H2/NDP kinase B [12]. Thus, crossreaction with nm23-H2/NDP kinase B cannot be excluded. In other studies, monoclonal nm23 antibody identifying only nm23-H1/NDP kinase has been used [20, 32, 35].

There was no significant correlation between the level of cathepsin D and lymph node metastases, distant me-

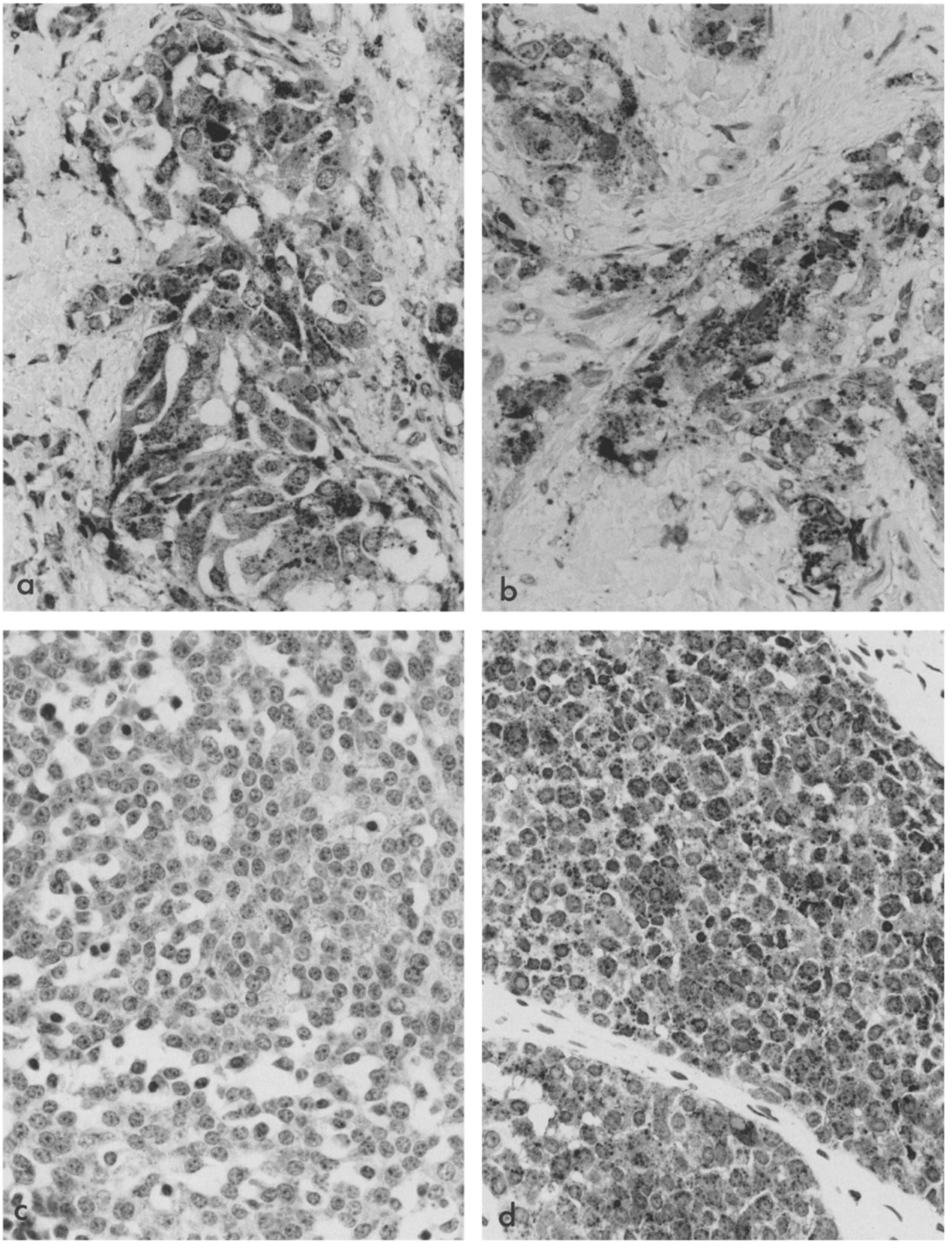

Fig. 2a–d Immunohistochemical staining for cathepsin D in two primary tumours and the corresponding lymph node metastases. **a** Primary tumour, **b** lymph node metastatic tumour, both with heavy cytoplasmic staining. **c** Cathepsin D-negative primary tumour, **d** lymph node metastatic tumour with heavy cytoplasmic staining. ×400

tastases or survival rate in patients with MCT in our study. This is in agreement with the immunohistochemical findings in breast carcinomas [2, 17] and cervical carcinomas [22]. However, in breast carcinomas positive cathepsin D immunostaining has been shown to be asso-

ciated with poor prognosis [18, 34]. Furthermore, Metaye et al. [21] have indicated that a high level of cathepsin D may be a potential marker for poor prognosis in thyroid carcinomas. However, only 14 carcinomas were included in their study.

Among the 32 cases of paired samples included in our series the levels of nm23/NDP kinase and cathepsin D expression were similar in the primary tumours and the corresponding lymph node metastases. Bertheau et al. [5] tested 9 cases of paired thyroid cancer and obtained no significant difference in nm23/NDP kinase staining of primary tumours and lymph node metastases. The histological subtypes of these 9 cases were not described. These results indicate that expression of nm23/NDP kinase and cathepsin D may not be important for progression of thyroid cancers.

We have found no indication that the nm23/NDP kinase level has any prognostic significance in MCT. The cathepsin D level is close to being prognostically significant according to the results of this study, and we cannot exclude the possibility that it is of prognostic value. However, it seems to be quite weak and therefore of little use in a clinical situation.

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