Immunohistological Study of NM 23 and C-erbB-2 Expression in Primary Tumor and Metastases of Colorectal Adenocarcinoma

V. V. Delektorskaya, A. G. Perevoshchikov, and N. E. Kushlinskii

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 135, No. 5, pp. 575-580, May, 2003
Original article submitted February 7, 2003

Immunohistochemical study of expression of nm 23 and c-erbB-2 protein markers in primary tumor and metastases of colorectal adenocarcinoma showed that the incidence and expression of both protein markers in primary tumor tissue tended to increase after the appearance of liver metastases. Peculiarities of protein expression in tumor metastases in lymph nodes and liver and the possibility of using these markers as additional prognostic criteria in colorectal cancer are discussed.

Key Words: *adenocarcinoma; colon; nm 23; c-erbB-2; immunohistochemistry*

Colorectal cancer is one of the most prevalent oncological diseases; the prognosis is determined by many clinical and morphological factors, in particular, by cell capacity to metastasize.

Many molecular genetic tumor markers serve as indicators of biological activity of cancer cells and can be used as prognostic criteria in clinical oncology. The presence or absence of expression of some markers allows more accurate evaluation of intricate many-staged process of tumor metastasizing. On the other hand, the role of the majority of these factors is not quite clear.

Protein nm 23 is a potentially important marker involved in cell proliferation, differentiation, and regulation of metastasizing. *nm 23-H1* gene is located in chromosome 17q21 and encodes protein with a relative molecular weight of 17 kDa. At first, *nm 23* gene was described as a suppressor of metastasizing, and some reports demonstrated decreased level of *nm 23* gene product expression during the progress of some human tumors [1,6]. However further studies demonstrated more intricate relationships between the appearance of protein marker in cancer cells and metastatic activity of the tumor [2,4]. Hyperexpression of nm 23 protein was detected in many human malignant tumors, such as lung cancer, colorectal cancer, melanoma, breast cancer, bladder, laryngeal, and stomach cancer [3,6].

Another important prognostic factor in colorectal cancer is c-erbB-2 protein belonging to the family of epidermal growth factor receptors with tyrokinase activity. *c-erbB-2* gene is located in chromosome 17q21 and encodes a transmembrane glycoprotein with a molecular weight of 185 kDa. Enhanced expression of this protein is associated with gene amplification and correlates with poor prognosis [7,8]. Hyperexpression of c-erbB-2 protein is observed in breast, lung, colorectal, pancreatic, gastric, and endometrial cancer.

We evaluated the relationship between the expression of protein markers nm 23 and c-erbB-2 and the prognosis and development of metastases of colorectal adenocarcinoma.

MATERIALS AND METHODS

The study was carried out on clinical data and biopsy specimens from 64 patients (43 men and 21 women aged 25-67 years) after radical surgery for colorectal cancer. Metastases to regional lymph nodes (T2-4N0 M0) were absent in 20 patients. Twelve patients had metastases in regional lymph nodes (T2-4N1-3M0). In

N. N. Blokhin Cancer Research Center, Russian Academy of Medical Sciences, Moscow

0007-4888/03/1355-0489$25.00

two patients local relapses occurred and surgical treatment was carried out 1 year after radical intervention. Resection of the liver for metastases of colorectal cancer (T2-4N0-3M1) was carried out in 32 cases. Simultaneous removal of metastases in the lungs and adrenals was carried out in 3 cases. The periods of the development of distant metastases varied: in 18 cases metastases were detected during surgery, in 12 patients 1-5 years after the intervention, and in 2 patients no relapses developed over 5 years postoperation. Repeated operations for removal of liver metastases were performed in 5 patients.

Histological type of the tumor, degree of its differentiation and level of invasion, and the presence of regional and distant metastases were evaluated on paraffin sections stained with hematoxylin and eosin and tested by Kreiberg's method (for mucus). All primary tumors had typical structure of adenocarcinoma (colorectal adenocarcinoma, CRAC) of different differentiation degree: 10 well-differentiated, 42 moderately differentiated, and 12 poorly differentiated tumors. According to Dukes classification, 2 patients had stage A, 18 stage B, 12 stage C, and 32 stage D. The presence of invasion in vessels, lymphoid infiltration, mucus production, and type of tumor growth (apposition, infiltrative, or mixed) were evaluated.

Specimens of 64 primary tumors, 19 metastases in regional lymph nodes, 32 liver metastases, 5 repeated metastases into the liver, 2 lung metastases, and 1 metastasis into the adrenal were selected for immunohistochemical analysis.

Immunohistochemical staining was carried out on serial paraffin sections of primary tumor and metastatic tissue pretreated in microwave oven; commercial monoclonal antibodies to nm 23-H1 and c-erbB-2 proteins (Dako) were used. Incubation with primary antibodies was carried out for 30 min at 20°C. Incubation with linker antibodies and streptavidine-labeled antibodies was carried out for 20 min at 20°C (LSAB+Kit, Dako). The reaction product was visualized with diaminobenzidine (Dako). Cell nuclei were post-stained with hematoxylin in all cases. Control reactions were carried out.

Results of reactions in primary tumor cells and in cells of local and distant metastases were evaluated by the semiquantitative method (by staining intensity and percentage of antigen-positive cells): "-" no stain; "+-" foci, weak staining; "+" strong staining of 25% tumor cells or moderate staining of <80% cells; "++" strong staining of 25-50% or moderate staining of >80% cells; "+++" strong staining of >50% tumor cells.

Sections with immunochemical staining "+" and more were considered as antigen-positive. The type of reaction, depending on the marker location in the cell (nuclear, cytoplasmic, membrane, mixed) was determined. The degree and type of immunohistochemical staining of cells of the primary tumor in the colon and metastases in the lymph nodes, liver, and other organs were compared. Statistical analysis of correlations between hyperexpression of protein and clinical and morphological parameters of the tumor was carried out using Fisher's test (the differences were significant at $p<0.05$).

RESULTS

Immunohistochemical analysis of primary colorectal tumors showed accumulation of nm 23 protein in 53% cases. Protein expression was seen as homogeneous staining of the tumor cell cytoplasm. The reaction intensity and percentage of antigen-positive cells varied in different tumors and sometimes at different sites within the same tumor. High level of expression ("++" and "+++") was observed in 54% positive cases (Fig. 1, *a*). Accumulation of c-erbB-2 protein in CRAC cells was less incident (45% cases). The type of reaction was mainly membrane or mixed (membrane and cytoplasmic). High level of expression ("++" or "+++") was detected in 62% positive CRAC cases (Fig. 1, *b*). Hence, both protein markers were present in CRAC cells and demonstrated a pronounced reaction in the majority of cases, but their incidence was different in patients with liver metastases and in patients without distant metastases.

Accumulation of nm 23 protein in the cytoplasm of primary tumor cells was observed in 38% patients without metastases (12/23) and in 63% patients with metastases in the liver. Hence, the incidence of nm 23 protein in patients with metastases in the liver was significantly higher than in those without distant metastases ($p=0.023$). High intensity of staining of the cytoplasm and more than 50% antigen-positive cells of primary CRAC was also more often observed in this group of patients (34%) than in the group without metastases (22%).

Expression of c-erbB-2 in primary tumor cells was detected in 31% patients (10/32) without signs of metastases and in 59% (21/32) patients with metastases in the liver. The incidence of protein detection and the degree of its expression were higher in patients with liver metastases than in those without distant metastases ($p=0.012$).

Expression of nm 23 did not depend on the presence or degree of c-erbB-2 expression, though there was a trend to increased expression of both markers in patients with liver metastases.

No relationships between the expression of nm 23 and c-erbB-2 proteins and other clinical morphological parameters of the tumor were detected. Epithelial cells of the borderline colorectal mucosa treated with anti-

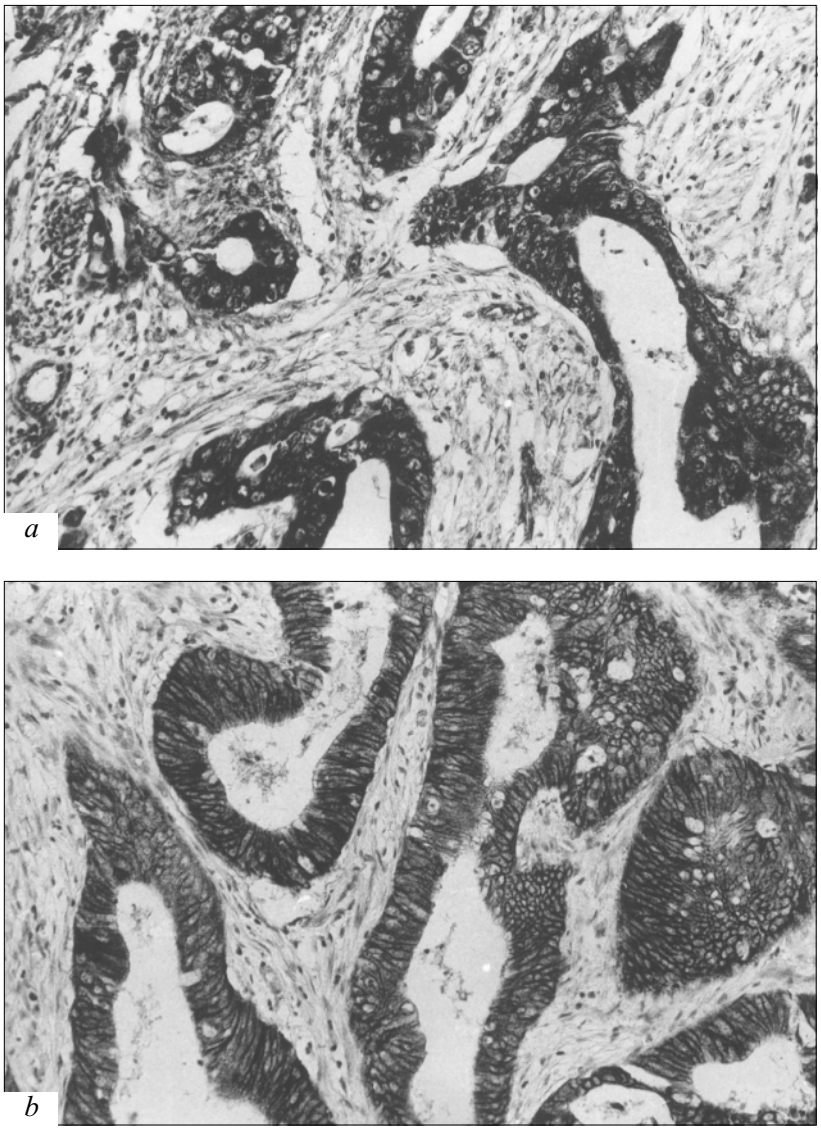

Fig. 1. Immunohistochemical detection of protein markers in primary adenocarcinoma cells. *a*) nm 23 protein; highly intensive cytoplasmic reaction in the majority of cancer cells, ×200; *b*) c-erbB-2: intensive expression of protein in cancer cells. Reaction of the membrane and cytoplasmic types, ×200.

bodies to nm 23 and c-erbB-2 were stained with moderate intensity.

Expression of proteins nm 23 and c-erbB-2 was studied in primary tumor cells and in cells of CRAC metastases. Protein nm 23 was detected in 58% of cancer metastases cells in lymph nodes. The expression of this marker in metastatic cells coincided with its expression in the primary tumor in 51% cases, was stronger in 14%, and weaker in 14% cases. In addition, moderate reaction was observed in metastatic cells in 2 cases (21%) without expression in the primary CRAC cells.

Accumulation of c-erbB-2 in this group was observed in 42% cases, the degree of the protein expression in the primary tumor and metastasis in the lymph node coincided. The reaction was moderate or pronounced in the majority of cases; cells within the same tumor were often stained heterogeneously (Fig. 2, *a*).

Staining of CRAC metastases in the liver revealed nm 23 protein in 66% cases, high level of expression ("++" or "+++") with diffuse even staining of the cell cytoplasm was observed in 52% positive cases. Accumulation of protein nm 23 in low levels was observed also in the hepatocyte cytoplasm (Fig. 2, *b*). The level of protein expression in metastatic cells coincided with the level of its expression in the primary tumor in 52% cases, was lower in 10%, and higher in 33% cases; the reaction in metastatic cells was negative in 5% cases with pronounced expression of the protein in the primary tumor cells.

Positive expression of c-erbB-2 protein in cells of CRAC metastases in the liver was detected in 59% cases. The reaction was weak in only 3 cases (Fig. 3, *a*), while in other cases the expression of the marker was pronounced (Fig. 3, *b*). The level of protein expression in metastases coincided with its expression in

the primary tumor in 74% cases, was lower in 11%, and higher in 16% cases.

Hence, comparison of expression of nm 23 and c-erbB-2 proteins in primary tumor and metastatic cells showed slight differences in the incidence of the studied markers. The types of reactions coincided in all cases. Expression of markers in metastases can decrease or increase. Examination of repeated metastases in the liver showed no specific features in expression of proteins nm 23 and c-erbB-2. Similar regularities in expression of protein markers were observed in CRAC metastases into the lungs and adrenal.

Peculiarities of expression of the two protein markers playing an important role in the development of colorectal cancer were analyzed in this study.

Enhanced expression of some genetic markers in the primary tumor can predict the disease outcome, but the role of these factors in cases with liver metastases is not yet quite clear. It is known that the phenotype of metastases can differ from that of the primary tumor. Since the involvement of the liver and lungs can rarely be surgically treated, studies of the antigenic structure of metastases attract special interest. Our findings indicate that nm 23 and c-erbB-2 proteins are accumulated in the lymph nodes, liver, lungs, and adrenal. Both proteins showed similar regularities of expression, which could coincide with expression in the primary tumor, be lower or higher, or absent in comparison with the primary tumor.

The role of nm 23 and c-erbB-2 proteins in colorectal cancer remains disputable [1,6,7]. Some studies [5,8] demonstrated an inverse relationship between nm 23 protein expression and progress of metastases. Pronounced expression of the protein is associated with less aggressive phenotype of the primary tumor. Some authors [4,10] reported that nm 23 protein cannot be

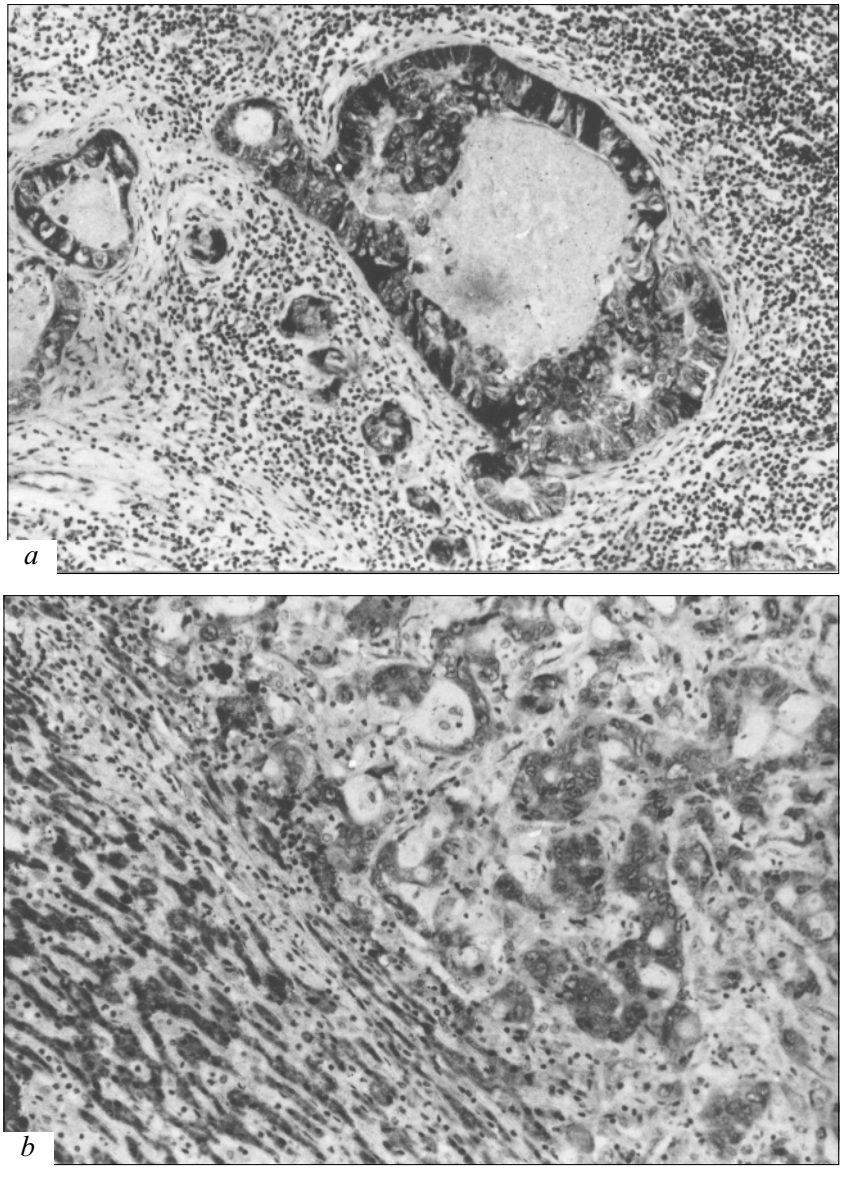

Fig. 2. Immunohistochemical detection of protein markers in cells of adenocarcinoma metastases. *a*) c-erbB-2; accumulation of protein in cells of metastasis into the lymph node. Highly intensive membrane and cytoplasmic reaction, ×200; *b*) nm 23; moderate cytoplasmic reaction in cells of liver metastasis, ×200.

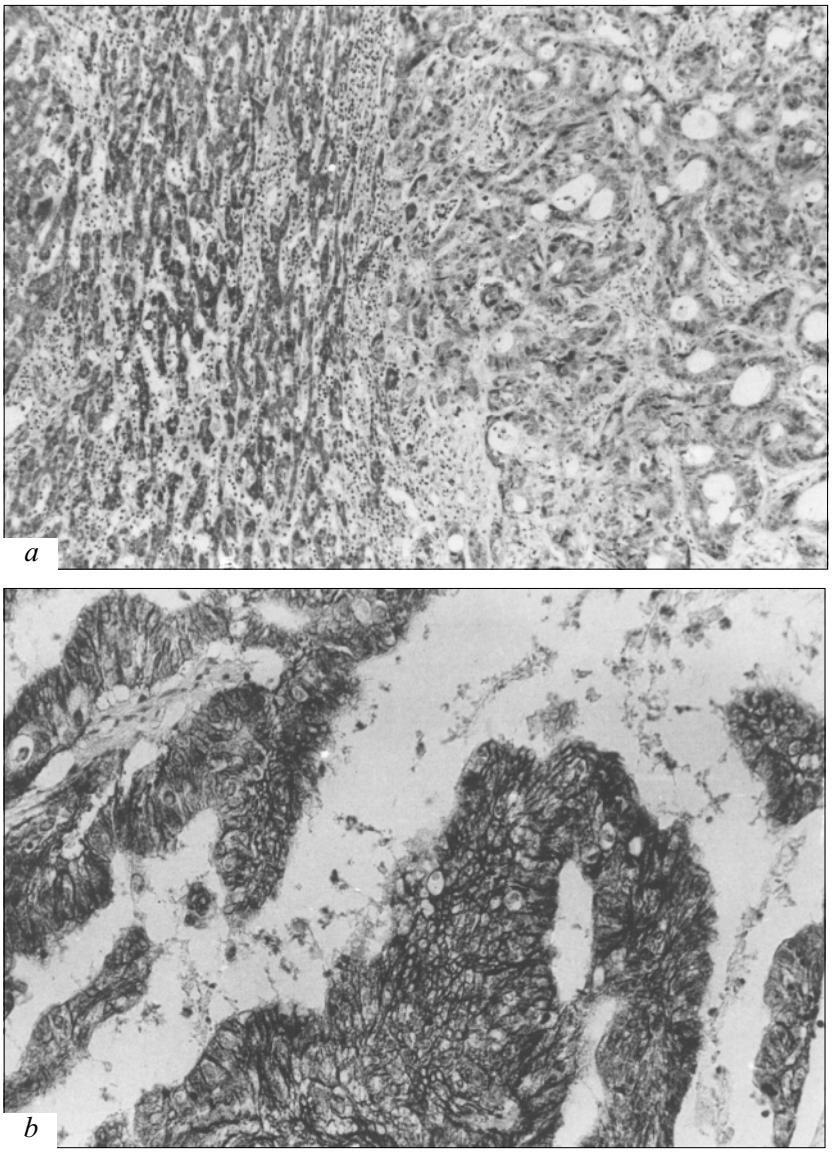

Fig. 3. Immunohistochemical detection of c-erbB-2 in cells of adenocarcinoma metastasis into the liver. *a*) low level of protein expression, ×100; *b*) high level of protein expression. Intensive staining of cancer cell membranes and cytoplasm, ×200. Paraffin sections: cell nuclei are post-stained with hematoxylin.

considered as a prognostic marker, because there is no relationship between the appearance of nm 23 gene product in cancer cells and clinical morphological parameters of the tumor. In contrary, some authors [9] showed that protein hyperexpression in the primary tumor correlated with more severe stage of the disease and higher risk of metastases into the liver. C. R. Berney *et al.* [3] showed that nm 23 can be regarded as the only independent marker, whose prognostic value does not depend on the presence of other positive markers, such as c-erbB-2, p53, u-PA, and VEGF. Our findings are in line with these data, because the level of positive expression of nm 23 protein in the primary tumor was significantly higher in cases with liver metastases, which appeared at different periods. Since the liver is the most frequent site of metastases of colorectal cancer, this information will help to detect patients at a high risk of metastases into the liver after surgery.

A high incidence of nm 23-positive tumors among metastases does not confirm the hypothesis on the inhibitory role of this protein in the metastatic process. Difficulties in the interpretation of specific features of expression of this marker are most likely due to the fact that metastatic process is characterized by a variety of genetic disorders, which can differ from disorders underlying malignant transformation of cells.

Our study demonstrated higher level of c-erbB-2 protein expression in the primary ATC cells during the disease progress and development of metastases, which is in line with published reports [9] and confirms the important role of this marker in the development of colorectal cancer metastases. However some authors noted [9] that after surgical treatment the expression of c-erbB-2 in colorectal cancer is much higher at earlier stages (Dukes' A and B) than at later stages (Dukes' C and D). Some scientists do not consider this

protein as a prognostic marker and claim that its expression does not correlate with the development of metastases into the lymph nodes and lungs, but correlates with the development of liver metastases [4,7].

Our results indicate that expression of nm 23 protein correlates with expression of c-erbB-2 protein and the incidence and levels of expression of both protein markers tend to increase with the appearance of liver metastases. Investigation of nm 23 and c-erbB-2 expression in CRAC primary tumors and metastases showed that these proteins play an important role in the disease progress and can predict the biological behavior of the tumor, but their role in the development of liver metastases requires further investigation. Evaluation of the role of these markers in prediction of the disease outcome after removal of liver metastases is one more interesting problem.

Hence, the use of immunohistochemical methods for evaluation of expression of genetic markers in CRAC provides more accurate criteria for prognosis of disease course and risk of liver metastases.

REFERENCES

1. V. Bazan, M. Migliavacca, I. Zanna, *et al.*, *Cancer Res. Clin. Oncol.*, **128**, No. 12, 650-658 (2002).
2. C. R. Berney, R. J. Fisher, J. Yang, *et al.*, *Ann. Surg.*, **230**, No. 2, 179-184 (1999).
3. C. R. Berney, J. Yang, R. J. Fisher, *et al.*, *Oncol. Res.*, **10**, No. 1, 47-54 (1998).
4. P. J. Crowe, J. Yang, and C. R. Berney, *World J. Surg.*, **25**, No. 8, 996-1001 (2001).
5. A. Dursun, N. Akyurek, N. Gunel, and D. Yamac, *Pathology*, **34**, No. 5, 427-432 (2002).
6. A. Forte, A. D'Urso, and S. Gallinarol.**,** *G. Chir.*, **23**, No. 3, 61-63 (2002).
7. J. Hayer, M. Engel, M. Seifent, *et al.*, *Anticancer Res.*, **21**, No. 4A, 2821-2825 (2001).
8. J. C. Lee, S. T. Wong, N. H. Chow, *et al.*, *Eur. J. Cancer*, **38**, No. 8, 1065-1071 (2002).
9. W. Muller, A. Schneiders, G. Hommel, *et al.*, *Cancer*, **1583**, No. 12, 2481-2487 (1998).
10. M. Sarris and C. S. Lee, *Eur. J. Surg. Oncol.*, **27**, No. 2, 170-174 (2001).