THE ROLE OF nm23 GENE IN COLORECTAL CARCINOGENESIS

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SUMMARY – The aim of this study was to investigate the correlation of immunohistochemical expression of nm23 gene in colorectal cancer cells with tumor stage according to Dukes, tumor differentiation, occurrence of distant metastases and patient survival. This retrospective study included 100 colorectal cancer patients who underwent surgical treatment. Both pathological and clinical data were analyzed according to sex, age, immunohistochemical expression of nm23, tumor stage, tumor differentiation, occurrence of distant metastases and patient survival. Overexpression of nm23 gene was related to both good tumor differentiation and Dukes' stage A, whereas no significant correlation was found between the occurrence of metastases and nm23 gene expression. There was no significant correlation between nm23 gene expression and 5-year survival of colorectal cancer patients either. Although the results of this study suggested that higher expression of nm23 gene correlated with an early stage of tumor and its good differentiation, this parameter cannot yet be taken as an independent and reliable prognostic indicator in colorectal cancer.

Key words: *Colorectal cancer; nm23 gene; Tumor suppressor genes; Dukes' stage; Metastases*

Introduction

Colorectal cancer is considered to be the third most common malignant disease in the world in both sexes[1,2]. Colorectal cancer prognosis is strongly related to the stage of disease at the time of diagnosis. Clinical and pathological classification of tumor according to Dukes is considered to be the most important indicator to predict prognosis.

The level of biological aggressiveness of a tumor is defined by its metastatic potential. The metastatic process itself is a very complex process consisting of many steps the molecular regulation of which is still unknown. The change of nm23 tumor suppressor gene could have an important role in the metastasis development. The nm23 protein is normally expressed in the nuclei and cytoplasm of all somatic cells as well as on the surface of most hematopoietic cells.

The role of nm23 gene has been examined in different types of tumor and the results suggest that the lower expression of nm23 at the protein/mRNA level is associated with metastatic tumors and poor prognosis when some types of tumors are concerned[3-7]. However, the role of nm23 gene in colorectal carcinoma has not yet been elucidated. According to some authors, a change of nm23 expression might be used as an important factor in prediction of the metastatic potential of colorectal carcinoma[8-14], while other studies suggest that there is no statistically significant relationship of nm23 expression with the metastatic stage of disease or survival[15-18].

The aim of this study was to investigate the correlation of immunohistochemical expression of nm23

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Received May 5, 2011, accepted April 16, 2012

protein in colorectal cancer cells with tumor stage according to Dukes, tumor differentiation, occurrence of distant metastases and patient survival.

Patients and Methods

This retrospective study included 100 colorectal cancer patients who underwent surgical treatment at University Department of Surgery, Split University Hospital Center in Split. Both pathological and clinical data were analyzed according to sex, age, immunohistochemical expression of nm23, tumor stage, tumor differentiation, occurrence of distant metastases and patient survival. Data on distant metastases were obtained by regular patient follow up in surgical and oncologic outpatient clinics including additional diagnostic work-up (determination of the levels of CEA and Ca19.9 tumor markers, abdominal ultrasonography and computerized tomography). Data on 5-year survival were obtained by inspection of the patients' medical chart and by telephone or personal contact with the study patients and their close relatives.

Histopathologic samples were analyzed at Department of Pathology and Cytology in the same University Hospital. Data used for determination of tumor differentiation were collected from the initial pathologic reports. Histologic differentiation of tumor was determined according to Broadman, while the original Dukes' classification from 1932 was used on tumor stage determination[19]. Tumor stage D was determined according to Astler-Coller classification[20], and in the results it is cited as Dukes' D stage of tumor.

The expression of nm23 in tumor epithelium was determined by immunohistochemistry followed by semi-quantitative (score 0-3) evaluation. Tissue for histologic analysis was fixed in 4% buffered formaldehyde for 24 hours and paraffin embedded. Paraffin blocks were cut into slices of 3-5 micrometers and stained both with the standard hemalaun-eosin method and immunohistochemically using monoclonal antibodies for nm23 protein (DAKO, rabbit anti human, AO 485 diluted 1:200). Antibodies were diluted with TRIS-NaCl with 1% bovine albumin 20 minutes before applying to the samples. For immunohistochemical analysis, previously deparaffinized and rehydrated histologic slides were heated in a microwave oven at 750 W at temperature of 110 °C in citrate buffer 3 times, 5 minutes each. Then the slides were stained by the LSAB (streptavidin-biotin labeled) method, washed with distilled water and PBS and incubated with primary antibodies for 30 minutes in a dark humid chamber. They were washed again with PBS and incubated in a humid chamber at room temperature for 30 minutes together with binding antibody, then washed with PBS and incubated with chromogen diaminobenzidine (DAKO) in a humid chamber for 15 minutes. They were stained using contrast hematoxylin, dehydrated in ethanol gradient, cleared in xylol and covered with Canada balsam. Cytoplasmic staining of tumor cells was considered as positive result. The immunohistochemical reaction was evaluated by the sum of intensity and proportion of the stain. Negative reaction without any cytoplasmic staining of tumor cells was marked as 0, weak as 1, moderate as 2

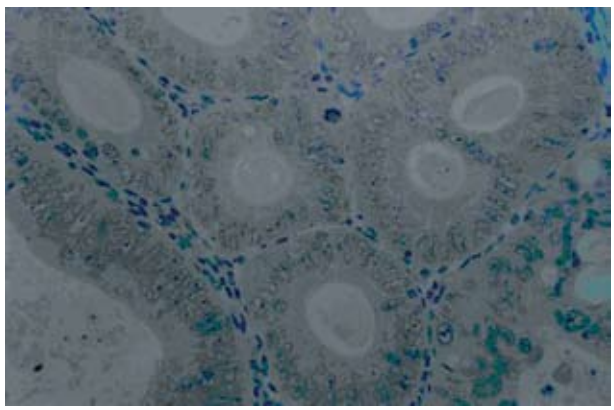

Fig. 1. Staining intensity 3-4.

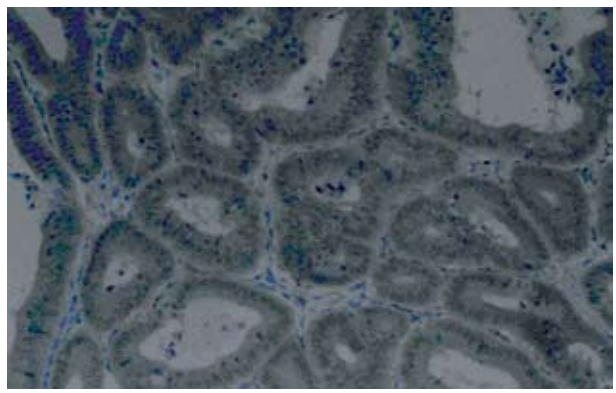

Fig. 1. Staining intensity 5-6.

and intensive as 3. The proportion of staining was also graded from 0 to 3. Samples that had no stained cells were marked as 0 and those with less than 1/3 of positively stained cells as 1. The samples that contained between 1/3 and 2/3 of stained cells were marked as 2, while those with more than 2/3 of stained cells were marked as 3. The level of staining intensity was added to the level of proportion of stained cells in order to get a sum from 0 to 6. When there was no stain at all, it was marked as 0, weak reaction was marked as 1 and 2, moderate reaction as 3 and 4 (Fig. 1), and strong reaction as 5 and 6 (Fig. 2). Normal colon mucosa stained in the same way as the study samples was used as a positive control, while samples where primary antibody was replaced with non immune sera were used as a negative control.

Statistics

Student's t-test was used to compare male and female patients according to age. Log-rank test was used to analyze patient survival. The χ^2-test was used to compare distribution of tumor stages according to Dukes, histologic differentiation and the occurrence of distant metastases relative to immunohistochemical expression of nm23. The results were interpreted at the level of significance of $P<0.05$.

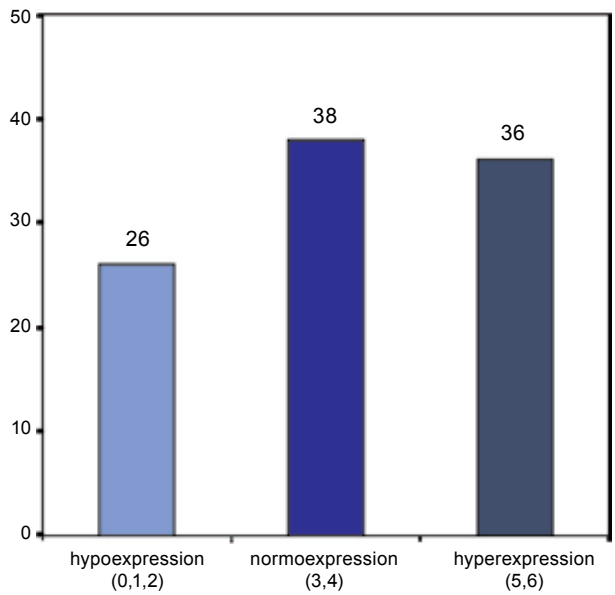

Fig. 3. Frequency of nm23 gene expression in colorectal cancer patients.

Results

Data on 100 colorectal cancer patients were collected. There were 59 (59%) female patients, mean age 65.9±11.5 and 41 (41%) male patients, mean age 62.1±11.5; the male and female patients did not differ significantly according to age (t=1.71; $P=0.090$).

Colorectal cancer patients were divided into 3 groups according to the level of nm23 gene expression in cancer cells (Fig. 3). There was no statistically significant difference between the observed and expected frequencies of nm23 expression in the study group of colorectal cancer patients ($\chi^2=2.48$; $P=0.289$). No type of nm23 gene expression was more frequent than the others in the group of patients with colorectal cancer ($\chi^2=2.48$; $P=0.289$).

In the study group of 100 patients, 13 (13%) showed a high, 32 (32%) medium and 55 (55%) low level of cancer differentiation.

The relation between histologic cancer differentiation and nm23 gene expression was established. In the subgroups of patients with overexpression, hypoexpression and normo-expression of nm23 gene, there were 9 (25%), 3 (11.5%) and 1 (2.6%) patients with histologically well differentiated tumor, respectively, and this difference was statistically significant ($\chi^2=9.93$; $P<0.042$) (Fig. 4).

A statistically significant difference was found in the distribution of Dukes' tumor stages according to

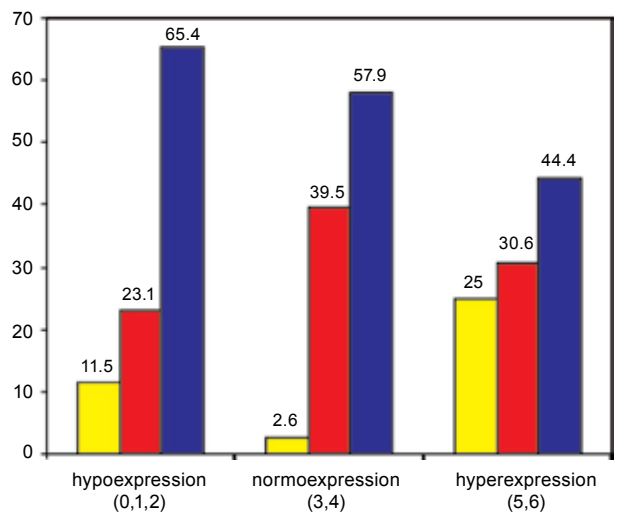

Fig. 4. Frequency of colorectal cancer patients according to histologic tumor differentiation and nm23 gene expression.

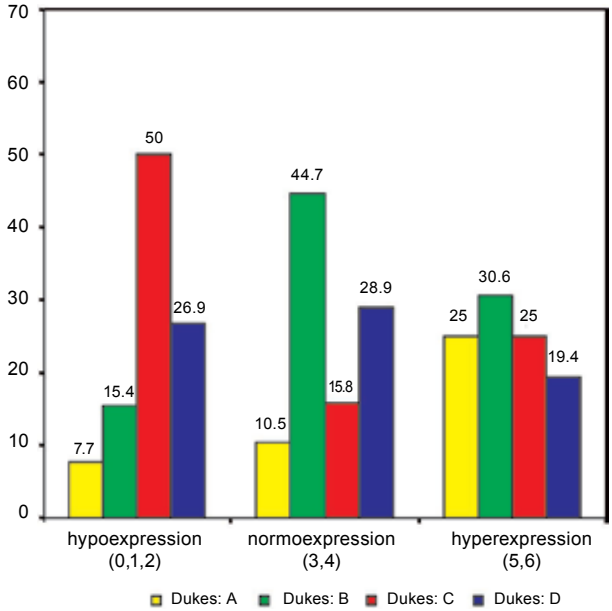

Fig. 5. Frequency of colorectal cancer patients according to Dukes' stage and nm23 gene expression.

the level of nm23 gene expression (χ^2=13.4; *P*=0.037) (Fig. 5). The patients with Dukes' stage A contributed to this difference. There were 9 (25%) such patients in the nm23 overexpression subgroup and 6 (9.4%) patients in the nm23 hypoexpression and nm23 normo-expression subgroup each (χ^2=4.41; *P*=0.036).

There was no statistically significant difference in the occurrence of distant metastases according to nm23 gene expression (χ^2=0.23; *P*=0.891). Distant metastases were present in 52 and absent in 48 patients. In the nm23 gene normo-expression subgroup, there were 20 (52.6%) patients with and 18 (47.4%) patients without distant metastases (Fig. 6).

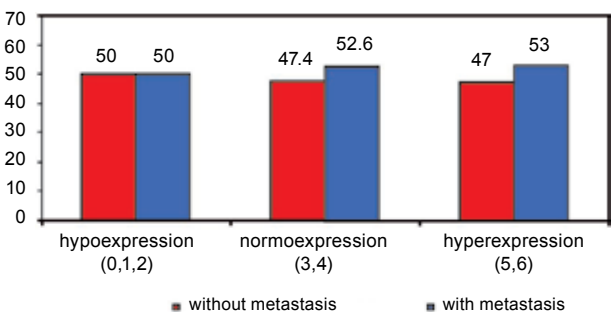

Fig. 6. Frequency of colorectal cancer patients according to the occurrence of distant metastases and nm23 gene expression.

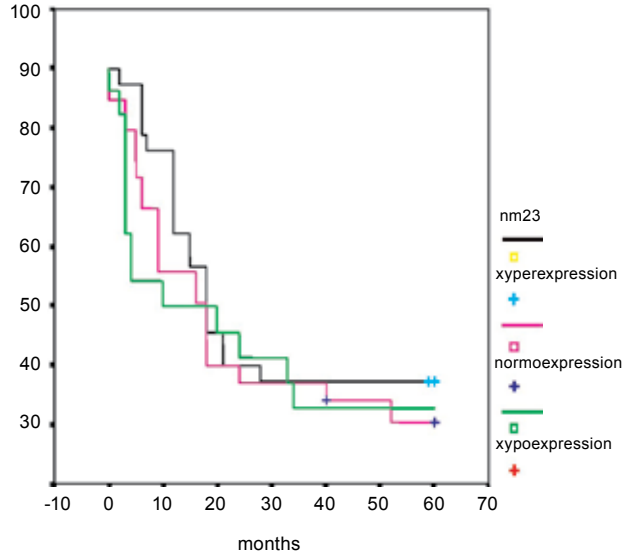

Fig. 7. Survival rate in colorectal cancer patients according to nm23 gene expression (Log Rank=0.74; P=0.690).

There was no statistically significant difference in the cumulative survival rate according to nm23 gene expression (Log Rank=0.74; *P*=0.690).

The average survival was 32, 33 and 36 months in the subgroups of patients with low or no nm23 expression, nm23 normo-expression and nm23 overexpression, respectively. The probability of 5-year survival was 43% in the subgroup of patients with low or no nm23 expression, 40% in the subgroup with nm23 normo-expression and 47% in the subgroup with nm23 overexpression (Fig. 7).

Discussion

Colorectal cancer is considered as one of the most common types of cancer in humans and its frequency is constantly rising. The prognosis of colorectal cancer patients is closely related to the depth of penetration to the intestine wall as well as to the presence of regional lymph nodes and distant metastases.

The level of biological aggressiveness of a tumor is defined by its metastatic potential and the occurrence of distant metastases is the final result of tumor progression, which is the most common reason for unsuccessful treatment. Therefore, great effort is made to discover genes that are involved in the metastatic process. Identification of such genes, able to enhance or inhibit metastatic phenotype in primary tumor,

would be of great importance for prognosis and therapy. Some researches have shown that variations in nm23 gene might influence the occurrence of metastases. In 1988, Steeg *et al*. showed that nm23 gene expression was considerably lower in tumors with high metastatic potential comparing to tumors with low metastatic potential[21], and since then nm23 gene has been considered as a possible tumor suppressor gene. Nevertheless, the results of various researches are not uniform, so reduced expression of nm23 gene has been related to the occurrence of metastasis and worse prognosis of breast cancer[3,22-25], hepatocellular cancers[4,26], malignant melanoma[27], and gastric cancer[28], while at the same time the situation is completely opposite in case of pancreas cancer[29], adenocarcinoma of the lung[30], and neuroblastoma[31].

The role of nm23 gene has not yet been elucidated when colorectal cancer is concerned. According to some studies[8-14], a change in nm23 gene expression can be used as an important indicator for prognosis of metastatic potential, whereas other researchers[15-18] claim that there is no significant relation between nm 23 expression and the occurrence of metastasis.

In this study, the change in nm23 gene expression in primary colon carcinoma was investigated in relation to normal colon mucosa, and there was no difference in the level of nm23 expression in the analyzed samples. Nevertheless, it was found that overexpression of nm23 gene correlated with both good tumor differentiation and Dukes' A stage. Furthermore, there was no significant correlation of nm23 gene expression with the occurrence of metastasis and 5-year survival of colorectal cancer patients.

The results of this study indicated that nm23 gene could be involved in the control of metastatic potential of a tumor in its early stage, when histologically well differentiated tumors are concerned. The correlation of nm23 gene overexpression with both clinical stage (Dukes' A) and good differentiation of a tumor is suggesting the possible protective role of nm23 gene in the early process of tumorigenesis. It is possible that, in later phases of tumor progression, the effect of nm23 gene is suppressed by other oncogenes and tumor suppressor genes that stimulate more malignant phenotypes characterized by weaker differentiation, higher invasive ability and metastatic activity of tumor cells. Furthermore, nm23 gene might be one of the factors responsible for the prevention of early stages of metastasis formation, since it was found that it was able to stop motility and colonization in the cultures of melanoma and breast cancer cells[32,33]. Finally, according to the results of this study, it can be concluded that there is a correlation between higher expression of nm23 gene and the early stage of tumor as well as its good differentiation, but it still cannot be taken as an independent and reliable prognostic indicator when colorectal cancer is concerned. For such a finding, additional studies including more colorectal cancer patients are necessary.

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Sažetak

ULOGA GENA nm23 U KOLOREKTALNOM KARCINOMU

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Cilj ovoga rada bio je istražiti povezanost između promjene ekspresije gena nm23 u stanicama kolorektalnog karcinoma sa stadijem tumora po Dukesu, diferenciranosti tumora, pojavom udaljenih metastaza i petogodišnjim preživljenjem. U ovom istraživanju obrađeno je 100 bolesnika oboljelih od karcinoma debelog crijeva koji su operirani u Klinici za kirurgiju KBC Split. Patohistološki i klinički podaci analizirani su prema spolu, dobi, imunohistokemijskoj ekspresiji gena nm23, stadiju tumora po Dukesu, stupnju histološke diferenciranosti tumora, pojavi udaljenih metastaza i petogodišnjem preživljenju. Utvrđeno je da dobro diferencirani karcinomi pokazuju statistički značajnu prekomjernu ekspresiju gena nm23. Isto tako, nađena je prekomjerna ekspresija gena nm23 u stadiju tumora Dukes A. Nije nađena povezanost između promjene ekspresije gena nm23 i pojave udaljenih metastaza ni petogodišnjeg preživljenja. Promjena ekspresije gena nm23, prema rezultatima ovoga istraživanja, ne može se rabiti za predviđanje pojave metastaza i procjenu petogodišnjeg preživljenja, ali povezanost prekomjerne ekspresije gena nm23 s ranim kliničkim stadijem (Dukes A) i dobrom diferencijacijom tumora upućuje na mogućnost zaštitnog djelovanja gena nm23 u ranom procesu tumorigeneze.

Ključne riječi: *Kolorektalni karcinom; gen nm23; Geni tumorske supresije; Stadij tumora po Dukesu; Metastaze*