Abstract. The function and prognostic significance of the nm23 gene is controversial in colorectal cancer (CRC). The aim of this study was to determine if nm23 protein expression correlated with the subsequent development of liver metastasis. Paraffin-embedded sections of 30 metastasizing CRC primaries and their subsequently resected liver secondaries were compared with those of 28 nonmetastasizing CRCs, 20 adenomas, and 20 cases of normal colonic mucosa. Expression of nm23 protein, assayed by immunohistochemistry, was measured using a standard semiquantitative scaling system and compared with a microcomputer-based color video image analysis (VIA). There was good correlation between color VIA and semiquantitative evaluation of nm23 immunoreactivity, confirming the validity of quantitative analysis (Pearson’s r = 0.88; p < 0.001). Metastasizing CRC primaries and secondaries overexpressed nm23 protein when compared with the other clinical groups, particularly nonmetastasizing CRC (Student’s t-test, p < 0.001). Furthermore, more nm23 immunoreactivity was associated with a higher risk of death from CRC (log-rank test, p = 0.002). These results suggest that overexpression of nm23 is highly associated with liver metastases from CRC and reduced survival.

Colorectal cancer (CRC) remains one of the most common malignancies in the Western world. Despite surgery and other treatments, the 5-year relative survival rate is 60% in white Americans but only 50% in African Americans [1]. Most patients who die of the disease succumb to liver failure secondary to extensive hepatic metastases. The metastatic cascade is a complex process involving many genetic alterations, which are probably regulated independently from those that confer tumorigenicity [2]. Angiogenesis [2–4], proteolysis [5], and probably apoptosis inhibition [6] are known to facilitate tumor progression, which is affected by these alterations. Several genetic alterations have been reported to occur in CRC [7, 8], including abnormalities within specific oncogenes (e.g., K-ras, c-myc, APC, and c-erbB2), tumor suppressor genes p53 and DCC, and the putative tumor metastasis-suppressor gene nm23.

The nm23 is an important gene family, originally cloned by hybridization experiments in murine melanoma cell lines [9]; it is located on chromosome 17q, region 21.3 [10]. Two nm23 genes encoding distinct nm23/NDP (nucleoside diphosphate) kinase proteins [11] have been reported: nm23-H1 encodes a polypeptide with a molecular weight of 18.5 kDa, whereas nm23-H2 encodes a protein of molecular weight 17 kDa [12]. The two proteins exhibit 88% amino acid sequence homology [11]. 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 tumors of high metastatic potential compared with those of low metastatic potential [9]. Evidence suggests that the nm23/NDP kinase proteins are expressed on the cell surface [13] and are important in the formation of basement membrane [14]. These proteins may therefore alter the microenvironment of the basement membrane, thus modulating cellular responsiveness. The nm23 protein also appears to have growth inhibitory effects [14]. In some human tumors, such as breast cancer [15], gastric carcinoma [16], malignant melanoma [17], and hepatocellular carcinoma [18], its expression has been shown to be inversely correlated with metastatic potential. In contrast, increased expression of nm23, at the mRNA level, has been associated with worsening prognosis in thyroid cancer [19] and squamous lung cancer [20].

The place of nm23 in CRC is controversial. Most researchers have reported that reduced expression of nm23, at both protein and mRNA levels, is associated with advanced tumor stage [21, 22] and liver metastasis [21, 23]. Loss of heterozygosity (LOH) and deletions either in the coding sequence of nm23-H1, or in its genomic DNA have been reported in 10% to 57% of CRCs [21, 24–26] but in 40% to 73% when associated with metastasis [24, 25, 27]. Others have failed to show any genetic mutations or deletions [28–32].

In summary, the function of nm23 in CRC, as either a tumor promoter or suppressor gene, is unclear. The purpose of this study was to assess and compare the expression of nm23 protein in various colonic tissues, including normal mucosa, adenoma, non-metastatic CRC, and primary and secondary metastatic CRC. In addition, we wanted to see if nm23 expression was related to liver metastasis-free interval and cancer-specific survival.

Overexpression of nm23 Protein Assessed by Color Video Image Analysis in Metastatic Colorectal Cancer: Correlation with Reduced Patient Survival

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Materials and Methods

Patients and Samples

Phosphate-buffered saline (PBS)–formalin fixed and paraffin-embedded specimens from 58 patients with CRC were studied, 28 of them with no evidence of metastatic disease. The remaining 30 patients had all undergone liver resection for metastatic CRC; both the original primary tumor and liver secondary tissue blocks were available for the study. Twelve of these patients underwent liver resection at the time of the initial operation (synchronous secondaries). The mean follow-up was 9.5 years (range 3.7–17.4 years). Controls were 20 samples of normal colonic mucosa taken from patients having resections for diverticular disease (DD) and 20 adenomas removed from patients having colonoscopy who did not have invasive cancer. Altogether 128 specimens were studied and were distributed within five clinical groups: (A) DD; (B) adenoma; (C) nonmetastatic CRC; (D) primary metastatic CRC; (E) secondary metastatic CRC.

Immunohistochemistry

For every procedure samples were randomly chosen from each of the five groups. The 5-μm sections of paraffin-embedded tissues were routinely dewaxed. Immunohistochemistry was performed on the tissue sections using previously described methods [33]. A monoclonal antibody (0.08 μg/ml) that recognized both nm23-H1 and nm23-H2 was used (Clone 56; Transduction Laboratories, Lexington, KY, USA). This antibody was developed using as immunogen a 12.3-kDa protein fragment corresponding to amino acids 44 to 152 of the human nm23 protein. As negative controls, the antibody was replaced by either PBS to rule out the effect of endogenous peroxidase or isotype-matched irrelevant mouse immunoglobulin to test for specificity. All samples were stained for 20 minutes with dianimobenzidine tetrahydrochloride (DAB).

Color Video Image Analysis

The immunostained sections were examined using a Leica (Leitz Laborlux S; Leica, Wetzlar, Germany) microscope (×400) coupled to a video camera (Sony Hyper HAD, color CCD-IRIS/RGB, model DXC-151AP), connected to a computer-aided color video image analysis (VIA) system (Multi-Sync 3V, model JC-1535VMR; NEC Corporation). After being captured and digitized onto the video screen, microscopic images were analyzed using an image analysis software program (Leica Q500MC, Microsoft Windows hosted image analysis system, Leica Australia P/L). The images stored in the software were composed of up to 512 × 512 pixels separated into 8-bit brightness. The transmitted light intensity was standardized by using a fixed rheostat setting at the microscope light source. The stability of the light output was frequently checked during all procedures.

Due to the high cellularity of the samples, sequential fields were systematically examined using a ×40 objective and a ×10 eyepiece within a fixed frame of 450 × 450 pixels (0.2025 mm²) to increase the number of cells per field. Color values for each of the red, green, and blue components were calculated for each pixel from 0 to 255. After counterstaining with Harris’s hematoxylin, which allowed excellent separation from DAB staining, a threshold of 160 (range 0–255) was determined for the blue color component as a cutoff range. With this setting, false or weakly positive staining could not be recorded. Ten slides (two of each group) were originally analyzed for this purpose.

Due to the heterogeneity of the tumor samples, it was necessary to examine 15 fields per slide. This number was determined using a progressive mean graph [34] to achieve a meaningful result in statistical terms. Each slide was initially examined at ×10 magnification for an overall view of the lesion and counterstain intensity. This practice allowed an area to be chosen that was the most representative, with no tissue folding or overlapping, and minimal background staining. The fields were then randomly selected within this area. The degree of staining intensity was estimated as the mean DAB area stained from a minimum of 900 cells in 15 microscopic fields at ×400 magnification. Evaluation of the staining reaction, which is usually cytoplasmic [22, 23], was performed using the immunoreactivity (IR) score, which is equal to the staining intensity (SI) in pixels multiplied by the percentage area stained.

Data from the VIA were divided into five distinct IR groups similar to the semiquantitative evaluation of staining. Cutoff points defining these groups were determined prior to any analysis to ensure roughly equal numbers per group. The IR scale was as follows: IR 0 = [0, 1), IR 1 = [1, 5), IR 2 = [5, 80), IR 3 = [80, 110), IR 4 = [110, ∞).

Semiquantitative Analysis

The degree of expression of nm23 was then estimated by semiquantitative analysis and classified into one of five grades according to our previous work [33]: 0 = no staining; 1 = focal, weak staining; 2 = strong staining of 25% or moderate staining of < 80%; 3 = strong staining of 25% to 50% or moderate staining of > 80%; 4 = strong staining of > 50% tumor cells. Slides were examined and scored independently by two of the authors blinded to any other pathologic information; in 50% of cases the data were similar, and the other half were reviewed until final agreement was achieved.

Comparison with Patient Outcome

Complete clinical follow-up data were available. The expression of nm23 protein was assessed for any association with pathologic staging (TNM classification according to the UICC criteria [35] and Dukes’ stage) and patient outcome within each clinical group.

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). Association between the semiquantitative estimation of nm23 expression and the quantitative value as measured by color VIA analysis was tested by Pearson’s correlation coefficient. The difference in nm23 expression across groups was determined by analysis of variance (ANOVA). Individual groups were then compared using the nonparametric Mann-Whitney U-test and the parametric Student’s t-test. As up to seven comparisons were made between these groups, each calculated p value was corrected according to the Bonferroni criterion [36]. An association between nm23 staining in paired primary (group D) and either synchronous or metachronous secondaries (group E) was tested using Pearson’s correlation coefficient. The relation between