Predictive Value of Ki-67 and Argyrophilic Nucleolar Organizer Region Staining for Lymph Node Metastasis in Gastric Cancer

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ABSTRACT

Proliferative activities in 91 primary gastric carcinomas and 36 corresponding metastatic perigastric lymph nodes were investigated using Ki-67 labeling percentage and an argyrophilic nucleolar organizer region (AgNOR) count. Tumors with a high proliferative activity often metastasized to lymph nodes, and the proliferative activities of the primary lesion and the perigastric lymph node metastases were similar. A significant correlation was recognized between the Ki-67 labeling percentage and the AgNOR count \((r = 0.744; \ P < 0.001)\). The Ki-67 labeling percentage and AgNOR count proved to be useful predictors of nodal metastasis regardless of tumor size, depth of invasion, and histological type. Even when tumors are smaller (<7 cm) or the stage of the disease is early (pT1, 2), the formation of metastasis increased with an increased Ki-67 labeling percentage or AgNOR count. The combination analysis of depth of invasion with Ki-67 labeling percentage or AgNOR count gives a more precise prediction of nodal metastasis, compared with histological analysis alone.

INTRODUCTION

Various methods are used to estimate the proliferative activity of gastric carcinoma, including mitotic index (1), the S-phase fraction obtained by the \([3H]thymidine (2) or bromodeoxyuridine labeling technique (3, 4), and flow cytometric analysis of the cell cycle phase distribution (4, 5). However, these approaches are not as applicable to routine clinical samples of gastric cancer because only a small fraction of proliferating cells are available, and external administration of mitogenic substances, such as bromodeoxyuridine or iododeoxyuridine, is complex and time consuming. The S-phase fractions determined by flow cytometric study are not always precise because the proliferating activity of not only tumor cells but also stromal cells, including plasma cells or lymphocytes, may be reflected.

The monoclonal antibody Ki-67 reacts with a nuclear antigen present throughout the cell cycle (late G1, S, G2, and M phases) of proliferating cells but is absent in quiescent (G0) cells (6). Although the exact nature of the antigen recognized by Ki-67 is unknown, there are similarities with monoclonal antibodies that recognize DNA polymerase \(\alpha\) (7). On the other hand, the NORs\(^2\) are loops of DNA (rDNA) encoded for rRNA production (8). Proteins associated with the NOR are so-called AgNOR proteins (the silver-stained nucleolar proteins), which are argyrophilic, acidic, nonhistone proteins (9) and may be considered a marker of the rDNA transcriptional activity and/or of the rDNA transcriptional potential (10–12). The correlation between these two parameters has been noted in cases of non-Hodgkin’s lymphoma (13) and breast cancer (14, 15). In a search for a more sensitive and pertinent estimation of the proliferative activity of gastric carcinoma, we analyzed the Ki-67 labeling percentage and Ag-NOR count. Our foregoing study revealed that microspectrophotometrically determined DNA content and mitotic activity of gastric carcinomas was higher in patients with lymph node metastasis than in those without a metastasis (16). We thus attempted to clarify the character of gastric carcinoma with high proliferative activity, using Ki-67 and AgNOR parameters, and to determine the precise and easiest method of determining the metastatic potential of human tumors.

MATERIALS AND METHODS

Patients. This prospective study included 91 randomly selected patients with primary gastric cancer who underwent gastrectomy with lymph node dissection in the Department of Surgery II in Kyushu University Hospital and affiliated hospitals, Fukuoka, Japan, from April 1989 to August 1990. No patient treated preoperatively with cytotoxic drugs was included in this study. A thorough histological examination was made on hematoxylin and eosin-stained preparations, and the histological classification was according to the tumor-node-metastasis classification system of the International Union Against Cancer (17). Tumor extension was classified as invasion of: the mucosa or the submucosa (pT1); the muscularis propria or the subserosa (pT2); the serosa (pT3); and the contiguous structure (pT4). Histological typing was assessed according to the classification of Sugano et al. (18); “differentiated-type” includes well- and moderately differentiated adenocarcinomas, and “undifferentiated-type” includes poorly differentiated adenocarcinoma and signet ring cell carcinoma.

Tissue Samples. Tissue samples from 91 primary tumors to be stained with Ki-67, 36 corresponding metastatic perigastric lymph nodes, and 13 normal gastric epithelia as controls were fixed in periodate-lysine-paraffinmaldehyde at 4°C for 5 h immediately after surgical resection, rinsed at 4°C in phosphate-buffered saline containing 10, 15, and 20% sucrose for 4 h, and embedded in OCT compound (Miles, Elkhart, IN), and stored at −80°C. The cryostat sections were cut at 6 μm and mounted on albumin-coated slides. Although AgNOR can be applied to paraffin sections, we used the same periodate-lysine-paraffinmaldehyde-fixed, OCT-embedded tissue for a comparison of Ki-67 and AgNOR parameters.

Immunohistochemical Staining of Proliferating Cells by Ki-67 Monoclonal Antibody. An avidin-biotin-peroxidase complex method was used. The sections were washed in phosphate-buffered saline, pH 7.2, and then incubated for 15 min at room temperature in normal horse serum (1:10; Vector Laboratories, Burlingame, CA). We then added mouse monoclonal Ki-67 antibody (1:100 overnight; Dako, Copenhagen, Denmark), biotinylated horse anti-mouse IgG (1:200 for 30 min; Vector Laboratories), and avidin-biotin-peroxidase complex (30 min; Vector Laboratories). Peroxidase labeling was developed with 3,3′-diaminobenzidine and H2O2, and the sections were counterstained with Mayer’s hematoxylin. All of the nuclei stained with 3,3′-diaminobenzidine were regarded as positive for Ki-67, regardless of the staining intensity (Fig. 1). The labeling index was determined by observing 1000 nuclei in areas of the section with the highest labeling rates, and the percentage of Ki-67-labeled nuclei was used for analyses.

Ag-NOR Staining. The one-step silver colloid method was used. The Net-gold staining solution was prepared according to the descriptions of Ploton et al. (19). We used 1 volume of 2% gelatin in 1% formic acid and 2 volumes of a 50% silver nitrate solution, poured it over the sections, and left the preparations for 1 h at room temperature in the...
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Fig. 1. Photomicrograph of human gastric carcinoma stained with Ki-67 monoclonal antibody (A). Note the scattered AgNORs in the nuclei (B).

Fig. 2. Scattergram showing the mean Ki-67 labeling percentage (left) and AgNOR count (right) in a normal epithelium, primary tumor, and nodal metastasis. For both parameters, the mean values showed statistical differences between normal epithelium and primary tumor (P < 0.01) but not between primary tumor and nodal metastasis.

Fig. 3. Correlation between Ki-67 labeling percentage and AgNOR count in gastric carcinoma (n = 91; r = 0.744; P < 0.001).

RESULTS

Proliferative Activity of Malignant Tissues Determined by Ki-67 and AgNORs. The extent of the proliferative activity of epithelial cells in normal mucosa, primary tumor, and metastatic lymph node is shown in Fig. 2. The Ki-67 labeling percentage and the AgNOR counts in the primary tumor were significantly higher than in the normal epithelium (P < 0.01). Cancer cells in the perigastric lymph node metastasis had a similar proliferative activity, compared with the primary lesion. The percentages of Ki-67 labeling cells in 36 cases of primary and metastatic lesions were 33.8 ± 9.4 and 35.4 ± 8.5 (SD), respectively. The difference was not statistically significant, as determined using a paired Student’s t test (P = 0.11). There was no significant difference for the AgNOR count. Fig. 3 shows the results of the linear regression analysis of Ki-67 labeling percentage and AgNOR count in the primary tumor. There was a significant correlation between the Ki-67 labeling percentage and the AgNOR count (r = 0.744; P < 0.001).

Metastatic Potential. To determine the metastatic behavior of gastric cancer, 91 tumors were classified into two groups according to the median values of Ki-67 labeling percentage and AgNOR count. The relationship between clinicopathological factors and these two parameters is summarized in Table 1. The advanced carcinoma (pT3, 4) had a higher proportion of Ki-67 labeling percentage than did the carcinoma in an early stage (pT1, 2) (P < 0.05). Tumors characterized by lymph node metastasis, lymphatic invasion, or venous invasion had a significantly higher proportion of Ki-67 labeling percentage (≥30.4%) or higher AgNOR count (≥4.05) than did those without such metastatic behavior. Table 2 summarizes quantitative results on proliferative activity in three groups with different degrees of nodal involvement. Tumors exhibiting metastatic spread (pN1, pN2, and pM1) had significantly higher values with respect to Ki-67 labeling percentage and AgNOR count than did those without metastatic spread (pN0) (P < 0.01). There was no statistical difference between the pN1 and the pN2 and pM1 groups. Hence, tumors with a high Ki-67 labeling percentage or AgNOR count are likely to metastasize.

Next, to determine the independent risk factors for lymph
node metastasis, we carried out a multivariate analysis, using stepwise logistic regression analysis. The information included sex, age, tumor location and size, gross appearance, depth of invasion, lymph node metastasis, histological type, growth pattern, lymphatic permeation, and blood vessel invasion. The multivariate analysis revealed that lymphatic permeation (P < 0.01) and Ki-67 labeling percentage (P < 0.01) were the independent risk factors for nodal metastasis.

Prediction of Lymph Node Metastasis. Table 3 shows the incidence of metastasis, as related to conventional histological characteristics and proliferative activities. The incidence of metastasis increased with tumor size and invasion into deeper layers. The combination of histological classifications and parameters of proliferative activity showed that even in the smaller tumors (<7 cm) or those in an early stage of disease (pT1, 2), the formation of metastasis increased with increases in Ki-67 labeling percentage or AgNOR count.

Table 4 summarizes the diagnostic values such as false positive and negative rates and overall accuracy for a single parameter based on the histological classification or proliferative activity, and their combination. There were surprising similarities with regard to these diagnostic values, as assessed by histological classifications, Ki-67 labeling percentage, and AgNOR count. Of great importance is that the accuracy of prediction of metastasis, in consideration of the combination of proliferative parameters, is considerably higher than it is for simple histological evaluations.

DISCUSSION

Our studies using Ki-67 and AgNORs showed that tumors with a high proliferative activity had a high nodal metastatic potential. There were quantitative differences in Ki-67 and AgNOR parameters between tumors with and without a metastasis. These findings are in close agreement with studies done by Korenaga et al. (16), who used the mitotic index and DNA ploidy, or those by Kamata et al. (22), who used bromodeoxyuridine and DNA ploidy.

We found that the proportion of proliferating cells in the primary and the metastatic perigastric lymph nodes was not significantly different. This would suggest that a gastric primary carcinoma and the related perigastric metastasis are in general made up of cell populations with similar characteristics. This similarity of tumor cells in primary and metastatic lesions has been noted using parameters such as [3H]thymidine in Lewis lung carcinoma (23), flow cytometric analysis of DNA aneuploidy in human solid tumors (24) and colon carcinomas (25), and cytophotometric measurements of DNA in breast carcinoma (26). Change in proliferative activity may occur during growth, as a result of selection of subpopulations with a high metastatic potential, due to heterogeneity of the primary tumor, genotypic instability, or clonal proliferation during the process of metastasis. Baba et al. (27) made a comparison of DNA content in gastric cancer between primary and nodal metastatic lesions and noted the same DNA contents in 25 of 61 patients and a reduction in DNA content in the remaining patients. All these observations taken together suggest that the proliferative activity of cancer cells is not always higher in the metastatic lesion compared with findings in the primary lesion; the activity...
ties of both lesions were much the same in approximately one-half the number of cases.

There was a significant relationship between the Ki-67 labeling percentage and AgNOR count in the case of gastric cancer; hence these two parameters are probably not independent. As for staining techniques, the one-step AgNOR staining method makes for easy processing of the paraffin-embedded tissue sections, while the Ki-67 antigen is not demonstrable in a formalin-fixed specimen. These parameters may be relevant to the classical microspectrophotometric analyses such as DNA ploidy or mitotic index and are likely to lead to a better understanding of growth fractions and to an easier scanning of the cancer cells with a high rate of proliferation.

To make use of Ki-67 and AgNORs in routine clinical work, we compared our observations with conventional histopathological data. In the prediction of lymph node metastasis, both Ki-67 and AgNORs were as useful as such histological factors as depth of invasion or tumor size. Multivariate analysis suggested that the Ki-67 labeling percentage was a significant risk factor for nodal metastasis. Furthermore, the combination analyses of proliferative activity and conventional histological factors provided advantages for the prediction of lymph node metastasis. Even when the tumor is smaller or the stage of the disease is early, the analyses of Ki-67 provided useful information about metastasis. These methods, if put to use in routine diagnostic pathology, will provide pertinent information for the surgeon and the chemotherapist, but the patient will be the beneficiary.

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