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Epithelial bone marrow cells in patients with advanced esophageal squamous cell carcinoma

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The aim of the current study was to examine epithelial cells in the bone marrow and peripheral blood of patients with various stages of esophageal squamous cell cancer prior to surgical treatment and to analyze the prognostic significance of these carcinoma cells deposits to the stage of the disease and applied surgical therapy. Thirty-two patients (25 men and 7 women), and 5 healthy bone marrow donors serving as controls were studied. Bone marrow samples were evaluated by light microscopy and examined by flow cytofluorometry. Cells were phenotypically analyzed for the antigens CD45- and CD18+ and/or EMA+. Results are presented as the number of cells revealing the investigated phenotype per 10^5 analyzed cells. CD18 was expressed in the bone marrow cells of 15 of the 32 (47%) patients and EMA in 20/32 (62%), but not in peripheral blood. In 13 of the 32 pts (41%), co-expression of CD18 and EMA was observed. Patients with the proportion of marrow erythroblasts below 15% had higher numbers of CD18+ and EMA+ cells and there was a negative correlation between the number of erythroblasts and EMA+ cells (r=0.54, p=0.01). In patients with esophageal cancer and anemia, the number of EMA+ cells was higher (p=0.05) and the percentage of erythropoietic cells in the bone marrow was lower (p=0.01). In conclusion, flow cytofluorometry using anti-cytokeratin and anti-EMA antibodies may be useful in evaluating microdeposits of esophageal squamous cells in bone marrow. A dysfunctioning erythropoietic system causing anemia can be a first signal for the presence of malignant cell microdeposits in the marrow of patients with esophageal carcinoma.

Key words: epithelial cells, bone marrow, esophageal carcinoma

Esophageal cancer is a malignancy with a particularly poor prognosis [1, 2, 3]. As distinct from the USA and Western Europe, Poland as well as from South America and Asia about 90% of esopageal cancers are squamous cell carcinoma [4, 5, 6, 7, 8]. Moreover, at diagnosis only about 5-10% of patients present with an early form of cancer, while the vast majority are in an advanced stage of disease, with simultaneous locoregional and distant lymph node involvement or organ metastasis [3, 9, 10]. Surgical treatment remains the central therapeutic modality [2, 11]. Extensive resection of the esophagus associated with wide excision of the lymph nodes constitutes the basic surgical treatment [2, 11, 12, 13]. Despite such extensive surgical intervention, five-year survival after surgery alone is observed in only about 20% of patients [2, 14]. Almost half of the patients with locally advanced tumors will die following cancer recurrence within the first two years after surgical resection [4, 10, 14, 15]. This means that at the time of diagnosis and surgical intervention the cancer has already spread far beyond its primary focus in the esophagus.

In the last decade the attention of researchers has focused on the detection of so-called micrometastases or deposits of carcinoma cells, especially in the bone marrow and peripheral blood of patients suffering from cancer of the esophagus, breast, lungs, or colon [16, 17, 18, 19, 20, 21, 22]. The detection of carcinoma cell deposits in the bone marrow and/or peripheral blood may have a crucial effect on determining the actual stage of disease advancement and thus on designing adequate therapeutic management [16, 17, 18, 23, 24].

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The aim of the current study was to investigate the presence of epithelial cells in the bone marrow and peripheral blood in patients with various stages of esophageal squamous cell cancer prior to surgical treatment, evaluate morphological changes in the bone marrow and peripheral blood in these patients, and to analyze the prognostic significance of carcinoma cell deposits with regard to the stage and applied surgical therapy.

Patients and methods

The analysis involved 32 patients treated for squamous cell carcinoma of the esophagus at the Department of Gastrointestinal and General Surgery, Wrocław Medical University, from January 2006 to July 2007. All patients were informed about the study protocol and gave their written consent. The patients group consisted of 25 males and 7 females aged 50–85 years (mean: 62.2 years). The control group comprised 5 healthy bone marrow donors, 3 males and 2 females, aged 21–46 years. Table 1 lists the demographic data of the 32 patients. All patients underwent standard staging examinations, including endoscopic evaluation together with biopsy studies, ultrasound examination of the neck and abdomen, and CT scans of the thorax and abdomen.

Twenty-six patients with advanced tumor were subjected to chemotherapy or palliative chemoradiotherapy. In six patients resection surgery (as described below) was followed by postoperative chemotherapy or chemoradiotherapy. The operations were performed under general anesthesia using standard techniques. The thoracic portion of the esophagus was resected in the right thoracic approach. The posterior mediastinal lymph nodes were removed together with the esophagus in one block. Then the abdominal portion of the esophagus together with the cardia and regional lymph nodes was resected in the abdominal approach and a gastric alimentary fistula was formed. The cervical esophagus was approached for incision on the left side of the neck and a salivary fistula was formed. The resected tissue was evaluated histopathologically and the postoperative stage was confirmed.

Table 1. Basic clinical data of patients with squamous cell carcinoma of the esophagus at diagnosis

Number of patients (F/M)	32 (7/25)
Age (years), range, mean	50-85 (mean: 62.2)
Clinical stage	
II, II/III, III	10 (31.25%)
IV	22 (68.75%)
Metastases on diagnosis	
Lack	2
Lymph nodes: periesophageal + mediastinal	7
Lymph nodes: epigastric	8
Generalized lymphadenopathy	10
Metastases to the lungs+ bronchoesophageal fistula	10
Metastases to the liver	2
Treatment	
Palliative	26
Total resection	6
Alive/dead (to 30 June 2007)	11/21
Survival (to 30 June 2007) M-ST, range	7.4 months (2-18 months)

In all patients, bone marrow samples were collected from the posterior iliac spine prior to treatment. The material, after standard G-G staining, was evaluated using a light microscope. In order to demonstrate epithelial carcinoma cells in the bone marrow (carcinoma cell microdeposits), the samples were examined by means of flow cytofluorometry. One hundred-µl bone marrow samples were stained with the following sets of antibodies 1) IgG 1/FITC, CD45/RPE, 2) IgG 2B/FITC, CD45/RPE, 3) CD18/FITC, CD45/RPE, and 4) EMA/FITC, CD45/RPE. IgG1 and IgG2B antibodies constituted isotope controls for the investigated antigens. The material was incubated for 30 minutes at 4°C. The cells were then lysed with Optilyse C (Beckman Coulter). The cells were analyzed using a Dako Galaxy flow cytofluorometer and $1x10^5$ events were read. The cell phenotypes identified were CD45- and CD18+ and/or epithelial membrane antigen (EMA)+. Cytokeratin 18 (CD18) is expressed on epithelial cells; however, no CD18 expression is found on mesenchymal cells [25, 26]. EMA is a marker for glandular epithelium and squamous epithelium. Results are presented as the number of cells revealing the investigated phenotype per 10^5 analyzed cells.

The statistical evaluation was performed using the Kaplan-Meier's Estimator and Spearman's, Student's *t*, and Fisher's exact tests.

Results

The CD18 was expressed on the bone marrow cells of 15 of the 32 (47%) patients with esophageal carcinoma (Fig. 1) and EMA was found in 20/32 (62%) patients (Fig. 2). In 13 of the 32 patients (41%), co-expression of CD18 and EMA was present. The number of CD18+ cells ranged from 0 to 280 per 10^5 analyzed cells (mean±*SD*: 26.6±53.2/10^5). The number of EMA+ cells varied between 0-568 per 10^5 analyzed cells (mean: 59.4±110/10^5). In the control group of healthy bone marrow donors, neither CD18+ nor EMA+ cells were present in bone marrow.

In all patients under study the mean percentage of erythroid cells in the bone marrow comprised 10.7±5.7% and varied from 4 to 21%. However, in majority of patients (21 of 32, 65.6%) the proportion of erythroblasts was significantly decreased and ranged from 4 to 12% (mean: 7.7%); paraerythroblasts were found in 5/32 (15.6%) and megaloblasts in one patient. Myeloid cells varied from 65 to 83% (mean: 61.4±17.6%). It was quantitatively normal in all patients, but in two some toxic granules in granulocytes were found. Lymphoid cells ranged from 33 to 46% (mean: 23±9.4%). In 2/32 patients the bone marrow

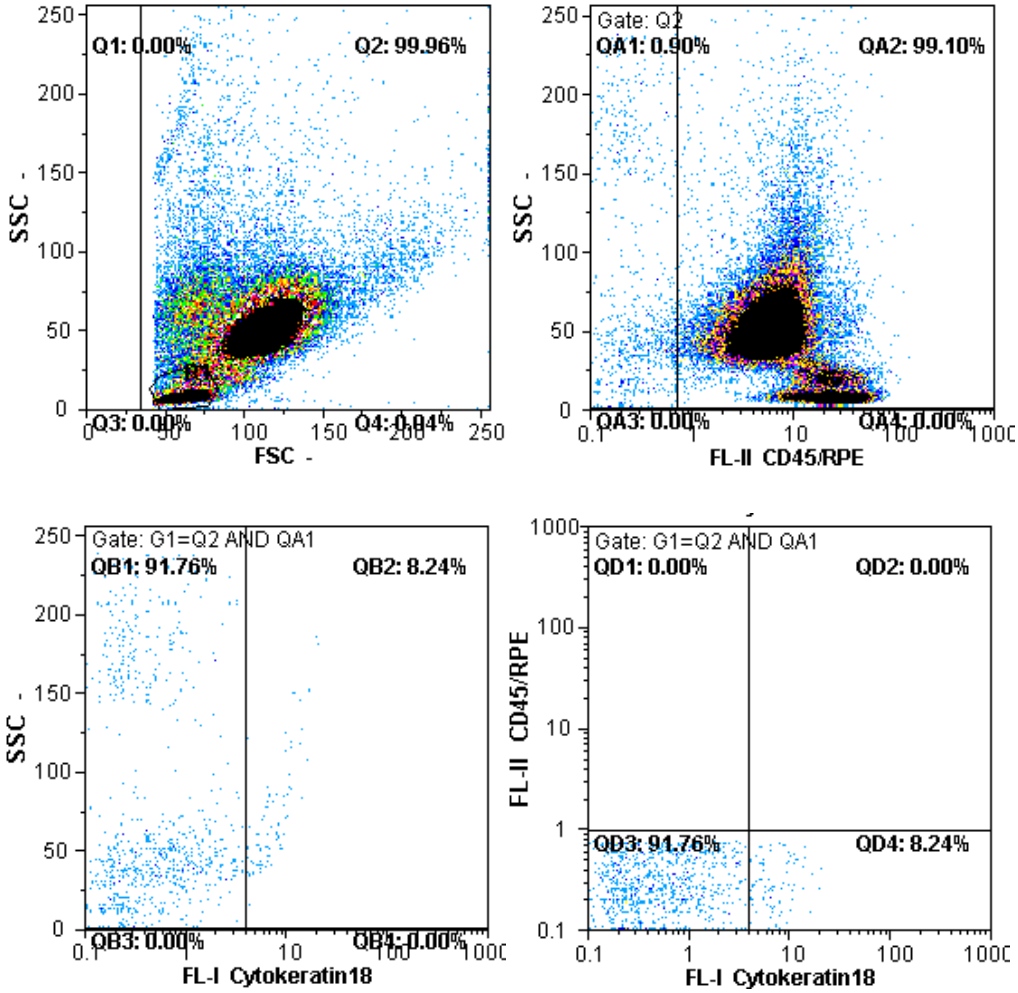

Figure 1. A two-colour flow cytometry showing expression of epithelial component positive for CD18 in bone marrow cells in oesophageal cancer
a) Gate on the whole marrow cells
b) Gate on CD45-positive whole leukocytes
c) Gate on CD18-positive/CD-45 negative marrow cells
d) Dot plot showing that the majority of cells express in membrane CD18-positive/CD45-negative marrow cells

was hypoplastic. No epithelial cells were found in bone marrow using light microscopy.

Hemoglobin levels ranged from 8.6 to 14.8 g/dl (mean: 12.3±1.4 g/dl). Seven patients were diagnosed with normocytic anemia (Hb: 8.6-11.0 g/dl). White cells count ranged from 4.3 to 24.7 × 10^9/l (mean: 8.8±4.2 10^9/l). In 10 patients it was above 9.0 10^9/l (range: 9.6-24.7 10^9/l). Platelets count ranged from 26 to 604 10^9/l (mean: 306±120 10^9/l). In 5 patients it was above 450 and in one below 150 10^9/l. None of the patients had epithelial cells in the peripheral blood.

Table 2. CD18 and EMA expression in the bone marrow of patients with squamous cell esophageal carcinoma according to stage of the disease

Data	Stage of the disease: II, II/III, and III n=10 range, mean±*SD*	Stage of the disease: IV n=22 range, mean±*SD*	*p* value
CD18+/10^5 cells	0-86 19.3±30.1	0-280 42.7±89.4	
EMA+/10^5 cells	0-235 51.4±67.4	0-568 77±115.1	0.03
m-ST (months)	9.0	6.5	

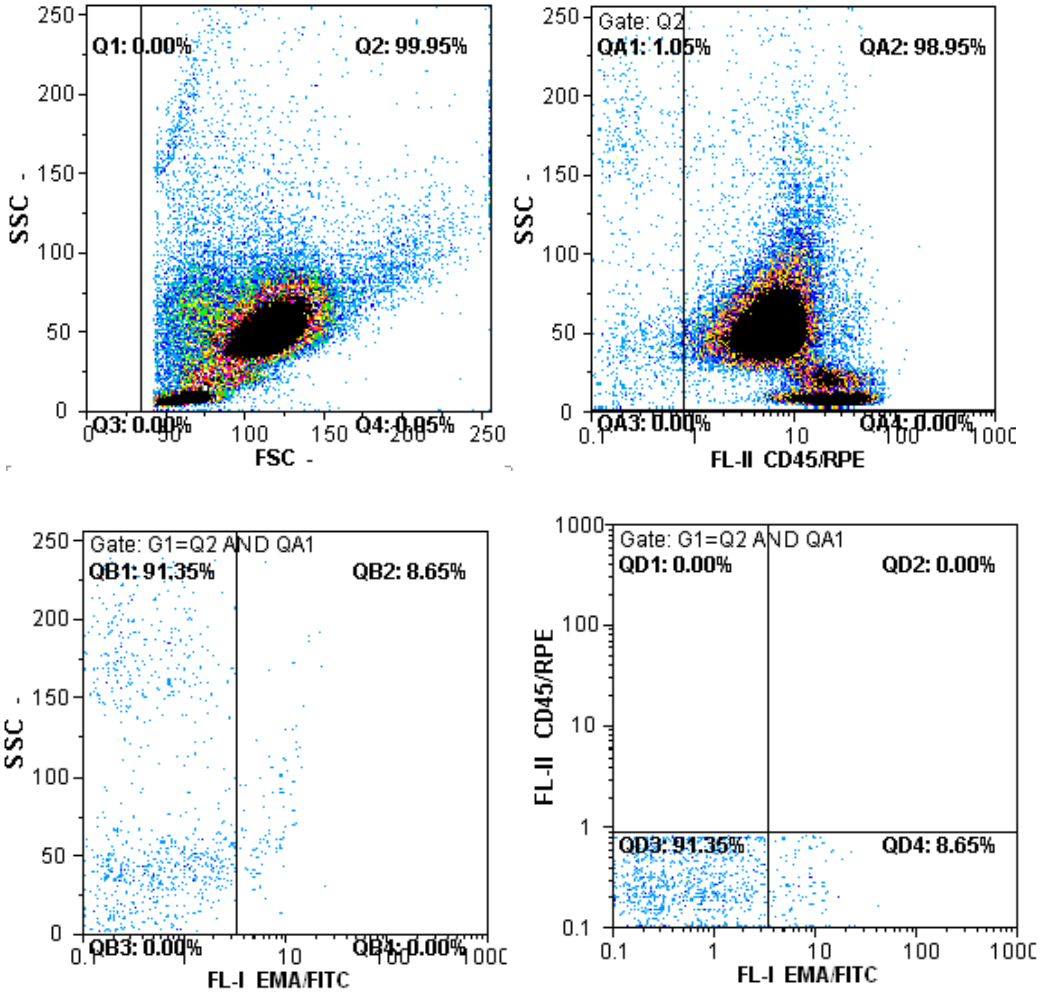

Figure 2. Flow cytometry dot plots using a two-colour method to detect EMA expression in bone marrow cells in oesophageal cancer
a) Gate on the whole marrow cells
b) Gate on CD45-positive whole leukocytes
c) Gate on Epithelial Membrane Antigen (EMA)-positive/CD45-negative marrow cells
d) Dot plot showing that the majority of cells express in membrane EMA-positive/CD45-negative marrow cells

Patients in the clinical stages II, II/III, and III (subgroup I) had a lower number of CD18+ cells than patients in stage IV (subgroup II), but the difference was not statistically significant. A similar trend was observed for EMA+ cells (Table 2). M-ST in subgroup I was 9.0 months, which was longer than in patients in subgroup II (6.5 months), but without statistical significance.

Six patients who underwent total resection of the neoplastic lesion were markedly younger (p=0.05) than those who did not qualify for resection, i.e. 55±3.7 years vs. 63±9.3 years.

Table 3. CD 18 and EMA expression in bone marrow of patients with squamous cell carcinoma of the esophagus qualified for surgery or palliative therapy

Data	Treatment: Surgical n=6 range, mean±*SD*	Treatment: Palliative n =26 range, mean±*SD*	*p* value
CD18+/10^5 cells	0-66 21.8±19.1	0-280 27.7±58.5	
EMA+/10^5 cells	0-97 26.3±37.4	0-568 67±97	0.05
m-ST (months)	12.8	7.0	0.05

Table 4. CD18 and EMA expression in bone marrow of patients with squamous cell carcinoma of the esophagus in relation to the percentage of erythropoietic bone marrow cells

Data	Percentage of erythropoietic bone marrow cells: decreased n=21 range, mean±*SD*	Percentage of erythropoietic bone marrow cells: normal n=11 range, mean±*SD*	*p* value
CD18+/10^5 cells	26-280 58.2±60.7	0-86 18.7±39.2	
EMA+/10^5 cells	27-586 79.6±129	0-134 48±78	0.03
m-ST (months)	6.4	9.9	0.05

Table 5. CD 18 and EMA expression in bone marrow of patients with squamous cell carcinoma of the esophagus in relation to the level of hemoglobin

Data	Hb level (g/dl): < 11g/dl n=7 range, mean±*SD*	Hb level (g/dl): >11 g/dl n=25 range, mean±*SD*	*p* value
CD18+/10^5 cells	15-280 39±43	0-126 29.3±60.3	
EMA+/10^5 cells	30-568 95.5±89	0-235 51.2±119.4	0.05
% of erythroblastic system in bone marrow	4-13 7.3±5.5	6-21 11.4±5.2	0.01
m-ST (months)	6.5	9.5	

The number of CD18+ cells in the patients who underwent resection was lower than in the patients who underwent palliative therapy (Table 3). EMA+ cells displayed a similar trend, with ranges of 0-97/10^5 cells (mean: 26.3±37.4/10^5 cells) and 0-568/10^5 cells (mean: 67.0±97/10^5 cells), respectively, and the difference was statistically significance (p=0.05). M-ST for the operated patients was much longer than for the other patients (12.8 vs. 7.0 months, p=0.05).

Patients in whom the erythropoietic system in the bone marrow was below 15% revealed higher numbers of CD18+ and EMA+ cells than patients in whom no quantitative changes were found in the system (Table 4). Moreover, a negative correlation was found between the number of erythroblasts in the bone marrow and the number of EMA+ cells (r=0.54, p=0.01). M-ST in the group of 21 patients in whom the erythropoietic system was below normal values was 6.4 months and was shorter than that of the remaining patients (M-ST: 9.9 months, p=0.05).

In seven patients with anemia (Hb <11 g/dl, subgroup A) the number of EMA+ cells was significantly higher (p=0.05) than in the patients with normal Hb levels (subgroup B), it being 30-568/10^5 cells (mean: 95.6±89.0/10^5 cells) and 0-235/10^5 cells (51.2±119.4/10^5 cells, respectively (Table 5). The percentage of the erythropoietic system in the bone marrow in subgroup A varied from 4 to 13% (mean: 7.3±5.5%) and was significantly lower (p=0.01) than in subgroup B, which ranged from 6 to 21% (mean: 11.4±5.2%). M-ST in the patients with anemia was shorter than in the patients with normal Hb level (6.5 and 9.5 months, respectively).

The median observation time was 8 months with range of 2 -18 months and in that time only 3 patients, who were followed up at 3,5 and 8th month respectively, did not develop new metastatic foci. None of the patients revealed the presence of CD18+ and EMA+ cells. Thirteen patients, who had both, CD18+ and EMA+ cells in their bone marrow, developed dissemination of the disease.

Discussion

Esophageal cancer develops insidiously and initially it does not produce any clinical symptoms. Dysphagia is associated usually only with advanced stages of the disease. Very good treatment outcomes are possible if the disease is discovered at an early stage when malignant lesions involve only the lamina propria of the mucosa. At this stage, metastases in the regional lymph nodes are observed in only 1.4% of patients. In the case of carcinoma penetrating the lamina muscularis of the mucosa, the incidence of metastases in the neighboring lymph nodes increases to 12.2% and five-year survival rates are significantly poorer [27, 28]. In the group of 32 patients of the present study, who at diagnosis predominantly presented with clinical stages III and IV, only 6 patients qualified for radical resection and only 2 did not reveal any metastatic lesions. Ten patients had generalized lympadenopathy and a further 10 patients had metastases in the lungs or fistulae in the respiratory tract. For this reason a comparison of CD18 and EMA expression in the bone marrow of patients with and without metastases was not possible.

In the material of Thorban, similar to the presented study, epithelial cells identified as cytokeratin-positive (CK+) were found in the bone marrow of about 40% of patients suffering from esophageal cancer and the disease-free survival time in

the CK+ group was shorter [29]. In O'Sullivan's studies, micrometastases in rib marrow were found in 88% and in the iliac crest marrow in 15% of patients undergoing radical resection of gastric or esophagastric cancer [30, 31]. The difference was statistically significant and it might have been associated with local infiltration of the rib marrow and remote micrometastases to the iliac crest marrow. According to the author, micrometastases in the bone marrow of patients who underwent radical resection of malignancy in alimentary tract represent a residual disease and are responsible for metastases to remote organs, irrespective of the histopathological type of the tumor, lymph node involvement, and neoadjuvant therapy. Other authors presented similar opinions [23, 24, 32].

The total number of tumor cells in the bone marrow of a 70-kg human may reach up to $1.5x10^8$, indicating that organ cancer may be a systemic disease [33]. The formation of metastases is a result of the migration of tumor cells, but only 0.05% of circulating cells colonize the bone marrow [34]. Incorporation of these cells in the marrow stroma depends on the presence of specific chemokine receptors, metalloproteases, and adhesion molecules on their surface, which join with active ligands in the marrow stroma [35, 36, 37, 38].

However, other authors did not confirm an association between the presence of micrometastases in the bone marrow evaluated by means of CEA-mRNA and the clinical condition of patients suffering from esophageal squamous carcinoma [39, 40]. In the present study, more CD18+ and EMA+ cells were found in patients in stage IV of the disease and with metastases in the lungs, but the differences were not statistically significant. However, it should be noted that the patients who qualified for resection revealed lower numbers of EMA+ and CD18+ cells than patients who did not qualify for resection due to their advanced stage of disease. Microdeposits of malignant cells in the bone marrow were found in all patients with enlarged lymph nodes in the abdominal cavity and metastases in the liver. Patients with micrometastases had shorter survival times in comparison with those free from this complication, but again the differences were not statistically significant.

Although light microscopy did not demonstrate the presence of malignant epithelial cells in the bone marrow in any of the patients, the erythroblastic system was below the norm in as many as 21 patients (65.6%). Five patients (16%) revealed the presence of paraerythroblasts and 1 patient was found to have megaloblastic regeneration. CD18+ and EMA+ cells were demonstrated in 18 patients with hypoplasia of the erythroblastic system. Thus it can be assumed that the decreased number of erythroblasts in the bone marrow may have been associated with the presence of epithelial cell micrometastases there. This hypothesis could be confirmed by a negative correlation between the percentage of erythroblasts in the bone marrow and the number of EMA+ cells. This observation requires further study in a larger group of patients.

Dysfunction of the erythroblastic system may be a reason for the anemia observed in 7 patients with esophageal carcinoma. In these patients the level of erythroblasts in the bone marrow was significantly decreased and the erythrocyte number in the peripheral blood was significantly lower (p=0.01) than in the remaining patients. All the patients had normocytic anemia, but they did not demonstrate bleeding from the digestive tract (negative benzidine test, normal iron level) or hemolysis (normal or decreased reticulocyte number, normal serum bilirubin level). The anemia associated with malignant diseases is far less clear than that associated with inflammatory and infectious diseases. Malignancy may cause the suppression of erythropoietin production and there may be a defect in the marrow's ability to respond to the erythropoietin and decreased availability of iron.

It has been shown that esophageal tumor cells which constitute micrometastases reveal a significant angiogenic and tumorigenic potential [36]. On the other hand, Japanese authors claim that malignant cells in bone marrow may have a limited metastatic potential and that neoadjuvant therapy does not affect the presence or absence of malignant cells there [39, 40].

The current data demonstrate that cytofluorometry with the use of anti-cytokeratin and anti-EMA antibodies allows an evaluation of microdeposits of esophageal squamous cancer cells in bone marrow. A dysfunctioning erythropoietic system in bone marrow leading to anemia may be a first signal for the presence of malignant cell microdeposits in marrow. These observations require further studies and should be confirmed in a larger group of patients.

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