Research Article

CXCR4 Expression in Gastric Cancer and Bone Marrow: Association with Hypoxia-Regulated Indices, Disseminated Tumor Cells, and Patients Survival

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Aim. The analysis of the association of CXCR4 expression in gastric cancer (GC) and bone marrow (BM) with clinical characteristics.

Patients and Methods. 65 patients with GC were investigated. Immunohistochemistry, immunocytochemistry, NMR-spectroscopy, and zymography were used.

Results. CXCR4 was expressed in 78.5% of GC specimens and correlated with tumor hypoxia \((P < 0.05)\), VEGF expression \((P < 0.01)\), and gelatinases activity \((P < 0.05)\). CXCR4-positive cells in GC were detected in 80% of patients with disseminated tumor cells (DTCs). Overall survival (OS) of patients with CXCR4-positive tumors was poorer than that of patients with CXCR4-negative tumors \((P = 0.037)\). The CXCR4-positive cells in BM were found in 46% of all patients and in 56% of patients with DTCs. CXCR4 expression in BM was not associated with OS. Risk of unfavourable outcome is increased in patients with CXCR4-positive tumors \((P < 0.05)\). CXCR4 expression in BM was positively associated with DTCs, especially in patients with M0 category. Risk of unfavourable outcome is increased in patients with M0 category and with both CXCR4-positive BM and DTCs \((P = 0.03)\).

Conclusions. CXCR4 expression in tumor was positively correlated with hypoxia level and VEGF expression in tumor as well as OS. CXCR4 expression in BM is associated with DTCs.

1. Introduction

CXCR4 is a chemokine receptor specific for stromal-derived factor-1 (SDF-1 also called CXCL12) with potent chemotactic activity for lymphocytes. Expression of CXCR4 is low or absent in many healthy tissues, but its expression is detected in many types of human cancer, including breast cancer [1], ovarian cancer [2], malignant melanoma [3], glioma [4], prostate cancer [5], oesophageal cancer [6], hepatocellular carcinoma [7], pancreatic cancer [8], colorectal cancer [9], renal cell carcinoma [10], and bladder cancer [11]. Moreover, CXCR4 expression was observed in human neuroblastoma [12] and small cell lung cancer [13] cell lines. It was shown by mentioned authors that CXCR4 expression in tumor tissue is associated with metastasis and poor outcome. But there are data that do not confirm the negative impact of CXCR4 on survival. So, Andre et al. [14] have shown that CXCR4 expression was not associated with clinical characteristics of breast cancer, and it was not prognostic factor for overall survival, but significantly higher risk for bone metastasis in patients with CXCR4-positive tumors which was observed.

CXCR4 expression was detected in gastric cancer as well. The significant correlation between the expression of CXCR4 and liver metastasis and lymphatic metastasis in the intestinal type of gastric cancer was found by Iwasa et al. [15]. Lee et al. [16] have shown that strong CXCR4 expression was significantly associated with lymph node metastases and higher stages III/IV and further tended to be correlated with a reduced 5-year survival rate. Our recent publication has confirmed the negative impact of CXCR4 expression in gastric cancer on patients survival [17]. Some studies have observed the expression of CXCR4 in gastric cancer...
with the development of peritoneal carcinomatosis in vivo and in vitro [18, 19]. Zieker et al. [20] have also revealed increased expression of CXCR4/SDF-1α in primary gastric cancer tissue from patients with peritoneal carcinomatosis. It was also shown that CXCR4 expression was significantly related to lymph node metastasis [21] and correlated with metastasis to distant organs and reduced overall survival [22]. Lee et al. [23] have observed that patients with high CXCR4/high SDF-1α expression had the worst prognosis. Nikzaban et al. [24] have shown that CXCR4 expression is significantly higher in patients with advanced stages of the disease. There is interesting observation of Nikkhow et al. [25] that CXCR4 is expressed both in cytoplasm and in nucleus of gastric cancer cells. Patients with nuclear CXCR4 expression have a better overall survival, although cytoplasmic pattern of CXCR4 expression tends to be associated with a shorter overall survival than nuclear staining. In this context it has to be noted that Spano et al. [26] have indicated strong nuclear staining of CXCR4 in 29.8% of patients with early stage non-small-cell lung cancer and a significantly better outcome in this case. The mechanisms of this phenomenon are not clear.

At the same time there are studies that have shown that CXCR4 expression does not influence the survival of gastric cancer patients. So, Mi et al. [27] have found no significant clinical implications of CXCR4 expression in gastric cancer; in particular CXCR4 expression was not linked with lymph node metastasis. Tsuibo et al. [28] have not shown the association between CXCR4 expression and peritoneal metastasis. Ingold et al. [29] have observed that CXCR4 expression in tumor cells of gastric carcinoma and in tumor microvessels had no impact on survival.

We have not found in the available literature the studies aiming at estimating the CXCR4 expression in bone marrow and its link with primary tumor and metastasis. At the same time it is known that CXCR4 is considered as a homing-protein that participates in the migration of tumor cells into the new sites to prolong the growth and form metastases [30, 31]. We assumed that it would be interesting and relevant to detect the CXCR4 expression in bone marrow of oncological patients and evaluate its connection with DTCs. Taking into account all what is mentioned we have aimed to analyze the association of CXCR4 expression in gastric cancer and in bone marrow with tumor hypoxia level, disseminated tumor cells, and patients survival. Preliminary results of our study were recently presented in abstract form.

2. Patients and Methods

2.1. Patients. A total of 65 patients (44 men and 21 women) with primary gastric cancer (GC) were diagnosed and treated at the City Clinical Oncological Center, Kiev, during the period of 2008–2013 (Table 1). No patient received chemotherapy or radiation prior to surgery, and the majority of patients with advanced cancer received the adjuvant chemotherapy. Tissue samples were taken immediately after tumor excision. 52 (80%) patients had undergone partial gastrectomy, 12 (18.5%) gastrectomy, and 1 (1.5%) explorative laparotomy. All patients had undergone standard lymph node dissection, in particular D1 (7 patients, 11%) and D2 (57 patients, 89%). The mean number of dissected lymph nodes was 32 (28–36). Tumors were classified and staged according to the 2002 version of the UICC staging system [32]. Histological types of tumor were evaluated by WHO histological classification (2000) [33]. All patients were thoroughly informed about the study that was approved by the local ethics committee.

2.2. Immunohistochemical Examination of Tumor Tissue. Expression of CXCR4 was evaluated on deparaffinized slides by means of immunohistochemical staining using specific monoclonal mouse antibodies: clone AB2074 (Abcam, UK). Immunoreactions were detected and visualized with the polymer-peroxidase method (EnVision+/HRP and 3,3-diaminobenzidine; DakoCytomation, Denmark) followed by counterstaining with Mayer hematoxylin. Negative control was employed in which the primary antibody was replaced by phosphate-buffered solution (PBS). Intratumoral CXCR4-positive cells were counted per 1000 cells in each slide and the number of CXCR4-positive cells was reported as percent. When the tumor consisted of more than 10% immunoreactive cells, the case was scored as positive.

Expression of VEGF was evaluated on deparaffinized slides by means of immunohistochemical staining using specific monoclonal mouse antibodies (clone VGI (1: 50), DakoCytomation, Denmark). Immunoreactions were detected and visualized with the polymer-peroxidase method (EnVision+/HRP, and 3,3-diaminobenzidine; DakoCytomation, Denmark) followed by counterstaining with Mayer hematoxylin. Positive controls were used as monoclonal antibodies against cytokeratins (clone MNF116, DakoCytomation, Denmark). Negative control was employed in which the primary antibody was replaced by nonimmunized serum or PBS.

VEGF expression was assessed by scoring the number of all positive cells per field (×400). When the tumor consisted of more than 10% immunoreactive cells, the case was scored as positive.

2.3. Immunocytochemical Examination of Bone Marrow. Preoperatively, 2.0-3.0 mL of bone marrow (BM) aspirates from the sternum with conventional cautions to avoid the hit of skin epithelial cells into the sample were taken into a heparinized syringe and transferred into a tube “Sarstedt” containing EDTA K. After Ficoll-Hypaque density centrifugation (density, 1.077; Sigma-Aldrich, USA) to isolate the mononuclear cell fraction (1105 g for 20 minutes), the interphase was washed twice in phosphate-buffered saline (PBS) with removing of erythrocytes (Uti-Lyse Erythrocyte Lysing Reagent, DakoCytomation, USA) and resuspended to a concentration of 570 · 10^3 cells/30 μL and cytocentrifuged on glass slides. Specimens were air-dried from 12 to 24 hours and stained immediately or stored at −20°C.

Detection of tumor cells (cytokeratin-positive cells, CK-positive cells) in BM cytosin preparations fixed in acetone was provided by APAAP method (alkaline phosphatase-anti-alkaline phosphatase) and visualization system EnVision G/2 System/AP Rabbit/Mouse (Permanent Red) (DakoCytomation, Denmark). Monoclonal mouse antibodies against
Table 1: Prevalence of CXCR4+ in gastric cancer tissue by clinical variables.

<table>
<thead>
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<th>Characteristics</th>
<th>All patients number (n = 65)</th>
<th>CXCR4+ number (n = 51)</th>
<th>CXCR4- number (n = 14)</th>
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<td>63 (34–73)</td>
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panCK (clone AE1/AE3, DakoCytomation, Denmark) were used as primary antibodies. Each assay was controlled negatively by staining of one cytospin preparation with nonspecific IgG₁ (MOPC21, Sigma). Number of tumor cells (CK-positive cells) was expressed on 10⁶ BM mononuclear cells. BM samples were scored “positive,” if the presence of two or more CK-positive cells per 10⁶ mononuclear cells was detected (from 6 to 12 slides per patient were screened).

Detection of CXCR4-positive cells in BM cytospin preparations fixed by formol-acetone solution (pH 6.6) in accordance with the instruction was performed by the next steps. Slides were treated by 0.3% Triton X-100 solution and washed by PBS and blocking of endogenous peroxidase followed by incubation in 3% bovine serum albumin to switch off nonspecific reaction antigen antibody. Cytospins were incubated with primary polyclonal rabbit antibodies against CXCR4 (clone AB2074, Abcam, UK) in optimal dilution 1:1000 within 1h. After washing of primary antibodies slides were processed with PolyVue HRP detection system components (Diagnostic Biosystems, USA) followed by counterstaining with the solution of methyl green (Methyl Green histological staining reagent, DakoCytomation, Denmark). Negative controls were employed in which the primary antibody was replaced by PBS. BM samples were scored “positive,” if the
presence of one or more CXCR4-positive cells was detected in the field.

2.4. $^{31}P$ NMR Spectroscopy. Level of tumor hypoxia was assessed with $^{31}P$ NMR spectroscopy. $^{31}P$ NMR spectra of perchloric acid (PCA) tumor extracts were acquired by means of a high-resolution Bruker 400 MHz spectrometer (Widobore Ultrashield, AV-400 electronics, Germany) using a probe of 5 mm inner diameter. All details of method were presented in our earlier publication [34].

2.5. Metalloproteinase-2 and Metalloproteinase-9 Activity Assay. Tumor specimens were placed into the liquid nitrogen up to processing. Activity of MMP-2 and MMP-9 was determined for each sample by zymography in 12% polyacrylamide gel with SDS and 0.1% of gelatin as substrate [34].

2.6. Statistical Analysis. All statistical analyses were conducted using the NCSS 2000/PASS 200 and Prism, version 4.03 software packages. Correlations were analyzed with the Pearson correlation coefficient. The $\chi^2$ test was performed to determine the correlation between the CXCR4 status of the gastric carcinomas and the clinicopathological characteristics. The survival proportion was estimated by using the Kaplan-Meier method and differences in survival were analyzed with the log-rank test. Prognostic values of relevant variables were analyzed by means of the Cox proportional hazards model using hazard ratio and $\chi^2$ test. Two-tailed $P$ values <0.05 were considered statistically significant.

3. Results

3.1. CXCR4-Positive Cells in Tumor Tissue and Their Correlation with Clinical Variables. Individual patient data from a total 65 histological confirmed gastric cancer patients were included in this study (Table 1). The median age was 63 years. Overall, 78.5% of patients had tumors with CXCR4-positive cells. The mean number of CXCR4-positive cells in tumor was $37.8 \pm 3.2$ cells. The median number of CXCR4-positive cells was 32.2% (range of 11.2–87.3). The association between clinicopathological characteristics of the gastric cancer patients and CXCR4 status was analyzed (Table 1). The same associations were observed between CXCR4 status in tumor and histological tumor type (adenocarcinomas dominated among CXCR4-positive tumors), grade of differentiation, lymph node, and distant metastasis; in particular 23 of 29 primary gastric tumors with N1 category and 7 of 8 ones with M1 category were positive for CXCR4 expression (79.3% and 87.5%, resp.). At the same time statistically significant correlation between CXCR4-positivity of tumors and clinical characteristics was not found (Table 1).

3.2. CXCR4-Positive Cells and Their Correlation with Hypoxia Level in Primary Tumor. The association between the CXCR4-positive cells and the level of hypoxia in primary tumor was shown. The correlation was observed between the number of CXCR4-positive cells in tumor and intratumoral PME/Pi ratio that indicates the level of hypoxia. In particular the number of CXCR4-positive cells was greater in tumors characterized by severe and moderate hypoxia (PME/Pi < 1.4) ($r = 0.492; P = 0.04$). Moreover, it was also shown that the probability of the appearance of high number of CXCR4-positive cells in tumor is increased by a factor of 5 (odds ratio 4.926, 95% CI 7.027–23.628, and $P = 0.046$), when tumors were characterized by severe and moderate hypoxia.

It was shown that the number of CXCR4-positive cells in tumors was positively correlated with the number of VEGF-positive cells ($r = 0.337; P = 0.006$). The association between CXCR4 expression and microvessel density was also observed; that is, tumors with high level of microvessel density were characterized by high number of CXCR4-positive cells, but this link was not statistically significant.

It was also found that the number of CXCR4-positive cells in tumor is positively correlated with gelatinases activity in tumor tissue, in particular with the concentration of active forms of MMP-2 ($r = 0.31; P < 0.05$) and MMP-9 ($r = 0.4; P < 0.05$).

3.3. CXCR4-Positive Cells in Tumor and Disseminated Tumor Cells in Bone Marrow. CXCR4-positive cells in tumor tissue were detected in 80% of patients with the presence of DTC in BM. The correlation was shown between CXCR4-positivity of primary tumors and appearance of DCT in BM: in patients with CXCR4-positive tumors DTCs in bone marrow were observed in 51.7% of cases, and with CXCR4-negative tumors in 40% of cases; in patients with the number of CXCR4-positive cells in tumors over median value DTCs in bone marrow were detected in 65% of cases, and in patients with the number of CXCR4-positive cells below median in 22.2% of cases.

It was detected that tumor cells in bone marrow were detected in 65% of patients with tumors which had the number of CXCR4-positive cells over median and were not detected in 35% of patients. At the same time when the number of CXCR4-positive cells was below median tumor cells in bone marrow were detected in 22.2% of patients and not detected in 77.8% of patients.

It was also determined that the probability of appearance of DTCs in BM of patients is increased by a factor of 4.0 (odds ratio 4.024, 95% CI 1.0597–15.2782, $\chi^2 = 4.367$, and $P = 0.0408$) and the relative risk of DTCs appearance was approximately 2.0 (RR = 1.955, 95% CI 0.999–3.824, and $P = 0.05$) when tumors were characterized by positivity for CXCR4 cells.

3.4. CXCR4-Positive Cells in Bone Marrow. The CXCR4-positive cells in BM were found in 46% of all patients and in 56% of patients with DTC. It was also detected that CXCR4-positive cells in BM in patients with M0 category were detected in 63% of patients with DTC.

It was shown that CXCR4-positive cells were registered in 46.1% of cases both in tumors and in bone marrow. CXCR4-positivity was absent both in tumor and in bone marrow in 20.5% of cases, and in 33.4% of cases the association between CXCR4 expression in tumor and bone marrow was not found.
It was detected also that CXCR4-positive cells in bone marrow were registered in 73.7% of cases when the number of CXCR4-positive cells in tumor was over median and in 40.0% of cases when the number of CXCR4-positive cells in tumor was below median. When CXCR4-positive cells were not observed in tumor, CXCR4-positive cells in bone marrow were found in 20% of patients only.

Moreover, it was also found that the presence of CXCR-positive cells in bone marrow was observed in 62.1% of patients with CXCR4-positive tumors.

3.5. Overall Survival of Patients with Positive Tumors and Bone Marrow for CXCR4. Overall survival (OS) was significantly longer in all patients with tumors characterized by CXCR4-negative status as compared to patients with CXCR4-positive tumors (log-rank test \( P = 0.0375 \), Figure 1). Moreover, it was shown that OS of patients with M0 category with CXCR4-negative tumors was significantly longer in comparison with patients with M0 category and with CXCR4-positive tumors (log-rank test \( P = 0.0137 \), Figure 2).

Median follow-up time was 15.1 (range of 0.56–68.02) months from diagnosis for all patients. Overall, 20 patients (30.8%) died during follow-up. In 19 patients (95.0%) death was related to gastric cancer at median of 23.4 months following operation (range of 3.9–36.4). Of these, 18 patients (94.7%) had CXCR4-positive tumors and 1 patient (5.3%) had CXCR4-negative tumor. Among patients with CXCR4-positive tumors, 72.32% and 27.7% deceased patients had tumors with the number of CXCR4-positive cells over and below median, respectively.

The association between tumor CXCR4-positivity and survival of patients in accordance with the type of treatment was analyzed separately: 20 patients (30.8%) were operated only and 45 patients (69.2%) were treated with adjuvant chemotherapy.

The survival in the group of “operation” was characterized by the following indices: 100% of patients with the number of CXCR4-positive cells over median have died, and 1 patient has died with CXCR4-negative tumor.

The analysis of the survival in the group “operation + chemotherapy” has shown the following: 28.6% of patients with the number of CXCR4-positive cells below median have died, and 71.4% over median (percentage was calculated for this group of patients).

OS was longer in all patients with BM characterized by CXCR4-negative status as compared to patients with CXCR4-positive BM (log-rank test \( P = 0.0441 \), Figure 3). Moreover, it was shown that OS of patients with M0 category with CXCR4-negative BM was also longer in comparison with patients with M0 category and with CXCR4-positive BM but this association was not statistically significant (log-rank test \( P = 0.089 \), Figure 4).

Moreover, it was evaluated that in all patients with CXCR4-positive tumors risk of unfavorable outcome was increased almost by a factor of 3.0 (HR = 2.82, 95% CI 1.162–6.832; \( P < 0.05 \)). CXCR4 expression in BM was positively associated with DTC, especially in patients with M0 category. It was observed that in patients with M0 category and with both DTC and CXCR4-positive BM risk of unfavorable outcome increased by a factor of 3.4 (HR = 3.4; 95% CI 1.156–12.054; \( P < 0.03 \)).

4. Discussion

CXCR4-positive cells were found in 78.5% of gastric cancer patients and 21.5% of patients were negative for CXCR4. The median number of CXCR4-positive cells was 32.2%; in 8% of tumors the number of CXCR4-positive cells reached 60% and over that indicates the relatively high CXCR4-positivity of gastric cancer. It was not possible to compare our data with the data of other authors because there is...
absence of the information about the number of CXCR4-positive cells in tumor in their studies. We have not found the strong association between CXCR4 expression in tumor and clinicopathological characteristics, although it may be paid attention to the more number of adenocarcinomas, tumors with grade G3 as well as with nodal involvement and M1 category in the cases with CXCR4-positive tumors.

The observation concerning the correlation between the number of CXCR4-positive cells and the level of hypoxia in primary tumor is relevant; in particular the level of CXCR4-positivity was higher in tumors characterized by severe and moderate hypoxia. This observation confirms the early data of Schioppa et al. [35] that the expression of CXCR4 was induced when tumor cells were cultured under hypoxia conditions. Interestingly, this year Staller et al. [31] have shown that negative regulation of CXCR4 expression by the von Hippel-Lindau tumor suppressor protein due to its capacity to target HIF for degradation under normoxia was suppressed under hypoxia conditions. It resulted in HIF-dependent CXCR4 activation. It was also shown that clear cell renal carcinoma that demonstrates the mutation of the VHL gene in most cases revealed an association of strong CXCR4 expression with poor survival. Obtained results have allowed suggesting that the expression of CXCR4 plays important role in the migration of tumor cells from primary tumor and homing in the distant organs that is regulated by the hypoxia [31].

Recently the link was shown between hypoxia and CXCR4 expression in breast cancer and suggested "that hypoxic condition selects the tumor cells which go on to proliferate and metastasize by activating the expression of CXCR4 in these cells" [36]. The suggestion concerning CXCR4 participation in the homing may be partly confirmed by our finding that CXCR4-positive cells in tumor were detected in 80% of patients with the presence of DTCs in bone marrow. It is remarkable that the CXCR4-positive cells in bone marrow were found in 46% of all patients, in 56% of patients with DTCs, and that is very important, in 63% of patients with M0 category and DTCs. The last finding may be discussed in the context of predisposition of BM to be a new site for tumor cells by CXCR4 expression. It may be noted that DTCs in bone marrow were detected in 51.4% of gastric cancer patients with M0 category as was published recently [37].

Kaifi et al. [6] found a statistically significant positive association between CXCR4-positive expression of esophageal cancer cells in the primary tumor and the presence of esophageal cancer cells in the bone marrow (P < 0.001; 43 (72%) of 60 patients with micrometastatic tumor cells in their bone marrow expressed CXCR4 in primary tumor.

At the same time Pituch-Noworolska et al. [38] evaluated the expression of CXCR4 on cytokeratin-positive (CK+) cells in the smears of CD45-negative cells isolated from the bone marrow of patients with gastric cancer and found that expression of CXCR4 was observed in 23.3% of CK+ samples and CXCR4 expression was higher on CK+ gastric cancer cells from the lymph nodes suggesting their role in homing to the lymph nodes. Yasumoto et al. [18] showed that 67% of primary gastric tumors with peritoneal metastasis were positive for CXCR4 expression, whereas with other distant metastases only 25% were positive. Our study indicated the association of CXCR4 expression in primary gastric cancer with DTCs, that is, with presence of tumor cells in bone marrow which may be considered as potential triggers for the distant metastasis.

It is known that tumor cells traffic into the bone marrow and can exist in bone marrow within long-time period before the formation of metastases in distant sites. Chemokine, in particular CXCL12 and CXCR4, plays a significant role in this process. Out data concerning association between CXCR4-positive cells and DTCs confirm this suggestion. In the context of CXCR4-positivity of bone marrow it has to be taken into account that CXCR4-positive cells in bone marrow may be tumor-derived as well hemopoietic cells attracted into
bone marrow that it was indicated in review of Chantrain et al. [39]. In any case the direct link there is between CXCR4 expression in primary tumor and presence of DTCs in bone marrow which are potential trigger for metastasis into the distant organs. On the basis of published data it is difficult to indicate the “preferable” role of CXCR4 in primary tumor in the metastasis into lymph nodes, peritoneum, or bone marrow due to different design of performed studies, in particular their methodological component.

At any rate, OS was longer in all patients with CXCR4-negative status than in patients with CXCR4-positive tumors. It is important that OS of patients with M₀ category was longer than in patients with M₀ category but with CXCR4-positive tumors. Prognostic relevance of CXCR4-positivity in tumor was also shown; in particular risk of unfavorable outcome was increased by a factor of 2.82 in patients with CXCR4-positive tumors. Our clinical observation is associated with the data of Zhang et al. [40] and Müller et al. [41] that CXCR4 overexpressing tumor cells have a higher propensity to form bone marrow metastasis when being injected intravenously in mice.

CXCR4-positive cells in BM were not associated with overall survival. At the same time CXCR4 expression in BM was positively correlated with DTCs, especially in patients with M₀ category, and CXCR4-positive BM is a risk factor of unfavorable outcome.

In conclusion, it has to be noted that our study has shown the direct correlation between CXCR4-positivity of tumor and tumor hypoxia level as well as some hypoxia-associated indices, in particular VEGF expression. It is important that positive association was observed between CXCR4 expression and activity of MMP-2 and MMP-9 in tumor that participate in remodeling of extracellular matrix providing the exit of tumor cells into circulation. High expression of CXCR4 in tumor is associated with appearance of DTCs in bone marrow. Moreover, the presence of CXCR4-positive cells in bone marrow was found that was associated with DTCs in bone marrow, especially in patients with M₀ category. CXCR4 expression in tumor was assessed as a prognostic factor of unfavorable clinical outcome.

Obtained results have clearly shown that hypoxia level in primary tumor is directly correlated with overexpression of homing-protein CXCR4” in tumor; as it is known, it mediates the migration of tumor cells to find the favorable sites for growth. This fact is confirmed by observed association between CXCR4-positive status of tumor, level of tumor hypoxia, and appearance of tumor cells in bone marrow. It is very important that this association was found in patients with M₀ category, that is, when distant metastases were not diagnosed, but available tumor cells in bone marrow there are the potential source of the formation of overt metastases. On this basis it seems important to evaluate the level of tumor hypoxia and expression of hypoxia-associated proteins, in particular VEGF and CXCR4 as being involved in metastasis. When the strong hypoxia and high level of CXCR4 expression both in tumor and in bone marrow with the presence of DTCs were found in patients with M₀ category even after curative surgery, the application of adjuvant chemotherapy or at least the selection of these patients at risk of early metastasis can be recommended.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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