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RESEARCH ARTICLES

Hypoxia, tumour-associated macrophages, microvessel density, VEGF and matrix metalloproteinases in human gastric cancer: interaction and impact on survival

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Abstract
Introduction Hypoxia is a key feature of the microenvironment of cancer cells actively participating in tumour progression. Our study was aimed to evaluate the impact of hypoxia and hypoxia-associated factors on tumour progression and survival of patients with gastric cancer.
Material and methods One hundred and five resected specimens were used. The level of tumour hypoxia was evaluated using ^{31}P NMR spectroscopy, CD68 (tumour-associated macrophages), CD34 (microvessel density, MVD) and VEGF expression, immunohistochemistry, MMP-2 and MMP-9 activity, zymography. Statistical analysis was conducted using Pearson's test, Kaplan–Meier survival analysis, log-rank test and Cox proportional hazards model.
Results Intratumoral hypoxia level has been significantly correlated with VEGF expression, TAM number and total protease activity. The overall survival rate of patients with strong tumour hypoxia, high level of MVD, VEGF expression, TAM and MMP activity was significantly lower than that of the patients without the mentioned tumour characteristics.
Conclusions The hypoxia-associated signalling that is activated in tumours promotes tumour progression through the recruitment of macrophages, remodelling of extracellular matrix and neoangiogenesis.

Keywords Hypoxia-associated events · Gastric cancer · Prognostic markers

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Introduction

Gastric cancer is one of the most common cancers in Europe, ranking fifth after lung, prostate, colorectal and bladder cancers in men and breast, colorectal, lung and cancer of the corpus uteri in women [1]. In Ukraine in 2007 the annual age-standardised incidence rate was 23.5/100,000, ranking third after lung and skin cancers, in men and 9.8/100,000, ranking sixth after breast, skin, corpus uteri, cervix uteri and colorectal cancers, in women [2]. The treatment outcome of gastric cancer is still not satisfactory because of objective difficulties in diagnosis and prediction of tumour response to treatment, as well as prognosis of disease outcome. It is well known that hypoxia is a key characteristic of solid tumours, mediating a poor outcome in a variety of human malignancies [3, 4]. Intratumoral hypoxia induces neoangiogenesis, recruitment of bone marrow-derived cells, polarisation of macrophages and degradation of extracellular matrix (ECM), resulting in the activation of proliferation, invasion and metastasis of cancer cells [5–7].

Earlier, correlations between VEGF expression, microvessel density (MVD), tumour-associated macrophage (TAM) infiltration, matrix metalloproteinase (MMP) activity in human gastric cancer and some clinicopathologic characteristics of patients were shown [8–14]. The prognostic significance of all these parameters was also determined. At the same time, in most studies dedicated to gastric cancer only individual indices, mainly MVD and VEGF, were

indicated and compared with each other [8–10, 14, 15]. MMPs [12] and TAM [16] were investigated in separate studies, but the attempt to study all these indices in one individual tumour specimen and to compare them with each other as well as with clinicopathologic tumour parameters has not been undertaken. Moreover no relationship has been found between all these indices and the level of tumour hypoxia, which is responsible for their expression.

Taking into account the above-mentioned, this study aimed to evaluate the correlations between intratumoral hypoxia, angiogenesis, TAM density and MMP activity in human gastric cancer and assess their prognostic relevance.

Patients and methods

Patients

A total of 105 patients (71 men and 34 women) with primary gastric cancer were diagnosed and treated at the Municipal Oncological Hospital, Kiev, during the period 2005–2007 (Table 1). No patient received chemotherapy or radiation prior to surgery. Tissue samples were taken immediately after tumour excision. Tumours were classified and staged according to the 2002 version of the UICC staging system [17]. Histological types of tumour were evaluated by the WHO histological classification (2000) [18]. All patients were thoroughly informed about the study, which was approved by the local ethics committee.

Table 1 Patient and tumour characteristics

Characteristics	Number, 105 (%)
Gender	
Male	71 (68)
Female	34 (32)
Age (years)	
Median	63
Range	31–79
Tumour location	
Upper third	17 (16)
Middle third	30 (29)
Lower third	53 (50)
Total	5 (5)
UICC stage	
I	17 (16)
II	31 (30)
III	33 (31)
IV	24 (23)
Histological type	
Adenocarcinoma	74 (70)
Mucinous adenocarcinoma	6 (6)
Signet-ring cell carcinoma	9 (9)
Undifferentiated carcinoma	16 (15)
Tumour grade	
Well differentiated	25 (24)
Poorly differentiated	80 (76)
T-classification	
T1	4 (4)
T2	17 (16)
T3	62 (59)
T4	22 (21)
Nodal involvement	
N0	52 (49.5)
N1	24 (22.9)
N2	27 (25.7)
N3	2 (1.9)
Distant metastasis	
M0	93 (89)
M1	12 (11)

^{31}P NMR spectroscopy

^{31}P NMR spectra of perchloric acid (PCA) tissue extracts were acquired by means of a high-resolution Bruker 400 MHz spectrometer (Widebore Ultrashield, AV-400 electronics, Germany) using a probe of 5 mm inner diameter. ^{31}P NMR spectra were measured at 161,976 MHz. Spectra were usually obtained up to 1000 transients with a spectral window of 64724.9, a 90° pulse angle and a line broadening of 10 Hz. As a standard, methylenediphosphonic acid, trisodium salt (Sigma, USA) was used. All ^{31}P chemical shifts in the spectra were set relative to PCr by setting the PCr signal to 0.00 ppm. Areas of the spectral signals were measured by the integration mode of the spectrometer. The metabolic ratio phosphomonoester (PME)/inorganic phosphate (Pi), as it is very sensitive to changes in the tissue oxygenation, was used as a parameter for estimation of changes in the tissue hypoxia level [19].

Immunohistochemical examination

Expression of CD34 (the endothelial cell marker), CD68 (the commonly used macrophage marker) and VEGF was evaluated on deparaffinised slides by means of immunohistochemical staining using specific monoclonal antibodies: clone QBEnd 10 (1:100), clone PG-M1 (1:80) and clone VG1 (1:30), respectively. All specific monoclonal antibodies belong to Dako Cytomation (Denmark).

Immunoreactions were detected and visualised with the polymer-peroxidase method (EnVision+/HRP, and 3,3-diaminobenzidin; Dako Cytomation, Denmark) followed by counterstaining with Mayer haematoxylin. Positive controls were used as monoclonal antibodies against cytokeratins (clone MNF116, DakoCytomation, Denmark). Nonimmunised serum or PBS were substituted by primary antibodies as the negative control.

MVD, detected by immunostaining for CD34, was assessed by the hot spot method [20]. CD34 staining was assessed using the ocular grid. MVD was expressed as the mean count of CD34 immunostained cells per 1 mm^2.

CD68-positive cells were counted per 1000 cells in each slide and the number of CD68-positive cells was reported as a percentage.