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PREMORPHOLOGICAL ALTERATIONS IN GASTRIC MUCOSA IN PATIENTS WITH GASTRIC CANCER: HYPOXIA LEVEL ASSESSED BY ^{31}P NMR SPECTROSCOPY

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The *aim* of this study was to detect the hypoxic status in adjacent histologically uninvolved gastric mucosa in gastric cancer (GC) patients. *Patients and Methods*: 50 naïve patients with primary GC, and one patient with stomach ulcer (gastric mucosa was used as control) were enrolled into the study. The tumor and mucosa samples of stomach (without muscularis and serosa layers) in patients with GC were obtained immediately after operation. Assessment of the hypoxia level has been provided using ^{31}P NMR spectroscopy in tissue perchloric acid extracts. Mucosa was examined by convenient histological method. Obtained results were analyzed statistically. *Results*: It was shown that gastric mucosa in 52% of patients with GC is under different levels of hypoxia, among them 23% of mucosa is under severe hypoxia (PME/Pi < 1.0) with average mean of PME/Pi 0.89 ± 0.04. It was revealed that there are definite associations between the hypoxia in mucosa tissue and pT as well as pN categories and G grade and stage of disease but not with M category. Overall survival of patients with gastric mucosa characterized by severe and mild hypoxia was significantly poorer as compared to that of patients with gastric mucosa with satisfactory oxygenation (p = 0.0035). *Conclusion*: Gastric mucosa uninvolved in tumor process is characterized by hypoxia in 52% of GC patients; severe hypoxia of mucosa was detected in 23% of patients having hypoxic mucosa. It was suggested that biochemical alterations in tissue surrounding tumor node may precede morphological ones.
Key Words: **gastric cancer, mucosa, hypoxia.**

Hypoxia is currently considered as a key factor in tumor pathogenesis and one of the main inducer of malignant progression. There are experimental and clinical data that the hypoxic fraction in solid tumors stimulates their growth and metastatic potential as well as their sensitivity to ionizing radiation and some chemotherapeutical agents [1–4]. It was also concluded that hypoxia may be considered as independent factor for prediction of disease outcome [1, 4].

It is known that tumor node is excised with the surrounding tissues in accordance with the rules of the margins of resection. Resected mucosa is investigated by histological method to control the existence of tumor cells. It has to be noted that this procedure may be performed intraoperatively to determine the status of margin and change, if the margin will be positive, the operation will be extended. It was shown that microscopic positive margin is an independent prognostic factor in gastric cancer (GC) patients [5–9].

At the same time the systemic influence of tumor on the normal tissues is known [10–15]. In this context it is interesting the observations about alterations in the noncancerous gastric mucosa in GC patients. Sugai et al. [16] have shown that genetic alteration already occur within the surrounding on the noncancerous mucosa, in particular microsatellite alterations were detected. It was determined the decrease of intensity of spontaneous apoptosis and its resistance to proapoptotic factors in extratumoral microfollicular thyroid tissue with absence of macro- or microscopical features of pathological changes in patients with thyroid carcinoma [17]. The abnormal methylation of *N33, CDH1,* and *RUNX3* genes in morphologically normal mucosa in gastric stump in patients operated on GC was observed [18]. The alterations in expression of Lewis antigen in adjacent uninvolved gastric mucosal in GC patients were shown by Kim et al. [19]. Currently the molecular assessment of surgical-resection margins was proposed to evaluate the margins status during GC operation to evaluate of volume operation [20]. Taking into account mentioned data we have aimed to detect the hypoxic status in adjacent histological uninvolved gastric mucosa in GC patients. The numerous data about positive impact of tumor hypoxia on malignant progression indicate the usefulness of providing of such study.

Meanwhile, early it was shown that oxygenation of gastric normal tissue in patients with GC was decreased in comparison with that of normal gastric tissue in patients with gastric ulcer [21]. The partial oxygen tension in this study was measured in gastric wall by microelectrode, but the information about the microelectrode location, i.e., in mucosa and submucosa or in full gastric wall including muscularis and serosa, was not presented. The histological examination of normal tissues was not performed.

It was shown that magnetic resonance spectroscopy is a useful technique that provides insight into the alterations of metabolic pathways in the pathological state and information on the early biochemical changes that are difficult to assess using as standard biochemical as well morphological methods. It is well known fact

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Abbreviations used: GC – gastric cancer; PCA – perchloric acid; PME/Pi – phosphomonoesters/inorganic phosphate.

that there is a strong dependency between bioenergetic status and oxygenation in tumor tissue since metabolic turnover in tissue is able to adapt to the level of tissue oxygenation, and changes in metabolic ratios may also indicate the hypoxia level, thus permitting their application not only for evaluation of tissue energy status but for hypoxia as well [22]. Nuclear magnetic resonance (NMR) spectroscopy, being a very convenient and informative technique *in vitro* and *in vivo* investigations, has been widely applied in oncological studies in recent decades.

The aim of the study was to characterize the gastric mucosa of patients with GC as to the level of its oxygenation.

PATIENTS AND METHODS

Patients. A total of 51 patients (30 men and 21 women) whose mucosa has been investigated were diagnosed and operated at the City Clinical Oncological Center (Kyiv), during period 2008–2013: 50 patients with primary GC, and one patient with stomach ulcer (gastric mucosa was used in this case as control). Tumors were classified and staged according to the 2002 version of the UICC staging system [23] (Table). All patients were thoroughly informed about the study that was approved by the local ethics committee.

Table. Patient and tumor characteristics

Characteristics	Number of patients, n (%)
Gender	
Male	30 (60.0)
Female	20 (40.0)
Age (years)	
Median	65.0
Range	33–79
Tumor location	
Upper third	9 (18.0)
Middle third	12 (24.0)
Lower third	27 (54.0)
Total	2 (4.0)
UICC stage	
I	5 (10.0)
II	12 (24.0)
III	17 (34.0)
IV	16 (32.0)
Histological type	
Adenocarcinoma	31 (62.0)
Mucinous adenocarcinoma	10 (20.0)
Signet-ring cell carcinoma	5 (10.0)
Undifferentiated carcinoma	4 (8.0)
Grade (G)	
1	1 (2.0)
2	9 (18.0)
3	29 (58.0)
4	11 (22.0)
T-classification	
T1	1 (2.0)
T2	6 (12.0)
T3	26 (52.0)
T4	17 (34.0)
Nodal involvement	
N0	22 (44.0)
N1–2	28 (56.0)
Distant metastasis	
M0	43 (86.0)
M1	7 (14.0)

The mucosa samples of stomach (without muscularis and serosa layers) in patients with GC were obtained immediately after operation. They were excised exclusively at a definite distance (approximately 8–10 cm) from the tumor node edge (beyond of obligatory operative resection) and frozen in liquid nitrogen immediately followed by NMR spectroscopy analysis. Moreover, the specimens of mucosa samples were fixed in 4% formalin and embedded in paraffin followed by convenient preparation and staining with hematoxylin and eosin.

NMR spectroscopy. Assessment of the hypoxia level has been provided using ^{31}P NMR spectroscopy in tissue perchloric acid (PCA) extracts. ^{31}P NMR spectra 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 have been obtained in the NMR Spectroscopy Center (G.V. Kurdyumov Institute for Metal Physics, NAS of Ukraine). The areas of the signals on the ^{31}P NMR spectra were determined by the integration mode of the spectrometer. All details of ^{31}P NMR method as well preparation of PCA tissue extracts have been presented in our earlier publication [24].

For the assessment of the hypoxia level in tumor as well in surrounding mucosa of patients with GC it was used the ^{31}P PME/Pi metabolic ratio (phosphomonoesters/inorganic phosphate) because it is very sensitive to tissue oxygenation. As it was shown there is a highly significant linear correlation ($p < 0.001$) between the mean tissue pO_2 value and the respective PME/Pi ratio [25]. In the Fig. 1 there is presented the ^{31}P NMR spectrum of mucosa as a typical.

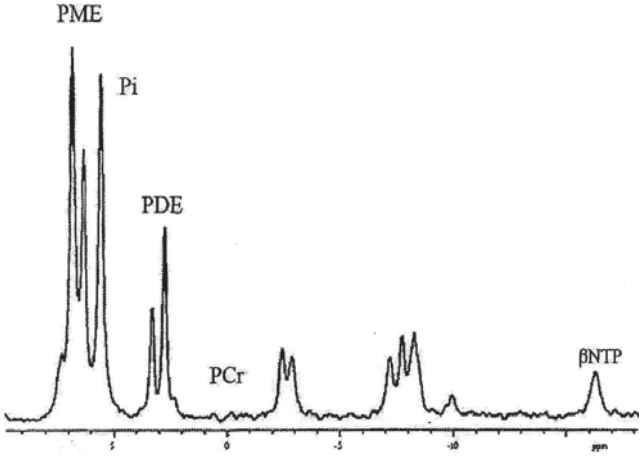

Fig. 1. ^{31}P NMR spectrum of PCA extract of gastric uninvolved mucosa. Copy of original spectra, patient Z.A.A.

Statistical analysis. All statistical analyses were conducted using the NCSS 2000/PASS 2000 and Prism, version 4.03 software packages. The survival proportion was estimated 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 Odds ratio (OR) and χ^2 test. Two-tailed p values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

A detailed analysis of obtained results of our recent investigations using ^{31}P NMR spectroscopy [24, 26, 27] allowed to divide gastric tumors into groups according to the hypoxia level: severe hypoxia (PME/Pi < 1.0), moderate and mild hypoxia (1.0 < PME/Pi < 2.0), and conditional "satisfactory" oxygenation (2.0 < PME/Pi < 2.4). The median value of the PME/Pi ratio for the GC tissue was 1.4 (range, 0.8–5.32) with average mean

1.49 ± 0.06, while for gastric surrounding uninvolved mucosa the median value of PME/Pi ratio is 2.125 (range, 0.78–5.27), with average mean 2.31 ± 0.13.

Analysis of ^{31}P NMR spectra of PCA extracts of mucosa tissue showed that gastric mucosa in 52% of patients with GC was characterized by hypoxia level below the median of the PME/Pi ratio for mucosa, i.e., they are under different levels of hypoxia, and gastric mucosa in 48% of patients was characterized by hypoxia level over the median, i.e., it is under "satisfactory" oxygenation.

In order to reveal distribution of mucosa tissue as to the level of hypoxia in the patients with GC it has been used the scheme of division as to the level of hypoxia for tumor tissue. It was obtained that 12% of mucosa are under severe hypoxia (PME/Pi < 1.0; with average mean of PME/Pi 0.89 ± 0.04, 34% of mucosa are under different levels of moderate hypoxia (1.0 < PME/P < 2.0; with average mean of PME/Pi 1.66 ± 0.06); and the rest ones have satisfactory oxygenation (PME/Pi > 2.0; with average mean of PME/Pi 2.93 ± 0.14).

As an example for the comparison we had the sample of gastric mucosa of patient who has been operated on gastric ulcer. It was characterized by very high level of oxygenation, i.e., PME/Pi 3.17.

In the group of patients with GC (n = 13) with the tumor characterized by severe hypoxia (PME/Pi < 1.0) mucosa has been found under the same condition in 38.5% of cases, under moderate hypoxia — in 38.5% also and satisfactory oxygenated mucosa — in 23.1%. In the group of patients with GC (n = 23) with tumor under different levels of hypoxia (1.0 < PME/P < 2.0) — only 4.3% of mucosa tissue was under severe hypoxia, 52.2% — under moderate hypoxia and 43.5% — satisfactory oxygenated. The complete coincidence as to the level of oxygenation there was in the group of patients with tumors that are satisfactory oxygenated (PME/P > 2.0).

The hypoxia level in GC was correlated neither with pT or M categories (TNM system) nor with stages of disease. At the same time, a very important interrelationship between high levels of hypoxia in primary tumors an appearance of metastases in lymph nodes of patients with GC has been established [28]. Taking into account the above mentioned it has been provided the analysis whether there is the association between the level of hypoxia in mucosa with clinico-pathological characteristics of patients with GC. It was also revealed that there are definite associations between the hypoxia in mucosa tissue and pT as well as pN categories and G grade and stage of disease but not with M category.

It was shown that patients with pT_4 category are 4.0 times more likely to have surrounding mucosa under hypoxic condition (PME/Pi < 2.125) than patients with pT_{1-3} categories (OR = 3.69, χ^2 = 4.37, 95% CI 1.0522–12.957, p = 0.0414); patients with node-positive tumors (pN_{1-2}) 5.0 times more (OR = 5.63, χ^2 = 8.11, 95% CI 1.6478–19.2329, p = 0.0058) than patients with node-negative tumors (pN_0) as well patients with G_{3-4} in comparison with patients with G_{1-2} categories (OR = 5.412, χ^2 = 4.51, 95% CI 1.0172–28.792, p = 0.0477).

It was not obtained any associations between the hypoxia level in adjacent "normal" mucosa of patients with GC and M category. At the same time it was observed that GC patients with III/IV stage have the more probability for surrounding mucosa to be hypoxic than patients with I/II stage (OR = 5.68, χ^2 = 7.215, 95% CI 1.5098–21.4245, p = 0.0102).

Overall, 21 patients with GC (42%) died during follow-up: among them 70% who had hypoxic mucosa (PME/P < 2.125) and 33.3% — when mucosa was satisfactory oxygenated (PME/P > 2.125). Analyzing the survival of these GC patients as to the level of hypoxia in surrounding mucosa it was obtained that mean survival time in the group of patients with hypoxic mucosa (PME/P < 2.125) was significantly shorter compared to that in the group of patients with satisfactory oxygenated mucosa: 17.2 ± 2.2 months and 32.4 ± 3.2 months, respectively. Survival time of patients with GC in accordance with the type of therapy has been detected: operation alone and operation with adjuvant chemotherapy (standard regimes). The following results were obtained: survival time of patients who were operated only was 15.5 ± 2.05 months when uninvolved mucosa was hypoxic (PME/Pi median < 2.125) and 35.4 ± 4.2 when uninvolved mucosa was satisfactory oxygenated (PME/Pi > 2.125); survival time of patients who have been treated with adjuvant chemotherapy was 14.6 ± 3.7 months when uninvolved mucosa was hypoxic and 23.4 ± 1.4 when mucosa was satisfactory oxygenated. It has to be noted that the same associations between survival time of patients treated by different methods and hypoxia level in GC were found.

Analysis of survival of all patients with GC as a function of the PME/Pi ratio (i.e., in dependence upon the level of hypoxia in GC mucosa) has revealed that overall survival of patients with gastric mucosa characterized by PME/Pi < 2.125 (severe and mild hypoxia) was significantly poorer as compared to patients with gastric mucosa characterized by satisfactory oxygenation (PME/Pi > 2.125) (p = 0.0035, Fig. 2).

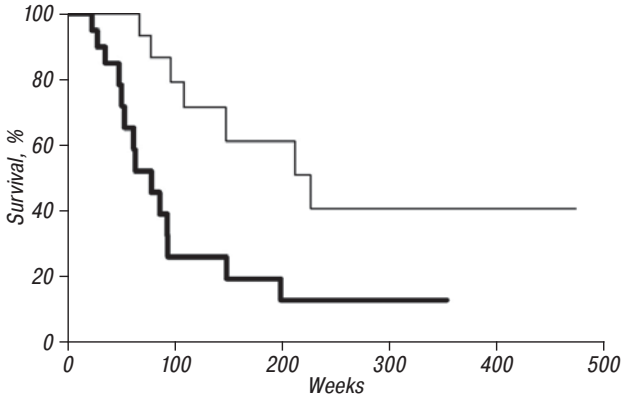

Fig. 2. Kaplan — Meier overall survival curves for GC patients as a function of PME/Pi ratio in adjacent uninvolved gastric mucosa (PME/Pi ratio over median, thin line; PME/Pi below median, bold line; median is 2.125; p < 0.0035)

Histological examination has not found any signs of neoplastic transformation in the surrounding gastric mucosa located at a distance of 8–10 cm from the tumor edge (Fig. 3, 4).

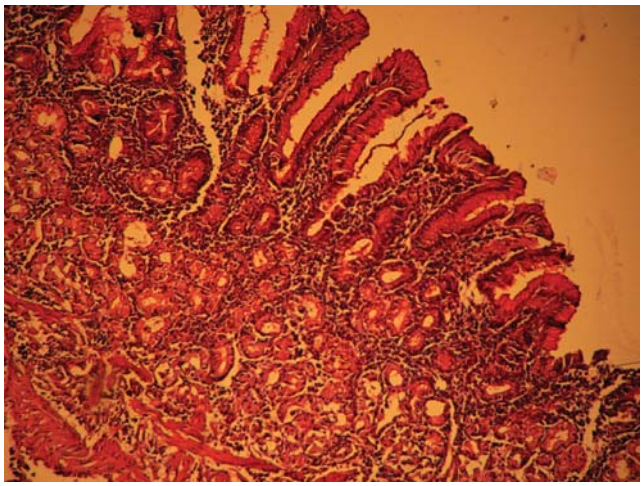

Fig. 3. Gastric mucosa surrounding gastric carcinoma. Gastric mucosa shows no signs of pathological alterations (hematoxylin and eosin; × 100)

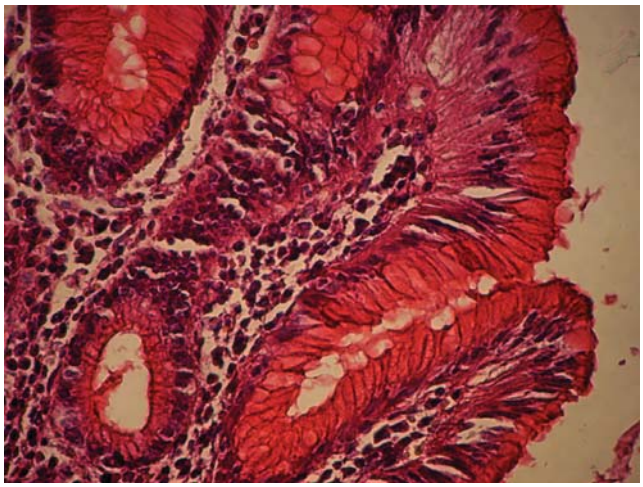

Fig. 4. Gastric mucosa surrounding gastric carcinoma. Surface and pit epithelium, and epithelium lining the glands show no signs of malignant transformation (hematoxylin and eosin; × 400)

At the same time the pathological alterations in the gastric mucosa surrounding tumors were observed. In some cases, mucosa demonstrated the evidence of atrophic gastritis manifested by the smoothness of folds, changes of the depth of pits and height of surface epithelium, reduction of secretory activity of epithelium (Fig. 5).

Moreover, the signs of the erosive gastritis were also observed (Fig. 6). It is notable that deeper located glands were covered by secretory cylindrical epithelium without signs of atypia. It has to be mentioned that Arista-Nasr et al. [29] have also shown that gastric carcinomas are frequently accompanied by atrophy, intestinal metaplasia and dysplasia of the surrounding non-neoplastic gastric mucosa.

Thus, taking into account the results of histological examination it could be supposed that metabolic changes in gastric mucosa surrounding gastric carcinoma, in particular increase of hypoxia level, appear before the morphological alterations in mucosa which can be assessed as neoplastic transformation. Observed metabolic rearrangement in gastric mucosa may be influenced by tumor which affects normal tissues confirming the existence of active tumor-host interaction. It has to be noted that the similar phenomenon, in particular the alterations of cytogenetic indices in surrounding normal tissues by the development of GC, endometrial carcinoma, and tumor from the pigment tissue were shown by Ganina et al. [30–32].

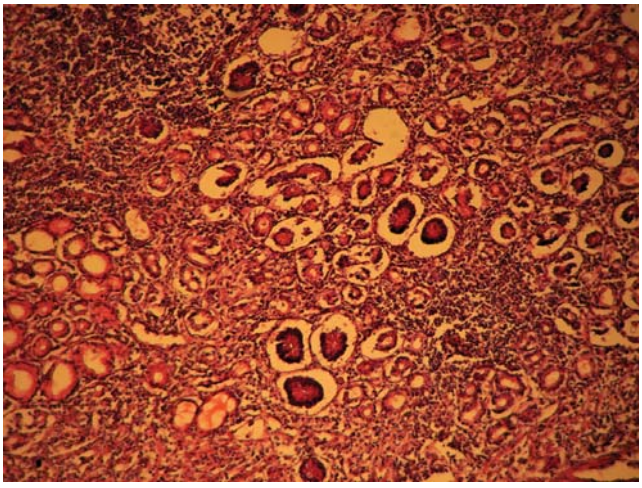

Fig. 5. Atrophy of the gastric mucosa surrounding gastric carcinoma. The decrease of the number of glands and damage of their architectonics and secretory activity are shown (hematoxylin and eosin; × 100)

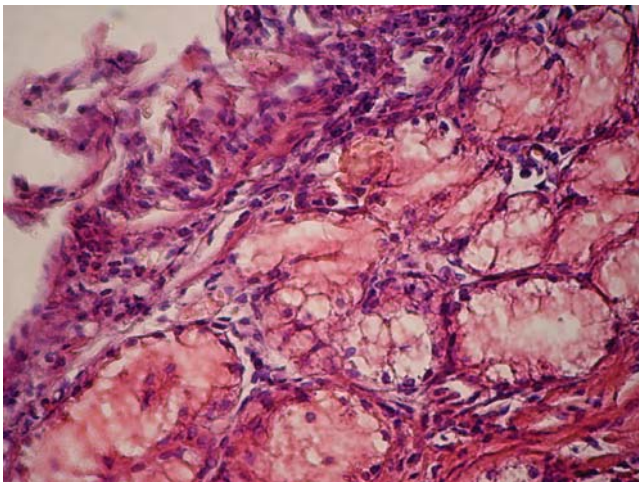

Fig. 6. Erosive gastritis. The disturbance of integrity of surface epithelium of gastric mucosa, extensive inflammatory infiltrate were observed. The glands located deeper are not altered, covered with cylindric epithelium with the signs of secretory activity (hematoxylin and eosin; × 400)

In conclusion, it may be summarized that gastric mucosa uninvolved in tumor process is characterized by hypoxia in 52% of GC patients. Histological examination of mucosa specimens has not found cancer cells or signs of malignization. It has to be noted that severe hypoxia of mucosa was detected in 23% of patients with hypoxic mucosa. It was also found that severe hypoxia in tumor was accompanied with severe hypoxic mucosa in 38.5% of cases. Taking into account obtained data and some data of other authors it may be suggested that biochemical alterations in tissue surrounding tumor node and uninvolved in malignant process may precede morphological ones. This phenomenon needs to be studied more widely, including decoding its mechanisms.

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REFERENCES

1. Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev 2007; **26**: 225–39.

2. Lara PC, Lloret M, Clavo B, ***et al.*** Severe hypoxia induces chemo-resistance in clinical cervical tumors through MVP over-expression. Radiat Oncol 2009; **4**: 29.

3. Nurwidya F, Takahashi F, Minakata K, ***et al***. From tumor hypoxia to cancer progression: the implications of hypoxia-inducible factor-1 expression in cancers. Anat Cell Biol 2012; **45**: 73–8.

4. Walsh JC, Lebedev A, Aten E, ***et al.*** The clinical importance of assessing tumor hypoxia: relationship of tumor hypoxia to prognosis and therapeutic opportunities. Antioxidants Redox Signaling 2014; **21**: 1516–54.

5. Nagata T, Ichikawa D, Komatsu S, ***et al.*** Prognostic impact of microscopic positive margin in gastric cancer patients. J Surg Oncol 2011; **194**: 592–7.

6. Catalano V, Labianca R, Beretta GD, ***et al.*** Gastric cancer. Crit Rev Oncol Hematol 2009; **71**: 127–64.

7. Shin D, Park S-S. Clinical importance and surgical decision-making regarding proximal resection margin for gastric cancer. World J Gastrointestinal Oncol 2013; **5**: 4–11.

8. Saka M, Morita S, Fukagawa T, Katai H. Present and future status of gastric cancer surgery. Jpn J Clin Oncol 2011; **41**: 307–13.

9. Waddell T, Verjeij M, Allum W, ***et al.*** Gastric cancer: ESMO-ESSO-ESTRO clinical practice guidelines for diagnosis, treatment and follow-up. Radiother Oncol 2014; **110**: 189–94.

10. Begg RW. Tumor-host relations. Adv Cancer Res 1958; **5**: 1–54.

11. Kavetsky RE. Tumor and Host. Kiev, Gosmedisdat UkrSSR, 1962. 301 p. (in Russian).

12. Shapot VS. Some biochemical aspects of the relationship between the tumor and the host. Adv Cancer Res 1972; **15**: 253–86.

13. Kerbel RS. Significance of tumor-host interactions in cancer growth and metastases. Cancer Met Rev 1995; **14**: 259–62.

14. McAllister SS, Weinberg RA. Tumor-host interactions: a far-reaching relationship. J Clin Oncol 2010; **28**: 4022–8.

15. Goodwin PJ, Meverhardt JA, Hursting SD. Host factors and cancer outcome. J Clin Oncol 2010; **28**: 4019–21.

16. Sugai T, Habano W, Jiao Y-F, ***et al.*** Molecular analysis of single isolated glands in gastric cancers and their surrounding gastric intestinal metaplastic mucosa. Oncol Rep 2010; **23**: 25–33.

17. Myshunina TM, Kalinichenko OV, Tronko ND. Abnormalities of apoptosis of the thyroid gland cells from extratumoral microfollicular tissue. Exp Oncol 2012; **34**: 112–5.

18. Chekunova NV. Molecular-genetic diagnostics of the alterations in gastric stump mucosa in patients operated on gastric cancer. PhD Thesis, M., 2014 (in Russian).

19. Kim MJ, Kim H-S, Song KS, ***et al.*** Altered expression of Lewis antigen on tissue and erythrocytes in gastric cancer patients. Yonsei Med J 2002; **43**: 427–34.

20. Eberlin LS, Tibshirani RJ, Zhang J, ***et al.*** Molecular assessment of surgical-resection margins of gastric cancer by mass-spectrometric imaging. Proc Natl Acad Sci USA 2014; **111:** 2436–41.

21. Kiryanov IYu, Olshevskaya OP, Sutyagin AG, Alipchenko LA. Study of the oxygen tension and reproductive ability of tumor cells in gastric cancer patients. Voprosy Onkologii 1979; **25**: 91–93 (in Russian).

22. Rofstad EK, DeMuth P, Fenton BM, ***et al***. ^{31}P nuclear magnetic spectroscopy studies of tumor energy metabolism and its relationship to intracapillary oxyhemoglobin saturation status and tumor hypoxia. Cancer Res 1988; **48**: 5440–6.

23. International Union Against Cancer, TNM Classification of Malignant Tumors. Ed. by LH Sobin and C Wittekind, Wiley-Liss, New York, NY, USA, 6th edition, 2002.

24. Bubnovska L, Kovelska A, Boldeskul I, ***et al.*** Prognostic relevance of metabolic ratios in tissue of human gastric cancer evaluated by NMR spectroscopy of perchloric extracts. Radiat Diagn Radiat Ther 2010; **1**: 13–22 (in Ukrainian).

25. Vaupel P, Okunieff P, Kallinowski F, ***et al***. Correlation between ^{31}P-NMR spectroscopy and tissue O_2 tension measurements in a murine fibrosarcoma. Radiat Res 1989; **120**: 477–93**.**

26. Bubnovska L, Kovelska A, Boldeskul I, ***et al.*** Hypoxia level in tissue of gastric cancer and clinical outcome. Onkologiya 2009; **11:** 39–44 (in Ukrainian).

27. Osinsky S, Bubnovskaya L, Ganusevich I, ***et al.*** Hypoxia, tumour-associated macrophages, microvessel density, VEGF and matrix metalloproteinases in human gastric cancer: interaction and impact on survival. Clin Transl Oncol 2011; **13:** 133–8.

28. Bubnovskaya L, Kovelskaya A, Boldeskul I, ***et al.*** Assessment of tumor hypoxia for prognosis: NMR spectroscopy study. In: Tumor hypoxia in the clinical setting. Osinsky S, Friess H, Vaupel P. (eds.), Akademperiodyka, Kyiv, 2011, 203–19.

29. Arista-Nasr J, Jiménez-Rosas F, Uribe-Uribe N, ***et al.*** Pathological disorders of the gastric mucosa surrounding carcinomas and primary lymphomas. Am J Gastroenterol 2001; **96**: 1746–50.

30. Ganina KP, Zinevich AK, Zhero SV. Special method of the investigation under premalignant processes in the stomach. Kiyv, Naukova Dumka, 1988. 158 p. (in Russian).

31. Ganina KP, Polishchuk LZ, Buchinskaya LG. Cytomorphology and cytogenetic of glandular hyperplasia and endometrial carcinoma. Kyiv, Naukova Dumka, 1990. 158 p. (in Russian).