5-ALA/PpIX photodiagnosis of stress-induced gastrointestinal metastatic tumours in laboratorial animals

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ABSTRACT

Combined spectroscopic and biochemical measurements were used to improve diagnostic accuracy and to evaluate gastrointestinal tract (GIT) neoplasia development parameters noninvasively.

Experiments were performed in mongrel male rats divided into 2 groups – control and experimental. To induce gastric cancer, the rats underwent to chronic stress (overpopulation during 9 months) and diet including the daily using of m-toluidine (25 µg/kg weight) in food and water with a solution of sodium nitrite (0.2%) for 9 months. We studied effectiveness of 5-ALA/PpIX fluorescent analysis of gastric carcinoma and biochemical stress-corresponding indices detection for early diagnosis of primary gastric tumours and their metastatic spreading in liver.

Affected by precancerous and cancerous alterations mucosa reveal red fluorescence, related to the accumulation of 5-ALA/PpIX. Liver tissues investigated also presented increase of the red fluorescence, which was used as an indicator for possible pathologic process detection there. The histological examination revealed liver metastases in 67.8 % of the rats with gastric cancer. Biochemical indicators detected malignant alterations presence in GIT, and fluorescent observation addressed the exact area and borders of neoplastic lesions.

5-ALA/PpIX fluorescence detection allow to find and precisely map premalignant and malignant areas of gastric mucosa and liver metastases of stress-induced gastric heterogeneous adenocarcinoma and biochemical evaluation of stress-related compounds increased the efficiency of such diagnosis and reveal information about the dynamics of lesions development. Diagnostic accuracy achieved using fluorescent detection reaches 93% for gastric carcinoma, and 87% for pre-cancerous mucosa alterations observed.

Keywords: gastric cancer, 5-ALA/PpIX, liver metastases, fluorescence mapping

1. INTRODUCTION

Fluorescence spectroscopy is a fast, sensitive and non-invasive tool for a diagnostics of cancerous lesions. It could be applied for in situ detection of tumours during primary endoscopic observations or as add-on measurement modality during microscopic observations of tissue histology slides for their initial or retrospective diagnosis. It could be based on endogenous signals detected from intrinsic fluorophores, such as amino acids, proteins, coenzymes, or due to the emission signal from fluorescent markers selectively accumulated in tumour areas, added to improve contrast between normal and abnormal tissue sites. Exogenous fluorescence detection based on photosensitizers’ compounds, such as porphyrins, phthalocyanines, chlorins, etc., as well known as photodynamic diagnostics (PDD) is often used for primary detection of soft tissues neoplasia, evaluation of lesions’ boundaries, and guidance tool during tumour resection procedures. High contrast is achieved due to significant spectral difference between red emitted exogenous fluorescence and blue-green autofluorescence, observed from the native tissue fluorophores. The fluorescent mapping of the lesions investigated also could be combined with other techniques for better differentiation of tissues’ type and/or stage of the lesions’ growth.1-4
Among oncological diseases, gastric cancer is still one of the most common forms of malignant neoplasms worldwide. Annually, up to 800 thousand new cases of this neoplasia are registered in the world. For example, in Korea, stomach cancer ranks first in incidence (21%) and second in mortality. Metastases occur in more than 80% of cancer patients; six-month survival is 65% in the case of early diagnosis and timely treatment. On average, the highest survival rate for gastric cancer is observed in Japan – about 53%, but in other countries it does not exceed 15-20%. The gastric carcinoma (GC) is characterized by high risk and early appearance of metastases. Therefore, different early diagnostic tools are under investigation for development of fast, highly-sensitive and precise diagnosis of gastric tumours.

From this point of view the optical fluorescence techniques could be very useful for monitoring of gastrointestinal lesions due to their easy combination with endoscopic equipment and high sensitivity to early malignancies detection. The tissue fluorescence signal analysis allows obtaining morphological and biochemical information, revealing differences in normal and abnormal tissue areas before visible to the naked eye changes. However, moderate specificity of this image technique requires special analysis of the spectral data received and/or combination with other diagnostic modalities for diagnostic accuracy improvement.

Biochemical analysis, used for evaluation of the alterations related to tumour development and growth is very appropriate low-invasive and relatively fast technique, which could be used as a secondary additive toll for stomach neoplasia evaluation. Sialic acids and mucins content in the stomach mucosa and internal content would be used as biochemical indicators of pathology development, related to Helicobacter pilori infection, typical for neoplastic gastric lesions.

Combined spectroscopic and biochemical measurements could be used to improve diagnostic accuracy and to evaluate gastrointestinal tract (GIT) neoplasia development parameters noninvasively.

In the current study we combined exogenous fluorescent detection using delta aminolevulinic acid/ protoporphyrin IX (5-ALA/PpIX) accumulated in the tumours and biochemical indicators detection (content of mucins, sialic acids, TBA-active and NO – products) for diagnosis of GC and metastatic spreading in laboratorial rats. The GC model developed in vivo is based on stress-induced adenocarcinoma in the animals due to overpopulation and nitrosamine diet stress factors.

2. METHODS AND MATERIALS

The normal and abnormal stomach mucosa areas were evaluated using photodynamic diagnostics based on application of 5-ALA/PpIX. 5-ALA in a dose of 20 mg/kg (ALASENS, Niopik Inc., Russia) was applied 2 hours before the spectroscopic observation. The stomach and liver organs were investigated in vivo and ex vivo, after decapitation of the animals, using excitation at 405 nm (AFS-405 LED light source, FWHM = 20 nm, P=25 mW, Polironik Ltd., Russia) using microspectrometer USB4000 (OceanOptics Inc., Dunedin, USA) for 1-D measurements. From each animal were detected from 5 to 7 spectra of normal and abnormal tissue areas for each organ and averaged. For 2-D format fluorescence imaging was used a digital microscope system DinoLite (model AM 4013 T-FWV, IDCP B.V., The Netherlands) with excitation at 405 nm (built-in LED sources).

The suspicious areas (with exogenous fluorescence observed) were placed in the groups of “cancerous mucosa” and “metastases” respectively, for the stomach and liver тиссусес. The absence of exogenous fluorescent signal from 5-ALA/PpIX was an indicator for healthy tissue areas in both organs. Histological verification was made for both “normal” and “abnormal” tissue areas, according standard procedure with eosin and hematoxylin staining. The results from histological analysis were used as a “gold standard” for tissue diagnosis.

Biochemical measurements were made on several compounds, related to carcinogenic alterations and indicators in stomach wall and content. Glycoproteins were extracted from the walls of the stomach using an 8M urea solution at room temperature for three days, then centrifuged for 20 minutes at 14,500 rpm. For evaluation of concentration of sialic acids and mucins in the stomach content, the samples were preliminarily centrifuged for 10 minutes at 10,000 rpm, and the obtained supernatant was used for further work. The concentration of sialic acids was determined using the SialoTest kit (SPC Eco-Service, Russia) after partial acid hydrolysis. The concentration of sialic acids was determined using the SialoTest kit (SPC Eco-Service, Russia) after partial acid hydrolysis. The concentration of sialic acids C, mmol / L, was calculated by the formula:

$$C = \frac{E_{\text{exp}} \times C_{\text{cal}}}{E_{\text{cal}}}$$

(1)
Where $E_{\text{exp}}$ – optical density of the test sample;

$C_{\text{cal}}$ – concentration of sialic acids in the calibrator, 2.2 mmol / L;

$E_{\text{cal}}$ – optical density of the calibration sample

The concentration of mucins was evaluated spectrophotometrically using a color reaction with bromophenol blue. The mucins concentration value was determined as the difference in optical density of the reaction products of the experimental sample and the same one after proteins precipitation (20% acetic acid).

The concentration of mucins - $C$ [g/l] calculation was carried out according to the formula:

$$C = C_{\text{in}} - C_{\text{res}}$$  \hspace{1cm} (2)

where $C_{\text{in}}$ – protein concentration in the initial supernatant, g/l, the calculation was carried out similarly to the calculation of $C_{\text{sup}}$;

$C_{\text{res}}$ - protein concentration in the resulting supernatant, g/l, the calculation was carried out according to the formula:

$$C_{\text{res}} = (OD_{\text{test}} \div OD_{\text{st}}) \times 0.25$$  \hspace{1cm} (3)

where $OD_{\text{test}}$ - optical density of the test probe;

$OD_{\text{st}}$ - optical density of the standard probe;

0.25 - protein concentration in a standard solution of albumin, g/l.

Assessment of the intensity of lipid peroxidation (LPO) was carried out by determining the amount of TBA-active products in the serum by a standard procedure. Calculation of the content of TBA - active products was carried out according to the formula:

$$C = \frac{D_{535} - D_{570}}{0.156} \times 0.16$$  \hspace{1cm} (4)

where: $C$ - the content of TBA-active products in the test sample of μmol/l;

$D_{535}$ – optical density of the test sample at 535 nm;

$D_{570}$ – optical density of the test sample at 570 nm;

0.156 – molar extinction coefficient of the malonic aldehyde complex-TBA in L/μmol/cm;

0.16 – Dilution factor of serum.

Nitrogen oxide (NO) was evaluated according Griess reagent protocol. The obtained results of biochemical analysis are processed by statistical methods with the use of Student’s t-test. Differences were considered reliable with a probability of difference exceeding 95%.

Stress-induced gastric carcinoma model is described in details elsewhere. Briefly, laboratorial white mongrel male rats (250-280 g) were used in these studies and treated according the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), protocols that were approved by the Institutional Review Boards of the SSU (Protocol № 7, 07.02.2017).

The rats were housed at 25±2°C, 55% humidity, and 12:12 h light - dark cycle. The animals were divided into control (n=10) and experimental one (n=40). The control group was kept in standard conditions and diet.

To induce gastric cancer in the animals we used an original model for neoplasia development, based on a combined chronic stress application: (1) overpopulation during 9 months and (2) nutrition with exceeding of nitrosamines levels – a diet including the daily usage of m-toluidine (25 µg/kg weight) in food and sodium nitrite solution in the water (0.2%) during 9 months of their life.
3. RESULTS AND DISCUSSION

At present time, it can be considered proven that a prolonged or excessively intense stress can serve as a trigger for the beginning of pathological processes and could cause a wide variety of diseases. The mechanisms of adaptation and protection existing in the body cannot cope with the load induced by stress factors. It was shown that the 9-months diet supplemented with sodium nitrite and aromatic amines leads to a formation of cancerous alterations in ~70% of the animals (28 from 40), which is confirmed by histological studies.

The appearance of additional ligands for interaction with *Helicobacter pylori* can be facilitated by various injuries of gastric mucosa that occur when exposed to external stress factors, such as prolonged intoxication, which can occur with persistent diet disturbance. It is well known that a number of products contain nitrates of acceptable concentrations, which are dangerous in themselves and especially in combination with aromatic amines form carcinogenic products. The effectiveness of the nitrate + amine– model for cancer development is indicated in publications of different research groups as well. In table 1 are presented biochemical indicators for control and experimental animal groups’ stomach mucosa without and with stress-induced neoplasia respectively.

<table>
<thead>
<tr>
<th>Biochemical indicator</th>
<th>Control group</th>
<th>Experimental group</th>
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<tbody>
<tr>
<td>TBA - active products in the blood serum (µmol/l)</td>
<td>0.78 ± 0.08</td>
<td>2.59 ± 0.17</td>
</tr>
<tr>
<td>NO-products content in the serum (µmol/l)</td>
<td>9.72 ± 2.45</td>
<td>4.19 ± 0.68</td>
</tr>
<tr>
<td>Concentration of mucins in the stomach content (g/l)</td>
<td>0.013 ± 0.004</td>
<td>0.02 ± 0.002</td>
</tr>
<tr>
<td>Concentration of mucins in the stomach wall (g/l)</td>
<td>0.041 ± 0.006</td>
<td>0.068 ± 0.009</td>
</tr>
<tr>
<td>Concentration of sialic acids in the stomach content (mmol/l)</td>
<td>0.751 ± 0.021</td>
<td>0.864 ± 0.036</td>
</tr>
<tr>
<td>Concentration of sialic acids in the stomach wall (mmol/l)</td>
<td>0.850 ± 0.046</td>
<td>0.829 ± 0.017</td>
</tr>
</tbody>
</table>

Oxidative and nitrosamine stresses play a key role in the cancerous pathogenesis. The long-term nitrite +m-toluidine intoxication of laboratorial animals leads to a significant accumulation of lipid peroxidation products in serum. The content of TBA-active products increases more than 3 times in the experimental group.

In the control group of animals’ serum, the NO concentration is about twice higher than in the experimental group, after the combined action of nitrates and amines, where the concentration of nitrogen oxide products decreased significantly.

Observed differences of the mucins concentration in the stomach walls of control and experimental groups could be related to vigorous mucin expression into gastric mucosa as a response to food toluidine and nitrite, as a protective reaction to their destroying actions. In general, the mucins should not be detected in the stomach content in normal conditions and their presence and especially their increase in the treated animals is an indicator for destruction of the mucous membrane.

The sialic acids concentration dynamics in the stomach walls and content is a result of mucins destruction and their desialylation in the zones of inflammatory and neoplastic changes, which result sialic acids exit into the stomach (see table 1).
High fluorescent contrast is achieved due to significant spectral difference between red emitted exogenous fluorescence and blue-green autofluorescence, observed from the native tissue fluorophores. 5-ALA/PpIX is proven to be a useful indicator of metastatic lesions of GIT. However, 5-ALA/PpIX is accumulated as well in dysplastic and inflammatory areas of GIT mucosa, which raise the number of false-positive results. Nevertheless, the red fluorescence is observable even by naked eye and could be used for a fluorescent mapping of the suspicious areas of gastric mucosa, as well as in a metastatic liver, see figure 1.

Figure 1. Averaged by tissue type fluorescence spectra of normal stomach mucosa, cancerous gastric mucosa and metastasis in liver, photosensitized by 5-ALA/PpIX. Normalized spectra with respect to maximum at 635 nm (right up) to reveal the broadening of 635 nm maximum in liver tissues observed.

The results obtained from 1-D spectral measurements showed a very good correlation between the fluorescence signals and the histological examination of the lesions investigated. Rapid detection of lesions’ boundaries using the exogenous fluorescence signal was observed. Another important issue is the contrast between pre-cancerous and malignant areas, see figure 2.

Figure 2. Ratio of \( R = I_{635}/I_{560} \) fluorescence intensities at 635 nm and 560 nm respectively, corresponding to the 5-ALA/PpIX exogenous signal from protoporphyrin IX vs. endogenous fluorescence of stomach mucosa in normal mucosa, and in stress-induced precancerous and cancerous gastric lesions in rats. Excitation at 405 nm was used.

The applied 5-ALA/PpIX sensitizer is accumulated in the inflammatory, dysplastic and malignant gastrointestinal tissues, due to its specific metabolism. In spectroscopic regime (1-D) the fluorescence intensities level differences on 635
nm, where is the primary maximum of PpIX, were used as discrimination factor for normal, pre-cancer and cancerous areas of the gastric mucosa.

Fluorescent spectra detected in metastatic areas of liver presented broader emission at the maximum on 635 nm, associated primarily with the PpIX. This widening could be related to the presence of other porphyrins in the liver tissue, such as coproporphyrin and uroporphyrin compounds.\textsuperscript{15-16} see figure 1. Emission intensity of 5-ALA/PpIX is significantly higher in case of malignant alteration of the tissues, in comparison with the precancerous changes observed in other part of the organ investigated and this difference could be used for selection and discrimination of malignant vs. benign and dysplastic stomach mucosa in the current animal model as well, see figure 2.

Absence of exogenous fluorescent signal from 5-ALA/ PpIX was used as an indicator for normal stomach mucosa. All tissue areas addressed spectrally were histologically verified. Diagnostic accuracy (DA) of 93 % was reached in evaluation of cancerous areas and DA was 87% in the case of “pre-cancer/inflammation” group, due to the presence of exogenous fluorophores in several cases valued as “normal” during histological verification. In general, the 5-ALA/PpIX photodiagnosis lead to some higher number of false-positive results than missed false-negative sampling.

4. CONCLUSIONS

Since one of the most important problems of oncology is the late detection of the neoplastic disease, the development of non-invasive diagnostic methods can greatly facilitate the solution of this problem, since they are characterized by high-speed, painlessness, simplicity, economic affordability and the absence of side effects, which allows for a quick preliminary monitoring of large groups of the population.

5-ALA/PpIX fluorescence detection allows us to find and precisely map premalignant and malignant areas of gastric mucosa and liver metastases of stress-induced gastric heterogeneous adenocarcinoma. The biochemical evaluation of stress-related compounds increased the efficiency of such diagnosis and reveal information about the dynamics of lesions development. Diagnostic accuracy achieved using fluorescent detection reaches 93% for gastric carcinoma, and 87% for pre-cancerous mucosa alterations observed. Dimensionless ratio, based on exogenous and endogenous fluorescent signals on 635 nm and 560 nm respectively, obtained from the gastric mucosa emission, in the case of stress-induced model on laboratorial animals (rats) (1) allows to discriminate normal from precancerous and cancerous mucosa and (2) strongly correspond to the data obtained earlier, in the case of photosensitization of human patients with 5-ALA/PpIX for detection of GIT neoplastic alterations observed clinically.

5. ACKNOWLEDGMENTS

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