Application of ellipsometry, spr-technic and raman-spectroscopy into diagnosis of colorectal cancer

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Abstract. Experiments with application of the “Ellips-SPEK" spectral ellipsometric SPR (Surface Plasmon Resonance) analyzer, validating the results on the basis of ProteOn XPR36, revealed the differences in the values of velocity constants of specific interactions of monoclonal highly specific antibodies with tumor M2-pyruvate kinase on the surface of blood serum biochips in patients with various stages colorectal carcinoma (CRC) and location of metastases (AUC 0.89). Scanning ellipsometry enabled to reveal a significant increase of biomolecule surface effective thickness resulting from the specific “antigen-antibody" interaction in the group of patients with extrahepatic and hepatic metastases, as compared to the control group with healthy patients (p<0.0001–0.05). Raman spectroscopy of blood serum demonstrated the differences in peak intensity within the range of 1005–1520 cm\textsuperscript{-1} in the same groups of patients (p<0.0001–0.05) with a 90% predictive accuracy of the method applied at early stages of the disease. The results obtained can be used for development of methods for early diagnosis of CRC, localization of different metastases, relapses and control of the quality of administered therapy.

1. Introduction
Colorectal cancer is known to be the fourth and most frequently diagnosed tumor in the world [1]. Currently the radiographic examination, comprising computer tomography, has become the most common method of preoperative evaluation of stages of the disease in patients with CRC [2]. Histological analysis of surgical samples often results in change of the initially diagnosed stage. In particular, surgery more accurately defines depth of tumorous invasion and involvement of lymphatic nodes. However, modern methods, used to determine a pathology stage, sometimes lead to omission of occult metastases. In postoperative period patients undergo thorough dynamic observation with the purpose of revealing the local or remote relapses as early detection leads to earlier treatment before the tumor expands [3]. Guidelines of the American Society of Clinical Oncology [4] recommend application of annual computer tomography in patients with the administered surgical alternatives as well as
evaluation of a carcino-embryonic antigen (CEA) in blood serum which is to be carried out every three months in patients with II and III stages of the disease for no less than 3 years in case such patients are to undergo surgery or chemotherapy due to metastatic disease [5]. This intense postoperative monitoring aims at detection of resectable metastases. For instance, local metastases into the liver, in case of absence of the extrahepatic ones, can be resected [6, 7].

Thus, biomarkers facilitating determination of occult metastasis prior to or after surgery will improve determination of the CRC stage, which potentially influences upon the therapeutic approach.

The aim of this study is to determine the possibilities of detection of blood serum antigens in patients with CRC of various location of metastases to highly specific monoclonal antibodies, i.e. the tumor M2-PK by means of spectral ellipsometry close to observation of surface plasmon resonance in order to improve sensitivity rate of determination of biomolecules in ultra-low concentrations; to reveal the differences in Raman-spectra of blood serum of the above groups of patients.

2. Materials and methods

Groups of patients. 49 patients (mean age - 52±9 years; 22 females, 27 males) with CRC of various localization i.e. transverse colon (n=3), descending parts of large intestine (n=3), sigmoid colon (n=9), recto-sigmoid part (n=6), rectum (n=22), numerous primary tumors of large intestine (n=6) have been included into the study. Histological studies revealed adenocarcinoma of various stage of differentiation in all patients under observation. Diagnosis was verified at the oncological hospital based on the complex of clinical and instrumental studies (including ultrasonography, KT, multi-spiral computer tomography, colonoscopy, fibrogastroduodenoscopy, histologic examination).

The patients were divided into three groups depending on localization of metastases, i.e. Group 1 comprised 15 patients with local regional CRC (7 subjects – stage II and 8 patients – stage III of the disease); Group 2 consisted of 18 patients with hepatic metastases only (12 patients had singular metastases, the rest – the multiple ones with the diameter of the nodes ranging from 16 to 92 mm); Group 3 comprised 16 patients with extrahepatic metastases (including the ones into supraclavicular lymphatic nodes, lungs, bones and brain). Thus, patients of Group 1 and Group 2 had the fourth stage of CRC. 3 patients (20%) from Group 1, 6 subjects (33%) from Group 2 and 11 (69%) from Group 3 have undergone the adjuvant polychemotherapy for 3 months.

Control group comprised 19 relatively healthy subjects (mean age - 53±8 years) without oncologic pathology and pathology of inner organs.

Samples preparation. Blood serum of patients under study, obtained by means of centrifugation (2000 rpm for 20 min.) of whole blood taken at fasting stage, has been examined.

The method of detection of biological macromolecules of small concentration by means of spectral ellipsometry under the conditions of observing the surface plasmon resonance has been developed and tested for application in spectral ellipsometric SPR-analyzer «Ellips-SPEK» [8]. Within the course of study we implemented optimization of ways of surface treatment in order to increase selectiveness to binding with certain macromolecules and surface adsorption ability.

A one-hour activation of silicon plate surface with carbonyl diimidazole was performed, following the stage of hydroxylation with sulfochromic mixture or «piranha» (i.e. the mixture of sulfuric acid with peroxide at ratio of 3:1). After that the plate was rinsed with H2O (distilled water; 5×10 ml), ethyl alcohol (3×10 ml), acetonitrile (1×10 ml). 200 mg of weighed sample of carbonyl diimidazole was dissolved in 20ml of acetonitrile. The silicon plate was submerged into the obtained solution, stirred slowly and stored for 12 hours at room temperature. The reaction being over, the plate was sequentially rinsed with acetonitrile (3×30 ml), acetone (1×10 ml) and dried under vacuum conditions.

Immobilization of protein probes. 0.3 mcI dots of buffer solution of protein molecules in concentration of 0.01–100 µg/ml (ranged from 0.3 pg to 360 ng) were applied to the activated surface, parent antibodies were diluted 10-fold, incubated for 12 hours at room temperature under the conditions of saturation with water vapors. Later on, the unconjugated biologic material was repeatedly rinsed from the surface with a 1x phosphate buffer (137 mM of NaCl, 2.7 mM of KCl, 10 mM of Na2HPO4, 1.76 mM of KH2PO4; pH 7.4), containing 0.1% of Tween-20.
**SPR-technics and Ellipsometry.** The Anti-PKM2 antibody [EPR10138(B)], (Abcam RabMab, USA) highly specific monoclonal rabbit antibodies were immobilized onto the surface for further study by means of spectral ellipsometry under the conditions of observing the surface plasmon resonance.

Surface response of silicon plates (initially covered with monoclonal antibodies) to Tumor M2-PK in reaction of interaction of blood serum of patients with CRC and healthy subjects, was studied applying the «Microscan» (ISP SB RAS) scanning HD ellipsometer [9]. Ellipsometric angles \( \Psi \) and \( \Delta \) were read in central areas of the plates (76 mm in diameter) of \( 15 \times 15 \) mm\(^2\) in size, X and Y step size of 0.1 mm. The tilt angle of the light to the sample equaled 60\(^\circ\).

**The-ProteOn XPR36 (BioRad)** surface plasmon resonance-based device was used for parallel detection of antigens to monoclonal antibodies to tumor

PKM2 in blood serum taken from healthy patients and patients suffering from CRC. Antibodies to human Tumor M2-PK were covalently bound with the surface of the GLC sensor chip. 0.2 mcg of mouse monoclonal antibodies to human Tumor M2-PK protein was applied to 230 mcl of acetate buffer (pH 5.0). Detection of Tumor M2-PK protein was made in PBST buffer (137 mM of NaCl, 2.7 mM of KCl, 10 mM of Na\(_2\)HPO\(_4\), 2 mM of KH\(_2\)PO\(_4\), 0.1% Tween-20, pH 7.4).

Initially, binding of serum, diluted 10, 50, 250 and 6250-fold in PBST, was tested using several samples taken from the healthy subjects and the patients with CRC. Concentrations of 1/50 and 1/250 were selected for screening, which would secure the clearest difference between the samples under evaluation. 200 mcl of diluted serum was run through the canal with conjugated antibody for 400 sec at 30 mcl/min. Dissociation of complex antigen-antibody was carried out in 10 mM buffer of glycine-HCl (pH 2.0). The curves obtained demonstrate that protein concentration to Tumor M2-PK in serum is proportional to the binding level.

**Raman spectroscopy.** Spectra of combinational scattering of light of liquid blood serum of patients were recorded by means of a spectrometer with the triple T64000 (Horiba Jobin Yvon) monochromator at room temperature. Ar\(^+\) laser line with a 514.5 nm wave length was used for excitation; spectral resolution equaled 1.5 cm\(^{-1}\). A silicon photo sensor array, cooled down by liquid nitrogen, served as a detector. A supplement for microscopic studies of Raman scattering was used. The power of laser beam, reaching the sample, comprised 2–3 megawatt (a spot size equaled 5-6 mcm).

### 3. Results and discussion

**Tumor markers** are known to be the substances, which at certain concentration in the organism signal about the presence of neoplasm [10]. Pyruvate kinase is one of such substances influencing upon the phosphoenolpyruvate metabolism. In normal cells it is present in several forms, i.e. L, R, M1, M2 that are located in kidneys and liver, erythrocytes, brain and muscles, lungs, correspondingly. They are highly specific. While tumor is growing the number of standard isoenzymes decreases and Tumor M2-PK appears. Primary isoenzyme, having four subunits in its structure, transforms into a low activity dimer, specific for tumorous cells [11].

In clinical practice, they make feces analysis aimed to reveal the presence of tumorous M2-pyruvate kinase, determined by enzyme (sensitivity \( 10^{-9} \)) in the following cases [12]:

- diagnosis of adenocarcinoma, recurrent tumors in postoperative period and dysplastic polyps;
- evaluate of the stage of adenocarcinoma;
- localization of bleeding tumors;
- routine examination of patients of risk group.

This study involved blood serum of the patients under study, as according to [13], studies of tumor pyruvate kinase of feces are not diagnostically efficient enough. Figure 1 demonstrates kinetics of interaction between highly specific monoclonal antibodies and Tumor M2-PK from blood serums of the patients from study groups defined by spectral ellipsometry in proximity to observation of plasmon resonance.
Figure 1. Sensograms of binding and decomposition of complexes upon interaction of antigens of blood serum and highly specific monoclonal antibodies to Tumor M2-PK in patients with CRC and control patients. Blood serum dilution rate equaled 1:250.

Figure 2. ROC-curve of serum tumor M2-PK levels in differentiating the patients suffering from CRC with metastases.

The mean level of serum Tu M2-PK, found in control, equaled 11.6 ± 4.5 RU/mL with no relation to sex (p = 0.51) and age (p = 0.61), which correlates with the findings of a number of studies [14, 15]. The mean values of serum level of Tu M2-PK (RU/mL) in patients with local regional CRC (Group 1) comprised 77.4±5.8; the level of 192.5±12.8 was registered in patients with CRC and liver metastases (Group 2) and 284.2±1466 – in the group of patients with extrahepatic metastases (Group 3) (p<0.0001-0.05), being approximately 6-23 times different as compared to the similar one in the controls. Tonus C., Neupert G. (2006) also revealed correlations of fecal Tu M2-PK level with the stage of the disease in compliance with the TNM classification and Duke's staging [16].

The defined low levels of serum Tu M2-PK in the control group and no apparent variations of readings in relation to sex and age can be explained by the fact that this enzyme is bound to tumor metabolism [11]. The obtained associations of serum level of Tu M2-PK with TNM classification of malignant tumors and Duke's staging of CRC have been proven by clinical studies by Mazurek S. et al.(2005), Christofk H.R. et al.(2008) [17, 18].

Diagnostic accuracy of detection of serum level of Tu M2-PK for determination of the difference between the cases of CRC and control comprised 100% which is consistent with the findings by Meng W. et al. (2012) [15] and is considerably higher than in case of applying colonoscopy, examination of feces for occult blood and detection of fecal tumor M2 pyruvate kinase [19, 20]. In case when the cut-off level of serum Tu M2-PK comprised 11.6 ± 4.5 RU/mL, none of the CRC cases were missed and 43.3% of colonoscopic examination could be avoided. Diagnostic sensitivity for all cases of colorectal lesions increased upon the reduction of cut-off level of serum Tu M2-PK. The results obtained speak in favor of viability of the proposed early stage of CRC diagnosis.

At the same time, the diagnostic value of the method was recognized in detection of various localizations of metastases. Carrying out the ROC-analysis for detection of metastases in patients with CRC, the value of AUC for serum Tu M2-PK comprised 0.89 (0.84, 0.94) (95% confidence interval) (Figure 2). The findings, obtained by applying a spectral ellipsometric SPR analyzer «Ellips-SPEK», correlated with those obtained using the ProteOn XPR36 (BioRad, USA) and demonstrated the same sensitivity and accuracy rate as the one achieved using similar foreign equipment [8]. Thus, the diagnostic method of a specific «antigen-antibody» interaction, based on the surface plasmon resonance phenomenon, enabled to increase the accuracy of detection of biomolecules up to ~10^{-11}–10^{-12} M/ml.
The scanning ellipsometry technique demonstrated a significant increase of bimolecular surface effective thickness resulting from a specific interaction «antigen-antibody», ranging from control (Figure 3a) to the group of patients with hepatic metastases (Figure 3d) (p<0.0001–0.05). The peak areas (1005–1520 cm⁻¹) of Raman spectra appeared to be significantly lower in patients with CRC as compared to the controls (p<0.0001–0.05) (Table 1, Figure 4), correlating with the process stage (r=-0.68, p<0.001) and the presence of metastases (r=-0.57, p<0.003). The accuracy rate of the above technique comprised 85%, 90%, 94%, 96% of serum of control and the patients with local regional CRC as well as CRC with only hepatic metastases and CRC with extrahepatic metastasis, correspondingly.

Various levels of peak intensity of Raman scattering spectra, to a certain extent, reflects the existing differences in integral metabolomic profiles of blood serum in patients with CRC [21].

The results, confirming the fact that changes in metabolomic profile tend to depend upon the tumor localization, seem to be astonishing. The question is, whether the changes in circulating metabolites will reflect the differences in biology of tumor or changes in the host reaction to tumor or a combination of both mechanisms. The host reaction to tumor can change along with the spread of metastases [22], as a metastatic disease is biologically deferent from cancer, not expanding outside the original tissues, and many other aggressive tumors can provoke a more (or less) abrupt reaction of the host [23]. The host reaction can also differ because of the local effect of the tumor. For instance, a tumor can cause numerous paracrine effects in a tumor microenvironment as well as metabolic or inflammatory reaction of surrounding normal tissues can differ in case of localization of metastases in intestine, liver and other locations [24].
Table 1. Peak areas of Raman scattering in patients suffering from CRC with various localization of metastases and control (M±m).

| Peak location, cm⁻¹ | Peak area, arb. units. |
|---------------------|------------------------|
|                     | Control (n=19)          | Group 1 with coloregional CRC (T2-3) (n=15) | Group with CRC and hepatic metastases (T4) (n=18) | Group with CRC and extrahepatic metastases (T4) (n=16) |
| 1005                | 990±                   | 557±       | 387±       | 129 ±    |
| 1157                | 2570±                  | 2004±      | 908±       | 712 ±    |
| 1520                | 3258 ±                 | 2404 ±     | 714 ±      | 474 ±    |
|                     | 190                    | 162 **     | 135 ***    | 108 ***  |

Notes: M – mean value, m – standard error of a mean value, *
* – statistical significance (p) of difference as compared to control (* - p<0.05, ** - p<0.02, *** - p<0.0001);
^ – statistical significance (p) of difference from Group 1 (^ - p<0.05, ^^ - p<0.02, ^^^ - p<0.0001);
$ – statistical significance (p) of difference from Group 2 ($ - p<0.05, $$ - p<0.01).

4. Conclusion
Studies with application of the «Ellips-SPEK» (ISP SB RAS, Russia) spectral ellipsometric SPR (Surface Plasmon Resonance) analyzer and validation of the results with ProteOn XPR36 (BioRad, USA) revealed distinction in velocity constants of specific interactions of monoclonal highly specific antibodies to the tumor M2-pyruvate kinase with blood serum tumor M2-PK on the surface of biochips in patients with colorectal carcinoma of various stages and location of metastases (AUC 0.89).

Application of scanning ellipsometry enabled to define a significant increase of biomolecule surface effective thickness, resulting from the specific «antigen-antibody» interaction in the group of patients with extrahepatic and hepatic metastases as compared to the control group of healthy patients (p<0.0001–0.05).

Raman spectroscopy of blood serum revealed the difference in peak intensity within the range of 1005-1520 cm⁻¹ in the same groups of patients (p<0.0001-0.05) with 90% predictive accuracy of the method at early stages of the disease.

The results obtained are perspective for development of methods for early stage diagnosis of colorectal cancer, detection of differently located metastases, relapses and control of administered therapy. Another alternative method for registering colorectal cancer based on dielectrophoresis is discussed in [25].

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References
[1] Jemal A. et al 2009 CA Cancer J. Clin. 59(4) 225–249 doi: 10.3322/caac.20006.
[2] Cai S.R. et al 2011 Cancer Prev. Res. (Phila) 4 1572-1579 doi: 10.1158/1940-6207.CAPR-10-0377
[3] Pawlik T.M. et al 2005 Ann. Surg. 241 715–724 doi: 10.1097/01.sla.0000160703.75808.7d
[4] Meyerhardt J.A. et al 2013 *J. Clin. Oncol.* **31**(35) 4465–4470 doi: 10.1200/JCO.2013.50.7442
[5] Bathe O.F. et al 2009 *BMC Cancer* **9** 156–163 doi: 10.1186/1471-2407-9-156
[6] Pawlik T.M. 2008 *Oncologist* **13** 51–64 doi: 10.1634/theoncologist.2007-0142
[7] Shah S.A. et al 2007 *J. Am. Coll. Surg.* **205** 676–683 doi: 10.1016/j.jamcollsurg.2007.06.283
[8] Rykhlitskiy S.V. et al 2010 *Pribory i tekhnika eksperimenta* **2** 1–2
[9] Spesivtsev E.V. et al 1997 *Avtometria* **1** 100–105
[10] Wild N. et al 2010 *Clin. Cancer Res.* **16** 6111–6121 doi: 10.1158/1078-0432.CCR-10-0119
[11] Kaura B. et al 2004 *J. Obstet. Gynaecol. Res.* **30** 193–196 doi: 10.1111/j.1447-0756.2004.00187.x
[12] Ewald N. et al 2007 *Anticancer Res.* **27** 1949–1952
[13] Shastri Y.M., Stein J.M. 2008. *Br. J. Cancer* **99** 1366–1374 doi: 10.1038/sj.bjc.6604679
[14] Demir A.S. et al 2013 *Turk. J. Gastroenterol.* **24**(1) 36–42 doi: 10.4318/tjg.2013.0607
[15] Meng W. et al 2012 *World J. Gastrointest. Oncol.* **4**(6) 145–151 doi: 10.4251/wjgo.v4.i6.145.
[16] Tonus C. et al 2006 *World J. Gastroenterol.* **12**(43) 7007–7011 doi: 10.3748/wjg.v12.i43.7007
[17] Mazurek S. et al 2005 *Semin. Cancer Biol.* **15** 300–308 doi: 10.1016/j.semcancer.2005.04.009
[18] Christofk H.R. et al 2008 *Nature* **452** 230–233 doi: 10.1038/nature06734
[19] Walkowiak J. et al 2005 *Scand. J. Gastroenterol.* **40** 1493–1494 doi: 10.1080/00365520500319112
[20] Helm J. et al 2003 *Cancer Control* **10** 193–204
[21] Feng S. et al 2015 *Biomedical Optics Express* **6**(9) 3494-3502 doi: 10.1364/BOE.6.003494
[22] Zhou Q. et al 2010 *J. Transl. Med.* **8** 13–19 doi: 10.1186/1479-5876-8-13
[23] Giusca S. E. et al 2010 *Rom J. Morphol. Embryol.* **51** 73–79
[24] Meyerhardt J.A. 2015 *J. Clin. Oncol.* **33**(16) 1717–1720 doi: 10.1200/JCO.2015.60.8661
[25] Kruchinina M. V. et al 2019 *Proc. SPIE*, in press.