# Microarray-based Simultaneous Quantitation of Six Serum Tumor Markers Improves Cancer Diagnostics

Tatiana P. Ryabykh[1], Tatiana V. Osipova[*,1], Zinaida A. Sokolova[1] and Nikolay B. Paklin[2]

[1]*N.N. Blokhin Russian Cancer Research Center, 115478, Moscow, Kashirskoye shosse, 24, Russia*

[2]*S.Yesenin Ryazan State University, 390000, Ryazan, Svoboda str., 46, Russia*

**Abstract:** Relative low sensitivity and specificity of the known protein tumor markers limit their application in cancer diagnosis and screening. Simultaneous use of their combination can improve diagnostic efficacy. The aim of the study was to estimate diagnostic characteristics of the hydrogel microarray-based test system "TM-Biochip" developed in EIMB RAS for a simultaneous quantitation of six tumor markers: alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), prostate-specific antigen (PSA total and free), neuron-specific enolase (NSE) and human chorionic gonadotropin (hCG) and to compare them with the diagnostic characteristics of systems used for estimating one tumor marker. Diagnostic performance of "TM-Biochip" system was evaluated using 170 serum samples from clinically confirmed cancer patients and control group patients. To evaluate the diagnostic efficiency of the test system we used linear regression and multiple logistic regression (LR), receiver operating characteristic (ROC) curve analysis and area under ROC curves (AUC). It was demonstrated that this new diagnostic system allows assessing the level of each marker and gives data comparable with the results obtained by measurement of each individual marker in a respective conventional ELISA system. ROC analysis and evaluation of diagnostic sensitivity and specificity of the system demonstrated that the new system did better than each of individual markers when classified: *(1)* cancer patients vs non-cancer patients; *(2)* patients with colorectal cancer (CRC) vs the other patients: patients with the other cancers and non-cancer patients; *(3)* patients with CRC vs patients with the other malignant tumors and *(4)* patients with prostate cancer (PC) vs patients with benign prostatic hyperplasia (BPH). In classification of the patients with PC and the patients with BPH AUC for the combination of six markers (LR6) was significantly higher than AUCs for each of individual markers, including the greatest AUC for PSAt (0.894 vs 0.771, p=0.032). On addition of such parameter as "age of patient" to the combination of six markers (LR7), AUC raised till 0.930 (p=0.01). Specificity at 90% sensitivity for PSAt, LR6 and LR7 accounted for 20 % (5.7-43.7), 60% (36.1-80.9) and 80.6 % (55.5-95.0), respectively. These results showed that "TM-Biochip" system is more efficient in revealing of cancer patients.

**Keywords:** Cancer diagnostics, logistic regression, protein microarrays, ROC analysis, tumor markers.

## INTRODUCTION

Protein biomarkers (tumor-associated antigens) provide a powerful and dynamic approach to studying the spectrum of malignancies. Potential uses of tumor markers are the following: (1) screening a healthy population or a high risk population for the presence of cancer; (2) making a diagnosis of cancer or a specific type of cancer; (3) determining the prognosis in a patient; (4) monitoring responses to therapy, etc. [1, 2].

Such tumor markers as alpha-fetoprotein (AFP), the main marker of hepatocellular carcinoma; carcino-embrionic antigen (CEA), the main marker of colorectal cancer (CRC); prostate-specific antigen (PSA), the main marker of prostatic cancer (PC); human chorionic gonadotropin (hCG), the main marker of trophoblastic tumors and neuron-specific enolase (NSE), the main marker of neuroblastoma, are widely used in oncological practice. The main use of these markers is the monitoring the effectiveness of cancer therapy. Currently, however, no individual marker (with rare exceptions) has been established as a practical cancer screening tool or is sufficiently accurate to be useful as diagnostic test, especially for identifying patients with small surgical resectable cancers. The reason for this is the relative low sensitivity and specificity of the available tests since increase in the level of these markers is also observed in patients with benign tumors [1, 2].

Combining assays of several tumor markers provides more precise diagnosis of as compared to single tumor marker [3-5]. So the future of cancer diagnostics may be based on multiparametric, miniaturized analysis of serum biomarkers with interpretation of data by new bioinformatics tools [2]. Microarray technology, which is being actively developed in recent years [6] allows simultaneous quantitation of multiple protein tumor markers [7, 8] in a minimum serum volume.

Gel-based microarrays with antibodies immobilized within highly hydrophilic gel-drops have an advantage of generating higher signal as compared to standard ELISA due to use of 3-D media which allows to employ less expensive equipment for reading [9]. The use of

*Address correspondence to this author at the N.N. Blokhin Russian Cancer Research Center, 115478, Moscow, Kashirskoye shosse, 24, Russia;
Tel: +7(499)3241065; E-mail: tvosipova2011@yandex.ru

E-ISSN: 2311-8792/14 

diagnostic systems based on this technology may help to improve cancer diagnostics.

We found earlier [10], that hydrogel microarray-based test system for simultaneous quantitation of total and free PSA forms (PSAt and PSAf) has analytic [11] and diagnostic [12] characteristics comparable with those of ELISA systems. A diagnostic system for simultaneous determination of six tumor markers was developed [13, 14]. It was shown that, by sensitivity and reproducibility of results, this system was comparable with the standard ELISA systems [14].

The aim of the study was to estimate diagnostic characteristics of the hydrogel microarray-based test system for a simultaneous quantitation of six tumor markers: AFP, CEA, PSAt, PSAf, NSE and hCG and to compare them with the diagnostic characteristics of systems used for estimating one tumor marker.

## MATERIALS AND METHODS

The study comprised 170 patients: 108 cancer patients with histologically proven diagnosis and 62 patients with no cancer. The first group involved patients with PC (n=35), CRC (n=43), trophoblastic disease (n=7), neuroblastoma (n=6), germ cell tumors (n=4), ovarian cancer (n=3), liver cancer (n=3), lymphoma (n=2), carcinoid (n=2), and other cancer types (n=3). The second (control) group included patients with benign prostatic hyperplasia (BPH) (n=21), intestinal polyposis (n=5), chronic colitis (n=4), chronic pancreatitis (n=9), liver cirrhosis (n=8), diverticulosis of the intestine (n=2), chronic rheumatic disease (n=2), and others (n=4), as well as healthy donors (n=7). Age information was obtained for 163 patients. Mean age of cancer patients was 50.5±21.6; mean age of the control group was 63.3±16.5.

Blood samples of the cancer patients were collected at the N.N.Blokhin Cancer Research Center, RAMS (Moscow, Russia), blood samples of the patients with benign tumors and non-tumor diseases - at Clinical Hospital № 60 (Moscow, Russia), blood samples of healthy donors - at Hematological Scientific Centre, RAMS (Moscow, Russia) and N.V. Sklifosovsky Scientific Research Institute of First Aid (Moscow, Russia)

Sera were obtained by blood centrifugation at 1500g for 15 min, harvested and snap-frozen. The samples were stored at -80°C until analysis.

Diagnostic system “TM-Biochip” for simultaneous quantitation of six tumor markers: PSAt, PSAf, AFP, CEA, NSE and hCG, developed in EIMB RAS [15], was used in the study. “TM-Biochip” detection kit approved for clinical application by the national regulatory agency, the Federal Agency of Supervision of Health Care and Social Development of Russian Federation (Registration Certification № ФСР 2008/03415) was purchased in the Ltd. “EIMB-Biochip”, Moscow, Russia. Analytical performance characteristics of the diagnostic test system claimed by the manufacturer were the following: (1) limit of detection for PSAt 0.3 ng/ml; PSAf 0.2 ng/ml; AFP 1.0 ng/ml; CEA 0.5 ng/ml; hCG 3.0 IU/L; NSE 2.0 ng/ml, (2) reproducibility within an assay (intra-assay precision) and between assays (inter-assay precision) was 8%. Recoveries of the antigens were in the range 90 - 110%. Each biochip included a microarray of 3-D gel elements (hemispherical drop) on a hydrophobic surface of glass slide. Monoclonal antibodies specific to appropriate antigens as well as the antigens were immobilized in gel elements [14].

The antigen concentrations in samples were determined by the single-stage variant of sandwich immunoassay with fluorescent detection. All assays were performed according to the manufacturer's instruction and with the reagents (diluents, calibrators, blocking reagents and detecting-antibody mixture) included with their kits. A mixture of fluorescently-labeled secondary mAbs was added to analyzed samples, calibration samples or control serum in concentrations recommended by manufacturer in ration of 1:5 and mixed thoroughly. The samples, calibrators and control sera were applied to biochips, and the biochips were incubated for 17 h at 37°C in humidity chamber. After incubation and washing biochips were dried, and theirs fluorescence images were registered by use of a Fluorescent Biochip Analyzer (EIMB, Russia) with laser excitation, using 650/750-nm filters (excitation/recording). To calculate the concentration of all tumor markers in the sample “ImaGelAssay” software was used (EIMB, Russia).

Conventional ELISAs were used as referent systems. Serum samples being tested by microarray system were analyzed also by ELISA diagnostic kits, designed for determination of single tumor marker. ELISAs were performed using commercially available kits for detection of PSAt, PSAf, AFP, CEA and NSE from Fujirebio Diagnostics (Sweden), and for detection of hCG from DRG Diagnostics (Germany) and employed the assay procedure recommended by the manufacturer.

Measurement of serum antigen concentrations in patients from different groups under study was done by

the use of calibration curves for each of six tumor markers (the dependence of fluorescence intensities on concentration of each marker in solution). We hypothesized that combining six tumor markers via multivariate logistic regression would improve diagnostic efficacy. Microarray results were correlated to conventional ELISA using linear regression. The ability of a new system to differentiate groups of patients (for example, cancer patients and noncancer patients, etc.) was considered. When evaluating the diagnostic efficiency of the test system, we used linear regression, multiple logistic regression, and receiver operating characteristic (ROC) curve analysis [16]. The efficiency of diagnostic systems was compared by measuring the area under ROC curves, which ranged from 0.5 (the lowest diagnostic efficiency) to 1 (the maximum diagnostic efficiency). Statistical calculations were performed using the MedCalc statistical software for Windows, version 9.3.5.

## RESULTS

At first, we compared the data obtained using the new system and the results obtained for the same samples using the commonly used well-established ELISA systems. Serum samples with different levels of each tumor marker were taken into the investigation: 149 for AFP; 146 for PSAt; 148 for PSAf; 155 for CEA, 126 for hCG and 139 for NSE. Linear regression analysis revealed a high degree of correlation between the levels of tumor markers measured in the two types of systems (Figure **1**). Correlation coefficients comprised 0.92 for AFP, 0.97 for CEA, 0.88 for PSAt, 0.96 for PSAf, 0.94 for hCG and 0.85 for NSE ($p<0.0001$ everywhere); $R^2$ comprised 0.85; 0.95; 0.77; 0.92; 0.89 and 0.72, respectively. The data obtained for each antigen when simultaneous evaluating by microarray system is consistent with the results derived when each biomarker is measured by appropriate ELISA system. The data allowed us to pass to the estimation of diagnostic efficiency of the new system.

### Classification into Cancer Patients and Noncancer Patients

One hundred seventy serum samples (108 from cancer patients and 62 - from patients with no cancer (control group)) were taken into the investigation. We assessed the ability of the new microarray system for quantitation of six tumor markers to discriminate between the patients with malignant neoplasms and the patients of the control group. For each individual marker and for combinations of tumor markers ROC curves showing the dependence of the proportion of true positive cases (diagnostic test sensitivity) on the proportion of false-positive cases (100% minus specifi-

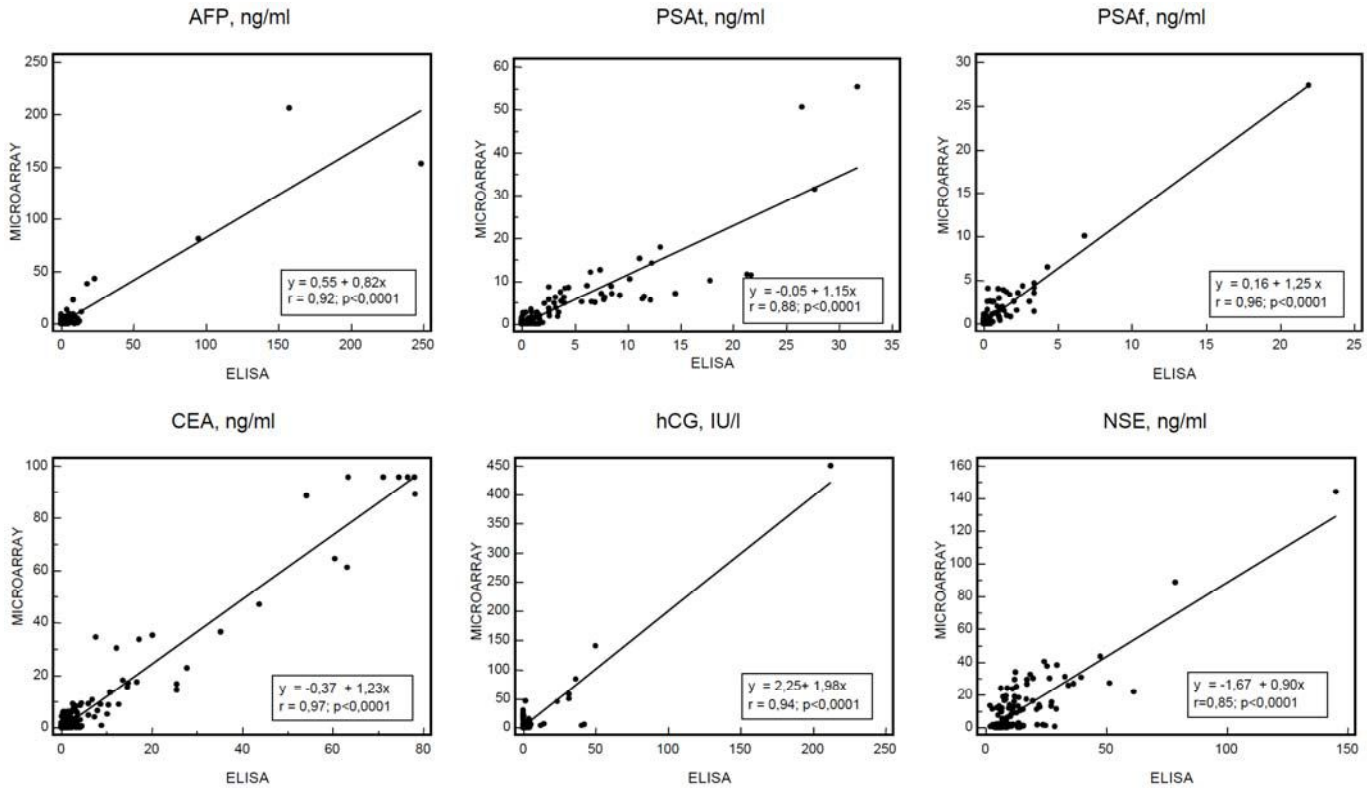


**Figure 1:** Regression lines for the levels of tumor markers measured in the same blood serum samples using the microchip format system and respective ELISA systems.

city) were constructed. The specificity is understood as the proportion of truly negative cases according to the test among all the healthy subjects at different threshold values.

Figure **2** shows the ROC curves for four individual tumor markers, which were measured on a microchip (AFP, CEA, PSAt and PSAf) and the curve obtained by using logistic regression for the combination of six tumor markers (LR6). It can be seen that the LR6 curve is located much closer to the upper left corner of the plot, which corresponds to 100% sensitivity and 100% specificity, as compared with the ROC curves for each individual marker. The ROC curve for the combination of the six tumor markers plus the 'patient's age' index (LR7) is even closer to the upper left corner of the chart (Figure **2**). The values of the area under the ROC curves (AUC) for all seven parameters are summarized in the Table **1**. As seen from the Table, AUC for each of the six tumor markers are in the range 0.518-0.605, indicating insufficient diagnostic efficiency of each individual marker. AUC corresponding to the combination of the six tumor markers (LR6) is 0.758, which significantly differs from the areas corresponding to each individual marker. The area under the LR7 curve is 0.865, which testifies to a very good diagnostic efficiency of the system. This value is significantly ($p <$ 0.001) higher than the area for each of the seven individual indicators (Table **1**). The significant difference still persists when experimental and control groups of patients have the same age (mean age 59.1±12.8 years and 59.7±16.3 years, respectively, n=129 patients) or when both groups are selected by sex and age by case-control method (44 pairs, mean age - 60.3 years).

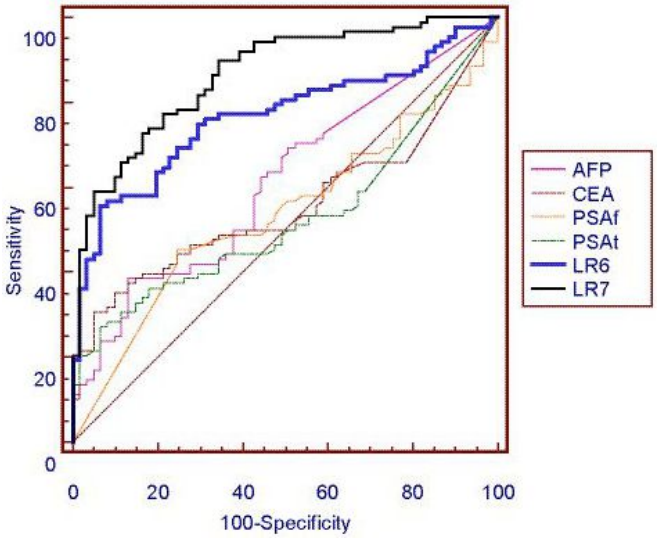


**Figure 2:** Results of ROC analysis of data obtained using the "TM-Biochip" test system. Classification into the patients with malignant neoplasms and the patients of the control group. LR6: AFP + hCG + CEA + PSAt + PSAf + NSE. LR7: the same plus the patient's age. The remaining curves correspond to individual markers: AFP, CEA, PSAt and PSAf. Dotted bold curve is the diagonal corresponding to the minimum diagnostic efficiency.

**Table 1: Evaluation of Diagnostic Efficiency by Measuring of the AUCs for each Variable and for Combinations of Six or Seven Variables. Classification: cancer patients versus noncancer patients**

| Marker | Six variables | | Seven variables | |
|---|---|---|---|---|
| | AUC | p[a)] | AUC | p[b)] |
| AFP | 0.605 | <0.001 | 0.614 | <0.001 |
| CEA | 0.547 | <0.001 | 0.556 | <0.001 |
| PSAt | 0.531 | <0.001 | 0.528 | <0.001 |
| PSAf | 0.540 | 0.001 | 0.539 | <0.001 |
| NSE | 0.571 | 0.003 | 0.573 | <0.001 |
| hCG | 0.518 | <0.001 | 0.530 | <0.001 |
| Age of patient | | | 0.700 | <0.001 |
| LR6 | 0.758 | | | |
| LR7 | | | 0.865 | |

a) *p*, significant differences from LR6;
b) *p*, significant differences from LR7.

Thus, in classification of the patients from group of interest to the patients with and without malignant tumors new test-system in microchip format for determination of six markers demonstrated much improved diagnostic efficacy over that of each individual tumor marker.

## Classification into Patients with CRC and the other Patients

Thirty one serum samples from patients with CRC and 125 serum samples from patients with the other malignant tumors and from non-cancer patients were analyzed with aim to model efficacy of the new system in selection of CRC from general population. Results of ROC-analysis are provided in Table **2**. The data provided in Table **2** evidence, that AUCs for all of the tumor markers with the exception of CEA are in the range 0.515-0.634, what points up to their poor diagnostic efficacy. AUC corresponding for CEA comprises 0.852; this means that CEA, the key marker for CRC, is highly efficient for revealing of this cancer. However AUC corresponding the combination of six tumor markers (LR6) comprises 0.935 (Table **2**) and

significantly differs from AUCs, relevant to each individual marker, including CEA (p<0.001-0.007). Inclusion of the seventh variable, age of patient, doesn't lead to improvement of diagnostic efficacy (AUC 0.930 vs 0.935).

**Table 2: Evaluation of Diagnostic Efficiency by Measuring of the AUCs for each Variable and for Combinations of Six or Seven Variables. Classification: CRC-patients versus the other patients**

| Marker | Six variables | | Seven variables | |
|---|---|---|---|---|
| | AUC | p[a)] | AUC | p[b)] |
| AFP | 0.515 | <0.001 | 0.516 | <0.001 |
| CEA | 0.852 | 0.007 | 0.850 | <0.008 |
| PSAt | 0.634 | <0.001 | 0.627 | <0.001 |
| PSAf | 0.607 | <0.001 | 0.606 | <0.001 |
| NSE | 0.613 | <0.001 | 0.604 | <0.001 |
| hCG | 0.611 | <0.001 | 0.607 | <0.001 |
| Age of patient | | | 0.555 | <0.001 |
| LR6 | 0.935 | | | |
| LR7 | | | 0.930 | |

a) *p*, significant differences from LR6;
b) *p*, significant differences from LR7.

Comparison of specificity of CEA and LR6 at the same sensitivity (90.6 and 90.3%, respectively) shows that it improves from 39.1% (30.6-48.1) for CEA to 75.6% (67.1-82.8) for (LR6).

Thus biochip-based system for six tumor markers makes possible to improve significantly diagnostic performance (AUC: 0.935 vs 0.852 for CEA, p= 0.007; specificity at 90% sensitivity: 75.6 vs 39.1%, respectively) when classify CRC patients and all the other patients.

## Classification into Patients with CRC and the other Cancer Patients

Thirty one serum samples from CRC patients and 62 serum samples from patients with the other malignant tumors were analyzed for evaluation of efficiency of microarray-based system in selection of CRC from the other kinds of cancer. ROC-curves for five individual cancer markers and for combination of six markers are shown in Figure **3**. It is evident from these curves that the most efficient individual marker is CEA. However, the ROC curve, corresponding combination of six markers (LR6) is situated closer to the upper left corner of the plot, which corresponds to 100% sensitivity and 100% specificity. AUC appropriate to LR6 is close to 1 and comprises 0.971 (Table **3**). It is significantly greater than AUCs for CEA and the other markers. Additional parameter, age of patient, does not improve significantly AUC (Table **3**). A comparison between specificity of CEA and LR6 on the same sensitivity (90%) demonstrates that combination of six markers raises specificity from 49.2 (36.5-62.1) for CEA to 88.7% (78.1-95.3) for LR6.

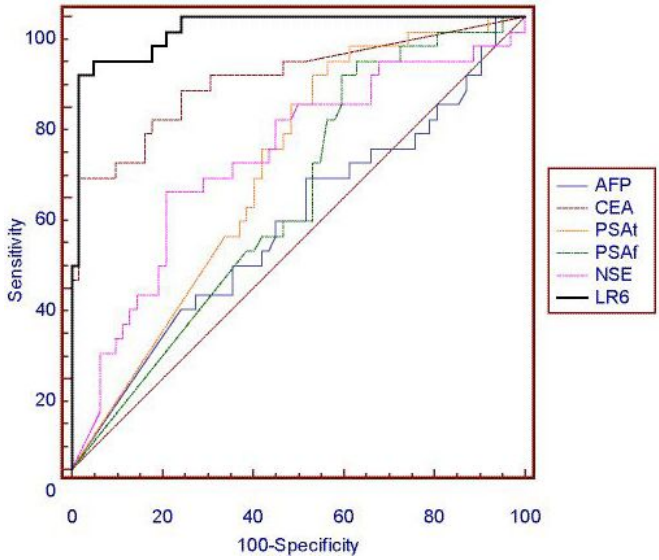


**Figure 3:** Results of ROC analysis of data obtained using the "TM-Biochip" test system. Classification into patients with CRC and the other cancer patients. LR6: AFP + hCG + CEA + PSAt + PSAf + NSE. The remaining curves correspond to individual markers: AFP, CEA, PSAt, PSAf and NSE. Dotted bold curve is the diagonal corresponding to the minimum diagnostic efficiency.

So microarray-based system "TM-Biochip" makes it possible to improve diagnostic efficiency significantly as compared to CEA, the main marker of CRC, as well as to the other individual markers (Table **3**) when classify patients with CRC and the other cancer patients.

Fifty six serum samples (15 - from patients with CRC and 41 - from patients with the other kinds of cancer) were analyzed by microarray-based system as well as by six relevant ELISA systems for comparison of their diagnostic efficacy. It was demonstrated that AUC for microarray-based system (LR6) was significantly higher, that AUC for the combination of six individual tumor markers evaluated in six independent ELISA systems ($LR6_{ELISA}$), p=0.031. A comparison between specificity of LR6 and $LR6_{ELISA}$ on the same sensitivity (86.7%) revealed that specificity of microarray-based system is significantly superior to

that for combination of six ELISA systems: 97.56 (87.1-99.9) and 68.29 (51.9-81.9) %, respectively, p<0.001.

**Table 3: Evaluation of Diagnostic Efficiency by Measuring of the AUCs for each Variable and for Combinations of Six or Seven Variables. Classification: CRC-patients versus the other cancer patients**

| Marker | Six variables | | Seven variables | |
|---|---|---|---|---|
| | AUC | p[a)] | AUC | p[b)] |
| AFP | 0.541 | <0.001 | 0.547 | <0.001 |
| CEA | 0.863 | 0.007 | 0.860 | 0.005 |
| PSAt | 0.663 | <0.001 | 0.657 | <0.001 |
| PSAf | 0.598 | <0.001 | 0.598 | <0.001 |
| NSE | 0.689 | <0.001 | 0.683 | <0.001 |
| hCG | 0.644 | <0.001 | 0.651 | <0.001 |
| Age of patient | | | 0.571 | <0.001 |
| LR6 | 0.971 | | | |
| LR7 | | | 0.975 | |

a) *p*, significant differences from LR6;
b) *p*, significant differences from LR7.

Consequently, system of six tumor markers combined onto the same support can offer advantages over six systems of appropriate markers not only in volumes of serum and reagents used, but in diagnostic efficacy as well.

### Classification into Patients with Prostate Cancer (PC) and Male Patients with the other Malignant Tumors

Thirty four serum samples from patients with PC and 27 serum samples from the male patients with the other malignant tumors were analyzed. ROC analysis revealed that the most efficient individual marker was PSAt (AUC 0.900), the main marker of PC. The use of combination of six markers (LR6) resulted in increase of AUC till 0.953, but difference was not statistically significant (p=0.232). The use of additional parameter, 'age of patient', did not result in appearance of statistically significant differences between LR7 and PSAt (AUCs 0.974 vs 0.898, p=0.109).

### Classification into Patients with PC and Patients with BPH

Thirty four serum samples from patients with PC and 20 serum samples from the patients with BPH were included into investigation. It was demonstrated by ROC-analysis that AUC for LR6 combination was significantly higher that AUCs for each of individual markers, including the greatest AUC for PSAt (0.894 vs 0.771; p=0.032) (Figure **4**, Table **4**). On addition of such parameter as 'age of patient' to the combination of six markers, AUC raised till 0,930 and significantly exceeded these for individual markers (Table **4**). Specificity at 90% sensitivity for PSAt, LR6 and LR7 accounted for 20 (5.7-43.7), 60 (36.1-80.9) and 80.6 (55.5-95.0), respectively.

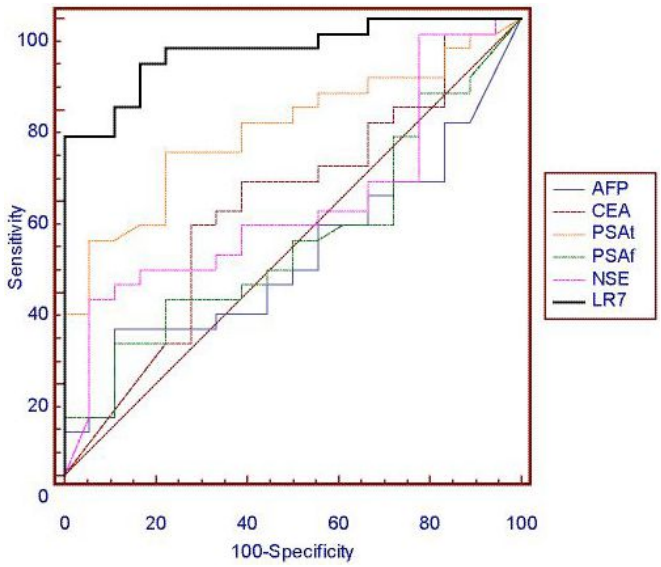


**Figure 4:** Results of ROC analysis of data obtained using the "TM-Biochip" test system. Classification into patients with PC and patients with BPH. LR7: AFP + hCG + CEA + PSAt + PSAf + NSE+ age of patient. The remaining curves correspond to individual markers: AFP, CEA, PSAt, and PSAf and NSE. Dotted bold curve is the diagonal corresponding to the minimum diagnostic efficiency.

Thus microarray-based system "TM-Biochip" can enhance diagnostic efficacy at distinction between patients with PC and patients with BPH as compare to each of individual markers, including PSAt, the main marker of PC.

Thirty three serum samples (16 - from patients with PC and 17 - from patients with BPH) were analyzed by "TM-Biochip" system as well as by six appropriate ELISA systems for the purpose of comparison of their diagnostic performance. AUCs for the combination of six tumor markers plus 'age of patient' index in "TM-Biochip" system (LR7) and for six independent ELISA systems ($LR7_{ELISA}$) were 0.971 and 0.879, respectively, but the difference was not statistically significant (p=0.09). At the same time specificity on the same sensitivity (93.7%) was significantly higher in microarray-based system: confidence intervals (85.5-100) and (18.4-67.1) %, respectively.

**Table 4: Evaluation of Diagnostic Efficiency by Measuring of the AUCs for each Variable and for Combinations of Six or Seven Variables. Classification: patients with PC versus patients with BPH**

| Marker | Six variables | | Seven variables | |
|---|---|---|---|---|
| | AUC | p[a)] | AUC | p[b)] |
| AFP | 0.530 | <0.001 | 0.480 | <0.001 |
| CEA | 0.579 | <0.001 | 0.590 | <0.001 |
| PSAt | 0.771 | 0.032 | 0.756 | 0.010 |
| PSAf | 0.536 | <0.001 | 0.517 | <0.001 |
| NSE | 0.583 | <0.001 | 0.595 | <0.001 |
| hCG | 0.658 | 0.001 | 0.646 | <0.001 |
| Age of patient | | | 0.741 | 0.006 |
| LR6 | 0.894 | | | |
| LR7 | | | 0.930 | |

a) *p*, significant differences from LR6;
b) *p*, significant differences from LR7

## DISCUSSION

Tumor markers are tools that clinicians use to help them answer clinical relevant questions, the first of which are: *1)* does a patient have cancer? and *2*) if yes, which organ is affected? [2]. Since no single marker is sensitive and specific enough to produce the answers to these questions, recent trends are toward increased use of microarrays, multiplex systems for simultaneous determination of several tumor markers [6, 7].

In our investigation a diagnostic performance of a new microarray-based system "TM-Biochip" for simultaneous quantitation of six tumor markers (AFP, CEA, PSAt, PSAf, NSE and hCG) were evaluate in several experimental models. Our study showed that the new diagnostic system allows assessing the level of each marker and gives data comparable with the results obtained by measurement of each individual marker in a respective conventional ELISA system. ROC analysis and evaluation of sensitivity and specificity of the system demonstrated that the new system did better than each of individual markers when classified: *(1)* cancer patients and non-cancer patients; *(2)* patients with CRC and the other patients: patients with the other cancers and non-cancer patients; *(3)* patients with CRC and patients with the other malignant tumors and *(4)* patients with PC and patients with BPH.

In classification of the patients of the experimental group to the patients with and without malignant tumors, effective was the combination of the six tumor markers (LR6) and the combination of the six markers plus the 'age of patient' index (LR7).

Thus, findings obtained with our set of sera (from 170 patients including 108 cancer patients and 62 patients with no cancer) suggest that new microarray-based system can help to find the answers to the above-listed questions.

It was showed earlier by us [17], that commercial system for six tumor markers determination (AFP; CA 125; CA 15-3; CA 19-9; CEA; prolactin), WideScreen$^{TM}$ Human Cancer Panel 1 (Tumor markers) from Novagen, USA, on a basis of suspension microarrays makes it possible to improve significantly diagnostic efficiency in relation to that of each individual marker. In the present investigation analogous results are obtained for the other combination of tumor markers and the other, hydrogel-based, diagnostic system ("TM-Biochip", Russia).

In the last few years several publications were demonstrated that microarray-based systems for several tumor markers determination were efficient at early detection of ovarian cancer [18, 19] and non-small cell lung cancer [20]. Here, we showed that "TM-Biochip" system is more efficient in revealing of cancer patients and, in specific cases, in diagnosing, than systems designed for determination of one tumor marker. In addition, the new system has improved diagnostic performance as compared to six relevant ELISA systems when discriminate between CRC-patients and the other cancer patients as well as between PC patients and BPH patients.

Further increase in the number of tumor markers measured by microarray-based diagnostic system will make it possible to expand the list of diagnoses of malignant neoplasms and eventually pass to the screening of the general population for the presence of tumors and to identify subjects with an increased cancer risk.

## CONCLUSION

The results presented in this article demonstrate the applicability of protein microarray for simultaneous quantitation immunoassay of several analytes per sample in a single assay. A new microarray-based system "TM-Biochip" (Russia) was employed for simultaneous quantitation of six tumor markers (AFP, CEA, PSAt, PSAf, NSE and hCG) in 170 serum samples from groups of cancer patients and control groups. It was demonstrated that this new diagnostic

system allows assessing the level of each marker and gives data comparable with the results obtained by measurement of each individual marker in a respective conventional ELISA system. Simultaneous measurement of six above-listed tumor markers in patients serum followed by the ROC analysis of the data resulted in better sensitivity and specificity as compared to each biomarker in patients classified as: *(1)* cancer patients and non-cancer patients; *(2)* patients with CRC and the other patients: patients with the other cancers and non-cancer patients; *(3)* patients with CRC and patients with the other malignant tumors and *(4)* patients with PC and patients with BPH. These results showed that "TM-Biochip" system is more efficient in revealing of cancer patients than systems designed for determination of one tumor marker.

"TM-Biochip" system using gel-based microchip technology has significant advantages as compared with ELISA system. It is compact in size and ease-to-use. Volume of biological material (blood serum) is small (50 µl for analysis of six tumor markers). The possibility exists of simultaneous quantitation of several (in perspective - many) tumor markers, and of the use the chip for large-scale statistical analysis. Reduction in the cost is associated with the use of little quantities of capture antibodies used in small volumes of gel elements (till 0.1 nl). The time taken to complete an analysis is significantly diminished.

Good correlation of the results obtained between "TM-Biochip" system and single ELISA system shows potential of the first one to replace ELISA as a cost-effective and high throughput screening tool.

## ACKNOWLEDGEMENTS

We gratefully acknowledge the Russian Academy of Medical Sciences (program on Application of Microarrays for simultaneous Quantitation of serum Tumor Markers in Diagnostics of malignant Tumors, № 01.2.007 01721) and the Moscow Government (Grant 17/09-Gen-M) for their financial support of this work.

We thank Dr. V.I. Karaseva for technical assistance.

## CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

## REFERENCES

[1] Voorzanger-Rousselot N, Garnero P. Biochemical markers in oncology. Part I: Molecular basis. Part II: Clinical uses. Cancer Treat Rev 2007; 33: 230-83. http://dx.doi.org/10.1016/j.ctrv.2007.01.008

[2] Diamandis EP. Tumor markers: Past, Present and Future. In: Diamandis EP, Fritsche HA, Lilja H, Chan D, Schwarth M, Eds. Tumor Markers: Physiology, Pathobiology, Technology, and Clinical Applications. Washington: AACC Press 2002; pp. 3-8.

[3] Ando S, Kimura H, Iwai N, Nomoto Y, Shima M, Ando M, *et al.* Optimal combination of seven tumor markers in prediction of advanced stage at first examination of patients with non-small cell lung cancer. Anticancer Res 2001; 21: 3085-92.

[4] Lahousen M, Stettner H, Pickel H, Urdl W, Pürstner P. The predictive value of a combination of tumor markers in monitoring patients with ovarian cancer. Cancer 1987; 60: 2228-32. http://dx.doi.org/10.1002/1097-0142(19871101)60:9<2228::AID-CNCR2820600921>3.0.CO;2-N

[5] Muzushima Y, Hirata H, Isumi S, Hoshino K, Konishi K, Morikage T, *et al.* Clinical significance of the number of positive tumor markers in assisting the diagnosis of lung cancer with multiple tumor marker assay. Oncology 1990; 47: 43-48. http://dx.doi.org/10.1159/000226783

[6] Chechetkin VR, Prokopenko DV, Makarov AA, Zasedatelev AS. Biochips for medical diagnostics. Nanotechologies in Russia 2006; 1: 13-28.

[7] Osipova TV, Ryabykh TP, Baryshnikov AYu. Diagnostic microchip: application in oncology. Russian Journal of Biotherapy, 2006; 5: 72-81.

[8] Sun Z, Fu X, Zhang L, Yang X, Liu F, Hu G. A protein chip system for parallel analysis of multi-tumor markers and its application in cancer detection. Anticancer Res 2004; 24: 1159-66.

[9] Rubina AYu, Kolchinsky A, Makarov AA, Zasedatelev AS. Why 3-D? Gel-based microarrays in proteomics. Proteomics 2008; 8: 817-31. http://dx.doi.org/10.1002/pmic.200700629

[10] Ryabykh T, Osipova T, Dement'eva E, Savvateeva E, Konovalova E, Sookolova Z, *et al.* Biochip-based test-system for simultaneous quantitative determination of prostate-specific antigen (total and free forms) in blood serum. Russian Journal of Biotherapy 2006; 5: 49-57.

[11] Osipova T, Ryabykh T, Rubina A, Dementieva E, Savvateeva E, Konovalova E, *et al.* Biochip-based test-system for prostate cancer diagnostics. In: Egorov AM, Zaikov GE, Eds. New Aspect of Biotechnology and Medicine. New York: Nova Science Publishers, Inc. 2007; Chapter 3: pp. 15-28.

[12] Osipova T, Sokolova Z, Ryabykh T, Karaseva V, Modorsky M, Matveev V, *et al.* Biochip-based Test-system for cancer Diagnostics. Simultaneous quantitation of total and free forms of prostate-specific antigen. Nanotechnology CRC Press, Boston 2008; 2: 30-33.

[13] Dementieva EI, Rubina AYu, Darii EL, Dyukova VI, Zasedatelev AS, Osipova TV, *et al.* Protein microchips in quantitative assays for tumor markers. Dokl Biochem Biophys 2004; 395: 88-92. http://dx.doi.org/10.1023/B:DOBI.0000025553.98757.be

[14] Savvateeva EN, Dementieva EI, Tsybul'skaya MV, Osipova TV, Ryabykh TP, Turygin AY, *et al.* Biological microchip for a simultaneous quantitative immunoassay of tumor markers in human serum. Bull Exp Biol Med 2009; 147: 679-83. http://dx.doi.org/10.1007/s10517-009-0591-2

[15] Mirzabekov AD, Rubina AYu, Pan'kov SV. Composition for polymerizing immobilization of biological molecules and method for producing said composition. Patent Russian Federation No. 2216547, Bull Izobr No. 32 (in Russian). Patent PCT/RU 01/00420 WO 03/033539.

[16] Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 1993; 39: 561-77.

[17] Ryabykh TP, Osipova TV, Sokolova ZA, Paklin NB. Assessment of diagnostic potentials of a commercial multiplex suspension microchip-based system for quantitative analysis of protein tumor markers. Journal of N.N. Blokhin Russian Cancer Research Center RAMS 2011; 22: 58-64.

[18] Hensley ML. A step forward for two-step screening for ovarian cancer. J Clin Oncol 2010; 28: 2128-30. http://dx.doi.org/10.1200/JCO.2009.26.6346

[19] Yurkovetsky Z, Skater S, Lomakin A, Nolen B, Pulsipher T, Modugno F, *et al.* Development of a multimarker assay for early detection of ovarian cancer. J Clin Oncol 2010; 28: 2159-66. http://dx.doi.org/10.1200/JCO.2008.19.2484

[20] Farlow EC, Vercillo MS, Coon JS, Basu S, Kim AW, Faber LP, *et al.* A multy-analyte serum test for the detection of non-small cell lung cancer. Br. J. Cancer 2010; 103: 1221-28. http://dx.doi.org/10.1038/sj.bjc.6605865

Received on 14-10-2014 Accepted on 07-11-2014 Published on 31-12-2014

DOI: http://dx.doi.org/10.12974/2311-8792.2014.02.02.1