THz laser spectroscopy exosome analysis of saliva and blood plasma

A.I. Knyazkova[1], N.V. Yunusova[2,3], E.A.Tugutova[2], , E.E. Ilyasova[1,3], E.A. Sandykova[1,3], V.S. Zasedatel[1]

[1]*Tomsk State University, Tomsk, Russia,*

[2]*Cancer Research Institute, Tomsk National Research Medical Center of the RAS, Tomsk, Russia*

[3]*Siberian State Medical University, Tomsk, Russia*

ABSTRACT

The study of exosomes of saliva and blood plasma by THz laser spectroscopy was carried out. Exosomes were sampled from patients with colorectal cancer (n = 6) and healthy volunteers (n = 5). A substantive examination of the samples absorption spectra was performed using the method of canonical correlation analysis. The presence of Glycine, L-Alanine, Mannose was revealed everywhere. The Mannose content was less in exosomes samples corresponding to colorectal cancer in comparison with exosomes samples from healthy volunteers.

Keywords: exosomes, colorectal cancer, THz laser spectroscopy, canonical correlation analysis

1 INTRODUCTION

In spite of progress in the diagnosis and treatment of cancer it is still one of the main problems for public health.[1] For early diagnostics it is necessary to find more specific cancer biomarkers. Exosomes play a significant role in intercellular communication. They derive from multivesicular bodies, special sorting complexes provide selective accumulation of extracellular vesicle proteins, lipids and RNA. Proteins are one of the main cargos in exosomes and they are associated with biological activities of exosomes such as cell-to-cell communication, biogenesis and so on. These particles of the size of 40-100 nm involved in various physiological pathways in normal and abnormal cells.

Recently it was shown that exosomes released from cancer cells can effect on development of cancer at different stages.[2] These important mediators could regulate, for example, tumor growth, metastasis, immune system response. Dissimilar kinds of extracellular vesicles including exosomes are released from various cell types but from cancer cells exosomes are secreted in substantially higher concentrations. It was showed that exosomes act in this case as a mediator in the transformation of normal cells to cancer cells. Exosomes extracted from serum of patients with breast cancer induced tumor formation in mouse models.

During recent research it was appeared that exosomes released from colorectal cancer cells carry cell cycle-related mRNAs.[3] Last ones can induce proliferation of endothelial cells. A systematic approach to the colorectal cancer cell-derived microvesicles using transcriptome analysis of the microvesicles derived from SW480 cells, a human CRC cell line, and the donor CRC cells was conducted. Such method helps to understand the potential role of cell-derived microvesicles in colorectal cancer-related processes. Namely, microvesicles reflect the physiology of the donor colorectal cancer cells, stimulated proliferation of endothelial cells. It was suggested that colorectal cancer-derived microvesicles can be involved in tumor growth and metastasis by means of stimulating angiogenesis-related processes.

Thus, exosomes can be used as diagnostic and therapeutic biomarkers in different types of cancers. Exosomes are attractive tools for this goal, because they can be isolated fast and easily from body fluids such as saliva, blood plasma, urine.[4]

Colorectal cancer is very frequent malignant tumor with a still increasing coverage both in Europe and United States.[5] In terms of mortality colorectal cancer is the second most common cancer in Europe. Metastasis is the most lethal attribute of colorectal cancer because about 90% of all cancer deaths arise from the metastatic dissemination of primary tumors. Here, metastasis is the result of early changes during tumor progression. Today much is known about molecules contributing to the pathways metastasis control. However, patient prognosis is mainly depended on extent of tumor spread within a surgical sample. Molecular determinants for metastasis of colorectal cancer potential markers for metastasis prognosis and as the result the metastasis prevention.

The aim of the paper was spectral analysis of paired samples of exosomes of saliva and plasma of patients with locally advanced forms of colorectal cancer and healthy volunteers using laser THz spectroscopy.

International Conference on Atomic and Molecular Pulsed Lasers XIII, edited by
Victor F. Tarasenko, Andrey M. Kabanov, Proc. of SPIE Vol. 10614, 106141Z
 · doi: 10.1117/12.2303629

The spectra of biological samples are complex so for data mining methods, Principal Component Analysis, Support Vector Machine, Canonical Correlation Analysis (CCA) are useful for content sample analysis.[6-9]

2 MATERIALS AND METHODS

The study was approved by the Local Ethics Committee of the Cancer Research Institute of Tomsk National Research Medical Center. The blood samples were obtained from 6 patients with Stage T2-4N0-2M0 colorectal cancer and 5 healthy donors. All patients were fully informed of the purpose and nature of the treatment and provided an informed written consent.

The exosomes from the blood plasma were isolated by ultra-filtration in combination with a double ultra-centrifugation.[10] In particular, to remove cellular debris, blood plasma samples were centrifuged at 17,000 g at 4°C for 20 minutes. The supernatants were diluted 5 times with phosphate buffer (PB, 10 mM phosphate buffer, 0.15 M NaCl, pH 7.5) and filtered through a filter with a pore diameter of 100 nm (Sartorius, USA). The filtrate was ultra-centrifuged (100,000 g, 90 min, 4°C), the precipitate was washed with PB. The exosomes were resuspended in 200 µl PB, frozen in liquid nitrogen and stored at -80°C. The samples were examined by means of transmission electron microscopy (TEM). Training included sorption of the exosomes sample onto a copper grid covered film for 1 minute and contrast of a 2% phosphotungstic acid-set 10. The grids were studied by transmission electron microscope Jem1400 (Jeol), images obtained with the Veleta digital camera.

Then the obtained exosomes were characterized using flow cytometry on the content of the most common exosomal membrane protein: CD9, CD63, CD81 and CD24.[11] Unconjugated antibodies against tetraspanins or CD24 (Abcam, UK) were adsorbed to latex beads (Invitrogen, USA) at the rate of 1 mg protein / 106 particles in MES-buffer at room temperature for 10 hours. Washed PB antibody coated beads were incubated with 30 µlexosomes that were obtained from 2.7 ml blood / $3.6x10^5$ particles in PB overnight at + 4°C. Then unreacted centers were blocked with 0.2 M glycine for 30 min at 4° C, the particle-antibody-exosomes complexes washed twice with 2% bovine serum, and then incubated with a blocking immunoglobulin G (BD, USA) for 10 min. To the complexes washed twice by PB with 2% bovine serum were added 20 µl of FITC-labeled anti-tetraspanins antibodies (BD, USA) and 80 µl PB with 2% bovine serum and incubated for 40 min. Fluorescence complexes of the exosomes with antibodies were measured by flow cytometry FACS Canto II (USA). The analysis of the median fluorescence intensity (MFI) was carried out single exosomes isolated population as compared to isotype control.

Samples of exosomes were kept in special cuvettes at a temperature of 193 K.[12] The measurement of the absorption spectra were obtained using Time-domain THz spectrometer (EKSPLA, Estonia) with tuning range 0.3-3 THz. The measurements were carried out at a temperature of 255 K. For each sample 12 scans of absorption spectra were recorded.

The component analysis of the exosome samples was carried out using the CCA. The CCA enables to identify and to estimate numerically associations between two sets of variables. The aim of CCA is to maximize measured by correlation connection between the low-dimensional projections of the two data sets, for example, between vectors of random quantities.[13]

The mathematical problem here is to find the closest approximation of the experimental spectrum $\mathbf{V}$ of the exosomes'sample by means of linear combinations of individual molecular components $\mathbf{U}$ (that can be contained in a sample) spectra set $\mathbf{X}_i$

$$\mathbf{U} = \sum_i \alpha_i \mathbf{X}_i \ .$$

Here α_i are the unknown coefficients characterizing the percentage of the components $\mathbf{X}_i$ in $\mathbf{U}$ .One can find maximum association between $\mathbf{U}$ and $\mathbf{V}$ vectors for the components set $\mathbf{X}_i$ (see Table 1) by applying CCA

$$\frac{\mathrm{cov}(\mathbf{U},\mathbf{V})}{\sigma_{\mathbf{U}}\sigma_{\mathbf{V}}}$$

Here cov is the covariance and σ is quality of standard deviation.The used set of individual molecular components characterized by absorption spectra $\mathbf{X}_i$ was generated expertly and is presented in Table 1. The absorption spectra $\mathbf{X}_i$ were taken from THz database 2.0. Note, that the set of substances in Table 1 is not complete.

Table 1. List of substances that can be present in exosomes of saliva and blood plasma.

2-Deoxy-D-glucose	Glycine
Acetylcholine Bromide	Histidine
Acetylcholine Chroride	Inositol
Acetylcholine Iodide	L-Alanine
Adenosin-5-Triphosphate(ATP)	L-Asparagine monohydrate 5K
Adenosine5Diphosphate(ADP)	L-Methionine 5K
Cysteine	L-Serine
D-Alanine	Lauric Acid
D-Mannitol	Leucine
Fructose	Mannose
Fructose 5K	Mannose 5K
Galactose	Methionine
Galactose 5K	Pyridoxine Hydrochloride
Glucose	Riboflavin
Glucose 5K	Riboflavin 4K
Glutamic Acid	H2O+cuvette

3 RESULTS

Figure 1 shows the absorption spectra of saliva exosomes samples of the patient with colorectal cancer (female) and of the healthy volunteer (female) and also their approximation by the CCA. In the case of colorectal cancer (Figure 1 on the left) the correlation coefficient is 0.899 and in the case of a healthy volunteer (Figure 1 on the right) the correlation coefficient is 0.935.

Table 2 shows the results of the component analysis of saliva exosomes samples (using the CCA) which correspond to the data presented in Figure 1. Concentrations are given in relative units.

Figure 2 shows the absorption spectra of blood plasma exosomes samples of the patient with colorectal cancer (female) and of the healthy volunteer (woman), and also their approximation by the CCA. In the case of colorectal cancer (Figure 2 on the left) the correlation coefficient is 0.935 and in the case of the healthy volunteer (Figure 2 on the right) the correlation coefficient is 0.884.

Table 3 shows the results of the component analysis of blood plasma exosomes samples (using the CCA) which correspond to the data presented in Figure 2. Concentrations are given in relative units.

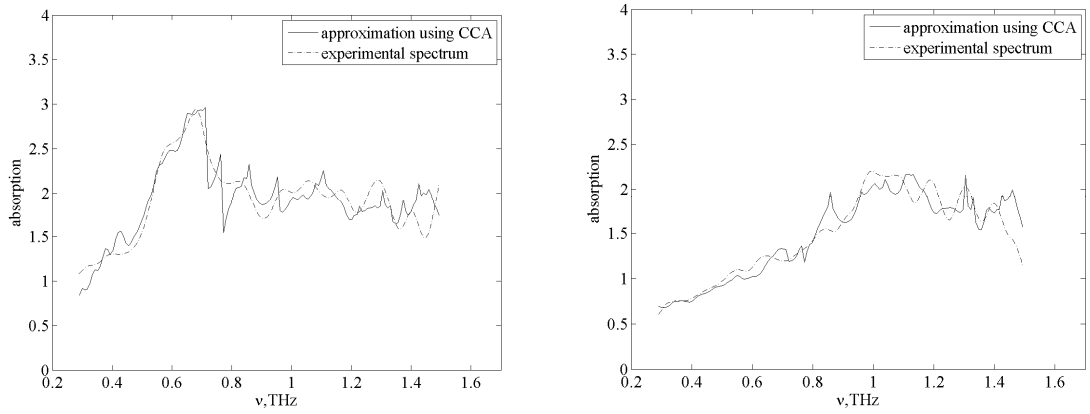

Figure 1. Absorption spectra corresponding to the saliva exosomes samples of the patient with colorectal cancer (female) on the left and of the healthy volunteer (female) on the right.

Table 2. Results of the component analysis of saliva exosomes samples by CCA.

Substance	Healthy volunteer (female)	Colorectal cancer (female)
H2O+cuvette	0.3492	0.3848
2-Deoxy-D-glucose	0.1074	0
Acetylcholine Chroride	0.1167	0
Cysteine	0.0416	0
Galactose 5K	0.0393	0
Glucose 5K	0	0.0083
Glycine	0.0278	0.0006
L-Alanine	0.0656	0.0589
L-Methionine 5K	0.2402	0.0331
Mannose 5K	0.4709	0.1311
Methionine	0.0261	0.0681

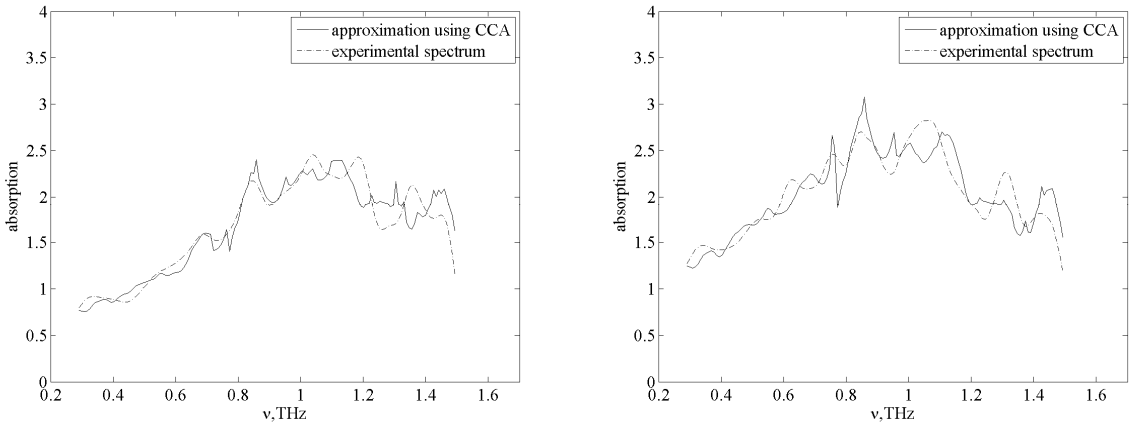

Figure 2. Absorption spectra corresponding to the blood plasma exosomes samples of the patient with colorectal cancer (female) on the left and of the healthy volunteer (female) on the right.

Table 3. Results of the component analysis of blood plasma exosomes samples by CCA

Substance	Healthy volunteer (female)	Colorectal cancer (female)
H2O+cuvette	0.5289	0.7678
Acetylcholine Chroride	0	0.0860
Cysteine	0	0.0164
Galactose 5K	0.0126	0.0336
Glucose 5K	0	0.1090
Glycine	0.0300	0.0157
Inositol	0.0393	0.0487
L-Alanine	0.0591	0.0662
L-Methionine 5K	0.0438	0
Mannose	0.0217	0
Mannose 5K	0.1545	0.1163
Methionine	0.0442	0

Almost all exosomes of both saliva and blood plasma were shown to contain the following substances: Glycine, L-Alanine, Mannose. The content of Mannose in exosomes samples (of both saliva and blood plasma) corresponding to colorectal cancer was less than in samples with exosomes corresponding to healthy volunteers. Also Methionine is regularly detected in exosomes of saliva and Inositol is regularly detected in the case of blood plasma exosomes.

4 CONCLUSION

In the study spectral analysis of paired exosomes samples of saliva and blood plasma of patients with locally advanced forms of colorectal cancer (n = 6) and healthy volunteers (n = 5) was performed. The component analysis of the samples was carried out using CCA method.

A correlation coefficient between the experimental absorption spectra and their approximations was found to be no less than 0.87 by means of approximating of the absorption spectra of samples curves by CCA using the spectra of individual components which are presented in Tables 2 and 3. This value is high enough. To improve the result of approximation we need to complete the correspondence database with the spectra of substances that can be presented in the samples under study.[14]

Thus exosomes can be used as potential non-invasive diagnostic biomarkers on various cancer stages.

ACKNOWLEDGEMENTS

The work was carried out under partial financial support of the Russian Fund of the Fundamental Research (grant No.17-00-00186\17).

References

[1] Mirzaei, H.R., Sahebkar, A., Salehi, R., Nahand, J.S., Karimi, E., Jaafari, M.R., et al. “Boron neutron capture therapy: Moving toward targeted cancer therapy,” Journal of Cancer Research and Therapeutics 12(2), 520-525 (2016).

[2] Mirzaei1, H., Sahebkar, A., Jaafari, M.R., Goodarzi, M., Mirzaei, H.R., “Diagnostic and therapeutic potential of exosomes in cancer: the beginning of a new tale?” Journal of Cellular Physiology 232(12), 3251–3260, (2017).

[3] Hong, B.S., Cho, J-H., Kim, H., Choi, E-J., Rho, S., Kim, J., et al. “Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells,” BMC genomics, 10(1), 556 (2009).

[4] Melo, S.A., Luecke, L.B., Kahlert, C., Fernandez, A.F., Gammon, S.T., Kaye, J., et al. “Glypican-1 identifies cancer exosomes and detects early pancreatic cancer.” Nature 523(7559), 177-82 (2015).

[5]Stein, U., Schlag, P.M., “Clinical, biological, and molecular aspects of metastasis in colorectal cancer,” Recent Results Cancer Res. 176, 61-80 (2007).

[6] Kistenev, Yu. V., Bukreeva, E.B., Bulanova, A. A., Kuzmin, D.A., Tuzikov, S.A., Yumov, E.L., “Laser spectroscopy and chemometric study of the specific features of air exhaled by patients with lung cancer and chronic obstructive pulmonary disease,” Physics of Wave Phenomena 22(3), 210-215 (2014).

[7] Kistenev, Yu.V., Shapovalov, A.V., Borisov, A.V., Vrazhnov, D.A., Nikolaev, V.V., Nikiforova, O.Y., “Applications of principal component analysis to breath air absorption spectra profiles classification,” Proceedings of SPIE 9810, 98101Y (2015).

[8] Bukreeva, E. B. Bulanova, A. A., Kistenev, Yu. V., Kuzmin, D. A., Tuzikov, S. A. , Yumov, E. L., “Analysis of the absorption spectra of gas emission of patients with lung cancer and chronic obstructive pulmonary disease by laser optoacoustic spectroscopy,” Proceedings of SPIE 8699, UNSP 86990K (2013).

[9] Kistenev, Y.V., Karapuzikov, A.I., Kostyukova, N.Y., Starikova, M.K., Boyko, A.A., Bukreeva, E.B., Bulanova, A.A., Kolker, D.B., Kuzmin, D.A., Zenov, K.G., Karapuzikov, A.A., “Screening of patients with bronchopulmonary diseases using methods of infrared laser photoacoustic spectroscopy and principal component analysis,” J Biomed Opt. 20(6), 065001 (2015).

[10] Yunusova, N.V., Tamkovich, S.N., Stakheeva, M.N., Afanas'ev. S.G., Frolova, A.Y., Kondakova, I.V., “The characterization of exosome from blood plasma of patients with colorectal cancer.” AIP Conference Proceedings, 020070 (2016).

[11] Tamkovich, S.N., Yunusova, N.V., Stakheeva, M.N., Somov, A.K., Frolova, A.Y., Kirushina, N.A, Afanasyev, S.G, Grigoryeva, A.E., Laktionov, P.P., Kondakova, I.V., “Isolation and characterization of exosomes from blood plasma of breast cancer and colorectal cancer patients,” Biomed Khim. 63(2), 165-169 (2017).

[12] Kistenev, Yu.V., Borisov, A. V., Shapovalov, A. V., Baydik, O. D., Titarenko, M. A., "Diagnostics of oral lichen planus based on analysis of volatile organic compounds in saliva", Proc. SPIE, 10063, 100630R (2017).

[13] Härdle, W., Simar, L., [Applied Multivariate Statistical Analysis], Berlin, Springer, 321–330 (2015).

[14] Kistenev, Yu.V., Borisov, A.V., Shapovalov, A.V., Nikiforova, O.Y., “Analysis of the component composition of exhaled air using laser spectroscopy and canonical correlation analysis," Proceedings of SPIE 9680, 96804C (2015).