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COLORECTAL CANCER DIAGNOSTICS VIA DETECTION OF TISSUE-SPECIFIC EXTRACELLULAR NANO-VESICLES

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The development of methods for effective diagnosis and monitoring of colorectal cancer (CRC) treatment is one of the basic scientific problem. The circulating plasma contains extracellular nanovesicles (EVs) secreted mainly by blood and endothelial cells. The minor fraction of plasma EVs is produced by cells of various tissues, including cells of the intestinal epithelium. The biochemical composition of such vesicles should have tissue-specific features. Presented study was aimed to identify surface markers of EVs secreted by intestinal epithelium cells and to assess the possibility of isolating and quantification of such vesicles for the diagnosis of CRC. The cell cultures (HCT-116, HT-29, COLO-320, HuTu-80, SW837), plasma of CRC patients and healthy donors were used in the study. The methods of nanoparticle tracking analysis (NTA), atomic force microscopy (AFM), dot-blotting and flow cytometry were applied for EVs characterization. With the original technology of immunosorption we have demonstrated an increased amount of CLRN3, GAL4 and Meprin A, i.e. positive EVs in plasma of CRC patients comparing to healthy donors. Based on the quantitative analysis of such EVs, new methods of diagnostics and monitoring of CRC therapy can be developed.

[Key words: colorectal cancer, extracellular nanovesicles, tissue-specific markers, exosomes, diagnostics]

CONFLICTS OF INTERESTS: The authors declare no conflicts of interest.

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INTRODUCTION

Colorectal cancer (CRC) occupies one of the leading positions in the structure of cancer mortality, which justifies the relevance of developing new methods of screening and early diagnosis. Clinical guidelines, Russian [1] and foreign [2], over the past decades have been reduced to various combinations of endoscopic technologies and methods for detecting occult blood in the stool, but both approaches havelimitations.

Active research is underway to develop methods for analyzing circulating plasma components [3], although none of the variants of the so-called "liquid biopsy" technology has yet been brought to practical use. One of the main reasons for this situation is the lack of reliable molecular markers of CRC.

Identification and creation of methods for analysis of

multi-molecular circulating complexes, for example, extracellular nanovesicles (EVs), is a solution to the problem. Circulating plasma contains about 1011-1012EVs/ml. This is a heterogeneous population of membrane vesicles secreted mainly by blood and endothelial cells [4]. The minor fraction of these vesicles enters the plasma stream from cells of various tissues [5]. The fraction of tissue-specific vesicles reflects the biochemical composition of the cells of the corresponding organs and it can have diagnostic potential. If this fraction is significant compared to the total population of circulating vesicles, its quantitative or qualitative changes may affect the number or composition of plasma vesicles. For example, an increase in the total concentration of all circulating nanovesicles of endosomal origin (or exosomes) has been described in patients with CRC [6]. Despite the apparently low diagnostic specificity of this approach, the study authors recommended quantitative analysis of circulating exosomes as a screening method. On the other hand, the detection of a specific fraction of EVs in plasma containing CRC-associated RNA [7], microRNA [8], or proteases [9] may have a higher diagnostic specificity than just counting vesicles. However, the complexity of detecting a minor fraction of the EVs with certain biochemical characteristics in the "background" population of all plasma vesicles compromises the diagnostic sensitivity and practical significance of this approach.

We can assume two fundamental solutions of the problem. First, the use of ultra-sensitive technologies for detecting vesicular markers using, for example, electrokinetic chips [10] or surface-enhanced raman spectroscopy [11]. Second, development of methods for concentration (ideally, specific isolation) of vesicles secreted by CRC cells. A simple example of this approach is the method of analyzing vesicles from a plasma sample obtained from mesentery veins [12], but the practical application of this method seems to be complicated. The concentration of a separate fraction of vesicles from plasma obtained in the traditional way using immune-sorption technologies is more promising, and it has already been implemented as part of the development of methods for the diagnosis of squamous cell carcinoma of the head and neck [13] and melanoma [14].

The present study is based on the assumption that intestinal epithelial cells secrete a specific set of membrane proteins, and these proteins can be secreted as part of the EVs membrane. During the development of CRC, cells of medium - or high-differentiated tumors retain tissue differentiation, their outer membrane and the membrane of their secreted EVs contain tissue-specific markers. These markers can be used to isolate and analyze tissue-specific EVs, and an increase in the concentration of such vesicles in plasma can serve as a sign of the development of CRC

MATERIALS AND METHODS

Biological Material

Biological material was obtained from donors and patients who were examined or treated at the N.N.Petrov National Medical Research Center of Oncology of Ministry of Health of Russia (Saint Petersburg) and the Ryzhikh National Medical Research Center of Coloproctology (Moscow). The venous blood was collected in vacutainers with EDTA, plasma was separated within 10 minutes after blood collection, frozen and stored at -80°C. The material of epithelial scrapings was obtained directly after surgeries from sites of the unchanged intestinal wall.

Cells

We used five stable cell lines HCT-116, HT-29, COLO-320, HuTu-80, SW837, obtained from the cell culture collection of the Institute of Cytology RAS (Saint-Petersburg). The cells were cultured in RPMI-1640 with the addition of 10% embryonic bovine serum and a mixture of Pen-Strep antibiotics, 100 mcg/ml (all from Biolot LLC, Russia) under standard conditions. For the experiments on flow cytometry and dot-blotting, the cells were grown to a density of no more than 70%, removed from the substrate with a mixture of trypsin (0.025%) and Versen (1:1) solutions, washed with a phosphate-salt buffer (FSB) and diluted to a concentration of $1x10^6$ cells/ml. To isolate EVs, the cells were cultured under standard conditions in a medium containing 10% veal serum without EVs, and the conditioned cell medium was accumulated over several passages to a volume of 200 ml.

Isolation of Nano-vesicles

To isolate EVs, the plasma (2 ml) was thawed to +4°C, diluted 1:1 in a phosphate-salt buffer (FSB), and sequentially centrifuged 300g – 10', 2,000g – 10', and 10,000g – 10' to precipitate cellular detritus and large membrane vesicles. Exosomes were isolated from the supernatant by ultracentrifugation (Beckman Coulter: Optima XPN 80, rotor 70.1 Ti/k-factor 36, rotor 45Ti/k-factor 133) according to the classical procedure [15] with minor modifications. The plasma solution was centrifuged for 110,000 g - 2 hours, the supernatant was removed, and the precipitate was dissolved in 100 ml of the FSB. To isolate exosomes secreted by the cells in vitro, the culture medium was also purified from cellular detritus and large membrane vesicles by differential centrifugation, then was ultracentrifuged at 110,000 g overnight, at +4°C. The sediment containing exosomes

was diluted in 100 µl FSB for subsequent analysis.

Dot-blotting

To detect the total content of "marker" proteins in biological samples (intestinal epithelial scrapings, cell cultures, or EVs), the material was lysed using a RIPA buffer solution (Thermo Scientific, USA). When studying the expression of proteins in the surface membrane, suspensions of cells or vesicles in the FSB were used. The total protein concentration in the samples was evaluated by the Bradford method and equalized to 1 microgram/microl. The samples were applied to a nitrocellulose membrane (each "dot" - 0.5 µl) with a pore size of 0.45 microns (BioRad Laboratories, USA), blocked (Tris-buffer salt solution (TBS), 0.05% Tween-20, 5% bovine serum albumin (all from Sigma, USA)), for 1 hour. Primary antibodies (shown in Table 1) were used in the concentrations recommended by the manufacturer. Incubation was performed for 30 minutes, then the membrane was "washed" in a TBS solution with 0.05% Tween-20 three times for 5 minutes, and was incubated with secondary antibodies labeled with horseradish peroxidase (Ab6721, Ab6789) at a dilution of 1:20,000 for 30 minutes. After three times "washing" of the membrane, detection of peroxidase activity was performed by chemiluminescent reaction using Pierce ECL Western Blotting Substrate (Thermo Scientific, USA) and Invitrogeni Bright FL1500 Imaging System (Thermo Fisher, USA). The data obtained was processed in the Image J software, USA.

Nanoparticles Tracking Analysis (NTA)

The measurements were performed using the Nanosight NS300 analyzer ("Malvern", USA). Each sample was studied in 4-5 different micro volumes by pumping the sample through a chamber. The duration of each measurement is 60 seconds. Camera level: 14, shutter slider: 1259, slider gain: 366, threshold level: for draft – 5, for VF2 – 6. The experimental data was analyzed using Nanosight NTA 3.2 Software.

Atomic Force Microscopy (AFM)

It was used to study the shape and size distribution of particles. The measurements were performed using a scanning probe microscope NT-MDT Solver Bio ("NT-MDT", Russia). The mode was semi - contact, the probe was NSG01_DLC ("NT-MDT", Russia). The samples were applied to the mica surface ("2SPI", USA) immediately after the top layer was removed. Then incubation was performed for 30 seconds, followed by double washing with distilled water.

Next, it was dried with compressed air. Image processing and analysis were performed in Gwyddion software (gwyddion.net) and Image Analysis ("NT-MDT", Russia).

Flow Cytometry (FC) for Cell Analysis

The cell suspension (1 x 10^6 C/ml) in the FSB was aliquoted to 100 µl, precipitated by centrifugation at 1,000g, the supernatant was removed, 100 µl of FACS-blocking buffer (1% BSA dissolved in the FSB) was added, incubated for 30 minutes on ice, then the supernatant was again precipitated and removed. In the case of analysis of the content of the "marker" proteins in the cytoplasm, the cells were processed using the BD Cytofix/CytoPerm Kit (Becton Dickinson and Company, USA). The cells were incubated with primary antibodies (Table1) in a dilution of 1:200 for 30' at +4°C. After three times "washing" in the FSB, the cells were incubated with secondary antibodies labeled with FITC at a dilution of 1:1,000 for 30' at +4°C and washed three times with the FSB. The cells prepared in this way were resuspended in 200 ml of the FSB and analyzed.

Flow Cytometry (FC) for Analysis of Total EVs Population

Sample preparation was carried out using a commercial Exo-FACS kit (Hansa BioMed, Estonia) according to the manufacturer's protocol and meant non-specific fixation of the EVs to latex micro-spheres. Detection of "exosmal" CD63 and CD9 markers was performed using antibodies conjugated with FITC fluorescent tags (CD63-FITC, Ab18235, Abcam, USA)PE (CD9-PE, 312105, Bio Legends, USA). Detection of tissue-specific proteins was performed using primary antibodies to the corresponding proteins (Table 1) and secondary fluorescentlylabeled antibodies (Ab6717, Ab6785 Abcam, USA).

Creation of Immuno-particles and Flow Cytometry (FC) for Analysis of EVs Isolated by Immuno-sorption

For this study, were made immuno-particles that bind vesicles with "marker" proteins (Meprin alpha, CLRN3, GAL4) on the surface (Fig. 6). The corresponding antibodies (Table1) were purified from preservative (sodium azide), concentrated using a set of Amicon Ultra-0.5 mL Centrifugal Filters for DNA and Protein Purification and Concentration (Millipore, Ireland), and conjugated with Biotin using a set of Pierce Antibody Biotinylation Kit for IP (Thermo Scientific, USA). The effectiveness of the biotin binding reaction was evaluated using the Pierce Biotin Quantification Kit (Thermo Scientific, USA). Biotinylated antibodies (10 µl) were incubated with superparamagnetic particles, SPMP (1 µl), whose surface was modified with streptavidin (Silex LLC, Moscow) for an hour at +4°C. As a result of the formation of the biotin-streptavidin bond during this incubation, SPMP-AB complexes or immuno-particles were formed (Fig.6A), the physical characteristics of which determine the possibility of

their analysis by flow cytometry.
The immuno-particles prepared in this way were incubated with the EVs isolated by ultra-centrifugation in a volume of 100 µl over the night at +4°C (Fig.6B). The resulting complex "SPMP-AB-EVs" (Fig.6B) was washed 3 times with the FSB on a magnetic tripod, blocked in 200 µl of 0.2% Tropixi-Block buffer (Thermo Fisher, USA) for 1 hour at +4°C, and washed twice with the FSB 2 times. To quantify the associated EVs, the complexes were incubated with antibodies to the classic "exosomal" marker CD63 (CD63-FITC, Ab18235, Abcam, USA) for 2 hours at +4°C in the dark. The resulting complexes were washed 2 times, diluted in 200 FSB and analyzed. As a negative control, the SPMP-AB complex without exosomes was used, "underwent" through similar blocking procedures, labeling with CD63-FITC antibodies, and washing.
All the tests were performed on a Cytoflex flow cytometer (Beckman Coulter, USA) equipped with a 488 nm argon laser for measuring forward scattering light (FSC) and side scattering (SSC). The results were analyzed using CytExpert (Beckman Coulter, USA) and Excel (Microsoft) software.

Funding and Ethical Justification

The study plan was approved by the local ethics committee of the Petrov NMRC of Oncology (St. Petersburg), the study was carried out as a research work AAAA-A18-118012390156-5 in cooperation with the Ryzhikh National Medical Research Center of Coloproctology (Moscow). All the patients signed an informed consent to participate in the study. The biological material was included in the study after depersonalization of the clinical data.

RESULTS

Selection of Potential Markers

In order to identify potential exosomal CRC markers, the available databases were analyzed (Human Protein Atlas [16], Uni Prot [17]). There were selected 12 molecules that were characterized by expression (exclusively or predominantly) in intestinal epithelial cells, membrane intracellular localization, and the absence of secreted (circulating) forms. Thus, the results of the analysis are summarized in Table 1. All the selected molecules (except CDH177 and DHRS11) were previously detected in the composition of EVs secreted by human cells, which is reflected in the Exo Carta database [18]. CDH177 and DHRS11 have so far been detected in the EVs secreted by ratline cells, indicating that they may be present in vesicles secreted by human intestinal epithelial cells.
In order to positively control the detection system, two proteins (EpCAM and CD47) were included in the analysis, the active expression of which is characteristic of all epithelial cells. The "classic" exosomal marker CD63 and VAMP1, a molecule detected in the membrane of various types of vesicles, not only exosomes, were used as positive controls for the detection of vesicular markers. In addition to information about the tissue-specific nature of expression and intracellular localization, the selection of potential markers took into account data on the function of the protein, which

Table 1. *The list of potential markers of EVs secreted by the cells of CRC.*

Gene Symbol	Uni Prot ID	Exo Carta ID	Name of protein	Cellular function	Antibodies
EPCAM	P16422	4072	Epithelial Cell Adhesion Molecule	Physical homophilic interaction molecule	ab20160
CD47	Q08722	282661	CD47 molecule	Cell adhesion	ab108415
CD63	P08962	967	TSPAN30	Lysosomal-Associated Membrane Protein	ab59479
VAMP1	P23763	6843	Vesicle Associated Membrane Protein 1	Endoplasmic reticulum unfolded protein response	ab151712
CEACAM5	P06731	1048	CEA-related cell adhesion molecule 5	Cell surface glycoprotein	ab190718
CD177	Q8N6Q3	(RAT)	CD177 molecule	Formation of with beta-2 integrin heterodimer	ab203025
CDH17	Q12864	1015	Cadherin 17	Cadherins are calcium-dependent cell adhesion proteins	ab109220
CDHR5	Q9HBB8	53841	Cadherin-related family member 5	Intermicrovillar adhesion molecule	ab189111
CLRN3	Q8NCR9	119467	Clarin 3	Integral component of membrane	ab177695
DHRS11	DHRS11	(RAT)	Dehydrogenase/reductase member 11	Steroid metabolism	ab98276
GCNT3	O95395	9245	Glucosaminyl (N-acetyl) transferase 3	Synthesis of mucin beta 6 N-acetylglucosaminides	ab98134
GPA33	Q99795	10223	Cellsurface A33 antigen	Cell-cell recognition and signaling	ab108938
LGALS4	P56470	3960	Lectin, galactoside-binding, soluble, 4	Assembly of adherens junctions	ab170638
MEP1A	Q16819	4224	Meprin A, alpha	Hydrolysis of protein and peptide substrates	ab107548
PIGY	Q3MUY2	84992	GPI-GlcNAc transferase	Modification of cell surface proteins	ab79453
REG4	Q9BYZ8	83998	Regenerating family member 4	Calcium-independentlectin	ab200723

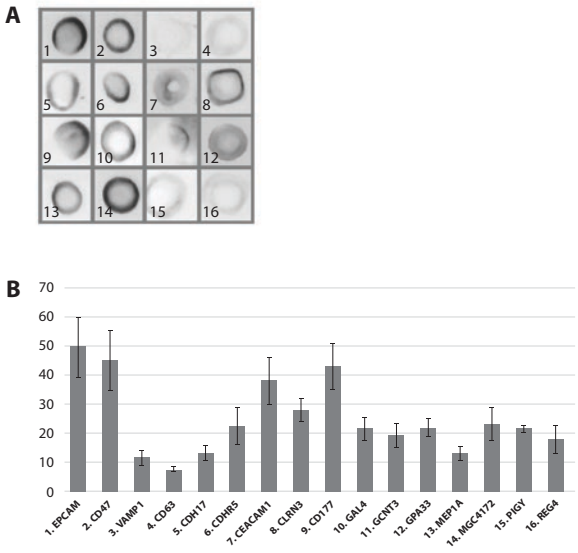


Figure 1. *Analysis of the expression of potential markers in large intestine epithelial scrapings dot-blotting method. A. Representative example of analysis of 16 molecules in one sample. B. Results averaged for 5 samples*

suggested the possibility of its "appearance" in the composition of the EVs membrane.

Evaluation of Expression of Selected Molecules in Colorectal Epithelium

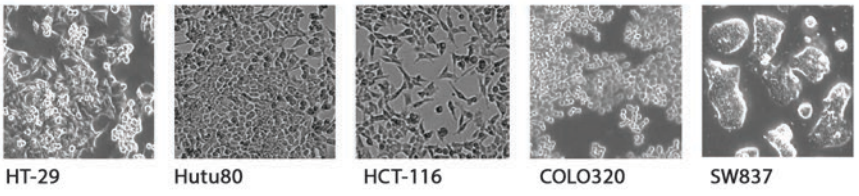
In order to verify the expression of the selected proteins, samples (n=5) of epithelial scraping from sites of the normal intestinal wall after hemicolectomy were collected. The protein lysates were normalized to the total protein content by the Bradford method, 2 µl of lysate was applied to the nitrocellulose membrane, and the relative content of each of the 16 selected molecules (including 4 controls) was analyzed using dot-blotting. Figure 1A shows a representative example of the results of a single sample analysis. The data for all the samples was digitized and normalized relative to the average signal intensity. The average values for the five samples are shown in Figure 1B. The presented results confirm the high level of EpCam and CD47 proteins, the expression of which is expected in cells of all epithelial types. Low expression of vesicular markers (VAMP1 and CD63) was detected. The expression of the remaining molecules was clearly determined, with varying degrees of intensity. Relatively high levels of expression were observed in CEACAM1 and CD177, while low levels were observed in CHD17 and MEP1A (Meprin A). The obtained results confirmed the fact of expression of the selected proteins in the colorectal epithelium and justified the assumption that they may be present in the composition of EVs secreted by cells of normal intestinal epithelium and transformed epithelium that preserved tissue differentiation.

Evaluation of Expression of Selected Molecules in Colorectal Carcinoma Cells In Vitro

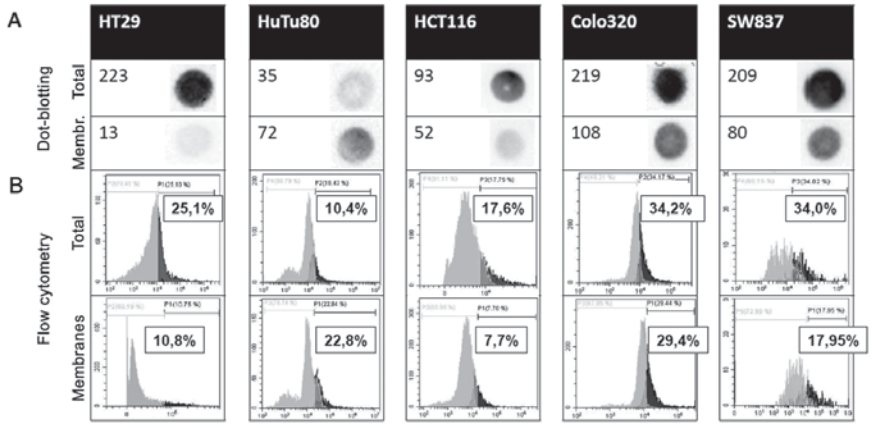
The task of the next stage of the study was to analyze the expression of selected proteins by intestinal epithelial cells in order to further evaluate their secretion in the composition of EVs in vitro. This approach made it possible to work with a "pure" population of EVs secreted by intestinal epithelial cells. Five stable lines obtained from the material of adenocarcinomas of various parts of the gastrointestinal tract were selected (Table2). The lines used had different genetic characteristics and morphology (Fig.2). All the lines were derivatives of highly or moderately differentiated intestinal epithelial adenocarcinomas and retained the

Table 2. *Cell lines.*

Cell line	Primary tumor		Features of xenograft tumor in vivo model
	Site	Histologicalform	
HT-29	Colon	Adenocarcinoma, G-II	Highly differentiated adenocarcinoma (G-I)
HuTu89	Duodenum	Adenocarcinoma, G-III	Highly differentiated adenocarcinoma (G-I)
HCT-116	Colon	Adenocarcinoma, G-III	Moderately differentiated tumor (G-II), distant metastases
COLO320	Colon	Adenocarcinoma, G-II	Highly differentiated adenocarcinoma (G-I)
SW837	Rectum	Adenocarcinoma, G-IV	Highly differentiated adenocarcinoma (G-III)



Fugure 2. *Morphology of the cell lines used*



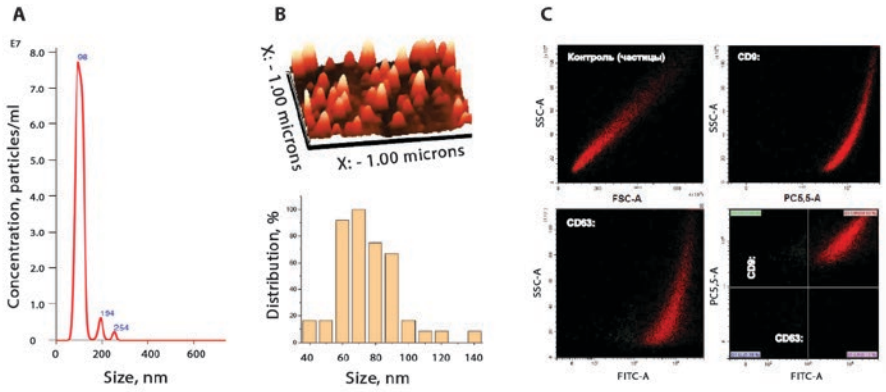
Fugure 3. *Results of analysis of the level of GAL3 molecule expression by cells of five lines*
A. Dot-blotting analysis using cell lysate (upper row of "Total" – total GAL3 protein content in cells) and using unchanged cells (lower row of "Membrane" – GAL4 protein content in the surface membrane)
B. Analysis performed using flow cytometry, after the cell membrane permobilization procedure (upper row of "Total" - the total content of GAL3 protein in cells) and when using cells with an unchanged membrane (lower row of "Membrane" – the content of GAL4 protein in the surface membrane)

ability to form xenograft tumors of similar morphology [19]. This fact allowed us to assume that the expression of tissue-specific proteins was preserved in the cells of these lines, and these proteins can be detected in the EVs secreted by cells. Before starting the time-consuming stage of isolation and analysis of EVs, we evaluated the relative level of expression of potential markers by CRC cells.

To increase the reliability of the results, we used two technologies: dot-blotting and flow cytometry. Both methods allow us to evaluate the total protein content in cells and separately analyze their content in the surface cell membrane. It was assumed that the detection of "potential markers" in the composition of the cell membrane increased the chances of "presence" of these proteins in the membrane of secreted EVs cells. Thus, total and membrane expression of each of the 16 "potential" markers was evaluated in cells of five lines using two technologies. The results obtained by dot-blotting are presented in Table 3 (columns "cells": M (membrane) and T (total)), a representative example of the data obtained for a single protein by dot-blotting and flow cytometry (GAL4) is shown in Figure 3. This example shows that both methods show similar ratios of the total and membrane fractions of the GAL4 protein in cells of different lines. For example, in HT29 cells, this protein is found mainly in the cytoplasm, and in HuTu80 cells – as part of the surface membrane. Thus, the ratios determined using dot-blotting were confirmed by the results of flow cytometry for other cell lines. Therefore, the dot-blotting technique was chosen for subsequent EVs studies as simpler and more reliable.

Isolation and Analysis of EVs Secreted by Cells In Vitro

Before studying the expression of "potential" CRC markers in the composition of EVs secreted by cells, vesicles were isolated from the culture medium and examined in accordance with the ISEV recommendations [20]. The size and concentration of the isolated nanoparticles were estimated using the nanoparticles tracking analysis (NTA). After recalculation of the obtained data taking into account the initial volume of the medium, the concentration of nanoparticles fluctuated in the range of 2-10x10^8/ml. The size of the major fraction of nanoparticles was estimated in the range of 80-110 nm. A representative example of the measurement results is shown in Figure 4A. The morphology and degree of heterogeneity of the isolated nanoparticles were evaluated using atomic force scanning microscopy (AFM). An example of the resulting image is shown in Figure 4B (upper panel). The particle sizes were digitized, and the particle size distribution is shown in the histogram (Fig. 4, lower panel). According to the data obtained, the isolated particles are represented by a relatively homogeneous population, with a monomodal size distribution (the size of the major fraction is 60-80 nm). The difference in the results of particle size estimation is determined by the features of the analysis methods: in the case of NTA, the hydrodynamic radius of the particles in the

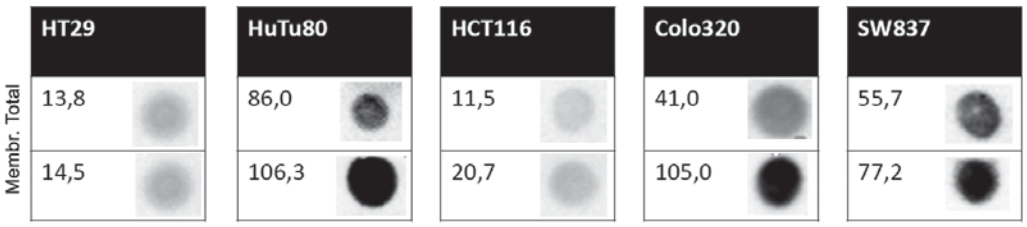


Fugure 4. *Analysis of EVs isolated from the cultural medium (HT-29)*
A. Evaluation of the size and concentration of EVs using nanoparticle tracking analysis (NTA)
B. Evaluation of the degree of homogeneity of the population of isolated EVs using atomic force microscopy (AFM). Above is an image of a fragment of the substrate with vesicles fixed on it. Below is a histogram of the vesicle size distribution.
C. Analysis of the expression of "exosomal" markers CD63 and CD9 on the vesicle membrane using the Exo FACS kit (Hansa BioMed, Estonia). Vesicles were isolated by ultra-centrifugation and non-specifically fixed to latex micro-spheres. After incubation with fluorescently labeled antibodies (CD9 or CD63), an analysis of the fluorescence intensity was performed for the corresponding channels (FITC, PC5.5). The lower right square is the result of combining data on two channels: 98.5% of particles with fixed EVs bind both antibodies, i.e. almost all vesicles are positive for two "exosomal" markers

suspension is calculated, and the AFM estimates the size of the particles fixed to the mica surface, washed and dried. In general, the results obtained for evaluating the physical characteristics of the particles isolated from the culture medium suggest their vesicular nature.
To assess the presence of classical "exosomal" markers on the particle surface, the particles were fixed tolatex micro-spheres and incubated with antibodies to CD63 and CD9 tetraspanins. Flow cytometry has shown that two channels (FITC-CD63, PE-CD9) detect a fluorescent signal on micro-spheres (98.5%), i.e. both markers are present in the membrane of particles fixed to micro-spheres (Fig.3B). Thus, the particles isolated from the medium are represented, atleast partially, by endosomal EVs, or exosomes.

Analysis of Expression of Selected Molecules in Composition of EVs Secreted by CRC Cells

The quantitative analysis of "potential" markers in the composition of EVs was carried out by the dot-blotting method, similar to the study of cells in two ways: by applying intact orlysed EVs to the membrane. The first method allows us to study mainly the composition of the surface membrane, and the second – the total composition of vesicular proteins. An example of the results obtained by analyzing the GAL4 protein in vesicles from all five celllines is shown in Figure 5.
Table 3 (columns "EVs") shows the results for the remaining proteins. In order to assess the fact of the concentration of the studied proteins in the EVs, the content of these molecules in vesicles was expressed in percents of the content in cells. The values of this parameter averaged for five celllines are shown in the table (column "EVs/C, %"). The concentration of seven (CLRN3, CD177, GAL4, GCNT3, Meprin A, MGC4172, PIGY) of 14 proteins, excluding controls (VAMP1, CD63), in EVs was higher in the composition oflysed EVs than in celllysate. This observation confirms the hypothesis of a possible specific secretion (concentration) of these proteins in the vesicles. Since the molecules in the surface membrane of vesicles can be used as diagnostic markers, we performed a comparative assessment of the content of the studied proteins in the surface membranes of EVs and in the composition of lysed vesicles. This ratio was also expressed in percents, the average values for the EVs of the five celllines are shown in Table 3 (column "M/T, %"). As expected, the classic "exosomal" marker tetraspan in CD63 is mainly present in the vesicular membrane (179%). Of the studied proteins, mainly membrane localization was determined in CEACAM6, CLRN3, GAL4, and Meprin A.
The results showed that CRC cells secrete CLRN3, GAL4, and Meprin A proteins as part of the EVs, and these proteins are localized mainly in the vesicular membrane.
This data suggested that the development of CRC may be accompanied by the appearance (increased concentration) in plasma of specific vesicles enriched with CLRN3, GAL4, and/or Meprin A. The membrane localization of these markers determined the possibility of developing a method for their isolation by immune-sorption and detection by flow cytometry.



Fugure 5. *Results of analysis of the AL3 concentration in EVs secreted by CRC cells.*
The analysis was carried out by dot-blotting method, when using the EVs lysate (the upper row of "Total" – the total GAL3 protein content in vesicles) and when using unchanged EVs (the lower row of "Membranes" – the GAL4 protein content in the vesicular membrane)

Table 3. *Results of dot-blotting analysis of the expression of "potential" tissue-specific markers in cells of CRC lines and extracellular vesicles secreted by these cells in vitro. Lysates of cells and vesicles reflecting the total protein content (columns "T") and suspensions of intact cells and vesicles reflecting the level of protein expression in the surface membrane (columns "M").*

Cell lines		HT29				HuTu80				HCT116				Colo320				SW837				EVs/ C, %	M/ T, %
Material		cells		EVs		cells		EVs		cells		EVs		cells		EVs		cells		EVs			
Cellfraction		M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T		
1	EpCAM	15	99	122	80	100	88	45	25	533	85	131	76	2	24	43	51	301	114	67	70	94,2	136,4
2	CD47	151	159	75	85	30	128	50	81	150	178	43	132	108	107	57	40	111	98	72	105	67,1	78,5
3	VAMP1	42	107	160	151	110	91	185	188	119	90	89	70	111	132	211	208	106	102	150	134	142,9	109,0
4	CD63	160	23	192	92	69	49	110	69	180	153	277	151	163	109	217	130	47	105	231	129	175,5	179,1
5	CDH17	20	85	83	71	305	243	18	219	149	57	107	61	361	67	103	96	325	66	83	73	94,6	104,4
6	CDHR5	199	51	54	87	2	111	30	16	44	100	69	133	115	76	35	100	63	103	54	44	89,4	93,0
7	CEACAM6	37	28	52	38	77	114	58	63	45	104	35	90	56	79	58	49	197	74	36	25	72,4	106,6
8	**CLRN3**	**90**	**197**	**320**	**260**	**276**	**95**	**145**	**122**	**87**	**111**	**91**	**48**	**36**	**105**	**238**	**212**	**51**	**99**	**156**	**149**	**131,2**	**129,5**
9	CD177	113	67	121	143	96	63	75	47	65	89	90	66	226	96	57	128	106	99	12	30	104,8	93,3
10	**GAL4**	**13**	**223**	**56**	**104**	**72**	**35**	**272**	**166**	**52**	**93**	**109**	**43**	**108**	**219**	**116**	**87**	**80**	**209**	**213**	**188**	**174,6**	**143,0**
11	GCNT3	127	81	67	154	20	113	72	81	78	73	63	91	83	95	75	34	57	58	79	132	110,9	96,2
12	GPA33	188	99	56	40	89	59	32	66	49	103	82	49	79	122	20	36	69	86	80	112	71,9	97,1
13	**Meprin A**	**7**	**79**	**102**	**55**	**93**	**69**	**129**	**88**	**22**	**62**	**156**	**136**	**15**	**128**	**138**	**136**	**48**	**124**	**160**	**177**	**133,5**	**128,2**
14	MGC4172	135	66	61	77	184	82	157	113	13	106	85	106	31	83	92	136	0	78	82	103	121,9	89,3
15	PIGY	186	53	4	50	48	152	72	135	15	89	69	212	59	101	79	96	39	87	60	65	117,9	53,7
16	REG4	116	184	73	115	29	109	150	122	15	109	110	136	36	66	59	60	1	112	66	66	89,8	93,5

Creation of Method for Immuno-sorption of CRC-exosomes and Quantitative Analysis of Tissue-Specific EVs in Plasma

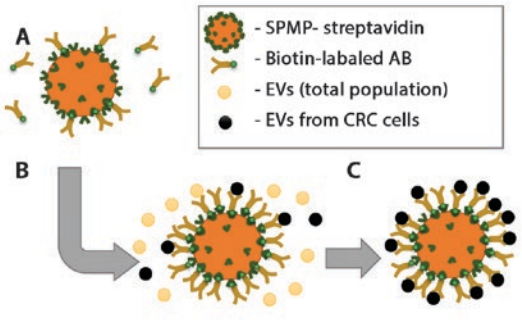
Quantitative analysis (or atleast detection) of tissue-specific EVs can be based on the technology of their specific isolation. To solve this problem, we created immuno-particles consisting of super-paramagnetic micro-spheres (1 µm) whose surface is "decorated" with anti-bodies to "potential" markers: CLRN3, GAL4, and/or Meprin A. The interaction of particles with antibodies was carried out due to the high affinity bond biotin-streptavidin (Fig.6A). During incubation of such immune-particles with EVs isolated from plasma, selective fixation of vesicles whose membrane contains a marker protein occurs (Fig.6B). Then the "fixed" vesicles are labeled with antibodies to any of the "classical" exosomal markers (in our case, CD63), and the quantitative evaluation of the "fixed" vesicles is performed by flow cytometry (Fig.6B).

The created technology was first tested to exclude false positive results due to non-specific interaction of antibodies. We then analyzed ten plasma samples from five patients with a verified diagnosis of CRC and five donors. An example of the result of quantitative evaluation of Meprin A (+) EVs in six plasma samples (3 patients with CRC and 3 donors) is shown in Figure 7.

Thus, an increased content of vesicles is clearly detected in the plasma of patients whose membrane contains the tissue - specific marker Meprin A.

The average results of the analysis of three markers selected during the preliminary stages of the work, and the epithelial "marker" EpCAM in ten samples are presented in table 4.

The concentration of EVs, the membrane of which



Fugure 6. *Scheme of creation and use of immuno-particles for isolation of a specific fraction of EVs*
A.Fixation of antibodies (AB) to superparamagnetic particles (SPMP) through the interaction of biotin withAB and streptavidin with SPMP
B.Incubation of immuno-particles (SPMP- AB complexes)with EVs plasma and interaction of immune-particles with a fraction of specific vesicles.
C. Separation of the SPMP-AB-EVs complex containing specific EVs from plasma vesicles. The resulting complexes can be used for subsequent analysis of the EVs fraction

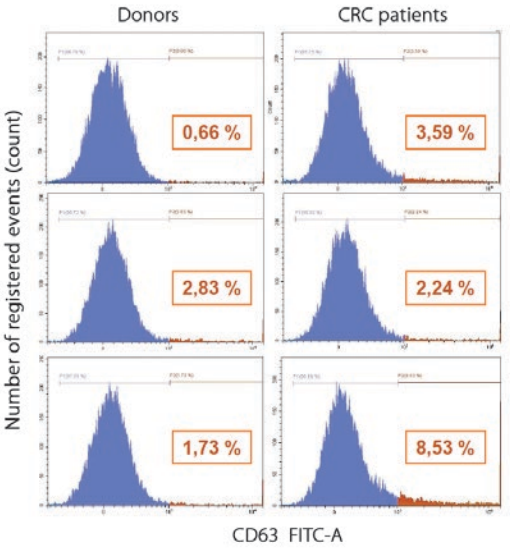
contains tissue-specific markers (CLRN3, GAL4, Meprin A) is significantly increased in the plasma of patients. The results confirm the hypothesis of possible diagnostic significance of tissue-specific vesicular markers. The strategy used for selecting and validating "potential" markers can be used in further research.

DISCUSSION

It is recognized that extracellular nano-vesicles secreted by CRC cells play a significant role in the development of the disease [21]. EVs participate in the process of invasive growth of the primary tumor by modifying the stroma structure, changing the morphology of fibroblasts, and stimulating pathological vascularization. Studies of the systemic effects of EVs secreted by tumor cells are of great practical interest. For example, it has been shown in experimental studies that the efficiency of metastatic dissemination of CRC cells through the mesenteric vascular system to the liver is regulated by the EVs. Among the possible mechanisms of such regulation, the effect of stimulating the differentiation of monocytes [22] and cells expressing the CXCR4 receptor is described [23]. So, against the background of many studies describing the biological effects of EVs secreted by CRC cells, it has not yet been possible to isolate them from plasma and compare their structural and functional features. Specific biochemical characteristics are not yet known that would allow physically separating this fraction of EVs from the total composition of plasma vesicles and elucidating the molecular mechanisms of the observed pathological effects. This situation reduces the practical value of the results of many experimental studies.
In addition, the development of diagnostic technologies based on the analysis of EVs plasma is not a promising direction, if we take into account the fact that only a minor fraction of the analyzed vesicles has diagnostic value.
Previously, we conducted a study to assess the diagnostic potential of exosomal microRNA [24]. As part of this work, the "determination" of the fraction of vesicles secreted by CRC cells was performed by comparing the plasma of patients before and after tumor removal. This approach was based on the assumption that after removal of the tumor, the fraction of vesicles secreted by the cells of this tumor in the plasma will decrease. Accordingly, differences in the profile of vesicular microRNA in the material obtained before and after surgery should reflect the composition of vesicles of tumor origin. This approach, supplemented by the results validation stage, made it possible to identify two molecules, miR-223 and miR-181A, whose combined analysis had a high diagnostic potential. However, these results did not solve the problem of "identifying" a diagnostically significant fraction of EVs. The task of physical isolation of vesicles secreted by CRC cells remained obvious, which would expand the panel of marker microRNA and improve the reliability of the method as a whole. To solve this problem, we assumed that vesicles secreted by cells of high- or medium-differentiated adenocarcinoma may have tissue-specific proteins in the membrane. To confirm this hypothesis, 12 candidate molecules were selected that meet a number of criteria: exclusive or predominant expression in intestinal epithelial cells, membrane localization, and proven expression in the composition of EVs. After analyzing the biopsy

Table 4. *Results of analysis of tissue-specific EVs in plasma of patients with CRC and healthy donors using immuno-particles by flow cytometry. The average data for 5 samples is presented. The significance of the difference between the group of patients and donors was assessed using the Mann-Whitney test*

Markers	CLRN3	GAL4	Meprin A	EpCAM
CRC (patients), n=5	1,7	2,5	4,8	6,2
Control (donors), n=5	0,3	0,9	1,7	11,6
Significance	**	*	**	NS



Fugure 7. *Representative results of quantitative evaluation of Meprin A –positive EVs in the total population of plasma vesicles of healthy donors and patients with CRC.*
Vesicles were isolated using immuno-particles carrying antibodies to Meprin A. After incubation with plasma vesicles and washing, % Meprin A (+) bound EVs were labeled with antibodies to the "exosomal" marker CD63-FITC. Highlighted values are:%, Meprin A (+) EVs

material from the normal colorectal epithelium, cell lines derived from intestinal adenocarcinomas and vesicles secreted by these cells in vitro, the candidate list narrowed to 3 molecules: CLRN3, GAL4, Meprin A. Quantitative evaluation of EVs with one of these markers in the plasma of patients with CRC and healthy donors showed the validity of our assumption. A similar study has recently been done by a group of Korean researchers [25]. This work solved a similar problem, but had a different design: the analysis of proteins in the composition of EVs secreted by cells of two lines (HT29 and HCT-116) was carried out by mass spectrometry, then the candidate molecules were selected from the list of identified molecules that met the criteria used in our work. Finally, the concentrations of selected molecules in the EVs of patients with CCR and healthy donors were compared. The result of the study was the identification of a new vesicular marker of CRC – tetraspain TSPAN1. According to The Protein Athlas database [16], the expression of this molecule is restricted to the intestines and urinary system, i.e. TSPAN1 is also a tissue-specific marker of EVs secreted by CRC cells.

CONCLUSION

The results of the study and the work of Korean colleagues confirm a number of conclusions: 1. CRC cells secrete EVs that include tissue-specific proteins. 2. Quantitative analysis of vesicles that include such proteins has diagnostic potential. 3. Immuno-sorption methods can be used for isolation (or enrichment) and subsequent analysis of the fraction of EVs secreted by CRC cells. Further study in this direction is necessary to expand the list of EVs "markers" secreted by CRC cells (1), optimize the technology for specific isolation of these vesicles (2), and apply methods for subsequent qualitative analysis of this specific vesicular fraction. The analysis of microRNA from the fraction of tissue-specific EVs seems to be the most promising. Achieving these objectives will be the basis for creating an effective method for non-invasive diagnosis of CRC.

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