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КАНЦЕРОГЕННОСТЬ МАЛАТИОНА И ЭСТРОГЕНА НА ЭКСПЕРИМЕНТАЛЬНОЙ МОДЕЛИ РАКА МОЛОЧНОЙ ЖЕЛЕЗЫ У КРЫС

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Аннотация

Рак молочной железы является самой распространенной злокачественной опухолью среди женщин как в развитых, так и в развивающихся странах. Этиология рака молочной железы всё еще остается не до конца изученной. Химические вещества, такие как фосфорорганическое соединение – малатион (карбофос), используемый для борьбы с вредителями растений, являются этиологическими факторами для рака молочной железы. Связь между инициацией рака молочной железы и длительным воздействием эстрогена позволяет предполагать, что этот гормон может также играть этиологическую роль в этом процессе. Тем не менее ключевые факторы, лежащие в основе формирования рака молочной железы, еще предстоит установить. Влияние таких субстанций, как малатион и эстроген, анализировалось в экспериментальной модели молочной железы у крыс. Различные цитоплазматические белки играют ключевую роль в превращении нормальной клетки в злокачественную, и среди них есть семейство Ras-super и Ras-гомологичный A (Rho-A). Уровень этих белков был выше у животных, получавших малатион, чем у животных, получавших эстроген. E-кадгерины составляют большое семейство мембранных белков. **Результаты** исследования показали, что экспрессия E-кадгерина и виментина была выше, чем экспрессия белков c-Ha-ras и Rho-A у крыс, получавших эстрогены. При раке молочной железы иммуногистохимический анализ является важным компонентом рутинных патологических исследований и играет существенную роль в диагностической и прогностической оценке тяжести заболевания. **Целью** настоящего исследования было выявление прогностических маркеров для пациентов с раком молочной железы.

Ключевые слова: канцерогенез, крысы, молочная железа, малатион, эстроген, этиология, белки.

CARCINOGENICITY OF MALATHION AND ESTROGEN IN AN EXPERIMENTAL RAT MAMMARY GLAND MODEL

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Abstract

Breast cancer is considered a major and common health problem in both developing and developed countries. The etiology of breast cancer, the most frequent malignancy diagnosed in women in the western world, has

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remained unidentified. Chemicals as the organophosphorous pesticide malathion have been used to control a wide range of sucking and chewing pests of field crops, and are involved in the etiology of breast cancers. The association between breast cancer initiation and prolonged exposure to estrogen suggests that this hormone may also have an etiologic role in such a process. However, the key factors behind the initiation of breast cancer remain to be elucidated. The effect of environmental substances, such as malathion and estrogen was analyzed in an experimental rat mammary gland model. Different cytoplasmic proteins are key in the transformation of a normal cell to a malignant tumor cell and among these are the Ras super family and Ras homologous A (Rho-A). Both types of proteins were greater in animals treated with malathion than those with estrogens. E-Cadherins constitute a large family of cell surface proteins. **Results** showed greater expression of E-Cadherin and vimentin than c-Ha-ras and Rho-A in rats treated by estrogens. In breast cancer, analysis using immunohistochemical markers is an essential component of routine pathological examinations, and plays an important role in the management of the disease by providing diagnostic and prognostic strategies. **The aim** of the present study was to identify markers that can be used as a prognostic tool for breast cancer patients.

Keywords: carcinogenesis, rat, mammary gland, malathion, estrogen, etiology, proteins.

Introduction

Breast cancer is considered a major and common health problem in both developing and developed countries. The etiology of breast cancer, the most frequent malignancy diagnosed in women in the western world, has remained unidentified. The initiation and progression of breast cancer follows a complex multistep process that depends on various endogenous (hormonal imbalances, proliferative lesions, inherited mutations) and exogenous (diet, smoking, radiation, chemical exposures as pesticides) factors [1, 2]. Some of them are widely used with a huge potential for human exposure. Environmental factors are considered to be among the major influencing components causing increase in the incidence of breast cancer risk [3]. An important but unclarified question is the effect that environmental substances may play in the neoplastic process. Epidemiological studies have demonstrated the association between cancer in humans and agriculture pesticide exposure [4, 5].

Breast cancer models, where normal human breast epithelium cells undergo stepwise transformation into malignant cells after treatment with several agents provide the opportunity to understand the cellular and molecular mechanisms involved in breast carcinogenesis. Knowledge of factors that control cell proliferation of normal and neoplastic mammary epithelium, and the molecular basis of such action, is essential for a deeper understanding of the progression of human breast cancer. In rat mammary gland models whereby individual neoplastic transformed stages can be dissected and studied provide an excellent opportunity to address cellular and molecular mechanisms involved in environmental-induced mammary carcinogenesis [6].

Experimental studies have shown that environmental substances (e.g., DDT, polychlorinated biphenyls, 4-nonylphenol,4-octylphenol) seem to be involved in the etiology of breast cancers and can promote mammary cancer [7, 8]. Such substances have been associated with the use of organophosphorous insecticides in agriculture and in non-occupational situations as exposure to contaminated clothing, soil, ground and surface water, as well as drifts from aerial spraying of pesticides [9-11] and it also has been associated with prolonged exposure to female hormones [12].

Chemicals as the organophosphorous pesticide malathion (M) have been used to control a wide range of sucking and chewing pests of field crops, and are involved in the etiology of breast cancers [13]. The association between breast cancer initiation and prolonged exposure to estrogen (E) suggests that this hormone may also have an etiologic role in such a process. However, the key factors behind the initiation of breast cancer remain to be elucidated. Studies have found an association between human cancer and exposure to agricultural pesticides as demonstrated by IARC [4, 5].

An adequate animal model system has been developed to study the morphological changes that occur during mammary gland carcinogenesis. Mammary gland development is an attractive experimental animal model for understanding the effect of carcinogens since cell is not a random event, but is related to the topography of the mammary parenchyma and changes affected by age, hormonal variations and parity history. The structure of a normal rat mammary gland is composed of a single primary or main lactiferous duct that branches into alveolar buds (ABs) and secondary ducts that are narrow and straight and end in small club-shaped terminals, called terminal end buds (TEBs) that are equivalent to the terminal ductal lobular unit in the human breast, considered the site of origin of human breast carcinomas [14, 15]. The susceptibility of the mammary gland to M alone and in the presence of E was previously analyzed in an established rat mammary gland model already reported [2, 12].

The Ras super family and Ras homologous A (Rho-A) have been shown to promote both cell proliferation and cell invasion [16, 17]. Genetic evolutionary changes may occur in a preferred sequence in solid tumors and among the steps in this sequences include c-Ha-ras oncogene over-expression in breast cancer [18, 19]. This oncogene plays an important role in the progression of mammary cancer. An over expression

of mutated c-Ha-ras oncogene has been previously reported in 10% of breast cancer patients [18]. The Ras gene has been reported to be involved in chemically induced mammary carcinomas, in breast cancer cell lines, and in primary breast cancers, in which point mutations, loss of heterozygosity are present [20].

Accumulating data indicate that Rho-A protein-dependent cell signaling is important for malignant transformation [21-23]. Once activated, Rho-A triggers a complex set of signal transduction pathways. Rho-A is over-expressed during tumorigenesis [24]. Authors [25] examined several breast cancers and found that the over-expression of Rho-A is involved in human carcinogenesis. All breast tumors analyzed contained large amounts of Rho-A protein, whereas was hardly found in adjacent normal tissue. Breast cancer progression from grade I to grade III, classification of World Health Organization (WHO) was accompanied by a significant increase in Rho-A protein levels.

E-cadherins constitute a large family of cell surface proteins, including E (epithelial), N (neural), VE (vascular endothelial), P (placental), R (retinal) and K (kidney) cadherins [26]. Classical cadherins are single-pass transmembrane proteins which participate in Ca^{2+}-dependent cell adhesion that is necessary to form solid tissues [27, 28]. E-cadherin is functionally linked to the generation of a polarized epithelial phenotype [29, 30]. The extracellular region of E-cadherin extends from the cell surface and binds to cadherins present on adjacent cells [31] whereas its intracellular region contains binding sites to interact with catenins and other regulatory proteins [32].

Different cytoplasmic proteins are important in the transformation of a normal cell to invasive tumor cell and among them is the vimentin. It is a 57-kDa intermediate filament protein which forms a part of the cytoskeleton. It is one of the cytoplasmic intermediate filament proteins, which are the major components of the cytoskeleton normally found in embryonic or mesenchymal stem cells [33, 34].

The aim of the present study was to evaluate Ras, Rho-A, E-Cadherin and Vimentin protein expression by immunohistochemistry in a transformed rat model induced by an environmental substance as malathion and an endogenous substance as estrogen to provide evidences that it can be used as good a prognostic tool for breast cancer patients.

Materials and Methods

Experimental designs: Thirty-nine-day-old virgin female Sprague-Dawley rats were obtained from the Catholic University of Chile (Santiago, Chile) and housed and bred in a barrier animal facility operated in accordance with the standards outlined in Guide for the Care and Use of Laboratory Animals [35]. All animals were allowed continuous access to a standard laboratory chow diet (Champion, Santiago, Chile). Experimental design: i) control group received saline solution, 250 µg/100 g body weight. Treated animals were injected subcutaneously (s.c.) for 5 days, twice a day with: ii) malathion (M) (Fyfanon TM, Cheminova, Denmark) that received 22 mg/100 g bw, iii) 17β-estradiol (E) (Sigma-Aldrich Chemical Co., Milwaukee, USA), 30 µg/100 g bw and iv) combination of both (M+E). The LD50 values of the substances were 1.000 mg/kg. However, the dose used in these experiments was $1/6^{th}$ of the LD50 for M, which allowed a 100% survival of animals after a 5-day treatment [2]. Animals were housed three per cage and palpated weekly to detect formation of tumors and sacrificed after 400 days following a 5-day treatment. Animals to be sacrificed were anesthetized by intraperitoneal injections of sodium pentobarbital (8 mg/100 g bw) and opened by a midline incision from the pubis to the sub-maxillary area to remove the tissues.

Immunohistochemistry

Mammary glands and palpable tumors were fixed in 10% neutral buffered, embedded in paraffin, then serially sectioned at a thickness of 5 µ and stained with hematoxylin-eosin (HE). Rat mammary gland tissues were excised to analyze protein expression by immunohistochemistry. Tissues were analyzed using a binocular microscope (Olympus CX31) with lens of 10 x in which a 1-mm^2 grid was installed in one of the oculars. The localization of the antibody was visualized using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and counterstaining with Mayer's hematoxylin (Sigma-Aldrich Chemical Co., Milwaukee, USA). All samples investigated were tested for anti-mouse monoclonal or polyclonal antibodies: H-Ras (mouse, sc-29), Rho-A (sc-418), E-Cadherin (mouse, sc-8426) and Vimentin (sc-6260) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immuno peroxidase staining of protein expression in the slides after treatment with pesticide and estrogen were examined in 10 fields of microscope. Five slides were counted per animal to determine per mm^2 the number of stained proliferative ducts and lobules filled with hyaline casts in the mammary gland from control and treated-animals. Fifty ducts in stage of proliferation (dsp/mm^2) and fifty lobules filled with secretion were counted. Such results were scored according to scale from 0 to 30 points. The ratings were: none (0 points), weak (10 points), slight (15 points), moderate (20 points) and intense (30 points) protein expression. Structures were graded as 0 when morphology of normal structure was present and there was not proliferative ducts and lobules with hyaline casts in the center of the structures. Immunochemical data were expressed as the average ± standard error (SE) of the mean. Statistical comparison between groups and controls were made by ANOVA and Dunnet´s test with $P \leq 0.05$ between groups was considered to be significant.

Results

Analysis of stage for treatment of breast cancer by using markers of immunohistochemistry remains an essential component of routine pathological

examinations, and plays an important role in the management of the disease by providing diagnostic and prognostic strategies. The evaluation of Ras, Rho-A, E-Cadherin and Vimentin protein expression by immunohistochemistry in a rat mammary gland cancer model of transformed cells by pesticides in the presence of hormones as estrogens gave us evidences that they can be used as a good prognostic tool for breast cancer patients.

We have previously reported a synergism in the type of structures by the effect of M and E in the structures present in rat mammary gland development by using morphological measurements [13]. In the present study such effect was confirmed by immunohistochemistry by determining Ras, Rho-A, E-Cadherin and Vimentin protein expression. Results indicated that M induced significantly ($p > 0.05$) higher number of ducts in stage of proliferation per mm^2 in mammary glands and had significantly greater ($P \leq 0.05$) c-Ha-ras (Figure 1A), Rho-A (Figure 1B), E-Cadherin (Figure 1C) and Vimentin (Figure 1D) protein expression at 400 days after a 5-day treatment in comparison to control, E and M+E treated rats, as seen in graphs. Figure 1E shows relative protein expression determined by peroxidase staining and correspond to M+E-treated animals on similar proteins where there was significantly ($P \leq 0.05$) greater c-Ha-ras and Rho-A than Cadherin and Vimentin expression.

Representative images of cross section of mammary gland immunostained with c-Ha-ras (Figure 2 B a-e), Rho-A (Figure 2 C a-e), E-Cadherin (Figure 2D a-e) and Vimentin (Figure 2E a-e) protein expression are seen. It can be observed that the control rats had normal mammary gland duct formation. A normal duct is shown in Figure 2 A a). Cross section of ducts in stage of proliferation can be seen in M-treated animals. Figure 2A b-d) represents ducts in stage of proliferation of M-treated rat stained with HE. Figure 2A e) corresponds to a cross section of a tumor formed in animal treated with M.

The relative c-Ha-ras, Rho-A, E-Cadherin and Vimentin protein expression in lobules can be seen in Figure 3A-D. Graphs correspond to the quantification of mammary glands of rats treated with E on relative average number of lobules with casts in stage of proliferation/mm^2 in immune-stained cells. It was studied the effect of control, malathion (M), estrogen (E) and combination of both on relative protein

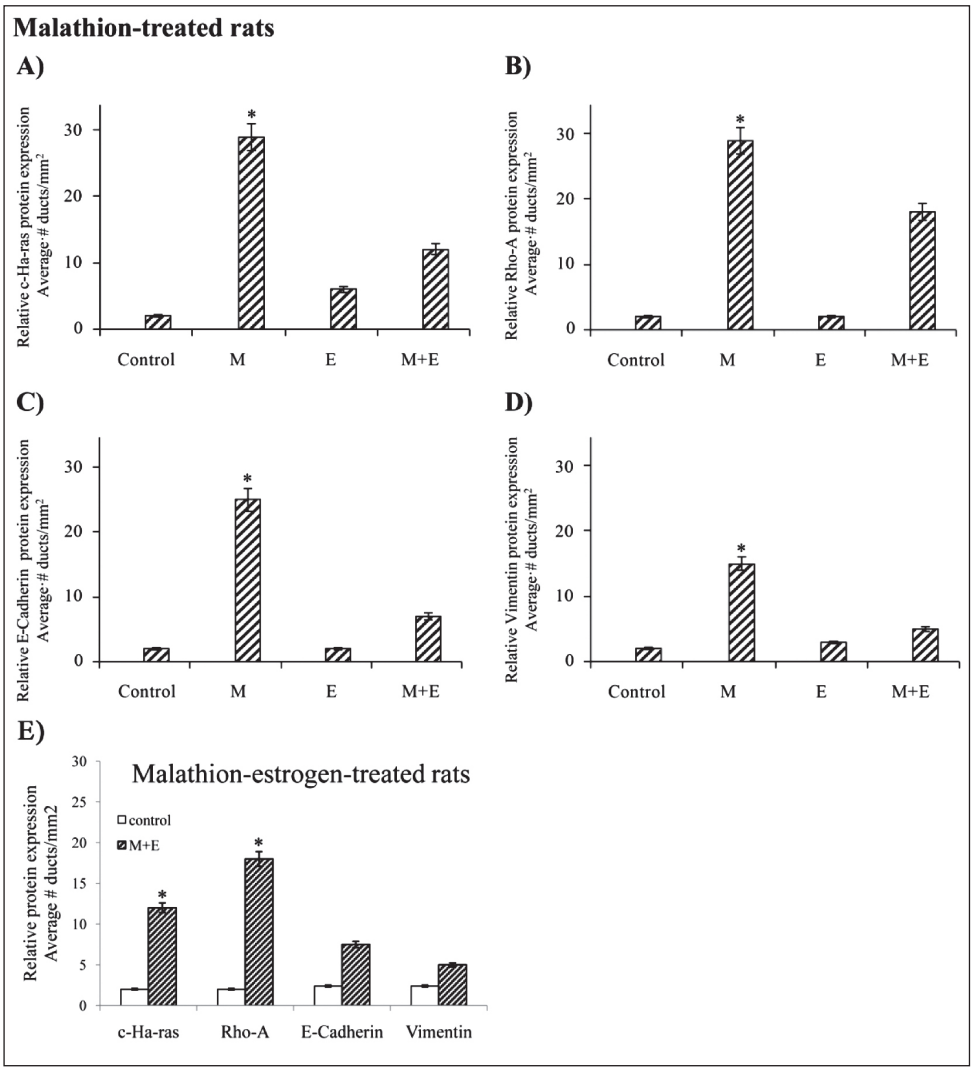

Figure 1. Effect of control, malathion (M), estrogen (E) and combination of both on relative protein expression determined by peroxidase immune-staining in cross sections of rat mammary gland tissues and tumors derived from such animals: Graphs correspond to the quantification of relative average number of ducts in stage of proliferation (dsp/mm^2) in sections immune-stained with: i) Malathion on: A) c-Ha-ras, B) Rho-A, C) E-Cadherin and D) Vimentin, and ii) Malathion+Estrogen on similar proteins. E) Graph corresponds to Malathion+Estrogen-treated animals on similar proteins where c-Ha-ras and Rho-A expression was greater than Cadherin and Vimentin ($P \leq 0.05$)

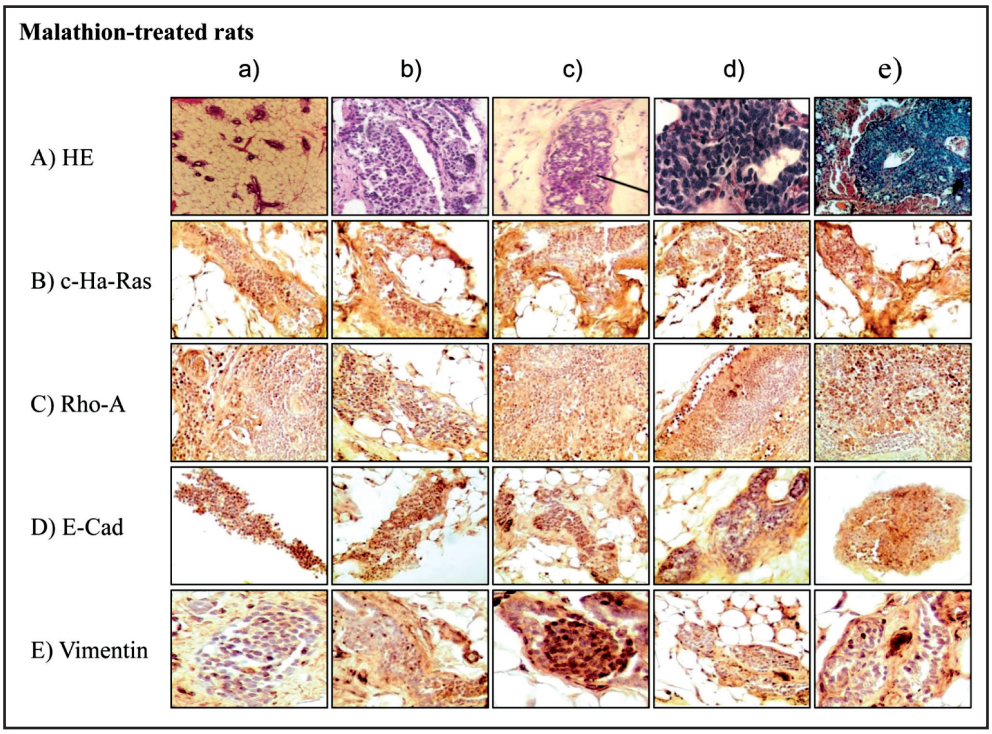

Figure 2. Representative images of cross section of rat mammary gland tissues and tumors stained with hematoxin and eosin) (2A a-e) and immune-peroxidase stained. Effect of M on: c-Ha-Ras (2B a-e), Rho-A (2C a-e), E-Cadherin (2D a-e) and Vimentin (2E a-e) protein expression

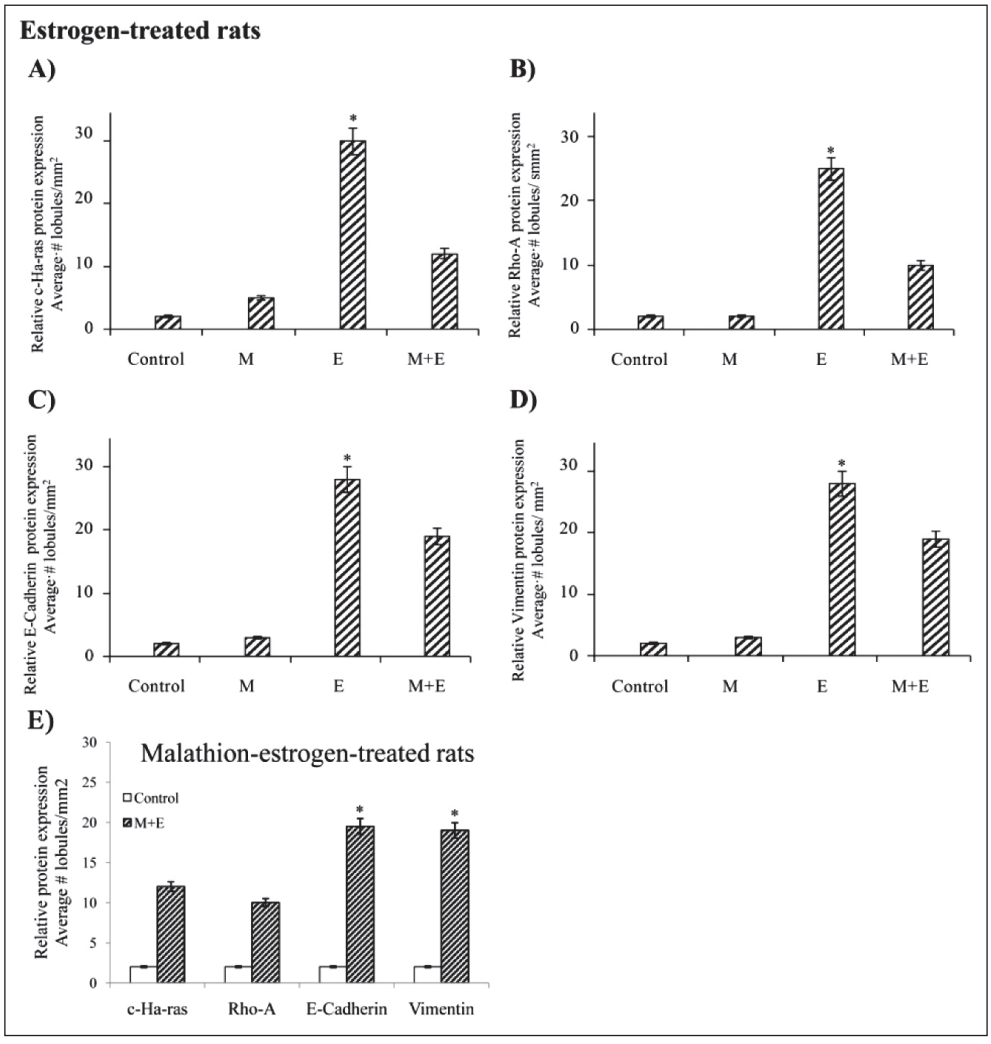

Figure 3. Effect of control, malathion (M), estrogen (E) and combination of both on relative protein expression determined by peroxidase immune-staining in cross sections of rat mammary gland tissues and tumors derived from such animals: Graphs correspond to the to the quantification of mammary glands of rats treated on relative average number of lobules with casts in stage of proliferation/mm² immune-stained with i) A) c-Ha-ras, B) Rho-A, C) E-Cadherin and D) Vimentin protein expression in control, M, E and combination of both-treated- group. ii) E) Graph corresponds to Malathion+Estrogen-treated animals on similar proteins where Cadherin and Vimentin expression was greater ($P \leq 0.05$) than c-Ha-ras and Rho-A

expression determined by peroxidase immune-staining in cross sections of rat mammary gland tissues and tumors derived from such animals. Results showed that E significantly ($P \leq 0.05$) increased the number of lobules filled with casts parallel to greater of A) c-Ha-ras, B) Rho-A, C) E-Cadherin and D) Vimentin protein expression than control, M, E and combination of both-treated- group at 400 days after a 5-day treatment in comparison to controls, M and M+E combined. In Figure 4A a can be seen that the control rat mammary tissue presented a single main lactiferous duct that branches into smaller ducts from which TEBs and ABs are formed. Figure 4A b) shows a normal mammary gland lobule. Cross section of lobules of mammary glands of E-treated rat stained with HE can be seen in Figure 4A c-d. Figure 4A e corresponds to a cross section of a tumor formed in animal treated with E. Figure 1E shows relative protein expression determined by peroxidase staining and correspond to M+E-treated animals on similar proteins where

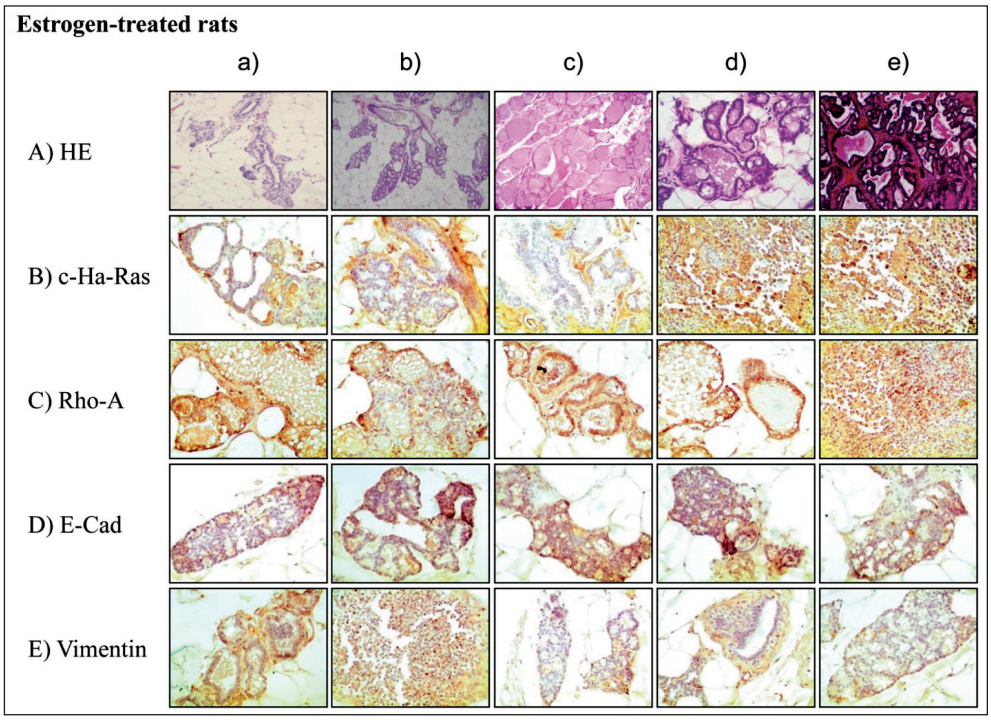

Figure 4. Representative images of cross section of rat mammary gland tissues and tumors stained with hematoxin and eosin) (4a-e) and immune-peroxidase stained. Effect of E on: c-Ha-Ras (4B a-e), Rho-A (4C a-e), E-Cadherin (4D a-e) and Vimentin (4E a-e) protein expression

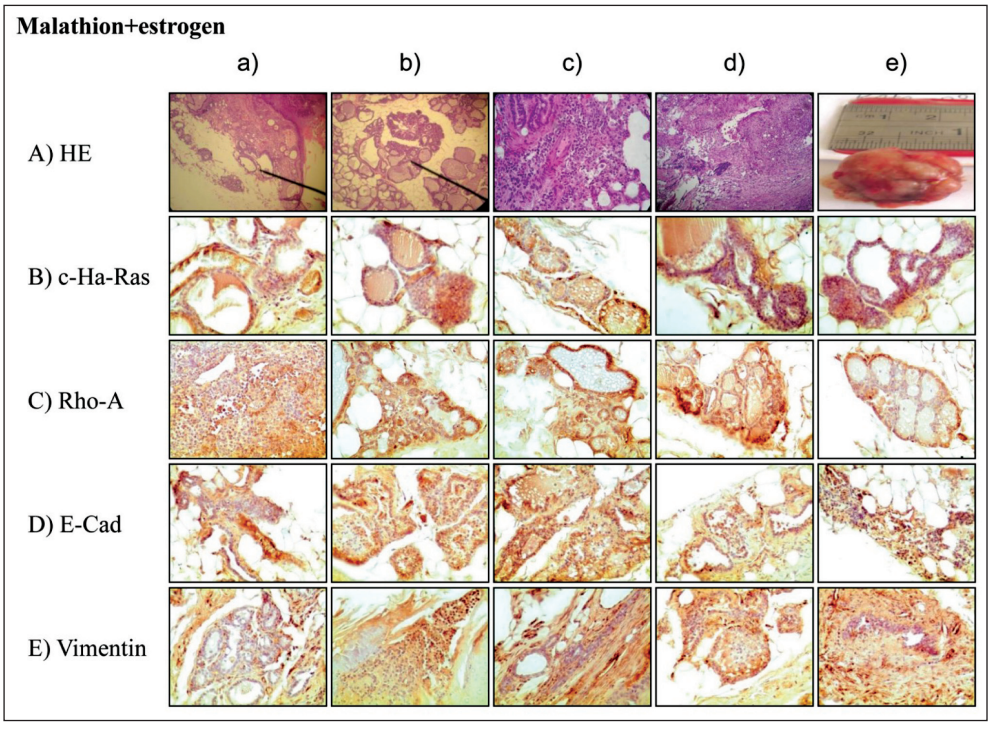

Figure 5. Representative images of cross section of rat mammary gland tissues and tumors stained with hematoxin and eosin) (4a-e) and immune-peroxidase stained. Effect of M and E on: c-Ha-Ras (5B a-e), Rho-A (5C a-e), E-Cadherin (5D a-e) and Vimentin (5E a-e) protein expression

E-Cadherin and Vimentin was significantly ($P \leq 0.05$) greater than c-Ha-ras and Rho-A expression.

Cross section of mammary glands of M+E-treated rat and stained with HE can be seen in Figure 5A a-d). Figure 5A e) corresponds to a tumor formed in animal treated with M+E. Results indicated that mammary glands of such animals showed numerous lobules with secretion or casts in stage of proliferation as well as ducts in stage of proliferation parallel to c-Ha-ras (Figure 5B), Rho-A (Figure 5C), E-cadherin (Figure 5D) and Vimentin (Figure 5E) protein expression at 400 days after a 5-day treatment in comparison to controls, E and M. The relative c-Ha-ras, Rho-A, E-Cadherin and Vimentin protein expression in ducts in stage of proliferation and lobules can be seen in graphs of Figures 5A-D. Figure 5E shows relative protein expression determined by peroxidase staining and correspond to M+E-treated animals on similar proteins where E-Cadherin and Vimentin was significantly ($P \leq 0.05$) greater than c-Ha-ras and Rho-A expression. Cross section of ducts in stage of proliferation and lobules filled with secretion can be seen in M+E-treated animals. Figure 5A a-d) stained with HE. Figure 5A e) corresponds to a tumor formed in animal treated with M+E. Representative images of cross section of mammary gland of these animals immunostained with c-Ha-ras (Figure 5B a-e), Rho-A (Figure 5C a-e), E-Cadherin (Figure 5D a-e) and Vimentin (Figure 5E a-e) protein expression are seen.

Discussion

The evaluation of Ras, Rho-A, E-Cadherin and Vimentin protein expression by immunohistochemistry in this transformed model induced by pesticides as malathion and an endogenous substances as estrogen provided evidence that it can be used as good a prognostic tool for breast cancer patients. The ducts markedly increased in size and number of cells per mm^2. Furthermore, such structures increased until tumors started to appear with similar type of cells and after 400 days of pesticide of 5 day treatment those structures were referred to as proliferative ducts. M alone induced changes exclusively at the level of ducts that increased in size and number of cells per mm^2, these were defined as ductal in stage of proliferation (dsp/mm^2) and the interesting finding was that as time progressed those structures were transformed in mammary gland tumors that revealed a similarity to ductal carcinomas as described by WHO [15] in comparison to controls. Sections of the ducts were filled with increased number of cells with dark nucleus.

E induced significant progressive alterations in lobules in comparison to control in the rat mammary gland after 400 days of the 5-day treatment. Congested tubules were filled with pink eosinophylic deposits. Furthermore, such structures increased in number per mm^2 and also in size until tumors started to appear. E-treated rats showed that the density of the number of terminal end buds per mm^2 decreased as time progressed and lobules became markedly abnormal, while large and dilated congested structures increased in size and number per mm^2. Results indicated that E alone increased the average number of lobules per mm^2 of rat mammary glands in comparison to control and M treatment alone at 400 days after a 5-day treatment. Such structures were referred to as secretory lobules since they were congested tubules filled with pink deposits until lobular carcinomas started to appear and revealed that the tumors originated were pathologically similar to lobular carcinomas according to WHO [15]. Mammary gland of E-treated animal had altered lobules full of hyaline casts; the control rats had normal lobule formation. The increase in the number of secretory lobules was higher in the E-treated animals after 400 days of the 5-day injections than in the other treated group. Lobules increased in size with the time and mammary gland tumors were induced by the effect of E alone.

Environmental endocrine disrupting chemicals, including pesticides and industrial chemicals, are also considered among the environmental agents that are released into the environment producing deleterious effects on wildlife and humans. Lacassagne in 1932 [36] was the first to demonstrate that the administration of estrogens to experimental animals increased the incidence of mammary cancer, indicating at the same time that sex hormones were involved in the development of neoplasias and their progression in hormone-target organs such as the prostate and the breast. This knowledge was soon applied to cancer treatment [13].

Combination of M+E induced greater cellular changes in the rat mammary glands than E or M alone in comparison to control animals after 400 days of the 5-day treatment. Increased amount of proliferative ducts and secretory lobules were induced by these two substances showing rat mammary gland tumor formation. Mammary gland tumor formed by the effect of these substances was characterized by the presence of both types of structures as ducts in stage of proliferation and secretory lobules [13].

Our studies showed that M induced greater c-Ha-ras protein expression in ducts in proliferative stage at 400 days after a 5-day treatment in comparison to control, E and M+E treated rats. High c-Ha-Ras expression in intraductal proliferative lesions is very relevant since these epithelial lesions have a risk of progression to invasive breast cancer and related issues, particularly atypical ductal hyperplasia [37]. However, the use of c-Ha-Ras expression as a diagnostic and prognostic marker should be selective and should preferably be used in conjunction with other markers. According to published data, the major value of c-Ha-Ras expression is in its clinical correlation with an improved prognosis of relapse [38-40]. Since Ras is often mutated in human cancers, much effort has been devoted to devising

means of controlling the activity of Ras [41]. Breast tumors have been shown to have an elevated expression of the Harvey Ras oncogene when compared to their respective normal tissue sections [42]. The Harvey Ras oncogene has also been shown to have a significant correlation with the clinicopathological characteristics of breast cancer [43]. Immunohistochemical analyses of Ras oncogene expression in human breast lesions have been carried out [44], as well as of the p21 Ras oncogene [45-47], with a high expression in breast cancer patients indicating clinical significance.

Our studies showed that E induced greater c-Ha-ras, Rho-A, Vimentin and E-Cadherin protein expression in lobules full of casts and secretion at 400 days after a 5-day treatment in comparison to control, E and M+E treated rats. Rho-A protein-dependent cell signaling is important for malignant transformation [21-23]. Once activated, Rho-A triggers a complex set of signal transduction pathways. Rho-A is over-expressed during human carcinogenesis [24, 48]. M also induced greater Vimentin protein expression that paralleled neoplastic cells with metastatic properties, including breast cancer cells as we have previosly reported in breast cancer patients [49]. E-Cadherins that constitute a large family of cell surface proteins was altered by these substances and its expression was greater than control. It can be concluded that Ras, Rho-A, E-Cadherin and Vimentin are good marker to be used in breast cancer patients and to predict response to therapy in such patients.

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Conflict of interest

The authors declare that they have no conflict of interest.

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ИЗУЧЕНИЕ ОСОБЕННОСТЕЙ МИГРАЦИИ ДЕНДРИТНЫХ КЛЕТОК В ЭКСПЕРИМЕНТАЛЬНОЙ АНАЛИТИЧЕСКОЙ СИСТЕМЕ CELL-IQ

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Аннотация

Дендритные клетки (ДК) – это специализированная группа антиген-презентирующих клеток с высокой функциональной пластичностью, которые проявляют иммуностимулирующий или иммуносупрессивный потенциал в зависимости от последовательности и комбинации стимулов микроокружения, определяющих их дифференцировку, созревание, активацию. Разработка противоопухолевых ДК-вакцин основана на способности ДК специфически активированных *in vitro* мигрировать для презентации антигенов Т-лимфоцитам *in vivo*. В работе проведено изучение элементов опухолевого микроокружения, способных оказывать влияние на миграцию ДК. Изучение подвижности ДК в экспериментальной аналитической системе Cell-IQ показало наличие обратной корреляции высокой силы между средней скоростью прохождения траектории ДК и содержанием иммуносупрессивных факторов (ИСФ) в супернатантах культивируемых клеток меланомы кожи ($TGF\beta_1$, IL-10, IL-18, VEGF-A, EGF, FGF, HGF, sFASL ($p<0,01$)). Была выявлена обратная зависимость высокой силы угла движения популяции ДК от экспрессии раково-тестикулярных антигенов (РТА) и других опухолеассоциированных антигенов (ОАА) на опухолевых клетках: Melan A, тирозиназы, семейств MAGE, BAGE, NY-ESO-1 ($p<0,05$). Скорость движения ДК в системе ко-культивирования с клетками меланомы №894 составила 30,10±2,23 мкм/ч и отличалась от таковой в присутствии 1 нг/мл IL-10 (10,45±0,52 мкм/ч), 10 нг/мл $TGF\beta_1$ (14,32±0,42 мкм/ч), 50 нг/мл VEGF (18,7±1,89 мкм/ч) ($p<0,05$). Можно предполагать, что содержание этих ИСФ в периферической крови больных является одним из факторов, определяющих клиническую эффективность противоопухолевой ДК-иммунотерапии.

Ключевые слова: дендритные клетки, миграция, иммуносупрессивные факторы, раково-тестикулярные антигены, Cell-IQ.

STUDY OF DENDRITIC CELL MIGRATION USING CELL-IQ ANALYSIS SYSTEM

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Abstract

Dendritic cells (DCs) belong to specialized pool of antigen-presenting cells with high functional plasticity and manifest with immunostimulatory or immunosuppressive potential depending on sequence and combination of microenvironment stimuli, which determine their differentiation, maturation and activation. The use of antitumor DCs vaccines is based on the ability of DCs specifically activated *in vitro* migrate for antigen presentation to T-lymphocytes. We studied the components of the tumor microenvironment that are capable of inhibiting DCs migration. The study of the mobility of DCs in Cell-IQ experimental analytical system showed the presence of an inverse correlation of high strength between the average trajectory speed and the level

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of immunosuppressive factors (ISFs) in supernatants of cultured skin melanoma cells ($TGF\beta_1$, IL-10, IL-18, VEGF-A, EGF, FGF, HGF, sFASL ($p<0.01$). An inverse relation of high force was found between the movement angle of the DCs population and the expression of cancer testis antigens (CTAs) and other tumor-associated antigens (TAAs) on tumor cells (Melan A, tyrosinase, families of MAGE, BAGE, NY-ESO-1 ($p<0.01$)). The speed of DCs movement in culture system with melanoma cells #894 was 30.10±2.23 μm/h and differed from that in the presence of IL-10 1 ng/ml (10.45±0.52 μm/h), $TGF\beta_1$ 10 ng/ml (14.32±0.42 μm/h), VEGF 50 ng/ml (18.7±1.89 μm/h) ($p<0.05$). One can assume that content of this ISFs in the blood is one of the factors determining clinical efficacy of DCs immune therapy.

Keywords: dendritic cells, migration, immunosuppressive factors, cancer testis antigens, Cell-IQ.

Introduction

Tumor cells are capable of synthetizing and producing a range of factors with inhibitory effect on the activity of immune cells, among which the effect on dendritic cells (DCs) system is particularly important. Cellular organisation of tumor microenvironment represents sophisticated living system with a complex of different stimuli, which influence all aspects of DCs biology and thus manage their functionality and vitality [1-3]. DCs belong to specialized pool of antigen-presenting cells with high functional plasticity and manifest with immunostimulatory or immunosuppressive potential depending on sequence and combination of microenvironment stimuli, which determine their differentiation, maturation, activation and polarisation. Immature or maturating DCs are found in different nonlymphoid tissues and organs but after activation they migrate towards lymphoid tissues to interact with T-lymphocytes and induce immune response [4]. A low level expression of the MHC antigens and costimulatory molecules is typical for the immature DCs. They are not capable to efficiently activate T-lymphocytes still they have ability for high degree endocytosis [5-7]. Activation of DCs under the influence of different stimuli of maturation is associated with the development of increased expression of a variety of intracellular and surface molecules providing migration of DCs to secondary lymphoid tissues and interaction with T-lymphocytes. However, activation and maturation of DCs are dependent on microenvironment and could be blocked by specific factors or their combinations leading to the formation of subpopulations of DCs with tolerogenic and immunosuppressive activity [8].

Experimental studies by K.M. Hargadon et al. (2016) on the model of murine melanoma have shown the influence of factors produced by malignant melanocytes on the maturation and subsequent activation of differentiated DCs from spleen and lungs [1]. A number of researchers have established facts of disturbed biophysical properties of DCs in particular ability to change shape of the cell under the influence of immunosuppressive molecules expressed by a tumor, such as interleukin 10 (IL-10), transforming growth factor ($TGF\beta_1$), vascular endothelial growth factor (VEGF) [9-11].

The use of antitumor dendritic cell vaccines is very relevant as it is based on the ability of the DCs, specifically activated *in vitro*, to migrate to T-lymphocytes for antigen presentation, therefore, the study of the tumor microenvironment components forming systemic and local immunosuppression in the patient's body is able to modify properties of the DCs, thus reducing clinical efficacy of immunotherapy.

Materials and methods

The study was done with immature DCs (iDCs) obtained from monocytes of peripheral blood samples of patients with skin melanoma and with skin melanoma cells obtained from surgical material of patients treated in NMRC of oncology named after N.N. Petrov from 2001 to 2017 years. Skin melanoma cells were maintained as cell lines after 20 or more passages, part of which was deposited in the Collection of cell cultures of the Institute of cytology of Russian Academy of Sciences, Saint-Petersburg, Russia.

Differentiation of DCs from the adhesion monocyte fraction from peripheral blood mononuclear cells of a patient with skin melanoma was carried out according to a standard procedure developed in the NMRC of oncology named after N.N. Petrov [12], in adhesive culture flasks (Sarstedt, Germany) under conditions of CO_2 incubator «Heracel» (Termo Electron LTD GmbH, Germany) at 37°C, 5 % CO_2 and 98 % humidity with culture medium «Cell-Gro DC», granulocyte-macrophage growth factor GM-CSF (72 ng/ml), interleukin 4 IL-4 (15 ng/ml) (CellGenix, Germany), which were introduced on the 1st, 3rd and 5th day of cultivation (iDCs). On the 7th day, DCs were removed with 0.25% trypsin solution and BD Falcon Cell Scrapers (BD Biosciences, USA) and analysed on a flow cytometer and DCs with $CD14^-CD1a^+CD83^-CD11c^+CD86^{\pm}HLA\text{-}DR^+CD80^{\pm}CD209^+$ immunophenotype on 92% of the DCs were used for the experiment.

Skin melanoma cell lines. After mechanical disaggregation of tumor samples, the isolated cells were plated in plastic flasks (Sarstedt, Germany) and placed into CO_2-incubator «Heracel» (Termo Electron LTDGmbH, Germany), continuously cultured and reseeded by the method of Frechney [13], DMEM/F12 culture medium was used (Biolot, Russia) containing 20% bovine embryo serum (Biolot, Russia), insulin, transferrin, selenium, penicillin and streptomycin (Invitrogen, USA). When the monolayer was reached at different passages, the cells were removed using a Trypsin-Versene solution (Biolot, Russia) and used for the experiments. Cell lines were cultivated continuously for 20 or more passages.

Cultivation in the Cell-IQ system. The cultures of the iDCs and skin melanoma cells were seeded into two well silicone inserts (Ibidi GmbH, Germany) at concentration 3 x 10^5 cell/ml in 70 mcl of culture medium containing 72 ng/ml GM-CSF, 15 ng/ml IL-4 and tumor necrosis factor TNFα (20 ng/ml) (BD Biosciences, USA). After the cells were attached to the substrate, the inserts were removed and the plates were placed in analytical cell analyser system Cell-IQ (Chip-Man Technologies Ltd, Finland) and observed by the phase contrast method for 22-47 hours. The distance between the cell populations was 500 μm. To quantify the migration of the DCs we created a special protocol based on the library of different types of cellular images, which was used for qualitative and quantitative analysis of images of moving cells in real time. For the cellular modelling allogeneic cells having a similar immunophenotype by HLA class I: HLA A2 were used.

Quantitative assessment of the production of immunosuppressive factors (ISFs). Quantitative content of ISFs (IL-6, 8, 10, 18, $TGFβ_1$, VEGF, follistatin, angiopoietin, placental growth factor PLGF, epidermal growth factor (EGF), stem cell growth factor (SFC), heparin-binding EGF-like growth factor (HB-EGF), insulin like growth factor binding protein 1 (IGFBP1), urokinase plasminogen activator inhibitor PAI-1, urokinase plasminogen activator uPA, fibroblast growth factor (FGF), hepatocyte growth factor (HGF), leptin, osteoponin, prolactin, CD95 receptor ligand sFASL) were determined in supernatants of cultured skin melanoma cells by an sandwich enzyme

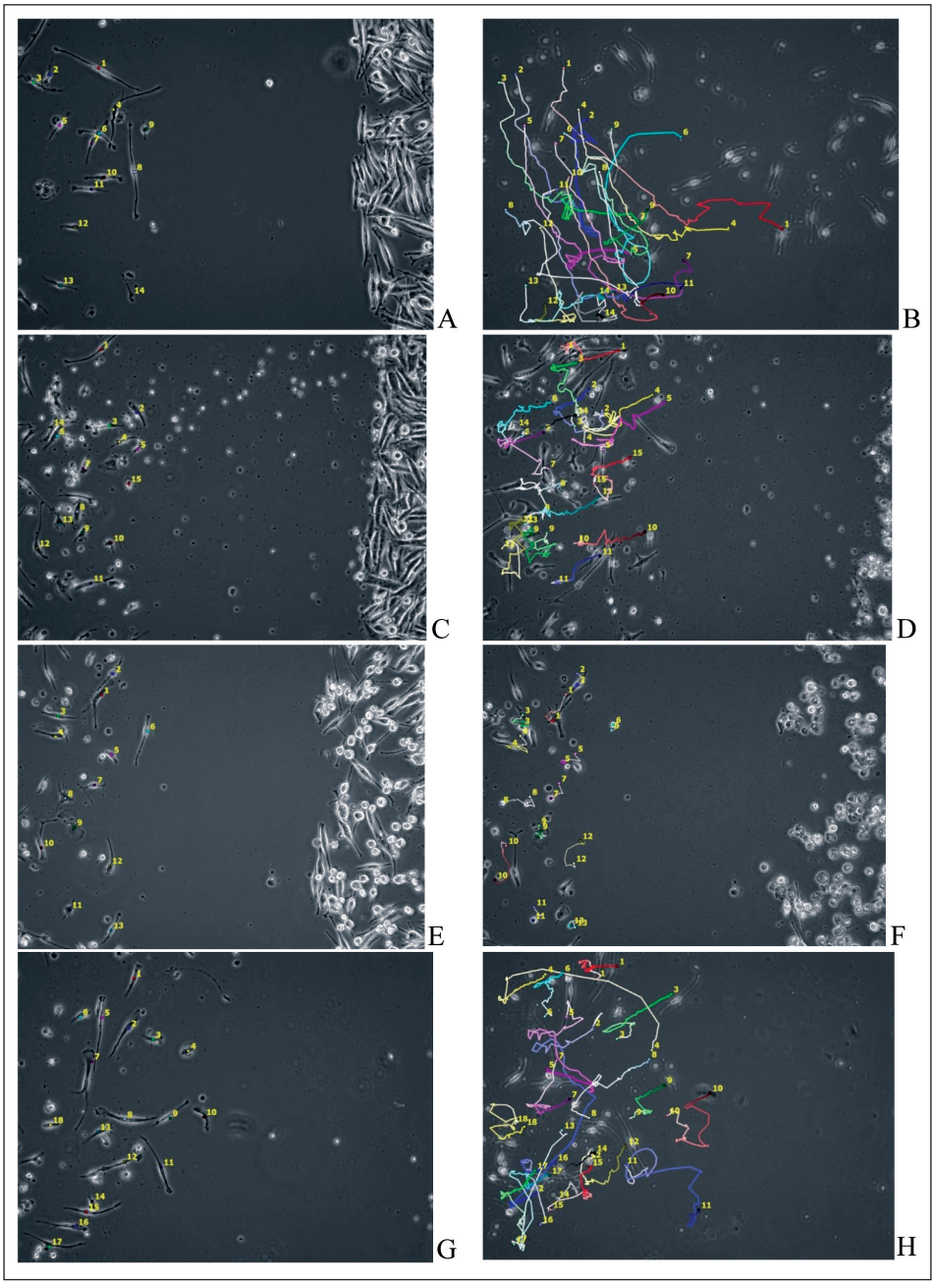

Figure. 1. Analysis of trajectory images of immature DCs movements in the presence of skin melanoma cells line #894.
A – start of DCs movement fixation and B – end of DCs movement fixation under standard conditions of cultivation;
C - start of DCs movement fixation and D - end of DCs movement fixation in the presence of TGFβ1 (10 ng/ml);
E - start of DCs movement fixation and F - end of DCs movement fixation in the presence of IL-10 (1 ng/ml); G - start of DCs movement fixation and H - end of DCs movement fixation in the presence of VEGF (50 ng/ml). Observation time 47 hours

Table 1

Parameters of movement trajectories of immature DCs co-cultivated with skin melanoma cells obtained from different patients

Cellular system: co-culture of immature DCs and skin melanoma cells line #	Observation time (h)	Number of view fields (n)	Average number of tested cells	Distance passed (μm)	Trajectory length (μm)	Average speed of trajectory pass (μm/h)	Angle of cell population movement (°)
226	24	10	13	213.5±6.67	2094.2±15.43	88.3±1.79	51.6±3.45
283	22	8	10	102.5±4.32	398.4±12.33	17.4±1.98	340.2±9.41
311	24	9	15	94.9±9.49	374.9±19.20	16.1±1.05	343.6±3.35
643	24	10	15	132.9±5.43	1121.2±74.73	41.4±1.69	130.6±10.72
685	34	10	12	155.0±8.92	1481.0±24.78	48.6±4.70	144.0±5.23
686	34	8	20	241.0±14.34	2556.0±56.11	75.26±1.65	135.7±2.25
694	26	8	15	168.0±18.0	1345.2±39.59	52.4±1.63	333.6±8.54
894	22	10	12	291.8±31.07	1423.3±105.21	30.1±2.23	324.5±18.30

immunoassay and multiplex analysis on the instrument Bio-Plex 200 System (BIO-RAD, USA). The kits from «BenderMedSystems» (Germany) and «BIO-RAD» (USA) were used.

Quantification of expression of tumor-associated antigens (TAAs). Cultured skin melanoma cells were analysed for the presence of differentiating TAAs (MelanA, tyrosinase, MITF, S100, gp100) and cancer testis antigens (CTAs) (NY-ESO-1, families MAGE, BAGE, GAGE) by the flow cytometry using FACSCanto II cytometer system (BD, USA) with BD Bioscience and Santa Cruz biotechnology reagents (USA).

Statistical data treatment. IBM SPSS 19.0 and Microsoft Excel 2010 software packages and the methods of descriptive and nonparametric statistics were used for the analysis [14]. Quantitative data are presented as mean with standard deviation (M±SD).

Results

To study the features of the motility of maturing DCs under the influence of products synthesized by melanoma cells, an *in vitro* system was created which allowed us to co-culture iDCs with human skin melanoma cell lines (n=8) and to evaluate speed, trajectory and direction of the DCs movement. At least 10 DCs were fixed in each view field and observed afterwards. The pattern of the movement of the DCs was determined by the distance passed by them during the observation period, the trajectory length, the speed of the trajectory pass in the selected period of time, and the angle of movement of the cell population, which determines the general direction of movement of the DCs (Table 1).

We observed great variability in all parameters: the average speed of trajectory pass was minimal when co-cultured with culture #311 (16.1±1.05 μm/h) and maximal in the presence of culture #226 (88.3±1.79 μm/h). Angle of movement of the DCs population that was co-cultured with melanoma cells #226 was the smallest and amounted to 51.6±3.45, while in the presence of melanoma cells #311 this parameter was defined as 343.6±3.35. In order to identify the factors that have the greatest impact on DCs migration we analysed the dependence of the average speed of DCs from the ISFs found in supernatants of cell lines under study. High strength inverse correlation was revealed between this parameter of the DCs motility and with the amount of ISFs in the supernatants of skin melanoma lines, namely for $TGF\beta_1$, IL-10, IL18, VEGF-A, EGF, FGF, HGF, sFASL ($p<0.01$) (Table 2). Under the influence of the ISFs, not only the speed of the DCs movement changed, but also the character of the movement, which became more chaotic and multidirectional. To test the hypothesis of a possible dependence of the direction of DCs movement from the level of expression of differentiating TAAs and CTAs by tumor cells, a correlation analysis was carried out, which revealed inverse correlation of high strength of angle of movement of the DCs population from the expression of antigens, such as Melan A, tyrosinase, families MAGE, BAGE, NY-ESO-1 ($p<0.05$) (Table 3).

To reveal more fully the nature of the effect of individual ISFs synthesized by the tumor on the motility of the DCs, additional defined concentrations of the IL-10 (1 ng/ml), TGFβ1 (10 ng/ml), VEGF-A (50 ng/ml) were added. Table 4 represents the results of the analysis of the movements of the DCs in different conditions in the system of co-cultivation with a culture of skin melanoma #894. The greatest impact was possessed by IL-10, in the presence of which the average speed of DCs movement decreased almost threefold (from 30.10±2.23 to 10.45±0.52 μm/h). Also in the presence of the ISFs, the distance between the beginning and the end of the movement observation and also the length of the trajectory passed decreased significantly, but no effect on the angle of movement of the cell population was detected, since statistically significant difference for this parameter was not found

Table 2

Dependence of the average speed of DCs movement on the production of immunosuppressive factors by cultured skin melanoma cells

Cell line #	$TGF\beta_1$ (ng/ml)	IL-10 (pg/ml)	VEGF-A (ng/ml)	IL6 (pg/ml)	IL8 (ng/ml)	Follistatin (ng/ml)	IL18 (pg/ml)	PLGF (pg/ml)	Angiopoetin (pg/ml)	EGF (pg/ml)	SCF (pg/ml)
226	0.73	1.53	0.79	0	0.37	0.24	0.12	116.90	0	0.43	5.02
283	23.15	48.15	5.59	8.04	0.94	2.09	25.49	632.41	538.89	419.56	2.65
311	19.84	40.70	10.05	17233.50	0.92	3.85	14.15	242.98	683.42	358.38	64.70
643	5.42	16.06	3.60	29.56	20.28	36.39	0.87	263.21	541.99	9.7	18.40
686	3.89	1.51	0.84	32.48	0.84	17.38	0.27	5266.42	12367.14	22.26	24.19
694	3.00	10.08	4.55	109.02	11.70	0.49	1.35	278.78	4999.91	191.00	22.32
685	2.43	36.67	12.70	89.86	11.01	5.17	1.29	46.53	16097.05	2.11	9.63
894	4.57	12.25	1.50	4.45	23.07	0.11	3.64	310.30	5943.86	134.00	11.87
Spearman correlation, r	-0.857	-0.857	-0.833	-0.262	-0.381	-0.143	-0.881	-0.095	-0.429	-0.714	-0.095
p	0.01	0.007	0.037	0.531	0.352	0.736	0.004	0.823	0.289	0.047	0.823
Cell line #	Endoglin (pg/ml)	HB-EGF (pg/ml)	IGFPB-1 (pg/ml)	PAI-1 (pg/ml)	uPA (pg/ml)	FGF (pg/ml)	HGF (pg/ml)	Leptin (pg/ml)	Osteoponin (ng/ml)	Prolactin (pg/ml)	sFASL (pg/ml)
226	0	0	0	39.16	0	40	52.05	34.17	0.47	15.75	0
283	3.00	0	18.30	4228.8	0.13	84.52	118.85	16.64	18.99	24.91	221.5
311	8.50	0	5.80	66.11	0	138.62	1353.74	296.95	17.22	393.69	368.93
643	10.70	262.56	0	1606.96	0	112.62	506.36	55.00	0.38	4.33	0
686	198.42	135.61	62.82	7096.80	54.42	60.75	38.44	104.97	1.82	59.36	3.5
694	17.27	214.45	4.34	4469.30	0	42.47	38.19	24.70	14.24	13.00	4.34
685	15.68	79.20	16.39	5187.17	5.41	26.8	13.48	30.66	3.95	26.28	3.54
894	50.29	20.62	4627.00	5206.00	9433.00	91.52	73.35	21.65	1.13	0	63.29
Spearman correlation, r	0.167	0.317	-0.287	0.119	-0.038	-0.738	-0.690	0.119	-0.500	-0.095	-0.790
p	0.693	0.444	0.49	0.779	0.929	0.037	0.05	0.779	0.207	0.823	0.02

Table 3

Dependence of the DCs direction of movement from TAAs expression by cultured skin melanoma cells (% cells)

Cell line #	melanA	Tyros	S100	Gp100	MITF	MAGE	BAGE	GAGE	NY-ESO-1
226	49.6	97.6	68.7	99.6	21.7	89.7	94.8	99.4	79.1
283	2.5	8.7	1.2	12.6	0	0.6	0.4	1.4	6.3
311	0	28.0	5.2	87.6	49.5	18.1	4.4	92.2	11.1
643	76.8	66.6	1.0	94.1	66.5	60.5	74.5	95.8	61.7
685	40.4	75.6	0	61.6	40.2	30.3	52.5	8.8	47.9
686	11.9	60.9	3.1	77.2	73.1	18.2	24.5	48.6	67.8
694	23.0	0	59.3	90.6	5.9	0	6.8	13.8	0
894	0.7	0	5.8	99.9	0	0	0	7.6	0
Spearman correlation, r	-0.810	-0.707	-0.024	-0.405	-0.383	-0.755	-0.810	-0.571	-0.755
p	0.015	0.050	0.955	0.320	0.349	0.031	0.015	0.139	0.031

under the influence of the studied ISFs (Table 4, Figures 1, 2). Nevertheless, the method of centring the trajectories of the DCs allowed us to determine more precisely the direction of movement of the DC populations and to reveal certain regularities: DCs in control samples migrated predominantly towards the skin melanoma cell population, while in the presence of TGFβ1 and VEGF-A, trajectories of the DCs were shortened, but the general direction was preserved (Figures 2, 3). Under the influence of IL-10, the distance passed by the DCs was minimal and the relative distribution of cells along the length of the trajectory

Table 4

Parameters of trajectories of movement of immature DCs, co-cultivated with skin melanoma cells line #894, evaluated in different conditions

Cellular system	Observation time (h)	Number of view fields (n)	Number of tested cells (min-max)	Distance passed (μm)	Trajectory length (μm)	Average speed of trajectory pass (μm/h)	Angle of cell population movement (°)
Control without exposure	47	8	12–15	291.75±31.07	1423.25±105.21	30.10±2.23	324.50±18.30
$TGF\beta_1$ (10 ng/ml)	47	8	15–18	136.0±7.32	676.0±20.46	14.32±0.42	234.0±3.85
p, vs Control				0.042	0.009	0.009	0.051
IL-10 (1 ng/ml)	47	8	11–15	44.45±1.84	224.25±10.76	10.45±0.52	254.25±11.01
p, vs Control				0.004	0.001	0.002	0.066
VEGF (50 ng/ml)	47	6	10–17	176.5±32.54	881.4±88.90	18.7±1.89	289.1±35.55
p, vs Control				0.068	0.005	0.006	0.067

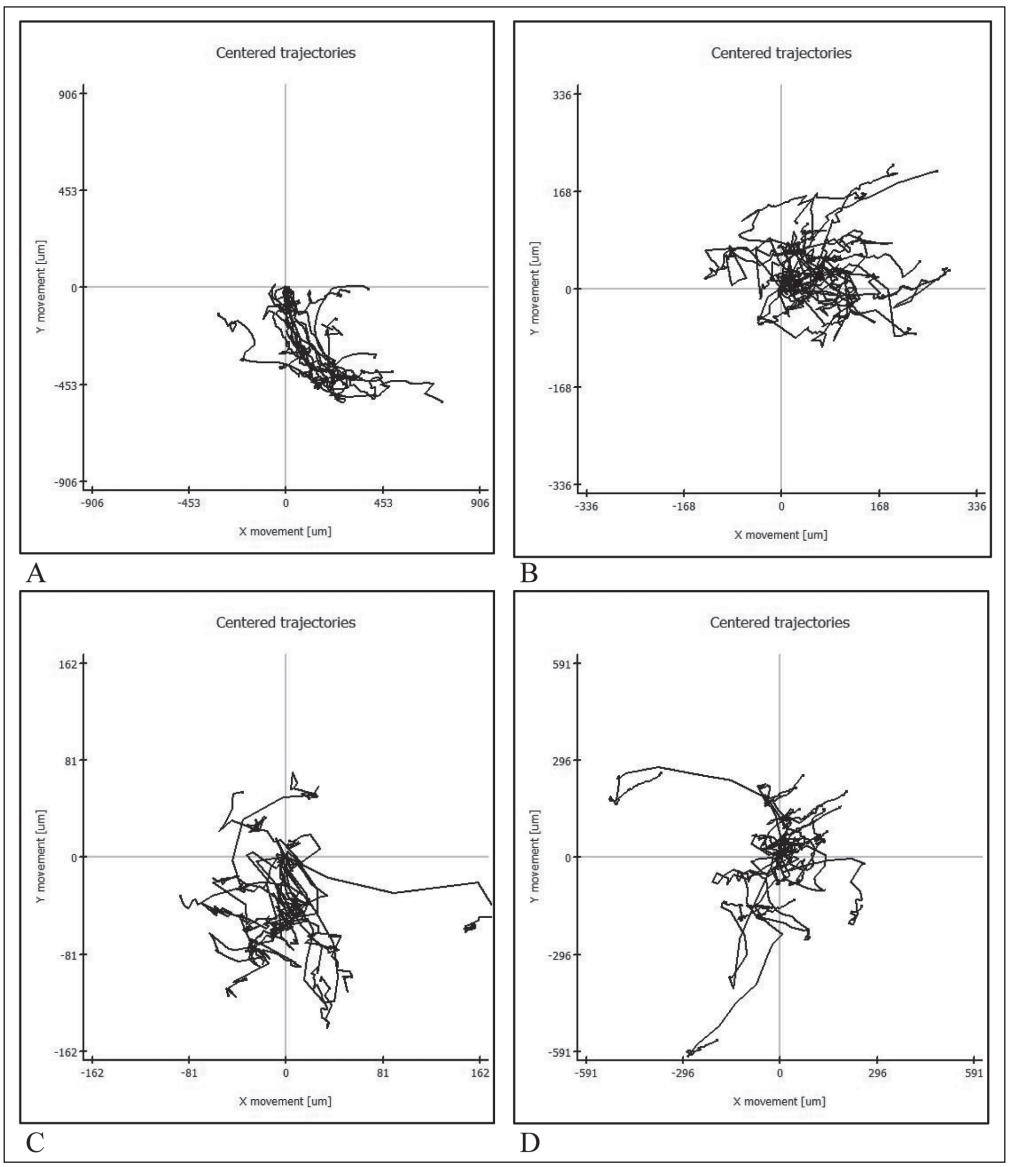

Figure 2. Centring the trajectories of movement of immature DCs in the presence of skin melanoma cells line #894. A – under standard conditions, B – in the presence of TGFβ1 (10 ng/ml), C – in the presence of IL-10 (1 ng/ml), D – in the presence of VEGF (50 ng/ml)

showed a decrease in the movement activity of the DCs and an increase in chaotic state of the motion (Figures 3, 4). The estimation of the motion pattern of the DCs in a two-dimensional coordinate system, taking into account the number of cells that overcome this distance at a certain point in time, made it possible to establish that all the studied ISFs had a similar inhibitory effect on the motility of DCs**,** the effect of IL-10 was maximal (Figure 4).

Discussion

DCs, as highly specialised antigen-presenting cells, are effective inducers of T-lymphocyte mediated responses and traditionally are considered as a decisive component in the implementation of an antitumor immune response [15]. DCs are found in various tumors, such as breast cancer, colorectal cancer, ovarian, lung, stomach, bladder cancer, etc. It is not surprising that in numerous publications of recent decades there are conflicting evidences of the association of tumor DCs infiltration with a positive or negative prognosis of the disease course and stage.

DCs in tumors are often characterized by a phenotype with a weak expression of co-stimulatory molecules, ineffective cross-presentation of antigens, as well as by the expression of regulatory molecules and receptors that often promote the development of immunosuppression [16, 17]. In animal models, it was shown that the type, phenotype and amount of DCs present in the tumor are dynamic during the tumor growth and are associated with the stage of the disease. J. Krempski et al. (2011) on the model of ovary cancer ID8 in mice demonstrated that the increase in tumor size resulted in a significant increase in the number of DCs in the tumor. Here, almost all DCs had immunosuppressive potential, and the number of T-lymphocytes infiltrating the tumor significantly decreased [18].

Tumor cells and other components of the tumor microenvironment synthesize and release factors that are capable of inhibiting or altering the maturation and activity of DCs. Besides, for a successful presentation of antigens, a high migration speed and a low degree of adhesion are necessary, and the change in these parameters must have a certain directionality during the DCs maturation process [19, 20].

A.J. Michielsen et al. (2011) showed that the conditioned medium of tissue explants of human colorectal cancer had a high content of VEGF and chemokines CCL2, CXCL1, CXCL5. Pre-incubation of DCs in this medium *in vitro* led to blockade of their maturation [21]. Moreover, as it turned out, in addition to the biochemical effects the ISFs produced by tumor cells influence the biophysical properties of the DCs preventing their effective migration. Most protocols of antitumor DCs immunotherapy are based on the

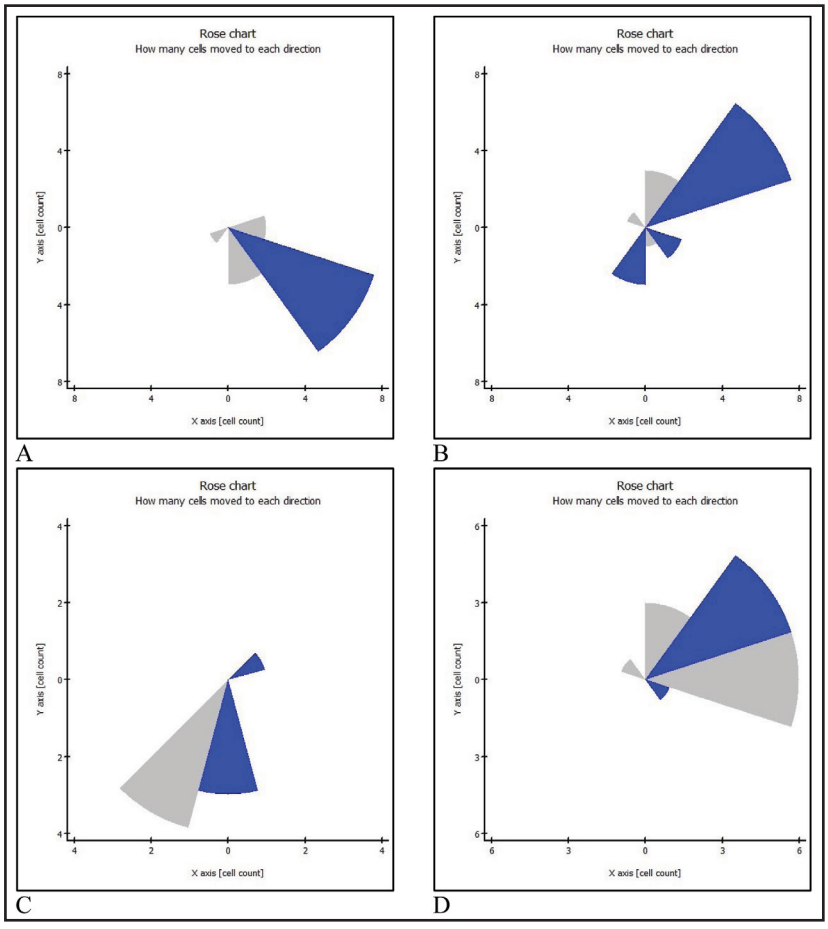

Figure 3. Graphical mapping of directions of movement of immature DCs and quantitative evaluation of the preferred direction of movement in the co-cultivation system with skin melanoma cells line #894. A – under standard conditions,
B– in the presence of TGFβ1 (10 ng/ml),
C – in the presence of IL-10 (1 ng/ml),
D – in the presence of VEGF (50 ng/ml)

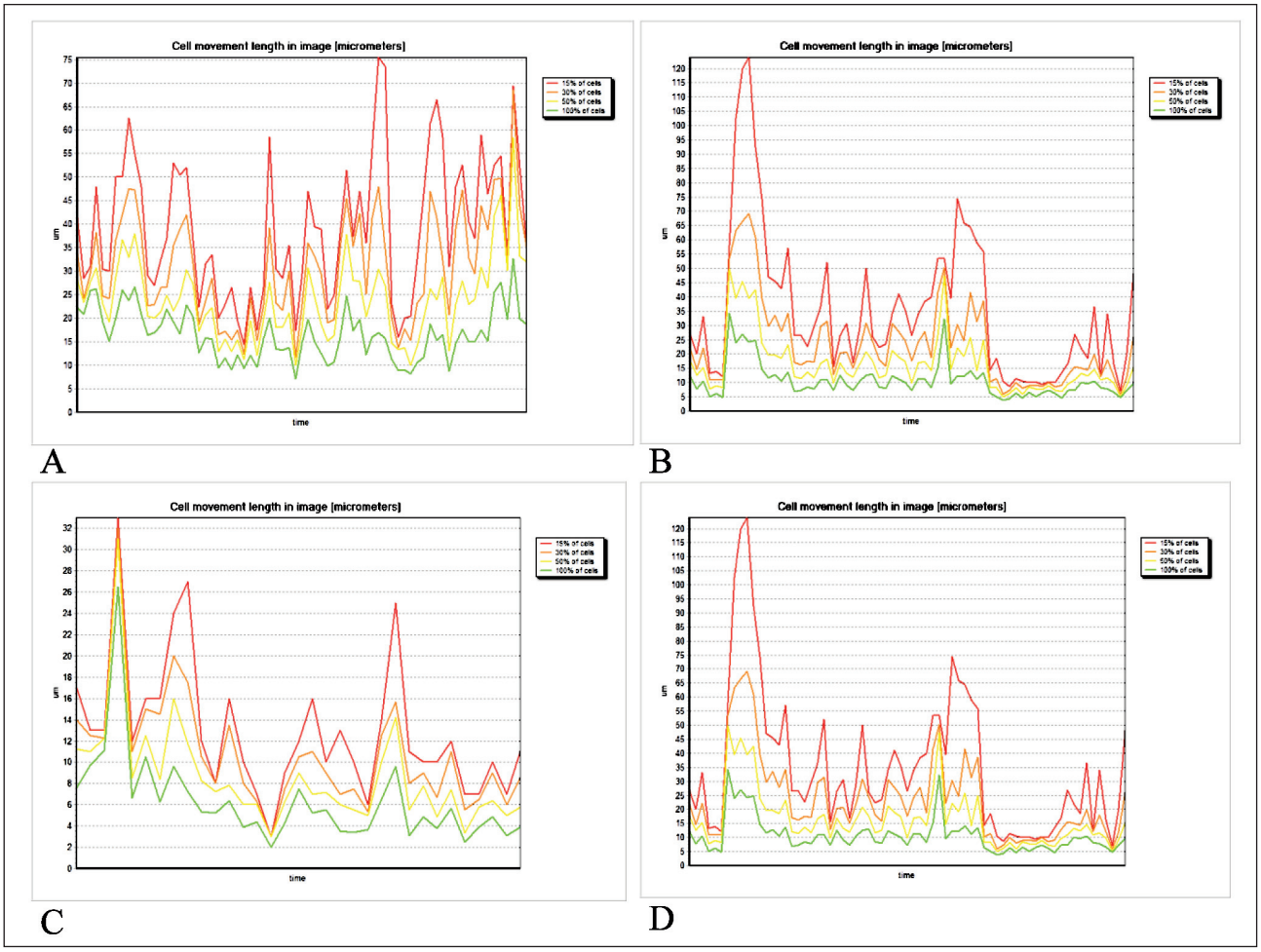

Figure 4. Graphical representation of 2D percentage distribution of immature DCs in movement in the co-cultivation system with skin melanoma cells line #894. A – under standard conditions, B – in the presence of TGFβ1 (10 ng/ml), C – in the presence of IL-10 (1 ng/ml), D – in the presence of VEGF (50 ng/ml). Red line ■ – 15 % of DCs, orange line ■ – 30 % of DCs, yellow line ■ – 50 % of DCs, green line ■ – 100 % of DCs

activation of DCs *in vitro* by loading with antigens contained in autologous and / or allogeneic tumor cells lysates. The activation of DCs is associated including the enhancement of expression of intracellular and surface molecules that provide DCs movability and ability to migrate. However, these processes are dependent on the microenvironment and can be blocked or modified by specific ISFs synthesised into the surrounding microenvironment, which may be the cause of the low efficacy of DCs-vaccine therapy in individual patients.

In this study, an *in vitro* model was created which allowed estimation of the influence of the ISFs produced by human skin melanoma cells on the efficacy of migration and activation of maturating DCs. It turned out that the most prominent inhibitory effect on the movability of maturing DCs had IL-10, and there was an inverse relationship between the production of IL-10 by skin melanoma cells and the speed of movement of the DCs ($p<0.05$) (Tables 2, 4). X. Xu et al. (2017) demonstrated the influence of IL-10 on biophysical characteristics of DCs, such as deformability, osmotic resistance and electrophoretic mobility [11]. It was found that IL-10 critically affected these parameters, contributing to their reduction. The electrophoretic mobility of DCs was associated with the presence of negative charges on their surface membrane. It has be established that the number of these charges continuously increases during the differentiation process (maturation and activation) of the DCs, which is directly linked with their movability [22]. Decrease of these charges on the surface of the cell membrane leads to an increase in the adhesion forces of the DCs to the components of the tumor microenvironment, and limits the motility of the DCs. The ability to change the shape of a cell is important in the migration process when passing a wall of blood or lymphatic vessel through the connections between the cells and the complex extracellular matrix. Studies of Z. Zeng et al. (2006) demonstrated that DCs showed a high capacity for deformation at different stages of differentiation, starting from a monocytic population [23]. Defects of properties to change the shape of cells that arise under the influence of IL-10 can contribute to the weakening of their migration, which can be considered as one of the elements of the phenomenon of «immune

escape» (evasion from the immune control) of tumor cells [9].

In our study, it was found that TGFβ1 and VEGF had a similar effect on DCs migration, blocking their movability. Differences between control samples and samples with increased number of these ISFs were statistically significant ($p<0.05$) in two parameters: trajectory length and average speed of trajectory pass (Table 4). Study done by Z.Q. Hu et al. (2016), identified that VEGF affected the biophysical properties of such structures of the DCs as the outer cytoplasmic membrane and the cytoskeleton, which led to immobilization of the DCs and disrupted their ability to antigen presentation [24]. It was suggested that the electrical potential of the cell membrane was changed by VEGF, thus influencing the effectiveness of immunological synapses formed by the DCs.

L.K. Spary et al. (2014) on the *in vitro* model of tumor and stromal cells of prostate cancer showed that DCs cultured in the presence of $TGFβ_1$ and secreted by stromal cellular elements lost expression of CD209, retained expression of CD14 and were unable to present antigens for T-lymphocytes [24]. These experiments demonstrated the role of the immunosuppressive microenvironment in the blockade of normal DCs maturation.

We obtained data on the inverse dependence of the movability of the DCs on the presence in the microenvironment of a number of growth factors, such as EGF, FGF, HGF (Table 2). In the literature, there are data on the effect of these factors on the mobility of eukaryotic cells [25, 26]. It is known that HGF changes the direction of DCs differentiation, contributing to the formation of a tolerogenic immunophenotype, but the mechanism of action of this factor on the DCs has not been studied sufficiently yet [27]. In addition, it was shown that under the influence of HGF, the adhesion of DCs to laminin was increased [28]. In our study, an inverse relationship was found between the production of FasL by skin melanoma cells and the speed of DCs movement in the studied system.

Among the numerous publications devoted to the analysis of elements of the tumor microenvironment that hamper the formation of the immunophenotype of the DCs, which is productive for the formation of an effective immune response, there are data on the presence in the tumor stroma of an increased amount VEGF, TGFβ1, IL-10, prostaglandin E2, phosphatidylserine, ligand of cytotoxic T-lymphocytes receptor and natural killers MICA, Fas receptor and its ligand FasL [29]. All these molecules are able to inhibit the interaction of DCs and T-lymphocytes.

Thus, it can be assumed, why in a comparative *in vivo* study of migration of mature and immature DCs labelled with radioactive iodine, when DCs vaccines were administered to patients with disseminated forms of skin melanoma, a very low number (<1%) of the DCs reached lymph nodes after intradermal administration [30].

Conclusions

DCs are a complex diversified cellular population with the goal to recognize, capture and deliver the foreign antigens to T-lymphocytes. This predetermines the ability of DCs to actively migrate inside a complex three-dimensional microenvironment, populated by other cellular elements. The study of the migration properties of the DCs using the *in vitro* model of co-cultivation with human melanoma cells under the influence of factors of a tumor microenvironment showed the following:

1. There is an inverse correlation between the quantitative content of $TGFβ_1$, IL-10, IL-18, VEGF-A, EGF, FGF, HGF, sFASL in the supernatants of skin melanoma cell lines and the average speed of the DCs movement, determined in analytical system Cell-IQ, ($p<0.01$).

2. There is an inverse high strength correlation between the angle of movement of the DCs population and the expression of antigens (Melan A, tyrosinase, families MAGE, BAGE, NY-ESO-1) by skin melanoma cells, ($p<0.05$).

3. When exposed to IL-10 (1 ng/ml), TGFβ1 (10 ng/ml), VEGF-A (50 ng/ml), the length of the trajectory and the average speed of trajectory pass of the DCs are decreased ($p<0.05$).

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РЕОРГАНИЗАЦИЯ МЕЖКЛЕТОЧНЫХ АДГЕЗИОННЫХ КОНТАКТОВ И ПОЯВЛЕНИЕ МИГРАЦИОННОЙ АКТИВНОСТИ У КЛЕТОК MCF-7-SNAI1 ПРИ ИНДУКЦИИ ЭПИТЕЛИАЛЬНО-МЕЗЕНХИМАЛЬНОГО ПЕРЕХОДА

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Аннотация

С помощью DIC-микроскопии и конфокальной микроскопии были проанализированы изменения морфологии, миграционных характеристик и межклеточных адгезионных контактов в культуре клеток рака молочной железы MCF-7-SNAI1 при активации экспрессии транскрипционного фактора ЭМП – SNAI1. Как показал Вестерн-блот анализ, экспрессия SNAI1 достигала максимальных значений через 24 часа после переноса клеток в среду без тетрациклина и поддерживалась на этом уровне в течение семи дней. В клетках в течение семи дней сохранялась экспрессия Е-кадхерина, при этом тангенциальные межклеточные адгезионные контакты, характерные для клеток со стабильной межклеточной адгезией, замещались радиальными контактами. В радиальных контактах в течение 24–72 часов отмывки от тетрациклина продолжалась аккумуляция Е-кадхерина. В результате активации SNAI1 клетки вступали в ЭМП и приобретали миграционную активность. На двумерном субстрате клетки мигрировали как индивидуально, так и коллективно. С увеличением продолжительности отмывки от тетрациклина повышался процент клеток, мигрировавших через поры в миграционных камерах, способность клеток инвазировать эпителиальный монослой, напротив, снижалась. Полученные данные свидетельствуют о том, что сохранение гибридного эпителиально-мезенхимального фенотипа и аккумуляция Е-кадхерина в межклеточных адгезионных контактах на ранних этапах ЭМП не препятствуют разрушению стабильной межклеточной адгезии и приобретению клетками миграционной активности.

Ключевые слова: эпителиально-мезенхимальный переход, опухолевые клетки, Snail, Е-кадхерин, миграция.

INDUCTION OF EPITHELIAL-TO-MESENCHYMAL TRANSITION IN MCF-7-SNAI1 CELLS LEADS TO REORGANIZATION OF ADHERENS JUNCTIONS AND ACQUISITION OF MIGRATORY ACTIVITY

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Abstract

Using DIC and confocal microscopy, changes in morphology, migratory characteristics and adherence junctions (AJs) were analyzed in the mammary carcinoma cell line MCF-7-SNAI1 after activation of the EMT transcription factor SNAI1. Western Blot analysis showed that after removal of tetracycline from the cell culture medium

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expression of SNAI1 reached its peak in 24 hours and then plateaued for 7 days. During the 7 days the cells continued to express E-cadherin; however, tangential AJs typical for cells with stable cell-cell adhesion, changed into radial AJs. The radial AJs continued to accumulate E-cadherin during 24-72 hours after tetracycline removal. As a result of SNAI1 activation, the cells underwent epithelial-mesenchymal transition (EMT) and became migratory. On a two-dimensional substrate, cells exhibited both individual and collective migration. As the tetracycline washout period progressed, the fraction of the cells capable of migrating through migration chamber membranes increased; on the contrary, cells' ability to invade an epithelial monolayer decreased. These results demonstrate that retaining a hybrid epithelial/mesenchymal phenotype and accumulation of E-cadherin in AJs during early stages of EMT do not impede disruption of stable cell-cell adhesion and cells' acquisition of migratory activity.

Keywords: epithelial-mesenchymal transition, tumor cells, SNAI1, E-cadherin, migration.

Introduction

Snail1 (human SNAI1) is a 29 kDa transcription factor belonging to the Snail superfamily. Snail1 comprises a SNAG domain and four Zn fingers [1]. Snail1 works as a transcription repressor for E-cadherin, occludin and claudins [2-4]. Snail1 also represses transcription of a Raf kinase inhibitor protein (RKIP) which suppresses metastasis by inhibiting Raf-MEK-ERK and NF-κB signaling cascades [5]. Snail1 induces expression of vimentin and fibronectin [6]. Snail1 positively regulates Snail12, MMP-1,2,3,9,13, Twist, Ets1, VEGF, and WAVE3. Snail1 together with Twist induce Zeb1 expression [6, 7].

It is considered that repression of E-cadherin expression induced by Snail1 weakens cell-cell adhesion during epithelial-mesenchymal transition (EMT) [2, 3], a process that promotes invasion and metastasis. During EMT, epithelial cells lose epithelial markers (E-cadherin, occludin, cytokeratins etc.) and begin to express mesenchymal markers (vimentin, fibronectin etc.). They also lose basal-apical polarity and acquire migratory capabilities [8]. Snail1 is expressed in many types of cancer. Snail1 is activated by multiple signaling pathways; in tumor cells, Snail1 can also be activated by the signals from the tumor microenvironment [9].

The goal of the present study was using DIC and confocal microscopy, to study changes in cell phenotype resulting from expression of the exogenous EMT transcription factor SNAI1 in the mammary tumor cell line MCF-7-SNAI1.

Materials and Methods

Cell lines, constructs and transfections MCF-7-SNAI1 cell line was established by Yatskouet al. [10]. Human breast cancer MCF-7 cells conditionally express SNAI1 in a tet-off expression system.

For some experiments, MCF-7-SNAI1 cells were transfected with EGFP (Evrogen) and human breast non-tumorigenic epithelial cells MCF-10A were transfected with mKate2 (Evrogen). All transfections were performed with LTX Plus (Thermo Fisher Scientific) according to the manufacturer's protocol.

Western blot analysis

Western blot analysis was performed as described in Rubtsova et al. [11].

Antibodies, fluorescent staining and microscopy

The following primary antibodies were used: monoclonal anti-E-cadherin (BD Transduction Labs) monoclonal anti-β-actin (AbD Serotec Bio-Rad), and anti-Snail (Cell Signaling). Mounted samples were examined with a Nikon Eclipse Ti-E microscope equipped with a Plan Fluor 40× objective and an ORCA-ER camera (Hamamatsu Photonics) controlled via Nis-Elements AR 2.30 software (Nikon). For live-cell imaging, cells were seeded into 35-mm glass bottom culture dishes (MatTeck Corporation) and observed with the same microscope.

Migration chamber and transepithelial migration assays

For the migration assay, migration chambers containing membrane inserts with 8-μm pores (Becton Dickinson) were used. MCF-7-SNAI1 cells were seeded into the upper wells of chambers. After 20 h of incubation at 37°C, cells from the upper surface of membranes were removed with a cotton swab; the cells that have migrated onto the lower surface of membranes were fixed with 100% methanol and stained with DAPI. Mounted membranes were examined at 20× magnification. Cells on the lower side of membranes were counted in 15 randomly selected fields.

Non-tumorigenic MCF-10A epithelial cells expressing mKate2 were seeded to form a confluent monolayer 24 h after seeding. At this time point, MCF-7-SNAI1 cells expressing EGFP were seeded at a low density onto the MCF-10A monolayer. The number of MCF-7-SNAI1 cells that had invaded the monolayer and spread on the glass substrate 20 h later was counted in 30 fields.

Results

MCF-7 cells stably expressing a tetracycline-regulated SNAI1 (MCF-7-SNAI1) were subjected to Western Blot analysis to determine the kinetics of expression of EMT transcription factor SNAI1 as well as the AJ protein E-cadherin after removal of tetracycline (Tet) which triggers SNAI1 expression. SNAI1 was first detected at 4 h after Tet removal. SNAI1 expression peaked at 24 h after Tet removal and then plateaued for 7 days. Despite the strong expression of exogenous SNAI1, expression of

E-cadherin decreased only very insignificantly (Figure 1A-C). Our data on kinetics of SNAI1 expression in these cells are in accordance with the RT-PCR data of Vettertaletal et al. [12]. Immunofluorescent staining for SNAI1 confirmed the Western Blot results: 24 h after Tet removal and beyond that time, a strong SNAI1 signal was detected in the nuclei of the MCF7-SNAI1 cells (Figure 1D).

Double immunofluorescence microscopy allowed us to observe changes in actin cytoskeleton organization and E-cadherin accumulation in AJs at different time points after Tet removal and hence, induction of SNAIl in the MCF7-SNAI1 cells. In control MCF-7-SNAI1 cells, cultured in the presence of Tet, continuous adhesion belts, formed by E-cadherin, were observed. These belts co-localized with underlying actin bundles (Figure 2) and resembled the tangential AJs of the immortalized mammary epithelial cells, such as MCF-10A (not shown).

At 24-48 h after Tet removal, the cells spread slightly. The adhesion belts became loose and discontinuous, with occasional radial elements. E-cadherin accumulation in these AJs was comparable to that in the control AJs. At 72 h after Tet removal, E-cadherin-based AJs acquired radial shape and became connected to straight actin bundles. At 96 h after Tet removal, E-cadherin accumulation at cell-cell borders significantly decreased to the point of occasionally observing cells in whose AJs E-cadherin was not detected at all.

DIC live cell imaging allowed us to compare the behavior of the MCF-7-SNAI1 cells in sparse culture in the presence of Tet vs. at 24 h after Tet removal when the SNAI1 expression was at its highest (Figure 3). In the presence of Tet, control MCF-7-SNAI1 in islands were connected by stable AJs which persisted during the 6 h of observation (Figure 3A). During the 24 h of Tet washout, the cells had undergone EMT and acquired a migratory phenotype. Stable cell-cell adhesion was disrupted. The cells migrated over the substrate individually or as small groups (Figure 3B). At later times (48-72 h of Tet washout), the migration became predominantly individual.

Further studies of migratory activity were conducted in migration chambers, where MCF-7-SNAI1 cells migrated through 8-μm pores to the bottom side of the chamber membrane during a 20-h period (Figure 4A). It was found that longer Tet washout times (up to 72 h) led to a significant increase in the number of migrated cells.

Earlier we have shown that retaining the ability to form E-cadherin-based AJs allows neoplastic cells to attach to normal epithelial cells, migrate over their surface and invade epithelial structures. In a cell culture system, developed by us earlier, we compared the ability to invade an MCF-10A monolayer by the MCF-7-SNAI1 cells after Tet removal. GFP-expressing MCF-7-SNAI1 cells at various times of Tet washout were seeded onto a monolayer of normal mammary epithelial cells MCF-10A. 20 h after seeding, using confocal microscopy, GFP-expressing cells which had invaded the monolayer, were detected on the substrate level. The average number of such cells per field gradually decreased with the increase of the Tet washout time (Figure 4B).

Discussion

The presented data demonstrate that activation of expression of exogenous SNAI1 in MCF-7 cells induces EMT which is characterized by loss of stable cell-cell adhesion and acquisition of a migratory phenotype. On a two-dimensional substrate cells could migrate either individually or as small groups. The weakening of cell-cell adhesion was not caused by a decrease in E-cadherin expression or its accumulation on the cell membrane, but was accompanied by a

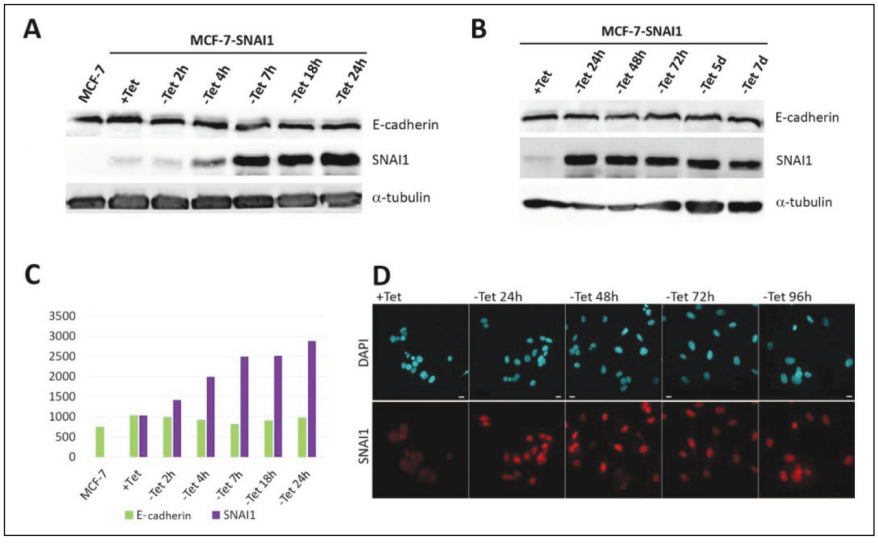

Figure 1. A-B – Western Blotting of the MCF-7-SNAI1 lysates at different time points of Tet washout. C – densitometric analysis of A. D – Immunofluorescent staining for DAPI (blue) and SNAI1 (red) in the MCF-7-SNAI1 cells at different time points of Tet washout. Scale, 10 μm

reorganization of tangential AJs into radial ones. Earlier we have observed these radial AJs in epithelial cells transformed *in vitro* by dimethylnitrosamine or the RAS oncogene. Radial AJs in these cells were very unstable and dynamic [13]. Radial AJs did not hinder the disruption of the stable cell-cell adhesion during neoplastic transformation and actively supported collective migration [14]. We propose that actin cytoskeleton reorganization underlies the acquisition of migratory phenotype and reorganization of AJs during EMT. However, the exact mechanisms of actin cytoskeleton reorganization after SNAI1 induction have not been elucidated. In certain transformed cell lines activation of SNAI1 led to a pronounced decrease of expression of a tumor suppressor protein maspin, which has been shown to negatively regulate mesenchymal migration induced by the small GTPase Rac [15, 16]. However, in MCF-7-SNAI1, maspin

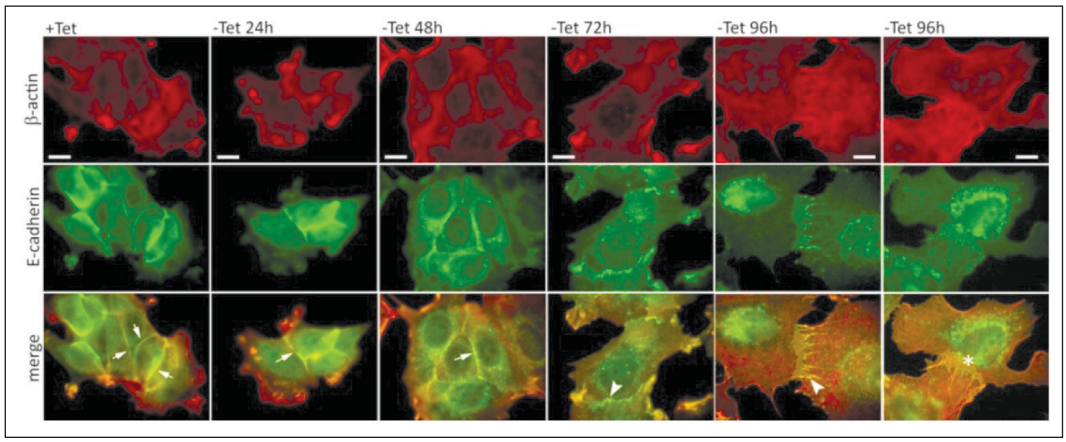

Figure 2. AJs and actin cytoskeleton of MCF-7-SNAI1 cells at different time points of Tet washout. Immunofluorescent staining, top – β-actin; middle – E-cadherin, bottom – merge. Two examples of AJs are given for - Tet 96h: left, normal E-cadherin accumulation, right, decreased E-cadherin accumulation. Adhesion belts are marked by arrows, radial AJs by arrowheads, AJs with no detectable E-cadherin by an asterisk. Scale, 10 μm

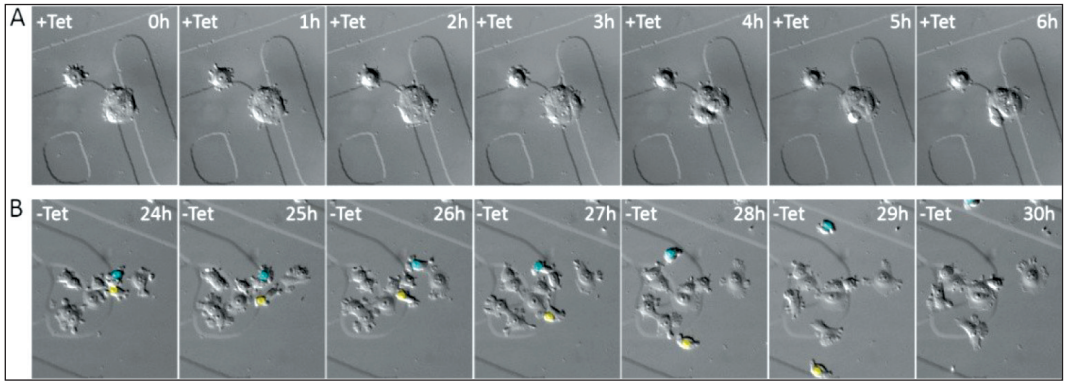

Figure 3. Migratory activity of the MCF-7-SNAI1 cells on a flat substrate. DIC live cell imaging; A – control cells, B – cells after activation of SNAI1 expression (24 h after Tet removal). The nuclei of the fastest migrating cells are marked blue

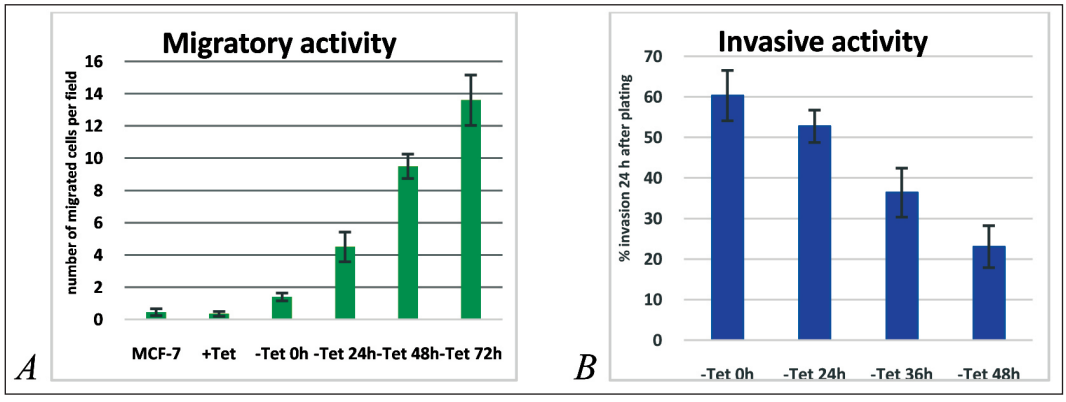

Figure 4. A – Migratory activity of the MCF-7-SNAI1 cells in migration chambers (20 h after plating). B – invasion of a monolayer formed by normal mammary epithelial cells MCF-10A by the MCF-7-SNAI1 cells at different time points of Tet washout

was not detected even in control cells cultured in the presence of Tet. Induction of SNAI1 expression in MCF7-7-SNAI1 cells led to heightened capability to migrate through 8-μm pores in a migration chamber assay. At the same time, longer Tet washout times led to poorer attachment of the MCF-7-SNAI1 cells to the MCF10A monolayer and 3-fold weaker invasion of the monolayer. These data correlate with gradual decrease of E-cadherin-based cell-cell adhesion during EMT. Retention of the hybrid phenotype during early stages of EMT may be an important factor, allowing cancer cells to migrate from the primary tumor following activation of an invasion/metastasis-promoting signaling cascade. Plasticity of the migratory phenotype provides additional adaptive capabilities during tumor cells dissemination.

Conclusion

Using a cellular model of EMT, consisting of human mammary tumor cell line MCF-7 inducibly expressing SNAI1, it was shown that activation of the exogenous SNAI1 led to weakening of cell-cell adhesion and acquisition of the migratory activity. These changes in cell phenotype were not caused by decrease in E-cadherin expression but were accompanied by reorganization of E-cadherin-based AJs.

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Conflict of interest

The authors declare that they have no conflict of interest.

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УСЛОВИЯ ЭФФЕКТИВНОГО ПОДАВЛЕНИЯ ПЦР С ПОМОЩЬЮ LNA-ОЛИГОНУКЛЕОТИДОВ ДЛЯ ПРОСТОЙ И ВЫСОКОЧУВСТВИТЕЛЬНОЙ ДЕТЕКЦИИ СОМАТИЧЕСКИХ МУТАЦИЙ

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Аннотация

Специфическое блокирование амплификации аллеля дикого типа в ПЦР с помощью олигонуклеотидов, модифицированных по остатку рибозы (закрытые нуклеиновые кислоты, locked nucleic acids, LNA), используется для высокочувствительной детекции соматических мутаций в опухолях. Описаны различные версии метода анализа мутаций с использованием LNA-олигонуклеотидов как с дополнительной модификацией фосфотиоатными группами, так и без таких групп, при этом использовались различные ДНК полимеразы. В работе проведен анализ оптимальных условий для успешного специфического блокирования ПЦР с помощью LNA-олигонуклеотидов при анализе мутаций в генах KRAS и BRAF. Мы обнаружили, что фосфотиоатная защита на 5'-конце олигонуклеотидов не влияет на эффективность блокирования аллеля дикого типа. Выявлено, что для большинства последовательностей эффективное блокирование наблюдается при проведении шага отжига и элонгации ПЦР при температуре на 20–25°C ниже температуры плавления LNA-олигонуклеотида. При таких условиях реакции возможна простая и высокочувствительная детекция мутаций в генах KRAS и BRAF с использованием как секвенирования по Сэнгеру, так и ПЦР в реальном времени с Taqman зондами.

Ключевые слова: запертая нуклеиновая кислота, зажим PCR, мутации, KRAS, BRAF, ДНК-полимераза Taq.

REQUIREMENTS FOR EFFICIENT PCR CLAMPING BY LOCKED NUCLEIC ACID OLIGONUCLEOTIES FOR SIMPLE AND SENSITIVE DETECTION OF SOMATIC MUTATIONS

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Abstract

PCR clamping/wild-type blocking PCR with non-extendable locked nucleic acid (LNA) oligonucleotides is used for sensitive detection of somatic mutations in tumors. Various versions of the technique use different DNA polymerases and LNA oligonucleotides with and without additional phosphorothioate modifications. Here we

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studied requirements for successful PCR clamping with LNA oligonucleotides and Taq DNA polymerase for analysis of mutations in KRAS and BRAF genes by means of real-time PCR and Sanger sequencing. We found that addition of phosphorothioate linkages at the 5'-end of LNA oligonucleotide to protect from 5'-exonuclease activity of Taq DNA polymerase did not improve clamping. For most target sequences, efficient clamping was observed at melting temperature of LNA oligonucleotide 20-25°C above annealing/extension temperature of the PCR with a 2-step protocol. Under such conditions, simple and sensitive detection of mutations in KRAS and BRAF genes was feasible using real-time PCR with TaqMan probes or Sanger sequencing.

Keywords: locked nucleic acid, PCR clamp, mutations, KRAS, BRAF, Taq DNA polymerase.

Introduction

Activating somatic mutations in certain genes (e.g. *BRAF, KRAS, EGFR*) are used to guide the choice of cancer therapy. The large excess of wild-type DNA often complicates detection of somatic mutations in tumor tissue. To detect small amount of a mutant allele within a large excess of wild-type DNA, a number of methods based on PCR, Sanger sequencing, pyrosequencing, mass spectrometry and next generation sequencing (NGS) were developed. PCR clamping or wild-type blocking (WTB) PCR with locked nucleic acid (LNA) oligonucleotide can be used for the sensitive detection of somatic mutations [1-4]. LNAs are nucleic acids with 2'-O-4'-C methylene bridge [5]. LNA modifications in an oligonucleotide increase melting temperature (Tm) and the specificity of binding to the target [5-7]. In WTB PCR non-extendable oligonucleotide (PCR clamp) corresponding to the wild-type sequence of expected mutation is added to PCR. Binding of PCR clamp to a target DNA inhibits amplification of the wild-type allele and results in the selective amplification of mutant allele(s) that can be detected by Sanger sequencing or by other methods.

It was proposed that clamping oligonucleotide can be degraded by the 5'-exonuclease activity of Taq DNA polymerase, and for efficient PCR clamping, the Stoffel fragment of DNA polymerase without 5'-exonuclease activity is required [1, 8]. Alternatively, some studies used Taq DNA polymerase and LNA oligonucleotides protected at the 5'-end with phosphorothioate modifications or employed Pfu DNA polymerase and its variants without 5'-exonuclease activity [9-11]. However, successful PCR clamping was also reported with regular Taq DNA polymerase and unprotected LNA oligonucleotides [2-4]. Thus, the need of PCR clamping to protect the 5'-end of LNA oligonucleotide and the need of DNA polymerase defective in 5'-exonuclease activity remains obscure. Here we studied the requirements for efficient PCR clamping with LNA oligonucleotides and Taq DNA polymerase for the simple and sensitive detection of somatic mutations in tumors.

Materials and Methods

LNA oligonucleotides

We designed PCR clamps KLNA1 (5'-GCCTACG+C+CA+C+CAGCTCCTT-p-3'; melting temperature (Tm) 80°C) and KLNA1S (5'-$G_SC_SC_S$TACG+C+CA+C+CAGCTCCTT-p-3'; Tm 80°C) corresponding to wild-type anti-sense KRAS sequence with codons 12 and 13. The clamps had identical nucleotide sequence but KLNA1S had phosphorothioate linkages for the first three internucleotide bonds at the 5'-end. For BRAF sequence including codon V600, we used PCR clamp BLNA2 (5'+G+C+T+A+C+A+G+T+G+AGGG-p-3'; Tm 77°C) [1]. Tm of LNA oligonucleotides was calculated by the on-line tool [12]. In the oligonucleotides LNA positions are preceded with "+" sign, while phosphorothioate linkages are designated by "S". Additionally LNA oligonucleotides carried at the 3'-end two or three nucleotide mismatches (underlined) and phosphate to block an extension. Oligonucleotides were synthesized by «DNK-sintez» (Moscow, Russia).

Wild-type and mutant human DNA standards

Recombinant plasmids with mutations KRAS-G12D (c.35G>A) and BRAF-V600E (c.1799T>A) were constructed by site-directed mutagenesis and mutations were confirmed by Sanger sequencing. Human placenta DNA without KRAS and BRAF mutation (Biolink, Russia) was diluted to 2 ng/µl in TE buffer (10 mmol/L Tris-HCl, pH 8.0; 1mmol/L EDTA) and used as wild-type DNA standard. Concentration of wild-type and mutant DNA was determined by real-time PCR. Wild-type DNA standard was spiked with the KRAS-G12D or BRAF-V600E plasmid DNA to prepare mutant DNA standards with different ratios of mutant/normal allele.

Real-time PCR

Real-time PCR KRAS was done in 25 µl mixture containing 1× buffer for Taq DNA polymerase (Medigen, Russia), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each of dATP, dGTP, dTTP, dCTP (Medigen, Russia), 0.5 µmol/L of forward primer KF1 (5'-GGCTGGTGGAGTATTTGATAGTGT-3'), 0.5 µmol/L reverse primer KR1 (5'-GGACAAGATTTACCTCTATTGTTGG-3'), 0.5 µmol/L TaqMan probe KTM6-2 (5'-FAM-CCTGC(dT-BHQ1)GAAAATGACTGAATATAAACTTGTGG-p-3'), and 1U of SuperHot Taq DNA polymerase (Bioron, Germany). SuperHot DNA polymerase is recombinant Taq DNA polymerase with anti-Taq Ab for hot-start. PCR clamps KLNA1 or KLNA1S were added to 2.0 µmol/L when required. Real-time PCR was performed in iCycler iQ5 or CFX96 thermocyclers (Bio-Rad) using

2-step protocol: 1 cycle 95 ºC for 3 min; followed by 50 cycles 95 ºC for 15 sec and 55 ºC for 90 sec with optical reading at 55 ºC. Difference in Ct values (dCt) in reactions with and without PCR clamp was calculated as the following: $dCt = Ct_{+LNA} - Ct_{-LNA}$, where Ct_{+LNA} – is the Ct of the DNA in PCR with clamp, Ct_{-LNA} – is the Ct of the DNA in PCR without clamp.

Unless otherwise indicated real-time PCR BRAF was done as described for KRAS with 2-step protocol but using forward primer BF2-2 (5'- AATGCTTGCTCTGATAGGAAAATG-3'), reverse primer BR2-3 (5'-AGTGGAAAAATAGCCTCAATTCTTA-3'), TaqMan probe BTM5-2 (5'-FAM-ATGAAGACC(dT-BHQ1) CACAGTAAAAATAGGTGATTTTGG-p-3') and PCR clamp BLNA2. When indicated the following 3-step protocol was used: 1 cycle 95 ºC for 3 min followed by 50 cycles 95 ºC for 15 sec, 55 ºC for 30 sec (with optic reading) and 72 ºC for 20 sec.

Sanger sequencing after PCR clamp

After PCR with clamp oligonucleotide amount of DNA may not be sufficient for Sanger sequencing. Therefore, DNA was amplified by nested PCR in two rounds. In the first round, reaction was done with PCR clamp to select for mutant allele essentially as described for the real-time PCR with primers KF1, KR1 and PCR clamps KLNA1 or KLNA1S without TaqMan probe; the following PCR protocol was used: 1 cycle 95 ºC for 3 min; followed by 25 cycles 95 ºC for 15 sec and 55 ºC for 90 sec. Product of the first round was diluted 1:50 in water and 5µl of diluted DNA was used in the second round without PCR clamp to obtain sufficient amount of mutation-enriched DNA for Sanger sequencing. In the second round, forward primer KF2 (5'-GCGTGTATTAACCTTATGTGTGACA-3') and reverse primer KR2 (5'-GGCAAGATTTACCTCTATTGTTGGA-3') were used with the following protocol: 1 cycle 95 ºC for 3 min; followed by 20 cycles 95 ºC for 15 sec, 60 ºC for 20 sec and 72 ºC for 20 sec. After the second round the DNA was purified on AMpure XP magnetic beads (Beckman Coulter) and sequenced using BigDye1.1 Kit (Applied Biosystems) with KF2 and KR2 primers.

Results

Protection of the 5'-end of LNA oligonucleotide is not required for efficient PCR clamp with Taq DNA polymerase.

We used initially WTB PCR to detect mutations at KRAS codons 12 and 13. These two codons have sequence GGTGGC with guanine nucleotides being mutation hot-spots in different cancers. We used sequence of the anti-sense strand of the KRAS gene for the clamp design to avoid LNA modification of guanine nucleotides that negatively impacts mismatch discrimination [6]. We designed clamp oligonucleotides KLNA1 and KLNA1S that had identical nucleotide sequence but the latter had phosphorothioate linkages for the first three internucleotide bonds at the 5'-end to protect from digestion by the 5'-exonuclease activity of Taq DNA polymerase. We tested both PCR clamps for the detection of mutation KRAS-G12D by real-time PCR and Sanger sequencing.

In real-time PCR both PCR clamps inhibited amplification of WT human KRAS DNA as evidenced by large increase in Ct (Figure 1A). As a quantitative parameter of performance of a PCR clamp we used

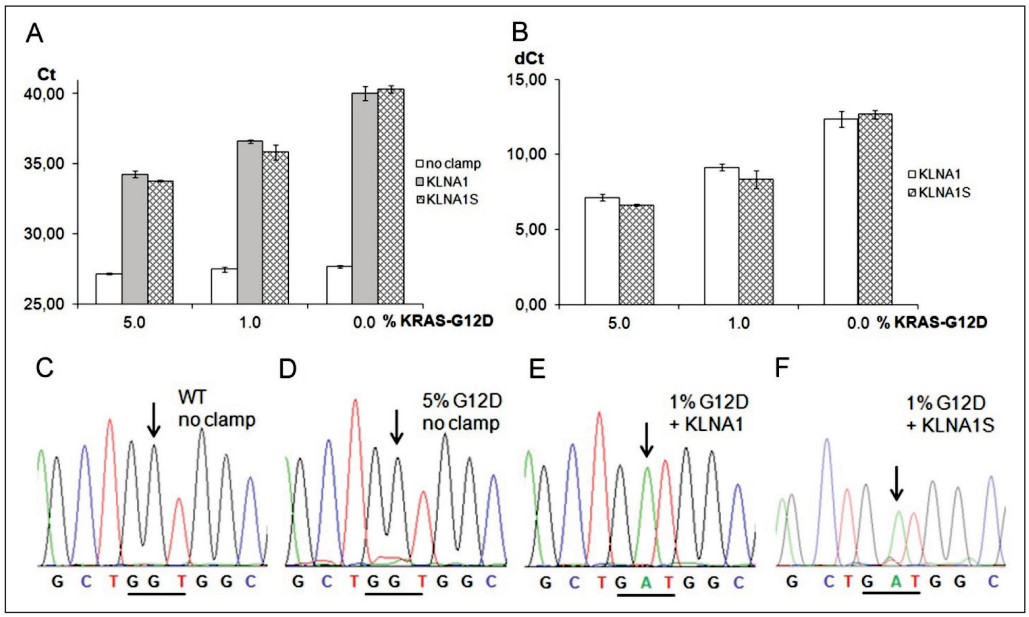

Figure 1. Detection of KRAS mutation G12D using Taq DNA polymerase and different PCR clamps. Ten nanogram human placenta DNA with wild-type KRAS or the same DNA spiked with 1% or 5% DNA copies KRAS-G12D was tested. PCR was done with and without PCR clamp KLNA1 without phosphorothioate linkages, or KLNA1S with phosphorothioate linkages. A, B - real-time PCR, plot of Ct and dCt, accordingly. C- F - Sanger sequencing after PCR without clamps (C, D) or with clamps KLNA1 (E) or KLNA1S (F). Codon 12 is underlined, nucleotide position corresponding to wild-type and mutation p.G12D (c.35G>A) is indicated by an arrow

the difference in Ct values (dCt) in reactions with and without PCR clamp. DNA samples spiked with KRAS-G12D plasmid had smaller dCt compared to wild-type, so that 1% mutant allele was readily distinguished (Figure 1B). Importantly, Ct as well as dCt values were similar in PCR with either clamp indicating similar performance of LNA oligonucleotides with and without phosphorothioate linkages.

Sanger sequencing of amplicons after PCR with either oligonucleotide clamp showed similar detection of 1% mutant allele (Figure 1D, F). Of note, 5% mutant allele was not visible by Sanger sequencing without PCR clamp (Figure 1E) and reliable detection required 20% or more mutant allele (data not shown).

These real-time PCR and Sanger sequencing data indicated that protection from 5'-exonuclease activity of Taq DNA polymerase did not improve PCR clamping by LNA oligonucleotides.

Efficient PCR block is observed at an annealing/extension temperature of 20-25 degrees below calculated Tm of the PCR clamp.

The strength of oligonucleotide binding to the template, and consequently efficiency of clamping should increase with higher Tm of LNA oligonucleotide and lower annealing/extension temperature (Tann/ext) during PCR. We investigated optimal Tann/ext for a PCR clamp with certain calculated Tm. Increase in Tann/ext from 54.6 to 62.8°C resulted in decrease in dCt of the wild-type DNA, while dCt of DNA with 5% allele KRAS-G12D was not appreciably changed (Figure 2). These data indicated less efficient PCR clamp and drop in discriminating power between wild-type and mutant alleles with increased Tann/ext. Further increase of Tann/ext to 65°C resulted in poor PCR performance, which was manifested in drop in fluorescence and large increase in Ct (data not shown).

Two-step PCR protocol is preferred for effective clamping.

Different studies use LNA-based PCR clamps either in 2-step PCR with 60-65°C annealing/extension temperature [2, 3, 10] or in classic 3-step PCR protocol with 56-64°C annealing and 72°C extension temperature [1, 4, 9, 11]. We speculated

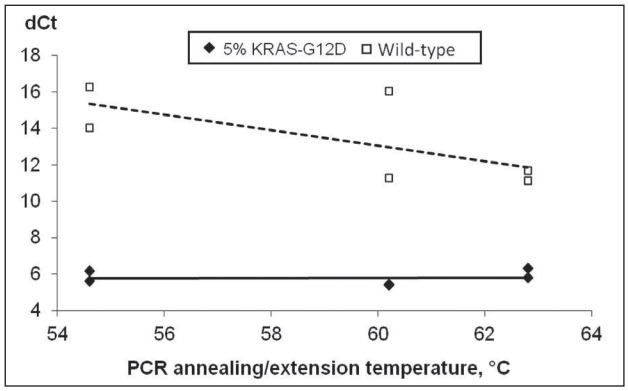

Figure 2. Performance of PCR clamp at different annealing/extension temperatures (Tann/ext). Real-time PCR of 5% KRAS-G12D and wild-type DNA with and without PCR clamp KLNA1. PCR was done using 2-step protocol with Tann/ext at 54.6; 60.2; or 62.8°C. Difference of Ct for reactions with and without clamp (dCt) and linear approximation of dCt is shown for 5% KRAS-G12D (solid line) and wild-type DNA (broken line)

that extension at 72°C can cause weaker binding of LNA oligonucleotide to the target and compromise clamping. Importantly, we described PCR clamp for BRAF-V600 that did not block amplification of the wild-type DNA in 3-step PCR protocol with Taq DNA polymerase [1]. We tested this PCR clamp in 2-step and 3-step PCR protocols. The amplification of wild type DNA was effectively blocked by PCR clamp that was manifested in large increase in Ct of the DNA if 2-step PCR protocol was applied with 55°C Tann/ext. Under such conditions DNA spiked with 1% BRAF-V600E allele was readily detected (Figure 3A). However, in agreement with Dominguez and Kolodney [1] the clamp did not block PCR with Taq DNA polymerase in 3-step PCR protocol (Figure 3B). These data showed that 2-step PCR protocol with 55 ºC Tann/ext was preferred for efficient clamping.

Furthermore, we designed and tested additional LNA oligonucleotides as PCR clamps to different loci in EGFR, KRAS and NRAS genes (data not shown). PCR was done using 2-step protocol with 55°C annealing temperature. We found that LNA oligonucleotides with Tm 72-73°C were poor blockers; in contrast, PCR was efficiently blocked by LNA

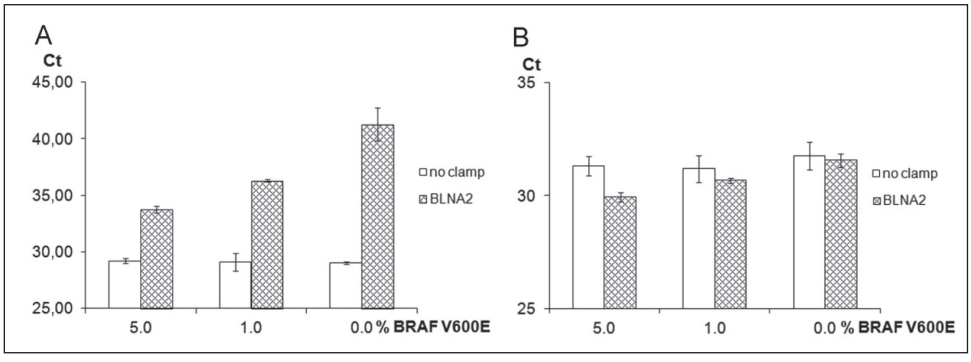

Figure 3. Detection of BRAF mutation V600E by PCR clamping using 2-step and 3-step protocol. Real-time PCR BRAF using 2-step protocol with Tann/ext 55°C (A), or 3-step protocol with 55°C annealing and 72°C extension (B). Ct of wild-type DNA and 1% or 5% BRAF-V600E is shown in reactions with and without PCR clamp BLNA2

oligonucleotides that had Tm about 80°C (range 76-80°C). The only exception was PCR clamp for sequence of NRAS including codons 12 and 13. In this case LNA oligonucleotide with Tm 80°C did not block PCR. Importantly, another oligonucleotide for the same sequence with additional LNA positions that increased Tm to 92°C blocked PCR of wild-type NRAS DNA and allowed detection of 1% NRAS-G12D (data not shown). Taken together these data indicated that for efficient clamping the Tm of oligonucleotide clamp should be at least 20°C above Tann/ext.

Discussion

WTB PCR using LNA oligonucleotides is a simple sensitive method for the detection of somatic mutations in tumors. Nafa et al. [13] reviewed the method; however, several important parameters of the technique remained unclear. In this study we evaluated requirements for effective PCR clamping by LNA oligonucleotides.

We found that phosphorothioate linkages in LNA oligonucleotide did not improve PCR clamping, indicating that protection of the clamp from 5'-exonuclease activity of the Taq DNA polymerase was not required. Interestingly, Stoffel fragment that lacks 5'-3' exonuclease activity was much more sensitive to PCR clamp in comparison to Taq DNA polymerase [1]. Our observation that phosphorothioate modifications of LNA oligonucleotide did not improve clamping indicated that the 5'-exonuclease activity of Taq DNA polymerase was not likely the reason for difference in sensitivity of these enzymes to PCR clamp. Most plausible explanation is weaker binding to the DNA template of Stoffel fragment in comparison to Taq DNA polymerase, which is manifested in about 10-fold difference in the binding constants of these enzymes to the DNA [14].

Tm of LNA oligonucleotide and annealing/extension temperature during PCR were critical parameters that had strong impact on clamping. There was a poor clamping if Tm of LNA oligonucleotide was 20°C lower than the annealing/extension temperature of the PCR. This observation provides simple and efficient rationale for design and optimization of WTB PCR with PCR clamps based on LNA oligonucleotides. Software to determine Tm of LNA oligonucleotides is available at the IDT and the Exiqon company sites [12, 15]. However, we noted that for different LNA oligonucleotides, the calculated Tm was 2.5-19.5°C lower using IDT software compared to the Exiqon one. We used Exiqon tool, however calculated Tm using either program should be used with caution for predicting real Tm for the design of PCR protocol. We think that poor clamping by some LNA oligonucleotides with the calculated Tm of about 80°C was the result of an error in the calculated Tm. In all cases when LNA oligonucleotide was not blocking PCR, adding more LNA positions to increase Tm incrementally by 5-12°C was sufficient for the effective PCR clamping.

Gel-electrophoresis, Sanger sequencing or real-time PCR can be used to evaluate performance of a PCR clamp. However, in our hands impact of PCR clamp on intensity of PCR bands sometimes was not clearly visible after gel-electrophoresis of PCR products. Importantly, real-time PCR and Sanger sequencing were in agreement with each other and provided more accurate estimate of performance of a PCR clamp.

Both real-time PCR and Sanger sequencing can be used to detect mutations by PCR clamping. In comparison to Sanger sequencing, real-time PCR had faster turnaround time and was less expensive, however exact genotyping was not possible. A limitation of Sanger sequencing after PCR clamping were occasional mutation artifacts on wild-type DNA. The problem was previously reported [4, 10] and is believed to result from mistakes of Taq DNA polymerase. The problem can be solved by the use of DNA polymerase with proofreading activity instead of Taq DNA polymerase [10]. Other potential options are a hi-fidelity version of Taq DNA polymerase [16], or use of less PCR cycles with PCR clamp.

In conclusion, our data showed efficient PCR clamping by LNA oligonucleotides with calculated Tm of at least 20°C above the annealing/extension temperature in a 2-step PCR protocol. Enrichment for target mutations was confirmed by the Sanger sequencing of PCR products. Importantly, Taq DNA polymerase and LNA oligonucleotides without phosphorothioate modifications were used without compromise in the efficiency of PCR clamp. This allows rational design of inexpensive, simple and sensitive assays for clinically actionable mutations in convenient format of real-time PCR with TaqMan probes.

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СРАВНИТЕЛЬНЫЙ АНАЛИЗ ЭКЗОСОМ КЛЕТОК ЭСТРОГЕН-РЕЗИСТЕНТНОГО РАКА МОЛОЧНОЙ ЖЕЛЕЗЫ

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Аннотация

Участие экзосом в патогенезе злокачественных опухолей основано на их способности проникать внутрь клеток-реципиентов, вызывая в последних каскад генетических и эпигенетических изменений. Ранее мы показали, что экзосомы, продуцируемые различными вариантами эстроген-независимых сублиний клеток рака молочной железы (MCF-7/T, полученной в результате длительного культивирования клеток в присутствии антиэстрогена тамоксифена, и MCF-7/M, полученной в результате культивирования клеток с метформином), способны индуцировать резистентность в родительских клетках MCF-7. В настоящей работе для исследования характерных особенностей состава экзосом резистентных клеток был проведен сравнительный анализ протеома и профиля микроРНК контрольных экзосом и экзосом, полученных от резистентных сублиний. В целом в образцах экзосом было идентифицировано более 400 белков, из которых только 2 белка, DMBT1 (Deleted in Malignant Brain Tumors 1) и THBS1 (Thrombospondin-1), были гиперэкспрессированы в обоих типах резистентных экзосом (менее 5 % от общего количества белков, дифференциально экспрессированных в экзосомах резистетных клеток), что свидетельствует об уникальном составе экзосомальных белков для каждого типа резистентных клеток. Сравнительный анализ состава микроРНК, дифференциально экспрессированных в обоих вариантах экзосом резистентных клеток, выявил 180 гиперэкспрессированных микроРНК и 202 микроРНК с пониженной экспрессией. Среди них 4 гиперэкспрессированных и 8 гипоэкспрессированных микроРНК оказались ассоциированы с развитием гормональной резистентности клеток рака молочной железы. Биоинформатический анализ 4 гиперэкспрессированных микроРНК выявил 2 микроРНК, mir-101и mir-181b, участвующих в стимуляции PI3K сигналинга, свидетельствуя о важной роли последнего в развитии гормональной резистентности клеток рака молочной железы.

Ключевые слова: рак молочной железы, тамоксифен, экзосомы, гормональная резистентность, микроРНК.

COMPARATIVE ANALYSIS OF THE EXOSOMAL CARGO OF THE ESTROGEN-RESISTANT BREAST CANCER CELLS

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Abstract

The exosomes involvement in the pathogenesis of tumors is based on their property to incorporate into the recipient cells resulting in the both genomic and epigenomic changes. Earlier we have shown that exosomes from different types of estrogen-independent breast cancer cells (MCF-7/T developed by long-term tamoxifen treatment, and MCF-7/M) developed by metformin treatment were able to transfer resistance to the parent MCF-7 cells. To elucidate the common features of the both types of resistant exosomes, the proteome and microRNA cargo of the control and both types of the resistant exosomes were analyzed. Totally, more than 400 proteins were identified in the exosome samples. Of these proteins, only two proteins, DMBT1 (Deleted in Malignant Brain Tumors 1) and THBS1 (Thrombospondin-1), were commonly expressed in the both resistant exosomes (less than 5% from total DEPs) demonstrating the unique protein composition of each type of the resistant exosomes. The comparative analysis of the miRNA differentially expressed in the both MCF-7/T and MCF-7/M resistant exosomes revealed 180 up-regulated and 202 down-regulated miRNAs. Among them, 4 up-regulated and 8 down-regulated miRNAs were associated with progression of hormonal resistance of breast tumors. The bioinformatical analysis of 4 up-regulated exosomal miRNAs revealed 2 miRNAs, mir-101and mir-181b, which up-regulated PI3K signaling supporting the key role of PI3K/Akt in the development of the resistant phenotype of breast cancer cells.

Keywords: breast cancer, tamoxifen, exosomes, hormonal resistance, microRNA.

Exosomes are 30-100 nm-sized microvesicles that are generated in the cells and released into the extracellular space accumulating in many biological fluids, including urine, milk, semen, cerebrospinal fluid, lymph, saliva, etc. [1]. It is noteworthy that tumor cells produce much more exosomes than normal cells [2]. The exosomes involvement in the pathogenesis of tumors is based on their property to incorporate into the recipient cells resulting in the both genomic and epigenomic changes [3-8]. Recently, the ability of the exosomes secreted by the drug- or hormone-resistant tumor cells to transfer the resistant properties to recipient cells has been demonstrated in different cell models [9, 10].

The main goal of the present study was to analyse the features of the exosomes of the estrogen-resistant breast cancer cells and to identify the exosomal factors respondent for transferring of the resistant phenotype to the donor cells.

Earlier, using the estrogen-dependent MCF-7 breast cancer cells and estrogen-independent MCF-7/T cells we have demonstrated the ability of the resistant cells-derived exosomes to initiate the estrogen-independent growth of the parent MCF-7 cells. The parallel experiments were performed on the MCF-7/M resistant subline developed under long-term cultivation of the parent MCF-7 cells with biguanide metformin and characterized by the cross-resistance to metformin and tamoxifen. The treatment of the parent MCF-7 cells with the MCF-7/M exosomes resulted in the cell cross-resistance to metformin and tamoxifen. Both types of resistant exosomes, MCF-7/T and MCF-7/M, induced the similar changes in the cell signaling: inhibition of the estrogen signaling and stimulation of the Akt protein kinase and transcription factors AP-1and NF-kB [11].

Here, to elucidate the common features of the both types of resistant exosomes, the proteome and microRNA cargo of the control and both types of the resistant exosomes were analyzed. Exosomes were prepared from the MCF-7, MCF-7/T and MCF-7/M' conditioned medium by the differential ultracentrifugation, and exosome imaging was carried out by transmission electron microscope. For proteome study, an AB Sciex 5800 MALDI TOF/TOF mass spectrometer (Sciex, Germany) was used. Analysis of MS and MS/MS spectra was done with Protein Pilot software using the UniProtKB/SwissProt/NCBI international protein databases. Then the differentially expressed proteins in the exosomes of MCF-7, MCF-7/T and MCF-7/M cells were detected. Totally, more than 400 proteins were identified in the exosome samples. Among them, 131 differentially expressed proteins (DEPs) were found in the exosomes of MCF-7/T cells versus MCF-7 exosomes and 97 DEPs were found in the MCF-7/M exosomes.

To find the common changes in the proteome of the resistant exosomes, DEPs in the exosomes from MCF-7/T and MCF-7/M cells were compared. As revealed, only two proteins, DMBT1 (Deleted in Malignant Brain Tumors 1) and THBS1 (Thrombospondin-1), were commonly expressed in the both resistant exosomes (less than 5% from total DEPs) demonstrating the unique protein composition of each type of the resistant exosomes. Noteworthy, the bioinformatical analysis showed correlation between expression of two identified proteins and breast cancer risk. Namely, single-nucleotide polymorphisms (SNPs) and overexpression of DMB1 were found to be associated with the breast cancer [12, 13]. Several studies demonstrated that high level of THBS1 mediates chemotherapy resistance through the integrin β1/mTOR pathway [14] and promotes aggressive phenotype via epithelial-mesenchymal transition (EMT) [15].

The analysis of exosomal microRNAs was performed by HiSeq2500 and at least 5 million reads per samples were obtained. Library preparation

Table 1

Differentially expressed miRNAs in the exosomes of the resistant MCF-7/T and MCF-7/M cells

Cell line	Total miRNAs	Up-regulated miRNAs	Down-regulated miRNAs
MCF-7/T	877	459	418
MCF-7/M	751	388	363
Common miRNAs	382	180	202

Table 2

Differentially expressed exosomal miRNAs associated with hormonal resistance

Up-regulated miRNAs	**Biological effects**	**Refs**
hsa-miR-101-3p	Upregulates the phosphorylated Akt (pAkt)	[16]
hsa-miR-210-5p	Up-regulated in TAM-R MCF-7	[17]
hsa-miR-7704	Up-regulated in TAM-R MCF-7	[18]
has-miR-181b	Up-regulated in TAM-R MCF-7cells	[19]
Down-regulated miRNAs	**Biological effects**	**Refs**
hsa-let-7b-3p	Induce tamoxifen sensitivity by downregulation of estrogen receptor	[20]
hsa-miR-10a-3p	Suppresses the levels of p-Akt, p-mTOR, p-p70S6K, and PIK3CA, and increases the expression of Cyt C, cleaves caspase-3, and the ratio of Bax/Bcl-2	[21]
hsa-miR-148a-3p	Increase drug sensitivity of breast cancer cells	[22]
hsa-miR-182-5p	Induces apoptosis through the upregulation of CASP9	[23]
hsa-miR-200b-5p	Suppresses the epithelial-mesenchymal transition	[24]
hsa-miR-27b-3p	Directly targets and inhibits the expression of nuclear receptor subfamily 5 group A member 2 (NR5A2) and cAMP-response element binding protein 1 (CREB1) and regulates ESR1, PGR1, FOXM1 and 14-3-3 family genes	[25, 26] [27]
hsa-miR-29a-3p	Suppresses proliferation of tamoxifen-resistant breast cancer cells	[28]
hsa-miR-503-5p	Suppresses proliferation by regulating the oncogene ZNF217	[29]

and sequencing was done by ZAO Genoanalytica (Moscow, Russia) as follow: microRNA was extracted from by PureLink RNA Micro Kit (#12183-016) according to manual. Library preparation was carried out with NEBNext® Small RNA Library Prep Set for Illumina® (E7330S) according to manual. More than 2500 miRNAs were identified in the exosomal samples. The comparison of miRNA profile of MCF-7 and MCF-7/T exosomes revealed 877 miRNA differentially expressed in MCF-7/T exosomes, among them 459 miRNA were up-regulated, and 418 miRNA were down-regulated. Study of miRNA of MCF-7/M exosomes showed 751 differentially expressed miRNA including 388 up-regulated and 363 down-regulated miRNAs. The comparative analysis of the miRNA differentially expressed in the both MCF-7/T and MCF-7/M resistant exosomes revealed 180 up-regulated and 202 down-regulated miRNAs (Table 1).

The following bioinformatical analysis of the common differentially expressed miRNAs revealed 4 up-regulated and 8 down-regulated miRNAs associated with progression of hormonal resistance of breast tumors. Importantly, we revealed the strong correlation between change vector of miRNA expression in the resistant exosomes and type of miRNA activity. Namely, all of 4 up-regulated miRNAs were described as resistance-associated mitogenic factors, whereas 8 down-regulated miRNAs were considered as the pro-apoptotic or hormone-sensitive- associated factors (Table 2).

As mentioned above, the parent cells response to the resistant exosomes involves the activation of Akt – one of the key signaling supporting the growth of the hormone-resistant cells [30]. The key role of the Akt signaling in the transferring of the resistant phenotype was substantiated in our experiments, showing the full block of the exosome-induced resistance of MCF-7 cells in the presence of PI3K inhibitor wortmannin [11]. Here, the analysis of 4 resistance-associated exosomal miRNAs revealed 2 miRNAs, mir-101, mir-181b, which up-regulated PI3K signaling. Both of miRNAs exert their effect via the suppression of PTEN phosphatase which is main physiological antagonist of PI3K [16].

Totally, we demonstrated the unique protein and miRNA composition of the exosomes of the resistant cells, identified the possible intercellular targets of exosomes and revealed the key exosomal miRNAs associated with hormonal resistance. Further studies are required to explore the role of the each of the identified miRNAs in the progression of the exosome-induced hormonal resistance.

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Conflict of interest

The authors declare that they have no conflict of interest.

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СВЯЗЬ МЕЖДУ РЕГУЛИРУЮЩИМИ БЕЛКАМИ АУТОФАГИИ M-TOR И BECLIN-1 И ПАРАМЕТРАМИ ЛИМФОГЕННОГО МЕТАСТАЗИРОВАНИЯ ПРИ КОЛОРЕКТАЛЬНОМ РАКЕ

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Аннотация

На сегодняшний день влияние процессов аутофагии на канцерогенез остается не до конца изученным. С одной стороны, аутофагия является опухолевым супрессором за счет активации разрушения онкогенных протеинов, токсичных белков и дефектных органелл, которые могут обладать агрессивными свойствами и способствовать повреждению ДНК клетки. С другой стороны, аутофагия может способствовать выживанию опухолевых клеток в условиях гипоксии и присутствия активных форм кислорода, что происходит преимущественно за счет блокировки механизмов апоптоза, увеличивая шансы на поддержание циркуляции опухолевого клона. Регуляция аутофагии является сложным, многоэтапным и комплексным процессом. Основным его регулятором является сигнальный путь, который активирует белок протеинкиназосерин-треониновой специфичности m-Tor (мишень рапамицина у млекопитающих). В литературе имеются данные о влиянии белков аутофагии ATG5, LC3A и LC3B, Beclin-1 на способность злокачественно трансформированных клеток к выживанию, а также на развитие опухоли и ее прогрессирование. Крайне актуальными являются исследования, направленные на поиск возможных взаимосвязей между процессами аутофагии и патогенетическими механизмами канцерогенеза. **Цель исследования** – изучить взаимосвязь экспрессионных параметров белков-регуляторов аутофагии m-TOR и Beclin-1 с параметрами лимфогенного метастазирования при колоректальном раке. **Материал и методы.** В исследование были включены 105 пациентов с колоректальным раком T1–4N0–3M0, находившихся на лечении в отделении торако-абдоминальной онкологии НИИ онкологии Томского НИМЦ в период с 2012 по 2015 г. Средний возраст больных составил 59,7±4,3 года. Морфологическая верификация диагноза колоректального рака проводилась на биопсийном материале фрагментов ткани первичной опухоли. Распространенность онкологического заболевания определялась согласно международной классификации по системе TNM (2002). **Результаты.** Анализ частоты лимфогенного метастазирования в зависимости от наличия или отсутствия экспрессии белков m-Tor и Beclin-1 в цитоплазме опухолевых клеток выявил статистически значимую связь между этими параметрами. **Заключение.** Полученные данные отчетливо демонстрируют тот факт, что снижение или утрата активности процессов аутофагии в опухоли сопровождается реализацией механизмов лимфогенной диссеминации, которая является предиктором неблагоприятного прогноза заболевания.

Ключевые слова: колоректальный рак, аутофагия, лимфогенное метастазирование, Beclin-1, m-Tor, прогноз.

A LINK BETWEEN AUTOPHAGY REGULATORY PROTEINS M-TOR AND BECLIN-1 AND PARAMETERS OF LYMPHOGENIC METASTASIS IN COLORECTAL CANCER

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Abstract

Currently the impact of autophagy on carcinogenesis remains understudied. On the one hand, autophagy acts as a tumor suppressor, as it activates degradation of oncoproteins, toxic proteins, and damaged cell organelles, that may be aggressive and lead to DNA damage. On the other hand, autophagy may promote tumor cell survival under hypoxia and in the presence of reactive oxygen species, which occurs primarily due to blocking of apoptosis mechanisms, raising the chances for maintaining tumor clone dynamics. Autophagy regulation is a complicated and multi-stage process. The main regulator here is a signaling pathway that activates serine/threonine protein kinase m-TOR (the mammalian target of rapamycin). Data on the impact of autophagic proteins ATG5, LC3A, LC3B, and Beclin-1 on malignant cell survival as well as on tumor growth and progression have been reported in literature. However, studies aimed at seeking possible relationships between autophagy and pathogenetic mechanisms of carcinogenesis are of great interest. **The aim of the study** is to investigate a relationship between the expression parameters of autophagy regulatory proteins m-TOR and Beclin-1 and the features of lymphogenic metastasis in colorectal cancer. **Materials and methods.** The study included 105 patients with T1-4N0-3M0 colorectal cancer treated in the Thoracic and Abdominal Department of Cancer Research Institute of Tomsk Research Medical Center from 2012 to 2015. The average age of patients was 59.7±4.3 years. Morphological verification of the diagnosis was performed on the biopsy samples of primary tumor tissue. Staging of colorectal cancer was determined according to the TNM classification of malignant tumors (2002). **Results.** Analysis of the frequency of lymphogenic metastasis depending on the presence or absence of m-Tor and Beclin-1 expression in tumor cell cytoplasm revealed a statistically significant link between these variables. **Conclusion.** The obtained findings clearly exhibit that deceleration or loss of autophagic activity in the tumor is accompanied by implementation of lymphogenic dissemination, which is a predictor of an unfavorable outcome of the disease.

Keywords: colorectal cancer, autophagy, lymphogenous metastasis, Beclin-1, m-Tor, prognosis.

Introduction

Currently, colorectal cancer is one of the most common cancers worldwide, and every year more and more new cases of this disease are recorded [1]. This cancer is characterized by low five-year survival rate, which is explained by frequent recurrences and high chances for development of lymphogenic and hematogenic metastases [2]. Differences in biological parameters of a primary tumor, variety in the types of carcinoma, differences in metastasis in organs, and resistance to therapy make difficult early detection of a tumor. Shortcomings of existing treatment algorithms dictate the necessity of studying clinical features of colorectal cancer as well as molecular and morphologic parameters of the disease.

According to the literature data, non-surgical treatment options, such as chemotherapy, radiation therapy, hormone therapy and immunotherapy, are the most common treatment strategies for patients with colorectal cancer [3, 4]. Special attention is given to the study of molecular and genetic features of the tumor, that are associated with different options of tumor progression as well as with disease prognosis. Autophagy is one of the processes involved in tumor pathogenesis. Its role in tumor pathogenesis is understudied and data that have been already published in literature are controversial. Autophagy is a catabolic process characterized by degradation of cell organelles. This process is necessary for growth and proliferation of healthy cells and maintenance of intracellular homeostasis [5]. Autophagy is regulated by more than 30 proteins encoded by ATG genes found in yeast fungi as well as by a big number of homologous proteins found in mammals [6].

The impact of autophagy on carcinogenesis remains unclear [7]. On the one hand, autophagy acts as a suppressor of tumor cell development due to degradation of oncoproteins, toxic proteins, and damaged cell organelles that may be aggressive and lead to DNA damage [8, 9]. On the other hand, autophagy may promote tumor cell survival under hypoxia, in the presence of reactive oxygen species, and under starvation by blocking apoptosis, thus favoring tumor progression [9-11]. The main autophagy regulator is a signaling pathway that activates serine/threonine protein kinase m-TOR (the mammalian target of rapamycin), which is involved in cell metabolism, growth, proliferation, and differentiation [12].

According to E.J. Write et.al., autophagy suppression in the tumor is associated with the oncogenic function of the PI3-K pathway, which triggers the mTORC1 cascade of reactions [13]. According to other authors, tumor cells with activated autophagy are characterized by increased aggressiveness and resistance to chemotherapy. After chemo- and radiation therapy, a rise in the number of autophagosomes in the tumor was observed [8, 14].

Impairment of m-TOR pathway functioning, determined by genetic variations in key genes, was identified in investigations on bladder cancer, breast cancer, lung cancer, and hepatocellular carcinoma [15-18]. According to L. Lei et. al., over the past few years, considerable attention has been given to the impact of the m-TOR pathway on tumor formation [19]. According to the findings of L. Xiao yan L. Zhou, increased proliferative activity in tumor cells in breast cancer is associated with elevated p-mTOR expression [20]. Another study exhibited that p-mTOR expression

was associated with a worse prognosis of stomach cancer, however, the survival rate in patients with positive m-TOR expression was significantly higher than that in patients with negative expression of this marker in the tumor [21, 22].

Literature contains data on the impact of autophagy-related proteins ATG5, LC3A, LC3B, and Beclin-1 on cell survival, carcinogenesis, and invasive features of the tumor [23-25]. The Beclin-1 protein plays an essential role in regulating autophagy [26] and is involved in implementation of various signaling pathways. Beclin-1 may act both as an inductor and suppressor of carcinogenesis. Till recently, the gene that encoded Beclin-1 had been viewed as a tumor suppressor gene. However, latest research has not revealed any evidence on a relationship between a loss of this gene or its mutation and development of tumors in different localities [27]. J. Gao et al. reported that transformations in the gene encoding the Beclin-1 protein were identified in only 2.5% of patients with colorectal cancer [28]. In the study of K.J. Schmits et al., a correlation between the level of Beclin-1 expression and low survival rate in patients with stage III and IV colorectal cancer was found [10]. The function of Beclin-1 is associated with many key cell molecules, for instance, with m-TOR and HIFs (a transcription factor that reacts to a decrease in cellular oxygen level) [29].

In recent years, the prognostic significance of Beclin-1 level in stomach cancer [30], epidermoid laryngeal cancer [31], and testicular cancer [32] has been actively studied. In case of monoallelic deletion of the gene encoding Beclin-1, in 40-75% of patients with malignant tumors deceleration of autophagy was observed. Such findings were obtained in investigations on testicular, breast, prostate, and brain cancers [33]. To date, data on the impact of Beclin-1 on tumor progression and disease prognosis remain controversial. According to the findings of Ahn, increased expression of Beclin-1 was observed in 95% of patients with intestinal adenocarcinoma. However, in this study no statistically significant data on the relationship between the level of this marker and clinical and morphological features of the tumor were obtained, thus leading to a hypothesis that Beclin-1 does not play an essential role in tumor suppression [33]. A number of other studies point to a relationship between the level of Beclin-1 expression and the depth of tumor invasion as well as metastasis parameters. Thus, high Beclin-1 expression in patients with colorectal cancer was considered as an indicator of a favorable prognosis and good survival rate [34]. Therefore, the study of autophagy-related proteins is a topical area in modern oncology, since the impact of these proteins on signaling pathways regulating autophagy in healthy and neoplastic cells may become a prospective therapeutic target for cancer.

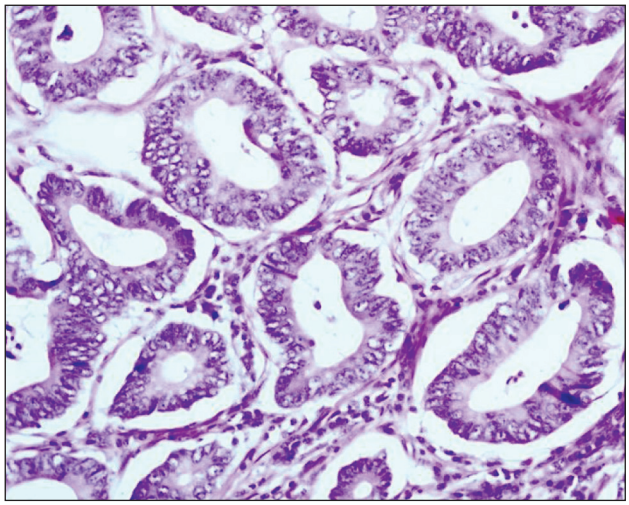

Figure 1. Well-differentiated adenocarcinoma with the minimal stromal component. Hematoxylin and eosin staining (200× magnification)

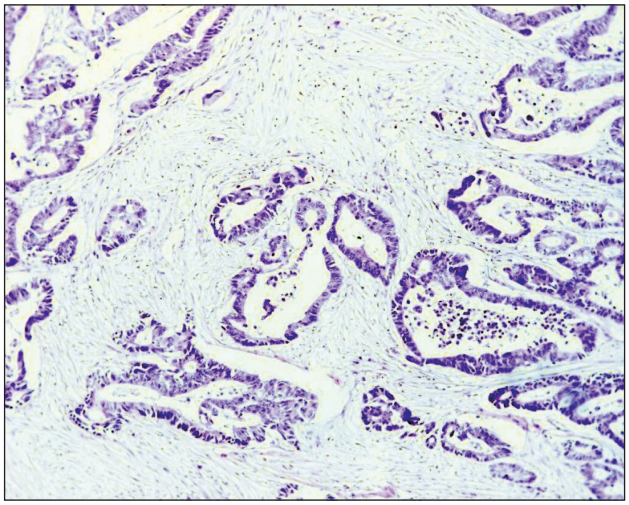

Figure 2. Moderately differentiated adenocarcinoma with pronounced stroma. Hematoxylin and eosin staining (100× magnification)

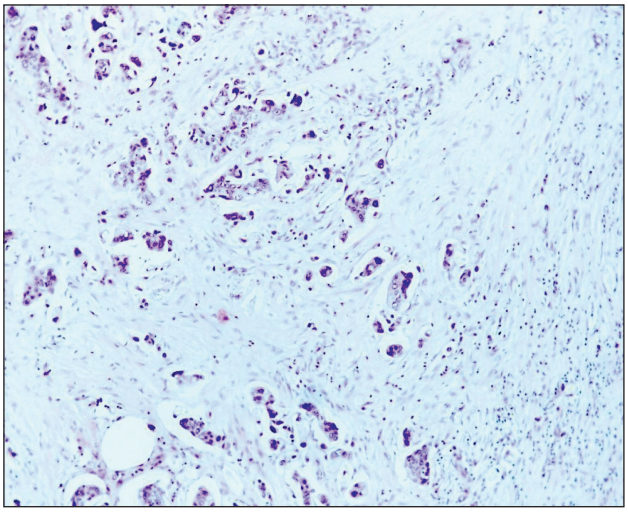

Figure 3. Poorly differentiated adenocarcinoma. Discrete tumor cells, small tumor cell clusters. Hematoxylin and eosin staining (100× magnification)

Material and Methods

The study included 105 patients with T1-4N0-3M0 colorectal cancer treated in the Thoracic and Abdominal Oncology Unit at the Research Institute of Oncology of Tomsk Research Medical Center from 2012 to 2015. The average age of patients was 59.7±4.3 years. Morphological verification of the diagnosis was performed on the biopsy samples of primary tumor tissue. Staging of colorectal cancer was determined according to the TNM classification of malignant tumors (2002). Stage I cancer was detected in 8 patients (7.6%), stage IIA in 16 patients (15.2%), stage IIB in 43 patients (41%), stage IIIA in 33 patients (31.5%), stage IIIB in 4 patients (3.8%), and stage IIIC in 1 case (0.9%). Tumor localization in the colon was the following: the cecum in 16 patients (15%), ascending colon in 8 patients (7%), hepatic flexure in 3 patients (3%), transverse colon in 2 patients (2%), splenic flexure in 23 patients (22%), descending colon in 6 patients (5%), sigmoid colon in 31 patients (30%), and rectosigmoid junction in 16 patients (16%). All patients underwent hemicolectomy or colon resection. In the post-operative period, 48 patients received different adjuvant treatments according to clinical recommendations. The patients had been followed up for 3 years. To evaluate the disease activity, patients' medical records were analyzed. The surgical specimens were sent to further morphological examination. Fixation, processing to a paraffin block, preparation of slices, and staining were performed in compliance with standard instructions. Morphological examination of the specimens was conducted using the light microscope ("Axioscope A1" Carl Zeiss). Digital images of the stained histology and immunohistochemistry slides were acquired using AxioCam MRc5 camera with AxioVision 4.6.3 (Carl Zeiss) software for digital image processing. When investigating the surgical specimens, we performed gross examination of the primary tumor: we evaluated the size and form of the tumor, the depth of tumor invasion, and the status of resection margins. To assess the status of regional lymph nodes, all lymph nodes were investigated with respect to the presence or absence of metastases. The diagnosis of colorectal cancer was made according to the WHO Histological classification of Gastrointestinal Tumors (WHO, 2013). In 92 patients (87.6%), the tumor histotype corresponded to colon adenocarcinoma with various degrees of differentiation (Figures 1-3). In 13 patients (12.4%), other histological subtypes were detected (mucinous cancer, cancer with neuroendocrine differentiation). Histological examination of the tumor tissue was conducted with assessment of the following parameters: the degree of differentiation, depth of colon wall invasion, presence of perineural or lymphovascular invasion, presence of tumor necrosis, presence and intensity of inflammatory infiltration, and degree of stroma representation in the tumor.

Various expression parameters of the tumor in colorectal cancer were investigated on the paraffin sections using the immunohistochemical assay according to the standard procedure. The following antibodies were used in the investigation: Anti-Beclin 1 antibody ab 62472 Abcam (polyclonal, 1:100), and Rabbit Anti-human m-Tor Antibody (polyclonal, 1:100). Application of antibodies was preceded by control reactions with external controls according to the manufacturer's instructions. The expression of Beclin-1 (cytoplasmic expression in the Golgi complex) and m-Tor (membrane and cytoplasmic staining) was evaluated as the percentage of positively-stained cells in the tumor detected per 10 fields of vision at 400× magnification. Along with quantitative assessment of the studied marker

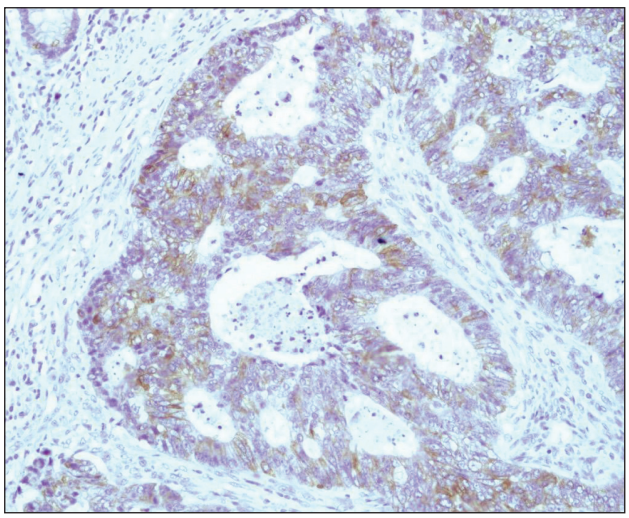

Figure 4. Moderately pronounced heterogeneous cytoplasmic expression of m-Tor in colon adenocarcinoma. The immunohistochemical reaction. Diaminobenzidine and hematoxylin staining (200× magnification)

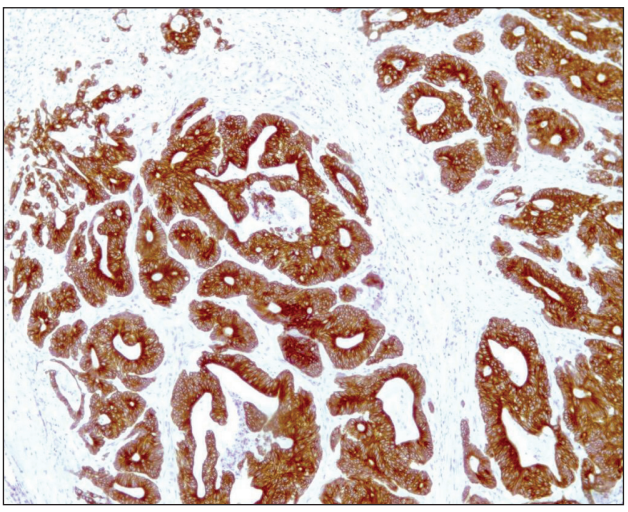

Figure 5. Strongly apparent homogeneous cytoplasmic expression of m-Tor in colon adenocarcinoma. The immunohistochemical reaction. Diaminobenzidine and hematoxylin staining (100× magnification)

Table 1

Distribution of the patients according to the number of lymph nodes affected by metastases

Number of metastatic lymph nodes	Number of patients (abs.num., %)
Up to 4 lymph nodes (N1)	39 (88 %)
More than 4 lymph nodes (N2)	5 (12 %)

Table 2

A link between the presence of m-Tor expression and the state of regional lymph nodes in the patients with colorectal cancer

m-TOR expression in the tumor cells	Presence of metastases in the regional lymph nodes (abs. num., %)		
	N0	N+	
Negative expression	1 (1.7 %)	5 (10.8)	χ^2=4.4
Positive expression	58 (98.3 %)	41 (89.2 %)	p=0.04

Table 3

Percentage of Beclin-1 and m-Tor expression depending on the presence of lymphogenic metastases

Presence of metastases	Percentage of m-TOR expression	Percentage of Beclin-1 expression
N0	79.6±25.1	24.5±26.1
N+	67.3±33.9	19.2±20.9
	F=4.5 p=0.03	F=1.2 p=0.2

expression in the tumor, heterogeneity of staining was investigated at 150× magnification. In cases of positive marker expression in the tumor cells with equal staining intensity, the expression was estimated as homogeneous. Cases where the tumor had regions with both positive and negative marker expression as well as regions with different staining intensity were considered as cases with heterogeneous expression distribution (Figures 4-5).

The obtained data were processed using "Statistica 10" software. The findings were analyzed with descriptive statistics and dispersion analysis. To calculate statistical significance of the differences, the non-parametric Chi-square test and Spearman's correlation coefficient were used. The differences between the studied variables were considered statistically significant at $p<0.05$.

Results

Since the presence of lymphogenic metastases is one of the key prognostic criteria for the course of colorectal cancer, we analyzed the frequency of metastases depending on the parameters of autophagy marker expression.

Table 1 represents the data on the distribution of the patients depending on the number of lymph nodes affected by metastases. In the study, most patients had less than 4 metastatic lymph nodes; it corresponded to N1 parameter in the TNM classification.

Analysis of the frequency of lymphogenic metastasis depending on the presence or absence of m-Tor expression in tumor cell cytoplasm revealed a statistically significant link between these variables. We found that metastases in regional lymph nodes were more often observed under negative m-Tor expression in tumor cells (Table 2).

Moreover, we identified a relationship between the percentage of the tumor cells with positive m-Tor expression and the presence of lymphogenic metastases. In patients with metastatic regional lymph nodes, lower m-Tor expression in tumor cells was detected (Table 3).

Following the obtained findings, we found that the presence or absence of Beclin-1 expression in tumor cells had no statistically significant link with the number of metastatic lymph nodes. What is more, we did not reveal a statistically significant relationship between the percentage of Beclin-1 expression and the parameters of lymphogenic metastasis (F=1.2, p=0.2) (Table 3).

Conclusion

The present study allowed us to identify the link between the expression features of m-Tor protein and the parameters of lymphogenic metastasis in patients with colorectal cancer. Thus, the presence of metastatic lymph nodes was more frequently registered under negative expression of the marker in tumor cells. We revealed that a statistically significant decrease in m-Tor expression in tumor cells occurred in the presence of metastases in regional lymph nodes. The obtained findings clearly exhibit that deceleration or loss of autophagic activity in the tumor is accompanied by implementation of lymphogenic dissemination, which is a predictor of an unfavorable outcome of the disease.

The expression characteristics of Beclin-1 appeared to have no relation to the parameters of lymphogenic metastasis. It may be explained by less involvement of this molecular and biologic marker in carcinogenesis and tumor progression in colorectal cancer.

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НАСЛЕДСТВЕННЫЙ РАК ЖЕЛУДКА ДИФФУЗНОГО ТИПА: ГЕНЕТИЧЕСКИЕ АСПЕКТЫ И ПРОФИЛАКТИЧЕСКАЯ ТОТАЛЬНАЯ ГАСТРЭКТОМИЯ

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Аннотация

Для пациентов с выявленной наследственной мутацией E-cadherin-1 (*CDH1*) терапией выбора является профилактическая гастрэктомия для устранения риска развития рака диффузного типа. Представленный клинический пример описывает редкий случай наследственного рака желудка диффузного типа (HDGC), ассоциированного с мутацией в гене *CDH1,* впервые описанного в России. В 2013 году 28-летняя пациентка была поставлена на учет в клинической онкогенетической лаборатории с семейной историей рака желудка. Молекулярно-генетический анализ выявил наследственную мутацию в гене *CDH1.* Риск заболеть раком в течение жизни у пациентов с выявленной мутацией превышает 80 %. Гистологическое исследование биопсийного образца, полученного при эндоскопии, обнаружило изолированные мелко-круглые клетки в lamina propria. В отделе абдоминальной онкологии пациентке было проведено хирургическое вмешательство: лимфодиссекция в объеме D2 с сохранением селезенки и тотальная гастрэктомия с Roux-en-Y реконструкцией с формированием тощекишечного резервуара.

Ключевые слова: наследственный рак желудка диффузного типа (HDGC), ген *CDH1,* гастрэктомия, молекулярно-генетическая диагностика.

HEREDITARY DIFFUSE GASTRIC CANCER: GENETIC ASPECTS AND PROPHYLACTIC TOTAL GASTRECTOMY

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Abstract

For patients with an identified germline E-cadherin-1 (*CDH1*) mutation, prophylactic gastrectomy is the treatment of choice to eliminate the high risk of developing diffuse gastric cancer. The case report describes a rare case of hereditary diffuse gastric cancer (HDGC) associated with *CDH1* gene mutation, which is reported in the Russian population for the first time. In 2013, a 28-year- old woman was referred to Clinical Oncogenetics Laboratory with a family history of gastric cancer. Molecular genetic analysis revealed *CDH1* gene mutation. The lifetime risk of cancer in mutation positive members is more than 80. Histological examination of gastric biopsy specimens obtained during endoscopy revealed isolated signet ring cells in the lamina propria. Spleen-preserving D2-lymphodissection and total gastrectomy with Roux-en-Y reconstruction with a jejunal reservoir formation were performed at the Abdominal Oncology Surgery Department.

Keywords: hereditary diffuse gastric cancer (HDGC), *CDH1* gene, gastrectomy, molecular genetic diagnostics.

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Most cases of gastric cancer are sporadic, but 1–3 % cases occur in syndromes with a high hereditary predisposition to gastric cancer [1]. The most frequent hereditary gastric cancer is a diffuse type or litinitis plastica, it is commonly referred to hereditary diffuse gastric cancer (HDGC). To classify a familial case as HDGC, the international gastric cancer consortium (IGCLC) formulates the following criteria: (1) two or more documented cases of diffuse gastric cancer diagnosed in first- or second-degree relatives of age under 50 years old, in one case, at least (2) three or more documented cases of diffuse gastric cancer in relatives of the first - or second – degree relatives, regardless of the age of the disease oneset [2, 3].

HDGC is associated with germinal mutations in E-cadherin 1 (*CDH1* gene), that encodes the cell adhesion protein E-cadherin [4]. About 25–30 % of the families meeting the HDGC criteria according to the criteria of international consortium (Internatinal Gastric Canser Linkage Consortium – IGCLC) have constitutional aberrations in *CDH1* gene [4]. More than 100 mutations in *CDH1* gene have been described now in the families originating from different ethnic groups [5].

Although there are no major mutational hotspots, some mutations, including 1003C>T [6–9], 1901C>T [7, 10, 11], and 1137G>A [7, 11, 12], (2398delC) [7] have been observed in several unrelated families. The most common types of mutation are small insertions or deletions (35 %). The other mutations are missense (28 %), nonsense (16 %), splice site (16 %), and large exonic deletions (5 %) [13]. In addition to these major mutations, two regulatory sequence variants,−160C−>A [14] and the intron 2 variant 163+37235G>A [15] have been associated with an elevated risk of DGC, although these polymorphisms are rarely associated with a strong familial clustering.

No correlations between the location or type of germline *CDH1* mutation and phenotype have been ascertained. Particularly, there is no obvious correlation between genotype and the presence of lobular breast cancer in HDGC families [16]. However, somatic *CDH1* mutations in sporadic DGC are predominantly splice site mutations resulting in exon skipping – particularly of exons 8–9, whereas most *CDH1* mutations identified in sporadic lobular breast cancer result in premature stop codons [17, 18].

The *CDH1* gene mutations originated de novo, are also identified in sporadic cases of early gastric cancer, at least, in 4 % of the patients with manifestation age under 35 years. Germinal mutations in the *CDH1* gene have a high penetrance: cancer risk throughout the life is 67 % in men and 83 % - in women. The average age at diagnosis is 38-40 years old, it varies from 14 to 85 years old [19].

The female patient of 28 years old was consulted on familial history of gastric cancer cases among her young relatives (Figure 1). The study of familial anamnesis followed by molecular genetic analysis - determination of *CDH1* gene sequence encoded suppressor E-cadherin, involved in HDGC carcinogenesis was performed at Clinical Oncogenetics Laboratory.

PCR analysis of DNA isolated from peripheral blood lymphocytes followed by conformation-sensitive gel electrophoresis and sequencing has revealed a germinal mutation c.1005delA - deletion in exon 7 of *CDH1* gene, resulting in open reading frame shift.

Conclusion: given the high penetrance of CHD1 gene, lifetime gastric cancer risk is 80 %. Risk of infiltrative lobular breast cancer risk is much higher (60%) than the common population risk. The risk of colon cancer is also significantly higher than common population risk. This points to the need for dynamic follow up in N.N. Blokhin Medical Research Center of Oncology.

The risk of the germinal mutation inheritance by first-degree relatives is 50 %. According to the recommendations on gastric cancer of the IGCLC, a total gastrectomy is justified as a preventive measure. Ultrasound, CT, mammography and/or MRI of the breast; consultations of geneticist, gastroenterologist, mammologist, proctologist, nutritionist; DNA - diagnostics in first-degree relatives are recommended for follow-up of patients.

A planned EGDS, which was performed simultaneously with the molecular genetic study detected a gastric tumor in its antral part. Biopsy also revealed gastric tumor – isolated signet ring cells in mucosa of lamina propria. For further examination and treatment, the patient was hospitalized at the Abdominal Oncology Surgery Department. Total gastrectomy with Roux-en-Y reconstruction with formation of jejunal reservoir and D2 limphodissection were performed on 14.03.2013. Histological verification and immunohistochemical examination of the surgical material obtained on 05.04.2013 confirmed the invasive ring-cell gastric cancer of the pyloric part within mucous membrane own plate - early gastric cancer of 0.9×0.2×0.1 cm, without vascular invasion and without metastases (Figure 2, 3). The Her2-neu overexpression was not detected («0»), the proliferation index Ki 67 was about 22 %.

This patient was monitored for 60 months. In the first 6 months there was a moderate weight loss and dumping syndrome of moderate severity, which were then compensated. According to clinical examination from 20.03.2018, no progression was observed.

Discussion

The patient pedigree represents a rare case of families that fully meets the HDGC criteria (Figure 1). Germinal mutation 1005delA in 7 exon of *CDH1* gene, which the patient most likely inherited from her mother, was described in Russia for the first time [20]. Molecular genetics testing of available relatives for *CDH1* 1005delA mutation was performed for the first- and second-degree siblings (IV-2, IV-3); the mutation was not detected.

Thirty-eight publications on gastrectomy in carriers of *CDH1* gene mutations were summarized. From

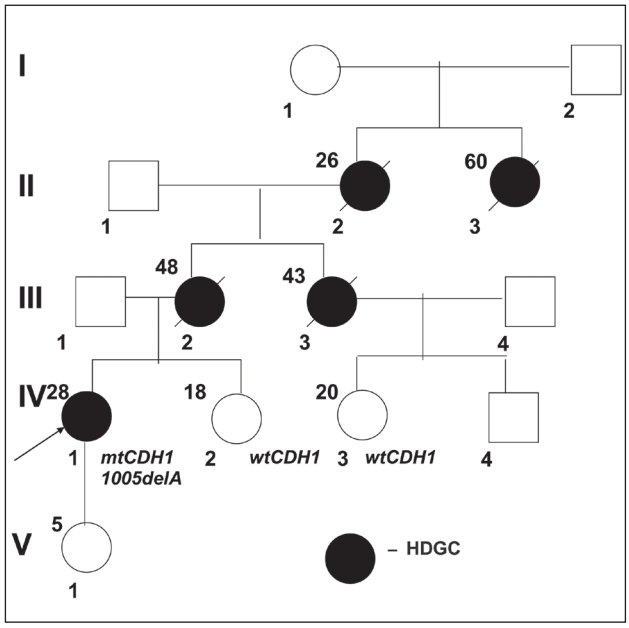

Figure 1. HDGC pedigree of proband.
mtCDH1 – CDH1 1005delA mutation
wtCDH1 – wild type CDH

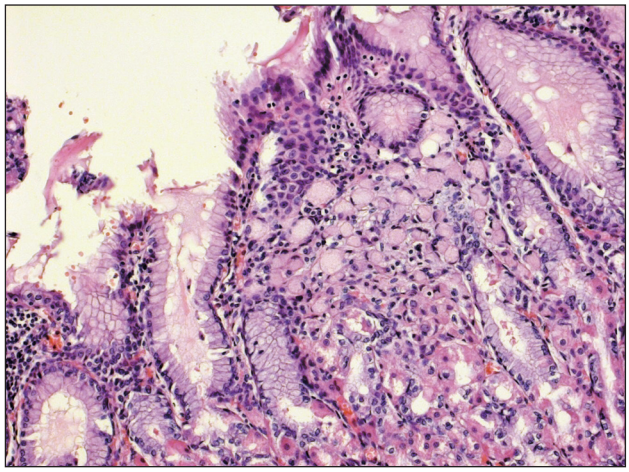

Figure 2. Signet ring cells carcinoma

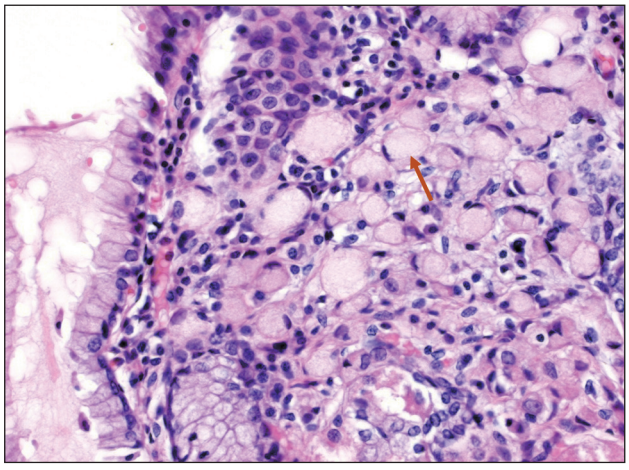

Figure 3. Signet ring cells carcinoma

the 169 patients, the symptoms at the preoperative stage were absent in 106 (62.7 %) cases. Diagnosis of HDGC was confirmed in 21 (12.4 %) patients before operation, there were no data for 42 patients. According to postoperative histological examination, ring-cell cancer was detected in 147 (87 %) patients and it was not revealed in 17 patients only [21]. Similar data were obtained in a recent study that described postoperative outcomes of total gastrectomy in 41 patients. Thirty-five patients (85 %) demonstrated 1 or more foci of intramucosal signet ring cell gastric cancer in the examined specimen [22].

Laparoscopic total gastrectomy with jejunal pouch reconstruction as a novel approach that may be especially suitable in these patients have been performed by the surgeons from the Netherlands. A total of 11 patients with a median age of 40 (22–61) years were included. The 60-day mortality rate was 0 %. Multiple foci of intramucosal diffuse gastric signet ring cell carcinoma were found in the resection specimen of 9/11 (82 %) patients. All 11/11 (100 %) resections were microscopically radical. Thus, they proved that prophylactic laparoscopic total gastrectomy with jejunal pouch reconstruction in patients with a *CDH1* germline mutation is feasible and safe [23]. In gastric cancer, laparoscopic total gastrectomy showed diminished blood loss, fewer postoperative complications, and shorter postoperative hospital stay [13]. This technique may therefore be especially suitable for prophylactic surgery [24].

This is a rare clinical case, when the diagnosis of HDGC was confirmed during routine EGDS with dynamic follow-up based on a family history and young age of disease onset in affected patient relatives.

However, the published data show that in most cases, ring-cell cancer remains undiagnosed even by repeated EGDS and biopsies. Two-year survival of the patients without any symptoms before gastrectomy was 100 %, it was 40 % among the patients with HDGC revealed before surgery [25].

High risk (>80 %) of HDGC occurrence, insufficient effectiveness of the regular EGDS and the proof of the microscopic foci with ring-shaped cells in all the *CDH1* gene mutation carriers with family histories fully justifies preventive gastrectomy in this group [21, 25].

The optimal age of preventive gastrectomy in such patients is under discussion. The following aspects should be taken into account. Prophylactic gastrectomy is not warranted before the final body formation, at least, up to 20 years old. However, the patients with detected signet ring cell carcinoma at the age of about 40 years old, have only 10 % chance of the favorable disease course [26].

Mortality associated with preventive gastrectomy varies from 0 to 6 %. Mortality rate among young patients without symptoms before surgery is less than 1 %. Thus, prophylactic gastrectomy is recommended for carriers of *CDH1* gene mutations with family accumulation of HDGC, taking into account the high disease risk during their life, late clinical detection and unfavorable prognosis.

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Conflict of interest

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СПЕКТР МУТАЦИЙ ГЕНА *BRCA1* У БОЛЬНЫХ РАКОМ МОЛОЧНОЙ ЖЕЛЕЗЫ В МОЛОДОМ ВОЗРАСТЕ В РОССИИ

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Аннотация

Цель исследования – оценить частоту встречаемости патогенных мутаций в BRCA1 гене у женщин с раком молочной железы, проживающих в России. **Материал и методы.** Проведён анализ полной кодирующей части гена BRCA1 у 445 больных раком молочной железы на ранней стадии (возраст больных до 40 лет), проживающих в Новосибирской области (Россия), с помощью метода таргетного секвенирования на платформе Ion Torrent. Результаты. Выявлено 40 (9 %) носительниц различных патогенных мутаций. У 35 (7,9 %) пациенток обнаружена мутация 5382insC, описанная ранее как «мутация-основателя» в славянской популяции. У 5 (1,1 %) пациенток были выявлены другие различные патогенные мутации, а именно C61G, 462delCC, E143X, 4153delA и IVS18 + 1G> T. Кроме того, 29 генетических вариантов с отсутствующей или неясной клинической значимостью были обнаружены в гене BRCA1 у 445 больных раком молочной железы на ранней стадии. Выводы. Получены данные о частоте генетических вариаций гена BRCA1 у больных раком молочной железы на ранней стадии, проживающих в Новосибирской области (Россия). Доля мутации 5382insC составляет 87,5 % от всех патогенных мутаций в гене BRCA1, обнаруженных у пациенток.

Ключевые слова: ген BRCA1, мутация, рак молочной железы в молодом возрасте, наследственный рак, секвенирование следующего поколения, таргетное секвенирование.

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THE SPECTRUM OF BRCA1 GENE MUTATIONS IN EARLY ONSET BREAST CANCER PATIENTS FROM RUSSIA

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Abstract

Aim of the study. Aim of the study was to estimate the occurrence of pathogenic mutations in the *BRCA1* gene in Russian breast cancer patients. **Material and methods.** Complete coding sequence of the *BRCA1* gene of 445 early onset breast cancer patients (under 40 years) from Novosibirsk region (Russia) were analyzed by targeted Next Generation Sequencing (NGS) using Ion Torrent platform. **Results.** Forty (9%) carriers of various pathogenic mutations were revealed. Thirty five (7,9%) patients carried 5382insC mutation, described earlier as a founder mutation for Slavic population. Five (1.1%) patients carried various pathogenic mutations, namely C61G, 462delCC, E143X, 4153delA, and IVS18+1G>T. Besides, 29 genetic variants with no clinical significance or with unknown clinical significance were detected in *BRCA1* gene among 445 early onset breast cancer patients. **Conclusions.** Data on the frequency of genetic variations in the *BRCA1* gene among early onset breast cancer patients in the Novosibirsk Region (Russia) were obtained. Proportion of the 5382insC mutation is 87.5% of all pathogenic mutations in the *BRCA1* gene found in patients.

Keywords: *BRCA1* gene, mutation, early onset breast cancer, hereditary cancer, NGS, targeted sequencing.

Introduction

Hereditary factors account for up to 10% of all breast cancer cases [1]. A significant part of the hereditary forms of breast cancer is caused by mutations in the *BRCA1* and BRCA2 genes. The likelihood of a malignancy during the lifespan for *BRCA1/2* mutation carriers is very high (up to 90%) [2].

Several studies demonstrated the prevalence of *BRCA1* 5382insC mutation among breast/ovarian cancer patients in Russia [3–5]. Frequency of 185delAG, C61G, and 4153delA mutations is significantly less compared to 5382insC, the frequency of other mutations in *BRCA1* gene remains unexplored [6, 7]. However, there are no exact figures regarding frequency of *BRCA1* mutation in Russian population and among cancer patients, these data can be useful for screening programs and/or for placing objectives on the mutation analysis guidelines. According to our previous results, population study can reveal only frequent mutations (as *BRCA1* 5382insC), only single cases of other frequent mutations can be found even in large (about 8000) population samples [8]. Early onset cancer patient's cohort contains increased proportion of hereditary cancer cases, so analysis of this group can provide information on the spectrum and frequencies of pathogenic mutations in population. This information can be of value for the guidelines for the analysis of mutations among Russian citizens. In this study, we analyzed a complete coding sequence of *BRCA1* gene of 445 early onset breast cancer patients from Novosibirsk region with the aim to estimate the occurrence of pathologic mutations in the *BRCA1* gene in Russia.

Material and Methods

Blood samples were collected from 445 breast cancer patients of the Novosibirsk Regional Oncology Clinic between April 2013 and June 2016. The age of the patients at the time of diagnosis was from 19 to 40 years.

DNA was isolated from the blood samples using RealBest extraction 100 Kit (Vector-Best, Russia).

Analysis of the complete coding sequence of the

Table 1

Genetic variants found in early onset breast cancer patients in the Novosibirsk region

Exon/ Intron	HGVS genomic (GRSh37 assembly)	Mutation type	Base change	Designation	HGVS cDNA	HGVS protein	dbSNP	Clinical importance (BIC)	Number of homozygous carriers	Homozygous carriers frequency	Number of heterozygous carriers	Heterozygous carriers frequency	Allele frequency	Allele frequency (ExAC)	Allele frequency (1000 genomes)
In 2	*41267810G>A*	*IVS*	*C>T*	*IVS2-14C>T*	*c.81-14C>T*	-	*rs80358006*	*Unknown*	*0*	*0*	*1*	*0.0022*	*0.0011*	*0.0005*	-
Ex 5	**41258504A>C**	**Missense**	**T>G**	**C61G**	**c.181T>G**	**p.Cys61Gly**	**rs28897672**	**Yes**	**0**	**0**	**1**	**0.0022**	**0.0011**	**0.00007**	**-**
In 5	41258417T>A	IVS	A>T	IVS5+56A>T	c.212+56A>T	-	-	-	0	0	1	0.0022	0.0011	-	-
Ex 7	**41256236_41256237delGG**	**Frameshift**	**delCC**	**462delCC**	**c.343_344delCC**	**p.Pro115Terfs**	**-**	**-**	**0**	**0**	**1**	**0.0022**	**0.0011**	**-**	**-**
Ex 7	**41256153C>A**	**Nonsense**	**G>T**	**E143X**	**c.427G>T**	**p.Glu143Ter**	**rs80356991**	**Yes**	**0**	**0**	**1**	**0.0022**	**0.0011**	**0.0001**	**-**
In 7	41251931G>A	IVS	C>T	IVS7-34C>T	c.442-34C>T	-	rs799923	No	11	0.0247	139	0.3124	0.1809	0.1738	0.0986
Ex 8	*41251803T>C*	*Missense*	*A>G*	*Y179C*	*c.536A>G*	*p.Tyr179Cys*	*rs56187033*	*Unknown*	0	0	3	0.0067	0.0034	0.0003	0.0002
In 8	41251778delC	IVS	delG	IVS8+14delG	c.547+14_547+14delG	-	rs273902771	Unknown	0	0	1	0.0022	*0.0011*	*0.0001*	-
Ex 9	41249263G>A	Synonymous	C>T	710C>T	c.591C>T	p.Cys197=	rs1799965	No	0	0	1	0.0022	0.0011	0.0015	0.0004
Ex 11	41246481T>C	Missense	A>G	Q356R	c.1067A>G	p.Gln356Arg	rs1799950	Unknown	2	0.0045	37	0.0831	0.0461	0.0441	0.0218
Ex 11	41246298T>C	Missense	A>G	N417S	c.1250A>G	p.Asn417Ser	rs80357113	Unknown	0	0	1	0.0022	0.0011	-	-
Ex 11	*41246092A>G*	*Missense*	*T>C*	*F486L*	*c.1456T>C*	*p.Phe486Leu*	*rs55906931*	*Unknown*	0	0	1	0.0022	0.0011	0.0003	0.0002
Ex 11	41245900T>G	Missense	A>C	N550H	c.1648A>C	p.Asn550His	rs56012641	Unknown	0	0	3	0.0067	*0.0034*	*0.0003*	*0.0002*
Ex 11	41245471C>T	Missense	G>A	D693N	c.2077G>A	p.Asp693Asn	rs4986850	No	0	0	32	0.0719	0.0360	0.0568	0.0335
Ex 11	41245466G>A	Synonymous	C>T	2201C>T	c.2082C>T	p.Ser694=	rs1799949	No	31	0.0697	193	0.4337	0.2865	0.3483	0.3365
Ex 11	41245237A>G	Synonymous	T>C	2430T>C	c.2311T>C	p.Leu771=	rs16940	No	27	0.0607	221	0.4966	0.3090	0.3420	0.3353
Ex 11	*41244952G>A*	*Missense*	*C>T*	*R866C*	*c.2596C>T*	*p.Arg866Cys*	*rs41286300*	*No*	*0*	*0*	*1*	*0.0022*	*0.0011*	*0.0001*	-
Ex 11	41244936G>A	Missense	C>T	P871L	c.2612C>T	p.Pro871Leu	rs799917	No	26	0.0584	223	0.5011	0.3090	0.4100	0.4561
Ex 11	41244435T>C	Missense	A>G	E1038G	c.3113A>G	p.Glu1038Gly	rs16941	No	23	0.0517	152	0.3416	0.2225	0.3429	0.3357
Ex 11	41244429C>T	Missense	G>A	S1040N	c.3119G>A	p.Ser1040Asn	rs4986852	Unknown	0	0	4	0.0090	0.0045	0.0132	0.0098
Ex 11	41244029A>C	Missense	T>G	S1173R	c.3519T>G	p.Ser1173Arg	-	-	0	0	1	0.0022	0.0011	-	-
Ex 11	41244000T>C	Missense	A>G	K1183R	c.3548A>G	p.Lys1183Arg	rs16942	No	30	0.0674	174	0.3910	0.2629	0.3490	0.3526
Ex 11	**41243513delT**	**Frameshift**	**delA**	**4153delA**	**c.4035_4035delA**	**p.Glu1345=fs**	**rs80357711**	**Yes**	**0**	**0**	**1**	**0.0022**	**0.0011**	**0.00004**	**-**
Ex 11	41243509T>C	Missense	A>G	R1347G	c.4039A>G	p.Arg1347Gly	rs28897689	Unknown	0	0	4	0.0090	0.0045	0.0040	0.0006
Ex 13	41234470A>G	Synonymous	T>C	4427T>C	c.4308T>C	p.Ser1436=	rs1060915	No	25	0.0562	214	0.4809	0.2966	0.3431	0.3363
Ex 15	41226488C>A	Missense	G>T	S1512I	c.4535G>T	p.Ser1512Ile	rs1800744	No	0	0	1	0.0022	0.0011	0.0022	0.0006
Ex 16	41223094T>C	Missense	A>G	S1613G	c.4837A>G	p.Ser1613Gly	rs1799966	No	25	0.0562	226	0.5079	0.3101	0.3496	0.3558
Ex 16	*41223048A>G*	*Missense*	*T>C*	*M1628T*	*c.4883T>C*	*p.Met1628Thr*	*rs4986854*	*Unknown*	*0*	*0*	*5*	*0.0112*	*0.0056*	*0.0015*	*0.0026*
Ex 16	41222975C>T	Missense	G>A	M1652I	c.4956G>A	p.Met1652Ile	rs1799967	Unknown	0	0	22	0.0494	0.0247	0.0176	0.0112
Ex 17	*41219694C>A*	*Missense*	*G>T*	*A1669S*	*c.5005G>T*	*p.Ala1669Ser*	*rs80357087*	*Unknown*	*0*	*0*	*2*	*0.0045*	*0.0022*	*0.00005*	-
In 17	41216021G>A	IVS	C>T	IVS17-53C>T	c.5075-53C>T	-	rs8176258	No	0	0	9	0.0202	0.0101	-	0.0110
In 18	**41215890C>A**	**IVS**	**G>T**	**IVS18+1G>T**	**c.5152+1G>T**	**-**	**rs80358094**	**Yes**	**0**	**0**	**1**	**0.0022**	**0.0011**	**-**	**-**
In 18	41215825C>T	IVS	G>A	IVS18+66G>A	c.5152+66G>A	-	rs3092994	No	31	0.0697	149	0.3348	0.2371	-	0.3425
Ex 20	**41209082_41209083insG**	**Frameshift**	**insC**	**5382insC**	**c.5263_5264insC**	**p.Gln1756Profs**	**rs80357906**	**Yes**	**0**	**0**	**35**	**0.0787**	**0.0393**	**-**	**-**
In 20	41208991C>T	IVS	G>A	IVS20+78G>A	c.5277+78G>A	-	rs80358107	Unknown	0	0	3	0.0067	0.0034	-	0.0002

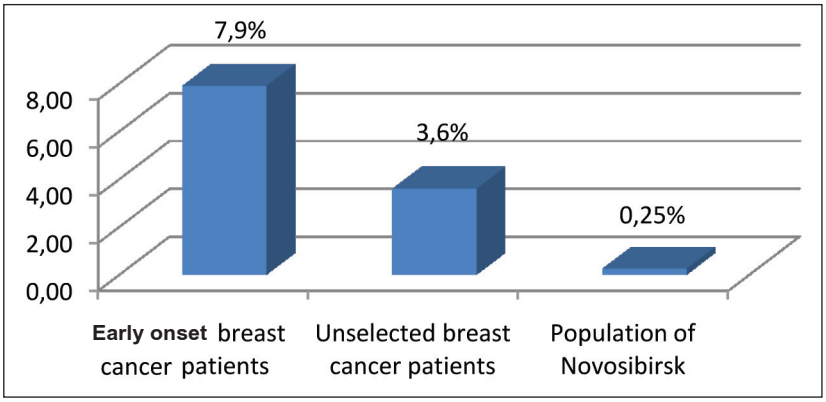

Figure 1. Frequency of the *BRCA1* 5382insC mutation in early onset breast cancer patients, unselected breast cancer patients, and in general population of Novosibirsk city

BRCA1 gene (22 exons, 5592 bp) was performed by targeted sequencing on IonTorrent platform. All exons with adjacent intron regions (20–80 bp) were completely covered with 68 amplicons (140–190 bp). Primers were designed by using Ion AmpliSeq Designer and Primer 3 software. The primer pairs were combined into 3 pools. Three multiplex PCR reactions were used to amplify the 68 selected fragments. Multiplex reaction products were combined and purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, USA). The amplicons were ligated with bar codes and A/P1 adapters. Then enrichment was carried out with the primer pair complementary to adapters A and P1. Concentration of the purified enriched amplicons was measured using Qubit dsDNA HS Assay kit on the Qubit 3.0 fluorimeter (Life Technologies, USA). The normalized DNA libraries were then amplified by emulsion PCR using Ion PGM Hi-Q OT2 kit and sequenced using Ion PGM instrument (Life Technologies, USA) according to the manufacturer's instructions.

Bioinformatic analysis of the raw data was based on PRINSEQ technique [9]. The nucleotide sequences obtained in the analysis were compared with the reference sequence of the human genome GRCh37/hg19 using the BWA-MEM software version 0.7.5 [10]. The search for genetic variants was carried out using the SAM tools software version 0.1.19 [11].

Results and discussion

Analysis of 445 early onset breast cancer patients revealed 35 genetic variants in *BRCA1* gene. Results of the study are presented in Table 1.

We detected 40 carriers (9%) of various pathogenic mutations, including 35 carriers (7.9%) of 5382insC mutation. *BRCA1* 5382 insC was described as founder mutation for Slavic population [5]. Five patients (1.1%) carried pathogenic mutations C61G, 462delCC, E143X, 4153delA, and IVS18+1G>T (each particular mutation was found in a single patient in a heterozygote state). All pathogenic mutations are depicted in bold in Table 1. According to our previous results, the frequency of the 5382insC mutation among residents of Novosibirsk city is 0.25% [8], the frequency of this mutation among unselected breast cancer patients in the Novosibirsk region is 3.6% [12]. Figure 1 shows the frequency of the 5382insC mutation among unselected breast cancer patients and in the cohort of early onset breast cancer patients in comparison with the frequency of this mutation in general population.

Remarkably, allele frequency of 9 genetic variants is at least twice higher than the frequency provided in the database for general population (ExAC or/and 1000 genomes), these variants are marked in italics in Table 1.

The frequency of three genetic variants has not assigned in dbSNP yet and are not specified in Table 1. Two of these variants were undisclosed for the first time. Ermolenko N.A. et al. found the 462delCC (p.Pro115Terfs) mutation in Russian breast cancer patients [13].

The obtained data on *BRCA1* mutation frequencies can be the basis for the guidelines for mutation analysis in various cohort of breast cancer patients (patients with family history, early onset breast cancer patients etc.). Indeed, our data indicate the absence of hot-spot mutation except *BRCA1* 5382insC, but a very strong prevalence of this mutation in early onset cancer patients (87.5 % of all found *BRCA1* mutations). A frequency of the *BRCA1* 5382insC mutation among non-selected breast cancer patients in Russia was reported in several studies [4, 12, 14]. Similar frequency of the *BRCA1* 5382insC mutation was reported for Ukranian breast cancer patients [15].

In spite of the high frequency of the *BRCA1* 5382insC mutation among unselected cancer patients in Poland [16] the mutation occurrence is more than two times less than in Russia (1.9 %) and just slightly higher than the frequency of the mutation C61G (1.2 %). A frequency of *BRCA1* 5382insC mutation in Germany among unselected breast cancer patients is even less (1%) [17].

Thus, the frequency of the *BRCA1* 5382C mutation among unselected breast cancer patients from Europe is maximal in Russia (3.6–4 %), intermediate in Poland

and Germany (1.9 % and 1.0 %, correspondingly) and quite rare in France [18] and in Spain [19].

So, this data leads to the logical considerations regarding the workflow of the *BRCA1* gene mutations analysis specifically for Russia. The frequencies of *BRCA1* gene mutations in Russia dictates the need to analyze *BRCA1* 5382insC mutation as the first step of analysis and, if not found, to analyze a complete coding region of *BRCA1* gene. This workflow is in contrast with the accepted idea to analyze 4–8 mutations which were ever found in cancer patients with family history. Moreover, due to the low cost and relative simplicity analysis of the *BRCA1* 5382insC mutation can be offered to all breast cancer patients with and without family history since a number of publications demonstrate a limited significance of family history in the present study.

Conclusion

As a result of the study, data on the frequency of genetic variations in the *BRCA1* gene among early onset breast cancer patients in the Novosibirsk Region were obtained. Important, that the proportion of the 5382insC mutation is 87.5 % of all pathogenic mutations in the *BRCA1* gene found in patients. Frequency of the 4153delA mutation, which was previously characterized as a founder mutation for Russian breast/ovarian cancer patients is not higher than frequency of other pathogenic mutations found for *BRCA1* mutations appearance [16].

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Conflict of interest

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ТРАНСКРИПТОМНЫЙ АНАЛИЗ КЛЕТОК МЕЛАНОМЫ, ПОЛУЧЕННЫХ ИЗ РАЗЛИЧНЫХ УЧАСТКОВ ПЕРВИЧНОЙ ОПУХОЛИ

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Аннотация

Введение. Внутриопухолевая гетерогенность представляет собой характерную черту большинства злокачественных новообразований, в том числе и меланомы кожи. Данное свойство является одним из препятствий для проведения эффективной таргетной терапии, поскольку у различных субклонов опухолевых клеток наблюдается вариабельная чувствительность к данным препаратам. С современных позиций терапия злокачественных новообразований требует персонифицированного подхода для каждого конкретного пациента. **Цель исследования** – оценка возможных различий между тканями меланомы, выделенными из различных участков первичной опухоли одного пациента на транскриптомном уровне. **Материал и методы.** В работе были использованы культуры клеток меланомы, полученные из центральной и периферической частей первичной опухоли двух пациентов. Исследование транскриптомов клеток проводили методом микрочипирования с последующим биоинформатическим анализом. **Результаты.** В клетках меланомы первого пациента, полученных из центрального и периферического участков одной опухоли, не было выявлено различий по транскриптомному профилю. У второго пациента имели место существенные различия (по 2953 транскриптам из 48226). В клетках, полученных из центрального участка опухоли, выявлено повышение мРНК генов, кодирующих белки, ассоциированные с иммунным ответом опухоли, транспортные белки ABC-семейства, сигнальные молекулы класса цитокинов. В культуре клеток, выделенной из периферического участка этой же опухоли, зарегистрировано увеличение уровня мРНК генов, кодирующих белки внеклеточного матрикса и воспалительного ответа. В целом различия между субклонами клеток второго пациента касались ряда сигнальных каскадов, играющих ведущую роль в онкогенезе (MAPK, PI3K-Akt-mTOR, VEGFA-VEGFR2 и др). **Заключение.** Проведенное исследование позволяет оценить возможные различия между клетками внутри опухоли на транскрипционном уровне с целью поиска новых подходов для персонифицированной терапии.

Ключевые слова: меланома, гетерогенность, транскриптом, микроокружение.

TRANSCRIPTOMIC ANALYSIS OF MELANOMA CELLS EXTRACTED FROM DIFFERENT SITES OF THE PRIMARY TUMOR

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Abstract

Introduction. Intratumor heterogeneity is a characteristic feature for most malignant tumors, including cutaneous melanoma. This property represents one of the main obstacles for effective targeted therapy, due to the different sensitivity to chemotherapeutic agents on various tumor cells subclones. Treatment of malignant tumors requires an individual approach to choose the most appropriate treatment regimen. **The purpose of the study** was to evaluate differences in melanoma tissue samples obtained from different parts of one patient's primary tumor at the transcriptomic level. **Material and Methods.** Melanoma cell cultures obtained from both central and peripheral parts of the primary tumor of two patients were used in the study. **Results.** Subclones from different parts of the first patient's tumor were similar, whereas the second patient demonstrated significant differences at the transcriptomic level (in 2953 transcripts out of 48226). In the cells of the central zone of the second patient's tumor, an increase in mRNA of the genes encoding proteins associated with tumor-specific immune response, as well as ABC-family transport proteins and cytokine signaling molecules, were noted. In the cells from the peripheral area of the same tumor, a more intensive transcription of genes encoding extracellular matrix and inflammatory response proteins was observed. Taken all round, the differences between the subclones of the second patient's cells were relevant to some signaling cascades playing a leading role in oncogenesis (MAPK, PI3K-Akt-mTOR, VEGFA-VEGFR2, etc.). **Conclusion**. The study allowed evaluation of differences between cancer cells within a tumor at the transcriptional level in order to search for further approaches to personalized melanoma therapy.

Keywords: melanoma, heterogeneity, transcriptome, microenvironment.

Introduction

Intratumor heterogeneity is a serious problem in terms of cancer treatment. With the development of a tumor cell genome, genetically heterogeneous subclones with different biological characteristics and variable sensitivity to chemotherapy appear in the primary tumor [1]. The development of malignant tumor and its growth is an active evolutionary process that results in a tumor consisting of cells with heterogeneous molecular characteristics [2]. This diversity entails the development of resistance to treatment in cancer patients [3].

The diverse molecular portrait of the cells that form one tumor can be a result of a set of changes at the genetic and epigenetic levels [4]. Among such changes, special attention should be given to differences in transcriptome profile of the cell. This is due to the fact that the transcription is a dynamic process that projects functional cell characteristics and predetermines different aspects of cells' biological activity, forming an individual molecular landscape of the development of cancer cells' subclones [5].

The aim of this research is a comparative analysis of transcriptomic profiles of melanoma cells derived from different parts of one patient's primary tumor.

Material and Methods

In the present study we used surgical specimens of two patients treated at the General Oncosurgery Department of A.I. Kryzhanovsky Krasnoyarsk Region Clinical Oncology Center, Krasnoyarsk, Russia.

The study was approved by the ethical Committee of Professor V.F. Voino-Yasenetsky Krasnoyarsk State Medical University (record No. 73/2016 dated 16.12.2016) and the ethical Committee of A.I. Kryzhanovsky Krasnoyarsk Region Clinical Oncology Center (record No. 8 dated 14.062.2017). Patients' clinical characteristics are shown in Table 1.

Immediately after surgical excision, a tumor fragment of at least 8 mm^3 and weighing at least 300 mg was immersed in a tube containing a culture medium RPMI-1640 (Gibco, Life Technologies, Paisley, UK) with the addition of 20% fetal bovine serum (Gibco, Life Technologies, Paisley, UK) and antibiotic-antimicotic complex: 100 U/ml penicillin G, 100 µg/ml streptomycin, 0.025 µg/ml amphotericin B (HyClone laboratories, USA) and was transported in ice. Two pieces were then aseptically separated from the tumor tissue: one from the central part of the tumor and one from the peripheral part of the tumor. If necessary, the obtained fragments were mechanically cleaned of necrotic tissue, washed in a sterile balanced Hanks salt solution (HBSS) (Gibco®, Life Technologies, Paisley, UK) and crushed by crossed scalpels.

The obtained tumor fragments were disaggregated by incubation in 0.25% trypsin solution with EDTA (Gibco, Life Technologies, Paisley, UK) in an amount of 1 ml of trypsin for every 100 mg of tissue at +37°C for 15-20 minutes. At the end of incubation, the suspension was centrifuged on a centrifuge CLMN-P10-01-Elecon ("Liston", Zhukov, Russia) at a speed

of 1000 rpm for 5 minutes. The obtained cellular precipitate was resuspended in 5 ml of RPMI-1640 nutrient medium with L-glutamine (Gibco®, Life Technologies, Paisley, UK), 10% fetal bovine serum (Gibco®, Life Technologies, Paisley, UK) and a mixture of antibiotic-antimicotic complex (HyClone laboratories, USA) and placed in a culture tube, in which further cultivation was carried out in CO_2-incubator (Sanyo, Osaka, Japan) with 5% of carbon dioxide, at +37°C. The first change of the nutrient medium was carried out 48 hours after the beginning of cultivation after the visual cells' adhesion to the adhesive surface of the tube. The first cell passage was carried out 4 days later and subsequent cell passages were carried out every 3 days of incubation.

To study the transcriptome of melanoma cells isolated from the central and peripheral parts of the tumor, the cells were transplanted into a 24-well plate at a concentration of 2×10^5 cells/ml. The culture medium was changed 24 hours after the cells' adhesion to the adhesive surface, with a subsequent culturing during 72 hours. At the end cells were removed using 0.25% trypsin with EDTA, washed in 0.01M phosphate-buffered saline and then the total RNA was isolated. RecoverAll™ Total Nucleic Acid Isolation kit (Ambion, Lithuania) was used for isolation according to the manufacturer's protocol. The concentration of purified total RNA was determined by Qubit® 2.0 fluorimeter (Singapore) using Qubit™ RNA HS Assay Kit (Ref. Q32852, Invitrogen™, Eugene, Oregon, USA).

The transcriptome of melanoma cells isolated from different parts of the tumor was studied using the GeneAtlas® system (Affymetrix, USA). For this purpose, 10 ng of purified total RNA were pretreated with the sample preparation kit titled GeneChip™ WT Pico Kit (Applied Biosystems™, Santa Clara, California, USA) in accordance with the manufacturer's protocol. Then, molecules were hybridized to GeneChip™ Human Gene 2.1 ST Array Strip microchips (Applied Biosystems™, Santa Clara, California, USA) with the reagents from GeneAtlas Hybridization, Wash, and Stain Kit for WT Array Strips (Applied Biosystems™, Santa Clara, California, USA). After 20 hours of hybridization microchips were washed and stained with solutions for washing and staining from the same set in the Fluidic station of the GeneAtlas® system. The detection of the microarray results was made in the Imaging station of this system.

Quality control (QC) of microarray was performed by the software system automatically due to the introduction of control RNA molecules into the study at the stages of sample preparation and hybridization. The fluorescence data analysis with its transformation into relative mRNA expression levels was carried out using the Expression Console ("Affymetrix", USA). Transcriptome Analysis Console 3.0 ("Affymetrix", USA) was used for statistical processing of microarray data. The non-parametric t-test ANOVA was used for data comparing. The statistical significance in transcriptomic profile differences was evaluated after an adjustment to the false discovery rate (FDR) criterion proposed by Benjamin, Hochberg for multiple comparisons [6]. The differences were statistically significant at $p_{FDR}<0.05$.

To assess the biological significance of individual genes, the GO (Gene ontology) project databases were used (https://www.ebi.ac.uk/QuickGO/, last access 06.11.2017), the analysis of genes' ontology of differentially expressed genes was carried out using Panther 13.1 database. Analysis of the clusters' biological role based on the microarray results was performed in the database DAVID 6.8 (https://david.ncifcrf.gov/home.jsp, last access 10.11.2017).

Results

The analysis of transcriptomic profiles of melanoma cells obtained from different parts of the first patient's tumor (Krsgmu-HS-Mel-CD-T-230317-Centr and Krsgmu-HS-Mel-CD-T-230317-K) revealed no differences between them. However, different results were shown in melanoma cells of the second patient – Krsgmu-HS-Mel-RI-T-040417-Centr and Krsgmu-HS-Mel-RI-T-040417-K. Profiles of these cultures

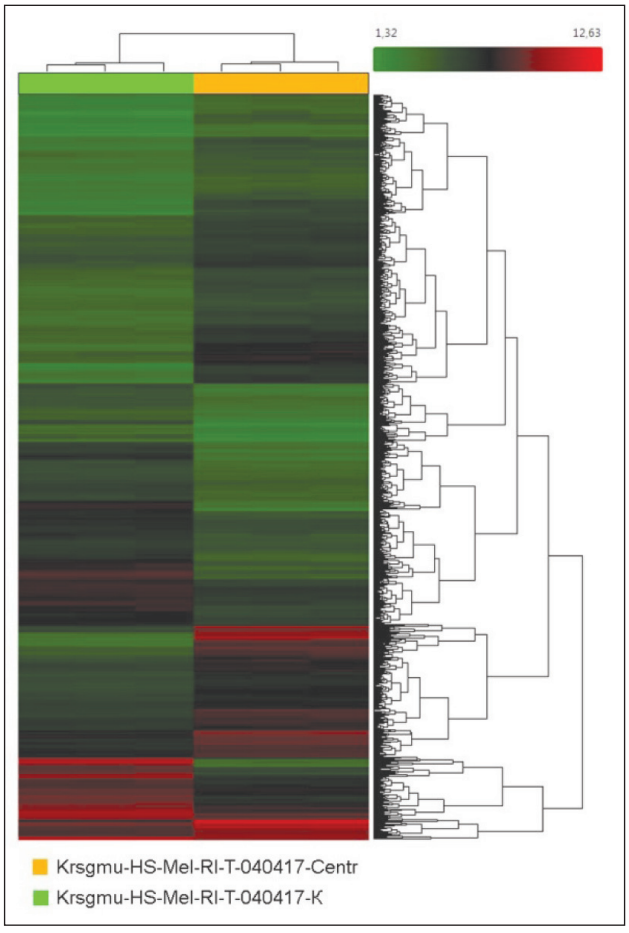

Figure 1. Hierarchical clustering of genes with different levels of transcription in melanoma cells isolated from the central part (Krsgmu-HS-Mel-RI-T-040417-Centr) and peripheral part (Krsgmu-HS-Mel-RI-T-040417-K) of the same tumor

differed in the level of 2953 transcripts. Out of them, the level of 1735 transcripts was two or more times higher, while the level of 1218 transcripts was two or more times lower in the cells of the central part of the tumor compared to the cells of the peripheral part of the tumor (Figure 1).

The transcript levels of genes encoding 42 proteins were more than 25 times higher, whereas transcripts of genes encoding 43 proteins were more than 25 times lower in cells from the central tumor part than in cells from the peripheral part. Proteins with increased mRNA levels in cells obtained from the central tumor parts belonged to the proteins responsible for various intracellular processes, including intercellular adhesion, transport of ions and metabolites. The most significant changes were detected in mRNA levels encoding proteins ABCC2, SNAP25, CCL2, OAS1, MAGEA3, MAGEB2, MAGEA6, DNER, TPTE, HLA-DRB5, DNER. Proteins, in which the mRNA levels were 25 times lower in cells from the central tumor part than in cells from the peripheral tumor part, included intercellular adhesion molecules, extracellular matrix proteins, growth factors, signal transduction proteins, apoptotic proteins (NEXN, PPP1R14A, POSTN, chipboard, B3GALT2, SEMA7A, FGF7, RIMS1, FBLN5, SCUBE3, PCDH18, COL1A2, TNFRSF10D, ADAMTS5, BGN).

Among the transcripts of genes that have a moderate increase in the level of cells obtained from the central part of the tumor compared with the periphery of the tumor (more than 5 times but less than 25 times), 423 transcripts were identified, with a 5-25 fold decrease in the level of 275 transcripts.

Proteins, which had increased mRNA levels in cells from the central part of the tumor compared to its periphery, were represented by the proteins of the Wnt-signaling cascade, proteins for intercellular contacts and adhesion, cadherins, regulators of the p53 signaling pathway, molecules of the EGF signaling pathways, and various cytokines. A similar trend was observed for the proteins with mRNA levels, which were lower in the culture of cells obtained from the central tumor sites compared to its periphery. The most common were the transcripts of genes of intercellular adhesion molecules, cadherin: CDH8, PCDHB3, PCDHB2, EN1, TGFBR1, ACTA2, TCF7L2, EDN1, PLCB4, SFRP4, and genes encoding proteins involved in angiogenesis: PRKD1, CRYAB, TCF7L2, PDGFRB, tissue factor F3.

Compared to the peripheral part of the tumor, genes' transcripts with a moderate decrease (a 5-25-fold) in mRNA levels in the central part represented the largest group since it contained 1249 altered transcripts of isolated genes, and 896 transcripts had a 2-5-fold decrease in mRNA levels. Among the altered gene transcripts, there were mRNAs of proteins involved in angiogenesis (the corresponding proteins are components of the integrin and endothelin signaling pathways.

There were identified 950 genes' transcripts with slightly elevated levels (more than 2 times, but less than 5 times) in cells obtained from the central tumor part compared to its periphery, and a 2-5-fold decrease was observed in the levels of 750 genes' transcripts. Proteins, the mRNA levels of which were elevated in the cells from the central parts of the tumor compared to its periphery, were represented by proteins-participants of the inflammatory response: PIK3CB, PLA2G4A, CXCL8, COL14A1, IL1B, NFATC2, PRKX, PLCG2, CCL5, RELB, RGS17, NFKBIA, ARRB1, GNA1, PDK1, as well as T-lymphocytes' activators: HLA-DQA1, MAP3K1, NFATC2, LCP2. Proteins of intercellular adhesion, cadherins and integrins dominated among proteins with a 2-5-fold decrease in the mRNA levels.

The results obtained showed that the altered genes' transcripts were the components of 642 signaling pathways. Among these signaling cascades, we identified signaling pathways, which included 10 or more proteins, the mRNA expression level of which was changed according to our findings. The transcriptomic profile revealed that mRNAs of genes with the increased transcription level in cells obtained from the central part of the tumor compared to cells obtained from the peripheral part of the tumor were involved into 40 signaling cascades**,** and mRNAs of genes with the decreased transcription level were involved only into 19 signaling pathways (Table 2).

Discussion

Transcriptomic profiling data analysis showed an increase in mRNAs of proteins, such as: melanoma-associated antigen-3 and melanoma-associated antigen B2. A 93.81-fold increase in the mRNA level of these proteins was observed in the center of the tumor. According to the literature review results, these genes are considered to be target genes recognized by cytotoxic T-cells under antigen-specific immunotherapy in skin melanoma patients [7].

Differences in the transcriptomic profile were revealed in ABC transporter proteins family. These proteins are considered to be associated with the formation of a multiple drug resistance. The ABCC2 protein mRNA level was 29.9 times higher in the center of the tumor. It is known that an increase in the expression activity of this protein on the tumor cells membrane can result in more intensive elimination of therapeutic agents from the cell, causing a decrease in the intracellular drug concentration, which is necessary for the efficient tumor growth inhibition [8]. This fact can explain why different tumor subclones yield a different therapeutic response to the anti-tumor agent.

A 98.14-fold increase in CCL2 chemokine mRNA expression in the center of the tumor indicates that there are different types of immunological reactions in central and peripheral parts of the tumor. This protein is known to be actively expressed by tumor-

Table 1

Characteristics of the studied samples

No	Patients' clinical data	Code number of the cell cultures, obtained from the tumor fragment	Tumor fragment localization
1	Patient K., a 30-year-old female, superficial spreading melanoma of the right lower leg	Krsgmu-HS-Mel-CD-T-230317-Centr	Center
		Krsgmu-HS-Mel-CD-T-230317-K	Peripheral part
2	Patient B., a 64-year-old male, superficial spreading melanoma of the interscapular area	Krsgmu-HS-Mel-RI-T-040417-Centr	Center
		Krsgmu-HS-Mel-RI-T-040417-K	Peripheral part

Table 2

Signaling pathways, involved in the regulation of differentially altered genes, according to the transcriptome analysis in the cell culture, obtained from different parts of the tumor

Signaling pathway *	Number of transcripts increased in the central tumor part compared to the peripheral one	Signaling pathway *	Number of transcripts decreased in the central tumor part compared to the peripheral one
Nuclear Receptors Meta-Pathway	33	miR-targeted genes in lymphocytes	37
miR-targeted genes in lymphocytes	27	miR-targeted genes in muscle cell	36
Retinoblastoma (RB) in Cancer	26	Focal Adhesion-PI3K-Akt-mTOR-signaling pathway	27
Cell Cycle	19	Focal Adhesion	21
Circadian rythm related genes	19	Mesodermal Commitment Pathway	20
PI3K-Akt Signaling Pathway	17	MAPK Signaling Pathway	19
MAPK Signaling Pathway	17	Wnt Signaling Pathway	14
Ectoderm Differentiation	17	ESC Pluripotency Pathways	14
Endoderm Differentiation	17	Vitamin D Receptor Pathway	13
Mesodermal Commitment Pathway	15	Regulation of Actin Cytoskeleton	13
VEGFA-VEGFR2 Signaling Pathway	15	miR-targeted genes in leukocytes	10
Chemokine Signaling Pathway	15	EGFR1 Signaling Pathway	10

* This table shows a total of 15 signaling pathways for both increased and decreased transcripts with the largest number of mRNAs involved into the signaling cascade.

associated macrophages, as well as by tumor cells themselves. In addition, the biological effect of this protein correlates with its concentration in the tumor tissue: at high concentrations of CCL2, a stimulation of classically activated M1 macrophages takes place, and a cytostatic effect in tumor cells occurs, whereas at low concentrations of CCL2, an accumulation of already alternatively activated M2 macrophages in the tissue takes place, which produces an inverse effect and induces an enhanced proliferation activity of the tumor cells [9].

The proteins whose mRNA levels were more than 25 times lower in the center than in the peripheral part of the tumor were mainly proteins-components of extracellular matrix. This fact can be due to a higher level of stromal components in the peripheral part of the tumor than in its central part. The change of the expression profile corresponds to data obtained from colorectal cancer research that reported the predominance of proteins forming components of extracellular matrix: collagens, tissue inhibitors of matrix metalloproteins (COL6A3, COL1A2, POSTN, TIMP2 and others) at the peripheral part of the tumor [10].

Among both increased and decreased gene transcripts in the central part of the tumor in comparison with its periphery, there were mRNAs, coding proteins that were components of the Wnt signaling pathway. The components of the Wnt signaling pathway are known to take part at all stages of skin melanoma development. For example, the transcription of a well-known c-myc oncogene whose enhanced expression is characteristic for various neoplasms, including melanoma of the skin, is induced by means of the β-catenin signaling cascade [11], the gene expression of negative regulators of a canonical Wnt signaling pathway being often suppressed. For example, the production of Dkk-1, 2 and 3 that inhibit the β-catenin signaling cascade by means of binding with co-receptor LRP5/6 is significantly reduced or absent in melanoma cells [12].

The transcriptome profile revealed that, mRNAs of genes with the elevated transcription level in cells obtained from the tumor center were involved in 40 signaling cascades, whereas mRNAs of genes with decreased transcription level were involved in only 19 signaling pathways. According to the bioinformatics analysis data, mRNA profile changes in melanoma

cells obtained from both the central and peripheral parts of the tumor affected genes-components of signaling pathways traditionally associated with cancerogenesis (MAPK, PI3K-Akt-mTOR, VEGFA-VEGFR2, Focal adhesion, Wnt signaling pathway). Proteins of the MAPK signaling cascade are one of the key targets in skin melanoma therapy although many patients suffering from metastatic melanoma showed acquired resistance to MAPK inhibitors.

One of the major mechanisms of MAPK-inhibitor resistance formation is the realization of the intratumor heterogeneity phenomenon. High plasticity of melanoma cells provokes both a clonal evolution of genetic resistance (for example due to a mutation in genes that code components of the MAPK or PI3K/AKT/PTEN signaling cascades) and an appearance of cel phenotypes that are functionally and metabolically resistant to various therapeutic agents [13]. Thus, melanoma cells, like 'a moving target', drift among different metabolic influences, cell cycles and states of cell differentiation, this projecting a high dynamical potential for adaptation to exogenous stress factors including medicaments.

Among the altered transcripts, there were mRNAs coding proteins responsible for the interaction between mRNAs and their genes-targets in different cells: lymphocytes, myocytes, and epithelial cells. This is consistent with the fact that mRNAs can act as paracrine and autocrine regulators of a biological behavior of the tumor micro-environment [14].

The changes in the mRNA profile in melanoma cells originated both from the center and the periphery of the tumor showed dysregulation of VEGFA-VEGFR2 signaling pathway, which took part in melanoma pathogenesis; the up-regulation of this signaling pathway proteins maintained a proliferation activity of melanoma cells [15].

The expression of mRNAs, coding THBS1 and SPARC proteins, was significantly lower in cells obtained from the center of the tumor than in cells obtained from the peripheral part of the tumor. These genes are components of autophagy signaling pathway, and according to previous reports, they can take part in a chemoresistance formation associated with autophagy [16].

At the same time, for the cells obtained from the tumor central part, some unique signaling pathways were determined (Cell Cycle, Circadian rhythm related genes) which were not determined for the cells obtained from the tumor peripheral part. The change of an expression profile of Circadian rhythm genes is known to be an early occurrence in case of skin melanoma and is connected with the involvement of tumor microenvironment into this process [17].

A special role is given to melatonin that prevents DNA potential damage and can provide an endogenous enzymatic system to protect from oxidative stress by means of growth factor regulators and through activating some antioxidant enzymes, such as superoxide dismutase and catalase. Besides, melatonin in pharmacological concentrations suppresses proliferation of the melanoma cells [18].

For the tumor periphery cells, there were determined signaling cascades that were not characteristic for the cells from central part of the tumor: miR-targeted genes in leukocytes, Vitamin D Receptor Pathway, Regulation of Actin Cytoskeleton. Apart from calcium metabolism regulation, vitamin D3 active forms are also known to have an anticarcinogenic effect, whereas alterations in vitamin D signaling, including both D3 activation and inactivation, as well as expression and activity of the corresponding receptors, influence the melanoma progression and the outcome of the disease [19].

Conclusion

Cells obtained from the central part of the tumor demonstrated the increased mRNA levels of genes indicating a high sensitivity of cells to immunotherapy; in particular, these cells were characterized by enhanced expression of CT-antigens, as well as MAGE-A3 and MAGE B2 genes. These genes were considered to be gene-targets detected by cytotoxic T-cells in antigen-specific immunotherapy of melanoma patients.

Melanoma cells derived from the peripheral part of the same tumor, had increased level of mRNA genes coding an extracellular matrix and inflammation-related proteins. According to bioinformatic analysis, the changes in the mRNA profile in the melanoma cells from the tumor periphery were observed in genes-components of the signaling pathways that took part in the antioxidant response. This fact suggested that the peripheral part of the tumor was more sensitive to antioxidant therapy.

Thus, the study results showed a difference between melanoma cells obtained from various parts of the primary tumor at the transcriptomic level. This fact should be taken into consideration when administering anticancer therapy.

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Conflict of interest

The authors declare that they have no conflict of interest.

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ВЛИЯНИЕ ТРАНСКРИПЦИОННЫХ ФАКТОРОВ, VEGF И ПРОТЕИНАЗ НА ПРОГРЕССИРОВАНИЕ РАКА ПОЧКИ

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Аннотация

Введение. Эффективность противоракового лечения зависит от биологических факторов опухоли. **Цель исследования** – определить активность протеасом и кальпаинов и выявить их связь с содержанием VEGF, HIF-1α и NF-κB в опухолевых, неизмененных и метастатических тканях карциномы почек (RCC). **Материал и методы.** В исследование были включены 93 пациента с почечно-клеточным раком. Содержание транскрипционных факторов и VEGF определяли методом ИФА. Количественный состав протеасом исследовали методом Вестерн-блоттинг. Активность протеасомы и кальпаина определяли с использованием специфического флюорогенного субстрата. **Результаты.** Выявлена инактивация протеолиза у пациентов с раком почки. Прогрессирование заболевания было связано со значительным снижением уровня клеточного протеолиза и ростом содержания транскрипционных и ростовых факторов в тканях первичной опухоли. Активация протеолиза была обнаружена в метастатических тканях. **Выводы.** В результате проведенного исследования показано, что факторы транскрипции NF-κB, HIF-1α, VEGF и внутриклеточные протеолитические системы участвуют в прогрессировании рака почки.

Ключевые слова: рак почки, метастазы, NF-κB, HIF-1α, VEGF.

IMPACT OF TRANSCRIPTION FACTORS, VEGF AND PROTEASES ON KIDNEY CANCER PROGRESSION

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Abstract

Introduction. The efficacy of anticancer treatment depends on biological factors of tumor. **The aim of the study** was to determine the activity of proteasomes and calpains and to reveal their association with VEGF, HIF-1α and NF-κB expressions in normal, primary and metastatic renal cell carcinoma (RCC) tissues. **Methods.** Ninety-three patients with renal cell carcinoma were included into the study. The expression levels of transcription factor and VEGF were measured using ELISA kits. The levels of proteasome subunits were measured by Western Blotting. Proteasome and calpain activities were determined using specific fluorogenic

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substrates. **Results.** We revealed inactivation of proteolysis in patients with kidney cancer. Disease advance was associated with a significant depression of cellular proteolysis and increase in transcription and growth factor levels in primary kidney cancer tissues. The proteolysis activation was found in metastatic tissues. **Conclusions.** Our results suggest that NF-κB, HIF-1α and VEGF transcription factors and intracellular proteolytic systems are involved in kidney cancer progression.

Keywords: renal cell carcinoma, metastasis, NF-κB, HIF-1α, VEGF.

Introduction

Kidney cancer is among the cancers that have the highest growth rate and it is known as the most deadly urinary tract caner [1]. In the past years, there has been an increased understanding of the tumor biology of renal cell carcinoma (RCC) [2–4]. Hypoxia-inducible factor (HIF-1) is a heterodimeric transcription factor consisting of an alpha and beta subunits. Hydroxylation of proline and asparagine residues in HIF-1α results in their binding to the von Hippel-Lindau protein (pVHL), and is followed by HIF-1α polyubiquitination and degradation in the proteasome [5, 6]. The HIV activation results in vascular endothelial growth factor (VEGF) production [3].

NF-κB is the key transcription factor that controls the transcription of 150 target genes [7]. The processing of p105 and p100 (NF-κB precursors) is mediated by the proteasome pathway [8]. The active form of the NF-κB transcription factor is p65/p50 heterodimers [9–12]. It is known that NF-κB can regulate the HIF-1α level [13].

Proteolysis is one of the mechanisms for regulation of NF-κB and HIF-1α transcription factors, and the proteasome and calpain systems are the most possible factors to be involved. Proteasome is the multicatalytic complex that consists of the catalytic core (20S) with one or two regulatory particles attached to it [14]. Both the 20S and 26S proteasomes may be divided into two large groups of immune and constitutive forms that consist of either constitutive (β1β2β3β4β5β6β7) or immune (LMP2, LMP7, MECL-1) subunits, respectively [15–19]. Calpains belong to the calcium-dependent proteases [20]. The calpain activity is implicated in several fundamental physiological processes, including cytoskeletal remodelling, cellular signalling, apoptosis, and cell survival [21–25].

A decrease in HIF-1α degradation in the presence of proteasome inhibitors or under hypoxia leads to a significant increase in both VEGF and its mRNA expression in tumor cells [26, 27]. Moreover, calpains were shown to be involved in HIF-1α destruction [5]. Activation of NF-κB is effected by proteasomes. The key moment of NF-κB activation belongs to breaking the ties between the transcription factor and repressor protein known as I-κB [8, 11]. Currently, even greater significance is attached to the investigation on the participation of calpains in I-κB destruction [28]. The additional mechanism in NF-κB regulation serves the NF-κB protein forming from precursors, which are mediated by proteasomes through the modification of p105 protein [29].

Our preliminary data showed the role of proteolytic regulation in kidney cancer progression [18]. The aim of our study was to determine the proteasome and calpain activities in normal, primary and metastatic human RCC tissues and to reveal their association with VEGF, HIF-1α and NF-κB expression levels.

Material and Methods

A total of 93 patients with RCC were treated at the Cancer Research Institute of Tomsk National Research Medical Center, Russian Academy of Sciences, Tomsk, Russian Federation (mean age 57.6 ± 2.2 years). Localized RCC (T1-3N0M0) was revealed in 50 patients. Forty-three patients had metastatic RCC (T2-4N0-1M1). All patients with localized RCC underwent surgery (partial nephrectomy or simple nephrectomy). Diagnosis verification and cancer stage estimation for patients with metastatic RCC included the biopsy analysis. The combined modality treatment included pre-operative pazopanib targeted therapy administered at a dose of 800 mg every day for 2 months. After completion of therapy, tumor response was evaluated according to the RECIST criteria, and radical nephrectomy was performed.

The study was approved by the Local Committee for Medical Ethics and all patients provided written informed consent. Specimens were reviewed by two pathologists separately. Normal, malignant and metastatic tissue samples taken at a distance of not less than 2 cm from the tumor border were used. The frozen samples were stored at -80°C.

Preparation of tissue homogenates. Tissue samples (100 mg) were homogenized and then resuspended in 300 μL of 50 mM Tris-HCl buffer (pH=7.5) containing 2 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, and 100 mM NaCl. The homogenate was centrifuged at 10000×g for 60 minutes at 4°C.

Proteasome fractiation. All procedures were carried out at 4°C. Proteins from tissue homogenates were fractionated with stepwise concentrations of ammonium sulfate [30]. The fractions were assayed for the proteasome activity.

Proteasome activity assay. Chymotrypsin-like activity of the total proteasome, 26S and 20S pools was measured in cancer, metastatic and non-transformed tissue homogenates, and in the proteasome fractions, using the fluorogenic substrate N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin (Suc-LLVY-AMC) in a Hitachi-850 (Japan) fluorimeter at an excitation wavelength of 380 nm and an emission of 440 nm [31]. The 20S proteasome activity solution contained 20 mM Tris-HCl (pH=7.5), 1 mM dithio-

threitol, and 30 μM Suc-LLVY-AMC. The 26S proteasome activity solution additionally contained 5 mM $MgCl_2$ and 1 mM ATP. The reaction was carried out for 20 minutes at 37°C and then was stopped by the addition of 1 % sodium dodecyl sulfate. We used the proteasome inhibitor MG-132 to estimate the influence of other proteases.

Calpains activity assay. The calpains activity was performed in tissue homogenates using the fluorogenic substrate N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin (Suc-LLVY-AMC) in a Hitachi-850 (Japan) fluorimeter at an excitation wavelength of 380 nm and an emission of 440 nm [32]. The calpains activity solution contained 100 мМ Tris-HCl (pH=7.3), 145 мМ NaCl and 30 μM Suc-LLVY-AMC. Incubations were performed at room temperature for 30 minutes in absence or presence of 10 mM $CaCl_2$ and N-Acetyl-L-leucyl-L-leucyl-L-norleucinal (calpain inhibitor I).

Electrophoresis. SDS-PAGE was used, according to the method of Laemmli [33]. The samples were incubated for 5 to 10 min in 62.5 mM Tris-HCl buffer (pH 6.8), containing 2.0 % (w/v) SDS, 5.0 % (v/v) mercaptoethanol, 10 % (v/v) glycerol, and 0.0012 % Bromophenol blue.

Western Blot Analysis. After SDS-PAGE, the gels were allowed to equilibrate for 10 min in 25 mM Tris and 192 mM glycine in 20 % (v/v) methanol. The protein was transferred to 0.2-/xm pore-sized PVDF membrane (GE Healthcare, UK), either at 150 mA or 100 V for 1 h by using a Bio-Rad Mini Trans-Blot electrophoresis cell according to the method described in the manual accompanying the unit. Before incubation with antibodies the PVDF membrane was incubated in 10 mM Tris-HC1 buffer (pH 7.5), containing 150 mM NaC1 and 0.1 % (v/v) tween-20 for 2 hours. Then it was incubated in a 1:2500 dilution of monoclonal mouse anti-human α1α2α3α5α6α7, LMP7(Santa Cruz, USA), Rpt6 (Enzo Life Science, USA) and of polyclonal rabbit anti-human LMP2, PA28β (Santa Cruz, USA) at 20 ºC for 1 h, followed by three consecutive washes in the 10 mM Tris-HC1 buffer (pH 7.5), containing 150 mM NaCI. PVDF samples were incubated in Amersham ECL western blotting detection analysis system according to the method described in the manual accompanying the unit and then were exposed to ECL-films (Amersham, USA). The results were standardized using the beta-actin expression in a sample and were expressed in percentages to the proteasome subunits content in non-transformed tissues. The expression of proteasome subunit in normal non-altered tissue was indicated as 100 %.

VEGF, HIF-1α and NF-κB (p65 and p50) determination. The pellets left after preparing tissue homogenates were resuspended in 50 μL of 50 mM Tris-HCl buffer (pH=7.5) containing 2 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, and 100 mM NaCl and then centrifugated at 14000×g for 10 minutes at 4 °C. HIF-1α and NF-κB (p50 and p65) expression were measured with Caymanchem ELISA kits (USA) in Anthos 2020 ELISA-microplate reader (Biochrom, UK). Nuclear extracts were prepared and purified according to manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using SPSS 19.0 software. Data were expressed as median (interquartile ranges). To evaluate the difference, either the Student t test or the Mann-Whitney test was applied. Correlation analysis on data was carried out with Spearman Rank Correlation test. Kruskal-Wallis ANOVA and median Test were used for comparing two or more independent groups.

Results

High expression levels of NF-κB, HIF-1α and VEGF were found in RCC tissues. The NF-κBp50 expression was 1.5 times higher in cancer tissues than in normal tissues (Table 1). Our results estimated the prevalence of active forms of NF-κB transcription factor in tumors. The NF-κBp65 and NF-κBp50 expression levels were 5.5 (2.8-8.7) and 6.3 (3.3-10.0) RLU per mg protein in a well, respectively. The_NF-κB p65 to NF-κBp50 ratio of less than 1.0 is known to be a sign of nonactive dimmers of NF-kB [12]. The NF-κB p65/p50 ratio of 1.1 in cancer tissues indicated NF-κB activation. In cancer tissues, the HIF-1α and VEGF expression levels were 4.2 and 12.45 times higher than those observed in non-transformed tissues (4.2 (2.2-7.9)) RLU per mg protein and 128.0 (81.8-168.2) pg /per mg protein, respectively). Thus, the high levels of transcription and growth factors were revealed in RCC tissues.

Proteasome and calpain activities were decreased in kidney cancer tissues. The total proteasome and 26S and 20S proteasome activities were found to be lower in RCC tissues than in non-transformed tissues. The proteasome activity is known to be associated with the content of proteasome subunits [16, 34]. The expression of the proteasome subunits α1α2α3α5α6α7 was higher in cancer tissues than in non-transformed tissues (agreed standard) ($p<0.05$). However, the expression levels of LMP2 immune proteasome subunit and PA28β proteasome activator subunit were decreased in cancer tissues. The difference in their levels between cancer tissues and normal surrounding tissues was 30% (Figure 1). The decreased 20S proteasome activity was likely dependent on a low level of PA28β subunit of the proteolytic complexes. A nonparametric correlation was revealed between the LMP2 level and proteasome activity ($r=0.26$; $p=0.04$). Proteasome activity depression was accompanied by a low calpain activity. The calpain activity was 1.4 times lower in RCC tissues than in normal tissues. It should be noted that proteasomes and calpains play a significant role in cancer development, tumor progression and metastatic spread due to their impact on transcription and growth factor levels. Thus, changes in the protease activity in kidney cancer tissues could be followed by the altered expression of transcription and growth factors.

Development of metastasis leads to changes in the protease activity and expression of transcription

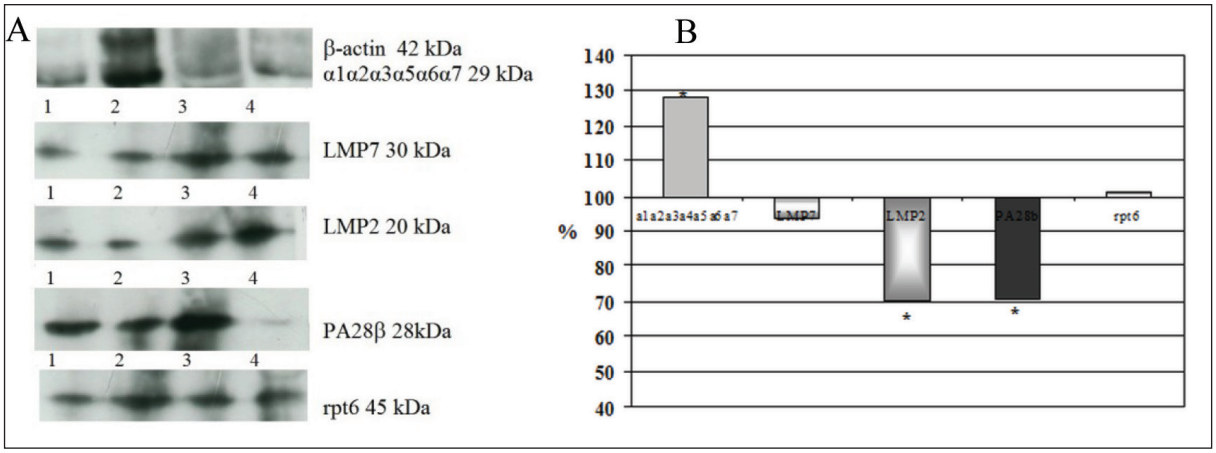

Figure 1. Proteasome subunit content in RCC.
A - Western Blotting analysis shows the expression of proteasome subunits in cancer and non-transformed tissues:
2, 3 – Western Blots of cancer tissues; 1, 4 - Western Blots of non-transformed tissues.
B – The expression of proteasome subunit in normal non-altered tissue is indicated as 100%.
* - in comparison with normal tissue, p<0.05; the results are standardized using the β-actin expression in a sample and are expressed in percentages to the proteasome subunits content in non-transformed tissues

and growth factors in primary cancer tissues. Kidney cancer progression and metastasis are followed by significant changes in proteolysis. Table 2 shows the activity of proteasomes and calpains in metastatic and non-metastatic tissues. Patients with T2-4N0-1M1 (metastatic cancer) had a 1.3-fold decrease in the total proteasome activity and a 1.3-fold increase in the calpain activity compared to T1-3N0-1M0 patients (non-metastatic tumors). Calpains are known to be responsible for limited protein proteolysis in cells. In the absence of proteasomes, they could damage and modify proteins and peptides [19]. I-κB, the endogenous inhibitor of NF-κB and HIF-1α transcription factors, is a substrate of proteases, and its incomplete proteolysis could lead to the accumulation of angiogenic factors in cancer cells and metastasis development [5, 19].

The development of metastatic spread was found to be accompanied by increased levels of transcription factors and VEGF expression (Table 3). The production of NF-κBp65 was 3.2 times higher in tumor tissues than in normal non-transformed tissues. So, this fact was accompanied by a 5.0-fold increase in the NF-κBp65 to NF-κBp50 ratio. The HIF-1α and VEGF expression levels were respectively 9- and 2.8 times higher in metastatic RCC tissues than in non-metastatic tissues.Thus, the progressive depression of protease activity in RCC tissues was associated with high expression levels of transcription factors and VEGF. Their accumulation in tumors led to cancer progression and metastasis.

Changes in NF-κB, HIF-1α and VEGF expression levels in primary cancer and metastatic tissues. NF-κBp50 expression in metastatic and primary RCC tissues was higher than in normal non-transformed tissues. The NF-κBp65 level was low both in metastatic and normal kidney parenchyma. And this molecular factor is reduced in 2.0 fold in comparison to RCC tissue. Interestingly, the HIF-1α content was 1.7 times higher in metastatic than in normal tissues. The VEGF expression was higher in metastatic and primary RCC tissues than in normal kidney tissues. We used the Kruskal Wallis (nonparametric ANOVA) Test and Median Test to analyze variances of multiple groups. We found significant differences in HIF-1 (p=0.0001; p=0.0001), NF-kBp50 (p=0.0002; p=0.0004), NF-kBp65 (p=0.02; p=0.01) and VEGF (p=0.0002; p=0.004) expression levels between normal, primary and metastatic RCC tissues.

Proteasome and calpain activities in primary and metastatic RCC tissues. Figure 2 shows the protease activity in distant metastatic tissues. Proteasome activity was 2.2 times lower in metastatic RCC tissues than in normal kidney tissues and was 1.5 times lower in metastatic than in primary RCC tissues. No difference in calpain activity was found between metastatic tis-

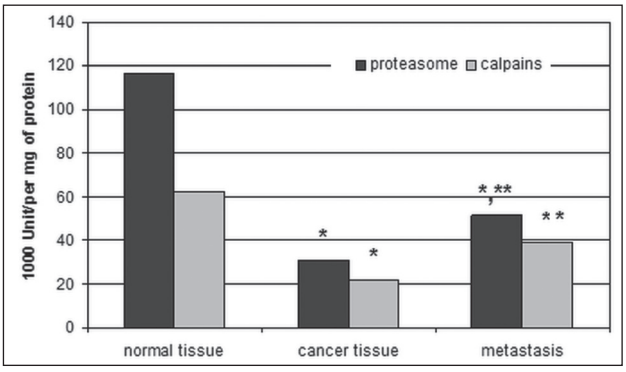

Figure 2. Total proteasome activity, 26S and 20S proteasome activity and calpain activity in kidney cancers. The results represent the Me (Q1-Q3); * in comparison with normal tissue, p<0.05. ** in comparison with cancer tissues. Proteasome depression is accompanied with fall of calpains activity. It is detected the growth in total proteasome and calpain activity in metastasis tissues compared to primary cancer tissues

Table 1

NF-κBp65, NF-κBp50, HIF-1α and VEGF expressions in normal and kidney cancer tissues

Samples	NF-κBp65 expression, RLU /per mg of protein in well	NF-κBp50 expression, RLU /per mg of protein in well	HIF-1α expression, RLU /per mg of protein in well	VEGF expression, pg/mg of protein
Non-transformed normal tissue, n=16	4,88 (4,66–5,21)	4,16 (3,76–4,66)	0,98 (0,62–1,18)	10,28 (8,46–14,3)
Kidney cancer tissue, n=69	7,0 (4,7–15,4)*	7,0 (4,7–14,7)*	4,88 (2,56–8,6)*	69,3 (27,5–139,75)*

Note: the results represent the Me (Q1-Q3); * in comparison with normal tissue, p<0,05.

Table 2

Total proteasome activity, 26S and 20S proteasome activities and calpain activity in metastatic and non-metastatic RCC

Samples	Proteasome activity, ·103 Unit/per mg of protein			Calpains activity, ·103 Unit/per mg of protein
	Total proteasome activity	26S proteasome activity	20S proteasome activity	
Non-metastatic RCC tissue, n=63	36.1 (20.0–102.0)	12.5 (7.3–21.3)	28.0 (16.7–65.7)	33.5 (16.8–33.5)
Metastatic RCC tissue, n=30	27.0 (5.5–43.1)*	10.2 (3.3–23.3)	30.1 (20.0–49.4)	44.7 (18.6–54.3)*

Note: the results represent Me (Q1-Q3); * – in comparison to non-metastatic RCC, p<0,05.

Table 3

NF-κBp65, NF-κBp50, HIF-1α and VEGF expressions in metastatic and non-metastatic RCC

Samples	NF-κBp65 expression, RLU / per mg of protein in well	NF-κBp50 expression, RLU / per mg of protein in well	Coefficient NF-κBp65/ p50	HIF-1α expression, RLU /per mg of protein in well	VEGF expression, pg/mg of protein
Non-metastatic RCC, n=45	5.0 (2.0–7.9)	6.4 (3.3–10.0)	0.6 (0.3–2.0)	4.2 (2.2–7.8)	71.5 (38.3–139.7)
Metastatic RCC, n=30	11.3 (6.8–15.7)*	6.2 (4.4–9.7)	3.0 (1.6–3.0)*	7.9 (6.2–8.5)*	205.4 (131.7–261.5)*

Note: the results represent the Me (Q1–Q3); * in comparison with non-metastatic RCC tissue, p<0,05.

sues and normal kidney tissues. However, the calpain activity was 1.75 times higher in metastatic RCC tissues than in primary kidney cancer tissues. The results of non-parametric ANOVA analysis (Kruskal-Wallis test and Median test) showed significant changes in the total proteasome (p=0.0001; p=0.0001) and calpain (p=0.03; p=0.06) activities in primary non-metastatic and metastatic tissues. Despite the fact that the contribution of the cellular protease system to tumorigenesis remains unclear, we decided to evaluate the NF-κB, HIF-1α and VEGF expression levels in primary and metastatic kidney cancer tissues. The recent study have documented that the increase of proteasome and calpains activity is required for sufficient carcinogenesis [20]. Thus, the protease activation in metastatic tissues is accompanied with increased NF-κBp50, HIF-1α and VEGF expression levels. Metastatic cancer cells are known to have the altered biological features. In our study we found the correlation between the aggressive behavior of cancer cells and proteolysis activity.

Correlations between the proteasome activity, transcription factors and VEGF expression were found in RCC tissues. To further examine the potential importance of proteases in RCC metastasis development, we analyzed the correlations between them and transcription and growth factor levels. Positive relationships between the activities of all proteasome pools in tumors were found. We observed statistically significant correlations between the 20S and 26S proteasome activities (r=0.60; p<0.05), total proteasome and 20S activities (r=0.80; p<0.05), and between the total and 26S proteasome activities (r=0.78; p<0.05). A positive correlation was found between the total proteasome activity, 26S proteasome activity and calpain activity (r1=0.56, p=0.00001; r2=0.57, p=0.00002; r3=0.63, p=0.00001, respectively). Goldberg A.L. [8] reported that the HIF-1α expression level was influenced by NF-kB transcription factors. The analysis of our data showed the relationships between NF-κB p65 and HIF-1α expression levels (r=0.9; p=0.0001) and between VEGF and HIF-1α expression levels (r=0.8; p=0.001). The effect of NF-κB on angiogenic growth factor was likely to be mediated through HIF-1 expression changes. The association between HIF-1α and VEGF expression was also detected in cancer tissues (r=0.97; p=0.0001).

Discussion

The proteolytic regulation of transcription and growth factors is of great importance in kidney cancer progression. In RCC, HIF-1α level was associated with low 26S proteasome activity (r= -0.36; p=0.04).

The loss of von Hippel-Lindau gene was followed by HIF-1α overexpression through blockage of its proteasome degradation [26, 27]. The intensive angiogenesis is the result of this process. The NF-κBp50 level was correlated with 20S proteasome activity. The post-translational modification of p105 was performed by proteasome [28]. The nonparametric nonlinear regression analysis was carried out to study the impact of proteases on transcription factor expression. It was found that the induced 20S proteasome activity (Beta=0.93; $p<0.05$) and depressed 26S proteasome activity (Beta=–0.58; $p<0.05$) led to the increased NF-κBp50 level in cancer cells.

There are miltiple crosstalks between the expression of transcription and growth factors in RCC. Proteolysis is considered the most significant. The 26S proteasome plays the main role in HIF-1α degradation, and 20S proteasome participates in NF-κBp50 production.

Low proteasome and calpain activities are specific to RCC. The increase in the protease activity is followed by RCC metastasis development. The blockage of HIF-1α degradation and the increased level of VEGF result in non-effective proteolysis. In RCC culture, P. van Uden [13] revealed the influence of NF-κB on HIF-1α transcription. Our findings highlight the potential contribution of intracellular proteases to kidney cancer metastasis. The increased NF-κBp65 expression is likely can influence on HIF-1 production in metastatic RCC and can be the main factor of cancer progression. There are reports on the association between the NF-κB activity and VEGF m RNA expression in breast cancer cells [35]. Our results are consistent with other recent studies, which indicate that the VEGF level is positively correlated with NF-κB p65 expression in cancer tissues.

Conclusion

Our results showed the relationship between the proteasome/calpain activity and transcription factor expression in primary and metastatic RCC. The hypothetic schema of proteolytic regulation of transcription factors and VEGF expression in RCC was obtained. The depression of cellular proteolytic systems and increase in the levels of transcription and growth factors were found in primary cancer tissues, resulting in metastasis development. High levels of NF-κB, HIF-1α and VEGF were found in primary metastatic tumors. A significant difference in cancer cells was found between primary and metastatic tissues. In distant metastatic tissues, proteolysis activation was accompanied by high levels of transcription and growth factors. The altered biological features were shown in metastatic tissues. The increased protease activity in metastatic tissues was followed by high NF-κBp50, HIF-1α and VEGF levels. Thus, proteasomes and calpains play a significant role in metastasis development. A decreased protease activity is necessary for the aggressive behavior of cancer cells and kidney cancer dissemination. Induced proteolysis in metastatic tissues is required for RCC progression.

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ЭКСПРЕССИЯ CXCR4 В РАЗЛИЧНЫХ ПОПУЛЯЦИЯХ ЦИРКУЛИРУЮЩИХ И ОДИНОЧНЫХ ОПУХОЛЕВЫХ КЛЕТОК РАКА МОЛОЧНОЙ ЖЕЛЕЗЫ

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Аннотация

Целью исследования явилось определение экспрессии CXCR4 в различных популяциях циркулирующих (ЦОК) и одиночных (дискретных) опухолевых клеток рака молочной железы. **Материал и методы.** В исследование были включены 35 пациенток с инвазивной карциномой неспецифического типа молочной железы (T1–4N0–2M0) в возрасте от 29 до 69 лет. Экспрессию CXCR4 в различных популяциях ЦОК оценивали методом проточной цитометрии. Для оценки экспрессии CXCR4 в аналогичных популяциях одиночных (дискретных) опухолевых клеток в первичной опухоли использовали метод конфокальной микроскопии. **Результаты.** Нами было установлено, что CXCR4 экспрессировался ЦОК без признаков стволовости и эпителиально-мезенхимального перехода (ЭМП), ЦОК с признаками ЭМП, но без маркеров стволовости, а также ЦОК с признаками стволовости, но без признаков ЭМП. У всех пациенток в крови ЦОК с признаками стволовости и ЭМП не экспрессировался CXCR4. В первичной опухоли молочной железы CXCR4 обнаруживался как на одиночных (дискретных) опухолевых клетках без признаков стволовости с признаками ЭМП, так и на клетках с маркерами стволовости и ЭМП. У всех пациенток в образцах первичной опухоли отсутствовали стволовые и нестволовые клетки без признаков ЭМП. **Заключение.** Таким образом, CXCR4 экспрессируются на различных популяциях ЦОК. Экспрессия CXCR4 не зависит от наличия или отсутствия признаков стволовости и/или ЭМП в опухолевых клетках. Также мы показали, что некоторые популяции одиночных (дискретных) опухолевых клеток в первичной опухоли характеризуются способностью презентировать на своей мембране CXCR4 и могут являться источником соответствующих популяций ЦОК.

Ключевые слова: CXCR4, циркулирующие опухолевые клетки, стволовые опухолевые клетки, одиночные (дискретные) опухолевые клетки, эпителиально-мезенхимальный переход, рак молочной железы.

CXCR4 EXPRESSION IN DIFFERENT SUBSETS OF CTCs AND SINGLE (DETACHED) BREAST CANCER CELLS

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Abstract

The aim of this study was to assess CXCR4 expression in different subsets of CTCs and single (detached) breast cancer cells. **Materials and methods**. Thirty five patients with invasive breast carcinoma of no special type (IC NST) (T1-4N0-2M0), between 29 and 69 years of age were included in this study. Different subsets of CTCs with CXCR4 expression were evaluated by flow cytometry. A confocal microscopy was used to assess CXCR4 expression in different subsets of single (detached) cancer cells in breast tissue. **Results.** The CXCR4 was expressed in CTCs without stem-like and EMT phenotype, in CTCs with EMT but not stem markers and in stem-like CTCs without EMT features. In all blood samples, the CXCR4 expression in CTCs with stem-like and EMT phenotype was absent. In breast tumor the CXCR4 was expressed in the non stem-like single (detached) breast cancer cells with EMT features, in the single (detached) breast cancer cells with stem and EMT features. In all tumor samples the stem-like or non stem-like single (detached) breast cancer cells without EMT features were absent. **Conclusions.** Different subsets of the CTCs exhibited CXCR4. The CXCR4 expression did not depend on the presence or absence of stem or/and EMT features in tumor cells. We showed that some subsets of single (detached) breast cancer cells in the primary tumor were characterized by the ability to express CXCR4 and may be a source of the respective CTC subsets.

Keywords: CXCR4, circulating tumor cells, cancer stem-like cells, single (detached) cancer cells, epithelial-mesenchymal transition, breast cancer.

Introduction

Circulating tumor cells (CTCs) mediate tumor dissemination and play the key role in the metastatic cascade [1, 2]. The prognostic value of CTCs in breast cancer has been previously shown. Thus, presence of CTCs in the peripheral blood is associated with the poor prognosis in breast cancer [3, 4]. However, little is known about exact mechanism of migration and trafficking of CTCs in the peripheral blood circulation [2]. Metastasis of cancer cells to distant locations needs intravasation from the primary tumor sites into blood vessels, survival in the circulation, migration to secondary organs, adhesion, and proliferation of cancer cells in targeting organs and tissues [5]. Chemokines might affect the selection of target organs for metastases formation. For example, the CXCR4/CXCL12 axis makes breast cancer cells move out of the circulation and traffic into organs with high amounts of chemokines, and thus forming metastases [6, 7]. In our previous study, we showed that CTCs were represented by heterogeneous population. Some cancer cells had stem-like or/and EMT (epithelialmesenchymal transition) phenotype, other cancer cells did not have EMT and stemness features [8]. We suggested that CTCs did not always precede metastasis, because they could not have the necessary features, including their CXCR4 expression. So, in present study we investigated the CXCR4 expression in different subsets of CTCs and single (detached) breast cancer cells in primary tumor.

Material and Methods

Patients (n=35) with invasive breast carcinoma of no special type (IC NST) (T1-4N0-2M0) within the range of 29 - 69 years of age (mean age: 49.06 ± 9.78) were treated at the Cancer Research Institute, Tomsk NRMC (Tomsk, Russia) between 2011 and 2017 included (Table 1). IC NST was defined according to the World Health Organization's recommendations [9]. The venous blood samples were collected in EDTA-treated tubes before surgical intervention and were used for flow cytometry. The formalin-fixed, paraffin-embedded (FFPE) primary tumor samples were used for immunofluorescence analysis. This study was approved by the institutional review board, all patients signed an informed consent for voluntary participation.

Flow Cytometry

Different subsets of CTCs were evaluated by flow cytometry (BD FACSCanto II (BD, USA)) using anti-CD45 (clone HI30, PE/Cy7) (BD Pharmingen, USA), anti-CD44 (clone G44-26, APC-H7) (BD Pharmingen, USA), anti-CD24 (clone ML5, PerCP-Cy5.5) (BD Pharmingen, USA), anti-CK7 (clone CAM5.2, AF647) (BD Pharmingen, USA), anti-N-cadherin (CD325) (clone 8C11, PE) (BD Pharmingen, USA) and anti-CXCR4 (clone 44717, AF488) (R&D Systems, USA) antibodies. Then, erythrocytes were lysed in BD FACS lysing solution.

Confocal microscopy

The FFPE tumor sections were incubated with the primary antibody cocktail against CK7 (polyclonal, 1:50, guinea pig, Acris Antibodies, Germany), CD133 (clone 3F10, 1:800, mouse, MyBioSource, USA), N-cadherin (CD325) (polyclonal, 1:400, rabbit, Abcam, UK), CXCR4 (polyclonal, 1:50, goat, Thermo, USA) in 1% BSA. As secondary antibodies were used anti-guinea pig IgG H&L (CF405) (Biotium, USA),

Table 1

The clinicopathological parameters of the patients with breast cancer

Clinicopathological parameters	N (%)
Age (year) (Me (Q1–Q3))	
49 (29–69)	35 (100%)
Molecular type of breast cancer	
Luminal A	12/35 (34%)
Luminal B	13/35 (37%)
HER2-positive	3/35 (9%)
Triple-negative	7/35 (20%)
Tumor size	
T1	8/35 (22.8%)
T2	25/35 (71.4%)
T3	1/35 (2.9%)
T4	1/35 (2.9%)
Lymph node status	
N0	21/35 (60.0%)
N1	9/35 (25.7%)
N2	1/35 (2.9%)
N3	1/35 (2.9%)
Neoadjuvant chemotherapy (NACT)	
NO	24/35 (69%)
YES	11/35 (31%)

Table 2

The CXCR4 expression in different subsets of circulating or single (detached) cancer cells

The breast cancer cell subsets	Frequency of cell detection	Cell count
The circulating tumor cells in patient's blood		
CK7+CD45-CD44-CD24+/-N-cadherin-CXCR4+	31.8 % (7/22)	0.00 (0.00–0.95) cell/ml
CK7+CD45-CD44-CD24+/-N-cadherin+CXCR4+	68.2 % (15/22)	1.96 (0.13–3.59) cell/ml
CK7+CD45-CD44+CD24-N-cadherin-CXCR4+	27.3 % (6/22)	0.00 (0.00–0.13) cell/ml
CK7+CD45-CD44+CD24-N-cadherin+CXCR4+	0.0 %(0/22)	–
The single (detached) cells in tumor		
CK7+N-cadherin-CD133-CXCR4+	0.0 % (0/25)	–
CK7+N-cadherin+CD133-CXCR4+	44.0 % (11/25)	0.00 (0.00–8.00)%
CK7+N-cadherin-CD133+CXCR4+	0.0 % (0/25)	–
CK7+N-cadherin+CD133+CXCR4+	28.0 % (7/25)	0.00 (0.00–1.05)%

anti-mouse IgG H&L (AF488) (Abcam, UK), anti-rabbit IgG H&L (Cy3) (Abcam, UK), anti-goat IgG H&L (AF647) (Abcam, UK). Nuclei were stained with DRAQ5. The single (detached) breast cancer cells were visualized using the LSM 780 NLO confocal microscope (Carl Zeiss, Germany) with a 63 plan apochromat objective, numerical aperture (z1), and immersion oil.

Results

As CXCR4 promotes breast cancer metastasis to distant organs where its ligand, SDF-1, is generated in large quantity [10, 11], we examined the expression of these molecules in different subsets of CTCs in the blood of breast cancer patients (Figure 1). The CXCR4 expression in CTCs without stem-like and EMT phenotype (CK7+CD45-CD44-CD24+/-N-cadherin-CXCR4+) was present in 31.8% (7/22) patients (0.00(0.00-0.95) cell/ml). In 68.2% (15/22) patients, CTCs with CXCR4 exhibited EMT but not stem markers (CK7+CD45-CD44-CD24+/-N-cadherin+CXCR4+) (1.96(0.13-3.59) cell/ml). The stem-like CTCs expressed CXCR4 in 27.3% (6/22) patients. All this cells (0.00(0.00-0.13) cell/ml) didn't have EMT features (CK7+CD45-CD44+CD24-N-cadherin-CXCR4+). In all patient blood samples (22/22) the CXCR4 expression in CTCs with stem-like and EMT phenotype (CK7+CD45-CD44+CD24-N-cadherin+CXCR4+) was absent (Table 2).

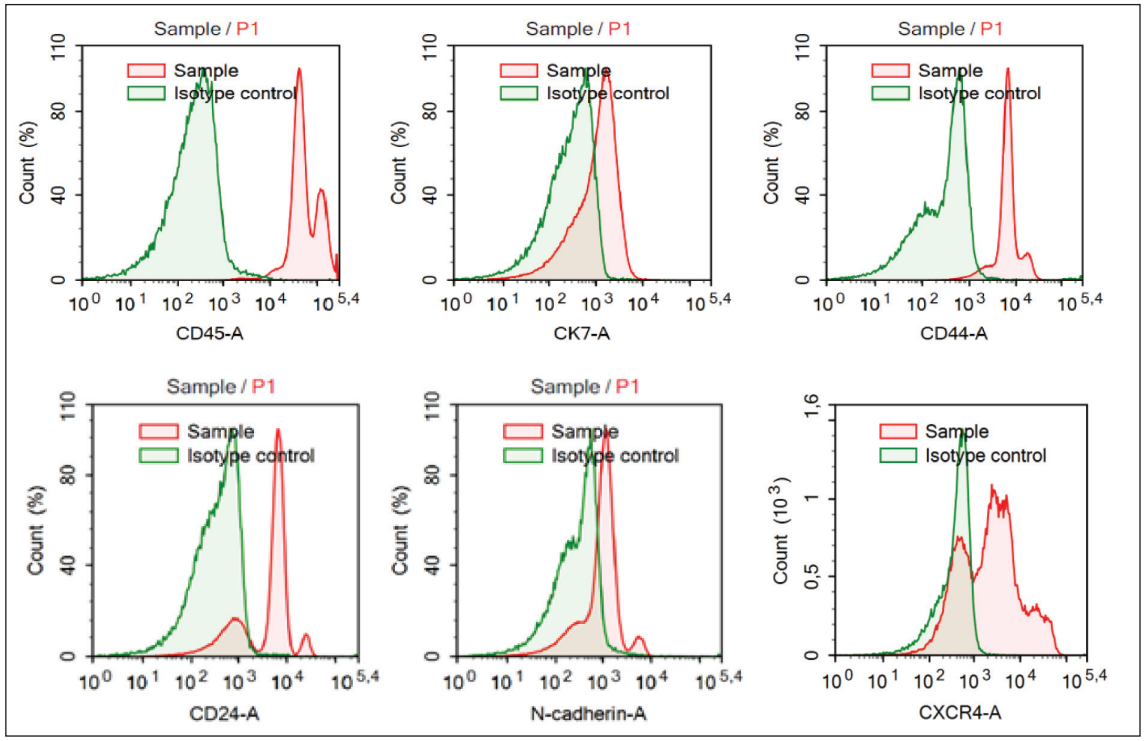

Figure 1. Flow cytometry analysis of the circulating tumor cells with CXCR4 expression

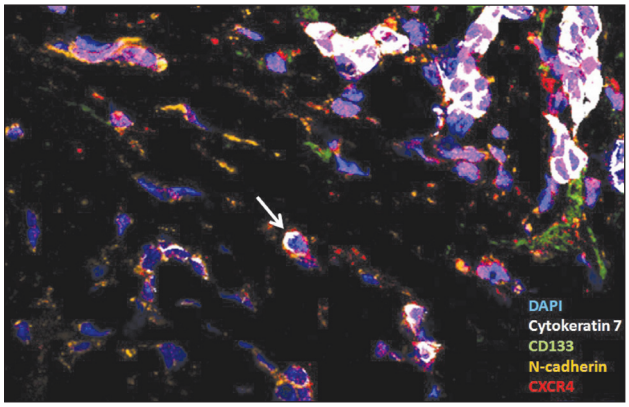

Figure 2. The single (detached) breast cancer cells with CXCR4 expression. The white cursor points to the non stem single (detached) breast cancer cell with EMT features and CXCR4 expression

The non stem-like single (detached) breast cancer cells with EMT features (CK7+CD133-N-cadherin+CXCR4+) expressed CXCR4 in 44.0% (11/25) tumor samples (Figure 2). Our results suggested that only 0.00(0.00-8.00)% of single (detached) CK7+CD133-N-cadherin+ cells exhibited CXCR4 in primary tumor. The single (detached) breast cancer cells with stem and EMT features (CK7+CD133+N-cadherin+CXCR4+) demonstrated an expression of CXCR4 with 28.0% (11/25) tumor samples. Only 0.00(0.00-1.05)% of CK7+CD133+N-cadherin+ cells were positive for CXCR4 in primary tumor. In all tumor samples (25/25) the stem-like (CK7+CD133+N-cadherin-CXCR4+) or non stem-like (CK7+CD133-N-cadherin-CXCR4+) single (detached) breast cancer cells without EMT features were absent (Table 2).

Conclusion

Thus, different subsets of the CTCs exhibit CXCR4 that provide their selective adhesion to the site of future hematogenous metastasis. CXCR4 expression does not depend on the presence or absence of stem or/and EMT features in tumor cells. This study showed that, the tumor cells with stem or/and EMT features can become disseminated cells in case of survival in peripheral blood. In particular, our results explain why not all disseminated cells become a source of clinically manifested hematogenous metastases. Finally, we showed that some subsets of single (detached) breast cancer cells in the primary tumor are characterized by the ability to CXCR4 expression and may be a source of the respectively CTC subsets.

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Conflict of interest

The authors declare that they have no conflict of interest.

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ОДНОФОТОННАЯ ЭМИССИОННАЯ КОМПЬЮТЕРНАЯ ТОМОГРАФИЯ С ^{99m}ТС-1-ТИО-D-ГЛЮКОЗОЙ В ДИАГНОСТИКЕ И СТАДИРОВАНИИ ЗЛОКАЧЕСТВЕННЫХ ЛИМФОМ: ПЕРВЫЙ ОПЫТ ИСПОЛЬЗОВАНИЯ

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Аннотация

Целью исследования явилась оценка возможности применения однофотонной эмиссионной компьютерной томографии (ОФЭКТ) с инновационным отечественным радиофармпрепаратом (РФП) ^{99m}Tc-1-тио-D-глюкоза (^{99m}Tc-ТГ) в диагностике и стадировании злокачественных лимфом. **Материал и методы.** В исследование включены 15 пациентов (средний возраст 50,7±18,3 года) с впервые диагностированными злокачественными лимфомами. Сцинтиграфическое исследование проводили на гамма-камере E.CAM 180 фирмы “Siemens” (Германия) через 4 часа после введения РФП в дозе 500 МБк. **Результаты.** По данным ОФЭКТ, патологическое включение ^{99m}Tc-ТГ в лимфатические узлы наблюдалось у 14 (93 %) из 15 пациентов. У одной пациентки не удалось визуализировать определяемый с помощью компьютерной томографии (КТ) единичный, увеличенный поднижнечелюстной лимфатический узел. Этот ложноотрицательный результат исследования связан с физиологичным усилением аккумуляции ^{99m}Tc-ТГ в орофарингеальной области. Сложности, связанные с высокой фоновой активностью крови, отмечались при визуализации паратрахеальных, парааортальных и паракардиальных лимфатических узлов. Наиболее часто патологическое накопление РФП отмечалось в аксиллярных, над- и подключичных, а также шейных лимфатических узлах. С помощью ОФЭКТ с ^{99m}Tc-ТГ экстралимфатические гиперметаболические участки определялись у 7 (78 %) из 9 пациентов с ранее диагностированным экстранодальным поражением. Кроме того, у одной пациентки по данным ОФЭКТ был выявлен гиперметаболический очаг в легком, не обнаруженный по результатам КТ. В исследуемой выборке пациентов по данным КТ и ОФЭКТ с ^{99m}Tc-ТГ наличие поражения костного мозга наблюдалось в одном случае. Кроме того, сцинтиграфическое исследование позволило выявить гиперметаболические участки в лопатке у пациента с интактной по результатам КТ костной тканью. **Заключение.** Результаты исследования позволяют рекомендовать использование ^{99m}Tc-ТГ ОФЭКТ в качестве дополнительного диагностического метода для обследования пациентов со злокачественными лимфомами для стадирования заболевания.

Ключевые слова: лимфомы, лимфома Ходжкина, неходжкинские лимфомы, однофотонная эмиссионная компьютерная томография, ^{99m}Tc-1-Thio-D-глюкоза.

THE FIRST EXPERIENCE OF USING ^{99M}TC-1-THIO-D-GLUCOSE FOR SINGLE-PHOTON EMISSION COMPUTED TOMOGRAPHY IMAGING OF LYMPHOMAS

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Abstract

Introduction. The purpose of this study was to evaluate the feasibility of using ^{99m}Tc-TG SPECT in the detection and staging of malignant lymphoma. **Materials and methods.** Fifteen patients with newly diagnosed malignant lymphoma underwent ^{99m}Tc-TG SPECT. Six patients had *Hodgkin's lymphoma* and 9 patients had aggressive forms of *non-Hodgkin's lymphoma (NHL)*: diffuse large B-cell lymphoma (7 cases), B-cell follicular lymphoma (1 case), and lymphoma from B cells in the marginal zone (1 case). Stage IIA was diagnosed in 5 patients, stage IIB in 1, stage IIIA in 1, stage IVA in 4 and stage IVB in 4 patients. **Results.** Pathological ^{99m}Tc-TG uptake in lymph nodes was observed in 14 (93 %) of the 15 patients**.** In one patient, the enlarged submandibular lymph node (16 mm in size) detected by CT was not visualized by ^{99m}Tc-TG SPECT. This false-negative result was likely to be associated with increased accumulation of ^{99m}Tc-TG in the oropharyngeal region. There were difficulties in the visualization of paratracheal, para-aortic and paracardial lymph nodes. These difficulties were associated with a high blood background activity, which persisted even 4 hours after intravenous injection of ^{99m}Tc-TG. Software-based SPECT and CT image fusion allowed visualization of these lymph nodes. The pathological ^{99m}Tc-TG accumulation in axillary, supraclavicular, infraclavicular and cervical lymph nodes was observed most often. Extranodal involvement was seen in 9 patients. ^{99m}Tc-TG SPECT identified extranodal hypermetabolic lesions in 7 (78 %) of these patients. In one patient, hypermetabolic lesion in the lung detected by ^{99m}Tc-TG SPECT was not detected on CT image. CT identified bone marrow involvement in the pelvic and scapula in 1 patient. The use of ^{99m}Tc-TG SPECT allowed the visualization of hypermetabolic bone tissue lesions in this patient (Figure 4). In addition, in a patient with intact bone tissue on CT, ^{99m}Tc-TG SPECT detected hypermetabolic lesions in the iliac bone. **Conclusion.** ^{99m}Tc-1-Thio-D-glucose demonstrated increased uptake in nodal and extranodal sites of lymphoma. The results indicate that SPECT with ^{99m}Tc-1-Thio-D-glucose is a feasible and useful tool in the detection and staging malignant lymphoma.

Keywords: lymphomas, Hodgkin's lymphoma, non-Hodgkin's lymphomas, single-photon emission computed tomography, ^{99m}Tc-1-Thio-D-glucose.

Introduction

Tumors of the hematopoietic and lymphoid tissues are relatively frequent in Russia, accounting for approximately 5 % of all cancers in men and 4.6% in women, and 5 % of all cancer deaths. In 2016, the incidence rate for tumors of the hematopoietic and lymphoid tissues was 19.58 per 100 000 population. The average annual growth rate is 1.78 % [1]. Among lymphoid and hematopoietic tissue tumors, Hodgkin lymphoma (HL) is observed most frequently (30 %) [2]. Among non-Hodgkin's lymphomas (NHLs), large B-cell lymphoma (33 %) and B-cell follicular lymphoma (22 %) are the most common lymphomas. Other types of lymphomas occur with a frequency of less than 10 % [3].

Accurate staging for lymphoma enables better prognostication and choice of the most appropriate treatment [4]. Thus, the estimated recurrence-free 10-year survival is highest in patients with stage I disease (90–95 %), slightly lower (80–85 %) in patients with stage II, significantly lower (70 %) in stage III and does not exceed 30–50 % in stage IV [4]. The Ann Arbor staging system for the assessment of lymphoma includes computed tomography (CT) scans [5]. However, diagnostic capabilities of CT are limited when visualizing lymph nodes of normal size. Moreover, in patients with retroperitoneal lymph nodes measuring from 1 to 3 cm, the likelihood of detection of a specific lesion with CT is only 50 %. Even a significant increase in the lymph nodes size (more than 3 cm) is associated with their benign hyperplasia in 25 % of cases. CT has low sensitivity in the diagnosis of diffuse lesions of the liver, spleen and bone marrow [6]. 18-F-fluorodeoxyglucose (^{18}F-FDG) – positron emission tomography (PET), and more recently PET/ computed tomography (CT), is the most sensitive and specific imaging technique currently available for patients with lymphoma.

Numerous studies indicate that PET has proved very useful for staging and therapy response of lymphoma [4–10]. The widespread use of PET in our country is limited due to the high cost and low number of PET centers, which are located mainly in the central regions of the Russian Federation. At the same time, in Russia there are more than 200 nuclear medicine departments equipped with SPECT scanners. Therefore, the use of radiopharmaceuticals labeled with gamma-emitting nuclides remains relevant. ^{99m}Tc-1-thio-D-glucose

(^{99m}Tc-TG) is the promising radiopharmaceutical for lymphoma imaging. This radiopharmaceutical is a glucose derivative complex in the form of 1-thio-D-glucose and ^{99m}Tc, in which 1-thio-D-glucose works as a radioisotope label transport (^{99m}Tc). A special feature of the pharmacokinetics of the radiopharmaceutical is the absence of its accumulation in the brain and myocardium. Tomsk NIIC and Tomsk Polytechnic University successfully completed the project «Preclinical studies of radiopharmaceutical on the basis of ^{99m}Tc labeled glucose derivative for tumor imaging» (№ 14.N08.11.0033). The laboratory regulations for ^{99m}Tc-TG preparation were developed, the analytical methods for ^{99m}Tc-TG quality control were elaborated and samples of the radiopharmaceutical were synthesized and analyzed in accordance with established methodology.

It was proved that ^{99m}Tc-TG was characterized by high accumulation in tumor cells in vivo and in vitro [11–15].

The purpose of this study was to evaluate the feasibility of using ^{99m}Tc-TG SPECT in the detection and staging of malignant lymphoma.

Materials and Methods

The study included 15 patients (mean age 50.7±18.3, range: 25 to 75 years) with newly diagnosed malignant lymphomas. None patients had previously received chemotherapy. The diagnosis was verified by immunohistochemical study. Six patients had HL and nine had aggressive forms of NHL: diffuse large B-cell lymphoma (7 cases), B-cell follicular lymphoma (1 case), and lymphoma from B cells in the marginal zone (1 case). Stage IIA was diagnosed in 5 patients, stage IIB in 1, stage IIIA in 1, stage IVA in 4 and stage IVB in 4 patients.

Single photon emission computed tomography was performed using a double-head gamma-camera (E.CAM 180, Siemens) equipped with parallel high energy collimators. The injection of ^{99m}Tc-TG at a dose of 500 MBq was made intravenously into the antecubital vein. Planar imaging began 4 hours after injection. Images were obtained with the patients lying in the supine position with the arms raised over the head and with the face, skull, chest, neck, abdomen, pelvis and groin included into the field of view. A total of 32 projection images were recorded into a 64×64 matrix (30 seconds per projection) without hardware magnification. The scan images were analyzed using the manufacturer software (e.soft, Siemens, Germany). Three-dimensional images of the chest, sagittal, transverse and coronal sections were obtained. Single photon emission computed tomography scans were visually assessed. Images of the contralateral areas were compared, and asymmetrically increased radiotracer uptake was considered pathological.

Results and Discussion

The pathological ^{99m}Tc-TG uptake in lymph nodes was observed in 14 (93%) of the 15 patients. In a patient with diffuse large B-cell lymphoma, a single enlarged submandibular lymph node (16 mm in size) detected by CT was not visualized by ^{99m}Tc-TG SPECT. This false-negative result is likely to be associated with the physiological enhancement of ^{99m}Tc-TG accumulation in the oropharyngeal region. There were difficulties in the visualization of paratracheal, para-aortic and paracardial lymph nodes. These difficulties were associated with a high background activity, which persisted even 4 hours after intravenous injection of ^{99m}Tc-TG. Software-based SPECT and CT image fusion allowed visualization of these lymph nodes. The pathological ^{99m}Tc-TG accumulation in axillary, supraclavicular, infraclavicular and cervical lymph nodes was observed most often Extranodal lesions were observed in 9 patients (lung in 3 patients, liver in 2, spleen in 2, stomach in 2 and parotid salivary gland in 1). ^{99m}Tc-TG SPECT identified extranodal hypermetabolic lesions in 7 (78%) of these patients. ^{99m}Tc-TG SPECT detected lesions in the lungs in 2 cases, in the liver in one, in the spleen in one, in the stomach in one and in the parotid salivary gland in one (Figure 2). In one patient, ^{99m}Tc-TG SPECT identified hypermetabolic lesion in the lung that was not detected by CT (Figure 3).

18F-FDG PET/CT was found to be superior to CT in the detection of bone marrow lesions in patients with lymphomas [16]. CT allows visualization of such pathological lesions only in the presence of bone destruction. CT detected bone marrow involvement in the pelvic and scapula in 1 patient. The use of ^{99m}Tc-TG SPECT allowed the visualization of hypermetabolic

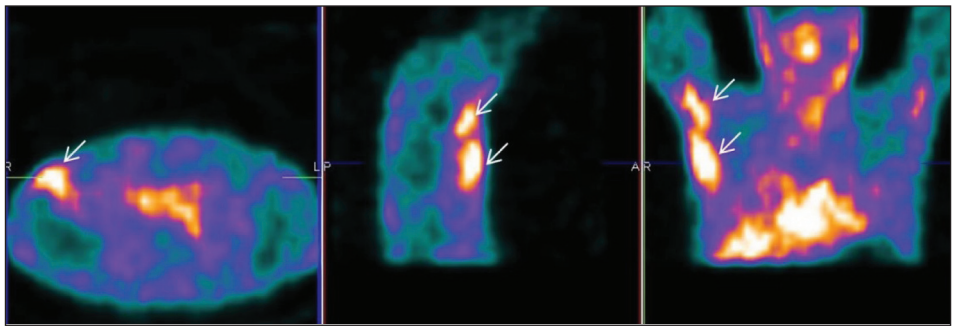

Figure 1. ^{99m}Tc-TG SPECT images of a patient with Hodgkin's lymphoma, nodular sclerosis, stage IIA, lesions of right axillar and right pectoral lymph nodes (arrows)

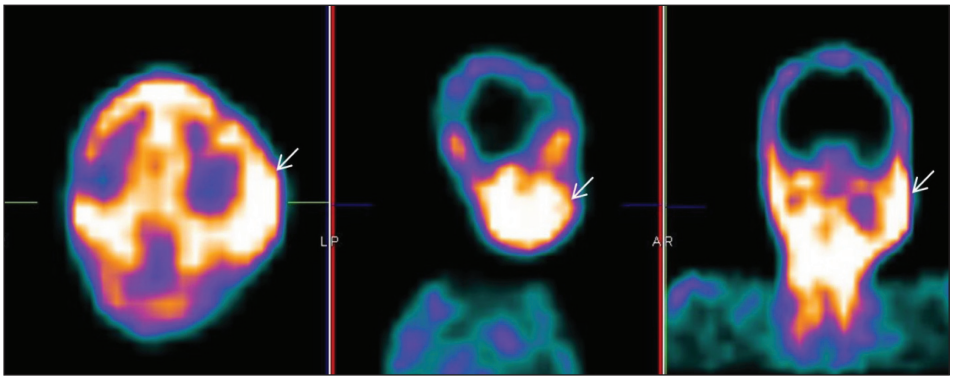

Figure 2. 99mTc-TG SPECT images of a patient with diffuse large B-cell lymphoma, germinogenic type, stage IIA (bulky) with lesions of the left parotid gland (arrows)

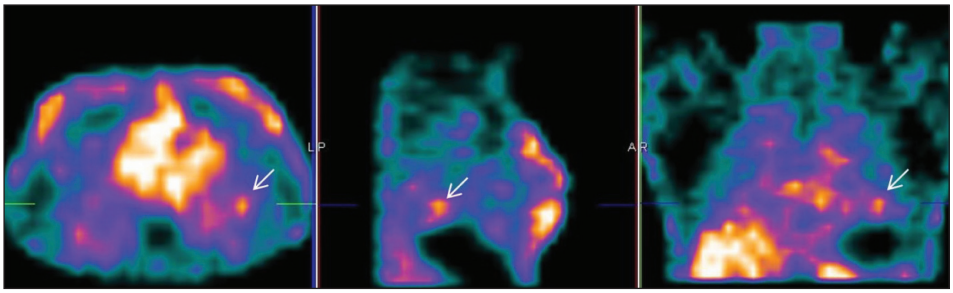

Figure 3. 99mTc-TG SPECT images of a patient with Hodgkin's lymphoma, nodular sclerosis, IIA stage with lesions of the left lung (arrows)

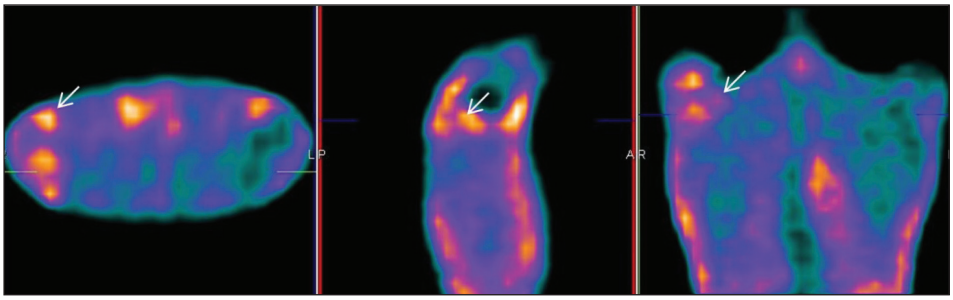

Figure 4. 99mTc-TG SPECT images of a patient with B-diffuse large-cell lymphoma, stage IVB, with lesions of cervical lymph nodes, pelvic bones and right scapula. Hypermetabolic lesion in the right scapula is seen (arrows)

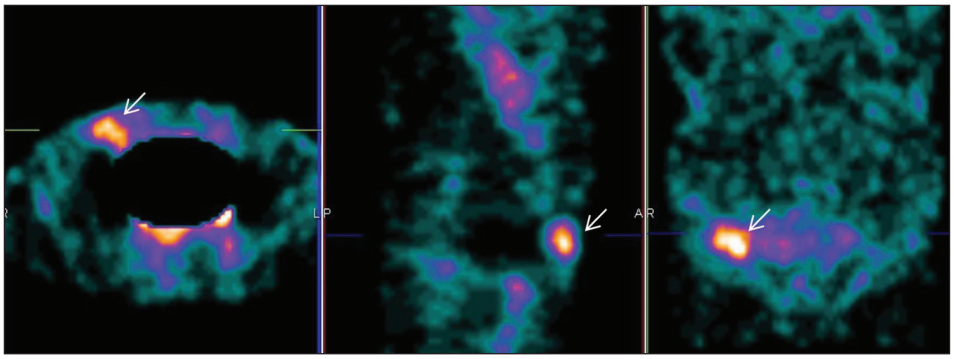

Figure 5 99mTc-TG SPECT images of a patient with diffuse large B-cell lymphoma, germinogenic type, stage IVB with lesion of the left submandibular salivary gland, right inguinal lymph node, lymph nodes of the abdominal cavity, spleen. Hypermetabolic lesion the iliac bone is visualized (arrows)

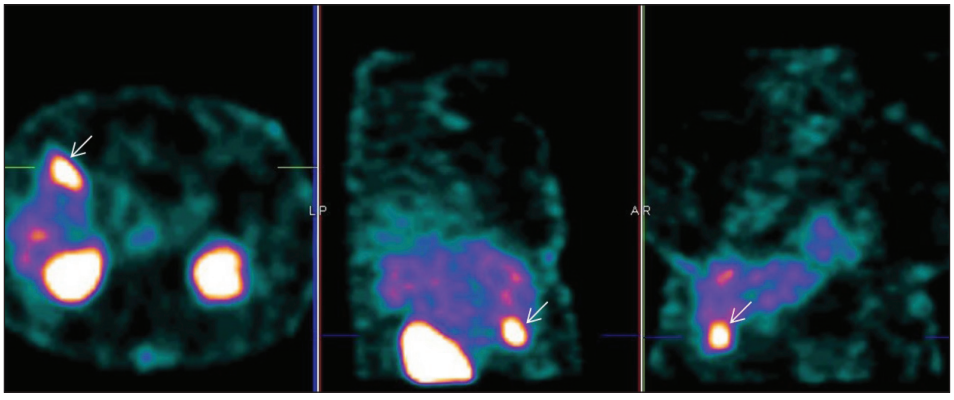

Figure 6. 99mTc-TG SPECT images of a patient with diffuse large B-cell lymphoma, germinogenic type, stage IIB with lesion of the left submandibular and paratracheal lymph nodes. Concomitant diagnosis: chronic cholecystitis. Hypermetabolic lesion in the gallbladder is visualized (arrows)

bone tissue lesions in this patient (Figure 4). In addition, in a patient with intact bone tissue on CT, ^{99m}Tc-TG SPECT detected hypermetabolic lesions in the iliac bone (Figure 5).

18F-FDG is characterized by its active accumulation in inflammation lesions, reducing the specificity of 18F-FDG PET/CT in cancer patients. In our study, two patients had cholecystitis as a concomitant disease; all of them had pathological ^{99m}Tc-TG uptake in the gallbladder (Figure 6). These observations indicate that it is necessary to take into account clinical patient data for the correct analysis of ^{99m}Tc-TG SPECT findings.

The mechanism of cellular uptake of colorectal carcinoma cell line HCT-116 and human lung adenocarcinoma cell line A549 was studied [17]. The levels of ^{99m}Tc-TG and 18F-FDG accumulation in HCT-116 cells were nearly similar, while the 18F-FDG uptake in A549 cell line was almost twice as much as ^{99m}Tc-TG. ^{99m}Tc-TG and 18F-FDG cellular accumulation decreased when the glucose concentration increased, and was enhanced in the presence of insulin. These facts suggest that both 18F-FDG and ^{99m}Tc-TG penetrate into the cell with the participation of glucose transport proteins. Physiologically, glucose is transported by the facilitated sodium-independent glucose transporters (GLUT1-GLUT6, and GLUT8) and by the sodium-dependent transporters (SGLT1 and SGLT2), with a variable expression level of these transporters in different human tissues. Most solid tumors exist in a hypoxic environment and prefer anaerobic glycolysis rather than aerobic glycolysis, converting glucose to lactate and producing less ATP with a smaller oxygen consumption. Therefore, glucose accumulation is frequently enhanced in tumors by overexpression of glucose transporters, generally GLUT1 and SGLT1 [18]. Considering the fact that both 18F-FDG and ^{99m}Tc-TG cellular uptakes are reduced in the presence of cytochalasin B, which blocks the GLUT1 channel, the authors conclude that these agents transferred into the cells mainly by GLUT1. This assumption is supported by the fact that a significant overexpression of GLUT1 in A549 and HCT-116 is described in the literature [19–22]. Seidensticker et al. found a failed uptake inhibition of ^{99m}Tc-TG and 18F-FDG when SGLT1-3 was blocked by phloretin, suggesting that these transporter proteins are not involved in the uptake process [17]. It should be noted that in contrast to 18F-FDG, which is almost completely localized in the cytoplasm, ^{99m}Tc-TG accumulates approximately equally in the cytoplasm and cell membrane. Seidensticker et al explain this feature by the large size of the ^{99m}Tc-TG molecule, which is partially bound to GLUT1 [17]. The chemical structure of ^{99m}Tc-TG does not allow this molecule to interact with hexokinase, so ^{99m}Tc-TG is not phosphorylated (in contrast to 18F-FDG) making it unlikely that ^{99m}Tc 1-TG can be further metabolized.

Conclusion

The ^{99m}Tc-labeled glucose derivative ^{99m}Tc-TG showed a high uptake in the nodal and extranodal lesions in patients with malignant lymphoma. Therefore, we can consider ^{99m}Tc-TG SPECT as an additional diagnostic method for staging malignant lymphoma.

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РАЗЛИЧИЕ РАКА ТОЛСТОЙ И ПРЯМОЙ КИШКИ С ТОЧКИ ЗРЕНИЯ ЭПИДЕМИОЛОГИИ, КАНЦЕРОГЕНЕЗА, МОЛЕКУЛЯРНОЙ БИОЛОГИИ, ПЕРВИЧНОЙ И ВТОРИЧНОЙ ПРОФИЛАКТИКИ: ДОКЛИНИЧЕСКИЕ ИССЛЕДОВАНИЯ

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Аннотация

Введение. Рак толстой и прямой кишки – разные опухоли с клинической и биологической точек зрения. В настоящее время рак толстой и прямой кишки синонимично называют колоректальным раком. Основываясь на нашем опыте в фундаментальных и клинических исследованиях в этой области, мы пришли к выводу, что термин «колоректальный рак» необходимо пересмотреть, его нельзя использовать как обобщающее понятие. **Материал и методы.** Были проанализировали данные литературы и собственные результаты исследований, чтобы доказать или отклонить эту гипотезу. **Результаты.** Выявлены следующие очевидные различия: риск развития рака прямой кишки в 4 раза выше, чем рака толстой кишки; молекулярный канцерогенез при раке толстой кишки отличается от рака прямой кишки; физическая активность помогает предотвратить рак толстой кишки, но не прямой кишки; существуют патогистологические различия между раком прямой и толстой кишки. Кроме того, имеются значительные клинические отличия между этими злокачественными новообразованиями, такие как различная хирургическая топография и объемы операций, разные показания для назначения комбинированного лечения, поскольку рак прямой кишки менее чувствителен к химиотерапии, чем рак толстой кишки, и отличаются прогностические факторы эффективности мультимодальной терапии (например, тимидилат синтетаза и дигидропиримидин дегидрогеназа). **Дискуссия.** Рак толстой и прямой кишки определенно различаются по этиологии и формальному канцерогенезу, эффективности первичной профилактики, связанной с физической активностью, обычной и по параметрам молекулярной патологии. Согласно нашим данным, можно утверждать, что с доклинической точки зрения рак толстой и прямой кишки являются двумя разными опухолями, поскольку обладают различными репрезентативными биологическими характеристиками. Рак толстой и прямой кишки также существенно различаются по многим клиническим признакам, что было указано в отдельной статье, представленной нашей исследовательской группой. **Заключение.** Термин «колоректальный рак» не должен использоваться в фундаментальных и клинических исследованиях, как определение единого заболевания. Рак толстой кишки не одно и то же, что и рак прямой кишки. Злокачественные новообразования толстой кишки могут быть разделены на рак правой и левой половины ободочной кишки.

Ключевые слова: колоректальный рак, рак толстой кишки, рак прямой кишки, молекулярные маркеры, эпидемиология, профилактика, доклинические исследования.

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COLON AND RECTAL CANCER ARE DIFFERENT TUMOR ENTITIES ACCORDING TO EPIDEMIOLOGY, CARCINOGENESIS, MOLECULAR- AND TUMOR BIOLOGY, PRIMARY AND SECONDARY PREVENTION: PRECLINICAL EVIDENCE

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Abstract

Introduction. Colon and rectal cancer (CC, RC) are different entities from a clinical and tumor biological point of view. Up to now, both, CC and RC, are synonymously called “Colorectal Cancer” (CRC). With our experience in basic and clinical research and routine work in this field we now have come to the opinion, that the term “CRC” should definitely be questioned, and if justified, be abandoned. **Materials/Methods.** We analyzed the actual available data from the literature and our own results from the Ulm based study group FOGT to proof or reject our hypothesis. **Results.** The following evident differences were recognized: Anatomically, the risk to develop RC is 4× higher than for CC. Molecular changes in carcinogenesis in CC are different from RC. Physical activity helps to prevent CC, not RC. Pathologically there are differences between RC and CC. In addition, there are also major clinical differences between CC and RC, such as in surgical topography and– procedures, multimodal treatment (MMT) approaches (RC in MMT is less sensitive to chemotherapy than CC), and prognostic factors for the spontaneous course and for success of MMT (e.g. TS or DPD). **Discussion.** CC´sand RC´s definitely are different in parameters of causal and formal carcinogenesis, effectivity of primary prevention by physical activity, conventional and molecular pathology.According to our findings we can demand from the preclinical point of view that CC and RC are two different tumor entities in terms of various representative biological characteristics.CC and RC are also differing substantially in many clinical features, as outlined in a separate paper from our group. **Conclusion.** “CRC” should no longer be used in basic and clinical research and other fields of cancer classification as a single disease entity. CC is not the same as RC. CC might even be divided into right and left CC.

Keywords: colorectal cancer, colon cancer, rectal cancer, molecular markers, epidemiology, prevention, pre-clinical trials.

Introduction

Colon- and rectal cancer (CC and RC) up to now are regarded as a single tumor entity, “Colorectal Cancer”/CRC, in all fields of basic- and clinical research as well as in clinical practice. This is based on the assumption that CC and RC develop in the large bowel, thought to be a similar organ from the ileocecal valve up to the dentate line as boundaries to the small bowel on the oral edge and to the anal canal, sphincter ani, and skin, aborally. The term “CRC” has been based on the similar anatomical structure (Mucosa, muscular layer, serosa +/-), function (stool concentration, fluid resorption, stool transportation and excretion) of the organ, and histology of CC and RC. Our groups for decades have worked on colon- and rectal cancer in basic-, translational-, and clinical research projects. We also have been involved in national projects to structure and improve treatment of colon- and rectal cancer patients (Interdisciplinary Ulm-based “Forschungsgruppe Oncologie Gastrointestinale Tumoren” (FOGT); German Cancer Society (DKG) -S3 Guide lines, DKG structural commision for DKG Large Bowel Centers, Surgical Group for Visceral Oncology (CAO-V)) and organized in part nationwide activities/projects for disease prevention for the Hessian and German Cancer Societies (HKG, DKG): (“1000 Mutige Männer” (a project to motivate for preventive colonoscopy), and “du bist kostbar”(nationwide DKG-projects for cancer prevention and living with cancer (www.dubistkostbar.de)). With those activities, and the associated knowledge and experience we came to the conclusion that summarizing CC and RC to “CRC” must be questioned. Therefore, we analyzed the

literature to landmark characteristics of CC and RC, such as epidemiology, carcinogenesis, prevention, clinical symptoms, diagnosis, multimodal therapy and clinical results to find out, whether there are significant homogeneities between CC and RC justifying the definition "CRC" or rejecting it. Our experience and the results of two large multimodal treatment studies, FOGT 1 (adjuvant chemotherapy of colon cancer) and FOGT 2 (adjuvant radiochemotherapy of rectal cancer) were included in the analytic set up at highest evidence levels. Rejection of the term "CRC" would divide CC and RC as self standing tumor entities. Recent publications concerning the molecular biology of these tumors support this hypothesis. In this paper we present the preclinical differences between CC and RC.

Materials and Methods

For recapitulating the basic known information we described the current anatomical/topographical definitions of the colon and the rectum, the macro- and histopathology of colon and rectal cancers in standard literature/books/actual S3 guide lines on "colorectal cancer". Then we analyzed actual reports (papers and abstracts)in English language on the epidemiology, etiology, formal and molecular carcinogenesis, hereditary syndromes, preventive possibilities, clinical symptoms, diagnostic procedures, surgical procedures, multimodal therapies, follow up, and short term results/long term results. We analyzed more than 2000 publications available from Pubmed, Medline etc. concerning these fields between 2000-2018 using the keywords "colorectal cancer", "colon cancer", "rectal cancer", "chemosensitivity of colon and rectal cancer", "chemotherapy", "surgery", "radiochemotherapy", "randomized clinical trials", "molecular biology", "prognostic factors", and others. We also took informations from the German S3 Guide Lines "Colorectal Cancer" from the versions 2008 and 2013. Results from the data bases from the FOGT trials on improvement of multimodal adjuvant chemotherapy in CC (FOGT1) and adjuvant radiochemotherapy in RC (FOGT2) and associated publications [1, 2] were used to substantiate or reject our hypothesis. The results concerning the preclinical informations are presented.

Results

Anatomically/topographically the rectum is defined as large bowel up to the edge of 16 cm from the anocutaneous line. The lower third reaches up to <6 cm, the middle third from 6-12 cm, and the upper third from >12-16 cm. The upper third has an intraperitoneal position, while the two lower two thirds are located extraperitoneally in the small pelvis. The upper edge of the rectum may also be defined by the confluens of the three colon tenias to a single rectal tenia. The topography of the upper third is varying between males and females. The two lower thirds have a sophisticated topography concerning the mesorectal structures, fascias, nerval and vascular anatomy to/from and around the rectum and position adjacent to the male/female ventrally located sexual organs and pelvic vessels and – nerve structures [3–6]. The venous blood from the lower two rectal thirds is flowing via the internal iliac veins and the inferior caval vein into the lungs, from the upper third via the inferior mesenteric vein into the liver. The venous outflow of the colon is directed into the liver via the inferior (colon sigmoideum and colon descendens) and superior (transverse colon and colon ascendens) mesenteric veins into the liver. The arterial blood supply of the colon descendens, sigmoid colon and the upper rectal third comes from the inferior mesenteric artery, while the rest of the colon is supplied by the superior mesenteric artery. The lower two rectal thirds receive their arterial blood supply via the internal iliac arteries [7–10]. The lymphatic drainages of the rectum are led in parallel to the inferior mesenteric vein (upper third) or along the pararectal/internal iliac lymph streets (middle third) or along the inferior rectal artery (lower third) [11, 12]. The innervation of the rectum is supplied by the superior and inferior hypogastric plexus (superior plexus = N.sympaticus; inferior plexus = N.sympaticus and N.parasympaticus). These plexus not only are responsible for the pelvic organ function, including the lower two thirds of the rectum [12–15]. The nerve supply of the colon runs along the arterial vascular supplies as described above. Regarding the surrounding structures, the topography of the rectum, especially of the lower two thirds, is much more hazardous to the surgeon (plexus, internal iliac vessels, ureters, sexual organs etc.) than that of the colon (ureters, portal- and splenic veins, lower pancreatic edge) – of course with consequences to the skills required for the surgical procedures and the curative limits in T4 stages. Surgery of rectal cancers with the aim of sphincter preservation is significantly more demanding than surgery of colon cancer and the difficulties, such as anastomotic insufficiencies and/or nerval injuries, increase the further down the tumor is located.

The topographic position of the rectum and its function for the patient imposes more perception than the colon and for surgical treatment imposes more challenges and risks for malfunction and irreversible damage concerning continence and lesions of surrounding structures resulting in major bleeding or malfunction pelvic organs.

Macroscopically there are four forms of colon cancers: Bowel shaped and ulcerating (55-60%), polyp-cauliflower form of growth (25%), flat forms (15-20%, and diffuse infiltrating (1%). Exophytic growth is predominant in the proximal colon, while growth is endophytic ring shaped in the distal colon [16, 17]. RC may be growing exophytically, endophytically with ulcerations and intramural expansion or diffuse infiltrating with linitis plastica [12].

Colon and rectal cancers may have different macroscopic appearances.

In **formal carcinogenesis** 85-90% of the cancers arise from low grade or high grade intraepithelial neoplasias (LGIN, HGIN [18]) mainly in form of adenomas. These are classified as tubular (75%), villous (10%) or mixed (15%) with a malignant transformation risk of 4.8% (tubular), 19% (mixed), and 38.4% (villous) [14, 19]. Most of the malignant tumors are mucinous or non-mucinous adenocarcinomas, others have signet ring, anaplastic or squamous cell differentiation with worse prognosis [20]. Low risk cancers (L0) have high or intermediate differentiation (G1, G2) high risk cancers (L1) have a bad or undefinable differentiation (G3, 4) [3, 17, 18, 21].

CC´s and RC´s **epidemiologically** usually are registered as CRC´s. The incidence of CRC in Europe is higher than in Africa or Asia, but lower than in the US [22, 23] and is associated to nutritional habits concerning fat- and meat consumption [24]. The sex distribution in CRC disease favours male (53%) vs. female (47%), but the risk to develop a RC in males is 1.5 the risk in females, while females are predominant in developing cancers in the proximal colon (46% females vs. 38% males (1.2:1)). In the last four decades there was a "shift to the right" with increasing incidences in the right hemicolon; currently 15-35% of the cancers are located in the rectum, 25% in the right hemicolon [25–30]. According to the statistics of the American Cancer Society in 2015 [31] out of 129 700 newly registered CRC´s 93.090 (72%) cancers were diagnosed in the colon, and 39.610 (28%) in the rectum, resulting in a proportion of 2.5:1 (CC:RC). This may suggest that the carcinogenic risk in the colon is higher than in the rectum.

There is a shift to the right meaning that the incidence of CC is increasing, that of RC decreasing.

To our analytic calculation, however, the **carcinogenic risk** in the rectal mucosa by far exceeds that in the colon mucosa due to the fact that the area at risk in the colon definitely is larger than that of the rectum. The area simply can be related to the length of the colon (150 cm) or rectum (16cm). Thus the incidence of CC in the US in 2015 per cm of the colon is 621cm^{-1} (93.090/150=620.6) vs. 2479cm rectum^{-1} (39.610/16=2478.6) resulting in a relation of at least 1:4. In other words, the rectal mucosa has at least 4x higher risk for malignant transformation than the colon mucosa, which either depends on various susceptibilities to carcinogens or to various carcinogenic processes in the colon and in the rectum.

Histopathologically in early CC´s and RC´s mucosal lesions (polypoid, nondepressed type) are more frequently located in the colon than in the rectum (right hemicolon 51%, left hemicolon 35%, rectum 14%), while submucosal lesions more frequently occur in the rectum (Figure 1a); mucosal lesions with villous components were found more frequently in the rectum (right hemicolon 2%, left hemicolon 5%, rectum 13% [32]. The absolute values for frequency of mucosal/submucosal lesions of the depressed type are higher in the rectum than in the right colon. (Figure 1b) [32]. The Japanese authors describing this phenomenon suggest that different carcinogenic mechanisms are the reason for this differing histopathologic appearance of colon vs. rectal cancers [32].

In CC mucosal lesions are more frequent than inRC, in RC submucosal lesions are more frequent.

The difference in the incidences of **hereditary syndromes** involved in the development of CC vs. RC implicates that the **molecular carcinogenesis** in CC seems to be different from that in RC. HNPCC manifests predominantly in the colon/proximal colon, while FAP predominantly is causing the cancer in the distal colon or rectum, but also occurs in the rest of the colon [33, 34]. Various characteristic differences between HNPCC and FAP are summarized in Table 1.

Macroscopically the APC type shows polypoid growth, while the growth pattern of the MSI type is flat. MSI types more frequently occur in the right colon (44% in the right colon vs. 25% in the left colon; $p<0.01$), while polypoid cancers are more frequent in the left (59%) vs. right (40%) colon ($p<0.01$) [35]. The flat growing early precursors of early cancers are significantly more difficult to detect than the polypoid growing early cancers [35–37].

HNPCC predominantly occurs in the right colon, for APC there is no predominance. CC and RC from a molecular biological point of view may be regarded as MSI- or APC types. MSI types are more frequent in the proximal colon and flat, APC types are polypoid. CC and RC differ in their chromosomal and molecular profiles as well as in enzyme expressions. There is no clear cut boundary between rectum and descending colon [38].

When regarding all CC´s vs. RC´s in their molecular carcinogenic alterations, differences in molecular profiles and enzyme expressions between CC and RC become evident (Table 2). For example, MSI more frequently is detected in proximal CC´s than in RC´s [39–43] which is also the case in HNPCC patients [34, 44–46]. When compared to RC, proximal CC´s more frequently show mutations in BRAF (Serin/Threoinin-Kinase- V600E [41, 47, 48], the expression of the CPG-Island Methylator-Phänotype (CIMP) [49–51], high gene expression of HOX [50] and CDX2 [52], increased mutation of KRAS [53] and higher levels ofactivated MAPK-signal transduction pathways [54]. In distal CC and in RC the following changes/molecular characteristics are more frequent when compared to proximal CC: Positivity of chromosome instability / CIN) [49], stability of microsatellites (MSS) [33, 34, 55], which is also the case in FAP [56]. The genes for EGFR or HER2 are amplified [57], p53 is mutated [58, 59], Wnt-signal-pathways in carcinogenesis activated [44, 60–62] in favour of distal CC´s and RC´s. The importance of p53 in carcinogenesis of colon and

rectal cancer has been extensively described by Harris [63]. CC´s and RC´s also differ in protein expressions significantly, which are higher for Cyclin D3 and cMyc in CC ($p<0.001$) and for Cyclin D1, Cyclin E and Nuclear beta-Catenin in RC ($p<0.001$) [59]. High TS expression in CC correlates to better survival in the spontaneous course [58, 64, 65] or after adjuvant chemotherapy in CC [66–69]. Vice versa in RC, high TS either correlates to worse survival [70–72] or is meaningless [73]. The hereditary cancer syndromes HNPCC (2-7% of all "CRC"´s) and FAP (1% of all "CRC"´s) are differing in their molecular chromosomal changes [38]. FAP has an APC-Gene mutation (APC type; 60% of all "CRC"´s), while in HNPCC the germ chromosomes are mutated in their DNA information for MMR-genes (MLH1, MSH2, MSH6, PMS1, and PMS2) leading to MSI (MSI type) [44, 45, 74]. CC´s and RC´s may be categorized according to the features of the APC type (about 2/3 of CC+RC) and the MSI type [21, 75, 76].

Innovative for the classification of "Colorectal Cancer" were the findings of an international consortium analyzing molecular, enzymatic and immunogenic features and microscopic growth patterns including angiogenesis. With their data collection, the CRC Subtyping Consortium (CRCSC) defined four

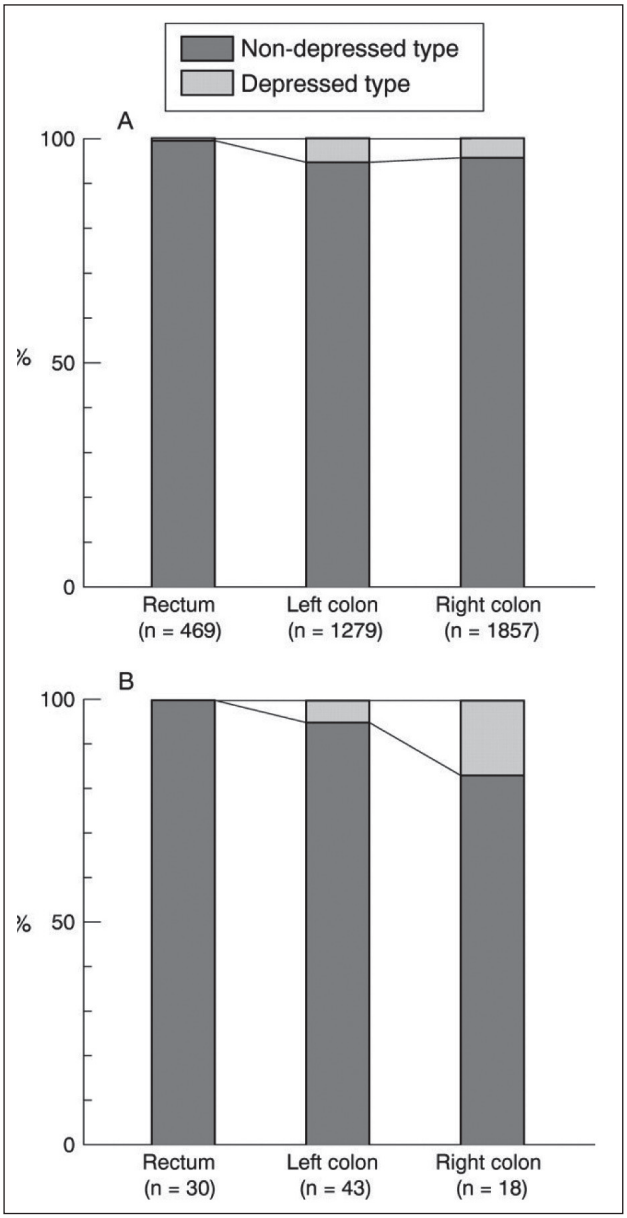

Figure 1a: Comparison of the incidences of depressed and non-depressed types of neoplastic lesions in the rectum, the left colon, and the right colon. (A) Mucosal lesions, (B) submucosal cancers. A significant difference in the macroscopic type was noted between the rectum and the colon ($p < 0.001$). The incidence of depressed submucosal cancers in the right colon was significantly higher than that in the rectum ($p = 0.0472$). [32]

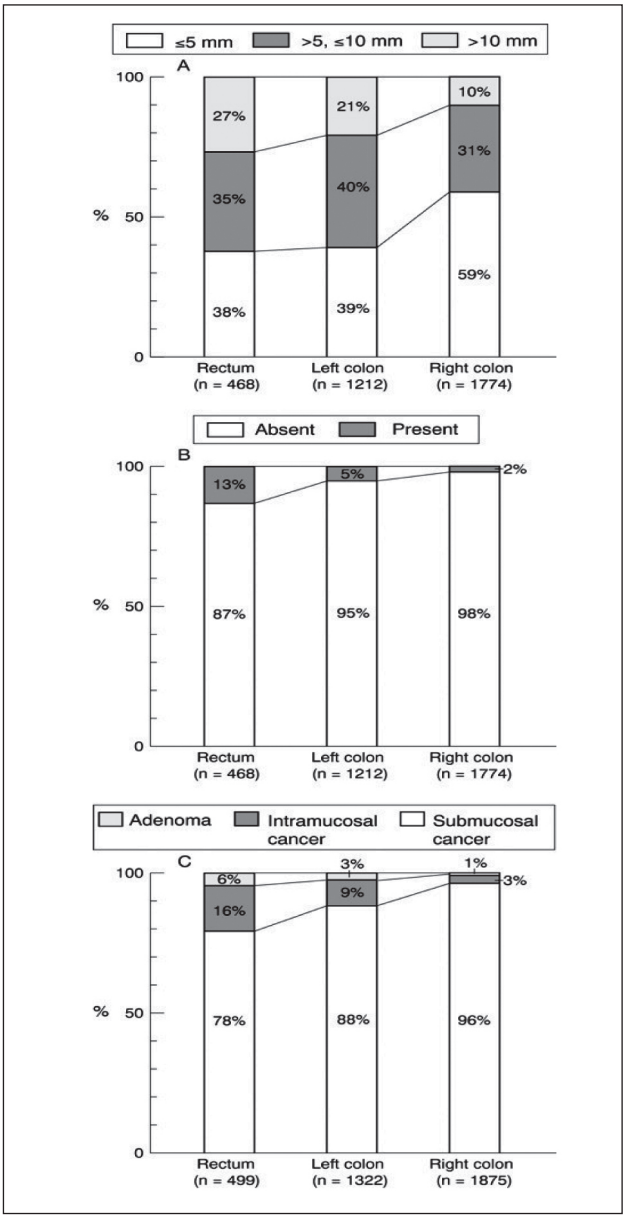

Figure 1b: (A) Relationship between the location and the size of non-depressed mucosal lesions. (B) Relationship between the location and the incidence of villous components in non-depressed mucosal lesions. (C) Locations of mucosal lesions and submucosal cancers. [32]

Table 1

Differences between FAP (familial adenomatous polyposis coli) and HNPCC (hereditary non-polyposis colorectal cancer)

	FAP	HNPCC
Prevalence rate	1 % of all colon and rectal cancers	5 % "CRC"
Phenotype	> 100 polyps	Only a few polyps can be present
Genotype	APC gene mutations	Germline mutations of the DNA MMR genes
Age of onset	In most cases, from 20 to 25 years	From, on average,year44 onwards
Localization	Colon, rectum; associated disease locations: bones, eyes, duodenum	Right colon; associated disease locations: endometrium and also (considerably rarer) stomach, ovaries, pancreas, ureter, renal pelvis, cystic ducts
Transformation to colon and/or rectum cancer	100 % to colon and/or rectum cancer	50–70 % to colon cancer

Table 2

Differences in carcinogenesis, molecular genetic profile, histopathology, and biology of sporadic colorectal cancer compared with rectal cancer

Mutation/Expression	Proximal colon cancer	Distal colon and rectum cancer	Author(s)
Chromosome instability (CIN)	NO	YES	[49, 56]
Microsatellite instability (MSI)	YES	NO	[39]
EGFR and HER2 amplification	NO	YES	[57]
CpG hypermethylation(CIMP)	YES	NO	[49]
BRAF mutation (BRAF-like)	YES	NO	[48]
KRAS	YES	NO	[59]
p53	NO	YES	[58]
HOX gene	YES	NO	[50]
CDX2 gene	YES	NO	[52]
Thymidylate synthase	YES	NO	[70, 71]
Cyclin D3 and c-Myc	YES	NO	[59]
Cyclin D1, cyclin E and nuclear β-catenin	NO	YES	[59]
Activation of MAPK-pathways	YES	NO	[54]
Activation of Wnt-pathways	NO	YES	[44]
Mucosal lesions (non-depressed type)	YES	NO	[32]
Submucosal lesions (non-depressed type)	NO	YES	[32]
Mucosal and submucosal lesions (depressed type)	YES	NO	[32]

YES –Often positive or frequent incidence, NO – often negative or rare incidence

Table 3

Effects of different prevention measures on the two cancer entities

	Decreased incidence	
Prevention measure	Colon cancer	Rectum cancer
Physical activity	YES [53, 80, 81, 84–86]	NO [81, 84–86]
Low BMI	YES [53, 86, 87]	NO [86, 87]
Reduced energy uptake	YES [53]	NO [87]
COX-2 inhibitors	NO in case of HNPCC (there are no sufficient data)	YES in case of FAP [88–90]
Aspirin	YES [91]	NO [91]

HNPCC – Hereditary non-polyposis colorectal cancer; FAP – familial adenomatous polyposis coli.

robust consensus molecular subtypes CMS1-4 [77]. Most interestingly, CMS1 tumors were frequently diagnosed in females with right sided lesions and presented with higher histopathological grade. CMS2 tumors were mainly left sided. CMS4 tumors tended to be diagnosed at more advanced stages (UICC III and IV) and displayed worse overall and relapse free survival (in the multimodal PETACCC-3 trial involving adjuvant CT in CC UICC III). After relapse (and treatment of relapse), survival was superior in CMS2 patients and very poor in the CMS1 population [77]. In our literature analysis looking at various factors relevant for CC and RC on the molecular and protein level, many of them included in the CRCSC-analysis, we found out, that the proximal colon and the distal colon+rectum show evident differences in expression (Table 2). The multi-omic analysis of CC´s confirmed the substantial differences between right- and left sided CC on the molecular genetic level [78].

A difference in carcinogenic principles might also be the reason for the different effectivity of primary preventive measures by physical activity, as pointed out in a review in 2000 on the impact of nutrition and physical activity on the development of CC/RC [79]. It now has been reported by various groups that physical activity at higher levels might reduce the incidence/risk for CC [80–83] by up to 40%, but has no influence on the incidence/risk for RC [81, 84–86]. In the Cancer Prevention Study II Nutrition Cohort involving 70.403 men and 80.771 women, the risk for CC was significantly reduced by 16% (RR 0.84, 95% KI 0.59-1.20) in participants practicing actively sports (79% of all study participants), while this was not observed in RC [85]. The results of various preventive trials involving sports activities, BMI, reduced energy intake or medical intervention with COX-2-inhibitors or aspirin are summarized in Table 3.

In summary, CC differs from RC in terms of molecular biological parameters. CC may be prevented by physical activity, while this cannot be achieved to prevent RC.

Discussion

In terms of epidemiology "CRC" has varying incidences, when continents/civilizations are compared. The male: female ratio in US statistics for incidences 2006-2010 was 1.3:1 in all CRC, but 1.6:1 in RC [29]. The location during the decades shifted from the left/rectum to the right [26, 92], and meanwhile patients with cancer in the right colon are older and more frequently females than males [28]. The most frequent locations are the right hemicolon (48%) and the rectum (28%) [29]. Up to now there are no exact data to show, whether the proportion CC:RC shows a difference in the various continents analyzed. In the Western countries, two thirds of "CRC" is located in the colon, one third in the rectum [3, 23, 29, 92]. This implicates, that the colon is more susceptible to develop cancer than the rectum, which is not the case. We set the 2015 US-incidence in proportion to the length of the organ at risk (colon 150 cm, rectum 16 cm) and came to the conclusion, that the rectum mucosa is four times more prone to malignant transformation than the colon mucosa!.

In the Western countries from a macroscopic/histological point of view colon cancers may have different growth patterns than rectal cancers [22, 23, 92]. The appearance of flat lesions (depressed type) is more frequent in the colon than in the rectum [32], thus more difficult to detect as early cancers, while the polypoid nondepressed types with villous components (easier to detect) were more frequent in the rectum [32]. The authors contributed their observation to possible differences in carcinogenesis colon vs. rectum [32]. When looking at the formal carcinogenesis, one first has to begin with analyzing differences in the most frequent autosomal dominant inheritable "CRC" cancers, HNPCC and FAP. HNPCC preferably is located in the right hemicolon, with FAP cancers there is not such a clear preference, but there is a tendency to left hemicolon- or towards the rectum cancers. Both entities differ substantially in their abnormalities on the chromosomal/DNA-mutational and enzymatic levels. FAP is an obligate precancerous disease; HNPCC may still be regarded as facultative, but has an expression rate of 50-70% [21]. The basis for FAP is an inherited mutation in the APC Gene (initiation) with the consequence of several carcinogenic steps (promotion). About 60-70% of sporadic CC and RC´s have the same "APC-type" formal carcinogenic pathway [21, 93–96]. In HNPCC, a mutation in the mismatch repair gene family (MMR) is the germ defect responsible for a sequence of molecular changes which eventually lead to CC or, rarely, to RC and, in addition to cancers of extracolic adenocarcinomas (CC only: Lynch-Syndrome I, CC and extracolonic cancers: Lynch-Syndrome II) [44, 45, 74, 95, 97, 98]. The gene-defect, responsible for the HNPCC type of cancer is detected pathologically (PCR or Immunohistology) in the tumor tissue as "Microsatellite"in the inheritable syndromes, but also in sporadic cancers which are classified as MSI-type CC or RC.The in male to female proportions in tumor locations (e.g. males get more RC than females, and females more proximal CC´s than males and the shift of "CRC" incidences to the right, and the preferred locations either in the (proximal) colon (HNPCC´s or MSI-type noninheritable "CRC") or in the distal colon/rectum (APC type of "CRC") and our hypothesis to carcinogenic susceptibility (see above) all support our hypothesis, that CC and RC are different tumor entities in terms of carcinogenic processes. When various alterations on the chromosomal- , gene- or protein levels were analyzed in the tumor tissue, marked differences between the "proximal colon" and the "distal colon and rectum" appeared (see Table 2). The possibility to prevent CC by (high) physical activity, but not RC indirectly supports our conclusion, that major carcinogenetic processes in CC are dissimilar from RC.

Very interesting and new findings concerning the classification of CRC´s were generated by the CRCSC Subtyping Consortium in 2015: The group subclassified "CRC´s" from 4151 samples/patients (according to various features from the molecular up to the histopathologic and immunogenic levels (27 unique subtype labels)) into the four distinct subtypes CMS1-4. (and an additional "mixed group") using a very heterogenous tumor population from CC and RC patients with/without surgery, with/without multimodal therapy, with a variety of multimodal treatments, and applied various analytical methods and very sophisticated biometrics. The groups CMS1-4 had various distinct biological properties. Most interestingly, two of the CMS-groups were associated to embryologically different parts of the colon: CMS to right sided lesions, and CMS2 mainly to the left sided lesions [77]. The tumor tissues/data were supplied by 6 different working groups who either had their data from "CRC"- or CC- samples/patients. There was no distinct differentiation between CC and RC [77]. In spite the facts that no separate views seem to have been shed on CC primary tumors as a whole vs. RC primary tumors, and that 858 patients/samples from primary tumors were excluded from the primary analysis, the new system implies, that the large bowel cancers seem to have significantly different characteristics, which eventually may become relevant for treatment individualization accordingto these groups. CRCSC proposes a new taxonomy of colorectal cancer reflecting significant biological differences in the gene expression-based molecular subtypes, which is supported by others [78, 99, 100]. We are thinking that this demand for a change in looking at "CRC" with a very complicated classification system is generalizing too early and mainly based on a molecular primed classification view. More facts need to be respected for dividing the term "CRC" in organ related taxonomic entities.

Various prognostic molecular or enzymatic factors have been tested in CC, RC, and "CRC" in the spontaneous courses or in multimodal therapy with the aim to have a better individualized treatment- and/or patient selection to avoid unnecessary potentially toxic CT´s or RCT´s. We and a few other groups[101, 102] were the first to study the potential role of TS and DPD to individualize patient selection for adjuvant/neoadjuvant and for palliative treatment in "CRC" [67, 79, 103] and conducted the first prospective randomized trial for treatment of metastases [104], always in translational projects with the USC Cancer Center in Los Angeles and the laboratory of P.V. and K.Danenberg, coworkers of the late Charles Heidelberger (teacher and inspirator of one of the authors (K.H.L.) to introduce individualization to surgical oncology). Up to now, there is a tendency in results from multimodal trials, that these molecular prognostic factors, e.g. TS and DPD, may be used for treatment individualization – with different results in CC- vs. RC-multimodal treatment. E.g. low TS might be associated with a benefit from RCT+CT in RC, and high TS in adjuvant CT of CC. Reimers et al. have suggested a cocktail of modern prognostic factors for patient selection in neoadjuvant treatment of RC [43], however, this approach due to the lack of unanimous convincing data obtained by best methods determined in translational research consensus is far from routine yet.

Regarding our findings, we strongly suggest to accept CC and RC as different tumor entities in all aspects of experimental and clinical research. The term "CRC" should be historical.

Summary and conclusion

We collected data on various relevant levels to question the term "colorectal cancer" and, if indicated, suggest to replace it by "colon cancer" (CC) and by "rectal cancer" (RC) separately, if the tumor is located/has been the origin of the primary tumor in the corresponding location. Basic and clinical research groups should respect this change of nomenclature. With our ample experience in carcinogenesis, prevention, surgery and multimodal therapy of primary CC and RC primary tumors and their metastases and in treatment individualization by molecular/cell culture methods and on the basis of the following collected data/experiences we think, that this recommendation is justified. The CMS system suggested by the CRCSC group also suggests taking a distinct look at the broad nondifferentiating term "CRC". Our opinion, that CC and RC are distinct tumor entities is supported by the facts that CC and RC seem to be submit two different pathways in initiation and promotion (carcinogenesis: HNPPC and MSI type of CC mainly located in the proximal colon, FAP and APC type without clear preference, but a tendency to the left colon and to the rectum), have a different susceptibility to/way of carcinogenesis (rectal mucosa is four times more susceptible to malignant transformation than colon mucosa) and to preventive principles in carcinogenesis (active sports may prevent CC (up to 40%), but not RC), that tumors shift to the right and female sex is dominating in proximal (right) CC´s (due to a change of carcinogenic principles). The clinical parameters of differences between CC and RC, such as in surgical techniques with morbidity/mortality and long term results, the responses and toxicities (= the benefit) of multimodal therapy (MMT) and molecular/clinical prognostic factors in the spontaneous course and after MMT, are analyzed and reported in a separate paper [105]. CC and RC have different profiles from the view of many preclinical (and clinical) parameters.In basic and translational research concerning "CRC", Colon Cancer (CC) and Rectal Cancer (CC) should be regarded as different tumor entities. CC may even be subdivided in right sided CC and left sided CC (and male vs. female).

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ХИТИНАЗОПОДОБНЫЕ БЕЛКИ КАК ПЕРСПЕКТИВНЫЕ МАРКЕРЫ ПРИ ЗЛОКАЧЕСТВЕННЫХ НОВООБРАЗОВАНИЯХ

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Аннотация

В обзоре проанализированы данные о роли хитиназоподобных белков (CLP), принадлежащих к семейству белков, содержащих Glyco_18 домен и не обладающих ферментативной активностью, при различных злокачественных новообразованиях. У человека идентифицировано 3 таких белка: YKL-40 (CHI3L1), YKL-39 (CHI3L2) и стабилин-связывающий CLP (SI-CLP). Хитиназоподобные белки, продуцируемые различными типами клеток, в том числе опухолевыми, проявляют активность как цитокины и ростовые факторы, а также они вовлечены в процессы воспаления. Высокий уровень CLP определяется в циркулирующей крови при воспалительных заболеваниях и разных локализациях злокачественных опухолей. Освещены данные о ключевых функциях CLP в физиологических и патологических условиях. Проанализированы сведения о вовлечении CLP в процессы инвазии, метастазирования, ангиогенеза, их связи с опухолевой прогрессией. Представлены собственные результаты, подтверждающие перспективность разработки прогностических и предсказательных маркеров на основе CLP при злокачественных новообразованиях.

Ключевые слова: хитиназоподобные белки, CLP, YKL-30, YKL-40, SI-CLP, злокачественные новообразования, опухолевая прогрессия.

CHITINASE-LIKE PROTEINS AS PROMISING MARKERS IN CANCER PATIENTS

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Abstract

In the present review we collected the main studies regarding the role of chitinase-like proteins (CLPs), belonging to the family of Glyco_18 domain-containing proteins, in different cancers. In humans, 3 chitinase-like proteins have been identified: YKL-40 (CHI3L1), YKL-39 (CHI3L2) and stabilin-1-interacting chitinase-like protein (SI-CLP). CLPs are produced by several types of cells and combine the properties of cytokines and growth factors. The high levels of CLPs were identified in the circulation of the patients with inflammatory diseases and various types of tumors. We highlighted the main known functions of CLPs in normal and pathological conditions, their contribution to metastasis development, angiogenesis, invasion and other processes in cancer, the correlation of the levels of CLPs with tumour progression. Our data also contribute to the understanding of question how CLP could be useful for cancer patient benefit.

Keywords: chitinase-like proteins, CLP, YKL-30, YKL-40, SI-CLP, cancer, tumor progression.

Common features of CLPs family

Chitinase-like proteins (CLPs) are structurally resemble chitinases that belong to a group of proteins, which are widely expressed in nature, and distributed in a wide range of organisms, including mammals, bacteria, plants, insects, viruses. Proteins with chitinase activity represent evolutionary ancient enzymes responsible for degradation of chitin, which is the second most abundant natural compound [1].

Mammalian chitinases and CLPs belong to glycosyl hydrolase family 18 (GH18) [2] due to presence of highly conserved Glyco_18 domain, responsible for sugar-binding, and catalytic site, which is essential for hydrolysis of chitin. The prehistoric purpose of GH18 existence is the degradation of complex sugar compounds, such as cellulose or chitin, via disruption of strong covalent or glycosylic bonds in polysaccharidic chains that compose polymer molecules. There are only two mammalian chitinases identified as functionally active enzymes, which are known as Acidic Mammalian Chitinase (AMCase) and Chitotriosidase (CHIT1) and they are both expressed in human [3, 4]. AMCase was firstly revealed in macrophages from patients with Gaucher disease [5]. The source of secreted chitotriosidase is abnormal lipid-laden macrophages that can be classified as a variation of alternatively activated macrophages, expressing CD68, CD14, HLA class II, CD163, CCL18 and IL-1-receptor antagonist, but not CD11b, CD40 and pro-inflammatory cytokines [6].

Chitinase-lake proteins predominantly contain Glyco-18 domain but not catalytic site (glycosyl hydrolase function). These proteins are also known as enzymatically inactive chi-lectins [2]. There are 4 known mammalian CLPs: YKL-40 (CHI3L1), YKL-39 (CHI3L2), stabilin-1-interacting chitinase-like protein (SI-CLP), and YM1/YM2 [7-10]. YM1/YM2 proteins are only found in rodents. YKL-39 is only present in humans and absent in rodents. All CLPs have specific characteristics in carbohydrate binding site. The binding region is located in (α/β)8 TIM-barrel domain, which allows CLPs to interact with chitin oligosaccharides with high affinity [10, 11]. It is crucial to be aware about the binding characteristics of CLP, because it allows prediction of the binding partners and, therefore, prediction of biological functions related to that binding.

YKL-39 is known to bind to chitooligosaccharides (GlcNac)5 and (GlcNac)6 [11, 12], that was demonstrated by glycan array screening, intrinsic tryptophan fluorescence and isothermal titration calorimetry (ITC). There are more binding targets known for YKL-40; it can bind to type I collagen that was revealed by affinity chromatography and surface plasmon resonance [13], to chitooligosaccharides, that shown in protein X-ray crystallography assay [14]; (GlcNac)5 and (GlcNac)4 that revealed by the Western blot [15] and heparin demonstrated by heparin affinity and HPLC chromatography [16]. ITC analysis showed that SI-CLP can bind to galactosamine, glucosamine, chitooligosacharide, (GlcNac)4, ribose and mannose [17]. It was demonstrated by surface plasmon resonance analysis that YM1 can bind to glucosamine, galactosamine and glucosamine polymers [18].

The main sources and functions of CLPs

The secretion of chitinase-like proteins was found in macrophages, neutrophils, epithelial

cells, chondrocytes and synovial cells, vascular smooth muscle cells as well as tumor cells (including breast, colon, kidney, lung, ovarian, prostate, uterine, osteosarcoma, glioblastoma) and their expression was regulated by various cytokines and hormones [1, 12].

YKL-39 is predominantly secreted by chondrocytes and synoviocytes and is recognized as a biochemical marker for the activation of chondrocytes and progression of osteoarthritis in humans [19].

YKL-40 is secreted by chondrocytes, synoviocytes, differentiated vascular smooth muscle cells, fibroblast-like synovial cells, by macrophages in the atherosclerotic plaque, tumor cells in many cancers [1, 20, 21]. In vivo, YKL-40 expression was found in places with intensive tissue remodeling [1]. YKL-40 regulates cell proliferation, migration, adhesion, macrophage differentiation, as well as extracellular matrix assembly and correlates with an elevated level of YKL-40 in chronic inflammation and connective tissue turnover [1, 22]. YKL-40 promotes the proliferation of chondrocytes and fibroblasts, migration and reorganization of vascular endothelial cells as well as inflammation and remodeling of extracellular matrix [1, 16]. It induces the migration of vascular smooth muscle cells (VSMC) [16] and promotes the growth of human synovial cells, skin and fibroblasts. High YKL-40 level was detected in serum of patients with rheumatoid arthritis (RA) and in patients with type 2 diabetes [1].

SI-CLP expression was found in various tumor cell lines, Raji cells, Jurkat cells, as well as in CD3+ T-cells, in the synovial fluid of patients with osteoarthritis or rheumatoid arthritis [17].

Expression of YKL-40 mRNA in human monocyte was strongly upregulated by IFN-gamma, and inhibited by IL-4 and dexamethasone [9]. There are also evidences that YKL-40 is secreted by macrophages during the late stages of differentiation. YKL-40 gene expression was up-regulated in monocytes stimulated with granulocyte-macrophage colony-stimulating factor, in colony-stimulating factor stimulated monocytes and in lipopolysaccharide stimulated monocytes [23, 24].

For YKL-39, no specific effects of IFN-gamma, IL-4 or dexamethasone were detected, but YKL-39 was upregulated in macrophages differentiated in the presence of IL-4+TGF-beta, but not IL-4 alone [25].

Human macrophages produce also SI-CLP and its expression is induced by Th2 cytokine IL-4 and glucocorticoid dexamethasone [9]. In vivo, high amounts of SI-CLP were detected in macrophages from bronchoalveolar lavage of patients with chronic airway inflammation [17].

In macrophages, SI-CLP is primarily localized in the secretory lysosomes. Kzhyshkowska et al. demonstrated that the intracellular sorting of SI-CLP in alternatively activated macrophages was mediated by the scavenger receptor stabilin-1, which was specifically expressed on subpopulations of tissue macrophages and sinusoidal endothelial cells in liver, spleen, lymph node and bone marrow. Stabilin-1 recognized SI-CLP in trans-Golgi network and delivered it to the late endosomes and consequently into Lamp1-positive and CD63-positive lysosomes [9]. The pattern of intracellular YKL-39 distribution was similar to the pattern demonstrated for SI-CLP suggesting that YKL-39 can be secreted by the lysosomal secretory pathway. Endogenous YKL-39 was found in the trans-Golgi network, where it was partially co-localized with stabilin-1. Using GST pull-down assay we showed that stabilin-1 can act as an intracellular sorting receptor for YKL-39 [25].

The role of CLPs in angiogenesis and chemotaxis

Among all chitinases and chitinase-like proteins, the pro-angiogenic activity and function of YKL-40 in various types of cancer progression were well studied. Angiogenic properties of YKL-40 in cancer development were demonstrated in breast and brain cancers where the expression level of YKL-40 was associated with tumor vascular formation [26, 27]. Immunohistochemical analysis of human breast cancer demonstrated a correlation between blood vessel density and YKL-40 protein expression [26]. In mouse model of breast cancer, YKL-40 was demonstrated to promote tumor growth by supporting angiogenesis. In mouse model of melanoma and glioblastoma, the inhibiting effect of anti-YKL-40 monoclonal antibody on tumor growth was shown [28, 29]. It was revealed that YKL-40 can facilitate tumor angiogenesis by interacting with syndecan-1 on endothelial cells and metastasis by stimulating production of MMP-9, CCL2 and CXCL2 [30].

Several studies on in vitro tube formation and endothelial cell migration have demonstrated that YKL-40 has stimulating effect on the endothelial cells that is similar to the effect of endothelial growth factor (VEGF) [27]. YKL-40-heparin interaction promotes the interaction with syndecan-1 and αvβ3 integrin, leading to activation of the ERK1/2 pathway and stimulation of VEGF [26, 27]. In glioblastoma, transient suppression of VEGF substantially increased YKL-40 expression and promoted tumor angiogenesis [31]. Anti-VEGF neutralizing antibody did not improve HMVECs tube formation and migration induced by YKL-40, thus confirming that pro-angiogenic effects of YKL-40 on HMVECs were not affected by VEGF [26].

Chitinase-like proteins can influence chemotactic activity of various cells directly or indirectly. Using an in vitro microchemotaxis transwell system model, Nio et al. demonstrated that YM1 acted as a chemotactic factor for eosinophils, T-lymphocytes and bone marrow cells [32]. YKL-40 was shown to affect chemotaxis of VSMC [16], THP-1 cells [33] and bronchial smooth muscle cells [34]. For THP-1 and VSMC cells, purified YKL-40 induced chemotaxis directly. In contrary, for

bronchial smooth muscle cells and SW480, YKL-40 enhanced secreted levels of IL-8, thus providing a chemotactic effect.

YKL-40 significantly increased the migration and invasion ability of CL1-1 NSCLC (non-small cell lung cancer) cell lines by regulating EMT (Epithelial Mesenchimal Transition) genes. In YKL-40 overexpressed cell line, the expression of E-cadherin, a marker of epithelial cells, was significantly lower; and the expression of markers of mesenchymal cells (N-cadherin, Vimentin) was significantly higher as well as other EMT regulators (Snail, Slug, and Twist) [35]. Moreover, inhibition of YKL-40 reduced the tube formation in vitro and suppressed tumor growth, angiogenesis, and progression of brain tumors [28].

Analysis of biological functions of YKL-39 demonstrated that it is unique that CLP combines monocyte attracting and pro-angiogenic activities, which essential for tumor progression [25]. The angiogenesis assay showed that recombinant YKL-39 induced tube formation of HUVEC cells 6 times higher than that observed in the negative control group, and this induction was more than 60% of positive control. The chemotactic effect of YKL-39 on primary monocytes was approximately 2 times higher after 1 h and more than 5 times higher after 3 h compared to control, and this effect was comparable with the effect of MCP-1/CCL2 chemokines [25].

CLPs in cancer

YKL-40 is expressed by several types of solid tumors including breast, colon, lung, kidney, head and neck, liver, bladder, prostate, stomach, ovary, pancreas, osteosarcoma, thyroid, glioblastoma and endometrial cancers. Microarray analysis identified YKL-40 gene as one of the most overexpressed genes in glioblastoma, papillary thyroid carcinoma, and chondrosarcoma [36]. YKL-40 protein expression was found in biopsies of glioblastomas, breast cancer and colon cancer. In vitro YKL-40 was secreted by the following human cancer cell lines: osteosarcoma, glioblastoma, colon cancer, ovarian cancer, prostate cancer and malignant melanoma [37]. YKL-40 protein expression was found in tumor associated macrophages (TAM) in patients with melanoma [37]. YKL-40 protein was not expressed in small cell lung cancer cells, but YKL-40 mRNA expression was elevated in TAM [36].

In tumors, YKL-40 may contribute to the proliferation and differentiation of malignant cells, protect the cancer cells from apoptosis, stimulate angiogenesis, and regulate extracellular tissue remodeling [23]. In non-small cell lung cancer, YKL-40 may also regulate (PI3K)/AKT/mTOR pathway, which is related with cell transformation, tumor survival, invasion and metastasis, and is a central feature of EMT [23]. In breast cancer, YKL-40 levels were inversely correlated with expression of GATA3 and E-cadherin, which regulate cell-cell contacts and act as tumor inhibitors [37]. The high risk of tumor progression may be explained either by the fact that cancer cells and TAM produce YKL-40, or that chronic inflammation causes both elevated plasma YKL-40 and cancer.

In our study we showed that the elevated levels of YKL-39 expression in tumors after neoadjuvant chemotherapy (NAC) were associated with increased risk of distant metastasis and poor response to NAC in patients with nonspecific invasive breast carcinoma [25]. Moreover, in the study of gene expression of M2 macrophage markers (YKL-39 and CCL18) we found that in breast cancer patients, who received anthracycline-containing NAC, the absence of clinical response was associated with the presence of M2+ macrophage phenotype (YKL-39-CCL18+or YKL-39+CCL18-) [38]. Kavsan et al. reported the increased expression of CHI3L2 gene in glioblastoma [39]. However, there is still insufficient data on the association of both YKL-39 gene and protein level with tumor progression, and no data on SI-CLP in tumor progression are available.

YKL-40 is a marker of late stages of cancer

Elevated plasma YKL-40 was found in patients with metastatic pancreatic and ovarian adenocarcinoma [36]. In patients with gastric cancer, serum levels of YKL-40 were significantly higher compared to those observed in healthy population, and the increased YKL-40 level indicated more aggressive phenotype of tumor [40]. Plasma YKL-40 level was elevated in approximately 80% of patients with metastatic renal cell carcinoma [37]. Dupont et al. showed that serum YKL-40 was upregulated in 65% of patients with stage I and II ovarian cancer in contrast to 74-91% of patients with stage III and IV cancer [41]. In patients with small cell lung cancer, the highest percentage of the patients who had elevated serum YKL-40 level was associated with advanced disease compared to local one. More than 80% of patients with metastatic renal cell cancer and more that 40% of patients with metastatic malignant melanoma and metastatic prostate cancer had also elevated serum YKL-40. In patients with glioblastoma, the serum YKL-40 level was higher in patients with glioblastoma multiforme compared to patients with lower grade gliomas [23]. In breast cancer, increased serum levels of YKL-40 were found more frequently in patients with metastatic cancer compared to patients with early cancer [23]. YKL-40 is associated with cancer aggressiveness. It was reported that not serum but urine YKL-40 level can be helpful in the diagnosis of bladder cancer in the assistance to BTA protein. Urine YKL-40 level was significantly higher in all invasive subgroups (T1, T2–T4, and T1–T4) compared to low stage (Ta) and can help determine treatment regimen in early invasive stages [42].

YKL-40 as an independent marker of tumor progression

Serum YKL-40 as a prognostic marker was independent of serum carcinoembryonic antigen in patients with colorectal cancer, of serum CA-125 and CA15-3 in patients with ovarian cancer, of estrogen receptor status, KRAS mutation status, of serum HER2 in patients with metastatic breast cancer, of serum prostate-specific antigen in patients with metastatic prostate cancer, and of serum lactate dehydrogenase in patients with small cell lung cancer or metastatic malignant melanoma and of clinical parameters (*i.e.*, age, performance status, tumor stage, histology), indicating that serum YKL-40 reflects other pathogenic aspects of tumor progression than these tumor markers [23]. Plasma YKL-40 in pre-treatment patients was shown to be an independent prognostic biomarker of short overall survival both at time of first cancer diagnosis and at time of relapse in patients with different types of adenocarcinoma (breast, colorectal, endometrial, non-small cell lung, ovary, cervix and prostate), in patients with head and neck and cervix squamous cell carcinoma [36].

In gastric cancer, high YKL-40 protein level was an independent biomarker of short survival and was associated with tumor invasion, lymph node metastasis [43]. In patients with localized or advanced small cell lung carcinoma, high plasma YKL-40 levels before chemotherapy independently predicted short survival [44]. Pre-treatment plasma and serum level of YKL-40 was an independent prognostic biomarker in patients with metastatic prostate cancer [36]. Serum level of YKL-40 is also an independent marker for the aggressiveness of metastatic breast cancer [1]. High plasma YKL-40 in patients with metastatic colorectal cancer before treatment was associated with short progression free survival and short overall survival, independently of KRAS status [45]. However, serum concentrations of YKL-40 do not show high sensitivity for early diagnostics of cancer and YKL-40 cannot be used as a single screening marker for diagnosis of cancer [1, 23].

Elevated YKL-40 level may serve as a useful potential prognostic biomarker for cancer patients

Serum levels of YKL-40 are indicative for the poor prognosis of metastatic process. Increased plasma concentration of YKL-40 is related to poor prognosis and shorter survival in patients with breast cancer, gastric cancer, ovarian cancer, colorectal carcinoma, metastatic prostate carcinoma, melanoma and many other cancers. High plasma YKL-40 levels predicted an absolute 10-year risk of gastrointestinal cancer in patients who were >70 years and smokers [46].

For advanced pancreatic cancer it was shown that the combination of plasma YKL-40, CA-19-9 and osteopontin was more sensitive compared to CA-19-9 alone [47]. High plasma YKL-40 was a predictor of chemoresistance in patients with ovarian cancer and breast cancer during treatment [36]. In recurrent breast cancer, high serum YKL-40 was associated with metastasis and high tumor grade and was elevated mostly in case of visceral and bone metastasis and less in case of lymph nodes metastasis. Moreover, the highest serum YKL-40 levels were found in patients with more than two different metastatic sites [23].

It was found that colorectal cancer patients with elevated serum YKL-40 after surgery had significantly shorter recurrence-free period and overall survival than patients with normal serum YKL-40, indicating that YKL-40 may be useful for the monitoring of cancer patients [23].

Conclusion remarks

In the present review we shortly highlighted the main features of CLPs, their key function and their ability to contribute to tumor progression. Nowadays we have clear evidences about the correlation with survival, invasion, metastasis etc. only for YKL-40 protein. There are a lot of studies related to the YKL-40 serum levels with cancer aggressiveness and disease progression. However, many fundamental aspects regarding the function, mechanisms of action and regulation of YKL-40 as well as YKL-39 and SI-CLP in cancer remain unclear. Problems regarding the direct or indirect contribution of YKL-39 and SI-CLP to tumor progression remain to be solved.

Future translational researches combining basic and clinical basis are needed and should give the answers on the main questions: “Are CLPs useful clinical biomarker for patients with cancer?” and “Can CLPs potentially become new targets for cancer therapy?”.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Dear Readers,

You've just turned over the last page of the regular issue of Siberian Journal of Oncology. All papers accepted for publication in this issue are presented in English.

The participants of the 22-nd International Charles Heidelberger Symposium on Cancer Research, the leading researchers from Russia and other countries are the authors of these papers.

I hope that the papers presented in this issue will prove to be interesting and useful, will serve as an occasion for new scientific discoveries.

With best regards,
Prof. E.L. Choynzonov
Editor-in-Chief