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ORIGINAL ARTICLE

PROSPECTS OF DIFFERENTIAL DIAGNOSIS OF FOCAL LESION OF PANCREAS BY THE MICRORNA ASSESSMENT

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ABSTRACT

Purpose of the study. Identification of potential miRNA markers in material of focal pancreatic lesions.

Materials and methods. Samples of focal pancreatic lesions after histological evaluation were enrolled in the study including chronic pancreatitis (ChP) (*n* = 23), low-grade pancreatic intraepithelial neoplasia /PanIN-1/2 (*n* = 19), high-grade pancreatic intraepithelial neoplasia /PanIN-3 (*n* = 8), and invasive pancreatic ductal adenocarcinoma PDAC (*n* = 26). Workflow of research included the profiling of cancer-associated miRNA in pooled samples, the selection of potential marker miRNAs, the assessment of selected miRNAs expression in total collection of specimens, the identification of differentially expressed miRNAs, and the approbation of new algorithm of data interpretation via ratio of "reciprocal miRNA pair". Consequent reactions of revers transcription and quantitative teal-time PCR were used.

Results. The expression levels of miR-216a and miR-217 were decreased in the following order: PanIN-1/2 > PanIN-3 > PDAC. Moreover, miR-375 was up-regulated while miR-143 was down-regulated in the PDAC. Differential diagnostics of PDAC versus focal chronic pancreatitis might be performed with high accuracy (AUC > 0.95) by assessment panel of four molecules: miR-216a, miR-217, miR-1246 and Let-7a.

Conclusion. The assessment of microRNAs in pancreatic lesions is a promising approach for the differential diagnosis of PDAC, but this technology requires further validation with an increase in the number of samples.

Keywords: microRNA, pancreatic cancer, ductal neoplasia, chronic pancreatitis, RT-PCR, diagnostics

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Compliance with ethical standards: the ethical principles presented by the World Medical Association Declaration of Helsinki (1964, ed. 2013) were observed in the work. The study was approved by the local Ethics committee of the N. N. Petrov National Medical Research Center of Oncology (extract 27/27 from the protocol of meeting No. 1 dated 01/28/2021). Informed consent was received from all participants of the study.

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ОРИГИНАЛЬНАЯ СТАТЬЯ

ВОЗМОЖНОСТЬ ДИФФЕРЕНЦИАЛЬНОЙ ДИАГНОСТИКИ ОЧАГОВЫХ ОБРАЗОВАНИЙ ПОДЖЕЛУДОЧНОЙ ЖЕЛЕЗЫ ПУТЕМ АНАЛИЗА МИКРОРНК

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РЕЗЮМЕ

Цель исследования. Поиск потенциально маркерных молекул микроРНК в материале узловых образований поджелудочной железы.

Материалы и методы. В исследование были включены образцы ткани очаговых образований поджелудочной железы с гистологическим заключением: хронический панкреатит (*n* = 23), интраэпителиальная неоплазия low grade / PanIN-1/2 (*n* = 19) и high grade степени /PanIN-3 (*n* = 8), инвазивная протоковая карцинома (*n* = 26). В рамках работы был проведен широкий профайлинг пулов образцов разных гистологических типов, выбор потенциально маркерных микроРНК, анализ экспрессии выбранных молекул микроРНК во всех образцах, включенных в исследование, поиск статистически значимых различий между группами образцов, апробация нового алгоритма интерпретации полученных результатов путем вычисления соотношений концентраций «реципрокных пар» микроРНК. Метод анализа: обратная транскрипция с последующей количественной ПЦР в режиме реального времени.

Результаты. Уровень экспрессии молекул miR-216a и miR-217 снижается в ряду: PanIN-1/2 > PanIN-3 > протоковая карцинома ПЖ. Также в клетках инвазивной протоковой карциномы поджелудочной железы повышена экспрессия miR-375 и снижена экспрессия miR-143. Высокая точность дифференциальной диагностики (AUC > 0,95) очагов хронического панкреатита и инвазивной протоковой карциномы ПЖ может быть обеспечена с помощью панели из четырех молекул miR-216a, miR-217, miR-1246 и let-7a.

Заключение. Оценка экспрессии молекул микроРНК в материале очаговых образований ПЖ с целью дифференциальной диагностики протоковой карциномы имеет диагностический потенциал, но метод требует валидации с использованием большей коллекции биологических образцов.

Ключевые слова: микроРНК, рак поджелудочной железы, протоковая неоплазия, хронический панкреатит, ОТ-ПЦР, диагностика

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Соблюдение этических стандартов: в работе соблюдались этические принципы, предъявляемые Хельсинкской декларацией Всемирной медицинской ассоциации (World Medical Association Declaration of Helsinki, 1964, ред. 2013). Исследование одобрено локальным этическим комитетом ФГБУ «НМИЦ онкологии им. Н.Н. Петрова» Минздрава России (выписка 27/27 из протокола заседания № 1 от 28.01.2021 г.). Информированное согласие получено от всех участников исследования.

Финансирование: исследование выполнено в рамках государственного задания Министерства Здравоохранения Российской Федерации «Разработка и клиническая апробация методов диагностики и прогнозирования эффекта терапии онкологических заболеваний на основе анализа микроРНК в биопсийном материале» (№ 121032300206-4) 2021–2023 гг. и в рамках проекта по соглашению № 075-15-2021-709, уникальный (идентификатор проекта RF-2296.61321X0037), финансируемого Министерством науки и высшего образования Российской федерации.

Конфликт интересов: все авторы заявляют об отсутствии явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

In the structure of oncological morbidity in Russia, pancreatic ductal adenocarcinoma (PDAC) accounts for 3.4 % of cases, this disease ranks 11th in incidence. During the period from 2010 to 2020, there was an increase in the absolute number of incidences of PDAC from 7522 to 9275 cases. In the structure of oncological mortality of the Russian population, the proportion of diagnoses of PDAC was 6.8 %, which corresponded to the 5th position in the list of the most "lethal" diagnoses. The absolute number of deaths from PDAC in the period 2010-2020 increased from 7783 to 9625 [1]. The cumulative five-year survival rate of patients with PDAC (M/W) is 22.2 / 33.3 % – at stage I; 14.9 / 13.7 % – at stage II; 8.4 / 5.9 % – at III; 3.4 / 3.9 % – at stage IV of the first diagnosis [2]. The presented statistical data confirm the well-known fact of low "curability" of PDAC and indicate a deterioration in the epidemiological situation observed despite the development of diagnostic and therapeutic technologies.

Modern diagnostic standards are based on methods of physical examination, laboratory and instrumental research methods, such as endoscopic ultrasound (EUS), dynamic multispiral computed tomography (MSCT) and magnetic resonance imaging (MRI) of the abdominal cavity [3]. Biopsy with subsequent morphological examination of the biopsy material is recommended for all patients with suspected PDAC, while surgical intervention in some cases can be performed without morphological confirmation; the use of conservative treatment requires mandatory morphological verification. Modern diagnostic algorithms do not fully solve the problem of timely diagnosis of PDAC. The task of introducing screening for certain risk groups and improving the effectiveness of the diagnosis of PDAC seems important and involves various approaches [4], including predictive analytics methods [5], innovative liquid biopsy technologies [6; 7], high-tech methods of pathology visualization. Modern instruments allow you to combine endo-ultrasound with contrast technologies [8], elastography, fine needle aspiration biopsy [9]. Endoscopic ultrasound-guided fine needle aspiration biopsy (EUS-FNA) is gradually entering into wide clinical practice, opening up prospects, firstly, for expanding the scope of morphological analysis methods and, secondly, for developing new technologies for molecular diagnostics of biopsy material.

The main advantage of the EUS-FNA method with subsequent production of cytological smears and cell block is relatively low invasiveness. The Russian authors showed high sensitivity (93 %) and specificity (100 %) of a complex morphological study of the EUS-FNA material using the technology of manufacturing cell blocks calculated taking into account the results of subsequent histological diagnostics of the surgical material [10]. Despite the obvious advantages, the technology and effectiveness of EUS-FNA is accompanied by known objective and subjective difficulties of subsequent cytological diagnosis. A fine needle puncture allows you to obtain microfragments of the tissue of the gland formations and a cellular suspension, the effectiveness of the analysis of which is determined by the volume and structure of the aspirate. Difficulties also arise in assessing the proliferative processes of the ductal epithelium, which are observed both in areas of intraepithelial neoplasia (primarily PanIN3) and in areas of reactive hyperplasia associated with the pancreatitis; cellular signs are similar and differential diagnosis is often difficult due to the small amount of material. In contrast to the histological examination of the surgical material, it is more difficult to assess the condition of the basement membrane, the structure and non-glandular patterns of the organ in the FNA material. In this regard, additional technologies of molecular diagnostics of epithelial changes can increase the informative value of a complex morphological diagnosis.

MicroRNAs are short regulatory molecules that control the stability of protein-coding RNAs and the efficiency of protein synthesis. The transformation of the ductal epithelium of the pancreas reflects a multi-stage process of development of ductal adenocarcinoma (the dominant morphological form of PDAC); this process is accompanied by a change in the expression profile (or composition) of microRNA molecules. Therefore, the analysis of the microRNA composition in the biopsy material seems to be a promising method for diagnosing focal lesions of the pancreas, which is confirmed by the activity of research and the number of relevant publications. A search in the PubMed scientific literature database for the keywords "pancreatic cancer AND miRNA" yields more than 2.8 thousand publications. The analysis of the available scientific information

is presented in a number of review articles [11-15], but in clinical practice, there are no examples of diagnostic test systems based on microRNA analysis of the material of pancreatic lesion biopsy.

Purpose of the study: To select potential microRNA markers of PDAC, to evaluate the diagnostic significance of the method and to develop an algorithm for PCR data interpretation, based on the analysis of the expression of tumor-associated microRNAs. To evaluate the possibility and expediency of developing and introducing into clinical practice the method of differential diagnosis of PDAC pathological patterns based on the analysis of microRNA in the material of EUS-FNA.

MATERIALS AND METHODS

Patients

The study plan was approved by the local Ethics Committee of the N. N. Petrov National Medical Research Center of Oncology, extract 27/27 No. 1 dated 01/28/2021. All patients signed an informed consent to participate in the study. Before being included in the study, biological samples and clinical data were depersonalized.

The study included patients who were treated at the L. G. Sokolov Federal State Medical Center and the N. N. Petrov National Medical Research Center of Oncology. In accordance with the standards of medical care and clinical recommendations (ID:355), all patients underwent pancreato-duodenal resection or pancreatectomy. The material of the pancreatic formations was immediately placed in a buffered solution of 10 % formalin in a ratio of 1:10 and after 24 hours of fixation was cut into cassettes for further histological wiring and subsequent light microscopy. Based on histological examination of hematoxylin-stained eosin preparations, the study included material from 47 patients. In most cases, the analysis of the preparations allowed us to identify areas of tissue with different morphological structures, so 76 representative samples were prepared for further study.

Isolation of RNA

Sections with a thickness of 3–4 microns were prepared from the tissue samples, the sections were dewaxed by incubation in 1 ml of mineral oil (MP Biomedicals, USA) at 65 °C – 15 min, then the oil and paraffin were removed using two washings with 96 % ethanol. Proteolysis was carried out in 100 µl of proteinase K solution, 2 mg/ml (activity: 30 units/mg, Algimed-Techno, Belarus) at 60 °C – 1 hour. The remaining tissue after proteolysis was precipitated by centrifugation (10,000 G, 4 °C – 10 min), the supernatant (~100 µl) was transferred to a clean test tube, 200 µl of buffer was added (0.8 M sodium acetate; pH 4.0; 0.5 % octanoic acid) and 100 µl of guanidine isothiocyanate (3M), mixed, and incubated for 5 minutes at room temperature. The sample was transferred to a spin column filled with sorbent (BioSilica, Russia), washed twice with buffer for washing No. 1 (500 µl; 0.5M guanidine isothiocyanate; 10mM tris-acetate; pH 6.5; 50 % ethanol; 1 % 2-mercaptoethanol) and twice with buffer for washing No. 2 (500 µl; 75 % ethanol; 0.1M sodium chloride, 10mM tris hydrochloride; pH 7.5. RNA from the surface of the sorbent was eluted using 50 µl of an elution buffer (10mM NaHCO3, 10 mM EDTA). The concentration and quality of the isolated RNA were evaluated using a NanoDrop 2000C spectrophotometer (Thermo Scientific, USA).

"Profiling" of tumor-associated microRNAs

In order to select potentially marker molecules, the expression analysis (profiling) of cancer-associated 85 microRNAs was carried out. For this purpose, "pools" were formed, which were mixtures of RNA samples of similar morphology: chronic pancreatitis (ChP), intraepithelial neoplasia of the pancreas (PanIN), invasive ductal adenocarcinoma of the pancreas (PDAC). The analysis was carried out using a set of reagents manufactured by Exiqon (miScript II RT Kit, miRCURY LNA miRNA Cancer-Focus PCR Panel) the analytic procedure involved poly-adenylation reaction, reverse transcription (RT) of all RNA molecules using a poly-T primer followed by real-time quantitative PCR reaction for 85 microRNA molecules. The intensity of amplification was evaluated using the DNA intercolating reagent SYBR-green. All reactions were carried out in accordance with the manufacturer's protocol on the CFX96 Touch™ amplifier (Bio-Rad, USA).

Analysis of individual microRNA molecules expression

The expression of individual microRNA molecules in the material of each sample (n = 76) was analyzed by RT-PCR using kits manufactured by

Algimed-Techno (Belarus). The set for the analysis of each microRNA involved conducting a "two-tailed" microRNA-specific reverse transcription reaction using an RT-primer, the "flanks" of which complementarily annealed on the 3' and 5' ends of the microRNA molecule, orienting "towards" each other. This technology of priming the reverse transcription reaction ensured its high specificity [16]. Then, a real-time quantitative PCR reaction was performed using two microRNA-specific PCR primers. The amplification intensity was evaluated using a fluorescently labeled PCR probe. All RT-PCR reactions were carried out in accordance with the manufacturer's protocols, each analysis was performed in three repeats on the CFX96 Touch™ amplifier (Bio-Rad, USA).

Statistics

Normalization of the "profiling" data was carried out according to the manufacturer's recommendations: the results of the analysis of each pooled sample were normalized versus averaged Ct values of the three reference molecules. Before comparing the results of the analysis of individual samples (pools), normalization was carried out versus "inter-plate" calibrators. The molecules for subsequent analysis were selected by simply comparing the normalized Ct values.

Normalization of the results of the individual molecules (n = 24) analysis in individual samples (n = 76) was carried out in two alternative ways: relative to the averaged Ct values of all analyzed samples (24 × 76 = 1842), so called total Ct, and relative to one of the 24 studied molecules, which was characterized by the most stable level of expression in the studied samples. To select the optimal normalizer, the stability (or variability) of the expression of the analyzed molecules was evaluated using the NormFinder algorithm [17]. Normalization was carried out according to the standard method of counting (formula 1):

$$dCt=2^{Ct(miRNA)-Ct(normalizer)}$$

The statistical significance of the difference in the expression levels of individual microRNAs in the compared groups was assessed using the nonparametric Mann-Whitney U-test. The prognostic significance of each molecule was evaluated using ROC analysis (constructing a ROC curve and calculating the AUC value).

We also searched for optimal ratios of the relative concentrations of the so-called "reciprocal pairs" of microRNA molecules (molecules with multidirectional and associated with the process of neoplastic transformation changes in expression levels). A software algorithm has been developed for the automated solution of this problem. Reciprocal pairs were formed as all possible combinations of 24 tested microRNAs. The total number of analyzed pairs, determined by the formula 2:

$$P_n^r = \frac{n!}{(n-r)! \times r!}$$

(where P – quantity of unique miRNAs combinations, n – quantity of analyzed miRNAs (24), r – quantity of miRNA in any particular combination (2)).

Then, for each mRNAs pair, the amplification efficiency ratio parameter (Ratio miR-1/miR-2) was determined by the formula 3:

$$dCt=2^{Ct(miR-1)-Ct(miR-2)}$$

The evaluation of the diagnostic significance of Ratio miR-1/miR-2 parameters was evaluated similarly to the significance of individual molecules: using ROC analysis and calculating the AUC value.

RESEARCH RESULTS

Research design, sample preparation

In the practice of analyzing the material of EUS-FNA focal formations of the pancreas, differential diagnosis between conditions having similar clinical and sonographic patterns is of clinical importance: chronic pancreatitis (ChP), intraepithelial lesions of varying degrees of malignancy (pancreatic intraepithelial neoplasia, PanIN), and invasive ductal adenocarcinoma (pancreatic ductal adenocarcinoma, PDAC). Taking into account the task of searching for markers of differential diagnosis of the listed conditions, the research plan assumed the sequential implementation of the following stages: 1) histological analysis of pancreatic pathology (surgical material), 2) selection and microdissection of sites with the appropriate morphological pattern and isolation of RNA, 3) formation of "pools" of RNA from samples of typical morphology and analysis of 85 tumor-associated microRNAs ("profiling") in these pools, 4) selection and analysis of potentially marker

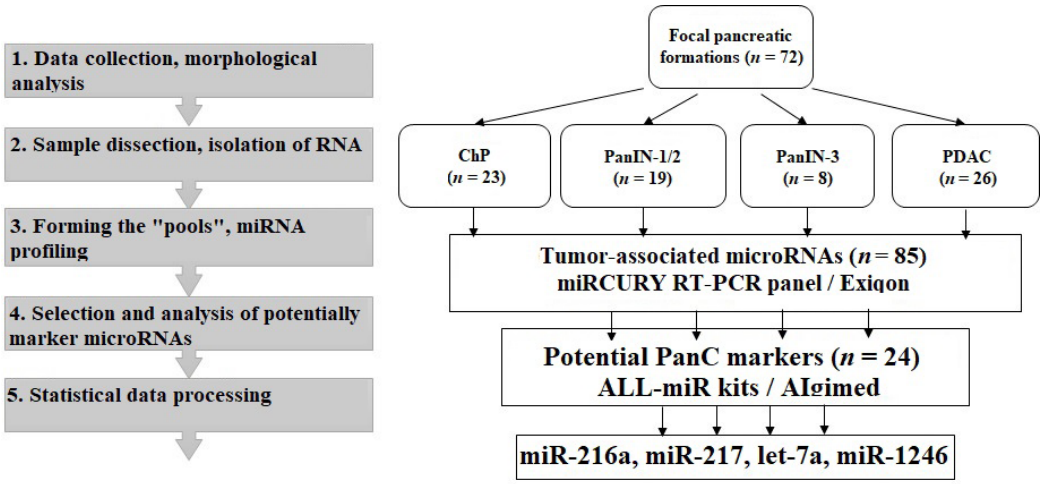

Fig. 1. Study design.

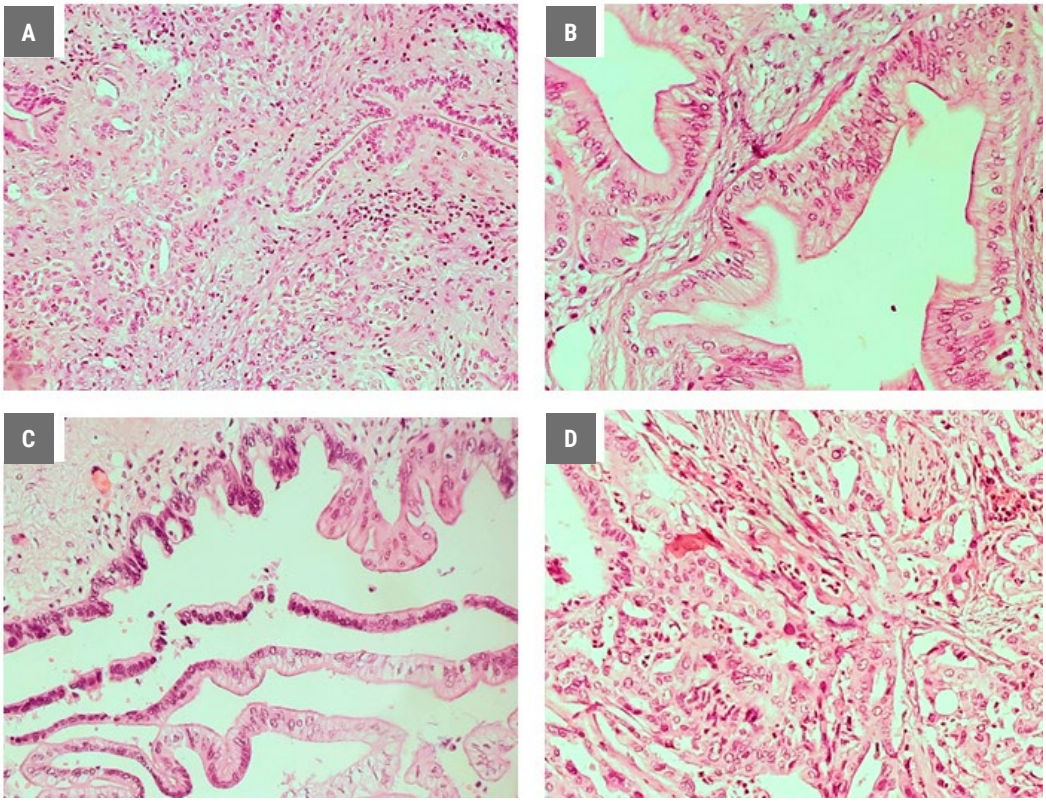

Fig. 2. Representative examples of sites of pathological formations of the pancreas.
Note: A – fibrosing chronic pancreatitis (ChP): the structure of the gland with pronounced structural changes, loss of acinar cells, pronounced fibrosis with inflammatory infiltration, compressed deformed ducts and areas of proliferation of small ducts. B – intraepithelial lesion (PanIN1/2): small pancreatic ducts lined with a single–layer cylindrical ductal epithelium with predominantly basally located nuclei (PanIN1), as well as areas with signs of pseudostratification – nuclei located at different levels (PanIN2). B – intraepithelial lesion (PanIN3): a large pancreatic duct lined mainly with cells with cellular and nuclear atypia, high nuclear-cytoplasmic ratio, hyperchromia of the nuclei, uneven nuclear contours, loss of polarity of the nuclei, mitosis figures, partly foamy-gland pattern (foamy cells), partly with mucus formation and oncocytic differentiation. Atypical cells are located within the basement membrane. G – invasive ductal adenocarcinoma (PDAC) with widespread perineural invasion. Staining in all cases with hematoxylin-eosin.

Table 1. The "profile" of 18 potentially marker microRNA molecules expression in normal pancreatic tissue and cells of focal formations of various morphologies

No.	microRNA	Relative expression level in pathological sample (miRNA Cancer-Focus PCR Panel)			Relative expression level in intact pancreatic cells (miRNATissueAtlas2)
		ChP	PanIN1/2/3	PDAC	
1	hsa-let-7a-5p	9.13	14.41	7.80	512.4
2	hsa-miR-10b-5p	0.50	0.40	0.60	111.5
3	hsa-miR-15a-5p	0.80	1.29	0.82	143.6
4	hsa-miR-23a-3p	6.96	4.43	5.21	462.7
5	hsa-miR-24-3p	10.03	7.67	8.31	177.2
6	hsa-miR-26b-5p	2.22	2.73	1.70	415.5
7	hsa-miR-27a-3p	10.25	9.31	7.08	229.1
8	hsa-miR-29c-3p	2.21	5.91	2.86	1298.3
9	hsa-miR-125b-5p	32.56	17.24	17.46	426.3
10	hsa-miR-126-3p	13.02	7.50	5.39	532.6
11	hsa-miR-141-3p	2.99	10.13	3.02	82
12	hsa-miR-143-3p	3.20	3.92	6.22	94.6
13	hsa-miR-145-5p	23.79	12.19	23.60	509.2
14	hsa-miR-146b-5p	0.51	0.80	0.61	82.1
15	hsa-miR-155-5p	0.32	0.39	0.28	74.7
16	hsa-miR-192-5p	1.38	4.25	4.08	24.6
17	hsa-miR-200a-3p	0.38	1.01	0.90	22.8
18	hsa-miR-200c-3p	7.56	10.96	5.16	17.4

Table 2. The list of molecules additionally included in the study

No.	microRNA	Relative expression level in intact pancreatic cells (miRNATissueAtlas2)	Literature sources
1	hsa-miR-375-3p	20.4	[19]
2	hsa-miR-451a-5p	4650.4	[13]
3	hsa-miR-1246	3416.7	[20]
4	hsa-miR-1290	886.3	[21]
5	hsa-miR-216a-5p	42.3	[22]
6	hsa-miR-217-5p	20.1	[23]

mircoRNA molecules, 5) statistical processing of the results. The design of the study is schematically presented in Figure 1.

The samples of ChP, when selected on the area of the entire slides, did not contain tumor tissue, the morphological picture varied from mild inflammatory infiltration with moderate fibrosis (both intra-lobular and periductal) to a pronounced inflammatory reaction with areas of fibrosing Ch P. The PanIN1/2 group included samples of intraductal papillary mucinous neoplasia (intraductal papillary mucinous neoplasm, IPMN) and mucinous cystic tumors (mucinous cystic neoplasm, MCN) with a low degree of dysplasia. Specimens of this group could include small fragments of normal pancreatic tissue. The PanIN3 group included images of severe dysplasia, including cases of IPMN and MCN, the presence of PDAC sites was excluded. The PDAC group was represented by typical histotypes and two cases of mucinous cystadenocarcinoma with moderate or low degree of differentiation. This category can contain small fragments of the PanIN3 and PDAC combination.

During light microscopy, zones with the corresponding morphological pattern were identified, microdissection of selected tissue fragments was performed for subsequent RNA isolation. In some cases, material of different morphology was obtained from the same sample and categorized in different groups. As a result, the following samples were included in the study: ChP (*n* = 23), PanIN1/2 (*n* = 19), PanIN3 (*n* = 8), PDAC (*n* = 26). Representative examples of sites with a characteristic morphological pattern selected for the study are shown in Figure 2.

Selection of potentially "marker" microRNAs

Total RNA was isolated from all samples. There was a large variation in RNA concentrations (0.5-350 ng/μl), which could be a result of tissue heterogeneity and/or different degrees of cellular RNA integrity. RNAs isolated from histologically similar samples were combined in equal mass ratio into so-called "pools" for preliminary analysis of the expression profile of potentially marker molecules. This approach makes it possible to conduct an inexpensive comparative analysis of groups of samples, but without the possibility of assessing the statistical significance of the results obtained. The analysis made it possible to assess the difference between three conditions: chronic pancreatitis (ChP), intraepithelial neoplasia of any degree (PanIN1/2/3) and invasive ductal adenocarcinoma of the pancreas (PDAC). MicroRNA molecules with relatively high and significantly different levels of expression in three groups were selected for subsequent analysis. An additional selection criterion was information on the previously estimated expression of these molecules in the pancreatic cell, presented

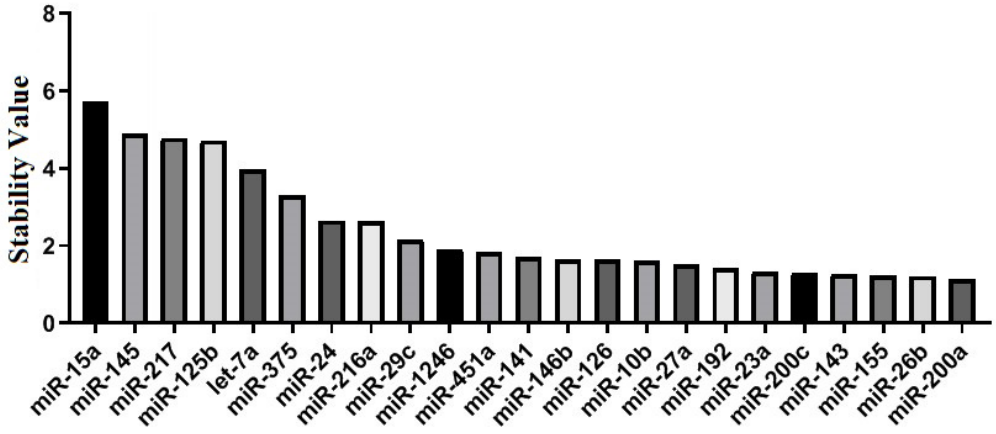

Fig. 3. Results of evaluation of expression variability of microRNA molecules (n.23) in the analyzed samples (n.76) using the NormFinder algorithm.
Note: the NormFinder algorithm [17] allows ranking the analyzed molecules according to the level of stability/variability of their expression in the group of samples under study. The stability coefficient = 1 corresponds to a molecule whose expression level is identical in all samples, such molecules can be used as normalizers. As part of the experiment, the most stable expression level was shown for miR-200a.

in the miRNATissueAtlas2 database [18]. A total of 18 molecules were selected. Table 1 presents normalized expression levels of selected molecules in samples of three variants of pathological focal formations: ChP, panIN1/2/3, PDAC and data on the expression of these molecules in cells of normal pancreatic tissue (according to miRNATissueAtlas2). It should be noted that the comparison of the presented results of the analysis of groups of pathological and normal samples is not adequate, because these are the results of different studies performed by different methods. But a comparative assessment of different molecules within a certain study can be informative. In general, the presented results suggest that the selected 18 molecules are indeed actively expressed in pancreatic cells, and the formation of focal formations by chronic inflammatory reaction or malignancy is probably associated with a change in their expression and functional activity.

An analysis of previous studies has shown that a number of mircoRNA molecules not included in the miRCURY LNA miRNA Cancer-Focus PCR Panel play an important role in the development of PDAC and may have diagnostic potential; they (n = 6) were also included in the study. The level of expression of these molecules in normal pancreatic tissue according to "miRNATissueAtlas2" and links to relevant studies are presented in Table 2. Thus, a "panel" of 24 potentially marker microRNA molecules was formed.

Table 3. The results of a comparative analysis of the expression of potentially marker microRNAs in samples of focal formations of pancreas of different morphology

MicroRNA	ChP (n = 23)	PanIN1/2 (n = 19)	PanIN3 (n = 8)	PDAC (n = 26)	Kruskal-Wallis test
hsa-miR-143-3p	0.1	0.16	0.09	0.04	0.0007 ***
hsa-miR-217-5p	0.87	0.42	0.28	0.07	0.001 ***
hsa-miR-216a-5p	1.48	0.84	0.83	0.38	0.0032 **
hsa-miR-375-3p	0.2	0.32	0.13	0.48	0.0137 *
hsa-miR-200c-3p	0.18	0.23	0.24	0.10	0.0259 *
hsa-miR-1246	0.07	0.09	0.09	0.03	0.0336 *
hsa-miR-155-5p	0.4	0.38	0.31	0.22	0.1098
hsa-miR-146b-5p	1.4	2.56	2.5	0.84	0.1131
hsa-miR-26b-5p	0.42	0.23	0.58	0.22	0.1485
hsa-miR-192-5p	0.5	0.38	0.19	0.29	0.1538
hsa-miR-125b-5p	0.09	0.17	0.34	0.20	0.2127
hsa-miR-451a-5p	0.11	0.12	0.04	0.52	0.2323
hsa-miR-29c-3p	0.84	5.97	17.65	0.62	0.2599
hsa-miR-24-3p	0.09	0.1	0.12	0.09	0.3167
hsa-let-7a-5p	0.34	0.31	0.27	0.24	0.3674
hsa-miR-126-3p	0.96	1.05	0.66	1.00	0.4304
hsa-miR-10b-5p	51.04	70.6	50.47	74.01	0.4911

Analysis of potentially marker molecules

The expression level of each of the selected 24 molecules was assessed using a microRNA-specific two-flank reaction and subsequent PCR in each sample included in the study. The specificity of RT was provided by an RT primer having two flanks for binding to a microRNA molecule. As a result of the RT reaction, a complementary DNA molecule was synthesized, both flanks of which had microRNA-specific sites for binding to PCR primers. The possibility of assessing microRNA expression levels and the high specificity of this approach were shown earlier [24]. On the base of obtained result, miR-1290 was excluded in further analysis since expression level of this molecules appeared under the limit of detection of used method in majority of samples.

In order to determine the optimal normalizer of RT-PCR data, the variability of expression of each of the 23 molecules was evaluated using the NormFinder algorithm, the results are shown in Figure 3.

The most stable expression level (stability value → 1) was shown for microRNA-200a, which was later used as a normalizer. An alternative normalization method involves calculating the averaged (total) Ct value of all molecules tested in all samples (76 samples × 24 molecules = 1824 values in total), and normalizing each particular results relative to this value [25]. The results of RT-PCR were normalized by two methods: relative to the value of total Ct and relative to Ct (miR-200a) for each individual sample. The normalized values were combined into groups (ChP, PanIN 1/2, PanIN 3, PDAC), which made it pos-

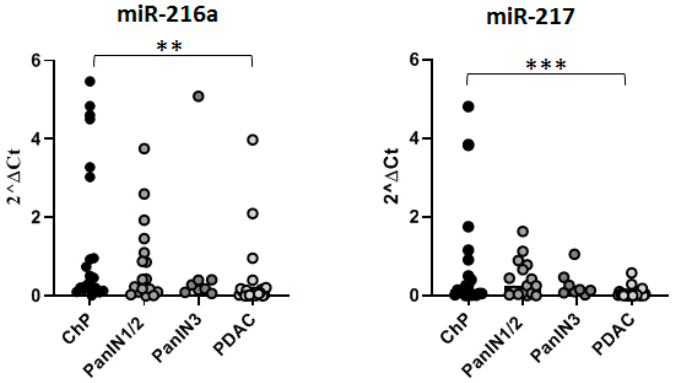

Fig. 4. Comparative analysis of miR-216a and miR-217 expression levels in tissue samples of pancreatic focal formations of various histological structures.
Note: groups of samples of chronic pancreatitis / ChP (n = 23), mild intra-epithelial neoplasia / PanIN-1/2 (n = 19), and severe / PanIN-3 (n = 8), ductal carcinoma / PDAC (n = 26). The statistical significance of the observed difference was estimated by calculating the Kruskal-Wallis criterion (** p < 0.005; *** p < 0.0005).

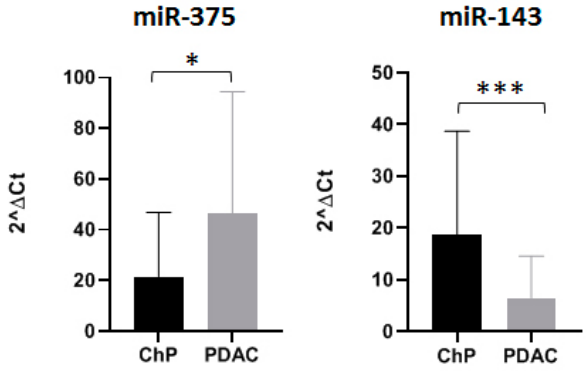

Fig. 5. Comparative analysis of miR-375 and miR-143 expression levels in the tissue of foci of chronic pancreatitis and PDAC.
Note: The graph represents arithmetic averages for a group of samples of chronic pancreatitis /ChP (n = 23) and ductal carcinoma/PDAC (n = 26). The statistical significance of the observed difference was estimated by calculating the Mann-Whitney criterion (* p < 0.05; *** p < 0.0005).

sible to evaluate the difference in the expression of individual molecules in the studied groups of samples. For a number of molecules, contradictions were observed between the results obtained using different normalization methods. Such molecules were excluded from the further analysis. Only those results that were similar with different normalization methods, i. e. minimally dependent on them, were taken into account; there were 17 of 24 such molecules.

The next step was to evaluate the statistical significance of the difference in the expression levels of individual microRNAs between clinical groups. The results are presented in Table 3.

For a number of molecules, a statistically significant change in expression activity was observed in the PanIN-1/2 – PanIN-3 – PDAC series. For example, an increase in the severity of ductal epithelial dysplasia was accompanied by a decrease in the expression level of miR-216a and miR-217 (Fig. 4).

The obtained results suggest that the low expression of these molecules in the material of the EUS-FNA of the focal lesion of the pancreas may be a marker of its malignant nature. Statistically significant difference between the studied groups was obtained for a number of molecules: miR-143, miR-375, miR-200c and miR-1246.

Interestingly, when comparing groups of ChP and PDAC samples, without taking into account the data obtained for samples of intraepithelial lesions of varying degrees of malignancy (PanIN1/2/3), an increase

Table 4. Indicators of efficacy of differential diagnosis of chronic pancreatitis (ChP) versus PDAC by means of individual microRNAs assessment

MicroRNA	AUC	Sens.	Spec.	PPV, %	NPV, %	Accuracy, %
hsa-miR-143-3p	0.75	70.59	82.61	79.17	75.00	77.50
hsa-miR-216a-5p	0.81	76.19	78.26	78.26	76.19	77.27
hsa-miR-217-5p	0.81	76.19	78.26	78.26	76.19	77.27
hsa-miR-375-3p	0.78	71.43	71.43	71.43	71.43	71.43

Table 5. Indicators of effectiveness of chronic pancreatitis (ChP) and PDAC differential diagnosis based on the assessment of the concentration ratios of microRNA "reciprocal pairs"

MicroRNA pair	AUC	Sens.	Spec.	PPV, %	NPV, %	Accuracy, %
miR-1246/miR-217	0.95	95.24	82.61	95.00	83.33	88.64
miR-1246/miR-216a	0.95	90.00	91.30	91.30	90.00	90.70
miR-1246/miR-375	0.81	85.00	73.91	85.00	73.91	79.07
miR-143/miR-216a	0.83	81.25	82.61	86.36	76.47	82.05
let-7a/miR-216a	0.97	94.44	94.74	94.74	94.44	94.59
miR-155/miR-217	0.93	88.89	90.48	90.48	88.89	89.74
let-7a/miR-217	0.96	88.24	100.00	90.48	100.00	94.44
miR-155/miR-216a	0.91	83.33	90.48	86.36	88.24	87.18
miR-192/miR-216a	0.93	83.33	100.00	87.50	100.00	92.31
miR-192/miR-217	0.91	80.95	95.24	83.33	94.44	88.10
miR-143/miR-217	0.85	68.75	95.65	81.48	91.67	84.62
miR-200c/miR-216a	0.88	80.95	78.26	81.82	77.27	79.55
miR-200c/miR-217	0.88	75.00	78.26	78.26	75.00	76.74
miR-451a/miR-216a	0.81	80.00	78.26	81.82	76.19	79.07
miR-451a/miR-217	0.82	85.71	73.91	85.00	75.00	79.55

in the expression level of miR-375 and a decrease in the expression level of miR-143 associated with the process of chronic inflammation was observed (Fig. 5).

In order to assess the diagnostic value of the individual potentially marker microRNAs (miR-143, miR-217, miR-216a, miR-375) for differential diagnostic of ChP and PDAC, a ROC analysis and calculation of standard indicators were carried out. The results presented in Table 4 and Figure 6 indicate a high diagnostic potential of these markers.

Differential diagnosis algorithm

Normalization of the results of RT-PCR analysis of microRNA and clinically applicable interpretation of the results of such analysis is a non-trivial task. One of the possible approaches is the search for molecules with a reciprocal (opposite) trend of tumor-associated expression changes and the evaluation of the ratio of concentrations of such molecules in tested samples. The effectiveness of this approach has been proven earlier: the values of such expression ratios may have a higher diagnostic potential than the diagnostic values of individual molecules [26].

As part of this work, a previously developed computational algorithm was used to search for promising "reciprocal pairs" of microRNAs. So, this algorithm assumed the selection of all possible pairs of microRNA molecules, calculation of their concentration ratios (miR-1/miR-2 Ratio) and ROC analysis of these parameters as markers of differential diagnosis of ChP vs. PDAC. Thus, diagnostic values were estimated for 276 pairs of molecules, including AUC, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy. The results presented in Table 5 demonstrated the high diagnostic potential of many "reciprocal pairs" of microRNAs (AUC > 0.8).

From the data in the table, it can be seen that the composition of "reciprocal pairs" includes molecules

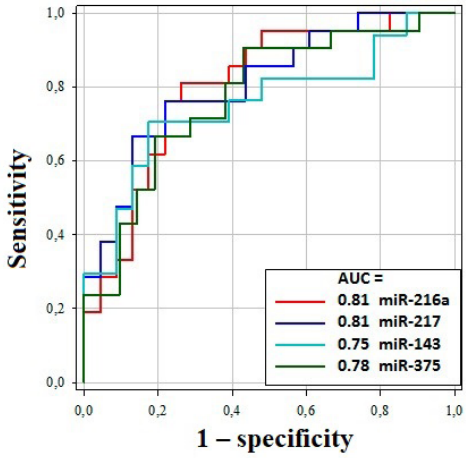

Fig. 6. Evaluation of individual microRNA diagnostic value in the framework of differential diagnosis of chronic pancreatitis (ChP) and PDAC.
Note: The results of the ROC (Receiver operating curve) analysis and the values of the area under the AUC (area under the curve) curves for four molecules are presented.

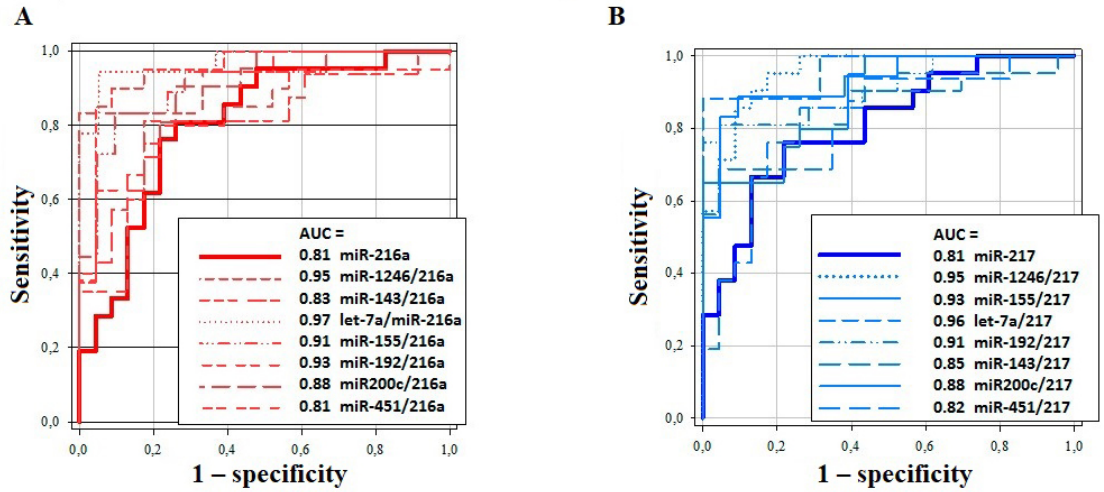

Fig. 7. Evaluation of the diagnostic value of the analysis of "reciprocal pairs" of microRNA molecules in the framework of differential diagnosis of chronic pancreatitis (ChP) and PDAC.
Note: A – the results of the evaluation of the diagnostic potential of miR-216a and several "reciprocal pairs" formed by this molecule. B – the results of the evaluation of the diagnostic potential of miR-217 and several "reciprocal pairs" formed by this molecule. The results of the ROC (Receiver operation curve) analysis and the values of the area under the AUC (area under curve) curves are presented.

with a relatively high diagnostic potential as individual markers. However, the ratio of the concentration of these molecules and molecules with a "reciprocal" expression behavior have significantly higher diagnostic value. Figure 7 shows the ROC curves confirming this conclusion for the "reciprocal pairs" that form the miR-216a and miR-217 molecules.

Thus, the AUC value for miR-216a is 0.81, and for the concentration ratios of this molecule and miR-192, miR-1246 or let-7a, the AUC values increase to 0.93, 0.95 and 0.97, respectively. Similarly, the AUC value for miR-217 is 0.81, and for the concentration ratios of this molecule and miR-155, miR-1246 or let-7a, the AUC values increase to 0.93, 0.95 and 0.96, respectively. In general, the analysis of a "panel" of four molecules, miR-216a, miR-217, miR-1246 or let-7a, provides the ability to calculate four parameters for the differential diagnosis of chronic pancreatitis and PDAC, and the AUC value of these parameters is in the range of 0.95–0.97, sensitivity: 0.88–0.95, specificity: 0.82–1.

DISCUSSION

The article presents the results of a pilot study conducted to assess the prospects for the development of a method for differential diagnosis of focal pancreatic pathology based on microRNA analysis in biopsy material. The materials of postoperative histological examination were used in the work. The authors suggest that the obtained results will serve as the basis for the development of an innovative method of molecular analysis of the EUS-FNA material, which will be able to complement and increase the diagnostic potential of a standard morphological study.

The study analyzed the expression of 85 cancer-associated microRNA molecules in the material of 76 samples obtained from 47 operated patients. Histological examination made it possible to form four groups of samples corresponding to the diagnoses: chronic pancreatitis (ChP), mild pancreatic intraepithelial neoplasia (PanIN-1/2), severe intra-epithelial neoplasia (PanIN-3) and the most common form of pancreatic cancer (invasive ductal adenocarcinoma, PDAC). MicroRNA expression analysis revealed statistically significant differences in expression levels of miR-143, -217, -216a, -375, -200c, -1246 between assayed groups of samples. The results obtained have both fundamental and applied significance.

Involvement of miR-217 [23], -216a [22], -375 [19], -1246 [20] in the process of malignant transformation of the ductal epithelium of the pancreas was shown earlier; these data were confirmed by the our results. Suppression of miR-143 in PDAC cells is an interesting finding, which is also in a line with the scientific literature. Thus, a comparative analysis of normal pancreatic tissue and PDAC, conducted by Chinese researchers using the material of 37 patients, showed similar results to ours [27]. In addition, ectopic expression of this molecule in various pancreatic cancercell lines (MIA PaCa-2 and PANS-1) had a therapeutic effect, inhibiting cell proliferation and metastatic potential [27; 28]. *In situ* experiments have shown that a decrease in the concentration/functional activity of miR-143 in PDAC cells is associated with activation of the expression of a number of oncogenes (ARHGEF1 (GEF1), ARHGEF2 (GEF2), K-RAS), which confirms the therapeutic potential of synthetic analogues of this molecule.

No statistically significant changes in the expression of miR-1246 were detected in our study, but the ratio of the concentration of this molecule and the concentration of miR-216a or miR-217 showed a high diagnostic potential. The results obtained indicate the possibility of over-expression of this molecule in PDAC cells, and this process is associated with inhibition of miR-216a or miR-217 expression. Whether this association is random or biologically justified event is still unknown. The analysis of scientific literature does not form an unambiguous idea of the participation of miR-1246 in the development of PDAC. A number of publications show the diagnostic potential circulating in the plasma miR-1246 [6; 29], but what is the relationship between the concentration of this molecule in plasma and its role in PDAC cells is not clear. The analysis of the biogenesis of this molecule in tumor cells was recently carried out by a group of American researchers [30]. It has been shown that miR-1246 is a product of degradation of small nuclear RNA (RNU2-1), which functions as part of the nuclear complex of the spliceosome. In this case, miR-1246 is a product of a non-canonical microRNA synthesis pathway, and an increase in intracellular concentration and/or secretion of this molecule by PDAC cells may not be related to its role in the process of post-transcriptional regulation of target genes expression. Our results shown that the expression of the let-7a molecule has a "reciprocal"

character relative to the expression of miR-216a and miR-217. The biological meaning of this phenomenon remains still unknown, but it can be used for clinically acceptable interpretation of RT-PCR results. In general, the results obtained complement the existing ideas about the role of individual microRNA molecules in the development of PDAC.

The practical value of the results obtained is important as well. We achieved to demonstrate, firstly, the technical possibility of isolating and analyzing microRNAs in biopsies of pancreatic formations using domestic reagents, and, secondly, the diagnostic potential of such an analysis. In terms of developing a technology suitable for clinical use, additional efforts should be made (1) to develop a method for preserving the EUS-FNA material and subsequent isolation of small RNAs and (2) to determine the optimal (minimum) set of marker microRNAs for inclusion in the diagnostic "panel" for differential diagnosis of focal pancreatic lesion. In order to ensure high and, most importantly, reliable indicators of the diagnostic significance of the test system, an important aspect is the development of an algorithm for interpreting RT-PCR data. This issue was successfully solved previously in the frame of test-system for the assessment the severity of cervical epithelial dysplasia [31]. Thus, the following analytical algorithm seems optimal: semi-quantitative analysis of a set of microRNA molecules with reciprocal (opposite) PDAC-associated expression changes, calculation of concentration ratios of "reciprocal pairs" of microRNAs, determination of the significance of each such parameter using a machine learning algorithm and calculation of the final diagnostic criterion reflecting the risk of pancreatic malignancy. After evaluating diagnostic parameters on a large collection of biopsy material, the developed test system can be registered as a medical device and offered for practical use.

CONCLUSION

In presented study we identified several potentially marker microRNA molecules and shown that the assessment of their expression in the sample of focal pancreatic formations is a promising diagnostic approach.

The development of a reliable and clinically-applicable diagnostic technology will require continued research in two main areas: (1) expanding the panel of marker molecules and developing an algorithm for their complex analysis, and (2) validating the diagnostic potential of the new technology using a larger collection of biological samples and developing approaches to clinically convenient and effective interpretation of the RT-PCR results.

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Vorobyev S. L. – the concept of the study, the organization of the laboratory part of the work, including the preparation and conduct of histological studies, scientific editing of the text;

Malek A. V. – concept of the study, organization of the laboratory part of the study, including isolation and analysis of miRNAs, analysis of RT-PCR results, final conclusions, preparation of illustrations, writing of the original text.