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Primary and Secondary micro-RNA Modulation the Extrinsic Pathway of Apoptosis in Hepatocellular Carcinoma

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Abstract—One of the most common malignant liver diseases is hepatocellular carcinoma, which has a high recurrence rate and a low five-year survival rate. It is very heterogeneous both in structure and between patients, which complicates the diagnosis, prognosis and response to treatment. In this regard, an individualized, patient-centered approach becomes important, in which the use of mimetics and hsa-miRNA inhibitors involved in the pathogenesis of the disease may be determinative. From this point of view hsa-miRNAs are of interest, their aberrant expression is associated with poor prognosis for patients and is associated with tumor progression due to dysregulation of programmed cell death (apoptosis). However, the effect of hsa-miRNA on tumor development depends not only on its direct effect on expression of genes, the primary targets, but also on secondary targets mediated by regulatory pathways. While the former are actively studied, the role of secondary targets of these hsa-miRNAs in modulating apoptosis is still unclear. The present work summarizes data on hsa-miRNAs whose primary targets are key genes of the extrinsic pathway of apoptosis. Their aberrant expression is associated with early disease relapse and poor patient outcome. For these hsa-miRNAs, using the software package ANDSystem, we reconstructed the regulation of the expression of secondary targets and analyzed their impact on the activity of the extrinsic pathway of apoptosis. The potential effect of hsa-miRNAs mediated by action on secondary targets is shown to negatively correlate with the number of primary targets. It is also shown that hsa-miR-373, hsa-miR-106b and hsa-miR-96 have the highest priority as markers of hepatocellular carcinoma, whose action on secondary targets enhances their anti-apoptotic effect.

Keywords: microRNA, primary and secondary targets, ANDSystem, hepatocellular carcinoma, programmed cell death, extrinsic pathway of apoptosis, regulatory pathways

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INTRODUCTION

MicroRNAs (miRNAs) are a class of small, endogenous, single-stranded, non-coding RNAs ~18−25 nucleotides in length that regulate the expression of their target genes through a variety of mechanisms, including mRNA degradation and translational inhibition through both direct binding to a complementary sequence in the 3'-untranslated region (3'UTR) mRNA [1−5] and regulation of gene expression mediated by regulatory pathways. microRNAs are involved in the regulation of many processes, including development, differentiation, cell survival, and functioning of the immune system [6−8]. The database hsa-miRTarBase (https://miRTarBase.cuhk.edu.cn/) has experimental data on the participation of more than 4000 dif-ferent miRNAs in the regulation of the activity of more than 27 thousand target genes in 37 species [9]. In man miRNA expression disorders are associated with various diseases: allergic diseases, cardiovascular diseases, respiratory diseases, diabetes mellitus, bronchial asthma [10−14], and oncological diseases [15−17].

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. Worldwide about 750000 people die from HCC every year [18]. HCC, which has a high recurrence rate and low five-year survival, is characterized by significant structural and interindividual heterogeneity, which complicates diagnosis, prognosis, and response to treatment. In this regard, an individual, patient-oriented approach is important. The use of miRNA mimetics and inhib-

itors, which play a significant role in the regulation of the expression of target genes involved in the pathogenesis of HCC, may be decisive [19−22]. From this point of view, of greatest interest are miRNAs whose high or low expression is associated with tumor proliferation. It should be noted that in the context of the development and progression of malignant tumors, miRNAs targeted to tumor suppressor genes function as oncogenes. If the aberrant expression of such onco-miRNAs is associated with early HCC recurrence and poor patient outcome, these miRNAs are of interest for use as biomarkers and therapeutic targets.

As a promising target for the development of new anticancer drugs, the inexternal pathway of apoptosis induced through cell death receptors, a separate a subgroup of the tumor necrosis factor (TNF) receptor family (see review [23]), is of interest. The most well studied CD95 receptor is (Fas/APO-1). Activation of this pathway is associated, among other things, with DNA damage and replicative stress, leading to dysregulation of cell division and the development of HCC. This pathway is realized through the interaction of FADD (Fas-associated DD-protein), cFLIP (cellular FLICE inhibitory protein) and procaspase-8, leading to the formation of the DISC (Death Inducing Signaling Complex) macromolecular complex, which ensures the activation of the external signaling pathway of apoptosis through the formation of active caspase-8 [23−26] and further activation of the caspase cascade involving caspase-3 and -7. Data on miRNAs, whose aberrant expression in HCC is directly related to impaired activity of the external apoptosis pathway and with a poor prognosis, are summarized in this work. However, the effect of miRNAs on tumor development depends not only on their direct effect on primary targets—gene expression of the external pathway of apoptosis, but also on secondary targets mediated by regulatory pathways. The mechanisms of modulation of the extrinsic apoptosis pathway in HCC through the action of miRNAs on primary targets are being actively studied, while little is known about the role of secondary targets. To solve this issue, we used the software and information system ANDSystem, developed by us earlier, designed for the reconstruction of gene networks based on information extracted from factographic databases or obtained by automatic analysis of the texts of scientific publications [27−30]. ANDSystem is widely used for the reconstruction and analysis of gene networks. In particular, the ANDSystem was used to reconstruct the preeclampsia association [31], identify new genes associated with susceptibility to tuberculosis [32], reconstruct and analyze the hepatitis C virus interact [33], in the search for new candidate genes potentially associated with asthma comorbidity and hypertension [34], and programmed cell death upon SARS-CoV-2 infection was analyzed [35].

In this work, ANDSystem was used to reconstruct and analyze the regulatory pathways that describe the effect of onco-miRNAs on the activity of key genes of the external apoptosis pathway, which are both primary targets of some and secondary targets of other miRNAs. It turned out that the expected modulating anti-apoptotic effect of these miRNAs on primary targets can be either enhanced or weakened by their action on secondary targets according to the reconstructed regulatory pathways. It has been shown that the expected potential effect of indirect modulation of the external pathway of apoptosis due to the regulation of the expression of secondary target genes correlates negatively with the number of miRNA primary targets: the more miRNA primary targets, the weaker the total anti-apoptotic effect of action on secondary targets.

EXPERIMENTAL

Reconstruction of molecular genetic pathways. Molecular genetic pathways for describing the effect of miRNAs on the expression of secondary target genes were reconstructed using the ANDSystem software and information system [26−29]. ANDSystem contains a global gene network that describes the interactions between objects of the ANDSystem ontology, for the formation of which a mass analysis of PubMed abstract texts and PubMed Central full-text articles was carried out. In total, ANDSystem contains 13 types of different objects (proteins, genes, metabolites, diseases, miRNAs, etc.) and 24 types of different interactions (physical interactions, regulation of expression, regulation of activity, regulation of stability, etc.). The ANDVisio software module provides a graphical interface for user access to the knowledge base, which allows you to reconstruct target gene networks, as well as search for regulatory pathways in the global gene network using templates. Templates are a linear chain of objects and the links between them. Objects can be specified by a list of specific names/identifiers, or only by the type of the object. In the first case, when searching for paths, only these specific objects will be considered, and in the second, all objects of a given type.

The program searches the global network for paths that satisfy the requirements of the template. In this work, we used a template that includes four participants: (1) miRNA; (2) a protein that is the primary target of miRNA; (3) a gene that is an miRNA secondary target whose expression can be regulated by a protein—primary target 2; and (4) a protein, gene expression product 3. Participants 1 and 4 were given a list of corresponding identifiers from miRbase (https://www.miRbase.org/) [36] and Swiss-Prot (https://www.expasy.org/resources/uniprotkb-swiss-prot) databases [37]. Participants 2 and 3 were given only by the object type, "gene" and "protein", respectively. The allowed types of interactions in the path chain are defined as follows: between participants 1 and 2—"microRNA regulation"; between participants 2 and 3—"expression regulation", "expression upregulation", "expression downregulation", "interaction" (when the program is running, a logical OR is applied

between the specified types of interactions); between participants 3 and 4—"expression" (gene expression).

Potential anti-apoptotic effect of miRNA. The potential anti-apoptotic effect of miRNAs in their action on the expression of secondary target genes was evaluated using the following formula:

$$Ea = \delta \left(\sum_{g \in AAP} Eg - \sum_{g \in PAP} Eg \right), \qquad (1)$$

where *AAP*—many anti-apoptotic genes, *PAP*—many pro-apoptotic genes, $\delta = 1$, if siRNA expression is increased in HCC, $\delta = -1$ if reduced.

$$Eg = N_1 - N_2, \qquad (2)$$

where Eg is the overall effect of miRNAs on the expression of the secondary target gene, N_1 is the number of regulatory pathways with upregulation of expression ("expression upregulation" type of connection between participants 2 and 3 in the template), N_2 is the number of regulatory pathways with negative regulation of expression (the "expression downregulation" type of connection between participants 2 and 3 in the template). Eg characterizes only the effect of miRNA action on the expression of the target gene, and not on apoptosis. The greater the value Eg, the more regulatory pathways provide activation of target gene expression. The pro- and anti-apoptotic effect of miRNA depending on the values Eg is determined by the fact that the target gene belongs to the set of pro- and anti-apoptotic genes.

According to equation (1), the total anti-apoptotic effect (*Ea*) siRNA on the activity of the process was assessed using the difference in the total contributions (*Eg*) regulatory genes in the expression of anti- and pro-apoptotic genes, which are secondary targets of miRNAs. With such an assessment of the effect, positive *Ea* values characterize an increased suppression of apoptosis (oncogenic activity), and negative values characterize an increased induction of apoptosis (oncosuppressive activity).

RESULTS AND DISCUSSION

Regulation of the Extrinsic Activation Pathway of Programmed Cell Death in Hepatocellular Carcinoma Mediated by the Action of Mirnas on Primary Targets

Figure 1 shows a diagram of the programmed cell death process mediated through death receptors (CD95, DR4/5; extrinsic apoptotic pathway). The components of this pathway are the FADD, cFLIP, and procaspase-8 proteins, the interaction of which leads to the formation of the DISC macromolecular complex, which ensures the process of activation of the external pathway of apoptosis through the formation of the active form of caspase-8 [23−26]. At the same time, the internal (mitochondrial) pathway of apoptosis is activated, the mediator of which is BID

protein (BH3 interacting domain death agonist). Activation of BID is associated with its cleavage by caspase-8 with subsequent association with the outer mitochondrial membrane, which leads to the formation of mitochondrial pores and induction of apoptosis [38−40]. The miRNAs involved in the regulation of the key genes of this pathway through direct (physical) interaction with their mRNAs, the expression of which is impaired in HCC, are indicated.

Data on miRNAs, whose aberrant expression in HCC is associated with impaired activity of the extrinsic apoptosis pathway and is associated with poor prognosis, were obtained from the analysis of patient-specific data on differential expression of miRNAs in HCC from the scientific literature. These data are summarized in Fig. 1 and in Table 1.

An analysis of these data showed that early HCC recurrence and a poor outcome of the disease correlate with a high expression level of hsa-miR-21-5p and hsa-miR-106b [41, 42], whose action is directed to the Fas ligand genes of the death receptor FASLG and death receptor DR-4, respectively, as well as a whole set of miRNAs that block the expression of caspase-8 and -3 genes (Fig. 1) [43, 44].

At the same time, in HCC, the level of hsa-miR-200c miRNA is reduced [45], which regulates gene expression of *fap-1* Fas-associated phosphatase [46], an inhibitor of the CD95 receptor [47, 48], hsa-miR-146a, which controls the expression of the FADD protein gene [49, 50], which is involved in the formation of the DISC macromolecular complex [25, 26, 51], as well as hsa-miR-512-3p, hsa-miR-382-3p, and hsa-miR-20a-3p blocking the expression of the cFLIP protein gene, which negatively affects the activation of pro-caspase-8 [52, 53] (Fig. 1). It is known that a change in the quantitative ratio of FADD and FLIP and pro-caspase-8 determines the balance between apoptotic death and cell survival [54, 55].

The c-FLIP protein within the DISC complex is also involved in the activation of the anti-apoptotic NF-κB signaling pathway. A special role in this process is played by the protein−protein interaction of c-FLIP with the NEMO (NF-kB essential modulator, known as IKKγ, IκB kinase γ) protein, a key activator of the NF-κB signaling pathway [56−58]. One of the mechanisms for increased tumor cell proliferation in HCC may be activation of the NF-κB signaling pathway through the interaction of c-FLIP/NEMO proteins. This possibility is confirmed by a decrease in the level of hsa-miR-342-3p siRNA [59], leading to the activation of NEMO protein gene expression and an increase in its content in hepatoma cells.

Thus, in HCC, miRNAs often block the first stages of the signaling pathway associated with the activation of death receptors on the cell membrane, and the caspase cascade itself through inhibition of the expression of the initiating caspase-8 and effector caspase-3 genes. At the same time, changes in the level of all the
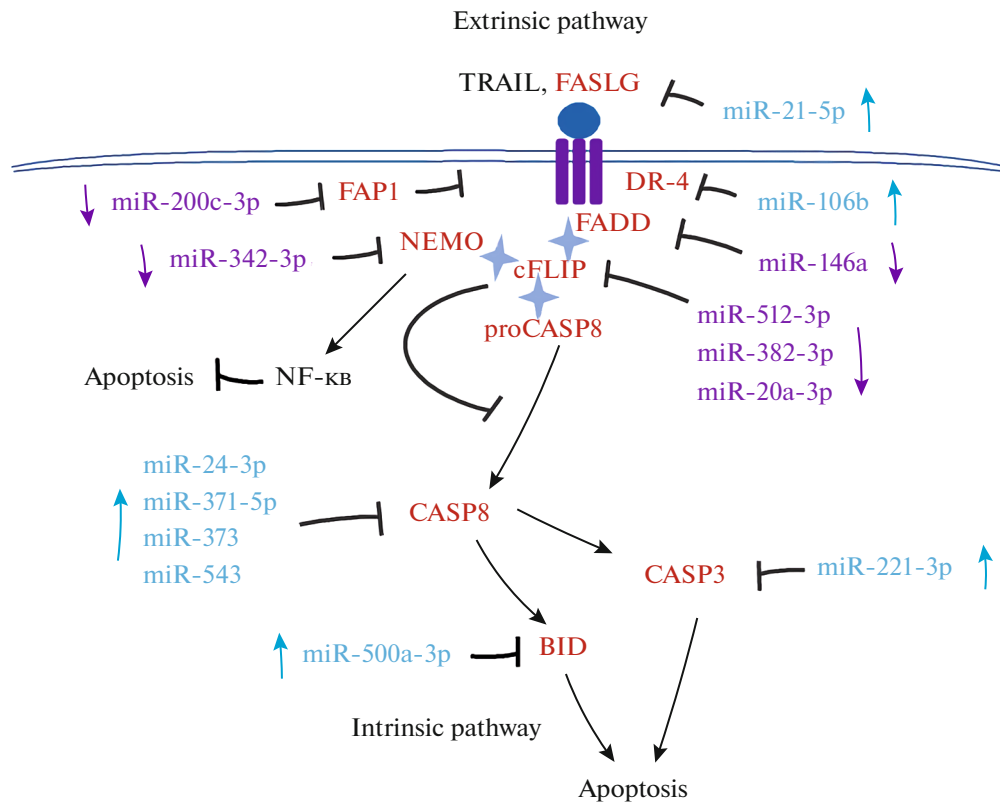
Extrinsic pathway

Fig. 1. Scheme of components of the external pathway of apoptosis and miRNAs involved in its regulation in HCC. Designations: BID (Bcl-2 homolog (BH)3-only protein, BH3 interacting domain death agonist), CD95/Fas/APO-1 (Death receptor), CASP-3, -8 (caspase-3, -8), cFLIP / CFLAR (cellular FADD-like interleukin-1b-converting enzyme (FLICE)-inhibitory protein/CASP8 and FADD like apoptosis regulator), DR-4 (death receptor 4), FADD (Fas associated via death domain), FAP1 (Fas-associated phosphatase-1), FASLG (FAS ligand), NF-κB (Nuclear factor kappa-B), NEMO (NF-kB essential modulator/IκB kinase γ), TRAIL (TNF-related apoptosis-inducing ligand). Extrinsic pathway—external pathway of apoptosis, Intrinsic pathway—internal pathway of apoptosis. Black arrows - activation of the function of the corresponding protein or expression of its gene, arrows with a blunt end—blockade. Proteins whose gene expression is controlled by miRNAs are marked in red, onco-miRNAs are highlighted in blue, suppressors are highlighted in purple. Colored arrows pointing up indicate an increased level of expression of the corresponding miRNAs in HCC, arrows pointing down indicate a reduced level. Icon ✦ indicates an interaction between the marked proteins.

described miRNAs that control the activity of the components of the external apoptosis pathway are logically associated with its blockade in hepatoma cells.

On the other hand, it should be noted that in heterogeneous tumors, such as HCC, the effect of each miRNA can also be associated with its other targets, which can not only modulate apoptosis activity, but also affect tumor progression via other mechanisms. As an example, consider the secondary targets hsa-miR-24, hsa-miR-21, hsa-miR-200s and hsa-miR-373. It turned out that the target of hsa-miR-24, in addition to the caspase gene-8 (initiating caspase of the extrinsic pathway of apoptosis), is the p53 protein gene — a key regulator of the mitochondrial pathway of apoptosis [64]. In this way, an increase in the level of this miRNA in HCC leads to blockade of both pathways of apoptosis and contributes to an increase in tumor progression.

Among the targets of hsa-miR-21, in addition to the Fas ligand gene, activator of the external pathway

of apoptosis, are the *PDCD4* (programmed cell death factor 4) gene coding negative regulator of anti-apoptotic proteins of the intrinsic pathway of apoptosis Bcl-x$_L$ and XIAP [65], and the phosphatase gene PTEN, a negative regulator of the PI3K/AKT signaling pathway [66−68]. In the considered example an increased level of this miRNA in HCC contributes not only to blocking the external and internal pathways of apoptosis, but also to the proliferation of tumor cells through activation of the PI3K/AKT signaling pathway and its effectors [69, 70]. In both cases, miRNAs act as oncogenes.

miRNA hsa-miR-200c generally acts as an oncosuppressor and its low level of expression in HCC is associated with a poor prognosis of the disease. In addition to the gene for Fas-associated phosphatase 1, an inhibitor of the CD95 receptor of the external apoptosis pathway, its targets are the genes for cyclin E2, RIP2 kinase (Receptor-interacting protein 2), and the proapoptotic protein Noxa [46, 71−73]. Thus, a low

Table 1. List of miRNAs involved in the regulation of the external pathway of apoptosis, the expression of which is impaired in hepatocellular carcinoma

miRNA	Nucleotide sequence*	Expression change	Target	Link
miR-21-5p	5'-UUGUAAACCCAUUGUUAUUCGAU	↑	FASLG	[42]
miR-106b	3'-UAGACGUGACAGU**CGUGAAA**U-5'	↑	DR-4	[41]
miR-200c	3'- AGGUAGUAAUGGGCCGUCAUAAU-5'	↓	fap-1	[45, 46]
miR-146a	3'-UAGUC**AGACUAUU**CGAU-5'	Same	FADD	[49, 50]
miR-20a-3p	3'-GAAAUUCACGAG**AUUACGU**CA-5'	"–"	c-FLIP	[60]
miR-512-3p	3'-CUGGAGUCCGAUACU**GUCGUGAA**-5'	"–"	c-FLIP	[61]
miR-382-3p	3'-UUCACACAGGC**ACUUACU**AA-5'	"–"	c-FLIP	[62]
miR-342-3p	3'-UGCCCACGCUAAA**GACACACUC**U-5'	"–"	NEMO (IKKγ)	[59]
miR-24-3p	3'-GACAAGGACGACUUG**ACUCGGU**-5'	↑	Caspase-eight	[43]
miR-543	3'-UUCUUCACGUGGCG**CUUACAA**-5'	Same	Same	[44]
miR-371-5p	3'-UCACGGGGGUGUC**AAACUC**A-5'	"–"	"–"	
miR-373	3'-UGUGGGGUUUUAGCUU**CGUGAA**G-5'	"–"	"–"	
miR-96-5p	3'-UCGUUUUUACACGAU**CACGGU**UU-5'	"–"	Caspase-9	[63]
miR-221-3p	3'-CUUUGGGUCGUCUGUU**ACAUCGA**-5'	"–"	Caspase-3	[43]

*The siRNA binding site with the mRNA of the target gene is highlighted in bold;
↓ Decreased expression;
↑ Increased expression.

level of this miRNA in HCC contributes to the activation of Noxa and the internal pathway of apoptosis; however, it is associated with a poor outcome of the disease [45], which, apparently, is associated with the action of this miRNA on other target genes, activation which contributes to the proliferation of tumor cells, the development of an inflammatory process in the liver, and blockade of the external pathway of apoptosis [74–77]. It is possible that there are other reasons for the poor prognosis in HCC associated with a low level of hsa-miR-200c siRNA.

An exception to this series is hsa-miR-373, which in some studies is positioned as an onco-miRNA, the level of which is increased in malignant tumors, including HCC [44, 78], and in others, as a tumor suppressor with a reduced level in HCC cells [79]. In the first case, hsa-miR-373 affects the activity of the caspase-8 [44] and CD44 genes, a marker of tumor stem cells [78], while in the second case, the action of this miRNA is directed to the transcription factor activating enhancer binding protein 4 (TFAP4), whose expression activation in HCC promotes tumor cell proliferation and blocks apoptosis via the PI3K/AKT signaling pathway [79]. Thus, the disruption of hsa-miR-373 expression in malignant tumors does not depend on whether it acts as an oncogene or as a tumor suppressor, and the specific manifestation of its effect depends on the set of target genes involved in this response [80].

In general, this means that when choosing a marker associated with an unfavorable developmental prognosis in HCC, additional analysis of the entire range of targets of the miRNA of interest is required. This kind of analysis was performed for miRNAs, whose aberrant expression in HCC blocks key genes of the external pathway of apoptosis and is associated with a poor prognosis of the disease (Table 1).

Regulation of the Extrinsic Activation Pathway of Programmed Cell Death in Hepatocellular Carcinoma Mediated by the Action of miRNAs on Secondary Targets

At this stage of the study, the search for secondary miRNA targets, presented in Table 1 was carried out, whose aberrant expression in HCC negatively affects the activity of the external apoptosis pathway. The potential influence of miRNAs on the extrinsic pathway of apoptosis through these targets was evaluated. The search space for secondary targets was limited to primary targets. We assumed that the primary target of one miRNA could be the secondary target of another miRNA. Obviously, such a network interaction of different miRNAs can lead to an increase or decrease in their action on their targets and, as a result, to modulation of the external pathway of apoptosis.

Using the ANDSystem, for each miRNA according to a given template, regulatory pathways were reconstructed, through which miRNA can potentially influence the expression of genes from the set of primary targets of other miRNAs (Fig. 2).

As we expected, many miRNAs use several primary targets of other miRNAs as secondary targets (Table 2). However, not all miRNAs are able to regulate the expression of primary targets of other miRNAs. It
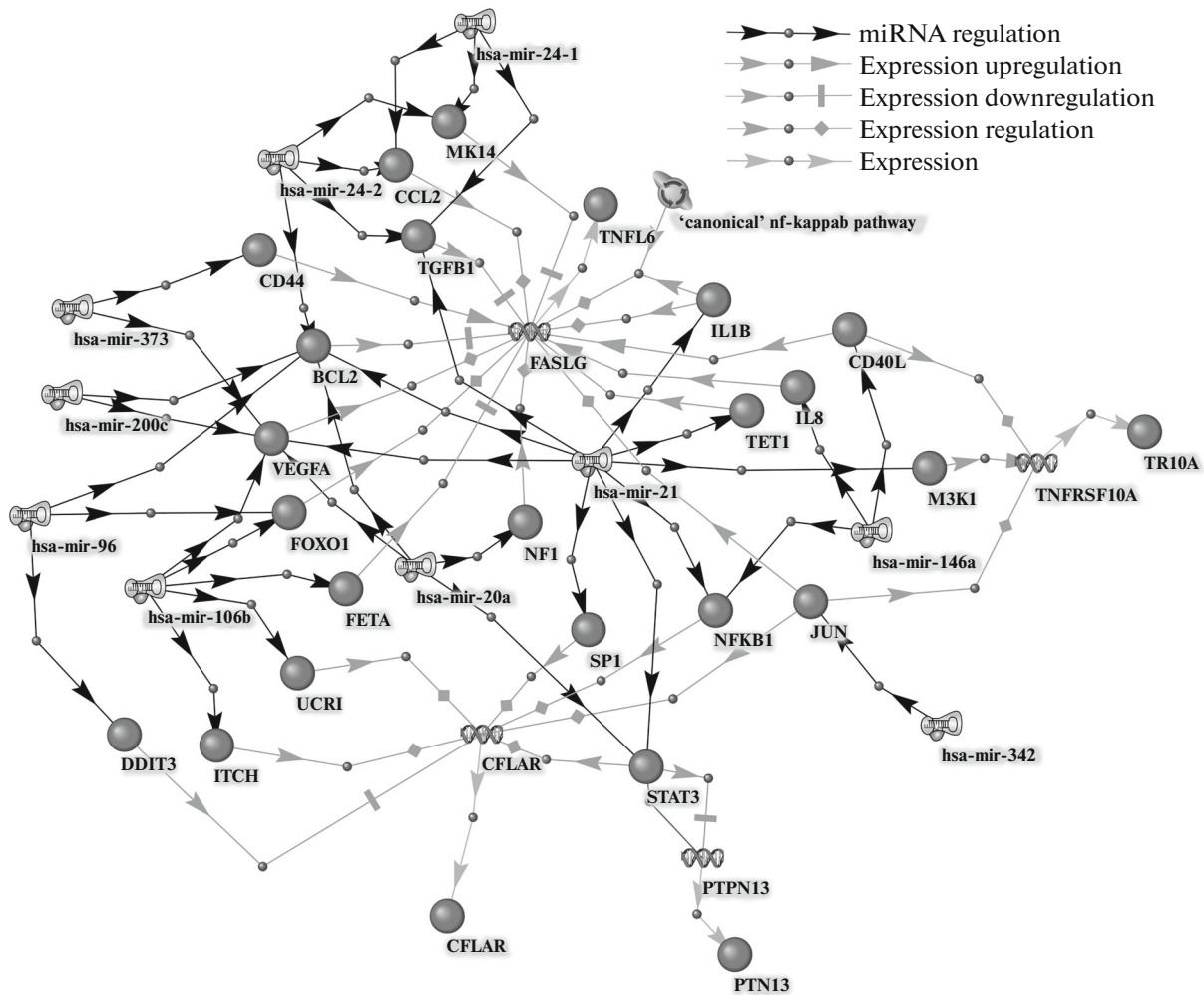
Fig. 2. Regulatory expression pathways for miRNA secondary target genes constructed using the ANDSystem. Designations: BCL2 (B-cell lymphoma 2), CCL2 (CC Motif Chemokine Ligand 2), CD (Crohn's disease, receptor), CD40L (CD40 ligand), CFLAR (CASP8 and FADD like apoptosis regulator), DDIT3 (DNA damage induced transcript 3), FASLG (FAS ligand), FETA (α-fetoprotein), FOXO1 (Forkhead box O protein 1), IL1B/8 (Interleukin 1B/8), ITCH (Itchy E3 ubiquitin-protein ligase), JUN (proto-oncogene, AP-1 transcription factor subunit), M3K1 (Mitogen-activated protein kinase kinase kinase 1), MK14 (Mitogen-activated protein kinase 14), hsa-miR (microRNA *Homo sapiens*), NF1 (Nuclear factor 1), NFKB1 (Nuclear factor kappa-B 1), PTPN13/PTN13 (Tyrosine-protein phosphatase nonreceptor type 13), SP1 (Specificity protein 1, transcription factor), STAT3 (Signal transducer and activator of transcription 3), TGFB1 (Transforming growth factor beta 1), TET1 (Ten-eleven translocation 1), TNFRSF10A/TR10A (Tumor necrosis factor receptor superfamily 10A), UCRI (Ubiquinol-cytochrome C reductase iron-sulfur subunits), VEGFA (Vascular endothelial growth factor receptor), 'canonical' nf-kappab pathway

turned out that regulatory pathways were found only for 10 out of 14 onco-miRNAs (Table 2). Only four of the seven primary target genes are present in these regulatory pathways. Among them were two proapoptotic genes—*FASLG* (FAS ligand) and *TNFRSF10A* (DR-4 receptor), as well as two anti-apoptotic genes - *PTPN13* (Fas-associated phosphatase 1) and *CFLAR* (negative regulator of caspase-8—protein c-FLIP).

The modulating effect of miRNAs on the extrinsic apoptosis pathway of secondary targets was assessed by summing the potential contributions of each secondary target according to equation (1). It has been shown that the action of miRNAs on secondary targets can potentially lead to both an increase and a decrease in the antiapoptotic effect.

For example, the anti-apoptotic effect index ($Ea = 2$), mediated by the action of secondary targets, was the highest for hsa-miR-373 miRNAs (Table 2). Caspase-8 is the primary target of this siRNA, which is directly associated with the extrinsic pathway of apoptosis (Table 1. According to ANDSystem, this miRNA has 59 other primary targets. As can be seen from Fig. 2, two of these targets (VEGFA and CD44 proteins) can upregulate gene expression. *FASLG*, which leads to the activation of apoptosis [81, 82]. Thus, by suppressing the expression of VEGFA and CD44 proteins, hsa-miR-373 can reduce the expression of its secondary target, the gene *FASLG*, which in turn has the effect of negatively modulating the extrinsic apoptosis pathway or an increased anti-apoptotic effect. However, it

Table 2. Regulation of miRNA expression of target genes of the extrinsic pathway of apoptosis in hepatocellular carcinoma through regulatory pathways

miRNA	Expression	Primary targets, total	Secondary targets	Regulatory pathways, number		Eg	Ea
				N_1	N_2		
hsa-miR-373	↑	60	FASLG	0	2	−2	2
hsa-miR-106b	Same	203	FASLG	1	2	−1	1
			CFLAR (c-FLIP)	1	1	0	
hsa-miR-96	"−"	74	FASLG	1	1	0	1
			CFLAR (c-FLIP)	1	0	1	
hsa-miR-24-1 (miR-24-3p)	"−"	249	FASLG	2	1	1	−1
hsa-miR-24-2 (miR-24-3p)	"−"	249	FASLG	3	1	2	−2
hsa-miR-21-5p	"−"	398	FASLG	3	2	1	−2
			CFLAR (c-FLIP)	0	3	−3	
			PTN13 (Fap-1)	1	0	1	
			TNFRSF10A (DR4)	0	1	−1	
hsa-miR-200c	↓	44	FASLG	1	1	0	0
hsa-miR-342	Same	91	FASLG	0	1	−1	−1
			CFLAR (c-FLIP)	0	1	−1	
			TNFRSF10A (DR4)	0	1	−1	
hsa-miR-20a	"−"	155	CFLAR (c-FLIP)	0	1	−1	−1
			PTN13 (Fap-1)	1	0	1	
			FASLG	1	2	−1	
hsa-miR-146a	"−"	52	FASLG	0	2	−2	−2
			CFLAR (c-FLIP)	0	1	−1	
			TNFRSF10A (DR4)	0	1	−1	

N_1—the number of regulatory pathways with positive regulation of expression; N_2—the number of regulatory pathways with negative regulation of expression; Eg—the effect of miRNAs on the expression of the secondary target gene; Ea—the total anti-apoptotic effect of siRNA when acting on the expression of all secondary target genes; ↓—expression is reduced, ↑—expression is increased.

should be noted that all reconstructed regulatory pathways represent a description of potential molecular genetic events in the cell and require further experimental confirmation.

The magnitude of the anti-apoptotic effect of miRNAs, the expression of which is increased in HCC (Table 2) correlated negatively with the total number of their primary targets (Fig. 3). This dependence is in good agreement with the observations, according to which the high polyfunctionality of genes or proteins can lead to interference of regulatory pathways [83]. Interestingly, miRNAs downregulated in HCC showed neither a positive nor a negative correlation. This result may be due to the fact that the absence of an active effect of downregulated miRNAs on primary and secondary targets can be considered as a kind of passive signal. It can be assumed that the patterns of propagation of such passive signals along regulatory pathways

have their own characteristics. However, our use of a very limited sample of miRNAs does not allow us to draw any firm conclusions. To conduct such an analysis, large datasets should be used.

CONCLUSIONS

Our analysis of patient/tissue specific expression of miRNAs differentially expressed in HCC cells showed that almost all components of the receptor-mediated (external) pathway of programmed cell death are subject to regulation by miRNAs, and the change in their expression level in HCC is logically associated with blockade of apoptosis activity. All identified miRNAs are closely associated with an unfavorable prognosis and are actively discussed as HCC markers.

Thus, the spectrum of primary and secondary targets of some miRNAs in HCC may be associated with
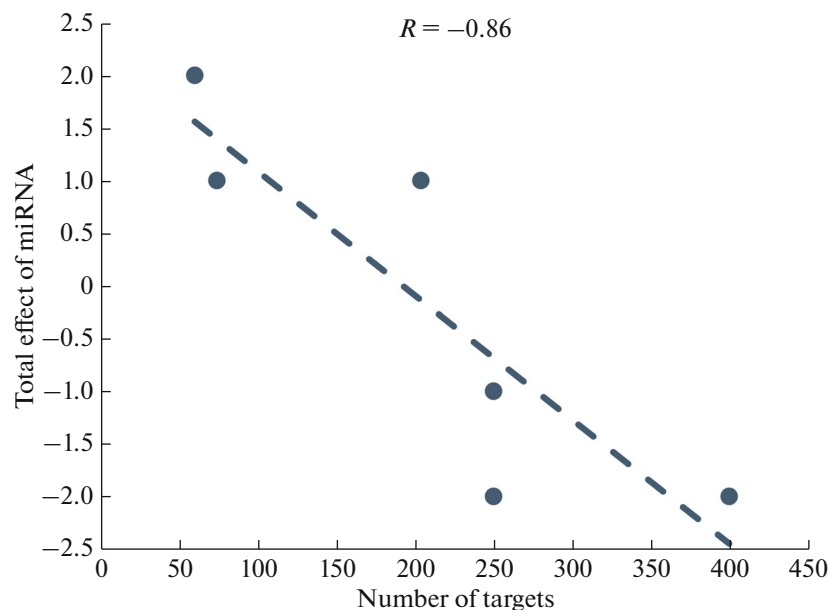
Fig. 3. The relationship between the number of miRNA targets, the expression of which is increased in HCC, and their total effect (*Ea*) to the external pathway of apoptosis through regulatory pathways. Pairwise correlation coefficient *R* amounted to -0.86 ($P = 0.025$).

the regulation of processes that make both unidirectional and multidirectional contributions to disease progression.

An analysis of the effects of secondary targets of the miRNAs considered showed that the potential effect of modulating the external pathway of apoptosis through secondary targets of miRNAs negatively correlates with the number of their primary targets: the antiapoptotic effect decreases with an increase in the number of targets. This pattern is well explained by the fact that with an increase in the number of targets, the number of regulatory pathways associated with them in the global gene network inevitably increases, which, in turn, increases the likelihood of interference. The problem of potential conflicts between individual functions of polyfunctional macromolecules is broad in nature.

The results obtained can help in the selection of targets for reducing tumor aggressiveness, and the analysis of secondary targets allows us to consider hsa-miR-373, hsa-miR-106b, and hsa-miR-96 as priority targets whose action on secondary targets enhances their antiapoptotic effect.

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