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Design of crRNA to Regulate MicroRNAs Related to Metastasis in Colorectal Cancer Using CRISPR-C2c2 (Cas13a) Technique

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Abstract

Colorectal cancer (CRC) is the third most prevalent cancer with the second-highest mortality rate worldwide. microRNAs (miRNAs) of cancer-derived exosomes have shown promising diagnosis potential. Recent studies have shown the metastatic potential of a specific group of microRNAs called metastasis. Therefore, down-regulation of miRNAs at the transcriptional level can reduce metastasis probability. The aim of this bioinformatics research is targeting of miRNAs precursors using CRISPR-C2c2 (Cas13a) technique. The C2c2 (Cas13a) enzyme structure was downloaded from the RCSB database, the sequence miRNAs and their precursors were collected from miRbase. The crRNAs were designed and evaluated for their specificity by using CRISPR-RT server. The modeling 3D structure of the designed crRNA was performed by RNAComposer server. Finally, HDOCK server was used to perform molecular docking to evaluate docked molecules' energy level and position. The crRNAs designed for miR-1280, miR-206, miR-195, miR-371a, miR-34a, miR-27a, miR-224, miR-99b, miR-877, miR-495 and miR-384 that showed high structural similarity with the situation observed in normal and appropriate orientation was obtained. Despite high specificity, the correct orientation was not established in the case of crRNAs that designed to target miR-145, miR-378a, miR-199a, miR-320a and miR-543. The predicted interactions between crRNAs and Cas13a enzyme showed that crRNAs have a strong potential to inhibit metastasis. Therefore, crRNAs may be considered as an effective anticancer agent for further research in drug development.

Keywords: Colorectal Cancer, Computational Biology, crRNA

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According to the International Agency for Research on Cancer and the World Health Organization, colorectal cancer (CRC) is the third most common cancer having the second-highest mortality rate in the world, (1, 2). When a polyp grows out of the inner wall of the colon and rectum, it can become malignant over time, causing multiple metastasis to the liver and, less frequently, the lungs, bones, spinal cord, and brain (3). Unique RNA-guided mechanism with the ability to target RNA, Cas13a (also known as C2c2), was recently found (4). CRISPR-based RNA-targeting techniques have already been used in biomedical applications, such as recognizing specific viral RNA sequences and tumor circulating RNA in patients (5). By modifying essential RNA molecules including mRNAs and noncoding RNAs such as microRNAs, lncRNAs, and others, RNA-targeting gene editing systems show enormous potential in cancer treatment (6). Interesting researches have indicated that Wnt/β-catenin pathway is closely correlated to chemoresistance in CRC patients (7). Notch signaling is one of the most important pathways in cancer metastasis (8). Drug-resistant CRC CSCs must be eliminated, and Notch signaling can cause colon adenoma along with Wnt signaling (9). The expression of Notch1 is dysregulated during the CRC initiating step (10). Inhibiting this pathway might improve the therapeutic efficiency of cancer treatment by eliminating CSCs (8, 11). Previous research has demonstrated that Transforming growth factor beta (TGF-β) signaling, through both Smad-dependent and Smad-independent pathways, is critical for the spread of cancer (12). Tumor occurrence and development are significantly influenced by the (EGFR)/PI3K/Akt nuclear factor kB (NF-kB) signaling pathway, which is particularly critical for the growth, invasion, and metastasis of tumor cells (13). In this study, using bioinformatic approaches and targeting microRNA

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precursors involved in CRC metastasis, a new method was proposed for post-transcriptional regulation of this cancer. Also, for increasing the accuracy and specificity of the method proposed in this study (CRISPR-C2c2), the analyses were performed at the design level of the target crRNA, and structures predicated. CRISPR-C2c2 system could significantly reduce the costs, frequency of trial and researcher errors.

The microRNAs chosen for this bioinformatics analysis related to multiple signaling pathways associated with CRC metastasis, based on previous research. First, the target's precursor miRNA sequences were downloaded in Fasta format from the mirBase website (https://www.mirbase.org/). Due to their small size and potential for off-targets, adult miRNAs were not a good target. Thus, miRNAs associated with metastasis were modulated at the precursor stage (Table 1). To create and simulate the target crRNAs, the CRISPR-RT (RNA Targeting) server (http://bioinfolab.miamioh.edu/CRISPR-RT/) was used. For the CRISPR-C2c2 method, the crRNA design is provided by the CRISPR-RT algorithm. This approach directs the most precise performance of crRNA design across a broad range of parameters. These parameters can include the protospacer flanking site (PFS), the number of incorrect pairings or gaps that this system will tolerate, and the length of the complementary region of the crRNA. The CRISPR-RT system displays a list of crRNA sequences with various features based on the input data for the anticipated target sequence after specifying and establishing the required parameters (14). The CRISPR-RT server was utilized to establish specified parameters, resulting in the presentation of candidates. These candidates determined by the input RNA sequence and precise search algorithms of non-target sites, executed for each input sequence in Transcriptome and Genome. The CRISPR-RT server findings informed the selection of suitable crRNAs, taking into account various parameters including the start and end site of the intended crRNA, GC%, as well as the minimized presence of non-target sites within both the Genome and Transcriptome. Such considerations were imperative to ensure that the desired crRNA sequence demonstrated optimal specificity in design. The designed CRISPR RNA molecules, commonly referred to as crRNAs, are integrated into a structural entity known as the scaffold. The CRISPR-RT component is utilized to selectively target the desired region, while the scaffold component allosterically modulates the enzymatic activity of the C2c2 molecule by virtue of its domain structures in two and three dimensions, ergo. The present investigation employs the proposed framework of the CRISPR-RT algorithm, whereby the scaffold under scrutiny features the ring stem structure. The said scaffold sequence has been duly authenticated and made known through prior research (4). Following the prescribed structural framework, the crRNA's three-dimensional architecture was executed in accordance with its designated component and intended recipient. To investigate the intricacies of the interplay between clustered regularly interspaced short palindromic repeats (CRISPR) RNAs (crRNAs) and the CRISPR-associated protein C2c2 (Cas13a) enzyme, three-dimensional simulations of the crRNA sequences proposed for the precursors of microRNAs were conducted. The RNAComposer system presents a novel and user-friendly means for automated prediction of substantial RNA tertiary structures. The approach relies on the machine translation principle and functions by utilizing the RNA FRABASE database as a lexicon connecting the RNA tertiary structure and secondary structure elements. RNA Composer operates in two distinct modes: Interactive mode enabling users to manipulate a singular RNA molecule of interest in a sequential manner. The usability of this mode is restricted to a maximum of 500 nucleotide (nt) residues yielding a solitary 3D-RNA structural model outcome. The Batch mode is intended to facilitate high-volume, automated modeling of RNA structures, encompassing up to 500 nucleotide residues, that hinge on RNA secondary structures defined by the user. A collection comprising a maximum of 10 RNA sequences may be employed. This particular mode is exclusively accessible to individuals who have completed the registration process (15). The crystallographic structure of the C2c2 enzyme (Cas13a) was acquired from the protein database, located at https://www.rcsb.org/, through the accession code 5wtj (https://www.rcsb.org/structure/5wtj). In the present study, molecular docking was executed by using the HDOCK server, accessible via http://hdock.phys.hust.edu.cn/, to analyze RNA molecules that were simulated utilizing C2c2 enzyme (cas13a) (16). The present server was utilized for conducting molecular docking simulations involving protein-protein, protein-DNA, and protein-RNA interactions. HDOCK employs a global docking approach for the generation of molecular complexes. PyMol (https://pymol.org/2/) was employed to conduct a comparative analysis of docking complexes.

The crRNAs that were devised for the chosen miRNAs have been collated in both Table 2 and Table S1 (See Supplementary Online Information at www.celljournal.org). 100 docking model simulations of C2c2 (Cas13a) with crRNA were presented for each target microRNA, utilizing the HDOCK server. From a set of 100 anticipated models, the 10 models that possessed the highest energy level were identified and deemed as the most exemplary (Table 3). The examination and analysis of the docking complex between the C2c2 enzyme and crRNAs specifically designed for this purpose, were conducted utilizing

the PyMoL software. Investigation of the formed complexes showed that the crRNA designed for *miR-1280* has a very high structural similarity in model 1 with binding energy -316.63 and for model 2 and 3 with binding energy -310.59 and -305.15 to the state observed in the normal condition. Also, the complexes of crRNAs designed for *miR-206*, *miR-195*, *miR-371a*, *miR-34a*, *miR-27a*, *miR-224*, *miR-99b*, *miR-877*, *miR-495* and *miR-384* had high structural similarity with binding energy -359.12, -329.82, -352.98, -358.99, -342.89, -291.73, -318.87, -355.59, -307.80 and -312.46 of respectively. In other words, the orientation of the hairpin-loop section to activate the C2c2 (Cas13a) enzyme prediction has happened correctly (Fig.1). In the case of crRNAs designed to target *miR-145*, *miR-378a*, *miR-199a*, *miR-320a* and *miR-543* with binding energy -345.32, -347.22, -447.03, -351.74 and -321.94 respectively, was observed that the orientation of the hairpin-loop section did not occur correctly and therefore it is very likely that the C2c2 (Cas13a) enzyme will not be activated properly (Fig.2).

Table 1: miRNAs involved in precursor

miRNA	Precursor (5′-3′)
hsa-miR-1280	UCUGUCCCACCGCUGCCACCCUCCCCUCUGCCUCAGUGUGCCAGGCAUCAGCACUCACUCACAGAG-GCAGGCUGGAUGGCGGGUGGGACAACAG
hsa-miR-206	UGCUUCCCGAGGCCACAUGCUUCUUUAUAUCCCCAUAUGGAUUACUUUGCUAUGGAAUGUAAGGAAGU-GUGUGGUUUCGGCAAGUG
hsa-miR-195	AGCUUCCCUGGCUCUAGCAGCACAGAAAUAUUGGCACAGGGAAGCGAGUCUGCCAAUAUUGGCU-GUGCUGCUCCAGGCAGGGUGGUG
hsa-miR-371a	GUGGCACUCAAACUGUGGGGGCACUUUCUGCUCUCUGGUGAAAGUGCCGCCAUCUUUUGAGUGUUAC
hsa-miR-145	CACCUUGUCCUCACGGUCCAGUUUUCCCAGGAAUCCCUUAGAUGCUAAGAUGGGGAUUCCUGGAAAU-ACUGUUCUUGAGGUCAUGGUU
hsa-miR-378a	AGGGCUCCUGACUCCAGGUCCUGUGUGUUACCUAGAAAUAGCACUGGACUUGGAGUCAGAAGGCCU
hsa-miR-34a	GGCCAGCUGUGAGUGUUUCUUUGGCAGUGUCUUAGCUGGUUGUUGUGAGCAAUAGUAAGGAAGCAAU-CAGCAAGUAUACUGCCCUAGAAGUGCUGCACGUUGUGGGGCCC
hsa-miR-199a	GCCAACCCAGUGUUCAGACUACCUGUUCAGGAGGCUCUCAAUGUGUACAGUAGUCUGCACAUUG-GUUAGGC
hsa-miR-27a	CUGAGGAGCAGGGCUUAGCUGCUUGUGAGCAGGGUCCACACCAAGUCGUGUUCACAGUGGCUAAGUUC-CGCCCCCCAG
hsa-miR-320a	CUCCCCUCCGCCUUCUCUUCCCGGUUCUUCCCGGAGUCGGGAAAAGCUGGGUUGAGAGGGC-GAAAAAGGAUG
hsa-miR-224	GGGCUUUCAAGUCACUAGUGGUUCCGUUUAGUAGAUGAUUGUGCAUUGUUUCAAAAUGGUGCCCU-AGUGACUACAAAGCCC
hsa-miR-99b	GGCACCCACCCGUAGAACCGACCUUGCGGGGCCUUCGCCGCACACAAGCUCGUGUCUGUGGGUCCGU-GUC
hsa-miR-877	GUAGAGGAGAUGGCGCAGGGGACACGGGCAAAGACUUGGGGGUUCCUGGGACCCUCAGACGUGUGUC-CUCUUCUCCCUCCUCCCAG
hsa-miR-495	UGGUACCUGAAAAGAAGUUGCCCAUGUUAUUUUCGCUUUAUAUGUGACGAAACAAACAUGGUGCA-CUUCUUUUUCGGUAUCA
hsa-miR-543	UACUUAAUGAGAAGUUGCCCGUGUUUUUUUCGCUUUAUUUGUGACGAAACAUUCGCGGUGCA-CUUCUUUUUCAGUAUC
hsa-miR-384	UGUUAAAUCAGGAAUUUUAAACAAUUCCUAGACAAUAUGUAUAAUGUUCAUAAGUCAUUCCUA-GAAAUUGUUCAUAAUGCCUGUAACA

Table 2: crRNAs designed for miRNA using CRISPR-RT server

No	miRNA name	Protospacer flanking site (crRNA)	Start	End	GC	Transcript target	Gene target
1	hsa-miR-1280	GUCCCACCGCUGCCACCCUCCCCUCUGCC [crRNA]	4	32	0.75	0	0
2	hsa-miR-206	CCGAGGCCACAUGCUUCUUUAUAUCCCCA [crRNA]	7	35	0.54	1	1
3	hsa-miR-195-5p	AUUGGCUGUGCUGCUCCAGGCAGGGUGGU [crRNA]	58	86	0.64	2	1
4	hsa-miR-371a	CUGUGGGGGCACUUUCUGCUCUCUGGUGA [crRNA]	13	41	0.61	1	1
5	hsa-miR-145	CUCACGGUCCAGUUUUCCCAGGAAUCCCU [crRNA]	10	38	0.57	1	1
6	hsa-miR-378a	GGGCUCCUGACUCCAGGUCCUGUGUGUUA [crRNA]	2	30	0.61	1	1
7	hsa-miR-34a	CCCUAGAAGUGCUGCACGUUGUGGGGCCC [crRNA]	82	110	0.64	2	1
8	hsa-miR-199a-5p	GUGUUCAGACUACCUGUUCAGGAGGCUCU [crRNA]	10	38	0.54	1	1
9	hsa-miR-27a	GCUUGUGAGCAGGGUCCACACCAAGUCGU [crRNA]	21	49	0.61	1	1
10	hsa-miR-320a	UCCGCCUUCUCUUCCCGGUUCUUCCCGGA [crRNA]	7	35	0.64	1	1
11	hsa-miR-224	GGGCUUUCAAGUCACUAGUGGUUCCGUUU [crRNA]	1	29	0.50	2	1
12	hsa-miR-99b	CCGACCUUGCGGGGCCUUCGCCGCACACA[crRNA]	18	46	0.75	1	1
13	hsa-miR-877	GGGGGUUCCUGGGACCCUCAGACGUGUGU[crRNA]	38	66	0.68	1	1
14	hsa-miR-495	UGUGACGAAACAAACAUGGUGCACUUCUU[crRNA]	43	71	0.43	1	1
15	hsa-miR-543	UUGUGACGAAACAUUCGCGGUGCACUUCU[crRNA]	39	67	0.50	1	1
16	hsa-miR-384	CCUAGAAAUUGUUCAUAAUGCCUGUAACA[crRNA]	60	88	0.36	1	1

Unfortunately, there are major problems with therapy methods: Severe patient toxicities and non-compliance are strongly correlated with all CRC treatment methods (17), Some cancer cells have demonstrated considerable resistance to common therapeutic methods including chemotherapy (18). A large number of CRC cases are also discovered at an advanced stage, frequently with metastases leading to patient death despite increased efforts of early screening programs (18). Additionally, in contrast with other types of cancer, CRC develops and advances gradually over time; it can be up to decades before normal colorectal epithelium transform into an adenoma (19, 20). Nevertheless, despite organized public education campaigns on CRC and efforts to enhance early diagnosis, 50% of patients with CRC diagnoses already have metastases (21). Biochemical analysis showed that the C2c2 enzyme is driven by crRNA and makes cuts in the target RNA. So the accuracy and ability of this technique depend on the correct and specific design of the crRNA (22). The Cas13a enzyme family continues to be developed as a platform for RNA detection and programmable RNA binding (5, 23). Based on a study by Liu et al, it was found that the enzyme Cas13a (C2c2) behaves based on sequence and structure in identifying crRNA. On the other hand, in the same study, it was found that the correct placement of the stem-loop section of the crRNA designed in the REC domain of the enzyme is quite effective in activating it (24). We investigated the expression of miRNAs in seventeen types of cancer (pan cancer) in signaling pathways including: Notch signaling, Wnt/β catenin signaling, TGF-β signaling and EGFR signaling and present them in Table 4. According to the above results, CRISPR/Cas 13a is needed for CRC that support our hypothesis. CRISPR/Cas13a based diagnosis can play a pivotal role in helping secondary prevention measures to meet current needs for CRC early diagnosis and prognosis, whereas early screenings have been shown to help assess and reduce CRC mortality (25). Molecular docking studies provided good information about the binding affinity of crRNAs with the Cas13a CRISPR enzyme and helped to understand the interactions, which require further biochemical experiments. results showed that crRNAs can reduce or inhibit metastasis in different signaling pathways, but this inhibition of metastasis should be confirmed at the protein level. The results showed that the effect of crRNAs in the regulation of miRNAs includes: miR-1280, miR-206, miR-195, miR-371a, miR-34a, miR-27a, miR-224, miR-99b, miR-877, miR-495 and miR-384 regulates Notch signaling, Wnt/β catenin signaling, TGF-β signaling, and EGFR signaling and thus reduces or completely inhibits CRC metastasis.

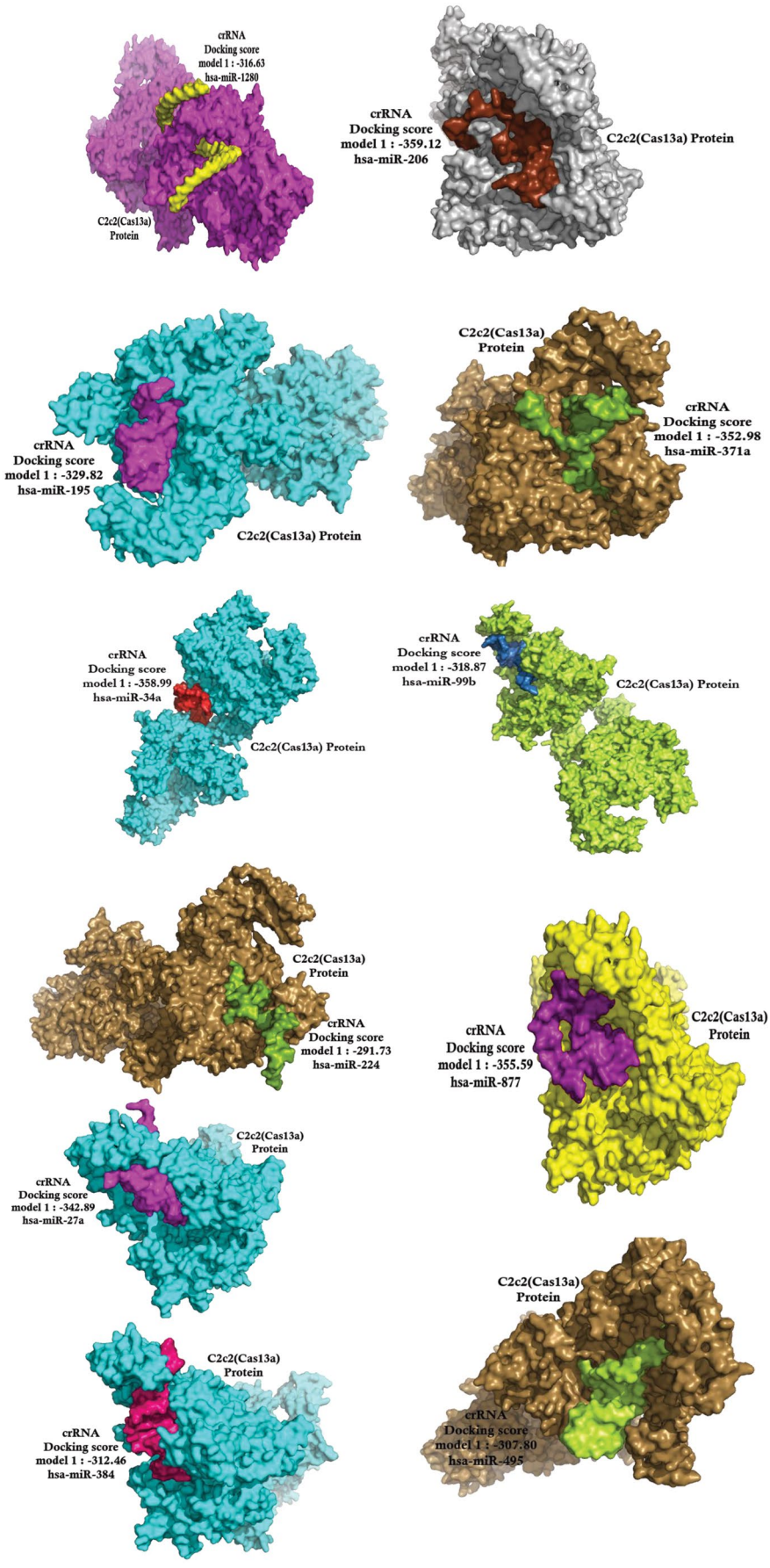

Fig.1: Simulated complexes of C2c2 protein with crRNA designed for miR-1280, miR-206, miR-195, miR-371a, miR-34a, miR-27a, miR-224, miR-99b, miR-877, miR-495, and miR-384 using PyMoL software.

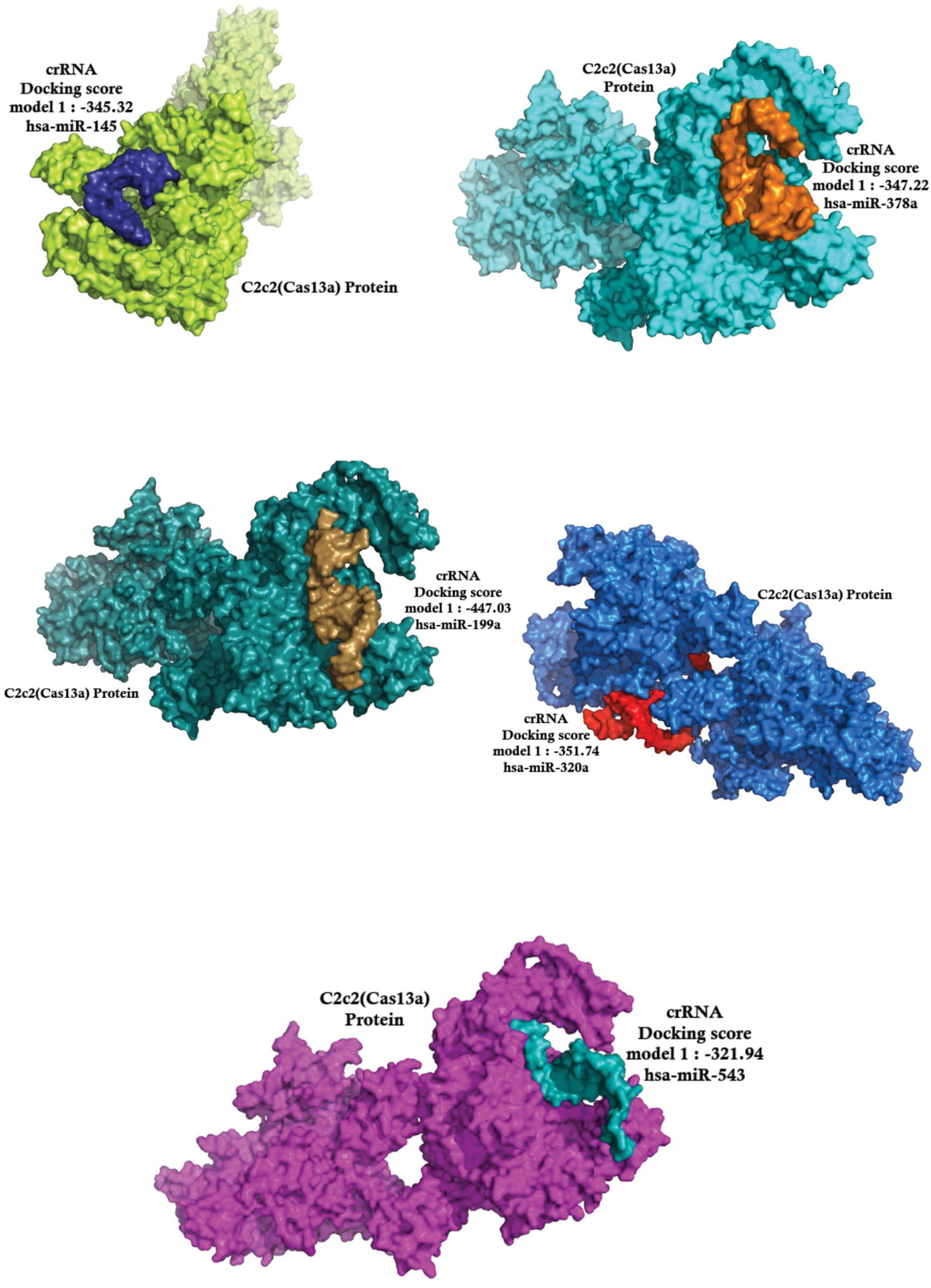

Fig.2: Simulated complexes of C2c2 protein with crRNA designed for miR-145, miR-378a, miR-199a, miR-320a, and miR-543 using PyMoL software.

Table 3: HDOCK server results based on interaction between protein Cas13a (C2c2) and crRNA designed by energy level

No	miRNA	Rank	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6	Model 7	Model 8	Model 9	Model 10
1	hsa-miR-1280	Docking score	-316.63	-310.59	-305.15	-305.12	-302.34	-299.93	-288.86	-287.97	-286.91	-286.40
		Ligand rmsd (Å)	157.80	104.97	143.45	145.80	167.79	170.37	166.31	186.35	152.33	204.57
2	hsa-miR-206	Docking score	-359.12	-313.01	-310.22	-300.38	-298.18	-284.78	-276.00	-274.24	-272.34	-268.10
		Ligand rmsd (Å)	109.85	191.21	104.17	147.13	99.60	173.84	161.16	184.80	108.32	183.90
3	hsa-miR-195	Docking score	-329.82	-327.15	-313.12	-302.48	-290.35	-286.96	-278.27	-275.67	-273.11	-271.59
		Ligand rmsd (Å)	117.09	117.18	192.21	113.48	119.22	206.95	179.19	118.80	198.97	111.76
4	hsa-miR-371a	Docking score	-352.98	-325.76	-321.37	-315.00	-302.39	-292.88	-286.83	-281.40	-278.32	-274.94
		Ligand rmsd (Å)	108.35	120.27	103.51	150.55	112.21	122.14	105.86	126.92	108.04	153.58
5	hsa-miR-145	Docking score	-345.32	-325.37	-316.13	-315.95	-314.71	-312.87	-307.77	-307.12	-304.68	-302.04
		Ligand rmsd (Å)	178.44	180.95	184.54	163.43	104.56	177.18	102.44	187.38	110.03	114.70
6	hsa-miR-378a	Docking score	-347.22	-298.32	-288.10	-287.75	-284.98	-277.95	-260.80	-258.46	-257.41	-255.34
		Ligand rmsd (Å)	110.88	197.26	173.13	118.25	187.33	175.65	195.16	107.36	107.77	108.18
7	hsa-miR-34a	Docking score	-358.99	-351.05	-338.79	-333.10	-311.28	-294.08	-290.27	-281.97	-277.77	-269.35
		Ligand rmsd (Å)	147.35	112.54	118.45	107.70	114.52	109.02	173.62	173.22	115.09	114.61
8	hsa-miR-199a	Docking score	-447.03	-346.76	-314.84	-288.88	-288.71	-285.34	-285.23	-283.72	-279.48	-276.34
		Ligand rmsd (Å)	133.57	187.80	137.53	133.65	131.41	144.88	185.85	152.34	191.49	156.14
9	hsa-miR-27a	Docking score	-342.89	-293.90	-287.04	-286.03	-285.78	-284.47	-280.49	-277.62	-273.98	-273.54
		Ligand rmsd (Å)	100.64	102.94	192.93	120.46	168.18	202.39	114.77	97.85	142.84	116.71
10	hsa-miR-320a	Docking score	-351.74	-292.35	-289.60	-274.89	-264.46	-261.91	-260.52	-256.19	-253.89	-252.82
		Ligand rmsd (Å)	115.13	104.20	183.27	166.90	137.94	160.49	98.29	191.14	125.88	189.38
11	hsa-miR-224	Docking score	-291.73	-291.25	-290.87	-288.19	-276.23	-273.50	-272.39	-268.50	-266.69	-263.69
		Ligand rmsd (Å)	140.86	129.15	183.53	186.81	189.77	126.14	142.01	196.65	135.56	176.10
12	hsa-miR-99b	Docking score	-318.87	-316.90	-309.55	-295.77	-290.76	-288.33	-287.71	-286.30	-284.70	-284.62
		Ligand rmsd (Å)	154.98	159.58	86.09	137.72	77.99	136.08	71.69	75.97	82.39	146.84
13	hsa-miR-877	Docking score	-355.59	-349.99	-345.58	-333.17	-323.35	-301.05	-300.79	-296.74	-292.59	-290.23
		Ligand rmsd (Å)	114.53	177.82	174.32	163.00	173.12	115.11	123.41	110.87	110.90	138.71
14	hsa-miR-495	Docking score	-307.80	-305.46	-305.17	-303.36	-296.00	-295.83	-294.08	-284.17	-278.86	-275.76
		Ligand rmsd (Å)	174.26	112.05	184.54	170.53	172.03	108.34	169.87	113.17	116.38	165.80
15	hsa-miR-543	Docking score	-321.94	-293.89	-289.21	-288.99	-284.81	-283.14	-280.02	-278.68	-275.04	-267.77
		Ligand rmsd (Å)	111.73	164.50	167.42	195.38	125.49	113.71	127.52	122.25	162.51	165.58
16	hsa-miR-384	Docking score	-312.46	-305.50	-302.68	-299.47	-295.31	-289.83	-282.15	-281.03	-278.10	-274.98
		Ligand rmsd (Å)	111.98	112.46	111.39	172.15	135.49	184.53	168.84	112.40	178.23	194.59

The top ten models (model 1, model 2, model 3, … model 10) selected as the best interaction between crRNA and Cas13a enzyme: hsa-miR-1280 (-316.63), hsa-miR-206 (-359.12), hsa-miR-195 (-329.82), hsa-miR-371a (-352.98), hsa-miR-145 (-345.32), hsa-miR-378a (-347.22), hsa-miR-34a (-358.99), hsa-miR-199a (-447.03), hsa-miR-27a (-342.89), hsa-miR-320a (-351.74), hsa-miR-224 (-291.73), hsa-miR-99b (-318.87), hsa-miR-877 (-355.59), hsa-miR-495 (-307.80), hsa-miR-543 -321.94), and hsa-miR-384 (-312.46).

Table 4: Pan cancer analysis of miRNAs in seventeen cancer type

No		miRNA name															
		Notch signaling			Wnt/β catenin signaling				TGF-β signaling				EGFR signaling				
	Cancer name	miR-1280	miR-206	miR-195-5p	miR-371a	miR-145	miR-378a	miR-34a	miR-199a-5p	miR-27a	miR-320a	miR-224	miR-99b-5p	miR-877	miR-495	miR-543	miR-384
1	Bladder																
2	Bone																
3	Brain																
4	Breast																
5	Cervix																
6	Colorectal																
7	Esophagus																
8	Head and Neck																
9	Kidney																
10	Liver																
11	Lung																
12	Ovary																
13	Pancreas																
14	Prostate																
15	Skin																
16	Stomach																
17	Thyroid																

Red color indicated upregulated and blue color indicated downregulated in signaling pathways.

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Authors' Contributions

S.T.H.; Conceptualization, Methodology, Data curation, Writing original draft, Software, and Formal analysis. A.S., M.A., R.B.; Formal analysis, Data curation, Writing review and Editing. F.N.; Supervision, Project administration, Writing review and Editing. All authors read and approved the final manuscript.

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