

**hsa-miR-497 AS A MODULATOR OF THE EXPRESSION IN THE PRESENCE
OR ABSENCE OF CHRNA5 IN BREAST CANCER**

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By
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CHRNA5 in Breast Cancer**

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June, 2017

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Abstract

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CHRNA5 is an important ligand-gated receptor with roles in addiction and in cancer. In lung cancer, CHRNA5 dysregulation is well known. There is also expression of CHRNA5 in breast cancer cell lines. microRNAs regulate mRNA expression; and different regulatory microRNAs are involved in different cancer types. microRNAs are thus potential biomarkers to diagnose the diseases (e.g. cancer). However, there is no study testing interactions between microRNAs and CHRNA5 in breast cancer. In the present study, mir-497 was found to be one of the most downregulated microRNAs with testable expression levels upon analysis of expression in the breast cancer cell line MCF7 when exposed to CHRNA5 siRNA. RT-qPCR was performed to test the expression level of mir-497. Validated target genes of mir-497 were found to be significantly related to a list of different KEGG pathways significantly (p value < 0.001) among which there were P53 and PI3K-Akt signalling pathways.

Mimic-mir-497 treatment, alone or together with CHRNA5 siRNA, was applied on MCF7 cells to understand the interaction between the miRNA and siRNA under investigation. Selected target genes of mir-497 were tested; the most significantly modulated genes were involved in P53 pathway. The results indicated interactions between mir-497 and CHRNA5 however selected targets were not affected by mimic-mir-497.

GSE41079 and GSE41074 public datasets containing mRNA and microRNA expression profiles of liver cancer cells treated with mimic-mir-497. Treatment were used to identify

novel targets of mir-497 for future use. Immune system was detected in the second place upon REACTOME analysis of GSE41079 and GSE41074. Using multiple online microRNA-mRNA network tools mir-497 mRNA-miRNA networks were extracted for all or only immune genes. The results from network based analyses helped identify additional targets for later use in our mimic-siRNA system.

Keywords: microRNA, breast cancer, CHRNA5, mir-497, P53, PI3K-Akt, immune system



Özet

mir-497 MEME KANSERİNDE, CHRNA5 İLE ALAKALI, ÖNEMLİ BİR REGÜLATÖR MÜ?

Başak ÖZGÜR SOY

Moleküler Biyoloji ve Genetik Bölümü Yüksek Lisans

Tez Danışmanı: Özlen KÖNÜ KARAKAYALI

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CHRNA5 bağımlılık ve kanserde rol oynayan önemli bir kimyasal kapılı iyon kanalıdır. Akciğer kanserinde bozulmaya uğradığı bilinen CHRNA5, meme kanserlerinde de ifade profile olan bir gendir. mikroRNAlar mRNA ifadesinde düzenleyici görevdedir, değişik mikroRNAlar değişik kanser tiplerinde düzenleyici görev alırlar. mikroRNAlar potansiyel biyo-belirteç görevi görerek kanser gibi hastalıkların teşhisinde kullanılırlar. Fakat meme kanserinde CHRNA5 ve onu hedefleyen mikroRNAlarla ilgili çalışma bulunmamaktadır.

Bu çalışmada, mir-497, siRNA CHRNA5 uygulanmış MCF7 hücrelerinde, en çok azalan ve test edilebilir ifade düzeyine sahip bir mikroRNA olarak saptanmıştır. Ters transkriptaz polimeraz zincir reaksiyonu gerçekleştirilmiş ve mir-497 ifadesi görülmüştür. MCF7 meme kanseri hücre hattında CHRNA5 susturulunca azalan ifadesiyle mir-497, meme kanserinde CHRNA5 bağlantılı önemli bir molekül olmaya adaydır. Kanıtlanmış mir-497 hedef genleri incelendiğinde, KEGG yollarının bu hedef genlerle belirgin bir seviyede ($p < 0.001$) ilgili olduğu görülmüştür. P53 and PI3K-Akt sinyal yolağı bunlardan ikisidir.

MCF7 hücrelerine mimik-mir-497 (tek başına veya siRNA CHRNA5 ile birlikte) uygulaması sonuçlarına dayanarak meme kanserinde mir-497 ve CHRNA5 arasındaki bağlantı incelenmiştir. Mir-497 hedef genleri deneyleri sonucu en çok belirgin bir değişim gösteren genler P53 yolağında bulunmaktadır. Sonuçlar mir-497 ve CHRNA5 arasındaki ilişkiyi gösterse de seçilen hedef genler mimik-mir-497'den etkilenmemektedir.

GSE41074 ve GSE41079'da mimik-mir-497 HCC hücresine uygulanan mimik-mir-497 ile değişen mikroRNA ve mRNA profilleri bulunmaktadır. Devam eden çalışmalarda

kullanılmak üzere mir-497 için daha önce çalışılmayan hedef genler belirlenmiştir. Analizleri sonrası ifadesi artan genler araştırıldığında bağışıklık sistemi REACTOME tarafından öne çıkarılmıştır. Çeşitli mRNA-mikroRNA odaklı harita oluşturma aracı kullanılarak tüm genler için ve sadece bağışıklık sistemi genleri için haritalar çıkartılmıştır. Bu haritalar sayesinde devam deneylerinde test edilebilecek aday moleküller elde edilmiştir.

Anahtar kelimeler: mikroRNA, meme kanseri, CHRNA5, mir-497, P53, PI3K-Akt, bağışıklık sistemi





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Abbreviations

BRCA: breast carcinomas

CRC: colorectal cancers

GBM: glioblastoma

UCEC: endometrial carcinomas

BLCA: bladder carcinomas

OVCA: ovarian serous adenocarcinomas

LAML: acute myelogenous leukemia

LUAD: lung adenocarcinoma

LGG: low grade glioma

STAD: stomach adenocarcinoma

LogFC: log2 fold change

Chapter 1. Introduction

1.1. Cancer

Cancer can be defined as the production of abnormal immortal cells unfettered in different parts of body. The next step is metastasis which means that cancer invades and spreads to other body parts. Physical (e.g. UV light), chemical (e.g. smoking) and biological (e.g. viruses) effects can produce a tumour cell from a normal cell by alteration of genes or expression of genes in the cell. Affected genes can be separated into two categories: tumour suppressors and oncogenes. Tumour suppressors prevent cancer formation while oncogenes potentially cause cancer. Additionally, aging is another important factor for cancer formation. Cancer is one of top diseases causing death in the world. There are different cancer types referring to the origin of tumour cells, e.g., lung cancer, liver cancer, breast cancer, stomach cancer, colorectal cancer and oesophageal cancer. Different cancer types have different behaviours: some are slowly developing some are fast, some cancer types give better results with chemotherapy and some others are treated with surgery. Therefore each should be investigated separately; treatment methods and importance of diagnosis differ among them ¹. Moreover, depending on gender, some cancer types are more common than the others. The most dramatic one is the breast cancer since it is seen much more commonly in females. The rate of incidence for different cancer are seen in Table 1.1 ².

Table 1.1: Cancer Types and Features of Different Cancer Types

Cancer type	Cohort	Samples (N)	Samples with clinical data (N)	Age (mean $\pm$ s.d.)	Gender		Ethnicity					Vital status		
					Male (%)	Female (%)	American Indian, Alaska Native, Hawaiian, Pacific Islander (N)	Asian (N)	African American (N)	Caucasians (N)	NA (N)	Alive (N)	Deceased (N)	NA (N)
BRCA	Discovery	770	770	58.2 $\pm$ 13.2	1.04	98.96	1	50	53	578	88	682	88	0
	Validation	217	200	59.5 $\pm$ 12.8	0.5	99.5	0	6	64	124	6	189	11	0
GBM	Discovery	267	267	59.6 $\pm$ 14.0	60.9	39.1	0	4	21	237	5	89	176	1
	Validation	124	118	61.0 $\pm$ 12.4	62.71	37.29	0	3	3	107	5	36	81	1
HNSC	Discovery	291	261	60.9 $\pm$ 12.4	71.26	28.74	1	4	26	223	7	153	108	0
	Validation	222	192	60.3 $\pm$ 11.3	73.44	26.56	0	5	9	174	4	155	37	0
KIRC	Discovery	452	452	60.7 $\pm$ 12.1	64.82	35.18	0	7	20	419	6	306	146	0
	Validation	42	39	58.5 $\pm$ 12.2	76.92	23.08	0	1	7	31	0	27	12	0
LGG	Discovery	200	200	55.0 $\pm$ 16.1	54.50	45.50	0	2	15	181	2	67	133	0
	Validation	223	220	43.0 $\pm$ 13.5	57.73	42.27	0	0	9	209	2	169	51	0
LUAD	Discovery	240	181	43.5 $\pm$ 13.5	51.93	48.07	1	4	6	167	3	167	14	0
	Validation	462	387	65.2 $\pm$ 9.9	46.25	53.75	1	5	23	297	61	294	93	0
LUSC	Discovery	94	76	66.7 $\pm$ 9.5	46.05	53.95	0	1	3	67	5	51	25	0
	Validation	193	139	67.7 $\pm$ 9.3	74.82	25.18	0	0	4	113	22	93	46	0
OV	Discovery	183	139	66.1 $\pm$ 8.4	78.42	21.58	0	6	3	79	51	103	36	0
	Validation	429	429	59.4 $\pm$ 11.8	0	100	2	15	19	370	23	207	218	4
PRAD	Discovery	68	68	61.2 $\pm$ 10.3	0	100	1	3	2	58	4	40	27	1
	Validation	178	148	60.4 $\pm$ 6.9	100	0	0	2	6	130	10	147	1	0
STAD	Discovery	157	128	60.4 $\pm$ 6.7	100	0	0	0	1	9	118	127	1	0
	Validation	321	306	66.0 $\pm$ 10.7	61.11	38.89	0	81	4	175	46	281	25	0
UCEC	Discovery	248	248	63.1 $\pm$ 11.1	0	100	10	13	25	193	7	231	17	0
	Validation	280	247	64.7 $\pm$ 11.3	0	100	3	6	52	167	19	221	26	0
TOTAL	Discovery	4,034	3,827	59.9 $\pm$ 13.2	39.29	60.71	15	183	225	3,125	279	2,719	1,102	5
	Validation	1,627	1,388	59.7 $\pm$ 13.1	44.09	55.91	5	35	150	983	215	1,116	270	2

AML, acute myeloid leukaemia; BRCA, breast adenocarcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KIRC, kidney renal clear cell carcinoma; LGG, low grade glioma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NA, not available; OV, ovarian carcinoma; PRAD, prostate adenocarcinoma; STAD, stomach adenocarcinoma; UCEC, uterine corpus endometrial carcinoma.

Taken from “Lu, C., et al., *Patterns and functional implications of rare germline variants across 12 cancer types*. Nat Commun, 2015. **6**: p. 10086.” by permission of Creative Commons.

1.1.1. Breast Cancer

For women breast cancer is a common cancer type (Table 1.1).

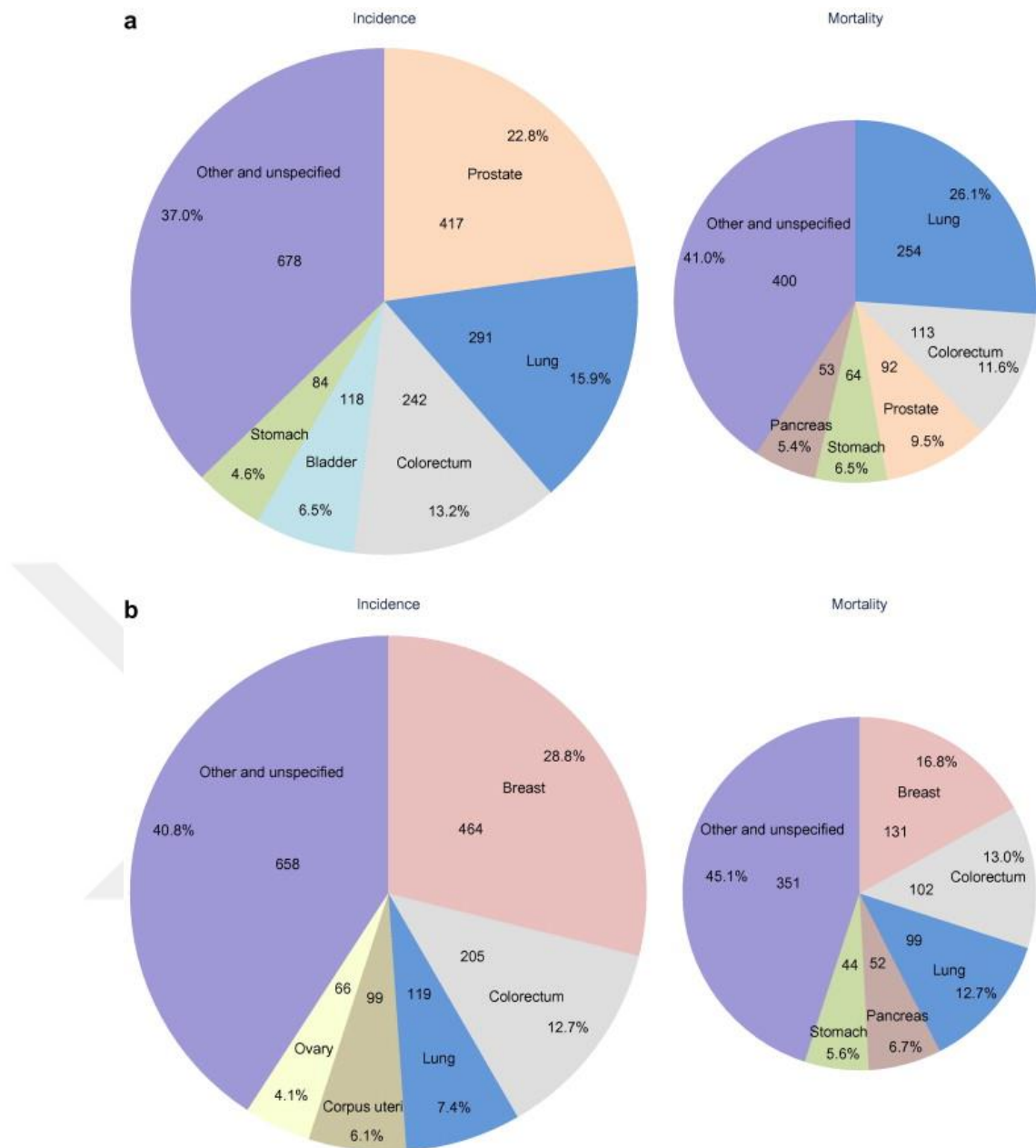


Figure 1.1: 2012 Europe results of the rate of incidence and case of death for top five common cancer types a) Males B) Females (proportion and number of cases are seen). (Figure was taken from “Ferlay, J., et al., *Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012*. Eur J Cancer, 2013. 49(6): p. 1374-403.” by permission of Elsevier.)

Cancer is one of the most common causes of death and in women breast cancer is the most common type of cancer: 28.8% of cancer cases are formed in breast, and 16.8% of deaths from cancer is due to breast cancer (Figure1.1). Factors that can contribute to breast cancer are being overweight, exposure to hormonal therapies, alcohol usage, radiation, not having a child and having a positive family history. These factors are physical, chemical or biological. Around ten percent of breast cancer cases occur because of an inherited gene mutation (BRCA1 or BRCA2 etc.)³.

Breast cancer cell lines obtained from different stages and types of cancers with different features exist for use by researchers. MCF7, BT20, T47D, MDAMB231 are examples to these cell lines. MCF7 whose expression profile belongs to luminal gene cluster, is Estrogen Receptor Positive (ER+) and Progesterone Receptor Positive (PR+) cell line while it is obtained from pleural effusion sourced invasive ductal carcinoma. BT20 falls into Basal A gene cluster. This is ER and PR negative invasive ductal carcinoma; it's source is primary breast. MDA-MB-231 is from ER and PR negative adenocarcinoma. It's source is pleural effusion and it clusters with Basal B type breast cancer samples ⁴.

1.1.2. Hepatocellular Carcinoma (HCC)

Third cause of death from cancer is liver originated cancers and hepatocellular carcinoma (HCC) is the most common type of liver cancer. Most common cause of liver cancer is cirrhosis because of alcohol, hepatitis B (by HBV: hepatitis B virus) or hepatitis C (HCV: hepatitis C virus). Hepatocellular carcinoma is not common in developed countries generally, but prevalence of hepatitis B infection makes the disease common ⁵. Molecular level of disease includes changes in gene regulation. Biomarkers ⁶, protein markers ⁷ and non-coding RNAs ⁸ are some categories of affected genes from HCC. Seven hundred thousand human deaths occur in a year because of this lethal disease ⁹.

1.2. microRNAs

Small non-coding RNAs are around 22 nucleotides length and have roles in regulation of mRNA expression. They are named as “microRNAs (miRNAs)” and they regulate mRNA/protein expression upon cutting of targeted mRNA or only repression of translation of the gene ¹⁰. Reinhart et al found that microRNAs exist also in *Arabidopsis* therefore it is a proof that microRNA existence is originated in early eukaryotic evolution ¹¹. And later, multiple microRNAs are identified in different organisms ¹² (even in viruses ¹³) by *in-silico* analysis and experiments.

On this point, understanding of mechanism of synthesis and involvement of microRNAs in the gene expression regulation is important. microRNA genes encode a longer RNA than mature microRNA molecule. There are processing steps for the mature microRNA occurrence. For example, there are pre-mRNA host genes and many of microRNAs are in introns of these host genes. These microRNAs use regulatory elements of host genes and have relatively similar expression ¹⁴. RNA polymerase II transcribes microRNAs as

pri-miRNA (large RNA precursor) which has a polyA tail and five-prime cap structures¹⁵. There is a complex in nucleus consisting of Drosha and DGCR8/Pasha where Drosha is the name of ribonuclease III enzyme and DGCR8 is a protein and subunit of microprocessor complex in human (Pasha is the name given to the same protein in other organisms especially model organisms)¹⁶. The complex makes pri-miRNAs processed to pre-miRNAs as hairpin loop of folded 70 nucleotide length sequence¹⁷. Exportation is then started by recognition of this RNA sequence by Exp5 which is a receptor¹⁸. The release of pre-miRNAs happens through nuclear pore complex as a result of GTP hydrolysis; and it is possible in the presence of Ran-GTP cofactor. After pre-miRNAs are in cytoplasm; there are more processing steps occurring with Dicer and RNase III to get mature microRNAs. Mature microRNAs bind to Ago protein (Argonaut) and RISC is composed.¹⁹ Dicer is encoded by DICER1 gene in human. It cuts pre-miRNA and double stranded RNA (dsRNA) leading to siRNA and mature microRNAs. The resulting fragments are around twenty-two base pairs long with two base pair extension on the 3' end²⁰. RISC is a ribonucleoprotein complex which has an ability to target and silence a nucleic acid sequence (degrade mRNA)²¹. As explained in Figure 1.2, after RISC complex formation there are different outcomes depending on which part of mRNA is targeted. Possible results are: mRNA degradation, translational repression when 3' UTR is targeted; translational repression, translational activation when 5' UTR is targeted; translational repression when coding sequence is targeted. Additionally, microRNAs cannot be in RISC complex but interact with some proteins (AGO2 & FXR1) which have effects on regulation so those microRNAs upregulate expression of target gene as a second-hand effector²².

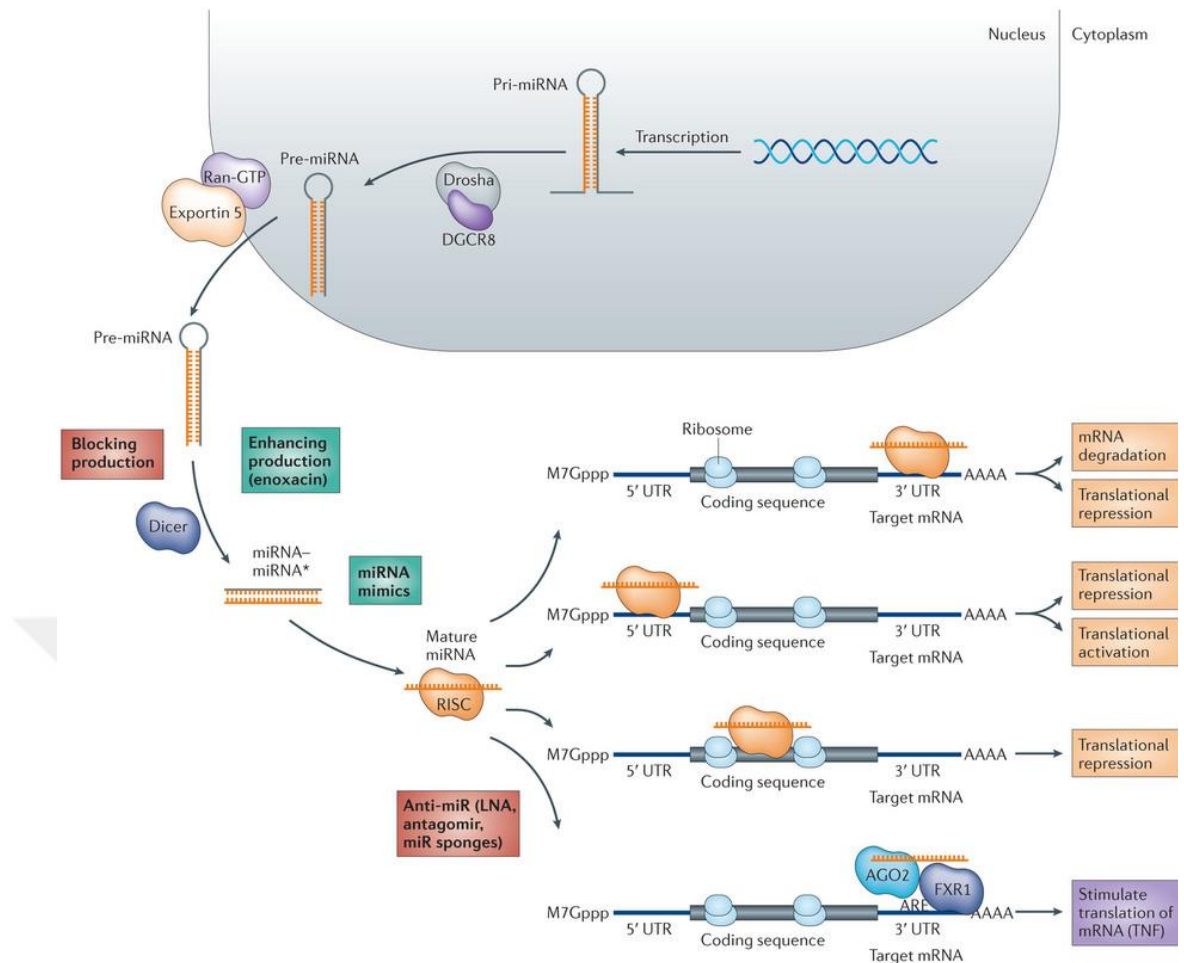


Figure 1.2: microRNA action mechanism and therapeutic blocker or activator of their function. (Figure was taken from “Ling, H., M. Fabbri, and G.A. Calin, *MicroRNAs and other non-coding RNAs as targets for anticancer drug development*. Nat Rev Drug Discov, 2013. 12(11): p. 847-65.” by permission of Nature Publishing Group.)

Regulation of microRNAs does not only happen in development²³, proliferation, differentiation²⁴ or apoptosis but also during tumorigenesis²⁵. microRNA gene locations seem near to important parts of chromosomes that correlate with tumour progression or chromosome abnormality²⁶. Since microRNAs have regulatory effects on gene expression, changes in microRNA expression tend to be related to diseases. As expected, current data suggest that microRNAs can be considered as potential biomarkers to use in the diagnosis of diseases (e.g., cancer²⁷, diabetes²⁸ or cardiovascular²⁹ disorders etc.). More than one mRNA can be targeted by a single microRNA. Also one mRNA can be regulated by multiple microRNAs. Thus network production by using integration between microRNAs-mRNAs expression has led researchers to discover cancer biomarkers (microRNAs as cancer biomarker). microRNAs can be useful not only at the treatment level but also help detection of disease phase. In Cambridge Healthtech

Institute's 10th Annual microRNA as Biomarkers and Diagnostics Conference, in 2014, the main question to answer was how to detect a disease earlier by the help microRNAs. The resulting achievement of the conference has been that microRNAs don't just help diagnose the disease but give hints about stratification of patients and treatment response³⁰. There are many different processes which microRNAs are involved such as cell cycle³¹, metabolism³², development³³. In development microRNAs have effective roles because of their potential to control cell growth and differentiation. Therefore, microRNAs tend to be used also in cancer therapy which share control of the same processes. There are different ways to modulate microRNAs and thus the related processes: microRNA can have a single point mutation preventing their binding, or mRNA which is targeted can have a mutation, or microRNA transcription can be silenced epigenetically³⁴. Lastly there is a wide range of research on microRNAs which supports that they can be used in treatment of diseases and or to diagnose them³⁵.

1.2.1. microRNAs Related to Cancer

In different human cancer types, there is an expression difference between patient and healthy groups depicted as an increase or decrease³⁶. Cleavage of targets is seen more commonly in oncogenic microRNAs' functional patterns. Although oncogenic microRNAs are placed in the amplified sequences, tumour suppressor microRNAs can be found in deleted parts of the genome in general³⁷. Two tables consisting of different microRNAs show expression changes in different cancer types. Common or uncommon microRNAs are seen in different cancer types. For example, the expression of mir-191, in both ovary and breast cancers, is upregulated. On the other hand, mir-199a is not seen to be changing in breast cancer³⁶.

Table 1.2: microRNAs and related cancer types (breast, ovary, colon, AML, CML, CLL, endometrioid adenocarcinoma)

Cancer Type	miRNA	Up/Down Regulation	References
Breast	miR-21 ,miR-155, miR-23, and miR-191	Up	(57,58)
	miR-205,miR-145,miR-10b,and miR-125b	Down	
Ovary	miR-200a,miR-200c, and miR-141	Up	(61)
	miR-199a,miR-140,miR-145,and miR125b1	Down	
Colon	miR-135,miR-21,miR-15b,miR-181b,miR-191,miR-200c	Up	(72,79)
	miR143,miR145,miR-133b,and miR-126	Down	
AML	Has-miR-191,199a,miR155	Up	(83,86)
CML	miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-19b-1,	Up	(91)
	miR- 20a, and miR-92a-1		
CLL	miR-21,miR-155	Up	(96)
	miR-15a,miR16,miR-29, miR143,miR-45,miR-30d,miR-let7a,miR-181a	Down	(32, 40, 94, 96)
	miR-205,miR-449, miR-429	Up	
Endometrioid adenocarcinoma	miR-193a,miR-204, miR-99b	Down	(100)

Taken from “Naeini, M.M. and A.M. Ardekani, *Noncoding RNAs and Cancer*. Avicenna J Med Biotechnol, 2009. 1(2): p. 55-70.” by permission of Creative Commons.

Table 1.3: microRNAs and related cancer types (Esophagus, Gastrointestinal, lung, bladder, thyroid tumours)

Cancer Type	miRNA
Esophagus	miR_194 miR_192,miR_200c
Gastrointestinal	miR-106b-25
	miR-15b,miR-16
Lung	miR-17-92
	let- 7
Bladder	miR-29c, miR-26a, miR-30c, miR-30e-5p,miR-145, miR-30a-3p, miR-133a, miR-133b, miR-195, miR-125b and miR-199a
Thyroid tumors	
-PC	miR-146b, miR-221, miR-222, miR-181b, miR-155,miR-224
-AC	miR-30d, miR-125b, miR-26a, miR-30a-5p

Taken from “Naeini, M.M. and A.M. Ardekani, *Noncoding RNAs and Cancer*. Avicenna J Med Biotechnol, 2009. 1(2): p. 55-70.” by permission of Creative Commons.

1.2.1.1. microRNAs related to Breast Cancer

Several microRNAs have been shown to be related to breast cancer formation due to their effects on breast cancer specific genes³⁸. These microRNAs are separated into different classes: tumour suppressors, oncogenic (oncomiRs) and lastly metastatic microRNAs. Tumour suppressor microRNAs' targets consist of oncogenes. Metastatic microRNAs

have roles in the process of changing a tissue from epithelial to mesenchymal, e.g., mir-9, mir-10b, mir-21, miR-29a are some of the metastatic microRNAs³⁹. Some microRNAs provide protection from metastases: miR-20, miR-30, miR-193b, miR-146, mir-31 while Let-7, mir-200 microRNA families and mir-205, mir145 are tumour suppressors⁴⁰. Examples to OncomiRs include mir-15, mir-16, mir155, mir-21 family etc.⁴¹.

1.2.1.2. microRNAs related to immune system and cancer

There are specific microRNAs related to the immune system. Innate and adaptive immune systems may be regulated by microRNAs specifically. mir-144⁴², mir-150⁴³, mir-10⁴⁴, mir21⁴⁵, mir-155⁴⁶, mir-146a⁴⁷, mir-125b⁴⁸ and let-7⁴⁸ are involved in innate immune cell development. mir-132⁴⁹, mir-150⁵⁰, mir-34a⁵¹, mir-155⁵², mir-146a⁵³, mir-21⁵⁴ are involved in immune function and B/T cell development. The general map of map of microRNAs in the immune system and where each one has role are shown⁵⁵.

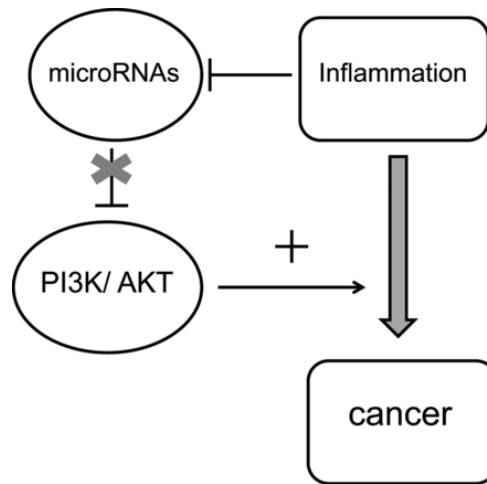


Figure 1.4: Connection among microRNAs inflammation and cancer by the expression change of elements are involved in PI3K/Akt Signaling pathway in colon cancer. (Figure was taken from “Josse, C., et al., *Identification of a microRNA landscape targeting the PI3K/Akt signaling pathway in inflammation-induced colorectal carcinogenesis*. Am J Physiol Gastrointest Liver Physiol, 2014. 306(3): p. G229-43.” Permission was not required.)

Table 1.4: In Tumour cancer and immune related functions of microRNAs.

Cancer type	miRNA	Expression status and cell localization ^b	Target	Immune-related role	Cancer-related role	Ref ^c
Breast	↑miR-10b	Cancer cells	↓MICB	Suppression of NK-mediated killing of tumor cells	Metastasis development ^d	[107]
	↑miR-19a-3p	M ₂ Macrophages	↓FRA-1	Macrophage polarization	Inhibition of cancer progression and metastasis development	[95]
	↑miR-21	Cancer cells	↓PIAS3	Reduced chemokine production and lymphocyte migration, immunoresistance to cancer immunotherapy ^d	Cancer cell survival, cell proliferation	[137]
	↓miR-23a/27a/24-2	Macrophages	↑A20 ↑JAK1 ↑STAT6	M ₂ Macrophage polarization	Xenograft tumor growth	[97]
	↓miR-126/126 ^a	Cancer cells	↑SDF-1a	Downregulation of Ccl2 expression, Suppression of Inflammatory monocyte recruitment	Repression of MSC recruitment, lung metastasis promotion	[100]
	↓miR-146a	Cancer cells	↑IRAK1 ↑TRAF6	Modulation of inflammation ^c	Cell Invasion and Migration impairment (NF-κB signaling block)	[92]
	↑miR-155	Cancer cells	↓SOCS1	STAT3 signaling activation	Cancer cell proliferation, colony formation, and xenograft tumor growth	[141]
		Myeloid cells	↓SHIP1	Tumor-infiltrating innate immune cell recruitment	Antitumor activity	[98]
	↑miR-223 ^a	M ₂ macrophages and cancer cells	↓MEF2C ^d	Macrophage differentiation ^d	Promotion of cancer cell invasion	[163]
	↑miR-494	MDSCs	↓PTEN	Accumulation of MDSCs	Tumor cell invasion and metastasis development	[117]
Gastric	↓miR-146a	Cancer cells	↑IRAK1 ↑TRAF6 ↓IL8	Modulation of inflammation ^d	Antitumor activity	[146]
Ovarian	↑miR-20a	Cancer cells	↓MICA/B	Suppression of NK-mediated killing of tumor cells	Long-term cellular proliferation, invasion capabilities	[104]
	↓miR-199a	Cancer cells	↑IKKβ	Cytokine production	Tumor progression, chemosensitivity (NF-κB signaling modulation)	[148]
Colorectal	↑miR-424	Cancer cells	↓PDL1 ↓CD80	T cell activation	Chemosensitivity	[151]
	↓miR-17-5p/ miR-20a/miR-124	MDSCs	↑STAT3	Inhibition of immunosuppressive potential of MDSCs	Tumor growth	[142–144]
Hepatocellular	↑miR-21/miR-29b ^a	Cancer cells and immune cells	↑IL-6 (Indirectly)	Activation of pro inflammatory immune cells ^d	Promotion of cancer cell invasion, tumor progression ^d	[164]
	↑miR-20a, miR-96, miR-106b	Cancer cells	↓MICA	Suppression of NK-mediated killing of tumor cells	Long-term cellular proliferation, invasion capabilities ^d	[108]
HBV ^e /Hepatocellular	↓miR-34a	Cancer cells	↑CCL2	Regulation of Treg recruitment	Suppression of tumor growth/metastasis development	[113]
Melanoma	↓miR-34a/c	Cancer cells	↑ULP2	Suppression of NK-mediated killing of tumor cells	Cell cycle arrest, senescence, apoptosis	[111–112]
	↓miR-17	T cells	↑STAT3	Impairment of T cell response ^d	Tumor growth ^d	[142]
Melanoma and Lewis lung cancer	↓miR-155	Immune cells	↑HIF1a	Recruitment of MDSC cells to tumor microenvironment	Promotion of tumor growth ^d	[118]
Lung	↑miR-23a	T cells	↓BLIMP1	Suppression of CD8 ⁺ T cell function ^d	Tumor progression, TGF-β-mediated immune evasion ^d	[145]
Glioma	↓miR-124	T cells	↑STAT3	Impairment of T cell responses	Tumor growth	[143]
Various solid tumors	↑miR-29	Cancer cells	↑B7-H3	Inhibition of NK and T cell function ^d	Protumor activity ^d	[114–115]
	↓miR-214 ^a	Cancer cells and CD4 ⁺ CD25 ⁺ T cells	↓PTEN	Expansion of Treg cells	Promotion of tumor growth	[166]

^a Detailed mechanism involving microvesicles-cell interactions (see also subsection "MicroRNAs and cell-to-cell communication")

^b These data are referred to studies in either tissue samples or in vitro/in vivo models

^c Reference number listed in bibliography

^d Further investigation is needed

Upregulation of miRNA or miRNA target, Downregulation of miRNA or miRNA target

HBV, Hepatitis B Virus; MDSCs, Myeloid-Derived Suppressor Cells; NKs, Natural Killer cells; TAMs, Tumor Associated Macrophages

Modified from “Paladini, L., et al., *Targeting microRNAs as key modulators of tumor immune response*. J Exp Clin Cancer Res, 2016. 35: p. 103.” by permission of Creative Commons.

1.2.2. microRNAs Related to Cholinergic Receptor/ Nicotine

Several microRNAs have roles in nicotinic acetylcholine receptor signalling. mir-140-3p is a nicotine sensitive microRNA ⁶⁰. Expression of mir-124 increases after LPS exposure to cells by cholinergic agonists. Mir-124 stops TACE and cause TNFa downregulation and also targets STAT3 activator and IL6 expression decreases ⁶¹. Mir-132 and mir-212 also have roles in cholinergic signalling ⁶². In mesocorticolimbic reward pathway,

nicotine arranges mir-542-3p level. When mir-542 is upregulated, CHRNB2 is downregulated⁶³. For primates and rodents, acetylcholine formation is regulated by mir-149⁶⁴. miR-199a-5p, miR-200b and miR-429 are predicted target butyrylcholinesterase and acetylcholinesterase receptor⁶⁵. There are specific mRNA-miRNA couple in primates: mir-608 targets acetylcholinesterase⁶⁶. Additionally, in mammals, mir-132 targets acetylcholinesterase and it is conserved couple⁶⁷. SNPS in CHRNA5 and NRXN1 are related with smoking. For NRXN1 SNP, the sequence is homolog to mir-6740-5p when common allele is seen and mir-6866-5p when risk allele exists⁶⁸. Mir-10a and mir-615-3p target CHRNA5⁶⁹.

1.2.3. hsa-mir-497

1.2.3.1. Features and Relationship with Breast Cancer

Hsa-mir-497 is the miRBase name of a microRNA found in human. MIR497HG (ENSG00000267532) is the host gene of the microRNA and is also named as mir-195. It is found on chromosome 17 from 7,015,818bp to 7,019,659bp, its size is around 4000 bases⁹. From the first intron of MIR497HG gene, mir-497 microRNA is encoded. It is categorized as a mir-15 family member⁷⁰, or miR-16/15/195/424/497⁷¹. In NCBI GeneRIFs hsa-mir-497 seems to be related to bladder cancer, colorectal cancer, gastric cancer, ovarian cancer, lung cancer, renal cancer, pancreatic cancer, cervical cancer and breast cancer.

Firstly, a breast cancer study indicates a change in mir-497 expression⁷². In a study of breast cancers in males mir-497 has been shown as one of the most downregulated microRNAs⁷³. Another paper indicates that mir-497 is downregulated in HCC⁷⁴. Also in other cancer types mir-497 is downregulated, e.g., colorectal cancer⁷⁵, bladder cancer⁷⁶, ovarian cancer⁷⁷. These suggest that this microRNA acts as a tumour suppressive in general. However, in new studies an opposite role (oncogenic) also has been recognized in some other cancer types: large B-cell lymphoma⁷⁸ and chronic lymphocytic leukaemia⁷⁹. When recent studies are combined with old ones the result is that, hsa-mir-497 can act both of the roles, as tumour suppressor or promoter.

In breast cancer, miR-497 correlates negatively with the size of tumour, metastasis and HER-2 status but seems not correlated with ER or PR status and p53 gene expression⁴⁰. Upregulation of the microRNA causes a decrease in expression of anti-apoptotic Bcl-2

family member (Bcl-w) whose downregulation is inhibited in cellular growth and enhanced apoptosis so mir-497 acts as a tumour suppressor. Therefore, mir-497 expression level can be used as a biomarker for diagnosis ⁸⁰. In breast cancer, mir-497 targets different genes and has a role in different processes: VEGFR-2/angiogenesis ⁸¹, CCNE1/Cell cycle ⁸² and Bcl-2/apoptosis ⁸³.

1.2.3.2. hsa-mir-497 Targets and Related pathways

Apoptosis

Bcl-2 decreases apoptosis rate; thus, it is anti-apoptotic. In breast cancer Bcl-2 is targeted by mir-497 ⁸³; and also in chronic lymphocytic leukaemia ⁷⁹ and gastric cancer ⁸⁴. A similar functioning molecule Bcl-w is another target of mir-497. Mir-497 binds to 3'UTR of Bcl-w and inhibits its effect on reducing apoptosis. The expression of Bcl-w in mRNA and protein level decreases via the effect of mir-497 ⁸⁵. These two anti-apoptotic molecules also promote migration and invasion of cells by involvement of downstream molecules as EGFR, PI3K and MMP-2 ⁸⁶.

Cell Cycle

There are genes which have roles in cell cycle regulation. In addition they are targets of mir-497: eIF4E (Eukaryotic translation initiation factor 4E) ⁸⁷, CCNE1 (G1/S-specific cyclin-E1) and CDK4 (Cyclin-dependent kinase 4) ⁹. In different cancer types, there is an increase in the expression of mir-497 level ⁸⁸. CCNE1 binds to CDK2 (Cyclin-dependent kinase 2) and activates it to change G1 to S phase ⁸⁹. In breast cancer and hepatocellular carcinoma, mir-497 targets CCNE1 and blocks CCNE1 mRNA. Thus, G1 arrest happens and tumour growth is inhibited ^{9, 82}.

PI3K/AKT, MAPK/ERK and NF-kappa B signalling pathways

Another critical target of mir-497 is VEGF-A, since it has a role in angiogenesis. In HCC, angiogenesis and metastasis are reduced by mir-497 by the help of targeting VEGF-A ⁹⁰. mir-497 inhibitors induce p-Akt upregulation because of VEGF-A upregulation. The increase of p-Akt upregulates Bcl-2 and CCND1; also promotes osteosarcoma cell growth ⁹¹. When an ovarian cancer cell is transfected with mir-497, the expression levels of downstream molecules of VEGF-A in signalling (AKT, ERK1, ERK2) display reductions. These reductions result in an inhibition of the rate of angiogenesis ⁹². VEGF-A, together with VEGFR-2 are important in the anti-angiogenesis actions; The expression levels of both of them are estimated to be reduced by mir-497 via pathways: PI3K/AKT

and MAPK/ERK ⁹³. Mir-497 inhibits cell growth and human umbilical vein endothelial cells' apoptosis in breast cancer by targeting VEGFR-2 ⁸¹. There are two more receptors: IGF-1R (insulin like growth factor receptor 1) and insulin receptor (IR) regulated by mir-497. Mir-497/IR or mir-497/IGF-1R are tumour suppressor/oncogene couples. mir-195 and mir-497 targets include Igf1r, Insr, Ccnd2 and Ccne1 in myoblasts. In myogenesis NF- κ B-miR-195/497-Igf1r/Insr-Ccnd2/Ccne1 signalling pathway is important ⁹⁴. In cervical cancer, mir-497 also is downregulated. The microRNA binds to 3' UTR of IGF-1R and reduces its expression level ⁹⁵. In colorectal cancer, inhibition of metastasis, proliferation and invasion are provided by mir-497 targeting IGF-1R and PI3K/Akt signalling pathway regulation ⁹⁶. In cytoplasm, when IKK β (Ik β kinase) phosphorylates, phosphorylated Ik β (NF κ B inhibitor) releases NF κ B and translocation signal is formed then NF κ B goes into the nucleus and binds to DNA. As a result of the binding, several steps in cancer are promoted: metastasis and invasion, tumour growth or angiogenesis ⁹⁷. There is a binding site on IKK β mRNA 3' UTR and when mir-497 binds to this site, it blocks NF κ B release and hence mir-497 acts as a tumour suppressor. There is also a feedback mechanism: mir-497 blocks the NF κ B release from IKK and also NF κ B can block mir-497 ⁹⁴.

mir-497 Relation to Immune System and Inflammation

There are examples that mir-497 has a relation to immune system regulation. It is one of eight microRNAs found to be down-regulated (significantly) in glaucomatous retinae causes up-regulation of immune system related genes ⁹⁸. Type 2 diabetes is a complex disease; microarray result show mir-497 is one of the microRNAs up-regulated in diabetic GK rats more than two fold. Additionally, statistical tests with genes show that genes (differently expressed in disease) maybe related to immune system ⁹⁹. Granulocytic airway inflammation has subgroups (e.g. allergic airway diseases) and there is a connection between granulocyte recruitment and microRNAs. miR-497 is one of the candidate microRNAs with a role in neutrophil recruitment and as a regulator of inflammation on the airway ¹⁰⁰.

Mir-497 and Calcium

There are microRNAs regulate cardiac calcium signalling (e.g. mir-494, mir-145, mir-1) ¹⁰¹. Also, in a study related with stroke, there are microRNAs shown related with Ca²⁺ influx. Mir-223 has role in the reduction of excitotoxicity of the influx. microRNAs (mir-

497, mir-320, mir-29b, mir-181) block pro-survival genes as exitotoxic Ca²⁺ influx¹⁰². On the other hand, mir-34a blocks pro-cell death gene which is promoted by exitotoxic Ca²⁺ influx. Antagomirs effect on mir-497 reduces severity when there is a neurological damage¹⁰³.

More Targets

There are more targets of mir-497. SMURF1, ANLN, NRDP1, BIRC5, WNT7A are directly repressed by mir-497. YAP1 is repressed by mir-497¹⁰⁴. mTOR¹⁰⁵ and KSR1¹⁰⁶ are also repressed by mir-497 and leading to less chemosensitive cells. WEE1 (retinoblastoma)¹⁰⁷, PDCD4 (glioma)⁷⁸, HDGF (lung cancer)¹⁰⁸ and AMOT (osteosarcoma)¹⁰⁹ are some other targets of mir-497. As it was explained under the title “microRNAs” DICER is important in mature microRNA formation. There is another molecule “TARBP2” which is bound to DICER in the process of binding non-mature microRNA together with Ago2. It is suggested that in adrenocortical carcinoma DICER and TARBP2 are direct targets of mir-497 by the help of Ago2 IP; mir-497 can down-regulate both of them. There is a feedback: pre-mir-497→ mature mir-497--| TARBP2&DICER→ transition from pre-mir-497 to mature mir-497¹¹⁰.

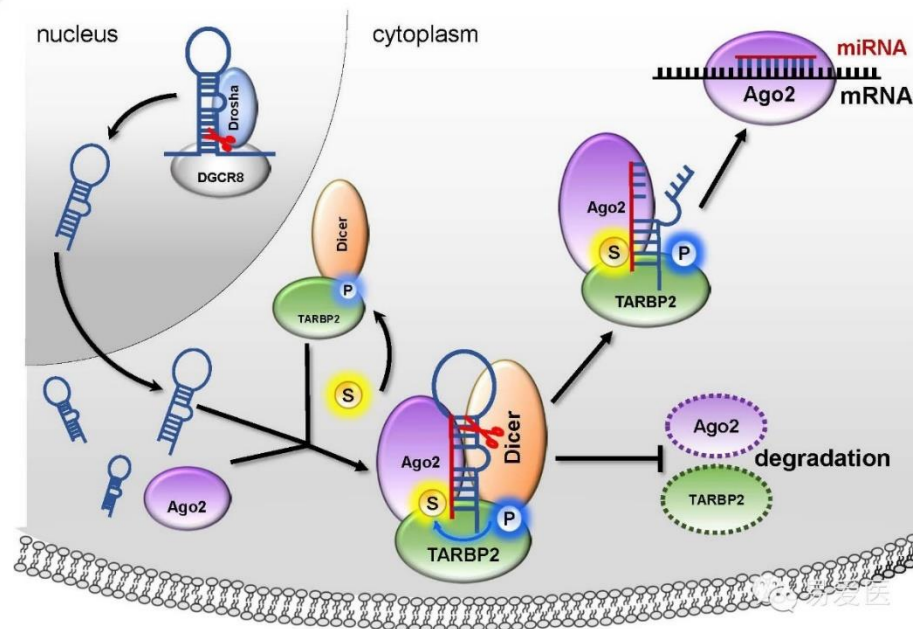


Figure 1.5: SUMOylation of TARBP2 model (S: SUMOylation and P:Phosphorylation
Figure was taken from “Chen, C., et al., *SUMOylation of TARBP2 regulates miRNA/siRNA efficiency*. Nat Commun, 2015. 6: p. 8899.” by permission of Creative Commons.)

1.3. Cholinergic Receptor Nicotinic Alpha

One of the important factors leading to lung cancer and even other cancer formations is smoking. Nicotine is contained in smoke and is a ligand of ligand gated ion channels/receptors (nAChRs: neuronal nicotinic acetylcholine receptors) to which ligand acetylcholine also binds. CHRNA5 is one of the nAChRs. It is encoded from Chromosome 15. On this chromosome CHRNA4 and CHRNA3 are also encoded (15q25). CHRNA5 affects cell proliferation in lung cancer ¹¹¹. There are two common SNPs on CHRNA5 and CHRNA3 in lung cancer ¹¹². Rs16969968 is a SNP on CHRNA5; CHRNA5 containing this SNP has less protectiveness against nicotine/CHRNA7 mediated signalling. CHRNA5 and CHRNA3 knockdown make Ca influx increase. The cause can be explained as more CHRNA7 formation to support the lack of other nAChRs ¹¹³. Other subunits which are involved in acetylcholine receptor with CHRNA5 are $\beta 2$ and $\alpha 4$. From miRNet.ca, for $\alpha 4$ and $\alpha 5$, there is no microRNA validation. However, there are thirteen microRNAs target $\beta 2$. Most of the microRNAs were not studied deeply. After nicotine binds to nAChRs, calcium influx happens and signalling pathways (e.g. PI3K/Akt) are affected. Proliferation and blocking on apoptosis are two examples of the result of the nicotine binding ¹¹⁴. Many lung and breast cancer cell lines have expression of CHRNA9, CHRNA5 and CHRNA10. Nicotine is not a carcinogen. However, in cancer cells nicotine induces cell growth (e.g. breast cancer). Nicotine exposure level promotes CHRNA9 expression in breast cancer ¹¹⁵. CHRNA5 might be involved in estrogen signalling ¹¹⁶. Nicotinic receptors show some connection to breast cancer but the relations between CHRNA5 and microRNAs are not known.

1.4. Association of CHRNA5 and microRNAs from Unpublished/Previous Studies

CHRNA5 was detected as an important gene for breast cancer in our previous unpublished data. In the study CHRNA5 was silenced resulting in phenotypic changes and differential expression of genes related to proliferation or apoptosis under siRNA CHRNA5 treatment in MCF7 cells (Ermira Jahja et al. submitted; TUBITAK 111T316; Affymetrix HGU133 Plus 2 microarrays for siRNA and scrambled oligo treated cells). Differentially expressed mRNAs were shown as targets of microRNAs and microRNA array was performed in the context of 114S367 project (MCF7 cells treated with siRNA

CHRNA5 / scrambled oligo). The aim was to find microRNAs antagonist or synergistic with CHRNA5. The achievement can be a biomarker and used in a treatment with CHRNA5. The microRNA array results gave a group of candidate microRNAs; they and their targets are being analysed to confirm the hypothesis (Tubitak Project 114S367; Cıngır Köker et al. unpublished).

1.5. Expression Measurement Technologies

1.5.1. Microarray

Nowadays the information obtained from expression levels is very important for diagnosis and treatment of human diseases. Microarray is a chip-based technology to obtain the expression levels of molecules (mRNA, microRNA) simultaneously. The chip consists of a surface (glass or silicon), and genetic materials to differentiate different molecules. The array is 2D. The array technology has become popular in recent years ¹¹⁷. Different companies provide different chips as Affymetrix, Agilent, etc. ¹¹⁸.

1.5.2. qRT-PCR using Sybr Green

Real-time PCR or qRT-PCR is a quantification technology of RNA molecules when converted into cDNA and amplified in real-time in molecular biology. Fluorescent dyes (eg. Sybr Green) bind to cDNA are measured by a fluorescent detector at each cycle of the amplification by giving values for a process period and not just at the plateau level. Quantification cycle C_q is set for the log linear phase of the amplification process and they can be compared to obtain relative expression of an RNA in comparison with other RNAs and an internal standard. Primers can be designed for specific gene locations ¹¹⁹ while using housekeeping molecules as GAPDH ¹¹⁹ for genes and RNU6 ¹²⁰ for microRNAs.

1.6. In Silico Analysis by Using Expression Matrices

There are three main categories on analysing expression matrices results together: a) online tools, b) desktop tools, c) customized programming (R, matlab etc.). Online tools are most time saving and user-friendly although the choices are limited. Desktop tools give more detailed results, have more options to choose. They require more time but give

better results if there is a need for a deeper understanding. Programming is the best to develop a novel idea; however, it needs bioinformatics knowledge. In this section of introduction, I will briefly describe online tools that I have used to identify a) targets of hsa-mir-497; b) hsa-mir-497 target networks in HCC; and c) related pathways to a group of gene d) immune gene and microRNA networks in HCC. Among these tools; MSigDB¹²¹, miRWalk¹²², miRNet¹²³, MAGIA2¹²⁴ and MirTarVis^{125, 126} are the most useful ones as a guide for further in vitro experimental steps.

1.6.1. miRWalk

miRWalk 2.0 is a new version of miRWalk. In this tool, user does not need to load in any microRNA and mRNA expression data while he/she needs to upload a gene list or a microRNA list (or individual mRNA or microRNA name). After mRNA-miRNA network tools (e.g., MAGIA2) miRWalk 2.0 can be used to see if chosen microRNA-mRNA integration has been validated in the literature. Another option is to use predicted target search. It provides a new algorithm and also a chance to see targets from different predictors in a list. miRWalk 2.0 provides the information from 14 species in addition to human: monkey, chimpanzee, orangutan, rat, mouse, pig, chicken, dog, cow, opossum, fruit fly, zebrafish, worm and frog. There are two modules: validated and predicted. There are eleven predictors, predicting integration between mRNA and microRNA: mirDB¹²⁷, PITA¹²⁸, microT4¹²⁹, miRMap¹³⁰, RNA22¹³¹, miRanda¹³², miRNAMap¹³³, RNAhybrid¹³⁴, miRBridge¹³⁵, PICTAR2¹³⁶ and TargetScan¹³⁷. For valid integrations, automated text mining is the method combined with the data of existing programs: miRTarBase¹³⁸, PhenomiR¹³⁹, miR2Disease¹⁴⁰ and HMDD¹⁴¹. Nearly fourteen thousand publications, three thousand microRNAs, nineteen thousand genes and their 151.5 million relations are used in miRWalk. Five hundred forty-six pathways and twenty-eight protein classes and four hundred and fifty diseases are accessed by miRWalk 2.0 as related concepts of integrated mRNA-microRNA¹²².

1.6.2. MAGIA2

MAGIA2 is one of the most useful and fast tools for mRNA-microRNA data integration and prediction. MAGIA2 was reconsidered and redesigned to improve the quality of the results by giving chance for more user defined options. MAGIA2 combines the results of

the different target predictors and detect better integrations. The organism content is bigger in MAGIA2 as human, rat, *Drosophila* and mouse microRNAs are included. MAGIA2 has a novel component since it identifies microRNAs and transcription factors that regulate microRNA-mRNA networks. MAGIA2 also has advantages over target prediction algorithms since it uses both the mRNA and microRNA data obtained from the same conditions (paired) or different conditions/experiments (unpaired). To use MAGIA2, ensemble IDs of genes and miRBase names of microRNAs are uploaded in the .csv files format for both microarray experiments. The tool gives the information on how many of the IDs in each file are valid among all IDs uploaded. Once files are uploaded, user can do analyses immediately or later since the data could be saved for later use. microRNA target prediction is done by using the knowledge of TF binding sites between microRNA and promoter of the target gene. Matched and unmatched samples can be used and depending on what kind of sample is used a different analysis method can be selected on the next step (i.e., Spearman Correlation, Pearson Correlation, Mutual Information or Meta-Analysis). When there are unmatched samples, sole analysis method is Meta-Analysis. microRNA-mRNA couples are found by using Inverse-Chi-Squared Distribution of differentially expressions p-values by LIMMA in Meta-Analysis. If gene and microRNA data are matched; there are three choices and the choice depends on the sample size. From small to bigger sample size, the order is as in the following lines: Spearman Correlation is used for three to 5 samples; Pearson Correlation is for more than five samples; Mutual Information is used for bigger data (more than twenty samples). There are differences between these three methods. Spearman Correlation is a non-parametric while Pearson is a parametric method; Spearman uses rank-based linear correlation and it is useful when the data do not have normal distribution inversely to Pearson Correlation. Mutual information method is again for non-linear relations and mutually dependent variables. The tool gives the user a warning when uploaded data and the analysis method don't match to each other. The next step is to choose a target predictor: DIANA microT ¹²⁹, TargetScan ¹³⁷, Pita ¹²⁸, Microcosm ¹⁴², PicTar ¹⁴³, mirDB ¹²⁷, RNA22 ¹⁴⁴ and microrna.org ¹²⁶. There is a possibility to choose more than one predictor. For each target predictor, one should decide on the stringency of the prediction and produce more than one list which are then combined by a Booleand operator. User gives a name to each analysis and the results are saved on MAGIA2. MAGIA2 results in not only the network but also text files that contain downloadable lists of interactions. The result figure gives 200 best interaction depend on the options chosen between TFs,

genes and microRNAs. There is a direct link to DAVID for functional enrichment. For only matched samples, positive or negative correlation can be distinguished by the colour of the arrow between hubs (red arrow: positive correlation, blue arrow: negative correlation). TFs, microRNAs and target genes can be distinguished by the shape and the colour of the hubs (TF: Green circle, target genes: blue circle, microRNA: orange triangle). Direction of the arrow states which regulates which molecule ¹²⁴.

1.6.3. MSigDB

The Molecular Signatures Database is a database which has more than ten thousand gene sets which are divided collections as: hallmark, curated, positional, computational, GO, motif gene sets and oncogenic, immunologic signatures. Gene set enrichment analysis use MSigDB. In “Investigate Gene Sets” option you can import a set of gene names and get the most related pathways, diseases or molecular function kind of information about the group of gene which you load. It directs the search by showing the most common feature of a group of gene ¹²¹.

1.6.4. MiRNet

Previously done microRNA studies guide the new studies on commenting and analysing statistically in miRNet ¹²³. In the tool the input options are various: gene set, microRNA set, microarray, RNA seq, lncRNA, small molecule, disease, epigenetic modifier or RT-qPCR. Eleven databases, three differential analysis and two enrichment analysis methods are used. It is more of an improved online tool (resembles a desktop tool) with options of different input types, analysis methods and last step modifications on downloaded images. Zooming and different network styles are allowed. For example, when there is a network, members of network can be analysed depending on the function and then can be coloured ¹²³. MiRNet can be used to get experimental targets of a given microRNA or microRNAs bind to a list of mRNAs modulated at the same time.

1.6.5. MirTarVis

MirTarVis is one of the desktop tools to make the mRNA-microRNA microarray integration analysis. There are four steps in MirTarVis analysis: data loading, filtering the data separately, integrated mRNA-microRNA prediction and visualization. First step asks

the kind of file to be uploaded because there are different choices: paired/unpaired, expressions for each sample/ directly fold changes and p-values, multi sample, RNA-seq and miRNA-seq type. Files should be .csv, microRNA names should be miRBase IDs and gene names should be gene symbols. First step should be downloading of the tool. The folder provides tool “miRTarVis.jar” and example input files for different data (multiple samples/ paired two sample/ unpaired two sample / p value and fold-change). At least Java Runtime Environment 7+ and 1280*720 screen resolution is required. After files are arranged as in the example input files which are provided inside of the downloaded folder, they directly uploaded without filtration. Analysis starts with the filtration separately on microRNA or mRNA and filtering may depend on p-value, fold change or both in the tool. User decides on the cut-off value (e.g. $p < 0.05$). Prediction is the third step after filtration. MirTarVis uses 2 algorithms for the prediction which depend on sequences: TargetScan¹³⁷ and microrna.org¹²⁶ and 4 algorithms which depend on expression: Correlation Analysis, MINE, Mutual Information and GenMiR. Correlation Analysis is the simplest and the best working one among them. Information of which resource or which species, and how many interactions from top are seen are decided by the user. Additionally, correlation (positive or negative) and direction of the change can be chosen before starting the analysis. Negative correlation could be selected when a microRNA targets a gene because the presence of this microRNA blocks the expression of that particular gene. The list composed of mRNA fold change, mRNA ID, microRNA ID, mRNA p- value, microRNA fold change, microRNA p-value and the correlation value cannot be downloaded but can be copied. Visualization is the last step containing two different visualization methods: Node Link Diagram or Treemap. Node Link Diagrams are done by different algorithms (ISOM layout, KK layout, Circle layout, force directed layout). The images are colour coded and able to edit.¹²⁵

Chapter 2. Aim

The aims of this thesis are multifold:

- 1) To understand the relationship between mir-497-5p and CHRNA5/cancer a) identify mRNA targets of mir-497-5p and b) analyse the functional enrichment of mir-497-5p using MSigDB tools.
- 2) To test by qPCR a) whether mimic mir-497 modulates expected targets obtained in above aim in MCF7 cells and b) whether, CHRNA5, a previously identified regulator of mir-497-5p modulates the expression of mir-497 family in MCF7; c) whether, mimic alone or mimic together with CHRNA5 siRNA modulates selected targets and d) whether chromosomal location of mir-497-5p has any effect on its expression and expression/regulation.
- 3) To understand the relationship between mir-497-5p and immune system a) filter the immune gene set from GSE41074 and make mRNA-miRNA network for specific immune genes b) to test immune microRNAs by qPCR.
- 4) To analyse mimic treated cancer samples (GSE41079 and GSE41079) in silico a) for a general understanding of mimic-mir-497 impacts on mRNAs and microRNAs in HCC by using mRNA-microRNA integration tools (MAGIA and MIRTARVIS) b) to compare Ago2-IP and Total RNA results and mimic-mir195 and mimic-mir-497 results.

Chapter 3. Materials and Methods

3.1. In Silico Analysis

3.1.1. Msigdb, MiRWalk, miRNet

MSigDB: “Investigate Gene Sets” option was used. The genes uploaded were obtained from GSE41074. After log₂ fold changes were found by subtracting non-treated samples from mimic treated samples, the log₂ fold change values are sorted for each treatment. Total RNA mimic-mir-497 treatment and Ago2-IP mimic-mir-497 treatment results were used. 0.5 and -0.5 are limits for log₂ fold change. Upregulated genes and downregulated genes by these treatments were taken as 4 groups: Total RNA mimic-mir-497 treated samples upregulated genes, Total RNA mimic-mir-497 treated samples downregulated genes, Ago2-IP mimic-mir-497 treated samples upregulated genes and Ago2-IP mimic-mir-497 treated samples downregulated genes. When one of the groups were loaded with the gene identifiers REACTOME was chosen under “Compute Overlaps” and significant 50 pathways were found in order of significance.

MiRWalk: miRWalk 2.0 tool was used to get the information about validated/predicted targets of microRNAs. To find predicted targets of mir-497-5p candidate genes’ official gene symbols were uploaded in “Predicted Target Model (Gene-miRNA Targets)” of miRWalk. There are 12 target-predictors and all of them were chosen for each gene. The result was analysed to see if mir-497-5p targeted the candidate gene or not by how many predictors.

miRNet: It gives information of validated targets of microRNAs in a network. Moreover, genes can be grouped by looking for features of targets as pathways that the genes were involved. Mir-497-5p was uploaded and target genes were shown. Selections were human (organism), miRBase ID (ID type), Genes (target type). Without any filtering there were many genes, therefore most related pathways to mir-497-5p were chosen to focus on. Pathways were found by KEGG/REACTOME by hypergeometric test of all target genes. To find which microRNAs are related to CHRNA5 mRNA was chosen by selection human (organism) and official symbol (ID type). There were just three microRNAs so no further limitation was needed.

3.1.2. MAGIA2

There is a need for two files: mRNA and microRNA with same treatments. GSE41079 and GSE41074 consisted mRNA and microRNA arrays for Ago2IP samples for same treatments (mimic-mir-497 and non-treated sample). Data should be put as a tab delimited text file with sample names and gene/microRNA ID in the first column. “Homo sapiens” was chosen as organism. Target predictor was chosen as TargetScan, correlation method was Spearman and there was no limitation on data. There was no mandatory sample name but the order should be same for both files. Ensembl ID or Entrez gene ID can be used for mRNAs and mirBase names are used for microRNAs. Sample file format was as in Table 3.1.

Table 3.1: File format for gene and microRNA data in MAGIA.

gene						
id	C	C	C	T	T	T
ENSG...						
microRNA						
id	C	C	C	T	T	T
hsa-mir-...						

3.1.3. MirTarVis

The first step was to arrange data suitable to upload into the tool. There had to be two files should be uploaded before starting analysis: microRNA and mRNA. There were two choices to make if expression values were uploaded directly: unpaired analysis and paired analysis. Column names labelled differently when it is paired or unpaired. Column names of paired analysis were 0_pre, 0_post 1_pre, 1_post... Pre and post meant two different conditions as tumour vs. normal samples. Paired values were near to each other in paired analysis. Unpaired analysis did not need pre and post values near to each other because they were not paired they cannot match to each other. The first column started with “1_” and columns continue as 2, 3, 4... Both files contained columns with the same name and the same sample at the same location.

Table 3.2: File format for paired and unpaired samples in MirTarVis.

For paired analysis

gene ID	0_pre	0_post	1_pre	1_post

miRNA ID	0_pre	0_post	1_pre	1_post

For unpaired analysis

gene	1_pre	2_pre	3_post	4_pre

miRNA	1_pre	2_pre	3_post	4_pre

Load Data, Filter, Predict and Visualize are four subtitles of MirTarVis analysis. Prepared files were uploaded under “Load Data”. No filter on fold-change or p value was used before uploading into the tool because there is a subtitle as “Filter” in MirTarVis. If only significant expression values and more filtered fold changes was needed to continue, filtration could be done during analysis. Third subtitle of MirTarVis analysis was “Predict”. Prediction style as correlation analysis, MINE, Mutual Information etc. could be selected. Correlation analysis was performed in this study.

3.1.4. MATLAB

GSE41079 and GSE41074⁹ were analysed by comparing samples and the results were reported in the Appendix. Ago2-IP vs. Total RNA results for the same treatment or different treatments in same collection conditions were compared (e.g. Ago2IP mimic-mir-497 vs mimic-mir-195, Total RNA mimic-mir-497 vs. mimic-mir-195). There were 6 samples for mRNA: Ago2-IP non-treated, Ago2-IP mimic-mir-497, Ago2-IP mimic-mir-195, Total RNA non-treated, Total RNA mimic-mir-497, Total RNA mimic-mir-195. There were three samples in microRNA array result: Ago2-IP non-treated, Ago2-IP mimic-mir-497, Ago2-IP mimic-mir-195. Plots, heatmaps and histograms of the samples with different combinations are handled from MATLAB. There are limitations on some plots, and the rest used all values/unlimited. Limitation was done by taking sum of LogFC values under two treatments for a molecule and limit the sum for being more than a value (indicated under figure).

3.2. In Vitro Analyses

3.2.1. Cell culture

3.2.1.1. Cell Culture Materials and Preparation of Reagents

For this study, MCF7 cell line was used (ATCC; Manassas, USA); cells have been cultured in Laboratories of Molecular Biology and Genetics Department, Bilkent University. MCF7 is a breast cancer cell line and features of MCF7 are as in the following lines: source is pleural effusion, tumour is invasive ductal carcinoma (IDC), age is 69, and ethnicity is white. MCF7 cells are ER+, PR+ and HER2-; and its molecular subtype is luminal A. Cell culture condition is provided by the media and here is the preparation of the complete growth medium: Into the bottle of DMEM (BE12-707F, Lonza, Basel, Switzerland) following reagents were added: 10% Filtered FBS (S181G, Biowest, Nuaille, France), 1% Penicillin/Streptomycin Solution (DE17-602E, Lonza, Basel, Switzerland), 2% L-glutamine (BE17-605E, Lonza, Basel, Switzerland), %1 NEAA (BE13-114E, Lonza, Basel, Switzerland), %1 Sodium Pyruvate (BE13-115E, Lonza, Basel, Switzerland). Mixture of 90% FBS and 10% DMSO was used as freezing medium. Other reagents are: MTT (M-6494, Invitrogen, Carlsbad, CA, ABD), Trypsin/EDTA solution (BE17-161E, Lonza, Basel, Switzerland), PBS (BE17-516F, Lonza, Basel, Switzerland), Dimethyl sulfoxide Cell culture grade (A3672,0100, AppliChem, Darmstadt, Germany), Phenol Red Free DMEM (11880-028, Gibco, ABD).

3.2.1.2. Thawing Cells

Frozen cells were stored in liquid nitrogen tanks. A vial of frozen cells (or vials, according to the need) was taken from liquid nitrogen cell stock and were put in the ice bucket. The vial of frozen cells immediately was transferred to the cell culture. The vial was placed into the water bath (37°C) until the cells melted (less than ~1 min.). Cells were added slowly into the centrifuge tube containing 5ml of complete medium. Cells were centrifuged for 5 min. at 1500 rpm for the removal of DMSO. The supernatant was discarded without disturbing the cell pellet; 7ml of complete growth medium was added in the tube. The pellet was dissolved in the medium gently; seeded in a T25 flask (Greiner Bio One, Frickenhausen, Germany) and the flask was transferred to the incubator. The next day the flask confluency was checked. After reaching at least 70% confluency; cells were transferred to the T75 flask (Greiner Bio One, Frickenhausen, Germany) by using routine passaging procedure.

3.2.1.3. Passaging Cells

Continuous cell culture experiments accompany passaging cells. T75 flask containing 80% or more confluent cells (not more than 100%) had been passaged. Complete growth medium, PBS, and trypsin were kept waiting into the water bath (37°C). The flask confluency was checked under the microscope. Old medium from the confluent flask was discarded. PBS was poured onto the cells; swished gently and aspirated from the flask. Added 5ml PBS made cells washed with the aim of the removal of DMEM's inhibitory effect on trypsin which was used in the following step. Cells were trypsinized with 1.5 ml and they were transferred into the incubator and placed 3 minutes or until the cells detached (exceed amount or incubation time for trypsin is harmful to the cells). The flask was removed from the incubator and checked under the microscope to be sure that cells were detached. Complete media volume was at least twice of the trypsin volume (>3ml) to suppress trypsin effect (not to harm cell membrane with long duration of trypsin). Before putting into the centrifuge tube (15ml) cells was pipetted carefully and re-suspended in the media. Cells were centrifuged for 5 minutes at 1500 rpm. Cells re-suspended in the new complete growth medium by pipetting. Depend on the next experiment, required cell amount changed.

3.2.1.4. Counting Cells

Hemocytometer and glass coverslips were cleaned by using 70% alcohol. Old medium was aspirated and cells were washed twice by PBS (5ml), then PBS was aspirated. Cells were trypsinized in 1.5 ml of trypsin solution and incubated (37C) for 3 minutes. Flask was removed from the incubator; complete medium was added (at least twice of the volume of trypsin) and cells were re-suspended. Cells were spinning at 1500 rpm for 5 minutes; supernatant was discarded. Cells dissolved in complete growth medium (pipetting is needed not to leave cells clamped to get an accurate result). 10µl from the suspended cells were taken into the new Eppendorf tube and 10µl Trypan blue solution 0.4% (T8154-20, Sigma Aldrich, Missouri, ABD) were mixed and 10µl from the mixture was applied to the hemocytometer (death cells take up Trypan blue solution). Cells were counted in each 4 (4x4) squares (not by taking outsider edges into account) and average of the counts is handled.

$$\text{Cell count} = \text{Average} \times 2 \text{ (because of trypan blue dilution)} \times (10^4) \times (\text{how much growth media used for the last re-suspension in ml})$$

3.2.1.5. Freezing Cells

Passaging steps were repeated however cells were not re-suspended in the complete growth medium but they were dissolved in the freezing medium after centrifugation (the amount of the freezing medium depend on the cell count as 1 million cells = 1ml freezing medium addition in this step). To each cryovial 1million cells were split and vials were stored for the first one to two hours at -20°C; they were carried over to - 80°C. The last stop was the liquid nitrogen on the purpose of the long-term storage.

3.2.1.6. Treatment (siRNA, Scrambled oligo, mimic microRNA)

MCF7 cells were seeded in 6 well plates (200.000 cells/well) one day before the treatment. Treatment reagents are transfection reagent and siRNA/ mimic of microRNAs/ scrambled oligo or combinations of them. Required reagents are siRNA CHRNA5 (SI03051111, Qiagen, Hilden, Germany), Syn-hsa-miR-497-5p miScript miRNA Mimic (MS00031906, Qiagen, Hilden, Germany) and scrambled oligo negative control siRNA (1022076, Qiagen, Hilden, Germany) and HiPerfect Transfection Reagent (301705, Qiagen, Hilden, Germany). There are four groups of samples: siRNA CHRNA5 (10nM, si111) and scrambled oligo (10nM) were used on MCF7 cells as treatment for 72 hours (five biological replicates for each treatment); siRNA CHRNA5 (si53, 10nM), siRNA CHRNA5 (si40, 10nM) and scrambled oligo (10nM) are used on MCF7 cells as treatments for 72 hours (2 biological replicates); siRNA CHRNA5 (10nM, si111) and scrambled oligo (10nM) are used on MDA-MB-231 cells as treatment for 72 hours (2 biological replicates). The last treatment was done with siRNA CHRNA5 (si111), mimic-mir-497 and scrambled oligo, details are in Table 3.3.

Table 3.3: Mimic treatment for further RNA isolation procedure

Name of the treatment	Ingredients per well
Scrambled 10nM	12ul transfection reagent + 1 ul scrambled oligo /well
siRNA CHRNA5 10nM	12ul transfection reagent + 1 ul siRNA CHRNA5 /well
mimic mir-497-5p 10nM	12ul transfection reagent + 1 ul mimic mir-497-5p /well
Scrambled 20nM	12ul transfection reagent + 2 ul scrambled oligo /well
siRNA CHRNA5 20nM	12ul transfection reagent + 2 ul siRNA /well
Scrambled 10nM & mimic-mir-497-5p 10nM	12ul transfection reagent + 1 ul scrambled oligo + 1 ul mimic mir-497-5p /well
siRNA CHRNA5 10nM & mimic-mir-497-5p 10nM	12ul transfection reagent + 1 ul siRNA CHRNA5 + 1 ul mimic mir-497-5p /well

The treatments could be done as well in 96 well-plates after formulas reduced down as 1/10 ratio.

3.2.1.7. MTT assay

MCF7 cells were seeded in 96 well plates (3.000 cells/well) one day before the treatment. Treatment reagents were transfection reagent and siRNA of the gene/ mimic of

microRNAs/ scrambled oligo or combinations of them. Required reagents were siRNA CHRNA5 (SI03051111/ SI03096940/ SI03099453, Qiagen, Hilden, Germany), Syn-hsa-miR-497-5p miScript miRNA Mimic (MS00031906, Qiagen, Hilden, Germany) and scrambled oligo negative control siRNA (1022076, Qiagen, Hilden, Germany) and HiPerfect Transfection Reagent (301705, Qiagen, Hilden, Germany). Treatments are done as in the table below.

Table 3.4: Treatments for further MTT procedure

Name of the treatment	Ingredients per well
DMEM	No treatment at all
TR	1.2 ul transfection reagent + 0.2 ul ddH ₂ O /well
Scrambled 10nM	1.2ul transfection reagent + 0.1 ul scrambled oligo /well
siRNA CHRNA5 10nM	1.2ul transfection reagent + 0.1 ul siRNA CHRNA5 /well
mimic mir-497-5p 10nM	1.2ul transfection reagent + 0.1 ul mimic mir-497-5p /well
Scrambled 20nM	1.2ul transfection reagent +0. 2 ul scrambled oligo /well
siRNA CHRNA5 20nM	1.2ul transfection reagent + 0.2 ul siRNA /well
scrambled & mimic mir-497-5p (total 20nM)	1.2ul transfection reagent + 0.1 ul scrambled oligo + 0.1ul mimic mir-497-5p /well
siRNA CHRNA5 & mimic mir-497-5p (total 20nM)	1.2ul transfection reagent + 0.1 ul siRNA CHRNA5 + 0.1ul mimic mir-497-5p /well

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	-	-	-	-
B	-	DMEM	TR	scrambled	siRNA CHRNA5	mimic mir-497-5p	sc	si	sc +mimic	si+mimic	-	-
C	-	DMEM	TR	scrambled	siRNA CHRNA5	mimic mir-497-5p	sc	si	sc +mimic	si+mimic	-	-
D	-	DMEM	TR	scrambled	siRNA CHRNA5	mimic mir-497-5p	sc	si	sc +mimic	si+mimic	-	-
E	-	DMEM	TR	scrambled	siRNA CHRNA5	mimic mir-497-5p	sc	si	sc +mimic	si+mimic	-	-
F	-	DMEM	TR	scrambled	siRNA CHRNA5	mimic mir-497-5p	sc	si	sc +mimic	si+mimic	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

Figure 3.1: Experimental design of treatment before MTT (grey parts should not be included in the experiments, - means no treatment there only DMEM to avoid vaporization)

Three days (72 hours) after treatment MTT protocol started. Experimental protocol of Vybrant® MTT Cell Proliferation Assay Kit (V-13154) was performed. MTT (M-6494, Invitrogen, Carlsbad, CA, ABD) was prepared as 12mM stock by mixing 1 mL sterile PBS and 5mg MTT in a tube covered by aluminium foil. They were vortexed and filtered before the usage and stored at most 4 weeks at 4°C. SDS-HCl was prepared by mixing 10 mL of 0.01M HCl and 1gram SDS (71725, Sigma Aldrich, USA) in a glass bottle by inversion until SDS powder solved. Firstly, media was discarded and 100µl new media is added (phenol free complete DMEM) to each well. 10µl MTT reagent is added on top of it. After four hours incubation at 37 °C 100µl/well SDS-HCl was added. At most eighteen hours incubation at 37 °C was necessary before taking results. Absorbance was read at 570 nm.

3.2.2. Gene Expression Analysis

3.2.2.1. RNA Extraction

miRNeasy mini kit (217004, Qiagen, Hilden, Germany) was used for the total RNA extraction (mRNA at the same time miRNA) from untreated and treated samples (with CHRNA5 siRNA, scrambled, mimic of candidate microRNA (hsa-mir-497-5p) separately or in a combination. After treatment (because MCF7 was grown in a monolayer) cells were directly lysed in the cell culture by aspirating medium and Qiazol lysis reagent was poured onto the samples and samples were homogenized. Five minutes incubation at room temperature was applied. Chloroform was added on top of the homogenate (as 1/5 volume of qiazol). Three minutes incubation at room temperature was applied. Sample was centrifuged at 12.000g at 4C for 15 minutes for phase separation. Without any interface, upper (aqueous) phase was transferred to the collection

tube and 100% ethanol (1.5x volume of the transferred upper phase) was poured into the tube. The spin column was placed into a new collection tube (2ml) and sample (700µl) applied to the column. Centrifuge was run at room temperature for 15 secs at $\geq 8.000g$. Last two steps were repeated for the remainder. 700µl RWT buffer was put on the column and centrifuge was run same as the last time. Liquid into the tube was discarded and column was placed onto the 2ml tube back. 500µl RPE buffer was poured on top of the column and centrifuge was run same as the last step. Same amount of RPE was added after flow-through was discarded and centrifuge was run this time for 2 minutes with the same speed. Until this step total RNA was bound to the column and other compounds were washed away. RNase-free water was applied in the middle of the column for elution of the RNA from the column in a new collection tube (1.5ml). NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA) was used for the measurements of the samples. RNA was stored at -80°C .

3.2.2.2. cDNA synthesis

Reverse transcription of total RNA containing miRNA (was handled by using miRNeasy mini kit as in the part 3.1.2.1) was performed by two different kits for different purposes: miScript II RT Kit (218160, Qiagen, Hilden, Germany) and RevertAid First Strand cDNA Synthesis Kit (K1621) (Thermo Fisher Scientific, Massachusetts, USA).

RevertAid First Strand cDNA Synthesis Kit:

Necessary RNA volume for 1ng cDNA synthesis for each sample is calculated at the first step. Afterwards, H₂O is added to complete the volume up to 11µl. 1µl oligo(dT), 4 µl Reaction Buffer (5x), 1 µl RiboLock RNase Inhibitor, 2 µl dNTP Mix (10mM), 1 µl RevertAid M-MuLV Reverse Transcriptase are added in this order into the tube. Mixtures were incubated for sixty minutes at 42°C . In the next step they were incubated for five minutes at 70°C . Samples were stored at -20°C .

miScript II RT Kit:

There are two buffers for different extractions. HiSpec buffer was used in this study. The obtained cDNA by using HiSpec buffer was used to make qPCRs to profile expression level of the mature microRNA and snoRNAs. Firstly, RNA samples were thawed on ice and kit reagents (miScript Nucleics Mix (10x) and miScript HiSpec buffer (5x)) were thawed at room temperature. The solutions should be mixed by finger flicking and briefly centrifuged. 4 µl miScript HiSpec buffer, 2 µl 10x nucleics mix, 2 µl reverse transcriptase

mix, RNA (vary between 10ng- 2µg) were mixed and RNase free water was added to complete volume up to 20 µl into a tube on ice. Reverse transcriptase should be taken from the freezer before addition and should be back immediately after usage. Mixture should be centrifuged briefly and incubated at 37 °C for sixty minutes, 95 °C for five minutes respectively. Store the solution on ice. If qPCR was the next step, cDNA was diluted (1:10).

3.2.2.3. Primer

Primer design was done by identifying the sequence of interested gene by NCBI. Gene name was written after “Gene” was selected from the Resource List of NCBI. The coming page showed different species that included the gene of interest, interested species is selected. REFSEQ ID which included “NM_” was selected to reach the mRNA sequence. There is a possibility to have more than one mRNA RefSeq ID for the same gene. In such a case, longest sequence was chosen to get FASTA sequence. By clicking on “CDS”, only coding sequence was shown on the full sequence. By clicking bottom right “FASTA”, fasta sequence of coding region was handled. (There is a second possibility: necessity of specific primers to two exons of the gene. In this case, exons for forward primer and reverse primer were chosen on the main page and FASTA sequences of them are taken to further design.) There are different tools as Primer3web version 4.0.0 or Primer-BLAST. Fasta sequence of the gene of interest was pasted in the tool and possible primers were observed. One of the options was chosen by caring following criteria: One primer on exon-exon junction was needed, GC content should have been 50-60%, TM value should have been between 50-65 °C, no more than three Gs or Cs existed, end of primers had Gs or Cs, amplicon size were between 70-200bp and primer size were between 18-30bp. Additionally self-complementarity should be low and primers should match perfectly to a region of the Fasta sequence that put by user unlike any random Fasta sequence by chance. For this reason, primers were checked after being designed if primer blast was not used during the process.

Table 3.5: Primer sequences of genes negatively correlated to hsa-mir-497-5p

Primer name	Sequence 5'→3'
CCNE1_F	GCCAGCCTTGGGACAATAATG
CCNE1_R	CTTGACGTTGAGTTTGGGT
TNRC6B_F	GAAGCACTCGCCCTCTCATC
TNRC6B_R	ACCAACCATGCCATAGTCCAT
ZMAT3_F	AGAAGCCTTTTGGGCAGGAG
ZMAT3_R	TGCTGCATAGTAATTTCCGAGTT
CADM1_F	GTCCCACCACGTAATCTGATG
CADM1_R	CCACCTCCGATTTGCCTTTTA
LAMC1_F	ACTGCCACTGACATAGAGTA
LAMC1_R	GCTTGCGTGTCCATTACATTTAC
PAM_F	TTCGTGATTGACTTCAAGCCTC
PAM_R	CTCGCCCAGGCATACAGAAT
Hs_CCNG1 Fwd	GTCTGCACACGATAATGGCC
Hs_CCNG1 Rev	AAAGCAGCTCAGTCCAACAC
HsC17ORF49_F	CCTTTCTCCTGCACCCCAA
HsC17ORF49_R	TGCTGATCCTCCTCTCCTCT

Table 3.6: Qiagen Primer Assays

Primer name	Cat.no.	Details
		targets mature miRNA: hsa-miR-23a-3p
Hs_miR-23a_1 miScript Primer Assay	MS00031633	MIMAT0000078: 5'AUCACAUUGCCAGGGAUUUCC
		targets mature miRNA: hsa-miR-34c-3p
Hs_miR-34c-3p_1 miScript Primer Assay	MS00009548	MIMAT0004677: 5'AAUCACUAACCACACGGCCAGG
		targets mature miRNA: hsa-miR-146a-5p
Hs_miR-146a_1 miScript Primer Assay	MS00003535	MIMAT0000449: 5'UGAGAACUGAAUCCAUGGGUU
		targets mature miRNA: hsa-miR-221-3p
Hs_miR-221_1 miScript Primer Assay	MS00003857	MIMAT0000278: 5'AGCUACAUUGUCUGCUGGGUUUC
		targets mature miRNA: hsa-miR-497-5p
Hs_miR-497_1 miScript Primer Assay	MS00004361	MIMAT0002820: 5'CAGCAGCACACUGUGGUUUGU
		targets mature miRNA: hsa-miR-497-3p
Hs_miR-497*_2 miScript Primer Assay	MS00031906	MIMAT0004768: 5'CAAACCACACUGUGGUGUUAGA

3.2.2.4. qPCR

Two methods were used (gene expression, microRNA expression).

For gene expression:

A mixture for each gene was prepared by putting the following reagents by multiplying two more than needed well number. 5µl LightCycler 480 SYBR Green I Master (04887352001, Roche Applied Science, Germany) + 1µl nuclease free water + 1µl reverse primer(1/10diluted) + 1µl forward primer (1/10 diluted). They were distributed to each well on 96 well plate (Light Cyclor 480 Multiwell plate 96) as 8µl mixtures and 2µl cDNA (1/10 diluted) was added on top of it. There were technical replicates and a control without cDNA but nuclease free water. The plate was put in Light Cyclor 480 PCR Instrument and the program was as in the table.

Table 3.7: qPCR Program for gene expression in Light Cycler 480 PCR Instrument		
Name of the qPCR steps	Temperature	Time
Initial Denaturation	95°C	5min
Denaturation (45cycles)	94°C	20sec
Annealing	Specific for primers	15sec
Extension	72°C	15sec
Acquisition	95°C	5sec
	55°C	1min
	95°C	Continuous
Final Extension	40°C	30sec

For microRNA expression:

A mixture for each gene was prepared by putting the following reagents by multiplying two more than needed well number. miScript SYBR Green PCR Kit (200) (218073, Qiagen, Germany) was used. 2x QuantiTect SYBR Green PCR Master Mix + 2µl nuclease free water + 1µl primer assay + 1µl 10x miScript Universal Primer. They were distributed to each well on 96 well plate (Light Cycler 480 Multiwell plate 96) as 9µl mixture and 1µl cDNA (1/10 diluted) is added on top of it. There were technical replicates and a control without cDNA but nuclease free water. The plate was put in Light Cycler 480 PCR Instrument and the program was as in the table.

Table 3.8: qPCR Program for microRNA expression in Light Cycler 480 PCR Instrument		
Name of the qPCR steps	Temperature	Time

Initial Denaturation	95°C	15min
Denaturation (45cycles)	94°C	15sec
Annealing	55°C	30sec
Extension	70°C	30sec
Acquisition	95°C 55°C 95°C	5sec 1min Continuous
Final Extension	40°C	30sec

Analysis of the data was done by relative quantification method. Averages of technical replicates were calculated. Average of all biological replicates of scrambles (sc10nM sc20nM separately) were calculated and used as control/s. There were two scrambles for mimic treatment and they belonged to different treatment amounts (10nM, 20nM). Following calculations were done and results were used for further analysis.

$$\text{Log}_2 (\text{Gene} [[\text{Control(sc)}\text{-treatment(siRNA/mimic)}]^2] / \text{TPT} [[\text{Control(sc)}\text{-treatment(siRNA/mimic)}]^2])$$

$$\text{Log}_2 (\text{microRNA} [[\text{Control(sc)}\text{-treatment(siRNA/mimic)}]^2] / \text{U6} [[\text{Control(sc)}\text{-treatment(siRNA/mimic)}]^2])$$

After calculation of log2 values for each treatment, the significance was checked with statistical tests.

3.2.2.5. Statistical Analysis

GraphPad Prism 6.0 was used for statistical analysis. For only siRNA CHRNA5 and scrambled qPCR results unpaired ttest was used (two-tailed). More samples existed in mimic treatment. Therefore, one-way ANOVA was done by using Tukey's multiple comparison test. Significance was seen in the test results by looking p values. Results of calculations explained in qPCR part were used in graphpad. Tukey's multiple comparison test was used to see the relation and different between each sample in the experiment.

3.2.2.6. Microarray Study

microRNA array and mRNA microarray of siRNA CHRNA5 treatment were analysed for the mir-497 and selected microRNAs as well as targets of mir-497. Two biological replicates for each treatment resulted RNA (2 siRNA and 2 scramble) were sent for microarray study to AYKA (Cingir-Koker, unpublished; 114S367). Affymetrix miRNA 4_0 was used as a microRNA array. 2 biological replicates for each siRNA CHRNA5 and scrambled oligo treatments were used for mRNA microarray (111T316; Jahja et al. unpublished) using Affymetrix HGU133 plus 2 arrays. RMA normalization and Affymetrix Expression Console used for normalization and annotations.



Chapter 4. Results

4.1. Integrated mRNA-microRNA Couples by siRNA and mimic treatments

4.1.1. mir-497 as a candidate microRNA related to CHRNA5 in breast cancer

Hsa-mir-497-5p is a microRNA which shows up as a downregulated microRNA in the absence of CHRNA5 based on studies conducted in Konu lab (114S367). As explained in microRNA microarray analysis of siRNA CHRNA5 & scrambled experiment the affected microRNAs have been sorted according to LogFC (unpublished). mir-497-5p was within top 10 of affected microRNAs in the list of negatively affected genes from of siRNA CHRNA5 treatment.

Hsa-mir-497-5p showed a decrease when the microRNA microarray results were analysed (average log2 fold change: ~ -0.9). Same as in the microarray result, CHRNA5 siRNA reduced the expression level of hsa-mir-497-5p by qPCR experiment significantly (p value = 0.0012) (Figure 4.1). The same trend, as in the Figure 4.1 A, was observed when different siRNA reagents were used (si53 p value = 0.0699, si40 p value = 0.0666) (Figure 4.1 B).

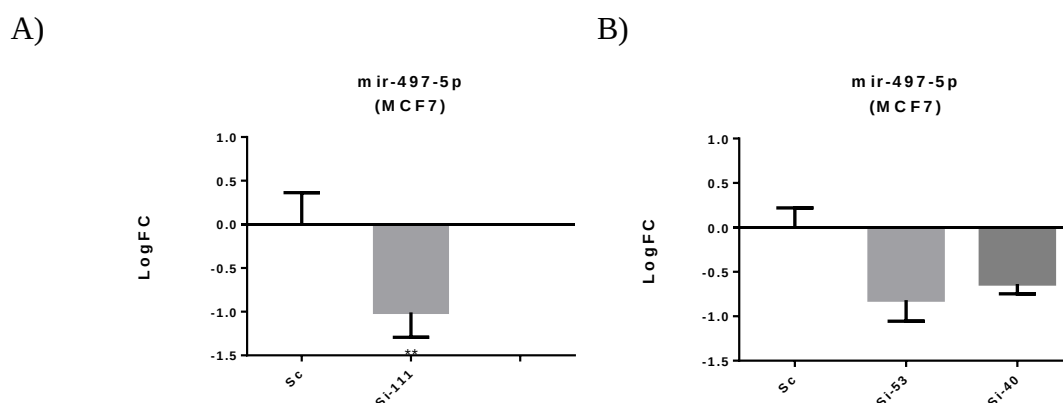


Figure 4.1: mir-497-5p expression level in siRNA CHRNA5 treated MCF7 cells A) Si-111 reagent was used for five biological replicates of scrambled oligo and siRNA CHRNA5 treatments B) Si-40 and Si-53 were used for two biological replicates of scrambled oligo and siRNA CHRNA5 treatments. (* significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ val} \leq 0.0001$)

As in different siRNA reagent, a different cell line (MDA-MB-231) showed the same decreasing trend with p value 0.1173

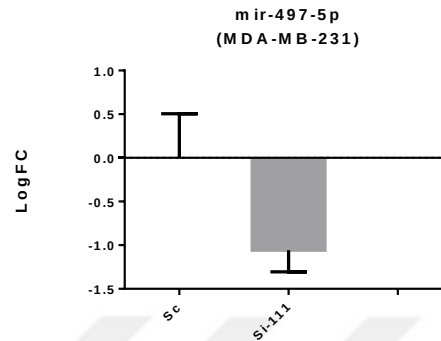


Figure 4.2: Mir-497-5p expression levels when SiRNA CHRNA5 treatment was applied with the same siRNA CHRNA5 reagent (Si-111) in MDA-MB-231 cell line. (p val. = 0.1173)

After mir-497 was chosen as a candidate, GSE41079 and GSE41074 public datasets were found by a literature search from GEO ⁹. Mimic-mir-497 and mimic-mir-195 treatments on HepG2 HCC cells were used in both series to identify mRNA and microRNA expression profiles. These datasets were used to identify targets to test in the presence of miR-497 mimic application in the future sections of this thesis.

4.1.2. Determination of target Genes of mir-497

For further experiments, there is a need to select candidate related target mRNAs and coexpressed microRNAs. Therefore, *in silico* leading points were achieved to create a list of target genes and also a set of immune related microRNAs were selected by literature search. hsa-mir-497-5p and related genes were also obtained through miRNet. There were 45 significant related KEGG pathways for mir-497-5p in the miRNet result and 14 of them had a p-value less than 0.001. The most related pathway was PI3K-Akt signalling pathway. P53 Signaling Pathway was related to breast cancer and it was also highly significantly in relationship to mir-497-5p according to miRNet (Table 4.1). Several mir-497 targets related to these two pathways were further investigated.

Table 4.1 : List of KEGG pathways related to mir-497-5p obtained from miRNet analysis
(P value < 0.001)

KEGG pathway name	Hits	P value
PI3K-Akt signalling pathway	28	0.0000324
Pathways in cancer	29	0.0000891
Cell cycle	15	0.0000901
Acute myeloid leukaemia	10	0.000157
Signaling pathways regulating pluripotency of stem cells	15	0.000304
Viral carcinogenesis	18	0.000356
Renal cell carcinoma	10	0.000356
p53 signalling pathway	10	0.00041
Small cell lung cancer	11	0.000469
Non-small cell lung cancer	9	0.000469
Insulin signalling pathway	14	0.000498
Chronic myeloid leukaemia	10	0.00052
Hepatitis B	14	0.000735
Focal adhesion	17	0.000742

CCNE1 and LAMC1 were found to be targets of mir-497 and also were related to PI3K-Akt signalling pathway that was most significantly connected to mir-497 (Figure 4.3).



Figure 4.3: hsa-mir-497-5p network with PI3K-Akt Signaling Pathway genes by miRNet (KEGG).

CCNE1 and ZMAT3 also were targets of mir-497 and found within the P53 Signaling pathway (Figure 4.4).

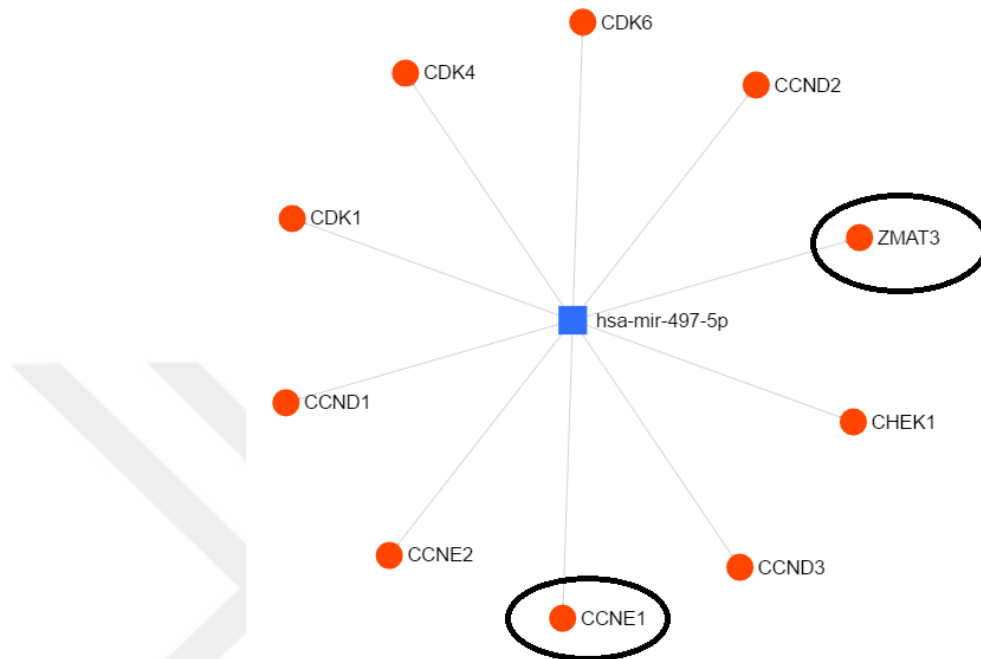


Figure 4.4: hsa-mir-497-5p network with P53 Signaling Pathway genes by miRNet (KEGG).

There were two options to see related pathways to the central microRNA (or gene), KEGG and REACTOME. Same steps were repeated with the REACTOME option in MiRNet. Gene Expression included maximum number of genes related to mir-497 significantly and TNRC6B was selected as candidate from the network (Figure 4.5).

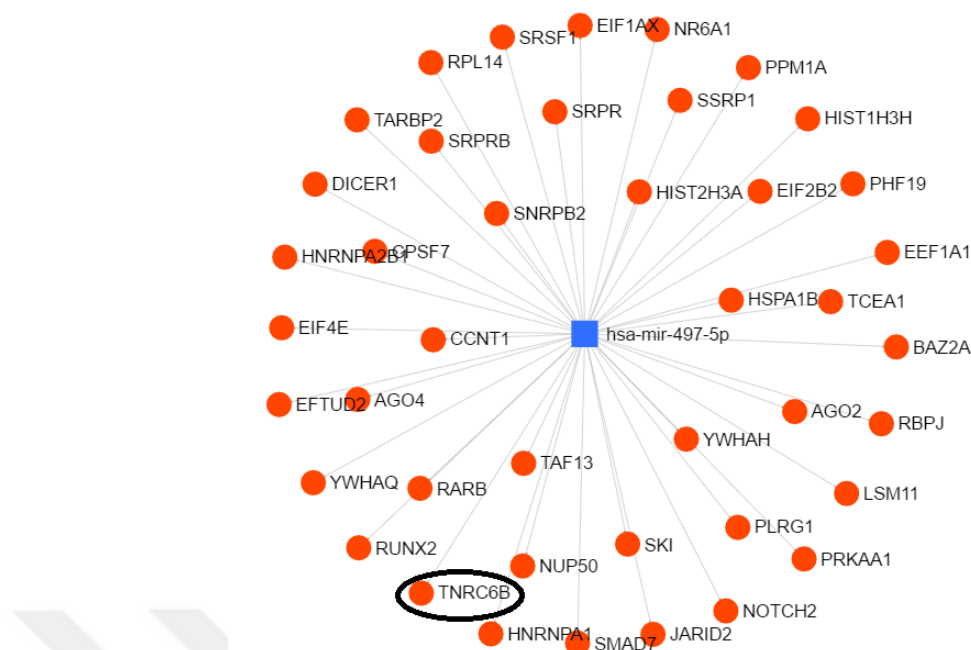


Figure 4.5: hsa-mir-497-5p network with genes from REACTOME maximum hits involved pathway (Gene Expression) in miRNet.

Next, miRWalk was used as a tool to predict targets or show validated targets. Different lists were obtained for a starting point. Table 4.2 shows one way of selecting the genes used in *in vitro* qPCR experiments in the future sections. LAMC1 was highly predicted (10/12 ratio) as a target of mir-497 also in miRWalk. All genes were predicted at least in 4 predictors (Table 4.3). In addition, microarray mRNA expression results from the siRNA treated MCF7 cells (111T316 unpublished results) were used to finalize the list of targets (Table 4.2). When the genes were sorted depending on the log2 fold changes under siRNA CHRNA5 treatment values ZMAT3 and LAMC1 were the most upregulated genes among the genes under siRNA CHRNA5 treatment. CCNE1 was slightly downregulated in under CHRNA5 depletion (Table 4.2).

Table 4.2: : Expression Profiles of mir-497 target genes from mRNA microarray (siRNA CHRNA5 Treatment; Data obtained from Tubitak Project111T316)

HGNC symbol	siRNA CHRNA5-1	siRNA CHRNA5-2	Scrambled oligo-1	Scrambled oligo-2	Avg LogFC
TNRC6B	7.423	7.567	6.385	6.383	1.111
LAMC1	9.587	9.631	7.809	7.843	1.783
ZMAT3	9.214	9.417	7.487	7.259	1.943
CADM1	7.580	7.476	6.337	6.187	1.266
CCNG1	11.696	11.835	10.348	10.314	1.434
CCNE1	7.801	7.585	8.273	8.224	-0.556
CHRNA5	4.478	4.401	6.952	7.158	-2.616
PAM	9.278	9.284	8.066	7.882	1.307

These genes were predicted by at least 4 predictors in miRWalk (Table 4.3). Also they showed disease relations (Table 4.4).

Table 4.3: Experimental genes' prediction out of twelve different predictors in miRWalk.		
Gene	miRNA	#Predictor
LAMC1	hsa-miR-497-5p	10
CCNE1	hsa-miR-497-5p	9
CADM1	hsa-miR-497-5p	8
ZMAT3	hsa-miR-497-5p	8
TNRC6B	hsa-miR-497-5p	6
PAM	hsa-miR-497-5p	5
CCNG1	hsa-miR-497-5p	4

In Table 4.4 disease classes related to selected genes were shown.

Table 4.4: Related disease classes to selected genes in DAVID ^{145, 146}.

Gene Symbol	Disease Class
CADM1	CARDIOVASCULAR, CHEMDEPENDENCY, IMMUNE, METABOLIC, NEUROLOGICAL, PHARMACOGENOMIC,
CCNE1	CANCER, METABOLIC, NEUROLOGICAL, RENAL,
CCNG1	CANCER,
LAMC1	AGING, CANCER, METABOLIC, UNKNOWN, VISION,
TNRC6B	CANCER, CARDIOVASCULAR, DEVELOPMENTAL, HEMATOLOGICAL, METABOLIC,
PAM	CARDIOVASCULAR, CHEMDEPENDENCY, HEMATOLOGICAL, METABOLIC

Two GEO datasets used herein contained paired mRNA and microarray expression profiles upon exposure to mimic mir-497 and mir-195 (GSE41079, GSE41074). GSE41074 has an expression profile of genes under mimic-mir-497 and mimic-mir-195. Supplementary results demonstrated the distribution of LogFC under different treatments or with different RNA handling procedures (Appendix). Using public dataset GSE41074 in HEPG2 cells CADM1 was identified as upregulated with mimic-mir-497 (total RNA). Ago2-IP result had three upregulated genes: CADM1, CCNE1 and ZMAT3. The most downregulated genes were CCNG1 and CCNE1 (total RNA). They were less downregulated in Ago2-IP samples. The change between Total RNA and Ago2-IP was CCNG1>CCNE1>TNRC6B>CADM1 (Table 4.5).

Table 4.5: Expression Profile of mir-497 target genes from GSE41074 (mimic-mir-497 or mimic-mir-195 Treatment)

HGNC symbol	Total RNA non-treated	Total RNA mimic-mir-195	Total RNA mimic-mir-497	Total RNA LogFC mimic-mir-195	Total RNA LogFC mimic-mir-497	Ago2-IP non treated	Ago2-IP mimic-mir-195	Ago2-IP mimic-mir-497	Ago2-IP LogFC mimic-mir-195	Ago2-IP LogFC mimic-mir-497
TNRC6B	2.852	2.484	2.560	-0.368	-0.293	9.802	10.662	3.730	0.860	-6.071
LAMC1	47.578	44.631	46.155	-2.948	-1.424	10.786	11.935	9.316	1.149	-1.469
ZMAT3	5.961	4.505	4.676	-1.456	-1.285	2.418	5.462	2.708	3.044	0.290
CADM1	36.271	41.234	40.523	4.964	4.252	4.125	6.428	5.928	2.304	1.803
CCNG1	124.224	79.604	89.141	-44.620	-35.083	31.415	39.109	24.100	7.693	-7.315
CCNE1	20.968	6.761	10.243	-14.206	-10.724	8.527	34.539	9.736	26.012	1.209
CHRNA5	2.571	1.736	1.736	-0.835	-0.835	0.590	0.667	0.373	0.077	-0.217
PAM	4.401	4.879	4.235	0.478	-0.166	0.991	2.160	0.915	1.170	-0.076

4.1.3. Expression Analyses of mir-497 Targets under CHRNA5 siRNA or mimic-mir-497 Treatment

qPCR Experiments of mir-497 Predicted Targets (Upregulated with siRNA CHRNA5)

Primers were designed for eight predicted target genes of hsa-mir-497-5p as explained in the methods section. qPCR experiments were performed to see how the expression level of those genes, targeted by the candidate microRNA, changed upon siRNA treatment as validation of microarray experiments (Table 4.2) Gene expressions of most genes were confirmed to increase in the depletion of CHRNA5 as expected from mRNA microarray study. Target gene group consisted of LAMC1 (Laminin Subunit Gamma 1), CADM1 (Cell Adhesion Molecule 1), CCNG1 (Cyclin G1), TNRC6B (Trinucleotide Repeat Containing 6B) and ZMAT3 (Zinc Finger Matrin-Type 3). Only PAM and TNRC6B were not significantly upregulated (Figures 4.10-11), others were significantly upregulated (Figures 4.6-4.9).

The two genes of P53 signalling pathway related to mir-497 were ZMAT3 and LAMC1 (Figure 4.6); they were the two most upregulated genes (ZMAT3, 225725_at microarray LogFC= 1.943) and it was also significantly upregulated in qPCR result (logfc~2; n=5 per group). It was also in the network of mir-497 and P53 Signaling Pathway related genes (Figure 4.6). LAMC1 was another gene in the p53 pathway related target of mir-497-5p (LAMC1/200771_at; microarray LogFC =1.783). qPCR result showed LAMC1 was significantly upregulated compatibly to microarray results. (Figure 4.7)

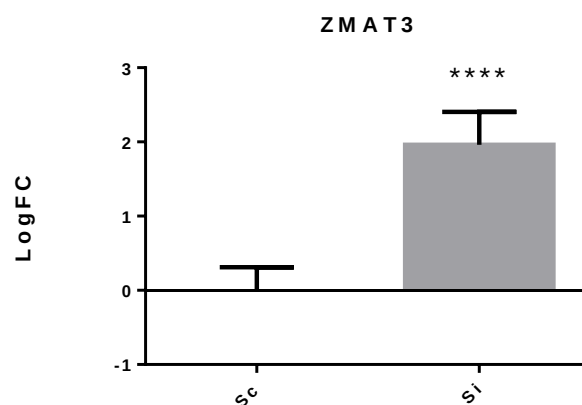


Figure 4.6: ZMAT3 expression level under siRNA CHRNA5 treatment by using 5 biological replicates. (ZMAT3 p val. <0.0001, * significant 0.01<p value< 0.05, ** very significant 0.001 < p value < 0.01, *** extremely significant 0.0001 < p value < 0.001, **** extremely significant p val≤0.0001)

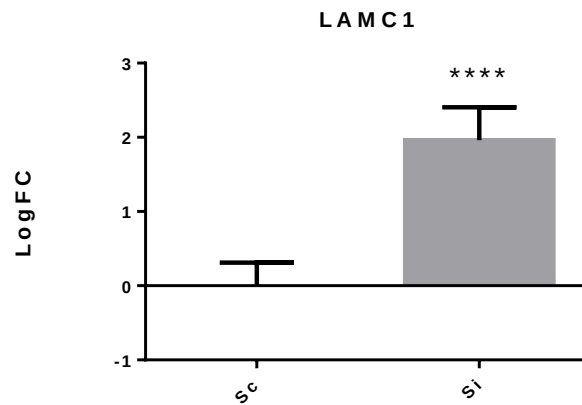


Figure 4.7: LAMC1 expression level under siRNA CHRNA5 treatment by using 5 biological replicates. (LAMC1 p val. <0.0001, * significant 0.01<p value< 0.05, ** very significant 0.001 < p value < 0.01, *** extremely significant 0.0001 < p value < 0.001, **** extremely significant p val≤0.0001)

CCNG1 and CADM1 were also upregulated (LogFC =1.434, 1.266, 209031_at) in microarray of siRNA CHRNA5 (Table 4.2) and they were significantly upregulated in qPCR results. Experimental results were close to each other according to Figure 4.8 and Figure 4.9.

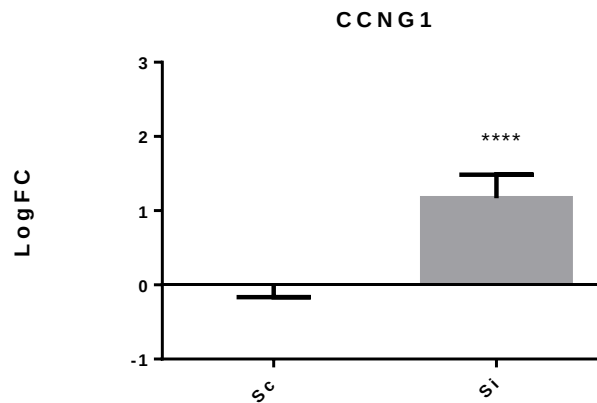


Figure 4.8: CCNG1 expression level under siRNA CHRNA5 treatment by using 5 biological replicates. (p val. <0.0001, * significant 0.01<p value< 0.05, ** very significant 0.001 < p value < 0.01, *** extremely significant 0.0001 < p value < 0.001, **** extremely significant p val≤0.0001)

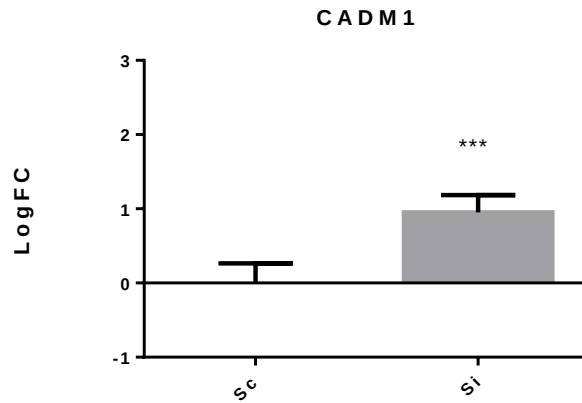


Figure 4.9: CADM1 expression level under siRNA CHRNA5 treatment by using 5 biological replicates. (p val. = 0.0003, * significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ val} \leq 0.0001$)

TNRC6B and PAM were not significantly upregulated by qPCR but again the qPCR results were similar to the LogFC values of mRNA microarray (siRNA CHRNA5; Table 4.2). (Figure 4.11)

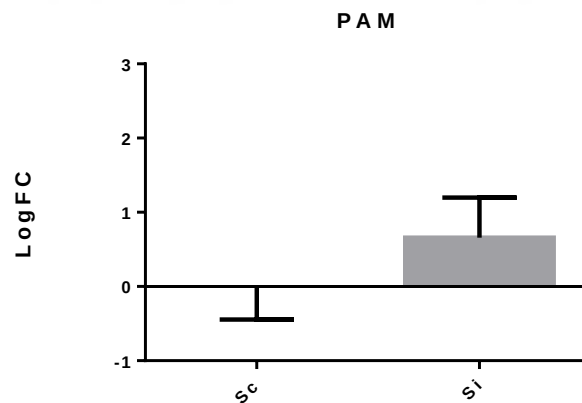


Figure 4.10: PAM expression level under siRNA CHRNA5 treatment by using 5 biological replicates. (p value = 0.0679)

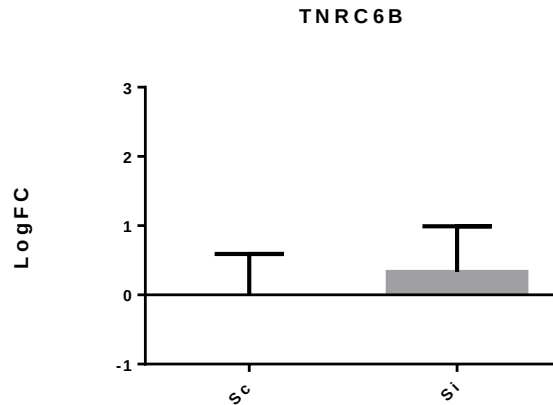


Figure 4.11: TNRC6B expression level under siRNA CHRNA5 treatment by using 5 biological replicates. (p val. = 0.4251)

CCNE1 (208796_s_at) on the other hand was downregulated according to mRNA microarray with a low LogFC value under siRNA treatment (Table 4.2). The result of qPCR was non-significant yet a negative trend was apparent (Figure 4.12).

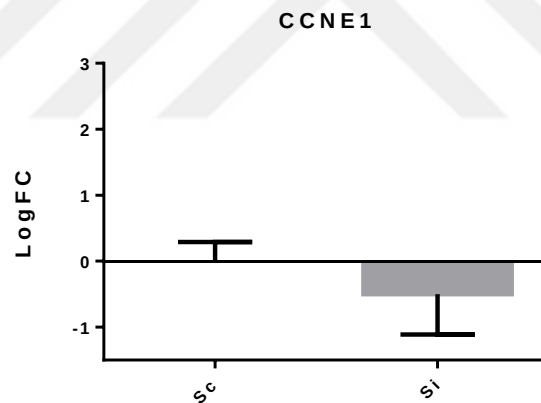


Figure 4.12: CCNE1 expression level under siRNA CHRNA5 treatment by using 5 biological replicates. (p val. = 0.1354)

Expression of Chromosome Location Related Genes to mir-497

MIR497HG is a host gene of a cluster which includes mir-497 and mir-195 while it is not a coding region. There is yet another gene near these two genes are located: C17orf49; this is a protein coding gene. The part in 17th chromosome covering microRNAs and these two genes can be seen in Figure 4.13..

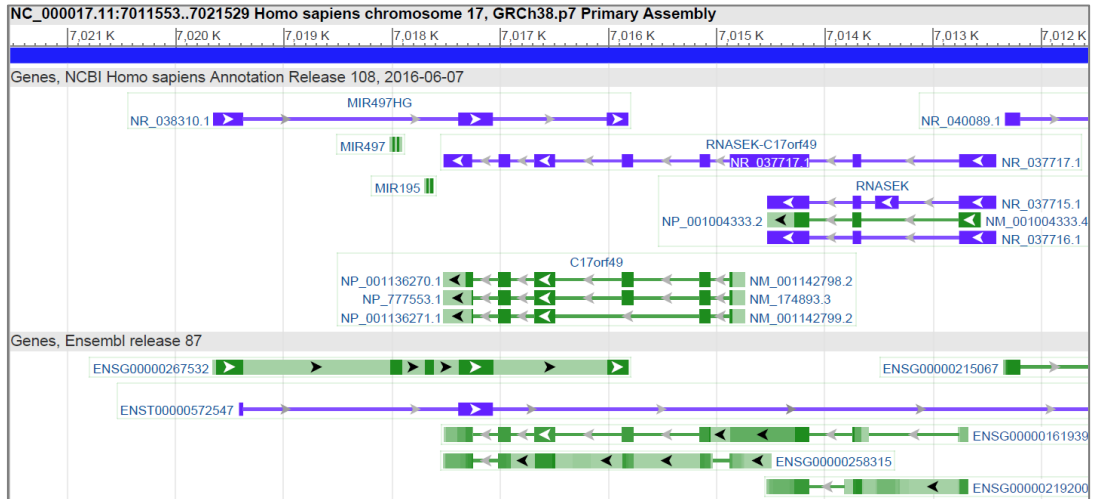


Figure 4.13: Map of a part of chromosome 17 (containing MIR497HG, C17orfHG and mir-497 cluster) from Genome Data Viewer.

Since C17orf49 is antisense with miR-497, the expectation is to that it would be upregulated after the siRNA CHRNA5 treatment. On the other hand, MIR497HG is sense with mir-497 (precursor of mir497) so downregulation has been expected. mRNA microarray of siRNA CHRNA5 gave overlapping results with expectation (Table 4.6).

Table 4.6: Analysis of Expression Profile of C17orf49 and MIR497 under siRNA CHRNA5 treatment (Tubitak project 111T316).

profile ID (affy_hg_u133_plus_2)	hgnc_symbol	avg LogFC siRNA CHRNA5	P.Value
224573_at	C17orf49	0.070293785	0.407556263
224574_at	C17orf49	0.483678054	0.003113738
230353_at	MIR497HG	-0.167813039	0.130144639

qPCR was done to test microarray result. The result was not statistically significant but the expected trends for both genes were demonstrated; C17orf49 exhibited an upregulated and MIR497HG a downregulated trend.

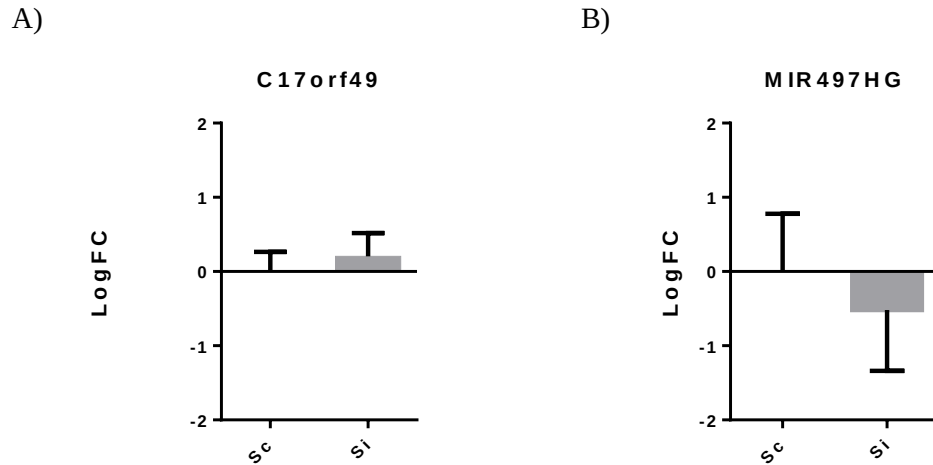


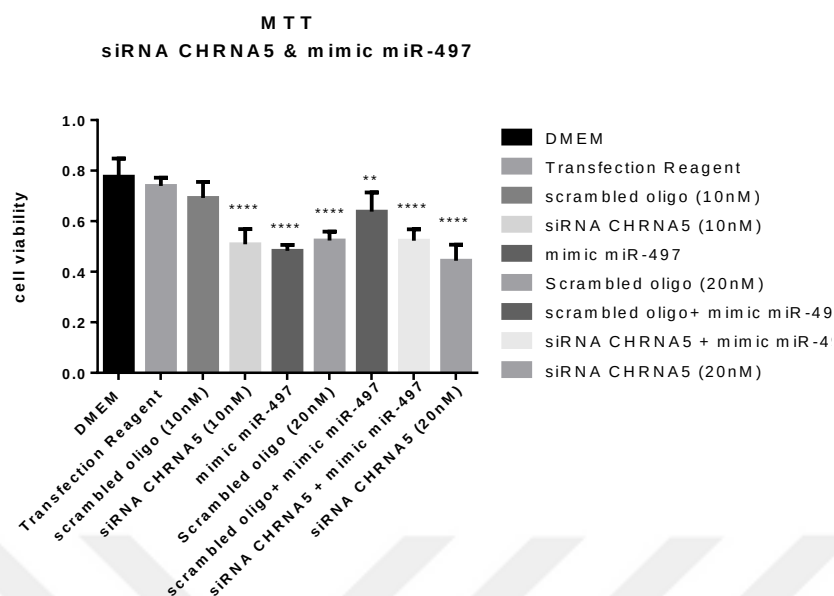
Figure 4.14: Expression of A) antagonistic gene to mir-497 and B) synergistic gene to mir-497 in the absence of CHRNA5 (5 biological replicates for each treatment were used)

4.1.4. Results of mimic-mir-497 in-vitro Treatment

4.1.4.1. MTT assay

mir-497-5p is an important downregulated microRNA under siRNA CHRNA5 because of the reasons previously explained in 4.1.1. It is expressed enough to be used in *in-vitro* experiments and related to other most down regulated microRNAs. MCF7 cells were thus treated with mimic-mir-497-5p and/or siRNA CHRNA5, alone or in combination with each other. There were eight experimental groups: DMEM, transfection reagent only, 10nM scrambled oligo treated, 10nM siRNA CHRNA5 treated, 10nM mimic-mir-497-5p treated, 20nM scrambled oligo, 20nM siRNA CHRNA5 treated, 10nM scrambled oligo + 10nM mimic-mir497 treated and 10nM siRNA CHRNA5 + 10nM mimic-mir-497 treated samples. This experiment design was repeated with half of transfection reagent (6μl). Less transfection reagent was found to be less toxic with mimic-mir-497; especially it was seen on mimic-mir-497 and scrambles oligo 20nM treatments. However, we continued with 12μl transfection reagent as previous studies were performed as such. SiRNA and mimic treatments were both effective as well as siRNA+mimic treatments (Figure 4.15).

A)



B)

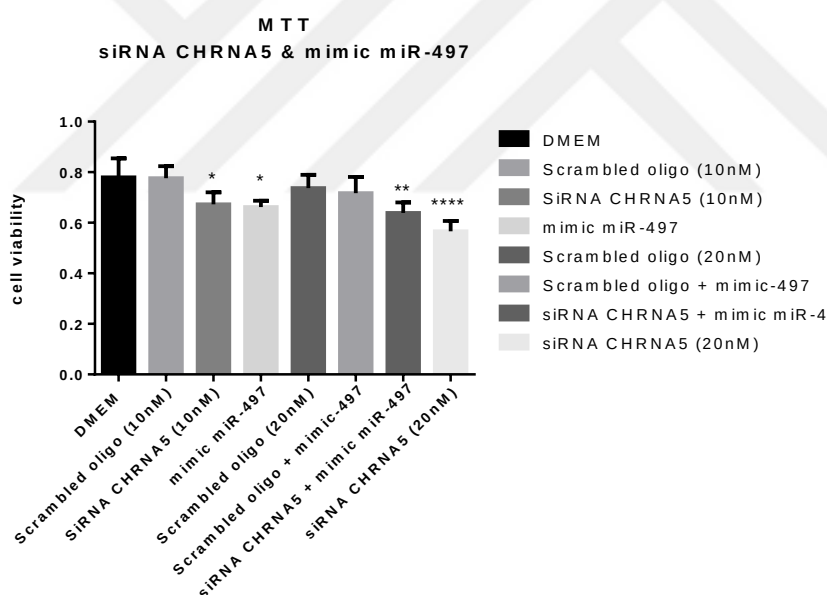


Figure 4.15: Cell viability data (MTT assay) of MCF7 cells treated with siRNA CHRNA5 and mimic-mir-497 single or alone with different doses A) by using 1.2µl transfection reagent B) by using 0.6µl transfection reagent. (, * significant 0.01<p value< 0.05, ** very significant 0.001 < p value < 0.01, *** extremely significant 0.0001 < p value < 0.001, **** extremely significant p val≤0.0001 depend on DMEM)

4.1.4.2. qPCR Analyses of Mimic and siRNA experiments

mimic-mir-497 and siRNA CHRNA5 effects were compared with each other based on their influence on CHRNA5 mRNA expression. They behaved similarly yet mimic had

less effect than siRNA. When both mimic-mir-497 and siRNA CHRNA5 reagents were used together, it was not obvious how they affected the outcome. CHRNA5 was significantly downregulated under mimic-mir-497 treatment (p val. = 0.0054). When mimic-mir-497 was used together with siRNA CHRNA5, siRNA effect became less. Scramble oligo and mimic-mir-497 were affecting each other because when they were used together the result was nearly equal to that of scramble alone (20nM).

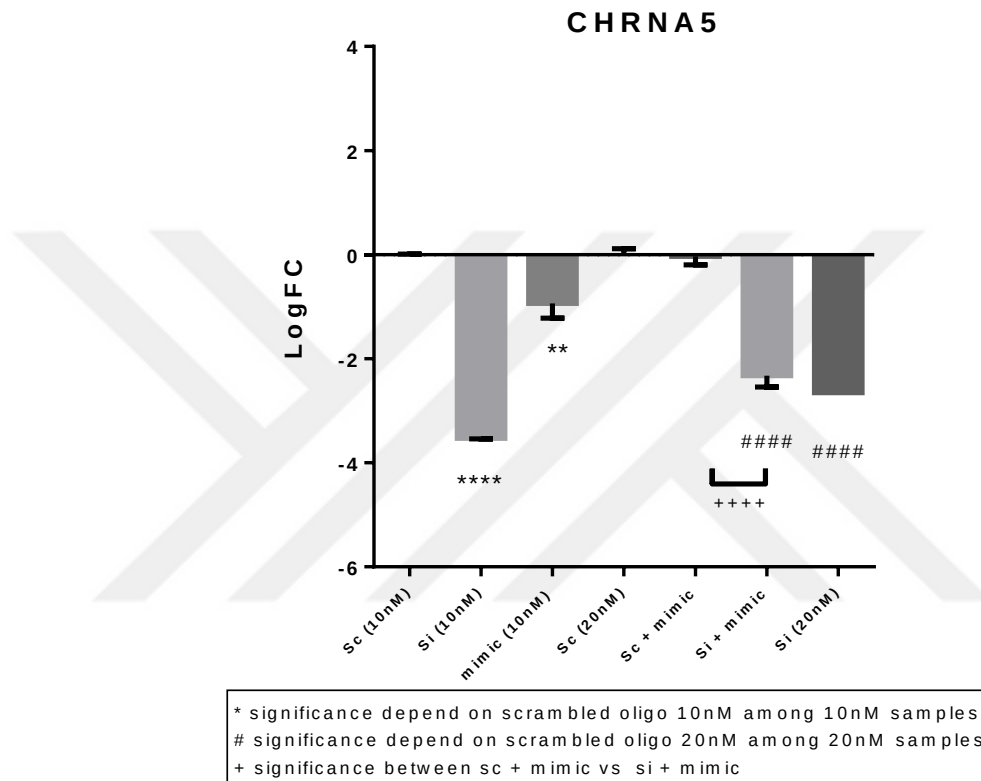


Figure 4.16: Expression of CHRNA5 in mimic-mir-497 and siRNA CHRAN5 treated samples A) 10nM reagents are used B) total treatment reagent is 20nM. (* significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ val} \leq 0.0001$)

Figure 4.17 showed that there was a non-significant yet a decreasing trend of mir-497 expression in siRNA CHRNA5 sample. Mimic was effective since, there was nearly 15-fold increase in expression of microRNA under mimic-mir-497 treatment. Mir-497 expression was significantly affecting if scrambled oligo was used with mimic.

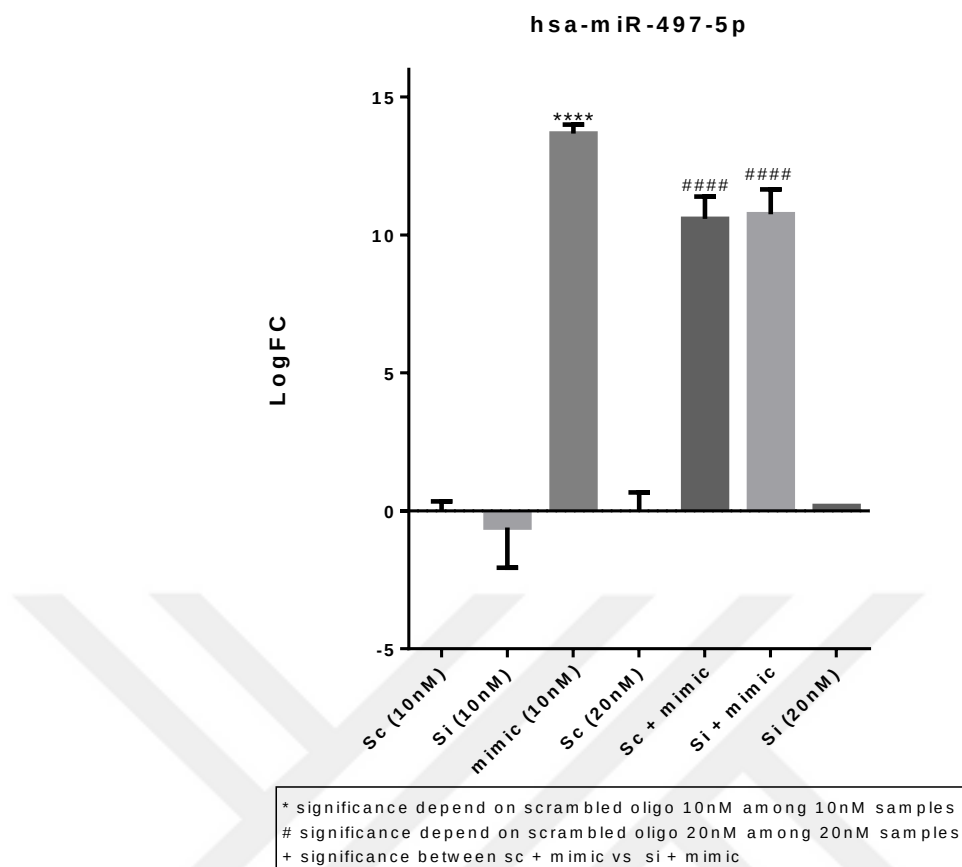


Figure 4.17: Expression of mir-497-5p in siRNA CHRNA5 and mimic-mir-497 treated samples. (* significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ value} \leq 0.0001$)

qPCR analysis of mir-497 Predicted Target Genes in mimic treated cells

Expressions of target genes were studied in the mimic, siRNA, and mimic+siRNA design. All targets except CCNG1 showed increased expression under mimic-mir-497 treatment as in the case of CHRNA5 siRNA treatment but with less effect. siRNA CHRNA5 + mimic-mir-497-5p provided the same effect on upregulated genes under siRNA CHRNA5 treatment (LogFC ~2). The increase was significant for more upregulated genes (ZMAT3, CCNG1) in siRNA CHRNA5 presence.

ZMAT3 was selected as one the most upregulated genes in siRNA CHRNA5 mRNA array (LogFC=1.943) and the expression of ZMAT3 in GSE41079 differed between total RNA and Ago2-IP mimic-mir-497 samples. Total RNA result gives the expected downregulation (LogFC=-1.285) but in Ago2-IP ZMAT3 expression slightly increased (LogFC=0.290) showing displacement to the RISC. qPCR in the siRNA/mimic

experiment showed ZMAT3 was upregulated in the siRNA treatment group significantly and was unaffected under mimic-mir-497 treatment in MCF7 cells (Figure 4.18).

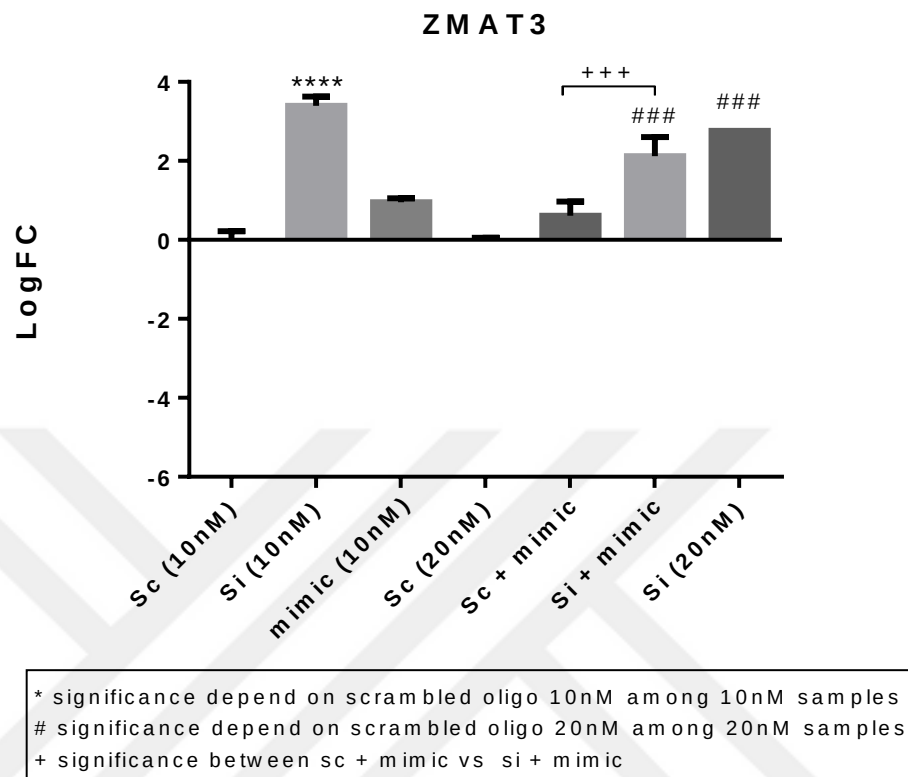
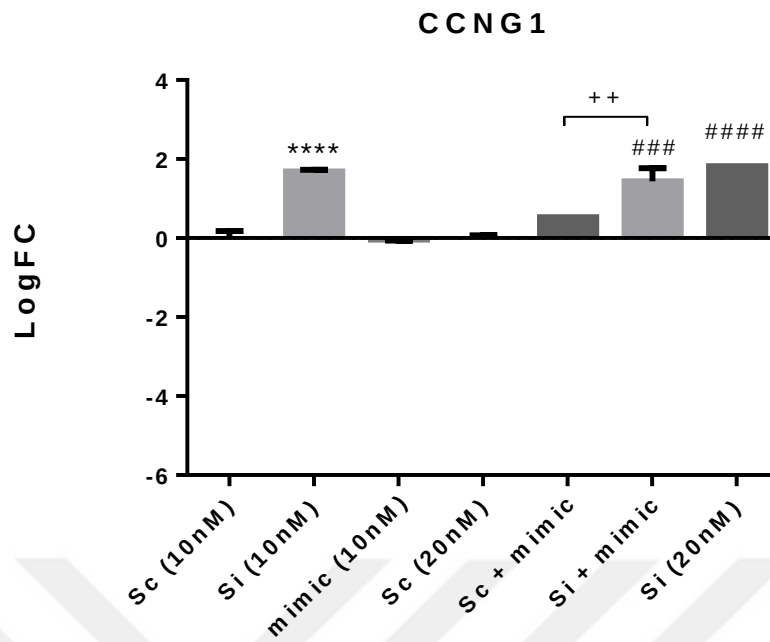


Figure 4.18: Expression of ZMAT3 in mimic-mir-497 and siRNA CHRNA5 treated samples. (* significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ value} \leq 0.0001$)

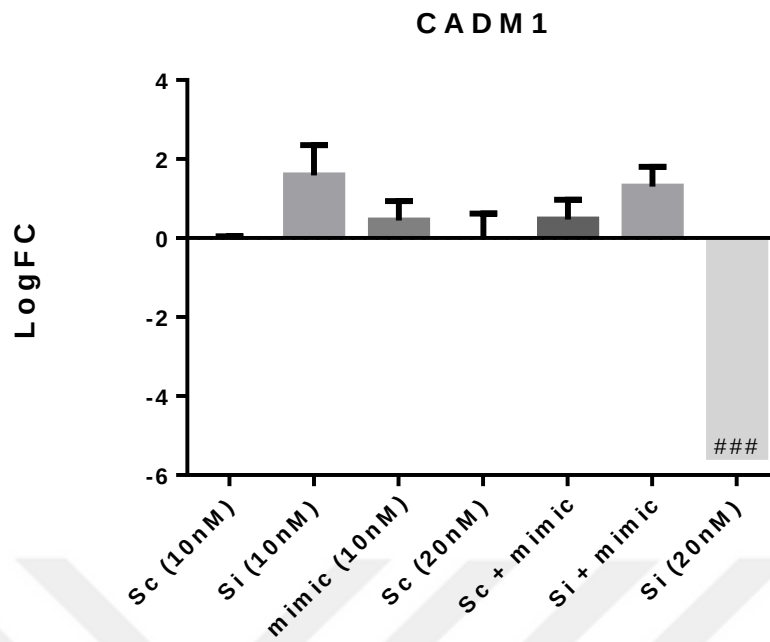
CCNG1 expression increased in the siRNA microarray experiment and it showed a decrease in both Ago2-IP and Total RNA samples in the GSE41074 study (LogFC Total = -35.083, LogFC Ago = -7.315). In the qPCR result from mimic experiment, CCNG1 was significantly upregulated under the siRNA treatment, it was not affected by the mimic treatment (10nM) unexpectedly (Figure 4.19). When siRNA CHRNA5 and mimic-mir-497 were given together CCNG1 was not expressed any different than siRNA CHRNA5 only treatment group. There was a non-significant yet an increasing trend in scrambled oligo+mimic treated samples.



* significance depend on scrambled oligo 10nM among 10nM samples
 # significance depend on scrambled oligo 20nM among 20nM samples
 + significance between sc + mimic vs si + mimic

Figure 4.19: Expression of CCNG1 in mimic-mir-497 and siRNA CHRNA5 treated samples. (* significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ val} \leq 0.0001$)

CADM1 was upregulated in both the CHRAN5 siRNA assay and GSE41074 (mimic-mir-497 treatment in HEPG2 cells). Figure 4.20 implied a potential upregulation trend under four treatments except scrambled oligo treated samples. However, none of the groups were significant. Scrambled oligo did not manipulate mimic effect for CADM1 when only mimic and mimic + scrambled oligo treatments were compared. The experiment showed an unexpected result for 20nM siRNA treatment: a very significant downregulation.



* significance depend on scrambled oligo 10nM among 10nM samples
 # significance depend on scrambled oligo 20nM among 20nM samples
 + significance between sc + mimic vs si + mimic

Figure 4.20: Expression of CADM1 in mimic-mir-497 and siRNA CHRNA5 treated samples. (* significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ val} \leq 0.0001$)

TNRC6B increased under the CHRNA5 siRNA treatment in microarray experiment and decreased for both Total RNA and Ago2-IP samples in GSE41074 under mimic-mir-497 treatment (LogFC Ago=-6.071, LogFC Total = -0.293). In qPCR result of the mimic experiment, mimic decreased siRNA effect although non-significantly, scrambled oligo increased mimic effect when they were used together for TNRC6B. None of the groups showed significant changes however (Figure 4.21).

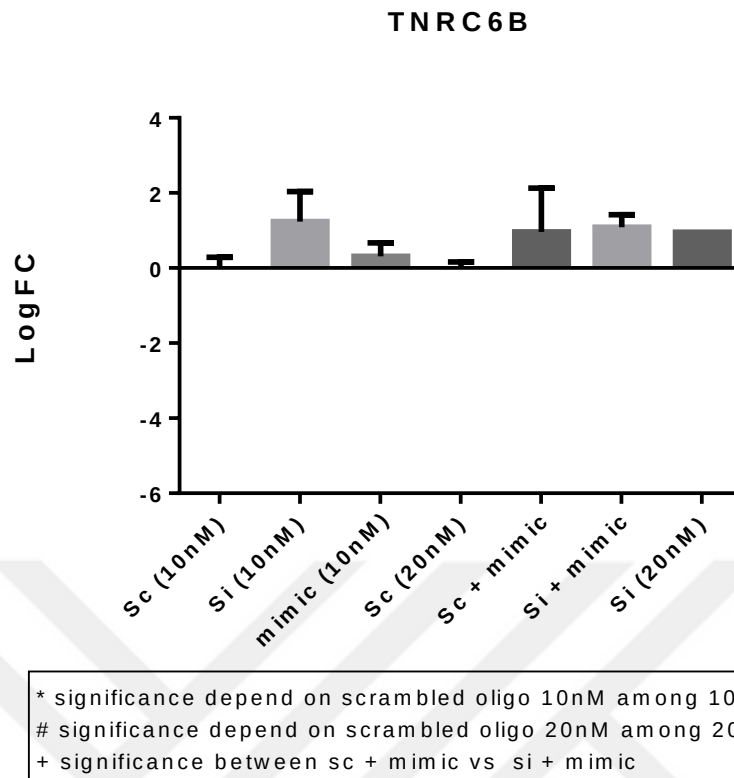
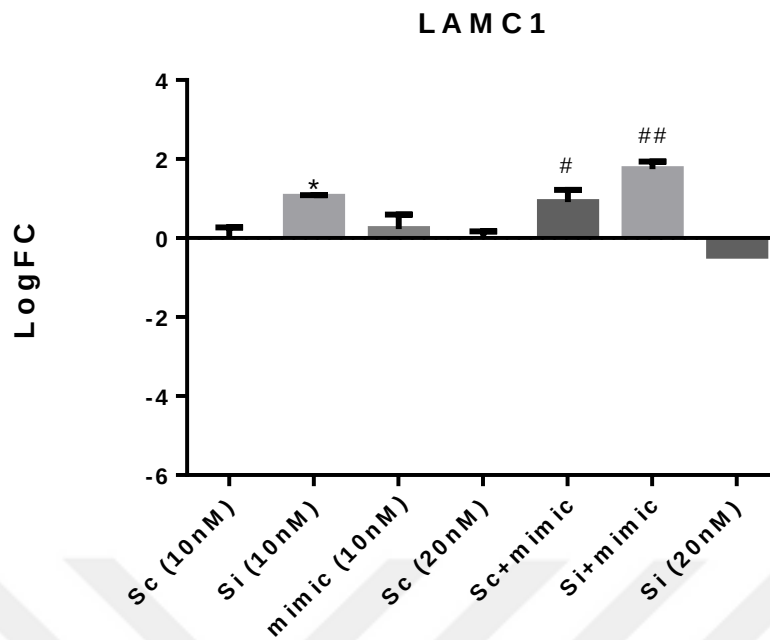


Figure 4.21: Expression of TNRC6B in mimic-mir-497 and siRNA CHRNA5 treated samples. (* significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ val} \leq 0.0001$)

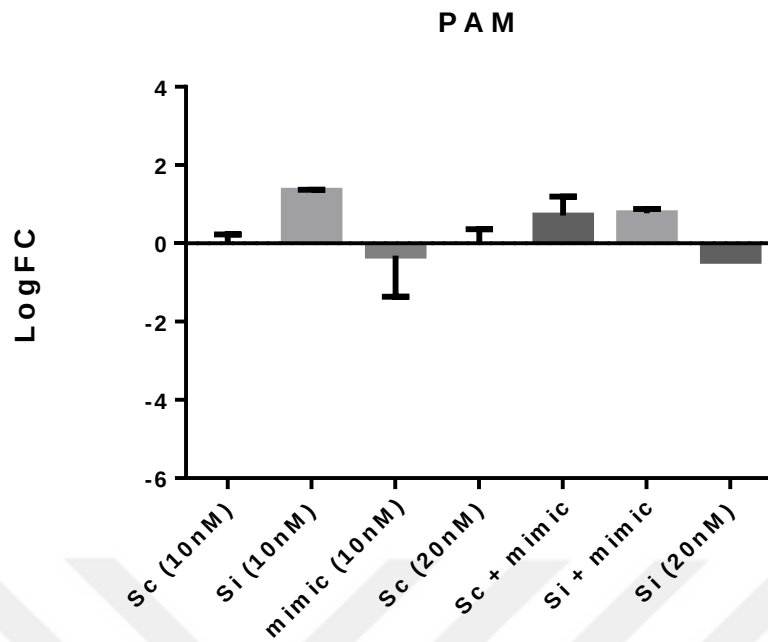
LAMC1 is a validated target of mir-497-5p, in microarray experiment siRNA treatment increased the expression of LAMC1 ($\text{LogFC} = 1.783$) and mimic decreased the expression ($\text{LogFC} = -1.4$) in HEPG2. qPCR experiments showed that CHRNA5 siRNA increased expression expectedly yet mimic mir-497 did not decrease as expectedly. Instead mimic and siRNA together might have enhanced the expression of LAMC1 (Figure 4.22). Interestingly, this effect might be additive.



* significance depend on scrambled oligo 10nM among 10nM samples
 # significance depend on scrambled oligo 20nM among 20nM samples
 + significance between sc + mimic vs si + mimic

Figure 4.22: Expression of LAMC1 in mimic-mir-497 and siRNA CHRN5 treated samples. (* significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ val} \leq 0.0001$)

PAM is increasing under siRNA treatment in microarray result (LogFC = 1.307) and slightly decreasing under mimic treatment (LogFC = -0.166). The result of qPCR fits the expectations (Figure 4.23).



* significance depend on scrambled oligo 10nM among 10nM samples
 # significance depend on scrambled oligo 20nM among 20nM samples
 + significance between sc + mimic vs si + mimic

Figure 4.23: Expression of PAM in mimic-mir-497 and siRNA CHRNA5 treated samples. (* significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ val} \leq 0.0001$)

CCNE1 was only the downregulated mir-497-5p target that we studied based on mRNA array under CHRNA5 siRNA; and qPCR result below confirmed this decrease of expression but the result was not significant (Figure 4.24). mRNA array gave -0.556 fold change value and in qPCR result fold change was between -0.5 and -1 ($p \text{ val.} = 0.1327$). mimic-mir-497 affected CCNE1 in a downward direction in both qPCRs but was not significant; and mimic-mir-497 treated total RNA sample of GSE41074 was also showing a downregulated. Scrambled oligo and mimic-mir-497 affected each other as seen in figures.

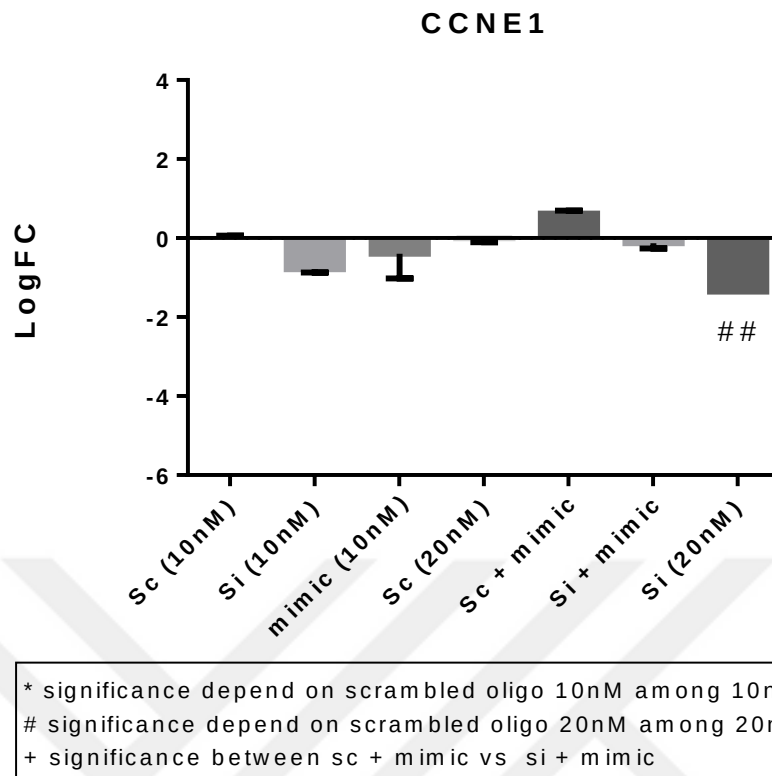
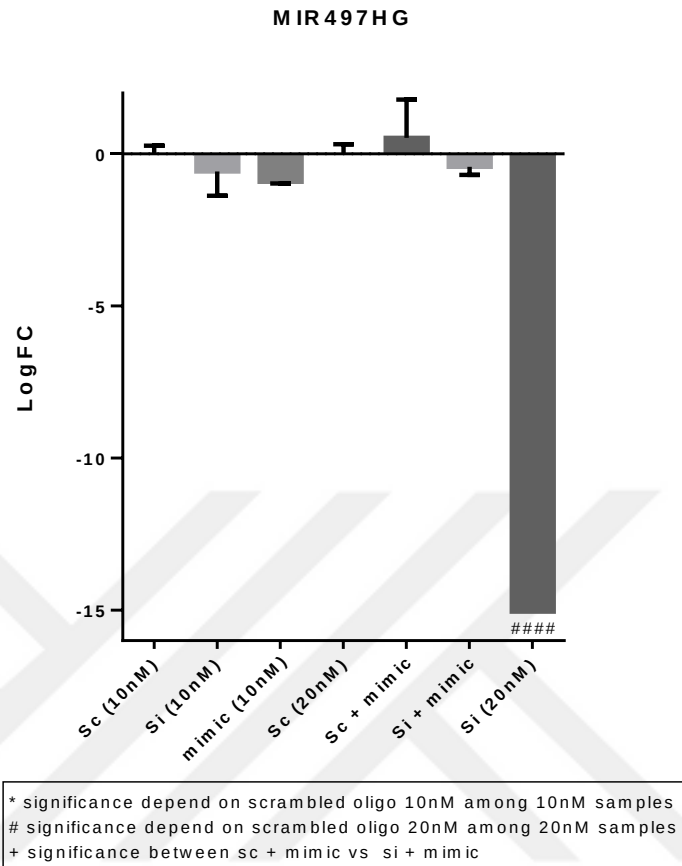


Figure 4.24: Expression of CCNE1 in mimic-mir-497 and siRNA CHRNA5 treated samples. (* significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ val} \leq 0.0001$)

qPCR analysis of Chromosomal Location Related Genes to mir-497

There was no significant change on the expression of MIR497HG except si20nM. It was expected after no significant LogFC for five siRNA 5scrambled oligo results (Figure 4.14). MIR497HG was effected by mimic treatment more than C17orf49, but both of the results for mimic 10nM were nonspecific. There is no change and similar pattern for 20nM treatments for both genes. There is a significant result under siRNA CHRNA5 for 20nM in combined experiment but the other confirmatory experiment also showed non-significant result for siRNA 20nM (Figure 4.25).

A)



B)

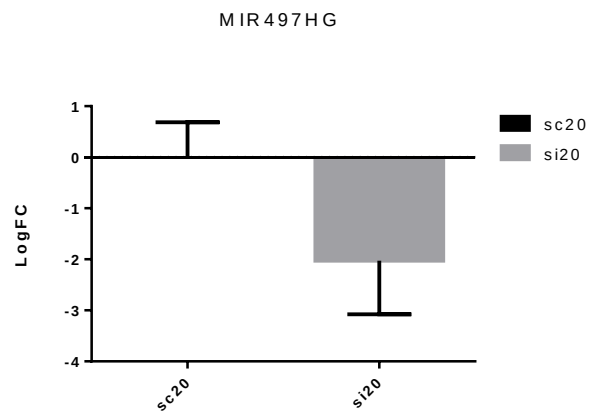
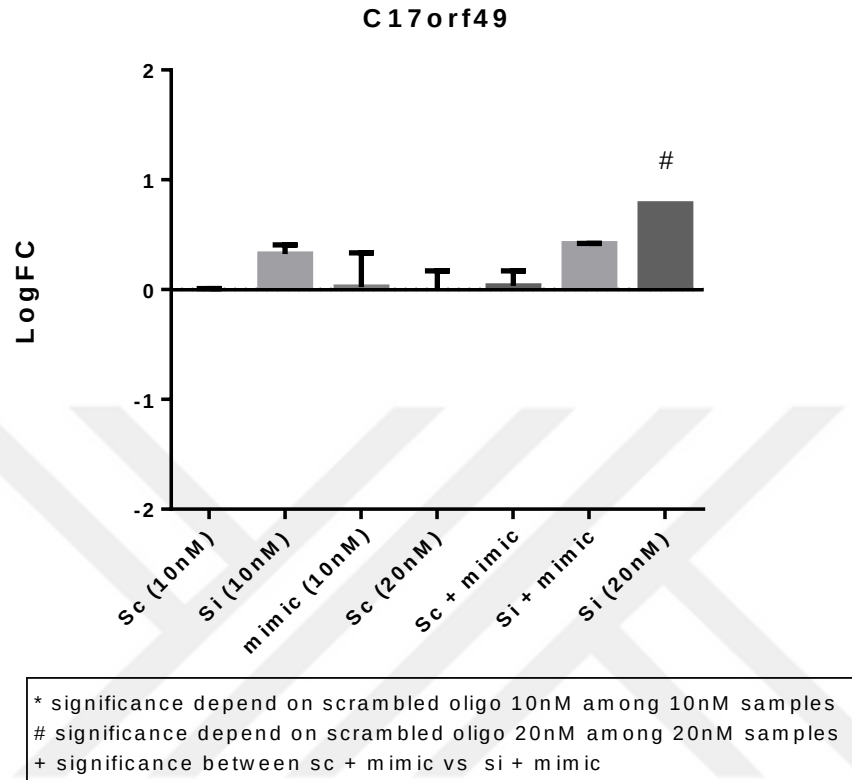


Figure 4.25: Expression of MIR497HG in mimic-mir-497 and siRNA CHRN5 treated A) samples from combined experiment B) samples from a new setup (p-val = 0.1491). (* significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ val} \leq 0.0001$)

A)



B)

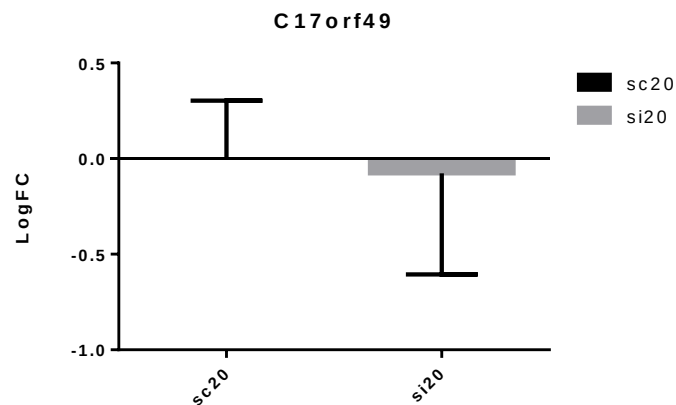


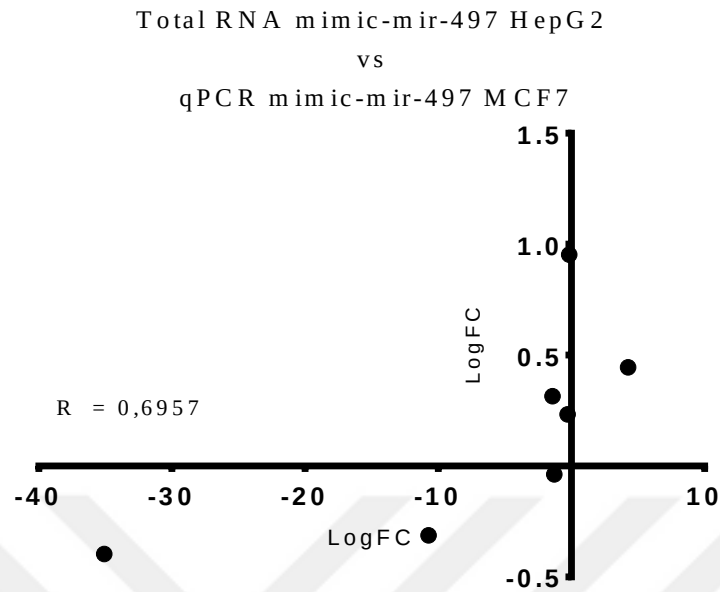
Figure 4.26: Expression of C17orf49 in mimic-mir-497 and siRNA CHRNA5 treated A) samples from combined experiment B) samples from a new setup (p-value= 0.8737). (* significant $0.01 < p \text{ value} < 0.05$, ** very significant $0.001 < p \text{ value} < 0.01$, *** extremely significant $0.0001 < p \text{ value} < 0.001$, **** extremely significant $p \text{ val} \leq 0.0001$)

4.1.5. Correlation between GEO Data and qPCR Results under mimic-mir-497 Treatment

GEO data we used consisted of both Ago2-IP and Total RNA. Mimic-mir-497 was applied on HepG2 cells in these GEO experiments (GSE41079, GSE41074)⁹. The qPCR results of selected target genes were compared as correlation between HepG2 fold changes and MCF7 fold changes upon mir-497 mimic exposure. Total RNA results of mimic treated HCC cells were more correlated to the qPCR results of mimic treated MCF7 cells than Ago2-IP results (Figure 4.26).



A)



B)

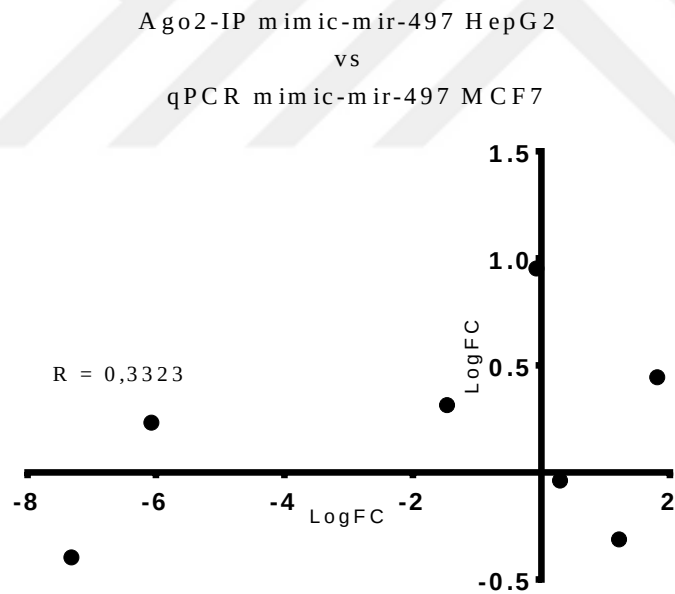


Figure 4.27: Target genes' scatter plots and Pearson correlation results under mimic-mir-497 treatment to compare A) GEO Total RNA result (HepG2) and qPCR result (MCF7) (p value = 0.0826) B) GEO Ago2-IP result (HepG2) and qPCR result (MCF7) (p value = 0.4665)

4.2. Immune System and mir-497 Relationship

4.2.1. Immune System Pathway Relation with mir-497

4.2.1.1. Msigdb (REACTOME)

The main element to search was mir-497, so mir-497 related pathways led the next steps of the research in this study. Investigation of gene sets were used in the following tables and REACTOME was used to see related pathways of gene sets. There were Total RNA and AGO2-IP results for mimic-mir-497 treatment in HCC and upregulated and downregulated genes were extracted. Therefore, there were four groups to analyse: upregulated genes (Ago2-IP), downregulated genes (Ago2-IP), upregulated genes (Total RNA) and downregulated genes (Total RNA) under mimic-mir-497 treatment. In Table 4.7, most downregulated genes in Total RNA result were analysed and immune system was seen in 27th rank with high number genes overlap and significance. In the next list of pathways (Table 4.8) which shows top 50 pathways using upregulated genes, immune system was the second pathway (80 genes overlap). In Table 4.9, Ago2-IP downregulated genes were used and in top 50 pathways 7th pathway was immune system. P values for all three list until here was far less than 0.05. For upregulated genes from Ago2-IP, there were only three pathways (Table 4.10) and none of them was immune system. Immune System pathway was one of the most gene including pathways significantly modulated in three lists. Therefore, mir-497/ immune system relationship was interrogated in the study.

Table 4.7: Top 50 pathways of downregulated genes (fc<-0.5, pathway database: REACTOME, data: Total RNA)

	Gene Set Name [# Genes (K)]	# Genes in Overlap (k)	p-value	FDR q-value
1	REACTOME_CELL_CYCLE [421]	57	3.22 e ⁻⁵²	2.17 e ⁻⁵⁰
2	REACTOME_CELL_CYCLE_MITOTIC [325]	52	1.56 e ⁻⁵¹	5.24 e ⁻⁵⁰
3	REACTOME_DNA_REPLICATION [192]	40	1.27 e ⁻⁴⁴	2.86 e ⁻⁴³
4	REACTOME_MITOTIC_M_M_G1_PHASES [172]	33	1.09 e ⁻³⁵	1.84 e ⁻³⁴
5	REACTOME_MITOTIC_PROMETAPHASE [87]	21	2.09 e ⁻²⁵	2.82 e ⁻²⁴
6	REACTOME_MITOTIC_G1_G1_S_PHASES [137]	19	2.95 e ⁻¹⁸	3.31 e ⁻¹⁷
7	REACTOME_CHROMOSOME_MAINTENANCE [122]	18	7.35 e ⁻¹⁸	7.08 e ⁻¹⁷
8	REACTOME_S_PHASE [109]	17	2.31 e ⁻¹⁷	1.94 e ⁻¹⁶
9	REACTOME_CELL_CYCLE_CHECKPOINTS [124]	17	2.19 e ⁻¹⁶	1.64 e ⁻¹⁵
10	REACTOME_G1_S_TRANSITION [112]	16	8.61 e ⁻¹⁶	5.8 e ⁻¹⁵
11	REACTOME_G2_M_CHECKPOINTS [45]	12	1.2 e ⁻¹⁵	7.32 e ⁻¹⁴
12	REACTOME_SYNTHESIS_OF_DNA [92]	14	2.23 e ⁻¹⁴	1.25 e ⁻¹³
13	REACTOME_E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION [35]	10	1.36 e ⁻¹³	7.05 e ⁻¹²
14	REACTOME_DNA_STRAND_ELONGATION [30]	9	1.41 e ⁻¹²	6.78 e ⁻¹¹
15	REACTOME_DNA_REPAIR [112]	13	6.77 e ⁻¹²	3.04 e ⁻¹¹
16	REACTOME_G0_AND_EARLY_G1 [25]	8	1.41 e ⁻¹¹	5.94 e ⁻¹⁰
17	REACTOME_ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS [38]	9	1.52 e ⁻¹¹	6.01 e ⁻¹⁰
18	REACTOME_DEPOSITION_OF_NEW_CENPA_CONTAINING_NUCLEOSOMES_AT_THE_CENTROMERE [64]	10	9.14 e ⁻¹¹	3.42 e ⁻¹⁰
19	REACTOME_ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX [31]	8	9.87 e ⁻¹¹	3.5 e ⁻¹⁰
20	REACTOME_M_G1_TRANSITION [81]	10	1.01 e ⁻⁹	3.39 e ⁻⁹
21	REACTOME_EXTENSION_OF_TELOMERES [27]	7	1.46 e ⁻⁹	4.68 e ⁻⁹
22	REACTOME_G1_S_SPECIFIC_TRANSCRIPTION [19]	6	5.99 e ⁻⁹	1.76 e ⁻⁸
23	REACTOME_LAGGING_STRAND_SYNTHESIS [19]	6	5.99 e ⁻⁹	1.76 e ⁻⁸
24	REACTOME_KINESINS [24]	6	2.87 e ⁻⁸	8.07 e ⁻⁸
25	REACTOME_POL_SWITCHING [13]	5	3.76 e ⁻⁸	1.01 e ⁻⁷
26	REACTOME_REGULATION_OF_MITOTIC_CELL_CYCLE [85]	8	4.15 e ⁻⁷	1.07 e ⁻⁶
27	REACTOME_IMMUNE_SYSTEM [933]	24	6.14 e ⁻⁷	1.53 e ⁻⁶
28	REACTOME_E2F_ENABLED_INHIBITION_OF_PRE_REPLICATION_COMPLEX_FORMATION [10]	4	7.92 e ⁻⁷	1.91 e ⁻⁶
29	REACTOME_DOUBLE_STRAND_BREAK_REPAIR [24]	5	1.15 e ⁻⁶	2.68 e ⁻⁶
30	REACTOME_FANCONI_ANEMIA_PATHWAY [25]	5	1.43 e ⁻⁶	3.22 e ⁻⁶
31	REACTOME_ACTIVATION_OF_GENES_BY_ATF4 [26]	5	1.76 e ⁻⁶	3.83 e ⁻⁶
32	REACTOME_G2_M_DNA_DAMAGE_CHECKPOINT [12]	4	1.84 e ⁻⁶	3.88 e ⁻⁶
33	REACTOME_ADAPTIVE_IMMUNE_SYSTEM [539]	17	1.93 e ⁻⁶	3.94 e ⁻⁶
34	REACTOME_TELOMERE_MAINTENANCE [75]	7	2.35 e ⁻⁶	4.66 e ⁻⁶
35	REACTOME_PERK_REGULATED_GENE_EXPRESSION [29]	5	3.12 e ⁻⁶	6.01 e ⁻⁶
36	REACTOME_REPAIR_SYNTHESIS_FOR_GAP_FILLING_BY_DNA_POL_IN_TC_NER [14]	4	3.68 e ⁻⁶	6.89 e ⁻⁶
37	REACTOME_MITOTIC_G2_G2_M_PHASES [81]	7	3.95 e ⁻⁶	7.2 e ⁻⁶
38	REACTOME_CYCLIN_A_B1_ASSOCIATED_EVENTS_DURING_G2_M_TRANSITION [15]	4	4.99 e ⁻⁶	8.62 e ⁻⁶
39	REACTOME_PROCESSIVE_SYNTHESIS_ON_THE_LAGGING_STRAND [15]	4	4.99 e ⁻⁶	8.62 e ⁻⁶
40	REACTOME_GLOBAL_GENOMIC_NER_GG_NER [35]	5	8.21 e ⁻⁶	1.38 e ⁻⁵
41	REACTOME_HOMOLOGOUS_RECOMBINATION_REPAIR_OF_REPLICATION_INDEPENDENT_DOUBLE_STRAND_BREAKS [17]	4	8.58 e ⁻⁶	1.38 e ⁻⁵
42	REACTOME_MHC_CLASS_II_ANTIGEN_PRESENTATION [91]	7	8.6 e ⁻⁶	1.38 e ⁻⁵
43	REACTOME_FACTORS_INVOLVED_IN_MEGAKARYO	8	1.15 e ⁻⁵	1.8 e ⁻⁵
44	REACTOME_TRANSCRIPTION_COUPLED_NER_TC_NER[45]	5	2.89 e ⁻⁵	4.43 e ⁻⁵
45	REACTOME_NUCLEOTIDE_EXCISION_REPAIR [51]	5	5.35 e ⁻⁵	8.01 e ⁻⁵
46	REACTOME_APC_CDC20_MEDIATED_DEGRADATION_OF_NEK2A[28]	4	6.89 e ⁻⁵	1.01 e ⁻⁴
47	REACTOME_CDC6_ASSOCIATION_WITH_THE_ORC_ORIGIN_COMPLEX[11]	3	7.82 e ⁻⁵	1.1 e ⁻⁴
48	REACTOME_UNWINDING_OF_DNA [11]	3	7.82 e ⁻⁵	1.1 e ⁻⁴
49	REACTOME_CDT1_ASSOCIATION_WITH_THE_CDC6_ORC_ORIGIN_COMPLEX[56]	5	8.42 e ⁻⁵	1.16 e ⁻⁴
50	REACTOME_DIABETES_PATHWAYS [133]	7	9.94 e ⁻⁵	1.34 e ⁻⁴

Table 4.8: Top 50 pathways of upregulated genes (fc>0.5, pathway database: REACTOME, data: Total RNA)

	Gene Set Name [# Genes (K)]	# Genes in Overlap (k)	p-value	FDR q-value
1	REACTOME_METABOLISM_OF_LIPIDS_AND_LIPOPROTEINS [478]	61	2.53 e ⁻²¹	1.7 e ⁻¹⁸
2	REACTOME_IMMUNE_SYSTEM [933]	80	8.02 e ⁻¹⁷	2.7 e ⁻¹⁴
3	REACTOME_ADAPTIVE_IMMUNE_SYSTEM [539]	54	1.81 e ⁻¹⁴	4.06 e ⁻¹²
4	REACTOME_INTERFERON_ALPHA_BETA_SIGNALING [64]	17	4.49 e ⁻¹²	7.56 e ⁻¹⁰
5	REACTOME_AMYLOIDS [83]	18	4.31 e ⁻¹¹	5.82 e ⁻⁹
6	REACTOME_ACTIVATION_OF_CHAPERONE_GENES_BY_XBP1S [46]	13	6.34 e ⁻¹⁰	7.13 e ⁻⁸
7	REACTOME_HEMOSTASIS [466]	41	1.3 e ⁻⁹	1.25 e ⁻⁷
8	REACTOME_FATTY_ACID_TRIACYLGLYCEROL_AND_KETONE_BODY_METABOLISM [168]	23	1.6 e ⁻⁹	1.35 e ⁻⁷
9	REACTOME_METABOLISM_OF_CARBOHYDRATES [247]	28	2.29 e ⁻⁹	1.71 e ⁻⁷
10	REACTOME_METABOLISM_OF_PROTEINS [518]	42	9.1 e ⁻⁹	6.13 e ⁻⁷
11	REACTOME_UNFOLDED_PROTEIN_RESPONSE [80]	15	1.48 e ⁻⁸	8.35 e ⁻⁷
12	REACTOME_CYTOKINE_SIGNALING_IN_IMMUNE_SYSTEM [270]	28	1.64 e ⁻⁸	8.35 e ⁻⁷
13	REACTOME_CHOLESTEROL_BIOSYNTHESIS [24]	9	1.72 e ⁻⁸	8.35 e ⁻⁷
14	REACTOME_CHONDROITIN_SULFATE_DERMATAN_SULFATE_METABOLISM [49]	12	1.73 e ⁻⁸	8.35 e ⁻⁷
15	REACTOME_RNA_POL_I_PROMOTER_OPENING [62]	13	3.31 e ⁻⁸	1.49 e ⁻⁶
16	REACTOME_GLYCOSAMINOGLYCAN_METABOLISM [111]	17	3.92 e ⁻⁸	1.65 e ⁻⁶
17	REACTOME_LIPOPROTEIN_METABOLISM [28]	9	8.13 e ⁻⁸	3.22 e ⁻⁶
18	REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS [79]	14	9.39 e ⁻⁸	3.52 e ⁻⁶
19	REACTOME_TRANSCRIPTION [210]	23	1.13 e ⁻⁷	4.02 e ⁻⁶
20	REACTOME_RNA_POL_I_RNA_POL_III_AND_MITOCHONDRIAL_TRANSCRIPTION [122]	17	1.62 e ⁻⁷	5.45 e ⁻⁶
21	REACTOME_INFLUENZA_VIRAL_RNA_TRANSCRIPTION_AND_REPLICATION [169]	20	2.13 e ⁻⁷	6.82 e ⁻⁶
22	REACTOME_INTERFERON_SIGNALING [159]	19	3.65 e ⁻⁷	1.12 e ⁻⁵
23	REACTOME_RNA_POL_I_TRANSCRIPTION [89]	14	4.35 e ⁻⁷	1.28 e ⁻⁵
24	REACTOME_SIGNALING_BY_SCF_KIT [78]	13	5.63 e ⁻⁷	1.53 e ⁻⁵
25	REACTOME_DIABETES_PATHWAYS [133]	17	5.69 e ⁻⁷	1.53 e ⁻⁵
26	REACTOME_SEMAPHORIN_INTERACTIONS [68]	12	8.08 e ⁻⁷	2.1 e ⁻⁵
27	REACTOME_PEPTIDE_CHAIN_ELONGATION [153]	18	9.25 e ⁻⁷	2.31 e ⁻⁵
28	REACTOME_TCA_CYCLE_AND_RESPIRATORY_ELECTRON_TRANSPORT [141]	17	1.31 e ⁻⁶	3.14 e ⁻⁵
29	REACTOME_PACKAGING_OF_TELOMERE_ENDS [48]	10	1.36 e ⁻⁶	3.16 e ⁻⁵
30	REACTOME_MEIOTIC_RECOMBINATION [86]	13	1.78 e ⁻⁶	4 e ⁻⁵
31	REACTOME_HDL_MEDIATED_LIPID_TRANSPORT [15]	6	2.91 e ⁻⁶	6.22 e ⁻⁵
32	REACTOME_HEPARAN_SULFATE_HEPARIN_HS_GAG_METABOLISM [52]	10	2.95 e ⁻⁶	6.22 e ⁻⁵
33	REACTOME_INFLUENZA_LIFE_CYCLE [203]	20	3.88 e ⁻⁶	7.92 e ⁻⁵
34	REACTOME_DEVELOPMENTAL_BIOLOGY [396]	30	4.54 e ⁻⁶	8.99 e ⁻⁵
35	REACTOME_SIGNALING_BY_EGFR_IN_CANCER [109]	14	5.24 e ⁻⁶	1.01 e ⁻⁴
36	REACTOME_METABOLISM_OF_MRNA [284]	24	6.51 e ⁻⁶	1.2 e ⁻⁴
37	REACTOME_A_TETRASACCHARIDE_LINKER_SEQUENCE_IS_REQUIRED_FOR_GAG_SYNTHESIS [25]	7	6.58 e ⁻⁶	1.2 e ⁻⁴
38	REACTOME_LIPID_DIGESTION_MOBILIZATION_AND_TRANSPORT [46]	9	7.97 e ⁻⁶	1.38 e ⁻⁴
39	REACTOME_SIGNALING_BY_RHO_GTPASES [113]	14	8.01 e ⁻⁶	1.38 e ⁻⁴
40	REACTOME_CLASS_I_MHC_MEDIATED_ANTIGEN_PROCESSING_PRESENTATION [251]	22	8.75 e ⁻⁶	1.44 e ⁻⁴
41	REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE [179]	18	8.75 e ⁻⁶	1.44 e ⁻⁴
42	REACTOME_PHOSPHOLIPID_METABOLISM [198]	19	9.81 e ⁻⁶	1.57 e ⁻⁴
43	REACTOME_SIGNALING_BY_NGF [217]	20	1.06 e ⁻⁵	1.66 e ⁻⁴
44	REACTOME_MEIOSIS [116]	14	1.09 e ⁻⁵	1.66 e ⁻⁴
45	REACTOME_METABOLISM_OF_AMINO_ACIDS_AND_DERIVATIVES [200]	19	1.13 e ⁻⁵	1.66 e ⁻⁴
46	REACTOME_MEIOTIC_SYNAPSIS [73]	11	1.13 e ⁻⁵	1.66 e ⁻⁴
47	REACTOME_GAB1_SIGNALOSOME [38]	8	1.44 e ⁻⁵	1.99 e ⁻⁴
48	REACTOME_PI3K_AKT_ACTIVATION [38]	8	1.44 e ⁻⁵	1.99 e ⁻⁴
49	REACTOME_TRANSLATION [222]	20	1.48 e ⁻⁵	1.99 e ⁻⁴
50	REACTOME_TELOMERE_MAINTENANCE [75]	11	1.48 e ⁻⁵	1.99 e ⁻⁴

Table 4.9: Top 50 pathways of downregulated genes (fc<-0.5, pathway database: REACTOME data: Ago2-IP)

	Gene Set Name [# Genes (K)]	# Genes in Overlap (k)	p-value	FDR q-value
1	REACTOME_CELL_CYCLE [421]	100	2.21 e ⁻⁶⁴	1.49 e ⁻⁶
2	REACTOME_CELL_CYCLE_MITOTIC [325]	79	8.34 e ⁻⁵²	2.81 e ⁻⁴
3	REACTOME_DNA_REPLICATION [192]	53	3.34 e ⁻³⁸	7.49 e ⁻³
4	REACTOME_MITOTIC_M_M_G1_PHASES [172]	41	4.21 e ⁻²⁷	7.09 e ⁻²
5	REACTOME_CHROMOSOME_MAINTENANCE [122]	35	2.58 e ⁻²⁶	3.48 e ⁻²
6	REACTOME_S_PHASE [109]	31	2.43 e ⁻²³	2.73 e ⁻²
7	REACTOME_IMMUNE_SYSTEM [933]	84	4.08 e ⁻²²	3.93 e ⁻²
8	REACTOME_CELL_CYCLE_CHECKPOINTS [124]	31	1.69 e ⁻²¹	1.42 e ⁻¹
9	REACTOME_MITOTIC_G1_G1_S_PHASES [137]	32	3.6 e ⁻²¹	2.7 e ⁻¹
10	REACTOME_TELOMERE_MAINTENANCE [75]	25	5.32 e ⁻²¹	3.59 e ⁻¹
11	REACTOME_SYNTHESIS_OF_DNA [92]	27	6.53 e ⁻²¹	4 e ⁻¹
12	REACTOME_DEPOSITION_OF_NEW_CENPA_CONTAINING_NUCLEOSOMES_AT_THE_CENTROMERE [64]	23	2.73 e ⁻²⁰	1.53 e ⁻¹
13	REACTOME_G1_S_TRANSITION [112]	28	1.48 e ⁻¹⁹	7.68 e ⁻¹
14	REACTOME_DNA_STRAND_ELONGATION [30]	16	6.2 e ⁻¹⁸	2.99 e ⁻¹
15	REACTOME_ADAPTIVE_IMMUNE_SYSTEM [539]	55	3.01 e ⁻¹⁷	1.35 e ⁻¹
16	REACTOME_MEIOTIC_RECOMBINATION [86]	22	6.72 e ⁻¹⁶	2.83 e ⁻¹
17	REACTOME_G2_M_CHECKPOINTS [45]	17	8.71 e ⁻¹⁶	3.28 e ⁻¹
18	REACTOME_MITOTIC_PROMETAPHASE [87]	22	8.77 e ⁻¹⁶	3.28 e ⁻¹
19	REACTOME_METABOLISM_OF_PROTEINS [518]	51	1.85 e ⁻¹⁵	6.57 e ⁻¹
20	REACTOME_TRANSCRIPTION [210]	31	1.37 e ⁻¹⁴	4.62 e ⁻¹
21	REACTOME_RNA_POL_I_PROMOTER_OPENING [62]	18	2.57 e ⁻¹⁴	8.23 e ⁻¹
22	REACTOME_MEIOSIS [116]	23	5.63 e ⁻¹⁴	1.72 e ⁻¹
23	REACTOME_DNA_REPAIR [112]	22	2.42 e ⁻¹³	7.09 e ⁻¹
24	REACTOME_ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS [38]	14	4.63 e ⁻¹³	1.3 e ⁻¹
25	REACTOME_METABOLISM_OF_RNA [330]	36	1.28 e ⁻¹²	3.45 e ⁻¹
26	REACTOME_M_G1_TRANSITION [81]	18	3.93 e ⁻¹²	1.02 e ⁻¹
27	REACTOME_AMYLOIDS [83]	18	6.11 e ⁻¹²	1.53 e ⁻¹
28	REACTOME_PACKAGING_OF_TELOMERE_ENDS [48]	14	1.8 e ⁻¹¹	4.33 e ⁻¹
29	REACTOME_ORC1_REMOVAL_FROM_CHROMATIN [67]	16	1.9 e ⁻¹¹	4.41 e ⁻¹
30	REACTOME_RNA_POL_I_TRANSCRIPTION [89]	18	2.13 e ⁻¹¹	4.79 e ⁻¹
31	REACTOME_UNWINDING_OF_DNA [11]	8	3.88 e ⁻¹¹	8.43 e ⁻¹
32	REACTOME_MITOTIC_G2_G2_M_PHASES [81]	17	4.11 e ⁻¹¹	8.46 e ⁻¹
33	REACTOME_EXTENSION_OF_TELOMERES [27]	11	4.14 e ⁻¹¹	8.46 e ⁻¹
34	REACTOME_MEIOTIC_SYNAPSIS [73]	16	7.65 e ⁻¹¹	1.52 e ⁻¹
35	REACTOME_ASSEMBLY_OF_THE_PRE_REPLICATIVE_COMPLEX [65]	15	1.37 e ⁻¹⁰	2.63 e ⁻¹
36	REACTOME_SIGNALING_BY_NGF [217]	26	2.14 e ⁻¹⁰	4 e ⁻¹
37	REACTOME_FACTORS_INVOLVED_IN_MEGAKARYOCYTE_DEVELOPMENT_AND_PLATELET_PRODUCTION [132]	20	3.99 e ⁻¹⁰	7.15 e ⁻¹
38	REACTOME_G0_AND_EARLY_G1 [25]	10	4.03 e ⁻¹⁰	7.15 e ⁻¹
39	REACTOME_RNA_POL_I_RNA_POL_III_AND_MITOCHONDRIAL_TRANSCRIPTION [122]	19	6.75 e ⁻¹⁰	1.17 e ⁻¹
40	REACTOME_REGULATION_OF_MITOTIC_CELL_CYCLE [85]	16	8.46 e ⁻¹⁰	1.43 e ⁻¹
41	REACTOME_METABOLISM_OF_MRNA [284]	29	9.14 e ⁻¹⁰	1.5 e ⁻¹
42	REACTOME_CYCLIN_A_B1_ASSOCIATED_EVENTS_DURING_G2_M_TRANSITION [15]	8	1.38 e ⁻⁹	2.21 e ⁻¹
43	REACTOME_CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION [65]	14	1.49 e ⁻⁹	2.34 e ⁻¹
44	REACTOME_HIV_INFECTION [207]	24	2.04 e ⁻⁹	3.12 e ⁻¹
45	REACTOME_ANTIGEN_PROCESSING_UBIQUITINATION_PROTEASOME_DEGRADATION [212]	24	3.3 e ⁻⁹	4.94 e ⁻¹
46	REACTOME_ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX [31]	10	4.73 e ⁻⁹	6.93 e ⁻¹
47	REACTOME_CLASS_I_MHC_MEDIATED_ANTIGEN_PROCESSING_PRESENTATION [251]	26	4.94 e ⁻⁹	7.08 e ⁻¹
48	REACTOME_POL_SWITCHING [13]	7	1.42 e ⁻⁸	1.99 e ⁻¹
49	REACTOME_LAGGING_STRAND_SYNTHESIS [19]	8	1.47 e ⁻⁸	2.02 e ⁻¹
50	REACTOME_E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION [35]	10	1.78 e ⁻⁸	2.39 e ⁻¹

Table 4.10: Top 50 pathways of upregulated genes (fc>0.5, pathway database: REACTOME data: Ago2-IP)

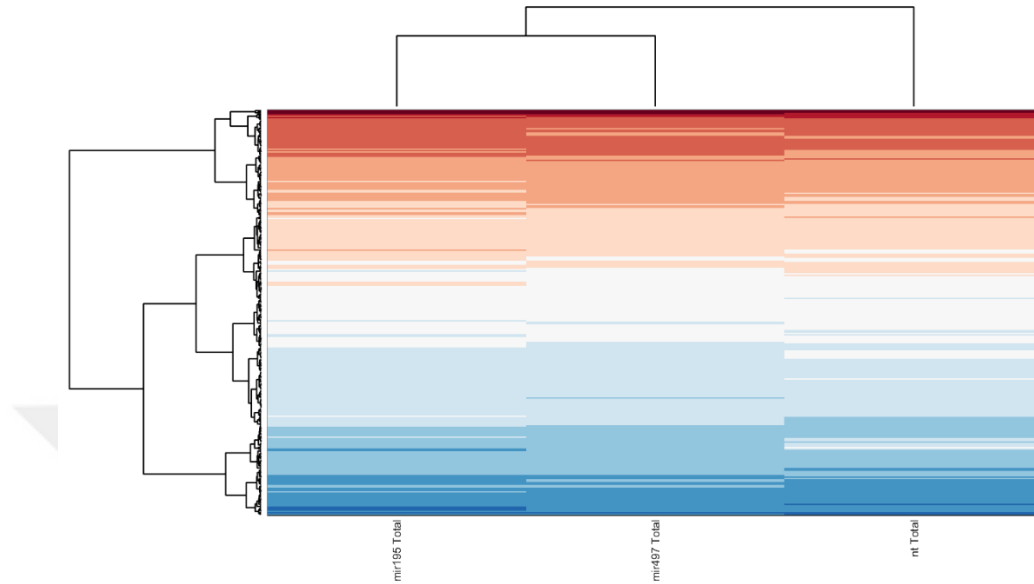
	Gene Set Name [# Genes (K)]	# Genes in Overlap (k)	p-value	FDR q-value
1	REACTOME_CHOLESTEROL_BIOSYNTHESIS [24]	6	5.48 e ⁻¹⁰	3.69 e ⁻⁷
2	REACTOME_METABOLISM_OF_LIPIDS_AND_LIPOPROTEINS [478]	10	3.1 e ⁻⁵	1.04 e ⁻²
3	REACTOME_FATTY_ACID_TRIACYLGLYCEROL_AND_KETONE_BODY_METABOLISM [168]	6	7.13 e ⁻⁵	1.6 e ⁻²

4.2.1.2. General Analysis of GSE41079 and GSE4107 to Compare All Genes and only Immune Genes Clustergrams

One of the best visual comparisons was obtained from clustergrams and in the following two figures comparisons were done by clustergrams. In Figure 4.28 B non-treated result

was closer to mimic-mir-497 treated samples than mimic-mir-195; in A non-treated result was closer to mimic-mir-195.

A)



B)

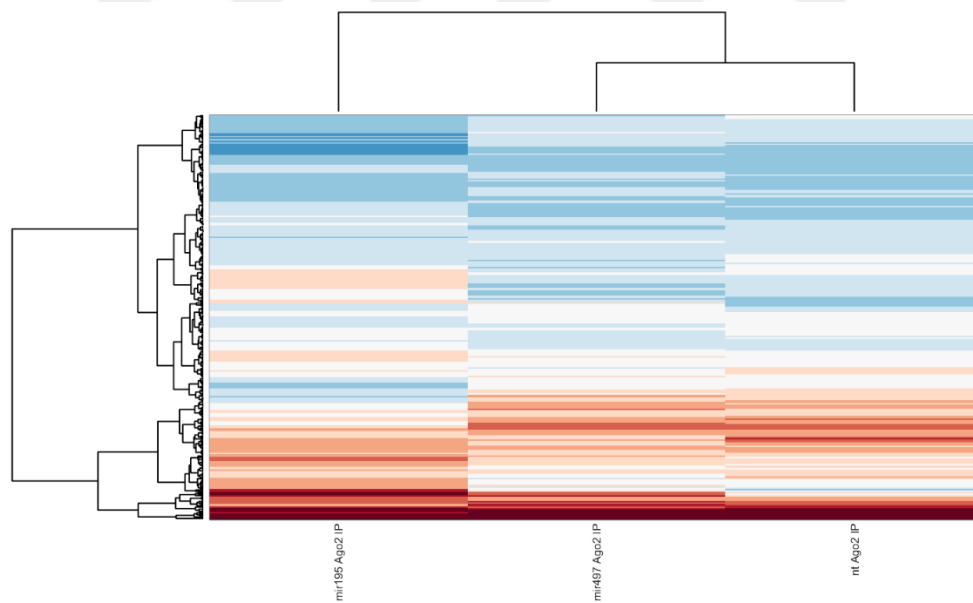
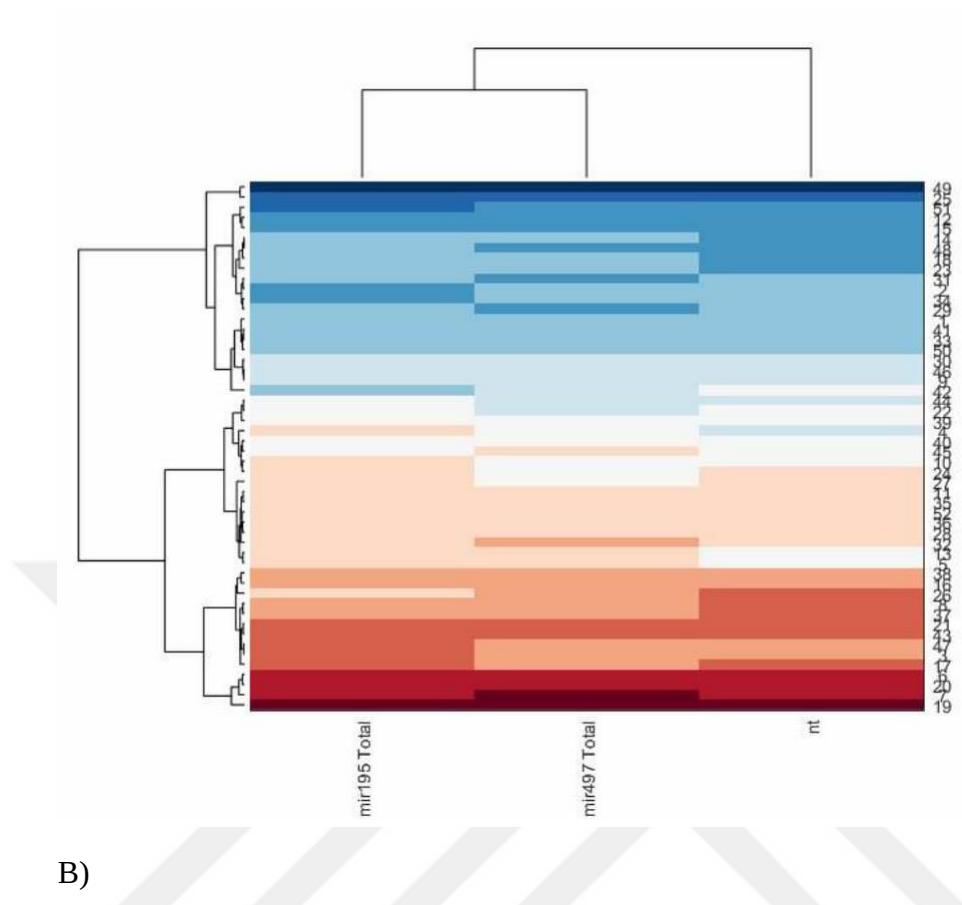


Figure 4.28: Analysis of GSE41074 linkage A)Total RNA B) Ago2-IP

Immune gene expression levels in GSE41074 under mimic-mir-195 and mimic-mir-497 were used to see the clustergrams of immune genes in GSE41074 (Figure 4.29).

A)



B)

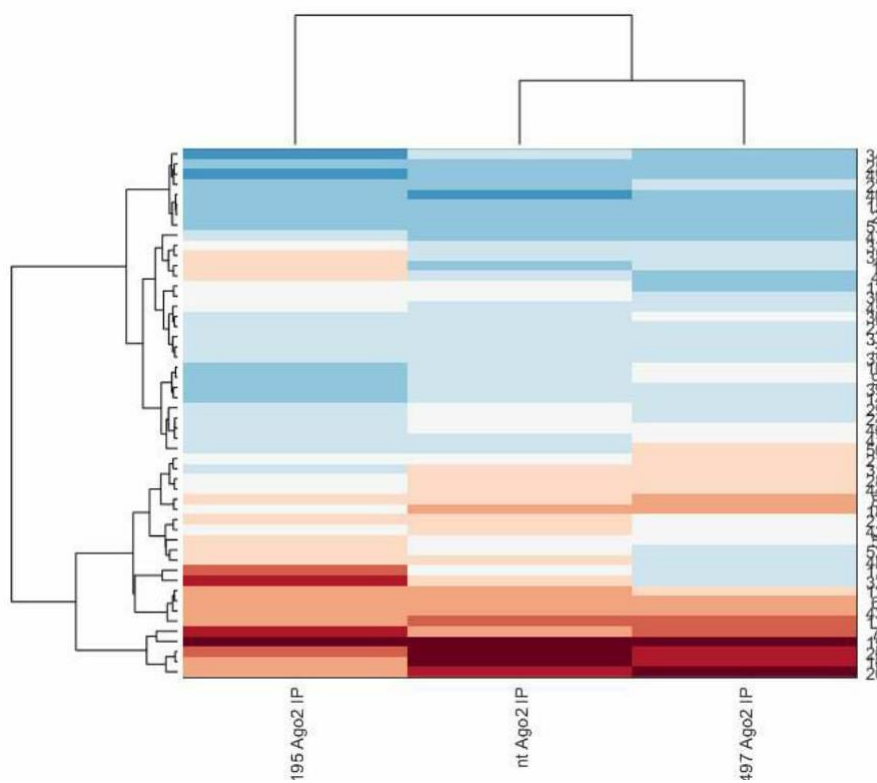


Figure 4.29: Analysis of immune genes in GSE41074 linkage A) Total RNA B) Ago2-IP

After using direct values, LogFC were used to show similarities and difference between mimic treatments. There was more difference between Ago2-IP and Total RNA under mimic-mir-195 treatment than mimic-mir-497 treatment (Figure 4.30).

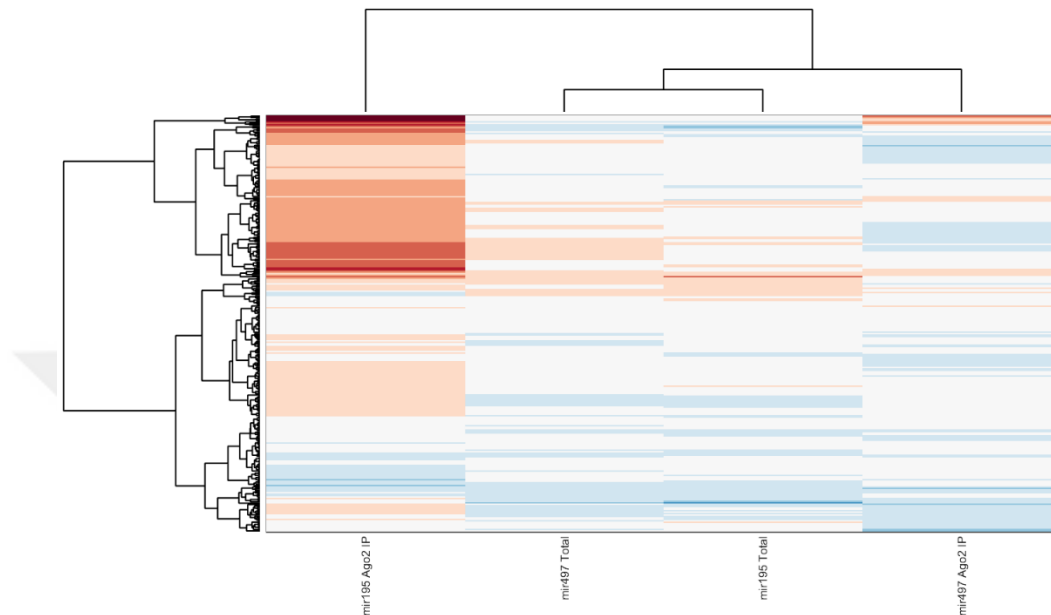


Figure 4.30: Clustergram of GSE41074 according to log2 fold changes.

4.2.2. Expression Results of Immune Genes under siRNA CHRNA5 and mimic-mir-497 Treatment

mRNA and microRNA microarray Results

A group of microRNAs were selected based on Paladini et al. ⁵⁵. According to Table 1.4 mir-146a has role in both immune (inflammation modulation) and breast cancer; mir-23 has a role in both immune (macrophage polarization) and breast cancer (tumour growth); mir-34c is immune and cancer related. Mir-221 has been shown in tumour associated immune cell (Figure 1.3) ⁵⁵. Under siRNA CHRNA5 treatment only mir-34c-3p and mir-23a-3p were upregulated; under mimic-mir-497 treatment, mir-497-5 and mir-497-3p were upregulated in Ago2-IP samples. Mir-184 and mir-34c-3p were not expressed in any sample. In addition to mir-195-3p/5p, mir-497-5p and mir-221-3p were upregulated under mimic-mir-195 treatment. Mimic-mir-497 decrease the expression of mir-146a and mir-23a and decrease mir-221-3p and mir-195-5p lesser. Mimic-mir-195 makes not that much

of a difference for those microRNAs except mir-497-5p, it was upregulated. (Table 4.11 and Table 4.12)

Table 4.11: Expression Profile of microRNAs under siRNA CHRNA5 Treatment

Probe Set ID	miRNA ID	siRNA CHRNA5-1	siRNA CHRNA5-2	Scrambled oligo-1	Scrambled oligo-2	Avg LogFC
20503809	hsa-mir-497-5p	8.021	8.044	7.195	7.078	-0.896
20503810	hsa-mir-497-3p	0.778	0.805	0.720	0.490	-0.186
20500484	hsa-miR-221-3p	7.836	7.917	7.919	7.832	-0.001
20501171	hsa-mir-34c-3p	0.752	0.986	1.095	1.405	0.381
20500778	hsa-mir-146a-5p	1.618	1.490	1.471	1.390	-0.123
20500146	hsa-miR-23a-3p	13.529	13.628	13.699	13.668	0.105
20500798	hsa-miR-195-5p	10.525	10.664	10.237	10.183	-0.385
20500799	hsa-miR-195-3p	2.856	2.260	1.837	1.183	-1.048

Table 4.12: Expression Profile of microRNAs under mimic-mir-497 or mimic-mir-195 Treatment

miRNA ID from GSE41079	Ago2-IP mimic-mir-195	Ago2-IP mimic-mir-497	Ago2-IP non treated	Ago2-IP LogFC mimic-mir-195	Ago2-IP LogFC mimic-mir-497
hsa-miR-497 (5p)	4.996	12.035	3.484	1.511	8.550
hsa-miR-497* (3p)	0.000	6.172	0.000	0.000	6.172
hsa-miR-221 (3p)	11.285	9.640	10.911	0.374	-1.270
hsa-miR-34c-3p	0.000	0.000	0.000	0.000	0.000
hsa-miR-146a (5p)	8.394	7.058	8.499	-0.105	-1.441
hsa-miR-23a (3p)	8.408	6.907	8.622	-0.214	-1.715
hsa-miR-195 (5p)	14.275	3.775	4.556	9.719	-0.781
hsa-miR-195* (3p)	11.375	0.000	0.000	11.375	0.000

microRNA qPCR Results

microRNAs related to immune system (mir-184, mir-146a, mir-221, mir-23a, mir-34c) were not highly effected by siRNA CHRNA5 and downregulated (except mir-34c) in GSE41079 under mimic-mir-497 treatment. There were interesting interactions in microRNA networks; microRNAs and genes were effected by transcription factors (either up or downward). Basically, when treatments done by itself (mimic only for example) microRNA expression was higher than the scrambled. On the other hand, mir-497 was upregulated after mimic-mir-497 treatment and downregulated in the absence of siRNA CHRNA5. By using different agents in the experiment, the result was that CHRNA5 and mir-497-5p might interact indirectly. The results indicated that mir-497-5p shows

significant alteration with both treatments (mimic and siRNA) in breast cancer and it might have a connection to immune system.

There was an expected decrease on expression of mir-497-5p under siRNA CHRNA5 treatment and upregulation in mimic-497 treatment. When scrambled oligo used with mimic, mir-497 was upregulated less. When siRNA and mimic used together, mir-497 was upregulated less than only mimic.

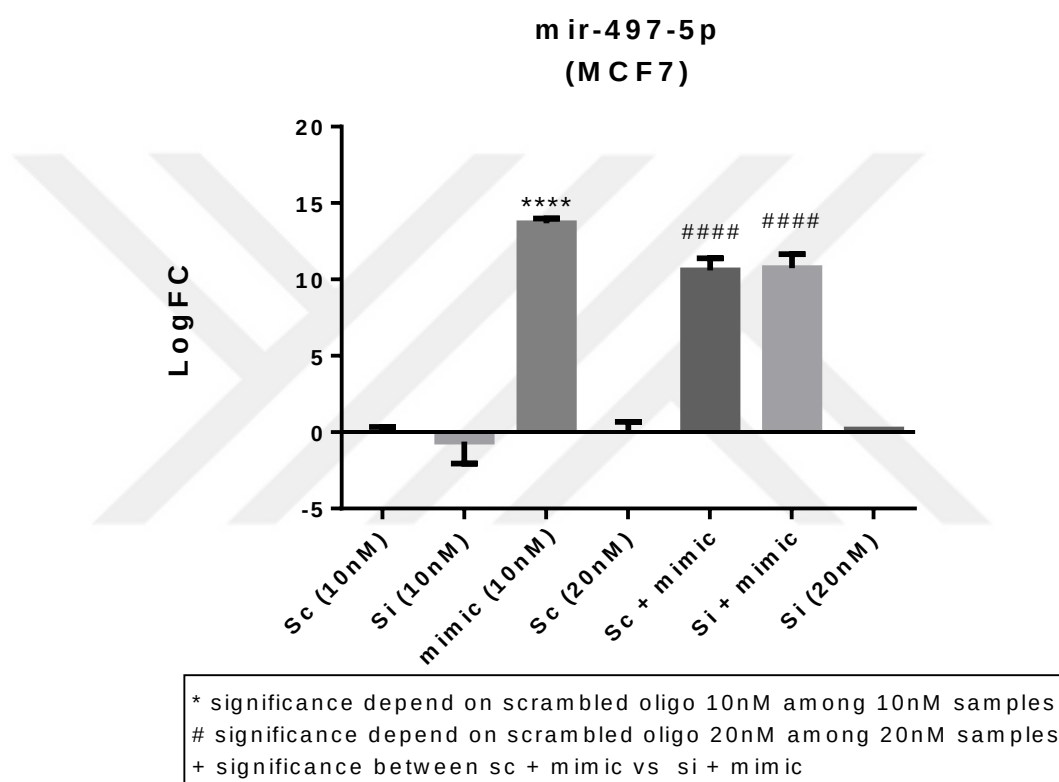
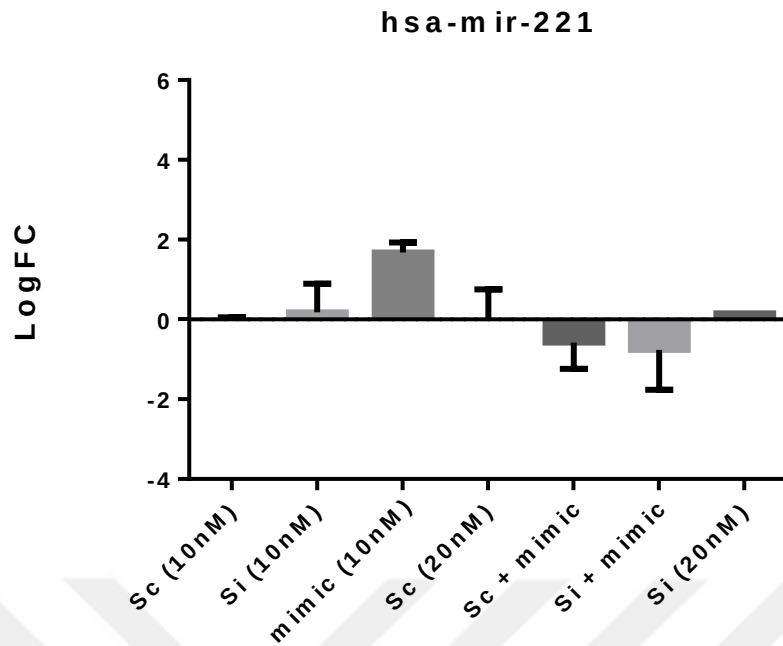


Figure 4.31: Expression of mir-497-5p in mimic-mir-497 and siRNA CHRAN5 treated samples.

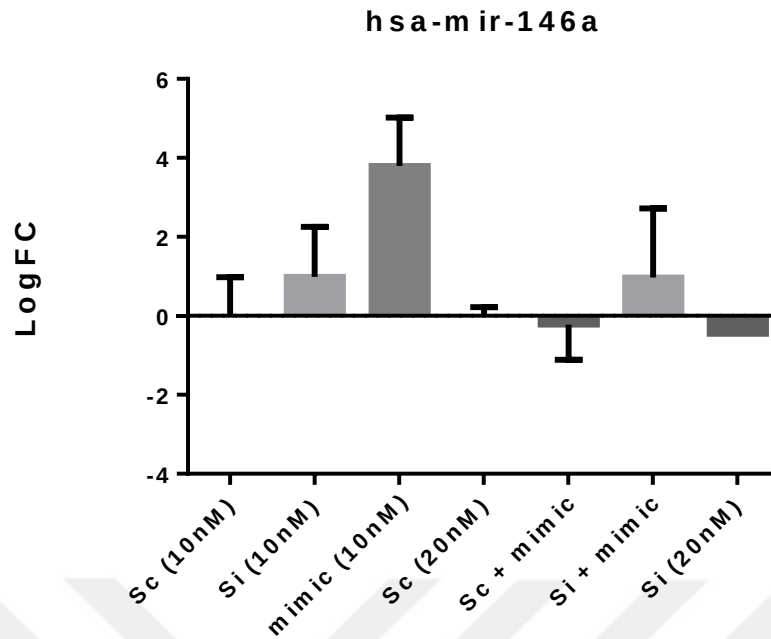
There was no change of mir-221 expression under siRNA CHRNA5 treatment in microarray (Figure 4.32). In GSE41074, it was downregulated. qPCR was showing that under each treatment separately there was a non-significant upregulation. If both mimic-497 and siRNA CHRNA5 were used together its expression decreases. When mimic was used together with siRNA or scrambled oligo, the result was decrease in expression as expected.



* significance depend on scrambled oligo 10nM among 10nM samples
 # significance depend on scrambled oligo 20nM among 20nM samples
 + significance between sc + mimic vs si + mimic

Figure 4.32: Expression of mir-221-3p in mimic-mir-497 and siRNA CHRNA5 treated samples.

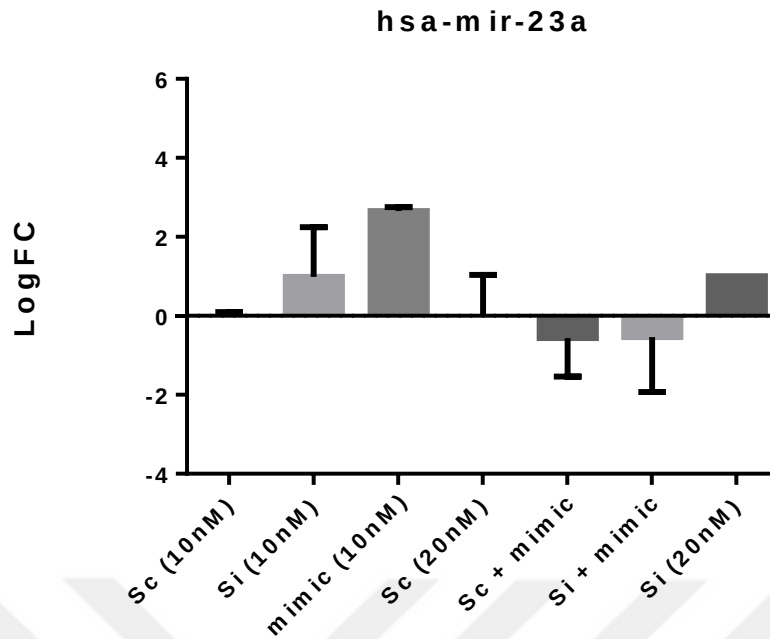
siRNA CHRNA5 and mimic-mir-497 made mir-146a downregulate in microRNA microarrays. However, in qPCR result they both were upregulated. Mimic did not show an impact on siRNA CHRNA5 effect for mir-146a expression.



* significance depend on scrambled oligo 10nM among 10nM samples
 # significance depend on scrambled oligo 20nM among 20nM samples
 + significance between sc + mimic vs si + mimic

Figure 4.33: Expression of mir-146a in mimic-mir-497 and siRNA CHRAN5 treated samples. (p value (sc/mimic) = 0.0811)

Mir-23a was slightly upregulated in siRNA CHRAN5 microarray and downregulated in mimic-mir-497 array. It was upregulated under both treatment (qPCR result) yet not significantly (Figure 4.34). When siRNA and mimic were used together, mir-23a was downregulated. Scrambled oligo changes mimic-mir-497 effect on mir-23a.



* significance depend on scrambled oligo 10nM among 10nM samples
 # significance depend on scrambled oligo 20nM among 20nM samples
 + significance between sc + mimic vs si + mimic

Figure 4.34: Expression of mir-23a in mimic-mir-497 and siRNA CHRAN5 treated samples

mir-34c was upregulated in siRNA CHRAN5 microRNA microarray and non-changed in mimic-497 microRNA microarray. In qPCR result mir-34c was upregulated, but mimic-mir-497 made it increase yet non significantly (Figure 4.35). Scrambled oligo effects mimic treatment result when they were used together.

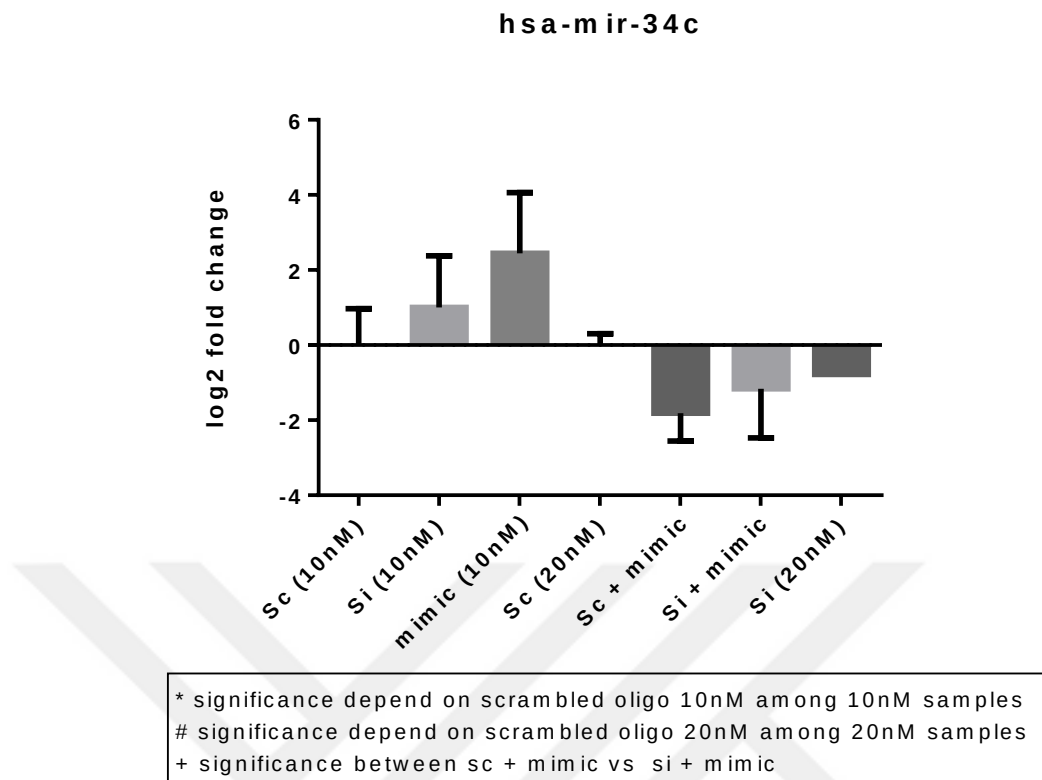


Figure 4.35: Expression of mir-34c in mimic-mir-497 and siRNA CHRN5 treated samples

4.3.mir-497 Related GEO Data Analysis (GSE41079 and GSE41074) by mRNA-microRNA Integration Tools

4.3.1. General GEO data analysis to see candidate microRNA under mimic-mir-497/195 treatment

The following graph shows the most upregulated microRNA under both treatments. For example, mir-483-3p, mir-494, mir-455-5p, mir-33a, mir-30e* (mir-30e-3p), mir-32, mir923_v12.0 and mir-497 were upregulated with both mimic treatments.

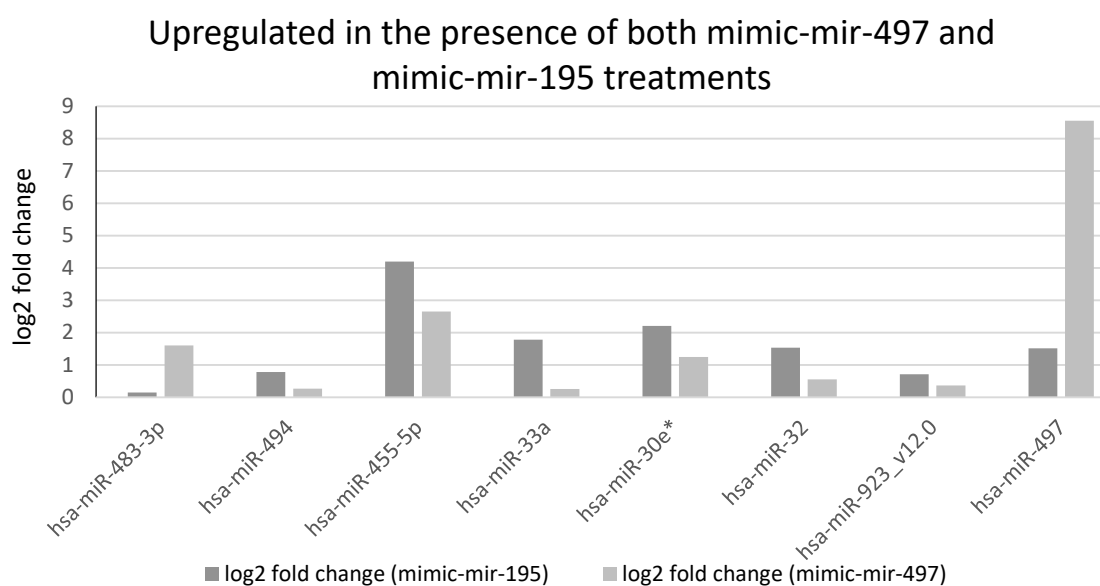


Figure 4.36: Upregulated microRNAs in both mimic treatments.

Many microRNAs were upregulated under mimic-mir-195 and downregulated or unaffected under mimic-mir-497. (Figure 4.37)

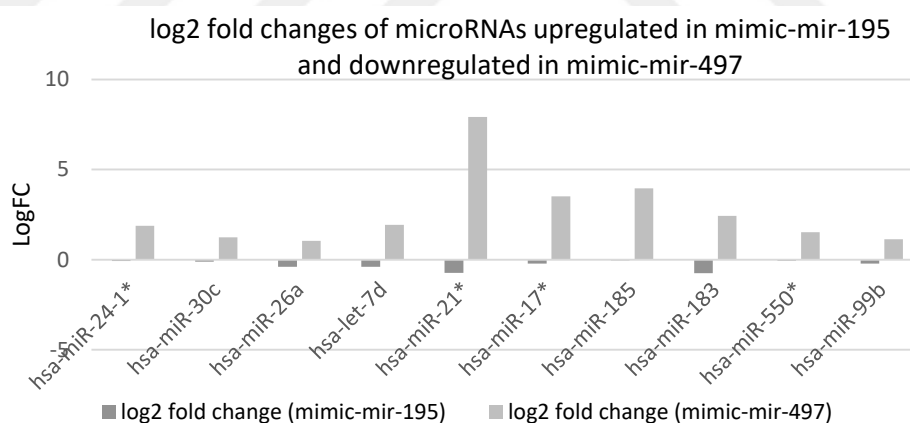


Figure 4.37: Log2 fold changes of upregulated microRNAs in mimic-mir-195 and down-regulated in mimic-mir-497.

When microRNAs were sorted depend on log2 fold change, 16 most upregulated and 24 most downregulated microRNAs were shown.

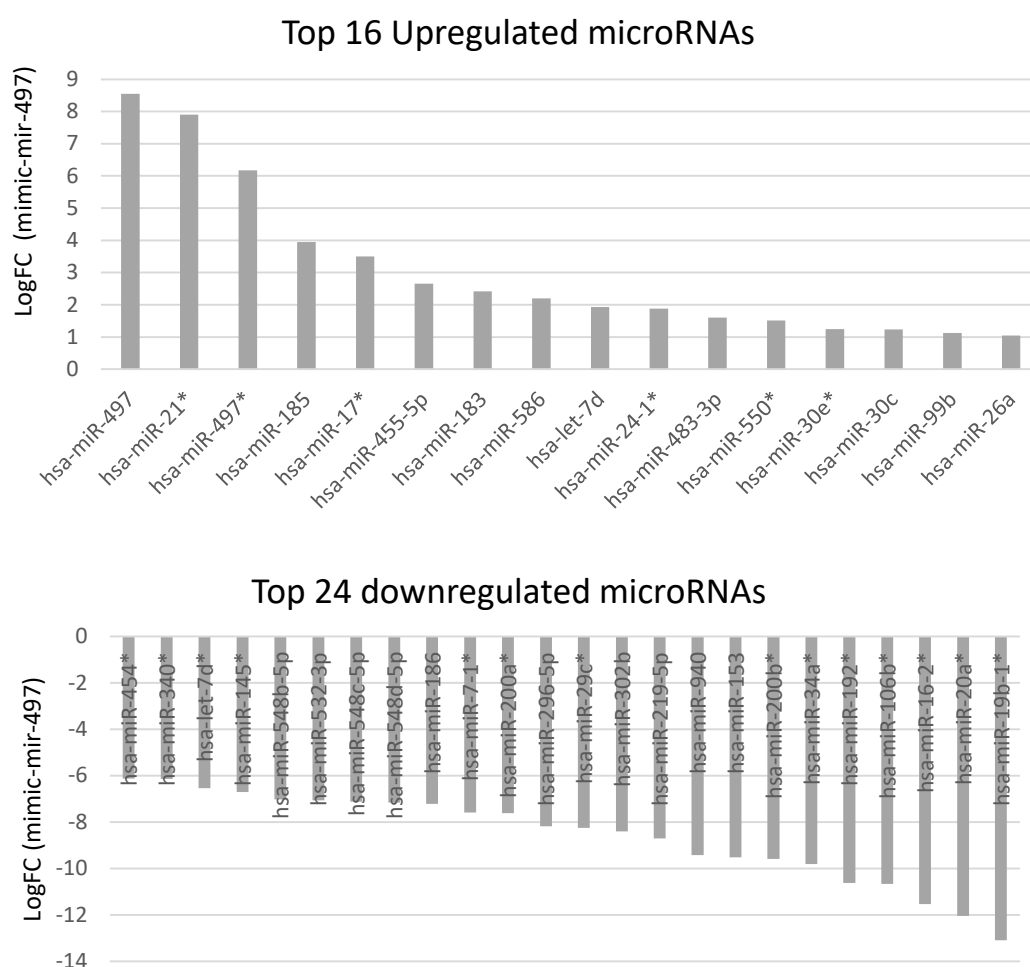


Figure 4.38 Most A) upregulated and B) downregulated microRNAs present in the sort of % difference for mimic-mir-497 treatment from most upregulated to most downregulated.

Predicted microRNAs that target CHRNA5, by miRWalk using twelve different algorithms in GSE41079

There are twelve algorithms in miRWalk to predict targets. Therefore, a list of predicted targets by at least one of the algorithms was handled. Afterwards the microRNAs which were predicted as targeting elements to CHRNA5 were searched in GSE41079 which showed the effected microRNAs from mimic-mir-497 and mimic-mir195 treatments to build a relationship between mir-497 and CHRNA5. In 4, first five microRNAs which are predicted to target CHRNA5 were affected by only mimic-mir-195 and mir-586 was affected by only mimic-mir-497 treatments.

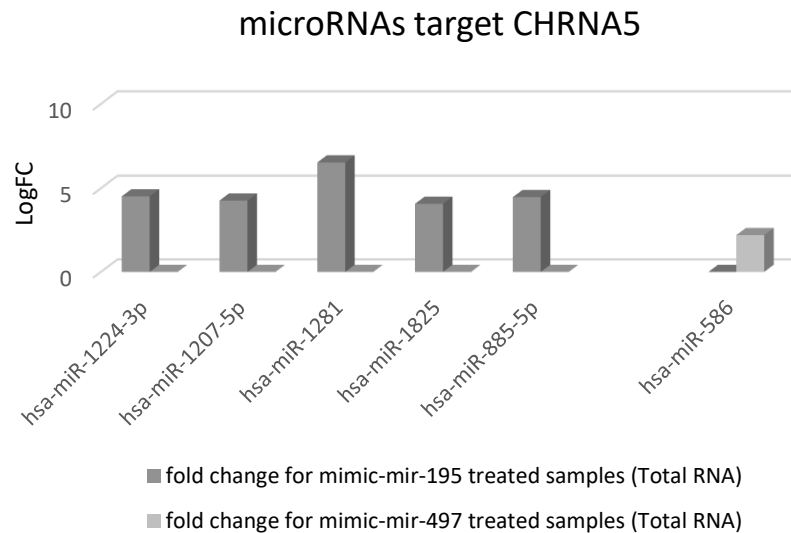


Figure 4.39: Predicted CHRNA5 targeted microRNAs which are affected by one of the treatments mimic-mir-195 or mimic-mir-497.

Twenty-two microRNAs were predicted in 5/12 algorithms, it was considered highly predicted microRNAs in this study and nearly half of highly predicted microRNAs had more than $\pm$ one-fold change under at least one of the mimic treatments. Except mir-1224-3p, mir-1202, mir-1207-5p and mir-638; mimic-mir-497 was more effective on microRNAs. (Figure 4.40)

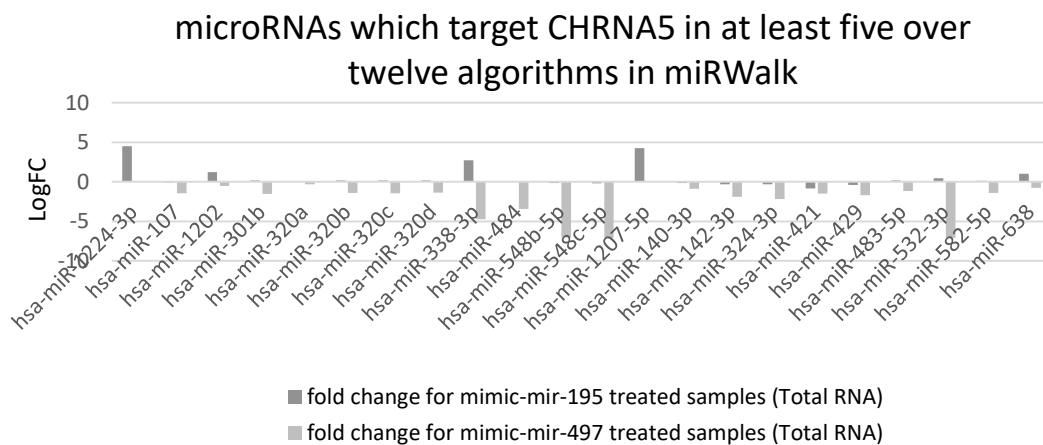


Figure 4.40: CHRNA5 is targeted by microRNAs which have log2 fold change in Ago2-IP mimic-mir-497 samples are less than Ago2-IP mimic-mir-195 in at least 5 predictors out of 12 in miRWalk. Future studies will focus on testing these findings.

4.3.2. MirTarVis

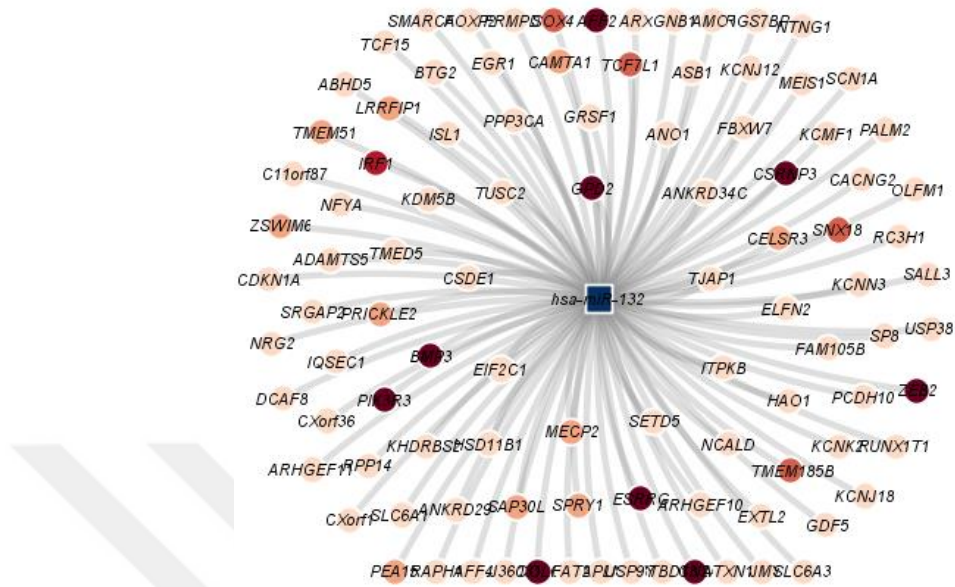
GSE41079 and GSE41074 had expression values of three common samples: Ago2-IP mimic-mir-497, Ago2-IP mimic-mir-195, Ago2-IP non-treated. Therefore, the values were used to see the best interactions on networks by MirTarVis. Additionally, only immune related mRNAs filtered from all mRNA list (GSE41074). Only immune related mRNAs and all microRNAs were used by MirTarVis so immune related networks under mimic treatments (mir-195 and mir-497) were handled. There are four subtitles to analyse: networks structured by mimic-mir-497 and mimic-mir-195 and immune versions of both.

mimic-mir-195

Mimic-mir-195 treatment results gave three microRNAs: mir-132, mir-381 and mir-373. LRRC7 was the most downregulated gene related to upregulated of mir-381 in the network without filtering (Figure 4.41 A). When filtering was done by choosing only negative correlation and opposite direction downregulated genes and upregulated microRNA was obtained. Genes tended to be downregulated in MirTarVis networks. When only downregulated microRNA and upregulated mRNA were chosen for network construct (Figure 4.41C), there was a different microRNA than other three networks: mir-132 and it was highly downregulated. There were highly upregulated genes: AFF2, GPD2, BMP3, PIK3R3, ESRRC, ZEB2, COLQ, IRF1 and SCN2A. In Figure 4.11 D there were only downregulated genes; most downregulated genes: NR4A2, UC5C, ESR1, GRIP2, STXBP5L, NPR3, MEF2C, RPS6KA6, GALNT7, GABRG1, LRRC7 and TFAP2B.



C)



D)

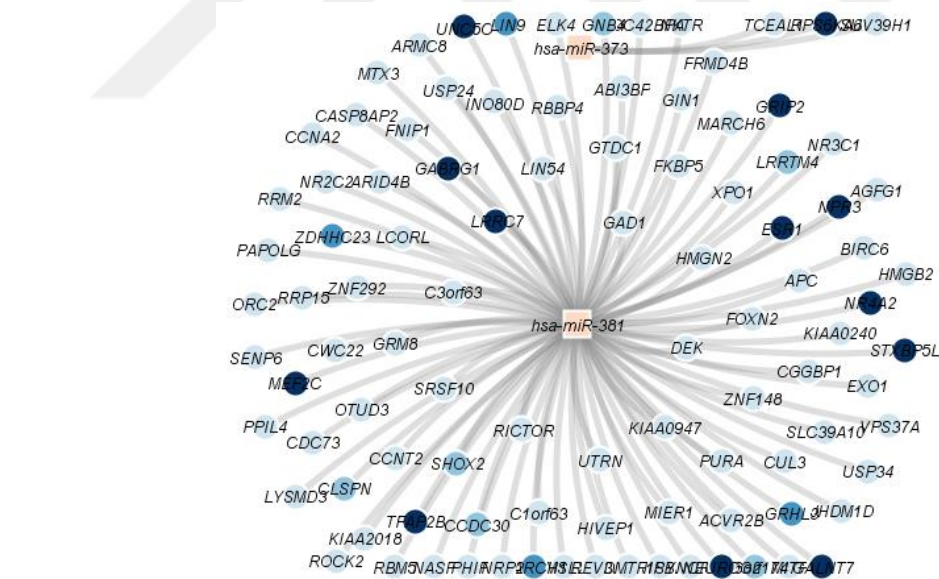
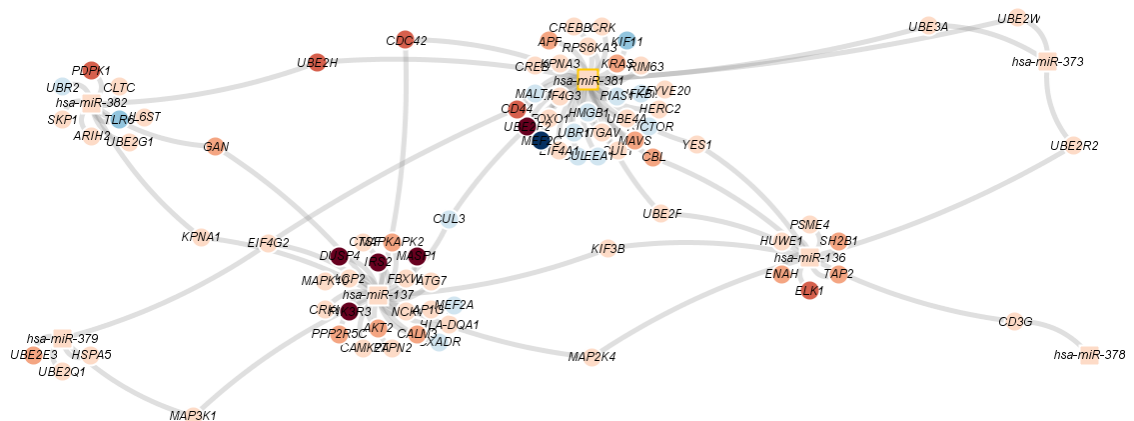


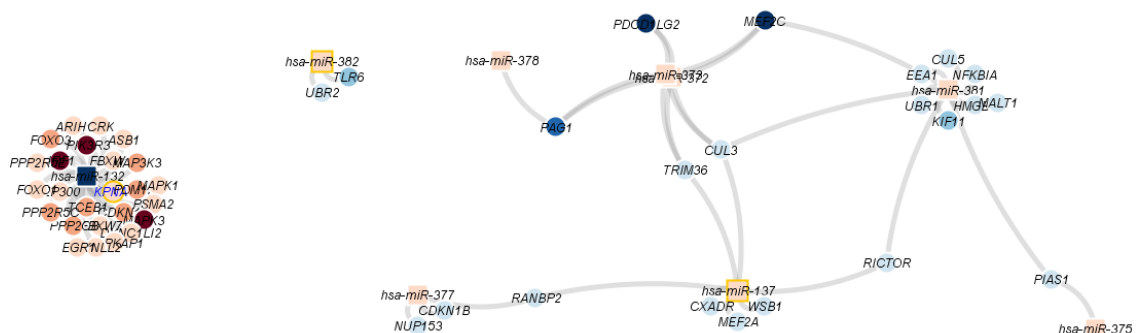
Figure 4.41: Top 100 interactions' network of mimic-mir-195 treatment by MirTarVis (Correlation Analysis) with no data filter by using Ago2-IP samples A) No negative correlation no filtering on direction B) Only negative correlation and opposite direction C) No filtering on correlation but downregulated microRNA and upregulated mRNA chosen as direction D) No filtering on correlation but downregulated mRNA and upregulated microRNA chosen as direction

mimic-mir-195 (immune related)

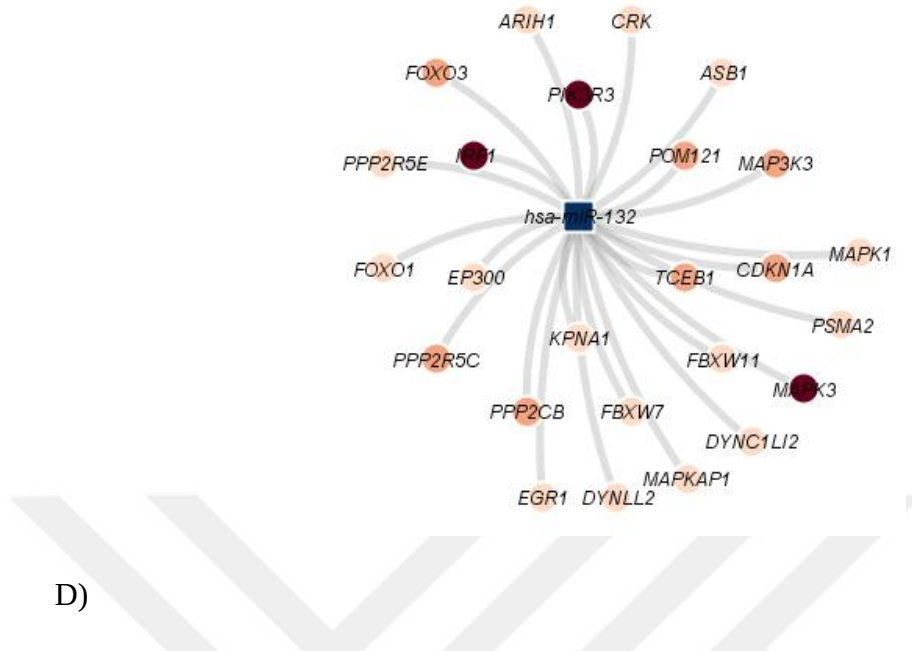
In Figure 4.42A there was no filter and B has only opposite direction only negative correlation filters. From A to B majority of microRNAs were same, mir-136 and mir-379 were not seen anymore and mir-132, mir-375, mir-377 and mir-372 show up. Filtering makes network has less related genes. In Figure 4.42 A, a great majority of elements (genes and microRNAs) were upregulated. MEF2C and KIF11 were outstanding downregulated genes from the figure. In Figure 4.42 B, these genes were staying and more downregulated genes show up: TLR6, PAG1 and PDCD1LG2. Downregulated mir-132 was another hub microRNA and most upregulated genes around it were: IRF1, PIK3R3 and MAPK3. Figure 4.42 C and D seem they were handled from Figure 4.42 B by separating upregulated and downregulated microRNAs.



B)



C)



D)

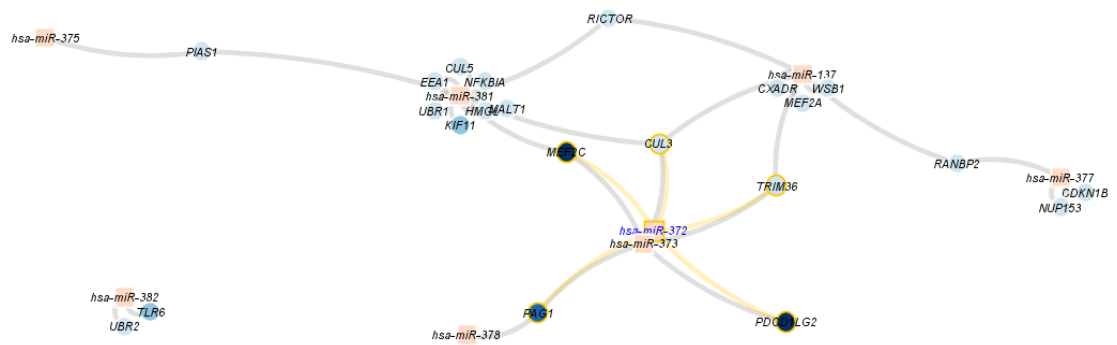
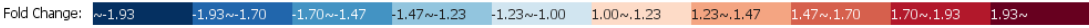


Figure 4.42: Top 100 interactions' immune related network of mimic-mir-195 treatment by MirTarVis (Correlation Analysis) with no data filter by using Ago2-IP samples A) No negative correlation no filtering on direction B) Only negative correlation and opposite direction C) No filtering on correlation but downregulated microRNA and upregulated mRNA chosen as direction D) No filtering on correlation but downregulated mRNA and upregulated microRNA chosen as direction

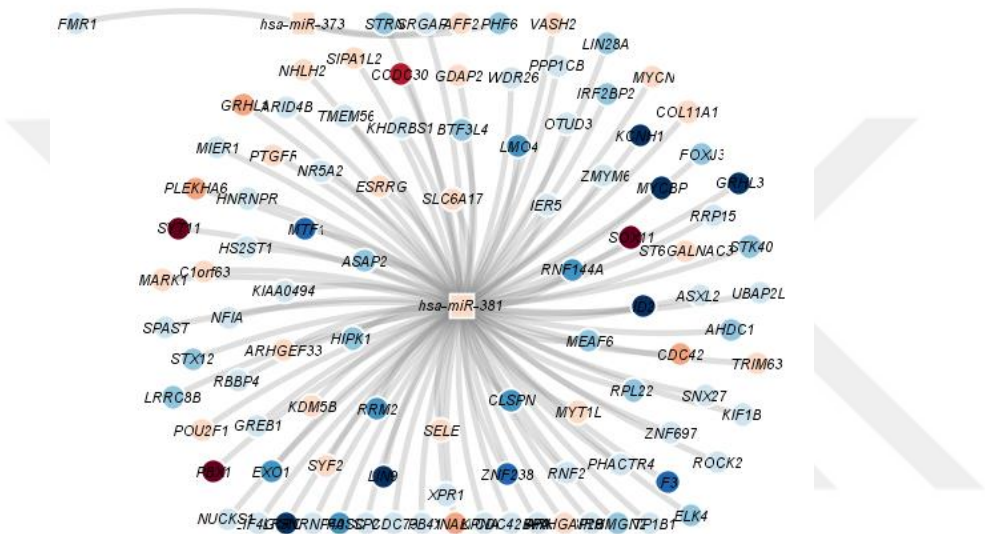
mimic-mir-497

mir-497 mimic treatment gave three same hub microRNAs: mir-132, mir-381 and mir-373 and additional one microRNA mir-378 not included in mimic-mir-195 networks (Figure 4.41, Figure 4.43). Genes tended to be downregulated. No filter gave low upregulated mir-381; highly downregulated genes related to this microRNA were: GPHL3, LIN9, ID2, GRHL3, MYCBP and KCNH1 (Figure 4.43B). Figure 4.43D

resembles to B. Figure 4.43 C gives different microRNAs mir-132 and mir-378. Mir-132 was highly downregulated and mir-378 showed low downregulation. PIK3R3 was highly upregulated as a common gene of both microRNAs. LMLN was upregulated and related to mir-132 only; mir-378 related and highly upregulated genes are: SLC26A9, BMP2, PAPP, CD226 and GOLT.



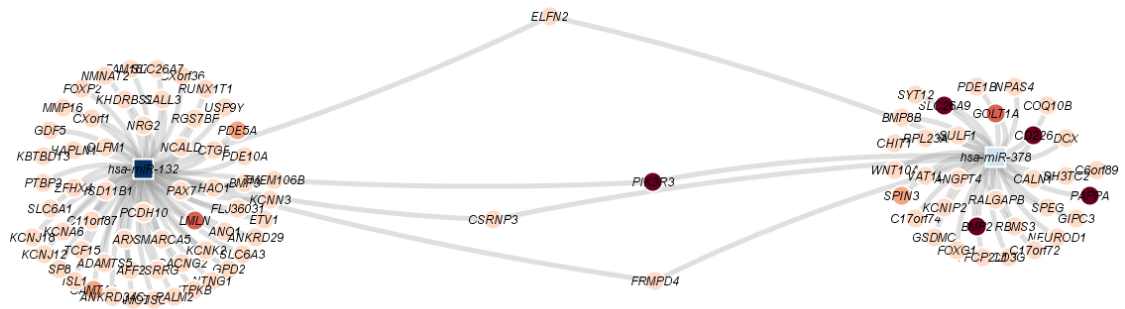
A)



B)



C)



D)

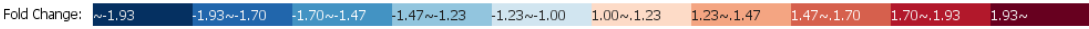


Figure 4.43: Top 100 interactions' related network of mimic-mir-497 treatment by MirTarVis (Correlation Analysis) with no data filter by using Ago2-IP samples A)No negative correlation no filtering on direction B) Only negative correlation and opposite direction C) No filtering on correlation but downregulated microRNA and upregulated mRNA chosen as direction D) No filtering on correlation but downregulated mRNA and upregulated microRNA chosen as direction

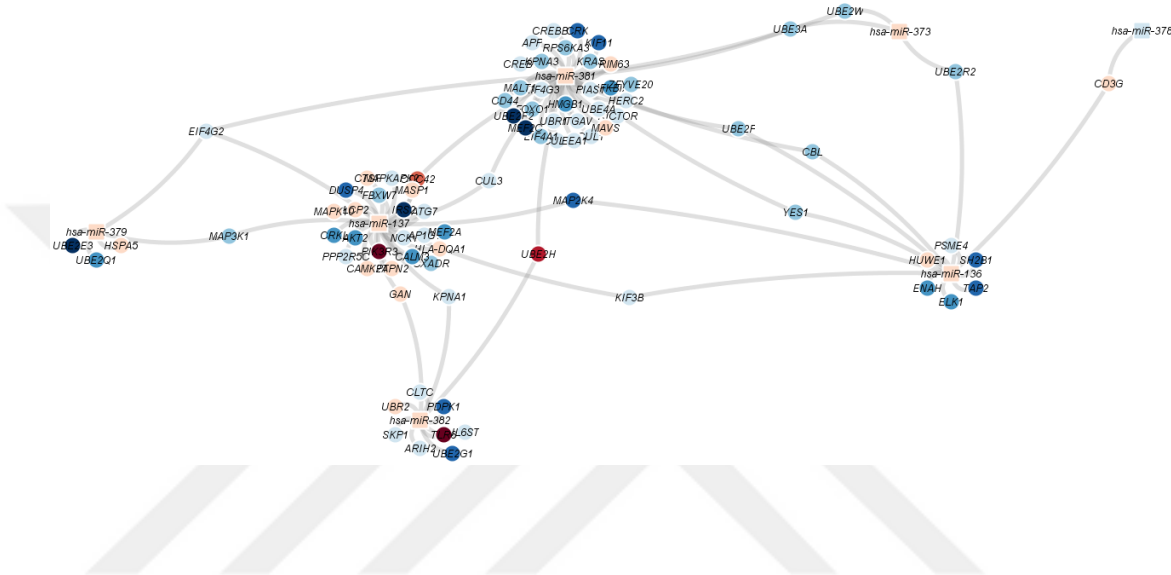
mimic-mir-497(immune related)

Figures related to immune genes are more complex than all genes under also mimic-mir-497. For both mimic treatments from immune perspective similar microRNAs built the networks (Figure 4.42, Figure 4.44). Same hub microRNAs are including in both no filter and only opposite direction and only negative correlation figures, filtered one has mir-134 additionally (Figure 4.44 A, B). Genes tended to be downregulated for best interactions but there were few upregulated genes: UBE2H, TLR6 and PIK3R3 (Figure 4.44 A). PIK3R3 was a common upregulated gene for mir-132 and mir-378. RNF144B /mir-378 and CD226/mir-378 were best interactions as upregulated gene/downregulated microRNA (Figure 4.44 C). Most downregulated common genes were UBE2G1 (mir-137 and mir-382) and MAP2K4 (mir-136 and mir-137). Additional best interactions were mir-

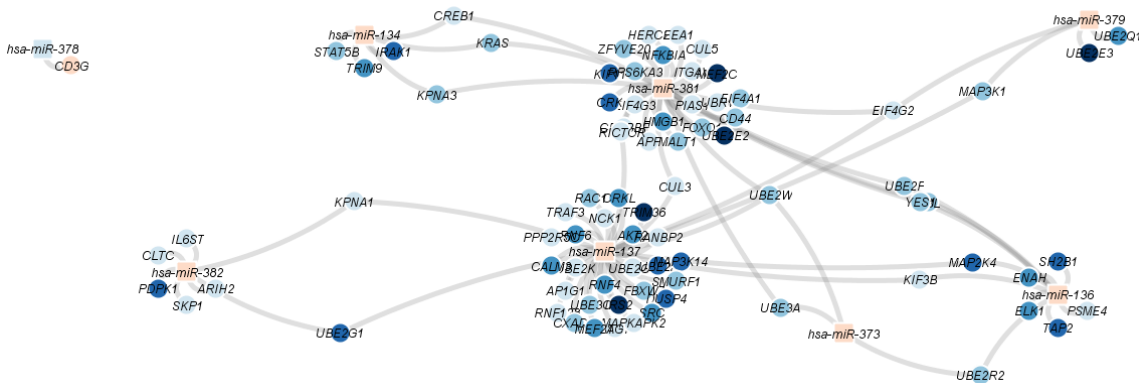
379/UBE2E3, mir-134/IRAK1, mir-382/PDPK1, mir-137/TRIM36, mir-381/MEF2C, mir-381/UBE2E2 as upregulated microRNAs and downregulated genes (Figure 4.44 D).



A)



B)



mir-615-3p, mir-132-3p and mir-10a-5p target CHRNA5, so miRNet result for CHRNA5 indicated that mir-132-3p was an important microRNA for CHRNA5 regulation.

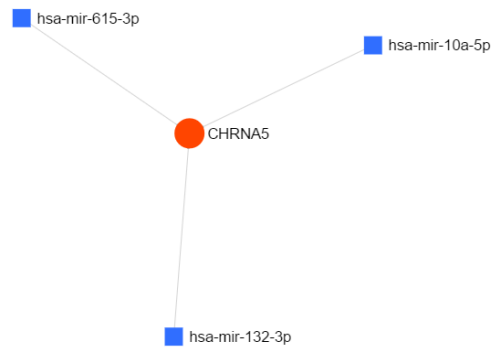


Figure 4.45: Network of microRNAs which targets CHRNA5 by miRNet.

4.3.3. MAGIA2

Basically interested topics were mir-497 and immune targets. Therefore, MAGIA2 networks were done on these subjects. There were two major networks: first one showing interactions by using all genes (Figure 4.46 A and Figure 4.47 A) and second one showing only immune genes (Figure 4.46 B, Figure 4.47 B). In MAGIA2 there was a second step after handling networks: networks of circuits. Circuits consisted of three elements which can be either a transcription factor, a regulated microRNA by this transcription factor and target gene of the microRNA or a microRNA and its regulated genes (a transcription factor and its regulated gene by this transcription factor). Therefore, the circuit networks gave more solid leading points. microRNAs from Figure 4.46 B were mir-125a-5p, mir-301b, mir-590-5p, mir-338-3p, mir-429 and mir-142-3p. They all were included in Figure 4.46. A and there are additional eleven microRNAs. In circuits network, the following genes became prominent: POU3F2, REL, EGR1, CEBPA, HNF1A and IRF1 (Figure 4.46 B). Non-immune direct network showed ZMAT3 as positively correlated with PBX1. (Figure 4.46))

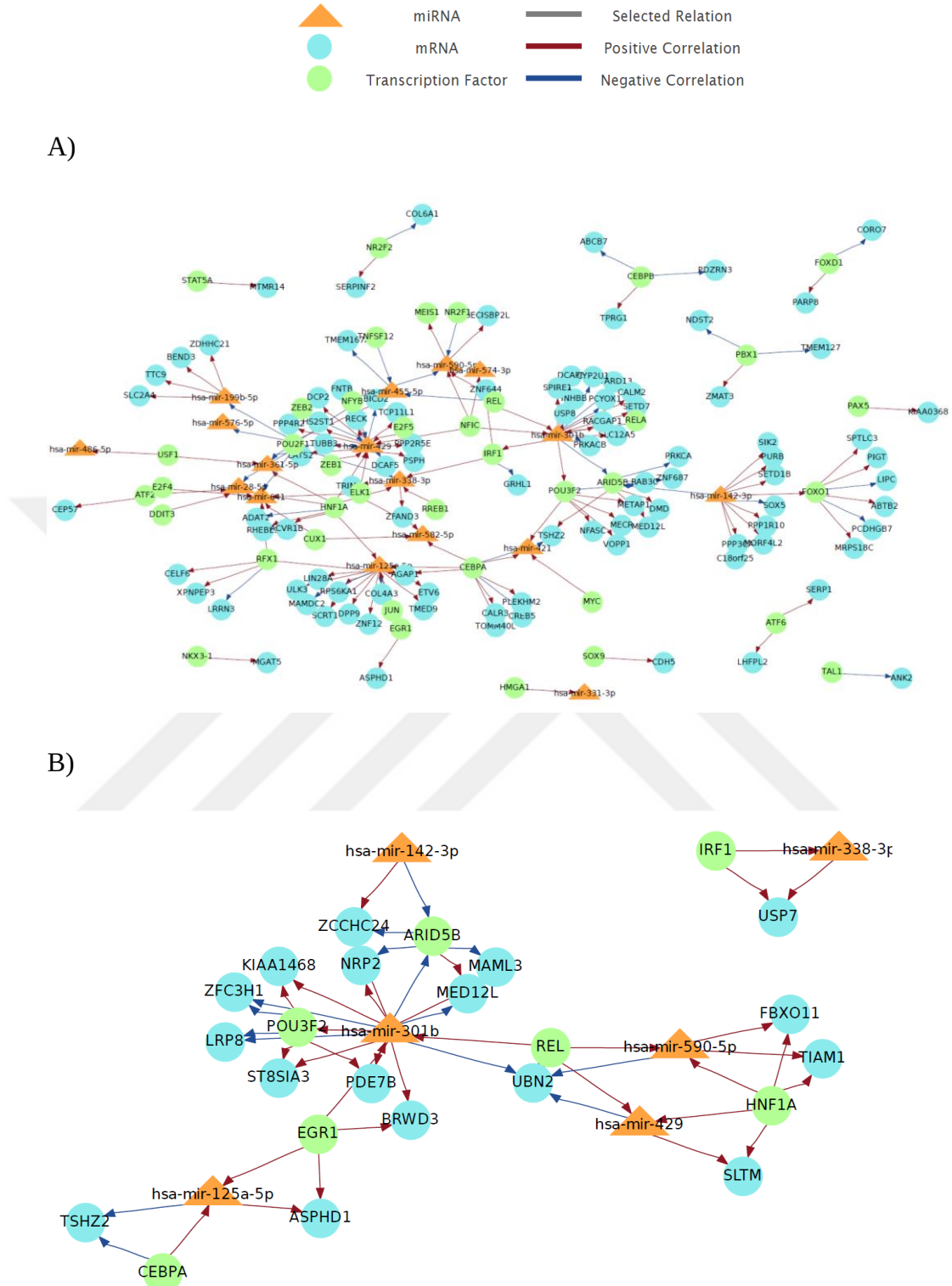


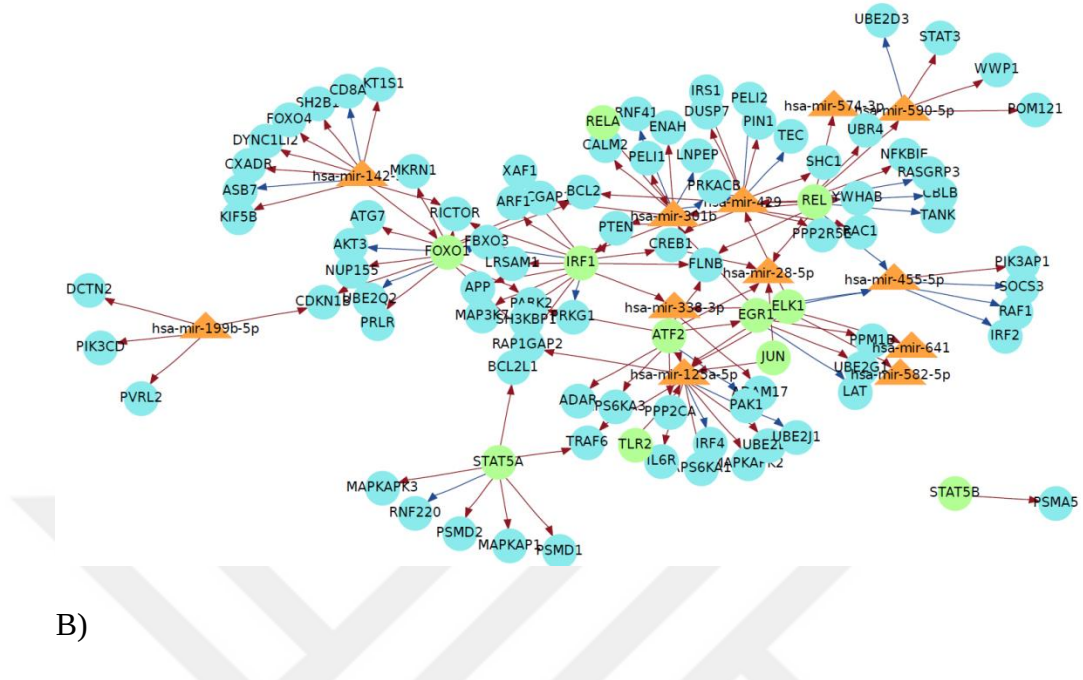
Figure 4.46: MAGIA2 networks of GSE41079 and GSE41074 A) direct network B) circuits network (Analysis: Matched, target predictor: TargetScan, correlation: spearman, limitation: nothing, data: Ago2-IP mimic-mir-497).

There were less microRNAs for immune direct network; mir-486-5p, mir-381-5p, mir-576-5p, mir-331 and mir-421 were lacking in immune network of mimic-mir-497 treatment result. The genes included in immune direct network were: FOXO1, RELA, REL, ELK1, EGR1, JUN, ATF2, STAT5B, STAT5A, IRF1 AND TLR2. For direct networks, immune one has $\frac{1}{4}$ th of genes of non-immune network. (Figure 4.46 A and Figure 4.47 A)

For immune circuits network, mir-590-5p and mir-338-3p were missing when it is compared to non-immune circuits pathway. There are five transcription factors: FOXO1, IRF1, ATF2, REL and EGR1. Three of them (IRF1, EGR1 and FOXO1) were common with non-immune circuits network. (Figure 4.46 B, Figure 4.47 B)



A)



B)

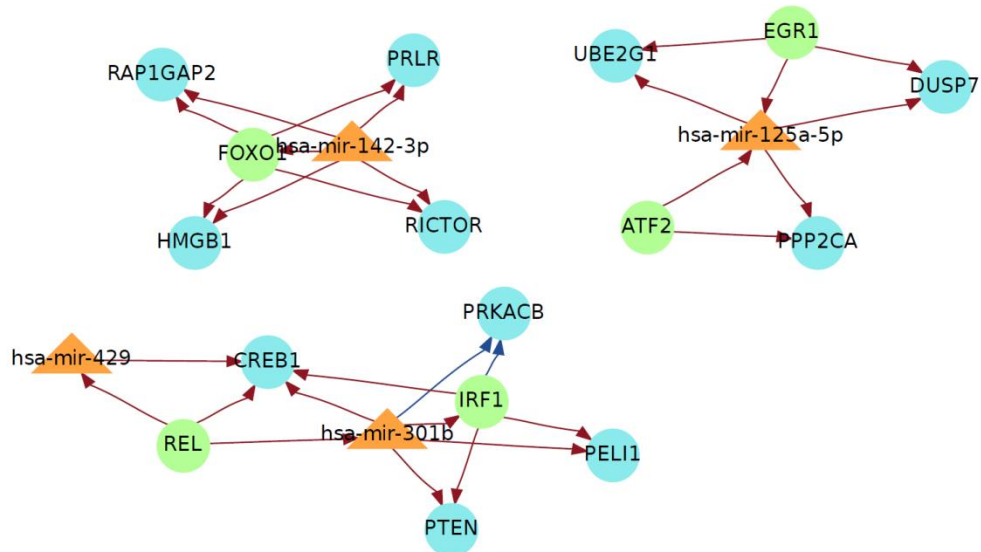


Figure 4.47: MAGIA2 immune networks of GSE41079 and GSE41074 A) direct network B) circuits network (Analysis: Matched, target predictor: TargetScan, correlation: spearman, limitation: nothing, data: Ago2-IP mimic-mir-497).

Chapter 5. Conclusion & Discussion

CHRNA5 is a member of a gene cluster associated with lung cancer¹⁴⁷. In breast cancer cells, cell growth was induced by nicotine and CHRNA9 expression increases, additionally CHRNA5 might be involved in oestrogen signalling^{115, 116}. The relationship between CHRNA5 and breast cancer is highly interesting to work on. The relationship and the differentially expressed microRNAs in the absence of CHRNA5 in breast cancer cells can give further clues for candidate biomarkers. The first step in my thesis was to obtain a differentially expressed candidate microRNA from an experiment done by using siRNA CHRNA5 and scrambled oligo (control) (unpublished). This analysis provided *mir-497-5p* as a candidate because of a notable level of expression in MCF7 cells and log2 fold change between siRNA treatment and scrambled control samples. We found that several mRNA targets of this microRNA were also affected by CHRNA5 depletion making this microRNA as a leading one for future tests. Mir-497 target genes were indicated as cancer, breast cancer and immune system related genes. In this study, mir-497 association with CHRNA5 in breast cancer was studied. The aim was to find differential expressions (in mRNA and miRNA) in the presence of mimic miR-497-5p in the presence or absence of CHRNA5 RNAi. The results were collected under three groups: mir-497 target gene in silico and qPCR results, candidate immune related microRNA results and general view of mRNA and microRNA expression change in silico.

In the present study, mir-497-5p expression under siRNA CHRNA5 was significantly decreased in the MCF7 cell line. Two other siRNA reagents (si53, si40) also suggested the downregulated status of mir-497-5p upon CHRNA5 depletion yet insignificantly. These siRNA reagents were used only on this step; the following experiments were done by using si111. These results with different cell lines and different reagents indicated that mir-497-5p expression level was affected by absence/presence of CHRNA5 in breast cancer cells. Also mir-497 mimic increased significantly mir-497 amount in the presence or absence of CHRNA5. Four of tested genes in this thesis (ZMAT3, LAMC1, TNRC6B and CCNE1) were validated targets of mir-497-5p while the rest (CADM1, CCNG1 and PAM) were predicted by at least 4/12 microRNA-target predictors (Table 4.4) . All of these genes were also modulated by CHRNA5 siRNA. In the literature there is no study

done about showing the association between CHRNA5 RNAi with mir-497-5p and its target genes; thus this study is the first investigating this interaction.

Despite all used genes were at least the predicted targets of mir-497, under mimic mir-497 treatment their expression was not modulated significantly. There can be a mechanism consisting more than one gene/miRNA. These microRNAs and mRNAs can regulate each other's functioning. As it was mentioned in Chapter 4 (4.1.1) mir-497 seemed to be a hub microRNA related to most downregulated microRNAs under siRNA treatment via target genes.

In this study, we also proposed an effective design for testing the association between an siRNA and mimic by using single or double treatments and their relevant controls. The groups were: Testing individual siRNA or mimic effects: sc(10nM), si(10nM), mimic(10nM); testing combination effects: sc(20nM), sc+mimic, si+mimic, si(20nM). This type of design allowed for comparison of single exposure to siRNA or the mimic and at the same time allowed for comparison of each treatment with a combination treatment. There were examples on combined treatments of nucleic acid modulators in the literature. In a paper related with nasopharyngeal carcinoma, mir-185 and WNT2B effects on metastasis and invasion were studied ¹⁴⁸. Only inhibitor of microRNA and inhibitor of microRNA combined with siRNA WNT2B were used to transfect cells. siRNA rescued the cells from effect of microRNA inhibitor ¹⁴⁸. In another paper related with lung cancer, a drug and a mimic were tested. Mimic-mir-146 and gefitinib combination gave the best reduction on invasion when it was compared with untreated, only gefitinib treated or only mimic treated samples ¹⁴⁹. As it is seen from these papers, combination of effective elements provided stronger significant results.

The validated targets of mir-497 (ZMAT3, LAMC1, TNRC6B and CCNE1) were selected since the interaction had been shown before (Figure 4.3-4.5). Among these, ZMAT3 showed si+mimic combination treatment effective. The others did not give significant results which can lead to make a connection with mir-497 and CHRNA5 about synergism or antagonism. For predicted targets of mir-497, CCNG1 showed a significant change for si+mimic combination as well. CHRNA5 RNAi seemed effective on 5 targets of 7 we used at least for one dose of siRNA treatment (either 10nM or 20nM). There could be another mechanisms or other targeting microRNAs between those genes and CHRNA5.

siRNA CHRNA5 treatment mRNA microarray analysis results indicated upregulation in six target genes as following: ZMAT3>LAMC1>CCNG1>PAM>CADM1>TNRC6B and downregulation in CCNE1 only (the ranking obtained from Tubitak Project 111T316 mRNA microarray experiments). In my validation qPCR results with 5 biological replicates, I achieved a similar order in significantly differentially expressed gene order: ZMAT3, LAMC1 and CCNG1 were most significantly upregulated genes. LogFC level was less in CCNG1 than other two. CADM1 and PAM had nearly same level of fold change but CADM1 was significantly upregulated. TNRC6B had non-significant slight upregulation. Mimic-mir-497 treatment result (GSE41074) indicated upregulation in CADM1 and downregulation for others: CCNG1<CCNE1<LAMC1<ZMAT3<TNRC6B<PAM in total RNA result. When Ago2-IP samples gene expression differentiations were considered, TNRC6B and CADM1 were more downregulated, CCNG1, CCNE1, PAM and ZMAT3 were more upregulated than in total RNA samples. LAMC1 was not very different. In CCNG1 and CCNE1, there was the biggest difference between ago2-IP and total RNA samples. CCNE1 is a direct target of mir-497. Therefore, when siRNA CHRNA5 used, CCNE1 upregulation was expected because mir-497 was downregulated by siRNA CHRNA5. However, the opposite result was shown for CCNE1. There is a possible other mechanism(s) which affects CCNE1 expression. In a paper related with glaucomatous retinae, P53 pathway related genes were shown as a target gene of mir-497⁹⁸. Primary peritoneal carcinoma and ovarian carcinoma showed p53 overexpression (more common in ovarian carcinoma). Also average p53 immunoreactivity was 4.6 and 6 in the same order (significantly different). For both carcinomas p53 and mir-497/195 downregulation was shown. The microRNA cluster's members were shown as candidates to be tumour suppressors. miR-195 and miR-497 as potential tumour-suppressor genes. For other cancer types as prostate or leukaemia change in the expression of mir-195/497 was observed¹⁵⁰. CCNE1 and ZMAT3 were shown as targets of mir-497 and they are p53 pathway members. Therefore, the expectation was that, in the absence of CHRNA5 mir-497 decreases and these targets increase. The experimental results showed that ZMAT3 was upregulated and CCNE1 was downregulated. Combined experiment for CCNE1 showed non-significant results in general. On the other hand, ZMAT3 expression level change was significant under si and si+mimic treatments. Not CCNE1 but ZMAT3 is a good candidate.

Before going into results of target genes, I want to mention the comparison of qPCR data and GEO data results. Under mimic-mir-497 treatment Total RNA sample (HepG2 cells) from GEO data showed a strong uphill (positive) linear relationship with qPCR data with the same treatment on MCF7 cells. Mimic-mir-497 treatment on HepG2 Ago2-IP results from GEO data had a weak uphill (positive) linear relationship with the same qPCR data.

ZMAT3 was involved in p53 pathway genes related with mir-497. Non-small cell lung cancer treatment was done by pemetrexed (chemotherapy drug), but it was shown having some adverse effects and one potential predictor for pemetrexed response was ZMAT3 (decrease with increasing pemetrexed) ¹⁵¹. TP53 mutation were indicated in nearly half of human cancers. For all BRCA, CRC, GBM, UCEC, BLCA, OVCA, LAML, LUAD, STAD and LGG (Abbreviations, xx), ZMAT3 was significantly upregulated ¹⁵². ZMAT3 was a hub gene in the list of 8 genes which have top different degrees for malignant-normal, malignant-benign and benign-normal for breast cancer ¹⁵³. ZMAT3 was significantly upregulated under CHRNA5 siRNA treatment and significantly upregulated for si(20nM) and si+mimic. ZMAT3 has been significantly affected by siRNA treatment as an expected way however no significant difference was observed under mimic-mir-497 treatment. However, si+mimic treatment made a significant difference when compared with sc(20nM) alone or sc+ mimic, the difference seemed to have arisen from only siRNA effect. This result implicates that effects of mir-497 may not be significant alone however there might be an interaction between CHRNA5 expression and miR-497-5p effect for ZMAT3. For cancers with low expression of CHRNA5, the induction of ZMAT3 could be higher through miR-497-5p thus this might have therapeutic significance when treated with pemetrexed. Pemetrexed and mimic-mir-497 has similar effects on ZMAT3. Mimic-mir-497 can be a candidate for being a therapeutical reagent for lung or breast cancer.

CCNG1 also was shown to be involved in the P53 pathway and in the cancer microRNA pathways found in KEGG Pathways. CCNG1 was targeted by microRNAs in cancer cells: for example, miR-1271 targets CCNG1 and the growth on ovarian cancer cells was inhibited ¹⁵⁴. CCNG1 also was targeted by miR-23b blocking the tumorigenesis in ovarian cancer ¹⁵⁵. A decrease in mir-122 may cause upregulation of CCNG1 expression which mediates p53 decrease ¹⁵⁶. CHRNA5 siRNA treatment increases CCNG1 highly significantly. However, no change under mimic treatment was observed while there was a significant difference between sc+mimic and si+mimic. Therefore, si and mimic

combination was effective on expression level of CCNG1. Mir-497 has no direct effect on CCNG1 but CHRNA5 expression differentiation mechanism increased its expression. The effects of CHRNA5 depletion was not as strong as in the case of ZMAT3 however CCNG1 also could be an important tumour suppressor affected by CHRNA5 expression status of cancer cell.

P53 related genes as ZMAT3, CCNG1, CCNE1 or LAMC1 were upregulated in the absence of CHRNA5. P53 is a predicted target of mir-497 as indicated before. Two mimic related responses were handled from ZMAT3 and CCNG1 which were both related to P53. P53 pathway seems be related to both CHRNA5 and mir-497 in breast cancer.

CADM1 was found in Cell adhesion molecules (CAMs) category of the KEGG pathway. A suppressor role in epithelial cells for breast cancer¹⁵⁷ and oncogenic role in ATL (adult T leukaemia) of CADM1 have been shown¹⁵⁷. We could not identify any significant interaction between mimic and siRNA for CADM1. Under siRNA treatment the expression level was significantly different than control for five biological replicates but not significant for two biological replicated experiments. Therefore, we saw that sample size was effective. There could be a connection between CHRNA5 and CADM1 by another mechanism than through mir-497.

TNRC6B action was indicated as related with Ago proteins and it was needed for gene silencing by microRNA¹⁵⁸. TNRC6B was shown as a paralog of GW182, both were required for miRNA stability¹⁵⁹. TNRC6 was shown to interact with CCR4–NOT complex and PAN2–PAN3 complex (deadenylation of mRNA, translational initiation inhibition)¹⁶⁰. Immune cells were activated by eIF4F activity enhancement by GW182 upregulation. Without change in mRNA expression of GW182, protein expression was enhanced by PI3K/Akt/mTOR and Jak/Stat/Pim signalling pathways¹⁶¹. TNRC6B expression change also could be related to cancer: an increase in mir-18b caused short relapse free time for HCC patients and TNRC6B was one of the target genes of mir-18b¹⁶². GW182 was a protein with glycine and tryptophan repeats. There are target autoantigens in GW bodies: TNRC6B/C, RAP55, LSm4 and Ago2¹⁶³. GW body number increases when cells stimulated with LPS (lipopolysaccharide). If Ago2 or Rck/ P54 was lacking, such an increase cannot be constituted. Also mir-132, mir-155 and mir-146a were upregulated after LPS stimulation¹⁶⁴. In Ago2-IP sample from GEO dataset, the expression of TNRC6B under mimic-mir-497 treatment decreases highly in HCC cell

line. Ago2-IP result showed 20 times more downregulation than total RNA sample with mimic-mir-497 treatment, it fits with the knowledge above. From microarray and qPCR (breast cancer) we did not see not a significant change under any treatments. There was a non-significant upregulation under siRNA treatment and si+mimic treatment, it seemed only caused from siRNA CHRNA5. From microarrays, there was an increased expression of TNRC6B with decreased CHRNA5. However, there was a contrast between MCF7 and HCC cells. It could be caused because of the lack of a mechanism which was affected by mir-497 in MCF7 but present in HCC to target TNRC6B.

LAMC1 was involved in eight different pathways of KEGG: PI3K-Akt signalling pathway, Pathways in cancer, Small cell lung cancer etc. LAMC1 was indicated direct target of these microRNAs of mir29a and mir-22, suggesting these microRNAs might have tumour suppressor roles ¹⁶⁵. mir29s (miR-29a/b/c) targets LAMC1 and in prostate cancer inhibits invasion/migration ¹⁶⁶. In colorectal cancer cells, when mir-506 overexpressed, it targets LAMC1 (low expression) and prevents malignancy ¹⁶⁷. Under mimic-mir-200c treatment on MDA-MB-231 cells, LAMC1 expression was decreased ¹⁶⁸. Under mimic-mir-497-5p treatment LAMC1 was downregulated for HCC (Table 4.5). Under siRNA CHRNA5 treatment LAMC1 was upregulated on MCF7 cells and not significantly upregulated for mimic and scr+mimic. There was a significant difference when siRNA and mimic used together than sc+mimic, this may indicate a synergy existing between siRNA and mimic-mir-497 in breast cancer cell line that needs further study.

AM (Adrenomedullin) was produced in prostate and cell growth can be regulated independently of androgen. PAM amidates AM and activates it ¹⁶⁹. Adrenomedullin was highly expressed in colorectal cancer ¹⁷⁰. In prostate cancer, PAM activates autocrine growth and generation of drug resistance (antiandrogen) in prostate cancer ca be related with PAM ¹⁷¹. The microarray result of PAM expression under siRNA CHRNA5 was up-regulation (LogFC ~ 1.3). Under any treatment there was no significant change on PAM expression level with 2 biological sampled mimic-mir-497 and siRNA CHRNA5 combined experiment. For 5 biological replicates used only scrambled oligo vs siRNA CHRNA5 experiment PAM p value was 0.0679. Sample size can cause low power in detecting significance future studies can involve increases in sample sizes.

PI3K was an important molecule for mir-497 which was related to target genes. Bcl-w was targeted by mir-497; Bcl-w works together with some molecules as PI3K and apoptosis decrease and invasion and migration was promoted. When mir-497 targets Bcl-w and decrease its inhibitory effect on apoptosis. LAMC1, CCNE1 and TNRC6B are all related to PI3K. In MAGIA under mimic-mir-497 treatment PIK3R3 was one of top upregulated genes. There was another point here related with PI3K. IL6 activates both JAK/STAT pathway and PI3K/Akt/NFkB pathway. PI3K/Akt/NFkB pathway could be related to mir-497 (explained mechanism in 1.2.3.2. hsa-mir-497 Targets and Related pathways). JAK/STAT pathway also can be linked to cholinergic receptors (explained in detail in 1.2.2) while IL6 can be related to both molecules.

The overall results until here show that there is a need for supportive reagents together with mimic or different dose of mimic may be needed for effect. There are different microRNAs targeting the same gene and an overexpression of a single microRNA cannot be enough for a significant change. We could confirm CHRNA5 depletion effect on validated and predicted target genes of mir-497-5p. However not all of these targets were modulated by 497 mimics.

A gene can also be influenced by its chromosomal localization since there are antisense genes against the gene itself or chromosomal clusters that are transcribed together. Therefore, we also investigated whether the chromosomal environment the mir-497 produced from have affected its expression. In the region where mir-497 was located there are two other genes, one of them was sense and the other was antisense to mir-497. In the absence of CHRNA5, MIR497HG tended to be downregulated and C17orf49 tended to be upregulated in mRNA microarray. qPCR result gave the same direction yet non-significantly upregulated c17orf49 and downregulated MIR497HG under siRNA CHRNA5 (10nM) treatment. miR-497-5p and miR-195-5p are contained in a cluster and found at 17p13.1 in the intronic part of the host gene (MIR497HG). In the literature, there are studies performed with this cluster. One of two downregulated miRNA clusters was found to be mir-497/195 in triple negative breast cancer. There were five upregulated microRNA clusters and one of them, the mir-301b/130b, included mir-130b which targeting CCNG2. CCNG2 is a paralog of CCNG1 which was the most important candidate in my thesis. CCNG2 regulates cell cycle in triple negative breast cancer. The sequence on 3' untranslated end effects the inhibition of CCNG2 by mir-130b-5p¹⁷². MIR497HG host gene intragenic cluster had two sites in HCC which were

hypermethylated: at 5.2 kb upstream (in CpG island), at 9.6 kb upstream¹⁷³. In the papers related with mir-497 and mir-195, MIR497HG was not searched, only the map of the chromosome region includes MIR497HG were shown with microRNAs on it. Therefore, the host gene was searched in my thesis. In literature, expression correlation was shown significant between the host gene and target genes. In synergistic or antagonistic association studies host gene and target gene similarities were confirmed¹⁷⁴. MIR497HG was not studied well on this subject so we performed experiments and analysis to see expression levels of MIR497HG and C17orf49. MIR497HG is sense and C17orf49 is antisense with mir-497; thus the expectation was an increase in C17orf49 and decrease in MIR497HG expression level under siRNA CHRNA5 treatment. The result showed that expectations could be confirmed with siRNA 20nM significantly. However, si20nM was the only treatment with one biological replicate, thus si20nM was analysed again qPCRs were re-performed for C17orf40 and MIR497HG under si20nM and sc20nM treatments with two replicates. The result of the qPCR showed that combined experiment data for siRNA20nM could not be replicated. Sense and antisense genes to mir-497 did not show significance with CHRNA5 by using high or low dose siRNA.

There are other members of the microRNA cluster which include mir-497. These microRNAs and their expression differences under mimic treatments or siRNA CHRNA5 treatment were listed in Table S1 and Table S2. From these lists, we see that none of them were highly effected. Mir-195-3p was affected from siRNA CHRNA5 more than other microRNA in the family but the expressions under siRNA/sc were not at testable levels.

For the second part of the study, mir-497 and its association with immune system related genes was questioned by searching immune related microRNAs' expression differentiation under siRNA CHRNA5 and mimic-mir-497 treated samples. microRNAs can affect each other's activity. As an example, there are microRNAs target DICER1 which makes mature microRNAs, so these microRNAs can inhibit formation of mature microRNA. There were four immune related microRNAs used in this study: mir-23a-3p, mir-221-3p, mir-146a-5p and mir-34c-3p. There was a not significant downregulation under siRNA treatment in MCF7 cells from microarray study (avg. FC ~ -1.5) except mir-34c. mir-34c was not changed in the microarray study. qPCR result indicated that all microRNAs had the same pattern for the group consist of sc (10nM) si (10nM) and mimic (10nM) group treatments. A general increase in response to mimic-497 for all

microRNAs tested was apparent. Future studies should focus on mimic effects on microRNA expressions comparing Ago2 and total compartments.

mir-23 is known to be upregulated in breast cancer. Mir-23a/27a/24-2 cluster also is shown to be associated with immune system (polarize macrophage) and breast cancer (tumour growth) ³⁶. Expression of target genes of these microRNAs JAK1 and STAT1 are increasing while microRNAs themselves are decreasing in breast cancer ⁵⁵. mir-23a was upregulated under both si or mimic treatments but mimic effect was more on the MCF7 cell lines. qPCR result showed the same level downregulation under si+mimic and sc+mimic. Either both were not effective together with mimic or sc/si made the same effect when it was used together with mimic (sc can be effective on mimic because of other qPCR results gave very different result if mimic was used alone or together with sc). Si(20nM) and si(10nM) results were not different significantly.

mir-146a was indicated as related to thyroid tumours ³⁶. mir-146a was shown related with T Reg Cell, TH17, aDC and active macrophage formation of functioning. In breast cancer, mir-146a was downregulated and IRAK1, TRAF6 (targets of mir-146a) were upregulated. Mir-146a was seen related to inflammation and cancer (defect on migration and invasion/ NF- κ B signal blocking). Also, mir-146a, IRAK1 and TRAF6 behaviours were the same in gastric cancer. In addition, IL8 was downregulated. In gastric cancer, mir-146a differentiation was related with inflammation and antitumour function ⁵⁵. There was an upregulation under both si and mimic treatments. Mir-146a was affected by the treatments more than other three microRNAs. In si+mimic, the increase seemed to be caused from only siRNA; since there was no change under sc+mimic and also mimic itself gave a higher effect.

mir-221 was shown related to thyroid tumours ³⁶. Mir-221 was related with MEP formation or functioning according to Figure 1.3. In breast cancer cells, ER-alpha repress-mir-221 ¹⁷⁵. Upregulation in mir-221 increased metastasis for the downstream of RAS-RAF-MEK pathway ¹⁷⁶. Mir-221/222 affected PTEN/Akt pathway and made impacts on breast cancer invasion, growth and migration ¹⁷⁷. Mir-221 KD supported apoptosis (BIM-Bax-Bak) ¹⁷⁸. Tamoxifen was more effective in breast cancer when mir-221/222 was downregulated by upregulation of TIMP3 ¹⁷⁹. Mir-221 was shown as a tumour suppressor in lung cancer ¹⁸⁰. There was a slight upregulation of mir-221 under siRNA treatment and there was an upregulation under the mimic treatment but both were insignificant.

In melanoma, mir-34a/c expression decreased and the expression of target gene ULBP2 increased⁵⁵. Mir-34a/c has shown a role in apoptosis and cell cycle arrest. ULBP2 was a ligand of NKG2D (immunoreceptor)¹⁸¹. In qPCR, there was no significant change, but expression differentiation directions were an increase under si and mimic (mimic effect was more), a decrease if both treatment were used together (si+mimic). For mir-34c, only mimic showed an increase and sc+mimic showed a decrease insignificantly, there was a possible effect of scrambled oligo on mimic reagent.

From integrated mRNA-miRNA couple handling tools, following results have been reached. There were hub genes from MirTarVis images. Mir-132 had been highly downregulated and was integrated with different genes under mimic-mir-195 or mimic-mir-497 treatments. For both miRNA mimic treatments there was mir-381 which showed a mild upregulation but integrated genes were highly downregulated. mir-132 was a candidate microRNA which was handled from in silico results (mimic-mir-497). CHRNA5 is a validated target of mir-132 (only two more microRNAs target CHRNA5). When microarray result for HCC for mir-132 was checked I saw that it was downregulated under mimic-mir-497 treatment. The expectation was such: since mir-132 was reduced could not target CHRNA5 properly under mimic treatment hence we saw upregulation on CHRNA5. PIK3R3 was the most upregulated gene related to downregulated mir-132 under mimic-mir-497 treatment. Another CHRNA5 targeting microRNA was mir-10a-5p. This microRNA also is a good candidate for further analysis because it is related breast tumours¹⁸² and immune system⁵⁵.

For the last step the public GEO data we used to identify targets to test in our system were reanalysed. Under different treatments Ago2IP results changed but Total RNA results were similar to each other. There is a wider range of values under mimic-mir-195 treatment by Ago2-IP result. This important finding suggested that mir-195 mimic was a more effective one in HCC cells. CHRNA5 siRNA affected mir-497 as much as mir-195 or more thus its effects we observed in the total RNA so AGO2 bound RNA could be also studied. Also the effects of mir-195 should be tested. When histograms and plots were combined, following comments can be made. Ago2-IP values among genes were more variable than Total RNA samples under both mimic treatments in GEO data. Total RNA gave a better correlation for mimic-mir-195 and mimic-mir-497 treatments. Target genes had difference between Total RNA and Ago2-IP because they bind to Ago2 and only a subset of mRNAs could be tested in Ago2IP bound fraction. Mimic-mir-195 gave more

significant differentially expressed genes values than mimic-mir-497 especially for Ago2-IP sample results. Distribution of mimic-mir-195 histogram LogFC values spread on a large area that means it effected genes to different directions. It can be explained because of it shows downregulation normally in breast cancer as mir-497 but circulating miR-195 was upregulated in breast cancer patients ¹⁸³. From kstest results, we can say that only Total RNA samples under different treatments were not significantly different. The other as the same treatment with different RNA collection or Ago2IP for different treatments were significantly different than each other.

When RNA collection types were compared, mimic-mir-195 caused a difference between Ago2-IP and Total RNA samples. Ago2-IP samples tended to be upregulated under mimic-mir-195 treatment and downregulated under mimic-mir-497 treatment as in histograms. Additionally, microRNA LogFC distribution was wider under mimic-mir-497 Ago2-IP samples than mimic-mir-195 Ago2-IP samples. However, less differentially modulated genes were present in the mir-497.

This can be related to the target gene number since there are more validated target genes of mir-195-5p than mir-497-5p ¹²³. There are more publications in PubMed related to mir-195 and breast cancer than mir-497 and breast cancer. Mir-195 targets IRS1 and prevent tumour growth ¹⁸⁴. Mir-195 was a biomarker for a therapeutic aim for breast cancer patients in Turkey ¹⁸⁵. Both mir-195 and mir-497 are downregulated in breast cancer ¹⁸³.

Chapter 6. Future Perspectives

Firstly, different doses of mimic-mir-497 can be studied with or without siRNA treatment. Higher sample size is a good option to try further, not two but at least three biological replicates could be needed for statistical power. Experiments may be repeated with a bigger sample size for genes which showed a higher difference in being significant between five and two biological replicated experiments when siRNA LogFCs were compared. Additional idea for mimic treatment would be that: mimic reagents should be used as in a combination to support a silenced gene or they can be overexpressed together if some known same functioning microRNAs were searched. In that case, mir-497 related and top downregulated microRNAs which has detectable expression for samples can be overexpressed together with mir-497.

P53 pathway seems related to target genes of mir-497. More target genes which are involved in P53 pathway, upregulated in siRNA CHRNA5 treatment microarray results and also were downregulated in mimic-mir-497 treatment microarray (it can be old GEO data on HCC or suggested microarray on MCF7 for a better idea). Other targets of P53 can be selected and investigated further.

However, for a more distinctive result/ specific achievement, PI3K/Akt pathway can be chosen for future in-vitro experiments. PI3K/Akt/NFkB pathway was important, for example, VEGFR2 is a key gene affected by mir-497 and involved in PI3K/Akt pathway as explained in introduction part. IL6 activates two pathways (JAK/STAT and PI3K/Akt/NFkB) and one is related to CHRNA5 (JAK/STAT) and the other is related to mir-497. Therefore, VEGFR2 and IL6 can be investigated by qPCR for further understanding of the effects of miR-497 and association with CHRNA5.

The most effected genes from both treatments were ZMAT3 and CCNG1. There are microRNAs which target CCNG1: mir-122, mir-23b and mir-1271. They are possible candidate microRNAs to search further. Mir-122 found in the literature targeting IGF1R (tumorigenesis and proliferation decreases) and is a key regulator of PI3K/Akt pathway¹⁸⁶. miR-1271 is again related to IGF1R and also to BCL2¹⁸⁷. mir-23b is the most promising microRNA related to CCNG1 since the cluster it is involved in was found to target PSAP (suppress metastasis) and promote breast cancer lung metastasis. If the

expression profile overlaps with the expectations, mir-23b/CCNG1 could become a bridge to connect CHRNA5 and mir-497 under breast cancer view ¹⁸⁸.

Immune microRNAs didn't show high significance or fold changes in response to siRNA yet there was a trend for upregulation upon mimic exposure in general. microRNA stability could be one of the factors that can explain this finding ¹⁸⁹. For further studies, a combination of GEO data with different cancer types mRNA and microRNA expression profiles can be analysed. There is another possibility, a bigger sample sized experiment can be performed for the same treatment in different times. mRNA and microRNA microarrays can be compared if mRNA is more stable than microRNAs in response to an agent.

There was a hypermethylation on MIR497HG for HCC as explained in the results part, in our case we can also search methylation patterns in breast cancer cells on this host gene to see if the same situation could be validated for breast cancer.

A combined transfection with immune microRNAs is another possible experiment and immune related target genes of the microRNAs (common and unique targets). Again sample size can be higher for a chance to get a significant differentiation. Also mimic-mir-497 can be used with related immune microRNAs because they give a better increase under mimic treatment. CHRNA5 expression can be compared between only mimic-mir-497 and the mimic-cocktail. Another combined experiment can be with mimic-mir-195 and mimic-mir-497. Because host gene seems synergistic to mir-497 and mir-195/497 are downregulated in breast cancer. CHRNA5, promising targets (CCNG1, LAMC1 and ZMAT3) expression levels can be compared to see if the effect is higher by using all microRNAs in that location. GEO data can be searched for a general idea of cluster microRNA effects on each other.

As in the GEO data Ago2-IP results can be handled for our experiment setup (mimic-mir-497 on MCF7, siRNA CHRNA5 on MCF7). From these result, we obtain data on the mature microRNAs. However, there can be precursor microRNAs which regulate a gene's function and can be related to a disease. In the following studies, precursor microRNAs of candidate microRNAs can be investigated to find the exact step of regulation; sometimes, precursor signal can be more than the mature.

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Appendix I

Supplementary Data

Reanalysis of GSE41079

There were three treatments in GSE41079: mimic-mir-497, mimic-mir-195 and non-treated samples. In this GSE, Agilent 021827 human miRNA microarray (G4470C) was used and log2 values of the expression of microRNAs under each treatment was obtained an excel file in GEO. There was a significant positive relationship between non-treated sample and mimic-mir-195, non-treated sample and mimic-mir-497, mimic-mir-497 and mimic-mir-195 samples as shown in Figure S1.

..

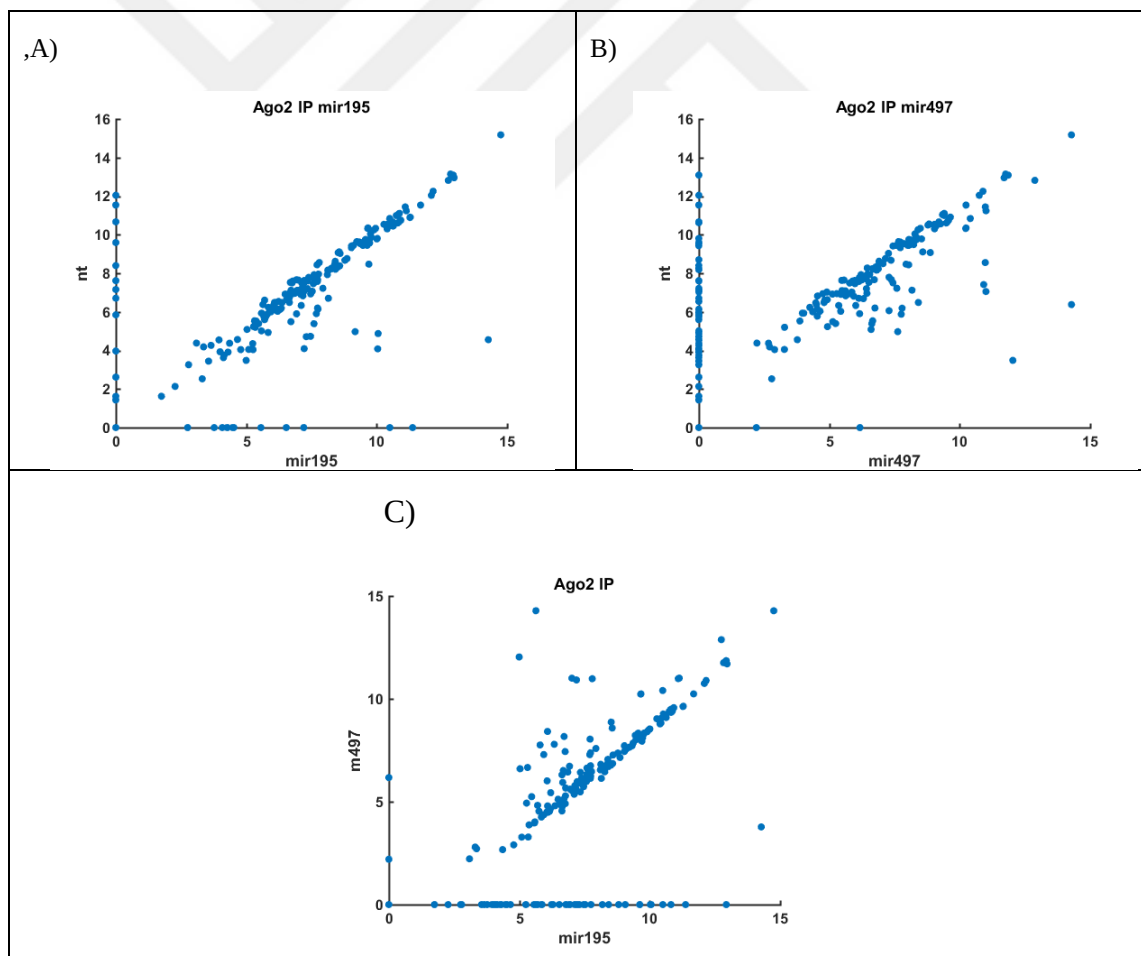


Figure S1. Scattered plots (Ago2-IP samples log2 values are used and if fold changes of the same microRNA for both mimics are zero it is filtered out.) A) mimic-mir-195 vs control/non-treated sample ($R = 0.9283$ P val. $< 2.2251e-308$) B) mimic-mir-497 treated sample vs non-treated sample ($R = 0.8616$ P val. $= 1.0005e-284$) C) mimic-mir-195 treated sample vs. mimic-mir-497 treated sample (Pearson Correlation is used, $R = 0.8575$ P val. $= 4.9583e-279$)

LogFC values of mimic-mir-195 and mimic-mir-497 treatments were compared. There were less than a thousand microRNAs, P value was very low and R was around 0.35. They were significantly positive correlated to each other.

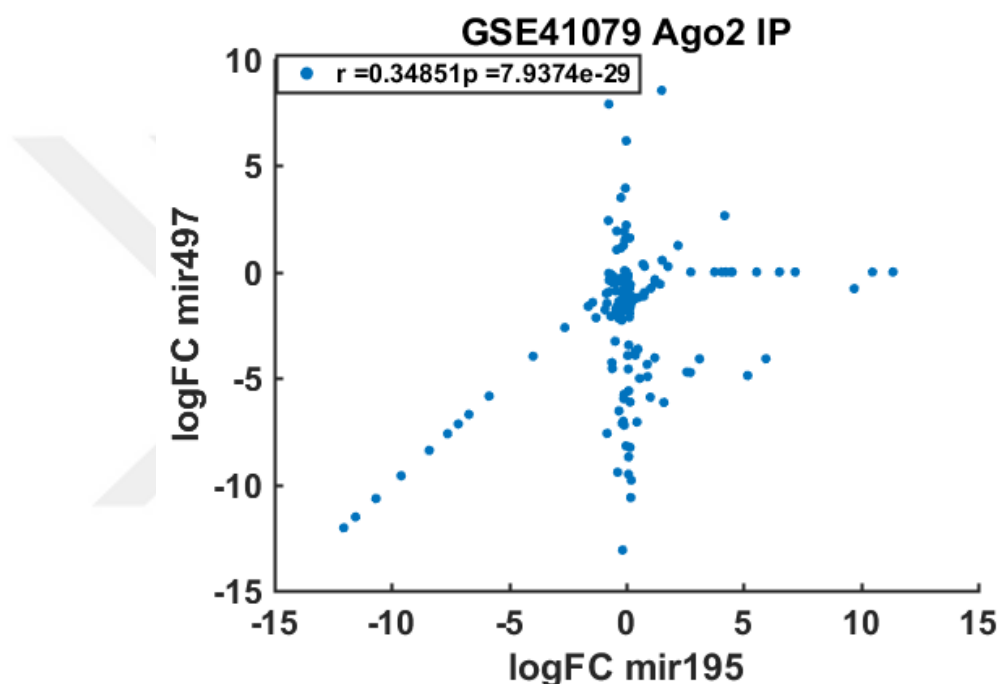


Figure S2. Scattered Plot of LogFC values of Ago2IP samples to compare different treatments in GSE41079.

Re-analysis of GSE41074

GSE41074 obtained from GEO (REF for GSE41974) had 6 different treatments: total RNA non-treated sample, total RNA mimic-mir-497 treated sample, total RNA mimic-mir-195 treated sample and Ago2-IP of all.

Comparison by Scattered Plots

There were two microRNAs which were used as mimic treatment in GSE41074 and the result was duplicated for the same treatment because of collection type of molecules

(Ago2-IP or Total RNA). The correlation between mir-497 and mir-195 were higher for the total RNA considered than the AGO2 bound mRNA (Figures 4.46A, B). There was a mild correlation between the AGO2 bound mRNA amount and total RNA amount for mir-195 but not for mir-497 (Figure 4.46C, D; Figure 4.47).

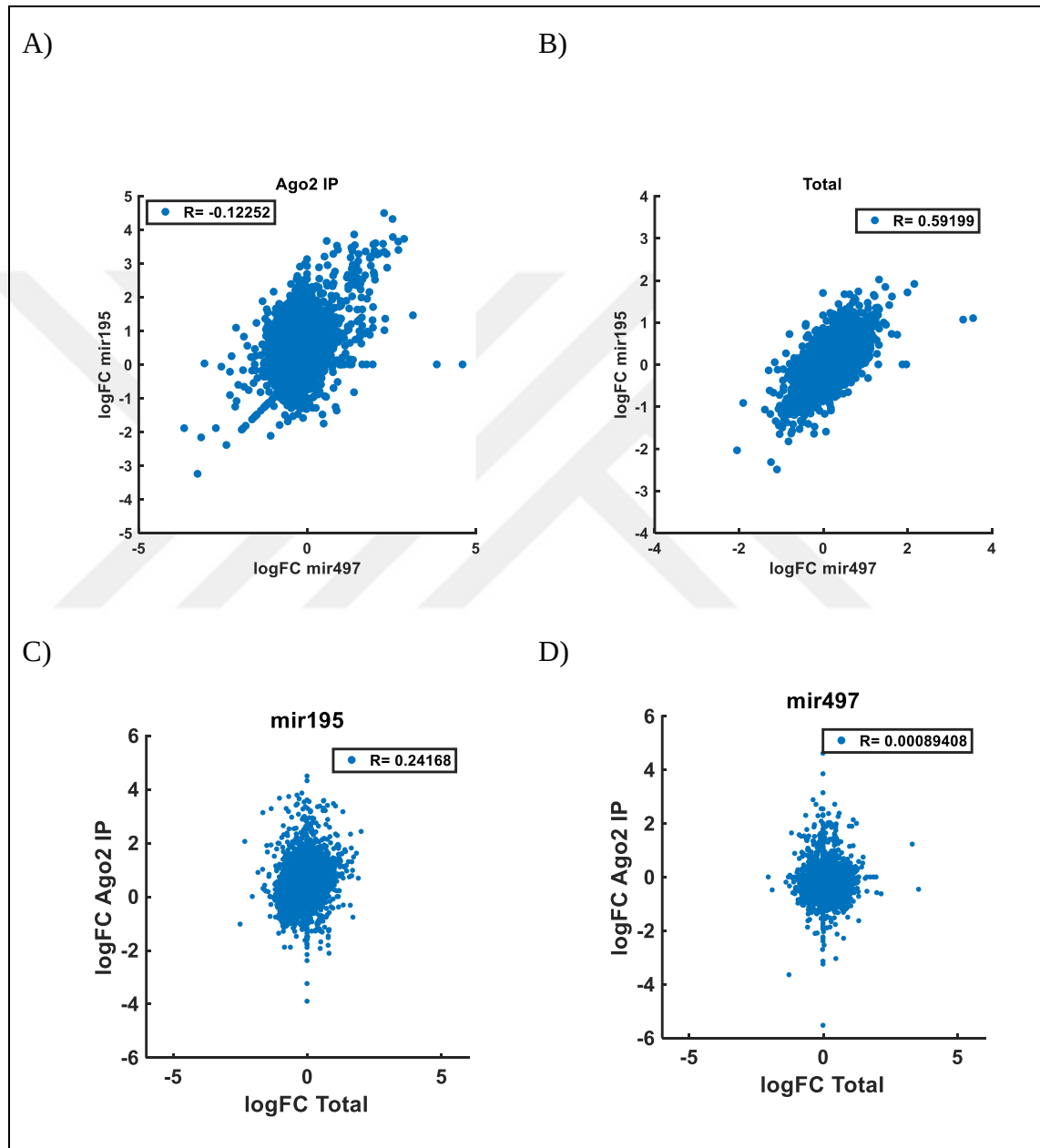


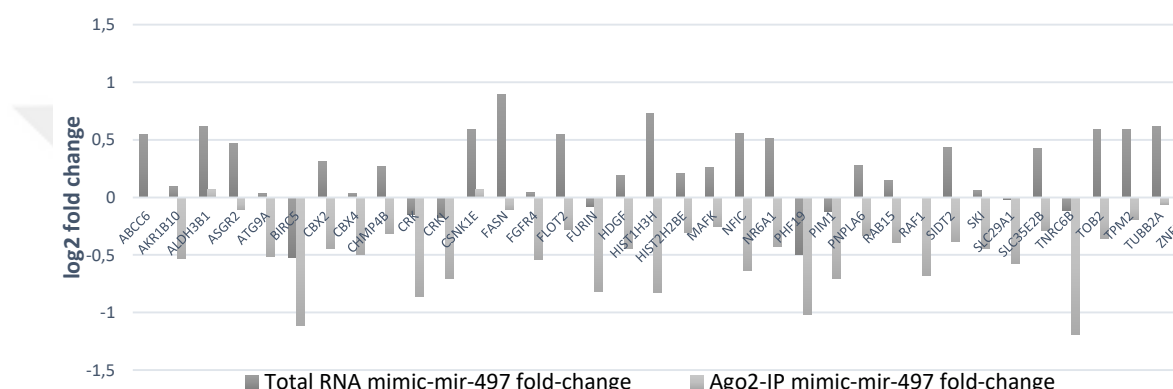
Figure S3. Scattered Plots of GSE41074 a) to compare different mimic treatments on Ago2-IP samples b) to compare different mimic treatments on Total RNA c) to compare Total RNA and Ago2-IP results under mimic mir-195 treatment d) to compare Total RNA and Ago2-IP results under mimic mir-497 treatment.

Analysis of Validated Targets of hsa-mir-497 in GSE41074

The list of validated targets of mir-497 was downloaded from miRWalk and the values of genes were taken out from GSE41074 from GEO and plotted. Filtering on fold changes showed that TNRC6B had more fold change in Ago2-IP samples and CCNE had more fold change value in total RNA samples. These genes were used in experimental parts.

A)

Genes have difference between fold changes in total RNA and Ago2-IP samples more than 0.5



B)

Genes has difference between fold changes in total RNA and Ago2-IP samples less than -0.5

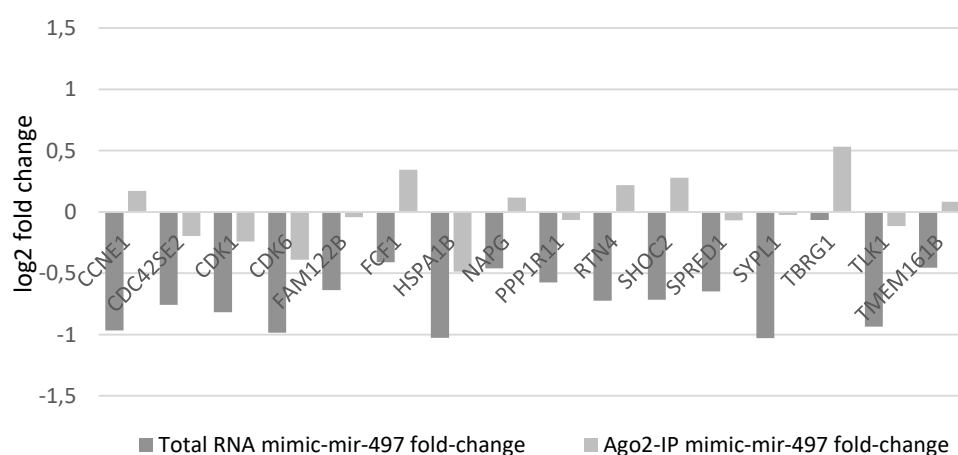
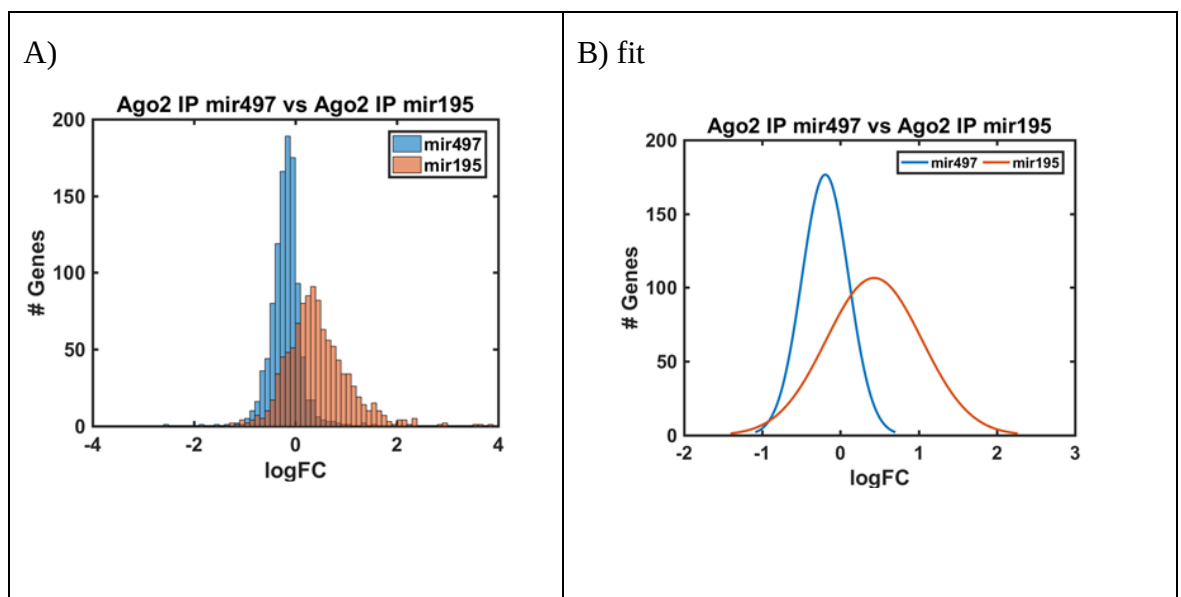


Figure S4. Differential fold changes (mimic-mir-497) for mir-497 validated targets A) Total RNA log2 fold-change (mimic-mir-497-5p-NT) - log2 fold-change (mimic-mir-497-5p -

non treated sample) Ago2-IP > 0.5 B) Total RNA log2 fold-change (mimic-mir-497-5p-NT)
- log2 fold-change (mimic-mir-497-5p – non treated sample) Ago2-IP < -0.5

Histograms

Histograms were plotted to understand whether AGO2 bound and total RNA under mimic exposure differ between mir-195 and mir-497. We showed that mir-497 and mir-195 mimics showed their affect more on the AGO2 bound population than the total RNA (Figure 4.51A-D). Similarly, mir-195 mimic affected the AGO2 bound RNA population more than mir-497 did (Figure 4.51E-H). There were more genes affected by mir-195 than by mir-497 as also was indicated in the original finding. Figure 4.52 A and C showed that Ago2IP results differ under different treatments, but total RNA results seem like have no significant difference under different treatments. For Ago2-IP mimic-mir-195 achieved a wider range of values than mimic-mir-497. Ago2-IP gave expanded range for especially mimic-mir-195 treatment. In the paper of the GSE data there is no LogFC distribution compared to each other on the plot. This result focus on how two different microRNA differ from each other or how they resemble to each other. Kstest2 indicates except “Total RNA mimic-mir497 vs Total RNA mimic-mir-195” others are not significant (1%). p value (fig.51 A) = 1.2305e-174, p value (fig.51 C) = 0.2342, p value (fig.51 E) = 3.6854e-15, p value (fig.51 G) = 1.1138e-115. Total RNA under different samples are not significantly different from each other. Others are significantly different from each other.



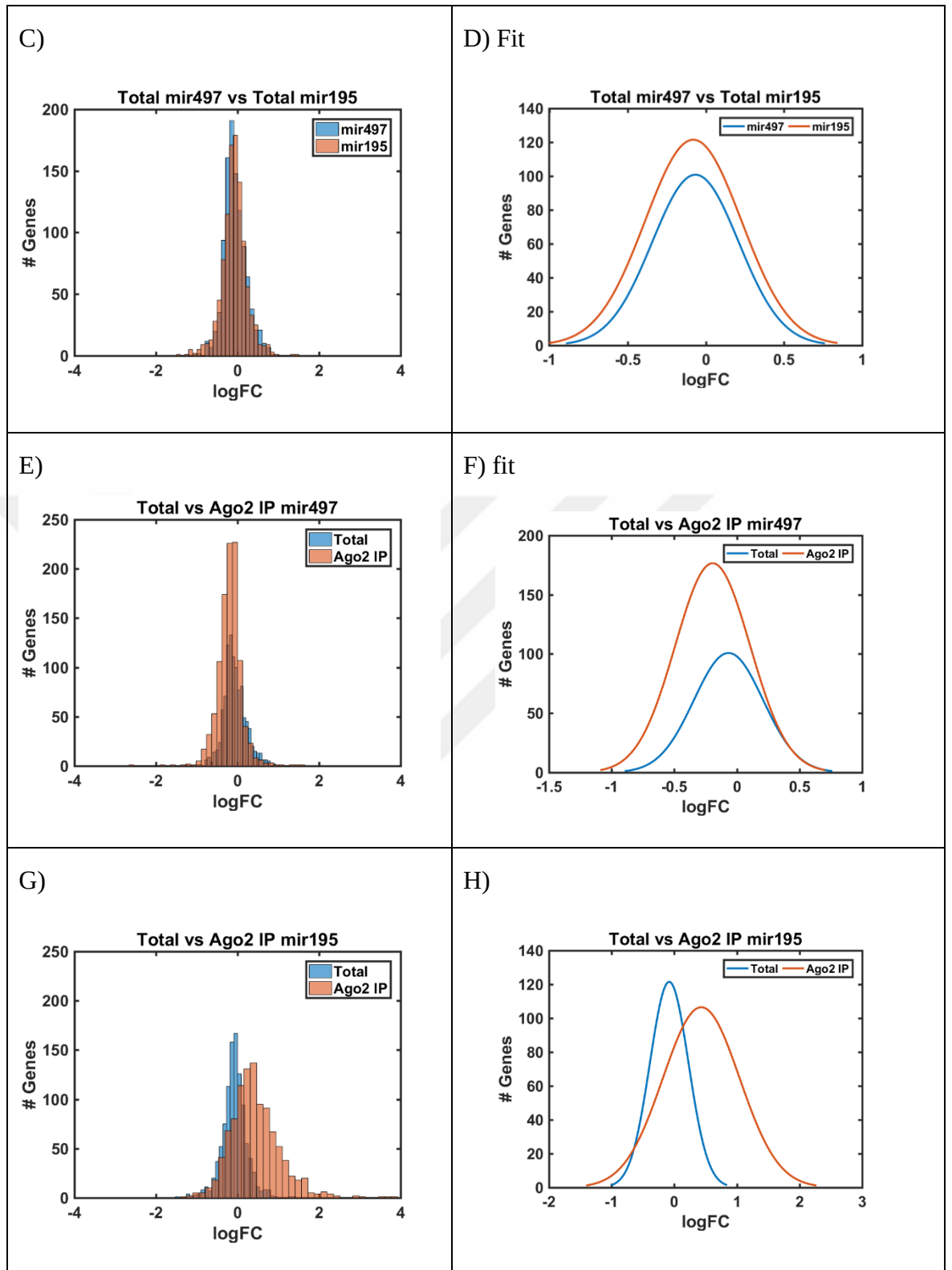


Figure S5. GSE41074 histograms to compare A, B) mimic-mir-195 and mimic-mir-497 treatments from Ago2-IP data C, D) mimic-mir-195 and mimic-mir-497 treatments from Total RNA data E, F) Total RNA with Ago2-IP under mimic-mir-497 treatment G, H) Total RNA with Ago2-IP under mimic-mir-195 treatment. (Threshold for sum of all six sample in GSE41074 is more than 10)

microRNA microarray had only Ago2-IP data therefore there was only one comparison between mimic-mir-195 (Ago2-IP) and mimic-mir-497 (Ago2-IP). There was a balance in mimic-mir-195 treatment data on direction but, mimic-mir-497 created a downward effect according to fold changes of microRNAs (Figure S6).

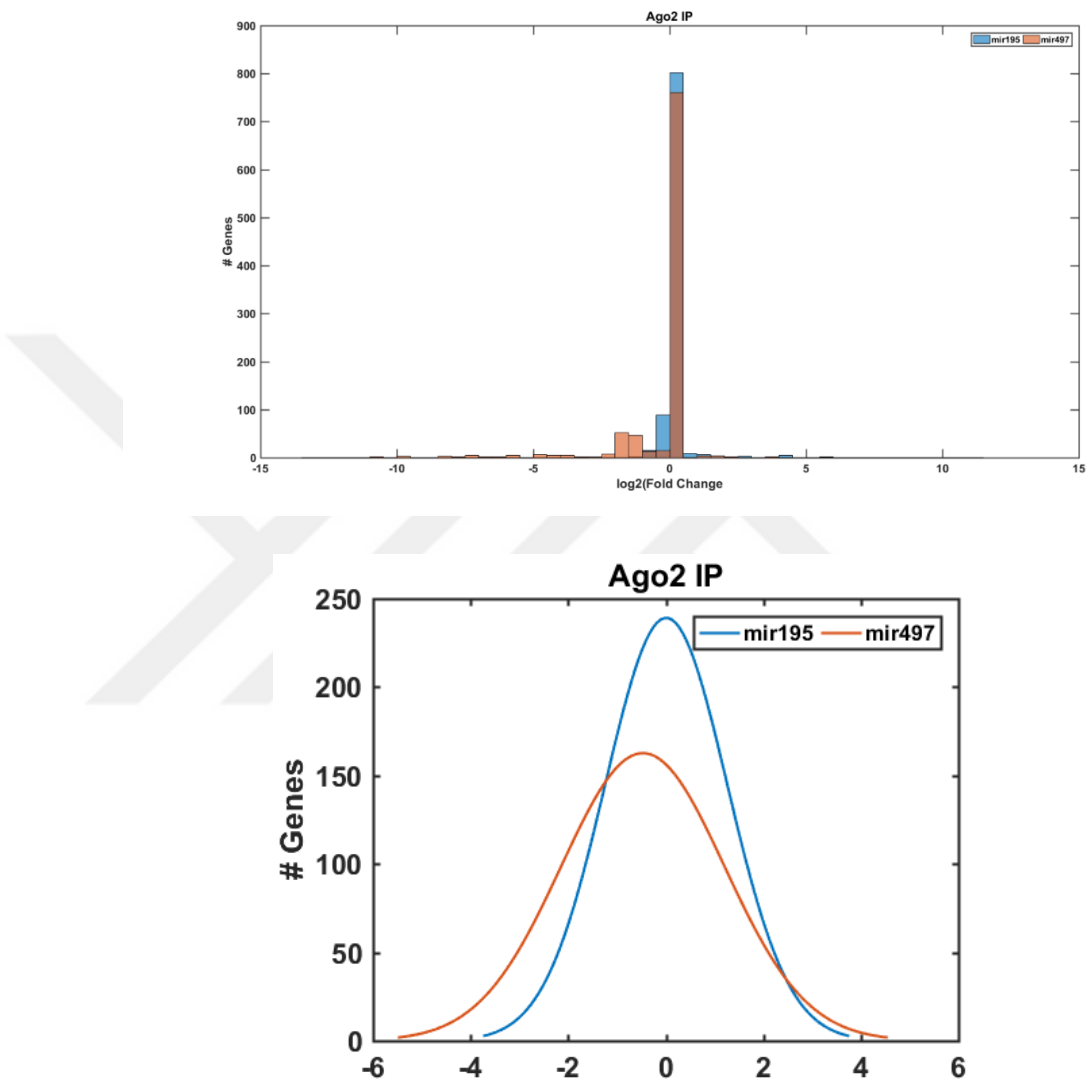


Figure S6. Histogram of data from GSE41079 (x: LogFC, no limitation on data)

Expressions Differentiation of the miRNA Cluster, involves mir-497 and mir-195, Members under siRNA CHRNA5 or under mimic-mir-195/497 Treatments

Table S1. Expression changes under mimic-mir-195/497 treatments of the microRNAs within the same cluster with mir-497/195

microRNAs	Hep G2_miR- 195_24h_ Ago2-IP	Hep G2_miR- 497_24h_A go2-IP	Hep G2_NT_Ag o2-IP	Ago 195 LogFC	Ago 497 LogFC
hsa-miR-424	0	0	0	0	0
hsa-miR-424*	0	0	0	0	0
hsa-miR-15a	9.363937	7.8539834	9.539998	-0.176061	-1.6860146
hsa-miR-15a*	0	0	0	0	0
hsa-miR-15b	10.75117	9.39788	10.997787	-0.2466195	-1.5999065
hsa-miR-15b*	0	0	0	0	0
hsa-miR-16	11.68244	10.240458	11.534945	0.147493	-1.294487

Table S2. Expression changes under siRNA CHRNA5 treatment of the microRNAs within the same cluster with mir-497/195

microRNAs	sc avg	si avg	LogFC (siRNA CHRNA5)
hsa-miR-424-5p	5.690919	5.917439	0.2265195
hsa-miR-424-5p	7.1801455	7.149411	-0.0307345
hsa-miR-15a-5p	11.17963	10.83961	-0.340025
hsa-miR-15a-3p	1.30898075	1.668542	0.35956125
hsa-miR-15b-5p	11.138035	11.35612	0.218085
hsa-miR-15b-3p	0.59528725	0.567763	-0.02752455
hsa-miR-16-5p	13.791135	13.66488	-0.126255

Immune Genes in GSE41081

Table S3. The most downregulated immune genes depend on Total RNA mimic-mir-497 LogFC (LogFC <-20).

immune genes	NT_mRNA	X195_mRNA	X497_mRNA	NT_Ago2IP	X195_Ago2IP	X497_Ago2IP	Total 195 FC	Total 497 FC	Ago 195 FC	Ago 497 FC
HSP90AB1	1075.386	1012.923	845.5066	90.78958	234.0684	83.4	-62.463	-229.88	143.2788	-7.39243
KIF5B	414.1907	206.6389	260.022	98.60664	88.93003	72.98	-207.55	-154.17	-9.67661	-25.623
EIF4G2	350.2744	372.4674	252.1495	58.24597	76.30535	47.91	22.193	-98.125	18.05938	-10.3345
CALM2	560.2268	564.8226	473.824	47.63906	67.11425	38.76	4.5958	-86.403	19.47519	-8.87575
KPNA2	189.6085	165.1036	109.7893	39.52213	45.99345	24.13	-24.505	-79.819	6.47132	-15.3926
CANX	352.6807	255.9449	278.3285	37.70358	49.55424	39.78	-96.736	-74.352	11.85066	2.07932
RPS6KA3	142.4877	51.57239	68.44343	35.48141	40.34978	22.69	-90.915	-74.044	4.86837	-12.7886
PSMA6	341.9827	288.9201	299.5461	23.06826	31.1045	17.3	-53.063	-42.437	8.03624	-5.76796
UBA2	92.60011	69.7222	55.78521	13.42205	13.03477	10.5	-22.878	-36.815	-0.38728	-2.92625
CDK1	78.31652	34.19818	43.99564	7.587793	4.039978	6.27	-44.118	-34.321	-3.54782	-1.31793
PSMA3	191.4168	168.383	162.883	20.95984	29.78843	22.28	-23.034	-28.534	8.82859	1.31626
PSMA4	190.773	173.6792	165.2189	14.81853	19.24222	12.24	-17.094	-25.554	4.42369	-2.58122
KPNA3	59.72672	30.32978	35.14046	11.02268	14.57342	8.433	-29.397	-24.586	3.55074	-2.58939

Table S4. The most upregulated immune genes depend on Total RNA mimic-mir-497 LogFC. (LogFC <-20)

immune genes	NT_mRNA	X195_mRNA	X497_mRNA	NT_Ago2IP	X195_Ago2IP	X497_Ago2IP	Total 195 FC	Total 497 FC	Ago 195 FC	Ago 497 FC
CALR	647.3101	604.4519	755.4665	52.60893	156.6822	53.34	-42.858	108.16	104.0733	0.73259
PSMA7	239.7397	260.3526	302.6621	20.34954	46.05	21.37	20.6129	62.922	25.70046	1.01773
IFITM3	65.0013	93.31462	120.366	4.269581	14.29245	2.951	28.3133	55.365	10.02287	-1.31878
CTSD	63.92376	93.48163	111.6475	5.213109	14.4111	4.915	29.5579	47.724	9.197991	-0.29855
C3	139.3184	170.9061	182.7753	25.56813	46.0717	25.85	31.5877	43.457	20.50357	0.28524
BCL2L1	92.26495	145.1806	132.003	20.43301	56.10687	16.1	52.9157	39.738	35.67386	-4.33062
MT2A	173.1987	152.0224	212.7042	36.58688	166.8639	17.92	-21.176	39.506	130.277	-18.6655
CALM3	130.2676	125.0169	168.6429	14.86113	30.1336	9.058	-5.2507	38.375	15.27247	-5.80305
CTSA	137.6137	150.4235	175.7578	27.08351	91.12401	20.36	12.8098	38.144	64.0405	-6.72214
CYBA	33.26	46.51463	68.66523	3.71364	10.4611	2.046	13.2546	35.405	6.74746	-1.66721
ARF1	113.1955	124.2621	147.2446	10.26392	26.31091	6.041	11.0666	34.049	16.04699	-4.22256
HLA-A	79.60542	99.27276	112.2783	7.940341	15.83561	7.09	19.6673	32.673	7.895269	-0.85061
DDOST	136.8238	145.6218	168.2441	11.34704	42.14403	11.92	8.798	31.42	30.79699	0.57353
PSMB4	144.321	150.067	174.5108	12.71872	28.02674	8.524	5.746	30.19	15.30802	-4.19491
CD74	22.02783	46.01502	52.18048	1.855582	5.562985	2.744	23.9872	30.153	3.707403	0.888592
SEC13	104.3369	108.3645	133.4336	8.002829	17.20853	6.812	4.0276	29.097	9.205701	-1.19109
AP2M1	123.641	133.7254	150.9241	11.0816	22.24802	8.705	10.0844	27.283	11.16642	-2.3771
PSMD3	80.44395	86.14701	107.1603	6.835723	13.64837	5.334	5.70306	26.716	6.812647	-1.50207
PSME1	191.7734	195.4629	217.1023	15.75301	31.31615	10.79	3.6895	25.329	15.56314	-4.96628
PSMB6	111.7919	112.8469	136.8261	9.567344	15.11501	4.545	1.055	25.034	5.547666	-5.02191
CDC34	55.98523	51.69936	79.80001	5.7979	18.44924	2.533	-4.2859	23.815	12.65134	-3.26455
IFITM2	26.47276	44.16525	49.01149	1.220929	7.142908	0.771	17.6925	22.539	5.921979	-0.44945
PSMC4	81.16814	87.13813	103.4211	6.459407	12.74345	4.775	5.96999	22.253	6.284043	-1.68447
UBA52	85.83456	88.65184	106.729	6.679535	15.41455	4.942	2.81728	20.894	8.735015	-1.7376
CTSH	61.1133	85.40653	81.89558	4.919286	13.15511	5.624	24.2932	20.782	8.235824	0.704435

Appendix II

MATLAB codes for mRNA Profile (GSE41074)

```
% GSE41074 data was uploaded and filtered for figures 4.47-
[num2, txt, ~]
=xlsread('GSE41074_HepG2_mRNAseq_Ago2IPseq_result.xlsx');
num2(sum(num2<15 ,2)~=0,:)=[];
num=log2(num2+1);
txt=txt(2:end,1);

Tnt=num(:,1);
T195=num(:,2);
T497=num(:,3);

T497nt=T497-Tnt;
T195nt=T195-Tnt;

Ant=num(:,4);
A195=num(:,5);
A497=num(:,6);

A497nt=A497-Ant;
A195nt=A195-Ant;

% Figure 4.47
scatter(T497nt, A497nt,15, 'filled')

hold on

scatter(T195nt, A195nt, 15 , 'filled')
axis([-2,4,-2,4])
axis square
xlabel('logFC Total')
ylabel('logFC Ago2 IP')
set(gca , 'fontsize',16, 'FontName', 'Arial','FontWeight',
'Bold','LineWidth',2);

legend({'mir497',
'mir195'}, 'Orientation','horizontal','FontWeight',
'Bold','FontSize',12, 'FontName', 'Arial' )

% Figure 4.49 A
CG=clustergram([T195, T497, Tnt], 'Standardize', 1,
'Linkage', 'ward','Colormap', 'redbluecmap',
'ColumnLabels', {'mir195 Total','mir497 Total', 'nt'});

% Figure 4.49 B
```

```

CG=clustergram([A195, A497, Ant], 'Standardize', 1,
'Linkage', 'ward','Colormap', 'redbluecmap',
'ColumnLabels', {'mir195 Ago2 IP','mir497 Ago2 IP','nt Ago2
IP'});

%GSE41074 data was uploaded and arranged for Figure 4.51
[num2, txt, ~]
=xlsread('GSE41074_HepG2_mRNAseq_Ago2IPseq_result.xlsx');
num2(sum(num2<10 ,2)~=0,:)=[];
num=log2(num2+1);
txt=txt(2:end,1);

Tnt=num(:,1);
T195=num(:,2);
T497=num(:,3);

T497nt=T497-Tnt;
T195nt=T195-Tnt;

Ant=num(:,4);
A195=num(:,5);
A497=num(:,6);

A497nt=A497-Ant;
A195nt=A195-Ant;

%Figure 4.51 A
histogram(A497nt);
hold on
histogram(A195nt);
xlabel( 'logFC')
ylabel( '# Genes')
title('Ago2 IP mir497 vs Ago2 IP mir195')
legend({'mir497',
'mir195'},'Orientation','horizontal','FontWeight',
'Bold','FontSize',12, 'FontName', 'Arial' )
set(gca, 'fontsize',16, 'FontName', 'Arial','FontWeight',
'Bold','LineWidth',2);

%Figure 4.51 B
h1=histfit(A497nt);
x1=h1(2).XData;
y1=h1(2).YData;
h2=histfit(A195nt);
x2=h2(2).XData;
y2=h2(2).YData;

plot(x1,y1, 'linewidth', 2)
hold on
plot(x2,y2, 'linewidth', 2)

```

```

xlabel( 'logFC')
ylabel( '# Genes')
title('Ago2 IP mir497 vs Ago2 IP mir195')
legend({'mir497',
'mir195'}, 'Orientation', 'horizontal', 'FontWeight',
'Bold', 'FontSize', 12, 'FontName', 'Arial' )
set(gca , 'fontsize', 16, 'FontName', 'Arial', 'FontWeight',
'Bold', 'LineWidth', 2);

%Figure 4.51 C
histogram(T497nt);
hold on
histogram(T195nt);
xlabel( 'logFC')
ylabel( '# Genes')
title('Total mir497 vs Total mir195')
legend({'mir497',
'mir195'}, 'Orientation', 'horizontal', 'FontWeight',
'Bold', 'FontSize', 12, 'FontName', 'Arial' )
set(gca , 'fontsize', 16, 'FontName', 'Arial', 'FontWeight',
'Bold', 'LineWidth', 2);

% Figure 4.51 D
h1=histfit(T497nt);
x1=h1(2).XData;
y1=h1(2).YData;
h2=histfit(T195nt);
x2=h2(2).XData;
y2=h2(2).YData;

plot(x1,y1, 'linewidth', 2)
hold on
plot(x2,y2, 'linewidth', 2)

xlabel( 'logFC')
ylabel( '# Genes')
title('Total mir497 vs Total mir195')
legend({'mir497',
'mir195'}, 'Orientation', 'horizontal', 'FontWeight',
'Bold', 'FontSize', 12, 'FontName', 'Arial' )
set(gca , 'fontsize', 16, 'FontName', 'Arial', 'FontWeight',
'Bold', 'LineWidth', 2);

% Figure 4.51 E
h1=histogram(T497nt);
hold on
h2=histogram(A497nt);
xlabel( 'logFC')
ylabel( '# Genes')
title('Total vs Ago2 IP mir497')

```

```

legend({'Total', 'Ago2
IP'}, 'Orientation', 'horizontal', 'FontWeight',
'Bold', 'FontSize', 12, 'FontName', 'Arial' )
set(gca, 'fontsize', 16, 'FontName', 'Arial', 'FontWeight',
'Bold', 'LineWidth', 2);

% Figure 4.51 F
h1=histfit(T497nt);
x1=h1(2).XData;
y1=h1(2).YData;
h2=histfit(A497nt);
x2=h2(2).XData;
y2=h2(2).YData;

plot(x1,y1, 'linewidth', 2)
hold on
plot(x2,y2, 'linewidth', 2)

xlabel( 'logFC')
ylabel( '# Genes')
title('Total vs Ago2 IP mir497')
legend({'Total', 'Ago2
IP'}, 'Orientation', 'horizontal', 'FontWeight',
'Bold', 'FontSize', 12, 'FontName', 'Arial' )
set(gca, 'fontsize', 16, 'FontName', 'Arial', 'FontWeight',
'Bold', 'LineWidth', 2);

%Figure 4.51 G
h1=histogram(T195nt);
hold on
h2=histogram(A195nt);
xlabel( 'logFC')
ylabel( '# Genes')
title('Total vs Ago2 IP mir195')
legend({'Total', 'Ago2
IP'}, 'Orientation', 'horizontal', 'FontWeight',
'Bold', 'FontSize', 12, 'FontName', 'Arial' )
set(gca, 'fontsize', 16, 'FontName', 'Arial', 'FontWeight',
'Bold', 'LineWidth', 2);

% Figure 4.51 H
h1=histfit(T195nt);
x1=h1(2).XData;
y1=h1(2).YData;
h2=histfit(A195nt);
x2=h2(2).XData;
y2=h2(2).YData;

plot(x1,y1, 'linewidth', 2)
hold on
plot(x2,y2, 'linewidth', 2)

```

```

xlabel( 'logFC')
ylabel( '# Genes')
title('Total vs Ago2 IP mir195')
legend({'Total', 'Ago2
IP'}, 'Orientation', 'horizontal', 'FontWeight',
'Bold', 'FontSize', 12, 'FontName', 'Arial' )
set(gca, 'fontsize', 16, 'FontName', 'Arial', 'FontWeight',
'Bold', 'LineWidth', 2);
% For histograms kstest2 was performed.
[h,p] = kstest2(T497nt,A497nt)
[h,p] = kstest2(T195nt,A195nt)
[h,p] = kstest2(T497nt,T195nt)
[h,p] = kstest2(A497nt,A195nt)

% GSE41074 data was uploaded for figures 4.46
[num2, txt, ~]
=xlsread('GSE41074_HepG2_mRNAseq_Ago2IPseq_result.xlsx');

num=log2(num2+1);
txt=txt(2:end,1);

Tnt=num(:,1);
T195=num(:,2);
T497=num(:,3);

T497nt=T497-Tnt;
T195nt=T195-Tnt;

Ant=num(:,4);
A195=num(:,5);
A497=num(:,6);

A497nt=A497-Ant;
A195nt=A195-Ant;

%Figure 4.46 A
scatter(A497nt, A195nt, 'filled')
axis equal
xlabel('logFC mir497')
ylabel('logFC mir195')
title('Ago2 IP')
set(gca, 'fontsize', 12, 'FontName', 'Arial', 'FontWeight',
'Bold', 'LineWidth', 2);
[r,p]=corr(A497nt, A195nt, 'type', 'Spearman');
legend(strcat('R=', {' '},
num2str(r)), 'Orientation', 'horizontal', 'FontWeight',
'Bold', 'FontSize', 12, 'FontName', 'Arial' )
axis([-5,5,-5,5])
axis square

```

```

%Figure 4.46 B
scatter(T497nt, T195nt, 'filled')

xlabel('logFC mir497')
ylabel('logFC mir195')
title('Total')
set(gca, 'fontsize', 12, 'FontName', 'Arial', 'FontWeight',
'Bold', 'LineWidth', 2);
[r, ~] = corr(T497nt, T195nt, 'type', 'Spearman');
legend(strcat('R=', {' '}),
num2str(r), 'Orientation', 'horizontal', 'FontWeight',
'Bold', 'FontSize', 12, 'FontName', 'Arial' )
axis([-4, 4, -4, 4])
axis square
%Figure 4.46 C
scatter(T195nt, A195nt, 15, 'filled')
axis([-6, 6, -6, 6])
axis square
xlabel('logFC Total')
ylabel('logFC Ago2 IP')
title('mir195 vs mir497')
set(gca, 'fontsize', 16, 'FontName', 'Arial', 'FontWeight',
'Bold', 'LineWidth', 2);
[r, p] = corr(T195nt, A195nt, 'type', 'Spearman');
legend(strcat('R=', {' '}),
num2str(r), 'Orientation', 'horizontal', 'FontWeight',
'Bold', 'FontSize', 12, 'FontName', 'Arial' )

%Figure 4.46 D
scatter(T497nt, A497nt, 15, 'filled')
axis([-6, 6, -6, 6])
axis square
xlabel('logFC Total')
ylabel('logFC Ago2 IP')
title('mir497')
set(gca, 'fontsize', 16, 'FontName', 'Arial', 'FontWeight',
'Bold', 'LineWidth', 2);
[r, p] = corr(T497nt, A497nt, 'type', 'Spearman');
legend(strcat('R=', {' '}),
num2str(r), 'Orientation', 'horizontal', 'FontWeight',
'Bold', 'FontSize', 12, 'FontName', 'Arial' )

```

Matlab Codes for miRNA Profile (GSE41079)

```

%GSE41079 data was uploaded for figures 4.39, 4.40, S
[num2, txt, ~] = xlsread('GSE41079_normalized.xlsx');
txt(1,:) = [];

```

```

m195=num2(:,1);
m497=num2(:,2);
nt=num2(:,3);

%Figure 4.39 A
scatter(m195, nt, 'filled')
set(gca, 'fontsize',12, 'FontName', 'Arial','FontWeight',
'Bold','LineWidth',2);
xlabel('mir195')
ylabel('nt')
title('Ago2 IP mir195')
axis square
[r,p]=corr(m195, nt, 'type', 'Pearson');

%Figure 4.39 B
scatter(m497, nt, 'filled')
set(gca, 'fontsize',12, 'FontName', 'Arial','FontWeight',
'Bold','LineWidth',2);
xlabel('mir497')
ylabel('nt')
title('Ago2 IP mir497')
axis square
[r,p]=corr(m497, nt, 'type', 'Pearson');

%Figure 4.39 C
scatter(m195, m497, 'filled')
set(gca, 'fontsize',12, 'FontName', 'Arial','FontWeight',
'Bold','LineWidth',2);
xlabel('mir195')
ylabel('mir497')
title('Ago2 IP')
axis square
[r,p]=corr(m497, m195, 'type', 'Pearson');

%Figure 4.40
scatter(m195-nt, m497-nt, 'filled')
set(gca, 'fontsize',12, 'FontName', 'Arial','FontWeight',
'Bold','LineWidth',2);
xlabel('logFC mir195')
ylabel('logFC mir497')
title('GSE41079 Ago2 IP')

%Figure 4.52 A
histogram(m497-nt)
hold on
histogram(m195-nt)
xlabel('log FC')
ylabel('# Genes')
title('Ago2 IP')

```

```

legend({'mir497','mir195'
}, 'Orientation','horizontal','FontWeight',
'Bold','FontSize',12, 'FontName', 'Arial' )
set(gca , 'fontsize',16, 'FontName', 'Arial','FontWeight',
'Bold','LineWidth',2);

%Figure 4.52 B
h1=histfit(m497-nt);
x1=h1(2).XData;
y1=h1(2).YData;
h2=histfit(m195-nt);
x2=h2(2).XData;
y2=h2(2).YData;
plot(x1,y1, 'linewidth', 2)
hold on
plot(x2,y2, 'linewidth', 2)

xlabel( 'logFC')
ylabel( '# Genes')
title('Ago2 IP')
legend({'mir497','mir195'
}, 'Orientation','horizontal','FontWeight',
'Bold','FontSize',12, 'FontName', 'Arial' )
set(gca , 'fontsize',16, 'FontName', 'Arial','FontWeight',
'Bold','LineWidth',2);

```

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[Avicenna J Med Biotechnol](#). 2009 Jul-Sep; 1(2): 55–70.

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Noncoding RNAs and Cancer

[Mozhgan Moslemi Naeini](#) and [Ali M. Ardekani](#)*

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Publication: Nature Communications
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Targeting microRNAs as key modulators of tumor immune response

[Laura Paladini](#), [Linda Fabris](#), [Giulia Bottai](#), [Carlotta Raschioni](#), [George A. Calin](#),[✉] and [Libero Santarpia](#)[✉]

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