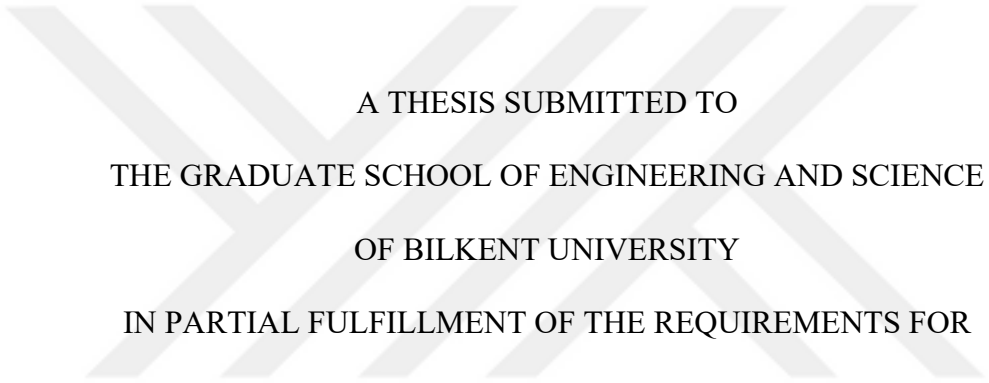


A SHINY APPLICATION FOR PANCAN SURVIVAL ANALYSIS WITH
PARALOG/MIRNA PAIRS AND *IN VITRO* VALIDATION OF MIRNA SYNERGISM
IN LIVER CANCER



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BY
MELIKE TOMBAZ
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IN LIVER CANCER

By Melike Tombaz

September 2022

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

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Abstract

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PARALOG/MIRNA PAIRS AND *IN VITRO* VALIDATION OF MIRNA SYNERGISM
IN LIVER CANCER

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M.Sc. in Molecular Biology and Genetics

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September, 2022

Emerging cancer survival tools can predict risk of disease and identify prognostic biomarkers. Multivariable Cox proportional hazards models with mRNA and microRNAs (miRNAs) expression can differentiate survival outcomes. Previous studies showed that genes that belong to the same pathways/families may act independently, and in a cancer-specific manner. In this thesis, cancer-dependent hazard ratios of paralog genes and sense-antisense strands of miRNAs were tested for TCGA PANCAN. The results were presented in a R/Shiny web application that provides gene-by-survival networks. The gene-by-survival network approach also was applied to the plasma membrane-endoplasmic reticulum (PM-ER) calcium channel geneset. Among paralogs, cancer-specific prognostic signatures and functional compartmentalization were observed. Some cancers like UVM, MESO emerged as hub cancers for PM-ER signalling. Further the proposed gene-by-survival network approach has been extended for miRNA-mRNA triplets that may act in synergy in hepatocellular carcinoma (HCC). Next, the effects of synergistic miRNA pairs provided by miRCoop algorithm were tested on cell viability and target gene expression for selected triplets. The results have revealed that the combinatorial miRNA treatments show promising results as RNAi therapeutics yet future studies with different doses and triplets are needed.

Keywords: survival, miRNAs, paralogs, TCGA-PANCAN, prognosis, gene network

Özet

PARALOG/MİRNA ÇİFTLERİ İLE PANCAN SAĞKALIM ANALİZ SHINY APLİKASYONU VE KARACİĞER KANSERİNDE MIRNA SİNERJİZMİNİN İN VİTRO DOĞRULANMASI

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Geliştirilmekte olan kanser sağkalım araçları, hastalık riskini tahmin edebilir ve prognostik biyobelirteçleri tanımlayabilir. mRNA ve mikroRNA (miRNA) ifadeleri ile yapılan çok değişkenli Cox orantılı tehlike modeller, sağkalım sonuçlarını farklılaştırabilir. Önceki çalışmalar, aynı yollara/ailelere ait genlerin bağımsız olarak ve kansere özgü bir şekilde hareket ettiğini göstermiştir. Bu tezde, miRNA'ların paraloglarının ve sense-antisense iplikçiklerinin kansere bağlı risk oranları TCGA PANCAN için test edilmiştir. Sonuçlar, gen bazında hayatta kalma ağları da sağlayan bir R/Shiny web uygulamasında sunulmuştur. Gen bazında hayatta kalma ağı yaklaşımı plazma membranı-endoplazmik retikulum (PM-ER) kalsiyum kanallarına uygulanmıştır. Paraloglar arasında, kansere özgü prognostik imzalar ve fonksiyonel kompartmanlaşma gözlemlenmiştir. UVM, MESO gibi bazı kanserler PM-ER sinyali için merkez kanserler olarak ortaya çıkmıştır. Ayrıca, önerilen gen bazında hayatta kalma ağı hepatoselüler kanserde, miRNA-mRNA üçlüleri için genişletilmiştir. Ardından, miRCoop algoritması tarasından öne sürülen sinerjik bazı miRNA çiftlerinin hücre canlılığı ve hedef gen ifadesi üzerindeki etkisi test edilmiştir. Sonuçlar, kombinatoriyal miRNA tedavilerinin RNAi terapötikleri için umut verici sonuçlar gösterse de farklı doz ve hedeflerle yeni çalışmalar yapılması gerektiğini de göstermiştir.

Anahtar kelimeler: sağkalım, mikroRNA, paralog, TCGA-PANCAN, prognoz, gen ağı



*“I stand on the sacrifices
of a million women before
me thinking what can I do
to make this mountain
taller
so the women after me
can see farther”
-Rupi Kaur*

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Abbreviations

ACC: Adrenocortical carcinoma

BLCA: Bladder urothelial carcinoma

BRCA: Breast invasive carcinoma

CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma

CHOL: Cholangiocarcinoma

COAD: Colon adenocarcinoma

DLBC: Lymphoid neoplasm Diffuse large B-cell lymphoma

ESCA: Esophageal carcinoma

GBM: Glioblastoma multiforme

HNSC: Head and neck squamous cell carcinoma

KICH: Kidney chromophobe

KIRC: Kidney renal clear cell carcinoma

KIRP: Kidney renal papillary cell carcinoma

LGG: Brain lower grade glioma

LIHC: Liver hepatocellular carcinoma

LUAD: Lung adenocarcinoma

LUSC: Lung squamous cell carcinoma

MESO: Mesothelioma

OV: Ovarian serous cystadenocarcinoma

PAAD: Pancreatic adenocarcinoma

PCPG: Pheochromocytoma and paraganglioma

PRAD: Prostate adenocarcinoma

READ: Rectum adenocarcinoma

SARC: Sarcoma

SKCM: Skin cutaneous Melanoma

STAD: Stomach adenocarcinoma

TGCT: Testicular germ cell tumours

THCA: Thyroid carcinoma

THYM: Thymoma

UCEC: Uterine corpus endometrial carcinoma

UCS: Uterine carcinoma

UVM: Uveal melanoma

OS: Overall Survival

DSS: Disease-Specific Survival

KM: Kaplan-Meier

1 Introduction

1.1 Cancer

Cancer is one of the leading causes of death worldwide [1]. Abnormal genetic variations and accumulative interactions amongst environmental factors determine the biological processes and phenotypes that result in cancer. Cancer is a disease that is heterogeneous; thus, a tumour can consist of a diverse collection of cells, each having distinct molecular signatures. Heterogeneity can originate from several factors, including transcriptomic, genetic, and epigenetic changes [2]. Tumour heterogeneity is one of the reasons to limit the therapy efficacy [3]. Cancer heterogeneity can cause drug and chemotherapy resistance. Besides tumour heterogeneity, the tumour microenvironment can be used to explain the therapeutic limitations. The tumour microenvironment is responsible for the tumour growth, initiation, response to therapy, and metastasis [4]. A tumour can alter the ecosystem that surrounds the tumour and consists of stromal cells (cellular) and extracellular matrix components (non-cellular) [5].

By studying the altered gene expressions and associated biological pathways, one can determine the cancer origin and identify potential therapeutic targets [6]. Prognostic studies on gene expression have become more popular as high-throughput technologies became more available [7]. Gene expression signatures are used for survival prediction for cancer patients [8]. Several studies use gene expression signatures to predict survival. For example, four genes' expressions were used to evaluate patient overall survival and aggressiveness of ovarian cancer [9]. Accordingly, prioritization of candidate genes can serve as biomarkers for early diagnosis and prognosis [10].

The role of miRNAs was first identified when Calin et al. showed that miR-15 and miR-16 were frequently deleted in patients with chronic lymphocytic leukaemia [11]. Later, miRNA profiling became more popular as miRNAs can be used as cancer classifiers and prognosis predictors [12]. miRNA expression levels are associated with prognostic results like overall survival through survival estimate methods. Sathipati and colleagues used a 29-miRNA signature in bladder cancer to estimate the survival [13]. Similarly, Wu, Ding and Lin showed that a three-miRNA expression signature could predict survival in patients with triple-negative breast cancer [14].

1.2 Survival Analysis

The proportional hazards models are used to estimate the effects of different variates, affecting the time for an event to occur [8]. In cancer studies, the time is the survival time, or relapse or progression between response to treatment of the disease and death is also widely used as the event of interest in cancer studies [15].

1.2.1 Kaplan-Meier Survival Estimate

In order to estimate the survival probability nonparametric Kaplan Meier (K-M) method is generally used. In the K-M method, it is assumed that the events occur independently from one another, and a cumulative survival probability can be calculated from the events. K-M survival curves are plots of K-M survival probability over time and are a very useful way to estimate measures [15]. The log-rank test is used to test the survival differences between different groups, such as treatment and control, or the case of expression high expression or low.

Univariate models in survival analysis take into one factor under investigation to estimate the survival and ignore the impact of other factors. However, in clinical investigations, multiple factors affect the clinical outcome, such as age and gender [16]. Considering multiple genes at a time has also been explored in estimating the prognostic capability of gene sets [17]. Indeed, genes that belong to the same pathway provide excellent hypotheses to test whether they act independent of each other in prediction of survival of cancer patients [18]. Netrin and Netrin receptors and Robo-Slit receptors, have been tested using multivariable regression and were found to be associated with different cancers in pairs or modules and often in the opposite directions, suggesting genes that act together may compensate for each other [17], [18].

One limitation of the log-rank test is it does not provide a clinical assessment of the factor but only a statistical estimate. Cox, proportional hazards model is the most analysing survival data with multivariable quantitative and categorical factors. Mathematically, the Cox model can be explained as follows:

Equation 1

$$h(t) = h_0(t) \times \exp\{a_1x_1 + a_2x_2 + \dots + a_px_p\}$$

where the hazard function $h(t)$ is dependent on different covariates $(x_1, x_2, \dots, x_p)$, which have coefficients $(a_1, a_2, \dots, a_p)$. H_0 equals zero when all covariates are equal to zero and is the baseline hazard. In summary, the hazard curves calculated by the Cox regression model should be proportional to the hazard of covariates. The exponential of coefficients are called hazard ratios. Thus, if the hazard ratio is greater than one, that means increased risk and the length of survival are negatively correlated [19].

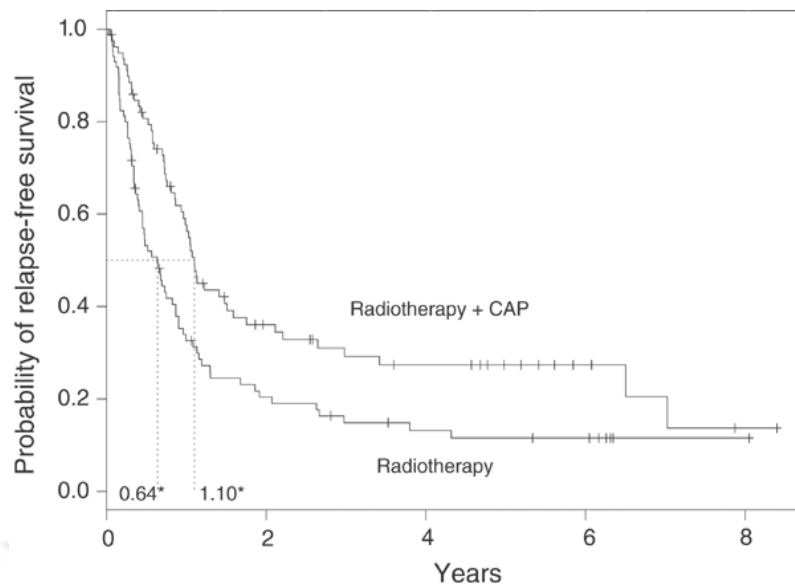


Figure 1.1. An example K-M plot for relapse-free survival curves for lung cancer (Adapted from [15], distributed under a Creative Commons license (Attribution-Noncommercial))

Another important approach for survival analysis is stratification. For instance, the covariate for survival is gender; the differences between each gender in terms of survival can be compared to one another (the strata) by the log-rank test. An important feature of the survival analysis is the sample size; due to the small number of individuals in the cohort, the model and covariates may be imprecise to give reliable results. A smaller cohort may not have enough events and thus have biased regression coefficients [20].

1.3 Paralog Genes

Paralog genes arise from gene duplication events and lead to the creation of new genes. Duplicated genes may degenerate through a process called non-functionalization or pseudogenization [21]. Paralogs can have entirely different functions, but they can also have some similar functions [22]. This overlap between paralogs can suggest a buffering capacity

for paralogs, and they can potentially compensate for the loss of each other [23]. A study by Lian et al. showed paralogs have cancer type-dependent expressions, and the networks of the paralogs can be used to identify cancer stages of lung cancers [24].

For instance, RAD51 paralogs are essential for homologous recombination by regulating the BRCA2-RAD51 dependent HR in several diseases like Fanconi anaemia and Bloom's syndrome [25], [26]. The human translation factor, eIF5A, is essential for cell proliferation and constitutively expressed in most cells, whereas its paralog eIFA2 is highly expressed in cancers and functions as an oncogene [27]. *COL1A1* gene mutations were more pathogenetic than *COL1A2* mutations in osteogenesis imperfecta [28]. Also, *COL1A1* and *COL1A2* have been proposed as biomarkers for hypopharyngeal squamous cell carcinoma, and *COL1A2* levels were used to predict poor survival [29]. *SMARCA2* knock-down reduced cell proliferation in *SMARCA4* mutated cells. However, *SMARCA2* silencing did not affect cell proliferation in *SMARCA4* wild-type cells [30]. *CDH1* and *CDH3* showed similar results in breast cancer cell line ZR75-30; the inhibition of *CDH3* showed a decrease in cell proliferation in *CDH1*-mutated cells only [30]. Silencing of *PRKCE* and *PRKC* family members suppressed the fitness in uveal cell line MEL202 [31]. Ito et al. also showed that DUSP4/6 dual knockout inhibited cell growth, while individual knockouts had no significant growth effects in the MAPK pathway [31]. These results suggest the presence of functional compensatory roles of these paralog genes.

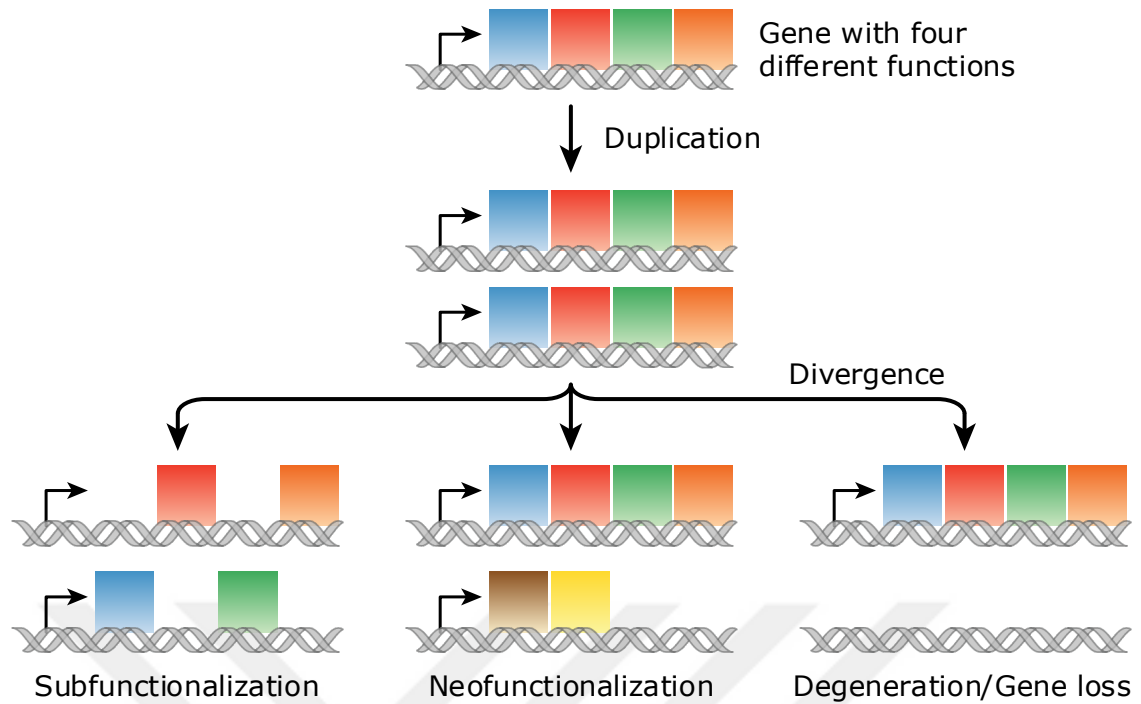


Figure 1.2. Evolutionary fate of duplicate genes. (From Wikimedia Commons, the free media repository)

1.4 MicroRNAs (miRNAs)

1.4.1 miRNA Biogenesis

microRNAs (miRNAs) are long non-coding RNAs that play a role in regulating mRNA levels [32]. miRNAs are first transcribed as pri-microRNAs and cleaved by Drosha into pre-miRNAs [33]. Pre-miRNAs are thermodynamically stable and exported to the cytoplasm, and the hairpin structure is processed into an RNA duplex by Dicer [34]. Following this process, miRNA duplexes are loaded into AGO proteins. There are more than 8000 miRNAs identified in humans with two mature miRNA arms (sense and antisense) which target different genes [35]. Following the AGO loading, one of the strands can be degraded. However, recent literature showed that one of the strands might not be degraded and both strands can co-exist. [36]. Relative expressions of coexisting arms are tissue-specific, suggesting tissue-dependent regulatory roles for 5p/3p arms [37]. It is important to

understand how the functions and expressions are altered for coexisting pairs in diseases, particularly cancer. Several studies show that both strands have functions in specific cancers. Let-7 and miR-126 families play different roles in embryonic stem cells [38], [39]. Both arms can accumulate in tissues while being subject to arm switching in others [40], [41]. Mature miRNA strands that are dysregulated in cancers and these oncogenic pathways are still being investigated. Whether both strands cooperatively regulate the oncogenic pathways and target and dysregulate genes in the pathways is still unclear.

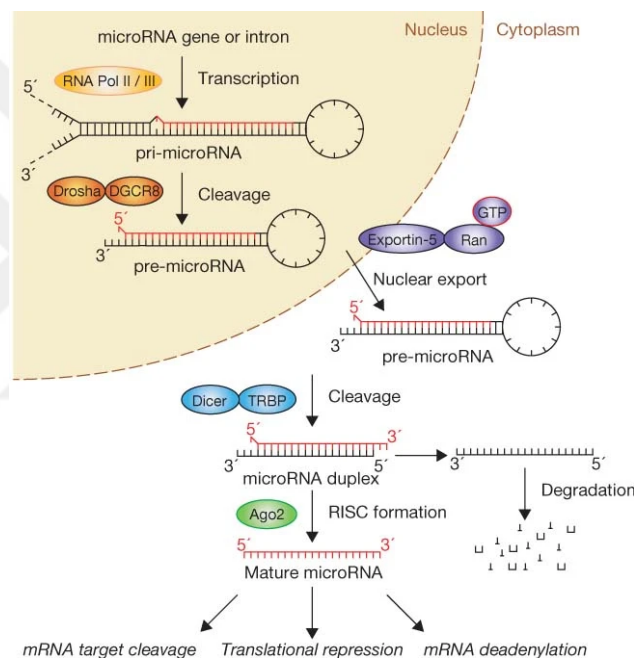


Figure 1.3. An overview of the miRNA processing pathway (Adapted from [42], Licence Number 5384910176416).

As mentioned before, two arms of a miRNA from the same precursor can target different sites. This arm switching can provide an evolutionary mechanism for novel functions for miRNAs and cause diversification [43]. For example, both strands of has-miR-31 are upregulated in oral squamous cell carcinoma, while one employs a tumour suppressor function, and the other is an oncogene [44], [45]. The miRNAs are shown to be dysregulated in human cancers, affecting the hallmarks of cancer [46]. miRNAs are continuously being

identified as potential biomarkers for human cancer. They can function as therapeutic targets and tools. miRNAs can function as a oncogene or tumour suppressor depending on the circumstances and the tissues [47]. In urothelial bladder carcinoma, the miRNA signatures have been used to estimate survival in patients [13]. Similarly, a three-miRNA signature was used as a prognostic biomarker to predict the overall survival of triple-negative breast cancer [14]. However, comparative and/or multivariable survival analysis of arms of a specific miRNA across multiple cancer has not been studied systematically.

1.4.2 miRNA Cooperativity

One mRNA can be targeted by more than one miRNA, and one miRNA can target many mRNAs, raising the possibility of cooperative relationships among miRNAs [48]. Complex diseases, including cancers, are affected by several miRNAs rather than one. Several studies showed that miRNAs from different clusters work together to regulate target gene expression. For example, Kandi et al. showed that miR-99a and miR-125b regulated drug resistance in acute megakaryoblastic leukaemia [49].

Several bioinformatics studies investigate miRNA cooperativity. These studies have focused on miRNA-miRNA networks and identified hub miRNAs that might be crucial for breast and small cell lung cancer [50]. These methodologies are either sequence-based, correlation-based or causality-based. The sequence data are used in sequence-based methods to identify miRNA-target interactions and significant common target genes. To determine the synergistic relationship of a miRNA-miRNA pair, enrichment analysis can be performed [51]–[53]. However, this method has limitations in using static data to study the synergism to understand the coregulation between different miRNAs heterogeneous cancers [54]. Correlation-based studies use the expression of miRNAs to identify differentially expressed miRNAs to correlate miRNA and mRNA expression data. Yet, this method does not

consider causal relationships of gene regulation [54]. The last method is causality-based to understand single miRNAs' causal effects by knocking them down each time [55]. Unfortunately, this method is limited due to miRNA coregulation that involves more than one miRNA.

miRSynergy tool detects synergistic miRNAs in ovarian, breast and thyroid cancers [56]. miRsyn is another tool to identify miRNA synergism, which uses sequence and expression data [54]. miRCoop uses kernel-based tests to detect synergistic miRNAs that have weak effects on their targets individually but show strong repression together [57].

1.4.3 miRNAs in HCC

Hepatocellular carcinoma, the liver's primary cancer, and its development is associated with environmental and genetic factors. Hepatitis B and hepatitis C virus infection, excessive alcohol consumption, and non-alcoholic steatohepatitis (NASH) are important risk factors for HCC development [58]. Early diagnosis and surveillance for HCC are important for mortality and improving patient outcomes. Early diagnosis can benefit survival for patients with HCC [59]. Unfortunately, at later stages, there is no effective treatment to improve survival [59]. Several treatment options can improve survival, both surgical and nonsurgical. Dysregulation of miRNA expression can be observed in many cancer types. Aberrant expression of miRNAs has been detected in both HCC cell lines and tissues [60]. Several studies show that miRNAs can serve as therapeutic targets and potential biomarkers for HCC [61]–[63]. miRNAs involved in lipid metabolism are important for the HCC. miRNAs such can modulate lipid and cholesterol levels [64]. miRNAs that target tumour suppressor genes are showed increased expressions in HCC [65]–[67]. miR-21, miR-221 and miR-222 are upregulated in HCC and miR-145, miR-199a and miR-223 are decreased [68]–[70]. HCC

alters several cancer pathways, such as PI3K/MAPK and the WNT/ β -catenin pathway. Downregulation of miR-1, miR-23b, and miR-34a causes promote cell proliferation by losing the suppressive effect in PI3K and MAPK pathways [71]. The WNT/ β -catenin pathway is frequently upregulated in HCC, and the upregulation of several miRNAs, such as miR-106b, miR-135 and miR-315, results in the release of activated β -catenin, which promotes the expression of c-Myc, c-jun, and Cyclin D [72], [73]. However, there is a need to investigate and identify and then validate synergistic miRNAs that can be better prognostic biomarkers [74]–[76].

1.4.4 miRNAs used in this study and their role in HCC

1.4.4.1 miR-19a-3p

miR-19a-3p, a frequently dysregulated member of the miR-17-92 cluster, is important for several cellular processes including metastasis and proliferation [77]. miR-19a-3p is dysregulated in liver cancer and induces sorafenib resistance [78]. Silencing of miR-19a-3p with inhibitors in hepatocellular cell lines showed increased apoptosis and suppression in cell proliferation [79].

1.4.4.2 miR-20a-5p

miR-20a-5p, also a member of the miR-17-92 cluster, functions as a tumour suppressor, and its deficiency promotes HCC metastasis [80], [81]. Downregulation of miR-20a-5p is also involved in liver fibrosis by regulating TGF- β signalling [82].

1.4.4.3 let-7c-5p

let-7c-5p is downregulated in HCC cell lines and inhibits migration [83]. Opposite to miR-19a-3p, let-7c-5p increases sorafenib sensitivity. By regulating *CDC25A*, let-7c induces cell cycle arrest and inhibits cell proliferation [84].

1.4.4.4 miR-125a-5p

miR-125a-5p also acts as a tumour suppressor in HCC. The overexpression of miR-125a-5p inhibits HCC cell proliferation through the MAPK signalling pathway [84], [85]. miR-125a-5p overexpression increases protein levels of caspase-9 and APAF1 to induce cell apoptosis [86].

1.4.4.5 miR-203a-3p

miR-203-3p is an oncogene in HCC and targets IL-24 [87]. Inhibition of miR-203a-3p reverses the HCC cell proliferation, and metastasis miR-203a also targets PTEN and by regulating PI3K/Akt pathway promotes cell proliferation [88], [89].

1.4.4.6 miR-195-5p

miR-195-5p expression is downregulated in HCC cells compared to controls, and its expression is associated with lymph node metastasis. Overexpression of miR-195 inhibits migration and inhibition [90].

1.5 Plasma membrane – Endoplasmic reticulum (PM-ER) Calcium Channels

Calcium channels play essential roles in biological systems by generating membrane potentials and acting as cell signalling molecules [91]. Alterations in calcium channels and homeostasis are involved in tumorigenesis and tumour progression [92]. The intracellular calcium concentrations significantly influence the tumour microenvironment [93]. Many components of PM-ER proteins are paralogous, and as discussed before, they may overlap in their function and/or be functionally compartmentalized or neo-functionalized [94]. We hypothesized that analyzing the prognostic significance of PM-ER genes with respect to their paralogous relationships can provide insight into our understanding of their functional

evolution. The PM-ER genes studied in this thesis and cancers found to be associated with calcium signalling are summarized in the following sections.

1.5.1 ORAIs and STIMs

Orai proteins are plasma membrane proteins that are coupled with endoplasmic reticulum (ER) STIM proteins [95]. STIM proteins consist of STIM1 and STIM2. Orai proteins include highly conserved three homologs, Orai1-3 [96]. STIM and Orai proteins have emerged as potential targets for cancer therapeutics [97].

1.5.2 IP3Rs and RYRs

IP3Rs are calcium uniporters, and IP3Rs mediate calcium release from the ER [98]. Different isoforms of IP3Rs have redundant functions and show different activation patterns. Several oncogenes and tumour suppressors target IP3Rs; thus, the alterations are seen in many cancer types [99]. Ryanodine receptors are homotetrameric complexes that regulate calcium release from the sarcoplasmic reticulum [99]. The mutations in three isoforms (RyR1-3) are associated with cancers like prostate, breast, head and neck and thyroid carcinoma [100].

1.5.3 SERCA/ATP2As

The sarco/endoplasmic reticulum ATPases are membrane-bound ATPases that transport calcium across the membrane [101]. These ATPases have critical roles in proliferation and secretion and are necessary for normal cellular function [102]. Dysregulated ATPase expression can serve as markers of differentiation in colon, lung, and breast cancers and novel targets for therapeutics [103].

1.5.4 Uveal melanoma (UVM)

Uveal melanoma is a tumour arising from the melanocytes located in the ocular region [104]. The BRCA1-associated protein 1 (*BAP1*) mutations predispose patients to develop uveal melanoma [105]. Changes in the calcium channel regulations contribute to the onset and progression of UVM [106]. Fibroblast growth factor 2 (*FGF2*) increases intracellular calcium concentrations by regulating *ORAI1* and *STIM1* and increases cell proliferation in uveal melanoma [107]. Shaughnessy et al. proposed calcium channel blockade as a therapeutic strategy for malignant uveal melanoma [108].

1.5.5 Mesothelioma (MESO)

Exposure to asbestos causes an increase in mesothelioma incidence. Mesothelioma is a cancer that occurs in abdominal linings and thoracic [109]. If diagnosed and treated in the early stages, mesothelioma can be treated successfully [110]. The alterations in calcium channel activity or expression are characteristics of mesothelioma cells [111].

1.6 The Cancer Genome Atlas Program (TCGA)

TCGA is established by the National Cancer Institute and National Human Genome Research Institute of the National Institute of Health to explore human cancer and involved genomic changes systematically [112]. The project explores 33 human cancers for primary tumours and their matched normal samples, approximately 11,000 samples. TCGA data is publicly available through several platforms [113], [114]. One of the many advantages of the TCGA project is that it can provide detailed information on DNA, RNA, protein and epigenetic levels to understand differences and similarities across tumour types [115]. Large cohorts like TCGA enable the identification of known and novel genomic aberrations. It can also help in the identification of similarities and differences among tumour subtypes. For

example, *TP53*, a tumour suppressor, is important for malignancies with common transcriptional regulation mechanisms [116], [117]. However, the same alteration has different effects depending on the tissue. An example of such a case is that *NOTCH* is activated in liquid tumours but inactivated in some cell cancers of the HNSC, CESC, and SKCM [115].

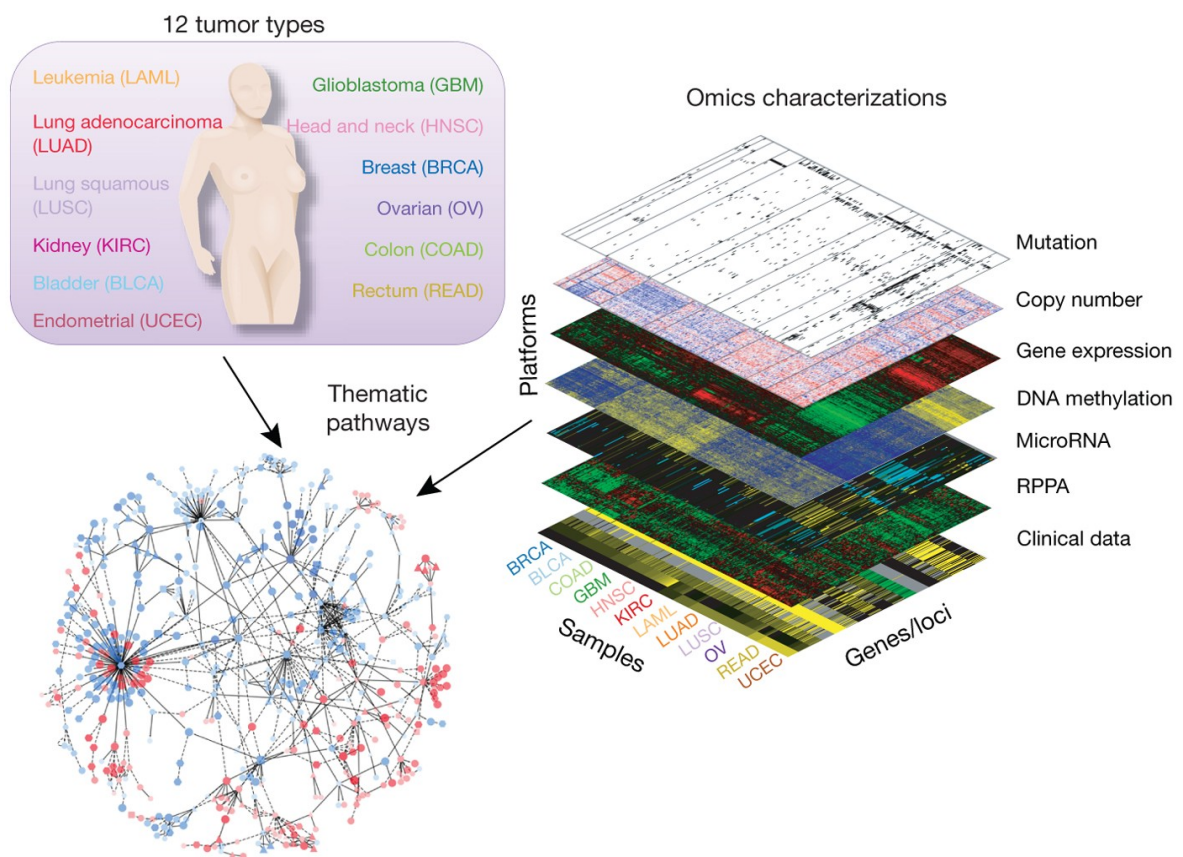


Figure 1.4. The TCGA Pan-Cancer project assembled data and a summary. (Adapted from [117])

1.7 R/Shiny

R is a programming language frequently used in data science, especially in bioinformatics and biological data analysis [119]. More than 1900 R packages are available through Bioconductor, making R one of bioinformatics' most commonly used environments [120]. R Shiny makes it possible to build web applications by exporting R codes into other web

development languages. R/Shiny can be utilized not only to build web applications but also large databases or servers that can be used by many users simultaneously. R is utilized more in building biological applications than programming languages like Java. Some popular biological web applications built in R include BoxPlotR [121], EndoDB [122], and CELLector [123] a visualization database and web server. Among different survival packages, SmulTCan emerges as an online multivariate R Shiny-based tool in the literature [124]. These applications allow users without requiring any coding skills from the users.

1.7.1 Structure of R/Shiny applications

R/Shiny applications, similar to other interactive web applications, consist of two parts, a graphical user interface (UI) for the users and a server for the calculations and processes [125]. The UI can be configured to change the appearance and layout and include various widgets to receive user inputs, parameter settings, file uploading etc. The outputs, such as figures and tables, can be displayed in the UI. User inputs and data are conveyed on the server side and processed by the server.

UI has different layouts, and the main panel can be split into several sections. Header and title panels are allocated for the header region of the Shiny app. The sidebar panel is used to collect inputs and set parameters. The main panel is where the outputs are rendered in the desired formats, such as plots, tables, texts, etc. Pre-built widgets are used to add functions like file input and download buttons. Developers can also add HTML tags to customize the widgets and UI. In the server, based on user inputs and data stored in the app, various calculations and operations are performed to produce outputs in the UI. The operations can be defined using the R functions and packages.

2 Aims and Rationale

Since the high-throughput data have become more available, prognostic studies have become more prevalent. Ozhan et al. showed the cancer-specific prognostic interactions of the Slit-Robo family [126]. However, paralogous genes and sense and antisense strands of a miRNA species have not been tested at the PANCAN level yet using multivariable Cox Regression and log-rank tests. Therefore, I focused on the paralog pairs and miRNA arms to study their cancer-specific prognostic significance across TCGA cancers. Moreover, cooperation of miRNAs in regulating synergistically their common targets can be predicted by algorithms such as miRCoop [57] however there needs to be in vitro studies for testing the most promising candidates in cancers such as HCC, which treatment options are limited. Therefore, in this thesis,

- I aimed to develop a Shiny web application that can allow users to perform multivariable survival analyses based on the expression of the paralogous gene pairs or two miRNA arms by:
 - Providing data exploration in the means of expression
 - Comparing hazard ratios of pairs of genes/miRNAs across TCGA cancers
 - Providing a gene-by-survival network visualization capability
- Furthermore, I aimed to generate a gene-cancer-by-survival network for the PM-ER gene set that extends the pairwise network in the Shiny app. This network approach can help the identification of hub genes and cancers and be integrated.
- Finally, I aimed to generate a gene-by-survival network for miRNA-mRNA triplets identified by the web tool developed based on miRCoop [57]. Then, I explored the effect of candidate miRNA transfections alone or in combination on cell viability as well as mRNA target expression in Hep3B cells.

3 Materials and Methods

3.1 Materials

3.1.1 Cell Culture Products and Reagents

Table 3-1. Cell growth & maintenance products

Product Name	Catalogue Number	Company
DMEM, low glucose	B-L0064-500	Biowest
Dulbecco's PBS	L0615	Biowest
FBS	S1400-500	Biowest
NEAA (100x)	B.X0557-100	Biowest
Pen/Strep	DE-17602E	Lonza
L-Glutamine	B.X0550-100	Biowest
Trypsin	BE-17161E	Lonza

Table 3-2. Viability assay reagents

Product Name	Catalogue Number	Company
MTT	M6494	Invitrogen
SDS	71725	Sigma Aldrich
HCl	7102	Sigma Aldrich

3.1.2 Transfection Reagents and Oligos

Table 3-3. miRNA mimics and PCR assays

Product Name	Description	Catalogue Number
miRCURY LNA hsa-miR-19a-3p mimic	miR-19a-3p mimic	339173 YM00472044-ADA

miRCURY LNA hsa-miR-20a-5p mimic	miR-20a-5p mimic	339173 YM00472205-ADA
miRCURY LNA hsa-miR-125-5p mimic	miR-125-5p mimic	339173 YM00473474-ADA
miRCURY LNA hsa-miR-203a-3p mimic	miR-203a-3p mimic	339173 YM00472239-ADA
miRCURY LNA hsa-miR-195-5p mimic	miR-195-5p mimic	339173 YM00472128-ADA
miRCURY LNA hsa-let-7c-5p mimic	let-7c-5p mimic	339173 YM00471696-ADA
miRCURY LNA hsa-miR-19-3p inhibitor	miR-19a-3p inhibitor	339131 YI04101300-DDA
miRCURY LNA has-miR-20a-5p inhibitor	miR-20a-5p inhibitor	339131 YI04100414-DDA
miRCURY LNA hsa-miR-125-5p inhibitor	miR-125-5p inhibitor	339131 YI04101665-DDA
miRCURY LNA miRNA mimic Negative Control 4	Mimic negative control	339173 YM00479903-ADA
miRCURY LNA miRNA inhibitor Negative Control B	Inhibitor negative control	339136 YI00199007-DDA
hsa-miR-19a-3p LNA PCR assay	miR-19a-3p primer mix	YP00205862
hsa-miR-20a-5p LNA PCR assay	miR-20a-5p primer mix	YP00204292

hsa-miR-203a-3p LNA PCR assay	miR-203a-3p primer mix	YP00205914
hsa-miR-125-5p LNA PCR assay	miR-125-5p primer mix	YP00204339
hsa-let-7c-5p LNA PCR assay	let-7c-5p primer mix	YP00204767
hsa-miR-195-5p LNA PCR assay	miR-195-5p primer mix	YP00205869
U6 snRNA (v2) PCR assay	U6 primer mix	YP02119464
HiPerfect	Transfection reagent	301705

3.1.3 RT-PCR Primers

Table 3-4. List of primer pairs

Name	Primer sequence (5'-3')
MYC	F: TGAGGAGACACCGCCCAC
	R: CAACATCGATTTCTTCCTCATCTT
SLC38A2	F: AGAACTGAAAGACCGCAGCC
	R: ACAGCCAGACGGACAATGAG
TPT1	F: GATCGCGGACGGGTTGT
	R: TTCAGCGGAGGCATTTC

Table 3-5. qRT-PCR related reagents

Product Name	Catalogue Number	Company
Water, molecular biology grade, nuclease-free	SH30538.01	HyClone

LightCycler 480 SYBR Green I Master	4887352001	Roche
LightCycler 480 Multiwell Plate 96, White	4729692001	Roche
RevertAid First Strand cDNA Synthesis Kit	K1622	Thermo Scientific
miScript II RT Kit	218160	Qiagen
miRNeasy Mini Kit	217004	Qiagen
miScript SYBR Green Kit	218073	Qiagen
QIAzol	79306	Qiagen

3.1.4 Laboratory Equipment

Table 3-6. Laboratory equipment

Name of the instrument	Procedure	Company
LightCycler 480 Instrument	Real-time PCR	Roche (Switzerland)
Synergy Multi-Mode Reader	MTT assay	BioTek (USA)
NanoDrop One	RNA quantification	Thermo (USA)
2720 Thermal Cycler	cDNA synthesis	Applied Biosystems (USA)

3.1.5 Solutions and Media Preparations

Table 3-7. Recipes for buffers and solutions

Solution	Contents	Total Volume
MTT	0.05 g MTT, 1 ml PBS	1 ml

SDS-HCl	1 g SDS, 10 ml 0.01 M HCl	10 ml
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Table 3-8. DMEM Complete Medium recipe

Reagent	Volume	Ratio
DMEM, low glucose	500 ml	
Fetal Bovine Serum (FBS)	50 ml	10%
Sodium pyruvate	10 ml	2%
L-glutamine	10 ml	2%
NEAA (100X)	5 ml	1%
Penicillin/Streptomycin	5 ml	1%

3.1.6 Packages

Table 3-9. List of R packages used in the analyses and Shiny app

Name	Reference
shinycustomloader	Tanaka et al., 2018
S4Vectors	Pagès et al., 2022
Biobase	Huber et al., 2015
BiocGenerics	Huber et al., 2015
survminer	Kassambara et al., 2021
ggpubr	Kassambara A., 2020
survival	Therneau T., 2022
tidyr	Wickham & Girlich, 2022
corrplot	Wei & Simko, 2021
visNetwork	Almende et al., 2021

DT	Xie et al., 2022
plotly	Sievert C., 2020
ggplot2	Wickham H, 2016
data.table	Dowle & Srinivasan, 2021
bslib	Sievert & Cheng, 2022
shinycssloaders	Sali & Attali, 2020
shinyjs	Attali D., 2021
shinyhelper	Mason-Thom C., 2019
shinyWidgets	Perrier et al., 2022
shinyBS	Bailey E., 2022
shinydashboard	Chang & Borges, 2021
shinythemes	Chang W., 2021
shiny	Chang et al., 2022
riskRegression	Gerds & Kattan, 2021
UCSCXenaTools	Wang et al., 2019

3.2 In vitro methods

3.2.1 Cell Culture Maintenance and Handling

3.2.1.1 Thawing Cells

Nitrogen tanks were used to maintain frozen cell stocks and cells were thawed before the cell culture experiments using water bath. The rest of the cells were thawed by mixing with complete fresh media prepared according to **Table 3-8**. The cell mixture containing the freezing media was spined down to remove the DMSO. The cell pellet was then resuspended in the fresh media and transferred to new cell culture plates, maintained in the incubator at 37°C at 5% CO₂.

3.2.1.2 Passaging Cells

When cells become confluent, they were transferred into a new flask to continue the cell lines. First, the old medium was removed, and cells were washed with PBS to remove any residue. Cells were detached using Trypsin-EDTA (0.25%) by incubating at 37°C 5% CO₂ for 3-5 minutes. Trypsin was deactivated by adding fresh complete DMEM and mixing. Detached cells were transferred to a new flask with suitable ratios depending on the cell line.

3.2.1.3 Freezing Cells

Cryopreservation was performed to maintain cell stocks. Trypsin was used to detached adherent cells and centrifuged for 5 minutes at 1500 rpm. The cell pellets were mixed with freezing media and transferred into cryovials. Cryovials were transferred to the freezing container, and transferred to nitrogen tanks.

3.2.1.4 miRNA mimic transfections

One day before the treatment, cells were seeded to new plates (6-well or 96-well). Trypsin was used to detached adherent cells, and after the deactivation of trypsin, the cells were centrifuged. Cell pellets were resuspended in fresh media and counted using a haemocytometer. Cells were seeded to 6- or 96-well plates. Transfection reagent concentrations for each plate were optimized for the experiments. For 6-well plates, 6 µL of HiPerfect and, for 96-well plates, 0.6 µL HiPerfect transfection reagent was used. To optimize the transfection conditions and determine the minimum required mimic concentration manufacturer's recommendations were followed. It has been recommended to use 0.05-5nM final concentrations of miRNA mimics for potent activity, and the miRNA mimic effects can be assessed 24-72 hours after transfection.

For biological replicates, a single transfection mixture was prepared. Transfection reagent and miRNA mimic/inhibitors were mixed through gentle pipetting. After a quick spin down, the transfection mixture was incubated for at least ten minutes for transfection complexes

to form. Old media from the wells were removed and replaced with new media. After the incubation, the transfection mixture was distributed dropwise onto the cells and mixed by swirling for equal distribution. After 24- or 48-, hour incubation, cells were used for the following experimental procedures, i.e., RNA isolation or MTT. For RNA isolation and MTT, duplicates and five replicates were used, respectively.

3.2.2 mRNA-miRNA Expression Analyses

3.2.2.1 Total RNA isolation

For the RNA experiments, cell pellets were collected from the 6-well plates. Cell pellets were used to obtain lysates and they were collected with PBS and transferred to liquid nitrogen for instant freezing and stored at -80°C until RNA extraction. 700 µl QIAzol and 140 µl chloroform were added to cell pellets, and isolation was performed following the manufacturer's instructions. In the final step, RNA was eluted in 30 µl nuclease-free water. RNA concentrations and their quality were measured using NanoDrop.

3.2.2.2 cDNA synthesis from mRNA

The RevertAid cDNA Synthesis Kit (Thermo Fisher, USA) was used to synthesise mRNA from total RNA. 800 ng RNA was used for the cDNA synthesis and synthesized accordingly. 800 ng of RNA was suspended, and a final volume of 11 µl with nuclease-free water and 1 µl oligo(dT) was added to each sample. 4 µl 5X Reaction buffer, 2 µl dNTP mix and 1 µl of RT and RiboLock RNase inhibitor were added for each sample to the master mix. The mixture was incubated at an hour at 42°C, and the mixture was then incubated for 5 minutes at 70°C. cDNA samples were diluted at 1:20 for qPCR synthesised and stored at -20°C.

3.2.2.3 cDNA synthesis from miRNA

For miRNA mimic treated samples, cDNA was synthesized using the miScript II RT KIT (Qiagen, Germany). The reaction mixture was incubated for an hour minutes at 37°C, and

incubated at 95°C for 5 minutes after the first step to inactivate the enzymes. For qPCR experiments, the cDNAs were diluted at 1:60 and stored at -20°C.

3.2.2.4 mRNA qRT-PCRs

The master mix for the mRNA qPCR consisted of the following 5 µl SYBR Green, 1 µl forward primer, 1 µl reverse primer, and 1 µl nuclease-free water. 8 µl master mix was distributed to qPCR plates, and 2 µl of 1:20 diluted cDNA was added. qPCR 96-well plates were then briefly centrifuged for 2 minutes at 1500 rpm. The reaction was conducted at LightCycler 96 qPCR instrument with the appropriate annealing temperature of the primer pairs for 50 cycles with the following conditions in **Table 3-10**. TPT1 gene was used as a housekeeping gene for mRNA qPCRs to normalize gene expression.

Table 3-10. Cycling conditions for mRNA qPCRs

Step	Time (mm:ss)	Temperature	Number of Cycles
Pre-incubation	05:00	95°C	1
3-Step Amplification	00:10	95°C	50
	00:20	Primer-dependent (58-62°C)	
	00:20	72°C	
Melting	00:05	95°C	1
	01:00	55°C	
	Continuous Acquisition per 5C	95°C	
Cooling	00:30	40°C	1

3.2.2.5 miRNA qRT-PCRs

miRCURY PCR assay primers and QuantiTect SYBR Green were used for the miRNA qPCRs. First, the master mix was prepared with the recipe per sample of 5 μ l SYBR Green, 1 μ l primer mix, and 1 μ l nuclease-free water. 7 μ l master mix was distributed to each well, and 3 μ l of 1:60 diluted cDNA was added to the wells. After 2 minutes of centrifugation at 1500 rpm, the qPCR reaction is performed at LightCycler 96 qPCR instrument with conditions. snora66 and U6 sRNA were used as a reference.

Table 3-11. Cycling conditions for miRNA qPCR

Step	Time (mm:ss)	Temperature	Number of Cycles
Pre-incubation	15:00	95°C	1
3-Step Amplification	00:15	94°C	45
	00:30	55°C	
	00:30	70°C	
Melting	00:05	95°C	1
	01:00	55°C	
	Continuous Acquisition per 5°C	95°C	
Cooling	00:30	40°C	1

$\Delta\Delta$ CT method was used to analyse the relative gene/miRNA expression. The mean for each technical replicate was calculated and normalized by the control group's average. Gene and miRNA expressions were normalized with the reference gene TPT1 and U6 for mRNA and miRNA, respectively. Log2-transformed values were used for statistical analyses.

3.2.3 MTT Viability Assay

Initially, the number of cells seeded to 96-well plates for MTT assays was selected as 2000 or 5000 cells per well. For 2000 cells/well, the absorbance values were low. As fewer cells can cause background high absorbance values, 5000 cells/well were later used. After miRNA mimic treatments, the MTT-PBS solution was mixed with phenol-free DMEM. The MTT-media mixture was distributed 110 µl per well on the cells and incubated at 37°C, 5% CO₂ for 4 hours. After 4 hours, 100 µl of SDS HCl solution was added to each well, and after 12-18 hours, the Synergy Multi-Mode reader was used to obtain MTT readings at 570 nm.

3.3 In silico methods

3.3.1 Data Acquisition

The miRNA and mRNA expression files that were PANCAN normalized and log2-transformed were obtained from UCSC Xena (<https://xenabrowser.net>). The study TCGA – PANCAN was selected, and only the primary tumour samples with survival data were used. The survival data for each cancer type were obtained using the UCSCXenaTools package. Due to a lack of information on survival, LAML (Acute Myeloid Leukaemia) was removed from the analyses. Also, the GBM (Glioblastoma) dataset does not have expression information for miRNA analyses; thus, it was excluded from the miRNA analyses parts of the app.

3.3.2 Shiny Pair-HR Application Design

Pair-HR is designed as an app, developed using R Shiny, uses miRNA arm pairs' and mRNA paralog pairs' expression data along with survival data and calculates hazard ratios of each pair member using multivariable cox regression function *coxph()* from the survival R

package. Paralogous pair ID data were obtained from a study by Dandage and Landry [127], while miRNA arm ID information and data were obtained from the publicly available database miRBase [128]. The Pair-HR app consists of several tabs with different functionalities. These include *Expression*, *K-M plots*, and *multiHRs*.

3.3.2.1 Tab 1. *Expression*

The *Expression* tab consists of three sub-tabs for exploring mRNA/miRNA expressions. In the first expression tab, two inputs for the pair members are taken along with the cancer type abbreviation. The users can pick sample types for the scatter plot using the check boxes given in the sidebar panel for primary tumour and solid tissue normal samples. The scatter plot is drawn using the interactive *plot_ly()* function from the plotly package. The point sizes indicate the Overall Survival (OS) time in months for each patient, whereas the colour indicates the sample type. Built-in functions of the plotly package can be used to save plots and zoom in/out. Also, users can switch the x- and y-axes of the plot by checking the checkbox for “Do you want to swap axes?” for plotting purposes.

In the second sub-tab, the boxplots for the selected pair’s expression are drawn using the Plotly package. When hovered on the boxes, the plot gives detailed information about the median, quartiles, and maximum and minimum values.

There is a correlation plot in the last tab under the *Expression* module. Pearson correlation values between the pairs are calculated and plotted using the *corrplot()* function with the following parameters, `method = number`, and `type = lower`.

3.3.2.2 Tab 2. *K-M plots*

Under the *K-M plots* tab, users can plot different Kaplan-Meier plots with different parameters. Users can select between univariate and multivariate methods for the selected pair within the selected cancer. Individual KM plots for the pair members can be plotted,

along with the arithmetic means of the pairs or the total expression of the pairs. Under the multivariate tab, multivariate cox proportional hazards are calculated for the pair, and the K-M plot is generated accordingly using the *ggsurvplot()* function from the survival package. Users can select two different stratification methods (median and quartiles) for the K-M plots.

3.3.2.3 Tab 3. *multiHRs*

The *multiHRs* tab gives the results for the multivariate Cox proportional hazard ratios for each pair in 32 cancers for Overall Survival (OS) and Disease-Specific Survival (DSS). In the first tab, the user can select a pair of interest, and under the dropdown menu, multiple cancers can be selected. Using the DT package data table, the first tab will give a table output for the pre-calculated hazard ratios (HRs). HR values with significant p-values are given with white background, whereas not significant ($p\text{-value} > 0.05$) HRs with a grey background can be seen. Also, for significant p-values, a bold font type is used to differentiate between hazardous ($HR > 1$) and protective ($HR < 1$) values, pink and blue colours are used, respectively, and all HR values are log2-transformed for ease of interpretation.

HR values of the samples can also be visualized under the *HR plot* tab as a scatter plot. Log2-transformed HR values are plotted using the interactive *plot_ly* function from the plotly package. Different colours are used for different cancers. Hovering on each point will give extra information about the hazard ratios of the pair and their relative p-values. A green and red background for the plotting area is used to distinguish points where both HRs are protective and hazardous. Default package options for the saving lasso/box selection are available. A second tab offers alternative plotting options for the HRs. Users can select 2D or 3D plotting options. 2D plotting options include Cancer-Expression, HR-Expression, and Cancer-HR for the selected pair. For Cancer-Expression and Cancer-HR plots, different

colours and shapes indicate different members of the pair. For the Expression-HR plot, different shapes indicate genes, and colour indicates different cancers. The 3D plot option generates a Cancer-HR-Expression plot of the pair. However, due to the limitations of the package, selecting more than eight cancers is not recommended in this option.

Lastly, the *HR network* tab generates a gene-cancer-survival network for the pair of interests. Each orange node indicates gene/miRNAs, whereas each pink node indicates cancer. The edge colours indicate the HR values for that cancer. If $\log_2(\text{HR}) > 0$, the edges are selected as red and dashed and if $\log_2(\text{HR}) < 0$, the edges are green and smooth. *visNetwork()* function from the visNetwork package is used for gene-cancer-survival networks.

3.3.3 Multivariable Cox regression

PANCAN normalized expression values for genes were obtained from UCSC Xena (<https://xenabrowser.net>) for LIHC. Each patient's survival data were obtained using the “UCSCXenaTools” package. Only the primary tumour samples with matched survival data were used in the analyses. The survival package function *coxph()* was used for multivariable Cox regression analyses.

3.3.4 K-M plots

Kaplan-Meier survival curves are generated using the *ggsurvplot()* function from the *survminer* package. For miRCoop triplets, the LIHC patients were divided into three groups based on the expression. miRNAs with “Up” (expression > median) expressions and their mRNA target with “Down” (expression < median) expression were assigned to the “Up-Up-Down” group. miRNAs with “Down” expressions and their mRNA target with “Up” expressions were assigned to the “Down-Down-Up” group, and the rest of the patients were assigned to “Rest”.

3.3.5 Gene-cancer-survival networks

Gene-cancer-survival networks were generated using Cytoscape (v.3.8.2) platform [129]. The network is narrowed down for genes with significant ($p\text{-value} < 0.05$) HR values for the PM-ER gene set. The node size represents the AUC values calculated by the riskRegression R package. Edge weights represent the $\log_2(\text{HR})$, and edge colours represent the HR value (red: $\text{HR} > 0$ and green: $\text{HR} < 0$).

Markov clustering function of Cytoscape was used to prune the original PM-ER network with the following parameters granularity parameter = 2.5, array source = $\log_2(\text{HR})$.

For miRCoop triplets, dashed edges were used for $p\text{-values} > 0.05$, whereas solid lines were used for $p\text{-values} < 0.05$. Diamond and rectangle shape nodes were used for mRNAs and miRNAs, respectively. Blue nodes represent a significant downregulation, and red nodes represent a significant upregulation in primary tumour samples compared to solid tissue normal. Each triplet member was connected to a dummy node.

3.3.6 Protein-protein interaction network of PM-ER gene set

For the PM-ER gene set, protein-protein interaction information has been obtained from the STRING database using the experimental evidence category only [130]. PPI-network was generated on Cytoscape, and edge weights represent the interaction values between genes.

4 Results

4.1 Pair-HR Application

4.1.1 Expression Tab

The screenshot from the “Scatter Plot” subtab of the “Expression” tab of Pair-HR is given in **Figure 4.1** and **Figure 4.2**. Three sub-tabs of this module can be accessed by clicking the titles in the main panel. For exemplary purposes, paralog genes *MYC* and *MYCN* were selected. Their expressions in Breast Invasive Carcinoma (BRCA) are plotted using the *plot_ly()* function with appropriate legends showing the sample types and regression lines. The samples included in the scatter plot can be selected using the checkboxes in the sidebar panel. If desired, users can change the plot’s x- and y- axes for regression purposes (**Figure 4.1**). To get the coordinates of a point, users can simply hover over the points, showing the x and y values as well as Overall Survival in months. Under the *Expression* tab, users can select the second module, which shows miRNA expression, from the dropdown menu. Users can select arm-1 and arm-2 for the miRNAs from the sidebar panel and plot the scatter plot similar to the mRNA expression plots (**Figure 4.2**).

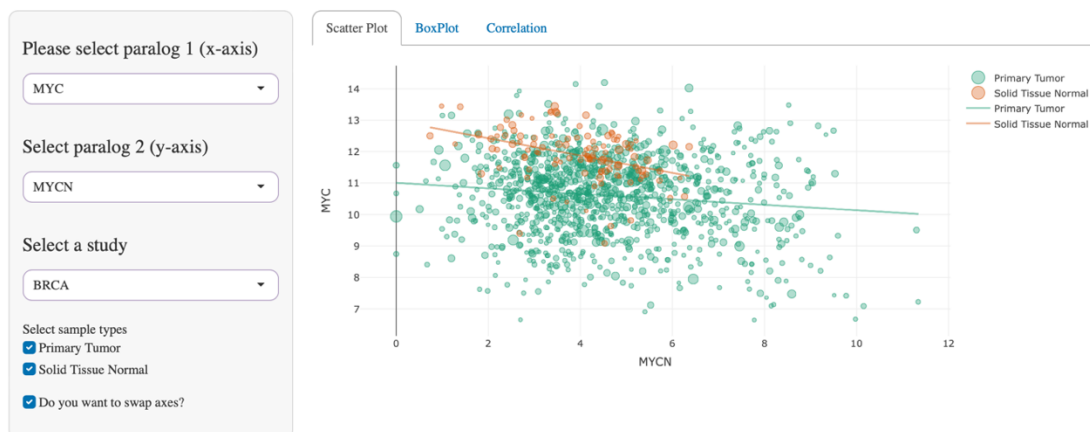


Figure 4.1. A screenshot from the “Scatter Plot” subtab of the “Expression” tab for mRNAs. Green dots indicate Primary Tumour samples, whereas orange dots indicate Solid Tissue Normal samples. Sizes for each point indicate Overall Survival in months.

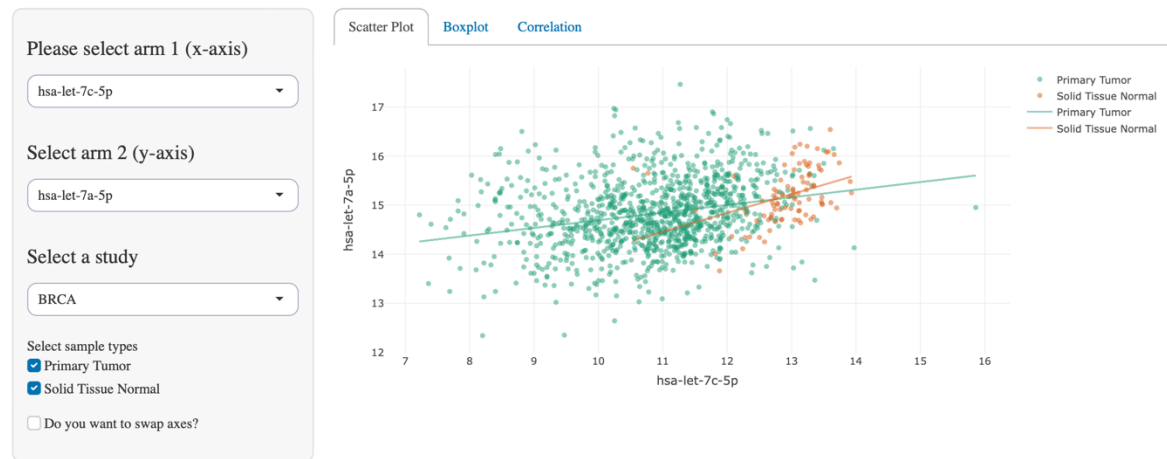


Figure 4.2. A screenshot from the “Scatter Plot” subtab of the “Expression” tab for miRNAs. Green dots indicate Primary Tumour samples, whereas orange dots indicate Solid Tissue Normal samples. Sizes for each point indicate Overall Survival in months.

The second part of the *Expression* tab is *BoxPlot*. Users can generate boxplots for their input gene pair by clicking on the *BoxPlot* tab, as shown in **Figure 4.3**. Like the *Scatter Plot* tab, users can select which sample types to include in the boxplot. The interactive boxplot gives important summary information for the genes of interest. Users can click on the boxplots to obtain the following information; minimum-maximum, median, q1-q3, upper and lower fence (**Figure 4.3**). Also, using the default *plotly* functions, users can zoom in and out on the plot. By clicking on the camera icon from the top right corner, users can save the plot to their local directories (**Figure 4.3**). The BoxPlot is also available for the miRNA arms under the miRNA Expression tab (**Figure 4.4**).

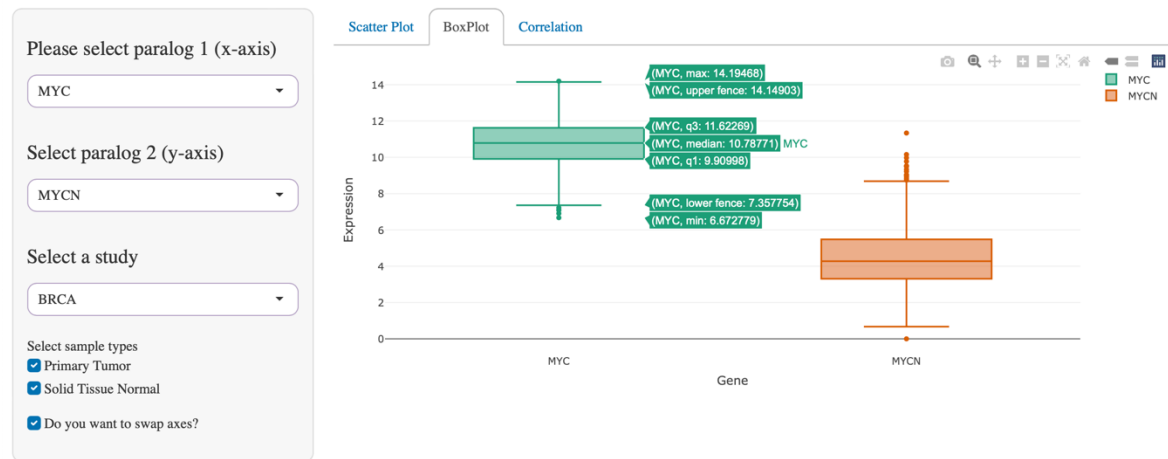


Figure 4.3. A screenshot from the “BoxPlot” subtab of the “Expression” tab for mRNAs.



Figure 4.4. A screenshot from the “BoxPlot” subtab of the “Expression” tab for miRNAs.

The last part of the *Expression* tab is “Correlation”. This tab calculates the Pearson correlation coefficients of two genes or miRNAs in the selected cancer for the selected sample types. The correlation is calculated using the *cor()* base function, and the plot is generated using the *corrplot()* function from the package with the same name. The *corrplot()* method is selected as “number”, and the type is selected as “lower” for the lower triangle plot with correlation coefficients. The correlation plot can be downloaded by clicking the download button at the bottom of the screen. Examples of correlation plots for mRNAs and miRNAs are given in **Figure 4.5-Figure 4.6**.

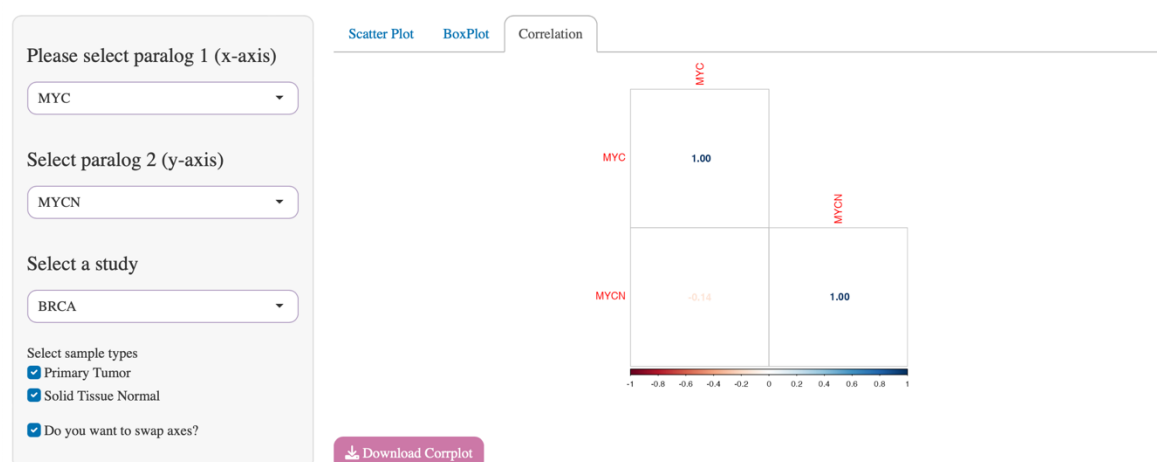


Figure 4.5. A screenshot from the “Correlation” subtab of the “Expression” tab for mRNAs.

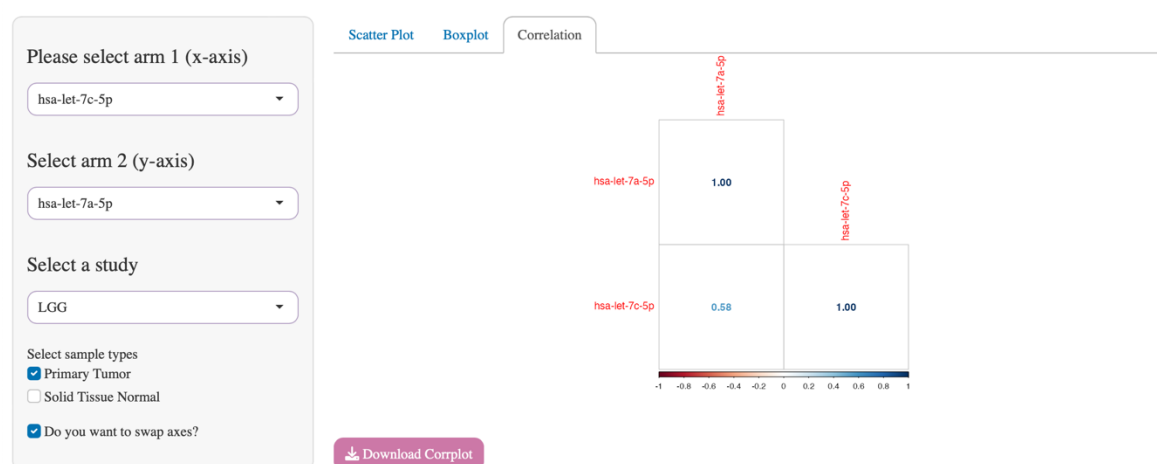


Figure 4.6. A screenshot from the “Correlation” subtab of the “Expression” tab for miRNAs.

4.1.2 K-M plots tab

K-M plots tab has two sub-tabs for the univariate and multivariate Cox proportional hazards model. The main output of this tab is K-M plots with different stratification methods. Under the *Univariate* Tab, users can plot individual K-M plots of the selected gene pair. Gene expression strata can be selected either as Median or Quartiles. If the expression is higher than the median, it is labelled as high, and if the expression is lower than the median, it is labelled as low. It is also possible to select the paralog pair’s total or average expression as the variate for univariate K-M plots. If the Quartiles option is selected as the stratification,

gene expression of the samples is labelled for the first quartile, fourth quartile and between the first and fourth quartile.

An example of the individual K-M plots with median stratification is given in **Figure 4.7** for *CREB3-CREB3L4* and *FGR-SRC* pairs in LGG. The median overall survival for the former pair of paralogs showed differences between high and low-expression groups, suggesting a better survival for High Expression groups (**Figure 4.7A**). Contrary, lower expression predicted better survival for *FGR*, and higher expression predicted better survival for *SRC* (**Figure 4.7B**). The log-rank tests for the difference in both paralogs give a p-value of 0.029 and <0.0001, indicating that the expression groups differ significantly in lower-grade glioma patients (**Figure 4.7A**). The samples can be divided into different groups based on quartiles. Patients that fall into the first quartile are assigned to the “≤25%” group, whereas patients that fall into the upper quartile are assigned to the “≥75%” group (**Figure 4.8**). For *CREB3*, patients between the ≥25% - ≤75% showed better survival, whereas, for *CREB3L4*, upper quartile patients showed better overall survival (**Figure 4.8**).

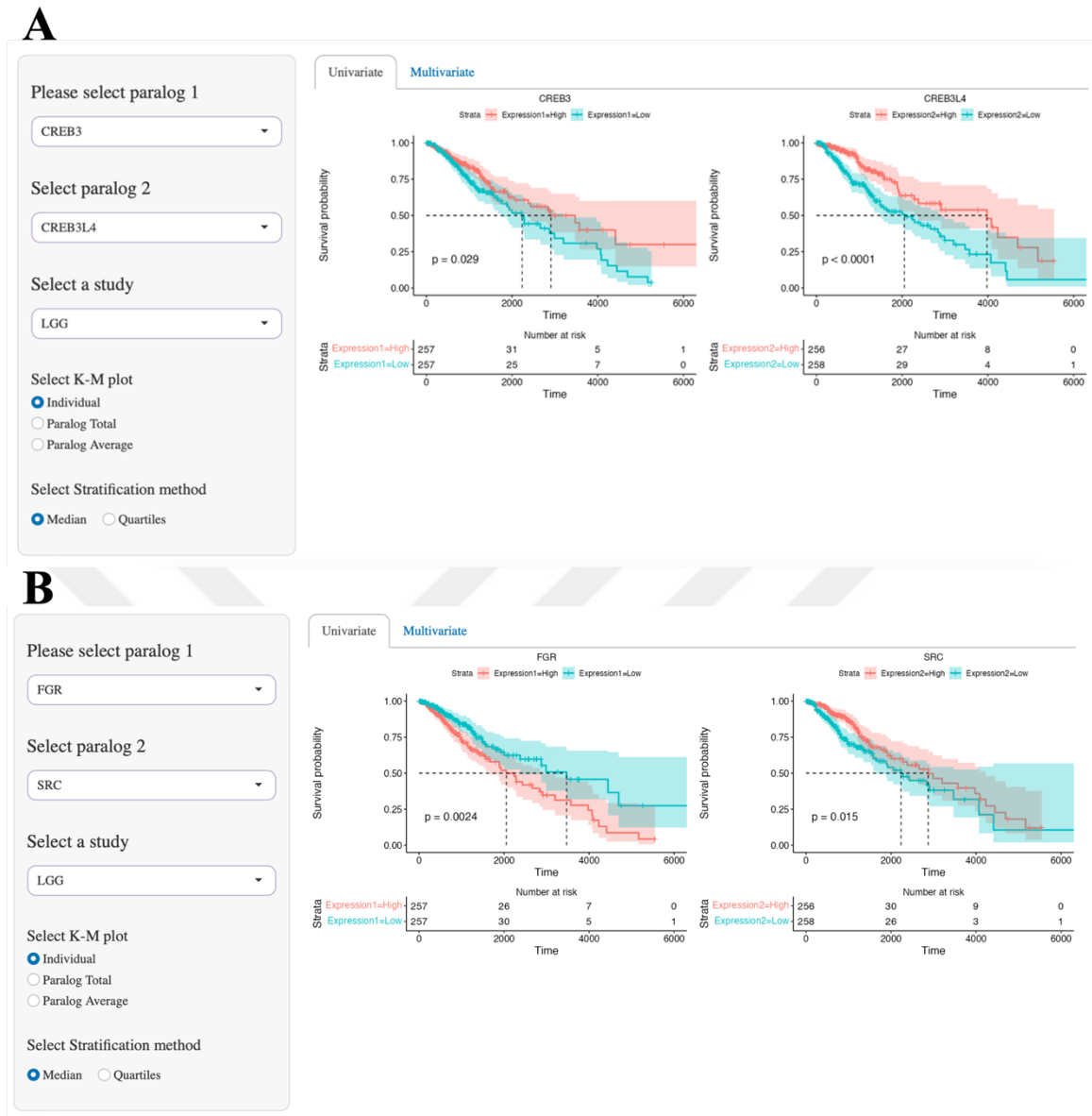


Figure 4.7. A screenshot from the “Univariate” subtab of the “K-M plots” tab for mRNAs. The stratification is selected as the median. (A) CREB3-CREB3L4 (B) FGR-SRC.

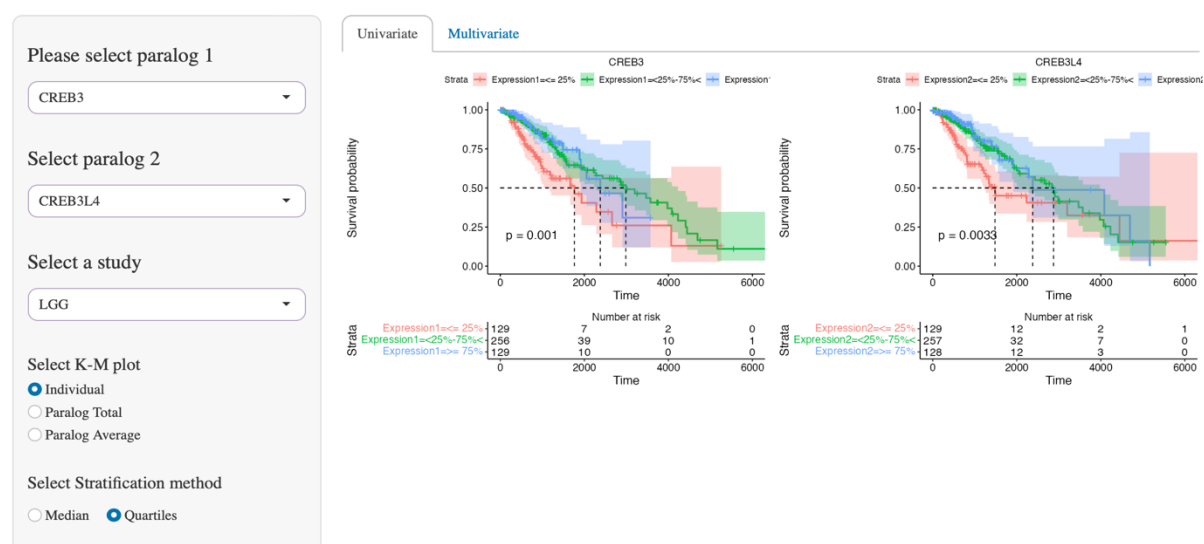


Figure 4.8. A screenshot from the “Univariate” subtab of the “K-M plots” tab for mRNAs. The stratification is selected as quartiles.

Paralog Total and Paralog Average K-M plots for *CREB3* and *CREB3L4* in LGG are also given as examples in **Figure 4.9** and **Figure 4.10**, respectively.

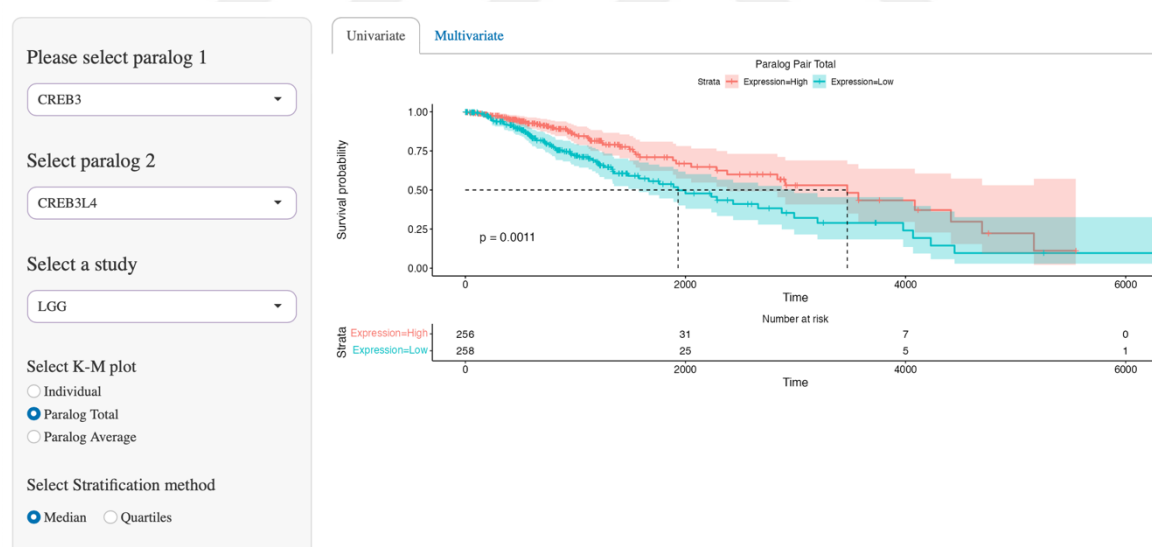


Figure 4.9. A screenshot of the Paralog Pair Total K-M plot. The stratification is selected as the median.

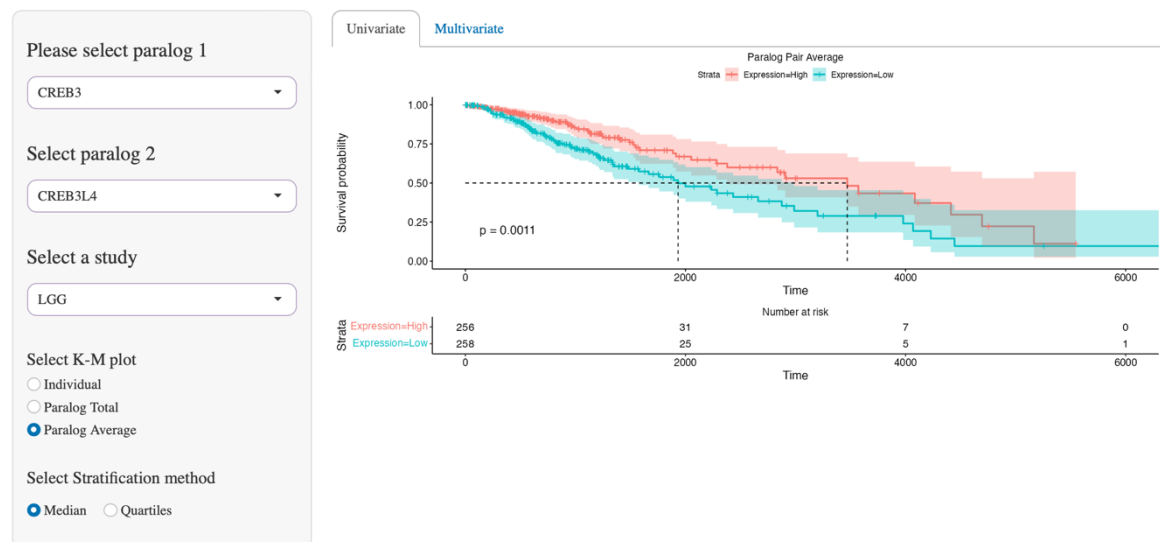


Figure 4.10. A screenshot of the Paralog Pair Average K-M plot. The stratification is selected as the median.

The “Multivariate” tab can be used to perform multivariate Cox regression for the gene/miRNA pair. Log-rank tests and K-M plots can be calculated for the categorical variables. Both variables’ median or quartile expressions are used to assign patients to different groups. An example of the multivariate K-M plot is given in **Figure 4.11**.

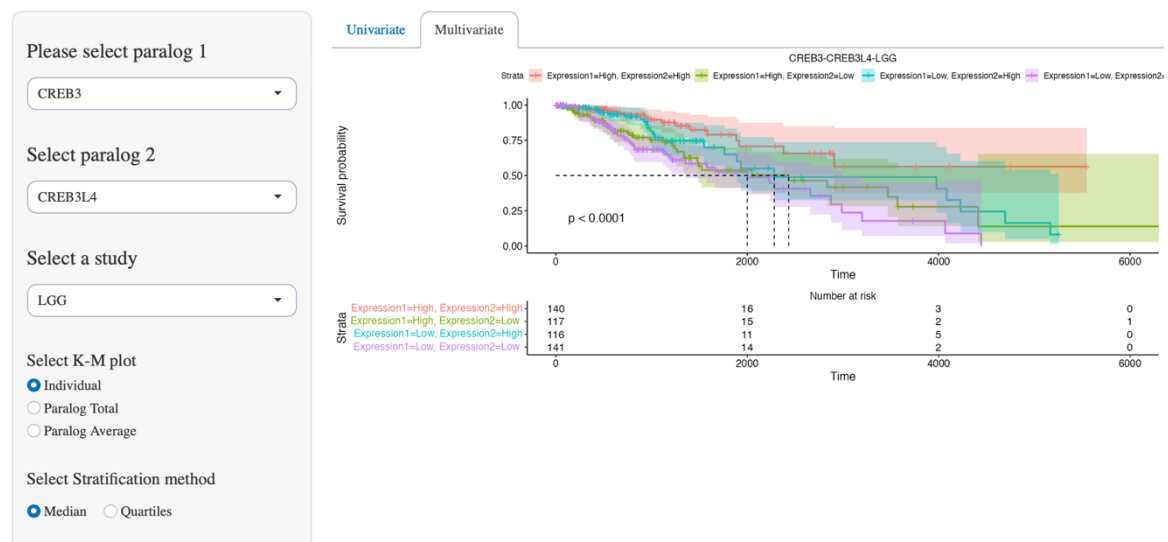


Figure 4.11. A screenshot of the “Multivariate” subtab of the “K-M plots” tab. The stratification method is selected as the median.

Similarly, miRNA K-M plots can be generated for different arms of the miRNAs by selecting the miRNA K-M plots from the dropdown menu. Individual K-M plots of hsa-

miR-200a-3p and has-miR-200a-5p and the pair average and total K-M plots are given in

Figure 4.12-Figure 4.13-Figure 4.14. For miR-200a-3p, higher expression predicted

better overall survival in bladder carcinoma with a p-value of 0.042 (**Figure 4.12A**).

However, higher expression of miR-200a-3p and that of miR-200a-5p predicted a worse

prognosis (**Figure 4.12B**) Multivariate K-M plot of the miRNA pair is also available under

the “Multivariate” tab (**Figure 4.15**).

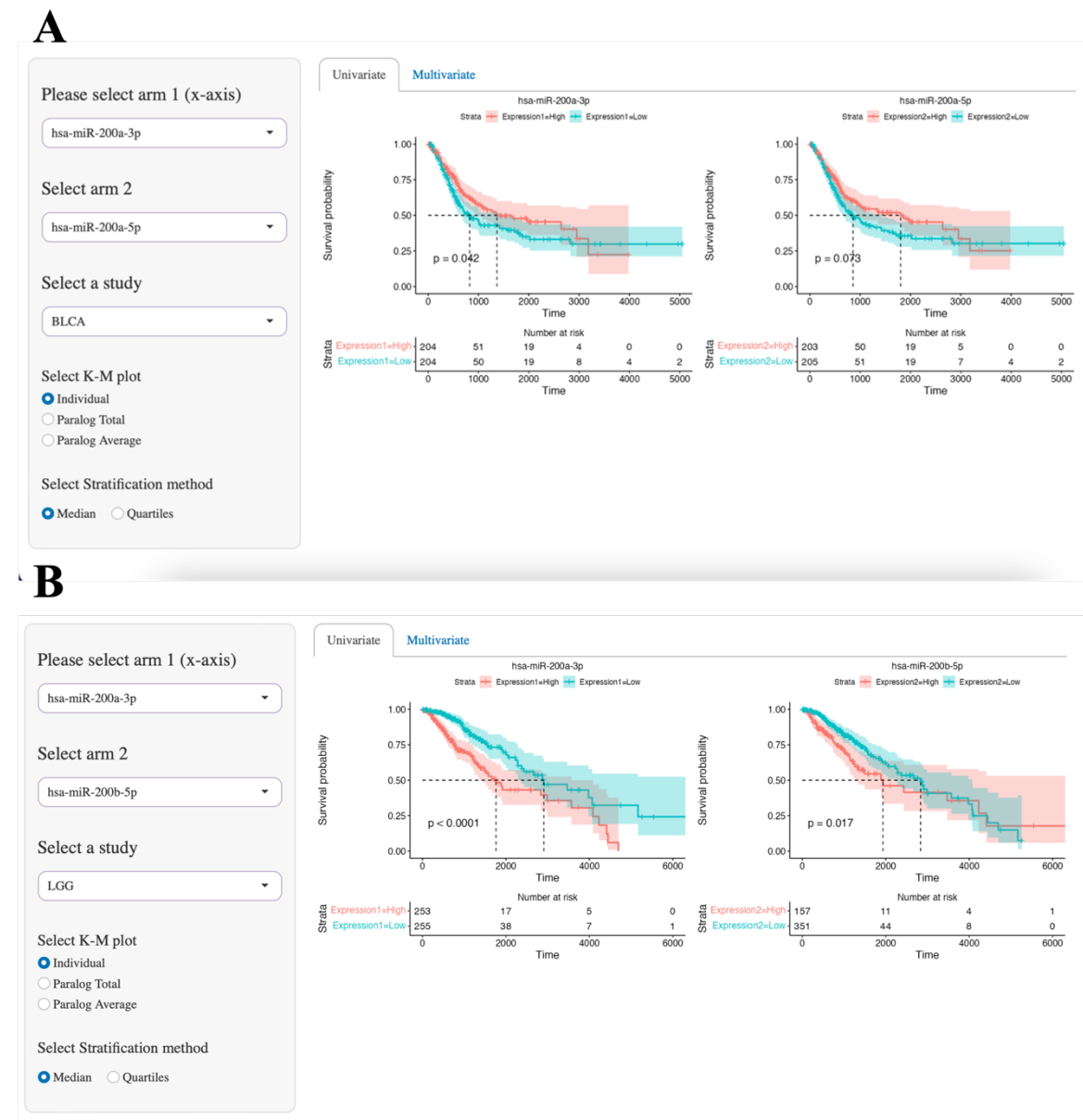


Figure 4.12. A screenshot of the “Univariate” subtab of the “K-M plots” tab for miRNAs for (A) BLCA and (B) LGG.

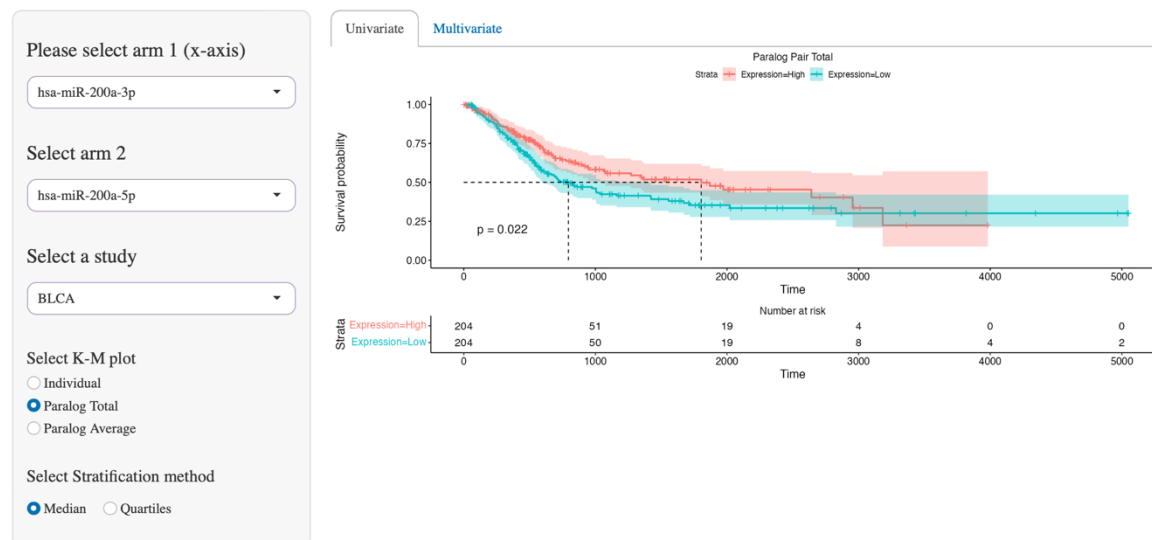


Figure 4.13. A screenshot of the miRNA pair total K-M plot from the “K-M plots” tab for miRNAs.

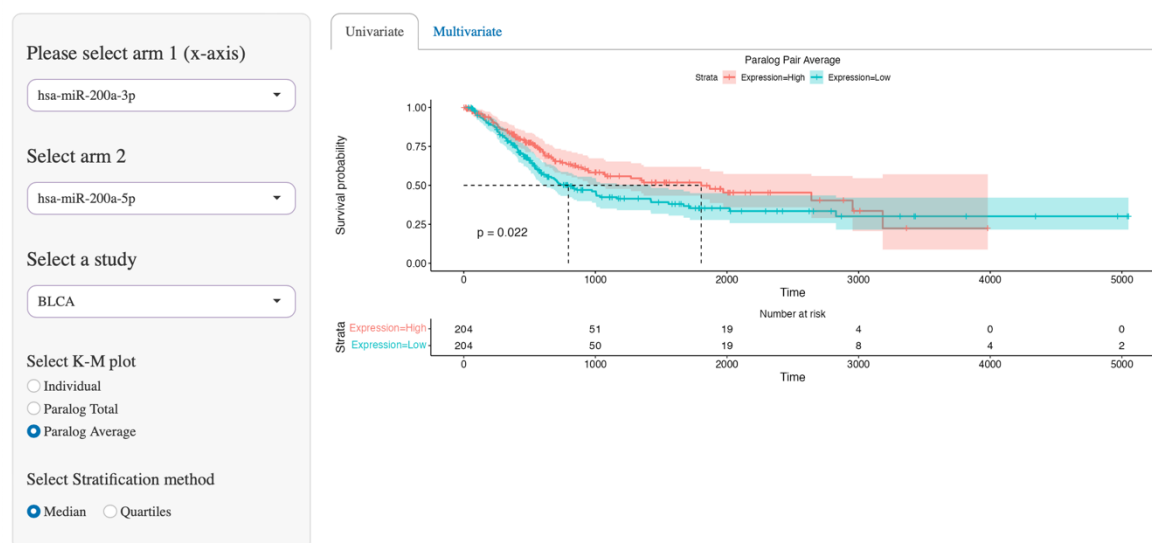


Figure 4.14. A screenshot of the miRNA pair average K-M plot from the “K-M plots” tab for miRNAs.

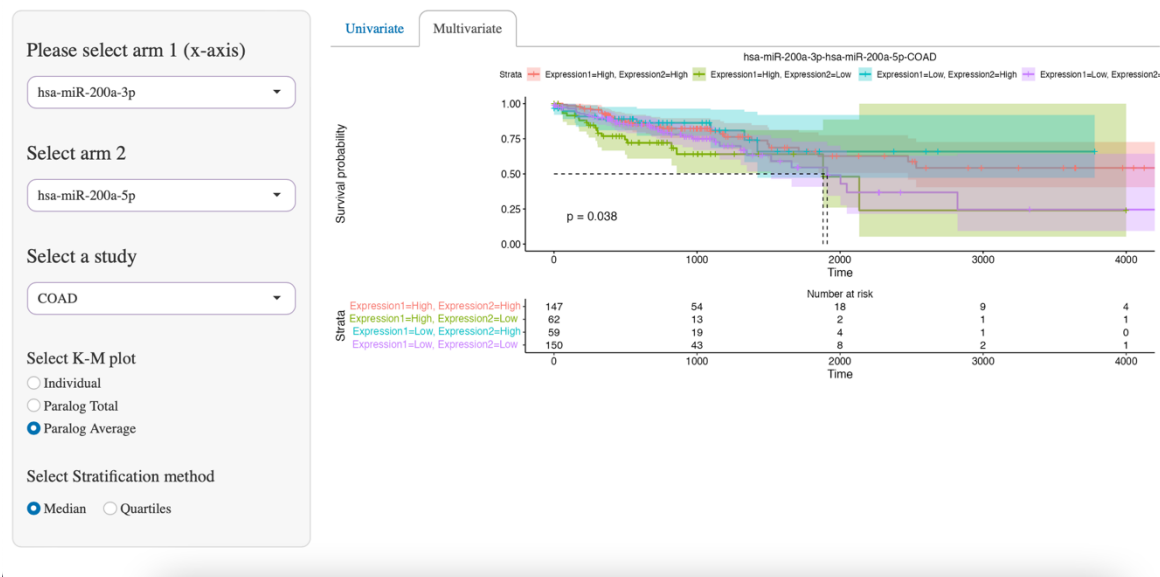


Figure 4.15. A screenshot of the “Multivariate” subtab of the “K-M plots” tab for miRNAs. The stratification method is selected as the median.

4.1.3 *multiHRs* tab

multiHRs tab is the main module of the Pair-HR app. Users can obtain pre-calculated HR values from the multivariate Cox models for each pair in 32 and 31 cancers for mRNAs and miRNAs, respectively. The *multiHRs* tab has several sub-tabs as well as different survival types. From the sidebar panel, users can select their pair of interests and multiple studies to obtain a data table and several interactive plots and networks. The first tab, HR data, is a table that shows the HR values of the pairs, their p-values and the expression correlation coefficient. The HR Data table is customized to give information about the HR values. By default, the background colour of the rows for HRs and p-values is grey. For significant HRs ($p\text{-value} < 0.05$), the font is selected as bold, and the background colour is switched to white. Log₂-transformed HR values with significant p-values are labelled with different font colours. If $\log_2(\text{HR}) > 0$, the variate is considered hazardous in that cancer and thus labelled red. However, if $\log_2(\text{HR}) < 0$, the variate is considered protective in cancer and labelled blue. The table is interactable and searchable with a search box on top. Also, users can filter

by using sliders for numeric columns. An example HR table for *EIF5A* and *EIF5AL1* genes is given in **Figure 4.16** for seven cancers.

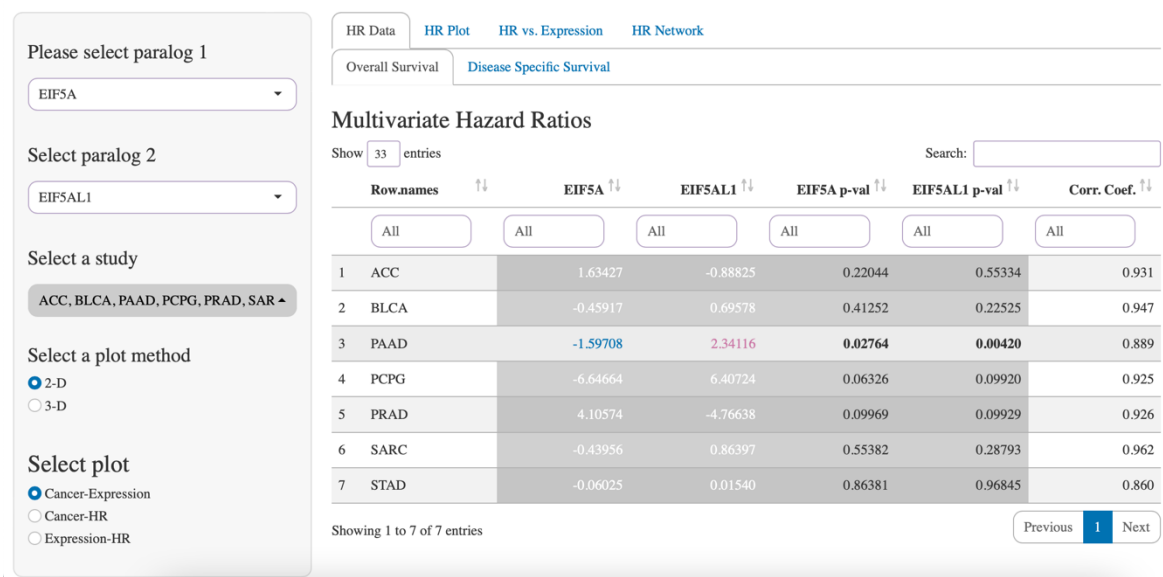


Figure 4.16. A screenshot of the “HR Data” subtab from the “multiHRs” tab for the log₂-transformed OS HRs for selected TCGA studies. The background is set as grey for HR values that are not significant. Significant HR p-values are written in bold. Log₂(HR) > 0 and significant are indicated as pink, whereas log₂(HR) < 0 and significant values are indicated as blue. Besides HRs and their relative p-values, users can also see correlation coefficients of expressions of the genes.

The second sub-tab of the multiHR tab is an interactive HR plot generated using the *plot_ly()* function. Log₂-transformed HR values of the pair are plotted across x- and y- axes for the selected studies. The background area for the plots is green and red for HR values that are both protective and hazardous in the plot (**Figure 4.17**). The sizes of the dots show the absolute value of the correlation coefficient for expression. Each cancer is indicated in different colours. For 32 TCGA cancers, even though the expressions are positively correlated, *EIF5A* and *EIF5AL1* showed a negative correlation across all HRs obtained for OS (**Figure 4.17**).

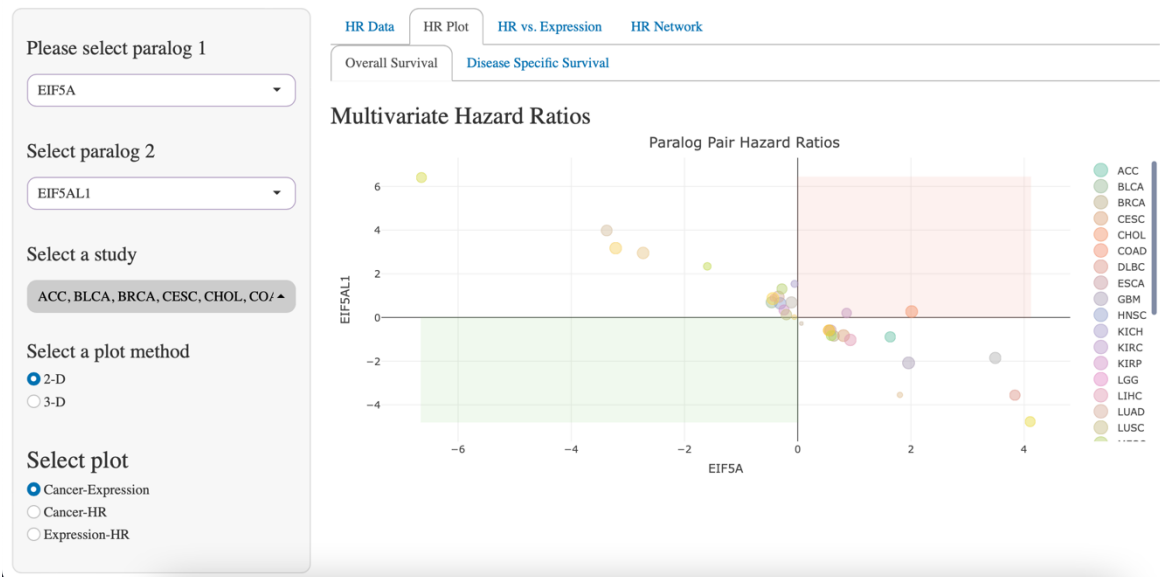


Figure 4.17. A screenshot of the “HR Plot” subtab from the “multiHRs” tab. The sizes of each point indicate the absolute correlation coefficients of the pair. The x-axis shows the log2-transformed HR values for EIF5A, while y-axis shows the log2-transformed HR values of EIF5AL1. The point sizes indicate the correlations coefficients of the expressions.

The third section of the multiHRs tab offers several plots for expression means, HRs and cancer types. Users can select 2-D or 3-D options for their plots. Users can select among three plots if the 2-D plot is selected. First, the Cancer vs Expression plot gives information about the mean expression for each selected cancer type (**Figure 4.18**). Each pair member is indicated with different shapes and colours. The second option is Cancer vs HR. Similar to the Cancer-Expression plot, this plot gives HR values for each selected cancer for pair members (**Figure 4.19**). For *EIF5A* and *EIF5AL1*, users can observe the opposite directions of the HRs (**Figure 4.19**).

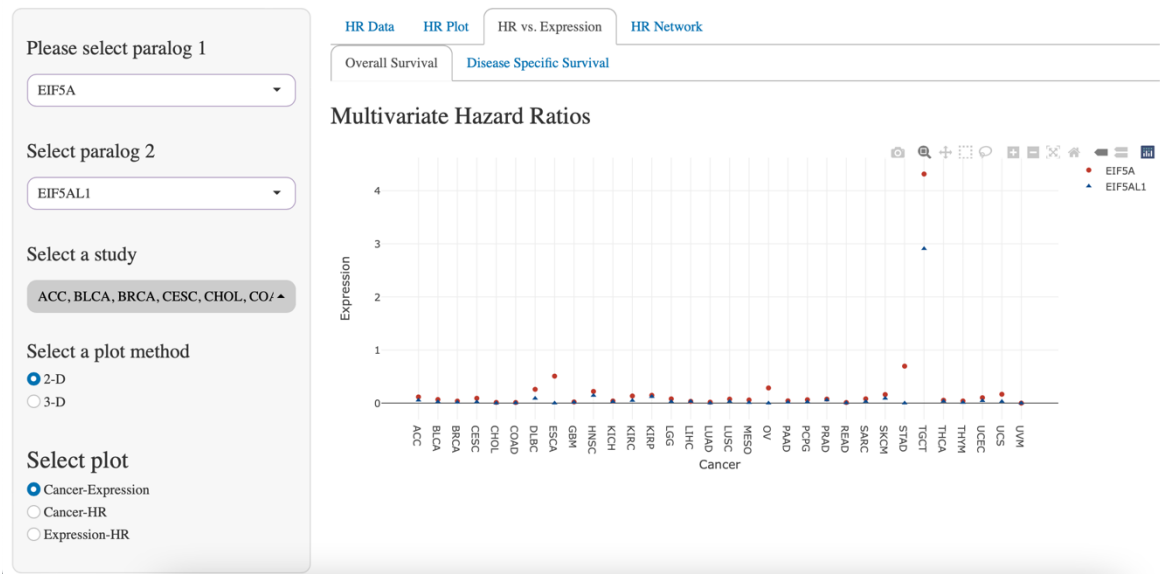


Figure 4.18. A screenshot of the “HR vs Expression” subtab of the “multiHRs” tab showing the Cancer-Expression plot.

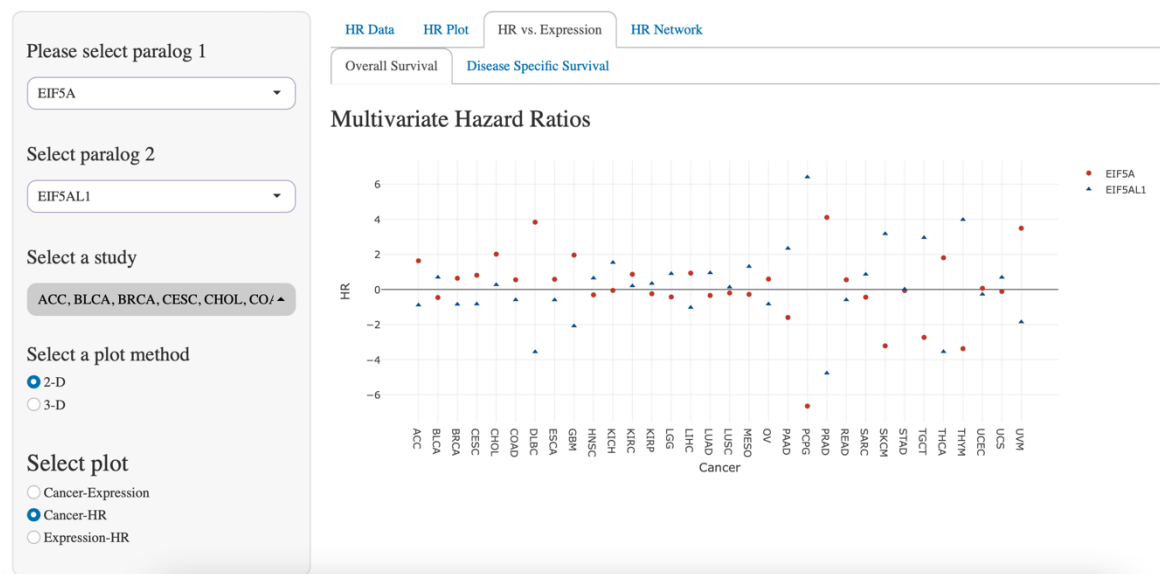


Figure 4.19. A screenshot of the “HR vs Expression” subtab of the “multiHRs” tab showing the Cancer-HR plot.

The third option is Expression vs HR, where each point's mean expression and HR are plotted along the axes (**Figure 4.20**).

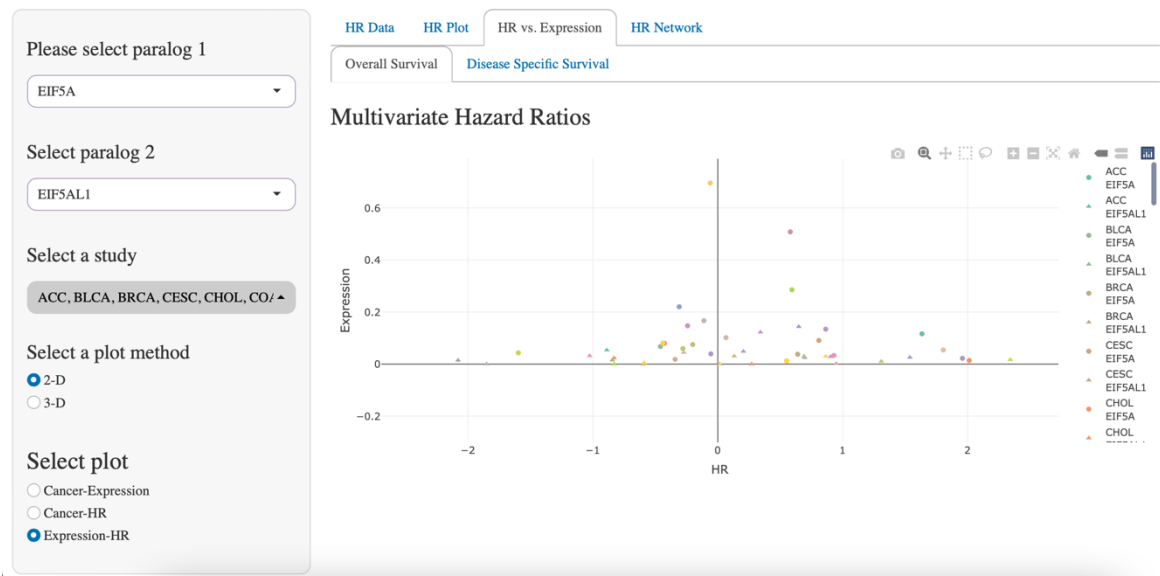


Figure 4.20. A screenshot of the “HR vs Expression” subtab of the “multiHRs” tab showing the Expression-HR plot.

Lastly, in the HR vs Expression tab, users can plot a 3-D plot for HR-Cancer-Expression, as shown in **Figure 4.21**. Due to the limitations of the package, it is recommended not to choose more than eight cancers for 3-D plots. All plots under the HR Plot and HR vs Expression tabs are interactive. Thus, when hovered, give detailed information about the points, allow selections, pan, and downloading options.

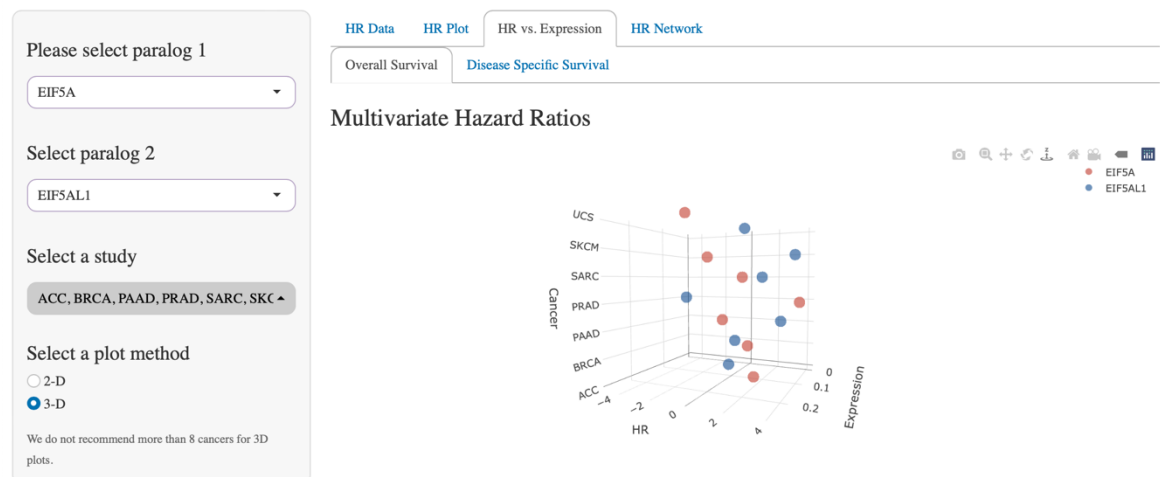


Figure 4.21. A screenshot of the “HR vs Expression” subtab of the “multiHRs” tab showing a 3-D HR-Cancer-Expression plot.

Lastly, users can plot HR-Cancer-by-Gene networks under the HR networks tab by the *visNetwork()* function from the *visNetwork* package. These networks show the HR values of each pair in selected cancers. Each edge between the gene node and cancer node shows the HR value of the gene in that cancer. For visualization purposes, hazard ratios higher than 0 are shown with red-dashed edges, whereas hazard ratios lower than 0 are shown with green-smooth edges (**Figure 4.22**). The “forceAtlas2Based” layout is selected to avoid overlapping, resulting in a circular layout as more cancer nodes are added. However, the user can arrange an interactive network, and edge lengths can be manually changed. The same data table, HR plots, HR expression and HR network functions are available for miRNAs in Pair-HR. The HR network of the *EIF5A* and *EIF5AL1* serves as a visual representation of the results to describe the biological interactions of the genes with each cancer (**Figure 4.22**).

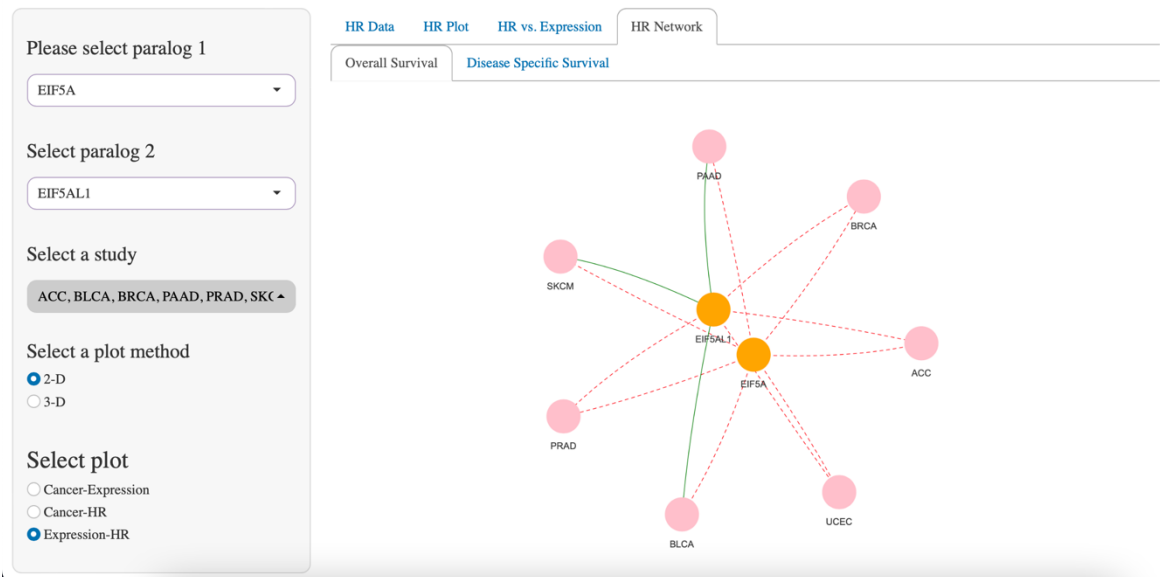


Figure 4.22. A screenshot of the “HR Network” subtab of the “multiHRs” tab. Green smooth edges represent HRs < 0, whereas red dashed edges represent HRs > 0.

4.2 Gene-cancer-by-survival network of PM-ER gene set shows cancer-specific prognostic signatures

To further examine the potential of hazard ratio networks, we have used a plasma membrane–endoplasmic reticulum (PM-ER) gene set consisting of several paralog genes. The network in **Figure 4.23** shows the overall survival HRs of 26 cancers with at least one member of the PM-ER gene set with significant HR. In the network, the number of edges for each cancer differs. UVM, THYM, KICH and ESCA displayed the highest number of edges. These paralogs showed unique interactions for cancers like UVM (**Figure 4.23**, **Appendix Figure 3**). Paralogs of RYR, *RYR2* and *RYR3* showed the highest number of interactions, eight and six, respectively. Even though a higher expression of these two paralogs predicted poor survival in OS, their effect was cancer-specific (**Figure 4.23**).

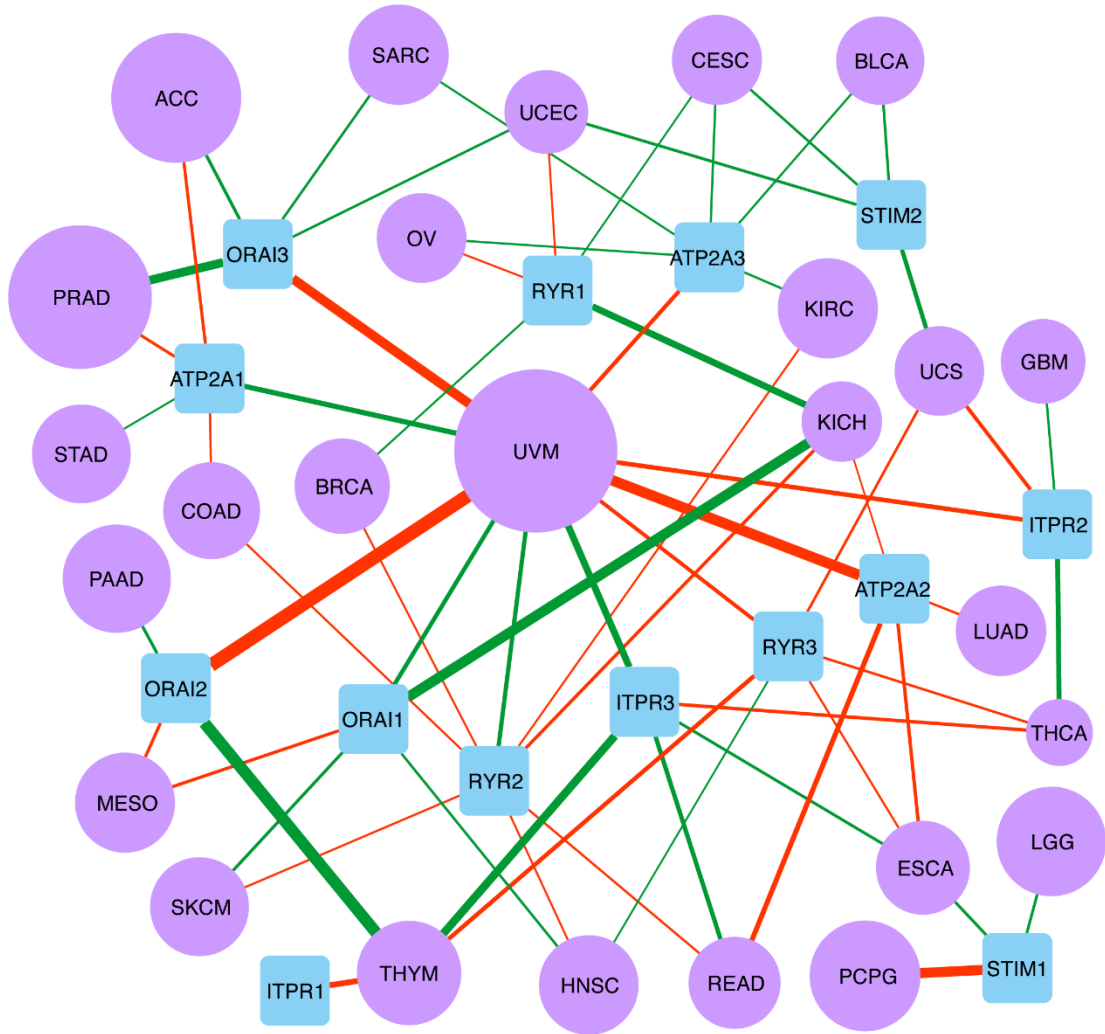


Figure 4.23. Overall survival hazard ratio network for PMER genes. The node weights represent the AUC, whereas edge weights represent each gene's $\log_2(\text{HR})$ values. Edge colours represent HR; $\text{HR} > 1$: red and $\text{HR} < 1$: green. [131]

The paralogs in the PM-ER gene set showed compartmentalization across cancers, and the paralogs acted as prognostic markers in opposite directions for the same cancers. For instance, *ITPR2* and *ITPR3* showed opposite HRs for UVM and THCA (**Figure 4.24**). UVM was still the most connected cancer after MCL clustering (**Figure 4.25**). The opposite HRs on mutual cancers or the mutual exclusivity of cancers except for UVM supports the compartmentalization of paralogs. Experimentally determined interactions for the PM-ER gene set also showed compartmentalization among paralogs (**Figure 4.26**).

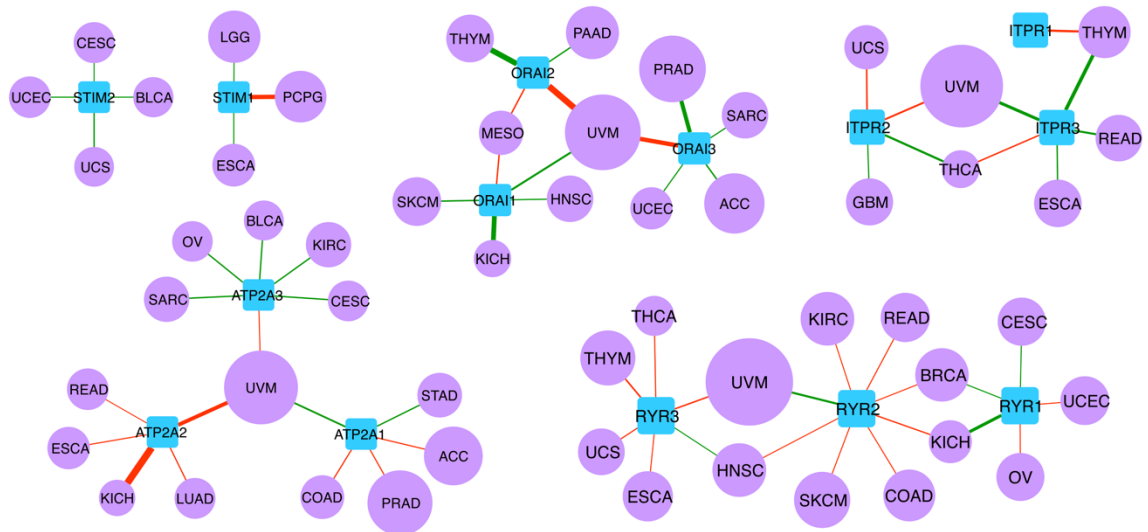


Figure 4.24. Paralog networks of PM-ER gene set. The node weights represent the AUC, whereas edge weights represent each gene's $\log_2(\text{HR})$ values. Edge colours represent HR; $\text{HR} > 1$: red and $\text{HR} < 1$: green. [131]

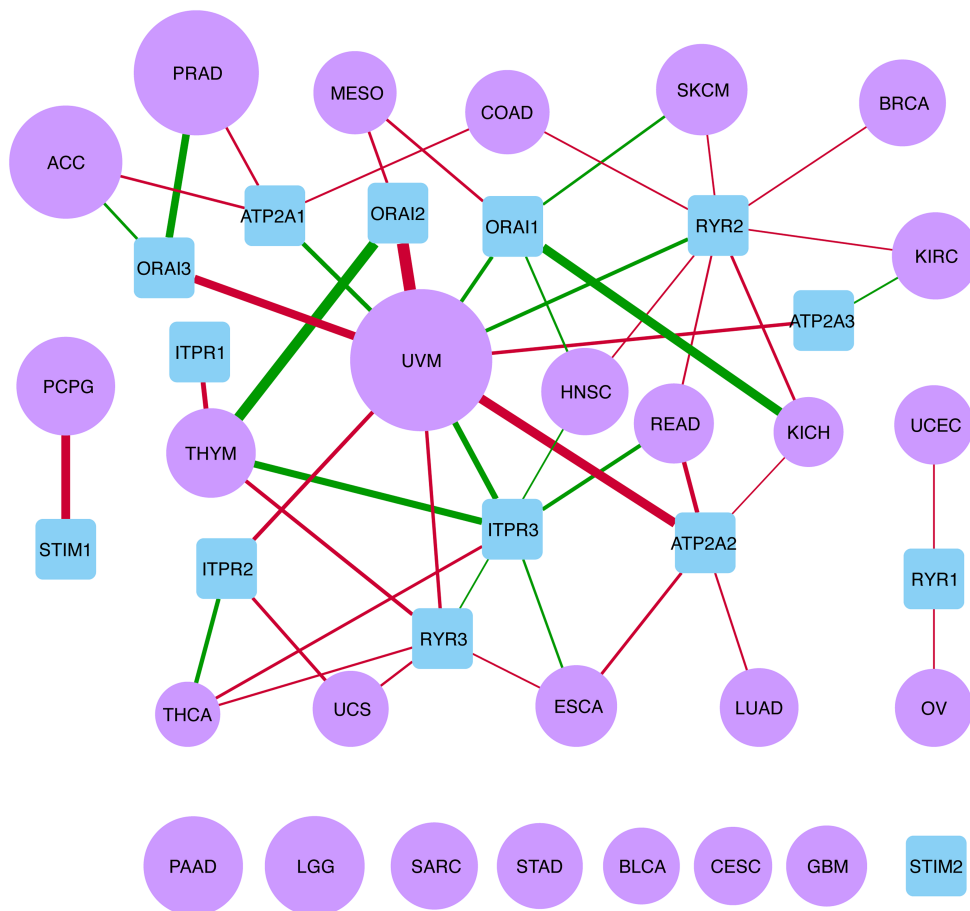


Figure 4.25. MCL clustering of the PM-ER gene set.

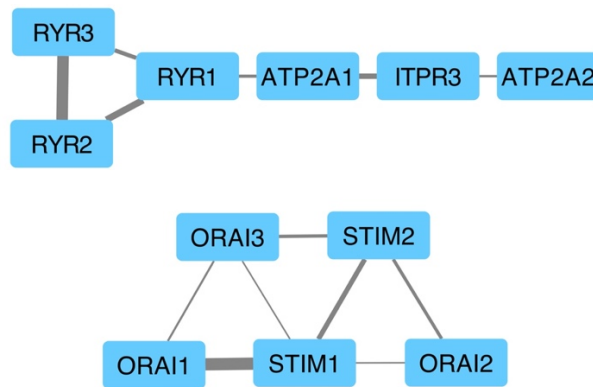


Figure 4.26. Protein-protein interactions of PM-ER gene set. Edge weights represent the number of experimentally determined interactions from STRING. [131]

4.3 Gene-by-survival network of miRNA-miRNA-mRNA synergistic triplets for HCC miRCoop network

We've used the miRCoop algorithm to detect the miRNA-miRNA-mRNA triplets that can be used as therapeutics. The algorithm resulted in 62 triplets for LIHC. Based on statistical tests, literature evidence, and differential expression, we have narrowed down the network for potential strong triplets for experimental validation (**Figure 4.27**). The hazard ratio networks of OS and DSS for these triplets were given in **Figure 4.27A** and **Figure 4.27B**, respectively.

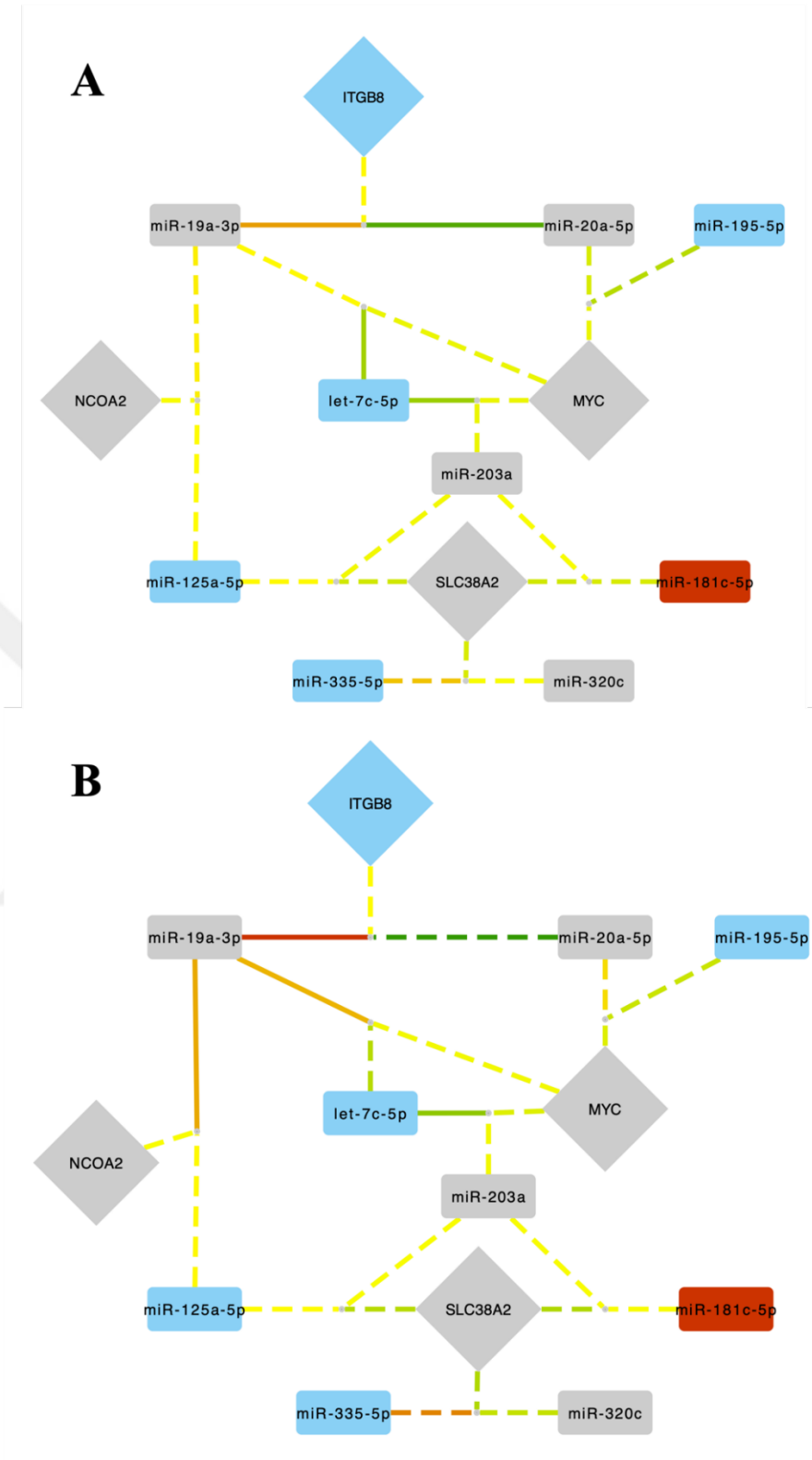


Figure 4.27. Hazard Ratio network of strong miRNA-mRNA triplets in LIHC for OS. The green edges represent $HR < 1$, while the red edges represent $HR > 1$. Red nodes represent upregulated, and blue nodes show downregulated expression in primary tumours compared to solid tissue normal. (A) OS and (B) DSS.

For overall survival, high *let-7c-5p* expression predicted better survival in its triplets for OS (**Figure 4.27A**). High *miR-19a* expression predicted worse survival, whereas high *miR-20a-5p* expression predicted better survival in the *ITGB8* triplet (**Figure 4.27A**). Similarly, *miR-335* and *miR-320c* have hazard ratios in opposite directions (**Figure 4.27A**). In DSS, *miR-19a-3p* mainly showed a hazardous effect in its triplets with significant p-values (**Figure 4.27B**). Opposite HR directions of *miR-19a* and *miR-20a-5p* were observed in DSS (**Figure 4.27B**).

The K-M plots of the miRCoop triplets showed differences in survival for expression groups. *miR-19-3p&let-7c-5p&MYC* triplet showed significant differences between groups in survival for both OS and DSS (**Figure 4.28-Figure 4.29**). Up-Up-Down groups showed better survival. *miR-181c-5p&miR-203a&SLC38A2* triplet showed significant differences for DSS only, and the Down-Down-Up group predicted better survival (**Figure 4.30 and Figure 4.31**). Similarly, *NCOA2* triplets showed significant differences between groups (**Figure 4.32 and Figure 4.33**).

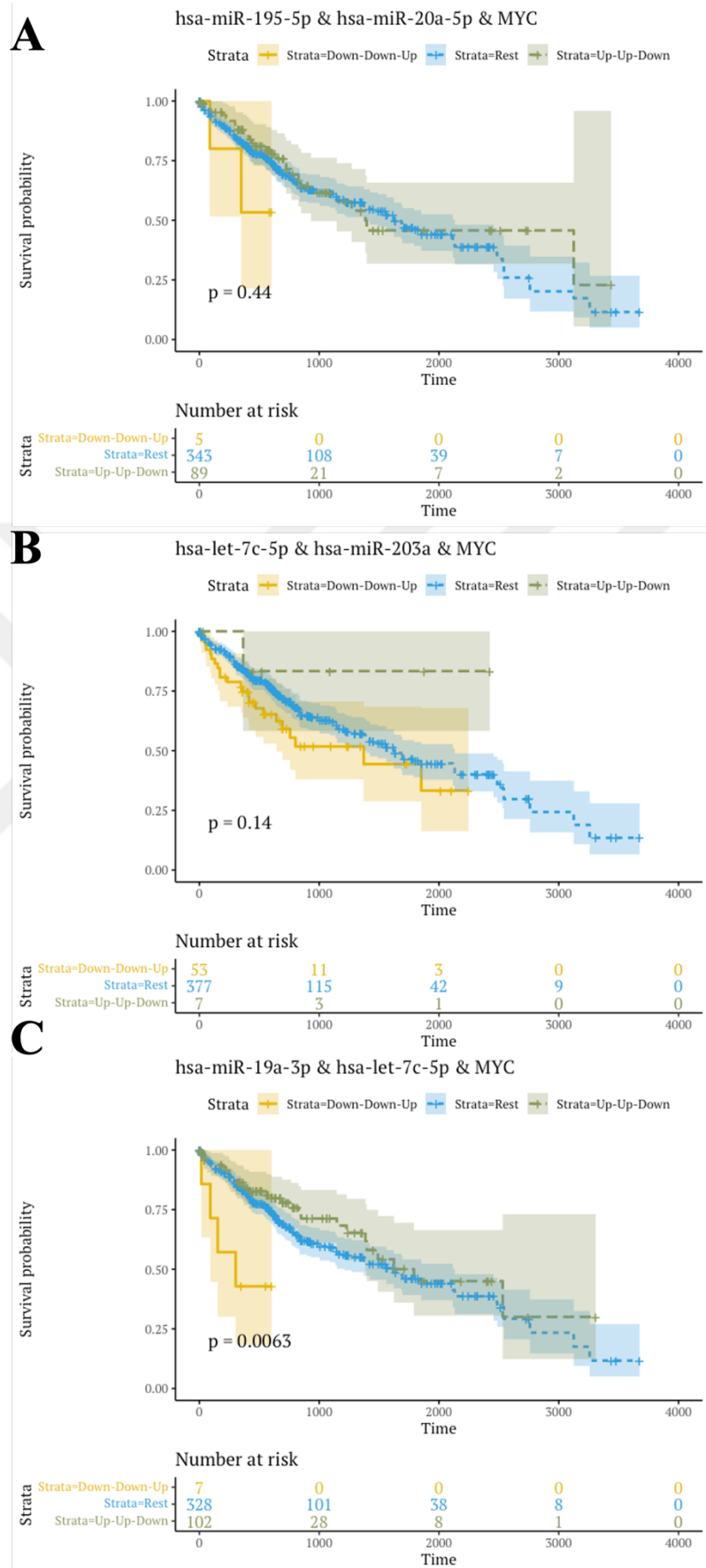


Figure 4.28. K-M plots of MYC triplets for OS.

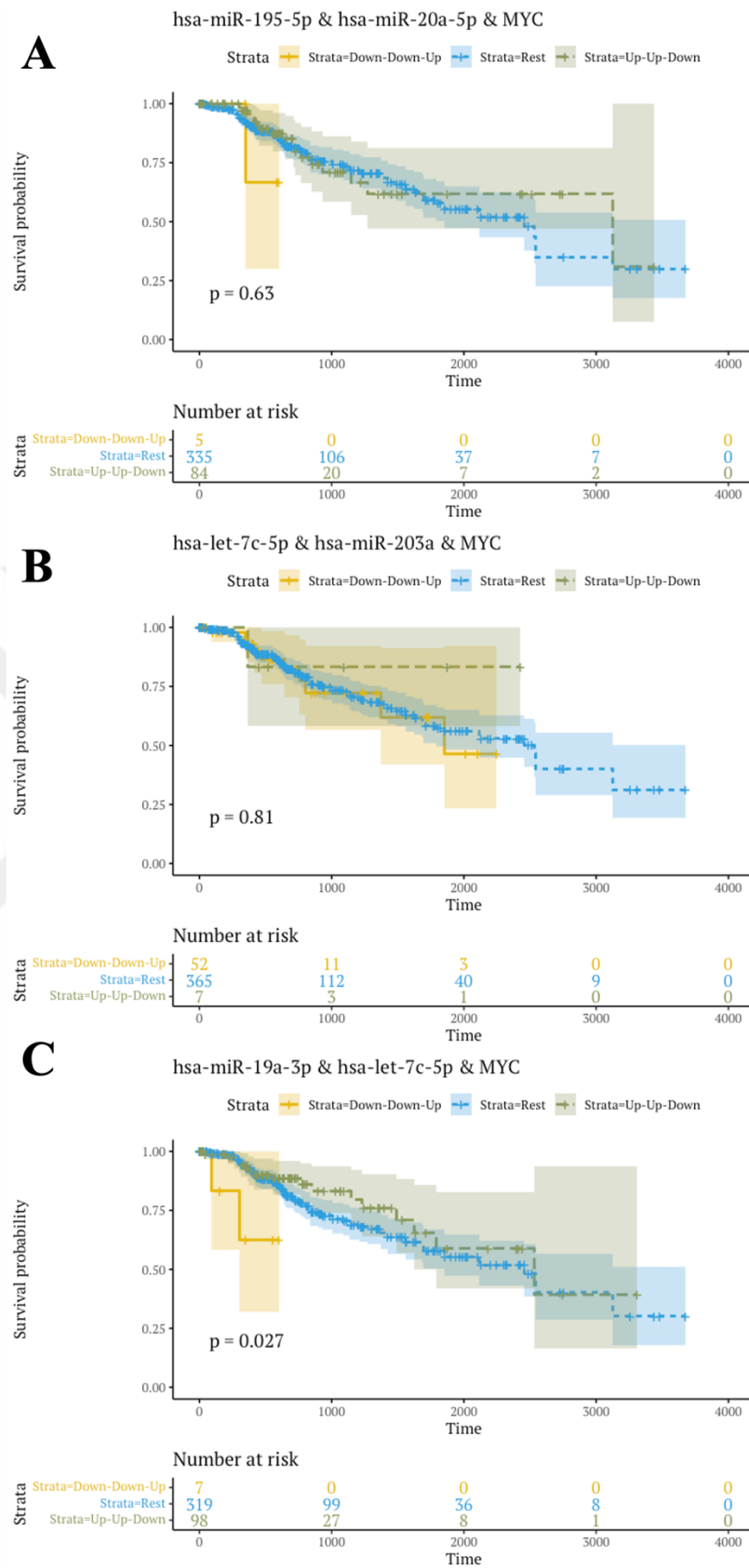


Figure 4.29. K-M plots of *MYC* triplets for DSS.

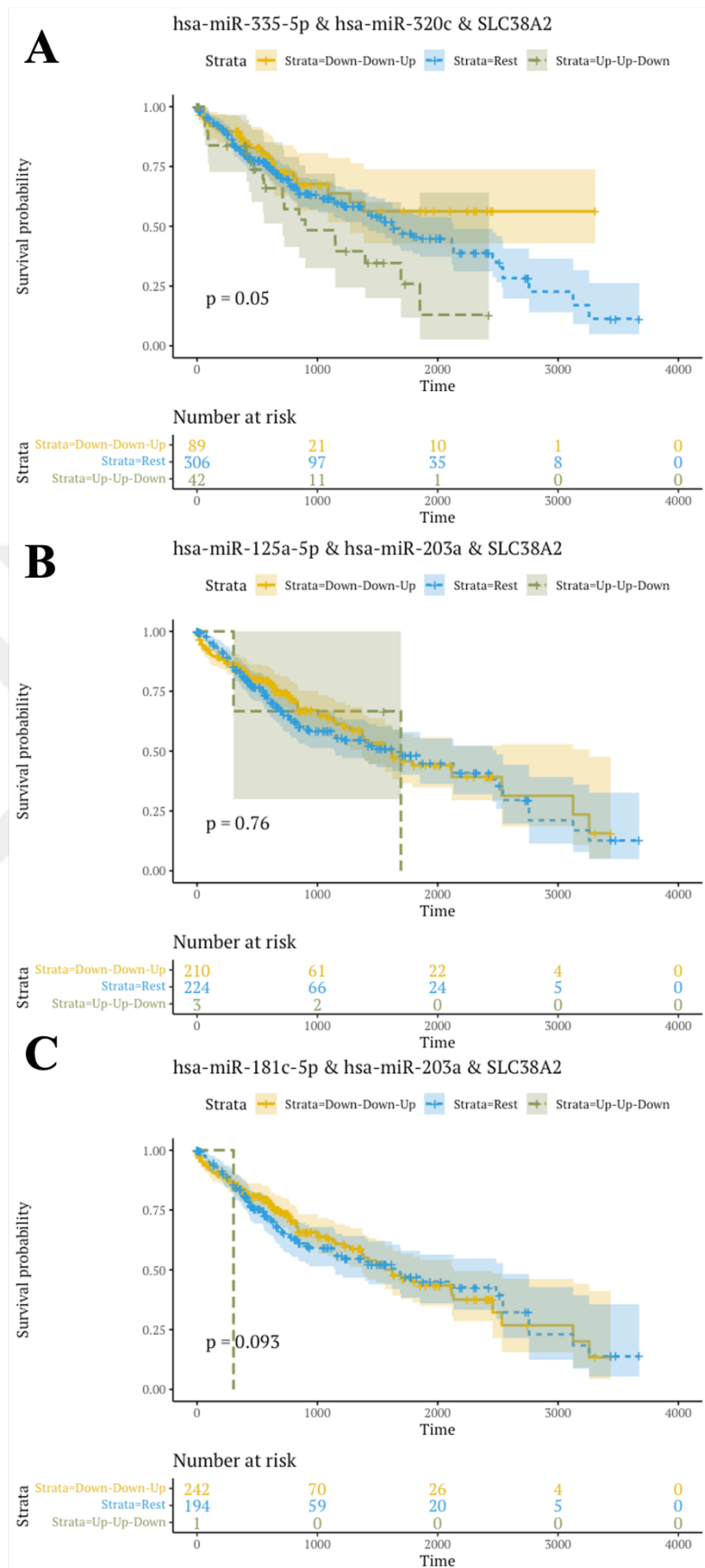


Figure 4.30. K-M plots of SLC38A2 triplets for OS.

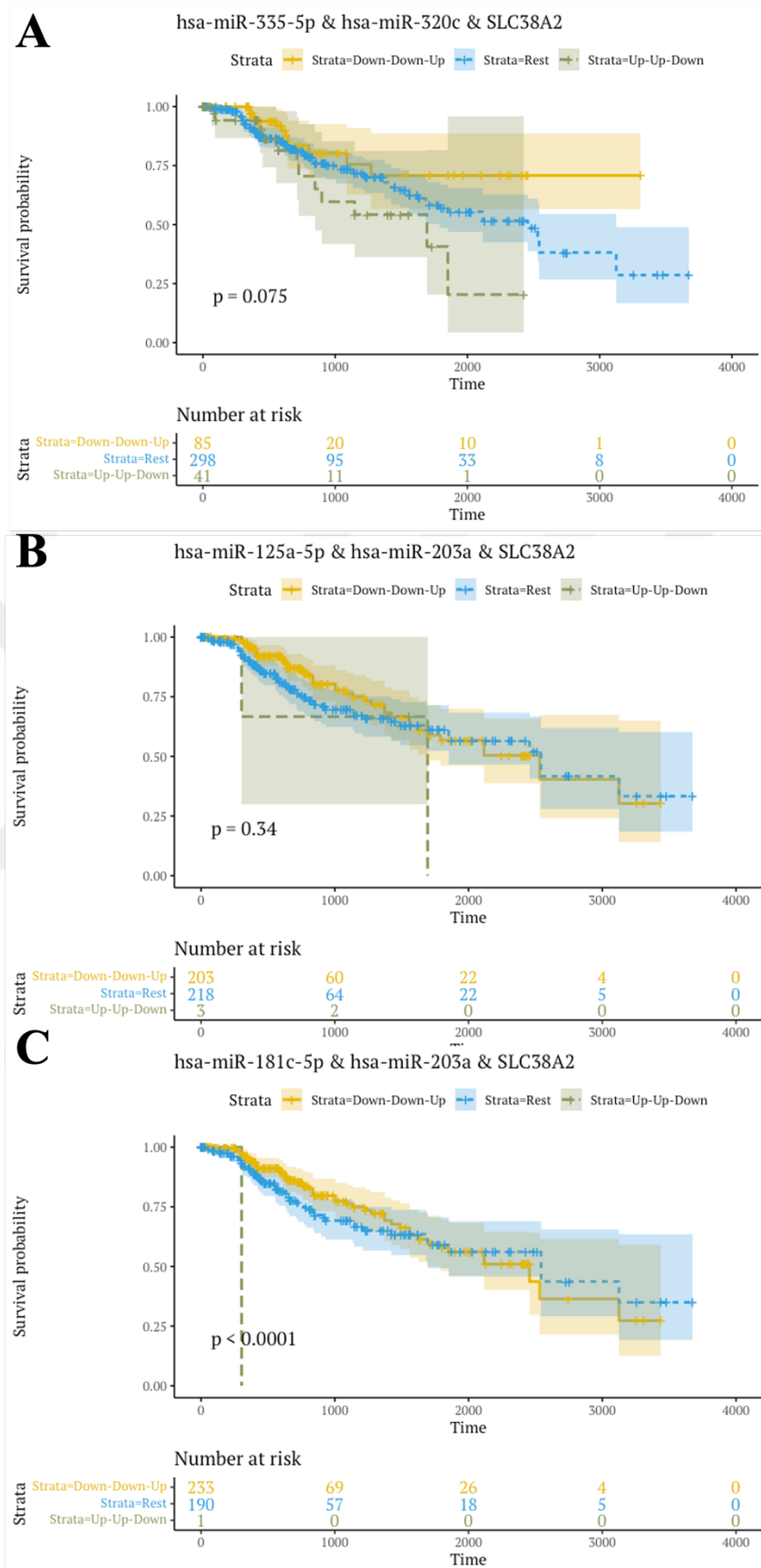


Figure 4.31. K-M plots of *SLC38A2* triplets for DSS.

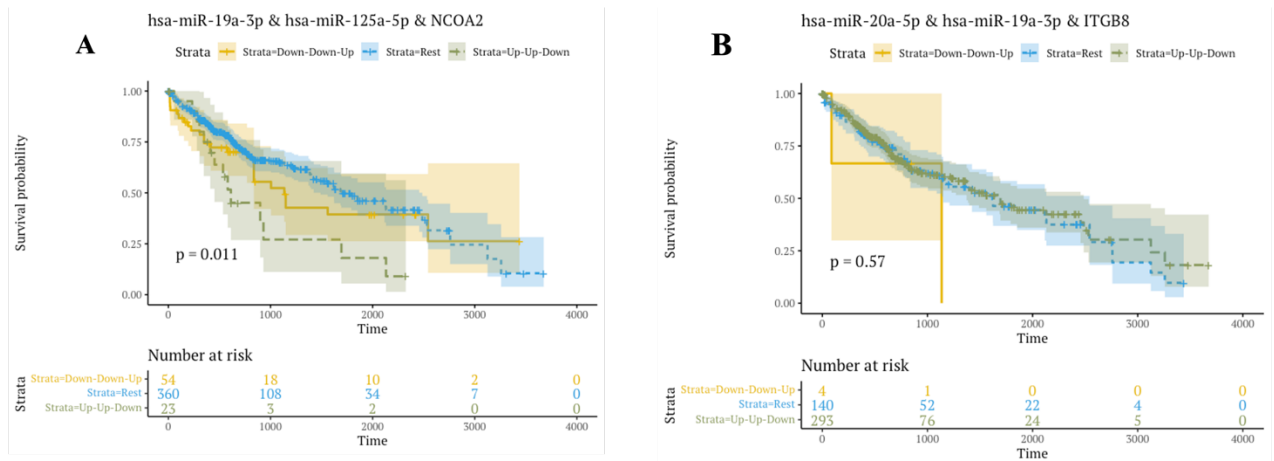


Figure 4.32. K-M plots of (A) *NCOA2* and (B) *ITGB8* for OS.

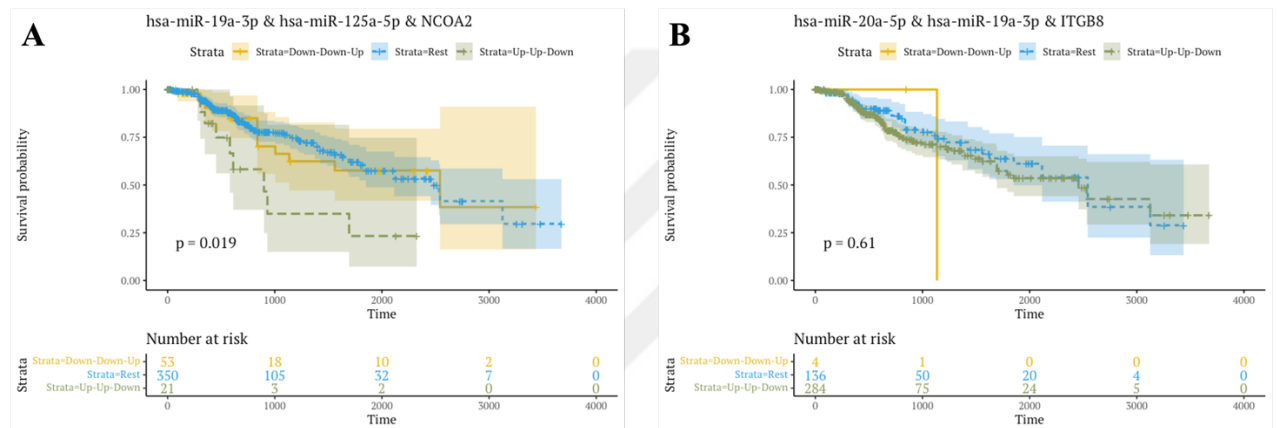


Figure 4.33. K-M plots of (A) *NCOA2* and (B) *ITGB8* for DSS.

4.4 *In vitro* validation of triplets using miRNA mimics

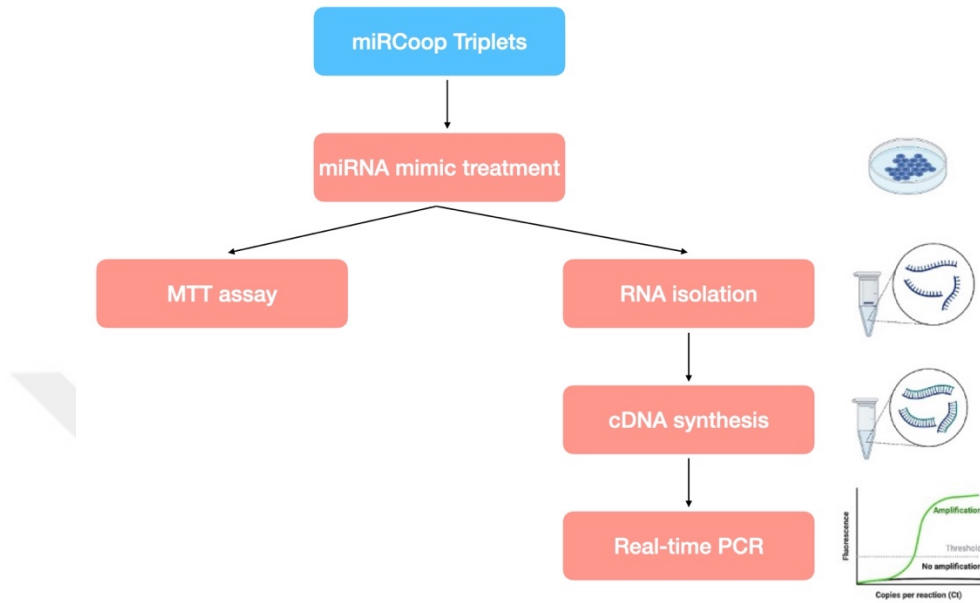


Figure 4.34. Diagram of experimental plans for the validation of miRCoop triplets.

miRNA mimic treatments were performed to check the effects of synergistic miRNA pairs on the detected triplet target genes, as shown in **Figure 4.34**. Initially, for the viability assays, Hep3B cells were treated with single miRNA or miRNA+miRNA combinations. The viability of the cells after miRNA mimic treatments were measured using the MTT assays. Secondly, RNA isolation was performed on cells treated with miRNAs to measure the relative miRNA and their target mRNA expressions. RT-PCR experiments were performed to measure the quantitative differences between single and combination miRNA treatments. MTT results indicated that miRNA doses were important for viability. First, 2000 cells/well were seeded to 96-well plates for MTT analysis.

4.4.1 Dose and time dependences of miRNA mimic treatment in HCC cells

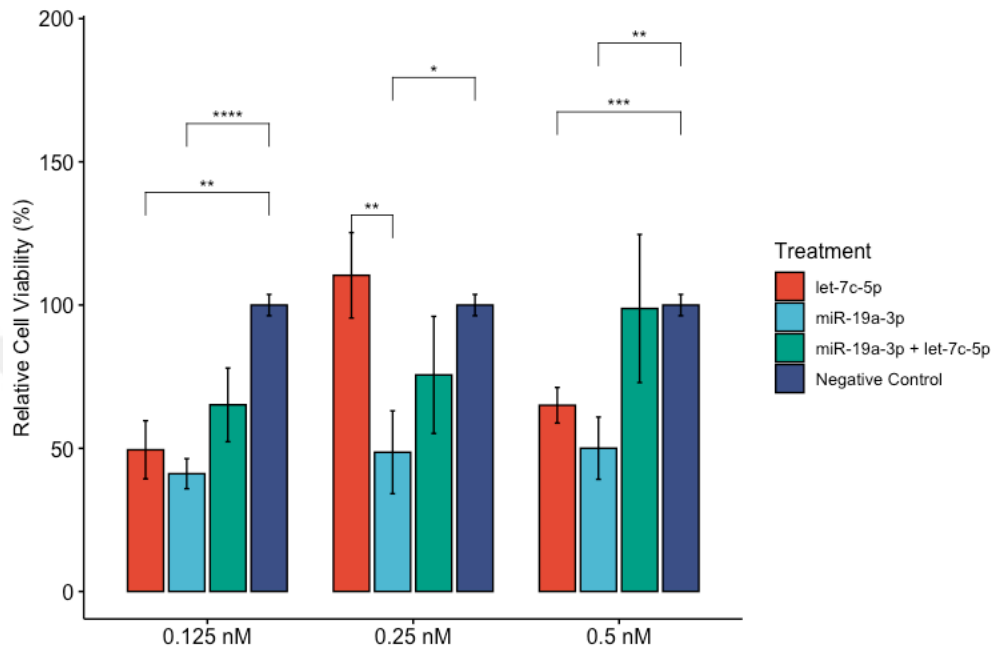
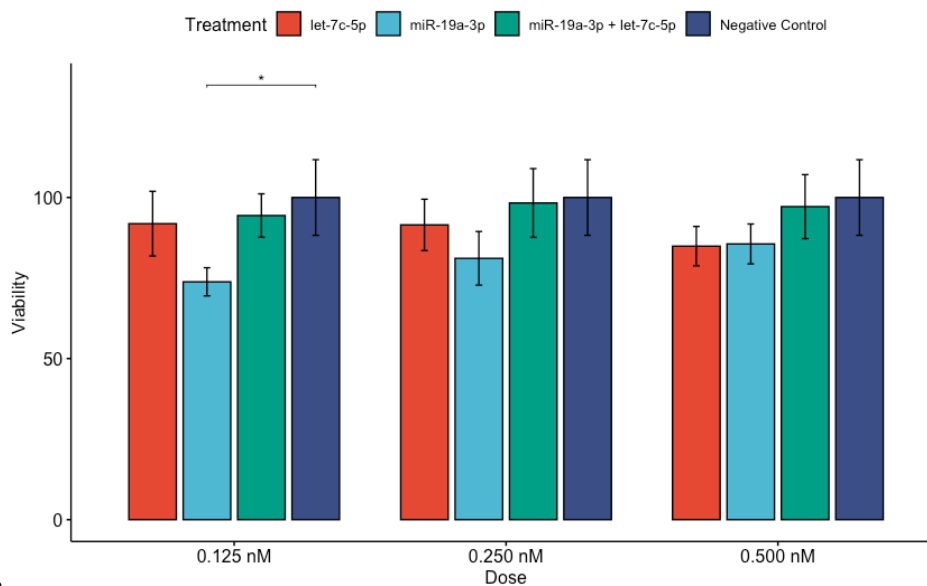


Figure 4.35. Results of synergistic miRNA treatment. 2000 cells/well Hep3B cells treated with miR-19 and let-7c for 24 hours ($n = 5$). Same negative control was used for more than one dose and replotted on each dose graph for showing significant differences. *, **, ***, **** indicate an adjusted p-value of ≤ 0.05 , ≤ 0.01 , ≤ 0.001 and ≤ 0.0001 , respectively. The tests were performed for each dose separately. The relative cell viabilities for samples are grouped by dose and t-test is performed for viability and treatments within groups. p-values are Bonferroni adjusted.



h

Figure 4.36. Results of synergistic miRNA treatment on 5000 cells/well Hep3B cells. Cells were treated for 24 hours with miRNA mimics. Same negative control was used for more than one dose and replotted on each dose graph for showing significant differences. *, **, ***, **** indicate an adjusted p-value of ≤ 0.05 , ≤ 0.01 , ≤ 0.001 and ≤ 0.0001 , respectively. The tests were performed for each dose separately. The relative cell viabilities for samples are grouped by dose and t-test is performed for viability and treatments within groups. p-values are Bonferroni adjusted.

Between combination groups and single miRNA treatments, there were no significant difference (**Figure 4.35**). On the other hand, each single miRNA was effective in the lowest and highest doses with respect to negative control. To determine the optimal time for analysing the effect of miRNA treatment, the same miRNA mimic treatments were repeated for 24-48-72 hours of treatment. 24h treatment of miR-19a-3p and let-7c-5p did not show significant differences between combination and miRNA groups for 0.125-0.250-0.500 nM (**Figure 4.36**). 0.500 nM treatments of miR-19a and let-7c showed differences in cell viability at 48h (**Figure 4.37**). The cells treated with combination of miRNAs showed a higher cell viability compared the single mimic treatments and negative control (**Figure 4.37**). The effects of combination and single mimic treatments were lost at 72h (**Figure 4.38**).

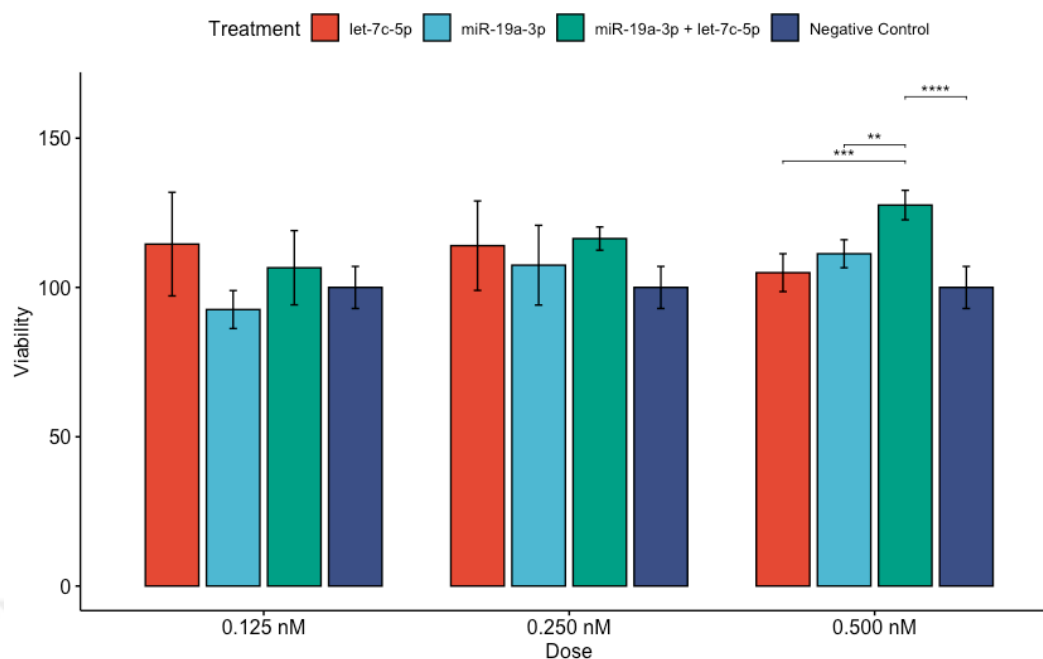


Figure 4.37. Effects of miRNA mimic treatment in Hep3B cells for 48 hours (n=5). Same negative control was used for more than one dose and replotted on each dose graph for showing significant differences. *, **, ***, **** indicate an adjusted p-value of ≤ 0.05 , ≤ 0.01 , ≤ 0.001 and ≤ 0.0001 , respectively. The tests were performed for each dose separately. The relative cell viabilities for samples are grouped by dose and t-test is performed for viability and treatments within groups. p-values are Bonferroni adjusted.

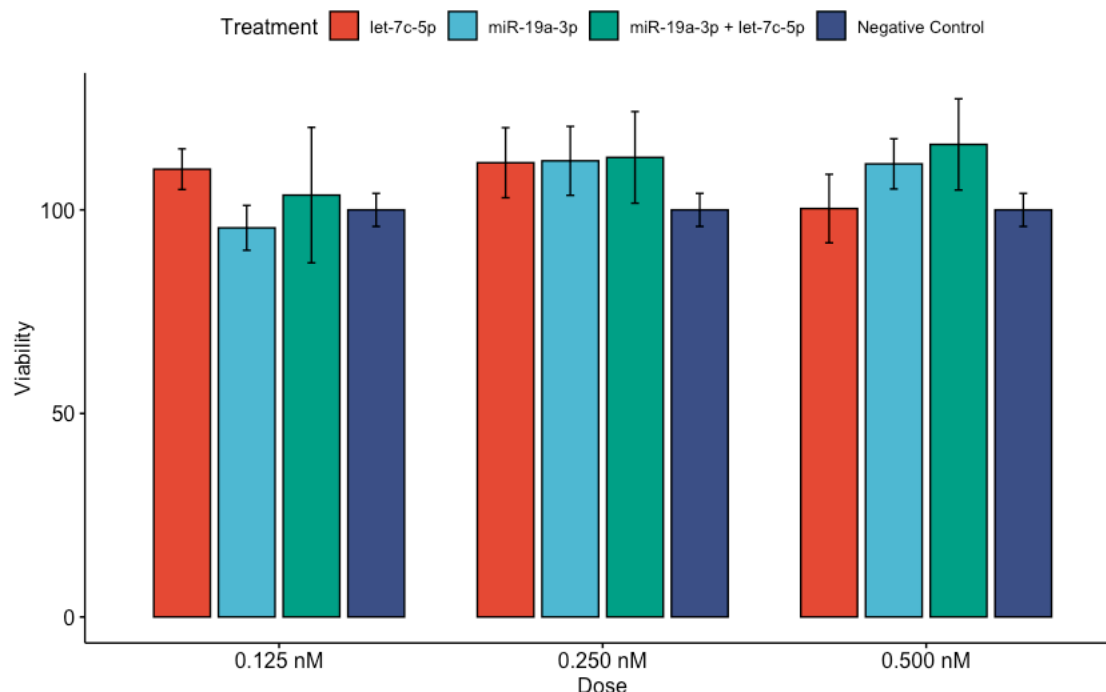


Figure 4.38. Effects of miRNA mimic treatment in Hep3B cells for 72 hours. (n=5). Same negative control was used for more than one dose and replotted on each dose graph for showing significant differences. *, **, ***, **** indicate an adjusted p-value of ≤ 0.05 , ≤ 0.01 , ≤ 0.001 and ≤ 0.0001 , respectively. The tests were performed for each dose

separately. The relative cell viabilities for samples are grouped by dose and t-test is performed for viability and treatments within groups. p-values are Bonferroni adjusted.

To optimize transfection doses, a wider range of doses of 1.0-20.0 nM was used in the MTT assay. There were no significant differences between doses 1.0 – 2.5 and 5.0 nM. However, for 7.5 nM and 10.0 nM treatments, the miR-19+let-7c combination treatment showed changes in relative cell viability between single mimic treatments (**Figure 4.39**). For 20.0 nM, the combination treatment showed a significant decrease when compared with negative control however this was not different than single treatments (**Figure 4.39**). I also tested whether mimic treatments were affected from use of different doses. Single mimic treatment of miR-19a-3p showed a 16-fold increase in miR-19 treated cells compared to the negative control at all doses tested even at the lowest, i.e., 2.5 (**Figure 4.40**).

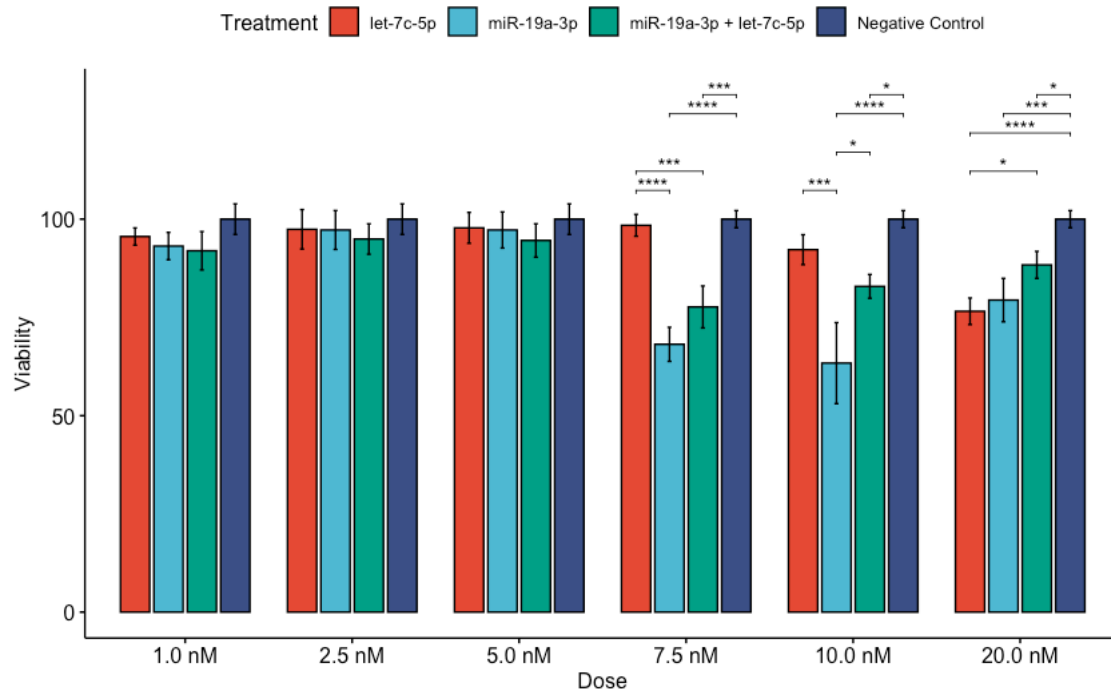


Figure 4.39. Results of synergistic mimic treatment in Hep3B cells. The cell viability of Hep3B cells at the end of 48 h treatment is measured for 1.0 – 20.0 nM. (n=5) Same negative control was used for more than one dose and replotted on each dose graph for showing significant differences. *, **, ***, **** indicate an adjusted p-value of ≤ 0.05 , ≤ 0.01 , ≤ 0.001 and ≤ 0.0001 , respectively. The tests were performed for each dose

separately. The relative cell viabilities for samples are grouped by dose and t-test is performed for viability and treatments within groups. p-values are Bonferroni adjusted.

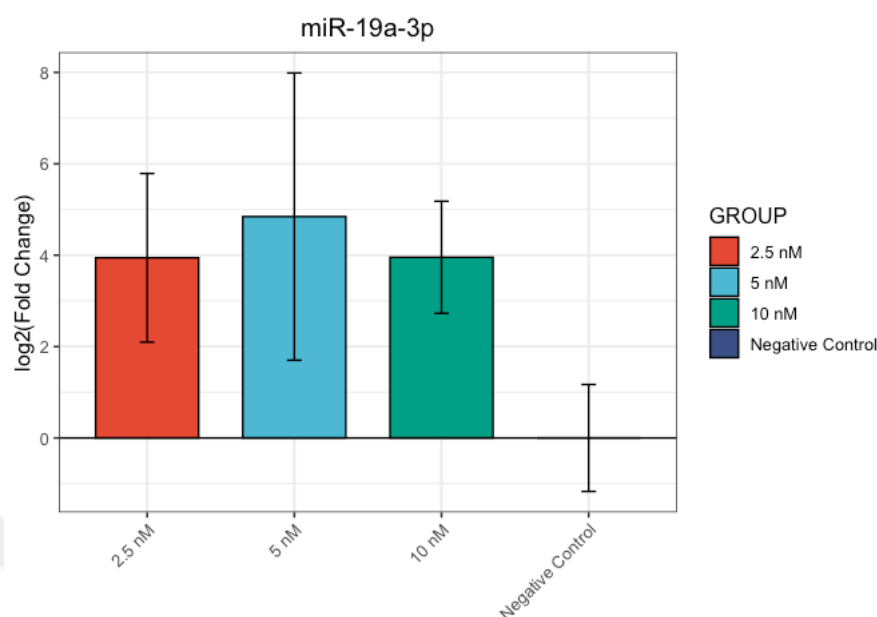


Figure 4.40. miR-19a-3p levels of Hep3B cells treated with miR-19 for 48h.

Treatment with miR-203a-3p and let-7c-5p for 24-72h showed no significant difference in cell viability between single and combination groups when tested at doses of 0.125-0.250-0.500 nM (**Figure 4.41**). Similar to miR-19a/let-7c treatment, there were no significant differences between doses 1.0 – 2.5 and 5.0 nM (**Figure 4.42**). For 20.0 nM treatments, the combination treatment showed lower cell viability compared to single miR-203a-3p treatment (**Figure 4.42**). Another miRNA pair, miR-203a-3p and miR-125a-5p treatment, also showed significant differences in cell viability for higher doses upon 48h treatment; the combination group for 7.5 nM dose showed a significant decrease in cell viability (**Figure 4.43**).

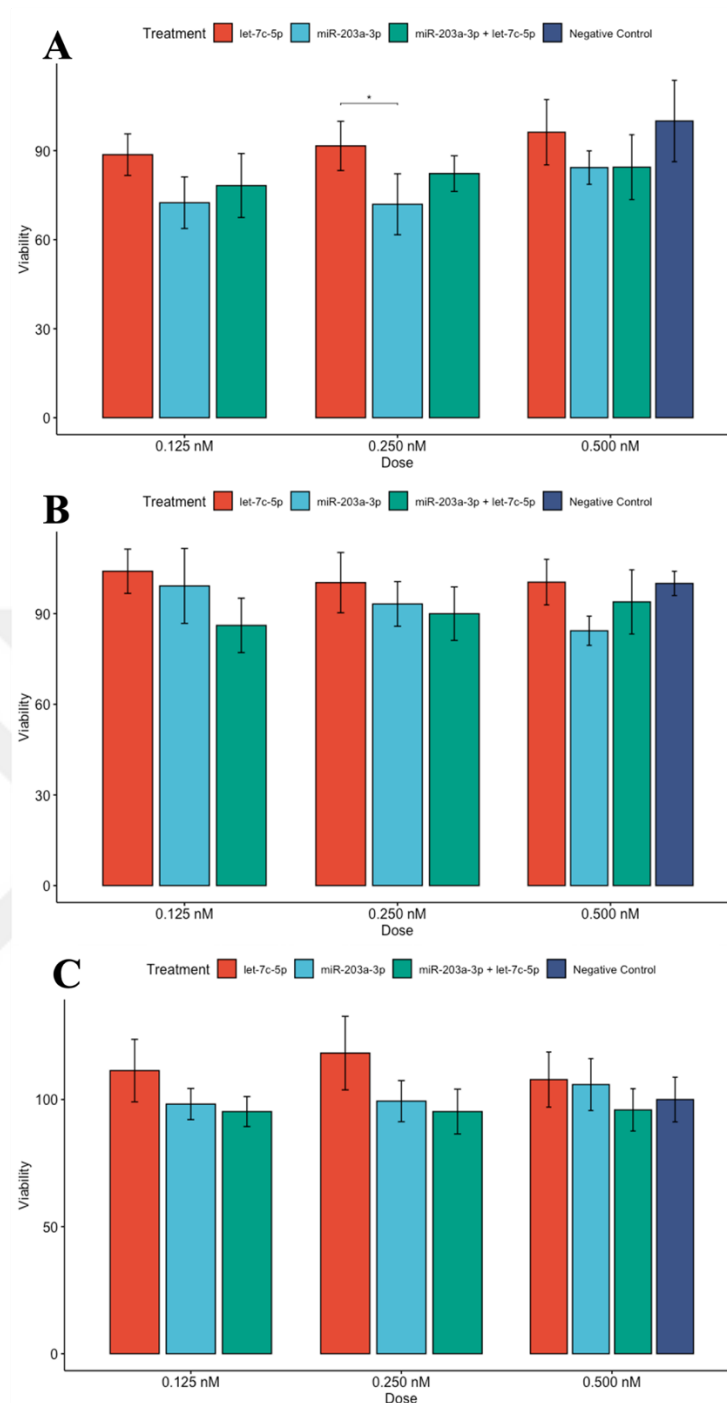


Figure 4.41. Effects of miRNA mimic treatment in Hep3B cells for (A) 24h, (B) 48h, and (C) 72h (n = 5). Same negative control was used for more than one dose and replotted on each dose graph for showing significant differences. *, **, ***, **** indicate an adjusted p-value of ≤ 0.05 , ≤ 0.01 , ≤ 0.001 and ≤ 0.0001 , respectively. The tests were performed for each dose separately. The relative cell viabilities for samples are grouped by dose and t-test is performed for viability and treatments within groups. p-values are Bonferroni adjusted.

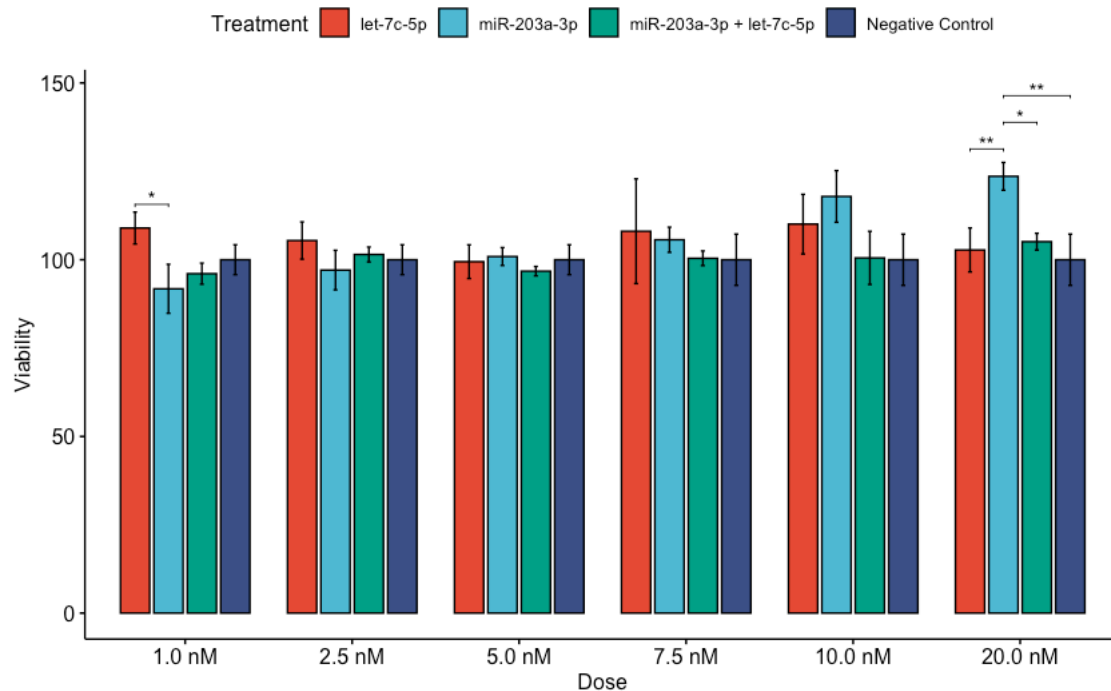


Figure 4.42. Results of synergistic mimic treatment in Hep3B cells. The cell viability of Hep3B cells at the end of 48 h treatment is measured for 1.0 – 20.0 nM (n=5). Same negative control was used for more than one dose and replotted on each dose graph for showing significant differences. *, **, ***, **** indicate an adjusted p-value of ≤ 0.05 , ≤ 0.01 , ≤ 0.001 and ≤ 0.0001 , respectively. The tests were performed for each dose separately. The relative cell viabilities for samples are grouped by dose and t-test is performed for viability and treatments within groups. p-values are Bonferroni adjusted.

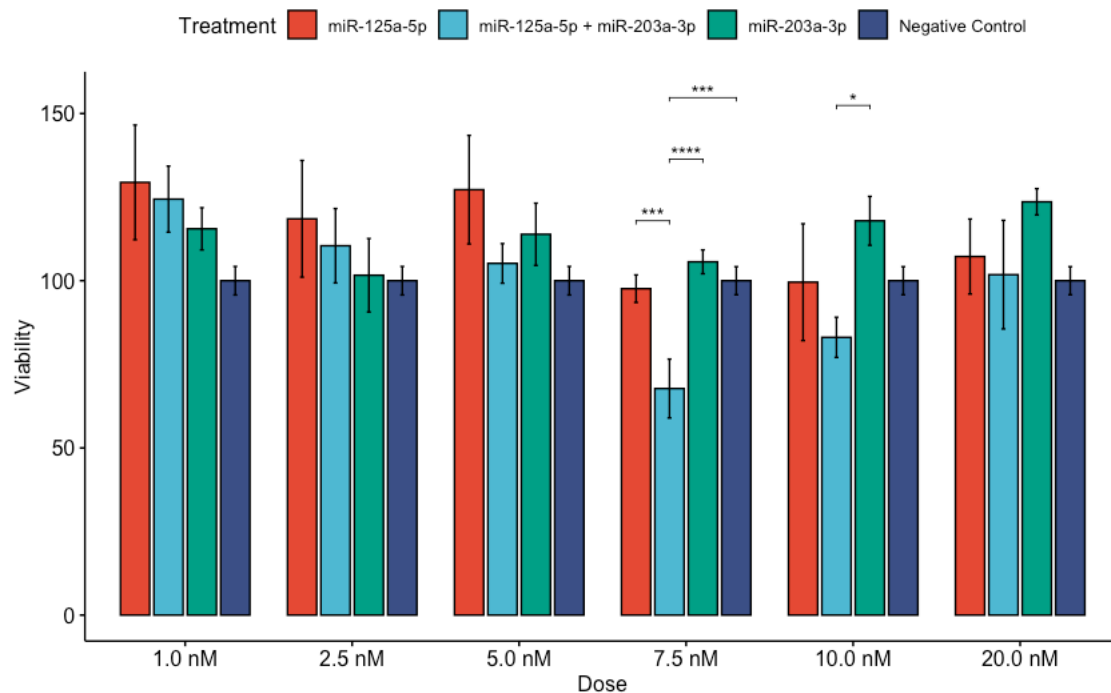


Figure 4.43. Results of synergistic mimic treatment in Hep3B cells. The cell viability of Hep3B cells at the end of 48 h treatment is measured for 1.0 – 20.0 nM (n=5). Same negative control was used for more than one dose and replotted on each dose graph for

showing significant differences. *, **, ***, **** indicate an adjusted p-value of ≤ 0.05 , ≤ 0.01 , ≤ 0.001 and ≤ 0.0001 , respectively. The tests were performed for each dose separately. The relative cell viabilities for samples are grouped by dose and t-test is performed for viability and treatments within groups. p-values are Bonferroni adjusted.

The RT-qPCR results to test changes in mimic and target gene expression showed that upon treatment with miR-19a-3p and miR-125a-5p, the levels of these miRNAs increased up to 3 and 5 fold for their respective single and combinations treatment groups (**Figure 4.44**). Similarly, miR-19a-3p and let-7c-5p treatments increased miRNA levels to 5-fold (**Figure 4.45**). Again, miR-125a and miR-203a treatments resulted in the upregulation of these mimics in Hep3B cells (**Figure 4.46**). The gene expression levels targeted by the candidate miRNA showed a decrease by 48h miRNA treatments (**Figure 4.47** and **Figure 4.49**). However, miR-19a-3p (7.5 nM) single treatment and its combination with let-7c-5p (7.5 nM) did not show a significant decrease in MYC levels (**Figure 4.48**).

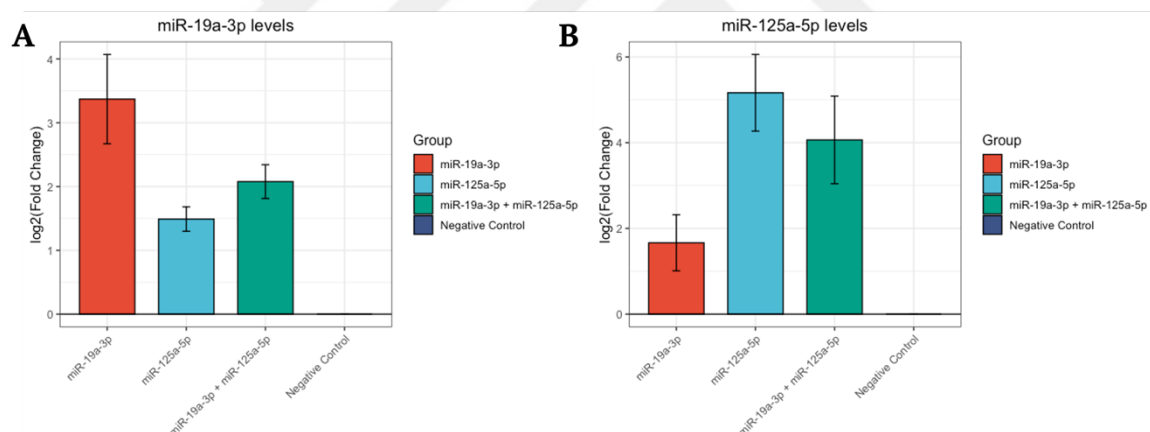


Figure 4.44. RT-qPCR validation of miRNA levels from mimic treatments. Cells were treated with miR-19a-3p (7.5 nM) and miR-125a-5p (7.5 nM) doses for 48h.

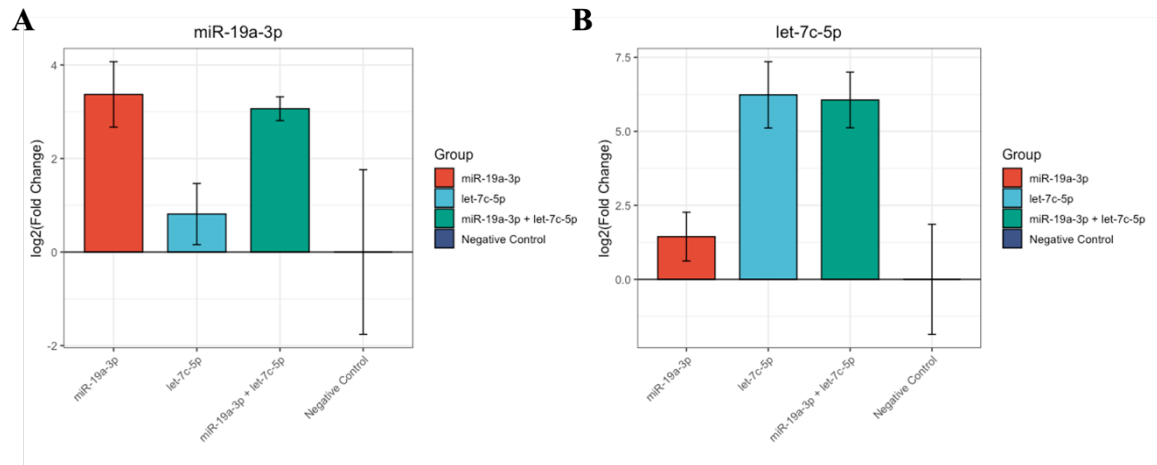


Figure 4.45. RT-qPCR validation of miRNA levels from mimic treatments. Cells were treated with miR-19a-3p (7.5 nM) and let-7c-5p (7.5 nM) doses for 48h.

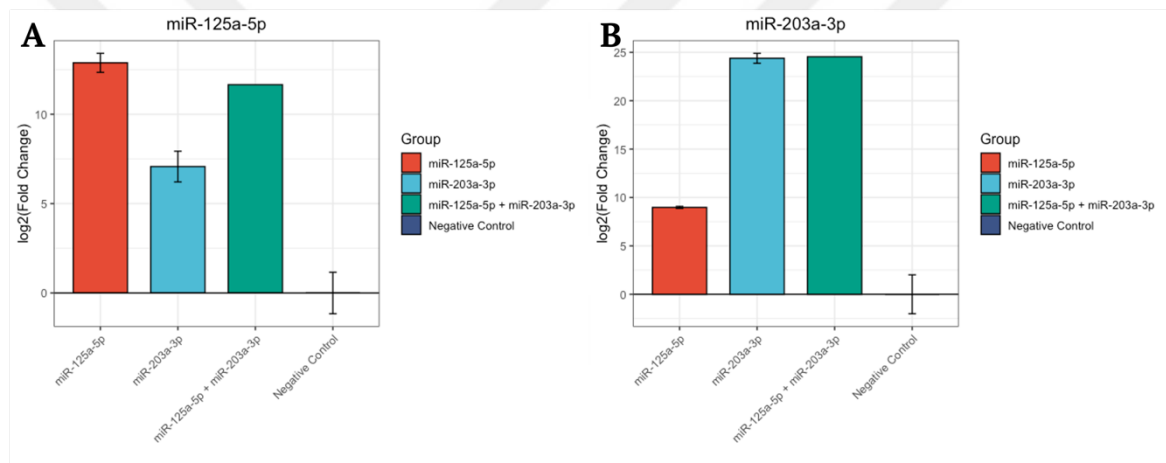


Figure 4.46. RT-qPCR validation of miRNA levels from mimic treatments. Cells were treated with miR-19a-3p (7.5 nM) and let-7c-5p (7.5 nM) doses for 48h.

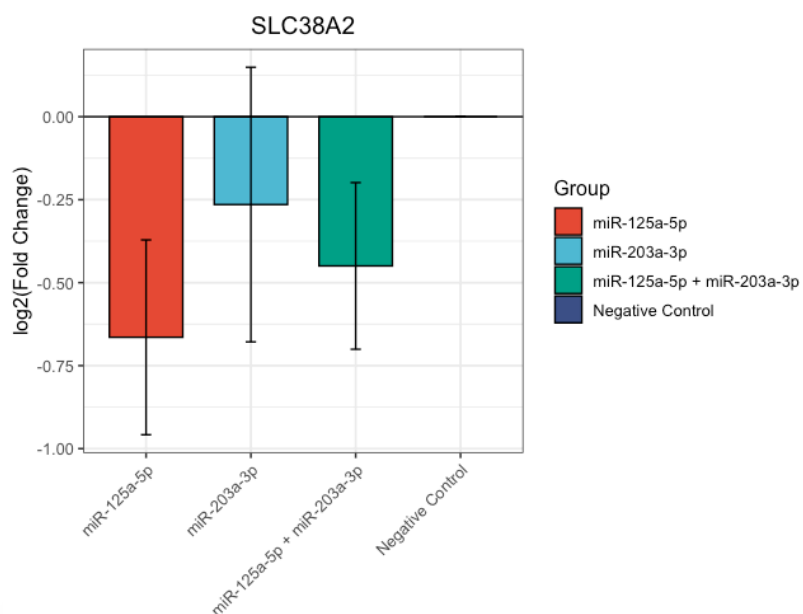


Figure 4.47. Expression levels of SLC38A2 upon 48h treatment with miR-125a (7.5 nM) and miR-203a (7.5 nM).

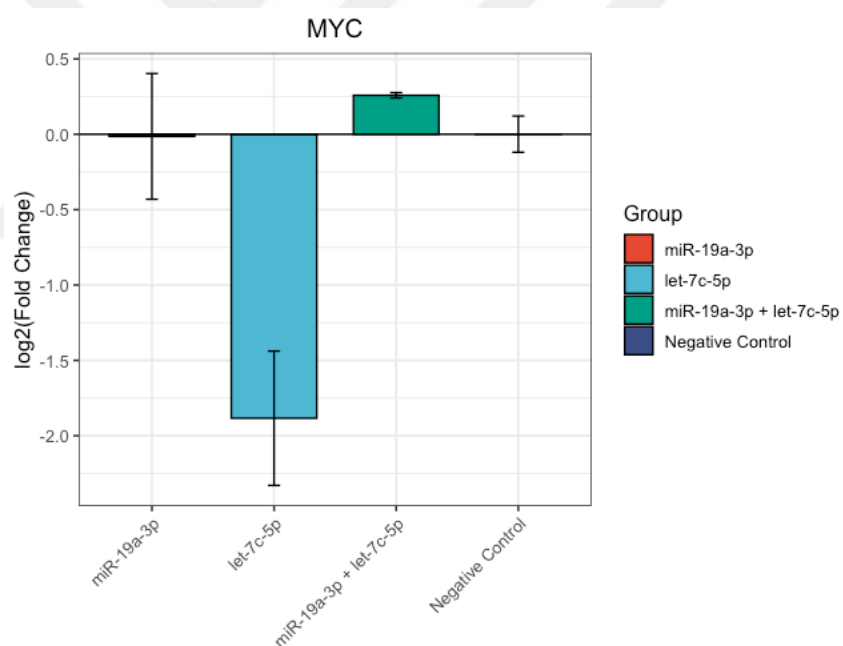


Figure 4.48. Expression levels of MYC upon 48h treatment with miR-19a (7.5 nM) and let-7c (7.5 nM).

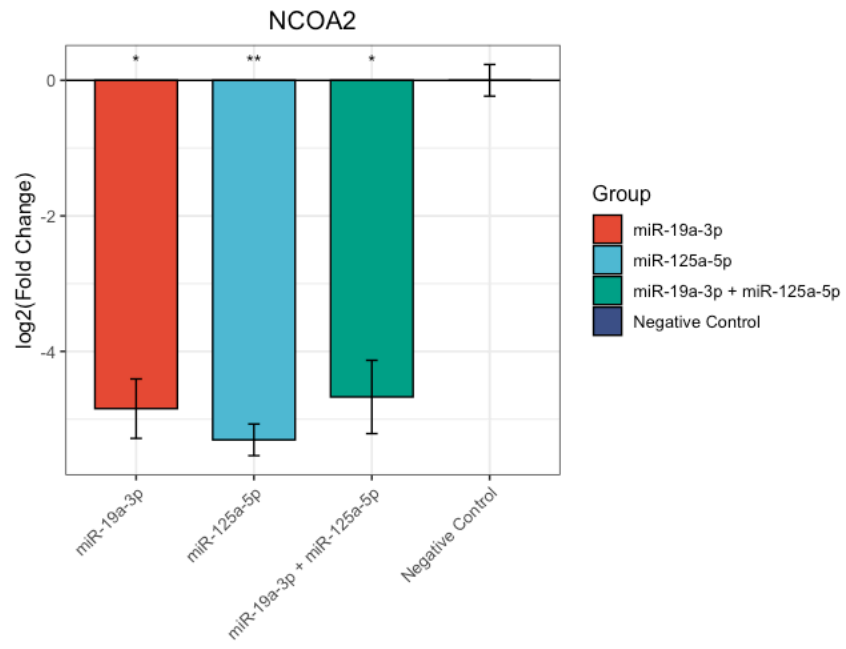


Figure 4.49. Expression levels of NCOA2 upon 48h treatment with miR-19a (7.5 nM) and miR-125a (7.5 nM).

5 Conclusions, Discussion, and Future Perspectives

5.1 Pair-HR app

Pair-HR apps enable alternative methods for survival analysis of the paralogous pairs of genes without any programming background. Cox Proportional Hazards methods and Kaplan-Meier are frequently used to analyse the prognostic factors associated with survival [132]. Prognosis in cancer is important to understand the likely course of the disease and make informed treatment choices [133]. Hazard ratios can imply how a factor changes the rate of experiencing an event, like the recurrence of the disease or response to treatment [134]. A hazard ratio greater than 1 ($\log_2, >0$) indicates a worse prognosis, while lower than 1 ($\log_2, <0$) suggests better survival. The hazard ratio calculations using the app revealed that paralogs/miRNAs could have opposite hazard ratios suggesting potential complementary functions for prognosis. There are several online applications available that perform survival analysis [135]–[137]. Still, as far as we know, no Shiny application can perform multivariate survival analysis for miRNA arms and paralog pairs using TCGA PANCAN datasets to generate gene/miRNA-Cancer-HR networks. The app can be used to explore the differences in expression between paralogs and miRNA arms in different cancers and their correlation. Users can use the “Expression” tab to visualize the survival information of each patient and further investigate the hazard ratios in the relative modules. This Shiny app I’ve developed can help identify paralog/miRNA genes’ effect on prognosis. The tool can identify paralogs and/or miRNAs with different or similar prognostic signatures in survival. Users can also investigate the cancer-specific behaviours of genes across TCGA cancers. Gene/miRNA-cancer-HR networks serve as a visualization option for the prognostic signatures of the pairs.

CREB3 and its paralog *CREB3L4* showed better survival for high expression groups in LGG. *CREB3* is an endoplasmic reticulum transcription factor and plays important roles in cellular functions like cell proliferation, migration, and tumour suppression [138]. It is also shown that *CREB3* is an important regulator of the malignant progression of glioblastoma [139]. (Hu et al., 2019). The finding that *CREB3L4* was also prognostically similar to its paralog *CREB3* is a novel finding. On the other hand, lower expressions of *FGR* predicted better overall survival, while its paralog, *SRC*, showed a higher survival probability for the high expression group in LGG. *SRC* is overexpressed in several malignancies, including ovarian cancer, and *FGR* might adjust its expression by lowering it to allow for dosage compensation [140].

For the miRNAs, higher expression of miR-200a-3p and miR-200a-5p predicted better survival for OS in bladder cancer patients. On the contrary, lower expression of the miR-200a pair estimated a better survival for LGG. These emphasize the presence of the cancer-specific prognostic effects of the miRNA arms. In literature, miR-200a-3p has been shown to suppress cell proliferation and invasion in non-small lung cancer [141]. A study by Li et al. showed that miR-200a-3p inhibits bladder cancer cell proliferation by regulating *STAT4* [142].

EIF5A is the eukaryotic initiation factor 5A and is essential for cell proliferation [143]. Its highly homologous paralog *EIF5AL1* is only validated at the transcript level and has not been studied before [143]. The hazard ratios of *EIF5A* and *EIF5AL1* were highly negatively correlated across many cancers ($r(32) = -0.9$, $p = 5.53E-16$). These opposite HRs might be due to genetic buffering that might arise from paralogous gene compensation [144]. An example from the literature is *DDX3Y* and *DDX3X*; these two paralogous helicases were inversely correlated in terms of essentiality with the expression levels, suggesting dosage-dependent functional redundancy [145]. Pair-HR app allows for testing whether a paralog

pair or two arms of a miRNA are concordant in their prognostic estimates in univariate and multivariate manners. This novel approach can help identify whether paralogs are neo-functionalized, compartmentalized, and/or amplified or dosage compensated expression/functions in a gene-pair cancer specific manner.

In the future, users can perform survival analysis of more than two genes/miRNAs and generate cancer-by-survival networks for their gene/miRNA set of interest, as demonstrated by the PM-ER gene set. Nevertheless, the pairs that can be studied by Pair-HR are now limited to pre-determined miRNA and gene pairs. I plan to integrate other datasets with survival information and make the app available so users can upload their data for analysis and use the modules. Incorporating additional clinical variables, such as stage and molecular subtypes, is also planned. The app can be modified to simultaneously include survival analysis for more than two genes/miRNAs. Also, currently, the app does not support the customization of the networks and can only generate networks with limited information due to the limitations of available packages.

The PM-ER gene set was used to give an idea of the potential of gene-cancer-by-survival networks [130]. For PM-ER paralogs, the hazard ratios for shared cancers were frequently opposite, and paralogs showed extensive compartmentalization across all cancers and less for uveal melanoma (UVM). Uveal melanoma is a form of malignant eye tumour [146]. Downstream signalling of the mutant GNAQ, a frequent UVM driver mutation, is mediated by calcium signalling [147]. Several studies show altered calcium signalling in malignant mesothelioma [148], [149]. This extended network method can identify pairs or modules of gene families. This network specific for calcium signalling helped identify hub genes and cancers with high connectivity. For the PM-ER gene set, I've identified that intracellular

calcium concentrations are determinant of prognosis in many cancers, but especially in UVM. The hub genes and cancers might have important implications for cancer prognosis and personalized medicine research. The proposed extended network model can be integrated into the Shiny app for detailed paralog/miRNA survival exploration for TCGA PANCAN.

5.2 In vitro validation of miRCoop triplets

miRNAs change their targets' levels post-transcriptionally via degradation and/or repression [150]. Synergistic gene regulation of miRNAs is important for overcoming the dependence on miRNA abundance and maximizing miRNA efficiency. Due to synergism, transfections with low concentrations of miRNA mimics may show more potent effects on cells [151]. Overall, combination miRNA treatment might significantly affect tumorigenesis more than single-miRNA-based treatments. Several studies also showed that specific miRNAs could alter the drug sensitivity in hepatocellular carcinoma and breast cancer [78], [152]. In order to identify cooperating miRNAs, the miRCoop algorithm and newly developed miRCoop Web (TUSEB project ongoing) was used. Based on literature evidence, the network was pruned to include eight triplets targeting four different genes among the miRNA-miRNA-mRNA triplets. The hazard ratio network for miRCoop triplets revealed that miRNAs that target the same gene could have opposite hazard ratios (**Figure 4.27**). For example, miR-19a and let-7c have opposite hazard ratios in their triplets with MYC. Let-7c and miR-203a, on the other hand, have parallel hazard ratios and their high expressions act protective with respect to survival their MYC triplet. miR-19a-3p has been shown to promote liver tumour growth [153], and its expression was associated with poor overall survival in my analysis. Let-7c-5p inhibits metastasis in liver cancer [83], and its downregulation is associated with poor survival [84], confirming the miRCoop HR network results.

For miRCoop triplets, I've generated Kaplan-Meier plots to test the differences between experimental pattern groups similar to the miRCoop method. Among MYC triplets, miR-19a-3p and let-7c-5p pair showed significant differences between expression groups (**Figure 4.29**). Among SLC38A2 triplets, miR-335 and miR-320c triplets showed significant differences between groups. miR-320 family has functions in epithelial-mesenchymal transition (REF) and miR-320c is upregulated in HCC (REF). NCOA2 triplet with miR-125 and miR-19 also showed differences in survival between expression groups (**Figure 4.33**). miR-125a-5p acts as a tumour suppressor in HCC and inhibits cell proliferation by targeting several factors, including MMP11 and VEGF (REF). These four genes show importance in survival for LIHC based on K-M analyses. Besides MYC, the importance of SLC38A2, NCOA2 and ITGB8 have not been studied in HCC in terms of prognosis. Therefore, it is crucial to study these triplet elements as potential therapeutic targets for HCC.

In miRNA mimic treatment experiments, there are multiple challenges that include effectiveness and time of delivery, potential toxicity, and lack/loss of stability. Therefore, MTT assays were carried out with different doses and times of miRNA pairs on Hep3B cells to test the effect of synergistic miRNA treatment on cell viability, a potential proxy for prognostic effects of miRNAs. MTT assay is a colourimetric test used to measure cellular activity as an indicator of cytotoxicity and cell proliferation by reducing yellow salt to purple formazan by the active cells [154]. The transfection doses and the duration of miRNA treatment showed variability on the cell viability for different pairs. To our knowledge, no study uses miRCURY LNA miRNA mimics (QIAGEN) for the synergistic effects of miRNAs. It is important to use optimal and comparable concentrations of miRNA mimics that have led to predicted synergy in the human patient data. Moreover, high concentrations

of miRNA mimics or other oligos can saturate the RISC complex, thus affecting endogenous miRNAs [155]. Also, high concentrations of miRNAs can have cytotoxic effects [156]. The optimal miRNA doses can depend on the endogenous miRNA abundance, cell type and target site [157], [158]. As a validation for the miRCoop, I've tested the effects of selected miRNA mimic treatments on cell viability and gene expression.

For optimization purposes, 2000 cells/well were seeded to 96-well plates for MTT assays. However, the readings obtained for MTT at 570 nM showed low absorbance. The reduction of MTT depends on the number of cells. Thus, five thousand cells/well were used in the following MTT assays. The MTT results in this thesis did not show significant differences between single treatment groups versus combination treatments for lower doses except for miR-19 (0.500 nM) and let-7c (0.500) treatment (**Figure 4.37**). 0.500 nM combination treatment for 48h showed a significant increase in relative cell viability compared to single mimic treatments of miR-19a and let-7c. The increase in cell viability was lost at 72h (**Figure 4.38**). To test a higher and wider range of doses, mimic treatments for 1 nM to 20 nM were performed for 48h(**Figure 4.39**). For 7.5 nM and 10.0 nM doses of miR-19a and let-7c, cell viability decreased in combination groups when compared to single miR-19a-3p treatments. However, combination groups' relative cell viability was higher than single let-7c-5p groups (**Figure 4.39**). For 20.0 nM, the combination showed higher cell viability than single mimic treatments. These results suggest that the effect of miRNA treatment on cell viability can be dose-dependent. Low doses of miR-203a-3p and let-7c-5p were also tested for three different time points. The effect of combination groups on cell viability did not affect the cell viability compared to single treatments or negative control groups (**Figure 4.41**). In order to see the effects of miRNA treatments on cell viability, in vitro experiments need further examination. The effect of mimic treatments on cell viability can be tested upon

cellular stress, e.g., hypoxia or DNA damage inducing agents mimicking tumour microenvironment and chemotherapy, respectively. How miRNA treatments affect the target gene levels under basal and stress conditions for miRNA pairs has not been studied in this thesis yet; however, the combinations of miRNAs in the presence or absence of stress can have different regulatory effects on cell viability.

Based on the miRNA pair, the effect on viability changed for higher doses. Relative cell viability for 7.5 nM doses showed significant differences and might be further studied for dose optimization. miRNA mimic treatments with a combination of miR-203a-3p and let-7c-5p were performed for 1.0 nM to 20.0 nM. I did not observe significant differences in cell viability between groups except for 20.0 nM (**Figure 4.42**). The combination of the miR-125a-5p and miR-203a-3p pair showed a significant decrease in cell viability compared to both single miRNA treatments for 7.5 nM (**Figure 4.43**). NCOA2 levels were significantly downregulated in single miRNA and combination groups compared to negative control groups (**Figure 4.49**). However, I did not observe a synergistic difference in the combination group compared to single miRNA groups. The findings of this thesis indicate that the doses selected for the miRNA treatments are important for modulation of the cell viability and expression of their gene targets.

Several studies tested the effects of ranging concentrations of miRNA mimics in the literature. It was shown that viable cell cultures could not be maintained for higher concentrations, and cells started to die. The same study showed that cell growth was inhibited with mimic doses higher than 5 nM [155]. HEK293T cells were transfected with increasing doses of miR-466, and doses higher than 10 nM caused non-specific effects [159]. Doses are also important for the target site competitions of miRNAs, limiting RISC

availability [160]. The doses used for the miRNA mimic treatment need to be further studied for transfection efficiency. Dose-dependent effects of the miRNA treatments need to be studied for doses for a broad range of miRNA concentrations. For some miRNAs, higher doses of mimic concentrations, e.g., 19a, might be needed to observe the repression and changes in viability. Examining other miRNA targets and how downstream elements might be affected upon mimic treatment could be important.

Moreover, the transfection duration needs to be further optimised by following the cellular miRNA concentrations after the treatment. The dilution effect caused by cell division might also be important for miRNA treatments [155]. Some challenges of miRNA therapeutics include avoiding potential toxicities caused by the increased doses of miRNAs and the off-target effects [161], [162]. Also, one must consider the tumour heterogeneity of miRNA expression [163]. miRNAs can be tumour suppressive, oncogenic or both, depending on the tumour type and their targets [164]. New techniques like miR-CLIP can determine more precisely the miRNA-mRNA associations by pull-down assays [165]. It is also important to consider the sponging effects caused by the long non-coding RNAs (lncRNAs). lncRNA H19 showed sponging effects on miR-106a [165]. We have focused on miRNAs that do not have overlapping binding sites. However, synergistic or antagonistic interactions of miRNAs may depend on the target site [166]. miRNAs target multiple genes ranging from 30 to 250; compared to siRNAs, the sequence complementarity with the target sequence is between 20 to 90% [161]. Therefore, multiple targets of miRNAs are a major challenge in miRNA therapeutics.

MYC is a proto-oncogene that promotes cellular growth and proliferation [167]. Knockdown of *MYC* induces proliferation and induces apoptosis in cancer cell lines. Suppression of *MYC*

has reversing effects on tumorigenesis. Therefore, efforts have been made to target *MYC* using siRNAs, shRNAs and antisense oligonucleotides [168]–[170]. In my studies, upon treatment with 7.5 nM miR-19a-3p and let-7c-5p, the miR-19 levels increased in the single miR-19a treatment and combination groups, although to variable degrees. Similarly, mimic treatments increased the let-7c-5p levels in single and combination groups (**Figure 4.44**). However, their target gene *MYC* expression did not show the expected decrease for miR-19a-3p single or combination treatment (**Figure 4.48**). Jin et al. showed that mRNA levels in HeLa cells that are transfected with the miR-17-92 cluster started to recover 12 hours post-transfection [155]. *MYC* levels too might start to recover until 48h post transcription. Transfections with lower doses might not be effective in suppressing the target gene expression for certain miRNAs. In literature, it is also shown that higher doses of miRNA concentrations may be required for adequate silencing of the mRNA target [158]. In particular, miRNAs with many targets can repress their target mRNAs to a lesser extent [157]. Even though miRNA mimic treatments increased the miRNA concentrations in the cells based on the qPCR results, the decrease in *MYC* levels was not as expected. miR-19a-3p has many targets [171] and might need higher doses for *MYC* repression. One important factor in miRNA therapeutics is that miRNAs are unstable and transient in cells [172]. Increasing doses of miR-19a treatment showed increased miR-19a levels, but the increase was not proportional to the doses, suggesting that miR-19 might be degraded in the cell [173].

SLC38A2 is another gene identified by the miRCoop algorithm, is essential for glutamine transportation across the plasma membrane, and its overexpression promotes cancer progression [174]. *SLC38A2* controls cellular proliferation through the mTOR pathway. *SLC38A2* is upregulated in breast cancer, especially the triple-negative breast cancers [175];

yet its role in hepatocellular carcinoma remains unclear. Upon miR-125a-5p mimic treatment, miR-125a-5p levels were upregulated up to 12-fold in combination and single miR-125a treatment. miR-203a-3p levels were upregulated compared to the negative control group upon mimic treatment (**Figure 4.46**). Their target gene, SLC38A2, was downregulated in single mimic and combination groups (**Figure 4.47**). Yet this decrease was not significant. Higher doses of miRNAs might be necessary for better repression of the gene.

NCOA2 promotes the growth of breast cancer cells by positively regulating the MAPK/ERK pathway [176]. Depletion of *NCOA2* inhibits the WNT signalling pathway in gastric cancers [177]. *NCOA2* is targeted by miR-19a-3p and miR-125a-5p. miR-19a-3p levels were upregulated upon 48h mimic treatment, but for the combination group, the levels were not upregulated as in the single mimic group (**Figure 4.44**). miR-125a-5p levels were on the other hand upregulated up to 4-5-fold in treatment groups (**Figure 4.44**). Due to p-value adjustment method, Bonferroni, I did not observe the significance between groups. For multiple test corrections, other test methods can be tried. Genes identified by miRCoop have been dysregulated in several diseases, including cancers. These genes can be potential targets for therapy, and synergistic miRNA pairs are an interesting option for RNA therapeutics. Both MYC and NCOA2 are transcription factors that are targeted by microRNAs, In the future, for miRCoop triplets activator and repressor roles of the transcription factors must be considered and tested in vitro. Targets of the transcription factors should be investigated in terms of expression and qPCR results shouldn't be limited to their direct targets. RNA-sequencing experiments can be incorporated to miRCoop algorithm for triplet prioritization with differentially expressed miRNA and mRNAs. Based on the RNA-seq results genes that are downregulated and share common binding sites with

the transfected miRNA mimics can be studied for their synergistic relationship. Differentially expressed genes that are targeted by miRNA pairs can be also used in functional enrichment analyses to investigate the biological processes that are regulated by their synergism and in vitro tests can be designed according, e.g., apoptotic measurements in addition to viability assays. Also, in order to avoid off-target effects of miRNA mimics, miRNAs with unique targets can be utilized in the experiments with ranging combination doses. Such approaches can help us compare the abundance of miRNAs between different cell lines as well. In addition, the miRNA mimic treatments should be tested in different cell lines. The findings regarding the cell lines can be useful for selecting appropriate miRNA pairs for the treatments. Finally, mimics and inhibitors can be used alone or in combination to demonstrate the specificity of the mimics. miRCoop triplets provide a novel outlet for discovering RNAi therapies in HCC cell line in vitro studies and can be extended to other cancers.

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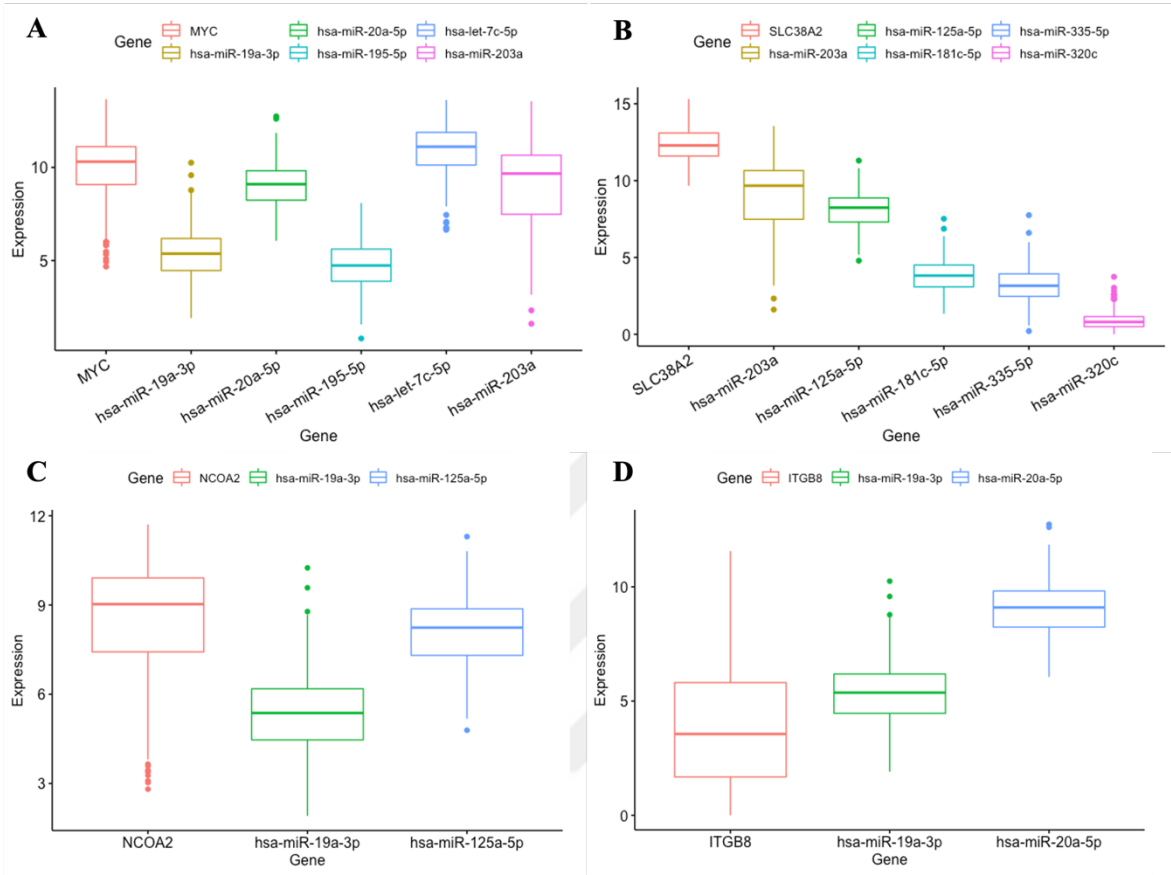
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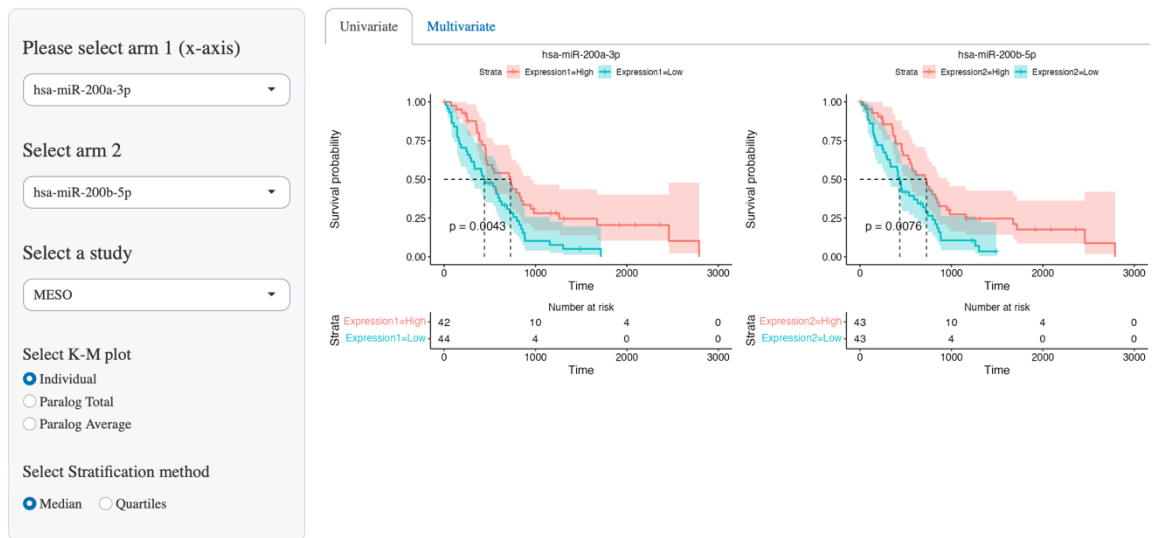
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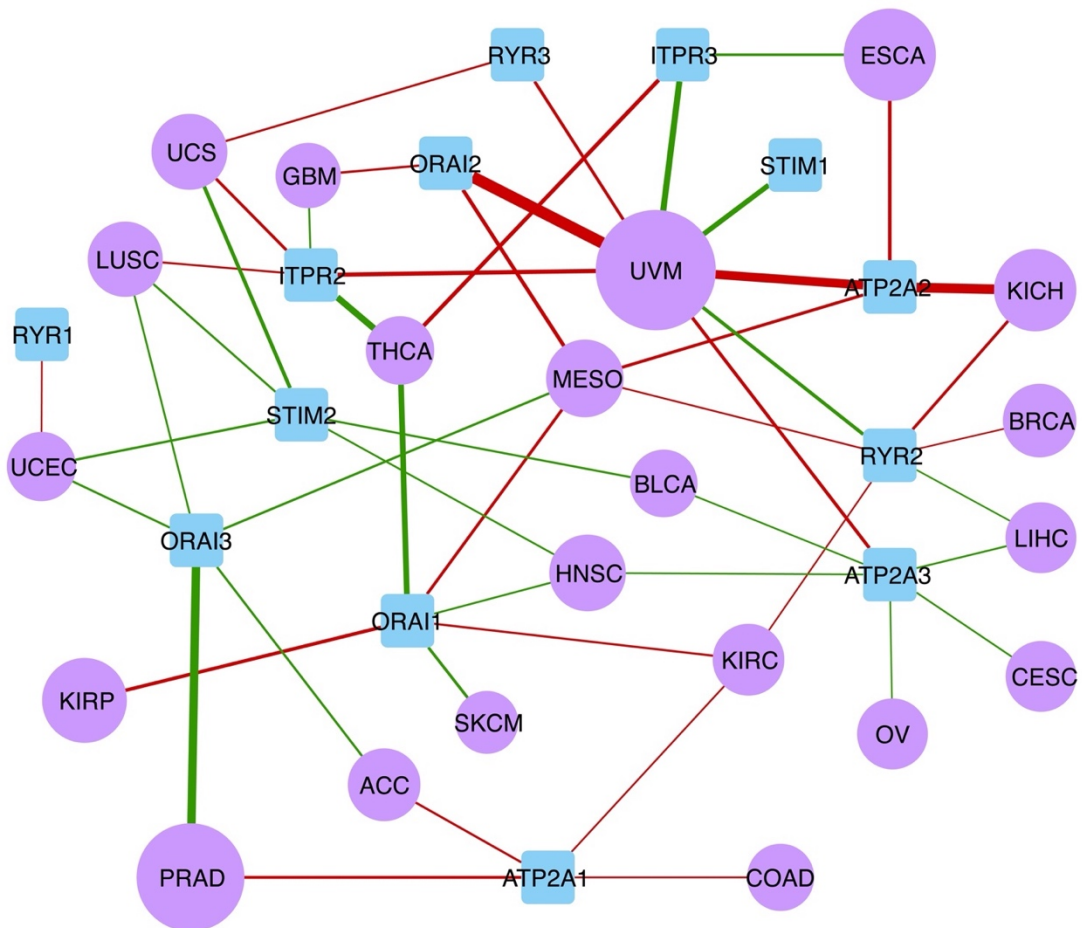
Appendix



Appendix Figure 1. Expressions of miRCoop mRNAs and miRNAs in LIHC. ($\log_2(\text{norm_value}+1)$)



Appendix Figure 2. A screenshot of the “Univariate” subtab from the “K-M plots” tab for miR-200a-3p/-5p for MESO.



Appendix Figure 3. Gene-cancer-by survival network of the PM-ER gene set for DSS. Network showing the survival network of 20 cancers. The node weights represent the AUC, whereas edge weights represent each gene's log2(HR) values. Edge-color represents HR; red, HR > 1 and green, HR < 1. HR, hazard ratio; AUC, area under ROC.

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For Figure 1.1



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