Malaysian Applied Biology (2023) 52(1): 87-100
https://doi.org/10.55230/mabjournal.v52i1.2608

Research

mIR-99a-5p and mIR-148a-3p as Candidate Molecular Biomarkers for the Survival of Lung Cancer Patients

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ABSTRACT

MicroRNA (miRNA) has emerged as a promising biomarker for improving the current state of an early lung cancer diagnosis. Multiple studies have reported that circulating miRNAs are usually combined in a single panel to determine lung cancer risk. In this study, we sought to assess the prognostic predictive values of the potential miRNAs for lung cancer survival among Malaysian patients. The microarray analysis was performed on the isolated miRNA samples of formalin-fixed lung cancer tissues from Malaysian populations. The correlation between miRNA expression and lung adenocarcinoma (LUAD) patient survival was predicted using TGGA data, followed by extensive *in silico* analyses, including miRNA target gene identification, protein-protein interaction (PPI) network construction, subnetwork (SN) detection, functional enrichment analysis, gene-disease associations, and survival analysis in advanced-stage LUAD. Overall, two promising *miR-99a-5p* and *miR-148a-3p* were upregulated in the patients with good survival. We found that 64 *miR-99a-5p* and 95 *miR-148a-3p* target genes were associated with poor prognosis and highly participated in cancer-associated processes, such as apoptosis, mRNA transport and cell-cell adhesion. The density score of 4.667, 3.333, and 3.000 in respective SN1, SN2, and SN3 showed the significant subnetworks of constructed PPI leading to the identification of 17 targets, of which ~79% of them involved in neoplastic diseases. Four high-confidence target genes (*SUDS3*, *TOMM22*, *KPNA4*, and *HMGB1*) were associated with worse overall survival in LUAD patients, implying their critical roles in LUAD pathogenesis. These findings shed additional light on the roles of *miR-99a-5p* and *miR-148a-3p* as potential biomarkers for LUAD survival.

Key words: Bioinformatics, lung cancer, microRNA, molecular biomarker, prognostication

Article History
Accepted: 1 April 2023
First version online: 3 April 2023

Cite This Article:
Abdullah-Zawawi, M.-R., Mohamad-Mokhtar, M.-F., Syafruddin, S.E., Ibrahim, F.F., Mohamed Rose, I., Harun, R. & Abdul Murad, N.A. 2023. MIR-99A-5P and MIR-148A-3P as candidate molecular biomarkers for the survival of lung cancer patients. Malaysian Applied Biology, 52(1): 87-100. https://doi.org/10.55230/mabjournal.v52i1.2608

INTRODUCTION

Lung cancer is among the most diagnosed cancer-related diseases that cause the highest incidence and mortality rates worldwide (Siegel *et al*., 2021). Non-small cell lung cancer (NSCLC) makes up 80 – 85% of lung cancer cases (Siegel *et al*., 2021). It begins in mucus-producing gland cells in the airway lining and progresses into adenocarcinoma (Travis *et al*., 2015). In Malaysia, lung cancer was one of the ten most common cancers accounting for 11,256 (9.8%) cases from 2012 to 2016 (Ab Manan *et al*., 2019). Based on ethnicity, the frequency of lung cancer is high in Chinese (16.0%), followed by Malay (12.5%) and Indian (5.7%) (Ab Manan *et al*., 2019). In 2018, 4,686 (10.7%) new cases of lung cancer in both sexes within all ages were reported in the GLOBOCAN database, with an estimated total death of 4,057 (14.4%) (Ferlay *et al*., 2019).

The high mortality rate among lung cancer patients is due to an ineffective screening strategy, poor five-year prognosis, and high recurrences (Lu *et al*., 2006). Therefore, it is critical to determine the molecular signatures for effective screening strategy, prediction of recurrent disease and enhanced prognosis in lung cancer patients (Liu *et al*., 2020). To date, various treatments are being used to slow cancer cell progression and increase overall patient survival, including chemotherapy, radiotherapy, targeted therapy, and surgical and traditional methods (Kan & Chan, 2016). However, many challenges occur during the treatment process, including the lack of specialists, especially thoracic surgeons, clinical and radiation oncologists,

insufficient data in the national cancer registry, expensive treatment costs that specifically involve molecular testing and most importantly, lack of awareness among Malaysians to do screening test especially when they already in the late stage III and IV (Rajadurai *et al*., 2019).

The miRNAs have been reported to regulate numerous biological processes related to cancer, including apoptosis, proliferation, stress response, survival, and metastasis (Garofalo & Croce, 2011). Loss and gain of miRNA function may contribute to cancer progression through upregulation and silencing of target mRNAs (Iorio & Croce, 2009). The changes in target mRNA cause genetic changes that lead to the initiation of cancer activities, such as inhibition of growth signals, evading programmed cell death pathways and tumour growth (MacFarlane & Murphy, 2010). In the past few years, several studies have identified the role of miRNA in predicting a good prognosis for lung cancer. Yang *et al*., (2018) reported that *miRNA-183* suppresses tumours in NSCLC by down-regulating the oncogene Metastasis Associated 1 (*MTA1*). Confirmation of reduced cell migration and invasion abilities in the *miR-183* mimic and increased abilities in the *miR-183* inhibitor group was achieved through luciferase reporter gene assay. Another miRNA study found that *microRNA-148b* regulates tumour cell inhibition by blocking the pathways of the Mitogen-activated protein kinase (*MAPK*) or c-Jun NH2-terminal Kinase (*JNK*) (Lu et al., 2019). Although NSCLC research is expanding yearly, the knowledge of the progression and therapy of the tumour in most cancers, including LUAD, is still not fully understood, and most published miRNA biomarkers are detected in the Caucasian population. Therefore, the characterisation of miRNA in our lung cancer patients is crucial as they may serve as molecular biomarkers to predict the future survival of the Malaysian population.

In this present study, the miRNA expression profiles of the formalin-fixed paraffin-embedded (FFPE) lung cancer tissues were obtained from UKM Medical Centre (UKMMC), comprising samples from patients who had either (i) good (n=13) or (ii) poor survival (n=9). The screening was conducted to identify significantly upregulated miRNAs at least 2-fold and $P < 0.05$ in the patients with good survival. Survival analysis was performed to determine our patient's associated miRNAs with good survival concordance with the TCGA LUAD patient cohort. Then, miRNA-mRNA data and filtering were employed to obtain the target genes of miRNA owing to developing a PPI network for survival-related genes and clustering them into a functionally associated subnetwork. GO and pathway enrichment was conducted on miRNA target genes to discover associated biological activities of genes in LUAD development. Furthermore, survival analysis of miRNA putative targets was performed to validate their lung cancer survival rate. Altogether, we used the abovementioned analyses to discover candidate miRNA as molecular biomarkers for LUAD patient survival that were previously unreported.

MATERIALS AND METHODS

Samples collection

This retrospective study used the formalin-fixed paraffin-embedded (FFPE) lung cancer tissues from the Department of Pathology, UKM Medical Centre (UKMMC). The ethical approval for this study was obtained from the Universiti Kebangsaan Malaysia Medical Research Ethics Committee (UKM1.5.3.5/244/UMBI-002-2012). These FFPE lung cancer tissues were collected and archived from patients who underwent treatments at the UKMMC between 2003-2013. The FFPE blocks were processed, stained with haematoxylin and eosin, and examined by pathologists to determine the cancer cells' representation. Only tissues representing 80% of cancerous cells were selected for downstream analysis. The patients' demographic and survival data were collected from the Malaysia Department of Registration database. The patients were stratified into good and poor survival groups; patients who were still alive 1-year post-diagnosis were classified as having good survival.

miRNAs extraction from FFPE lung cancer tissues

miRNAs were extracted from the FFPE lung cancer tissues using the High Pure miRNA Isolation kit (Roche, Germany) according to the manufacturer's protocol. The yield and quality of the extracted miRNAs were determined using the Bioanalyzer Small RNA kit (Agilent Technologies, USA).

miRNAs expression profiling

cDNAs were synthesised from the extracted miRNAs using the cDNA Universal Synthesis Kit II (Qiagen). In total, 10ng of miRNA starting material was converted into cDNA following the manufacturer's recommendation. The miRNA expression level in these archived lung cancer tissues was profiled using the miRCURY LNATM Universal RT microRNA Ready-to-Use PCR, Cancer Focus Panel (Qiagen) according to the manufacturer's protocol. The miRNA expression quantitative real-time PCR (qPCR) data were analysed using the ExiqonGenEx Software version 2.5 (Qiagen, Germany).

miRNAs expression and patient's survival status correlation analysis

The OncoLnc webtool (http://www.oncolnc.org) (Anaya, 2016) was utilised to determine the correlation between miRNAs expression level and lung adenocarcinoma (LUAD) patients' survival status. OncoLnc is a publicly available webtool that curates and allows the correlation analysis between TCGA patients' survival status and the expression level of mRNAs, miRNAs or lncRNAs to be conducted and visualised. Specifically, the OncoLnc webtool was employed to check the TCGA LUAD patient's survival status who had either high or low expression of *miR-146a-5p*, *miR-99a-5p*, *miR-126-3p*, *miR-125b-5p* and *miR-148a-3p*.

In-silico prediction of high-confidence miRNAs target genes

The miRWalk webtool (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) (Dweep *et al*., 2014) was first employed to identify the putative genes that are targeted by these identified miRNAs. The miRWalk webtool utilises eight different miRNAs prediction algorithms and databases: the Diana-microT, miRanda, miRDB, PICTAR, PITA, RNA22, RNA hybrid and Target Scan. GEPIA2 webtool (http://gepia2.cancer-pku.cn/) (Tang *et al*., 2019) was utilised to narrow the list of putative target genes. In brief, the significantly associated putative target genes with low survival status in the TCGA LUAD patient cohort were selected for further analysis (Tomczak *et al*., 2015). Next, to obtain the high-confidence putative target genes, we overlapped the genes identified from the GEPIA2 analysis with miRTarBase. This miRNA prediction webtool curates the functionally validated miRNA-target gene interactions (MTIs). The list of genes identified from the miRWalk-GEPIA2 and miRTarBase analysis were overlapped using the Venn diagram webtool, VENNY2.1 (https://bioinfogp.cnb.csic.es/tools/venny/). The survival analysis of high-confidence miRNAs target genes was performed using Kaplan-Meier Plotter. The survival results by Kaplan-Meier curves, along with 95% confidence intervals (Cis) of hazard ratios (HRs) and log-rank test < 0.05, were considered statistically significant.

Functional enrichment analysis of gene ontology (GO) and cancer-related pathway

The analysis of GO and the related pathway was conducted using publicly available resources from Gene Ontology (http://geneontology.org/) (Gene Ontology Consortium, 2015) and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/pathway.html) (Kanehisa *et al*., 2016) to decipher the biological significance of the miRWalk-GEPIA2 target genes. To functionally enrich the GO biological process and pathways of miRNA target genes, we used the Database for Annotation, Visualization, and Integrated Discovery tool (DAVID v6.8; https://david.ncifcrf.gov/home.jsp) (Huang *et al*., 2007). Parameters for functional enrichment analysis were as follows: Fisher Exact P-value < 0.05, Min. Count ≥ 2 and FDR ≤ 1.

Protein-protein interaction (PPI) analysis and subnetwork detection

A PPI network of miRWalk-GEPIA2 target genes was constructed by retrieving PPI information from the Search Tool for the Retrieval of Interacting Genes database (STRING v11.5; https://string-db.org/) (Szklarczyk *et al*., 2019). The PPI information was extracted using StringApp, a data import plugin installed in Cytoscape v3.7 software, to decipher the interaction among the potential miRNAs target gene at the protein level (Doncheva *et al*., 2019; Shannon *et al*., 2003). To discard the inconsistent interactions from the constructed PPI, we applied a confidence (score) cutoff of ≥ 0.4. Further, the PPI evidence of target genes encoding proteins by STRING was derived from physical and functional associations interactions. To scrutinise the highly connected regions (also known as subnetworks or clusters) in the PPI network, we applied the Molecular Complex Detection (MCODE v2.0) algorithm (Bader & Hogue, 2003). The density (D) of the PPI subnetwork was exploited based on $|E|max = |V|(|V| - 1)/2$, where $|E|max$ represents the maximum number of subnetwork edges and $|V|$ denotes the number of nodes. Meanwhile, the score of the subnetwork, S, is ranked according to $S = D \times |V|$. The default parameters used to generate the subnetworks are as follows: Degree Cutoff = 2, Node Score Cutoff = 0.2, Kappa-score (K-core) = 2 and Maximum Depth = 100 (Bader & Hogue, 2003). We then used Cytoscape v3.7 to visualise the constructed PPI network and subnetworks (Shannon *et al*., 2003). Therefore, the distributions of node degrees were calculated using NetworkAnalyzer to detect high-degree nodes in the subnetwork (Assenov *et al*., 2008). For subnetwork-based gene-disease association, all curated biomarker associations between target genes and associated diseases were retrieved from DisGeNET (https://www.disgenet.org) (Piñero *et al*., 2019) using the DisGeNET Cytoscape plugin (Piñero *et al*., 2021).

RESULTS

Patients' demographic and clinical data

In total, we performed miRNA expression profiling in twenty-two lung cancer FFPE tissues collected from patients who had undergone treatment at the UKMMC between 2003-2013. Of these twenty-two lung cancer patients, thirteen were male (59.1%), while nine were female (40.9%). The patients' mean age was 67.59 $\pm$ 10.42 years old. In terms of ethnic groups, twelve patients were Chinese (54.5%), nine were Malay (40.9%), and one was Indian (4.6%). All the profiled lung cancer samples were the non-small cells subtype (NSCLC). On top of this, most of the analysed samples were advanced-stage lung cancer; stage 3B (n=13), 4A (n=3) and 4B (n=5). The patients' demographic and clinical data are summarised in Table 1.

miRNAs profiling in good and poor survival patient groups

The lung cancer FFPE tissues analysed in this study were stratified into two groups; samples collected from patients with either (i) good or (ii) poor survival. Patients who survived at least 1-year post-diagnosis were classified as having good survival. Next, we were prompted to profile and compare the miRNAs expression landscape between these good and poor survival patient groups. The aim was to identify the miRNAs that could be developed and utilised as novel and robust lung cancer prognostic markers. To achieve this objective, the cDNA-converted miRNAs were subjected to qPCR-mediated miRNAs expression profiling using the miRCURY LNATM Universal RT miRNAs Ready-to-Use PCR Cancer Focus Panel. This cancer focus panel contained primer pairs for miRNAs that have been previously reported to involve in cancer pathogenesis. Overall, we discovered five miRNAs that were significantly upregulated at least 2-fold (P-value < 0.05) in the patients with good survival. These miRNAs were *miR-146a-5p*, *miR-99a-5p*, *miR-126-3p*, *miR-125b-5p* and *miR-148a-3p* (Table 2).

Table 1. Demographic data of lung cancer patients from the UKMMC.

Patient	Prognosis	Survival (days)	Gender	Age	Race	Diagnosis
1	Good	927	Male	64	Malay	Adenocarcinoma
2		549	Male	65	Chinese	Adenocarcinoma well differentiated
3		700	Female	72	Chinese	Primary adenocarcinoma
4		2261	Female	49	Chinese	Squamous cell carcinoma, poorly differentiated
5		1134	Female	69	Chinese	Adenocarcinoma
6		707	Male	74	Chinese	Lung adenocarcinoma, moderately differentiated
7	Poor	53	Male	51	Chinese	Squamous cell carcinoma, poorly differentiated
8		13	Male	77	Malay	Primary adenocarcinoma
9		21	Female	84	Chinese	Squamous cell carcinoma
10		56	Male	74	Chinese	Squamous cell carcinoma, moderately differentiated
11		13	Female	65	Malay	Adenocarcinoma with bronchioalveolar carcinoma compartment
12		1	Male	75	Malay	Poorly differentiated adenocarcinoma
13		49	Female	76	Chinese	Lung Adenocarcinoma
14		21	Male	69	Malay	Small cell carcinoma
15		35	Male	70	Chinese	Adenocarcinoma, poorly differentiated
16		98	Male	84	Malay	Poorly differentiated, adenocarcinoma
17		249	Male	75	Chinese	Large cell carcinoma, undifferentiated
18		175	Male	70	Chinese	Adenocarcinoma
19		198	Female	55	Malay	Squamous cell carcinoma
20		69	Male	59	Indian	Large cell carcinoma
21		322	Female	64	Malay	Adenocarcinoma, moderately differentiated
22		140	Female	46	Malay	Adenocarcinoma

Table 2. List of significantly upregulated miRNAs with good survival.

miRNAs	Normality test	Fold change (High vs low)	p-value
miR-146a-5p	Passed	3.40	0.0104
miR-99a-5p	Passed	2.48	0.0285
miR-126-3p	Passed	2.14	0.0331
miR-125b-5p	Passed	2.47	0.0335
miR-148a-3p	Passed	2.36	0.0346

Validating the miRNAs profiling findings

Next, we checked whether our present findings were concordant with what had been previously reported. To this end, we utilised the TCGA LUAD dataset (https://portal.gdc.cancer.gov/projects/TCGA-LUAD), which is the most comprehensive molecular analysis of lung cancer samples to date (n=585 cases). By utilising the OncoLnc webtool, we observed that high expression of *miR-146a-5p*, *miR-99a-5p*, and *miR-148a-3p* was significantly associated with better prognosis in the TCGA LUAD patient cohort. However, we found that the expression levels of *miR-125b-5p* and *miR-126-3p* did not correlate with the prognosis status of TCGA LUAD patients due to a non-significant P-value of > 0.05 (Figure 1). Three out of five miRNAs we found to be significantly associated with good survival in our patient cohort were concordant with better prognoses in the TCGA LUAD patient cohort. As the P-value of *miR-146a-5p* is close to 0.05, we focused on *miR-99a-5p* (P-value = 0.0123) and *miR-148a-3p* (P-value = 0.0269) as the significant miRNA in the following analysis.

Predicting *miR-99a-5p* and *miR-148a-3p* target genes

Next, we aimed to identify high-confidence putative target genes for *miR-99a-5p* and *miR-148a-38p.* The analysis pipeline to attain this objective is depicted in Figure 2. We first performed a parallel analysis using two different miRNA target genes prediction webtools, miRWalk and MirTarBase. After removing the redundant genes, the miRWalk predicted 611 and 806 putative target genes for *miR-99a-5p* and *miR148a-3p*, respectively. The MirTarBase, on the other hand, predicted that *miR-99a-5p* and *miR148a-3p* had 133 and 213 putative target genes, respectively. In the subsequent analysis step, we focused on narrowing down the miRWalk prediction gene lists. Since high expression of *miR-99a-5p* and *miR148a-3p* were associated with good survival, we postulated that these miRNA's target genes should be downregulated in patients with better prognoses and vice versa. Therefore, we employed this rationale to prioritise the *miR-99a-5p* and *miR148a-3p* target genes further, as predicted by the miRWalk webtool. To achieve this objective, we utilised the GEPIA2 webtool to observe if there was any correlation between the expression level of these genes and the TCGA LUAD patients' prognosis. The GEPIA2 analysis found that high expression of 64 out of the 611 *miR-99a-5p* putative target genes was significantly associated with poor prognosis. Meanwhile, for the *miR-148a-3p*, high expression of 95 putative target genes was associated with poor survival, bringing the total number of unique target genes to 154.

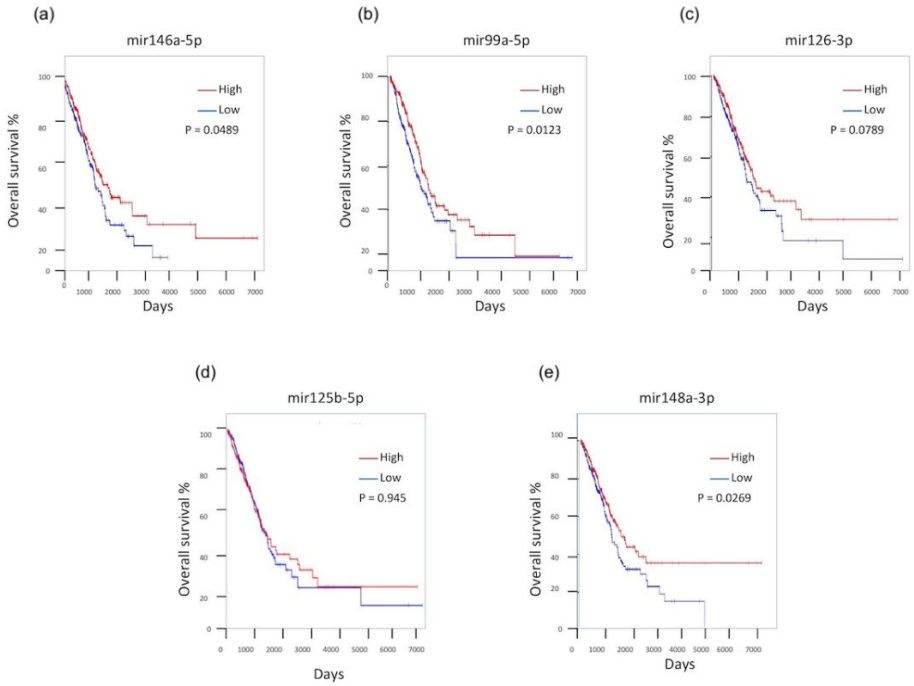

Fig. 1. Overall survival analysis for TCGA LUAD patient cohorts. High (red line) and low (blue line) expression of (a) *mir146a-5p*, (b) *mir99a-5p*, (c) *mir126-3p*, (d) *mir125b-5p*, and (e) *mir148a-3p* was analyzed using Kaplan-Meier plot

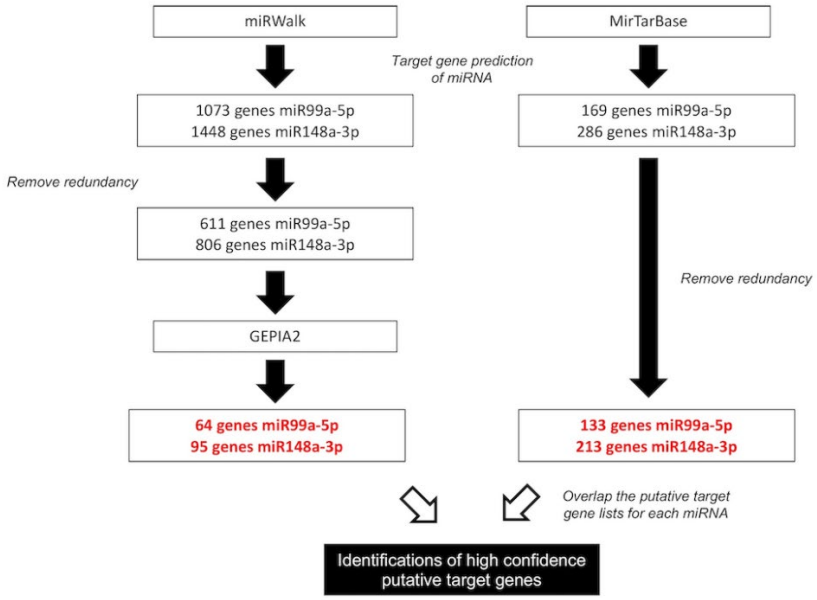

Fig. 2. Analysis pipeline for obtaining high confidence mir99a-5p and mir148a-3p putative target genes

GO and pathway enrichment analysis of *miR-99a-5p* and *miR148a-3p* target genes

Functional enrichment analysis was conducted on the target gene sets of *miR-99a-5p* and *miR148a-3p* to identify biological processes and pathways that are statistically associated with lung cancer. By analysing the BP, we found the putative target genes of *miR-99a-5p* we enriched in positive regulation of apoptotic process (GO:0043065), mRNA transport (GO:0051028), regulation of translation (GO:0006417), response to virus (GO:0009615), histone H4 deacetylation (GO:0070933) and G1/S transition of the mitotic cell cycle (GO:0000082) (Figure 3a). Meanwhile, stem cell division (GO:0017145), neuron migration (GO:0001764), positive regulation of DNA ligation (GO:0051106), modulation by virus of host process (GO:0019054), cerebral cortex radially oriented cell migration (GO:0021799), nucleobase-containing compound metabolic process (GO:0006139), G2/M transition of mitotic cell cycle (GO:0000086). cell-cell adhesion (GO:0098609) and lipid

biosynthetic process (GO:0008610) are among the BP that were enriched among the target gene sets of *miR-148a-3p* (Figure 3b). The enrichment analysis of KEGG pathways demonstrated the association of chronic myeloid leukaemia (hsa05220), small cell lung cancer (hsa05222), hepatitis B (hsa05161) and Huntington's disease (hsa05016) among *miR99a-5p* target genes (Figure 4c), whilst *miR148a-3p* target genes were closely associated with sphingolipid signalling pathway (hsa04071), hippo signalling pathway (hsa04390) and pancreatic cancer (hsa05212) (Figure 4d). The detailed results for the following BP and pathway are listed in Table 3. Our functional enrichment analysis revealed that the identified biological function and pathways could significantly affect lung cancer survival. However, further study is required to validate our findings experimentally.

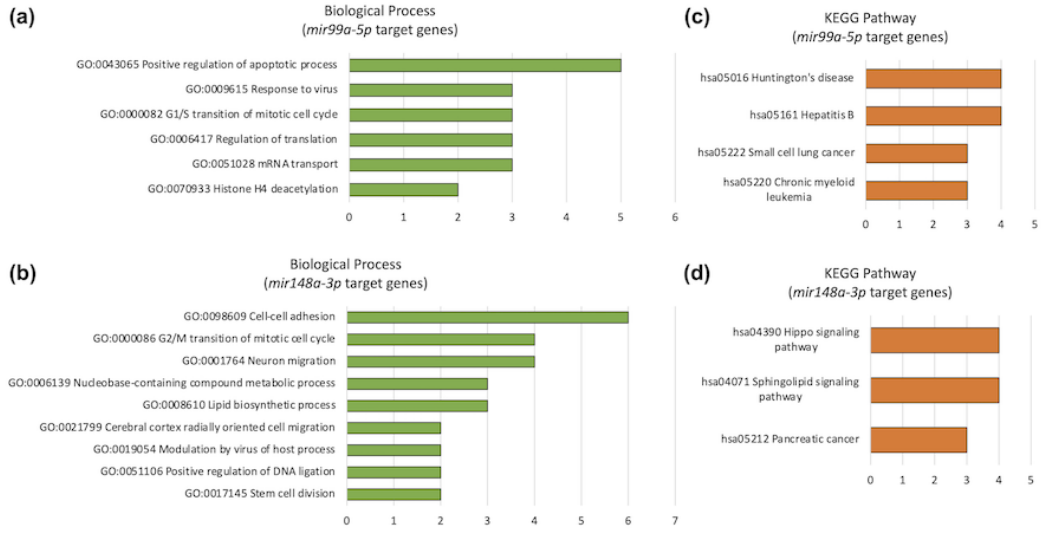

Fig. 3. Functional enrichment analysis of target genes. The bar graph depicts the biological process (BP) involved in both target genes of (a) *miR99a-5p* and (b) *miR148a-3p*. Meanwhile, (c) and (d) denote KEGG pathway of target genes from *miR-99a-5p* and *miR148a-3p*, respectively. The count of target genes is assigned to the x-axis and GO term of BP and pathway to the y-axis

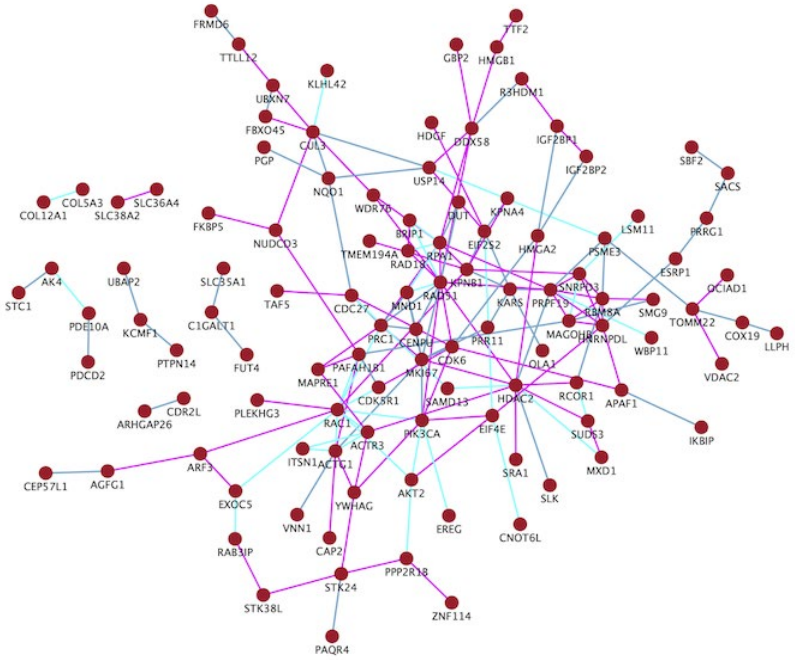

Fig. 4. PPI network highlights the physical and functional associations of *miR-99a-5p* and *miR148a-3p* target genes from the STRING database. The nodes represent proteins while the edges are their interactions. Different colouring of edges represents the evidence of known interactions from curated databases (turquoise), physical interactions/experimentally validated (purple) and functional associations (grey)

Table 3. GO biological process and KEGG pathways of enriched *miR-99a-5p* and *miR148a-3p* downstream targets

Identity	Term	Count	Genes	%	*p*-value	Fold enrichment
GO:0051028	mRNA transport	3	RBM8A, IGF2BP1, MAGOHB	4.6875	0.009649	19.8487
GO:0006417	Regulation of translation	3	RBM8A, AKT2, EIF4E	4.6875	0.011718	17.94017
GO:0043065	Positive regulation of apoptotic process	5	APAF1, ITSN1, HMGA2, PDCD2, SUDS3	7.8125	0.014724	5.182716
GO:0070933	Histone H4 deacetylation	2	HDAC2, RCOR1	3.125	0.028057	69.10288
GO:0000082	G1/S transition of mitotic cell cycle	3	CDK6, RPA1, EIF4E	4.6875	0.041199	9.145969
GO:0009615	Response to virus	3	CDK6, DDX58, HMGA2	4.6875	0.047196	8.480808
GO:0008610	Lipid biosynthetic process	3	AGPS, ACSL4, PRPF19	3.157895	0.002425	39.98095
GO:0098609	Cell-cell adhesion	6	SLK, TES, STK24, OLA1, UBAP2, MAPRE1	6.315789	0.01459	4.130873
GO:0001764	Neuron migration	4	SATB2, FBXO45, CDK5R1, PAFAH1B1	4.210526	0.0183	7.107725
GO:0017145	Stem cell division	2	CUL3, PAFAH1B1	2.105263	0.021034	93.28889
GO:0051106	Positive regulation of DNA ligation	2	RAD51, HMGB1	2.105263	0.021034	93.28889
GO:0006139	Nucleobase-containing compound metabolic process	3	BRIP1, DUT, AK4	3.157895	0.027804	11.42313
GO:0000086	G2/M transition of mitotic cell cycle	4	MAPRE1, HAUS2, YWHAG, PAFAH1B1	4.210526	0.0363	5.447526
GO:0019054	Modulation by virus of host process	2	KPNA4, KPNB1	2.105263	0.036523	53.30794
GO:0021799	Cerebral cortex radially oriented cell migration	2	FBXO45, RAC1	2.105263	0.046713	41.46173
hsa05161	Hepatitis B	4	CDK6, APAF1, DDX58, AKT2	6.25	0.018532	6.77734
hsa05220	Chronic myeloid leukemia	3	HDAC2, CDK6, AKT2	4.6875	0.032037	10.23661
hsa05016	Huntington's disease	4	HDAC2, APAF1, VDAC2, RCOR1	6.25	0.038291	5.118304
hsa05222	Small cell lung cancer	3	CDK6, APAF1, AKT2	4.6875	0.043379	8.671008
hsa04071	Sphingolipid signaling pathway	4	CERS6, PIK3CA, PPP2R1B, RAC1	4.210526	0.026148	6.034211
hsa04390	Hippo signaling pathway	4	FRMD6, PPP2R1B, ID1, YWHAG	4.210526	0.046782	4.795399
hsa05212	Pancreatic cancer	3	RAD51, PIK3CA, RAC1	3.157895	0.047373	8.355061

PPI network and subnetworks detection of *miR-99a-5p* and *miR148a-3p* target genes

The physical and functional associations of 154 *miR-99a-5p* and *miR148a-3p* target genes were retrieved from the STRING database to build the PPI network. In total, we discovered 172 interactions among 109 putative downstream genes. The remaining 45 genes were identified as single nodes and removed due to the absence of interactions among the genes. Following the elimination of single nodes, a PPI network of the target genes was established based on a confidence score of ≥ 0.4 (Figure 4). In addition, three significant subnetworks representing densely connected regions were identified with the density (D) of the subnetwork (SN) 1 = 4.667, SN2 (D = 3.333) and SN3 (D = 3.0) (Table 4).

PRPF19 and *RAD51* had the highest degree of connectivity in SN1, where the number of edges was n=5, followed by *EIF2S2* and *KPNB1* in SN2 (n=3), and *IGF2BP2*, *IGF2BP1* and *HMGA2* in SN3 (n=3) (Figure 5). The 17 shortlisted genes from subnetworks were examined further by peeking into their associations with LUAD or/and related diseases. However, only 14 genes were curated and associated with 14 disease categories based on DisGeNET scores ≥ 0.3. Among them, ~79% of the genes are involved in multiple types of neoplastic diseases caused by abnormal cell growth that can be benign or malignant.

SN1 highlighted the association of several *miR-99a-5p* and *miR148a-3p* target genes with neoplasms, especially *MKI67* (n = 29), *RAD51* (n = 23), *SNRPD3* (n = 10), *PRC1* (n = 9), *PRPF19* (n = 4), *MAGOHB* (n = 3) and *CENPU* (n = 1). SN1 was derived from *PRPF19* and *RAD51*, enriched in lipid biosynthetic process and positive regulation of DNA ligation, respectively. In SN2, an enriched *KPNB1* (n = 3) in modulation by virus of host process was found associated with infections, immune and neoplasm, suggesting its function in virus-mediated oncogenesis and antiviral immune responses as reported in DisGeNET. For SN3, *HMGA2* (n = 14) and *IGF2BP1* (n = 1) were associated with neoplasms, whereas *IGF2BP1* with a mental disorder, endocrine systems, and nutritional and metabolic diseases. We suggested association of reported genes with neoplastic diseases and other disease-related categories may directly or indirectly contribute to the advanced stage LUAD. All the target genes and their ranked associated disease categories are presented in Table 5.

Table 4. Three significant subnetworks identified from the PPI network by MCODE.

Subnetwork	Density score	No. of target genes	No. of interactions	Target genes
1	4.667	10	21	MKI67, PRPF19, MAGOHB, HNRNPDL, RBM8A, RAD51, SNRPD3, PRC1, CENPU, MND1
2	3.333	4	5	KARS, KPNA4, EIF2S2, KPNB1
3	3.000	3	3	IGF2BP2, IGF2BP1, HMGA2

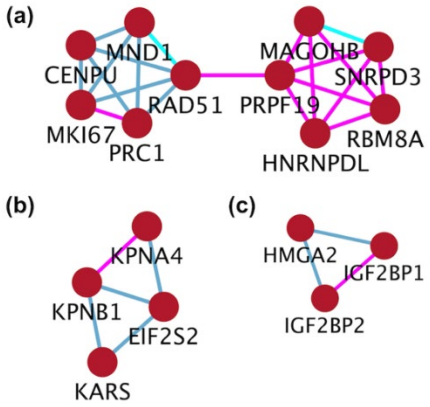

Fig. 5. Subnetworks of the densely connected region from the PPI network using MCODE. (a) Subnetwork 1; (b) Subnetwork 2; (c) Subnetwork 3. Purple and turquoise colours of edges represented physical interactions and grey as functional association interaction

Table 5. The shortlist of 14 genes from three subnetworks based on their significance in the top 2 highest disease categories

Subnetwork	Gene	No. of degree in the subnetwork	Disease category	Disease count
1	RAD51	5	Neoplasms Skin and connective tissue	23
	MAGOHB	4	Neoplasms Endocrine system	3
	PRC1	4	Neoplasms Skin and connective tissue	9
	MKI67	4	Neoplasms Digestive system	29
	PRPF19	5	Neoplasms Digestive system	4
	CENPU	4	Neoplasms Digestive system	1
	SNRPD3	4	Neoplasms Digestive system	10
	RBM8A	4	Musculoskeletal Hemic and Lymphatic	3
	HNRNPDL	4	Musculoskeletal Nervous system	2
2	EIF2S2	3	Neoplasms Skin and connective tissue	5
	KPNB1	3	Infections Immune Neoplasms	3
3	IGF2BP1	2	Neoplasms Digestive system	1
	IGF2BP2	2	Mental disorders Nutritional and metabolic Endocrine system	3
	HMGA2	2	Neoplasms Pathological condition Congenital, hereditary, and neonatal	14

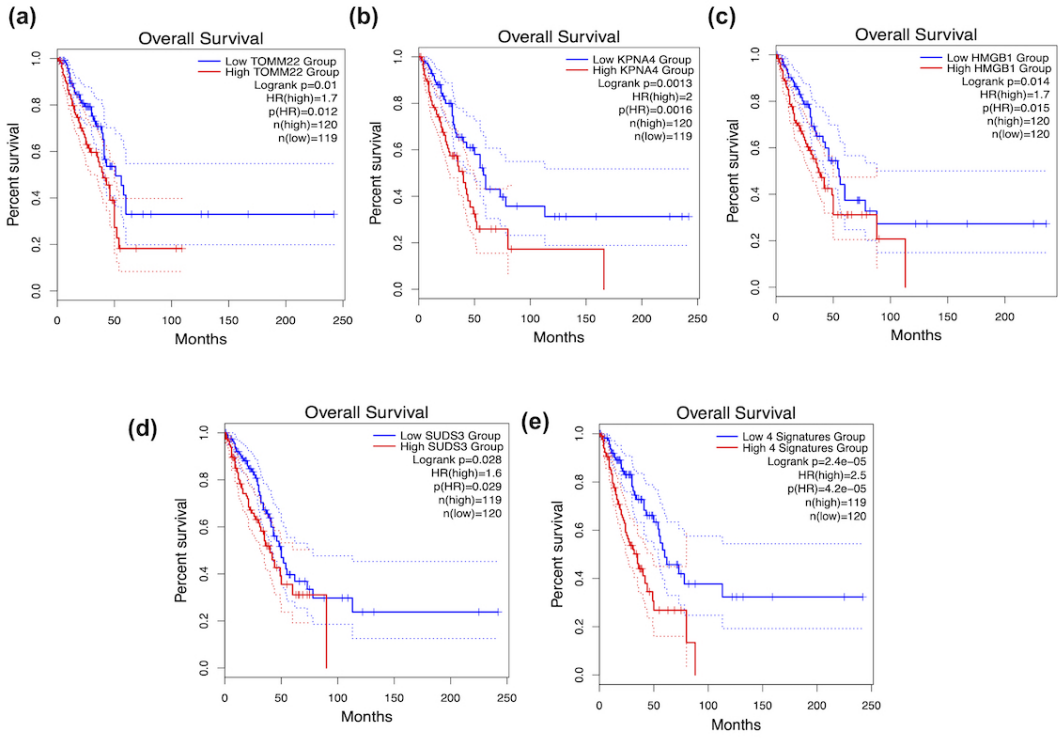

Fig. 6. Prognostic analysis of gene expression in advanced stage LUAD using GEPIA2 database. The overall survival analysis was based on mRNA expression levels of (a) *TOMM22*, (b) *KPNA4*, (c) *HMGB1*, (d) *SUDS3* and (e) signature survival status in the TCGA LUAD patient cohort. The dotted line represents the 95% confidence interval. Gene expression levels of high and low expression groups demonstrate in solid red and blue lines, respectively

Identification of high-confidence *miR-99a-5p* and *miR148a-3p* putative downstream targets

To infer high-confidence putative downstream targets for *miR-99a-5p* and *miR148a-3p*, we combined the miRWalk-GEPIA2 target genes with functionally validated miRNA-mRNA interactions from miRTarBase-curated target genes employed the miRTarBase webtool, which curates the functionally validated miRNA-mRNA interactions. By utilising this webtool, we found 13 and 21 experimentally validated target genes for *miR-99a-5p* and *miR148a-3p*, respectively. Upon overlapping these two gene lists for each miRNA, we found *SUDS3* and *TOMM22* were the putative target genes for *miR-99a-5p*, each of the miRNAs. *KPNA4* and *HMGB1* were the identified putative target genes for *miR148a-3p*.

High expression of *SUDS3*, *TOMM22*, *KPNA4* and *HMGB1* in advanced stage LUAD

By utilising the GEPIA2 webtool, we found that these genes' expression and clinical outcomes were higher in advanced-stage lung adenocarcinoma (Figure 6). The results demonstrated that high expression of *TOMM22* (HR = 1.7, P = 0.01), *KPNA4* (HR = 2.0, P = 0.0013), *HMGB1* (HR = 1.7, P = 0.014), and *SUDS3* (HR = 1.6, P = 0.028) were associated with worse overall survival for LUAD patients. Based on these observations, these genes could play important roles in supporting lung adenocarcinoma pathogenesis and are worth comprehensively interrogating in future studies. Also, to functionally validate whether *SUDS3/TOMM22* and *KPNA4/HMGB1* are the direct downstream targets of *miR-99a-5p* and *miR-148a-3p* in lung adenocarcinoma, respectively.

DISCUSSION

In lung cancer, miRNAs have been shown to function as either tumour suppressors or oncogenes. Dysregulation of miRNAs may lead to aberrant expression of their target genes, ultimately resulting in tumorigenesis (Bartel, 2004; Bracken *et al*., 2016). Regulating genetic changes in cancer is a complex mechanism, as a single miRNA can target hundreds or thousands of mRNA molecules. Hence, the potential of miRNA as a specific target for treating lung cancer remains uncertain. In this study, *miR99a-5p* and *miR148a-3p* were identified as potential biomarkers of survival in lung cancer patients. Several studies have shown that *miR148a-3p* acts as a tumour-suppressive miRNA by targeting oncogenic pathways, such as Ras, MAPK Erk and PI3K/AKT signalling (Xie *et al*., 2019; Yin *et al*., 2020), whereas *miR99a-5p* suppressed mTOR signalling (Tsai *et al*., 2018). Identifying the target genes regulated by the passenger strands of *miR-99a-5p* and *miR-148a-3p* could offer valuable insights into the molecular mechanisms of LUAD.

To investigate the role of target genes in the GO biological process (BP) and pathway of LUAD, functional enrichment analysis was conducted by querying a list of target genes to DAVID v6.8. The enrichment

analysis of BP indicated that *miR-99a-5p* targets were predominantly enriched in positive regulation of apoptotic process, response to virus, G1/S transition of mitotic cell cycle, regulation of translation and mRNA transport. In cancer cells, miRNA-mediated regulation of apoptosis control cell survival by negatively regulating the oncogenes or genes involved in cell differentiation or apoptosis (Othman & Nagoor, 2014). Apoptosis and cell proliferation have also been linked with aberration in mitotic cell cycles (Li *et al*., 2017). Following our findings, *HMGA2* and *PDCD2* were significantly enriched in the apoptosis process. Shi *et al*., (2016) reported that knocking out *HMGA2* increased the expression of pro-apoptotic genes Bax and reduced cell proliferation, indicating the anti-apoptotic ability of *HMGA2* in cancer cells. A recent study by Leonardi *et al*., (2021) reported that CDK was dysregulated during virus infection to bypass the cell cycle in tumorigenesis. Thus, our target genes are consistent with enrichment in response to viruses and G1/S and G2/M transition of mitotic cell cycles.

Furthermore, we discovered that regulation of translation and mRNA transport were significant among the enriched BP and are crucial as key drivers of cancer initiation and progression. In our findings, *EIF4E* and *IGF2BP1* were associated with good survival in the TCGA LUAD patient cohort. *EIF4E*, a cap-binding protein, interacts with the mRNA and undergoes recruitment of ribosomes, initiating translation and activating downstream oncogenes (Amorim *et al*., 2018; Jiang *et al*., 2013). *EIF4E* promotes cancer cell survival by translating mRNAs that regulate the production of intracellular reactive oxygen species (ROS) (Song *et al*., 2021). ROS levels in cancer cells are higher than in normal cells. According to Truitt *et al*., (2015), the reduction of *EIF4E* results in excessive ROS production, revealing the importance of translation regulation under stress conditions. *IGF2BP1* is a recognition protein that plays a role in the 6-methyladenine (m^6A) modification that influences RNA transport and translation of tumour cells (Song *et al*., 2021). Altogether, dysregulation of m^6A reader *IGF2BP1* is closely associated with cancer progression, where the oncopeptide RBRP of *IGF2BP1* promotes tumorigenesis by enhancing m^6A recognition and increasing the stability of target oncogenic c-Myc mRNAs (Zhu *et al*., 2020).

The enrichment of cancer-related pathways, including small cell lung cancer (SCLC), pancreatic cancer, chronic myeloid leukaemia (CML), Hippo and sphingolipid signalling pathways, offers valuable insights into the potential mechanisms involved in the development and progression of cancer. The Hippo and sphingolipid signaling pathways regulate cell proliferation and can serve as potential targets for treating various carcinoma cancers by either promoting tumor suppression or survival (Han, 2019; Ogretmen, 2018), as reported in LUAD (Wang *et al*., 2015), breast (Wei *et al*., 2018), pancreatic (Wu *et al*., 2021), and leukaemia (Noorbakhsh *et al*., 2021). Our findings suggest that the target genes are also associated with the Hippo and sphingolipid signalling pathways, which may impact the survival of patients with LUAD.

The protein-protein interaction (PPI) network is an effective tool for comprehending the intricate biological pathways contributing to various cellular processes. Constructing a PPI network entails retrieving interaction data from the STRING database, searching for known interactions, building the network, and analysing it to identify crucial nodes or subnetworks. Subnetwork detection from the PPI network is frequently used in determining the critical function of associated proteins in the pathogenesis and progression of diseases (Hu & Chen, 2012; Luo *et al*., 2013). The target genes of SN1, SN2, and SN3 exhibited significant associations with neoplastic diseases. SN1 is derived from two hub nodes, namely *RAD51* and *PRPF19* genes. *RAD51* and *PRPF19* genes are the key players in DNA damage response. A clinical trial by Nogueira *et al*., (2010) discovered that the *RAD51* G135C polymorphism predicts a better prognosis of lung cancer after first-line chemotherapy. To date, the role of *PRPF19* remained unelucidated in LUAD. However, the *PRPF19* gene was significantly upregulated in tongue tissue, as He *et al.*, (2021) discovered in their study, where *PRPF19* promotes cell proliferation and migration in tongue cancer cells. In addition, *PRPF19* expression is associated with a poor prognosis due to the presence of *PRPF19* that influences chemoradiotherapy resistance by regulating the expression of *SLC40A1*, an iron transporter gene, and *MACROD2*, a DNA damage responsive gene (He *et al*., 2021).

KARS, *KPNA4*, *EIF2S2*, and *KPNB1* were clustered in SN2. The interaction of SN2 genes, direct or indirect, is strongly supported by evidence of their association with the progression of LUAD. For example, LUAD patients with higher expression of *KPNB1* and *EIF2S2* have a poorer prognosis than those with a knockdown of *KPNB1* and *EIF2S2* (Du *et al*., 2021; Tanaka *et al*., 2018). *KPNA4* is a nuclear import protein that is vital in promoting malignant phenotypes in LUAD cells (Hu *et al*., 2020). Both *KPNA4* and *KPNB1* were enriched in the modulation of the virus-mediated host process, and autophagy has been reported to influence tumour behaviour, especially at the early stages of oncogenesis (Leonardi *et al*., 2021). KARS encodes aminoacyl-tRNA synthetase, which is required for mRNA translation and has become a target of autoantibodies in autoimmune diseases (Vargas *et al*., 2020). It was found that autoimmune diseases are associated with LUAD. Zhou *et al*., (2019) suggested that autoimmune diseases may share risk factors with LUAD rather than the autoimmune disease alone.

The SN3 suggested the association of *HMGA2*, *IGF2BP1*, and *IGF2BP2* in mRNA transport and apoptosis of LUAD. This result is supported by Müller *et al*., (2018) from their study, where the upregulation of *IGF2BP1* levels contributes to the downregulation of miRNA-regulated target mRNAs. *IGF2BP2* maintains cancer cells by interfering with the inhibition of let-7 miRNAs-regulated target mRNAs, whereas *IGF2BP3* contributes to the downregulation of the apoptotic-related gene *HMGA2*. *HMGA2* plays a role in cancer development by enhancing the cell cycle and inhibiting apoptosis (Mansoori *et al*., 2021). It is suggested that *IGF2BP1*, *IGF2BP2*, and *HMGA2* might be candidate targets for effective therapeutic intervention of aggressive tumour cells in LUAD patients.

To summarise, this study has identified two miRNAs, namely *miR-99a-5p* and *miR-148a-3p*, as potential biomarkers for predicting survival in lung cancer patients. Moreover, these miRNAs appear to target

certain genes, including *SUDS3*, *TOMM22*, *KPNA4*, and *HMGB1*, which have been linked to poor overall survival rates in individuals with LUAD. These findings suggest that these miRNAs and their target genes may play crucial roles in the development and progression of LUAD. These findings suggest that both miRNAs may have important diagnostic and therapeutic implications for lung cancer management. However, further studies are needed to validate these miRNAs as prognostic biomarkers and to explore their potential as targets for novel cancer therapies.

CONCLUSION, LIMITATION AND FUTURE PROSPECT

In summary, a miRNAs profile between high and low survival of LUAD patients in the Hospital Canselor Tuanku Muhriz UKM (HCTM), Kuala Lumpur was generated and demonstrated both *miR99a-5p* and *miR148a-3p* as potential biomarkers that could predict survival of LUAD. These miRNAs were highly expressed in high survival LUAD patients. Our findings demonstrated an essential role of the target genes of *miR99a-5p* and *miR148a-3p* in the progression of LUAD based on their involvement in significant biological processes (BPs), including positive regulation of apoptotic process, mRNA transport, regulation transport and G1/S transition of mitotic cell cycle, cell-cell adhesion, G2/M transition of mitotic cell cycle and lipid biosynthetic process. The study of subnetwork detection from PPIN of *miR99a-5p* and *miR148a-3p* target genes and their association with neoplastic diseases and cancer-related BPs support the function played by these genes to participate in LUAD progression and pathogenesis. Four candidate target genes, *SUD53*, *TOMM22*, *KPN4*, and *HMGB1*, were significantly associated with the poor prognosis of LUAD patients and could be used as therapeutic biomarkers in preventing LUAD. This integrated analysis will assist many clinicians in understanding the multidimensional nature of cancer to discover new biomarkers and expedite the development of effective therapies in the future.

Nevertheless, to determine the exact role of the target genes in LUAD, further molecular biological experiments are necessary. Also, it is important to note that the small number of samples from LUAD patients between 2003–2013 was a limiting factor in our study. Our miRNA predictive markers were obtained based on the available lung cancer dataset. This hindered our ability to advance our understanding of biomarkers that are differentially expressed based on the aspect of the tumour. A larger dataset may provide more accurate results. Obtaining more samples and performing the validation would be valuable in furthering our research in this area.

In recent years, bioinformatics research has become increasingly capable of analysing vast and intricate genomic information with a broad range of applications. Our research findings on the miRNA as potential biomarkers and their target genes in the survival of LUAD may enhance comprehension of the fundamental molecular mechanisms of LUAD and furnish valuable insights for forthcoming investigations on new LUAD anticancer therapies. In the coming years, the accessibility of biological databases tailored to LUAD, predicted genes linked to LUAD, and solid experimental data obtained from the Malaysian population are expected to boost the precision and efficacy of lung cancer patient survival.

ACKNOWLEDGEMENTS

We want to thank the Ministry of Higher Education (MOHE), Malaysia, for the funding and the lung cancer patients involved in this study. This research was funded by the Fundamental Grant Research Scheme (FRGS) (Grant no: FRGS/1/2011/SKK/UKM/03/16/02) from the Ministry of Higher Education (MOHE), Malaysia.

ETHICAL STATEMENT

This retrospective study used the formalin-fixed paraffin-embedded (FFPE) lung cancer tissues from the Department of Pathology, UKM Medical Centre (UKMMC). This study was approved by the UKM Research Ethics Committee and granted in January 2012 (File No: UKM 1.5.3.5/244/SPP/ UMBI-002-2012).

CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states there is no conflict of interest.

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