TITLE: Circulating microRNAs modulating glycolysis as non-invasive prognostic biomarkers of HNSCC

RUNNING TITLE: Circulating miRNAs in head-neck cancer prognosis

ABSTRACT

BACKGROUND: The identification of prognostic non-invasive biomarkers is a priority for cancer patients' care. Circulating microRNA (miRNAs) have been described in numerous human malignancies as diagnostic, prognostic, and therapeutic cancer biomarkers. The aim of our study was to analyze the expression profile of a set of miRNAs, involved in the modulation of the glycolytic pathway, as prognostic factors in human head and neck squamous cell carcinomas (HNSCC).

METHODS: Serum samples of 54 patients with untreated HNSCC were obtained at the time of diagnosis. The prognostic value of circulating miR-26b, miR-124, miR-155 and miR-375 was evaluated towards disease-free survival.

RESULTS: We found that there were optimal miRNAs cut-off values for lower risk of recurrence in HNSCC patients. Kaplan-Meier curves showed that higher levels of miR-26b and lower levels of miR-155 were associated with better disease-free survival rates. In the multivariate analysis, patients with serum miR-26b>0.062 and miR-155<0.159 presented more than 2.9 times lower risk of poor outcome.

CONCLUSION: Our results suggest that two miRNAs that modulate the glycolytic pathway, miR-26b and miR-155, are independently associated with the risk of recurrence in patients with HNSCC. The overall results in this study

supports the evidence that the glucose homeostasis may be a target to improve the outcomes for patients with HNSCC.

KEYWORDS: head and neck cancer; prognosis; miRNAs; glycolysis; non-invasive biomarker

LEVEL OF EVIDENCE: Individual retrospective cohort study (2b)

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. HNSCC is associated with severe disease and treatment-related morbidity, with 5-year survival rates of approximately 50%[1]. Treatments of HNSCC include radiotherapy, chemotherapy and surgery, in different strategies and combinations. Currently, prognostic factors able to efficiently predict the outcome after treatment are not ready for use[2]. The identification of pre-treatment molecular markers of response to therapy would be of great interest from the clinical point of view, because they would help us to develop personalized treatments that maximize survival while minimizing morbidity.

MicroRNA (miRNAs) are small non-coding RNA species that are transcriptionally processed in the host cell and released extracellularly into the bloodstream. From its intracellular origin, miRNAs can be secreted extracellularly bounded to lipoproteins or secreted in cell-derived extracellular vesicles as a method of cell-to-cell communication[3]. The deregulation of miRNA usually involves post-transcriptional gene silencing, and has been shown to influence pathogenesis of a number of diseases, including cancer[3]. Recently miRNAs have emerged as tumor-related biomarkers that reflect the

presence of early-stage tumors, the dynamics and status of advanced stage tumors, the presence of tumor recurrence, and also drug sensitivities[4].

Some miRNAs have shown to modulate the secretion, action and sensitivity of insulin and may affect glucose uptake and production[5]. This fact makes miRNAs involved in glycemic metabolism potential targets, because in cancer cells, glucose is preferentially metabolized by aerobic glycolysis, whereas non-tumorigenic cells use the mitochondrial oxidative phosphorylation. This phenomenon, termed as the Warburg effect, is a signature of cancer cells, characterized by increased glycolysis and lactate production regardless of oxygen availability[6]. We have selected a set of four miRNAs (miR-375, miR-124, miR-26b and miR-155) involved in different crucial steps of the glucose metabolism pathway (**Figure 1**) to study their role as prognostic factors in HNSCC. The overexpression of miR-375 results in suppressed glucose-stimulated insulin secretion and its inhibition enhances insulin secretion[7]. MiR-375 is frequently downregulated in HNSCC cancer cells compared with those in healthy controls, and functions as a tumor suppressor[8]. Mir-124, reduces glucose consumption and lactate production, decreasing ATP formation and increasing the NAD+/NADH ratio[9]. This way, miR-124 suppresses energy metabolism in cancer cells. In line with these roles, miR-124 has been considered as a tumor suppressor in several types of cancer[9] and also in HNSCC[10]. Studies also show that some miRNAs can regulate key transporters and enzymes involved in glycolysis[11]. GLUT1 is a glucose transporter that mediates glucose influx into a cell which is the first rate-limiting process involved in glucose metabolism. Hexokinase 2 (HK2) is a key enzyme

that catalyzes the first step in the glycolysis pathway. MiR-124 has been described to suppress proliferation and glycolysis by targeting GLUT1/HK2[9]. MiR-155 also appears to upregulate the expression of HK2 through distinct mechanisms. MiR-26b inhibits proliferation, migration, invasion and apoptosis induction via the downregulation of another key enzyme in the glycolytic pathway, the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB) in osteosarcoma cells[12] (**Figure 1**).

In a previous study we evaluated the prognostic relevance of altered glycemic metabolism on disease-free survival in HNSCC[13]. Our results suggested that improving the glycemic control in patients with HNSCC, the outcome of treatment would be better. In the present work, we focused in the analysis of circulating miRNAs related to glucose metabolism enzymes that are deregulated in several cancers, in order to evaluate their usefulness as novel non-invasive biomarkers in patients with HNSCC.

MATERIALS AND METHODS

Patients

Fifty-four consecutive patients with HNSCC pathologically confirmed, and untreated advanced tumors (stages III-IV) from Hospital Universitari Joan XXIII in Tarragona and Hospital de la Santa Creu i Sant Pau, in Barcelona were included in this study. Tumor boards evaluated all the patients, and the decision to treat with radiotherapy, chemoradiotherapy or surgery was made according to the standard protocols and guidelines of both centers. In general, these patients were treated with chemoradiotherapy. Human papilloma virus (HPV) status was available for those patients that presented an oropharyngeal carcinoma (n=17). HPV status was detected by a multiplex polymerase chain reaction (PCR) assay.

External-beam RT was administered by continuous-course radiotherapy 5 days a week, 2 Gy per session in normo-fractionated treatments, and 1.2 Gy twice daily in hyper- fractionated treatments. Treatment was administered in total doses of 65 to 74 Gy to the primary site, 50 Gy to the neck in all patients with N0 nodes except, and 70 Gy to the neck in patients with clinical metastatic neck nodes (N+). Chemoradiotherapy (ChRT) consisted of radiotherapy at the same doses plus 3 cycles of cisplatin at a dose of 100 mg/m2 on day 1 every 3 weeks.

An evaluation of symptoms and locoregional examinations at 2-month intervals during the first year, 3-month intervals in the second year, and 4-month intervals over years 3-5 was the routine follow-up. The mean follow-up of the patients included in the study was 3.7 years (95% CI 2.4–5.0 years).

The research study was reviewed and approved by the Institutional Review Board of both institutions. The investigation conforms to the principles outlined in the Declaration of Helsinki. All patients gave informed consent.

Quantification of circulating miRNAs in serum

Pre-treatment blood samples were obtained from 54 patients after an overnight fast. Blood was drawn in a 10 mL vacutainer tube from an antecubital vein. Within 1 h of drawing, the serum was separated by centrifugation at 1500×g for 15 min at 4ºC. Serum samples were stored at − 80 °C until analytical measurements were performed. Total RNA was isolated from HNSCC serum samples using the miRNeasy mini kit Qiagen using Qiazol according to the manufacturer´s protocol. RNA concentrations were determined using absorbance readings at 260 nm, while RNA purity was evaluated using the optical density (OD)260/(OD)280 absortion ratio.

Reverse transcription was achieved using a Taqman microRNA reverse transcription kit. The expression levels of the microRNAs were determined using the Taqman miRNA assay kit according to the manufacturer´s instructions. Briefly, cDNA samples were incubated at 50ºC for 2 min and at 95ºC for 10 min followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min using the Step One Real-time PCR System. The qPCR analysis of miRNA was performed using Taqman probes for: hsa-mir-375 (Assay ID: 000564); hsa-mir-124 (Assay ID: 001182); hsa-mir-26b (Assay ID: 000407); hsa-mir-155 (Assay ID: 002623) and U6 snRNA (Assay ID: 001973) was used as the housekeeping

expression. MiRNAs expression was calculated by normalization to the signal for U6 expression using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Circulating levels of the different miRNAs according to the clinic-pathological variables were compared using Student's t test. The Chi-Squared Test was used to analyze the relationship between categorical variables. As proposed by Chiesa et al.[14] for studies on predictive factors in HNSCC, we evaluated the outcome by the locoregional control with a follow-up of at least 2 years. Disease-free survival was defined as the period of time from the completion of the primary treatment of the tumor to any local, regional or distant recurrence. The variable profile was defined according to the control of the disease (local-regional-distant recurrence) using the Classification and Regression Tree (CRT) method. Variables included to generate the Regression tree were the stage, primary location, HPV status, age, and the levels of miRNAs in serum. CRT analysis splits the data into segments that are as homogeneous as possible with respect to the dependent variable. The disease-free survival according to the variable profile was calculated using the Kaplan-Meier method. Differences in survival rates were compared using the log-rank test. Univariate and multivariate analysis was made with a Cox regression analysis considering the disease-free survival as the dependent variable, and location of the tumor (oropharynx-oral cavity vs larynx-hypopharynx), age, tobacco and alcohol consumption, Eastern Cooperative Oncology Group (ECOG) general status index, Stage (III vs IV), T (T1-2 vs T3-4) and N category (N0 vs N+) and the categorized variable profile (1, 2 or 3) as the independent variables. All

statistical analyses were made using SPSS software v. 20.0 (IBM, Madrid, Spain).

RESULTS

Clinical and pathological characteristics of the patients included in the study

The characteristics of the patients are summarized in Table 1. There were no significant differences in the distribution of patients by the clinic and pathologic parameters evaluated. We did not find any difference in the disease-free survival when the patients were subclassified. HPV status was evaluated in 17 oropharyngeal patients. Most of them presented HPV-negative tumors (78%). There were no differential expression in any miRNA studied between HPV positive and HPV negative oropharyngeal tumours.

Figure 2 shows the median of serum miRNAs according to the disease free survival. No differences were found between both groups in any of the miRNAs analysed.

Prediction of locoregional control by miRNAs expression using classification trees

The CRT method was used to obtain the best cut-off values of each variable in order to predict the outcome. We created a tree entering, on one hand, the clinico-pathological variables (age, location of the tumor, HPV status and Stage), and on the other hand, the levels of serum expression of miR-26b, miR-124, miR-155 and miR-375 as independent variables. Only serum expression of miR-26b and miR-155 were finally selected to create the decision tree. These miRNAs together correctly discriminated 79.6% of the cases according to the disease free survival achieved after treatment. The tree created shows that patients with higher serum expression levels of miR26b had a better

locoregional control than patients with lower levels (71% vs 37%, respectively). In this case, the classification tree analysis revealed a cut-off value of 0.062 for miR26b to be optimal to discriminate between patients' disease-free survival (Figure 3). In addition, the tree also selected miR155 serum expression with a cut-off value of 0.159, being patients with lower levels, those with higher survival rates. Using this classification method, we generated a categorized variable "Profile", which classifies the patients according to their miRNAs expression. Profile 1 corresponded to patients with miR26b lower than 0.062, profile 2 included patients with high miR26b (>0.062) and high miR155 (>0.159), and finally, profile 3 comprised patients with high miR26b and low miR155 (<0.159).

The disease-free survival values are shown in Table2. Patients are distributed according to the profile categories defined by the CRT analysis, the local and regional extension of the tumour and the location of the primary tumour. The disease-free survival rate was higher in the patients with Profile 3, reaching statistical significance for patients with Stage IV tumors, T3-4 tumor size, with node metastasis and located in the oral cavity-oropharynx.

COX regression analysis

The results of the univariate and multivariate study considering disease-free survival as the dependent variable are shown in Table 3. In the univariate study, the stage, T and N categories, primary site, tobacco and alcohol consume, age and the profile were included as categorical variables. According to the results, the profile was significantly related to the local control of the disease. Patients

with profile 3 are considered the reference category, then those with lower profiles had a higher risk of poor outcome after treatment (Table 3). Concretely, those patients included in profile 2 had 7.3 times higher risk of recurrence of the tumor (CI 95%: 1.95-27.60, P=0.003), and patients with profile 1 presented 2.9 times higher risk of locoregional recurrence (CI 95%: 1.05-8.00, P=0.004). Interestingly, significant results were obtained even when the profile was adjusted for Stage and age in the multivariate analysis. After adjustment, the recurrence risk increases significantly as the profile value increases (Table 3).

Kaplan-Meier curve according to the profiles defined with CRT method

For patients included in profile 3, the disease-free survival rate was 81.6% (95% CI: 69.2-98.4%), for profile 2 was 18.5% (95% CI: 0.0-67.4%) and it was 32.4% (95% CI: 7.4-57.4%) for patients in profile 1, indicating a lower locoregional recurrence-free survival rate for patients in low profiles (Figure 4). We found significant differences in the disease-free survival of the patients as a function of the profile groups (P=0.033).

DISCUSSION

The identification of novel non-invasive biomarkers to ameliorate early-diagnosis, and disease prognosis, as well as to support targeted treatment is a priority for cancer patients' care. Circulating miRNAs can be utilized as diagnostic, prognostic, and therapeutic biomarkers, since deregulated miRNA levels have been described in numerous human malignancies[15]. However their potential as prognostic biomarkers in HNSCC has not been evaluated so far. Circulating tumor-derived miRNAs might reflect miRNA expression of donor tumor cells, in fact, several studies have assessed the relationship between serum or plasma miRNAs and tissue miRNAs[16]. Identifying the correlation between circulating miRNAs and tissue miRNAs would support the hypothesis that circulating miRNAs can serve as ideal biomarkers[15].

The Warburg effect, which was described as the propensity of cancer cells and tissues to take up glucose avidly and convert it almost exclusively to lactate (aerobic glycolysis), was the central principle of cancer cell metabolism and it is a common feature of tumor cells. In the last decade, numerous miRNAs have been described to play important roles in diverse cellular processes, including the modulation of aerobic glycolysis of tumor cells, by modulating gene expression at the post-transcriptional level. We have previously found that in patients with more advanced tumors, the highest locoregional control was achieved by patients with lower insulin resistance. This finding supports the fact that HNSCC tumor cells are dependent on glucose for energy production and survival, which provides a rationale for treatment and diagnosis strategies that target glucose catabolism[17]. In the current work, we aimed to focus in the miRNAs that direct target enzymes involved in glucose metabolism, and to

evaluate its application as prognostic. We studied miR-375, miR-124, miR-26b and miR-155, that participate in cancer cell metabolism control by regulating the expression of genes that either, directly regulate metabolic machinery or indirectly modulate the expression of metabolic enzymes (**Figure 1**).

In a recent meta-analysis, miR-375 has been identified as a potentially promising biomarker to predict the prognosis of patients with head and neck or esophageal squamous cell carcinoma[17]. The meta-analysis, includes 13 studies (1340 patients) with important heterogeneity between them, and concluded that the low expression of miR-375 in the tumors was significantly correlated with poor overall survival. In our study, we could not find this association between low circulating miRNA 375 and poor survival in HNSCC.

Data from Sun et al[18] indicated that miR-124 alters the expression of genes involved in glucose metabolism in colorectal cancer cells. Also Zhao et al[9] described that miR-124 suppresses proliferation and glycolysis in non-small cell lung cancer by targeting glucose transporter 1 and hexokinase pathway. In HNSCC, the anti-tumor effects of miR-124 have been described previously. miR-124 intravenous injection inhibited oral tumor growth in mice[19], its up-regulation is involved in mitochondrial apoptosis[20], and Yuan Zhao et al[21] found that miR-124 presented a significantly reduced expression in HNSCC compared with normal tissues. Although its anti-tumoral effects have been established, its association with prognosis in HNSCC remains unclear. The expression and role of miR-124 in HNSCC is still poorly understood and the molecular mechanisms by which miR-124 acts on tumor cells remain largely

unknown. In our cohort of HNSCC, the circulating levels of this miRNA were not associated with prognosis.

In this cohort, we found that circulating mir-155 predicted HNSCC patient outcome. Previous reports indicated that miR-155 was up-regulated in several malignancies[22–25]. Some authors evaluated the value of miR-155 as a diagnostic tool, comparing tumor with healthy tissue/plasma, however the prognostic impact was not evaluated. Importantly, Wang et al[26] found that the expression of miR-155 in plasma and tissue was matched in human laryngeal squamous cell carcinoma. If we assume this circumstance in our cohort, miR-155 may be a non-invasive prognostic biomarker that can reflect tumor status.

Regarding miR-26b, previous studies in vitro showed that the reduction of this miRNA was associated with chemo-resistance in cisplatin-resistant laryngeal cancer cell model, and recovery of miR-26b was able to decrease this cisplatin resistance[27]. The study of Fukumoto et al[28] demonstrated that the restoration of both miR-26a and miR-26b in oral squamous cell carcinoma cell lines significantly inhibited cancer cell migration and invasion. Furthermore, Li et al[29] showed that miR-26 family can suppress esophageal cancer cell proliferation and that esophageal squamous cell carcinoma tissues presented an > 50% reduction, even in the early-staged tumors. Despite all of them evaluated only the tumor levels of miR-26b, their results are in agreement with our findings, where lower circulating levels of miR-26b were associated with poor prognosis.

Taken together, our results showed that patients with higher serum expression levels of miR26b had a better locoregional control than patients with lower

levels. In addition, miR155 serum expression with a cut-off value under 0.159 classified patients with higher survival rates. These two miRNAs regulate rate-limiting enzymes in the glycolytic pathway. MiR-155 appears to upregulate the expression of hexokinase 2 (HK2) through distinct mechanisms. HK2 is the major isozyme that is overexpressed in tumors and contributes to aerobic glycolysis, and thus it is documented as a pivotal player in the Warburg effect[30, 31]. MiR-26b inhibits proliferation, migration, invasion and apoptosis induction via the downregulation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 driven glycolysis in osteosarcoma cells[32]. Then our results might support the mechanistic hypothesis that the high levels of miR-26b and the low miR-155 levels modulate the glycolytic pathway, reducing aerobic glycolysis in this HNSCC patients that present better prognosis.

Some of the advantages of miRNAs is that they can be easily collected through a blood test, are analytically stable and, can be measured using assays that are specific, sensitive and reproducible. In addition, although the majority of studies have assessed circulating miRNAs in serum and plasma, recent studies have confirmed the potential use of tumor-specific miRNAs as diagnostic markers for cancer in other body fluids, such as urine[33] or saliva[34]. Thus, the presence of miR-26b and miR-155 in such body fluids should be studied in the future, because they may represent excellent noninvasive biomarkers in cancer[15].

This study has potential limitations that should be addressed. The main limitation of the study is the relatively small number of patients, together with the heterogeneity of the sample, which limits the statistical power of our analysis.

However, we were able to collect data from a prospective and real cohort of patients from two different hospitals and adjust for most potential confounding

factors, such as stage. After this adjustment, we obtained similar results, which strengthen our conclusions. Surprisingly, the nodal classification was not a significant prognostic factor. However, the outcome in this work was response to first treatment (disease-free survival yes or no) and disease-free survival of the index tumor rather than overall survival.

Globally, our preliminary results are still far for implementation in clinical practice and should be first validated in larger and external cohorts. On the other side, the present study is focused on cancer metabolism, which is an emerging field in several solid tumors, but has been little explored in head and neck. Our results, in the case of being validated, may open a new scenario in terms of de-intensifying treatment or even developing new target therapies for head and neck cancer. To our knowledge, this is the first study that addresses the potential relevance of a set of metabolic microRNAs in a clinical cohort of HNSCC patients.

In conclusion, our findings suggest that two miRNAs that modulate the glycolytic pathway, miR-26b and miR-155, are independently associated with the risk of recurrence in patients with HNSCC. Moreover, we found that there are ideal cut-off values and higher levels of miR-26b and lower levels of miR-155 were associated with better outcome. The overall results in this study supports the evidence that the glucose homeostasis may be of interest to improve the outcomes for patients with HNSCC.

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FIGURE AND TABLE LEGENDS

Figure 1. Selected miRNAs target the glycolytic and insulin signaling pathways in cancer cells. Akt, protein kinase B; F6P, fructose-6-phosphate; GLUT, glucose transporter; G6P, glucose-6-phosphate; HK2, hexokinase 2; IR, insulin receptor; IRS, insulin receptor substrate; LDHA, lactate dehydrogenase A; MCT, monocarboxylate transporter; PDH, pyruvate dehydrogenase complex; PFKFB, 6-phosphofructo-2-kinase/fructose2,6-biphosphatase;PI3K, phosphatidylinositol-4,5-bisphosphate 3- kinase; TCA, tricarboxylic acid.

Figure 2. Serum levels of the different miRNAs analysed acording to the disease-free survival. YES: patients are free of disease at the last follow-up; NO: patients are not free of disease at the last follow up.

Figure 3. Classification and regression tree for disease-free survival rates based on serum levels of miR-26b and miR-155. Pie charts represent the proportion of patients who met the disease-free survival (light grey) or not (dark grey) at each node of the tree.

Figure 3. Kaplan Meier curves showing the disease-free survival according to the defined profile.

Table 1. Characteristics of the patients included in the study, and results of univariate analysis regarding disease-free survival.

Table 2. Disease free-survival in function of clinical variables according to the defined profile.

Table 3. Results of the univariate and multivariate Cox regression analysis according to disease-free survival.

Table 1. Characteristics of the patients included in the study, and results of univariate analysis regarding disease-free survival.

Characteristics	Num. Patients (%)	Disease-free survival % (CI95%)	p-value
Age (years)			0.548
<50	16 (29.6)	50.0 (24.7-75.3)	
50-60	19 (35.2)	63.2 (38.4-83.7)	
60-70	16 (29.6)	68.8 (41.3-89.0)	
>70	3 (5.6)	33.3 (0.0-90.6)	
Sex			0.653
Male	52 (96.3)	59.6 (45.1-73.0)	
Female	2 (3.7)	50.0 (1.2-98.7)	
Tobacco consumption			0.795
Never	7 (9.9)	71.4 (29.0-96.3)	
<20 cigarettes per day	5 (7)	80.0 (28.4-99.5)	
>20 cigarettes per day	59 (83.1)	66.1 (44.8-77.6)	
Alcohol consumption			0.338
Never	13 (24.1)	69.2 (38.6-90.9)	
Mild-Moderate	11 (22.2)	41.7 (15.2-72.3)	
Severe	41 (53.7)	62.2 (42.3-79.3)	
ECOG Index			0.586
0	11 (20.4)	72.7 (39.0-94.0)	
>0	16 (29.6)	68.8 (41.3-89.0)	
Tumor location			*0. 053*
Oral cavity–oropharynx	31 (57.4)	48.4 (30.1-66.9)	
Larynx-hypopharynx	23 (42.6)	73.9 (51.6-89.8)	
T category			0.207
T1-T2	6 (11.1)	83.3 (35.9-99.6)	
T3-T4	48 (88.9)	56.3 (41.2-70.5)	
N category			0.640
N0	3 (5.6)	66.7 (9.4-99.1)	
N+	51 (94.4)	58.8 (44.2-72.4)	
Tumor differentiation			0.412
Good	8 (11.3)	87.5 (47.3-99.7)	
Moderate	48 (67.6)	64.6 (49.4-77.8)	
Poor	11 (15.5)	72.7 (39.0-94.0)	
Treatment			0.752
ChRT	37 (68.5)	59.5 (42.1-75.2)	
Surgical	4 (7.4)	75.0 (19.4-99.4)	
Both	13 (24.1)	53.8 (25.1-80.8)	

Abbreviations: CI, confidence interval; ECOG, Eastern Cooperative Oncology Group. ChRT: chemoradiotherapy

Table 2. Disease free-survival in function of clinical variables according to the defined profile.

Clinico-pathological variables	Profile			
	1	2	3	p-value
Stage				
III	25.0%	33.3%	80.0%	0.209
IV	38.5%	33.3%	84.2%	**0.011***
T category				
T1-T2	100.0%	50.0%	100.0%	0.301
T3-T4	31.3%	28.6%	81.0%	**0.003***
N category				
N0	0.0%	100.0%	0.0%	0.157
N+	37.5%	25.0%	83.3%	**0.002***
Tumor location				
Oral cavity–oropharynx	25.0%	16.7%	81.8%	**0.007***
Larynx-hypopharynx	60.0%	66.7%	84.6%	0.501

Differences between groups were calculated using the Chi Squared test. *$p<0.05$, statistically significant. Profile 1, serum miR26b<0.062; Profile 2, miR26b>0.062 and miR155>0.159, and Profile 3, miR26b>0.062 and miR155<0.159.

Table 3. Results of the univariate and multivariate Cox regression analysis according to disease-free survival.

Variables	Categories	HR	CI95%	p-value
Univariate analysis				
Profile	1 vs 3	2.900	1.051-8.007	**0.040***
	2 vs 3	7.339	1.952-27.596	**0.003***
	1 vs 2	2.530	0.758-8.442	0.131
ECOG	2-4 vs 0-1	1.032	0.226-4.714	0.968
T category	T3-4 vsT1-2	3.998	0.530-30.154	0.179
N category	N+ vs N0	1.725	0.231-12.907	0.595
Primary location	LH vs OCO	0.627	0.242-1.624	0.336
Stage	IV vs III	1.294	0.469-3.568	0.619
Alcohol consume	Moderate vs no	1.991	0.580-6.841	0.274
	Severe vs no	0.986	0.308-3.151	0.981
Tobaco smoking (cig/day)		1.004	0.994-1.014	0.435
Age (years)		0.982	0.939-1.026	0.420
Multivariate analysis				
Profile	1 vs 3	2.908	1.020-8.288	**0.046***
	2 vs 3	7.499	1.983-28.351	**0.003***
Age (years)		0.993	0.944-1.043	0.768
Stage	IV vs III	1.291	0.412-4.044	0.661

Dependent Variable: Disease-free survival. *$p < 0.05$ were considered to be statistically significant.

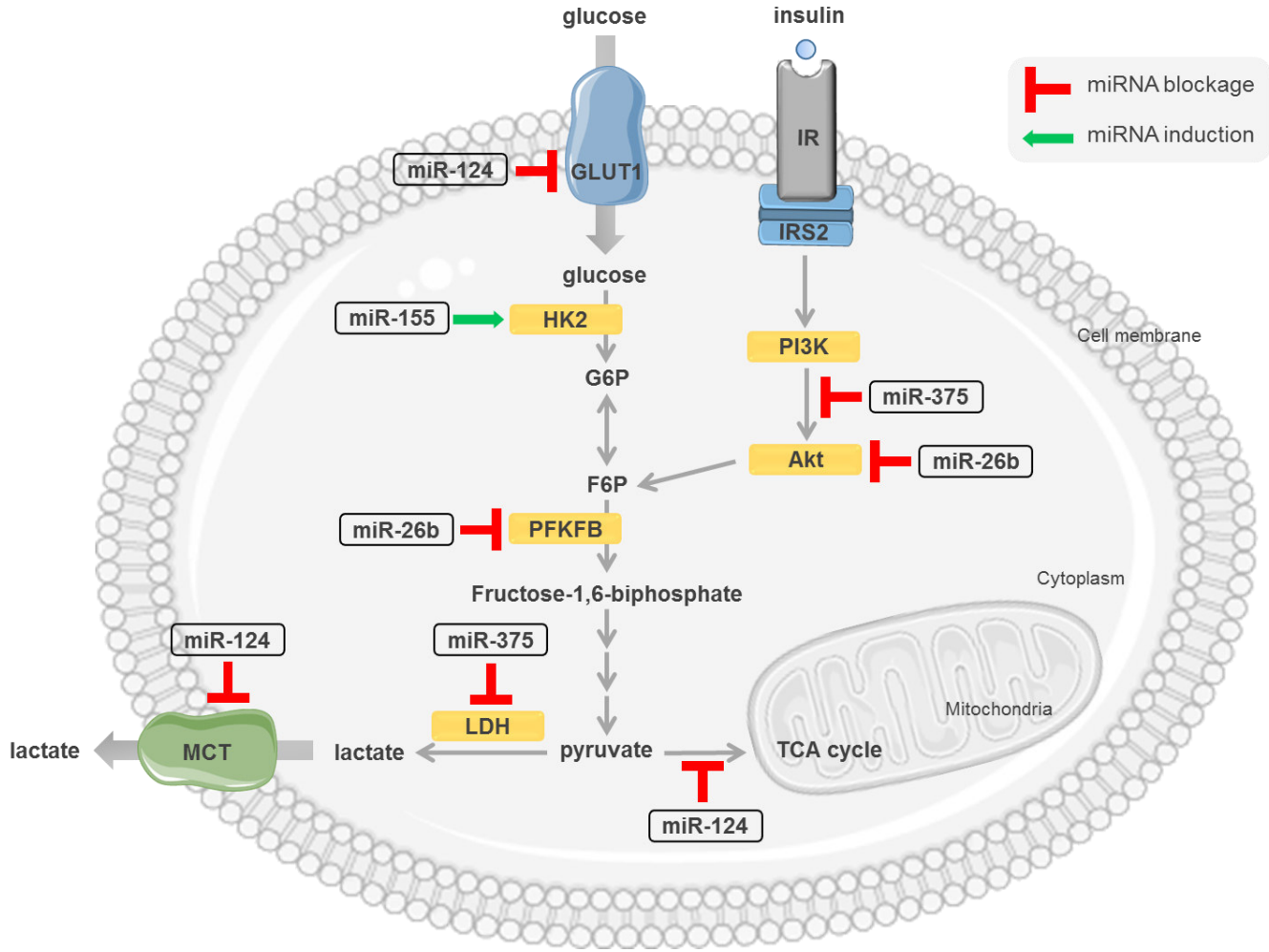


Figure 1. Selected miRNAs target the glycolytic and insulin signaling pathways in cancer cells. Akt, protein kinase B; F6P, fructose-6-phosphate; GLUT, glucose transporter; G6P, glucose-6-phosphate; HK2, hexokinase 2; IR, insulin receptor; IRS, insulin receptor substrate; LDHA, lactate dehydrogenase A; MCT, monocarboxylate transporter; PDH, pyruvate dehydrogenase complex; PFKFB, 6-phosphofructo-2-kinase/fructose2,6-biphosphatase; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; TCA, tricarboxylic acid.

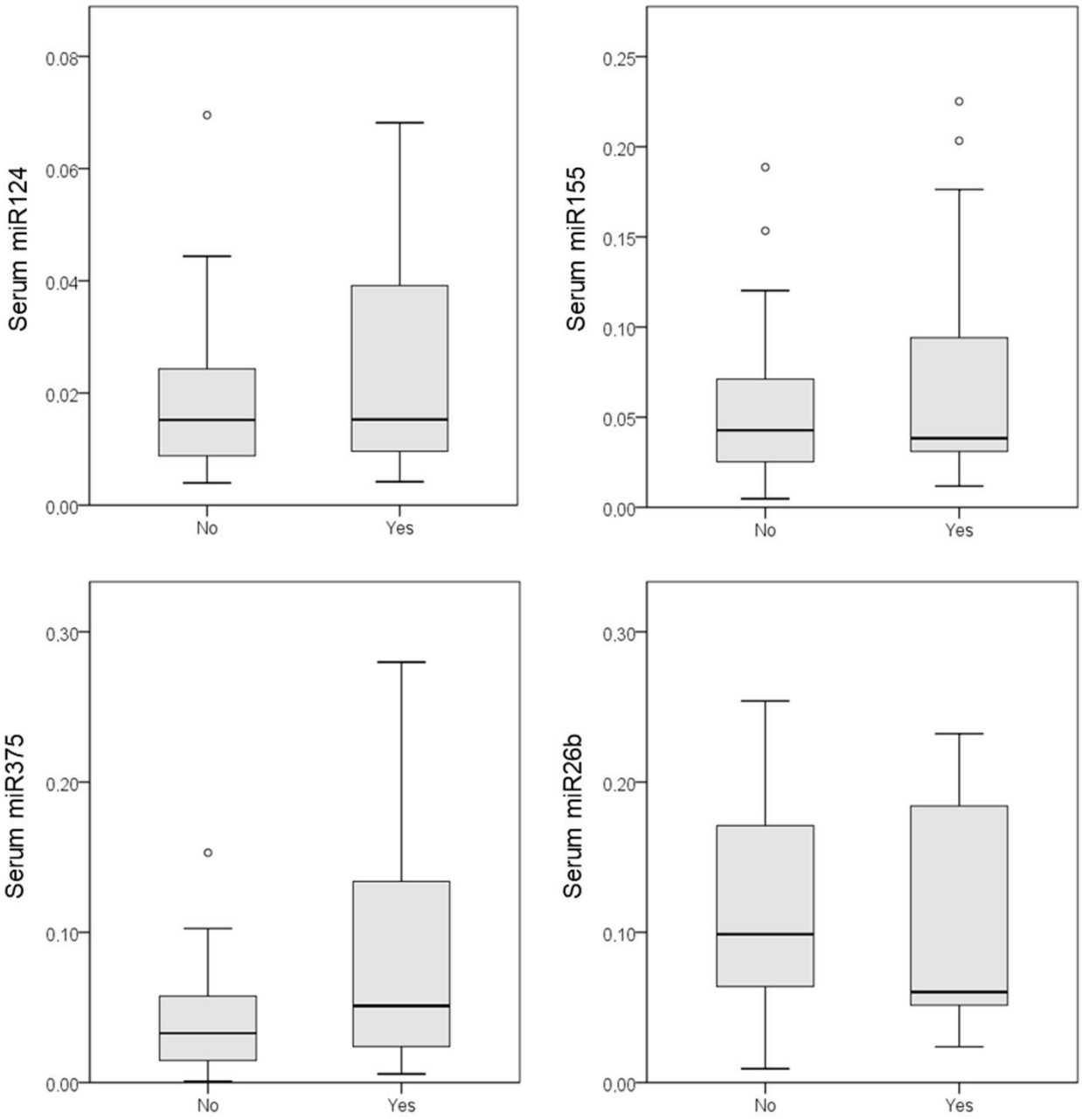


Figure 2. Serum levels of the different miRNAs analysed acording to the disease-free survival. YES: patients are free of disease at the last follow-up; NO: patients are not free of disease at the last follow up.

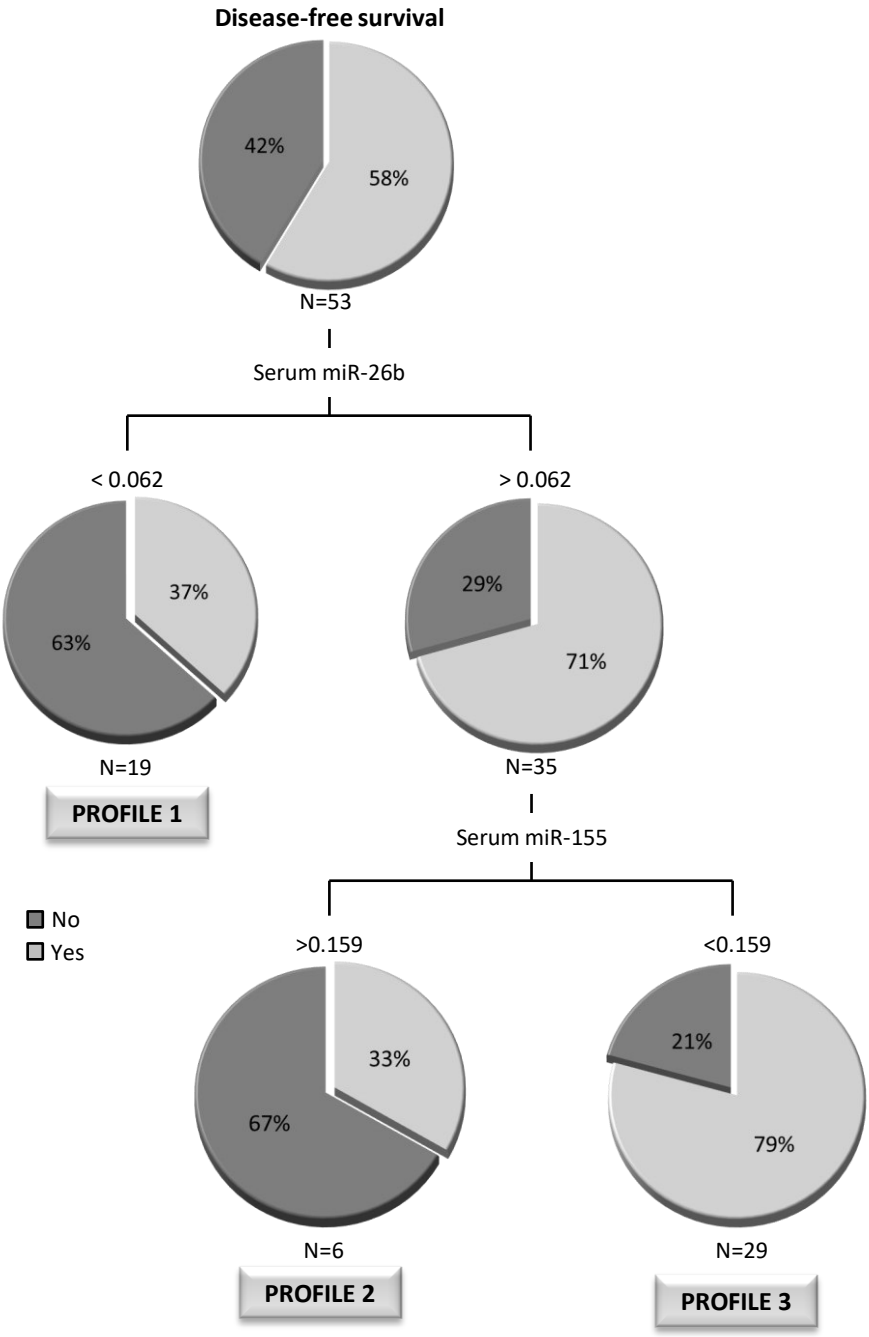


Figure 3. Classification and regression tree for disease-free survival rates based on serum levels of miR-26b and miR-155. Pie charts represent the proportion of patients who met the disease-free survival (light grey; yes) or not (dark grey; no) at each node of the tree, then "Yes" include patients that are free of disease at the last follow-up; and "No" include patients that are not free of disease at the last follow up.

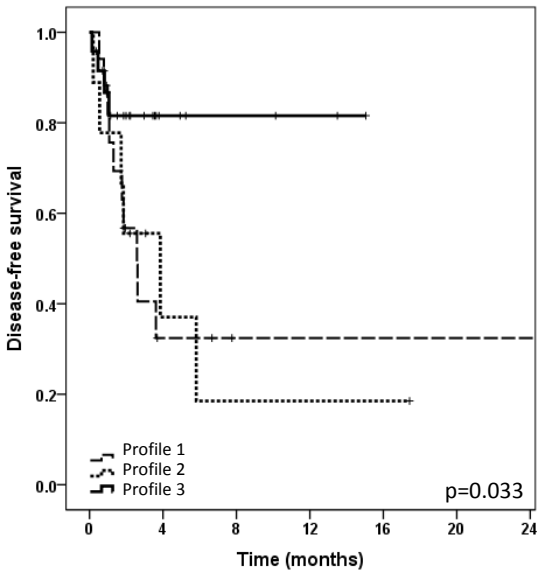
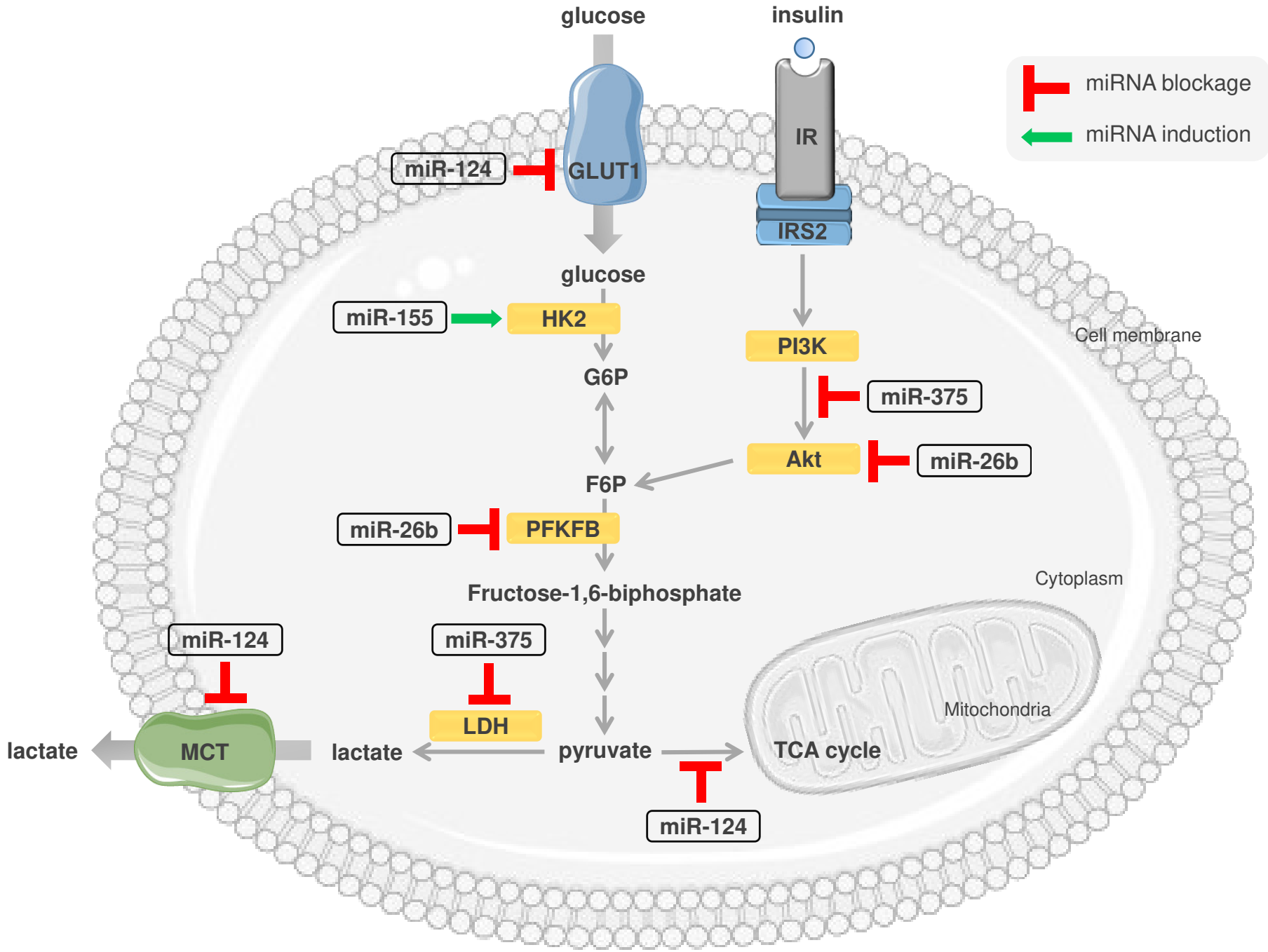


Figure 4. Kaplan Meier curves showing the disease-free survival according to the defined profile.


glucose
insulin
miRNA blockage
miRNA induction
miR-124
GLUT1
IR
IRS2
glucose
miR-155
HK2
PI3K
Cell membrane
G6P
miR-375
Akt
miR-26b
F6P
miR-26b
PFKFB
Cytoplasm
Fructose-1,6-biphosphate
miR-124
miR-375
Mitochondria
LDH
lactate
MCT
lactate
pyruvate
TCA cycle
miR-124