

MicroRNA-146a as a Prognostic Biomarker for Esophageal Squamous Cell Carcinoma

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Background and Aims: MicroRNAs including miR146a have a regulatory role on the expression of genes and act with binding to 3'-UTR region of the genes. Cyclooxygenase-2 (COX-2) is involved in carcinogenesis as an inflammatory marker, and microRNA-146a (miR-146a) as a negative regulatory factor. We aimed to evaluate miR146a expression as a prognostic or diagnostic biomarker for esophageal squamous cell carcinoma (ESCC) and also an association between miR146a and COX2 expression.

Materials and Methods: We quantified the level of miR-146a and COX-2 expression in cancerous and adjacent normal tissue samples obtained from 34 patients with ESCC, using real-time-PCR. Statistical analyses were conducted using one-sample *t*-test. Receiver-operating characteristic (ROC) curve and Kaplan-Meier analysis were applied to assay miR146a as a diagnostic and prognostic marker, respectively, during 4 years of the study. Furthermore, the Cox regression model was performed to assay the hazard ratio (HR). The association between miR-146a and COX2 expression level in ESCC patients was evaluated by nonparametric Spearman's rho analysis.

Results: The results revealed a reduction of miR-146a expression in 50% of cancerous tissue when compared with adjacent normal regions (P -value=0.127). COX-2 expression in 80% of ESCC patients was higher than in the controls (P -value=0.001). Overall, in 60% of cases, direct association was seen between microRNA-146a and COX-2 expression level (correlation coefficient= 0.438, P -value=0.011). COX2 can be considered as a diagnostic biomarker (AUC=0.834, sensitivity=72%, specificity =83%, P -value<0.0001) but miR146a cannot be considered as a diagnostic biomarker (AUC=0.553, sensitivity=88%, specificity =28%, P -value=0.453). Survival analysis by Kaplan-Meier method showed miR146a and COX2 expression can be probably considered as prognostic biomarkers for ESCC because patients with high expression of miR146a had 7 months shorter life span and patients with low expression of COX2 had 8 months shorter life span.

Conclusion: COX2 expression is a diagnostic biomarker. MiR-146a and COX2 expression can probably be considered as prognostic biomarkers for survival in ESCC.

Keywords: miR-146a, cyclooxygenase-2, esophageal cancer

Introduction

Esophageal cancer is the eighth most common cancer worldwide and the sixth cause of mortality due to cancer. The overall 5-year survival is 15% to 25% in patients with esophageal cancer.¹ Early diagnosis was shown to be promising to improve overall 5-year survival in more than 90% of the ESCC cases. Therefore, the finding of early diagnostic, as well as prognostic biomarkers is

important for ESCC to predict the survival and effectiveness of treatment in patients. MicroRNAs have been introduced as a biomarker in different cancers.²

MicroRNAs (miRNA) are belonging to small non-coding regulatory RNA inhibit the expression of specific genes. MicroRNAs prevent protein expression by cleavage of the genes' mRNA after binding to their 3'-UTR or translational inhibition of the mRNA.³ Nowadays, more than 9000 microRNAs have been known in plants, animals, and viruses.⁴ Over 700 microRNAs have been detected in humans.^{5,6} MicroRNAs can regulate most of the cellular processes (eg, cellular proliferation, differentiation, and apoptosis) via mRNA degradation or protein synthesis distribution functions.⁷⁻⁹ MiRNAs may play an important role as a tumor suppressor or as oncogenes.¹⁰⁻¹⁴ Recent studies reported microRNAs and COX-2 involvement in esophageal cancer.¹⁵⁻¹⁹ Mir-146a was shown to have roles in the development of breast, lung, pancreatic, esophageal squamous and gastric carcinomas. Up- and down-regulation of miRNA-146a are reported in the mentioned cancers.²⁰⁻²³

Numerous microRNAs were observed in esophageal cancer patients including miR-145, miR-133a, miR-133b, miR-375, miR-21, miR-184, miR-221 and mir-146a. Each of them acts in the specific pathways in the pathogenesis of esophageal cancer.^{24,25} There were two copies of the genes encoding miR-146, so-called miR-146a and miR-146b.²⁶ MiR-146a directly binds to 3'-UTR COX-2 gene and has a key regulatory role on COX-2 expression. Deletion of miR146a by antagomiR (complementary sequence of miR-146a that cut off binding miR146a to 3' UTR COX-2) or existence of mutation in 3'-UTR COX2 upregulated COX2 and subsequently prostaglandin that control cell proliferation.²⁷ Polymorphism in 3'-UTR COX2 may delete the miR-binding site and upregulates COX2 expression.²⁸ In this study, we assessed miR-146-a and COX-2 expression level in the patients with ESCC who 44% had 8473 SNP in 3'-UTR COX2. Furthermore, we analyzed miR146a and COX2 expression levels as a diagnostic or prognostic biomarker.

Materials and Methods

Samples

We collected fresh cancerous and adjacent noncancerous marginal tissues from 34 ESCC patients during 2015–2017. Patients had informed consent to sampling in

this study as well as long-term follow-up for the evaluation of the prognosis.

RNA Isolation

Total RNA was extracted from the tissue by Trizole reagent by the manufacturer's protocol. RNA was treated with DNase-I (Thermo scientific) to reduce or eliminate DNA debris and was incubated 30 min at 37°C. Consequently, the DNase-I was inactivated by adding 1 µL 0.5M EDTA and heating at 80°C for 2 min.

Poly (A)/cDNA Synthesis Reaction

Mir-X miRNA First-Strand Synthesis Kit (Clontech Laboratories, Inc. cat.no.638515) was applied for cDNA synthesis. According to the manufacturer, 5µL mRQ Buffer (2x), 3.75 µL RNA sample (0.25–8 µg), 1.25 µL mRQEnzyme (including polyA polymerase and Reverse Transcriptase) were mixed and incubated for 60 mins at 37°C. The enzymes were inactivated at 85°C for 5 min, and the final volume was reached to 100µL by adding 90 µL ddH₂O.

Quantification of miRNA146a and U6 by Real-Time-PCR

Real time-PCR (using a “sequence detection system the ABI Prism 7300, Applied Biosystems”) condition for mir146a expression was set as following by 12.5 µl2X qPCR Master Mix Green high Rox (Amplicon, Denmark), 0.5 µL miR-specific primer (10 µM) (MystiCq[®] microRNAs qPCR Assay Primer, hsa-miR-146a-5p, MIRAP00182, Sigma Aldrich, Manchester, UK), 0.5 µL mRQ 3' Primer, 9.5µl dd H₂O, 2 µL cDNA. We used the U6 gene as the internal control. The qPCR conditions were set as 95°C 15min, 40 Cycles: 95°C 15 sec, 60°C 1 min. The expression of mir146a was determined relative to the expression of U6 in tumor and normal tissues, using $2^{-\Delta\Delta Ct}$ formula.²⁹

Quantification of COX2 and GAPDH by Real-Time –PCR

The real time-PCR condition for COX2 was set the same as mir146 expression evaluation. GAPDH gene was used as internal control. Initially, 1µg of total RNA was used for the synthesis of first-strand cDNA, using the “cDNA synthesis kit” (Thermo Fisher Scientific, USA). Consequently, COX2 mRNA-specific fragments were amplified by the specific primers (Table 1). The expression of COX-2 was determined

Table 1 The Primer Sequences Were Used for Real-Time PCR

Gene Name	Forward Primer Sequence	Reverse Primer Sequence
miR146a ²⁷ COX2 ³¹ GAPDH ³¹	5'-UGAGAACUGAAUCCAUGGGUU-3' GGGGATCAGGGATGAACTTT CATCAAGAAGGTGGTGAAGCAG	Universal 3' primer (included in the kit) TGGCTACAAAAGCTGGGAAG TGTAGCCAAATTCGTTGTCATACC

relative to the expression of GAPDH in tumor and normal tissues, using $2^{-\Delta\Delta Ct}$ formula.³⁰

Statistical Analysis

We applied one-sample *t*-test for evaluating miR146a and COX2 expression levels between cases and controls. The association between miR-146a and COX2 expression level in ESCC patients was evaluated by nonparametric Spearman's rho analysis and the correlation coefficient was assayed. Pearson Chi-Square test was used to evaluate the association between clinicopathologic variables including age, gender, smoking and histological grade with the expression of miR-146a in tumor tissue samples. ROC and survival analysis were applied to assay miR-146a as a diagnostic or prognostic marker, respectively. These statistical analyses were performed using the SPSS software (version 16.0; SPSS, Chicago, IL, USA).

Results

MicroRNA-146a Expression

The Student's *t*-test was used to determine differences in miR-146a level between ESCC and adjacent paracancerous tissue. The miR-146a expression was increased in

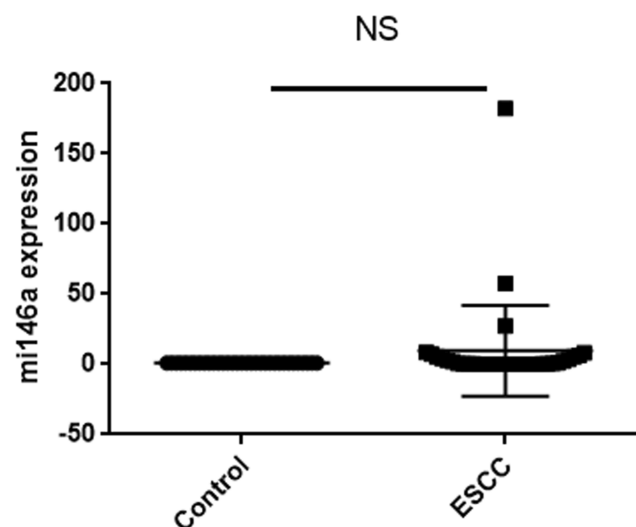


Figure 1 MiR-146a expression in cancerous tissue and marginal normal tissue.

50% cancerous tissue rather and decreased in other 50% cancerous tissue than adjacent normal tissue (fold change mean $\pm$ SE: 7.68 ± 2.92 , P -value=0.127; [Figure 1](#)).

In our work, 44% of the patients had 8473 T>C polymorphism in 3'-UTR COX2 and miR146a expression was lower in patients with 8473 TC (heterozygote) and CC (mutant) genotypes than TT wild type genotype (Table 2). This result was not reported in any of the previous studies.

COX2 Expression

The COX-2 expression level was significantly increased in 80% esophageal cancerous tissues rather than adjacent normal tissues with fold change (mean $\pm$ SE: 9.51 ± 2.42 , $P=0.001$; [Figure 2](#)).

The Association Between miR-146a and COX2 Expressions

There was a direct association between miR-146a and COX2 expression level in ESCC patients by nonparametric Spearman's rho test and the correlation coefficient was 0.438 (P -value=0.011, [95% confidence interval (0.1013–0.6850)]; **Figure 3, Table 3**.

The Association Between miR-146a Expression with Clinicopathologic Variables

Pearson Chi-Square and Spearman's rho tests were conducted to evaluate the association of age, gender, smoking and histological grade variable with the expression of miR-146a in tumor tissue samples. It seems that miR-146 expression was more increased in age $\geq$ 65 (P -value=0.09). MicroRNA-146a expression had no association with gender, smoking and histological grade (Table 4).

The Association of COX2 Expression and Clinicopathologic Variables

Pearson Chi-Square and Spearman's rho test was conducted to evaluate the association of age, gender, smoking and histological grade variable with COX2 expression in tumor tissue samples. It seems that COX2 expression was

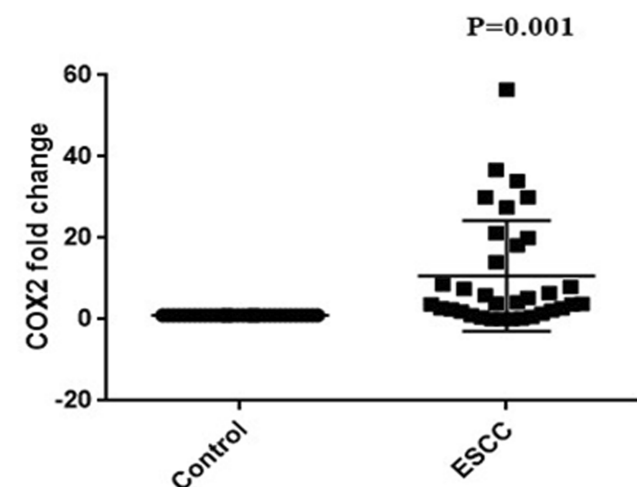
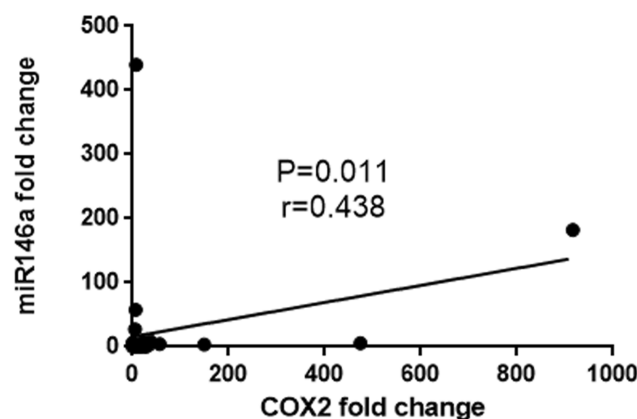
Table 2 The Association of Mir146a Expression and 8473 T>C Polymorphism in ESCC

SNP	Genotypes	Number	Fold Change miR146a Mean $\pm$ SE	95% CI (Confidence Interval)	P-value
8473T>C (rs5275)	8473 CC (mutant)	3	1.25 $\pm$ 1.05	-3.30-5.80	0.538
	8473 TC (heterozygote)	12	2.47 $\pm$ 0.91	0.41-4.54	
	8473 TT (wild type)	19	15.80 $\pm$ 9.78	-4.75-36.36	

more increased in age<65 (P -value=0.031). COX2 expression had no significant association with gender, smoking and histological grade (Table 5).

ROC Curve for miR146a

MicroRNA-146a expression was increased in 50% cancerous tissue compared with marginal normal tissue ($P=0.127$). MicroRNA-146a expression cannot be

**Figure 2** COX2 expression in cancerous tissue and marginal normal tissue.**Figure 3** Correlation between miR146a and COX2 expression.**Table 3** The Association Between Mir146a and COX2 Expression

Gene	Number (%)	Correlation Coefficient	P-value
COX2 High expression/ miR146a High expression	15 (45%)	0.438*	0.011
COX2 Low expression/ miR146a Low expression	5 (15%)		
COX2 High expression/ miR146a Low expression	12 (37%)		
COX2 Low expression/ miR146a High expression	1 (3%)		
Total	33 (100%)	-	-

Notes: *Correlation is significant at the 0.05 level (2-tailed).

Table 4 The Association of miR-146a Expression with Clinicopathologic Variables

Clinicopathologic Features	Low Expression (n=17)	High Expression (n=17)	P-value
Age (years)			0.096
<65	13	7	
≥65	4	10	
Gender			0.813
Male	7	6	
Female	10	11	
Smoking			0.716
Never or light	16	15	
Heavy	1	2	
Histological Grade			0.528
Well	13	11	
Differentiation			
Moderately	5	2	
Differentiation			
Poorly	2	3	
Differentiation			

considered as a diagnostic biomarker for ESCC, because it has no sufficient specificity (AUC = 0.553, sensitivity=88%, specificity=28%, 95% CI=0.413-0.692, P -value=0.453) (Figure 4).

Table 5 The Association of COX2 Expression with Clinicopathologic Variables

Clinicopathologic Features	COX2 Low Expression (N=6)	COX2 High Expression (N=28)	P-value
Age (years)			
<65	4	16	0.031*
≥65	2	12	
Gender			
Male	3	9	0.539
Female	3	19	
Smoking			
Never or light	4	26	0.587
Heavy	2	2	
Histological Grade			
Well	3	20	0.182
Moderately	3	4	
Poorly	0	4	

Note: *Correlation is significant at the 0.05 level (2-tailed).

ROC Curve for COX2

COX2 expression was increased in 80% cancerous tissue compared with marginal normal tissue ($P=0.001$). COX2 expression can be considered as a diagnostic biomarker for ESCC (AUC = 0.834, sensitivity=72%, specificity=83%, 95% CI=0.736 –0.932, P -value<0.0001) (Figure 5).

Survival Analysis Based on miR146a

Kaplan–Meier curve revealed that individuals with high expression of miR146a had a worse overall survival (OS) rather than who have miR146a low expression. Therefore, miR-146a expression can be an independent prognostic factor for overall survival in ESCC (Table 6, Figure 6). The mean overall survival time of patients was 24.4 months (95% CI: 19.09–29.7 months). Eleven patients (30.6%) were alive and 23 patients (63.9%) died and two patients were missed (5.6%) during our follow-up period of 4 years. Furthermore, univariate Cox survival analysis was used to assess the hazard ratios (HRs). Hazard ratio (HR) in patients with high expression miR146a was 1.59 [95% CI=0.66–3.62, P -value=0.269] which represents a higher risk when miR146a expression is higher.

Survival Analysis Based on COX2 Expression

Survival analysis based on COX2 expression showed patients with low expression COX2 had 8 months shorter life span than high expression (P -value= 0.125) (Table 7, Figure 7).

Discussion

In this study, we evaluated levels of miR-146a and COX-2 expression in 34 cancerous and marginal normal tissues with ESCC. The expression level of miR-146a

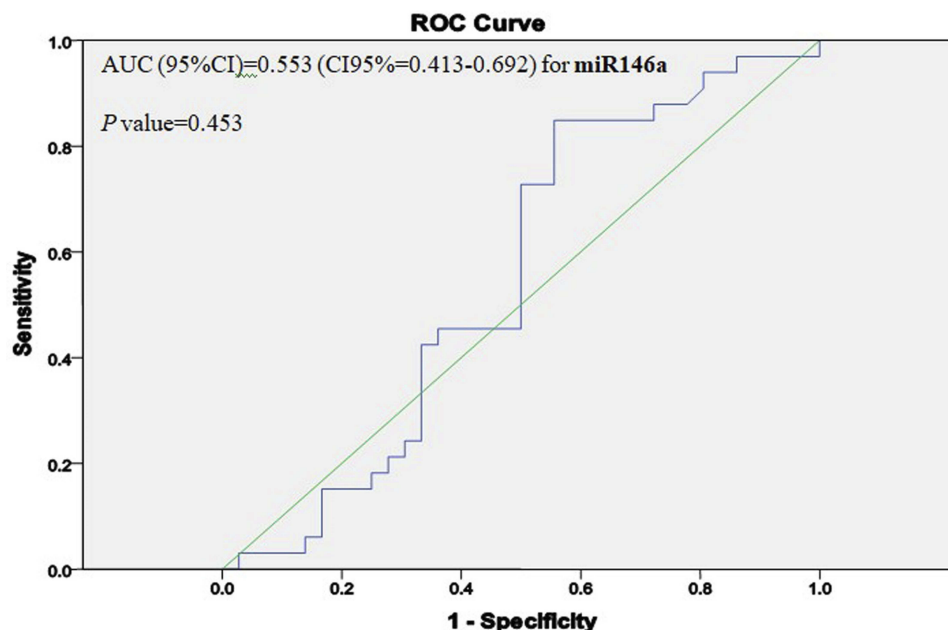


Figure 4 The ROC curve shows that miR146a cannot be a diagnostic biomarker for ESCC (P -value=0.453). (AUC=area under the curve).

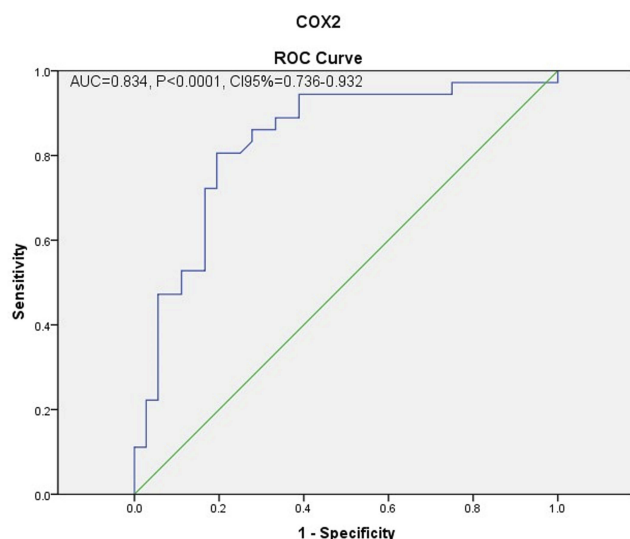


Figure 5 The ROC curve shows that COX2 can be a diagnostic biomarker for ESCC (P-value<0.0001). (AUC= area under the curve).

was approximately associated with age and was upregulated in 75% men and 70% women age 65 and above. Therefore, age $\geq$ 65 can be a risk factor for ESCC. But, there was no association between miR146a expression and other clinicopathologic variables including gender, smoking and histological grade. Both miR146a and COX2 expression were upregulated in 45% cases and downregulated in 15% cases. Despite our expectation, patients who had high expression miR146a, they had high expression COX2 as well. This may be described by having a +8473 (TC/CC) SNP (into 3'-UTR COX2) in 44% of the samples, based on our previous project.³²

Researchers have previously described that miR-146a directly binds into 3'-UTR COX2 and downregulate COX2 expression and subsequently decreased prostaglandin level. However, the mutation in 3'-UTR COX2 disrupts the miR-binding site somehow prevents the regulatory effect of miR146a on COX2.²⁷ Notably, Ashley et al found an inverse relationship between

Table 6 Survival Analysis for miR146a

Survival Analysis (Months)				
ESCC Patients		95% Confidence Interval		P-value
miR-146a Expression	Survival (Mean $\pm$ SE)	Lower Bound	Upper Bound	0.257
Low	27.8 $\pm$ 3.5	20.93	34.66	
High	20.69 $\pm$ 3.81	13.22	28.16	
Overall	24.40 $\pm$ 2.70	19.09	29.7	

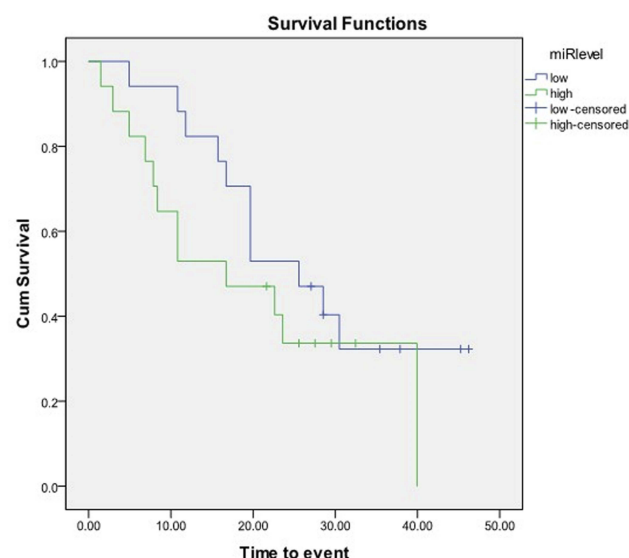


Figure 6 Kaplan–Meier curve revealed that individuals with high expression of miR146a had a worse survival rather than low expression.

miR146a and COX-2 expression.²⁷ Wise versa, the direct correlation between miR146a with COX2 expression in our results may discuss based on the presence of 8473 TC/CC SNPs at 3'-UTR COX2 which eliminates miR-146a binding site and subsequently its inhibitory effect. As a result, prostaglandin E2 levels increase and probably the risk of ESCC.

Wong et al reported MiR146a was significantly downregulated in cancerous tissue and serum of ESCC patients. They introduced miR146a as a prognostic and diagnostic biomarker for ESCC.¹ Other research mentioned that miR-21 and miR-375 can be used as prognostic biomarkers in esophageal cancer. MicroRNAs expression level can help us the detection of high-risk subjects and designing of sufficient treatment.³³ MicroRNAs expression level can help us design drugs against transcription of microRNA or select appropriate therapies for ESCC.³⁴

In our study, the Kaplan–Meier curve demonstrated a worse overall survival (OS) for individuals with high

Table 7 Survival Analysis for ESCC Based on COX2 Expression

Survival Analysis for ESCC Patients			
COX2 Expression	Survival (Mean $\pm$ SE)	95% Confidence Interval	P-value
Low	18.33 $\pm$ 3.41	11.64–25.02	0.125
High	26.34 $\pm$ 3.23	20.00–32.67	
Overall	24.75 $\pm$ 2.75	19.36–30.14	

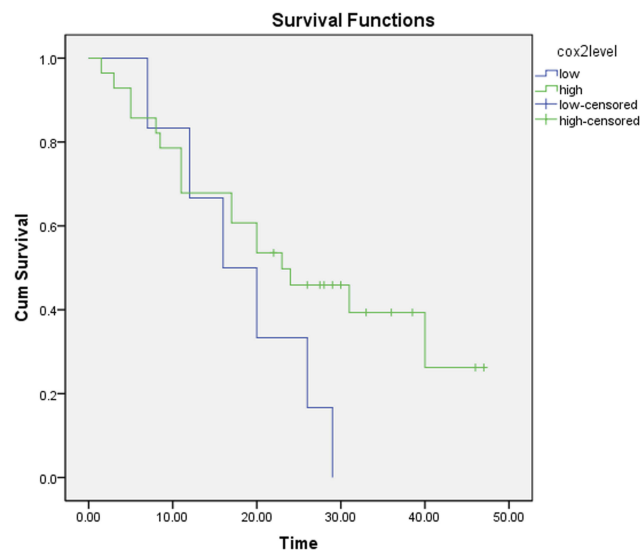


Figure 7 Kaplan–Meier curve revealed that individuals with low expression of COX2 had a worse survival rather than low expression.

expression of miR-146a so they had 7 months shorter life span rather than patients with low expression miR146a that our results are reversed to Wang et al¹. Furthermore, survival analysis based on COX2 expression showed patients with low expression COX2 had 8 months shorter life span than the high expression that our results are not in line with Nozoe et al³⁵. These results probably suggest a high miR-146a level and low COX2 level as a worse prognostic biological marker for ESCC. ROC curve analysis revealed miR146a cannot be a diagnostic biomarker for ESCC but ROC curve analysis showed COX2 expression can be considered as a diagnostic biomarker for ESCC.

Notably, there are computational models to predict the association of miRNAs with diseases and also as a biomarker for the detection of diseases.^{36–38} Therefore, we suggest using system biology because of decrease cost and time for the detection of ESCC.

Conclusions

MiR146a expression levels cannot be a diagnostic biomarker but COX2 expression can be considered as a diagnostic biomarker for ESCC. MiR146a and COX2 expression may be considered as prognostic biomarkers for ESCC.

Ethics and Consent

This study was approved by the institutional ethics committee; the ethical number is ir.goums.rec.1394.146. All

patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

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Disclosure

The authors declare no conflicts of interest.

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