A comprehensive analysis for associations between multiple microRNAs and prognosis of osteosarcoma patients

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Background. Osteosarcoma (OS) is the most common malignant primary bone tumor occurring in children and young adults, which occupies the second important cause of tumor-associated deaths among children and young adults. Recent studies have demonstrated that many microRNAs (miRNAs) have abnormal expression in OS, and can function as prognostic factors of OS patients. However, no previous studies have comprehensively analyzed the relationship between multiple miRNAs and prognosis of OS patients. **Methods.** A total of 63 OS patients were retrospectively enrolled. The clinical characteristics were collected, and the expression levels of miRNA-21, miRNA-30c, miRNA-34a, miRNA-101, miRNA-133a, miRNA-214, miRNA-218, miRNA-433 and miRNA-539 in tumor tissues were measured through quantitative real-time PCR (qRT-PCR). Kaplan-Meier analysis was used to perform univariate survival analysis, and Cox regression model was used to perform multivariate survival analysis which included the variables with $P<0.1$ in univariate survival analysis. **Results.** The cumulative survival for 1, 2 and 5 years was 90.48%, 68.25% and 38.10%, respectively, and mean survival time was (45.39±3.60) months (95%*CI*: 38.34-52.45). Kaplan-Meier analysis demonstrated that TNM stage, metastasis or recurrence, miRNA-21, miRNA-214, miRNA-34a, miRNA-133a and miRNA-539 were correlated with cum survival, but gender, age, tumor diameter, differentiation, miRNA-30c, miRNA-433, miRNA-101 and miRNA-218 were not. Multivariate survival analysis demonstrated that miRNA-21 (*HR*: 3.457, 95%*CI*: 2.165-11.518), miRNA-214 (*HR*: 3.138, 95%*CI*: 2.014-10.259), miRNA-34a (*HR*: 0.452, 95%*CI*: 0.202-0.915), miRNA-133a (*HR*: 0.307, 95%*CI*: 0.113-0.874) and miRNA-539 (*HR*: 0.358, 95%*CI*: 0.155-0.896) were independent prognostic markers of OS patients after adjusting for TNM stage (*HR*: 2.893, 95%*CI*: 1.496-8.125), metastasis or recurrence (*HR*: 3.628, 95%*CI*: 2.217-12.316) and miRNA-30c (*HR*: 0.689, 95%*CI*: 0.445-1.828). **Conclusions.** High expression of miRNA-21 and miRNA-214 and low expression of miRNA-34a, miRNA-133a and miRNA-539 were

associated with poor prognosis of OS patients after adjusting for TNM stage, metastasis or recurrence and miRNA-30c.

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Abstract

Background. Osteosarcoma (OS) is the most common malignant primary bone tumor occurring in children and young adults, which occupies the second important cause of tumor-associated deaths among children and young adults. Recent studies have demonstrated that many microRNAs (miRNAs) have abnormal expression in OS, and can function as prognostic factors of OS patients. However, no previous studies have comprehensively analyzed the relationship between multiple miRNAs and prognosis of OS patients.

Methods. A total of 63 OS patients were retrospectively enrolled. The clinical characteristics were collected, and the expression levels of miRNA-21, miRNA-30c, miRNA-34a, miRNA-101, miRNA-133a, miRNA-214, miRNA-218, miRNA-433 and miRNA-539 in tumor tissues were measured through quantitative real-time PCR (qRT-PCR). Kaplan-Meier analysis was used to perform univariate survival analysis, and Cox regression model was used to perform multivariate survival analysis which included the variables with $P<0.1$ in univariate survival analysis.

Results. The cumulative survival for 1, 2 and 5 years was 90.48%, 68.25% and 38.10%, respectively, and mean survival time was (45.39±3.60) months (95%*CI*: 38.34-52.45). Kaplan-Meier analysis demonstrated that TNM stage, metastasis or recurrence, miRNA-21, miRNA-214, miRNA-34a, miRNA-133a and miRNA-539 were correlated with cum survival, but gender, age, tumor diameter, differentiation, miRNA-30c, miRNA-433, miRNA-101 and miRNA-218 were not. Multivariate survival analysis demonstrated that miRNA-21 (*HR*: 3.457, 95%*CI*: 2.165-11.518), miRNA-214 (*HR*: 3.138, 95%*CI*: 2.014-10.259), miRNA-34a (*HR*: 0.452, 95%*CI*:

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Conclusions. High expression of miRNA-21 and miRNA-214 and low expression of miRNA-34a, miRNA-133a and miRNA-539 were associated with poor prognosis of OS patients after adjusting for TNM stage, metastasis or recurrence and miRNA-30c.

Key words: MicroRNAs; Survival; Kaplan-Meier analysis; Multivariate Cox regression analysis

Introduction

Osteosarcoma (OS) is the most common malignant primary bone tumor occurring in children and young adults, which occupies the second important cause of tumor-associated deaths among children and young adults (Mirabello et al., 2009; Mirabello et al., 2009; Biermann et al., 2013; Yu et al., 2017). It is highly aggressive and occurs mainly in the proximal tibia, proximal humerus, and metaphyseal regions of the distal femur, with an incidence of 4.4 per million people around the world (Zhu et al., 2016). OS responds poorly to chemotherapy and the 5-year survival rate is still very low for OS patients with metastasis or recurrence (Hutanu et al., 2017; Zhou et al., 2016), although its prognosis has been improved gradually over the past 30 years (Rytting et al., 2000; Kunz et al., 2015). Therefore, it is crucial to identify new biomarkers that can exactly evaluate the prognosis of OS.

MicroRNAs (miRNAs) are a group of non-coding RNAs, which consist of 18-25 nucleotides

(Ambros, 2004; Chang et al., 2016; Jamieson et al., 2012). They widely exist in animals, plants and even some viruses, and have an important role in post-transcriptional modulation of gene expression and gene silencing (Bartel, 2004; Hayes et al., 2014; Griffihs-Jones et al., 2008; Liu et al., 2017). Approximately 50% of miRNAs are confirmed to be associated with human tumorigenesis through directly targeting tumor suppressor genes or oncogenes (Li & Rana, 2014; Bracken et al., 2016). Recent studies have demonstrated that many miRNAs have abnormal expression in OS, and can function as prognostic factors of OS patients (Cheng et al., 2017). However, no previous studies have comprehensively analyzed the relationship between multiple miRNAs and prognosis of OS patients. In this study, the expression levels of miRNA-21, miRNA-30c, miRNA-34a, miRNA-101, miRNA-133a, miRNA-214, miRNA-218, miRNA-433 and miRNA-539 in tumor tissues of OS patients were measured through quantitative real-time PCR (qRT-PCR). Kaplan-Meier method was employed to determine the survival rate of OS patients, and long-rank test was employed to compare the survival rates between groups. Multivariate Cox regression analysis was finally performed to identify the independent prognostic factors with adjusting for confounders.

Materials & Methods

Patients

A total of 63 OS patients were retrospectively collected in Heze Municipal Hospital between January 2012 and January 2018. Surgery was performed in all of them, and tumor tissues and adjacent normal bone tissues were sampled. None of them received chemotherapy and

radiotherapy before surgery. All tissue samples, obtained during surgery, were frozen immediately in liquid nitrogen and stored at -80℃. The diagnosis and histological grading were determined with histopathological examination. This study received the approval of the ethic committee of Heze Municipal Hospital (20185261), and was performed according to the Declaration of Helsinki. All patients provided written informed consents.

Quantitative real-time PCR

Total RNA was extract from tumor tissues and adjacent normal bone tissues through miRNeasy kit (Qiagen, Germany) in accordance with instructions of the manufacturer. The TaqMan miRNA assey kit (Applied Biosystems, USA) was used to quantitate the expression levels of miRNAs. Rotor Gene 6000 Real-Time PCR (Qiagen, Germany) was used to perform Real-Time PCR with a TaqMan universal PCR master mix and an invitrogen kit. U6 was chosen as the reference gene, and the $2^{-\Delta\Delta Ct}$ method was used to assess the relative expression levels of miRNAs. The primers of miRNAs and U6 were designed and chemosynthesized by Shanghai Jima Biotech Ltd (Shanghai, China).

Statistical analysis

Statistical analysis was conducted using the SPSS version 20.0 for Windows (SPSS Inc., USA). Kolmogorov-Smirnov test was used to determine the normality of quantitative data. Normal data were expressed as mean ± standard deviation (SD), and non-normal data were expressed as median (interquartile range). Qualitative data were expressed as percentages or ratios (%). Kaplan-Meier analysis was used to perform univariate survival analysis, and Cox regression model was used to perform multivariate survival analysis which included the variables with

106 $P<0.1$ in univariate survival analysis. Significance was set at $P<0.05$.

107

108 **Results**

109 **General data**

110 These 63 OS patients included 36 males and 27 females, and the age of onset was 17 (10) years.

111 The other detailed clinical characteristics were demonstrated in *Table 1*. The follow-up was up to

112 January 2019. The cumulative survival for 1, 2 and 5 years was 90.48%, 68.25% and 38.10%,

113 respectively, and mean survival time was (45.39±3.60) months (95% *CI*: 38.34-52.45).

114 **Expression levels of miRNAs in tumor tissues and adjacent normal bone tissues**

115 According to the results of qRT-PCR (*Table 2*), the expression levels of miRNA-21, miRNA-

116 214 and miRNA-433 were higher in tumor tissues than in adjacent normal bone tissues, and the

117 expression levels of miRNA-30c, miRNA-34a, miRNA-101, miRNA-133a and miRNA-539 was

118 lower in tumor tissues than in adjacent normal bone tissues, and the expression level of miRNA-

119 218 was not statistically different.

120 **Univariate survival analysis**

121 The OS patients were divided into high expression group and low expression group according to

122 the median expression levels of miRNAs. Kaplan-Meier analysis demonstrated that TNM stage,

123 metastasis or recurrence, miRNA-21, miRNA-214, miRNA-34a, miRNA-133a and miRNA-539

124 were correlated with cum survival (*Fig. 1-7*), but gender, age, tumor diameter, differentiation,

125 miRNA-30c (*Fig. 8*), miRNA-433, miRNA-101 and miRNA-218 were not.

126 **Multivariate survival analysis**

TNM stage, metastasis or recurrence, miRNA-21, miRNA-214, miRNA-30c, miRNA-34a, miRNA-133a and miRNA-539 were included in Cox proportional hadards model. Multivariate survival analysis demonstrated that miRNA-21 (*HR*: 3.457, 95%*CI*: 2.165-11.518), miRNA-214 (*HR*: 3.138, 95%*CI*: 2.014-10.259), miRNA-34a (*HR*: 0.452, 95%*CI*: 0.202-0.915), miRNA-133a (*HR*: 0.307, 95%*CI*: 0.113-0.874) and miRNA-539 (*HR*: 0.358, 95%*CI*: 0.155-0.896) were independent prognostic markers of OS patients after adjusting for TNM stage (*HR*: 2.893, 95%*CI*: 1.496-8.125), metastasis or recurrence (*HR*: 3.628, 95%*CI*: 2.217-12.316) and miRNA-30c (*HR*: 0.689, 95%*CI*: 0.445-1.828). In other words, high expression of miRNA-21 and miRNA-214 and low expression of miRNA-34a, miRNA-133a and miRNA-539 were associated with poor prognosis of OS patients.

Discussion

The prognosis of OS patients has been significantly improved with the development of multiple chemotherapy regimens. However, OS patients receiving the same treatment often demonstrate different clinical outcomes, suggesting an urgent need for developing reliable prognostic biomarkers to improve the prognosis of OS patients. MiRNAs modulate protein expression through regulating the degradation and translation of mRNAs at post-transcriptional level (Chang et al., 2016; Jamieson et al., 2012). They play a critical role in various biological processes which are involved in the development and progression of tumors, including proliferation, apoptosis, differentiation and metastasis (Hayes et al., 2014; Ebert & Sharp, 2012; Rogers & Chen, 2013; Liu et al., 2012).

148 Additionally, they are very stable and easily detected in the blood and tissues (Gilad et al., 2008).
149 Therefore, plenty of miRNAs are employed as new biomarkers for the diagnosis and prognosis
150 of tumors. Regarding to OS, a variety of miRNAs has been reported to be associated with its
151 prognosis. In our study, high expression of miRNA-21 and miRNA-214 and low expression of
152 miRNA-34a, miRNA-133a and miRNA-539 were associated with poor prognosis of OS patients.
153 MiRNA-21has been confirmed to act as tumor oncogene in many types of tumors. For OS, it
154 may regulate the proliferation, invasion and metastasis of OS cells through directly targeting
155 PTEN and RECK (Ziyan et al., 2011; Lv et al., 2016). Li et al. demonstrated that the elevated
156 expression of miRNA-21 might lead to elevated expression of the proteins in the PI3K/AKT
157 signaling pathway and decreased expression of PTEN, which was associated with the increased
158 invasiveness of OS cells (Li et al., 2018). Hu et al. indicated that inhibition of miRNA-21 might
159 reduce the proliferation of OS cells through modulating the TGF-β1 signaling pathway and
160 targeting PTEN (Hu et al., 2018). Additionally, miRNA-21 might decrease the anti-tumor effect
161 of cisplatin through modulating the expression of Bcl-2 (Ziyan & Yang, 2016). MiRNA-214
162 may act as either a tumor suppressor gene or an oncogene. For OS, the elevated expression of
163 miRNA-214 is associated with enhanced invasion and proliferation of OS cells through
164 modulating the expression of LZTS1 (Xu & Wang, 2014). However, Rehei et al. found that the
165 expression of miRNA-214 was negatively associated with the expression of TRAF3 in OS
166 tissues, and over-expression of miRNA-214 could inhibit the invasion and metastasis of OS cells
167 through targeting TRAF3 (Rehei et al., 2018).
168 MiRNA-34a has various target genes which play important roles in biological function of OS

169 cells, such as Fag1, Wnt, p53 and Notch (Wu et al., 2013; Yan et al., 2012). Gang et al.
170 demonstrated that miRNA-34a was correlated with the apoptosis, proliferation and adhesion of
171 OS cells, and could function as a new tumor suppressor gene by reducing the expression of
172 DUSP1 (Gang et al., 2017). Zhang et al. proved that miRNA-34a was a crucial regulator in the
173 dedifferentiation of OS cells through modulating PAI-1-Sox2 axis (Zhang et al., 2018). In
174 addition, Wang et al. showed that down-modulated expression of miRNA-34a was a prognostic
175 biomarker for poor prognosis of OS patients through a meta-analysis (Wang et al., 2018).
176 MiRNA-133a has been proved to be a crucial modulator for osteogenesis, and have a key role in
177 osteoblast differentiation (Bao et al., 2010). It can act as an antionco-miRNA or a tumor
178 suppressor gene in the development and progression of tumors (Ji et al., 2013). It has been
179 reported to be associated with many cancers, including esophagus cancer, bladder cancer and
180 prostate cancer. The underlying mechanisms of pro-apoptotic function of miRNA-133a may be
181 associated with the inhibition ofMcl-1 and Bcl-xL expression (Wang et al., 2010). Few reports
182 have investigated the biological functions of miRNA-539. Muthusamy et al. found that miRNA-
183 539 could inhibit *O*-GlcNAcase expression (Muthusamy et al., 2014). Wang et al. demonstrated
184 that miRNA-539 was involved in the regulation of apoptosis and mitochondrial activity by
185 means of targeting PHB2 (Wang et al., 2014). The expression of miRNA-539 is down-regulated
186 in thyroid cancer, and moreover, it has a suppressor role in the invasion and metastasis of thyroid
187 cancer cells through targeting CARMA1 (Gu & Sun, 2015).
188
189 **Conclusions**

High expression of miRNA-21 and miRNA-214 and low expression of miRNA-34a, miRNA-133a and miRNA-539 were associated with poor prognosis of OS patients after adjusting for TNM stage, metastasis or recurrence and miRNA-30c.

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None.

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Table 1(on next page)

Clinical characteristics of OS patients

1 **Table 1** Clinical characteristics of OS patients

Clinical characteristics	No. of patients	Percentages (%)
Gender		
Male	36	57.14%
Female	27	42.86%
Age (years)		
≤25	55	87.30%
>25	8	12.70%
Tumor diameter (cm)		
≤5	37	58.73%
>5	26	41.27%
TNM stage		
Ⅰ+Ⅱ	25	39.68%
Ⅲ+Ⅳ	38	60.32%
Metastasis or recurrence		
Yes	37	58.73%
No	26	41.27%
Differentiation		
Well and moderate	31	49.21%
Poor	32	50.79%

2

3

Table 2(on next page)

Expression levels of miRNAs in tumor tissues and adjacent normal bone tissues

Table 2 Expression levels of miRNAs in tumor tissues and adjacent normal bone tissues

	miRNA-21	miRNA-214	miRNA-433	miRNA-30c	miRNA-34a	miRNA-101	miRNA-133a	miRNA-539	miRNA-218
Tumor tissues	7.35±2.96	6.12±2.25	2.26±1.34	3.93±1.77	3.09±0.94	3.16±1.72	3.78±2.17	2.35±1.08	2.16±1.07
Adjacent normal bone tissues	3.14±1.58	3.37±1.49	1.17±0.91	5.34±1.32	5.24±1.35	5.19±2.74	11.89±4.16	5.23±1.84	2.31±1.18
t	9.959	8.088	5.341	-5.069	-10.374	-4.981	-13.719	-10.714	-0.747
P	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	>0.05

Figure 1

Kaplan-Meier analysis of cumulative survival for TNM stage using Log Rank test.

χ^2=4.199, P=0.040

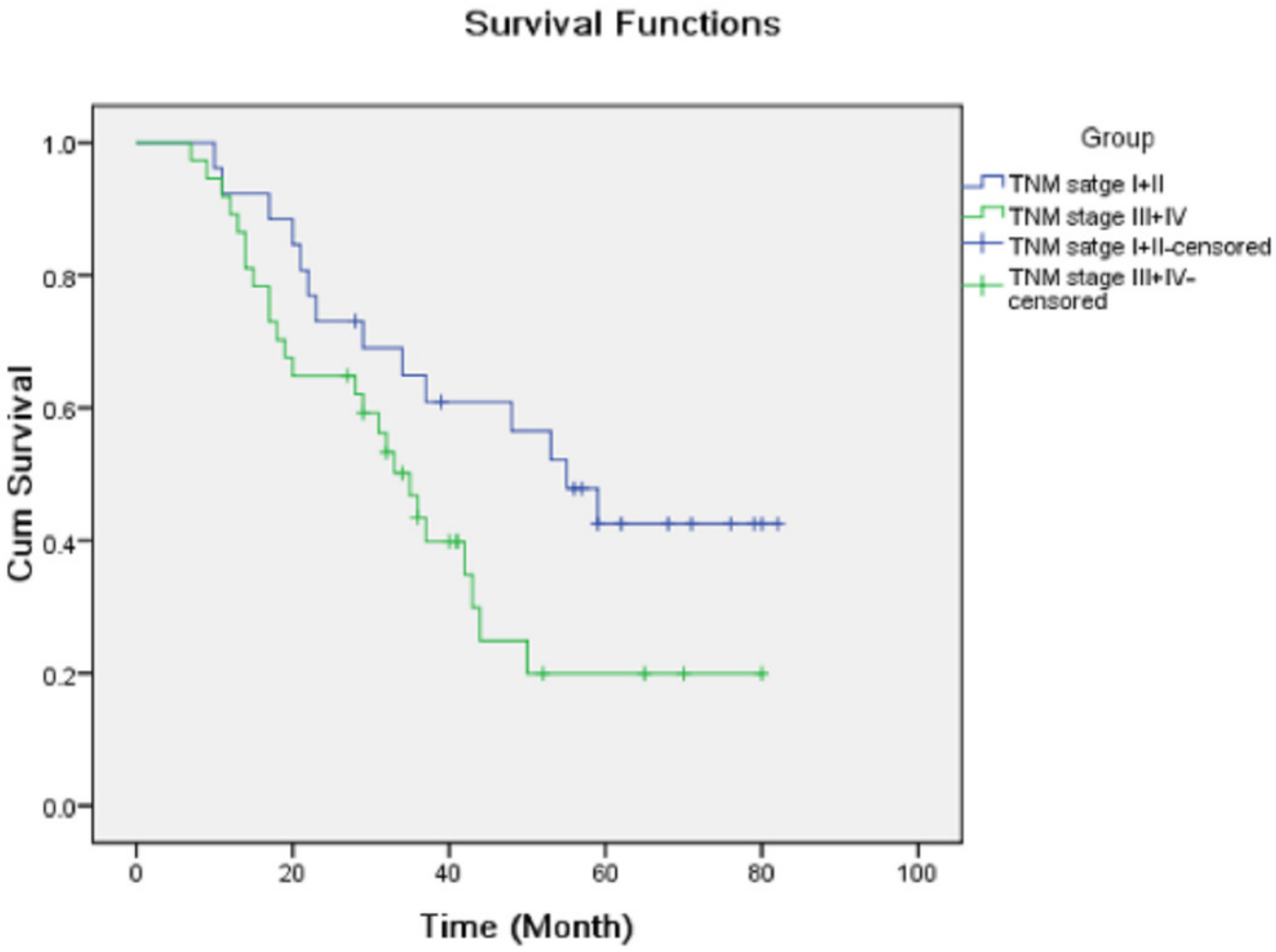

Figure 2

Kaplan-Meier analysis of cumulative survival for metastasis or recurrence using Log Rank test. χ^2=28.970, P<0.001

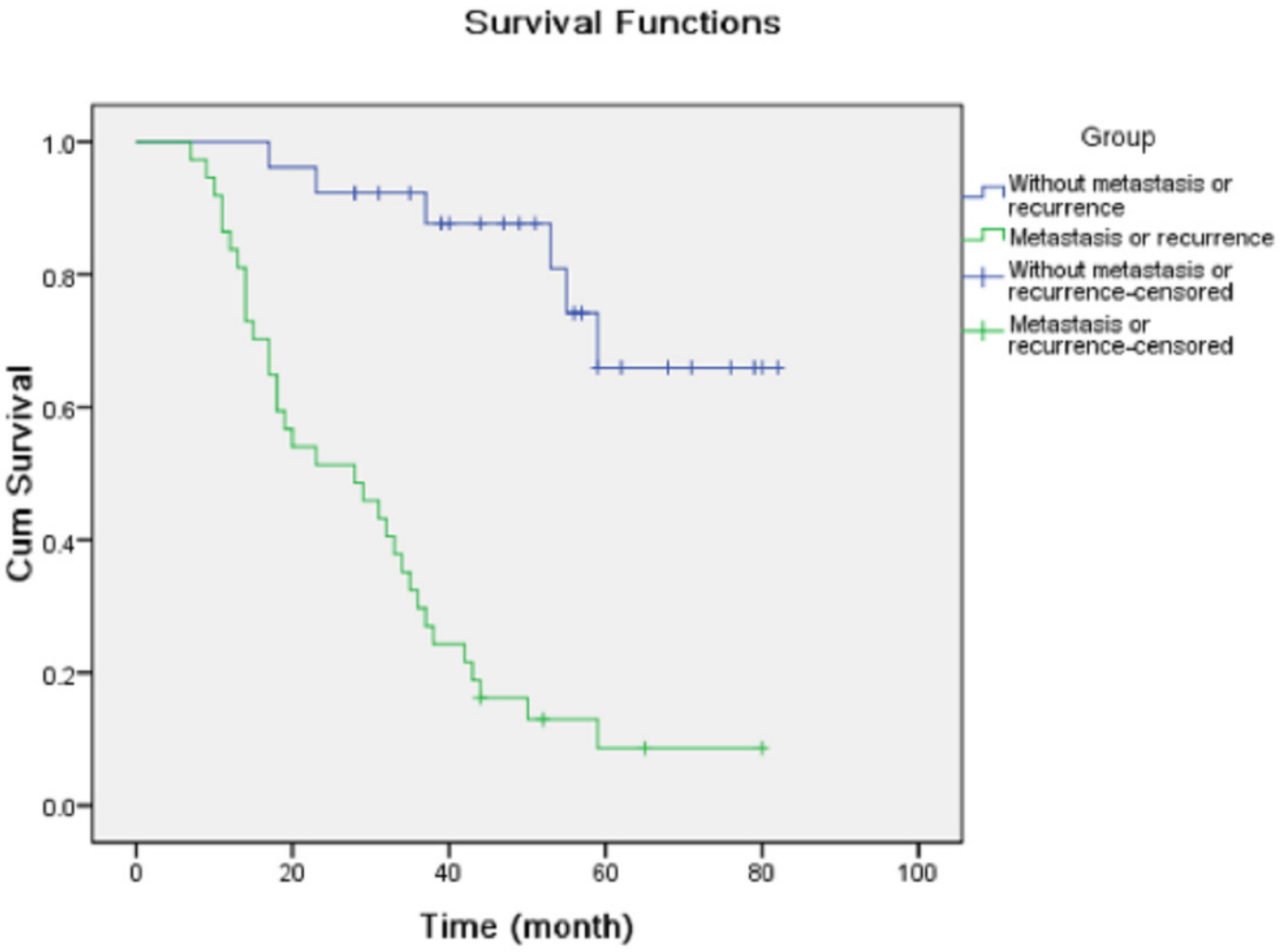

Figure 3

Kaplan-Meier analysis of cumulative survival for miRNA-21 using Log Rank test.

χ^2=11.847, P=0.001

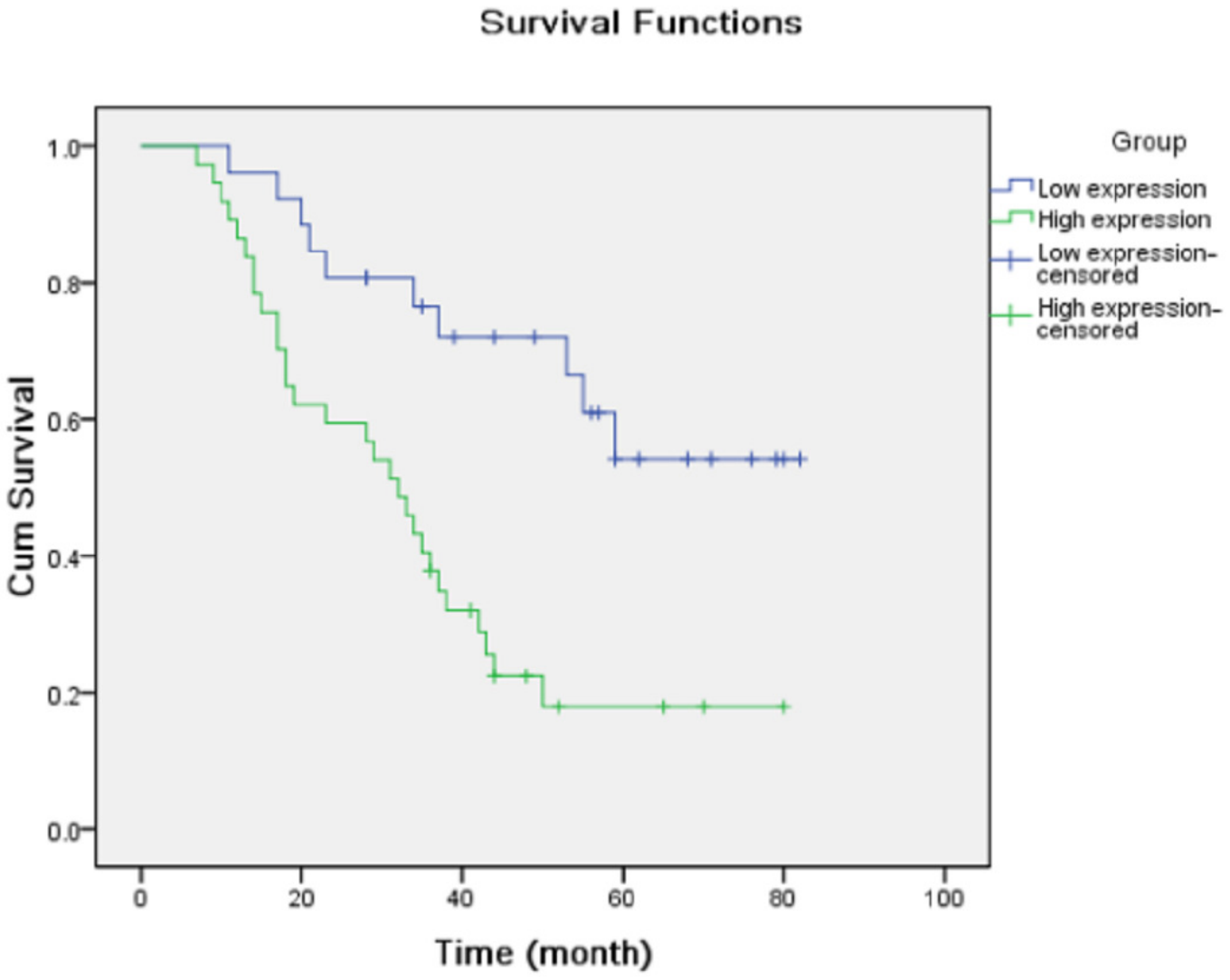

Figure 4

Kaplan-Meier analysis of cumulative survival for miRNA-214 using Log Rank test.

χ^2=7.338, P=0.007

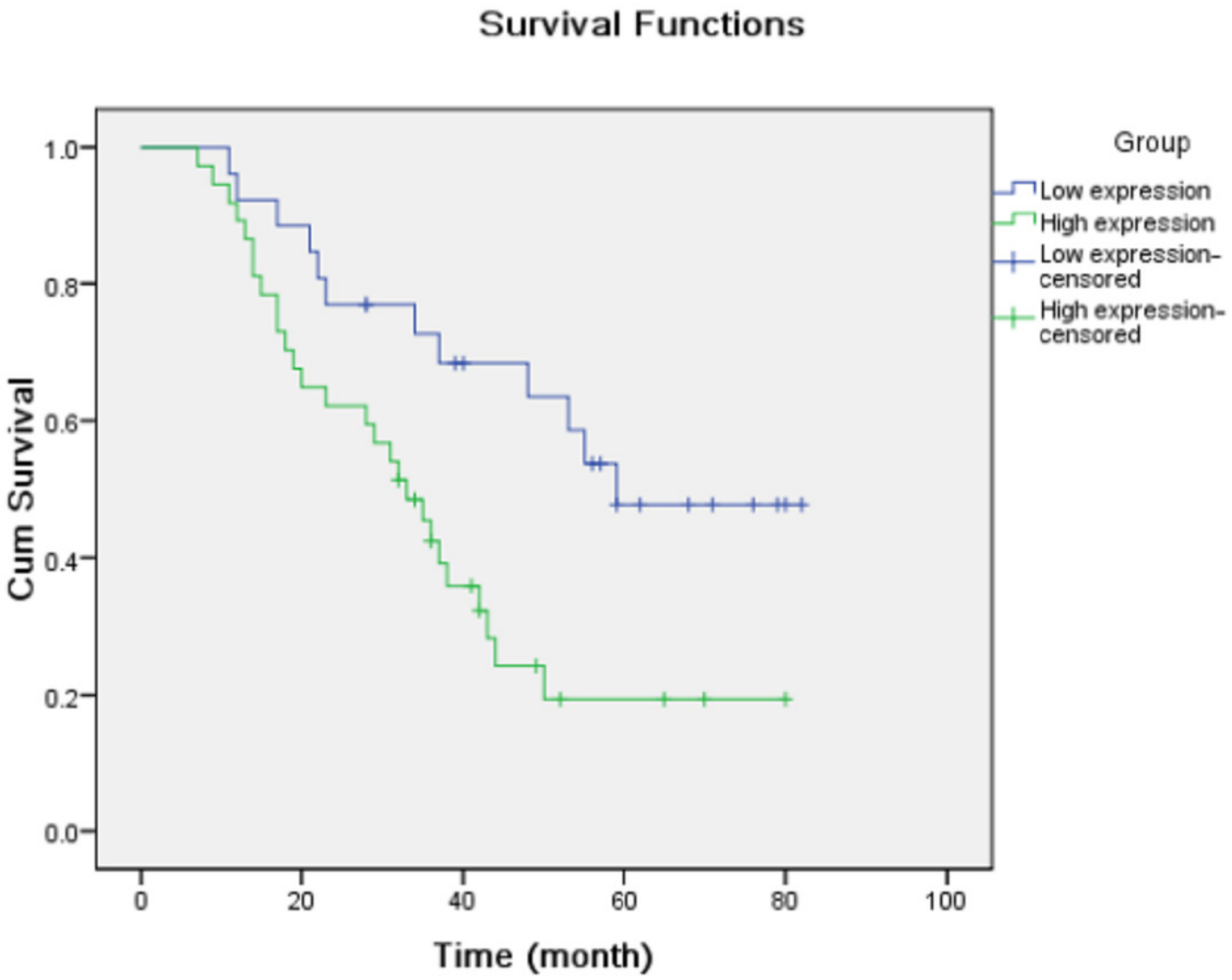

Figure 5

Kaplan-Meier analysis of cumulative survival for miRNA-34a using Log Rank test.

χ^2=5.372, P=0.020

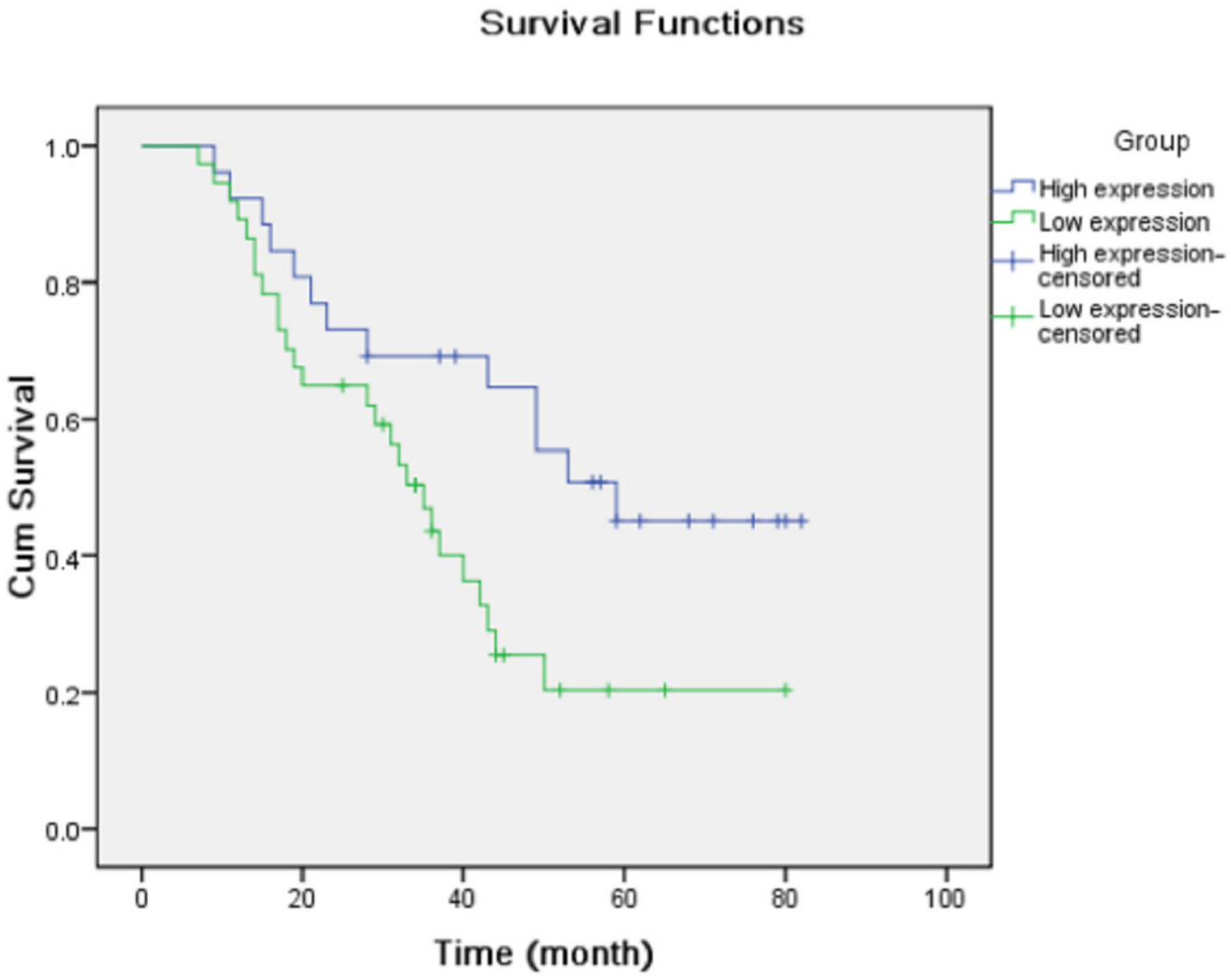

Figure 6

Kaplan-Meier analysis of cumulative survival for miRNA-133a using Log Rank test.

χ^2=16.258, P<0.001

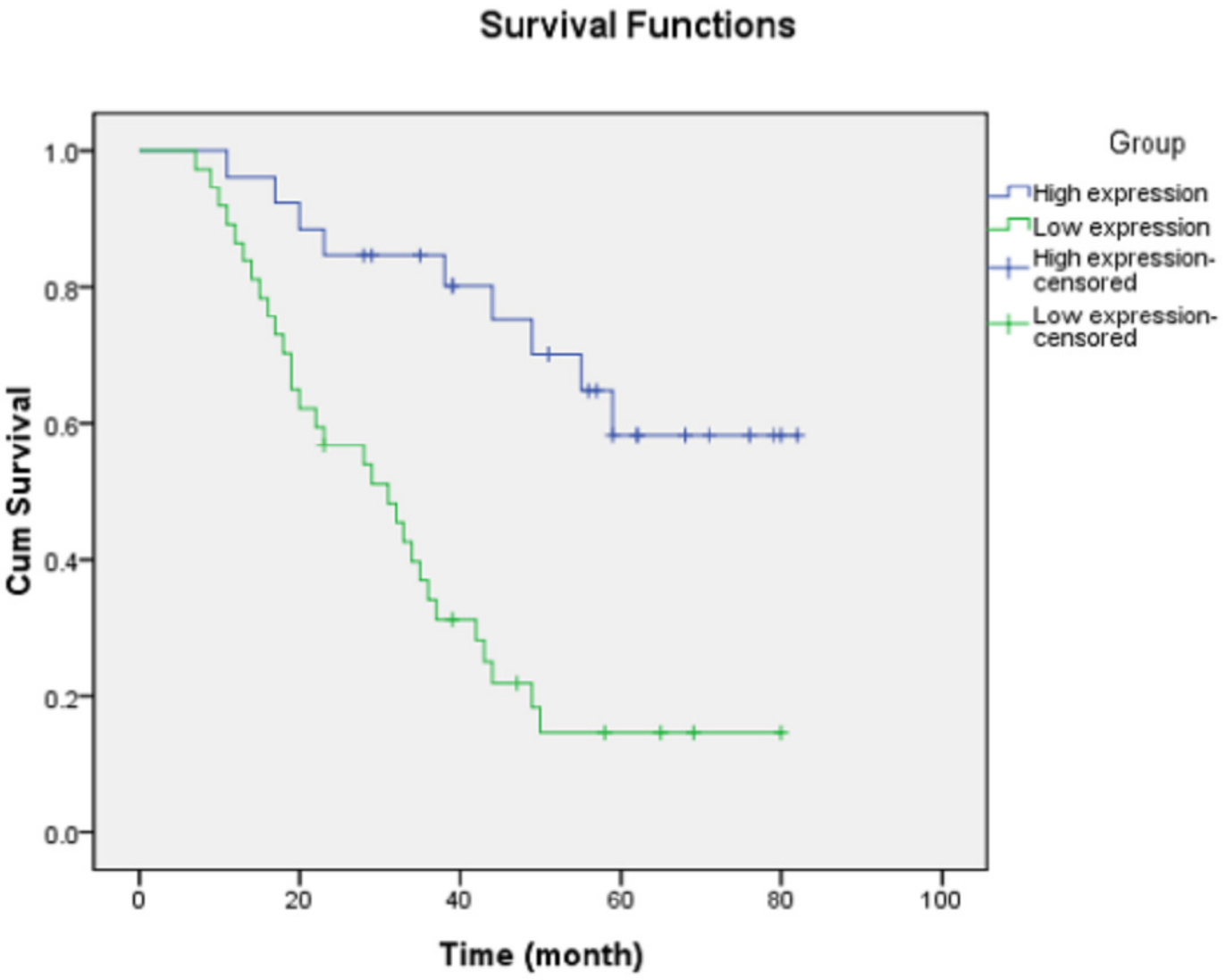

Figure 7

Kaplan-Meier analysis of cumulative survival for miRNA-539 using Log Rank test.

χ^2=7.390, P=0.007

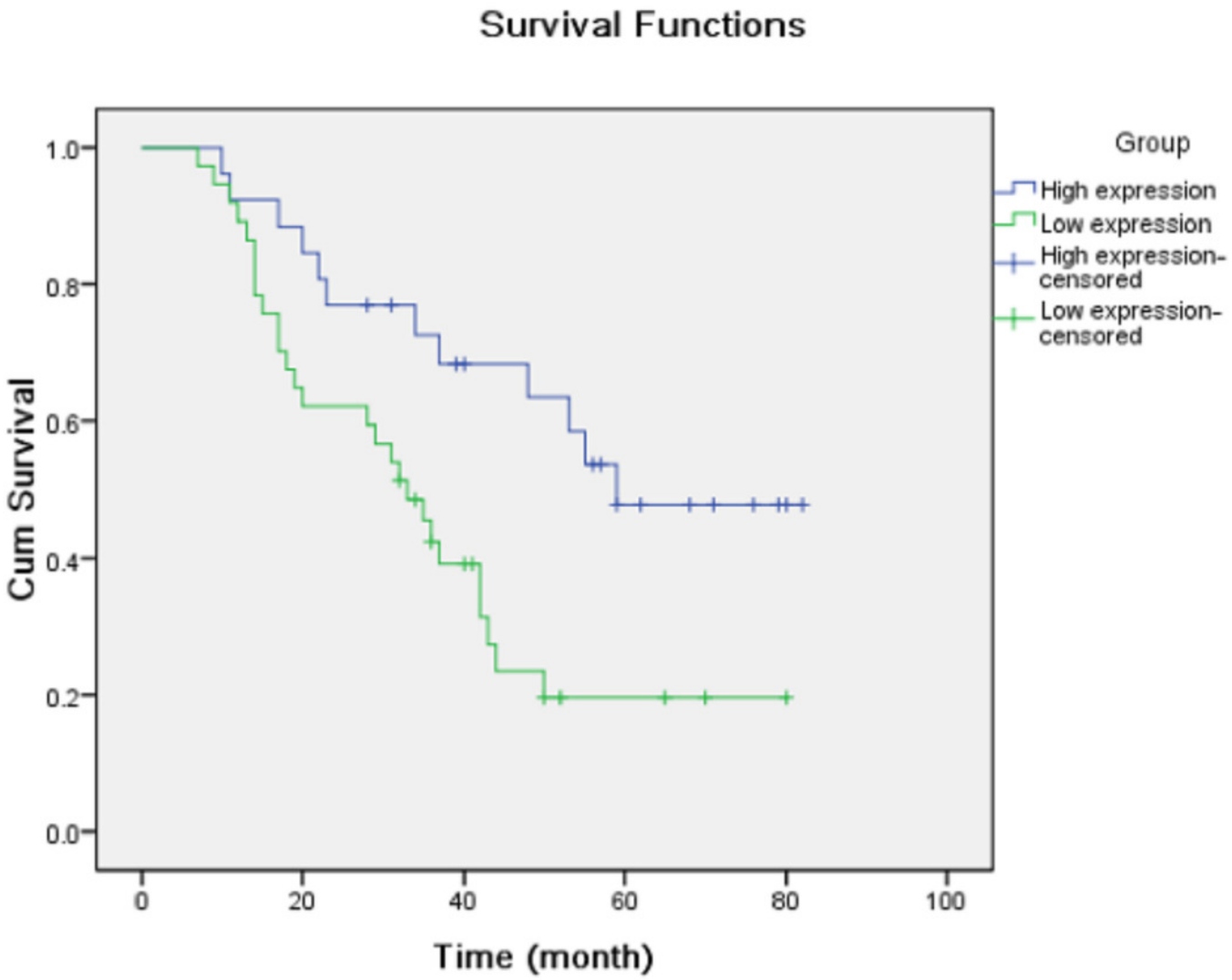

Figure 8

Kaplan-Meier analysis of cumulative survival for miRNA-30c using Log Rank test.

χ^2=3.378, P=0.066