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ORIGINAL ARTICLE

# Association Between Polymorphisms in the Promoter Region of microRNA-34b/c and the Chemoradiotherapy Efficacy for Locally Advanced Esophageal Squamous Cell Carcinoma in Chinese Han Population

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## Abstract

The study aims to explore the association between polymorphisms in the promoter region of microRNA-34b/c (miR-34b/c) and the chemoradiotherapy efficacy for locally advanced esophageal squamous cell carcinoma (ESCC) in Chinese Han population. A total of 175 locally advanced ESCC cases and 186 healthy individuals were enrolled as the case and control groups. Denaturing high performance liquid chromatography (DHPLC) was applied to determine the genotypes of subjects. Subjects in the case group were classified into complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). CR + PR were defined as the sensitive group, and SD + PD were defined as the resistance group. All patients were followed up for 3 ~ 36 months. Receiver operating characteristic (ROC) curve was used to evaluate the predictive value of rs4938723 in the promoter region of miR-34b/c in the chemoradiotherapy efficacy for patients with locally advanced ESCC. The distribution of genotype and allele of rs4938723 in the promoter region of miR-34b/c was significantly different between the case and control group (both $P < 0.05$), and CC genotype and C allele could decrease the risk of ESCC (CC genotype: OR = 0.57, 95%CI = 0.32 ~ 0.99, $P = 0.045$; C allele: OR = 0.72, 95%CI = 0.54 ~ 0.97, $P = 0.032$). MiR-34b/c rs4938723 was associated with ESCC TNM staging, differentiation degree, and lymph node metastasis (LNM) for ES CC patients (all $P < 0.05$). The chemoradiotherapy efficacy of patients with CC genotype was better than that of patients with (TT + TC) genotypes ($P < 0.05$). ROC curve results showed that the area under curve (AUC), sensitivity and specificity were 0.777, 85.1% and 71.3%, respectively. The average median progression free survival (PFS) of patients with (TT + TC) genotypes was significantly shorter than those patients with CC genotype ($P < 0.05$). Our study provides evidence that miR-34b/c rs4938723 is closely related with the chemoradiotherapy efficacy for locally advanced ESCC.

**Keywords** MicroRNA-34b/c · Esophageal squamous cell carcinoma · Promoter region · Polymorphism · Chemoradiotherapy efficacy · Chinese Han population

## Introduction

Esophageal squamous cell carcinoma (ESCC) is the most common type of esophageal cancer, ranking as the sixth leading cause of cancer mortality in the world [1]. The risk factors of ESCC range from smoking, excessive drinking, micronutrient deficiency to dietary carcinogen exposure, and one's genetic makeup could also play an important part in the development of ESCC [2]. It is reported that lymph node metastases (LNM) is prevalent during the development of ESCC, and that is the reason why more than 50% of ESCC cases are diagnosed at locally advanced stage with enlargement node and obvious esophageal invasion [3]. Surgical resection is regarded as curative only at the early stage, while chemoradiotherapy is widely accepted by ESCC patients to improve their clinical survival rate and life quality [4]. However, it is indicated in recent published literature that at

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least 50% of ESCC patients receiving chemoradiotherapy would suffer from serious toxicities, therefore, more efforts are expected to be devoted into enhancing the efficacy of chemoradiotherapy for ESCC patients [5].

MicroRNAs (miRs) are known as a class of small noncoding RNAs (21–25 nucleotides in length) which control gene expression at posttranscriptional level, among which miR-21 is reported to regulate cell proliferation and invasion process in ESCC through targeting the programmed cell death 4 (PDCD4) gene [6]. MiR-145, miR-133a and miR-133b are also revealed to be tumor-suppressive miRs in ESCC targeting at Fascin homolog 1 (FSCN1) gene [7]. MiR-34b/c is member of the tumor suppressor miR-34 family, which is revealed as a downstream transcriptional target of p53, and miR-34a and miR-34b are believed to cooperate in inhibiting cell proliferation and adhesion-independent growth [8]. MiR-34b and miR-34c are frequently silenced through DNA methylation in gastric cancer [9]. MiR-34b/c also acts as tumor suppressor in colorectal cancer and is considered as a potential target of epigenetic silencing in colorectal cancer [10]. Single nucleotide polymorphisms (SNPs) refer to genetic variations located in miRNA genes region which could alter the expression or maturation of miRNAs and then affect cancer risk, in other words, SNPs in miRNAs are valuable predictive factors for cancer risk [11]. The polymorphism rs4938723 in the promoter region of miR-34b/c is found to be potentially associated with the susceptibility to a variety of human cancers, such as hepatocellular carcinoma, colorectal cancer and breast cancer [12]. However, researches about the relationship between miR-34b/c and ESCC are still unclear; therefore, our study aims to elucidate the association of SNP rs4938723 in the promoter region of miR-34b/c with the chemoradiotherapy efficacy for locally advanced ESCC, in the hope of finding out a novel approach to improve the efficacy of chemoradiotherapy for ESCC patients.

## Materials and Methods

### Ethical Statement

This study was approved and supervised by the Ethical Committee of the Affiliated Tumor Hospital of Xinjiang Medical University, and informed consent has been obtained from each participant.

### Subjects

From March 2011 to October 2013, a total of 175 patients diagnosed as locally advanced ESCC in III-IV stage in the Affiliated Tumor Hospital of Xinjiang Medical University in accordance with the standards of esophageal cancer tumor node metastasis (TNM) staging jointly made by American Joint Committee on Cancer (AJCC) and International Union Against Cancer (UICC) [13] were recruited as the case group. Inclusion criteria: (1) patients had an expected survival period of more than 6 months and without complication of esophageal fistula; (2) patients with normal hemogram, liver, kidney and heart functions and without other diseases requiring hospitalization; (3) patients in Chinese Han population were pathologically diagnosed as primary ESCC at first diagnosis; (4) patients without any surgical treatment, radiotherapy or chemotherapy before sampling; (5) patients aging between 30 and 65 years old whose general physical conditions were acceptable; (6) patients without disease or dysfunction in other major organs, and no apparent abnormality was detected in their blood routine, urine routine, stool routine, liver function, kidney function or heart function. Exclusion criteria: (1) patients in Chinese Han population suffered from secondary ESCC; (2) patients who have received surgical treatment, radiotherapy or chemotherapy; (3) patients with other malignancies. Additionally, 186 individuals in the same area whose health examination results were normal were randomly enrolled in the control group.

### Denaturing High Performance Liquid Chromatography (DHPLC)

Five milliliters of peripheral blood were extracted from the subjects in the case group and the control group with empty stomach in the morning respectively and then anticoagulated by ethylene diamine tetraacetic acid (EDTA). The procedures were in accordance with DNA kit instructions (Qiagen company, Hilden, Germany). An ultraviolet spectrophotometer was applied to detect DNA content, and the absorbance value (A value), A260 and A280, were examined, and the ratio was between 1.8 and 2.0 Premer Premier 5.0 software was employed to design and determine the PCR amplification primer of rs4938723, and the primer was synthesized by Shanghai Biological Engineering Technology Co., Ltd. (Shanghai, China). The primer sequences of rs4938723 were: forward 5′-CTCACCTCCTCTGGGAACCTT-3, reverse 5′-AAGGCCATACCATTCAAGACAGTAT-3′. PCR reaction conditions were: 94 °C for 2 min, 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, with 35 cycles; extending at 72 °C for 5 min, and the product was preserved at 4 °C. DHPLC was applied to examine all genotypes under partial denaturation. The column temperature was 59.3 °C, and the flow velocity was 0.9 ml/min. There were two steps in the process of genotyping: first step, the genotype presented two peaks in DHPLC was heterozygous genotype; second step, the PCR samples presented single peak in DHPLC were equally mixed with the wild homozygous genotype samples confirmed by sequencing, and DHPLC was then performed on the mixture, and then the genotype presented single peak was considered as wild homozygous genotype, while the

genotype presented two peaks was mutant homozygote genotype. The mutation was confirmed by sequencing.

### Treatment Regime and Efficacy Evaluation

All patients received radiotherapy combined with chemotherapy. Radiotherapy dose: 60 ~ 64 Gy/30 ~ 32 times; treatment regime was platinum-based combination chemotherapy: FP regimen: 5-fluorouracil (5-FU) + cis-dichlorodiamineplatinum (DDP); TP regimen: paclitaxel or docetaxel + platinum. Chest computed tomography (CT) or 18F–fluorodeoxyglucose polythylene terephthalate (18F–FDG PET) examination was performed on the patients in 4 ~ 6 weeks after chemoradiotherapy to evaluate the short-term efficacy. According to the standards of Response Evaluation Criteria in Solid Tumors (RECIST) (version 1.0) [14], the short-term efficacy was classified into complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). CR + PR were defined as the sensitive group, and SD + PD were defined as the resistance group.

### Follow Up

All patients were followed up for 3 ~ 36 months through telephone, outpatient service and medical record review until October 2016. Among which, 6 cases were lost to follow up, with the follow up rate showing 93.0%. Progression free survival (PFS) is defined as the period from the beginning of treatment to carcinoma recurrence or death of the patients.

### Statistical Analysis

All data were analyzed by SPSS 21.0 (IBM Corp. Armonk, NY, USA) statistical software. The data in line with normal distribution was examined by t-test, while the data not in line with normal distribution was analyzed by nonparametric rank sum test. Nonparametric rank sum test was applied to analyze the ranked data, Kaplan-Meier method combined with Log-rank test were employed in survival analysis, and receiver operating characteristic (ROC) curve was used to evaluate the predictive value of microRNA-34b/c genetic polymorphism in promoter region in the chemoradiotherapy efficacy for patients with locally advanced ESCC. $P$ represented two-tailed probability, and $P < 0.05$ was considered as significantly different.

## Results

### Baseline Characteristics of Subjects Between the Case Group and the Control Group

There were 175 ESCC patients in the case group (123 males and 52 females, the average age was 52.41 ± 6.79 years old) and 186 healthy controls in the control group (124 males and 62 females, the average age was 52.12 ± 6.76 years old). There was no significant difference in age, gender smoking history or drinking history between the two groups (all $P > 0.05$) (Table 1).

### Distribution of Genotype and Allele of rs4938723 in the Promoter Region of miR-34b/c

Hardy-Weinberg Equilibrium (HWE) test based on goodness of fit test was performed on the subjects in the control group. The results revealed that in the control group, the genotype distribution of rs4938723 in the promoter region of miR-34b/c was in line with HWE test ($P > 0.05$). The samples in the study were randomly selected so that the samples were representative.

The distribution of genotype and allele of rs4938723 in the promoter region of miR-34b/c was significantly different between the case and control group (both $P < 0.05$). For rs4938723 in the promoter region of miR-34b/c, CC genotype could decrease the risk of ESCC (OR = 0.57, 95%CI = 0.32 ~ 0.99, $P = 0.045$), and C allele could also decrease ESCC risk (OR = 0.72, 95%CI = 0.54 ~ 0.97, $P = 0.032$) (Table 2).

### Association of miR-34b/c rs4938723 with the Clinicopathological Characteristics of Locally Advanced ESCC Patients

MiR-34b/c rs4938723 was associated with ESCC TNM staging, differentiation degree and LNM (all $P < 0.05$), while no significant difference in gender, age, smoking history, drinking history and ESCC site was found between the case and control groups (all $P > 0.05$) (Table 3).

**Table 1** Baseline characteristics of subjects in the case and control group

| Baseline characteristics | Case group ($n = 175$) | Control group ($n = 186$) | t/χ2 | $P$ |
|---|---|---|---|---|
| Average age | 52.41 ± 6.79 | 52.12 ± 6.76 | 0.323 | 0.747 |
| Gender (Male/Female) | 123/52 | 124/62 | 0.547 | 0.46 |
| Smoking history (With/Without) | 77/98 | 84/102 | 0.049 | 0.824 |
| Drinking history (With/Without) | 68/107 | 74/112 | 0.033 | 0.857 |

**Table 2** Distribution of genotype and allele of rs4938723 in the promoter region of miR-34b/c (n/%)

| SNP | Genotype | Case group (n = 175) | Control group (n = 186) | *P* | OR (95%CI) |
|---|---|---|---|---|---|
| rs4938723 | TT | 64 (36.7%) | 52 (28.0%) | Ref. | |
| | TC | 74 (42.2%) | 81 (43.5%) | 0.226 | 0.74 (0.46–1.20) |
| | CC | 37 (21.1%) | 53 (28.5%) | 0.045 | 0.57 (0.32–0.99) |
| | T | 202 (57.7%) | 185 (49.7%) | Ref. | |
| | C | 148 (42.3%) | 187 (50.3%) | 0.032 | 0.72 (0.54–0.97) |

*SNP* single nucleotide polymorphism

## Association of miR-34b/c rs4938723 with the Chemoradiotherapy Efficacy for Locally Advanced ESCC Patients

In the sensitive group, the patients with (TT + TC) genotypes and the patients with CC genotype accounted for 26.0% and 74.0%, respectively. In the resistance group, the patients with (TT + TC) genotypes and the patients with CC genotype accounted for 66.7% and 33.3%, respectively. The chemoradiotherapy efficacy of patients with CC genotype was better than that of patients with (TT + TC) genotypes ($P < 0.05$) (Table 4).

## Predictive Value of miR-34b/c rs4938723 in the Chemoradiotherapy Efficacy for Locally Advanced ESCC Patients

ROC curve was applied to evaluate the predictive value of miR-34b/c rs4938723 in the chemoradiotherapy efficacy for locally advanced ESCC patients. The area under curve

**Table 3** Association of miR-34b/c rs4938723 with the clinicopathological characteristics of locally advanced ESCC patients

| Clinicopathological characteristics | Cases | rs4938723 | | $\chi 2$ | *P* |
|---|---|---|---|---|---|
| | | CC (*n* = 37) | TT + TC (*n* = 138) | | |
| Gender | | | | | |
| Male | 123 | 29 | 94 | 1.471 | 0.225 |
| Female | 52 | 8 | 44 | | |
| Age | | | | | |
| ≤ 50 | 72 | 15 | 57 | 0.007 | 0.933 |
| > 50 | 103 | 22 | 81 | | |
| Smoking history | | | | | |
| With | 77 | 12 | 65 | 2.548 | 0.11 |
| Without | 98 | 25 | 73 | | |
| Drinking history | | | | | |
| With | 68 | 10 | 58 | 2.764 | 0.096 |
| Without | 107 | 27 | 80 | | |
| ESCC site | | | | | |
| Upper | 34 | 7 | 27 | 0.07 | 0.966 |
| Middle | 115 | 24 | 91 | | |
| Lower | 26 | 6 | 20 | | |
| Lymph node metastasis | | | | | |
| With | 123 | 15 | 108 | 19.88 | < 0.001 |
| Without | 52 | 22 | 30 | | |
| Differentiation degree | | | | | |
| Well-differentiated | 34 | 15 | 19 | 47.44 | < 0.001 |
| Moderately differentiated | 40 | 19 | 21 | | |
| Poorly differentiated | 101 | 3 | 98 | | |
| TNM aging | | | | | |
| Stage III | 92 | 25 | 67 | 4.232 | 0.04 |
| Stage IV | 83 | 12 | 71 | | |

*ESCC* esophageal squamous cell carcinoma

**Table 4** Association of miR-34b/c rs4938723 with the chemoradiotherapy efficacy for locally advanced ESCC patients

| Groups | rs4938723 | | $\chi 2$ | *P* |
|---|---|---|---|---|
| | CC (n = 37) | TT + TC (n = 138) | | |
| Sensitive group | 20 | 73 | 21.27 | < 0.001 |
| Resistance group | 17 | 65 | | |

*SNP* single nucleotide polymorphism, *ESCC* esophageal squamous cell carcinoma

(AUC), sensitivity and specificity were 0.777, 85.1% and 71.3%, respectively (Fig. 1).

### Association of miR-34b/c rs4938723 with the Prognosis of Locally Advanced ESCC Patients

The average median PFS of patients in the case group was 24.0 months. The average median PFS of patients with (TT + TC) genotypes was 22.6 months, which is significantly shorter than those patients with CC genotype (29.3 months) ($P < 0.01$) (Fig. 2).

## Discussion

China is reported to be one of the high-risk areas of ESCC, whose incidence is 20-fold greater than low-risk western Africa, prompting the search for new approaches to enhance the clinical outcome of ESCC based on Chinese population [15]. MiR-34b/c is widely reported

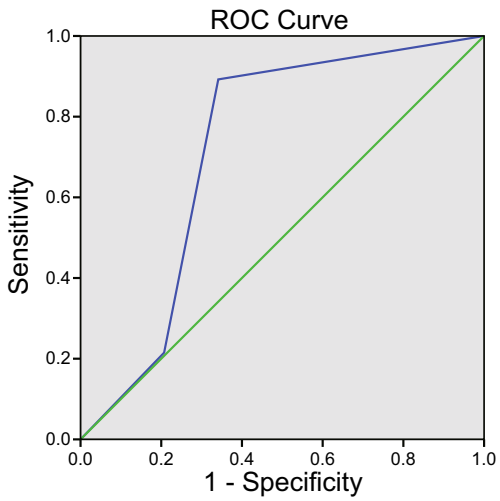


**Fig. 1** Evaluation of the predictive value of miR-34b/c rs4938723 in the chemoradiotherapy efficacy for locally advanced ESCC patients by ROC curve. Note: ESCC, esophageal squamous cell carcinoma; ROC curve, receiver operating characteristic curve

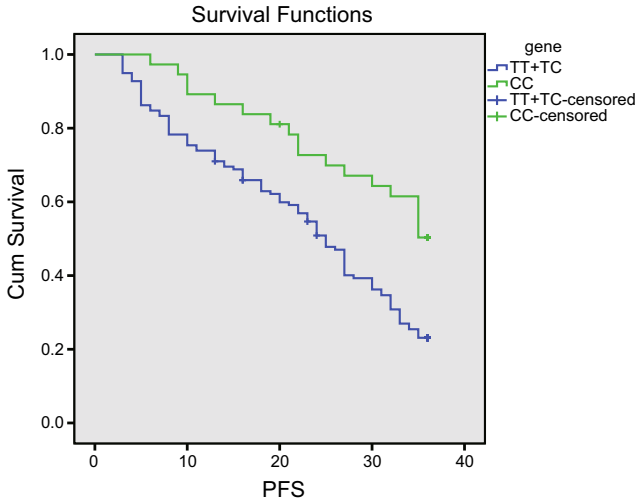


**Fig. 2** Survival rates of locally advanced ESCC patients with different genotypes in miR-34b/c rs4938723. Note: ESCC, esophageal squamous cell carcinoma

to play a crucial role in diseases such as digestive tract cancer [16]. Therefore, our study aims to find out the association of miR-34b/c rs4938723 with the chemoradiotherapy efficacy for locally advanced ESCC in Chinese Han population.

First of all, we found that CC genotype and C allele of miR-34b/c rs4938723 could decrease the risk of ESCC. As revealed in published literature, miR-34 family members act as direct targets of tumor suppressor p53, and inhibition of miR-34 expression would impair the process of p53-mediated cell cycle arrest and apoptosis, aggravating the progression of malignancy [17]. MiR-34b/c rs4938723 (T > C) is an extensively studied polymorphism which is located in CpG islands in miR-34b/c promoter region and may take part in the epigenetic silence of miR-34b/c [18]. In the past decades, several studies concentrated on the association between miR34b/c rs4938723 and susceptibility to various cancers, however, the findings remain inconclusive and controversial [12, 19]. It is pointed out by Lin et al. that this T > C variant is also predicted to impact the binding sites of GATA-X transcription factors to miR-34b/c promoter region, in other words, only when the polymorphic location is occupied by C allele, it can bind to GATA-X, otherwise it cannot bind to GATA-X. Hence rs4938723 polymorphism may alter miR-34b/c expression by transcriptional mechanisms, and subsequently change the susceptibility to cancer [11]. Consistent with our findings, Yin et al. clarify that miR-34b/c rs4938723 CC genotype declines individual susceptibility to ESCC [20]. Therefore, it is safe to speculate that CC genotype and C allele of miR-34b/c rs4938723 could decrease ESCC risk.

In addition, the findings demonstrated that miR-34b/c is closely related with the chemoradiotherapy efficacy and prognosis of locally advanced ESCC patients. MiRNAs are

acknowledged to function as key regulatory molecules in the regulation of extensive fundamental cellular processes, including cell proliferation, death, differentiation and invasiveness, and aberrant expression of miRNAs has been observed in a wide range of pathological events [21]. Considering the tumor suppressive functions of the miR-34 family members, it will be worthy of figuring out whether the expression of miR-34 also has diagnostic or prognostic value in cancers [22]. The mechanism of miR-34b/c in the regulation of the chemoradiotherapy efficacy and prognosis of locally advanced ESCC patients remains obscure, Kumamoto et al. suggested Nutlin-3a, and its downstream effectors p53 and inhibitor of growth 2 (*ING2*), may take their parts in the process. On the one hand, Nutlin-3a-induced p53 activation induces miR-34b/c overexpression and then increase the rate of cell senescence; on the other hand, p53 activation induced by Nutlin-3a could suppress *ING2* mRNA transcription [23]. Moreover, a recent research about the link between miR-34 and lung cancer cells apoptosis put forwarded a possible negative feedback loop which may provide a reasonable explanation for the question. AXL receptor tyrosine kinase, which is discovered to be frequently overexpressed in cancers, plays vital role in cancer invasion/metastasis and chemoradiotherapy resistance. And miR-34 is found to be capable of inhibiting AXL expression through targeting the 3'UTR of AXL mRNA, therefore, the chemoradiotherapy efficacy as well as the prognosis of lung cancer patients are improved by miR-34 [24].

## Conclusion

In conclusion, our study demonstrated that miR-34b/c rs4938723 CC genotype and C allele could reduce ESCC risk and improve the prognosis of locally advanced ESCC patients. Besides, miR-34b/c is found to be closely related with the chemoradiotherapy efficacy and prognosis of locally advanced ESCC patients, indicating that miR-34b/c rs4938723 could be a valuable chemoradiotherapy efficacy predictor as well as prognostic factor of locally advanced ESCC patients. However, future well-organized studies with larger sample sizes are still expected to determine the target of miR-34 regulating the chemotherapy efficacy and prognosis of locally advanced ESCC patients.

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**Author Contribution** JB and YT designed the study. WW and KS collated the data, designed and developed the database, carried out data analyses and produced the initial draft of the manuscript. NL contributed to drafting the manuscript. All authors have read and approved the final submitted manuscript.

**Compliance with Ethical Standards**

**Conflict of Interest** None.

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