Hsa-miR-34b/c rs4938723 T>C and hsa-miR-423 rs6505162 C>A Polymorphisms Are Associated with the Risk of Esophageal Cancer in a Chinese Population

Jun Yin1*, Xu Wang1, Liang Zheng2, Yijun Shi1, Liming Wang3, Aizhong Shao1, Weifeng Tang1, Guowen Ding1, Chao Liu1, Ruiping Liu4, Suocheng Chen1*, Haiyong Gu1*

1 Department of Cardiothoracic Surgery, Affiliated People's Hospital of Jiangsu University, Zhenjiang, China, 2 Department of Cardiothoracic Surgery, The First People's Hospital of Changzhou and The Third Affiliated Hospital of Suzhou University, Changzhou, China, 3 Cancer Institute, Department of Chemotherapy, Affiliated People's Hospital of Jiangsu University, Zhenjiang, Jiangsu, China, 4 Department of Orthopedics, Affiliated Hospital of Nanjing Medical University, Changzhou Second People's Hospital, Changzhou, China

Abstract

Esophageal cancer is the eighth most common cancer and sixth leading cause of cancer associated death worldwide. Besides environmental risk factors, genetic factors might play an important role in the esophageal cancer carcinogenesis. We conducted a hospital based case–control study to evaluate the genetic susceptibility of functional single nucleotide polymorphisms (SNPs) in the microRNAs on the development of esophageal cancer. A total of 629 esophageal squamous cell carcinoma (ESCC) cases and 686 controls were recruited for this study. The hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G, pre-miR-125a rs12975333 G>T and hsa-miR-423 rs6505162 C>A genotypes were determined using Ligation Detection Reaction (LDR) method. Our results demonstrated that hsa-miR-34b/c rs4938723 CC genotype had a decreased risk of ESCC. The association was evident among patients who never drinking. Hsa-miR-423 rs6505162 C>A might associated with an increased risk of ESCC in patients who smoking. These findings indicated that functional polymorphisms hsa-miR-34b/c rs4938723 T>C and hsa-miR-423 rs6505162 C>A might alter individual susceptibility to ESCC. However, our results were obtained with a limited sample size. Future larger studies with other ethnic populations are required to confirm current findings.

Citation: Yin J, Wang X, Zheng L, Shi Y, Wang L, et al. (2013) Hsa-miR-34b/c rs4938723 T>C and hsa-miR-423 rs6505162 C>A Polymorphisms Are Associated with the Risk of Esophageal Cancer in a Chinese Population. PLoS ONE 8(11): e80570. doi:10.1371/journal.pone.0080570

Editor: Xin-Yuan Guan, The University of Hong Kong, China

Received August 28, 2013; Accepted October 4, 2013; Published November 18, 2013

Copyright: © 2013 Yin et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported in part by National Natural Science Foundation of China (81101889, 81000028), Jiangsu Province Natural Science Foundation (BK2010333, BK2011481), Social Development Foundation of Zhenjiang (SH2010017) and Changzhou Young Talents and Science-Technology Foundation of Health Bureau (QN201102). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: chensuocheng@sina.com (SC); haiyong_gu@hotmail.com (HG)
† These authors contributed equally to this work.

Introduction

MicroRNAs (miRNAs) are tiny noncoding RNAs that act as posttranscriptional gene regulatory elements [1]. Specifically, miRNAs act by binding to the 3’-untranslated region of target genes and to consequently down-regulate their expression [2]. miRNAs are important players in carcinogenesis [3].

Genetic factors, such as single nucleotide polymorphisms (SNPs), may contribute to carcinogenesis [4]. The SNPs in the genomic miRNA sequences could influence miRNA-dependent regulation, affect the final level and function of miRNAs and alter consequently tumor susceptibility [5].

Members of the miR-34 family are direct p53 targets, and their expression is directly induced by p53 in response to DNA damage or oncogenic stress [6]. In previous studies in colorectal cancer [7], oral cancer [8] and malignant melanoma [9], down-regulation of mir-34b/c by methylation was found. Hsa-miR-34b/c rs4938723 polymorphism is located within the CpG island of pri-miR-34b/c, and might be the predicted binding site for GATA-X transcription factors [10]. Hsa-miR-34b/c rs4938723 T>C polymorphism was associated with the risk of nasopharyngeal carcinoma [11], hepatocellular carcinoma [12], colorectal cancer [13] and breast cancer survival [14].

Besides hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G, pre-miR-125a rs12975333 G>T and hsa-miR-423 rs6505162 C>A were also associated with the risk of different types of cancers. The rs531564 C>G SNP in pre-miR-124-1 was associated with an increased risk of bladder cancer [15] and esophageal cancer in males [16]. The pre-miR-125a rs12975333 G>T polymorphism was a founder mutation specific to the Antwerp area and associated with high risk for breast cancer [17]. Hsa-miR-423 rs6505162 C>A polymorphism was associated with reduced breast cancer risk [18]. Hsa-miR-423 rs6505162 C>A polymorphism was also significantly associated with both the overall survival and the recurrence-free survival of colorectal cancer [19].

We previously investigated miR-156a2 rs11614913 T>C, miR-146a rs2910164 C>G, miR-499 rs37346444 T>C, miR-26a-1 rs7372209 C>T and miR-27a rs859819 T>C SNPs and esophageal squamous cell carcinoma (ESCC) risk in 308 cancer cases and 390 controls [20]. We found miR-156a2 rs11614913
T>C might contribute to decreased ESCC risk among women patients and patients who never smoking or drinking [20]. Now, the objective of this investigation was to evaluate the association between hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G, pri-miR-125a rs12975333 G>T and hsa-miR-423 rs6505162 C>A genotypes and ESCC risk. We performed genotyping analyses for the four miRNA SNPs with 629 ESCC cases and 686 controls in a Chinese population.

Materials and Methods

Ethical approval of the study protocol

This hospital-based case-control study was approved by the Review Board of Jiangsu University (Zhenjiang, China). We have complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals. All subjects provided written informed consent to be included in the study.

Patients and Controls

629 subjects with esophageal cancer were consecutively recruited from the Affiliated People’s Hospital of Jiangsu University and Affiliated Hospital of Jiangsu University (Zhenjiang, China) between October 2008 and December 2010. Diagnosis was done by biopsy and all cases of esophageal cancer were ESCC. The exclusion criteria were patients who previously had: cancer; any metastasized cancer; radiotherapy or chemotherapy. The 686 controls were patients without cancer frequency-matched to the cases with regard to age (±5 years) and sex recruited from the two hospitals mentioned above during the same time period. Most of the controls were admitted to the hospitals for the treatment of trauma.

Each subject was personally questioned by trained interviewers using a pre-tested questionnaire to obtain information on demographic data (e.g., age, sex) and related risk factors (including tobacco smoking and alcohol consumption). After the interview, 2-mL samples of venous blood were collected from each subject. Individuals who smoked one cigarette per day for >1 year were defined as “smokers”. Subjects who consumed ≥3 alcoholic drinks a week for >6 months were considered to be “alcohol drinkers”.

Isolation of DNA and genotyping by Ligation Detection Reaction

Blood samples were collected from patients using Vacutainers and transferred to tubes lined with ethylenediamine tetra-acetic acid (EDTA). Genomic DNA was isolated from whole blood with the QIAamp DNA Blood Mini Kit (Qiagen, Berlin, Germany) [21]. Sample DNA were amplified by PCR according to the manufacturer’s recommendations. The samples were genotyped using the Ligation Detection Reaction (LDR) method with technical support from the Shanghai Biowing Applied Biotechnology Company as previously described [22]. For quality control, repeated analyses were done for 100 (12.17%) randomly selected samples with high DNA quality.

Statistical Analyses

Differences in the distributions of demographic characteristics, selected variables, and genotypes of the hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G, pri-miR-125a rs12975333 G>T, and hsa-miR-423 rs6505162 C>A variables between the cases and controls were evaluated using student t test and the χ² test. The associations between the four SNPs and risk of ESCC were estimated by computing the ORs and their 95% CIs using logistic regression analyses for crude ORs and adjusted ORs when adjusting for age, sex, smoking and drinking status. The Hardy-Weinberg equilibrium (HWE) was tested by a goodness-of-fit χ² test to compare the observed genotype frequencies to the expected ones among the control subjects. All statistical analyses were performed with SAS 9.1.3 (SAS Institute, Cary, NC, USA).

Results

Characteristics of the study population

Characteristics of cases and controls included in the study were summarized in Table 1. The cases and controls appeared to be adequately matched on age and sex as suggested by the χ² tests (p = 0.541 and p = 0.183, respectively). As shown in Table 1, significant difference was detected on smoking status between the cases and the controls (p < 0.001), and drinking rate was higher in ESCC patients than in control subjects (p < 0.001). The primary information for four genotyped SNPs was in Table 2. The genotyping success rate is ranging from 95.13% to 96.81% in all 1315 samples. The concordance rates of repeated analyses were 100% for all four SNPs. Minor allele frequency (MAF) in our controls was similar to MAF for Chinese in database for all four SNPs (Table 2). The observed genotype frequencies for hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G, hsa-miR-423 rs6505162 C>A polymorphisms in the controls were in HWE (p = 0.675, p = 0.400 and p = 0.299) except pre-miR-125a rs12975333 G>T (not available) (Table 2).

Associations between hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G, pre-miR-125a rs12975333 G>T and hsa-miR-423 rs6505162 C>A polymorphisms and risk of ESCC

The genotype distributions of hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G, pre-miR-125a rs12975333 G>T and hsa-miR-423 rs6505162 C>A in the cases and the controls are shown in Table 3. In the single locus analyses, the genotype frequencies of hsa-miR-34b/c rs4938723 T>G were 46.2% (TT), 46.3% (TC), and 7.5% (CC) in the case patients and 46.1% (TT), 43.1% (TC), and 10.8% (CC) in the control subjects, and the difference was not statistically significant (p = 0.101). In the recessive model, when the hsa-miR-34b/c rs4938723 TT/TC genotypes were used as the reference group, the CC homozygote genotype was associated with a statistically significantly decreased risk for ESCC (CC vs. TT/TC: adjusted OR = 0.65, 95% CI = 0.44–0.97, p = 0.036). When the hsa-miR-423 rs6505162 TT homozygote genotype was used as the reference group, the TC genotype was not associated with the risk for ESCC (CC vs. TT/TC: adjusted OR = 1.11, 95% CI = 0.88–1.40, p = 0.397); the CC genotype was not associated with the risk for ESCC (CC vs. TT/TC: adjusted OR = 0.69, 95% CI = 0.45–1.04, p = 0.076). In the dominant model, the hsa-miR-34b/c rs4938723 TT/CC variants were not associated with the risk for ESCC, compared with the hsa-miR-34b/c rs4938723 TT genotype (adjusted OR = 1.02, 95% CI = 0.82–1.28, p = 0.853) (Table 3).

No association was observed between pri-miR-124-1 rs531564 C>G and hsa-miR-423 rs6505162 C>A polymorphisms and the risk of ESCC (Table 3). For pre-miR-125a rs12975333 G>T, all genotypes are GG homozygotes (Table 3).

Stratification analyses of hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G and hsa-miR-423 rs6505162 C>A and risk of ESCC

To evaluate the effects of hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G and hsa-miR-423 rs6505162 C>A
genotypes on ESCC risk according to different age, sex, smoking and alcohol drinking status; we performed the stratification analyses (Table 4). A significantly decreased risk of ESCC associated with the hsa-miR-34b/c T>C polymorphism was evident among patients who never drinking (CC vs. TT/TC: adjusted OR = 0.57, 95% CI = 0.34–0.94, p = 0.029) (Table 4). In patients who smoking, hsa-miR-423 C>A might associated with a significantly increased risk of ESCC (AA vs. CC/CA: adjusted OR = 4.94, 95% CI = 1.42–17.21, p = 0.012) (Table 4).

### Discussion

In this hospital-based case-control study of ESCC, we investigated the associations of hsa-miR-34b/c T>C, pri-miR-124-1 rs531564 C>G, pre-miR-125a rs12975333 G>T and hsa-miR-423 rs6505162 C>A with risk of ESCC in a high risk Chinese population. Our multivariable logistic analysis revealed that hsa-miR-34b/c CC genotype had a decreased risk of ESCC. The association was evident among patients who never drinking. Hsa-miR-423 rs6505162 C>A might associated with a significantly increased risk of ESCC in patients who smoking.

### Table 1. Distribution of selected demographic variables and risk factors in ESCC cases and controls.

| Variable          | Cases (n = 629) n % | Controls (n = 686) n % | p *  |
|-------------------|---------------------|------------------------|------|
| Age (years) mean ± SD | 62.85 (±8.13)       | 62.58 (±7.89)          | 0.541|
| Age (years)       |                     |                        | 0.155|
| < 63              | 310                 | 49.28                  | 365  | 53.21|
| ≥ 63              | 319                 | 50.72                  | 321  | 46.79|
| Sex               |                     |                        | 0.185|
| Male              | 444                 | 70.99                  | 461  | 67.20|
| Female            | 185                 | 29.01                  | 225  | 32.80|
| Tobacco use       |                     |                        | <0.001|
| Never             | 355                 | 56.44                  | 499  | 72.74|
| Ever              | 274                 | 43.56                  | 187  | 27.26|
| Alcohol use       |                     |                        | <0.001|
| Never             | 428                 | 68.04                  | 526  | 76.68|
| Ever              | 201                 | 31.96                  | 160  | 23.32|

*pTwo-sided χ² test and student t test; Bold values are statistically significant (p <0.05).

doi:10.1371/journal.pone.0080570.t001

### Table 2. Primary information for hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G, pre-miR-125a rs12975333 G>T and hsa-miR-423 rs6505162 C>A polymorphisms.

| Genotyped SNPs | hsa-miR-34b/c rs4938723 | pri-miR-124-1 rs531564 | pre-miR-125a rs12975333 | hsa-miR-423 rs6505162 |
|----------------|-------------------------|------------------------|------------------------|----------------------|
| T>C            | 11                      | 8                      | 19                     | 17                   |
| C>G            | 8                       | 19                     | 5                      | 1f                   |
| G>T            | 19                      | 5                      | 5                      | 1f                   |
| C>A            | 17                      | 1f                     | 1f                     |                      |
| Chromosome     |                         |                        |                        |                      |
| Gene Official Symbol | MIR34B/C                | MIR124-1               | MIR125A                | MIR423               |
| Function       | ncRNA                   | ncRNA                  | ncRNA                  | ncRNA                |
| Chr Pos (Genome Build 36.3) | 110887775               | 9798109                | 56888340               | 25468309             |
| Regulome DB Score* | 5                      | 5                      | 5                      | 1f                   |
| TFBSb          | Y                       | Y                      | Y                      | Y                    |
| Splicing (ESE or ESS) | —                      | —                      | —                      | Y                    |
| MAF* for Chinese in database | 0.400                  | 0.178                  | Unknown                | 0.200                |
| MAF in our controls (n = 686) | 0.324                  | 0.157                  | 0.000                  | 0.188                |
| p value for HWEδ in our controls | 0.675                  | 0.400                  | —                      | 0.299                |
| Genotyping method* | LDR                    | LDR                    | LDR                    | LDR                  |
| % Genotyping value | 96.81%                 | 96.43%                 | 96.43%                 | 95.13%               |

*http://www.regulomedb.org/;

†TFBS: Transcription Factor Binding Site (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm);
‡MAF: minor allele frequency;
§HWE: Hardy–Weinberg equilibrium;
‖LDR: Ligation Detection Reaction.

doi:10.1371/journal.pone.0080570.t002
It is well known that members of the miR-34 family expression is directly induced by p53 in response to DNA damage or oncogenic stress [6]. A rat model experiment indicated that the alteration of miR-34b/c under an inflammatory microenvironment can be influenced by p53 [23]. By DNA methylation of its own promoter, miR-34 family has been silenced in numerous cancers [24]. By triggering Wnt signaling cascades, the loss of miR-34 impairs p53-mediated cell death. Overexpression of miR-34 can induce apoptosis [25–27]. The role of miR-34a in the suppression of tumor growth has also been detected in vivo [28].

In a previous study, down-regulation of mir-34b/c by methylation was found in colorectal cancer [7], oral cancer [8] and malignant melanoma [9]. The hsa-miR-34b/c rs4938723 T>C SNP is located within the CpG island of pri-miR-34b/c and may affect a predicted GATA-X transcription factor binding [10]. Hsa-miR-34b/c rs4938723 T>C polymorphism was associated with the risk of nasopharyngeal carcinoma [11], hepatocellular carcinoma [12], colorectal cancer [13] and breast cancer survival [14]. Hsa-miR-423 rs6505162 C>A polymorphism was associated with reduced breast cancer risk [18] and both the overall survival and the

### Table 3. Logistic regression analyses of associations between hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G, pre-miR-125a rs12975333 G>T and hsa-miR-423 rs6505162 C>A polymorphisms and risk of ESCC.

| Genotype     | Cases (n=629) n % | Controls (n=686) n % | Crude OR (95%CI) | p  | Adjusted OR * (95%CI) | p  |
|--------------|-------------------|----------------------|------------------|----|----------------------|----|
| **hsa-miR-34b/c rs4938723 T>C** |                   |                      |                  |    |                      |    |
| TT           | 277   46.2         | 310  46.1            | 1.00             | 1.00 |                      |    |
| TC           | 278   46.3         | 290  43.1            | 1.07 (0.85–1.35) | 0.551 | 1.11 (0.88–1.40) | 0.397 |
| CC           | 45    7.5          | 73   10.8            | 0.69 (0.46–1.04) | 0.073 | 0.69 (0.45–1.04) | 0.076 |
| CC vs. TC vs. TT |       |                     |                  |    |                      |    |
| TC+CC        | 323   53.8         | 363  53.9            | 1.00 (0.80–1.24) | 0.970 | 1.02 (0.82–1.28) | 0.853 |
| TT+TC        | 555   92.5         | 600  89.2            | 1.00             | 1.00 |                      |    |
| **T allele** |                   |                      |                  |    |                      |    |
| 832 69.3     | 910  67.6          | 1.00                |      |    |                      |    |
| **C allele** |                   |                      |                  |    |                      |    |
| 368 30.7     | 436  32.4          | 0.92 (0.78–1.09)    | 0.350 | |
| **pri-miR-124-1 rs531564 C>G** |                   |                      |                  |    |                      |    |
| CC           | 454   74.3         | 470  71.5            | 1.00             | 1.00 |                      |    |
| CG           | 146   23.9         | 168  25.6            | 0.90 (0.70–1.16) | 0.726 | 0.96 (0.74–1.25) | 0.768 |
| GG           | 11    1.8           | 19   2.9             | 0.60 (0.28–1.27) | 0.183 | 0.63 (0.29–1.36) | 0.237 |
| GG vs. CG vs. CC |       |                     |                  |    |                      |    |
| CG+GG        | 157   25.7         | 187  28.5            | 0.87 (0.68–1.11) | 0.269 | 0.93 (0.72–1.19) | 0.559 |
| CC+CG        | 600   98.2         | 638  97.1            | 1.00             | 1.00 |                      |    |
| GG           | 11    1.8           | 19   2.9             | 0.62 (0.29–1.30) | 0.205 | 0.64 (0.30–1.37) | 0.245 |
| C allele     | 1054  86.3         | 1108 84.3           | 1.00             | 1.00 |                      |    |
| G allele     | 168   13.7         | 206  15.7            | 0.86 (0.69–1.07) | 0.171 | |
| **pre-miR-125a rs12975333 G>T** |                   |                      |                  |    |                      |    |
| GG           | 611   100.0         | 657  100.0           | 1.00             | 1.00 |                      |    |
| GT           | 0     0.0           | 0   0.0             | —                | —   | —                    | —   |
| TT           | 0     0.0           | 0   0.0             | —                | —   | —                    | —   |
| **hsa-miR-423 rs6505162 C>A** |                   |                      |                  |    |                      |    |
| CC           | 374   62.3         | 425  65.3            | 1.00             | 1.00 |                      |    |
| CA           | 197   32.8         | 207  31.8            | 1.08 (0.85–1.37) | 0.522 | 1.09 (0.86–1.40) | 0.476 |
| AA           | 29    4.8           | 19   2.9             | 1.73 (0.96–3.14) | 0.070 | 1.70 (0.92–3.12) | 0.089 |
| AA vs. CA vs. CC |       |                     |                  |    |                      |    |
| CA+AA        | 226   37.7         | 226  34.7            | 1.14 (0.90–1.43) | 0.278 | 1.14 (0.90–1.45) | 0.264 |
| CC+CA        | 571   95.2         | 632  97.1            | 1.00             | 1.00 |                      |    |
| AA           | 29    4.8           | 19   2.9             | 1.69 (0.94–3.05) | 0.081 | 1.65 (0.90–3.01) | 0.106 |
| C allele     | 945   78.8         | 1057 81.2           | 1.00             | 1.00 |                      |    |
| A allele     | 255   21.3         | 245  18.8            | 1.16 (0.96–1.42) | 0.128 | |

*Adjusted for age, sex, smoking status and alcohol consumption.

doi:10.1371/journal.pone.0080570.t003
Table 4. Stratified analyses between hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G and hsa-miR-423 rs6505162 C>A polymorphism and ESCC risk by sex, age, smoking status and alcohol consumption.

| Variable          | hsa-miR-34b/c rs4938723 T>C (case/control) | Adjusted OR (95% CI) * | pri-miR-124-1 rs531564 C>G (case/control) | Adjusted OR (95% CI) | hsa-miR-423 rs6505162 C>A (case/control) | Adjusted OR (95% CI) |
|-------------------|------------------------------------------|------------------------|------------------------------------------|------------------------|------------------------------------------|------------------------|
|                   | TT+TC                                    | CC                     | Adjusted OR (95% CI) *                   |                        | CC+CA                                    | AA                     | CC+CA                                    | AA                     |
| Sex               |                                          |                        |                                          |                        |                                          |                        |                                          |                        |
| Male              | 396/402                                  | 32/47                  | 1.00                                     | 0.68(0.42–1.10)        | 420/430                                  | 9/14                   | 1.00                                     | 0.69(0.29–1.63)        |
|                   |                                          |                        |                                          |                        | 402/419                                  | 24/16                  | 1.00                                     | 1.54(0.79–2.98)        |
| Female            | 159/198                                  | 13/26                  | 1.00                                     | 0.60(0.30–1.21)        | 180/208                                  | 2/5                    | 1.00                                     | 0.51(0.10–2.67)        |
|                   |                                          |                        |                                          |                        | 169/213                                  | 5/3                    | 1.00                                     | 2.26(0.53–9.66)        |
| Age               |                                          |                        |                                          |                        |                                          |                        |                                          |                        |
| <63               | 274/319                                  | 20/37                  | 1.00                                     | 0.64(0.35–1.15)        | 297/333                                  | 4/14                   | 1.00                                     | 0.35(0.11–1.11)        |
|                   |                                          |                        |                                          |                        | 278/333                                  | 16/12                  | 1.00                                     | 1.75(0.79–3.86)        |
| ≥63               | 281/281                                  | 25/36                  | 1.00                                     | 0.68(0.39–1.16)        | 303/305                                  | 7/5                    | 1.00                                     | 1.33(0.41–4.31)        |
|                   |                                          |                        |                                          |                        | 293/299                                  | 13/7                   | 1.00                                     | 1.91(0.74–4.94)        |
| Smoking status    |                                          |                        |                                          |                        |                                          |                        |                                          |                        |
| Never             | 309/441                                  | 24/51                  | 1.00                                     | 0.62(0.37–1.04)        | 341/460                                  | 6/14                   | 1.00                                     | 0.68(0.26–1.79)        |
|                   |                                          |                        |                                          |                        | 327/456                                  | 10/16                  | 1.00                                     | 0.96(0.42–2.20)        |
| Ever              | 246/159                                  | 21/22                  | 1.00                                     | 0.69(0.36–1.30)        | 259/178                                  | 5/5                    | 1.00                                     | 0.74(0.21–2.64)        |
|                   |                                          |                        |                                          |                        | 244/176                                  | 19/3                   | 1.00                                     | 4.94(1.42–17.21)       |
| Alcohol consumption|                                         |                        |                                          |                        |                                          |                        |                                          |                        |
| Never             | 379/465                                  | 25/54                  | 1.00                                     | 0.57(0.34–0.94)        | 408/486                                  | 8/14                   | 1.00                                     | 0.77(0.31–1.91)        |
|                   |                                          |                        |                                          |                        | 391/484                                  | 16/15                  | 1.00                                     | 1.41(0.66–2.99)        |
| Ever              | 176/135                                  | 20/19                  | 1.00                                     | 0.79(0.39–1.57)        | 192/152                                  | 3/5                    | 1.00                                     | 0.50(0.12–2.17)        |
|                   |                                          |                        |                                          |                        | 180/148                                  | 13/4                   | 1.00                                     | 3.00(0.94–9.57)        |

*Adjusted for age, sex, smoking status and alcohol consumption (besides stratified factors accordingly) in a logistic regression model; Bold values are statistically significant (p < 0.05). doi:10.1371/journal.pone.0080570.t004
recurrence-free survival of colorectal cancer [19]. In our study, we found hsa-miR-34b/c rs4938723 C allele had a decreased risk of ESCC among patients who never drinking, hsa-miR-423 rs5051622 C>A might associated with a significantly increased risk of ESCC in patients who smoking, indicating gene-environment interaction.

The frequencies of genetic polymorphisms often vary between ethnic groups. In the present Chinese study, the allele frequency of hsa-miR-34b/c rs4938723 C was 0.324 among 686 control subjects, which is slightly higher than that of Japanese population (0.261) and similar to that of European population (0.310) and Sub-Saharan African population (0.305). The allele frequency of hsa-miR-423 rs505162 A was 0.188 among 686 control subjects, which is in accordance with that of Chinese population (0.200) and Japanese population (0.178). But the allele frequency is significantly lower than that of European population (0.575) and Sub-Saharan African population (0.783) [http://www.ncbi.nlm.nih.gov/SNP, http://hapmap.ncbi.nlm.nih.gov/].

Using Power and Sample Size Calculation (PS, version 3.0, 2009, http://biostat.mc.vanderbilt.edu/wiki/bin/view/Main/PowerSampleSize), considering hsa-miR-34b/c rs4938723 T>C mutant alleles in the control group, OR, ESCC samples and control samples, the power of our analysis (α = 0.05) was 0.929 in 600 ESCC cases and 675 controls with adjusted OR 0.65. The control samples, the power of our analysis (α = 0.05) was 0.962 in 404 ESCC cases and 519 controls with adjusted OR 0.57 in non- drinking subgroup. For hsa-miR-423 rs505162 C>A, the power of our analysis (α = 0.05) was 1.000 in 263 ESCC cases and 179 controls with adjusted OR 4.94 in smoking subgroup.

In this case-control study, several limitations need to be addressed. Firstly, the patients and controls were enrolled from hospitals and may not represent the general population. Secondly, statistical power of our study was limited especially in stratification analyses, it is better that the control group being larger than the case group; therefore it is possible to have a more statistical power. Thirdly, detailed information on cancer metastasis and survival were not recruited till now, which restricted further analyses on the role of the four polymorphisms in ESCC progression and prognosis. Finally, the information about viral infections and immune parameters was not available, which restricted the power of our analyses.

In conclusion, our study provides evidence that functional polymorphisms hsa-miR-34b/c rs4938723 T>C and hsa-miR-423 rs6505162 C>A might alter individual susceptibility to ESCC. Future larger studies with other ethnic populations and functional analysis are required to confirm current findings.

### Author Contributions
Conceived and designed the experiments: JYWTSCHG. Performed the experiments: XWYSLWAS. Analyzed the data: HGJYSC. Contributed reagents/materials/analysis tools: XWYLDCLRL. Wrote the paper: JYWTSCHG. Critical review of the manuscript: YCGSHG.

### References
1. Sand M, Gambichler T, Sand D, Skrygan M, Altmeyer P, et al. (2009) MicroRNAs and the skin: tiny players in the body's largest organ. J Dermatol Sci 53: 169–173.
2. Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9: 102–114.
3. Mathe E, Nguyen GH, Funamizu N, He P, Moake M, et al. (2012) Genome-wide association study identifies multiple alleles in the promoter region of miR-34b/c and pre-miR-196a2 polymorphisms and their multiplicative interactions with TP-53 polymorphisms on the risk of nasopharyngeal carcinoma. Tumour Biol 34: 156–160.
4. Shen H, Jin G (2013) Human genome epidemiology, progress and future. J Biomed Res 27: 167–169.
5. Simon MS, Sun H, Spizzo R, Kim H, Wickramasinghe P, et al. (2010) Single-nucleotide polymorphisms inside microRNA target sites influence tumor susceptibility. Cancer Res 70: 2789–2798.
6. Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, et al. (2007) p53-mediated activation of miRNA34 candidate tumor-suppressor genes. Curr Biol 17: 1290–1307.
7. Toyama M, Suzuki H, Sasaki Y, Maruyama R, Inai K, et al. (2008) Epigenetic silencing of microRNA-143/145 and B-cell translocation gene 6 is associated with CpG island methylation in colorectal cancer. Cancer Res 68: 4121–4132.
8. Kozaki K, Imoto I, Mogi S, Oomura K, Inazawa J (2008) Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. Cancer Res 68: 2094–2105.
9. Lujambio A, Rovero S, Ballestar E, Fraga MF, Cerrato C, et al. (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res 67: 1143–1129.
10. Son MS, Jung MJ, Jeon YJ, Kim WH, Kwon CJ, et al. (2013) Promoter polymorphisms of pri-miR-34b/h-c are associated with hepatocellular carcinoma. Genet 524: 156–160.
11. Li L, Wu J, Sima X, Bai P, Deng W, et al. (2013) Interactions of miR-34b/c and TP-35 polymorphisms on the risk of nasopharyngeal carcinoma. Tumour Biol 34: 1919–1923.
12. Han Y, Lu K, Han X, Zhao J, Zhang Y, et al. (2013) Associations of pre-miR-34b/c and pre-miR-196a2 polymorphisms and their multiplicative interactions with hepatitis B virus mutations with hepatocellular carcinoma risk. PLoS One 9: e85354.
13. Gao LB, Li LJ, Pan XM, Li ZH, Liang WB, et al. (2013) A genetic variant in the promoter region of miR-34b/c is associated with a reduced risk of colorectal cancer. Biol Chem 394: 415–420.
14. Jensen JT, Tse CK, Nyante SJ, Barlough-Sloan JS, Cole SR, et al. (2013) Association of germline microRNA SNPs in pre-miRNA flanking region and breast cancer risk and survival the Carolina Breast Cancer Study. Cancer Causes Control 24: 1099–1109.
15. Wang H, Jinne CP, Ye Y, Zhu Y, Grossman HB, et al. (2008) Evaluation of genetic variants in microRNA-related genes and risk of bladder cancer. Cancer Res 68: 2530–2537.
16. Ye Y, Wang KK, Gu J, Yang H, Lin J, et al. (2008) Genetic variations in microRNA-related genes are novel susceptibility loci for esophageal cancer risk. Cancer Prev Res (Phila) 1: 460–469.
17. Petersolo P, Caleca L, Cattaneo E, Ravagamini F, Bianchi T, et al. (2011) The rs1297533 variant in the miR-125a and breast cancer risk in Germany, Italy, Australia and Spain. J Med Genet 48: 707–704.
18. Smith RA, Jedelski DJ, Gabrovska PN, Weinstein SR, Haupt L, et al. (2012) A genetic variant located in miR-423 is associated with reduced breast cancer risk. Cancer Genomics Proteomics 9: 115–118.
19. Xing J, Wan S, Zhou F, Qi F, Li B, et al. (2012) Genetic polymorphisms in pre-microRNA genes as prognostic markers of colorectal cancer. Cancer Epidemiol Biomarkers Prev 21: 217–227.
20. Wei J, Zheng L, Liu S, Yin J, Wang L, et al. (2013) MiR-196a2 rs11614913 T>C polymorphism and risk of esophageal cancer in a Chinese population. Hum Immunol 74: 1199–1205.
21. Gu H, Ding G, Zhang W, Liu G, Chen Y, et al. (2012) Replication study of PLCE1 and C20orf54 polymorphism and risk of esophageal cancer in a Chinese population. Mol Biol Rep 39: 9105–9111.
22. Chen AJ, Zhao H, He L, et al. (2011) Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. Nat Genet 43: 55.
23. Mathe E, Nguyen GH, Fumanizu N, He P, Moake M, et al. (2012) Inflammation regulates microRNA expression in cooperation with p33 and nitric oxide. Int J Cancer 131: 760–765.
24. Vogt M, Mundling J, Grauner M, Löffers ST, Verdooth B, et al. (2011) Frequent concomitant inactivation of miR-34a and miR-34b/c by CpG methylation in colorectal, pancreatic, mammary, ovarian, urothelial, and renal cell carcinomas and soft tissue sarcomas. Virchows Arch 458: 313–322.
25. He I, He X, Lim LP, de Stanchina E, Xuan Z, et al. (2007) A microRNA component of the p53 tumour suppressor network. Nature 447: 1130–1134.
26. He I, He X, Lowe SW, Hanlon GJ (2007) microRNAs join the p53 network--another piece in the tumour-suppression puzzle. Nat Rev Cancer 7: 819–822.
27. Kim NH, Kim HS, Kim NG, Lee I, Choi HS, et al. (2011) MiR-196a2 rs11614913 T>C is a suppressors of canonical Wnt signaling. Sci Signal 4: ra71.
28. Tazawa H, Tsuchiya N, Izumiya M, Nakagama H (2007) Tumor-suppressive microRNA Polymorphism and Esophageal Cancer Risk. Cancer Res 67: 1099–1109.