Original Paper

MiRNAs Associated Polymorphisms in the 3'UTR of MET Promote the Risk of Non-Small Cell Lung Cancer

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Key Words

SNP • MET • Non-small lung cancer • MiRNA

Abstract

Background/Aims: MET can act as an oncogene and its signaling server has essential roles in regulating tumorigenesis. Polymorphisms in MET have been reported to be associated with poor prognosis in human cancer, but an association with the risk of human non-small-cell lung cancer (NSCLC) has not been found so far. In this study rs41281081 and rs76322625, located in the 3'UTR of MET, were selected to evaluate their relationship with the risk of NSCLC among the Chinese population. **Methods:** A questionnaire, SNaPshot genotype assay, real time PCR assay, cell transfection and the dual luciferase reporter assay were used. Single-nucleotide polymorphisms (SNPs) of rs41281081 and rs76322625 in the 3' untranslated region (UTR) of MET was involved as a risk factor in the occurrence of NSCLC. **Results:** SNP rs41281081 could be regulated by miR-335 and rs76322625 could be regulated by miR-1026 to cause an up-regulation of MET in patients with NSCLC. Furthermore, the carriers of the GA and AA genotypes in rs41281081, and the CU and UU genotypes in rs76322625 presented with poor cell differentiation and large tumor size, as well as a high probability of metastasis. **Conclusion:** Our findings have shown that the SNPs rs41281081 and rs76322625 in MET 3'UTR, through disruption of the regulatory role of miR-335 and miR-1026 in MET expression, may act as promoting factors in the pathogenesis of NSCLC.

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Introduction

Non-small-cell lung carcinoma (NSCLC) is any type of epithelial lung cancer other than small-cell lung carcinoma (SCLC). It accounts for approximately 85% of all lung cancers [1-3]. Histologically, NSCLC is divided into adenocarcinoma and squamous cell carcinoma (SCC). Smoking is the major causes of NSCLC (78% in men and 90% in women); besides smoking, other causes of NSCLC including asbestos exposure, radon exposure, halogen ether

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exposure and chronic interstitial pneumonitis [4, 5]. Traditional treatment for NSCLC is not sufficient, given the increasing number of people suffering with NSCLC [6-9]. Improving results with the candidate gene approach have led to its growing acceptance as a potentially useful method for investigating genetic risk factors for NSCLC among Chinese people.

MET (MET proto-oncogene, receptor tyrosine kinase), also called c-Met and hepatocyte growth factor receptor (HGFR), is a protein that in humans is encoded by the MET gene. MET is a membrane receptor that is essential for embryonic development and wound healing. It is normally expressed by cells of epithelial origin, while expression of HGF is restricted to cells of mesenchymal origin. Upon HGF stimulation, MET induces several biological responses that collectively give rise to a program known as invasive growth. Abnormal MET activation has been found in several cancers, including liver cancer, gastric cancer and colon cancer, as well as leukemia. Expression of MET can serve as a ideal indicator of poor prognosis of NSCLC. Prolonged or continuous activity of the receptor leads to excessive cell proliferation and is related to the development or progression of neoplastic disease. C-MET inhibitors can be classified into three groups: small-molecule tyrosine kinase inhibitors of the c-MET receptor (crizotinib, tivantinib, cabozantinib, foretinib), as well as monoclonal antibodies against c-MET (onartuzumab) and against the HGF ligand (ficlatuzumab, rilotumumab). The efficacy and safety of these agents is assessed both in monotherapy and in combination with other molecularly targeted agents [10-12]. Furthermore, the toxicity profile of c-MET inhibitors is completely different from that of standard chemotherapy [13]. Aberrantly active MET can trigger tumor growth, angiogenesis and metastasis. In addition to mutation and abnormal activation of its upstream, overexpression of MET has also been reported to be associated with miRNA [14-16].

MiRNAs are small, non-coding RNA molecules of 19–25 nucleotides which have been reported to play important roles by regulating cell differentiation, proliferation, migration and apoptosis [17-20]. MiRNAs not only negatively regulate expression of their target genes at the posttranscription level, through binding to 3' untranslated regions (UTRs) of their targets' messenger RNAs [21, 22], but they also regulate the 3'UTR region, which harbors single-nucleotide polymorphisms (SNPs). Increasing numbers of studies have provided evidence that SNPs located in the miRNA (miRSNPs) binding sites affect the binding of miRNAs with the target genes, resulting in the reduction or increase in target mRNA translation, and thus are associated with the susceptibility to cancers[23-25].

In this study, we focused on the SNPs in the 3'UTR of MET, which have rarely been reported before. By using bioinformatics software (<http://www.bioguo.org/miRNASNP/>), we obtained all the SNPs which could be regulated by different miRNAs as candidate SNPs, and further investigated the allele distribution in a case-control study.

Materials and Methods

Study subjects

The hospital-based case-control study consists of 873 patients newly diagnosed with NSCLC and 711 cancer-free controls. All the subjects were genetically unrelated Han Chinese recruited from the first people's hospital of Zhangjiagang City (Zhangjiagang China), January 2010 and September 2014. Patients with other hematological disorders, previous history of cancers, radiotherapy and chemotherapy were excluded. The cancer-free control subjects from the same geographic area showed no evidence of genetic relationship with the cases. The patients were classified according to World Health Organization classification. This study was approved by the Ethical Committee of the first people's hospital of Zhangjiagang City, and every patient had written informed consent.

Genotype

We extracted genomic DNA from peripheral whole blood of every validation subject by using QIAamp DNA blood mini kits (Qiagen, Germany) according to the manufacturer's instructions. Genotyping was performed with the TaqMan SNP Genotyping Assay. The PCR reactions were carried out in a total volume of

5 µL containing TaqMan Universal Master Mix, 80X SNP Genotyping AssayMix, Dnase-free water and 10-ng genomic DNA. The PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The 384-well ABI 7900HT Real Time PCR System.

Real time PCR assay

Real time polymerase chain reaction (RT-PCR) was performed to determine whether the C to T mutation changed the expression level of MET. The amplification conditions were 95°C for 10 minutes, followed by 40cycles of 95°C for 30 seconds, 55°C for 40seconds, and 72°C for 30 seconds, and finally 4°C for 30 minutes for cooling.

Cell lines and cell culture

NSCLC cell lines and A549 and NCIH1975 were purchased from the Chinese Academy of Sciences Cell Bank. All cells were cultured in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, USA) and grown in humidified 5% CO₂ at 37°C. MiR-355 and miR-1026 mimics and normal control were obtained from Genepharma (Shanghai, China).The transfection was conducted by using Lipofectamine 2000 (Invitrogen Corp, CA, USA).

Prediction of miRNAs binding to the SNP

Based on our bioinformatics analysis by using the bioinformatics software (<http://www.bioguo.org/miRNASNP/>) to predict the related SNPs in the 3'UTR of MET which could regulated by different miRNAs.

Construction of luciferase-based reporter plasmids

All the fragment of the 3'UTR containing the mutated alleles we desired were amplified and constructed. The PCR production was cloned into the pGL3-promoterless luciferase-based plasmid (Promega, WI, USA) at the cloning site between KpnI and XhoI. The amplified fragment was verified by DNA sequencing.

Dual-luciferase reporter assay

The 3'-UTR sequence of MET predicted to interact with miR-603 or a mutated sequence with the predicted target sites were inserted into the KpnI and XhoI sites of pGL3 promoter vector (Genscript, Nanjing, China). For reporter assay, cells were plated onto 24-well plates and transfected with 100 ng of pGL3-MET wild, pGL3-MET mutant, miRNA and their mimics, respectively by using Lipofectamine 2000 (Invitrogen Corp, CA, USA). A Renilla luciferase vector pRL-SV40 (5 ng) was also co-transfected to normalize the differences in transfection efficiency. Transfection was repeated three times in triplicate

Statistical analysis

The association between rs41281081 and rs76322625 genotypes and the risk of NSCLC was evaluated by calculating the odds ratios (ORs) and their 95% confidence intervals (CIs) using univariate and multivariate logistic regression analysis. Stratification analysis was performed according to the clinical characteristic and risk classification to determine the genotype distribution in cases and controls as well as their association with the risk of NSCLC. The difference of the expression levels of MET with three genotypes and the difference of the relative luciferase activities between the wild and mutant genotype were evaluated by independent-sample t test. All statistical tests were two-sided and P<0.05 was considered statistically significant. Statistical analysis was performed with SPSS 13.0 (SPSS Ltd.) and SAS software (version 9.1.3; SAS Institute, Cary, NC, USA). The graphs were generated by Graphpad Prism 5.0 (Graphpad Software, Inc.).

Results

Participant characteristics

The characteristics of the 873 patients with NSCLC and 711 healthy controls are summarized in Table 1. No statistically significant differences were observed between cases and controls in terms of sex and age (both P>0.05). This indicates that the frequency matching was adequate. Patients suffering from exposure to smoking were indicated to be the susceptible population by comparison with controls (Table 1).

Table 1. Frequency distributions of selected variables in patients and cancer-free controls. *Two-sided chi-square test for either genotype distributions or allele frequencies between cases and controls

Variables	Cases (n = 873)		Controls (n = 711)		P*
	N	%	N	%	
Age (years)					0.0314
≤ 50	413	47.31	375	52.74	
> 50	460	52.69	336	47.26	
Gender					0.527
Male	433	49.59	364	51.20	
Female	440	50.41	347	48.80	
Parental smoking status					<0.0001
Negative	318	36.43	353	49.65	
Positive	555	63.57	358	50.35	
Differentiation grade					
Well	318	36.43			
Moderate	253	28.98			
Poorly	302	34.59			
Tumor Size(cm)					
≤3cm	416	47.65			
>3cm	457	52.35			
Metastasis					
Yes	489	56.01			
No	384	43.99			

Table 2. Association between SNPs and NSCLC. Chr = chromosome; UTR = Untranslated Regions

SNP	chr	3'UTR position	Associated miRNA	Allele
rs59399612	7	212-234	miR-122	U/G
rs41281081	7	68-94	miR-335	G/A
rs75361918	7	1369-1391	miR-767-3p	C/G
rs1129355	7	182-203	miR-1204	C/G
rs76322625	7	1968-1988	miR-1026	C/U
rs59399612	7	214-233	miR-3659	U/G

Table 3. Genotype frequencies of the MET rs41281081 and rs76322625 polymorphism among NSCLC cases and controls. ^aThe ORs, 95% CIs and P value were calculated after adjusting for age, gender, parental smoking, drinking and family cancer history

Genotype	Cases (n =873)		Controls (n = 711)		OR (95% CI) ^a	P Value ^a
	N	%	N	%		
rs41281081						
GG	275	31.50	419	58.93	1.00	<0.0001
GA	326	37.34	195	27.43	2.54 (1.34-1.93)	
AA	272	31.16	97	11.11	2.73 (1.27-1.79)	
A Carrier	598	68.50	292	38.54	3.12(1.55-1.98)	<0.0001
rs76322625						
CC	378	43.30	518	72.86	1.00	<0.0001
CU	347	39.75	143	20.11	3.32 (1.45-1.79)	
UU	148	16.95	50	5.73	4.05 (1.36-1.79)	
U Carrier	495	56.70	193	25.84	3.51(1.05-1.85)	<0.0001

The miRSNPs in the MET gene 3'UTR

In order to investigate the miRNA-associated SNPs in the 3'UTR of MET, we first searched the SNP databases NCBI db SNP BUILED 129 and ENSEMBL v58 to find all the SNPs in the 3'UTR of the MET gene with the minor allele frequency (MAF) >0.05. We then used bioinformatics software (Diana-Micro, RNAhybrid) to predict miRNAs that can bind to the MET 3'UTR. The miRNASNP database was also applied to explore the miRNAs which could also bind to the 3'UTR of patients harboring the SNP, as shown in Table 2. Finally, we obtained six SNPs in the 3'UTR which could be regulated by three different miRNAs (miR-

Fig. 1. SNP rs41281081 in 3'UTR of NSCLC patients with GA/AA genotype can up-regulate of MET transcription by deregulated by miR-335. (A) The expression level of MET was determined by RT-PCR in patients with GG, GA and AA genotypes. (B) Bioinformatics predicted the binding site between the miR-335 with MET and the mutation types were conducted into the pGL3 plasmid as presented. (C) Cells were co-transfected with miR-335 mimics or control, Renilla luciferase vector pRL-SV40 for 48 h. Both firefly and Renilla luciferase activities were measured in the same sample. Firefly luciferase signals were normalized with Renilla luciferase signals. Left panel indicated the A549 cell line while the right indicated NCIH1975 cell lines. Data was presented as the mean $\pm$ SEM. * indicates a significant difference ($P < 0.05$).

122, miR-335, miR-767-3p, miR-1024, miR-1026 and miR-3659). The positions of the SNPs in the 3'UTR of MET as well as the variants were listed. Further genotyping was performed to detect the distribution of the alleles of the six SNPs in our research.

Correlation of rs76322625 and rs41281081 with NSCLC

Interestingly, two SNPs (rs76322625 and rs41281081) showed a significant difference between NSCLC patients and controls, as listed in Table 3. The results showed that the genotypes of both rs41281081 and rs76322625 were in a Hardy–Weinberg equilibrium distribution pattern in the healthy control group ($P < 0.0001$). Further, logistic regression analysis revealed that the NSCLC genotype GG in SNP rs41281081 presented a significantly decreased risk of NSCLC when compared with the A carrier genotypes (GA and AA; $P < 0.0001$). Similarly, rs76322625 presented a significantly decreased risk of NSCLC when compared with the U carrier genotype (CU and UU; $P < 0.0001$). Logistic regression analyses indicated that the rs41281081 GA and AA genotypes were significantly associated with NSCLC risk (OR = 2.54, 95% CI = 1.34–1.93 for GA; OR = 2.73, 95% CI = 1.27–1.79 for AA; and OR = 3.12; 95% CI = 1.55–1.98 for A carriers). Similarly, the rs76322625 CU and UU genotypes were also significantly associated with NSCLC risk (OR = 3.32, 95% CI = 1.45–1.79 for CU; OR = 4.05, 95% CI = 1.36–1.79 for UU; and OR = 3.51; 95% CI = 1.05–1.85 for U carriers). All ORs were adjusted for sex, age, and smoking status, drinking history and family cancer history.

Fig. 2. SNP rs76322625 in 3'UTR of NSCLC patients with CU/UU genotype can up-regulate of MET transcription by deregulated by miR-1026. (A) The expression level of MET was determined by RT-PCR in patients with CC, CU and UU genotypes. (B) Bioinformatics predicted the binding site between the miR-1026 with MET and the mutation types were conducted into the pGL3 plasmid as presented. (C) Cells were co-transfected with miR-1026 mimics or control, Renilla luciferase vector pRL-SV40 for 48 h. Both firefly and Renilla luciferase activities were measured in the same sample. Firefly luciferase signals were normalized with Renilla luciferase signals. Left panel indicated the A549 cell line while the right indicated NCIH1975 cell lines. Data was presented as the mean $\pm$ SEM. * indicates a significant difference ($P < 0.05$).

The effect of rs76322625 and rs41281081 on the regulatory role of miRNAs in MET expression

The SNPs rs41281081 and rs76322625 were predicted to be located in the binding sites of miR-335 and miR-1026, respectively. We hypothesized that the expression of MET might be regulated by these miRNAs, and could be affected by rs41281081 and rs76322625. To determine whether or not the inhibitory role of these miRNAs was impacted by the two SNPs, we first measured the level of expression of MET in patients harboring the GG, GA and AA genotypes, as well as the patients with CC, CU and UU genotypes. We found that patients with GA or AA genotypes presented a significantly higher level of MET in comparison with the patients with the GG genotype. Similarly, for rs76322625 of MET 3'UTR, the patients with genotype CC or CU also had a significantly greater level of MET than those with the UU genotype (Fig. 1A and 2A). We constructed pGL3 vectors including the allele-specific binding sequences (Fig. 1B and 2B), and then used them to co-transfect, with miR-335 and miR-1026 as well as controls, the NSCLC cell lines A549 and NCIH1975. As presented in Fig. 1 (C and D) and Fig. 2 (C and D), we found that the expression of both A-allele-specific and U-allele-specific pGL3 constructs was not significantly suppressed by miR-335 or miR-1026 on rs76322625 and rs41281081 (Fig. 1 (C and D) and Fig. 2 (C and D)). These findings suggest that rs76322625 and rs41281081 are regulated by miR-335 or miR-1026.

Stratified analyses of the association between MET polymorphism and NSCLC risk

Stratified analysis was used to investigate the association of the rs76322625 and rs41281081 genotypes with the clinicopathological parameters of NSCLC (Table 4 and 5). We found a significant association of the rs76322625 and rs41281081 genotypes with tumor size, differentiation and metastasis. Comparing with GG and CC homozygotes, the carriers of

Table 4. Stratified analysis of rs41281081 genotype with clinicopathological parameters of NSCLC. *Two-sided chi-square test for either genotype distributions or allele frequencies between cases and controls

Feather	GG	GA	AA	GG vs GA P Value*	GG vs AA P Value*
Age (years)					
≤ 50	243	105	65	0.108	0.402
> 50	246	137	77		
Gender					
Male	231	125	77	0.261	0.143
Female	258	117	65		
Differentiation grade					
Well	175	125	18	<0.0001	<0.0001
Moderate	147	48	58		
Poorly	167	69	66		
Tumor Size(cm)					
≤3cm	176	118	122	0.0009	<0.0001
>3cm	313	124	20		
Metastasis					
Yes	208	163	118	<0.0001	<0.0001
No	281	79	24		

Table 5. Stratified analysis of rs76322625 genotype with clinicopathological parameters of NSCLC. *Two-sided chi-square test for either genotype distributions or allele frequencies between cases and controls

Feather	UU	UC	CC	UU vs UC P Value*	UU vs CC P Value*
Age (years)					
≤ 50	174	166	73	0.63	0.50
> 50	204	181	75		
Gender					
Male	185	185	84	0.24	0.11
Female	193	162	64		
Differentiation grade					
Well	201	139	35	0.0012	<0.0001
Moderate	101	108	56		
Poorly	76	100	57		
Tumor Size(cm)					
≤3cm	231	139	119	<0.0001	<0.0001
>3cm	147	208	29		
Tumor Number					
Solitary	201	149	139	0.0059	<0.0001
Multiple	177	198	9		
Metastasis					
Yes	216	163	110	0.0062	0.0003
No	162	184	38		

the A or U genotype presented significantly larger tumors, poorer cellular differentiation and a high potential for metastasis.

Discussion

In this study, we investigated the relationship between miRSNPs within the 3'UTR of the MET gene and the risk of NSCLC. We observed that MET rs76322625 and rs41281081 with the GA, AA or CU, UU genotypes were associated with significantly increased tumor size, poor cell differentiation and metastasis of NSCLC. We further found that the SNPs rs76322625 and rs41281081, located in the binding sites of miR-1026 and miR-335, can disrupt their inhibitory role on MET expression, and play an important role in the development of NSCLC.

Several studies have reported increased expression of MET in human NSCLC. For example, overexpression of c-MET is frequently detected in adenocarcinoma cells, whereas upregulated HGF was detected in alveolar type II (ATII) cells. Overexpression of HGF has been correlated with cigarette smoking and tumor stages. An *in vitro* study showed that nicotine can activate HGF expression in ATII cells and lung cancer cells, which imply that HGF/c-MET signaling can be abnormally activated by environmental stimuli [26]. In addition, Xu et al. indicated that c-MET can be up-regulated in the response to hypoxia. EGFR signaling can regulate MET levels through HIF-1 α , and MET is a key downstream mediator of EGFR-induced invasiveness in EGFR-dependent NSCLC cells [27]. However, little research has been carried out on the SNP of MET and its influence on tumorigenesis.

Furthermore, c-MET can be regulated by miRNA. For example, MiR-335 was found to negatively regulate the c-MET protein level by directly targeting its 3'UTR. Forced expression of miR-335 decreased c-MET expression at the protein level and consequently diminished hepatocyte growth factor (HGF)-induced phosphorylation of c-MET, and subsequently inhibited HGF promotion of breast cancer cell migration in a c-MET-dependent manner [28]. MiR-335 expression was increased after 5-aza-2'-deoxycytidine (5-AZA-CdR) treatment, and 5-AZA-CdR treatment resulted in the same phenotype as the effect of miR-335 overexpression. MiR-34a inhibited cell growth and induced apoptosis partly by targeting MET in HGF-induced gefitinib-resistant HCC827 and PC-9 cells.

In summary, we have reported the first evidence that the SNPs rs76322625 and rs41281081 in the MET 3'UTR are involved in the occurrence of NSCLC by acting as a stimulating factor, and this may be one of the reasons for overexpression of MET in human NSCLC. The SNP rs41281081 can be regulated by miR-1026 and rs76322625 can be regulated by miR-335 to cause an up-regulation of MET in patients. These SNPs were also found to be related to the clinicopathological features of NSCLC, suggesting that they may have important roles in promoting tumor development. Our results support the hypothesis that genetic variants interrupting miRNA-mediated regulation and tumor suppressors are involved in the etiology of NSCLC.

Disclosure Statement

None declared.

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