

***AGTR1* promoter hypermethylation in lung squamous cell carcinoma but not in lung adenocarcinoma**

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Received December 12, 2016; Accepted August 3, 2017

DOI: 10.3892/ol.2017.6824

Abstract. Aberrant DNA methylation is associated with non-small cell lung cancer (NSCLC), suggesting that gene promoter methylation may be a potential biomarker for the detection or risk prediction of NSCLC. The present study aimed to evaluate the potential usage of angiotensin II receptor type 1 (*AGTR1*) methylation in two major pathologic subtypes: Lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC). Quantitative methylation-specific polymerase chain reaction was used to investigate the effect of *AGTR1* promoter methylation in the tumor and the paired adjacent non-tumor tissue samples from 42 patients with LUSC, and 69 with LUAD. The percentage of methylated reference was calculated and presented as the median (interquartile range 25th-75th percentile). The results of the current study revealed that there was significantly increased *AGTR1* promoter methylation in the tumor tissues compared with the paired adjacent non-tumor tissue [97.4 (57.22-130.5) vs. 85 (48.25-123); $P=0.024$]. Furthermore, higher *AGTR1* promoter methylation was observed in patients

with LUSC compared with LUAD (odds ratio=2.483; 95% confidence interval=1.125-5.480; $P=0.023$). Significant differences were identified in *AGTR1* methylation between non-tumor and the tumor tissues in LUSC [113.5 (68.33-148.73) vs. 93.04 (45.94-140); $P=0.008$]. In addition, the Cancer Genome Atlas data of 378 patients with LUSC and 477 with LUAD revealed an inverse correlation between gene expression and the methylation status of *AGTR1* promoter. These data suggest that *AGTR1* hypermethylation is a promising biomarker to assist in LUSC detection and diagnosis.

Introduction

Lung cancer is a kind of malignancy that arises from epithelial cells. It is the leading cause of cancer-related death worldwide (1). There were nearly 1.8 million new patients and caused 159 million deaths in 2012, of which China accounted for more than one third (2). According to the size and appearance of the malignant cells, lung cancer is categorized as non small cell lung cancer (NSCLC) and small cell lung cancer (3). NSCLC represents approximately 85% of lung cancer (4), and it can be further subdivided into large cell carcinoma, lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC). There are a lot of differences in molecular profiling, characteristics and therapeutic methods between LUAD and LUSC (5).

Promoter hypermethylation of the tumor suppressor genes has been recognized as an important factor in inducing oncogenesis (6). For example, SRY-box 17 (*SOX17*) methylation was found in 60.2% of primary lung cancer samples, and promoter methylation of *SOX17* silenced gene expression, leading to the elimination of cell proliferation suppression in lung cancer (7). Identification of specific gene hypermethylation may explain the genomic instability and complexity of NSCLC and provide a basis for targeted therapy or risk prediction.

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Key words: angiotensin II receptor type 1, lung adenocarcinoma, lung squamous cell carcinoma, quantitative methylation specific polymerase chain reaction, promoter methylation

AGTR1 encodes the angiotensin II (Ang II) type I receptor that belongs to the family of G-protein coupled receptors (8). Ang II is a major effector controlling blood pressure in cardiovascular system and induces diverse signal transduction pathways such as the biphasic activation of Raf-1, MEK, and ERK via both Gq and Gi proteins (9). It regulates the aldosterone secretion and is involved in vascular remodeling, inflammation and endothelial dysfunction (10). Hypomethylation in the *AGTR1* promoter had been validated to be inversely correlated with uric acid levels, which can be a significant risk predictor of essential hypertension (EH) (11). Besides, *AGTR1* methylation had been extensively studied in human cancers, such as oral squamous cell carcinoma (12), colorectal cancer (13), breast cancer (14), ovarian cancer (15) and oral cancer (12). For example, several studies support a possible role for *AGTR1* in regulating cell growth and proliferation during cancer development in breast cancer (16), which was shown to be amplified and overexpressed in 10-20% of breast cancer cases, and was even markedly overexpressed more than 100 fold (14). Besides, the *AGTR1* promoter methylation is associated with oral squamous cell carcinoma (OSCC) development (12). And *AGTR1* was also validated in stool DNA with a high detection sensitivity for noninvasive diagnosis of colorectal cancer (13). In addition, DNA methylation microarray datasets showed that methylation of *AGTR1* might be an effective biomarker for NSCLC diagnosis (17).

Since the detection of *AGTR1* methylation in different pathologic subtypes and stages have not been conducted, we used LUSC and LUAD samples to study the effects of *AGTR1* methylation on the risk of the disease in this study.

Materials and methods

Patients. Tumor tissues and paired adjacent non-tumor tissues from 111 patients were collected from Affiliated Wujiang Hospital of Nantong University (Jiangsu, China) between August 2010 and October 2013. There were 73 male and 38 female patients with a mean age of 63.59 ± 10.19 years (range, 33-82 years), including 42 patients with LUSC and 69 patients with LUAD. Clinical pathological data and isoforms were obtained from the patients' medical records and pathology files. Clinical stage was classified by the third edition of the American College of Chest Physicians (ACCP) Lung Cancer Guidelines (LC III) (18). The study protocol was approved by the Ethics Committee of Affiliated Wujiang Hospital of Nantong University. All the patients had signed the written informed consent forms.

DNA extraction and bisulphite conversion. DNA was isolated from the formalin-fixed and paraffin-embedded (FFPE) cancer sample using QIAamp DNA FFPE Tissue Kit (Qiagen Inc., Hilden, Germany). DNA concentrations were measured using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Then DNA were converted using the EZ DNA Methylation-Gold Kit™ (Zymo Research Corporation, Irvine, CA, USA).

Quantitative methylation-specific PCR. SYBR green-based quantitative methylation-specific PCR (qMSP) was conducted to detect the methylation level. PCR was carried out in a final

volume of 20 μ l containing 5 μ l SYBR mix, 4 μ l H₂O, 0.5 μ l primer and 0.5 μ l modified DNA. PCR amplification were run in triplicate for every sample, and the reactions was performed on Light Cycler 480 system (Roche Applied Science, Mannheim, Germany) under the following conditions: 10 min of denaturation at 95°C followed by 45 cycles of 20 sec at 95°C, 20 sec at 58°C and 30 sec at 72°C. Melting curves system was as follows: 15 sec at 95°C, 1 min at 58°C, 95°C continuous prior to drop the temperature to 40°C for 4 min. The Actin Beta gene (*ACTB*) was used as an internal reference by amplifying non-CpG sequences. Results with cycle threshold values (Ct values) of *ACTB* > 40 were defined as detection failures. *M.SssI* catalyzes the deamination of target cytosine to uracil to generate DNAs that differ only in their CpG methylation status (19). The methylated DNA was prepared to be the positive internal control. Each set of amplifications included a positive control, a negative control, and a non-template control.

The sequences of primers for target and internal reference genes were summarized in Table I, and the genomic location of *AGTR1* gene was shown in Fig. 1A. Some of the PCR products were analysed using to the Qsep100 DNA Analyzer (Bioptic Inc, Taiwan, China) to validate the methylation status, and ultimately the visible peaks were exported by the Q-analyzer software (Fig. 1B). Besides, some of the products were sequenced randomly using the Applied Bio systems® 3730 DNA Analyzer (Applied Biosystems, Warrington, UK) to confirm a complete bisulphite conversion (Fig. 1B).

Statistical analysis. For each sample the comparative Ct ($\Delta\Delta$ Ct) method was used to determine the relative methylation values. And the percentage of methylated reference (PMR) was shown by using the following formula (substituted the actual methylation value here): [(gene/*AGTR1*) sample/(gene/*AGTR1*) positive] x100%. The median of PMR (97.4) was set as the cutoff value and defined methylation as hypermethylation (positive) and hypomethylation (negative) subgroups. Statistical analyses were performed using SPSS 18.0 version (SPSS Inc., Chicago, IL, USA). The wilcoxon signed ranks test was used to determine the difference of the methylation index between tumor tissues and non-tumor tissues. χ^2 test was used to evaluate the association between promoter methylation and clinical parameters. Overall survival in relation to methylation status was calculated by Kaplan-Meier survival curves, and survival differences were assessed in the log-rank test. P<0.05 was considered to indicate statistical significance.

Results

The result of sequenced PCR products showed that all the non-CpG cytosines were converted to thymine, whereas the cytosines of CG dinucleotide remained unchanged (Fig. 1). And the results of capillary electrophoresis experiments showed the length of products were correct.

As the results revealed, there existed statistical differences in *AGTR1* methylation between tumor tissues and the adjacent non-tumor tissues (PMR: 97.4 vs. 85, P=0.024). A subgroup analysis indicated that there was a significantly induced hypermethylation of *AGTR1* in LUSC tumors (P=0.008) but not in LUAD tumors (P=0.449, Fig. 2).

Table I. Primer sequences for quantitative methylation-specific polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product (bp)	T _m (°C)
<i>AGTR1</i>	GGAGGAGGAGGGAATGTAA	CCTATCACTCGCTACTACCT	142	58
<i>ACTB</i>	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA	133	58

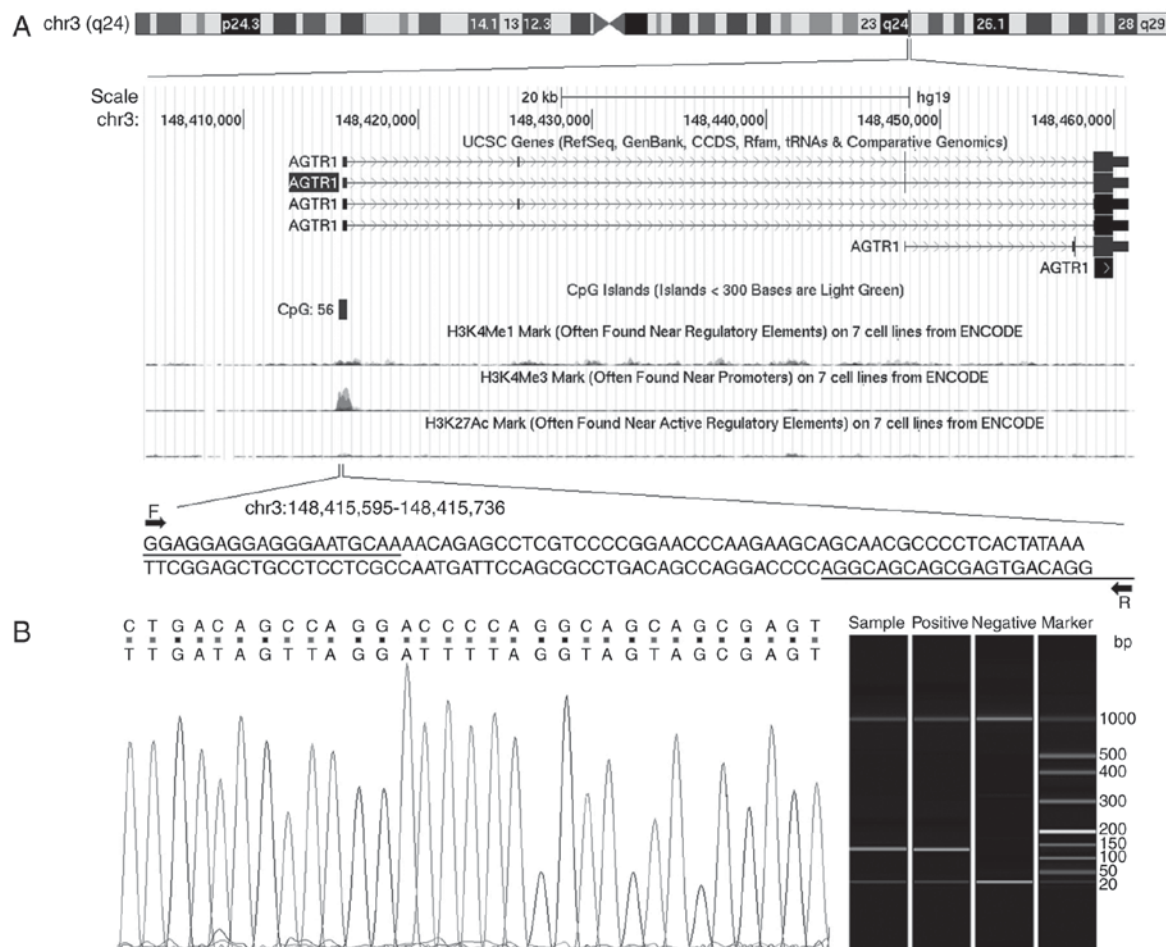


Figure 1. *AGTR1* and the validation for sequencing and electrophoretic results. (A) The location of *AGTR1* on the chromosome and the sequences of primer for target gene. (B) The top row of sequencing validation result represents the original gene sequence, and the second row shows the converted one. The electrophoresis experiments reveal the results of methylated sample, the positive internal control and the negative control.

Further correlation analysis of clinicopathologic characteristics in patients with promoter methylation status was also performed (Table II). There was higher *AGTR1* promoter methylation in LUSC patients than LUAD (Odds ratio=2.483, 95% CI=1.125-5.480, $P=0.023$). In addition, *AGTR1* promoter methylation was not associated with gender, age, smoking history, clinical stage and lesion location in NSCLC (Table II).

Previous study showed that *AGTR1* is predictor of progression-free survival (PFS) and response to advanced/metastatic breast cancer (20). However, the correlation of *AGTR1* methylation with overall survival of cancer was unknown. In this study, we examined the overall survival of individuals according to *AGTR1* PMR value, and no significant difference was observed between LUSC and the LUAD patients ($P=0.293$, Fig. 3).

To further reveal the correlation between expression and methylation status of *AGTR1* in LUSC and LUAD, the MEXPRESS system (21) (<http://mexpress.be>) was used. And the Cancer Genome Atlas (TCGA) data of 378 LUSC and 477 LUAD patients (21) indicated a lower expression in cancer tissues, supporting an inverse correlation between gene expression and *AGTR1* methylation.

Discussion

NSCLC is a huge threat to human health, and the study of the methylation and its relation to NSCLC is a field of growing interest. *AGTR1* acts as a novel component of the renin-angiotensin system (RAS) to affect the blood pressure and heart hypertrophy (15), targeting of renin-angiotensin system (RAS) through *AGTR1* blocker is associated with the resistance

Table II. Association between gene methylation and clinicopathological characteristics in patients with lung cancer.

Variables	n	<i>AGTR1</i> hypermethylation	<i>AGTR1</i> hypomethylation	OR (95% CI)	P-value
Gender					
Male	73	38	35	1.206 (0.550-2.645)	0.639
Female	38	18	20	1	
Age (years)					
≤65	62	31	31	0.960 (0.454-2.031)	0.915
>65	49	25	24	1	
Smoking history					
Nonsmoker	50	26	24	1.119 (0.530-2.366)	0.768
Smoker	61	30	31	1	
Histological type					
LUSC	42	27	15	2.483 (1.125-5.480)	0.023
LUAD	69	29	40	1	
Clinical stage					
I+II	88	41	47	0.465 (0.179-1.209)	0.112
III+IV	23	15	8	1	
Tumor location					
Left lung	46	25	21	1.306 (0.612-2.784)	0.490
Right lung	65	31	34		

LUSC, lung squamous carcinoma; LUAD, lung adenocarcinoma; The median of percentage of methylated reference (PMR) (97.4%) was set as the cutoff value, the PMR >97.4% was considered to be hypermethylation and the PMR ≤97.4% was considered to be hypomethylation. Bold fonts displayed the statistical differences.

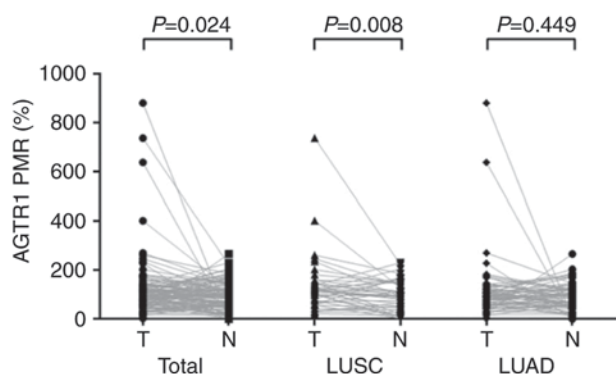


Figure 2. Comparisons of methylation levels of *AGTR1* between tumor tissues and paired adjacent non-tumor tissue.

phenomenon in treatment of resistant breast cancers. And now the *AGTR1* blocker has been applied in the clinical oncology, such as the losartan for breast cancer (22) and candesartan for prostate cancer (23). And now emerging evidences showed that *AGTR1* regulates the cell proliferation during cancer development, and promotes tumor invasion, migration, metastasis and angiogenesis (24). In consideration of the unfavorable treatment status of NSCLC, detection of biomarkers like the *AGTR1* is really necessary and urgent.

In the present study, DNA methylation differences of *AGTR1* between 111 paired tumor sample and adjacent non-tumor tissues were assessed using quantitative

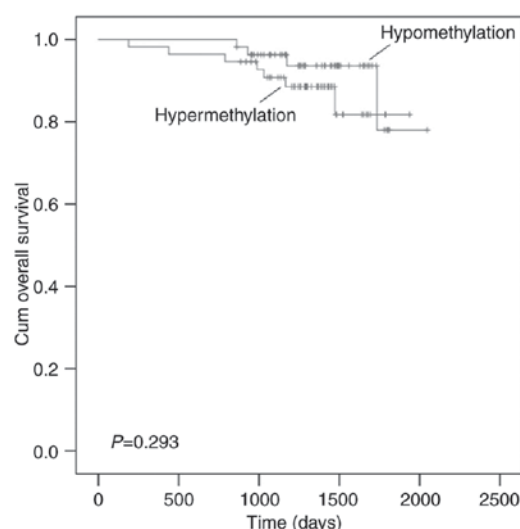


Figure 3. Kaplan-Meier curve for cum overall survival of individuals.

methylation-specific PCR. We identified significant differences in *AGTR1* methylation between tumor tissues and the adjacent non-tumor tissues, this observation lead us to suggest that CpG island methylation phenotype in *AGTR1* may be an early event during NSCLC development. Besides, *AGTR1* were hypermethylated in LUSC but not in LUAD, and there were significant differences in *AGTR1* promoter methylation

between LUAD and LUSC patients, suggesting a potential usage of this epigenetic biomarker in the diagnosis of LUSC. In addition, we found that *AGTR1* promoter methylation was not associated with gender, age, smoking history, clinical stage and lesion location in NSCLC. And research on the data of TCGA suggested a negative correlation between gene expression and *AGTR1* methylation.

Our finding of *AGTR1* promoter methylation as a diagnostic marker of NSCLC was consistent with a study of high-throughput DNA methylation microarray dataset for Chinese Han NSCLC retrospective cohort which showed the significant association between methylation and NSCLC (17). This was also consistent with the result of TCGA for LUSC and LUAD patients, suggesting that *AGTR1* might be associated with increased risk of NSCLC.

Our results showed that there were significant differences in *AGTR1* promoter methylation between LUAD and LUSC patients, and there was a significantly induced hypermethylation of *AGTR1* in LUSC tumors compared with the normal tissues but not in LUAD. Previous study declared differentially methylated genes were specially differential methylation in unique cancer, and the DNA methylation correlation network had been built based on the methylation correlation, such as seven biomarkers (*PCDH15*, *IGF1*, *PRRT1*, *CYGB*, *WBSCR17*, *ACTG2* and *GYPC*) can distinguish the breast cancer into high-risk group and low-risk group. Likewise, different methylation status in eight biomarkers (*ZBTB32*, *GPSM1*, *SALL1*, *OR51B4*, *MAGEA8*, *SALL3*, *CCL8* and *TMEFF2*) in colon cancer showed the different risk level (25). So the methylated *AGTR1* might be identified as the specific driver gene for LUSC, which might be implicated in cancer type specific pathway and be expected to be used to explain part of the heterogeneity between these two NSCLC types.

NSCLC was regarded as a complex diseases, and some clinicopathologic characteristics were involved in cancer, such as the smoking history, which was identified as the main risk factor of lung cancer (26), and association between tobacco smoking and promoter DNA hypermethylation had been demonstrated for several genes, such as the transcription factor 21 (*TCF21*) and *MicroRNA Let-7a-3* (27). The previous study on primary lung cancer cases in China (N=40,022) showed that males were 1.5 times more likely to have lung cancer than females (28), but the incidence of lung cancer among women was rising exponentially as a consequence of recent changes in gender-specific smoking patterns. Besides, women with lung cancer were diagnosed at younger ages than men, suggesting the different gender and age susceptibility existed in patients (29). Notably, the clinical stages were important prognostic factors in NSCLC. The advanced disease stages affected survival negatively (30). Besides, even though apparently symmetric, previous study showed that higher incidence of lung cancer was found on the right side (31), just as the breast cancer, which was about 5% more likely to be diagnosed in the left breast than the right (32). Breast cancer that arising on different sides of the body presented different cancer traits inferred from methylation and expression profiles (31), and this result contributed to serve as proof of principle for other bilateral cancers like the lung cancer. In our study, *AGTR1* promoter methylation was shown likely to be not linked with gender, age, smoking history, clinical stage

and tumor location. But the further verification is required to ensure the results considering of the limitation factors existed in our experimental design.

In the present study, we enrolled the relatively large cohorts that contained 111 patients, and we collected the precious surgery tissues to perform the survey by general methodology that easily to be applied to different cancer subtypes. However, there existed several limitations here. Firstly, because of the limited time, the large cell carcinoma was not included in our study, the study design may still be too simple to uncover the complicated trait of NSCLC. Secondly, because of the limited amount of sample, we did not detect the expression of different methylated *AGTR1* and replaced by a database analysis of TCGA, which showed the negative relevance between methylation and expression of *AGTR1*. However, one previous studies showed there was no correlation between the degree of methylation and mRNA abundance of *AGTR1* in early gestation amnion and placenta (33), so the further studies are needed. Thirdly, the mechanisms by which *AGTR1* methylation affect the LUSC remained largely elusive. Fourthly, we just detect the part of the content area, it could be possible that some more CpG sites are required for gene function, besides, additional epigenetic alteration, such as the histone modifications that closely connected with the gene methylation are needed for study on NSCLC progression. In addition, only the *AGTR1* gene was investigated in the present study, and additional relevant genes should be explored in the future.

In conclusion, our findings of the association between *AGTR1* methylation and LUSC provided a potential biomarker for detection, diagnosis and risk prediction for LUSC.

Acknowledgements

The research and publication was supported by the grants from the National Natural Science Foundation of China (31100919, 81371469), K. C. Wong Magna Fund of the Ningbo University, National Natural Science Foundation for the Youth of China (81402220) and Suzhou Planning Project of Science and Technology (SYS201301).

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