

Received: 2015.04.16

Accepted: 2015.06.03

Published: 2015.10.12

The Correlation Relationship between P14^{ARF} Gene DNA Methylation and Primary Liver Cancer

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Source of support: Departmental sources

Background: Primary liver cancer is a common malignant tumor that causes serious damage to human health. DNA methylation is common in epigenetics. DNA methylation plays an important role in the process of primary liver cancer occurrence and development. The P14^{ARF} gene is an important tumor suppressor gene. It was found that P14^{ARF} methylation is associated with the degree of malignancy in multiple tumors. Therefore, this study aimed to investigate the relationship between P14^{ARF} methylation level and primary liver cancer malignant degree.

Material/Methods: Carcinoma tissues and adjacent tissues were collected from 87 primary liver cancer patients. Pyrosequencing was applied to obtain P14^{ARF} methylation. Real-time PCR was used to detect P14^{ARF} mRNA level.

Results: P14^{ARF} methylation level in cancerous tissue was significantly higher than in the adjacent tissue ($t=76.54$, $P<0.001$). P14^{ARF} methylation showed no significant difference in patients with different age, sex, smoking status, or drinking status. It did not present an obvious difference in tumors with different size. Its methylation level increased following the improvement of TNM stage ($P<0.05$). Compared with the adjacent tissue, P14^{ARF} mRNA in carcinoma tissue decreased by 31% ($t=28.91$, $P<0.001$). P14^{ARF} methylation showed a significant negative correlation with mRNA expression in cancerous tissue ($r=-0.43$, $P<0.01$).

Conclusions: P14^{ARF} mRNA level is regulated by DNA methylation in primary liver cancer. P14^{ARF} gene DNA methylation may be associated with the occurrence of primary liver cancer occurrence and TNM staging.

MeSH Keywords: **DNA Methylation • Pneumonia, Mycoplasma • Sarcoglycanopathies**

Full-text PDF: <http://www.medscimonit.com/abstract/index/idArt/894395>

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Background

Primary liver cancer is a common malignant tumor with great impacts on human health and economics and its incidence is continuously rising [1]. Following the development of serum AFP clinical application and imaging techniques, more liver cancers are found in the early stage. Early liver cancer treatment also has experienced great progress, but the 5-year survival rate has not significantly improved. Currently, researchers found that, in addition to genetic factors and environmental factors, epigenetic changes also play an important role in tumor development [2].

DNA methylation is a common epigenetic modification that is closely related to tumor occurrence and development, and even potential biomarkers for tumor [3]. Abnormal DNA methylation in tumors is mainly presented as specific gene hypermethylation and genome-wide hypomethylation. It can increase chromosome instability or cause tumor suppressor gene inactivation, resulting in tumor occurrence. Studies have shown that DNA methylation change is related to primary liver cancer occurrence. Multiple genes in liver tissue, such as SASH1 [4], GPX3 [5], RASSF1A [6], GSTP1 [7], and P16 genes [8], are found as DNA methylation levels increase, while genome-wide alternative gene LINE-1 methylation level decrease [9,10]. The P14^{ARF} gene is an important tumor suppressor gene that has a negative regulatory role in the cell cycle. Kawamoto [11] found that P14^{ARF} gene DNA methylation level is associated with the pathological staging of bladder cancer. The present study investigated the relationship between P14^{ARF} methylation level and primary liver cancer malignant degree by pyrosequencing to provide a basis for liver cancer early diagnosis, prognosis, and treatment.

Material and Methods

Main reagents and instruments

EpiTect Fast DNA Bisulfite Kit (QIAGEN), PyroMark PCR Kit (QIAGEN), PyroMarkCpG Assay 96 well (QIAGEN), PyroMark Q96 ID sequencer (QIAGEN), Trizol (Invitrogen), Real-time PCR kit (Takara), gel-imaging system, ViiA7 real-time PCR amplifier (ABI).

Objects and samples

We enrolled 87 liver cancer patients that needed surgery from Third Xiangya Hospital between Jan 2012 and Oct 2014. All of the selected patients received no radiotherapy or chemotherapy preoperatively. Carcinoma tissues and adjacent tissues that were at least 2 cm away from the cancer edge were collected. Liver cancer staging was according to the international standard of TNM stage. Smoking status was defined as at least 1

Table 1. RT-PCR primers.

Gene	Primer sequence
P14ARF	F: GGGTTTCGTGGTTCACATCCCGG
	R: CAGGAAGCCCTCCCGGGCAGC
β-actin	F: GGGTCAGAAGGACTCCTATG
	R: GTAACAATGCCATGTTCAT

cigarette a day for more than 1 year. Drinking status was defined as at least 1 drink per month.

The experimental protocol was pre-approved by the ethics committee of our hospital and written consents were obtained from all patients and healthy volunteers.

DNA extraction and Bisulfite modification

Salt fractionation was adopted to extract the whole genome DNA. Sample absorbance of A260/A280 between 1.8 and 2.0 could be used in the subsequent experiment. 2 µg DNA was modified and purified using EpiTect Bisulfite Kit (QIAGEN), according to the manual. Genomic DNA treated by *M.SssI* methylation transferase and transformed by bisulfite was selected as the positive control (universal methylation, UM) to modify DNA as a template.

P14^{ARF} gene methylation level detection

As previously reported [12], PCR amplification and sequencing primers were synthesized by Shanghai Generay biological engineering co., LTD. PCR primers were as follows: F: TTTATTTGGTTTTTAGGAAG, R: Biotin-CAAATCTTAATAACCTCC, sequencing primers: GTTGGTTTTTAGTAGTATTAGTA. P14^{ARF} gene target segment was amplified according to the PyroMark PCR Kit instructions. The cycling conditions consisted of an initial, single cycle of 15 min at 95°C, followed by 45 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, and single cycle of 10 min at 72°C. Five µl of amplification product was identified by agarose gel electrophoresis. One chain of the PCR product with biotin was mixed with magnetic beads carrying streptavidin. After separated by vacuum preprocessor, single-stranded DNA was mixed with P14^{ARF} sequencing primer and sequenced in the PyroMark Q96 ID System (Qiagen) sequencer. Three independent experiments were performed.

RNA extraction and P14^{ARF} mRNA detection

Total RNA was extracted by TRizol. The cDNA was synthesized using reverse transcriptase (TaKaRa), oligo (dT) primers with 1 µg RNA. P14^{ARF} primer was according to Zhang's report [13]. The primers used are listed in Table 1. Each real-time RT-PCR

Table 2. P14^{ARF} gene DNA methylation level comparison.

TNM stage	Cases	Adjacent tissue (mean ±SD)%	Carcinoma tissue (mean ±SD)%	t value	P value
Stage I	51	20.41±0.78	40.32±2.85	56.20	<0.001
Stage II	20	20.23±0.56	46.78±3.12	34.22	<0.001
Stage III	16	21.12±0.43	49.86±3.34	40.06	<0.001
Total	87	20.50±0.67	43.56±3.00	76.54	<0.001

reaction (in 20 µL) contained 2×SYBR Green Mixture (TIANGEN), 0.5 µL primers, and 0.5 µL template cDNA. The cycling conditions consisted of an initial, single cycle of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. PCR amplifications were performed in 3 duplicates for each sample. Gene expression levels were quantified relative to the expression of β-actin using an optimized comparative Ct ($\Delta\Delta C_t$) value method. The differences in gene expression levels between groups were compared using the t test. P values <0.05 were considered statistically significant.

Statistical analysis

All statistical analyses were performed using SPSS13.0 software (SPSS Inc., USA). Differences between multiple groups were analyzed by t test or one-way ANOVA. Linear regression analysis was used for the trend analysis. Pearson correlation analysis was applied when the double variable complied with normal distribution, inspection level $\alpha=0.05$.

Results

P14^{ARF} gene DNA methylation comparison

P14^{ARF} gene DNA methylation level in carcinoma tissue was 43.56±3.00%, and it was 20.50±0.67% in adjacent tissue; the paired t test showed that P14^{ARF} gene DNA methylation level was significantly higher than in the adjacent tissue ($t=76.54$, $P<0.001$). When comparing P14^{ARF} gene DNA methylation level in different TNM stages, we found that P14^{ARF} gene DNA methylation level was obviously higher in carcinoma tissue in stage I, II, and III ($P<0.001$) (Table 2).

P14^{ARF} gene DNA methylation correlation analysis with clinicopathological characteristics

As shown in Table 3, P14^{ARF} methylation showed no significant difference in patients with different age, sex, smoking status, or drinking status. It did not present obvious difference in tumors with different sizes. It showed a significant difference among patients in stage I, II, and III by one-way ANOVA

analysis ($F=76.30$, $P<0.001$). To avoid the interference of possible confounding factors, multiple linear regression results showed that P14^{ARF} methylation level increased following the improvement of TNM stage ($P<0.05$).

P14^{ARF} gene mRNA expression

Real-time PCR was used to detect P14^{ARF} mRNA expression level (Figure 1). Compared with the adjacent tissue, P14^{ARF} mRNA expression decreased by about 31% in carcinoma tissue ($t=28.91$, $P<0.001$). One-way ANOVA results revealed that P14^{ARF} mRNA level was different in patients with different TNM stage ($F=24.15$, $P<0.001$). Linear regression analysis suggested that its level reduced gradually following TNM staging increase ($P<0.05$).

The correlation between P14^{ARF} gene mRNA expression and DNA methylation level

Pearson correlation analysis indicated that P14^{ARF} methylation showed a significant negative correlation with mRNA expression in cancerous tissue ($r=-0.43$, $P<0.01$). Following the P14^{ARF} gene methylation level increase, P14^{ARF} mRNA expression declined gradually, suggesting that P14^{ARF} mRNA expression changes in primary liver cancer might be regulated by DNA methylation.

Discussion

Epigenetics is a concept corresponding to genetics, which mainly includes DNA methylation, histone covalent modification, and RNA editing. Abnormal DNA methylation is closely related to tumor occurrence, such as lung cancer [14], gastric cancer [15], and colorectal cancer [16]. It is even closely associated with tumor prognosis [17]. Thus, more and more researchers committed to DNA methylation for tumor early diagnosis and treatment. There were numerous DNA methylation detection methods, mainly including methylation-specific PCR, methylation fluorescence PCR, and bisulfite sequencing PCR. However, the abovementioned methods can only detect qualitative, semi-quantitative, or relative quantitative levels

Table 3. P14^{ARF} gene DNA methylation correlation analysis with clinicopathological characteristics.

Clinicopathological characteristics	Cases	DNA methylation (Mean±SD)%	Statistics	P value
Gender				
Male	45	43.18±3.34	1.22	0.11
Female	42	43.97±2.64		
Age				
<60 years	36	43.21±2.97	0.92	0.18
≥60 years	51	43.81±3.02		
Smoking				
Yes	19	44.23±3.12	1.10	0.14
No	68	43.37±2.97		
Drinking				
Yes	72	43.33±3.14	1.53	0.07
No	15	44.67±2.78		
Tumor size				
≥5 cm	30	44.01±2.85	1.02	0.16
<5 cm	57	43.32±3.08		
TNM stage				
Stage I	51	40.32±2.85	76.30	<0.001
Stage II	20	46.78±3.12		
Stage III	16	49.86±3.34		

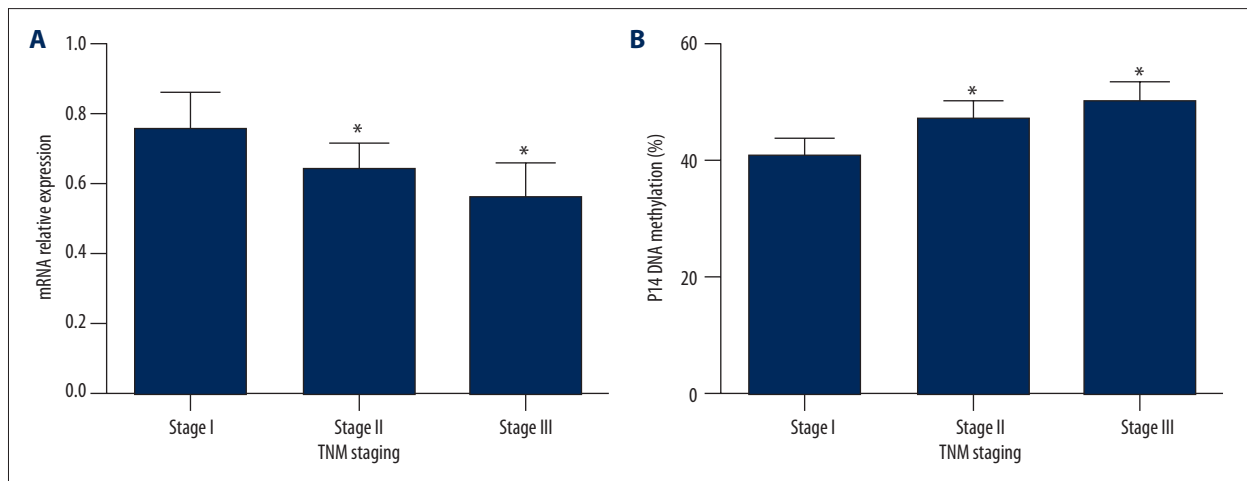


Figure 1. P14^{ARF} gene mRNA expression (A) and DNA methylation level (B) in patients with different TNM stages. * p<0.01 compared with stage I.

of DNA methylation. Pyrosequencing is a technique based on DNA sequence analysis [18] that could be used for accurate DNA methylation quantification. Thus, it is the criterion standard for DNA methylation analysis.

P14^{ARF} gene is a tumor suppressor gene located on short arm 21th segment of chromosome 9. It codes a 14 kD protein that can bind with MDM2 protein to speed its degradation, further stabilize p53 protein, and play a role as cell cycle check point [19]. Therefore, this study aimed to investigate the relationship between P14^{ARF} methylation level and primary liver cancer TNM staging by pyrosequencing. The results showed that P14^{ARF} methylation level in the cancer tissue was significantly higher than that of adjacent tissue, while P14^{ARF} mRNA level was reduced in cancerous tissue compared with the adjacent tissue and exhibited a negative correlation with DNA methylation. This indicates that P14^{ARF} gene DNA methylation is associated with primary liver cancer occurrence. P14^{ARF} gene mRNA expression in carcinoma tissues could be affected by DNA methylation, which is consistent with previous results. Zhang et al. used methylation-specific PCR to detect P14^{ARF} gene DNA methylation level in liver cancer tissue and found that P14^{ARF} DNA methylation level in liver cancer tissue (53.3%) was higher than in normal tissue (33.3%) [13]. DNA methylation level increase may cause P14^{ARF} gene expression silencing. Many other studies also suggested that P14^{ARF} methylation was higher in liver cancer tissue than in normal tissue [20–23]. The meta-analysis results reported by Li indicated

that primary liver cancer risk was associated with P14^{ARF} gene DNA methylation level [24]. All of these research results revealed that P14^{ARF} gene DNA methylation may be associated with primary liver cancer occurrence.

Our study found that P14^{ARF} gene DNA methylation level in carcinoma tissue was gradually up-regulated following the improvement of TNM staging, suggesting that P14^{ARF} gene DNA methylation might be associated with TNM stage, malignant degree, and prognosis. However, Anzola reported that P14^{ARF} gene DNA methylation showed no association with TNM staging [23]. They analyzed the correlation between cancer tissue differentiation degree and DNA methylation, and used methylation-specific PCR method. These may cause the different results of the 2 studies.

Conclusions

To the best of our knowledge, this study is the first to use pyrosequencing to analyze the correlation between P14^{ARF} gene DNA methylation and primary liver cancer. Compared with the previous study, we applied a more accurate method for analysis. However, since only 87 cases were included, further studies with larger sample sizes are needed to determine whether the P14^{ARF} gene can be used as a biomarker for primary liver cancer early diagnosis and prognosis evaluation.

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