Int J Clin Exp Med 2015;8(8):14070-14074
www.ijcem.com /ISSN:1940-5901/IJCEM0008015

Original Article

Liver kinase B1 promoter CpG island methylation is related to lung cancer and smoking

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Received March 15, 2015; Accepted May 20, 2015; Epub August 15, 2015; Published August 30, 2015

Abstract: The aim of this study was to explore the association of CpG islands methylation of liver kinase B1 (LKB1) with primary lung cancer and smoking, providing a theoretical basis for the demethylating drug to treat lung cancer by detecting the LKB1 promoter CpG methylation. mRNA expression of LKB1 were detected by in situ hybridization and methylation status on Hap II locus of the promoter of LKB1 was analyzed by methylation-specific polymerase chain reaction (PCR). 7 of 80 LKB1 positive cases had methylation on CpG islands while 18 of 44 LKB1 negative cases had methylation on CpG islands. The difference was significant between CpG island methylation and LKB1 expression. 8 of 54 cases of early and middle lung cancer were detected LKB1 promoter CpG island methylation while 30 controls were not detected, the difference was significant. 5 of 64 more-than-5-year cases had methylation on CpG islands while 20 of 60 less-than-5-year cases had methylation. The difference was significant between of 5-year survival and CpG island methylation of LKB1. 22 of 74 smoking cases of lung cancer had methylation on CpG islands of LKB1 while only 3 of 50 non-smoking cases had methylation. The difference of smoking and CpG island methylation of LKB1 was significant. LKB1 promoter CpG islands aberrant methylation is closely associated with the occurrence, development and prognosis of lung cancer, especially with smoking history including clinical early diagnosis and prognosis. CpG islands methylation in the promoter of LKB1 is likely important one of the mechanism of smoking-associated lung cancer.

Keywords: Liver kinase B1, methylation, smoking, lung cancer, methylation specific PCR

Introduction

With air pollution and environmental deterioration, lung cancer has become one of the fastest increasing in morbidity and mortality of lung cancer accounting for the first of all malignancies in China. The occurrence and development of lung cancer is a complex biological process involved in multiple steps and multiple factors such as oncogene activation and tumor suppressor gene inactivation [1]. In normal mammalian cells, 70-90% scattered CpG are modified methylation in DNA sequences while highly aggregated CpG islands are non-methylated. CpG island with rich CpG dinucleotide in human genome is always in a non-methylated state and located near the transcriptional regulatory region. About 28890 CpG islands are in human genome that average 1 Mb chromosome contains about 10 CpG islands and the number of CpG islands have good correspondence with gene density [2].

Suppression of oncogene-encoded protein product LKB1 is a serine/threonine kinase regulating a variety of physiological and pathological processes [3]. Human LKB1 gene or called STK II (Serine-threonine kinase II) located on human chromosome 19p 13.3 locus, comprising 10 exons, encoding LKB1 protein containing 433 amino acids including the kinase domain (44-309), N terminal regulatory domain and a C-terminal regulatory region. N-terminal regulatory domain contains a nuclear localization sequence making LKB1 localized in the nucleus. Embryonic inactivating mutations of LKB1 causes cancer susceptible Piglet's syndrome (Peutz-Jeghers syndrome, PJS) with multiple

hamartomas and an increased risk of cancer [4-7]. LKB1 mutants are widely distributed in a variety of cancers such as lung cancer, colon cancer and breast cancer [8, 9]. Fernandez et al [10] reported LKB1 mutants including point mutations, deletions and promoter methylation in primary lung adenocarcinomas. But LKB1 methylation inactivation in Asians especially in North China has not been reported. In this paper, 124 cases of lung adenocarcinomas or squamous cell carcinomas from the north China were randomly collected to study the association of LKB1 promoter CpG island methylation with early diagnosis, prognosis and smoking.

Material and methods

Materials

124 cases of confirmed lung cancer were collected during 1999-2010 including 78 squamous and 46 adenocarcinoma cell carcinoma cases. All 124 cases were male and 74 cases had the exact history of smoking. Another 30 cases were collected as control with benign lung inflammatory diseases such as pneumonia, but not including Peutz-Jeghers syndrome, hamartoma and adenomatous hyperplasia. Mouse anti-LKB1 IgG antibody (E-9, Santa Cruz). LKB1 gene promoter primer, sense: 5'-ACT TAA TCG CCG AAC ATC ACG CA-3', antisense: 5'-ACG ATC CCG TAT CTG GAC CTA GTC A-3'. In situ hybridization: probe A-CCAGATGTCTATGCCGGA labeled 3' end of the probe, B-AAGATACGTACACCGCGCATT labeled 5' end of digoxin antibody labeled probes were synthesized by Beijing Institute of Microbiology, Chinese Academy of Sciences. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of General Hospital of PLA, Beijing. Written informed consent was obtained from all participants.

LKB1 gene expression

mRNA of LKB1 gene was detected by In situ hybridization that the probes hybridized with tissue sections (3-4 microns) of lung cancer and control groups and then detected by NBT-BCIP in dark environment. The positive cells were ones within cytoplasmic dark blue granules. The percentage of positive cells were counted and staining intensity were calculated by image analyzer.

Methylation-specific polymerase chain reaction (PCR)

With hydrogen sulfite salt-treated genomic DNA, all unmethylated cytosines are converted to uracil whereas methylated cytosine unchanged. If DNA treated by bisulfite can be amplified fragment with specific primers, it shows that specific sites methylated. Briefly, 50 ηg was taken from 1 μg DNA incubated with 1M bisulfite at 37°C for PCR. About 50 μL PCR product was subjected to 1.0% agarose gel electrophoresis. PCR reaction conditions were: 94°C for 1 min, 56°C for 60 s, 72°C for 70 s and repeated 20 cycles, the last cycle extended for 10 min.

Statistical analysis

All data are expressed as the mean ± S.E.M. (n = 4). Statistical analysis was performed by using Student's test or one-way ANOVA followed by Bonferroni test when applicable ($^{*}P < 0.05$ was considered significant and $^{**}P < 0.01$ was considered highly significant). Test level $\alpha = 0.05$.

Results

KB1 promoter aberrant methylation inhibiting the expression of LKB1 in lung cancer

In all 124 cases of lung cancer, 25 cases occurred methylation in the promoter CpG islands of LKB1. The methylation rate of lung cancer group was 20.16% while only 1 of 30 control cases was detected methylation in the CpG islands. The methylation rate of control group was only 6.67%. The difference of CpG island methylation in two groups was significant ($\chi^2 = 4.7311$, $P = 0.024$). LKB1 protein expressed in 80 of all 124 cases and LKB1 positive rate was 64.52% while all 30 benign controls expressed LKB1. The difference of LKB1 expressing rates in both groups was statistically significant ($\chi^2 = 14.9$, $P = 0.0006$). Only 7 of 80 lung cancer cases expressing LKB1 had CpG islands methylation in the promoter of LKB1 with 8.75% of methylation rate while 18 of 44 cases non-expressing LKB1 methylated in the promoter CpG islands with high 40.9% of methylation rate. The difference of methylation rates of both groups was clearly significant ($\chi^2 = 18.2388$, $P = 0.0006$) which showing the closing association of CpG islands methylation in the promoter of LKB1 with its

Table 1. The relationship of LKB1 promoter CpG island methylation with protein expression in lung cancer

LKB1 expression	CpG island methylation state		Total
	Methylation	Non-methylation	
Positive	7	73	80
Negative	18	26	44
Total	25	99	124

$\chi^2 = 18.2388$, $P = 0.0006$.

Table 2. The relationship of LKB1 promoter CpG island methylation with prognosis in lung cancer

Prognosis	LKB1 methylation state		Total
	Methylation	Non-methylation	
More than 5-year survival	5	59	64
Less than 5-year survival	20	40	60
Total	25	99	124

$\chi^2 = 12.5305$, $P = 0.005$.

Table 3. The relationship of CpG island methylation in LKB1 promoter with smoking

Smoking	LKB1 CpG island methylation		Total
	Methylation	Non-methylation	
Smoking	22	52	74
Non-smoking	3	47	50
Total	25	99	124

$\chi^2 = 10.4384$, $P = 0.001$.

expression (**Table 1**). LKB1 expressing rate was very low in the cases that CpG island methylated in the promoter of LKB1.

LKB1 promoter CpG island methylation present in early stage of lung cancer

54 peripheral blood samples were successfully obtained from the patients with the early and middle (I-II phase) lung cancer of 124 cases. 30 peripheral blood samples from lung inflammatory diseases were also obtained as control. LKB1 methylation analysis was conducted by the methylation-specific PCR with genomic DNA from those blood samples. The results showed that 8 of 54 lung cancer cases occurred LKB1 promoter CpG island methylation in peripheral blood and the methylation rate was 14.81% while no methylation was detected in the promoter CpG island of LKB1 of 30 lung inflammatory diseases. The difference was significant ($\chi^2 = 4.9123$, $P = 0.03$) which suggesting LKB1 promoter CpG island methylation occurred in the early stage of lung cancer.

LKB1 promoter CpG island methylation effect on prognosis of lung cancer

64 of 124 lung cancer cases had more than 5-year survival and only 5 of these 64 cases occurred CpG island methylation in the promoter of LKB1 while 20 of the remaining 60 cases with less than 5-year survival occurred methylation in the LKB1 promoter. The difference was significant ($\chi^2 = 12.5305$, $P = 0.005$) which suggesting the likely association of LKB1 promoter CpG island methylation with the survival of lung cancer patients, and CpG island methylation with a poor prognosis (**Table 2**).

LKB1 promoter CpG island methylation closely related to smoking

74 of 124 lung cancer cases had exact history of smoking and the smoking rate was 59.68%. 22 of 74 cases with smoking occurred CpG island methylation in the promoter of LKB1 and the methylation rate was 29.73%. Only 3 of the remaining 50 non-smoking lung cancer cases had CpG island methylation in the promoter of LKB1 and the methylation rate was 6%. The difference was significant compared of the CpG island in the promoter of LKB1 to smoking ($\chi^2 = 10.4384$, $P = 0.001$) (**Table 3**) which suggesting high CpG island methylation rate of smoking cases and closing association of smoking with CpG island methylation of LKB1 promoter.

Discussion

LKB1 gene is not only a causative gene of the autosomal dominant genetic disease Peutz-Jeghers syndrome (PJS) but also an important suppressor gene in lung cancer [11-14]. LKB1 mainly functions in inhibiting cell proliferation and inducing apoptosis through the G1 phase arrest [15]. Sanchez-Cespedes and his group first reported that a substantial proportion of functional LKB1 deletion mutants existed in non-small cell carcinoma [16]. Koivunen et al [17] reported in North America and East Asia with primary lung cancer LKB1 inactivation mutation rate is different from the North American people higher than Asians. Fernandez et al [10] examined 19 cases of primary lung

adenocarcinoma LKB1 gene mutations, including point mutations, deletions and promoter methylations. But in Asians especially in the North China region CpG islands methylation in the promoter of LKB1 in lung cancer has not been reported.

124 patients with primary lung cancer from north China were randomly chosen to detect LKB1 promoter CpG island methylation states. The results showed that 25 of all 124 lung cancer cases occurred CpG island methylation in the promoter of LKB1 and 20.61% of the methylation rate had significant difference compared to that of control which suggesting CpG island methylation of LKB1 likely common events in lung cancer. The results in situ hybridization showed that existence of the phenomenon of loss of LKB1 expression and LKB1 expression positive rate was 64.52%. Further, 7 of 80 LKB1 positive cases occurred CpG island methylation in the promoter of LKB1 and the methylation rate was 8.7% while 18 of 44 LKB1 negative cases had CpG island methylation and the methylation rate was highly 40.9%. LKB1 promoter CpG island methylation is closely related to the LKB1 protein expression. CpG island methylation is prone to silence of LKB1 expression. These results suggested that LKB1 promoter CpG island aberrant methylation is likely one of the mechanism leading to loss of expression of LKB1 in lung cancer.

In our results, LKB1 promoter CpG island methylation can be detected in the early to middle phase of lung cancer, suggesting that LKB1 promoter methylation is likely to be an early event in lung cancer, and LKB1 promoter CpG island methylation leading loss of LKB1 expression may contribute to the pathogenesis of lung cancer. LKB1 is likely to play an important role in the initial stages of lung cancer. We found that the percentage of LKB1 promoter CpG island methylation of cases with less than 5-year survival was significantly higher than that of cases with more than 5-year survival, suggesting a poor prognosis of lung cancer occurred CpG island methylation. These results suggest LKB1 promoter CpG island methylation plays an important role in the progression of lung cancer and LKB1 as suppressor gene functions indispensably in resisting tumorigenesis.

Furthermore, LKB1 promoter CpG island methylation in cases with smoking history occurred significantly higher than that of non-smokers, suggesting that smoking is closely related to LKB1 promoter CpG island methylation and smoking itself is likely important factor to lead to CpG island methylation of LKB1 promoter while the latter likely plays an important in the mechanism of smoking leading lung cancer. Large numbers of epidemiological studies have confirmed that smoking is the leading risk factor for lung cancer, about 87% of lung cancer deaths are caused by smoking [18-21]. However, the mechanism of lung cancer led by smoking is not clarified. More than 40 kinds of carcinogens produced by smoking may damage DNA of epithelial cell, activate oncogenes and inactivate tumor suppressor genes [22]. Particular gene methylation mechanism caused by smoking need to be further studied. The clarified clinical significance of LKB1 methylation mechanism is helpful to find inhibiting tumor drugs such as demethylating drug to effectively reverse the expressing silence of functional protein.

Acknowledgements

This paper were funded by the National Natural Science project of China (No. 81272088), and the Special Scientific Research Foundation of Health Sector from the National Health and Family Planning Commission of China (No. 201302016, No. 201302017) and PLA Medical Technology key project of scientific research in the 12th research projects in 12th Five-Year-Plan (No. BWS12J049).

Disclosure of conflict of interest

None.

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