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WT1 CpG islands methylation in human lung cancer: A pilot study

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ABSTRACT

Background: CpG island hypermethylation of gene promoters and regulatory regions is a well-known mechanism of epigenetic silencing of tumor suppressors and is directly linked to carcinogenesis. Wilm's tumor gene (WT1) is a tumor suppressor protein involved in the regulation of human cell growth and differentiation and a modulator of oncogenic K Ras signaling in lung cancer. Changes in the pattern of methylation of the WT1 gene have not yet been studied in detail in human lung cancer. In this study we compared the methylation profile of WT1 gene in samples of neoplastic and non-neoplastic lung tissue taken from the same patients.

Methods: DNA was extracted from neoplastic and normal lung tissue obtained from 16 patients with non small cell lung cancer (NSCLC). The methylation status of 29 CpG islands in the 5′ region of WT1 was determined by pyrosequencing. Statistical analysis was carried out by T test and Mann Whitney test.

Results: The mean percentage of methylation, considering all CpG islands of WT1 in the neoplastic tissues of the 16 NSCLC patients, was 16.2 ± 3.4, whereas in the normal lung tissue from the same patients it was 5.6 ± 1.7 ($p < 0.001$). Adenocarcinomas presented higher methylation levels than squamous cell carcinomas ($p < 0,001$).

Conclusions: Methylation of WT1 gene is significantly increased in NSCLC. Both histotype and exposure to cigarette smoke heavily influence the pattern of CpG islands which undergo hypermethylation.

1. Introduction

Lung cancer is the main cause of death from cancer in Western countries, accounting for 30% all cancer deaths. Its high mortality rate is mainly due to the lack of effective treatment in the advanced stages of the illness and to the lack of screening methods [1]. Recently, some progress has been made in the treatment of non small cell lung cancer (NSCLC) thanks to new drugs such as bevacizumab, erlotinib and gefitinib which however target only specific subgroups such as the non-squamous histotype and lung adenocarcinoma with mutations of the epidermal growth factor receptor (EGFR) [2,3].

CpG island hypermethylation (CpG islands: regions of DNA with a high G + C content and a high frequency of CpG dinucleotides relative to the bulk genome) of the promoters and regulatory regions of the genes that modulate cell growth is a well-known mechanism of epigenetic alteration that affects the function of genes involved in carcinogenesis [4].

Recently, light has been shed on the role of methylation in the functional silencing of oncosuppressors in lung cancer, by investigating several genes such as *p16*, *RASSF1A*, *RARbeta*, *MGMT*, *GSTP1*, *CDH13*, *APC*, *DAPK*, *TIMP3*, *BHLHB4*. In the various studies frequency of methylation (percentage of analyzed tumors presenting methylated alleles) ranged between 1% and 80% approximately [5–12,24–26].

Wilm's tumor gene (WT1), mapping to chromosome 11p13, encodes a transcription factor involved in the regulation of human cell growth and differentiation [13]. Such locus is frequently deleted in patients with Wilm's tumor [14], however its alterations have been recognized also in cancer cell lines of the stomach,

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Table 1
Patients characteristics.

Pts	16
M/F	8/8
Age	66 ± 8
Smokers	6
Ex smokers > 2 aa	4
Never smokers	6
Histology	
Adenocarcinomas	8
Large cells	2
Squamous	6
Stage	
I	10
II	4
III	2

colon, breast, liver and lung [15]. WT1 was identified to be a modulator of oncogenic K Ras signaling in lung cancer; in both mouse and human lung cells WT1 regulates the proliferative potential of oncogenic K Ras and loss of WT1 drives cells expressing oncogenic K Ras toward a senescence program [16].

The aim of this pilot study was to compare the methylation profile of WT1 in samples of neoplastic and non-neoplastic lung tissue taken from the same patients, in order to establish whether there is a relationship between the methylation features observed and the patient's histology, age, smoking habit.

2. Materials and methods

2.1. Patients

In the first part of this study we analyzed lung tissue samples obtained from 16 patients with NSCLC; a portion of neoplastic lung and a portion of lung without tumor have been histologically selected in each patient. The patients' characteristics with NSCLC have been summarized in Table 1: eight men and eight women, mean age 66 ± 8 years, of which six were smokers, four ex-smokers (>2 years), six non-smokers. The histological findings revealed eight adenocarcinomas and six squamous cell carcinomas, two large cells, 10 at stage I, four at stage II and two at stage III.

The patients' informed consent was recorded and further studies were conducted according to ethical guidelines of Sant'Andrea Hospital.

2.2. Methods

WT1 methylation profile in non small cell lung cancer (NSCLC) was investigated by studying the methylation status of 29 CpG islands in the 5′ region of the gene by means of pyrosequencing.

2.3. CpG island analysis and primer design

PCR assay was designed to amplify a part of the CpG islands in the 5′ region of WT1 gene (NCBI Reference Sequence on chromosome 11: NG_009272.1). Primers targeted CpG-free regions to ensure that the PCR product would proportionally represent the methylation characteristics of the source DNA. Pyrosequencing primers were designed using PSQ Assay Design (Biotage AB, Charlotte, NC) and focused on 29 CpG dinucleotides located between positions +481 and +760 from the transcription start of WT1 gene. A fragment of 323 nucleotides was amplified using: 5′-GGTTGTGTTTTGTTTGTGA-3′ as forward primer and biotin - 5′-TTAAAAAACATCCTAACCTA-3′ as reverse primer. The forward primer was also used as sequencing primer for methylation analysis of the first 23 CpG sites. An additional sequencing primer (5′-GTTTTATTTTTTTATTAAATAG-3′) allowed the analysis of further six CpG islands.

2.4. Pyrosequencing methylation analysis

Genomic DNA was isolated from biopsies using the QIAamp DNA Mini kit (Qiagen, Valencia, CA). Bisulfite modification was performed with 300–500 ng of DNA using the EZ DNA Methylation Gold kit (Zymo Research, Orange, CA). Three microliters of bisulfite-modified DNA was used as a template for the PCR amplification of WT1 fragment. PCR was performed in 25 microliters of 1.5 mM MgCl2, 1× PCR buffer, 200 μM dNTPs and 1 U Taq Polymerase (TaKaRa Ex Taq, TaKaRa Bio INC. Otsu, Japan).

The following cycling conditions were used: an initial denaturing step of 15 min at 95 °C; 45 cycles of 20 s at 95 °C, 20 s at 48 °C and 20 s at 72 °C; a final elongation step of 10 min at 72 °C.

After confirmation of successful PCR amplification by 1.5% agarose gel electrophoresis, the amplicon was sequenced using the Pyrosequencer PyroMark ID system (Biotage AB and Biosystems, Uppsala, Sweden) according to instrument's protocol. In details, singlestranded DNA was isolated from the PCR reaction using the Pyrosequencing Vacuum Prep Workstation (Biotage) and Streptavidin Sepharose TM High Performance beads (Amersham Biosciences) that bind to the biotinylated primer. After washing in 70% ethanol, incubation in denaturation buffer and flushing with wash buffer, the beads were then released into a 96-well plate containing annealing buffer and the specific sequencing primer (Diatech, Italy). Annealing was performed at 80 °C for 2 min followed by room temperature. Then real-time sequencing was performed.

Methylation level of target CpGs was evaluated by Pyro Q-CpG Software (Biotage AB and Biosystems, Uppsala, Sweden). Each sample was run in duplicate.

2.5. Statistical analysis

For the comparison of methylation in healthy and in neoplastic tissues, the T test was used to determine the significance of the differences between the means, where distributions were normal;

Table 2
Means and standard deviation of the 29 CpG islands methylation of WT1 gene studied in 16 patients with NSCLC.

CpG islands	Control% methilation	SD	Tumor% methylation	SD
1	5.08	2.9	16.46	9.99
2	3.04	3.25	12.38	8.87
3	7.23	2.19	16.89	7.96
4	8.01	2.52	19.7	10.14
5	3.66	3.04	10	7.91
6	3.75	3.55	16.91	8.35
7	8.69	3.19	20.21	15.23
8	4.05	3.66	15	8.6
9	4.7	2.66	11.44	5.14
10	6.01	3.87	22.91	13.17
11	3.65	3.51	13.44	8.73
12	2.66	3.12	16.95	10.27
13	6.7	2.65	14.39	7.31
14	8.78	4.6	19.39	13.08
15	4.56	3.27	11.42	7.56
16	6.39	4.49	19.75	11.10
17	7.14	4.53	22.56	12.13
18	9.14	3.44	23.47	13.78
19	6.91	3.57	17.9	10.44
20	4.48	4.95	14.09	8.69
21	6.04	5.35	15.6	9.39
22	4.67	4.22	16.82	10.2
23	5.63	4.98	14.4	9
24	5.55	3.85	15.43	8.39
25	7.46	4.23	16.19	9.2
26	4.78	3.52	14.21	7.5
27	4.09	3.57	13.45	9.66
28	6.40	4.6	16.03	8.92
29	4.43	4.16	12.34	9.23