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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.

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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

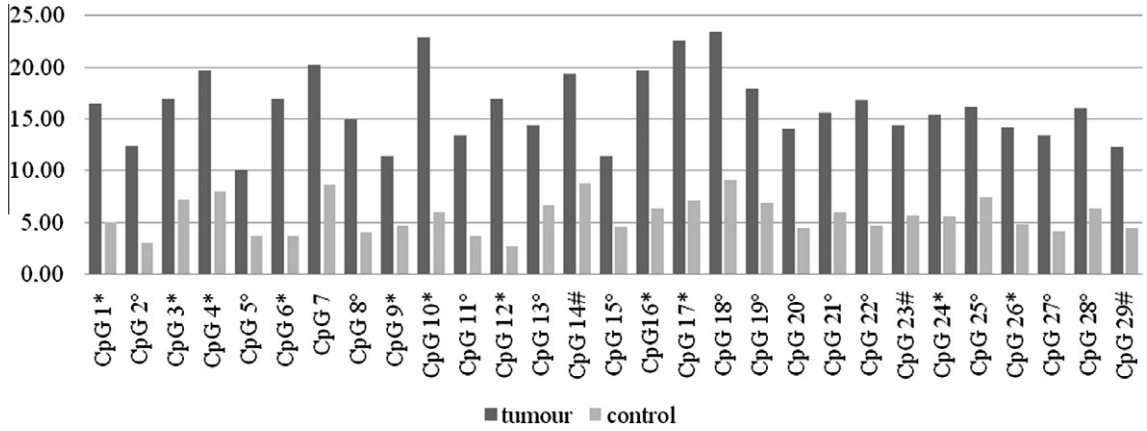

Fig. 1. Mean percentage of WT1 CpG island methylation in 16 NSCLC pts.

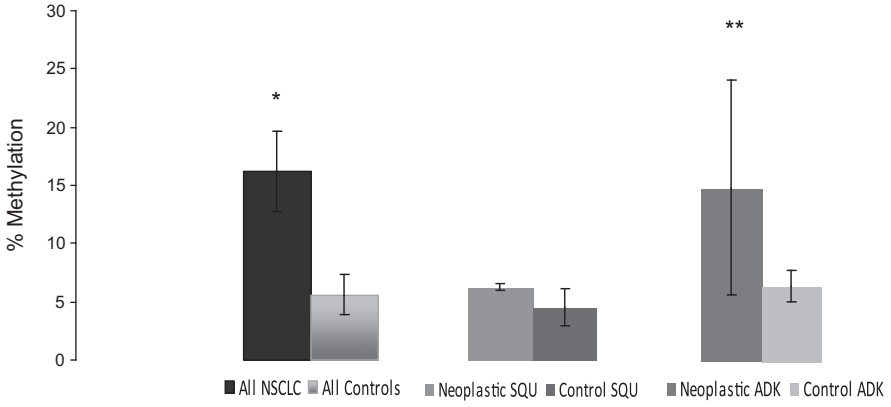

Fig. 2. WT1 methylation in all NSCLC patients and in the adenocarcinoma (8 pts) and epidermoidal (6 pts) sub-groups.

otherwise the Mann Whitney test was used. The same approach was applied as regards the relationships between methylation and patients' characteristics. The data have been processed with SPSS software, version 17.0 (SPSS, Chicago, IL, USA).

3. Results

The means and standard deviations of the 29 analyzed CpG islands of WT1 promoter and the comparison between healthy and neoplastic tissue of NSCLC are reported in Table 2. The mean percentage of methylation, considering all CpG islands of WT1 in the neoplastic tissues of all NSCLC patients, was 16.2 ± 3.4, whereas in the control tissue from the same patients it was 5.6 ± 1.7 ($p < 0.001$) (Fig. 2).

Fig. 1 summarizes the comparison between the percentage of methylation in the control and in the neoplastic tissue of NSCLC patients, analyzed for the individual CpG islands of WT1; the difference is statistically significant: in particular, for positions 1,3,4,6,9,10,12,16,17,24,26 ($p < 0.001$), for positions 2,5,8,11,13, 15,18,19,20,21,22,25,27,28 ($p < 0.01$) and for positions 14,23,29 ($p < 0.05$) respectively.

Methylation in normal lung tissues showed no correlation with smoking history, age, stage and chemotherapy. When we analyzed methylation in the cancer tissue samples with respect to the characteristics of NSCLC patients the results were as follows: the analysis of mean dependence between CpG island methylation and histotype showed that adenocarcinomas present grater methylation *vs* squamous cell carcinomas in positions: 4,24,25,26,28 ($p < 0{,}001$), 1,2,3,6,7,8,9,10,12,13,14,15,16,17,18,19,21,29 ($p < 0{,}01$) and 11,22,27 ($p < 0{,}05$) respectively; if we consider only the squamous cell carcinomas, there is no statistically significant difference in methylation between neoplastic and healthy tissue (Fig. 2). Large cell histotype was not considered for statistical analysis because we enrolled only two patients with this pathology.

In NSCLC a statistically negative correlation was found between packs/year and CpG islands methylation at 1,3,4,9,10, 11,12,15,16,17,18,19,21,22,23 ($p < 0.01$) and at 2,6,7,13,14,27 sites ($p < 0.05$); this means that, as the smoking habit increases, the percent methylation of the above mentioned CpG islands decreases.

NSCLC non-smoker patients present greater methylation of CpG islands 2,12,15,17,21,23 ($p < 0{,}01$) and 1,3,4,8,9,11,16,18,19,21,23, 27 ($p < 0{,}05$) *vs.* smoker patients. No statistically significant correlation was found between CpG island methylation of WT1 and the age of patients and stage of tumor in NSCLC.

4. Discussion

Methylation of cytosines in regulatory regions is an epigenetic change that profoundly affects gene expression [4]. In this paper we have analyzed WT1 methylation in lung cancer samples *vs* samples of non tumor lung tissue from the same patients. The WT1 gene encodes a protein that regulates the expression of

several factors involved in tumor growth [17]; recently, WT1 has been considered as the target of vaccines aiming at improving the body immune response to neoplasia [18]. In NSCLC the difference in the methylation profile of neoplastic tissue *vs.* control lung tissue is statistically significant and this supports the hypothesis that WT1 methylation could be involved in the carcinogenesis of NSCLC.

In our case survey, albeit carried out on a small number of patients, WT1 hypermethylation was detected in all cases analyzed. However, the degree of methylation in the various loci analyzed varied according to the patient's smoking habit and to the histological type; in fact adenocarcinomas presented greater methylation *vs.* squamous cell carcinomas, and never smokers showed greater methylation of WT1 CpG islands than smokers, which confirms the hypothesis that we are facing different diseases with different pathogeneic mechanisms [19–23]. This information may be used to single out the most effective treatment, based on the factors to which the patient has been exposed and on his/her WT1 methylation profile. We have not examined more closely the correlation between WT1 methylation and the patients' follow-up, because the NSCLC patients were at an initial stage of the illness and surgical treatment offered a good prognosis. At the time of the drafting of this paper, the follow-up has lasted 1 year and no patient has been relapsing either at the same site or elsewhere.

Based on the preliminary observations above reported, we can conclude that hypermethylation of WT1 gene could be involved in the carcinogenesis of NSCLC, and in particular the most frequent form, i.e. adenocarcinomas; histotype, exposure to cigarette smoke are all factors influencing the gene CpG islands which become hypermethylated in NSCLC. Thus, the WT1 methylation profile could be used as a criterion for classification of different forms of NSCLC presenting different clinical outcome and response to therapy.

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