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IGFBP7 is a p53 target gene inactivated in human lung cancer by DNA hypermethylation

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ABSTRACT

Insulin-like growth factor binding protein 7 (IGFBP7) was considered a tumor suppressor gene in lung cancer. However, the mechanism responsible for the downregulation of this gene has not yet been fully understood. In this study, we analyzed the epigenetic inactivation of IGFBP7 expression in human lung cancer. We found that 14 out of 16 lung cancer cell lines showed decreased expression of IGFBP7 compared to control cells by real-time RT-PCR, and 42 out of 90 patients (46.7%) with primary lung tumor exhibited negative staining of IGFBP7 by immunohistochemistry analysis. The IGFBP7 expression could be restored by demethylation agent 5-aza-2′-deoxycytidine (DAC) in 7 cancer cell lines. Methylation status of IGFBP7 was further evaluated by bisulfite sequencing (BS) and methylation-specific-PCR (MSP). It turned out that low expression of IGFBP7 was associated with DNA methylation in lung cancer cell lines and in primary lung tumors ($P = 0.019$). To explore the regulatory role of p53 on IGFBP7, we transfected a wild type p53 expression vector into lung cancer cell lines H1299, H2228, and H82. Forced expression of p53 increased IGFBP7 expression only in H82 harboring no IGFBP7 methylation, while transfection in combination with DAC induced the expression of IGFBP7 in H1299 and H2228, in which IGFBP7 was methylated. Additionally, treatment with p53 inducer adriamycin (ADR) alone or in combination with DAC increased the expression of IGFBP7 in the 3 cell lines. Our data suggest that IGFBP7 is inactivated in lung cancer by DNA hypermethylation in both lung cancer cell lines and primary lung tumors, and IGFBP7 might be regulated by p53 in lung cancer cells.

1. Introduction

Lung cancer is a predominant cause of cancer death in both men and women worldwide [1]. Despite the major progress made in cancer treatment during the last decades, currently still more than 85% of all cases of lung cancer diagnosed in Europe die within 5 years, a proportion that compares poorly with the mortality of breast or colon cancer [2]. Improving the survival rate of patients with this disease requires a better understanding of tumor biology, pathogenesis, and the ultimate development of novel therapeutic strategies.

Lung carcinogenesis, like development of other cancers, is a multistage process involving alteration in multiple genes and diverse pathways. Epigenetic alteration like aberrant methylation of CpG islands in promoter region has been frequently detected and is associated with transcriptional inhibition of certain genes in various cancers including lung cancer. For instance, cohypermethylation of p16 and FHIT promoters was a prognostic factor of recurrence in surgically resected stage I non-small cell lung cancer [3]. Epigenetic inactivation of the RAS-effector gene RASSF2 was frequently found in lung cancer [4]. Downregulation of the ARLTS1 gene could be explained by DNA methylation in its promoter region [5]. In a previous study, we characterized insulin-like growth factor binding protein 7 (IGFBP7) as a potential tumor suppressor, since forced expression of IGFBP7 inhibited cell proliferation, induced apoptosis, and reduced tumor formation in nude mice [6].

IGFBP7 belongs to the IGFBP superfamily, a large group of secreted proteins. A total of 16 IGFBP family members have been identified, 6 of which bind IGFs with a high affinity (IGFBP1–6), and the other 10 members including IGFBP7 bind to IGFs with a low affinity [7]. IGFBP7 has been shown to regulate cell proliferation, cell adhesion, cellular senescence, differentiation, and angiogenesis in various cancer cells, and it functions as a tumor suppressor gene in numerous cancers [7–11].

IGFBP7 expression could be induced by retinoic acid [12], glucocorticoids [13], and TGF-beta [14]. Recently, it has been reported that expression of BRAF oncogene (BRAFV600E) in primary fibroblasts and melanocytes led to synthesis and secretion of IGFBP7 which acted through autocrine/paracrine pathways to inhibit BRAF–MEK–ERK signalling [15], and recombinant protein of IGFBP7 markedly suppressed the growth of BRAF-positive primary tumors in xenografted mice cancer [16]. Most recently, IGFBP7 was

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identified as a downstream direct target gene of p53 in colon cancer cells by silico analysis using p53 response elements (p53REs) as probe, together with chromatin immunoprecipitation (ChIP), and luciferase assays cancer [17]. This result raised the possibility that IGFBP7 could be a mediator of p53-dependent growth suppression in this type of cancer.

In this study, we extended the methylation analysis of IGFBP7 in a panel of lung cancer cell lines and in primary lung tumors. Additionally, the regulatory role of p53 on IGFBP7 was investigated in lung cancer cells.

2. Materials and methods

2.1. Cell lines and cell culture

Human bronchial epithelia cells (HBECs) and human small airway epithelial cells (SAEC) were purchased from Clonetics (San Diego, CA) and cultured in BEG or SAB media (Clonetics) until population doubling of maximal 10. Human lung carcinoma cell lines, including small cell lung carcinomas (SCLCs: COLO677, SHP77, CPC-N, COLO668, H82, and H526) and non-small cell lung carcinomas (NSCLCs: H2170, H125, H2030, H2228, BEN, H226, and H23), were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany). One NSCLC cell line H1299 was kindly provided by Dr. Bastians (Marburg, Germany), and additional 2 NSCLC cell lines, D51 and D54, were established in our lab. They were derived from primary tumors of patients who were operated at the Charité University Hospital. These cells were grown in Leibovitz 15 media supplemented with 10% FCS and 1% glutamine.

To analyze restoration of IGFBP7 gene expression, cell line H2228, COLO668, H23, H226, H1299, H2170, and SHP77 were treated with 10 μM 5-aza-2′-deoxycytidine (DAC) (Sigma–Aldrich) for 96 h, replacing the drug and medium every 48 h. To determine whether IGFBP7 could be upregulated by p53 inducer adriamycin (ADR), lung cancer cell line H1299 and H2228 and H82 were treated with 1 μM DAC for 48 h, then ADR was added to a final concentration of 0.5 mg/ml, and the cells were incubated for an additional 24 h. After incubation, the treated cells were harvested for total RNA isolation.

2.2. Real-time RT-PCR

Total RNA was prepared by using Trizol (Invitrogen), and then incubated with RQ DNase I (Promega) for 40 min at 37 °C to eliminate genomic DNA contamination before cDNA reverse-transcription.

First-strand cDNA was reverse-transcribed by 15 U ThermoScript RT (Invitrogen) from 1 μg of total RNA, in the presence of 1× RT buffer, 100 mM DTT, 20 U RNase inhibitor, and 10 mM dNTPs, using the random hexamer primers supplied in the kit. Real-time PCR was performed in 0.1 ml tubes on the Rotor-Gene 6000 (Qiagen) in the presence of the FastStart universal SYBR green Master (Roche). Twenty-five ng of RNA was used for PCR amplification of IGFBP7 with the primer sequence: forward: 5′-CACTGGTGCCCAGGTGTACT-3′ and reverse: 5′-TTGGATGCATGGCACTCACA-3′. Controls were performed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer: 5′-TCAAGGGCATCCTGGGCTACA-3′ (sense) and 5′-CCAGCCCC-AGCGTCAAAGGT-3′ (antisense). The relative expression value of IGFBP7 to GAPDH in each sample was calculated and compared.

2.3. Transient transfection

Three lung cancer cell lines H1299, H2228, and H82 were seeded at a density of 0.5×10^5, 2×10^5, and 6×10^5, respectively, in a 12-well dish, cultured for 24 h, and transfected with 1.6 μg of pcDNA3.1/p53 (gift from Dr. Liu, Oxford, England) and 4 μl of lipofectamin 2000 (Invitrogen) according to the manufacturer's recommendation. Empty vector was transfected as control. For the cell line H1299 and H2228, cells were exposed to DAC with a concentration of 5 μM on day 0 and day 2 before transfection. Transfection efficiency was assessed under a fluorescent microscope after cells were transfected with a pEGFP vector (gift from Dr. Liu). Forty-eight hours after transfection, total RNA was isolated for the real-time PCR analysis. All experiments were performed in duplicate and repeated three times.

Table 1
Study cohort.

Total no (n = 90)	IGFBP7 (M) 46 (51%)	IGFBP7 (U) 44 (49%)	P
Protein (−)	27 (30.0%)	15 (16.7%)	**0.019***
Protein (+)	19 (21.1%)	29 (32.2%)	
≤62	23 (26.4%)	23 (26.4%)	0.91
>62	20 (23.0%)	21 (24.2%)	
SCC	21 (23.3%)	18 (20.0%)	0.674
ADC	25 (27.8%)	26 (28.9)	
Grade 0–2	22 (26.2%)	26 (31%)	0.705
Grade 3	18 (21.4%)	18 (21.4%)	
pT1	9 (10.6%)	9 (10.6%)	0.955
pT2+	33 (38.8%)	34 (40%)	
pN0	21 (25%)	25 (29.8%)	0.524
pN1+	20 (23.8%)	18 (21.4%)	

ADC: adenocarcinoma; SCC: squamous cell carcinoma; pT2+: pT2–pT4; pN1+: pN1–pN3; M: methylated; and U: unmethylated.
* Statistically significant.

2.4. Tumor tissues and genomic DNA isolation

In total, 90 tumor specimens from patients with lung cancer (51 adenocarcinoma and 39 squamous cell carcinoma) were included for this study (Table 1). All of these patients were undergoing surgical operation of lung cancer at the Department of Surgery of Charité University Hospital from 1995 to 2000. No adjuvant radiotherapy or chemotherapy was administered before surgery. The study was approved by the local ethical committee.

Genomic DNA was isolated from the 90 tumor samples by using a QiAamp DNA mini kit (Qiagen) according to the manufacturer's instruction. In order to minimize the contamination of normal fibroblast and stromal cells, microdissection was performed before DNA isolation.

2.5. Bisulfite sequencing

Bisulfite treatment was carried out by using an EZ DNA Methylation kit (Zymo Research) according to the manufacturer's guide. After bisulfite treatment, unmethylated cytosine residues were converted to uracil, whereas methylated cytosine residues remain unchanged. The bisulfite modified genomic DNA was applied for bisulfite sequencing and/or methylation-specific-PCR (MSP). HBEC were used as control.

Two pairs of primer from the exon/intron 1 and intron 1 of the IGFBP7 gene were designed to amplify both bisulfite modified methylated and unmethylated DNA but not unmodified DNA. One of the target-fragment of 186-bp (+464 to + 649), spanning 19 CpG dinucleotides within exon/intron 1, was amplified by using the primer pair: 5′-GGGGAGAAGGTTATTATTTAGGTTAGTAA-3′ (sense) and 5′-CCCTCCCATCTAACTCCTAAAATAC-3′ (antisense). The other target-fragment of 192-bp (+1501 to +1693) near the putative p53 binding site, spanning 5 CpG sites within intron 1, was amplified by the primer pair: 5′-GTTTGTTGTAAATGAAATAAGGAAATAGGT-3′