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ZEB1-responsive genes in non-small cell lung cancer

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ABSTRACT

The epithelial to mesenchymal transition (EMT) is a developmental process enabling epithelial cells to gain a migratory mesenchymal phenotype. In cancer, this process contributes to metastases; however the regulatory signals and mechanistic details are not fully elucidated. Here, we sought to identify the subset of genes regulated in lung cancer by ZEB1, an E-box transcriptional repressor known to induce EMT. Using an Affymetrix-based expression database of 38 non-small cell lung cancer (NSCLC) cell lines, we identified 324 genes that correlated negatively with ZEB1 and 142 that were positively correlated. A mesenchymal gene pattern (low E-cadherin, high Vimentin or N-cadherin) was significantly associated with ZEB1 and ZEB2, but not with Snail, Slug, Twist1 or Twist2. Among eight genes selected for validation, seven were confirmed to correlate with ZEB1 by quantitative real-time RT-PCR in a series of 22 NSCLC cell lines, either negatively (CDS1, EpCAM, ESRP1, ESRP2, ST14) or positively (FGFR1, Vimentin). In addition, over-expression or knockdown of ZEB1 led to corresponding changes in gene expression, demonstrating that these genes are also regulated by ZEB1, either directly or indirectly. Of note, the combined knockdown of ZEB1 and ZEB2 led to apparent synergistic responses in gene expression. Furthermore, these responses were not restricted to artificial settings, since most genes were similarly regulated during a physiologic induction of EMT by TGF-β plus EGF. Finally, the absence of ST14 (matriptase) was linked to ZEB1 positivity in lung cancer tissue microarrays, implying that the regulation observed *in vitro* applies to the human disease. In summary, this study identifies a new set of ZEB-regulated genes in human lung cancer cells and supports the hypothesis that ZEB1 and ZEB2 are key regulators of the EMT process in this disease.

1. Introduction

According to data from the World Health Organization, lung cancer accounts for 1.3 million deaths per year, or 18% of the total cancer deaths worldwide. The lung cancer mortality age-adjusted standardized rates increased rapidly from 1960 but then reached a plateau in 1990. Subsequently, lung cancer mortality has declined, although not in all countries and there has been little reduction in women [1]. Approximately half of patients undergoing complete surgical resection for low-stage lung cancer die from distant metastases. Therefore, targeting mechanisms that underlie the metastatic process might positively impact the outcome of patients with this disease.

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The epithelial to mesenchymal transition (EMT), which converts epithelial cells into an elongated, motile and invasive phenotype, is thought to be a critical step in the dissemination of tumor cells [2–6]. Among hundreds of genes affected by EMT, the loss of E-cadherin and upregulation of Vimentin or N-cadherin have been most frequently described. Other frequent changes include the loss of cytokeratins, increased MMP activity and increased fibronectin. In epithelial cancers, EMT has been associated with resistance to therapy, the acquisition of tumor-initiating cell properties, and increased cell migration and invasion, all of which are associated with a poor prognosis [3,5].

By immunostaining, severe E-cadherin loss occurs in approximately 10% of resected NSCLCs [7]. Using a low E-cadherin/high N-cadherin ratio and other markers including integrin αvβ6 and MMP-9, Prudkin et al. [8] concluded that a majority of lung adenocarcinomas and squamous cancers had evidence of EMT. These and other data suggest that EMT reflects a continuum with its assessment dependent on the markers used. Although E-cadherin loss is a major feature of EMT, this has often been considered a late event in tumor progression. E-cadherin loss can also be transient or occur preferentially at the tumor periphery [5]. Thus, a better understanding of the spectrum of EMT phenotypes and its regulation is indicated.

In part, EMT is mediated by transcription factors including ZEB [9], Snail [10], Slug, Twist [11] and E12/E47 that bind E-box elements (i.e., CANNTG) in genomic DNA [2]. In lung cancer, ZEB1 appears to be a major factor in the EMT process. In NSCLC cell lines, we previously found that loss of E-cadherin was inversely and specifically correlated with ZEB1 mRNA expression [12]. Takeyama et al. [13] recently confirmed these findings and demonstrated that ZEB1 positively influences anchorage-independent growth. In addition, we reported that the tumor suppressor gene, *SEMA3F*, was specifically repressed by ZEB1 in NSCLC cell lines [14], providing additional support for the importance of this factor in lung cancer.

In the present report, we aimed to identify genes that responded to ZEB1 in lung cancer cell lines. With Affymetrix data from 38 NSCLCs, we ranked genes depending on the correlation between their expression and that of ZEB1. A subset of genes was examined in more detail and their correlation and responsiveness to ZEB1 was validated. We also verified that the same variations in gene expression were obtained after treatment with TGF-β plus EGF to induce EMT. In addition, the expression of ST14, coding for matriptase, was compared to that of E-cadherin and ZEB1 in a tissue microarray (TMA) of lung cancers.

2. Materials and methods

2.1. Cell lines and primary tumor tissues

NSCLC cell lines were obtained from the Colorado Lung Cancer SPORE Cell Line Repository. They were grown in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics (InVitrogen, Inc., Carlsbad, CA). Non-immortalized normal human broncho-epithelial cells (NHBE) were grown in BEBM supplemented with cytokines and growth factors according to manufacturer's instructions (Lonza, Basel, Switzerland). The FC6625-2 3KT cell line, kindly provided by Dr. John Minna, was derived from human broncho-epithelial cells immortalized by telomerase. BEAS2B cells were derived from SV40-immortalized broncho-epithelia. FC6625-2 3KT and BEAS2B cells were also grown in BEBM supplemented with cytokines and growth factors. H358 FlpIn cells were engineered to express six Myc-tagged ZEB1 after induction with doxycycline [14]. TMA slides containing 109 lung tumor samples and 10 normal lung tissues were obtained from US Biomax (Rockville, MD, ref: BC041115).

2.2. RNA isolation and quantitative real-time RT-PCR analysis

Total RNA was extracted from cell lines using the RNeasy Plus Isolation kit (Qiagen, Inc., Valencia, CA). RT-PCR was performed with the Superscript III reverse transcriptase (InVitrogen, Inc.) using the procedure supplied by the manufacturer. mRNA levels were measured by quantitative real-time PCR using the GeneAmp 7500 quantitative PCR system with SYBR-Green chemistry (Applied Biosystems, Foster City, CA). Intron-spanning gene-specific PCR primers were designed to avoid genomic DNA amplification and the PCR products were confirmed by DNA sequencing (not shown). The primers are described in Supplementary Table S1. The results are displayed in terms of the relative expression (100×) compared to GAPDH or actin expression. RT-PCR was done in duplicate.

2.3. Western blot analysis

Protein lysates and SDS/PAGE were performed using standard techniques, as previously described [15]. Primary antibodies were incubated overnight at 4 °C. These included rabbit anti-ZEB1 (1:1000; clone H-102; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti E-cadherin (1:1000; Cell Signaling Technology, Inc., Danvers, MA), rabbit anti-ST14 (1:1000; Bethyl Laboratories, Inc., Montgomery, TX), mouse anti-EpCAM (1:1000; clone VU-1D9, ThermoFisher Scientific, Inc., Fremont, CA), rabbit anti-N-cadherin (1:1000; Abcam, Inc., Cambridge, MA), rabbit anti-Vimentin (1:1000; Cell Signaling Technology, Inc.), mouse anti-CDS1 (1:1000, clone 2D10; Novus Biologicals, Inc., Littleton, CO) and mouse anti-β-actin (1:20,000; Sigma–Aldrich, Inc., St. Louis, MO). Secondary antibodies were goat anti-mouse HRP or goat anti-rabbit HRP (1:5000; Perkin Elmer, Inc., Waltham, MA) with ECL (Perkin Elmer, Inc.) detection. For β-actin and E-cadherin, the filters were incubated at 4 °C with Alexa 488-conjugated chicken anti-mouse secondary antibodies (1:1000; InVitrogen, Inc.) and detected using a Typhoon 9400 Image system (GE Healthcare Bio-sciences Corp., Piscataway, NJ).

2.4. Transfection of short-interfering RNA

Pre-validated siRNAs (InVitrogen, Inc., cat# HSS110549 and HSS190654) were used to inhibit endogenous ZEB1 and ZEB2, respectively, as previously reported [15]. Transfections were performed using HiPerFect according to manufacturer's recommendations (Qiagen, Inc.). Cells were