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Re-expression of *CXCL14*, a common target for epigenetic silencing in lung cancer, induces tumor necrosis

Mathewos Tessema[1], **Donna M. Klinge**[1], **Christin M. Yingling**[1], **Kieu Do**[1], **Leander Van Neste**[2], and **Steven A. Belinsky**[1]

[1]Lung Cancer Program, Lovelace Respiratory Research Institute, Albuquerque, NM 87108

[2]Department of Molecular Biotechnology, Faculty of Biosciences Engineering, Ghent University Ghent, Belgium

Abstract

Chemokines are important regulators of directional cell migration and tumor metastasis. A genome-wide transcriptome array designed to uncover novel genes silenced by methylation in lung cancer identified the CXC-subfamily of chemokines. Expression of eleven of the sixteen known human CXC-chemokines was increased in lung adenocarcinoma cell lines after treatment with 5-aza-2deoxycytidine (DAC). Tumor-specific methylation leading to silencing of *CXCL5*, *12* and *14* was found in over 75% of primary lung adenocarcinomas and DAC treatment restored expression of each silenced gene. Forced expression of *CXCL14* in H23 cells where this gene is silenced by methylation increased cell death *in vitro* and dramatically reduced *in vivo* growth of lung tumor xenografts through necrosis of up to 90% of the tumor mass. CXCL14 re-expression had a profound effect on the genome altering the transcription of over 1,000 genes, including increased expression of 30 cell cycle inhibitor and pro-apoptosis genes. In addition, *CXCL14* methylation in sputum from asymptomatic early stage lung cancer cases was associated with a 2.9-fold elevated risk for this disease compared to controls, substantiating its potential as a biomarker for early detection of lung cancer. Together these findings identify *CXCL14* as an important tumor suppressor gene epigenetically silenced during lung carcinogenesis.

Keywords

CXCL14; Chemokines; lung cancer; DNA methylation; CXCL5; CXCL12

Correspondence should be addressed to S. A. Belinsky, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr. SE, Albuquerque, NM 87108, Phone: 505-348-9465, Fax: 505-348-4990, sbelinsk@LRRI.org.

Conflict of interest

S.A. Belinsky is a consultant to Oncomethylome Sciences. Under a licensing agreement between Lovelace Respiratory Research Institute and Oncomethylome Sciences, nested MSP was licensed to Oncomethylome Sciences, and the author is entitled to a share of the royalties received by the Institute from sales of the licensed technology. The Institute, in accordance with its conflict-of-interest policies, is managing the terms of these arrangements.

Introduction

Aberrant DNA methylation has been established as one of the major mechanisms by which tumor suppressor genes are silenced in cancer (Baylin and Ohm, 2006). Several genome-wide methylation assays have identified a large number of abnormal gene methylation in various malignancies (Bennett *et al.*, 2008; Jacinto *et al.*, 2007; Kim *et al.*, 2006; Meissner and Jaenisch, 2006; Shames *et al.*, 2006). A genome-wide transcriptome based approach that can identify novel genes silenced by methylation in cancer has been developed (Schuebel et al., 2007). This approach relies on the differential response of densely methylated promoters to the demethylating agent DAC as compared to the histone deacetylase inhibitor trichostatin A (TSA). Using this approach we interrogated six lung tumor-derived cell lines and identified the CXC subfamily of chemokines as potential candidates for epigenetic silencing.

Chemokines are a superfamily of small chemotactic cytokines that direct the migration of leukocytes (Moser and Loetscher, 2001). In addition, they regulate cellular processes such as proliferation, migration, angiogenesis, and tumor related immunity (Muller *et al.*, 2001; Shellenberger *et al.*, 2004; Shurin *et al.*, 2005; Strieter *et al.*, 2004). Chemokines are classified into four subfamilies: C, CC, CXC, and CX_3C chemokines based on the location of conserved cysteine residues (Strieter et al., 2004). The CXC subfamily in humans consists of sixteen members (CXCL1-14, 16, and 17) that are important regulators of tumor angiogenesis, immunity, and tissue-specific cancer metastasis (Darash-Yahana et al., 2009; Hromas et al., 1999; Mu et al., 2009; Strieter et al., 2004; Yuvaraj et al., 2009). CXC chemokines share four cysteine residues in a highly conserved location that determine the 3-dimensional structure of these heparin-binding proteins. A glutamate-leucine-arginine (ELR) motif near the NH2 terminus of the molecule determines the property of a specific chemokine. The ELR-positive chemokines, including CXCL1-3, 5-8, are proangiogenic, whereas members lacking the ELR motif such as CXCL4, 9-11 are interferon-inducible and are potential inhibitors of angiogenesis (Strieter et al., 2004).

CXCL14, also known as BRAK, is an ELR-negative chemokine abundantly expressed in most normal tissue including lung (Kurth et al., 2001; Ozawa et al., 2006; Parsanejad et al., 2008; Schwarze et al., 2005; Shurin et al., 2005). In contrast, the majority of established epithelial cancer cell lines and many primary carcinomas do not express CXCL14 suggesting a tumor suppressor function (McKinnon et al., 2008; Ozawa et al., 2006; Shellenberger et al., 2004; Shurin et al., 2005). Interestingly, CXCL14 expression was suppressed by epidermal growth factor (EGF) and restored by a EGF receptor tyrosine kinase inhibitor (gefitinib) in head and neck squamous cell carcinoma (HNSCC) cells (Ozawa et al., 2009). In addition, gefitinib-mediated re-expression of CXCL14 is strongly associated with the anti-tumor efficacy of this drug in HNSCC cell xenografts (Ozawa et al., 2009).

The purpose of this study was to evaluate primary lung adenocarcinomas from current, former, and never smokers for promoter hypermethylation of the CXC subfamily of chemokines identified by a genome-wide transcriptome array. Cancer specificity was determined by comparing methylation in lung cancer cell lines to normal human bronchial

epithelial cells (NHBEC) from bronchoscopy of cancer free smokers and normal peripheral blood mononuclear cells (PBMC) from healthy donors. The potential use of aberrant methylation of these genes as biomarkers for early lung cancer detection was assessed using sputum samples from early stage lung cancer cases and controls. Finally, *in vitro* and *in vivo* studies were also conducted to evaluate the functional consequences associated with silencing of *CXCL14*.

Results

DNA methylation regulates transcription of CXC-chemokines in lung cancer

A genome-wide transcriptome array designed to discover novel aberrantly methylated genes identified 11 of the 16 known *CXC* genes (*CXCL2-6, 8, 10-12, 14* and *16*) in human lung cancer cell lines (Figure 1a and Table 1). Nine genes (*CXCL1-6, 12, 14,* and *16*), including seven identified by the array, have promoter CpG islands (Table 1) (Gardiner-Garden and Frommer, 1987). The specificity of promoter methylation to tumor cells was validated by combined bisulfite restriction analysis (COBRA) using lung cancer cell lines, PBMC from healthy donors, and NHBEC obtained from bronchoscopy of cancer free smokers. Tumor-specific methylation (not detected in NHBEC) of *CXCL5*, *CXCL6*, *CXCL12,* and *CXCL14* was found in 65, 65, 63 and 59% of lung cancer cell lines, respectively. In contrast, *CXCL4* was methylated in 82% of the lung cancer cell lines, 90% of NHBEC and 100% of PBMC, and *CXCL6* was methylated in 55% of PBMC. Although, *CXCL4* and *CXCL6* were methylated in lung cancer cell lines, the frequent methylation of these genes in PBMC negated their evaluation in primary adenocarcinomas. The promoter CpG islands of the remaining four genes (*CXCL1, 2, 3,* and *16*) were unmethylated in all lung cancer cell lines (Table 1).

Methylation of *CXCL5*, *12,* and *14* is common in primary lung adenocarcinomas

The methylation status of *CXCL5*, *12*, and *14* promoters was evaluated in primary lung adenocarcinomas using methylation-specific PCR (MSP) and methylation was found in 80, 75, and 78% of the tumors, respectively (Figures 1b-d). All three genes were methylated in 107 (61%) of the primary tumors whereas only 7 (4%) showed unmethylated promoter in all three genes. Although the prevalence for methylation of *CXCL14* in never smokers is slightly higher than current and former smokers, the difference was not statistically significant (Figure 1d). Similarly, the prevalence for *CXCL5* and *CXCL12* methylation among the different smoking groups was similar (Figures 1b and 1c). There was no difference in prevalence for gene methylation by tumor stage and methylation of these chemokines alone or in combination was not prognostic for survival (not shown).

Methylation of *CXCL5, 12*, and *14* silences gene expression

The effect of methylation on gene expression was compared between samples with and without methylation of *CXCL5*, *12* and *14* promoters using RT-PCR. Complete methylation of these genes (determined by a complete digestion of the multiple CGCG sites by the *BstUI* restriction enzyme) strongly correlated with loss of gene expression. In lung cancer cell lines with completely methylated *CXCL5* (H1568, H1993, and Calu-6), *CXCL12* (Calu-6 and SKLU-1) and *CXCL14* (H23, Calu-6, and SKLU-1), transcription of these genes was absent

(Figure 2a-c). In contrast, all three genes were readily transcribed in samples with unmethylated promoters such as NHBEC and H2228.

DAC treatment restores expression of genes silenced by methylation

The causality of promoter hypermethylation and/or histone modification to gene silencing was evaluated using drugs to inhibit DNA methylation (DAC) and histone deacetylation (TSA). Lung cancer cell lines with or without methylation of *CXCL5*, *12*, and *14* promoters were treated with vehicle (sham), TSA, or DAC and gene expression was evaluated by RT-PCR. DAC treatment restored the expression of *CXCL5* (H1568, H1993, and Calu-6), *CXCL12* (Calu-6 and SKLU-1), and *CXCL14* (H23, Calu-6, and SKLU-1) to a level comparable to cell lines without methylation (Figure 2a-c). TSA was unable to restore expression of these genes in cell lines where dense methylation within the promoter CpG islands was detected by the COBRA assay. The only exceptions to this scenario were *CXCL5* in H1993 and *CXCL14* in Calu-6 where response to DAC and TSA was similar. Interestingly, *CXCL14* was completely silenced in H2023 and H1568 where the promoter CpG island is unmethylated or weakly methylated, and expression was restored primarily by TSA suggesting histone modification is the predominant cause of *CXCL14* silencing in these cell lines (Figure 2c).

Because epigenetic regulation of *CXCL14* is unknown, unlike *CXCL5* and *CXCL12* (Speetjens et al., 2008; Wendt et al., 2008), studies on this cytokine were extended to map the distribution of methylation across the promoter CpG island. Out of 82 CpGs present within the promoter CpG island of *CXCL14*, 52 were analyzed using sodium bisulfite sequencing. Primary lung adenocarcinomas and lung cancer cell lines that were strongly positive for methylation in the MSP and COBRA (#54, #66, #100, H23 and Calu-6) were methylated for 58-100% of the 260 CpG islands evaluated (52 CpGs per clone and 5 clones per sample) (Figure 2d). In contrast, samples with no methylation (#18, H1435, and the NHBEC N255 and N256) or weak methylation (#35) in the MSP and COBRA assays were methylated in 0-8% and 33% of the CpGs, respectively (Figure 2d).

Methylation of *CXCL14* is a diagnostic marker for early stage lung cancer

Detection of aberrant gene methylation in sputum is emerging as a promising early biomarker for lung cancer (Belinsky et al., 2006). Therefore, methylation of *CXCL12* and *CXCL14* was evaluated using sputum samples collected from asymptomatic stage I lung cancer cases (n = 40) and cancer-free smokers (control, n = 80) matched by age, gender, smoking status (current, former), and enrollment site. The low number of CpGs within *CXCL5* promoter CpG island (Table 1) prohibited the development of a sensitive and specific nested, MSP assay required for interrogating DNA recovered from sputum samples. *CXCL14* was methylated in 55% of cases compared to 33.8% in controls indicating a 2.9-fold increased risk (95% CI: 1.7, 7.3; p = 0.026) for lung cancer. Similarly, *CXCL12* was methylated in 50% cases to 37.5% in controls suggesting an elevated, albeit not significant, 1.7-fold increased risk for lung cancer (95% CI: 0.7, 3.3; p = 0.24). As expected, the prevalence for methylation of these chemokines in sputum samples that contain cells from both the deep lung parenchyma and airways reflecting field cancerization was greater than seen in cytologically normal bronchial epithelial cells obtained by bronchoscopy.

CXCL14 expression increases cell death

The effect of *CXCL14* expression on cell survival and proliferation was evaluated using H23 and SKLU1 cells where this gene is completely silenced by methylation. Transient expression of *CXCL14-GFP* increased cell death in H23 by 20% ($p < 0.01$) (Figure 3a-d) and in SKLU1 by 40% ($p < 0.001$) (Figure S1) as compared to expression of GFP. Although expression of *CXCL14-GFP* also led to a 6% reduction in the number of actively cycling (G2-M phase) cells compared to the GFP control, the difference was not statistically significant ($p = 0.28$) (data not shown). In addition, migration of serum starved H23 cells with or without stable *CXCL14* expression was compared using cell migration chambers with 8μm pores and H23 growth media containing 10% FBS as a chemo-attractant. Stable expression of *CXCL14* increased cell migration by 40% ($p < 0.01$) compared to the parental cells (Figure S2).

CXCL14 expression dramatically suppresses tumor growth in nude mice through induction of tumor necrosis

The candidate tumor suppressor function of *CXCL14* was examined *in vivo* by comparing the tumorigenicity in nude mice of H23 cells with or without stable *CXCL14* expression. Parental H23 cells with silenced *CXCL14* and H23 clone (H23-CXCL14) stably expressing this chemokine (Figure 4a inset) were subcutaneously injected into nude mice and tumor growth was monitored for 10 wks. Tumor growth was comparable between the two cell lines for the first 4 wks. However, H23-CXCL14 tumors showed little additional increase in size while the parental line continued to grow (Figure 4a). The mice in both groups were sacrificed 10 wks post inoculation, the tumors were collected, and tumor volume and weight were measured. Tumors derived from the H23-CXCL14 cells were significantly smaller in size and weight than tumors from the parental H23 cell line (Figure 4a-c). Consistent with the flow cytometric cell death assay, H&E stained tumor sections revealed large areas of necrosis encompassing 50-90% of the *CXCL14* expressing cell tumor mass compared to 10-30% tumor necrosis in *CXCL14* negative tumors (Figure 4d).

CXCL14 re-expression modulates pathways leading to cell cycle arrest and cell death

Genome-wide transcription patterns of H23 cells with or without stable *CXCL14* expression were compared to identify pathways that might be altered by *CXCL14*. As compared to the parental H23, the *CXCL14* expressing clone showed 659 and 445 genes with over 2-fold increased and decreased expression, respectively (data not shown). Because functional studies suggest a role for *CXCL14* in cell cycle and cell death, we focused our evaluation of the microarray data on genes regulating these two pathways. Consistent with the *in vitro* and *in vivo* studies, expression of 30 genes that directly or indirectly inhibit cell cycle progression or promote apoptosis was increased in the *CXCL14* expressing cells (Table 2). Moreover, expression of 41 genes that promote DNA replication, cell cycle progression and cytokinesis, or genes with anti-apoptosis and/or oncogenic properties was significantly reduced in the *CXCL14* expressing cells. Fold expression changes and gene function are detailed in Table 2. Most notable, were the 4 – 7.6-fold increase in expression of caspases and the 20-fold increase in expression of TXNIP, an inducer of G_1 cell cycle arrest. In

contrast, expression of the cyclin family of genes (A2, A3, B1, D3, and E2) that promote cell cycle progression was reduced by 45 – 70% (Table 2).

Discussion

Chemokines regulate cell proliferation, apoptosis, angiogenesis, metastasis, and tumor immunity, pathways that are critical in carcinogenesis (Darash-Yahana et al., 2009; Hromas et al., 1999; Mu et al., 2009; Strieter et al., 2004). A genome-wide transcriptome array identified the *CXCL5*, *CXCL12*, and *CXLC14* chemokines as common targets for silencing by promoter methylation in adenocarcinomas. Dense methylation that was reversible by treatment with a demethylating agent accounted for silencing of all three genes. Strong support for *CXCL14* as a tumor suppressor gene was provided by its marked effect on growth of tumor xenografts, induction of tumor necrosis, and likely influence on many genes central to cell cycle control and apoptosis. The commonality and diverse function of the multitude of genes silenced by methylation in lung tumors has generated intense interest by our group and others for assessing their potential as biomarkers through detection of methylated genes in sputum from asymptomatic lung cancer patients (Belinsky et al., 2006; Belinsky et al., 1998; Cirincione et al., 2006; Hsu et al., 2007). *CXCL14* methylation in sputum was associated with a 2.9-fold elevated risk for lung cancer, making it a potential marker for inclusion in our developing diagnostic gene panel (Belinsky et al., 2006).

The genome-wide transcriptome array developed to discover novel methylated genes in cancer identified eleven of the 16 CXC-chemokines as potential targets of DNA methylation in lung cancer. Five of the eight genes identified by the array that contained promoter CpG islands were methylated in lung cancer cell lines, a 62.5% accuracy for identifying genes regulated by methylation. However, two of the five methylated genes were also methylated in normal cells (*CXCL4* in NHBEC and PBMC, and *CXCL6* in PBMC) indicating that gene regulation through methylation can be cell-specific and related to state of differentiation. This conclusion is supported by our previous study identifying four genes within the lung cancer susceptibility locus 6q23-25 that were methylated in lymphocytes and two genes methylated in NHBEC (Tessema et al., 2008). Recently, a genome-wide screen for methylation also demonstrated that approximately 10% of all promoters were methylated in differentiated B cells (Rauch et al., 2009).

The expression of *CXCL2, 3,* and *16* that contain promoter CpG islands was significantly increased by DAC treatment; however, methylation of these genes was not seen in any lung cancer cell line. One explanation for this epigenetic regulation is re-expression of methylated genes whose proteins function as transcription factors to regulate expression of these chemokines. While detailed studies have not been conducted to map transcription factor binding sites within these chemokines, our own studies on regulation of the *PAX5 β* gene support this premise. *PAX5 β* is commonly silenced in lung cancer through methylation and encodes for the transcription factor B cell-specific activating protein that in turn, directly regulates *CD19*, a gene not containing a CpG island. A strong association was observed between *PAX5 β* methylation and loss of expression of CD19 and treatment with a demethylating agent restored expression of both genes (Palmisano et al., 2003). Unlike lung cancer, *CXCL16* is commonly silenced by methylation in kidney tumors (Morris et al.,

2008). In addition, RNA interference mediated knockdown of DNMT3a in melanoma cells resulted in CXCL9 and CXCL16 re-expression that was associated with suppression of tumor growth and metastasis suggesting methylation-mediated regulation of these chemokines in other tumors (Deng et al., 2009).

Among the CXC-chemokines with tumor-specific methylation in lung cancer, the role of *CXCL12* in carcinogenesis is well established. Whereas *CXCL12* is frequently methylated in various carcinomas, its cell surface receptor (CXCR4) is abundantly expressed in most tumors (Wendt et al., 2008; Wendt et al., 2006; Yoshino et al., 2009; Zhou et al., 2008). This limits the *CXCL12-CXCR4* autocrine signaling and promotes directional migration of carcinoma cells toward organs/tissues with high CXCL12 expression. Lung is one of the organs expressing higher levels of CXCL12 and is a primary site for metastasis of carcinomas with low CXCL12 expression such as breast and colorectal cancers (Muller et al., 2001). Conversely, when CXCL12 is silenced in lung cancer, it may enhance local invasion as well as metastasis to other organs with high CXCL12 expression such as the adrenal glands, liver, and bone marrow (Phillips et al., 2003). Therefore, methylation-mediated silencing of *CXCL12* in lung cancer could play a major role in deregulating the autocrine *CXCL12-CXCR4* signaling pathway to promote tumor invasion and metastasis.

Unlike *CXCL12*, the role of *CXCL5* in cancer is unclear and likely varies in different malignancies. ShRNA knockdown of CXCL5 in colon cancer increased tumor growth and metastasis that is reversed by restoring CXCL5 expression suggesting a tumor suppressor function (Speetjens et al., 2008). In contrast, expression of CXCL5 is increased in metastatic HNSCC and promotes cell proliferation, while shRNA knockdown of CXCL5 suppresses cell migration, proliferation, and tumorigenicity in nude mice suggesting an oncogenic role (Miyazaki et al., 2006). While *CXCL5* is clearly silenced through methylation in lung cancer, its elevated expression in NSCLC could be from stromal cells within the tumor (Arenberg et al., 1998).

CXCL14 is a potent angiostatic chemokine that blocks *CXCL8* (*IL-8*), *VEGF* and *bFGF* mediated angiogenesis (Shellenberger et al., 2004). It is also a chemo-attractant mediating infiltration of dendritic cells into tumors, which is critical for tumor immunity (Shurin et al., 2005). As shown in this study, re-expression of *CXCL14* enhances cell death *in vitro* and induces marked necrosis in lung tumor xenografts, due in part through increased expression of multiple apoptosis effector proteins including the caspases. Moreover, re-expression of *CXCL14* has a profound effect on the genome altering the expression of more than 1,000 genes. Taken together these results suggest *CXCL14* expression is central for various anti-tumor mechanisms and its silencing could be critical for carcinogenesis.

Aberrant methylation may ultimately serve two vital roles in cancer: as biomarkers for detection and prognosis and targets for epigenetic therapy (Belinsky et al., 2003; Belinsky et al., 2006; Gore et al., 2006; Yang et al., 2006). *CXCL14* may participate in both arenas. We are developing a gene panel whose methylation in sputum could identify people at high risk for cancer incidence. Previously, six of 14 genes evaluated in a lung cancer case-control study were associated with over 50% increase in lung cancer risk. Importantly, concomitant methylation of three or more of these six genes increased risk 6.5-fold with a sensitivity and

specificity of 64% for classifying lung cancer cases. While, this sensitivity and specificity is not high enough for prospective population-based screening, additional genes such as *CXLC14* whose methylation in sputum conferred a 190% increased risk, could contribute significantly to our gene panel.

Rather than targeting single pathway alterations in cancer, epigenetic therapy may circumvent the problem of tumor heterogeneity by inducing the re-expression of multiple tumor suppressor genes; several may be essential for abrogating cancer cell survival and proliferation. Demethylating agents used at doses far below their maximum tolerated dose have proven to be a potent therapy for a precursor state to acute myelogenous leukemia (AML) and myelodyplasia (MDS) and are now approved by the FDA for treatment of these cancers (Yang et al., 2006). Clinical trials with demethylating agents combined with histone deacetylase inhibitors also are showing promising responses in the treatment of myeloid malignancies (Gore et al., 2006). The extension of this targeted approach to solid tumors such as those in the lung may also hold promise as a therapy. Our recent work in which combined treatment with DAC and sodium phenylbutyrate reduced the number of developing lung tumors in a murine model by more than 50% support this supposition and Phase II trials in lung cancer are underway (Belinsky et al., 2003). The effective awakening of silenced genes such as *CXCL14* that can affect the activity of many critical pathways could profoundly impact the growth and survival of tumor cells setting the stage for using epigenetic therapy in the management of lung cancer in some patients.

Materials and Methods

Samples

Frozen lung adenocarcinomas from current (n = 37), former (n = 59) and never (n=75) smokers were obtained from tumor banks at Johns Hopkins and the Mayo Clinic. Demographic variables including age, gender, and stage of lung cancer and selection criteria for suitable adenocarcinomas has been described previously (Tessema et al., 2008). NHBEC isolated from bronchoscopy of cancer-free smokers (n = 20) and PBMC from healthy donors (n = 20) were used as controls. Seventeen lung cancer-derived cell lines (H23, H358, H1435, H1568, H1993, H2023, H2085, H2228, H2009, Calu-3, Calu-6, SKLU-1, SKMES1, H1299, H1975, HCC827, and HCC4006) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Sputum samples collected from stage I NSCLC cases prior to surgery (n = 40) and cancer free smokers (n = 80) and were matched on age (+/- 5 years), gender, smoking status (current, former) and location of hospital as described (Chin et al., 2008).

Treatment and Genome-Wide Transcriptome Array

Three lung adenocarcinoma cell lines (H23, H1568, and H1993) from smokers and three (H2023, H2085, and H2228) from never smokers were used for the transcriptome array as described (Tessema et al., 2008). Briefly, cells at log phase of growth were treated as follows: Control (culture medium), TSA (culture medium containing 300 nM of trichostatin A, [Sigma, St. Louis, MO; stock solution 5 μM in ethanol] for 18 h), or DAC (500nM 5-aza-2-deoxycytidine [Sigma; stock solution 10 mM in PBS] for 96 h with fresh medium

containing the drug changed every 24 h). Cells were harvested in TRI Reagent (Sigma-Aldrich, Steinheim, Germany) and genome-wide transcriptome array analysis was conducted using the Agilent 44K expression array as described (Tessema et al., 2008).

DNA methylation analysis

DNA was extracted and modified as described (Tessema et al., 2009) and 40 ng of modified DNA was used per PCR. Methylation in NHBEC, PBMC, primary lung tumors and cancer cell lines was studied using COBRA and MSP as described (Tessema et al., 2009). Methylation of DNA isolated from sputum was assessed using a nested, MSP assay developed as described (Belinsky et al., 2006). Primer sequences and PCR conditions are described in Tables S1 and S2. Bisulfite sequencing of *CXCL14* promoter CpG island was conducted using modified DNA and COBRA primers (Table S1) that do not discriminate between methylated and unmethylated DNA. PCR products were cloned into pCR II cloning vector (Invitrogen, Santa Clara CA) and five clones were sequenced per sample.

Gene Expression Analysis

Total RNA (3 μg) isolated from sham (vehicle), TSA, and DAC treated cells as described (Tessema et al., 2009) was reverse transcribed using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) according to the protocol from Invitrogen®. Transcription of *CXCL5, 12* and *14* was evaluated using RT-PCR and electrophoresis in 3% agarose gels. To avoid PCR products from DNA, RNA was treated with DNase and PCR primers were located in exons separated by a large intron. For *CXCL14* transcripts originating from plasmid vectors (no intron), RT-negative (RT-) PCR was done in parallel using cDNA synthesized in the absence of Superscriptase-II. RT-PCR primers and amplification conditions are described in Table S3.

Cloning, transfection, and establishment of stable CXCL14 expressing clones

Full-length *CXCL14* transcript amplified by PCR using cDNA from NHBEC 255 CXCL14 F2/R2 primer pairs (Table S3) was directly ligated into pcDNA3.1/NT-GFP-TOPO® vector (Invitrogen) for transient transfection and into pTARGET™ Mammalian Expression Vector System (Promega, Madison, WI) for stable expression and cloned by TA cloning strategy. The sense orientation and sequences of the cloned *CXCL14* cDNA was confirmed by DNA sequencing. H23 cells (1×10^5/well in 6-well plate) were transfected with CXCL14-GFP or the GFP expression vector (Mock) using Lipofectamine™ LTX (Invitrogen). Stable transformants were selected from CXCL14 and Mock pTARGET™ vector transfected H23 cells using 400 μg/ml Geneticin (Geneticin® Liquid (G-418 sulfate), Invitrogen).

Cell death, cell cycle, and cell migration assays

H23 cells were plated at a density of 1×10^5/well in 6-well plates and transfected 24 hours later with Mock (GFP) or CXCL14-GFP vectors. For cell death analysis, cells were harvested (48 hours post transfection) with trypsin, washed once with PBS, stained in the dark with 10 μg/ml Propidium iodide (PI) (Sigma-Aldrich) at 37°C for 1 hr, washed, resuspended in 1 ml PBS and the PI and GFP fluorescence were evaluated using flow cytometry (Becton Dickinson FACScalibur Flow Cytometer). The proportion of PI and/or

GFP positive cells was calculated from 10,000 events. For cell cycle analysis, cells were plated, transfected, harvested, and washed as described above and resuspended in 500 μl ice-cold PBS, fixed by adding 500 μl of ice cold 2% buffered paraformaldehyde, and incubated at 4°C for 30 min. The cells were then washed, permeablized with 1 ml ethanol at 4°C overnight, stained with 1 ml PI solution containing 40 μg/ml PI, 100 μg/ml RNase in PBS at 37°C for 1 hr in the dark, washed, resuspended in PBS and cells were analyzed with flow cytometry as described above. For cell migration, H23 cells with or without stable *CXCL14* expression were serum starved for 48 hrs and cell migration was measured using CytoSelect™ 24-Well Cell Migration Assay (8 μm, Colorimetric Format) (Cell Biolabs, San Diego, CA) and 10% serum containing growth medium as a chemo-attractant for 24 hrs according to the protocol.

In vivo tumor growth

Matrigel™ Basement Membrane Matrix (BD Biosciences, San Jose, CA) was mixed 1:1 with H23-CXCL14 clone stably expressing CXCL14 and the parental H23 cell line and subcutaneously injected (5×10^6/site) into both sides of the dorso-lateral region of four female athymic nude mice (Athymic NCr-nu/nu, Frederick, MD) per group. Tumor size was quantified once a wk from 2^{nd} to 10^{th} wk post injection and tumor volume was calculated as: $(a \times b^2)/2$, where a and b represent the longer and shorter dimensions respectively. On the 10^{th} wk, all the mice were sacrificed, tumors collected, and weighed. Tumors were formalin fixed, paraffin embedded, sectioned, and stained with hematoxylin and eosin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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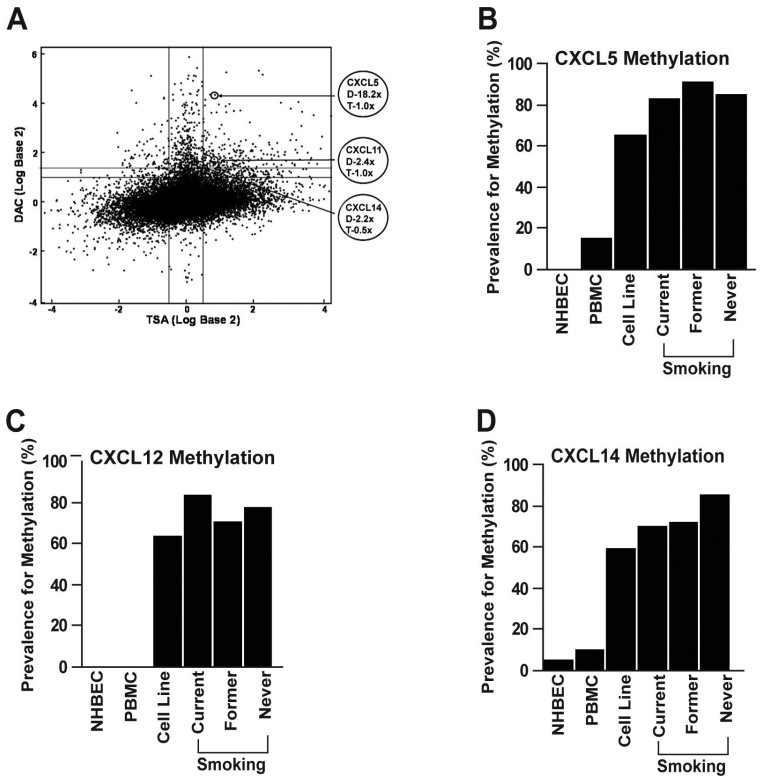

Figure 1.

Transcription of the CXC-chemokines in lung cancer is regulated by methylation. a) Genes that are most likely regulated by promoter hypermethylation are expected in the top tier group (2-fold or higher change with DAC treatment (Y-axis > 1.4) and little or no change with TSA treatment (X-axis = -0.5 to 0.5). Treatment of H23 cells with DAC led to an 18.2, 2.4, and 2.2-fold increase in expression of *CXCL5*, *CXCL11*, and *CXCL14* respectively on Agilent 44K expression array. In contrast, H23 cells treated with trichostatin A (TSA) showed little or no change in the expression of these genes suggesting DNA methylation is the primary regulator of the transcription of these genes in H23. Primary lung adenocarcinomas from current, former and never smokers showed tumor specific and highly prevalent methylation in the promoter CpG island of *CXCL5* (b), *CXCL12* (c), and *CXCL14* (d).

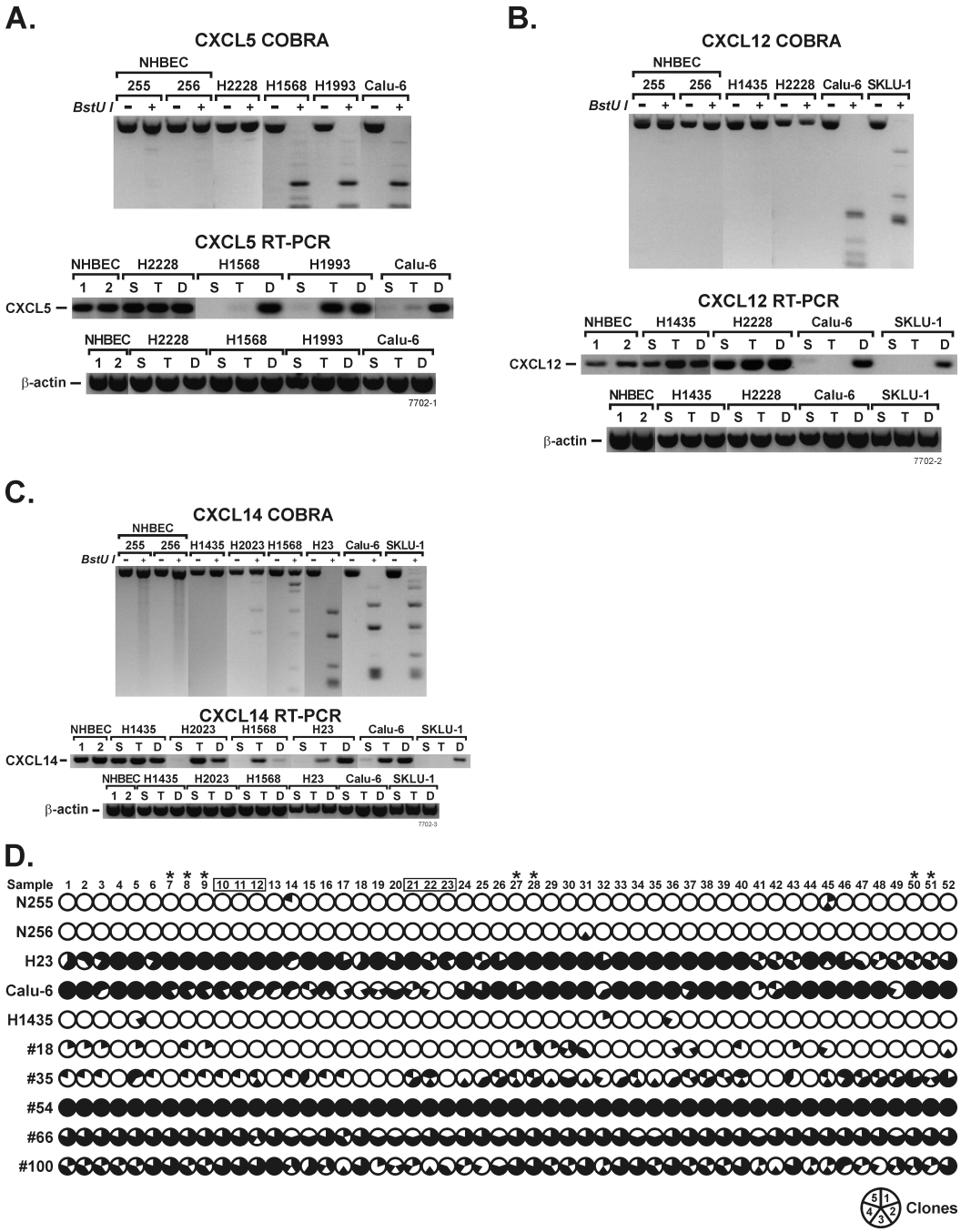

Figure 2.

DAC treatment restores expression of genes silenced by methylation. Expression of *CXCL5* (a), *CXCL12* (b), and *CXCL14* (c) was silenced in untreated control (S) lung cancer cell lines with methylated promoter CpG islands and could be restored primarily with DAC (D) treatment. In some cell lines (*CXCL5* in H1993 and *CXCL14* in Calu-6) gene expression could also be restored by TSA (T) treatment. NHBEC and lung cancer cell lines with unmethylated promoter CpG islands, readily expressed these chemokines and their expression was not affected by treatment with either drug. *CXCL14* expression in H2023

and H1568 cell lines was silenced in the absence of methylation and expression was primarily restored by TSA treatment. d) Dense methylation that corresponds with gene silencing was found in the promoter CpG island of *CXCL14*. Modified DNA was amplified using primers that do not discriminate between methylated and unmethylated alleles (the same used for COBRA), cloned, and five clones per sample were sequenced. The shaded area of each circle indicates the extent of methylation at that CpG and one clone represents 1/5th of the circle. CpGs 10-12 and 20-23 (placed in boxes) indicate the primer binding sites for the MSP assay and the CpGs recognized by BstU1 restriction enzyme used for COBRA are indicated by asterisks. Nearly all CpGs of samples that were unmethylated by COBRA and MSP assays (NHBEC 255, and 256, H1435, and primary lung adenocarcinoma sample #18) were free of methylation. Only 0-8% of the 260 CpGs (52 CpGs per clone and 5 clones per sample) were methylated in these samples. In contrast, 58-100% of the CpGs were methylated in samples that were strongly methylated by COBRA and MSP (H23, Calu-6 and primary tumor #54, #66, and #100). Primary adenocarcinoma #35 that was weakly methylated by the two assays was methylated for 33% of the CpGs.

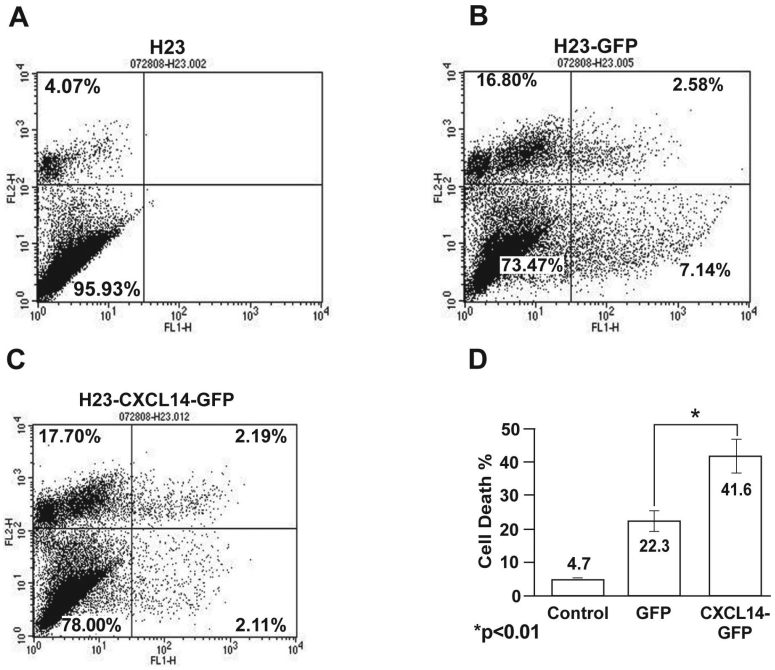

Figure 3.

Transient re-expression of *CXCL14* induces cell death. Cell survival was evaluated by flow cytometry using a GFP containing expression vector. The normal level of cell death which occurs in 4-5% of H23 cells (a) was increased to 15-20% due to exposure to the transfection reagent alone (data not shown). GFP expression in the transfected cells increased cell death by 20-25% which was only marginally greater than seen with the transfection media (b). In contrast, expression of CXCL14-GFP led to a 40-45% cell death (c). This indicates that re-expression of CXCL14 in H23 cells where the endogenous expression is silenced by methylation increased cell death by 19.3 % compared to the GFP control (d).

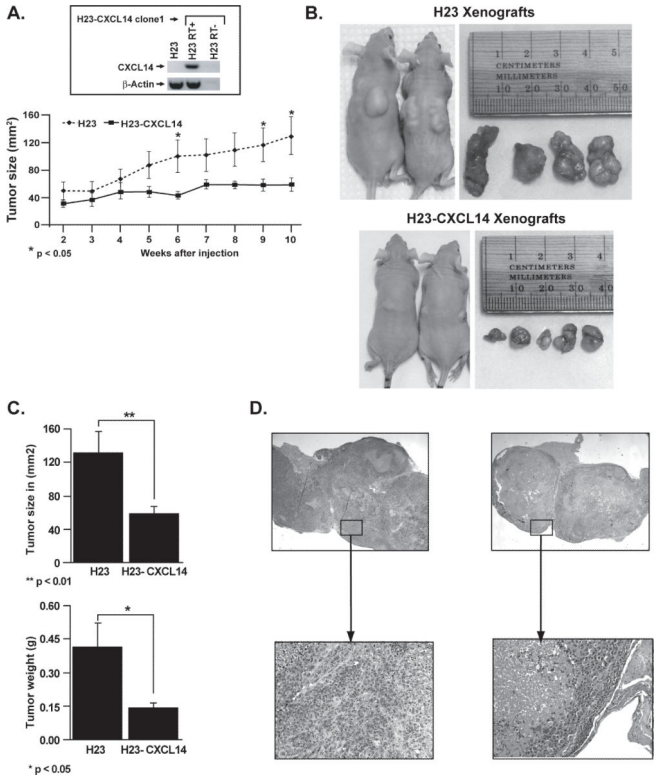

Figure 4.

Stable re-expression of *CXCL14* significantly reduced tumor growth and induced necrosis of H23 xenografts in nude mice. a) Both the parental and *CXCL14* expressing H23 cells formed detectable tumors within two wks post inoculation and showed comparable rates of tumor growth in the first four wks. After wk four, tumors from the parental H23 kept growing while those from *CXCL14* expressing cells barely increased in size. The asterisks at wk 6, 9, and 10 indicate significant differences in tumor size. b) The size differences between the tumors in the two groups was obvious under the skin when the mice were sacrificed ten wks post-injection. c) Similarly, the size and weight of the tumors harvested from the *CXCL14* expressing cells were significantly smaller than tumors from the parental H23 cells. d) Histological examination of H&E stained slides revealed tumors from the *CXCL14* expressing cells contain large necrotic foci that involved up to 90% of the tumor mass as compared to tumor necrosis in the range of 20-30% of the tumor mass from the parental H23 cells.

Table 1

Promoter methylation of CXCL family genes in lung cancer

No.	Gene	CpG island property				Methylated (%)			Array** positive (#)
		CpG* (#)	Size (bp)	GC (%)	Obs/exp	Cell lines	NHBEC	PBMC	
1	CXCL1	54	626	69.3	0.74	0	-	-	0
2	CXCL2	62	623	69.2	0.84	0	-	-	1
3	CXCL3	62	636	69.0	0.84	0	-	-	1
4	CXCL4 (PF4)	22	302	65.6	0.68	82	90	100	2
5	CXCL5	26	216	70.8	0.69	65	0	15	3
6	CXCL6	23	207	73.4	0.85	65	0	55	1
7	CXCL7 (PPBP)	0	-	-	-	-	-	-	0
8	CXCL8 (IL-8)	0	-	-	-	-	-	-	1
9	CXCL9	0	-	-	-	-	-	-	0
10	CXCL10	0	-	-	-	-	-	-	1
11	CXCL11	0	-	-	-	-	-	-	3
12	CXCL12	223	2677	60.9	0.90	63	0	0	1
13	CXCL13	0	-	-	-	-	-	-	0
14	CXCL14	82	803	68.0	0.90	59	5	10	2
15	CXCL16	134	1372	69.2	0.82	0	-	-	1
16	CXCL17	0	-	-	-	-	-	-	0

* When a gene has no CpG island the CpG number was shown as 0.

** Array positive indicates the number of lung adenocarcinoma cell lines (out of 6 cell lines) that after DAC treatment showed a 2-fold or higher increase in expression.

Table 2

Stable expression of *CXCL14* in H23 cells promotes pathways that activate cell death and suppresses cell proliferation.

Inhibitors of cell cycle progression		
CDKN1C	2.89	p57 (KIP2) is a tumor suppressor gene with reduced expression in many cancers
CEBPD	8.14	plays important role in IL-6/STAT3 mediated growth arrest
FOXN3	2.36	S-phase checkpoint pathway gene arresting cell cycle in case of DNA damage
GAS1	3.18	blocks entry to S phase and prevents cycling of normal and transformed cells
GAS5	4.30	induces growth arrest and apoptosis and its expression is reduced in breast cancer
NDRG2	2.44	leads to G1/S cell cycle arrest by attenuating AP-1 and downregulating of cyclin D1
NEIL1	2.66	damage sensor activating checkpoint control and involved in base excision repair.
NOTCH2	2.12	potent inhibitor of NOTCH1 induced cell cycle progression and induces apoptosis
RBP1	4.30	inhibits cell proliferation and induces expression of a senescence marker
RBP7	2.33	internalization and degradation of nutrient transporters triggering nutrient starvation and induce cell death
SEPP1	4.33	involved in hydrogen peroxide mediated oxidative stress induced cell cycle arrest
SPRY1	2.35	suppresses proliferation and promote terminal differentiation via p21 and STAT1 upregulation and sustains ERK activation
SSBP2	2.48	causes G1 arrest, partial differentiation and downregulation of c-MYC expression
Inducers of apoptosis		
BAI1	2.13	p53 regulated receptor for recognition and engulfment of apoptotic cells
BCL3	3.04	increases apoptosis of multiple myeloma cells
CASP1	4.44	caspase 1 promotes apoptosis
CASP4	7.68	caspase 4 is effector of apoptosis
IFITM1	2.20	promotes STAT1 and p53 crosstalk for the antiproliferative action of IFN-gamma
IFITM2	2.11	interferon induced, p53 independent pro-apoptotic transmembrane protein
IFITM3	2.58	human 1-8D gene is a novel p53 independent pro-apoptotic gene
GLTSCR2	2.92	phosphorylates PTEN for caspase-independent PTEN-modulated apoptosis
NDRG1	5.30	transcriptionally activated by p53 leading to caspase activation and apoptosis
OGT	2.74	modifies the anti-apoptotic Akt1 and induces apoptosis
PLAU	3.11	plasminogen activator induces apoptosis in brain endothelial cells (HBMEC).
PPARG	2.60	induces apoptotic cell death in NSCLC via ROS formation and POX induction
RHOBTB2	8.08	required for CXCL14 expression and is a direct target of E2F1 with a short term increase in cell cycle but in the long term induces apoptosis
SALL2	2.30	increase p21 and BAX for p53-independent regulation of growth and survival
TLE1	2.02	promotes cell death through caspase-independent apoptosis
TRB2	4.38	pro-apoptotic molecule leading to apoptosis of mainly hematopoietic cells
TXNIP	20.02	induces G1 cell cycle arrest via ASK1 activation, ER stress, p38 and JNK phosphorylation and stabilization of p27(kip1)
Promoters of cell cycle progression		
CCNA2	0.31	Cyclin A, critical for G2/M cell cycle progression

CCNB1	0.46	Cyclin B1, required for the progression of M-phase of the cell cycle
CCND3	0.55	Cyclin D3, important for G1/S cell cycle progression
CCNE2	0.46	Cyclin E2, required for S/G2 cell cycle progression
CDCA3	0.50	mediates the ubiquitination and degradation of the cdk1 inhibitor (WEE1) at G2/M leading to cdk1/cyclin B activation and mitotic entry
CDC2	0.45	M-phase promoting factor (MPF) essential for G1/S and G2/M phase)
CDCA8	0.39	key regulator of mitosis required for stability of the bipolar mitotic spindle
CDC20	0.48	along with APC, required for cell cylce progression form metaphase to anaphase
CDC25A	0.39	required for progression from G1 to the S
CDC27	0.49	component of APC, promotes ubiquitin-mediated degradation of B-type cyclins
PLK1	0.45	promotes mitotic exit and cytokinesis via centrosome maturation, spindle assembly, removal of cohesins and inactivation of APC/C inhibitors
PLK2	0.49	serine/threonine protein kinase that plays a role in normal cell division
Promoters of DNA replication		
CDC6	0.41	initiates DNA replication and ensures its completion before the initiation of mitosis
CDC45L	0.48	important for early steps of DNA replication in eukaryotes
ENDOG	0.41	generates the RNA primers for the initiation of mitochondrial DNA replication
MCM10	0.30	helps form the pre-initiation complex for DNA replication and S to G2 transition
POLA2	0.49	DNA primase that is a replicative polymerase
POLE4	0.48	allows polymerase epsilon to carry out its replication and/or repair function
Promoters of Chromosomal assembly and cytokinesis		
CDCA5	0.50	Sororin, a regulator of sister chromatid cohesion in mitosis
CENPA	0.48	required for recruitment and assembly of kinetochore proteins, mitotic progression and chromosome segregation
CENPE	0.41	kinesin-like motor protein that accumulates in the G2 phase of the cell cycle
CENPO	0.50	component of the CENPA-CAD complex recruited to centromeres and involved in the assembly of kinetochore, mitotic progression, and chromosome segregation
CEP27	0.50	centrosomal protein required for spindle assembly and completion of cytokinesis
CEP55	0.39	centrosomal protein required for mitotic exit and cytokinesis
DSCC1	0.34	involved in the establishment sister chromatid cohesion
NCAPG	0.41	helps to convert interphase chromatin into condensed mitotic chromosomes
NCAPH2	0.43	play essential roles in mitotic chromosome assembly and segregation
NDC80	0.41	expression peaks in mitosis and is required for the organization of microtubule binding sites, integrity of kinetochore, and chromosome segregation
STAG3	0.34	cohesin complex required for the cohesion of sister chromatids.
Inhibitors of apoptosis		
BIRC3	0.44	suppresses apoptosis via interaction with TRAF1, TRAF2 and, TNFR2
BIRC5	0.34	Survivin, prevents apoptotic cell death and is overexpressed in many tumors including NSCLC and embryos, but is low in adult cells
IL7R	0.37	blocks apoptosis, differentiation, and activation of T lymphocytes
PTTG1	0.50	suppresses transcriptional and apoptotic activity of TP53 and is tumorigenic
Oncogenes		

ID1	0.42	Ids are overexpressed in many tumors and correlate with advanced tumor stages. Id1 dimerizes with bHLH transcription factors, induces cell proliferation and inhibits differentiation and apoptosis. ID2 promotes cell survival and suppresses apoptosis of tumor cell by reducing the expression of p21 and the pro-apoptotic protein Bim/Bod, and preventing cleavage of caspase-7. Id1, 2, and 3 increase self-renewal and proliferation of cortical neural stem cells and inhibit neuronal differentiation.
ID2	0.09	
ID3	0.34	
ID4	0.05	
EPGN	0.27	ligand for ERBB family receptors, promotes epithelial cell growth
MYB	0.35	oncogene in leukemia, breast and colon cancers
MYBL1	0.37	Transcription factors required for cell proliferation and cell cycle progression. MYBL2 activates cell division cycle 2 and cyclin D1
MYBL2	0.33	