Wnt Pathway Regulation by DOT1L Mediated Methylation of H3K79 in Non-Small Cell Lung Cancer

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Abstract

The Wnt pathway is a developmental pathway that is abnormally upregulated in cancer. Our current lab results suggest that decreased H3K79 methylation levels are associated with decreased DNMT1 activity and binding to the WIF1 5' region, upregulation of WIF1 protein levels, and suppression of Wnt signaling in lung cancer cell lines. We therefore hypothesize that the silencing of the WIF1 gene seen in many lung cancers involves upregulation of H3K79 methylation. We aim to knock down DOT1L and AF10, H3K79me3 mediators, to diminish Wnt expression in non-small cell lung cancers (NSCLCs). NSCLC lines including the A549 and H460 lines will be transfected with shRNAs for DOT1L and AF10; Western blotting will be used to confirm knockdown of DOT1L, AF10, and WIF1 expression. Wnt signaling will be measured by TOPFlash reporter assay, and by western blotting of nuclear β -catenin from total and fractionated cell lysates. We will then measure lung cancer cell growth, apoptosis and senescence.

Introduction

Lung cancer is the leading cause of cancer-related deaths in the United States and globally; over 200,000 patients are diagnosed with it in the U.S. every year. Despite recent advances in lung cancer treatment with targeted therapies and early detection, 5-year survival rates remain at only 15%.

The Wnt signaling pathway is a strong candidate target for developing new drugs against lung cancer. Wnts are secreted proteins that regulate cell differentiation, migration, and survival during embryogenesis and adult tissue homeostasis. Wnt protein expression and/or Wnt signaling is upregulated in most lung cancer cell lines and resected lung cancers. Activation of the Wnt signaling pathway is associated with increased cell proliferation, expression of stem cell markers, and invasiveness in lung cancer cell lines and/or xenografts, as well as with worse clinical prognoses in patients.

Wnt inhibitory factor-1 (WIF1) is one of several secreted proteins that bind Wnts and block their activity in vivo, and is expressed at high levels in the developing and adult lung; hypermethylation of the WIF1 promoter decreases WIF1 expression in lung cancer. WIF1 has been shown to be downregulated in non-small cell lung cancer (NSCLC); prior research has shown that WIF1 is able to inhibit lung cancer cell growth both in vitro and in vivo.

Our long-term goal is to develop novel, druggable routes into the canonical Wnt pathway by targeting the epigenetic regulators of WIF1 expression, such as DOT1L and AF10.

Background

Histone methyltransferase DOT1L (disruptor of telomeric-silencing 1-like) is the sole enzyme methylating lysine 79 of histone H3 (H3K79), whose di- and tri-methylation directly affects gene expression and induces lung cancer cell senescence; Wnt-targeted genes depend on H3K79 methylation. Acute Lymphoid Leukemia 1-fused gene from chromosome 10 (AF10) is an interaction partner and activator of DOT1L, both of which are essential coactivators of Wnt-dependent transcription.

Hypothesis

We hypothesize that WIF1 suppression in lung cancers results from increased trimethylation of H3K79me3, which in turn increases the binding and activity of DNA methyltransferase 1 (DNMT1) at the WIF1 promoter.

Figure 1. DOT1L and AF10 function in WIF1 DNA. AF10 is a transcription factor that regulates DOT1L expression; our model suggests that DOT1L mediates H3K79 methylation, increasing DNMT1 methylation of WIF1 DNA, resulting in Wnt activation. We propose that Wnt inhibition will result if DOT1L or its fusion protein activator AF10 are knocked down, reducing H3K79 methylation, decreasing DNMT1 methylation of WIF1 DNA, and allowing WIF1 to inhibit Wnt.

We propose that knocking down DOT1L and AF10 will result in decreased Wnt pathway expression, impairing lung cancer cell growth and promoting apoptosis and cellular senescence.

Materials and Methods

Generation of DOT1L and AF10 Knockdown Stable Cell Lines

- HEK293T cells were transfected with shRNAs, targeting DOT1L and AF10 mRNA and lentiviral plasmids using Polyethylenimine (PEI)
- Supernatant containing lentiviral particles harvested at 48 hours

Materials and Methods (cont.)

Figure 2. Transfection of 293T cells. (a) shRNA crosses cell membrane within lentiviral vector; (b) Reverse transcription of shRNA; (c) Pre-integration complex and entrance into nucleus; (d) DNA polymerase III inserts lentiviral shRNA; (e) Droscha processes product and (f) Exportin 5 releases product into cytoplasm; (g) Dicer removes shRNA loop and (h) processed siRNA enters RISC (RNA-induced silencing complex) where siRNA strands are unwound and separated, then (i) entering supernatant.

H460 and A549 Transduction

- Supernatant is added to H460 and A549 cells with polybrene (aids in retrovirus introduction)
- shRNA leads to target gene silencing by cleavage, if perfectly complementary to mRNA, or bypass of DOT1L/AF10 if imperfectly complementary
- Silencing gene leads to DOT1L/AF10 downregulation
- Knockdown is verified by western blot and RT-PCR

Results

Figure 3. RFP images depict lentiviral plasmid incorporation into host following transduction.

Figure 4 and 5. DOT1L mRNA Expression in H460 knockdown cells. Four combinations of shRNAs show reduced DOT1L mRNA expression with reference to a non-targeting shRNA control. All values have been normalized to GAPDH levels. Results were verified by western blot (above). sh#2,3 displays greatest knockdown.

Conclusion

Knockdown of DOT1L in H460 has been successful, as confirmed by western blot and RT-PCR. DOT1L knockdown in A549 will be conducted establish method; AF10 knockdown in H460 and A549 will follow. WIF1 expression and Wnt pathway activation will be measured to determine whether DOT1L and AF10 are necessary in NSCLCs for Wnt expression and resultant cancer proliferation and invasion.

If DOT1L and AF10 knockdown do not lead to loss of binding of DNMT1 to WIF1, and if DOT1L knockdown still lead to WIF1 promoter demethylation and Wnt pathway suppression, this would indicate that alternative mechanisms of WIF1 promoter demethylation exist. We would examine the role of other DNA methyltransferases on WIF1 promoter methylation and Wnt pathway activity in the knockdown cell lines.

Future Directions

A DOT1L inhibitor, EPZ-5676 (Epizyme), is currently in Phase I clinical trials in MLL-r leukemia and may be of promise in Wnt pathway-driven lung cancers. Although these drugs hold promise for targeting the Wnt pathway in lung cancer, they are still very early in clinical development and nothing is known about their efficacy in lung cancer. By understanding the mechanism by which H3K79 methylation regulates WIF1 expression in lung cancer, we may be able identify alternative or more effective targets for drug development.

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