Identification of chromosome 7 myeloid tumor suppressor genes

Abstract:

Monosomy 7 and del(7q) are among the most common cytogenetic abnormalities in myeloid malignancies, yet their underlying pathogenesis remains unclear. Herein we comprehensively identify chromosome 7 tumor suppressor genes (TSGs) in an unbiased manner. We selected candidate TSGs via datamining of genome-scale studies. In an array-based screen, we individually CRISPR-edited 112 candidates and measured the subsequent impact on proliferation and erythroid differentiation of primary, human CD34+ cells. An unexpected 36% of genes increased proliferation when edited. TSGs were significantly enriched in commonly deleted regions, a finding we corroborated computationally through an orthogonal machine learning approach. The only two genes that increased proliferation and decreased differentiation when edited were the *CUX1* transcription factor and *ACHE*, encoding acetylcholinesterase, both in the 7q22.1 commonly deleted region. As the function of ACHE (Cartwright blood group) on hematopoietic cells was previously uncharacterized, we determined that ACHE regulates erythropoiesis through acetylcholine receptor signaling. The loss of ACHE was rescued by a muscarinic receptor inhibitor, implicating muscarinic antagonists as potential treatments for -7/del(7q)-associated anemia. While chromosome-level deletions were historically thought to harbor a single TSG, we provide evidence for a contiguous gene syndrome, wherein combinatorial loss of multiple neighboring genes drives disease.

Introduction:

Monosomy 7 and del(7q) are adverse-risk cytogenetic abnormalities prevalent in myeloid malignancies in both pediatric and adult patients (1). Despite its clear clinical import, the underlying mechanism by which -7/del(7q) promotes transformation is incompletely understood. It has been postulated that critical tumor suppressor genes (TSGs) are encoded on chromosome 7, however identifying these genes has been challenging. Approaches have included mapping commonly deleted regions (CDRs), which helped identify *CUX1* (2), and searching for second-hit mutations, which pinpointed *EZH2* (3). The third method of large-scale cancer re-sequencing has shown that, with the exception of *EZH2* and *CUX1*, recurrent somatic mutations in 7q genes are rare. This raises the question of whether additional 7q TSGs remain to be revealed. Here we implement *in silico* and *in vitro* screening as an alternative means to systematically uncover latent 7q TSGs.

Methods:

*Arrayed CRISPR screen:* gRNAs were designed for 161 chromosome 7 genes and transfected into peripheral blood mobilized CD34+ hematopoietic stem cells (HSCs). Cells were split into proliferation and erythroid differentiation cultures, and Sanger sequencing for editing (Figure 1B). Proliferation was assayed by CelltiterGlo. Differentiation was measured by flow cytometry for CD71 and GlyA.

*Machine Learning*: We used a classification random forest model to predict putative tumor suppressors on chromosome 7, based on publicly available genome-wide datasets.

See Supplemental Methods for details.

Results and Discussion:

To comprehensively identify chromosome 7 TSGs in an unbiased manner, we leveraged published genome-scale data. We mined genome-wide proliferation screens using shRNA, CRISPR, gene-trap, and ORF libraries performed in hematopoietic and non-hematopoietic cells. We included TSGs predicted from pan-cancer analyses of mutational patterns across thousands of primary patient cancers. TSGs from the Cancer Gene Census list were incorporated. We excluded studies with insufficient datasets available for analysis. From these 11 datasets (4-14), we identified 96 coding genes with evidence of TSG activity that are also expressed in human HSCs (Supplemental Table 1). For completeness, we included all HSC-expressed coding genes within the CDRs of 7q (q21.3, q22.1, q34, and q35-36), resulting in a total of 161 genes (15)(Figure 1A).

We chose an array-based CRISPR screen in lieu of a pooled approach for increased power to detect features of TSGs, namely proliferation and differentiation (16). We transfected Cas9-gRNA ribonucleoprotein complexes into primary human CD34+ HSCs in a 96-well format, with one targeted gene per well. The cells were assessed for increased proliferation and impaired erythroid differentiation as indicators of myeloid TSGs (17). *AAVS1* gRNA was used as a negative control (18). gPTEN was included as a positive control for increased proliferation, and gGATA1 was used as a control for decreased differentiation (Supplemental Figure 1)(19,20). Each gRNA was tested in 3-4 biological replicates with 108 guides (excluding controls) passing a threshold of >25% mean editing across all replicates (Figure 1C). We chose this threshold based on the assumption that knockout of a TSG causes edited cell outgrowth, which was borne out (Supplemental Figure 2). We selected day 5 for proliferation measurements as that time point showed the greatest separation of genes without plateau of growth (Supplemental Figure 3).

39 target genes significantly increased proliferation when edited (Figure 1D). Conversely, only two genes, *CUX1* and *ACHE*, had a significant impairment of erythroid differentiation (Figure 1D,E). This discrepant number of hits may partially relate to the lack of technical replicates for each erythroid experiment, but may also indicate that *CUX1* and *ACHE* are most critical for differentiation defects in -7/del7q myeloid malignancies. Across all genes, accelerated proliferation correlated with impaired erythropoiesis, consistent with the known link between these two processes (Figure 1F). Using a combined proliferation and erythroid impairment metric, 12 target genes scored significantly different from the AAVS1 control (Figure 1F). Overall, while an unexpectedly high number of genes increased HSPC proliferation, only two of these, *CUX1* and *ACHE*, also decreased erythropoiesis when edited.

Across all genes, their effect on proliferation was non-random, with a significant bias towards increased proliferation (Supplemental Figure 9A). This is perhaps a consequence of our inclusion criteria (Fig. 1A). The effect size for genes within CDRs was significantly more pronounced (Figure 1H), as measured by either proliferation alone or through the combined score (Figure 1I). This later result is remarkable because the CDR genes were not pre-selected via datamining. That the CDR regions are significantly enriched for TSGs implies that the deletion of these regions in myeloid malignancies contributes to disease progression through the combined loss of several contributing genes.

We next sought to validate and extend our finding of increased TSGs within CDRs through an orthogonal approach assessing all chromosome 7 coding-genes. Advances in genome wide perturbation screens such as RNAi-based and CRISPR-based screens have generated enormous amount of data for us to mine putative TSGs. We sought to utilize these data to learn and predict genes whose perturbation leads to cell growth phenotypes that are consistent with tumor suppressors. In recent years, machine learning has become a powerful tool in uncovering biological associations hidden in large genomic datasets (21). We implemented a random forest classification model using 29 variables from eight genome-wide perturbation screens in cancer cell lines, along with mutational signatures from *Davoli et al. 2013,* to assign a TSG score for each gene (Supplemental Figures 7 and 8). We trained the data on all non-chromosome 7 genes and measured the performance of the classification system using the Cancer Gene Census TSG designations as the ground truth (average AUC of 0.777[0.747 – 0.806, 95% CI]). We then ran the classifier on chromosome 7 genes, and many highly scoring genes overlapped with experimental hits, such as *CUX1, LUC7L2, and TRIM24* (Figure 1G)(22,23). Using the classifier scores, genes within CDRs are again significantly enriched for TSGs (Figure 2D). This result from disparate datasets, across tumor types, mirrors our experimental results. To our knowledge, the use of machine learning with genomic and CRISPR screen data to identify TSGs has not been previously reported. Furthermore, our result buttresses the concept of del(7q) as a contiguous gene syndrome.

Other than *CUX1*, *ACHE* was the only gene with significant experimental effects on both proliferation and differentiation. Located within the 7q22.1 CDR, *ACHE* encodes extracellular membrane-associated acetylcholinesterase, also known as the Cartwright blood group on erythrocytes. While its function on erythrocytes is unclear, acetylcholinesterase’s canonical role is to degrade acetylcholine and abrogate acetylcholine receptor signaling (24). As the acetylcholine pathway is a druggable target with multiple antagonists FDA-approved or in clinical trials (25), we chose this candidate for further investigation. We first validated our findings with independent gRNAs targeting *CUX1* and *ACHE*. All four gRNAs edited their targets*,* resulting in decreased protein (Figure 2E,H) and recapitulated the effects on proliferation and erythroid differentiation seen in the screen (Figure 2F,G and K,L). We then tested the effect of the muscarinic acetycholine receptor inhibitor oxyphenonium bromide in our proliferation and differentiation assays and found that it had the opposite effect of loss of *ACHE*, causing decreased proliferation and increased erythroid differentiation (Figure 2I,J). Importantly, oxyphenonium bromide treatment in gACHE cells restored proliferation and differentiation to the same level as the AAVS1 control (Figure 2K,L). This is congruent with a model wherein loss of ACHE in HSCs causes increased muscarinic acetylcholine receptor signaling, leading to increased proliferation and impaired erythroid differentiation, contributing to the myeloid expansion and anemia seen in -7/del7q myeloid malignancies (Figure 2M). Therefore, pharmaceutical targeting of acetylcholine signaling may be a novel treatment avenue in -7 myeloid disease.

Overall, this study identified several novel Chromosome 7 TSGs for further investigation and demonstrated a strong TSG enrichment within the 7q CDRs, bringing us closer to understanding the complex etiology of -7/del(7q) myeloid malignancies.

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