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The role of DNMT3A and HOXA9 hypomethylation in acute myeloid leukemia (AML)

Introduction

Epigenetic modifications of DNA and histones are key to gene regulation throughout development. DNA methylation is an epigenetic modification in which a methyl group is transferred from the universal methyl donor, S-adenosyl-methionine (SAM), and covalently bonded to the C5 position of the cytosine moiety[1]. DNA methylation occurs mainly at CG residues, with 60-80% of CG residues methylated across the genome[2]. Non-CG methylation is also observed, but only on actively transcribed genes within somatic stem cell lineages[2]. Less than 10% of the CpG residues are located in CpG islands, regions of high CG density that are typically located at transcription start sites of developmentally important genes[2]. Methylation typically acts as an epigenetic repressor of gene expression, with methylation within the promoter of a gene preventing its transcription.

The process of DNA methylation is carried out by the DNA methyltransferase family of enzymes, which in mammals includes DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L[4]. DNMT1 is involved in maintaining the parental methylation patterns in newly synthesized DNA strands, and preferentially binds to hemimethylated substrates[5]. DNMT3L is a non-catalytic accessory protein to DNMT3A, and assists with methylation during imprinting and embryonic development[4]. DNMT3A and DNMT3B are responsible for *de novo* DNA methylation, and the two enzymes have overlapping regions in which they act. DNMT3A and 3B are critical to differentiation of ESCs, as deletion of both genes results in continuous self-renewal and reduced differentiation capacity. DNMT3A$^{-/-}$ hematopoietic stem cells (HSCs) have an overall loss in DNA methylation, particularly at the edge of CpG "canyon" regions that contain genes involved in self-renewal and cancer[4].

Many studies over the previous 20 years have found that anomalous global DNA methylation patterns are associated with cancers[6]. However, in spite of this knowledge, the first malignancy-associated mutations in DNMT3A were only identified in 2010. Three separate groups demonstrated that DNMT3A was mutated in acute myeloid leukemia (AML) with mutation frequencies of up to 22%[4]. Mutations in DNMT3A have since been found in other types of cancers, including lung cancer and acute lymphoblastic leukemia[7]. However, the mechanisms through which defective DNMT3A affects leukemia development remain unclear.

A recent study showed a correlation between the DNMT3A mutation and hypomethylation of homeobox-containing (HOX) genes in cytogenetically normal AML (CN-AML) patients[8]. HOX genes are critical to embryogenesis and hematopoietic development, and their overexpression has been implicated in the development of AML. Of particular interest is HOXA9, a transcription factor that is 2-8 fold overexpressed in approximately 50% of AML cases[9]. The dysregulation of HOXA9 also strongly correlates with poor disease prognosis[10]. This research study will examine the connection between DNMT3A mutation and HOX9A methylation and the downstream effects on tumorigenesis.

Hypothesis & Predictions

I hypothesize that loss of DNMT3A increases propensity for leukemia through the dysregulation of HOXA9 epigenetic marks. This is supported by the observation that DNMT3A$^{-/-}$ cancers frequently show hypomethylation of HOX genes, and that overexpression of HOXA9 is associated with increased incidence of leukemia.

I predict that knockout of the DNMT3A gene in human myeloid cells will result in hypomethylation of the HOXA9 gene promoter and overexpression of the HOXA9 gene. Cells possessing this altered methylation and expression pattern will have an increased propensity towards leukemia development when transplanted into a mouse model.

Experimental Plan

1. Cell culture and myeloid differentiation

The *in vitro* experiments in this study will be performing in Hoxb8-FL cells. These cells are hematopoietic progenitor cells that have lost the potential to differentiation into erythrocytes or megakaryotes, but can still generate myeloid and lymphoid lineages[14]. The cells will then be predisposed towards a myeloid cell fate by treating cells with GM-CSF and M-CSF, which support the generation of dendritic cells and granulocytes. The cell identity will be confirmed through flow cytometry, using the myeloid markers IL-3R alpha, CD34, c-kit, and Flk-2.

2. Knockout of DNMT3A gene

Following differentiation, the DNMT3A gene will be knocked out using the CRISPR-Cas9 system. The sgRNA sequence corresponding to the DNMT3A methyltransferase domain will be cloned into a plasmid containing the essential CRISPR elements. This plasmid will then be delivered along with a Cas9 plasmid to target cells. The knockout of DNMT3A will be confirmed via polymerase chain reaction (PCR), amplifying the region of interest using primers that flank the methyltransferase gene. As a control, cells will receive a scramble plasmid rather than the DNMT3A sequence.

3. Assess methylation status of the HOXA9 gene

When it has been confirmed that DNMT3A has been knocked out, the methylation state of the HOXA9 gene must be assessed. This will be achieved through bisulfite conversion and pyrosequencing. Bisulfite conversion deaminates unmethylated cytosines, resulting in uracil. The uracil residues are amplified as thymines during subsequent PCR reactions[15]. Methylated cytosine residues are not deaminated and therefore remain cytosines through subsequent PCR and sequencing. Pyrosequencing will be performed using the QIGEN platform, and the percent methylation of the entire HOXA9 gene will be determined in the control and DNMT3A$^{-/-}$ cells.

4. Quantify expression of HOXA9 gene

To determine whether differences in methylation of the HOXA9 gene have actually resulted in changes in gene expression, the expression levels of the HOXA9 transcript and protein will be measured. qPCR will be performed on the DNA from both the wild-type and DNMT$^{-/-}$ cells, using primers specific to HOXA9 and a control housekeeping gene such as GAPDH. Protein expression will be measured by Western blot, using an antibody specific to HOXA9. A negative control lysate from a cell line known not to express HOXA9 and the positive control lysate

provided by the antibody producer will be run alongside the cell lysate from both transfection conditions.

5. Xenografts to assess cells' tumourigenic capacity

Finally, I will investigate the tumorigenic capacity of the $DNMT^{-/-}$ cells relative to wild-type when transplanted into irradiated NOD/LtSz-$Prkdc^{scid}$ $Il2r\gamma^{tmlWjl}$/J (NSG) mice. This strain was chosen since it is immunodeficient, which reduces the risk of transplant rejection, and has the highest engraftment success in AML studies[16]. One group of mice will receive a sham surgery to control for the effects of the transplant. The blood cell counts of the mice will be monitored weekly, looking for signs of leukemic disease. Survival (time-to-event) analysis will be performed, with the "event" being defined as the point at which the blood count meets the diagnostic criteria for AML – specifically, the accumulation of blasts within the bone marrow, resulting from the differentiation block that is characteristic of the disease[11]. AML will be diagnosed when the bone marrow contains 20% blasts, according to the World Health Organization classification system[11].

Possible Results and Discussion

1. Cell culture and myeloid differentiation

Upon the withdrawal of estrogen from the cell culture media, M-CSF and GM-CSF will be added to the cells to promote differentiation into granulocytes and dendritic cells. *In vitro*, this has been shown to successfully differentiate the HoxB8-FL lines into myeloid cells. Upon flow cytometry, the cells should be positive for markers that are characteristic of the myeloid lineage, and be negative for markers of the lymphoid lineage. If the cells are positive for markers of both lineages, it is possible that the concentrations of GM-CSF and M-CSF should be increased to promote more robust differentiation. Another possibility is to pursue differentiation into more specific myeloid cell types, such as dendritic cells alone (through the addition of Flt3L to the cell culture medium) or to macrophages (by adding M-CSF to the culture medium).

2. Knockout of DNMT3A gene

The cloning of the plasmids can be determined to be successful through growth of the bacteria on a medium containing an antibiotic, such as puromycin, to which the plasmid is resistant. If there are no colonies that grow on the plate following transformation, it can be assumed that the transformation was unsuccessful. Once cloning has been successfully achieved, the plasmids will be introduced to the cells, and excision of the Dnmt3a catalytic domain by Cas9 will be attempted. If the knockout is successful, running PCR on the extracted DNA using primers that flank the Dnmt3a region of interest will yield a small band that does not include the Dnmt3a catalytic sequence. However, if the knockout is unsuccessful, the band will be larger, as it will still contain the sequence of interest. In this case, the scramble and Dnmt3a sgRNA conditions will be identical. Should this be the case, troubleshooting for the CRISPR-Cas9 process should take place.

3. Assess methylation status of the HOXA9 gene

If the DNMT3A gene has been successfully knocked out during the previous step in the experimental procedure, there are three possible outcomes for this experiment. The first is that loss of DNMT3A results in hypomethylation of the HOXA9 gene. This explanation would support the experimental hypothesis and the existing data, which shows that homeobox-

containing genes are hypomethylated in DNMT3A knockout cells. The second possible outcome is that loss of DNMT3A results in hypermethylation of the HOXA9 gene. This result would be surprising, as previous studies have shown the opposite outcome. Should this arise, the methylation status of other homeobox-containing genes would be assessed to see if this was a result unique to HOXA9. The third possible outcome is that there is no difference in methylation status between $DNMT3A^{WT}$ and $DNMT3A^{-/-}$. A possible explanation for this could be that DNMT3B, the other *de novo* methyltransferase in mammals, compensates for the loss of DNMT3A activity. This could be tested by measuring the expression levels of DNMT3B mRNA transcripts and proteins to see if they are elevated in the DNMT3A knockout cells relative to wild-type.

4. Quantify expression of the HOXA9 gene

The HOXA9 gene expression will be quantified at the mRNA and protein levels. mRNA will be assessed through qPCR. If the HOXA9 gene was hypomethylated upon the knockout of DNMT3A, there are three possible outcomes. The first is that hypomethylation is correlated with increased transcription relative to wild-type. This would agree with the experimental hypothesis, and would make logical sense as hypomethylation is typically associated with increased expression of the target gene. The other possible outcomes are that hypomethylation results in either unchanged or reduced mRNA expression. If this were the case, I would examine the precise locations where methylation was taking place on the gene, and determine if they were in non-regulatory regions such as introns. Protein expression levels would generally be expected to correlate with mRNA expression levels, but it is possible that mRNA levels are overexpressed while protein levels are low. In this case, I would consider whether miRNAs target the mRNA transcript of the HOXA9 gene, and would examine their expression in the cells.

5. Xenograft transplants to assess leukogenic potential

Finally, the cells would be transplanted into the bone marrow of a mouse model. The mice receiving the $DNMT3A^{-/-}$ cells would be expected to develop AML at a higher rate relative to the controls. Even if no correlation is found between DNMT3A knockout and HOXA9 expression, this experiment would be worth pursuing, as studies to date involving DNMT3A mutations have mainly characterized clinical samples with pre-existing mutations. It is also possible that the mice receiving DNMT3A KO cells will not develop leukemia at a higher rate relative to controls. This would be unexpected, as DNMT3A is associated with an increased predisposition towards AML. However, since studies have shown that DNMT3A mutation is not sufficient to induce leukemia, it may be that another factor (mutation, epigenetic change, etc.) is required in order to make the transition towards malignancy.

Relevance and potential impact

Acute myeloid leukemia (AML) is the most common cancer of the myeloid lineage, characterized by the dysregulation of differentiation and proliferation in myeloid progenitor cells ("blasts")[11]. Its prevalence is 3.8 cases per 100,000, but the risk increases with age, rising to 17.9 cases per 100,000 in adults over age 65. The five-year survival rate for the condition is 26%, with even lower survival rates for the elderly, who typically do not respond well to treatment[12].

Deep sequencing of malignancies suggests that DNMT3A mutations are among the first to originate in hematological cancers[4]. $DNMT3A^{-/-}$ hematopoietic stem cells have been firmly established as pre-leukemic stem cells, frequently acting as the founding clones in AML[13]. The $DNMT3A^{-/-}$ mutation alone is not sufficient to induce leukemia, but it is associated with an

increased risk for leukemia development[4]. By understanding the downstream effects of this enzyme, we will gain a better idea of how leukemia develops, and may identify novel therapeutic targets to prevent malignancy.

Lay Person Summary

Small molecules called enzymes carry out a wide variety of important processes in the body. One such molecule is DNMT3A, which adds modifications to DNA regulatory regions to prevent genes from being expressed as proteins. This process is called DNA methylation, and it is used to regulate the expression of different genes in the cells of the body.

Studies have shown that DNA methylation patterns are altered in many types of cancer, including acute myeloid leukemia (AML). This is a type of blood cancer in which blood stem cells fail to grow into mature blood cells, leaving immature cells called "blasts" crowding the bone marrow and impairing its function. It is a fast-developing cancer with a five-year survival rate of less than 1 in 4. Mutations in the DNMT3A gene that prevent it from functioning properly has been observed in up to 22% of AML cases, as well as in other types of cancer. DNMT3A is one of the earliest mutations that occurs in AML, which suggests that its dysfunction might predispose cells to leukemia development.

But how does this happen? Few studies have investigated the mechanisms through which defective DNMT3A can affect AML. However, a recent study showed that there may be a link between the DNMT3A mutation and decreased methylation of a group of genes called the homeobox-containing (HOX) genes. One member of this family is HOX9A, . Since decreased methylation of a gene can cause increased expression of the gene's protein product, it is possible that the dysfunctional DNMT3A protein is unable to methylate the HOXA9 gene, leading to overexpression and AML. This study will investigate this possibility by destoying the DNMT3A gene in blood cells, assessing the methylation levels of the HOXA9 gene, and transplanting the cells into mice to determine their ability to cause leukemia. By developing a better understanding of DNMT3A's role in leukemia development, it will be possible to identify new targets for therapies to help treat those with AML.

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