Molecular Dissection of the 5q Deletion in Myelodysplastic Syndrome

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The 5q-syndrome is a subtype of myelodysplastic syndrome (MDS) with a defined clinical phenotype associated with heterozygous deletions of chromosome 5q. While no genes have been identified that undergo recurrent homozygous inactivation, functional studies have revealed individual genes that contribute to the clinical phenotype of MDS through haplo-insufficient gene expression. Heterozygous loss of the *RPS14* gene on 5q leads to activation of p53 in the erythroid lineage and the macrocytic anemia characteristic of the 5q-syndrome. The megakaryocytic and platelet phenotype of the 5q-syndrome has been attributed to heterozygous deletion of miR145 and miR146a. Murine models have implicated heterozygous loss of *APC*, *EGR1*, *DIAPH1*, and *NPM1* in the pathophysiology of del(5q) MDS. These findings indicate that the phenotype of MDS patients with deletions of chromosome 5q is due to haplo-insufficiency of multiple genes.
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Among the diverse clinical presentations of myelodysplastic syndromes (MDS), a subset of patients has isolated deletion of chromosome 5q and a distinctive set of features. This hematologic phenotype, termed the 5q-syndrome, includes a severe macrocytic anemia, a normal or elevated platelet count with hypolobated micromegakaryocytes, a normal or slightly decreased neutrophil count, and a low rate of progression of acute myeloid leukemia (AML) relative to other types of MDS.[1-6] The association of a clinical phenotype with a chromosomal deletion has provided a scientific opportunity to attribute individual clinical features with deletion of particular genes.

Deletions of chromosome 5q in MDS are somatically acquired, heterozygous, and encompass many genes.[7] Heterozygous chromosomal deletions in cancer often highlight the locus of a tumor-suppressor gene that undergoes homozygous inactivation, but in other cases, disease is caused directly by mono-allelic deletions due to haplo-insufficiency for one or more genes. In the case of del(5q) MDS, genetic lesions on the nondeleted allele have not been identified, despite extensive searches for microdeletions or mutations that lead to homozygous inactivation.

While the vast majority of patients with del(5q) MDS have large deletions, rare patients have smaller chromosomal deletions that have enabled geneticists to localize common deleted regions (CDRs) that are minimally necessary for a clinical phenotype. Two CDRs have been reported (Figure 1). The CDR located more distally on chromosome 5q is minimally sufficient for the 5q-syndrome and is located at 5q52–33.[8,9] The CDR located more proximal to the centromere is located at 5q31.[10-12] Most patients have deletions that encompass both loci, but the CDRs provide a starting place for the search for critical genes.[7]

PATHOGENESIS OF ANEMIA IN DEL(5Q) MDS

Anemia is the most prominent cytopenia in patients with the 5q-syndrome. The anemia is macrocytic, and patients are generally transfusion-dependent. Given the stability of the disease, with low rates of progression to AML, iron overload from chronic transfusions can be a significant cause of morbidity and mortality.[7,13-15]

The distal CDR, associated with the 5q-syndrome, contains 40 genes.[9] Conditional deletion of this entire region recapitulates the severe macrocytic anemia in a murine model.[16] Sequencing of genes and microarray-based analysis of copy number have failed to identify mutations or microdeletions that would lead to homozygous inactivation, fulfilling Knudson's two-hit hypothesis for a tumor suppressor.[17] An alternative explanation is that heterozygous deletion of a critical gene is pathogenic. Since deletions of one allele are large, and no abnormalities have been reported on the allele, genetic studies have not been able to identify a crucial gene. Functional studies are an alternative approach to

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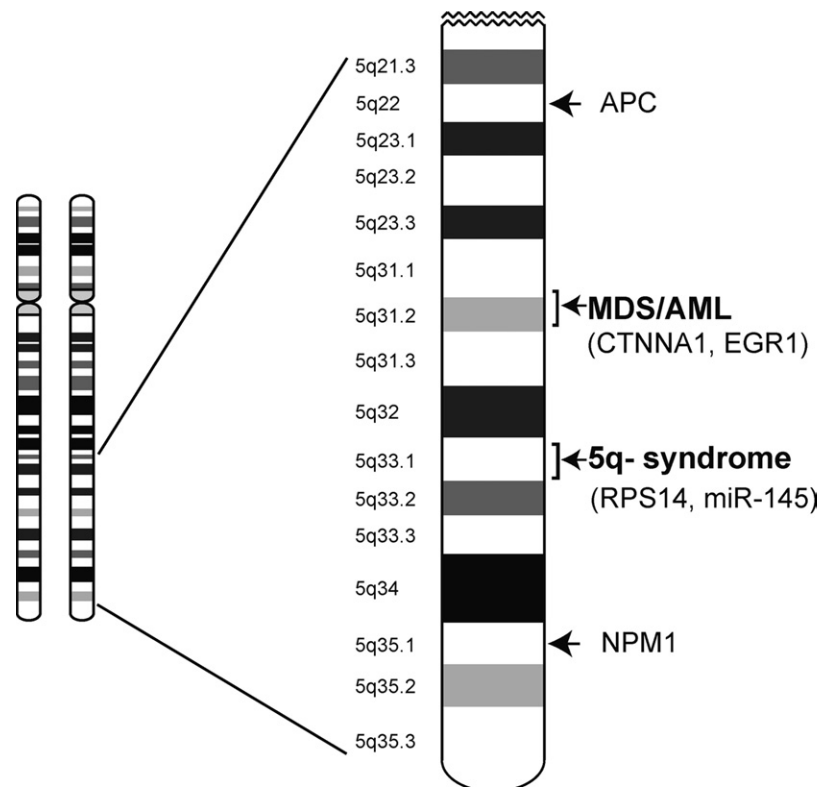

Figure 1. Schema of the CDRs on chromosome 5q for MDS and key genes.

the molecular dissection of the 5q deletion and the identification of pathologic roles for individual genes.

The *RPS14* gene was identified as a critical gene for the erythroid phenotype of the 5q-syndrome using an RNA interference screen.[18] Each of the 40 genes in the 5q32–33 CDR was targeted by short hairpin (sh)RNAs, enabling a systematic evaluation of the effects of decreased expression of each gene. The shRNAs were introduced into primary human hematopoietic stem and progenitor cells using lentiviral vectors in order to examine the effects on hematopoietic differentiation. Only shRNAs targeting the *RPS14* gene caused a severe block in erythroid differentiation. Moreover, forced overexpression of *RPS14* in cells with del(5q) MDS rescued erythroid differentiation. Expression of *RPS14* in del(5q) MDS is approximately 50% of expression in non-del(5q) MDS, and the nondeleted allele is not deleted, demonstrating that *RPS14* is a haplo-insufficiency disease gene.[18–21]

Heterozygous inactivating mutations have been described in a congenital syndrome, Diamond Blackfan anemia (DBA).[22,23] DBA patients have a severe macrocytic anemia, analogous to the erythroid defect in the 5q-syndrome. Approximately 25% of cases of DBA have mutations in the *RPS19* gene, and mutations have now been reported in more than eight ribosomal genes.[22,24–27] The mutations are universally heterozygous, and functional studies indicate that homozygous inactivation of most if not all ribosomal genes would not be tolerated in a mammalian cell. In aggregate, the genetic data strongly implicate haplo-insufficiency for ribosomal genes in the pathogenesis of macrocytic anemias of both DBA and the 5q-syndrome.[23]

Studies of both del(5q) MDS and DBA have demonstrated that induction of p53 is essential for the erythroid failure in the setting of ribosomal gene haplo-insufficiency.[16,28,29] Decreased expression of *RPS14* and *RPS19* causes a dramatic increase in total p53 levels, expression of p53 target genes including p21, and cell cycle arrest.[30] In murine models, the macrocytic anemia associated with heterozygous loss of *RPS14* (in combination with eight adjacent genes) or heterozygous mutation of *RPS19* in a p53 null background.[16] These studies indicate that haplo-insufficiency for ribosomal protein genes causes a p53-mediated cell cycle arrest in erythroid progenitor cells and a consequent macrocytic anemia.

PATHOGENESIS OF DYSMEGAKARYOCYTOPOIESIS

While thrombocytopenia is common in MDS in general, some patients with del(5q) MDS have an elevated platelet count, and patients commonly have hypolobated micromegakaryocytes.[13] Patients with DBA do not have thrombocytosis, indicating that haplo-insufficiency for a ribosomal gene does not generally cause an

elevated platelet count or the distinctive megakaryocyte morphology.[23]

A microRNA cluster is located within the CDR associated with the 5q-syndrome, containing miR-143 and miR-145. In addition, miR146a is located just telomeric to the CDR. Expression of miR-145 and miR-146a is lower in patients with del(5q) MDS compared with MDS patients with an intact chromosome 5. In both human and murine cells, decreased expression of miR-145, or miR145 and miR-146a together, cause an increased expression of miR-145.[31,32]

While miR-145 and miR-146a likely regulate the expression of many genes, several critical targets have been identified. Two key regulators of the innate immune response, Toll-interluekin-1 receptor domain-containing adaptor protein (TIRAP) and tumor necrosis factor receptor-associated factor-6 (TRAF6) are targeted by miR-145 and miR-146a, respectively. In mice, decreased expression of both miR-145 and miR146, or forced overexpression of TRAF6, causes thrombocytosis and the hypolobated micromegaryocyte characteristic of the 5q-syndrome.[32]

The *FLI-1* gene, encoding a critical transcriptional regulator of megakaryocyte differentiation, is also a target of miR-145.[31] Decreased expression of miR-145 causes increased FLI-1 levels, with consequent increase in megkaryocyte production. In aggregate, these data indicate that the effects of haplo-insufficiency for RPS14 and heterozygous deletion of miR145 and miR146a are integrated in the phenotype of the 5q-syndrome, and that the 5q-syndrome is a contiguous gene syndrome.

CLONAL SELECTION, DISEASE PROGRESSION, AND OTHER ASPECTS OF DISEASE PHENOTYPE

The del(5q) lesion is present in the hematopoietic stem cell compartment and can be found in all lineages.[33,34] Cells harboring del(5q) gain clonal advantage in the bone marrow, and the deletion remains present in leukemias that result from progression of del(5q) MDS. A systematic examination of all genes on 5q for genes that might contribute to clonal advantage of the del(5q) clone has not been reported. However, experiments in murine models have provided functional evidence for the role of individual genes in hematopoietic stem cell function and leukemic progression.[7]

The *EGR1* gene is located in the 5q31 CDR. In a murine model with heterozygous inactivation of *EGR1*, hematopoietic stem cells have increased proliferation and mobilization from the bone marrow.[35] In addition, mice with heterozygous loss of *EGR1* develop leukemia with increased frequency and decreased latency in mice treated with a DNA alkylating agent, N-ethyl-nitrosourea (ENU), to induce secondary mutations.[36] These findings indicate that heterozygous deletion of *EGR1* may play a functional role in the pathogenesis of MDS and AML in patients with del(5q).

The *APC* gene is located on 5q23 is deleted in more than 95% of cases with del(5q) MDS. *APC* is a negative regulator of beta catenin function, and inactivating mutations in *APC* are pathogenic in colon cancer. Heterozygous deletion of *APC* in a mouse model causes expansion of the long-term hematopoietic stem cell population, but decreased repopulation of secondary recipients in bone marrow transplantation assays.[37] Similarly, mice bearing the *APC*(min) allele, resulting in heterozygous loss of function for APC, have increased repopulating potential in primary bone marrow transplants, but decreased repopulation potential of secondary recipients due to loss of quiescence in the hematopoietic stem cell compartment.[38] In both models, heterozygous inactivation of *APC* results in MDS or MDS/myeloproliferative neoplasms phenotype. Given the role of beta catenin in stem cell self-renewal and hematologic malignancy, *APC* haplo-insufficiency may contribute to the pathogenesis of del(5q) MDS through increased beta catenin activity.[37,38]

Multiple other genes have been implicated in the pathogenesis of del(5q) MDS, but the precise role of these genes in the phenotype of patients with del(5q) has not been established. The *SPARC* gene is located in the CDR for the 5q-syndrome, and homozygous inactivation of the *SPARC* gene in mice causes thrombocytopenia and decreased erythroid colony formation.[39] *SPARC* has haplo-insufficient expression in del(5q) MDS, and treatment with lenalidomide increases expression of the gene.[19,40] The *DIAPH1* gene encodes a protein involved in actin dynamics.[41] In one proposed model, this gene may integrate the effects of haplo-insufficiency for multiple genes on 5q, including RPS14 and EGR1.[41]

PHENOTYPIC HETEROGENEITY IN PATIENTS WITH DEL(5Q) MDS

The vast majority of patients (>95%) with del(5q) MDS have large deletions that encompass both defined CDRs and additional segments of the chromosome, but not all patients with isolated deletions of chromosome 5q have the full collection of features ascribed to the 5q-syndrome. Additional molecular abnormalities, independent of chromosome 5q, also contribute to the phenotype of patients with del(5q), including other cytogenetic abnormalities, somatic mutations in individual genes, and aberrant epigenetic alterations.[42,43] Each of these abnormalities has the potential to alter hematopoietic differentiation and blood counts, the percentage of blast cells in the bone marrow, progression to leukemia, and overall survival. Thus the phenotypic heterogeneity of patients with del(5q) MDS is not surprising, but further research is required to define

the precise contributions of these additional molecular abnormalities.

The size of deletions on chromosome 5q is likely to alter disease phenotype, but this has yet to be demonstrated conclusively. Rare patients with small deletions have been extremely informative for the identification of CDRs, but the number of patients with these deletions is small, making strong genotype–phenotype associations difficult. Monosomy for chromosome 5 is nearly always associated with multiple cytogenetic abnormalities, while del(5q) occurs commonly in isolation. It is also possible that deletions that encompass *NPM1* on distal 5q may have a more aggressive phenotype given the genetic instability caused by *NPM1* haplo-insufficiency.[44] Future studies with precise mapping of deletion boundaries in large numbers of patients will be necessary to determine whether the extent of deletions correlate with clinical phenotype.

The state of the bone marrow microenvironment in MDS pathogenesis is poorly understood, but is a possible contributor to disease phenotype. For example, osteoblasts are thought to be a critical component of the hematopoietic stem cell niche.[45,46] Mice with dysfunctional osteoblasts due to selective inactivation of micro-RNA processing in osteoblast progenitor cells develop dysplasia of myeloid lineage cells with ineffective hematopoiesis.[47] The role of the bone marrow microenvironment in patients with MDS remains to be determined.

Phenotypic heterogeneity in MDS patients with del(5q) is the inevitable consequence of the numerous influences on the del(5q) clone. These influences include additional clonal genetic abnormalities, epigenetic alterations, and perhaps an aberrant microenvironment and the status of normal (non-MDS) hematopoietic stem and progenitor cells.

CONCLUSIONS: VALIDATING HAPLO-INSUFFICIENCY DISEASE GENES

Increasing evidence supports the hypothesis that heterozygous deletions of chromosome 5q in MDS causes haplo-insufficiency for multiple genes that alter hematopoiesis. The phenotype encompassed by the 5q-syndrome is likely generated by the integration of effects from decreased expression of multiple genes. Specific aspects of the clinical phenotype have now been ascribed to distinct genes.

Validation of the functional importance of candidate genes within heterozygous deletions presents particular challenges. In general, genetic evidence of recurrent somatic mutations, translocations, or copy number alterations for a particular gene has provided incontrovertable evidence of the importance of this gene in a type of cancer. This type of evidence has not been obtained for genes within the 5q deletion in MDS, despite a great deal of effort, and may never be found. Large heterozygous deletions may be the only recurrent genetic event. In this case, only functional studies can demonstrate the importance of a particular gene, but these studies are challenging and can lead to false conclusions.

A particularly appealing approach to heterozygous deletions is to evaluate all genes within a CDR systematically. This has been accomplished in the 5q32–33 CDR using two approaches, RNA interference and a series of conditionally floxed mice.[18,48] The examination of all genes within a CDR removes the bias inherent in the examination of single candidate gene in isolation.

In theory, haplo-insufficiency can be rescued by forced overexpression of the gene. In the case of del(5q) MDS, rescue of some aspect of the MDS phenotype by overexpression of a candidate gene in hematopoietic stem and progenitor cells that bear the 5q deletion provides powerful evidence in support of a pathogenic role for that gene. This experiment is associated with a number of technical challenges: viable progenitor cells from MDS patient bone marrow aspirates must be obtained, purified, and cultured; these cells must be efficiently transduced with a virus expressing the relevant cDNA or control; and expression of the cDNA must approximate endogenous expression, rescuing haplo-insufficiency. For example, overexpression of the *RPS14* gene in del(5q) MDS cells rescues erythroid differentiation.[18] Such experiments are important for the validation of key candidate genes.

Murine models of heterozygous inactivation of candidate genes can provide a critical demonstration of the effects of haplo-insufficiency for a gene in vivo. While the phenotype of murine models of hematologic malignancy does not always fully recapitulate the human disease, mice with conditional deletion of the 5q-syndrome CDR causes the expected macrocytic anemia.[48] Murine models also have provided support for the effects of heterozygous loss of *NPM1*, *EGR1*, and *APC*.[35–38,44]

Functional studies, in vitro and in vivo, have provided critical insights into the biology of del(5q) MDS. Activation of p53 in response to heterozygous loss of *RPS14*, or other ribosomal genes that are mutated in DBA, leads to a macrocytic anemia in multiple models. Anemia, thrombocytosis, and clonal advantage are likely due to the combined effects of haplo-insufficiency for multiple genes. These studies provide an example for the elucidation of critical genes within other recurrent heterozygous deletions in human malignancies.

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