

**Second hit mutations in the RTK/RAS signaling pathway in acute myeloid leukemia with inv(16)**

We report that 69% of patients with acute myeloid leukemia (AML) and inv(16) possess either a RAS or receptor tyrosine kinase (RTK) mutation (e.g. *c-KIT* or *FLT3*). These findings, together with the fact that 90% of the mutations were mutually exclusive, lend support for the *two-hit* model of leukemogenesis and suggest possibilities of targeted therapy for patients with AML and inv(16).

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Inversion of chromosome 16, inv(16)(p13q22) is found in a subset of patients with AML, especially the M4Eo subtype, and results in the molecular fusion of the *CBFβ* gene on 16q22 and the *MYH11* gene on 16p13.<sup>1</sup> The mechanism by which the resultant chimeric *CBFβ*-*SMMHC* protein induces leukemogenesis is unclear, although fusion gene expression by itself is not sufficient to cause leukemia.<sup>2</sup> For example, mice transgenic for the chimeric gene *CBFβ*/*MYH11* exhibit a myeloid maturation block but require additional mutations for the development of leukemia, suggesting that additional hits are required for transformation. Recently, Gilliland has proposed a pathogenetic model of AML based upon the interaction of at least two classes of mutation; a class I mutation that confers a proliferative signal (e.g. a RTK/RAS mutation), and a class II mutation that inhibits hematopoietic differentiation, such as a CBF fusion gene.<sup>3</sup> We have shown that 40% of AML with inv(16) present with mutually exclusive RTK mutations involving either *c-KIT* or *FLT3*.<sup>4</sup> Since RAS and RTK have important interactions, we extended these studies to RAS mutations in the same cohort of patients.

Genomic DNA and RNA was obtained, at presentation, from the bone marrow or peripheral blood of 58 patients with AML and inv(16) (34 males, 24 females, mean age 43.4 years, range 15-74). The methods and preliminary results of *c-KIT* and *FLT3* mutational analysis of these cases have been published previously.<sup>4,5</sup> N- and K-*Ras* mutational screening, involving codons 12, 13 and 61, was undertaken on 44 cases using heteroduplex analysis (WAVE technology, Transgenomic Inc. San José, California, USA) as previously described,<sup>6</sup> while 14 cases were analyzed using a cDNA based approach. Quality control was carried out by exchanging blinded samples between the different centers involved. Mutation detection was 100% concordant.

A total of 19 patients (33%) possessed one or more activating *RAS* mutations involving either N-*RAS* (n=15) or K-*RAS* (n=5). In three individual cases more than one *RAS* mutation was noted N-*RAS*12/13 (n=1); N-*RAS*12/61 (n=1) and N-*RAS*12/K-*RAS* 12 (n=1). The mutational frequencies for individual codons were as follows: N-*RAS* codon 12 (7%, 4 of 58), codon 13 (12%, 7 of 58) and codon 61 (12%, 7 of 58); K-*RAS* codon 12 (7%, 4 of 58). Fifteen patients (26%) had *c-KIT* exon 8 mutations, four (7%) had *c-KIT* Asp816 mutations, two (3%) had *FLT3*-ITD mutations, while three (5%) had *FLT3* Asp835 mutations. In only four cases were the *RAS* mutations found in association with these RTK mutations: N-*RAS*61/*c-KIT*816 (n=1); N-*RAS*12/K-*RAS*12/*FLT3* 835 (n=1); N-*RAS*61/*c-KIT*exon8 (n=1); N-*RAS*61/*FLT3* 835 (n=1). Thus, 40 patients (69%) with AML (inv16) possessed at least one class I mutation (*RAS*, *c-KIT* or *FLT3*) and these were mutually exclusive in 36 (90%) of cases.

Recently, a *two-hit* model for the pathogenesis of AML has

been proposed in which the critical events are an activating mutation, or class I mutation, conferring a proliferative and/or survival advantage and a class II mutation impairing hematopoietic differentiation. This hypothesis provides a unified molecular theme in AML explaining how a variety of distinctly different mutations can give rise to an essentially similar phenotype.<sup>3</sup> Recently, we reported supportive evidence for this hypothesis by showing that 40% of patients with AML and inv(16) have mutually exclusive *c-KIT* or *FLT3* mutations.<sup>4</sup> *RAS* mutations are frequent events in AML, with their reported frequency being up to 30%.<sup>7,8</sup> Most mutations are point mutations involving codons 12, 13 and 61, with the N-*RAS* gene being mutated most frequently in hematopoietic malignancy.<sup>9</sup> In this study, the incorporation of *RAS* and RTK mutational analysis has increased the class I mutational rate to nearly 70%, the highest frequency reported for any AML subclass. Interestingly, class I mutations tended to be mutually exclusive, with only four cases possessing both a *RAS* and a RTK mutation. In conclusion, we believe that the above findings in patients with AML and inv(16) further support the *two-hit* model of leukemogenesis. In the future, it will be possible to confirm the relevance of such interactions using animal models, as has already been reported for *FLT3*-ITD and *PML/RARα*.<sup>10</sup> These results open up the possibility of incorporating targeted therapy for high risk patients with AML and inv(16), since specific inhibitors to activate *RAS* and RTK are already being employed in clinical trials.

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