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THE DETECTION AND SIGNIFICANCE OF MINIMAL RESIDUAL DISEASE IN CHRONIC MYELOID LEUKEMIA

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Abstract The focus of the study of minimal residual disease (MRD) is to redefine the concept of remission by using more sensitive molecular techniques to detect level of disease burden below that of conventional pathology. The detection of the chimeric *bcr-abl* mRNA transcript in chronic myeloid leukemia (CML) is a paradigm of the use of molecular biology for clinical applications. The qualitative (yes vs no) detection of MRD is associated with a relative increase in relapse rate, and the magnitude of the relative risk appears dependent on the time from transplant, and the type of transplant. The quantification of disease burden by quantitative PCR (Q-PCR) can greatly strengthen the relationship of MRD and subsequent relapse. In addition, the promise of genomics offers hope that in the near future, leukemia may be sub-classified by the genetic profile of an individual patient's particular leukemia, allowing truly "tailored" individual therapy.

Key words: mínimal residual disease, chronic myeloid leukemia

Resumen ***Detección y significado de enfermedad mínima residual en leucemia mieloide crónica.*** El punto central en el estudio de la enfermedad mínima residual (MRD) es redefinir el concepto de remisión utilizando técnicas moleculares más sensibles para detectar el nivel de carga de enfermedad subyacente por debajo de los detectados por la patología convencional. La detección del transcripto quimérico bcr/abl ARNm en leucemia mieloide crónica (LMC) es el paradigma del uso de la biología molecular en la aplicación clínica. La detección cualitativa (sí *vs* no) de la MRD está asociado a un relativo incremento en el índice de recaída, y la magnitud del riesgo relativo parece depender del tiempo y tipo de trasplante. La cuantificación de la carga de enfermedad con PCR cuantitativa puede estrechar ampliamente la relación entre MRD y la subsiguiente recaída. Además la promesa de *genomics* ofrece la esperanza de que en un futuro cercano la leucemia podrá ser sub-clasificada de acuerdo al perfil genético de la leucemia de cada paciente, permitiendo una verdadera terapia individual a medida.

The molecular biology of CML

The Philadelphia chromosome (Ph) results from the reciprocal translocation of the long arm of chromosome 9 with the long arm of chromosome 22. This translocation causes the 5' upstream domains of the BCR gene from chromosome 22 to be placed in juxtaposition with the 3' tyrosine kinase domains of the ABL gene from chromosome 9[1-3]. This unique chimeric fusion gene produces the chimeric BCR-ABL mRNA, which is translated to the functional chimeric BCR-ABL protein. This BCR-ABL fusion protein has an elevated tyrosine kinase activity relative to the wild type ABL activity[4, 5], and various mouse models have implicated the abnormal BCR-ABL activity as being sufficient to cause leukemia.

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The detection of minimal residual disease

The chimeric *bcr-abl* mRNA is an attractive target for monitoring minimal residual disease (MRD), since it should be found only in CML cells, and not in normal hematopoetic cells (however, see below). The most sensitive method to detect the *bcr-abl* fusion transcript is by use of reverse transcriptase-polymerase chain reaction (RT-PCR) amplification[6]. The sensitivity of this assay can

Abbreviations

AML:	acute myeloblastic leukemia
ASH:	American Society of Hematology
BMT:	bone marrow transplantation
CML:	chronic myeloid leukemia
GVHD:	graft-versus-host disease
GVL:	graft-versus-leukemia
MRD:	minimal residual disease
NHL:	non-Hodgkin leukemia
PCR:	polymerase chain reaction
Ph:	Philadelphia chromosome
Q-PCR:	PCR quantification
RR:	relative risk of relape
RT-PCR:	reverse transcriptase PCR
URD:	unrelated donor

detect one CML cell in a background of 10^4-10^6 normal cells[7]. Initially all studies of the relationship of MRD and relapse focused on the qualitative (yes vs no) *bcr-abl* RT-PCR assay. Recent technologic innovations have allowed a quantification of *bcr-abl* burden.

Qualitative determination of MRD and relapse in CML

Studies have examined the association of *bcr-abl* detection after bone marrow transplantation (BMT) and subsequent relapse. In general, the qualitative detection MRD (yes vs no) is strongly associated with relapse, though not absolutely predictive. Because of this, many investigators feel that a qualitative assay for *bcr-abl* has limited clinical utility. However, as outlined below, the presence of MRD is strongly associated with relapse; the difficulty lies in the compromise of relative vs absolute risk. The risk of relapse associated with MRD is influenced by the time at which the patient is tested post-transplant, and the type of transplant[8, 15].

Timing of MRD detection and relapse

The significance of qualitative *bcr-abl* detection varies with the time of detection from transplant. We studied 346 CML patients post-BMT and found that a single positive assay for *bcr-abl* was associated with an elevated risk of relapse[11]. Overall, the relative risk (RR) of relapse in MRD positive patients compared to negative was 30; multivariate analysis including type of donor, phase of disease, and presence or absence of graft-versus-host disease (GVHD) indicated that only MRD detection and type of transplant had independent association with relapse. From 6 months post-transplant on approximately 25% of patients had detectable MRD at some time. The detection of *bcr-abl* at 6-12 months post-BMT was associated with a 42% risk of relapse at a median of 200 days from the first *bcr-abl*-positive result, as opposed to a 3% risk of relapse in *bcr-abl*-negative patients. At later times, however, the RR of MRD and relapse decreased. After 3 years post-transplant, nearly 25% of patients tested were positive for MRD, but only 10% relapsed.

The demonstration of late residual disease persisting years after transplant has been reported by others, as well. Costello et al found that 66/117 (56%) of patients were MRD-positive > 36 months post-BMT, but only 8% relapsed[15]. In addition, Van Rhee et al reported 19 patients in complete remission for more that 10 years post-BMT, 2 of whom were still positive for *bcr-abl*[16]. Last year we reported at ASH a larger study of patients with late MRD detection. Overall we studied 321 patients who had survived > 18 months post-transplant. 81/321 (25%) had detectable MRD at some time > 18 months post-BMT[17]. Of these 81, 16 relapsed at a median of 961 days after their first MRD detection. The RR of relapse in MRD patients was 28, and the cumulative risk of relapse at 3 years first MRD detection was 20%. To further define the risk of relapse associated with molecular relapse in these long-term CML survivors, we studied 379 CML patients alive ≥ 18 months without hematologic or cytogenetic relapse. Ninety of 379 patients (24%) had at least one positive *bcr-abl* test ≥ 18 months post-transplant. Thirteen of these 90 *bcr-abl* positive patients (14%) relapsed, compared to 3/289 (1.0%) relapses in *bcr-abl* negative patients. The cumulative incidence of relapse among the 90 *bcr-abl* positive patients was 19.9% at 3 years after the first positive test. The median time from the detection of *bcr-abl* to relapse was 916 days (range 251-2654 days). The hazard ratio of relapse associated with *bcr-abl* positivity was 19.2 ($p < 0.0001$). Multivariable regression revealed that the stage of disease at transplant, the presence of chronic graft-versus-host disease (cGVHD), and the donor type did not qualitatively change the association between *bcr-abl* and relapse.

Type of transplant, MRD, and relapse

The significance of MRD detection is influenced by the type of transplant (eg., unmanipulated related; T-cell depleted; unrelated). This presumably reflects various amounts of immunological effect on the residual CML cells (the graft versus leukemia effect). The effect of donor type on *bcr-abl*-positivity and relapse was illustrated by the experience of unrelated donor (URD) transplants, which have a much lower risk of relapse after BMT compared to allogeneic-related matched transplants. Thus, while URD patients had a similar prevalence of *bcr-abl*-positivity at 6-12 months post-BMT compared to related BMT patients (25% vs 30%), the subsequent relapse rate was much greater in the PCR-positive related donor group compared to the PCR-positive URD recipients (~60% vs ~10%). This suggests that in the URD the GVL effect may be working to control the leukemia clones that have escaped the conditioning regimen. Pichert et al[36] also demonstrated the power of an immunologic effect in controlling relapse in 48 T-cell depleted and 44 of unmanipulated graft recipients[12]. They found that > 80% of patients who received a T-cell depleted marrow were MRD-positive 6-24 months post-BMT, compared to 25% of patients who received an unmanipulated graft. MRD was highly associated with relapse, especially in the T-cell depleted graft recipients. All patients with persistent MRD relapsed, compared to a 30% relapse rate in patients with intermittently positive *bcr-abl* assays, and a 0% relapse rate in always *bcr-abl*

negative patients. Mackinnon et al studied the relationship of T-cell depletion, MRD, and relapse in 36 patients following T-cell depleted marrow transplan-tation[13]; 30/36 (83%) were MRD-positive post-transplant, and 60% relapsed. Fifteen of 20 patients (84%) who became MRD-positive within 6 months post-transplant progressed to cytogenetic or hematologic relapse.

Quantification of MRD burden and relapse in CML

The predictive value of MRD detection in CML may be strenghthened by *bcr-abl* quantification (so called Q-PCR)[14, 18, 19]. Lin an colleagues studied 69 patients with a competitive Q-PCR and demonstrated that the kinetics of *bcr-abl* level over time described both impending relapse and response to donor leukocyte infusion after relapse[14]. Low (or no) residual *bcr-abl* was associated with a very low risk of relapse (1%), compared to 75% relapse rate in patients with increasing or persistently high *bcr-abl* levels. Patients who relapsed had doubling times of the *bcr-abl* transcript level twice that of patients who failed to relapse (15 vs 25 days).

The studies of MRD quantification have been handicapped by technical methods capable of harnessing the power of the PCR into a reliable quantification assay. However, the method of real time Q-PCR has been forwarded by the development of the new Taqman system (ABI PRISM 7700, Perkin-Elmer Applied Biosystems, Foster City, CA). This system uses fluorescence chemistry and sophisticated reporter technology to perform real time calculations of PCR product accumulation during amplification[20, 21]. The PCR takes place with the usual upstream 5' primer and downstream antisense 3' primer, with an internal probe that anneals downstream of the 5' primer. This internal probe includes a 5' fluorescente reporter molecule and a 3' quencher molecule. At the annealing temperature the PCR primers and the labeled probe anneal to their complimentary sequences in the target molecule. With polymerization from the upstream 5' PCR primer, the Taq polymerase enzyme encounters the internal probe, and the 5' nuclease activity of the polymerase chews off and releases the reporter. The liberated reporter molecule can then be excited by the internal laser, and the subsequent liberated energy detected in real time. The use of quantitative PCR (Q-PCR) has been pioneered in the study of MRD in chronic myeloid leukemia[22, 24]. In these studies the increase in MRD burden over time clearly heralds relapse. We are currently completing Q-PCR of *bcr-abl* on > 400 CML patients post-transplant. Our preliminary data, presented at ASH[17], reported on the quantification of *bcr-abl* burden in 344 samples from 85 *bcr-abl* positive patients. The median *bcr-abl* level over time was compared between patients who relapsed against those did not, and the difference was statistically significant (p = 0.002). The median level of *bcr-abl* at relapse was 40,443 *bcr-abl* copies/µg RNA (range, 960-299,552). Of the 73 patients who were *bcr-abl* and failed to relapse, 69% had only one positive test at a median level of 24 copies *bcr-abl*/µg RNA.

These above qualitative and quantitative MRD data confirm that molecular relapse of *bcr-abl* is common in CML patients following transplantation. A qualitative *bcr-abl* result has prognostic importance, and refinement by quantitative assays may target patients who would benefit from early intervention. Many patients remain with detectable molecular relapse, and their study may further our insight into the mechanisms that dictate remission or relapse.

The persistence of MRD

Not all patients with MRD relapse. In CML patients post-transplant the *bcr-abl* mRNA is often detected post-transplant, and can persist for years despite the appearance of hematologic remission[11, 15, 16]. In $t^{8, 21}$ AML, most long-term survivors have detectable AML1-ETO mRNA, even years after achieving remission[25, 26]. Lastly, even patients with ALL have can have persisting low levels of clonal cells detected by their leukemia-specific IgH V-D-J rearrangements[27]. Recently, there have been documented cases of ALL relapsing > 10 from initial remission with the same IgH V-D-J rearrangement[28]. This suggests that even an aggressive disease such as ALL may lay dormant for years before progression to frank relapse. What does this tell us about the biology of leukemia, and what is really needed for a cure? "Dormancy" of leukemia in a "cured" patient might be due to various influences, such as: 1) genetic, such as the lack of other secondary genetic hits necessary for progression to frank leukemia; 2) epigenetic factors, such as growth factor exposure in the marrow stroma that may promote or inhibit the outgrowth of leukemia clones; and 3) immunologic response, whereby the patient's immune system manages to harass and control the proliferation of the malignancy. The dissection of which process(es) are at work may yield new insights and approaches to "curing" leukemia.

Translocations in normals

The common assumption was that translocations would be disease-specific that is, only found in leukemic, not normal, cells. It has become clear that reality is not so simple. Several studies have documented the surprising finding of disease-specific translocations in normal

people. Both the BCL2/IgH rearrangement found in follicular lymphoma, and the *bcr-abl* chimeric mRNA of CML have been found in nearly 40% of adults[29, 32]. These studies have used modified PCR assays to effectively increase the sensitivity of detection more than a log over that of conventional assays employed in MRD studies. The frequency of these translocations in normal people increased with age, and semi-quantitative PCR assays suggested that positive cells were very rare, generally beneath the threshold of PCR positivity in routine PCR assays. Thus, the presence of translocations in normals may have little consequence towards generating false positive assays of MRD. An interesting question is why translocations occur in normals. Certainly this percentage of the population is not going to get CML, or NHL. The increasing prevalence of translocation with increasing age begs the question if these genetic events represent a bio-marker of increasing damage from environmental sources with years, and/or decreased genetic repair occurring with increasing patient age.

The genetics of progression and response

The investigation of the genetics of response, relapse, and leukemogenesis has been limited by the painstaking process of gene identification and understanding the complicated networks that drive normal (and abnormal) cellular function. However, we are in the midst of a revolution. The Human Genome Project is providing enormous genetic information, and equally impressive technical feats have occurred that enable us to use this abundance of genetic data. It has become possible to investigate the simultaneous expression of thousands of genes by the use of microarray expression arrays[33]. In this technology, nucleotide sequences homologous to genes are spotted on a glass or synthetic platform. This platform becomes the probe to investigate gene expression in cell samples. It becomes possible to examine and compare gene expression profiles from any cell populations: cells before and after a specific drug treatment, cells from responsive and unresponsive patients; cells from two different stages of a malignancy; etc. Depending on the technology, RNA from two different cell populations can be labeled with different color fluorescence and mixed, then hybridized to the probe, or separate experiments can be performed, and the two samples compared computationally.

Obviously, the computaional obstacles to simultaneous interrogation of > 10,000 genes is daunting. However, it is clear that this method can yield fruitful insight into normal and abnormal cell biology. While this technology is in its infancy, it has already been demonstrated that it may be powerful in finding new biological classification methods in leukemia and lymphoma[34, 35]. In a separate application, our lab is using this approach to determine the genes involved in the progression of chronic phase CML to accelerate and blast phases. The long-term promise of such technology includes identifying new genes that are involved in treatment response, relapse, and progression. It is not unreasonable to expect in the near future that gene expression patterns will denote patients particularly susceptible to different types of treatment.

Conclusion

CML has been the paradigm for the integration of molecular biology with clinical care. In the future gene expression array studies will classify the biologic state of the disease, and suggest routes of tailored therapy based on the profile or response genes. Quantification of MRD will then sensitively monitor response, and signal when a new intervention must be entertained. The future is exciting, and it is about... now.

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What has been learned from oncogenes represents the first peep behind the curtain that for so long has obscured the mechanisms of cancer. In one respect, the first look is unnerving, because the chemical mechanisms that seem to drive the cancer cell astray are not different in kind from mechanisms at work in the normal cell.

Lo que se aprendió con los oncogenes representa el primer vistazo detrás de la cortina que durante tanto tiempo ha ocultado los mecanismos del cáncer. En cierto sentido, la primera mirada es decepcionante, porque los mecanismos químicos que parecen llevar a la célula cancerosa por el mal camino no son distintos de los mecanismos que encaminan a la célula normal.

Michael Bishop, 1982