Genome wide characterization of minimally differentiated acute myeloid leukemia

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Discussion

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Minimally differentiated acute myeloid leukemia, known in the French-American-British classification as AML-M0, is defined as a leukemia subtype that shows some heterogeneity. This leukemia type represents less than 5 percent of AML cases, frequently occurs in elderly patients and has a bad prognosis. By expanding our knowledge on the causes and characteristics of this leukemia we enhance the chance of improving the outcome of this disease.

Though this thesis deals first and foremost with the characterization of AML-M0, it is worth to mention that this project was originally thought as a proof of principal for the detection of putative tumor suppressor genes (TSG) by loss of heterozygosity (LOH) analysis. Progression of normal cells into a neoplasm is associated with genetic alterations. The main two groups of genes altered are either proto-oncogenes or TSGs. Until recently, translocations leading to the activation of proto-oncogenes, were the predominant form of mutations reported in leukemia. However, AML-M0 is not associated with any specific translocations and translocations are not frequent, which makes it a good case study for the search of TSGs. LOH detection used in the search of TSGs in other cancers was successfully applied for the same purpose in AML-M0. Using microsatellite analysis we were able to delimit a particular region of chromosome 21 harboring a single gene: *RUNX1*. Following contemporary studies we detected mutations in *RUNX1*, including for the first time homozygous deletions of this gene. As discussed in chapter 2, most of the *RUNX1* mutations detected by us were biallelic and resulted from the duplication of a first mutation (like a point mutation) by a mechanism leading to uniparental disomy (UPD) (or isodisomy) such as mitotic recombination or chromosome loss and duplication. This observation was later supported by the LOH patterns detected by whole genome SNP analysis discussed in chapter 3. The latter result supports mitotic recombination as the major mechanism leading to uniparental disomy in chromosome 21. Whole genome SNP analysis (chapter 3) also showed that the mechanisms leading to LOH vary with chromosome. Our results show that in chromosome 21 the main mechanism of LOH leads to UPD while in other chromosomes, like chromosomes 5 and 7, the mechanism of LOH is mainly hemizygous deletion. This observation suggests a selection process for a specific LOH mechanism in each chromosome. Hemizygous deletions can either be a second hit, according to Knudson "two-hit" TSG inactivation hypothesis, or simply lead to haploinsufficiency of one to several genes as a one hit event, without the need for further mutations. This could be the reason why until this date no TSG was found in chromosomes 5 and 7, which are frequently affected by hemizygous deletions in hematological disorders. On the other hand, it is possible that certain chromosomal regions are essential for cell survival and hemizygosity is not tolerated. In these situations hemizygous deletions would be rare. That could be the case of chromosome 21, where the great majority of LOH occurrences were related to UPD. UPD events only lead to loss of normal function of a gene by duplicating a first mutation, i.e., they are a second hit. However, UPD can also be negatively selected by different factors. For instance, mitotic recombination (see above) has an increased cumulative frequency towards the telomere. Thus, UPD by mitotic recombination would be a less effective way of targeting genes closer to the centrosome. That could be the case for *TP53* located in the short arm of chromosome 17, where we only detected deletions. Another possible negative selector of

UPD is gene imprinting and the occurrence of recessive lethal gene variants, as duplication of these genes could lead to cell death. These are basic mechanistic questions that deserve further exploring.
Our SNP data (chapter 3) reinforced the role of *RUNX1* in the pathogenesis of AML-M0. It also showed that there is no other TSG with such an important role in this leukemia as *RUNX1*. Nevertheless, a few regions were found in a limited number of patients that can harbor a putative TSG, such as chromosome 3. SNP analysis also showed that *NF1* and *TP53* are likely involved in this neoplasm.

RUNX1 has a preponderant role in AML-M0, as shown by us and others. We detected mutations in this gene in near 40 percent of the patients. In chapter 3, we showed for the first time the occurrence of mutations in the transactivation domain of *RUNX1* in AML-M0. Mutations in the transactivation domain of *RUNX1* had only been found in myeloproliferative syndrome, maybe because screening of this region has been neglected in most AML studies. Another important finding was the association between increased expression of TdT (terminal deoxynucleotidyl transferase) and *RUNX1* mutation. Since *RUNX1* is implied in lymphoid maturation it is possible that this correlation has a close molecular basis. TdT analysis is commonly done during diagnoses making it an easy assay for detection of *RUNX1* mutation.

Most recent studies in AML and in leukemia in general, search for two types of mutations. One leads to a block in differentiation and usually involves a transcription factor such as *RUNX1*, *ETV6*, *CEBPA*, etc… The second leads to a proliferative advantage and occurs in members of pathways controlling proliferation, such as *FLT3*, *RAS* and *PTPN11*. These two types of mutations seem to occur in tandem. Our results did not show a major alternative to *RUNX1* mutations as key differentiation blockers in AML-M0. Instead, they point to the existence of different factors with a role similar to *RUNX1* which seem to present an alternative to it (chapter 3). Among these factors are loss of genetic information in specific chromosomes (like in chromosome 3), various translocations and *ETV6* mutations.
ETV6 is a major player in acute lymphoid leukemia (ALL). Our work, and others, showed that it is also involved in AML (chapter 4). While in ALL the *ETV6* mutations are predominantly translocations leading to oncoproteins, in AML we found point mutations and insertions in addition to translocations. It is not clear if these mutations lead simply to loss of the DNA binding activity of ETV6 or if the mutated proteins have a residual dominant-negative effect, a point which needs additional attention. Also, contrary to translocations found in ALL, the translocations detected by us and others in AML-M0 did not lead to the formation of oncogenic fusion proteins. Finally, we observed hemizygous loss of a small area of chromosome 12 containing the *ETV6* locus in some patients. All these results suggested that the *ETV6* role in the leukemogenic process in AML-M0 is related to the loss of one allele, leading to haploinsufficiency. We also detected biallelic mutation of *ETV6* in one case. Haploinsufficiency and biallelic loss are inactivation mechanisms related to TSGs and not to proto-oncogenes, which implies *ETV6* as a TSG. Either way, *ETV6* mutation seems to be important as an alternative to *RUNX1* mutation in AML-M0.

We detected mutations in several genes with roles in cell proliferation in a high percentage of AML-M0 cases. Mutations were observed in *FLT3*, *RAS*, *PTPN11* and, for the first time, *JAK2*. We also detected loss of the *NF1* locus in chromosome 17 implying this gene in the etiology of AML-M0.
Trisomy 13 associated with high *FLT3* expression could be another anomaly related with cell proliferation. In chapter 4, we showed that trisomy 13 was associated to mutations in *RUNX1*. We also observed that *FLT3* transcription was increased when a *RUNX1* mutation was present, and that this increase was much higher when the *RUNX1* mutation was associated to trisomy 13. A limited study of the protein expression in patients carrying a *RUNX1* mutation and trisomy 13 indicated a similar increase in both the cell fraction expressing *FLT3* and the number of *FLT3* receptors at the cell surface. It would be of interest to confirm this result in a larger cohort. The association between FLT3 expression, *RUNX1* mutation and trisomy 13 could be explained by two points. First, chromosome 13 harbors *FLT3*. Second, gene expression analysis, discussed in chapter 5, showed that AML-M0 cells with *RUNX1* mutation have early B-cell characteristics and it is known that FLT3 expression is part of the early developmental program of B-cells. The two factors could have a synergistic effect. In any case, the increase in expression of FLT3 receptor, activated either by ligand or other mechanisms, is probably an alternative to the well studied mutations of FLT3 which lead to its ubiquitous activation. A comparative study on the activation of downstream targets of FLT3 in the two types of anomalies could be useful in finding out if there is a similar effect. As a consequence of the findings discussed in chapter 4, therapies used in cases with *FLT3* mutations could be explored for patients with *RUNX1* mutation associated with trisomy 13.

AML-M0 has been reported as a heterogeneous subtype. Nevertheless, our gene expression profiling showed that the AML-M0 samples have characteristics that discriminate them from other AML subtypes and make them a distinct entity (chapter 6). Among the differences found between AML-M0 and other subtypes are the low expression of key hematopoietic transcription regulators and the high expression of mitochondrion related genes.
In agreement with the heterogeneity within the AML-M0 subtype we detected two distinct subgroups within this leukemia. One of the subgroups was not linked to any particular abnormality. The second subgroup was associated with *RUNX1* mutations. This subgroup was characterized by the expression of B-cell related genes, some of which are only seen in early stages of development of this lineage. This result is consistent with studies reporting lymphoid characteristics in AML-M0. The expression of B-cell genes can have a critical role in the development of AML-M0. A more detailed study of the protein expression of these genes, for example at the cell surface, could help establishing their importance.
Another outcome of gene expression profiling is a gene signature. These can be useful at diagnostics, especially if included in a dedicated diagnostic microarray. In addition, gene expression profiles can potentially be used in drug target discovery. Unfortunately, this is a promising area which is lagging behind. Discovery of new drug targets in AML-M0 would be a great consequence in this leukemia with a notably poor outcome.

For a rational approach in the improvement of available therapies, the characterization of leukemia is an important step. Without being able to discriminate between different types of

leukemia it is not possible to tailor the therapies. Ultimately, it is expected that these therapies will have also to target the cancer stem cells for successful treatment. Current therapies are aimed at the bulk of the cancer cells. Targeting the leukemic stem cells would potentially reduce the risk of relapse. In this respect, finding the first step in the leukemogenic process can have important consequences, as targeting the first event will probably also result in the targeting of the leukemic stem cells. From a theoretical point of view a case can be made both for mutations leading to a proliferative advantage and for a block in differentiation, as the primary event. Thus, a mutation leading to a proliferative advantage can induce a (fast) growing population. With each new cell division the probability of further mutations targeting other genes increases and with it the chance of a full blown leukemia. On the other hand, a mutation providing a block or impairment in differentiation in a hematopoietic stem cell can be expected to have the same end effect if we consider the self-renewal properties of these cells, and the amplification process associated with hematopoiesis. Creating an (even limitedly) expanding cell population unable to differentiate will also increase the chances for additional mutations within that population with time. Furthermore, mutation in genes necessary for the stability of the cell, such as gate-keepers and caretakers, can likely precede both types of mutations in some cases or be a intermediate step between them. It is possible that each of these models will be reflected in different types of leukemia, or it might even be the case that there is no rule. Only more work can provide the answers we are looking for.

In conclusion, this work helped to prove the importance of *RUNX1* mutation in AML-M0, provided alternatives for it and broadened the number of collaborating mutations known. It also showed that AML-M0 is a distinct entity, though one with a manifest subdivision.