Kinases and miRNAs in the pathogenesis of small B cell lymphomas

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Chapter 8

Summary, discussion and perspectives
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CLL and MCL are two types of small cell lymphoma for which no effective treatment protocols exist. The mainstays of the treatment for these lymphomas are varying between a “wait and see” or monochemotherapy in the indolent form of CLL to polychemotherapy combined with molecular therapy followed by allogeneic stem cell rescue or even allogeneic stem cell transplantation in MCL. However, many of these modalities have limitations because of severe side effects and limited capacities to discriminate between healthy and cancerous cells, which are in particular relevant since both diseases mainly affect elderly patients with a limited capacity to recover from these therapies.

Research on signal transduction pathways and key players therein can contribute to our understanding of the molecular individual cancers, the identification of possible molecular targets for pharmacological inhibitors or activators, and thus might lead to novel therapeutic strategies and molecular therapies.

MiRNAs are a new group of regulators relatively recently discovered which have been shown to play important roles in the pathogenesis of cancer by regulating both oncogenes and tumor suppressor genes.

This thesis focuses on the signal transduction profile and miRNA expression of these two diseases.

Part 1: Kinases

In this part, we investigated the role of kinases in CLL and MCL. Signaling through BCR and CD40L are the most important signal transduction pathways in B cells and also play an important role in B cell malignancies. In these two signaling pathways, the mitogen-activated protein kinases (MAPK) are an extensively studied group of protein kinases, as reviewed in chapter 1.

Kinome profiling is an array-based method to identify all active kinases in cells by monitoring phosphorylation of consensus peptides. This method is an efficient tool to compare the whole kinome between different groups of samples. Based on the notion that most kinase inhibitors have broad kinase inhibition functions, kinome profiling can be used to study the mechanisms of kinase inhibitors by comparing the kinase activity between an uninhibited and inhibited population of cells. By using this method, we compared the kinase activity between ZAP-70+ CLL cells and ZAP-70- cases (chapter 2). We found that there is higher kinase activity of Syk in ZAP-70+ cases. These findings were confirmed by conventional techniques. Furthermore, Syk inhibition can lead to apoptosis in cell lines as well as patient samples, so Syk could be a potential therapy target for CLL cells. Syk is a protein tyrosine kinase
which plays an important role in the initiation of signaling from the B-cell receptor (BCR)\(^4\). Syk is required for the survival of many kinds of B cell lymphomas and has been identified as a treatment target for several lymphomas such as diffuse large B cell lymphoma (DLBCL), MCL, Burkitt lymphoma (BL) and follicular lymphoma (FL)\(^5-8\).

MAPK signal transduction is very important for cell proliferation and cell cycle progression and has become an important target in many kinds of cancers\(^9;10\). In chapter 2, we found that there were higher kinase activities of JNK and S6K in ZAP-70+ cases compared to ZAP-70-cases. JNK inhibition with SP600125 induced G2/M cell cycle arrest and inhibition of S6K through rapamycin led to G0/G1 arrest in cell lines. Recently, Decker\(^11\) et al reported a phase II trial with the oral rapamycin RAD001 in seven patients with advanced B-CLL. After two cycles of treatment, one patient was in partial remission and three patients had stable disease. Thus, targeting the cell cycle can be an attractive therapeutic approach for CLL.

In chapter 3, we identify Raf-1 as a critical anti-apoptotic and cell cycle regulation kinase in CLL cells, by activating the p-ERK and p-BAD pathways. By using specific inhibitors, we demonstrated that Raf-1 is also important for G0/G1 cell cycle progression by regulation of cyclin D3 and cyclin E. By Annexin V staining, we found that GW5074 can lead to apoptosis of CLL cells, both in cell lines and patient samples. Raf-1 inhibition could thus be an important therapy target for CLL. The methods of targeting Raf-1 in cancer include antisense oligodeoxynucleotides (ASON) and Raf-1 inhibitors (GW5074) or destabilizers (Geldanamycin). The anti-tumor activity of ASON has been evaluated in phase II studies in patients with ovarian, small-cell lung and non–small-cell lung carcinomas. The strategy to be used in CLL to target Raf-1 needs to be further studied\(^12\).

In comparison with normal tonsil B cells, we identified JNK as an important MAPK in MCL cells (chapter 4). Inhibition of JNK with SP600125 resulted in G2-M phase arrest on day 1 and a strikingly increased endoreduplication on day 2 and day 3, which was confirmed by karyotype analysis. G2-M arrest was associated with downregulation of EGR1. SP600125 induced polyploidy, that could be blocked by the BCL-2 inhibitor YC 137. These data suggest that constitutive JNK activity is necessary to promote proliferation and maintain diploidy in MCL and that disruption of this MAPK leads to a profound cell cycle deregulation and endoreduplication. Our data also provide a model to study polyploid MCL cells.

We found that Syk inhibition can lead to apoptosis in CLL cells. Rinaldi \(^13\) et al showed that Syk is over-expressed in MCL and constitutes a possible treatment target for MCL. We investigated the mRNA expression level in eleven B cell lines and found that Syk is highly expressed in MCL cell lines (Figure 1). The function of Syk in MCL can be
studied in MCL cell lines, the effect of Syk inhibition in MCL needs to be studied in primary cases.

We found that Raf-1 is overexpressed in CLL and that inhibition of Raf-1 can lead to apoptosis of CLL cells. Since overexpression of Raf-1 at the mRNA level is associated with a bad prognosis in MCL\textsuperscript{14}, it is interesting to gain insight into the function of Raf-1 kinase in this disease. Our results also showed that Raf-1 inhibition can lead to cell cycle arrest by downregulating cyclin D3 and cyclin E expression in CLL cell lines. In MCL, where cyclin D1 is overexpressed due to the t (11, 14) translocation, Raf-1 inhibition may only be effective if it leads to degradation of cyclin D1.

**Part 2: miRNA**

In this part, we analyzed miRNA expression in CLL and normal B cell subsets. Recent studies have shown that miRNAs play an important role in normal B cell differentiation and affect the prognosis of CLL, however, there is some controversy which may have been caused by different experimental methods. We reviewed the miRNA study methods and recent miRNA studies in CLL (chapter 5). The miRNA field has grown exponentially and many CLL related miRNAs have been identified. To solve the problem of discordant results in CLL, several factors should be taken into account. First, as different results can be generated from different methods, miRNA study methods should be improved and standardized. Second, miRNA expression is usually tissue specific and individual miRNAs may have a different function in different tissues. Thus, miRNA expression studies in cancer samples should also compare the normal tissues as counterpart, which would help in defining those miRNAs that are functionally significant.
Summary, discussion and perspective

In chapter 6, we analyzed the expression of fifteen miRNAs in thirty-three lymph nodes and thirty-seven blood samples of CLL patients. There were similar miRNA expression patterns in the lymph node and blood samples and three miRNAs (miR-16, miR-21 and miR-150) showed a high expression level in all samples. Contrary to some reports, we did not find a correlation between miRNA expression and ZAP-70/VH mutation status, which might be due to the different detection methods. Our RNA in situ hybridization (ISH) experiments revealed a strong homogeneous staining of miR-150 in the tumor cells but a very weak staining within the proliferation centers, which are a hallmark of CLL. In reverse BIC/pri-miR-155 expression was observed mainly in individual cells including prolymphocytes of the proliferation centers. This reciprocal pattern likely reflects the different functions and targets of miR-150 and miR-155. Recent reports in transgenic mouse models showed that miR-150 plays a key role in B cell differentiation by targeting c-myb and foxp1 protein expression. Ectopic expression of miR-150 in breast cancer and leukemic cells repressed endogenous c-Myb at both the mRNA and protein levels, which shows that c-Myb is an evolutionary conserved target of miR-150. The function of miR-150 in CLL needs to be further studied.

In chapter 7, we identified some miRNAs that are differentially expressed in three subsets of B cells (naive B cells, germinal center B cells and memory B cells) by miRNA profiling. Naive and memory B cells had very similar expression patterns, which were different from germinal center B cells. These findings were confirmed by RNA in situ hybridization (ISH). MiR-150 was the most obvious discriminatory marker for GC and non-GC B cells. By transfecting the precursor of miR-150, we confirmed that c-Myb and Foxp1 are targets and found that miR-150 plays an important role in cell proliferation by regulating survivin expression.

The potentially most interesting observation was that RNA in situ hybridization (ISH) revealed a low expression of miR-150 and high expression of BIC (the precursor of miR-155) in the PCs in CLL. In CLL, the neoplastic cells in these structures are more activated than the surrounding neoplastic cells and circulating cells with an active BCR or CD40L signaling pathway. So it would be interesting to correlate the expression and function of miR-150 and BIC with BCR and CD40L signaling. We analyzed the change in miR-150 expression after IgM stimulation in purified B cells of three tonsils and three CLL cases. After IgM stimulation for 6 hours, miR-150 expression was downregulated in two CLL cases, whereas no obvious changes were found in another case and in the tonsil B cells.

We also need to investigate the BIC/miR-155 expression after IgM stimulation. We should correlate the changes in miRNA expression levels with a proteomics study to find the targets of a specific miRNA.
Chapter 8

Future perspectives

Our studies demonstrate that deregulation of the kinase activity and miRNAs is involved in the pathogenesis of lymphomas and leukemias. Thus, an important step for future studies is to introduce specific kinase inhibitors, anti-miRNA or pre-miRNA oligonucleotides into clinical trials. However, there are a few obstacles that need to be considered.

The first obstacle is the question whether the selected targets are essential or not for kinase inhibition and to what extent redundancy exists. It is tempting to speculate on the future application of MAP kinase inhibitors in clinical lymphoma treatment. The design of kinase inhibitors is difficult because there is much cross-talk between the MAPK pathways as well as double-edged functions. For instance, different functions (targeting apoptosis and proliferation) have been ascribed to the three MAPK - ERK, JNK and p38. Piceatannol was already identified as a tyrosine kinase inhibitor in 1989. However, it can also lead to apoptosis of human leukemic U937 cells by downregulation of Bcl-2 and activation of caspases. Because of its anti-proliferative property, Sorafenib (BAY 43-9006) has been developed as an inhibitor of RAF kinase. However, it also inhibits receptor tyrosine kinases of multiple pro-angiogenic factors such as VEGFR-2/3, Flt-3/ and PDGFR-beta. 17-N-Allylamino-17-demethoxygeldanamycin (17-AAG) binds to Hsp90 and alters levels of proteins regulated by Hsp90. Hsp90 is not only the chaperone for Raf-1 and Bcl-2, but also for many other molecules important for cell survival and cell cycle progression, such as p53, survivin and c-myc. SP600125 has been identified as a specific inhibitor of JNK, but also inhibits voltage dependent K+ channels in a JNK-independent way.

Thus, improvements in specific kinase inhibitor design and development are still very much needed. Another fact is that kinase activity is also regulated by phosphatases.
Thus, targeting phosphatases is also a method to regulate MAPK signaling\textsuperscript{27}.

MiRNAs can function not only as a tumor suppressor gene but also as oncogene by their posttranscriptional regulatory functions. Thus, miRNAs can be used as drugs or as therapy targets. The advantage but possibly also the disadvantage of miRNAs as a focus for research on novel drugs is the fact that many miRNAs are tissue specific and miRNAs may have different functions in different tissues. Furthermore, there may be many interactions between individual miRNAs. Therefore, it may be difficult to qualify a particular miRNA as a specific drug or drug target.

Another obstacle is drug resistance and safety concerns. The selective inhibition of protein kinases becomes more and more important as an effective therapeutic approach. Imatinib is a specific inhibitor of a number of tyrosine kinase enzymes and is used to decrease bcr-abl activity which is caused by the Philadelphia chromosome. However, clinical resistance caused by mutations in the targeted oncogene has been observed, especially in imatinib-treated leukaemia patients\textsuperscript{28}. Based on the concerns about the toxicity of RNAi\textsuperscript{29}, we can imagine that a main concern in miRNA treatment will also be safety. Thus, mechanistic studies are still very important to test and develop new ideas for treatment.

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