MIRNAs and their target genes in B cell lymphomas
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Chapter 8

Summary and Discussions

Future perspectives

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8.1 Summary and Discussions

Since the discovery of BIC (pri-miR-155) and miR-155 in Hodgkin lymphoma (HL) (1;2), the role of miRNAs in B cell development and in the pathogenesis of B cell lymphomas has become an important issue. In order to identify miRNAs which are involved in B cell differentiation and the germinal center response, we sorted naive B cells, germinal center B cells and memory B cells from tonsils and evaluated their miRNA profiles (Chapter 2). Among the 183 miRNAs assessed, the expression levels of miR-17-5p, miR-106a, miR-146a, miR-150 and miR-181b were most clearly changed during the germinal center response. These miRNAs were mostly upregulated in germinal center B cells, with the exception of miR-150, which showed a more than 10 fold downregulation in germinal center B cells compared to naïve and memory B cells. Unlike most publications which reported miRNA profiling data solely based on microarray analysis or quantitative RT-PCR, we validated our profiling results with miRNA in situ hybridization (ISH) in tonsil sections. Also, with immunohistochemistry staining and Western blotting, we demonstrated an inversed expression pattern of c-Myb and survivin with miR-150. This suggested that miR-150 may play a role in B cell homeostasis, which is consistent with the phenotype in transgenic mice(3;4).

Based on existing knowledge of which miRNAs can be important in B cells we studied the expression of 15 miRNAs in Chronic lymphocytic leukemia (CLL) (Chapter 3). In line with its non-germinal center B cell origin(5-7), CLL cases exhibit high expression of miR-150 irrespective of their site of presentation (blood or lymph node). In lymph node CLL cases, the neoplastic cells located in the proliferation centers are known to be more activated. This has been demonstrated by a higher CD38, Ki67, CD23 and IRF4 expression(8;9). Strikingly, our miRNA ISH revealed a reduced expression of miR-150 in proliferation centers while strong homogeneous staining was present in the tumor cells outside of proliferation centers. In contrast, BIC staining was only positive in proliferation centers. This inversed staining pattern in proliferation centers is similar to the staining pattern observed in germinal centers in normal lymph nodes(2;10), which suggests that miR-150 and miR-155 may be
involved in homeostasis of normal B cells. Thus, imbalance of the expression levels of these miRNAs may contribute to lymphomagenesis.

Using microarray as well as quantitative RT-PCR we examined the miRNA profile of HL, primary mediastinal B-cell lymphoma and germinal center B cell–derived lymphoblastoid cell lines (Chapter 4). In HL, 35 significantly different and/or highly expressed miRNAs were examined and compared to a panel of 33 B cell lymphoma cell lines. In agreement with previous reports(1;11), the expression level of miR-155 was high in HL. In addition, all Epstein-Barr virus (EBV) transformed cell lines, including the CLL cell lines had elevated levels of miR-155. Compared to the lymphoma cell lines of germinal center B cell origin, cell lines of CLL derived from memory B cells had the highest level of miR-150. A significant downregulation of miR-150 was observed in HL, accompanied by upregulation of a series of oncogenic miRNAs, including miR-21 and miRNAs from the miR-17-92 cluster and its paralogous clusters (miR-17-5p, miR-19a, miR-19b, miR-20a, miR-20b, miR-25, miR-92, miR-93, miR-106a and miR-106b). In addition, we reported the current limitations for miRNA target gene validation, as demonstrated by our pursuit of identifying miR-155 targets relevant for HL. In different HL cell lines with distinct endogenous levels of miR-155, marked differences were observed for validation of target genes using luciferase reporter assay. This warrants the use of relevant cell types and consideration of endogenous miRNA expression levels for luciferase reporter assays. Despite the difficulties, we showed that AGTR1, ZNF537, FGF7, ZIC3, MAF and IKBKE can be regulated by miR-155 in HL cell lines.

Because of the limitations of the luciferase assay we examined the miRNA regulated gene set (miRNA-targetome) of HL with an approach called Ribonucleoprotein ImmunoPrecipitation - gene Chip (RIP-Chip) (Chapter 6). In this approach, antibodies against wild type human Ago2 were utilized to immunoprecipitate the RNA induced silencing complex for unbiased high throughput identification of miRNA target genes. The miRNA-targetome of HL identified in our study is enriched with genes involved in the regulation of cell cycle, apoptosis and p53 signaling pathway, all of which are characteristic features of Hodgkin Reed-Sternberg (HRS) cells. Our approach gives an advantage over current methods of large scale miRNA
target gene identification because cells were untreated and physiological relevance is taken into account. In order to identify specific miRNA targets, we combined the RIP-Chip approach with anti-miRNA strategy for the miR-17 seed family. This lead to the identification of about 900 target genes including CCL1, FBXO31, GPR137B, NPAT, OBFC2A, RAB12, RBJ, YES1, and ZNFX1. We showed that current knowledge of the miRNA targeting mechanism which is applied by most of the prediction programs, like conservation of target sites among species and restriction of the target sites to the 3’ UTR, may not always be a good indicator to predict actual miRNA targeting. As much of the miRNA targeting mechanism still remain to be explored, we proposed the use of this approach for unbiased miRNA target gene identification in the cell type of interest. An increasing amount of evidence showed that this biochemical approach is practical for identification of miRNA targets not only in human cells but also in different species like in Caenorhabditis elegans(12) and in Drosophila melanogaster(13).

Knowing that the miRNA-targetome of HL was significantly enriched with genes involved in the regulation of cell cycle, we performed cell proliferation and cell cycle analysis in HL cells with or without specific miRNA inhibitors (Chapter 7). We showed that inhibition of miRNAs of the miR-17 seed family and miR-155 contribute to reduced numbers of metabolically active cells and increased percentages of cells in the G2/M phase. Interestingly, we found several genes in the miRNA-targetome of HL cell lines that were known to be inactivated in the HRS cells. The genes included NFKB1A, NFKB1E, TNFAIP3, SOCS1, FAS and PERP. However, only a few B cell specific genes (MYBL1 and CXCR4) were observed in the miRNA-targetome of HL. This suggests that miRNAs may not be responsible for the loss of B cell phenotype in HL. However, we cannot exclude the possibility that miRNA mediated silencing of the B cell phenotype in HL is exerted by other Ago proteins.
8.2 Future Perspectives
In many recent publications(14) and as a part of the scope of this thesis, the expression profile of miRNAs in B cells of various differentiation stages and in B cell lymphomas was established. However, in HL, a piece of the puzzle is still missing – the expression of EBV encoded miRNAs in HL cases has not yet been characterized. Although the work is rather tedious, this is a challenge which can be met by miRNA ISH for 39 of the viral encoded miRNAs and/or quantitative RT-PCR with laser microdissected Hodgkin Reed-Sternberg (HRS) cells. To further the identification of new, HRS specific miRNAs, short RNA cloning and/or deep sequencing may be applied.

Importantly, merely knowing the expression level of miRNAs is not enough. The corresponding miRNA target genes need to be identified to elucidate the (patho)physiological significance of the miRNAs involved. In the future, we expect application of RIP-Chip in frozen cells as well as isolated cell populations. When appropriate antibodies become available for immunoprecipitation, RIP-Chip can also be performed using antibodies against many other protein members from the Argonaute family, including Ago1, Ago3 and Ago4(15). It has already been shown in plants that different Ago proteins preferentially bind to different miRNAs depending on the first nucleotide of the miRNA(16;17). Members of the Ago family can be differentially expressed across cell lines(18), tend to associate with different protein complexes(19) and are functionally distinct(20). Mammalian Ago2 is the only Ago protein among the family known to have catalytic activities(21). The precise functions and differences among the other Ago proteins remain to be discovered. Hence, it is interesting to use the RIP-Chip approach to correlate regulatory specificity of the miRNAs and genes with specific Ago proteins. In combination with an anti-miRNA strategy, the RIP-Chip approach allows high throughput identification of miRNA specific targets. We also foresee the use of this RIP-Chip approach in combination with the induction of miRNAs that are downregulated in the cell type of interest. The advantage of the RIP-Chip approach over current methods is apparent. However, since miRNAs ultimately affect protein levels, an effective high throughput proteomics approach for the identification of miRNA targets needs to be established.
In the meantime, while awaiting advancement from the proteomics field, the RIP-Chip approach is satisfactory.
In HL we identified about 900 miR-17 seed family targets but miRNA targets of other important miRNAs are yet to be identified. miRNA mediated loss of B cell phenotype in HRS cells is still possible with Ago proteins other than Ago2. In CLL, the apoptosis regulator BCL-2 has been demonstrated to be a target of miR-15/16(22) and the ubiquitin ligase Itch has been demonstrated to be a target of miR-106b. Upon upregulation of miR-106b, downregulation of Itch would increase the expression level of the proapoptotic transcription factor, p73 leading to apoptosis of CLL cells(23). The issue of miRNAs exerting their impact by regulating one major target or by affecting many components of the entire network awaits to be answered. As the mechanisms by which miRNAs regulate cell biological processes is far more complex than initially presumed(24), the physiological impact of specific miRNAs should be carefully assessed. Nevertheless, several ongoing clinical trials have begun to evaluate the use of miRNAs as therapeutic agent for cancer treatment.
Reference List


Summary, discussions and future perspectives