A proteomic approach for leukemia epigenetics research

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CHAPTER 8

SUMMARY, DISCUSSION & FUTURE PERSPECTIVES

Summary

Leukemia is a complex disease that occurs across all ages and remains a leading cause of cancer death. Due to its heterogeneity, choosing the best treatment option for individual patients is an everyday challenge for pediatric oncologists and hematologists. Currently, patients are generally well-characterized by chromosomal abnormalities and mutations, but there is a disconnect between these classifications and current therapies, as most of these cannot currently be targeted. Additionally, emerging evidence suggests that mutated or otherwise aberrantly expressed epigenetic modifiers are critical to the development of leukemia and have potential therapeutic promise. Despite this potential and the introduction of methylation agents plus small molecular inhibitors for a variety of HME, current approaches to profile epigenetic states have not yet translated to the clinic due to lack of trials showing their efficacy and the inability to tell which patients might benefit from them pre-treatment. One size does not fit all and therefore we aimed to use RPPA-based proteomics to improve epigenetic-focused target identification, prognostication and therapeutic guidance in AML, ALL and CLL.

After a general introduction in leukemia, epigenetics and proteomics, we hypothesized for the first time that chromatin and histone modifying enzymes form patterns in AML and that these have clinical impact. While individual aberrantly expressed epigenetic proteins have shown to be prognostic, in **Chapter 2** we performed simultaneous integrated analysis of 20 HME and HMM in 205 adult AML derived samples by RPPA. Expressions of the HME and HMM were measured relatively to its expression in normal CD34+ bone marrow derived samples. Then, we identified five distinct clusters that were classified as “normal-like”, “higher” (more *on*) or “lower” (more *off*) based on their relative HME expression. We observed that patients with more *on* HME had shorter median overall survival (OS), independently of cytogenetic risk group. Also, this group presented with higher proliferative potential, manifesting in higher white blood counts and peripheral and bone marrow blast percentages, and greater resistance to therapy. The identified patterns were unassociated with age, sex or remission duration. In more detail, the more *on* grouped patients were characterized by relatively high levels of BRD4, KDM1A, NCL, SIRT1 and hnRNPK. We provide a short discussion on these proteins and argue how these could have contributed to the poor prognostic performance of these patients. In conclusion, these data support RPPA-based profiling as potential tool to identify new epigenetic targets for treatment in AML.

Epigenetic dysregulation by histone modification marks is well-recognized to contribute to cancer development. Best-known is that loss of the repressive trimethylated mark H3K27 (H3K27me3) predicts poor outcome in multiple solid tumors.[1-6] H3K4 di- and trimethylation, that is associated with open and active chromatin, has also been described predictive for outcome in different cancers.[7,8] In **Chapter 3** we examined the prognostic value of these HMM in adult AML and ALL by RPPA. Similar as found in solid cancers, greater loss of H3K27me3 was associated with a poor prognosis in AML. Low-H3K27me3 independently predicted shorter OS in the whole patient population, as well as in subsets with DNA methylation mutations. Based on protein correlations revealed by the RPPA, AML patients with decreased H3K27me3 demonstrated an upregulated anti-apoptotic phenotype. In ALL, patients who carried the Philadelphia cytogenetic aberration (Ph+ ALL) and had resistance against tyrosine kinase inhibitors showed significantly reduced levels of methylation marks at diagnosis compared to long-term survivors. Noteworthy was that 2/3 patients who were resistant against TKI were prior smokers, compared to none of the 8 responders. The concept that smoking alters the epigenetic machinery and associates with response to TKI in Ph+ ALL has been proposed previously and warrants further investigation. Thus, our data supports that proteomic profiling of histone methylation levels improves outcome predication in AML and Ph+ ALL and suggests potential targets to further investigate diagnostically and therapeutically.

In **Chapter 4** we performed the same RPPA-based proteomic approach to investigate the effect of histone modification related protein levels in pediatric AML. A protein array was built using peripheral blood samples of 483 children with AML who were treated in the COG AAML1031 clinical trial. Similar as in the adult population, histone and chromatin modifying protein and histone methylation mark expression is heterogeneous among *de novo* pediatric AML. Also, in the patient group who received the standard chemotherapeutic regime we observed that higher expression of 16 HME (high-HME) was an independent variable that predicted a higher relapse risk (RR) 3 year after second induction therapy compared to those with lower HME expression (low-HME). This finding was independent of HMM expression. No significant difference between standard and standard+bortezomib treated patients in terms of RR in the low- or high-HME group separately, but 1 year after the end of second induction therapy, RR was 47% in the high-HME standard-treated and only 29% in the high-HME who received additional bortezomib. Also, there was significantly improved 3-years OS for those with the high-HME who were treated with the bortezomib-

containing chemotherapy compared to high-HME patients without bortezomib. Next, we demonstrate that the high-HME patients had more open chromatin surrounding promotor sites compared to low-HME patients using ATAC-seq. High-HME inversely correlated with FOXO3 protein expression on the array. In line with this finding, patients with lower expression of FOXO3 had adverse outcome after ADE treatment, but this was ameliorated by the bortezomib addition. As validated by *in vitro* experimentation, knockdown of FOXO3 leads to higher leukemic cell counts and cells that are more resistant to doxorubicin and etoposide combination therapy. Additionally, we report here for the first time a FOXO3-vimentin correlation in AML. Thus, although bortezomib-containing chemotherapy did not improve outcomes in the entire population in the AAML1031 study, there is a group of patients that may benefit from bortezomib addition based on histone and chromatin modification. We speculate that enhanced proteasome inhibitor sensitivity in AML is related to high-HME expression that conveys more open chromatin and higher protein load. In **Chapter 5** we showed that the prognostic significance of another protein PTM in the AAML1031 clinical trial. Low expression of the RelA-pSer^{536} was favorably prognostic when compared to those with high RelA-pSer^{536} after standard chemotherapy plus the proteasome inhibitor bortezomib. Additionally, we found a strong correlation between RelA-pSer^{536} and HSF1-pSer^{326}, which was strengthened by a similarly strong association in a cohort of 358 T-ALL patients, increasing the probability that there is a real, but previously unrecognized, relationship between these proteins. The prognostic value of HSF1-pSer^{326} in bortezomib treated pediatric AML have been shown previously by Hoff et al.[9] In addition to low-RelA-pSer^{536}, we here show that the combination of low-RelA-pSer^{536} and low-HSF1-pSer^{326} improves prognosis compared with either protein alone. We suggest that the combination of these two proteins could be used to a priori identify a group of AML patients (38% of patients in this study) that benefit from additional bortezomib. Of note, while the combination with low/high expression of HME did not affect patient prognosis with both low-RelA-pSer^{536} and low-HSF1-pSer^{326} expression, HME stratification can potentially identify another small group of low-RelA-pSer^{536} and high-HSF1-pSer^{326} expression with a favorable prognosis after standard treatment plus bortezomib, while the low-RelA-pSer^{536} and high-HSF1-pSer^{326} following standard treatment did not.

In **Chapter 6**, we identified four distinct epigenetic signatures based on RPPA proteomics in a large cohort of CLL and related diseases related samples. Again, these signatures associated with clinical features and outcome. In detail, the proteomic

signatures associated with clinical features including age, cytogenetic abnormalities [trisomy 12, del(13q) and del(17p)], immunoglobulin heavy-chain locus (IGHV) mutational load, ZAP-70 status, Binet and Rai staging as well as with the outcome measures of time to treatment and overall survival. Protein signature membership was identified as the predictive marker for overall survival regardless of other clinical features. Among the analyzed epigenetic proteins, EZH2, HDAC6 and loss of H3K27me3 levels were the most independently associated with poor survival. Additionally, standard CLL therapy has been rapidly evolving since the introduction of Bruton's tyrosine kinase inhibitors (BTKi) and more recently the availability of Bcl-2 blockade with venetoclax. Therefore we were keen to investigate whether the epigenetic proteomic signatures were prognostic for both old and/or new treatment regimes. To study this, we analyzed outcome (overall survival after first treatment and time to second treatment) after chemotherapy-, BTKi- and antibody/targeted-based therapies separately. Patients with a proteomic signature similar to mature small B-cell lymphomas (MSBL)-like malignancies had poorest outcomes after all three regimes compared to the other signatures, but best with chemotherapy-based treatments. Overall, "bulk" CLL patients within the poor prognostic proteomic signature with low HMM had poor prognosis after chemotherapy-based treatments, but not after BTK inhibitors. We conclude that an analysis of epigenetic protein biomarkers is valuable for prognostication and treatment selection in CLL and complements prior genomic and methylomic CLL classifications.

In **Chapter 7** we discuss currently used therapeutic approaches in AML, including targeted treatment strategies that have recently entered the clinic. Then we will introduce the general concept of quantitative proteomics and debate why we think this technology may hold promise in identifying new diagnostic and prognostic biomarkers and in providing new therapeutic leads, especially in a heterogeneous disease as AML.

Discussion and future perspectives

Every answered question leads to new smarter questions. So does this thesis. The combined findings of this thesis raise numerous questions for furter investigation. What are the gaps that have been bridged and what are the gaps that have been created?

The current WHO classification incorporates cytogenetics and molecular changes, and most prognostic stratification systems are based on these in association with clinical

features, typically dividing patients into favorable, intermediate and unfavorable groups. However, within these groups there is significant heterogeneity of response suggesting that these prognostic classifications could be improved on. Furthermore, despite the usage of mutations in predicting disease progression, few of the most common mutations, for example FLT3, DNMT3A, NPM1 in AML, have no drugs that just targets the mutation the way the IDH inhibitors do. This limits the ability to convert knowledge of molecular abnormalities into specific therapeutic interventions. Besides chromosomal and genetic abnormalities, epigenetic events contribute to the pathophysiology of leukemia. The improved understanding of how epigenetics (both DNA methylation and histone modification) impacts the biology and prognosis in leukemia have resulted in the translational exploration of epigenetic modifying drugs in various stages of clinical development. But, as aforementioned, epigenetic profiling is not yet incorporated in the daily practice of newly admitted leukemia patients. This thesis shows that dysfunctional epigenetic protein expressions confer prognostic information beyond the existence of mutations or chromosomal abnormalities. Therefore, clinical proteomic tests to measure epigenetic protein status could be a powerful means to add and to improve risk stratification in this complex disease.

We hypothesize that adding proteomics can likely help to identify more refined subgroups that better correlate with risk. Clinically, this is important as it can help by the selection of patients that need more aggressive chemotherapy regimens, for example stem cell transplantation, as well as patients that can be treated with less toxic regimens. If we can discover an epigenetic modifying protein or a limited set of proteins or PTMs as a kind of biomarker that is associated with outcome or disease progression with a high sensitivity and specificity, we can develop a rapid test that quickly provides information about the protein expression in these patients. If this supposition of therapy selection appears to be working, real-time measurement of protein expression of a limited panel of proteins at time of diagnosis is needed to facilitate its potential clinical application by identifying the right patient based on their matching protein profile. For instance, immunohistochemistry (IHC) which quantifies proteins in samples fixed to slides, or enzyme-linked immunosorbent assays (ELISA), that quantifies protein concentration using antibodies can be used, enabling rapid classification.[10,11]

Protein candidates for real-time measurement at time of diagnosis based on our findings include BRD4, SIRT1 and KDM1A (**Chapter 2**), H3K27me3 **(Chapter 3**

and **6**), FOXO3 (**Chapter 4),** phosphorylated RelA (**Chapter 5**), HDAC6 and EZH2 (**Chapter 6**). Preclinical studies have already demonstrated the efficacy of BRD4 inhibition in AML as these inhibit transcription of downstream genes involved in proliferation and NF-kB inflammation as well as increase apoptosis by downregulation of anti-apoptotic Bcl-2 protein.[12] Although clinical studies are underway with no response data yet available, previous studies suggest that BRD4 inhibition show synergistic effects in combination with other chemotherapeutics. Unfortunately, a recent study in AML testing a small molecule inhibitor of bromodomain and extra-terminal domain (BET) discontinued early based on limited efficacy (NCT02698189). As subsets of patients with high BRD4 (part of the BET protein family) had the poorest outcome, we argue that BRD4 inhibition might have better clinical effect in this subset of patients. Furthermore, high SIRT1 expression also contributed to the adverse outcome in adult AML. SIRT1 acts as histone deacetylator for proteins including p53 and FOXO3. We advocate that exploring the value of HDAC inhibitors in those with the highest SIRT1 levels is worthier compared to those with lower levels.[13] Identification of KDM1A level at time of diagnosis might identify eligible candidates for histone lysine demethylase inhibition. Inhibitors have shown therapeutic potential and are currently being tested in clinical trials.[14] In both AML and CLL we found that loss of H3K27me3 levels predicted poor outcomes. Inhibition of H3K27 by methyltransferases inhibitors contributed to chemoresistance in both *in vitro* and *in vivo* xenograft mouse AML models which was rescued by EZH2 restoration using proteasome inhibitors.[15] This might provide a rationale for the addition of proteasome inhibitors in the AML treatment regime for identified patient subgroups with H3K27me3 loss and poor outcomes, i.e. intermediate and unfavorable AML, p53 and DNA methylation or spliceosome mutated patients. Another approach that may lead to an increase in H3K27me3 levels is by the inhibition of H3K27me3 demethylators, for example KDM6B. Pharmacological inhibition of KDM6 with GSK-J4 reduces AML cell survival and is under consideration as treatment option in AML.[16,17] In Chapter 4, we observe that higher HME at time of diagnosis predicts adverse outcome in standard treated pediatric AML patients, similar as in our adult population. The high-HME signature correlated inversely with FOXO3 protein expression and low-FOXO3 was identified as a poor prognostic factor in the patients who received standard chemotherapy. These patients had better outcomes when bortezomib was added to their treatment regime. In addition, bortezomib is significantly beneficial for pediatric AML patients with low phosphorylation of the NF-kB subunit RelA (low-RelA-pSer^{536}, Chapter 5). If we can

identify which patients express higher levels of FOXO3 and/or RelA-pSer^{536} at time of diagnosis, we likely could select pediatric AML patients that are more sensitive to additional bortezomib which can greatly contribute to rational selection of the right patients for the right treatments and improve outcomes. More extensive studies are warranted to explain why high-HME, low-FOXO3 or low-RelA-pSer^{536} benefitted more from additional bortezomib than their opponents. In Chapter 6, we identified a group of CLL patients with an epigenetic distinct proteomic signature that had adverse outcome with chemotherapy-based treatment, but not with BTK inhibitors. Patients with this signature were characterized by high EZH2 and HDAC6 among others. Selective HDAC6 inhibition have already showed therapeutic efficacy in pre-clinical CLL models[18] and synergy between HDAC6 inhibitors and ibrutinib (BTK inhibitor) have been reported in follicular lymphoma.[19] These data show that RPPA can be used to improve individual response prediction to modern treatments in CLL focusing on epigenetics. More research is needed to confirm and validate these findings before these can be brought to the clinic.

Validation of results

In Chapter 4 we observed an association between HME and bortezomib sensitivity indicating that this effect is related to variation in histone regulated DNA accessibility. To test this hypothesis, we have performed ATAC-seq on a panel of 24 patient samples who participated in the AAML1031 study. Increased sensitivity to bortezomib was associated with the high-HME signature. Patients with this signature had more open chromatin at promotor loci than those with low-HME. We hypothesize that this potentially could reflect increased protein production. An increased protein load has been suggested as predictor for enhanced sensitivity to proteasome inhibition. To validate our findings biologically, we aim to delineate chromatin accessibility, but also transcription factor binding (ChIP-seq) and gene expression (RNA-seq) differences in cell lines that mimic HME states. Previously, we have quantitated AML cell line sensitivity to bortezomib, and we have identified cell lines that have high-HME (HL60, AML-ML2) or low-HME (OCI-AML3, OCI-AML5). After comparing bortezomib sensitivity in these cell lines, we would ideally examine the differences in ATAC-seq, but also ChIP-seq and RNA-seq before and after bortezomib treatment. Subsequently, a comparison between high-HME (bortezomib sensitive) and low-HME (bortezomib resistant) should be made. Chromatin accessibility analysis in Chapter 4 suggests more open regions for high-HME that were enriched for proteins that act in the Wnt pathway

(WNT3, WNT5A, WNT6) suggesting that baseline upregulation of this pathway may sensitize cells to undergo apoptosis when treated with bortezomib. These results should be validated by *in vitro* experiments as outlined above.

We further hypothesize that treatment of low-HME cell lines with HDAC inhibitors (HDACi) and hypomethylating agents (HMA) or specific targets (for example KDM1A or BET inhibitors) might mimic a more high-HME state. If proven, we will compare bortezomib sensitivity in low-HME cell lines with or without histone modification modifying treatment. Once a shift in the bortezomib sensitivity is detected, we should test whether the low-HME cell lines with bortezomib and HDACi/HMA demonstrate a similar response as the high-HME with bortezomib only. Finally, when successful, we would compare bortezomib sensitivity between high-HME patient samples and low-HME samples+HMA/HDACi *in vitro,* and in PDX-mice. Unfortunately, due to the COVID-19 pandemic any additional validations could not have been performed.

Challenges in target identification

RPPA is an antibody-based proteomic tool and its reliability relies on the quality of the antibodies. However, the number of proteins that have strictly validated antibodies available for RPPA is still limited, considering the existence of ~20,000-25,000 proteins when even excluding their PTMs, and therefore RPPA is not applicable for *de novo* identification of PTMs that might represent markers for specific signal-transduction pathways. Many HME and HMM interact with each other and although strong correlations in expressions were observed in the panel, there may be other, not analyzed HME or non-HME that have influenced certain HMM modifications. Technical and methodological issues about the RPPA technique will be further discussed in more detail later in this chapter.

Challenges in prognostication

This thesis provides a number of epigenetic modifying proteins that have shown to improve prognostication in various leukemic populations. In general, we have performed multivariate analysis to analyze their prognostic power in light of other known risk factors. Although we have validated some of our findings in more detail, there are still many other biological processes underlying leukemia which are not yet tested for in the clinical diagnostic setting. Acute leukemia is a very chaotic disease

and the biology underlying a patient's leukemia is likely more complex than known risk factors. For example, we did not have the tools to take into account the potential influences of the microenvironment, the stress, metabolic or immune responses and the degree of genome instability.

Nevertheless, many researchers around the world are currently working together with the same goal and we are experiencing an exponential rise in big data generation and dataset availability globally. The integration of datasets is just beginning to be performed, but there are some complicating factors, such as differences in methodology between laboratories and platforms. The most used and reviewed 'omics' approaches are the genetic code along with mRNA expression data which, in AML, is indisputably helpful in risk stratification but unfortunately less significant in target therapy selection.[20] As most developed drugs target proteins rather than genes, studying the proteome at time of diagnoses as well as post-treatment hold promise to eventually support the realization of precision medicine. The integration of datasets at larger scales is necessary to achieve such goals, but we believe that there is great potential in combining (epi)genomic, transcriptomic and proteomic data for a chaotic disease as leukemia.

Challenges in therapeutic guidance

One of the biggest remaining challenges we are facing is the implementation of our findings into routine clinical use. Current platforms utilize methodologies appropriate to the research laboratory but not to the routine clinical molecular laboratory. First, rapidly performed tests that enable classification of the epigenetic protein signature membership, that can be Clinical Laboratory Improvement Amendments (CLIA) certified, are required. To do so, clinically validated cut-off levels need to be determined for each protein/PTM marker. Secondly, verification is needed to confirm suggestions of which targeted agents would increase therapeutic efficacy when used in a particular signature. Knowledge of the epigenetic signature membership only has utility if a reliable knowledge based on which targeted agent should be used in each signature is available. As new epigenetic agents that are not currently covered by the proteomic analyses performed to date are developed, there must be ways to incorporate that growing list of available targeted agents.

This study provide insight into epigenetic regulators and histone methylation marks can improve outcome prediction in different leukemias. In every study, treatment options were two or more, and it depended on the trial (AAML1031, Chapter 4 and 5), or doctor's expertise (Chapter 2, 3, 6) to determine what regimen a patient would get. We therefore could not fully examine the effect of epigenetic modifying agents according to the epigenetic proteomic profiles. Current epigenetic treatment options in leukemia include hypomethylating agents (HMA) and histone deacetylase inhibitors (HDACi). HMA encompasses azacytidine and decitabine, and work by inhibiting methylation of the DNA, which affects the coiling of the DNA, thereby causing re-expression of silenced genes and reprogram cancer cells to a more normal-like state. Nowadays they are mainly used in elderly patients who are ineligible for intensive treatment such as SCT. The advantage of the use of HMA in elder patients is that side effects are relatively well-tolerated. HDACi work by inhibiting histone deacetylases, resulting in a more transcriptionally silenced chromatin. Although they have been intensively studied in AML, their success remains limited.[21] There was a small portion of the analyzed adult AML patients that were treated with HMA and HDACi agents. Interestingly, we observed that all responders in the dataset to these agents had the FLT3-ITD mutation. Since FLT3-ITD mutation was independent of outcome assembled in the clusters with high HME expression, it shows a possibility that HMA and HDACi may contribute in treatment approaches for AML patients with both the high-HME and FLT3-ITD. Further studies should be performed to test HMA and HDACi sensitivity according to epigenetic modifying protein and methylation mark expressions.

Technological challenges of RPPA

In comparison to mass spec-based methods (See Chapter 7 for detailed information), the disadvantage of RPPA is that it cannot be used as a *de novo* discovery platform. In theory MS can be used to identify all proteins although in practice this requires depletion of highly abundant proteins, to enable the detection of low abundance proteins. RPPA has some advantage over MS: 1) it requires less material (approximately 3 x 105 cells to test 400 different proteins), which makes it highly suitable for clinical applications; 2) it analyzes all samples at once, allowing a direct comparison of protein expression across samples, a process that is much more difficult in MS where each sample is studied individually at multiple timepoints; and 3) it is far more economical when studying a large number of samples. In particular its potential to analyze large sets of patients

simultaneously is very important, as analyzing small groups of patients may not fully represent the heterogeneous AML population and thus may miss important protein utilizations in subgroups of AML. The disadvantages of RPPA are that 1) all samples must be printed at one time, making it impractical for real-time individual patient analysis; and 2) RPPA is biased to proteins and isoforms for which a strictly validated antibody is available. As RPPA is a high-throughput antibody-based technique, the reliability is highly dependent on the quality of the antibodies that are used. Unlike in a Western blot, with RPPA there is no separation of the proteins according to molecular weight, which has the consequence of making signals from potential cross-reactivities indistinguishable from the intended signals. Antibodies used on RPPA must all be strictly validated, and must be shown to be specific, selective, and reproducible in the context for which it is to be used, must perform robustly across different sample types and must acts consistently over time. However, once an antibody is validated, RPPA allows not only detection of total proteins, but can also yield information about PTMs since a broad spectrum of antibodies that detect phosphorylated, acetylated, methylated, and cleaved forms of proteins is available.

Methodological challenges of RPPA

Although having great promise, certain studies should help optimize the use of proteomics in the future. The first question that should be considered is the use of an appropriate control cell to define the range of 'normal' expression. While the most frequently used control sample is the CD34+ cell population, it would even be more preferable to use normal bone marrow CD34+ CD38− stem cells to compare against the AML blast. However, as only a very minor fraction of the mononuclear cells in the bone marrow is a hematopoietic stem cell, this complicates its use. Secondly, it would be interesting to study the protein expression in blast cells derived from therapy resistant patients or from relapsed leukemia patients, and compare those to cell from patients that were relapse-free. Evidence suggests that despite the ability of chemotherapy to kill the vast majority of leukemic cells, the rare leukemic stem cell that survives the chemotherapy, is responsible for the outgrowth of the leukemia cells which is manifested as relapse or primary resistant disease. Proteomic analysis of these cells might be more informative than the analysis of the bulk leukemia population, but without a current means to a priori identify those few cells, isolation of (enough of) those cells remain a real challenge.

Another interesting approach would be to study proteomics in single cells, rather than studying the average abundance of protein expression across bulk cells. Although it was expected that the same genome and the same environment give rise to identical proteomes, it is known that individual cells differ in their proteomes, with sometimes significantly different functional consequences. As described by Simpson's paradox, it is likely true that if you take a population-average protein level, there seems to be a particular correlation between proteins, whereas if you look at the individual cells, proteins can have the same correlation in one cell but the inverse relationship in the other.[22] So, if we can detect and study such differences between cells from a single patient, this can provide useful information about cell to cell heterogeneity. Mass cytometry time-of-flight (CyTOF) methodology has already enabled to characterize rare AML stem/progenitor cells in AML and to identify proteins that play key role in drug resistance and AML relapse. Recently, Palii et al. utilized CyTOF to capture temporal dynamics of protein expression of transcription factors in individual cells during human erythropoiesis, showing that most transcription factors from different lineages are co-expressed and that their expression changes gradually during differentiation rather than abruptly.[23] However, so far this method is not cost-effective to perform on large scale. A variant of this would be to study single cell proteomics in different cell populations, for minimal residual disease detection or in cells before and after chemotherapy exposure to evaluate the effect of treatment on the protein expression or activation dynamic over time. Knowing how different cells respond to chemotherapy, would then likely to raise new biological questions about why different cells behave differently, and why or how cells are able to circumvent chemotherapy.

Challenges of studying epigenetics using proteomics

Proteomics is not the standard to study epigenetics. Nonetheless, mass spectrometry (MS)-based analysis of chromatin is expanding and have shown to be promising in order to study the chromatin-bound proteome.[24] The advantage of using MS for proteomic analysis is that the aberrant chromatin landscape of cancers is marked by abnormal histone tail PTM and by abnormal distribution of histone and chromatin modifying protein (complexes) to specific promotor loci, which is both covered using this technique.[24,25] The latter is one limitation of our study using RPPA. While we are able to analyze multiple HME and HMM simultaneously in large cohorts of patient derived material and link patterns to chromatin accessibility, RPPA does not directly

allow the mapping of where in the genome the protein/PTM changes are localized. Also, the effects of aberrant HME/HMM expression on transcriptional programs are not fully elaborated. Additional chromatin immunoprecipitation sequencing procedures (ChIP-seq) and transcriptome analysis (RNA-seq) to validate findings are thus necessary.

Conclusion

Leukemia is an epigenetically heterogeneous, chaotic disease. Choice and intensity of treatment is currently guided by cytogenetic and molecular genetic risk classifications. Yet, these incompletely predict outcomes, requiring additional information for more accurate risk stratification and response to therapy predictions. This thesis shows that proteomic profiling of epigenetic modifications has clinical implications in acute and chronic leukemia and supports the idea that epigenetic patterns contribute to a more accurate picture of the leukemic state that complements cytogenetic and molecular genetic subgrouping. A combination of these variables may offer more accurate outcome prediction and we suggest that histone methylation mark measurement at time of diagnosis might be a suitable method to improve patient outcome prediction and subsequent treatment intensity stratification in selected subgroups.

References

1. Yu J, Rhodes DR, Tomlins SA, et al. A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. *Cancer Res*. 2007;67(22):10657-63. https://rug.on.worldcat.org/oclc/181652137.
2. Wei Y, Xia W, Zhang Z, et al. Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. *Mol Carcinog*. 2008;47(9):701-6. https://rug.on.worldcat.org/oclc/264689191. doi: 10.1002/mc.20413.
3. Schlesinger Y, Straussman R, Keshet I, et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat Genet*. 2007;39(2):232-6. https://rug.on.worldcat.org/oclc/110093554.
4. Arjen H G Cleven, Ghadah A Al Sannaa, Inge Briaire-de Bruijn, et al. Loss of H3K27 tri-methylation is a diagnostic marker for malignant peripheral nerve sheath tumors and an indicator for an inferior survival. *Modern Pathology*. 2016;29:582-590. https://rug.on.worldcat.org/oclc/8253530389. doi: 10.1038/modpathol.2016.45.
5. Panwalkar P, Clark J, Ramaswamy V, et al. Immunohistochemical analysis of H3K27me3 demonstrates global reduction in group-A childhood posterior fossa ependymoma and is a powerful predictor of outcome. *Acta Neuropathol*. 2017;134(5):705-714. https://rug.on.worldcat.org/oclc/7183698666.
6. Katz LM, Hielscher T, Liechty B, et al. Loss of histone H3K27me3 identifies a subset of meningiomas with increased risk of recurrence. *Acta Neuropathol*. 2018;135(6):955-963. https://rug.on.worldcat.org/oclc/7453654627. doi: 10.1007/s00401-018-1844-9.
7. Seligson DB, Horvath S, McBrian MA, et al. Global levels of histone modifications predict prognosis in different cancers. *The American journal of pathology*. 2009;174(5):1619-28. https://rug.on.worldcat.org/oclc/319540815. doi: 10.2353/ajpath.2009.080874.
8. Li S, Shen L, Chen KN. Association between H3K4 methylation and cancer prognosis: A meta-analysis. *Thoracic cancer*. 2018;9(7):794-799. https://rug.on.worldcat.org/oclc/7584212371. doi: 10.1111/1759-7714.12647.
9. Hoff FW, van Dijk AD, Qiu YH, et al. Heat shock factor 1 (HSF1-pSer326) predicts response to bortezomib-containing chemotherapy in pediatric AML: A COG study. *Blood*. 2020. https://rug.on.worldcat.org/oclc/8667700660. doi: 10.1182/blood.2020005208.
10. Ferrier CM, de Witte HH, Straatman H, et al. Comparison of immunohistochemistry with immunoassay (ELISA) for the detection of components of the plasminogen activation system in human tumour tissue. *Br J Cancer*. 1999;79(9-10):1534-41. https://rug.on.worldcat.org/oclc/119409807.
11. Afaf Abdel-Aziz Abdel-Ghafar, Manal Ahmed Shams El Din El Telbany, Hanan Mohamed Mahmoud, Yasmin Nabil El-Sakhawy. Immunophenotyping of chronic B-cell neoplasms: Flow cytometry versus immunohistochemistry. *Hematology Reports*. 2012;1:e3. https://rug.on.worldcat.org/oclc/8772717672. doi: 10.4081/hr.2012.e3.

12. Diana Reyes-Garau, Marcelo L. Ribeiro, Gaël Roué. Pharmacological targeting of BET bromodomain proteins in acute myeloid leukemia and malignant lymphomas: From molecular characterization to clinical applications. *Cancers*. 2019;11(10). https://rug.on.worldcat.org/oclc/8286324945. doi: 10.3390/cancers11101483.

13. C A Bradbury, F L Khanim, R Hayden, et al. Histone deacetylases in acute myeloid leukaemia show a distinctive pattern of expression that changes selectively in response to deacetylase inhibitors. *Leukemia*. 2005;19:1751-1759. https://rug.on.worldcat.org/oclc/8254526975. doi: 10.1038/sj.leu.2403910.

14. Fang Y, Liao G, Yu B. LSD1/KDM1A inhibitors in clinical trials: Advances and prospects. *Journal of hematology & oncology*. 2019;12(1):129. https://rug.on.worldcat.org/oclc/8392995934. doi: 10.1186/s13045-019-0811-9.

15. Göllner S, Oellerich T, Agrawal-Singh S, et al. Loss of the histone methyltransferase EZH2 induces resistance to multiple drugs in acute myeloid leukemia. *Nat Med*. 2017;23(1):69-78. https://rug.on.worldcat.org/oclc/6905067175. doi: 10.1038/nm.4247.

16. Li Y, Zhang M, Sheng M, et al. Therapeutic potential of GSK-J4, a histone demethylase KDM6B/JMJD3 inhibitor, for acute myeloid leukemia. *J Cancer Res Clin Oncol*. 2018;144(6):1065-1077. https://rug.on.worldcat.org/oclc/7408370674. doi: 10.1007/s00432-018-2631-7.

17. Boila LD, Chatterjee SS, Banerjee D, Sengupta A. KDM6 and KDM4 histone lysine demethylases emerge as molecular therapeutic targets in human acute myeloid leukemia. *Exp Hematol*. 2018;58:44-51. https://rug.on.worldcat.org/oclc/7181778234. doi: 10.1016/j.exphem.2017.10.002.

18. Maharaj K, Powers JJ, Achille A, et al. Silencing of HDAC6 as a therapeutic target in chronic lymphocytic leukemia. *Blood advances*. 2018;2(21):3012-3024. https://rug.on.worldcat.org/oclc/7912265597. doi: 10.1182/bloodadvances.2018020065.

19. Lee DH, Kim GW, Kwon SH. The HDAC6-selective inhibitor is effective against non-hodgkin lymphoma and synergizes with ibrutinib in follicular lymphoma. *Mol Carcinog*. 2019;58(6):944-956. https://rug.on.worldcat.org/oclc/8106869086. doi: 10.1002/mc.22983.

20. López de Maturana E, Alonso L, Alarcón P, et al. Challenges in the integration of omics and non-omics data. *Genes*. 2019;10(3):238. https://rug.on.worldcat.org/oclc/8034866084. doi: 10.3390/genes10030238.

21. San José-Enériz E, Gimenez-Camino N, Agirre X, Prosper F. HDAC inhibitors in acute myeloid leukemia. *Cancers*. 2019;11(11). https://rug.on.worldcat.org/oclc/8324237781. doi: 10.3390/cancers11111794.

22. Blyth CR. On simpson's paradox and the sure-thing principle. *Journal of the American Statistical Association*. 1972;67(338):364-366. https://rug.on.worldcat.org/oclc/5545465793.

23. Palii CG, Cheng Q, Gillespie MA, et al. Single-cell proteomics reveal that quantitative changes in co-expressed lineage-specific transcription factors determine cell fate. *Cell Stem Cell*. 2019;24(5):812-820. https://rug.on.worldcat.org/oclc/8096621175. doi: 10.1016/j.stem.2019.02.006.

24. van Mierlo G, Vermeulen M. Chromatin proteomics to study epigenetics - challenges and opportunities. *Molecular & cellular proteomics : MCP*. 2021;20:100056. https://rug.on.worldcat.org/oclc/8914363467. doi: 10.1074/mcp.R120.002208.

25. Marchione DM, Garcia BA, Wojcik J. Proteomic approaches for cancer epigenetics research. *Expert Review of Proteomics*. 2019;16(1):33-47. https://rug.on.worldcat.org/oclc/7952406471. doi: 10.1080/14789450.2019.1550363.