5 What Have We Learnt from Genomics and Transcriptomics in Classic Hodgkin Lymphoma

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Contents

5.1 Introduction

A prominent pathological feature of cHL is the abnormal immune response represented by the abundant TME. It is thought that the majority of the immune cells in the TME are recruited by a variety of cytokines expressed by the HRS cells [1]. Cytokines are low-molecular-weight proteins with a wide variety of functions that work either in a paracrine manner to modulate the activity of surrounding cells or in an autocrine fashion to affect the cells that produce them. Furthermore, it is a widely accepted concept that the overexpression of regulatory cytokines and TGFβ leads to a microenvironment that suppresses cell-mediated immunity and in return favors HRS cell survival highlighting the bidirectional crosstalk of cells involved in the pathogenesis of HL [2].

The recent advances in HRS cell genomics and profiling the tumor microenvironment have already led to better insight into the molecular underpinnings of the disease, and we are anticipating discovery of additional clues explaining

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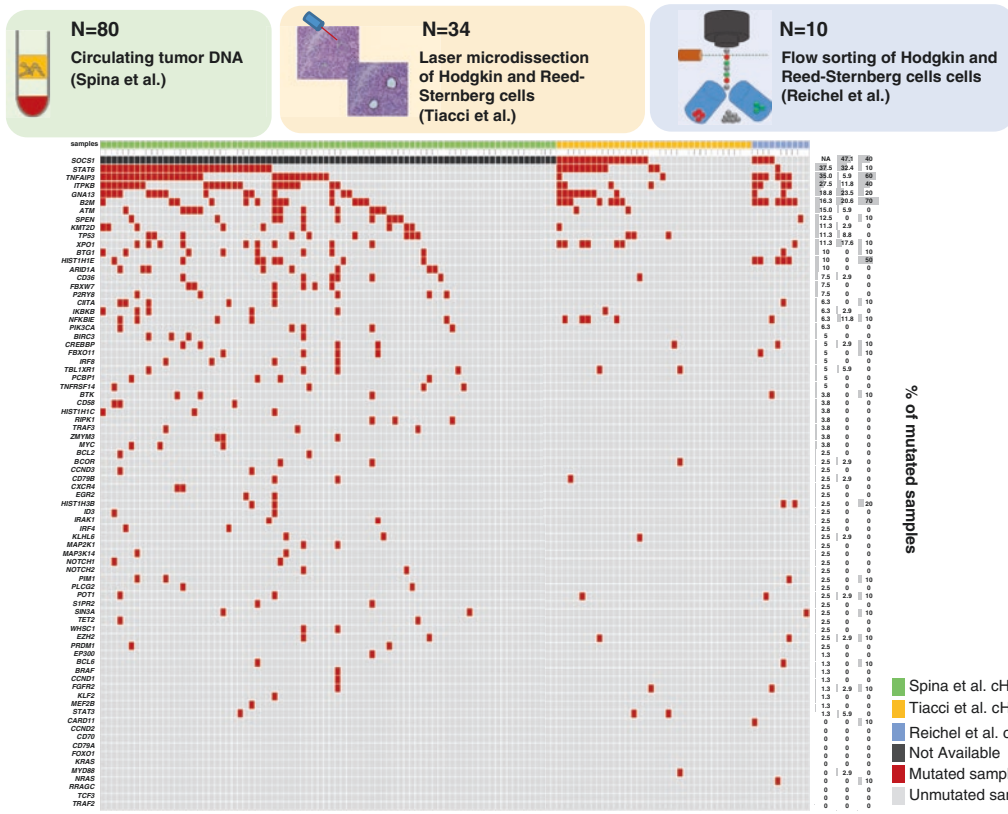

Fig. 5.1 The mutational profile of newly diagnosed cHL. The heatmap shows individual non-synonymous somatic mutations detected in three different cohorts (Spine et al., green; Tiacci et al., yellow; Reichel et al., blue). Each cohort has a different source of tumor DNA (i.e., circulating tumor DNA, DNA from laser microdissected Hodgkin and Reed-Sternberg cells, and DNA from flow-sorted Hodgkin and Reed-Sternberg cells). Each row represents a gene and each column represents a primary tumor. The heatmap was manually clustered to emphasize mutational co-occurrence. Mutations are color-coded in red. The horizontal bar graph shows the gene mutation frequency found in each different cohort

the unique crosstalk and symbiosis of the malignant cells with the non-malignant cells in the TME. In the following, we will highlight recent advances and future directions in (1) HRS cell genomics (Fig. 5.1) and (2) gene expression profiling.

5.2 Genomics of Hodgkin and Reed-Sternberg Cells

5.2.1 Cytokine Signaling

Constitutive activation of cytokine signaling pathways is a long recognized molecular hallmark of HRS cells. A number of studies provided evidence that various molecular mechanisms, including gene mutations and chromosomal alterations, can converge along with deregulated surface receptor signaling to lead to exuberant activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway [3–5].

Chromosomal aberrations of the *JAK2* locus on 9p24.1 in HRS cells were reported in one study in the large majority of cHL cases, including copy gain in 60% of cases, amplification in 30%, and polysomy in 10% [5]. Almost ubiquitous (~90% of cases) are genetic alterations of a variety of other JAK-STAT pathway members, which goes beyond previous estimates based on the presence of copy number gains of *JAK2*.

These include mutational disruption of the *SOCS1* (40%) and *PTPN1* (20%) negative pathway regulators, activating mutations of *JAK1* (10%), and multiple STAT transcription factors (*STAT6*, 30%; *STAT3*, 10% *STAT5B*, 10%) [4].

The association between convergent and recurrent point mutations in genes coding for interacting proteins of the JAK-STAT pathway is a common mechanism shared by CD30+ lymphomas, in particular cHL and anaplastic large cell lymphoma. Concurrence of these multiple somatic events indicates that these synergistic mutations are strongly selected for beyond single alterations to sustain pathway activation [6].

The pervasive targeting of JAK-STAT signaling genes in cHL, along with functional genomic studies, confirmed that JAK-STAT pathway activation represents a vulnerability of cHL and makes clinically available JAK or STAT inhibitors an attractive therapeutic approach in this disease [4].

5.2.2 NF-κB Signaling

Overall, genetic lesions in the NF-κB pathway occur in most of cHL cases, confirming their important role in the pathogenesis of this disease. Genomic gains/amplifications of the NF-κB transcription factor *REL* have been described in about 70% of cHL cases causing protein overexpression [7].

Mutations in negative regulators of NF-κB constitute a second important mechanism of pathway activation. *NFKBIA*, encoding IκBα, an inhibitor that binds NF-κB factors and prevents their nuclear translocation, is mutated in about 20% of cHL [8]. *NFKBIE*, encoding IκBε, an inhibitor that binds NF-κB factors and prevents their nuclear translocation, has been found in 30% of cases [9]. *TNFAIP3* the master negative regulator of NF-κB pathway is mutated in 30% of cases [3, 10].

Overall, NF-κB pathway mutations have been described in cHL with a higher frequency in EBV-negative cases, consistent with data establishing expression of the EBV-latent membrane protein 1 (LMP-1) as an independent contributor to constitutive activation of NF-κB in cHL [11, 12].

5.2.3 PI3K/AKT/mTOR Signaling

Mutations within the PI3K/AKT/mammalian target of rapamycin (mTOR) pathway occur in 50% of cHL, consistent with the pre-clinical evidence that cHL is addicted to this actionable cellular program [13]. *ITPKB* is mutated in 25% of cases. ITPKB is a non-canonical antagonist of PI3K. Physiologically, ITPKB dampens PI3K/AKT signaling by producing IP4, a soluble antagonist of the AKT-activating PI3K-product PIP3.

ITPKB mutations are quite specific for cHL, being rare or absent in other lymphomas, and cause the subcellular delocalization of the mutated protein in primary HRS cells. Moreover, *ITPKB* mutations correlate with PI3K/AKT signaling activation at both the gene expression and protein levels and, consistent with linkages to the downstream PI3K pathway, associate with resistance to PI3K inhibitors [3, 4].

The Ga13 G-protein subunit encoded by *GNA13* is mutated in 10% of cHL [3, 4]. By transmitting signals from the G-protein-coupled receptors S1PR2 and P2RY8 that result in the inhibition of AKT phosphorylation, Ga13 ensures the proper confinement of proliferating germinal center (CG) B cells within secondary lymphoid follicles and at the same time constrains their expansion by facilitating apoptosis in this potentially dangerous niche. Inactivating *GNA13* mutations promote altered GC B-cell migration within and beyond the GC, as well as impaired cellular adhesion, resulting in cells that may have a reduced ability to establish interactions with GC helper cells. Under normal conditions, a GC cell that is unable to form these helper cell interactions, due to either GC exit or ineffective cellular adhesion, would undergo apoptosis. However, *GNA13*-mutated GC B cells are resistant to programmed cell death by leading to elevated levels of pAKT [14].

Importantly, the genomic studies of microdissected HRS cells and ctDNA strongly suggest that mutations of *STAT6*, *TNFAIP3*, *GNA13*, and *ITPKB* are preferentially occurring in the ancestral clones, indicating that they are an early event in cHL pathogenesis [3, 4].

5.2.4 Immune Escape

Classical HL leverages multiple genetic mechanisms to escape immunosurveillance. First, reduction or loss of antigen presentation through *B2M* inactivating mutations/deletion has been described in 30% of cases [4, 15]. *B2M* encodes β2 microglobulin, a key component of the major histocompatibility complex (MHC) class I which is required for its expression and antigen presentation on the cell surface. Consistently, genetic disruption of *B2M* results in the loss of MHC class I protein expression on lymphoma cells [16, 17].

Second, gene rearrangements involving the MHC class II transactivator *CIITA* were found in 15% of cases. *CIITA* rearrangements result in the disruption of its transcriptional proprieties and loss of MHC class II expression on cHL cells. Both MHC class I and MHC class II losses are predicted to abrogate the interaction of the T-cell receptor (TCR) with a MHC-bound antigen presented on the cell surface, which is the first signal required to activate T-cell antitumor response [18]. Loss of both MHC I and II expression and related lack of neoantigen expression have been consistently found to induce "cold" immune microenvironments in lymphoma and other cancers [19, 20].

Third, PD-L1 and PD-L2 overexpression driven by copy gain of 9p24.1 is a frequent event in cHL. Alterations of the *PD-L1* and *PD-L2* loci were reported to include polysomy in 5% of cHL, copy gain in 56%, and amplification in 36%. The 9p24.1 amplification in cHL acts through two distinct mechanisms resulting in copy number-dependent increases of PD-L1 and PD-L2 expression and increased JAK/STAT signaling promoted by JAK2 protein expression which is almost exclusively co-regulated with PD-L1 and PD-L2 in the 9p24.1 amplicon [21].

5.3 The Transcriptome of HRS Cells

Overall, gene expression profiling experiments have contributed substantially to an improved understanding of the disease with respect to the inherent phenotypic features of the malignant HRS cells and the specific composition of the tumor microenvironment. Furthermore, first steps could be made to establish outcome correlations with the potential to improve treatment outcome prediction. However, many questions remain including often contradictory results derived from different patient cohorts. Focusing on HRS cells, the first major contribution of gene expression profiling was made by investigating HL-derived cell lines. These pivotal studies first established a transcriptome-wide view of the malignant cell compartment describing a unifying gene signature for cHL [22]. Together with other important similar studies, this gene expression work helped to elucidate the loss of B-cell signature phenotypes and the deregulated expression of transcription factor networks in comparison to the normal germinal center B-cell counterparts [23–26]. Major advances have also been made examining microdissected HRS cells from clinical biopsy material that further characterized transcriptional changes in primary cells [27–29]. Steidl and colleagues identified significant phenotypic heterogeneity within cHL and described for the first time genome-wide association with treatment outcome [28] (Fig. 5.2). The second study by Tiacci and colleagues added significant texture to the primary HRS cell expression phenotype emphasizing the differences in comparison to HL-derived cell lines [29]. Furthermore, two molecularly distinct cHL subtypes were discovered related to the transcription factor activity of NOTCH1, MYC, and IRF4. Another study for the first time also focused on gene expression profiling of microdissected cells from nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) describing a close relationship to classical Hodgkin lymphoma and T-cell-rich B-cell lymphoma [27].

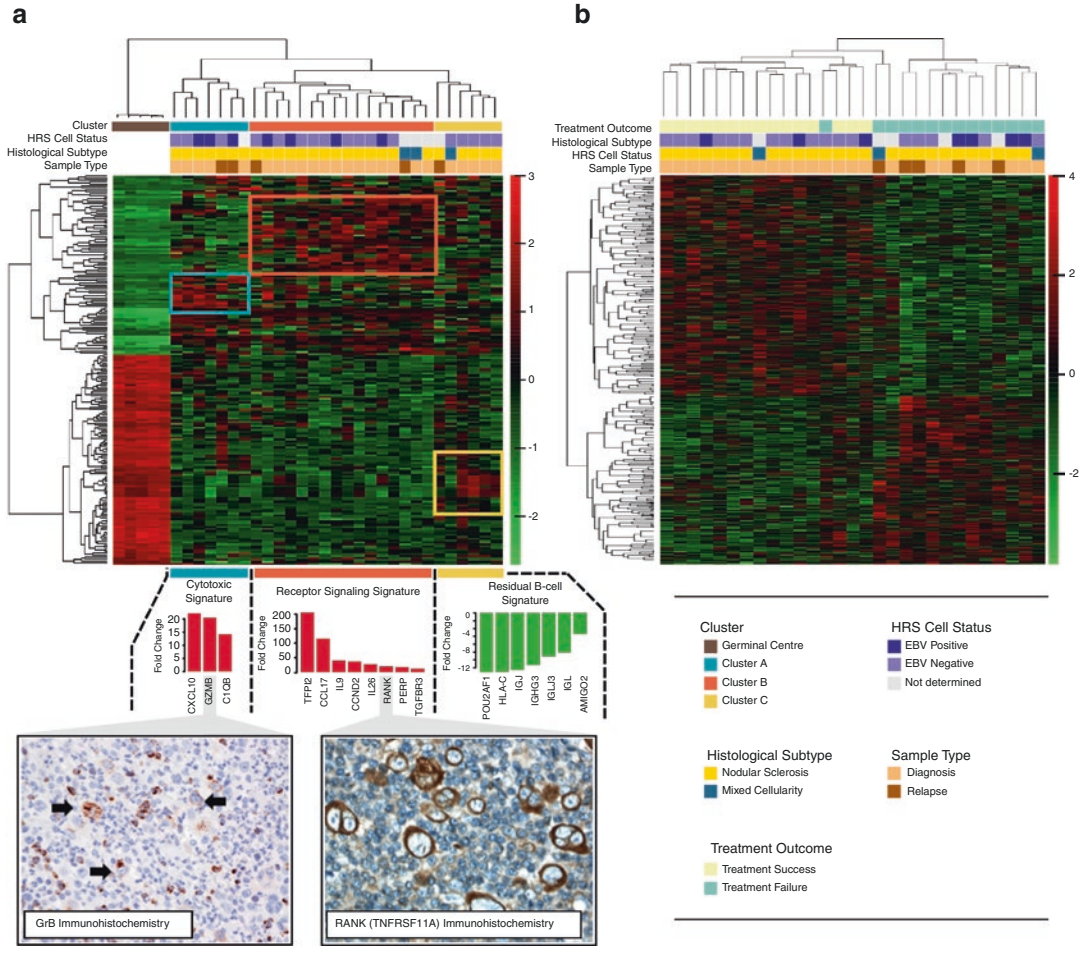

Fig. 5.2 Expression profiling of 29 samples of microdissected Hodgkin and Reed-Sternberg cells. (**a**) Unsupervised hierarchical clustering of gene expression profiles is shown using high variance genes. Red indicates relative overexpression and green relative under-expression. Patient clusters, histological subtype, EBV positivity of HRS cells by EBER in situ hybridization, and sample type are shown. The average fold changes of genes representative of the three main signatures are shown in the bar plots. Representative immunohistochemistry images are depicted demonstrating cytoplasmic positivity of Granzyme B (GrB, black arrows) and RANK in HRS cells. (**b**) Unsupervised hierarchical clustering of the cohort using the most differentially expressed genes between primary treatment failure and success. Treatment outcome, histological subtype, EBV positivity of HRS cells by EBER in situ hybridization, and sample type are shown. Cases cluster according to the outcome groups (two main clusters)

5.4 Microenvironment Profiling

Focusing on the HL microenvironment, a number of genome-wide gene expression studies have been published to date analyzing whole tissue lymph node biopsy material. Since the HRS cells are largely outnumbered by reactive cells in most biopsies, these studies on whole frozen biopsies are regarded as a reflection of the microenvironment [30–33]. However, some of these data provide evidence that at least parts of the apparent signatures are derived from HRS cells [31, 33]. In one study a specific gene expression signature could be linked to EBV positivity with genes overexpressed indicative of an increased Th1/antiviral response in comparison to the EBV-negative cases [32]. In addition to a better characterization of certain Hodgkin lymphoma subtypes

defined by specific gene signatures, these experiments also allowed for the study of outcome correlations using supervised analyses.

5.5 Biomarker-Driven Prognostication and Risk Stratification in cHL

The lack of extensive genotyping of microdissected HRS cells from large cHL patient cohorts has so far limited the identification of mutations affecting cHL outcome. ctDNA has been established as a source of tumor DNA for cHL mutational profiling. By overcoming the major technical hurdles that have so far limited cHL genotyping, ctDNA technology will allow large-scale assessment of mutations in different clinical phases ranging from newly diagnosed to refractory disease, and longitudinally during disease treatment, which in turn can reveal yet unknown prognostic and predictive biomarkers for cHL [3] (Fig. 5.3).

Beside disclosing tumor mutation profiles, ctDNA can also provide an estimate of the levels of residual disease during treatment in cHL. Consistently, ctDNA quantification after two chemotherapy courses has prognostic implications. A drop of 100-fold or 2-log drop in ctDNA after two chemotherapy courses, a threshold proposed and validated also in DLBCL, associates with complete response and cure in advanced-stage cHL treated with ABVD [3]. Conversely, a drop of less than 2-log in ctDNA after two ABVD courses associates with progression and inferior survival. Quantification of ctDNA complements interim PET/CT in determining residual disease. Indeed, cured patients who are inconsistently judged as interim PET/CT positive have a >2-log drop in ctDNA, while relapsing patients who are inconsistently judged as interim PET/CT negative have a <2-log drop in ctDNA. On this basis, incorporation of both PET/CT and ctDNA monitoring into clinical trials should allow to precisely define their cumulative sensitivity and specificity in anticipating the clinical course of cHL patients. Indeed, though interim PET/CT response assessment is a novel approach to refine management strategies before completing treatment in cHL, meta-analyses demonstrated a certain degree of inaccuracy of this application. In order to fill this gap, an area of growing interest is pairing interim PET/CT with biomarkers,

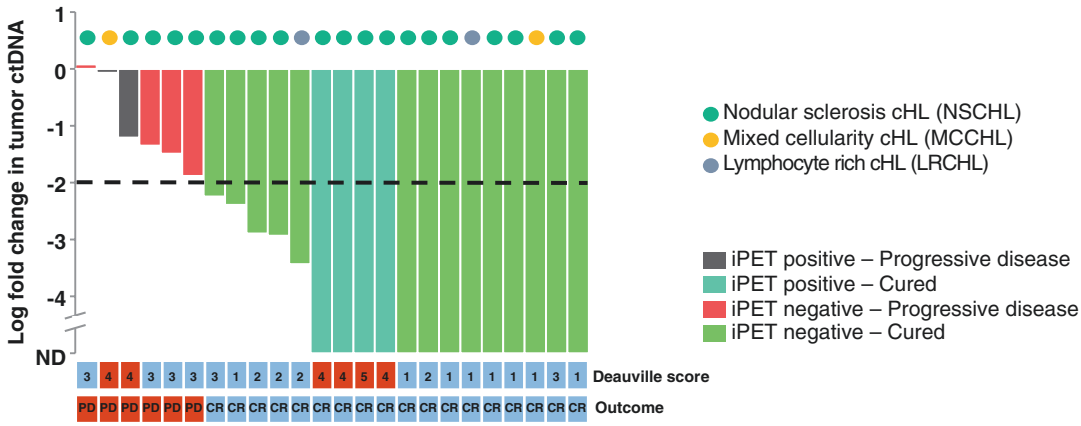

Fig. 5.3 Change in tumor ctDNA is a prognostic biomarker in cHL treated with chemotherapy. Waterfall plot of the log-fold change in ctDNA load after two courses of ABVD in 24 advanced-stage cHL cases. At the bottom of the graph, the interim PET/CT response scored according to the Deauville criteria, and the final outcome of the patient is indicated. Histological subtype of cHL is shown above the plot. Each column is color-coded according to the interim PET/CT results and the final patient outcome. Levels of ctDNA are normalized to baseline levels. The dashed line tracks the −2-log threshold (*iPET* interim PET/CT, *ND* not detectable, *PD* progressive disease, *CR* complete remission and cure)

such as ctDNA or serum TARC, to enhance their cumulative predictive value.

The type of 9p24.1 chromosomal aberration affects cHL outcome in both chemotherapy and immunotherapy treatment settings. Among chemotherapy-treated cHL, 9p24.1 amplification, but not polysomy or copy gain, associates with inferior progression-free survival [21]. Among patients treated with checkpoint blockade antibodies, those with higher-level 9p24.1 alterations and PD-L1 expression on HRS cells had superior PFS [34]. These analyses highlight the importance of quantifying and specifically delineating PD-L1 expression in malignant HRS cells for prognostic purposes.

Beside genetics, the tumor/TME phenotype has been prominently involved in past and ongoing biomarker considerations in cHL. Studies have used dichotomized clinical data sets based on slightly different definitions of clinical extremes according to the outcome after systemic treatment (i.e., treatment success versus treatment failure). However, these types of analyses have in part yielded conflicting results regarding the specific signatures that best define these clinical extremes. While one study found overexpression of genes involved in fibroblast activation, angiogenesis, extracellular matrix remodeling, and downregulation of tumor suppressor genes to be linked with an unfavorable prognosis, another study found a correlation of fibroblast activation, fibroblast chemotaxis, and matrix remodeling with improved outcome [30, 31]. While small sample sizes in both studies might have hampered interpretation, a more recent study investigated gene expression profiles of 130 patients including 38 patients whose primary treatments failed [33]. This study validated previously reported outcome correlations and furthermore showed that a gene signature of macrophages was linked to primary treatment failure. In a number of immunohistochemistry-based follow-up studies, multiple groups demonstrated that the enumeration of CD68+ macrophages in lymph node biopsies was a strong and independent predictor of disease-specific survival [35]. Specifically, an elegant retrospective study using Intergroup E2496 trial material (comparing ABVD to the Stanford V regimen) showed that high abundance of both CD68+ and CD163+ cells was correlated with shorter progression-free and overall survival independent of the IPS [36]. Importantly, the latter study used a computer-based scoring algorithm (Aperio) and systematically derived scoring thresholds that were tested in an independent validation cohort. Maximizing the concept of combining markers for building outcome predictors, a recent study used the same E2496 trial material to train a predictive model using intermediate density digital gene expression profiling developed in and applicable to routinely collected formalin-fixed paraffin-embedded tissue [37]. In this study the authors developed a 23-gene predictive model and associated thresholds to distinguish high-risk from low-risk advanced-stage Hodgkin lymphoma using overall survival as the end point. Encouragingly, when applied to an independent cohort treated with ABVD chemotherapy, the model validated the results in the E2496 training cohort identifying the patient at high risk of death. Follow-up studies are needed to further validate and implement biomarker assays for potential routine clinical use, risk stratification, and assessment as a predictive biomarker possibly guiding initial treatment decisions.

To date, cHL research has been for the most part focused on primary specimens, and only a few studies have explored the biology of relapse. However recently, the feasibility of biomarker studies and assay development at the time point of relapse was demonstrated in the context of outcome prediction of salvage therapy and ASCT [38]. The authors demonstrated that gene expression patterns, reflecting TME composition, differ significantly between matched primary and relapse specimens in a subset of cHL patients. Based on the superior predictive properties of gene expression measurements in relapse specimens, a novel clinically applicable prognostic model/assay (RHL30) was developed that identifies a subset of patients at high risk of treatment failure following salvage therapy and ASCT. Specifically, RHL30 identifies a high-risk group of patients with significantly inferior post-ASCT-FFS compared to the low-

risk group (5-year: 23.8% high-risk vs. 77.5% low-risk) and also inferior post-ASCT-OS (5-year: 28.7% high-risk vs. 85.4% low-risk). Importantly, the prognostic power of RHL30 was reproduced in two separate validation cohorts of relapse specimens, and the RHL30 was statistically independent of all previously described prognostic markers in the validation cohorts, including post-salvage therapy response assessment by PET/CT [38].

5.6 Conclusions and Future Perspective

The advent of next-generation sequencing has significantly added to the armamentarium of genomics techniques interrogating tumor genetics of cHL and elucidating the molecular underpinnings of the unique crosstalk of the malignant HRS cells with their immune microenvironment. The sequencing studies of ctDNA and enrichment of HRS cells confirmed the importance of, and added texture to, the known molecular hallmarks of NFκB, JAK-STAT, and PI3K signaling as well as immune privilege phenotypes. Moreover, gene expression profiling studies of the microenvironment have reached more maturity in comprehensively describing cellular compartments in the TME and validated key correlations to pathologic and clinical outcome data. In particular, effective biomarker assay translation appears more and more realistic with the emergence of methods that are compatible with FFPE tissues that can be applied to relapse biopsies and are minimally invasive (e.g., serial peripheral blood draws) for dynamic biomarker testing. Despite these most recent advances, a number of challenges and open questions remain that need to be addressed in future studies. First, with respect to cHL biology, no unique and specific somatic gene mutations have been identified that would explain the unique histopathology of cHL in contrast to other lymphomas, leaving room for future discoveries. Second, systematic integration of HRS cell genomics with features and cellular components of the TME are lacking. Third, sample numbers for genomic landscape studies are still limited to be fully powered for mutational pattern analysis and robust outcome correlates in patients treated with standard of care. Finally, with the emergence of targeted therapies (e.g., brentuximab vedotin [39]) and modern immunotherapies (e.g., checkpoint inhibitors [40] or bispecific antibodies [41]), predictive biomarker development using genomics has to be prioritized alongside the next generation of clinical trials and population-based outcome studies of patients receiving these novel therapies in the standard of care setting. Excitingly, novel cutting-edge genomics techniques might also overcome some of the described obstacles, including HRS cell sequencing, to interrogate the non-coding space (e.g., whole genome sequencing), epigenetic profiling (e.g., ATAC-seq, bisulfite sequencing), and RNAseq at the single cell level to characterize the TME. Integrating these novel genomics approaches for dynamic, multi-time point biomarker testing alongside existing and novel therapeutic approaches holds the great promise to fully realize the benefits of precision medicine by genomics-driven clinical decision-making.

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