



Review

Hodgkin Lymphoma: A Special Microenvironment

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Abstract: Classical Hodgkin’s lymphoma (cHL) is one of the most particular lymphomas for the few tumor cells surrounded by an inflammatory microenvironment. Reed-Sternberg (RS) and Hodgkin (H) cells reprogram and evade antitumor mechanisms of the normal cells present in the microenvironment. The cells of microenvironment are essential for growth and survival of the RS/H cells and are recruited through the effect of cytokines/chemokines. We summarize recent advances in gene expression profiling (GEP) analysis applied to study microenvironment component in cHL. We also describe the main therapies that target not only the neoplastic cells but also the cellular components of the background.

Keywords: Classical Hodgkin’s lymphoma; microenvironment; gene expression profiling; therapy



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1. Classical Hodgkin’s Lymphoma

Classical Hodgkin’s lymphoma (cHL) accounts for about 10% of all annual lymphoma diagnosis worldwide. cHL is one of the most singular lymphomas for the presence of large multi- and mononucleated cells named Reed-Sternberg (RS) and Hodgkin (H) cells respectively, admixed with an inflammatory background. Four histological subtypes of cHL are recognized: nodular sclerosis (60–70%), mixed cellularity (20–25%), lymphocyte rich (5%) and lymphocyte-depleted (<5%) subtypes [1]. RS and H detection in an appropriate microenvironment (ME) is the key of cHL diagnosis [2].

Rengstl et al. [3], by filming Hodgkin cell lines in real-time by long-term time-lapse microscopy, suggested that RS cells generate from mitosis and incomplete cytokinesis of H cells followed by subsequent re-fusion of identical mononuclear daughter cells [3].

Microdissection experiments have revealed that RS and H cells carry clonal Ig rearrangements and Ig genes somatic hyper mutations [4], suggesting their origin from a pre-apoptotic germinal center (GC) B cell [5,6]. The expression of the tumor necrosis factor receptor family (TNFR) CD30 is a typical feature of cHL tumoral cells [7]. However, RS cells have an unusual immunophenotype characterized by absence of B cell markers, associated to possible coexpression of molecules of various hematopoietic lineage. [7]

The early event that produces this reprogramming is unknown; several studies reported the hypermethylation of the promoter regions of the transcription factors PU.1, BOB.1/OBF.1, that led to their down regulation [8]. Oct-2, BOB.1/OBF.1 and PU.1 regulate the expression of several genes like CD20 [9], BCL-2 [10], CD19 [11] and CD79A, [12] and furthermore they activate Ig genes transcription. This latter function explains the low level of Ig transcripts in cHL neoplastic cells [13]. Other characteristic B cell transcriptional

factors are expressed at low levels (EBF) or inactivated (E2A) by the aberrant expression of competitive inhibitors as Notch-1, Id2 and ABF1 [14,15]. An essential mark of the B cell differentiation of the tumoral clone is the expression of the transcription factor PAX5, although many of its target genes are downregulated [16].

RS and H cells are also characterized by the constitutive activation of both the canonical and non-canonical NF-κB signaling pathways [17]. This permanent stimulation is due to the complex network of paracrine and direct interactions between neoplastic cells and microenvironment, mediated by CD30, CD40, BCMA and other surface receptors [18–21], to recurrent somatic genetic lesions inactivating mutations of negative regulators of NF-κB like TNFAIP3 and NFKBIA in about 40% and 20% respectively [22–24], or to copy number gains of genes encoding positive regulators like REL and MAP3K14 in about 40% and 30% of cHL [25,26]. Finally, the latent membrane protein 1 encoded by Epstein-Barr virus, present in RS and H cells of 40% of cHL cases in western country, causes NF-κB activation by mimicking an active CD40 [17].

cHL is also characterized by Jak/Stat pathway activation, principally caused by autocrine and/or paracrine signaling events via interleukin receptors and receptor tyrosine kinases [27–30], PI3K-AKT and MAPK/ERK pathways [31–34].

2. The Main Component of Tumor Microenvironment (TME)

RS and H cells represent just 1% to 10% of total tumor mass; the remaining 99–90% of the cellular infiltrate is formed by a TME consisting of non-malignant T and B lymphocytes, plasma cells, histiocytes/macrophages, granulocytes, eosinophils, mast cells, mesenchymal stromal cells (MSCs) and endothelial cells [35] (Figure 1).

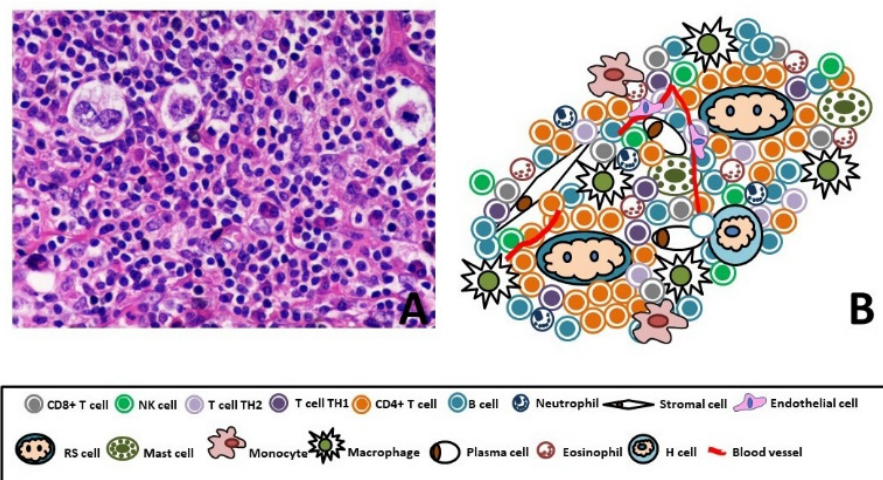


Figure 1. (A) Representative figure of Hodgkin Lymphoma (40X, Hematoxylin Eosin). (B) Cartoon of Hodgkin Lymphoma, few RS and H cells (1–10%) immerse in a special microenvironment. The 90% of the tumor mass is composed by CD4⁺ Th cells, CD4⁺ Tregs, CD8 T cells, B lymphocytes, plasma cells, histiocytes/macrophages, granulocytes, eosinophils, mast cells, MSCs and endothelial cells.

TME is essential for growth and survival of neoplastic cells and when they metastasize into non-lymphoid organs, they reproduce their cellular background [36]. The TME construction depends on the effect of cytokines/chemokines, including IL-5, IL-7, IL-8, CCL5 (RANTES), CCL17 (TARC), CCL20 and CCL28 produced by RS, H cells and the recruited immune cells [37]. In detail, IL-5, CCL5, CCL28 and the granulocyte-macrophage colony-stimulating factor (GM-CSF) attract the eosinophils [36,38,39], IL-8 is chemotactic for neutrophils [36]; IL-7 increases the proliferation of regulatory T cells [40], CCL5 recruits T-cells, eosinophils and mast cells [18,41], whereas CCL17 and CCL20 attract the T helper (Th) 2 cells and the regulatory T cells [36,42]. Other cytokines and in particular IL-3, IL-4, IL-6, IL-13, IL-15, TGFβ, BAFF, APRIL, RANKL influence the survival of the same

tumor cells [36,43]. The dynamics of the TME are also conditioned by cytokines produced by the immune infiltrates at the tumoral site: T cells secrete IL-3, IL-10 and RANKL. IL-3 influences the formation of the inflammatory infiltrate and support the neoplastic cell growth [44], IL-10 promotes strong anti-inflammatory properties [45] and RANKL contributes to activation and survival of dendritic cells [46]. Stromal cells may secrete CCL11 that attracts eosinophils and Th2 cells, IL-7 and CCL5 involved in the growth or survival of RS cells, while dendritic cells may liberate TARC involved in the recruitment of Th2 cells and regulatory T cells [37]. Macrophages have been shown to secrete cytokines involved in tumor progression such as macrophage migration inhibitory factor (MIF), IL-8 and TNF α . MIF may contribute to the proliferation of RS cells in TME [47,48] and IL-8 may increase neutrophilic infiltration [49,50]. Moreover, mast cell may increase survival signals of tumor cells through CD30L [51].

2.1. T Cell Subsets

T cells are the main component of the cHL tumor TME. Several subsets were recognized: CD4⁺ Th cells, CD4⁺ T-regulatory (Tregs) and CD8 cytotoxic T lymphocyte (CTL) [52]. CD4⁺ T cells sometimes are in close contact with RS cells as they can form rosettes around the neoplastic blasts [53,54]. A recent study has been described rosette formation and T cell activation in cHL, using coculture model. In the immune synapse model between RS cells and CD4⁺ T cells, the two interactions TCR-MHCII and CD2-CD58 were needed for T cell activations, while CD2-C58 axis was associated with cell adhesion and rosette formation [55].

Previous studies have suggested that the prevalent Th phenotype present in cHL TME is the Th2 [56,57]. This T cell subset is physiologically involved in the eradication of extracellular parasites, but in a tumoral background seems to contribute to the tumor growth [58]. The Th1 subpopulation, is indeed important in host defense against intracellular pathogens and is an effective mediator of anti-tumor immunity [58], seem to be reduced in HL TME. However, a recent study found predominance of an activated, proliferative, and pro-inflammatory cytokine-secretory phenotype, typically of Th1 cells [59]. Normally, CD4⁺ Th cells do not directly demolish malignant tumor cells, but they support the development of tumor immunity by recognizing tumor antigen peptides presented by MHC class II molecules and amplifying the activation and clonal expansion of CTL [60]. In general, CD8⁺ CTL or Natural Killer cells (NK) are the most important effectors of antitumor immunity. The CD8⁺ T cells are a proportion of T-cell infiltrate of Hodgkin lymphoma and are not in close contact with the tumor cells [61]. However paradoxically, an increased numbers of cytotoxic T cells positive for cytotoxic granule-associated RNA-binding protein (TIA1) in the TME correlated with poor outcomes [62,63].

In the TME there is also an accumulation of Treg [60], expressing factor forkhead box P3 (FOXP3) [64]. Treg cells play important roles in limiting immune response and their dysfunction was reported in autoimmune disease [65]. They act their immunomodulatory function through several mechanisms, including overexpression of CTLA4, consumption of IL-2, secretion of IL-10, TGF- β , IL-35 and galectin-1 [66–68]. Moreover, Treg cells may facilitate tumor spread, probably inhibiting the antitumor immunity [65]. In several localized or metastatic human carcinomas high infiltration of Treg cells FOXP3⁺ was associated with an unfavorable outcome [69–74]. However, in cHL patients, higher number of intra-tumoral FOXP3⁺ Treg cells was, associated with longer DFS and OS, even in multivariate analyses [60].

2.2. B Lymphocytes

Large quantities of non-malignant B cells are present in the microenvironment of HL but their role in TME is still not well established [75]. Dates of gene profiling provide the association of high intratumoral B-cell counts with better outcome in patients with cHL [76]. Some authors reported that antitumor immunity is highly reduced in the presence of B cells

due to down-regulation of both innate and adaptive immunity, in cases where CD40L is expressed by tumor cells. [77].

2.3. Macrophages

In many cancers, macrophages of the tumoral TME (Tumor Associated Macrophages: TAM) seems to support tumor progression. Steidl et. al. [78] found a macrophage gene signature correlated with the failure of primary treatment and then in an independent cohort of patients they demonstrated by immunohistochemistry (IHC), that higher number of CD68⁺ TAM was associated with shortened survival and with the outcome of secondary treatments such as autologous stem-cell transplantation [78]. Later many, but not all [79–81] IHC studies, conducted by Tan et. al., Greaves et. al., Tzankov A et. al., Gotti et. al., [82–85] confirmed the relationship among TAM and inferior outcomes after upfront treatment [82–85]. Moreover, the molecular characterization of HR cells reported in the neoplastic clone the over-expression of CSF1R (colony stimulating factor 1 receptor), a gene of the macrophage signature and the latter gene resulted associated with primary treatment failure [86]. Macrophages are versatile cells that can have an immune-stimulatory or an immune suppressive function [87]. Mantovani et. al. [87], to stigmatize the plasticity of macrophages, proposed their distinction in two functional sub-type: the M1 and the M2. The M1, or classically activated macrophages, includes mononuclear phagocytes activated by bacterial moieties and Th1 cytokines. The M2-type macrophages, or alternatively activated macrophages, express several proteins like CD206 (mannose receptor), CD204 (scavenger receptor A), IL-1, IL-10, CC ligand 22 (CCL22), and CD163 [87–89]. They suppress immune response, promote angiogenesis and tumor progression and metastasis. The M1 and M2 are present in the TME of cHL. Zaki et. al. [90] observed a correlation among number of M1 cells and favorable prognosis in mixed cellularity subtype of cHL [90].

In a recent study was described the cellular components of microenvironment underlying topographical aspects of the immune-niche surround RS cells. According to this model, in TME was observed abundant PD-L1+TAMs and PD-1+ CD4 T cells, that were in contact with PD-L1+ tumor cells. These observations supported a possible role also of the macrophages in the mechanism of action of checkpoint inhibitor therapy [91].

2.4. Mast Cells

Inflammatory infiltrate of cHL contains also mast cells. These cells are positive for CD30L and tend to activate the RS cells through the interaction between CD30-CD30L [92,93]. Moreover, mast cells can promote tumor development thanks to the release of pro-angiogenic factor that increased the vasculature [94], the release of protease that enhance fibroblast proliferation and the secretion of tumor-promoting cytokines that induce an immunotolerant status of the TME [95].

The association between the number of mast cells and survival of patients with cHL is controversial. Molin et. al. [92] found a correlation between higher number of mast cells and poor prognosis [92], whereas retrospective study of 104 patients did not find any association between the degree of mast cells infiltration and outcome [96]. However, in further study Andersen et. al. [97] showed a significant association between high mast cells counts and poorer EFS as well as OS in mixed cellularity but not in nodular sclerosis histological subtype [97].

3. cHL and Immunoescape

RS cells have developed different mechanisms to escape from antitumoral immune response. Several studies reported the down regulation of molecules involved in antigen presentation to cytotoxic cells including HLA-I and HLA-II. The down regulation of HLA-I in Epstein-Barr virus (EBV)-cases or the presence of a polymorphism in HLA-I in EBV+ cases represent mechanisms that allow RS cells to escape from CD8-mediated cytotoxicity [98]. Furthermore, an association between the expression of HLA-G by RS cells, EBV negative status and absence of MHC-I was found. HLA-G is a ligand for

inhibitory receptor presents on NK cells and other immune cells, may contribute to escape from immunosurveillance [99].

Moreover, alterations of gene CD58 contribute to immune evasion of RS cells. CD58 is relevant for the activation and adhesion of CTL and NK cells through the interaction between the protein and the receptor CD2 [100,101]. Numerous studies reported mutations of the CD58-gene in HL, with concurrent loss of expression of the protein [102–105]. CD58 mutations are observed in three Hodgkin's lymphoma cell lines by whole-exome sequencing [104]. Probably the absence of CD58 allow RS cells evasion from immune recognition, particularly during advanced disease when the tumor cells become less dependent on the immune infiltrate and more immunogenic [102].

APC cells use the CD137 receptor/ligand system to costimulate activated T cells and the ectopic expression and release of CD137 by RS impaired T cells activation [106]. Many other molecules secreted by H/RS such as PGE2, LGALS1 and TGF β are immunosuppressive factors [57,107,108]. The immunosuppressive activity is also mediated by the low levels of natural killer group 2D (NKG2D) expressed by RS [109]. The bond between NKG2DL and his receptor modulates lymphocyte activation and promotes immunity [109].

Another important mechanism by which RS cells evade host immuneresponse is by the expression of FASL, which induce apoptosis of activated CD8⁺ and Th1 cells [98]. RS also express FAS, but the tumor cells, thanks to mutations of the FAS gene, are resistant to the induction of apoptosis mediated by FAS-FASL complex. [110–112].

Moreover, in HL there is an overexpression of programmed cell death ligand 1 (PD-L1) and ligand 2 (PD-L2) [113,114]. RS cells present membranous expression of PD-L1 and the amplification of a region 9p24.1 that includes PD-L1 and PD-L2 explains the overexpression of PD-Ls in more than 85% of cHL patients [113]. For Roemer et al. PD-L1/PD-L2 alterations are a defining feature of cHL, 97% of the patients had concordant alterations of the PD-L1 and PD-L2 loci (including polysomy, copy gain and amplification) and this amplification was connected to a short PFS and a more advanced stage of disease [115]. In addition, alternative mechanisms promoting overexpression of PD-L1 and PD-L2 by RS include amplification of gene JAK2, leading to JAK2 protein overexpression and subsequent transcriptional activation of PD-L1, Epstein-Barr virus infection and activation of AP-1 [113,116]. Moreover Steidl et al. found a highly expressed chromosomal fusion gene involving CIITA, that caused the downregulation of MHCII expression and overexpression of PD-L1 and PD-L2 [117].

The binding of PD-1 ligands to PD-1, present on T cells, has been shown to contribute to the inhibition of the antitumoral function [114]. This phenomenon is called T cell "exhaustion" and is associated with an altered metabolism and singular transcriptional program compared with memory and effector T cells. A main characteristic of T cell exhaustion is the elevated expression of multiple inhibitory co-receptors, including PD-1, cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte-activation gene 3 (Lag-3) T cells immunoglobulin and mucin domain 3 (Tim-3) [118]. These group of proteins called "immune checkpoints", in physiologic conditions limit aberrant immune cell activities following chronic activation or preventing autoimmune responses, but in the setting of cancer TME they diminish immune responses against malignant cells, allowing to the latter the escape from immune surveillance [119].

4. GEP Signature and TME

Previous immunohistochemistry (IHC) studies, have described the morphology and immunophenotype of TME. However, IHC results published generally are based on the use of single IHC biomarker that does not show simultaneously the predictive role of multiple cellular elements in the TME. Moreover, IHC technique presented interpretation bias and limitations.

Several groups have tried to define the molecular characteristics of the TME through high throughput technologies. Gene expression-profiling (GEP) results could be used to simultaneously measure the expression levels of thousands of gene. In many cases, the GEP

results provided quantity genes level to building a gene expression signature suggesting how the cellular components of TME influenced the state of disease. Furthermore, expression array represented an opportunity to discover gene expression signatures associated with treatment outcomes (Figure 2).

A first group profiled the total tissue of 21 cases of cHL. In this study, they observed different transcriptional patterns with distinct response to therapy and clinical outcome. They noted how genes involved to fibroblast activation or function, but also to angiogenesis extracellular matrix remodeling and cell proliferation were overspread in sample with bad outcome. In the same group most genes related to tumor suppressor were underexpressed. While patient with a good prognosis were characterized by the up regulation of genes involved in apoptosis activation and cell signaling [120].

Sanchez et al. [121] identified specific gene signatures associated with outcome. Gene expressed by a group of T cells, macrophages and plasmacytoid dendritic cells were overexpressed in group of patients with unfavorable outcome. In the same unfavorable outcome groups, other genes related to apoptosis, signal transduction and cell growth were overspread. Using immunohistochemical analysis, representative markers of the immune response signatures and cell-cycle signatures were validated in an independent cohort. In agreement with the data of the gene expression, the results demonstrated a relation between increased number of macrophages markers (ALDH1, LYZ), T cells (SH2D1A) and inferior disease specific survival (DSS) [121].

Later, Chetaille et al. [76] identified new prognostic factors in cases of cHL with EBV infection. Gene profiling data showed in EBV+ cHL, Th1 activity and overexpression of macrophage genes. They also searched an association between outcome and specific gene expression signatures. In the group of patients with favorable outcome, they noted an overexpression of genes for B cells and genes involved in apoptotic pathway. To validate the signatures, they performed IHC analysis in an independent series of 146 cHL samples. High percentage of either TIA-1 reactive cells or topo-II positive cells had an adverse influence on OS. Whereas high count of BCL11A⁺, CD20⁺, FOXP3⁺ reactive cells showed a favorable influence [76].

Another study was designed to find a prognostic model from 130 frozen samples obtained from patients with cHL. Although, the results of unsupervised hierarchical clustering analysis did not identify an association with the effect of treatment, the study of Steidl et al. provided a relation between a microenvironment gene signature and outcome. In the treatment-failure group, they observed an overexpression of gene signatures of TAMs, monocytes, adipocytes and for angiogenic cells. Using immunohistochemical analysis, they tried to confirm the findings of the gene expression analysis, the results showed an increased number of CD68⁺ cells associated with an adverse outcome [78]. Later, the same authors, investigated the gene expression profiles of microdissected H/RS cells and identified a link between the transcriptional program of the tumor cells and microenvironment. A signature of macrophage function in H/RS cells was correlated with first-line treatment failure. They focused their attention to CSF1R, a representative gene of macrophages signature. In an independent patient cohort, CSF1R *ISH* expressed by H/RS was correlated with inferior PFS, OS and the abundance of macrophages in microenvironment [86].

A new probe-based technology, the NanoString nCounter platform allow gene expression quantification using also low amounts of highly fragmented RNA isolated from routinely formalin-fixed paraffin embedded biopsies FFPE. NanoString method is based on direct measurement of gene expression level, eliminating enzymatic reactions and amplification bias. NanoString's nCounter chemistry utilizes target-specific probes, collectively referred to as a CodeSet, that directly hybridize to a target of interest.

Scott et. al. [122] developed a predictive model of OS associated with outcomes in advanced stage cHL, the levels of gene expression were determined with NanoString Platform. 259 genes were selected from data of literature previously reported to be associated with outcome in cHL. Among these genes, a model with 23 genes was generated, involving components of the microenvironment and tumor. The study was conducted in

290 patients with advanced stage enrolled onto the E2496 intergroup trial company ABVD and Stanford regimens. The model and the threshold were tested in a validation cohort of patients with advanced stage cHL. Gene associated with macrophages program, activation of Th1 response, cytotoxic T cells/NK were overexpressed in patients with an increased risk of death [122].

In a recent study, the same group, applied the previously published 23-gene in a distinct cohort of 401 patients with advanced-stage cHL, treated with BEACOPP based regimens. The 23-gene predictor was not prognostic for PFS and OS in the context of BEACOPP-treated advanced stage cHL.

However, they identified that three individual genes PDGFRA, TNFRSF8 and CCL17 after multiple testing, were correlated with PFS in patients treated with BEACOPP based regimens.

This result highlighted how different therapeutic approaches may require the necessity to develop different predictors for risk assessment [123].

Another gene expression analysis explored the TME composition of 245 FFPE samples with cHL, including 71 paired primary and relapse specimens, to investigate temporal gene expression difference and association with post autologous stem cell transplant (ASCT) outcomes. Chan et al. observed a TME dynamism between primary and relapse specimens, moreover they showed that the biology at relapse, compared with primary diagnosis, contained more prognostic information for predicting treatment outcomes after ASCT. The authors developed a new prognostic model, RHL30 based on the expression of gene associated with tumor cells and immune cells type of TME (macrophage, neutrophil and natural killer). A high RHL30 score identified patients with unfavorable outcomes (worse FFS and OS) after ASCT [124].

Later, the same authors validated the RHL30 assay, in an additional independent cohort of 41 patients with relapsed cHL. In part, the latest results were different from those presented in the first work. The RHL30 risk score was associated with FFS post-ASCT, but the same cohort of patients didn't present an association with OS [125].

The Interim PET (iPET), after 2 cycles of Chemotherapy is a good predictor of outcome in cHL.

Luminari et. al. [126], identified the biological features of patients iPET+ developing 13-gene signature. They evaluated the expression profile by NanoString using a commercial panel of 770 genes, filtered the 241 genes differentially expressed and developed a stringent gene signature. The authors found a predictive score associated with iPET status composed of genes (ITGA5, SAA1, CXCL2, SPP1, and TREM1) and Lymphocytes T-monocytes ratio (LMR) with the aim to define the right treatment strategy upfront without waiting two months from treatment start [126].

In a retrospective study was studied the association of 25-hydroxy vitamin D (VitD) blood level with data of gene expression also in cHL. VitD deficiency reactivated genes that mediate tumor cell survival and resistance to stress, contributing to promote cHL aggressiveness [127].

STUDY	CASES AND MATERIAL	TECHNIQUE	TREATMENT	PROGNOSIS	PROCESS, CELLULAR COMPONENT	BIOMARKERS	OUTCOME CORRELATIONS	REF
Devillard et al	21/FF from initial diagnosis and first relapse	Microarray (~1000 genes)	Standard Therapies	Favorable	Apoptosis Cell signaling	▲APAF, BAX, BID, Caspase 8, TP53, TRAIL ▲STAT3, MAP2K6		[120]
				Unfavorable	Angiogenesis Fibroblast Extracellular remodeling Cell proliferation Tumor suppressor genes	▲Endostatin ▲PDGFRB, Collagen ▲MFAP2, MMP2, MMP3, TIMP1 ▲MK167, TNF α ▼PTEN, DCC ▲TIMP4, SPON1, LAMB1		
Sanchez et al	29/FF (Training set)	Microarray (9348 gene)	Standard Therapies (ABVD or similar regimens)	Favorable	Extracellular remodeling and adhesion Fibroblast Antigen-presenting cells B cells	▲TACR1, CCL26 ▲CR1, HLA-DRB3 ▲IRTA2, VDR ▲CD8B1, CD3D, CD26, SH2D1A ▲ALDH1A1, LYZ, SPON1, LAMB1 ▲ITIEA ▲CYCS, CASP14, HSPA1L ▲PDCD10, PRKACB ▲COX7A2, MYC, DCK		[121]
				Unfavorable	T cells Macrophages Plasmacytoid dendritic cells Apoptosis Signal transduction Cell growth	▲ALDH1A1, STAT1 ▲SH2D1A ▲RRM2, MAD2L1, CDC2 ▲BCL11A, BANK1, STAP ▲YWHAB, CASP8, PTPRC ▲THBS1/2, FNI, EDNRA	Inferior DSS Inferior DSS Inferior DSS	
Chetaille et al	63/FF	Microarray (14500 genes)	Standard Therapies	Favorable	Macrophages T cells HRS B cells Apoptosis Extracellular matrix	▲TIA-1 ▲FOXP3 ▲BCL11A, CD20		[76]
				Unfavorable	T cells B cells	▲TIA-1 ▲FOXP3 ▲BCL11A, CD20	Adverse OS/Adverse EFS Favorable OS e EFS	
Steidl et al	130/FF	Microarray (~39000 genes)	Standard Therapies (ABVD or similar regimens)	Favorable	GC B cells	▲GC B cells signature		[78]
				Unfavorable	Macrophages Monocyte Adipocytes Angiogenic cells	▲Tumor associated macrophage signature ▲Resting monocyte signature ▲Adipocyte signature ▲Angiogenesis signature ▲CD68 ▲Macrophage-like signature	Adverse PFS, DSS	
Steidl et al	166/FFPE 29/FF	IHC Microarray (~39000 genes)	Standard Therapies (ABVD or similar regimens)	HRS cells		▲Macrophage-like signature		[86]
Scott et al	166/FFPE 290/FFPE	IHS NanoString (259 genes)		Unfavorable	Macrophages Th1 cytotoxic T cells/NK HRS cells	▲CSF1R 23-gene model ▲PDGFRA, TNFRSF8, CCL17	Inferior PFS, OS High risk group inferior OS	[122]
				Jachimowicz et al	401/FFPE	NanoString	BEACOPP	Unfavorable
Chan et al	245/FFPE	NanoString (784 genes)	Standard Therapies (ABVD or similar regimens)	Unfavorable	B cells Macrophages HRS cells Neutrophils NK	RHL30 model	High score inferior PFS, OS	[124]
Luminari et al	206/FFPE	NanoString (770 genes)	ABVD	Unfavorable	Macrophages Monocytes Myeloid cells Angiogenesis	iPET+ predictive score	iPET+	[126]

Figure 2. Published studies examining GEP and outcome in classical Hodgkin lymphoma.

5. New TME Based Therapeutic Strategy

Approximately 80% of patients are cured with standard first line chemotherapy [128]. In patients with early-stage, the first line therapy made up of cycles of Adriamycin, Bleomycin, Vinblastine Sulfate, Dacarbazine (ABVD) chemotherapy, followed by radiotherapy in some cases. While Patients with advanced-stage disease usually receive a prolonged or more intense chemotherapy consisting of either ABVD or a regimen of bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone (BEACOPP), with the possible inclusion of radiation treatment [129].

However 15% of patients with early stage disease and 30% with advanced stage disease relapse or have primary refractory disease after initial treatment [128,130].

Patients with relapsed or refractory are treated with salvage chemotherapy followed by ASCT [131].

Brentuximab-vedotin (BV) a monoclonal antibody directed against the CD30 expressed by HRS cells, is another therapeutic opportunity for the treatment of cHL [132]. Originally BV has been used as second line therapy or a consolidation of the ASCT in high risk patient [133] recently BV was proposed into frontline treatment [134].

In this scenario, the growing interest in tumor TME has contributed to the development of exciting novel therapy that target not only the neoplastic cells but also the cellular components of the background. There are antibodies direct against PD-1 or PD-L1 and CTLA-4, which reverse the down regulation of T cell function induced by tumor condition and allow T cells activation in the TME.

Nivolumab is a fully immunoglobulin IgG4 anti-PD-1 antibody and blocks the binding between PD-1 and PD-L1/PD-L2. Nivolumab has been studied in 23 patients with Hodgkin's lymphoma, 78% were enrolled in the study after a relapse following autologous stem-cell transplantation and 78% after a relapse following the receipt of BV (CheckMate-039). The monoclonal antibody had an ORR of 87%, a CR rate of 17%, a partial response (PR) of 70% and PFS of 86% at 24 weeks [135]. Given these encouraging preliminary results,

a subsequent phase II study (CheckMate-205) was conducted on 80 patients, who had received prior ASCT and BV. Nivolumab was administered at the dose of 3 mg/kg every 2 weeks and demonstrated a therapeutic activity with ORR of 66% at a median follow-up of 8.9 months [136]. Based on these results, FDA approved nivolumab for the treatment of patients with cHL for whom ASCT and BV failed.

Pembrolizumab is another IgG4 monoclonal antibody to PD-1. In a phase 1b multicenter study (KEYNOTE-013), pembrolizumab was administered at the dose of 10 mg/kg every 2 weeks for up to 2 years, to 31 patients with cHL unresponsive to treatment with BV (100%) and with relapsed after ASCT (71%). The ORR was of 65%, the CR rate was of 16% and PR rate was of 48%. The PFS was 69% at 24 weeks and 46% at 52 weeks. The treatment with pembrolizumab was well tolerated [137].

A study in phase II (KEYNOTE-087) was designed to evaluate the clinical activity of pembrolizumab in 3 cohorts of patients with relapsed/refractory cHL. In all cohorts were observed substantial clinical activity and a high ORR. Pembrolizumab received FDA approval for the treatment of adult and pediatric patients with refractory cHL, or those who have relapsed after three or more prior lines of therapy [138,139].

Results of long term follow up analysis of KEYNOTE-013 [140] and KEYNOTE-087 [141] showed durable antitumor activity of Pembrolizumab in phase 1 and phase 2 studies.

In solid tumors, antibodies anti-CTLA-4 were the first immune checkpoint inhibitors to receive FDA approval for the treatment of melanoma. In addition, also antibodies against PD-1 have been approved for the treatment of melanoma and non-small-cell-lung cancer [142]. CTLA-4 is a negative regulator of T cell activation and a CD28 homologue [143]. CTLA-4 interacts with CD80 and CD86 with a higher affinity than CD28, leading to impaired T cell costimulation and functional inactivation [144]. CTLA-4 is expressed by T cells, B cells, fibroblasts, but is unclear the role of CTLA-4 for non-T cells [145]. Ipilimumab is an antibody anti-CTLA-4 and it was the first checkpoint inhibitor examined in patients with HL. In a phase I study of 29 patients with hematologic malignancies, that also including 14 patients with relapsed/refractory HL after allogeneic stem cell transplantation were treated with anti-CTLA-4. Ipilimumab was well tolerated, case of relevant graft-versus-host disease was absent and 2 patients showed a complete remission (CR) [146]. CTLA4 antibodies have been surpassed by antibodies direct against PD-1 and PD-L1, but they have been effective in combination therapies with other agents [147].

Other innovative therapeutic strategies have been developed targeting TAM and NK [148]. In a phase 1/2 trial was evaluated the effect of JNJ-40346527, an inhibitor of CSF1R expressed by RS cells and correlated to an increased number of TAM [86,149]. CSF1R inhibitor presented limited activity when used in the relapsed/refractory setting [150].

AFM13 is a bispecific antibodies against CD30 and CD16A, that recruits NK cells via binding to CD16A [151]. A phase I study (AFM13-101) was conducted in patients with cHL who relapsed or were refractory after standard therapies [152].

Although the AFM13 represents a new target therapy, the study demonstrated limited efficacy (ORR 12% to 23%, no CR) [152].

Even in patients with relapsed cHL, chimeric antigen receptor T cells (CAR T) represent a potential treatment. CAR T therapy against CD30 has not produced encouraging results [153]. While anti-CD123 CAR T cells for the treatment of cHL represents a more promising CAR T cell approach [154]. CD123 is expressed on RS cells, macrophages, plasmacytoid dendritic cells, eosinophils, basophils, and mast cells [155]. In cHL CD123 CAR T were capable of targeting malignant cells and TAMs eliminating a crucial immunosuppressive component of TME [154].

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