**SMAD1 promoter hypermethylation and lack of SMAD1 expression in Hodgkin lymphoma: a potential target for hypomethylating drug therapy**

Hodgkin lymphoma (HL) is an immunologically active lymphoid neoplasm composed of a few (usually 1-10%) neoplastic Hodgkin and Reed-Sternberg (HRS) cells or lymphocyte-predominant (LP) cells and >90% non-neoplastic cells, mainly T- and B-lymphocytes, plasma cells, macrophages, eosinophils and fibroblasts. The substantial amount of reactive cells in HL is supposed to be the net effect of a complex signaling network of cytokines and chemokines secreted by either the HRS cells or non-neoplastic cells. One component of this network is transforming growth factor beta (TGF-β), which is produced by HRS cells and cancer-associated fibroblasts. TGF-β unfolds its immunosuppressive impact by stimulating tumor-infiltrating T-lymphocytes (TIL) to differentiate into anergic, tumor-promoting, regulatory T cells (Treg). Additionally, TGF-β inhibits natural killer cells - one of the key components of the innate anticancer immunity. Interestingly and still poorly understood, the HRS cells themselves seem to remain unaffected by the tumor-suppressive properties of TGF-β. Recent studies on diffuse large B-cell lymphoma (DLBCL) revealed a previously unknown tumor-suppressive signaling axis involving SMAD1 as a downstream messenger of TGF-β. SMAD1 functions as an intracellular signal transducer between extracellular TGF-β and the nucleus, where it modulates the transcription of target genes. This signaling cascade was shown to be recurrently inactivated in DLBCL, mainly by hypermethylation of five promoter regions surrounding the SMAD1 transcription start site, which finally generates a significant growth advantage for lymphoma cells. In the course of these investigations, we noted that SMAD1 was not expressed in HRS cells of screened HL cases. This led us to hypothesize that the absence of SMAD1 expression in HRS cells may mechanistically be linked to their resistance to the tumor-suppressive effects of TGF-β.

In order to further elucidate this finding, we analyzed 132 well-characterized archival tissue-microarrayed cases, and 11 conventional routine lymphadenectomy specimens from patients suffering from all subtypes of classic HL (77 nodular sclerosis [NS]; 48 mixed cellularity [MC]; 7 lymphocyte-rich [LR]; 5 lymphocyte-depleted; and 6 unclassifiable classic HL) and 14 routine samples from patients suffering from nodular lymphocyte-predominant HL (NLPHL). We analyzed all these instances for immunohistochemical expression of SMAD1. Importantly, to guarantee retained antigenicity, only cases containing (physiologically) SMAD1-positive endothelia were considered. We found that all NLPHL (14/14 cases; 100%) and the great majority of classic HL (138/143 cases; 97%) displayed SMAD1-negative LP and HRS cells, respectively (Figure 1A and B). Single HRS cells stained faintly for SMAD1 in five cases only (2 NS; 2 MC; and 1 LR classic HL). With respect to non-neoplastic cells, 65/143 classic HL (45%) showed moderate (15-49% of TIL) up to abundant (≥50% of TIL) amounts of SMAD1-positive surrounding TIL, thus being potentially susceptible to the suppressive influence of TGF-β (Figure 1A and B); in NLPHL, 11/14 (79%) cases displayed abundant SMAD1-expressing TIL, including TIL involved in rosetting around LP cells (Online Supplementary Figure S1). The presence of abundant SMAD1-expressing TIL did not correlate with disease stage, patients’ age, gender, presence of B symptoms, association with Epstein-Barr virus (EBV) or outcome, while showing significant correlations with the NS subtype (45/77 NS cases, i.e. 58%, compared to 20/66 non-NS cases, i.e. 30%, P=0.025 χ² test) and with the amount of FOXP3-positive Treg (Rho=0.351, P=0.000053 Spearman correlation), which both, in turn, may be directly linked to the effects of TGF-β, promoting sclerosis and a shift towards Treg differentiation. In contrast, surrounding plasma cells seemed to lack SMAD1 expression, potentially rendering them insensitive to the pro-apoptotic and anti-proliferative signals of TGF-β. With regard to plasma cells, this largely fits with the newly described negative prognostic impact of their increased numbers in classic HL.

To strengthen our hypothesis, we investigated the promoter methylation status of the SMAD1 gene in six different HL cell lines, including one NLPHL cell line (DEV) exactly as described elsewhere. Methylation analysis by bisulfite sequencing was successful for three regions of

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**Figure 1. Expression of SMAD1 in classic Hodgkin lymphoma.** (A) Tissue microarrayed archival mixed cellularity classic Hodgkin lymphoma with moderate numbers of SMAD1-positive tumor-infiltrating lymphocytes (TIL) and a few strongly staining endothelia. Note that all Hodgkin and Reed-Sternberg (HRS) cells are negative. (B) Diagnostic lymphadenectomy of a nodular sclerosis HL with abundant SMAD1-positive TIL and a few strongly staining endothelia. Note that all HRS cells are negative. Immunoperoxidase staining, original magnification 400x.
the SMAD1 promoter and yielded four hypermethylated cell lines (L428, KMH-2, DEV, HDLM-2), that differed substantially, particularly regarding their methylation of region A4(3), from cell lines showing no evidence of promoter hypermethylation (L1236, L540) (Figure 2A). Importantly, KMH-2 is known to be SMAD1 p.ADTP220fs mutant (https://portals.broadinstitute.org/ccle/page?cell_line=KMH2_haematopoietic_and_lymphoid_tissue), which additionally points towards a potential role of this gene silencing in lymphomagenesis.

The impact of the promoter methylation status of SMAD1 on protein expression was further addressed by western blot analysis, comparing one hypermethylated cell line (DEV) with a cell line without hypermethylation (L1236). Concordantly, the expression of SMAD1 differed clearly, without detectable protein in the DEV cell line (Figure 2B). When treated with the DNA methyltransferase inhibitor decitabine, which reverses, among others, the hypermethylation of SMAD1 promoters, the SMAD1-negative cell line DEV died immediately after exposure. As expected, the expression of SMAD1 was not affected by treatment in the L1236 cell line, which is not hypermethylated.

To obtain further clinical evidence, the promoter methylation status of SMAD1 was assessed in samples from three patients with classic HL. To do this, we used our newly developed, flow sorting-assisted technique for HRS cell enrichment from formalin-fixed and paraffin-embedded tissues, allowing for targeted genetic analysis of DNA isolated from classic HL tumor cells. In this collective, not only the A4(3) promoter region of SMAD1, but also the A1(1) region was substantially hypermethylated (Figure 2C). Furthermore, SMAD1 promoter hypermethylation was identified in sorted tumor-infiltrating plasma cells, fitting with the immunohistochemically noted lack of SMAD1 in plasma cells.

Although risk-adjusted standard treatment of HL is successful in over 90% of patients, relapses after salvage therapy and refractory cases represent oncological challenges and run an unfavorable clinical course with limited therapeutic options. In this context, demethylating agents such as decitabine and azacytidine may be of potential therapeutic interest and have already shown promising effects. At clinically relevant concentrations, decitabine has been documented to inhibit the growth of classic HL cell lines in vitro and a single case observation of regressing relapsed classic HL as an unexpected “side effect” of azacytidine has been reported in a patient suffering from concomitant myelodysplastic syndrome. Importantly, decitabine-treated samples of the DEV cell line are not shown because of lack of protein after demethylating treatment. (C) Methylation analysis by bisulfite sequencing of regions A1(1), A4(3) and B2(5) in samples from three patients with classic HL. Each circle represents one CG dinucleotide; black circles indicate methylated, white circles indicate unmethylated cytosines. Each line represents one clone. Two or three clones were sequenced per sample. X indicates aligned mismatches between genomic and bisulfite sequences.
cells express TGFβ and associated proteins (ACVR1, ACVR1B, ACVR1C, ACVR2A, ACVR2B, ACVR1L, AMHR2, BMPR2, TGFBR1, TGFBR2, TGFBR3, TGFBRAP1). The classic HL cell line KM-H2 contained relevant transcript levels of all TGFβ types, HDLM-2 expressed TGFBR1 and TGFBR3, and the NLPHL cell line DEV expressed TGFBR1; the classic HL cell lines L1236 and L428 exhibited TGFBRAP1 transcripts. In all these cell lines SMAD1 transcripts were decreased.

In summary, our data suggest a likely important, not yet described role of SMAD1 hypermethylation in HL, potentially causing an imbalance of TGF-β signaling axis responses in involved tissues. SMAD1 has been demonstrated to be part of the TGF-β-mediated anti-proliferative pathway in different B-cell lymphomas. Intriguingly, lymphomas with mutated or knocked-out SMAD1 were protected from the tumor-suppressive effects of TGF-β. Lack of SMAD1 expression in HRS and LP cells due to promoter hypermethylation or gene mutation may analogously contribute to their resistance towards the pro-apoptotic and anti-proliferative effects of TGF-β, despite the presence of TGFBR transcripts. This hypothesis is further supported by observations in EBV-positive classic HL, in which decreased SMAD2 levels due to EBNA1-mediated increased protein turnover disable TGF-β signaling, being congruent with our data regarding SMAD1 downregulation in HRS cells.

In contrast, retained SMAD1 expression in surrounding TIL may contribute to immune escape, as intact TGF-β signaling promotes T-cell differentiation into tumor-supporting Treg, which is reflected by the observed correlation between higher numbers of FOXP3-positive Treg and expression of SMAD1 in TIL. The tumor-suppressor effects of TGF-β on TIL have recently been challenged by a promising clinical study in which the infusion of TGFβ-positive regulatory T cells was successfully used in patients with EBV-positive relapsed classic HL.

Our data suggest a possible rationale for the application of a more tailored treatment with hypomethylating agents in HL, which may be worth of prospective investigations, as agents such as decitabine have already shown promising results in patients with EBV-positive relapsed classic HL.15

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References