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Commentary

Epigenetics modulates the complexity of the response to Immune Checkpoint Blockade

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The emergence of Immune Checkpoint Blockade (ICB) therapy has unleashed an abundance of promising cancer treatment alternatives that focus on the re-activation of an immunosuppressed tumour environment. These strategies, initially applied to advanced stages of cancer with durable responses and acceptable toxicity, are also transforming the clinical practice in the adjuvant and neoadjuvant settings and are indicated in more than 15 types of tumours. However, the frequent occurrence of innate or acquired resistance, of up to 85% of the patients, has turned the focus on precision medicine to decipher which patients will benefit from the treatment, and on combination therapies with multiple targets that can holistically combat the disease [1]. In this context, despite a nascent instrument in clinical practice, epigenetic marks stand as putative biomarkers informative both of response to immunotherapy and of the disease or therapy-induced dynamic landscape. In addition, such mouldable nature of the epigenetic system is the cornerstone of the development of several epigenetic drugs that are currently combined with Checkpoint Inhibitors in Phase I and II Clinical Trials [2]. Amongst the plethora of receptors, ligands, cytokines and immune cells that interact in the tumour microenvironment to promote or counteract immunosuppression, *Lymphocyte- associated gene 3* (*LAG3*) stands as a promising checkpoint target for the promotion of tumour immunosurveillance when combined with other checkpoint molecules [3]. Given the increasing evidence on the insufficient immune-reactivation as one of the main reasons for low response rates in ICB in cancer, and the emerging relevance of epigenetic regulation of T lymphocytes after antigen presentation [4], in this article of *EBioMedicine*, Fröhlich et al. [5] address the evaluation of *LAG3* methylation as a prognostic factor and predictor of response to anti-PD-1 and anti-CTLA4 ICB, also in combination with anti-IDO immunotherapy in melanoma. Remarkably, they identify a pattern of differential methylation in the promoter region of *LAG3* with respect to the gene body and 3′ UTR regions, and trace its association to mRNA expression, a "hot" tumour signature of pro-inflammatory immune cells and cytokines, as well as to improved overall survival in melanoma and progression-free survival in the ICB treated cohort. In addition, they demonstrate *in vitro* that *LAG3* is also expressed to a certain extent in melanoma cells, and, relevantly, that expression is inducible under exposure to 5-azacytidine.

Until recently, *LAG3* epigenetic control had been restricted to histone modifications and miRNA inhibition; however, earlier this year, Klümper *et al.* reported for the first time the association of *LAG3* methylation with gene expression in clear cell renal cell carcinoma [6]. Fröhlich et al. [5] extend this association to TCGA bulk melanoma samples and delineate region specific correlations between DNA methylation and mRNA expression, associating the hypomethylation of the promoter region of *LAG3*, which contains a CpG island (CGI), and the hypermethylation of the gene body and 3′UTR region outside the CGI, with expression. Interestingly, they are able to assign this specific methylation pattern to the tumour immune infiltrate by correlating it with the leukocyte fraction, lymphocyte score and Interferon gamma, and the opposite pattern to the tumour cell fraction by means of association with the tumour content and purity. Given that bulk, normally formalin-fixed paraffin-embedded tissues, are the most abundant deposited clinical samples, *LAG3* methylation could be of use as a surrogate marker of immune infiltration. In addition, this hypothesis is reinforced by the identification of similar patterns of deconvoluted TCGA methylation in pro-inflammatory and activated immune cells, as well as in regulatory T lymphocytes. Moreover, these correlations are also validated in a separate melanoma cohort with bulk tissues where they associate the N-shore, first exon, promoter CpG hypomethylation with the immune infiltrate, as well as the respective hypermethylation with the tumour fraction.

While it is known that gene body methylation can be a mark of transcriptional activity, gene body regions and enhancers of actively transcribed genes can also be enriched in DNA hydroxymethylation [7]. Moreover, DNA hydroxymethylation is typical of intermediate

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density CpG regions. According to that, and given that DNA hydroxymethylation cannot be distinguished from DNA methylation with the methods used in this work, one could also speculate that the hypermethylation observed in the non-CGI gene body and 3′UTR regions of *LAG3* is actually increased DNA hydroxymethylation, which is why the correlation with expression is positive in those regions. The identification of a CCCTC-binding factor (CTCF) binding site in the 3′UTR region of *LAG3*, led the authors to hypothesize that DNA methylation in this region could affect the binding of this dual Transcription Factor. Noteworthy, CTCF binding sites have also been associated to DNA hydroxymethylation, so the question arises whether *LAG3* upregulation in the context of the T lymphocyte exhaustion phenotype induced by persistent tumour antigen presentation could be associated with the re-patterning of both DNA methylation and hydroxymethylation. Interestingly, the regions that tend to have higher variation in DNA methylation are the shores and shelves, and indeed Fröhlich et al. [5], when comparing *LAG3* methylation across different cell types (peripheral blood mononuclear cells (PBMCs), melanocytes and melanoma cell lines), unmask DNA methylation variability between melanoma cell lines and melanocytes at precisely CpG sites located in *LAG3* regions of non high CpG density.

In addition to the cell type specific dissection of the correlations between *LAG3* methylation and expression, the current work by Fröhlich et al. [5] includes another major asset that is the novel prognostic and response predictive value of *LAG3* methylation in the context of anti-PD-1 and anti-CTLA-4 ICB, being hypomethylation associated with better outcomes. This is particularly relevant given that only in few genes, methylation has been previously associated to drug response to ICB (*PD-L1*, *CTLA-4*, *FOXP3*) [2], and evidences the myriad of players in the regulation of the dynamics of the immunosuppression in the tumour microenvironment that can influence the clinical benefit of this kind of therapies.

Finally, another important finding in Fröhlich et al.s work [5] is the detection of a low basal but epigenetically inducible *LAG3* expression in melanoma cell lines, opening a new avenue of investigation on the putative interference of the tumour co-expression of LAG3 and its ligands MHC-II in general, and FGL1 o LSectin in melanoma cells [3], in the response to ICB, as it occurs with the tumour co-expression of PD-1 and anti-PD-L1, where they mediate the resistance to anti-PD-1 and anti-PD-L1 treatments by activation of AKT and ERK1/2 [8].

In summary, the work of Fröhlich et al. [5] introduces several findings relevant to the field of ICB, where melanoma *LAG3* region-specific methylation deserves further attention with respect to the prediction of prognosis and the response to ICB, and the intrinsic tumour *LAG3* expression inducible by pharmacological de-methylation raises the need to decipher its interaction with ICB and with epigenetic drugs that are currently being tested in combination with ICB.

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Declaration of Competing interest

The author declares no conflicts of interest.

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