The Role of HOXA4 in Chronic Lymphocytic Leukaemia Progression and Response to Therapy

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Chronic lymphocytic leukaemia (CLL) is the most common form of adult leukaemia worldwide. Patients display a highly variable clinical course, with some requiring immediate therapeutic intervention while others can remain untreated for years. We have previously reported that DNA methylation of the homeobox A4 (*HOXA4)* promoter can serve as part of a three gene prognostic signature with *CD38* and *BTG4* to predict time to first treatment (TTT) in Stage A patients. *HOXA4* encodes a transcription factor that is expressed in haematopoietic progenitor cells and is involved in embryonic development and B-cell differentiation, and its aberrant epigenetic regulation has been identified in multiple forms of leukaemia. In this study we have sought to elucidate the role of *HOXA4* in the progression of CLL and determine the functional consequences of its expression.

We analysed DNA methylation of the *HOXA4* promoter by pyrosequencing in a heterogeneous cohort of 163 CLL patients (median age: 70; median follow-up: 10 years), of whom 60% were Binet Stage A, 16% Stage B and 24% Stage C. Data was collected regarding treatment history, TTT and overall survival, as well as cytogenetic abnormalities and IGVH mutation status. *HOXA4* methylation increased with disease progression and was significantly higher in Stage C patients (median 74%) than those with Stage A (62%; $p = 0.03$) and Stage B disease (65%; $p < 0.05$). *HOXA4* methylation was positively correlated with IGVH sequence homology ($r = 0.34$, $p < 0.0001$) and negatively associated with TTT among patients who have started chemotherapy ($p = 0.04$) and with overall survival ($p = 0.04$). No associations were observed between *HOXA4* methylation and 11q, 13q or 17p deletions, or *TP53* and *ATM* mutations.

To investigate the role of *HOXA4* in the evolution of the disease, we analysed samples taken at multiple timepoints from 42 patients, of whom 29 were undergoing treatment and 13 remained untreated. *HOXA4* methylation significantly increased in patients undergoing treatment ($p = 0.01$), but did not differ in untreated patients ($p = 0.19$).

We hypothesised that silencing of *HOXA4* may be selected for during treatment due to its expression conferring increased sensitivity to chemotherapy. Using a lentiviral

system, we observed that re-expression of *HOXA4* increased drug sensitivity in a malignant differentiated B-cell line (Raji). Significantly higher apoptosis was identified after treatment with 3 μM and 10 μM fludarabine (both $p < 0.001$) and 1 μM and 10 μM ibrutinib ($p < 0.01$ and $p < 0.001$), but not 1 μM and 10 μM idelalisib.

To confirm the translational relevance our observations, we overexpressed HOXA4 in primary CLL cells derived from four patients and confirmed increased apoptosis in response to 3 μM and 10 μM fludarabine treatment in comparison to control cells ($p = 0.02$ and $p < 0.01$). Further work is underway in primary CLL cells to elucidate the pathways under the control of HOXA4 that may confer this drug sensitivity.

Our ongoing work may indicate that HOXA4 is also implicated in the progression of CLL through directing malignant cells to the protective bone marrow niche, thereby further reducing sensitivity to antimetabolites. In cell lines HOXA4 up-regulates the expression of RGS2 and RGS16, which are negative regulators of the CXCR4-CXCL12 signalling axis, and we have identified selection for biallelic *HOXA4* methylation in primary acute lymphoblastic leukaemia cells following engraftment in mice (median in primary cells: 80%; engrafted cells: 92%; $p < 0.0001$).

To determine the origins of *HOXA4* dysregulation during the course of the disease, we analysed prospective blood samples from the European Prospective Investigation into Cancer and Nutrition (EPIC) from 20 individuals diagnosed with CLL up to 17 years after blood draw (median: 7 years) and 20 age-matched controls who remained free of cancer. We observed that *HOXA4* methylation was significantly higher among future CLL patients (median: 49% vs 42%; $p = 0.01$) and was inversely correlated with time to diagnosis, but did not reach statistical significance ($r = -0.39$, $p = 0.09$).

Together, our findings suggest that silencing of the *HOXA4* gene is an early event in CLL which is selected for during the course of disease through reduced sensitivity to chemotherapeutic agents. Our ongoing work will identify downstream targets that may be implicated in conferring sensitivity, and which may serve as biomarkers to predict prognosis and inform treatment strategies.