Assessment of Pathological Role of Large E3 Ubiquitin Ligase HERC1 in Myeloid Haematological Malignancies.

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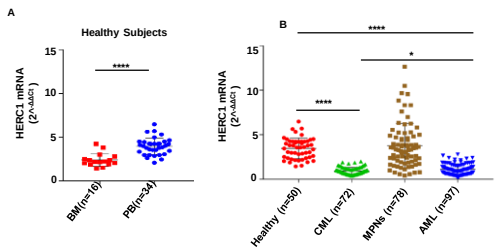
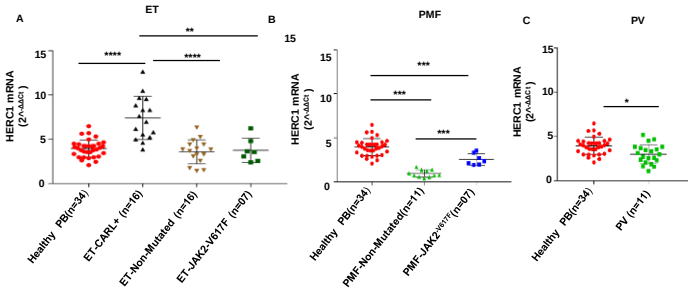
Accumulating evidence shows the HERC-E3 ubiquitin ligase family proteins are key components of a wide range of cellular functions including pivotal roles in cancer-related pathways. By using a simple model organism such as the social amoeba *Dictyostelium*, we identified a novel E3-HECT-Ubiquitin Ligase- can be considered a non-conventional HERC1 member that suppresses the mTORC2-dependent activities. Besides the HERC1 roles played in the nervous system of higher organisms like human beings, in the past few years, it has emerged that few hematological neoplasms harbor somatic mutations affecting the HERC1 locus in different kinds of Leukemia. Protein degradation by the ubiquitin-proteasome system (UPS) regulates hematopoietic stem cell (HSC) self-renewal, differentiation, and survival. However, the roles played by HERC1 in blood cells, under physiological and pathological conditions, remain at the moment unknown. Hence, we have recently started to assess whether HERC1 might be, or not, associated to a pathological and physiological conditions. We have identified for the first time an association of large E3 ligase HERC1 with hematological malignancies in which *HERC1* transcript level was mostly down-regulated, thus likely acting as an onco-suppressor. Moreover, we noticed that there are few remarkable exceptions suggesting that its role is tightly context-dependent. HERC1 protein also represents a novel BCR-ABL1 interacting partner and substrate. We also observed a differential HERC1 gene expression when leukemic cell lines were induced to differentiate. These preliminary observations highlighted the pivotal role of HERC1 in the regulation of blood cell differentiation.

THE *HERC1* GENE EXPRESSION IS DYSREGULATED IN HEAMATOLOGICAL MALIGNANCIES



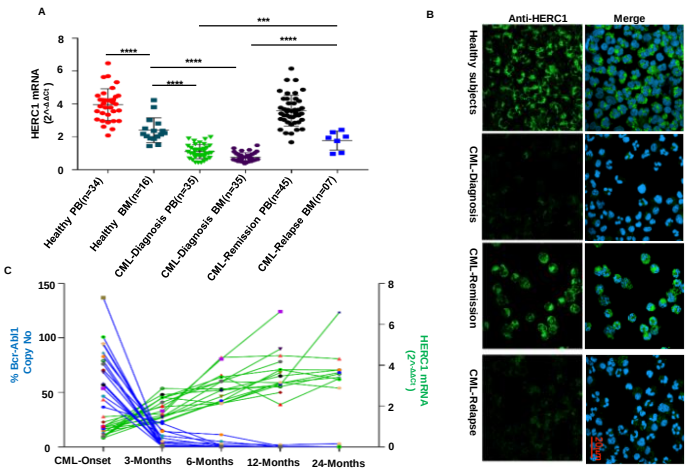
The expression of the *HERC1* gene is differently regulated in healthy blood specimens. PB samples exhibited higher *HERC1* amount (median=4.01) when compared to the BM (median=2.2) **(A)**. *HERC1* transcript was assayed in healthy donors and in a panel of myeloid related disorders at diagnosis, namely Chronic Myeloid Leukemia (CML), Acute Myeloid Leukemia (AML), MyeloProliferative Neoplasms (MPNs), by RT-qPCR. The *HERC1* mRNA quantity was expressed as $2^{-\Delta\Delta Ct}$ after normalization against *GUSB*. *HERC1* gene expression was significantly down-regulated in newly diagnosed CML and AML, with median values of 0.9 and 1 respectively, while in MPNs the *HERC1* transcript amount fluctuated from 0.4 to 12.65 with a median value of 3.3 and thus not significantly differing from healthy subjects. *P*-values were calculated on the differences in *HERC1* gene expression observed between patients and the healthy control specimens **(B)**.

HERC1 mRNA IS DIFFERENTIALLY AND PECULIARLY EXPRESSED IN MPNs PATIENTS



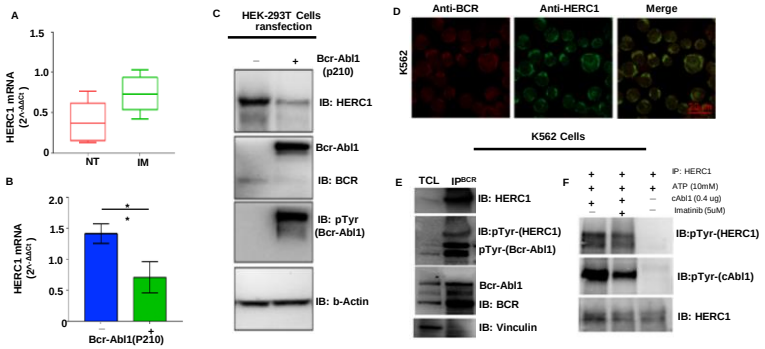
On the whole, the expression pattern of *HERC1* in MPNs appeared to be rather scattered and variable. Among MPNs, when compared to the healthy subjects the CARL mutated Essential Thrombocythemia (ET) specimens **(A)** displayed a significantly higher amount of *HERC1* transcript, ($P \leqslant 0.0001$). On the contrary, the *JAK2* mutated, and non-mutated, specimens showed levels comparable to the control counterpart. In Polycythemia Vera (PV) patients the *HERC1* mRNA level was slightly, but significantly, lower than that of the healthy subjects **(B)**. Differently, the Primary Myelofibrosis (PMF) patients displayed a rather steep downregulation of *HERC1* gene expression. However, the JAK2V617F mutated samples showed higher *HERC1* levels in comparison to the mutation free cohort **(C)**.

HERC1 GENE EXPRESSION IS DIFFERENTLY REGULATED DURING THE EVOLUTION IN CHRONIC MYLOID LEUKEMIA (CML) PATIENTS



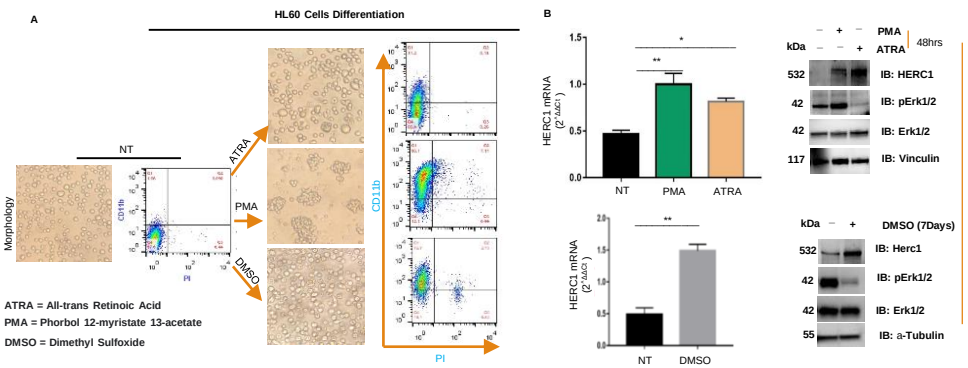
HERC1 gene was differentially expressed during the different CML phases being severely downregulated at diagnosis, both in BM and PB CML specimens, while its transcript level arose at remission and declined again at the onset of relapse **(A).** During the evolution of CML patients an inverse correlation exists between *HERC1* and *BCR-ABL1* gene expression at different time points of disease **(B).** Similarly to the mRNA, in primary CML cells the HERC1 protein amount was barely detectable at diagnosis and at the relapse onset. On the contrary, at remission the protein level was comparable to that of healthy control. Immunofluorescence was performed on cytospun cells by using specific anti-HERC1 antibody. DAPI staining (blue) indicated the cells nuclei. The green signal, corresponding to HERC1 protein **(C)**.

HERC1 IS A NOVEL INTERACTOR AND A POTENTIAL SUBSTATE OF BCR-ABL1



Primary bone marrow-derived leukemic cells from newly diagnosed CML patients showed a moderate increase in HERC1 mRNA level after in-vitro Imatinib treatment **(A)**. HEK-293T cells transiently and exogenously expressing the constitutively active p210 Bcr-Abl1 tyrosine kinase exhibited a severe down-modulation of either the HERC1 transcript **(B)** and protein **(C)** levels. The subcellular localization of either the HERC1 or Bcr-Abl1 (p210) proteins were assessed by staining cytospun K562 cells with anti-HERC1 and anti-Bcr specific antibodies. The immunofluorescence assay revealed that the localization of the two proteins was mostly restricted to the cytoplasmic compartment and they co-localized **(D)**. In K562 cells Bcr-Abl1 and HERC1 proteins form a complex. The immunoprecipitated Bcr-Abl1 was employed to pull-down HERC1 from K562 total cell lysate. The presence of HERC1 in the co-immunoprecipitated complex was determined by immunoblotting the membrane with HERC1 antibody **(E)**. Subsequently probing with a tyrosine phospho-specific antibody proved the phosphorylated HERC1 status **(E)** In vitro kinase assay with purified c-ABL and HERC1 substrate (obtained after HERC1 immunoprecipitation on 293T), performed in the absence or presence of 5 µM Imatinib treatment for 30 min which confirmed HERC1 is phosphorylated by cAbl1 **(F)**. Actin and Vinculin were used as loading control.

THE *HERC1* GENE EXPRESSION AND ITS ASSOCIATION WITH DIFFERENTIATION IN MYELOID LEUKEMIA CELLS



Upon incubation with differentiation agents the HL-60 cells change the morphology and adhesion properties, being capable to adhere to the substratum. Morphological changes and differentiation-specific cell surface marker expression was confirmed by quantifying CD11b neutrophil cell surface marker with flow cytometry (FACS) analysis, after incubation of HL60 cells with phorbol 12 myristate 13-acetate (PMA), All Trans-Retinoic Acid (ATRA) and Dimethyl Sulfoxide (DMSO). Cell treated with PMA and DMSO showed an increase by approximately 75% and 85% of CD11b, associated with morphological changes respectively, while with ATRA differentiating marker CD11b raised approximately by 30%, with elongated cell morphology and weakly attached to the substratum **(A)**. *HERC1* gene expression both at mRNA and protein level upregulated in differentiated cells. Remarkably, the phosphorylation of Erk1/2 was severely impaired in differentiated cells and is tightly linked with the HERC1 protein level when compared to undifferentiated control. These findings revealed that the downregulated HERC1 gene expression might be linked to differentiation arrest at the onset of myelogenous leukemia. The vinculin and a-tubulin were used as loading control in Western Blot **(B)**.

CONCLUSION: Our findings revealed that *HERC1* gene expression was down-regulated both in acute and in chronic myelogenous leukemia patients, while its expression is peculiarly regulated in Ph negative neoplasms. Additionally, we observed that in CML cells the regulation of the *HERC1* gene expression is sensitive to the Bcr-Abl1 kinase activity and that a physical interaction between HERC1 and Bcr-Abl1 proteins occurred. On the whole our findings indicate that HERC1 is associated with hematological malignancies and a novel player in the regulation of blood cells differentiation in lineage specific manner.

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