Original Article HMGA1 induces EZH2 overexpression in human B-cell lymphomas

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Abstract: EZH2 is an enzymatic subunit of PRC2, an epigenetic regulator that triggers the methylation of the histone H3 lysine 27 silencing the transcription of several genes. EZH2 has a critical role in cancer progression, since its overexpression has been associated with increased cancer cell invasiveness, drug resistance and poor patient survival. However, the mechanisms accounting for EZH2 overexpression in cancer remain still unclear. Intriguingly, also HMGA protein overexpression is a feature of many human malignancies and correlates with the presence of metastases and a poor outcome. The HMGA proteins, including HMGA1 and HMGA2, belong to the architectural transcription factors that play a key role in the organization of chromatin structure. Here, we report a statistically significant correlation between HMGA1 and EZH2 expression in human lymphomas. We demonstrate that HMGA1 is able to bind *EZH2* promoter and induce its activity. Consistently, silencing of *HMGA1* expression results in the downregulation of the *EZH2* levels leading to a decreased proliferation and migration rate of human lymphoma cell lines. Therefore, these data identify HMGA1 as an *EZH2* activator, suggesting a novel molecular mechanism contributing to EZH2 overexpression in human malignancies and a synergism of these proteins in cancer progression.

Keywords: HMGA1, EZH2, B-cell lymphomas

Introduction

Enhancer of zeste-homolog 2 (EZH2) regulates gene silencing by catalysing trimethylation of lysine 27 residue of histone H3 (H3K27Me3) within the epigenetic regulator polycomb repressive complex 2 (PRC2) [1]. EZH2 is frequently overexpressed in several human malignancies, and its expression correlates with the presence of metastases and invasiveness [2]. An evident correlation between increased levels of EZH2, H3K27me3 and a poor outcome in numerous human tumours comprising breast [3], endometrial [4], ovarian [5], melanoma [6], bladder [7], glioblastoma [8], kidney [9], colorectal [10], lung cancer [11] has been reported. As far as the role of EZH2 in haematological neoplasias is concerned, its activation occurs in T-cell and B-cell lymphomas also through molecular mechanisms other than gene overexpression [12, 13]. Indeed, EZH2 increasedenzymatic activity has been frequently due to a somatic mutation that replaces the aminoacid tyrosine 641 (Y641) [14] in a subclass of follicular lymphomas (FL) and germinal center subtype of diffuse large B-cell lymphomas (DLBCL). Moreover, other gain-of-function mutations of EZH2 (A687V and A677G) have been identified in B-cell lymphomas [15, 16].

In adult T-Cell leukemia/lymphoma and mantle cell lymphoma (MCL), EZH2 levels have also

been found upregulated, whereas the overexpression of MYC contributed to enhance EZH2 expression in Burkitt lymphomas. Hodgkin/ Reed-Sternberg cells co-express BMI-1/EZH2 [17]. Consistently, it has been demonstrated that EZH2 is abundantly expressed in lymphoid progenitors, and its deficiency induces defects in early lymphopoiesis [18] suggesting a critical role of EZH2 in lymphocyte development and differentiation. Therefore, EZH2 overexpression or activation, through somatic mutations, is a feature of human haematological malignancies. However, the molecular mechanisms underlying upregulation of EZH2 need still to be defined. Recent studies have provided a detailed promoter sequence analyses identifying the transcription factors that affect the EZH2 gene, which include pRB-E2F [19], MEK-ERK-ELK1 [20], KRAS mutations and downstream ERK or Akt [21], hypoxia-induced HIF-1α [22], and NF-κB [23].

Similarly to EZH2, a family of chromatin architectural factors named high-mobility group A (HMGA) is involved in cancer progression. This family encompasses two main proteins, HM-GA1 and HMGA2 [24], that, through their DNA binding domains, known as "AT-hooks", bind to AT-rich DNA sequences at the minor groove. Even though HMGA proteins do not have transcriptional skills, they modify the chromatin organization, cooperating with the transcription apparatus, thereby regulating the expression of numerous genes [25, 26]. The amount of HMGA proteins detected in normal cells and adult tissues is low or absent [27], whereas is exceptionally high in embryonic cells and in neoplastically transformed cells [28]. HMGA proteins represent a poor prognostic index since their overexpression habitually associates with the presence of metastases and a reduced survival [29]. Moreover, both in vitro and in vivo studies support the oncogenic role of HMGA genes [30-33]. Indeed, transgenic mice overexpressing HMGA develop T-cell leukemia with a natural killer (NK) phenotype [34]. Consistently, HMGA1 overexpression has been found in human T- and B-acute lymphoblastic leukemia [35] and HMGA2 locus was found translocated in a case of chronic lymphocytic leukemia [36].

Therefore, since HMGA and EZH2 are both involved in several aspects of human carcinogenesis (proliferation, pluripotency, EpithelialMesenchymal Transition (EMT), tumor-acquired resistance [37]), resulting also key protagonists in haematological malignancies, we studied the possible molecular mechanisms by which HMGA may regulate EZH2 transcription in this disease.

Here, we report a clear correlation between the elevated *HMGA1* and *EZH2* expression levels in human lymphomas, also confirmed by the data available in Cancer Genome Atlas (TCGA). Then, we show that HMGA1 protein can upregulate *EZH2* expression by binding to its promoter. Consistently, the overexpression or the silencing of HMGA1 affects EZH2 levels, modulating lymphoma cell line growth and motility. Therefore, our data suggest that HMGA1 can contribute to EZH2 overexpression in human lymphomas indicating a critical role of both these proteins in these human malignancies.

Methods

Cell culture and transfections

In order to culture HEK293 and Hmga1^{-/-} Mouse Embryonic Fibroblasts (MEFs) (from 12.5dav-old embryos) cells, DMEM (Sigma-Aldrich, Saint Louis, MO, USA) medium was supplemented with 10% foetal calf serum (Thermo Fisher, Waltham, MA, USA), glutamine and antibiotics (Sigma-Aldrich, Saint Louis, MO, USA). RIVA and SU-DHL4 lymphoma-derived cell lines were grown in RPMI (Sigma-Aldrich, Saint Louis, MO, USA) medium with 20% foetal calf serum (Thermo Fisher, Waltham, MA, USA). To detect mycoplasma contamination in the employed cells, MycoAlert (Lonza, Basel, Switzerland) reagent was used. HEK293 cells were transfected by Lipofectamine plus reagent (Thermo Fisher, Waltham, MA, USA), whereas Neon Transfection System MPK5000 was utilized for SU-DHL4 and RIVA cells.

Plasmids

The HMGA1 expression construct (pCEFL-HA-HMGA1) was described elsewhere [38]. For the EZH2 promoter luciferase reporter construct, the human EZH2 promoter region from nucleotides -235 to +142 related to TSS was amplified using the primers: promEZH2-Fw 5'-gcgaaagaacaaagagacgg-3' and promEZH2-Rv 5'actcgcgttgttcccgcgcgtc-3'. The EZH2 expression construct was previously described [39].

The obtained sequence was inserted into pGL3-Basic Firefly luciferase vector (Promega, Madison, WI, USA). In order to confirm the DNA sequence of the cloned vector, it was controlled by sequencing. The Renilla luciferase vector (pRL-CMV) was purchased from Promega (Madison, WI, USA). The HMGA1 and EZH2 inhibiting small interfering RNAs (siRNAs) and their relative scramble controls were synthetized and transfected as suggested by the manufacturer (RIBOXX, Radebeul, Germany). To stably inhibit HMGA1 expression short hairpin RNA (shRNA) vector and its relative scramble control were designed and used as suggested by the manufacturer (Sigma-Aldrich. Saint Louis, MO, USA).

The cancer genome atlas (TCGA) database

HMGA1 and EZH2 expression data for 48 DL-BCL samples were obtained from The Cancer Genome Atlas (TCGA) genomic data Commons portal (https://portal.gdc.cancer.gov/repository) as data for validation of gene expression correlations.

Western blotting

Western blotting procedures were performed following a protocol reported elsewhere [40]. Western blotting antibodies were: anti-EZH2 #3147 (Cell Signalling Technology, Danvers, MA, USA), anti-Tubulin T518 (Sigma-Aldrich, Saint Louis, MO, USA), anti-Actin sc-1616 (Santa-Cruz Biotechnology, Dallas, TX, USA). The customized HMGA1 antibody characteristics were described elsewhere [41, 42].

RNA extraction and qRT-PCR

Total RNA was extracted by using Trizol reagent (Thermo Fisher, Waltham, MA, USA) in accordance with the manufacturer's instructions. The expression of mRNAs was valued by qRT-PCR as described elsewhere [43], using the following primers: HMGA1-Fw 5'-aaggggcagacccaaaaa-3', HMGA1-Rev 5'-tccagtcccagaaggaagc-3'; EZH2-Fw 5'-gtctcccctacagcagaa-3', EZH2-Rev 5'-cctttgctccctcaaa-3'; G6PD-Fw 5'-acagagtgagcccttcttcaa-3', G6PD-Rev 5'-ataggagttgcgggcaaag-3'; Hmga1-Fw 5'-ggcagacccaagaaactgg-3', Hmga1-Rev 5'-ggcactgcgagtggtgat-3'; Ezh2-Fw 5'-tggaagcagcggaggata-3', Ezh2-Rev 5'-gtcactggtgactgaacactcc-3'; G6pd-Fw 5'-cagcggcaactaaactcaga-3', G6pd-Rev 5'ttccctcaggatcccacac-3'.

Gene expression Fold Change was assessed by the 2- $\Delta\Delta$ CT formula [44].

Lymphoma tissue samples

The human lymphomas samples were collected at the Centre de Ressources Biologiques des Hospices Civils de Lyon, France. Immediately after the resection, tumour samples were frozen until the RNA extraction. We declare that informed consent for the scientific use of biological material was obtained from all patients and in accordance with the ethic declaration of Helsinki.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) samples were processed as reported elsewhere [45] and immunoprecipitated with anti-HMGA1 antibody [41]. Immunoprecipitated fragments were amplified by PCR using the following primers: EZH2-prom-FW 5'-actcagaaggcagtggagc-3', EZH2-prom-Rev 5'-ccaacaaactggtcccttctc-3'.

Immunohistochemistry

A human lymphomas Tissue Microarray (TMA) was purchased from Biomax.us (#LY800a). After the permeabilization with a solution of PBS-0.2% triton and two wash passages in PBS, the sections were unmasked by using a citrate buffer (0.01 M pH 6) 15' in the microwave. To saturate the endogenous peroxidases methanol and 1.5% oxygen peroxide were used and, after PBS-0.2% triton solution to permeabilize tissues, they were blocked by blocking solution (5% Normal goat serum (Vector Laboratories S-1000), 3% BSA, 20 mM MgCl., 0.3% tween 20 in PBS) 1 h at room temperature. Anti-EZH2 #3147 (Cell Signalling Technology, Danvers, MA, USA) and anti-HM-GA1 [41] were used as primary antibodies. They were diluted in blocking solution and used overnight at 4°C. To stain the TMA the following protocol was used: 5' PBS-0.2% triton, 2 × 5' PBS, 1 h secondary antibody (biotinylated α -rabbit and α -mouse IgG (H+L) Vector Laboratories BA-1000) 1:100 in blocking solution 1 h at room temperature, 5' PBS-0.2% triton, $2 \times 5'$ PBS, 30' ABC (Vector Laboratories SK-4000) RT, 5' PBS-0.2% triton, $3 \times 5'$ PBS, DAB substrate (Vector Laboratories SK-4100). The samples were then de-hydrated and covered with cover glasses using D. P. X. Mountant (liquid) (GRM655). HMGA1 and EZH2 proteins were considered overexpressed when more than 30% of the neoplastic cells presented a strong immunoreaction.

Dual-luciferase reporter assay

 3×10^5 HEK293 cells, plated in 6-well plates, were transfected with the luciferase reporter vector for EZH2 promoter, simultaneously with pCEFL-HA-HMGA1 plasmid and the Renilla luciferase vector (Promega, Madison, WI, USA). To normalize number of cells and transfection efficiency, the pRL-CMV control plasmid expressing Renilla luciferase (Promega, Madison, WI, USA) was utilized. Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used, 48 hours after transfection, to detect the activity of Luciferase using a Lumat LB 9507 device (Berthold Technologies, Bad Wildbad, Germany).

Migration assay

To assess cell migration, Transwell chambers with a pore size of 5 μ m (Corning, NY, USA) were employed. 4 × 10⁵ cells were resuspended in a medium without serum and then plated in the transwell upper chamber, meanwhile in the bottom chamber was added the complete culture medium. After 24 hours, the migrated cells were counted in the lower chamber.

Flow cytometric analysis

Flow cytometry was used to study the cell cycle of shRNA-HMGA1 and scrambled control stably transfected RIVA cells. Cells were collected in PBS containing 2 mmol/I EDTA, cleaned with PBS, and then fixed in 70% ethanol for 2 hours. Following a wash in PBS, fixed cells were processed with 40 μ g/ml RNase A in PBS for 30 minutes. After a wash passage in PBS, fixed cells were stained with 50 μ g/ml propidium iodide (Roche, Basel, Switzerland). A Calibur (Becton-Dickinson, Franklin Lakes, NJ, USA) fluorescence activated cell sorter (FACS) was used to analyze the stained cells and the results were analyzed using a mod-fit cell cycle analysis programme.

Statistical analysis

Two-sided unpaired Student's t test and Mann-Whitney test were performed to analyze data (GraphPad Prism, GraphPad Software, Inc.). Results with P<0.05 were classified as statistically significant. The mean ± standard deviation of three or more independent experiments is reported. GraphPad Prism was used to generate regression and correlation analyses.

Results

HMGA1 and EZH2 expression positively correlates in human lymphomas

Firstly, we analyzed the expression of the *HMGA1* and *EZH2* gene, by qRT-PCR, in a set of samples constituted by FL, MCL and DLBCL. As shown in **Figure 1**, *HMGA1* is overexpressed in almost all lymphoma samples analyzed (**Figure 1A**). *EZH2* gene showed a similar behaviour being overexpressed in most of the neoplastic tumor samples with respect to normal human lymphocytes (**Figure 1B**). Then, a significant linear correlation was found between *HMGA1* and *EZH2* expression (r=0.5438, P<0.005), suggesting that these genes are co-regulated (**Figure 1C**).

To further confirm a correlation between HMGA1 and EZH2 expression in haematological malignancies we analyzed the data available in The Cancer Genome Atlas (TCGA). Consistently with the results obtained by our group, the TCGA data confirmed the overexpression of both genes in 48 DLBCL samples with direct correlation between HMGA1 and EZH2 (r=0.49, P<0.003) (Figure 1D). In the same TCGA cohort, EZH2 and HMGA1P1 expression (Figure 1E) levels were directly correlated. Noteworthy, HMGA1P1 is a translated HMGA1-pseudogene that could contribute to lymphomagenesis in a HMGA1-like manner [46]. To further verify whether HMGA1 and EZH2 are co-regulated also at protein level we performed an immunohistochemistry analysis on TMA comprising 40 cases of DLBCL, five FL, 15 reactive hyperplasia of lymph node, 10 lymph nodes (Figure 2A-D). As shown in Figure 2E, HMGA1 and EZH2 were co-regulated in 61.5% of the analyzed samples (46.1% upregulated and 15.4% downregulated), whereas only HMGA1 or EZH2 were found upregulated



Figure 1. HMGA1 and EZH2 expression positively correlates in human lymphomas. (A) *HMGA1* and (B) *EZH2* qRT-PCR analyses in samples derived from FL, MCL and in DLBCL. The fold change indicates the relative change in expression levels between each tumor samples and normal samples (n=3), assuming that the value of normal samples is equal to 1. (C) The obtained values were then combined to generate a linear regression of *HMGA1* vs. *EZH2* in the whole series of human lymphomas analyzed (r=0.5438; P<0.05). (D) Direct correlation between *HMGA1* and *EZH2* in 48 TCGA DLBCL samples (r=0.49; P<0.0003). (E) Direct correlation between *HMGA1* and *EZH2* in 48 TCGA DLBCL samples (r=0.49; P<0.0003). (E) Direct correlation between *HMGA1* P1 and *EZH2* in 48 TCGA DLBCL samples (r=0.59; P<0.0001).



Figure 2. HMGA1 and EZH2 protein expression in human DLBC lymphoma samples. (A and B) Gross view of immunohistochemical staining of a tissue microarray slide. Scale bar, 5 mm. (C) Representative immunohistochemical staining of HMGA1 and EZH2 protein in three representative samples with weak, moderate and strong signal, respectively. Scale bar, 200 μ m. (D) HMGA1 and EZH2 signals in normal and tumoral representative samples. Scale bar, 200 μ m. (E) Graphic distribution of 65 bioptic tissues from the TMA analyzed for HMGA1 and EZH2 protein expression by immunohistochemistry.

in 26.1% or 12.3% of the analyzed samples, respectively.

HMGA1 protein binds to and activates human EZH2 promoter

On the basis of the results shown above, we envisaged the hypothesis that HMGA1 might regulate *EZH2* expression. Then, we first investigated whether HMGA1 was able to bind to

EZH2 gene regulatory regions performing a ChIP assay. To this aim, we transiently transfected HEK 293 cells with HA-tagged-HMGA1 expression vector or empty vector. Then, the crosslinked DNA-protein complexes were immunoprecipitated with anti-HMGA1 or IgG antibodies. In particular, by qPCR analysis, we examined a region spanning nucleotides from -88 to +53 related to the transcription start site (TSS) of the human *EZH2* gene.

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Figure 3. HMGA1 binds to *EZH2* promoter enhancing its transcription. A. HEK293 cells were transiently transfected with either a *HMGA1*-overexpressing vector or an empty vector (EV). The chromatin was immunoprecipitated by using antibodies against HMGA1. IgG were used as negative control (IgG). The IP chromatin was analyzed by qPCR assay performed with primers specific for the *EZH2* promoter. B. HEK293 were transfected with the *EZH2* promoter luciferase reporter construct and with increasing doses of *HMGA1*-overexpressing vector. Relative luciferase activity was calculated relative to that observed in cells transfected with the *EZH2* promoter luciferase reporter construct and the empty vector alone, assumed equal to 1. C. qRT-PCR analysis of *Ezh2* mRNA levels in *Hmga1*^{+/+} and *Hmga1*^{-/-} MEFs. The results are reported as the mean of values. The error bars represent mean ± SD; **P<0.01; ***P<0.001 (t test).

As shown in **Figure 3A**, anti-HMGA1 antibodies precipitated human *EZH2* promoter in both empty vector-transfected cells and *HMGA1*-transfected cells. Almost undetectable amplification was observed in samples immunoprecipitated with IgG.

To examine the consequences of HMGA1 binding to *EZH2* promoter, HEK 293 cells were transiently transfected with a vector carrying the human *EZH2* promoter region, from nucleotides -235 to +142 related to TSS, followed by the luciferase gene. An induction of the luciferase activity was observed in a dose-dependent manner due to *HMGA1* overexpression (**Figure 3B**). These observations point out not only that HMGA1 protein was able to bind to *EZH2* promoter but also to promote its transcription.

HMGA1 regulates lymphoma cell proliferation and migration modulating EZH2 expression

Looking for a possible correlation between HMGA1 and EZH2 protein expression, we analyzed their expression levels in MEFs derived

from hmga1 knock-out (hmga1^{-/-}) mice. Hmga1^{-/-} MEFs express lower ezh2 mRNA levels in comparison with the wild-type MEFs (Figure 3C). Subsequently, we examined the ability of HMGA1 to modulate EZH2 expression in the human lymphoma cell lines SU-DHL4 and RIVA. As expected, qRT-PCR and western blot analyses revealed the overexpression of HM-GA1-induced EZH2 expression, at both RNA and protein levels (Figure 4A). Conversely, EZH2 levels decreased following HMGA1 transient silencing in SU-DHL4 cells achieved by HMGA1-siRNA oligos (Figure 4B). Consistently, RIVA cells stably transfected with HMGA1-targeting shRNAs showed lower amount of both EZH2 mRNA and protein when compared to scrambled-transfected cells (Figure 5A). Then, we investigated the functional consequences of EZH2 downregulation induced by HMGA1 silencing on cell proliferation. To this aim, both scrambled-transfected and HMGA1-silenced RIVA cells were counted each day for 6 days. As shown in Figure 5B HMGA1-silenced RIVA cell growth rate was lower compared with the

HMGA1 and EZH2 correlation in human B-cell lymphoma



Figure 4. EZH2 is positively regulated by HMGA1 in SU-DHL4 cells. A. (Upper panel) qRT-PCR analysis of *EZH2* mRNA levels in SU-DHL4 cells transfected with the empty vector and HMGA1. (Lower panels) Western blot analysis of EZH2 and HMGA1 protein levels from the same samples shown in the upper panel. B. (Upper panel) qRT-PCR analysis of *HMGA1* and *EZH2* mRNA levels in SU-DHL4 cells transfected with the scrambled oligonucleotide and HMGA1-siRNA. (Lower panel) Western blot analysis of EZH2 and HMGA1 protein levels from the same samples shown in the upper panel. The results are reported as the mean of values. The error bars represent mean \pm SD; **P<0.01; ***P<0.001 (t test).

HMGA1 and EZH2 correlation in human B-cell lymphoma



Figure 5. *HMGA1* silencing impairs lymphoma cell line proliferation. A. (Upper panel) qRT-PCR analysis of *HMGA1* and *EZH2* mRNA levels in RIVA cells transfected with the scrambled oligonucleotide and shHMGA1. (Lower panel) Western blot analysis of EZH2 and HMGA1 protein levels from the same samples shown in the upper panel. B. RIVA cell proliferation curve in shHMGA1 transfected cells. C. The DNA content of the transfected RIVA cells was analyzed by flow cytometry after propidium iodine staining. The results are reported as the mean of values. The error bars represent mean ± SD; *P<0.05; **P<0.01 (t test).



1

0,8

0,6

0,4

0,2

0

Scr

Ξ

y-Tubulin expression was used as a control for equal protein loading. B. RIVA cell proliferation curve in shH-MGA1 and shHMGA1 + EZH2 transfected cells. C. shEZH2, shHMGA1 and shHMGA1 + EZH2 transfected RIVA cells were assessed for migration capability in Transwell chambers assay. Histograms represent mean ± SD from, *P<0.05.

cells transfected with the scrambled vector. Finally, we investigated the cell cycle phase distribution of the HMGA1-silenced RIVA cells by FACS analysis. As it can be observed in Figure 5C, the HMGA1-silenced cells showed a delay in the transition to the G2/M phase since they remain blocked in the S phase in comparison with scrambled-transfected RIVA cells. These results are consistent with the critical role of the EZH2 protein in the S/G2-M transition of the cell cycle [39]. The growth inhibition exerted by the HMGA1-silencing in RIVA cells was significantly attenuated by restoring the EZH2 expression in *HMGA1*-silenced RIVA cells (Figure 6A and 6B). Moreover, since EZH2 is a master regulator of cell migration capability, we have analysed this property in HMGA1-silenced RIVA cells. As reported in Figure 6C, the migration capability of these cells was significantly

Consistently, when EZH2 expression was restored in HMGA1-silenced RIVA cells, the reduction of the migration index induced by HMGA1silencing was abolished, suggesting that EZH2

reduced when compared to the control cells.

regulation by HMGA1 has a pivotal role in cell growth and migration capability.

shHMGA1

shHMGA1 +

EZH2

Discussion

shEZH2

Several studies have reported that EZH2 is upregulated in several non-haematological solid cancers and its high levels are linked to cancer progression and aggressiveness [19, 47]. Moreover, the inhibition of the PRC2/ EZH2 complex activity either genetically or pharmacologically dramatically reduces the proliferative rate of cancer cells expressing high EZH2 levels and tumors developing in EZH2-transgenic mouse models [48, 49]. Importantly, EZH2 overexpression has also a key role in the development of several haematological malignancies [18]. Indeed, Yan et al. have reported that EZH2 levels were found upregulated in NK/T-cell lymphomas as a result of MYC-mediated downregulation of two tumor suppressor microRNAs (miR-26 and miR-101) [50]. Moreover, EZH2 was found upregulated in adult T-Cell leukaemia/lymphoma cells, in mantle cell and in Burkitt lymphomas, where the c-MYC upregulation enhances EZH2 expression [50-52].

Interestingly, the HMGA proteins show several properties in common with EZH2. Indeed, their overexpression is a feature of the experimental and human malignancies, and inflammatory pathways induced by HMGA1 have been described in lymphoid tumorigenesis and pathways taking part in stem cells and in cell cycle progression [53]. Moreover, *HMGA1* deregulates genes and pathways at both early and late stages of tumorigenesis in stem cells, cellular development and haematopoiesis [53].

Therefore, we decided to study the expression of both HMGA1 and EZH2 in a panel of haematological neoplasias including a range of FL, MCL and DLBCL. Both the genes were highly expressed in most of the analyzed neoplastic samples with a significant correlation between their expression levels. These data were also supported by the analysis of TCGA database regarding 48 DLBCL cases. Importantly, HMG-A1P1, a recently identified protein-coding HM-GA1 pseudogene [46], has been found overexpressed in several TCGA samples and directly correlated with EZH2 expression levels. Consistently, preliminary data have shown a chromosomal amplification in the HMGA1P1 genetic locus, suggesting a HMGA1-like role for this pseudogene in lymphomagenesis. Moreover, since we have recently demonstrated that transgenic mice overexpressing HMGA1P7 develop B-cell lymphoma [54], we investigated also the expression of these HMGA1-pseudogenes which contribute to HMGA1 overexpression in several human carcinomas through a competitive endogenous RNA (ceRNA) mechanism [54-57]. However, we did not find any significant expression of these HMGA1-pseudogenes in the analysed human lymphoma samples, thus suggesting different molecular mechanisms responsible for HMGA1 overexpression.

Then, in the attempt to unveil the possible mechanisms accounting for this correlation, we demonstrated that HMGA1 binds to *EZH2* promoter and sustains its transcription. Subsequently, functional studies demonstrate that the HMGA1-EZH2 axis controls the lymphoma cell proliferation and migration rate. In fact, silencing of *HMGA1* results in EZH2 downregulation and in the block of the cell cycle at the S-phase.

Therefore, these results support an oncogenic role for EZH2 and HMGA1 in B-cell neoplasms, similar to their role in non-haematological solid neoplasias, and then they can be considered as critical therapeutic targets in haematological malignancies. Accordingly, studies are in progress to test compounds able to target EZH2 and HMGA, representing key targets for cancer treatment. Indeed, several inhibitors of DNA methyltransferases and histone deacetylases [58, 59] have revealed hopeful antitumor effects as well as compound with the ability to impair the function of the HMGA proteins [41].

In conclusion, the results reported here provide important insights into the biology of human lymphomas and support an innovative therapeutic approach for these diseases based on EZH2-HMGA1 targeting compounds alone or in combination for the treatment of lymphomas.

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Disclosure of conflict of interest

None.

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