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Transcriptional deregulation of homeobox gene ZHX2 in Hodgkin lymphoma

Stefan Nagel*, Björn Schneider[1], Corinna Meyer, Maren Kaufmann, Hans G. Drexler, Roderick A.F. MacLeod

Department of Human and Animal Cell Lines, DSMZ – German Collection of Microorganisms and Cell Cultures, Inhoffenstr. 7B, 38124 Braunschweig, Germany

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ABSTRACT

Recently, we identified a novel chromosomal rearrangement in Hodgkin lymphoma (HL), t(4;8)(q27;q24), which targets homeobox gene ZHX2 at the recurrent breakpoint 8q24. This aberration deletes the far upstream region of ZHX2 and results in silenced transcription pinpointing loss of activatory elements. Here, we have looked for potential binding sites within this deleted region to analyze the transcriptional deregulation of this tumor suppressor gene in B-cell malignancies. SiRNA-mediated knockdown and reporter gene analyses identified two transcription factors, homeodomain protein MSX1 and bZIP protein XBP1, directly regulating ZHX2 expression. Furthermore, MSX1-cofactor histone H1C mediated repression of ZHX2 and showed enhanced expression levels in cell line L-1236. As demonstrated by fluorescence in situ hybridization and genomic array analysis, the gene loci of MSX1 at 4p16 and H1C at 6p22 were rearranged in several HL cell lines, correlating with their altered expression activity. The expression of XBP1 was reduced in 6/7 HL cell lines as compared to primary hematopoietic cells. Taken together, our results demonstrate multiple mechanisms decreasing expression of tumor suppressor gene ZHX2 in HL cell lines: loss of enhancing binding sites, reduced expression of activators MSX1 and XBP1, and overexpression of MSX1-corepressor H1C. Moreover, chromosomal deregulations of genes involved in this regulative network highlight their role in development and malignancy of B-cells.

1. Introduction

Hodgkin/Reed-Sternberg (HRS) cells represent the malignant fraction of infiltrated lymph nodes in Hodgkin lymphoma (HL). These cells constitute aberrant B-cells although they express some T-cell or myeloid associated genes [1]. Therefore, deregulation of B-cell development represents a major aspect of aberrant gene expression in HRS cells. Accordingly, downregulation of B-cell transcription factors BOB1 and OCT2 contribute to the loss of immunoglobulin (IGH) expression [2]. Furthermore, repression of TCF3/E2A activity by overexpressed ID2 and ABF1 proteins and reduced expression of BCL6 and B-cell receptor signaling component BLNK are also features of disturbed B-cell differentiation in HL [3–5]. Malfunction of plasma cell differentiation has been observed in HL as well, indicating gene deregulations corresponding to multiple stages of B-cell development [6,7]. Additional pathologically expressed genes and pathways in HL comprise NFkB, A20, ERK- and JAK-STAT-signaling [1].

Homeobox genes encode transcription factors with a great impact in developmental processes including embryogenesis and cell differentiation [8]. Due to their distinct and tight regulation in developing tissues homeobox genes are often embedded in large non-coding regions, containing several activatory and inhibitory elements. Homeobox genes are frequently deregulated in cancer, including leukemia and lymphoma [9,10]. In HL homeobox genes HLXB9 and HOXB9 have been identified as transcriptional deregulated downstream targets of aberrant PI3K- and ERK5-signaling, respectively [11,12].

Chromosomal aberrations in leukemia and lymphoma target regulators of oncogenesis. Although HRS cells display multiple chromosomal aberrations, few are recurrent and the targeted genes unknown. However, understanding the pathology of HL and developing rational therapies may well require identifying putative target genes and their deregulating mechanisms. Recently, we have identified t(4;8)(q27;q24) in HL cell line L-1236, activating phosphodiesterase PDE5A at 4q27 and inhibiting homeobox gene ZHX2 at 8q24 [13]. Expression analysis in hematopoietic cell lines and primary cells and identification of downstream target genes indicated ZHX2 as a tumor suppressor gene in HL. The breakpoint at ZHX2 is located in the far upstream region and may remove activating regulatory elements. The aim of this study was to examine in more detail the transcriptional deregulation of ZHX2 in HL. Our

* Corresponding author. Tel.: +49 531 2616 167; fax: +49 531 2616 150.
E-mail address: sna@dsmz.de (S. Nagel).
[1] Present Address: Institute of Pathology, University of Rostock, Rostock, Germany.

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analyses identified two transcription factors, MSX1 and XBP1, itself deregulated by chromosomal rearrangements.

2. Materials and methods

2.1. Cell lines and treatments

Cell lines are held by the DSMZ (Braunschweig, Germany) and cultivated as described previously [14]. SiRNA oligonucleotides (siMSX1, siH1C, siXBP1, and AllStars negative Control siRNA) were obtained from Qiagen (Hilden, Germany). Expression constructs (pCMV6-MSX1, pCMV6-XBP1) were obtained from Origene (Wiesbaden, Germany) or cloned as described below (pcDNA3-H1C). Both, siRNAs and expression constructs, were transfected into the cells by electroporation using the EPI-2500 impulse generator (Fischer, Heidelberg, Germany) at 350 V for 10 ms. CD40-stimulation was performed by treatment with recombinant human CD40 ligand for 16 h with 500 ng/ml (R & D Systems, Wiesbaden, Germany). For stimulation with cyclic monophosphate nucleotides we used 8-bromoguanosine 3′,5′-cyclic monophosphate and 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (Sigma, Taufkirchen, Germany) for 16 h at concentrations of 100 μM.

2.2. Genomic array analyses

Affymetrix SNP 6.0 datasets used for genome wide copy number analysis of HL cell lines HDLM-2 and L-1236 were obtained from the Gene Expression Omnibus data base of the National Center for Biotechnology information NCBI-GEO (accession code GSM381297 for L-1236, GSM381298 for HDLM-2), http://www.ncbi.nlm.nih.gov/geo/ [15]. The CEL-files were downloaded and copy number analyses were performed using the Affymetrix Genotyping Console GTC Software version 4.0 (Affymetrix, High Wycombe, UK) and visualized by the Affymetrix GTC-Browser program. Genomic array data for HL cell line L-540 were obtained from the Sanger Centre, Hinxton, UK (http://www.sanger.ac.uk/cgi-bin/genetics/CGP/cghviewer/CghViewer.cgi).

For genomic array analysis via Agilent 180K-arrays the preparation of genomic DNA was performed using the DNeasy Blood and Tissue Kit (Qiagen). Genomic DNA of cell line SUP-HD1 and of reference human male DNA (Promega, Mannheim, Germany) was labelled with Cy3 and Cy5, respectively, using the Genomic DNA ULS labelling kit (Agilent, Waldbronn, Germany). Genomic copy number calculation was performed using SurePrint G3 Human CGH 4 × 180 K arrays (Agilent). Hybridization and washing of the arrays was performed according to the protocol of the manufacturer. Data analyses were performed using the software Genomic Workbench Lite Edition, version 6.5 (Agilent).

2.3. Chromosomal analyses

Fluorescent in situ-hybridization (FISH) analyses were performed as described recently [16]. Fosmid-clones and Rp11-BAC-clones were obtained from the Childrenïs Hospital Oakland Research Institute (California, USA), prepared using the Big BAC DNA Kit (Princeton Separations, Adelphia, New Jersey, USA) and directly labelled by nick translation with dUTP-fluors (Dyomics, Jena, Germany). Fluorescence images were captured using an Axioskop 2 plus microscope (Zeiss, Göttingen, Germany) and analyzed with Cytovision 2 software (Genetix, Newcastle, UK).

2.4. Polymerase chain-reaction (PCR) analyses

Total RNA from cells was extracted using TRIzol reagent (Invitrogen, Karlsruhe, Germany). Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors using Lymphoprep (Axis Shield PoC AS, Oslo, Norway). The donors are coauthors of this manuscript and gave their blood by choice. Total RNA isolated from primary bone marrow (BM), representing a pool of 3 healthy donors was obtained from Clontech (Saint-Germain-en-Laye, France), and from CD19-positive B-cells isolated from PBMC using corresponding magnetic beads from Miltenyi Biotec (Bergisch Gladbach, Germany). cDNA was subsequently synthesized from 5 μg RNA by random priming, using Superscript II (Invitrogen). Genomic DNA used for PCR was prepared by the DNeasy Blood and Tissue Kit (Qiagen).

Reverse transcription (RT)-PCR and genomic PCR was performed using taqpol (Qiagen) and thermocycler TGradient (Biometra, Göttingen, Germany). Oligonucleotides were obtained from MWG Eurofins (Martinsried, Germany). Their sequences are listed in Suppl. Table 1. Restriction of the XBP1 PCR product was performed with *Pst*I according to the manufactors protocol (Thermo Scientific Fermentas, St. Leon-Rot, Germany)

Real-time quantitative expression analysis (RQ-PCR) was performed by the 7500 Real-time System, using commercial buffer and primer sets (Applied Biosystems, Darmstadt, Germany) or oligonucleotides as listed in Suppl. Table 1. For normalization of expression levels we used TBP, for normalization of copy numbers we used MEF2C. Quantitative analyses were performed in triplicate and repeated twice.

2.5. Protein analysis

Western blot analysis was performed by the semi-dry method. Proteins obtained from cell lysates were transferred onto nitrocellulose membranes (Bio-Rad, München, Germany) which were blocked with 5% bovine serum albumin (BSA) dissolved in phosphate-buffered-saline buffer (PBS). The following antibodies were obtained from Santa Cruz (Heidelberg, Germany): MSX1 (F-22), XBP1 (H6E5), ERK1 (K-23) and from Abcam (Cambridge, UK): H1C (ab17677).

2.6. Cloning procedures and reporter gene analysis

For creation of an expression construct for histone H1C we cloned an RT-PCR product obtained from L-1236 cDNA into the *Eco*RI site of vector pcDNA3. The sequences of the oligonucleotides are listed in Suppl. Table 1. The validity of the construct (pcDNA3-H1C) was confirmed by sequence analysis (MWG Eurofins).

For creation of the reporter gene constructs we combined a reporter with genomic fragments, containing binding sites for MSX1 or XBP1. We cloned genomic PCR products (oligonucleotides are listed in Suppl. Table 1) of the corresponding ZHX2 upstream region (regulator) and of the HOXA9 gene, comprising exon1-intron1-exon2 (reporter), into the *Hind*III/*Bam*HI and *Eco*RI sites, respectively, of the expression vector pcDNA3 downstream of the CMV enhancer. The validity of the constructs was confirmed by sequence analysis (MWG Eurofins). Taqman real-time PCR using the commercial HOXA9 assay quantifies the spliced reporter-transcript, corresponding to promoter activity [17]. A cotransfected luciferase construct served as transfection control, quantified by the Luciferase Assay System (Promega) using the luminometer Lumat LB9501 (Berthold Technologies, Bad Wildbad, Germany).

2.7. Expression profiling

For quantification of gene expression via profiling we used gene chips HG U133 Plus 2.0 obtained from Affymetrix (Buckinghamshire, UK). The analysis of transcription factor activity in L-1236 was performed by a cut-off of the profiling data: genes showing higher values than −3.0 were set as positively expressed (Suppl. Table 2). Chip-data analysis was performed as described recently [12]. Analysis of expression data was performed using Microsoft Excel and online programs. For creation of heat maps we used CLUSTER version 2.11 and TREEVIEW version 1.60 (http://rana.lbl.gov/EisenSoftware.htm).

3. Results

3.1. ZHX2 upstream deletion contains binding sites for MSX1 and XBP1

In accordance with genomic array data of HL cell line L-1236 [13], high resolution fluorescence in situ hybridization (FISH) analysis using adjacent fosmid clones confirmed the chromosomal breakpoint at 8q24 nearby ZHX2 as shown in Fig. 1. Depletion of the red signal on chromosome der(4) demonstrated loss of ZHX2 upstream material, mapping the chromosomal breakpoint at position 123.0 Mbp. This aberration deletes the distal part of the regulatory upstream region at one ZHX2 allele which may contain binding sites for transcriptional activator proteins. To identify such proteins we looked for transcription factor consensus sites located in the region between the mapped breakpoint and the neighboring gene HAS2 (Fig. 1). For this purpose we used online data obtained from the UCSC genome browser (http://genome.cse.ucsc.edu, release NCBI36/hg18). Accordingly, this region contains 53 potential binding sites of 40 distinct transcription factors (Suppl. Table 2). Expression profiling data indicated that 20/40 factors are expressed in L-1236 cells (Suppl. Table 2) and represent, therefore, potential ZHX2 regulators. These candidates included several factors described in B-cell lymphomagenesis e.g. CEBPB, MYC, OCT1 and REL. In the following we focused on two of these 20 factors, MSX1 and XBP1, the binding-sites of which are conserved between human and mouse (Suppl. Fig. 1), and which have been described in lymphatic malignancies [18,19].

3.2. MSX1 and H1C co-regulate ZHX2 expression

MSX1 is a family member of the homeobox transcription factors, regulating basic developmental processes in embryogenesis and the adult [20–22]. The expression of MSX1 in HL cell lines was determined by RQ-PCR and Western blot analysis (Fig. 2A). All cell lines except L-540 expressed MSX1 with varying intensities. The highest expression levels of both, RNA and protein were detected