

# **Nuclear repositioning of the non-translocated *HLXB9* allele in the leukaemia cell line GDM-1 harbouring a t(6;7)(q23;q36)**

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**Key Words:** gene nuclear repositioning; *MNX1* gene; *HLXB9* gene; isochores; LADs.

## **Abstract**

*Background/Aims.* Transcriptionally active and inactive topologically associated domains (TADs) occupy different areas in the cell nucleus, and chromosomal rearrangements relocating TADs could determine ectopic expression of the repositioned genes. In this study, we investigated the *HLXB9* gene in a myeloid leukaemia cell line, GDM-1, known to harbour a rearrangement involving the chromosome 7 with breakpoint distal to *HLXB9*, highly expressed in these cells.

*Methods.* We used fluorescence *in situ* hybridisation (FISH) to target the regions involved in the translocation and to distinguish the translocated chromosome from the non-translocated one in interphase nuclei.

*Results.* Two-dimensional (2D) analysis of the interphase FISH data indicated that the two *HLXB9* alleles had a different localisation in the cell nuclei, with the translocated allele consistently positioned in the nuclear periphery and the normal one in the more internal portion of the nucleus, known as the transcriptionally active compartment.

*Conclusion.* Our data may indicate that *HLXB9* transcripts in GDM-1 cell line do not arise from the allele located in the rearranged chromosome 7, suggesting that regulation of gene expression in cancer cells harbouring chromosomal translocations might be more complex than previously thought, paving the path to further investigations on mechanisms of gene expression.

## Introduction

For more than two decades, a significant proportion of genome research has focused on the understanding of the nuclear architecture, where genes and chromosomal regions are positioned according to a precise order that depends mostly on gene density. Seminal work showed that in the interphase nuclei, human chromosomes are arranged in the so-called chromosome territories [Croft et al., 1999; Cremer and Cremer, 2001], whose organization and gene distribution define nuclear compartments endowed with different structural and functional properties [Saccone et al., 2002; Federico et al., 2008; Bernà et al., 2012], with the transcriptionally active chromatin preferentially located more internally in the nucleus, whereas the transcriptionally inactive one, particularly the heterochromatin, occupies the nuclear or the nucleolar periphery [Andrulis et al., 1998; Lukasova et al., 2002; Foster and Bridger, 2005; Dekker et al., 2013; Mattout, et al., 2015; Bernardi, 2015]. The correct maintenance of the higher order chromatin structure is crucial for cellular health, and its alteration is an emerging factor in human cancer, including leukaemia [Bártová et al., 2000; Ballabio et al., 2009; Tosi et al., 2015].

In the past decade or so, the observations obtained using microscope imaging and analysis of FISH data were further supported by the introduction of chromosome conformation capture (3C) technique [Dekker et al., 2002] and its variants, such as the high-throughput chromosome conformation capture (Hi-C) [Lieberman-Aiden et al., 2009]. These methods allowed a better understanding of the complex interplay between different parts of the genome, their positioning within the nuclear space and how changes in the relative genomic DNA positioning can influence transcription. 3C based studies highlighted the presence, in the cell nucleus, of topologically associated domains (TADs), namely genomic DNA sequences identified by their large degree of reciprocal physical interactions and endowed with different properties. Transcriptionally active or inactive TADs are located in the more internal or in the more peripheral nuclear compartment respectively [Stevens et al., 2017], as previously defined by molecular cytogenetic methods. Moreover, TADs located at the periphery of the cell nucleus are endowed with relevant properties that favour association to the nuclear lamina (lamina associated domains or LADs) [Kind et al., 2015]

*HLXB9*, a gene mapping on chromosome 7q36.3, also known as *MNX1* (motor neuron and pancreas homeobox), belongs to the family of EHG homeobox genes which also includes *EN1*, *EN2*, *GBX1* and *GBX2* [Holland, 2001]. Human *HLXB9* is a gene of 12,801 bp, composed of 3 exons, and the corresponding protein is a transcription factor, HB9, of 401 aminoacids [Harrison et al., 1994; Wildenhain et al. 2012]. During development, HB9 controls pancreas and motoneuronal differentiation [Arber et al., 1999; Harrison et al., 1999]. The *HLXB9* gene is mutated in constitutional disorders such as the Currarino syndrome, characterized by anorectal malformations and sacral agenesis [Ross et al., 1998]. Physiologically, *HLXB9* is transiently over-expressed during the early

stages of *in vitro* neuronal-like differentiation of neuroblastoma cells and this overexpression is associated with *HLXB9* gene repositioning towards the nuclear interior [Leotta et al., 2014].

HB9 is selectively present at high levels in bone marrow CD34-positive cells. The differentiation of hematopoietic progenitor cells along lineage-specific lines is accompanied by a *HLXB9* down-regulation [Deguchi and Kehrl, 1991]. Interestingly, homeobox genes overexpression is well documented in leukaemia [Alharbi et al., 2013]. Indeed ectopic expression of *GBX2* has been shown in avian leukaemia, whereas *HLXB9* overexpression has been detected in acute myeloid leukaemia (AML) [Deguchi et al., 1993; Kowenz-Leutz et al., 1997; Beverloo et al., 2001]. In particular, overexpression of the *HLXB9* gene was found in association with its altered positioning in the nucleus of leukaemic cells carrying the t(7;12)(q36;p13) rearrangement [Ballabio et al., 2009]. Leukemia derived cell lines are a useful tool for the study of genome organisation in relation to gene expression in this pathology. In particular, the acute myeloid leukaemia derived cell line GDM-1 [Ben-Bassat et al., 1982] was already subject of investigations that showed over-expression of the *HLXB9* gene [Nagel et al., 2005]. This cell line harbours a translocation between chromosomes 6 and 7, precisely t(6;7)(q23;q36), with the breakpoint on chromosome 7 distal to *HLXB9* and upstream of *MYB* at 6q23, a gene often activated in leukaemia [Nagel et al., 2005; Mostafa Kamel et al., 2014]. The mechanism for *HLXB9* activation in the GDM-1 cell line is yet to be elucidated and so is the impact of ectopic overexpression of *HLXB9* in the pathogenesis of leukaemia. In the work here presented, we used dual colour interphase fluorescence *in situ* hybridisation (FISH) to obtain a map of the cell nucleus in which both alleles of *HLXB9* could be visualised and identified simultaneously. We observed that the derivative chromosome containing the translocated *HLXB9* allele maintained a peripheral position, whereas the non-translocated homolog was repositioned towards the nuclear interior.

## Materials and methods

### *Cell cultures*

Human leukaemia GDM-1 cell line (from “Biological bank and Cell Factory” IST, Genova, Italy - code number HTL01008) was grown at 37 °C and 5% CO<sub>2</sub>, in RPMI 1640 supplemented with foetal bovine serum (FBS) to a final concentration of 20%, 1% Penicillin/Streptomycin (P/S) and 1% L-Glutamine [Ben-Bassat et al., 1982].

Human lymphocytes were obtained from whole peripheral blood samples of healthy volunteers who signed the informed consent, and cell separation with Ficoll solution. Phytohaemagglutinin (PHA) stimulated lymphocytes were obtained by 72 hours culture in RPMI 1640 supplemented with 10% foetal bovine serum, 1% Penicillin/Streptomycin, 1% L-Glutamine, 3% PHA at 37 °C, with 5% CO<sub>2</sub>.

### *In situ hybridization on chromosomes and nuclei*

To prepare metaphase chromosomes, colcemid (0.05 µg/ml) was added to cell cultures 1 hour before harvesting. Then, cells were harvested in hypotonic solution (KCl 0.075 M) and fixed with methanol-acetic acid (3:1). Interphase nuclei were obtained using a protocol to preserve the 3D chromatin structure, as previously described [Leotta et al., 2014]. Briefly, cells were fixed in 4% paraformaldehyde in PBS for 10 min, washed in PBS, incubated in 0.5% Triton X-100 in PBS for 15 min, and then in 20% glycerol in PBS for 30 min. Cells were then frozen in liquid nitrogen and thawed at room temperature, washed in PBS, and finally incubated for 5 min in 0.1 N HCl.

Fluorescence *in situ* hybridization (FISH) experiments were performed using specific probes (Table 1) for loci flanking the breakpoint regions on chromosomes 6 and 7. One of the probes, PAC RP5-1121A15 (GenBank accession no. AC006357.5), contains the entire *HLXB9* gene, the other two probes are the PAC RP1-48H15 (kindly provided by Stephen Scherer and Jeff MacDonald Toronto Hospital for Sick Children, Canada) and RP11-474A9 (kindly provided by M. Rocchi, University of Bari, Italy), mapping at 7q36.3 and 6q24.3 respectively. Each probe DNA was extracted from bacteria using a commercial kit (Qiagen, Milan, Italy), digoxigenin or biotin-labelled by nick translation (Roche, Mannheim, Germany), and hybridized as previously described [Leotta et al., 2014]. Detection was carried out using rhodamine conjugated avidin (for biotin-labelled probes), and anti-digoxigenin secondary antibody conjugated with fluorescein (for digoxigenin-labelled probes).

### *Radial nuclear location analysis*

Radial nuclear location (RNL) of *HLXB9* gene was determined using the two dimensional (2D) FISH analysis as previously described [Federico et al., 2008]. Briefly, images of hybridised nuclei were randomly acquired using an epifluorescence microscopy (Olympus AX70) equipped with a CCD camera (COHU 4910 series), and recorded using MacProbe v4.3 software (Applied Imaging,

Newcastle, UK). Each hybridized nucleus was analysed using a dedicated software developed at the University of Catania [Federico et al., 2008] in order to obtain the RNL for each hybridization signal. This was the value defined by the ratio of its position relative to the nuclear radius (0 and 1 indicate the centre and the periphery of the nucleus, respectively). The assessment of RNL was then based on the statistical analysis of at least 300 hybridization signals from the randomly recorded nuclei on at least three different experiments for each probe. RNL was statistically defined as the median value  $\pm$  confidence interval (C.I.) of all the analysed hybridization signals. It was established, analysing a high number of probes [Federico et al., 2008; Ballabio et al. 2009; Federico et al., 2017 and other our unpublished data], that median values of 0.65 could be considered a landmark between loci located at the nuclear periphery and at the nuclear interior. Differences in the RNLs were statistically evaluated by two tails T-test. Statistical values and analyses were performed with Microsoft Excel and StatView softwares.

#### *Expression analysis and immunodetection*

*HLXB9* total RNA was extracted from GDM-1 cells using the TRI Reagent (SIGMA-ALDRICH, St. Louis, USA), according to the manufacturer's instructions, and used in Real Time quantitative PCR (RT-Q-PCR) experiments (StepOne, Applied Biosystems) with primers (SIGMA-ALDRICH, St. Louis, USA) specifically designed for target and control genes (Table 2) using One-step SYBR Green RT-Q-PCR mix (Invitrogen, Carlsbad, CA, USA). RT-Q-PCR experiments, performed according to the manufacturer instructions, were repeated at least three times. Data were analysed using the Ct value and comparison with the endogenous *ACTB* gene used as control. Total RNA extracted as above from whole blood samples, of healthy subjects, were used as negative control.

Experiments of indirect immunofluorescence (IIF) were performed as previously described [Maugeri et al., 2016]. Briefly, GDM-1 and control cells were fixed with 4% paraformaldehyde for 20 min at room temperature, washed with PBS, incubated 15 min in PBS containing 0.5% Triton X-100, and for 20 min in blocking solution. The cells were then incubated with HB9 antibody (Sigma-Aldrich, 1:100 dilution) overnight at 37°C. After PBS/tween-20 washes, the cells were incubated for 1 hour with anti-rabbit secondary antibody conjugated with FITC (Invitrogen, 1:100 dilution) at 37°C, washed in PBS/tween-20, dehydrated in alcohol and stained with DAPI. Images were captured using a Confocal Laser Scanning microscopy CLSM (Zeiss LSM 700) equipped with the ZEN2011 software.

## Results

GDM-1 cells were previously shown to overexpress *HLXB9* gene [Nagel et al., 2005]. This was confirmed in the present work by RT-Q-PCR and by IIF that showed high levels of *HLXB9* transcript as well as high amount of HB9 protein (Fig. 1). In addition, GDM-1 cell line validation was obtained by identification of the typical chromosomal translocation t(6;7)(q23;q36), present in these cells, by karyotype analysis (data not shown) and by FISH results with probes specific for this chromosomal translocation (see Fig. 2).

RNL of the *HLXB9* gene has been assessed in the leukaemia derived cell line GDM-1 using dual colour FISH with probes that consented discrimination of the two alleles in the nuclei hybridized (Fig. 2). We used the digoxigenin-labelled PAC RP5-1121A15 (containing *HLXB9*) in combination with biotin-labelled BAC RP11-474A9 close to the breakpoint on chromosome 6 (probe set-A), or with the PAC RP1-48H15 telomeric to *HLXB9* on chromosome 7 (probe set-B) (Fig. 2). This latter probe, not previously described, is telomeric respect to the *HLXB9/MNX1* gene and is precisely detailed in Fig. 2E and in Table. 1. Using these specific probes, it was possible to distinguish the *HLXB9* wild-type allele (*HLXB9* in normal chr7) from the one located on the translocated chromosome, the der(7), making it possible to obtain a specific RNL for each of the two alleles by 2D analysis (Fig. 3).

Median values obtained for *HLXB9* allele on the der(7) were 0.663 and 0.652, with the probe set-A and the probe set-B respectively. For the *HLXB9* allele in the non-translocated chromosome 7 the median values were 0.595 and 0.610, with the probe set-A and the probe set-B respectively. The shift, in the cell nucleus of the GDM-1 cells, of the *HLXB9* allele (present in the not rearranged chromosome 7) from a peripheral location (RNL median value higher than 0.65) to a more internal one (RNL median value close to 0.60) is statistically highly significant ( $P < 0.001$ ) (Fig. 3). This is also schematically visualised in the cartoon presented in Fig. 4. It should be stressed that the RNL of the *HLXB9* located on the der(7) is similar to RNL of the gene in the nuclei of PHA-induced lymphocytes used as controls (Fig. 3) and is different from the one located on the non-rearranged chromosome 7.

Analysis of the genomic features of the terminal q arm of chromosomes 6 and 7, showed that the segment of chromosome 6 translocated onto chromosome 7 contains a large number of LADs. Moreover the rearrangement determines a juxtaposition of a GC-poor region of chromosome 6 with a very GC-rich region of chromosome 7 (Fig. 5), where the *HLXB9* gene is located.

## Discussion

Our previous studies showed that ectopic overexpression of the homeobox gene *HLXB9* was driven by the t(7;12)(q36;p13) rearrangement found in infant acute myeloid leukaemia. This chromosomal translocation implied a repositioning of the translocated *HLXB9* allele to a more internal area of the cell nucleus, where transcription is generally more active [Ballabio et al., 2009]. Using the myeloid leukaemia derived cell line GDM-1, also known to overexpress *HLXB9* [Nagel et al., 2005], we investigated whether nuclear repositioning of *HLXB9* was also detectable. GDM-1 karyotype was reported to contain a translocation between chromosome 6 and chromosome 7, precisely a t(6;7)(q23;q36), with chromosome 7 breakpoint distal to the *HLXB9* gene at 7q36.3 [Mostafa Kamel et al., 2014; Nagel et al., 2005]. Although both t(7;12) and t(6;7) rearrangements are associated with acute myeloid leukaemia and disrupt chromosome 7 in the 7q36 region, the breakpoints are slightly different and map precisely proximal, in the t(7;12) leukaemias, and distal, in the t(6;7) observed in GDM-1, to the *HLXB9* gene. In both cases *HLXB9* itself is not disrupted by the translocation, but ends up close to a gene with potential to drive transcription, that is *ETV6* on chromosome 12 or *MYB* on chromosome 6. However, we could speculate that *HLXB9* transcripts in these two leukaemia scenarios might have different origin.

Through radial nuclear localisation, it was deduced that the translocated *HLXB9* allele in the t(7;12) leukaemia cells was responsible for transcription, because it was repositioned to a more internal area of the cell nucleus, where transcription is more likely to occur. Similar RNL studies on GDM-1 presented here, showed a more interior localisation of the non-translocated *HLXB9* allele. The rearranged chromosome 7 remains in the same peripheral nuclear area where the *HLXB9* gene is physiologically located and transcriptionally inactive, in the PHA-stimulated lymphocytes [Federico et al., 2008; Ballabio et al., 2009]. Moreover, the chromosome 6 segment (about 36 Mb of genomic DNA) translocated to the telomeric end of the chromosome 7, is very GC-poor and contains a large number of LADs (see Fig. 5). Thus, the translocation of this chromosomal segment could be responsible for the preservation of the peripheral location of *HLXB9*.

These findings may suggest that there are different ways to instigate transcription arising from a chromosomal translocation. Traditionally, gene overexpression due to juxtaposition of an active one is a widely accepted mechanism in cancer biology. However, one could suppose that in GDM-1 gene regulation may be somehow driven by the der(7) via activation of the *HLXB9* gene located on the non-translocated chromosome 7. This might imply that the chromosomal rearrangement had a broader effect on the genome organisation and not only an ectopic interaction between elements *in cis*. It is also possible that an alteration of the nuclear architecture due to the presence of additional chromosomal abnormalities could be inducive to altered expression patterns, including *HLXB9* overexpression. Altogether this study has generated few more questions on the role of *HLXB9* in leukaemogenesis, opening new avenues for research on the topic. Further epigenetics studies would



clarify methylation patterns responsible for gene silencing. Additionally, chromosome conformation capture methods would help understanding the physical interactions between distal regulatory elements and promoters hopefully elucidating the role, if any, of *MYB* in the activation of *HLXB9*.

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**TABLE 1.** List of probes used in FISH experiments

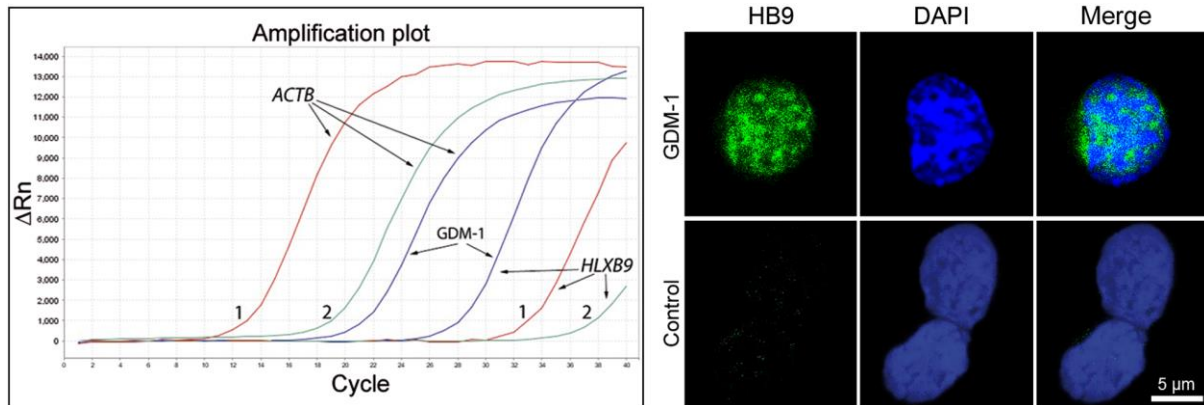
<i>Probe</i>	<i>Vector type</i>	<i>Chr. band</i>	<i>Start<sup>(a)</sup></i>	<i>End<sup>(a)</sup></i>	<i>Length, bp</i>
RP5-1121A15	PAC	7q36.3	156,689,179	156,819,479	130,301
RP1-48H15	PAC	7q36.3	156,805,535	156,877,583	72,049
RP11-474A9	BAC	6q24.3	145,609,951	145,804,203	194,253

<sup>(a)</sup>Positional data are from the Human Genome Assembly hg19 (GRCh37, Feb. 2009).

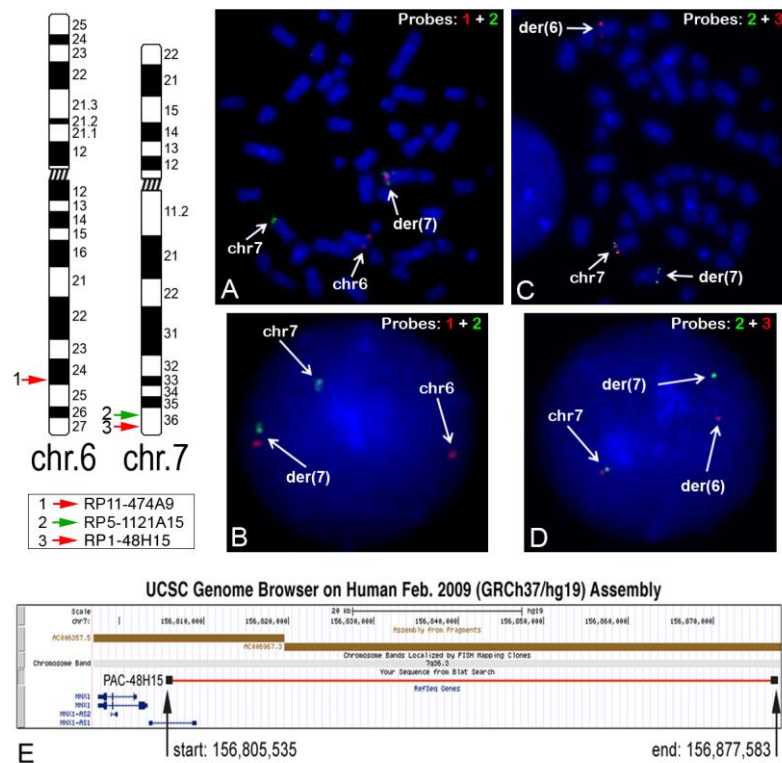
**TABLE 2.** PCR primers used in the present study

<i>Human Gene</i>	<i>PCR segment Size</i>	<i>Nucleotide sequence (5'-3')</i>	
<i>HLXB9</i>	98 bp	Forward	GTTCAAGCTCAACAAGTACC
		Reverse	GGTTCTGGAACCAAATCTTC
<i>ACTB</i>	131 bp	Forward	GACGACATGGAGAAAATCTG
		Reverse	ATGATCTGGGTCATCTTCTC

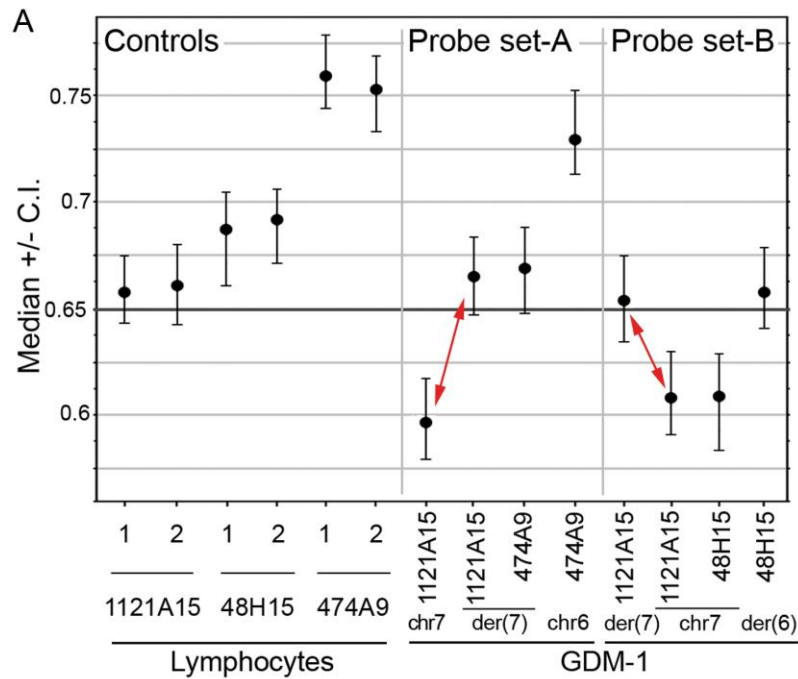
## FIGURE LEGENDS



**Fig. 1. Expression of the *HLXB9* gene in GDM-1 cell line.** Left: RT-Q-PCR showing the relative expression of *HLXB9* gene in the GDM-1 cells compared to the internal control *ACTB* gene. 1 and 2 show data from two negative controls (total RNA from human peripheral blood). *Right*: IIF of the HB9 protein (green signals), in the GDM-1 (upper part) and negative control (bottom part) cells. Negative controls are cells from human peripheral whole blood. Nuclei were counterstained with DAPI (blue).



**Fig. 2. Localization of *HLXB9* alleles in chromosomes and nuclei of the GDM-1 cell line.** *Upper left:* ideograms of the human chromosomes 6 and 7 indicating the position of the probes used in this study. Probe no. 1 (RP11-474A9) is telomeric to the breakpoint on chromosome 6 and probes no. 2 (RP5-1121A15, containing the entire *HLXB9/MNX1* gene) and no. 3 (RP1-48H15) flank the breakpoint on chromosome 7. A and B: representative metaphase and nucleus hybridized in the GDM-1 cells with probes no. 1 and no. 2 (probe set-A) detected with rhodamine (red) and fluorescein (green) respectively. C and D: representative metaphase and nucleus hybridized with probes no. 2 and no. 3 (probe set-B) detected with fluorescein (green) and rhodamine (red) respectively. Chromosomes and nuclei were stained with DAPI (blue). E: description of the PAC probe RP1-48H15, not previously published and not present in public databases. PAC RP1-48H15 was precisely positioned in the hg19 assembly by localization of the end sequences (described in the additional file-1). RP5-1121A15 (AC006357.5) and RP1-48H15 show an overlapping region of 13.9 kb. Further data and the format used are from UCSC genome browser (<http://genome.ucsc.edu/>).

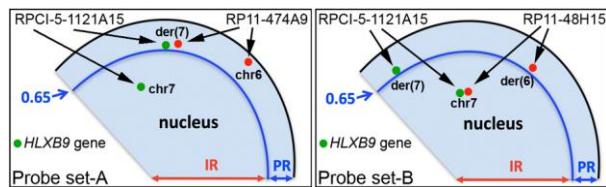


**B**

		Lymphocytes			GDM-1								
		Subject-1			Probe set-A				Probe set-B				
Probe		1121A15	48H15	474A9	1125A15	1125A15	474A9	474A9	1125A15	1125A15	48H15	48H15	
chr		chr7	chr7	chr6	chr7	der(7)	der(7)	chr6	der(7)	chr7	chr7	der(6)	
Lymphocytes	Subject-1	1121A15	chr7	NS	< 0.0001	< 0.0001	NS	NS	< 0.0001	NS	< 0.001	< 0.01	NS
		48H15	chr7		< 0.0001	< 0.0001	NS	NS	< 0.0001	NS	< 0.001	< 0.005	NS
		474A9	chr6		< 0.0001	< 0.0001	< 0.001	NS	< 0.001	< 0.0001	< 0.0001	< 0.001	
GDM-1	Probe set-A	1125A15	chr7			<u>&lt; 0.0001</u>	< 0.0001	< 0.0001	< 0.0001	NS	NS	< 0.0001	
		1125A15	der(7)					NS	< 0.001	NS	< 0.0001	< 0.005	NS
		474A9	der(7)						< 0.005	NS	< 0.0001	< 0.005	NS
		474A9	chr6							< 0.005	< 0.0001	< 0.0001	< 0.005
	Probe set-B	1125A15	der(7)								<u>&lt; 0.001</u>	< 0.005	NS
		1125A15	chr7								NS	< 0.0001	< 0.0001
		48H15	chr7										< 0.005
		48H15	der(6)										

**Fig. 3. Radial nuclear localization of *HLXB9* alleles.** A: median values (and the relative confidence interval, C.I.) of the nuclear location relative to the hybridised probes (see figure 2 for maps of probes on chromosomes). On the left side of the graph, 1 and 2 indicate lymphocyte samples from two different healthy subjects used as controls, whereas values relative to GDM-1 are on the right. Chr7 and der(7) indicate the chromosome 7, normal or rearranged respectively. The thickest line indicates the median value (0.65) roughly delimitating the peripheral/inner nuclear compartment. The double red arrows compare the values corresponding to *HLXB9* allele located in the normal and in the rearranged chromosome 7. B: Statistical significance (P values) of the differences in the RNL data obtained for each probe respect to the others. Two tails T-test were applied for all pairs of data and the obtained P value is indicated in the corresponding box. NS: statistically not-significant. The underlined values correspond to the comparison between the *HLXB9* alleles on the normal and the rearranged chromosome 7, and indicated by the arrows in the upper chart.





**Fig. 4. Schematic representation of RNL data shown in figure 3.** The location of RP5-1121A15 probe (containing the *HLXB9* gene) in the non-translocated chromosome 7 (chr7) is always localised more internally in the nucleus, whereas the same probe on the der(7) is consistently localised in a more peripheral area of the nucleus. IR, and PR: internal and peripheral nuclear region respectively.



**ADDITIONAL FILE-1**

Additional information on PAC RPCI-5-48H15

Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly

Start sequence

tttccctcccacattgcctastaraggttctccatgagggctctgcccctgcagtggayttctgctt  
ggacattcaggccttttcatacaacctctgaaattaggkkggaggctcccaagcctcaactcttgccc  
yctgggaacccacaggcttaacaccatgtggaagccatcatagctkgkkgcttgcmcccyctgagcas  
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ctgasgcaggagga

End sequence

gggtttcaccatgtkggctcmggctggtytkamytcccagacctcaggtgatccaccacttcagcctc  
ccaaagkgctgggattacaggcatgagcgaccgtgcccgggctatcatctcattttctagagtacaa  
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