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MicroRNA-223 is a novel negative regulator of HSP90B1 in CLL

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Abstract

Background: MicroRNAs are known to inhibit gene expression by binding to the 3'UTR of the target transcript. Downregulation of miR-223 has been recently reported to have prognostic significance in CLL. However, there is no evidence of the pathogenetic mechanism of this miRNA in CLL patients.

Methods: By applying next-generation sequencing techniques we have detected a common polymorphism (rs2307842), in 24% of CLL patients, which disrupts the binding site for miR-223 in *HSP90B1* 3'UTR. We investigated whether miR-223 directly targets *HSP90B1* through luciferase assays and ectopic expression of miR-223. Quantitative real-time polymerase chain reaction and western blot were used to determine *HSP90B1* expression in CLL patients. The relationship between rs2307842 status, *HSP90B1* expression and clinico-biological data were assessed.

Results: *HSP90B1* is a direct target for miR-223 by interaction with the putative miR-223 binding site. The analysis in paired samples (CD19+ fraction cell and non-CD19+ fraction cell) showed that the presence of rs2307842 and IGHV unmutated genes determined *HSP90B1* overexpression in B lymphocytes from CLL patients. These results were confirmed at the protein level by western blot. Of note, *HSP90B1* overexpression was independently predictive of shorter time to the first therapy in CLL patients. By contrast, the presence of rs2307842 was not related to the outcome.

Conclusions: *HSP90B1* is a direct target gene of miR-223. Our results provide a plausible explanation of why CLL patients harboring miR-223 downregulation are associated with a poor outcome, pointing out *HSP90B1* as a new pathogenetic mechanism in CLL and a promising therapeutic target.

Keywords: Chronic lymphocytic leukemia, MicroRNAs, Next-generation sequencing

Background

MicroRNAs (miRNAs) are endogenously expressed small RNA molecules that mediate posttranscriptional gene silencing through complimentary binding of the 3' untranslated regions (3'UTR) of target genes [1]. Over half of the human transcriptome is predicted to be under miRNA regulation, embedding this post-transcriptional control pathway within nearly every biological process [2-4]. Thus, miRNAs are involved in almost all aspects of cancer biology, such as proliferation, apoptosis, invasion/metastasis, and angiogenesis [5].

Over the past few years several studies have shown that miRNAs play an important role in CLL [6-9]. Distinct microRNA signatures are associated with prognosis, disease progression [9-14] and response to treatment [15,16]. In CLL, the downregulation of miR-223 is associated with disease aggressiveness and poor prognostic factors [13,14], which may become this miRNA a new reliable prognostic predictor. However, unlike other miRNAs with prognostic value in CLL such as miR-181b and miR-29c, there is no evidence of its pathogenetic role, and no target has so far been proposed or validated for miR-223 in CLL.

Over the last decade, several studies have implicated heat shock proteins (HSPs) as major contributors to cancer progression and the development of chemoresistance. HSPs are upregulated in many cancers, including CLL, and may

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contribute to prolonged tumor cell survival via several mechanisms that remain to be fully described [17-19]. Pre-clinical studies in CLL have shown that *HSP90* inhibition causes the degradation of ZAP-70 and other proteins associated with poor survival, and this may ultimately lead to apoptosis [20-24]. Targeting *HSP90* is an attractive strategy in CLL as this could represent a therapeutic option to drug resistance in CLL associated with lesions in the *ATM/TP53* pathway [25-27]. Thus, inhibitors of *HSP90* have been proposed as a novel therapeutic option for CLL [28-30].

By applying next-generation sequencing (NGS) techniques we have detected a common polymorphism (rs2307842), in 24% of CLL patients, which disrupts the binding site for miR-223 in *HSP90B1* 3'UTR, leading to its overexpression in clonal B lymphocytes. This finding has helped us to identify miR-223 as a regulator of *HSP90B1* levels in CLL patients, with therapeutic consequences.

Methods

Patients and controls

Four patients with CLL were selected for a Targeted Sequence Capture and DNA Sequencing assay. CLL diagnosis was performed according to World Health Organization (WHO) classification [31] and Working Group of National Cancer Institute (NCI) criteria [32]. CD19+ fraction cells were used for sequencing and were obtained before administration of any treatment. To determine the clinical impact of *HSP90B1* 3'UTR polymorphism, we expanded the study to 165 additional patients with CLL and 32 healthy controls. FISH studies and *IGHV* mutational status were assessed. Details on the main characteristics of the 169 CLL patients included in the study are reported in Table 1 and Additional file 1: Supplementary Methods. The study was approved by the local ethical committee "Comité Ético de Investigación Clínica, Hospital Universitario de Salamanca". Written informed consent was obtained from each patient before they entered the study.

Cells and culture conditions

The human cell lines NCI-H929 and MM1S were acquired from the ATCC (American Type Culture Collection). Cell lines identity was confirmed periodically by STR analysis, PowerPlex 16 HS System kit (www.promega.com) and online STR matching analysis (www.dsmz.de/fp/cgi-bin/str.html). The human STR profile database includes data sets of 2455 cell lines from ATCC, DSMZ, JCRB and RIKEN. Both cell lines were cultured in RPMI 1640 medium supplemented with 10% of fetal bovine serum and antibiotics (Gibco). Cells were routinely checked for the presence of mycoplasma with MycoAlert kit (Lonza GmbH) and only mycoplasma-free cells were used in the experiments. The phenotypic and cytogenetic identities of the cell

Table 1 Clinical and biological features of the CLL patients included in the study

Parameter	Category	
Age (years), median (range)		66 (34-90)
Gender	Male	66.0%
White blood cells/mL (range)		21 545 (7 080-188 020)
Lymphocytes/mL (range)		15 741 (1 580-180 000)
Hemoglobin, g/dL (range)		14.1 (4.4-16.8)
Platelet count/mL (range)		171 500 (23 000-399 000)
<i>IGHV</i>	Unmutated	50.3%
Binet stage	A	65.9%
	B	23.2%
	C	10.9%
LDH	Normal	81.6%
	High	18.4%
b ₂ microglobulin	Normal	55.9%
	High	44.1%
Bone marrow pattern	Diffuse	41.9%
	Other	58.1%
Hepatomegaly	Yes	10.5%
	No	89.5%
Splenomegaly	Yes	26.5%
	No	73.5%
B symptoms	Yes	13.5%
	No	86.5%
Dead during follow-up	Yes	21.6%
	No	78.4%
Therapy during follow-up	Yes	45.7%
	No	78.4%

Results expressed as median or percentages.

IGHV: immunoglobulin heavy variable gene; LDH: lactate dehydrogenase.

lines were verified by flow cytometry and FISH before the experiments.

Details on collection and preparation of patients and cell culture samples are available in Additional file 1: Supplementary Methods.

Targeted sequence capture and DNA sequencing assays

We applied array-based sequence capture (Roche NimbleGen) followed by next-generation sequencing (Roche GS FLX Titanium sequencing platform) to analyze a large panel of genes of relevance in CLL (Additional file 2: Table S1) and two chromosomal regions: 13q14.3 (50043128–50382849 bp) and 17p13.1 (7500000–7535000). The genes had been selected according to published data and our previous gene expression data and included, for example *HSP90B1*, *TP53*, *ATM*, *PHLPP1*, *E2F1*, *RAPGEF2* and *PI3K*. Pyrosequencing assays were performed to analyze the sequence for 3'UTR

region of the *HSP90B1* gene. Details of the design of the array, 454 sequencing, coverage statistics and data analysis, as well as the pyrosequencing assays are provided in the Additional file 1: Supplementary Methods and Additional file 2: Table S2. The sequencing data are uploaded to the Sequence Read Archive (SRA) (<http://trace.ncbi.nlm.nih.gov/Traces/sra/>) under accession number PRJNA275978. All the information is accessible with the following link <http://www.ncbi.nlm.nih.gov/bioproject/275978>.

Luciferase reporter assay

HEK293 cells were transfected with 500 ng of the constructs detailed in the Additional file 1: Supplementary Methods and Additional file 2: Table S3, and cotransfected with 25 nM miRNA precursor molecule by nucleofection, using the HEK293 cell line program in the Amaxa II nucleofactor system. Cells were collected 24 hours after transfection and Firefly and Renilla luciferase activities were measured using the Dual-Glo® Luciferase Assay System (Promega) according to the manufacturer's protocol. Measurements were performed on a Tekan Infinite® F500 microplate reader. Firefly luciferase activity was normalized with respect to Renilla luciferase activity.

Transfection with synthetic miRNAs

H929 and MM1S cell lines were transfected with Pre-miR™ miRNA precursors pre-miR-223 or pre-miR™ miRNA-negative, non-targeting control#1 (Ambion) at 50 nM concentration, using the nucleofactor II system with C-16 program and Q-023 program, respectively (Amaxa). Transfection efficiency was assessed with Block-iT™ Fluorescent Oligo (Invitrogen) by flow cytometry.

Quantitative real-time polymerase chain reaction analysis and Immunoblotting

This methodology is provided in Additional file 1: Supplementary Methods.

Statistical analysis

Statistical analysis was performed using SPSS (v20). The two-sided Student's *t* test was used to analyze differences between means (presented here with SD) of different experiments, based on triplicate determinations. Differences between the results of the qRT-PCR experiments with CLL patients were analyzed with the Mann-Whitney *U*. Kaplan-Meier analysis with the Log Rank test and Cox regression were used for survival analysis examining the impact of *HSP90B1* expression on OS and TFT. Chi-squared and Mann-Whitney *U* tests were employed when appropriate to correlate a range of biomarkers and clinical data according to rs2307842 status and *HSP90B1* expression. The results were considered statistically significant at $P < 0.05$.

Results

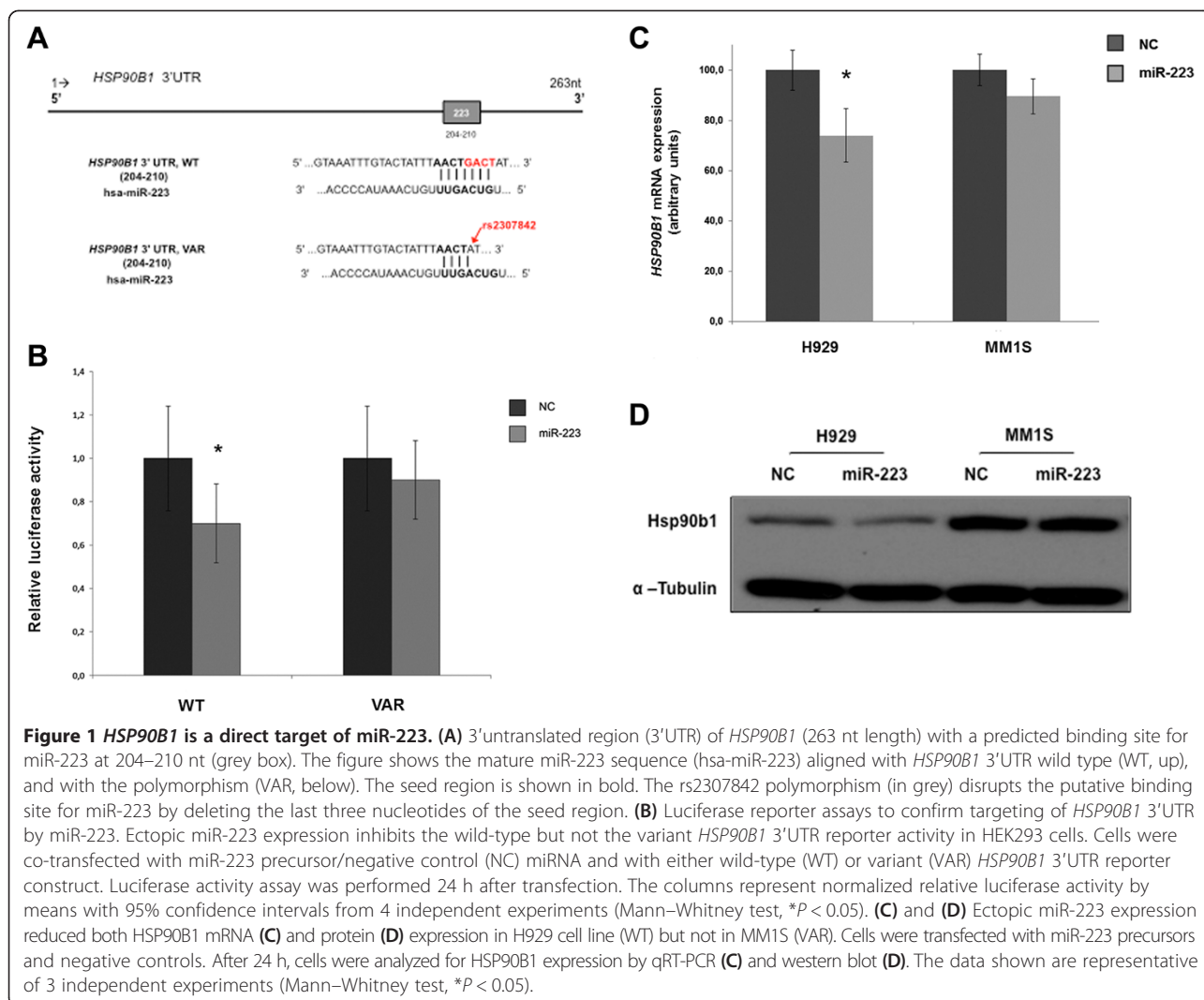
A targeted genome capture and next-generation sequencing strategy identifies a common polymorphism in 3'UTR of *HSP90B1*

Using a custom NimbleGen array we captured and sequenced 93 genes and two entire chromosomal regions of four CLL patients. The enrichment assay followed by NGS allowed the detection of over 1600 variations/sample (median 1721, range 1618–1823). All putative variants were first compared with published single nucleotide polymorphism (SNP) data (dbSNP build 130; <http://www.ncbi.nlm.nih.gov/projects/SNP>). Most of the variants detected were identified as known SNPs and 226 variants were present in all the patients, so these were discarded. Overall, 10% of variants detected in each sample were not previously described mutations. Seventy-three missense variations affecting 33 genes were detected. Most of the genes had one (70%) or two (12%) variations. Results are summarized in Additional file 2: Table S4.

By applying a custom-made data analysis pipeline, we have annotated the detected variants, including reported single-nucleotide polymorphisms (SNPs), genomic location, predicted miRNA binding sites, consequences of the variant in transcripts (i.e. synonymous, missense) and protein function prediction for those variants that are predicted to result in an amino acid substitution. In one out of four CLL patients (25%) we identified a 4-bp insertion/deletion polymorphism (–/GACT) in 3'UTR of *HSP90B1*, filled as rs2307842 (102865778–102865781b) in the NCBI SNP database. Rs2307842 results in the deletion of four nucleotides in 3'UTR sequence, three of them being part of the predicted binding site for miR-223 (Figure 1A). According to the databases, UCSC Genome Browser, NCBI and Ensembl, the reference genome contains the 'GACT' sequence. The major allele in the European population, according to the NCBI SNP database, is 'GACT' (allele frequency: 0.79 ± 0.06), whereas the 4-bp deletion has a minor allele frequency of 0.21 ± 0.06 . Thus, we considered the individuals carrying the 'GACT' sequence as *wild-type* (WT) and the individuals with the 4 bp-deletion as *variants* (VAR). We hypothesized that this deletion disrupts the binding site for miR-223, thereby increasing the translation of *HSP90B1*.

HSP90B1 is a direct target gene of miR-223

We have confirmed that miR-223 regulates *HSP90B1* expression by 3'UTR reporter assays. First, the double-stranded oligonucleotides, corresponding to the wild-type (WT-3'UTR) or variant (VAR-3'UTR) miR-223 binding site in the 3'UTR of *HSP90B1* (NM_003299), were synthesized. PmirGLO Vectors made up of an SV40 promoter, the Renilla luciferase gene, and the 3'UTRs of *HSP90B1* were transfected into HEK293 cells along with miR-223 or negative control (NC) mimics. Relative luciferase activity



was measured at 24 h. The relative luciferase activity of the construct with wild-type 3'UTR was significantly repressed following miR-223 transfection ($P < 0.05$) (Figure 1B). However, the presence of rs2307842 polymorphism in 3'UTR of *HSP90B1* (VAR-3'UTR) abolished this suppression (Figure 1B), suggesting that miR-223 directly binds to this site.

We also validated *HSP90B1* as a target gene of miR-223 by transfecting MM1S and H929 cell lines with miR-223/NC mimics and then measuring *HSP90B1* expression by qRT-PCR and western blot. Sequencing assays showed that H929 cell line has WT-3'UTR, whereas rs2307842 polymorphism was present in *HSP90B1* 3'UTR of MM1S cell line (VAR-3'UTR). All experiments were done in triplicate. Exogenous expression of miR-223 downregulated the expression levels of *HSP90B1* in H929 cell line (WT-3'UTR) in both mRNA ($P < 0.05$) and protein levels (Figure 1C and D). By contrast, *HSP90B1* expression

was not modified in the MM1S cell line (VAR-3'UTR) (Figure 1C and D). Taken together, all these results demonstrate that *HSP90B1* is a *bona fide* target gene of miR-223 and that the rs2307842 polymorphism abolishes the miR-223 regulation on *HSP90B1* expression.

rs2307842 is a common polymorphism in CLL patients

To determine the clinical impact of *HSP90B1* 3'UTR polymorphism in CLL, we screened 165 additional patients with CLL and 32 healthy controls for this polymorphism by pyrosequencing. A total of 50 paired DNA samples (CD19+ and non-CD19+ fraction cells) immunomagnetically purified from CLL patients showed complete concordance in their 3'UTR sequence, confirming that rs2307842 was the result of a SNP and not an acquired mutation. The polymorphism was found at a similar frequency in CLLs and healthy controls: 41/169 (24%) in CLL patients and 8/32 (25%) in healthy controls. These results are consistent with the

data obtained from NCBI SNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP>). Of note, no major differences regarding clinical, biological and genetic features were found between CLLs cases with the polymorphism (VAR) and wild-type (WT) (Additional file 2: Table S5).

miR-223 is downregulated in CLL patients with IGHV unmutated genes

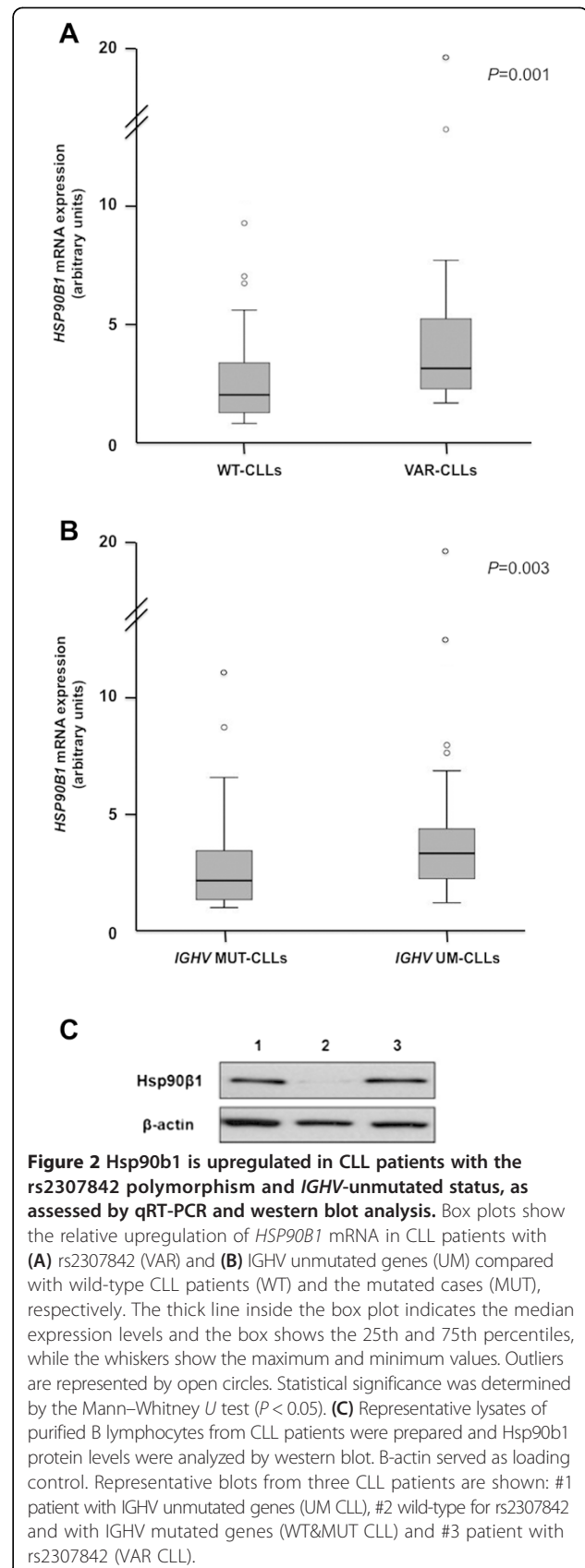
In order to corroborate the down-regulation of miR-223 previously reported in CLL patients with IGHV unmutated (UM) genes, 53 samples were subjected to miRNA Taqman qRT-PCR to measure miR-223 expression according to IGHV mutation status. As expected, miR-223 was down-regulated in UM CLL patients when compared to mutated IGHV cases ($P = 0.036$).

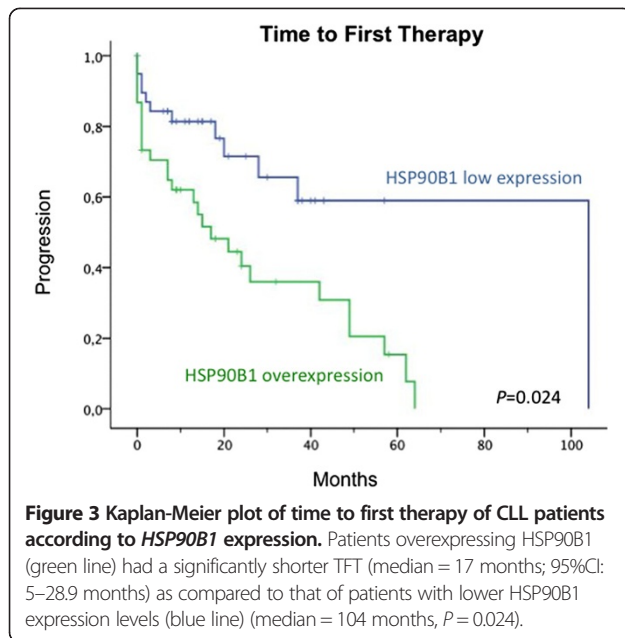
HSP90B1 overexpression is observed in B lymphocytes from CLL patients with the rs2307842 polymorphism and IGHV-unmutated status

To test the hypothesis that *HSP90B1* overexpression may be due to a defective miR-223 regulation in CLL patients, we analyzed *HSP90B1* expression in a subgroup of patients previously characterized for the presence of the polymorphism and IGHV mutation status.

We have performed qRT-PCR in a total of 97 CLL samples: 25 out of them were CLL patients with rs2307842 (VAR-CLLs) and 72 were wild-type (WT-CLLs). qRT-PCR results showed that *HSP90B1* was overexpressed in VAR-CLLs ($P = 0.001$) (Figure 2A). To gain insight into its influence on gene expression, we have measured *HSP90B1* mRNA levels in the paired normal fraction of 50 cases (13 VAR-CLLs and 37 WT-CLLs). As expected, the results showed that B lymphocytes (tumor fraction) from VAR-CLLs showed a higher level of *HSP90B1* expression than B lymphocytes from WT-CLLs ($P = 0.001$), and also from the normal cells from the same patients (VAR-CLLs) ($P < 0.001$) (Additional file 3: Figure S1). However, no changes in *HSP90B1* mRNA expression were observed between tumor and normal fractions in CLLs without the SNP ($P = 0.201$). Thus, rs2307842 influenced *HSP90B1* overexpression only in the tumor fraction of the CLL patients with the polymorphism. Of note, we also observed overexpression of *HSP90B1* in patients with Figure 2B). The overexpression was also confirmed in the tumor fraction of the purified paired samples (data not shown). IGHV unmutated genes (UM-CLLs, $n = 52$) in comparison with mutated cases (MUT-CLLs, $n = 45$) ($P = 0.003$) (Figure 2B).

Hsp90b1 protein expression was also measured by Western blot analysis in the B lymphocytes from CLL patients harboring the variant, unmutated IGHV genes and wild-type CLLs (Figure 2C). As expected, Hsp90b1 expression was higher in CLL with *HSP90B1* the SNP and in unmutated CLL.





HSP90B1 overexpression is associated with a shorter time to treatment

The relationship between clinical and biological characteristics of CLL patients and *HSP90B1* gene expression was analyzed. A higher *HSP90B1* mRNA expression was

correlated with the presence of rs2307842 ($P = 0.003$), unmutated status of the *IGHV* gene ($P = 0.008$) and need for treatment ($P = 0.001$) compared to that of patients with lower *HSP90B1* mRNA expression levels.

A significantly shorter time to first therapy (TFT) was observed in the patients with *HSP90B1* overexpression (median of 17 months; 95% CI: 5–28.9 months) as compared to those cases without *HSP90B1* overexpression (median of 104 months) ($p = 0.024$) (Figure 3). Thus, 71% of patients in the group with *HSP90B1* overexpression required treatment vs. 31% of patients in the non-overexpressed group. Other variables associated with shorter TFT were age, non-mutated *IGHV*, lymphocyte count, adverse cytogenetics and the presence of B symptoms (Table 2). Multivariate analysis selected *HSP90B1* overexpression as an independent risk factor of TFT (HR: 2.63; 95% CI: 1.15–5.98; $P = 0.021$), after adjusting for *IGHV* mutation status, lymphocyte count (< vs >30000), cytogenetics (good prognosis vs high-risk), age (< vs > 65 years) and the presence of B symptoms.

Discussion

MicroRNAs are known to inhibit gene expression by binding to the 3'UTR of the target transcript. In the present study *HSP90B1* was validated as a miR-223 direct target by 3'UTR reporter assays and transfection with synthetic miR-223 (Figure 1B and D). Thus *HSP90B1* was overexpressed

Table 2 Univariate and multivariate analysis for time to first therapy (TFT) in this series

Characteristics	Events	Total	Univariate analysis				Multivariate analysis				
			Median	LCI	UCI	<i>P</i>	HR	LCI	UCI	<i>P</i>	
HSP90B1 expression											
Normal	12	39	104.0	-	-	-	-	-	-	-	-
High	28	39	17.0	5.0	28.9	0.024	2.7	1.18	6.46	0.026	
IGHV identity											
<98%	15	60	104.0	11.3	196.7	-	-	-	-	-	-
≥98%	39	57	14.0	6.8	21.2	<0.001	2.34	1.03	5.35	0.043	
Lymphocyte											
<30000	35	90	53.0	35.1	70.8	-	-	-	-	-	-
≥30000	25	37	8.0	0.0	17.5	<0.001	4.2	1.75	10.05	0.001	
Cytogenetics											
Good prognosis	28	82	57.0	38.3	75.7	-	-	-	-	-	-
Poor prognosis	16	21	9.0	1.7	16.2	<0.001	1.65	1.907	2.54	0.023	
Age (years)											
≥65	29	71	42.0	18.3	65.7	-	-	-	-	-	-
<65	29	53	24.0	6.6	41.4	0.04	0.37	0.17	0.83	0.015	
B symptoms											
No	42	194	49.0	34.3	63.7	-	-	-	-	-	-
Yes	15	18	1.0	0.0	2.1	<0.001	0.17	0.06	0.53	0.002	

IGHV: immunoglobulin heavy variable gene; LCI: 95% lower confidence interval; UCI: 95% upper confidence interval; HR: Hazard ratio. Time to first therapy (TFT) was defined as the interval between diagnosis and the treatment.

in CLL patients harboring unmutated *IGHV* genes and rs2307842, a common polymorphism located in *HSP90B1* 3'UTR, which disrupts the binding site of miR-223. More importantly, *HSP90B1* overexpression was independently predictive of shorter time to the first therapy. We propose that this overexpression could represent a pathogenic mechanism for miR-223 in CLL.

Functional polymorphisms in 3'UTRs of several genes (also known as miRSNPs or miR-polymorphisms) are associated with diseases affecting gene expression. Loss of microRNA function due to defective miRNA-mRNA binding results in overexpression of the target mRNA, which can be involved in key biological processes, oncogenic mechanisms or drug resistance [33-36]. Moreover, the presence of some SNPs has been suggested to influence disease progression and clinical outcome in CLL [37-42], although the results are discrepant [43-46]. Our results showed that the presence of rs2307842, a common polymorphism located in the 3'UTR of *HSP90B1* (Figure 1A), alters the interaction between the target site in *HSP90B1* and miR-223 in CLL, resulting in *HSP90B1* overexpression (Figure 2A). However, no major differences regarding clinical, biological and genetic features were found between CLLs harbouring rs2307842 and wild-type cases (Additional file 2: Table S5).

We have also performed qRT-PCR using CD19+ peripheral blood lymphocytes from CLL patients displaying the polymorphism and wild-type cases (Additional file 3: Figure S1). As expected, B lymphocytes from CLL patients with the polymorphism had higher levels of *HSP90B1* than B lymphocytes from wild-type CLL patients. Surprisingly, non-clonal cells from CLL patients with the polymorphism showed levels of *HSP90B1* mRNA similar to that of wild-type CLL patients (both CD19+ and non-CD19+ fraction cells). These findings suggest that a regulatory mechanism of *HSP90B1* expression could be present in cells with rs2307842. Further work is needed to understand the relevance and functional consequences of this common polymorphism in CLL patients. Of note, our study shows that the presence of variants that alter the 3' UTR-site targeted by the miRNA could be an alternative mechanism to the presence of mutations inside or surrounding microRNA genetic loci.

Although miR-223 has been related to HSP90 in osteosarcoma [47], miR-223 function is not well characterized in CLL. However the expression levels significantly decrease with the progression of the disease and miR-223 downregulation has been associated with higher tumor burden, disease aggressiveness, and poor prognostic factors, such as *IGHV* unmutated genes (UM CLL) [8,13,14]. Despite the proven implication of miR-223 expression in CLL prognosis, little is known about the molecular mechanisms that may be responsible for the poor outcome of CLL patients showing miR-223 downregulation and,

unlike other miRNAs with prognostic value in CLL, such as miR-181b and miR-29c, the target of miR-223 in CLL is still unknown [48,49]. Our results confirmed the downregulation of miR-223 in *IGHV* UM CLLs. Moreover, the present results, demonstrating that *HSP90B1* is a direct target gene of miR-223, provide more information about how the downregulation of miR-223 could determine the poor outcome of *IGHV* UM CLLs, possibly by upregulation of *HSP90B1* expression (Figure 2B and C). Limited data are available regarding the expression of HSP90 in CLL. In myelodysplastic syndromes, high levels of HSP90 were associated with shorter survival and increased risk of progression into acute myeloid leukemia (AML) [50,51]. In AML, the percentage of HSP90-positive cells was correlated with that of Bcl2-positive cells and higher expression of HSPs was associated with lower complete remission rate and poor survival [52,53]. Of note, we also observed a correlation between *HSP90B1* and *BCL2* overexpression in CLL patients (data not shown). HSP90 has been proposed to have a role in the modulation of apoptosis and is implicated in the resistance of leukemic cells to chemotherapeutic agents and recent evidence suggests that HSP90 inhibitors such as 17-AAG and 17-DMAG [23], which have shown preclinical efficacy, could be a therapeutic option in CLL [25]. More importantly, our data suggest that *HSP90B1* overexpression is independently predictive of shorter time to first therapy in CLL (Table 2).

Conclusions

Our study highlights the relevance of miRNAs as critical players in the pathogenesis of CLL and shows for the first time that miR-223 modulates *HSP90B1* expression in B lymphocytes of CLL. These results provide a plausible explanation of why CLL patients harboring miR-223 downregulation are associated with a poor outcome. Our work also points out *HSP90B1* overexpression as a new pathogenic mechanism in CLL and a promising therapeutic target, at least in a subgroup of CLL patients.

Additional files

Additional file 1: Supplementary Method.

Additional file 2: Tables S1-S5.

Additional file 3: Figure S1. Differential expression of *HSP90B1* in purified paired samples (CD19+ and non-CD19) from CLL patients assessed by qRT-PCR analysis. Box plots show the relative upregulation of *HSP90B1* in B lymphocytes (CD19+ fraction cell, CD19+) from CLL patients with rs2307842 (VAR-CLLs) compared with the non-CD19+ fraction cell (non-CD19) from the same patients ($P < 0.001$) and the wild-type CLL patients (WT-CLLs) ($P = 0.001$). The thick line inside the box plot indicates the median expression levels and the box shows the 25th and 75th percentiles, while the whiskers show the maximum and minimum values. Outliers are represented by open circles. Statistical significance was determined by the Mann-Whitney U test ($P < 0.05$).

Abbreviations

3'UTR: 3'untranslated region; ATCC: American Type Culture Collection; Bp: Base pair; CI: Confidence interval; CLL: Chronic lymphocytic leukemia; FISH: Fluorescence in situ hybridization; HR: Hazard ratio; HSP: Heat shock protein; IGHV: Immunoglobulin heavy chain variable; miRNA: MicroRNA; MUT: Mutated; NCI: National Cancer Institute; NGS: Next generation sequencing; OS: Overall survival; SNP: Single nucleotide polymorphism; TFT: Time to first therapy; UM: Unmutated; VAR: Variant; Vs: Versus; WT: Wild type.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

A-ERV designed the research, collected and prepared the samples, performed qRT-PCR assays, helped analyze and interpret the data, and wrote the manuscript; DQ and IM performed the miRNA transfections and the luciferase and immunoblotting assays; RB and MHS designed and performed the sequencing assays; AGC, RF and J-MA provided patients' data; CZ, JFP and JMC performed the sequencing data analysis; M-ES performed the Taqman assays; J-LG, J-AH and N-CG collected data, participated in discussions and critically reviewed the manuscript; MG performed the IGHV mutational status analysis and critically reviewed the manuscript and J-MHR designed and supervised the study, did some of the research and wrote the manuscript. All authors read and approved the final manuscript.

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References

- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281–97.
- Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009;136(2):215–33.
- Bartel DP, Chen CZ. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat Rev Genet*. 2004;5(5):396–400.
- Lim LP, Lau NC, Garrett-Engle P, Grimson A, Schelter JM, Castle J, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*. 2005;433(7027):769–73.
- Lee YS, Dutta A. MicroRNAs in cancer. *Annu Rev Pathol*. 2009;4:199–227.
- Calin GA, Liu CG, Sevignani C, Ferracin M, Felli N, Dumitru CD, et al. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. *Proc Natl Acad Sci U S A*. 2004;101(32):11755–60.
- Barbarotto E, Calin GA. Potential therapeutic applications of miRNA-based technology in hematological malignancies. *Curr Pharm Des*. 2008;14(21):2040–50.
- Visone R, Rassenti LZ, Veronese A, Taccioli C, Costinean S, Aguda BD, et al. Karyotype-specific microRNA signature in chronic lymphocytic leukemia. *Blood*. 2009;114(18):3872–9.
- Rossi S, Shimizu M, Barbarotto E, Nicoloso MS, Dimitri F, Sampath D, et al. microRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. *Blood*. 2010;116(6):945–52.
- Fulci V, Chiaretti S, Goldoni M, Azzalin G, Carucci N, Tavolaro S, et al. Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. *Blood*. 2007;109(11):4944–51.
- Marton S, Garcia MR, Robello C, Persson H, Trajtenberg F, Pritsch O, et al. Small RNAs analysis in CLL reveals a deregulation of miRNA expression and novel miRNA candidates of putative relevance in CLL pathogenesis. *Leukemia*. 2008;22(2):330–8.
- Pallasch CP, Patz M, Park YJ, Hagist S, Eggle D, Claus R, et al. miRNA deregulation by epigenetic silencing disrupts suppression of the oncogene PLAG1 in chronic lymphocytic leukemia. *Blood*. 2009;114(15):3255–64.
- Stamatopoulos B, Meuleman N, Haibe-Kains B, Saussoy P, Van Den NE, Michaux L, et al. MicroRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukemia and improves disease risk stratification. *Blood*. 2009;113(21):5237–45.
- Calin GA, Ferracin M, Cimmino A, Di LG, Shimizu M, Wojcik SE, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med*. 2005;353(17):1793–801.
- Ferracin M, Zagatti B, Rizzotto L, Cavazzini F, Veronese A, Ciccone M, et al. MicroRNAs involvement in fludarabine refractory chronic lymphocytic leukemia. *Mol Cancer*. 2010;9:123.
- Moussay E, Palissot V, Vallar L, Poirel HA, Wenner T, El Khoury V, et al. Determination of genes and microRNAs involved in the resistance to fludarabine in vivo in chronic lymphocytic leukemia. *Mol Cancer*. 2010;9:115.
- Nylandsted J, Brand K, Jaattela M. Heat shock protein 70 is required for the survival of cancer cells. *Ann N Y Acad Sci*. 2000;926:122–5.
- Broemer M, Krappmann D, Scheidereit C. Requirement of Hsp90 activity for IκappaB kinase (IKK) biosynthesis and for constitutive and inducible IKK and NF-κappaB activation. *Oncogene*. 2004;23(31):5378–86.
- Sato S, Fujita N, Tsuruo T. Modulation of Akt kinase activity by binding to Hsp90. *Proc Natl Acad Sci USA*. 2000;97(20):10832–7.
- Johnson AJ, Wagner AJ, Cheney CM, Smith LL, Lucas DM, Guster SK, et al. Rituximab and 17-allylamino-17-demethoxygeldanamycin induce synergistic apoptosis in B-cell chronic lymphocytic leukaemia. *Br J Haematol*. 2007;139(5):837–44.
- Trentin L, Frasson M, Donella-Deana A, Frezzato F, Pagano MA, Tibaldi E, et al. Geldanamycin-induced Lyn dissociation from aberrant Hsp90-stabilized cytosolic complex is an early event in apoptotic mechanisms in B-chronic lymphocytic leukemia. *Blood*. 2008;112(12):4665–74.
- Hertlein E, Wagner AJ, Jones J, Lin TS, Maddocks KJ, Towns III WH, et al. 17-DMAG targets the nuclear factor-κappaB family of proteins to induce apoptosis in chronic lymphocytic leukemia: clinical implications of HSP90 inhibition. *Blood*. 2010;116(1):45–53.
- Castro JE, Prada CE, Loria O, Kamal A, Chen L, Burrows FJ, et al. ZAP-70 is a novel conditional heat shock protein 90 (Hsp90) client: inhibition of Hsp90 leads to ZAP-70 degradation, apoptosis, and impaired signaling in chronic lymphocytic leukemia. *Blood*. 2005;106(7):2506–12.
- Jones DT, Addison E, North JM, Lowdell MW, Hoffbrand AV, Mehta AB, et al. Geldanamycin and herbimycin A induce apoptotic killing of B chronic lymphocytic leukemia cells and augment the cells' sensitivity to cytotoxic drugs. *Blood*. 2004;103(5):1855–61.
- Best OG, Che Y, Singh N, Forsyth C, Christopherson RI, Mulligan SP. The Hsp90 inhibitor SNX-7081 synergizes with and restores sensitivity to fludarabine in chronic lymphocytic leukemia cells with lesions in the TP53 pathway: a potential treatment strategy for fludarabine refractory disease. *Leuk Lymphoma*. 2012;53(7):1367–75.
- Best OG, Singh N, Forsyth C, Mulligan SP. The novel Hsp-90 inhibitor SNX7081 is significantly more potent than 17-AAG against primary CLL cells and a range of hematological cell lines, irrespective of lesions in the TP53 pathway. *Br J Haematol*. 2010;151(2):185–8.

27. Lin K, Rockliffe N, Johnson GG, Sherrington PD, Pettitt AR. Hsp90 inhibition has opposing effects on wild-type and mutant p53 and induces p21 expression and cytotoxicity irrespective of p53/ATM status in chronic lymphocytic leukaemia cells. *Oncogene*. 2008;27(17):2445–55.
28. Best OG, Mulligan SP. Heat shock protein-90 inhibitor, NVP-AUY922, is effective in combination with fludarabine against chronic lymphocytic leukemia cells cultured on CD40L-stromal layer and inhibits their activated/proliferative phenotype. *Leuk Lymphoma*. 2012;53(11):2314–20.
29. Walsby E, Pearce L, Burnett AK, Fegan C, Pepper C. The Hsp90 inhibitor NVP-AUY922-AG inhibits NF-kappaB signaling, overcomes microenvironmental cytoprotection and is highly synergistic with fludarabine in primary CLL cells. *Oncotarget*. 2012;3(5):525–34.
30. McCaig AM, Cosimo E, Leach MT, Michie AM. Dasatinib inhibits B cell receptor signalling in chronic lymphocytic leukaemia but novel combination approaches are required to overcome additional pro-survival microenvironmental signals. *Br J Haematol*. 2011;153(2):199–211.
31. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. *J Clin Oncol*. 1999;17(12):3835–49.
32. Binet JL, Caligaris-Cappio F, Catovsky D, Cheson B, Davis T, Dighiero G, et al. Perspectives on the use of new diagnostic tools in the treatment of chronic lymphocytic leukemia. *Blood*. 2006;107(3):859–61.
33. Mayr C, Hemann MT, Bartel DP. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. *Science*. 2007;315(5818):1576–9.
34. Mishra PJ, Humeniuk R, Longo-Sorbello GS, Banerjee D, Bertino JR. A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. *Proc Natl Acad Sci U S A*. 2007;104(33):13513–8.
35. Bertino JR, Banerjee D, Mishra PJ. Pharmacogenomics of microRNA: a miRSNP towards individualized therapy. *Pharmacogenomics*. 2007;8(12):1625–7.
36. Zhou X, Chen X, Hu L, Han S, Qiang F, Wu Y, et al. Polymorphisms involved in the miR-218-LAMB3 pathway and susceptibility of cervical cancer, a case-control study in Chinese women. *Gynecol Oncol*. 2010;117(2):287–90.
37. Starczynski J, Pepper C, Pratt G, Hooper L, Thomas A, Milligan D, et al. Common polymorphism G(-248)A in the promoter region of the bax gene results in significantly shorter survival in patients with chronic lymphocytic Leukemia once treatment is initiated. *J Clin Oncol*. 2005;23(7):1514–21.
38. Saxena A, Moshynska O, Sankaran K, Viswanathan S, Sheridan DP. Association of a novel single nucleotide polymorphism, G(-248)A, in the 5'-UTR of BAX gene in chronic lymphocytic leukemia with disease progression and treatment resistance. *Cancer Lett*. 2002;187(1–2):199–205.
39. Thunberg U, Tobin G, Johnson A, Soderberg O, Padyukov L, Hultdin M, et al. Polymorphism in the P2X7 receptor gene and survival in chronic lymphocytic leukaemia. *Lancet*. 2002;360(9349):1935–9.
40. Moshynska O, Sankaran K, Pahwa P, Saxena A. Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia. *J Natl Cancer Inst*. 2004;96(9):673–82.
41. Nuckel H, Frey UH, Bau M, Sellmann L, Stanelle J, Durig J, et al. Association of a novel regulatory polymorphism (-938C > A) in the BCL2 gene promoter with disease progression and survival in chronic lymphocytic leukemia. *Blood*. 2007;109(1):290–7.
42. Gryshchenko I, Hofbauer S, Stoecher M, Daniel PT, Steurer M, Gaiger A, et al. MDM2 SNP309 is associated with poor outcome in B-cell chronic lymphocytic leukemia. *J Clin Oncol*. 2008;26(14):2252–7.
43. Skogsberg S, Tobin G, Krober A, Kienle D, Thunberg U, Aleskog A, et al. The G(-248)A polymorphism in the promoter region of the Bax gene does not correlate with prognostic markers or overall survival in chronic lymphocytic leukemia. *Leukemia*. 2006;20(1):77–81.
44. Tobin G, Skogsberg A, Thunberg U, Laurell A, Aleskog A, Merup M, et al. Mcl-1 gene promoter insertions do not correlate with disease outcome, stage or VH gene mutation status in chronic lymphocytic leukaemia. *Leukemia*. 2005;19(5):871–3.
45. Nuckel H, Frey UH, Durig J, Duhrsen U, Siffert W. 1513A/C polymorphism in the P2X7 receptor gene in chronic lymphocytic leukemia: absence of correlation with clinical outcome. *Eur J Haematol*. 2004;72(4):259–63.
46. Zenz T, Habe S, Benner A, Kienle D, Dohner H, Stilgenbauer S. The MDM2-309 T/G promoter single nucleotide polymorphism does not alter disease characteristics in chronic lymphocytic leukemia. *Haematologica*. 2008;93(7):1111–3.
47. Li G, Cai M, Fu D, Chen K, Sun M, Cai Z, et al. Heat shock protein 90B1 plays an oncogenic role and is a target of microRNA-223 in human osteosarcoma. *Cell Physiol Biochem*. 2012;30(6):1481–90.
48. Pekarsky Y, Santanam U, Cimmino A, Palamarchuk A, Efanov A, Maximov V, et al. Tc1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. *Cancer Res*. 2006;66(24):11590–3.
49. Mott JL, Kobayashi S, Bronk SF, Gores GJ. miR-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene*. 2007;26(42):6133–40.
50. Duval A, Oлару D, Campos L, Flandrin P, Nadal N, Guyotat D. Expression and prognostic significance of heat-shock proteins in myelodysplastic syndromes. *Haematologica*. 2006;91(5):713–4.
51. Flandrin-Gresta P, Solly F, Aanei CM, Cornillon J, Tavernier E, Nadal N, et al. Heat Shock Protein 90 is overexpressed in high-risk myelodysplastic syndromes and associated with higher expression and activation of Focal Adhesion Kinase. *Oncotarget*. 2012;3(10):1158–68.
52. Thomas X, Campos L, Mounier C, Cornillon J, Flandrin P, Le QH, et al. Expression of heat-shock proteins is associated with major adverse prognostic factors in acute myeloid leukemia. *Leuk Res*. 2005;29(9):1049–58.
53. Flandrin P, Guyotat D, Duval A, Cornillon J, Tavernier E, Nadal N, et al. Significance of heat-shock protein (HSP) 90 expression in acute myeloid leukemia cells. *Cell Stress Chaperones*. 2008;13(3):357–64.

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