Opposite expression pattern of Src kinase Lyn in acute and chronic haematological malignancies

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ORIGINAL ARTICLE

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Abstract Lck/yes-related novel (Lyn) tyrosine kinase overexpression has been suggested to be important for leukaemic cell growth making it an attractive target for therapy. By contrast, Lyn deficiency was shown to be responsible for a phenotype resembling myeloproliferative neoplasm (MPN) in mice. We aimed to shed more light on Lyn's role in haematological neoplasm and systematically investigated Lyn expression in MPN, acute and chronic leukaemia subtypes (n=236). On top, B-cell chronic lymphocytic leukaemia (B-CLL) and chronic myeloid leukaemia significantly overexpressed Lyn when compared to de novo acute lymphoblastic leukaemia, de novo acute myeloid leukaemia (AML) and Philadelphia-chromosome-negative myeloproliferative neoplasms (p<0.001). Most of acute leukaemia subtypes showed a notable down-regulation of Lyn mRNA but anyhow individual cases were labelled for the active form of Lyn protein. Intriguingly, secondary AML evolved in myelodysplastic syndromes revealed almost undetectable Lyn. Overexpression of Lyn in B-CLL was associated with a significant down-regulation of microRNA-337-5p suggesting that aberrant expression of this particular microRNA could be involved in post-transcriptional control of Lyn mRNA fate. We conclude that tyrosine kinase Lyn contributes to the malignant phenotype in certain leukaemia subtypes and therefore attracts targeted therapy.

Keywords Src kinase Lyn · Myeloproliferative neoplasm · Lymphoproliferative leukaemia · Acute leukaemia · Chronic leukaemia · Bone marrow cells · Real-time RT-PCR · MicroRNA-337-5p

Introduction

The Src family kinase (SFK) member Lck/yes-related novel (Lyn) protein kinase is a cytoplasmic non-receptor tyrosine kinase which was first described as an inhibitor of B cell receptor signalling [1]. Its inhibitory role was also demonstrated in Lyn deficient ($Lyn^{-/-}$) B cells which showed antigen-driven hyperproliferation with aberrant responsiveness to antigen stimulation and a failure in down-modulation of responses to immune complexes bound to the B cell receptor [2]. $Lyn^{-/-}$ mast cells exhibited an uncontrolled Fcε-receptor stimulation via bound IgE with constitutive cellular degranulation [3]. As a matter of fact, $Lyn^{-/-}$ mice were prone to evolve autoimmune diseases [4].

Besides perturbation of immune regulatory mechanisms, $Lyn^{-/-}$ deficient mice also developed severe abnormalities in haematopoiesis [5]. The animals showed a massive splenomegaly, myeloid lineage neoplasia and, with rising age, an increase of myeloid and erythroid progenitors. In a related model, $Lyn^{-/-}$ mice showed a prominent expansion of megakaryopoiesis in the bone marrow, an increase in platelet counts, and a severe splenomegaly [6]. Moreover, $Lyn^{-/-}$ megakaryocytes strikingly resembled features demonstrable in Philadelphia-chromosome-negative chronic

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myeloproliferative neoplasms (Ph^- MPN) such as primary myelofibrosis (PMF) [7] including high ploidy (>8N), a significant increase of colony-forming units-megakaryocyte, and large colonies of clustered megakaryocytes phenotypically similar to bone marrows in Ph^- MPN [6, 8].

Lyn has also been described to be a potent negative regulator of G-CSF stimulated granulopoiesis, however, in leukaemic cells Lyn was suggested to promote cell growth and proliferation [9–11]. Thus, Lyn per se apparently exhibits a relevant potential to contribute to an abnormal state of cellular proliferation and consequently represents a target for specific therapies [12].

However, a comprehensive analysis of Lyn expression in different types of MPN, acute and chronic leukaemia is still pending.

To this end, we first tested if aberrantly expressed Lyn, as suggested by the $Lyn^{-/-}$ models, might be involved in the pathogenesis of myeloproliferative neoplasia in humans. We then focussed on the two most common types of chronic leukaemia, i.e. Ph^+ chronic myelogenous leukaemia (CML) and B-cell chronic lymphatic leukaemia (B-CLL) to further look for Lyn expression pattern in myeloid and lymphoid lineages. Lastly, we systematically investigated subtypes of de novo and secondary acute leukaemia likewise representing either a myeloid or a lymphoid origin.

Materials and methods

Bone marrow study group

Formalin-fixed and paraffin-embedded (FFPE) bone marrow trephines were retrieved from the bone marrow registry of the Institute of Pathology, Hannover Medical School. The study group comprised 236 bone marrow biopsies. We selected primary and secondary acute myeloid leukaemia (AML, $n=60$), acute lymphoblastic leukaemia (ALL, $n=25$); B-CLL ($n=15$), Ph^+ CML ($n=17$), 76 Ph^- MPN (40 PMF, 20 essential thrombocythaemia—ET, 16 polycythaemia vera—PV), and 43 control cases. According to the WHO classification in close agreement with clinical data, diagnoses were made in the years 2000–2006. Bone marrows showing acute leukaemia were carefully selected in order to fulfil the major criteria of at least 80% infiltration by bone marrow blasts. B-CLL cases (immunophenotype $CD5^+$, $CD20^+$, $CD23^+$, $CD79a^+$, BCL-2^+, $CD10^-$, Cyclin $D1^-$, ZAP-$70^{+/-}$) exhibit a nodular to diffuse bone marrow infiltration pattern by neoplastic cells ranging from 60–90% of total cellularity. Metaphase cytogenetics and/or RT-PCR were performed to determine the presence of the Philadelphia chromosome and/or BCR-ABL fusion transcripts and to confirm the histopathological diagnosis of CML.

PMF cases represent different degrees of myelofibrosis (mf) determined by silver impregnation (Gomori) as previously described [13]. Biopsies in the control group were indicated for several reasons such as peripheral thrombocytopenia or exclusion of involvement by peripheral lymphoma. All control cases showed normal haematopoiesis in line with age or a mild reactive hyperplasia of megakaryocytes and granulopoiesis. For a summary of patients' characteristics, see Tables 1 and 2. A comprehensive review on the molecular characteristics of acute leukaemia subtypes investigated by this study is shown in Table 3.

Table 1 Molecular and clinical parameters of MPN and B-CLL

Diagnosis (n)	Molecular status (n)	♀/♂	Age	Erythrocytes (10^6/μl)	Haemoglobin (g/dl)	Platelets (10^3/μl)	Leukocytes (10^3/μl)
Cellular PMF (20)	$JAK2^{V617F}$ +/+ (2) +/− (8)	5/5	72 (47–85)	4.9 (3.9–5.8)	14 (10.2–14.6)	819 (660–1317)	12 (8.4–19.5)
	JAK2 wild-type (9)	1/8	66 (42–79)	4.4 (3.6–5.3)	12 (10.2–14.1)	816 (520–1919)	9.6 (7.2–32.7)
	MPL^{W515L} +/− (1)	0/1	60	3.9	12.3	883	8.3
Advanced PMF (20)	$JAK2^{V617F}$ +/+ (4) +/− (6)	7/3	75 (66–83)	3.6 (2.7–8.1)	9.2 (5.3–16.8)	348 (72–1415)	8.5 (6.7–48.1)
	JAK2 wild-type (10)	5/5	58 (37–79)	3.4 (2.9–4)	10.1 (7.9–13.1)	373 (16–800)	8.3 (3.4–31.4)
ET (20)	$JAK2^{V617F}$ +/+ (1) +/− (11)	9/3	63 (34–82)	5 (4.3–6.3)	14.7 (13.2–16.3)	863 (512–1953)	10.2 (7.1–47.2)
	JAK2 wild-type (8)	4/4	53 (29–77)	4.6 (4.3–5.4)	13 (7.9–15.8)	1007 (613–1515)	8.4 (5.9–13.0)
PV (16)	$JAK2^{V617F}$ +/+ (8)	7/1	65 (51–82)	6.7 (5.7–8.5)	16.9 (14.7–19.2)	648 (404–1089)	12 (9.3–19.8)
	$JAK2^{V617F}$ +/− (8)	3/5	69 (50–79)	6.6 (6.1–7.2)	17.7 (14.1–19.8)	379 (289–741)	10.9 (6.3–28.2)
Ph^+ CML (17)	JAK2 wild-type, BCR-ABL positive	7/10	51 (17-85)	4.7 (3.9–5.5)	13.4 (7.1–16.2)	373 (287–673)	57.3 (15.8–380)
B-CLL (15)	JAK2 wild-type	3/12	69 (50–78)	4.6 (3.4–5.1)	13.8 (9.5–15.3)	142 (58–380)	23.2 (4.1–103)

Median values are followed by range. Samples were scored as heterozygous (+/−) for the $JAK2^{V617}$/MPL^{W515L} mutation if the percentage of mutant alleles exceeded 5%. Homozygosity (+/+) was considered if the percentage of mutant T alleles exceeded 50% [14]

Table 2 Clinical parameter in acute leukaemia

Diagnosis	♀/♂ *n*	Age	Erythrocytes (10^6/μl)	Haemoglobin (g/dl)	Platelets (10^3/μl)	Leukocytes (10^3/μl)
1° AML (normal karyotype)	10/10	58 (26–84)	2.7 (1.3–3.9)	8.6 (4.6–12.9)	172 (16–470)	6.6 (1.5–18)
1° AML (abnormal karyotype)	14/6	62 (30–75)	2.2 (1.8–3.3)	7.8 (6.7–9.8)	131 (42.6–379)	5.9 (2.2–64)
2° AML (evolved in MDS)	10/10	70 (47–77)	2.7 (1.5–4.2)	8.6 (5.8–12.8)	66 (19–541)	2.1 (0.8–59.7)
1° ALL	14/11	55 (4–76)	3.3 (1.8–5)	10.6 (5.6–13.4)	42.3 (2–387)	7.7 (0.7–193.1)

RNA extraction and real-time RT-PCR

RNA was extracted from total bone marrow cells as previously described [14]. Megakaryocyte-derived RNA was retrieved by application of the PALM Laser-MicroBeam System (P.A.L.M., Wolfratshausen, Germany) which allows their precise isolation by microdissection from bone marrow sections (~4 μm thick). Megakaryocytes (pools of ~600 megakaryocytes derived from five different Ph^- MPN or control samples) were directly catapulted into the lid of a 500 μl tube in which the cells were further processed for RNA extraction [15].

Total RNA (100 ng–1 μg), pretreated with RNase free ($Rnase^-$) DNase (1 U/μg RNA; RQ1, Promega, Madison, WI, USA), was transcribed into the complementary DNA using 500 ng random hexamer primer (Amersham Pharmacia, Picattaway, CA, USA) and 200 U of Super-Script II $Rnase^-$ Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Real-time RT-PCR was performed on an ABI PRISM 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using PCR primers and TaqMan™ probes for Lyn and the house-keeping gene β-Glucuronidase ((β-GUS) as follows: Lyn forward 5′-TGGATTCTCCACTCAGTTGCA-3′, Lyn reverse 5′-GGCTGCACTCAGATGTTGTCA-3′, Lyn probe 5′-ACTTGTCCTCAGCAGCTGGTAATCTTGCTC-3′ generating an 82-bp product [16] and β-GUS forward 5′-CTCATTTGGAATTTTGCCGATT-3′, β-GUS reverse 5′-CCGAGTGAGATCCCCTTTTTA-3′, and β-GUS probe 5′-TGAACAGTCACCGACGAGAGTGCTGG-3′ generating an 81-bp product (GenBank NM 000181) [17]. The probes were labelled with 6-carboxy-fluorescein (FAM) as the reporter dye and 6-carboxy-tetramethyl-rhodamine (TAMRA) as the fluorescent quencher. Linearity of amplification for Lyn and β-GUS could be demonstrated over a broad concentration range enabling relative quantification in at least two independent runs using the ΔΔCT method as previously described [18].

Genotyping for a potential underlying $JAK2^{V617F}$ and MPL^{W515L} mutation by Pyrosequencing®

We used pyrosequencing assays which allow quantification of the percentage of mutant alleles (allele burden) of potential $JAK2^{V617F}$ or MPL^{W515L} mutations in bone marrow cells [19]. Amplicons covering the hotspots ($JAK2^{G1849T}$ and MPL^{G1544T}, respectively) were amplified by PCR from 25 ng genomic DNA extracted by means of the Qiagen DNeasy kit (Qiagen, Hilden Germany) according to the manufacturer's instruction using primers JAK2 forward 5′-TATGATGAGCAAGCTTTCTCACAAG-3′, JAK2 reverse 5′-AGAAAGGCATTAGAAAGCCTG TAGTT-3′ (GenBank AL161450) generating a 102 bp product, and MPL forward 5′-ATCTCCTTGGTGAC

Table 3 Classification and molecular characteristics in acute leukaemia

Acute leukaemia subtype	FAB	*n*	FLT3/ITD n (%)	$JAK2^{V617F}$ n (%)
AML Normal karyotype	All FAB subtypes but M0 & M6 (incl. 1 hyploplastic variant)	20	2 (10%)	2 (10%)
AML Abnormal karyotype	All FAB subtypes but M3, M6, M7	20		
Complex aberrations* *(3 or more numerical and/or structural chromosomal aberrations)		6	n.d.	1 (<1%)
Isolated aberrations −5/5q− −7/7q− +8, +21, others −Y		12	n.d.	0 (0%)
t(5 ;22) t(8 ;21) t(10 ;11)		2	n.d.	0 (0%)
Secondary AML (evolved from MDS)		20	n.d.	1 (5%)
ALL	Common-ALL, *n*=22; incl. two cases with t(9;22) and one case with *t*(12;21); Pre-B-ALL: *n*=3	25	n.d.	n.d.

n.d. Not determined

CGCTCTG-3′ and MPL reverse 5′-TGGTCCACCGC CAGTCTG-3′ (GenBank U68161) generating a 130-bp product with an additional 5′-biotin tag on respective reverse primers. Pyrosequencing® was performed as previously described [19]. In brief, single-stranded PCR products were prepared by streptavidin sepharose beat sorting and JAK2 and MPL specific sequencing (S) primer (JAK2-S 5′-GGTTTTAAATTATGGAGTATGT-3′, nt 55039–55060 in GenBank AL161450, MPL-S 5′-GCCTGC TGCTGCTGAGGT-3′, nt 1085–1102 in GenBank U68161) were used for sequencing analysis in a PSQ 96MA instrument (Biotage, Uppsala, Sweden). Allele burden was quantified using the SNP Software (Biotage, Uppsala, Sweden).

Samples under investigation were strictly accompanied by a positive control ($JAK2^{V617F}$ cell lines HEL and SET-2; PMF patient with MPL^{W515L} as evidenced by direct sequencing) and a negative control ($JAK2^{wild\text{-}type}$/ $MPL^{wild\text{-}type}$ cell line HL-60).

Determination of FLT3-ITD

For genotypic FLT3, DNA was extracted from bone marrow samples using the DNA Isolation Kit for Mammalian Blood (Roche Molecular Biochemicals, Indianapolis, IN). DNA was amplified for the FLT3 locus using the previously described primers 11F, 5′- GCA ATT TAG GTA TGA AAG CCA GC-3′, and 12R, 5′-CTT TCA GCA TTT TGA CGG CAA CC-3′[20]. PCR amplicons with ITD mutations were sequenced to confirm the presence of and to further characterise the internal duplication. Automated DNA sequencing of PCR products was performed using dideoxynucleotide termination chemistry (DTCS Quick Start MasterMix, Beckman Coulter, Krefeld, Germany) and the CEQ8000 Genetic Analysis System (Beckman Coulter, Krefeld, Germany).

Immunohistochemistry

Detection of total Lyn protein and its phosphorylated isoform (in Ph^- MPN and controls) was performed on 130 cases (17 AML, seven ALL, 15 B-CLL, nine CML, 40 PMF, all degrees of mf, 18 ET, six PV, and 18 controls). Bone marrow sections (~1 μm) were dewaxed, treated with 0.3% hydrogen peroxidase in methanol and were then pretreated in a microwave oven in 10 mmol/l citrate buffer (pH 6.0). Sections were incubated overnight at 4 °C with a monoclonal mouse anti-Lyn antibody (1:200 dilution, Lyn H-6, sc-7274, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and a monoclonal rabbit anti-Lyn-pY antibody (1:100 dilution; Epitomics, Inc., Burlingame, CA, USA). The anti-Lyn-pY antibody specifically detects Lyn's phosphorylation on tyrosine residue 507 (pY507) which is localised in the C-terminal regulatory domain of Lyn. Positivity for anti-Lyn-pY therefore stands for the inactive isoform. Visualisation of the immunoreaction was carried out with the ABC kit and DAB (Vector Lab., Inc. Burlingame, CA, USA) according to the manufacturer's instructions and by counterstaining with haematoxylin. The protocol was accompanied by negative controls (samples were processed by omission of primary antibodies) and positive controls (non-neoplastic lymph nodes).

Quantitative expression analysis of microRNA-337-5p

RNA samples which were already analysed for Lyn mRNA expression level in 1° ALL (n=9), B-CLL (n=10) and controls (n=9) were subsequently used for real-time RT-PCR analyses of human miRNA hsa-miR-337-5p (AB Assay ID 002156, part no. 4395267) and the small nuclear RNA, C/D box 49A "RNU 49" as the endogenous control (AB Assay ID 001005, part no. 4373376) according to the manufacturer's instructions (both assays from Applied Biosystems, Foster City, CA, USA).

Statistical analyses

To compare Lyn mRNA expression in malignant and non-neoplastic haematopoiesis, Kruskal–Wallis tests were performed followed by Dunn's post tests for pairwise group differences. One-way analysis of variance (ANOVA) followed by Tukey's tests were applied for investigation of differences in miRNA-337-5p expression. p values$\leq$0.05 were considered as statistically significant. Statistical analyses and graphic design was made by using GraphPadPrism, Version 5.0 (GraphPad Software, San Diego, CA, USA).

Results

Significant overexpression of Lyn mRNA level in chronic leukaemia of myeloid (Ph^+ CML) and lymphoid (B-CLL) origin—down-regulation of Lyn mRNA in acute leukaemia

B-CLL significantly overexpressed Lyn mRNA (median 4.4, range 2.5–8.9) when compared to all other entities under investigation (p<0.001, Ph^+ CML p<0.01, respectively). Ph^+ CML likewise overexpressed Lyn mRNA (median 2.5, range 1.0–8.9) compared to all entities (p<0.001).

Acute leukaemia subtypes underexpressed Lyn mRNA with secondary AML (evolved in myelodysplastic syndromes, MDS) showing nearly undetectable levels (median 0.02, range 0.003–0.2). Any molecular defect in the study group, i.e. JAK2 (V617F), MPL (W515L)

or FLT3/ITD could not be correlated with a particular aberrant Lyn mRNA level.

For a comprehensive illustration of Lyn mRNA data, see Fig. 1 a + b and Table 4.

Megakaryocytes isolated from bone marrow trephines in PMF, ET, and PV showed no clear-cut difference of Lyn mRNA expression when compared to non-neoplastic megakaryocytes and therefore no hint for a Lyn deficiency (data not shown).

Overexpression of Lyn protein in B-CLL–megakaryocyte-lineage-restricted Lyn expression in CML

In B-CLL the population of neoplastic lymphocytes showed strong labelling of membranous and cytoplasmatic Lyn in the entirety of cases independent of the predominant infiltration pattern, i.e. nodular or diffuse (Fig. 2c + d).

CML cases showed positivity for Lyn restricted to the megakaryocyte lineage and rarely in granulopoiesis (Fig. 2a).

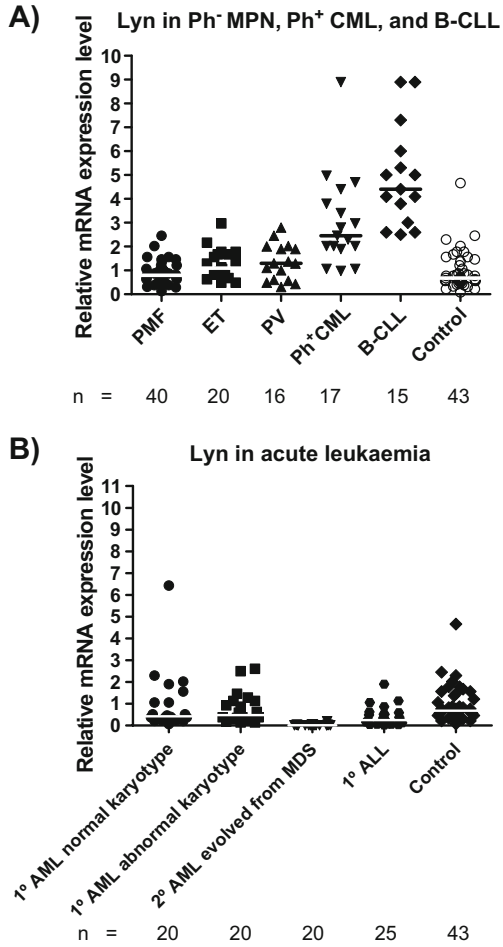

Fig. 1 Point plots highlighting Lyn mRNA expression in Ph⁻ MPN and chronic leukaemia (Ph⁺ CML, B-CLL) and in subtypes of acute leukaemia (AML and ALL) compared to the control group (**a** and **b**, respectively). **a** + **b** show the identical control group. PMF cases were combined regardless the grade of myelofibrosis (**a**). *1°* Primary, *2°* secondary. AML with abnormal karyotype includes either complex (three or more numerical and/or structural chromosomal aberrations) as well as single chromosomal defects. Note that the expression level in the control group was arbitrarily set to 1. *White or black horizontal bars* represent median values of Lyn mRNA expression

Table 4 Lyn mRNA expression in bone marrow cells

Diagnosis (*n*)	Lyn mRNA expression Median (range)	Probability values (*p*)
PMF (40; all degrees of mf)	0.8 (0.1–2.5)	0.01 (vs CML)
		0.001 (vs B-CLL)
		0.001 (vs 2° AML)
ET (20)	1.1 (0.5–3.0)	0.001 (vs 2° AML)
		0.01 (vs 1° ALL)
PV (16)	1.3 (0.3–2.8)	0.001 (vs 2° AML)
		0.01 (vs 1° ALL)
Ph⁺ CML (17)	2.5 (1.0–8.9)	0.001 (vs 1° AML, normal karyotype)
		0.001 (vs 1° AML, complex karyotype)
		0.001 (vs 2° AML)
		0.001 (vs 1° ALL)
		0.01 (vs Control group)
B- CLL (15)	4.4 (2.5–8.9)	0.001 (vs 1° AML, normal karyotype)
		0.001 (vs 1° AML, complex karyotype)
		0.001 (vs 2° AML)
		0.001 (vs 1° ALL)
		0.001 (vs Control group)
1° AML (20) (normal karyotype)	0.4 (0.07–6.4)	0.01 (vs 2° AML)
1° AML (20) (abnormal karyotype)	0.5 (0.1–2.6)	0.01 (vs 2° AML)
2° AML (20) (evolved in MDS)	0.02 (0.003–0.2)	0.001 (vs control group)
1° ALL (25)	0.3 (0.02–1.9)	–
Control group (43)	0.7 (0.08–4.7)	–

1° Primary, *2°* secondary, *mf* myelofibrosis

No Lyn deficiency in Ph⁻ MPN

Typically enlarged and clustered megakaryocytes in PMF but also some myeloid progenitors were strongly labelled for Lyn arguing against a Lyn deficiency in PMF and other Ph⁻ MPN (Fig. 2b).

Heterogenous Lyn expression in AML subtypes

In de novo AML (independent of the subtype), some bone marrows revealed strong (Fig. 2e) and some even an absent (Fig. 2f) Lyn expression in myeloblasts.

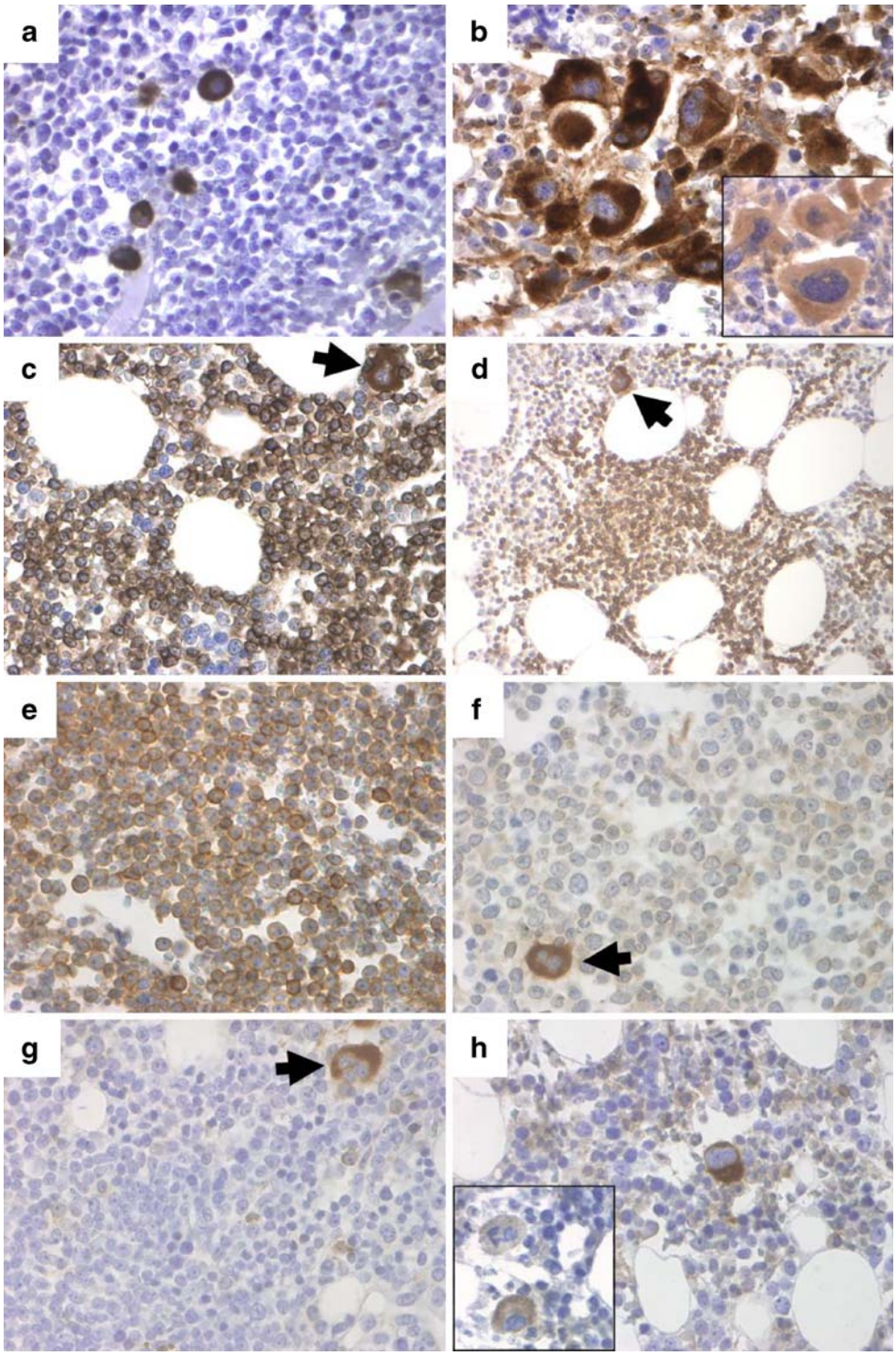

Fig. 2 In CML, total Lyn protein showed a strong cytoplasmatic expression predominantly in megakaryocytes but was almost undetectable in granulopoiesis (**a**). Megakaryocyte cluster in PMF were likewise labelled (**b**). Neoplastic lymphocytes in B-CLL were consistently Lyn positive (**c**—diffuse infiltration pattern, **d**—nodular infiltration pattern). In acute leukaemia, some AML cases revealed strong (**e**) or absent (**f**) Lyn expression in myeloblasts while in all ALL cases bone marrow lymphoblasts were Lyn-negative (**g**). Megakaryocytes in all cases served as internal positive controls even though intensity of staining varied between individual cases (*arrows* in **c**, **d**, **f** and **g**). Megakaryocytes from Ph^- MPN revealed comparable Lyn protein expression compared to non-neoplastic haematopoiesis (**h**). Immunostaining of tyrosin 507 phosphorylation in Lyn's C-terminal regulatory domain (=inactive Lyn isoform) was frequently weaker or even absent in megakaryocytes indicating that most of total Lyn represented the active form (*representative insert* in **b** and **h**). Original magnification in micrographs **a**–**c**, **e**–**h** (including inserts): ×400; in **d**: × 200. Images were produced with a DP71 Camera (Olympus, Germany) on an Axiophot microscope with a Plan-Neofluar ×40/0.75 objective (both Zeiss, Germany) and were processed with Soft Imaging System software (Olympus, Germany)

Almost undetectable Lyn in ALL and secondary AML

By contrast, all ALL cases under investigation showed bone marrow lymphoblasts negative for Lyn protein (Fig. 2g). Megakaryocytes in all cases served as internal positive controls even though intensity of staining varied between individual cases (arrows in Fig. 2c, d, f and g). Megakaryocytes from Ph^- MPN revealed an almost similar Lyn protein expression when compared to non-neoplastic haematopoiesis (Fig. 2h). In the majority of cases, some megakaryocytes were labelled weak while adjacent megakaryocytes showed a strong expression indicating a variable activation state of Lyn among individual cells (not shown). Immunostaining of tyrosin 507 phosphorylation in Lyn's

C-terminal regulatory domain (=inactive Lyn isoform) was frequently weaker or even absent in megakaryocytes indicating that most of total Lyn represented the active form (representative insert in Fig. 2b and h).

Expression of miRNA 337-5p is significantly down-regulated in B-CLL

The miRBase (http://microrna.sanger.ac.uk/targets/v5/) was used for mRNA/miRNA target prediction and revealed that the mature miRNA-337-5p has a highly complementary binding site in the 3′-untranslated region (UTR) of LYN mRNA (Fig. 3). Quantitative expression analysis in ALL, B-CLL and controls determined a significantly decreased expression of miRNA 337-5p in B-CLL (median 0.06, range 0.02–0.24) compared to controls (median 1.2, range 0.33–2.3, $p \leq 0.05$) but not to 1° ALL (median 0.24, range 0.11–4.1), Fig. 4.

Discussion

The Src kinase Lyn is an important player in the network of regulatory elements in signal transduction in haematopoiesis. Any aberrant state, i.e. deficiency or abundance, might interfere with normal cellular growth and function and potentially leads to distinct haematological phenotypes.

Recent data on aberrant megakaryopoiesis in Lyn deficient cells and mice [6, 8] attracted our intention, because abnormal megakaryocyte proliferation is also the predominant feature of Ph^- MPN. The entirety of Ph^- MPN under investigation showed a constitutive Lyn mRNA expression in total cellularity compared to control cases independent of an underlying mutation in JAK2 or MPL. Moreover, no clear hint for a Lyn deficiency restricted to the megakaryocyte population could be spotted. Megakaryocytes in a given case frequently showed no Lyn-pY staining indicating non-phosphorylation of the regulatory domain and thus strongly activated Lyn. Therefore, data from the $Lyn^{-/-}$ model could not be transferred to human Ph^- MPN.

By contrast, chronic and acute leukaemia subtypes showed notable aberrations of Lyn expression. On top, B-CLL and Ph^+ CML revealed Lyn mRNA expression levels significantly higher than those determined in Ph^-

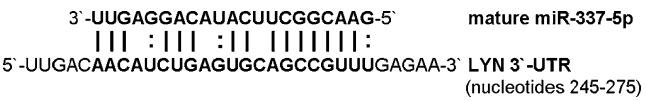

Fig. 3 Prediction of high complementary between miRNA-337-5p and parts of the 3′-UTR of Lyn mRNA as determined by http://microrna.sanger.ac.uk/targets/v5/ .The 21-nucleotide short mature miRNA-337-5p matches at 15 positions including eight G/C base-pairs

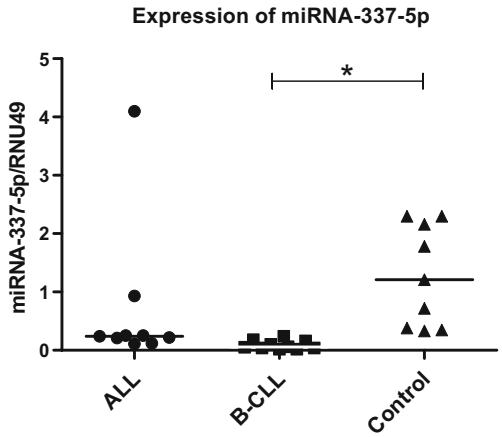

Fig. 4 Point plot showing the expression level of mature miRNA-337-5p relative to the reference (RNU49) in 1° ALL, B-CLL and controls. B-CLL showed significant down-regulation of this miRNA species compared to controls

MPN and, much more prominent, in acute leukaemia. Lyn overexpression in B-CLL (independent of the infiltration pattern) could be reproduced also on the protein level in the neoplastic lymphocyte population, whereas Ph^+ CML showed a strong Lyn protein expression predominantly restricted to the megakaryocytic lineage by sparing the granulopoiesis (Fig. 2a). Interestingly, a previous study showed overexpression and activation of Lyn in CML patients resistant to imatinib (formerly STI571) [21]. Since resistance to imatinib provokes acceleration of the disease the increasing population of blasts might account for the overexpression and activation of Lyn. Moreover, a recent study showed that Lyn in CML might act in a complex with BCR-ABL but that even in imatinib-sensitive cases Lyn per se is powerful enough to mediate signals leading to autonomous proliferation [22]. As shown here (Fig. 2a), the chronic phase of CML exhibits the megakaryocyte population but not the granulopoiesis as a major source for Lyn.

In B-CLL previous work showed that abnormal Lyn activation is apparently responsible for impaired apoptosis of neoplastic lymphocytes isolated from peripheral blood [10]. These lymphocytes not only showed membranous Lyn protein (as expected and demonstrable in normal B lymphocytes) but also distributed in the cytoplasm thereby corresponding to the increased total Lyn in B-CLL. These data on Lyn overexpression were unexceptionally reproducible in our study analysing the neoplastic bone marrow infiltration instead of peripheral blood cells. Neoplastic lymphocytes showed a strong membranous and cytoplasmatic staining for Lyn corresponding to the increased mRNA level (Figs. 1a and 2 c + d). Because of the important roles of cellular miRNA species in the control of target mRNA levels [23] we postulated that a certain miRNA might be dysregulated in B-CLL. The mature miRNA-337-5p was identified to highly match the

3′-untranslated region (UTR) of Lyn mRNA (Fig. 3). We hypothesised that overexpression of Lyn mRNA could be associated with a reduced level of miRNA-337-5p. In an explorative analysis we determined a significant lower level of miRNA-337-5p in B-CLL (Fig. 4) compared to controls. Even though speculative at this stage, this experiment suggests that low miRNA-337-5p might no longer control the level of Lyn mRNA, i.e. binding the mRNA and initiating its degradation in B-CLL cells. Increased Lyn in turn might then disturb mechanisms of cellular apoptosis in B-CLL [10]. However, this potential molecular mechanism might be restricted to chronic leukaemia of B cell type because in de novo ALL lymphoid blasts frequently showed a total absence of Lyn expression which was also accompanied by reduced miRNA-337-5p level. This might be due to a totally different effect of Src kinase Lyn in immature cells compared with the mature but neoplastic population in B-CLL. A reason for decreased Lyn mRNA and consecutively protein expression in ALL also could be the aberrant hypermethylation of promoter regions but recent data demonstrated that the Src kinase Hck but not Lyn showed epigenetic regulation associated with poorer prognosis [24]. Interestingly, in our study the 2 cases of *de novo* ALL with Philadelphia-chromosome (Ph^+ ALL) showed very low mRNA level (0.02; 0.07, respectively) and almost no Lyn protein. The mechanisms responsible for this down-regulation remain elusive. However, targeting BCR-ABL positive cells with or without Imatinib resistance by using Lyn kinase inhibitors as suggested earlier [21, 25] may require the prior demonstration of Lyn expression in an individual patient.

Besides ALL, the majority of AML in our study also showed significantly down-regulated mRNA levels of Lyn (Fig. 1b). In secondary AML evolved in patients with a history of MDS Lyn mRNA level were lowest and Lyn protein was almost undetectable. Accordingly, absence or down-regulation of Lyn might pave the way for leukaemic transformation, a hypothesis supported by the fact that also primary AML subtypes, regardless the underlying complexity of cytogenetic aberrations and molecular defects, showed low Lyn mRNA levels (Fig. 1b). However, in these cases, Lyn protein expression could not consistently be correlated with the mRNA level. We found cases showing a considerable strong labelling of bone marrow blasts whilst in other cases blasts were negative. Because megakaryocytes in AML were labelled for total Lyn in the entirety of cases (Fig. 2g) a technical reason could be excluded. Therefore, Lyn protein expression and stability in AML blasts might be individually regulated by yet unknown mechanisms. Recent data suggested that Lyn is important for survival and proliferation of AML blasts sorted from the bone marrow tap [12]. Independent of the FAB classification, underlying cytogenetic status (normal/abnormal karyotype), and molecular defect (e.g. FLT3/ITD) selected AML samples of this study showed constitutively active Lyn. Lyn's distribution within AML cells was both membranous and cytoplasmatic. We were unable to demonstrate this continuity of Lyn expression in AML, but the similar distribution pattern was demonstrable in bone marrow blasts of positive cases (Fig. 2e). A previous study suggested Lyn to be one factor valuable for determination of disease activity in AML because AML patients in remission showed significantly lower Lyn mRNA expression [16]. Because in our study, Lyn mRNA expression in AML subtypes rarely reached notable levels we initially found Lyn not being appropriate for disease monitoring. However, in patients with Ph^- MPN showing transformation into AML preliminary analyses in our hands showed an increasing Lyn mRNA level during disease acceleration. Conversely, in secondary AML evolved in patients with a history of MDS Lyn is either dispensable for transformation or Lyn's down-regulation is part of the leukaemic switch. Accordingly, it would be of merit to investigate the role of Lyn in disease acceleration and transformation of MPN and MDS in individual courses.

In summary, Lyn overexpression is common in B-CLL, Ph^+ CML and in certain cases of de novo AML. However, independent of demonstrable cytogenetic aberrations and due to yet unknown pathomechanisms the latter shows a great variability of expression among cases and subtypes. Thus, Lyn might be considered as a potential target for upcoming specific therapies but expression should be determined first in every individual case.

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