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RESEARCH PAPER

Colorimetric assessment of *BCR-ABL1* transcripts in clinical samples via gold nanoprobes

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Abstract Gold nanoparticles functionalized with thiolated oligonucleotides (Au-nanoprobes) have been used in a range of applications for the detection of bioanalytes of interest, from ions to proteins and DNA targets. These detection strategies are based on the unique optical properties of gold nanoparticles, in particular, the intense color that is subject to modulation by modification of the medium dieletric. Au-nanoprobes have been applied for the detection and characterization of specific DNA sequences of interest, namely pathogens and disease biomarkers. Nevertheless, despite its relevance, only a few reports exist on the detection of RNA targets. Among these strategies, the colorimetric detection of DNA has been proven to work for several different targets in controlled samples but demonstration in real clinical bioanalysis has been elusive. Here, we used a colorimetric method based on Au-nanoprobes for the direct detection of the e14a2 *BCR-ABL* fusion transcript in myeloid leukemia patient samples without the need for retro-transcription. Au-nanoprobes directly assessed total RNA from 38 clinical samples, and results were validated against reverse transcription-nested polymerase chain reaction (RT-nested PCR) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The colorimetric Au-nanoprobe assay is a simple yet reliable strategy to scrutinize myeloid leukemia patients at diagnosis and evaluate progression, with obvious advantages in terms of time and cost, particularly in low- to medium-income countries where molecular screening is not routinely feasible.

Keywords Chronic myeloid leukemia · Leukemia · RNA · Gold nanoparticles · Colorimetric detection

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Introduction

Gold nanoparticles functionalized with thiolated oligonucleotides (Au-nanoprobes) due to their unique optical proprieties have been widely used for nucleic acid detection with high sensibility and sensitivity at much lower costs when compared with conventional molecular methods. Disperse Au-nanoprobe solutions show a characteristic surface plasmon resonance (SPR) band at around 525 nm (red color), which is red-shifted upon salt-induced aggregation (blue color); hybridization to a complementary nucleic acid target sequence prevents aggregation and the solution remains red [1]. This non-cross-linking colorimetric detection method has been efficiently applied to the detection of pathogens and other DNA sequences of interest, where hybridization to a specific complementary target leads to Au-nanoprobe stabilization and resistance to salt-induced aggregation [2, 3].

Chronic myeloid leukemia (CML) is a clonal bone marrow stem cell disease with an annual incidence of 1–2 new cases per 100,000 adults and accounting for approximately 15 % of leukemia cases. CML is one of the rarest forms of leukemia, which is expected to become the most prevalent hematologic malignancy in the world by 2020 [4–6]. Management of CML therapy with tyrosine kinase inhibitors (TKIs) has been successfully achieved [7–10]. The Philadelphia chromosome

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(Ph), a reciprocal translocation of the long arms of chromosomes 9 and 22, t(9;22), is found in more than 90 % patients with CML, 15–25 % of patients with acute lymphoblastic leukemia (ALL), and 1 % of newly diagnosed adults with acute myeloid leukemia (AML) [11–13]. This translocation transposes the *ABL1* oncogene from chromosome 9q34 to the *BCR* gene on chromosome 22q11 (Fig. 1) [14–16].

This reciprocal translocation during hematopoiesis combined with alternative splicing events may originate at least eight different reported *BCR-ABL1* transcripts, all encoding proteins with high tyrosine kinase activity. The vast majority of patients with CML (~95 %) express either e13a2 or e14a2 mRNAs that result from a rearrangement of the major breakpoint cluster regions (M-BCR) generating 210-kDa fusion proteins (Fig. 1c). Several studies suggest that not only qualitative differences in the type of BCR-ABL1 proteins expressed but also quantitative variations in their total level within the cells may have an important role in determining CML phenotype [17–20]. The fused *BCR-ABL1* gene and its gene products provide specific markers for diagnosis and disease monitoring. Currently, CML diagnosis is primarily based on clinical symptoms, complete blood count, and bone marrow biopsy. Cytogenetic analysis, such as karyotype and fluorescence in situ hybridization (FISH), may be carried out to check the presence of the Ph chromosome, and molecular tests, e.g., reverse transcription-polymerase chain reaction (RT-PCR) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR), are used for assessing type and levels of fusion transcript on RNA from bone marrow or peripheral blood specimen [21, 22]. Monitoring CML by RT-qPCR and RT-nested PCR depends on local facilities and on the degree of molecular standardization of the local laboratory [23–25].

Based on their size-dependent surface plasmon resonance (SPR) absorption and the high surface-to-volume ratio allowing for surface functionalization with a variety of molecules, gold nanoparticles (AuNPs) are frequently used as probes in biological detections [1, 2]. Baptista and colleagues developed a proof-of-concept colorimetric Au-nanoprobe method for direct detection and quantification of a *BCR-ABL1* e14a2 fusion suitable for identification of BCR-ABL targets in lab-controlled conditions [3]. Contrary to the existing molecular tools for the diagnostics of CML, the Au-nanoprobe methodology requires neither retrotranscription of the sample RNA into cDNA nor its amplification in a nested-PCR format with extreme complexity. Despite several reports on the use of BCR-ABL target as proof-of-concept for nanotechnology-based detection, the use of nanoparticles for CML diagnostics in clinical samples has never been performed before.

Here, we were able to directly screen RNA extracted from blood or bone marrow samples from patients admitted to the clinics for CML or AML via the Au-nanoprobe assay and validated results against the gold standard, RT-nested PCR and qPCR, without loss of information.

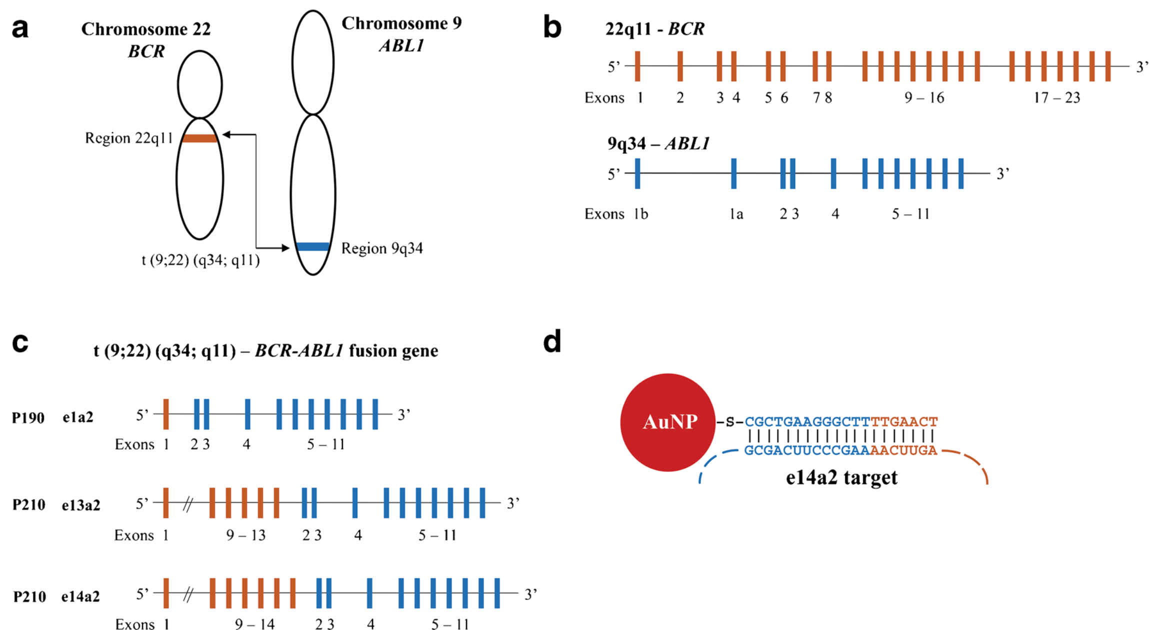

Fig. 1 Genetics of CML. **a** Chromosomes reciprocal translocation that originates the *BCR-ABL1* oncogene during hematopoiesis. **b** Structure of *BCR* and *ABL1* genes. **c** Structure of the two most common *BCR-ABL1* fusion transcripts (e14a2 and e13a2) and a rare one (e1a2). e14a2 and e13a2 isoforms encode 210 kDa fusion proteins, and the e1a2 isoform encodes a 190-kDa protein. **d** Au-nanoprobe designed to target e14a2 mRNA

Patients and methods

Patient samples

All CML patients were confirmed to harbor the t(9;22) by FISH. Levels of *BCR-ABL1* were assessed using RT-qPCR, according to the 2015 European LeukemiaNet (ELN) guidelines [26]. Treatment with TKIs was initiated immediately and clinical, hematological, and molecular remission was evaluated. All 16 AML bone marrow samples were collected at diagnosis, presenting a normal karyotype and negative t(9;22) FISH analysis (Table 1). Bone marrow and peripheral blood patient samples were collected in RNAlater® (Thermo Fisher Scientific, Carlsbad, CA, USA) for maximum RNA stabilization and used directly for RNA extraction without any culture expansion and/or any cell culture method.

Au-nanoprobe synthesis

The probe sequence and the complementary target derive from the *BCR-ABL1* e14a2 (also known as b3a2) chimeric protein mRNA. This target was selected because it represents the most frequent breakpoint in CML, accounting for 55 % of cases [27, 28]. Probe selectivity was assessed against two other *BCR-ABL1* breakpoint regions: e13a2 and e1a2. Control oligonucleotide target sequences included *BCR*, *ABL1*, and an unrelated target—see Electronic Supplementary Material Table S1.

Au-nanoprobe design and synthesis were performed as previously described [3]. In brief, 14-nm gold nanoparticles were prepared by the citrate reduction method and functionalized with the respective thiolated oligonucleotide in an aqueous solution of AuNPs at a 1:150 (AuNP:oligonucleotide) ratio for 16 h. After centrifugation (20 min at 14,500*g*), the oily precipitate was washed with 10 mM phosphate buffer (pH 8.0), 0.1 M NaCl, centrifuged, and resuspended in the same buffer. The resulting Au-nanoprobes were stored in the dark at 4 °C.

Characterization of AuNPs and Au-nanoprobes was performed by transmission electron microscopy and dynamic light scattering (see Electronic Supplementary Material Table S2).

Cell culture

Immortalized cell lines derived from CML patients in blast crisis, K562 (*BCR-ABL1* e14a2 fusion transcript positive cell line) and BV173 (*BCR-ABL1* e13a2 fusion transcript positive cell line; e14a2 negative) [28] were cultured, respectively, in DMEM and RPMI with 10 % FBS, at 37 °C with 5 % CO_2. Human acute promyelocytic leukemia cell line (HL60), negative for *BCR-ABL1*, was cultured in RPMI with 10 % FBS, at 37 °C with 5 % CO_2. These cell lines were used as positive and negative controls for the presence and absence of *BCR-ABL1* transcript, respectively.

Total RNA isolation

Total RNA was extracted from (1) K562, BV173, and HL60 cell pellets and from (2) bone marrow and peripheral blood patient samples previously stored in RNAlater® (Thermo Fisher Scientific, Carlsbad, CA, USA). Patients' bone marrow or white blood cells were used directly for RNA extraction and not expanded by any cell culture method. RNA extraction was performed by the guanidine thiocyanate procedure (SV Total RNA Isolation System, Promega, Madison, WI, USA). Cell pellets were lysed in a solution containing 4 M guanidine thiocyanate, 0.1 M Tris-HCl pH 7.5, and 1 % β-mercaptoethanol and were subsequently centrifuged at 13,000×*g* for 10 min to clear the lysate of precipitated proteins and cellular debris. Nucleic acids were selectively precipitated with ethanol; bound to the silica surface of glass fibers; and washed with 60 mM potassium acetate, 10 mM Tris-HCl pH 7.5, and 60 % (*v*/*v*) ethanol. On-column DNase I treatment lasted for 15 min at room temperature to remove genomic DNA. After several washing steps, RNA was resuspended in DEPC-treated water and stored at −80 °C until use. RNA concentration and purity were determined by UV spectrophotometry.

RT and PCR amplification

Total RNA extracted from samples was reverse transcribed using the NZY M-MuLV First-Strand cDNA Synthesis kit (Nzytech, Lisbon, Portugal) followed by nested-PCR amplification (see primer sequences under Electronic Supplementary Material Table S1). Outer PCR was performed under the following conditions: initial denaturation at 95 °C for 5 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and a final extension step at 72 °C for 10 min. Inner PCR included the following conditions: initial denaturation at 95 °C for 5 min; 30 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 1 min; and a final extension step at 72 °C for 10 min.

Colorimetric detection with Au-nanoprobes

The Au-nanoprobe assay was performed in a total volume of 30 μL containing the Au-nanoprobe at a final concentration of 2.5 nM and the appropriate targets at a final concentration of 0.33 μM in 10 mM phosphate buffer (pH 8.0). A blank reaction was made in exactly the same conditions but replacing

Table 1 Performance of Au-nanoprobe diagnostic test

	Sample	Tissue	FISH or RT-qPCR[a] (IS)	RT-nested PCR	e14a2 Au-nanoprobe assay
AML at diagnostics	1	BM	n/a	No amp	−
	2	BM	n/a	No amp	−
	3	BM	n/a	No amp	−
	4	BM	n/a	No amp	−
	5	BM	n/a	No amp	−
	6	BM	n/a	No amp	−
	7	BM	n/a	No amp	−
	8	BM	n/a	No amp	−
	9	BM	n/a	No amp	−
	10	BM	n/a	No amp	−
	11	BM	n/a	No amp	−
	12	BM	n/a	No amp	−
	13	BM	n/a	No amp	−
	14	BM	n/a	No amp	−
	15	BM	n/a	No amp	−
	16	BM	n/a	e14a2	+
CML at diagnostics	17	BM	n/a	e14a2	+
	18	BM	n/a	e13a2	−
	19	BM	n/a	e14a2	+
	20	BM	n/a	e14a2	+
	21	BM	n/a	e13a2	−
	22	BM	n/a	e14a2	+
	23	BM	98 %	e13a2	−
	24	BM	87 %	e14a2	+
	25	BM	98 %	e14a2	+
	26	BM	97 %	e14a2	+
	27	BM	95 %	e13a2	−
	28	BM	99 %	e13a2	−
	29	BM	77 %	e14a2	+
	30	BM	95 %	e14a2	+
	31	BM	98 %	e14a2	+
	32	PB	90 %	e13a2	−
CML at follow-up	33	PB	0.017 %	No amp	−
	34	PB	0.34 %	e14a2	+
	35	PB	0.00031 %	No amp	−
	36	PB	0.11 %	e13a2	−
	37	PB	1 %	No amp	−
	38	PB	3.4 %	e13a2	−

IS international scale, *n/a* not available, *No amp* no amplification, *BM* bone marrow, *PB* peripheral blood

[a] FISH analysis for diagnostic samples (1–32) and RT-qPCR analysis for follow-up samples (33–38)

target or total RNA by an equivalent volume of 10 mM phosphate buffer (pH 8.0). Following 5 min of denaturation at 95 °C, the mixtures were cooled down to 25 °C and 80 mM $MgCl_2$ added for color revelation. Absorption spectra were run in a Tecan Infinite® M200 microplate reader (Männedorf, Switzerland). Aggregation profiles were interpreted based on the areas under the curve ($rAbs = AUC_{500–530\ nm}/AUC_{570–600\ nm}$) using the trapezoidal rule. Au-nanoprobe aggregation is portrayed by the natural logarithm of rAbs: a value of 0 for the *ln (rAbs)* is considered

as the threshold for distinction between non-aggregated [*ln (rAbs)* ≥ 0] and aggregated [*ln (rAbs)* < 0] Au-nanoprobe, corresponding to a positive and a negative sample, respectively. The accuracy of the Au-nanoprobe assay was assessed calculating positive and negative predictive values (PPV and NPV, respectively), by comparison to the gold-standard methodology, RT-PCR. These proportions determine the positive results that are true-positive hits [PPV = number of positives $_{\text{(gold standard)}}$/number of positives $_{\text{(Au-nanoprobe)}}$] and the negative results that are true negative hits [NPV = number of negatives $_{\text{(gold standard)}}$/number of negatives $_{\text{(Au-nanoprobe)}}$].

Statistical analysis

A one-way analysis of variance (ANOVA) and a post hoc Tukey's test were carried and mean differences (*P* value <0.001) between groups determined with a confidence interval (CI) of 99 %.

Results

Au-nanoprobe calibration

Molecular diagnostics of CML usually involves the detection of the *BCR-ABL1* e14a2 fusion transcript. First, we calibrated the Au-nanoprobe to selectively identify this fusion transcript using synthetic oligonucleotides as targets for hybridization. Figure 2 shows that the Au-nanoprobe was able to discriminate between the fully complementary target and two other *BCR-ABL1* fusion transcripts that share the same *ABL1* region as the e14a2 transcript (e13a2 and e1a2) at 100 fmol/μl of ssDNA oligo. One can observe the selectivity of the Au-nanoprobe towards the e14a2 transcript, as the values for *BCR*, *ABL1*, and non-complementary targets are below the defined threshold.

Total RNA extracted from K562 (harboring the target e14a2 fusion transcript), BV173 (harboring the e13a2 fusion transcript), and HL60 (promyelocytic leukemia cell line without fusion transcript) was used to calibrate for complex sample mixtures. The Au-nanoprobe was able to recognize the e14a2 sequence present in K562 cells, with identical specificity as that attained for synthetic oligonucleotides (Fig. 3).

RNA extracted from HL60 and BV173 cell lines was scored as negative despite the presence of partially complementary targets: *BCR*, *ABL1*, and e13a2 mRNA (Fig. 3b). The obtained value is clearly below the established threshold indicating that the system is capable to selectively detect only the correct target sequence even in presence of similar sequences with more than 50 % homology to the target. The colorimetric data is in clear agreement with that of the gold standard, RT-PCR [26, 27]. The LOD = 15 ng/μl is ideal and critical for optimal performance and sequence discrimination directly from RNA samples.

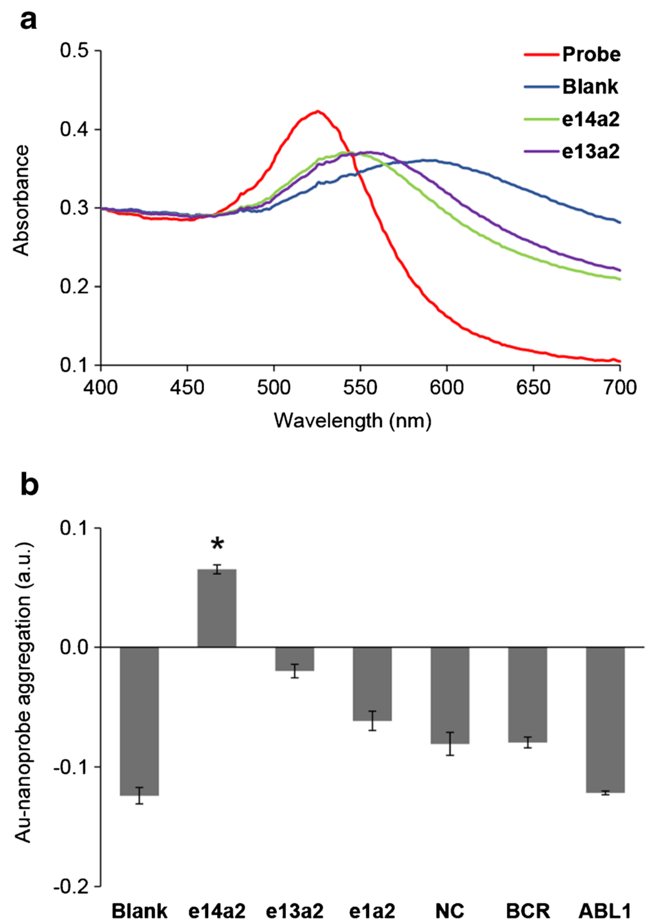

Fig. 2 Au-nanoprobe detection of the e14a2 *BCR-ABL1* transcript variant. **a** UV-vis spectra before salt addition (Probe, *red*); after the addition of salt in absence of any target (blank, *blue*), presence of the e14a2 (*green*) or e13a2 (*purple*) *BCR-ABL1* synthetic oligonucleotides. **b** Specificity of the Au-nanoprobe towards different synthetic oligonucleotides. A threshold of 0 for Au-nanoprobe aggregation [*ln (rAbs)*] was considered for discrimination between positive and negative detection. The *error bars* represent the standard error of the mean from three independent assays. *Statistically significant difference with *P* value <0.001, in comparison to e13a2 condition (post hoc Tukey's test)

Au-nanoprobe assay for CML diagnostics

Following calibration of the Au-nanoprobe assay, 38 myeloid leukemia samples (16 AML sample and 22 CML samples), previously characterized at the cell and molecular levels, were blindly tested via the Au-nanoprobe approach and compared to RT-nested PCR. From the attained data, we determined the sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) for the Au-nanoprobe assay (Fig. 4; Table 1).

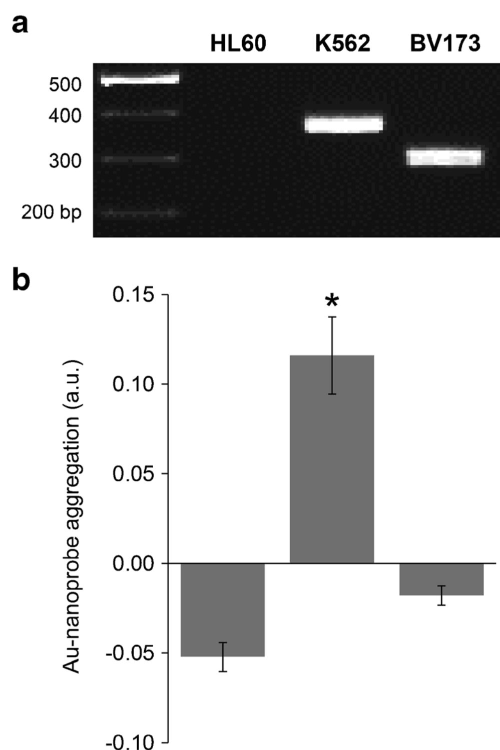

Fig. 3 Au-nanoprobe detection of the e14a2 *BCR-ABL1* transcript variant in total RNA samples. RNA was extracted from cell lines (HL60, K562, and BV173). **a** Agarose (2 %) gel electrophoresis showing nested-PCR products using standardized primers for the detection of the e14a2 (360 bp) and e13a2 (285 bp) transcripts. First lane is DNA ladder (GeneRuler, Fermentas). **b** e14a2 Au-nanoprobe detection assay. A threshold of 0 for Au-nanoprobe aggregation [*ln (rAbs)*] was considered for discrimination between positive and negative detection. The *error bars* represent the standard error of the mean from at least three independent assays. *Statistically significant difference with *P* value <0.001, in comparison to HL60 or BV173 samples (post hoc Tukey's test)

Samples were scored negative for e14a2 based on *ln(rAbs)* <0. Twelve clinical samples were correctly scored as positive for the e14a2 transcript and the remaining scored as negative. Even though all AML bone marrow samples were found negative for t(9;22) by FISH analysis, the Au-nanoprobe method was sensitive enough to detect one AML sample harboring the e14a2 fusion transcript, confirmed by RT-nested PCR. Moreover, the Au-nanoprobe procedure allowed to scrutinize peripheral blood follow-up samples expressing the e14a2 transcript. Sample 34, previously determined to express *BCR-ABL1* levels of 0.34 % (International Scale, IS), was correctly scored as positive. According to the 2015 ELN guidelines, these levels define a complete cytogentic response (CCyR) [26].

Care should be taken when borderline values are obtained, as seen in samples 28 and 38, to avoid the occurrence of false-positives. However, results based on *ln(rAbs)* are in accordance with RT-nested PCR results. PPV (12 true positive hits out of 12 positive calls) and NPV (26 true negative hits out of 26 negative calls) of 100 % indicate that the Au-nanoprobe assay under these conditions is a reliable test to scrutinize leukemia patients expressing the e14a2 fusion transcript.

Discussion

The rapid and fast molecular characterization of CML patients is of utmost relevance if suitable therapy is to be initiated. Routine molecular diagnostics requires cumbersome equipment and specialized technicians to evaluate the presence of the characteristic *BCR-ABL1* fusion transcripts that constitute

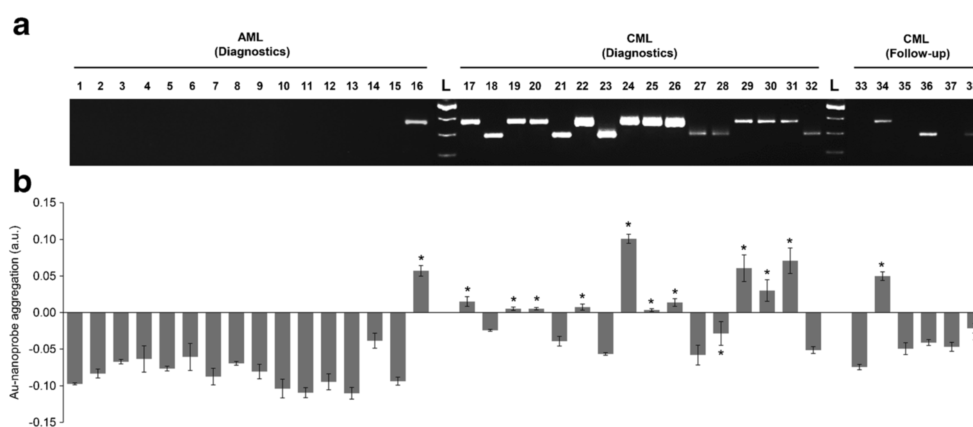

Fig. 4 Au-nanoprobe detection of the e14a2 *BCR-ABL1* transcript variant in total RNA clinical samples. RNA extracted from bone marrow or peripheral blood samples from patients previously diagnosed with AML (1–16) or CML (17–38). **a** Agarose (2 %) gel electrophoresis showing nested-PCR products using standardized primers for the detection of the e14a2 (360 bp) and e13a2 (285 bp) transcripts. Lanes *L* refer to DNA ladder (GeneRuler, Fermentas): 200, 300, 400, and 500 bp (from bottom to top). **b** e14a2 Au-nanoprobe assay. A threshold of 0 for Au-nanoprobe aggregation [*ln (rAbs)*] was considered for discrimination between positive and negative detection. The *error bars* represent the standard error of the mean from at least three independent assays. *Statistically significant difference with *P* value <0.001, in comparison to sample 1 (post hoc Tukey's test)

the hallmark of this disease. Here, we demonstrated for the first time that the Au-nanoprobe system based on the non-cross-linking mechanism may be used to rapidly identify e14a2-positive patients. Besides being able to detect a clinical sample with a *BCR-ABL1* fusion transcript, this approach allowed to discriminate between the two most frequent isoforms of this genetic abnormality—e13a2 and e14a2—that alone account for more than 95 % of CML cases. The attained PPV and NPV indicate that the Au-nanoprobe assay is suitable for the direct screening of patients' mRNA with obvious advantages in terms of time. It should be noted that the Au-nanoprobe assay detects the fusion transcript directly from RNA extracted from patient samples and, thus, is free from the stochastic bias introduced by the standard methodologies involving enzymatic retro-transcription into cDNA followed by PCR. Because it avoids these reaction steps, the Au-nanoprobe approach is much faster and simple, reducing the detection time frame from 9 h, required for RT-nested PCR alone, to 1 h and 30 min.

Diagnostic confirmation of myeloid leukemia patients where the Ph chromosome is undetectable by chromosome banding analysis or FISH is a major concern. Results achieved with an e14a2 expressing AML sample (negative for t(9;22) negative by cytogenetic analysis) and with follow-up samples with *BCR-ABL1* levels as low as 0.34 % (IS) indicate the Au-nanoprobe methodology is suitable for screening patients that exhibit a CCyR but still did not achieve a major molecular response (MMR). Note that ELN recommends the assessment and monitoring of the disease by RT-qPCR on buffy coat blood cells every 3 months, not only after a CCyR has been achieved but also from the beginning [26]. The Au-nanoprobe methodology would be highly advantageous in these consecutive tests due to its simplicity and low cost.

The Au-nanoprobe assay performance suggests that it might be useful for a combined diagnostics strategy: early screening via the Au-nanoprobes for point-of-care to quickly identify CML samples, followed by standard protocol on negative samples.

The possibility of fast and cheap screening technology based on RNA samples at admission is clearly advantageous. This is particular relevant for countries where the economic burden of molecular diagnostics of rare diseases is way too high to allow routine testing via proposed guidelines. Considerable time would be saved from the time of admission to the start of therapy, which in turn would impact on therapy success with advantages for patients.

The overall cost of the Au-nanoprobe assay is approximately €0.20 per sample without the need of dedicated instrumentation. This is particularly relevant considering that the percentage of newly reported cases of CML occurring in low- to middle-income countries has more than tripled over the past 40 years and that only about 5 % of the global resources spent on cancer are deployed in these countries [29, 30]. CML is one of the commonest adult leukemia in Indian population accounting for 30 to 60 % of all adult leukemia, yet little is known about the epidemiology of CML in such regions mostly due to poor registries, lack of conclusive molecular diagnosis, and the prohibitive cost of CML molecular characterization [31, 32]. Indeed, the first molecular test to measure *BCR-ABL1* in sub-Saharan Africa was performed in 2011 in Ethiopia [33]. The availability of rapid and cost-effective tests should result in reducing the rate of misclassifications for leukemia and will likely improve CML incidence and mortality rates in low- to medium-income countries.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Informed consent Patients were sent to the hematology department of the Hospital dos Capuchos (CHLC, Lisbon, Portugal) for leukemia diagnosis. Written informed consent was obtained from all participants and the study was approved by the Hospital dos Capuchos Ethics Committee. All approved ethical requirements for sample collection and assortment, processing, and analysis required by the Hospital dos Capuchos Ethics Committee have been strictly followed.

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