Ann Hematol (2013) 92:1319–1323
DOI 10.1007/s00277-013-1868-0

ORIGINAL ARTICLE

Rapid detection of *IDH2* (R140Q and R172K) mutations in acute myeloid leukemia

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Received: 11 June 2013 / Accepted: 29 July 2013 / Published online: 15 August 2013

Abstract NADP-dependent enzyme isocitrate dehydrogenase (*IDH*) mutations, *IDH1* and *IDH2*, have been described in acute myeloid leukemia (AML) using next generation sequencing approaches. *IDH2* mutations are heterozygous; they alter a single arginine residue at position 140 or 172 and have distinct prognostic significance. The current detection methods of *IDH2* mutations are laborious and time consuming as they require DNA sequencing. Herein, we report a new allele-specific oligonucleotide–polymerase chain reaction (ASO-PCR) method to detect the *IDH2* mutations. Analysis of leukemic DNA samples from 120 AML patients enabled to identify *IDH2* mutations in 22 cases which were confirmed by direct DNA sequencing. Of these, 17 harbored *IDH2* (R140Q) and 5 *IDH2* (R172K) mutations. Serial dilution experiments showed that the assay enable to detect mutations in 10^{-3} dilutions. Our ASO-PCR method appears useful for routine diagnostic screening of these prognostically relevant alterations in AML and may be conveniently included in the diagnostic workup.

Keywords Acute myeloid leukemia · Direct DNA sequencing · IDH2 mutations · Allele-specific PCR

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Introduction

Isocitrate dehydrogenase (*IDH*) is a component of the citric acid cycle which catalyzes oxidative decarboxylation of isocitrate. Two isocitrate dehydrogenases have been reported which utilizes NADP(+) as the electron acceptor one of which is mitochondrial (*IDH2*) and the other predominantly cytosolic (*IDH1*). *IDH* utilizes isocitrate as a substrate to convert $NADP^+$ into NADPH, which is necessary for nucleotide and fatty acid biosynthesis and cellular antioxidant activity. It has been shown that mutations in *IDH2* result in loss of function towards its ability to use isocitrate as a substrate. However, mutated form of *IDH2* is more effective in converting alpha ketoglutarate into an oncogenic metabolite 2-hydroxy glutarate (2-HG) [1–3]. A recent study has shown high levels of 2-HG in IDH mutants as compared to wild-type IDH. No significant difference was found in the levels of 2-HG between IDH1 and IDH2 mutated patients [4]. Arginine-based mutations in *IDH1* and *IDH2* genes have been reported in AML by whole genome sequencing [5, 6]. The available literature shows that 15–33 % of AML patients harbor in their leukemic cells somatic mutations in *IDH* genes [7–9] with *IDH2* mutations occurring more frequently than *IDH1* (19 and 6–14 %, respectively)[9, 10]. Mutations in *IDH2* gene are located in exon 4 at two positions, i.e. codon R140Q and codon R172K.

The prognostic impact of *IDH2* mutations in AML is controversial. However, recent findings showed that *IDH2*-mutated AML patients with normal karyotype (AML-NK) tend to show better overall survival as compared to patients with other *IDH* AML-NK mutations [11, 12]. Given the prevalence and prognostic relevance of *IDH2* mutations in AML, there is a need to develop simpler techniques for identifying these alterations in routine diagnostic screening of AML. Currently available methods are direct sequencing and PCR-based high-resolution melting (HRM) analysis [13]. Here, we developed an allele-specific PCR (ASO-PCR) technique for the detection of *IDH2* R140Q and R172K point mutations.

Table 1 Primer sequences used in the study

Name	Sequence	Nucleotide position	Application	Fragment size
IDH2-F	5′-AATTTTAGGACCCCCGTCTG-3′	90632139-90632158	Direct sequencing	345 bp
IDH2-R	5′-CTGCAGAGACAAGAGGATGG-3′	90631736-90631755		
*IDH2*M140F	5′-GAAAAGTCCCAATGGAACT***G***TCC***A***-3′	90631957-90631934	ASO-PCR	376 bp
IDH2 R	5′-CAGCCTCACCTCGTCGGTGTT-3′	90631583-90631603		
*IDH2*M172F	5′-CAAGCCCATCACCATTG***C***CA***A*** -3′	90631858-90631838	ASO-PCR	277 bp
IDH2 R	5′-CAGCCTCACCTCGTCGGTGTT-3′	90631583-90631603		
ABL-ENF	5′-GGAGATAACACTCTAAGCATAACTAAAGGT-3′	140329-140359	Primers for internal control	686 bp
ABL-ENR	5′-GATGTAGTTGCTTGGGACCCA-3′	140995-141015		

The bold and underlined letters represent mismatch

Patients and methods

One hundred and twenty AML patients (males 71, females 49, median age 69.5 years, range 60–90 years) consecutively diagnosed at the Department of Biopathology, Policlinico Tor Vergata, University of Rome, were included in the study. It has previously been reported that frequency of *IDH2* mutations increases with the age [7]; therefore, only elderly AML patients were selected in order to increase the potential number of mutated cases. Written informed consent was obtained from all patients according to the declaration of Helsinki and the study was approved by the IRB of Policlinico Tor Vergata.

DNA Extraction and ASO-PCR

DNA was extracted from Ficoll-Hypaque-isolated mononuclear cells obtained from patient bone marrow samples using Nucleospin DNA extraction kit (Macherey-Nagel, GmbH & Co., Germany). Specific forward primers containing a mismatch

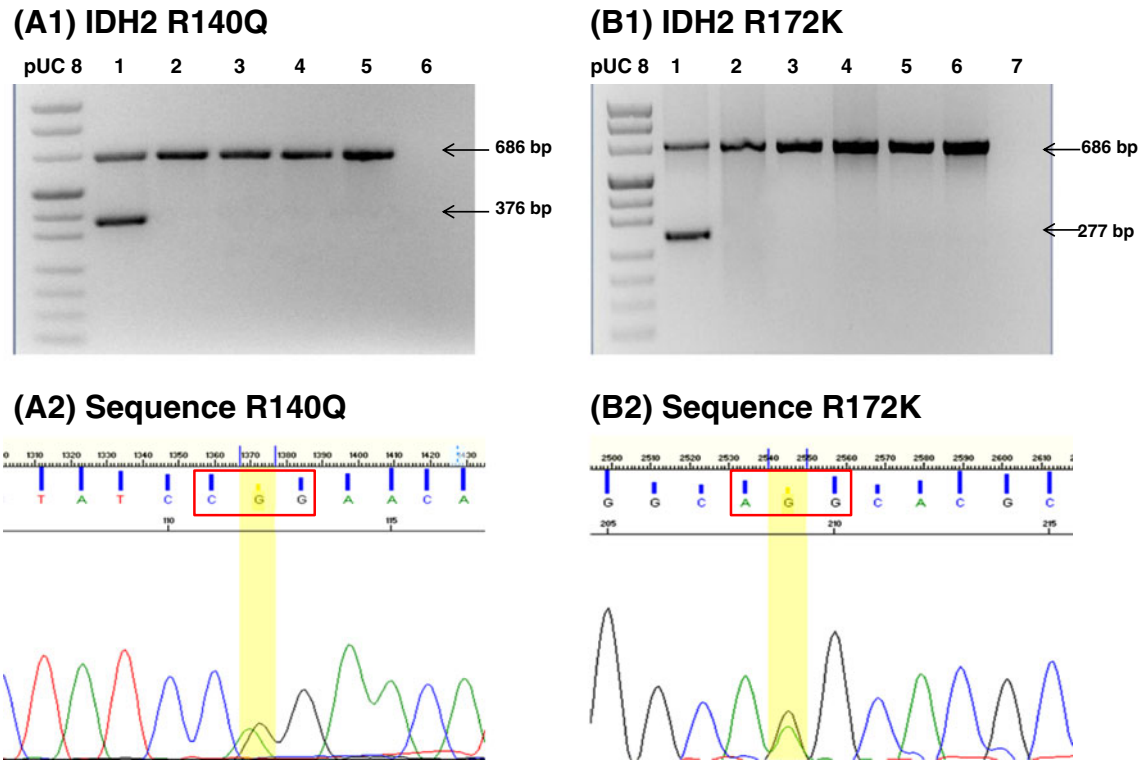

Fig. 1 (*A1*) ASO-PCR amplification band of 376 bp containing the mutation of *IDH2* (R140Q) is visualized in *lane 1*. *Lanes 2–5* show the amplification band of about 686 bp containing the *IDH2* wild-type sample showing only amplification of the ABL gene used as internal control. *Lane 6* is the blank (without DNA). (*A2*) Sequence result showing the IDH2 gene substitution CGG→CAG. (*B1*) Amplification band of 277 bp containing the mutation of *IDH2* (R172K) is visualized in positive patients sample in *lane 1*. *Lane 2–6* shows the *IDH2* wild-type amplification band. *Lane 7* is the blank (without DNA). (*B2*) Sequence result showing the substitution AGG→AAG

Table 2 Patient's detail

Variable	Wild type n=98	IDH2(R140Q) n=17	IDH2(R172K) n=5
Age			
Median	69	72	70
Range	60–90	62–84	61–76
Sex			
Male	58	11	2
Female	40	6	3
AML history[a]			
Denovo	82	15	5
Secondary	8	2	0
WBC count[b]			
Median	5300	2540	700
Range	1,140–130,170	990–89,000	600–2,280
Platelet count[b]			
Median	39,000	87,500	100,000
Range	3,000–635,000	14,000–213,000	33,000–142,000
Hemoglobin[b]			
Median	8.25	8	9
Range	6.2–19	4.9–12.9	6.4–11
NPM1 positive[c]	10	5	0
FLT3-ITD positive[c]	11	2	0

[a] Only 112 patient's data were available; 8 patient's data were missing

[b] Only 91 patient's data were available

[c] Only 109 patient's data was available

to improve specificity and a common reverse primer were designed to specifically amplify *IDH2* (R140Q) and *IDH2* (R172K) mutations on exon 4. *ABL* was used as an internal control (using *ABL*-ENF as forward and *ABL*-ENR as a reverse primer). Primer sequences and PCR product size obtained after amplification are shown in Table 1. One hundred nanograms of DNA was amplified in a total volume of 20 μl using HotStarTaq-Plus Master-Mix Kit (QIAGEN, Germany). Ten picomoles of each *IDH2* specific and common primer and 5 pmol of forward and reverse ABL primers were included. The PCR conditions included preheating of the mixture at 95 °C for 5 min followed by 35 cycles for 30 s at 95 °C, annealing for 1 min at 62 °C for *IDH2* (R140Q), and 1 min at 60 °C for *IDH2* (R172K); elongation for 45 s at 72 °C with a final extension of 5 min was carried out at 72 °C. PCR products were visualized by electrophoresis on a 2 % (*w*/*v*) agarose gel by loading 5 μl of PCR product and then the remaining PCR product was purified using a QIAquick PCR purification kit (Qiagen, Chatsworth, CA) for sequencing analysis.

Direct DNA sequencing

All patient samples showing *IDH2* mutations by ASO-PCR were subject to direct sequencing using the primers *IDH2*F and *IDH2*R [9] (Table 1). In brief, all purified amplicons were directly sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in conjunction with GeneAmp 9700 PCR Systems (Applied Biosystems). Each 10 μl of the sequencing reaction contained the following: 1 μl of BigDye v3.1 (Applied Biosystems), 2 μl of BigDye® Terminator v1.1/3.1 Sequencing Buffer (5×), 1 μl of PCR primer (5 μM), approximately 3 ng/200 bp of purified PCR product, and enough Gibco distilled water (Invitrogen, Grand Island, NY) to bring the total volume to 10 μl. Thermocycling parameters for PCR product sequencing were as follows: 1 min at 96 °C, 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C for 40 cycles. Sequencing reaction products were purified using the Centri·Sep columns (Applied Biosystems) according to the manufacturer's recommendations. Samples were diluted with 16 μl of ABI HiDi Formamide (Applied

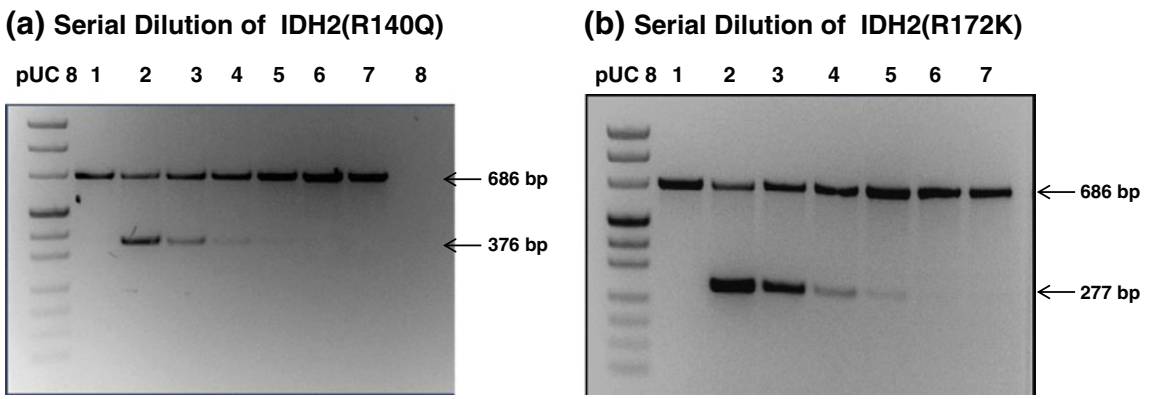

Fig. 2 ASO_PCR: serial dilution of patient DNAs containing *IDH2* (R140Q) (**a**) or *IDH2* (R172K) (**b**) mutation. *Lane 1* shows negative control; *lane 2*, undiluted positive control. *Lanes 3–7* mutated samples with dilutions of 10^{-1} to 10^{-5}. *Lane 8* shows the blank (without DNA)

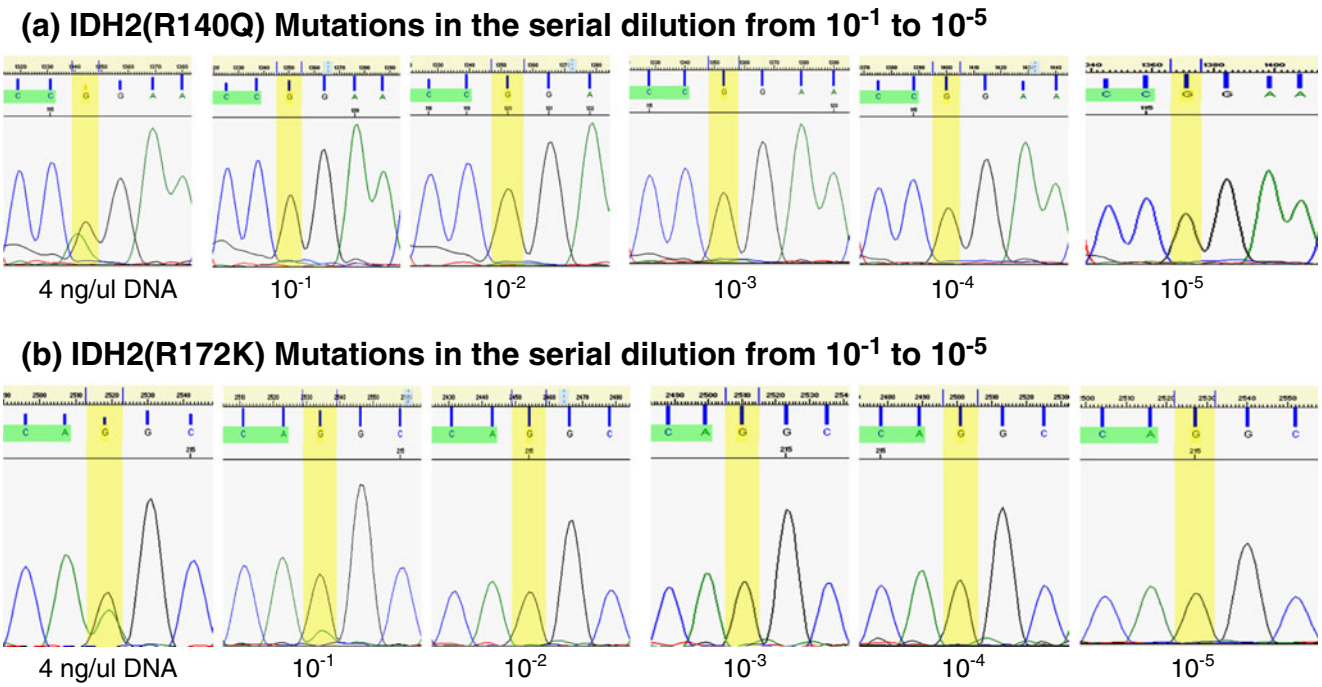

Fig. 3 Sanger sequencing in serially diluted DNA samples. **a** *IDH2* (R140Q): double peak is visible only in 4 ng/μl DNA; **b** *IDH2* (R172K): mutation is detectable till 10^{-1} dilution

Biosystems) and resolved on an ABI 3130 automated sequencer (Applied Biosystems).

Results and discussion

ASO-PCR allowed us to identify *IDH2* mutations in 22 out of total 120 (18.3 %) AML cases, a frequency similar to that published by Paschka et al. in a larger study [8].

Of these, 17 harbored *IDH2*: R140Q (14.16 %) and 5 *IDH2*: R172K (4.16 %) mutations. Direct DNA sequencing in both directions was performed in all 120 (wild-type and mutated) cases to confirm the results obtained by ASO-PCR. Following Sanger sequencing, no other substitutions were detected in exon 4 of *IDH2* except for *IDH2* 140 or *IDH2* 172 positions. All mutated patients of *IDH2* 140 revealed the substitution CGG→CAG and *IDH2* 172 showed the substitution AGG→AAG. The DNA sequencing results of *IDH2*: R140Q and *IDH2*: R172K in two representative patients are shown in Fig. 1

Clinical and biologic features of AML patients at diagnosis according to IDH2 mutations are shown in Table 2. We observed that (1) *IDH2* R140Q mutation is more frequent (13.6 %) than *IDH2* R172K (4 %); (2) out of 15 *NPM1* type A mutated patients, five (33 %) were also positive for *IDH2* R140Q; (3) with regard to *FLT3-ITD* positive patients (n=13), two patients had *IDH2* R140Q mutation. We did not find any *IDH2* R172K-positive patient carrying either *NPM1* type A or *FLT3-ITD* mutation.

To assess the sensitivity of the ASO-PCR assay, we performed serial dilution experiments using DNA of *IDH2* wild-type and mutated patients. The median blasts at diagnosis in *IDH2*-mutated patients were 80 %. R140Q was weakly detected at 10^{-3} but amplification was clearly evident up to 10^{-2} dilution, whereas in case of R172K amplification was clearly visible until the 10^{-3} dilution (Fig. 2a, b). Direct sequencing of serially diluted *IDH* mutated patient's DNA did not reveal any positivity from 10^{-1} to 10^{-5} dilutions while undiluted DNA showed double peaks confirming positivity for R140Q mutation. In case of R172K mutation, the double peak remains detectable until 10^{-1} dilution (Fig. 3a, b)

Following the discovery of *IDH* mutations in 2009, more than 40 additional studies on this alteration and its prognostic significance in AML have been reported in literature [14, 15]. In recent past, several studies have reported various strategies for *IDH2* mutation detection yet no study has published ASO-PCR-based detection of *IDH2* mutations. This report in terms of its simplicity is equivalent to the one designed by Baxter et al. [16] for *JAK2* mutational screening in myeloproliferative syndromes, which has become extremely popular and widespread due to its easy execution and reliability. Patel et al. [13] recently utilized HRM curve analysis for *IDH1* and *IDH2* mutational screening in 146 AML patients and identified 12 patients with *IDH2* R140Q and 4 patients *IDH2* R172K mutations. In conclusion, the ASO-PCR method described here represents a specific and sensitive method for the screening of *IDH2* (R140Q and R172K) mutations in AML patients at diagnosis. Our method is convenient, easily applicable, and

rapid. Finally, this ASO-PCR-based detection method for *IDH2* mutations would be useful in countries with limited resources and no access to either DNA sequencer or DHPLC instruments.

Acknowledgments This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro and Associazione Italiana contro le Leucemie, Linfomi e Mieloma (to F.L.C.).

Conflict of interest The authors declare that they have no conflict of interest.

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