Minimal Residual Disease assessment of IDH1/2 mutations in Acute Myeloid Leukemia by LNA-RQ-PCR

Suivi de la maladie résiduelle par les mutations IDH1/2 dans les Leucémies Aiguës Myéloïdes par LNA-RQ-PCR

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RÉSUMÉ

Prérequis : Avec l'importance croissante de la maladie résiduelle minimale (MRD) et la découverte récente des mutations des gènes IDH dans les leucémies aiguës myéloïdes (LAM), la quantification de ce marqueur moléculaire fournit la possibilité de contrôler la progression de maladie et l'efficacité thérapeutique dans les LAM de l'adulte.

Objectif : Notre but est d'évaluer la MRD en utilisant pour la première fois les mutations géniques IDH1 et IDH2 chez 15 patients présentant une LAM.

Méthodes : Nous avons examiné les mutations R132 IDH1, R140 IDH2 et R172 IDH2 par amplification et séquençage direct et nous les avons quantifiées en utilisant, pour la première fois, des amorces reverses modifiées par un analogue d'acide nucléique ou LNA. Une bonne sensibilité a été obtenue. Les taux de MRD obtenus par LNA-RQ-PCR ont été utilisés pour tracer la cinétique d'évolution de la maladie au cours du suivi des patients.

Résultats : Les résultats de IDH1/2 ont été comparés avec ceux de la mutation du gène NPM1 et de la surexpression du gène WT1 montrant une cohérence des taux de MRD dans 7/11 cas. Pour les cas restant, la technique de séquençage direct et l'essai de fusion à haute résolution (HRM) ont confirmé les résultats de quantification au diagnostic, mais pas pour les échantillons résiduels.

Conclusion : Quelques optimisations sont nécessaires pour améliorer l'amplification de l'allèle muté. La LNA-RQ-PCR peu coûteuse, pourrait alors être la méthode de choix utilisable dans un petit laboratoire pour l'évaluation de la MRD dans les LAM en utilisant les mutations IDH1/2.

Mots-clés

LAM, MRD, LNA-RQ-PCR, gènes IDH1/2.

SUMMARY

Background: With the growing importance of minimal residual disease (MRD) monitoring and the recent discover of IDH mutations in acute myeloid leukemia (AML), the quantification of this molecular marker provides the possibility to monitor the disease progression and the therapy efficacy.

Objective: The aim of this study is to assess the MRD in AML for the first time with IDH1 and IDH2 gene mutations in 15 AML patients.

Methods: We have screened R132 IDH1, R140 IDH2 and R172 IDH2 mutations by PCR amplification and direct sequencing and we have quantified them for the first time by RQ-PCR using reverse primers modified by an LNA. A good sensitivity has been obtained. MRD rates obtained by LNA-RQ-PCR were used to draw kinetics of the disease evolution during the follow-up.

Results: IDH1/2 results were compared to NPM1 mutation and WT1 over expression and have showed coherent kinetic between MRD rates in 7/11 cases. For the rest, the direct sequencing and the high resolution melting (HRM) assay have confirmed the quantification results in diagnosis but not in residual samples.

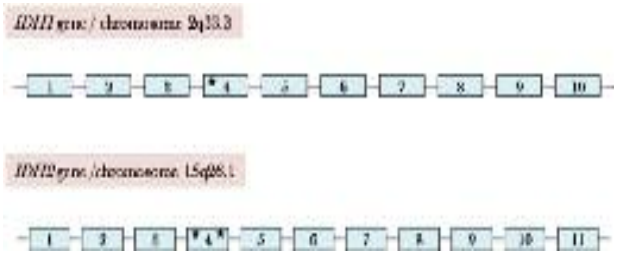
Conclusion: Some optimization will be necessary to improve the mutated allele amplification. The LNA-RQ-PCR might be an easy and less cost method used in a small laboratory for myeloid leukemia MRD assessment using IDH1/2 mutations.

Key-words

AML, MRD, LNA-RQ-PCR, IDH1/2 gene

Acute myeloid leukemia (AML) patients without cytogenetic aberration called normal cytogenetic AML (CN-AML) represent 50% of all AML cases and are stratified in the intermediate risk group (1). A number of gene mutations have been identified in these cases (2). Among them, the mutations of isocitrate dehydrogenase 1 (*IDH1*) and isocitrate dehydrogenase 2 (*IDH2*) genes (3,4) which are located in the human 2q33.3 and 15q26.1 chromosome region respectively (Figure 1).

Figure 1 : IDH1/2 gene stucture and localization of IDH1 and IDH2 mutations Boxes designed exons and stars designed mutation site



Mutations in *IDH1* and *IDH2* genes were first reported in gliomas then are described as frequent genetic alteration in AML (5,6,7). They are mutually exclusive and affect generally in the exon 4 the Arginine residue at position 132 (R132) of *IDH1* gene and positions 140 (R140) or 172 (R172) of *IDH2* gene (8). These three highly conserved residues are located within the isocitrate binding site (3,9,10). Interestingly, while the R132 *IDH1* and R172 *IDH2* mutation have been previously found in gliomas, the R140 *IDH2* mutation has not been reported in the begining in human cancer or in normal tissue (5,6). Contrarily to gliomas, *IDH1* and *IDH2* mutations are associated with a poor prognosis in AML (5,11).

The minimal residual disease (MRD) evaluation becomes increasingly important in the risk-adapted management of patients with AML (12,13). Since the mutations of *IDH1* and *IDH2* genes provided useful markers for AML diagnosis, monitoring response to therapy and also prognosis (8,14,15), they can be used as potential target for MRD assessment, especially they are stable during disease evolution (16). The mutations are heterozygous in almost all AML and the mutated allele is weakly present and so not easy to amplify by classical RQ-PCR. Improved allele-specific PCR methods have been reported in particular using locked nucleic acid (LNA) which displays unprecedented hybridization affinity and higher specificity towards complementary DNA and RNA (17,18).

LNA-RQ-PCR approach is successfully used for mutation quantification and can be easily implemented in clinical molecular diagnostic laboratories (19).

The aim of this study was to quantify the MRD rates in 15 AML patients using R132 *IDH1*, R140 *IDH2* and R172 *IDH2* genes mutations and test LNA-primers approach in the RQ-PCR essay. We have compared the *IDH* results to the quantification of *NPM1* gene mutation and *WT1* gene overexpression previously investigated in these AML.

PATIENTS

Patients

The study included 15 adult patients (median age of 60 years) with non M3-AML diagnosed in laboratory of Hematology of Lille (France). All patients were treated according to the Acute Leukemia French Association (ALFA) trials and received an intensive chemotherapy based on anthracycline and cytarabine for induction therapy and high-dose of cytarabine for consolidation therapy. Eleven patients, with available information, reached complete remission (CR), five of them relapsed and one died (UPN4) during the follow-up.

The study was approved by the ethics committees of the participating institutions before its initiation and was performed in accordance with the declaration of Helsinki. The characteristics of 15 patients studied are summarized in Table 1.

Mutation analysis of IDH1 and IDH2 genes

Genomic DNA was extracted from peripheral blood and/or bone marrow specimens from all patients at diagnosis and during follow-up using the Qiagen kit. While total RNA was extracted and reverse transcribed using the standardized protocol developed within the Europe Against Cancer (EAC) program (20). The screening of *IDH1* (R132) and *IDH2* (R140 and R172) mutations was performed by polymerase chain reaction (PCR) followed by direct sequencing. The exon 4 of *IDH1* gene was amplified from genomic DNA by PCR using the HotStar HiFidelity Polymerase kit (Qiagen) and the following forward and reverse primers:

IDH1-F (5'-GTGGCACGGTCTTCAGAGA-3')

and *IDH1*-R (5'-TTCATACCTTGCTTAATGGGTGT-3'). The total reaction volume of 50 µl contained 50 ng of genomic DNA, 0.2 mM of each dNTP, 1× Hotstar buffer, 2.5 mM of $MgCl_2$, 0.026 U of Taq Hotstar and 0.5 µM of each primer. The mixture was initially preheated at 95°C for 15 min, followed by 40 cycles of 95°C for 30s, 48°C for 30s, 72°C 1min, completed by a final elongation step of 72°C for 10 min. The detection of *IDH2* mutations was performed using the FastStart Taq DNA Polymerase kit (Roche) and the following primers:

IDH2-F (5'-TGAAAGATGGCGGCTGCAGT-3')

and *IDH2*-R (5'-GGGGTGAAGACCATTTTGAA-3'). The total reaction volume of 50 µl contained 50 ng of genomic DNA, 0.2 mM of each dNTP, 1× Hotstar buffer, 1× CG rich solution, 1.1 mM of $MgCl_2$, 0.03 U of Taq FastStart and 1 µM of each primer. The mixture was initially preheated at 95°C for 12 min, followed by 40 cycles of 95°C for 1min,

Tableau 1: Clinical and biological characteristics of AML patients UPN: unique patient number, CR: complete remission, WT: wild type allele, na: not available, A: insertion of TCTG, D: insertion of CCTG, F: female, M: male, U: unknown

UPN	Age / Gender	CR	Relapse	*IDH1/IDH2* mutation	*NPM1* mutation type	*WT1* expression
1	61 / U	yes	na	R132H	A	1162.48
2	53 / M	yes	no	R132H	A	105.05
3	59 / F	yes	no	R132H	A	17.80
4	68 / F	na	no	R132G	A	2.90
5	73 / F	na	na	R132G	A	190.52
6	67 / F	yes	yes	R132G	A	165.76
7	58 / M	yes	no	R132S	A	81.90
8	60 / F	yes	no	R132S	A	53.76
9	42 / M	yes	no	R140Q	A	82.67
10	69 / M	yes	yes	R140Q	D	20.25
11	45 / M	yes	yes	R140Q	A	53.23
12	61 / F	yes	yes	R172K	WT	0.55
13	65 / M	yes	yes	R172K	WT	1.82
14	43 / F	na	na	R172K	WT	na
15	59 / F	na	na	R172K	WT	na

55°C for 1min, 72°C 1min, completed by a final elongation step of 72°C for 10 min. Sequencing reaction was performed using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). The mixture was initially preheated at 98°C for 1 min, followed by 30 PCR cycles including 98°C for 10 s, 50°C for 10 s, 60°C for 4 min on GeneAmp PCR system 9700 (Applied Biosystems). Purified PCR products on sephadex resin were subsequently sequenced on an automated sequencer (3130 xL, Genetic Analyser, Applied Biosystems). Data were analyzed with the SeqScape software version 2.5.

LNA-RQ-PCR assay

In order to monitor the MRD in hematological malignancies using *IDH1* and *IDH2* mutations, we amplified the mutant allele that is weakly present in the neoplastic DNA studied by the RQ-PCR technique. The reverse primer was designed and modified by addition of LNA (Eurogentec) which is a synthetic analogue of nucleic acid characterized by the presence of a methylen between the positions 2'O and 4'C of the ribose. LNA enhances the duplex DNA stability during the hybridization step of PCR. Contrary to the wild type allele, only the mutated allele will be amplified.

The primer sequences used to amplify *R132 IDH1*, *R140 IDH2* and *R172 IDH2* mutations are summarized in the Table 2. For each mutation, two reverse LNA-primers types were designed and only the more specific will be retained for the study. The reverse primers contained an LNA fixed in the last 3'nucleotid (LNA-0) or in the second position from 3' nucleotide (LNA-2) were used for Taqman real-time quantitative PCR. Both reactions were performed into 25 µl volume containing 50 ng of cDNA, 1x of master mix (Applied Biosystems),

400 nM of each primer and 200 nM of minor groove binder (MGB) Taqman probe. The annealing temperature was of 56°C for 1 min and the amplification reaction was repeated 40 times in the Applied Biosysystems® 7900. Each sample was analyzed in triplicate. To provide a quantification assay, standard curves were needed and drawn with serial dilution of plasmids containing a pure *IDH* mutation of each type. These were obtained by cloning *IDH1/IDH2* mutations into the pUC pCR2.1 TOPO vector as previously described (21).

For each patient, specificity was performed using the two LNA-reverse primers while sensitivity was performed with the serial dilution of the plasmids.

The MRD rates with these two markers were evaluated by the relation of the copie number of the mutation of the target marker (*IDH1* or *IDH2*) to the copie number of standard *ABL* gene as :

MRD Rate = (target copie / *ABL* copie) x 100

While *NPM1* mutant transcripts and *WT1* mRNA levels were quantified as previously mentioned (21).

Examination points

A total of 88 samples of cDNA collected at diagnosis (11 cases) and at different time points of the follow-up were analyzed by LNA-RQ-PCR for both *IDH1* and *IDH2*

Tableau 2: Primers designed for MRD monitoring with IDH1/2 mutations

Gene	Mutation type	Reverse LNA-primer	Common Forward primer	Common MGB probe
IDH1	R132S	R-LNA-0: 5'-GATCCCCATAAGCATGACT-3'	5'-CGGTCTTCAGAGAAGCCATT-3'	5'-FAM-ATGGGTAAAACCTATCATC-3'
		R-LNA-2: 5'-GATCCCCATAAGCATGACT-3'		
	R132G	R-LNA-0:5'-GATCCCCATAAGCATGACC-3'		
		R-LNA-2: 5'-GATCCCCATAAGCATGACC-3'		
	R132H	R-LNA-0:5'-CTTGATCCCCATAAGCATGAT-3'		
		R-LNA-2:5'-CTTGATCCCCATAAGCATGAT-3'		
IDH2	R140Q	R-LNA-0: 5'-GTCCCCCCCAGGATGTTCT-3'	5'-AGTTCAAGCTGAAGAAGATGTGG-3'	5'-FAM-AGTCCCAATGGAACTA-3'
		R-LNA-2: 5'-GTCCCCCCCAGGATGTTCT-3'		
	R172K	R-LNA-0 :5'-GTCGCCATGGGCGTGCT-3'	5'-TCCCACGCCTAGTCCCTGGCTG-3'	5'-FAM-AGCCCATCACCAT-3'
		R-LNA-2 :5'-GTCGCCATGGGCGTGCT-3'		

markers. 26 samples of follow-up corresponding to mutated R140 *IDH2* (7.7 points of follow-up per patient) and 51 samples for the other patients mutated in *R132 IDH1 (*5.4 points of follow-up per patient) were included.

Tableau 3 : HRM and direct sequencing results versus LNA-RQ-PCR results
UPN: unique patient number, R: residual, D: diagnosis, WT: wild-type allele, Mut: mutated allele, na: not available

UPN	Disease stage	LNA-RQ-PCR	HRM	Sequencing
4	D	236.27	Mut	R132G
	R	144.29	WT	WT
	R	92.79	WT	WT
	R	239.71	WT	WT
	R	471.33	Mut	R132G
6	R	4.42	WT	WT
	R	22.82	WT	WT
	R	22.72	WT	WT
	R	38.37	WT	WT
	R	45.37	WT	WT
	R	254.02	WT	WT
	R	na	WT	WT
	R	na	WT	WT
7	D	110.48	Mut	R132S
	R	21.59	WT	WT
	R	56.98	WT	WT
	R	25.1	WT	WT
	R	33.12	WT	WT
	R	na	WT	WT
11	D	1414.71	Mut	R140Q
	R	1.255	WT	WT

High resolution melting assay (HRM)

HRM has been used to identify somatic mutations (22). It is a PCR-based method for detecting DNA sequence variation by measuring changes in the melting of a DNA duplex. After the PCR assay, carried out in the presence of a suitable fluorescent dye, the amplified product is heated and the level of fluorescence is measured as a melting curve in the presence of mutation (23). In case of heterozygous sample containing four duplex species , four individual melting curves were obtained (23,24). In order to verify the presence of *IDH* mutated allele in AML samples, we performed the HRM assay into 96-well plate containing 28µl of mixture including 5ng of genomic DNA, 0.05 µM of each primer, and the mastermix (Roche), 1.7 mM of $MgCl_2$. The assay was performed in the Light Cycler®480 instrument (Roche).

Statistical analysis

MRD rates of *IDH*, *NPM1* and *WT1* molecular markers at different time points of follow-up were considered as continuous variables. Correlation between two continuous variables was calculated by the Spearman rank correlation test. Statistical analysis was carried out using the Statistical Package for Social Sciences, version 20 (IBM, SPSS Statistics 20). A p value of less than 0.05 was considered statistically significant.

RESULTS

We have sequenced the amplified DNA of 15 AML studied. We have identified different types of R132 *IDH1* mutation for eight patients: three R132H, three R132G and two R132S mutations. *IDH2* mutations were identifed in the remaining AML: three R140Q and four R172K mutations (Table1).

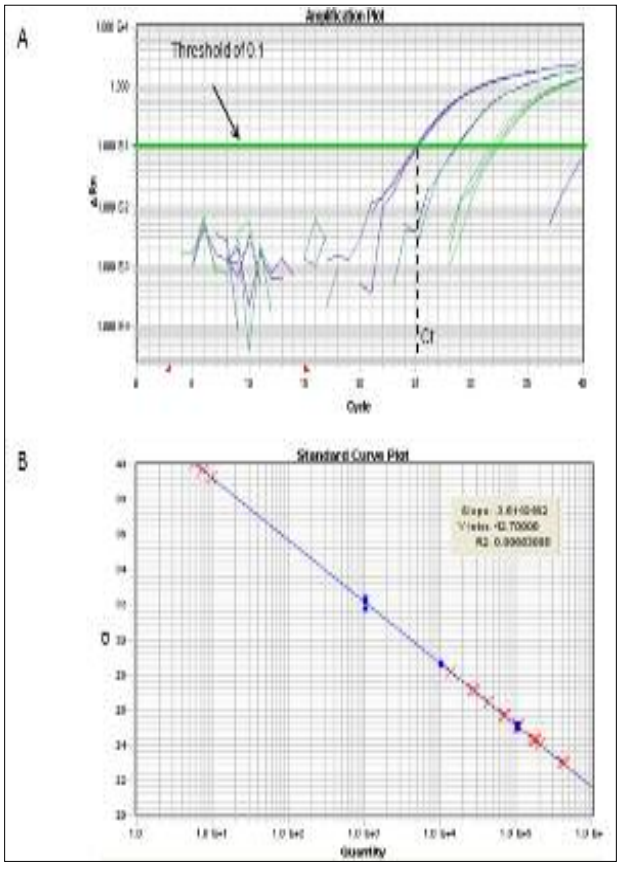
Only eleven patients (UPN1-UPN11) were assessed by LNA-RQ-PCR. The remaining patients showing R172K *IDH2* mutation were not assessed because of the exclusivity of this mutation (it is not associated with any known molecular marker).

LNA-RQ-PCR assay

The locked nucleic acid (LNA) primers were used to potentially improve sensitivity and specificity of quantitative PCR assay. To choose the more sensitive and suitable reverse LNA-primer, we tested simultaneously two types of LNA: LNA-0 and LNA-2 by RQ-PCR to investigate R132 *IDH1* and R140 *IDH2* mutations, using a common forward primer and a common MGB probe for each mutation. When we compared cycle number Ct values between mutated and wild type alleles we obtained best results with LNA-0. LNA-0 were then retained for the rest of the study.

The RQ-PCR assay was performed with the cDNA of each patient. Its quality was assessed by comparison of amplification results of housekeeping Abelson gene (*ABL*). We analysed 88 cDNA corresponding for each patient to dignostic and residual points. All samples were traited in triplicate wells in one run with appropiate water controls.

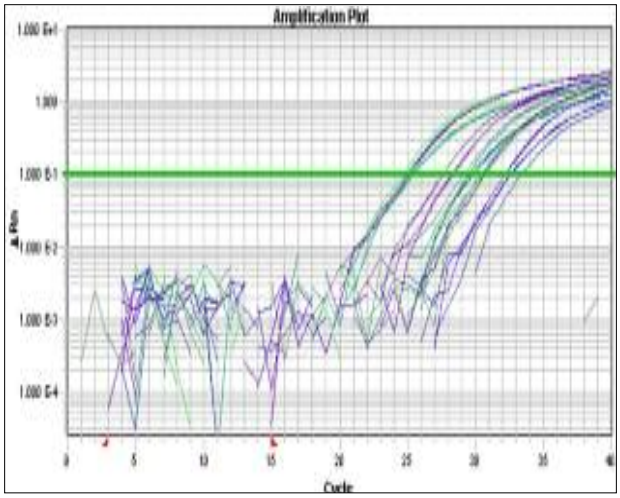
Figure 2 : Amplification curve (A) and standard curve (B) obtained with R132G IDH1 in UPN6



Data were reported using a common threshold of 0.1, where the Ct (cycle threshold) value denotes the PCR cycle at which fluorescence is detected above the background level (Figure 2 A). To define sensitivities for the LNA-RQ-PCR assay, the detection limit was designated as the lowest dilution of the plasmids giving rise to amplification above the background level.

For all patients, standard curves exhibit with a slope of -3.5 (Figure 2B), and the RQ-PCR assay showed a reproductible sensitivity going to 10^{-4} of the mutation into wild type background. An example of an amplification curve was giving in the Figure 3 in which Ct of each point will be extrapoled in the standard curve for the evaluation of the MRD level.

Figure 3 : Amplification curve obtained with R132G IDH1 in UPN6



Comparison of IDH1/2 results to NPM1 and WT1 markers

The MRD rates obtained by LNA-RQ-PCR for *IDH1/2* mutation were used to draw the disease evolution kinetic during the follow-up. Curves obtained were compared to those of two other molecular markers: *NPM1* mutation and *WT1* overexpression which have been already investigated in previous study (21). The MRD levels were also assessed and compared at different follow-up time-points to investigate the correlation between these three markers. The results of *IDH1* were not correlated significantly with the *NPM1* mutation either with peripheral blood or bone marrow samples (r =0.44, p =.15 and r = 0.53, p =.07 respectively). Similarly, *IDH1* results were not correlated significantly with the *WT1* expression with either peripheral blood or bone marrow (r =-0.13, p =.73 and r = 0.25, p =.53 respectively). While the results obtained with *IDH2* did not allowed for a statistical study.

For the eight patients R132 *IDH1* positive, five showed

Figure 4 : Comparison of MRD profiles obtained with (A) R132H IDH1, NPM1, WT1 markers (UPN2) and with (B) R140Q IDH2, NPM1, WT1 markers (UPN10) UPN: unique patient number, MRD: minimal residual disease, PB: peripheral blood, BM: bone marrow

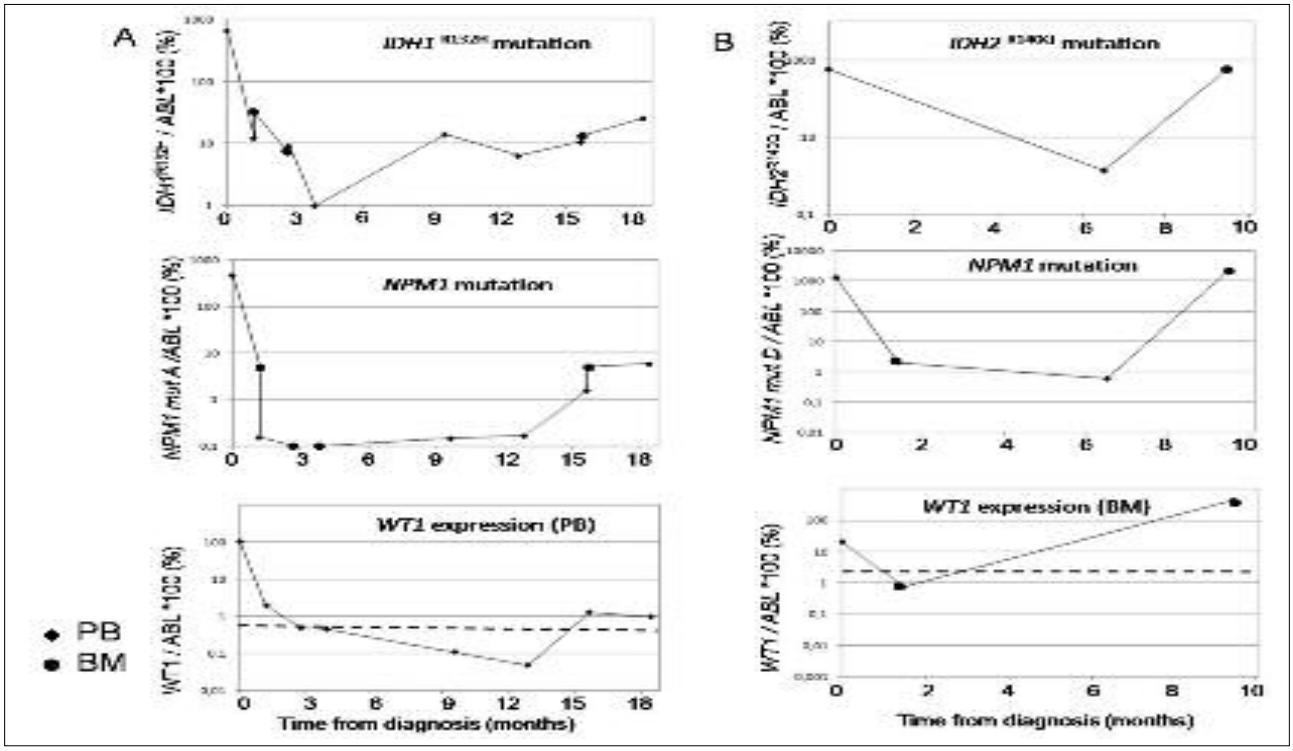
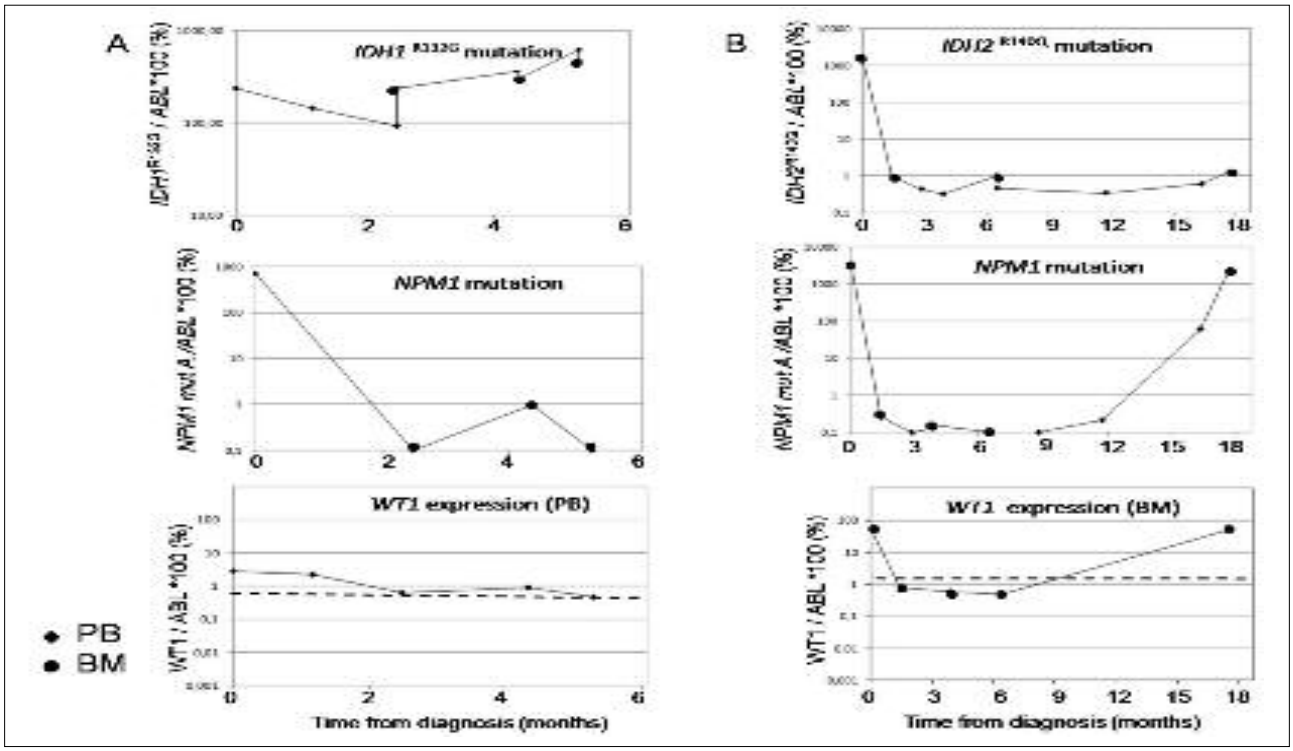


Figure 5 : Comparison of MRD profiles obtained with (A) R132G IDH1, NPM1, WT1 markers (UPN4) and with (B) R140Q IDH2, NPM1, WT1 markers (UPN11) UPN: unique patient number, MRD: minimal residual disease, PB: peripheral blood, BM: bone marrow



the same kinetic of the MRD during the evolution of the disease (Figure 4A). Kinetic curves looked also concordant for two cases among three patients R140Q *IDH2* positive (Figure 4B). They have similar kinetics of *IDH*, *NPM1*, *WT1* markers concerning the positivity or the negativity of the MRD rates. For the remaining cases of R132 *IDH1* positive, the MRD rate is still positive with *IDH1* whereas with *NPM1* and *WT1* the MRD rates are low or undetectable (Figure 5A). One patient (UPN6), who relapsed, showed by LNA-RQ-PCR increased MRD rates with only *NPM1* mutation and *WT1* overexpression indicating the molecular relapse. In one case of R140Q *IDH2* positive, after a complete remission period, the rates of the MRD obtained with *NPM1* and *WT1* increase considerably indicating an eventual relapse but remain low with *IDH2* (Figure 5B).

We have found different MRD rates were in four patients (UPN4, 6, 7 and 11) as they showed high MRD rate with *IDH1* or *IDH2* and negatives or undetectable MRD rate with *NPM1* and *WT1* markers. To verify our results of LNA-RQ-PCR, twenty-one samples issued from these 4 patients were analyzed by direct sequencing and by HRM assay (Table 3). Only 3 diagnosis DNA from 4 patients were available to perform these approaches. Sequencing as well as HRM showed the presence of *IDH* mutation which confirmed the LNA-RQ-PCR result at the diagnostic point. For the *IDH* residual samples, in only one case (UPN4) HRM assay revealed the mutated allele and sequencing confirmed the R132G *IDH1* mutation. For the remaining cases, MRD rates with LNA-RQ-PCR were high indicating the presence of the mutation during the disease follow-up, but sequencing or HRM assay showed *IDH* wild type allele.

DISCUSSION

In the present study, we have developed a screening method such LNA-RQ-PCR to assess for the first time the *IDH1/2* genes mutations in fifteen newly diagnosed AML patients. We have used reverse primers modified by addition of LNA to amplify preferentially *IDH* mutated allele which could be a suitable target for MRD monitoring in AML. Even though this approach has shown a good sensitivity, no sufficient specificity was obtained to detect and quantify a trace amount of mutated *IDH* gene. Few optimizations are needed to improve the marker amplification and use LNA-RQ-PCR as a simple, less costly method for MRD evaluation in a small laboratory.

Fifteen AML patients were included in this study. *IDH1/2* mutations were identified by direct sequencing. Eight cases of *IDH1* mutations and seven cases of *IDH2* mutations were found. Among 8 patients, three R132 *IDH1* mutation types were identified as following: 3 cases of R132H, 3 cases of R132G, 2 cases of R132S. For *IDH2* mutation cases, three patients presented only R140Q type and four patients exhibited the same R172K mutation. For the residual disease evaluation, the quantification of each *IDH* mutation type was conducted by LNA-RQ-PCR using reverse primers modified by LNA. Before MRD assessment, sensitivity and specificity of primers were performed. Since LNA bases can be placed at any position allowing thermal stability of oligonucleotides, we have chosen to add it in the last nucleotide (LNA-0) or in the second one (LNA-2). LNA-0 showed more specific amplification and the ΔCt was greater than 10 Ct compared to wild type and mutated alleles. A maximal reproducible sensitivity of 10^{-4} was reached with LNA-0 which we used for the rest of the study.

All patients were assessed by LNA-RQ-PCR except the four R172K cases which didn't present any other known molecular alteration so they didn't allow a comparative study. MRD rates of *IDH* mutations obtained in 11 AML cases were compared to those of *NPM1* mutation and *WT1* overexpression obtained by RQ-PCR. These molecular markers are usually stable during the disease follow-up (25,26,27), and are routinely used in hematology laboratory of Lille. Compared kinetic profiles of *IDH* mutation, *NPM1* and *WT1* alterations were coherent for the majority of patients and were concordant with the disease evolution. Nevertheless, discrepancies of results were obtained with these markers for only 4/11 patients which may reflect the non specific amplification of the wild type allele of *IDH1* or *IDH2* during the run. We have verified these false positive LNA-RQ-PCR results by the direct sequencing and the HRM assay. MRD rates were correlated to the presence of *IDH* mutation for diagnostic samples using the three approaches. While for residual samples, only one bone marrow sample (1/18) showed the presence of the mutated allele by the three techniques suggesting the specificity and the feasibility of the LNA-RQ-PCR technique for detecting of *IDH* mutation in bone marrow. For the rest, HRM or sequencing methods did not confirm the high MRD rates found with LNA-RQ-PCR. These results may be explained by the predominant wild type allele amplification because of the small proportion of the *IDH* mutation versus the wild type background. LNA primer has been used to block the wild type allele allowing more sensitivity and specificity (18,28,29); we have reached a good sensitivity but specificity has to be still improved.

We have tried in this study to evaluate for the first time the suitability of *IDH* mutations as a target for MRD monitoring by LNA-RQ-PCR. This method is simple, convenient for quick less time and cost consuming than others. Despite further elaboration, it's useful and a good alternative for a small laboratory which cannot easily develop the next generation sequencing. This novel approach should be used for the MRD quantification in AML patients which plays a key role in therapeutic decision and prognosis relevance of molecular markers.

Conflict of interest
The authors declare no conflict of interest.

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