High-resolution melting analysis for rapid and sensitive *NOTCH1* screening in chronic lymphocytic leukemia

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Abstract. Chronic lymphocytic leukemia (CLL) is a biological and clinical heterogeneous disease. Activating mutations of *NOTCH1* have been implicated to be associated with adverse prognosis in CLL. The objective of the present study was to develop an effective high-resolution melting (HRM) assay for detecting *NOTCH1* mutations. Genomic DNA (gDNA) extracted from 133 CLL patients was screened by HRM assay, and the results were compared with the data obtained using direct sequencing. The relative sensitivity of the HRM assay and direct sequencing was evaluated using diluted gDNA with different *NOTCH1* mutational frequencies. The HRM assay was able to detect and discriminate samples with *NOTCH1* mutations from the wild-type template in CLL. Eight of the 133 CLL patients (6.02%) were scored positively for *NOTCH1* mutations in the HRM assay. The results of the *NOTCH1* mutations detected by HRM analysis achieved 100% concordance with those determined from direct sequencing. HRM had a higher sensitivity (1%) and shorter turn-around time (TAT), compared to direct sequencing. In conclusion, the HRM assay developed by us was confirmed to be a rapid, sensitive, and promising approach for high-throughput prognostic *NOTCH1* screening in CLL. It enables real-time *NOTCH1* evaluation, which is of great significance in clinical practice and may facilitate the decision-making of clinicians in CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the proliferation and accumulation of malignant $CD5^+$ $CD19^+$ and $CD23^+$ mature, monoclonal B lymphocytes in the peripheral blood, bone marrow, lymph nodes, and other secondary lymphoid organs (1,2). The biological behaviors and the clinical features of the disease are significantly heterogeneous (3,4). Several factors, including immunoglobulin heavy chain variable region (*IGHV*) gene mutation, cytogenetic abnormalities, zeta-chain-associated protein kinase 70 (ZAP70) and CD38 expression at diagnosis, predict prognosis and help guide therapeutic decisions (1). Recurrent gene mutations such as *NOTCH1*, *MYD88*, *BIRC3* and *SF3B1* confer drug resistance and adverse prognosis in CLL (2,5,6).

Notch homolog 1, translocation-associated (*Drosophila*), also known as *NOTCH1*, encodes a single-pass class I transmembrane protein which exists as a non-covalently linked heterodimer (7). Functioning as a ligand-activation transcription factor, it is composed of an extracellular domain mediating ligand binding and an intracellular domain mediating signaling. Ligands, Delta-like and Jagged, following binding to the NOTCH receptor, induce proteolytic cleavage of the receptor, and result in the release and translocation of the intracellular domain to the nucleus, thus leading to transcriptional activation of multiple target genes including nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) (8-10).

NOTCH1 mutations in exon 34, selectively disrupt the carboxy-terminal proline-glutamate-serine-threonine-rich (PEST) domain of the protein, resulting in a truncated protein which is more stable, impaired NOTCH1 degradation, and constitutive transcriptional activation of the NOTCH1 downstream signaling including canonical and noncanonical NF-κB pathways (5,11-18). Mutations in *NOTCH1* are reported to be associated with a particularly poor outcome and may play a pivotal role in the pathogenesis and treatment resistance of CLL, and contribute to poor patient prognosis (19). Based on current research, activating mutations of *NOTCH1* occur at a low frequency in CLL patients at diagnosis (8.3-12.6%), but at a significantly higher frequency in patients with the more clinically aggressive *IGHV* unmutated subtype of CLL (20.4%), Richter syndrome (31.0%) and chemo-refractory CLL (20.8%) (5,13-15,20-23). Among all *NOTCH1* mutations, ~80% are located in exon 34 which are selected to disrupt the PEST domain.

High-resolution melting (HRM) assay is a technique for fast genotyping and high-throughput mutation of germ-line and somatic mutation analysis, which has been established since 2003 (24-26). The HRM melting profile provides a

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specific sequence-related pattern that differentiates wild-type sequences from homozygote or heterozygote variants. This method is based on real-time PCR amplification in the presence of a saturating intercalating fluorescent dye (27-29); mutations residing in the melting domain are visualized as alteration of the melting curve derived after PCR amplification by increasing the temperature and measuring the decrease of fluorescence emitted from within the double helix while the DNA strands separate (25,30,31).

In the present study, we developed an accurate and sensitive HRM assay for detecting somatic *NOTCH1* mutations in a total of 133 CLL patients. These gene mutations were further confirmed by direct sequencing, the gold standard. The results of HRM analysis achieved 100% concordance with those from direct sequencing. The HRM method we developed proved to be an effective, rapid and sensitive approach for *NOTCH1* screening with higher sensitivity and shorter turn-around time (TAT). It may be routinely used for the high-throughput screening of *NOTCH1* in clinical CLL patients at diagnosis or at any clinical course of CLL, which is significant for decision-making regarding therapeutic strategies.

Materials and methods

Clinical specimens. A total of 133 CLL bone marrow samples were obtained after informed consent from patients fulfilling diagnostic and immune-phenotypic criteria for CLL at the Hematologic Department, The First Affiliated Hospital of Soochow University. Only patients at initial diagnosis without prior therapy were included in the study. Positive cut-off values were 30 and 20% for CD38 and ZAP70 expression. The mutation status of the *IGHV* gene and cytogenetic alterations for all the patients included in this study were also analyzed. Germline *IGHV* was defined as ≥98% homology. Research was performed upon approval of the Ethics Committee of The First Affiliated Hospital of Soochow University.

Genomic DNA (gDNA) extraction. To obtain somatic DNA from CLL cells, the cells were isolated from bone marrow of CLL patients by density gradient centrifugation over lymphocyte cell separation media (Cedarlane Laboratories, Shanghai, China). After isolation, the cells were stained with anti-CD19, anti-CD5 and anti-CD20 antibodies (all from BD Biosciences, Shanghai, China), which were further analyzed by flow cytometry. Only CLL samples containing ≥95% $CD5^+CD19^+$ cells were included in the study. gDNA was extracted using gDNA isolation kits (Omega BioTek Guangzhou, Ltd., Guangzhou, China) according to the manufacturer's instructions. DNA was quantified using a NanoDrop ND-1000 fluorospectrometer (Thermo Fisher Scientific, Shanghai, China) and the A260/280 value was ensured between 1.8 and 2.0.

Cell culture. The acute lymphoblastic leukemia cell line Molt4 and the acute T cell leukemia cell line Jurkat, purchased from the Shanghai Institute for Biological Sciences (Shanghai, China), were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate at 37˚C in a humidified incubator at 5% CO_2.

HRM assay. The primers of *NOTCH1* used in this study are listed in Table I. The PEST domain of *NOTCH1* was amplified in two fragments (Ex34a and Ex34b) with product sizes of 131 and 116 bp. The HRM assay was performed using Fast EvaGreen® qPCR Master Mix (Biotium, Hayward, CA, USA) on a LightCycler 480 instrument (Roche Diagnostics, Beijing, China). The reaction mixture in a 20 μl final volume contained 1X Fast EvaGreen qPCR Master Mix, 200 nM forward primer and 200 nM reverse primer, 50 ng of genomic DNA and PCR grade water. The cycling and melting conditions were as follows: 95˚C for 2 min (95˚C for 5 sec, 60˚C for 35 sec, 72˚C for 25 sec) x50 cycles; HRM: 95˚C for 1 min, 40˚C for 1 min, 65˚C for 1 sec, with a continuous increase in temperature from 65˚C to 95˚C at the rate of 0.02˚C/sec with 25 signal acquisitions per degree; and cooling: 40˚C for 30 sec. The melting profiles of the amplicons were analyzed using LightCycler 480 Gene-Scanning software to detect wild-type and mutations. All samples were tested in triplicate.

Direct sequencing. *NOTCH1* screening (PEST domain; RefSeq NM_017617.4) in CLL was carried out by PCR amplification and direct sequencing. The primers used are listed in Table I. The PCR reaction was amplified using Platinum *Taq* DNA polymerase (Invitrogen, Beijing, China) and conducted under the following conditions: 94˚C for 5 min, (94˚C for 30 sec, 60˚C for 30 sec, 72˚C for 45 sec) x40 cycles; 72˚C for 10 min. The PCR products were checked on 2% agarose gels. PCR products were purified and followed by bi-directional sequencing using an ABI 3730 DNA Analyzer (Applied Biosystems, Inc., Beijing, China). Sequencing chromatograms were analyzed using DNA Baser 3.0. Nucleotide changes detected by sequencing were all checked in Sanger's COSMIC database, and diagnosed as mutations accordingly.

Sensitivity determination. Cancer cell lines, Molt4 and Jurkat, were used. Molt4 cells harbor deletion of a CT dinucleotide in the PEST domain of the *NOTCH1* gene (heterozygous for c.7541_7542delCT). Jurkat cells are wild-type of the *NOTCH1* gene. Serial dilutions of the *NOTCH1* mutant Molt4 cells with Jurkat cells were used to determine the sensitivity of the direct sequencing and HRM.

Statistical analysis. Data analysis was performed using GraphPad Prism 5 program (GraphPad Software, Inc., La Jolla, CA, USA). The Chi-square test was used to analyze biological features between *NOTCH1*-mutated and unmutated CLL groups. Differences were considered to be statistically significant when the p-value was <0.05.

Results

Detection of NOTCH1 mutations by HRM analysis. For the HRM assay, we chose the optimal temperature and the primer concentration to generate specific products with efficient amplification and melting with an acceptable profile. The normalized and shifted melting curves provide the basic representation of the different genotypes, while difference plots show the difference between fluorescence of a patient's sample and a wild-type template at each temperature transition. The normalized and shifted melting curves and difference plots of

Table I. Primers for direct sequencing or HRM assay of the PEST domain of the *NOTCH1* gene.

Method	Primer name	Sequences	Amplicon size
Sequencing	*NOTCH1* Ex34_F	5'-CTGGCGGTGCACACTATTCTG-3'	327 bp
	NOTCH1 Ex34_R	5'-GCGCGCCGTTTACTTGAAG-3'	
HRM	*NOTCH1* Ex34a_F	5'-ACAGCTACTCCTCGCCTGTG-3'	131 bp
	NOTCH1 Ex34a_R	5'-GTCGGAGACGTTGGAATGCG-3'	
	NOTCH1 Ex34b_F	5'-GTGCACACTATTCTGCCCCAG-3'	116 bp
	NOTCH1 Ex34b_R	5'-GAGTAGCTGTGCTGCGAGG-3'	

HRM, high-resolution melting; PEST, proline-glutamate-serine-threonine-rich.

Table II. *NOTCH1* mutations of the CLL patients.

Mutation	CLL patients	Direct sequencing	HRM analysis
CLL-48	c.7541_7542delCT	p.P2514fs*4	Mutated
CLL-108	c.7541_7542delCT	p.P2514fs*4	Mutated
CLL-109	c.7541_7542delCT	p.P2514fs*4	Mutated
CLL-185	c.7541_7542delCT	p.P2514fs*4	Mutated
CLL-213	c.7541_7542delCT	p.P2514fs*4	Mutated
CLL-232	c.7535_7536insC	p.S2513fs*3	Mutated
CLL-250	c.7541_7542delCT	p.P2514fs*4	Mutated
CLL-295	c.7541_7542delCT	p.P2514fs*4	Mutated

CLL, chronic lymphocytic leukemia; HRM, high-resolution melting.

Table III. Characteristics of the CLL patients according to *NOTCH1* mutations.

Characteristics	All (n=133) N (%)	*NOTCH1* wild-type (n=125) N (%)	*NOTCH1* mutated (n=8) N (%)	P-value
IGHV unmutated	41 (30.8)	35 (28)	6 (75)	0.0053
Trisomy 12	30 (22.6)	25 (20)	5 (62.5)	0.0053
$CD38^+$ expression	21 (15.8)	19 (15.2)	2 (25)	0.4612
$ZAP70^+$ expression	22 (16.5)	20 (15)	2 (25)	0.5066

CLL, chronic lymphocytic leukemia.

6.02% (8/133) of the patients were significantly different from those of the wild-type control. There were two types of shifted melting curves and difference plots in the 8 CLL samples with *NOTCH1* mutations, represented by CLL-108 and CLL-232 (Fig. 1A and B). The melting curves of 7 out of 8 *NOTCH1*-mutated patients were exactly the same. The 7 patients were confirmed to be c.7541_7542delCT (p. P2514fs*4) mutated by direct sequencing represented by CLL-108 (Fig. 1C). The melting curve of 1 out of 8 *NOTCH1*-mutated patients was different. This patient was confirmed to be c.7535_7536insC mutated (p.S2513fs*3) represented by CLL-232 (Fig. 1D).

Screening for NOTCH1 mutations in the PEST domain by direct sequencing. We also analyzed the *NOTCH1* mutation status of 133 samples with CLL by direct sequencing and then compared the findings to that of the HRM assay. Among the 133 patients, 8 (6.02%) patients, who scored positively in the HRM assay, also turned out to carry somatic mutations of the *NOTCH1* gene, indicated by direct sequencing (Table II). All the mutations were heterozygous. Of them, 7 (87.5%) cases had the 2-bp frame-shift deletion, c.7541_7542delCT (p.P2514fs*4) and 1 case had a frame-shift insertion, c.7535_7536insC (p.S2513fs*3). Another 93.98% (125/133) of the CLL samples, showing consistency in melting curves and temp-shifted plots from those generated by the wild-type template, were implicated to be *NOTCH1* wild-type in the direct sequencing. Above all, the results of the *NOTCH1* mutations detected by HRM analysis were 100% consistent with the findings from the direct sequencing.

NOTCH1 mutations are associated with adverse biological features in CLL patients. The main biological features of the CLL cohort according to *NOTCH1* mutations are listed in Table III. *NOTCH1*-mutated CLL patients presented with higher frequencies of germline *IGHV* unmutated (6/8, 75%) status and trisomy 12 (5/8, 62.5%) than these frequencies noted in the *NOTCH1*-unmutated patients. It is noteworthy that there were significant correlations between *NOTCH1* mutations and *IGHV* status (p=0.0053) or trisomy 12 (p=0.0053). There were no significant correlations between expression level of CD38 (p=0.4612) or ZAP70 (p=0.5066) with *NOTCH1* mutations.

Sensitivity evaluation by HRM and direct sequencing. Cancer cell lines with known *NOTCH1* genotype were used for the validation and sensitivity testing for the HRM assay and direct sequencing. The acute lymphoblastic leukemia cell line Molt4 harboring a heterozygous 7541_7542delCT (p.P2514fs*4) in *NOTCH1* was used as a positive control and the acute T-cell leukemia cell line Jurkat which has a wild-type *NOTCH1* genotype was used as a negative control. The gDNA of the Molt4 cells was serially diluted into Jurkat gDNA at ratios of 100, 40, 20, 10 and 2% to yield mutant allele frequencies of 50, 20, 10, 5 and 1%. The relative sensitivities of direct sequencing and HRM were evaluated using the diluted gDNA. The

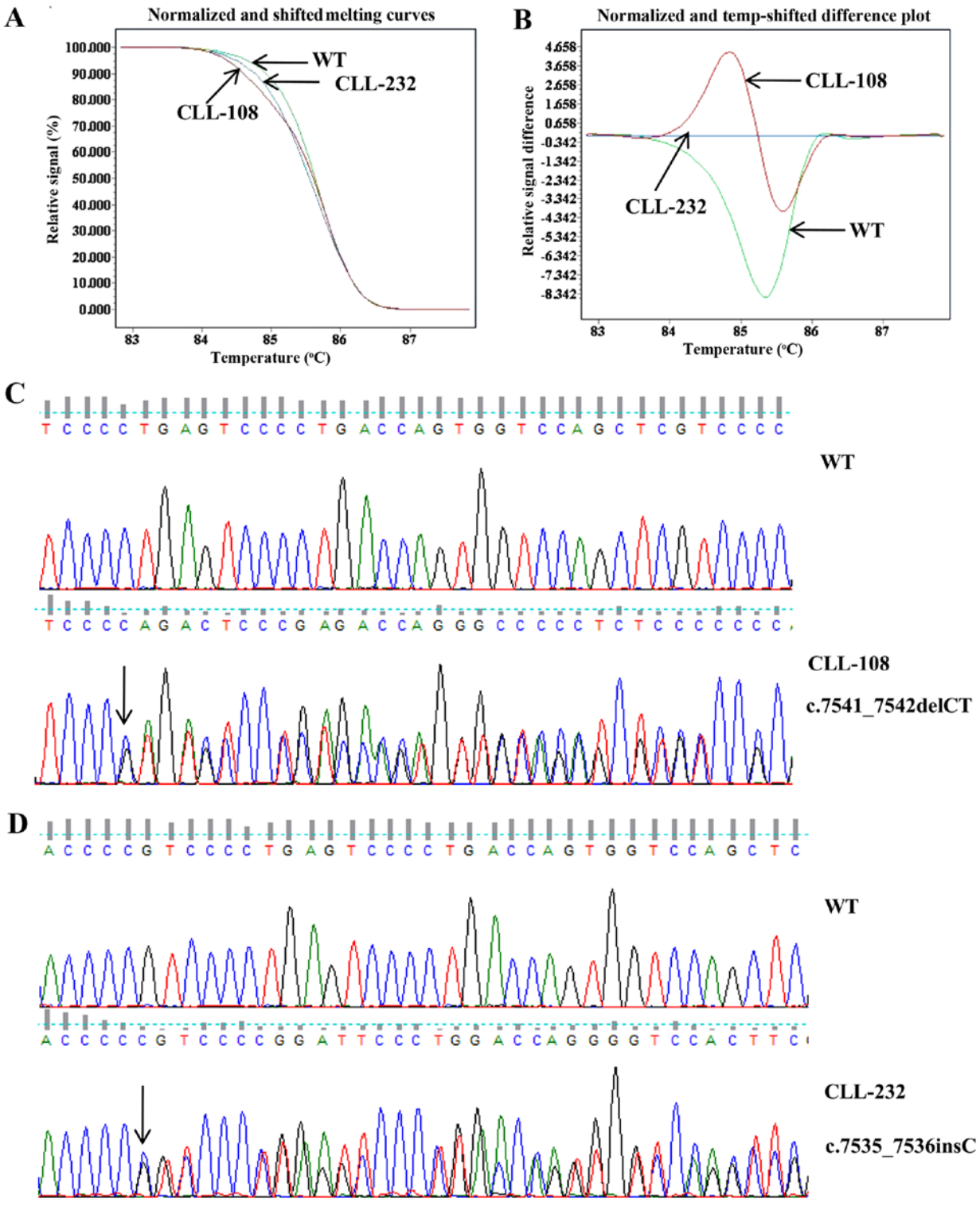

Figure 1. Melting curves and difference plots and sequencing traces for the PEST domain of *NOTCH1*. (A) The normalized and shifted melting curves of *NOTCH1* showed that the HRM assay could distinguish the heterozygous genotype c.7541_7542delCT (CLL-108 in red) and heterozygous genotype c.7535_7536insC (CLL-232 in blue) from the wild-type. (B) The normalized and temp-shifted difference plots showed that the HRM could distinguish the heterozygous genotype c.7541_7542delCT (CLL-108 in red) and heterozygous genotype c.7535_7536insC (CLL-232 in blue) from the wild-type. Sequencing chromatograms confirmed (C) the heterozygous genotype c.7541_7542delCT in CLL-108) and (D) the heterozygous genotype c.7535_7536insC in CLL-232. HRM, high-resolution melting; CLL, chronic lymphocytic leukemia; WT wild-type.

mutation was detectable at a low peak by direct sequencing when the mutant frequency was >10%. However, when the mutation frequency was at 5%, it was only distinguishable from the background. When the mutant frequency was <5%, the mutation was not detectable (Fig. 2A). Fig. 2B shows a normalized and shifted melting curves plot and normalized and temp-shifted difference plot of the HRM data. The melting curve from 1% mutant template sufficiently differed

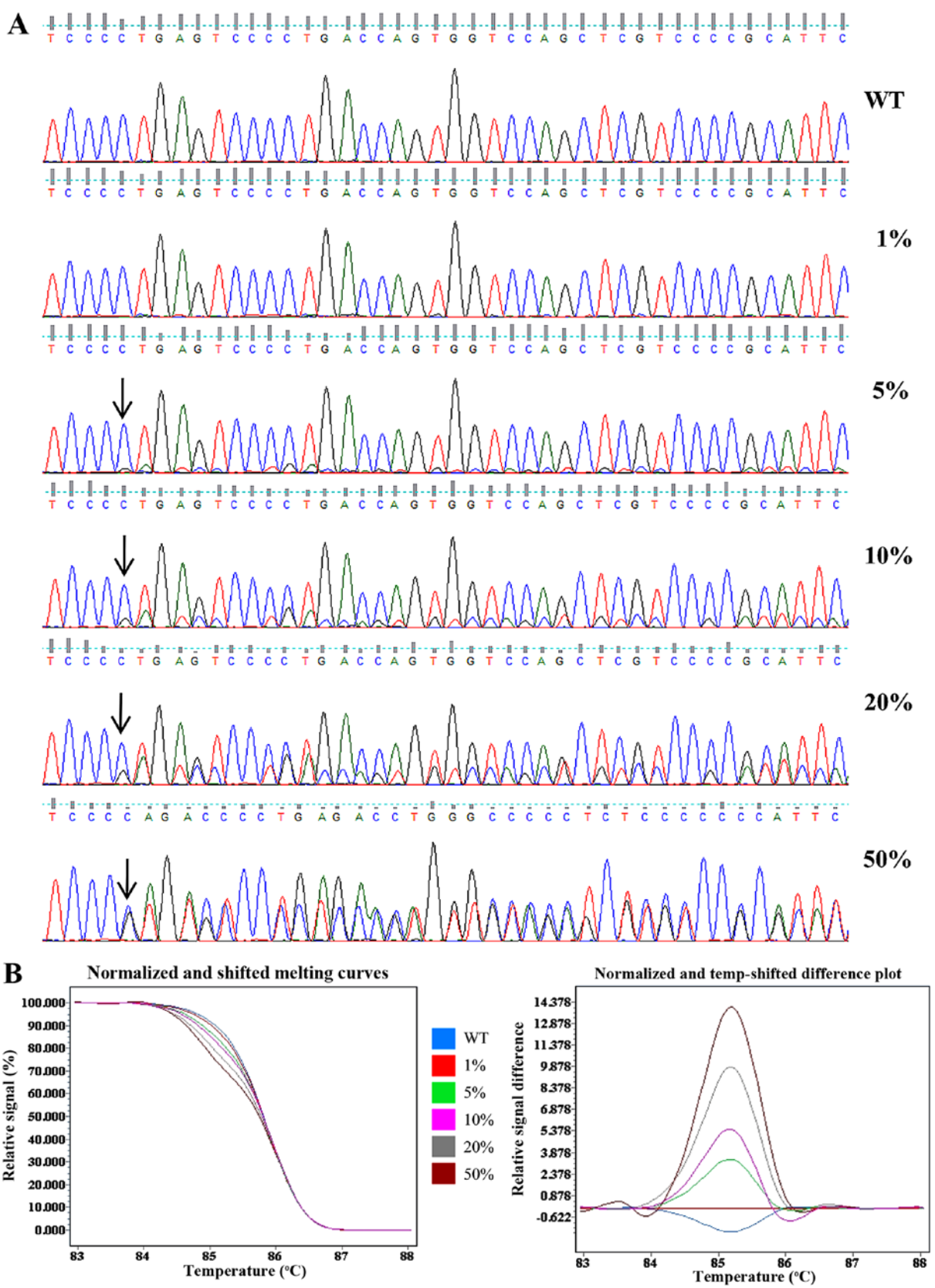

Figure 2. Validation and sensitivity testing for the HRM assay and direct sequencing. (A) Direct sequencing. At least 10% mutant gDNA was necessary to detect *NOTCH1* mutations. (B) HRM. One percent mutant gDNA was necessary to be plotted differently from wild-type gDNA. HRM, high-resolution melting; gDNA, genomic DNA; WT, wild-type.

from the wild-type template, and this distinct melting profile was consistently observed across all other templates measured (5, 10, 20 and 50%). Thus, the sensitivity of direct sequencing and HRM was found to be 10 and 1%, respectively.

Table IV. Comparison of direct sequencing and HRM assay.

Methods	Facts	Sensitivity (%)	Mutation analysis methods TAT, d[a]
HRM	Detects both known and unknown mutations; inexpensive; requires sequencing validation	1	0.5
Direct sequencing	Detects every nucleotide change; expensive; criterion standard	10	3

[a]The *NOTCH1* mutation analysis method TAT is defined as the time spent from measuring DNA extraction to the time that the results are generated from the instrument; it excludes the time spent for DNA extraction. TAT, turn-around time (d, days); HRM, high-resolution melting.

Discussion

The presence of *NOTCH1* mutations disrupting the carboxy-terminal PEST domain appears to confer adverse prognosis in CLL (11,12,14,15). Evaluating the *NOTCH1* mutation status is useful in clinical practice for patients with CLL and may facilitate therapeutic decision-making (16,32,33). Therefore, selection of method for detecting somatic mutation with high sensitivity and specificity are of great importance. In this study, we successfully developed a powerful, highly sensitive, cost-effective and easy to perform approach for *NOTCH1* mutation screening in CLL. Most important, this is the first study to report *NOTCH1* mutation detection in bone marrow samples of Chinese CLL patients using the HRM assay.

In the 133 newly diagnosed CLL patients, the melting curves of 6.02% (8/133) of the CLL patients were markedly different compared with the other 125 patients. The melting curves of 7 out of 8 *NOTCH1*-mutated patients were exactly the same. The 7 patients were confirmed to be c.7541_7542delCT mutated. The melting curve of 1 out of 8 *NOTCH1*-mutated patients was different. This patient was confirmed to be c.7535_7536insC mutated. All of the 133 CLL samples lacked single nucleotide polymorphisms (SNPs) according to the direct sequencing. SNPs are the result of genomic variation or changes of a single nucleotide in the genomic DNA. The HRM method has been successfully used in SNP genotyping and mutation detection (34,35). Indeed, there are certain SNPs in the region of our PCR amplicons according to the dbSNP database. Unexpected SNPs may generated unique amplicon melting patterns (36,37) (shifted melting curves and difference plot patterns) and be differentiated from either the c.7541_7542delCT or c.7535_7536insC mutation. Altogether, the HRM analysis used by us generated specific melting profiles that allowed the discrimination between wild-type and mutated samples (Fig. 1). Our HRM assay was confirmed to be reliable since all mutations detected by the HRM assay were also confirmed by direct sequencing.

In the present study, direct sequencing confirmed that 6.02% of the 133 Chinese CLL cases at diagnosis harbored *NOTCH1* mutations in the PEST domain, and there were two genotypic heterozygous variations, c.7541_7542delCT (p.P2514fs*4) and c.7535_7536insC (p.S2513fs*3). The former mutation type comprised up to 87.5% (7/8) of the mutations. This frequency is consistent with a report in the Chinese population by Xia *et al* (38), but lower than that found in a series of studies of European CLL patients (13,14,39). Therefore, there are discrepancies between Asian and European populations. CLL patients with mutated *NOTCH1* showed a higher frequency of unmutated *IGHV* and trisomy 12, with statistically significant differences. This is in concordance with several previous studies (40-42).

The high sensitivity of the method was also confirmed in our experiments. Using cell lines with wild-type and heterozygous c.7541_7542delCT *NOTCH1* mutation, we found that the sensitivity of HRM in our experimental setting was 1%, while direct sequencing analysis had a sensitivity of 10%. However, the frequency of the *NOTCH1* mutation detected by HRM was the same as that of direct sequencing, indicating that the mutational burden in our CLL cohort was >10%. Actually, the samples we analyzed were CLL monoclonal cells. Only CLL samples containing ≥95% of $CD5^+CD19^+$ cells were included in the study.

Direct sequencing, known as the 'gold standard', has been used to detect somatic mutations for many years. It is able to detect any mutation in the DNA sequence being analyzed. But its limited sensitivity, high cost and long TAT have prompted the development of alternative methods for routine clinical testing which have greater diagnostic practicality for somatic mutation detection.

HRM is more sensitive, faster, less expensive and time saving than direct sequencing. In this study, the entire procedure needs ~50 ng gDNA and requires 0.5 days for the TAT, one sixth of the time compared to direct sequencing (Table IV). Another major advantage for HRM over direct sequencing is that it is performed in a 'closed tube' system. This eliminates the risk of post-PCR product contamination during scanning while also reducing processing time, since our PCR and HRM assay were performed within one instrument in the present study.

However, it has also been reported that this method can produce false-positive results due to bad DNA quality, particularly when the starting material is formalin-fixed, paraffin-embedded (FFPE) tissue (43). In the present study, the gDNA we detected was extracted from the bone marrow of the CLL patients, therefore the DNA quality was controllable. The concordance rate between HRM and sequencing was 100%. There was only a slight false-positive chance for our analysis.

To the best of our knowledge, HRM analysis has already been applied for the diagnosis of tumors and genetic diseases. HRM analysis has been confirmed to a reliable, accurate, and rapid screening method for APC mutations in oral squamous cell carcinoma (44). BRCA1 gene screening using HRM

analysis has been demonstrated to be useful for the diagnosis of lung adenocarcinoma (45) and Moroccan breast cancer (46); Studies using HRM analysis showed heterozygous mutations in COL1A1 and COL1A2 genes are associated with osteogenesis imperfecta (47).

In conclusion, our HRM assay opens a new avenue for the detection of *NOTCH1* gene mutations in CLL. It is a valid and promising tool for high-throughput *NOTCH1* screening, enabling real-time evaluation of CLL progression, which is significant for decision-making regarding treatment.

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