Detection of NPM1 exon 12 mutations and FLT3 – internal tandem duplications by high resolution melting analysis in normal karyotype acute myeloid leukemia

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Abstract

Background: Molecular characterisation of normal karyotype acute myeloid leukemia (NK-AML) allows prognostic stratification and potentially can alter treatment choices and pathways. Approximately 45–60% of patients with NK-AML carry NPM1 gene mutations and are associated with a favourable clinical outcome when FLT3-internal tandem duplications (ITD) are absent. High resolution melting (HRM) is a novel screening method that enables rapid identification of mutation positive DNA samples.

Results: We developed HRM assays to detect NPM1 mutations and FLT3-ITD and tested diagnostic samples from 44 NK-AML patients. Eight were NPM1 mutation positive only, 4 were both NPM1 mutation and FLT3-ITD positive and 4 were FLT3-ITD positive only. A novel point mutation Y572C (c.1715A>G) in exon 14 of FLT3 was also detected. In the group with de novo NK-AML, 40% (12/29) were NPM1 mutation positive whereas NPM1 mutations were observed in 20% (3/15) of secondary NK-AML cases. Sequencing was performed and demonstrated 100% concordance with the HRM results.

Conclusion: HRM is a rapid and efficient method of screening NK-AML samples for both novel and known NPM1 and FLT3 mutations. NPM1 mutations can be observed in both primary and secondary NK-AML cases.

Background

Acute myeloid leukemia with a normal karyotype (NK-AML) is considered to have an intermediate prognostic risk with 5 year disease free survival (DFS) ranging between 24–42% [1,2]. However, there is marked variability in outcome suggesting significant biological and molecular heterogeneity within this group of AML [3].

In 2005, Falini et al. described a set of common mutations within the final exon of the NPM1 gene in primary NK-AML cases.
AML patients, which alter the N-terminal domain nuclear localisation signal leading to abnormal cytoplasmic accumulation of the NPM1 phosphoprotein [4]. While the precise functional effect of the NPM1 mutation is incompletely understood, several groups confirmed that NK-AML patients have a high incidence of NPM1 exon 12 mutations (~24% – 60%) [5-9]. Mutations in NPM1 are the most frequent genetic change known in patients with NK-AML and a number of studies have shown that NPM1 mutation positive patients have a better prognosis with longer event-free and overall survival (OS) [10].

Schnittger et al. demonstrated that the favourable prognostic implications of NPM1 mutation status are overridden in FLT3-ITD positive cases which have a uniformly poor prognosis [7]. These findings demonstrate the need to screen patients for mutations in FLT3-ITD alongside NPM1 [10]. However, such a molecular screening program can be demanding on the resources of a diagnostic laboratory. Therefore, in this study we assessed the use of high resolution melting (HRM) analysis as a rapid method to screen NK-AML patient samples for the critical molecular changes in NPM1 and FLT3.

Results and Discussion
In this study, we developed HRM assays allowing rapid assessment of the mutation status of NPM1 and the presence of the FLT3-ITD in the same run. In HRM, the PCR product is subjected to melting in the presence of a dye that only fluoresces when bound to double stranded DNA [11]. As melting is sequence dependent, monitoring the precise melting behaviour by observing the change in fluorescence allows the detection of variant sequences. In addition, sequence variants in the DNA such as mutations give rise to heteroduplexes that form earlier melting products allowing ready detection of mutations even at comparatively low concentrations.

Samples from 44 patients with NK-AML were analysed. The median age of the patients was 62 years (range 18–89 years) and 27 (61%) patients were male. Twenty nine (66%) had de novo AML and 15 (34%) had secondary AML. Sixteen patients generated an abnormal melting

![Figure 1](http://www.jhoonline.org/content/1/1/10)

**Figure 1**
Detection of NPM1 mutations and FLT3-ITD using high resolution melting analysis. (A) The melt curve of NPM1 exon 12 and (B) The difference plot of NPM1 exon 12. Six patient samples are shown in comparison to five normal controls. Four patients (16, 12, 14 and 38) are NPM1 mutation positive and two patients (33 and 43) are NPM1 mutation negative. (C) The melt curve of FLT3 exon 14 and (D) The difference plot of FLT3 exon 14 - Six patient samples are shown in comparison to five normal controls. Three patients (6, 33 and 43) are FLT3-ITD positive and three patients (12, 14 and 48) are FLT3-ITD negative. (E) The melt curve of FLT3 exon 14 and (F) The difference plot of FLT3 exon 14 - Eight patient samples are shown in comparison to five normal controls. One patient (19) is positive for FLT3 Y572C and seven patients (4, 5, 10, 24, 25, 26 and 30) are FLT3 mutation negative. All samples are shown in duplicate.
profile in one of the two tested amplicons, 8 were NPM1 mutation positive only, 4 were NPM1 positive and FLT3-ITD positive and 4 were FLT3-ITD positive only (Figure 1.).

Sequencing confirmed all the HRM detected mutations and did not reveal any further mutations, indicating that HRM was capable of detecting mutations with 100% sensitivity in this cohort.

Table 1: Patient demographics and list of NPM1 and FLT3-ITD mutations detected

| #  | Age | Sex | FAB | Prior Disease† | HRM – NPM1‡ | Seq – NPM1§ | HRM – FLT3-ITD | Seq-FLT3-ITD| |
|----|-----|-----|-----|----------------|-------------|-------------|----------------|-------------|
| 1  | 64  | M   | M1  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 2  | 63  | M   | M6  | MDS            | Normal      | Neg         | Normal         | Normal      |
| 3  | 36  | M   | M2  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 4  | 69  | M   | M2  | basophilic leukemia | RAEB-T     | Normal      | Neg           | Normal      |
| 5  | 68  | M   | M2  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 6  | 81  | M   | M5  | Nil            | Aberrant    | 860_863dupTCTG | Aberrant     | 1754_1798dup |
| 7  | 19  | M   | M2  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 8  | 68  | M   | M6  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 9  | 72  | M   | M4  | CMML           | Normal      | Neg         | Normal         | Normal      |
| 10 | 58  | F   | M2  | Aberrant       | 860_863dupTCTG | Normal    | Normal         |
| 11 | 66  | F   | M4/5| MDS transformed | Aberrant    | 860_863dupTCTG | Normal     |
| 12 | 51  | F   | M4  | Aberrant       | 860_863dupTCTG | Normal     | Normal         |
| 13 | 42  | F   | M2  | MDS/pelvic chloroma | Normal   | Neg         | Normal         | Normal      |
| 14 | 53  | M   | M4  | Aberrant       | 860_863dupTCTG | Normal    | Normal         |
| 15 | 59  | F   | M1  | Aberrant       | 860_863dupTCTG | Aberrant   | 1811_1837dup   |
|    |     |     |     |                |             |             | 1838_1867ins  |            |
| 16 | 69  | M   | M1  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 17 | 52  | M   | M1  | MDS            | Normal      | Neg         | Normal         | Normal      |
| 18 | 74  | M   | M1  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 19 | 66  | M   | M0  | ca prostate    | Aberrant    | 860_863dupTCTG | Aberrant     | 1715A>G     |
| 20 | 52  | M   | M1  | MDS            | Normal      | Neg         | Normal         | Normal      |
| 21 | 75  | M   | M1  | MDS            | Normal      | Neg         | Normal         | Normal      |
| 22 | 56  | M   | M0  | NHL on TX      | Normal      | Neg         | Normal         | Normal      |
| 23 | 18  | F   | M1  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 24 | 73  | M   | M4  | N1             | Normal      | Neg         | Normal         | Normal      |
| 25 | 56  | F   | M1  | therapy related| Normal      | Neg         | Normal         | Normal      |
| 26 | 53  | F   | M4  | Aberrant       | 860_863dupTCTG | Normal    |
| 27 | 64  | M   | M0  | MDS            | Normal      | Neg         | Normal         | Normal      |
| 28 | 63  | F   | M5a | Aberrant       | 861_864insCTGC | Normal    |
| 29 | 71  | F   | M5b | MDS transformed | Aberrant    | 860_863dupTCTG | Aberrant     | 1754_1789dup |
| 30 | 89  | F   | M6  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 31 | 28  | F   | M4  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 32 | 69  | F   | M4  | nil            | Normal      | Neg         | Normal         | Normal      |
| 33 | 36  | F   | M5b | Nil            | Normal      | Neg         | Aberrant       | 1783_1812dup |
| 34 | 54  | M   | M1  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 35 | 52  | M   | M1  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 36 | 82  | M   | M4  | Nil            | Normal      | Neg         | Aberrant       | 1786G>C, 1787_1818dup |
| 37 | 68  | M   | M5b | CMML           | Normal      | Neg         | Normal         | Normal      |
| 38 | 61  | F   | M4  | Aberrant       | 860_863dupTCTG | Normal    |
| 39 | 49  | M   | M4  | Nil            | Normal      | Neg         | Aberrant       | ins ? bp ¶| |
| 40 | 83  | F   | M1  | Aberrant       | 860_863dupTCTG | Normal    |
| 41 | 72  | F   | M4  | CMML           | Normal      | Neg         | Normal         | Normal      |
| 42 | 57  | M   | M4  | Nil            | Normal      | Neg         | Normal         | Normal      |
| 43 | 42  | M   | M1  | Nil            | Normal      | Neg         | Aberrant       | 1741_1831dup |
|    |     |     |     |                |             |             | 1832_1842ins  |            |
| 44 | 49  | M   | M6  | Nil            | Normal      | Neg         | Normal         | Normal      |

† MDS = myelodysplastic syndrome; RAEB-T = refractory anemia with excess of blasts in transformation; CMML = chronic myelomonocytic leukemia; Ca prostate = prostate cancer; NHL = Non-Hodgkin lymphoma.
‡Normal = normal melt profile, Aberrant = abnormal melt profile.
§Neg = no mutation detected in the sequence; numbering according to NPM1 reference sequence NM_002520.5
| Numbering according to FLT3 reference sequence NM_004119.2 (5'UTR not included)
¶ The size of the internal tandem duplication could not be determined due to the low levels of mutant peaks in the sequence,
All the NPM1 mutations detected involved one of two 4 base insertions that altered the tryptophan at amino acid position 288 and the FLT3-ITD ranged from 33–102 bases (Table 1). These mutations were similar to those previously described [4,12,13]. All 12 NPM1 mutation positive patients were also positive by immunohistochemistry (IHC) on bone marrow trephine sections, showing typical cytoplasmic localisation (data not shown).

The incidence of NPM1 mutations in the de novo AML cases was 40% (12/29), consistent with the incidence reported in previous studies [5-9]. Interestingly, 3/15 of the secondary AML cases were NPM1 mutation positive which contrasts with an earlier study, where cytoplasmic localisation of NPM indicative of NPM1 mutations was not seen in 135 secondary AML samples by IHC [4].

A novel point mutation Y572C in exon 14 of FLT3 was also detected. This tyrosine residue within the juxtamembrane domain of FLT3 has been shown to be phosphorylated in vivo [14] and could be included in the newly described class of FLT3 juxtamembrane domain point mutations for which the similar mutation Y591C has been reported [15]. This illustrates the power of HRM to detect novel as well known mutations. The use of HRM to screen for FLT3-ITD has been previously reported [16].

HRM is rapidly becoming the most important mutation scanning methodology. It is an in-tube method, meaning that PCR amplification and subsequent analysis are sequentially performed in the one tube or well. This makes it more convenient than other scanning methodologies such as denaturing high-performance liquid chromatography [17]. We used a real-time PCR machine with HRM capability rather than a stand-alone HRM instrument. This facilitates quality control as the success of the amplification can be assessed on the same platform as the melting analysis.

HRM has no real disadvantages in mutation scanning except that extra care needs to be taken in designing PCR reactions to avoid primer dimers and non-specific amplification. Secondly, DNA needs to be prepared in a uniform fashion to avoid variation in salt concentration that will affect the melting. In addition, the exact nature of any mutation cannot be determined without sequencing. Nevertheless, performing HRM as an initial screen for potential mutations significantly reduces the volume of samples requiring sequencing with consequent reduction of cost and labour, and improvements to turn around time.

**Conclusion**

HRM is likely to play a major role in clinical applications as it enables rapid detection of defined and novel molecular changes in clinical samples. In this study, the conditions have been optimised to enable screening of normal karyotype AML patients for both NPM1 and FLT3-ITD in the same run. This has enhanced patient prognostication and clinical decision making regarding therapeutic approaches. The assays are suitable both for individual patient diagnosis and for large scale clinical trials.

**Methods**

**Patients and samples**

DNA was extracted from archival bone marrow smears from 44 NK-AML patients from 1999–2007 sent to the Pathology Department of The Peter MacCallum Cancer Centre. Normal peripheral blood samples were obtained from 11 healthy volunteers. All samples were collected and were obtained in accordance with the Peter MacCallum Cancer Centre Ethics of Human Research guidelines. DNA was extracted from bone marrow smears using a standard phenol/chloroform extraction technique. DNA was extracted from peripheral blood using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI).

**High resolution melting analysis**

The PCR and melting analysis for NPM1 and FLT3 mutations were all performed on the LightCycler 480 (Roche Diagnostics, Penzberg, Germany) a real-time PCR machine with HRM capability and a 96/384 well capacity. All samples were tested in duplicate. At least 5 different normal controls for each gene were included in each run. Approximately 10 ng of DNA was amplified in a total volume of 10 μL containing 400 nM each of the relevant forward and reverse primer (NPMex12FRCACAATTATAAAAAGGACAGCCAG; or FLT3ex14F-GCTCGAAGCCTCACTTTCTCACTCATCA; FLT3ex14R-CTTGCTCAGCAGCTGTCC, 4 mM (NPM1) or 3 mM (FLT3) MgCl2, and LightCycler 480 High-Resolution Melting Master (Roche Diagnostics). The cycling conditions were the same for both amplicons allowing them to be performed in the one run. The conditions were 95°C (10 min) and a touch down of 10 cycles of 95°C (10 sec), 65°C–55°C (10 sec, 1°C/step), 72°C (30 sec) and a further 45 cycles. The melting program was 95°C (1 min) 45°C (1 min), then 65°C–95°C (5 sec, 1°C/sec). Thirty acquisitions were collected per °C. Upon completion of the run (approximately 2 hours), analysis was performed using the software supplied with the LightCycler 480. The melting curves were normalised and temperature shifted to allow samples to be directly compared. Difference plots were generated by selecting a negative control as the baseline and the fluorescence of all other samples was plotted relative to this sample. Significant differences in fluorescence were indicative of mutations.
Sequencing

Sequencing was performed on all samples. Approximately 10 ng of DNA was amplified in a total volume of 25 µL containing 200 nM each of M13 tagged primers, 2 mM MgCl₂, 200 µM each dNTPs, 0.5 units FastStart Taq (Roche Diagnostics) and 1 × Buffer. The primers used were the same as stated above except that the M13 sequences 5’ TGTAAAAACGACGGCCAGT and 5’ CAGGAAACGCTATGACC were tagged to the forward and reverse primers respectively. The cycling conditions were 95 °C (10 min) and 45 cycles of 94°C (30 sec), 64°C (30 sec), 72°C (30 sec) and 72°C for 10 min. The products were checked on a 2% ethidium bromide stained agarose gel before sequencing.

Competing interests

Alex Dobrovic has received honoraria from Roche Diagnostics for speaking about HRM.

Authors' contributions

AYCT wrote the paper and performed the experiments, AD developed the assay with AYCT, co-wrote the paper and revised the paper in accordance with the reviewers' comments, DAW, DC, and JFS initiated the project, provided the specimens and assisted with writing, SJ provided specimens, and previously established gene expression signatures and their favorable prognostic significance. Blood 2005, 106:3747-3754.

Note added in proof

After this manuscript was submitted, another report of NPM1 mutations in secondary AML has appeared [18].

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