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# Development of a High-Resolution Melting Curve Analysis Screening Test for *SRSF2* Splicing Factor Gene Mutations in Myelodysplastic Syndromes

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Somatic mutations of the spliceosome machinery have been recently identified by whole genome analysis in hematologic diseases, such as myelodysplastic syndrome, chronic lymphocytic leukemia, myeloproliferative neoplasms, acute myeloid leukemia, and advanced forms of mastocytosis, and also in nonhematologic conditions. *SRSF2* is a member of the serine/arginine–rich family pre-mRNA splicing factors that plays a role in mRNA export from the nucleus and translation. We describe a high-resolution melting (HRM) curve analysis to screen for *SRSF2* hotspot mutations in a fast, sensitive, and reliable way. Fifty bone marrow samples from patients with myelodysplastic syndrome were analyzed by the HRM assay and by direct sequencing. HRM screening identified four melting patterns corresponding to a negative (wild-type) group and three different mutated groups. Each mutated group was identified according to the positive control used: P95H, P95L, and P95R, respectively. An HRM mutated pattern was identified in seven patients. Positive and negative results from HRM were compared with direct sequencing results with a sensitivity and specificity of 100% (95% CI, 0.56–1, and 95% CI, 0.89–1, respectively). Analytical sensitivity analysis revealed a detection threshold of up to 1:9 (mutated/wild type) dilution. This rapid screening method may provide useful information for clinical decision making and be helpful to optimize laboratory resources and reduce turnaround time. *(J Mol Diagn 2015, 17: 85–89; http://dx.doi.org/10.1016/j.jmoldx.2014.08.002)*

Recently, somatic mutations of the spliceosome machinery have been identified using whole genome analysis in several hematologic diseases, such as myelodysplastic syndrome (MDS), chronic lymphocytic leukemia, myeloproliferative neoplasms, acute myeloid leukemia (AML), and solid tumors, such as breast, pancreatic cancers, uveal melanomas, and lung adenocarcinomas.[1–11] *SRSF2* is a member of the serine/arginine–rich family pre-mRNA splicing factors that is involved in mRNA export from the nucleus and translation. Besides the RNA recognition domain, SRSF2 protein contains a serine/arginine–rich domain that promotes interaction with other splicing factors. Therefore, it constitutes a critical

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player in the process of mRNA splicing. The *SRSF2* gene is located on the long arm of chromosome 17 subregion 25.1.

Earlier reports on *SRSF2* mutations in hematologic diseases described a frequency of 11.6% ($n = 155$) in MDS without ringed sideroblasts, 5.5% ($n = 73$) in refractory anemia with ringed sideroblasts and refractory cytopenia with multilineage dysplasia with ringed sideroblasts, 28.4% ($n = 88$) in chronic myelomonocytic leukemia, 6.5% ($n = 62$) in AML and MDS, 0.7% ($n = 151$) in *de novo* AML, and 1.9% ($n = 53$) in myeloproliferative neoplasms.[1] In another study, the frequency of *SRSF2* mutations in MDS was 12.4% in a cohort of 193 MDS cases, in which 80% were classified as missense and the rest as frameshift deletion.[2] Moreover, 95% of the mutations were located at the amino acid position P95 and only one at position P96. Subsequent studies have had the same results regarding this hotspot localization corresponding to the exon 1 of the *SRSF2* gene. In chronic myelomonocytic leukemia, *SRSF2* mutations are present in almost half of the cases, with a frequency of 47% in a cohort of 275 patients.[3] Among the 129 mutated cases identified, 120 were missense mutations in which the amino acid substitution P95H was the most common at 47.5%, P95L was present in 31.6%, P95R was present in 19.2%, and the remaining two cases were P95A, and P95T. In primary myelofibrosis, *SRSF2* monoallelic mutations were reported in 32 of 187 patients (17%), affecting residue P95. When examining this subset of mutated cases, the authors reported cooperating mutations with *JAK2* mutation V617F in 72%, *IDH1/2* in 28%, and *SF3B1* in 4% and concluded that *SRSF2* mutations were significantly associated with advanced age, high-risk category, and worse prognostic outcome compared with nonmutated primary myelofibrosis.[5]

Regarding the prognostic effect in MDS, a study found that patients with *SRSF2* mutations have shorter overall survival and shorter time to AML progression compared with patients without the mutation.[2] Similar results were obtained in other studies that involved MDS patients.[7] In another study, *SRSF2* mutation was present in 18.9% of AML cases derived from myeloproliferative neoplasms. Moreover, in these cases, *SRSF2* mutations were associated with a worse overall survival in univariate (hazard ratio, 2.77; 95% CI, 1.10−7.00; $P = 0.03$) and multivariate analysis (hazard ratio, 2.11; 95% CI, 1.01−4.42; $P < 0.05$).[4]

Our aim was to develop a rapid, sensitive, and reliable screening test for *SRSF2* mutations that can be performed on samples from patients with hematologic diseases and solid tumors and provide clinically useful information. By performing a rapid screening test, positive results can be confirmed by a highly specific test, such as direct DNA sequencing, thus saving time and laboratory resources.

## Materials and Methods

### Patient Samples and DNA Extraction

Bone marrow samples collected from 50 patients who underwent cytogenetic analysis as a part of the laboratory workup for the diagnosis of MDS were used. Written informed consent for the study was obtained from all patients, and the study was approved by the IRB of Policlinico Tor Vergata, Rome. Five DNA samples from patients diagnosed as having MDS in which *SRSF2* mutations were identified by whole genome amplification and confirmed by direct Sanger sequencing were used as positive controls. The method was also tested in 14 cases of chronic myelomonocytic leukemia and different cell lines derived from hematologic diseases, such as U937, NB4, NB4-R4 (all trans-retinoic acid resistant), NB4 arsenic trioxide−resistant, MV-4:11, HL-60, OCI-AML2, and OCI-AML3.

Bone marrow mononuclear cells were obtained from 2 to 5 mL of bone marrow by density-gradient Ficoll centrifugation using Lympholyte (Cedarlane Laboratories Ltd., Ontario, Canada). Cells from cell lines were washed and suspended in 200 μL of phosphate buffered saline. Genomic DNA was extracted using a column-based purification kit (DNeasy Blood & Tissue kit; Qiagen, Valencia, CA), and quantified by spectrophotometric analysis.

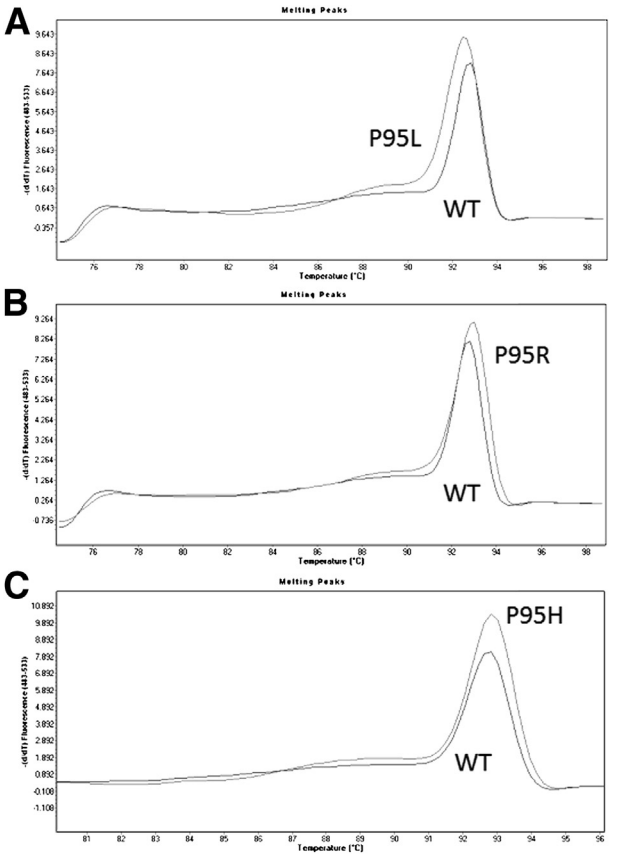


**Figure 1** Melting peak analysis. Discrimination of mutated samples from wild-type (WT) was possible by analyzing differences between the melting peaks individually using positive and negative controls as a reference. P95L (**A**), P95R (**B**), and P95H (**C**) mutations have different melting peaks compared with WT mutations. d/dT, negative derivative of the change in fluorescence divided by the change in temperature in relation to the temperature.

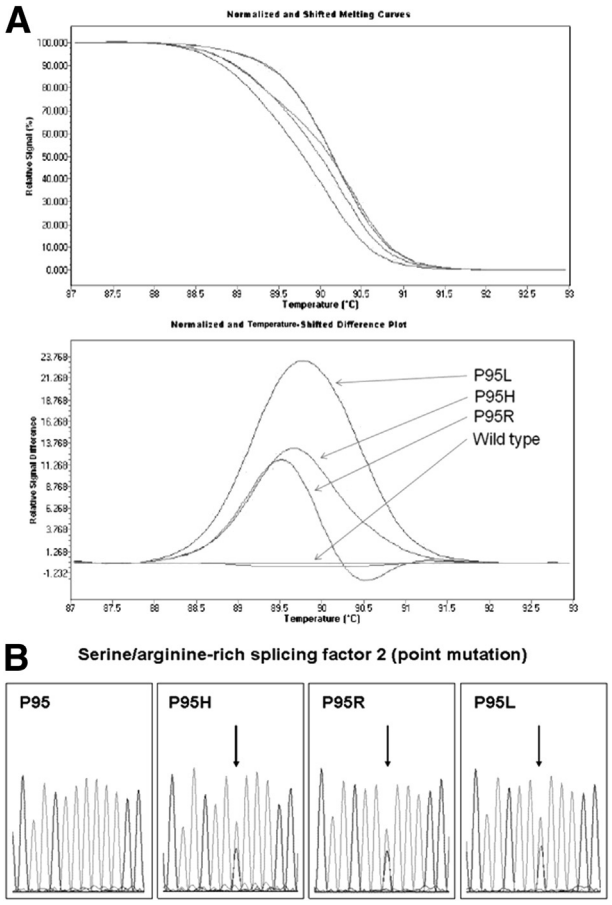


**Figure 2** **A:** High-resolution melting curve analysis gene scanning test reveals each mutated group identified according to the positive control used: P95H, P95L, and P95R, respectively. **B:** Sequencing analysis from positive controls. **Arrows** indicate the presence of a single point mutation represented as a double spike in the sequencing analysis.

### Primer Design, PCR, and Melting Conditions

A single pair of oligonucleotide primers was used to amplify the hotspot mutation region of the *SRSF2* gene located at exon 1. Primers were designed using Internet-based software Primer3-web version 4.0.0[12,13] and aligned with the National Center for Biotechnology Information Basic Local Alignment Search Tool using *SRSF2* genomic reference sequence (*http://www.ncbi.nlm.nih.gov/refseq/rsg*; Accession number NG_032095.1). Primers were purified by high-performance liquid chromatography. The primer sequences were as follows: 5′-GCTGAGGACGCTATGGATG-3′ forward and 5′-GCGGCTGTGGTGTGAGTC-3′ reverse. The size of the amplicon was 105 base pairs. The HRM reaction was performed in a 20-μL reaction, and the following reagent concentrations were used: 2× high-resolution melting master (LightCycler 480; Roche Diagnostics GmbH, Mannheim, Germany), 3.5 mmol/L $MgCl_2$, 0.5 μmol/L primers, 3% dimethyl sulfoxide, and DNA diluted to a concentration of 10 ng/μL (in water). The HRM cycling protocol was composed by a single hot start cycle with a temperature of 95°C for 10 minutes followed by 50 cycles of 95°C for 10 seconds, 61°C for 10 seconds, and 72°C for 10 seconds. Melting temperature was raised from 65°C to 95°C with a ramp of 0.02°C per second. LightCycler 480 (Roche Diagnostics GmbH) real-time platform was used to perform the reactions and LightCycler 480 software version 1.5.0 SP4 (Roche Diagnostics GmbH) to conduct the analysis.

### DNA Sequencing Analysis

All samples were analyzed by direct Sanger sequencing analysis to confirm the HRM results using the ABI Genetic Analyzer 3130 platform (Applied Biosystems Inc., Foster City, CA). A different amplified product was developed using the following primers: 5′-CAACCTGACCTACCGCACCTC-3′ forward and 5′-GCCCGCACCACGTGCTTCG-3′ reverse with a size of 402 base pairs. The PCR mix was composed of 20 μL of HotStartTaq Master Mix (Qiagen), 10 μmol/L of each forward and reverse primer, 100 ng of genomic DNA, and water to a final volume of 40 μL. The PCR products were visualized under UV light lamp to confirm the presence of a 402 base pairs band. The amplified products were purified using a HiYield DNA fragment extraction kit (Real Genomics, Taiwan, Republic of China). Sequencing analysis was performed unidirectionally with the following forward internal primer: 5′-ATCCCGCGGGACCGCTACAC-3′. An addition reverse internal primer was used for bidirectional confirmation of the mutated cases: 5′-CCCCGTTTACCTGCGGCTCC-3′. All primers were designed with the same considerations as mentioned before. The sequencing reaction was performed using BigDye terminator version 3.1 Cycle Sequencing kit (Applied Biosystems Inc.) according to the manufacturer specifications. Sequencing results were analyzed by Seq Scanner software version 1.0 (Applied Biosystems Inc.).

## Results

All amplification curves reached a plateau before the 45th cycle, and the cycle cutoff value ranged from 20 to 25

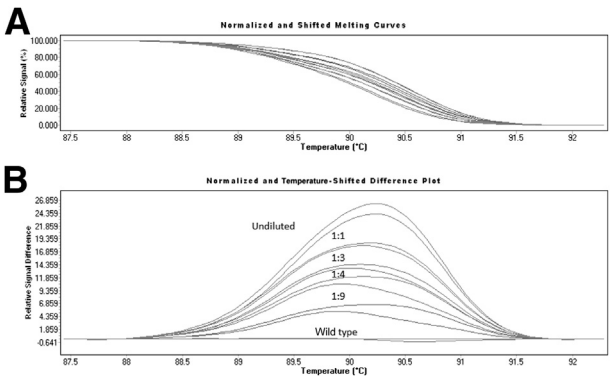


**Figure 3** Sensitivity analysis was performed in duplicate using a known mutated DNA sample subjected to serial dilutions: undiluted, 1:1, 1:3, 1:4, and 1:9 with a wild-type sample. **A:** Normalized and shifted melting curves. **B:** Normalized and temperature-shifted difference plot.

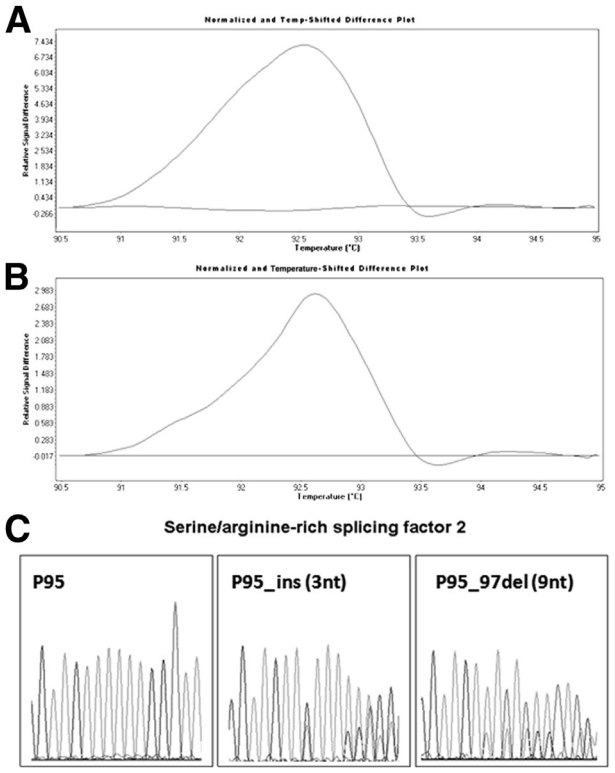


**Figure 4** Gene scanning analysis of P95_ins (3nt) (**A**) and P95_97del (9nt) (**B**) mutations reveals abnormal melting pattern compared with a wild-type control. **C:** Sequencing analysis of the WT control and the insertion/deletion mutations.

crossing points. Melting peak analysis revealed a single melting peak at a temperature between 91°C and 94°C, corresponding to the specific *SRSF2* amplicon targeted. A different melting temperature was noted on mutated cases, whereas all wild-type (WT) cases had the same temperature. Discrimination of mutated samples from WT was possible by analyzing differences between the melting peaks individually using positive and negative controls as a reference (Figure 1). Melting genotype analysis was not included in this study.

The HRM gene scanning test was able to successfully identify four melting patterns corresponding to a negative (WT) group and three different positive (mutated) groups. Each mutated group was identified according to the positive control used: P95H, P95L, and P95R, respectively (Figure 2A). Of the selected MDS patients, seven were found to have mutations (14%). Regarding the chronic myelomonocytic leukemia patients studied, 5 of 14 were identified as having mutations (36%). All cell lines were negative for mutations. Positive and negative results from HRM were compared with direct sequencing (Figure 2B). For the MDS group, sensitivity and specificity were calculated with 95% CIs using Vassar College's VassarStats Website for Statistical Computation (*http://www.vassarstats.net*, last accessed July 24, 2014). Sanger sequencing was selected as the reference method. Both the sensitivity and specificity were 100% (95% CI, 0.56−1, and 95% CI, 0.89−1, respectively).

Analytical sensitivity analysis was performed in duplicate using a known mutated DNA sample subjected to serial dilutions: 1:1, 1:3, 1:4, and 1:9 with a WT sample. The test revealed detection sensitivity up to 1:9, indicating high analytical sensitivity (Figure 3).

Because a small percentage of *SRSF2* mutated cases represent insertions or deletions, we included two additional controls: one with an insertion and another with a deletion, both encompassed within the *SRSF2* amplicon. Gene scanning analysis revealed an abnormal melting pattern for these two samples compared with the WT control (Figure 4).

## Discussion

Because P95 point mutations may be present with a different nucleotide (A, G, or T), the expected melting

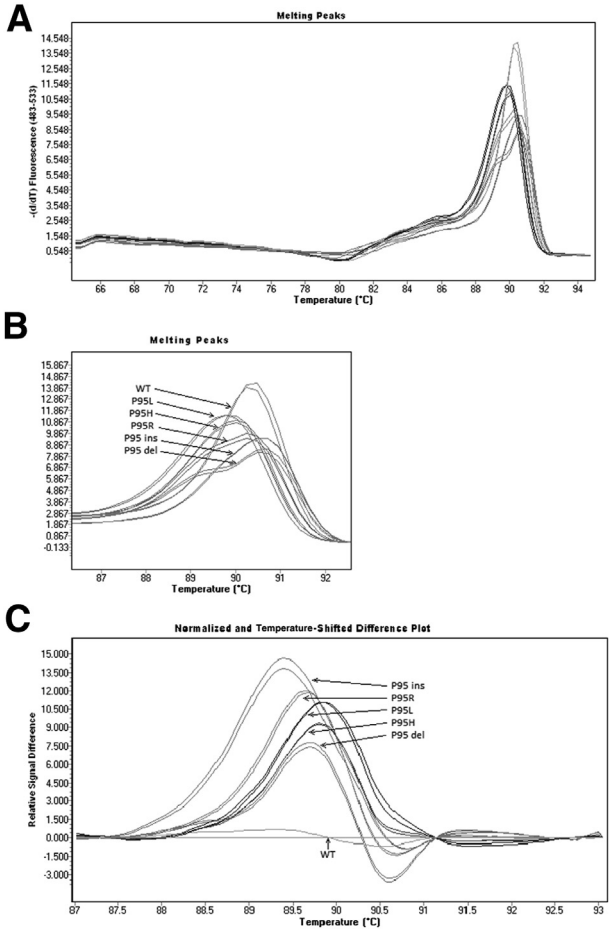


**Figure 5** Melting peaks and difference plots performed in duplicated reveal distinct characteristic patterns of different P95 mutations. **A:** Melting peaks ranging from 64°C to 95°C. **B:** A close-up of the melting peaks reveals each P95 mutation melting peak and the corresponding mutation. **C:** Difference plot reveals different positive groups corresponding to P95 point mutations, insertion, and deletion compared with the baseline negative wild-type (WT) control. d/dT, negative derivative of the change in fluorescence divided by the change in temperature in relation to the temperature.

pattern varies as a function of the melting temperature. This characteristic allowed us to recognize three different melting groups by adjusting the normalization settings of the software's gene scanning program. Nevertheless, any change in the nucleotide position of the codon corresponding to P95 and 96 can be possibly identified by this test when a negative WT control is included.

During the development stage of this test, we experienced some difficulties with the efficiency of the amplification mainly because the *SRSF2* hotspot 105 base pairs fragment has a 72% guanidine-cytidine content. To overcome this problem, we used dimethyl sulfoxide as an additive at different concentrations until we reached the optimal amplification conditions without altering the melting pattern characteristics.[14]

Although this method is aimed to identify *SRSF2* point mutations because >95% of cases are of this type, our test effectively identified an insertion and a deletion mutation as shown in Figure 5. A limitation of this test might be the identification of other alterations, such as deletions, occurring in the primer annealing region or outside the amplicon.

Since the recent discovery of the splicing gene mutations in hematologic diseases, an increasing interest in the study of these genes has emerged. Many publications have described an important role of some splicing factors in the pathogenesis of MDS, leading to the development of diagnostic tests, such as *SF3B1* screening methods to detect point mutations.[15] Given the clinical implications of *SRSF2* mutations, this rapid screening method may provide useful information for clinical decision making.

In conclusion, we propose the use of this HRM method to optimize laboratory resources by selecting only the positive cases to be confirmed by sequencing, thus reducing turnaround time by screening out negative cases. Although this method can be used in laboratories where HRM is available but Sanger sequencing is not accessible, larger studies are needed to determine diagnostic performance.

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