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NRAS Mutations with Low Allele Burden have Independent Prognostic Significance for Patients with Lower Risk Myelodysplastic Syndromes

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Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematopoietic disorders characterized by inefficient hematopoiesis, cytopenias, and an increased risk of progression to acute myeloid leukemia (AML).[1] Common prognostic scoring methods, such as the International Prognostic Scoring System for MDS (IPSS), use clinical features and karyotypic abnormalities to stratify patients into risk groups that form the basis for clinical treatment guidelines.[2] However, more than half of all MDS patients display a normal karyotype and patients with identical chromosomal abnormalities often have varied clinical outcomes. Therefore, additional prognostic indicators that can refine prognosis in MDS patients could help determine the optimal form and timing of therapy. Recent analysis of bone marrow aspirates from 439 MDS patients using various methods, including mass-extend mass spectrometry-based genotyping (MALDI), revealed that somatic point mutations in specific genes are associated with clinical features and are independent prognostic factors.[3] Of these patients, 295 were classified as low/intermediate-1 IPSS risk, yet some displayed an unexpectedly short survival even in the absence of adverse prognostic mutations.

The clinical heterogeneity in MDS patients is partially explained by the diversity of genetic abnormalities acquired by their disease cells.[4] For example, somatic mutations in specific genes such as *NRAS* and *TP53* are associated with clinical features and overall survival in patients with MDS.[5–7] However, even within individual patients, these mutations are not

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uniformly distributed. The abnormal cells in the bone marrow of patients with MDS evolve over time giving rise to sub-clonal populations. Different sub-clones that have acquired and selected for additional genetic abnormalities therefore often coexist. Mutations present in a small fraction of cells can retain important clinical significance, e.g. low-level *TP53* mutations have been associated with resistance to therapy using lenalidomide.[8]

In studies by Bejar *et al.*[3,9] patients with *NRAS* mutations were similarly found to have a poor prognosis, particularly in lower risk individuals where they predicted a shorter overall survival independently of the IPSS. Since the majority of these patients had *NRAS* mutant allele frequencies of less than 25%, and in several cases below 10%,[3] we hypothesized that even rarer *NRAS* mutant sub-clones with comparable prognostic significance might be present but were not detected by MALDI. Indeed, mutations at the ~5–10% level or below are essentially invisible to MALDI or Sanger sequencing, potentially generating false-negatives and hindering the ability to accurately predict prognosis.[10] In perspective, there has been burgeoning interest in the clinical significance of low-abundance mutations in tumors with data pointing different directions in distinct clinical situations.

In an attempt to address these points, we performed the first high-resolution mutational analysis of a clinically significant target in MDS samples, *NRAS,* examining the effect of mutations on overall survival. We employed Co-amplification at Lower Denaturation Temperature COLD-PCR, (Figure 1A), for a highly sensitive re-analysis of *NRAS* mutations in the 295 low/intermediate-1 IPSS risk samples. COLD-PCR is capable of enriching low-abundance mutations during PCR, irrespective of the type or position of mutation on the amplicon.[11,12] COLD-PCR technology was recently refined further with the incorporation of specifically modified reference sequence (RS) oligonucleotides for Improved and Complete mutation Enrichment, ICE COLD-PCR (Figure 1B).[13] By combining COLD/ICE COLD-PCR technology with High Resolution Melting (HRM) analysis and Sanger sequencing or next generation sequencing the mutation detection sensitivity improves to the extent that mutant allele frequencies of 0.02–0.5% can be detected.[14,15]

The experimental work-flow we followed is displayed in Figure 1C. Briefly, we pre-amplified DNA using a multiplex-PCR primer set that includes *NRAS* exon 2. Both unamplified genomic DNA and whole-genome-amplified DNA were used as starting material, where available, for comparison. The amplified material was diluted and subjected to *fast*-COLD PCR amplification using an *NRAS* exon 2 nested primer set. Conventional-PCR was also performed in parallel for a direct comparison. The products from these parallel PCR reactions were subjected to HRM analysis. Amplification and HRM analysis of samples was repeated at least three independent times. Repeated testing using high fidelity polymerase (Phusion™) and run in parallel with wild type samples were performed to reduce the possibility for PCR misincorporations. Samples which displayed variant HRM profiles in the more sensitive of the two approaches, i.e. *fast*-COLD-PCR, were processed for Sanger sequencing analysis. Samples with non-variant HRM profiles following *fast*-COLD-PCR were considered wild type and not examined further. Those samples that had a variant HRM profile following *fast*-COLD-PCR but demonstrated absence of mutations in Sanger sequencing chromatographs were processed for further analysis via the most sensitive technique, ICE COLD-PCR. A commercial version of ICE COLD-PCR (Reveal™ kit, Transgenomic Inc) was employed, followed by Sanger sequencing. ICE COLD-PCR-Sanger sequencing was repeated several independent times for each sample examined. Once *NRAS* exon 2 missense mutations at codons 12–13 were identified and verified by independent repetition from unamplified genomic DNA, we then examined whether these mutations were associated with clinical variables such as specific cytopenias and overall survival. The sensitivity of these techniques to detect low-level mutations is illustrated in Figure 1D. The

team that performed the mutational COLD-PCR analysis was blinded to the clinical data during the course of the experiments.

Overall, conventional PCR followed by Sanger sequencing detected *NRAS* exon 2 hotspot mutations in 8 MDS patient samples confirming the results of the previous MALDI testing.[3] Analysis with *fast*-COLD-PCR with a limit of detection of ~0.5% mutation abundance revealed an additional 9 samples containing *NRAS* exon 2 hotspot missense mutations. ICE COLD-PCR testing, with a limit of detection of 0.02%–0.1% mutation abundance, detected 6 additional mutation-positive samples (Table 1A). The most sensitive of these approaches, ICE COLD-PCR, identified *NRAS* exon 2 mutations at a nearly three-fold higher rate than MALDI or conventional PCR-Sanger sequencing. The data indicate that improving the mutation detection capabilities of the approach used, leads to discovery of additional codon 12–13 *NRAS* mutations in MDS.

To determine if the lower level (<5%) occult exon 2 *NRAS* mutations detected by COLD-PCR techniques were clinically relevant we examined their relationship to overall survival. Patients with mutations evident in both *fast*-COLD-PCR and ICE COLD-PCR chromatograms, but not by conventional methods had similarly poor survival outcomes to those with mutations discovered using MALDI (Figure 1E–G). This indicates that the prognostic relevance of lower-level NRAS mutations that fall below the detection limits of currently employed conventional screening methods can be comparable to that of higher level mutations.

Multivariable analysis that considered IPSS risk group, sex, age, and the mutation status of 16 other recurrently mutated genes demonstrates that the prognostic significance of *NRAS* mutations detected by *fast*-COLD-PCR is independent of other known risk factors (Table 1B). These findings demonstrate that overall survival can be affected by *NRAS* mutant allele frequencies as low as 0.5%, potentially because a minor clone may evolve over time to become the dominant clone that determines outcome. This association with shorter overall survival was not present in patients with the rarest *NRAS* mutations, those falling below 0.5% mutant allele frequency detected only by ICE COLD-PCR, indicating a threshold above which the mutant allele frequency becomes clinically relevant. As the technical limit of sensitivity should preferably be lower than the clinically relevant threshold, detection of *NRAS* with ICE COLD-PCR followed by determination of whether the mutation is clinically relevant (higher or lower than 0.5%) would be appropriate.

In summary, by employing highly sensitive mutational analysis to assess for *NRAS* mutations in a group of low/intermediate-1 MDS patients, we demonstrated that (a) mutations down to the 0.02–0.1% mutation allele frequency could be identified in a total of 24 out of 295 patients, tripling the original number of mutations detected with less sensitive methods; (b) patients with *NRAS* mutations at allele mutation frequencies down to ~0.5% have a worse outcome than would be expected based on their clinical features, demonstrating the independent prognostic significance of these otherwise occult lesions; (c) assays with a detection limit down to 0.1% are needed for effective capture of clinically significant low-level mutations.

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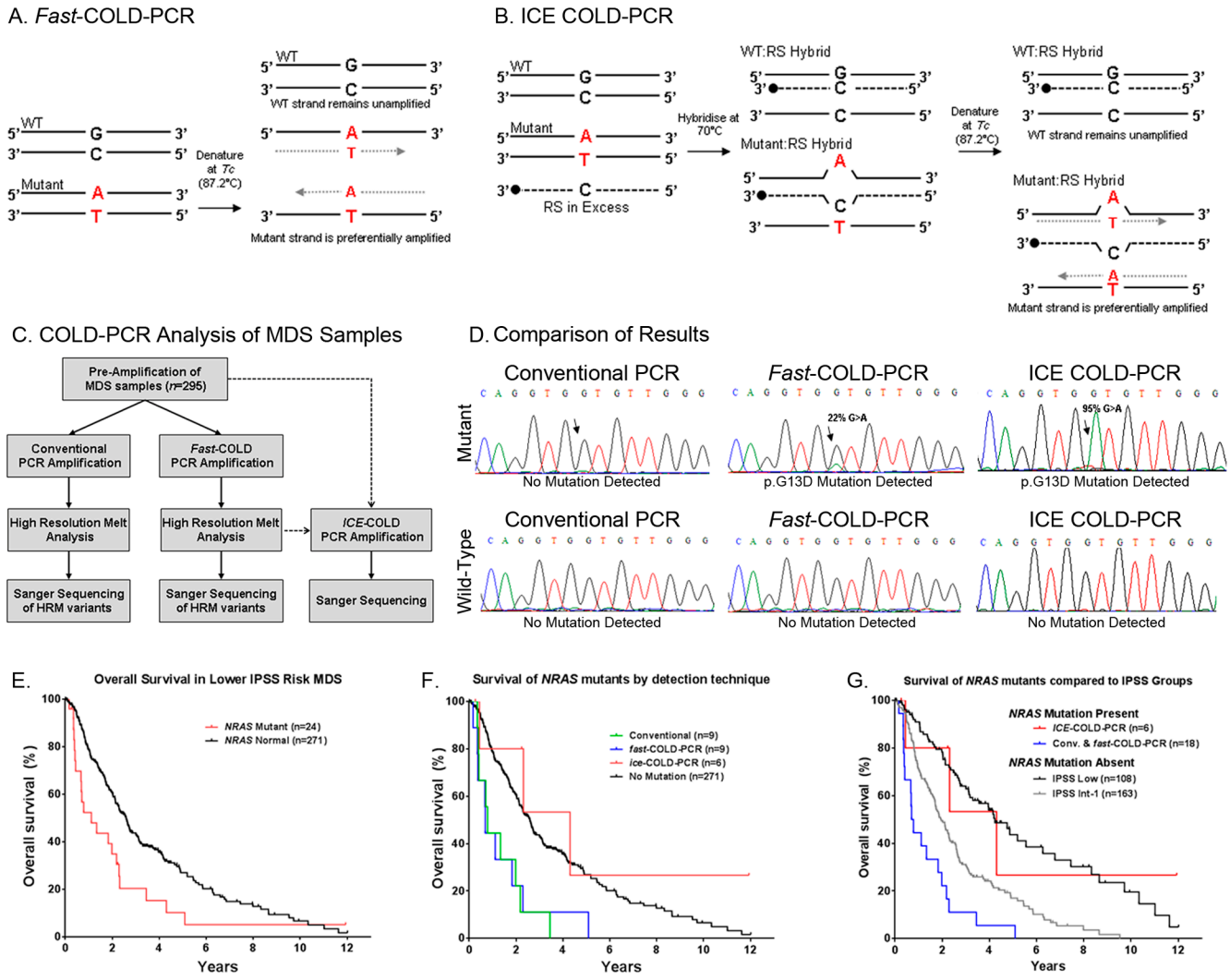

Figure 1.

COLD-PCR Analysis of MDS Samples **(A)** *Fast*-COLD PCR preferentially amplifies mutant DNA strands containing mutations which lower the melting temperature (*Tm)* of the amplicon, by incorporating the critical denaturation temperature (*Tc*) for the amplicon of interest in the PCR reaction. **(B)** ICE COLD-PCR preferentially enriches mutant DNA sequences in an excess of wild-type DNA through selective amplification of the mutant DNA population using a reference sequence oligonucleotide (RS) complementary to wild-type sequence. The RS prevents the amplification of the wild-type sequences while allowing amplification of any mutation covered by the RS region. **(C)** A flow-diagram of the experimental design behind the analysis of MDS samples with COLD-PCR. An initial pre-amplification step was performed on all MDS samples using a *TP53*/*NRAS* multiplex primer set. Samples were then subjected to conventional and *fast*-Cold PCR amplification of exon 2 of the *NRAS* oncogene. HRM analysis was used to screen the amplified products for any potential mutations and samples with altered HRM profiles were examined via Sanger sequencing. Patient samples with altered HRM profiles were also sent for further analysis with ICE COLD-PCR-Sanger sequencing. **(D)** The sensitivity of the *fast*-COLD and ICE COLD-PCR approaches followed by Sanger sequencing is illustrated. Sample #11 (Table 1A) contains a previously undetected p.G13D mutation, conventional PCR is insufficient to

detect this mutation, but it is readily detected with both *fast*- (22% G>A) and ICE COLD-PCR (95% G>A). **(E)** *NRAS* mutations detected by conventional or COLD-PCR techniques identify patients within the low/intermediate-1 IPSS risk groups that have significantly shorter overall survival compared with patients without mutations (P=0.010). **(F)** The overall survival of patients with occult *NRAS* mutations detectable by *fast*-COLD-PCR (limit of detection = 0.5% mutation abundance) is similar to the overall survival of *NRAS*-mutant patients identified by conventional methods (P=0.95) and significantly shorter than that of patients without mutations (P<0.001). **(G)** Patients with mutations detected by either *fast*-COLD-PCR or conventional methods were mostly from the Intermediate-1 (Int-1) risk group (16/18), yet have a shorter overall survival than Int-1 patients without *NRAS* mutations (P=0.001). Patients with *NRAS* mutations below the 0.5% limit of detection of *fast*-COLD-PCR and detectable only by ICE COLD-PCR (limit of detection = 0.1% mutation abundance) have no difference in overall survival compared with patients without *NRAS* mutations (P=0.23).

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Table 1A

Samples with *NRAS* Exon 2 Hotspot Mutations identified by Methodologies with Different Limits of Detection

	Sample ID	Known NRAS Mutations[+]	Conventional PCR	*Fast*-Cold PCR	*Fast-COLD PCR*	ICECOLD-PCR
		(MALDI)	Sanger	Sanger	HRM	Sanger
Conventional	1	p.G13V	p.G13V	p.G13V	+	*
	2	p.G13R	p.G13R	p.G13R	+	p.G13R
	3	p.G12D	p.G12D	p.G12D	+	p.G13D
	4	p.G13V	p.G13V	p.G13V	+	p.G13V
	5	p.G12D	p.G12D	p.G12D	+	p.G12D
	6**	p.G12S	p.G12D	p.G12D	+	*
	7	p.G12D	p.G12D	p.G12D	+	p.G12D
	8	–	p.G13D	p.G13D	+	p.G13D
	9	p.Q61K	*	*	+	*
***fast*-COLD-PCR**	**10**	–	–	p.G12D	+	p.G12D
	11	–	–	p.G13D	+	p.G13D
	12	–	–	p.G12C	+	p.G12C
	13†	–	–	p.G12D	+	p.G12D; p.G13D
	14	–	–	p.G12V p.G13D	+	p.G12V, p.G13D
	15	–	–	p.G13V	+	p.G13V
	16	–	–	p.G12V	+	p.G12V
	17**	p.G12S	–	p.G12D	+	p.G12D
	18	–	–	p.G12C	+	p.G12C
***ICE*-ONLY**	19	–	–	–	+	p.G12D, p.G13S
	20	–	–	–	+	p.G13D
	21	–	–	–	+	p.G12D
	22	–	–	–	+	p.G13V
	23	–	–	HBKG	+	p.G12C
	24	–	–	HBKG	+	p.G12C

Sample ID	Known NRAS Mutations[+]	Conventional PCR	*Fast*-Cold PCR	*Fast-COLD PCR*	ICECOLD-PCR
	(MALDI)	Sanger	Sanger	HRM	Sanger
25***	−	−	−	+	p.G13G
26****	−	−	−	+	−

(−) = Wild Type; (+) = Variant; (*) = Not Tested; (HBKG) = High Background/unclear sequencing; (ICE) ICE-COLD-PCR

** Samples 6 and 17, classified by MALDI as p.G12S (G>T), were re-classified as p.G12D (G>A) mutations with Sanger sequencing.

*** Sample 25 contained a low level mutation detectable solely by ICD-COLD-PCR. The mutation is synonymous hence it was not compared to clinical data

**** A comparison of the results obtained from the Whole-genome amplified (WGA) material with available unamplified genomic DNA demonstrated concordance between all samples, except in two cases. A p.G13D mutation was detected within the WGA DNA of sample 26, but not from genomic DNA, hence this was not included in the clinical correlation. Another sample (#13) showed the reverse, i.e. it was positive only from unamplified genomic DNA and this sample was included.

+ An additional codon 61 mutant was detected by MALDI, but not examined in this study

Table 1B

Final Survival Model from Stepwise Regression Cox Modeling

Variable	Unadjusted	p-value	Adjusted†	p-value
	HR (95% CI)		HR (95% CI)	
IPSS Risk Classification				
Intermediate 1 vs. Low	2.40 (1.77–3.26)	<0.001	2.61 (1.90–3.57)	<0.001
Mutational Status				
EZH2 Present vs. Absent	3.25 (2.11–5.02)	<0.001	3.56 (2.29–5.52)	<0.001
NRAS††				
Conventional PCR vs. WT	3.19 (1.63–6.27)	<0.001	2.41 (1.22–4.76)	0.011
Fast-Cold PCR vs. WT	2.96 (1.51–5.79)	0.002	3.43 (1.74–6.76)	<0.001
ICE-Cold PCR vs. WT	0.50 (0.16–1.59)	0.24	0.33 (0.10–1.06)	0.062
U2AF1 Present vs. Absent	1.51 (1.06–2.14)	0.022	1.47 (1.04–2.09)	0.031

† Final model obtained from stepwise regression algorithm shown, HR=hazard ratio.

†† Categories include additionally detected mutations by each method (*Fast*-Cold PCR n=9; ICE-COLD n=6).