Brief report

Development of an allele-specific minimal residual disease assay for patients with juvenile myelomonocytic leukemia

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Juvenile myelomonocytic leukemia is an aggressive and frequently lethal myeloproliferative disorder of childhood. Somatic mutations in *NRAS*, *KRAS*, or *PTPN11* occur in 60% of cases. Monitoring disease status is difficult because of the lack of characteristic leukemic blasts at diagnosis. We designed a fluorescently based, allele-specific polymerase chain reaction assay called TaqMAMA to detect the most common *RAS* or *PTPN11* mutations. We analyzed peripheral blood and/or bone marrow of 25 patients for levels of mutant alleles over time. Analysis of pre–hematopoietic stem-cell transplantation, samples revealed a broad distribution of the quantity of the mutant alleles. After hematopoietic stem-cell transplantation, the level of the mutant allele rose rapidly in patients who relapsed and correlated well with falling donor chimerism. Simultaneously analyzed peripheral blood and bone marrow samples demonstrate that blood can be monitored for residual disease. Importantly, these assays provide a sensitive strategy to evaluate molecular responses to new therapeutic strategies. (Blood. 2008;111:1124-1127)

Introduction

Juvenile myelomonocytic leukemia (JMML) is an aggressive clonal malignancy of young children characterized by overproduction of myeloid lineage cells that infiltrate hematopoietic and nonhematopoietic tissues.[1,2] More than 70% of patients have a mutation in the *NF1*, *RAS,* or *PTPN11* genes, which encode proteins involved in Ras signaling.[3] Animal models demonstrate that $Nf1^{-/-}$, $Kras^{G12D}$, and $Ptpn11^{D61G}$ mutant mice all develop fatal myeloproliferative disorders that model JMML in vivo and in vitro.[4-8] These observations strongly support the role of hyperactive Ras in initiating JMML.

The diagnosis and treatment of patients present great challenges to physicians. Currently, the diagnosis is made by meeting a constellation of clinical and laboratory criteria,[1,9] including the demonstration of fewer than 20% blasts in the bone marrow (BM). Historically, patients with JMML treated with conventional chemotherapy have an event-free survival of less than 15%.[9,10] Allogeneic hematopoietic stem cell transplantation (HSCT) will cure up to 50% of patients.[11] Novel treatment approaches are urgently needed for this disease, given the dismal survival with traditional chemotherapy and the severity of late effects that HSCT confers on these young children.

However, defining response to therapy is another major challenge for clinicians.[12] Current response criteria are broadly defined as a decreasing white blood cell count and lessening organomegaly. Detecting minimal residual disease (MRD) in JMML relies on tracking donor chimerism after HSCT. One quantitative donor chimerism tracking method used and reported in JMML involves polymerase chain reaction (PCR) amplification of short tandem repeats with comparison of informative loci.[13] Thus, delivering chemotherapy or novel agents before HSCT is severely limited by our inability to follow disease burden in these patients.

Allele-specific oligonucleotide–PCR has been developed to detect single nucleotide substitution levels in various cancers[14] and involves designing primers that will preferentially amplify the mutant allele over the wild-type (WT) allele by exploiting differences in the amplification efficiency of DNA polymerase in the presence of mismatched base pairs. By combining TaqMan chemistry with a mismatched amplification mutation assay (MAMA), a form of allele-specific oligonucleotide–PCR, TaqMAMA allows for the quantification of mutant DNA, thereby making it possible to quantify mutant copy number in the presence of a 1000-fold excess of WT DNA.[15] We have developed quantitative TaqMAMA assays to track the most common mutations in *PTPN11*, *KRAS*, and *NRAS*. Using this method, it is now possible to quantify disease burden before HSCT and to measure MRD after HSCT in JMML patients.

Methods

Patients and samples

The University of California San Francisco Institutional Review Board approved this research. Written informed consent was obtained in accordance with the Declaration of Helsinki.

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Table 1. Patient characteristics

Characteristic	No. of patients	Mean false positive rate*
Patients	25	—
Girls	8	—
Boys	17	—
Pre-HSCT therapy	21	—
Chemotherapy only	10	—
Splenectomy only	3	—
Chemotherapy and splenectomy	8	—
Remission following first HSCT	11	—
Transplantation-related mortality	2	—
Relapse following first HSCT	12	—
Second HSCT	4	—
Median duration of post-HSCT follow-up	1.8 y	—
Mutation		
PTPN11 g226G>A	13	0.0000
PTPN11 214G>A	3	0.0059
PTPN11 227A>G	2	0.0712
NRAS 38G>A	2	0.0203
KRAS 38G>A	2	0.0237
PTPN11 1508G>C	1	0.0162
NRAS 37G>C	1	0.0000
KRAS 35G>A	1	0.3449

— indicates not applicable.
*See Figure S1 for graphic depiction of false positive rates.

The 25 patients studied were enrolled in the European Working Group of Myelodysplastic Syndromes in Childhood study group trials or the North American Juvenile Myelomonocytic Leukemia Project or were treated at the University of California San Francisco. Table 1 lists clinical and genetic features at diagnosis, pre-HSCT therapy, and treatment outcomes. Pretransplantation samples were obtained for 24 patients. Post-HSCT samples were collected weekly or monthly for 19 patients who received 22 transplants. Peripheral blood (PB) and BM samples were obtained on the same day or on consecutive days when possible (n = 16). DNA was extracted from whole BM or PB or from mononuclear cells isolated from BM, PB, or spleen, and diluted to 50 ng/μL. Genomic DNA from normal subjects served as negative controls.

Preparation of standard plasmids

DNA segments containing each of the 8 mutations were cloned according to the Invitrogen TA Cloning Kit (Invitrogen, Carlsbad, CA). Mutation containing plasmids were confirmed by sequencing, quantified using pico green, and serially diluted into yeast tRNA, 100 ng/μL.

Sample digestion

To maximize amplification of the mutant allele, residual WT sequences containing appropriate sites were digested with restriction enzymes when possible. Table S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article) lists the 8 primer/probe sets, relevant enzymatic digestions for the samples, and the enzyme used to linearize the plasmid standards (New England BioLabs, Ipswich, MA).

TaqMAMA PCR reactions

Each assay was run with a 5-point standard curve of a plasmid standard ranging from 100 000 to 10 copies, patient samples, 2 WT samples, and no template controls, all in duplicate. A total copy number (TCN) forward and reverse primer set was designed to amplify all copies (mutant + WT) of the exon being quantified as a control for DNA integrity. The mutant allele was amplified using a MAMA primer and one of the TCN primers to minimize variability between reactions. TaqMAMA reaction buffers are listed in Table S2, and cycling conditions are available on request.

The percentage of mutant DNA was calculated by dividing the mean MAMA copy number by the mean TCN (calculated from the Ct values by the SDS 1.9.1 software) and multiplying by 100. This percentage was also calculated for WT samples as a false positive rate and used as a threshold for negative results. Any sample with a total copy number less than 5000 was discarded for insufficient DNA integrity.

Results and discussion

Sensitivity and specificity of the TaqMAMA assay

To test the sensitivity of the MAMA primers to detect mutant DNA, JMML patient DNA from diagnosis that harbored either the *PTPNII* 214G > A or 226G > A mutation was spiked into WT DNA at serial dilutions of 100 ng patient DNA (100%), 10%, 1%, 0.1%, and down to 0% (100 ng WT DNA). Linear regression of the 226G > A and 214G > A dilution assays gave slopes of 0.827 and 0.344, and r^2 values of 0.998 and 0.999, respectively (data not shown). The amplification of the mutant compared with WT DNA at the lowest dilution of mutant DNA (0.1%) is not shown. A 1-tailed *t* test using Welch's correction found significant differences in amplification of the mutant allele between the 0.1% patient DNA and the WT samples ($P < .001$ and $P = .008$ for the 226G > A and 214G > A assays, respectively), thus validating the sensitivity of these assays for the mutant DNA. The standard curve method reported here negates the necessity to accurately quantify DNA samples before testing.

False positive amplification of the WT allele was less than 0.1% for 7 of the assays (Figure S1). Any percent mutant DNA that was equal to or lower than the average false positive rate on that plate was considered 0%. The highest false positive rate was for the *KRAS* 35G > A assay, and approached 1 in 300. However, the reemergence of the mutant allele occurred at a level of 1 in 100 rapidly in the patient who relapsed, and we therefore concluded that detection of mutant *KRAS* 35G > A DNA at these levels remained highly informative.

Concordance of PB versus BM samples

Sixteen pairs of BM and PB were taken on the same or consecutive days and analyzed. Of these, 6 pairs had 0% mutant DNA in both PB and BM. Of the remaining 10 pairs, 7 had reasonable concordance between PB and BM values (Figure S2). Ninety percent of the blood samples were positive simultaneously with the BM. It is unquestionably easier to obtain PB samples from these young children than BM samples, which allows for more frequent sampling. Monitoring the blood frequently during the first 6 months after HSCT is particularly important because most relapses occur during this time[11] and can thus be tracked with rapidly rising mutant DNA levels.

Response to pre-HSCT treatment

We investigated whether traditional pre-HSCT chemotherapy would reduce MRD before HSCT and improve chances of complete remission after HSCT. It has been reported that no particular pre-HSCT therapy impacts the likelihood of achieving a CR after HSCT.[11] Pre-HSCT samples were collected for 24 patients receiving first transplant. Median percent mutant DNA before HSCT is listed by mutation and type of pre-HSCT therapy in Table S3, along with achievement of clinical remission (CR) after HSCT. Complete remission was defined as patients who did not meet clinical criteria for relapse and who displayed no mixed chimerism (> 5% autologous cells) for the time period followed (0.2-6.4 years).

Table 2. Earliest detected TaqMAMA versus chimerism in relapsing patients after HSCT

Patient no., mutation	Time after HSCT, d	Mutant DNA by TaqMAMA, %*	Autologous cells, %
D098			
KRAS 38G>A	36	0.063	0
KRAS 38G>A	57	0.019	0
KRAS 38G>A	77	0.034	0
KRAS 38G>A	100	0.203	0
Clinical relapse	140	36.11	90
D292			
PTPN11 227A>G	18	0.159	0
PTPN11 227A>G	32	0.200	10
Clinical relapse	140		
D361			
NRAS 38G>A	28	0.050	0
NRAS 38G>A	32	0.044	0
NRAS 38G>A	64	0.374	5
Clinical relapse	366		
D405			
NRAS 37G>C	29	0.110	0
NRAS 37G>C	29	0.408	0
NRAS 37G>C	35	0.187	0
NRAS 37G>C	41	1.033	5
Clinical relapse	57		
I160			
PTPN11 226G>A	33	0.069	0
PTPN11 226G>A	64	0.313	0
PTPN11 226G>A	77	0.044	0
PTPN11 226G>A	107	0.074	0
PTPN11 226G>A	180	0.000	0
PTPN11 226G>A	215	0.177	0
PTPN11 226G>A	229	0.472	0
PTPN11 226G>A	245	5.469	15
Clinical relapse	298		

*Samples are adjusted for any false positive amplification.

There was no difference in the achievement of CR between patients who received pre-HSCT chemotherapy (9 of 17) versus those who did not (4 of 7). The median percent mutant DNA pre-HSCT for patients who achieved CR was 46% (n = 13); and for patients who relapsed, the median was 49% (n = 11). We did not observe a significant reduction of disease burden in response to any of the pre-HSCT chemotherapy strategies, although admittedly, there was little information about the choices of pre-HSCT chemotherapy and the indications for treatment. In addition, the number of patients analyzed was small. Despite the low number of patients, however, the wide range of pre-HSCT disease burden in all patients indicates no particular relationship between pre-HSCT therapies with achievement of CR after HSCT. Taken together, this supports the conclusion that HSCT remains the only curative therapy currently available for these patients.

TaqMAMA versus autologous cells

In all, 149 after HSCT samples were analyzed for mutant DNA by the TaqMAMA method and for autologous cells by the chimerism method described previously.[13] Taq-MAMA levels were then plotted vs autologous cells. The resulting correlation was strong ($P < .01$). For 8 patients who relapsed after HSCT, the time point of earliest detection of mutant DNA was compared with rising levels of autologous cells. The TaqMAMA assay detected mutant cells earlier than the chimerism method for 5 of the 8 patients (Table 2; Figure S3) and thus may be a more sensitive method of detecting minimal disease burden. The other 3 patients had simultaneously detected mutant DNA levels and rising autologous cells (data not shown). Taken together, however, both methods predicted relapse long before the diagnosis of clinical relapse occurred.

Because the TaqMAMA method investigates disease burden by looking at the presence of JMML specific mutant cells, rather than donor versus recipient cells, it is the only way to track disease burden before HSCT. Thus, this assay will be critical during the testing of novel agents before HSCT for the treatment of JMML. Although the validity of our assay against traditional donor chimerism is statistically robust, further validation on prospectively acquired specimens is planned for the next generation of clinical trials.

We have shown that the TaqMAMA method of quantifying mutant DNA is a useful tool to assess disease burden in JMML patients. Although HSCT is currently the only known curative option for JMML, the late effects sustained by these young survivors are serious enough to warrant a concerted effort to treat this disease with targeted inhibitors of components of the Ras pathway. Thus, this mutation-specific MRD technique will become increasingly important for clinical care as novel targeted therapies for patients with JMML are developed and tested in future clinical trials.

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Authorship

Contribution: S.A. performed research, analyzed data, and wrote the paper; A.Y. contributed samples and wrote the paper; N.J.F., M.R., A.F., and P.S. performed research and analyzed data; C.P.K. analyzed data and wrote the paper; P.N. and C.F. analyzed data; F.L., M.Z., and R.P.C. contributed samples; P.D.E. contributed samples and analyzed data; C.M.N. contributed samples, analyzed data, and wrote the paper; P.B. contributed samples, performed research, and analyzed data; M.L.L. designed research, analyzed data, and wrote the paper.

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