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Development of real-time quantitative polymerase chain reaction assays to track treatment response in retinoid resistant acute promyelocytic leukemia

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Molecular detection of minimal residual disease (MRD) has become established to assess remission status and guide therapy in patients with ProMyelocytic Leukemia–*RARA*+ acute promyelocytic leukemia (APL). However, there are few data on tracking disease response in patients with rarer retinoid resistant subtypes of APL, characterized by *PLZF–RARA* and *STAT5b–RARA*. Despite their rarity (<1% of APL) we identified 6 cases (*PLZF–RARA*, $n = 5$; *STAT5b–RARA*, $n = 1$), established the respective breakpoint junction regions and designed reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) assays to detect leukemic transcripts. The relative level of fusion gene expression in diagnostic samples was comparable to that observed in t(15;17) – associated APL, affording assay sensitivities of ~1 in 10^4–10^5. Serial samples were available from two *PLZF–RARA* APL patients. One showed persistent polymerase chain reaction positivity, predicting subsequent relapse, and remains in CR2, ~11 years post-autograft. The other, achieved molecular remission (CRm) with combination chemotherapy, remaining in CR1 at 6 years. The *STAT5b–RARA* patient failed to achieve CRm following frontline combination chemotherapy and ultimately proceeded to allogeneic transplant on the basis of a steadily rising fusion transcript level. These data highlight the potential of RT-qPCR detection of MRD to facilitate development of more individualized approaches to the management of rarer molecularly defined subsets of acute leukemia.

Keywords: minimal residual disease, acute myeloid leukemia

INTRODUCTION

Acute promyelocytic leukemia (APL) is characterized by rearrangements of the gene encoding retinoic acid receptor alpha (RARα), which is most commonly fused to the ProMyelocytic Leukemia (*PML*) gene consequent upon the t(15;17)(q22;21) (reviewed Mistry et al., 2003). In approximately 10% of APL cases the t(15;17) is not detected, due to cytogenetic failures, simple variant translocations involving 15q22 or 17q21 and another partner chromosome, or more complex rearrangements (Grimwade et al., 2000). The majority of these cases lacking the classic t(15;17) nevertheless still harbor an underlying *PML–RARA* fusion gene, while in ~1–2% of cases presenting with APL an alternative fusion partner is involved (Grimwade et al., 2000). These include PLZF (ZBTB16), NPM1, NuMA, FIP1L1, and BCOR, formed as a result of the t(11;17)(q23;q21), t(5;17)(q35;q21), t(11;17)(q13;q21), t(4;17)(q12;q21), and t(X;17)(p11;q21), respectively; while the *PRKAR1A*, and *STAT5b* genes are fused to *RARA* following rearrangements involving 17q (Chen et al., 1993; Redner et al., 1996; Wells et al., 1997; Arnould et al., 1999; Catalano et al., 2007; Kondo et al., 2008; Yamamoto et al., 2010). The nature of the fusion partner has an important bearing on disease biology, particularly the likely response to molecularly targeted therapies in the form of all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO). ATRA sensitivity has been documented in APL subtypes involving PML, NPM1, NuMA, and FIP1L1 (reviewed Grimwade et al., 2010); whereas, PLZF–RARα and STAT5b–RARα have both been associated with primary resistance to retinoids and a poorer prognosis (Licht et al., 1995; Arnould et al., 1999; Dong and Tweardy, 2002). In the case of PLZF–RARα associated APL with the t(11;17)(q23;q21), the retinoid insensitivity is compounded by expression of the reciprocal RARα–PLZF fusion product from the derivative chromosome 17 [der(17)], which functions as a transcriptional activator targeting PLZF-binding sites leading to upregulation of cellular retinoic acid binding protein I (CRABP1), which sequesters retinoic acid, limiting its access to the nucleus (Guidez et al., 2007). To date, sensitivity to arsenic has only been demonstrated in PML–RARα positive APL, reflecting the capacity of ATO to bind directly to the PML moiety of the fusion

protein inducing its degradation via the proteosome (Zhang et al., 2010).

Molecular diagnostics to establish the nature of the fusion partner are therefore important for appropriate management, but in addition the application of sensitive minimal residual disease (MRD) assays to track treatment response has been found to be clinically useful in patients with PML–RARα+ disease, with previous studies showing that achievement of molecular remission (CRm) as determined by qualitative or quantitative polymerase chain reaction (PCR) assays (with a sensitivity of 10–4) is a prerequisite for long-term remission and disease cure (reviewed Sanz et al., 2009; Grimwade and Tallman, 2011). These assays when applied at the post-consolidation timepoint are not sufficiently sensitive to identify all patients destined to relapse (Grimwade et al., 1996; Burnett et al., 1999). However, sequential molecular monitoring studies have shown that in patients who achieve CRm, recurrence of PCR positivity heralds disease relapse (Diverio et al., 1998; Jurcic et al., 2001). Prediction is further refined by the use of reverse transcription-quantitative real-time PCR (RT-qPCR) which enables parallel quantification of endogenous control genes (e.g., Abelson, *ABL*) and leukemic transcripts, such that poor quality samples that could otherwise give rise to "false negative" results can be more reliably identified (Grimwade et al., 2009). Importantly, it also provides data on the kinetics of disease relapse, informing development of optimized MRD monitoring schedules (reviewed Freeman et al., 2008).

A significant complication of relapsed APL is death from hemorrhage due to the associated coagulopathy (Sanz et al., 2009). Therefore, Italian GIMEMA and Spanish PETHEMA groups explored the use of serial MRD monitoring as a tool to identify patients with impending relapse of APL (based upon persistent PCR positivity during therapy or recurrent PCR positivity in patients showing an initial response) to guide pre-emptive therapy to prevent disease progression (Lo Coco et al., 1999; Esteve et al., 2007). These studies, which were conducted before the availability of ATO for the treatment of relapse, suggested a survival benefit for early treatment intervention. More recently, we have shown in the Medical Research Council (MRC) AML15 trial that sequential monitoring using standardized RT-qPCR assays [developed within the Europe Against Cancer (EAC) program; Gabert et al., 2003)] provides the most powerful independent prognostic factor in APL (Grimwade et al., 2009). In addition we clearly demonstrated that these assays could be used to pinpoint particular patients destined to relapse, allowing successful delivery of pre-emptive therapy (Grimwade et al., 2009). This led to a significant reduction in the rate of frank relapse and improved survival, which was most marked in patients with high risk disease, i.e., with presenting white blood cell count above 10×10^9/l. Moreover, we have shown that use of MRD monitoring to allow early deployment of ATO is associated with a significant reduction in treatment-related complications – substantially decreasing the risk of hyperleukocytosis and the associated life-threatening differentiation syndrome (Grimwade et al., 2009). Accordingly molecular monitoring of MRD has become widely recognized as a standard component of care for patients with *PML–RARA*+ APL, as reflected in recent disease guidelines (Sanz et al., 2009).

While treatment is increasingly being tailored to the needs of individual patients, there are virtually no data on molecular monitoring in PLZF–RARα and STAT5b–RARα associated APL, which have been associated with a poorer prognosis. We have developed sensitive RT-qPCR assays suitable for tracking treatment response in these patients and which could be used to assess novel therapeutic approaches in retinoid insensitive disease.

MATERIALS AND METHODS

PATIENTS

Our laboratory has served as the reference center for molecular diagnosis of APL for successive MRC/National Cancer Research Institute (NCRI) trials since 1994 and also receives samples for diagnosis and MRD monitoring from non-trial patients from across the UK (Burnett et al., 1999; Grimwade et al., 2009). To date, we have identified six cases of morphologically suspected APL presenting in the UK that lacked the t(15;17) and were subsequently found to have an underlying *PLZF–RARA* ($n = 5$) or *STAT5b–RARA* ($n = 1$) fusion (**Table 1**). These include a previously unreported case (UPN 5) with the t(11;17)(q23;q21) giving rise to the *PLZF–RARA* fusion, treated within the UK MRC AML12 trial. Clinical details of the APL patient with the STAT5b–RARα fusion, who presented with pancytopenia and intracardiac thrombus have recently been described (Cahill et al., 2011). Samples were taken for molecular analysis following informed patient consent in accordance with the Declaration of Helsinki and the study was subject to Local Research Ethics Committee approval (St Thomas' Hospital Research Ethics Committee ref 06/Q0702/140).

CHARACTERIZATION OF APL FUSION PARTNER

Total RNA was extracted using the TRIzol reagent (Invitrogen Ltd., UK) according to the manufacturer's instructions, and 2 μg were used for cDNA synthesis with random hexamers (Invitrogen Ltd., UK) and either M-MLV or SuperScript II reverse transcriptases (both Invitrogen Ltd., UK). In four cases with documented t(11;17)(q23;q21) on diagnostic cytogenetic assessment, diagnostic samples were screened for expression of *PLZF–RARA* and reciprocal *RARA–PLZF* fusion transcripts by nested RT-PCR, as previously described (Grimwade et al., 1997). In 2 patients with simple variant translocations, i.e., t(7;17)(q36;q21) and t(3;17)(q26;q21) in UPN4 and UPN6, respectively, 5′ rapid amplification of cDNA ends (RACE) PCR was performed to identify the *RARA* fusion partner, using 2 μg of total RNA and the 5′/3′ RACE Kit, second generation (Roche Diagnostics Ltd., UK) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA using an antisense gene-specific primer located in *RARA* exon 4 (SP1, 5′-CGGTGACACGTGTACACCATGTTC-3′) and the homopolymeric A-tail was added to its 3′ end as per manufacturer's instructions. Tailed cDNA was then amplified by PCR using a second gene-specific primer located in *RARA* exon 4 upstream of the SP1 primer (SP2, 5′-TGGATGCTGCGGCGGAAGAAGC-3′), and the supplied Oligo dT-anchor primer (5′-GACCACGCGTATCGATGTCGAC$(T)_{16}$V-3′, where V = A, C or G) which binds to the 5′ end of the poly(A)-tail. First round PCR product was then used as a template in a second PCR reaction with a nested PCR primer (SP3, 5′-CCATAGTGGTAGCCTGAGGACTTG-3′)

Table 1 | Clinical details and disease characteristics of patients with *PLZF–RARA* or *STAT5b–RARA* associated APL.

Patient	Age at diagnosis (years)	Presenting WBC (10^9/l)	Cytogenetics	Fusion genes expressed	Treatment	Outcome	RQ-PCR assay sensitivity
UPN1	53	4.5	46,XY,t(11;17)(q23;q21)	*PLZF–RARA* (2ZF) *RARA–PLZF*	ADE/G-CSF/ATRA, 3 consolidation courses MRC AML12*	Relapse at 45mo, FLAGx2 + ATRA, Cy TBI autograft. Alive in second CR at 177mo from diagnosis	1 in $10^{5.1}$ (*PLZF–RARA*), 1 in $10^{4.3}$ (*RARA–PLZF*)
UPN2	50	6.8	46,XY,t(11;17)(q23;q21)/45,X–Y,t(11;17)(q23;q21)	*PLZF–RARA* (2ZF)	ADE/ATRA, ADE MACE, MiDAC	1st CR 73mo from diagnosis	1 in $10^{4.3}$
UPN3	75	2.0	46,XY,t(11;17)(q23;q21)/46,idem,del(12)(p1?)/46,idem,−6,+r	*PLZF–RARA* (3ZF) *RARA–PLZF*	DAT2 + 7/ATRA, DAT2 + 7, MACE	Relapse at 55mo, Dauno + Ara-C. Died in second CR 88mo from diagnosis	1 in $10^{4.3}$ (*PLZF–RARA*), 1 in $10^{4.3}$ (*RARA–PLZF*)
UPN4	58	7.4	46,XY,t(7;17)(q36;q21)	*PLZF–RARA* (3ZF)	DAT3 + 10/ATRA, DAT3 + 8/ATRA, MACE	Died in relapse 3.5mo from diagnosis	1 in $10^{4.6}$
UPN5	62	1.2	47,XY,+8[3]/47,XY,+8,t(11;17)(q23;q21)[23]	*PLZF–RARA* (3ZF) *RARA–PLZF*	MRC AML12	Relapsed at 7mo. Died in relapse at 15mo	1 in $10^{4.6}$ (*PLZF–RARA*), 1 in $10^{4.6}$ (*RARA–PLZF*)
UPN6	29	5.6	46,XX,t(3;17)(q26;q21)	*STAT5b–RARA*	AIDA #1, DA 3 + 8, Ara-C 1.5g/m^2 × 2	Persistent PCR positivity → FLA, BuCy sibling allograft. Died 15mo from diagnosis – respiratory failure	1 in $10^{5.4}$

*UPN5 is a previously unreported case. Clinical details and information regarding further molecular characterization of UPNs1–4 has been reported elsewhere (Culligan et al., 1998; Grimwade et al., 1997; Grimwade et al., 2000; Guidez et al., 2007). Details of the clinical presentation of the STAT5b–RARA case (UPN6) have been described previously (Cahill et al., 2011). *Details of the MRC AML12 protocol have been published previously (Burnett et al., 1999). Dauno, daunorubicin; Ara-C, cytosine arabinoside.*

located in *RARA* exon 3 and the PCR anchor primer (5′-GACCACGCGTATCGATGTCGAC-3′) from the kit which anneals to the sequence introduced by the non-T portion of the Oligo d(T)-anchor primer in the previous PCR round. Upon visualization in 1% agarose gels, purified 5′ RACE PCR products were cloned using the pGEM-T Easy Vector System (Promega, UK) and identified by sequence analysis (**Figure 1**). Breakpoint location was further verified by sequencing of nested RT-PCR products which were obtained from independent RNA aliquots.

DEVELOPMENT OF RT-qPCR ASSAYS FOR APL FUSION TRANSCRIPTS

The assay designs to amplify *PLZF–RARA* and *STAT5b–RARA* fusion transcripts were adapted from the standardized *PML–RARA* assay developed in the EAC program (Gabert et al., 2003), using the EAC probe and reverse primer located in *RARA* exon 3 in conjunction with newly designed forward primers located within *PLZF* (exon 3 or 4, depending upon the breakpoint) and *STAT5b* (exon 15), respectively (**Figure 2**; **Table 2**). In addition, reciprocal *RARA–PLZF* transcripts expressed from the der(17) were detected using a common forward primer and probe located in *RARA* exon 2, which were previously described for amplification of reciprocal *RARA–PML* transcripts in APL with the classic t(15;17) (Grimwade et al., 2009), used in conjunction with newly designed reverse primers located in *PLZF* exons 4 and 5, according to patient breakpoint (**Figure 2**; **Table 2**). Assays were designed using Primer Express software (Applied Biosystems, Warrington, UK). RT-qPCR reactions were run on the ABI7900 platform under the standard EAC conditions (Gabert et al., 2003), with expression of leukemic fusion transcripts normalized to the *ABL* control gene using the ΔCt method, as described previously (Flora and Grimwade, 2004; Grimwade et al., 2009). All assays were confirmed to be fusion transcript specific based on lack of detectable amplification in normal control ($n = 5$) or diagnostic *PML–RARA*+ APL ($n = 5$) blood and bone marrow (BM) samples. RT-qPCR assay sensitivity was calculated, based upon the level of expression of leukemic transcripts in the diagnostic sample in relation to the *ABL* control gene, as described (Freeman et al., 2008; Grimwade et al., 2009). Assays were run in triplicate; amplification in at least two of three replicates with Cycle Threshold (Ct) values ≤40 (threshold 0.05) was required to define a result as PCR positive for the fusion transcript in question, according to EAC criteria (Gabert et al., 2003). No-template controls (NTCs) for each assay were run in duplicate to exclude possible contamination, while patients' diagnostic samples served as positive controls.

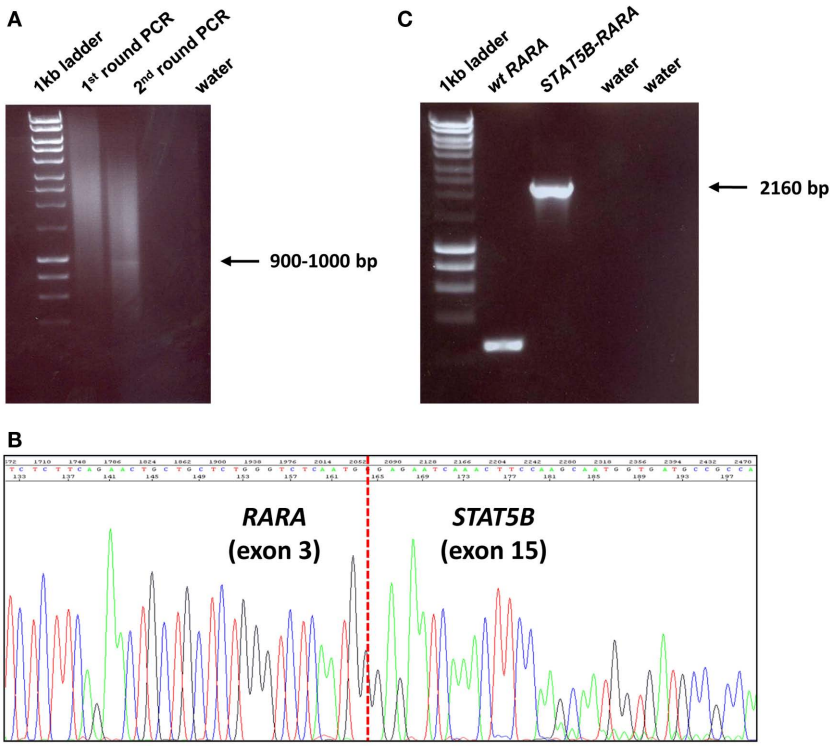

FIGURE 1 | Identification of *STAT5b–RARA* fusion underlying APL in UPN6 with t(3;17)(q26;q21) variant translocation. (A) 5′ RACE was undertaken, which showed a weak band on second round PCR; the amplification product was cloned, sequenced and found to be a fusion between *STAT5b* exon 15 and *RARA* exon 3 **(B)**, in accordance with the breakpoints identified in 4 previously reported cases with this rearrangement (Arnould et al., 1999; Kusakabe et al., 2008; Iwanaga et al., 2009; Qiao et al., 2011). Detection of *STAT5b–RARA* fusion transcripts was confirmed by nested RT-PCR using a fresh aliquot of RNA **(C)**.

MRD level in follow-up samples was calculated using the ΔΔCt method as described by Beillard et al. (2003). Briefly, the difference in expression between the fusion transcript (FT) and *ABL* in a follow-up (FUP) sample ($\Delta Ct_{FUP} = Ct_{FT} - Ct_{ABL}$) was normalized to the difference between their expression at diagnosis (D_x) ($\Delta Ct_{Dx} = Ct_{FT} - Ct_{ABL}$) using the following formula: $10^{[(\Delta CtFUP - \Delta CtDx)/-3.5]}$, where −3.5 represents the mean slope observed in the EAC program for plasmid standard curves (Beillard et al., 2003). Persistent PCR positivity was defined by the presence of leukemic transcripts throughout frontline therapy including the post-consolidation timepoint. CRm was defined as lack of detection of leukemic fusion transcripts in a BM sample affording a sensitivity of at least 1 in 10^4.

RESULTS

MOLECULAR CHARACTERIZATION OF APL CASES WITH ALTERNATIVE FUSION PARTNERS

Molecular analysis was undertaken in six cases of *PML–RARA* negative APL. In four cases (UPN1-3, UPN5), cytogenetics showed the t(11;17)(q23;q21), and presence of a *PLZF/RARA* rearrangement was confirmed by conventional nested RT-PCR (**Table 1**). In two cases (UPN4, UPN6) with t(7;17)(q36;q21) and t(3;17)(q26;q21) we postulated occurrence of a novel APL fusion; however, in both cases 5′ RACE revealed involvement of a known fusion partner, i.e., *PLZF* and *STAT5b*, respectively (**Table 1**; **Figures 1** and **2**), which was confirmed by nested RT-PCR performed on fresh aliquots of RNA from the diagnostic samples. In two cases with *PLZF/RARA* rearrangements, the chromosome 11 breakpoint fell within *PLZF* intron 3, leading to retention of 2 zinc fingers (2ZF) in the PLZF moiety of the PLZF–RARα fusion protein. In the other three patients, the *PLZF* breakpoint fell within intron 4, leading to inclusion of 3 zinc fingers (3ZF) in the PLZF component of PLZF–RARα (**Table 1**). Reciprocal *RARA–PLZF* fusion transcripts were co-expressed in three of five cases (**Table 1**). In UPN6 with the *STAT5b–RARA* fusion, the breakpoint location within the *STAT5b* locus was found to be identical to that reported previously (Arnould et al., 1999; Kusakabe et al., 2008; Iwanaga et al., 2009; Qiao et al., 2011; **Figure 1**). In accordance with the findings reported in the index case (Arnould et al., 1999), reciprocal *RARA–STAT5b* transcripts were not detected in UPN6.

DEVELOPMENT OF RT-qPCR ASSAYS TO TRACK TREATMENT RESPONSE IN PATIENTS WITH *PLZF–RARA* AND *STAT5B–RARA* ASSOCIATED APL

In order to detect *PLZF–RARA* and *STAT5b–RARA* transcripts by RT-qPCR, forward primers were designed to be used in conjunction with the common probe and reverse primer developed within the EAC program to amplify *PML–RARA* fusion transcripts (**Figure 2**; Gabert et al., 2003). To amplify reciprocal *RARA–PLZF* transcripts by RT-qPCR, reverse primers were designed within *PLZF* exon 4 or 5 (according to patient breakpoint location), used

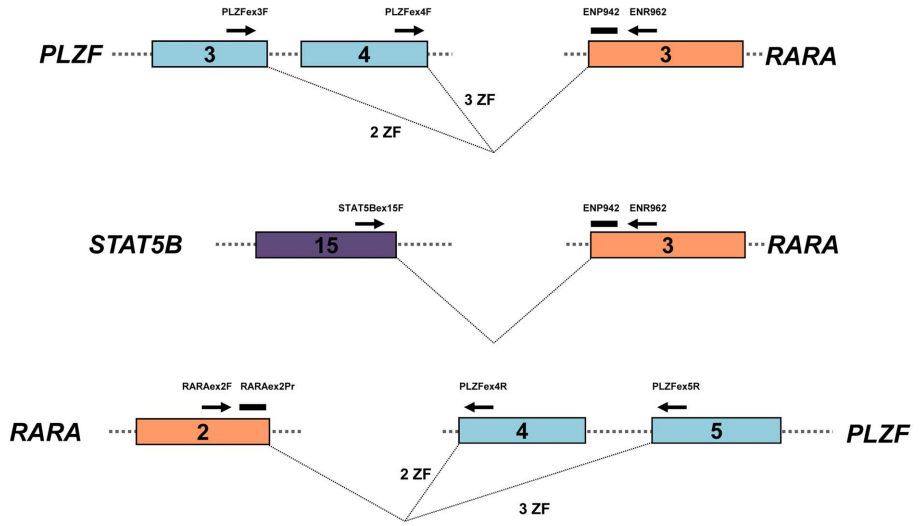

FIGURE 2 | Design of RT-qPCR assays to detect *PLZF–RARA*, *RARA–PLZF*, and *STAT5b–RARA* fusion transcripts. *PLZF–RARA* and *STAT5b–RARA* transcripts were detected with the common Europe Against Cancer probe (ENP942) and reverse primer (ENR962) located in *RARA* exon 3, used in conjunction with fusion-specific forward primers (see **Table 2**). For *PLZF–RARA* cases in which the chromosome 11 breakpoint fell within intron 3 of *PLZF* [retaining 2 zinc fingers (ZF) in the PLZF moiety of the resultant PLZF–RARα fusion protein] the forward primer was located in *PLZF* exon 3 (upper panel). Whereas for cases in which the breakpoint fell within intron 4 (retaining 3 ZF in the PLZF moiety of PLZF–RARα), the forward primer was located within exon 4 (upper panel). For amplification of *STAT5b–RARA*, the forward primer was placed in *STAT5b* exon 15 (middle panel). In UPN1, UPN3, and UPN5, reciprocal *RARA–PLZF* transcripts were co-expressed; RT-qPCR assays were designed for this target using a previously published common forward primer and probe located in *RARA* exon 2 (Grimwade et al., 2009), used in conjunction with a reverse primer located in *PLZF* exon 4 or 5, according to chromosome 11 breakpoint location (bottom panel).

Table 2 | Primers and probes used to detect APL fusion transcripts.

Primer/probe	Sequence (5′–3′)
PLZF–RARA* AND *STAT5B–RARA	
PLZFex3F	TGGATAGTTTGCGGCTGAGA
PLZFex4F	GAGACACACAGGCAGACCCATA
STAT5Bex15F	GCATCACCATTGCTTGGAAG
ENR962*	GCTTGTAGATGCGGGGTAGAG
ENP942*	FAM–AGTGCCCAGCCCTCCCTCGC–TAMRA
RARA–PLZF	
RARAex2F‡	CCCCTATGCTGGGTGGACT
PLZFex4R	CACCGCACTGATCACAGACAA
PLZFex5R	AGACAGAAGACGGCCATGTCA
RARAex2Pr‡	FAM–CCGCCAGGCGCTCTGACCAC–TAMRA

Sequences of the primers and probes used to detect PLZF–RARA, RARA–PLZF, and STAT5b–RARA fusion transcripts.

**Europe Against Cancer common reverse primer and probe (Gabert et al., 2003).*

‡ Published common forward primer and probe, used previously to amplify RARA–PML (Grimwade et al., 2009).

in conjunction with the common forward primer and probe both located within *RARA* exon 2 (**Figure 2**), which we have recently validated for amplification of reciprocal *RARA–PML* transcripts in patients with t(15;17) APL within the UK MRC AML15 trial (Grimwade et al., 2009). The relative expression of *PLZF–RARA* at diagnosis was comparable to that observed for *PML–RARA* transcripts in t(15;17) associated APL (Grimwade et al., 2009). The ΔCt_{Dx} ranged from −2 to +1, corresponding to assay sensitivities for detection of *PLZF–RARA* transcripts of between 1 in $10^{4.3}$ and 1 in $10^{5.1}$ (**Table 1**). In the three patients who were informative for the reciprocal *RARA–PLZF* assay, this was not found to improve the sensitivity to detect MRD as compared to detection of *PLZF–RARA* alone (**Table 1**). In UPN6, *STAT5b–RARA* transcripts were found to be very highly expressed at diagnosis ($\Delta Ct_{Dx} = -3$), affording an assay sensitivity of 1 in $10^{5.4}$.

In two *PLZF–RARA* patients (UPN1, UPN2), follow-up samples were available for analysis. Samples from UPN1 had originally been tested by conventional nested RT-PCR, with BMs taken at 5 and 10 months from diagnosis found to test PCR negative. However, in accordance with our experience with *PML–RARA*+ APL, the RT-qPCR assay afforded greater sensitivity, with *PLZF–RARA* transcripts detected at both timepoints (**Figure 3A**). Reciprocal *RARA–PLZF* transcripts were not detectable in these follow-up samples in accordance with the poorer sensitivity afforded by this assay (**Figure 3A**; **Table 1**). Failure to achieve CRm following frontline therapy predicted subsequent disease relapse, which occurred at 45 months from original diagnosis (**Figure 3A**). In UPN2, MRD monitoring was undertaken by RT-qPCR in real time; in this case, CRm was achieved with combination chemotherapy, *PLZF–RARA* transcripts remained undetected in subsequent surveillance MRD samples and this patient is in ongoing remission of APL at 73 months (**Figure 3A**). UPN6 with *STAT5b–RARA* was also monitored by RT-qPCR in real time (**Figure 3B**); this patient exhibited a 2-log reduction in fusion transcripts (i.e., 10^{-2} MRD level) following AIDA induction (ATRA + idarubicin). However, treatment response was much poorer than typically seen in *PML–RARA*+ APL (Grimwade et al., 2009), with no significant further decline in

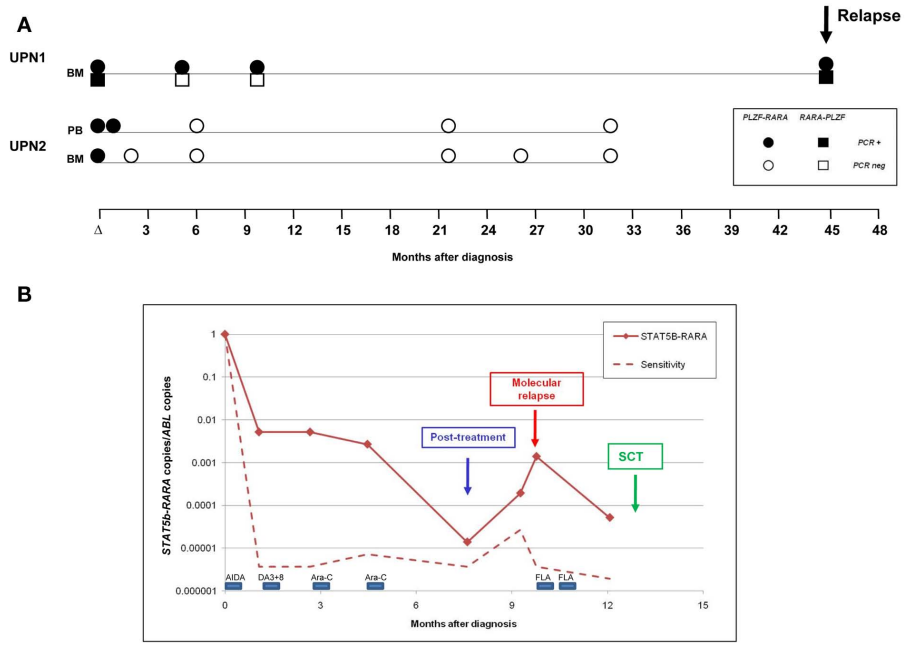

FIGURE 3 | Detection of minimal residual disease (MRD) by RT-qPCR assay in *PLZF–RARA* and *STAT5b–RARA* associated APL. (A) Serial samples were available from two patients (UPN1, UPN2) with t(11;17)(q23;q21)-associated APL. In UPN1, *PLZF–RARA* transcripts were still detectable at the post-treatment timepoint, predicting subsequent disease relapse. Reciprocal *RARA–PLZF* were not detectable in early follow-up samples, consistent with the poorer sensitivity of this assay in this patient (see **Table 1**). UPN2, who was not informative for *RARA–PLZF*, achieved molecular remission following frontline therapy and remains in ongoing remission of their leukemia. **(B)** Serial monitoring of *STAT5b–RARA* transcripts normalized to the *ABL* control gene in UPN6. The patient failed to achieve molecular remission following frontline therapy and showed a rapidly rising fusion transcript level indicative of impending full blown relapse (labeled "molecular relapse"). The patient received two courses of Fludarabine and cytosine arabinoside (FLA) as pre-emptive therapy and then proceeded to a sibling myeloablative allogeneic stem cell transplant (SCT).

fusion transcript level following two further courses of chemotherapy (DA3 + 8; cytarabine 1.5g/m^2). A 2-log decline in fusion transcripts was documented following the fourth course of chemotherapy (cytarabine 1.5g/m^2); however *STAT5b–RARA* transcripts remained detectable at the post-treatment timepoint and exhibited a steady rise of ∼2-logs over the following 2 months. The patient was deemed to be in molecular relapse and received further therapy (Fludarabine, cytarabine) which led to a further decline in fusion transcripts. However, the patient never achieved a CRm and therefore proceeded to a myeloablative sibling donor allogeneic transplant with busulphan and cyclophosphamide conditioning, which was unfortunately complicated by respiratory failure, leading to the patient's demise while still in clinical remission.

DISCUSSION

Application of molecular monitoring by RT-qPCR to establish remission status and identify patients needing additional therapy to achieve disease cure is now firmly established as a key component of the management of patients with *PML–RARA*+ APL (Sanz et al., 2009). However, there remains considerable uncertainty regarding the clinical utility of MRD monitoring in other forms of acute myeloid leukemia (AML). While there is evidence that RT-qPCR can be used to predict disease relapse in patients with nucleophosmin (NPM1) mutant AML (Schnittger et al., 2009; Krönke et al., 2011) and core binding factor (CBF) leukemia (Corbacioglu et al., 2010; Ommen et al., 2010), there are very limited data in patients with other molecularly defined subsets of disease.

To date, over 100 balanced chromosomal rearrangements which are considered to be primary events in leukemogenesis have been cloned (Mitelman et al., 2011). The characterization of the resulting chimeric fusion genes is not only important to achieve a greater understanding of disease biology, but has concomitantly yielded an extensive array of leukemia-specific targets that can effectively be used to track MRD by RT-qPCR. A number of genes (e.g., *MLL*, *RUNX1*, *RARA*, *NUP98*) are recurrently involved, fused to a range of potential partner genes. Depending upon breakpoint location, this allows common primers and probes located in the exon immediately adjacent to the breakpoint to be used in conjunction with an appropriate partner-gene specific primer to amplify the leukemic fusion transcript. In APL, translocation breakpoints consistently involve *RARA* intron 2, meaning that for cases with alternative fusion partners (e.g., *PLZF*, *STAT5b*, as described here), it is possible to use partner-specific forward primers in conjunction with an extensively validated probe and reverse primer located in *RARA* exon 3, that were designed in the EAC program (Gabert et al., 2003). Based on the expression of the fusion gene transcripts relative to the validated endogenous control gene *ABL* in diagnostic samples, it was established that the sensitivity of the assays was comparable to those used in *PML–RARA*+ APL, capable of detecting MRD at a sensitivity of at least 1 in 10^4. Due to the rarity of *PLZF–RARA*-associated APL, experience of MRD detection in

patients with this subset of leukemia is extremely limited (Cassinat et al., 2006); nevertheless, the significance of the MRD results seems to parallel those observed in patients with classic *PML–RARA*+ disease. In particular, CRm can be achieved with frontline therapy and is a prerequisite for disease cure.

As has been clearly demonstrated in *PML–RARA*+ APL, in order to reliably predict relapse it is important to adopt a sequential MRD monitoring approach (reviewed Grimwade and Tallman, 2011). This was applied in the patient with *STAT5b–RARA*+ APL, showing a failure to achieve CRm following intensive frontline therapy. Based upon the rising transcript level, further therapy was given to prevent impending relapse followed by a sibling allogeneic transplant. This approach was based on published data showing that patients with *PML–RARA*+ APL with persistent PCR positivity can potentially be salvaged by allogeneic transplant (Lo-Coco et al., 2003; Grimwade et al., 2009; Kishore et al., 2010). However, unfortunately there was an unsuccessful outcome in our patient with *STAT5b–RARA* due to transplant-related complications.

Therefore in conclusion, we have used 5′ RACE PCR to characterize simple variant translocations in APL, identifying cases involving the *PLZF* and *STAT5b* genes. Having defined breakpoint regions by sequence analysis, we adapted standardized RT-qPCR assays used for disease monitoring in patients with the classic t(15;17) in order to detect leukemic transcripts in these retinoid resistant subtypes of APL. As a consequence of the rarity of these disease entities, the number of cases analyzed was very small and study of further patients is merited to identify thresholds that may be useful to predict risk of relapse. Nevertheless, this study highlights the potential of RT-qPCR to guide management in patients with infrequent recurring translocations for which there is currently a paucity of robust prognostic information on which to base treatment decisions, particularly with respect to the role of allogeneic transplant in first remission.

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