Evolution of a *FLT3*-TKD mutated subclone at meningeal relapse in acute promyelocytic leukemia

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Abstract Here, we report the case of an acute promyelocytic leukemia (APL) patient who—although negative for *FLT3* mutations at diagnosis—developed isolated *FLT3* tyrosine kinase II domain (*FLT3*-TKD)-positive meningeal relapse, which, in retrospect, could be traced back to a minute bone marrow subclone present at first diagnosis. Initially, the 48-yr-old female diagnosed with high-risk APL had achieved complete molecular remission after standard treatment with all-*trans* retinoic acid (ATRA) and chemotherapy according to the AIDA (ATRA plus idarubicin) protocol. Thirteen months after the start of ATRA maintenance, the patient suffered clinically overt meningeal relapse along with minute molecular traces of PML/RARA (promyelocytic leukemia/retinoic acid receptor alpha) in the bone marrow. Following treatment with arsenic trioxide and ATRA in combination with intrathecal cytarabine and methotrexate, the patient achieved a complete molecular remission in both cerebrospinal fluid (CSF) and bone marrow, which currently lasts for 2 yr after completion of therapy. Whole-exome sequencing and subsequent ultradeep targeted resequencing revealed a heterozygous *FLT3*-TKD mutation in CSF leukemic cells (p.D835Y, c.2503G>T, 1000/1961 reads [51%]), which was undetectable in the concurrent bone marrow sample. Interestingly, the *FLT3*-TKD mutated meningeal clone originated from a small bone marrow subclone present in a variant allele frequency of 0.4% (6/1553 reads) at initial diagnosis. This case highlights the concept of clonal evolution with a subclone harboring an additional mutation being selected as the “fittest” and leading to meningeal relapse. It also further supports earlier suggestions that *FLT3* mutations may play a role for migration and clonal expansion in the CSF sanctuary site.

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INTRODUCTION

Acute promyelocytic leukemia (APL) is a distinct subset of acute myeloid leukemia (AML), associated with the chromosomal translocation t(15;17)(q22;q12), resulting in *PML*-*RARA* gene fusion and chimeric protein, which interferes with the maturation process of myeloid cells

(Grimwade et al. 2010). Clinical trials report long-term disease-free survival in ~85% of patients treated with all-*trans* retinoic acid (ATRA) and anthracycline-based chemotherapy (Lo-Coco et al. 2016). However, ~10% of patients will suffer relapse. Recently, the therapeutic spectrum has been widened by the introduction of arsenic trioxide (ATO), which in combination with ATRA produced 2-yr event-free survival rates of 97% in patients with low-to-intermediate-risk APL (Lo-Coco et al. 2013).

The Sanz score, which is based on peripheral leukocyte and platelet counts at presentation, forms the mainstay for risk stratification in APL (Sanz et al. 2000). Other factors that have been associated with an adverse outcome include CD56 expression and a promoter polymorphism of the *FAS* (also known as *CD95*) gene (Montesinos et al. 2011; Sunter et al. 2012).

Internal tandem duplications of the *FLT3* gene (*FLT3*-ITD) and point mutations of the *FLT3* tyrosine kinase II domain (*FLT3*-TKD), typically at Asp 835, are detected in 15%–40% and 10%–20% of APL cases, respectively (Noguera et al. 2002; Arrigoni et al. 2003; Au et al. 2004; Callens et al. 2005; Gale et al. 2005; Kuchenbauer et al. 2005; Chillon et al. 2010; Barragan et al. 2011; Schnittger et al. 2011; Welch et al. 2012; Shen et al. 2015). In APL, *FLT3*-ITD mutations are associated with leukocytosis at presentation but did not independently correlate with response to treatment or survival (Noguera et al. 2002; Au et al. 2004; Gale et al. 2005; Kutny et al. 2012; Breccia et al. 2013; Poire et al. 2014; Shen et al. 2015). No distinct clinical phenotype or consistent impact on prognosis has been identified for *FLT3*-TKD mutations in APL so far, perhaps because of their relative rarity (Shih et al. 2003; Barragan et al. 2011; Schnittger et al. 2011; Kutny et al. 2012).

Similar to other types of AML, extramedullary manifestations are rare in APL. However, extramedullary relapses have been reported with increasing frequency during the last 20 years, corresponding to the time when ATRA, which penetrates the central nervous system (CNS) poorly, became the mainstay of APL treatment (Byrd et al. 1995; Bakst et al. 2011). Approximately 3%–5% of APL patients will experience extramedullary relapse, most commonly in the CNS, with CNS relapse being either isolated or associated with bone marrow relapse (de Botton et al. 2006; Albano and Specchia 2011; Wang et al. 2014). Although high white blood cell counts at diagnosis and prior CNS hemorrhage are established risk factors for CNS relapse, the significance of increased age, differentiation syndrome, CD56 expression, and *FLT3*-ITD positivity remains unclear (Montesinos et al. 2009; Tashiro et al. 2011; Colovic et al. 2012).

RESULTS

Clinical Presentation and Treatment Outcome

The 48-yr-old female patient was diagnosed with high-risk APL in January 2012. After treatment according to the high-risk arm of the AIDA (ATRA plus idarubicin) protocol with ATRA and chemotherapy (Lo-Coco et al. 2010), complete molecular remission was achieved by the end of consolidation chemotherapy. Subsequently, maintenance therapy with ATRA was administered. In August 2013, 13 mo after initiation of maintenance, the patient developed meningeal relapse with pleocytosis of 597/ μ L in the cerebrospinal fluid (CSF) (Fig. 1), which caused headaches and paresthesia of the arms. Meningeal relapse was confirmed by immunophenotyping (CD33⁺, CD13^{+/−}, CD34^{+/−}, CD11b[−], HLA-DR[−], CD117[−], CD56[−]; Fig. 1), interphase fluorescence in situ hybridization, *PML-RAR α* (promyelocytic leukemia/retinoic acid receptor- α) reverse transcription–polymerase chain reaction (RT-PCR), and brain magnetic resonance imaging. At the time of meningeal relapse, the bone marrow continued to be in complete hematologic remission, although minimal residual disease (MRD) testing was positive for *PML-RARA* by nested RT-PCR without quantifiable load. The patient was treated with intrathecal applications of cytarabine and methotrexate and received

Figure 1. Clinical manifestations of meningeal APL relapse with Giemsa staining (*upper left panel*) and fluorescence-activated cell sorting analysis of CD33, CD34, HLA-DR, CD13, CD11b, CD117, CD56, and CD3 expression from cerebrospinal fluid blasts at relapse. Cells were gated in the side scatter (SSC)/forward scatter (FSC) analysis as shown in the *upper right panel*.

Figure 2. Treatment and course of cerebrospinal fluid (CSF) cell counts during 3 mo following relapse. The normalization of the CSF cell count sets in with delay. Ara-C, cytosine arabinoside; MTX, methotrexate; PCR, polymerase chain reaction; PML, promyelocytic leukemia; RARA, retinoic acid receptor- α ; iFISH, interphase fluorescence in situ hybridization; ATO, arsenic trioxide; ATRA, all-trans retinoic acid.

ATO/ATRA according to the APL0406 protocol (Lo-Coco et al. 2013), with the first cycle given over 2 mo and intervals between consolidation cycles shortened to 1 wk to increase dose density. At Day 26 of ATO/ATRA induction, elemental arsenic levels in peripheral blood and CSF were 114 $\mu\text{g/L}$ and 13 $\mu\text{g/L}$ as measured by inductively coupled plasma mass spectrometry. Following neutrophilic differentiation of meningeal APL blasts by Day 42, CSF cell counts normalized by week 8 and complete molecular remission was achieved by week 11 (Fig. 2). The patient continued on ATO/ATRA until February 2014 and on intrathecal chemotherapy until April 2014. For consolidation, the patient was recommended autologous hematopoietic stem cell transplantation with busulfan-based conditioning but opted for a watch-and-wait strategy instead. As of March 2016, with 2 yr of follow-up after the end of salvage treatment, the patient continues to be in complete molecular remission in bone marrow and CSF alike.

Genomic Analysis

To identify molecular drivers of meningeal relapse, we performed whole-exome sequencing of CSF-derived leukemic cells within NCT MASTER (Molecularly Aided Stratification for Tumor Eradication Research), a clinical sequencing program for advanced-stage cancer across all histologies, which identified a *FLT3*-TKD mutation (p.D835Y, c.2503G>T) (Table 1). Ultradeep targeted resequencing revealed the heterozygous presence of this mutation, which had not been found at first diagnosis, in virtually all CSF cells (51%, 1000/1961 reads). Of note, the mutation was undetectable (0/1666 reads) in the corresponding MRD-positive bone marrow sample drawn concurrently at meningeal relapse. Next, ultradeep targeted sequencing was performed to trace back the meningeal relapse clone to bone marrow samples obtained during the course of the disease. Interestingly, a small subclone comprising 0.4% (6/1553 reads) of bone marrow cells at first diagnosis already harbored

Table 1. Somatic mutations in CSF-derived leukemic cells identified by whole-exome sequencing

Chromosome	Position	Gene	RefSeq accession	Exon	Nucleotide change	VAF	Protein change
SNVs							
1q22	155,931,985	ARHGEF2	NM_001162383	10	c.G1130T	0.6	p.R377L
3q25.32	158,449,993	RARRES1	NM_002888	1	c.A212G	0.33	p.N71S
5q31.3	140,215,358	PCDHA7	NM_018910	1	c.G1390A	0.35	p.V464M
9q21.2	79,320,582	PRUNE2	NM_015225	8	c.T6608A	0.43	p.L2203X
9q33.1	118,974,010	PAPPA	NM_002581	4	c.G1717A	0.32	p.V573I
11p15.1	21,135,209	NELL1	NM_006157	13	c.C1375T	0.57	p.R459C
13q12.2	28,592,642	FLT3	NM_004119	20	c.G2503T	0.4	p.D835Y
13q14.3	52,678,680	NEK5	NM_199289	9	c.G563T	0.38	p.W188L
14q23.2	62,194,324	HIF1A	NM_001243084	6	c.T796G	0.36	p.F266V
16q24.2	87,678,376	JPH3	NM_020655	2	c.G895A	0.55	p.G299S
19p13.2	10,091,528	COL5A3	NM_015719	34	c.G2513A	0.5	p.R838H
20p11.23	18,474,662	RBBP9	NM_006606	3	c.T188C	0.49	p.L63P
Xq24	117,676,802	DOCK11	NM_144658	2	c.T217C	0.43	p.S73P
Indels							
8p22	17,067,557	ZDHHC2	NM_016353	9	c.848dupT	0.5	p.I283fs
8q24.21	128,750,873	MYC	NM_002467	2	c.411_412insGACCTTCTG	0.34	p.E137delinsEDLL
14q32.32	103,592,943	TNFAIP2	NM_006291	1	c.150_152del	0.12	p.50_51del
15q26.1	90,378,824	AP3S2	NM_001199058	10	c.1107delC	0.49	p.I369fs

CSF, cerebrospinal fluid; SNVs, single-nucleotide variants; Indels, insertion/deletion mutations; VAF, variant allele frequency.

the *FLT3*-TKD mutation, whereas it was undetectable in the bone marrow at all other time points—that is, during ATRA maintenance (0/874 reads), at meningeal relapse (0/1666 reads), and after the first cycle of ATO/ATRA (0/1198 reads) (Fig. 3).

DISCUSSION

From a clinical perspective, this case corroborates the effectiveness of ATO as a relapse treatment and, because of its permeability across the blood–brain barrier (Au et al. 2006, 2008), as a treatment for meningeal disease. ATO has been described to exert dose-dependent

Figure 3. *FLT3*-TKD subclone architecture based on ultradeep sequencing results, inserted into the time line of the disease course. A heterozygous *FLT3*-TKD mutation was present in virtually all cerebrospinal fluid (CSF) cells at meningeal relapse. Interestingly, the mutation could be traced back to a tiny *FLT3*-TKD clone present in the bone marrow sample from first diagnosis. BM, bone marrow; AIDA, ATRA plus idarubicin; ATRA, all-*trans* retinoic acid; ATO, arsenic trioxide.

dual effects on APL cells, inducing preferentially apoptosis at high concentrations and differentiation at low concentrations (Chen et al. 1997). ATO significantly penetrates into the CSF, leading to CSF arsenic concentrations of ~20% of the respective plasma levels, with considerable variation between individual patients (Au et al. 2006, 2008; Helwig et al. 2007). In our patient, the CSF arsenic concentration reached only 11% of the respective plasma concentration at day 26 of induction treatment, explaining the delayed differentiation effect that became detectable only after several weeks and cautioning us not to stop ATO-based protocols prematurely because of a perceived lack of efficacy.

From a molecular perspective, this case highlights the concept of genetic heterogeneity and selection of the “fittest” subclone as a driving mechanism of leukemia progression and relapse. This concept has recently been illustrated by whole-genome sequencing on paired diagnostic and relapse samples from patients with normal karyotype AML, establishing that relapse clones can originate from a minor subclone at initial diagnosis (Ding et al. 2012). Our case corroborates and extends this concept of clonal evolution as pathogenetic mechanism in AML.

Similar to normal karyotype AML, both *FLT3*-ITD and *FLT3*-TKD mutations were recurrently found as cooperating mutations in APL (Au et al. 2004; Callens et al. 2005; Gale et al. 2005; Kuchenbauer et al. 2005; Chillon et al. 2010; Barragan et al. 2011; Schnittger et al. 2011; Welch et al. 2012; Shen et al. 2015). Several *FLT3*-mutant APL cases with extramedullary relapse manifestations have been published (Housman et al. 2010; Tashiro et al. 2011; Colovic et al. 2012; Zou et al. 2013; Gill et al. 2015; Liu et al. 2015). In the majority of these cases, *FLT3* mutational analysis has only been performed in bone marrow or peripheral blood samples and either at initial diagnosis or relapse, respectively. Interestingly, however, in one patient, a *FLT3*-ITD mutation was detected at the time of combined CNS and bone marrow relapse, whereas the bone marrow at initial APL diagnosis was *FLT3*-ITD-negative (Tashiro et al. 2011). Additionally, a case of meningeal involvement in second relapse of APL was reported recently, which harbored a *FLT3*-ITD mutation, whereas the concurrently drawn bone marrow sample was *FLT3*-ITD-negative (Gill et al. 2015). Analogous to our case, the *FLT3*-ITD-positive meningeal clone could be traced back to a minor, although not quantified, subclone in the bone marrow at first relapse.

Recently, it was shown that, analogous to normal hematopoietic cells, the level of C-X-C chemokine receptor 4 (CXCR4) expression on AML cells correlates with stromal cell-derived factor-1 (SDF1, encoded by *CXCL12*)-induced chemotaxis and poor prognosis (Rombouts et al. 2004). Importantly, CXCR4 expression was significantly higher in *FLT3*-ITD mutated when compared with *FLT3* wild-type AML, and cells expressing constitutively active *FLT3*-ITD displayed dramatically enhanced migration toward SDF1/CXCL12 (Fukuda et al. 2005). In addition, SDF1/CXCL12 is highly expressed on the vasculature of the blood–brain barrier where it regulates the entry of leukocytes into the CNS (Williams et al. 2014). In conclusion, it appears that *FLT3* mutations favor migration and seeding of APL cells to the CSF sanctuary. The mechanisms of clonal selection in this case are closely intertwined with the biology of this privileged CSF site.

APL cases with meningeal involvement are rare, thereby precluding more systematic analyses on the role of specific mutations for extramedullary disease manifestations. Nevertheless, the above cases, together with the data reported here, strongly suggest a role for *FLT3* mutations in establishment and expansion of APL CNS manifestations.

METHODS

Isolation of DNA

Tissue samples were provided in accordance with the regulations of the National Center for Tumor Diseases (NCT) Heidelberg Tissue Bank and the approval of the Ethics Committee of

Heidelberg University. DNA from APL cells and DNA from the normal blood sample were isolated using the AllPrep DNA/RNA/Protein Mini Kit (QIAGEN), followed by quality control and quantification using a Qubit 2.0 Fluorometer (Life Technologies), a 2200 TapeStation system (Agilent), and a 2100 Bioanalyzer system (Agilent).

Whole-Exome Sequencing

High-throughput sequencing and data analysis were performed as described (Kordes et al. 2016). In brief, exome capturing was performed using SureSelect Human All Exon V5+UTRs in-solution capture reagents (Agilent). Briefly, 1.5 µg genomic DNA were fragmented to 150–200 bp (paired-end) insert size with a Covaris S2 device, and 250 ng of Illumina adapter-containing libraries were hybridized with exome baits at 65°C for 16 h. Paired-end sequencing (101 bp) was performed with a HiSeq 2500 instrument (Illumina) in rapid mode.

Mapping and Analysis of Whole-Exome Sequencing Data

Reads were mapped to the 1000 Genomes Phase 2 assembly of the human reference genome (NCBI build 37.1) using BWA (version 0.6.2) with default parameters and maximum insert size set to 1000 bp (Li and Durbin 2009). BAM files were sorted with SAMtools (version 0.1.19) (Li et al. 2009), and duplicates were marked with Picard tools (version 1.90). Average target coverage was 116.13× for the CSF leukemia and 113.41× for the bone marrow control. In both, >80% of the targets had a coverage of at least 40× (see Table 2).

For the detection of single-nucleotide variants, we applied our in-house analysis pipeline based on SAMtools mpileup and BCFtools with parameter adjustments to allow for calling of somatic variants with heuristic filtering as previously described (Jones et al. 2012, 2013; Yaktapour et al. 2014). After annotation with RefSeq (version September 2013) using ANNOVAR (Wang et al. 2010), somatic, nonsilent coding variants of high confidence were selected.

Ultradeep Targeted Resequencing

For the *FLT3*-D835Y-TKD mutation, ultradeep targeted resequencing was performed.

Table 2. Sequencing coverage for the leukemic CSF cells and the bone marrow control

Sample	Percentage of reads aligned	Average read coverage	Number of CCDS r15 sites with ≥10-fold coverage	Percentage of CCDS r15 with ≥10-fold coverage	Percentage of CCDS r15 with ≥20-fold coverage	Percentage of <i>FLT3</i> sites (2958 bases) with ≥10-fold coverage
Bone marrow	99.65%	113.41	46,754,560	94.78%	89.84%	98.72%
CSF blasts	99.64%	116.13	46,758,736	94.79%	89.84%	97.73%

The 49 Mbp of consensus coding sequence (CCDS release 15, [ftp://ftp.ncbi.nlm.nih.gov/pub/CCDS/archive/15/CCDS.current.txt](http://ftp.ncbi.nlm.nih.gov/pub/CCDS/archive/15/CCDS.current.txt) dated August 3, 2014) is used as the reference for protein-coding sequence coverage. The CCDS release was downloaded and converted into a BED file containing the exon coordinates with a custom Perl script. Only genes with *ccds_status* "Public" were considered. Using SAMtools version 0.1.19 (Li et al. 2009) *view* and *coverageBed* v2.16.2 (Quinlan and Hall 2010), the read number per site was determined for nonduplicate reads with a minimal mapping quality of 1.

CSF, cerebrospinal fluid; CCDS, Consensus Coding DNA Sequence.

Library Preparation and Semiconductor Sequencing

For library preparation, the multiplex PCR-based Ion Torrent AmpliSeq technology (Life Technologies) with the Cancer HotSpot Panel v2 (IonTorrent/Thermo Fisher Scientific) was used. Amplicon library preparation was performed with the Ion AmpliSeq Library Kit v2.0 using ~10 ng of DNA. Briefly, the DNA was mixed with the primer pool, containing all primers for generating the 207 amplicons and the AmpliSeq HiFi Master Mix and transferred to a PCR cyclor (Bio-Rad). After the end of the PCR, primer end sequences were partially digested using FuPa reagent, followed by the ligation of barcoded sequencing adapters (Ion Xpress Barcode Adapters, Life Technologies). The final library was purified using AMPure XP magnetic beads (Beckman Coulter) and quantified using qPCR (Ion Library Quantitation Kit, Thermo Fisher Scientific) on a StepOne qPCR machine (Thermo Fisher Scientific). The individual libraries were diluted to a final concentration of 100 pM and eight to 10 libraries were pooled and processed to library amplification on Ion Spheres using Ion PGM Template OT2 200 Kit. Unenriched libraries were quality-controlled using Ion Sphere quality control measurement on a QuBit instrument. After library enrichment (Ion OneTouch ES), the library was processed for sequencing using the Ion Torrent 200 bp sequencing v2 chemistry and the barcoded libraries were loaded onto a chip. Our way of pooling eight samples on a 318 chip resulted in a mean coverage of 3000-fold per amplicon.

Variant Calling and Annotation

Data analysis was performed using the Ion Torrent Suite Software (version 4.4). After base calling, the reads were aligned against the human genome (hg19) using the TMAP algorithm within the Torrent Suite. Variant calling was performed with the variant caller plugin within the Torrent Suite Software and the IonReporter package using a corresponding BED file containing the coordinates of the amplified regions. Only variants with an allele frequency >5% and minimum coverage >100 reads were taken into account. Variant annotation was performed using ANNOVAR (Wang et al. 2010). Annotations included information about nucleotide and amino acid changes of RefSeq annotated genes, COSMIC and dbSNP entries, and detection of possible splice site mutations. For data interpretation and verification, the aligned reads were visualized using the IGV browser (Broad Institute) (Robinson et al. 2011).

ADDITIONAL INFORMATION

Data Deposition and Access

The raw analytical data have been deposited at the European Genome-phenome Archive (<https://www.ebi.ac.uk/ega/datasets>), accession number EGAS00001001848.

Ethics Statement

APL cells and a matched normal blood sample were obtained following written informed consent under an institutional review board–approved protocol covering all aspects relevant to clinical cancer genome sequencing. This study was conducted in accordance with the Declaration of Helsinki. The patient also granted written consent to the publication of her case in *Molecular Case Studies*.

Author Contributions

T.B. and A.K. designed the research. T.B., S.F., W.W., B.H., M.H., and V.E. performed the research and analyzed the data. A.D.H. contributed to clinical data collection. C.T. contributed to sample preparation. T.B. and A.K. wrote the manuscript. S.F. and W.W. edited the

Competing Interest Statement

T.B. and A.K. have received travel grants from Teva, the manufacturer of arsenic trioxide (ATO).

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manuscript. All authors made significant contributions to, reviewed, and approved the final version of the manuscript.

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