Abstract. Background/Aim: Fusion of histone-lysine N-methyltransferase 2A gene (KMT2A) with the Rho guanine nucleotide exchange factor 12 gene (ARHGEF12), both located in 11q23, was reported in some leukemic patients. We report a KMT2A-ARHGEF12 fusion occurring during treatment of a pediatric acute myeloid leukemia (AML) with topoisomerase II inhibitors leading to a secondary acute lymphoblastic leukemia (ALL). Materials and Methods: Multiple genetic analyses were performed on bone marrow cells of a girl initially diagnosed with AML. Results: At the time of diagnosis with AML, the t(9;11)(p21;q23)/KMT2A-MLLT3 genetic abnormality was found. After chemotherapy resulting in AML clinical remission, a 2 Mb deletion in 11q23 was found generating a KMT2A-ARHGEF12 fusion gene. When the patient later developed B lineage ALL, a t(14;19)(q32;q13), loss of one chromosome 9, and KMT2A-ARHGEF12 were detected. Conclusion: The patient sequentially developed AML and ALL with three leukemia-specific genomic abnormalities in her bone marrow cells, two of which were KMT2A-rearrangements.

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The histone-lysine N-methyltransferase 2A (KMT2A, also known as MLL) gene in 11q23 (1, 2) may fuse with more than 100 different partners in acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myeloid leukemia, myelodysplastic syndromes, lymphomas, and solid tumors (3). Some of the resulting chimeras are common, such as the fusions with the AF4/FMR2 family member 1 (AFF1) and MLLT3 super elongation complex subunit (MLLT3) genes generated by t(4;11)(q21;q23) in ALL (KMT2A-AFF1) and t(9;11)(p21;q23) in AML (KMT2A-MLLT3), while others have only been reported in few or single cases (4, 5). The prognostic impact of the frequent KMT2A fusions is well known (6, 7); however, knowledge about the clinical consequences of the infrequent chimeras is inadequate. For this reason, not just because of biological curiosity, the reporting of cases involving uncommon KMT2A fusions is important as recently exemplified by the description of rare KMT2A-ELL fusion transcripts in pediatric AML associated with myeloid sarcomas (8-11).

We herein report the first pediatric leukemia, and the sixth case overall, in which fusion of KMT2A with the Rho guanine nucleotide exchange factor 12 (ARHGEF12) gene was detected.

Materials and Methods

Ethics statement. The study was approved by the regional ethics committee (Regional komité for medisinsk forskningsetikk Sør-Øst, Norge, http://helseforskning.etikkom.no), and written informed consent was obtained from the patient’s parents. The Ethics Committee’s approval included a review of the consent procedure. All patient information has been anonymized.

Case report. The patient was a girl diagnosed with AML at an age of 9.5 years (Table I, Sample S0), after a period of three months
Table I. *G-banding, fluorescence in situ hybridization (FISH) and molecular genetic results of the patient.*

| Sample | Days from AML-diagnosis | Karyotype | Nuclear in situ hybridization (nuc ish) based on KMT2A break-apart probe | Aberrant nuclei (%) | Additional experiments |
|--------|--------------------------|-----------|--------------------------------------------------------------------------------|---------------------|------------------------|
| S0     | 0                        | 46,XX t(9;11) (p21;q23)[9]/46,XX[2] | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[195/204] | 96% | FISH with a KMT2A-MLLT3 dual fusion probe: Fusion in metaphase spreads and in 184 out of 190 (97%) nuclei | Neither RT-PCR nor genomic PCR amplified KMT2A-ARHGEF12 fusion fragments |
| S1     | 300                      | Fail      | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[160/200] | 80% | Not done |
| S2     | 328                      | 46,XX     | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[181/204] | 89% | Not done |
| S3     | 389                      | 46,XX     | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[184/200] | 92% | RNA sequencing: Detection of a KMT2A-ARHGEF12 fusion transcript |
| S4     | 466                      | 46,XX     | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[199/219] | 91% | RT-PCR/Sanger sequencing: Confirmation of the presence of the KMT2A-ARHGEF12 fusion transcript |
| S5     | 571                      | 46,XX     | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[164/219] | 89% | Not done |
| S6     | 718                      | 46,XX     | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[158/200] | 79% | FISH with a KMT2A-MLLT3 dual fusion: No fusion in 200 nuclei |
| S7     | 914                      | 46,XX     | Not done | | FISH with a KMT2A-MLLT3 dual fusion probe: No fusion in 200 nuclei |
| S8     | 1,198                    | 45,XX,-9, t(14;19) (q32;q13)[8]/46,XX[3] | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[194/200] | 97% | Genomic PCR/Sanger sequencing: Detection of the genomic breakpoint/junction of KMT2A-ARHGEF12 fusion gene |
| S9     | 1,294                    | Fail      | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[17/200] | 8.5% | FISH with IGH break-apart probe: No split of IGH in 200 nuclei |
| S10    | 1,539                    | 46,XX     | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[164/203] | 81% | Genomic PCR/Sanger sequencing: Detection of the genomic breakpoint/junction of KMT2A-ARHGEF12 fusion gene aCGH detected a 2Mbp deletion between KMT2A and ARHGEF12 genes |
| S11-A  | 1,686A                   | 46,XX     | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[87/212] | 41% | Not done |
| S11-B  | 1,686B                   | Not done  | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[85/110] | 77% | Not done |
| S11-C  | 1,686C                   | Not done  | nuc ish (5’KMT2Ax2,3’KMT2Ax1) (5’KMT2A con 3’KMT2Ax1)[115/335] | 34% | Not done |

Note: The table continues with additional entries not fully transcribed here.
with fatigue, pallor, headache, and dizziness. There was no history of hematologic diseases, leukemia or frequent cancers in the family. At admittance, she was in good shape. Physical examination was normal except for pallor. Spleen and liver size were normal. Her blood tests were abnormal with hemoglobin 5.8 g/dl, platelet count 95×10^9/l, white blood cell count 4.9×10^9/l, and neutrophils 0.1×10^9/l. Bone marrow investigation revealed findings indicative of acute myelomonocytic leukemia (AML M4), and cytogenetic analysis showed a t(9;11)(p21;q23) chromosome translocation, however one that was different from the original KMT2A-MLLT3 (Table I, sample S0, see below).

The girl was treated according to the NOPHO-DBH 2012 protocol (12) where she was classified as a standard risk patient. She received five chemotherapy courses including the drugs liposomal daunorubicin, mitoxantrone, etoposide, cytarabine, and fludarabine. Following completion of therapy, she entered a prolonged phase with moderate pancytopenia. During this period which lasted two years and three months, repeated bone marrow examinations were performed (Table I, samples S1-S7). They revealed hematological remission and a normal karyotype but with a persisting KMT2A rearrangement, however one that was different from the original KMT2A-ARHGEF12 Fusion, and Pediatric B Lineage ALL.

**Table I. Continued**

| Sample  | Days from AML-diagnosis | Karyotype | Nuclear in situ hybridization (nuc ish) based on KMT2A break-apart probe | Aberrant nuclei (%) | Additional experiments |
|---------|-------------------------|-----------|------------------------------------------------------------------------|---------------------|------------------------|
| S12     | 1,945                   | Not done  | nuc ish (5′KMT2A,3′KMT2A)x 2(5′KMT2A con 3′KMT2Ax2)[200]              | 100%                | Not done               |

Clinical diagnosis of acute myeloid leukemia (AML) at time point (sample) S0, diagnosis of B lineage acute lymphoblastic leukemia (ALL) at time point S8. Sample 11 (S11-A, S11-B, and S-11C) represents the last bone marrow before stem cell transplantation. Sample 12 represents bone marrow 3 months after stem cell transplantation. RT-PCR: reverse transcription-polymerase chain reaction. aCGH: array comparative genomic hybridization. A: Bone marrow. B: CD34+/CD117+ cells. C: Negative fraction.

G-banding and fluorescence in situ hybridization (FISH) analyses. Bone marrow cells were short-term cultured, G-banded, and analyzed cytogenetically as previously described (14).

FISH analyses of bone marrow interphase nuclei and metaphase spreads were performed with the CytoCell KMT2A (MLL) and IGH break-apart probes, as well as with KMT2A-MLLT3 translocation dual fusion probes (CytoCell, Oxford Gene Technology, Begbroke, Oxfordshire, UK). Fluorescent signals were captured and analyzed using the CytoVision system (Leica Biosystems, Newcastle, UK).

DNA and RNA isolation and complementary DNA (cDNA) synthesis. Genomic DNA was extracted from the patient’s bone marrow samples at diagnosis as well as 1,198 and 1,539 days after the diagnosis (Table I, samples S0, S8, and S10) using the Maxwell 16 Instrument System and the Maxwell 16 Cell DNA Purification Kit (Promega, Madison, WI, USA). Total RNA was extracted from the patient’s bone marrow at diagnosis and after 389 days (Table I, samples S0, and S3) using the mirRNeasy Mini Kit (Qiagen, Hilden, Germany) and the QuiCube automated purification system according to the manufacturer’s instructions (Qiagen). The concentration and purity of DNA and RNA treatment and persisting ever since, was still detectable. It was therefore decided to give her one dose of Daratumomab (a CD38-antibody) 16 mg/kg with the aim of eradicating the abnormal clone before stem cell transplantation. After the one dose of Daratumomab CD38 was not detected on any CD34+ cells, using the standard monoclonal anti-CD38. However, using a multiepitope anti-CD38 the antigen could still be detected, indicating down-regulation of the epitope detected by the monoclonal antibody. Other markers demonstrated that the abnormal population was still present in the first controls after Daratumomab. The first bone marrow negative for CD34+ cells was confirmed 11 weeks after initiation of Daratumomab treatment, i.e., more than four years after the clone was first detected. Six weeks after the first negative bone marrow, autologous stem cell transplantation was performed with a human leukocyte antigen (HLA) - matched, unrelated donor after standard ALL-conditioning regimen consisting of total body irradiation (TBI) and etoposide. The patient is presently in complete remission, three months after stem cell transplantation. The current bone marrow assessment did not detect abnormal CD34+CD38+ cells or rearrangement of the KMT2A gene (see below).
were measured with the QIAxpert microfluidic UV/VIS spectrophotometer (Qiagen). In addition, the Agilent 2100 bioanalyzer and the RNA Integrity Number (RIN) were used to assess the RNA quality (15). RIN of RNA was 6.6. cDNA was synthesized from one µg of total RNA using the iScript Advanced cDNA Synthesis Kit for RT-qPCR according to the manufacturer’s instructions (Bio-Rad, Hercules, CA, USA). The quality of the cDNA synthesis was assessed by amplification of a cDNA fragment of the ABL protooncogene 1, non-receptor tyrosine kinase (ABL1) gene using the primer combination ABL1-91F1/ABL1-404R1 (16).

RNA sequencing. High-throughput paired-end RNA-sequencing was performed at the Genomics Core Facility, Norwegian Radium Hospital, Oslo University Hospital (http://genomics.no/oslo/). For library preparation from total RNA, the Illumina TruSeq RNA Access Library Prep kit was used according to Illumina’s protocol (Illumina, San Diego, CA, USA). Sequencing was performed on a NextSeq 550 System (Illumina) and 76 million reads were generated. The FASTQC software was used for quality control of the raw sequence data (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The software was used for quality control of the raw sequence data. The FASTQC software was used for quality control of the raw sequence data.

PCR analyses. The primers used for PCR amplification and Sanger sequencing are listed in Table II. For reverse transcription-polymerase chain reaction (RT-PCR) and cycle Sanger sequencing, the BigDye Direct Cycle Sequencing Kit was used (ThermoFisher Scientific, Waltham, MA, USA) according to the company’s recommendations. As template, cDNA corresponding to 20 ng total RNA was used. The primer combinations were M13For-MLL-4115F1/M13Rev-ARHGEF12-1579R1 and M13For-MLL-4115F1/M13Rev-ARHGEF12-1515R1. For the detection of possible chimeric cDNA fragments at initial diagnosis, the primer combination MLL-4116-F1/ARHGEF12-1502-R1 was used as described below.

Genomic PCR amplifications were performed in 25 µl reaction volume which contained 12.5 µl Premix Ex Taq™ DNA Polymerase Hot Start Version (Takara Bio Europe/SAS, Saint-Germain-en-Laye, France), 100 ng of genomic DNA, and 0.4 µM of each of the forward and reverse primers. The primer combinations were MLL-4116-F1/ARHGEF12-1502-R1 and MLL-4202-F1/ARHGEF12-1437-R1. The PCR cycling conditions were an initial denaturation step at 94˚C for 30 sec followed by 35 cycles of 7 sec at 98˚C and 2 min at 68˚C, and a final extension for 5 min at 68˚C. Three µl of the PCR products were stained with GelRed (Biotium, Fremont, CA, USA), analyzed by electrophoresis through 1% agarose gel, and photographed. DNA gel electrophoresis was performed using lithium borate buffer (19). The remaining PCR products were purified using the MinElute PCR Purification Kit (Qiagen) and direct sequenced using the dyeoxy procedure with the BigDye terminator v1.1 cycle sequencing kit following the company’s recommendations (ThermoFisher Scientific).

Sequence analyses were performed on the Applied Biosystems SeqStudio Genetic Analyzer system (ThermoFisher Scientific). The basic local alignment search tool (BLAST) software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for computer analysis of sequence data (20). The BLAT alignment tool and the human genome browser at UCSC were also used to map the sequences on the Human GRCh37/hg19 assembly (21, 22).

Array comparative genomic hybridization (aCGH) analysis. aCGH was performed using the CytoSure array products (Oxford Gene Technology, Begbroke, Oxfordshire, UK) following the company’s protocols. Thus, the CytoSure Genomic DNA Labelling Kit was used for the labelling of 1 µg of patient’s and reference DNAs and the CytoSure Cancer +SNP array for hybridization. The patient’s DNA was that isolated from a sample drawn 1,539 days after the initial diagnosis of AML (Table I, S10). The reference DNA was Promega’s human genomic female DNA (Promega, Madison, WI, USA). The slides were scanned by an Agilent scanner using Agilent Feature Extraction Software (version 10.7.3.1). Data were analysed using the CytoSure Interpret analysis software (version 4.9.40). Annotations are based on human genome build 19.

Results

G-band analyses. The data from G-banding and FISH analyses are summarized in Table I. The G-banding analysis of bone marrow cells at diagnosis yielded the karyotype 46,XX,t(9;11)(p21;q23)[9]/46,XX[2] (Figure 1A). Subsequently, bone marrow G-band analyses always showed a normal 46,XX karyotype except for the sample obtained 1,198 days after diagnosis. At that point, the karyotype had become 45,XX,-9, t(14;19)(q32;q13)[8]/46,XX[3] and the patient had developed B lineage ALL (Table I, sample S8) (Figure 1B).
Figure 1. Cytogenetic analyses of the two pediatric leukemias. (A) Karyogram showing the t(9;11)(p21;q23) found at diagnosis, when the patient had acute myeloid leukemia (AML). (B) Karyogram showing the loss of chromosome 9 and the t(14;19)(q32;q13) found when the patient was diagnosed with acute lymphoblastic leukemia (ALL) 1,198 days after the initial AML. Breakpoint positions are indicated by arrows.
FISH analyses. Three FISH analyses were performed at initial diagnosis of AML. Interphase FISH with the KMT2A break-apart probe showed a normal (yellow) as well as split (separated red and green) signals of the probe in 195 out of 204 examined nuclei (96%) (Table I, sample S0) (Figure 2A). FISH analysis with a KMT2A-MLLT3 translocation dual fusion probe on metaphase spreads showed the KMT2A-MLLT3 and MLLT3-KMT2A fusion genes on chromosomes der(11) and der(9), respectively (Figure 2B). Interphase FISH with the same KMT2A-MLLT3 translocation dual fusion probe showed a normal red signal corresponding to the MLLT3 gene, a normal green signal corresponding to the KMT2A gene, and two yellow fusion signals corresponding to KMT2A-MLLT3 and MLLT3-KMT2A in 184 out of 190 (97%) examined nuclei (Figure 2C). In addition, at another diagnostic laboratory, a KMT2A-MLLT3 fusion transcript was detected (data not shown) which was in agreement with the G-banding and interphase FISH results.

In the sample obtained 300 days after diagnosis, interphase FISH with a break-apart KMT2A probe for the first time showed loss of the distal part of the KMT2A probe, namely in 160 out of 200 nuclei (80%) (Table I, sample S1, Figure 2D). This pattern with deletion or loss of the distal part of KMT2A has since persisted throughout the entire follow-up period (Table I, samples S1-S11-A) until the patient was transplanted. Deletion of the distal part of the KMT2A probe was also found in 77% of the CD34+CD117+ cells and in 34% of the negative fraction of the sorted cells 1,686 days after the diagnosis (Table I, samples S11-B and S11-C).

In the sample obtained 90 days after allogenic stem cell transplantation the KMT2A break-apart probe showed two normal (yellow) signals in 200 interphase nuclei (Table I, sample S12).

Interphase FISH with a KMT2A-MLLT3 translocation dual fusion probe on samples obtained 718 days and 914 days after the initial diagnosis did not detect any KMT2A-MLLT3 fusion in 200 examined nuclei (Table I, samples S6 and S7).

In the sample obtained 1,198 days after diagnosis, when a CGH analysis detected a deletion in the q arm of chromosome 11 (Figure 4), the deletion was from the probe at position Chr11:118355288-118355347 in KMT2A to the probe at position Chr11:120290981-120291040 in ARHGEF12 (Figure 4). Thus, the aCGH data agreed with the results of FISH analyses, genomic PCR, and RT-PCR; they suggested that the KMT2A-ARHGEF12 fusion gene was the result of a deletion within chromosome band 11q23.

Discussion
A fusion of KMT2A with the ARHGEF12 gene was first reported in a 38-year-old male with a history of occupational exposure to herbicides who had developed AML (FAB-M4) and the abnormal karyotype 51,XY,+8,+19,+3mar (23). The patient received standard induction chemotherapy and achieved complete remission by morphological bone marrow...
Figure 2. Fluorescence in situ hybridization (FISH) analyses of pediatric leukemia. (A) Interphase FISH at initial diagnosis of AML with the KMT2A break-apart probe showing a normal (yellow) and split (separated red and green) signals of the probe in 3 nuclei. (B) FISH analysis at initial diagnosis of AML with the KMT2A-MLLT3 translocation dual fusion probe on metaphase spreads showing a normal green signal on chromosome 11, corresponding to KMT2A, a normal red signal on chromosome 9, corresponding to MLLT3, and two yellow fusion signals on der(11) and der(9) corresponding to the KMT2A-MLLT3 and MLLT3-KMT2A fusion genes, respectively. (C) Interphase FISH at initial diagnosis of AML on two nuclei using the KMT2A-MLLT3 translocation dual fusion probe showing a normal green signal, corresponding to KMT2A, a normal red signal, corresponding to MLLT3, and two yellow fusion signals corresponding to the KMT2A-MLLT3 and MLLT3-KMT2A fusion genes. (D) Interphase FISH with the break-apart KMT2A probe on the sample obtained 300 days after diagnosis showing deletion of the distal part of the KMT2A probe (lack of red signal) in two nuclei and two normal (yellow) KMT2A signals in one nucleus. (E) FISH analysis with the break-apart KMT2A probe on metaphase spread from the sample obtained 1,198 days after diagnosis, when a t(14;19)(q32;q13) was seen by karyotyping and the patient had developed ALL. The distal part (red signal) of the probe is absent in one of the two copies of chromosome 11. (F) Interphase FISH with the IGH break-apart probe on the sample obtained 1,198 days after diagnosis, when a t(14;19)(q32;q13) was seen by karyotyping and the patient had developed ALL. A normal (yellow) and split (separated red and green) signals of the probe are shown in 2 nuclei. (G) FISH analysis with the IGH break-apart probe on a metaphase spread from the sample obtained 1,198 days after diagnosis, when a t(14;19)(q32;q13) was seen by karyotyping and the patient had developed ALL. A normal (yellow) signal on chromosome 14 together with separate red, on der(14), and green, on der(19), probe signals are shown.
analysis, whereupon he received successful allogeneic bone marrow transplantation from an HLA identical sibling. He died 6 months later from interstitial pneumonia. There were no signs of relapse at autopsy (23).

The second case was a 77-year-old female with AML (FAB-M5a) and the karyotype 53,XX,+6,+8,+8,+9,+11,+13,+22 (24). The third patient with KMT2A-ARHGEF12 was an adult female with therapy-related AML (t-AML) (4, 5). No
information on the treatment and clinical outcome was provided for the second and third patients. The fourth patient with KMT2A-ARHGEF12 fusion was a 69-year-old male with B-cell ALL and a normal karyotype (25). This patient did not achieve complete remission after two courses of induction therapy and died from the disease (25). The fifth patient was a female with B-cell ALL and the bone marrow karyotype 46,XX,der(9)t(3;9)(p21;q34)/46,idem,der(9)t(3;9) (26). No further information was provided.

The present, sixth patient is the first reported pediatric case with a KMT2A-ARHGEF12 fusion gene. The fusion was detected in the patient’s bone marrow after completion of treatment for an AML with t(9;11)(p21;q23) and a KMT2A-MLLT3 fusion gene. The chemotherapy included, among other drugs, daunorubicin, mitoxantrone and etoposide which all are topoisomerase II inhibitors and associated with DNA double-strand breaks, the generation of KMT2A rearrangements, the formation of KMT2A-fusion genes, and therapy-related acute leukemias (27-33). B-ALL with 11q23 abnormalities/KMT2A rearrangements developing after treatment of a primary malignancy with topoisomerase II inhibitors have been reported (34-40). The majority of cases had the t(4;11)(q21;q23) translocation resulting in formation of a KMT2A-AFF1 fusion gene, but fusions of KMT2A with MLLT1 (19p13.3), FOXO3A (6q21), MAML2 (11q21), ACTN4 (19q13), CEP164 (11q23.3), and PRRC1 (5q23) were also found (5, 39). Therefore, we consider the chemotherapy to have caused the KMT2A-ARHGEF12 fusion.

The first indication of a KMT2A-ARHGEF12 fusion gene was seen in the patient’s bone marrow cells at days 300 and 328 after the initial AML diagnosis when, in FISH experiments, deletion of the distal part of the KMT2A probe was detected in spite of a normal karyotype (Table I, samples S1 and S2). RNA sequencing performed later detected the

Figure 4. aCGH showing the deletion in the q arm of chromosome 11. Based on the hg19 assembly, the deletion started from the probe at position Chr11:118355288-118355347 in KMT2A and ended at position Chr11:120290981-120291040 in ARHGEF12. The deletion is approximately 2 Mbp.
KMT2A-ARHGEF12 fusion gene, PCR amplifications verified it both at the transcriptional and genomic level (Figure 3), and aCGH showed that the cause of the fusion was indeed a submicroscopic deletion starting in intron 9 of KMT2A extending to intron 13 of ARHGEF12 (Figure 4). These retrospective findings fit perfectly the early detection of a deleted distal part of the KMT2A probe, linking it to the emergence of the KMT2A-ARHGEF12 fusion. Because the aberration was found in 77% of the CD34+/CD117+ cells (Table I, sample S11-B), we concluded that the treatment-induced generation of KMT2A-ARHGEF12 fusion probably took place in a multipotent progenitor cell. Indeed, in vitro experiments have shown that etoposide-induced rearrangements of KMT2A resulting in fusion genes can occur in mouse embryonic stem cells (27), human CD34+ hematopoietic stem cells from fetal liver (41), human CD34+ cells isolated from umbilical cord blood (28, 42), and in human embryonic stem cells (43). Furthermore, Libura et al. (42) showed that the etoposide-induced-KMT2A rearrangements were found at high frequency in human primitive hematopoietic stem cells with, in vitro and in vivo, long-term repopolulating potential.

During a period of two years and three months (after completion of therapy of the initial AML), repeated bone marrow examinations were performed in and all of them, although there was no sign of malignancy, the genetic pattern was the same: normal karyotype and deletion of the distal part of the KMT2A probe in FISH experiments indicating, as we only later realized, the formation of KMT2A-ARHGEF12 (Table I, samples S1-S7). Searching the relevant literature, we found two reports describing similar findings to ours (44, 45). In the first, a 5-year-old girl was diagnosed with AML carrying a variant of t(8;21) (44). The patient achieved clinical and hematologic remission which, 4 years later when the authors reported the case, was still unquestionable. Bone marrow contained 90% blasts of the pre-B phenotype, cytogenetic analysis revealed a t(14;19)(q32;q13) translocation together with loss of one chromosome 9 (Figure 1B, Table I, sample S8, see below), and FISH analyses showed splitting of the IGH probe in 92% of the nuclei together with deletion of the distal part of the KMT2A probe in 97% of the nuclei. In addition, later molecular analysis demonstrated presence of the KMT2A-ARHGEF12 fusion gene also at this time. Given the fact that so many bone marrow cells had the aberrations, most of them must have had both, leading us to conclude that the t(14;19)(q32;q13) translocation and loss of one chromosome 9 were secondary to the submicroscopic deletion in 11q23. Three months after treatment for B-ALL had been instituted, the (14;19)(q32;q13)/IGH aberration was no longer detectable while the KMT2A rearrangement was found in only 8.5% of the examined interphase nuclei. Evidently, the treatment given had considerable effect also on the cells carrying KMT2A rearrangement, but not enough to completely eradicate them. Summing up the extensive and somewhat complicating data – the high frequency of t(14;19)(q32;q13)/IGH rearrangement and KMT2A rearrangement in bone marrow cells when the patient developed B-ALL, and the absence of t(14;19)(q32;q13)/IGH rearrangement in bone marrow cells when the patient developed B-ALL, and the absence of t(14;19)(q32;q13)/IGH rearrangement together with the low frequency of KMT2A rearrangement after treatment for this disease – strongly indicate that (most of) the cells carrying KMT2A-rearrangement were part of the leukemic clone which carried the t(14;19)(q32;q13)/IGH rearrangement when the patient had B-ALL.

Our data are also in agreement with the findings reported by Jeffries et al. (46). Examining 20 B-ALL patients, they showed that translocations involving the IGH locus coexisted with other primary chromosomal rearrangements either in the same or separate clones. In one of the studied patients, KMT2A rearrangement was the primary aberration whereas the IGH-translocation the secondary. Most cells had only KMT2A rearrangement but cells with only IGH-translocation and cells with both KMT2A aberration and IGH-translocation were also found (46). The second case was a 3-year-old boy with B-ALL and a hyperdiploid karyotype: 58<2n>,XY,+X,+4,+6,+10,+15,+17,+18,+18,+20,+21,+21,+22 (45). During chemotherapy, a t(3;11)(p21;q23) developed in bone marrow cells giving rise to a KMT2A-SACM1L fusion gene. The KMT2A-SACM1L fusion was detected in many samples over a 7-year period in which the patient was in hematological remission. A difference between the KMT2A-ARHGEF17 and KMT2A-SACM1L fusion genes was that whereas the first resulted in a KMT2A-ARHGEF17 fusion protein, the second encodes a truncated KMT2A protein. Our patient was, two years and nine months after the start of the last AML chemotherapy course (or 3 years and 3 months after the initial diagnosis of AML), diagnosed with B-ALL (Table I, sample S8). The bone marrow contained 90% blasts of the pre-B phenotype, cytogenetic analysis revealed a t(14;19)(q32;q13) translocation together with loss of one chromosome 9 (Figure 2B, Table I, sample S8, see below), and FISH analyses showed splitting of the IGH probe in 92% of the nuclei together with deletion of the distal part of the KMT2A probe in 97% of the nuclei. In addition, later molecular analysis demonstrated presence of the KMT2A-ARHGEF12 fusion gene also at this time. Given the fact that so many bone marrow cells had the aberrations, most of them must have had both, leading us to conclude that the t(14;19)(q32;q13) translocation and loss of one chromosome 9 were secondary to the submicroscopic deletion in 11q23. Three months after treatment for B-ALL had been instituted, the (14;19)(q32;q13)/IGH aberration was no longer detectable while the KMT2A rearrangement was found in only 8.5% of the examined interphase nuclei. Evidently, the treatment given had considerable effect also on the cells carrying KMT2A rearrangement, but not enough to completely eradicate them. Summing up the extensive and somewhat complicating data – the high frequency of t(14;19)(q32;q13)/IGH rearrangement and KMT2A rearrangement in bone marrow cells when the patient developed B-ALL, and the absence of t(14;19)(q32;q13)/IGH rearrangement together with the low frequency of KMT2A rearrangement after treatment for this disease – strongly indicate that (most of) the cells carrying KMT2A-rearrangement were part of the leukemic clone which carried the t(14;19)(q32;q13)/IGH rearrangement when the patient had B-ALL.

The case we present has extensive similarities with the case reported by Żuna et al. (47). In brief, a 15-year-old girl with acute promyelocytic leukemia (APL, AML-M3) carrying
a PML-RARA fusion gene together with internal tandem duplication of FLT3 (FLT3/ITD) was treated with drugs that included the topoisomerase II inhibitors idarubicin and etoposide. Thirty months after the diagnosis of APL and 13 months after completion of therapy, she was diagnosed with B-ALL. Cytogenetic analysis revealed a t(6;11)(q21;q23) translocation aberration with molecular analyses showing the presence of a KMT2A-FOXO3A fusion gene whereas both PML-RARA fusion gene and FLT3/ITD were absent (47).

Backtracking the B-ALL, just as we did in the present study, the authors found the KMT2A-FOXO3A fusion gene in up to 90% of bone marrow cells, including cells of the myeloid lineage, more than one year before the B-ALL diagnosis which suggested that the generation of the KMT2A-FOXO3A fusion occurred in a multipotent progenitor. During this preleukemic phase, no blasts were found in the bone marrow but a small population of CD19+ B-cells, comprising 0.2% of the bone marrow, was detected (47). In our case, similarly a small persisting aberrant CD34+, CD38+ cell population, decreasing in a proportion from 1.5% to 0.05% over eight months, was found whereas the deletion of the distal part of KMT2A was found in 79-92% of the cells (Table 1, samples S1-S7). Zuna et al. (47) identified a 10 Mb gain on 19q13.32 as a potential “second hit” for the development of the B-ALL. The gain was present in B-ALL but was not found in the preleukemic specimen. By analogy, in our case t(14;19)(q32;q13) and loss of one chromosome 9 were secondary aberrations found at diagnosis of ALL but not in any previously examined samples (Table I, samples S1-S8).

Our patient also has similarities with the case presented by Jonveaux et al (48): A 35-year-old male had an AML (FAB M5b) with the chromosome aberration t(6;11)(q27;q23). FISH analysis showed that presence of a 400-600 kbp deletion downstream of the 11q23 breakpoint. Using Southern blot methodology, the breakpoint of KMT2A was found within intron 7 of the gene. The patient received chemotherapy which, among the drugs, included mitoxantrone and etoposide. Ten and a half months after complete remission, he was diagnosed with B-ALL. The cytogenetic analysis now revealed a t(4;11)(q21;q23) chromosome translocation, FISH showed absence of the deletion, and Southern blot detected a breakpoint in intron 9 of KMT2A (48). The authors considered etoposide as responsible for the development of the secondary B-ALL.

At the transcription level, the fusion of the present case was found to recombine exon 9 of KMT2A with exon 14 of ARHGFE12. This is at odds with what was reported by Kourlas et al. (23) who found fusion between exons 8 of KMT2A and 12 of ARHGFE12. No information on the exact KMT2A-ARHGFE12 fusion point was given for the other three reported patients (4, 5, 24, 25). The KMT2A-ARHGFE12 fusion of the present patient was brought about by a 2Mbp deletion stretching from intron 9 of KMT2A to intron 13 of ARHGFE12. Deletions were also reported as the cause of KMT2A-ARHGFE12 formation in the patients described by Kourlas (23) and Meyer (4, 5).

The exact genomic breakpoint position in KMT2A has been shown to correlate with clinical outcome in acute leukemias caused by fusions between KMT2A and some of the more common partner genes (5, 49, 50). Breakpoints in intron 10 (listed as intron 11 in the references (5, 49, 50)) interfere with the dimerization capacity of KMT2A plant homeodomains (PHD) 1-3, disabling binding to the BMI1 (B cell-specific Moloney murine leukemia virus integration site 1) repressor complex, and are associated with worse prognosis (5, 49-51). Breakpoints within KMT2A introns 8 and 9 (introns 9 and 10 in references (5, 49, 50), on the other hand, do not affect the three PHD finger domains and are associated with somewhat better clinical outcomes. The breakpoint in intron 9 of the present case could explain the relatively benign clinical behavior observed with long-term survival in spite of the late detection of a KMT2A-ARHGFE12 fusion gene and the therapy-related leukemia it signals.

The ARHGFE12 gene codes for the Rho guanine nucleotide exchange factor 12 which is a guanine nucleotide exchange factor that activates small GTPases of the Rho family (see RHOA, 165390) and catalyzes the exchange of GDP for GTP (52-54). ARHGFE12 was shown to be involved in cell polarization, morphology, invasion, and cytokinetic abscission (55-61). ARHGFE12 together with ARHGFE2 (also known as GEF-H1) were found to be key molecules for cellular adaptation to force and regulate the mechanical response to force on integrins (62). ARHGFE12 was found to regulate human megakaryocyte maturation, is critical for platelet function, regulates erythropoiesis, and is involved in erythroid regeneration after chemotherapy in ALL patients (63, 64). ARHGFE12 was also reported as a candidate tumor suppressor gene in breast and colorectal cancers (65). Mutations in the coding region of the gene are registered in COSMIC, the catalogue of somatic mutations in cancer (cancer.sanger.ac.uk), in various disease types (66).

In conclusion, we herein report the complex and sequential genetic steps of a therapy-related pediatric B-ALL. The patient was initially diagnosed with AML carrying a t(9;11)(p21;q23) chromosome translocation and a KMT2A-MLLT3 fusion gene. The first secondary genetic step, resulting from chemotherapy that included treatment with topoisomerase II-inhibitors, was a submicroscopic 2 Mb deletion in 11q23 generating a KMT2A-ARHGFE12 fusion gene in a multipotent progenitor cell. At that point, the patient entered a long "preleukemic" phase which lasted until yet another genetic aberration occurred, t(14;19)(q32;q13) accompanied by the loss of one chromosome 9. This resulted in the development of B-ALL.
Conflicts of Interest

The Authors declare that they have no potential conflicts of interest in regard to this study.

Authors’ Contributions

IP designed and supervised the experiments, performed bioinformatics analysis, molecular genetic experiments, evaluated the data, and drafted the manuscript. KA performed cytogenetic, FISH and molecular experiments and evaluated the data. ME-O evaluated the cytogenetic and FISH data. SH evaluated the data and assisted with writing of the manuscript. All Authors read and approved the final manuscript.

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