EWSR1/ELF5 induces acute myeloid leukemia by inhibiting p53/p21 pathway

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The Ewing sarcoma breakpoint region 1 (*EWSR1*) gene is known to fuse with various partner genes to promote the development of the Ewing sarcoma family of tumors and other sarcomas. In contrast, the association of *EWSR1* chimeric fusion genes with leukemia has rarely been reported. We identified a novel *EWSR1*-associated chimeric fusion gene in a patient with acute myeloid leukemia harboring 46, XY, t (11; 22) (p13; q12) karyotype abnormality. The patient was refractory to intensified chemotherapy including hematopoietic stem cell transplantation. Total RNA paired-end sequencing identified a novel chimeric fusion gene as *EWSR1/ELF5*, a member of the E26 transformation-specific transcription factor family. Transduction of *EWSR1/ELF5* to NIH3T3 cells induced transformation by attenuating with the p53/p21-dependent pathway. The injection of *EWSR1/ELF5*-transduced NIH3T3 cells into NSG-SCID mice systematically induced the development of tumors *in vivo*. These results revealed the oncogenic potency of *EWSR1/ELF5*.

Ewing sarcoma breakpoint region 1 (*EWSR1*) belongs to a small family of RNA binding proteins, including FUS, EWSR1 and TAFII68. They are involved in multiple cellular processes, including gene expression, cell signaling and RNA processing and transport.[1,2] The encoding genes are known to fuse with several partner genes encoding transcription factors which are associated with sarcoma and leukemia. The phenotype of the tumor is defined by the fusion partner. For example, the Ewing sarcoma family of tumors (EFT) carries *EWSR1* fused to a member of the E26 transformation-specific (ETS) transcription factor family, such as *FLI1*, *ERG*, *ETV1*, *ETV4* or *FEV*.[3–7] In other types of tumors, *EWSR1* fuses with *WT1* in desmoplastic small round cell sarcoma, with *CHN* in myxoid chondrosarcoma, with *ATF1* in clear cell sarcoma, and with *CHOP* in myxoid liposarcoma.[8–11] Although *EWSR1* is mainly associated with sarcoma, one report showed the fusion of *EWSR1* with *ZNF384* (*CIZ/NMP4*) in patients diagnosed with acute undifferentiated leukemia and acute lymphoblastic leukemia (ALL).[12]

The molecular mechanism of oncogenic transformation induced by a chimeric gene is relatively well characterized for EWSR1/FLI1. *In vitro* studies suggest that a disruption of senescence or DNA damage responses, both associated with the tumor barrier, may play a critical role in the tumorigenic effects of *EWSR1/ETS*.[13,14] In addition, the tumor phenotype is determined by the cell type expressing *EWSR1/ETS*. Expression of EWSR1/FLI1 in NIH3T3 cells and primary murine bone marrow derived mesenchymal stem cells (MSC) induced

oncogenesis, but not in Rat-1 cells, primary mouse embryonic fibroblasts (MEF) and hTERT-immortalized human primary fibroblasts. Intriguingly, EWSR1/FLI1 can direct partial neuroectodermal differentiation of primary mesenchymal stem cells.[15] However, *EWSR1/ETS* is rarely associated with leukemia,[16,17] thus preventing hematopoietic lineage analysis in clinical specimens. However, conditional *EWSR1/ETS* transgenic mice exhibit a leukemia phenotype, suggesting that the expression of *EWSR1/ETS* in the hematopoietic lineage has leukemogenic potential.[18,19]

We identified a 2-year-old boy who developed acute myeloid leukemia (AML) and carried a novel *EWSR1/ETS* chimeric fusion gene, *EWSR1/ELF5*. Chromosomal and functional assays demonstrate that this fusion gene promotes oncogenesis by interfering with the p53/p21-dependent pathway.

Materials and Methods

Cytogenetic analysis. FISH analysis was performed following the standard method. The probe used for *EWSR1* was the LSI *EWSR1* dual-color break-apart probe (Abbott Molecular/Vysis, Des Plaines, IL, USA).

Establishment of an Epstein–Barr virus-transformed lymphoblastoid cell line. An Epstein–Barr virus-transformed lymphoblastoid cell line (EB-LCL) was established using peripheral lymphocytes from a patient when they had first achieved remission. The Epstein–Barr virus from the B95-8 strain was used to infect the lymphocytes, and the cells were cultured with RPMI 1640 (Thermo Fisher Scientific, Waltham,

MA, USA) supplemented with 20% FBS and cyclosporin 200 ng/mL, as described previously.[20] EB-LCL were maintained in RPMI 1640 with 15% FBS at approximately 3–5 $\times$ 10^5 cells/mL at 37°C in 5% CO$_2$.

Total RNA paired-end sequencing. The RNA paired-end sequencing (RNA-seq) experiments were performed as previously described.[21] All samples collected from the patient were obtained after obtaining written informed consent from the parents. The research protocol was approved by the Institutional Review Board of the Tokyo Medical and Dental University (No. 103). Total RNA was extracted from the cells of AML patients, and the patient's Epstein–Barr virus-transformed lymphoblastoid cell line (EB-LCL) using Sepagene (Eidia, Tokyo, Japan). The cDNA was generated using the SmartPCR cDNA kit (Clontech Laboratories, Mountain View, CA, USA) and fragmented using the Covaris instrument (Covaris, Woburn, MA, USA). The cDNA fragments were used to prepare an Illumina library with the NEBNext reagents (New England Biolabs, Ipswich, MA, USA). The libraries were then submitted for Illumina HiSeq2000 sequencing, according to the standard protocols. Paired-end 100 nucleotide reads were generated and verified for data quality using the FASTQC software (Babraham Institute, Cambridge, UK) and mapped using the reference human genome (Homo sapiens hg19 sequence). Fusion transcript discovery was performed using the CLC genomics Workbench software 6.0.2 (CLC-bio, Aarhus, Denmark), which identifies the fusion transcripts by clustering discordantly the aligning paired-end reads spanning a fusion breakpoint.

RT-PCR and direct sequencing. The RT-PCR experiments were performed using standard protocols. The mRNA from the patient's AML cells were reverse-transcribed into cDNA using SuperScript III (Thermo Fisher Scientific). The *EWSR1/ELF5* fusion transcript was confirmed by RT-PCR using patient cDNA and specific primers for *EWSR1* (5′-CAGCCACTG CACCTACAAGA) and *ELF5* (5′-AATGAGCTTGATGCCTG GAG). The cDNA PCR-amplicon was detected after electrophoresis on a 1% agarose gel and was then purified and sequenced using a BigDye Terminator kit (version 3.1, Applied Biosystems, Foster City, CA, USA).

Plasmid constructs. FLAG-tagged *EWSR1/ELF5* was generated by PCR amplification of the cDNA of the patient's AML cells using Phusion high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) and specific primers for *EcoRI-FLAG*-tagged *EWSR1* (5′-ATGCGAATTCGCCAC CATGGATTACAAGGATGACGACGATAAGGCGTCCACG-GATTACA) and *XhoI*-tagged *ELF5* (5′-AGACTCGAGTCAT AGCTTGTCTTCCTGCCA). The PCR product was cloned into the pCR2.1-TOPO TA vector (Invitrogen, Carlsbad, CA, USA) and verified by sequence analysis. Then, the insert was transferred into the *EcoRI-SalI* site of the pBABE-Puro retroviral vector in the correct orientation downstream to the 5′ long terminal repeat.[22]

Cell lines and transduction of DNA. NIH3T3 cells, H1299 and U2OS cells were purchased from ATCC (Manassas, VA, USA) and grown in DMEM, supplemented with 10% FBS and penicillin–streptomycin (100 units/mL). The patient's EB-LCL was grown in RPMI medium supplemented with 10% FBS and penicillin–streptomycin (100 units/mL). All cell lines were maintained at 37°C in an atmosphere of 5% CO$_2$. The pBABE-Puro vectors containing *FLAG-EWSR1/ELF5* or empty vectors (MOCK) were transfected using a polyethyleneimine into PlatE cells, an ecotropic packaging cell line.[23] Supernatants containing high titers of retrovirus were collected at 48 and 72 h and used to infect the NIH3T3 cell line. NIH3T3 cells were seeded at a density of 2 $\times$ 10^5 cells/well in a 6-well plate during 24 h before adding viral supernatant containing 4-µg/mL protamine. Although infection efficacy was >90%, the infected cells were selected with 3-µg/mL puromycin during the 48 h after the infection. The U2OS and the H1299 cells were directly transfected using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's protocol.

Transformation and tumorigenesis assays *in vitro*. For the focus formation assay, *FLAG-EWSR1/ELF5*-transduced or MOCK-transduced NIH3T3 cells were seeded at a density of 10^6 cells/10-cm culture dish over 3 weeks. The culture medium was replaced twice a week. Soft agar assays were performed as previously described.[24] *FLAG-EWSR1/ELF5*-transduced or MOCK-transduced NIH3T3 cells were plated at a density of 1 $\times$ 10^4 cells/3.5-cm dish in 0.3% agar. The number of colonies measuring >150 µm in diameter was scored on day 14.

Apoptosis analysis and senescence associated-β-gal staining. Apoptotic cells were identified by flow cytometry using Annexin V-PE (MBL, Nagoya, Japan). Flow cytometry analysis was conducted on a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ, USA) using the CellQuest software. The detection protocol for senescence associated (SA)-β-gal activity was as previously described.[25]

***In vivo* mouse model.** Six-week-old female NSG-SCID mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Experimental and animal care protocols were approved by the Tokyo Medical and Dental University Animal Care and Use Committee (protocol numbers 0150358A and 0150004A). NIH3T3 cells (1 $\times$ 10^6) transduced with *FLAG-EWSR1/ELF5* or MOCK vectors were inoculated subcutaneously into NSG-SCID mice, and their tumorigenicity was monitored over 6 weeks.

Gene expression profiling and Gene mutation analysis. Gene expression levels were determined according to the entire coverage of a gene, which was defined as the sum of the coverage of each non-redundant exonic nucleotide normalized by all mapped nucleotides. The CLC genomics Workbench software 6.0.2 (Qiagen, Rewood City, CA, USA) was used to calculate the expression level of the UniProtKB genes annotated in reads per kilobase of transcript per million mapped reads (RPKM) and to identify gene mutations.

Western blotting. Cell line lysates were prepared in TGN buffer (50-mM Tris-HCl pH 7.5, 150-mM NaCl, 1% Tween 20 and 0.5% NP40) containing the protease inhibitor PMSA. The lysates were electrophoresed on SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% nonfat milk and then incubated with primary antibodies against the following proteins: FLAG M2 (F3165, Sigma-Aldrich, St. Louis, MO, USA), β-Actin (AC-15, Santa Cruz Biotechnology, Dallas, TX, USA), p53 (Ab-6, Santa Cruz Biotechnology) and p21 (c-19, Santa Cruz Biotechnology). The primary antibodies were detected with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies and visualized using an enhanced chemiluminescence (ECL) kit (GE Healthcare, Little Chalfont, UK).

Luciferase assay. Luciferase assay was performed as described previously.[26] Briefly, p53 null H1299 cells were cultured in 24-well plates and transfected with pCMV wild-type p53 vector, pG13 p21 promoter-luciferase reporter plasmid or TM861-2 BAX, pRL-renilla-luciferase expression vector, and pBABE-Puro FLAG-EWSR1/ELF5 or pBABE-Puro MOCK vector. Then, 24 h after transfection, firefly and renilla luciferase activities were measured using the Dual Luciferase Assay Kit (Promega, Madison, WI, USA). The firefly
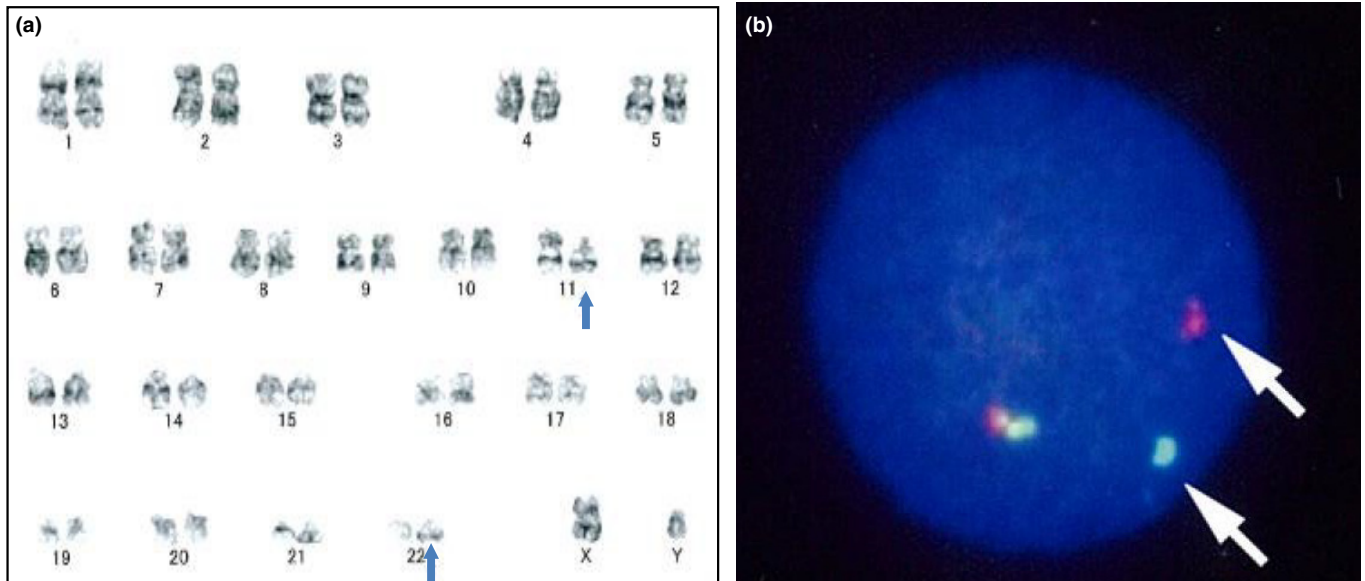
Fig. 1. Chromosome analysis providing evidence of *EWSR1* rearrangement. (a) Complete G-banding chromosomal analysis identified a translocation between 11p13 and 22q12 (blue arrows). (b) FISH analysis revealed *EWSR1* split signals (green or red) on the acute myeloid leukemia (AML) cells, whereas intact *EWSR1* genes appear as fused yellow signals. The *EWSR1* rearrangement-positive cells show one yellow, one red and one green signal pattern.
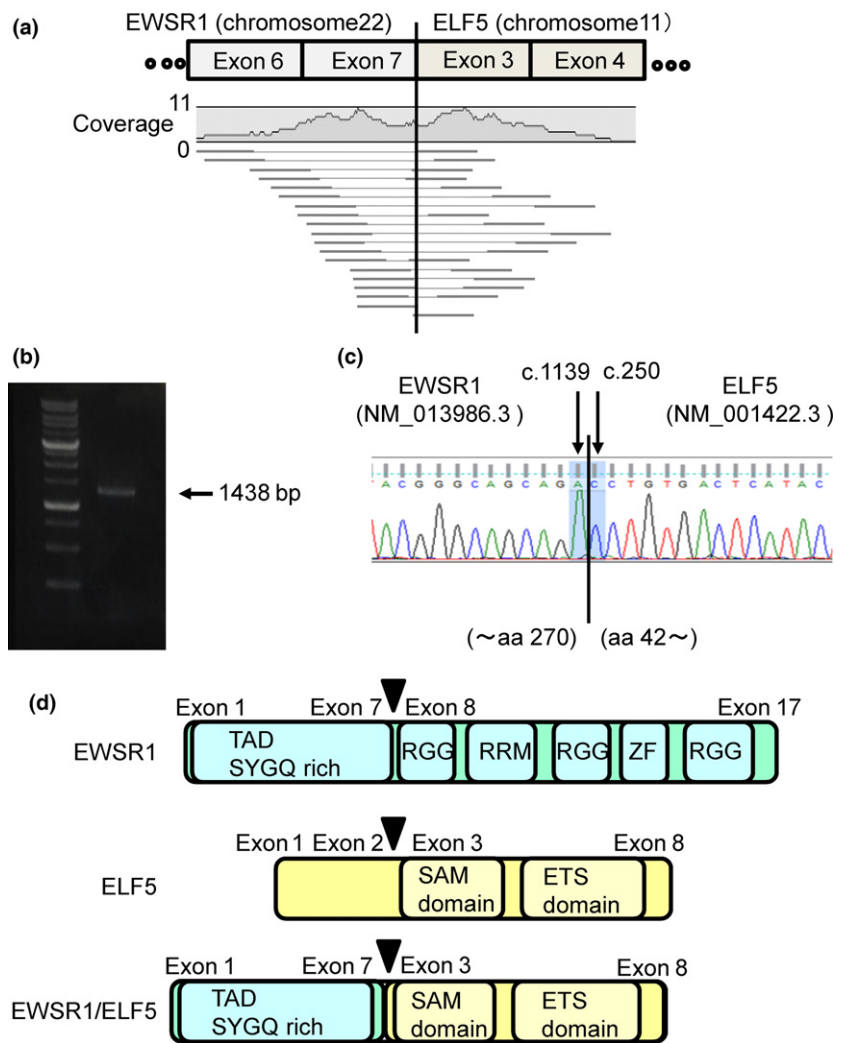

Fig. 2. Total RNA paired-end sequencing (RNA-seq) identified the *EWSR1/ELF5* transcript fusion. (a) Fragments of *EWSR1/ELF5* fusion mRNA observed by RNA-seq. Alignment of 17 mate-pairs and two single sequences on either side of the breakpoint (pairing *EWSR1* and *ELF5*). The histogram shows the absolute coverage of the sequence reads, and the mean numbers of reads. (b) RT-PCR with a *EWSR1* sense primer and an *ELF5* antisense primer showing the *EWSR1/ELF5* fusion mRNA product. (c) Direct sequencing of the RT-PCR product confirmed the identity of the *EWSR1/ELF5* fusion. aa, amino acid. (d) Schematic representation of the EWSR1 wild-type protein (top panel), the ELF5 wild-type protein (middle panel) and the EWSR1/ELF5 fusion protein (bottom panel). Arrows indicate the breakpoint. SAM, sterile alpha motif; ZF, zinc finger.

luciferase values of each sample were normalized by renilla luciferase activity.

Results

Case presentation. A 2-year-old boy was admitted to our hospital with skin lesions presenting extramedullary infiltrations. He was diagnosed with low blast-count AML immunologically positive for CD7, CD33, CD34, CD45dull, CD61, CD99 and CD117 (c-kit) but negative for the sarcoma markers desmin, MyoD1, myogenin, neuron-specific enolase (NSE) and paired box gene 5 (PAX5). The leukemia karyotype was identified as 46, XY, t (11; 22) (p13; q12), and FISH analysis detected *EWSR1* split signal (Fig. 1a,b). The patient was given conventional induction therapy, including daunorubicin, cytosine arabinoside, mitoxantrone and etoposide but failed to achieve complete remission. Therefore, he received a hematopoietic stem cell transplantation (HSCT) from his mother, who expressed identical human leukocyte antigen (HLA). The conditioning regimen using melphalan and total body irradiation (total dose: 12 Gy) was employed. However, the AML relapsed 1 year after HSCT. A second HSCT was performed, but remission was not achieved and the patient died.

Total RNA paired-end sequencing identifies the new *EWSR1/ELF5* chimeric fusion transcript. Chromosomal G-banding analysis using leukemic cell revealed translocation between 22q12 and 11p13 in leukemia cells (Fig. 1a). FISH analysis provided evidence of gene translocation involving *EWSR1*, the gene located at 22q12 (Fig. 1b). Chromosomal G-banding analysis suggests that the partner gene is located at 11p (Fig. 1a). The only gene which has been known to fuse with *EWSR1* located at 11p was *WT1*. However, RT-PCR analysis failed to detect *EWSR1/WT1* fusion mRNA (the partner gene(s) fused with *EWSR1*. The RNA samples were obtained from primary cells data not shown). Therefore, total RNA paired-end sequencing (RNA-seq) was performed to identify the patient's AML cells, and EB-LCL cells were derived from the patient for the internal control. A total of 28.9 million paired-end AML-derived RNA fragments and 29.0 million paired-end EB-LCL-derived
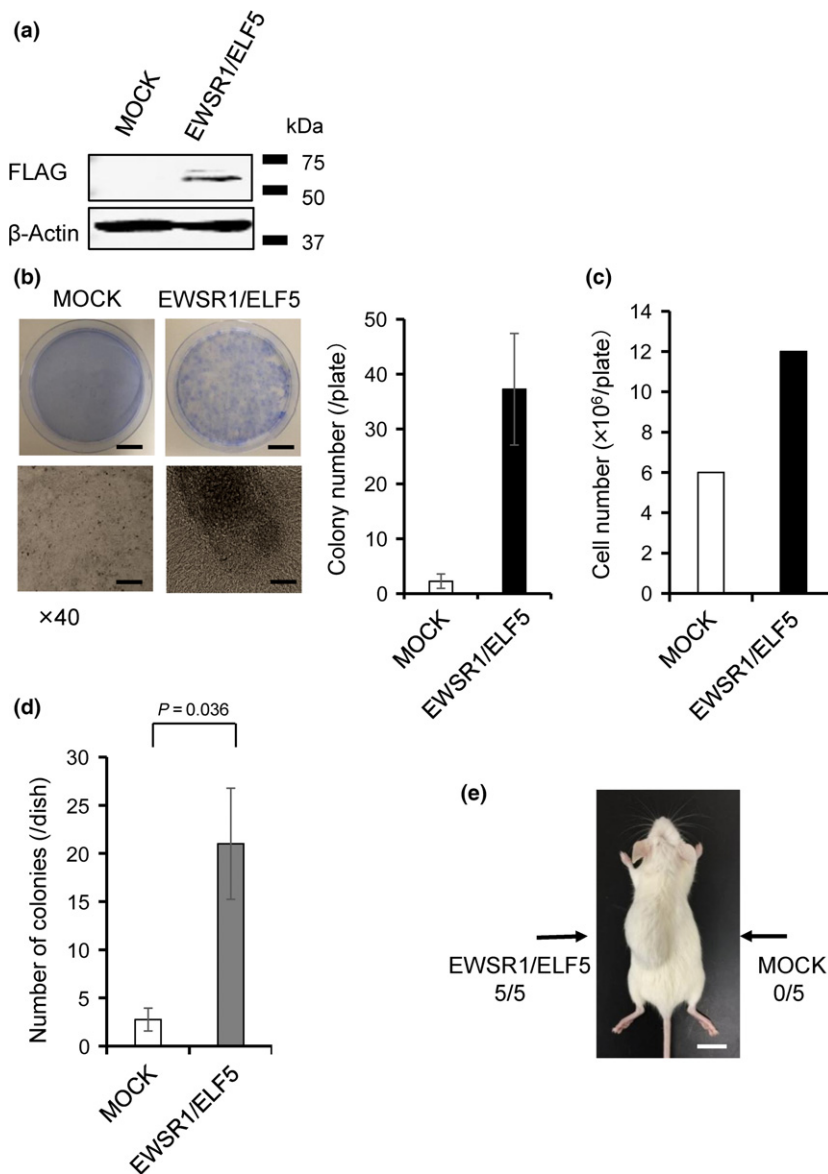

Fig. 3. Transforming and tumorigenic properties of the EWSR1/ELF5 fusion protein. (a) Expression of FLAG-tagged EWSR1/ELF5 in NIH3T3 cells confirmed by immunoblot analysis. (b) Macroscopic images of the focus formation assay taken with crystal violet staining after 3 weeks culture (top panels). Microscopic images (bottom panels) taken at indicated magnifications. Scale bars, top panels 20 mm; bottom panels 1000 μm. Average colony number from four independent experiments are shown as a bar graph. (c) Comparison of the number of NIH3T3 cells observed in (b). (d) Colony assay in soft agar showing that EWSR1/ELF5 promotes the anchorage-independent growth of NIH3T3 cells. The number of colonies was scored on day 14. The data are presented as the mean ± SE (*n* = 3). The Student *t*-test was used to compare differences. (e) *In vivo* tumorigenicity assay using NSG-SCID mice (*n* = 5). The EWSR1/ELF5-transduced and MOCK-transduced cells were injected on opposite sides of the same mice (arrows). Scale bar, 10 mm.
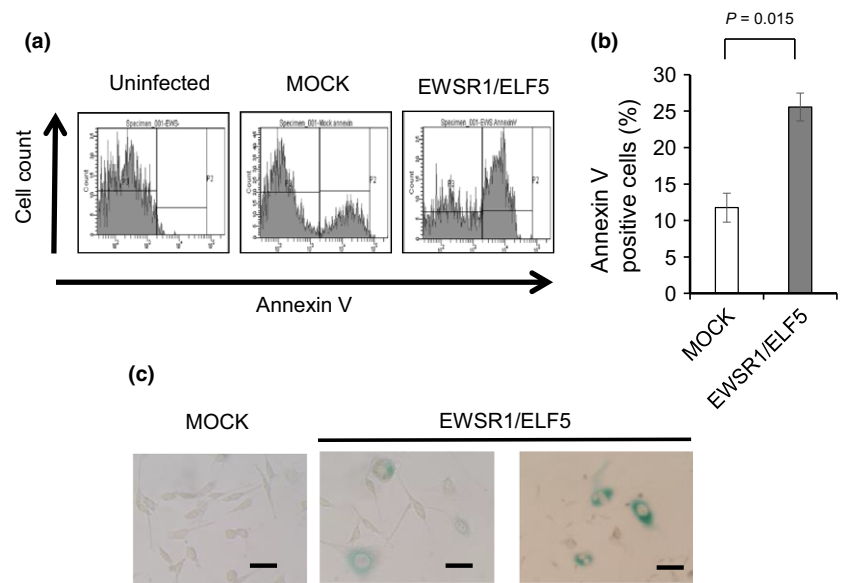
Fig. 4. The expression of *EWSR1/ELF5* induces toxicity in NIH3T3 cells. The cells were either uninfected, transduced with the empty vector (MOCK) or transduced with EWSR1/ELF5. (a) Flow cytometry analysis of apoptotic cells detected using Annexin V. (b) Comparison of the percentages of apoptotic cells. The data are presented as the mean $\pm$ SE ($n = 3$). The Student *t*-test was used to compare differences. (c) Senescence associated (SA)-β-gal staining of MOCK-transduced and EWSR1/ELF5-transduced cells. Senescent cells were stained in blue. Scale bar, 50 μm.
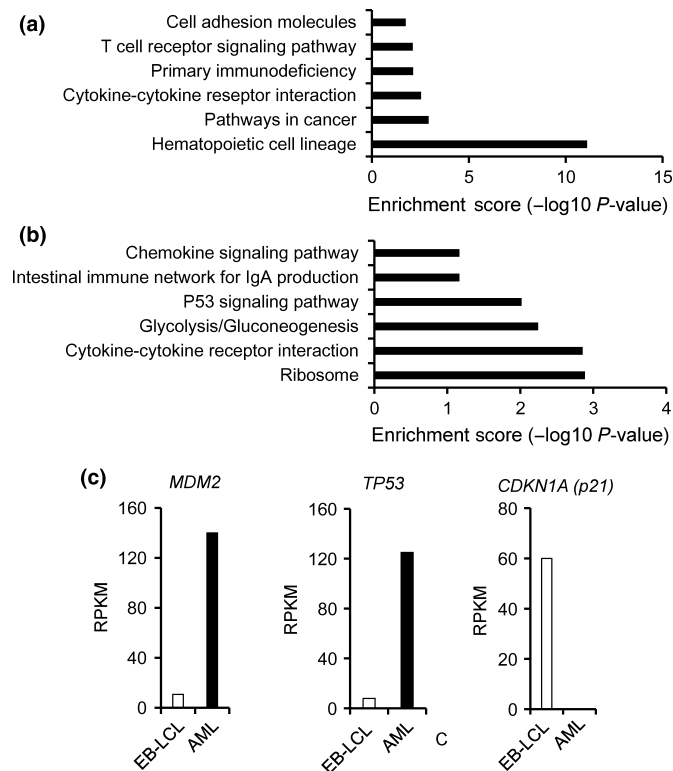

Fig. 5. Gene expression profile of acute myeloid leukemia (AML) cells. DAVID analysis of the most (a) upregulated and (b) downregulated 200 genes in the patient's AML cells, compared to the patient's Epstein–Barr virus-transformed lymphoblastoid cell line (EB-LCL). (c) mRNA expression level of *MDM2*, *TP53* and *CDKN1A* in the patient's AML cells, compared to the patient's EB-LCL control cells. RPKM, reads per kilobase of transcript per million mapped reads.

RNA fragments were sequenced. Among them, the fusion of *ELF5* and *EWSR1* (19 reads) was detected only in the AML samples (Fig. 2a). The presence of *EWSR1/ELF5* fusion mRNA was confirmed by RT-PCR and Sanger sequencing (Fig. 2b,c). The exon 7 of *EWSR1* (NM_013986.3, c.1139)

was fused to the exon 3 of *ELF5* (NM_001422.3, c250) (Fig. 2c,d). EWSR1 has a Ser–Tyr–Gln–Gly-rich (SYQG) transactivation domain at the NH₂ terminus, followed by an RNA-binding domain composed of three Arg–Gly–Gly-rich (RGG) regions and an RNA recognition motif (RRM) at the COOH terminus. ELF5 contains an ETS DNA-binding domain at the COOH terminus. In the *EWSR1/ELF5* fusion transcript, the RNA-binding domain of *EWSR1* is replaced by the ETS DNA-binding domain of *ELF5*, as reported for other *EWSR1/ETS* gene fusions.[3–7]

Mutation or altered expressed genes in leukemic cells. By comparing with EB-LCL, tumor-specific mutations and the altered expression of genes were investigated using RNA-seq data. In the leukemic cells, 223 mutations were identified, although mutation of the well-characterized tumor suppressor gene *TP53* was not detected. Instead, mutation of *CASP9*, a central player in common apoptosis pathways, was identified (Data S1). Analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) revealed several patterns of tumor-specific gene alterations. Among them, gene ontology classification revealed that genes involved in proteolysis were highly mutated (Data S2). Pathway analysis extracted two pathways: protein processing in the endoplasmic reticulum and control of gene expression by the vitamin D receptor (Data S3). Various non-expressed genes were also identified. This gene silencing likely occurs due to gene deletion or gene silencing by epigenetic alteration (Data S1 and S4). Among them, changes in expression of *CDKN1A*, a cell cycle inhibitor, and *NPM1*, known to be mutated in AML, were both observed (Data S1). Intriguingly, pathway analysis revealed that several genes associated with DNA replication were also silenced (Data S5).

The EWSR1/ELF5 chimeric protein possesses transformation properties and tumorigenic potential *in vitro* and *in vivo*. The oncogenic potential of *EWSR1/ELF5* was investigated by transformation assay using NIH3T3 cells, the cell line traditionally used to test the oncogenicity of other *EWSR1/ETS* fusion transcripts.[27,28] FLAG-tagged *EWSR1/ELF5* or empty vectors (MOCK) were retrovirally transduced into NIH3T3 cells (Fig. 3a). The *EWSR1/ELF5* fusion transcript consistently induced the transformation of NIH3T3 cells, as evidenced by a
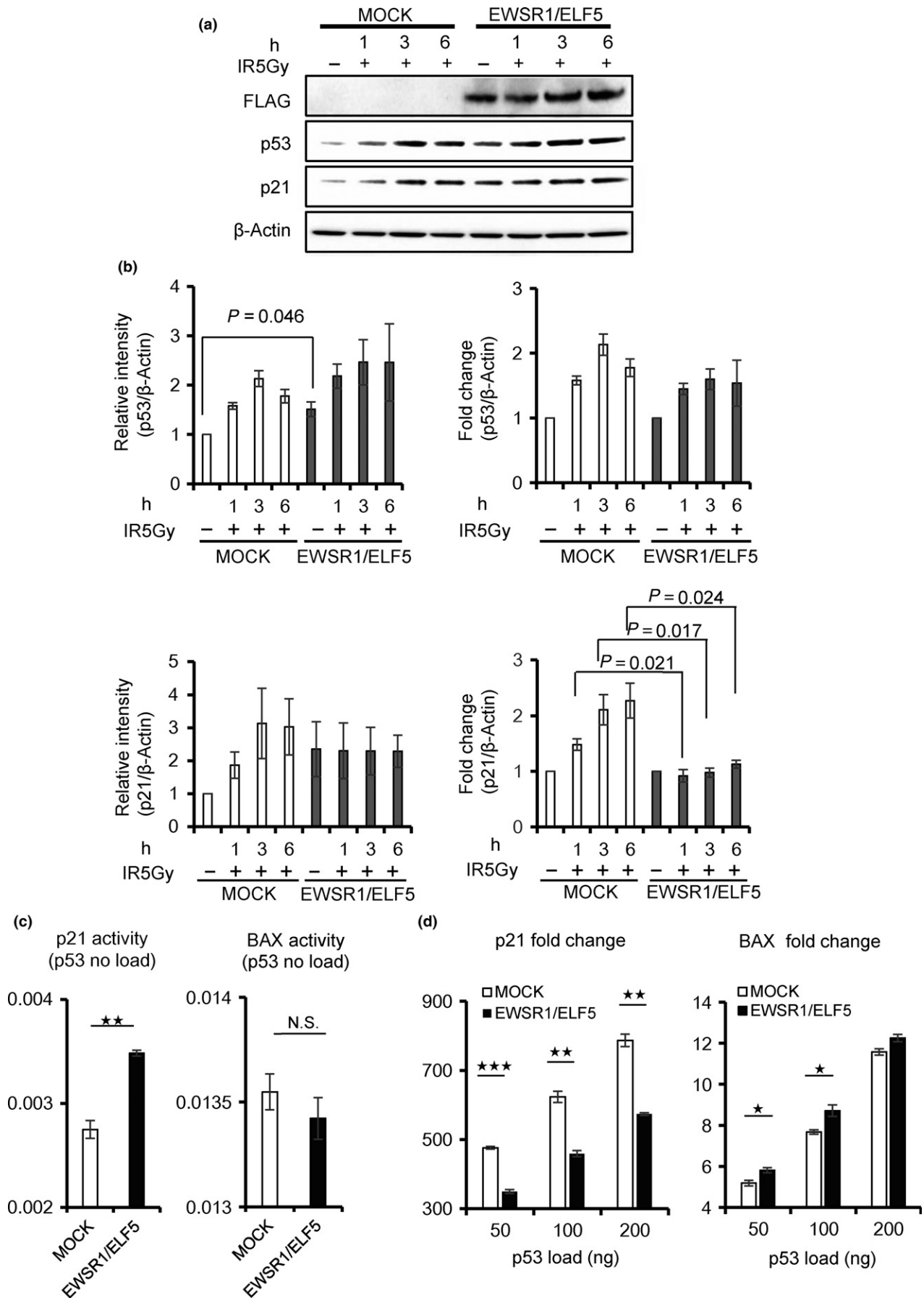
Fig. 6. Impact of the EWSR1/ELF5 fusion transcript on the p53/p21 pathway promoting apoptosis. (a) Western blot analysis of U2OS cells transduced with *EWSR1/ELF5* or the empty vector (MOCK). Cells were irradiated with 5 Gy, then harvested after 1, 3 or 6 h. (b) Left panel, quantitative analysis of the Western blot from three independent experiments. The intensities of p53 and p21 expression were normalized to β-Actin expression. Right panel, fold increase in p53 and p21 expression. The values obtained from unirradiated samples are set as 1. The data are presented as the mean ± SE. (c) p53 reporter (p21 or BAX) promoter activity was measured using a luciferase reporter system. Luciferase activity was shown 24 h after the transfection of MOCK (white column) or EWSR1/ELF5 (black column). (d) Fold increase of luciferase activity was shown 24 h after the transfection of MOCK (white column) or EWSR1/ELF5 (black column) with various amounts of p53 expression vectors in H1299 cells. The values obtained from no load of p53 samples are set as 1. The Student t-test was used to compare differences. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.

loss of contact inhibition with more proliferative activity than the MOCK-transduced cells (Fig. 3b,c). The tumorigenic potential of *EWSR1/ELF5* was also analyzed by the substrate-independent growth assay using *EWSR1/ELF5* and MOCK transduced NIH3T3 cells in soft agar. The *EWSR1/ELF5*-transduced cells formed over six times more colonies than the MOCK-transduced cells (Fig. 3d).

The *in vivo* oncogenic effect was tested by injecting *EWSR1/ELF5*-transduced or MOCK-transduced NIH3T3 cells into the left and right sides of five NSG-SCID mice, respectively. After 6 weeks, all the mice presented a large tumor on the left side only (Fig. 3e). The *in vitro* leukemogenic potential of *EWSR1/ELF5* was first investigated using the Ba/F3 hematopoietic cell line. In this murine pro-B-cell line, oncogenic transformation was reported to induce growth factor-independent cell growth or enhanced resistance to apoptosis.[29] However, *EWSR1/ELF5* transduction did not elicit these transformations (data not shown). Next, the *EWSR1/ELF5* fusion gene was transduced into murine bone marrow cells to conduct colony assays, as previously described.[30] *EWSR1/ELF5* transduction did not stimulate significant colony formation (Fig. S1). In addition, HSCT of *EWSR1/ELF5*-transduced murine bone marrow cells into lethally-irradiated mice did not induce leukemia over a period of 12 months, based on the normal blood counts of low-level reporter genes in the peripheral blood (data not shown). These experiments suggest that despite the structural differences between the partner genes, *EWSR1/ELF5* has transformation potential as the other *EWSR1* fusions in NIH3T3 cells but not in hematopoietic cell lineages.

Aberrant *EWSR1/ELF5* expression had toxicity in NIH3T3 cells. The oncogenic potential of *EWSR1/ELF5* was supported by transductions conducted in NIH3T3 cells but not in the Ba/F3 hematopoietic lineage cell line. We hypothesized that the expression of *EWSR1/ELF5* is toxic in primary cells cultured *in vitro*. Therefore, we investigated whether the transduction of *EWSR1/ELF5* induces apoptosis or cellular senescence in NIH3T3 cells. Apoptosis was increased twofold in *EWSR1/ELF5*-transduced cells, compared to MOCK-transduced cells (Fig. 4a,b). In addition, SA-β-gal staining was enhanced in *EWSR1/ELF5* transduced NIH3T3 cells (Fig. 4c). Together, these data suggest that *EWSR1/ELF5* induces senescence and apoptosis, as reported for *EWSR1/FLI1*, yet is associated with transformation activity.[14]

***EWSR1/ELF5* drives an expression signature of transformation.** RNA-seq analysis not only detects fusion mRNA but also provides genome-wide mapping of quantitative mRNA expressions. A comparison of the expression profiles of the patient's AML cells and the patient's EB-LCL revealed the alignment of 17 797 annotated genes. DAVID analysis of the most significantly upregulated and downregulated 200 genes in the patient's AML cells in comparison with the patient's EB-LCL identified several informative Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, including those expressed by the hematopoietic cell lineage pathway, the cytokine–cytokine

receptor interaction pathway, and the p53 signaling pathway (Fig. 5a,b; gene lists in Tables S1 and S2).[31]

The expression of *EWSR1/ELF5* activates p53 but inhibits p21. Given the senescence and apoptotic phenotypes induced by *EWSR1/ELF5* (Fig. 4a–c) and the identification of the p53 signaling pathway involved in apoptosis by DAVID analysis (Fig. 5b), we focused on major molecules implicated in the p53 signaling pathway: *TP53*, which encodes p53; *MDM2* and p21 (*CDKN1A*), which operates downstream of p53. In the patient's AML cells, *TP53* and *MDM2* are upregulated, whereas p21 (*CDKN1A*) is downregulated, compared to the patient's control EB-LCL cells (Fig. 5c). The *EWSR1/FLI1* fusion transcript was reported to inhibit p21 activity.[32–34] To determine whether *EWSR1/ELF5* also suppresses p21 expression, EWSR1/ELF5 was transduced into p53-competent U2OS cells. Intriguingly, *EWSR1/ELF5* stimulated the baseline expression of p53 and p21, compared to MOCK-transduced U2OS cells (Fig. 6a,b). In contrast, p21 induction was inhibited by EWSR1/ELF5 after 5-Gy irradiation, suggesting that the fusion transcript functions as a suppressor of p21 transcription after DNA damage. The effect of EWSR1/ELF5 to p53 downstream gene promoters (p21 and BAX promoter) were investigated using a luciferase reporter assay. Wild-type p53 expression vectors were co-transfected with MOCK or EWSR1/ELF5 expressing vector into H1299 cells. Interestingly, basal p21 reporter promoter activity was upregulated by EWSR1/ELF5 expression (Fig. 6c). p21 reporter promoter activity induced by p53 transfection was attenuated by EWSR1/ELF5 expression. In contrast, BAX reporter promoter activity was not suppressed by EWSR1/ELF5 expression (Fig. 6d). These data indicate that the novel *EWSR1/ELF5* fusion transcript promotes oncogenesis through a dysregulation of the p53/p21-dependent pathway (Fig. 7), similar to other EFT.

Discussion

In the present study, we identified *EWSR1/ELF5*, a new *EWSR1/ETS* chimeric fusion gene that is associated with
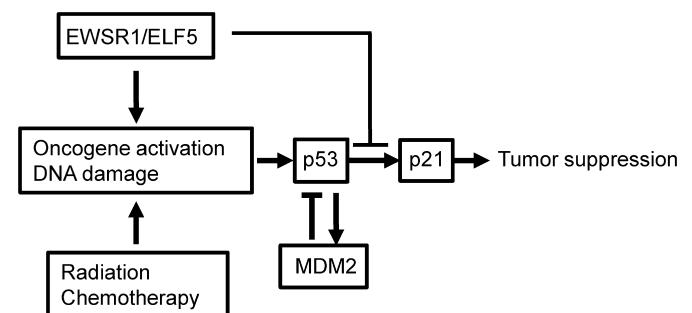

Fig. 7. Proposed schematic model of tumorigenicity by EWSR1/ELF5.

AML. *EWSR1/ELF5* was identified from the chromosomal translocation t(11;22)(p13;q12). Cytogenetically, the same translocation t(11;22)(p13;q12) also creates the *EWSR1/WT1* fusion in the desmoplastic small round cell tumor. Interestingly, both genes are closely located, with only 2.1 Mb separating them at the genomic level. This region contains various genes involved in oncogenesis, such as *LMO2*, *WT1* and *PAX6*. In addition, this region is known to be altered in various types of tumors, as well as the inherited cancer-prone WAGR syndrome. We hypothesized that this region may, therefore, be susceptible to gene rearrangement.

The EWSR1/ELF5 fusion protein exhibits oncogenic potential *in vitro* and *in vivo*. The molecular mechanism of oncogenesis involves an aberrant regulation of the p53/p21-dependent pathway by EWSR1/ELF5. However, the expression of EWSR1/ELF5 in murine hematopoietic cells does not confer leukemia, suggesting that some additional mechanisms or yet identified prerequisite genetic background is needed for full transformation in the hematopoietic system.

The molecular mechanism of the transformation induced by the family of *EWSR1/ETS* gene fusions is not fully understood. Among them, *EWSR1/FLI1* is relatively well characterized owing to the strong association between this chimeric gene fusion and human EFT. The oncogenic potency of *EWSR1/FLI1* was demonstrated by its ability to transform NIH3T3 cells, to grow in semisolid medium, and to form tumors in immunodeficient mice.[35,36] Conversely, the ectopic expression of *EWSR1/ETS* proteins in primary cells induces growth arrest or cell death, rather than promoting cellular transformation, suggesting that the cellular context is critical for the oncogenic potential of *EWSR1/ETS* fusions.[13,14] Despite the rare associations of *EWSR1/ETS* fusion genes with human leukemia,[16,17] the development of leukemia was observed in transgenic mouse models of *EWSR1/FLI1*[18] and *EWSR1/ERG*.[19] Torchia *et al.*[18] emphasized that a high expression level of *EWS/FLI1*, driven by the retroviral long terminal repeat (LTR), was toxic to murine hematopoietic cells. This might also be the case in our experiments, because retroviral transduction did not induce leukemia even after 12 months in our model. We speculate that the retroviral LTR-driven *EWSR1/ELF5* expression system may not be suitable for leukemogenesis study in murine model, as has been observed in *EWSR1/FLI1*. Under the light of the finding that *EWSR1* is essential for stem cell quiescence and the maintenance of hematopoietic stem cells,[37] endogenous EWSR1 promoter dependent expression of *EWSR1/FLI1* might be essential for the leukemogenic transformation.

The ETS transcription factor family is a diverse group of proteins cooperating with other factors to regulate a wide range of cellular processes, such as proliferation, differentiation, apoptosis and senescence; members also possess oncogenic or tumor suppressive activities.[38] Chromosomal translocations involving ETS family members are associated with several forms of human cancers. Among the various EWSR1 fusion partners, only *EWSR1/ZNF384* (CIZ/NMP4) has been identified in acute leukemia.[12] *ZNF384* is known to fuse with various partner genes, including *EWSR1*, *TAF15*, *TCF3* (*E2A*) and *EP300*, mainly in acute lymphoblastic leukemia.[39–41] ZNF384 exerts its function as a transcription factor and regulates matrix-related proteins and/or represses PTH-induced bone formation.[42,43] However, the role of this gene in the hematopoietic lineage has not been fully elucidated yet, although fusion genes have been identified in leukemia. As for ZNF384, the function of ELF5 in hematopoietic lineage

is also uncertain. The protein encoded by *ELF5* belongs to an epithelium-specific subclass of ETS and regulates the later stages of terminal differentiation in keratinocytes, and participates in the regulation of cell fate, beginning with the specification of the trophectoderm in the blastocyst.[44,45] Most ETS genes appear to be functional and expressed during hematopoiesis. However, *ELF5* is not expressed in hematopoietic cells. In normal human tissues, ELF5 is expressed in the kidney, prostate, lung, mammary gland, salivary gland, placenta and stomach. However, in cancer tissue, dysregulated expression of ELF5 has been reported, including in leukemia/lymphoma.[46] Regarding the fusion gene, only the *ZFPM2-ELF5* fusion gene in multicystic mesothelioma has been reported.[47] The only similarity between ZNF384 and FLF5 is the DNA binding transcription factor. These results suggest that an EWSR1 fusion with any DNA binding transcription factor in the hematopoietic lineage may be sufficient for leukemogenesis.

The fusion of *ELF5* with *EWSR1* in hematopoietic cell lineages makes it difficult to explain the leukemogenic mechanism on its own. Further study is needed to show whether *ELF5* aberrantly expressed by a chimeric protein may induce tumorigenesis, or this fusion may play a role in malignant transformation in the hematopoietic system.

In this study we demonstrated that the novel *EWSR1/ELF5* fusion gene disrupts the p53/p21-dependent pathway, similar to the case of sarcoma genesis induced by other members of the *EWSR1/ETS* family. In most primary cells, the ectopic expression of *EWSR1/ETS* leads to cell cycle arrest or apoptosis, whereas an inhibition of p53 activity may rescue the cells from *EWSR1/ETS*-induced toxicity and facilitate tumorigenesis.[13,14] This observation suggests that the p53/p21-dependent pathway plays an important role in the prevention of tumorigenesis by *EWSR1/ETS*. However, 90% of the tumors of EFT express wild-type p53, suggesting that the function of p53 is blocked in the downstream of p53 by EFT-specific cancer-related molecular changes. In the EFT cell line, *EWSR1/FLI1* was reported to modulate p21 activity by several mechanisms.[32–34] The expression of p21 is induced by wild-type p53 in the presence of DNA damage, leading to cell cycle arrest at the G1 checkpoint. The activity of the p21 promoter is negatively regulated by the EWSR1/FLI1 fusion protein through ETS-binding sites located within the promoter region of *p21*.[32] In addition, EWSR1/FLI1 interacted with the cotransactivator of p300 and suppressed its histone acetyltransferase activity. The epigenetic regulation of the *p21* promotor by EWSR1/FLI1 also contributed to the dysregulation of p21 transactivation. In the present study, the AML patient did not show any mutation in *TP53* (data not shown), suggesting that the same interfering mechanism was exerted by *EWSR1/ELF5* (Fig. 7). In fact, p21 was reported to inhibit the proliferation of leukemic cells, and the suppression of p21 expression fostered the progression of leukemia.[48]

Although *EWSR1/ETS* is rarely detected in hematopoietic diseases, the homologue *FUS/ERG* was identified in several cases of acute myeloid or lymphoblastic leukemia and in Ewing tumors.[17,49–51] There is considerable amino acid sequence homology (56%) between *FUS* and *EWSR1*.[52] Incidentally, *EWSR1* and *FUS* are functionally interchangeable in the context of fusion oncogenes, as shown by the identification of *FUS/ERG* gene fusions in Ewing tumors and *FUS/CHOP* fusions in myxoid liposarcoma.[51,52] Therefore, it is not surprising that rare *EWSR1/ETS* family members, such as *EWSR1/ELF5*, may contribute to leukemic development. Unfortunately, our young patient died after the second round

of HSCT. Likewise, a poor outcome was reported for *FUS/ERG*-positive leukemia patients.[53] As the present study is based on a single patient, additional patients expressing this novel *EWSR1/ELF5* fusion gene are required to evaluate the relationship between the presence of the chimeric gene and patient outcome in leukemia. A novel therapy based on the understanding of molecular mechanism may be required for this *EWSR1/ETS* positive group.

In conclusion, *EWSR1/ELF5*, a novel *EWSR1/ETS* chimeric gene, was identified in a patient diagnosed with refractory AML, suggesting a potential role of leukemogenesis in rare cases of AML. This fusion gene is very likely to exhibit oncogenic potential by interfering with the p53/p21-dependent pathway.

Disclosure Statement

The authors have no conflict of interest to declare.

References

1 Erkizan HV, Uversky VN, Toretsky JA. Oncogenic partnerships: EWS-FLI1 protein interactions initiate key pathways of Ewing's sarcoma. *Clin Cancer Res* 2010; **16**: 4077–83.

2 Cantile M, Marra L, Franco R *et al.* Molecular detection and targeting of EWSR1 fusion transcripts in soft tissue tumors. *Med Oncol* 2013; **30**: 412.

3 Delattre O, Zucman J, Plougastel B *et al.* Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. *Nature* 1992; **359**: 162–5.

4 Sorensen PH, Lessnick SL, Lopez-Terrada D, Liu XF, Triche TJ, Denny CT. A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG. *Nat Genet* 1994; **6**: 146–51.

5 Jeon IS, Davis JN, Braun BS *et al.* A variant Ewing's sarcoma translocation (7;22) fuses the EWS gene to the ETS gene ETV1. *Oncogene* 1995; **10**: 1229–34.

6 Kaneko Y, Yoshida K, Handa M *et al.* Fusion of an ETS-family gene, EIAF, to EWS by t(17;22)(q12;q12) chromosome translocation in an undifferentiated sarcoma of infancy. *Genes Chromosom Cancer* 1996; **15**: 115–21.

7 Peter M, Couturier J, Pacquement H *et al.* A new member of the ETS family fused to EWS in Ewing tumors. *Oncogene* 1997; **14**: 1159–64.

8 Ladanyi M, Gerald W. Fusion of the EWS and WT1 genes in the desmoplastic small round cell tumor. *Cancer Res* 1994; **54**: 2837–40.

9 Clark J, Benjamin H, Gill S *et al.* Fusion of the EWS gene to CHN, a member of the steroid/thyroid receptor gene superfamily, in a human myxoid chondrosarcoma. *Oncogene* 1996; **12**: 229–35.

10 Zucman J, Delattre O, Desmaze C *et al.* EWS and ATF-1 gene fusion induced by t(12;22) translocation in malignant melanoma of soft parts. *Nat Genet* 1993; **4**: 341–5.

11 Panagopoulos I, Hoglund M, Mertens F, Mandahl N, Mitelman F, Aman P. Fusion of the EWS and CHOP genes in myxoid liposarcoma. *Oncogene* 1996; **12**: 489–94.

12 Martini A, La Starza R, Janssen H *et al.* Recurrent rearrangement of the Ewing's sarcoma gene, EWSR1, or its homologue, TAF15, with the transcription factor CIZ/NMP4 in acute leukemia. *Cancer Res* 2002; **62**: 5408–12.

13 Deneen B, Denny CT. Loss of p16 pathways stabilizes EWS/FLI1 expression and complements EWS/FLI1 mediated transformation. *Oncogene* 2001; **20**: 6731–41.

14 Lessnick SL, Dacwag CS, Golub TR. The Ewing's sarcoma oncoprotein EWS/FLI induces a p53-dependent growth arrest in primary human fibroblasts. *Cancer Cell* 2002; **1**: 393–401.

15 Riggi N, Suva ML, Stamenkovic I. Ewing's sarcoma origin: from duel to duality. *Exp Rev Anticancer Ther* 2009; **9**: 1025–30.

16 Hawkins JM, Craig JM, Secker-Walker LM, Prentice HG, Mehta AB. Ewing's sarcoma t(11;22) in a case of acute nonlymphocytic leukemia. *Cancer Genet Cytogenet* 1991; **55**: 157–62.

17 Jakovljevic G, Nakic M, Rogosic S *et al.* Pre-B-cell acute lymphoblastic leukemia with bulk extramedullary disease and chromosome 22 (EWSR1) rearrangement masquerading as Ewing sarcoma. *Pediatr Blood Cancer* 2010; **54**: 606–9.

18 Torchia EC, Boyd K, Rehg JE, Qu C, Baker SJ. EWS/FLI-1 induces rapid onset of myeloid/erythroid leukemia in mice. *Mol Cell Biol* 2007; **27**: 7918–34.

19 Codrington R, Pannell R, Forster A *et al.* The Ews-ERG fusion protein can initiate neoplasia from lineage-committed haematopoietic cells. *PLoS Biol* 2005; **3**: e242.

20 Pelloquin F, Lamelin JP, Lenoir GM. Human B lymphocytes immortalization by Epstein-Barr virus in the presence of cyclosporin A. *In vitro Cell Dev Biol* 1986; **22**: 689–94.

21 Steidl C, Shah SP, Woolcock BW *et al.* MHC class II transactivator CIITA is a recurrent gene fusion partner in lymphoid cancers. *Nature* 2011; **471**: 377–81.

22 Morgenstern JP, Land H. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res* 1990; **18**: 3587–96.

23 Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 2000; **7**: 1063–6.

24 Welford SM, Hebert SP, Deneen B, Arvand A, Denny CT. DNA binding domain-independent pathways are involved in EWS/FLI1-mediated oncogenesis. *J Biol Chem* 2001; **276**: 41977–84.

25 Itahana K, Campisi J, Dimri GP. Methods to detect biomarkers of cellular senescence: the senescence-associated beta-galactosidase assay. *Methods Mol Biol* 2007; **371**: 21–31.

26 Piao S, Sakurai M, Iwamoto S *et al.* Functional studies of a novel germline p53 splicing mutation identified in a patient with Li-Fraumeni-like syndrome. *Mol Carcinog* 2013; **52**: 770–6.

27 Im YH, Kim HT, Lee C *et al.* EWS-FLI1, EWS-ERG, and EWS-ETV1 oncoproteins of Ewing tumor family all suppress transcription of transforming growth factor beta type II receptor gene. *Cancer Res* 2000; **60**: 1536–40.

28 Thompson AD, Teitell MA, Arvand A, Denny CT. Divergent Ewing's sarcoma EWS/ETS fusions confer a common tumorigenic phenotype on NIH3T3 cells. *Oncogene* 1999; **18**: 5506–13.

29 Tomita O, Iijima K, Ishibashi T *et al.* Sensitivity of SNX2-ABL1 toward tyrosine kinase inhibitors distinct from that of BCR-ABL1. *Leuk Res* 2014; **38**: 361–70.

30 Lavau C, Szilvassy SJ, Slany R, Cleary ML. Immortalization and leukemic transformation of a myelomonocytic precursor by retrovirally transduced HRX-ENL. *EMBO J* 1997; **16**: 4226–37.

31 da Huang W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; **4**: 44–57.

32 Nakatani F, Tanaka K, Sakimura R *et al.* Identification of p21WAF1/CIP1 as a direct target of EWS-Fli1 oncogenic fusion protein. *J Biol Chem* 2003; **278**: 15105–15.

33 Li Y, Tanaka K, Fan X *et al.* Inhibition of the transcriptional function of p53 by EWS-Fli1 chimeric protein in Ewing Family Tumors. *Cancer Lett* 2010; **294**: 57–65.

34 Li Y, Li X, Fan G *et al.* Impairment of p53 acetylation by EWS-Fli1 chimeric protein in Ewing family tumors. *Cancer Lett* 2012; **320**: 14–22.

35 May WA, Gishizky ML, Lessnick SL *et al.* Ewing sarcoma 11;22 translocation produces a chimeric transcription factor that requires the DNA-binding domain encoded by FLI1 for transformation. *Proc Nat Acad Sci USA* 1993; **90**: 5752–6.

36 May WA, Arvand A, Thompson AD, Braun BS, Wright M, Denny CT. EWS/FLI1-induced manic fringe renders NIH 3T3 cells tumorigenic. *Nat Genet* 1997; **17**: 495–7.

37 Cho J, Shen H, Yu H *et al.* Ewing sarcoma gene Ews regulates hematopoietic stem cell senescence. *Blood* 2011; **117**: 1156–66.

38 Zhou J, Ng AY, Tymms MJ *et al.* A novel transcription factor, ELF5, belongs to the ELF subfamily of ETS genes and maps to human chromosome 11p13-15, a region subject to LOH and rearrangement in human carcinoma cell lines. *Oncogene* 1998; **17**: 2719–32.

39 Gocho Y, Kiyokawa N, Ichikawa H *et al.* A novel recurrent EP300-ZNF384 gene fusion in B-cell precursor acute lymphoblastic leukemia. *Leukemia* 2015; **29**: 2445–8.

40 Nyquist KB, Thorsen J, Zeller B *et al.* Identification of the TAF15-ZNF384 fusion gene in two new cases of acute lymphoblastic leukemia with a t (12;17)(p13;q12). *Cancer Genet* 2011; **204**: 147–52.

41 Zhong CH, Prima V, Liang X *et al.* E2A-ZNF384 and NOL1-E2A fusion created by a cryptic t(12;19)(p13.3; p13.3) in acute leukemia. *Leukemia* 2008; **22**: 723–9.

42 Bidwell JP, Childress P, Alvarez MB *et al.* Nmp4/CIZ closes the parathyroid hormone anabolic window. *Crit Rev Eukaryot Gene Expr* 2012; **22**: 205–18.

43 Bidwell JP, Torrungruang K, Alvarez M *et al.* Involvement of the nuclear matrix in the control of skeletal genes: the NMP1 (YY1), NMP2 (Cbfa1), and NMP4 (Nmp4/CIZ) transcription factors. *Crit Rev Eukaryot Gene Expr* 2001; **11**: 279–97.

44 Oettgen P, Kas K, Dube A *et al.* Characterization of ESE-2, a novel ESE-1-related Ets transcription factor that is restricted to glandular epithelium and differentiated keratinocytes. *J Biol Chem* 1999; **274**: 29439–52.

45 Donnison M, Beaton A, Davey HW, Broadhurst R, L'Huillier P, Pfeffer PL. Loss of the extraembryonic ectoderm in Elf5 mutants leads to defects in embryonic patterning. *Development* 2005; **132**: 2299–308.

46 Piggin CL, Roden DL, Gallego-Ortega D, Lee HJ, Oakes SR, Ormandy CJ. ELF5 isoform expression is tissue-specific and significantly altered in cancer. *Breast Cancer Res* 2016; **18**: 4.

47 Panagopoulos I, Gorunova L, Davidson B, Heim S. Novel TNS3-MAP3K3 and ZFPM2-ELF5 fusion genes identified by RNA sequencing in multicystic mesothelioma with t(7;17)(p12;q23) and t(8;11)(q23;p13). *Cancer Lett* 2015; **357**: 502–9.

48 Roman-Gomez J, Castillejo JA, Jimenez A *et al.* 5′ CpG island hypermethylation is associated with transcriptional silencing of the p21(CIP1/WAF1/SDI1) gene and confers poor prognosis in acute lymphoblastic leukemia. *Blood* 2002; **99**: 2291–6.

49 Ichikawa H, Shimizu K, Hayashi Y, Ohki M. An RNA-binding protein gene, TLS/FUS, is fused to ERG in human myeloid leukemia with t(16;21) chromosomal translocation. *Cancer Res* 1994; **54**: 2865–8.

50 Kanazawa T, Ogawa C, Taketani T, Taki T, Hayashi Y, Morikawa A. TLS/FUS-ERG fusion gene in acute lymphoblastic leukemia with t(16;21)(p11; q22) and monitoring of minimal residual disease. *Leuk Lymphoma* 2005; **46**: 1833–5.

51 Shing DC, McMullan DJ, Roberts P *et al.* FUS/ERG gene fusions in Ewing's tumors. *Cancer Res* 2003; **63**: 4568–76.

52 Crozat A, Aman P, Mandahl N, Ron D. Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. *Nature* 1993; **363**: 640–4.

53 Kong XT, Ida K, Ichikawa H *et al.* Consistent detection of TLS/FUS-ERG chimeric transcripts in acute myeloid leukemia with t(16;21)(p11;q22) and identification of a novel transcript. *Blood* 1997; **90**: 1192–9.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. The *EWSR1/ELF5* fusion transcript does not stimulate colony formation in murine bone marrow cells.

Table S1. Top 200 significantly upregulated genes in acute myeloid leukemia (AML) cells compared to Epstein–Barr virus-transformed lymphoblastoid cell line (EB-LCL).

Table S2. Top 200 significantly down regulated genes in acute myeloid leukemia (AML) cells compared to Epstein–Barr virus-transformed lymphoblastoid cell line (EB-LCL).

Data S1 Mutation or silenced gene candidates in leukemic cell.

Data S2 Gene ontology terms list of mutations.

Data S3 Pathway list of mutations.

Data S4 Gene ontology terms list of gene silencing.

Data S5 Pathway list of gene silencing.