Reduced expression of the WW domain-containing oxidoreductase in human hematopoietic malignancies

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Abstract. The role of the WW domain-containing oxidoreductase (*WWOX*) gene in multiple types of solid human cancers has been documented extensively thus far. Recently, we investigated the *in vitro* effects of *WWOX* overexpression and observed marked growth arrest in human leukemia cells; however, the clinical characterization of *WWOX* in leukemia remains poorly investigated. The present study evaluated the *WWOX* expression profiles of 182 patients with leukemia of different types and 5 leukemic cell lines, using reverse transcription-polymerase chain reaction and immunofluorescence analysis. The results found that *WWOX* mRNA and WWOX protein expression was significantly reduced or absent in the leukemia cases and cell lines compared with paired controls. The *WWOX*-positive rate was also lower in the leukemia cases compared with the rate of the normal controls. Notably, the *WWOX* level was reduced in newly diagnosed and relapsed cases, or in chronic myelogenous leukemia in the blastic phase, yet elevated in remission samples. Moreover, *WWOX*-negative cases exhibited *WWOX* expression restoration following induced remission. These findings suggest that *WWOX* may contribute to the occurrence and development of leukemia, and that it has potential to be a good biomarker or predictor for leukemia therapy.

Introduction

In 2000, Bednarek *et al* identified a novel tumor suppressor gene termed WW domain-containing oxidoreductase (*WWOX*) (1). The *WWOX* gene maps at the 16p23.3-24.1 chromosomal region and spans the second most common fragile site in the human genome, FRA16D (1). Since its discovery, *WWOX*/WWOX has been shown to exhibit reduced or no expression in a range of neoplastic diseases of varying locations, such as the lungs, breasts, bladder, ovaries, esophagus and pancreas (2-7). Moreover, abnormal *WWOX*/WWOX expression profiles had been reported to be associated with a poor prognosis in carcinomas (8-10). Therefore, *WWOX* is expected to be a potential target for the gene-targeted therapy of human carcinomas.

The role of *WWOX*/WWOX in leukemia has also been reported, suggesting that WWOX may exert antineoplastic activity in human hematopoietic malignancies (11-16). Our previous studies have demonstrated that *WWOX* exerts a role as an tumor suppressor gene in leukemia, inhibiting cell proliferation and promoting apoptosis via binding with p73 and triggering of the mitochondrial pathway (14,15). However, the clinical characterization of *WWOX* in leukemia remains unclear thus far. In order to obtain more information on the role of *WWOX* with regard to the occurrence, development and therapeutic effect of leukemia, the present study evaluated the expression of *WWOX* in 182 primary leukemia patients and 5 leukemic cell lines. Additionally, the study attempted to assess the association between *WWOX* expression and clinical features. It was demonstrated that a low expression level of *WWOX* is present in leukemia, and that *WWOX* expression varies among the different phases of leukemia. Moreover, *WWOX* expression was shown to have an association with the treatment outcome. These findings may aid in providing a better understanding of the role of *WWOX* in leukemia.

Materials and methods

Agents. RPMI-1640, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific, Inc., (Waltham, MA, USA) and Lymphocyte Separation Medium was obtained from TBD Sciences (Tianjin, China). The reverse transcription-polymerase chain reaction (RT-PCR) kit, radioimmunoprecipitation assay (RIPA) lysis buffer, TRIzol reagent

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and 2X PCR Master Mix were all purchased from Thermo Fisher Scientific, Inc. The 4',6-diamidino-2-phenylindole (DAPI) and fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-rabbit immunoglobulin G (IgG) antibodies (cat. no. P0186) were obtained from the Beyotime Institute of Biotechnology (Shanghai, China), while the polyclonal rabbit anti-human WWOX (cat. no. ab189410) and monoclonal mouse anti-human β-actin (cat. no. sc-8432) antibodies were purchased from Abcam (Cambridge, MA, USA) and Santa Cruz Biotechnology Inc. (Dallas, TX, USA), respectively.

Patients and samples. Between October 2010 and June 2012, blood samples were collected from the diagnosed leukemia cases at Fujian Medical University Union Hospital (Fujian, China) and 182 cases were enrolled in the study. Among these patients, 101 were male. The mean age ± standard deviation of the total patients was 32.0±11.68 years (range, 6-76 years). The cohort included 61 cases of acute lymphoblastic leukemia (ALL), 89 cases of acute myelogenous leukemia (AML) and 32 cases of chronic myelogenous leukemia (CML), which were diagnosed based on morphology, histopathology, leukocyte differentiation antigens and the French-American-British classification (17). Mononuclear cells purified from 43 healthy volunteers were used as paired controls. Prior consent was obtained from the patients for the use of these clinical materials for research purposes and study approval was granted by the project approval authorities (Fujian Medical University & Fujian Provincial Department of Science and Technology).

Cell lines and cell culture. Human Jurkat (acute T-lymphoblastic leukemia), K562 (chronic myelogenous leukemia in erythroid crisis), HL-60 (acute myelogenous leukemia), HL-60/ADR (HL-60 resistance to doxorubicin), K562/ADR (K562 resistance to doxorubicin) and 293a cell lines were all purchased from the cell bank of the Chinese Academy of Medical Sciences (Beijing, China). The cells were maintained in RPMI-1640 supplemented with 10% FBS, and cultured at 37˚C in a 5% CO_2 humidified atmosphere. The human 293a cell line was maintained in DMEM containing 10% FBS and was utilized as a positive control.

RT-PCR analysis. Mononuclear cells were purified with the aid of Lymphocyte Separation Medium. Total RNA was extracted with TRIzol reagent and was reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). Briefly, total RNA (1-2 μg), hexamer primer (1 μl) and ddH_2O were added to a total volume of 12 μl, and incubated at 65˚C for 5 min. Subsequently, 10 mM dNTP mix (2 μl), 5X reaction buffer (4 μl), 1 μl RNase inhibitor and 1 μl Reverse Transcriptase were added, followed by incubation at 25˚C for 5 min, 45˚C for 1 h and 70˚C for 5 min. The primer sequences for *WWOX* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as previously described (14): WWOX forward, 5'-CACGCATTTTAGAAGAATGG-3' and reverse, 5'-GACAGCAGCACAGTACACG-3'; and GAPDH forward, 5'-CAAGGTCATCCATGACAACTTTG-3' and reverse, 5'-GTCCACCACCCTGTTGCTGTAG-3'. cDNA was then amplified by PCR using the 2X Green Master Mix (Thermo Fisher Scientific, Inc). The reaction system (25 μl) contained 2 μl cDNA template, 12.5 μl Master Mix (2X), 8.5 μl ddH_2O and 2 μl primers (20 μM). The cDNA template was replaced with ddH_2O for the negative control. PCR was performed using an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, Inc.), under the recommended conditions of an initial denaturation for 5 min at 94˚C, followed by 30 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 30 sec, and a final extension for 7 min at 72˚C. Quantified data were normalized to *GAPDH*. Each PCR experiment was performed twice.

Western blotting analysis. Cell lysates were prepared using RIPA protein lysis buffer, and the protein extracts were quantified and subjected to electrophoresis on a 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. The proteins were transferred onto polyvinylidene difluoride membranes and blocked in Tris-buffered saline containing 5% skimmed milk powder. The primary antibodies were used at 1:1,000 dilutions for both the rabbit anti-WWOX and mouse monoclonal anti-β-actin antibodies.

Immunofluorescence staining assay. In brief, cell monolayers were fixed with 4% paraformaldehyde and incubated at 4˚C overnight with rabbit anti-human WWOX (1:500). FITC-conjugated goat anti-rabbit IgG was diluted to 1:1,000. DAPI was used to stain the nuclei. The stained cells were washed with PBS and observed with a fluorescence microscope (Olympus, Tokyo, Japan) at x400 magnification.

Statistical analysis. Data are presented as the mean ± standard deviation and all statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) software. The χ^2 test or corrected χ^2 test was used to analyze the association between the *WWOX*-positive rate and the clinical features. $P<0.05$ (confidence level >95%) was considered to indicate a statistically significant difference.

Results

Expression of WWOX in primary leukemia cases. The expression level of *WWOX* mRNA and the WWOX protein was examined in the leukemia patients via RT-PCR and western blotting analysis. As shown in Fig. 1A, D and E, the expression of *WWOX*/WWOX was reduced or lost in the leukemia cases when compared with that in the normal paired controls ($P<0.000$); the positive rates of *WWOX* for the AML, ALL and CML patients were 47.19% (42/89), 50.81% (31/61) and 34.38% (11/32), respectively, which was significant (AML, $P<0.000$, $\chi^2=23.15$; ALL, $P<0.000$, $\chi^2=18.23$; CML, $P<0.000$, $\chi^2=26.19$) compared with the normal controls (90.70%, 39/43) (Fig. 1B). Notably, the *WWOX*-positive rates of newly diagnosed and relapsed cases in AML, ALL and CML were significantly lower than those of remission cases, accompanied by a P-value of <0.05 or <0.01 (Fig. 1B; AML, P=0.008; ALL, P=0.036; CML, P=0.011). Nevertheless, the *WWOX*-positive rates of remission cases in AL (involving ALL and AML) and CML exhibited no significance with the normal controls (AL, P=0.299; CML, P=0.051; corrected $\chi^2=3.763$ or 3.811). The quantified data of *WWOX* levels for AML, ALL and CML are displayed in Fig. 1C. As is shown, the expression levels of *WWOX* in AML, ALL and CML were distinctly reduced when compared with the normal paired controls (AML, $P<0.000$; ALL, P=0.025; CML, $P<0.000$). Significantly, *WWOX*

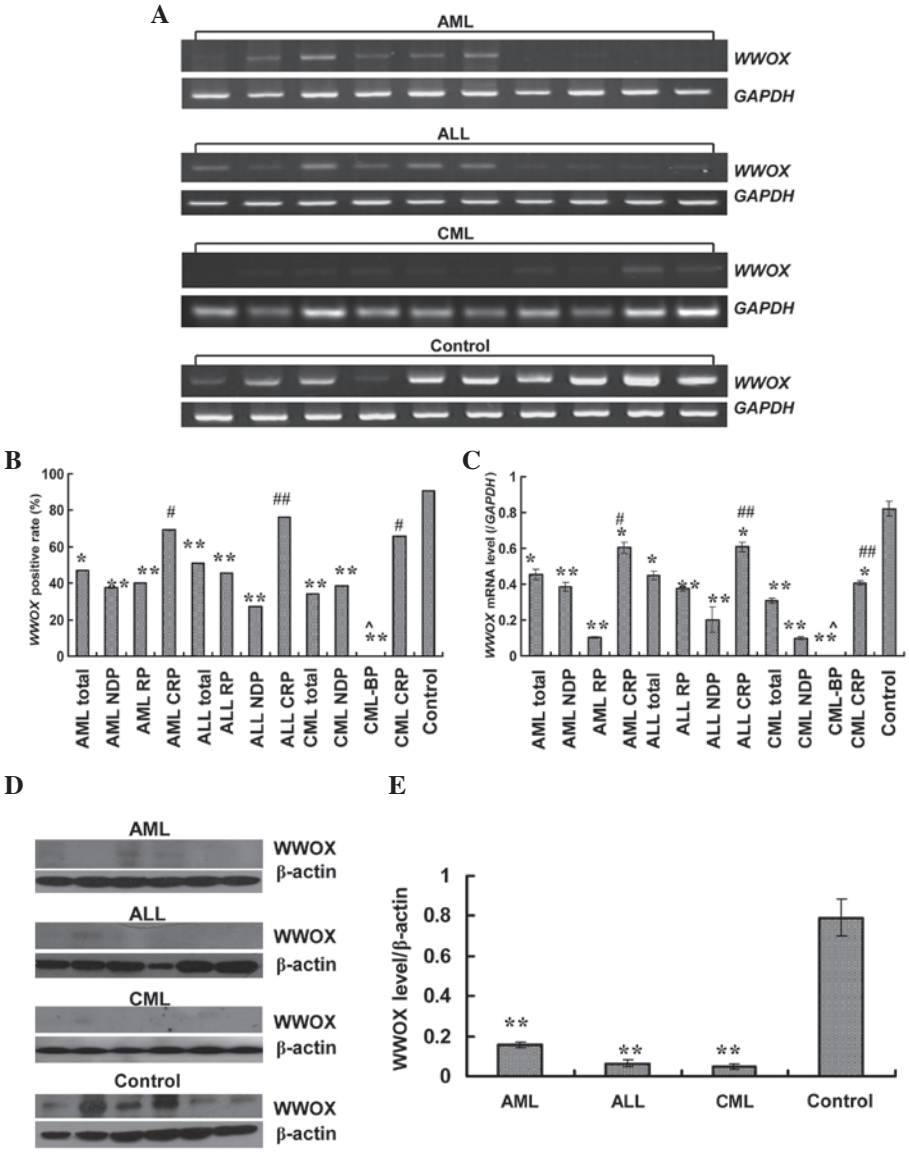

Figure 1. Expression of *WWOX* in primary leukemia cases. (A) Expression of *WWOX* mRNA analyzed by reverse transcription-polymerase chain reaction. (B) *WWOX*-positive rate in different types or stages of leukemia. (C) Expression level of *WWOX* in different types or stages of leukemia, normalized to GAPDH. (D) Expression of WWOX protein examined by western blotting. (E) Expression level of WWOX in different types of leukemia, quantified to β-actin. Data shown are the mean ± standard deviation (n=182 for reverse transcription-polymerase chain reaction, and n=18 for western blotting). *P<0.05 and **P<0.01 vs. normal volunteers; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. newly diagnosed cases; and ^P<0.01 vs. remission cases. WWOX, WW domain-containing oxidoreductase; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; NDP, newly diagnosed patients; CRP, complete remission patients; RP, relapsed patients; CML-BP, chronic myelogenous leukemia in blastic phase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

expression were relatively elevated in the remission cases (AML, P=0.022; ALL, P=0.010; CML, P=0.002; all vs. newly diagnosed cases), yet silenced in the relapsed or CML in blastic phase (CML-BP) cases (P<0.000 vs. remission cases).

Expression of WWOX in leukemia cell lines. Expression status of *WWOX* mRNA and WWOX protein in the leukemia cell lines was similar to that in the leukemia patients. As indicated in Fig. 2A, 4/5 leukemic cell lines (80%) showed absent or reduced *WWOX* expression; Jurkat, K562, HL-60/ADR and K562/ADR cell lines were *WWOX*-negative or exhibited a low expression level, and only HL-60 cells exhibited a high expression level of *WWOX* similar to the 293a controls (Fig. 2A). Western blotting and immunofluorescence assays showed that endogenous WWOX protein in all leukemic cells was undetectable, whereas the 293a cells yielded a positive expression level of WWOX protein (Fig. 2B and C).

Association between WWOX-positive rate and clinical features. The χ^2 test or corrected χ^2 test was utilized to analyze the association between the *WWOX*-positive rate and the clinical features. The clinical characteristics of the newly diagnosed patient cohort are summarized in Table I. There were no significant differences between *WWOX* positive rate and age, gender, leukocytes or immature cells in the newly diagnosed patients (all P>0.05; see Table I for exact P-values).

Association between remission rate and WWOX expression. In order to trace whether WWOX expression was correlated with the therapeutic effect, a total of 99 newly diagnosed patients

Table I. Association between *WWOX*-positive rate and clinical features.

Parameters	n	*WWOX*-positive, n	Positive rate, %	χ^2	P-value
Age, years					
≤40	61	26	42.62	0.531	0.446
>40	38	14	36.84		
Gender					
Male	59	24	40.68	0.005	0.946
Female	40	16	40.00		
Immature cells					
≤50%	47	16	34.04	1.504	0.220
>50%	52	24	46.15		
Leukocytes					
$\leq 5x10^{10}$	57	23	40.35	<0.000	0.990
$>5x10^{10}$	42	17	40.48		

WWOX, WW domain-containing oxidoreductase.

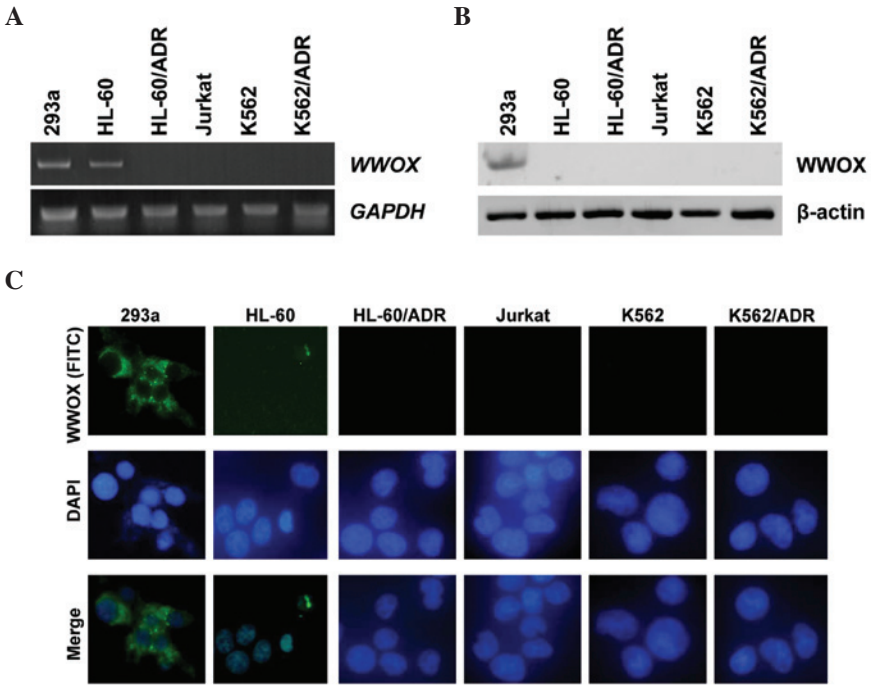

Figure 2. Expression of *WWOX*/WWOX in leukemia cell lines. (A) Expression of WWOX in leukemia cells, as measured by reverse transcription-polymerase chain reaction. (B) Expression level of WWOX in leukemia cells, as detected by western blotting. (C) Immunofluorescence staining assay, observed by fluorescence microscopy (x400 magnification). A rabbit anti-human WWOX antibody was used, which was marked by green fluorescence FITC-conjugated anti-rabbit immunoglobulin G. The nuclei were stained with DAPI (blue fluorescence). Human 293a cells were utilized as a WWOX-positive control. WWOX, WW domain-containing oxidoreductase; FITC, fluorescein isothiocyanate; DAPI, 4',6-diamidino-2-phenylindole.

were followed up. A total of 94 peripheral blood samples were collected and 59.57% (56/94) were revealed to be *WWOX*-negative prior to treatment. The data indicated that the remission rate of the 56 *WWOX*-negative hospitalized cases was 35.71% (20/56), compared with 76.32% (29/38) in the *WWOX*-positive cases (χ^2=14.96; P<0.000). Moreover, 73.68% (14/19) *WWOX*-negative cases exhibited restored *WWOX* expression following treatment, and the mean level of *WWOX* expression was 0.2180±0.1251 vs . 0.5457±0.3859, prior to and following treatment, respectively. Additionally, *WWOX* expression in the originally positive cases yielded a value of 0.3932±0.2083 prior to treatment, which was elevated to a level of 0.8696±0.3216 after corresponding chemotherapies, which included intensive chemotherapy for AML and vincristine, daunorubicin, cyclophosphamide, L-asparaginase, and prednisone chemotherapy for ALL (data not shown).

Discussion

In the present study, the expression of *WWOX*/WWOX in 182 primary leukemia patients and 5 leukemia cell lines was

evaluated, and mRNA and protein expression were found to be significantly reduced or absent in the two sources. Notably, the *WWOX*-positive rates of newly diagnosed and relapsed cases were significantly lower than those of remission cases, indicating a prognostic role of *WWOX* expression in leukemia. Moreover, *WWOX* expression was correlated with the treatment effect, which further suggests that the *WWOX* gene has a potential to be a biomarker or target for the therapy of leukemia.

Leukemia is an oligoclonal or monoclonal disease and the carcinogenesis is generally a complicated multipathway process, including the activation of oncogenes and/or the inactivation of tumor suppressor genes (18). *WWOX* is a tumor suppressor gene that maps to the common fragile site FRA16D and is involved in carcinogenesis and cancer progression in a number of carcinoma types (1,19,20). The bulk of the data documented have shown that the expression of *WWOX*/WWOX was lost or reduced in various types of solid cancers (2-10). Similarly, the aberration or absence of *WWOX*/WWOX expression in primary hematopoietic malignancies has also been reported (11-16). A recently published study detected the expression of *WWOX*/WWOX in a small cohort of 38 primary leukemia patients and observed low expression levels (14). Chen *et al* (13) recently validated the expression of *WWOX* in ALL, and observed that the mRNA expression of *WWOX*, fragile histidine triad and tumor protein p73 was significantly lower in the ALL samples compared with the controls. Correspondingly, the current study showed that *WWOX*/WWOX expression was reduced or lost in a range of different leukemia types and cell lines in comparison to the normal controls. The present findings are consistent with the published studies.

Reduced *WWOX*/WWOX expression is associated with more aggressive phenotypes or the development of carcinomas (21-26). Guler *et al* (22) reported that WWOX protein expression was more frequently reduced in high-grade lesions, and Donati *et al* (26) presented similar results. In addition, other studies showed that the expression of *WWOX*/WWOX exhibited an inverse correlation with clinical or clinicopathological stage, implying that *WWOX*/WWOX expression varies during the development of carcinomas (8,26,27). For instance, Nunez *et al* (27) evaluated the WWOX protein expression levels in ovarian carcinomas and observed that the WWOX-negative rate in stage I patients was 23%, which increased to 48% in stage IV patients. Another study by Huang *et al* (8) also demonstrated that the downregulation of *WWOX*/WWOX was an unfavorable predictor for overall survival and cumulative recurrence rates. In agreement with these reported studies, the present study observed that the *WWOX*-positive rates of newly diagnosed and relapsed cases in AML, ALL and CML were significantly lower than those of remission cases; *WWOX* expression was reduced or silenced in newly diagnosed, relapsed and CML-BP cases, but was relatively elevated when remission was induced. These data strongly demonstrated that *WWOX* may play an important role in the development of cancer. Nevertheless, data from the study by Nunez *et al* showed no statistical difference between the relapsing and non-relapsing cases of ovarian carcinoma with regard to WWOX expression (27). Hence, further larger studies are required to confirm these findings.

Another noteworthy finding of the present study was the association that was found between *WWOX* level and the curative effect among patients. In the present data, the remission rate in expression-negative and -positive hospitalized patients was 35.71 vs. 76.32%. Moreover, *WWOX*-negative cases exhibited restored *WWOX* expression following treatment, and the mean expression level in the *WWOX*-positive patients was relatively elevated after receiving clinical therapy, indicating that *WWOX* has the potential to be a candidate predictive marker for treatment. Supporting evidence from another study similarly showed that WWOX protein expression was significantly elevated in the 5-Aza-CdR-treated ovarian cancer group (28). Recently published data indicated that in the treated breast cancer cases with a tumor expressing moderate or strong WWOX protein, recurrence-free survival was improved (29). Additionally, a previous study conducted by Guler *et al* (30) revealed that reduced or absent WWOX expression was associated with tamoxifen resistance in breast cancer, hinting of the potential of WWOX in predicting tamoxifen response.

In summary, the current study suggested that the loss or silencing of *WWOX*/WWOX expression may contribute to the occurrence and development of leukemia, and that detecting the expression level of *WWOX*/WWOX may aid in the development of future treatment approaches for leukemia. Despite these promising results, the number of cases examined in the present study may not be sufficient, and the study did not evaluate the expression of *WWOX* in chronic lymphoblastic leukemia, multiple myeloma and other hematopoietic malignancies. Even though no significant association was observed between the *WWOX*-positive rate and clinical features in leukemia, additional prospective and retrospective studies are warranted.

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