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TYRO3 Knockdown Suppresses the Growth of Myeloid Leukaemia Cells

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Abstract. *Background/Aim: TYRO3 is a member of the TAM family (TYRO3, AXL, and MERTK) of receptor tyrosine kinases. While the roles of activated AXL and MERTK in the growth of leukaemia cells have been reported, the effect of TYRO3 has not been determined. Therefore, we examined the effects of TYRO3 knockdown on the growth of leukaemia cell lines. Materials and Methods: Three human leukaemia cell lines (AA derived from pure erythroid leukaemia, OCI/AML2, and K562), which express TYRO3 protein were used in this study. To induce TYRO3 knockdown, small interfering RNA (siRNA) against TYRO3 was transfected using an electroporation system. Cell growth was assessed by a colorimetric assay. The expression levels and activation of various signalling proteins were examined by immunoblotting. Changes in comprehensive gene expression after TYRO3 knockdown were examined by microarray analysis. Results: TYRO3 knockdown suppressed cell growth in the leukaemia cell lines tested. Additionally, the knockdown suppressed phosphorylation of signal transducer and activator of transcription-3 in AA cells, and extracellular signal-regulated kinase (ERK) 1/2 in AA and OCI/AML2 cells; both are downstream molecules of TYRO3 signalling. TYRO3 knockdown also suppressed the expression of survivin in all the cell lines. TYRO3 knockdown potently suppressed TYRO3 mRNA expression but not that of AXL and MERTK. Furthermore, TYRO3 knockdown suppressed cyclin D1 mRNA expression, which is a downstream molecule of ERK. Conclusion: TYRO3 plays a role in leukaemia cell growth and is a potential therapeutic target for leukaemia.*

Acute myeloid leukaemia (AML) is a highly aggressive disease characterized by the rapid growth of leukaemia cells. Cytotoxic drugs are used to treat AML. In recent years, small molecule inhibitors targeting FMS-like tyrosine kinase receptor 3 (FLT3) and AXL have been used. However, resistance or relapse is frequently observed; Therefore, novel molecular targeted drugs are needed to improve patient outcomes.

Tyrosine-protein kinase receptor 3 (TYRO3) is a member of the TYRO3, AXL and MER proto-oncogene tyrosine kinase (MERTK) (known collectively as TAM) family receptor tyrosine kinases (RTKs). These RTKs have similar extracellular structures composed of two N-terminal immunoglobulin-like and two fibronectin type III-like motifs (1). These are activated mainly by the growth arrest-specific gene 6 (GAS6) and protein S. TYRO3 is more effectively activated by protein S than GAS6 (2).

TAM RTKs regulate various pathways involved in cell survival and proliferation. Moreover, TAM RTKs play an important role in cancer cell survival and drug resistance (3). The roles of activated AXL and MERTK in AML cell growth have been previously reported by us and others (4-6). We have also reported that metformin, an antidiabetic drug, suppressed AML cell growth partly by down-regulating the phosphorylation of AXL (7). However, the role of TYRO3 in this mechanism has not yet been elucidated. Moreover, the development of small molecule inhibitors selective for TYRO3 is limited (8).

In this study, we performed *TYRO3* knockdown experiments using small interfering RNA (siRNA) to investigate the roles of TYRO3 in the growth of leukaemia cells. We also evaluated the effects of downstream signalling molecules using immunoblotting and microarray analyses to clarify the underlying mechanisms involved in this process.

Materials and Methods

Cell lines and cell cultures. Eight AML cell lines (OCI/AML2, OCI/AML3, OCI/AML5, U937, THP-1, TMD7, HEL, and AA) and one chronic myelogenous leukaemia (CML) cell line K562 were used in this study. The AA cell line was established by Dr. A. Arai from blast cells taken from a patient with acute pure erythroid leukaemia (9). The OCI/AML2 cell line was established from acute myelomonocytic leukaemia cells at the Ontario Cancer Institute. K562

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Key Words: TYRO3, TAM family, small interfering RNA, leukaemia.

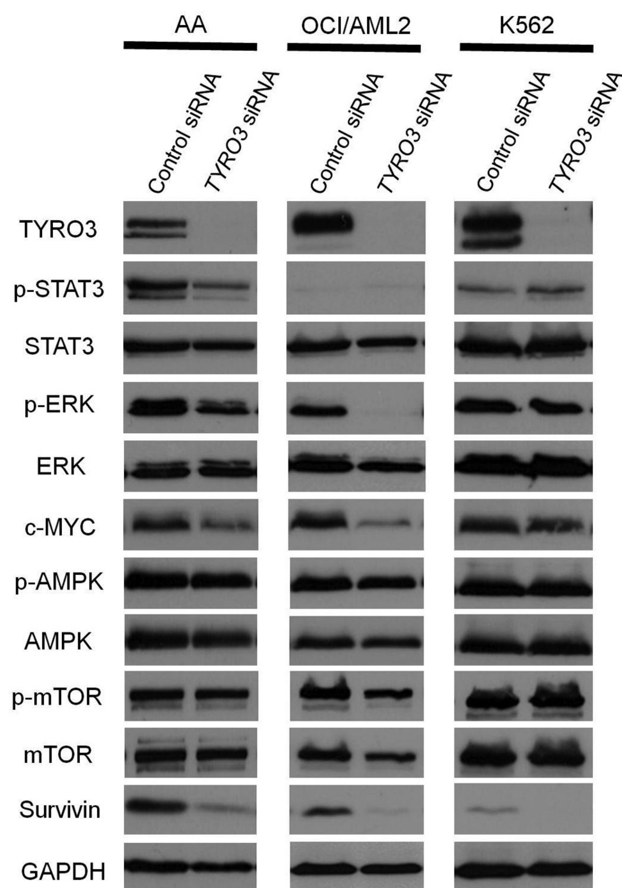

Figure 1. *Protein expression of TYRO3 and its associated signalling proteins in AA, OCI/AML2, and K562 cells after TYRO3 knockdown. Cells were transfected with 20 nM TYRO3 siRNA or control siRNA for 24 h and analysed for the expression of the indicated proteins using immunoblotting.*

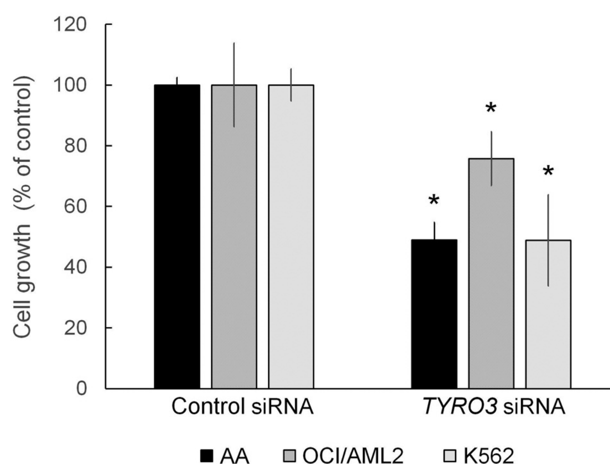

Figure 2. *Effects of TYRO3 knockdown on the growth of AA, OCI/AML2, and K562 leukaemia cells. Cells were transfected with 20 nM TYRO3 siRNA or control siRNA and analysed after 72 h using a colorimetric assay. *p<0.05 compared to each control.*

cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Osaka, Japan). The other cell lines were obtained as previously reported (6, 7, 10). All cell lines were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum at 37˚C in a humidified atmosphere with 5% CO_2.

TYRO3 knockdown by siRNA. Cells were transfected with 20 nM *TYRO3* siRNA (Stealth siRNA™ , Life Technologies, Carlsbad, CA, USA) or a negative control duplex using the Neon™ pipette tip chamber-based electroporation system (Life Technologies) according to the manufacturer's instructions. The *TYRO3* siRNA sequence was 5'-GCUGUGCCUCCAAACUGCCUGUCAA-3'. The cells were immediately transferred to culture medium following transfection.

Cell growth assay. Short-term cell growth was evaluated using a colorimetric water-soluble tetrazolium 8 (WST-8) assay kit (Dojindo Laboratories, Kumamoto, Japan). The transfected cells were cultured for 72 h before WST-8 was added. The optical density (OD) was measured using an enzyme-linked immunosorbent assay plate reader to determine the relative cell number. Relative cell proliferation was calculated as the percentage of the mean OD value normalized to that of control cells transfected with negative control siRNA. Cell morphology was analysed in cytospin preparations stained with Wright's stain and observed under a microscope.

Immunoblot analysis. Cells were harvested 24 h after transfection. Whole cell lysates were prepared from the cells using lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Nonidet® P-40 (Nacalai Tesque, Kyoto, Japan), 100 mM sodium fluoride, 2 mM sodium orthovanadate, and a cOmplete™ mini protease inhibitor cocktail tablet (Sigma-Aldrich, St. Louis, MO, USA). The lysates were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene fluoride membranes. The membranes were then probed with primary and secondary antibodies. Immunoreactive bands were detected using a Pierce enhanced chemiluminescent western blotting substrate (Pierce Biotechnology, Rockford, IL, USA).

Antibodies. Antibodies against TYRO3 (#5585), p-STAT3 (Tyr705) (#9145), STAT3 (#12640), p-ERK1/2 (#4370), ERK1/2 (#4695), c-MYC (#13987), p-AMPK (Thr172) (#2535), AMPK (#2532), p-mTOR (Ser2448) (#5536), mTOR (#2983), and survivin (#2808), along with the anti-rabbit HRP-linked IgG antibody (#7074) were purchased from Cell Signaling Technology (CST, Danvers, MA, USA). GAPDH (#015-25473) was purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan).

Microarray analysis. Microarray analysis was performed to assess the effects of *TYRO3* siRNA on comprehensive gene expression. Total RNA was extracted using a High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany). The RNA was then used to prepare cyanine-3-labelled cRNA, which was hybridized to a SurePrint G3 Human GE 8× 60K v3 microarray (Agilent Technologies, Santa Clara, CA, USA). The expression profile was analysed using Agilent Feature Extraction 11.5.1.1 software.

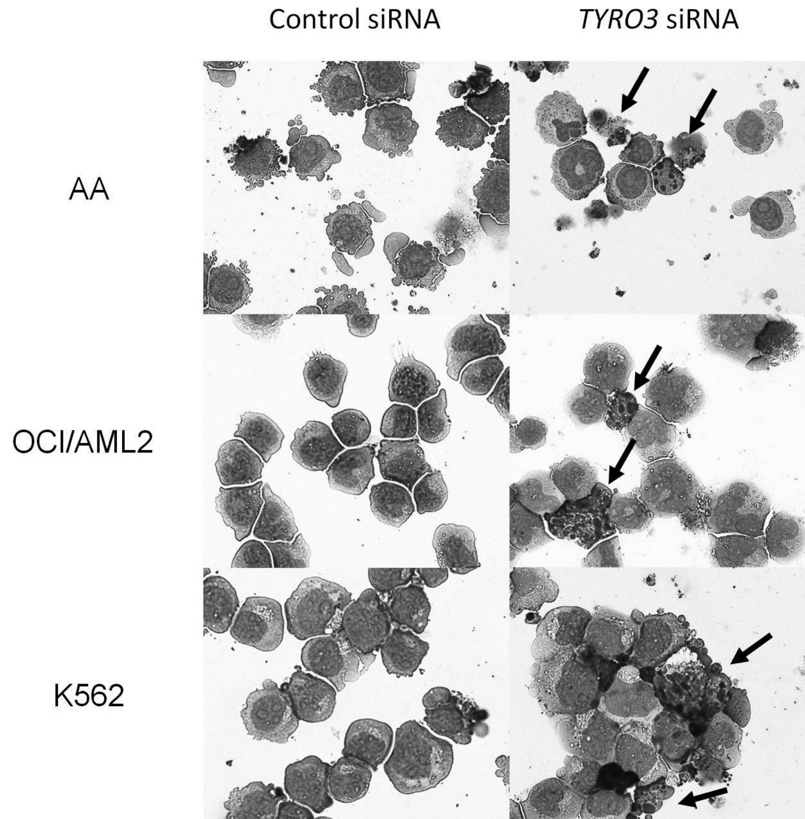

Figure 3. *Morphology of leukaemia cells transfected with 20 nM TYRO3 siRNA or control siRNA after 72 h. Cytospin preparations were stained with Wright's stain and observed under a microscope (original magnifications, ×600). Arrows point on nuclear condensation and fragmentation.*

Statistical analysis. Data are expressed as mean±standard deviation of the samples. Each experiment was repeated three times. Statistical analysis was performed using the Student's *t*-test and two-tailed distribution (IBM SPSS 28).

Results

Expression of TYRO3 in leukaemia cells. TYRO3 expression in eight AML cell lines and K562 cells was examined by immunoblot analysis. Seven cell lines, except for THP-1 and TMD7, expressed TYRO3 (data not shown). The OCI/AML2, AA, and K562 cell lines strongly expressed TYRO3 and had a relatively high knockdown efficiency. Therefore, we chose these three cell lines for use in subsequent experiments.

Effects of TYRO3 knockdown on cell growth and morphology. We knocked down *TYRO3* expression by siRNA, as shown in the top panels of Figure 1. Cell growth was assessed 72 h after *TYRO3* knockdown. The growth rate was significantly suppressed to 48.9% (±5.7%), 75.8% (±8.9%), and 48.8% (±15.0%) in AA, OCI/AML2, and K562 cells, respectively ($p<0.05$; Figure 2). Cytospin preparations indicated that the knockdown caused nuclear condensation and fragmentation, which suggests the induction of apoptosis (Figure 3).

Effects of TYRO3 knockdown on the expression of signalling proteins. To investigate the mechanisms of growth suppression by *TYRO3* knockdown, the expression and phosphorylation status of signalling proteins were examined (Figure 1). The knockdown suppressed the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in AA cells and extracellular signal-regulated kinase (ERK) 1/2 in AA and OCI/AML2 cell lines. It also suppressed c-MYC expression in AA and OCI/AML2 cell lines but did not change the phosphorylation of AMP-activated protein kinase (AMPK) or mammalian target of rapamycin (mTOR). The expression of survivin, an inhibitor of apoptosis, was suppressed in all cell lines.

Comprehensive mRNA expression analysis. The changes in comprehensive gene expression following *TYRO3* knockdown was examined using AA cells. As shown in Table I, *TYRO3*

Table I. *Effects of TYRO3 knockdown on representative gene expression in AA cells analysed with microarray.*

Gene symbol	Log2 ratio
TYRO3	–1.76
AXL	–0.04
MERTK	–0.07
GAS6	–0.18
PROS1	0.31
CCND1	–1.59
MYC	–0.57
BIRC5	–1.61
HK2	–2.15

Log2 ratios of mRNA expression in TYRO3 siRNA-transfected cells were normalized to those in control siRNA-transfected cells.

knockdown potently suppressed *TYRO3* mRNA expression without affecting the expression of *AXL* and *MERTK*. The expression of *GAS6* and *PROS1,* which encode TYRO3 ligands, was not suppressed. The mRNA expression of cyclin D1 (*CCND1)*, a baculoviral inhibitor of apoptosis repeat-containing 5 *(BIRC5)*, which encodes survivin, and hexokinase-2 *(HK2)*, a key enzyme for glycolysis, was also suppressed.

Discussion

This study revealed that *TYRO3* knockdown suppressed the growth of leukaemia cells. Recent studies have shown a relationship between TYRO3 and tumorigenesis. For instance, inhibition of TYRO3 using siRNA suppressed the growth of various cancer cell types, such as colorectal cancer (11), hepatocellular carcinoma (12), and breast cancer (13). Therefore, TYRO3 has emerged as a potential therapeutic target for various cancers (8). However, little is known about the effect of TYRO3 in leukaemia, although approximately half of AML patients' samples expressed *TYRO3* mRNA (14).

Immunoblot analysis revealed that *TYRO3* knockdown suppressed signalling proteins, such as STAT3, ERK1/2, and c-MYC. It has been well defined that STAT3, ERK1/2, and mTOR are downstream molecules of AXL and MERTK in AML cells (5, 15, 16). However, the expression of TYRO3 in AML has not been well studied. Our results suggest that STAT3, ERK1/2, and c-MYC are downstream molecules of TYRO3 in some leukaemia cell lines (Figure 4). However, these molecules were not suppressed in K562 cells, although *TYRO3* knockdown suppressed growth. This suggests that other signalling molecules may be involved in this suppression.

TYRO3 knockdown also suppressed survivin expression in all cell lines. Survivin regulates apoptosis and its inhibition suppresses cell growth and induces apoptosis in AML cells (17). Additionally, *MERTK* knockdown decreases the levels of survivin in cancer cells (18). Therefore, it is possible that *TYRO3* knockdown induces apoptosis of cancer cells through the down-regulation of survivin.

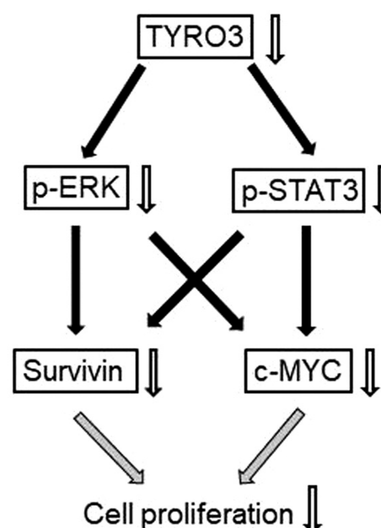

Figure 4. *Schematic representation of TYRO3 signalling pathways.*

Microarray analysis showed that *TYRO3* knockdown suppressed the mRNA expression of *TYRO3* but not that of *AXL* and *MERTK*. This indicated that *TYRO3* siRNA did not cause off-target effects on other TAMs. Additionally, the mRNA expression of *GAS6* and *PROS1*, the ligands of TAMs, was also not affected *by TYRO3* knockdown. This suggests that the down-regulation of TAM signalling molecules was not caused by a decrease in the ligands as an off-target effect.

In addition to survivin, *TYRO3* knockdown resulted in the down-regulation of *BIRC5* mRNA. STAT3 and ERK are regulators of survivin (19). Therefore, it is proposed that the down-regulation of STAT3 and ERK by *TYRO3* knockdown leads to the suppression of survivin (Figure 4). Moreover, the suppression of *CCND1* by *TYRO3* knockdown may lead to inhibition of the cell cycle.

HK2 expression was also suppressed by *TYRO3* knockdown. *HK2* encodes hexokinase, which is an enzyme that catalyses the first step of glycolysis. Over-expression of HK2 is related to poor prognosis in many cancers (20), and HK2 inhibition suppresses AML cell growth by apoptosis (21). Therefore, HK2 inhibition may be related to the suppression of AA cell growth in this study.

Our study has some limitations. First, we did not confirm the microarray data on *CCND1* and *HK2* using quantitative reverse transcription polymerase chain reaction and immunoblot analysis. Second, we did not perform experiments with samples from leukaemia patients. Further experiments are needed to clarify whether our findings are clinically applicable.

In conclusion, we show that TYRO3 plays a role in leukaemia cell growth and is a promising molecular target for the treatment of leukaemia.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

TS and ST designed the study. TS and MI performed the analysis. TS, MI, and ST wrote the article.

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