De novo Identification of MIZ-1 (ZBTB17) Encoding a MYC-Interacting Zinc-Finger Protein as a New Favorable Neuroblastoma Gene

Naohiko Ikegaki, Takahiro Gotoh, Bing Kung, Justin S. Riceberg, David Y. Kim, Huaqing Zhao, Eric F. Rappaport, Sakeenah L. Hicks, Robert C. Seeger, and Xiao X. Tang

Abstract

Purpose: Neuroblastoma is a childhood cancer that exhibits either a favorable or an unfavorable phenotype. Favorable neuroblastoma genes (EPHB6, EPHB2, EFNB3, NTRK1, and CD44) are genes whose high-level expression predicts favorable neuroblastoma disease outcome. Accordingly, the forced expression of these genes or their reactivation by gene silencing inhibitors in unfavorable neuroblastoma cells results in suppression of tumor growth and metastases. This study was undertaken to design an experimental strategy to identify additional favorable neuroblastoma genes.

Experimental Design: Favorable neuroblastoma gene candidates were first identified by gene expression profiling analysis on IMR5 neuroblastoma cells treated with inhibitors of DNA methylation and histone deacetylase against the untreated control cells. Among the candidates, we focused on MIZ-1, which encodes a MYC-interacting zinc-finger protein, because it is known to enhance the expression of growth suppressive genes, such as CDKN1A.

Results: High-level MIZ-1 expression was associated with favorable disease outcome of neuroblastoma (P = 0.0048). Forced MIZ-1 expression suppressed in vitro growth of neuroblastoma cell lines. High MIZ-1 expression was correlated with the small-size neuroblastoma xenografts treated with gene silencing inhibitors or a glucocorticoid. In addition, forced MIZ-1 expression enhanced the expression of CD44 and EFN B2 in neuroblastoma cell lines in vitro. Furthermore, MIZ-1 expression was positively correlated with the expression of favorable neuroblastoma genes (EFNB2, EFNB3, EPHB6, and NTRK1) in the human neuroblastoma xenograft therapeutic models.

Conclusion: MIZ-1 is a new favorable neuroblastoma gene, which may directly or indirectly regulate the expression of other favorable neuroblastoma genes.

Neuroblastoma is the most common extracranial solid tumor in children. The tumor is unique because of its striking clinical bipolarity. Favorable neuroblastomas undergo regression or maturation with little or no treatment. Unfavorable neuroblastomas exhibit unrestrained growth despite very aggressive treatment. Increasing evidence indicates that expression levels of favorable neuroblastoma genes determine biological and clinical behaviors of neuroblastoma (1–5). Favorable neuroblastoma genes are genes whose high-level expression is associated with good neuroblastoma disease outcome. Accordingly, the forced expression of these genes in unfavorable neuroblastoma cells results in tumor growth suppression (1). Moreover, when one of the favorable neuroblastoma genes is expressed at high levels, the clinical outcome is favorable (1). EPHB6, NTRK1 or TrkA, and CD44, encoding cell surface receptors, and EFN B2 and EFN B3, encoding cell surface ligands, are currently known favorable neuroblastoma genes. Our previous studies have shown that inhibitors of DNA methylation and histone deacetylase enhance the expression of favorable neuroblastoma genes in neuroblastoma cell lines, which are derived from unfavorable neuroblastoma (6). These compounds also suppress growth and metastases of human neuroblastoma xenografts grown in immune-suppressed mice (6). In this study, we report (a) an experimental strategy to identify favorable neuroblastoma genes and (b) the identification of MIZ-1 as a favorable neuroblastoma gene by using such an approach. In addition, we showed that forced MIZ-1 expression in neuroblastoma cells in vitro enhanced the expression of other favorable neuroblastoma genes, as well as...
EGR1, a differentiation/apoptosis-associated gene (7–11). MIZ-1 expression was also positively correlated with the expression of favorable neuroblastoma genes in human neuroblastoma xenografts in mice treated with gene silencing inhibitors or a glucocorticoid. These observations suggest that MIZ-1 directly or indirectly enhances the expression of favorable neuroblastoma genes and growth suppressive genes for neuroblastoma.

Materials and Methods

Neuroblastoma cell lines. The neuroblastoma cell lines were grown in RPMI 1640 supplemented with 5% fetal bovine serum and 1% OPI (Life Technologies). These cell lines were tested negative for Mycoplasma, and their identity was validated by the original source or by microsatellites analysis. SYSY was from Dr. Robert Ross (Fordham University); IMR5 (a clone of IMR32), CHP134, and Nbh9 were from Dr. Roger H. Bennett (Department of Biology, Wheaton College; a former faculty member of Department of Human Genetics, The University of Pennsylvania School of Medicine). CHP901 was established by Dr. Naohiko Ikegaki. LAN-1, LAN-5, SMS-KAN, SMS-KCN, SK-N-AS, and OAN were from Dr. C. Patrick Reynolds (The Children’s Hospital of Los Angeles). NGP, NMB, and NLF were from Dr. Garrett M. Brodeur (The Children’s Hospital of Philadelphia). NBL-S was from Dr. Susan L. Cohn (Tapestry Pharmaceuticals, Inc.). SK-N-DZ was obtained from American Type Culture Collection.

Primary neuroblastoma tumor samples. Seventy-three neuroblastoma tumor specimens were obtained from the tumor bank of the former Pediatric Oncology Group, the tumor bank of the former Children Cancer Group, and Memorial Sloan-Kettering Cancer Center. The neuroblastoma cohort included nine of stage 1, twelve of stage 2, eight of stage 4S, fourteen of stage 3, and thirty of stage 4. Among these, 19 (26%) are stage 4S, 14 (20%) are stage 3, and 30 (40%) are stage 4. Known favorable neuroblastoma genes have been shown to be silenced in unfavorable neuroblastoma cells (6). We thus first identified favorable neuroblastoma gene candidates by performing two independent gene-profiling analyses on IMR5 cells treated with a combination of inhibitors of DNA methylation (5-aza-2'-deoxycytidine) and histone deacetylase (4-phenylbutyrate) – a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterrazolium bromide assay was used to assess the viable cells in each culture (see below) at day 7. To obtain RNA and protein samples for expression studies, the cells were harvested at three different time points after MIZ-1 transfection: 24 h, 48 h after electroporation without drug selection, and 4 days after electroporation with drug selection (0.5 μg/ml puromycin).

Affymetrix microarray analysis. Affymetrix cDNA microarray analysis. The xenograft study of IMR5 treated with 4-phenylbutyrate and/or 5-aza-2'-deoxycytidine was described earlier (6). The SYSY xenograft study was done in the same way as the IMR5 study. Briefly, SYSY cells were suspended in Matrigel (BD Bioscience) and injected s.c. in the flank of nude mice (107 cells per 0.25 ml per mouse). Treatment of prednisolone-hemisuccinate sodium salt (Sigma) at 200 mg/kg/day started when the tumor volume reached 0.2 to 0.25 cm3 in size. Animals in the control group were given PBS. The total amount of drug or PBS given daily was divided into two i.p. injections (two injections per day and 7 days a week). Tumors were measured in anesthetized animals every other day using a Vernier caliper. Briefly, a sterile gauge was placed at the bottom of a 50-mL tube, and anesthetized animals were placed in the tube for a few seconds, which allowed the animal to inhale the anesthetic agent. Tumor size was calculated by the formula (tumor volume = d1 x d2 x d3 x π / 6). The P values for significance in xenograft experiments were determined by Student’s t test.

Results

MIZ-1 expression is silenced in neuroblastoma cell lines derived from unfavorable neuroblastomas. Known favorable neuroblastoma genes have been shown to be silenced in unfavorable neuroblastoma cells (6). We thus first identified favorable neuroblastoma gene candidates by performing two independent gene-profiling analyses on IMR5 cells treated with a combination of inhibitors of DNA methylation (5-aza-2'-deoxycytidine) and histone deacetylase (4-phenylbutyrate) and untreated control cells. In each experiment, the U95Av2 DE expression signal as an internal control and fetal brain as a reference chip was used. The TaqMan probe sequence was CAG CTG CTG CTG CTG GTT 3′. The TaqMan probe sequence was CAC AGC CAG AGC CTC AGT ACC-3′ and 5′-TGA CCA CGT CGA TTT CTT CCT-3′. The TaqMan probe sequence was 5′-GAC CAC AAG GCC CTC AGT ACC-3′ and 5′-TGA CCA CGT CGA TTT CTT CCT CFF-3′. The TaqMan probe sequence was 5′-CCG GAG AGC ACA CCC TGA GGC A-3′. Relative quantification of MIZ-1 and MYCN expression was done by the ΔΔCt method using GAPD signal as an internal control and fetal brain as a reference chip. The TaqMan real-time PCR. MIZ-1 and MYCN expression in primary neuroblastoma tumors was examined using TaqMan real-time PCR. The primer sequences for MIZ-1 were 5′-TGT CTG CAA ATA ATC TCA GCC AT T-3′ and 5′-CAC CGC TTC TGC GTC GTT TT T-3′. The TaqMan probe sequence for MIZ-1 was 5′-CAC AGC CAC CAT GTC TTC GAA CAC GT C-3′. The MYCN primer sequences were 5′-GAC CAC AAG GCC CTC AGT ACC-3′ and 5′-TGA CCA CGT CGA TTT CTT CCT CFF-3′. The TaqMan probe sequence was 5′-CCG GAG AGC ACA CCC TGA GGC A-3′. Relative quantification of MIZ-1 and MYCN expression was done by the ΔΔCt method using GAPD signal as an internal control and fetal brain as a reference chip. The TaqMan real-time PCR. MIZ-1 and MYCN expression in primary neuroblastoma tumors was examined using TaqMan real-time PCR. The primer sequences for MIZ-1 were 5′-TGT CTG CAA ATA ATC TCA GCC AT T-3′ and 5′-CAC CGC TTC TGC GTC GTT TT T-3′. The TaqMan probe sequence for MIZ-1 was 5′-CAC AGC CAC CAT GTC TTC GAA CAC GT C-3′. The MYCN primer sequences were 5′-GAC CAC AAG GCC CTC AGT ACC-3′ and 5′-TGA CCA CGT CGA TTT CTT CCT CFF-3′. The TaqMan probe sequence was 5′-CCG GAG AGC ACA CCC TGA GGC A-3′. Relative quantification of MIZ-1 and MYCN expression was done by the ΔΔCt method using GAPD signal as an internal control and fetal brain as a reference chip. The TaqMan real-time PCR. MIZ-1 and MYCN expression in primary neuroblastoma tumors was examined using TaqMan real-time PCR. The primer sequences for MIZ-1 were 5′-TGT CTG CAA ATA ATC TCA GCC AT T-3′ and 5′-CAC CGC TTC TGC GTC GTT TT T-3′. The TaqMan probe sequence for MIZ-1 was 5′-CAC AGC CAC CAT GTC TTC GAA CAC GT C-3′. The MYCN primer sequences were 5′-GAC CAC AAG GCC CTC AGT ACC-3′ and 5′-TGA CCA CGT CGA TTT CTT CCT CFF-3′. The TaqMan probe sequence was 5′-CCG GAG AGC ACA CCC TGA GGC A-3′. Relative quantification of MIZ-1 and MYCN expression was done by the ΔΔCt method using GAPD signal as an internal control and fetal brain as a reference chip.
4-phenylbutyrate (2.5 mmol/L) for 2 days in vitro. Genes that showed an increased expression in the treated cells compared with the control cells were considered favorable neuroblastoma gene candidates. From these experiments, we identified 423 genes that consistently showed more than a 2-fold increase or decrease in expression in the 2-day treated samples. Among these genes, we identified CDKN1A (encoding p21WAF1), CD95, and Bax, which are induced by inhibitors of DNA methylation and histone deacetylase (17–19), indicating that this approach was feasible. MIZ-1, encoding a MYC-interacting protein, was one of the candidate genes because its expression was enhanced in IMR5 cells treated with 5-aza-2’-deoxycytidine and 4-phenylbutyrate. TaqMan real-time reverse transcription–PCR further confirmed this observation using three neuroblastoma cell lines (SY5Y, CHP134, and IMR5) treated with 5-aza-2’-deoxycytidine and/or 4-phenylbutyrate (Fig. 1). These data indicate that MIZ-1 is silenced in unfavorable neuroblastoma cells, a characteristic of currently known favorable neuroblastoma genes.

Neuroblastoma cell lines express low levels of MIZ-1, and forced MIZ-1 expression results in growth suppression in vitro. Neuroblastoma cell lines express low levels of favorable neuroblastoma genes. We therefore examined the expression of MIZ-1 in a panel of 16 cell lines by real-time reverse transcription–PCR. MIZ-1 expression was significantly low in all neuroblastoma cell lines compared with normal tissues, including fetal brain (P < 0.05; Fig. 2A and B). To further confirm that MIZ-1 is in fact a favorable neuroblastoma gene, we examined whether forced expression of MIZ-1 suppressed growth of neuroblastoma cell lines (IMR5 and SY5Y) in vitro. As shown in Fig. 2C, MIZ-1 significantly inhibits the growth of both IMR5 (MYCN-amplified) and SY5Y (MYCN-nonamplified; P < 0.01).

High MIZ-1 expression is associated with favorable disease outcome of neuroblastoma. MIZ-1 expression was next analyzed for correlation with age at diagnosis, disease stage, or MYCN amplification in a cohort of 73 primary neuroblastoma cases. Lower MIZ-1 expression was significantly associated with MYCN amplification (P < 0.00005) and advanced stage (P = 0.032), but not with age at diagnosis (P = 0.193). This feature was similar to those observed for EPHB6 and EFNB3 (1). Figure 3A shows the Kaplan-Meier analysis of the two
neuroblastoma subgroups dichotomized by the median MIZ-1 expression value of the entire cohort (n = 73), of which clinical data were available for 66 samples. The MIZ-1 high group had a 5-year survival of 80.3% with 95% confidence interval of 60.3% to 90.9%, whereas the MIZ-1 low group had a 5-year survival of 48.5% with 95% confidence interval of 29.7% to 65.0%. This difference in survival rate was statistically significant by the log-rank test (P = 0.0048). Single-variable Cox regression analysis using MIZ-1 expression as a continuous variable further confirmed this observation (P = 0.007). These results show that MIZ-1 expression is associated with favorable disease outcome of neuroblastoma.

In addition, as shown in Table 1, MIZ-1 expression remains prognostic in the presence of age and/or stage. However, in the presence of MYCN amplification, MIZ-1 expression was no longer prognostic (see Discussion). Due to the sample size, further Cox analysis was not attempted to examine prognostic relationships among the variables.

**Relationship between MIZ-1 and MYCN expression in primary neuroblastomas.** MIZ-1 is known to interact with MYCN (20), whose expression significantly influences the biology of neuroblastoma (21). We therefore investigated a possible relationship between MIZ-1 and MYCN expression in primary neuroblastomas. No significant relationship between MIZ-1 and MYCN expression was found when the overall neuroblastoma cohort...

### Table 1. Cox models with MIZ-1 expression, age, stage, and MYCN amplification of the neuroblastoma cohort

<table>
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<tr>
<th>Model</th>
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<td>0.106</td>
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<td>Age* 1</td>
<td>0.234</td>
<td>Stage 0.370</td>
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<tr>
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<td>MYCN</td>
<td>0.009</td>
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*Two categories: ages <1 and >1 y.

*Two categories: low and advanced stages.
was analyzed. However, when MYCN-amplified and MYCN-nonamplified cases were examined separately, MIZ-1 and MYCN expressions showed interesting relationships. As shown in Fig. 3B, MIZ-1 expression positively correlated with MYCN expression in MYCN-nonamplified cases \((n=54; r=0.485; P=0.0002)\), whereas there was a trend that MIZ-1 expression was inversely correlated with MYCN expression in MYCN-amplified cases \((n=19; r=-0.366; P=0.123)\). The MYCN-nonamplified neuroblastoma population shown in Fig. 3B (solid square) contains a mixture of both favorable (age, <1 year) and unfavorable tumors (age, >1 year). We thus divided the MYCN-nonamplified neuroblastoma into two groups based on age (<1 and >1 year) and examined MIZ-1 and MYCN expression. As shown in Fig. 3C and D, both groups (<1 and >1 year) expressed similar levels of MIZ-1. However, the favorable neuroblastoma group with ages of <1 year had higher expression of MYCN compared with that of the unfavorable neuroblastoma group with ages >1 year (see Discussion).

High MIZ-1 expression is correlated with the small size of human neuroblastoma xenografts treated with gene silencing inhibitors or a glucocorticoid. We previously showed that growth of IMR5 xenografts in nude mice was significantly suppressed by 4-phenylbutyrate or a 4-phenylbutyrate/5-aza-2′-deoxycytidine combination (6). To address the growth suppressive effect of MIZ-1 in vivo, we reexamined these IMR5 xenografts for MIZ-1 expression. As shown in Fig. 4A, MIZ-1 expression exhibited a significant inverse correlation with the size of tumors among the control and 4-phenylbutyrate groups \((r=0.856; P=0.0004)\). There was also a trend that MIZ-1 expression was higher in smaller tumors among the 4-phenylbutyrate/5-aza-2′-deoxycytidine combination group \((r=0.697; P=0.124)\). Similarly, the size of these xenografts was inversely correlated with expression levels of CDKN1A \((r=-0.7413; P=0.0058)\), NTRK1 or TrkA \((r=-0.8681; P=0.0002)\); EFNB2 \((r=-0.7236; P=0.0078)\), and EFNB3 \((r=-0.7376; P=0.0062)\) in the control and 4-phenylbutyrate groups (Supplementary Fig. S1). In addition, there was a tendency that expression levels of these genes were higher in smaller tumors in the 4-phenylbutyrate/5-aza-2′-deoxycytidine combination group (Supplementary Fig. S1).

To further confirm this observation, we examined MIZ-1 expression in SY5Y xenografts treated with a glucocorticoid (i.e., prednisolone). Glucocorticoids have been known to induce differentiation and suppress growth of neuroblastoma
in vivo (22–24), yet its potential as therapy for neuroblastoma has not been tested in xenograft models. Moreover, glucocorticoids are also epigenetic modifiers that induce site-specific DNA demethylation (25). The neuroblastoma cell line SY5Y was used because, unlike IMR5 cells, SY5Y does not carry 1p deletion, and thus, both alleles of MIZ-1 are intact in these cells. As shown in Fig. 4B, we confirmed the growth suppressive effect of prednisolone on growth of SY5Y xenografts in nude mice. The actual mean tumor size effect of prednisolone on growth of SY5Y xenografts in nude cells. As shown in Fig. 4B, we confirmed the growth suppressive deletion, and thus, both alleles of used because, unlike IMR5 cells, SY5Y does not carry 1p demethylation (25). The neuroblastoma cell line SY5Y was has not been tested in xenograft models. Moreover, glucocorticoids are also epigenetic modifiers that induce site-specific DNA invitro expression were found in the prednisolone-treated MIZ-1 xenografts that responded to the treatment. There was a statistically significant correlation between high MIZ-1 expression and the small tumor size ($r = -0.714; P = 0.0006; \text{Fig. 4C}$). A negative correlation was also found between a tumor size and $EFNB2$ expression ($r = -0.539; P = 0.0172$), as well as $EPHB6$ expression ($r = -0.508; P = 0.0264; \text{Fig. 4C}$). Similarly, there was a trend that $CDKN1A$ and $CD44$ expression inversely correlated with tumor size (Supplementary Fig. S2). The IMR5 and SY5Y xenograft studies thus suggest that the growth of these neuroblastoma xenografts is an inverse function of $MIZ-1$ and other favorable neuroblastoma gene expression. Collectively, our results show that $MIZ-1$ is a favorable neuroblastoma gene: its high expression is associated with a good disease outcome (Fig. 3A) and suppresses growth of unfavorable neuroblastoma in vivo (Fig. 2C) and in vivo (Fig. 4A and C).

**Forced $MIZ-1$ expression enhances the expression of other favorable neuroblastoma genes in neuroblastoma cell lines.** Because $MIZ-1$ functions as a transcription factor, it was of interest to examine whether $MIZ-1$ could influence the expression of other favorable neuroblastoma genes in neuroblastoma cells. As neuroblastoma cell lines express little or no $MIZ-1$, we first transfected $MIZ-1$ into these cells and examined the expression of favorable neuroblastoma genes. As shown in Fig. 5A, $MIZ-1$ transcripts were expressed at high levels in the transfected cells. Western blot analysis also confirmed the expression of $MIZ-1$ protein (Fig. 5B). The highest $MIZ-1$ expression was found at 1 day after transfection in SY5Y cells and at 4 days after transfection in IMR5 and CHP134 cells (Fig. 5A). The expression of $CDKN2B$ served as a positive control because $MIZ-1$ is known to activate $CDKN2B$ transcription (26).

As shown in Fig. 5A, forced expression of $MIZ-1$ enhanced $CD44$ expression in SY5Y, CHP134, and IMR5 cells. The highest $CD44$ expression was found after 1 day of transfection in SY5Y and at 4 days of transfection in CHP134 and IMR5. On the other hand, $MIZ-1$ enhanced $EFNB2$ expression in IMR5 but not SY5Y and CHP134 (Fig. 5A). This observation may be related to the fact that the $MIZ-1$ protein expression is higher in IMR5 than those in the other two cell lines (Fig. 5B). These data revealed a functional interaction of $MIZ-1$ with other favorable neuroblastoma genes, which may in turn provide the basis for its growth suppressive effect on neuroblastoma cells. We therefore examined whether transfection of $MIZ-1$ could up-regulate the expression of $EGR1$, whose expression has been shown to promote apoptosis and differentiation of neuroblastoma cells (7–11). As shown in Fig. 5A, $MIZ-1$ enhanced $EGR1$ expression in IMR5 and CHP134, but not SY5Y. As expected, $CDKN2B$ expression was increased in the $MIZ-1$-transfected cells, and the pattern of induction was similar to the levels of $MIZ-1$ in these cells (Fig. 5A). There was little or no change in expression of $EPHB6$, $EFNB3$, $NTRK1$, and $CDKN1A$ in the transient $MIZ-1$ transfectants (data not shown).

**Fig. 5.** Effect of ectopic $MIZ-1$ expression on neuroblastoma cell lines. A. $MIZ-1$ enhances the expression of $CD44$, $EFNB2$, and $EGR1$ genes in neuroblastoma cell lines. Neuroblastoma cell lines indicated were transfected either with the vector alone (pEAK12) or the vector containing a full-length $MIZ-1$ cDNA. $MIZ-1$ expression was the highest at 1 d after transfection for SY5Y and at 4 d after transfection for CHP134 and IMR5. The expression of $EPHB6$, $EFNB2$, $EFNB3$, $CD44$, and $NTRK1$ (TrkA) was examined by quantitative reverse transcription–PCR assay using $GAPD$ as an internal standard. There was little or no change in expression of $EPHB6$, $EFNB3$, and $NTRK1$ in response to the forced $MIZ-1$ expression (not shown). B. expression of the $MIZ-1$ protein in three neuroblastoma cell lines (SY5Y, CHP134, and IMR5) transfected with $MIZ-1$.
Positive correlation of MIZ-1 expression with the expression of favorable neuroblastoma genes [EFNB2, EFNB3, EPHB6, and TrkA (NTRK1)] in neuroblastoma xenograft therapeutic models. To further confirm our in vitro data shown in Fig. 5, we examined whether there was any correlation between MIZ-1 expression and favorable neuroblastoma gene expression in the human neuroblastoma xenografts described above. CDKN1A expression was included in the analysis as a control because MIZ-1 is known to activate CDKN1A (27, 28). As shown in Fig. 6A, there was a positive correlation between MIZ-1 and CDKN1A expression ($r = 0.839; P < 0.00005$), between MIZ-1 and EFNB2 expression ($r = 0.462; P = 0.0536$), between MIZ-1 and EPHB3 expression ($r = 0.701; P = 0.0012$), and between MIZ-1 and TrkA (NTRK1) expression ($r = 0.708; P = 0.001$) in the IMR5 xenografts treated with 5-aza-2′-deoxycytidine and/or 4-phenylbutyrate. None of these IMR5 xenografts expressed EPHB6 at detectable levels, and thus, EPHB6 expression was not available to the above analysis shown in Fig. 6A. Furthermore, as shown in Fig. 6B, there was a positive correlation between MIZ-1 and CDKN1A expression ($r = 0.610; P = 0.0056$), between MIZ-1 and EFNB2 expression ($r = 0.683; P = 0.0012$), and between MIZ-1 and EPHB6 expression ($r = 0.658; P = 0.0022$) in the SY5Y xenografts treated with prednisolone.

**Discussion**

Historically, known favorable neuroblastoma genes have been identified by the observations that their expression predicts neuroblastoma disease outcome, followed by experimental data demonstrating the growth suppressive effect of these genes on neuroblastoma cell lines (1–5). Subsequently, inhibitors of DNA methylation and histone deacetylase are shown to enhance the expression of favorable neuroblastoma genes in unfavorable neuroblastoma cells (6). Based on these characteristics, we have designed a strategy to identify additional favorable neuroblastoma genes. This study shows the feasibility of such an approach as we present preliminary data demonstrating the growth suppressive effect of MIZ-1 expression with the expression of favorable neuroblastoma genes in human neuroblastoma xenografts treated with gene silencing inhibitors and prednisolone.

**A**, MIZ-1 expression is correlated with CDKN1A, EFNB2, EFNB3, and NTRK1 (TrkA) expression in the IMR5 xenografts treated with gene silencing inhibitors. **B**, MIZ-1 expression is correlated with CDKN1A, EFNB2, and EPHB6 expression in the SY5Y xenografts treated with prednisolone. The expression level of CDKN1A, EFNB2, EFNB3, TrkA (NTRK1), or EPHB6 was plotted against that of MIZ-1. Pearson correlation coefficient and $P$ value were then calculated for each combination.
induce cell death in MYCN-nonamplified neuroblastomas with high MIZ-1 and MYCN expression (Fig. 3C). Therefore, these tumors exhibit a favorable phenotype. In addition, our in vitro data and xenograft studies indicate that MIZ-1 can induce CDKN1A and CDKN2B in neuroblastoma cells (Figs. 5 and 6). Thus, MIZ-1 could trigger both apoptosis and growth arrest in neuroblastoma when expressed at high levels.

In contrast, MYCN-amplified neuroblastomas express low levels of MIZ-1 and high levels of MYCN (Fig. 3B, gray circle), suggesting that high MIZ-1 expression is incompatible with high MYCN expression in MYCN-amplified cases as high-level expression of both MYCN and MIZ-1 may otherwise trigger massive cell death. To sustain growth of the most aggressive form of neuroblastoma, these MYCN-amplified neuroblastoma cells may acquire mechanisms that suppress MIZ-1 expression. Because MIZ-1 resides in 1p36 and the deletion of this chromosomal region is associated with MYCN amplification (31, 32), a copy of MIZ-1 is likely to be deleted in MYCN-amplified neuroblastoma, resulting in low MIZ-1 expression in these tumors. It was therefore not surprising that MIZ-1 expression lost its prognostic significance in a two-variable Cox regression analysis against expression (Fig. 3C). Therefore, these tumors exhibit a favorable phenotype. In addition, our in vitro data and xenograft studies indicate that MIZ-1 can induce cell death in MYCN-amplified neuroblastomas with high MIZ-1 and MYCN expression (Fig. 3C). Therefore, these tumors exhibit a favorable phenotype. In addition, our in vitro data and xenograft studies indicate that MIZ-1 can induce CDKN1A and CDKN2B in neuroblastoma cells (Figs. 5 and 6). Thus, MIZ-1 could trigger both apoptosis and growth arrest in neuroblastoma when expressed at high levels.

MIZ-1 binds the initiator element (Inr) of target genes to activate transcription, and these initiator elements can be found in several favorable neuroblastoma genes (CD44, EFNB3, EPHB6, and NTRK1). It remains to be proved that favorable neuroblastoma genes are MIZ-1 target genes. Nonetheless, results of this study (Figs. 5 and 6) support the idea that MIZ-1 may directly or indirectly enhance favorable neuroblastoma gene expression. It should also be mentioned that, in contrast to the data shown in Fig. 5, transfection of EPHB6 and EFN3 into neuroblastoma cell lines does not increase MIZ-1 expression.

Lastly, the in vivo studies presented in this report have an important clinical implication. We have found a significant correlation between the high expression of favorable neuroblastoma genes and the small size of IMR5 and SY5Y xenografts treated with gene silencing inhibitors and prednisolone, respectively (Fig. 4A and C). These data reemphasize that favorable neuroblastoma gene expression is important for growth suppression of neuroblastoma in vivo. In addition, our data (Fig. 4) suggest that glucocorticoids can be considered potential treatment for neuroblastoma in combination with gene silencing inhibitors, which alone can enhance the expression of favorable neuroblastoma genes (6). However, it remains to be seen whether favorable neuroblastoma genes, such as MIZ-1, EFN3, and EPHB6, are glucocorticoid-responsive genes (Fig. 4C). Furthermore, results of our mouse xenograft experiments (Fig. 4A and C) suggest that favorable neuroblastoma gene expressions can be considered molecular indicators to assess whether or not experimental chemotherapeutic agents are active against neuroblastoma. In fact, logistically it is very difficult to study an actual response of neuroblastoma to experimental drugs in human patients because the tumor specimens after or during the treatment are rarely available for analysis. Therefore, drug-treated xenografts are the only realistic source of such materials. Consequently, such in vivo studies would be a promising approach to predict the efficacy of a drug in treatment for children with unfavorable neuroblastoma.

In conclusion, we anticipate that there will be additional favorable neuroblastoma genes identified through the strategy presented in this study. Alternatively, one may identify potential favorable neuroblastoma genes by directly identifying MIZ-1 target genes. Further elucidation of functional interactions among favorable neuroblastoma genes would provide insight into the biology of the tumor and pathways that are important for neuroblastoma phenotypes.

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De novo Identification of MIZ-1 (ZBTB17) Encoding a MYC-Interacting Zinc-Finger Protein as a New Favorable Neuroblastoma Gene

Naohiko Ikegaki, Takahiro Gotoh, Bing Kung, et al.


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