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RAPID COMMUNICATION

# Overactivation of histone deacetylases and EZH2 in Wilms tumorigenesis

Wilms tumor (WT) is the most common childhood kidney cancer. Although WT is largely curable, current treatments fail in up to 15 percent of patients. Moreover, survivors suffer from the complications and late effects of the aggressive treatments. Thus, there is a critical need to improve our understanding of tumorigenesis to develop novel therapies to reduce the treatment burden while maintaining excellent survival rates. WT is believed to arise from the immature kidney cells, nephron progenitor cells (NPCs), which have failed to differentiate properly. Previous studies revealed that Wilms cells share a transcriptional and epigenetic landscape with normal renal stem cells.[1] Although several studies have shown the positive associations between WT in children and embryonic exposure to adverse environments, the underlying mechanisms remain unknown. Altered epigenetics is central to oncogenesis in many pediatric cancers. The critical contribution of epigenetic dysregulation to pediatric tumors provides a compelling rationale for the therapeutic potential of epigenetic drugs. Histone deacetylases (HDACs) and Enhancer of Zeste Homolog 2 (EZH2, a histone H3K27 methyltransferase), have been demonstrated to play a critical role in self-renewal and differentiation of mouse NPCs.[2,3] In addition, altered expression and mutations of HDACs and EZH2 have been linked to many human cancers, including WT. Thus, they are among the most promising therapeutic targets for cancer treatment. We reasoned that WT would result from the unrestrained proliferation of progenitor cells due to overactive HDAC1/2 (HDAC1 and HDAC2), and EZH2. We tested this hypothesis by analyzing a clinical specimen received from the left kidney tumor of an 11-year-old male patient diagnosed with WT and four other human WT specimens. We have the approval from Tulane Human Research Protection Office & Institutional Review Boards (Study number: 2019–623) to study WT specimens. The tumor (9.5 cm × 8 cm × 7.5 cm) is classified as favorable for the histology of indeterminate cell tumors. As shown in Figure 1A, the tumor exhibits a biphasic pattern with significant blastemal component admixed with stromal component. No significant epithelial component is present. The blastema represents the undifferentiated and malignant component, consisting of small round blue cells with overlapping nuclei and brisk mitotic activity. The blastemal component shows somewhat a basaloid growth pattern. The stromal component is also prominent and includes hypercellular undifferentiated mesenchymal cells.

Phosphorylation of HDAC1 and HDAC2 was well characterized biochemically and positively correlated with the deacetylase activity of HDAC1/2. Immunofluorescence (IF) demonstrated dramatically higher levels of HDAC1, p-HDAC1 (phosphorylated at Ser 421, 423), HDAC2, and p-HDAC2 (phosphorylated at Ser 394) (Fig. 1B1, 3, 5, 7) in the tumor tissues compared with adjacent normal tissues (Fig. 1B2, 4, 6, 8), strongly suggesting a role of the overactive HDAC1/2 in Wilms tumorigenesis. Neural cell adhesion molecule (NCAM, a cell surface marker of the WT stem cell) was also highly expressed in the tumor (Fig. 1B3), indicating the presence of undifferentiated and proliferating WT blastemal cells. Of note, DBA-staining is a sensitive indicator of the morphogenetic activity of the collecting duct system. The lack of DBA-staining (Fig. 1B7) revealed the developmental deficiency of kidney tumor tissue. Consistent with a higher level of HDAC1/2 (Fig. 1B), histone H3 acetylation on Lysine 27 (H3K27Ac) was markedly reduced in the tumor specimen (Fig. 1D1) compared with adjacent normal tissues (Fig. 1D2). We also detected higher H3 histone tri-methylation on Lysine 27 (H3K27Me3) in the tumor (Fig. 1D3), suggesting the overactivation of

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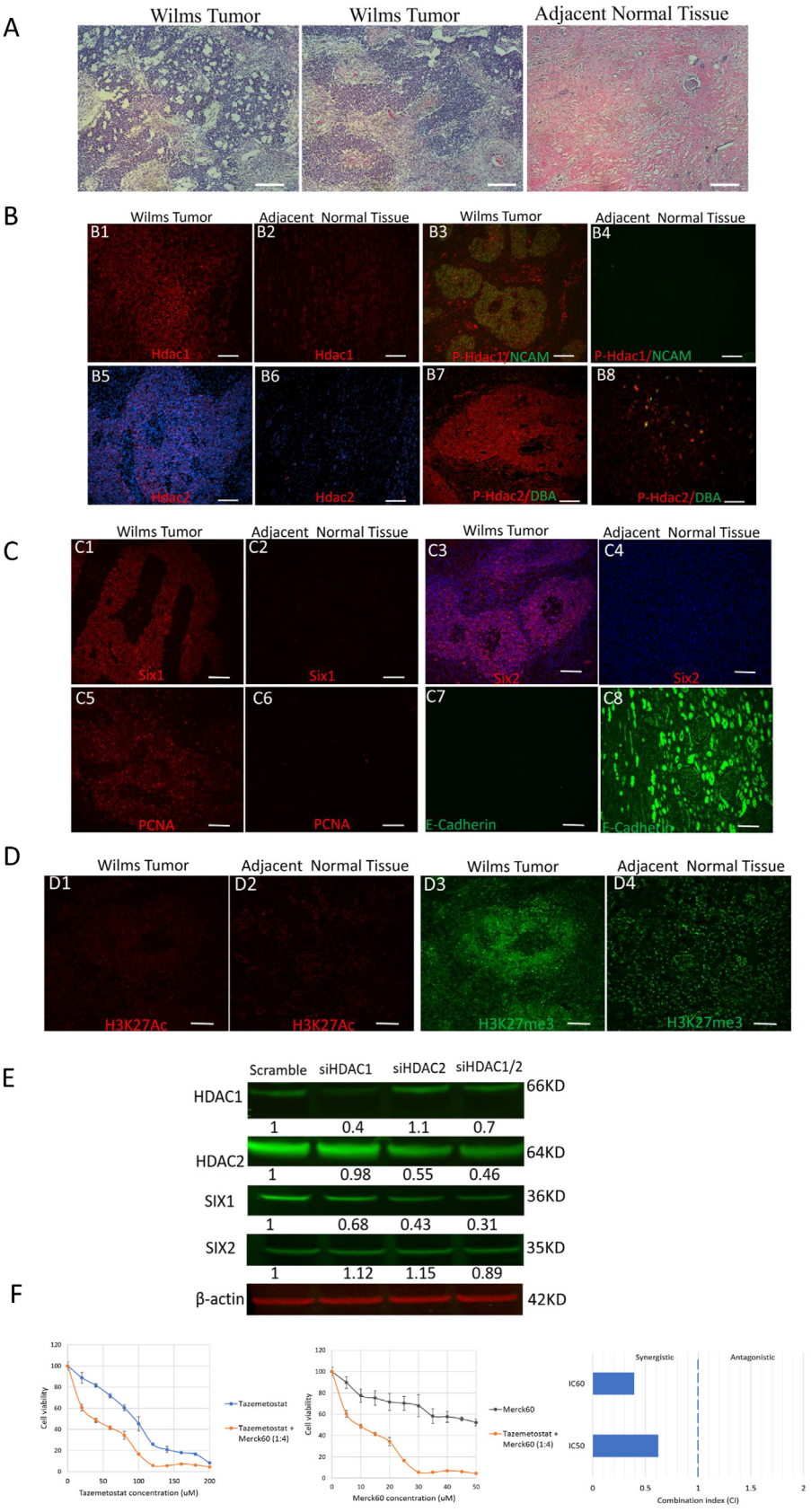
A
Wilms Tumor
Wilms Tumor
Adjacent Normal Tissue
B
Wilms Tumor
Adjacent Normal Tissue
Wilms Tumor
Adjacent Normal Tissue
B1
Hdac1
B2
Hdac1
B3
P-Hdac1/NCAM
B4
P-Hdac1/NCAM
B5
Hdac2
B6
Hdac2
B7
P-Hdac2/DBA
B8
P-Hdac2/DBA
C
Wilms Tumor
Adjacent Normal Tissue
Wilms Tumor
Adjacent Normal Tissue
C1
Six1
C2
Six1
C3
Six2
C4
Six2
C5
PCNA
C6
PCNA
C7
E-Cadherin
C8
D
Wilms Tumor
Adjacent Normal Tissue
Wilms Tumor
Adjacent Normal Tissue
D1
H3K27Ac
D2
H3K27Ac
D3
H3K27me3
D4
H3K27me3
E
Scramble
siHDAC1
siHDAC2
siHDAC1/2
HDAC1
66KD
1
0.4
1.1
0.7
HDAC2
64KD
1
0.98
0.55
0.46
SIX1
36KD
1
0.68
0.43
0.31
SIX2
35KD
1
1.12
1.15
0.89
β-actin
42KD
F
Cell viability
Tazemetostat
Tazemetostat + Merck60 (1:4)
Tazemetostat concentration (uM)
Cell viability
Merck60
Tazemetostat + Merck60 (1:4)
Merck60 concentration (uM)
Synergistic
Antagonistic
IC60
IC50
Combination index (CI)

EZH2. EZH2 was reported to be highly expressed in WT and has been associated with WT progression.

We also examined the level of the sine oculis (SIX) family members in these WT tissues. The SIX family of transcription factors are key regulators for developmental processes and tumorigenesis. Members of this family control gene expression to promote cell proliferation and govern cell differentiation. SIX1/2 are the master transcription factors especially expressed in the human nephron progenitors and play a critical role in balancing the self-renewal and differentiation of progenitor cells.[4] Persistent SIX2 in human WT cells shifts the balance from a differentiation path toward a cell proliferation pathway. SIX1 is predominantly expressed in blastemal cells of WT and is identified as a candidate maker for blastemal. As predicted, we detected higher levels of SIX1 and SIX2. Cumulative evidence suggests that SIX1 and SIX2 drive the proliferation of the metanephric mesenchyme of developing human kidneys and have an oncogenic function in certain tumors. Consistently, IF for PCNA showed dramatically increased cell proliferation in tumor tissue (Fig. 1C5, 6). E-cadherin is a tumor suppressor protein, and the loss of its expression in association with the epithelial–mesenchymal transition (EMT) occurs frequently during tumorigenesis. Interestingly, E-cadherin was drastically reduced in the tumor compared to its adjacent normal tissues (Fig. 1C7, 8).

To study the regulation of SIX1/2 expression by HDAC1/2, we did HDAC1/2 knockdown in human embryonic kidney 293 (HEK 293) cells. The cells were transfected with siRNA-HDAC1 and/or siRNA-HDAC2, and HDAC1/2, SIX1/2 level was evaluated by WB analysis and quantified against the endogenous β-actin level. As a result (Fig. 1E), the knockdown of HDAC1/2 together significantly downregulated the expression of SIX1 and slightly downregulated the expression of SIX2, indicating the positive regulation of SIX1/SIX2 by HDAC1/2. Of note, in humans, SIX1 is the direct target of SIX2.[4] The more marked downregulation of SIX1 may partially result from impaired SIX2 activity after HDAC1/2 knockdown. A previous study showed that *SIX1* and *SIX2*, which encode transcription factors with non-redundant roles in renal development, are responsible for Wilms tumorigenesis.[5] Furthermore, SIX1 and SIX2 are associated with the high-risk blastemal subtype and with the presence of undifferentiated blastema in WT. Our study in the mouse model also revealed that the deletion of *Hdac1/2* resulted in the deletion of the progenitor pool and downregulation of key progenitor genes that are mutated in WT, such as *Six2* and *Wt1*.[2] We also demonstrated that Ezh2 is the dominant H3K27 methyltransferase in $Six2^{+}$ NPCs and is required for NPC proliferation.[3] Based on these findings, we reason that SIX1/2 upregulation by HDAC1/2 and EZH2 overactivation may play an important role in Wilms tumorigenesis.

Studies have demonstrated that EZH2 interacts with HDAC1/2 and that HDACs are required for the transcriptional repression by EZH2. Concomitant inhibition of HDACs and EZH2 has proven highly synergistic and very potent for the treatment of many types of human cancers. To develop a potentially effective epigenetic therapy for WT, we examined the effects of co-treatment with benzamide-based HDAC1/2-selective inhibitor Merck 60 and FDA-approved EZH2 inhibitor Tazemetostat in G401 cells. The line was utilized for studies of chromosomal changes in WT and formerly classified as a WT cell line. Due to a change in the classification, the cell line was found to be more appropriately classified as derived from a rhabdoid tumor of the kidney. Our result showed that the co-treatment synergistically suppresses the proliferation of G401 kidney cancer cells (Fig. 1F).

In summary, our studies show the elevated expression and activity of HDAC1/2 and EZH2 in WT samples, positive regulation of SIX1/SIX2 by HDAC1/2 during tumorigenesis, and synergic effect of HDAC1/2 and EZH2 inhibition in suppressing tumor cell proliferation. Thus, specifically co-targeting HDAC1/2 and EZH2 may provide a promising therapeutic approach to treat WT and other kidney cancer with low toxicity and low side effects. Epigenetic therapies have been proven to be effective in well-defined clinical contexts. The availability of epigenetic drugs will facilitate the translation of this research into promising therapies.

**Figure 1** Overactivation of histone deacetylases and EZH2 in Wilms tumor and cell proliferation. **(A)** Histological morphology of Wilms tumor (right two panels) and the adjacent normal tissues (left panel), revealing the tumor's biphasic pattern with significant blastemal component admixed with the stromal component. Scale bar = 100 μm. **(B)** Immunostaining revealed the significantly elevated expression and activity of HDAC1 and HDAC2 in Wilms tumor compared with adjacent normal tissues. NCAM, a critical cell surface marker of a putative WT stem cell line, was also highly expressed in the tumor. Scale bar = 100 μm. **(C)** Higher expression of SIX1 and SIX2 in Wilms tumor. PCNA staining also demonstrated the dramatically increased cell proliferation of tumor tissue. Scale bar = 100 μm. **(D)** Loss of H3K27Ac and gain of H3K27Me2/3 in Wilms tumor compared with adjacent normal tissues. Scale bar = 100 μm. **(E)** Western blotting showed the HDAC1/2 knockdown downregulated SIX1/2 expression in HEK293 cells. The number below the protein band is the relative band intensity normalized with the band intensity of β-actin. **(F)** Merck 60 and Tazemetostat demonstrated synergistic growth-suppressive effect in G401 kidney cancer cells. Cells were plated in 96-well plates and treated with Tazemetostat or Merck60 alone or both at the ratio of 1:4 for 72 h. Cell viability was determined using an MTT assay. The data represent the mean of three independent experiments ± standard error of the mean. The presented CI values indicate the interaction of Merck60 with Tazemetostat when the combined treatment inhibits cell growth by 50% or 60%, compared to the control for their treatment alone.

## Author contributions

Hongbing Liu designed the study, performed the experiments and data analysis, and wrote the manuscript. Chao Hui Chen and Nguyen Yen Nhi Ngo performed the experiments and data analysis. Alun Wang and Samir El-Dahr performed data analysis. All authors approved the final version of the manuscript.

## Conflict of interests

All authors declare no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.10.026.

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