
Tumour suppressive function of HUWE1 in thyroid cancer

WEIYUAN MA, PENGXIN ZHAO, LEILEI ZANG, KAILI ZHANG, HAIYING LIAO* and ZHIGANG HU*

The Second Hospital of Hebei Medical University, Shijiazhuang 050000 Hebei Province, China

**Corresponding author (Emails, ZH– zhiganghug@sina.com; HL– 18731103921@163.com)*

HUWE1 (the HECT, UBA, and WWE domain-containing protein 1) is an ubiquitin E3 ligase which plays an important role in coordinating diverse cellular processes. It has been found to be dysregulated in various cancer type and its functions in tumorigenesis remain controversial. The potential tumour suppressive role of HUWE1 in thyroid cancer development was investigated by knocking down HUWE1 in three authentic thyroid cancer cell lines, WRO, FTC133 and BCPAP, followed by various functional assays, including cell proliferation, scratch wound healing and invasion assays. Xenograft experiment was performed to examine *in vivo* tumour suppressive properties of HUWE1. Small-interfering RNA mediated knockdown of HUWE1 promoted cell proliferation, cell migration and invasion in thyroid cancer cells. Overexpression of HUWE1 conferred partial sensitivity to chemo drugs interfering with DNA replication in these cells. Moreover, HUWE1 was found to be down-regulated in human thyroid cancer tissues compared with matched normal thyroid tissues. In addition, overexpression of HUWE1 significantly inhibited tumour growth *in vivo* using xenograft mouse models. Mechanistic investigation revealed that HUWE1 can regulate p53 protein level through its stabilization. HUWE1 functions as a tumour suppressor in thyroid cancer progression, which may represent a novel therapeutic target for prevention or intervention of thyroid cancer.

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1. Introduction

Thyroid cancer is the most common human endocrine malignancy with increasing incidence worldwide. It is estimated that approximately 64,300 new cases of thyroid cancer will occur in 2016 with around 2,000 resultant death (Siegel *et al.* 2016). Different from the other cancer types, thyroid cancer is commonly diagnosed at a younger age than most other adult cancers. Nearly two thirds of thyroid cancers are found in people younger than 55 years of age and around 2% of the cases occur in children and teens. Papillary thyroid carcinomas (PTC) and follicular thyroid carcinomas (FTC) are well-differentiated thyroid cancers (WDTC) accounting for 95% of all thyroid cancer cases (Paricharttanakul *et al.* 2016). The survival rate is almost 100% when diagnosed early. However, mechanistic investigations regarding thyroid cancer tumorigenesis have been limited. Identification of critical genes involved in thyroid cancer development will

be of great significance to improve our understanding of thyroid cancer etiology.

The HECT, UBA, and WWE domain-containing protein 1 (HUWE1), also known as Mule or ARF-BP1, is a HECT (homologous to E6-AP carboxyl terminus)-domain containing ubiquitin E3 ligase. It plays an important role in coordinating diverse cellular processes such as DNA replication, DNA damage repair, transcription regulation and apoptosis (Adhikary *et al.* 2005; Zhong *et al.* 2005; Zhao *et al.* 2008; Parsons *et al.* 2009; Markkanen *et al.* 2012; Wang *et al.* 2014). The known substrates for this E3 enzyme include Cdc6 (Hall *et al.* 2007), Mcl-1 (Zhong *et al.* 2005), histones (Liu *et al.* 2005), c-Myc (Adhikary *et al.* 2005), and p53 (Chen *et al.* 2005). Due to the varying nature of its substrates, discrepant results have been reported regarding the phenotypes after HUWE1 silencing and its functions in tumorigenesis remain controversial. Recently, a functional screen using a combination of shRNA mediated knockdown

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and soft agar assay identified HUWE1 as a candidate tumour suppressor in partially transformed human colonic epithelial cells harboring TP53 knockdown and it has been shown to be mutated in these pre-malignant cells (Zhang *et al.* 2013; Di *et al.* 2015). However, its role in tumorigenesis has not been investigated in thyroid cancer.

In this study, we investigated the potential role of HUWE1 in thyroid cancer tumorigenesis and we found that down-regulation of HUWE1 promoted cell proliferation, cell migration and invasion. Importantly, overexpression of HUWE1 inhibited tumour growth in a xenograft mouse model. Moreover, expression level of HUWE1 was down-regulated in thyroid cancer tissues and associated with patient survival.

2. Methods

2.1 Cell culture and treatment

Human thyroid cancer cell lines (WRO, FTC133, BCPAP, ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% serum at 37°C and 5% CO₂. Cycloheximide (CHX) (Sigma, St Louis, MO) was used at the final concentration of 100 µg/ml. All the cancer samples and normal tissues were retrieved from The Second Hospital of Hebei Medical University. All patients agreed with our data utilization, and they also signed informed, written consent forms. This study was approved by the ethics committee of The Second Hospital of Hebei Medical University. Thyroid cancer tissue samples and matched non-tumour adjacent tissues (NATs) were obtained from patients who underwent surgical resection at The Second Hospital of Hebei Medical University, between March 2012 and October 2014 and were diagnosed with thyroid cancer based on histopathological evaluation. All tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until use. In addition, the patients with any other tumour were excluded from the study. A total of 50 pairs of patients were examined in the study. None of the subjects had received any therapeutic procedures prior to this study, including surgery, chemotherapy, and radiotherapy. Overall survival was defined as the time from cancer onset until death or by censoring at the last follow-up date.

2.2 Plasmids and transfections

pLenti-C-Myc-DDK HUWE1 cDNA, pCMV6-XL5 TP53 cDNA and 3 individual siRNAs targeting HUWE1 were obtained from Origene and siRNA sequences were provided in supplementary table 1. siRNAs were transfected with Lipofectamine RNAi Max reagent (Invitrogen, Grand Island, NY, USA) as per manufacturer's protocol. cDNA

transfections were performed with Lipofectamine LTX reagent (Invitrogen) as per manufacturer's protocol.

2.3 Viral transductions and stable selections

For lentivirus production, 1 µg of pLenti-C-Myc-DDK HUWE1 plasmid together with 1 µg of helper plasmids (0.4 µg pMD2G and 0.6 µg psPAX2) were transfected into 293FT cells with Effectene reagent (Qiagen, Valencia, CA, USA). Viral supernatants were collected 48 h after transfections and cleared through a 0.45-µm filter. Cells were infected with viral supernatants containing 4 µg/mL polybrene (Sigma) and selected with puromycin for 7 days.

2.4 Immunoblot analysis

Total cell lysates were prepared by harvesting cells in Laemmli S.D.S reducing buffer (50 mM Tris-HCl (pH 6.8), 2% S.D.S, and 10% glycerol), boiled and resolved on an 8% to 10% polyacrylamide gel, and transferred to polyvinylidene difluoride. Antibodies against HUWE1 (Abcam), H2AX, gH2AX, c-Myc and β-actin (Sigma) were used. The blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit (HUWE1) or anti-mouse (β-actin) IgG (Santa Cruz Biotechnology) at a dilution of 1:5000 and detected with SuperSignalWest Pico or Femto Chemiluminescent Substrate Kit (Thermo Scientific).

2.5 Luciferase assay

The fragments containing the p53-RE of the fragments containing the p53-REs of p21 and Bax genes were amplified from human genomic DNA and cloned upstream of the reporter luciferase gene in the pGL-3 Basic plasmid (Promega). WRO cells were co-transfected with these constructs plus pRLSV40 (Promega), followed by dual luciferase assay (Promega).

2.6 Proliferation rate assay

Cells were seeded at a density of 10,000 cells/well in a 6-well plate in triplicate on day 0. Cells were trypsinized and counted on day 1, 3, 5 and 7. Each experiment was performed twice using cells from different cell suspension.

2.7 Wound healing Assay

Cells were grown in normal growth media to monolayer confluence in 6-well tissue culture plates. They were then treated with mitomycin C (10 µg/mL) (Sigma, St. Louis, MO, USA) for 2 hours prior to scratching with a 1 mL

pipette tip. A perpendicular pen mark on the tissue culture plate was used to mark the place for repeated imaging with an EVOS microscope (AMG/Life Technologies, Grand Prairie, TX, USA).

2.8 Invasion assays

10^5 cells were serum-starved overnight, suspended in basal medium and plated onto 8.0- μm pore transwell PET membranes (BD Biosciences, Bedford, MA). Five hundred microliters of medium containing 10% serum was added to the bottom well. Nonmigratory cells were scraped off 24 hours later, and migratory cells were stained with 6% glutaraldehyde/0.5% crystal violet solution. Experiments were performed in triplicate transwells for biological replicates and quantified by averaging the number of stained cells per $4\times$ field of view counting five fields per chamber.

2.9 Xenograft experiments

All animal experiments were approved by Institutional Animal Care and Use Committee of National Cancer Center. The protocol was approved by the Committee on the Ethics of Animal Experiments of The Second Hospital of Hebei Medical University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. WRO cells expressing vector control or HUWE1 (3×10^6 cells/injection) were subcutaneously injected into both flanks of 10 weeks old female nude mice ($n = 5/\text{group}$). Tumour volumes were measured using caliper and determined by a formula [volume = (length \times width 2)/2] from day 6 to day 18 post implantation. The results were expressed as mean tumour volumes with SD.

2.10 Total RNA extraction

Tissue sections were minced with scissors into small fragments (1-2 mm^3) and homogenized with TRIzolTM reagent (Takara Bio, Inc., Otsu, Japan). Chloroform (200 μl ; Sigma-Aldrich, Santa Clara, CA, USA) was added to the TRIzol homogenate. The preparations were then centrifuged at 12,000g for 15 min at 4°C, and the upper aqueous layer was transferred to a clean Eppendorf tube, containing an equal volume of isopropanol (Sigma-Aldrich). The mixed suspensions were centrifuged at 12,000g for a further 15 min at 4°C. The precipitations were then collected. After washing with 70% ethanol, total RNA was dissolved in RNase-free water and the quality of RNA was evaluated by gel electrophoresis. RNA concentrations were measured by optical density (260 nm, Q5000, Quawell, San Jose, CA, USA) and the preparations stored at -80°C for subsequent analysis.

2.11 RT-qPCR analysis

cDNA was reverse transcribed on the Bio-Rad S1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using oligo (dT) as primers. Briefly, the total RNA (1 μg) from each sample was reverse transcribed in a 20 μL reaction volume, containing 0.5 μg of oligo (dT) and 200 U M-MLV (MBI Fermentas, Vilnius, Lithuania). All samples were amplified in triplicate under the following conditions: 95°C for 2 min, 35 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 20 s. qPCR reaction was performed on the Bio-Rad C1000 Real-Time Fluorescence Thermal Cycler (Bio-Rad Laboratories), using the following cycling conditions: Initiation at 95°C for 10 min; amplification for 35 cycles, with denaturation at 95°C for 30 s; annealing at 56°C for 30 s; and elongation at 72°C for 30 s. A final extension at 72°C was performed for 10 min. GAPDH mRNA level was used for normalization. Log(T/N) greater than 0 is considered HUWE1 high expression whereas smaller than 0 is considered low expression.

2.12 Statistical analysis

Quantitative data are expressed as mean \pm SD. Statistical significance was assessed by the Student's *t*-test. Differences were considered to be significant when $P < 0.05$.

3. Results

3.1 Knockdown of HUWE1 promoted cell proliferation in thyroid cancer cells

To assess the potential role of HUWE1 in thyroid tumorigenesis, we first investigated the effect of HUWE1 on cell proliferation. Transfection of small-interfering RNA (siRNAs) targeting HUWE1 led to efficient knockdown of HUWE1 in WRO, FTC133 and BCPAP cells with siRNA-2 giving the best knockdown efficiency (figure 1A, C&E, supplementary figure 1). Growth rates were monitored in these three cell lines transfected with either non silencing siRNA or HUWE1 siRNA. As shown in figure 1B, D&F, transient knockdown of HUWE1 significantly increased cell growth rate in all three cell lines. On the contrary, overexpression of HUWE1 reduced cell growth rate in WRO cell line (supplementary figure 2). A recent study reported that inhibition of HUWE1 expression can lead to stabilization of H2AX, which is required for active cell growth and efficient DNA damage response (Atsumi *et al.* 2015). Consistently, we observed an up-regulation of H2AX as well as gH2AX expression upon transient knockdown of HUWE1, which may contribute to the increased proliferation in the knockdown cells (supplementary figure 3).

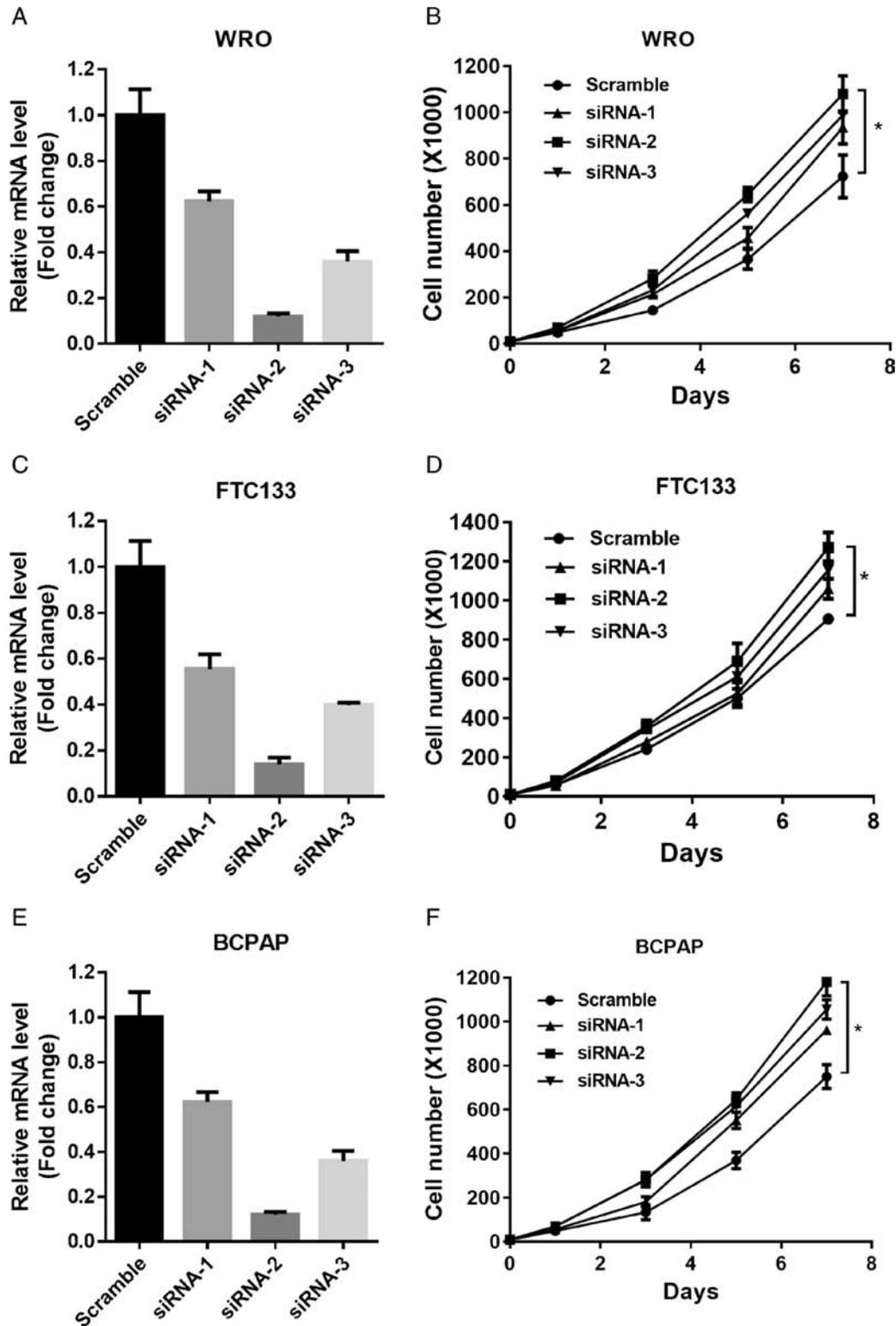


Figure 1. Knockdown of HUWE1 promotes cell proliferation in thyroid cancer cells. (A, C & E) Silencing of HUWE1 in WRO (A), FTC133 (C) and BCPAP (E) cell lines by siRNAs targeting HUWE1 was confirmed by quantitative real-time PCR. (B, D & F) Growth curves of WRO (B), FTC133 (D) and BCPAP (F) with transfection of HUWE1 siRNAs. The bars represent the mean values of triplicates (mean \pm SD). *, $P < 0.05$.

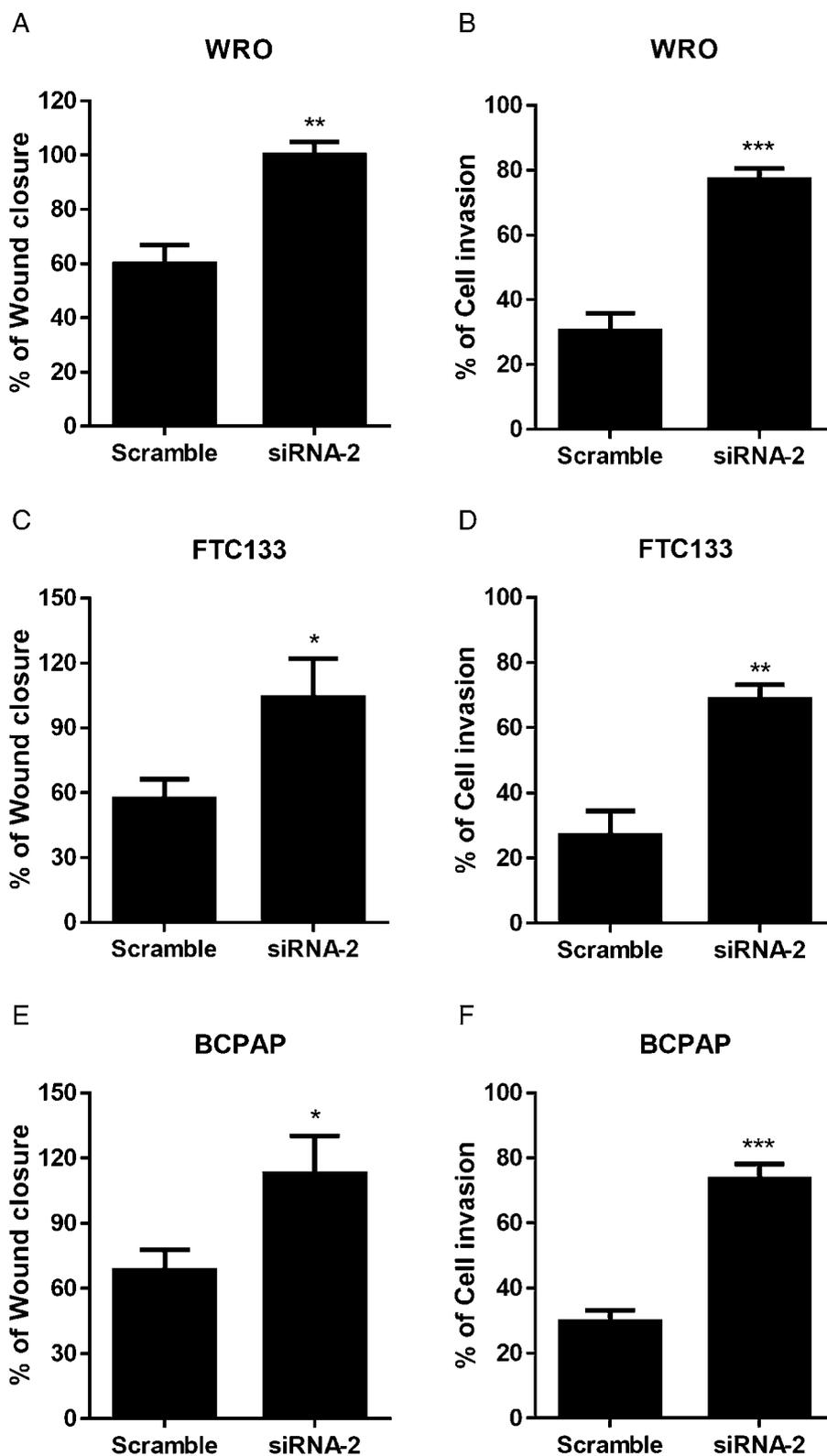


Figure 2. Stable knockdown of HUWE1 promotes cell migration and invasion in thyroid cancer cells. (A, C & E) Knockdown of HUWE1 enhanced cell migration of thyroid cancer cells. A wound healing assay was performed in WRO (A), FTC133 (C) and BCPAP (E) cell lines transfected with siRNA targeting HUWE1. (A, C & E) Knockdown of HUWE1 enhanced invasion through Matrigel® in thyroid cancer cells. An invasion assay was performed in WRO (B), FTC133 (D) and BCPAP (F) cell lines transfected with siRNA targeting HUWE1. The bars represent the mean values of triplicates (mean ± SD). *, $P < 0.05$, **, $P < 0.01$, *** $P < 0.001$.

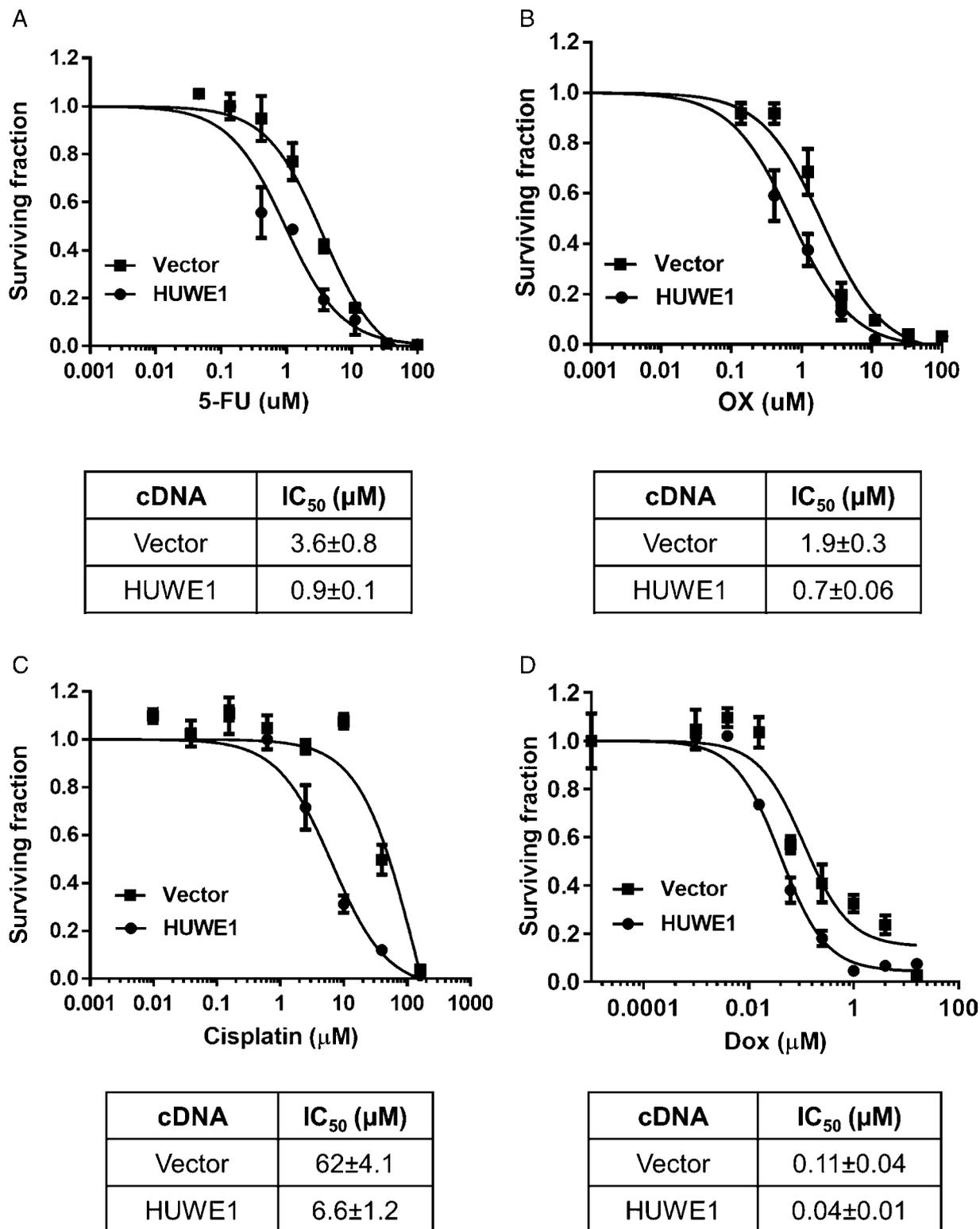


Figure 3. Overexpression of HUWE1 sensitized WRO cells to a subset of chemotherapeutic agents. Dose response curves of 5-FU (A), OX (B), Cisplatin (C), and Dox (D) in WRO cells expressing vector or HUWE1 cDNA. Data represent mean \pm SD, $n=3$. 5-FU: 5-fluouracil; OX: oxaliplatin; Dox; doxorubicin.

3.2 Knockdown of HUWE1 enhanced cell migration and invasion in thyroid cancer cells

We next examined the functions of HUWE1 in regulating migration and invasion in thyroid cancer cells. Wound healing assay and invasion assay were performed in WRO, FTC133 and BCPAP cells expressing non silencing siRNA or HUWE1 siRNA. Consistently, transient knockdown of HUWE1 resulted in significant enhancement in both cell migration and invasion through Matrigel® (figure 2), supporting the effects of HUWE1 on transformative properties of thyroid cancer cells. Since a previous study revealed that HUWE1 suppresses tumorigenesis by preventing an accumulation of c-Myc/Miz1 complexes (Inoue *et al.* 2013), we examined the effect of HUWE1 knockdown on c-Myc expression in WRO cells. As shown in supplementary figure 4, c-Myc was up-regulated in HUWE1 knockdown cells, which may contribute to the enhanced cell migration and invasion.

3.3 Overexpression of HUWE1 conferred sensitivity to chemotherapeutic drugs in thyroid cancer cells

Since HUWE1 has been reported to regulate protein level of several proteins involved in cell proliferation and DNA damage response, we investigated whether HUWE1 plays a role in chemosensitivity. Interestingly, we observed that ectopic expression of HUWE1 sensitized WRO cells to 5-fluouracil (5-FU), oxaliplatin (OX), cisplatin, doxorubicin (Dox) (figure 3), suggesting that chemo drugs interfering with DNA replication may exhibit synergistic effects with HUWE1 overexpression.

3.4 Ectopic expression of HUWE1 suppressed tumour growth *in vivo* and it was down-regulated in thyroid cancer patient samples

To investigate the tumour suppressive role of HUWE1 *in vivo*, we injected stable HUWE1 overexpressing WRO or control cells into nude mice and monitored tumour growth. As shown in figure 4A&B, the growth of tumours derived from HUWE1 overexpressing cells was significantly suppressed and the tumour weights were reduced in HUWE1 overexpressing xenografts. To investigate the clinical relevance of HUWE1 in thyroid cancer, we examined the HUWE1 expression level in human patient samples. This study was approved by the ethics committee of The Second Hospital of Hebei Medical University. Quantitative PCR analysis showed that expression level of HUWE1 was significantly down-regulated in thyroid cancer tissues compared with their NATs. Importantly, Kaplan-Meier curve analysis showed that patients with high HUWE1 expression had

better survival, suggesting that HUWE1 may serve as a potential prognostic biomarker in thyroid cancer (figure 4D).

3.5 HUWE1 exerted its tumour suppressive effects partially through regulation of p53 stabilization

Since several HUWE1 proteins have been shown mediate p53 protein level and its signaling pathways in B cell (Hao *et al.* 2012), we hypothesized that HUWE1 may exert its tumour suppressive effects through regulation of p53 protein in thyroid cancer cells. We first examined the effects of HUWE1 on p53 protein expression level. As shown in figure 5A, knockdown of HUWE1 increased MDM2 and reduced p53 protein level whereas overexpression of HUWE1 down-regulated MDM2 and up-regulated p53 in WRO cells. Additionally, HUWE1 overexpression increased p53 half-life from ~30 minutes to ~90 minutes (figure 5B). Correspondingly, we observed induction of transcriptional activity of two p53 downstream targets, p21 and Bax (figure 5C&D). Moreover, overexpression of p53 rescued the reduced cell proliferation rate induced by knockdown of HUWE1 (figure 5E&F). Taken together, these results suggest that growth inhibitory effects of HUWE1 on thyroid cancer cells may be partially due to its regulation of p53 protein stabilization.

4. Discussion

There are various types of thyroid carcinomas and the most frequent ones are papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), anaplastic thyroid carcinoma (ATC) and an intermediate form between PTC/FTC and ATC, the poorly differentiated thyroid cancer (Catalano *et al.* 2010). The genetic alterations most commonly found in PTC are BRAF point mutations which are present in 40-60% of the cases, and RET/PTC rearrangements accounting for about 20% of the cases (Xing 2010). For FTC, the genetic changes most often observed are RAS point mutations (about 45% of the cases) and PAX8/PPAR γ rearrangements (about 35% of the cases) (Saiselet *et al.* 2012). However, it is not clear what other genes are implicated in thyroid tumorigenesis.

Accumulating evidence suggest that HECT-type E3s have oncogenic functions due to a number of tumour suppressor molecules among their protein substrates. Genetic aberrations and altered expression patterns of some of the family members have also been observed in a number of human cancers. For example, WWP1 (WW domain-containing protein 1), an HECT E3 family member, has been found to be amplified and overexpressed in prostate and breast cancer via its regulation of multiple substrates, including, p53, Smad2, Notch1, etc. (Chen *et al.* 2007a; Chen *et al.*

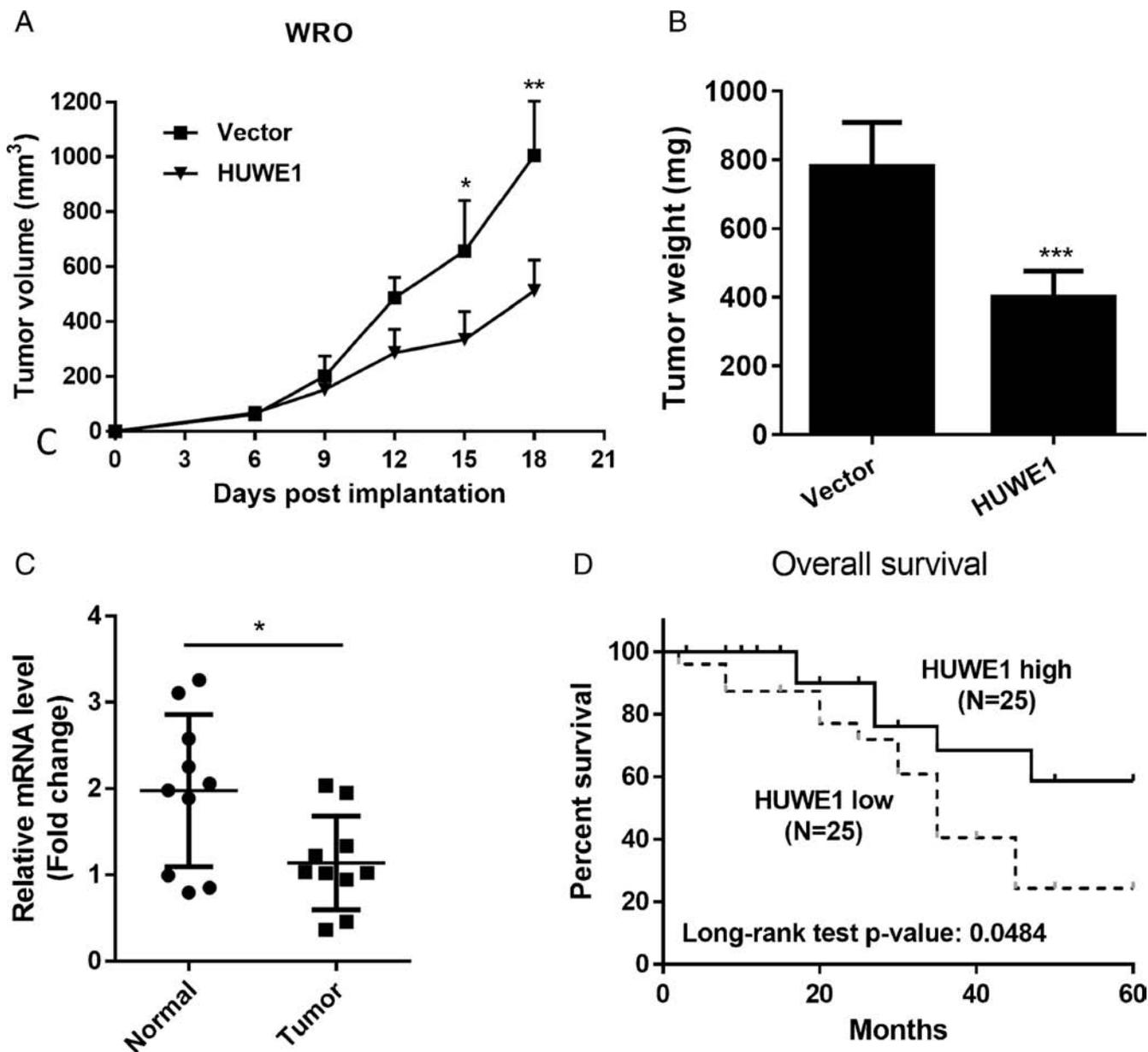


Figure 4. HUWE1 was down-regulated in thyroid carcinoma and ectopic expression of HUWE1 inhibited tumour growth *in vivo*. (A) Inhibitory effect of HUWE1 on tumour growth *in vivo*. WRO cells stably expressing vector ($n = 5$) or HUWE1 ($n = 5$) were subcutaneously implanted into nude mice. Tumour volumes (mean \pm SD) were obtained from 5 mice for each group and plotted. *, $P < 0.05$, **, $P < 0.01$ versus the vector control. (B) Tumour weight of xenografts expressing vector only or HUWE1 cDNA. The bars represent the mean \pm SD of 8 tumours. *** $P < 0.001$. (C) Quantitative real-time PCR analysis of the HUWE1 mRNA levels in ten paired human thyroid cancer and adjacent normal tissues. HUWE1 mRNA level was normalized to the GAPDH (mean \pm SD). *, $P < 0.05$ versus normal control. (D) Overall survival curves of thyroid cancer patients with low or high expression of HUWE1.

2007b). HUWE1 is a member of this family and is found to be overexpressed in lung, breast, colon, prostate, liver, pancreas, and thyroid cancers but down-regulated in stomach and uterine cancer as well as glioblastomas (Adhikary *et al.* 2005; Confalonieri *et al.* 2009; Zhao *et al.* 2009). Both oncogenic and tumour suppressive roles have been attributed

to this protein because its substrates include key proteins involved in tumorigenesis process such as p53 and MYC (Adhikary *et al.* 2005 and Chen *et al.* 2005), which regulate diverse cellular responses including proliferation and survival with opposite effects. An *in vivo* study revealed that HUWE1 suppressed Ras-driven tumorigenesis by preventing

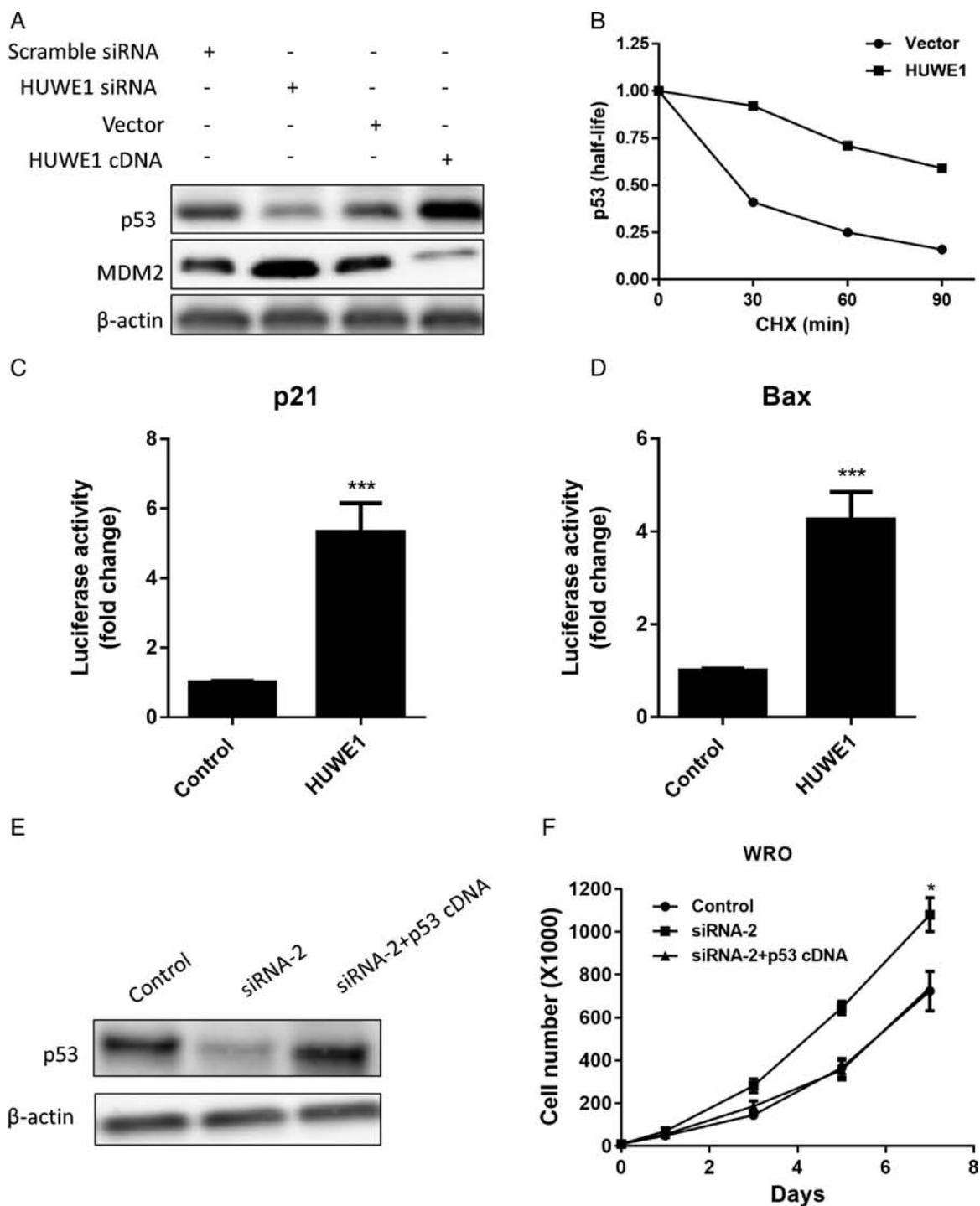


Figure 5. HUWE1 exerts its tumour suppressive effects partially through regulation of p53 stabilization. (A) Western Blot analysis of p53 and MDM2 protein level in WRO cells transfected with HUWE1 siRNA or stably expressing HUWE1 cDNA. β -actin was used as an loading control. (B) Half-life of p53 in WRO cells transfected with control or HUWE1 cDNA. Cycloheximide (CHX) was treated for the indicated time. (C & D) Luciferase assay. WRO cells were co-transfected with control vector or HUWE1 cDNA with pGL3-basic-luciferase reporter constructs containing p21(C) or Bax (D) p53REs. Transfection efficacy was normalized to renilla luciferase activity. The bars represent the mean values of triplicates (mean \pm SD). ***, $P < 0.001$. (E) Western Blot analysis of p53 protein level in WRO cells transfected with HUWE1 siRNA with or without transient overexpression of HUWE1. β -actin was used as an loading control. (F) Growth curves of WRO cells with transfection of HUWE1 siRNA-2 together with p53 cDNA. The bars represent the mean values of triplicates (mean \pm SD). *, $P < 0.05$.

an accumulation of c-Myc/Miz1 complexes that mediated p21 and p15 down-regulation (Inoue *et al.* 2013). However, the potential role of HUWE1 in thyroid cancer cells has not been well established. In this study, we observed that down-regulation of HUWE1 conferred growth advantage and tumorigenic properties in terms of cell proliferative, cell migratory and invasive capability in thyroid cancer cells, partially due to stabilization of H2AX and up-regulation of c-Myc protein (figure 1 and 2, supplementary figure 3 and 4). Additionally, overexpression of HUWE1 reduced tumour growth compared with control cells in a xenograft mouse model (figure 4A&B). Moreover, HUWE1 was found to be down-regulated in thyroid cancer patient samples and its down-regulation was associated with worse survival (figure 4C&D). Taken together, these results support that HUWE1 plays a tumour suppressive role in thyroid cancer.

Numerous evidence support that HUWE1 is implicated in tumorigenesis via its diverse functions in various aspects of tumorigenesis processes. HUWE1 functions in cell cycle progression and apoptosis primarily through its regulation of p53 (a proapoptotic protein) and Mcl-1 protein (an anti-apoptotic protein) expression (Bernassola *et al.* 2008). Ectopic expression of the HECT domain of HUWE1 has been reported to reduce the protein level and transcriptional activity of the p53 tumour suppressor via its HECT domain (Yoon *et al.* 2005). Importantly, it has been shown that HUWE1 plays a critical role in mediating both the p53-independent and p53-dependent functions of ARF tumour suppressor (Chen *et al.* 2005). HUWE1 also plays an important role in regulation of base excision repair (BER) proteins and maintenance of genome stability through its regulation of Pol β , the central component of the BER pathway (Parsons *et al.* 2009). In this study, we found that overexpression of HUWE1 sensitized thyroid cancer cells to chemo drugs interfering with DNA replication (figure 3). Importantly, our results showed that knockdown of HUWE1 led to down-regulation of p53 whereas overexpression of HUWE1 resulted in up-regulation of p53 with concomitant induction of transcriptional activity of downstream target genes, p21 and Bax (figure 5A-D). This stabilization of p53 protein may be partially due to down-regulation of MDM2, which is a well-established p53-specific E3 ubiquitin ligase protein. Overexpression of p53 reversed the growth inhibitory effects of HUWE1 knockdown (figure 5E&F). Interestingly, we also observed an up-regulation of H2AX as well as gH2AX expression in HUWE1 knockdown cells (Supplementary Figure S3). Therefore, HUWE1 may exert its tumour suppressive function and chemo sensitizing effects partially by regulating MDM2-p53 axis.

In conclusion, we have established the tumour suppressive role of HUWE1 in thyroid cancer. This function is exerted partially through regulation of p53 protein stability. Identifying and understanding the function of novel tumour

suppressors will provide better insight into the molecular mechanism underlying thyroid cancer development and these proteins may represent novel therapeutic targets for thyroid cancer management.

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