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Original Paper

WT1 Enhances Proliferation and Impedes Apoptosis in *KRAS* Mutant NSCLC via Targeting *cMyc*

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Key Words
KRAS mutation • *WT1* • *cMyc* • NSCLC • Apoptosis

Abstract
Background: A novel link between oncogenic *KRAS* signalling and *WT1* was recently identified. We sought to investigate the role of *WT1* and *KRAS* in proliferation and apoptosis. ***Methods:*** *KRAS* mutations and *WT1* (*cMyc*) expression were detected using Sanger sequencing and real-time PCR in 77 patients with non-small cell lung cancer (NSCLC). Overexpression and knockdown of WT1 were generated with plasmid and siRNA via transient transfection technology in H1299 and H1568 cells. MTT assay for detection of cell proliferation, and TUNEL assay and proteomic profiler assay for apoptosis evaluation were carried out. Dual luciferase reporter assay and ChIP-PCR were performed to validate the effect of WT1 on the cMyc promoter. ***Results:*** *KRAS* mutations showed a negative impact on overall survival (OS). High expressions of *WT1* and *cMyc* were associated with poor OS in *KRAS* mutant subgroup. The potential mechanisms that WT1 promotes proliferation and impedes apoptosis through affecting multiple apoptosis-related regulators in *KRAS* mutant NSCLC cells were identified. WT1 could activate *cMyc* promoter directly in *KRAS* mutant cells. ***Conclusion:*** The results suggest that *WT1* and *c-MYC* expression is important for survival in *KRAS* mutant tumors as opposed to *KRAS* wild-type tumors. For treatment of *KRAS* mutant NSCLC, targeting *WT1* and *cMyc* may provide alternative therapeutic strategies.

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Introduction

In lung cancer patients, five-year survival rates still remain low, approximately 10-15% [1]. Remarkable effort has been made to improve the outcome of these patients and promising results have been achieved using target therapies direct against tumor's driver mutations. Indeed, patients harboring mutations of the epidermal growth factor receptor (EGFR) benefit from treatment with tyrosine kinase inhibitors (TKIs) targeting EGFR [2-4]. Since *KRAS* is a downstream target of EGFR, increasing clinical interest has been shown in *KRAS* mutation as a predictive biomarker for EGFR-targeted therapies. *KRAS* mutations are present in 15–30% of NSCLC [5, 6]. Recent clinical trials have demonstrated that *KRAS* mutant tumors respond poorly to EGFR inhibitor (erlotinib or gefitinib) being a predictor of innate resistance [7-9].

EGFR functions as an upstream activator of RAS. Cell proliferation and survival are regulated by RAS through several pathways including MAPK, STAT and PI3K signaling cascades [10-12]. The Wilms' tumor gene (*WT1*) was recently found as a key regulator of the genetic network of oncogenic *KRAS* [13]. However, the precise mechanisms for mutant *KRAS*-related signaling pathways remain elusive.

At least 24 isoforms of WT1 are generated by a combination of alternative translation start sites, alternative RNA splicing and RNA editing. Exons 5 (a 17-amino-acid insertion) and 9 (inclusion or exclusion of three amino acids, lysine, threonine and serine (KTS)) are subjected to alternative splicing, generating four different major splice isoforms designated as A (-/-), B (+/-), C (-/+) and D (+/+) [14]. These four isoforms are also the best studied. Initially, *WT1* was described as a tumor suppressor gene in Wilms' tumor [15]. In clear cell renal carcinoma (ccRCC), we have demonstrated that *WT1* can act as a tumor suppressor via multiple pathways leading to downregulation of *hTERT* [16]. Moreover, WT1 was found to bind directly the promoters of the downstream targets *hTERT, cMyc* and *SMAD3* promoters [16]. Accumulating evidence have demonstrated the function of *WT1* as an oncogene in other types of cancers including leukemia and breast cancer (reviewed in [17]). Thus, the *WT1* gene was recently proposed to act as a chameleon gene in human malignancies [18].

We have recently reported that *WT1* expression was correlated with clinical stage, metastasis, and survival rate in NSCLC patients. By *in vitro* experiments, we showed that WT1 can promote NSCLC invasion through direct binding to the *CDH1* promoter [19]. Furthermore, WT1 was found to promote NSCLC cell proliferation by upregulating Cyclin D1 and p-pRb expression *in vitro* and *in vivo* [20] . The present study aimed to investigate the functional link between *WT1*, *KRAS* mutation and a WT1 downstream target (*cMyc*) in NSCLC. We report our evaluation of three markers in relation to clinical outcome in NSCLC and describe WT1 function in cell proliferation and apoptosis in *KRAS* mutant cells.

Materials and Methods

Patient and tissue samples

The study was performed on a total of 77 NSCLC patients, of which 62 were adenocarcinomas and the remaining 15 specimens were squamous cell carcinomas. These patients were treated at the People's Hospital of Jiangsu Province between September, 2007 and October, 2009. The specimens were collected under a protocol approved by the Human Ethics Committee of Nanjing Medical University. Each patient participated after providing informed consent. The median age of the patients was 68 years (range 54 – 86 years) and median survival time was 35.5 months (range 15.5 – 44.0 months).

A total of 154 tissue specimens were included, with one tumor sample and one corresponding adjacent sample (within 3 cm of the incised margin) for each patient. All fresh tissues were cut into two parts and one was frozen in nitrogen until DNA and RNA extraction, the other was embedded for pathological confirmation. These specimens were histologically classified according to the National Comprehensive Cancer Network (NCCN) guidelines for NSCLC [21].

DNA and RNA preparation

DNA and total RNA were prepared from fresh frozen tissues as previously described [16]. cDNA was synthesized using reverse transcriptase kit (TAKARA, Tokyo, Japan).

Real-time quantitative PCR for WT1 and cMyc RNA expression

WT1 and *cMyc* mRNA levels were measured by RQ-PCR using SYBR Premix Ex Taq (TAKARA, Tokyo, Japan). *WT1* transcription values were normalized against the expression of β-*actin* to adjust for variations in RNA and cDNA synthesis. Amplification conditions, primers and probes for *WT1* were previously described [16]. Standard curves were generated using a series of dilutions of plasmid DNA carrying the *WT1* or β-*actin* gene with copy numbers from 10^0 to 10^7. The mean of triplicates of the *WT1* gene copy numbers was divided by the mean of duplicates of copy numbers of the β-*actin* gene. Primers for *cMyc* were used as previously described [16]. Values of target gene expression were calculated with template and normalized to the β-*actin* gene.

Sequence analysis for identification of KRAS mutation

The primers spanning codon 12 and 13 in exon 2 of the *KRAS* gene to detect mutation status were used as previously described [22]. Sequence analysis was performed on ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, USA). The derived sequences were identified using GenBank (EMBL) searches (http://www.ncbi.nlm.nih.gov/genbank/).

Cell culture, WT1A and WT1D plasmid and siRNA transfection

NSCLC cell lines, H1299 (CRL-5803™) and H1568 (CRL-5876 ™, LGC Standards, Middlesex, UK) were employed in transfection experiments. Cells were cultured according to the manufacturer's instructions. The WT1 isoforms A and D have been demonstrated to induce morphological changes in ovarian cancer cells [23] and in osteosarcoma cell lines [24] acting as an oncogene. These isoforms were therefore selected in this study. pcDNA 3.1(+) vectors (Invitrogen) ligating with *WT1* variant A or D were constructed as described previously [23]. In order to induce *WT1* overexpression, cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with 2μg per well of *WT1A* or *WT1D* pcDNA 3.1(+) vectors in six-well plate (1.5 x 10^5 cells) or with 0.2μg per well of *WT1A* or *WT1D* pcDNA 3.1(+) vectors in 96-well plate (3.0 x 10^3 cells). pcDNA 3.1(+) vector without insert of *WT1A* or *WT1D* was used as control. For suppression of WT1 expression, pooled siGENOME SMART pool *WT1* siRNA (50nM, Dhamacon, Chicago, USA) was used for transfection. Cells were collected at 24, 48 and 72 hours after transfection for further analysis.

Protein extraction and western blot analysis

Total proteins were extracted using CHAPS lysis buffer. Protein expressions of WT1 and cMyc were evaluated using western blot as previously described [16]. The antibodies used were the following: WT1 (1 : 500; Dako, Glostrup, Denmark), cMyc (1 : 1000, Cell Signalling Technology Inc. Danvers, MA, USA), Bcl-2 (1 : 1000, Cell Signalling Technology Inc.), β-actin (1 : 10 000, Dako) and peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1 : 5000, Dako).

MTT assay

Vybrant MTT Cell Proliferation Assay Kit (Invitrogen) was applied to measure cell proliferation. Cells were collected at 0, 24 and 48 hours after *WT1* siRNA or plasmid transfection were labeled and incubated with MTT solution, mixed with SDS-HCL. Absorbance was read at 570nm on a spectrometer. The experiment was performed in triplicates.

Immunocytochemistry

Cytospins were prepared from culturing H1299 and H1568 cell lines and fixed in acetone for 10 minutes after air drying on slides, blocked with 0.05% PBST (0.5ml Tween-20 in 1L Phosphate buffer solution) for 20 minutes and treated with hydrogen peroxide for 10 minutes to inactivate endogenous peroxidase. The Ki-67 staining, with monoclonal antibody (1 : 200, Abcam, Cambridge, UK) supplied in its own blocking solution, was performed by a fully automated slide preparation system (Ventana Medical Systems, Arizona, USA). For each slide, 200 cells were analyzed.

Terminal deoxynucleotidyltransferas dUTP nick end labeling (TUNEL) analysis

TUNEL staining using In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics GmbH, Mannheim, Germany) was performed to detect oligonucleosomal DNA fragmentation. At 48 hours after transfection, cells were fixed in 2% paraformaldehyde. After permeabilization with 0.1% Triton X-100 in 0.1% sodium citrate, cells were stained for DNA strands breaks with TMR red. The staining was analyzed in a FACSCaliburTM flow cytometer using FL3 channel for detection of TMR red (BD Biosciences, San Jose, USA).

Proteomic profiler assay

The Proteome ProfilerTM Human Apoptotic Array Kit (R&D System, Oxford, UK) was applied to determine the relative levels of proteins or kinases related to apoptosis. The assay was performed in ChemiDoc XRS System (Bio-Rad) using Quantity One 1-D Analysis Software. 300μg of proteins were used on each membrane and spots were visualized using chemiluminescent detection system (GE Healthcare).

Luciferase reporter gene assay

The wild-type and mutant *cMyc* promoters were cloned using overlap PCR method as previously described [25]. H1299 and H1568 cells were co-transfected using Lipofectamine 2000 (Invitrogen) and the following transfection mix: 250 ng of the reporter plasmid containing promoter response elements, 3 ng of pRL-TK encoding *Renilla* luciferase cDNA and 500 ng of the pcDNA3 vector either empty as control or containing *WT1A* and *WT1D* variants. Luciferase activity was measured by a dual luciferase reporter assay system (Promega) and the transfection efficiency standardized against *Renilla* luciferase activity.

Chromatin immunoprecipitation (ChIP) analysis

ChIPs were performed using the Chromatin Immunoprecipitation Kit (Upstate Millipore, Billerica, MA, USA). Approximately 2 - 3 x 10^6 cells were cross-linked with 1% formaldehyde, followed by glycine to quench unreacted formaldehyde. Chromatin was sonicated on ice to shear cross-linked DNA to about 200 – 1 000 base pairs in length using a Sonifier ultrasonic cell disruptor (Branson, Danbury, CT, USA) with 12 × 10s pulses. The sheared chromatin was resuspended in dilution buffer and 1% of the chromatin was removed as input, followed by immunoprecipitation using protein G magnetic beads with 2 μg of either anti-WT1 (C-19) antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA,) or normal rabbit IgG (Cell Signalling Technology Inc, Danvers, MA, USA) at 4°C overnight with rotation. After the reversal of cross-links by incubation in ChIP elution buffer containing proteinase K at 62°C for 2 h, DNA was purified using spin columns.

PCR reactions containing 2 μl of the immunoprecipitated DNA or input chromatin, primers and AmpliTaq Gold (Applied Biosystems) in a 50 μl volume were performed with initial denaturation at 95°C for 10 min, followed by 35 cycles (95° for 30 s, 55°C for 30 s and 72°C for 45 s) and a final extension at 72°C for 10 min. The primer sequences for *cMyc* promoter were described previously [25]. PCR products were fractionated on 1% agarose gel, and ethidium bromide stained DNA was visualised on Ultraviolet Transilluminator (Spectroline, Westbury, NY, USA).

SKOV-3 cell line with undetectable endogenous WT1 protein was used as an additional negative control in the experiment.

Immunofluorescence

H1299 and H1568 cells were seeded at a cell density of 0.5 × 10^5 cells/cm2, grown on coverslips for 24 hours. Next, they were washed once in PBS, fixed in 4% formaldehyde, permeabilized with 2% Triton X-100, and then blocked in 5% bovine serum albumin. Incubations with anti-WT1 antibodies (1:50, DAKO) and anti-cMyc (1:100, Abcam) were performed overnight at 4°C. Then, secondary antibodies Alexa Fluor 555 and 488 (Invitrogen) were added. The preparations were mounted with coverslips in mounting medium with DAPI according to the manufacturer's recommendations. Images were captured using a NikonEFD3 microscope (Boyce Scientific, Gray Summit, MO, USA) and Nikon camera (100Eplan [160/0.17] objective, Nikon, Melville, NY, USA).

Statistical analysis

Statistical analysis was performed using SPSS (version 17, SPSS Inc., Chicago, IL, USA). The Mann-Whitney U-test was used to calculate differences in the expression of two independent variables. Fisher's

Table 1. KRAS mutations at codon 12 and 13 in 19 NSCLC patients

Codon	Mutation	Amino acid change	Number of patients
12	GGT-TGT	Gly-Cys	5
12	GGT-GAT	Gly-Asp	6
12	GGT-GTT	Gly-Val	2
12	GGT-GCT	Gly-Ala	1
13	GGT-AGT	Gly-Ser	3
13	GGC-GAT	Gly-Asp	2

Table 2. Correlation between *KRAS* mutation status and clinical and pathologic characteristics in patients with NSCLC Pathology Stage was based on NCCN (National Comprehensive Cancer Network) Guidelines™ Version 3.2011 Non-Small Cell Lung Cancer

Characteristics	All patients	KRAS mutant	KRAS wild type	p
n	77	19 (24.6%)	58 (75.3%)	
Age (years)				0.352
Median (range)	68（54-86）	71 （56-86）	68 （54-86）	
Sex				0.539
Male	62 (80.5%)	15 (19.5%)	47 (61%)	
Female	15 (19.5%)	4 (5.2%)	11 (14.3%)	
Histology				0.867
Adenocarcinoma	55 (71.4%)	15 (19.4%)	40 (52%)	
Suqamous cell carcinoma	20 (26%)	4 (5.2%)	16 (20.8%)	
Large cell carcinoma	2 (2.6%)	0 (0%)	2 (2.6%)	
Pathological stage*				0.001
IA	27 (35%)	4 (5.2%)	23 (29.8%)	
IB	26 (33.8%)	6 (7.8%)	20 (26%)	
IIA	5 (6.5%)	1 (1.3%)	4 (5.2%)	
IIB	10 (13%)	6 (7.8%)	4 (5.2%)	
IIIA	5 (6.5%)	2 (2.6%)	3 (3.9%)	
IIIB	4 (5.2%)	0 (0%)	4 (5.2%)	
WT1 RNA level (WT/β-actin x 10^{-3})				0.518
Median	3.48	3.15	3.78	
(range)	(0.02-11.51)	(0.03-7.58)	(0.02-11.51)	
cMyc RNA level (cMyc/β-actin x 10^{-3})				0.726
Median	4.09	3.51	4.66	
(range)	(0.06-23.64)	(0.17-22.62)	(0.06-23.64)	

exact tests (when cell size was < 5) were used for comparison of proportions. The χ^2 test was used to test the significance of observed differences in proportions. The Kaplan-Meier method was used to estimate the distribution of overall survival (OS). Differences in survival distribution between groups (*WT1*, *cMyc* expression level and *KRAS* mutation status) were compared using the log-rank test.

Results

Correlation of KRAS mutations with patients' clinical and pathological characteristics in NSCLC

KRAS mutation was identified in 19 of 77 tumor specimens (24.7%). Single amino acid substitutions in codon 12 were detected in 14 samples, whereas mutations in codon 13 were found in 5 samples (Table 1).

KRAS mutation status and clinical and pathological characteristics in patients were summarized in Table 2. *KRAS* mutation was less frequently detected in pathological stage I ($p = 0.001$). No significant differences could be found in patients with and without *KRAS* mutations with regard to age, sex and histology.

Overexpression of WT1 and cMyc in NSCLC

Significantly higher RNA levels of *WT1* and *cMyc* were detected in tumor specimens with *KRAS* mutations or *KRAS* wild-type than their paired adjacent tissues (Fig. 1A and B),

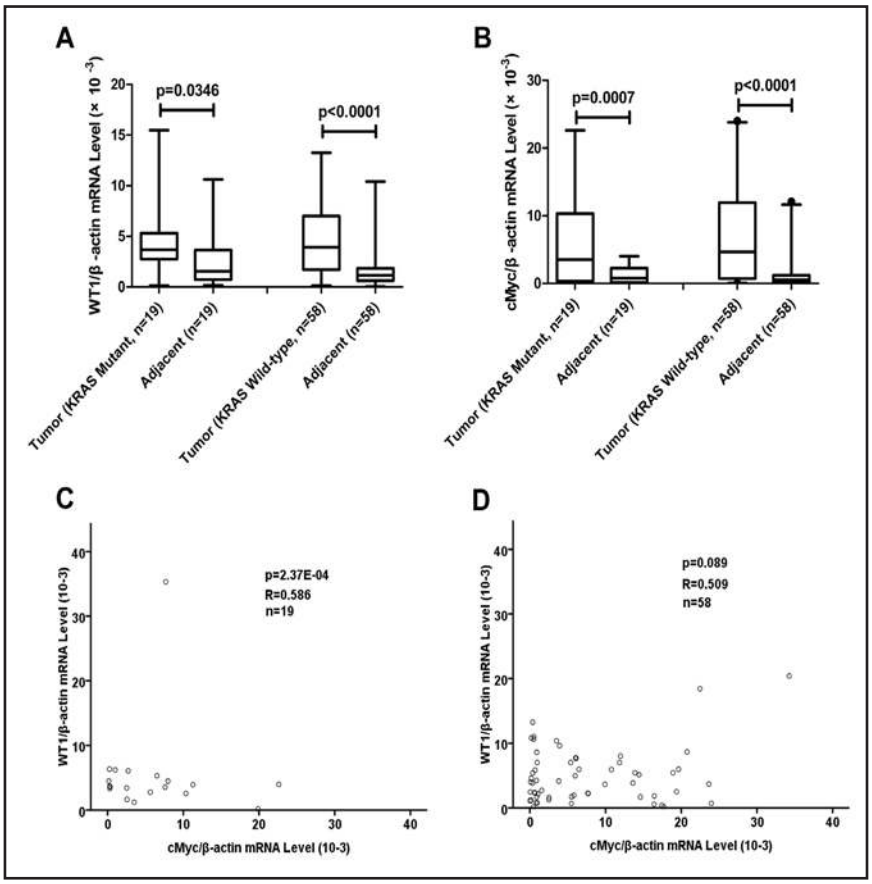


Fig. 1. Expression of *WT1* and *cMyc* transcripts and correlation between expressions of these two genes in non-small cell lung cancer (NSCLC) patients. A, RNA expression of *WT1* in paired tumour and adjacent tissue samples. B, *cMyc* RNA levels in paired tumour and adjacent tumour free tissues. C, Correlation between RNA expression of *WT1* and *cMyc* in *KRAS* mutant tumour samples. D, Correlation between RNA expression of *WT1* and *cMyc* in *KRAS* wild-type tumour samples.

indicating upregulation of both genes in NSCLC. Moreover, a significant correlation was found between *WT1* and *cMyc* in tumor samples with *KRAS* mutations as shown in Fig. 1C but only a trend in *KRAS* wild type tumors (Fig. 1D), suggesting a functional link between them may exist in *KRAS* mutant tumors. It is of note that the coefficiency was mild but it may be explained by some other factors that may affect these gene expressions because both genes act as oncogene in NSCLC in our limited sample materials.

Impact of KRAS mutations, WT1 and cMyc expressions on overall survival

Patients with *KRAS* mutant tumors had shorter OS compared to patients with wild-type *KRAS* ($p < 0.001$, Fig. 2A). By using cutoffs at a median of *WT1* RNA level (median = 3.48) and a median of *cMyc* RNA level (median = 4.09), patients were divided into subgroups. A poor OS was demonstrated in patients with both *KRAS* mutation and a high *WT1* expression ($p = 0.008$, Fig. 2B) but not in patients with wild-type *KRAS* ($p = 0.347$). Interestingly, a similar pattern was detected in the *cMyc* RNA expression subgroup. Survival analysis showed no significant differences in comparison with a cutoff at a median *cMyc* RNA level in all NSCLC subjects ($p = 0.942$) or the wild-type *KRAS* subjects ($p = 0.737$). However, patients with mutant *KRAS* and higher *cMyc* expression had shorter OS compared to those with lower *cMyc* expression levels ($p = 0.001$, Fig. 2C).

Further analysis was performed combining *WT1* and *cMyc* RNA expression levels. We found that patients with mutant *KRAS*, and higher *WT1* and *cMyc* expression levels (both > median) had worse OS ($p = 0.003$, Fig. 2D) but no differences were found in wild-type *KRAS* patients ($p = 0.515$). The results indicated that prognosis for clinical outcome was affected by tumors with *KRAS* mutation itself and together with upregulated *WT1* and *cMyc* RNA expression.

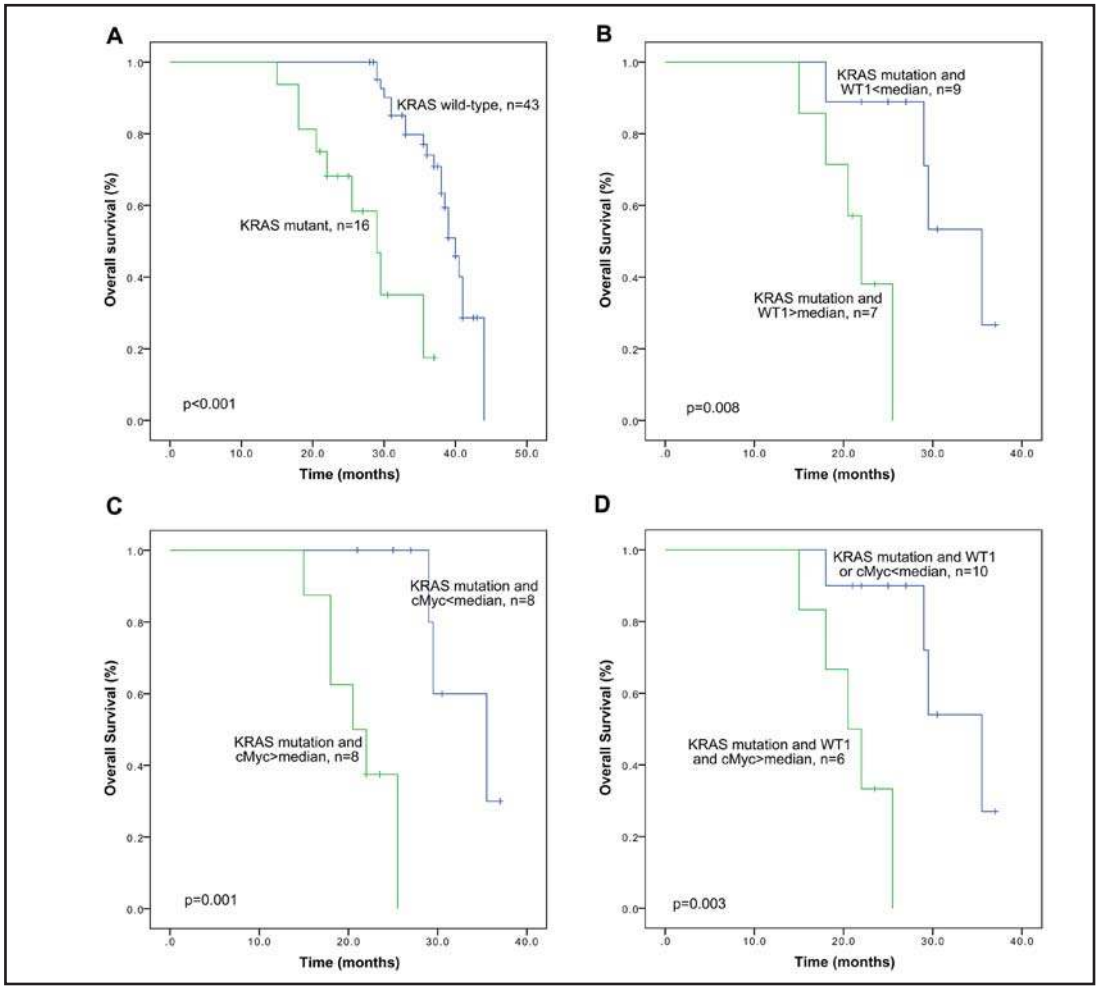


Fig. 2. Impact of *KRAS* mutations, *WT1* and *cMyc* expressions on overall survival. A, Overall survival (OS) using the Kaplan-Meier analysis in patients with *KRAS* mutant tumours compared to patients with wild-type *KRAS*. B, Survival analysis in the subgroup of patients with *KRAS* mutation and *WT1* expression at a cut off with median (3.48×10^{-3}). C, Survival analysis in the subgroup of patients with *KRAS* mutation and *cMyc* RNA expression at a cut off with median (4.09×10^{-3}). D, Survival analysis in the subgroup of patients with *KRAS* mutation and both *WT1* and *cMyc* expression levels.

WT1 promotes cell proliferation in KRAS mutant NSCLC cell line

To further investigate the potential mechanism of *WT1* and *KRAS* functions, *KRAS* mutant cell line H1299 and *KRAS* wild-type cell line H1568 were used to perform transcriptional experiments. *WT1* RNA silencing demonstrated a marked decreased expression of WT1 protein in both cell lines (Fig. 3A). The effect of WT1 silencing on in vitro proliferation was examined using MTT assay. The H1299 cells proliferated at a significantly lower rate with *WT1* siRNA compared to control (Fig. 3B). The WT1 silencing did not significantly affect H1568 cell proliferation. Moreover, a cellular marker for proliferation, Ki-67, was stained on both cell lines collected at 48 hours. In H1299 cells, much less positive Ki-67 staining in *WT1* siRNA (39%) was shown compared to control (96% of cells with positive Ki-67 staining, Fig. 3C). However, both *WT1* siRNA and control H1568 cells showed intense staining patterns without marked differences (81% vs 84%, Fig. 3D).

Additionally, transfection experiments using plasmids carrying *WT1* variant A or D was performed to confirm the effect of WT1 and *KRAS* on cell proliferation. High expression levels of WT1 isoforms A and D were demonstrated in both cell lines (Fig. 3E). Cell proliferation

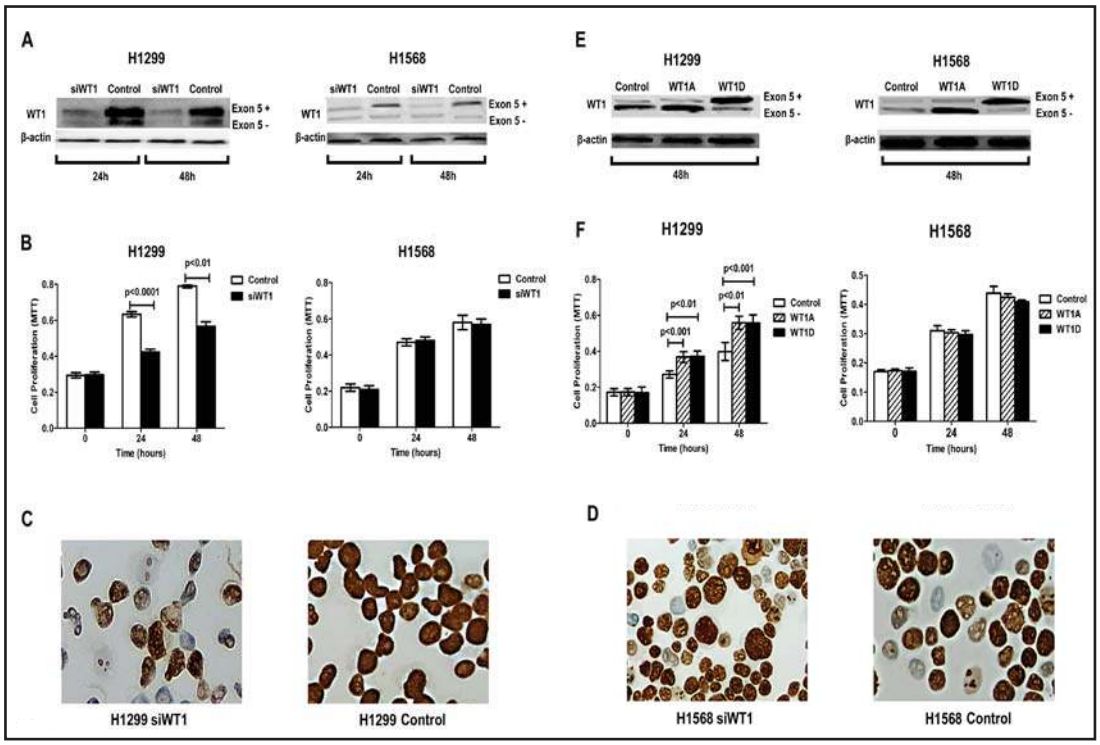
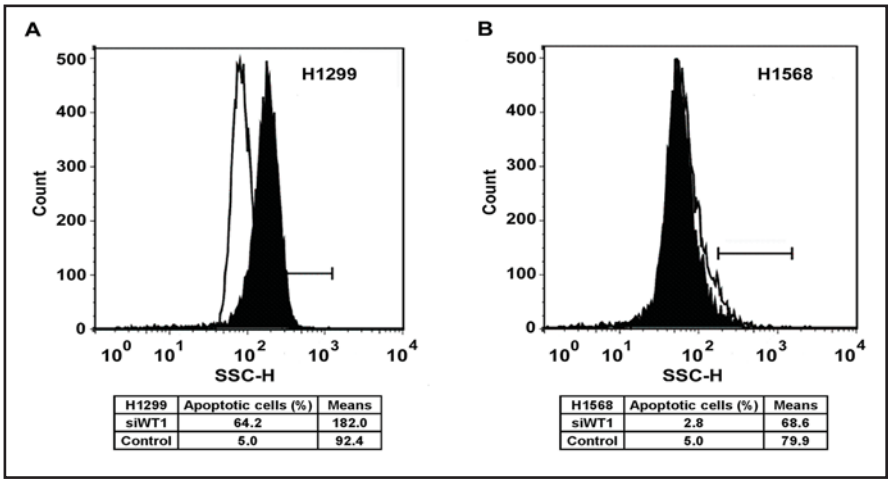


Fig. 3. WT1 affected cell proliferation in the *KRAS* mutant NSCLC cell line. A, WT1 RNA silencing demonstrated a marked decreased expression of WT1 protein in NSCLC cell lines H1299 (*KRAS* mutant) and H1568 (*KRAS* wild-type) at 24 and 48 hours after transfection. B, The effect of *WT1* silencing on cell proliferation was examined using MTT assay in H1299 cells and in H1568. C, Ki-67 positive staining in SiWT1 (39%) and in control (96%) in H1299. Cells were collected at 48 hours after transfection. D, Ki-67 positive staining in SiWT1 (81%) and in control (84%) in H1568. Cells were collected at 48 hours after transfection. E, The effect of WT1 on cell proliferation by transfection experiments using plasmids carrying *WT1* variant A (-/-) or D (+/+). WT1 isoforms A and D were presented at 48 hours after transfection in both H1299 and H1568 cell lines. F, Effect on cell proliferation by forced overexpression of WT1 in H1299 and H 1568 cells.



H1299	Apoptotic cells (%)	Means
siWT1	64.2	182.0
Control	5.0	92.4

H1568	Apoptotic cells (%)	Means
siWT1	2.8	68.6
Control	5.0	79.9

Fig. 4. *WT1* RNA silencing induced apoptosis in *KRAS* mutant NSCLC cells. Cells were collected at 48 hours after transfection. A, TUNEL analysis in *KRAS* mutant H1299 cells and B in *KRAS* wild-type H1568 cells.

was significantly increased in H1299 cells compared to control but not in H1568 cells (Fig. 3F). These results indicated that *WT1* promotes cell proliferation in *KRAS* mutant tumors but not in *KRAS* wild-type.

Table 3. Silencing WT1 induced fold changes in the expression of apoptosis-related regulator genes by proteome profiler analysis in H1299 and H1568 cells**Statistical significance ($p < 0.01$). *Statistical significance ($p < 0.05$)

Gene name	Expected effect on apoptosis	Fold change (vs control)	
		H1299	H1568
FasTNFSF6	Activator	1.408**	1.01
Cleaved caspase3	Activator	1.309*	1.046
ProCaspase3	Activator	1.176	1.068
SMACDiablo	Activator	1.168	0.973
HO1HMOX1HSP32	Activator	1.089	1.169
Bax	Activator	0.998	0.974
Phosphop53S15	Activator	0.996	1.066
TRAILR2DR4	Activator	0.935	1.006
P21CIPCDNK1A	Activator	0.921	1.08
Bad	Activator	0.91	0.97
HTRAOmi	Activator	0.901	1.09
Cytokromc	Activator	0.894	1.196
Phosphop53S46	Activator	0.892	1.08
Phosphop53S392	Activator	0.891	1.065
TRAILR2DR5	Activator	0.84	1.195
FADD	Activator	0.716*	1.075
TNFRITNFRSF1A	Activator	0.645	1.028
Bclx	Suppressor	1.079	1.265
PON2	Suppressor	0.947	1.056
HSP70	Suppressor	0.917	1.175
HSP27	Suppressor	0.896	1.098
HIF1a	Suppressor	0.848	1.078
P27Kip1	Suppressor	0.845	1.102
Livin	Suppressor	0.816	0.982
Survivin	Suppressor	0.698*	1.064
Clusterin	Suppressor	0.694*	1.186
Catalase	Suppressor	0.637	1.036
XIAP	Suppressor	0.622**	1.071
cIAP2	Suppressor	0.573*	1.097
cIAP1	Suppressor	0.503**	1.003
Bcl-2	Suppressor/Activator	1.215*	0.771*
HSP60	Suppressor/Activator	0.937	1.031
HO2HMOX2	Suppressor/Activator	0.926	1.169
PhosphoRad17S63S	Suppressor/Activator	0.829	1.019
Claspin	Suppressor/Activator	0.746*	1.157

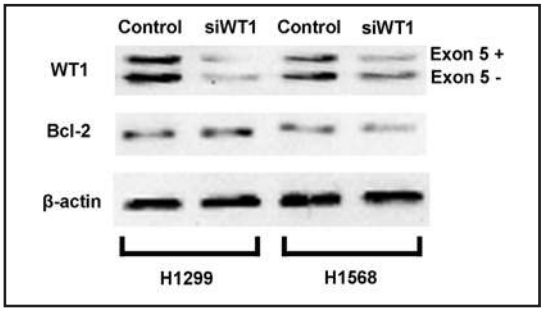


Fig. 5. Bcl-2 expression using western blot analysis in WT1 knock-down cells using silencing WT1 (SiWT1) RNA compared to controls in H1299 and H1568.

WT1 RNA silencing induces apoptosis in KRAS mutant NSCLC cells

Using TUNEL analysis, *WT1* RNA silencing induced an increased apoptotic cell population in H1299 cells (64.2%) compared to controls (5.0%, Fig. 4A), whereas in H1568 cells, *WT1* siRNA and control did not differ noticeably and showed only a small apoptotic cell population (2.8% for *WT1* siRNA and 5.0% for control, Fig. 4B).

Furthermore, a total of 35 genes related to apoptosis were analyzed using human apoptotic protein array. Altered protein expressions were significantly changed in WT1 knock-down cells in 10 out of the 35 apoptosis related genes in H1299 cells but only one gene in H1568 cells compared to their controls (Table 3). In H1299 cells, two positive regulators,

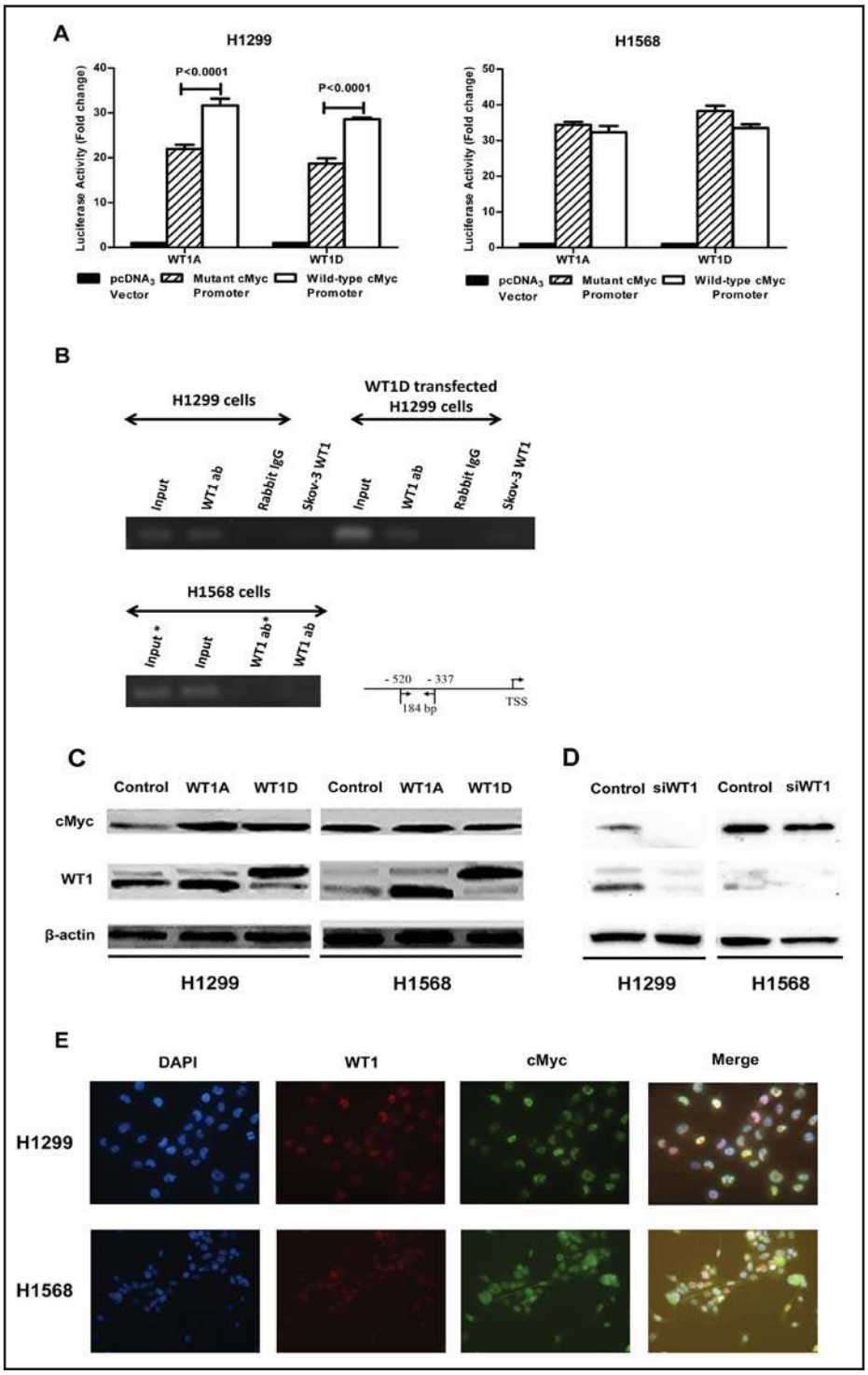


Fig. 6. WT1 can directly increase *cMyc* promoter activity and bind to the *cMyc* promoter WT1 binding cite in *KRAS* mutant NSCLC cells. A, Luciferase reporter gene assay in the two cell lines H1299 and H1568 together with transfection experiments. The WT1A and WT1D cDNA expression plasmids were each cotransfected with *cMyc* construct (wild or mutant type). Cells were collected at 48 hours after transfection. The value obtained by control transfection was arbitrarily set at 1. The results were shown with the fold change in luciferase activity of experimental cells versus control cells. Means and 95% confidence interval were calculated after at least three independent transfections. B, DNA binding of WT1 protein to the *cMyc* promoter. The location and size for each PCR product are illustrated on the right. TSS, transcriptional start site. *, WT1D transfected in H1568 cells. C, cMyc protein expression by western blot in WT1A and WT1D transfected H1299 and H1568 cells. Cells were collected at 48 hours after transfection. D. Decreased expression of cMyc by western blot in H1299 cells but not in H1568 cells after WT1 RNA silencing. Cells were collected at 48 hours after transfection. E, Predominant nuclear localization of endogenous WT1 (red) and endogenous cMyc (green) by double-immunofluorescence in H1299 and H1568 cells. Nuclei were visualized by DAPI (blue) staining.

FasTNFSF6 and cleaved Caspase 3, were significantly upregulated. Five negative regulators, including Survivin, Clusterin, XIAP, cIAP2 and cIAP1, and one positive regulator, FADD, demonstrated decreased expression. Alterations in two dual function genes were shown as decreased expression of Claspin and increased expression of Bcl-2. In contrast, decreased Bcl-2 expression was shown in H1568 cells. Alteration of Bcl-2 expression at protein level was also verified using western blot analysis (Fig. 5). The results demonstrated that *WT1* has a potential antiapoptotic function through affecting multiple regulators in *KRAS* mutant cells.

The cMyc gene is a target of WT1 in KRAS mutant NSCLC cells

A positive correlation between *WT1* and *cMyc* expression was presented in clinical NSCLC *KRAS* mutant tumor specimens as described above. To demonstrate the functional link between *WT1* and *cMyc*, we performed luciferase reporter gene assay. Plasmids carrying *WT1A* or *WT1D* variants were each cotransfected with *cMyc* construct. As shown in Fig. 6A, the ability of WT1 to activate the *cMyc* promoter was observed in H1299 cells but not in H1568 cells. To further demonstrate that the WT1 protein binds directly to the promoters of the *cMyc* gene, we performed ChIP experiments. As shown in Fig. 6B, a single band was observed when chromatin from H1299 cells and WT1D transfected H1299 cells were immunoprecipitated with WT1 antibody. This was not observed in negative controls when rabbit IgG antibody and SKOV-3 cell line were used. In H1568 cells and WT1D transfected H1568 cells, no specific band could be shown compared to negative control. Using ChIP/PCR, we could demonstrate direct binding of WT1 to the *cMyc* promoter in H1299 cells but not in H1568 cells. Western blot showed upregulated cMyc protein in WT1A and D transfected H1299 cells compared to control and no differences in expression of cMyc in H1568 cells (Fig. 6C). Additional experiments were performed to validate the effect of WT1 on *cMyc* using *WT1* RNA silencing. Decreased expression of cMyc was shown in H1299 but not in H1568 compared to their controls (Fig. 6D). The results demonstrated that the *cMyc* promoter is a target of the WT1 protein in *KRAS* mutant cells. Double immunofluorescences were performed, showing predominant nuclear localization of WT1 and cMyc in H1299 and H1568 cells (Fig. 6E).

Discussion

In the present study, *KRAS* mutation was less frequently detected in stage I tumors and patients with *KRAS* mutation had shorter OS compared to those with wild-type *KRAS*. A meta-analysis performed by Huncharek et al. has previously reported that *KRAS* mutation was associated with shortened survival in NSCLC [26]. A strong association between *KRAS* mutation and decreased survival in NSCLC patients was demonstrated by Nelson et al. and the correlation was specifically significant for patients diagnosed with stage I tumors [27]. These findings indicate that *KRAS* mutation analysis can be used as a prognostic molecular marker. Recent studies have shown that NSCLC patients with *KRAS* mutations are resistant to TKIs and have significantly poorer outcome [9, 28] and suggest that *KRAS* mutation should be added to the panel of resistance markers for EGFR-TKIs in NSCLC [5]. This is supported by a recent meta-analysis showing that KRAS codon 13 mutation is associated with a poorer response to adjuvant CT [29]. However, Tsao et al. found that *KRAS* mutation status had no prognostic impact on survival or benefits from adjuvant chemotherapy [30]. Mutations in *KRAS* did not significantly affect outcome in EGRF wild-type NSCLC patients, according to Jackman et al. [31].

Overexpression of *WT1* was demonstrated in 96% of *de novo* NSCLC [32]. Interestingly, low *WT1* expressions predicted poor OS in the entire cohort and a shorter disease-free survival in a subgroup of patients with stage III and IV NSCLC [33]. In this study, *WT1* overexpression had a significant negative effect on OS in patients with mutant *KRAS* tumors but not in wild-type *KRAS* tumors. Our data suggest that *WT1* may affect tumor progression only in *KRAS* mutant tumors.

The *cMyc* gene has been studied as a proto-oncogene in human malignancies. Overexpression of *cMyc* was shown in human lung cancer cell lines [34]. A recent study found that progressive lung cancer could be identified by several genetic pathways, including a *myc*-centered transcription network [35]. We demonstrated that *cMyc* was overexpressed in NSCLC tumor specimens and high *cMyc* expression in *KRAS* mutant tumors had negative correlation with OS. The collected data support the hypothesis that altered *myc* signaling is involved in disease progression. Considering the limited patient material, the survival analysis in this study was exploratory and our results need to be confirmed in larger study populations.

Using in vitro experiments, we found that cell proliferation decreased due to loss of *WT1* in *KRAS* mutant tumor cells. Forced overexpression of *WT1* in *KRAS* mutant tumor cells induced significantly increased cell proliferation. Wagner et al. showed that inhibition of *WT1* reduces cell proliferation, migration and vascular formation through transcriptional activation of the proto-oncogene ETS-1 [36]. A combination strategy of *WT1* gene silencing and chemotherapeutic agents cisplatin and doxorubicin was found to have a synergistic effect on inhibiting cell proliferation in B16F10 murine melanoma cells [37]. In human melanoma, WT1 protein expression was found to be associated with shorter overall survival[38]. Treatment using PS-341 (Bortezomib) on a megakaryoblastic cell line was able to retain NF-kB, inhibit cell growth and downregulate *WT1* expression [39]. Reduction of *WT1* RNA copies was tested in vitro as a predictor of sensitivity to imatinib in chronic myelogenous leukemia [40]. Thus, a compound that downregulates *WT1* expression would be of great interest in the treatment of *KRAS* mutant NSCLC.

WT1 is known to be a transcriptional factor involved in the regulation of apoptosis and cell cycle progression (reviewed in [17, 41]). In the present study, WT1 silencing induced a higher apoptotic cell population. This effect was observed only in *KRAS* mutant NSCLC cells. However, a study by Vicent S et al. did not show any difference in apoptosis using a group of NSCLC cell lines with and without *KRAS* mutation including H1299 and H1568. Furthermore, we demonstrated significant fold changes in multiple apoptosis-related genes using proteomic profiler assay. In *KRAS* mutant cells, the WT1 generated effect on the process of apoptosis seemed complex, as WT1 silencing caused reduced expression of several negative regulators such as Survivin, Clusterin, XIAP, cIAP2, and cIAP1. The expression levels of potential positive regulators, such as FasTNFSF6 and cleaved Caspase 3, were increased. The Bcl-2 gene was significantly upregulated and the expression of Claspin was decreased. These results suggested that the *WT1* gene contributes to suppression of apoptosis by affecting multiple apoptosis-related genes. Consistent with our findings, Zamora-Avila et al. demonstrated that loss of WT1 protein expression increased caspase-3 and poly-ADP-ribose polymerase activity, as well as apoptotic body formation, chromatin condensation and DNA fragmentation in the B16F10 murine melanoma cell line [42]. Algar et al. found that WT1 antisense nucleotide reduced cell proliferation, induced apoptosis and abrogated WT1 protein expression in myeloid leukemia cell lines [43]. By shRNA-targeted knockdown of *WT1*, resistant leukemia cells were sensitized to the cancer therapeutic agent tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL) to TRAIL-induced cell death [44]. The serin protease HtrA2 was identified to be able to cleave WT1 at multiple sites and was shown to be an important regulator of WT1 under proapoptotic conditions, such as cytotoxic drug treatment [45]. The collected data suggest that *WT1* has an antiapoptotic function and acts an oncogene in some tumors as described above. In combination with cytotoxic drugs, targeting *WT1* may provide a novel therapeutic alternative against cancer cells resistant to regular chemotherapy treatment.

It is known that oncogenic *KRAS* can lead to either cellular senescence under high oncogene signaling conditions or tumorigenesis when the negative feedback loops are blocked as reviewed in Collado [46]. *WT1* was recently identified as a critical regulator of senescence and proliferation downstream of oncogenic *KRAS* signaling [13]. In a mouse model of *KRAS*-driven lung cancer, loss of *WT1* decreased tumor burden and led to senescence of mouse primary cells expressing physiological level of oncogenic RAS [13]. These effects

were also found in human lung cancer cell lines. Furthermore, *WT1* high signature could be used to stratify lung cancer patients that express *KRAS* signature genes into a poor prognostic subgroup [13]. In accordance with their findings, our observations of clinical data showed patients with high expression of *WT1* and *KRAS* mutation had shorter survival time. In vitro experiments, cell proliferation and apoptosis were notably affected in only *KRAS* mutant NSCLC cells. However, the precise molecular mechanisms for mutant *KRAS*-related WT1 function remain unclear. Further studies are needed to find how WT1 regulates mutant *KRAS*-driven oncogenic function. Since RAS proteins have proven difficult to use as a direct therapeutic target, a recent phase 2 study on NSCLC patients with KRAS mutant tumors assessed the compound selumetinib, targeting RAS downstream effecter MEK with promising efficacy but a higher number of adverse events [47]. Therefore, *WT1* may provide a new indirect target to inhibit RAS-induced oncogenesis.

A previous study demonstrated that WT1 can bind to the GC-rich Egr1 site (5'-GNGNGGGNG-3') in the *cMyc* promoter [25]. The WT1 binding site near the second major transcription start site just upstream of nucleotides - 107 and + 36 was extensively involved in the regulation of *cMyc* [25]. Studies of WT1 effect on the *cMyc* promoter have given divergent results, including stimulatory function in breast cancer [25] and suppressive function in ccRCC [16]. Vicent et al. demonstrated that the experimentally derived independent gene sets for *Myc* pathway genes were upregulated in *KRAS* mutant tumors compared with control and enrichment of Myc target genes was further found to be up-regulated in *KRAS* mutant tumors, suggesting a role of *Myc* as a possible target of WT1 in *KRAS* mutant cells [13]. Using *cMyc*-promoter-driven luciferase constructs and ChIP-PCR, we confirmed in this study that WT1 can directly bind to *cMyc* promoter and act as a positive regulator of *cMyc* in *KRAS* mutant NSCLC. Using small molecule inhibitor of MYC-MAX heterodimerization, 10058-F4, cell death was efficiently induced in myeloma cell lines as well as primary myeloma cells [48]. 10058-F4-induced apoptosis and differentiation could also be observed in acute myeloid leukemia [49]. Taken together, an indirect approach by targeting *cMyc* may be a therapeutic alternative in *KRAS* mutant NSCLCs.

In summary, we found a correlation between overexpression of *WT1*, *cMyc* and poor survival in NSCLC patients with *KRAS* mutation. The results indicated that measuring *WT1* and *cMyc* expression may serve as useful biomarkers to identify subgroups with KRAS mutation for individualized therapy. Furthermore, in vitro experiments demonstrated that in *KRAS* mutant cells, WT1 promotes cell proliferation and has antiapoptotic function by affecting multiple apoptosis-related genes and by directly targeting the *cMyc* promoter. In agreement with findings by Vicent et al. [13], our results support the hypothesis that *WT1* acts as a key regulator and as an oncogene in *KRAS* mutant NSCLC. Together with previous studies on therapeutic alternatives [47-50], this study suggests that *WT1* and *cMyc* may be useful targets in *KRAS* mutant NSCLCs. These findings may warrant further investigation on the effects of *WT1* and *cMyc* inhibitors *in vitro* and *in vivo.*

Disclosure Statement

None declared.

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