Dermatology Practical & Conceptual

GRWD1 Drives Melanoma Growth Through NF-κB Signaling Pathway

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ABSTRACT

Introduction: Melanoma is an aggressive skin cancer with high metastatic potential. The oncogenic protein GRWD1 has been implicated in various cancer types, but its role in melanoma remains unclear.

Objectives: To examine the effects of GRWD1 knockdown on melanoma cell proliferation, apoptosis, and migration and to evaluate its prognostic significance in melanoma patients.

Methods: A combination of in vitro and clinical analyses was performed. A2058 melanoma cells were treated with GRWD1-specific siRNA, and cell proliferation, apoptosis, and migration assays were conducted. Western blotting was used to assess alterations in key oncogenic pathways. Additionally, clinical tissue samples from melanoma patients were analyzed for GRWD1 expression, and Kaplan-Meier survival analysis was performed to determine its prognostic value.

Results: GRWD1 was highly expressed in melanoma cells. GRWD1 knockdown significantly reduced cell proliferation (by 63%), impaired colony formation, and induced apoptosis (cleaved caspase-3 levels

increased by 17.3%). Migration capacity decreased by 70%, and NF-κB pathway activity was suppressed, leading to reduced expression of Bcl-2, Src, and MDM2, while stabilizing p53. TCGA-based analyses revealed that high GRWD1 expression was significantly associated with shorter survival in metastatic melanoma cases (P=0.00029) but showed no correlation with melanoma subtypes. However, in immunohistochemical analysis of clinical samples, no statistically significant correlation was found between GRWD1 staining intensity and survival.

Conclusions: GRWD1 plays a crucial role in melanoma progression by enhancing NF-κB activity, promoting proliferation, and suppressing apoptosis. While high GRWD1 expression is associated with poor prognosis in public datasets, further clinical validation with larger patient cohorts is needed to confirm its utility as a prognostic biomarker and therapeutic target.

Introduction

Melanomas are malignant skin tumors originating from melanocytes [1,2]. These tumors can arise in any tissue containing melanocytes, including the skin, mucosa, conjunctiva, uvea, and meninges, with the skin being the most commonly affected site [2]. Although melanomas constitute only 4% of all skin cancers, they account for 75% of skin cancer-related deaths [3]. Since the 1960s, the incidence of malignant melanoma has been increasing globally in both males and females, posing a significant socioeconomic burden [4,5].

In the United States, melanoma ranks as the fifth most common cancer among males and the sixth most common among females [4]. Unlike other solid tumors, it frequently affects younger and middle-aged individuals, with a median age at diagnosis of 57 years [4]. Melanoma pathogenesis is a multifactorial process integrating genetic and environmental factors [3]. Ultraviolet radiation (UVR) from natural or artificial sources is the most significant environmental risk factor. Individuals with lighter skin tones, characterized by lower melanin levels, are more susceptible to sunburns, thus increasing their risk of malignant melanoma. Additionally, a higher number of nevi correlates with an elevated risk of developing melanoma. A family history of malignant melanoma further amplifies this risk, potentially due to shared sun exposure habits or hereditary genetic mutations [3].

Early-stage primary amelanotic melanoma (AM) or melanotic melanoma (MM) is generally associated with a favorable prognosis [6]. However, due to challenging anatomical locations, most AM and MM cases are diagnosed at advanced or metastatic stages, leading to poor prognosis and necessitating systemic therapies, including chemotherapy, targeted therapies, or immunotherapy [6]. The five-year survival rate for stage 4 cases is reported to be between 15 and 20% [7]. This underscores the critical need for novel treatment strategies, particularly for advanced-stage melanoma. A thorough understanding of the molecular underpinnings of the disease is imperative for the development of effective therapeutic interventions.

Ribosomal/nucleolar proteins constitute a diverse family with various biological functions. While some members of this protein family exert tumor-suppressive effects, others promote tumorigenesis by inhibiting the activity of tumor-suppressive ribosomal/nucleolar proteins [8]. The glutamate-rich WD40 repeat-containing 1 (GRWD1) protein, a member of this family, interacts with RPL11, disrupting its association with MDM2, thereby facilitating p53 degradation via ubiquitination [8,9]. This mechanism significantly contributes to cancer progression. These findings suggest that GRWD1 is a multifunctional protein involved in multiple cellular regulatory pathways associated with cell growth control [9].

Recent studies have demonstrated that GRWD1 directly interacts with p53, suppressing its transcriptional activity. Additionally, elevated GRWD1 expression has been correlated with poor prognosis in patients with certain cancer types [9]. These findings support the oncogenic role of GRWD1, suggesting that its suppression may inhibit tumor progression. Nevertheless, further investigations are required to elucidate the precise oncogenic mechanisms of GRWD1 [10].

Objectives

The present study examined the effects of GRWD1 on cellular processes, including proliferation, cell cycle regulation, invasion, and apoptosis, in melanoma cancer cell line. Furthermore, GRWD1 expression was analyzed in melanoma tumor samples obtained from patients, and its association with survival rates, histological subtypes, tumor depth, and disease stage was assessed. Our findings suggest that GRWD1 is a promising therapeutic target and prognostic biomarker in melanoma.

Methods

Cell Line and Reagents

The human melanoma cancer cell line A2058 was commercially acquired from the American Type Culture Collection

(ATCC, USA). GRWD1 siRNA was purchased from Qiagen. The culture medium Dulbecco's Minimal Essential Medium (DMEM high glucose), fetal bovine serum (FBS), antibiotics (penicillin and streptomycin), and L-glutamine (Cat. No: 25030-081, Gibco) were obtained from Gibco (USA) with the following catalog numbers: DMEM (11965084, ThermoFisher, Waltham, MA, USA) and FBS (P2442, Sigma). The MTS Assay Kit for cell proliferation was obtained from Promega (Cat. No. G3582). BCA Protein Assay Kit (Abcam, Cambridge, UK, Cat. No: ab287853) were used.

Cell Culture

A2058 cells were cultured in DMEM high glucose supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine in T25 flasks at 37° C in a humidified environment with 5% CO_2. The culture medium was replaced every 48 hours, and cells were subcultured upon reaching 80% confluency.

Cell Proliferation Assay

GRWD1 knockdown was performed via transient siRNA transfection, and efficiency was confirmed 72 hours post-transfection by Western blotting. The effect of GRWD1 siRNA on tumor cell proliferation was evaluated using the MTS assay (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). Cells were initially seeded in 25 cm² cell culture flasks and treated with GRWD1 siRNA (75 nM) or control siRNA. After 24 hours of incubation, the cells were harvested and plated into 96-well plates with equal cell distribution per well in 100 µL of growth media. For proliferation assessment, CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega kit) was used. 25 µL of MTS labeling reagent was added to each well and incubated in a humidified environment at 37° C for two hours. Absorbance was measured at 490 nm using a microplate reader (E-Max Plus, Molecular Devices, USA).

Colony Formation Assay

To evaluate long-term proliferative capacity, cells were seeded in 6-well plates at a density of 3,000 cells per well. The following day, siRNA transfection was performed. The medium was replaced after 24 hours, and the cells were maintained for seven days. A second siRNA treatment was administered, followed by an additional 7-day incubation period. On day 15, colonies were fixed with methanol (5 min) and stained with 0.5% crystal violet (Sigma-Aldrich) for 10 minutes. Colonies were quantified using ImageJ software.

Flow Cytometric Analysis

Annexin V-PI Apoptosis Analysis

Cells were seeded in 6-well plates at a density of 2×10^5 cells per well and treated with GRWD1 siRNA. After 24 hours, fresh growth media was added to the wells. Following an additional 48 hours of incubation (total 72 hours), cells were collected via trypsinization and centrifugation (1500 rpm, 5 min). Apoptosis was assessed using the Annexin V-PI Apoptosis Detection Kit (BD Biosciences, Cat. No: 556547). Samples were analyzed using a Beckman Coulter CytoFLEX Flow Cytometer (Beckman Coulter, USA) [11].

Cell Cycle Analysis via Propidium Iodide (PI) Staining

To determine the effect of GRWD1 knockdown on the cell cycle, A2058 cells (200,000 per 25 cm² flask) were transfected with GRWD1 siRNA. After 24 hours, fresh growth media was added, and cells were further incubated for an additional 48 hours (total 72 hours). Cells were then harvested and fixed in 70% ethanol at -20° C overnight. The following day, cells were stained with propidium iodide (PI, BioLegend, Cat. No: 421301) and RNase A (100 µg/mL) for 30 minutes at 37° C. The cell cycle phase distribution was analyzed using a Beckman Coulter CytoFLEX Flow Cytometer (Beckman Coulter, USA).

Caspase-3 Activation Assay

Cells were seeded in 6-well plates and transfected with GRWD1 siRNA. After 24 hours, fresh growth media was added, and cells were further incubated for an additional 48 hours (total 72 hours). Cells were then collected via centrifugation (500 × g, 5 min), fixed, and permeabilized using BD Cytofix/Cytoperm buffer at 4° C for 20 minutes. Apoptosis was assessed using the FITC Active Caspase-3 Apoptosis Kit (BD Biosciences, Cat. No: 550480). For caspase-3 detection, cells were stained with FITC-conjugated active caspase-3 antibody in the dark for 30 minutes. Data acquisition was performed using a Beckman Coulter CytoFLEX Flow Cytometer (Beckman Coulter, USA), and 10,000 events per sample were analyzed.

Western Blotting

Western blotting was performed to analyze GRWD1 expression and signaling pathway alterations. This method was carried out as described elsewhere [12]. To confirm knockdown efficiency, GRWD1 band intensities were quantified via densitometric analysis using ImageJ software and normalized to β-actin levels. The relative expression was used to verify the silencing efficiency at the 72-hour post-transfection point. After protein extraction and quantification, samples were separated via SDS-PAGE and transferred onto PVDF membranes. Membranes were incubated overnight at 4°C with primary antibodies against GRWD1 (Biolegend, Cat. No: 645702), Bcl-2 (Elabscience, Cat. No: E-AB-22004), cleaved PARP (Cell Signaling, Cat. No: D214, 19F4), P53 (Biolegend, Cat. No: 645702), p21 (BT LAB, Cat. No: BT-AP12602), MDM2 (Biogend, Cat. No: E-AB-60076), NF-κB p65 (St John's Laboratory, Cat. No: STJ903553-100),

Src (Cell Signaling, Cat. No: 2108S), and β-Actin (Santa Cruz, Cat. No: sc-47778) as a loading control. After washing, membranes were incubated with HRP-conjugated secondary antibodies, including goat anti-rabbit HRP (Cell Signaling Technology, Cat. No: 7074S) and goat anti-mouse HRP (Cell Signaling Technology, Cat. No: 7076S). Protein bands were detected using the ChemiDoc Imaging System, and densitometric analysis was conducted using ImageJ software.

Histopathological Examination

The slides and blocks of the patients included in the histopathological study were evaluated. Appropriate samples were selected, and tissues were re-blocked after being taken from the blocks using the microarray method. Routine H&E staining and immunohistochemical staining with the GRWD1 antibody were applied to the sections obtained from the new blocks. Based on GRWD1 staining intensity, nuclear and cytoplasmic staining intensity was graded as strong (+3), moderate (+2), weak (+1), or negative (0). The medical records of the patients included in the study were thoroughly reviewed. Demographic, clinical, and biopsy data such as age, sex, melanoma type, Breslow score, lesion size, ulceration, localization, mitotic rate, and metastasis were recorded. The relationship between staining intensity and sex, melanoma type, Breslow score, lesion size, ulceration, localization, and metastasis was investigated. Numerical data such as Breslow score, mitotic rate, lesion size, and survival were compared among different groups. Correlation analysis was performed between age, Breslow score, mitotic rate, lesion size, survival, and staining intensity.

Statistical Analysis

In Vitro Analysis

For in vitro experiments (including MTS proliferation assay, Annexin V/PI apoptosis analysis, caspase-3 activation, cell cycle distribution, invasion assay, and Western blot quantifications), data were collected from three independent biological replicates (N=3), each performed under identical conditions. Results are presented as mean ± standard deviation (SD). Comparisons among the three groups (untreated control, non-targeting siRNA, and GRWD1 siRNA) were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test to determine statistical significance between specific pairs of groups. A p-value <0.05 was considered statistically significant. Student's t-test was used for colony formation ability. Western blot band intensities were quantified using ImageJ software and normalized to β-actin prior to statistical comparison. Flow cytometry data (Annexin V, Caspase-3, and cell cycle analyses) were collected from at least 10,000 events per sample, and gating strategies were consistently applied across replicates. All statistical analyses were conducted using GraphPad Prism v9.0 (GraphPad Software, San Diego, CA, USA).

Clinical Analysis

Correlations between GRWD1 expression and clinical parameters were analyzed. Qualitative data are summarized as counts and percentages, while quantitative data are presented as minimum and maximum values along with mean ± standard deviation (SD). For the comparison of categorical variables, Pearson's chi-square test and Yates' corrected chi-square test were used. Since the data did not follow a normal distribution, Spearman's correlation test was applied for correlation analysis. A p-value <0.05 was considered statistically significant. Nonparametric tests (Mann Whitney-U test) were used to compare Breslow score, mitotic rate, and lesion size between different groups. Experiments were performed in triplicate (N=3) unless otherwise specified.

This study was approved by Clinical Research Ethics Committee of Inonu University Medical Faculty (2022/121).

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Results

High GRWD1 Expression is Associated with Reduced Survival Time

Although the relationship between GRWD1 expression and survival is reported to be complex and cancer-type specific, in general, high GRWD1 expression has been linked to poor prognosis and decreased survival time in various cancer types [13]. To determine the relationship between GRWD1 expression and patient survival in melanoma cases, data from The Cancer Genome Atlas (TCGA) database were analyzed. However, TCGA data did not provide datasets that allowed a direct comparison of GRWD1 expression between normal skin and melanoma tumor tissues. Additionally, no significant difference in GRWD1 expression was observed between primary and metastatic tumors (Figure 1A). Nevertheless, survival analysis revealed that high GRWD1 expression in melanoma was significantly associated with reduced patient survival (Figure 1B). When examining primary and metastatic tumors separately, high GRWD1 expression in primary tumors was associated with decreased survival, although this finding was not statistically significant (Figure 1C). In contrast, in metastatic tumors, high GRWD1 expression was significantly correlated with markedly reduced survival (Figure 1D). These findings suggest that GRWD1 expression is associated with aggressive tumor characteristics and poor prognosis in melanoma.

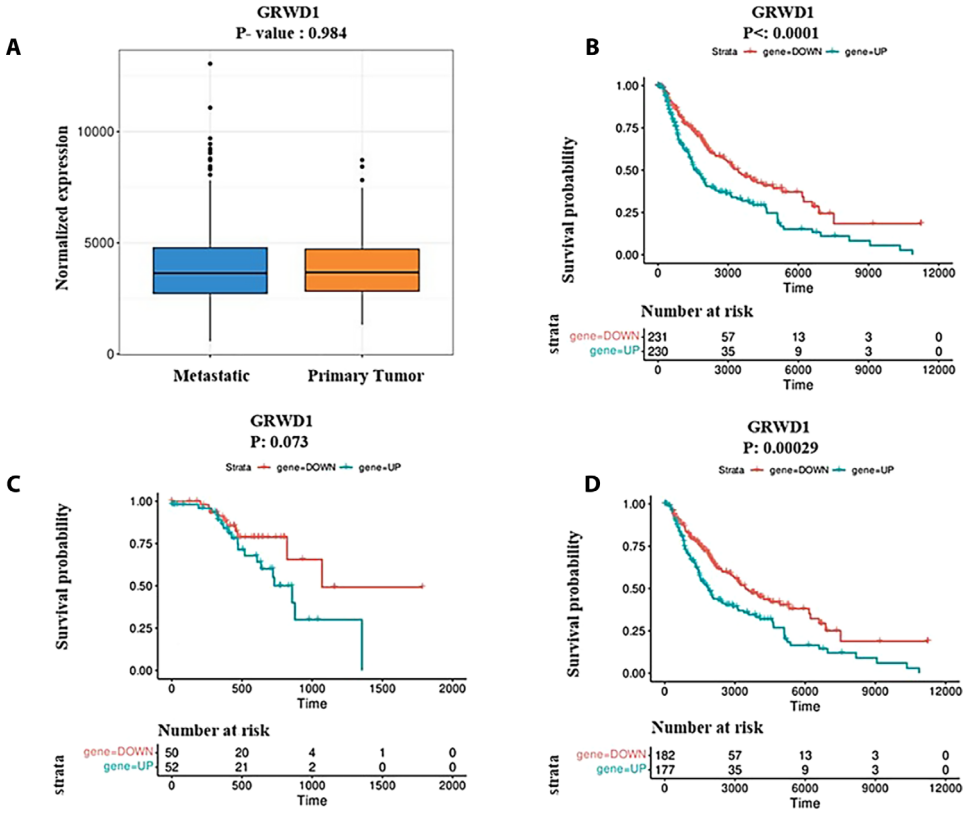

Figure 1. GRWD1 expression and survival analysis in melanoma patients. (A) GRWD1 expression levels in metastatic and primary melanoma tumors based on TCGA data. No significant difference was observed (P=0.984). (B) Kaplan-Meier survival analysis of melanoma patients stratified by GRWD1 expression. High GRWD1 expression correlates with significantly reduced survival (P<0.001). (C) Survival probability analysis for primary melanoma cases based on GRWD1 expression. A trend toward poorer survival in high-GRWD1 cases was observed despite the statistical insignificance (P=0.073). (D) Survival analysis of metastatic melanoma cases. High GRWD1 expression is significantly associated with decreased survival (P<0.001).

GRWD1 is Expressed in Melanoma Cells and Plays a Role in Cell Proliferation

GRWD1 is expressed at higher levels in many cancer cells compared to normal tissues. In breast cancer cells, GRWD1 mRNA and protein levels have been found to be elevated compared to normal human breast epithelial cells [14]. Wang et al. reported that GRWD1 is overexpressed in non-small cell lung cancer (NSCLC) [13]. Since there was no prior study demonstrating GRWD1 expression in melanoma cells, we first confirmed GRWD1 expression in A2058 cells, a highly invasive melanoma cell line (Figure 2A). To test our hypothesis that suppression of GRWD1 expression would reduce melanoma cell proliferation, we used GRWD1-specific siRNA to inhibit GRWD1 expression by 95%. Suppression of GRWD1 expression led to a noticeable decrease in cell viability (Figure 2B). After 72 hours of knockdown, a proliferation assay showed a 63% reduction in cell proliferation in the GRWD1 siRNA-treated group (Figure 2C). This reduction in proliferative capacity was further emphasized by a colony formation assay. Cells treated with 75 nM GRWD1 siRNA were stained after two weeks, and the number of colonies was quantified. The results showed a 74.4% decrease in colony formation ability in treated cells (Figure 2D). Our findings support the conclusion that GRWD1 overexpression promotes cell proliferation.

GRWD1 Knockdown Leads to an Increase in Cell Death

In a study, GRWD1 was found to support cell colony formation by affecting the expression of Cyclin B1, CDK1, and p27 and inducing the G2/M transition [13]. Additionally, research on gastric cancer (GC) demonstrated that GRWD1 overexpression promotes the proliferation, migration, and invasion of gastric cancer cells in vitro [15]. To determine whether the reduction in cell proliferation due to GRWD1 suppression was caused by slowed cell division or increased cell death, cell cycle analysis was first performed using flow cytometry. Cells treated with GRWD1-specific siRNA and control siRNA for 72 hours were stained with PI and analyzed by

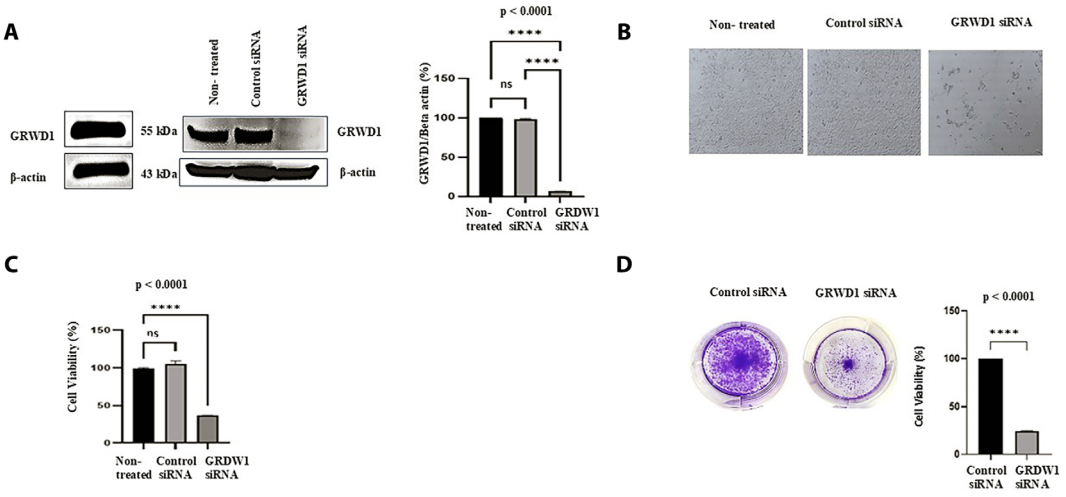

Figure 2. GRWD1 knockdown reduces melanoma cell proliferation and colony formation. (A) Western blot analysis confirming GRWD1 knockdown in A2058 melanoma cells treated with GRWD1 siRNA. (B) Phase-contrast microscopy images showing reduced cell density following GRWD1 knockdown compared to control. (C) MTS assay results demonstrate a significant decrease in cell viability after GRWD1 knockdown (*****P*<0.001). (D) Colony formation assay shows a reduction in the number of colonies formed by GRWD1 siRNA-treated cells. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test (N=3). Data are shown as mean ± SD.

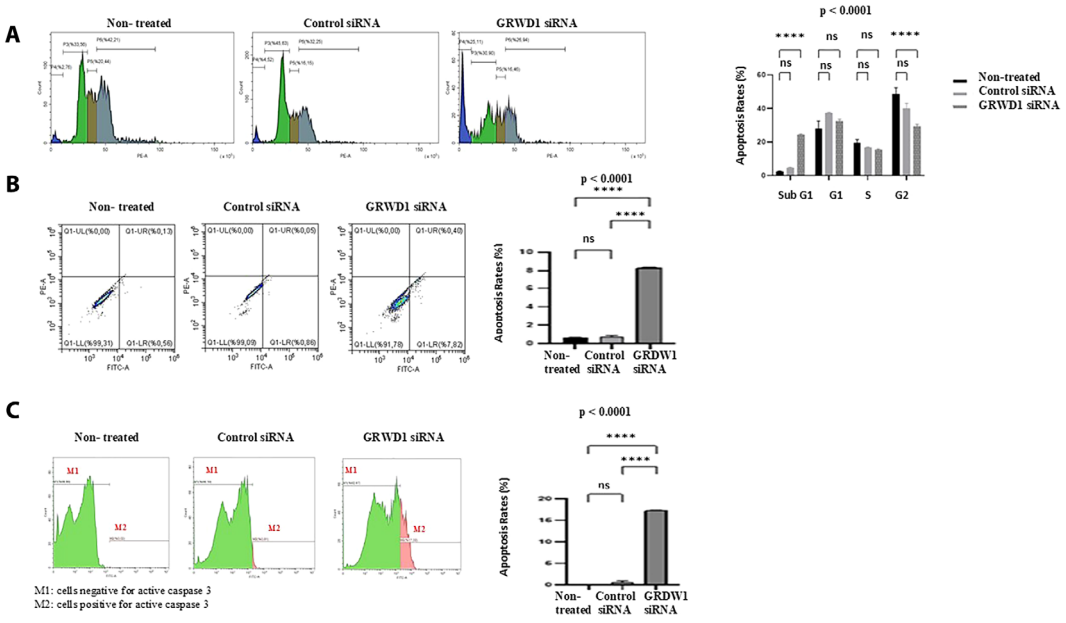

Figure 3. GRWD1 knockdown induces apoptosis in melanoma cells. (A) Flow cytometric analysis of cell cycle distribution. GRWD1 knockdown significantly increases the sub-G1 population, indicating apoptotic cell death (*****P*<0.001). (B) Annexin V-PI staining showing a significant increase in apoptotic cell population following GRWD1 knockdown (*****P*<0.001). (C) Caspase-3 activation assay confirming apoptosis induction in GRWD1 siRNA-treated cells Flow cytometry data represent mean ± SD from three independent experiments. Statistical comparisons were made using one-way ANOVA (Tukey's post hoc test). *****P*<0.0001.

flow cytometry. While no statistically significant change was observed in the number of cells in the G1 and S phases, a decrease in the G2 phase population and a significant increase in the sub-G1 phase cells were detected (Figure 3A). These results suggest that GRWD1 knockdown drives melanoma cells toward apoptosis. This finding was further supported by both Annexin V-PI staining and flow cytometric detection of cleaved caspase-3 levels. In Annexin V-PI staining, the apoptotic cell population increased from 0.91% to 7.82% in the GRWD1 siRNA-treated group (*P*<0.001) (Figure 3B). Additionally, the percentage of cleaved caspase-3-positive cells increased from 0.81% in the control siRNA group

to 17.33% in the GRWD1 siRNA group (P<0.0001) (Figure 3C). These findings indicate that GRWD1 plays a critical role in the survival of melanoma cells. Although these results show that suppression of GRWD1 in vitro stimulated cell death, no significant difference was found in the IHC analyses we performed on pathology samples taken from patients (Figures S1 and S2).

GRWD1 Regulates Cell Migration in Melanoma Cells

A study on non-small cell lung cancer reported that GRWD1 expression enhances the metastatic potential of cells [13]. Another study on triple-negative breast cancer demonstrated that suppression of GRWD1 expression increases apoptosis while reducing cell migration and invasion [14]. GRWD1 overexpression has been shown to be associated with lymph node metastasis in colorectal carcinoma. Suppression of GRWD1 significantly reduced the migration and invasion abilities of colorectal cancer cells in wound healing and transwell assays [16]. A similar study on gastric cancer also obtained comparable results, demonstrating that GRWD1 suppression significantly reduces cell migration and invasion [15]. GRWD1 has been reported to enhance the invasive ability of cancer cells in all these cancer types. To evaluate the invasion capacity of melanoma cells in vitro, we used matrigel-coated Boyden chambers. In A2058 melanoma cells, suppression of GRWD1 expression led to a 70% reduction in invasion capacity (Figure 4A). Our findings indicate that GRWD1 regulates melanoma cell proliferation, apoptosis, and metastasis. As the next step, we performed Western blot analysis to identify the molecular mechanisms underlying these effects. We examined PARP cleavage, a marker of apoptosis, and Bcl-2, a key regulator of apoptosis that is overexpressed in more than half of all cancers. Suppression of GRWD1 expression resulted in an increase in PARP cleavage and a decrease in Bcl-2 expression (Figure 4B). Additionally, we investigated the expression of Src kinase, a key regulator of invasion, and found that its expression was reduced following GRWD1 knockdown (Figure 4C). The relationship between MDM2 and p53 proteins is well known, and previous studies have reported that GRWD1 competes with RPL11 to inhibit MDM2's ability to stabilize p53 [8]. However, in our study, we demonstrate that GRWD1 positively regulates MDM2 expression in melanoma cells. When GRWD1 expression was suppressed via RNA interference (RNAi), MDM2 expression decreased in parallel, and as expected, p53 expression increased (Figure 4C). Furthermore, we analyzed changes in NF-κB expression, a transcription factor known to regulate the expression of Src, Bcl-2, and MDM2 proteins. We show for the first time that GRWD1 knockdown suppresses NF-κB expression (Figure 4C). To determine whether the effects of GRWD1

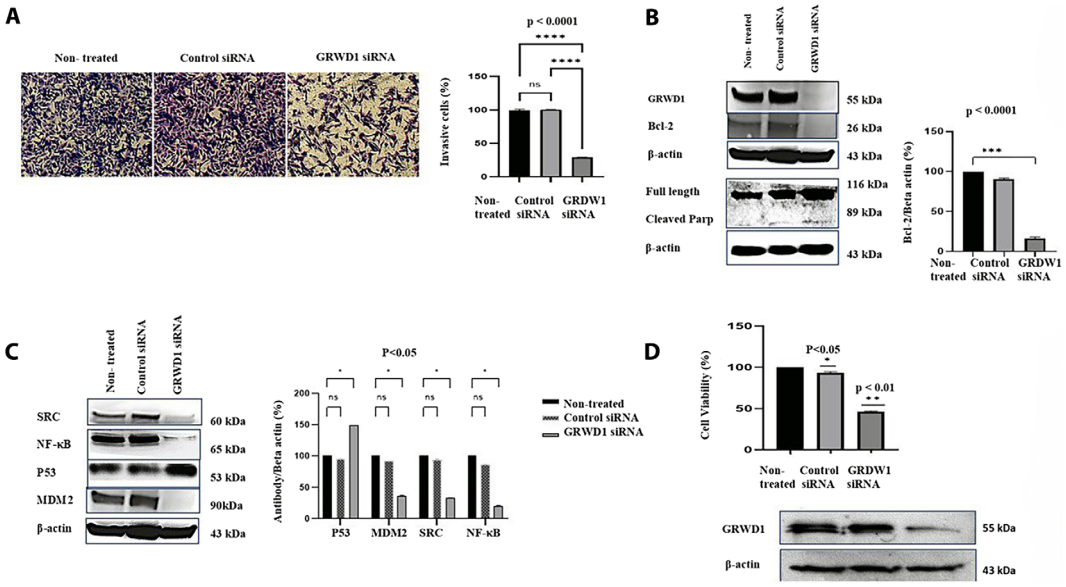

Figure 4. GRWD1 knockdown suppresses invasion and oncogenic signaling in melanoma cells. (A) Matrigel invasion assay shows significantly reduced invasion capacity in GRWD1 siRNA-treated melanoma cells (****P<0.001). (B) Western blot analysis shows reduced Bcl-2 expression and increased cleaved PARP levels in GRWD1 knockdown cells, indicating apoptosis. (C) Western blot analysis of key oncogenic signaling proteins. GRWD1 knockdown leads to decreased NF-κB, Src, and MDM2 expression while increasing p53 levels. (D) Validation of GRWD1 knockdown effects in A2780 ovarian cancer cells. Cell viability is significantly reduced after GRWD1 knockdown (*P<0.05, *P<0.01). Flow cytometry and Western blot data represent mean ± SD from three independent experiments. Statistical comparisons were made using one-way ANOVA (Tukey's post hoc test).

on melanoma cells are cancer-specific, we conducted a proliferation analysis using a second cancer cell line. Suppression of GRWD1 expression in A2780 ovarian cancer cells resulted in a similar inhibitory effect on cell proliferation, demonstrating that GRWD1 plays a critical role in cancer cell growth beyond melanoma (Figure 4D).

No Association was Found between GRWD1 Expression and Melanoma Subtypes

We analyzed the expression of GRWD1 in a cohort of melanoma FFPE tumor samples. Among the 37 patients included in the study, 15 (40.5%) were female, and 22 (59.5%) were male. The mean age was 68.1±14.2 years. Regarding the melanoma subtypes in the examined specimens, 54.1% were nodular melanoma, and 32.4% were acral lentiginous melanoma. Three specimens were from metastatic lesions. The tumor was localized in the extremities in 62.2% of cases, with the second most common localization being the head and neck region (24.3%). Metastasis was observed in 62.2% of the cases. The survival time of deceased patients after diagnosis ranged from one to 39 months, with an average of 13.1±12.7 months. We examined the staining status of melanoma tumor samples with GRWD1 and classified the staining intensity as mild, moderate, or severe (Figure S1). To compare the staining intensity among different subgroups, we divided the staining intensity into two groups as mild/moderate and severe. We could not obtain survival information for some of the patients whose biopsy specimens we examined. Perhaps due to the small sample size, there was no significant difference in survival between the two groups (Figure S2). GRWD1 staining intensity was mild/moderate in 59.5% of patients and severe in 40.5% (Table S1). In the comparison of staining intensity across different groups, severe staining was significantly more frequent in female patients than in male patients (P=0.020). However, no statistically significant difference was observed in staining intensity based on melanoma subtype, lesion size, localization, or metastasis (P>0.05) (Table S2).

Patients with metastasis had a significantly higher mean Breslow score compared to those without metastasis (P=0.029). In nodular melanoma, the mean Breslow score and mitotic rate were significantly higher compared to acral lentiginous melanoma (P=0.034, P=0.012, respectively) (Table S3). Correlation analysis revealed a significant positive relationship between age and Breslow score, mitotic rate, and lesion size. There was also a significant positive correlation between Breslow score and lesion size, while mitotic rate showed a significant negative correlation with both survival and staining intensity (Table S4). We also collected demographic and clinical data of all patients including age, sex, melanoma types, metastasis, localization, and GRWD1 staining intensity in a separate table (Table S5).

Discussion

GRWD1 is a multifunctional protein that has been implicated in various cellular processes, including ribosome biogenesis, DNA damage response, and chromatin remodeling. The regulation of protein expression via the suppression of GRWD1 gene led to a reduction in NF-κB expression. NF-κB, recognized as a transcription factor that orchestrates various cellular functions including immune response, inflammation, and cell viability, is extensively reported in scholarly literature for its role in modulating the expression of numerous proteins such as Bcl-2, Src, and MDM2. GRWD1 has been previously shown to participate in both ribosome biogenesis and chromatin remodeling processes through interactions with RPL11, WDR5, and other regulatory proteins [13,17,18]. In our study, we observed that GRWD1 knockdown led to a reduction in NF-κB protein levels. However, we did not assess NF-κB mRNA expression. Therefore, while our data support a link between GRWD1 and NF-κB activity, the underlying mechanism remains unclear.

Based on the existing literature, we propose that GRWD1 may influence NF-κB at the translational or chromatin remodeling level, although this remains speculative and was not directly tested in our study.

Our data demonstrate that GRWD1 silencing leads to a suppression of NF-κB expression, which likely results in transcriptional downregulation of downstream targets such as Bcl-2, Src, and MDM2. This is supported by the observed decrease in their protein levels (Figure 4C), consistent with NF-κB's known role as a transcriptional activator. Nevertheless, post-transcriptional regulatory mechanisms may also contribute to this downregulation, as GRWD1 is known to regulate ribosome biogenesis and mRNA translation processes. This action is also supported by Kayama et al. [19], who reported that elevated expression levels of GRWD1 were associated with SKCM as well as with certain other malignancies with unfavorable prognosis. The decrease in MDM2 expression upon GRWD1 knockdown, which results in the stabilization of p53, emphasizes the significance of this pathway in melanoma. Earlier studies have indicated that GRWD1 binds with ribosomal proteins to control translational mechanisms and promote protein synthesis in neoplastic cells [13]. These observations are a direct association between GRWD1 and tumor growth via the MDM2-p53 pathway. Deng et al.'s research confirmed that the long non-coding RNA (lncRNA) PiHL forms an interaction with GRWD1 and RPL11 that degrades p53 [20]. The mechanism by which GRWD1 facilitates cancer development through inhibition of the tumor suppressor p53 is further upheld by our data, which demonstrate that repression of GRWD1 caused stabilization of p53 and the activation of apoptotic pathways (Figure 4C).

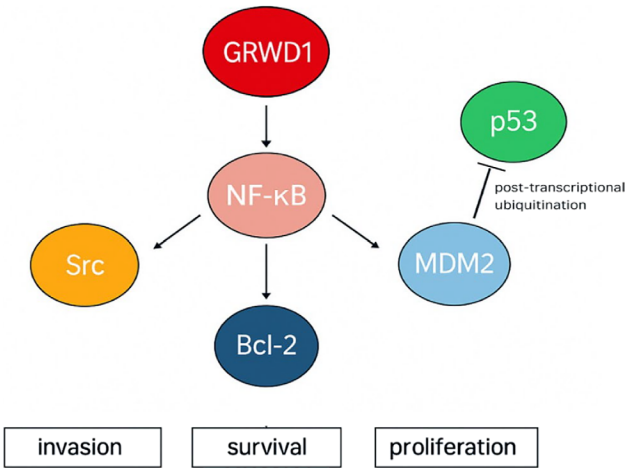

Figure 5. GRWD1 promotes the ability of proliferation, survival and invasion in melanoma cells through the activation of NF-κB pathway. Proposed mechanism underlying GRWD1-mediated melanoma progression. GRWD1 enhances NF-κB signaling, which transcriptionally upregulates Bcl-2 and activates Src. NF-κB also induces MDM2 expression, promoting p53 degradation. The downregulation of p53 relieves transcriptional repression of Bcl-2, further supporting cell survival and proliferation. This GRWD1–NF-κB–MDM2/p53–Bcl-2 axis contributes to melanoma pathogenesis. Arrows represent activation (solid) and inhibition (dashed).

Regarding its role in a variety of malignancies, Zhou and Shang [16] concluded that GRWD1 overexpression is linked to poor clinical outcomes in colorectal cancer. In contrast, in our immunohistochemical analyses conducted with clinical data, the lack of statistical significance in the relationship between GRWD1 expression and survival is likely due to the limited sample size in our local cohort. Therefore, while TCGA data suggest GRWD1 may serve as a potential prognostic marker, further validation in larger, independent patient cohorts is required to confirm its clinical relevance and accuracy. We acknowledge the limitations of our current sample and emphasize the need for future studies with greater statistical power.

Conclusion

Our results suggest that GRWD1 supports melanoma progression through the NF-κB pathway regulating MDM2-p53, Src, Bcl-2 expressions (Figure 5). Therefore, targeting GRWD1 holds promise for melanoma treatment. However, tumor-specific targeting strategies, in vivo studies, and clinical validation are required to confirm its therapeutic potential.

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