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Research Article

Nuclear Localization of Yes-Associated Protein Is Associated With Tumor Progression in Cutaneous Melanoma

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ABSTRACT

Yes-associated protein (YAP), an effector molecule of the Hippo signaling pathway, is expressed at high levels in cutaneous melanoma. However, the role of YAP in melanoma progression according to cellular localization is poorly understood. Tissues from 140 patients with invasive melanoma were evaluated by immunohistochemistry. Flow cytometry, western blotting, viability assays, wound healing assays, verteporfin treatment, and xenograft assays were conducted using melanoma cell lines B16F1 and B16F10 subjected to Yap^{S127A} transfection and *siYap* knockdown. Nuclear YAP localization was identified in 63 tumors (45.0%) and was more frequent than cytoplasmic YAP in acral lentiginous and nodular subtypes ($P = .007$). Compared with cytoplasmic YAP melanomas, melanomas with nuclear YAP had higher mitotic activity ($P = .016$), deeper invasion ($P < .001$), and more frequently metastasized to lymph nodes ($P < .001$) and distant organs ($P < .001$). Patients with nuclear YAP melanomas had poorer disease-free survival ($P < .001$) and overall survival ($P < .001$). Nuclear YAP was an independent risk factor for distant metastasis (hazard ratio: 3.206; 95% CI, 1.032-9.961; $P = .044$). Proliferative ability was decreased in *siYap*B16F1 ($P < .001$) and *siYap*B16F10 ($P = .001$) cells and increased in Yap^{S127A}B16F1 ($P = .003$) and Yap^{S127A}B16F10 ($P = .002$) cells. Cell cycle analysis demonstrated relative G1 retention in *siYap*B16F1 ($P < .001$) and *siYap*B16F10 ($P < .001$) cells and S retention in Yap^{S127A}B16F1 cells ($P = .008$). Wound healing assays showed that *Yap* knockdown inhibited cell invasion (*siYap*B16F1, $P = .001$; *siYap*B16F10, $P < .001$), whereas nuclear YAP promoted it (Yap^{S127A}B16F, $P < .001$; Yap^{S127A}B16F1, $P = .017$). Verteporfin, a direct YAP inhibitor, reduced cellular proliferation in B16F1 ($P = .003$) and B16F10 ($P < .001$) cells. Proliferative effects of nuclear YAP were confirmed in xenograft mice ($P < .001$). In conclusion, nuclear YAP in human melanomas showed subtype specificity and correlated with proliferative activity and proinvasiveness. It is expected that YAP becomes a useful prognostic marker, and its inhibition may be a potential therapy for melanoma patients.

Introduction

Despite its low incidence, cutaneous melanoma, a malignancy of melanocytes in the basal layer of the epidermis, is one of the most fatal tumors.[1,2] The prognosis of melanoma is affected by

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various pathological features, including tumor thickness, ulceration, microscopic satellites, tumor-infiltrating cells, lymphatic invasion, regression, and Clark level of invasion.[1,3] With an improved understanding of the biology and pathogenesis of melanoma in recent years, it has become evident that there is no single evolutionary pattern describing how neoplastic lesions progress into fully evolved, aggressive melanomas.[4] Each melanoma subtype may have various precursor lesions, different gene alterations, and different stages of transformation.[2] Therefore, investigators have been searching for melanoma-specific biomarkers to predict the behavior of this malignancy and act as therapeutic targets, despite the existence of different clinicopathologic varieties.[5,6]

Yes-associated protein (YAP) is a transcriptional coactivator that functions as the downstream effector of the Hippo signaling pathway.[7] The Hippo signaling system is essential for controlling organ size, promoting tissue regeneration, and preventing cancer growth.[8,9] In normal cells, YAP function is inhibited by the Hippo pathway through phosphorylation, which results in its cytoplasmic sequestration and destruction.[9] It has long been assumed that a specific 14-3-3 binding site created by large tumor suppressor kinase 1 (LATS1)-mediated phosphorylation of YAPS127 mediates YAP cytoplasmic anchoring, and YAP phosphorylation favors its cytoplasmic localization.[10] When wild-type YAP is substituted with YAPS112A, nuclear sequestration of YAP occurs.[11] Activation of target genes by YAP nuclear translocation and inhibition of the Hippo signaling pathway promote cell proliferation, survival, and migration.

In cancer cells, aberrant activation of YAP, which promotes tumor genesis, development, invasion, and metastasis, has been linked to multiple types of malignancies.[7,12] Studies have shown that YAP overexpression is associated with not only tumorigenesis but also poorer prognosis in various malignancies, including pancreatic, liver, breast, and lung cancers.[13-17] High-YAP expression has also been observed in patient samples and melanoma cell lines in several studies and has been suggested to be associated with tumor proinvasion and a poor prognosis.[18-20] However, the role of YAP in melanoma progression according to cellular localization of this protein has not been fully examined.

In this study, we aim to explore the role of YAP in cutaneous melanoma according to its cellular localization (nucleus or cytoplasm), highlight the implications of YAP in predicting aggressive tumor behavior, and evaluate the potential therapeutic effects of a direct YAP inhibitor in melanoma cells.

Materials and Methods

Patients and Tissues

We retrospectively recruited 140 patients diagnosed with invasive cutaneous melanoma from 2005 to 2013 at Severance Hospital, Yonsei University College of Medicine. Their medical records were reviewed for clinical features. To select the most representative formalin-fixed paraffin-embedded tissues, tumor samples were mounted on slides, stained with hematoxylin and eosin, and reviewed by two pathologists (H.J.R. and S.K.K.). The tumor-infiltrating lymphocyte (TIL) score was calculated using a previously described method, briefly outlined as the sum of the lymphocyte distribution and density scores.[21] The lymphocyte distribution score, spanning from 0 to 3, was defined as follows: 0 = absence of lymphocytes within the tissue, 1 = presence of lymphocytes occupying <25% of the tissue, 2 = presence of lymphocytes occupying 25% to 50% of the tissue, and 3 = presence of lymphocytes occupying >50% of the tissue. Additionally, the lymphocyte density score, ranging from 0 to 3, was defined as follows: 0 = absent, 1 = mild, 2 = moderate, and 3 = severe. Applying this scoring method, we calculated the TIL scores, considering scores from 0 to 2 as low and scores from 3 to 6 as high. All methods and experimental protocols using human tissues were conducted in accordance with relevant guidelines and regulations approved by the Institutional Review Board of Severance Hospital, Yonsei University Health System (IRB no. 4-2018-0469).

Immunohistochemistry

Formalin-fixed paraffin-embedded blocks were cut into 4-μm-thick sections and processed using heat-induced epitope retrieval. Immunohistochemical (IHC) staining was performed in an automated immunostainer (Ventana BenchMark XT; Roche Diagnostics), according to the manufacturer's instructions. To avoid color interference from brown melanin pigment in tumor cells, the Ultraview Universal Alkaline Phosphatase Red Detection Kit (Roche Diagnostics) was used to detect the anti-YAP antibody (#sc-101199, 1:200 dilution; Santa Cruz Biotechnology). Staining for YAP was considered positive when observed in >1% of tumor cells, and cases exhibiting no YAP expression were excluded from the study population. Positive YAP staining was classified as cytoplasmic or nuclear based on the subcellular staining pattern. We categorized the expression as nuclear when YAP was detected in the nucleus of tumor cells, regardless of whether cytoplasmic staining was also present. The expression was classified as cytoplasmic when YAP was detected only in the cytoplasm of tumor cells, with no evidence of nuclear staining. Two pathologists (H.J.R. and S.K.K.) who were blinded to the pathologic information independently evaluated the IHC staining results.

Cell Cultures

Mouse melanoma cell lines B16F1, B16F10, B16BL6, K-1735, and Clone M-3 (Cloudman S91 melanoma) were purchased from the Korean Cell Line Bank. B16F1 and B16F10 cells were maintained in Dulbecco's Modified Eagle Medium (Hyclone Laboratories) with 10% fetal bovine serum (Hyclone Laboratories), 100 unit/mL penicillin, and 100 μg/mL streptomycin (Hyclone Laboratories). B16BL6 cells were maintained in minimum essential medium with Earle's balanced salt solution (Hyclone Laboratories), and K-1735 and Clone M-3 cells were maintained in Roswell Park Memorial Institute-1640 medium (Hyclone Laboratories). All cell lines were grown in a humidified 5% CO_2 incubator at 37 °C.

Vectors and DNA Transfection

Substitution of wild-type *YAP* with the *YAPS112A* allele induces nuclear sequestration of YAP, and nuclear translocation of YAP activates target genes associated with cell proliferation, survival, and migration.[11] To establish melanoma cell lines with YAP nuclear translocation and overexpression, we used a Yap^{S127A} plasmid DNA vector (kindly provided by Dae-Sik Lim, KAIST), in which a human Yap^{S127A} fragment was cloned into the pcDNA3 Flag vector. Transfection was performed using the Lipofectamine 3000 Transfection Kit (Thermo Fisher Scientific). After transfection of Yap^{S127A} and control vectors into melanoma cell lines, the inserted DNA fragments were verified by sequencing, and YAP overexpression was identified by western blot analysis.

siRNA Transfection

To establish *Yap*-knockdown (KD) melanoma cell lines, melanoma cells were transfected with *siYAP* or control *siRNA* using the Lipofectamine RNAi MAX Kit (Invitrogen), according to the manufacturer's protocol. After 48 hours, cells were harvested and subjected to western blot analysis. The *siRNA* sequences were as follows: control *siRNA*, 5′-CGUACGCGGAAUACUUCGATT-3′; *siYAP*, 5′-GAAGCGCUGAGUUCCGAAAUC-3′.

Nuclear/Cytoplasmic Fractionation

Nuclear and cytosolic proteins in each melanoma cell line were fractionated using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific) to quantify cytoplasmic and nuclear YAP. Protein quantification was measured with Bradford solution, and 5 μg of each fraction was subjected to western blot analysis.

Wound Healing Assay

Tumor cells were grown for 24 hours in 60-mm culture plates at a cell density of 2×10^5, after which a gap was created by scratching with a 1000-μL disposable plastic pipette tip. Microscopy pictures were obtained every 12 hours, and the distance of tumor cell migration across the gap was measured and calculated using ImageJ software.

Viability Assay

Viability assays were performed using PrestoBlue dye (Invitrogen), according to the manufacturer's instructions. Samples were read by a microplate reader using 560 nm as the wavelength of absorbance. To evaluate the effects of YAP inhibition on melanoma cell lines according to cellular location, we used verteporfin, a direct YAP inhibitor (Sigma-Aldrich).

Confocal Microscopy Analysis

Slides were seeded at 5×10^3 cells per slide and mounted on cover glasses with DAPI containing solution (Vectashield; Vector Laboratories). Samples were visualized with an LSM700 confocal microscope (Zeiss), and images were analyzed with ZEN microscopy software (Zeiss).

Flow Cytometry

Flow cytometry was used for cell cycle analysis to estimate the percentages of a cell population in the different phases according to YAP cellular location. Cells were harvested and then fixed with freshly made 70% ethanol. They were washed, followed by treatment with 100 μg/mL RNase A (Sigma-Aldrich) for 15 minutes at 37 °C. After the addition of 50 μg/mL propidium iodide (Sigma-Aldrich), the incubated cells underwent flow cytometry using a FACS LSR II (BD Biosciences) and FlowJo software (BD Bioscience).

Western Blotting

Protein extracts were obtained from cells or tumor masses using a protein extraction solution (Pro-Prep; iNtRON), according to the manufacturer's instructions. The following antibodies were used for western blot analysis: anti-YAP (#4912; Cell Signaling Technology), antiphospho YAP ser127 (#4911; Cell Signaling Technology), anti-LATS1 (#3477; Cell Signaling Technology), antiphospho-LATS1 (Thr1079) (#8654; Cell Signaling Technology), anti-LATS2 (#5888; Cell Signaling Technology), anti-MST1 (#3682; Cell Signaling Technology), anti-MST2 (#3952; Cell Signaling Technology), antiphospho MST1 (Thr183)/MST2 (Thr180) (#3681; Cell Signaling Technology), anti-TAZ (#4883; Cell Signaling Technology), β-actin (#LF-PA020; Ab Frontier), goat antirabbit IgG (#LF-SA8002A; Ab Frontier), and anti-Flag (#018-22381; WAKO).

Mouse Experiments

Animal care and experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee of Yonsei University. We used 6-week-old male BALB/c-nude mice for xenograft assays (Orient Bio). B16F1 cells transfected with *siYap* or Yap^{S127A} vector or their corresponding control *siRNA* or vector were injected subcutaneously into the left flank of the mice. Tumor size was measured when the tumors were at least 100 mm^3. Tumor dimensions were measured with a caliper, and tumor volume was calculated using the standard formula: length $\times$ width^2 $\times$ 0.5 (mm^3). Cancer cell−injected mice were monitored daily until a tumor appeared, after which the tumor size was determined weekly for 3 to 5 weeks, as indicated.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software) or SPSS version 26 software (SPSS Inc). Cell viability and tumor volume according to YAP expression in cellular compartments were recorded as mean ± SD, and significant differences between means were determined using analysis of variance, followed by *t* tests. IHC and flow cytometry results were analyzed using *t* tests, and the ratio of tumors with nuclear YAP expression in surgically resected melanomas was compared using χ^2 analysis. All reported *P* values are two-sided, and *P* values of <.05 were considered statistically significant.

Results

Clinical Features of Cutaneous Melanoma Patients According to Yes-Associated Protein Cellular Location

We performed IHC staining for YAP on invasive cutaneous melanoma tissues from 140 patients (Fig. 1A, B). Based on the staining patterns, we divided the cases into two groups: melanoma with cytoplasmic YAP expression and melanoma with nuclear YAP expression. Cytoplasmic YAP expression was found in 77 cases (55.0%), and nuclear YAP expression was observed in 63 cases (45.0%).

The clinical characteristics of the two groups are shown in Table 1. Nuclear YAP expression was more frequent in men (40/75, 53.3%) than in women (23/65, 35.4%, $P = .033$). There was no difference in age at diagnosis between the nuclear and cytoplasmic expression groups. Metastasis to regional lymph nodes (LNs) and distant organs was more frequent in patients with nuclear YAP expression than in those with cytoplasmic YAP expression ($P = .0004$ and $P < .001$, respectively). To evaluate

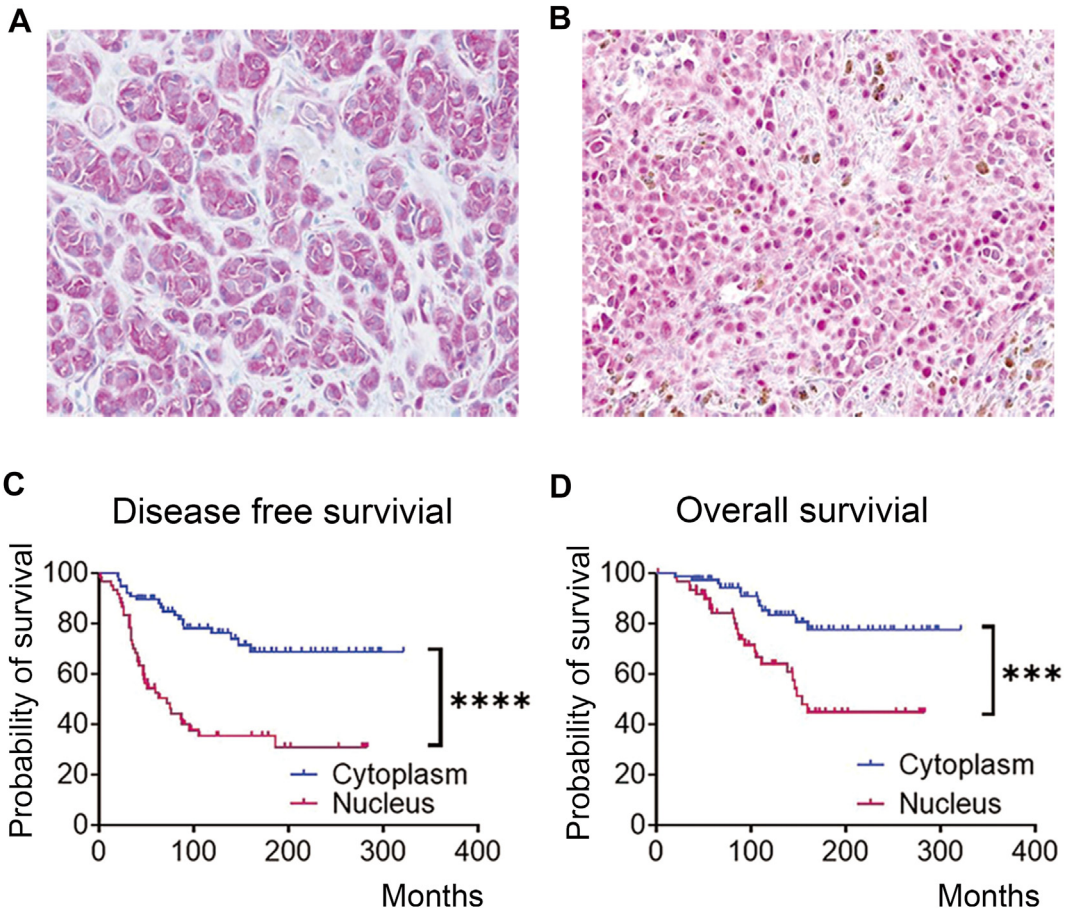

Figure 1.
YAP expression according to cellular compartments and survival curves of patients with melanoma. Cellular location of YAP in human cutaneous melanoma tissue: (A) cytoplasmic and (B) nuclear (magnification, ×200). Kaplan-Meier survival curves of patients with melanoma according to YAP cellular location: (C) disease-free survival and (D) overall survival. YAP, yes-associated protein.

clinicopathological factors associated with metastasis to LNs and distant organs, we performed logistic regression analysis (Table 2). Melanomas with nuclear YAP expression more frequently metastasized to distant organs than tumors with cytoplasmic YAP expression (hazard ratio [HR], 3.206; 95% CI, 1.032-9.961; $P = .044$). Deeper tumor invasion was also associated with LN metastasis (HR, 1.279; 95% CI, 1.076-1.520; $P = .005$) and distant organ metastasis (HR, 1.197; 95% CI, 1.016-1.409; $P = .031$). Tumor recurrence was more frequent in patients with nuclear YAP expression (37/63, 58.7%) than in those with cytoplasmic YAP expression (19/77, 24.7%; $P < .001$).

Patient Survival According to Yes-Associated Protein Cellular Location

Compared with patients with cytoplasmic YAP expression, individuals with nuclear YAP expression had a poorer prognosis for both disease-free survival (DFS) ($P < .001$; Fig. 1C) and overall survival (OS) ($P < .001$; Fig. 1D). We performed Cox regression analysis to identify factors independently associated with patient survival (Table 3). In univariate analysis, nuclear YAP expression was associated with worse DFS (HR, 3.433; 95% CI, 1.973-5.973; $P < .0001$) and OS (HR, 3.019; 95% CI, 1.508-6.045; $P = .002$), compared with cytoplasmic YAP expression. Breslow thickness of invasion was also associated with worse DFS (HR, 1.143; 95% CI, 1.074-1.217; $P < .0001$) and OS (HR, 1.148; 95% CI, 1.063-1.240; $P < .0001$). Patients with LN metastasis (HR, 5.783; 95% CI, 2.777-12.043; $P < .0001$) and distant organ metastasis (HR, 5.385; 95% CI, 2.750-10.546; $P < .0001$) had poorer OS than those with no metastasis. In multivariate analysis, nuclear YAP expression was an independent risk factor for worse DFS (HR, 2.493; 95% CI, 1.254-4.953; $P = .009$), whereas LN metastasis (HR, 3.867; 95% CI, 1.410-10.601; $P = .009$) and distant organ metastasis (HR, 3.207; 95% CI, 1.315-7.821; $P = .010$) were independent risk factors for worse OS.

Histopathologic Features of Human Cutaneous Melanoma According to Yes-Associated Protein Cellular Location

The histopathologic subtypes of cutaneous melanoma in the study population were as follows: acral lentiginous melanoma, $n = 85$ (60.7%); superficial spreading melanoma, $n = 30$ (18.8%); and nodular melanoma, $n = 25$ (15.6%; Table 1). YAP nuclear location was more frequent in acral lentiginous and nodular subtypes than in the superficial spreading subtype ($P = .007$). When we evaluated tumor invasion depth, cases with nuclear YAP expression had a higher Clark level (III and IV), greater Breslow thickness, a higher pT stage, and vertical growth phase than those with cytoplasmic YAP expression ($P < .001$ for each variable). We also counted the number of mitotic figures in the field with the highest mitotic activity per 1 mm^2 of each representative hematoxylin and eosin slide

Table 1
Clinicopathologic features of cutaneous melanoma according to YAP cellular location

Characteristic	YAP expression		*P*
	Cytoplasm (n = 77, 55.0%)	Nucleus (n = 63, 45.0%)	
Sex (male:female)	35:42	40:23	.033
Age at diagnosis, y (mean ± SD)	61.28 ± 1.371	59.82 ± 1.547	.482
Follow-up period, mo (mean ± SD)	141.2 ± 8.963	115.3 ± 8.982	.045
Metastasis to lymph nodes	18 (23.4%)	33 (52.4%)	<.001
Distant metastasis	6 (7.8%)	23 (36.5%)	<.001
Recurrence	19 (24.7%)	37 (58.7%)	<.001
Histopathologic subtype			.007
Acral lentiginous	42 (49.4%)	43 (50.6%)	
Nodular	11 (44.0%)	14 (56.0%)	
Superficial spreading	24 (80.0%)	6 (20.0%)	
Clark level			<.001
II	14 (93.3%)	1 (6.7%)	
III	21 (87.5%)	3 (4.8%)	
IV	33 (47.8%)	36 (52.2%)	
V	9 (40.9%)	13 (59.1%)	
Breslow thickness, mm (mean ± SD)	1.945 ± 0.2156	4.763 ± 0.4467	<.001
pT stage			<.001
pT1	28 (36.4%)	4 (6.3%)	
pT2	27 (35.1%)	18 (28.6%)	
pT3	16 (20.8%)	14 (22.2%)	
pT4	6 (7.8%)	27 (42.9%)	
Growth phase			<.001
Radial	35 (45.5%)	4 (6.3%)	
Vertical	42 (54.5%)	59 (93.7%)	
Mitotic count (mean ± SD)			
Per 1 mm^2	1.809 ± 2.201	3.293 ± 4.425	.016
Per 10 HPFs	4.206 ± 0.760	7.328 ± 1.449	.049
Ulceration	13 (16.9%)	23 (36.5%)	.011
Tumor-infiltrating lymphocytes	16 (20.8%)	4 (6.3%)	.016
Pigmentation	63 (81.8%)	56 (88.9%)	.342

HPF, high-power field; YAP, yes-associated protein.

to compare the proliferative activity of melanomas between YAP expression groups. Mean mitotic activity was higher in melanomas with nuclear YAP expression (3.293 ± 4.425) than in those with cytoplasmic YAP expression (1.809 ± 2.201) (P = .016). We conducted a microscopic examination of additional melanoma characteristics in the included cases, including tumor ulceration, TIL, and pigmentation. The findings indicated a higher prevalence of ulceration in melanomas exhibiting nuclear YAP expression in contrast to those with cytoplasmic YAP expression (P = .011). Conversely, melanomas exhibiting cytoplasmic YAP expression demonstrated a higher TIL score compared with those with nuclear YAP expression (P = .016). Nevertheless, there was no statistically significant difference in pigmentation among melanomas based on YAP expression (P =.342).

Features of Melanoma Cells According to Cellular Location of Yes-Associated Protein

After quantifying YAP in five melanoma cell lines (B16F1, B16F10, B16BL6, K-1735, and Clone M-3), in whole lysate and nuclear/cytoplasmic fractionations, we chose B16F1 and B16F10 to produce mutant cell lines (Supplementary Information S1). We used these mutant lines to explore the features of melanoma cells with nuclear YAP expression. We produced mutant B16F1 and B16F10 cell lines with YAP nuclear overexpression, in which YAP was translocated to the nucleus by substituting serine 127 with alanine (Yap^{S127A}). For comparison, we also created *Yap*-KD melanoma lines (*siYap*) using B16F1 and B16F10 cells.

Table 2
Univariate logistic regression analysis for metastasis to lymph nodes and distant organs

Factor	Lymph node metastasis			Distant metastasis		
	HR	95% CI	*P*	HR	95% CI	*P*
YAP location (cytoplasm vs nucleus)	2.270	0.914-5.637	.077	3.206	1.032-9.961	.044
Age at diagnosis	0.997	0.963-1.032	.858	0.969	0.931-1.010	.135
Subtype			.229			.806
vs NM	0.393	0.126-1.227	.108	1.222	0.385-3.875	.734
vs SSM	1.148	0.406-3.243	.794	0.709	0.163-3.077	.646
Breslow thickness	1.279	1.076-1.520	.005	1.197	1.016-1.409	.031
Mitosis	0.995	0.948-1.044	.831	0.965	0.903-1.032	.299

HR, hazard ratio; NM, nodular melanoma; SSM, superficial spreading melanoma; YAP, yes-associated protein.

Table 3
Cox regression analysis for survival of patients with cutaneous melanoma

Factor	Univariate analysis						Multivariate analysis					
	Overall survival			Disease-free survival			Overall survival			Disease-free survival		
	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
YAP location (cytoplasm vs. nucleus)	3.019	1.508-6.045	.002	3.433	1.973-5.973	<.0001	1.221	0.444-3.362	.699	2.493	1.254-4.953	.009
Age at diagnosis	1.02	0.992-1.049	.157	1.014	0.993-1.036	.182	1.028	0.990-1.068	.156	1.008	0.984-1.032	.528
Subtype			.25			.045			.593			.565
vs. NM	1.41	0.867-2.293	.166	1.322	0.702-2.490	.387	0.552	0.176-1.726	.307	1.033	0.519-2.055	.387
vs. SSM	1.128	0.579-2.198	.723	0.418	0.186-0.938	.034	0.886	0.301-2.605	.825	0.638	0.270-1.505	.304
Breslow thickness	1.148	1.063-1.240	<.0001	1.143	1.074-1.217	.000	1.009	0.897-1.135	.88	1.066	0.978-1.163	.146
Mitosis	1.021	0.994-1.049	.135	1.013	0.988-1.039	.308	1.021	0.982-1.062	.294	0.995	0.966-1.024	.715
LN metastasis	5.783	2.777-12.043	<.0001	–	–	–	3.867	1.410-10.601	.009	–	–	–
Distant metastasis	5.385	2.750-10.546	<.0001	–	–	–	3.207	1.315-7.821	.010	–	–	–

HR, hazard ratio; LN, lymph node; NM, nodular melanoma; SSM, superficial spreading melanoma; YAP, yes-associated protein.

Decreased YAP expression in *Yap*-KD cells was confirmed by western blotting (Fig. 2A) and immunofluorescence imaging (Fig. 2B). We also evaluated changes in the expression of Hippo signaling pathway kinases that inhibit YAP expression when they are activated. Decreased expression of LATS1 and phosphorylated LATS1/LATS2 was observed in *Yap*-KD cells (Fig. 2C).

In Yap^{S127A} cells, overexpression of YAP and nuclear expression of YAP were confirmed by western blotting (Fig. 2D) and immunofluorescence imaging (Fig. 2E). Analysis of changes in the expression of Hippo signaling kinases in Yap^{S127A} cells revealed an increased expression of LATS2 and phosphorylated LATS1 (Fig. 2F). These results suggest that YAP expression and its cellular location may be directly regulated by kinases of the Hippo signaling pathway in melanoma cells.

Proliferative Activity of Melanoma Cells According to Cellular Yes-Associated Protein Location

Based on our finding that mitotic activity was increased in human melanoma tissues with nuclear YAP expression (Table 1), we used flow cytometry to estimate the percentage of cells in each phase of the cell cycle in each melanoma cell line. In *Yap*-KD cells (*siYap*B16F1 and *siYap*B16F10), we observed relative gap1 phase retention, in comparison to control cells ($P < .001$ for both cell lines; Fig. 3A). In Yap^{S127A} B16F1 cells, more cells had entered the synthesis (S) phase, compared with control cells ($P = .008$), although no differences in cell cycle populations were observed between Yap^{S127A} B16F10 and control cells (Fig. 3B). These results suggest that changes in the cell cycle of melanoma cells with nuclear YAP localization may lead to increased cell division.

To assess proliferative activity according to nuclear YAP expression, we performed viability assays in *Yap*-KD and Yap^{S127A} cells. Compared with their corresponding controls, *Yap*-KD cells exhibited less proliferative activity ($P < .001$ for B16F1; $P = .001$ for *siYap*B16F10; Fig. 3C), whereas Yap^{S127A} cells exhibited increased proliferative activity ($P = .003$ for B16F1; $P = .002$ for B16F10; Fig. 3D). These in vitro results indicate that YAP cellular location affects proliferative activity of melanoma cells.

Next, we performed xenograft mouse experiments to confirm changes in proliferative activity in vivo according to the cellular location of YAP using *Yap*-KD cells and Yap^{S127A} mutant cells. Xenograft mice injected with *Yap*-KD B16F1 cells had smaller tumor volumes than mice injected with control cells ($P < .0001$; Fig. 3E). Reduced YAP expression in the tumor cells of *Yap*-KD B16F1 xenograft mice was confirmed by western blot analysis after sacrificing the mice. When we injected Yap^{S127A} B16F1 cells and corresponding control cells into BALB/c mice, animals receiving Yap^{S127A} B16F1 cells had larger tumor volumes than control mice ($P < .001$; Fig. 3F). Increased YAP expression in the tumor cells of Yap^{S127A} B16F1-injected mice was confirmed by western blotting.

Invasiveness of Melanoma Cells According to Yes-Associated Protein Expression

In our study population, invasion of melanoma cells was deeper in patients with nuclear YAP expression than in those with cytoplasmic YAP expression, according to Clark level and Breslow thickness (Table 1). To explore this further, we performed wound healing assays to evaluate differences in invasiveness of melanoma cells according to YAP cellular location. After producing a wound by scratching cell cultures with a pipette, we measured the migrated distance of tumor cells across the gap. The migrated

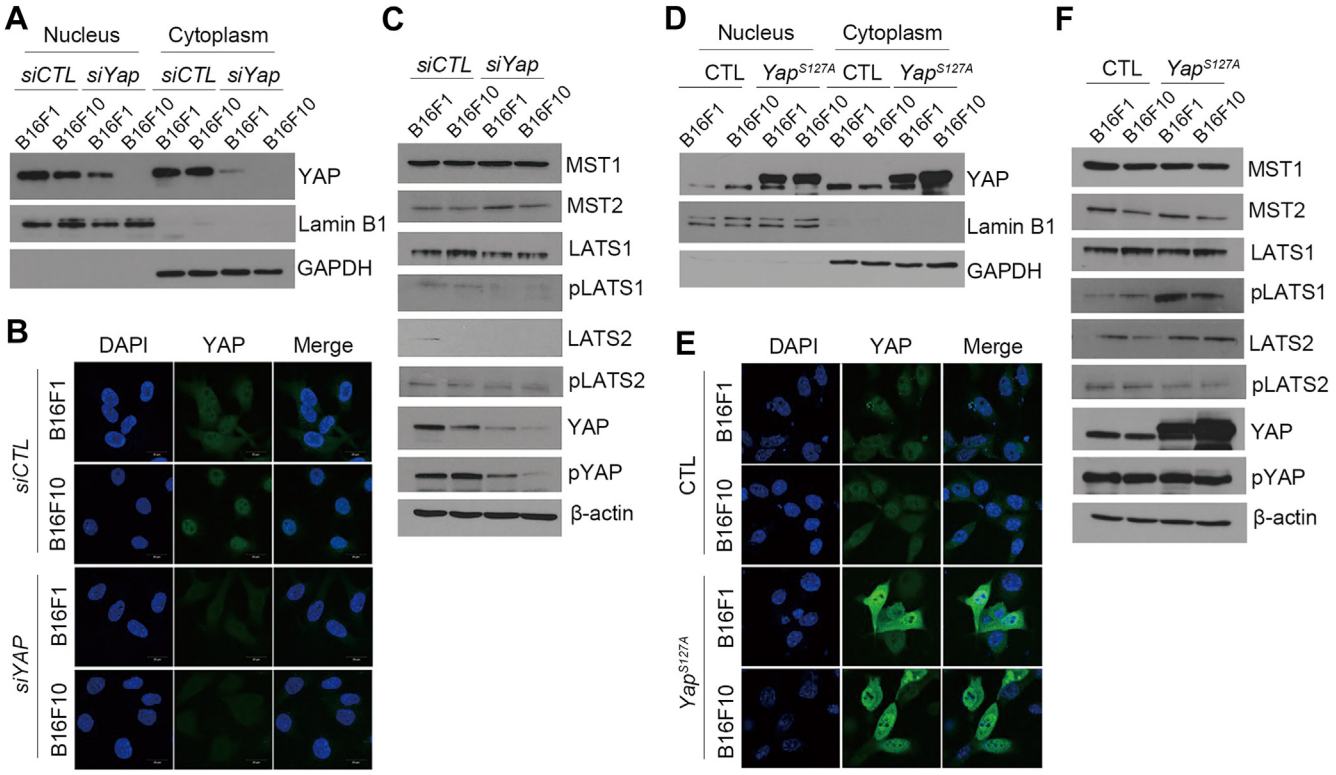

Figure 2.
Hippo signaling pathway in *Yap*-knock down (KD) and yes-associated protein (YAP)-overexpressing melanoma cells. (A) Nuclear/cytoplasmic (N/C) fraction of B16F1 and B16F10 cells with *Yap*-KD. (B) Immunofluorescent images of YAP expression in *Yap*-KD cell lines. (C) Kinases associated with Hippo signaling pathways of *Yap*-KD cell lines. (D) N/C fraction of B16F1 and B16F10 cells with YAP overexpression. (E) Immunofluorescent images of YAP expression in YAP-overexpressing cell lines. (F) Kinases associated with Hippo signaling pathways of YAP-overexpressing cell lines. CTL, control.

distance was shorter (ie, migration was less) in *Yap*-KD cells than in control cells ($P = .0006$ for *siYap*B16F1; $P < .0001$ for *siYap*B16F10; Fig. 4A). Conversely, the migrated distance was longer in mutant cells with nuclear YAP expression, compared with control cells ($P = .0001$ for Yap^{S127A}B16F1; $P = .0172$ for Yap^{S127A}B16F10; Fig. 4B). Thus, the wound healing assays demonstrated that invasion of melanoma cells was regulated by the cellular location of YAP, consistent with the results in human melanoma tissues.

Treatment Effects of a Direct Yes-Associated Protein Inhibitor on Melanoma Cells

We investigated the treatment effects of verteporfin, a direct inhibitor of YAP, on melanoma cells expressing YAP. We exposed B16F1 and B16F10 cells to 2 μM verteporfin and performed viability assays 24 hours later. Verteporfin inhibited cellular proliferation of melanoma cells more effectively than solvent ($P = .003$ for B16F1; $P < .001$ for B16F10; Fig. 5A). Western blot analysis confirmed reduced YAP expression in melanoma cells treated with verteporfin (Fig. 5B). These results suggest that verteporfin targeted YAP-expressing melanoma cells, directly inhibiting YAP expression in vitro.

Discussion

In this study, we demonstrated the proliferative and proinvasive properties of nuclear YAP localization in human cutaneous melanoma tissues and melanoma cell lines and evaluated the effects of a direct YAP inhibitor in melanoma cells. As shown in our results, human melanomas with nuclear YAP expression recurred and metastasized to LNs and distant organs more frequently than melanomas with cytoplasmic YAP expression. Accordingly, melanoma patients with nuclear YAP expression had poorer DFS and OS than patients with cytoplasmic YAP expression, but nuclear localization of YAP was also an independent risk factor for tumor recurrence. A previous report showed that YAP expression was significantly higher in patients with LN metastasis than in patients without metastases.[22] YAP has also been demonstrated to promote spontaneous melanoma metastasis in vivo, suggesting that tumor progression is regulated by YAP in human melanoma.[23] However, detailed mechanisms of the association of YAP with recurrence and metastasis have not been previously examined in human melanoma.

Activation of YAP has been shown to promote resistance to anticancer therapies in various malignancies.[24,25] In melanoma, high-YAP expression is negatively correlated with responsiveness to RAF/MEK inhibitors.[26] Despite recent progress in immune checkpoint therapies for treating melanoma and other cancers, it is not fully understood how immune tolerance is installed in the tumor infiltrate.[27] Previously, we reported a pivotal role of actin remodeling in YAP-dependent *BRAF* inhibitor resistance in *BRAF* V600E mutant melanoma cells.[28] We also reported that nuclear enrichment of YAP in clinical melanoma samples correlates with increased programmed death-ligand 1 (PD-L1) expression and that YAP directly mediates evasion of cytotoxic T-cell immune responses in *BRAFi*-resistant melanoma cells by upregulating PD-L1, suggesting that targeting YAP-mediated immune evasion may

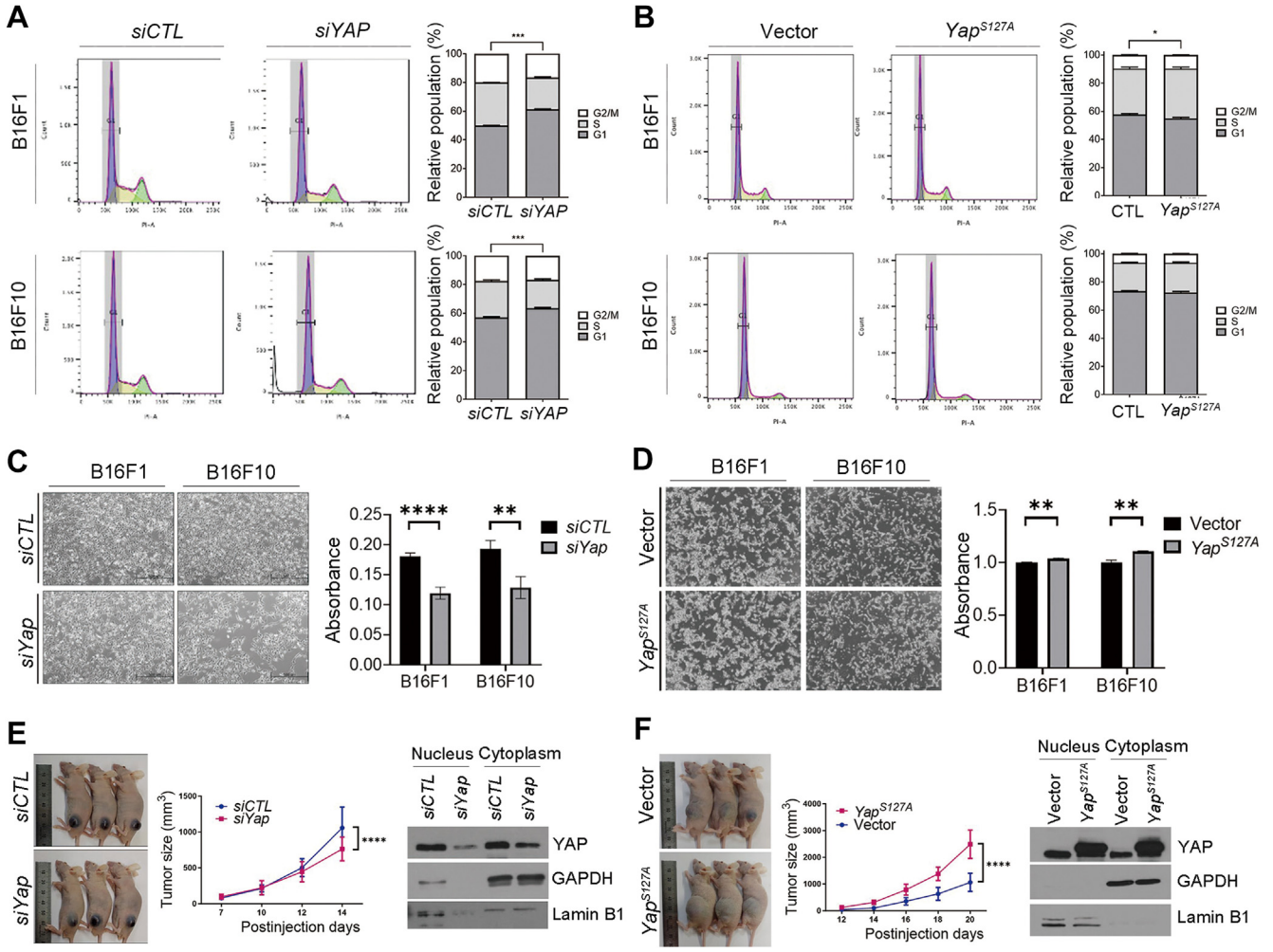

Figure 3.
Proliferative ability of melanoma cells according to yes-associated protein (YAP) expression. (A) Flow cytometry for cell cycle analysis in *Yap*-knock down (KD) melanoma cells. (B) Flow cytometry for cell cycle analysis in YAP-overexpressing melanoma cells. (C) Viability assay in *Yap*-KD melanoma cells. (D) Viability assay in YAP-overexpressing melanoma cells. (E) Xenograft assay for measuring tumor size in mice injected with *Yap*-KD melanoma cells. (F) Xenograft assay for measuring tumor size in mice injected with YAP-overexpressing melanoma cells. CTL, control.

improve the prognosis of patients with melanoma.[19] In the current study, we showed that a direct YAP inhibitor could affect the viability of melanoma cells expressing YAP. Together, our investigations suggest that directly targeting YAP-expressing tumor cells may enhance the therapeutic effectiveness of drugs to which melanoma cells are resistant.

Histopathologically, cellular location of YAP differed between melanoma subtypes, with nuclear YAP location being observed more frequently in acral lentiginous and nodular melanomas. The most common histopathologic subtype in our study population, as in other Asian populations, was acral lentiginous, a melanoma that occurs on glabrous acral skin.[29,30] Acral lentiginous melanoma is associated with higher regional recurrence rates and worse survival than nonacral melanomas,[31] and sex, race, sentinel LN status, and pathologic stage are independent risk factors for survival in patients with this melanoma subtype.[32,33] We showed that acral lentiginous subtype was significantly associated with worse DFS in univariate analysis, although it was not an independent prognostic factor in multivariate analysis. Thus, the unfavorable prognosis of acral lentiginous melanoma may be attributed to high nuclear YAP expression, which adversely affects patient survival.

Sun exposure is the main environmental risk factor for cutaneous melanoma development,[4,34] and melanomas can be categorized into high-chronically sun damaged (CSD), low-CSD, and non-CSD, along with their mutational signatures, anatomical site, and epidemiology.[2,35] High-CSD CMs encompass lentigo maligna and desmoplastic melanomas, and low-CSD CMs include superficial spreading melanomas. The non-CSD category includes not only acral melanomas, some melanomas in congenital nevi, melanomas in blue nevi, and Spitz melanomas but also noncutaneous melanomas, such as mucosal melanomas and uveal melanomas (UMs). Nodular melanomas are distributed in all the categories.[35] In our study, we included acral lentiginous, superficial spreading, and nodular melanomas, and the findings revealed a higher frequency of YAP nuclear location in acral lentiginous and nodular subtypes compared with the superficial spreading subtype.

Studies have been conducted to elucidate the progression of melanoma, considering the degree of CSD. Progression in both low- and high-CSD melanomas is typically linked to additional mutations, including TP53 and PTEN mutations, mutations in the telomerase reverse transcriptase promoter, and biallelic loss of CDKN2A.[2,4] Within the non-CSD category of cutaneous melanoma, Spitz melanomas are distinguished by driver fusion genes,

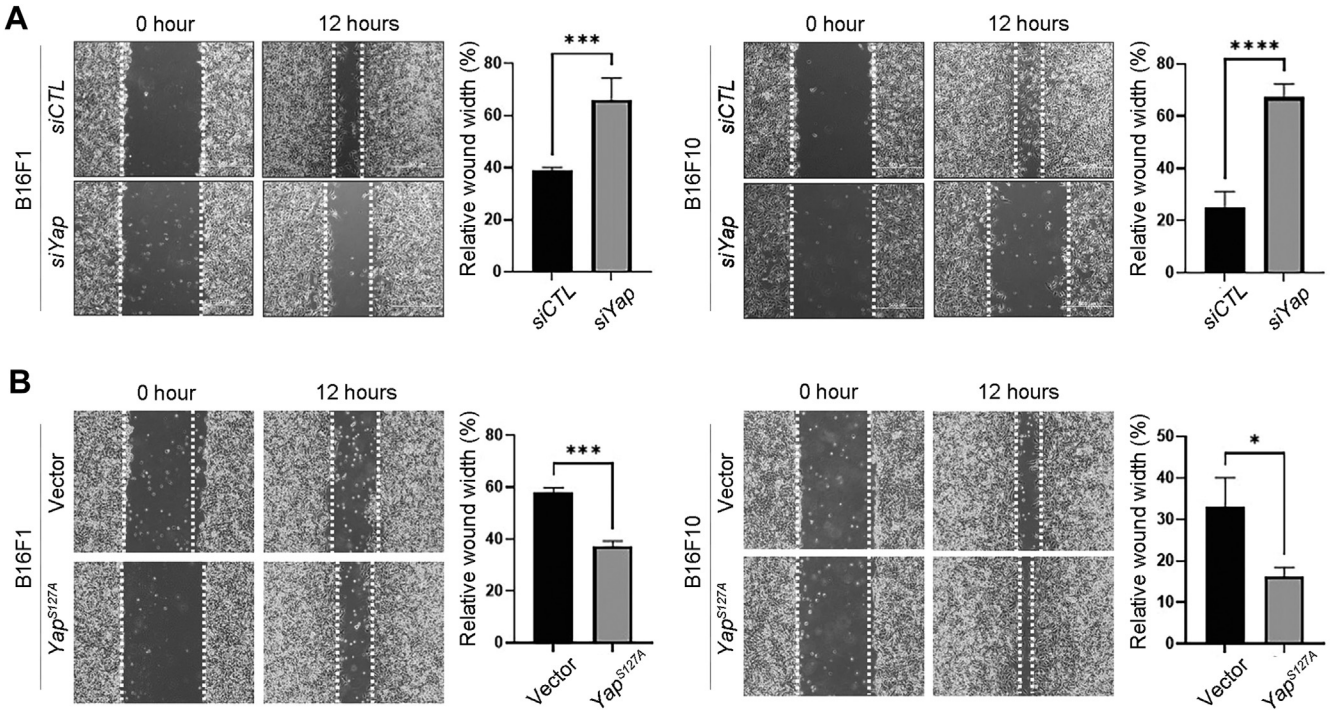

Figure 4.
Wound healing assay for *Yap*-knock down (KD) and yes-associated protein (YAP)-overexpressed melanoma cells. (A) Relative wound width of *Yap*-KD cells at 12 hours after wound creation. (B) Relative wound width of YAP-overexpressing cells at 12 hours after wound creation. CTL, control.

encompassing the kinase domains of ALK, ROS1, NTRK1, NTRK3, MET, RET, BRAF, and MAP3K8. On the other hand, acral melanomas exhibit a high frequency of copy number variations, with gene amplifications observed in CCND1 and KIT.[35] In association with the Hippo signaling pathway, although BRAF inhibitor-resistant melanoma cells were shown to depend on YAP/TAZ for their proliferation and survival,[28] YAP/TAZ activity is not associated with the mutation status of BRAF and NRAS.[23] This aligns with observations indicating that the sensitivity of YAP/TAZ in melanoma cells is not associated with either the BRAF or NRAS mutation status.[36] Meanwhile, UM, classified within non-CSD melanoma category, is identified by GNAQ/11 mutations, and it is recognized that the initiation and progression of UM are driven by YAP/TAZ activation;[37] however, there was no correlation found between YAP activity and the survival of UM patients or melanoma cell lines in our prior study.[38]

To investigate whether YAP could influence melanogenesis, we evaluated melanin pigmentation based on YAP expression, but the intracellular YAP localization did not have a significant impact on the pigmentation of melanoma cells in human melanoma tissues. It has been reported that when LATS1, an upstream kinase of Hippo signaling pathway that negatively regulates YAP, is ablated, a significantly decreased expression of melanogenesis markers and melanin synthesis in melanocyte and melanoma cell lines occurs.[39] Additionally, reduced melanin content in *LATS1* knocked down tumors was associated with increased tumor growth. However, YAP expression based on LATS1 level or changes in melanogenesis according to YAP level in melanocytes or melanoma cells has not been studied to date. Future research endeavors should be undertaken to delve into the direct relationship between YAP and melanoma pigmentation.

It has been demonstrated that YAP and TAZ, both effector molecules in the Hippo signaling pathway, directly enhance the expression of the immune checkpoint molecule PD-L1, and consequently, this suppression of T-cell function occurs.[40] In the context of melanoma, our previous research demonstrated that

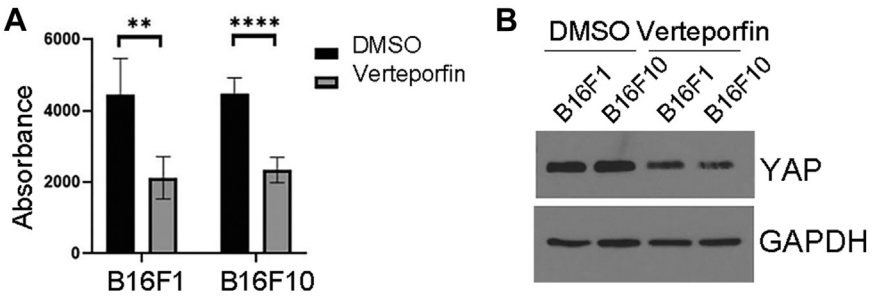

Figure 5.
Drug treatment assay of melanoma cell lines after exposure to verteporfin. (A) Viability assay of verteporfin-treated melanoma cells. (B) Western blot showing yes-associated protein (YAP) expression in verteporfin-treated cells.

YAP inhibition led to the regulation of PDL1 expression and directly inhibited cytotoxic T cells, which improved BRAF inhibition efficacy and patient survival as a result.[19] In this study, we found that melanomas with cytoplasmic YAP expression showed a higher TIL score compared with those with nuclear YAP expression. We suggest that the results presented in this study can be seen as consistent with the findings of previous researchers.

When we evaluated the number of mitotic figures in human melanoma tissues as an indicator of melanoma cell proliferation, our results showed that mitoses were more frequent in melanomas with nuclear YAP than in those with cytoplasmic YAP. We reproduced the increased mitotic activity of nuclear YAP-expressing melanomas by performing in vitro and in vivo experiments using YAP-overexpressing and underexpressing (*Yap*-KD) melanoma cell lines. When we examined the expression of Hippo signaling pathway kinases that inhibit YAP expression and nuclear translocation when activated, expressions of both LATS1 and LATS2 varied according to YAP expression. Expression of these kinases was increased when YAP was overexpressed and decreased when YAP was underexpressed. These findings suggest that YAP expression in melanoma is directly regulated by the Hippo signaling pathway.

Cell cycle analysis revealed that YAP nuclear overexpression induced changes in cellular populations, leading to more melanoma cells entering the S phase compared with YAP underexpression. In xenograft mice, tumors were larger in mice that were injected with Yap^{S127A} melanoma cells than in those injected with *Yap*-KD cells. Dysregulation of the Hippo pathway leads to hyperactivity of YAP, ultimately promoting the transcription of genes involved in cancer survival, proliferation, migration, and invasion,[11,41] and the role of the Hippo signaling pathway in cell cycle regulation of human cancers has been previously investigated.[42,43] One study showed that LATS1/2 double-knockout cells induced YAP overexpression, a prolonged S phase, and increased DNA replication, compared with control cells.[44] Another study showed that YAP is required for S phase entry, and its absence causes retention of cells in the relative gap1 phase.[45] Our results showing higher proliferative activity in nuclear YAP-expressing cells are consistent with these previous findings.

We found that deeper tumor cell invasion was more frequent in melanoma tissues with nuclear YAP expression than in those with cytoplasmic YAP expression. This finding is consistent with our wound healing assay results showing that cell migration was increased in melanoma cell lines overexpressing YAP but inhibited in lines underexpressing YAP. Statistical analysis revealed that deeper invasion was associated with LN and distant organ metastasis and, accordingly, was an unfavorable prognostic factor for DFS in patients with melanoma in univariate analysis. The proinvasive activity of YAP as the Hippo pathway effector in melanoma has been previously investigated,[18,46] although the underlying mechanisms are not fully understood. A previous study using unbiased genomic approaches followed by cell-based assays demonstrated that the role of YAP in melanoma invasiveness is dependent on transcriptional enhanced associate domain (TEAD) transcription factor 1, TEAD2, TEAD3, and TEAD4.[47] Another study reported that YAP-mediated transcriptional activity strongly correlated with published gene expression profiles linked to melanoma cell invasiveness and that YAP target genes (*AXL*, *THBS1*, and *CYR61*) are key mediators of YAP-induced melanoma cell invasion.[23] However, work remains to be done to understand exactly how YAP regulates the invasion of tumor cells in the context of melanoma.

We conducted both in vitro and in vivo experiments using murine B16F1 and B16F10 melanoma cell lines that originate in the syngeneic C57BL/6 (H-2b) mouse strain, although these cell lines are murine and may have limitations in studying human melanoma. We assessed YAP expression levels in various melanoma cell lines through western blotting, utilizing nuclear/cytoplasmic fractionation to identify cell lines expressing YAP in both the nucleus and cytoplasm. This selection was crucial for generating cell lines with knocked down and overexpressed YAP mutants. Following the quantification of YAP in melanoma cell lines, we opted for the B16F1 and B16F10 melanoma cell lines. These cell lines exhibited suitable nuclear and cytoplasmic expressions, allowing us to observe significant changes in their mutant cells with Yap^{S127A} or *siYap*. Additionally, we examined the treatment effects of verteporfin, a direct YAP inhibitor, on B16F1 and B16F10 melanoma cells. The B16 cell lines have been applied in many studies evaluating the efficacy and pharmacodynamics of anticancer therapeutics.[48]

We performed in vivo experiments with B16F1 melanoma cell line to make a subcutaneous xenografted mouse model. The subcutaneous model is widely used for the evaluation of therapy in many tumor models, including B16 melanoma cell lines.[48] Upon subcutaneous injection with B16 melanoma cell lines, tumor volume evolution throughout days after inoculation was well known.[49] It is important to note that for subcutaneous tumor growth experiments, consistent and reproducible results can be obtained when a well-established experimental protocol is utilized.[48,49] For these reasons, B16 melanoma cell lines have been widely used in melanoma research, and in our study, meaningful results were obtained by conducting xenograft experiments using B16F1 cell line. We decided to use nude mice for the xenograft experiments because it allows for direct observation of the formation of tumors after injecting melanoma cells into the mice, and after the formation of tumors, tumor dimensions were measured with a caliper. Therefore, we analyzed differences in tumor growth by injecting control and mutant murine melanoma cell lines into immune-compromised mice instead of injecting them into the corresponding C57BL/6J.

Verteporfin, a Food and Drug Administration–approved drug widely utilized as a photosensitizer in photodynamic therapy, has been demonstrated to directly bind to YAP, preventing its interaction with TEAD factors and thereby inhibiting its transcriptional activity.[50-53] The previous study showed that verteporfin decreased nuclear YAP levels and function as a consequence of trapping YAP in the cytosol and inhibiting cell proliferation.[54] Preclinical data also suggest the use of verteporfin as a treatment for various cancers, including endometrial cancer, ovarian cancer, neuroblastoma, breast cancer, and gastric cancer.[54-58] We explored the potential therapeutic role of verteporfin in YAP-expressing melanoma and observed a decrease in YAP levels in melanoma cell lines following verteporfin treatment. However, investigations into the intracellular mechanisms occurring in melanoma cell lines after verteporfin treatment, as well as assessments of the therapeutic effects on melanoma cells under physiological conditions, were not addressed in this study.

In this study, we investigated the function of YAP in cutaneous melanoma according to its cellular location to highlight its relevance in predicting tumor behavior and show the potential therapeutic benefits of administering a direct YAP inhibitor in melanoma cells. Our preclinical data can be used to advance the understanding of the pathophysiology underlying melanoma progression, indicate that nuclear YAP may be a useful prognostic marker in cutaneous melanoma, and suggest

the possibility of a YAP inhibitor as a therapeutic option for patients with melanoma.

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Author Contributions

S.K.K. performed study conception and design; H.J.R., C.K., C.T., and S.K.K. developed the methodology and wrote, reviewed, and revised the paper; H.J.R., C.K., and S.K.K. acquired, analyzed, and interpreted the data and performed the statistical analysis; and S.J.S. and K.Y.C provided technical and material support. All authors read and approved the final manuscript.

Data Availability

The data sets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Declaration of Competing Interest

The authors declare no competing financial interests.

Ethics Approval and Consent to Participate

All methods and experimental protocols using human tissues were conducted in accordance with relevant guidelines and regulations approved by the Institutional Review Board of Severance Hospital, Yonsei University Health System (IRB no. 4-2018-0469). All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Supplementary Material

The online version contains supplementary material available at https://doi.org/10.1016/j.labinv.2024.102048

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