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Targeting Bmi1 for Enhancing Anoikis Sensitivity and Inhibiting Metastasis in Colorectal Cancer

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Abstract. *Background/Aim: Patients diagnosed with advanced metastatic colorectal cancer (CRC) confront a bleak prognosis characterized by low survival rates. Anoikis, the programmed apoptosis resistance exhibited by metastatic cancer cells, is a crucial factor in this scenario. Materials and Methods: We employed bulk flow cytometry and RT-qPCR assays, conducted in vivo experiments with mice and zebrafish, and analyzed patient tissues to examine the effects of the B cell-specific Moloney murine leukemia virus insertion site 1 (Bmi1)-midkine (MDK) axis on the cellular response to anoikis. Bmi1 is pivotal in tumorigenesis. This study elucidated the involvement of Bmi1 in conferring anoikis resistance in CRC and explored its downstream targets associated with metastasis. Results: Elevated levels of Bmi1 expression correlated with distant metastasis in CRC. Suppression of Bmi1 significantly diminished the metastatic potential of CRC cells. Inhibition of Bmi1 led to an increase in the proportion of apoptotic SW620 cells detached from the matrix. This effect was further enhanced by the addition of irinotecan, a topoisomerase I inhibitor. Furthermore, Bmi1 was found to synergize with MDK in modulating CRC viability, with consistent expression patterns observed in in vivo models and clinical tissue specimens. In summary, Bmi1 acted as a*

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regulator of CRC metastatic capability by conferring anoikis resistance. Additionally, it collaborated with MDK to facilitate invasion and distant metastasis. Conclusion: Targeting Bmi1 may offer a promising adjunctive therapeutic strategy when administering traditional chemotherapy regimens to patients with advanced CRC.

Colorectal cancer (CRC) stands as the third most prevalent malignancy globally, with projections anticipating an increase to 3.2 million new cases and 1.6 million deaths by 2040 (1). CRC ranks second in cancer-related mortality worldwide and is the primary cause of death among men under 50 years old in the US (2). Recent statistics from the US indicate a concerning trend of CRC diagnoses occurring at younger ages, in more advanced stages, and predominantly in the left colon/rectum (2). Despite the widespread adoption of diagnostic modalities and endoscopic screenings for early cancer detection, approximately 20% of the patients are diagnosed with advanced metastatic CRC, bearing a grim five-year survival rate of 14–15% (2).

It has been documented that <0.1% of tumor cells disseminate into circulation and go on to form metastases (3). The process of cancer cell metastasis entails a highly orchestrated sequence, including invasion into surrounding tissue, survival within the circulatory system, infiltration of lymphatic or blood vessel walls, and proliferation in distant organs (3). An essential aspect of this process is programmed apoptosis triggered by cell detachment from the extracellular matrix, known as anoikis, crucial for maintaining tissue homeostasis (4, 5). Cancer cells can evade anoikis by activating pro-survival pathways, undergoing epithelial–mesenchymal transition (EMT), and altering integrin expression patterns (5). Notably, resistance to anoikis stands as an independent prognostic factor for poor outcomes in patients with CRC, although the underlying mechanisms remain incompletely understood (6-8).

The polycomb group protein B-lymphoma Moloney murine leukemia virus Insertion region-1 (Bmi1) functions as a gene silencer involved in various epigenetic modifications (9). Bmi1 plays a crucial role in maintaining cellular stemness, including self-renewal, senescence, and cell cycle regulation, as demonstrated by previous investigations (9, 10). Significantly, Bmi1 expression correlates with CRC progression, invasion, metastasis, and patient survival (11-13). Moreover, Bmi1 facilitates stemness, proliferation, and migration of CRC cells through the activation of EMT (14, 15). Prior studies have shown that Bmi1 drives cancer cell proliferation, invasion, and distant metastasis by imparting resistance to anoikis in melanoma and breast cancer cells (16, 17). In this study, we examined the role of Bmi1 in CRC anoikis resistance and investigated its potential downstream targets associated with CRC metastasis.

Materials and Methods

Cell lines and culture. Human colorectal cancer cell lines SW480 and SW620 were procured from BCRC (Hsinchu, Taiwan, ROC) and maintained in Dulbecco's Modified Eagle's medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). The cells were cultured in a humidified atmosphere at 37°C with 5% CO_2.

Western blotting. The antibody against Bmi1 (GTX114008) was acquired from GeneTex International (Irvine, CA, USA). Antibodies against Tubulin (#2144), GAPDH (#97166), β-actin (#3700), and cleaved caspase 3 (#9661) were purchased from Cell Signaling Technology (Danvers, MA, USA), whereas antibodies against MDK (ab52637) were obtained from Abcam (Cambridge, MA, USA). Cells were washed with ice-cold phosphate-buffered saline and lysed. Equal amounts of cellular proteins were collected, loaded onto a sodium dodecyl sulfate-polyacrylamide gel, and then transferred onto nitrocellulose membranes. Immunoblotting was performed using primary and secondary antibodies, followed by visualization and comparison of protein bands on the membranes using enhanced chemiluminescence reagents.

Immunohistochemistry staining and scoring. A human colorectal cancer tissue microarray (CDA3, Biomax Inc. and Super Bio Chips Laboratories, Seoul, Republic of Korea) was utilized. Detailed instructions regarding patient and tumor demographics and clinicopathological details are available on the manufacturer's website. Tissue sections were stained with primary antibodies against Bmi1 and midkine (MDK), appropriate secondary antibodies, and the Envision system (Dako, Glostrup, Denmark). Subsequently, the sections were counterstained with hematoxylin and examined under a microscope. Expression intensity was categorized as "low" or "high" based on the proportion of positively stained tumor cells (18). We collected 43 clinical tissue specimens from patients with varying stages of colorectal cancer, monitored from 2009 to 2019 at E-Da Hospital, Kaohsiung, Taiwan, ROC (EMRP-109-012 and BIRB-109-002). All patients underwent pathological diagnosis of CRC through endoscopic or surgical biopsy, followed by regular post-diagnosis follow-up until death or the conclusion of the study period. Clinical data, including age, sex, histological grade, comorbidities, treatment regimen (*e.g.*, irinotecan, bevacizumab), recurrence, and survival status, were retrieved from the Cancer Database of E-Da Hospital. Expression levels of Bmi1 and its target proteins in these specimens were assessed.

Zebrafish assay. Zebrafish [strain fli1: enhanced green fluorescent protein (EGFP)] were sourced from the Zebrafish Core Facility at the Center for Laboratory Animals, Kaohsiung Medical University, Kaohsiung, Taiwan. The care and maintenance of the zebrafish were conducted in strict compliance with standard animal care regulations and protocols of the Animal Center at Kaohsiung Medical University, Taiwan, ROC. These zebrafish feature eGFP under the fli1 promoter, specifically expressed in endothelial cells, enabling visualization of both the blood and lymphatic vascular systems. To elaborate, shLuci and shBmi1 SW-620 cancer cells were labeled using the PKH26 Red Fluorescent Cell Linker Kit (Merck KGaA, Darmstadt, Germany) and subsequently microinjected into the perivitelline cavity of 2-day-old zebrafish embryos. Upon confirmation of the localized PKH26-labeled cell mass at the

injection site, the zebrafish embryos were transferred to fresh water and maintained at 32.5˚C. Vascular sprouts were then observed utilizing a Nikon Eclipse Ti-S 217 microscope (Tokyo, Japan) (19).

Animal study. BALB/c-null mice were procured from the National Laboratory Animal Center in Taipei City, Taiwan, ROC. At 8 weeks of age, six mice from each group underwent anesthesia with isoflurane, followed by injection of SW620 cells (2×10^6; 1:1 mixed with Matrigel) into the cecum. The mice were carefully returned to the peritoneal cavity and sutured (20). After two months, the mice were weighed and anesthetized using CO_2. All experimental procedures were conducted in accordance with the Animal Care and Use Guidelines of Kaohsiung Medical University, Taiwan, ROC and approved by the Animal Care and Use Committee of Kaohsiung Medical University (IACUC protocol No. 107202).

Migration and invasion assay. Transwell units were employed for conducting migration and invasion assays. Briefly, 3,000 cells in 100 μl of medium with vehicle were placed in the upper part of the Transwell unit and allowed to invade for 24 h. The lower part of the Transwell unit was filled with lymphatic endothelial cells. Following incubation, invading cells on the bottom surface of the membrane were fixed in formaldehyde, stained with Giemsa solution, and enumerated under a microscope.

RNA interference transfection. To down-regulate Bmi1 expression in SW620 cells, small hairpin RNAs (shRNAs) were employed. The sh-Bmi1 and sh-luciferase (sh-Luci) plasmids were sourced from the National RNAi Core Facility (Academia Sinica, Taiwan, ROC). The sequences for sh-Bmi1#1 and sh-Bmi1#2 were as follows: sh-Bmi1#1: 5'-CAGATTGGATCGGAAAGTAAA-3'; sh-Bmi1#2: 5'-ATTGATGCCACAACCATAATA-3'; sh-Luci: 5'-CTTCGAAAT GTCCGTTCGGTT-3'. Cells were transfected with appropriate amounts of non-targeting and specific shRNAs using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. Transfected cells were selected using puromycin, and the efficacy of Bmi1 silencing was evaluated using real-time reverse transcription-polymerase chain reaction.

Flow cytometry analysis. SW620 cells transfected with sh-Bmi1 were harvested by trypsinization and fixed with 70% ice-cold ethanol overnight at –20˚C. The following day, the cell pellet was suspended in propidium iodide (PI) staining buffer (50 μl/ml PI, RNAse A, Beckman Coulter, Brea, CA, USA) and then incubated for 15 min at 37˚C for subsequent cell cycle analysis. The distribution of the cell cycle was assessed using FACSCalibur (BD Biosciences, San Diego, CA, USA) and analyzed with ModFit software.

Quantitative real-time polymerase chain reaction (qPCR). Total RNA was isolated using the TRIzol reagent (Invitrogen) and reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's protocol. The resulting cDNA was utilized as a template for PCR amplification. Quantitative real-time PCR was conducted in a 20 μl reaction volume using the standard protocols provided with the Roche LightCycler 480 II system (Basel, Switzerland). The primer sequences were as follows: MDK forward: 5'-AAGGAGTTTGGAGCCGACTG-3', reverse: 5'-CATT GTAGCGCGCCTTCTTC-3'.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays were conducted utilizing a Millipore ChIP kit (Merck Millipore, Darmstadt, Germany), with modifications to the manufacturer's protocol. During the DNA fragmentation step, SW620 cells, along with SW620 cells depleted of Bmi1 or treated with PTC209, underwent sonication for 45 min each using high and low sonication settings, respectively. Protein pull-down assays utilized specific antibodies against RNA polymerase II. Subsequently, DNA from the pull-down was purified employing the MiniElute PCR purification kit following the manufacturer's guidelines (Qiagen, VIC, Australia). The purification process was accompanied by the following PCR protocol: 95˚C for 2 min, followed by 35 cycles of 95˚C for 45 s, 58˚C for 45 s, and 72˚C for 45 s, with a final extension at 72˚C for 7 min. Primer pairs utilized for the MDK promoter chip sequences were as follows: (forward) 5'-GGCGGCCGGAGCGGGACGGG-3' and (reverse) 5'-GGGG CGGCCCCTCGCCGCTA-3'.

Statistical analysis. Associations among various groups of cell-based experiments were evaluated using a two-tailed Student's *t*-test. Differences in immunohistochemical staining intensity among subgroups were assessed using either the chi-square test or Fisher's exact test. Pearson analysis was employed to examine the correlation between Bmi1 and target proteins. Kaplan–Meier survival analysis was utilized to illustrate prognostic differences between the subgroups. Statistical significance was defined as $p<0.05$. Statistical analyses were conducted using the Statistical Package for the Social Sciences (Chicago, IL, USA) version 25.0.

Results

Impact of Bmi1 expression on CRC metastasis. Initially, we examined tissue microarrays through immunohistochemical staining, revealing consistently stronger Bmi1 expression in metastatic tumors compared to primary sites across all four representative specimens (Figure 1A). Subsequently, we evaluated Bmi1 protein expression in two CRC cell lines, SW480 and SW620, originating from primary and metastatic colon adenocarcinomas, respectively, in the same patient. As depicted in Figure 1B, SW620 cells exhibited elevated levels of Bmi1 protein, prompting their selection for further cell-based experiments due to their heightened Bmi1 expression and enhanced metastatic potential. Consistent with these observations, the zebrafish model demonstrated a significant reduction in metastatic ability within the SW620 Bmi1-knockdown groups (Figure 1C). Furthermore, we conducted western blot analysis on detached SW620 cells collected at various time points (0, 6, 16, and 24 h), revealing escalating levels of Bmi1 expression, underscoring its involvement in regulating metastatic capability (Figure 1D).

Bmi1's role in modulating anoikis resistance in CRC cells. To delve deeper into Bmi1's influence on anoikis resistance in SW620 cells, we generated Bmi1-depleted cells. The transwell migration assay demonstrated a significant reduction in migration ability in Bmi1-knockdown SW620 detached cells at 0 h and 24 h timeframes (Figure 2A). Additionally, flow cytometry analysis of detached SW620, SW620-shBmi1 #1, and SW620-shBmi1 #2 cells collected

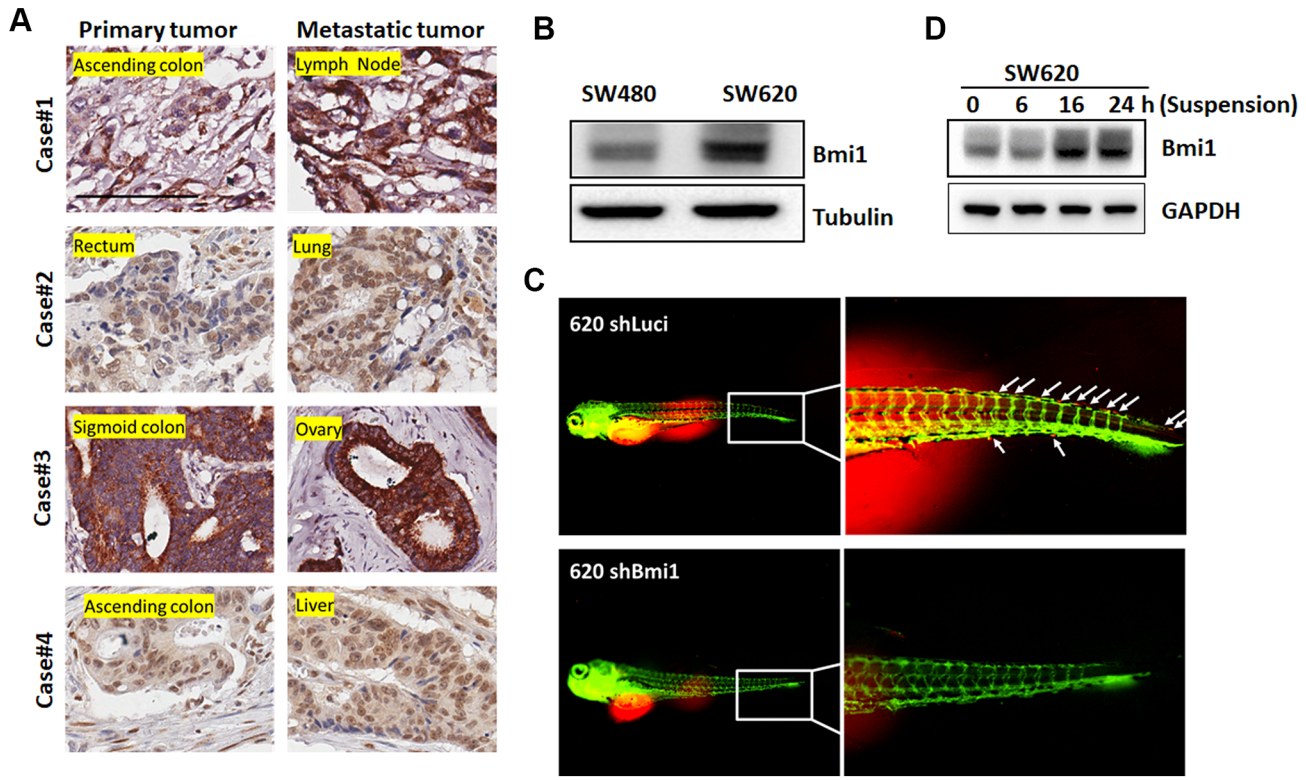

Figure 1. *The level of Bmi1 expression dictates the metastatic potential of colorectal cancer (CRC) cells. (A) Formalin-fixed paraffin-embedded samples from four pairs of CRC patients were stained with the anti-Bmi1 antibody (magnification, 200×). (B) Bmi1 expression was evaluated via western blotting using anti-Bmi1 antibodies in SW480 and SW620 cells. (C) Migration of SW620 cells and Bmi1-depleted cells in zebrafish. (D) Expression of Bmi1 was analyzed via western blotting using anti-Bmi1 antibodies in suspended SW620 cells at specified time points.*

at different time points (0 h, 24 h, 48 h) revealed a notable increase in apoptosis within the Bmi1-knockdown groups (Figure 2B). Similarly, pretreatment with PTC209 (21), a Bmi1 inhibitor, resulted in a dose-dependent increase in apoptosis in detached SW620 cells (Figure 2C). For protein expression analysis, detached cells from both the SW620 and knockdown groups were collected at 24 h. Upon pretreatment of SW620 detached cells with irinotecan, a topoisomerase I inhibitor widely used in metastatic CRC, the knockdown group exhibited heightened caspase-3 expression levels, indicative of increased apoptotic activity (Figure 2D).

Bmi1 up-regulates MDK expression transcriptionally. Previous mammary tumorigenesis models have demonstrated that anoikis resistance and increased angiogenesis are associated with enhanced metastatic spread efficiency (5). Several angiogenic factors and cytokines have been reported to correlate with tumors' ability to resist anoikis, including interleukin-6, chemokine (C-X-C motif) ligand 1, and tumor necrosis factor-α (5, 22, 23). Among these angiogenic factors, MDK, a heparin-binding growth factor, plays a pivotal role in various pro-tumorigenic processes, including those relevant to CRC (24, 25). MDK has been identified as a significant factor in enhancing resistance to anoikis and promoting metastasis in hepatocellular carcinoma cells (24, 25). Thus, our objective was to elucidate the association between Bmi1 and MDK expression in subsequent experiments. As depicted in Figure 3A and B, inhibition of Bmi1 also suppressed MDK expression at both protein and mRNA levels, indicating that Bmi1 regulates downstream MDK expression. Similarly, pretreatment of PTC209 in SW620 cells resulted in reduced MDK expression (Figure 3C and D). Moreover, ChIP assays revealed significantly diminished MDK promoter-binding activity in SW620 cells following Bmi1 knockdown (Figure 3E and F). In summary, Bmi1 directly influences MDK through transcriptional and translational mechanisms.

Positive correlation between Bmi1 and MDK in vivo and in clinical specimens. We further investigated the relationship between Bmi1 and MDK expression in clinical tissue specimens. SW620 sh-Luci and Bmi1-knockdown cells were introduced into the cecum of nude mice, resulting in

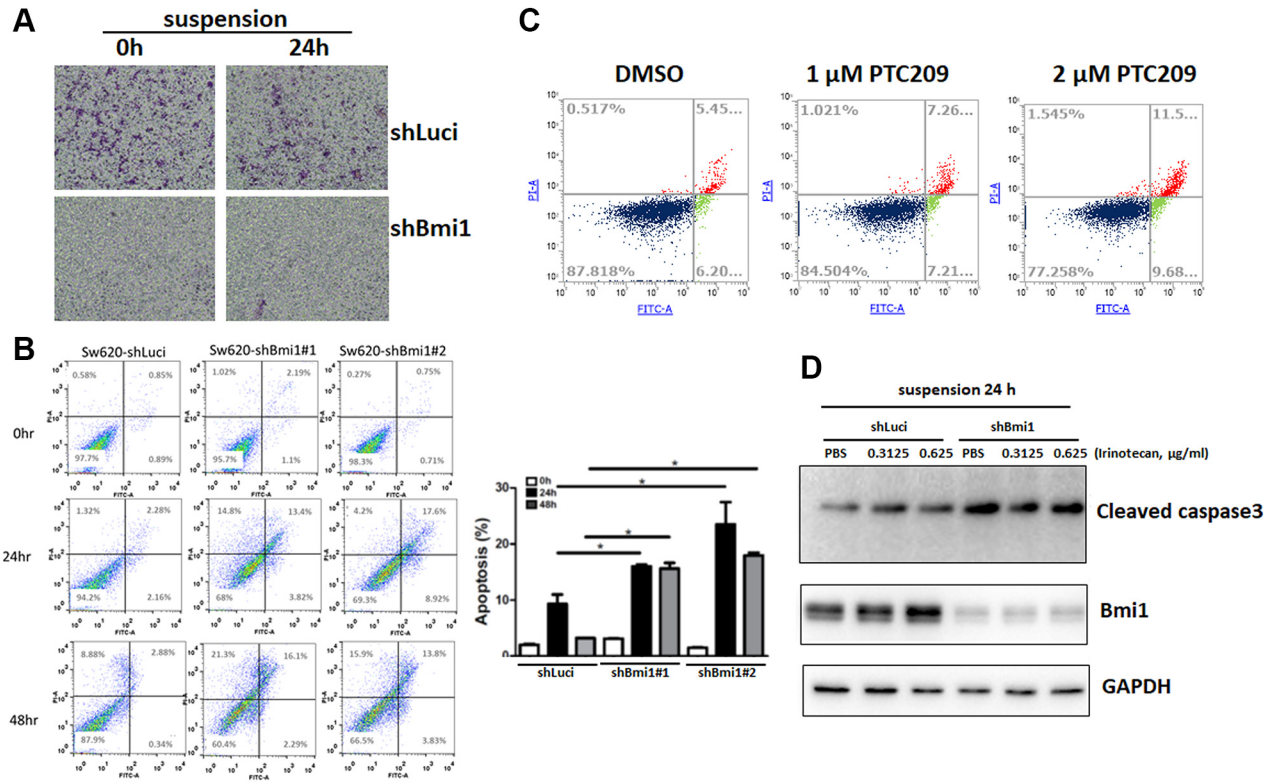

Figure 2. *Bmi1 regulates anoikis resistance in colorectal cancer (CRC) cells. (A) Migration of SW620 cells and Bmi1-depleted cells was assessed using a Transwell assay after 24 h of growth in suspension. (B) The percentage of apoptotic SW620 cells and Bmi1-depleted cells at specified time points in suspension was determined by dual staining with annexin V-FITC and PI. The experiment was replicated thrice, and between-group comparisons were performed using a one-way ANOVA (*p<0.05). (C) The percentage of apoptotic SW620 cells treated with the Bmi1 inhibitor PTC209 at specified time points in suspension was determined by dual staining with annexin V-FITC and PI. The experiments were replicated thrice, and between-group comparisons were performed using a one-way ANOVA (*p<0.05). (D) Expression levels of Bmi1 and cleaved caspase-3 were assessed via western blotting after 48 h of suspension.*

decreased distant metastasis in the knockdown group (Figure 4A). In alignment with this finding, immunohistochemical staining of mouse tissue exhibited reduced expression of MDK in the Bmi1-knockdown group (Figure 4B), underscoring its pivotal role in CRC metastasis. Subsequently, we analyzed clinical tissue specimens from CRC patients. We evaluated 43 CRC patients through immunohistochemical staining for Bmi1 and MDK expression, stratifying them into high and low expression groups. The baseline characteristics of the 43 CRC patients are detailed in Table I. As presented in Table II, the extent of Bmi1 and MDK expression correlated with the patients' survival status ($p<0.05$). Furthermore, high Bmi1 protein expression exhibited a positive correlation with MDK levels ($p<0.05$), indicating a consistent association in clinical tissue specimens (Figure 4C). Finally, both high Bmi1 and MDK expression groups were linked to significantly poorer overall survival and progression-free survival in survival curve analyses (Figure 4D).

Discussion

Abundant cancer cells detach from the primary tumor and enter circulation, yet only a fraction can evade apoptosis and survive (6). During metastasis, cancer cells acquire the ability to thrive and migrate independently of adhesion to the extracellular matrix, thus overcoming anoikis resistance (5). Bmi1 cooperates with H-RAS to promote proliferation, invasion, anoikis resistance, and distant organ metastasis in breast cancer (16). Additionally, Bmi1 induces the expression of invasive gene signatures in melanoma cells, conferring resistance to apoptotic stimuli and enhancing tumor cell survival (17). Our study reveals that Bmi1 collaborates with MDK to modulate anoikis resistance in CRC cells, consequently influencing their migration, invasion, and distant metastasis. Targeting Bmi1 significantly mitigated these processes, leading to a marked increase in apoptotic cell proportion. Thus, Bmi1 emerges as a promising therapeutic target in conjunction with traditional chemotherapy regimens such as irinotecan for treating CRC.

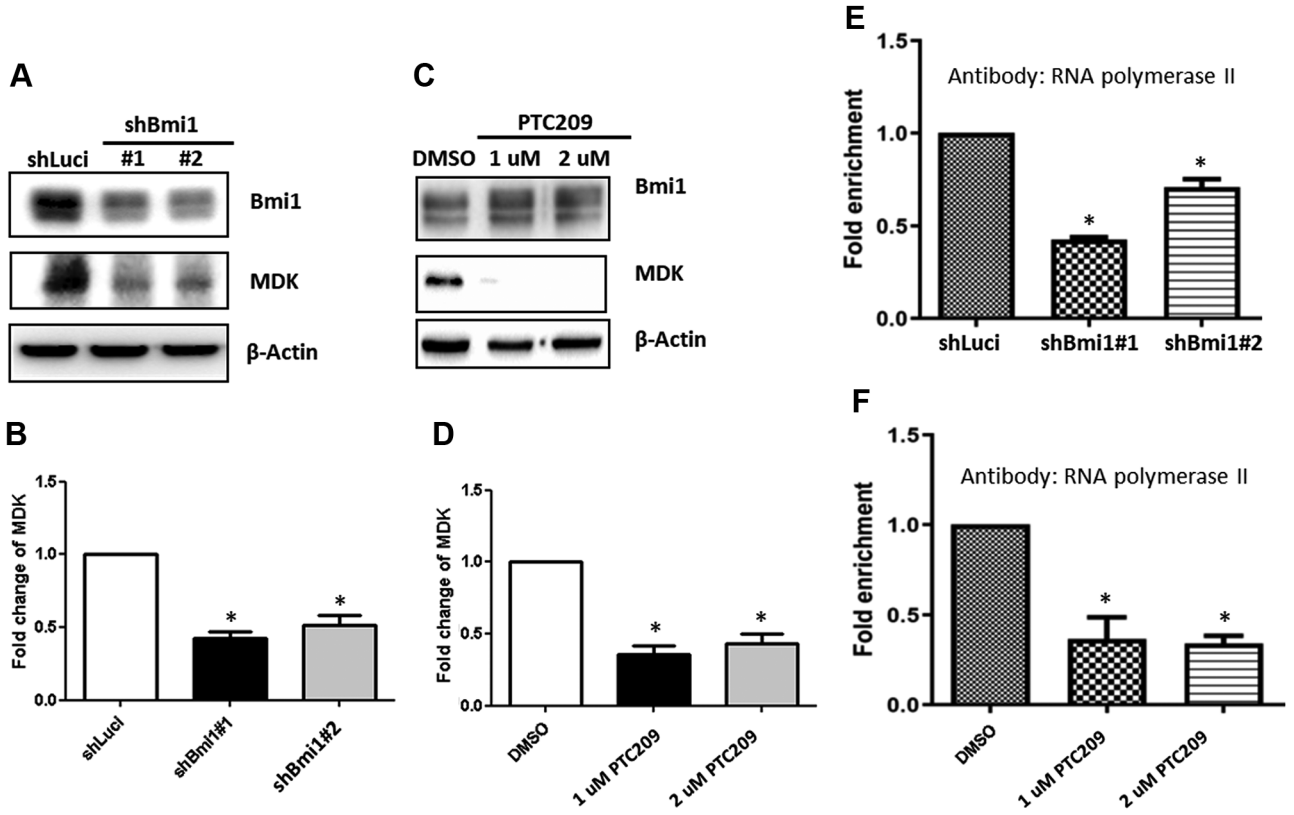

Figure 3. *Bmi1 up-regulates MDK expression transcriptionally. (A) Western blot analysis of Bmi1 and MDK expression in SW620 cells and Bmi1-depleted cells. (B) Quantification of MDK mRNA expression in SW620 cells and Bmi1-depleted cells using real-time qPCR. Columns represent the mean from triplicate PCR assays normalized to GAPDH (*p<0.05). (C) Western blot analysis of Bmi1 and MDK expression in SW620 cells treated with PTC209. (D) Quantification of MDK mRNA expression in SW620 cells treated with PTC209 using real-time qPCR. Columns represent the mean from triplicate PCR assays normalized to GAPDH (*p<0.05). (E) ChIP-qPCR analysis investigating the status of RNA polymerase II bound to the MDK promoter in SW620 cells, Bmi1-depleted cells (E), and PTC209-treated cells (F). The experiments were repeated thrice (*p<0.05).*

EMT, characterized by the transition from an epithelial to a mesenchymal phenotype in tumor cells, represents a pivotal event in tumor progression and metastasis (7). Various regulators and pathways, including WNT/β-catenin and TGF-β pathways, zinc finger E-box binding homeobox 1 and 2, and multiple microRNAs (26, 27), have been implicated in inducing the EMT process in CRC cells. Among these, Bmi1 facilitates CRC cell migration and invasion through the EMT process by modulating the AKT/GSK-3β/snail signaling pathway (15). In a previous investigation, we illustrated that the level of Bmi1 expression influences cancer stemness and associated transcription factors, including OCT4, SOX2, KLF4, and NANOG, which are implicated in the metastatic potential and chemo-radiosensitivity of human CRC cells (28). Consistent with these observations, our mouse and zebrafish models in this study demonstrated a correlation between Bmi1 expression and the metastatic capacity of SW620 cells, reinforcing its role in tumorigenesis and stemness maintenance in CRC cells.

The acquisition of anoikis resistance enables cancer cells to proliferate independently of anchorage, a pivotal step in the secondary phase of tumor metastasis (7). Cancer cells employ various mechanisms to develop resistance to anoikis, including modulation of integrin expression to adapt to diverse metastatic microenvironments (5, 29). Additionally, they exploit pro-survival signaling pathways such as those involving reactive oxygen species and hypoxia, influenced by intrinsic factors or environmental cues that down-regulate pro-apoptotic factors (5). Previous research has demonstrated that EZH2, a prominent member of the polycomb group protein family, can be up-regulated through hyper-activation of the ERK/AKT pathway and activation of the transcription factor FRA1/C-JUN (30). Elevated EZH2 expression results in the repression of integrin α2 transcription, consequently enhancing the mobility and anoikis resistance of CRC cells (30). Micropapillary structures (MIPs) in colorectal carcinoma exhibit lower proliferation and apoptosis rates compared to other cancer epithelial cells (non-MIPs), and

Table I. *Patients baseline characteristics (n=43).*

Variable	Value
Age (years)	
Mean (range)	57.21 (33.00-78.00)
Tumor size (cm)	
Mean (ranges)	6.06 (3.00-15.00)
Follow-up of the patient cohort (months)	
Mean (ranges)	16.38 (2.83-41.73)
Sex	
Female	30 (66.77)
Male	13 (30.23)
Tumor size	
<4 cm	7 (16.28)
≥4 cm	36 (83.72)
Tumor location	
Colon	21 (48.84)
Rectum	22 (51.16)
Histological grade	
I/II	35 (89.74)
III/IV	4 (10.26)
T stage	
T3	18 (41.86)
T4	25 (58.14)
N stage	
0	5 (11.63)
1/2	38 (88.37)
M stage	
0	6 (13.95)
1/2	37 (86.05)
Recurrence	
Absent	41 (95.35)
Present	2 (4.65)
Survival status	
Survived	21 (48.84)
Died	22 (51.16)
BMI	
<25	28 (65.12)
≥25	15 (34.88)
Diabetes mellitus	
Absent	32 (74.42)
Present	11 (25.58)
Serum CEA level (ng/ml)	
Mean (range)	201.11 (0.93-4076.35)
<5	9 (21.95)
≥5	32 (78.05)
Irinotecan	
No	8 (18.60)
Yes	35 (81.40)
Bevacizumab	
No	27 (62.79)
Yes	16 (37.21)

SD: Standard deviation.

are associated with poor prognosis due to low apoptosis rates. MIPs also show lower survivin expression, indicating a biologically distinct subpopulation with potential anoikis resistance and quiescence (31). In our investigation, we have identified another polycomb group protein, Bmi1, as a transcriptional regulator that collaborates with the growth factor MDK, modulating the anoikis resistance capability of CRC cells. Future studies should explore the interplay between these polycomb group proteins, as variations in their expression may impact the overall repression of downstream target genes (30).

Similar to prior investigations, we selected SW480 and SW620 cell lines as our primary models due to their common origin from the same patient, thereby circumventing genetic heterogeneity (7, 32). It is noteworthy that although both SW480 and SW620 cells derive from the same patient, they exhibit distinct characteristics, such as varying membrane protrusions, surface roughness, and actin skeletonization, all of which influence cell migration and adhesion (7). Specifically, SW620 cells, in comparison to SW480 cells, demonstrate heightened levels of E-cadherin and β-integrin activity, indicating reduced migratory capacity but enhanced cell-cell and cell-matrix adhesion, rendering them more resistant to anoikis (7). Our findings indicate that the presence of Bmi1 significantly impacts the proportion of apoptotically detached SW620 cells, implicating its role in metastasis.

MDK has been identified as an inducer of pro-inflammatory cytokine expression, promoting the infiltration of neutrophils and monocytes into tissues, thereby contributing to chronic inflammation (33). Additionally, MDK can stimulate neoangiogenesis and tumor cell proliferation, consequently fostering tumor growth and angiogenesis (25, 33). Moreover, it participates in extracellular matrix modulation, facilitating the dissemination of cancer cells (33). The concentration of MDK protein in bowel tissue correlates with lymph node involvement and increases with the degree of cancer cell dedifferentiation. A prospective study has further revealed a correlation between elevated serum MDK levels and CRC, indicative of a poorer prognosis (25).

A recent investigation unveiled that miR-1275 suppressed both Bmi1 and MDK in breast cancer cells, impacting cancer stemness maintenance and the PI3K/AKT phosphorylation pathways, thereby enhancing chemosensitivity (18). Our research contributes evidence supporting the notion that Bmi1 and MDK function within the same axis to govern anoikis resistance in CRC cells, a relationship validated in clinical tissue specimens. Notably, silencing Bmi1 not only inhibited MDK expression at both transcriptional and translational levels but also suggested its candidacy as a downstream target, holding promise for advanced CRC treatment.

In essence, our study affirms the pivotal role of Bmi1 in regulating CRC cell metastasis, particularly in terms of anoikis resistance, and demonstrates its collaborative role with downstream MDK in mediating migration and invasion capabilities. Notably, concurrent suppression of Bmi1 alongside the application of a topoisomerase I inhibitor (irinotecan) substantially augmented CRC cell anoikis and apoptosis, underscoring the potential of Bmi1 inhibition as a

Table II. *Correlation between Bmi1, MDK and clinicopathological parameters. in colorectal cancer.*

Parameters	Bmi1– (%)	Bmi1+ (%)	*p*-Value	MDK– (%)	MDK+ (%)	*p*-Value
N (%)	8 (1.60)	35 (81.40)		17 (39.53)	26 (60.47)	
Age			0.5970			0.8442
<70 years	6 (75.00)	30 (85.71)		14 (82.35)	22 (84.62)	
≥70 years	2 (25.00)	5 (14.29)		3 (17.65)	4 (15.38)	
Sex			0.0819			0.1462
Male	8 (100.00)	22 (62.86)		14 (82.35)	16 (61.54)	
Female	0 (0.00)	13 (37.14)		3 (17.65)	10 (38.46)	
Tumor size			0.5970			0.8442
<4 cm	2 (25.00)	5 (14.29)		3 (17.65)	4 (15.38)	
≥4 cm	6 (75.00)	30 (85.71)		14 (82.35)	22 (84.62)	
Tumor location			0.1324			0.2895
Colon	6 (75.00)	15 (42.86)		10 (58.82)	11 (42.31)	
Rectum	2 (25.00)	20 (57.14)		7 (41.18)	15 (57.69)	
Histological grade			0.5025			0.7001
I/II	5 (83.33)	30 (90.91)		14 (87.50)	21 (91.30)	
III/IV	1 (16.67)	3 (9.09)		2 (12.50)	2 (8.70)	
T stage			0.7010			0.9414
T3	4 (50.00)	14 (40.00)		7 (41.18)	11 (42.31)	
T4	4 (50.00)	21 (60.00)		10 (58.82)	15 (57.69)	
N stage			0.5648			0.3686
0	0 (0.00)	5 (14.29)		3 (17.65)	2 (7.69)	
1/2	8 (100.00)	30 (85.71)		14 (82.35)	24 (92.31)	
M stage			0.8954			0.7377
0	1 (12.50)	5 (14.29)		2 (11.76)	4 (15.38)	
1/2	7 (87.50)	30 (85.71)		15 (88.24)	22 (84.62)	
Recurrence			0.4887			0.7566
Absent	8 (100.00)	33 (94.29)		16 (94.12)	25 (96.15)	
Present	0 (0.00)	2 (5.71)		1 (5.88)	1 (3.85)	
Survival status			0.0212			0.0210
Survived	7 (87.50)	14 (40.00)		12 (70.59)	9 (34.62)	
Died	1 (12.50)	21 (60.00)		5 (29.41)	17 (65.38)	
BMI			0.0143			0.1756
<25	2 (25.00)	26 (74.29)		9 (52.94)	19 (73.08)	
≥25	6 (75.00)	9 (25.71)		8 (47.06)	7 (26.92)	
Diabetes mellitus			0.9667			0.2955
Absent	6 (75.00)	26 (74.29)		11 (64.71)	21 (80.77)	
Present	2 (25.00)	9 (25.71)		6 (35.29)	5 (19.23)	
Serum CEA (ng/ml)			0.5905			0.7010
<5.0	1 (14.29)	8 (23.53)		4 (26.67)	5 (19.23)	
≥5.0	6 (85.71)	26 (76.47)		11 (73.33)	21 (80.77)	
Irinotecan			0.6229			0.6915
No	1 (12.50)	7 (20.00)		4 (23.53)	4 (15.38)	
Yes	7 (87.50)	28 (80.00)		13 (76.47)	22 (84.62)	
Bevacizumab			0.2230			0.3923
No	7 (87.50)	20 (57.14)		12 (70.59)	15 (57.69)	
Yes	1 (12.50)	15 (42.86)		5 (29.41)	11 (42.31)	

Chi-square test was used for statistical analysis. $p<0.05$ was considered statistically significant.

novel therapeutic avenue for advanced and metastatic CRCs. Further validation through clinical studies is imperative to substantiate these findings.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

Yin-Chou Hsu, Chi-Wen Luo, Shu-Jyuan Chang, Chiao-Ying Lai, Yu-Tzu Yang, Yi-Zi Chen, Wang-ta Liu, Chun-Chieh Wu: Formal analysis, Data curation and Conceptualization. Yin-Chou Hsu, Chi-Wen Luo, Ming-Feng Hou and Mei-Ren Pan: Writing-original draft and writing-review & editing. Cheuk-Kwan Sun: Investigation. Ming-Feng Hou and Mei-Ren Pan: Funding acquisition.

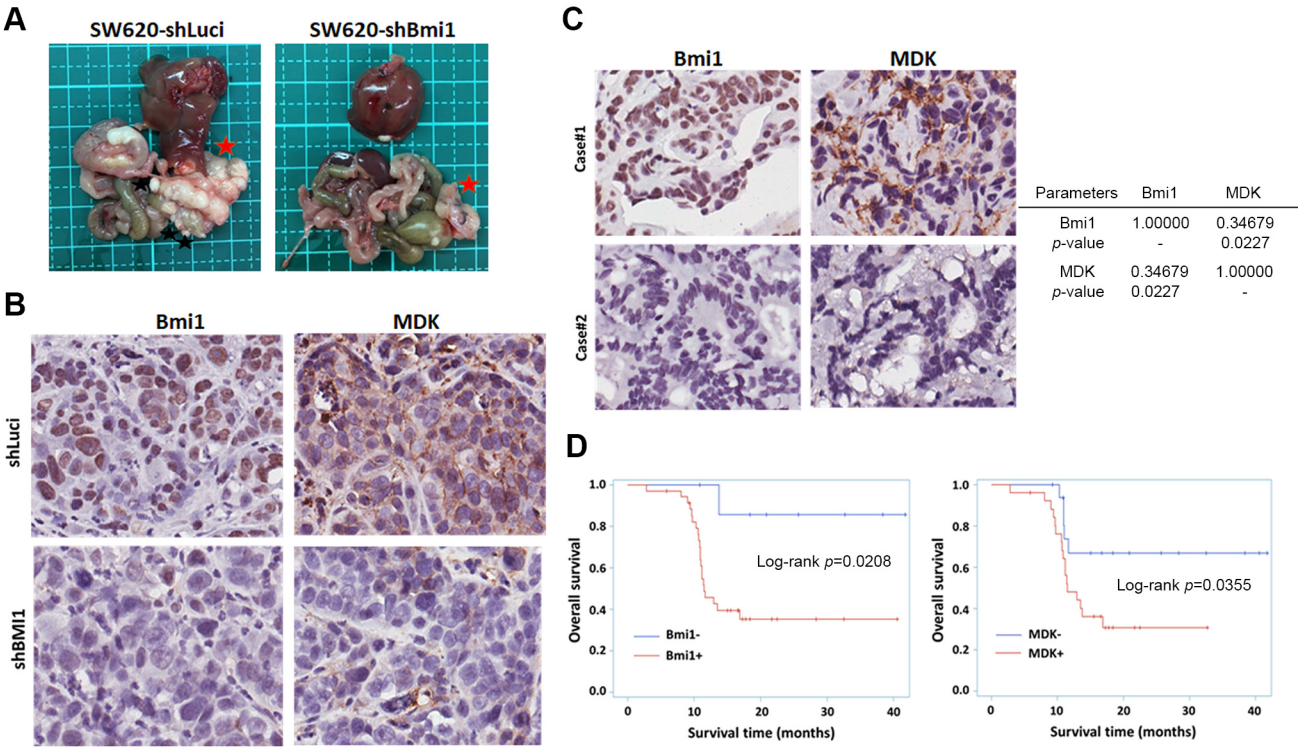

Figure 4. *Positive correlation between Bmi1 and MDK observed in vivo in mice and patient specimens. (A) At 8 weeks of age, six mice from each group were anesthetized using isoflurane, and SW620 and Bmi1-depleted cells (2×10^6; mixed 1:1 with Matrigel) were injected into the cecum. After two months, the mice were euthanized using CO_2. Images depict tumor growth in vivo. (B) Immunohistochemistry (IHC) images of Bmi1 (left) and MDK (right) in cancer tissue sections from mice implanted with SW620 and Bmi1-depleted cells. Magnification: 200×. (C) Representative IHC images of Bmi1 and MDK in two CRC tissue sections; original magnification, 200×; scale bar, 100 μm (left). Associations between Bmi1 and MDK calculated in 43 patients by Pearson analysis, with corresponding p-value (right). (D) Overall survival Kaplan–Meier curves of CRC patients with low and high Bmi1 and MDK expression.*

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References

1 Morgan E, Arnold M, Gini A, Lorenzoni V, Cabasag CJ, Laversanne M, Vignat J, Ferlay J, Murphy N, Bray F: Global burden of colorectal cancer in 2020 and 2040: incidence and mortality estimates from GLOBOCAN. Gut 72(2): 338-344, 2023. DOI: 10.1136/gutjnl-2022-327736

2 Siegel RL, Wagle NS, Cercek A, Smith RA, Jemal A: Colorectal cancer statistics, 2023. CA Cancer J Clin 73(3): 233-254, 2023. DOI: 10.3322/caac.21772

3 Fares J, Fares MY, Khachfe HH, Salhab HA, Fares Y: Molecular principles of metastasis: a hallmark of cancer revisited. Signal Transduct Target Ther 5(1): 28, 2020. DOI: 10.1038/s41392-020-0134-x

4 Frisch SM, Screaton RA: Anoikis mechanisms. Curr Opin Cell Biol 13(5): 555-562, 2001. DOI: 10.1016/s0955-0674(00)00251-9

5 Taddei M, Giannoni E, Fiaschi T, Chiarugi P: Anoikis: an emerging hallmark in health and diseases. J Pathol 226(2): 380-393, 2012. DOI: 10.1002/path.3000

6 Xiao YC, Yang ZB, Cheng XS, Fang XB, Shen T, Xia CF, Liu P, Qian HH, Sun B, Yin ZF, Li YF: CXCL8, overexpressed in colorectal cancer, enhances the resistance of colorectal cancer cells to anoikis. Cancer Lett 361(1): 22-32, 2015. DOI: 10.1016/j.canlet.2015.02.021

7 Liu Y, Zhang Y, Wu H, Li Y, Zhang Y, Liu M, Li X, Tang H: miR-10a suppresses colorectal cancer metastasis by modulating the epithelial-to-mesenchymal transition and anoikis. Cell Death Dis 8(4): e2739, 2017. DOI: 10.1038/cddis.2017.61

8 Liu J, Lu G, Liang C, Tian Y, Jiang Z: Roles of anoikis in colorectal cancer therapy and the assessment of anoikis-

regulatory molecules as therapeutic targets. Pathol Res Pract 241: 154256, 2023. DOI: 10.1016/j.prp.2022.154256

9 Siddique HR, Saleem M: Role of BMI1, a stem cell factor, in cancer recurrence and chemoresistance: preclinical and clinical evidences. Stem Cells 30(3): 372-378, 2012. DOI: 10.1002/stem.1035

10 Park IK, Morrison SJ, Clarke MF: Bmi1, stem cells, and senescence regulation. J Clin Invest 113(2): 175-179, 2004. DOI: 10.1172/JCI20800

11 Du J, Li Y, Li J, Zheng J: Polycomb group protein Bmi1 expression in colon cancers predicts the survival. Med Oncol 27(4): 1273-1276, 2010. DOI: 10.1007/s12032-009-9373-y

12 Pun JC, Chan JY, Chun BK, Ng KW, Tsui SY, Wan TM, Lo O, Poon JT, Ng L, Pang R: Plasma Bmi1 mRNA as a potential prognostic biomarker for distant metastasis in colorectal cancer patients. Mol Clin Oncol 2(5): 817-820, 2014. DOI: 10.3892/mco.2014.321

13 Benard A, Goossens-Beumer IJ, van Hoesel AQ, Horati H, Putter H, Zeestraten EC, van de Velde CJ, Kuppen PJ: Prognostic value of polycomb proteins EZH2, BMI1 and SUZ12 and histone modification H3K27me3 in colorectal cancer. PLoS One 9(9): e108265, 2014. DOI: 10.1371/journal.pone.0108265

14 Zhang Z, Bu X, Chen H, Wang Q, Sha W: Bmi-1 promotes the invasion and migration of colon cancer stem cells through the downregulation of E-cadherin. Int J Mol Med 38(4): 1199-1207, 2016. DOI: 10.3892/ijmm.2016.2730

15 Xu Z, Zhou Z, Zhang J, Xuan F, Fan M, Zhou D, Liuyang Z, Ma X, Hong Y, Wang Y, Sharma S, Dong Q, Wang G: Targeting BMI-1-mediated epithelial-mesenchymal transition to inhibit colorectal cancer liver metastasis. Acta Pharm Sin B 11(5): 1274-1285, 2021. DOI: 10.1016/j.apsb.2020.11.018

16 Hoenerhoff MJ, Chu I, Barkan D, Liu ZY, Datta S, Dimri GP, Green JE: BMI1 cooperates with H-RAS to induce an aggressive breast cancer phenotype with brain metastases. Oncogene 28(34): 3022-3032, 2009. DOI: 10.1038/onc.2009.165

17 Ferretti R, Bhutkar A, McNamara MC, Lees JA: BMI1 induces an invasive signature in melanoma that promotes metastasis and chemoresistance. Genes Dev 30(1): 18-33, 2016. DOI: 10.1101/gad.267757.115

18 Han X, Li M, Xu J, Fu J, Wang X, Wang J, Xia T, Wang S, Ma G: miR-1275 targets MDK/AKT signaling to inhibit breast cancer chemoresistance by lessening the properties of cancer stem cells. Int J Biol Sci 19(1): 89-103, 2023. DOI: 10.7150/ijbs.74227

19 Jhou AJ, Chang HC, Hung CC, Lin HC, Lee YC, Liu WT, Han KF, Lai YW, Lin MY, Lee CH: Chlorpromazine, an antipsychotic agent, induces G2/M phase arrest and apoptosis via regulation of the PI3K/AKT/mTOR-mediated autophagy pathways in human oral cancer. Biochem Pharmacol 184: 114403, 2021. DOI: 10.1016/j.bcp.2020.114403

20 Kasashima H, Duran A, Cid-Diaz T, Muta Y, Kinoshita H, Batlle E, Diaz-Meco MT, Moscat J: Mouse model of colorectal cancer: orthotopic co-implantation of tumor and stroma cells in cecum and rectum. STAR Protoc 2(1): 100297, 2021. DOI: 10.1016/j.xpro.2021.100297

21 Li J, Vangundy Z, Poi M: PTC209, a specific inhibitor of BMI1, promotes cell cycle arrest and apoptosis in cervical cancer cell lines. Anticancer Res 40(1): 133-141, 2020. DOI: 10.21873/anticanres.13934

22 Neiva KG, Zhang Z, Miyazawa M, Warner KA, Karl E, Nör JE: Cross talk initiated by endothelial cells enhances migration and inhibits anoikis of squamous cell carcinoma cells through STAT3/Akt/ERK signaling. Neoplasia 11(6): 583-593, 2009. DOI: 10.1593/neo.09266

23 Du L, Han XG, Tu B, Wang MQ, Qiao H, Zhang SH, Fan QM, Tang TT: CXCR1/Akt signaling activation induced by mesenchymal stem cell-derived IL-8 promotes osteosarcoma cell anoikis resistance and pulmonary metastasis. Cell Death Dis 9(7): 714, 2018. DOI: 10.1038/s41419-018-0745-0

24 Ye C, Qi M, Fan QW, Ito K, Akiyama S, Kasai Y, Matsuyama M, Muramatsu T, Kadomatsu K: Expression of midkine in the early stage of carcinogenesis in human colorectal cancer. Br J Cancer 79(1): 179-184, 1999. DOI: 10.1038/sj.bjc.6690030

25 Kemper M, Hentschel W, Graß JK, Stüben BO, Konczalla L, Rawnaq T, Ghadban T, Izbicki JR, Reeh M: Serum Midkine is a clinical significant biomarker for colorectal cancer and associated with poor survival. Cancer Med 9(6): 2010-2018, 2020. DOI: 10.1002/cam4.2884

26 Vu T, Datta PK: Regulation of EMT in colorectal cancer: a culprit in metastasis. Cancers (Basel) 9(12): 171, 2017. DOI: 10.3390/cancers9120171

27 Weidle UH, Brinkmann U, Auslaender S: microRNAs and corresponding targets involved in metastasis of colorectal cancer in preclinical in vivo models. Cancer Genomics Proteomics 17(5): 453-468, 2020. DOI: 10.21873/cgp.20204

28 Hsu YC, Luo CW, Huang WL, Wu CC, Chou CL, Chen CI, Chang SJ, Chai CY, Wang HC, Chen TY, Li CF, Pan MR: BMI1-KLF4 axis deficiency improves responses to neoadjuvant concurrent chemoradiotherapy in patients with rectal cancer. Radiother Oncol 149: 249-258, 2020. DOI: 10.1016/j.radonc.2020.06.023

29 Paoli P, Giannoni E, Chiarugi P: Anoikis molecular pathways and its role in cancer progression. Biochim Biophys Acta 1833(12): 3481-3498, 2013. DOI: 10.1016/j.bbamcr.2013.06.026

30 Ferraro A, Mourtzoukou D, Kosmidou V, Avlonitis S, Kontogeorgos G, Zografos G, Pintzas A: EZH2 is regulated by ERK/AKT and targets integrin alpha2 gene to control Epithelial–Mesenchymal Transition and anoikis in colon cancer cells. Int J Biochem Cell Biol 45(2): 243-254, 2013. DOI: 10.1016/j.biocel.2012.10.009

31 Patankar M, Väyrynen S, Tuomisto A, Mäkinen M, Eskelinen S, Karttunen TJ: Micropapillary structures in colorectal cancer: An anoikis-resistant subpopulation. Anticancer Res 38(5): 2915-2921, 2018. DOI: 10.21873/anticanres.12539

32 Maamer-Azzabi A, Ndozangue-Touriguine O, Bréard J: Metastatic SW620 colon cancer cells are primed for death when detached and can be sensitized to anoikis by the BH3-mimetic ABT-737. Cell Death Dis 4(9): e801, 2013. DOI: 10.1038/cddis.2013.328

33 Krzystek-Korpacka M, Gorska S, Diakowska D, Kapturkiewicz B, Podkowik M, Gamian A, Bednarz-Misa I: Midkine is up-regulated in both cancerous and inflamed bowel, reflecting lymph node metastasis in colorectal cancer and clinical activity of ulcerative colitis. Cytokine 89: 68-75, 2017. DOI: 10.1016/j.cyto.2016.09.020

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