Heat shock protein 90 is involved in the regulation of HMGA2-driven growth and epithelial-to-mesenchymal transition of colorectal cancer cells

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High mobility group AT-hook 2 (HMGA2) is the architectural nuclear factor involved in chromatin remodeling and gene transcription. In particular, the association of HMGA2 overexpression with the transformation and metastatic progression of neoplastic cells suggests its causal role in carcinogenesis and tumor progression. Heat shock protein 90 (Hsp90), one of the most abundant and highly conserved molecular chaperones, is required for the stability and function of multimutated, chimeric, and overexpressed signaling proteins that promote the growth, mobility, and survival of cancer cells. Moreover, using a combination of microarray gene expression of 132 colorectal cancer (CRC) patients and Connectivity Map data mining, extremely strong positive connections were observed between Hsp90 inhibitors and CRC, which indicated their potential for use in CRC treatment. However, little is known about the effect of Hsp90 inhibition on HMGA2 protein expression in CRC. In this study, we tested the hypothesis that Hsp90 may regulate HMGA2 expression and investigated the relationship between Hsp90 and HMGA2 signaling. The use of the second-generation Hsp90 inhibitor, NVP-AUY922, considerably knocked down HMGA2 expression, and the effects of Hsp90 and HMGA2 knockdown were similar. In addition, the interaction between Hsp90 and HMGA2 was examined, and HMGA2 was newly identified as the Hsp90 client protein. Moreover, our cell viability data clearly demonstrated that HMGA2 expression levels influence the NVP-AUY922 drug sensitivity in CRC cells. Our findings suggest that the NVP-AUY922-dependent mitigation of HMGA2 signaling occurred through the reactivation of the tumor suppressor microRNA (miRNA), let-7a, and the inhibition of HMGA2-associated kinases involved in regulating the growth and mobility of CRC cells. In conclusion, Hsp90 inhibition is a promising pharmacological approach to suppress HMGA2 expression and signaling, and Hsp90 inhibitors may have clinical application in antagonizing HMGA2-dependent tumorigenesis.

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ABSTRACT

High mobility group AT-hook 2 (HMGA2) is the architectural nuclear factor involved in chromatin remodeling and gene transcription. In particular, the association of HMGA2 overexpression with the transformation and metastatic progression of neoplastic cells suggests its causal role in carcinogenesis and tumor progression. Heat shock protein 90 (Hsp90), one of the most abundant and highly conserved molecular chaperones, is required for the stability and function of multimutated, chimeric, and overexpressed signaling proteins that promote the growth, mobility, and survival of cancer cells. Moreover, using a

combination of microarray gene expression of 132 colorectal cancer (CRC) patients and Connectivity Map data mining, extremely strong positive connections were observed between Hsp90 inhibitors and CRC, which indicated their potential for use in CRC treatment. However, little is known about the effect of Hsp90 inhibition on HMGA2 protein expression in CRC. In this study, we tested the hypothesis that Hsp90 may regulate HMGA2 expression and investigated the relationship between Hsp90 and HMGA2 signaling. The use of the second-generation Hsp90 inhibitor, NVP-AUY922, considerably knocked down HMGA2 expression, and the effects of Hsp90 and HMGA2 knockdown were similar. In addition, the interaction between Hsp90 and HMGA2 was examined, and HMGA2 was newly identified as the Hsp90 client protein. Moreover, our cell viability data clearly demonstrated that HMGA2 expression levels influence the NVP-AUY922 drug sensitivity in CRC cells. Our findings suggest that the NVP-AUY922-dependent mitigation of HMGA2 signaling occurred through the reactivation of the tumor suppressor microRNA (miRNA), let-7a, and the inhibition of HMGA2-associated kinases involved in regulating the growth and mobility of CRC cells. In conclusion, Hsp90 inhibition is a promising pharmacological approach to suppress HMGA2 expression and signaling, and Hsp90 inhibitors may have clinical application in antagonizing HMGA2-dependent tumorigenesis.

INTRODUCTION

High mobility group AT-hook (HMGA) nonhistone chromatin-binding proteins, including HMGA1 (isoforms HMGA1a and HMGA1b) and HMGA2, are architectural nuclear factors involved in chromatin remodeling and gene transcription (Reeves & Nissen, 1990). HMGA1 and HMGA2 have similar functions and are abundantly expressed in the early embryo, in which cells proliferate rapidly (Sgarra et al., 2004). However, *HMGA2* cannot be detected in adult human tissues, in which it is probably completely silenced (Gattas et al., 1999; Rogalla et al., 1996). In particular, HMGA2 is weakly expressed only in preadipocytic proliferating cells (Anand & Chada, 2000) and spermatocytes (Di Agostino et al., 2004). Conversely, several studies have reported that the association of HMGA2 overexpression with the transformation and metastatic progression of neoplastic cells suggests its causal role in carcinogenesis and tumor progression (Mahajan et al., 2010; Piscuoglio et al., 2012; Wang et al., 2011; Wend et al., 2013; Xu et al., 2004). Furthermore, the essential role of HMGA2 in cell proliferation and migration has been reported in various cancers (Malek et al., 2008; Sun et al., 2013; Xia et al., 2015; Yang et al., 2011). Thus, the HMGA2 protein is a promising biomarker for cancer detection as well as a potential molecular target in cancer therapy.

Heat shock protein 90 (Hsp90), one of the most abundant and highly conserved molecular chaperones, is essential for the stability and function of multimutated, chimeric, and overexpressed signaling proteins that promote the growth, mobility, and survival of cancer cells (Neckers, 2002). In addition, Hsp90 is involved in the maturation and stabilization of various oncogenic client proteins crucial for oncogenesis and malignant progression (Chiosis et al., 2006). Thus, Hsp90 is considered a valuable target for cancer therapy. Moreover, using a combination of microarray gene expression of 132 colorectal cancer (CRC) patients and Connectivity Map data mining, extremely strong positive connections were observed between Hsp90 inhibitors and CRC, which indicated their potential for use in CRC treatment (Su et al., 2015). To determine the HMGA2 expression levels in these CRC patients, we analyzed the gene expression of *HMGA2* in 132 CRC tumor samples: 67 primary CRC tissues, 65 metastatic tissues, and nine normal colon controls. As expected, HMGA2 expression was significantly upregulated in metastatic and primary CRC tissues compared with that in the normal colon controls (Fig. S1A). Similarly, the Hsp90 expression levels were analyzed in the same metastatic and primary CRC tissues, and the mRNA expression levels of Hsp90 were similar to those of HMGA2 (Fig. S1B). This result implies that HMGA2 expression can be affected by treatment with Hsp90 inhibitors of CRC

cells; however, whether HMGA2 signaling was dependent on Hsp90 function requires further clarification.

MATERIALS AND METHODS

Chemicals, reagents, antibodies, and expression constructs

NVP-AUY922 was purchased from Selleck Chemicals LLC (Houston, TX, USA). Crystal violet and DMSO were obtained from Sigma (St. Louis, MO, USA). Small interfering RNA (siRNA) targeting Hsp90 or HMGA2 mRNA, control siRNA, and the RNAiMax transfection reagent were purchased from Invitrogen (Carlsbad, CA, USA). Rabbit antibodies against Hsp90, CDK4, E-cadherin, vimentin, Twist, Snail, Slug, extracellular signal-regulated kinase (ERK), Thr(P)202/Tyr(P)204-ERK1/2, cAMP response element-binding protein (CREB), Ser(P)133-CREB, focal adhesion kinase (FAK), and Tyr(P)397-FAK were obtained from Cell Signaling (Beverly, MA, USA). HMGA2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody against β-actin was purchased from MP Biomedicals (Irvine, CA, USA).

Cell culture

CRC cell lines were provided by Prof. YW Cheng and Prof. H Lee. Stable DLD-HMGA2-GFP expression cell line was provided by Dr. PM Yang. All CRC cell lines were cultured in RPMI-1640 and supplemented with 10% fetal bovine serum (FBS) and antibiotics. CRL-1459/CCD-18Co (noncancerous human colon cells) was provided by Prof. PJ Lu and cultured in minimum essential Eagle's medium and supplemented with 10% FBS and antibiotics.

Cell viability assay

Cell viability was determined through crystal violet staining, as described by (*Kim et al., 1997*). In brief, the cells were plated in 96-well plates at 4000 cells/mL and subjected to DMSO or NVP-AUY922 treatment at the indicated concentrations. Viable cells were stained with 0.5% crystal violet in 30% ethanol for 10 min at room temperature. Subsequently, the plates were washed four times with tap water. After drying, the cells were lysed with a 0.1 M sodium citrate solution, and the dye uptake was measured at 550 nm using a 96-well plate reader. Cell viability was calculated by comparing the relative dye intensities of the treated and untreated samples.

Tissue microarray of CRC clinical specimens

A colon adenocarcinoma tissue array was purchased from US Biomax (CO1505, containing 50 cases of CRC tissues with matched adjacent tissues as the controls). All tissue sections were stained using a standard immunohistochemical (IHC) protocol. In brief, slides were deparaffinized using serial xylene–ethanol treatment. Antigens were retrieved through boiling in a sodium citrate buffer for 10 min. Slides were blocked in 5% normal goat serum for 1 h at room temperature. After blocking, the slides were incubated with a primary antibody against HMGA2, followed by a biotin-conjugated secondary antibody, horseradish peroxidase polymer (HRP), and a diaminobenzidine-tetrahydrochloride-dihydrate solution. The staining intensity was scored as follows: 0 point, negative; 1 point, weakly positive; 2 points, moderately positive; and 3 points, strongly positive.

Quantitative reverse-transcription polymerase chain reaction

Total RNA was extracted from the cell lines with or without drug treatment using a Qiagen RNeasy kit and Qiashredder columns according to manufacturer instructions (Valencia, CA, USA). One microgram of the total RNA was reverse transcribed to cDNA using a SABiosciences Reaction Ready™ First Strand cDNA Synthesis Kit (Frederick, MD, USA). Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed in an Applied Biosystems StepOne Plus™ Real-Time PCR System (Foster City, CA, USA) using an automated baseline and threshold cycle detection. For detecting the expression levels of let-7a, the amplification and detection of specific products were performed using the cycle profile of the Qiagen miScript PCR starter kit (Valencia, CA, USA). The relative gene expression level was calculated by comparing the cycle times for each target PCR. The target PCR Ct values were normalized by subtracting the U6 rRNA Ct value (internal control).

Western blotting

Cell lines were placed in a lysis buffer at 4 °C for 1 h. Protein samples were electrophoresed using 8%–15% SDS-polyacrylamide gel electrophoresis, as described by Su et al. (2015).

Human phospho-kinase array

HCT116 cell line was analyzed in the array panel of kinase phosphorylation profiles after DMSO or NVP-AUY922 treatment (Human Phospho-Kinase Array, ARY003; R&D Systems). This array specifically screens for relative phosphorylation levels of 42 individual proteins involved in cellular proliferation and survival. Each phospho-kinase array has duplicate signal spots for each gene. After DMSO or NVP-AUY922 (10 nM) treatment, cell lysates were incubated with the membrane. Thereafter, a cocktail of biotinylated detection antibodies, streptavidin-HRP, and chemiluminescent detection reagents were used for

detecting phosphorylated proteins. The dot density was scanned from the scanned X-ray film, and images were analyzed and quantified using image analysis software (NIH-Image J).

In vitro migration assay

Assays were performed using Falcon™ cell culture inserts (8-μm pore size) in a 24-well plate (BD Biosciences, San Jose, CA, USA) according to manufacturer instructions. In the migration assay, HCT116 cells (10^4cells/well) in 0.5 mL of serum-free medium were seeded onto the upper chamber membranes that received different treatment. These membranes were previously inserted into the 24-well plates containing 10% FBS-supplemented medium. After 12 h, the cells were fixed with 100% methanol and stained with 5% Giemsa stain (Merck, Darmstadt, Germany). Nonmigrated cells that remained in the upper chambers were removed by wiping the top of the inserted membranes using a damp cotton swab, leaving only those cells that migrated to the underside of the membranes. All experiments were performed in triplicate and photographed under a phase-contrast microscope (200×).

Statistical analysis

Statistical analyses were performed as recommended by an independent statistician. Unpaired Student's *t*-test was used for analyses. All statistical analyses were performed using SPSS (SPSS, Chicago, IL, USA), and all values are expressed as the mean ± standard error. $p<0.05$ was considered statistically significant.

RESULTS

Elevated expression of HMGA2 mRNA and protein in CRC cell lines and tissues

To understand the level of HMGA2 gene expression in human cancers, various cancer cell lines were selected from the National Cancer Institute Cancer Genome Anatomy Project gene expression database. CRC cell lines had relatively high levels of HMGA2 mRNA expression (Fig. 1A). Notably, HMGA2 was highly expressed in CRC cell lines among the nine cancer cell lines, thus validating its specificity in CRC. To further determine the level of HMGA2 protein expression in CRC cell lines, eight CRC cell lines and one noncancerous human colon cell line (CRL-1459) were chosen. Compared with CRL-1459, HMGA2 proteins were highly expressed in all CRC cell lines except SW480 (Fig. 1B). Thus, the high levels of HMGA2 protein expression in HCT116 cells was attributed to its high mRNA expression, as shown in Figures 1A and 1B. Therefore, HCT116 was chosen for further cell model experiments. HMGA2 protein expression was further examined in a colon adenocarcinoma tissue array (BioMax, Rockville, MD, USA). As shown in Fig. 1C, HMGA2 was upregulated in colon adenocarcinoma tissues of different tumor grades. The staining intensity score was

defined on the basis of immunoexpression, as outlined in the IHC protocol (Fig. S2), and the colon adenocarcinoma tissues of all grades exhibited strong positive staining (scores 2 and 3) for HMGA2 (Fig. 1C, right panel). These results indicate that HMGA2 expression was specific and elevated in CRC cells.

Inhibition of HMGA2 protein increased sensitivity of Hsp90 inhibitor

HMGA2 contributes to resistance against anticancer drugs in various cancer cell lines (Gyorffy et al., 2006). Thus, HMGA2 silencing was hypothesized to increase the sensitivity to anticancer drugs in cancer cells. To test this hypothesis, the HCT116 cell line with elevated HMGA2 expression was selected for transfection with HMGA2 small-interfering RNA oligomer (siHMGA2) or scrambled oligomer (control siRNA). HMGA2 protein expression and cell viability were subsequently examined. As shown in Fig. 2A (upper panel), HMGA2 protein expression was significantly inhibited in siHMGA2-transfected HCT116 cells. We have recently shown that second-generation Hsp90 inhibitor, NVP-AUY922, significantly inhibited cell growth and induced cell death in CRC cells (Su et al., 2015). To examine the NVP-AUY922 drug sensitivity in siHMGA2-transfected HCT116 cells, a cell viability assay was performed. NVP-AUY922 treatment significantly reduced the cell viability of siHMGA2-transfected HCT116 cells compared with the control siRNA-transfected HCT116 cells (Fig. 2A, bottom panel). Similarly, we examined the NVP-AUY922 drug sensitivity in HMGA2-overexpressed CRC cells. A stable cell line, DLD1 HMGA2-GFP, was established and characterized using an anti-GFP antibody for Western blotting (Fig. 2B, upper panel). As expected, the proliferation index of the stable DLD1 HMGA2-GFP cells significantly reduced on NVP-AUY922 treatment compared with the parental group (Fig. 2B, bottom panel). These results are consistent with the previous observation that the HMGA2 expression levels influence anticancer drug sensitivity.

Hsp90 regulates and interacts with HMGA2

Hsp90 is critical in regulating cell growth (Cheung et al., 2010; Ko et al., 2012; Miyata, 2003; Nagaraju et al., 2014), and HMGA2 has a well-documented role in this process (Di Cello et al., 2008; Malek et al., 2008; Sun et al., 2013; Wend et al., 2013); therefore, we examined whether HMGA2 may be regulated by Hsp90. Thus, we performed RNA interference to deplete Hsp90 in the HCT116 and HCT15 cell lines and examined the effect of its depletion. As shown in Fig. 3A, Hsp90 siRNA-mediated endogenous Hsp90 knockdown significantly reduced CDK4 (sensitive client protein to Hsp90) and HMGA2 expression in the siHsp90-transfected HCT116 and HCT15 cell lines. Furthermore, we examined whether the effects of the Hsp90 inhibitor were similar to those of the Hsp90 siRNA on Hsp90 inhibition.

As shown in Fig. 3B, NVP-AUY922 significantly reduced HMGA2 protein expression dose-dependently in both HCT116 and HCT15 cells. The above results indicate that HMGA2 can be regulated by Hsp90. We performed an immunoprecipitation assay to determine the effect of NVP-AUY922 on the physical interactions between Hsp90 and HMGA2. After NVP-AUY922 treatment, the HMGA2 protein was immunoprecipitated with an anti-HMGA2 antibody and analyzed through Western blotting with an anti-Hsp90 antibody. As shown in Fig. 3C, a single band was detected using anti-Hsp90 antibody in immunoprecipitates or input lysate from HCT116 cells (left panel); however, a low HMGA2 expression was observed only in the input lysate and not in immunoprecipitates of the HCT15 cells (right panel). In addition, NVP-AUY922 treatment did not significantly change the protein interactions between Hsp90 and HMGA2 (Fig. 3C). Recently, the tumor suppressor microRNA (miRNA), let-7a, was reported to regulate the expression of certain oncogenes, including HMGA2, in various cancers (Wang et al., 2013; Wu et al., 2015; Yang et al., 2014). To examine whether NVP-AUY922 caused HGMA2 downregulation through let-7a expression in CRC cells, the effect of NVP-AUY922 on let-7a expression was examined. HCT116 or HCT15 cell line treatment with 40 nM NVP-AUY922 significantly induced let-7a expression (Fig 3D). Thus, these results indicate that Hsp90 was the upstream regulator of HMGA2 and that the Hsp90 inhibitor can be used in cancer cells for inhibiting the interaction between Hsp90 and HMGA2 or for inducing the expression of let-7a.

Effects of gene-specific inhibition of HMGA2 or Hsp90 and pharmaceutical inhibition of Hsp90 were similar

Specific knockdown of HMGA2 inhibited cell proliferation, leading to an epithelial-state transition in human pancreatic cancer cells (Watanabe et al., 2009). To determine the effects of HMGA2 inhibition in CRC cells, cell proliferation and cell migration assays were performed; siHMGA2 knockdown effectively reduced the proliferation of siHMGA2-transfected HCT116 cells (Fig. 4A). The migration transwell assay was performed to determine the migratory abilities of the siHMGA2-transfected HCT116 cells. As shown in Fig.4B, the migratory abilities significantly reduced in approximately 43% of siHMGA2-transfected HCT116 cells compared with those in the control cells. According to the aforementioned results (Fig. 3), Hsp90 might have participated in HMGA2 upstream regulation. To investigate whether the phenotype of gene-specific or pharmaceutical Hsp90 inhibition is similar to that of gene-specific HMGA2 inhibition in the CRC cells, we performed cell proliferation and cell migration assays in HCT116 cells. The proliferation index in siHsp90- and NVP-AUY922-treated HCT116 cells significantly reduced on Hsp90

knockdown compared with that in the control cells (Figs. 4C and E). Moreover, cell migration significantly reduced in the siHsp90- and NVP-AUY922-treated HCT116 cells. The inhibition rate was approximately 85% and 65% after siHsp90 and 40 nM NVP-AUY922 treatments, respectively (Figs. 4D and 4F, respectively). Thus, the effect of Hsp90 inhibition is similar to that of HMGA2 inhibition, and Hsp90 is a strategic target for the inhibition of HMGA2-triggered CRC tumorigenesis.

Phospho-kinase array for investigating NVP-AUY922-induced altered activity of HMGA2-associated kinases that regulate growth and mobility of HCT116 cells

Several Hsp90 inhibitors have been identified to target Hsp90 client proteins, such as receptors, kinases, and transcription factors, which are involved in oncogenesis (Porter et al., 2010; Trepel et al., 2010). ERK1/2 and FAK were involved in HMGA2-regulated CRC cell growth and mobility, respectively (Li et al., 2014; Li et al., 2013; Zhang et al., 2015a). Our aforementioned results (Fig. 2 and 3) demonstrated that Hsp90 might be the upstream regulator of HMGA2. Therefore, using the human phospho-kinase array, we examined whether NVP-AUY922 treatment in HCT116 cells altered the regulatory activity of HMGA2-associated kinases. As shown in Fig. 5A, the phosphorylation levels of ERK1/2, FAK, and CREB were significantly inhibited in NVP-AUY922-treated HCT116 cells. The CREB/HMGA2 pathway is crucial in malignant transformation (Shibanuma et al., 2012). Furthermore, CREB is a transcription factor and a downstream target of the ERK1/2 pathway (Qi et al., 2008). Accordingly, we hypothesized that HMGA2-mediated cell growth can be inhibited using NVP-AUY922 treatment through NVP-AUY922-regulated ERK-CREB-HMGA2 signaling. Thus, HCT116 cells were dose-dependently treated with NVP-AUY922, and the ERK and CREB activity and protein expression were examined. As shown in Fig. 5B, Western blotting results revealed that the ERK and CREB activity and protein expression were significantly inhibited in the NVP-AUY922-treated HCT116 cells. Downregulated FAK expression results in the loss of mesenchymal markers and increased expression of the epithelial marker, E-cadherin, in breast tumor models (Kong et al., 2012). In addition, Hsp90 inhibition disrupts FAK signaling and inhibits tumor progression (Schwock et al., 2009). Moreover, mesenchymal markers, Twist, Snail, and Slug, are regulated by HMGA2 (Li et al., 2014; Tan et al., 2012; Thuault et al., 2008). Therefore, the phosphorylation of FAK and epithelial–mesenchymal transition (EMT) effectors were examined. NVP-AUY922 dose-dependently reduced the phosphorylation of FAK, accompanied by parallel changes in the expression of various EMT effectors, including E-cadherin, vimentin, Twist, Snail, and Slug in HCT116 cells, with the reversal from a

mesenchymal to an epithelial phenotype (Fig. 5C). On the basis of these findings, we conclude that targeting HMGA2, HMGA2-related molecular signaling pathway, or both using an Hsp90 inhibitor can inhibit the growth and mobility of CRC cells.

DISCUSSION

HMGA2 overexpression in various human neoplasias is associated with highly malignant phenotypes, such as chemoresistance, metastasis, and poor survival (Di Cello et al., 2008; Mahajan et al., 2010; Wang et al., 2011; Yang et al., 2011). HMGA2 or HMGA2-regulated signaling is the preferred therapeutic target in CRC. This is the first study to recognize HMGA2 as a newly identified Hsp90 client protein and to propose pharmacological Hsp90 inhibition as a promising strategy for impairing HMGA2 function. We demonstrated that the Hsp90 mRNA expression levels in primary and metastatic CRC tissues were similar to those of HMGA2, analyzed from the Gene Expression Omnibus repository (GSE21815), and reported that the Hsp90 inhibitor follows a rational therapeutic approach in inhibiting HMGA2-triggered tumorigenesis. Our cell viability data clearly demonstrated that HMGA2 expression levels influenced NVP-AUY922-induced drug sensitivity of the CRC cells. The knockdown of Hsp90 using Hsp90 siRNA significantly reduced HMGA2 expression, and the effects of Hsp90 and HMGA2 knockdown were similar. NVP-AUY922 treatment in CRC cells significantly downregulated the regulatory activities of HMGA2-associated kinases. Collectively, this is the first study to report that Hsp90 inhibitor significantly suppressed HMGA2 protein expression and HMGA2-mediated regulation of cell growth and mobility.

MiRNAs are critical in the regulation of HMGA2 protein expression (D'Angelo et al., 2015). Let-7a is one of the most critical tumor suppressor miRNA that regulates HMGA2 expression (Wang et al., 2013). In particular, let-7a dysregulation was observed in CRC (Pallante et al., 2015). In the present study, let-7a expression was significantly induced using NVP-AUY922 (40 nM) treatment in HCT116 and HCT15 cell lines, and HMGA2 protein expression was simultaneously inhibited (comparison of Figs. 3B and D). In clinical CRC specimens, quantitative RT-PCR and IHC analysis revealed downregulated let-7a expression levels and upregulated HMGA2 protein expression levels, respectively (unpublished data). These results show that let-7a acts as a suppressor of CRC tumorigenesis, and NVP-AUY92-induced let-7a reactivation can inhibit HMGA2-triggered cell growth and mobility of CRC cells.

HMGA2 is an architectural transcription factor and belongs to the high motility group A family. This family of proteins can modify the structure of its binding partners to generate a

conformation that facilitates various DNA-dependent activities and influences various biological processes, including cell growth, metastasis, and survival (Califano et al., 2014; Morishita et al., 2013). HMGA2 protein regulates the transcription of several EMT-related genes and thus is closely associated with tumor invasion and metastasis (Morishita et al., 2013). HMGA2 upregulated the expression of Snail and Twist and downregulated the expression of E-cadherin in normal murine mammary gland epithelial cells (Thuault et al., 2006). In addition, HMGA2 positively regulated Slug expression by directly binding to the regulatory region of the Slug promoter (Li et al., 2014). HMGA2 was involved in cordycepin-mediated suppression of late-stage melanoma metastasis through the modulation of the activation of FAK and expression of EMT markers (Zhang et al., 2015b). Furthermore, FAK expression downregulation results in the loss of mesenchymal markers and increased epithelial marker expression in breast tumor models (Kong et al., 2012). These results reveal the criticality of HMGA2 in cancer progression, and thus HMGA2 is a potential molecular target for preventing cancer progression. However, the molecular mechanism of the Hsp90 inhibitor in the inhibition of metastasis remains unclear. An Hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin, inhibited prostate cancer metastasis through Slug inhibition (Ding et al., 2013). This is the first study to examine the potency of a second generation Hsp90 inhibitor, NVP-AUY922, on the inhibition of metastasis in CRC cells through the simultaneous inhibition of EMT effectors regulated by HMGA2 and FAK.

In summary, NVP-AUY922 reduced the activity and expression of ERK and CREB and suppressed CRC cell growth. In addition, NVP-AUY922 downregulated the expression of HMGA2 and HMGA2-mediated EMT effectors, which suppressed cell motility, suggesting that NVP-AUY922 not only regulates the growth of CRC cells but also its dissemination.

CONCLUSIONS

Our study is the first to identify the interaction between Hsp90 and HMGA2 and that the Hsp90 inhibitor has therapeutic potential to inhibit HMGA2-triggered tumorigenesis. Moreover, our findings clarify the downregulation of HMGA2 through NVP-AUY922-induced let-7a expression and the inhibition of HMGA2-associated kinases for regulating the growth and mobility of CRC cells.

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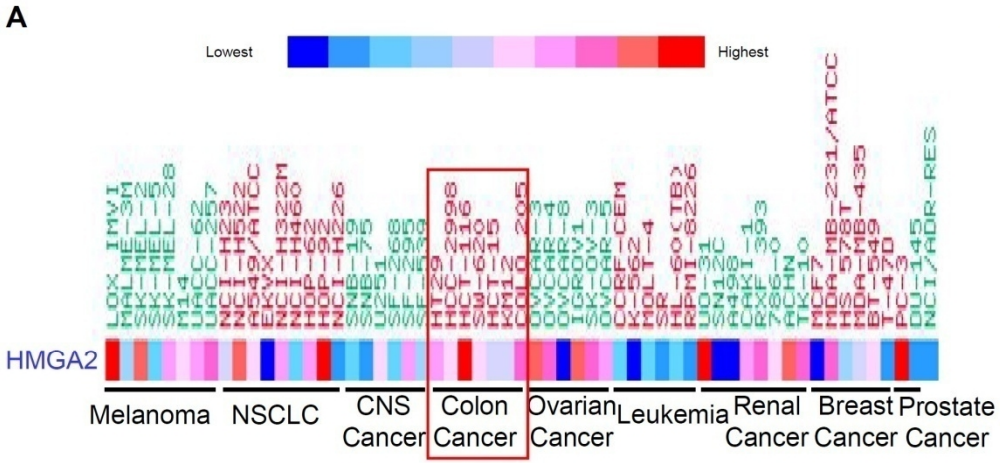
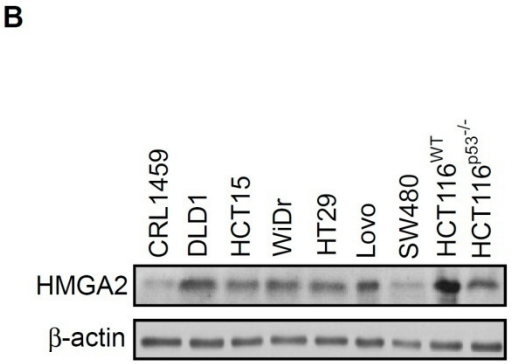

A
Lowest
Highest
HMGA2
Melanoma
NSCLC
CNS Cancer
Colon Cancer
Ovarian Cancer
Leukemia
Renal Cancer
Breast Cancer
Prostate Cancer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HCT15
WiDr
HT29
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HCT116WT
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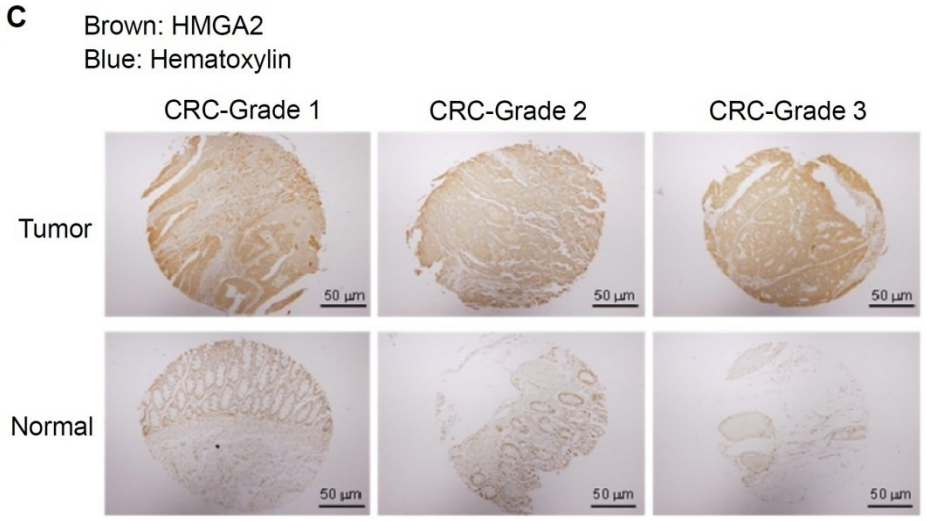
HMGA2
β-actin


C
Brown: HMGA2
Blue: Hematoxylin
CRC-Grade 1
CRC-Grade 2
CRC-Grade 3
Tumor
Normal
50 μm

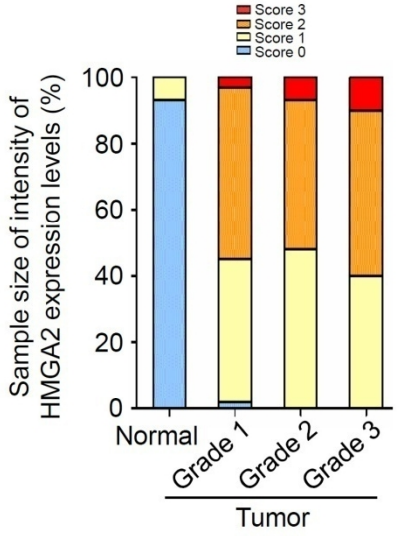

Score 3
Score 2
Score 1
Score 0
Sample size of intensity of HMGA2 expression levels (%)
100
80
60
40
20
0
Normal
Grade 1
Grade 2
Grade 3
Tumor

Fig.1

Figure 1 HMGA2 was overexpressed in colorectal cancer (CRC) cell lines and tumors. (A) Gene expression levels of HMGA2 protein in various human cancer cell lines. (B) HMGA2 protein analysis was conducted on proteins isolated from eight CRC cell lines and one noncancerous human colon cell line (CRL-1459). (C) Left panel: Representative immunohistochemical (IHC) images of HMGA2 expression in paired normal tissues and tumors of three CRC patients with different tumor grades. Right panel: HMGA2 protein expression levels obtained from the IHC results. The percentage of cases is plotted on the y-axis, and the type of sample is plotted on the x-axis; the color indicates the HMGA2 expression scores (0 to 3).

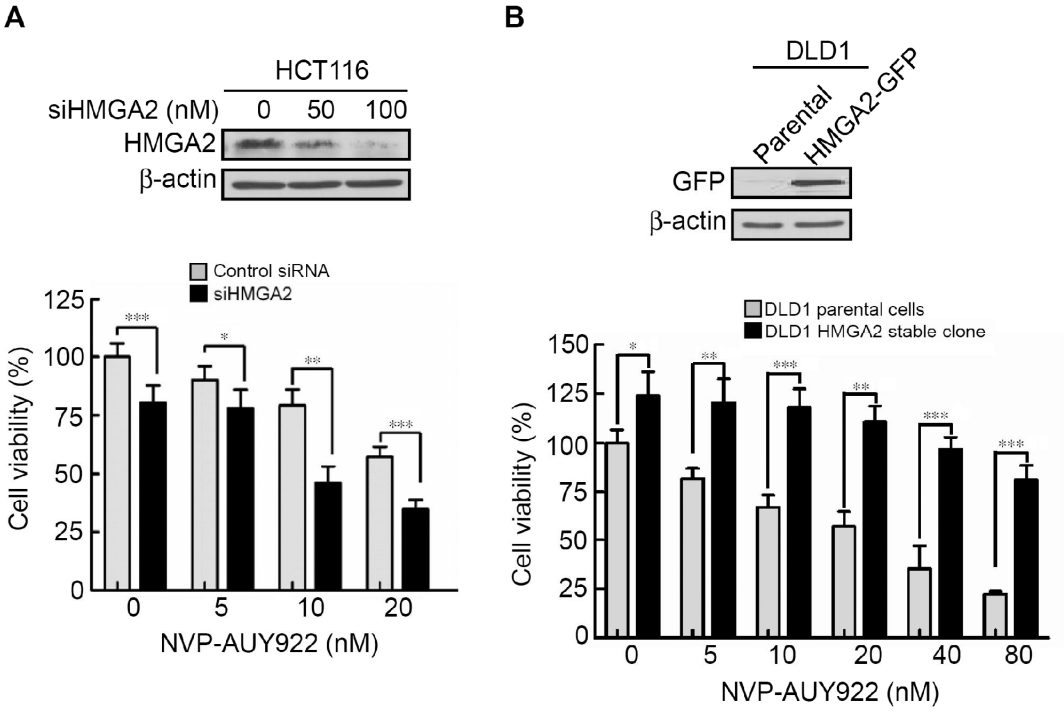


Fig.2

Figure 2 Expression levels of HMGA2 are responsible for NVP-AUY922 drug sensitivity. (A) Upper panel: HMGA2 was detected in siHMGA2-transfected HCT116 cells. Bottom panel: HCT116 cells were transfected with control siRNA and siHMGA2 for 48 h and subsequently incubated with NVP-AUY922 at the indicated concentrations for an additional 48 h. A cell viability assay was performed to determine the viability of cells treated with various NVP-AUY922 concentrations. Bars, SD (n = 6). (B) Upper panel: Western blotting with anti-GFP antibody of the parental and stable HMGA2-GFP groups of HCT116 cells. Bottom panel: Cell proliferation assays of the parental and stable HMGA2-GFP groups of HCT116 cells treated with NVP-AUY922 at the indicated concentrations for 48 h. Bars, SD (n = 6). *p<0.05, **p<0.01, ***p<0.001.

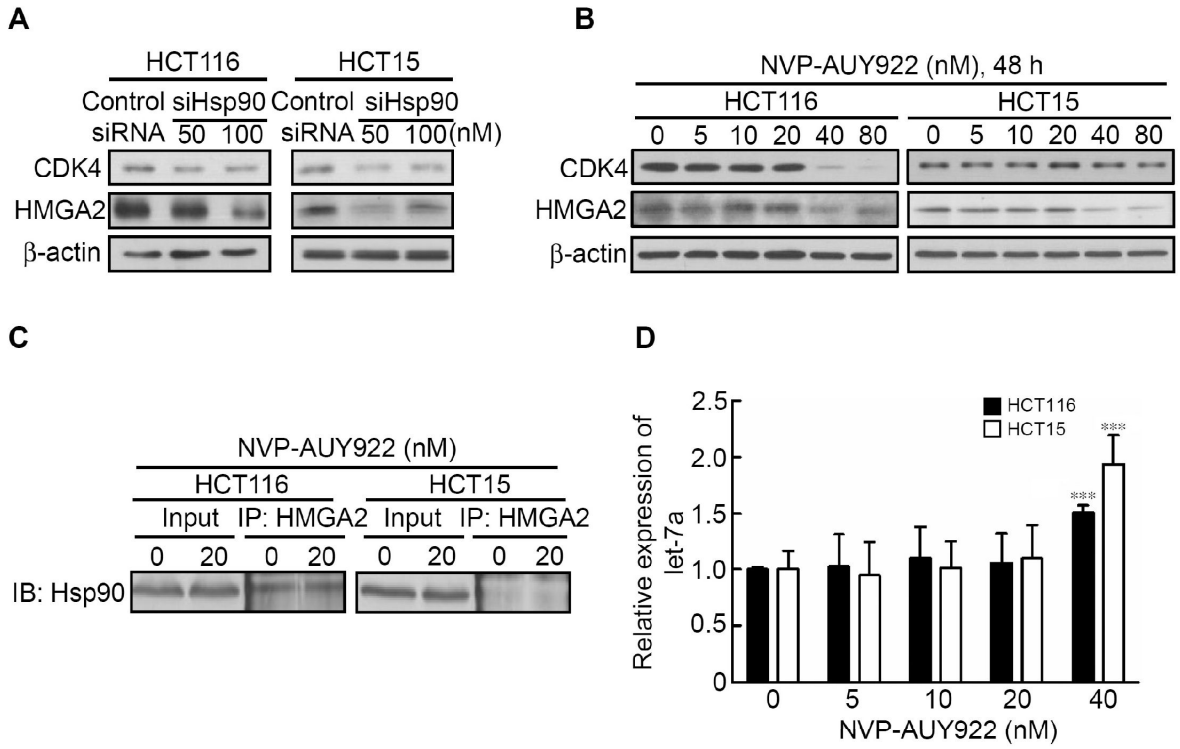


Fig.3

Figure 3 Hsp90 and Hsp90 inhibitor regulate the activity of HMGA2. (A) CDK4 and HMGA2 were detected in siHsp90-transfected HCT116 and HCT15 cells. (B) HCT116 and HCT15 cells were treated with NVP-AUY922 at the indicated concentrations for 48 h. Cell extracts were analyzed using Western blotting with the antibodies for CDK4 and HMGA2, respectively. (C) HCT116 and HCT15 cells were treated with NVP-AUY922 for 48 h, HMGA2 was immunoprecipitated from 500-μg cell lysate, and resultant blots were probed for Hsp90. (D) Let-7a expression in HCT116 and HCT15 cells treated with NVP-AUY922 at the indicated concentrations for 24 h was analyzed using quantitative RT-PCR. Let-7a expression was significantly upregulated on NVP-AUY922 treatment (40 nM) in both cell lines. *** $p<0.001$.

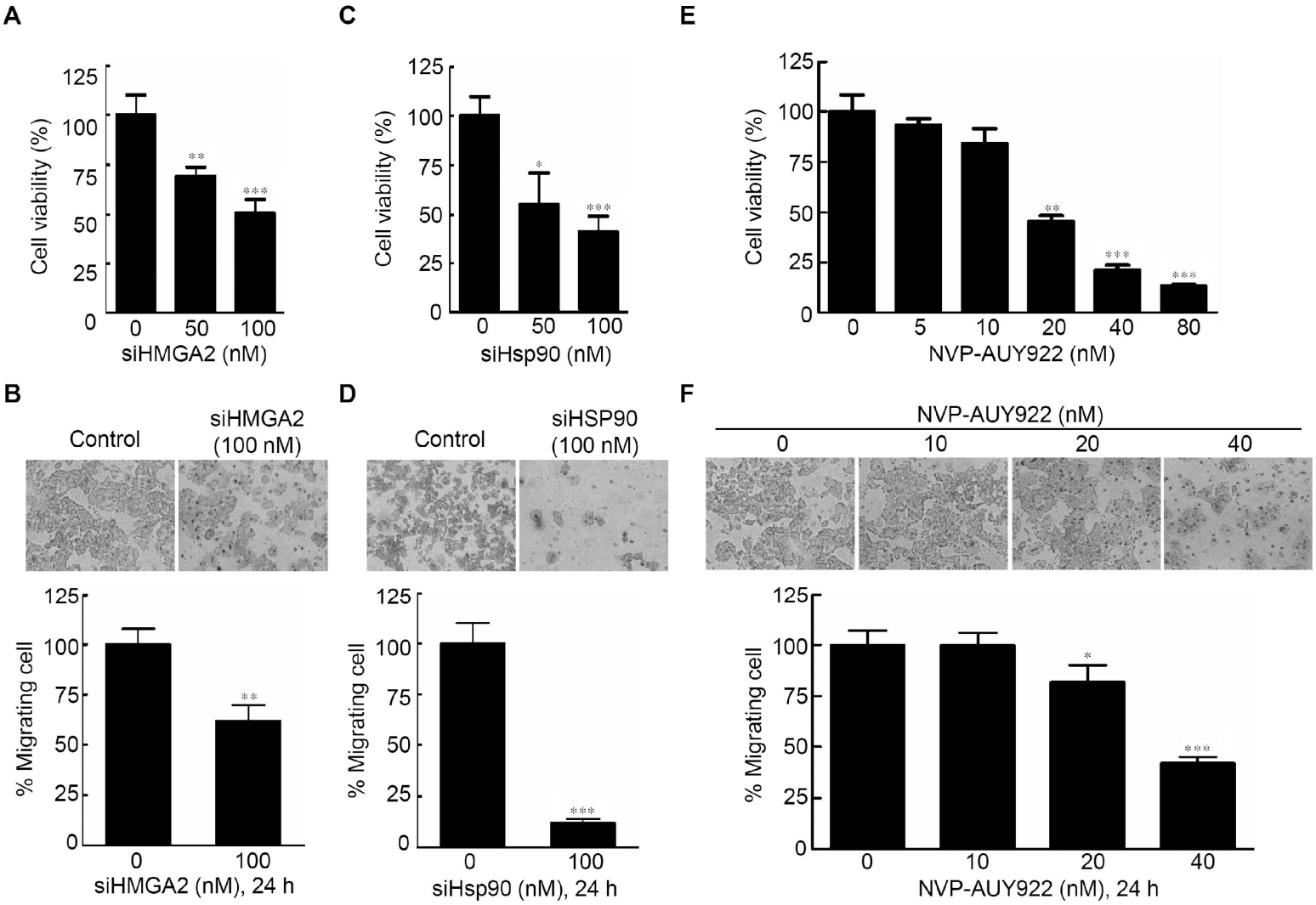

A
Cell viability (%)
125
100
75
50
25
0
0
50
100
siHMGA2 (nM)
C
Cell viability (%)
siHsp90 (nM)
E
Cell viability (%)
0
5
10
20
40
80
NVP-AUY922 (nM)
B
Control
siHMGA2
(100 nM)
% Migrating cell
siHMGA2 (nM), 24 h
D
Control
siHSP90
(100 nM)
% Migrating cell
siHsp90 (nM), 24 h
F
NVP-AUY922 (nM)
0
10
20
40
% Migrating cell
NVP-AUY922 (nM), 24 h

Fig.4

Figure 4 Effects of gene-specific inhibition of HMGA2 or Hsp90 and pharmaceutical inhibition of Hsp90 were similar. Cell viability assay (A, C, and E) and cell migration analysis (B, D, and F) were performed to determine the viability and migratory ability of HCT116 cells treated with siHMGA2, siHSp90, and various concentrations of NVP-AUY922, respectively. *p<0.05, **p<0.01, ***p<0.001.

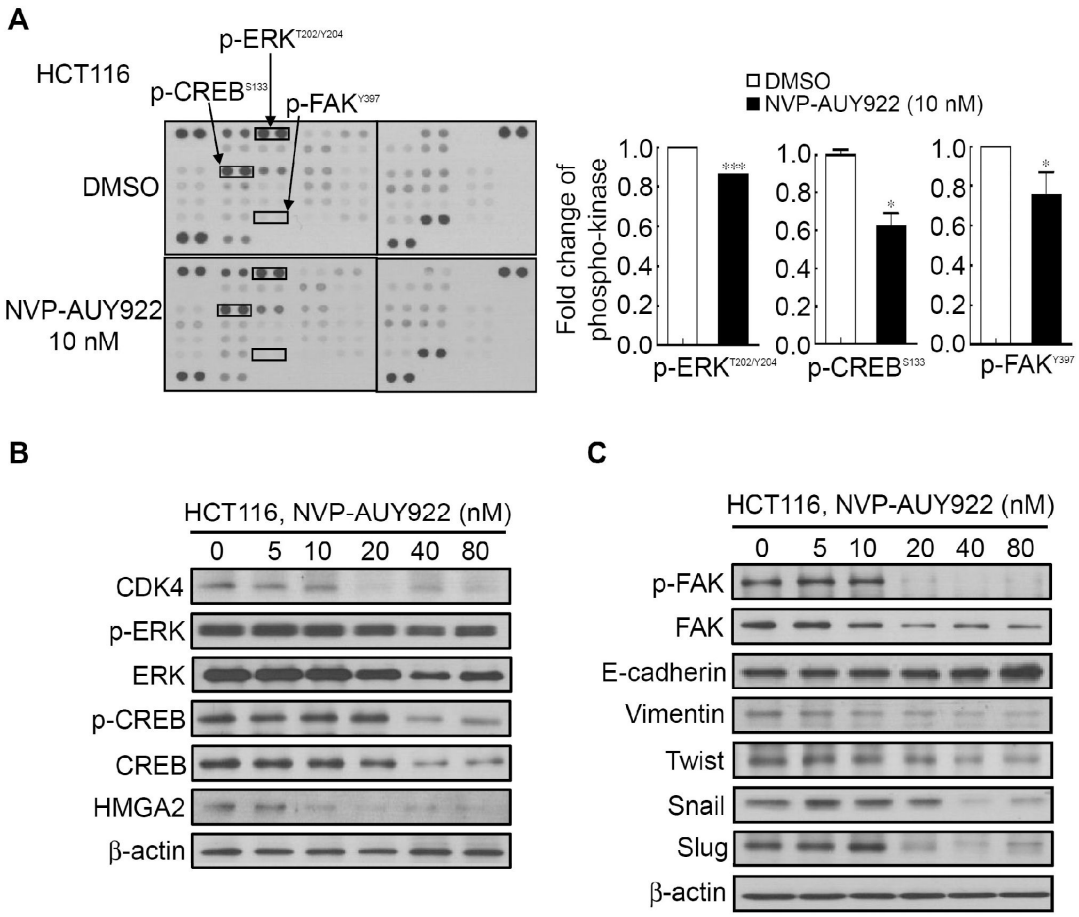
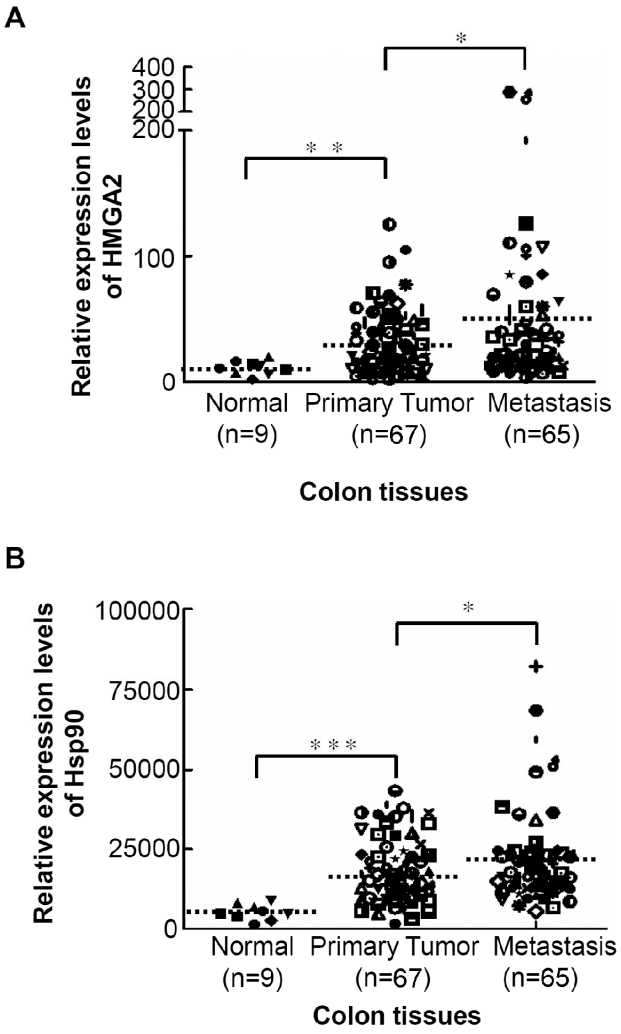


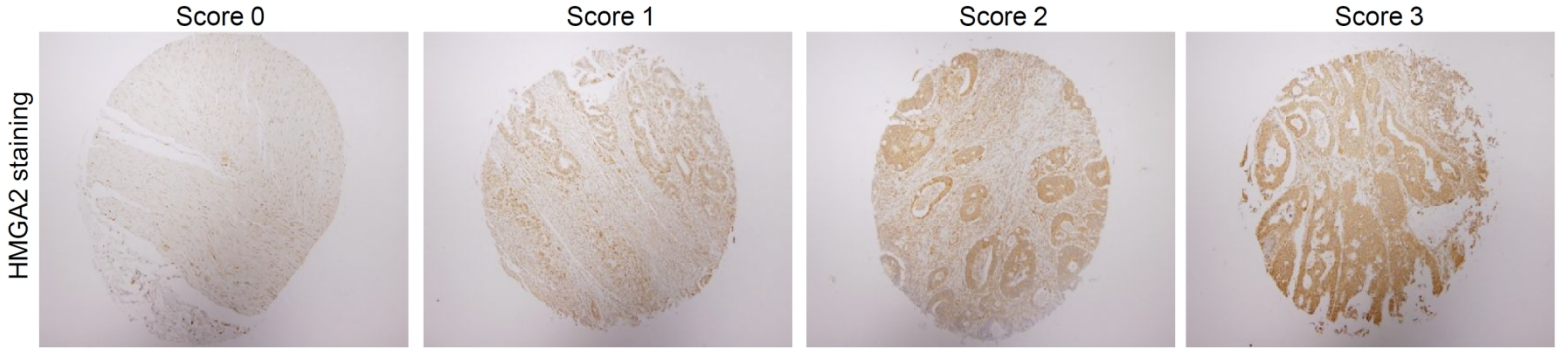
Fig.5

Figure 5 HMGA2-associated kinases and their downstream signaling intermediates are inactivated simultaneously in response to NVP-AUY922 treatment in HCT116 cells. (A) HMGA2-associated kinases, ERK, CREB, and FAK, were significantly downregulated on NVP-AUY922 treatment. (B) Western blotting results of the concentration-dependent effects of NVP-AUY922 on the phosphorylation and expression of ERK and CREB involved in HMGA2-mediated regulation of cell growth in HCT116 cells. (C) Western blotting results of the concentration-dependent effects of NVP-AUY922 on the phosphorylation and expression of FAK and various EMT effectors involved in HMGA2-mediated regulation of cell mobility in HCT116 cells.



Supplementary Fig. 1

Figure S1 mRNA expression levels of HMGA2 and Hsp90 in CRC tissues. Relative expression levels of HMGA2 (A) and Hsp90 (B) at different clinical stages of CRC tissues analyzed using the public Gene Expression Omnibus database. *p<0.05, **p<0.01, ***p<0.001.



Supplementary Fig. 2

Figure S2 Criterion of the staining level used to score the expression of HMGA2.