Hyperthermia reduces cancer cell invasion and combats chemoresistance and immune evasion in human bladder cancer

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Abstract. Bladder cancer (BC) is a common malignancy and its most prevalent type is urothelial carcinoma, which accounts for ~90% of all cases of BC. The current treatment options for BC are limited, which necessitates the development of alternative treatment strategies. Hyperthermia (HT), as an adjuvant cancer therapy, is known to improve the efficacy of chemotherapy or radiotherapy. The present study aimed to investigate the anti-tumor effects of HT on cell survival, invasiveness, chemoresistance and immune evasion in human BC cell lines (5637, T24 and UMUC3). Calcein AM staining was performed to analyze the cytotoxicity of natural killer (NK) cells against human BC cells following HT treatment. Cell migration and invasion affected by HT were analyzed using Transwell migration and invasion assays. It was found that HT inhibited the proliferation of BC cells by downregulating the phosphorylation of protein kinase B. Moreover, HT effectively enhanced the sensitivity of BC cells to the chemotherapy drug cisplatin (DDP) and reduced the chemoresistance of DDP-resistant cells by downregulating the expression of cadherin-11. It was further demonstrated that HT inhibited the migration and invasion of BC cells and enhanced the cytotoxic effects of NK cells. In summary, the antineoplastic effects of HT were mediated through three main mechanisms: Enhancement of the chemosensitivity of BC cells and mitigation of DDP-induced chemoresistance, suppression of the invasive potential of BC cells and reinforcement of the anticancer response of NK cells. Thus, HT appears to be a promising adjunctive therapy for human BC.

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Introduction

According to the International Agency for Research on Cancer, bladder cancer (BC) is a major global health concern, with >200,000 deaths and ~550,000 new cases reported annually worldwide (1). The mortality rate associated with BC varies considerably, with 5-year survival rates ranging from 96% for early-stage disease to only 5% for advanced-stage disease (2). Cancer recurrence within 5 years of diagnosis occurs in 60-70% of all patients with BC (3). Common treatment approaches for BC include transurethral resection, radiotherapy, intravesical chemotherapy and immunotherapy (4). Despite their efficacy against BC, these approaches have some limitations (5). In some patients with advanced BC, treatment leads to suboptimal outcomes (6); moreover, chemoresistance often complicates disease management (7). The rate of tumor response to immune checkpoint inhibitor-based immunotherapy is low at ~20% (8). Therefore, an improved therapeutic approach is needed for the effective management of BC.

Hyperthermia (HT) is a type of cancer therapy that involves exposing patients to high temperatures (41-45˚C) (9). This treatment approach includes local, regional and whole-body HT (10). The objective of HT treatment is to specifically target and damage or destroy cancer cells, with minimal damage to normal cells. Mechanistically, high temperatures directly damage cancer cells by denaturing proteins, destroying cell membrane and DNA and disrupting cellular metabolism (11). HT sensitizes cancer cells, enhancing their response to chemotherapy and radiotherapy (12,13). In patients with high-grade superficial BC, the combination of HT and intravesical mitomycin C is beneficial for inhibiting

recurrence (14). Furthermore, HT facilitates bladder preservation and function, demonstrating its viability as a therapeutic option for elderly patients with muscle-invasive BC who are unsuitable candidates for surgery or chemoradiotherapy (15). In patients with head and neck cancer, the combined use of HT and radiotherapy has been reported to increase the likelihood of achieving a complete response by ~25% compared with that observed when using conventional radiotherapy alone; notably, the combination therapy had no major acute or delayed side effects (16). Furthermore, HT can create a favorable microenvironment for antitumor immune responses (17), which makes it an attractive treatment option for use in combination with immunotherapies, such as intratumoral dendritic cell-based immunotherapy and natural killer (NK) cell-based adaptive immunotherapy (18,19). Overall, HT is promising as an adjunct to various standard treatments for cancers.

BC is a common malignancy of the urinary tract (20). The multifaceted molecular mechanisms underlying the progression of BC include cell proliferation, apoptosis regulation, cancer cell invasion, angiogenesis and metastasis (21-23). Matrix metalloproteinases (MMPs), which constitute a zinc-dependent endopeptidase family, play vital roles in different stages of cancer progression (24). Through the proteolytic degradation of the extracellular matrix and the release of matrix-bound proangiogenic factors (for example, vascular endothelial growth factor-A), MMPs facilitate the invasion of cancer cells into neighboring tissues (25,26). Moreover, MMPs play a role in the pathways involved in cell proliferation and survival signaling and thus promote tumor growth and metastasis (27).

The present study investigated the anti-tumoral effects of HT on human BC. HT inhibited the cell proliferation, suppressed cell migration and invasion through MMP2 downregulation, stimulated the antitumor immune response and reduced the chemoresistance of BC cells. These findings underscore the potential of HT as a viable adjunctive therapy for BC.

Materials and methods

Cell culture. The human BC cell lines 5637, UMUC3 and T24 and the normal epithelial cell line SV-HUC-1 were obtained from Bioresource Collection and Research Center. Each cell line was cultured in a specific medium: 5637, Roswell Park Memorial Institute-1640 (Gibco; Thermo Fisher Scientific, Inc.; Thermo Fisher Scientific, Inc.); UMUC3, Minimum Essential Medium (Gibco; Thermo Fisher Scientific, Inc.); T24, McCoy's 5A (Gibco; Thermo Fisher Scientific, Inc.); and SV-HUC-1, F12K (Gibco; Thermo Fisher Scientific, Inc.). All media were supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 2 mM GlutaMAX-1 and Penicillin/Streptomycin/Amphotericin B Solution (Sigma-Aldrich; Merck KGaA). The cells were incubated at 37˚C under 5% CO_2.

HT treatment in vitro. For the *in vitro* HT treatment, BC cells and normal epithelial cells were pretreated with varying concentrations of cisplatin (DDP; 0-25 μM) and exposed to 43˚C for 1 h, as previously described (28,29). Subsequently, all cells were maintained at 37˚C for 24 h. Following treatment, cell viability and colony formation were assessed.

Western blotting. Immunoblotting was performed using a previously reported method (30,31). Proteins from BC cells were extracted using RIPA lysis and extraction buffer (Thermo Fisher Scientific, Inc.) supplemented with 2% protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc.). Protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). A total of 30 μg of protein was applied to a 10-15% SDS-polyacrylamide gel for separation and then transferred onto PVDF membranes (MilliporeSigma). The membranes were blocked at room temperature for 1 h with 5% skimmed milk prepared in Tris-buffered saline with 0.1% Tween-20 (TBST), then washed three times with TBST. After blocking, the membranes were incubated overnight at 4˚C with the primary antibodies. Primary antibodies against the following proteins were used to measure their levels: heat shock protein (HSP)70, AKT, phosphorylated (p-)AKT, MMP2, MMP9, cadherin 11 (CDH11), programmed death ligand 1 (PD-L1), β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Following three washes with TBST, the membranes were incubated at room temperature for 1 h with HRP-conjugated secondary antibodies (anti-rabbit or anti-mouse; 1:3,000; GeneTex, Inc.). The proteins were detected by performing an enhanced chemiluminescence assay (Kodak X-OMAT LS Film; Kodak). Protein levels were quantified using a computing densitometer with ImageQuant LAS 4000 (Ge Healthcare Life Sciences). Additional information on the antibodies used for western blotting is presented in Table I.

Transfection. The negative control short hairpin (sh)RNA (shCon; 5'-CGCGATCGTAATCACCCGAGT-3') and CDH11 shRNA (shCDH11; 5'-GCAGATTTGTATGGT TCCAAA-3') were both purchased from RNAiCore, while pcDNA3.1(+)-scrambled plasmid and PD-L1 overexpressed plasmid were obtained from Addgene. Transient transfection was carried out using a ViaFect Transfection Reagent (Promega Corporation) with Opti-MEM (Thermo Fisher Scientific, Inc.) medium following to the manufacturer's instructions. BC cells were transfected with or without 2 μg/μl of each of the aforementioned plasmid, combined with 4 μl of transfection reagent, and incubated for 24 h at 37˚C in an incubator. Cell samples were then evaluated for the indicated protein expression, as well as their cell viability and adhesion abilities.

Reverse transcription-quantitative (RT-q) PCR. BC cells were seeded in a 6-well plate ($3x10^5$ cells/well) and treated with HT. Total RNA was extracted from BC cells using TRIzol® manufacturer's protocol (Thermo Fisher Scientific, Inc.). RNA concentration was measured using Nanodrop (Thermo Fisher Scientific, Inc.). A total of 1 μg of RNA was reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The obtained complementary DNA was subjected to RT-qPCR, which was performed using the SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primer pairs were used: human CDH11 (forward, 5'-ACCCTCACCATC AAAGTCTG-3'; reverse, 5'-TCAGGGTCACAAACAATA CT-3'), human E-cadherin (forward, 5'-CGAGAGC TACAC GTTCACGG-3'; reverse, 5'-GGGTGTCGAGGGAAAAA

Table I. Antibodies used in the present study.

Antibody	Application	Supplier	Catalog number
HSP70	Western blotting	Cell Signaling Technology, Inc.	4873
p-AKT	Western blotting	GeneTex, Inc.	GTX128414
AKT	Western blotting	GeneTex, Inc.	GTX121937
MMP2	Western blotting	Cell Signaling Technology, Inc.	40994
CDH11	Western blotting	Sigma-Aldrich; Merck KGaA	3112087
PD-L1	Western blotting	Cell Signaling Technology, Inc.	13684
Ki-67	Immunofluorescence	Cell Signaling Technology, Inc.	9449
β-actin	Western blotting	Sigma-Aldrich; Merck KGaA	A5441
GAPDH	Western blotting	Proteintech Group, Inc.	60004-1-lg

HSP, heat shock protein; p-, phosphorylated; MMP, matrix metalloproteinase; CDH11, cadherin 11; PD-L1, programmed death ligand 1.

TAGG-3'), human cytokeratin-8 (forward, 5'-ACAAGGTAG AGCTGGAGTCTCG-3'; reverse, 5'-AGCACCACAGATGTG TCCGAGA-3'), human snail1 (forward, 5'-TCGGAAG CCT AACTACAGCGA-3'; reverse, 5'-AGATGAGCATTGGCA GCGAG-3'), human ZEB1 (forward, 5'-GATGATGAATGC GAGTCAGATGC-3'; reverse, 5'-ACAG CAGTGTCTTGTT GTTGT-3'), human ZEB2 (forward, 5'-CAAGAG GCGCAAA CAAGCC-3'; reverse, 5'-GGTTGGCAATACCGTCATCC-3') and human β-actin (forward, 5'-CACCATTGGCAATGA GCGGTTC-3'; reverse, 5'-AGGTCTTT GCGGATGTCCACG T-3'). The reaction parameters were the same as those reported previously (31). The thermocycling protocol was as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, and annealing at 60˚C for 360 sec. The melting curve analysis was conducted with denaturation at 95˚C for 15 sec, annealing at 60˚C for 1 min, and a final high-resolution melt at 95˚C for 15 sec. Each RT-qPCR reaction was performed with three technical replicates. The expression levels of the indicated EMT markers were assessed using the $2^{-\Delta\Delta Cq}$ method to quantify transcript levels, with β-actin serving as the internal control (32).

Cell viability. BC cells were cultured in 48-well plates ($2x10^4$ cells/well). Then, they were subjected to HT and treated with different concentrations of DDP (0, 3.125, 6.25, 12.5 and 25 μM) for 24 h. Cell viability was assessed using a resazurin reagent (Biotium, Inc.). Briefly, the resazurin solution was added to the well, constituting 10% of the initial volume and the plate incubated for 6 h at 37˚C under 5% CO_2. Subsequently, the fluorescence signal was measured using a multimode microplate reader (Varioskan LUX Plate Reader; Thermo Fisher Scientific, Inc.) at an excitation wavelength of 550 nm and an emission wavelength of 600 nm.

Colony formation assay. BC cells were seeded in a 6-well plate ($3x10^3$ cells/well) and treated with HT and DDP. After 7 days of incubation, a colony was defined as having ≤50 BC cells. The cell colonies were then fixed with 3.7% formaldehyde for 20 min at room temperature, followed by staining with 0.05% crystal violet (w/v) for another 20 min at room temperature. The primary stain was extracted with 10% acetic acid. The absorbance of the resultant solution was measured to quantify the number of cells in the colonies.

Transwell migration and invasion assays. Transwell inserts (pore size, 8 μm; Costar; Corning, Inc.) were used in 24-well plates to perform cell migration and invasion assays. For the cell invasion assay, the upper chamber was precoated with 30 μl of Corning Matrigel matrix (Corning, Inc.) and incubated for 30 min at 37˚C. The upper chamber was seeded with $1x10^4$ BC cells suspended in 200 μl of serum-free medium, whereas the lower chamber was filled with 300 μl of 1% FBS medium. After 24 h of incubation at 37˚C, the cells that had migrated and invaded into the lower chamber were stained with 0.05% crystal violet for 30 min at room temperature, which was followed by the quantification of cell numbers.

Immunofluorescence (IF) assay. BC cells were placed on a chamber slide (Sigma-Aldrich; Merck KGaA) and treated as indicated. BC cells were incubated with a primary antibody against Ki-67 (1:50 dilution; Cell Signaling Technology, Inc.) for 1 h at room temperature and then counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. Ki-67-positive BC cells were visualized under the Nikon Ti2 fluorescence microscope (Nikon Corporation). Further information about the Ki-67 antibody is presented in Table I.

Cytotoxicity assay. BC cells were seeded in a 48-well plate ($1x10^4$ cells/well) and treated as indicated. The cells were pre-stained with calcein AM (a green fluorescent dye; 1 μg/μl) for 1 h at 37˚C in an incubator, then washed three times with phosphate-buffered saline (PBS). Next, the cells were mixed with NK-92MI cells at a ratio of 1:5 and incubated for 4 h at 37˚C in an incubator. Viable BC cells were identified based on the green fluorescence signal of calcein AM and the cytotoxicity of NK cells was assessed by measuring the corresponding fluorescence signal using a Varioskan LUX Plate Reader (Thermo Fisher Scientific, Inc.).

Cell adhesion assay. A cell monolayer was formed by seeding BC cells ($3.5x10^5$ cells/well) in a 6-well plate. The cells were

then subjected to HT treatment with or without PD-L1 plasmid (1 μg/μl) transfection. After 24 h, calcein AM-prestained NK-92MI cells were cocultured with the BC cells for 1 h at a 37˚C incubator. Nonadherent NK cells were removed by washing with PBS three times. Subsequently, the cell monolayer was fixed in 3.7% formaldehyde for 30 min at room temperature and the number of green fluorescence-emitting NK-92MI cells was counted to quantify NK cells adhesion on BC cells.

Statistical analysis. Each experiment was performed thrice in triplicate. Data are presented in terms of mean ± standard deviation (SD) values. Student's t-test was used to compare mean values between two experimental groups, whereas one-way analysis of variance followed by Bonferroni's post hoc test was used to compare mean values between >2 groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HT inhibits the proliferation of human BC cells. The present study first investigated the inhibitory effects of HT on proliferation of bladder epithelial cells (SV-HUC-1) and BC cells at various stages of cancer (5637, grade II; T24, grade III; UMUC3, grade III). Effective HT treatment was confirmed based on an increase in the level of HSP70 compared with the level at normal temperature (NT; 37˚C), as shown in Fig. 1A-D. It was observed that BC cells subjected to HT displayed a reduction in the expression level of Ki-67, a key biomarker of cell proliferation (Fig. 1E-G). Considering that AKT is essential for cell proliferation (33), it was further investigated whether HT affected the levels of AKT protein expression. The resulting data showed that HT inhibited phosphorylated and total AKT protein expression in BC cells (Fig. 1I-K). However, bladder epithelial cells did not show such effects (Fig. 1H and L). The colony formation assay revealed that HT reduced the survival rate of BC cells, while an AKT activator suppressed this phenomenon (Fig. 1M-O). Taken together, HT inhibited the proliferation of BC cells by downregulating the expression of AKT.

HT enhances the sensitivity of human BC cells to DDP. Platinum-based chemotherapy drugs are the preferred first-line treatment and are often effective in eliciting positive treatment responses (34,35). However, the adverse effects of chemotherapy remain a major concern. The present study found that HT enhanced the therapeutic efficacy of DDP for BC. The combination of HT and DDP dose-dependently reduced the survival rate of BC cells (Fig. 2A-C). To assess the effect of HT on the long-term effectiveness of DDP treatment, a colony formation assay was conducted. The resulting data demonstrated that HT enhanced the sensitivity of BC cells to DDP (Fig. 2E-G). As expected, the co-administration of HT and DDP showed minimal effects on normal epithelial cells (Fig. 2D and H). In conclusion, HT showed promise as a complementary approach alongside chemotherapy for human BC.

HT suppresses the DDP resistance in human BC cells. Morphological transition from a mesenchymal to an epithelial phenotype was observed in BC cells with HT treatment (Fig. 3A). Considering that epithelial-mesenchymal transition (EMT) activity is known to regulate drug resistance (36), it was hypothesized that HT treatment would counteract chemoresistance by promoting mesenchymal-epithelial transition (MET). Through HT treatment, DDP-resistant BC cells (UMUC3/DDP) exhibited an increase in chemosensitivity to DDP (Fig. 3B). It was found that HT reduced the levels of CDH11 expression (Fig. 3C and D), while the expressions of E-cadherin, cytokeratin-8, snail1 and ZEB1/2 showed no such effect (Fig. S1A). In bladder epithelial cells, neither CDH11 nor other EMT markers showed significant differences (Fig. S1B and C). *CDH11* knockdown significantly increased the sensitivity of BC cells to DDP (Fig. 3E), doxorubicin and epirubicin (Fig. S1B and C). Furthermore, the expression level of CDH11 protein was higher in UMUC3/DDP cells than in wild-type UMUC3 cells (Fig. 3F). Finally, through cell viability and colony formation assays, it was validated that the inhibition of CDH11 was associated with the enhanced chemosensitivity in UMUC3/DDP cells (Fig. 3G and H). Notably, the combination of low-dose DDP with CDH11 shRNA demonstrated anticancer effects comparable to those exerted by high-dose DDP alone (Fig. 3H), indicating the critical role of CDH11 in regulating chemoresistance in BC.

HT suppresses the migration and invasion of human BC cells. Treating cancer poses challenges, given the capacity of cancer cells to metastasize to distant locations (27). The Transwell migration and invasion assay demonstrated that HT significantly inhibited the migratory and invasive abilities of BC cells (Fig. 4A and B). MMP2 and MMP9 are crucial mediators of BC progression (37-39). The resulting data indicated that HT comprehensively suppressed MMP2 expression but had no effect on MMP9 in BC cells (Fig. 4C and D; Fig. S2A-D). In bladder epithelial cells, the expression of both MMP2 and MMP9 was unaffected by HT (Fig. S2E and F). Taken together, it appeared that HT might inhibit the migration and invasion of BC cells by downregulating the expression of MMP2.

HT enhances the cytotoxicity of NK cells. PD-L1 is a crucial immune checkpoint protein that inhibits the cytotoxicity of NK cells in cancer (40). The present study found that HT significantly suppressed the levels of PD-L1 protein expression (Fig. 5A-C), while bladder epithelial cells were not affected (Fig. S2G). Next, BC cells were overexpressed with PD-L1 (Fig. S2H-J) and it was found that the HT-enhanced adhesion and cytotoxicity of NK cells towards BC cells were impeded by the overexpression of PD-L1 in these cells (Fig. 5D and E). Therefore, HT enhances the cytotoxicity of NK cells against BC cells by downregulating the expression of PD-L1.

Discussion

As HT affects various biological processes in tumor cells and their surrounding microenvironments (41), it has emerged as a promising adjunctive therapy for different cancers. Clinical and preclinical studies have indicated that HT combined with chemotherapy enhances the rates of treatment response and overall survival in various cancers, such as non-small cell lung cancer (42), esophageal cancer (43) and soft tissue sarcomas (44).

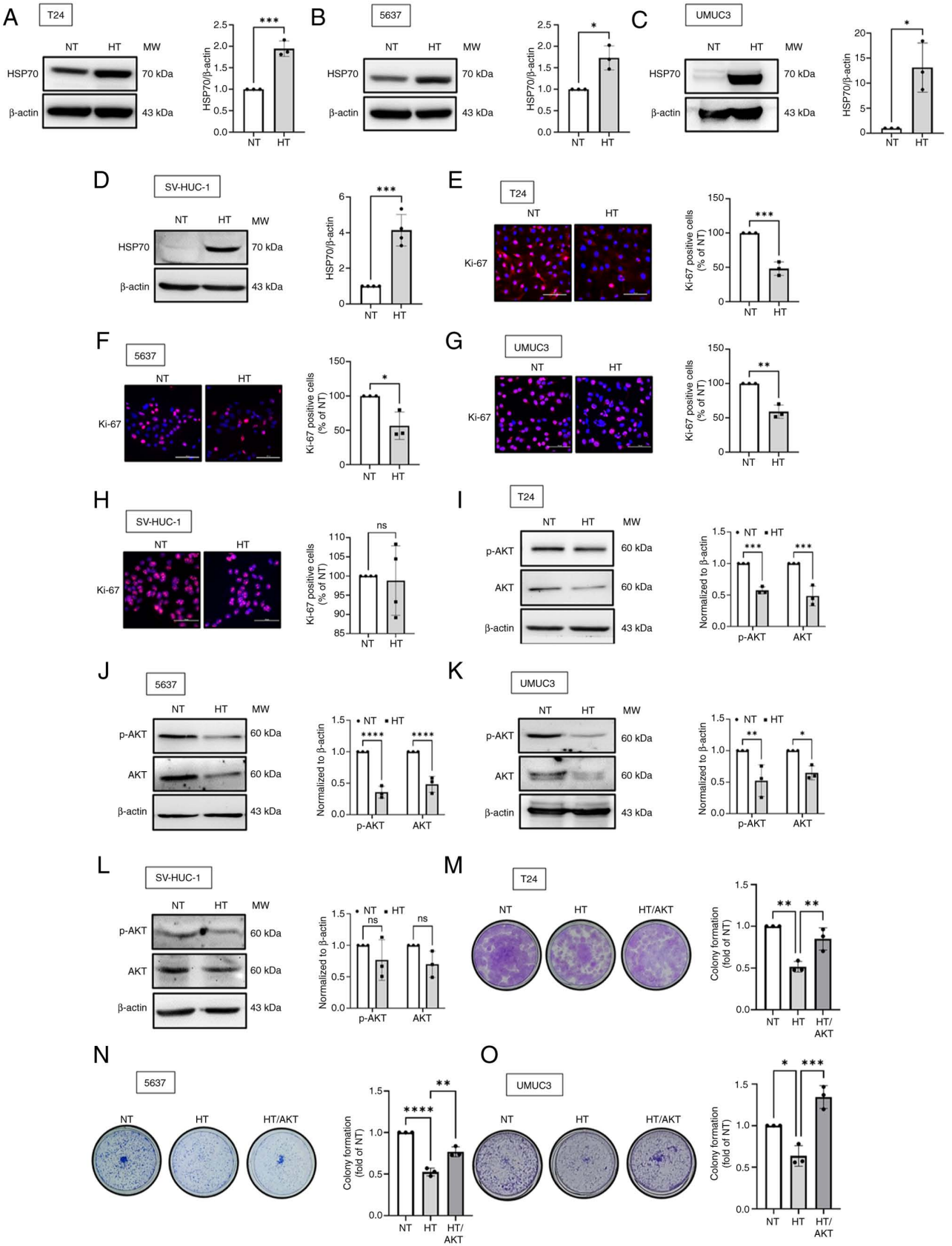

Figure 1. HT inhibits the proliferation of human BC cells. (A-D) The normal bladder epithelial cell line (SV-HUC-1) and BC cell lines (T24, 5637 and UMUC3) were subjected to HT for 1 h, followed by incubation at NT for 24 h. The level of HSP70 protein expression was evaluated through western blotting. (E-H) IF staining was performed to measure the expression level of Ki-67 in BC cells and normal bladder epithelial cell; Ki-67 was stained red, whereas the nuclei were stained blue (DAPI). (I-L) Western blotting was performed to measure the levels of AKT and p-AKT protein expression. (M-O) Colony formation assays were performed to assess the viability of BC cells subjected to HT and AKT activator (SC79; 0.5 μg/ml). Data are presented in terms of mean ± SD values. Two-groups comparisons were performed using Student's t-test, whereas one-way ANOVA followed by Bonferroni's post hoc test was used to compare mean values among multiple groups. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, vs. NT group. HT, hyperthermia; BC, bladder cancer; NT, normal temperature; HSP70, heat shock protein 70; DAPI, 4',6-diamidino-2-phenylindole; ns, not significant; p-, phosphorylated.

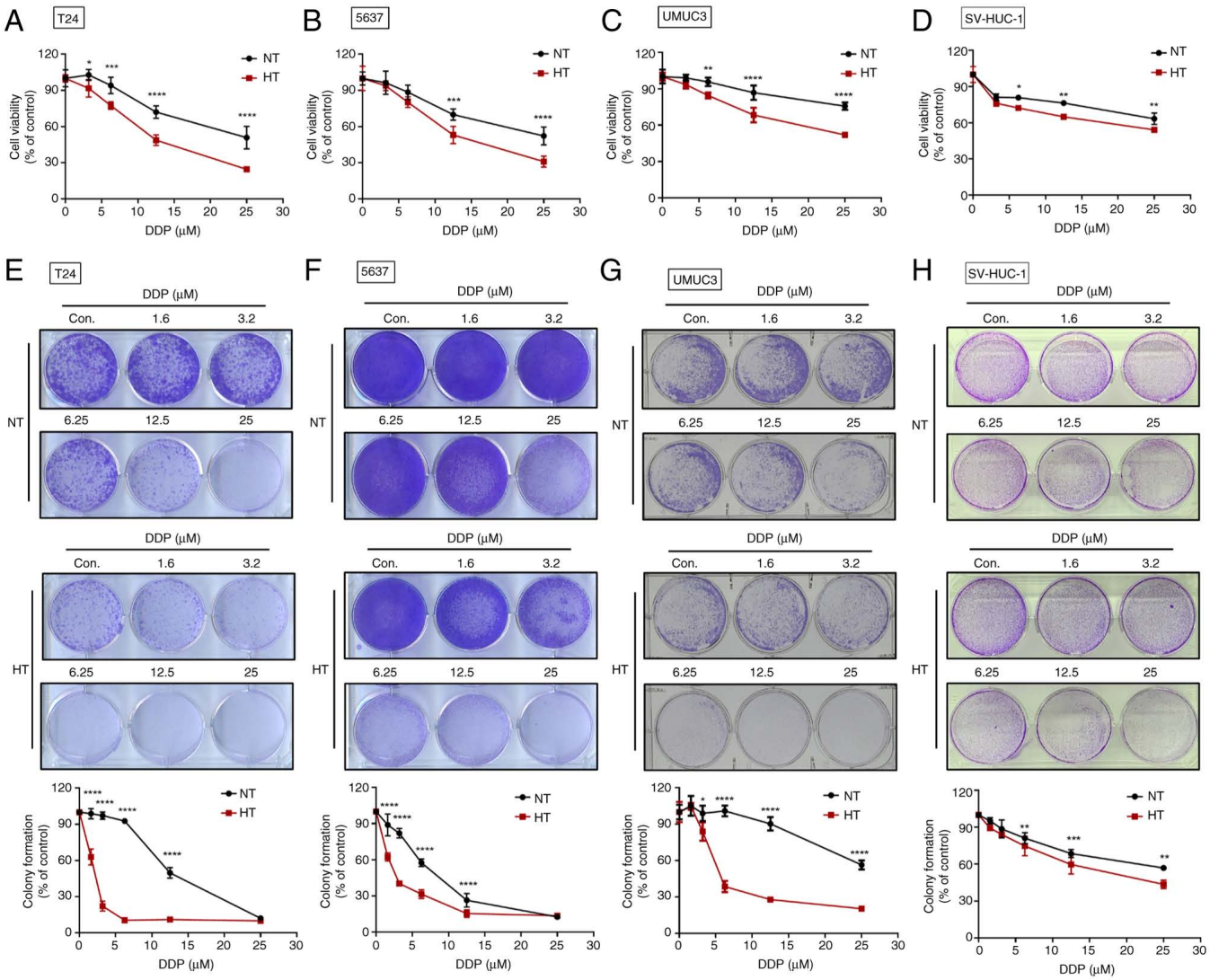

Figure 2. HT improves the sensitivity of human BC cells to DDP. (A-D) The BC cell lines (T24, 5637 and UMUC3) and normal bladder epithelial cell line (SV-HUC-1) were subjected to HT for 1 h, followed by incubation with varying concentrations of DDP for 24 h. Cell viability was assessed using a resazurin reagent. (E-H) After 1 week of incubation, the survival rate of cells treated with both HT and DDP was evaluated through colony formation assays. Data are presented in terms of mean ± SD values. Two-groups comparisons were performed using Student's t-test, whereas one-way ANOVA followed by Bonferroni's post hoc test was used to compare mean values among multiple groups. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, vs. NT group. HT, hyperthermia; BC, bladder cancer; DDP, cisplatin; NT, normal temperature.

The clinical bladder HT systems comprise external regional hyperthermia therapy (RHT) achieved through tissue penetration heating, microwave heating facilitated by a miniature antenna inserted via an intravesical catheter and intravesical circulation of heated fluid to warm the interior wall of the bladder (45). The BSD-2000, one of the most widely used devices for RHT, has been used as an adjunctive treatment in conjunction with surgery, radiation and chemotherapy for BC patients (46,47). A study in which patients were followed up for 2 years revealed that the efficacy of intravesical thermochemotherapy in treating of superficial transitional cell carcinoma of the bladder was higher than that of traditional intravesical therapy delivered using mitomycin C (48). Although the combination therapy results in an increase in the level of local toxicity, the level remains manageable and within acceptable thresholds (48). Furthermore, a study exploring the benefits of intravesical thermochemotherapy for patients with non-muscle invasive bladder cancer reported promising long-term outcomes; notably, 53% of all patients were tumor-free 10 years after treatment completion and 86% of all patients were able to retain their bladders (49). In terms of tumor recurrence, HT combined with intravesical chemotherapy is more effective than conventional bladder chemotherapy and equally effective as bacillus Calmette-Guérin perfusion (50). These findings further support the use of HT as an adjunctive therapy, which is effective in managing BC over extended periods.

Chemotherapy serves as the first-line treatment for human BC (51); however, chemoresistance remains a major hurdle in achieving effective treatment outcomes (7,52). A key factor contributing to chemoresistance is EMT, which refers to cellular transformation from an epithelial phenotype to a mesenchymal phenotype with reduced chemosensitivity (53,54). EMT mediates chemoresistance through a number of mechanisms, such as the activation of efflux pumps (55), inhibition of apoptosis and upregulation of the survival signaling pathway, ultimately leading to the acquisition of stem cell-like features with inherent

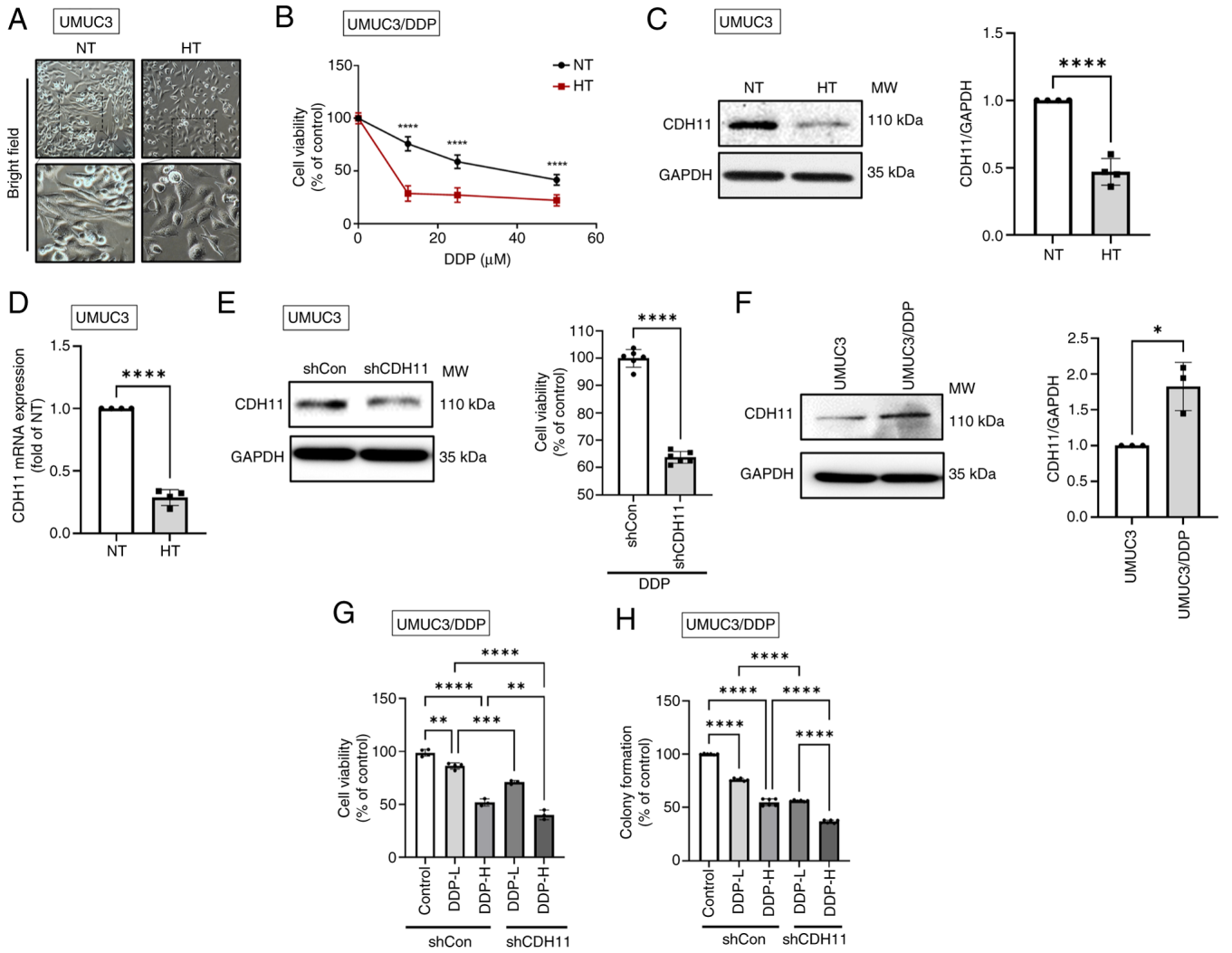

Figure 3. HT mitigates the DDP resistance of human BC cells by downregulating CDH11 expression. (A) Morphological characteristics of cells subjected to HT were observed under a bright-field microscope. (B) Following HT treatment for 1 h, DDP-resistant UMUC3 cells (UMUC3/DDP) were incubated with varying concentrations of cisplatin for 24 h. Cell viability was assessed using a resazurin reagent. (C) Western blotting and (D) RT-qPCR and were performed to evaluate the levels of CDH11 mRNA and protein in UMUC3 cells, respectively. (E) UMUC3 cells were transfected with either control shRNA (shCon; 1 μg/μl) or CDH11 shRNA (shCDH11; 1 μg/μl) for 24 h and then incubated with DDP (100 μM) for another 24 h. Cell viability was assessed using a resazurin reagent. (F) Western blotting was performed to measure the protein levels of CDH11 in both UMUC3 and UMUC3/DDP cells. (G) UMUC3/DDP cells were transfected with shCon or shCDH11 for 24 h and then incubated with high-dose (100 μM) and low-dose (25 μM) DDP for another 24 h. Cell viability was assessed using a resazurin reagent. (H) UMUC3/DDP cells were cotreated with shCDH11 and high-dose (6 μM) or low-dose (2 μM) DDP for 1 week; colony formation assays were performed to estimate the rate of cell survival. Data are presented in terms of mean ± SD values. Two-groups comparisons were performed using Student's t-test, whereas one-way ANOVA followed by Bonferroni's post hoc test was used to compare mean values among multiple groups. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, vs. control group. HT, hyperthermia; DDP, CDH11, cadherin 11; BC, bladder cancer; cadherin 11 NT, normal temperature; RT-qPCR, reverse transcription-quantitative PCR; sh, short hairpin; shCon, control shRNA; CDH11 shRNA, shCDH11.

chemoresistance potential (56-58). Therefore, targeting EMT may help overcome the challenges associated with the chemoresistance of BC cells. The present study revealed that HT increased the sensitivity of BC cells to the chemotherapy drugs DDP, doxorubicin and mitomycin C. Furthermore, this therapy mitigated the chemoresistance of DDP-resistant cells to DDP by negatively regulating EMT. The expression of CDH11, a biomarker of EMT, was upregulated in DDP-resistant BC cells. DDP resistance can be mitigated by reducing the level of CDH11 through the shRNA-mediated knockdown of *CDH11*; this indicates the importance of CDH11 in regulating DDP resistance. Notably, HT reduced the levels of CDH11 mRNA and protein in BC cells, increasing their susceptibility to chemotherapy drugs. In summary, the modulation of CDH11 by HT optimized the response of BC cells to chemotherapy. Thus, HT is an attractive adjunctive treatment strategy for BC. A study indicated that the induction of miR-409-3p expression through HT prevents EMT by reducing the expression levels of β-cadherin, vimentin and N-cadherin (59). Furthermore, HT can sensitize gemcitabine-resistant pancreatic cancer cells by reversing EMT through the regulation EMT-associated factors, such as the epithelial marker E-cadherin (upregulated) and the mesenchymal marker vimentin (downregulated) (60).

HT exerts direct and indirect effects on the immune cells present in tumor microenvironments (61). HSP70 and HSP90, released by cancer cells subjected to HT, activate various immune cells, such as macrophages, dendritic cells and T cells, thereby modulating immune response through HSP-mediated

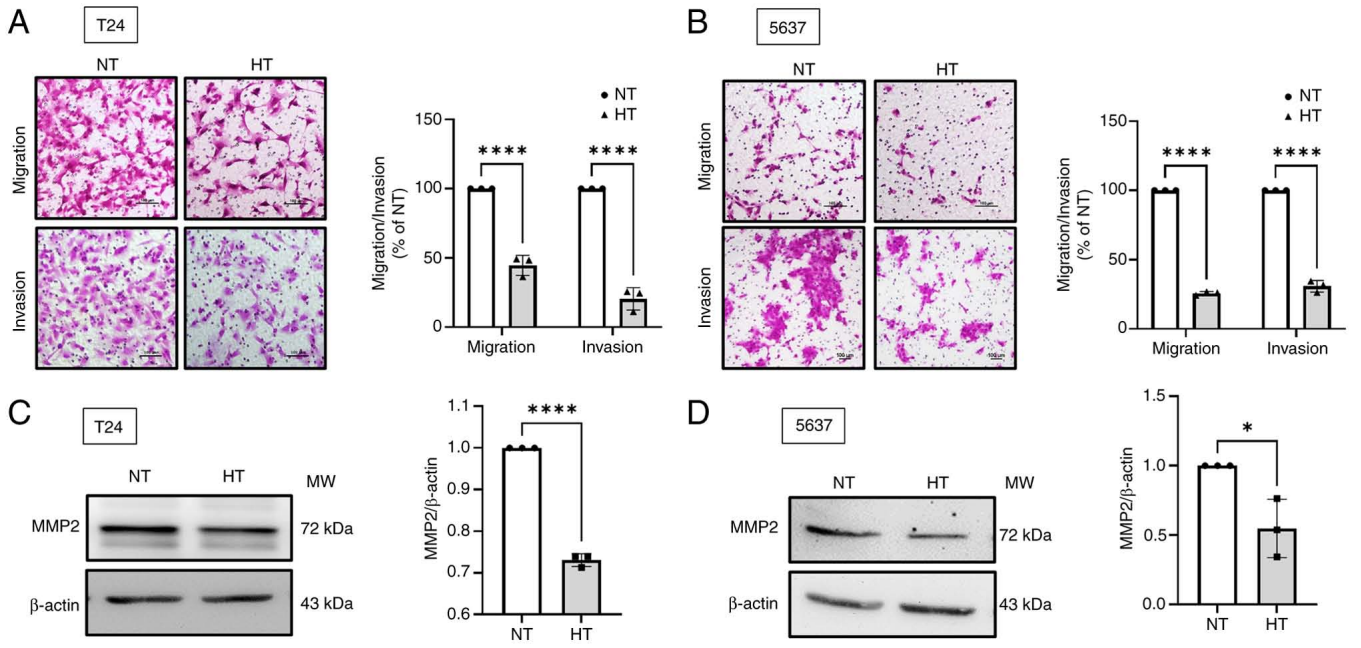

Figure 4. HT inhibits the migration and invasion of human BC cells. (A and B) Transwell migration and invasion assays were conducted to assess the migratory and invasive abilities of BC cells. Magnification, x200. (C and D) Western blotting was performed to measure the level of MMP2 protein expression in BC cells. Data are presented in terms of mean ± SD values. Two-groups comparisons were performed using Student's t-test, whereas one-way ANOVA followed by Bonferroni's post hoc test was used to compare mean values among multiple groups. *P<0.05 and ****P<0.0001, vs. NT group. HT, hyperthermia; BC, bladder cancer; NT, normal temperature; MMP, matrix metalloproteinase.

signaling pathways (62-64). The findings of the present study indicated that HT can reduce the expression level of PD-L1 in BC cells, thus enhancing the cytotoxicity of NK cells against the cancer cells. HT can upregulate the expression of the adhesion molecules L-selectin, P-selectin and intercellular cell adhesion molecule-1 on endothelial cells that favor T-cell infiltration into the tumor site (65). Additionally, HT can activate NK cells by increasing the activation of receptors such as NK group 2D, thereby enhancing the ability of NK cells to efficiently target and eliminate cancer cells (66). HT can also induce macrophages to secrete cytokines, such as interleukin-6 and tumor necrosis factor-alpha, into the tumor microenvironment, thereby activating the response of immune cells to tumors (62). Moreover, HT increases the blood flow to the affected tissues, thus enhancing delivery of antitumor agents and immune effectors to the cancer site (67,68). Collectively, HT can strengthen the immune response against malignant neoplasms by inhibiting the suppression of immune response and enhancing the ability of immune cells to recognize and target cancer cells.

The association between MMP2 and an unfavorable prognosis is well established (37) and the potential of MMP2 as a prognostic biomarker has been demonstrated in patients with BC (69). Suppressing MMP2 activity can reduce the invasiveness of BC cells, both *in vitro* (70) and *in vivo* (71). Approaches aimed at inhibiting various MMPs (such as, MMP2) hold promise for preventing cancer progression and distant metastasis. The present study demonstrated that HT limited the migration and invasion of BC cells by downregulating the expression of MMP2. HT has been reported to reduce cell invasiveness in various cancers, including breast cancer (72), pancreatic cancer (60), glioma (73), gastric cancer (59) and glioblastoma (74). An *in vivo* study conducted by Ma *et al* (75) revealed that the combination of a chemokine (C-C motif) ligand 3 derivative (enhanced macrophage inflammatory protein) and HT markedly inhibited the growth of colon adenocarcinoma cells and significantly reduced distant metastases to the lungs. In a study conducted using an *in vivo* breast tumor model, the combination of ultrasound-stimulated microbubble exposure with HT increased apoptosis and vascular disruption compared with the outcomes of ultrasound-stimulated microbubble exposure alone (76). This highlights the wide applicability of HT in inhibiting cell invasion and distant metastasis across cancers.

The present study offered a comprehensive analysis of the anticancer effects of HT in BC, highlighting its potential as an adjunctive therapy. Specifically, it demonstrated that HT inhibited cancer cell growth by reducing both phosphorylated AKT and total AKT signaling proteins, while decreasing the resistance of BC cells to DDP-based medications by suppressing CDH11, which could be beneficial for combination chemotherapy. Additionally, HT reduced cancer cell motility by inhibiting MMP2 expression. Finally, HT enhanced the immunotoxicity of NK cells against cancer cells by downregulating PD-L1 expression. It was hypothesized that these findings offered significant insights into the multifaceted antitumor effects of HT and highlighted its potential as a supplementary treatment in bladder cancer therapy (Fig. 6). However, further studies are needed to include normal bladder epithelial cells as a control group for comparison with bladder cancer cells, to demonstrate that HT primarily affects cancer cells rather than normal cells. Additionally, since the present study was conducted *in vitro* and did not fully simulate the complex tumor

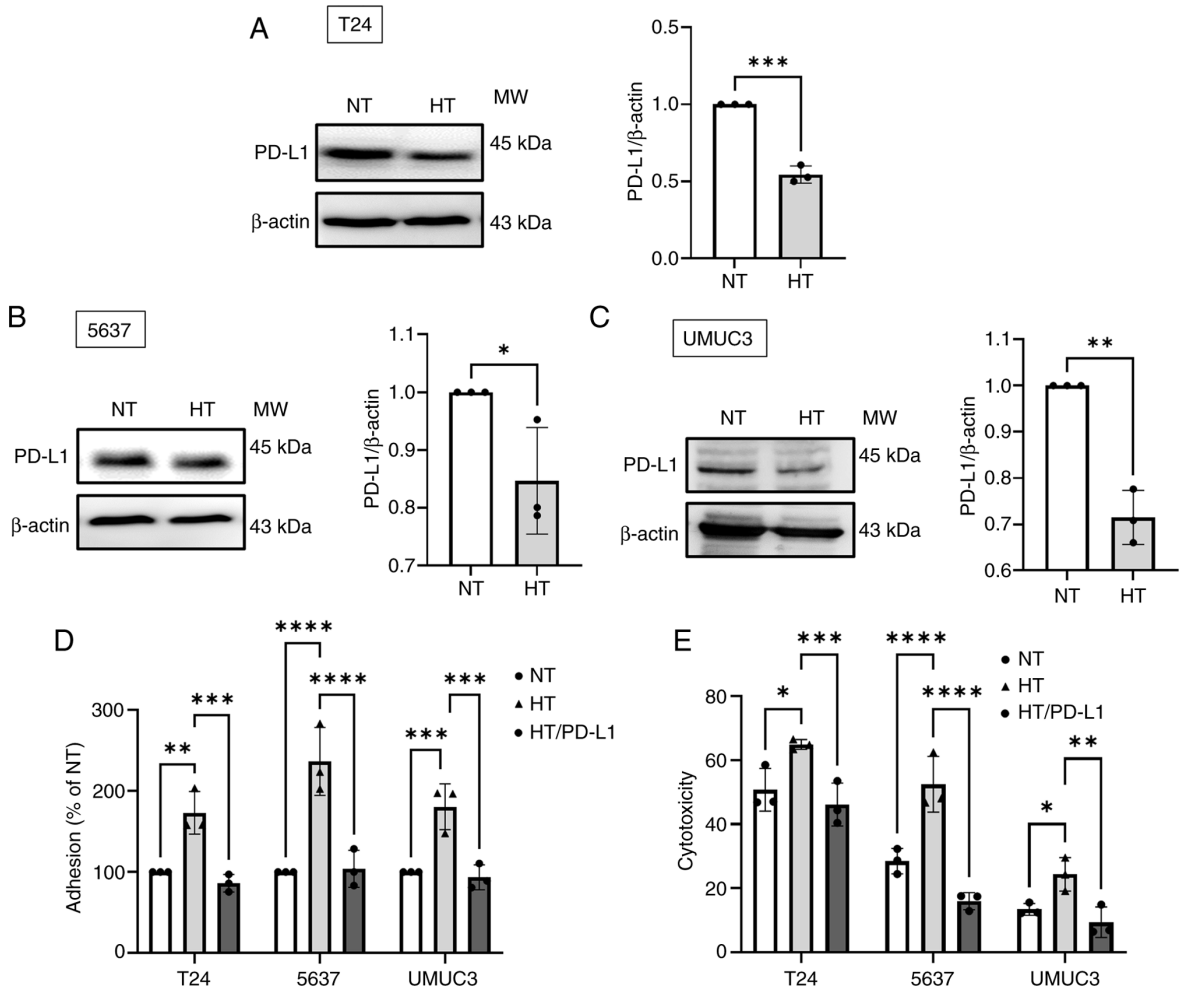

Figure 5. HT increases the cytotoxicity of NK cells against human BC cells by suppressing PD-L1 expression. (A-C) Western blotting was performed to measure the level of PD-L1 protein expression in BC cells subjected to HT. (D) BC cells were subjected to HT treatment for 24 h, with or without PD-L1 plasmid transfection. Subsequently, a cell adhesion assay was performed to evaluate the level of NK cell adhesion to BC cells. (E) The cytotoxicity of NK cells against BC cells was measured by detecting the fluorescence signal. Data are presented in terms of mean ± SD values. Two-groups comparisons were performed using Student's t-test, whereas one-way ANOVA followed by Bonferroni's post hoc test was used to compare mean values among multiple groups. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, vs. NT group. HT, hyperthermia; NK, natural killer; BC, bladder cancer; PD-L1, programmed cell death 1 ligand 1; NT, normal temperature.

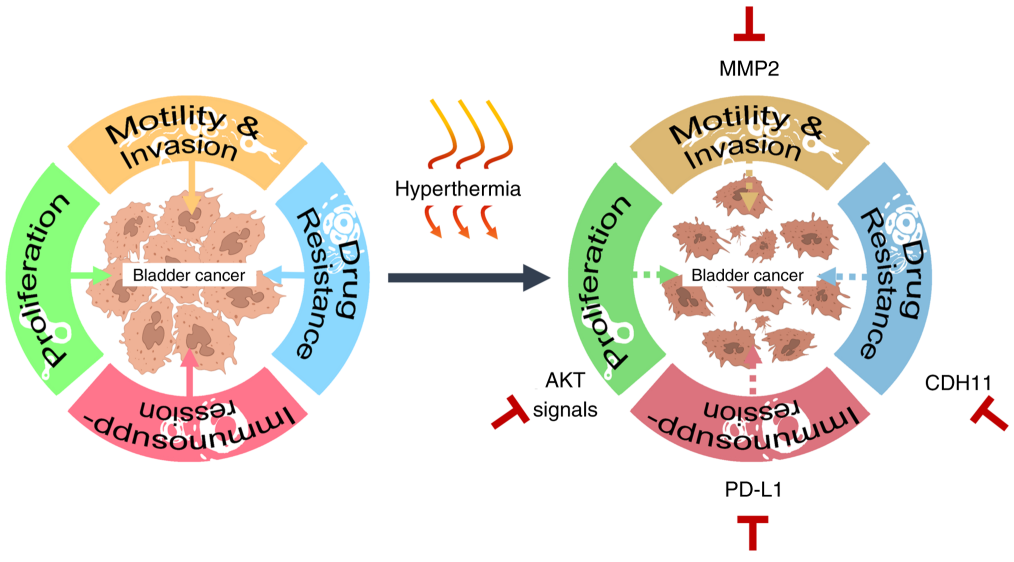

Figure 6. Schematic showing the potential mechanisms underlying the anticancer effects of HT. The anti-tumor effects of HT are mediated through the inhibition of cell proliferation, migration and invasion in BC cells. Additionally, HT may affect immunosuppression and the development of chemoresistance, revealing the potential of HT as an adjunctive therapy in human BC. HT, hyperthermia; BC, bladder cancer; MMP, matrix metalloproteinase; CDH11, cadherin 11; PD-L1, programmed cell death 1 ligand 1.

microenvironment, this is a limitation. Moreover, future research should focus on *in vivo* experiments for an improved understanding of the efficacy and potential side effects of HT in a more realistic biological context.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

Conceptualization was by TH and AC. Data curation was by TT and KC. Formal analysis was by TT and CH. Investigation was by TH and AC. Methodology was by KC and YC. Project administration was by HC and CH. Resource management was by PC and TT. Software was by YC and PC. Supervision was by AC and TH. Writing the original draft was by TT and PC. Writing, reviewing and editing was by AC and TT. AC and TT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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