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Original Article

Targeting gallbladder cancer: hyaluronan sensitizes cancer cells to chemo-therapeutics

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Abstract: Gallbladder cancer is the most common biliary tract malignancy and the fifth most common gastrointestinal malignancy. Chemo-resistance is the most remarkable characteristic of gallbladder cancer. The relatively dense extracellular space in tumor is the main barrier to nanotherapeutics' anticancer efficacy. Hyaluronan (HA) was shown in our previous study to significantly improve the myxoma virus distribution via promoting the MMP-9 production, which degrades collagen IV. We demonstrated that HA increased the chemo-sensitivity of gallbladder cancer cells both *in vitro* and *in vivo*. The *in vivo* chemo-sensitization effect of HA could partially be due to the penetration-promoting effect of HA via degrading collagen IV.

Keywords: Gallbladder cancer, hyaluronan, chemo-resistance, collagen IV

Introduction

Gallbladder cancer is the most common biliary tract malignancy and the fifth most common gastrointestinal malignancy [1]. The outcome of patients with more advanced disease is dismal with 5-year survival rates ranging from 20% to 40% [2]. Despite advances in chemo-radiation and adjuvant chemotherapy, chemotherapeutic options prolong life minimally as chemo-resistance and progression are the most remarkable characteristics of GBC [3].

Although advances in nanotherapeutics have casted light on cancer treatment, limited penetration of nanoscale therapeutics in solid tumors may be the main barrier to their anticancer efficacy [4-6]. This is partly due to the relatively dense extracellular space in tumor. Fibrous macromolecules, including collagen are main the components of extracellular matrix (ECM) in solid tumor. The small interfibrillar spacing in ECM retards the movement of particles larger than 10 nm [7].

Our previous study demonstrated that collagen IV was a critical factor hindering nanoscale intratumoral myxoma virus distribution in gallbladder cancer and hyaluronan (HA) significantly improved the myxoma virus distribution via promoting the MMP-9 production, which degrades collage IV [8]. Furthermore, Lee et al [9] has shown that HA increased the chemo-sensitivity of breast cancer cells. We would like to explore the effect of HA on gallbladder cancer both *in vitro* and *in vivo*.

Materials and methods

Cell lines

Two human GBC cell lines (GBC-SD and SGC-996) were used in this study. GBC-SD and SGC-996 were purchased from Cell Bank of the Chinese Academy of Science (Shanghai, China). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL), containing 10% fetal calf serum (FBS, HyClone) as well as 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cells were maintained in a humidified incubator at 37°C in the presence of 5% CO_2. All cell lines have been passaged for fewer than 6 months.

Reagents

Low-molecular-weight HA (LMW-HA) fragments were purchased from RD (Minneapolis, MN,

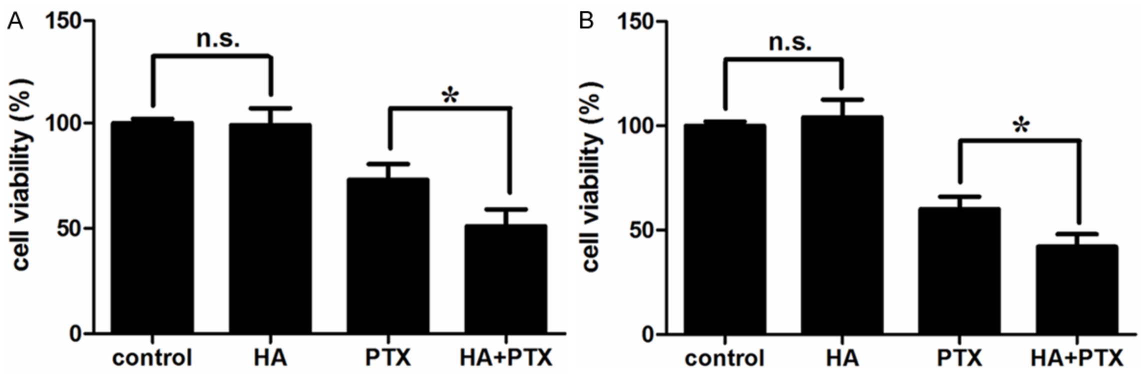

Figure 1. HA increased the chemo-sensitivity of gallbladder cancer cells. CCK8 assays were performed to determine the proliferation of gallbladder cancer cells GBC-SD (A) and SGC-996 (B) treated with control, HA, PTX only (10 nM) or HA (10 nM)-mixed PTX (10 nM). Data represent the mean ± S.D. from three independent experiments. **P* < 0.05.

Table 1. Dose-dependent viability of gallbladder cancer cells in both PTX only and HA + PTX treatment groups

(%)	Dose	1 nM	10 nM	100 nM	1 μM	10 μM
GBC-SD	PTX	93	74	65	54	43
	HA + PTX	81	55	51	42	31
SGC-996	PTX	85	60	52	41	32
	HA + PTX	60	42	33	26	20

USA). Paclitaxel was obtained from Wyeth Pharmaceuticals, nc. (Collegeville, PA, USA).

Cell viability assay

Cells transfected with the desired plasmid were allowed to grow in 96-well plates (3000/well). After 24 h, cells were washed with PBS twice, and desired agent was added into each plate. Cell proliferation was documented every 24 h following the manufacturer's protocol. Cell proliferation assay was conducted using Cell Proliferation Reagent Kit (CCK8) (Roche Applied Science).

Xenograft study

Female CD-1 nude mice (age: 5 weeks; weight: 20-25 g) were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China) and housed at 3-5/cage on a 12-h light/dark schedule at 22 ± 1°C and 50 ± 5% relative humidity. All animal experiments were performed in animal laboratory center of Xinhua Hospital and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The study protocol was approved by the Animal Care and use committee of Xinhua Hospital (approval ID: 2014005).

GBC-SD cells (1 × 10^7) were subcutaneously injected into were injected into either side of the flank area of female nude mice. All treatment regimens started 7 days after cell implantation. Gemcitabine treatments consisted of 5 doses of 50 mg/kg given IV once every four days beginning 7 days after tumor inoculation. Hyaluronan treatments consisted of 7 doses of 200 μg injected intratumorally at multiple points every other day for 3 weeks beginning 9 days after tumor inoculation. Tumor volumes were measured (0.5 × length × $width^2$) in mice on a weekly basis. After 5 weeks, the mice were sacrificed and the tumors were got.

Statistical analysis

GraphPad Prism 5 statistical software (GraphPad Software, La Jolla, CA) was used for statistical analyses. Difference in each two group comparison was determined with the *t*-test. A two-sided *P* value of less than 0.05 was considered to be statistically significant.

Results

HA increased the chemo-sensitivity of gallbladder cancer cells in vitro

Gallbladder cancer cells (GBC-SD and SGC-996) were treated with free-Paclitaxel (PTX) or HA-mixed PTX. The drug effect on cell growth was evaluated. As demonstrated in **Figure 1A**,

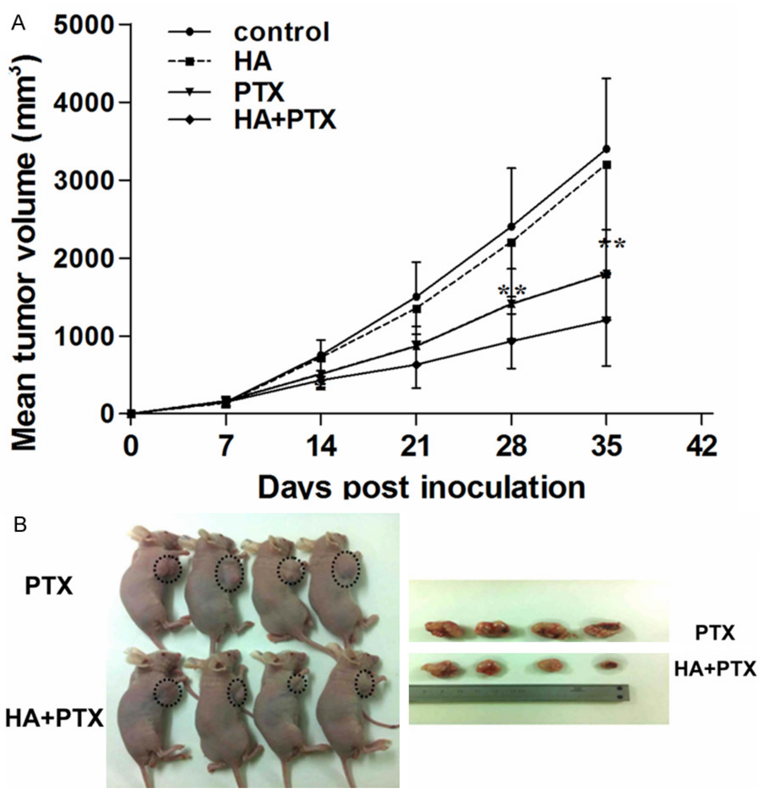

Figure 2. HA increased the chemo-sensitivity of gallbladder cancer cells *in vivo*. A. Mice bearing GBC-SD tumors were treated with either PTX alone or HA i.t. before the i.v. injection of PTX. Tumor growth curves of subcutaneous implantation models of gallbladder cancer are shown. B. Mice that received HA treatment along with i.v. PTX infusion have significantly smaller tumors than those that received PTX alone in GBC-SD tumors. $^*P < 0.05$, $^{**}P < 0.01$.

HA-mixed PTX was more efficient in inhibiting cell growth than free PTX. The cell viability of free-PTX (10 nM) treated GBC-SD cells was 74%, while it was 51% ($P = 0.026$, $P < 0.05$) in the HA-mixed PTX group. Similar effect was observed in SGC-996 cells (**Figure 1B**). The chemo-sensitizing effect of HA was observed in a dose-dependent manner with CCK8 assay in GBC-SD and SGC-996 cells (**Table 1**).

HA sensitized the gallbladder cancer cells to PTX in vivo

In our previous study, we demonstrated that HA significantly improved the myxoma virus distribution *in vivo* via promoting the MMP-9 production, which degrades collage IV [8]. We also demonstrated that the concentration of HA employed in this study had no significant effect on the invasiveness of gallbladder cancer cells [8]. Furthermore, we showed that HA had chemo-sensitization effect. We would like to explore whether i.t. injected HA could improve the efficacy of i.v. injected PTX *in vivo*.

GBC-SD cells were injected subcutaneously into nude mice. The injection of HA alone had no significant effect on tumor growth rate (**Figure 2A**). However, when nude mice were i.t. injected with HA along with i.v. injection of PTX, HA significantly delayed the tumor growth (**Figure 2A**). In mice treated with HA and PTX, the tumor was significantly smaller ($P \leq 0.01$) than in mice that received PTX alone (**Figure 2B**).

Immunohistochemical staining of tumor tissues showed that HA significantly decreased collagen distribution within tumors compared to the PTX group (**Figure 3A**, **3B**), suggesting that the *in vivo* chemo-sensitization effect of HA could partially be due to the penetration-promoting effect of HA via degrading collagen IV.

Discussion

In this study, we confirmed the HA as a complementary anti-tumor agent. Hyaluronan (HA), a large glycosaminoglycan (GAG) [8], is a chief extracellular matrix (ECM) component. HA-mixed PTX was shown to inhibit the tumor growth better. What's more, it has the potential of circumvent collagen barrier via promoting the production of MMP9 [8] in gallbladder cancer. We demonstrated that HA sensitized the gallbladder cancer to chemo-therapeutics such as PTX both *in vitro* and *in vivo*.

The *in vitro* chemo-sensitization effect of HA could be due to the interaction between HA and cell surface receptors, which may facilitated the entrance of drug into cancer cells [9]. The *in vivo* chemo-sensitization effect of HA could be a combination of the mechanism mentioned above and the penetration-promoting effect.

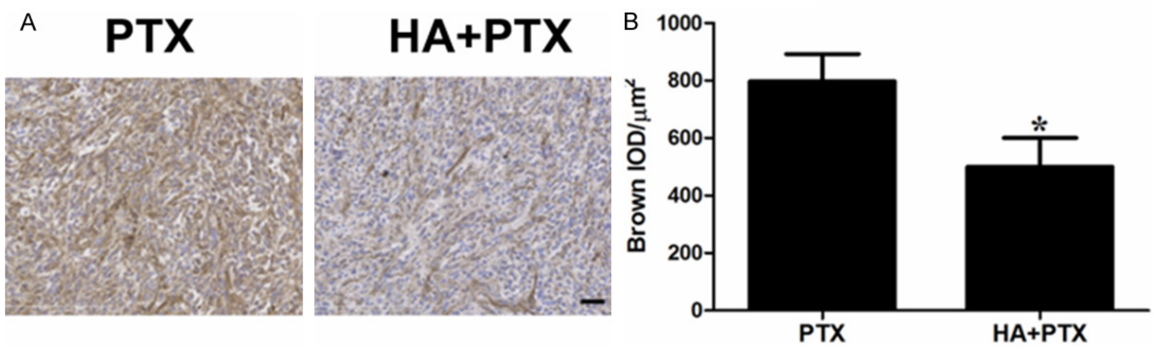

Figure 3. HA treatment alters the extracellular matrix (ECM) architecture of human gallbladder cancer xenograft in nude mice. A. Immunohistochemistry staining for Collagen IV. B. Quantification of staining intensity was done through a computer based scoring for each of the corresponding IHC slides (n = 4 animals for each group) and mean ± S.D are presented in bar diagrams. Bars equal 50 μm; *P < 0.05.

Due to the simplicity of HA-directed therapeutics, we propose that HA has the clinical application potential in the treatment of chemo-resistant cancer cells.

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Disclosure of conflict of interest

None.

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