Heparanase regulates EMT and cancer stem cell properties in prostate tumors

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Prostate cancer displays a certain phenotypic plasticity that allows for the transition of cells from the epithelial to the mesenchymal state. This process, known as epithelial–mesenchymal transition (EMT), is one of the factors that give the tumor cells greater invasive and migratory capacity with subsequent formation of metastases. In addition, many cancers, including prostate cancer, are derived from a cell population that shows the properties of stem cells. These cells, called cancer stem cells (CSCs) or tumor-initiating cells, not only initiate the tumor process and growth but are also able to mediate metastasis and drug resistance. However, the impact of EMT and CSCs in prostate cancer progression and patient survival is still far from fully understood. Heparanase (HPSE), the sole mammalian endoglycosidase capable of degrading heparan sulfate (HS), is also involved in prostate cancer progression. We had previously proved that HPSE regulates EMT in non-cancerous pathologies. Two prostate cancer cell lines (DU145 and PC3) were silenced and overexpressed for HPSE. Expression of EMT and stemness markers was evaluated. Results showed that the expression of several EMT markers is modified by HPSE expression in both the prostate cancer cell lines analyzed. In the same way, the stemness markers and features are also modulated by HPSE expression. Taken together, the present findings seem to prove a new mechanism of action of HPSE in sustaining prostate cancer growth and diffusion. As for other tumors, these results highlight the importance of HPSE as a potential pharmacological target in prostate cancer treatment.

KEYWORDS
prostate cancer, heparanase, epithelial to mesenchymal transition, cancer stem cells, in vitro
Introduction

Of all the existing cancers, prostate cancer is the one that has globally recorded the greatest growth in cases over the last 30 years in both developing and developed countries. Being a male cancer that occurs mainly in old age, the aging of the population is one of the factors that have contributed most to the increase in cases (1). It is estimated that prostate cancer will increase to nearly 2.3 million new cases and 740,000 deaths per year by 2040 simply as a result of population growth and aging (2, 3). The severity of the disease is conferred by the ability of cancer cells to disseminate and metastasize, affecting various organs and tissues—specifically, bone tissue in 84% of cases, distant lymph nodes in 10.6%, the liver in 10.2%, and the thorax in 9.1% (4).

Two phenomena that contribute to the progression and metastasis of this neoplasm are the epithelium–mesenchymal transition process (EMT) and the presence of tumor stem cells. The EMT is a reversible cell-differentiation process during which the morphological and phenotypic conversion of polarized epithelial cells into mesenchymal cells occurs. These cells have a greater migratory capacity, greater resistance to apoptosis, increased expression of mesenchymal markers, and resistance to senescence. This change involves the loss of the junction systems that hold epithelial cells together and the loss of baso-apical polarity and various rearrangements of the cytoskeletal apparatus (5, 6).

The EMT process has also been shown to be present in prostate cancer, and among the various factors that seem to be involved in promoting this change, androgens and estrogens with their related signaling, hypoxia, transforming growth factor beta (TGF-β), and epidermal growth factor (EGF) must be mentioned (7).

These factors promote the activation of the PI3K/AKT and MAPK signaling pathway—thus activating the downstream effectors such as GSK3β and NF-kB, which increase the activity of SNAI-1 and Twist and consequently induce the expression of mesenchymal proteins (8).

Cancer stem cells, according to the American Association for Cancer Research, are described as “a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor” (9).

Consequently, in line with this definition, CSCs possess both the ability to expand the population of cancer stem cells and, after differentiation, to give rise to other types of neoplastic cells that will make up a large part of the tumor mass. Since non-stem cells in cancer have a limited capacity for proliferation, the only cells with unlimited potential are cancer stem cells, which are thus able to guide the growth and metastatic process (10). For this reason, CSCs that have very long division times, and which are relatively insensitive to drug therapies aimed at targeting rapidly proliferating cells, may be one of the sources of cancer recurrence (11). Over the last few years, experimental evidence has accumulated in favor of the existence of CSC in prostate cancer and its role in tumor and metastatic progression (12). CSCs of the prostate can originate from basal or luminal-type progenitor/stem cells that will develop into tumors with markedly different biological and clinical characteristics in terms of aggressiveness and response to chemotherapy treatments and androgen-deprivation therapy (ADT) (13, 14). Since prostatic CSCs play a fundamental role in ADT resistance, it is currently considered essential to develop new anti-CSC strategies to compensate for treatment failure and disease recurrence (15).

More recently, it has been suggested that the expression of HPSE, the only enzyme capable of degrading the heparan sulfate (HS) chains of HS-proteoglycans (HSPGs), is associated with the characteristics of CSCs in Hodgkin’s lymphoma cells (16) and in myeloma (17).

HS is a highly sulfated linear polysaccharide, attached to the core protein of heparan sulfate proteoglycans (18, 19). HS proteoglycans are ubiquitously found both at the cell surface (i.e., syndecans and glypicans) and in the extracellular matrix (ECM) (18) where they regulate ECM structure and cell–ECM interaction (20, 21). In addition, HS regulates the activity of several molecules (cytokines, growth factors, etc.) (22–25). Currently, heparanase (HPSE) is defined as a multitasking protein capable of performing enzymatic-degradative activity towards HS chains, but which, at the same time, also manifests non-enzymatic activities (26). Through its cutting activity of the side chains of heparane sulfates (HS), it contributes both to the remodeling of the extracellular matrix and to the release and diffusion of various bioactive molecules linked to HS such as growth factors, cytokines, and enzymes. Considering that heparanase is not only produced and secreted by cancer cells but also by endothelial cells and activated immune cells and platelets, it is not surprising that its activity has a strong impact on the tumor microenvironment, thanks to those factors linked to HS, which, once released, promote tumor growth, neoangiogenesis, and the formation of a metastatic niche (27). As a proof of concept, it has been shown that heparanase overexpression in transgenic mice (Hpa-Tg) makes the tumor microenvironment more conducive to neoplastic development in various experimental models of in vivo tumorigenesis (28, 29).

Since prostate cancer also has an increased expression of HPSE (30), we decided to verify whether this increase could be able to regulate EMT and cancer stem cells properties of prostate cancer.

Methods

Cell lines

DU145 (ATCC® HTB-81™) and PC3 (ATCC® CRL-1435™) prostate cancer cell lines were cultured in Roswell...
Park Memorial Institute (RPMI) containing 10% fetal bovine serum and supplemented with 1% penicillin/streptomycin. Cells were maintained in a humidified environment containing 5% CO₂ at 37°C, and the culture medium was replaced every 2 days.

Transfection of HPSE overexpressing and shRNA plasmid

In order to stably obtain HPSE-overexpressing DU145 cells, we used a plasmid-coding HPSE ORF purchased from OriGene, and as a negative control, we used the corresponding empty vector.

To stably obtain HPSE-silenced PC3 cells, we used four different shRNAs targeting human heparanase (NM_006665) purchased from OriGene as described earlier (31, 32). As a negative control, we used an shRNA pRS non-effective GFP plasmid (TR30003).

DU145 and PC3 cells were seeded in six-well plates and when they reached 70%–80% of confluence, they were transfected with Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours after transfection, DU145 cells overexpressing HPSE were selected with 500 mg/ml G418 (Sigma), and PC3 cells silenced for HPSE were selected with 0.75 mg/ml of puromycin (Sigma). Single clones were isolated and analyzed for HPSE expression. The ones with the highest overexpression/silencing rate were used in the subsequent experiments.

RNA isolation and real-time qPCR analysis

Total RNA was extracted from cells by Trizol reagent (Invitrogen) according to the manufacturer’s instructions (33). RNA yield and purity were checked using a Nanodrop spectrophotometer (EuroClone), and total RNA from each sample was reverse transcribed into cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (Sigma-Aldrich). Real-time PCR was performed on a StepOne™ Real-Time PCR System (Thermo Fisher) using SensifAST SYBR Hi-Rox (Bioline). The comparative Ct method (ΔΔCt) was used to quantify gene expression, and the relative quantification was calculated as 2^−ΔΔCt. The presence of non-specific amplification products was excluded by melting curve analysis. Statistical analyses on real-time PCR data were performed using the Relative Expression Software Tool (REST) (34). The forward and reverse primer sequences were reported in Table 1.

Western blotting and immunofluorescence

Cells were lysed in radioimmunoprecipitation assay buffer (RIPA) buffer composed of 150 mM NaCl, 50 mM TRIS_HCl (pH 8), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1% Triton-X with Complete Protease Inhibitor Mixture (Roche Applied Science, Penzberg, Germany). In brief, equal amounts of proteins were treated in reducing sample buffer and denatured for 10 min at 100°C. Protein samples were then resolved in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred to nitrocellulose membranes. Non-specific binding was blocked for 1 h at room temperature with non-fat milk (5%) in TBST buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20). Membranes were exposed to primary antibodies GAPDH (sc-47778 Santa Cruz), HPSE (MA1-83806 HP3/17, Thermo Fisher), E-cadherin (E-CAD) (GT10443 GeneTex), vimentin (VIM) (sc-7557 Santa Cruz), α-SMA (A5228 Sigma), SOX2 (GTX101507 GeneTex), OCT4 (GTX627419 GeneTex), and NANOG (GTX100863 GeneTex), overnight at 4°C and incubated with a secondary peroxidase-

| Gene   | Forward Sequence (5’–3’) | Reverse Sequence (5’–3’) | Product Length (bp) |
|--------|--------------------------|--------------------------|---------------------|
| GAPDH  | ACACCCACTCTCCACCTTT     | TCCACCACCTCGTGGTA       | 112                 |
| HPSE   | ATTTGAATGGACGGACTGC      | TGGTCTCTAAACAGACATTTC   | 136                 |
| E-CAD  | TCTGTGCTCTCTGTGTTT      | TGGCTCAAGTAAAGCTGCT     | 142                 |
| VIM    | AAAAAACCTTTGCAATTCCTCAGA| CACCTGGTGTCTAAGGTAAGAC  | 74                  |
| α-SMA  | GAGAGAGAGGACAGACACTG    | TCCATCCCACCATCAC        | 143                 |
| TGF-β  | CCTGGGATGCATTACCAAAT    | GATAACCTCTGGCGAGTCC     | 90                  |
| SDC1   | GAAAGATCAAGATGGCTCTGGG  | GTTCTGGGAGCTGGAGATAG    | 145                 |
| SOX2   | AGCTGACAGATGTCAGAGA     | GGTCAATGGATGACTGCGA     | 126                 |
| OCT4   | CTCTCACTCTCAGCCTTGCA    | CAGAATTTACCTTCCCTAGT    | 164                 |
| NANO   | CAGCTCAGAAGACTGGCAAGGAA | CTCGCGTATTAGGCTTCCAC   | 149                 |
| CD133  | TCAGTGAGAAAGTGGCATGG    | GCTTTCATATTGCAACACCA   | 121                 |
conjugated antibody for 1 h at room temperature. The signal was detected with Luminata™ Forte Western HRP Substrate (Millipore) according to the manufacturer’s instructions, and the signal was acquired with Mini HD9 (UVItec, Cambridge, UK). Immunofluorescence cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 5 min, and blocked with 3% bovine serum albumin (BSA) in PBS at room temperature for 30 min. Cells were then incubated overnight at 4°C with the primary antibodies in PBS with 1% BSA and then washed three times for 5 min with PBS before incubation for 1 h at 37°C with the secondary antibody, again in PBS with 1% BSA. Cell nuclei were visualized with a Hoechst 33258. Images were acquired with Mini HD9 (UVItec, Cambridge, UK). Immuno-fluorescence cells were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. Cell colony-forming ability was assessed by counting the number of colonies. A colony was defined when the number of cells was more than 50.

Colony formation assay

WT and HPSE-silenced/overexpressing prostate cancer cells were seeded in 35-mm culture dishes (1,000 cells per well) and incubated with RPMI supplemented with 10% FBS for 7–10 days (35, 36). The media was renewed every 2 days. The colonies were fixed using paraformaldehyde and stained with 0.1% crystal violet. Cell colony-forming ability was assessed by counting the number of colonies. A colony was defined when the number of cells was more than 50.

Hanging drop assay

To assess the spheroid-formation capacity and compare the spheroid size of the cells, 500 cells in DMEM-F12 complete medium were placed as drops (20 μl each) into the lid of a Petri dish. The lid was then rapidly re-inverted onto the culture dish filled with 10 ml of sterile PBS to prevent evaporation of the drop. The hanging drop cultures were incubated at 37°C in a humidified atmosphere with 5% CO2 for 1 week. Pictures of the spheroids inside the drop were taken using a Leica MZ16F stereomicroscope, and their comparative size was obtained measuring the area occupied by the spheres using the software NIH Image.

Results

Establishment of HPSE overexpressing/silenced prostate cancer cell lines

In order to investigate the function of HPSE in prostate cancer, we choose to use two prostate cancer cell lines because they display very different morphological aspects related to EMT. DU145 has a more epithelial phenotype, whereas PC3 cells are elongated and spindle-shaped like mesenchymal cells. Starting from this evidence, we decided to overexpress HPSE in DU145 and to silence it in PC3 (Supplementary Figure S1). HPSE was overexpressed in DU145 cells and silenced in PC3 cells. Results confirmed a significant HPSE upregulation in DU145 cells both at gene and protein level confirmed by WB and immunofluorescence (Figure 1) with respect to DU145 control cells transfected with the empty vector (CTR). CTR cells displayed HPSE expression levels comparable to wt DU145 cells (data not shown). Furthermore, HPSE was silenced by shRNA in PC3 cells by more than 50% both at gene and protein levels (Figure 1).

HPSE regulates EMT in prostate cancer cells

In order to investigate whether HPSE is able to modulate EMT, the expression levels of epithelial and mesenchymal markers were measured in DU145 and PC3 cells. Gene expression analyses indicated a decrease in epithelial marker E-cadherin in HPSE overexpressing DU145 cells compared with the control. Also observed was a marked increase in the expression levels of vimentin, α-SMA, SNAI1, and TGF-β compared with the control (Figure 2A). By contrast, in HPSE-silenced PC3 cells, E-cadherin gene expression was increased, and the expression of mesenchymal markers was reduced (Figure 2C). The reduction in E-cadherin and the increase in vimentin and α-SMA in HPSE-overexpressing DU145 cells were also confirmed at protein level by WB and by immunofluorescence (Figures 2B, E). On the other hand, the reduction in vimentin and α-SMA in HPSE-silenced PC3 cells was also confirmed at protein level (Figures 2D, E). These results indicate that HPSE likely promotes EMT in prostate cancer cells.

HS proteoglycans has an important role on prostate epithelium–stroma architecture (34). In particular, syndecans, a family of heparan sulfate proteoglycans that are present on the cell surface, are involved in the control of cell proliferation, apoptosis, and transformation. In prostate cancer, the expression of syndecan-1 in epithelial cells decreases when cells are transformed and acquire invasive properties. This decreased expression is associated with a bad prognosis (37–39).

In addition, HPSE cleaves HS chains on syndecan-1, and a tight relationship between HPSE and syndecan-1 has been documented in tumor and non-tumor models (19, 32, 40, 41).

Results showed that syndecan-1 gene expression was reduced in HPSE-overexpressing DU145 cells, and, in contrast, syndecan-1 expression was increased in HPSE-silenced PC3 cells.

HPSE regulates stemness features in prostate cancer cells

Numerous studies have shown that the key regulators in maintaining the stemness of embryonic stem cells, including...
Oct4, Sox2, and Nanog, along with their activation targets, are commonly overexpressed in cancer stem cells in several malignancies (42–44). In addition, a series of molecules, including CD44, CD133, integrin α2β1, ALDH1A1, and Bmi1, involved in the regulation of cancer stem cell self-renewal, metastasis, and drug resistance, have also been confirmed in prostate cancer (45, 46). Results showed that the stemness markers SOX2, OCT4, NANOG, and CD133 were upregulated both at gene and protein level in HPSE-overexpressing DU145 cells compared with the control. By contrast, the same markers were significantly reduced in HPSE-silenced PC3 cells compared with the control (Figure 3).

Two important characteristics of cancer stem cells are the capacity to grow starting from a single cell and to form a sphere in an independent anchoring system.

Colony formation is the ability of cancer stem cells to form colonies when seeded on cell culture dishes at very low concentrations after limiting dilutions (47). Results showed that HPSE overexpression in DU145 cells increases the number of colonies compared to control cells and that HPSE silencing in PC3 significantly limited the ability to form colonies (Figures 4A, B).

To assess the spontaneous sphere formation efficiency of cancer stem cells, we used the hanging drop method (48, 49). Both cell lines were able to form similar circular spheres. HPSE overexpression in DU145 cells increased the sphere perimeter, whereas HPSE silencing in PC3 cells exerted the opposite effect (Figures 4C, D).

**Discussion**

The malignant growth and progression of tumor disease are supported by key features of cancer cells that have collectively been referred to as “hallmarks of cancer.” To the initial six hallmarks (supporting proliferative signaling, evading growth...
suppressors, replicative immortality, resistance to cell death, inducing angiogenesis, and activating invasion and metastasis), four other “enabling features” have more recently been added, such as instability and mutation of the genome, tumor-promoting inflammation, deregulated energy metabolism, and escape from immune destruction (50). Since it is now consolidated evidence that all tumors examined to date overexpress HPSE, some authors have proposed that with its multiple roles within the tumor microenvironment, heparanase can regulate each of these distinctive characteristics of cancer and, in turn, highlight the need for therapies aimed at its inhibition (51).

Starting from this evidence, our goal was to evaluate how the overexpression and silencing of heparanase in prostate cancer cells affect stemness characteristics and epithelium–mesenchymal transition (EMT)—two of the classic tumor “hallmarks.”

EMT is thought to be activated in cancer cells, linked to their dissociation from the primary tumor and their intravasation into blood vessels (52). However, the impact of EMT in cancer progression and patient survival is still far from fully understood.

EMT was originally described during morphogenesis and later was observed in several pathological events, including fibrosis and cancer metastasis (53). During EMT, epithelial cells lose adherence junctions and (54) apical–basal polarity and acquire a mesenchymal phenotype with an enhanced motility. In response to various signals (55–58), epithelial cells upregulate a group of transcription factors to orchestrate EMT, and the main ones are SNAI-1 and TWIST. EMT can be considered to be a continuum process (59), and cells with this hybrid phenotype have been referred to as “metastable” (60), reflecting the flexibility of these cells in inducing or reversing the EMT process (61).
FIGURE 3
Stemness markers expression in prostate cancer cells. SOX2, OCT4, NANOG, and CD133 gene expression were evaluated by real-time PCR in DU145 (A) and PC3 (B) cells. Data were normalized to GAPDH expression. Means ± SD (error bars), n=6. **p < 0.001, *p<0.05 vs. C. SOX2, OCT4, and NANOG protein level were quantified by WB in DU145 and PC3 cells. GAPDH was included as loading control.

FIGURE 4
Stemness properties in prostate cancer cells. Representative images of colony (A) and sphere (C) assays evaluated in DU145 and PC3 cells. (B, D) Bars represent the quantification of colony and sphere assay respectively and are expressed as mean ± SD values; n=8. **p < 0.001, *p<0.05 vs. CTR.
Heparanase is the sole mammalian endoglycosidase capable of degrading glycosaminoglycan HS. The enzymatic cleavage of HS by HPSE results in ECM remodeling and releasing these bioactive mediators, producing a rapid tissue response to local or systemic stimuli. These effects profoundly affect multiple pathophysiological processes such as tumor progression, inflammation, and fibrosis (62–65). Since uncontrolled HS degradation results in significant tissue damage, HPSE expression is tightly regulated (19, 66, 67), whereas it is overexpressed in malignant tumors (66, 68).

Prostate cancer is responsible for more gender-specific cancer-related deaths in men than any other cancer (1). Zhou et al. (69) have shown that HPSE overexpression can facilitate cancer-related deaths in men than any other cancer (1). Zhou degradation results in significant tissue damage, HPSE expression is tightly regulated (19, 66, 67), whereas it is overexpressed in malignant tumors (66, 68).

We and other previous literature support the contention that heparanase is needed for pathological organ fibrosis, and this action is exerted thorough the regulation of EMT. In vitro and in vivo studies have shown that HPSE regulates renal EMT induced by FGF-2, TGF-beta, and hypoxia (71–74). We have also proved that HPSE regulates high glucose-induced EMT of mesothelial cells (75). In addition, it has been proven that HPSE participates in lung fibrosis by regulating EMT (76), and it probably also has a role in the liver (33).

We induced HPSE overexpression in DU145 cell line and its silencing, by shRNA, in PC3 cell line. The silencing/overexpression rate was confirmed both at gene and protein levels. Results showed that HPSE is an EMT inducer in prostate cancer. Indeed, the epithelial marker E-cadherin was reduced in HPSE-overexpressing DU145 cells and upregulated in HPSE-silenced PC3 cells with respect to control. In contrast, the expression of mesenchymal markers a-SMA and vimentin was increased in HPSE-overexpressing DU145 cells and reduced in HPSE-silenced PC3 cells with respect to control.

Moreover, we have proved that HPSE regulates the expression of TGF-β (one of the principal EMT activators) and the levels of its associated transcription factor SNAI-1. Specifically, TGF-b and SNAI-1 were increased in HPSE-overexpressing DU145 cells and reduced in HPSE-silenced PC3 cells with respect to control.

During malignant transformation depletion of epithelial cell surface, syndecan-1 profoundly alters cell morphology and anchorage-dependent growth; thus, syndecan-1 is necessary in maintaining the epithelial phenotype. TGF-β can induce EMT by activating SNAI-1, which in turn, represses the expression of syndecan-1. A coordinated loss of syndecan-1 and E-cadherin has been documented in many epithelial malignancies compared to their benign counterparts. In the prostate, changes in syndecan-1 expression are linked to EMT (77). It has been described that syndecan-1 expression is lower in PC3 and DU145 prostate cancer cell lines than in normal prostate epithelial cells (78).

Here, we have shown that HPSE expression modulates syndecan-1: HPSE overexpression reduced syndecan-1 in DU145 cells, and HPSE silencing increased syndecan-1 expression in PC3 cell line.

Recently, EMT has been linked to stem cell phenotype (79), since cancer cells with EMT characteristics acquire stem-cell-like features, such as self-renewal and slow proliferation (80, 81). Cancer stem cells acquire more complete EMT molecular characteristics and exhibit more aggressive abilities. Specifically, prostate cancer stem cells display increased EMT markers and increased tumorigenesis, migration, and invasion ability (82). Cancer stem cells have a specific gene signature, and the principal markers are SOX2, OCT4, and NANOG. Additionally, other genes involved in maintaining self-renewal capacity in prostate cancer include CD-133 and CD-44 (83, 84).

Here, we have proved that HPSE expression modulates prostate CSCs. Specifically, the stemness markers SOX2, OCT4, NANOG, and CD133 were upregulated both at gene and protein levels in HPSE-overexpressing DU145 cells and reduced in HPSE-silenced PC3 cells compared with the control. In addition, functional assays confirmed a role of HPSE in prostate cancer stemness: HPSE increases the capacity to grow starting from a single cell and to form a sphere in an independent anchoring system. Future studies could also characterize the potential role of HPSE in self-renewal capacity of prostate CSCs.

Here, we report that the expression of several EMT markers is controlled by HPSE expression in prostate cancer. Moreover, stemness markers and features of CSCs are also modulated by HPSE. Collectively, these results proved an additional mechanism by means of which HPSE can contribute to prostate cancer progression and metastasis, and further studies will be necessary to clarify its potential as a pharmacological goal.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

VM, MO, and GG conceptualized this study. GZ and MF contributed to the design and implementation of the research. VM and FA carried out the experiment. VM and MO wrote the
manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.918419/full#supplementary-material
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