Role of hyaluronan in pancreatic cancer biology and therapy: Once again in the spotlight

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Pancreatic ductal adenocarcinoma (PDAC) remains the most deadly disease worldwide, with the lowest survival rate among all cancer types. Recent evidence suggests that hyaluronan (HA), a major component of ECM, provides a favorable microenvironment for cancer progression. Pancreatic ductal adenocarcinoma is typically characterized by a dense desmoplastic stroma containing a large amount of HA. Accumulation of HA promotes tumor growth in mice and correlates with poor prognosis in patients with PDAC. Because HA is involved in various malignant behaviors of cancer (such as increased cell proliferation, migration, invasion, angiogenesis, and chemoresistance), inhibiting HA synthesis/signaling or depleting HA in tumor stroma could represent a promising therapeutic strategy against PDAC. In this review article, we summarize our current understanding of the role of HA in the progression of PDAC and discuss possible therapeutic approaches targeting HA.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and intractable solid tumors, which often invades surrounding stromal components, including lymphatic, vascular, and perineural systems, ultimately metastasizing to distant organs. Despite recent advances in the clinical management, the survival rate in patients with PDAC remains the lowest among all cancer types, emphasizing the need for a better understanding of its biology. In particular, identification of molecular mechanisms underlying the aggressive behaviors of PDAC can provide the basis for the development of novel therapeutic intervention. Although substantial progress has been made in our understanding of the genetic and epigenetic alterations in PDAC, the identification of these molecular defects predominantly in cancer cells has led to little progress in developing new treatment strategies.

The progression of cancer is governed by complex mechanisms and is significantly accelerated by the tumor microenvironment, composed of a variety of stromal cells and ECM. The tumor-stroma interactions play a critical role in the progression of PDAC, which is typically characterized by a dense desmoplastic stroma. Of the major ECM components detected in tumor stroma, hyaluronan (HA) has been extensively studied in its relation to cancer progression. In normal physiological conditions, the amount of HA is controlled by a balance between synthesis and degradation; however, HA has been shown to be abundantly accumulated in the surrounding stroma of malignant tumor. The HA-rich microenvironment may promote tumor progression by enhancing cell proliferation, migration, invasion, metastasis, angiogenesis, and resistance to chemotherapeutic agents.

Several studies have shown increased expression of HA and its receptors in PDAC. Importantly, the abnormal accumulation of HA correlates with worsened prognosis in patients with PDAC. In an experimental model of PDAC, accumulation of extracellular HA by forced expression of synthesizing genes accelerated tumor growth. Taken together, these findings strongly suggest that HA may play a critical role in the progression of PDAC and could be a therapeutic target. In this review article, we summarize the current understanding of the role of HA in PDAC and discuss its potential therapeutic applications.

Role of HA in Initiation and Progression of Human Cancers

Regulatory mechanisms of HA. Hyaluronan is a linear glycosaminoglycan that consists of repeating disaccharide subunits of glucuronic acid and N-acetylgalcosamine, with molecular weights usually ranging from 10⁵ to 10⁶ Da. Hyaluronan is synthesized by HA synthases (HAS1, HAS2, and HAS3).
Newly synthesized HA molecules are extruded directly onto the cell surface for assembly into pericellular or extracellular matrices. Hyaluronan is degraded by hyaluronidases (HYAL1, HYAL2, HYAL3, HYAL4, HYALP1, and PH20). Among the known HYALs, HYAL1 and HYAL2 are widely distributed throughout tissues and are most likely to play key roles in degrading HA. HYAL2 initially cleaves high-molecular-weight (HMW)-HA into ~20-kDa fragments, which are further digested into small fragments of ~0.8 kDa by HYAL1. Extracellular HA binds to and interacts with specific cell surface receptors including CD44 and receptor for HA-mediated motility (RHAMM). CD44 is a major receptor for HA and is also a multifunctional receptor, having diverse roles in cell-cell and cell-matrix interactions. Due to alternative splicing, multiple forms of CD44 variants (CD44v) are generated that are further modified by N- and O-linked glycosylation. Importantly, variants of CD44, particularly CD44v6, have been shown to promote tumor progression linked glycosylation. Importantly, variants of CD44, specifically CD44v6, have been shown to promote tumor progression and metastatic spread in lung, breast, and colon cancer. (26) These clinical findings, there is considerable experimental evidence supporting the role of HA in cancer initiation and progression. For example, overexpression of Has2 in mammary glands resulted in the development of mammary tumors in a transgenic mouse model. (33) Likewise, other investigators showed that forced expression of HAS2 and HAS3 results in overproduction of HA and enhances the tumorigenic ability of fibrosarcoma and prostate cancer. (35) Together, these studies provided evidence for an involvement of HA in cancer initiation. In terms of cancer progression, suppression of HA production by antisense inhibition of HAS blocked growth of prostate cancer (36) and invasion of colon cancer cells. (37) Furthermore, abnormal HA production in non-malignant cells diminishes contact inhibition and enhances cell migration, suggesting that HA is required for the aggressive behaviors of cancer cells. A close link between HA and epithelial-mesenchymal transition also supports the role of HA in cancer initiation and progression.

Interestingly, the size of HA is important in terms of its effects on cancer initiation, growth, and progression, although the actions of different sizes of HA are diverse and complex. High-molecular-weight HA may play, at least in a certain species, an inhibitory role in cancer initiation. However, other studies have shown cancer-promoting effects of HMW-HA. For example, HMW-HA enhanced cell migration and angiogenesis of hepatocellular carcinoma (HCC) via CXCL12-induced CXCR4 activation. (42) Binding of HMW-HA to CD44 downregulated tumor suppressor protein PDCD4, leading to anti-apoptosis and chemotherapy resistance (43) and enhanced invasion (44) in breast cancer cells. However, several studies have shown that low-molecular-weight HA (LMW-HA) or oligosaccharides promote angiogenesis, suggesting that LMW-HA accelerates tumor progression through the stimulation of angiogenesis. In addition, LMW-HA has been shown to promote cancer growth and metastasis. (46,47) We also showed that direct addition of LMW-HA (25–75 kDa) to cultured PDAC cells increased their migration more robustly than HMW-HA (400–600 kDa) (Fig. 1). Thus, the effects of HA on cell behavior may depend on its size and cell type; however, the mechanism underlying these different effects of HA is not clearly understood.

Expression and distribution of HA and its receptors in PDAC.

There have been several studies investigating the degree of HA concentration and/or pattern of HA expression in PDAC. For example, a previous study showed that HA is secreted from cultured human pancreatic cancer cell lines. (10) In addition, the amount of HA is increased in human PDAC tissues (12-fold increase) compared to the normal pancreas. (13) Using
a biotinylated HA-binding protein isolated from bovine cartilage. Fries et al.\(^\text{(11)}\) showed that, in primary PDAC tissues, HA was found predominantly in the connective tissue immediately around tumor cells or at the border between the tumor and normal pancreatic tissue. A comprehensive analysis of the HA content in a variety of human malignant tumors revealed that PDAC had the highest incidence of detectable HA content, which was predominantly associated with the desmoplastic stroma rather than with tumor cells.\(^{49}\) We also used immunohistochemistry to analyze the expression of HA and its regulators (including HAS2 and HYAL1) in primary PDAC.\(^{15}\) We found that HA is strongly expressed in 80% of primary PDAC tissues, with positive staining being detected both in tumor and stromal components. Furthermore, strong expression of HAS2 and weak expression of HYAL1 were significantly associated with shorter survival time after surgery.\(^{15}\) Whatcott et al.\(^{14}\) reported that ECM components, such as collagen and HA, are found in high levels in both primary tumors and metastatic lesions in patients with PDAC. Importantly, we showed that strong HA expression was an independent prognostic factor in patients with PDAC undergoing resection, suggesting a prognostic significance of HA in PDAC.\(^{15}\) Similarly, Whatcott et al.\(^{14}\) reported that patients having primary PDAC tumors with low-level HA showed median survival times of 24.3 months compared with 9.3 months in those patients having tumors with high-level HA (\(P < 0.05\)).

It has also been shown that HA receptors are overexpressed in PDAC. Previous studies using immunohistochemistry have shown that CD44 is highly expressed on the membrane (mainly on the basolateral membrane) of PDAC cells.\(^{50,51}\) Interestingly, while the expression of standard CD44 (CD44s) was frequently observed in both PDAC and intraductal papillary neoplasms of the pancreas, the expression of CD44v5 and CD44v6 was exclusively seen in PDAC, suggesting a role of these splice variants in the invasive phenotype.\(^{52}\) Similarly, CD44v6 was not detected in any of the normal tissue or chronic pancreatitis specimens, whereas 54% of primary PDAC and 55% of metastases expressed this variant.\(^{53}\) Furthermore, the expression of CD44v6 and CD44v2 was correlated with decreased overall survival in patients with PDAC (\(P = 0.0160\) and \(P = 0.0125\), respectively).\(^{54}\) These results suggest that CD44, especially CD44v6, plays a role in the aggressive phenotype in PDAC. Similarly, RHAMM mRNA is overexpressed in PDAC cell lines showing a poorly differentiated phenotype and a high metastatic potential.\(^{12}\) Recently, we also showed that RHAMM is overexpressed in primary PDAC tissues with both membranous and cytoplasmic staining.\(^{17}\) Furthermore, high RHAMM expression correlated with poor survival in PDAC patients who underwent surgical resection.\(^{17}\)

**Functional significance of HA in PDAC progression.** There is only little experimental evidence supporting the role of HA in the progression of PDAC. Teranishi et al.\(^{55}\) reported that addition of exogenous HA increases the migratory ability of PDAC cells. Kuliti et al.\(^{18}\) showed that forced production and accumulation of HA by HAS3 overexpression promoted the growth of PDAC tumor in mice through inhibition of epithelial–mesenchymal transition, as evidenced by loss of plasma membrane E-cadherin and accumulation of cytoplasmic β-catenin. We used different models to investigate the effect of HA on PDAC cell motility by wound healing and Transwell migration assays.\(^{48}\) Inhibition of HA by 4-methylumbelliferone (4-MU) significantly decreased migration, whereas promotion of HA by 12-O-tetradecanoylphorbol-13-acetate or co-culture with tumor-derived fibroblasts significantly increased the migration of PDAC cells. Furthermore, addition of exogenous HA significantly increased the migration of PDAC cells. These studies may provide the first experimental evidence for a relationship between increased HA and progression of PDAC. Further studies are required to determine the exact mechanism by which HA promotes the progression of PDAC.

**Regulation of HA in PDAC and therapeutic strategies targeting HA.** *Hyaluronan synthesis.* The exact mechanism underlying the accumulation of HA in cancer is unclear. Because HA is synthesized by HAS and is degraded by HYAL, a possible mechanism may be overproduction of HA.\(^{19,21,56}\) In fact, many previous studies have shown overexpression of HAS in various cancers.\(^{57–62}\) Our previous study showed overexpression of one of the HA synthases, HAS2, in PDAC tissues.\(^{15}\) We recently investigated a possible association between DNA methylation and HA synthesis in PDAC cells using two different models, including treatment with a DNA methyltransferase inhibitor (5-aza-2′-deoxycytidine) and knockdown of the *DNMT1* gene by siRNA.\(^{63}\) In both models, decreased DNA methylation resulted in enhanced HA production in PDAC cells and was associated with increased expression of HAS2 and HAS3. These findings suggest, for the first time, that an epigenetic mechanism (namely DNA methylation) is involved in the regulation of HA synthesis in PDAC. Specifically, PDAC cells have reported increased expression of DNA methyltransferase in PDAC.\(^{64,65}\) raising concerns about a discrepancy between increased DNA methyltransferase activity and overexpression of HAS in PDAC. This could be explained partly by the fact that DNA methylation affects not only oncogenes but also cancer-promoting genes. It could be possible that HAS expression in PDAC cells is suppressed, to some extent, by increased DNA methyltransferase activity and is therefore reactivated by DNA methylation inhibition. In fact, a previous study reported a number of similar genes that are commonly overexpressed in PDAC but regulated by DNA methylation.\(^{66,67}\) Further studies are required to elucidate the complex mechanism regulating HA production in PDAC cells.

Another mechanism may be related to the enhanced secretion of HA from stromal cells, including fibroblasts. In support of this, it has been shown that HA staining was predominantly found within the desmoplastic stroma rather than tumor cells in human PDAC tissues.\(^{14,49}\) Interestingly, Knudsen et al.\(^{68}\) reported that direct coculture between tumor cells (including PDAC cells) and normal fibroblasts promotes the production of HA into the culture medium. Furthermore, a global gene expression analysis using microarrays identified an increased expression of HAS2 mRNA in PDAC cells in response to coculture with stromal fibroblasts.\(^{69}\) Taken together, these findings suggest that HA can be secreted from both tumor and stromal cells and that tumor–stroma interactions may play a pivotal role in the increased HA accumulation in PDAC.

Based on these findings, it is reasonable to suppose that inhibition of HA synthesis may be an ideal and straightforward treatment strategy. One agent that has been extensively studied as a potent inhibitor for HA is 4-MU, also known as hymecromone, which is already used in several countries as a drug to improve liver function or to treat biliary spasm.\(^{70}\) 4-Methylumbelliferone inhibits HA synthesis by acting as a competitive substrate for UDP-glucuronosyltransferase and by downregulating HAS2 and HAS3.\(^{71,72}\) Potent anticancer effects of 4-MU have been reported in various malignant tumors, including melanoma,\(^{73}\) breast cancer,\(^{74}\) esophageal cancer,\(^{75}\) and liver cancer (hepatocellular carcinoma).\(^{76}\) Pre-
vious studies have shown that 4-MU and its derivatives (including 4-methylesculetin) inhibit the growth and metastasis of PDAC in vitro and in vivo. In addition, Nakazawa et al. showed that 4-MU enhanced the anticancer activity of a commonly used chemotherapeutic drug, gemcitabine, in a PDAC cell line and animal model. This finding is consistent with a recent study showing that chemotherapy with carboplatin induces HA production, which can contribute to chemoresistance by regulating ABC transporter expression in ovarian cancer.

Recently, in addition to its anticancer efficacy, the chemopreventive efficacy of 4-MU has been shown in prostate cancer animal models. Therefore, this drug should be tested as a chemopreventive agent in individuals at high risk of developing PDAC and as a chemotherapeutic drug in patients with PDAC in the future.

Another HA inhibitor is Minnelide, a water-soluble pro-drug of triptolide, with antitumor activities against variety of cancers. Banerjee et al. showed that treatment with Minnelide decreases tumor HA thorough downregulating HAS activity, resulting in improved drug delivery and survival in both spontaneous KPC mice (a genetically engineered mouse model of PDAC) and patient-derived xenografts tumors.

**Hyaluronan-receptor signaling.** According to a number of previous studies, there are a variety of intracellular signaling pathways that are triggered by HA-receptor interactions (Fig. 2). Binding of HA to CD44 has been shown to activate MAPK, Rac, and phosphoinositide 3-kinase (PI3K) signaling, leading to enhanced cell survival, proliferation, migration, and invasion. The activated PI3K pathway also activates MRD1 to mediate multidrug resistance. RHAMM is known to induce focal adhesion kinase and also interacts with CD44 to enhance the signaling pathways, including the MAPK pathway. Furthermore, HA binding to CD44v10 induces CD44v10–EphA2 complex formation, recruitment, and activation of Src and Ssrc-mediated EphA2 tyrosine phosphorylation, resulting in RhoA activation and angiogenesis.

An alternative approach of targeting HA is to block signal transduction pathways induced by HA-receptor interactions. In many cancers including PDAC, HA–CD44 interactions are known to play an important role in tumor cell growth, survival, migration, invasion, multidrug resistance, and cancer stem cell self-renewal. Therefore, blocking this interaction may prevent progression of PDAC. Several studies have reported the efficacy of anti-CD44 therapies. For example, Li et al. used antibody against CD44s (standard isoform) to inhibit pancreatic tumor initiation and post-radiation recurrence in mice. Interestingly, anti-CD44s downregulated the stem cell self-renewal gene Nanog, Sox-2, and Rex-1, suggesting an important role of CD44 in cancer stem cell signatures.

Furthermore, a recent study used xenograft tumors in mice from human PDAC tissues to show that a residual PDAC tumor originated from a small number of CD44-positive cells present in the tumor and that systemic administration of anti-CD44 antibodies inhibited the growth of relapsed tumors, but not primary tumors. RHAMM may also be a promising target to block HA signaling but has not yet been investigated in terms of its therapeutic efficacy in PDAC.

Downstream of CD44–HA interactions, Ras–MAPK and PI3K–protein kinase B signaling pathways are known to be involved in the cancer-promoting effects of HA. These signaling pathways are thus a target for therapy. Teranishi et al. reported that enhanced cell motility and peritoneal metastasis of PDAC induced by HA were blocked by the PI3K inhibitor wortmannin in mice.

**Stromal HA accumulation.** Another major effect of HA on PDAC progression is as a barrier to access of chemotherapeutic agents to tumor cells. It has been suggested that abundant HA in cancer tissues causes elevated interstitial fluid pressure, vascular collapse, and decreased vascular permeability, thereby limiting chemotherapeutic drug delivery. Consequently, targeting the components of ECM, particularly HA, has been considered an attractive therapeutic strategy to overcome chemoresistance.

In recent years, several stroma-targeted agents have been developed for the treatment of PDAC. For example, a stroma-targeted drug, nab-paclitaxel, was tested in a pivotal phase III trial, which showed a significantly prolonged survival in the combination of nab-paclitaxel and gemcitabine as compared to the gemcitabine alone. There are several other stroma-targeted agents under preclinical or clinical evaluation. One best characterized agent to deplete stromal HA is a pegylated recombinant human hyaluronidase PH20 (PEGPH20), which is currently being evaluated in clinical trials. Several preclinical models, administration of PEGPH20 depleted stromal HA, reduced interstitial fluid pressure, re-expanded tumor microvessels, improved macromolecular permeability, and subsequently enhanced the effects of chemotherapeutic agents.

In addition, treatment with PEGPH20 increases the efficacy of mAb therapy and bacterial-based therapy targeting indoleamine 2,3-dioxygenase in murine PDAC models, supporting the potential...
utility of this agent as a sensitizer of various anticancer therapies.

Based on these promising results of preclinical studies, PEGPH20 is now being tested in clinical trials to determine its efficacy when used in combination with nab-paclitaxel plus gemcitabine (NCT01839487) and in combination with modified FOLFIRINOX (leucovorin calcium, 5-fluorouracil, irinotecan hydrochloride, and oxaliplatin) (NCT01959139) in patients with metastatic PDAC. These studies will reveal the clinical efficacy of PEGPH20 as an adjunct to anticancer therapies and offer a novel treatment option for otherwise untreatable patients with PDAC.

Summary

In PDAC tissues, HA is secreted from tumor cells and stromal cells through upregulation of HAS in response to tumor-stroma interactions. The secreted HA, or its smaller fragments, may promote tumor cell proliferation, migration, invasion, and angiogenesis through HA-receptor signaling and also serve as a barrier to access of chemotherapeutic agents to tumor cells. Given these links of HA with cancer progression, targeting HA represents a potential therapeutic approach for treatment of PDAC. There are different approaches of HA-targeted therapies that include strategies aimed at inhibiting HA synthesis, blocking HA-receptor signaling, and depleting stromal HA (Fig. 3).

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