Recent advances in research on aspartate β-hydroxylase (ASPH) in pancreatic cancer: A brief update

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ABSTRACT

Pancreatic cancer (PC) is a highly aggressive tumor, often difficult to diagnose and treat. Aspartate β-hydroxylase (ASPH) is a type II transmembrane protein and the member of α-ketoglutarate-dependent dioxygenase family, found to be overexpressed in different cancer types, including PC. ASPH appears to be involved in the regulation of proliferation, invasion and metastasis of PC cells through multiple signaling pathways, suggesting its role as a tumor biomarker and therapeutic target. In this review, we briefly summarize the possible mechanisms of action of ASPH in PC and recent progress in the therapeutic approaches targeting ASPH.

INTRODUCTION

Pancreatic cancer (PC) is an aggressive malignancy with a high mortality rate [1-4]. In the United States (US), a 5-year relative survival rate was estimated to be only 8% [4]. Despite improvements in the diagnosis and management of PC over the last few decades [1,2], PC was reported to be the seventh cause of cancer-related death in China [3]. According to the most recent American Cancer Society (ACS) report, in the US, the number of new PC cases and deaths was 55,440 and 44,330, respectively in 2018, and PC was the fourth leading cause of cancer death in 2015 [4]. Also, by 2030, PC is projected to become the second leading cause of cancer-related death in the US [5,6]. Radical surgery combined with neo-adjuvant chemotherapy is considered to be the most effective treatment for PC. However, due to the absence of early symptoms, 80–85% of PC patients are diagnosed at the stage of locally advanced or distant metastatic, unresectable disease. Moreover, clinical and preclinical data indicate that PC metastases develop during the early stages of pathogenesis, before the primary tumor can even be detected [5]. Thus, to improve the diagnosis, treatment and outcomes of PC patients it is necessary to better understand the molecular mechanisms of PC onset, progression and metastasis and to identify targetable pathways.

Aspartate β-hydroxylase (ASPH) is a highly conserved dioxygenase enzyme found to be overexpressed in multiple malignancies, including PC. ASPH appears to be involved in the regulation of proliferation, invasion and metastasis of PC cells through multiple signaling pathways, suggesting its role as a tumor biomarker and therapeutic target. In this review, we briefly summarize the possible mechanisms of action of ASPH in PC and recent progress in the therapeutic approaches targeting ASPH.

THE STRUCTURE AND FUNCTION OF ASPH

ASPH was first described in 1989, it is a type II transmembrane protein of ~86 kDa in size and the member of α-ketoglutarate-dependent dioxygenase family [7-13]. ASPH has a very low expression in normal adult tissue and is predominately expressed during embryogenesis, to promote cell migration for organ development [7,8]. The *ASPH* gene is 214,085 bases long and has 33 exons. By alternative splicing, it encodes four protein isoforms: ASPH, junctin (structural protein of sarcoplasmic reticulum), humbug (ASPH-type junctate that lacks the catalytic domain), and junctin-type junctate [14,15]. The ASPH protein consists of four domains: amino or N-terminal cytoplasmic domain, transmembrane domain, a highly charged region that projects into the lumen

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of endoplasmic reticulum (ER), and COOH-terminal catalytic domain [14]. Different studies showed that the Wnt/β-catenin, insulin (IN)/insulin-like growth factor 1 (IGF-1)/insulin receptor substrate 1 (IRS1)/phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt), and IN/IGF-1/IRS1/mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) signaling pathways play an important role in the transcriptional regulation of ASPH (Figure 1) [16,17]. When Wnt signaling is aberrantly activated, Wnt ligand binds to Frizzled (FZD) cell-surface receptors and low density lipoprotein (LDL)-receptor-related proteins 5 and 6 (LRP5 and LRP6) which leads to the degradation of the β-catenin destruction complex (contains adenomatous polyposis coli [APC] and AXIN) and inhibition of glycogen synthase kinase 3β (GSK3β), and consequent accumulation of β-catenin in the cytoplasm. Subsequently, β-catenin moves into the nucleus where it interacts with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) proteins to form a transcriptional regulatory complex and activate the transcription of target genes [16]. Among the proposed target genes is *IRS1*, where the TCF/LEF/β-catenin complex upregulates its expression possibly by binding to TCF consensus binding elements (enhancers) located in the first intron of the *IRS1* gene and downstream of its transcriptional start site [18,19]. The overexpressed IRS1 can relay signals from activated IN/IGF-1 receptors to downstream effector cascades such as the ERK/MAPK and PI3K/Akt signaling, and thus upregulate the expression of ASPH as a downstream target of these pathways [19]. Namely, binding of IN and IGF-1 to insulin receptor (IR) and IGF-1 receptor (IGF1R), respectively leads to the autophosphorylation of the receptor on tyrosine residues and activation of the intrinsic tyrosine kinase. The kinase catalyzes the phosphorylation of tyrosine (Y-P) on intracellular IRS1 and activates PI3K, which then phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) on the 3C position and generates phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 interacts with protein kinases such as phosphoinositide-dependent kinase 1 (PDK1) which initiates a cascade of phosphorylation events, finally leading to the activation of Akt and/or atypical protein kinase C (PKC) [18,20]. In addition to the PI3K cascade, tyrosine phosphorylation of IRS1 can result in the activation of the downstream MAPK pathway, i.e., PY-IRS1 interacts with growth factor receptor-bound protein 2 (Grb2) and synaptophysin (Syp) proteins leading to the sequential activation
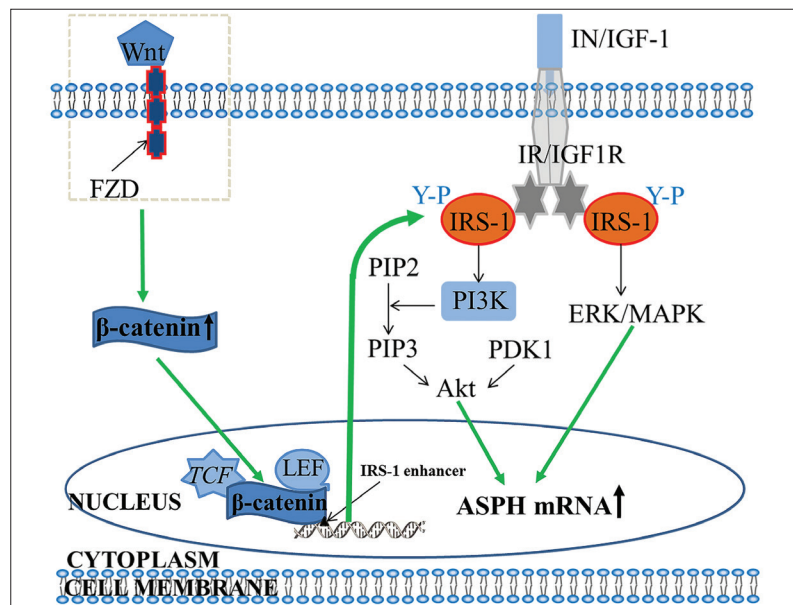

FIGURE 1. The role of the Wnt/β-catenin, insulin (IN)/insulin-like growth factor 1 (IGF-1)/insulin receptor substrate 1 (IRS1)/phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt), and IN/IGF-1/IRS1/mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) signaling pathways in the transcriptional regulation of aspartate β-hydroxylase (ASPH). The binding of Wnt ligand to Frizzled (FZD) cell-surface receptors and low density lipoprotein (LDL)-receptor-related proteins 5 and 6 (LRP5 and LRP6) leads to the accumulation of β-catenin in the cytoplasm. β-catenin then moves into the nucleus where it interacts with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) proteins to form a transcriptional regulatory complex and activate the transcription of target genes such as *IRS1*. The overexpressed IRS1 relays signals from activated IN/IGF-1 receptors to downstream effector cascades such as the ERK/MAPK and PI3K/Akt signaling, and thus upregulate the expression of ASPH as a downstream target of these pathways. Namely, binding of IN and IGF-1 to insulin receptor (IR) and IGF-1 receptor (IGF1R), respectively leads to the activation of the intrinsic tyrosine kinase. The kinase catalyzes the phosphorylation of tyrosine (Y-P) on intracellular IRS1 and activates PI3K, which then phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) and generates phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 interacts with phosphoinositide-dependent kinase 1 (PDK1), initiating a cascade of phosphorylation events and leading to the activation of Akt. The tyrosine phosphorylation of IRS1 can also result in the activation of the downstream MAPK pathway, i.e., PY-IRS1 interacts with growth factor receptor-bound protein 2 (Grb2) and synaptophysin (Syp) proteins leading to the sequential activation of p21ras, mitogen-activated protein kinase kinase (MAPKK), and MAPK [16,19,21,22].
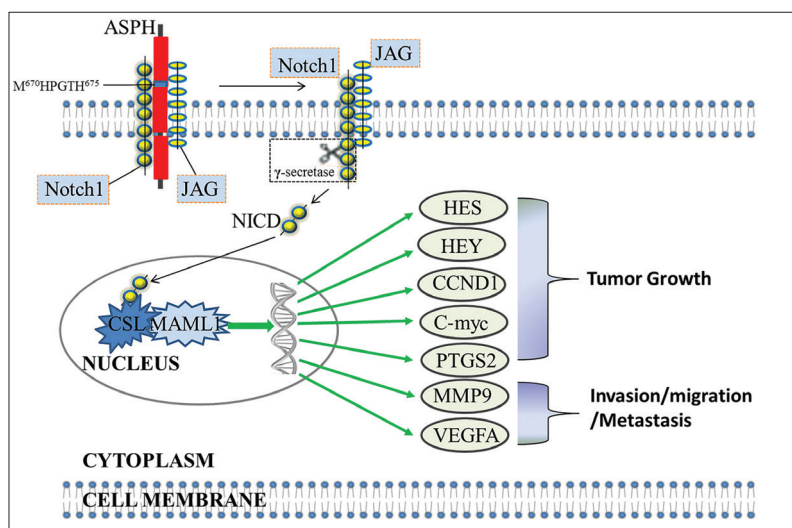
FIGURE 2. The proposed mechanism how aspartate β-hydroxylase (ASPH) may affect the progression of pancreatic cancer (PC) through the activation of Notch signaling pathway [27]. ASPH catalyzes the hydroxylation of aspartyl and asparaginyl residues present in epidermal growth factor (EGF)-like domains of Notch receptors and ligands. The C-terminal catalytic domain of ASPH contains the amino acid (AA) sequence M^{670}HPGTH^{675}. After ASPH overexpression is induced, the enzyme interacts with the EGF-like repeats in Notch receptor extracellular domain (ECD), promoting the interaction between Notch receptor and its ligand (e.g., Jagged [JAG]). The receptor-ligand interaction induces a conformational change of the Notch receptor, leading to S2 and S3 cleavage. The S3 cleavage releases the active Notch intracellular domain (NICD) from the plasma membrane, which then enters the nucleus and mediates the conversion of the CSL [CBF1–Su(H)–LAG1] repressor complex into a transcriptional activation complex and the recruitment of mastermind-like 1 (MAML1) coactivator protein, leading to the transcriptional activation of a number of downstream target genes, including those from hairy and enhancer of split (HES) and hairy-related transcription factor (HRT or HEY) families, cyclin D1 (*CCND1*), *c-myc*, cyclooxygenase-2 or prostaglandin-endoperoxide synthase 2 (*PTGS2*), matrix metalloproteinase-9 (*MMP9*) and vascular endothelial growth factor (*VEGFA*).

of p21ras, mitogen-activated protein kinase kinase (MAPKK), and MAPK [21,22].

One of the downstream targets of IRS1-mediated signaling pathways is ASPH. For example, de la Monte et al. [22] found that the stimulation of insulin and IGF-1 increased ASPH mRNA and protein expression, and consequently the motility of human hepatocellular carcinoma (HCC) cell lines, which was mediated by the ERK/MAPK and PI3K/Akt pathways [22].

ASPH is rarely expressed in normal adult tissue, except placental trophoblastic cells [23-25]; however, its overexpression has been observed in a number of malignancies, including cholangiocarcinoma, HCC, lung, breast and colon cancer, as well as in the neoplasms of the nervous system [22,26]. Moreover, in HCC patients, Wang et al. [25] showed a significant association between ASPH overexpression and higher recurrence and lower survival rate following surgery. Also, ASPH overexpression could predict worse surgical outcome in the early-stage HCC patients [25]. The overexpression of ASPH was also observed in PC, and its important role in the promotion of proliferation, migration, invasion, and malignant transformation of PC cells, through multiple signaling pathways, was suggested [27].

THE MECHANISM OF ASPH IN PC

ASPH activates the Notch signaling pathway

The mechanisms how ASPH affects cell proliferation and tumor invasion/metastasis in PC are not completely clear.

Dong et al. [27] indicated that ASPH activates the Notch signaling pathway as the mechanism of malignant transformation in PC cells. For example, they showed that activated Notch1 and hairy and enhancer of split-1 (HES1), which is a Notch responsive gene, were overexpressed in the cytoplasm and nuclei of pancreatic ductal adenocarcinoma (PDAC) cells compared to adjacent normal tissues [27].

The Notch signaling cascade is a highly conserved pathway with a critical role in cell-cell signaling and the control of cell fate determination during embryogenesis. Due to the diverse functions of Notch pathway, including the maintenance of stem cell populations and the regulation of cell proliferation, survival, apoptosis and differentiation, it plays an important role in the development and progression of human cancers. In mammals, four different Notch receptors exist (NOTCH1−4), which respond to five different ligands. Four of these ligands Jagged (JAG) 1 and 2 and Delta-like (DLL) 1 and 4 may act in *cis* to inhibit Notch receptor or in *trans* to interact with neighboring cells, whereas the fifth ligand DLL3 has the *cis*-inhibitory function. While the expression and activation of Notch receptors and ligands appear to be downregulated in normal adult pancreas tissues, as in many other cancer types, they are activated during pancreatic tumorigenesis and may act as oncogenes or tumor suppressors [28]. Numerous studies have shown that Notch signaling is associated with the occurrence and progression of PC [29-33]. For example, the activation of Notch signaling pathway can promote the

epithelial-to-mesenchymal transition (EMT) and facilitate the invasion and metastasis of cancer cells in PC tissues [34].

ASPH catalyzes the hydroxylation of aspartyl and asparaginyl residues present in epidermal growth factor (EGF)-like domains of various proteins, including Notch receptors and ligands (Figure 2) [27,35,36]. The C-terminal catalytic domain of ASPH contains the amino acid (AA) sequence $M^{670}HPGTH^{675}$. The AA H^{675} is involved in Fe^{2+} coordination and is critical for the enzymatic activity of ASPH [14,27]. After ASPH overexpression is induced, the enzyme interacts with the EGF-like repeats in Notch receptor extracellular domain (ECD), promoting the interaction between Notch receptor and its ligand. The receptor-ligand interaction induces a conformational change of the Notch receptor, leading to S2 cleavage by tumor necrosis factor-α-converting enzyme (TACE/ADAM17) and then S3 cleavage by the presenilin/γ-secretase complex. The S3 cleavage releases the active Notch intracellular domain (NICD) from the plasma membrane, which then enters the nucleus and mediates the conversion of the CSL [CBF1–Su(H)–LAG1] repressor complex into a transcriptional activation complex and the recruitment of mastermind-like 1 (MAML1) coactivator protein, leading to the transcriptional activation of a number of downstream target genes, including those from HES and hairy-related transcription factor (HRT or HEY) families, cyclin D1 (*CCND1*), *c-myc*, cyclooxygenase-2 or prostaglandin-endoperoxide synthase 2 (*PTGS2*), matrix metalloproteinase-9 (*MMP9*) and vascular endothelial growth factor (*VEGFA*). The overall effect of these processes in PC is the promotion of cell proliferation, migration, invasion, tumor growth, and metastasis [34,37].

ASPH promotes mitochondrial DNA D-loop mutations by inhibiting H2A histone family, member X (H2AX)-mitochondrial transcription factor A (mtTFA) signal

Somatic mitochondrial DNA (mtDNA) mutations have been detected in various tumor types, including PC [38-41]. In HCC tissues and cell lines, the overexpression of ASPH was significantly correlated with decreased copy numbers of displacement loop (D-loop) and nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 1, and increased somatic mutations in the D-loop [38]. The D-loop is a non-coding region of mtDNA which contains the origin of replication for heavy (H) mtDNA strand and the promoters for the transcription of H and light (L) strands [41].

In HCC cell lines, Tang et al. [38] demonstrated that the overexpressed ASPH disrupts the mtDNA integrity and affects mitochondrial function through H2AX-mtTFA signal (Figure 3) [38]. mtTFA is a key regulator of mtDNA transcription and is also important in the maintenance and repair of mtDNA. H2AX has been suggested to function as a shuttle protein transporter with a critical role in mitochondrial protein transport [42-44]. mtTFA was identified as protein that is transported by H2AX [44] from the cytoplasm to the mitochondria, to participate in the replication, transcription and repair of mtDNA (Figure 3A) [42,43]. However, it appears
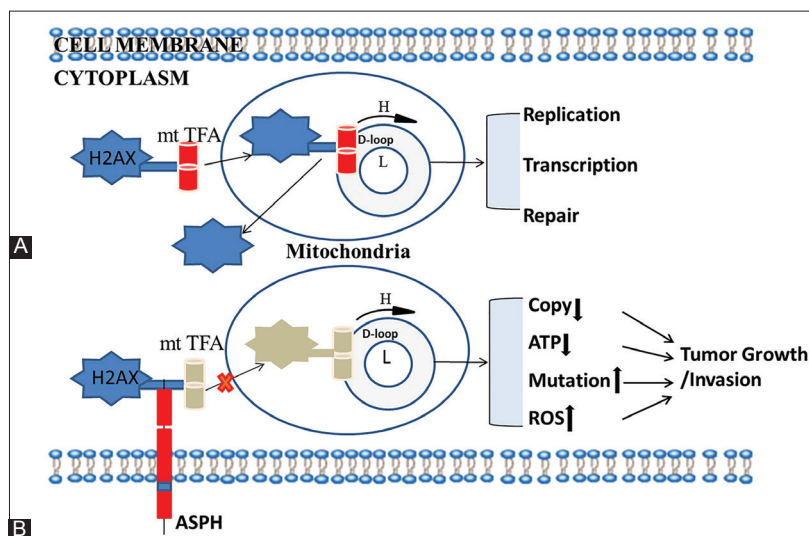

FIGURE 3. Aspartate β-hydroxylase (ASPH) disrupts the mitochondrial DNA (mtDNA) integrity and affects mitochondrial function through H2A histone family, member X (H2AX)-mitochondrial transcription factor A (mtTFA) signal in hepatocellular carcinoma (HCC) cells [38]. (A) H2AX transfers mtTFA from the cytoplasm to the mitochondria to participate in the replication, transcription, and repair of mtDNA. (B) ASPH competes with mtTFA for binding to H2AX, consequently blocking the binding between mtTFA and mtDNA displacement (D)-loop. This ultimately disrupts the function of mtTFA in mtDNA replication, transcription and repair, leading to increased mutations in the D-loop and other mtDNA regions, reduced mtDNA copy number and decreased expression of mitochondrial respiratory chain enzymes. These alterations affect the mitochondrial function, resulting in aberrant mitochondrial membrane potential, decreased adenosine triphosphate (ATP) production and increased levels of reactive oxygen species (ROS), and promoting tumor growth. H: Heavy mtDNA strand; L: Light mtDNA strand.

that overexpressed ASPH competes with mtTFA for binding to H2AX, thus blocking the translocation of mtTFA into the mitochondria and resulting in reduced binding of mtTFA to the D-loop [38,44]. This ultimately disrupts the function of mtTFA in mtDNA replication, transcription and repair (Figure 3B), leading to increased mutations in the D-loop and other mtDNA regions, reduced mtDNA copy number and decreased expression of mitochondrial respiratory chain enzymes. These alterations affect the mitochondrial function, resulting in aberrant mitochondrial membrane potential, decreased adenosine triphosphate (ATP) production and increased levels of reactive oxygen species (ROS) [38,45]. In primitive neuroectodermal tumor 2 (PNET2) human neuronal cells exposed to H_2O_2, Lawton et al. [46] showed that ASPH, hypoxia-inducible factor 1-alpha (HIF-1α) and neuronal migration were stimulated by the mild oxidative stress and suggested that the cross-talk between these molecules within a hydroxylation-regulated signaling pathway, which ultimately affects cell motility and migration, is transiently driven by fluctuations in oxidative stress and chronically regulated by the insulin/IGF signaling [46]. Changes in cellular microenvironment can lead to increased oxidative stress which is characterized by the overproduction of ROS in mitochondria, leading to increased mutations in mtDNA and disruption of its stability and function [45], possibly resulting in tumor development. However, due to low occurrence of somatic mtDNA D-loop mutations in a series of PC, Navaglia et al. [41] suggested that those molecular changes are epiphenomena, probably related to the damaging effects of ROS, rather than a direct cause of PC [41,47].

ASPH suppresses the natural killer (NK) cell-surveillance activity

NK cells play a pivotal role in immune surveillance of tumors and exert their function by producing cytokines such as interferon-γ (IFN-γ) and cytolytic proteins (perforin and granzymes), and by expressing NK cell-activating receptors (e.g., NKG2D, NKp30, and NKp44) [48]. IFN-γ is a critical molecule for innate and adaptive immunity and has antiviral, immunostimulatory, immunoregulatory, and antitumor properties [49]. Perforins do not only generate poly-perforin tubular channels (pores) on tumor cell membrane, increasing membrane permeability, but also mediate delivery of granzymes which induce apoptosis of target cells [48]. NKG2D triggers cytotoxicity by recognizing ligands (induced-self proteins) overexpressed by transformed and infected cells. Together with NKp30 and NKp44, it is the most important molecule in NK cell-mediated tumor cell lysis [50]. In MP2 PC cells, the cytotoxic activity of NK cells was enhanced by the combined effect of curcuminoids, omega-3 fatty acids, and antioxidants [51].

The activation and function of NK cells is controlled by two different categories of receptors, activating and inhibitory receptors, which are expressed on the surface of NK cells [52,53]. In normal conditions, the inhibitory receptors on NK cells interact with their ligands (mostly major histocompatibility complex [MHC] class I molecules), suppressing the activation of NK cells [54]. In cancer cells, these types of ligands can be downregulated and the ligands of activating receptors upregulated, leading to the engagement of activating receptors and consequent activation of NK cells. Finally,
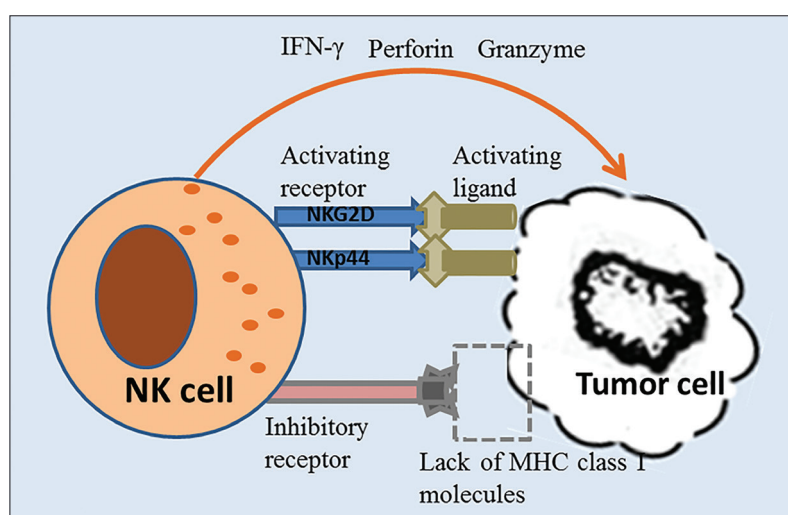

FIGURE 4. Natural killer (NK) cell-surveillance activity in tumor. The activation and function of NK cells is controlled by two different categories of receptors, activating and inhibitory receptors. In normal conditions, the inhibitory receptors on NK cells interact with their ligands (mostly major histocompatibility complex [MHC] class I molecules), suppressing the activation of NK cells. In cancer cells, these types of ligands can be downregulated and the ligands of activating receptors upregulated, leading to the engagement of activating receptors (e.g., NKG2D and NKp44) and consequent activation of NK cells. Activated NK cells can rapidly kill tumor cells by releasing molecules such as interferon-γ (IFN-γ), perforins and granzymes [52-55].

activated NK cells can rapidly kill tumor cells by releasing molecules such as IFN-γ, perforins and granzymes (Figure 4) [55].

A recent study showed that a recombinant ASPH (rASPH) had a negative effect on the activity and function of primary human NK cells. Namely, the rASPH reduced the viability and cytotoxicity of NK cells in a time and dose-dependent manner, inhibited NK cell aggregation, promoted apoptosis and necrosis, reduced the mRNA expression of INF-γ, mRNA and protein expression of activating receptors NKG2D and NKp44, and mRNA expression of inhibitory receptor NKG2A [56]. Overall, it appears that one of the mechanisms of ASPH in promoting tumor formation and viability is by inhibiting NK cell-surveillance activity [56]. Other studies also demonstrated that the inhibition of NK cell activating receptor expression and function, e.g., by methylprednisolone or histone deacetylase inhibitors, significantly reduces NK cell cytotoxicity [57,58], allowing tumor cells to escape immune surveillance.

THERAPEUTIC APPROACHES TARGETING ASPH

ASPH has been proposed as an important biological target to control tumor cell migration and invasion, as its overexpression has been observed in 70–90% of human tumors, including PC [22,27]. After ASPH overexpression is induced in tumor cells, the protein transfers from the ER to the plasma membrane, where it is exposed to extracellular environment and, thus, could be used as a tumor associated antigen (TAA) in immunotherapy [59].

The activation of both cluster of differentiation (CD)4+ T cells and CD8+ cytotoxic T cells (CTLs) is required for a sustained anti-tumor response [59-61]. In an experimental murine model, ASPH-loaded dendritic cells (DCs) had a substantial anti-tumor effect on HCC, and both CD4+ and CD8+ cells contributed to these effects [59]. Furthermore, in peripheral blood mononuclear cells (PBMCs) derived from HCC patients, ASPH protein-loaded DCs could also activate CD4+ T cell and CD8+ CTLs, via ASPH-derived human leukocyte antigen (HLA) class I- and class II-restricted peptides [61]. These findings indicate the usefulness of ASPH as a molecular target in immunotherapy, especially in HCC. For example, ASPH-DCs immunotherapy could potentially delay the recurrence of HCC following surgical resection [59]. Similarly, Noda et al. [62] showed that immunization with ASPH-loaded DCs had a cytotoxic effect on cholangiocarcinoma cells *in vitro*, suppressed intrahepatic tumor growth and metastasis in rats, and was associated with an increased infiltration of CD3+ lymphocytes into the tumor [62]. However, the effect of ASPH-loaded DCs on immune response in PC remains to be investigated.

Recently, molecular targeted therapy against ASPH has gained considerable attention. Dong et al. [27] reported that MO-I-1100, a small molecule inhibitor (SMI) of β-hydroxylase, reduced ASPH activity by 80%, inhibited ASPH-induced proliferation, migration, invasion and colony formation, and suppressed Notch signaling in PC [27]. Sturla et al. [63] showed that SMI MO-I-1100 and MO-I-1151 significantly reduced viability and directional motility of glioblastoma multiforme (GBM) cells, and similar effects were observed in GBM cells using lentivirus-sh-ASPH construct, confirming the role of ASPH in these processes [63]. Revskaya et al. indicated that radiolabeled human monoclonal antibody (mAb) PAN-622 targeting ASPH on the surface of cancer cells is a promising approach in imaging and, possibly, treatment of metastatic breast cancer [64]. In addition, antisense oligodeoxynucleotide inhibition of ASPH expression significantly reduced the motility of cholangiocarcinoma cells [65] and small interfering RNAs (siRNAs) targeting the exon 2 of *ASPH* gene inhibited the expression of ASPH and reduced directional motility in HCC cells [22]. In another study, mAb against the ASPH C-terminal (ASPH-C) increased antibody-dependent cellular cytotoxicity of NK cells on HeLa, MCF-7 and HepG2 cells, suggesting the use of this mAb in cancer immunotherapy [66]. Considering the above-described findings, ASPH may play an important role in the development of therapeutic agents for PC.

CONCLUSION

ASPH is overexpressed in many cancer types, including PC. It plays an important role in tumor development and progression by activating the Notch signaling pathway, promoting mtDNA D-loop mutations/disrupting mitochondrial function, and inhibiting the NK cell activity. Different studies demonstrated the potential of ASPH as a biomarker and therapeutic target in cancer. Recently, our study has found that the Notch signaling pathway is pivotal for exosome secretion and biological activity in MIA-Paca2 cell lines. Hence, we hypothesize that ASPH stimulates PC cells to secrete/release specific exosomes and acquire invasive properties by activating the Notch signaling. Overall, it appears that the ASPH-mediated regulation of PC development, progression and metastasis may be more complex than originally thought.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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