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Review

Biological functions of hyaluronan and cytokine-inducible deubiquitinating enzymes

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ABSTRACT

The modification of proteins through post-translation and degradation by the ubiquitin–proteasome system plays a pivotal role in a broad array of biological processes. Reversal of this process by deubiquitination is a central step in the maintenance and regulation of cellular homeostasis. It now appears that the regulation of ubiquitin pathways by deubiquitinating enzymes (DUBs) could be used as targets for anticancer therapy. Recent success in inducing apoptosis in cancerous cells by USP17, a cytokine-inducible DUB encoding two hyaluronan binding motifs (HABMs) showing direct interaction with hyaluronan (HA), could prove a promising step in the development of DUBs containing HABMs as agents in anticancer therapeutics. In this review, we summarize the importance of hyaluronan (HA) in cancer, the role played by DUBs in apoptosis, and a possible relationship between DUBs and HA in cancerous cells, suggesting new strategies for applying DUB enzymes as potential anticancer therapeutics.

Contents

1. Introduction

Covalent attachment of ubiquitin (Ub) to proteins is a significant regulatory step in a diverse array of cellular and biological processes, including embryonic development, cell cycle control, transcriptional regulation, immune response, apoptosis, oncogenesis, pre-implantation, and intracellular signaling pathways [1]. These ubiquitin molecules can be attached to their substrates as monomers or as polymers (Fig. 1). In general, a monoubiquitination process is involved in the regulation of diverse cellular processes including DNA repair, receptor endocytosis, vesicle sorting, gene silencing, and signal transduction [2–5]. Polyubiquitination processes are involved both in protein degradation

Abbreviations: DUB, deubiquitinating enzymes; HABMs, hyaluronan binding motifs; HA, hyaluronan; Ub, ubiquitin; UCH, ubiquitin C-terminal hydrolases; USP, ubiquitin-specific processing proteases; JAMM, Jab1/Pab1/MPN domain-containing metalloenzymes; OTU, Otu-domain ubiquitin aldehyde-binding proteins; ECM, extracellular matrix; GAGs, glycosaminoglycans; Has, hyaluronan synthesizing enzymes; HYAL, hyaluronidases; SPAM-1, sperm adhesion molecule 1; DOX, doxorubicin; HAUSP, herpes virus-associated ubiquitin-specific protease

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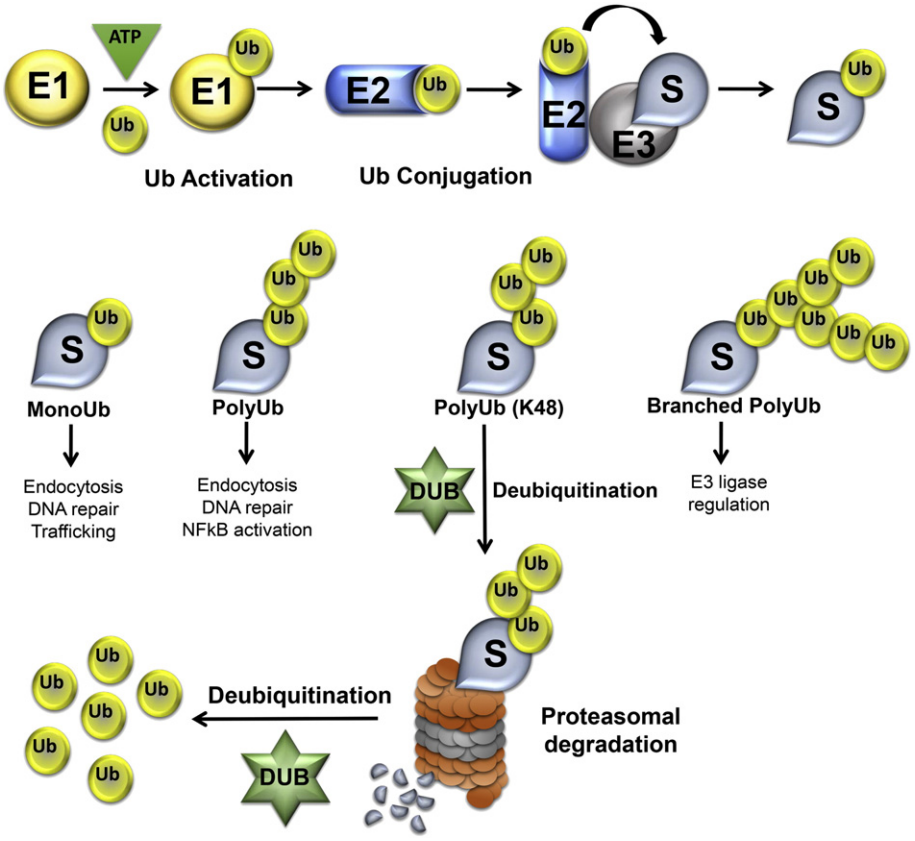

Fig. 1. The ubiquitin proteolytic pathway. The process of ubiquitination is regulated by an organized milieu of E1, E2 and E3 enzymes to mediate the ligation of ubiquitin to the lysine residues in the proteins targeted to the 26S proteasome for degradation. Ubiquitins are recycled by the action of DUB enzymes.

and signal transduction. Ubiquitin itself contains seven Lys residues, comprising K6, K11, K27, K29, K33, K48, and K63 [6], which serve as acceptor sites for other ubiquitin molecules during the formation of ubiquitin chains. Ubiquitin chains are arranged in several different ways. A conjugation of ubiquitin chains linked through lysine 48 serves primarily as a targeting signal for proteasomal degradation by sequential enzymatic actions via ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). A novel ubiquitination factor (E4), required for efficient multiubiquitination, has been identified in yeast [7–9]. K29- and K33-branched ubiquitin chains have been noted in the regulation of AMP-activated protein kinase-related kinases [10]. Recent studies have identified K33 linkages that can restrict T cell receptor (TCR) signaling by disengaging TCR-zeta in T cells [11]. K29-branched ubiquitin chains have also been shown to promote their substrates for proteasomal and lysosomal degradation [12,13]. K63-branched ubiquitin chains are not involved in the protein degradation process. Instead, they are involved in several cellular processes, such as DNA repair, signal transduction, intracellular trafficking of membrane proteins, endocytosis, and stress responses [14–17]. As a result, depending on the cellular function, proteins can be monoubiquitinated, multiubiquitinated, or polyubiquitinated (Fig. 1).

Deubiquitination is a critical step in the regulation of the proteasomal pathway. Deubiquitinating (DUB) enzymes, a large family of proteases, have primarily been instrumental in the renewal of the polyubiquitin chains for use during ubiquitination (Fig. 1). Almost 100 DUB enzyme genes have been identified from the human genome; these enzyme genes are mainly involved in recycling monomeric Ub, the release of Ub from Ub fusion precursors, inverse regulatory ubiquitination, and the editing of inappropriately ubiquitinated proteins [1,18]. Most DUB enzymes are cysteine proteases, which are classified into at least five families: the ubiquitin C-terminal hydrolases (UCH), the ubiquitin-specific processing proteases (USP), Jab1/Pab1/MPN domain-containing metallo-enzymes (JAMM), Otu-domain ubiquitin aldehyde-binding proteins (OTU), and Ataxin-3/Josephin [1,9,19,20].

A relatively recent upsurge in knowledge relating to DUB enzymes (including USP2, USP7, USP8, USP9X, USP15, USP16, USP17, USP28, USP41, CYLD, UCHL-1, Ataxin-2, Ataxin-3, and Ataxin-7) has highlighted their essential roles in promoting apoptosis in cells [21–23]. These DUBs mainly regulate the process between prosurvival signaling and cell death signaling. During cell stresses, such as DNA damage, unfolded protein, and oxidative stress signaling, the expression level of DUBs is either up- or down-regulated, leading to cell apoptosis. In addition, there are several DUBs, namely, DUB-1, DUB-1A, DUB-2, and DUB-2A, which belong to cytokine-inducible DUB subfamily members involved in the regulation of cell proliferation and apoptosis in lymphocytes [9,20]. Recently, we isolated cytokine-inducible DUB USP17 from chorionic villi tissue and various cancer cell lines [24] that induce apoptosis and the death of cancerous cells by negatively regulating histone deacetylases (HDAC) activity [24–26]. USP17, with two putative hyaluronan binding motifs (HABMs), directly interacts with hyaluronan (HA) to inhibit cell proliferation and anchorage-independent tumor growth [27].

An important connective tissue glycosaminoglycan, HA is enriched in pericellular matrices surrounding proliferating and migrating cells. Elevated HA biosynthesis is a common feature in many types of human cancers, and its constitutive interactions with tumor cells have a major influence on tumor growth and metastasis, promoting anchorage-independent growth and invasiveness in animal models [28–30]. Apart from its significant role in the extracellular matrix (ECM), HA can also be detected in the rough endoplasmic membrane, plasma membranes, cytoplasm and nuclei of cells in a number of tissues in vivo [31–34]. Intracellular HA tends to accumulate in the perinuclear region of the aortic smooth muscle cells during the premitotic and mitotic stages and facilitates the process of nuclei separation and subsequent cell

division [32,35]. Although, several functions of intracellular HA have been reported, the actual source of intracellular HA and its correlation with other proteins has not yet been revealed.

Research has shown that several intracellular HA-binding proteins are involved in the regulation of cell cycle or gene transcription [36–39]. Recent reports on USP17 with two HABMs have revealed similar behavior with regard to inducing apoptosis and anchorage-independent conditions [20,25–27]. This observation has led us to predict that DUBs containing HABMs in their sequence can act as HA-binding proteins and may be able to regulate cell cycle and cell viability. Thus, further clarification of the molecular mechanisms of DUBs and their implication in the regulation of apoptosis in tumor cells is highly desirable. This review summarizes the recent report on USP17, showing HA-mediated apoptosis and emphasizing the importance of HA in tumor progression, the role of HA polymers in targeting drug conjugates, the use of HA oligosaccharides in the inhibition of tumor growth, the effect of variation in CD44 expression in malignant cells, and the regulatory action of DUBs in the apoptosis of cancer cells.

2. HA and cancer

HA is commonly found in synovial fluid and the ECM. It is a natural polysaccharide composed of alternating (1 → 4)-β linked D-glucuronic and (1 → 3)-β linked *N*-acetyl-D-glucosamine residues and has a linear, high molar mass. HA belongs to a group of substances known as glycosaminoglycans (GAGs). Unlike other GAGs, HA is an unsulphated, unbranched member that is synthesized as a free polysaccharide. The molecular weights of HA polymers range from 10^5 to 10^7 Da with 2000–25,000 disaccharides depending on tissue source and physiological conditions [40]. In a free solution under physiological conditions of pH and ionic strength, it behaves as a long, stiffened random coil and interacts with its neighbors to create viscoelastic solutions. At a high concentration, neighboring molecules are entangled to form a continuous mesh-like structure. Interactions in tissues between HA and HA-binding proteins, termed hyaladherins, change its conformation compared to that in free solution conditions.

Although HA is almost omnipresent in the human body and in other vertebrates, the highest amount of HA is found in the ECM of soft connective tissues [40]. In addition to vertebrates, HA is also present in the capsules of some bacteria (e.g., strains of *Streptococci*), but is absent in fungi, plants, and insects. In order to understand the roles of HA in tumor progression, both the regulatory mechanisms balancing the HA synthesis and the degrading enzymes must also be understood. Hyaluronan synthesizing (HAS) enzymes were identified and characterized in 1996 [41–43]. Identification of eukaryotic hyaluronan synthases, termed *HAS1*, *HAS2*, and *HAS3*, has shown that they are functionally related [44–48]. These three HAS enzymes share a high degree of sequence and structural homology and are located on chromosomes 19q13.4, 8g24.12, and 16q22.1, respectively. Ablating the *HAS2* gene has proved to be lethal to embryonic mice at E9.5 [49] and is critical in HA biosynthesis during the development stage [50]. Although not yet fully established, newly synthesized HA at the inner face of the plasma membrane may be retained inside the cell or released into pericellular and extracellular matrices via ABC transporters [32,51,52]. In addition, regulation of HA biosynthesis is significantly affected by the interaction between different cytoplasmic proteins and HAS protein [53].

The enzymes involved in HA degradation are hyaluronidases (HYAL); the family of human HYAL genes includes *HYAL1*, *HYAL2*, *HYAL3*, *HYAL4*, *HYALP1* and *PH-20* (SPAM-1, sperm adhesion molecule 1). The *HYAL* genes are located on two human chromosomes: 3p21.3 and 7q31.3 [54]. *HYAL1* and *HYAL2* are ubiquitously expressed in most tissues and body fluids and are considered as major HA-degrading enzymes in somatic tissues [55]. Very little is known about *HYAL3*, *HYAL4*, and *HYALP1* in humans. Hyaluronidases may also play a major role in cell differentiation, especially in angiogenesis [56]. In vivo studies have found faster development of transplantable tumors when the *HAS2* gene was overexpressed, whereas overexpression of the *HYAL1* gene showed growth suppression [57]. Thus, hyaluronidases may play a critical role in suppressing tumors, sensitizing multidrug-resistant cells, and inducing TNF-mediated cancer cell death, as well as in tumor progression [58]. However, other studies indicate that the balance of HA synthesis and degradation plays a complex role in cancer progression [59–61]. Elevated levels of both HA and hyaluronidases in the urine form a clinically reliable marker for the presence and grade of bladder cancer [62]. Thus, the anticancer and tumor-progressing properties of hyaluronidases are like the two faces of the same coin.

HA has a high capacity for holding water and its high viscoelasticity provides a unique profile among biological materials. Accumulation of HA within the extracellular matrix leads to the expansion of tissue spaces due to hydration by osmotic pressure [63]. An HA-rich, hydrated extracellular matrix loses its compact architecture and facilitates cell migration and invasiveness into neighboring tissues [64–66] as well as providing an immunoprotective coat to cancer cells [67]. A decline in HA levels within the matrix interrupts cellular movements and allows the onset of cytodifferentiation. Histological studies of various tumors, using a specific HA affinity probe, have shown that almost all human epithelial tumors are surrounded by a connective tissue matrix (stroma) enriched in HA as compared with normal tissues. Elevated levels of HA are evident in several cancers including breast, stomach, ovarian, prostate, and colorectal cancer [68–72]. In the same way, urinary levels of HA and hyaluronidases are a reliable marker for bladder cancer [62]. A high level of HA has also been found in the serum or lung lavage fluids of tumor-bearing patients [73–75]. The average HA concentrations in the blood serum of a healthy, middle aged person are about 30–40 μg/ml. In the case of an abnormal condition, the HA level in serum can increase to above 250 μg/ml and acts as a clinical biomarker for diagnosis [76]. Similar results have also been observed in animal models; for example, the accumulation of HA correlates with tumor invasiveness in rabbit V2 carcinoma [28], and increased HA amounts during the early stages of invasion in the mouse model of melanoma [77]. Thus, HA is a central component of the distinct stroma that surrounds and influences tumor progression, in which overexpression is believed to accelerate tumor growth and metastasis [78,79].

Manipulation of HA levels and perturbation of endogenous HA interactions have a significant impact on tumor progression. HA interacts with numerous receptors, including CD44, RHAMM, LYVE-1, HARE, layilin, and Toll-4 [80,81], the most important of which is CD44, a single-pass transmembrane glycoprotein. The interaction between HA and CD44 has important physiological implications in cellular processes such as signal transduction and the arrangement of pericellular matrices [82]. HA also affects cell behaviors including migration, proliferation, and differentiation via binding to the specific cell surface receptor CD44 [83]. Interruption in interactions between HA and CD44 interferes directly with the catabolism of HA in chondrocytes [82] and keratinocytes [84]. Thus, the growth and metastasis of several tumor types can be inhibited by perturbing endogenous cell receptor interactions between HA and CD44, by the administration of HA oligosaccharides or soluble HA-binding proteins. For example, mouse mammary carcinoma cells and human malignant melanoma cells showed reduced in vivo tumor growth, invasion, and metastasis when soluble CD44 was overexpressed [83,85–87]. In addition, the treatment of HA oligomers competes for endogenous polymeric hyaluronan-receptor interactions resulting in the inhibition of tumor growth [83,88,89]. Thus, perturbation of endogenous polymeric HA by targeting smaller oligomers could result in a loss of HA-induced signaling for tumor progression. In conclusion, HA is considered to be a key regulator in enhancing cell motility and invasiveness, and acts as a characteristic sign of the malignant phenotype in various cancerous cells.

3. Intracellular HA

In general, HA is synthesized by the group of proteins called HA synthase at the plasma membrane and is later released into the

extracellular space. However, recent studies indicate that intracellular HA also exists, but its functions are not clearly understood. Previously, HA has been identified in smooth muscle cells and fibroblasts in the cytoplasm as a network-like structure, in vesicles, and in nuclear staining [32,35]. As a result, it is possible to predict a role for HA in chromosome condensation, the nuclear matrix and the cytoskeleton. In contrast, intracellular HA has also been found in cytoplasmic vesicles, without any nuclear staining, in keratinocytes treated with epidermal growth factor [90]. Intracellular HA has also been reported in microtubule distribution [91].

Intracellular HA undergoes endocytosis and is eventually destined for degradation. During this process, high molecular weight intracellular HA is trapped in larger vesicles, and some HA is diffused forming a network-like pattern in the perinuclear area [91]. During endoplasmic reticulum stress, activation of intracellular HA synthesis occurs, leading to the formation of HA cables inside the cell [32,35]. In addition, intracellular HA synthesis can give rise to HA cables that span through the cell during hyperglycemic conditions [92–94]. However, this intracellular HA has been synthesized by different HA synthases such as variant *HAS1*, which is involved in the production of intracellular HA in multiple myeloma [95], whereas *HAS2* is responsible for the production of intracellular HA in the osteosarcoma cell line MG-63 [96]. Many reports suggest that intracellular HA is capable of binding to several intracellular proteins such as RHAMM [97], HABP/P32 [98], CDC37 [37] and USP17 [24,27]. Although, there have been several studies on intracellular HA and binding proteins, the exact source, function, and biological relevance of protein binding to intracellular HA remain unclear.

4. CD44 and cancer

CD44 is a polymorphic glycoprotein with a myriad of functions including cell to cell interactions, cell adhesion, migration, and metastasis [99]. This versatility in function results from the differential splicing of a large number of additional exons in a region of mRNA corresponding to the extracellular domain of CD44, termed exons v1–v10, as well as glycosylation that is specific to a particular cell type [100,101]. CD44, the principal receptor for the ECM glycosaminoglycan, HA [40], plays a central role in the growth and metastasis of numerous tumor types [102,103]. Importantly, the sequential proteolytic cleavage of CD44 regulates cell migration and the translocation of the CD44 intracellular domain into the nucleus, leading to the transcription of several genes (including CD44), which are associated with inflammation and malignancy [104].

A direct relationship between CD44 and HA has been shown to promote the proliferation of cells into adjacent stromal tissues [105], augmentation of tumor cell motility on HA-coated substrates [106] and the enhancement of tumor growth and metastasis [85,103]. Interestingly, disruption of the interaction of HA with surface CD44 induces apoptosis in metastatic mammary carcinoma cells and invasive glioma cells in vivo [87,89]. However, in solid tumors, alterations in CD44 can lead to either up-regulation [107] or down-regulation [108] in variant isoform expression [109,110]. Metastatic proteins were identified by raising antibodies against a metastatic subline [102,111] and these were found to be reactive with two variant CD44 isoforms (CD44v4–7 and CD44v6–7) on these metastatic cells, by binding to the v6 exon present in both isoforms [111]. The metastatic efficiency of these two isoforms has been confirmed by in vivo studies in rats [112,113].

Modifications of CD44, affected by changing the composition of glycosylation or GAG modification, can lead to changes in the function of CD44 in normal cells [114]. The treatment of antibodies, which block the binding of CD44 to HA, results in the inhibition of tumor growth and invasion [115,116]. Administration of soluble CD44 into metastatic mammary carcinoma cells in vivo disrupts CD44 interaction on the tumor cell surface, thus inhibiting tumor progression [85,117] due to the induction of apoptosis [87]. To summarize, disturbance in the interaction between CD44 and HA could conceivably influence the metastatic behavior of tumor cells.

5. Hyaluronic acid as a drug-targeting moiety

Despite significant advances in the development of anticancer technology, there is still no promising cure for patients with malignant diseases. In reality, traditional chemotherapy that relies on treating cancerous cells with cytotoxic agents has resulted in systemic toxicity leading to severe side effects. Over the past decades, various drug delivery systems have been explored to overcome the drawbacks of using cytotoxic agents. A drug delivery system consisting of a tumor identifying moiety such as HA, combined through a linker to form a conjugate with a drug, is considered to be a new paradigm in targeting tumors [118–120].

Recently, it has been identified that HA can be coupled with an active cytotoxic agent directly to form a non-toxic drug. Direct conjugations of low molecular weight HA with cytotoxic drugs such as butyric acid [121], paclitaxel [122–124] and doxorubicin [125] have been studied. It is believed that these bioconjugates interact with cells in a manner that directly induces receptor-mediated endocytosis, followed by intracellular release of active drugs, thus restoring their cytotoxicity. Bioconjugates are dose dependent in terms of the efficiency of their administration to cancerous cells. The potency of bioconjugates relies on the level of cytotoxic agent loading to HA. The HA-butanoic acid conjugates were tested against the MCF-7 cell line with different degrees of butanoic acid attachments, ranging from 0.1 to 2.24 per HA molecule. Based on this study, a weak response was reported in the cytotoxicity of a highly-loaded conjugate when compared with use of the drug alone [121]. Similar results were observed for the HA-paclitaxel conjugates tested against SKOV-3 (ovarian), HBL-100 (breast), and HCT-116 (colon) cancer cell lines [123], probably due to a masking phenomenon occurring at the receptor recognition sites of HA, leading to a decrease in cytotoxicity.

HA-containing liposomes with entrapped doxorubicin (DOX) as a tumor-targeting moiety have proved to be an efficient drug-delivery system. The HA-containing DOX liposomes showed high cytotoxicity and double activity compared to non-targeting liposomes against B16F10 melanoma cells. In addition, the cytotoxicity of HA-containing DOX liposomes was negligible to CV-1 cells, which had a low level of CD44 expression, supporting the idea that HA was targeting the drug via interaction with CD44, thus leading to endocytosis and drug release [126]. In another trial, delivering HA with N-(2-hydroxypropyl) methacrylamide (HPMA) polymer showed efficient internalization and cytotoxicity [125]. These observations provide direct evidence that delivering HA conjugated with cytotoxic drugs or inhibiting HA interactions with CD44 in carcinoma cells leads to the suppression of tumor growth.

6. HA oligosaccharides as anticancer therapeutics

Another promising area of research in the development of anticancer therapy relates to treating tumor cells with small chains or HA oligosaccharides (6 to 18 sugar units). It has been observed that HA oligosaccharides competitively block binding of endogenous HA polymer to CD44 [127] and destroy several types of cancer cells by triggering apoptosis and eventually blocking tumor progression [83,88,128]. Perturbation of endogenous HA interaction by HA oligomers inhibits anchorage-independent growth of tumor cells in vitro and suppresses growth of tumors in vivo [88]. CD44, the principal receptor for the ECM glycosaminoglycan HA [40], plays a central role in the growth and metastasis of numerous tumor types [102,103]. It has been reported that there are 160 amino acid residues in CD44 receptor codes for the binding domain for HA [80,129]. The affinity between CD44 and HA binding is significantly affected by the size of the HA oligomers. Monovalent binding exists in oligomers ranging in size from 6 to 18 sugar

residues [128]. Hexamer is the minimum size of HA oligomers necessary for effective binding with CD44 [128], although in some cells, a decamer is required [130]. Larger oligomers show increased binding affinity in relation to smaller oligomers due to multiple interactions with more than one CD44 receptor [80,128]. HA oligosaccharides that interact monovalently with CD44 inhibit the growth of several types of tumors in vivo by suppressing receptor tyrosine kinase activities and the PI3-kinase/Akt cell survival pathway [78,88,120]. In addition, HA oligosaccharides have the capacity to sensitize cancer cells to chemotherapeutic agents [83,131]. Thus, the antagonism of HA interactions with small HA oligomers could be a basis for the future development of anticancer therapeutics.

7. Cytokine-inducible deubiquitinating enzymes with HABMs

Approximately 100 human DUB enzyme genes exist in the human genome; some of these are involved either in promoting or suppressing cell proliferation activity [22]. These DUBs mostly regulate the expression level of their binding partners or substrates to combat against various cell stresses, such as DNA damage, unfolded protein, and oxidative stress signaling to maintain cell homeostasis [22]. Although, DUBs are known to regulate apoptosis, the molecular mechanism regulating programmed cell death is not clearly understood. The study of factors involved in the DUBs that are associated with a series of biochemical signaling leading to cell death has made a strong impact on therapeutic biology and medicine.

Recent identification of HABMs in USP17, and their significance in regulating cell viability and apoptosis, are considered as the first evidence of cytokine-inducible DUBs associated with HA-mediated apoptosis [24,27]. Here, we emphasize the significance of USP17 with HABMs having antitumor effects on cancer cells. USP17 subfamily members have been previously identified [24,132]. They are regulated by IL-4 and IL-6 cytokines and have been found to block cell proliferation, leading to apoptosis under constitutive expressions [9,20,133]. Elevated expression of USP17 has been reported in several tumor-derived cell lines and tumor biopsies [134]. The balanced expression of USP17 protein is necessary for normal cell cycle progression because its depletion leads to a significantly impaired G1-S transition and blocked cell proliferation [134]. Four newly-discovered members of the USP17 subfamily, which encode a DUB enzyme, were identified in human chorionic villi tissues and classified as USP17K, USP17L, USP17M, and USP17N [24]. The conserved Cys, His and Asp domains, which are mainly responsible for the deubiquitinating function, are found in all subfamily members of USP17 [24] (Fig. 2A).

The amino acid sequence of the USP17 subfamily encodes the suggested HABMs—(R/K) X7(R/K)—found in HA-binding proteins such as RHAMM, CD44 and link protein [135]. These HABMs are found at the C-terminus of USP17 subfamily members, except in USP17N, which lacks the C-terminus region containing HABMs (Fig. 2A). We compared and aligned the amino acid sequences of the HA-binding regions for USP17 to the known HA-binding proteins containing HABMs, such as CD44, RHAMM, and hyaluronidases (Fig. 2B). It has been well established that peptides containing HA-binding motifs inhibit tumor growth and induce apoptosis [136]. In the same way, USP17 subfamily members with HABMs showed a similar negative regulation of cell viability and cell proliferation in cancer cells [24,25,27]. Overexpression of USP17 subfamily members in cancer cells induced apoptosis by elevating the expression level of apoptotic markers [24,25,27]. In contrast,

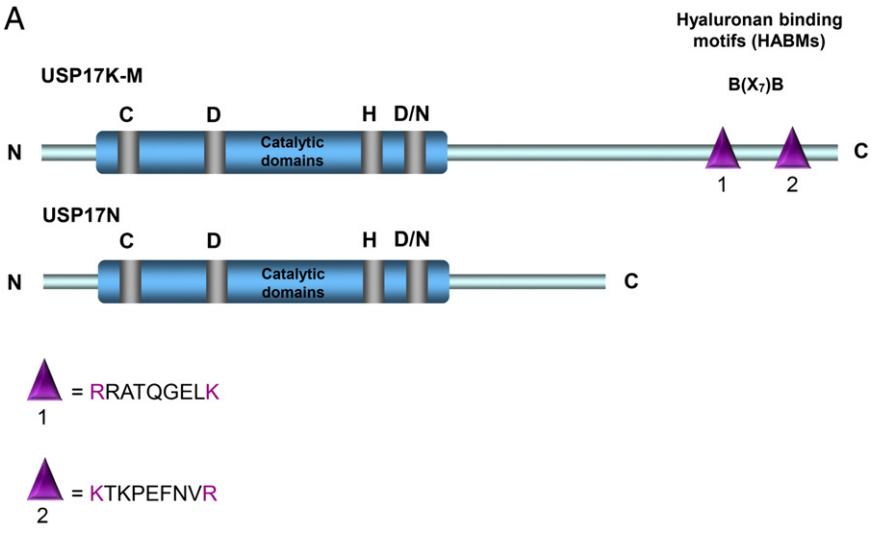

Fig. 2. (A) A schematic diagram of the structural features of USP17 subfamily members. The conserved catalytic domains (Cys, Asp(I), His, Asp/Asn(II)) required for DUB enzyme activity are located at the N-terminus and two HABMs are located at position 401–409 and 445–453 amino acids towards the C-terminus. (B) USP17 as an HA-binding protein. Comparison and alignment of USP17 amino acid sequence and HA-binding proteins at HA-binding regions.

overexpression of USP17N without HA-binding motifs was unable to alter cell viability. Further, characterization of HABMs in USP17 revealed that USP17 directly interacts with HA and that this interaction is essential to inhibit cell proliferation and anchorage-independent tumor growth. Where HABMs were deleted, USP17 lost its inhibitory effect on cell proliferation, indicating that the HABMs in USP17 are associated with HA-mediated cell apoptosis [27].

As with USP17, there are several other DUBs (including DUB-1, DUB-1A, DUB-2, and DUB-2A), which belong to cytokine-inducible DUB subfamily members; these are involved in the regulation of cell proliferation and apoptosis in murine lymphocytes [1,9,19,20,137,138]. DUB-1, induced by IL-3, IL-5 and GM-CSF, is responsible for growth arrest in the G1 phase of the cell cycle [137]. Manual prediction of HABMs in DUB-1 shows the existence of eight HABMs within its sequence (Fig. 3A). DUB-2, induced by IL-2 in CTLL-2, has been found to express in T cells with constitutive activation of the IL-2 signaling pathway [139]. Cytokine withdrawal leads to an inhibitory effect on DUB-2-induced apoptosis in Ba/F3 cells [140]. Manual prediction of HABMs in DUB-2 also reveals eight HABMs within its sequence (Fig. 3A). Taken together, these cytokine-inducible DUBs, with high homology within the members of the DUB enzyme subfamily, may be involved in the HA signaling pathway. Further research is essential to reveal the relationship between HA and cytokine-inducible DUBs.

Based both on our recent studies on the way DUBs regulate apoptosis and on previous studies by other groups [22], we propose that most DUBs that show anti-proliferative behavior may contain HABMs within their sequence. As a result, we made an attempt to manually identify HABMs among those DUBs which are involved primarily in promoting apoptosis. Identification of HABMs on the DUBs which are involved in the negative regulation of cell viability showed several HABMs within its sequence (Fig. 3B). This is in agreement with our observations supporting the involvement of the HABMs of DUBs in the promotion of an apoptotic signaling pathway. Thus, the characterization of DUBs with HABMs is essential to a greater understanding of the molecular mechanism regulating cell viability. In conclusion, DUBs that contain HABMs can also be considered as viable targets for future anticancer therapy. This suggests that the development of DUB enzymes containing HABMs as a future therapeutic research objective.

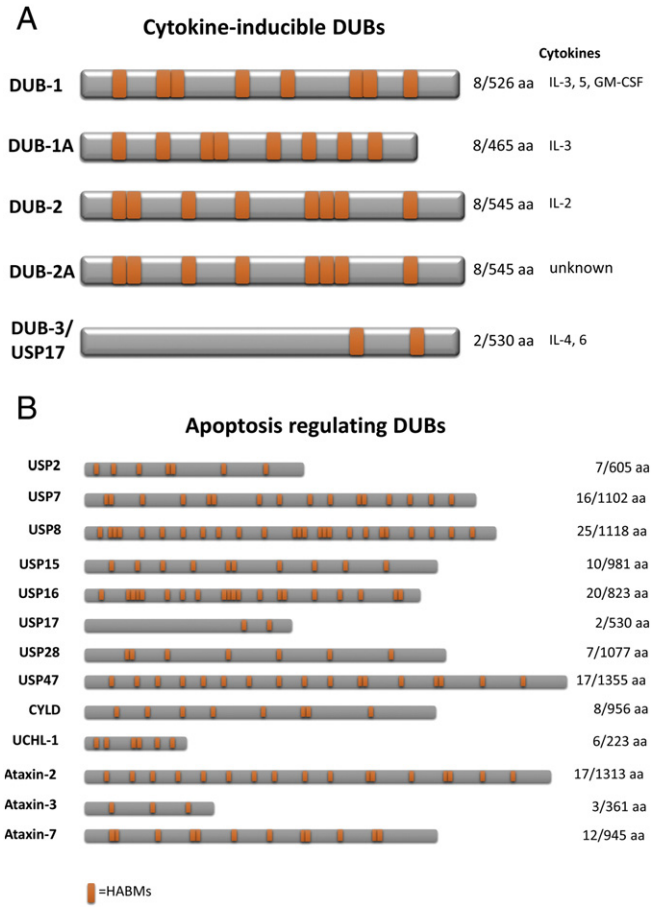

Fig. 3. (A) List of cytokine-inducible DUBs encoding several HABMs within its sequence. HABMs are represented in the red box. (B) List of apoptosis promoting DUBs encoding several HABMs within its sequence. HABMs are represented in the red box.

8. Deubiquitinating (DUB) enzymes as anticancer therapeutics

Post-translational modification by ubiquitin plays an essential role in numerous cellular functions such as protein degradation, cell cycle control, transcriptional regulation, immune response, apoptosis, oncogenesis, pre-implantation, and intracellular signaling pathways [7,9,20,22]. Deubiquitination, i.e., removal of ubiquitin from ubiquitin-conjugated protein substrates, is mediated by a number of DUB enzymes. The recently-discovered role played by DUB enzymes in cell viability suggests that regulation of deubiquitination in a cellular system could provide novel molecular drug targets for the treatment of cancer.

DUB-1 was identified as the first enzyme of the ubiquitin system to be associated with cytokine-regulated growth control [137]. Thereafter, a number of research studies have pointed out growth-suppressing activity in the ubiquitin–proteasome process. The most important discovery was cylindromatosis (CYLD), a tumor suppressor, which is mutated in familial cylindromatosis. The loss of CYLD inhibits apoptosis by activating NF-kB signaling [141]. CYLD directly deubiquitinates Bcl3, an oncogenic transcription factor, altering its nucleus localization and function. This suggests that CYLD could potentially block tumor formation by anti-proliferative and pro-apoptotic behavior [142].

USP7 has been shown to regulate several proteins that are mainly responsible for regulating cell cycle, apoptosis, tumor suppressors, and oncogenes [143–147]. The deubiquitinating action of USP7 on E3 ligase Hdm2 results in high levels of Hdm2 and low levels of the tumor suppressor p53 [148]. A mouse orthologue of herpes virus-associated ubiquitin-specific protease (HAUSP), a DUB enzyme, is also reported to be a regulator of apoptosis in cervical adenocarcinoma cells [149]. Thus, inhibiting the function of USP7 offers a promising strategy for cancer therapeutics. Recently, several research groups have been involved in developing functional USP7 inhibitors that act as antagonists of USP7 in cancer cell lines [143–147]. These inhibitors covalently bind to the active sites of USP7 resulting in the regulation of its substrates, which arrests growth in cancer cells [147]. In addition, virus-derived short peptides, such as vif1 and vif2, have been shown to act as USP7 antagonists. These peptides bind to the TRAF and catalytic domains of USP7 resulting in the suppression of its substrate binding ability and deubiquitinating activity [144]. Thus, USP7 inhibitors may also be a promising therapeutic for cancer.

In a similar way, there are several DUBs, such as USP28, USP2, UCHL1, and BAP1, which look to be promising candidates as tumor suppressors [21,23]. USP28 binds to 53BP1, an essential protein required for the stabilization of the Chk2-p53-PUMA repair pathway [150]. Thus, altering the USP28 expression level may act as a key regulator of DNA damage checkpoint. USP2 was found to act as a deubiquitinating enzyme for fatty acid synthase, which is found in elevated levels in prostate cancers [151]. USP2 shows a tendency to bind and deubiquitinate Mdm2 and thus suppress the expression of p53 [152]. As a result, development of an inhibitor for USP2 could suppress tumor progression. BAP1, a member of the DUB family, has been shown to directly upregulate the expression of BRCA1, a tumor suppressor in cancer cells [153]. Indeed, overexpression of BAP1 showed significant reduction in tumorigenecity in a mouse xenograft model [154]. UCHL1 is another DUB that shows high expression levels in several cancers [23,155,156]. Increased levels of UCHL1 are also associated with increased tumor

size or invasiveness [157]. Taken together, depending on various cell conditions, DUBs behave as oncogenes or tumor suppressors.

9. Conclusions

It is apparent that elevation of HA is a hallmark property of the malignant phenotype, including anchorage-independent growth and tumor invasion. Detailed mechanisms, whereby HA oligomers induce inhibition of tumor growth and perturbation of the HA interaction with CD44 leading to tumor suppression, and the way in which putative HA-binding motifs in the USP17 enzyme regulate apoptosis in tumor cells are necessary for the development of new approaches to cancer treatment using DUBs. However, recent advances in the characterization of the significant role played by HABMs in USP17 on antitumor activity have led us to predict a new way forward for cancer treatment.

The administration of HA oligomers and DUBs containing HABMs to cancerous cells may promote antitumor activity both by antagonizing endogenous HA interaction and by triggering apoptosis. The effect of exogenous DUBs encoding HABMs, and HA oligomers on cancerous cells probably relies on appropriate ratios between these two components to act as a potential inhibitor for tumorigenesis. In addition, it is known that the administration of HA oligosaccharides aborts oncogenic signaling and induces apoptosis, thereby reducing tumor progression [83,88,128]. Thus, a similar response can be expected when DUBs encoding HABMs are transfected into cancerous cells i.e. via competitive binding to endogenous HA.

Direct conjugations of HA with cytotoxic drugs such as butyric acid [121], paclitaxel [122–124] and doxorubicin [125] have been studied. An encouraging response from HA bioconjugates has led us to propose a possible therapeutic strategy for targeting tumors using a combination of conjugating polymers with DUBs containing HABMs to enhance the efficiency of tumor apoptosis. Interestingly, perturbation of HA signaling sensitizes multidrug-resistant cells [83,131]. Therefore, tumor targeting with HA shows great promise in cancer therapy, especially when tumor cells are treated with a combination of HA polymer conjugated with a cytotoxic drug and DUBs containing HABMs. This type of combined treatment for tumor cells offers many advantages, such as better drug solubilization, specific localization, controlled release, along with sensitizing chemoresistant cells, triggering apoptosis, and finally suppressing tumor growth. In conclusion, the application of DUBs containing HABMs may enhance the selective apoptosis of cancerous cells, while minimizing undesirable side effects and improving the quality of life for cancer patients.

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