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WJCO mainly publishes articles reporting research results and findings obtained in the field of oncology and covering a wide range of topics including art of oncology, biology of neoplasia, breast cancer, cancer prevention and control, cancer-related complications, diagnosis in oncology, gastrointestinal cancer, genetic testing for cancer, gynecologic cancer, head and neck cancer, hematologic malignancy, lung cancer, melanoma, molecular oncology, neurooncology, palliative and supportive care, pediatric oncology, surgical oncology, translational oncology, and urologic oncology.

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ORIGINAL ARTICLE

Basic Study

Tumor specifically internalizing peptide 'HN-1': Targeting the putative receptor retinoblastoma-regulated discoidin domain receptor 1 involved in metastasis

Frank-Un Hong, Miguel Castro, Klaus Linse

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Abstract

BACKGROUND

Less than 0.5% of intravenously injected drugs reach tumors, contributing to side effects. To limit damage to healthy cells, various delivery vectors have been formulated; yet, previously developed vectors suffer from poor penetration into solid tumors. This issue was resolved by the discovery of HN-1 peptide isolated *via* biopanning a phage-display library. HN-1 targets human head and neck squamous cell carcinoma (HNSCC) (breast, thyroid; potentially lung, cervix, uterine, colon cancer), translocates across the cell membrane, and efficiently infiltrates solid tumors. HN-1 peptide has been conjugated to various anticancer drugs and imaging agents though the identity of its receptor remained enigmatic.

AIM

To decipher the clues that pointed to retinoblastoma (Rb)-regulated discoidin-domain receptor 1 as the putative receptor for HN-1 is described.

METHODS

HN-1 peptide was synthesized and purified using reverse-phase high-performance liquid chromatography and gel electrophoresis. The predicted mass was confirmed by mass spectroscopy. To image the 3-dimensional structure of HN-1 peptide, PyMOL was used. Molecular modeling was also performed with PEP-FOLD3 software *via* RPBS bioinformatics web portal (INSERM, France). The immunohistochemistry results of discoidin domain receptor 1 (DDR1) protein were obtained from the publicly accessible database in the Human Protein Atlas portal, which contained the images of immunohistochemically labeled human cancers and the corresponding normal tissues.

RESULTS

The clues that led to DDR1 involved in metastasis as the putative receptor

mediating HN-1 endocytosis are the following: (1) HN-1 is internalized in phosphate-buffered saline and its uptake is competitively inhibited; (2) HN-1 (TSPLNIHNGQKL) exhibits similarity with a stretch of amino acids in alpha5 beta3 integrin (KLLITIHDRKEF). Aside from two identical residues (Ile-His) in the middle, the overall distribution of polar and nonpolar residues throughout the sequences is nearly identical. As HN-1 sequence lacks the Arg-Gly-Asp motif recognized by integrins, HN-1 may interact with an "integrin-like" molecule. The tertiary structure of both peptides showed similarity at the 3-dimensional level; (3) HN-1 is internalized by attached cells but not by suspended cells. As culture plates are typically coated with collagen, collagen-binding receptor (expressed by adherent but not suspended cells) may represent the receptor for HN-1; (4) DDR1 is highly expressed in head and neck cancer (or breast cancer) targeted by HN-1; (5) Upon activation by collagen, DDR1 becomes internalized and compartmentalized in endosomes consistent with the determination of 'energy-dependent clathrin-mediated endocytosis' as the HN-1 entry route and the identification of HN-1 entrapped vesicles as endosomes; and (6) DDR1 is essential for the development of mammary glands consistent with the common embryonic lineage rationale used to identify breast cancer as an additional target of HN-1. In summary, collagen-activated tyrosine kinase receptor DDR1 overexpressed in HNSCC assumes a critical role in metastasis. Further studies are warranted to assess HN-1 peptide's interaction with DDR1 and the therapeutic potential of treating metastatic cancer. Additionally, advances in delivery (conformation, endocytic mechanism, repertoire of targeted cancers of HN-1 peptide), tracking (HN-1 conjugated imaging agents), and activity (HN-1 conjugated therapeutic agents) are described.

CONCLUSION

The discovery of DDR1 as HN-1 peptide's putative receptor represents a significant advance as it enables identification of metastatic cancers or clinical application of previously developed therapeutics to block metastasis.

Key Words: HN-1 peptide; Solid tumor; Targeted drug delivery; Discoidin domain receptor 1; Tyrosine kinase; Metastasis

Core Tip: The side effects associated with current drugs are exacerbated by the accumulation of administered drugs in non-tumor tissues. To guide, various tumor-homing vectors have been developed though their delivery efficacy is limited by poor penetration into solid tumors. To resolve, the 'tumor specifically internalizing peptide' HN-1 was isolated *via* biopanning a phage-display library. HN-1 peptide targets human head and neck squamous cell carcinoma (breast, thyroid, potentially cervical, lung, uterine, colon cancer), translocates across the cell membrane and effectively penetrates solid tumors. Here, deciphering of the clues that pointed to discoidin domain receptor 1 as the putative receptor for HN-1 is described.

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INTRODUCTION

The genetic analysis of head and neck cancer has uncovered novel therapeutic targets. Head and neck cancer represents ~5% of all cancers diagnosed in the United States and the affected regions include the oral cavity, paranasal sinuses, pharynx, nasal cavity, larynx, thyroid gland, parathyroid gland, salivary gland, skin or cervical lymph nodes. Aesthetic loss or functional impairment (ex. difficulties with swallowing, speech, chewing) frequently accompany the disorder. Squamous cells constitute the mucosal membranes lining the lips, mouth, throat, breathing tubes, nose, eyelids, *etc.* and head and neck squamous cell carcinoma (HNSCC) (the second most common type of skin cancer) affects more men than women. Its mortality rate (~50%) has not changed significantly over the past several decades although early diagnosis may increase survival. Most HNSCC-associated deaths are caused by loco-regional recurrence or distant metastasis[1].

Epithelial carcinogenesis leading to head and neck cancer involves progression through multiple stages (from normal to hyperplasia to dysplasia to carcinoma to invasive carcinoma) accompanied by distinct genetic changes. Among the alterations are the loss of heterozygosity at chromosomal region 9p21 or 3p12-14 (squamous dysplasia, carcinoma *in situ*, invasive carcinoma), loss of heterozygosity at 9p21 or 3p14 (oral leukoplakia), and translocation between chromosomes 11 and 19 (mucoepidermoid carcinoma of the minor salivary gland). Microsatellite instability is observed in a subset of dysplastic, invasive, and aggressive lesions while aneuploidy is a frequent occurrence amongst HNSCC tumors[2].

The inactivation of retinoblastoma (Rb) function represents a critical step in HNSCC development. Briefly, following the identification of the prototypic tumor suppressor *RB* gene, its role in mammalian DNA damage checkpoint (in G1 or S phase) was elucidated[3]. Rb's ability to arrest at G1 is negatively regulated by distinct cyclin-dependent kinases (Cdks) through phosphorylation by cyclin D-Cdk4/6 at early G1 phase and cyclin E-Cdk2 late G1 phase[4,5]. Cdks are, in turn, negatively regulated by $p14^{Art}$, $p16^{INK4A}$, or other factors including $p21^{Cip1}$, which is transactivated by p53. The above 'Rb pathway' is dysregulated in nearly all human cancers and is also targeted by oncogenic viruses (ex. papillomavirus, adenovirus) to transform human cells. In HNSCC, the loss of $p16^{INK4A}$ is characteristic of hyperplasia and the loss of 3p21 and 17p13 (inactivates p53) is observed during progression to dysplasia. The amplification of the *CCND1* gene (encodes cyclin D) and the loss of 11q13, 14q32, or 13q21 is associated with carcinoma. The loss of 6p, 4q27, 8 or 10q23 as well as the inactivation of phosphatase and tensin homolog (PTEN) has been documented in invasive carcinoma[6]. In a significant fraction of HNSCC patients with poor prognosis, human papillomavirus DNA is found in tumors, whose gene product E7 inactivates Rb while E6 inactivates p53. Additionally, FAT1, NOTCH1, KMT2D, NSD1, TGFBR2, PIK3CA, and HER-2/neu are frequently upregulated in a subset of HNSCC.

The above advances have led to the development of targeted therapies, *i.e.*, Cdk4/6 inhibitor, epidermal growth factor receptor inhibitor, *etc*. However, even with targeted drugs, the problem of side effects persists. Furthermore, less than 0.5% of systemically administered drugs reach tumors, requiring a higher dose for treatment which exacerbates side effects[7]. To resolve, tumor-homing vectors are increasingly sought for targeted delivery of existing drugs. In the case of HNSCC, 'sac'-like structures contain tumor cells enclosed within the basement membrane. The parenchyma is comprised of tumor cells with intercellular spaces. A solid tumor may contain multiple such 'sacs' interspersed within the stroma. Stroma is a complex structure comprised of cytokeratin and normal cells, ex. fibroblasts, endothelial cells, immune cells. As such, an ideal delivery vector should be capable of penetrating these barriers to reach tumor cells located at the interior of the sac-like structures.

For targeted delivery, antibodies recognizing tumor-specific antigens such as CD20 (B-cell lymphoma), HER-2 (breast cancer), CD33 (acute myeloid leukemia), epidermal growth factor receptor have been developed. Anticancer agents conjugated to these antibodies include radioactive isotopes (non-Hodgkin's lymphoma) and chemotherapeutics, ex. doxorubicin, maytansine, and calicheamicin (acute myelogenous leukemia). Alternatively, larger molecules such as polyethylene glycol have been used as they can escape through leaky tumor vasculature to deposit on a tumor. The leakage occurs primarily at venules and small veins that are lined by a continuous endothelium. Their exiting route consists of a system of vesiculo-vacuolar organelles, cell junction, or endothelial fenestrae. Previously, polyethylene glycol has been conjugated to taxol or tumor-specific antibodies. Also, liposomes have been utilized as they can accumulate at the tumor due to enhanced permeability and retention. Liposomes deliver drugs *via* fusion, destabilization of the membrane or endocytosis. Liposomes conjugated to polyethylene glycol exhibit greater stability due to lesser removal by the reticuloendothelial system[8]. Pegylated liposomes containing doxorubicin or cisplatin or liposomes coupled to tumor-targeting molecules have been developed.

However, the above delivery vectors face several limitations. For antibody vectors, its preparation or purification is complex due to the relative ease with which antibodies can denature. Other potential issues are immunogenicity (requires humanization) and the lack of internalization by the cancer cells. Poor penetration of solid tumors by antibodies further limits their use and may contribute to a higher level of drugs in circulation contributing to toxicity, ex. Herceptin antibody penetrates mere 38 micrometers after exiting blood vessels[9]. For these reasons, its use has been limited to lymphocytic cancers. For minute micrometastasized tumors that rely on nutrients obtained from adjacent blood vessels *via* diffusion, nanoparticles such as polyethylene glycol or liposome-based vectors that escape through leaky tumor vasculature may find little utility. The use of liposomes is delimited by the inefficient release of the enclosed drugs[10]. Overall, inefficient penetration of solid tumors has been a major issue with larger nanoparticles. As the interior of solid tumors may harbor hypoxic regions that give rise to refractory metastatic cancer cells to which patients ultimately succumb, the need to develop delivery vectors with greater infiltrating potential remains a priority[11].

The above issues have been largely resolved through the discovery of the prototypic 'tumor specifically internalizing peptide' HN-1, which was reported in Cancer Research in 2000[12]. HN-1 peptide is unique as it provided multiple advantages including tumor-selectivity for HNSCC, the capacity to translocate across the cell membrane, and the ability to penetrate solid tumors to reach cancer cells located within the sac-like structures. Since its discovery, HN-1 has been conjugated to various agents for both cancer therapy (taxol, doxorubicin, protein kinase C inhibiting peptide, ribonucleotide reductase inhibiting siRNA, diphtheria toxin, polyethylene glycol linked to doxorubicin,

graphene oxide nanoparticle-containing doxorubicin) and imaging (gamma-ray emitting isotopes for radiotherapy, near-infrared fluorescent dyes for surgical navigation) (see below). Recently, the target of HN-1 has been extended to breast cancer, thyroid cancer, and potentially cervical, lung, uterine, or colon cancer. Here, we describe the deciphering of clues that helped to unravel a 20 year-old enigma regarding the identity of the receptor mediating HN-1 endocytosis. Discoidin domain receptor 1 (DDR1) is a collagen tyrosine kinase overexpressed in head and neck cancer (also breast cancer), and the transcription of *DDR1* gene is regulated by the RB-interacting protein E2F[13]. DDR1 differs from conventional tyrosine kinases as it is activated by collagen, and plays a critical role in metastasis by facilitating invasion or migration, promoting epithelial-to-mesenchymal transition, reactivating previously disseminated metastasis-initiating cancer cells, *etc.* As such, it has become a novel therapeutic target of high importance for the pharmacological management of metastatic cancers (see below).

MATERIALS AND METHODS

Peptide

HN-1 peptide was synthesized and purified using reverse-phase high-performance liquid chromatography to attain ~95% purity. For *in vivo* application, it was further purified using gel electrophoresis. The predicted mass was confirmed by mass spectroscopy.

Molecular modeling

PyMOL (Python-enhanced molecular graphics) was used to image the 3-dimensional structure of the HN-1 peptide. Additional molecular modeling was performed using PEP-FOLD3 software *via* RPBS (Ressource Parisienne en Bioinformatique Structurale) bioinformatics web portal (INSERM, France) (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/)[14,15].

Immunohistochemistry

The immunohistochemistry images of DDR1 protein in a panel of human head and neck cancer specimens *vs* normal human oral mucosa were obtained from the publicly accessible database in the Human Protein Atlas portal (http://www.proteinatlas.org/). It contained the images of immunohistochemically labeled human cancers and the corresponding normal tissues.

RESULTS

Structure of HN-1 peptide

F. Hong (a.k.a. Frank Un, Frank D. Hong)'s contributions to the *RB* gene field was previously described [16]. Briefly, for cancer biology and genetics, his works include the identification of the human *RB* gene and its sequence containing cyclin-dependent kinase recognition motifs[3], determination of RB genome structure including the promoter[17] and its mutant in prostate cancer[18], the discovery of the DNA binding property of Rb protein indicative of its function as a transcription factor[19], uncovering of RB-to-RB self-interaction to form higher-ordered structures implicating its role in epigenetics, DNA replication, histone modification, heterochromatin, DNA condensation, *etc*[20-23]. For cancer therapy, his works concerned elucidating the cytotoxic mechanism of anticancer drugs to solve the side effects or drug resistance problem. He discovered tumor-specific lytic path 'hyperploid progression mediated death' targeting G1 DNA damage checkpoint-defective RB or p53 mutants induced by antimicrotubule drugs (ex. Taxol)[24], RB's role as the mediator of DNA crosslinking drug cytotoxicity (ex. cisplatin) in G1 checkpoint retaining human cancers[25], and the reversal of drug resistance to antimetabolite drugs (ex. hydroxyurea) *via* attenuating the Rb-associating protein ICBP90 (UHRF1)[26].

Following the identification of the human *RB* gene, W. Lee (University of California at San Diego, La Jolla, United States) collaborated with T. Friedmann (University of California at San Diego, La Jolla, United States) to develop a recombinant retrovirus expressing human *RB* or *p53* gene during 1988-1993. The retrovirally expressed RB or p53 suppressed the formation of tumors derived from human retinoblastoma, breast, or prostate cancer cells in murine xenograft models[27-29]. These works led to gene therapy clinical trials testing the efficacy of p53 expressing retrovirus or adenovirus in non-small cell lung cancer patients by J. Roth (University of Texas M. D. Anderson Cancer Center, Houston, United States) in 1996 and HNSCC patients by G. Clayman (University of Texas M. D. Anderson Cancer Center, Houston, United States) in 1998[30,31]. The clinical trial was conducted in collaboration with W. K. Hong (University of Texas M. D. Anderson Cancer Center, Houston, United States), who pioneered the chemoprevention of cancer[32]. Despite the therapeutic gains made in gene transduction, gene expression, and clinical response, further improvement was necessary regarding tumor specificity, solid tumor penetration, and *in vivo* stability.

The side effects occurred as the RB or p53 expressing recombinant virus indiscriminately infects both cancer and normal cells. To provide tumor specificity, F. Hong sought to identify a human HNSCC-specific peptide to be displayed on the surface of viral vectors. Earlier In 1984-1985, he used M13 single-stranded bacteriophage to perform site-directed mutagenesis at the Salk Institute (Molecular Biology & Virology Laboratory). Later, in 1993-1995, he worked with normal human fibroblasts while studying the mechanism of aging at the Salk Institute (Neurobiology Laboratory). During 1998-2000, F. Hong screened M13 bacteriophage-displayed random peptide libraries (2.5×10^{12} random peptides) using live cancer cells at the University of Texas M. D. Anderson Cancer Center[12]. Filamentous phages displaying peptides fused to coat proteins were designed by G. Smith (Nobel prize, 2018). The biopanning involved 5 successive rounds of selection using human HNSCC cells, followed by 3 cycles of subtraction using normal human fibroblasts in the presence of serum to ensure stability *in vivo*. It led to the discovery of HN-1 peptide (TSPLNIHNGQKL; ~1.2 kDa) that meets multiple criteria for targeted drug delivery into solid tumors: (1) Translocates drugs across the cell membrane into the cytosol; (2) Tumor specifically internalized; and (3) Capable of penetrating solid tumors. Further, HN-1 peptide is nontoxic, nonimmunogenic, stable *in vivo*, transports payload efficiently within 48 h, and does not trigger biological responses.

Recent researches increasingly indicate the dynamic nature of protein structures, *i.e.*, 'intrinsically disordered protein'[33]. Despite the conformational flexibility, they can adopt a fixed or rigid structure upon recognizing the interacting target. Such properties have been harnessed by peptides, which provide further advantages over antibodies or small molecules for pharmaceutical treatment, resulting in the approval of > 60 peptide drugs worldwide in the last two decades. In the case of HN-1, computational modeling predicted several structural conformations. A 3-dimensional model depicting HN-1 peptide in a beta-sheet configuration is shown (Figure 1A). Alternate conformations of HN-1 peptide predicted by the PEP-FOLD modeling program are also shown (Figure 1B). Finally, a structural model consisting of a gamma-turn is shown (Figure 1C).

DDR1 may mediate HN-1 endocytosis

The treatment issue of recurrent metastatic cancer refractory to current drugs remains unresolved for HNSCC as well as breast cancer. The dysregulation of the extracellular matrix dynamics is thought to play a significant role in metastasis. The extracellular matrix is comprised of glycoproteins, proteoglycans, and other proteins that function in biomechanics, cell motility, growth factor reservoir, cell-to-cell communication, *etc.* An early event in tumor development involves neovascularization (for nutrient, oxygen, excretion), which requires the outgrowth of blood vessels *via* endothelial branching mediated by tip cells and stalk cells that depend on the extracellular matrix. The extracellular matrix also attracts immune cells and activates them to release cytokines and proteases (causes tumor-associated inflammation) while suppressing the activation of macrophages that lyse cancer cells. The dysregulated extracellular matrix exhibits altered activities of remodeling enzymes (ex. collagenase or metalloprotease secreted by stroma), excess deposition of the matrix components (ex. collagen types I, IV or XVII by tumor cells, cancer-associated fibroblasts or tumor-associated macrophages in colorectal cancer; heparan sulfate proteoglycan), stiffness through the crosslinking by lysyl oxidase, *etc.* Distinct collagen types found in the microenvironment of various cancers have been compiled, *e.g.*, type I (head and neck squamous cell carcinoma, non-small cell lung cancer, breast cancer), type III (breast cancer), type IV (oral squamous cell cancer, colorectal cancer)[34]. Other changes include the altered orientation (linearization) of collagen fibers, which facilitates tumor cell migration (for invasion into adjacent tissues following the breakdown of basement membrane by matrix metalloproteinase)[35]. Further, the extracellular matrix is involved in the development of lymphatic vessels that serve as a conduit (in addition to the leaky tumor vasculature) for metastasis.

Discoidin domain receptors DDR1 and DDR2 are cell membrane-associated receptors with tyrosine kinase activity, whose extracellular domain resembles discoidin of *Dictyostelium discoideum*. DDR1 is activated by most collagen types including I and IV (abundant in the basement membrane) whereas DDR2 is activated by fibrillar collagen types I, III, and X but not II or IV[36]. Discoidin domain receptors regulate cell adhesion, growth, polarity, and migration through sensing extracellular matrix and interacting with TGF-beta, Notch, or adhesive receptors for signaling. The stromal-epithelial interaction mediated by DDR1 is essential for the normal development of mammary glands in mice[37]. DDR1 promotes cancer progression by facilitating the migration of squamous cell carcinoma cells[38], bone metastasis by lung cancer[39], lung metastasis by breast cancer[40], stroma-induced peritoneal metastasis of gastric cancer[41], and tissue invasion by metastatic colorectal cancer cells[42]. Mechanistically, activation of DDR1 by collagen induces matrix metalloproteinase to degrade extracellular matrix [43]. DDR1 overexpressed in oral squamous carcinoma is involved in angiolymphatic invasion[44]. DDR1 is involved in invadosome formation *via* collagen-mediated activation of Rho-GTPase Cdc42[45]. DDR1 facilitates the invasion of a collective group of tumor cells by modulating actomyosin contractility at the cell-cell contacts[38]. Collagen type IV was shown to activate DDR1 to induce migration of breast cancer cells[46]. The epithelial-to-mesenchymal transition represents a critical step for metastasis and is triggered by extracellular matrix molecules or growth factors. The triggering by collagen type I is mediated by DDR1, whose signaling is transduced by proline-rich tyrosine kinase 2 to upregulate N-cadherin[47]. The DDR1 expression positively correlates with epithelial-to-mesenchymal transition in

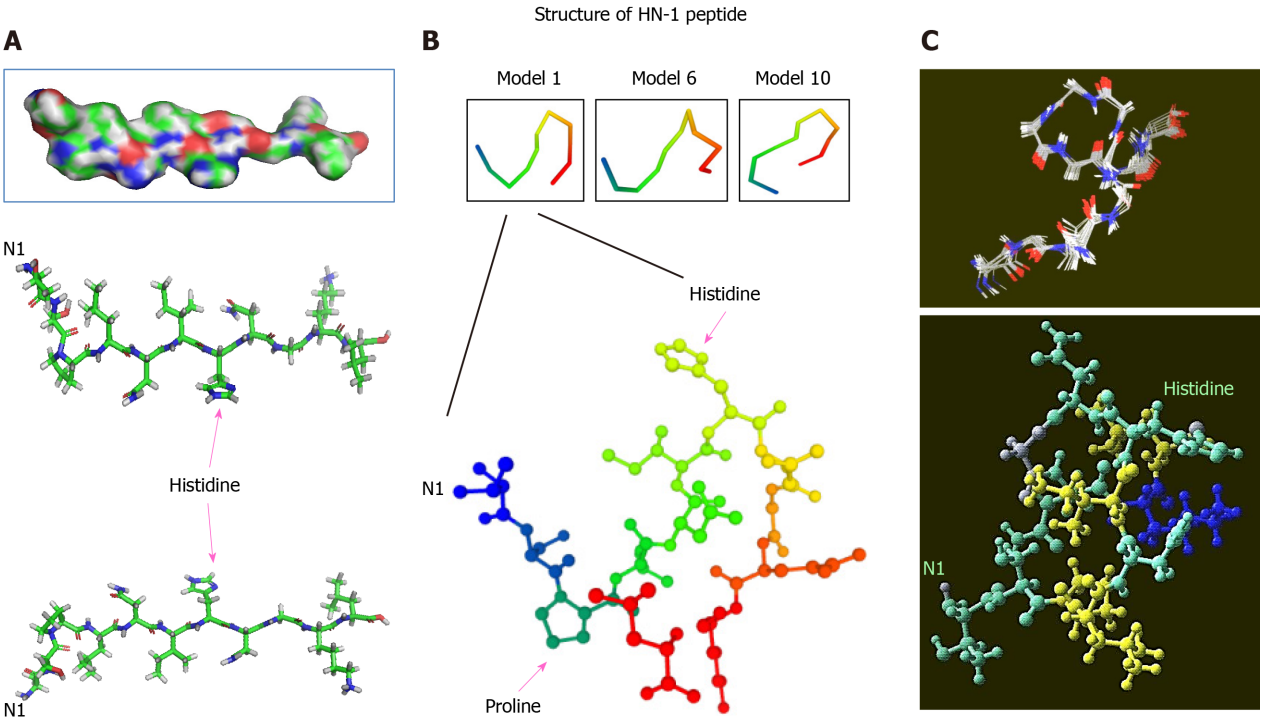

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Figure 1 Three-dimensional structure of HN-1 peptide. A: A 3D model of HN-1 peptide generated using PyMol molecular graphics system, version 1.2r3pre, Schrödinger, LLC. All graphics depict an identical configuration with the bottom two panels in the opposite orientation; B: An ensemble of *de novo* conformations generated by PEP-FOLD (INSERM, France) in RPBS bioinformatics web portal: https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/; C: A 3-dimensional profile of the lowest energy structure obtained for HN-1 peptide was viewed using Raswin computer modeling software. All structures were generated using TSPLNIHNGQKL as the raw input peptide sequence. N1: The N-terminal residue.

squamous cell carcinoma, breast cancer, ovarian cancer, and hepatoma[48]. Additionally, DDR1 (activated by collagen type I) functions in the reactivation of previously disseminated metastasis-initiating cancer cells after dormancy[13,40].

Rb's tumor-suppressing function extends to the genetic regulation of DDR1 to inhibit metastasis. Through associating with E2F, Rb inhibits the transactivation of DDR1 by E2F[49]. The transcription of DDR1 is also regulated by p53 of the 'Rb pathway'[50]. The human *DDR1* gene consists of 17 exons, which encode an extracellular domain (discoidin domain for ligand binding), a transmembrane domain, and an intracellular domain (tyrosine kinase). Alternative splicing generates multiple isoforms, which include DDR1c encoding the full-length receptor, DDR1b lacking 6 residues (between exon 13 and 14), DDR1a lacking these and additional 37 residues (juxtamembrane region), and DDR1d plus DDR1e (both tyrosine kinase-deficient due to C-terminal truncation)[48,51]. DDR1 remains a dimer (N-glycosylated) without the bound ligand. Binding to collagen activates DDR1 *via* clustering, causing autophosphorylation to initiate signaling[43,52]. DDR1 is overexpressed in head and neck, esophagus, lung, breast, ovarian, prostate, and brain cancers in addition to leukemia and lymphoma. DDR1 is also upregulated in osteosarcoma, endometrial cancer, primary central nervous system lymphoma, and liver cancer[13]. It correlated with a reduced overall survival (*e.g.*, metastatic colorectal cancer)[36] and a poor prognosis (*e.g.*, non-small cell lung cancer) for most cancers. Figure 2 shows the overexpression of DDR1 in human head and neck squamous cell carcinoma.

Multiple data indicate that the entry of HN-1 peptide is mediated by DDR1. First, HN-1 is internalized in phosphate-buffered saline (lacking the components of fetal bovine solution); plus, its uptake is competitively inhibited, indicating that specific interaction with cell surface receptor is necessary for its uptake[12]. Second, a high degree of similarity was discovered between HN-1 (TSPLNIHNGQKL) and a stretch of amino acids in alpha5 beta3 integrin (KLLITIHDRKEF). Aside from two identical residues (Ile-His) in the middle, the overall distribution of polar and nonpolar residues throughout the sequences was nearly identical (Figure 3A). The tertiary structure of both peptides exhibited similarity at the 3-dimensional level (Figure 3B). The found motif represents the membrane-proximal sequence of the beta subunit's cytoplasmic tail that interacts with the alpha subunit's cytoplasmic tail, whose disruption changes the conformation of the extracellular domain to engage the ligand[53,54]. As the HN-1 sequence lacks the Arg-Gly-Asp motif recognized by integrins, HN-1 may interact with an "integrin-like" molecule (instead of integrin). Third, HN-1 is uptaken by attached cells but not by suspended cells[55]. As tissue culture plates are typically coated with collagen (type I or IV), it suggests that collagen-binding receptor (expressed by adherent but not suspended cells) may represent the receptor for HN-1. Fourth, DDR1 is overexpressed by head and neck cancer or breast cancer targeted by HN-1. Fifth, upon

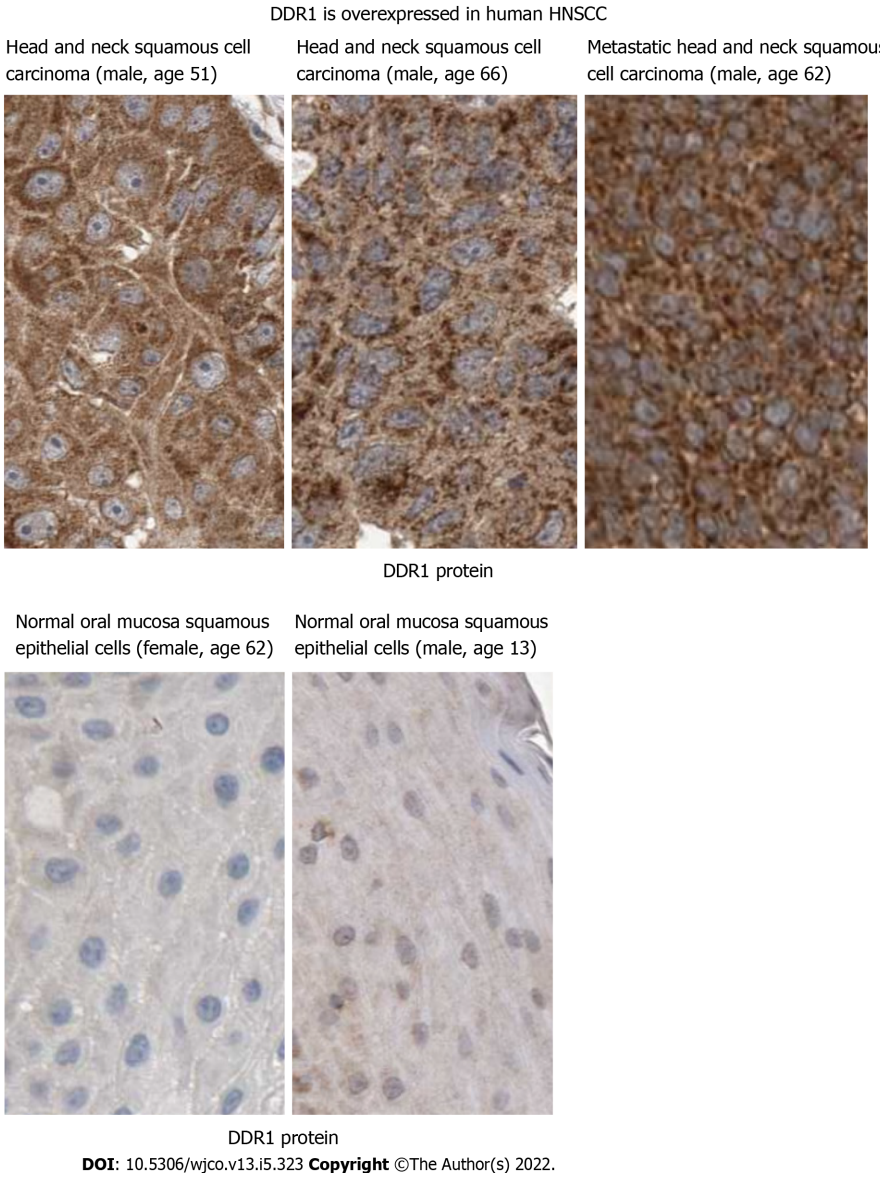

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Figure 2 Discoidin domain receptor 1 may mediate HN-1 endocytosis. Discoidin domain receptor 1 (DDR1) protein is upregulated in human head and neck squamous cell carcinoma. Immunohistochemical analysis of DDR1 was conducted by comparing tumor *vs* normal tissues in Human Protein Atlas database (http://www.proteinatlas.org/). The results showed positive cytoplasmic and membranous staining. Bar: 25 micrometers; DDR1: Discoidin domain receptor 1.

activation by collagen, DDR1 becomes internalized and compartmentalized in endosomes[56], which is consistent with the identification of energy-dependent clathrin-mediated endocytosis as the entry route of HN-1[57] and the determination of HN-1 entrapped vesicles as endosomes[38]. Sixth, DDR1 is essential for the development of mammary glands[37] in keeping with the common embryonic lineage rationale used to identify breast cancer as an additional cancer target of HN-1[58]. Taken together, these results suggest that DDR1 may mediate the endocytosis of the HN-1 peptide.

DISCUSSION

HN-1 peptide is preferentially internalized by cancer cells. HN-1 was internalized by almost all human HNSCC cell lines examined to date (MDA177Tu, MDA138Tu, MDA59Tu, MDA167Tu, MDA686Tu, MDA1986Tu, UMSCC1, UMSCC36)[12,59]. UMB-SCC-745, UT-SCC-36, UT-SCC-38[60], SCC-25, Detroit 562[61], CAL-27, and SCC-25 human HNSCC cells[62] also internalized HN-1. Recently, the uptake of HN-1 by human oral squamous cell carcinoma SCC-25 and CAL-27 cells was reported[63]. Further, HN-1 was selectively internalized by human head and neck squamous cell carcinoma derived SCC4, SCC9, and CAL27[57]. Little uptake of HN-1 was observed with MDA182Tu cells. Additionally, HN-1 peptide was internalized by human pharynx squamous cell carcinoma FaDu cells used in the mouse xenograft model study[64]. *In vivo,* intravenously administered HN-1 selectively localized to human HNSCC-

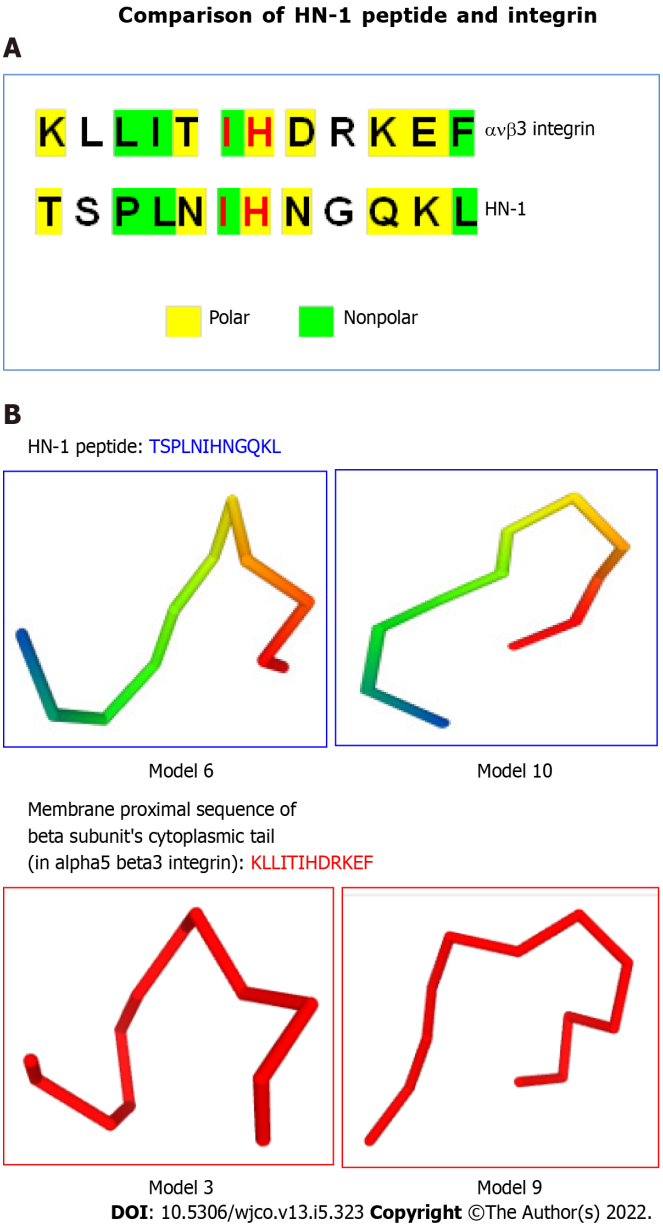

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Figure 3 HN-1 peptide exhibits similarity to integrin peptide. A: The similarities between the HN-1 sequence (TSPLNIHNGQKL) and a stretch of amino acids in alpha5 beta3 integrin (KLLITIHDRKEF) are highlighted. As HN-1 peptide lacks the recognition motif (RGD) of integrin, HN-1 may interact with an "integrin-like" molecule. HN-1 is internalized by attached cells but not by suspended cells. As tissue culture plates are typically coated with collagen, collagen-binding DDR1 receptor (expressed by adherent but not suspended cells) may represent the receptor for HN-1 consistent with that DDR1 is overexpressed in human head and neck cancer (also breast cancer) targeted by HN-1. avb3: alpha5 beta3; B: Comparison of 3D models generated by PEP-FOLD (INSERM, France) in RPBS bioinformatics web portal: https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/ TSPLNIHNGQKL: HN-1 peptide (top panels); KLLITIHDRKEF, membrane proximal sequence of beta subunit's cytoplasmic tail in alpha5 beta3 integrin (bottom panels); TSPLNIHNGQKL: Thr-Ser-Pro-Leu-Asn-Ile-His-Asn-Gly-Gln-Lys-Leu; KLLITIHDRKEF: Lys-Leu-Leu-Ile-Thr-Ile-His-Asp-Arg-Lys-Glu-Phe.

derived tumors in a mouse xenograft model[12,57,59,62,65]. In contrast, HN-1 was poorly uptaken by their normal counterpart (human oral keratinocytes HOK16B, NOE human normal oral epithelial cells, NHDF normal human dermal fibroblasts)[12,58,59].

Recent works have uncovered additional cancer types targeted by HN-1 peptide (Figure 4A). HN-1 uptake was also observed with human large cell lung carcinoma H460a cells[64]. Also, the HN-1 derived peptide HN17 (contains rearranged HN-1 sequence) was internalized by MZ-CRC 1 and TT human thyroid cancer cells[66]. HN-1 was also shown to target breast cancer irrespective of their 'triple status'. HN-1 or HN-1^{TYR} (HN-1 with two extra tyrosine residues added N-terminally) was internalized by MDA-MB231, SKBR3, MDA-MB-468, ZR-75-1, or MCF-7 breast cancer cells while MCF10A nontumorigenic mammary epithelial cells exhibited little HN-1 uptake[58,59]. Additionally, HN-1 was internalized by MDA-MB-435, MDA-MB-231, and MTLn3 breast cancer cells[64]. Further, HN-1 was internalized by KB cells[58] (originally human oral epidermoid carcinoma cells but subsequently found to contain HeLa human cervical adenocarcinoma cells)[67], raising the prospect that HN-1 may target

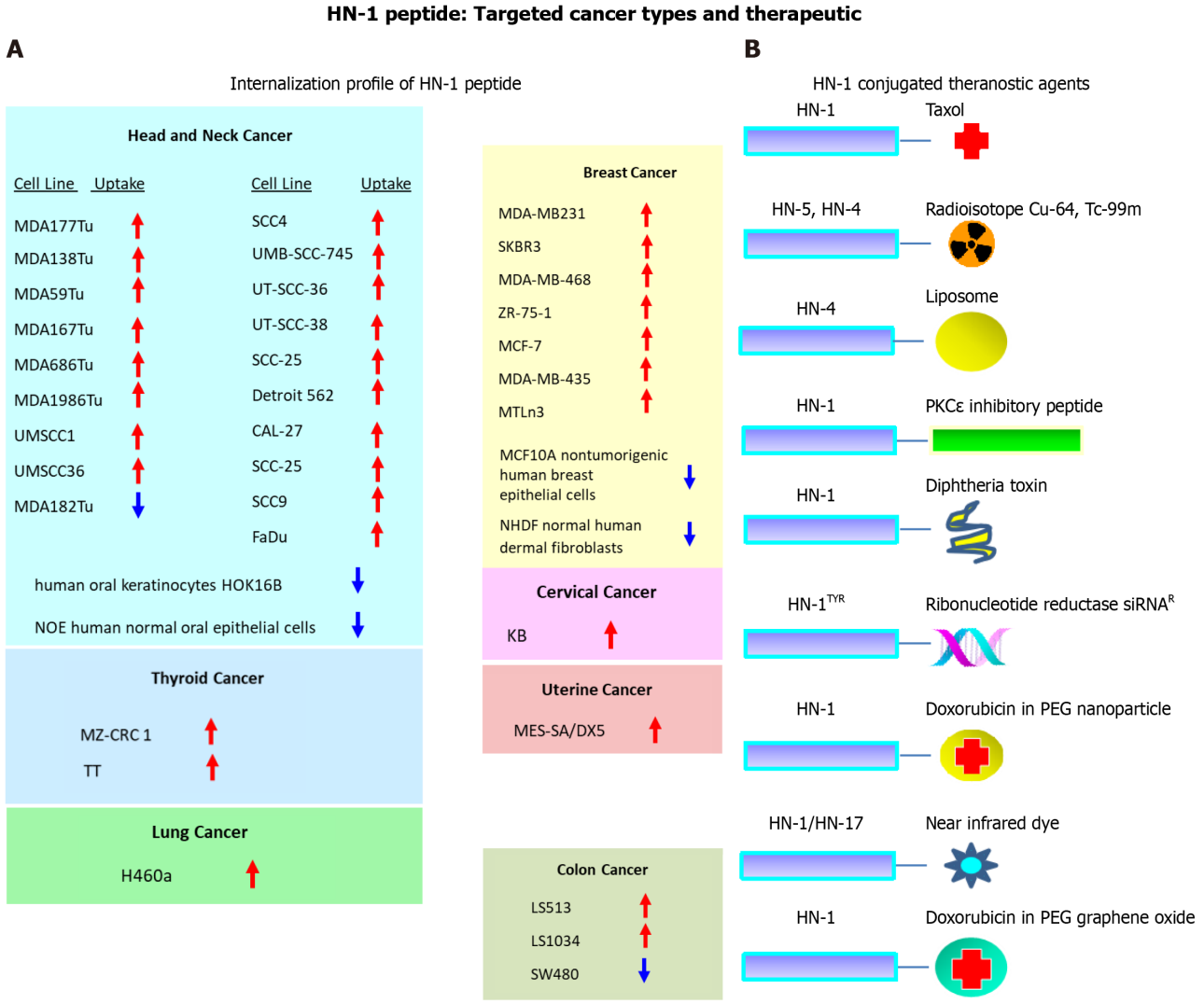

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Figure 4 The repertoire of targeted cancers and therapeutic application of HN-1 peptide. A: HN-1 uptake profile. The internalization of HN-1 peptide by various human cancer cells *vs* the corresponding normal human cells was compared. HN-1 uptake: red arrow (internalized), blue arrow (undetectable); B: Previously developed HN-1 based conjugates for therapy or diagnosis. Note: whether HN-17 peptide (consisting of a permutated version of HN-1 sequence) enters cells *via* the same route as HN-1 peptide or through a distinct route is not known. HOK: Human oral keratinocyte; NOE: Normal oral epithelial; NHDF: Normal human dermal fibroblasts; Cu: Copper; Tc: Technetium; PKCe: Protein kinase C epsilon; siRNA: Small interfering RNA; PEG: Polyethylene glycol.

cervical cancer. For uterine cancer, HN-1 uptake by MES-SA/Dx5, a multi-drug resistant cell-line derived from the human uterine sarcoma cell line MES-SA, was documented[64]. For gastrointestinal cancer, HN-1 uptake was observed with human colorectal carcinoma LS513 and LS1034 cells[64].

HN-1 peptide translocates across the cell membrane to facilitate drug delivery. Upon entry, HN-1 is localized in punctate particles, which were subsequently identified as endosomes[57,60]. Multiple reports suggest that HN-1 is internalized *via* the 'receptor-mediated endocytosis': (1) The internalization of HN-1 is 'specific' (based on competition assay); (2) HN-1 can be uptaken in a serum-free medium (*i.e.*, its receptor is cell-associated); (3) HN-1 is compartmentalized in endosomes after entry; and (4) Fusing the translocation domain of diphtheria toxin allows HN-1 to escape from the endosome into the cytosol. Using inhibitors of various entry routes (receptor-dependent endocytosis, pinocytosis, simple transmembrane diffusion, caveolae-mediated pathway), HN-1 uptake was shown to be mediated by 'energy-dependent clathrin-mediated endocytosis'[57].

HN-1 efficiently penetrates tumor mass, which is significant as > 90% of human cancers are comprised of solid tumors. The intravenously injected HN-1 was localized at the interior of solid tumors derived from MDA177Tu or MDA167Tu human HNSCC cells in a mouse xenograft model[12]. An independent report showed that the intravenously injected HN1 penetrates solid tumors derived from UMSCC1 human HNSCC cells *in vivo*[59]. Using radiolabeled HN-5 peptide, a derivative of HN-1, its penetration into solid tumors formed by SCC4 human HNSCC cells was documented using a nude rat model[57]. Further, nanoparticles displaying HN-1 peptide effectively penetrated solid tumors derived from human oral squamous cell carcinoma SCC-25 cells in a murine xenograft model[62]. Through these

works, the pharmacological properties of HN-1 have been confirmed globally.

The unique properties of HN-1 have been exploited to develop novel anticancer therapeutics (Figure 4B). In 2009, Henri *et al*[64] (Tapestry Pharmaceuticals, Boulder, United States) developed targeted chemotherapy comprised of HN-1 peptide and taxol. Taxol is an antimicrotubule drug that induces lethality by triggering 'hyperploid progression mediated death' in G1 DNA damage checkpoint defective human cancer cells due to mutant *Rb* or *p53* gene[16,24,68]. The authors delineated a series of complex organic chemistry synthesis steps to conjugate HN-1 to taxol without affecting the latter's antimicrotubule property. Using a murine xenograft model derived from human head and neck cancer FaDu cells, the authors demonstrated that the HN-1 peptide-to-taxol conjugate completely suppresses the growth of tumors. Further, it exhibited synergy with a second chemotherapeutic agent against breast, lung, uterine, or colon cancer cells. Of significance, the combined regimen increased growth inhibition from < 15% (by a single agent) to ~80% against breast cancer cells. For drug resistance, the conjugate showed activity against multi-drug resistant human uterine sarcoma cells.

In 2007, Zheng (University of Texas Health Science Center, San Antonio, United States) developed HN-5 (CYTSPLNIHNGQKL), a derivative of HN-1 peptide containing one cysteine and one tyrosine residue at the N terminus[57]. Using HN-5 peptide radiolabeled with Cu-64 gamma-ray emitter, human HNSCC cell-derived tumor xenograft in a nude rat model was successfully imaged *via* positron emission tomography. HN-5 peptide exhibited specificity and affinity to HNSCC and exhibited a high diffusion capacity within a solid tumor. More significantly, by conjugating to HN-5, a significant reduction in the accumulation of unconjugated Cu-64 radioisotopes in the liver, heart, kidney, lung, and small intestine was observed. Subsequently, in 2013, Nordquist (University of Texas Health Science Center, San Antonio, United States) quantitatively documented selective accumulation of the radiolabeled HN-5 peptide in HNSCC-derived tumor xenografts. Of relevance, HN-4 peptide linked to the radioisotope Tc-99m has been presented at the 2007 American Association for Advancement of Science annual meeting.

In 2008, the HN-4 peptide was used to develop a human HNSCC-targeting liposome for targeted drug delivery. The components used to construct the liposomes included the lipids DPPC, DMPG, and PEG. The synthesis involved the preparation of lipid film, reconstitution in sucrose solution, and extrusion to limit particle size to 100 nm, which was presented at the 2008 Texas Science & Engineering Fair.

In 2010, Bao *et al*[59] (Ohio State University Medical, Center, Columbus, United States) developed HN1-PKC(epsilon), a capped bi-functional peptide comprised of HN-1 peptide and PKC(epsilon)-inhibitory peptide connected through a linker. The bifunctional inhibitory peptide was selectively internalized by human HNSCC cells and suppressed the growth of HNSCC xenograft in nude mice. The report also extended the targets of HN1 peptide to breast cancer. Of clinical significance, whereas cisplatin alone inhibited the growth of HNSCC cells by 49%, combining cisplatin with the above bi-functional peptide suppressed growth by 72%.

In 2011, Potala *et al*[60] (Indian Institute of Technology Madras, Chennai, India) developed a novel fusion toxin comprised of HN-1 peptide and diphtheria toxin. The fusion toxin displayed a high degree of selectivity towards HNSCC and exhibited IC_{50} of 1-5 nM (nanomolar). They characterized 'energy-dependent clathrin-mediated endocytosis' as the HN-1 entry route and identified the intracellular punctate particles containing internalized HN-1 peptides as endosomes. The authors also found that the internalized fusion toxin utilized the translocation domain (of diphtheria toxin) to gain entry into the cytosol from endosomes.

In 2011, Dudas *et al*[55] (Medical University Innsbruck, Innsbruck, Austria) reported that the pharmacological properties of HN-1 peptide could be recapitulated using distinct human HNSCC cells. Further, the authors reported a seminal finding that the binding of HN-1 peptide to human HNSCC cells occurred in attached but not suspended cells[55]. It suggested that the expression level of the cognate cell surface receptor for HN-1 is governed by the presence of an extracellular matrix to which it adheres, providing an important clue for the discovery of DDR1 as the putative receptor for HN-1 peptide (see above).

In 2012, for gene therapy, Un *et al*[58] (Beckman Research Institute of City of Hope National Medical Center, Duarte, United States) developed a therapeutic conjugate composed of HN-1(Tyr) and a siRNA targeting human ribonucleotide reductase subunit M2. HN-1(Tyr) contains two tyrosine residues added N-terminally to the HN-1 peptide[58]. The siRNA(R) resistant to RNase degradation was designed by J. Rossi (City of Hope National Medical Center, Duarte, United States)[69] and was able to suppress endogenously expressed ribonucleotide reductase *in vivo* after delivering in a nanoparticle constructed by M. Davis (California Institute of Technology, Pasadena, United States)[70]. The clinical trial involving melanoma patients was conducted by Y. Yen (City of Hope National Medical Center, Duarte, United States), a former colleague of J. Doroshow (National Cancer Institute, Bethesda, United States). For RNA interference therapy, the HN-1(TYR)-anti-hRRM2 siRNA(R) construct moderatly suppressed the endogenously expressed ribonucleotide reductase M2 subunit in breast cancer cells.

In 2017, Wang *et al*[62] (Tianjin Medical University, Tianjin, China) constructed a nanoparticle comprised of polyethylene glycol *via* self-assembly, which contained the chemotherapeutic doxorubicin. Doxorubicin's primary anticancer activity is associated with its propensity to intercalate with double-stranded DNA and inhibit topoisomerase II, and it has been used to treat leukemia, breast, lung,

bladder, and other cancers. The nanoparticle was further modified to display HN-1 peptide at the exterior[62,71]. The spherical construct (~150 nanometers) exhibited HNSCC-specific uptake and cytotoxicity. *In vivo,* HN-1 peptide endowed greater HNSCC targeting capacity and tumor penetrating potential to the PEGylated nanoparticle. Whereas unconjugated doxorubicin remained at the periphery, HN-1 Linked nanoparticle readily infiltrated to deeper regions of the tumor. The clinical use of doxorubicin is hampered by side effects-especially, cardiac damage. Intriguingly, a significantly lower amount (~1/15 fold) of doxorubicin accumulated in the heart and other organs (ex. liver, kidney, spleen, lung) after administering the construct in a murine xenograft model.

In 2017, Rossfeld *et al*[66] (Arthur G. James Comprehensive Cancer Center and Ohio State University, Columbus, United States) developed a conjugate comprised of the near-infrared dye IRdye800 and HN-17 (TLPNSNHI KQGL), a derivative of HN-1 peptide, for intraoperative fluorescence imaging of tumors for optical surgical navigation. HN-17 (also called Compound-17) differs significantly from HN-1 at the primary structural level as it consists of a differentially permutated version of the HN-1 sequence. Though the sequence of HN-17 is comprised of identical amino acids (or their representation) as HN-1, it may not necessarily utilize the same entry mechanism as HN-1 (as indicated by its considerably faster rate of uptake). Further, whether HN-17 targets the same types of cancer as HN-1 is not known. Nevertheless, the study demonstrated that HN-1 peptide or its derivatives target medullary thyroid cancer. Subsequently, in 2019, Ding *et al*[65] (Ohio State University, Columbus, United States) demonstrated that IRdye800 conjugated HN-1 or HN-17 peptide could be used for fluorescence image guided resection of HNSCC during surgery using an animal model harboring an HNSCC-derived tumor xenograft.

In 2021, Li *et al*[63] (Shanxi Medical University School and Hospital of Stomatology, Taiyuan, China) developed a pH-sensitive (for drug release) delivery vector comprised of HN-1 peptide and nanoscale graphene oxide. After introducing polyethylene glycol to stabilize nanoscale graphene oxide, the construct was further modified to display HN-1 peptide to provide specificity for oral squamous cell cancer and achieve greater penetration into solid tumors. Doxorubicin was then loaded through the formation of hydrogen bond and pie-bond, which is weakened in an acidic milieu to allow drug release. Nearly 70% release of loaded drugs occurred at pH 5.6 compared to a significantly lower percentage at pH 7.4. As the tumor tissue exhibits an acidic environment, lower toxicity of the doxorubicin-containing nanoparticle to normal tissues (displaying higher pH) is expected.

CONCLUSION

In closing, we would like to reiterate that the accumulation of systemically administered cytotoxic anticancer drugs at various normal tissues following the intravenous injection can be debilitating for many cancer patients. To a large extent, the failure to guide drugs to tumors (resulting in < 0.5% of drugs reaching tumors) represents one of the major causes of side effects. Given the number of obstacles that the microenvironment of solid tumors poses (ex. stroma, basement membrane, interstitial pressure), overcoming these hindrances remains a major challenge in drug delivery. To reduce side effects, tumor-homing vectors are increasingly sought for targeted delivery. The use of previously developed vectors has been limited by inefficient penetration into solid tumors. Through the isolation of tumor specifically internalizing peptides such as HN-1, we have begun to chip away at the formidable problem of eliminating side effects associated with current drugs--hence, providing a molecular biological solution to the drug delivery problem. Following its discovery, multiple laboratories located globally have developed HN-1 conjugated therapeutics to mitigate side effects[61]. The international effort has been extended to developing imaging agents for tumor diagnosis as well as surgical navigation. Taken together, these works confirm the tumor specificity of the HN-1 peptide and underscore the expanding repertoire of its therapeutic application. Finally, the deciphering of clues that pointed to DDR1 tyrosine kinase as the putative receptor of HN1 may prove to be critical in moving toward clinical application. Further studies are planned to assess HN-1 peptide's interaction with DDR1 and the therapeutic potential of treating metastatic cancer.

ARTICLE HIGHLIGHTS

Research background

The genetic basis of human cancers was elucidated *via* the identification of the prototypic human tumor suppressor retinoblastoma (*Rb*) gene by F. Hong (previously worked on phosphate transferase system governing diauxie at the Johns Hopkins University, whose alternate interpretation inspired operon concept) at the University of California at San Diego. His determination of the *Rb* gene sequence helped to uncover the central role of Rb in regulating the cell cycle as a component of DNA damage checkpoint at the G1 or S phase, which is regulated by cyclin-dependent kinase (Cdk) resulting in FDA-approved Cdk4/6 inhibitors for treating advanced-stage breast cancer. His discovery of Rb's intrinsic properties to

interact with DNA as well as to form oligomers like the breast cancer type 1 susceptibility protein C-terminus (BRCT) laid the foundation for understanding Rb's function in regulating DNA replication, transcription (ex. E2F), epigenetics (histone modification), heterochromatin, and condensation. These works culminated in his discovery of the tumor-specific lytic path 'hyperploid progression mediated death' targeting Rb or p53 mutant cancers.

Research motivation

Metastatic cancer diagnosed in late-stage remains a formidable challenge, often resulting in mortality. Combinatorial regimens consisting of multiple chemotherapeutic agents administered to treat metastatic cancer incur an unacceptably high level of morbidity.

Research objectives

There is a great unmet need to direct or guide the intravenously injected drugs to tumors as less than 0.5% reach tumors currently, contributing to severe side effects.

Research methods

Harnessing the power of molecular biology, random peptide displaying M13 bacteriophage-based library was screened by F. Hong, who previously utilized the recombinant phages to determine the genomic sequence of avian infectious bronchitis virus' spike protein for vaccine development at the Salk Institute, which predated the emergence of COVID-19 coronavirus. The screening was conducted at the University of Texas M. D. Anderson Cancer Center using live surgically derived human head and neck squamous cell carcinoma cells. After screening 2.5×10^{12} random peptides, a single peptide TSPLNIHNGQKL (HN-1) was isolated, which is tumor-specific, translocates across the cell membrane, and capable of penetrating solid tumors for targeted drug delivery.

Research results

Through global participation, the above properties of the HN-1 peptide have been confirmed. The international endeavor also led to the development of numerous HN-1 peptide conjugated agents for therapy (taxol, doxorubicin, protein kinase C inhibiting peptide, ribonucleotide reductase inhibiting siRNA, diphtheria toxin, polyethylene glycol linked to doxorubicin, graphene oxide nanoparticle-containing doxorubicin) as well as imaging (gamma-ray emitting isotopes for radiotherapy, near-infrared fluorescent dyes for surgical navigation) of cancer. More significantly, we now know that HN-1 peptide also targets breast and thyroid (potentially cervical, lung, uterine, colon) cancers.

Research conclusions

While analyzing its amino acid content, an important clue was obtained that pointed to discoidin domain receptor 1 (DDR1) as the HN-1 peptide's cognate receptor. The finding is in alignment with previously accrued experimental data globally concerning the uptake route of HN-1. The identification of Rb-regulated DDR1 as the putative receptor for HN-1 opens unexpected opportunities to block cancer progression *via* targeting the very protein mediating metastasis.

Research perspectives

Through abrogating metastasis, it may preempt the recurrence of refractory metastatic cancers, which inevitably arise due to the acquiring of drug resistance.

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FOOTNOTES

Author contributions: Hong FU has worked on cancer genetics and molecular pharmacology; Castro M participated in therapeutics research; Linse K performed molecular modeling to predict the structure of peptides; Hong FU, Castro M, and Linse K meet the criteria for authorship established by the International Committee of Medical Journal Editors and verify the validity of the results reported; Hong FU wrote the manuscript; all authors approved the final version of the article.

Conflict-of-interest statement: Dr. Hong has received royalties from University of Texas M. D. Anderson Cancer Center patent covering materials related to HN-1 peptide. All other authors have nothing to disclose.

Data sharing statement: No additional data available.

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