Research Progress on GE11 Peptide-Modified EGFR-Targeting Nano Delivery System and the Delivery Barriers to Solid Tumor

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Abstract

In order to achieve the targeted accumulation of drug in tumor tissue, and reduce the distribution of cytotoxic drug in normal tissue, targeting ligands, which play the role of "warhead", are universally employed for the construction of tumor targeting nanoparticle drug delivery system to specifically combine with the tumor hallmarks. Peptide molecule, as a specific target ligand, has been a hotspot in the research field of nano-targeting precision medicine. This review focuses on the construction and application of GE11 peptide-modified EGFR-targeting nano-delivery system. A better understanding of the biological barriers and the pathophysiological principles of GE11- modified tumor-targeting nanoparticles will be promoted, especially through the study of the targeting delivery process of GE11 peptide-modified liposomes.

Keywords: *GE11 Peptide; Nano Drug Delivery System; Tumor-targeted Delivery Barriers*

With the rapid development of nanotechnology, ligand-modified tumor-targeting nanocarriers loaded with contrast agents, radionuclides and physiologically active drugs, have brought new era for tumor detection, diagnosis, prevention and treatment. Comparing with conventional therapies, nanomedicine in clinic trials have shown low toxicity, but some still have produced new side effects, such as hand foot syndrome and mucositis caused by pegylated doxorubicin liposomes [1], hypersensitivity reactions to paclitaxel micelle [2] and so on. How to further improve the tumor specific target efficiency of nanocarriers, significantly increase the aggregation in tumor sites, and directly act on tumor cells, is the "bottle-neck" problem needed to have a careful consideration.

The targeting molecules for fabricating tumor-targeting nanocarriers, include monoclonal antibody, antibody Fab fragment, peptides, sugar, aptamer., *et al*. Compared with antibody molecules, peptide is easier to obtain, has lower screening and production costs, and provides chemical group structure for specific site modification, such as carboxyl group, base group, etc. Targeting peptides can be obtained by computer-aided design screening [3], such as CDX [4] designed from the region of candoxin targeting nicotinic acetylcholine receptor (nAChR), or by phage display peptide library, such as Arg-Gly-Asp (RGD) targeting integrin (αVβ3) [5], A7R [6] targeting vascular endothelial growth factor receptor 2 (VEGFR-2) and neurociliary protein-1 (NRP-1), and GE11 [7] targeting epidermal growth factor receptor (EGFR), etc. In order to further improve the enzyme stability of ligand peptides in blood and increase the specific binding affinity

with target cells, the selected peptides can be further optimized and modified [8]. In this paper, we reviewed the research progress of EGFR-targeted GE11 peptide-modified nano drug delivery system in cancer diagnosis and treatment, and discussed the key "bottle-neck" technology and common issues existed in the transformation process of peptide-modified tumor targeted nanoparticles from laboratory to clinical application.

GE11 peptide targeting EGFR

Expression of EGFR in tumor cells

Compared with the cells in normal tissues, the growth and apoptosis mechanism of tumor cells are abnormal. The main feature is that the signal transduction pathway related to cell growth receptor is out of control, which leads to the unlimited proliferation and growth of tumor cells. The overexpression of epidermal growth factor receptor (EGFR) is one of them. The EGFR family belongs to tyrosine kinase receptor, containing EGFR, HerB2, ErbB3, ErbB4 subtypes, which are directly related to tumor metastasis, invasion and prognosis [9]. EGFR is typically overexpressed in epithelial tumors, such as lung cancer, breast cancer, and colorectal cancer. Herbst., et al. [10] showed that EGFR overexpression accounted for approximately 30% of human tumors, especially high in head and neck cancer (about 80 - 100%), renal cancer (about 50-90%) and lung cancer (about 40-80%). When epidermal growth factor (EGF), transforming growth factor-α (TGF- α) and other specific ligands bind to EGFR extracellular protein domain, they induce the production of dimer and phosphorylate tyrosine residues in the intracellular domain of receptor, lead to the activation of intracellular signal transduction, and enhance the gene transcription level related to cell growth. The development of EGFR inhibitors attracts intensive attention for cancer chemotherapy. In addition to small molecule drugs such as Erlotinib, Gefitinib and Afatinib, monoclonal antibodies, such as Trastuzumab and Cetuximab, which can inhibit the tyrosine kinase activity of EGFR, have been approved by Food and Drug Administration (FDA) and can be obtained in clinic. However, antibody drugs have poor permeability and high production cost in solid tumors [11]. Antibody fragments as target ligands modified on carriers by chemical bonds requires high synthesis and purification techniques.

Application of GE11 peptide in tumor target therapy

GE11 (YHWYGYTPQNVI) dodecapeptide screened by Zonghai Li., et al. [7] using phage display peptide library, can specifically bind EGFR over-expressing tumor cells. The research achievement was first published in FASEB Journal "Research Communication" in 2005. In the past 15 years, more systematic and in-depth studies on the EGFR-targeted GE11 peptide have been carried out in the prevention, diagnosis and treatment of tumors, as shown in figure 1, including: (1) design and synthesis of different radionuclides-labeled GE11 for early diagnosis and treatment of EGFR overexpressed tumors [12]; (2) GE11 conjugated with polymer materials for nucleic acid drug tumor-targeted delivery, such as GE11 modified polyethylenimine (PEI) [13]; (3) GE11-modified nano carriers used for tumor-targeting delivery of chemotherapeutic drugs and biological macromolecular drugs, such as GE11-decorated doxorubicin liposome [14], poly-lactic-co-glycolic acid (PLGA) nanoparticles with GE11 surface modification [15]; (4) GE11-drug conjugates by chemical covalent bonds, for tumor targeted delivery of single drug molecules, such as GE11-Dox [16], Pc-GE11 [17]; (5) GE11 expressed on the surface of exosomes for the tumor target delivery of nucleic acid drugs [18]. Although different EGFR ligands screened from different ways may exhibit different binding affinity to tumor cells overexpressing different surface density of EGFR. But existing research shows that GE11 can specifically target a series of tumor cells with high expression of EGFR, including SMMC-7721, MDA-MA-468, MDA-MB-231, U87MGwtEGFR, U-87 MG, H1299, Panc-1, HuH7, PC346C, A549, A431, SW480, HT29, HCC70, HCC1954, HepG2, MCF-7, EGFR, SPCA1, SKOV3, FTC-133 and other tumor cell lines [7,12,15,19-26]. The carrier system decorated with GE11 peptide can deliver the drug to the target tumor cells, without obvious effect on promoting mitotic cell proliferation [21], comparing with hEGF.
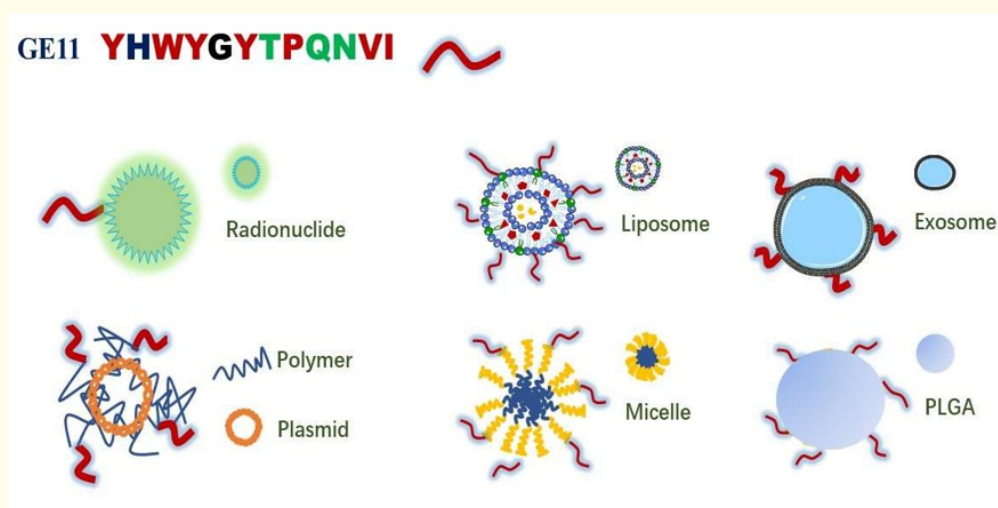

Figure 1: Schematic diagram of GE11-modified tumor-targeted drug delivery system
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Research Progress on GE11 Peptide-Modified EGFR-Targeting Nano Delivery System and the Delivery Barriers to Solid Tumor

33

GE11 peptide surface-modified tumor-targeted drug delivery system
Active targeting effect and tumor inhibition effect

Based on the specific targeting of GE11 to EGFR, more and more research work has been done on the design and fabrication of tumor targeted nano delivery system decorated by GE11. Table 1 gives the formulation information of GE11 peptide modified carrier materials and the types of different chemical bond conjugating various carrier materials with GE11 peptide.

Polymer	Linker	Formulation	Ref.
PEI	Dithiobis (succinimidylpropionate) (DSP)	PEI-GE11/pGL3	[7]
LPEI-PEG	N-hydroxy succinimidyl ester (NHS)	LPEI-PEG-GE11/NIS;	[12,21]
PEI-PEG	orthopyridyl disulfide (OPSS) NHS-PEG-OPSS	PEI-PEG-GE11/Poly IC	[22]
Adamantane-PEG	N-Hydroxysuccinimide (SCM) maleimido (MAL)	Ad-PEG-GE11/Ad-PEG (0.28nmol:1.69nm)/CD-PEI/ pDNA	[13]
DSPE-PEG2000	Maleimide (MAL)	DSPE-PEG2000-GE11(0-15%)/ SPC/Chol/DSPE- PEG2000/	[14]
DSPE-PEG2000	Maleimide (MAL)	DSPE-PEG2000-GE11 (0-2%)/ HSPC/Chol/DSPE- MPEG/DOX	[27,28]
PLGA	Carbodiimmide	PLGA-GE11/PLGA- PEG(1:1)/ DOX	[15]
PLGA-PEG	Maleimide (MAL)	PLGA-PEG-GE11/Cur- CAA-PEG/DTX	[26]
P-(GG-ONp)- HZBoc	ONp ester, C-terminal thioester, Boc	DOX-HPMA-GE11	[20]
GHDC	Epoxy chloropropane, cysteine	GE11-GHDC/Chol/HQCMC/ magnetic nanoparticles	[29]
DSPE-PEG2000	GGGSGGGSC spacer, maleimide (MAL)	DSPE-PEG2000-GE11/DSPE-PEG2000(5:1)/DOX	[16]
PEI-PEG	GGGGC spacer, maleimide-PEG-NHS	PEI-PEG-GE11/pORFh- TRAIL	[24]
GHDC	GGGSGGGSC spacer	GE11-GHDC/Chol/DOPE/DOX/ siRNA	[30]
PAMAM PAMAM-PLA	GGGGC spacer, Mal–PEG–COOH, NH2–PEG–COOH, OCH3–PEG– COOH	PAMAM–PLA–PEG– OCH3/ Cy5.5/GE11/Aminoflavone	[31]
PEG–STP Oligomer	Fmoc-N-amido-dPEG24-acid, Boc Fmoc-Oligoamino acid	B6–PEG–STP/GE11–PEG–STP/pDNA	[32]
DSPE-PEG2000	Maleimide (MAL)	DSPE-PEG-GE11(2~4%)/ DPPC/DSPC/DSPE-PEG/DOX	[33]
HA-ss-Chol	GGGGG-NH2 spacer	GE11–HA-ss-Chol/DOX	[34]
type B gelatin	CGG spacer, MAL-PEG-SCM	GE11-GENS/pDNA	[35]
PEG-P(TMC- DTC)- PAsp	Acetal group PAsp/GE11- PEG-P(TMC-DTC)	PEG-P(TMC-DTC)	[36,37]

Table 1: *Summary of GE11 peptide modified carrier materials and the formulation of EGFR active-targeting tumor nano carrier system.*

Research Progress on GE11 Peptide-Modified EGFR-Targeting Nano Delivery System and the Delivery Barriers to Solid Tumor

34

Zonghai Li., *et al*. [7] used ^{125}I-labeled GE11 peptide for the first time to observe the distribution behavior of GE11 *in vivo*, and confirmed GE11 peptide can specifically accumulate at tumor tissues of SMMC7721 xenograft tumor model *in vivo* through the determination of the radioactivity of ^{125}I in the tumor tissues and normal tissues at 0.5h and 4h, respectively. Compared with hEGF, GE11 had no obvious effect on the proliferation of SMMC7721 cells. The Scatchard analysis of GE11 with EGFR was 22.28 ± 0.4nm and hEGF was 1 ∼ 2 nm.

Initially, GE11-modified polymeric materials are employed for targeted delivery of nucleic acids. GE11 modified pH/redox responsive PBLA nanoparticles constructed by Chen G., *et al* [38] improved the silencing effect of siRNA in human breast cancer cells (MDA-MA-468 cells). Scha¨fer A., *et al*. [21] investigated the interaction between GE11 and EGFR and its effect on mitotic cell proliferation. GE11 and human EGF peptide MC were selected to synthesize LPEI-PEG-GE11, LPEI-PEG-MYI and LPEI-PEG- CMY to load luciferase plasmid DNA, respectively. The transfection effect was obvious in U87MGwtEGFR and U-87 MG with high expression of EGFR. WB analysis of downstream signal proteins activated by EGFR, including p-Akt, Akt, pErk and ErK, were performed. LPEI-PEG-GE11 had no EGFR signaling pathway activation. CLSM also observed that EGFR on the surface of HuH7 cells decreased due to receptor- mediated endocytosis after transfection of Huh7 cells with LPEI-PEG-EGF, while LPEI-PEG-GE11 did not. In PC346C prostate tumor situ model, the expression of LPEI-PEG-GE11/DNA was obvious in tumor tissue.

Lin W J., *et al*. [23] replaced histidine with arginine to optimize the structure of GE11 peptide, and synthesized GE11 (2R), and prepared pDNA/DOTAP/GE11 (2R)-PEG- PLGA micelles, which exhibits higher affinity to EGFR. Ren HL., *et al*. [24] synthesized GE11-PEG-PEI for encapsulation of pDNA, and prepared GE11-PEG-PEI/pEGFP-N2 and GE11-PEG-PEI/pORF-hTRAIL micelles, which showed obvious cytotoxic effect and antitumor effect. Uusi-Kerttula H., *et al*. [39] expressed GE11 on Ad5 adenovirus vector, which significantly improved the transfection effect of the vector.

Liang XF., *et al*. [30] constructed self-assembled peptide nanocapsules (SPV) using GE11-GHDC, phospholipid (DOPE) or cholesterol as carrier material for tumor targeted delivery of small molecule drugs and gene carriers. Lu S., *et al*. [40] synthesized Ad-PEG, CD-PEI, Ad-PAMAM, Ad-PEG-GE11/GALA molecular blocks to prepare GE11&GALA-pshVEGF@SNPs nanoparticles for loading pDNA and shRNA, using the hydrophobic assembly characteristics of β-cyclodextrin and adamantane, and can down regulate the expression of VEGF and angiogenesis in tumor cells.

Various chemotherapeutic drugs were also encapsulated in GE11 modified nanocarriers for tumor targeting and exhibited improved anti-tumor effect. Eva Kopansky., *et al*. [20] incubated GE11 peptide modified pH responsive doxorubicin hydroxypropyl methacrylate (DOX-HPMA) copolymer with A431 and SW480 tumor cells for 72 h. Due to the high expression of EGFR, IC50 values for both tumor cells decreased from 7.8 and 135 to 0.08 and 0.59, respectively, 100 times and 200 times lower than those of DOX-HPMA without targeted peptide. Lysotracker was used to label lysosomes to verify that GE11-modified HPMA copolymer was a receptor- mediated endocytosis pathway. Ashley M., *et al*. [31] prepared aminoflavone (AF) GE11 micelles with PAMAM-PLA-PEG-OCH3/GE11 as carrier material for the treatment of triple negative breast cancer. Galith Aborbeh., *et al*. [22] carefully evaluated the targeting affinity of GE11 conjugated poly-inosine-cytosine (polyIC)-PEI with tumor cells of high expression of EGFR, and confirmed that the binding capacity of GE11 modified pIC-PEI was significantly higher than that of free GE11 peptide. Jieke Yan., *et al*. [26] constructed GE11-modified PLGA nanoparticles loaded with paclitaxel and pH responsive curcumin prodrug. When curcumin prodrug actively reaches the tumorsite, curcumin is released in the local acidic environment of the tumor, realizing the synergistic effect of the combined administration of curcumin and paclitaxel in the tumor site. In the subcutaneous xenograft model of human prostate cancer (LNCaP cells), the tumor inhibition effect was obvious.

Chen L., *et al*. [14] prepared mixed micelles of GE11-Mal-MPEG-DSPE and MPEG-DSPE in different proportions, decorated GE11 on doxorubicin liposomes by post-insertion method, and exhibited specific cytotoxicity to A549 NSCLC. The liposomal surface density of GE11 was up to 15%. Tang H., *et al*. [27,28] observed that doxorubicin liposomes decorated by 2% GE11 and 4% PEG had obviously rapid tumor targeted aggregation *in vivo* and the antitumor effect was obvious. The mixed micelles of GE11- Mal-MPEG-DSPE and MPEG-DSPEGE11 were prepared to load free doxorubicin directly by Fan M., *et al*. [16].

Research Progress on GE11 Peptide-Modified EGFR-Targeting Nano Delivery System and the Delivery Barriers to Solid Tumor

35

Some studies have also attempted to use GE11 nanocarrier system to encapsulate biomacromolecule drugs. Barbara Colzani., *et al*. [15] constructed Myoglobin (Myo) - loaded PLGA/GE11-PLGA (1:1) nanoparticles to target A549 tumor cells with drug loading rate of 2.4% and particle size range of 143.9 ± 5.0nm. When the nanoparticles were placed in normal saline at 4°C (pH 7.4) and 37°C (pH5.0) for 30 days, the structure of nanoparticles was stable, and no significant difference was observed in particle size and PDI. In HEPES buffer at 37°C (pH 7.4), Myo sustained release for 60 days.

Some researchers have directly conjugated GE11 peptide with anti-tumor cytotoxic drugs and synthesized GE11-drug conjugates for tumor targeted therapy. Fan M L [16]., *et al*. synthesized GE11-doxorubicin conjugates (GE11-DOX) by using GSH responsive disulfide bond and investigated the cytotoxicity efficiency of GE11-DOX on SMMC7721 and MCF-7. The accumulation of GE11-DOX in SMMC7721 cells was significantly higher than that in MCF-7 cells, while no difference was observed for free doxorubicin group in the two cell lines. Yu LG., *et al*. [17] used photodynamic therapy to investigate the cytotoxic effect of GE11-conjugated zinc phthalocyanine (Pc-GE11) on human epidermal cancer cells (A431 cells). The target effect of Pc-GE11 in A431 subcutaneous xenograft tumor model could be observed in living animal imaging.

In conclusion, the construction of tumor nano carrier system decorated by GE11 peptide involves liposomes, self-assembled micelles, polymer nanoparticles, adenovirus vector and exosomes. These results effectively confirmed the specific EGFR targeting behavior of GE11 and the targeting effect can be significantly affected by formulation.

Particle size

The size distribution is closely related to the diffusion and penetration of nanoparticles in the tumor stroma and its contact feasibility to targeted tumor cells. With the reduction of the particle size, it will be easier for nanocarriers to cross the tumor interstitial barrier into tumor site and reach the tumor cells through the EPR effect [30]. The control of nano drug particle size is closely related to the preparation process. In the published research report of GE11 modified nanocarriers, the preparation process of nanoparticles for cell experiment and animal *in vivo* pharmacodynamics experiment include co-solvent evaporation method [41], ultrasonic dispersion rotary evaporation method [42], film hydration ultrasonic dispersion [27,28], film hydration extrusion method [43], ultrasonic dispersion dialysis [44], vortex mixed incubation [45]. Although microfluidic chip technology has been popular application in the construction of nanoparticles [46], there is few relevant research on the construction of GE11-modified nano carrier system. The particle size distribution of GE11 nanoparticles ranged from 30 nm to 200 nm, which showed good tumor targeting effect. Taking C18-EEG-GE11 micelles as an example, particles of about 30nm can be obtained by simple ultrasonic dispersion dialysis method [44].

Effect of GE11 peptide surface density of nanocarriers on tumor targeting

Among these GE11 decorated nanocarrier systems, the correlation between the surface density of GE11 modification and tumor targeting efficiency *in vivo* has attracted much attention. In the design of carrier materials, the cytotoxicity of cationic carrier materials can be reduced by increased PEG modification. The changes of hydration layer and surface potential of cationic materials after PEG modification will affect the contact capability between cationic carrier nanoparticles and cells. In general, PEG and GE11 are selected to co-modify the surface of nanoparticles. The molar ratio and surface density of PEG and GE11 were optimized to obtain the longer circulation characteristics and better EGFR active targeted effect [27,47]. Take the cationic polymer gene delivery nanocarriers as example, nanoparticles are fabricated by electrostatic interaction with nucleic acid drugs. The surface modification density of GE11 is mainly achieved by adjusting the mass ratio of carrier materials to nucleic acid drug, such as the amount of PEI-GE11, PAMAM–PLA–PEG–GE11, GE11–PEG–STP, etc. [7,32,48].

Among GE11-modified nano carrier systems with different GE11 surface densities, liposomes are the most deeply studied. Due to the liposomal special structure of phospholipid bilayer, the surface density of GE11 peptides varies with the preparation methods, thus affecting the targeting behavior of liposomes *in vivo* [14,19,27,28,33,43,49]. Song S., *et al*. [19] decorated DSPE-PEG-GE11 on the surface

Research Progress on GE11 Peptide-Modified EGFR-Targeting Nano Delivery System and the Delivery Barriers to Solid Tumor

36

of doxorubicin liposomes by post insertion of DSPE-PEG-GE11 micelles. *In vivo* animal imaging showed that 9% Cy5.5-labeled GE11-modified liposomes accumulated in tumor tissues for 54 hours. Compared with non-targeted doxorubicin liposomes, the intra-tumoral retention time was significantly prolonged. Chen L., *et al*. [14] prepared doxorubicin liposomes with different GE11 surface density by post insertion method. When the surface density of GE11 increased to 10% and 15%, the cytotoxicity of doxorubicin liposomes had no significant difference. *In vivo* animal imaging showed that the fluorescence intensity of Cy7 labeled 10% GE11 and 5% PEG modified liposomes in A549 tumor tissues was about 2.2 times higher than that of only PEG decorated doxorubicin liposomes at 24h. When the post insertion method is employed to decorate GE11 peptide on the surface of liposome, how to ensure the insertion efficiency of GE11-MPEG-DSPE in the lipid membrane, the uniformity of GE11 peptide distribution on the liposome surface, the stability of the lipid bilayer structure and the consistency of targeting behavior of GE11-MPEG-DSPE are key problems need to be solved in the transformation process of GE11-targeted liposomes from laboratory research to clinical application.

However, the accurate quantitative analysis of the distribution behavior of nano delivery system *in vivo* by animal imaging method is not yet possible. Due to the interference of the background fluorescence signal in animals, the different depth distribution of fluorescent labeled nanoparticles in tissues has a significant impact on the acquisition of fluorescence signals. Tang H., *et al*. [27] directly prepared doxorubicin liposomes modified with GE11 by film dispersion and ultrasonic hydration. Both GE11 peptide and PEG were distributed on the inner and outer surfaces of the lipid bilayer. Cell-binding experiments showed that it was not benefit to improve the targeting efficiency of GE11 when the total PEG density of liposomes increased to 8%. 4% GE11 and 2% PEG-modified liposomes showed the best targeting behavior. The distribution of doxorubicin in normal tissues and tumor tissues in the xenograft SMMC7721 tumor model was determined by HPLC. The results showed that GE11-modified doxorubicin liposomes had obvious aggregation in tumor tissues at 1h, which exhibited the active targeting effect. Only PEG-modified liposomes had more doxorubicin tumor aggregation than GE11-modified liposomes after administration for 24h, and exhibited

Longer t1/2 MRT. This is not consistent with the *in vivo* distribution data of fluorescence-labeled GE11-modified liposomes with different surface densities presented by Song S, Cheng L., *et al*. [14,19]. The half-life of liposomes modified with 2% GE11 and 4% PEG decreased from 1164.8min to 587.88min, compared with liposomes modified with only 4% PEG. With the increase of surface density of GE11, the leakage rate of doxorubicin encapsulated in liposomes also increased. To a certain extent, it can also explain the obvious antitumor mechanism of GE11-modified liposomes, that was related to the rapid accumulation of liposome in tumor neovascularization area and later the rapid release of drugs.

For the study on the EGFR-targeted behavior of GE11 peptide targeting liposomes with different surface densities, Tang H observed the dynamic changes of doxorubicin encapsulated in liposomes *in vivo* [27], while Song S and Cheng L observed the clearance and metabolism of fluorescent-labeled liposome system *in vivo* [14,19]. Direct detection and analysis of encapsulated drugs is often more objective and realistic to reflect the tumor-targeted delivery process and pharmacokinetic characteristics of GE11-modified nanocarriers *in vivo*, which is conducive to the establishment of correlation analysis between formulation and therapeutic effect.

Tumor targeting delivery barriers of GE11-decorated nano delivery system

After systemic administration, it takes three steps for GE11 peptides in blood to reach tumor cells: (1) gather in the neovascular area of tumor tissue during blood circulation, through the EPR effect and the "missile" effect of GE11 peptide; (2) across the vascular area of tumor tissue to reach the tumor stromal area; (3) diffuse and infiltrate in the tumor stromal area to reach the tumor cells.

The structure of tumor tissue was significantly different from that of normal tissue. In order to meet the nutritional needs of rapid proliferation of tumor cells, the formation of tumor neovascularization microstructure is often different from that of normal vascular tissue. The gap of endothelial cells of tumor neovascularization is wide and the structural integrity is poor. The nanoparticles that enter the blood circulation easily cross the loose endothelial space of tumor neovascularization and selectively stay in tumor tissue. At the same

Research Progress on GE11 Peptide-Modified EGFR-Targeting Nano Delivery System and the Delivery Barriers to Solid Tumor

37

time, due to the lack of lymphatic reflux in tumor tissue and the high permeability caused by incomplete vascular wall, the existence of tumor interstitial high pressure was caused, and the convective transport of drugs in tumor interstitium was reduced. Previous studies have confirmed that GE11 peptide -modified tumor targeting nano drug delivery system can effectively target and accumulate in tumor tissues with high expression of EGFR. However, due to the heterogeneity of tumor blood vessels after systemic administration, is it possible for GE11 peptide- modified nanocarriers to cross the vascular barrier and tumor interstitial barrier to reach tumor cells and evenly disperse in tumor tissues? How to exhibit the active targeting characteristics of GE11 peptide to EGFR-overexpressed tumor models? Animal imaging technology [13,38], micro PET-CT [41] and MR [50] presented the targeted aggregation of GE11-modified nano carrier system in tumor models in real time. However, these imaging technologies can't display and label the fine internal structure of tumor tissue, and can't provide clear and visual images to observe the distribution of nanocarriers in the vascular area and tumor stroma region of tumor tissue, and the way that finally directly act on tumor cells and exert tumor inhibitory effect.

Tang H [27] used tissue section technique and immunohistochemical method to observe the structure and morphology of tumor tissue in SMMC7721 xenografts. The effect of some specific components on the intra-tumoral diffusion of GE11-modified liposome and *in vivo* transport barrier of GE11-modified fluorescent liposomes (GE11- TLS) was studied in detail, as shown in figure 2. The nanodrugs gathered in tumor tissue by EPR effect needs to cross the tumor stromal cell population and tumor stromal components surrounding the tumor cells. These tumor stromal components include macrophages expressing CD68, tumor neovascularization endothelial cells expressing CD105, collagen I and collagen IV. Type I collagen fibers are widely distributed in liver tumor tissues, surrounding SMMC7721 tumor cells. If nano carriers contact with tumor cells through EPR effect of tumor blood vessels, they need to cross the space barrier of type I collagen fibers.
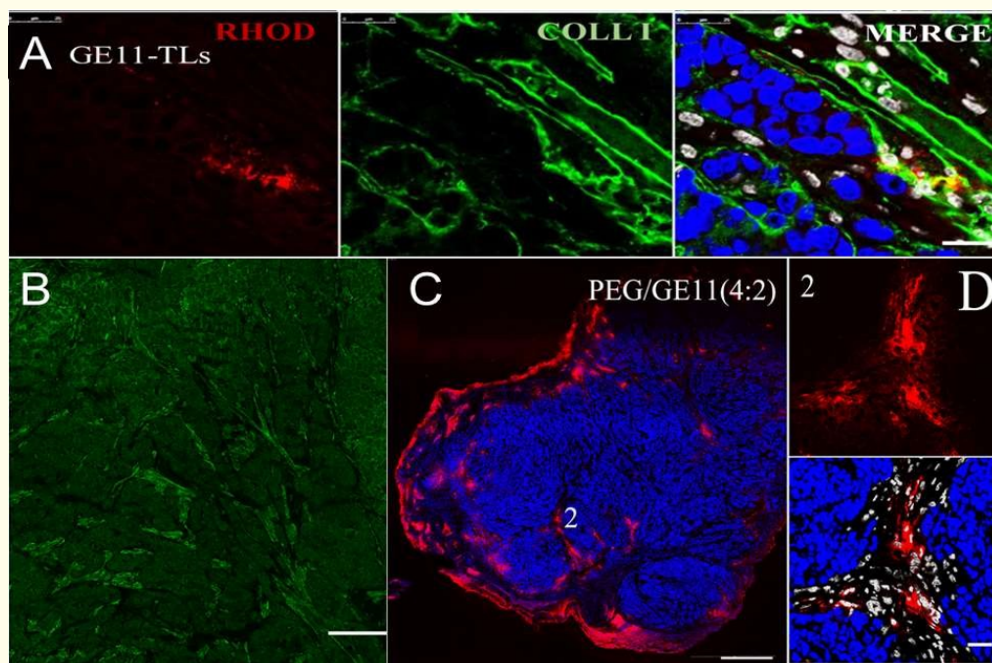

Figure 2: Distribution of GE11-modified rhodamine-labeled fluorescent liposomes PEG/GE11 (4:2)) in SMMC7721 subcutaneous xenografts (A and C). The overview image of the distribution of EGFR in SMMC7721 xenograft(B) [27].

Research Progress on GE11 Peptide-Modified EGFR-Targeting Nano Delivery System and the Delivery Barriers to Solid Tumor

38

The images of immunohistochemistry (IHC) presented that EGFR was highly expressed not only in SMMC7721 cells, but also in stromal cells. The whole tumor tissue seemed to be immersed in EGFR solution. The distribution of EGFR in the whole tumor tissue was highly heterogeneous and high expression EGFR mostly presented around the neovascular endothelial cells. In order to reach the tumor cells with high expression of EGFR, it is necessary to cross the "receptor" binding barrier formed by the tumor stromal cells with the same high expression of EGFR. Therefore, how to build a more intelligent nano preparation, achieve the specific response to the tumor microenvironment, promote the drug diffusion in tumor tissue more effectively, and finally acting on tumor cells, are important research contents for GE11-modified tumor targeting nanoparticles.

Based on the safety of phospholipid materials, GE11-modified liposomes for targeting drug delivery to tumor tissues with high expression of EGFR, exhibited better prospects. However, like most liposomal drugs, GE11 modified doxorubicin liposomes are mainly concentrated in liver, spleen, lung and kidney.

Construction of GE11 "SMART" nano carrier system for tumor microenvironment response can enable the loaded active drug molecules directly to target the tumor site, effectively act on tumor cells, and avoid damage to normal tissues. In the later research work, "SMART" GE11 nanocarriers need to be designed. Some tips are provided as following: (1) selecting disulfide bond and pH sensitive peptide as GE11 linkage to construct the tumor microenvironment response GE11-modified "intelligent" drug delivery nanocarriers; (2) multiple active components loaded into the carrier system at the same time, and simultaneously deliver to tumor tissues to play a synergistic anti-cancer effect; (3) the diffusion and penetration of nanoparticles in tumor tissues were realized by reducing particle size less than 50 nm.
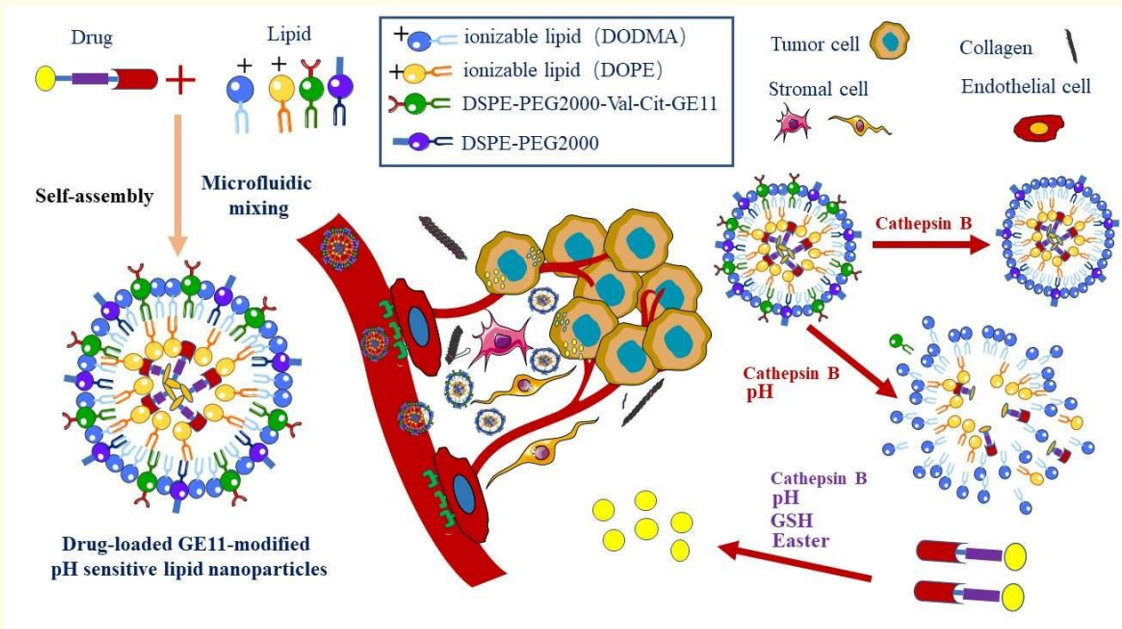

Figure 3: *Schematic diagram of delivery and release of GE11 "SMART" lipid nanoparticles across the "tumor stromal barrier" to target tumor cells (Servier Medical Art by Servier is licensed under a Creative Commons Attribute 3.0 Unported License).*

Research Progress on GE11 Peptide-Modified EGFR-Targeting Nano Delivery System and the Delivery Barriers to Solid Tumor

39

Summary and Conclusion

GE11 has been proved to be a specific EGFR ligand and has no EGF-like effect on cell proliferation and division. However, how to use the EPR effect of tumor tissue to realize the active target aggregation of GE11 mediated nanoparticles and promote the release, diffusion and penetration of loaded active drug molecules into tumor tissue, and finally act on tumor cells, is the key problem for the clinical application of GE11- modified nano carrier system, and also the common problem of peptide-modifiednano drug delivery system. Co-delivery of nucleic acid drugs, protein drugs or small molecule chemotherapy drugs with intelligent response to the tumor microenvironment and synergistic antitumor effect in tumor bearing mice model is the future research directions. Due to the diversity and safety of phospholipid materials, it has high feasibility for GE11-mediated "intelligent" liposomal drug from laboratory research to clinical application. Amounts of research activity now are focusing on the construction of "SMART" GE11-modified nanocarriers.

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Research Progress on GE11 Peptide-Modified EGFR-Targeting Nano Delivery System and the Delivery Barriers to Solid Tumor

40

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Research Progress on GE11 Peptide-Modified EGFR-Targeting Nano Delivery System and the Delivery Barriers to Solid Tumor

41

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Volume 8 Issue 11 November 2020