Introduction

Cancer is one of the major health-related issues in the United States and all around the world. In average, one in four deaths in America is caused by cancer [1]. Hence extensive efforts have been taken towards the related cancer therapy and cancer diagnostics. Whereas traditional approaches to locate, target and destroy tumor cells are limited by the restricted access to tumors [2, 3], a novel strategy to target the tumor vasculature turns out to be promising [4, 5]. The new strategy is expected to inhibit angiogenesis and the function of existing vasculatures of tumors, thereby leading to their starvation and eventual regression [4].

The NGR peptide is reported to have the greatest tumor selectivity [11] and NGR-based drug delivery has been under rapid developments over the past decades [14-17]. An anti-cancer drug doxorubicin (DOX) coupled to an NGR peptide displays enhanced anti-tumor effects with even lower toxicity than the free drug itself [13]. While intensive attention has been paid to therapeutic development, interest is increasingly being directed toward imaging-related research with NGR. Molecular imaging techniques permit direct visualization of targets and characterization of cellular activity by using contrast agents or so-called probes that specifically bind to targets in order to generate detectable signals in the target location [12].

Keywords: Asparagine-glycine-arginine (NGR), arginine-glycine-aspartic acid (RGD), isoaspartate-glycine-arginine (isoDGR), cancer, imaging, tumor angiogenesis, vasculature, aminopeptidase N (APN/CD13)
imaging in vivo would not only provide more insight into NGR’s targeting process, including bio-distribution and pharmacokinetics, but also reveal angiogenic activities related to tumor progression and malignancy [12]. Besides diagnostic purposes, the sensitive detection of tumor regions by the combination of labeled probes and quantitative imaging methods [18] also makes it feasible to monitor a tumor’s response to therapy, which is important to biomedical research.

In this review, we comprehensively discuss the current development of NGR peptides as tools for tumor imaging. NGR peptides in linear and cyclic forms have been conjugated either directly to imaging probes or indirectly to polymers that are later on modified or uploaded with probes. A wide variety of imaging methods such as magnetic resonance imaging (MRI), two-photon laser scanning microscopy (TPLSM), two-dimensional (2-D) planar fluorescence reflectance imaging (FRI), and three-dimensional (3-D) fluorescence mediated tomography (FMT) have been also included here.

The NGR motif for tumor targeting

Given that the NGR peptide can selectively bind to APN/CD13 either immune-captured or expressed on the surface of cells, the receptor of the tumor-homing NGR peptide was suspected to be APN/CD13. The receptor APN/CD13 was further confirmed by the finding that antibodies against APN/CD13 competed with NGR peptide in vivo tumor targeting [19]. Since APN/CD13 is widely-expressed in different cell lines including epithelial cells, mast cells, fibroblasts and muscle cells, as well as different locations such as cell membranes, cytoplasm, plasma, and stromal fibrillar components of some connective tissues, the detailed mechanism of NGR’s specific tumor-targeting still remained unclear [20]. Until recently, different APN/CD13 isoforms were disclosed to exist in different cells and organs based on the immune-reactive patterns [20]. Further, an APN/CD13 isoform expressed in tumor blood vessels was discovered to recognize NGR peptide while other isoforms in normal cells/organs did not [20]. Besides the special APN/CD13 isoform, an alternative tumor-homing mechanism for NGR peptide was also proposed following the observation that through rapid deamidation of asparagine [21, 22], NGR could be converted to isoaspartate-glycine-arginine (isoDGR) that antagonized the ανβ3 integrin, another upregulated biomarker in the endothelial cells of angiogenic vessels [14, 22]. The binding of isoDGR inhibited ανβ3 integrin-mediated endothelial cell adhesion, proliferation and related tumor development [22].

Regarding the antagonist, one pentapeptide containing NGR flanked by one amino acid at both ends turned out to be sufficient for binding activity (Figure 1) [13]. Both linear and cyclic forms of NGR peptides were initially reported, among which the cyclized CNGRC (cNGR) were constructed by the disulfide bonding of the two cysteines [13]. cNGR was revealed to provide not only increased affinity but also the specificity to APN/CD13 [23]. When both forms of NGR were coupled to the tumor necrosis factor (TNF-α), the cNGR-TNF-α exhibited more than 10-fold higher anti-tumor activities than the linear derivative [24]. Detailed studies revealed that a bent geometry involving glycine and arginine existed in the cyclic form, and was also favored thermodynamically in the linear form, which was believed to be essential for tumor targeting [24]. Up to now, a diverse set of NGR structures has been designed and applied in drug delivery and tumor imaging.

For the linear type, two linear NGR epitopes can be conjugated through their carboxyl ends to the amino groups in the branch and N-terminal of lysine, so that a dimeric NGR peptide is constructed [25] (Figure 1). While the disulfide bond of cNGR may be unstable to biodegradation and chemical modification, a more robust cyclization is created through the coupling of the amino group in lysine’s N-terminal to the carboxyl branch of the glutamic acid residue of KNGRE peptide [26] (Figure 1). Most of the NGR developed so far has shown promising efficacies in tumor targeting, thereby paving the avenue for NGR-directed tumor diagnoses and therapy.

NGR peptides directly conjugated to imaging agents

Fluorescent dye-conjugated NGR peptides

The very early design of NGR conjugated probes was reported by Dirksen et al [27], where they extended the carboxyl terminal of cNGR with glycines and a thioester. The N-terminal cys-
teine functionalized diethylenetriaminepentaacetic acid (DTPA) was then coupled to the NGR peptide’s thioester by a native chemical ligation. The resulting cysteine after coupling and spontaneous rearrangement could be further conjugated with a maleimide-modified fluorescent dye, Oregon Green 488 (OG488), for optical imaging. In addition, the DTPA ligand attached at the end of the peptide could be complexed to gadolinium (III) for MRI. This bimodal target-specific contrast agent was believed to afford both MRI and optical imaging of angiogenesis. Unfortunately, there has been no imaging results reported so far using this bimodal agent.

Lysine can be also incorporated into the N-terminal of linear or cyclized NGR peptide, where its amino-ended branch was coupled to OG488 [26]. The resulting OG488-cyclic KNGRE exhibited a 3.6 fold-higher affinity than the OG488-linear KNRRG in APN/CD13-positive tumor cells, which was in agreement with the reported trend [24]. Accordingly the cNGR derivative showed stronger punctuate fluorescence than the linear peptide derivatives in APN/CD13-positive cells, as characterized by epifluorescent microscopy (Figure 2A, 2B). It is noteworthy that both NGR-OG488 derivatives failed to stain APN/CD13-negative tumor cells, demonstrating their specificity to the APN/CD13 receptor. Moreover, the much reduced fluorescence of cNGR-OG488 treated cells at 4°C than at 37°C further revealed that the cellular uptake and internalization of NGR peptide was through endosomal uptake [26, 28-31] (Figure 2C, 2D).

Besides the OG488 dye, fluorescent dye Cy 5.5...
was also conjugated to the amino-ended branch of lysine at the carboxyl terminal of cNGR [18] (Figure 3A). The cNGR-Cy5.5 exerted distinct affinity to APN/CD13-positive HT-1080 cells but not to APN/CD13-negative MCF-7 cells. Revealed by fluorescence microscopy, cNGR-Cy5.5 was cell membrane-associated upon initial binding, but underwent endocytosis and nuclear staining with long-time incubation. cNGR-Cy5.5 could be clearly visualized by FRI and FMT imaging \textit{in vivo} in the HT-1080 xenografts and the target/background ratio can be decreased by competition with unlabelled cNGR peptide (Figure 3B, 3C). Direct imaging of the excised organs implied that the cNGR-Cy5.5 was distributed not only in the tumor but also in the kidney and liver (Figure 3D), indicating rapid blood-clearance and renal excretion that may reduce its toxicity during the clinical application of fluorescent cNGR [18]. Nonetheless, the over-accumulation of cNGR-Cy5.5 in kidney and liver, together with the medium affinity of cNGR-Cy5.5 to targets may limit its accumulation in tumor regions, thus reducing the sensitivity of detection [18].

\textit{Quantum dot-conjugated NGR peptides}

Quantum dot (QD) is a type of semiconductor nanocrystal that has electrons confined to mathematical points [32], and can be readily synthesized to bear diameters around 1nm-10nm [33]. Common QD features in core-shell architecture, with its core composed of heavy...
metals such as cadmium or lead to make a narrow band-gap for electron excitations [34]. The shell coating, on the contrary, is comprised of materials of higher band-gap to confine the excitation/emission only in the core, enhance the quantum yield of core emission and protect QD from photo-bleaching [34]. Compared to traditional dyes, QD’s emission is brighter, with narrow emission spectra and is tunable based upon its size [34, 35]. Moreover, QD’s optical performance is more stable [34] and durable [36]. Given these advantages, QD-based imaging has been applied in living cells and animal models [36-40], among which QD labeled with antibodies or targeting peptide can lead to specific imaging of various receptors [37, 38] and organs [39].

To develop QD-conjugated NGR peptides, the carboxyl terminal of cNGR was extended with glycines and a lysine whose amino branch was conjugated to OG488 or biotin [11]. The biotin-tagged NGR can then multi-valently label the QDs that had been pre-modified with streptavidin (Figure 4A). When applied to tumor imaging by in vivo fluorescence microscopy and TPLSM, the higher intrinsic fluorescence and lower bleaching rate of QD than OG488 dye made the cNGR-QD highly specific in APN/CD13-rich cells (Figure 4B, 4C). In vivo, the fluorescence of cNGR-QD is greater in intensity and longer in persistence than the cNGR-OG488 dye. Judged by fluorescent antibody, APN/CD13 was preferentially expressed in angiogenic areas of heart and the NGR-directed
probes can exclusively co-localize with APN/CD13 in cardiac angiogenesis [11].

The streptavidin-modified paramagnetic QD could be simultaneously coupled with biotin-tagged cNGR and biotin-tagged Gd(III)-DTPA, so that quantitative MRI was conducted to noninvasively assess the tumor’s angiogenic activity [12]. This dual-modified contrast agent was highly selective, showing a three-fold higher MRI contrast in the tumor rim than control QD, while giving little signal at muscle tissue and tumor core where no angiogenic activity was expected (Figure 5A, 5B). The accurate discrimination of the tumor’s rim-core heterogeneity by the dual-modified QD demonstrated its successful quantization of the extent of the tumor angiogenesis. Further, the dual-modified QD could be co-localized with endothelial cells in tumor vasculature but not in normal muscle, when characterized by ex vivo TPLSM, implying the contrast agent’s specificity [12] (Figure 5C, 5D).

### Polymer-NGR peptide conjugates

Instead of direct conjugation which in most cases can only allow a limited number of fluorescent probes to be attached to NGR peptide, the tumor-homing peptide can be conjugated to a polymer carrier. Serving as a harbor, the polymer carrier can be modified or encapsulated with multiple probes to produce amplified signals for imaging.

**Fluorophores encapsulated in polymers**

One of the most popular carriers used in current drug delivery system was liposome which was non-toxic and biodegradable [41-43]. Synthetically the NGR containing linear peptide was made with a cysteine at the C-terminal. The 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) can be conjugated with polyethylene glycol (PEG), and then further modified to have a maleimide end for final conjugation with the peptide cysteine [4]. The DSPE part of the final product was then dissolved in the solvent and specially treated to form the liposome [4], during which the DOX was added to make a DOX-encapsulated NGR-modified liposome. The cellular uptake and DOX activity could be characterized by fluorescent microscopy, making use of DOX’s intrinsic fluorescence. The NGR-targeted liposomal DOX specifically bound to the APN/CD13-expressing tumor cells and was internalized through the endosomal pathway. After the break-up of the liposome, DOX was released and observed to localize into the nuclei [4]. The linear or cyclic NGR peptides can be also conjugated to DSPE-PEG through the N-terminal lysine [26]. The resulting multi-valent NGR peptides on the surface of liposome displayed a 10-fold enhanced affinity towards APN/CD13-positive cancer cells versus the peptides alone [26]. Intriguingly, the DOX encapsulated in liposome was susceptible to controlled-release upon temperature changes. Unfortunately, there were no reports about DOX-based fluorescent imaging for this NGR-liposome agent [26].

In another example, additional glycines were added to the N-terminal of cNGR which was then conjugated to a biodegradable di-block copolymer, PEG-poly (D, L-lactide) [44]. The fluorescent probe “1,1-diocadecyl-3,3,3’-3’-
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Fluorophores conjugated to polymers

The N-(hydroxypropyl)methacrylamide (HPMA) copolymer was synthesized through copolymerization of HPMA with methacryloyl-glycine-glycine-p-nitrophenyl ester (MA-GG-ONp) and methacryloyl aminopropyl fluorescein-5-isothiocyanate (MAP-FITC) [25]. The fluorescein-isothiocyanate (FITC)-labeled HPMA copolymer was further modified with a series of NGR peptides (linear, dimeric, and cyclic) by replacement of the nitrophenol group at the branch sites of the polymer (Figure 6A). Confocal imaging revealed that the NGR-labeled FITC-HPMA conjugates selectively bound to APN/CD13-positive cells in a concentration-dependent manner, and were internalized to a greater extent in APN/CD13-positive cells through the lysosomal pathway (Figure 6B - 6G). On the other hand, whereas FITC-labeled NGR peptide was subjected to lysosomal trafficking after 4 hours, and then increasingly localized into mitochondria after 17 hours, the FITC-labeled HPMA copolymer seemed to be trapped in endosomal and lysosomal compartments [25]. The above comparison demonstrated NGR’s effect on improving the imaging agent’s cellular distribution by promoting their escape from lysosome and endosome.

NGR peptides fused to proteins

Cleavage of some cytoskeletal proteins such as actin was found to be involved in apoptosis [45, 46] and one fragment of the degraded actin – 15 kDa actin was shown to induce the morphological changes of apoptosis in tumor cells [47]. It is therefore postulated that once internalized, the 15 kDa actin may bind to the cytoskeleton of tumor cells and induce apoptosis.

While NGR can be chemically conjugated to synthetic polymers, Lei et al [5] illustrated that the NGR peptide can be fused to a 15 kDa actin by using recombinant DNA technology to construct the corresponding plasmid and express it in E. coli. The lysines on NGR-actin were then chemically labeled with FITC. Fluorescent imaging confirmed that NGR lead to the internalization of NGR-actin to HeLa cells and HepG2 cells, and the actin fragment further induced the binding of NGR-actin to cytoskeleton proteins. The re-
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A


cyclic NGR

HT-1080 cell
CD13-positive

A431 cell
CD13-negative

B
C
D
E
F
G

Figure 6. In vitro imaging of FITC-HMPA-cNGR in CD13-positive/negative cells. (A) Structure of FITC-HMPA-cNGR. (B)-(G) Confocal images of (B)(E) FITC-HMPA-cNGR, (C)(F) LysoTracker Red DND-99, and their co-localization image (D)(G) in HT-1080 and A431 tumor cells, respectively. Green color is from FITC; red color is from LysoTracker Red DND-99; blue color is the nuclear staining by DAPI. The figures are adapted from reference [25].

Resulting tumor apoptosis was then observed.

Conclusion

In this review, we have summarized the current development of NGR peptide-based agents for tumor imaging. For NGR directly conjugated with probes, the low sensitivity brought by the limited signal/background ratio, as well as the in vivo stability of peptide and fluorophores remain challenging issues. So far the introduction of QD seems to be the most effective solution, given its strong intrinsic fluorescence, its ability to carry multivalent dyes for signal amplification, and its endurable performance. One major concern of QD is its potential toxicity [48] and difficulty in being excreted out from body [49], which hopefully can be overcome by new generations of QDs that are claimed to be non-toxic and renally excretable [49-52].

For polymers conjugated with NGR peptides on the surface, the targeting efficiency and signal intensity are both expected to be increased, due to the multivalent modifications similar to what can happen with QDs. Despite the frequent trials of NGR-dyes for in vivo experiments, most NGR-polymer-dye conjugates are only tested in cells. The related confocal fluorescence was focused on cellular uptake mechanisms, instead of tumor imaging. Additionally, given their potential clinical applications, most polymers are made biodegradable to avoid any toxicity, thereby rendering other concerns about the leakage of encapsulated probes or stability of attached probes, once they are tested in vivo.
Finally for all the reported imaging studies, the NGR-based probes were co-localized with CD13 receptor, with assumption of no degradation of NGR, which otherwise would result in iso-DGR that binds to $\alpha_\beta_3$ integrin instead. Future imaging work may involve the localization of $\alpha_\beta_3$ integrin in cells or xenografts and to study its correlation with NGR-labeled probes. Despite current limits and concerns, the development of NGR-labeled probes and polymer conjugates for tumor-specific imaging has opened up a promising field in the research of cancer diagnostics and therapy. Boosted by the rapid progress of imaging techniques, it is conceivable that NGR-peptide related drugs will find profound future applications in the clinic.

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References

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