Development of a Targeted anti-HER2 scFv Chimeric Peptide for Gene Delivery into HER2-Positive Breast Cancer Cells

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Abstract

Chimeric polymers are known as suitable carriers for gene delivery. Certain properties are critical for a polymer to be used as a gene delivery vector. A new polymer was designed for the targeted delivery of genes into breast cancer cell lines, based on MPG peptide. It is composed of different functional domains, including HIV gp41, nuclear localization sequence of SV40 T-antigen, two C-terminus repeats of histone H1, and the scFv of anti-HER2 antibody. The results demonstrated that the vector can effectively condense plasmid DNA into nanoparticles with an average size of 250 nm. Moreover, fusion of the scFv portion to the carrier brought about the specific recognition of HER2. Overall, the transfection efficiency of the vector demonstrated that it could deliver the desired gene into BT-474 HER2-positive breast cancer cells.

1. Introduction

Gene therapy refers to the delivery of DNA as a drug into cells (Kamimura et al., 2011), and has been considered a therapeutic approach in different diseases, including cancer. The first gene therapy clinical trial was performed for the treatment of ADA_SCID (Sheridan, 2011). Gene therapy requires practical and non-toxic vectors that can deliver the gene of interest to target cells. Development of vectors that are able to transfer genes selectively and practically to target cells is a considerable step in gene therapy (Nishikawa and Huang, 2001). Viral vectors are the most efficient, but their toxicity and immunogenicity are major disadvantages. Nowadays, non-viral vectors with minimal toxicity and immunogenicity have been developed to mimic the cell entry mechanism of viruses (Tokatljan and Segura, 2010). Non-viral vectors are generally

cationic in nature. Cationic polymers such as polyethylenimine (PEI), poly-L-lysine (PLL), and cationic liposomes are the most commonly used cationic vectors. Some of the most important carriers used in gene transfer into cells are peptides, which are synthesized by gene engineering techniques (Canine and Hatefi, 2010). Peptide carriers are composed of several domains to mimic native peptide sequences found in nature, especially viruses.

MPG (27 residues) is one of these cell-penetrating peptides, containing a hydrophobic domain derived from the fusion sequence of HIV gp41 and a hydrophilic domain derived from the nuclear localization sequence (NLS) of SV40 T-antigen. It was designed for oligonucleotide delivery by Morris in 1997 (Morris et al., 1997). The interaction of MPG and DNA is mainly electrostatic, between the negatively charged phosphates of short nucleic acid strands and the positively charged residues of the peptide vector (arginine and lysine). It promotes the delivery of fluorescently labeled 18mer and 26mer oligonucleotides into cells, with more than 90 % efficiency in less than 1 h (Morris et al., 1997). However, to deliver plasmid DNA, MPG apparently needs another motif to condense pDNA for transfection into the cells (Churchill and Travers, 1991; Collins et al., 2007). The transfection efficiency of MPG-based nanoparticles was shown to be comparable with that of polyethylenimine (PEI) (Saleh et al., 2015), and MPG can condense plasmid DNA into stable nanoparticles. While MPG can condense DNA at high N/P ratio (Saleh et al., 2015), addition of a condensing motif can increase the capacity of MPG for interaction with DNA. Several DNA-binding motifs have been described in the C-terminus of histone H1. Khadake and Rao demonstrated that the condensation property of histone H1 is localized at its C-terminus tail (ATPKKSTKKTPKKAKK, residues 144 – 159), and that this can condense DNA as well as full-length histone H1 (Khadake and Rao, 1997). Therefore, two repeats of the DNA condensing motif of histone H1 were used to improve DNA packaging, as part of the fusion peptide in our construct (Sadeghian et al., 2012).

The human epidermal growth factor receptor 2 (HER2) is a transmembrane tyrosine kinase receptor. HER2 plays important roles in cell growth, survival, and differentiation (Tai et al., 2010). As it has a key role in cell survival, its expression level affects cell behavior. It was overexpressed in 30 % of human breast cancers (Roskoski, 2014). Its overexpression is associated with a poor prognosis for breast and ovarian cancer patients (Chang et al., 1997). High expression of HER2, and the accessibility of its extracellular domain, makes it a good candidate

for the targeting of nanoparticles in gene delivery systems (Tai et al., 2010). Trastuzumab, a humanized monoclonal antibody that recognizes an extracellular domain of HER2, is the most commonly used drug that targets HER2 (Tai et al., 2010). During the past decade, advances in recombinant antibody technology have greatly facilitated the genetic manipulation of antibody fragments (Kane, 1995; Sahdev et al., 2008). These advances led to the development of a wide variety of engineered antibody molecules for research, diagnosis, and therapy. Single-chain variable domain fragment (scFv) is one of several fragments used in research and clinical settings, due to better pharmacokinetic properties compared to whole antibodies. Besides, they are easy to produce in large quantities, and at low cost. They display improved pharmacokinetics properties, such as better tissue penetration and rapid blood clearance (Kreitman and Pastan, 2006).

The aim of this study was the design and production of a biomimetic vector containing the scFv of anti-HER2 antibody as a targeting motif, HIV gp41, the nuclear localization sequence (NLS) of SV40 T-antigen, and two repeats of the C-terminus of histone H1 as a condensing motif (Fig. 1). The ability of the newly designed vector to achieve gene delivery into an HER2-positive breast cancer cell line was then investigated.

2. Materials and methods

2.1. Materials

Escherichia coli strains were obtained from Invitrogen. *E. coli* Rosetta 2 host strains are BL21 derivatives designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. (Kane, 1995). *E. coli* Origami™ host strains are K-12 derivatives that have mutations in both the thioredoxin reductase (trxB) and glutathione reductase (gor) genes, which greatly enhance disulfide bond formation in the cytoplasm (Sahdev et al., 2008). *E. coli* C41(DE3) is designed for production of toxic recombinant proteins (Dumon-Seignover et al., 2004). Vectors used were pET28a (Novagen), pCold I (Takara, Japan), and psiCHECK (Promega). Fetal bovine serum (FBS), bovine serum albumin (BSA), and Roswell Park Memorial Institute medium (RPMI) were purchased from Gibco. Restriction enzymes were purchased from Fermentas; plasmid extraction kits and Ni-NTA were from Qiagen. High molecular weight (25000 Da) anhydrous branched PEI and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich.

2.2. Cell lines

BT-474 (HER2-positive) (Subik et al., 2010) and MDA-MB-468 (HER2-negative) (Phillips et al., 2008) breast cancer cells were obtained from Pasteur Institute of Iran, and cultivated in RPMI 1640 supplemented with 10 % fetal bovine serum at 37 °C in a humidified atmosphere of 5 % CO₂/95 % O₂.

2.3. Methods

2.3.1. Cloning, expression and purification of proteins

The gene encoding MPGHT was designed, codon-optimized, and synthesized with N-terminal *Nde*I and C-terminal *Xho*I restriction sites. The synthesized genes were double digested with *Nde*I and *Xho*I restriction enzymes and cloned into the digested, dephosphorylated pET28a vector. The scFv was cloned in the pCold I expression vector using two primers with N-terminal *Nde*I and C-terminal *Hind*III restriction sites. After verification by DNA sequencing, the expression vector was transformed into each host. For MPGHT production, cultures were started by inoculation of a single fresh colony of *E. coli* Rosetta 2(DE3), harboring expression plasmids, in 5 ml of 2XYT medium (tryptone 1.6 %, yeast extract 1 %, sodium chloride 0.5 %, pH 6.8 ± 0.2 at 25 °C) containing 50 µg/mL kanamycin, and incubated at 37 °C overnight. The next day, 200 µl of the starter culture was used to inoculate 500 mL of 2XYT medium containing 50 µg/mL kanamycin. The culture was grown at 37 °C until the OD₆₀₀ reached 0.6. Protein expression was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C. The cells were harvested after 4 h by centrifugation at 5000 × *g* for 20 min. In the case of MPGH, starter cultures were prepared in *E. coli* C41(DE3). Expression conditions were as for MPGHT protein, with a lower concentration (0.2 mM) of IPTG. For production of scFv, the gene of interest was expressed in the *E. coli* Origami™ (DE3) host, and expression was performed in TB (900 ml tryptone 1.2 %, yeast extract 2.4 %, and glycerol 0.5 %, with 100 ml KH₂PO₄ 0.17 M and K₂HPO₄ 0.72 M) containing 1 mM IPTG, at 15 °C for 24 h.

MPGH and MPGHT were purified by Ni-NTA affinity chromatography under denaturing conditions. Cells were collected, lysed (50 mM Tris, 1.0 M NaCl, 8 M urea, 5.0 mM imidazole, pH 12.0), and centrifuged for 40 min at 10000 × *g* to pellet the insoluble fraction. The supernatant was then transferred to a column with Ni-NTA resins and washed with decreasing

concentrations of urea (from 7.0 to 0 M), and increasing concentrations of imidazole (20 mM to 70 mM) to remove impurities. Finally, the proteins were eluted with elution buffer (0.5 M NaCl, 50 mM Tris, 250 mM imidazole). The scFv were purified using Ni-NTA affinity chromatography under native conditions. The pellet was suspended in lysis buffer (50 mM Tris, 300 mM NaCl, 5 mM imidazole, pH 8.0), sonicated, and centrifuged for 20 min at $10000 \times g$ at 4 °C to pellet the insoluble fraction. The supernatant was transferred to a column with Ni-NTA resins and washed with washing buffer (50 mM Tris, 300 mM NaCl, and 40 mM imidazole, pH 8) to remove impurities. Finally, the peptides were eluted with elution buffer (300 mM NaCl, 50 mM Tris, 250 mM imidazole, pH 8). The purity of proteins was analyzed by western blot using an anti-his-tag antibody and SDS-PAGE. Densitometric analysis and molecular weight determination of the purified fusion vectors were performed using AlphaEase FC Software (Version 5.0.1), with standard settings calculating AUC (area under curve) for each band. The protein concentration was determined by the Bradford protein assay method, using BSA as the standard (Bradford, 1976; Nazari et al., 2012) or by spectroscopy, using a calculated molar absorption coefficient at 280 nm (ϵ_{280}) of 66350, 5500, and 72225 $M^{-1} cm^{-1}$ for the purified protein scFv, MPGH, and MPGHT, respectively. Absorbance at 214 nm with a molar absorption coefficient of 193969 was also used to calculate the concentration of MPGH. It is possible that the fusion proteins had degraded automatically.

2.3.2. Gel retardation

A fusion vector that is used for gene delivery should have the ability to bind DNA. The ability of the fusion vectors to condense pDNA was confirmed by gel retardation assay. One microgram of plasmid, harboring the gene encoding luciferase, was mixed with increasing amounts of the fusion vectors at various charge ratios (nitrogen to phosphate (N/P) ratio), and incubated at room temperature for 30 min. Agarose gel was used to investigate the electrophoretic mobility of each complex, followed by visualization using UV illumination (UV tech, Germany).

2.3.3. Serum stability

The stability of the nanoparticles in the presence of serum was examined by gel retardation assay. The fusion vectors complexed with the desired plasmids at an N/P ratio of 15 were prepared. The complexes were incubated for 60 min at 37 °C in the presence of 10 % FBS. To

dissociate pDNA from nanoparticles, 10 % SDS was added. Samples were then electrophoresed on a 1 % agarose gel containing 1% ethidium bromide and visualized with UV.

2.3.4. Hemolysis

Whole human blood was collected in EDTA-containing vacutainers and the red blood cells (RBCs) were separated from the plasma by 2 min centrifugation at $10000 \times g$. RBCs were washed thoroughly with phosphate buffered saline (PBS) and spun down at $10000 \times g$ for 10 min. After the final wash, RBCs were suspended and diluted in PBS (pH 7.4 and 5.4) to yield a solution with 10^8 RBCs per 100 μ l. This solution was adjusted to 1 ml with PBS at the appropriate pH. Different amounts of fusion vectors (10 and 30 μ g) were added to the solution and incubated for 1 h at 37 °C. The tubes were centrifuged for 5 min at $10000 \times g$ and the absorbance of the supernatant was measured at 540 nm.

2.3.5. Size and zeta potential analysis of nanoparticles

The average particle size and size distribution of the complexes were determined by dynamic light scattering by photon correlation spectroscopy. A Zetasizer Nano ZS instrument (Malvern Instruments, UK) was used to measure both particle size and zeta potential at 25 °C. Various ratios (nitrogen to phosphate (N/P) ratio) were incubated at room temperature for 30 min in PBS buffer (pH 7.4).

2.3.6. Flow cytometric analysis of HER2, using FITC-conjugated protein

FITC conjugation was performed according to the manufacturer's instructions (Sigma-Aldrich, Wisconsin, USA). In brief, proteins were dialyzed against bicarbonate buffer (0.1 M, pH 8.3) at 4 °C, overnight. FITC was combined with different concentrations of proteins in a total volume of 1 ml. After mixing for 1 h at room temperature, the mixture was dialyzed against PBS at 4 °C overnight, with three changes of buffer (Holmes and Lantz, 2009). A fluorophore/protein ratio was determined for each sample. Human breast cancer cell lines BT-474 and MDA-MB-468 were stained with FITC-conjugated proteins. The binding ability of the conjugated probes was evaluated. Briefly, BT-474 and MDA-MB-468 cells were trypsinized, counted, and aliquoted to 2×10^5 cells per analyzed point. To estimate binding affinity, the cells were incubated with an increasing concentration of the probes in PBS containing 2 % BSA. The reaction was carried out on ice with intensive shaking for 1 h. Next, the cells were washed three times with PBS and

analyzed by Partec flow cytometer, using the blue laser (excitation = 488 nm). FlowJo (Tree Star Inc., Ashland, OR) software was used for data acquisition and analysis.

2.3.7. Immunocytochemical localization of HER-2 staining with FITC-conjugated probes

Briefly, acetone-fixed cytospin cells were washed in 0.1 % PBS-BSA. Blocking was done with 5 % normal human serum for 15 min. Fixed cells were then incubated with FITC-conjugated probes for 2 h on ice, and washed three times with 0.1 % PBS-BSA before they were counterstained and mounted with Vectashield mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., California, USA). Cells were then washed with PBS and directly observed under a fluorescence microscope (Olympus, Tokyo, Japan). This procedure was repeated three times.

2.3.8. Cytotoxicity Assay

BT-474 human cancer cell line cells were seeded in 96-well plates at 10^4 cells per well in RPMI media with 10 % FBS serum. Cells were treated with various amounts of complexes (containing 0.25 μ g psiCHECK) for 4 h. The media was removed and replaced with fresh media supplemented with 10 % serum, followed by 48 h incubation at 37 °C, humidified, with 5% CO₂. In a further assay, different amounts of fusion vectors were added to the cells and left for 48 h without exchanging the media. The control well was not treated and PEI was used as a positive control. Cell viability was assayed using MTT after 48 h. Briefly, 10 μ l MTT (5 mg/ml) was added to each well and the cells were incubated at 37 °C for 4 h. The formazan product was dissolved in 100 μ l dimethyl sulfoxide (DMSO). Color intensity was measured using a microplate reader (ELx800, Biotek, USA) at 570 nm.

2.3.9. Transfection

BT-474 and MDA-MB-468 cell lines were seeded at a density of 4×10^4 cells per well in a 24-well plate (SPL Life Sciences), and left to adhere for 24 h prior to transfection. Serum-free media was added to each well for 2 h. Transfection was performed with MPGHT and MPGH complexes (containing 1 μ g psiCHECK) at different N/P ratios for 4 h before addition of RPMI 1640 supplemented with 10 % FBS. As a positive control, cells were also transfected with PEI. Forty-eight hours after transfection each well was gently washed twice with PBS and the cells were lysed with 100 μ l of cell culture lysis buffer (Promega, WI, USA). Luciferase activity was assessed in the presence of luciferin, ATP and Mg²⁺ using a luminometer (Berthold Detection

Systems, GmbH) (Nazari and Hosseinkhani, 2011; Riahi-Madvar and Hosseinkhani, 2009; Samadikhah et al., 2011).

3. Results and Discussion

3.1. Cloning and protein expression

The structures of the recombinant fusion vectors (RFVs) are shown schematically in Fig 1. As mentioned previously, genetic engineering approaches were used to produce monodisperse recombinant fusion proteins (Canine and Hatefi, 2010). The desired genes were incorporated into the aforementioned vectors and optimized for synthesis in an *E. coli* expression system. Different expression conditions were used for production, as described above. MPGH and MPGHT were purified under denaturing conditions in the presence of urea, while scFv was purified under native conditions. The calculated MW of the MPGH peptide was 9815 Da, while the calculated MWs of MPGHT and scFv were 38075 Da and 29069 Da, respectively. A band was usually observed around the expected MW of each recombinant fusion protein, in both SDS-PAGE and western blot analysis (Fig. 2). Based on calculation, the expected molecular weight (MW) for MPGHT and scFv was 38075 Da and 29069 Da; respectively. As it is indicated, two smaller fragment of MPGHT (30, 15 kDa) and one smaller fragment (15kDa) for scFv was obtained. The low MW band (15 kDa) has the His tag sequence as detected by Western blotting, indicating that they might be proteolytic cleavage derivatives of the original 29 kDa scFvs. It is likely to say that the fusion proteins were degraded automatically similar to previous reports (Alonso-Camino et al., 2013; Kabir et al., 2009; Min et al., 2005).

3.2. Gel retardation assay

The ability of MPGH, scFv and MPGHT to bind DNA was tested. DNA (1 µg) was mixed with various amounts of fusion vectors and incubated at room temperature for 45 min. N/P ratio was calculated using the molar ratio of positive charge to negative charge.

To calculate the N/P ratio, the molecular weight and number of Lys and Arg residues were used:

To obtain an N/P value of 1 for MPGH, 1.4 μ g of MPGH was used.

As we checked the ability of scFv to retard DNA, and it has no ability to condense the DNA, we did not consider the number of Lys and Arg of this portion in the calculation of N/P for MPGHT.

For an N/P of 1 for MPGHT, 5.2 μ g of MPGHT was used. The reaction mixture was then analyzed on 1 % agarose gel. As shown in Fig. 3, both MPGH and MPGHT were able to bind DNA in a concentration-dependent manner (Fig. 3A, B), whereas scFv alone displayed no DNA binding capability (Fig. 3C).

3.3. Nanoparticles size and zeta potential analysis

As DNA is negatively charged, cationic fusion vectors must interact electrostatically with it and condense it into positively charged nanoscale particles. The characterization of nanoparticles, dynamic light scattering and zeta potential analysis, was performed to determine their properties. According to Fig. 4, with both fusion vectors, increasing the ratio of the fusion peptide to plasmid DNA decreased nanoparticle size to about 250 nm. As was expected from the gel retardation assay, at low N/P ratios, the size of the nanoparticles was increased and the fusion

vector was unable to condense DNA completely. Zeta potential is usually used for surface charge determination, and a similar pattern was observed in the nanoparticle zeta potential (Fig. 4); the highest N/P ratios were correlated with increasingly positive zeta potential. The polydispersity index (PDI) of all nanoparticles was around 0.4 or less.

3.4. Serum stability analysis

As serum has a high protein concentration, and some of these proteins could interact with the complexes to alter their ability to protect DNA from degradation, analyzing the ability of the complexes to prevent digestion of the DNA by serum nucleases was essential. Mobility of the plasmids on 1 % agarose gel was used as an indicator of the stability of the nanoparticles in 10 % serum. N/P 15 was chosen for this assay owing to its correct particle characteristics (Fig. 4) and transfection efficiency in comparison with other N/P ratios. Results showed that nanoparticles produced with MPGH and MPGHT retarded plasmid movement on the agarose gel in the presence of serum as well as in the absence of serum. DNA in complexes can be recovered by SDS due to their negative charge interacting with the positive charge of the vectors (Sadeghian et al., 2012; Wang et al., 2002). Plasmid DNA is completely protected against serum nucleases and the recovered plasmid DNA remained intact (Fig. 5). A naked DNA control was included in the test to ensure that serum nuclease activity was effective.

3.5. Hemolysis assay

Fusogenic peptides are capable of membrane disruption at acidic pH (Sadeghian et al., 2012). However, according to a study, cell-penetrating peptides are likely to avoid the endosomal pathway or facilitate escape of cargoes from early endosomes (Morris et al., 2008). Therefore, it is not necessary for them to be active at acidic pH. Our experiment shows that fusion proteins are more active in neutral pH than acidic pH. For both MPGH and MPGHT hemolytic activity was increased in neutral conditions compared with acidic conditions (Fig. 6). Accordingly, the fusion vectors are able to disrupt the RBC membrane at higher concentrations at pH 7.4, but at lower concentrations, this disruptive activity was not significant. The hemolysis activity of MPGHT was lower than with the same amount of MPGH. Triton X-100 (1 %) was used as the positive control, and PBS buffer at the appropriate pH was used as the negative control.

3.6. Flow cytometric analysis of HER2, using FITC-conjugated protein

BT-474 and MDA-MB-468 cell lines have the highest and lowest expression levels of HER2 respectively (Phillips et al., 2008; Subik et al., 2010). Determinations of the binding affinity of scFv to cells overexpressing HER2 were made using flow cytometric analysis. Different concentrations of FITC-conjugated scFv were examined. FITC-conjugated scFv was recognized by 96.4 % of BT-474 cells and 4.13 % of MDA-MB-468 cells (Fig. 7A). As shown in Fig. 7A, staining with the FITC-conjugated anti-HER2 scFv molecule resulted in increased fluorescence intensity and the entire population of BT-474 cells was shifted to the right (increased mean fluorescence intensity, MFI), compared to the control. In contrast, anti-HER2 scFv staining of the HER2 negative cell line (MDA-MB-468) did not cause a shift in fluorescence intensity. As a result of this study, it was concluded that the scFv structure was functional in the fusion protein. Overall, mean fluorescence intensity increased with increasing concentration of FITC-conjugated anti-HER2 scFv in BT-474 cells. Significant differences were not observed for the negative cell line MDA-MB-468 (Fig. 7B).

In a parallel experiment, the ability of the FITC-conjugated fusion vector MPGHT to bind to HER2(+) BT-474 cells was evaluated. It was shown that the scFv portion of MPGHT can recognize HER2 as well as scFv alone. As shown in Fig. 7C, staining with the FITC-conjugated MPGHT molecule resulted in recognition of a significant percentage of the HER2 positive cell population. The fact that 50 µg of MPGHT contains less functional units of scFv, compared with 50 µg of scFv alone, explains why it recognized a lower percentage of the BT-474 population.

3.7. Immunocytochemical localization of HER-2 staining with FITC-conjugated probes

The human breast cancer cell line BT-474 expresses high levels of HER2 and this cell line was used to demonstrate fluorescence staining. As described in previous sections, cells were incubated with 50 µg/ml of FITC-conjugated MPGHT for 2 h on ice, washed three times with PBS-BSA 0.1 %, and DAPI was used for nuclear staining. The immunofluorescence microscopy assay clearly showed that the scFv portion of MPGHT can bind to the cell surface receptor of HER2-positive cells. No accumulation of MPGH was observed at the cell surface (Fig. 8).

3.8. Cytotoxicity Assay

The toxicity of the vectors is one of the most challenging problems in their application in gene delivery. For example, PEI shows good transfection efficiency, but its application in vivo is limited due to its high toxicity. The MTT assay was performed to investigate the toxicity of the

fusion vectors, either in the form of nanoparticles with the plasmid DNA, or in their naked form. BT-474 cells were incubated with various amounts of the fusion vectors (1-20 μ g) for 48 h to examine their toxicity during longer exposure to the cells. Different N/P ratios (5-20) were also used to study the toxicity of the nanoparticles. Figure 9 indicates that no significant toxicity was observed, either from proteins or nanoparticles, in comparison with untreated cells. Moreover, PEI was used as a positive control. Results have shown that even after 48 h treatment with MPGH, more than 80 % of cells were viable. Addition of the scFv motif to MPGH increased its cytotoxicity, presumably by induction of cell death through activation of surface cell receptors (Nejatollahi et al., 2014). In the case of PEI, the cell viability was reduced to 15 % as the amount added exceeded 5 μ g (Fig. 9).

3.9. Cell transfection

Transfection efficiency of the peptides was measured using a firefly luciferase assay system and expressed as relative light units (RLU/s) per number of seeded cells. In a parallel study, the transfection efficiency of MPGH into HEK293T was measured, showing that this vector had good efficiency (Fig. 10A) (Majidi et al., 2016). Transfection efficiency of MPGH in BT-474, which is known as a difficult cell line to transfect (Pietilä, 2012), was very low. To investigate the efficiency of the targeting motif, the transfection was performed with MPGHT. The optimum ratio of biopolymer to plasmid for maximum gene transfer efficiency was determined by transfecting cells with fusion vectors/plasmid complexes at various N/P ratios. The highest rate of gene transfection efficiency was observed at N/P 15 (Fig. 10B). The luciferase activity in cell lysate transfected with MPGHT was 60 to 80 fold higher than MPGH in different assays. It should be noted, due to the presence of some free scfv in MPGHT upon autodigestion; we have already had anti-HER2 single chain peptide in our mixture. It may be suggested that if it had not been for the presence of free scfv in the mixture much higher transfection efficiency even in lower N/P ratio would have been obtained upon transfection by MPGHT in HER2 positive cells. Almost no transfection was observed in cells treated with naked plasmid. PEI at an N/P ratio of 12 (the highest transfection efficiency) was used as positive control. The HER2-negative cell line (MDA-MB-468) was transfected to examine HER2-targeted and cell-selective gene transfer. The

results showed that the percentage of transfected cells was low for both fusion vectors (Fig. 10C).

4. Conclusion

Recombinant peptide-based vectors, consisting of several functional domains, have been considered as an alternative to cationic synthetic polymers. Due to progress in recombinant DNA technology, the production of recombinant peptide-based vectors can be achieved at lower cost (Canine and Hatefi, 2010). A specific set of properties is critical for a polymer to be a gene carrier. Cell-penetrating peptide (CPP)-based delivery systems with combination of targeting ligand provides an effective tumor specific strategy to deliver nucleic acids into HER2 positive breast cancer cell lines (Govindarajan et al., 2012). Four kinds of functional motif in our construct were combined into a single-chain vector that allows targeted delivery of genes into the desired cell lines: DNA binding motif of 16mer histone H1, the scFv of anti-HER2 antibody, fusion sequence of HIV gp41 and a hydrophilic domain derived from the nuclear localization sequence (NLS) of SV40 T-antigen.

Taken together, these results demonstrate that the fusion vector MPGHT is bifunctional, the scFv portion can recognize HER2, and the MPGH portion can bind to DNA. MPGH was designed for transfection of the interested gene into normal cells. By addition of a targeting motif to MPGH, the efficiency of transfection of BT-474 was increased compared to MPGH alone. MPGHT and MPGH have shown 75% and 5% of PEI transfection for a desired gene into BT-474 HER2-positive cell line; respectively. No remarkable transfection was shown for both fusion vectors in the MDA-MB-468 cell line. Overall, according to the results of the present manuscript, it may be concluded that a multi-domain vector based on biomimetic properties can be designed and successfully used in targeted cell transfection.

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Figure legends

Figure1. Schematic representation of recombinant fusion-proteins.

MPG: Gp41; glycine-rich region of the fusion sequence of HIV gp41 is GALFLGFLGAAGSTMGA (green), linker is WSQ sequence (orange), nuclear localization signal (NLS) of the SV40 large T antigen is PKSKRKV(blue), **MPGH:** (Histone H1)₂ which is two repeating units of histone H1(red) was added to MPG, **MPGHT:** scFv anti ERbB2(gray) was added to MPGH.

Figure 2. (A) The coomassie blue stained proteins (lane: 1, 2, 3) and western blot with anti-6XHis antibody against His-tag of them (lane: 4, 5, 6) in 15% SDS-PAGE. The calculated molecular weight of the MPGHT was 38075 Da, the calculated MW of MPGH and scFv was 9815 Da and 29069 Da respectively. 1, 4) MPGHT, 2, 5) MPGH 3, 6) scFv. It is possible that the fusion proteins had degraded automatically.

Figure 3. Agarose gel electrophoresis (1%) indicates the mobility pattern of different N/P ratios of A) MPGHT and *psiCHECK* plasmid DNA; lane 1: naked plasmid, lane 2: N/P = 0.25, lane 3: N/P = 0.5, lane 4: N/P = 1, lane 5: N/P = 2, lane 6: N/P = 4, lane 7: N/P = 6, lane 8: N/P = 8, lane 9: N/P = 10, lane 10: N/P = 12, lane 11: Ladder. And B) MPGH and *psiCHECK* plasmid DNA; lane 1: naked plasmid, lane 2: N/P = 0.25, lane 3: N/P = 0.5, lane 4: N/P = 0.75, lane 5: N/P = 1, lane 6: N/P = 10, lane 7: N/P = 15. C) scFv and *psiCHECK* plasmid DNA; lane 1: naked plasmid, Lanes 2 through 11 are N/P = 1, 4,8,10,12,14,16,18,20,30 respectively, lane 12: DNA Ladder.

Figure 4. The particle size, zeta potential of A) MPGHT /pDNA; B) MPGH/pDNA; nanoparticles over a range of N:P ratios (0 - 20).

Figure 5. A) Serum stability assay of MPGHT, lane 1: DNA Ladder, lane 2; naked plasmid DNA, lane 3: naked plasmid DNA in the presence of 10% serum which is degraded by the nucleases, lane 4: peptide/plasmid complex (N/P 15) in the absence of serum, lane 5: peptide/plasmid complex (N/P 15) in the presence of serum, lane 6: recovered plasmid DNA from the complex. B) Serum stability assay of MPGH, lane 1: peptide/plasmid complex (N/P 15) in the absence of serum, lane 2; peptide/plasmid complex (N/P 15) in the presence of serum, lane 3: recovered plasmid DNA from the complex. Samples were electrophoresed on 1% agarose gel in TAE buffer and visualized by staining with ethidium bromide.

Figure 6. Hemolytic activity of uncomplexed A) MPGHT, B) MPGH in 10, 30 μg at pH 7.4 and 5.4. The hemolysis percentage of them was conveyed as relative to fully lysed cells by Triton X-100 (defined as 100%). The data is reported as mean $\pm$ s.d., n = 3.

Figure 7. A) Ability of FITC-conjugated scFv to bind to HER2 (+) BT474 cells were detected by flow cytometric analysis. MDA-MB-468 used as negative control. 1, 3, 5 show BT-474 and 2, 4, 6 show MDA-MB-468. 1 and 2 were obtained with 10 $\mu\text{g}/\text{ml}$, 3,4 with 50 $\mu\text{g}/\text{ml}$, 5,6 with 100 $\mu\text{g}/\text{ml}$ of purified FITC conjugated scFv. B) Mean fluorescence intensity of FITC conjugated Anti-HER2 scFv for BT-47T and MDA-MB-468 cell lines. C) Ability of FITC conjugated MPGHT to bind to HER2 (+) BT474 cells were detected by flow cytometric analysis. 5 and 50 $\mu\text{g}/\text{ml}$ purified FITC-conjugated MPGHT were incubated with HER2 expressing BT474 cells respectively in 1 and 2.

Figure 8. Bright green membrane was staining with FITC conjugated MPGHT. No accumulation was shown for FITC conjugated MPGH at the cell surface. The nucleus was counter stained with DAPI (blue fluorescence).

Figure 9. Cell viability profile of A) the fusion vectors/pDNA nanoparticles in different N:P ratio and B) uncomplexed fusion vectors in different concentration. In both experiments we assume untreated cells as 100% viability. PEI and naked DNA were used as a negative and positive control respectively. The data are shown as mean $\pm$ s.d. (n = 3).

Figure 10. A) Transfection of *psiCHECK* plasmid using MPGH/pDNA in HEK293T. B) Transfection of *psiCHECK* plasmid using MPGHT/pDNA, MPGH/pDNA in BT474 as HER2 positive cells in different N/P ratios. C) Transfection of *psiCHECK* plasmid using MPGHT/pDNA, MPGH/pDNA and PEI/pDNA nanoparticles in different cell lines BT474 as HER2 positive cells, MDA-MB-468 as negative one. PEI was used in N/P ratio of 12 while N/P ratio 15 was used for fusion vectors.

Fig. 1

Fig. 2

A)

B)

C)

Fig. 3

A)

B)

Fig. 4

A)

B)

Fig. 5

A)

B)

Fig. 6

A)

1)

2)

3)

4)

5)

6)

B)

C)

1)

2)

Fig. 7

Fig. 8

A)

B)

Fig. 9

A)

B)

C)

Fig. 10