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Selective suppression of tumor cells by a tumor-specific bicistronic lentiviral vector

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Abstract: Lentiviral gene delivery is now considered as a major candidate in the future of cancer therapy. To avoid common side-effects associated with methods of cancer therapy, the survivin promoter shows great promise due to its high expression level in multiple cancers. In this research, using a 2A peptide, Noxa was coexpressed with hemagglutinin neuraminidase (HN) driven by the survivin promoter (Surp). Coding genes of Noxa and HN were connected to the upstream and downstream of a 2A peptide using SOE PCR. After cloning of *Noxa-2A-HN* and *Surp* into the lentiviral vector, the final construct was transfected to SK-BR3 tumor cells and human embryonic kidney 293T cells. Quantitative real-time PCR implied efficient transfection and integration of the lentiviral vector in target cells. Western blotting analysis was performed to verify the expression of Noxa and HN and we determined cell viability by MTT calorimetric assay after transfection. Our results showed that the expression of Noxa and HN, though not utterly specific, was relatively higher in tumor cells and led to the selective suppression of tumor cells, which eventually resulted in a cell survival reduction of 76%. In conclusion, the novel pLN2AH-Surp lentiviral vector proved the potential for further in vivo and clinical experiments.

Key words: Lentiviral, gene delivery, Noxa, survivin, tumor cells

1. Introduction

Gene therapy plays an imperative role in the postgenomic fight against cancer. Because of the costs and time involved in producing and manufacturing monoclonal antibodies as the primary biological agents for cancer therapy and the indefinite demand for follow-up treatment, alternative platforms including immunotherapy and genetic delivery of therapeutics have been extensively investigated in recent years (Kafri et al., 2000; Liechtenstein et al., 2013). With their clinical studies recently having started (Levine et al., 2006), lentiviral vectors are now considered as effective means for the delivery, integration, and expression of exogenous genes in mammalian cells, providing attractive gene delivery vehicles in the context of both dividing and nondividing cells. These vectors have proven to be useful in stably transducing brain (Naldini et al., 1996), liver (Kafri et al., 1997), heart (Zhao et al., 2002), and retinal (Miyoshi et al., 1997) cells in vivo without toxicity or immune responses. Various strategies are applied in viral vectors to coexpress multiple genes, including dual promoters, mRNA splicing, internal ribosomal entry sites (IRES), and fusion proteins. Due to several disadvantages attributed to the mentioned methods, other possibilities have been investigated, of which viral-based 2A peptides have proven to be the best option (Luke, 2012; Yan et al., 2015). Many positive-strand RNA viruses including foot-and-mouth disease virus (F2A), porcine teschovirus-1 (P2A), and *Thosea asigna* virus (T2A) generally contain 2A or similar peptide coding sequences to mediate protein cleavage through a theoretically described ribosomal skip mechanism in order to produce several proteins from a single polypeptide (Luke, 2012). The cleavage occurs at the C-terminal end of the 2A peptide, leaving it fused to the upstream protein and releasing the downstream protein with the addition of an N-terminal proline, which generally does not appear to significantly affect the function of the fused protein. Previously, different 2A peptides have been successfully incorporated into AAV and retroviral vectors to construct multicistronic vectors (Yang et al., 2008; Li et al., 2012; Dong et al., 2015).

Showing high and specific activity in multiple cancers, the survivin promoter is a valuable tool in gene therapy to drive specific expression of toxic genes in cancer cells. Survivin has been efficiently used in several previous studies to specifically suppress various tumor cells with minimum impact on normal cells, which is considered an essential characteristic for an effective method of cancer therapy (Ambrosini et al., 1997; Yamamoto and Tanigawa, 2001; Chen et al., 2004).

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The *p53* tumor-suppressor gene, functionally inactivated in approximately 50% of human tumors, has been extensively studied for gene therapy purposes. Activation of *p53* by harmful stimuli such as DNA damage, hypoxia, and oncogenes normally leads to cell cycle arrest followed by DNA repair or induction of apoptosis as a cellular response. Apoptosis is induced by BH3-only proteins of the Bcl-2 family, of which Noxa and Puma have been well characterized (Shibue et al., 2006; Wang et al., 2009; Nakajima and Tanaka, 2011; Zhang et al., 2013). These two proteins function in cooperation as downstream factors to promote p53-mediated apoptotic response, with the distinction of Noxa selectively inducing apoptosis only in cancerous cells upon increased expression of other proapoptotic factors such as Bax and consequently more cell sensitivity, whereas Puma induces apoptosis in both transformed and untransformed cells. Thus, in contrast to most suicide genes commonly used in gene therapy applications, Noxa shows negligible side-effects on normal cells (Shibue et al., 2003, 2006; Suzuki et al., 2009). In this experiment, Noxa was introduced in combination with the hemagglutinin-neuraminidase (HN) of Newcastle disease virus (NDV) to enhance the inhibitory effects. As an immune adjuvant, by hydrolyzing sialic acid on the surface of host cells, HN can decompose sialic acid-containing receptors, expose the biological recognition sites of host cells, stimulate innate immune responses through increased natural killer (NK) cell activity in the tumor niche, and, finally, promote the release of new virus particles from the infected cell membrane (Ni et al., 2010, 2011; Ji et al., 2013; He et al., 2014).

Expecting the potential synergistic effect of survivin as a tumor-specific promoter and *Noxa* as a tumor-specific suicide gene coupled with HN as a molecular adjuvant, we hoped to achieve maximal and minimal apoptotic induction respectively in tumor cells and normal cells.

2. Materials and methods

2.1. Plasmids

The coding sequences of *Noxa*, *hemagglutinin neuraminidase* (*HN*), and *survivin promoter* (*Surp*) were PCR-amplified by proper primers. Using SOE PCR, amplified genes of *Noxa* (192 bp) and *HN* (1764 bp) were connected to the upstream and downstream of a 2A connecting peptide, respectively, to form the *Noxa-2A-HN* fusion gene of 1956 bp. Construction of the lentiviral vector pLN2AH-Surp was accomplished by cloning 1430 bp of the *Surp* gene, which was cloned into the vector, cutting out the original CMV promoter, using *BamHI* and *SfiI* restriction enzymes on both the vector and the amplified *Surp* gene. The order of genes and the survivin promoter is illustrated in Figure 1. Two helper plasmids, pMD2.G and psPAX2 (Addgene, USA), were used in this study, which helped with the virus particle packaging.

2.2. Cell culture

The human breast cancer cell line SK-BR-3 and human normal embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% FBS (Sigma-Aldrich, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin and incubated in 5% CO_2 at 37 °C. During passages, the cells were rinsed twice with 3 mL of PBS buffer (room temperature). To stablish the recombinant stable cell lines, 1.5 µg/mL puromycin was added to the culture medium. Digestion was conducted with a 0.25% trypsin solution at 37 °C for 2–3 min and terminated with 3 mL of DMEM culture solution (10% FBS). The day before transfection, 4×10^6 HEK-293T or SK-BR-3 cells were seeded in 100-mm culture dishes containing 10 mL of DMEM. On the day of transfection, the culture medium was replaced with 10 mL of normal growth medium containing serum without antibiotics. The respective cell lines were harvested at 1000 rpm and tested for cell viability and apoptosis at 1, 2, and 4 days after transfection.

2.3. Recombinant lentivirus packaging and titration

The lentiviral vector was packaged adopting the three-plasmid packaging system (Durocher et al., 2002). After purifying the three plasmids to adequate concentrations using the PureLink Quick Plasmid Miniprep Kit (Invitrogen, USA), a mix of the three plasmids and appropriate buffers was made as follows: a solution of 0.5 mL was first prepared consisting of 10.5 µg of shuttle plasmid pMD2.G, 21 µg of packaging plasmid psPAX2, 21 µg of pLN2AH-Surp (or pLEX as a control empty vector) plasmid DNA, and 125 µL of 2 mM CaCl2 and 0.5 mM HEPES in deionized distilled water, which was then added dropwise while vortexing to an equal volume of 2X HBS for a total of 1 mL. This mix was added to dishes at an MOI of 100 (100 viral genomes per cell), determined by real-time PCR using the primers FNox: 3'-atgcctgggaagaaggcgcg-5' and RNox: 3'-tcaggttcctgagcagaagag-5', as described elsewhere (Salmon and Trono, 2007), and the cells were maintained in 5% CO_2 at 37 °C. After 24 h, the transduced cells were trypsinized, collected at 1000 rpm, and resuspended with DMEM. The packaged recombinant lentiviruses were harvested from the supernatant of cell

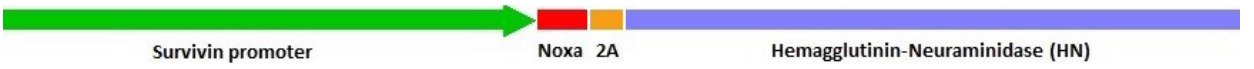

Figure 1. Diagram of the Noxa-2A-HN construct under the control of the survivin promoter.

cultures at 48 h after transfection and the lentivirus RNA was prepared with the RNeasy Mini Kit (QIAGEN, USA) and then treated by DNase I (Roche, the Netherlands) digestion.

2.4. Expression analysis

Quantitative real-time PCR was performed on extracted RNA of respective transformed cell lines to confirm the expression of the *Noxa*-2A-*HN* gene using specific primers LXG-F: 5'-attcacaaccgtagtatcca-3' and LXG-R 5'-cactcagacatccatacactgacg-3' and relative expression values of the target gene in different cell lines were calculated using the delta-delta CT method. For western blotting analysis, protein extracts of stably transduced and control cells were prepared in PBS supplemented with Complete Mini EDTA-Free Protease Inhibitor (Roche, Germany) through one freeze/thaw cycle. Equal amounts of total protein, as assayed by Bradford quantification, were separated in 12% sodium dodecyl sulfate polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Blocking of the membrane, dilution of mouse anti-His antibody with the sealing solution at a ratio of 1:500, washing of the membrane with PBS, reaction with the enzyme-labeled antimouse goat secondary antibody, and DAB color development were performed to analyze the expression of His-tagged HN and Noxa.

2.5. Cell viability test

The recombinant lentiviruses were added to a 96-well plate lined with SK-BR-3 and HEK-293T cells at an MOI of 100 and cultured at 37 °C for 48 h to determine cell viability.

2.5.1. MTT assay

Cells were treated according to the manufacturer's instructions (Roche, the Netherlands), and the optical density at 490 nm was detected with ELISA at various times after infection.

2.5.2. DAPI staining assay

After centrifugation at 10,000 rpm for 5 min, harvested transduced cells were fixed in a solution containing 3.7% formaldehyde, 0.5% Nonidet P-40, and 10 μg/mL 4',6-diamidino-2-phenylindole (Promega, USA) in PBS. Following 15 min of incubation in the dark, fluorescent microscopy was used to detect apoptotic cells.

3. Results

3.1. Cloning

SOE PCR was used to connect *Noxa* and *HN* genes flanking the 2A peptide sequence. As shown in Figure 2, PCR amplification and successful cloning of *Noxa*, *HN*, and *Surp* into the lentiviral vector was confirmed by agarose gel electrophoresis.

3.2. Expression analysis

The results of RT-PCR implied efficient viral load and transfection of the retroviral vector pLN2AH-Surp in SK-BR-3 and HEK-293T cells. Real-time quantitative PCR was also implemented to analyze the expression level of the two transgenes in tumor cells, which resulted in 22% expression in normal cells relative to tumor cells as displayed in Figure 3A. Western blotting analysis further confirmed the expression of Noxa (6 kDa) and HN (63 kDa) proteins and the proper cleavage of the 2A peptide in both cell lines, as shown in Figure 3B.

3.3. Cell viability assay

Following transfection, we assessed the viability of the transfected cells at various times (day 1, day 2, and day 4) to calculate the inhibitory effect of the recombinant lentiviral vector on tumor cell growth. Based on the results of the cellular survival assays, as illustrated in Figure 4, it was inferred that normal cells are hardly sensitive to the Noxa-HN-expressing lentiviral vector driven by the survivin promoter; meanwhile, a contrary response of the tumor

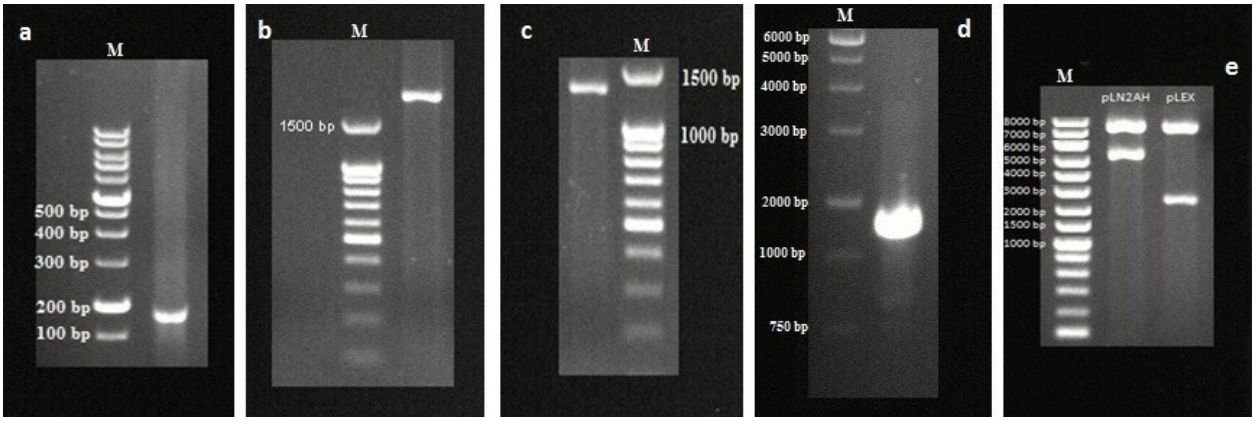

Figure 2. Cloning steps to construct lentiviral vector pLN2AH-Surp: (a) PCR amplification of *Noxa*, (b) PCR amplification of *HN*, (c) PCR amplification of *Surp*, (d) *Noxa-2A-HN*, the product of SOE PCR, (e) digestion of empty pLEX vs. pLN2AH-Surp with *NotI*/*NrulI*; M: DNA marker.

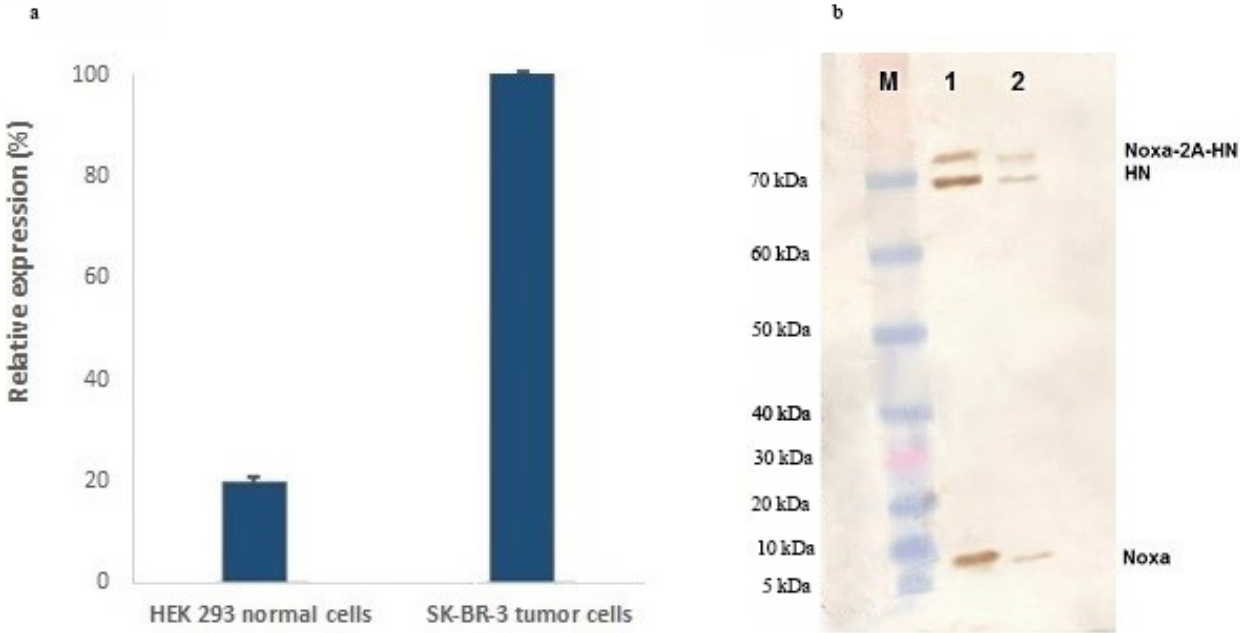

Figure 3. Expression analysis of the Noxa-2A-HN construct: (a) relative gene expression levels of Noxa-2A-HN in normal and tumor cells by quantitative RT-PCR indicates much higher expression of the recombinant gene in tumor cells driven by the survivin promoter; (b) western blotting analysis of SK-BR-3 tumor cells and HEK-293T normal cells transfected with lentiviral pLN2AH-Surp vector verified the expression of Noxa and HN and their separation by the 2A peptide in both cell lines; M: protein marker, Lane 1: SK-BR-3 cells, Lane 2: HEK-293T cells.

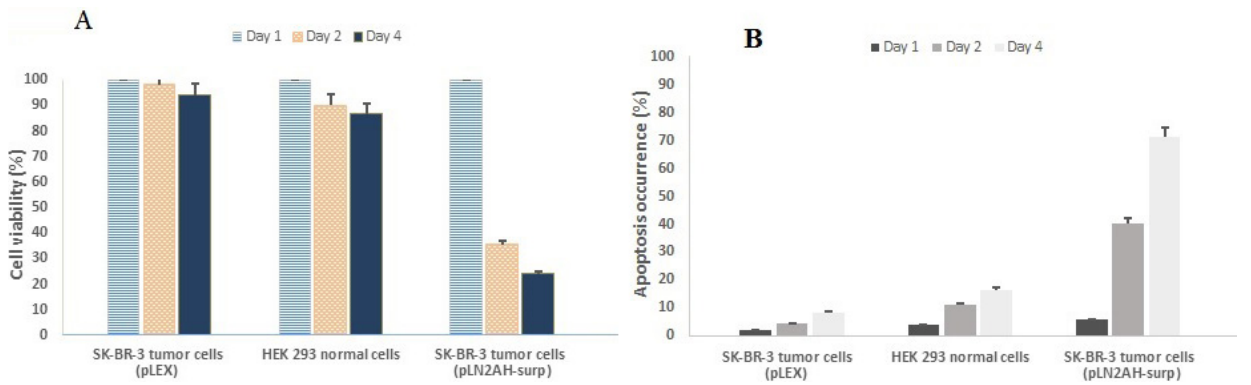

Figure 4. Cell survival assay of SK-BR-3 tumor cells transfected with either pLEX or recombinant pLN2AH-Surp vector and HEK-293T normal cells transfected with pLN2AH-Surp vector: A) MTT assay shows selective restriction of tumor cell growth after transfection with recombinant lentiviral pLN2AH-Surp vector and trivial effect of the construct on normal cells viability; B) DAPI staining results indicate higher apoptosis occurrence in SK-BR-3 tumor cells transfected with recombinant pLN2AH compared to normal and pLEX transfected cancer cells.

cells to the construct eventually resulted in cell survival reduction of 76%. The results obtained from DAPI staining indicated that 71% of tumor cells underwent apoptosis at 4 days after transfection; conversely, only a slight percentage of normal cells (16.5%) and pLEX transfected tumor cells (8.2%) were apoptotic at that time.

4. Discussion

Finding effective therapeutic platforms to treat cancer, as a significant threat to public health, has been the primary objective of scientists in recent years. DNA vaccines provide a valuable means to deliver exogenous genes to malignant or normal cells with lentiviral vectors considered as major

candidates (Yang et al., 2014). Several suicide genes have been reported to trigger apoptotic effects when delivered to tumor cells. Although similar apoptotic proteins such as Puma and t-Bid are also potent in the induction of apoptosis (Zhang et al., 2013), Noxa is currently the only example of this class of proteins with selective activity in cancerous cells causing no damage to normal cells (Albert et al., 2014). It has been proposed that Noxa may require a more sensitive cell state wherein the expression of other apoptotic factors such as Bax have increased (Shibue et al., 2003; Nakajima and Tanaka, 2011; Zhang et al., 2013). Suzuki et al. tested adenoviral, polymeric, and TAT fusion of Noxa on different malignant cell lines, which resulted in selective elimination of tumor cells (Suzuki et al., 2009).

Employing a tumor-specific promoter is another method to guarantee the selective expression of transgenes in cancerous cells and thus reduce the side-effects on normal cells. Bao et al. proposed cancer-specific expression of the survivin promoter to be a useful means in prognosis and gene therapy of multiple cancers (Bao et al., 2002). Along with Noxa, we coexpressed hemagglutinin neuraminidase as a molecular adjuvant to impair the cell membrane and help with the easy detection of tumor cells by the immune system (Ni et al., 2010; Ji et al., 2013), which will play a more important role during in vivo experiments (Ni et al., 2011; He et al., 2014). Undesirably low expression levels of the downstream gene in IRES-based bicistronic expression systems, possible interference problems associated with using two different promoters, and spatial limitations of fusion proteins have introduced 2A peptides as our best chance to coexpress transgenes in mammalian cells (de Felipe et al., 1999; Luke 2012). Expression of multiple genes linked with 2A peptides has previously been reported in retroviral constructs (Minskaia et al., 2006; Yang et al., 2008; Li et al., 2012; Dong et al., 2015). Since tumor cell targeting using protein ligands may lead to systemic immune responses, applying specificity at the genetic level via tumor-specific genes and promoters can be very useful to elude the damage imposed by common methods of cancer therapy (Yamamoto and Tanigawa, 2001; Chen et al., 2004).

In the present study, we developed a highly specific bicistronic lentiviral vector coexpressing the suicide gene Noxa along with HN, under the control of the tumor-specific survivin promoter. Data gathered by western blotting and real-time PCR indicated efficient expression of the two transgenes driven by the survivin promoter in SK-BR-3 cancer cells and relatively low expression levels of these proteins in normal HEK-293T cells leading to a much lower death rate in the latter, although the general expression level of Noxa and HN in normal cells was higher than expected, resulting in the occurrence of a slight percentage of apoptosis and population decrease in normal cells, which may be interpreted as unspecific function of HN, following the basic expression in this cell line.

Cell survival results showed a 56% reduction in tumor cells' viability at 2 days after transfection, whereas after 4 days, 76% reduction was achieved, which highlighted better results of the construct over time. The low apoptosis and death rate of pLEX transfected control tumor cells further supports the effectual role of the cancer-specific elements in the tumor-killing property of the construct. Additionally, it is noteworthy that the two analyzed cell lines are from the same origin but not the same tissue, presenting analysis of a broader spectrum of cells with different markers and surface properties as potential hosts of the construct; further examinations of systematic effect of the recombinant lentiviral vector seems logical.

In conclusion, our results corroborate previous reports (Suzuki et al., 2009; Nakajima and Tanaka, 2011; Albert et al., 2014) suggesting that Noxa can be effectively used in cancer gene therapy with negligible effect on normal cells. The combination of Noxa, HN, and the survivin promoter as cancer-specific elements in the novel pLN2AH-Surp lentiviral vector proved the potential for further in vivo and clinical experiments.

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