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Original Article

Downregulation of PI3Kcb utilizing adenovirus-mediated transfer of siRNA attenuates bone cancer pain

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Abstract: Phosphatidylinositol 3-kinase (PI3K) signaling plays a pivotal role in intracellular signal transduction pathways involved in chronic pain states. PI3K is implicated in pathomechanisms of enhanced synaptic strength, such as wind-up and central sensitization in the spinal dorsal horn. The PI3Kcb gene encoding the class 1A PI3K catalytic subunit p110beta is one of the most important molecular of the P13K signaling pathway. Here, we used small interfering RNA (siRNA) targeted to PI3Kcb by adenovirus-mediated transfer, to determine whether inhibition of PI3Kcb was a potential therapeutic target for bone cancer pain (BCP). In this study, treatment of BCP model in rats with PI3Kcb-specific siRNA resulted in inhibited pain-related behavior. Depletion of PI3Kcb decreased the protein levels of spinal PI3Kcb and phospho-Akt (P-Akt)-downstream targets of PI3K. Knockdown of PI3Kcb by siRNA also induced decreased expression of GFAP and OX42, suggesting that the upregulation of spinal PI3Kcb may increase glia excitability, at least in part by regulating glia message. Our findings suggest that siRNA-mediated gene silencing of PI3Kcb may be a useful therapeutic strategy for BCP.

Keywords: Bone cancer pain, phosphatidylinositol 3-kinase, phospho-Akt, small interfering RNA, adenovirus vector

Introduction

Pain associated with cancer that metastasizes to bone results in very poor quality of life, and its mechanisms are still not completely understood [1-3]. To clarify the pathomechanisms of bone cancer pain, rat models of bone cancer pain (BCP) using mammary gland carcinoma cells (MADB-106 cells) have been established [4, 5], and increasing evidence indicated that some signal pathways were involved in BCP [6-8].

The phosphatidylinositol kinase-3(PI3K) family of lipid kinases has been implicated in the transduction and regulation of pain signal acting as secondary messengers [9-15]. Mammalian PI3Ks are categorised into three classes, with the class-IA PI3Ks comprising a 110-kDa catalytic subunit (p110α, p110β or p110δ, encoded by the genes Pik3ca, Pik3cb and Pik3cd, respectively) coupled to a regulatory subunit (p85) [16-21]. These subtypes are thought to be relative to the activation of the receptors possessing protein-tyrosine kinase activity or the receptors coupling to Src-type protein-tyrosine kinases by phosphatidylinositol (3,4,5)-trisphosphate [22, 23]. Several lines of evidence suggested that PI3K and PI3K-PKB/Akt signal pathway activation contributed to the development of neuropathic pain [10, 24, 25]. So, development of knockdown for PI3K pathways to target neurons and glial cells may lead to new therapeutic strategy for pain management.

The small interfering RNA (siRNA) strategy is a better gene therapy technique and could be a useful tool for the study of endogenous gene regulation [26-28]. Recently, shRNA for p110β induced apoptosis and proliferation arrest in tumor cell lines [26, 29, 30]. Because targeted disruption of p110β caused death at the early embryonic stage [31, 32], this isoform of PI3K in signaling is poorly understood. Roles for PI3K catalytic beta polypeptide (PI3Kcb) in mediating chronic refractory pain have not been addressed. Thus, we pursued PI3Kcb as a potential therapeutic target for bone cancer pain. In this study, we used siRNA against

PI3Kcb to clarify whether specific down-regulation of PI3Kcb induced antinociception properties in bone cancer pain and to identify the downstream targets of this molecule.

Materials and methods

Cell preparation and intra-tibial injection

MADB-106 mammary gland carcinoma cells were donated by Page GG and LY Liu and prepared as described previously [5, 33]. Briefly, cells were grown in 75 cm^2 flasks containing RPMI 1640 (Gibco, Invitrogen Ltd.) with 10% fetal bovine serum, 1% L-glutamine and 2% penicillin/ streptomycin (Gibco, Invitrogen Ltd.). By creating a cell suspension with trypsin (0.1%w/v), they were then quenched with an equal volume of 10% fetal calf serum and centrifuged for 3 min at 1200 rpm. The resulting pellet was washed twice with 10 ml of Hank's balanced salt solution (Gibco, Invitrogen Ltd.) without Ca^{2+}, Mg^{2+}, and finally suspended in 1 ml Hank's solution. Cells were diluted in Hank's medium to the required concentration for injection and kept on ice. Rats were anesthetized and 3 μL solution was injected into the tibial cavitas medullaris of the left hind paw using an insulin syringe with a 29.5 gauge needle, as described previously [33, 34].

Recombinant adenoviral vectors

Construction of recombinant adenoviruses was described previously [35, 36]. Briefly, A specificity target sequence (5'-AAACTGCCGTATATGAGGAAC-3') from PI3Kcb mRNA sequence was designed according to the principle of design on siRNA, and PI3Kcb gene primer sets (Si-sense: 5'-GATCCAAACTGCCGTATATGAGGAACGAGTACTGGTTCCTCATATACGGCAGTTTTTTTTA-3'; Si-anti: 5'-AGCTTAAAAAAAACTGCCGTATATGAGGAACCAGTA CTCGTTCCTCATATACGGCAGTTTG-3') were designed via GenBank accession number NM_053481. PI3Kcb cDNA was cloned by PCR technique, and then the correspond hairpin-shaped DNA fragment was synthesized in vitro,annealed and handled with added A (adenosine acid). The product was cloned into siRNA vector pDC316-EGFP-U6. The insertion plasmid pDC316-PI3KcbsiRNA-EGFP was identified by PCR, and then DNA sequencing demonstrated the correct orientation and sequence. The reaction product was transformed into E. col i (DH5α), and the positive bacterial colony was selected. The recombinant eukaryotic expression vector pDC316-PI3KcbsiRNA-EGFP was identified by using restriction endonucleases digestive reaction and DNA sequencing. The HEK 293 cells were cotransfected by the shuttle plasmid of pDC316-PI3KcbsiRNA and the skeleton plasmid of pBHG35, and the recombinant plasmid of Ad5F35-PI3KcbsiRNA was obtained. The expression of the transfected genes was evaluated by PCR and immunocytochemical stain. Virus was purified by double CsCl centrifugation and subsequently dialysed as described previously [37-39]. Final yields as assessed by plaque assays on 293 cells were approximately 1×10^{12} plaque forming units (pfu)/ml [31]. Ad5F35 and Ad5F35-PI3KcbsiRNA stock solutions were stored in aliquots at -80°C.

Animals and intrathecal catheter implantation

Adult female rats weighing 250-300 g were housed on a 12-h light/12-h dark cycle and controlled temperature with free access to food and water. Efforts were made to minimize animal discomfort and reduce numbers of animals used. All experiments were carried out according to the National Institute of Health Guide for the Care and Use of Laboratory Animals [40], and the Institutional Animal Care and Use Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology University approved this study protocol.

The implantation technique of the intrathecal catheter was modified and performed [41-43]. A polyethylene-10 catheter was inserted into the subarachnoid space under pentobarbital anesthesia (50 mg/kg, i.p.). The catheter was passed 8.5 cm caudally to the level of the lumbar enlargement through an incision in the atlanto-occipital membrane. The external part of the catheter was tunneled subcutaneously to exit at the top of the head. The skin was closed with 3-0 silk sutures. After surgery, rats were housed in individual cages. To avoid occlusion of the catheter, 10 μl of normal saline was injected via a catheter on alternate days until the end of the experiment. The marker of successful catheterization was that rats showed no impaired movement or lower limb paralysis within 30 s after 2% lidocaine (10 μL) was injected intrathecally.

Rats were randomly assigned to five groups (10 in each). Group 1 received neither intra-tibial operation nor intrathecal catheter implantation, and served as Naive group. Group 2 received between intra-tibial injection of 3 μL Hank's solution and intrathecal injection of 10 μl artificial cerebrospinal fluid (aCSF), and served as Control group. Group 3 received between intra-tibial injection of 3 μL MADB-106 (7.2×10^9 cells μL) and intrathecal injection of 10 μl aCSF, and served as the BCP group. While rats in group 4 and 5 received intra-tibial injection of 3 μL MADB-106 as well as implanted intrathecally with Ad5F35, Ad5F35-Pik3cbsiRNA (10 μl), which served as Ad5F35 and PI3KcbsiRNA group, respectively. All groups received intra-tibial injection 5 days after intrathecal catheter implantation. From the 7^{th} day of intra-tibial injection, Ad5F35 and Ad5F35-PI3KcbsiRNA were injected intrathecally into group 4 and 5 respectively, and injections were given daily for six consecutive days.

Behavioral testing

Animals were acclimated to the testing room for 30 min in individual Plexiglass test chambers with wire mesh floors. Tactile sensitivity was evaluated with von Frey filaments (Stoelting, Wood Dale, IL, USA) having buckling forces between 0.4 and 15 g, and applied perpendicularly to the mid-plantar surface of the ipsilateral hind paws of intra-tibial injection in rats, with sufficient force to bend the filament slightly for 5 s. The threshold was determined using the up-down testing paradigm. Rapid lifting of the hindpaw or licking and vigorously shaking in response to stimulation were regarded as a positive responses [44]. The 50% paw withdrawal threshold (PWT) was calculated using the nonparametric Dixon test [45, 46]. Any rat with a basal paw withdrawal threshold below 10 g on either paw was excluded from the study. All testing was performed by an experimenter who was blinded to the contents of the intrathecal injection.

Quantitative PCR

Following the last behavioural testing day (day 14 after intra-tibial injection), animals (n = 6) were decapitated and L4-6 lumbar spinal segments were harvested and kept at -80°C. The methods for quantitative PCR (Q-PCR) were essentially as described previously [7, 47]. Briefly, RNA was extracted from frozen spinal segments. RNA was isolated using RNeasy columns (Qiagen), including an on-column DNase step. Reverse transcription was carried out on 1 μg RNA using the iScript kit with random primers (BioRad, Hercules, CA). Q-PCR was performed with an iCycler (BioRad) using the iQ SYBR supermix (Biorad). The Q-PCR primers were used: Rat PI3Kcb, 5'-GAAGATTGCAAGCAGTGATAGTGC-3' (forward) and 5'-CCTATCCTCCGATTACCAAGTCGTC-3' (reverse); rat GAPDH, 5'-AC-CACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. Quantitative PCR was carried out with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR reactions were prepared using the components from the Platinum qRT-PCR kit and assembled according to the manufacturer's instructions (Qiagen). The threshold cycle, C_T, which correlates inversely with the levels of target mRNA, was measured as the number of cycles at which the reporter fluorescence emission exceeds the preset threshold level. The amplified transcripts were quantified using the comparative C_T method [48], with the formula for relative fold change = 2^{-DDCT}. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimmers. Each experiment was run three times and each sample was run in triplicate.

Western blots

Spinal tissues were collected in the same way as the procedure of quantitative PCR. Tissues were homogenized in extraction buffer containing protease and phosphatase inhibitors (Sigma, St. Louis, MO, USA), 0.5% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 3% sodium dodecyl sulfate (SDS). The homogenate was centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant was used for Western immunoblotting. The protein concentration of the supernatant was determined using a bicinchoninic acid (BCA) kit (Pierce Biotechnology Inc., Rockford, IL, USA). Equivalent amounts (20 μg) of protein from each sample were loaded into a Nu-PAGE 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA) and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in Tris-HCl buffer containing 0.1% Tween 20, pH 7.4 (TBS-T) for 1 h at room temperature and then incubated

Table 1. Immunohistochemical results for GFAP and OX-42 activation in spinal cord from the tested rats on days 14 after operation

Group	Astrocyte	Microglia
Naive	0	0
Control	0	0
BCP	+++	+++
Ad5F35	+++	+++
PI3KcbsiRNA	+	+

Note: Scoring is as follows: 0, normal unactivated tissues; +, mild activation; ++, moderate activation; +++ intense activation. There was no significant difference for GFAP and OX-42 immunostaining both in spinal dorsal horns between rats from BCP group and Ad5F35 group, where there was significant difference for GFAP and OX-42 immunostaining both in spinal dorsal horns between rats from BCP group and PI3KcbsiRNA group.

overnight at 4°C with rabbit anti-P-Akt ser 473 (1:1000). The membrane was washed with TBS-T and then incubated with goat anti-rabbit HRP (horse-radish peroxidase)-linked secondary antibody (Cell Signaling) for 1 h on the next day. After incubation the membrane was exposed to SuperSignal West Femto substrate (Pierce Biotechnology, Inc.) to enhance the signal. Following exposure to X-ray film, membranes were stripped and reprocessed for one more protein of interest and then for β-actin (mouse anti-β-actin, 1:10,000, Sigma) as a loading control. Immunoblots were scanned and densitometric analysis performed using ImageQuant (AmershamBiosciences, Piscataway, NJ, USA). Immunoblot density was normalized to controls run on the same gel.

Immunohistochemistry

Following the last behavioural testing day, rats (n = 4) were deeply anesthetized and transcardially perfused with room temperature heparinized 0.9% saline, which contained phosphatase inhibitors (Sigma) followed by chilled 4% paraformaldehyde in 0.1 M phosphate buffer. The lumbar (L4-6) spinal segments were removed and post-fixed in perfusate for 6 h and transferred, first to 20% sucrose for 4 hs and then to 30% sucrose until they sank for cryoprotection. Tissue was kept at 4°C. The fixed lumbar enlargements were embedded in O.C.T. compound (Tissue-Tek, Torrance, CA, USA) snap frozen, and transverse sections (20 μm) from L3-S1 were cut on a Leica CM 1800 cryostat. Sections were mounted on Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA, USA) and labeled with rabbit anti-OX-42 (microglia, 1:100; BioSource International, Camarillo, CA, USA) and mouse anti-glial fibrillary acidic protein (GFAP) (astrocytes, 1:500; Chemicon). After a three-minute washing in PBS, sections were incubated for 2 h at R.T. with the secondary antibody, biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, USA) for 1 h at 4°C. Immunoreactive signals were further amplified by ABC solution (1:100; Vector Laboratories Inc.) [49]. Control sections were processed similarly, except that primary antibodies were omitted. Reported results were surveyed in a minimum of four animals under each condition and clearly immunopositive cells were counted, under blinded conditions. Cells were counted following staining five or more sections from each animal. A score was observed under low and medium magnifications based on the following scale: baseline staining (0), mild response (+), moderate response (++), and intense response (+++). The criteria for each score have been described in detail previously [50-52].

Statistical analysis

Paw data were expressed as means ± S.E.M. and subjected to statistic evaluation using two-way analysis of variance (two-way ANOVA) followed by post-hoc comparison (Student-Newman- Keuls test) to confirm significant differences between groups. Values of $P < 0.05$ were considered as statistically significant.

Results

Intrathecal PI3Kcb siRNA reverses mechanical allodynia

The functional consequence of knockdown of PI3Kcb was examined in BCP rats following local delivery of siRNA-PI3Kcb to spinal cord (n = 6 each group). No significant differences in PWT to tactile stimulation were found at all time points in Naive group and Control group, respectively. No significant differences in PWT were found at all time points in BCP group and Ad5F35 group. In first six days after intra-tibial operation, PWT in BCP group was not significantly different from that in Control group ($P < 0.05$), whereas on 8-14 day after operation, differences were remarkable between the two groups ($^*P < 0.01$). On 10-14 day after opera-

tion, PWT in PI3KcbsiRNA group was not significantly different from that in Control group ($P < 0.05$), whereas significant differences in PWT were found between PI3KcbsiRNA group and Ad5F35 group ($^{*}P < 0.05$, $^{**}P < 0.01$), and there were obvious differences in PWT between PI3KcbsiRNA group and BCP group ($^{*}P < 0.05$, $^{\#}P < 0.01$).

siRNA-mediated knockdown of PI3Kcb decreased bone cancer pain-induced PI3Kcb expression

The effect of Ad5F35-Pik3cbsiRNA on spinal Pik3cb protein expression was evaluated by quantitative PCR (n = 6 each group). No significant differences in Pik3cb protein expression were found at day 14 after operation between Naive group and Control group, between BCP group and Ad5F35 group, respectively. At day 14 after operation, Pik3cb protein in BCP group evidently augmented when compared with that in Control group ($^{**}P < 0.01$), whereas Pik3cb protein in PI3KcbsiRNA group significantly decreased when compared with that in BCP group ($^{*}P < 0.01$). Pik3cb protein in PI3KcbsiRNA group was not significantly different from that in Control group at day 14 after operation ($P < 0.05$).

PI3Kcb siRNA inhibited bone cancer pain-induced spinal P-Akt expression

In order to further determine the regulation of PI3Kcb siRNA on PI3K-PKB/Akt signal pathway in the spinal cord, western blot analysis was preformed to detect changes of downstream of PI3K (n = 6 each group). As PI3K is upstream of kinase Akt phosphorylation, we also used Akt phosphorylation as an indicator of the activation of PI3K-PKB/Akt pathway. Bone cancer pain produced a large increase of protein level of P-Akt in spinal cord at days 14 after operation ($^{**}P < 0.01$, versus control group). We examined the effect of intrathecal administration of Ad5F35-Pik3cbsiRNA on protein expression by western blot analysis, and found that Pik3cbsiRNA treatment resulted in marked decreases of P-Akt protein levels at days 14 after operation ($^{*}P < 0.01$, versus BCP group or siRNAPI3Kcb group). The results confirmed that specific disruption of PI3Kcb led to the reduction of well-known PI3K downstream target, P-Akt.

Intrathecal PI3Kcb siRNA suppressed glial activation in the bone cancer pain model

A low-power image showed that the activation of GFAP and OX-42 was observed between group BCP and group Ad5F35 at days 14 after operation (n = 4 each group). And activated astrocytes demonstrated profoundly cell proliferation and hypertrophy, whereas the resting astrocytes displayed small round nuclei and slender processes. The result found that Pik3cbsiRNA treatment markedly inhibited the activation of GFAP and OX-42 at days 14 after operation. In terms of immunohistochemical scores (**Table 1**), there was no significant difference for GFAP and OX-42 immunostaining in spinal dorsal horns between BCP group and Ad5F35 group, where there was significant difference for GFAP and OX-42 immunostaining between BCP group and PI3KcbsiRNA group.

Discussion

This study investigates the utility of a vector-based approach of siRNA to knockdown PI3Kcb in the spinal cord dorsal horn (SCDH) in adult rats of bone cancer pain. The PI3Kcb gene was selected because it is essential for PI3K family functions, and the PI3K and PI3K-PKB/Akt signal pathway may play very critical roles in the production and maintain of chronic pain, which makes it feasible to assess behaviorally the consequences of a decrease in PI3K-PKB/Akt signal function.

Several recent reports have demonstrated the intrathecal utility of adenovirus vectors for antinociceptive effect in the CNS [53] and the effective method for adenoviral-mediated delivery of siRNA to repair the diseased or injured tissue [54-56]. Adenovirus vectors can be produced in high titers, and have advantages of infection of both dividing and nondividing cells, reproducibility and safety, and can transduce postmitotic neurons in the brain and SCDH in a highly spatially localized manner [53]. This study demonstrated the utility of Ad5F35 vectors for gene silencing in the CNS using siRNA technology.

In the present study, we investigated the expression both spinal PI3Kcb and P-Akt in bone cancer pain induced by intra-tibial injection of MADB-106 mammary gland carcinoma cells, and the results indicated the protein lev-

els of spinal PI3Kcb and P-Akt were significantly increased, that which in accordance with other published data, for example, the p110β subunit was associated with a more aggressive profile of breast tumors, highly related to lymph node involvement and presence of distant metastasis [9, 20, 57]. Previous work also demonstrated that peripheral inflammation and nociceptive stimulation induced the activation of PI3K and PI3K-PKB/Akt signal pathway [12], and the increase between phospho-PKB/Akt-immunoreactive positive neurons [24] and P-Akt of dorsal spinal cord homogenates [10]. Our outcomes suggested that PI3Kcb and P-Akt play a major role in bone cancer pain.

After glia activation, they could release some pro-inflammatory cytokines, which were well-known to increase neuronal excitability through a variety of post-transcriptional mechanisms [58-60] and contributed to spinal sensitization and pain behavior [28, 45]. We observed that the up-regulation of GFAP and OX-42 was distributed through SCDH to the tibia with cancer, and knockdown of PI3Kcb by siRNA induced decreased expression of GFAP and OX42, suggesting that the upregulation of spinal PI3Kcb increased glia excitability, at least in part by regulating glia message.

It was evident that siRNA-mediated knockdown of PI3Kcb did not completely block mechanical allodynia in BCP rats. Quantitative PCR revealed about 55% knock down in the spinal PI3Kcb mRNA levels of rats. This magnitude of mRNA knockdown was very similar to that observed in cancer model rats [26]. Similarly, Western blot analysis revealed nearly 60% knockdown in P-Akt protein levels in the spinal cord dorsal horn of rats. Because complete PI3Kcb knockout produced death at the early embryonic stage [31, 32], siRNA was a facile approach to selectively target PI3Kcb gene expression. Our results demonstrated that siRNA targeting PI3Kcb prevented bone cancer pain hypersensitivity, and suggesting that this approach might provide a useful approach for both target validation and therapeutic intervention for bone cancer pain at CNS sites. Virus vector-delivered PI3Kcb siRNA provided an important new tool for pain research that extends beyond the gene expression targeted in this report.

In conclusion, this study revealed that the upregulation of spinal PI3Kcb induced by bone cancer pain increased glia excitability. Our results indicated that siRNA-mediated gene silencing of PI3Kcb reversed bone cancer pain hypersensitivity, and provided a useful therapeutic strategy for bone cancer pain.

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Disclosure of conflict of interest

None.

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