132

Available online at www.sciencedirect.com

SCIENCE DIRECT

ELSEVIER

JNMU

Journal of Nanjing Medical University, 2009, 23(2):132-136

www.elsevier.com/locate/jnmu

Research Paper

Recombinant adenovirus-mediated shRNA silencing of midkine gene in BxPC-3 cells

Mingyue Xiong, Kunzheng Wang*

Department of Orthopedics, the Second Affiliated Hospital of Xi' an Jiaotong University, Xi' an 710004, China

Received 8 January 2009

Abstract

Objective: To investigate the silencing effects of recombinant adenovirus Ad-shRNA-MK on midkine(MK) gene in pancreatic cancer cells. **Methods:**Ad-shRNA-MK was used to infect pancreatic cancer BxPC-3 cells. Assays were conducted for knockdown of the MK gene on the day of infection and on the 1st, 3rd, 5th, 7th, and 9th days post-infection by using immunocytochemistry, real-time RT-PCR, and Western blot analysis. **Results:**The adenoviral Ad-shRNA-PTN was constructed successfully, and infection was confirmed by electron microscopic observation. By using real-time RT-PCR, the inhibition rates of MK mRNA expression in the BxPC-3 cells were 20%, 80%, 55%, and 23% on the 1st, 3rd, 5th, and 7th days post-infection. Immunocytochemistry and Western blot analysis confirmed this effect at the gene product level. **Conclusion:**Efficient and specific knockdown of MK in pancreatic cancer cells by adenoviral Ad-shRNA-PTN is a potentially powerful tool for the study of gene therapy of pancreatic cancer nerve infiltration.

Keywords: BxPC-3 cell; neural invasion; midkine(MK); RNA interference(RNAi); short hairpin RNA(shRNA)

INTRODUCTION

Pancreatic cancer has an overall 5 year survival rate of less than 25% after partial pancreaticoduodenectomy[1-2], and it is still one of the most aggressive and intractable human malignant tumors[3-4]. Therefore, it is important to seek out novel targets for therapeutic intervention. Recent experimental data have revealed the involvement of a protein family of neurotrophic factors in the progression and neural invasion of pancreatic cancer[5-6]. Thus, this protein family is of interest in the study of pancreatic cancer. Midkine(MK) is a type of neurotrophic factor, and is also known as the neurite growth-promoting factor. It is a 136-amino-acid long secreted cytokine with diverse biological properties, including neurite outgrowth, angiogenesis, expansion, and metastasis of tumor cells[7-8]. MK proteins in humans, mice, and rats are identical and share a 45% amino acid homology with pleiotrophin, which is another member of this family[7]. MK is mainly expressed during early embryogenesis. In human adult tissues, it is markedly downregulated and present only at minimal levels in very few tissues[8]. MK is not expressed in normal pancreatic tissues, but is highly expressed in pancreatic cancer tissues and its presence correlates with the progression of pancreatic cancer. This would suggest that inhibition of MK protein would be an important technique in the study of the progression and neural invasion of pancreatic cancer.

RNA interference(RNAi) is a process during which double-stranded RNA induces the homology-dependent degradation of cognate mRNA. In some organisms, the introduction of double-stranded synthetic siRNA has been proven to be a powerful tool for suppressing genes[9]. Its silencing efficacy is higher than that of antisense RNA[10]. Nevertheless, therapeutic use of synthetic siRNAs is significantly limited by their rapid degradation in target cells, resulting in only transient gene silencing[11], severely restricting their application. To overcome this restriction, novel adenoviral vectors have been designed to express small hairpin RNA(shRNA) transcripts to suppress expression of endogenous genes

*Corresponding author
E-mail address: wkz588@sina.com

in mammalian cells[12].

In previous studies, we investigated levels of MK protein and its receptor in 38 patients with pancreatic cancer by immunohistochemistry, analyzed for its correlation with clinicopathological features, perineural invasion, and prognosis. We concluded that high expression of MK may contribute to the high degree of perineural invasion and poor prognosis of pancreatic cancer(unpublished data).

In the present study, we constructed the adenoviral vector Ad-shRNA-MK that could suppress MK gene expression in BxPC-3 cells. Such suppression was assessed by infecting the BxPC-3 cells, and assaying for knockdown of the MK gene and gene product post-infection by immunocytochemistry, real-time RT-PCR and Western blot analysis.

MATERIALS AND METHODS

Cell lines, medium, and reagents

The BLOCK-iT™ Adenoviral RNAi Kits(that include pAd/BLOCK-iT™ -DEST Gateway^R Vector Kit; Gateway^R LR Clonase™ Enzyme Mix; 293A cell line; and BLOCK-iT™ U6 RNAi Entry Vector Kit) and Lipofectamine™ 2000 were purchased from Invitrogen Corp. (CA, USA). Monoclonal mouse antihuman MK, anti-GAPDH antibodies, and the secondary antibody (peroxidase-coupled goat anti-mouse IgG) were purchased from Santa Cruz Biotechnology(CA, USA). The human pancreatic cancer cell line BxPC-3 was purchased from the American Type Culture Collection(VA, USA). The BxPC-3 cells were maintained in Dulbecco's modified Eagle's medium(DMEM; Life Technologies, CA, USA). Fetal calf serum(FCS) was purchased from Gibco BRL, USA.

Construction of recombinant adenovirus Ad-shRNA-MK

We have successfully constructed the adenoviral Ad-shRNA-MK. Briefly, according to data on the human midkine mRNA(GenBank accession no. NM002825), 4 pairs of complementary single-stranded oligonucleotides(ss oligos) were designed and synthesized by using Invitrogen's RNAi Designer online. Then, the ss oligos were annealed to create double-stranded oligonucleotides(ds oligos). The 4 ds oligos were cloned into the pENTR/U6 vector to produce 4 shuttle plasmid pENTR/U6-shRNAs that were transduced into the BxPC-3 cells by Lipofectamine™ 2000 after sequencing to identify and select the shuttle plasmids showing optimal silencing effect. Oligo-3 proved to have the optimal silencing effect. The sense and antisense strands of oligo-3 were 5′-CACCGCC-AGAA-GACTGTCACCATCTCGAAAGATGGT-GACA-GTCTTCTGGC-3′ and 5′-AAAAGCCA-GAA-GACTGTCACCATCTTTCGAGATGGTGAC-AGTCTTCTGGC-3′ . Then, the LR recombination reaction was performed. Recombinant adenovirus Ad-shRNA-MK was produced and amplified in HEK 293A cells; the viral titers were determined by TCID50 assays[13]. The construction of Ad-shRNA-MK was confirmed ultrastructurally by electron microscopy.

The procedure for infecting the BxPC-3 cells

The BxPC-3 cells were plated at a concentration of 1×10^6 cells per well in a 6-well plate, and 2 ml of normal DMEM along with 10% FCS was added in each well. The next day, cells were infected. On this day (Day 0), 5 μl adenoviral stocks(1×10^{10} pfu/ml) was added into 2 ml fresh culture medium with 2% FCS(at a multiplicity of infection, i.e., MOI of 50). The previous culture medium was removed from the cells, the medium containing virus gently mixed and added to each well, and incubated at 37℃ overnight. The cells were harvested on the day of infection and on the 1st, 3rd, 5th, 7th, and 9th days post-infection, and assayed for knockdown of the MK gene by immunocytochemistry, real-time RT-PCR and Western blot analysis. In this study, we regarded the cells harvested on the day of infection, prior to infection, as the control.

Immunocytochemistry

Immunostaining of the human pancreatic cancer cell line, BxPC-3, was performed on the day of infection (control), and on the 3rd, and 5th day post-infection. The BxPC-3 cells were grown on glass slides, fixed with acetone, and endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol. The sections were washed and incubated overnight with a 1:20 dilution of anti-MK antibody at 4℃. After subsequent washings in PBS, the secondary antibody was added and incubated for 1 h at room temperature. After another wash in PBS, the peroxidase activity was localized by staining with diaminobenzidine as the substrate, and sections were rinsed in water, dried, and mounted.

Total RNA isolation and quantitative realtime RT-PCR

On the day of infection and on the 1st, 3rd, 5th, 7th, and 9th days post-infection, the medium was removed from each well. Total RNA from each plate of BxPC-3 cells was extracted(RNeasy Mini Kit, Qiagen, USA). The extracted RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA was verified by calculating the ratio between the absorbance values at 260 and 280 nm. Values of this ratio ranged between 1.83 and 2.00, demonstrating the high quality of the RNA. Reverse transcription reactions were performed

at 50℃ for 30 min. The reaction mixtures were heated to 95℃ for 15 min to activate HotStarTaq (Qiagen). The forward and reverse primers of MK were 5′-TCCTAGTATTTTTTTCCTCAG-3′ and 5′-CTTGTTTTCTGCCAATAG-3′, respectively. The forward and reverse primers of GAPDH were 5′-TCATCCCTGCCTCTACTG-3′ and 5′-TGCTTCACCACCTTCTTG-3′, respectively. PCR amplification was performed in a total volume of 20 μl: 4.4 μl PCR master mix(TaKaRa Ex Taq R-PCR Version, TaKaRa), 10 pmol of each primer, 2 mmol/L $MgCl_2$, 2 μl(1:15 000 dilution) SYBr Green(SYBr Green Nucleic Acid Gel Stain, Takara Co. Japan), and distilled water. PCR was performed using the ABI PRISM 7700 Sequence Detection System under the following conditions:95℃ for 30 s and 35 cycles at 95℃ for 0 s, for instrument setting to 0 s, 57℃ for 5 s, and 72℃ for 10 s. Melting curve analysis was performed for all the positive results. The melting temperature range used to determine whether the specimen yielding a crossing point was a true positive was 86.5~87.5℃. GAPDH was used as the endogenous RNA control, in order to calibrate the variations in the amounts of input mRNA, and each sample was normalized based on its GAPDH content.

Western blot analysis

The cells were harvested on the day of infection and on the 3rd, 5th, 7th, and 9th days post-infection, washed once with cold PBS(pH 7.0), and lysed in a lysis buffer (150 mmol/l NaCl, 50 mmol/l Tris-HCl, pH 7.4, 2 mmol/l EDTA, and 1% NP-40) containing protease inhibitors(Boehringer Mannheim, Germany). The analysis was performed on all the lysates with equal amounts of protein(20 μg per lane), quantified by colorimetric detection based on the bicinchoninic acid (BCA) test. The samples were heated at 95℃ for 5 min and loaded on a 12% SDS-polyacrylamide gel. Following electrophoresis, the separated protein fractions were transferred onto a methanol-activated PVDF membrane and incubated with anti-MK and anti-GAPDH antibodies, followed by incubation with the corresponding secondary antibodies. The bands were visualized by using the enhanced chemiluminescence system.

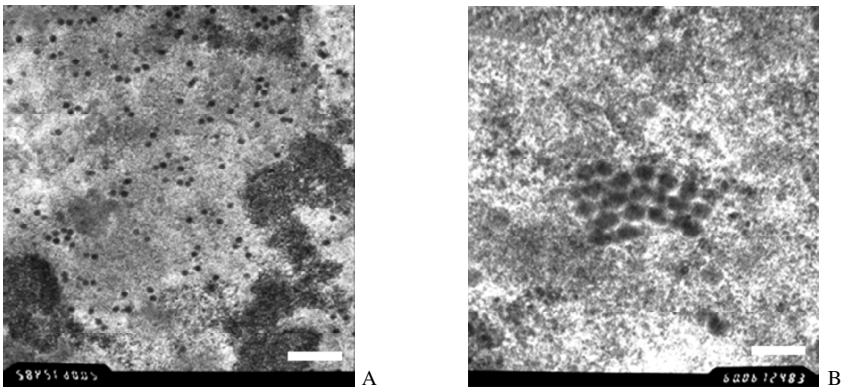
Statistical analysis

The images obtained from the western blot were scanned and analyzed using the Quantity One software. The optical density scores of bands were calculated to analyze the relative content of MK to GAPDH. The statistical significance between the control and infected groups was calculated using one-way ANOVA test. T tests were subsequently performed using 3 replicate experiments. P values of < 0.05 were considered statistically significant.

RESULTS

Transmission electron microscopy

Two days post-infection, we were able to find morphological changes characteristic of adenovirus infection. There were many scattered virus capsids in the nucleus, next to the nuclear membrane and few virus capsids in the cytoplasm(***Fig. 1A***). At a higher magnification, the capsids showed no connection to each other. Virus capsids measured 50~70 nm and had an almost round appearance(***Fig. 1B***).



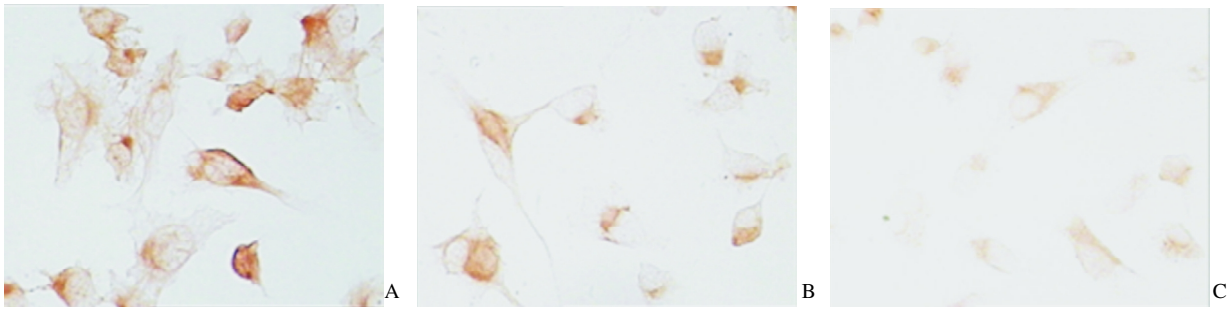
A (× 20 000) B (× 60 000)

Scale bars:600 nm(A) and 200 nm(B)

Fig. 1 Transmission electron microscopy showing recombinant adenovirus Ad-shRNA-MK in HEK 293A cells 2 days after infection

MK expression in pancreatic cancer cells

Using immunocytochemistry, we found a strong staining pattern for MK in the cytoplasm of normal BxPC-3 cells(control, ***Fig. 2A***). The MK gene silencing and reduced gene product expression was determined in comparison with the control. The expression of the MK protein in the cytoplasm was decreased by 30% (3 days, ***Fig. 2B***) and 70%(5 days, ***Fig. 2C***). The quantification of the gray values of these images was obtained using the Image Analysis System. The gray values of normal BxPC-3 cells group were used as the control and the other two groups compared with it to



A:Control; B:3 days post-infection; C: 5 days post-infection

Fig. 2 MK immunostaining of BxPC-3 human pancreatic cancer cells(400×)

obtain the above decreases in expression.

Down-regulation of MK mRNA expression in BxPC-3 cells

The efficacy of Ad-shRNA-MK with regard to knockdown of MK mRNA was confirmed through real-time RT-PCR analysis. The melting temperature range of MK was 86.5~87.5℃, and the melting temperature range of GAPDH was 89.5~90.5℃. No nonspecific amplification was observed. High expression levels of MK mRNA were observed in the control. Statistically significant inhibition of MK mRNA expression in the BxPC-3 cells of 20%, 80%, 55%, and 23% occurred on the 1st, 3rd, 5th, and 7th days post-infection, respectively. The MK mRNA level was decreased on the 1st day, and down-regulation of MK mRNA expression was most obvious on the 3rd post-infection day. On the 5th day, the MK mRNA level increased gradually, and the MK mRNA expression approximately returned to the level shown by normal BxPC-3 cells on the 9th post-infection day(***Fig. 3***).

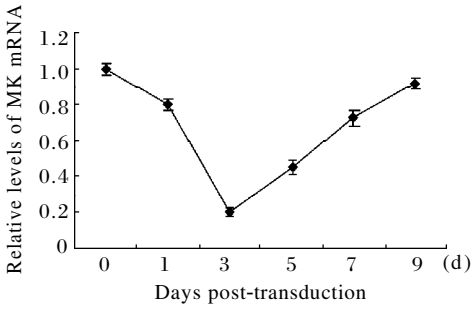
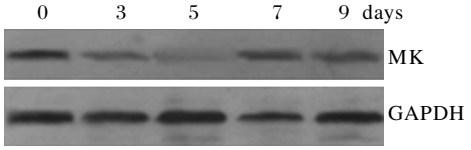


Fig. 3 The inhibitory effects on MK mRNA expression in the pancreatic cancer cell line BxPC-3 following MK gene silencing using Ad-shRNA-MK

Western blot analysis of the down-regulation of MK protein expression in BxPC-3 cells

Western blot analysis of the anti-MK-specific antibodies revealed that MK protein expression in BxPC-3 cells infected with Ad-shRNA-MK was markedly suppressed from 3~9 days compared to the control (0 day). Statistically significant inhibition of MK protein expression of 47.5%, 80.5%, and 20.0% occurred on the 3rd, 5th, and 7th days respectively following Ad-shRNA-MK infection. The maximal knockdown occurred on the 5th post-infection day. The MK protein levels began to rise on the 7th post-infection day(***Fig. 4***).



Molecular weight of MK is 18 ku

Fig. 4 The inhibitory effects on MK protein expression in the pancreatic cancer cell line BxPC-3 following Ad-shRNA-MK infection

DISCUSSION

Pancreatic cancer is characterized by perineural invasion, early lymph node metastasis, and poor prognosis[14]. As an essential autocrine growth factor and neurite growth-promoting factor, MK acts synergistically to promote the progression and perineural invasion of pancreatic cancer. Kadomatsu *et al.*[15] showed that MK is expressed in gastrointestinal and, particularly, in pancreatic cancer patients. In this study, we found that MK was clearly expressed in the human pancreatic adenocarcinoma cell line, BxPC-3, using immunocytochemistry.

RNAi is a powerful tool for gene functional analysis in mammals[12]. A 21~23 nt short hairpin RNAs (shRNAs) combined with the special mRNA, leading to the degradation of the target gene, followed by the down regulation of the target gene expression[16-17]. In this study, we constructed Ad-shRNA-MK expressing MK shRNA that could suppress MK gene expression in BxPC-3 cells, and results indicated that MK shRNA could efficiently inhibit the expression of MK genes. RNAi is a gene silencing mechanism of post-transcription[18]. Therefore, the analysis of RNAi is to investigate the efficiency of interference from the transcription and protein expression levels by using immunocytochemistry, PCR and western blot. The combination of these three methods can more accurately judge

the effectiveness of the RNAi. According to immunocytochemistry, there was a strong staining pattern for MK in the cytoplasm of uninfected BxPC-3 cells. The expression of the MK protein was decreased by 30% on the 3rd day and 70% on the 5th day post-infection; this is consistent with the results of the western blot analysis.

The pAd/BLOCK-iT™ adenoviral construct is replication-incompetent and does not integrate into the host genome. Therefore, once transduced into the mammalian cells of choice, the shRNA of interest will be expressed only as long as the viral genome is present [19]. In actively dividing cells, the adenovirus genome is gradually diluted as cell division occurs, resulting in an overall decrease in shRNA expression over time, i.e., the target protein levels generally return to background levels within 1~2 weeks following transduction. In our study, by using real-time RT-PCR we found that the MK gene knockdown in BxPC-3 cells was detectable (20%) on the 1st post-infection day, with maximal knockdown levels(80%) observed on the 3rd post-infection day. On the 5th day, as the adenovirus genome was diluted, the level of MK mRNA began to increase and the MK mRNA expression returned to the level observed in control BxPC-3 cells by the 9th post-infection day. Western blot analysis also revealed the same tendency, except that the maximal knockdown levels of MK protein were observed 2 days later. This may be related to the translation process of the protein. The results of quantitative RT-PCR analysis of MK revealed mRNA variations generally correlated with the Western blot data. Another interesting discovery was that Ad-shRNA-MK reduced MK mRNA levels by nearly 4-fold on the 3rd post-infection, almost equal to the decrease in the protein levels on the 5th post-infection. The tendency for variations between mRNA and protein was a common observation in our and other studies[20].

In conclusion, we proved that the adenoviral Ad-shRNA-MK could be constructed successfully and show efficient and specific knockdown of MK in the pancreatic cancer cell line BxPC-3. Therefore, adenoviral construct Ad-shRNA-MK may be an important research tool in the study of pancreatic cancer. In further studies, we will set up a model of pancreatic cancer in situ in nude mice. By injecting Ad-shRNA-MK stock, we can investigate the effects of the MK gene and adenoviral construct Ad-shRNA-MK on the progression and neural invasion of pancreatic cancer.

References

[1] Richter A, Niedergethmann M, Sturm JW, Lorenz D, Post S, Trede M. Long-term results of partial pancreaticoduodenectomy for ductal adenocarcinoma of the pancreatic head: 25-year experience. *World J Surg* 2003;27:324-9.

[2] Wagner M, Redaelli C, Lietz M, Seiler CA, Friess H, Buchler MW. Curative resection is the single most important factor determining outcome in patients with pancreatic adenocarcinoma. *Br J Surg* 2004;91:586-94.

[3] Li D, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. *Lancet* 2004; 363:1049-57.

[4] Laheru D, Jaffee EM. Immunotherapy for pancreatic cancer-science driving clinical progress. *Nat Rev Cancer* 2005;5:459-67.

[5] Ceyhan GO, Giese NA, Erkan M, Kerscher AG, Wente MN, Giese T, *et al.* The neurotrophic factor artemin promotes pancreatic cancer invasion. *Ann Surg* 2006;244:274-81.

[6] Veit C, Genze F, Menke A, Hoeffert S, Gress TM, Gierschik P, *et al.* Activation of phosphatidylinositol 3-kinase and extracellular signal-regulated kinase is required for glial cell line-derived neurotrophic factor-induced migration and invasion of pancreatic carcinoma cells. *Cancer Res* 2004;64:5291-300.

[7] Muramatsu T. Midkine and pleiotrophin: two related proteins involved in development, survival, inflammation and tumorigenesis. *J Biochem* 2002;132:359-71.

[8] Kaneda N, Talukder AH, Nishiyama H, Koizumi S, Muramatsu T. Midkine, a heparin-binding growth/differentiation factor, exhibits nerve cell adhesion and guidance activity for neurite outgrowth *in vitro*. *J Biochem* 1996;119:1150-6.

[9] Gaither A, Iourgenko V. RNA interference technologies and their use in cancer research. *Curr Opin Oncol* 2007;19:50-4.

[10] Barik S. Silence of the transcripts: RNA interference in medicine. *J Mol Med* 2005;83:764-73.

[11] Watanabe A, Arai M, Yamazaki M, Koitabashi N, Wuytack F, Kurabayashi M. Phospholamban ablation by RNA interference increases Ca^{2+} uptake into rat cardiac myocyte sarcoplasmic reticulum. *J Mol Cell Cardiol* 2004;37:691-8.

[12] Yuan J, Wang X, Zhang Y, Hu X, Deng X, Fei J, *et al.* shRNA transcribed by RNA Pol II promoter induce RNA interference in mammalian cell. *Mol Biol Rep* 2006;33:43-9.

[13] LaBarre DD, Lowy RJ. Improvements in methods for calculating virus titer estimates from TCID50 and plaque assays. *J Virol Methods* 2001;96:107-26.

[14] Kayahara M, Nakagawara H, Kitagawa H, Ohta T. The nature of neural invasion by pancreatic cancer. *Pancreas* 2007;35: 218-23.

[15] Kadomatsu K, Muramatsu T. Midkine and pleiotrophin in neural development and cancer. *Cancer Lett* 2004;204:127-43.

[16] Hao DL, Liu CM, Dong WJ, Gong H, Wu XS, Liu DP, *et al.* Knockdown of human p53 gene expression in 293-T cells by retroviral vector-mediated short hairpin RNA. *Acta Biochim Biophys Sin(Shanghai) JT-Acta biochimica et biophysica Sinica* 2005;37:779-83.

[17] Kobayashi S, Higuchi T, Anzai K. Application of the BC1 RNA gene promoter for short hairpin RNA expression in cultured neuronal cells. *Biochem Biophys Res Commun* 2005;334:1305-9.

[18] Li CX, Parker A, Menocal E, Xiang S, Borodyansky L, Fruehauf JH. Delivery of RNA interference. *Cell Cycle* 2006;5:2103-9.

[19] Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs(shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* 2002;16:948-58.

[20] Yuefeng Du, Yifei Xing, Fuqing Zeng, Peng Lu, Xianyin Liu. The inhibitory effects of siRNA expression vector on CXCR4 expression in prostate carcinoma cell lines. *JNMU* 2006;20: 104-8.