Adeno-associated virus mediated endostatin gene therapy in combination with topoisomerase inhibitor effectively controls liver tumor in mouse model

Sung Yi Hong, Myun Hee Lee, Kyung Sup Kim, Hyun Cheol Jung, Jae Kyung Roh, Woo J in Hyung, Sung Hoon Noh, Seung Ho Choi

INTRODUCTION

Antiangiogenic therapy for cancer has emerged as an exciting new therapeutic modality because tumors are angiogenesis-dependent during growth and metastasis.[7-10] One of the most potent endogenous angiogenic inhibitors, endostatin, has been reported to inhibit endothelial proliferation and regression of solid tumors.[7-10] Although endostatin induces and sustains the dormancy of tumor growth, large quantities of proteins are needed for prolonged periods.[9] Moreover, besides being difficulty to be purified, endostatin has a short half-life in vivo. In order to circumvent the obstacle presented by the pharmacokinetics of endostatin, delivery of the gene cassettes encoding endostatin has been attempted.[11-19]

Recombinant adeno-associated virus (rAAV) vector is a good candidate for antiangiogenesis-based cancer gene therapy[20]. rAAV vector is derived from a nonpathogenic parvovirus that is capable of integrating into the host DNA, which allows the long-term expression. In addition, removal of the viral coding sequences minimizes immunogenicity. The rAAV vector has a broad host tropism and transducts in dividing and non-dividing cells. The liver is an important target for gene therapy, because of its large size, its protein synthesizing capacity and because it is easily accessible to vectors. Although the rAAV is a promising vector for liver-directed gene therapy, its potential for therapeutic use has been limited due to its inefficient transduction into the liver[21,22]. In order to achieve high serum levels of endostatin with a stable expression, the transduction of non-dividing cell populations is essential in liver-directed gene therapy. Some topoisomerase inhibitors, such as etoposide or camptothecin, increase the transduction efficiency of the rAAV in non-dividing cells as well as in dividing cells[23-25].

Therefore, this study investigated the potential of a rAAV vector-mediated endostatin gene therapy in combination with topoisomerase inhibitor in a liver tumor model. This paper demonstrates that a topoisomerase inhibitor in a rAAV delivered endostatin gene therapy enhances the antiangiogenic effects, and that this method has the potential to be used as a new strategy for cancer gene therapy.

MATERIALS AND METHODS

Cells culture

Hepa1c1c7 mouse hepatoma cell line (ATCC CRL 2026), S-180 murine sarcoma cell line (ATCC CCL-8) and 293-EBNA cells (transformed human embryonic kidney, ATCC R620-07) were grown in DMEM (Gibco BRL, Grand Island, NY) with 100 mL/L...
DNA Master SYBR Green system (Roche Molecular Biochemicals, TGG-AGA-CAG-ACA, reverse primer: ACA-CTG-GAG-TCA-TTA-CTT-GGA-GAA-AGA-GGT), which was modified by site-directed mutagenesis (oligonucleotide primer 1: ACC-TCT-TTC-TCC-AAG-TAA-TGA-CTC-CAG-TGT-GGT-GGA, oligonucleotide primer 2: TCC-ACC-ACA-CTG-GAG-TCA-TTA-CTT-GGA-GAA-AGA-GGT), which mutated the sequence upstream of the c-myc epitope into stop codons to remove c-myc tag[25]. AAV-helper free system (Stratagene, La Jolla, CA) was used to produce rAAV. SaI – XhoI fragment from modified pEndoSTHB was subcloned into the pCMV-MCS vector (Stratagene). Once this expression construct was verified, the NotI fragment, containing the expression cassette of endostatin, was cloned into the pAAV-LacZ viral expression vector (Stratagene). The parental vector rAAV-LacZ was used as control. pAAV-endostatin containing the cytomegalovirus (CMV) promoter with a murine Ig κ-chain signal peptide was flanked by cDNA of murine endostatin. The rAAV vectors were produced using a standard triple-plasmid transfection method, and purified by a heparin sulfate column separation[26]. Briefly, the recombinant expression plasmid was co-transfected into 293-EBNA cells with pHelper (Stratagene) and pAAV-RC (Stratagene), which supply all the trans-acting factors required for AAV replication and packaging in 293-EBNA cells. rAAV stocks were subjected to 3 rounds of freezing and thawing. After cell debris was removed by centrifugation, the stocks were filtered using a low protein binding 5 µm syringe filter and subsequently by heparin agarose column (Sigma) supplemented with 100 ng/mL heparin (Gibco BRL), 200 µL/FBS, 100 µg/mL streptomycin, 100 U/mL penicillin, 3 ng/mL bFGF (Upstate, Waltham, MA).

Endostatin enzyme immunoassay
Endostatin levels in the conditioned medium of cultured cells or in the mouse serum were determined using a mouse endostatin immunoassay kit (Chemicon International, Temecula, CA, USA).

Endothelial cell proliferation assay
HUVECs were plated on 5x10^3 in 96-well gelatin-coated plates and allowed to attach in complete medium for 4 h. The medium was then replaced by the conditioned medium, where the endostatin concentration was measured by an ELISA assay. After 1 h, an equal volume of 2x complete medium was added, and the number of cells was quantified by a colorimetric MTT assay on the indicated days. The test was performed in triplicate.

Endothelial cell tube formation assay
Twenty-four well plates were coated with 50 µL of Matrigel (BD Biosciences, Bedford, MA) in an ice bath and then incubated at 37 °C for 1 h. HUVECs at a density of 5x10^5 cells in each well were seeded and cultured in the conditioned media. After 18-h incubation, the plates were photographed. All tests were performed in triplicate.

In vivo tumor model
Animal experiments were carried out in accordance with the policies of Animal Research Committee of the Yonsei University College of Medicine. Twenty-five 8-wk-old female ICR mice (Charles River Laboratories, Wilmington, MA) were randomly divided into 5 equal groups, namely no treatment and rAAV-LacZ treatment alone as the control, treated with rAAV-LacZ in combination with etoposide pretreatment, treated with rAAV-endostatin alone, and treated with rAAV-endostatin in combination with etoposide pretreatment. In the pretreatment group, etoposide (40 mg/kg) in 200 µL HBSS was administered 3 times for a week by an intraperitoneal injection beginning 7 d before rAAV injection. Hepatic tumors were induced by directly injecting 5x10^6 S -180 murine sarcoma cells into the liver. Simultaneously, 500 µL of rAAV-mEndostatin (1.5x10^{12} viral particles) was injected into the spleen in the endostatin treatment groups in order to deliver viral particles into the liver. The mice were sacrificed 7 d after tumor cell injections by a halothane overdose to examine hepatic tumors. The tumor volume (TV) was determined using the following formula: TV=(Length × width ^2)/2. In order to evaluate the long-term survival, the experiment was repeated and the mice were followed up for 2 mo. The survival time was defined from the day of tumor injection to death. The mice that were alive at the end of the follow-up period were estimated as the censored observation.
Localization of endostatin expression in liver

The livers were harvested and 5 µm-thick sections of the formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene and heated in a citrate buffer for 10 min. Endogenous peroxidase activity was blocked by incubation with 10 mL/L H₂O₂ and 10 g/L Triton X-100. Gout anti-mouse antibodies were applied at a dilution of 1:60 overnight at 4 °C. Biotinylated rabbit anti-gout antibodies (Vector, Burlingame, CA) were then applied for 1 h at room temperature at a 1:200 dilution. After incubation with streptavidin conjugated to horseradish peroxidase, a substrate containing chromogen, 3,3’ dianinobenzidine tetrahydrochloride, was added and the slides were counterstained with hematoxylin. All the slides were air-dried and kept in dark at 4 °C until evaluation.

Microvessel density (MVD) assessment

In order to analyze hepatic tumor microvessels, tissue sections (5 µm) of formalin-fixed, paraffin-embedded specimens were evaluated using rat anti-mouse CD34 antibodies (1:50; RAM34; Pharmingen, San Diego, CA) as the primary antibody and biotinylated rabbit anti-rat antibodies (Vector, Burlingame, CA) as the secondary antibody. After incubation of the tissue with streptavidin conjugated to horseradish peroxidase, the reactions were visualized by a substrate containing chromogen, 3,3’ dianinobenzidine tetrahydrochloride. The slides were counterstained with hematoxylin. At least 5 thin slices were made from each tumor and used for MVD assessment. MVD was estimated by counting the number of CD34-positive vessels in the tumor area, which was representative of the highest MVD at ×200 magnification. The counts were typically made in 3-5 hot spots, and the highest MVD was used to characterize the tumor.

Statistical analysis

The data were expressed as mean±SD. Student’s t test was used to analyze the statistical differences in endostatin levels in the conditioned medium of cultured cells or in mouse serum, endothelial cell proliferation, tumor size, or microvessel assessment among the groups. The Kaplan and Meier method was used to calculate the survival rate in the in vivo experiment and the survival differences between the groups were evaluated using the log-rank test. A P<0.05 was considered statistically significant.

RESULTS

Increased in vitro expression of endostatin molecules on rAAV-endostatin transduced hepatoma cells by pretreatment with topoisomerase inhibitors

Hepa1c1c7 mouse hepatoma cells in the 10 cm plate were incubated with either rAAV-endostatin or rAAV-LacZ vectors (1×10⁶ particles/cell) for 48 h. In the pretreatment group, etoposide (3 µmol/L) or camptothecin (10 µmol/L) was administered 6 h prior to transduction. Conditioned media were concentrated 10 times and used in both Western blotting and ELISA. The in vitro expression of endostatin was detected by Western blot analysis. The recombinant endostatin was visualized in the supernatant of Hepa1c1c7 cells transduced with rAAV-endostatin, but not in the supernatant of Hepa1c1c7 transduced with rAAV-LacZ. The endostatin expression level was enhanced as a result of the pretreatment with either etoposide or camptothecin (Figure 1A). ELISA was performed to quantify the expression level. Hepa1c1c7 cells transduced with rAAV-endostatin expressed endostatin (19.0±3.0 ng/mL) compared with the vector control (0.3±0.3 ng/mL) and mock control (0.3±0.2 ng/mL), and topoisomerase inhibitors enhanced significantly the endostatin expression level (P<0.05) (Figure 1B). In the range of concentrations used in this study, etoposide or camptothecin had little effect on Hepa1c1c7 cell growth (data not shown). Etoposide increased the endostatin expression level more than camptothecin (43.3±5.1 vs 30.7±5.7 ng/mL, P<0.05). Consequently, etoposide was chosen as a combination therapy in the in vivo experiment.

Figure 1

A

B

In vitro biological activities of endostatin produced by rAAV vectors and pretreatment with topoisomerase inhibitors

As expected, the conditioned media from Hepa1c1c7 cells transduced with the control rAAV-LacZ vector did not influence either endothelial cell proliferation or tube formation compared to the conditioned media from the non-treated control (data not shown). Etoposide and camptothecin had a minor effect on the growth of HUVECs, and recombinant endostatin actively reduced endothelial cell growth. However, the conditioned media from rAAV-endostatin combined with etoposide group showed very strong inhibition (Figure 2A). Similarly, the recombinant endostatin suppressed tube formation of endothelial cells, and the rAAV-endostatin combined with etoposide group had the highest effect (Figure 2B).

Synergic effect of rAAV-endostatin with topoisomerase inhibitors in a mouse liver tumor model

Hepatic tumors were formed by injecting S-180 murine sarcoma cells directly into the predetermined site of the liver, and rAAV-Lac-Z treatment mice had similar hepatic tumors compared to non-treated mice (170.0±38.6 and 159.7±27.7 mm³, respectively). rAAV-LacZ in combination with etoposide and rAAV-endostatin reduced hepatic tumor burden (52.0±9.4 and 9.3±4.5 mm³, respectively) (P<0.05, treated group vs non-treated or rAAV-Lac-Z treatment group). Interestingly, tumor nodules were barely present in the rAAV-endostatin treatment group (data not shown).
observed in the rAAV-endostatin plus etoposide group (Figure 3). Serum endostatin was hardly shown in non-treatment group (30.4±20.8 ng/mL), rAAV-LacZ treatment alone (34.2±21.4 ng/mL) and rAAV-LacZ plus etoposide groups (25.8±19.9 ng/mL), although rAAV-endostatin induced detectable serum endostatin level (191.1±54.7 ng/mL, \( P < 0.05 \), rAAV-endostatin group vs control groups). In contrast, rAAV-endostatin plus etoposide induced the highest endostatin level (321.5±54.3 ng/mL, \( P < 0.05 \), rAAV-endostatin plus etoposide group vs other groups) (Figure 4). The in vivo expression of endostatin was detected immunohistochemically. Staining with anti-endostatin antibodies revealed positive cells in vessels of the liver sections from the rAAV-endostatin treatment group, whereas the control sections were negative, and a dramatic increase was observed in rAAV-endostatin plus etoposide treatment mice (Figure 5). Moreover, endostatin was stained in hepatocytes of rAAV-endostatin plus etoposide treatment mice. The microvessel densities in tumor were estimated by CD34 staining, which were found to be decreased in the rAAV treatment group (19.3±4.5, \( P < 0.05 \), rAAV treatment group vs control groups) compared with the mock control (97.7±15.2) and the vector control (105.2±17.6). As expected, the rAAV-endostatin plus etoposide treatment mice had the lowest microvessel density (7.6±1.5) (Table 1). All the non-treated or rAAV-lacZ treated mice died within 30 d. rAAV-LacZ plus etoposide treatment barely affected the survival and rAAV-endostatin extended the survival time. However, the rAAV-endostatin plus etoposide treatment mice had the longest survival (Figure 6). In addition, significant endostatin expressions were detected in the surviving mice of rAAV-endostatin plus etoposide treatment group even after 2 mo (data not shown).

Table 1  Microvessel assessment of S-180 murine sarcoma tumors

| Group                        | Microvessel density |
|------------------------------|---------------------|
| No treatment                 | 97.7±15.2           |
| rAAV-LacZ alone              | 105.2±17.6          |
| rAAV-LacZ plus etoposide     | 50.2±9.4            |
| rAAV-endostatin alone        | 19.3±4.5            |
| rAAV-endostatin plus etoposide | 7.6±1.5            |

The microvessel density was measured with a light microscope in the tumor representative of the highest microvessel density at magnification ×200 ("hot spot"). *\( P < 0.05 \), rAAV-endostatin plus etoposide group against other groups.

Figure 2  In vitro biological activities of expressed endostatin. A: 5×10⁴ HUVECs in a 96-well were cultured in the conditioned media from Hepa1c1c7 mouse hepatoma cells without pre-treatment (Z), those from the cells pretreated with etoposide (Z/et) or camptothecin (Z/cpt), those from rAAV-endostatin transduced cells without pretreatment (E), those from rAAV-endostatin transduced cells pretreated with etoposide (E/et) or camptothecin (E/cpt). The number of cells was then calculated by a MTT assay. Each value represents mean±SD of 3 independent experiments (\( P < 0.05 \), rAAV-endostatin in combination with pretreatment groups versus other groups). B: Impact on tube formation of endothelial cells. HUVECs were seeded into 24-well plates coated with Matrigel at a density of 5x10⁴ cells in each well and cultured in the conditioned media. After 18 h incubation, the level of cell growth and differentiation was observed. All tests were performed in triplicate.
endostatin vector were injected into the spleen simultaneously with tumor cell inoculation (5×10⁶ S-180 cells) into the liver. The tumor volume was determined 7 d after injecting murine sarcoma cells (P <0.05, rAAV plus etoposide group versus other groups). Tumor volume = (Length×width²)/2.

Figure 3 Effect of rAAV-endostatin in combination with etoposide on murine sarcoma bearing mice. Twenty five mice bearing S-180 murine sarcoma cells were randomly divided into 5 groups, namely no treatment, rAAV-LacZ alone, rAAV-LacZ plus etoposide pretreatment, rAAV-endostatin alone, and rAAV-endostatin plus etoposide pretreatment. In the pretreatment group, etoposide (40 mg/kg) was administered 3 times for one week by an intraperitoneal injection beginning 7 d prior to rAAV injection, and 1.5×10¹² viral particles of rAAV-

Figure 4 Mouse endostatin levels determined in sera of mice inoculated with murine sarcoma cells. S-180 murine sarcoma cells were inoculated into liver. Seven days later, ELISA determined the endostatin concentration and the results were expressed as mean±SD of 5 animals (P <0.05, rAAV-endostatin plus etoposide group versus other groups).
and strongly expressed endostatin. Etoposide could irreversibly inhibit CMV replication and suppress viral DNA and late viral-protein synthesis. It is unclear whether or not etoposide inhibits the function of CMV promoter. This study did not evaluate the effect of etoposide on CMV promoter. However, topoisoamerase inhibitor increased the overall endostatin expression level.

This study examined the in vivo antitumor effects of combined therapy with rAAV-endostatin and etoposide. In this mouse model of a hepatic tumor, etoposide had little antitumor effect, and rAAV-endostatin alone was insufficient to control a hepatic tumor. However, the combined modality significantly enhanced the tumor response. Interestingly, endostatin expression was immunohistochemically detected in hepatocytes and was significantly increased around vessels in the liver of the rAAV-endostatin plus etoposide treatment group compared with those of the rAAV-endostatin alone group. The topoisoamerase inhibitor increased the transduction efficiency of AAV in both S-phase and non-S-phase cells, and hepatocytes were much more efficiently transduced than other cells. Overall, rAAV-endostatin in combination with etoposide increased the endostatin expression level in hepatocytes of mice, and induced sufficient control in the hepatic tumor model. One potential obstacle to the clinical application of rAAV-mediated antiangiogenesis is that it maintains high levels of the target molecules over a long-term. rAAV vector-mediated cancer gene therapy protocols combined with topoisoamerase inhibitor pretreatment might be a solution to this problem.

ACKNOWLEDGMENTS

The authors thank Dr. Woo Ik Yang in the Department of Pathology for his technical assistance.

REFERENCES

1. Guo XL, Lin GJ, Zhao H, Gao Y, Qian LP, Xu SR, Fu LN, Xu Q, Wang J. Inhibitory effects of docetaxel on expression of VEGF, bFGF and MMPs of LS174T cell, World J Gastroenterol 2003; 9: 1995-1998
2. Gupta MK, Qin Y. Mechanism and its regulation of tumor-induced angiogenesis, World J Gastroenterol 2003; 9: 1144-1155
3. Daly ME, Makris A, Reed M, Lewis CE. Hemostatic regulators of tumor angiogenesis: a source of angiogenic agents for cancer treatment? J Natl Cancer Inst 2003; 95: 1660-1673
4. Folkman J. Role of angiogenesis in tumour growth and metastasis, Semin Oncol 2002; 29(6 Suppl 16): 15-18
5. Folkman J. Angiogenesis inhibitors: a new class of drugs, Cancer Biol Ther 2003; 2(4 Suppl): S127-133
6. Kiselev SM, Lusenko SV, Severin SE, Severin ES. Tumor angiogenesis inhibitors, Biochemistry 2003; 68: 497-513
7. Boehm T, Folkman J, Bowdler T, O’Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance, Nature 1997; 390: 404-407
8. Li X, Fu GF, Fan YR, Shi CF, Liu XJ, Xu GX, Wang J. Potent inhibition of angiogenesis and liver tumor growth by administration of an aerosol containing a transferrin-liposome-endostatin complex, World J Gastroenterol 2003; 9: 262-266
9. O’Reilly MS, Boehm T, Shing Y, Fukai N, Vasiou G, Lane WS, Flynn E, Birkehead JR, Olsen BR, Folkman J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth, Cell 1997; 88: 277-285
10. Wang X, Liu FK, Li X, Li JS, Xu GX. Retrovirus-mediated gene transfer of human endostatin inhibits growth of human liver carcinoma cells SMCC7721 in nude mice, World J Gastroenterol 2002; 8: 1045-1049
11. Blezinger P, Wang J, Gondo M, Quezada A, Mehrens D, French M, Singhal A, Sullivan S, Rolland A, Raslan R, Min W. Systemic inhibition of tumor growth and tumor metastases by intramuscular administration of the endostatin gene, Nat Biotechnol 1999; 17: 343-348
12. Chen QR, Kumar D, Stass SA, Mixson AJ. Liposomes complexed
to plasmids encoding angiostatin and endostatin inhibit breast cancer in nude mice. Cancer Res 1999; 59: 3308-3312

13 Ding I, Sun JZ, Fenton B, Liu WM, Kimsely P, Okunieff P, Min W. Intratumoral administration of endostatin plasmid inhibits vascular growth and perfusion in MCa-4 murine mammary carcinomas. Cancer Res 2001; 61: 526-531

14 Feldman AL, Alexander HR, Hewitt SM, Lorang D, Thiruvathukal CE, Turner EM, Libutti SK. Effect of retroviral endostatin gene transfer on subcutaneous and intraperitoneal growth of murine tumors. J Natl Cancer Inst 2001; 93: 1014-1020

15 Langer JC, Klotman ME, Hanss B, Tulchin N, Bruggeman LA, Klotman PE, Lipkowitz MS. Adeno-associated virus gene delivery into renal cells: potential for in vivo gene delivery. Exp Nephrol 1998; 6: 189-194

16 Li S, Zhang X, Xia X, Zhou L, Breau R, Suen J, Hanna E. Intramuscular electroporation delivery of IFN-alpha gene therapy for inhibition of tumor growth located at a distant site. Gene Ther 2001; 8: 400-407

17 Nakashima Y, Yanoo M, Kobayashi Y, Moriyana S, Sasaki H, Toyama T, Yamashita H, Yamakawa Y, Fuji Y. Endostatin gene therapy on murine lung metastases model utilizing cationic vector-mediated intravenous gene delivery. Gene Ther 2003; 10: 123-130

18 Nguyen JT, Wu P, Clouse ME, Hlatky L, Terwilliger EF. Adeno-associated virus-mediated delivery of angiogenen inhibitors as an antitumor strategy. Cancer Res 1998; 58: 5673-5677

19 Wang X, Liu FK, Li X, Li JS, Xu GX. Inhibitory effect of endostatin expressed by human liver carcinoma SMMC7721 on endothelial cell proliferation in vitro. World J Gastroenterol 2002; 8: 253-257

20 Muzycka I. Use of adeno-associated virus as a general transduction vector for mammalian cells. Curr Top Microbiol Immunol 1992; 158: 97-129

21 Xiao W, Berta SC, Lu MM, Moschioni AD, Tazelaar J, Wilson JM. Adeno-associated virus as a vector for liver-directed gene therapy. J Virol 1998; 72: 10222-10226

22 High K. AAV-mediated gene transfer for hemophilia. Genet Med 2002; 4(6 Suppl): 565-615

23 Russell DW, Alexander IE, Miller AD. DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. Proc Natl Acad Sci U S A 1995; 92: 5719-5723

24 Koebel DD, Alexander IE, Halbert CL, Russell DW, Miller AD. Persistent expression of human clotting factor IX from mouse liver after intravenous injection of adeno-associated virus vectors. Proc Natl Acad Sci U S A 1997; 94: 1426-1431

25 Peng D, Qian C, Sun Y, Barajas MA, Prieto J. Transduction of hepatocellular carcinoma (HCC) using recombinant adeno-associated virus (rAAV): in vitro and in vivo effects of genotoxic agents. J Hepatol 2000; 32: 975-985

26 Yoon SS, Eto H, Lin CM, Nakamura H, Pawlik TM, Song SU, Tanabe KK. Mouse endostatin inhibits the formation of lung and liver metastases. Cancer Res 1999; 59: 6251-6256

27 Weiner MP, Costa GL, Schoettlin W, Cline J, Mathur E, Bauer JC. Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction. Gene 1994; 151: 119-123

28 Matsushita T, Elliger S, Elliger C, Podskaff F, Villarreal L, Kurtzman GJ, Iwaki Y, Colosi P. Adeno-associated virus vectors can be efficiently produced without helper virus. Gene Ther 1998; 5: 938-945

29 Russell DW, Miller AD, Alexander IE. Adeno-associated virus vectors preferentially transduce cells in S phase. Proc Natl Acad Sci U S A 1994; 91: 8915-8919

30 Alexander IE, Russell DW, Miller AD. DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors. J Virol 1994; 68: 8282-8287

31 Huang ES, Benson JD, Huong SM, Wilson B, van der Horst C. Irreversible inhibition of human cytomegalovirus replication by topoisomerase II inhibitor, etoposide: a new strategy for the treatment of human cytomegalovirus infection. Antiviral Res 1992; 17: 17-32

Edited by Xu JY and Wang XL  Proofread by Xu FM