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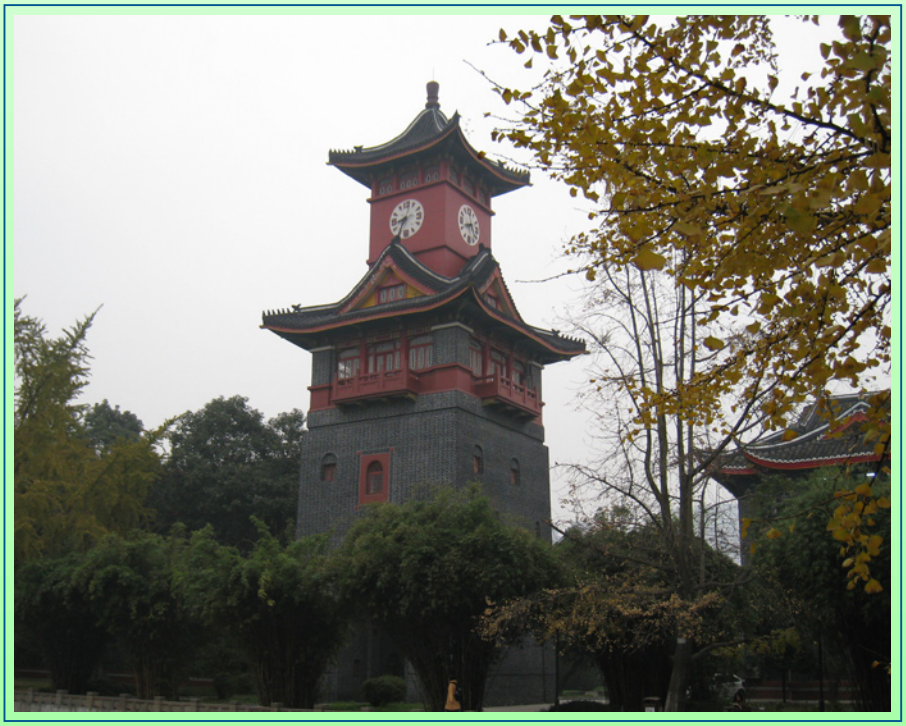

Clock Tower in the West China Medical Center
of Sichuan University

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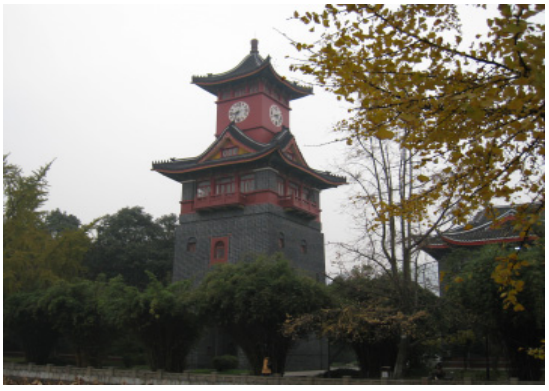
Cover Photo of this issue

Clock Tower in the West China Medical Center of Sichuan University

The Clock Tower (钟塔) in the West China Medical Center of Sichuan University is formerly known as the Coles Memorial Clock Tower (柯里斯纪念楼); funds for the tower were donated by J. Ackerman Coles of New York and the tower was built in 1925. The Clock Tower is the essence of architecture of Hua Xi Ba in Chengdu. The tower features a Western clock that was first rung by hand and later run electrically. In 1954, Nanping Zuo, an expert in ancient architecture, directed alterations to the tower starting from its base. While the Chinese and Western Clock Tower that people see today differs slightly from the original architectural style, it encourages people to ponder by conveying the historic implications of interaction between Chinese and Western culture. (*by Xiaoyan Cui*).



Review

Gene targeting using the human Nalm-6 pre-B cell line

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Summary **Gene targeting by homologous recombination is a powerful tool to precisely manipulate the genome in order to study the function of a gene of interest (*GOI*). Indeed, it has become a routine methodology in yeasts, murine embryonic stem cells, and a chicken DT40 cell line. However, gene targeting has not been used often in human somatic cells to date since the relative efficiency of gene targeting (the ratio of homologous integrations to random integrations) is remarkably low. In this review, we introduce a fundamental strategy and a protocol to generate a null allele and/or 'tetracycline-inducible conditional gene knockout' for the *GOI* by gene targeting in the human Nalm-6 pre-B cell line. The Nalm-6 is a rare cell line in which gene targeting by homologous recombination takes place efficiently, and it carries a stable near-diploid karyotype with a doubling time of around 20 h. In addition, the tetracycline-regulated gene depletion (Tet-Off) system is steadily applicable to this cell line. Therefore, gene targeting systems using the Nalm-6 cell are used increasingly and offer promise in the study of human gene functions. This review should prove useful to researchers in a wide rage of fields.**

Keywords: Gene targeting, Gene knockout, Nalm-6

1. Introduction

Although RNA interference is now used routinely, gene targeting by homologous recombination is a powerful approach to studying gene function. However, homologous recombination in human somatic cells occurs infrequently except for in a limited number of cell lines including the HCT116 colon cancer cell line (*1*) and the Nalm-6 pre-B cell line (*2*) (Figure 1A). The Nalm-6 cell line, originally established from the peripheral blood of a patient with acute lymphoblastic leukemia (*3*), is highly efficient for gene targeting by homologous recombination (*2*,*4*,*5*). It also carries a stable near-diploid karyotype (Figure 1B) (*6*), unlike the HCT116 cell line. In addition, a tetracycline-regulated gene depletion (Tet-Off) system (*7*), a highly convenient inducible gene expression system, can be efficiently applied to this cell line with some technical improvements (Ono *et al*., submitted). A 'tetracycline-inducible conditional gene knockout' can thus be generated for a human gene of interest (*GOI*) in this cell line, in which the expression of the *GOI* can be depleted in a tetracycline-dependent manner

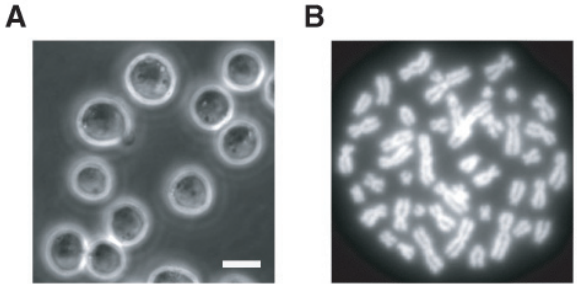

Figure 1. (A) Phase contrast images of the living Nalm-6 human pre-B cell line. Scale bar = 10 μm. (B) Staining metaphase chromosomes of the Nalm-6 cell with 4',6-diamidino-2-phenylindole (DAPI), showing the chromosome number of 2N = 46.

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on a knockout background. This method is useful and potentially applicable to any human genes, and thus gene-targeting systems using the Nalm-6 cell line will be used popularly in the near future in the study of human gene function.

2. General strategy

The general strategy described in this review uses a replacement-type vector as a targeting vector. The standard features of replacement-type vectors are a plasmid backbone containing a positive selection cassette placed between two regions of chromosomal homology (these regions are termed the 'left arm' and 'right arm' hereafter, they are also called the '5'-arm' and '3'-arm' for their position, or the 'long arm' and 'short arm' for their length) and a negative selection cassette adjacent to one of the homologous arms (*8*). With those negative and positive selections, the efficiency of homologous recombination between the targeting vector and the endogenous locus in the Nalm-6 cell line increases to a tractable range (the ratio between homologous recombinants and random integrants is around 1-5%). To design the targeting vector, a gene locus and the exon-intron structure of the *GOI* need to be defined. The University of California at Santa Cruz's Genome Browser web site is an extremely convenient way to obtain almost all of the information required for these purposes.

Unless the *GOI* is an essential gene, the first desired mutation to generate is a null allele. If protein-coding exons span a relatively small genomic distance (< 10 kb), almost all of the protein coding exons can be deleted. Using this strategy, the generation of a functional null allele is almost guaranteed because it is almost impossible to generate a protein product from the target locus. However, if the protein-coding exons of the *GOI* span a relatively large distance (greater than > 10 kb), more thought is required to determine which regions of the *GOI* should be deleted because conventional gene targeting strategies are less efficient at generating very large deletions (data not published). The first strategy suggested is to generate up to a 5-kb deletion (hopefully less than 3 kb) to remove as many of the protein coding exons as possible, including the exon containing the translation initiation codon (Figure 2A). Although this does not always guarantee the generation of a null allele, it increases its likelihood. Additionally, since neighboring genes may lie very close to the *GOI*, care must be taken not to delete portions beyond the known sequence of the gene. The second strategy suggested is to delete a coding region that encodes crucial functions of the proteins (Figure 2B). Again, the size of the deletion is limited up to 5 kb, and an in-frame stop codon can be placed in the downstream primer of the left arm when the left arm resides in the exon (Figure 3).

When the *GOI* is an essential gene or compensation experiments are required, a 'tetracycline-inducible conditional gene knockout' needs to be generated in which expression of the *GOI* can be depleted in a tetracycline-dependent manner on a knockout background. To generate a tetracycline-inducible conditional null allele, the tetracycline-inducible expression cassette of the *GOI* needs to be introduced into the cells before or after the targeting of the first allele, as outlined in Figure 5. A full-length protein or a tag-conjugated full-length protein can be expressed as the expression cassette of the *GOI* depending on plans for subsequent experimentation. Often expressed are FLAG-HA-tagged fusion proteins for the subsequent immuno-affinity purification of the functional protein complex or green fluorescence protein (GFP) conjugates to examine their cellular distribution. Expressing tagged-fusion proteins, instead of full-length proteins, is also beneficial because it facilitates confirmation of the tetracycline-regulated expression of the transgenes by Western blot analysis, as described in section 6.4. However, the most critical point here is that tagged-fusion proteins must be functionally intact to rescue the knockout phenotype.

3. Design of the targeting vector

3.1. *Finding the target locus to be deleted*

This is the most critical step to design the targeting vector as described above.

3.2. *The size and location of the target arms*

One of the most important parameters of the gene-targeting vector that influences targeting efficiency at a given genetic locus is generally assumed to be the length and the sequence identity of the homologous DNA regions on the vector (*9*). Shorter homologous arms might reduce the targeting efficiency and longer homologous arms hamper the construction of the targeting vector. In Nalm-6 cells, gene targeting is practicable with relatively shorter arms. The shorter arm should be 1.0~2.5 kb and the longer arm should be 2.5~6.0 kb in the Nalm-6 system. Genomic PCR is usually performed as the first screening to select the targeted clones, and thus one of the primer for the PCR is preset inside the drug resistance cassette (Figure 2). Given the efficacy of PCR amplification, the length of the DNA fragment amplified in a mass screening of PCR should be less than 3 kb (see section 6.3.2). Homologous arms generally include intron sequences or other untranslated segments of the endogenous gene. Untranslated regions of the genes often contain stretches of interspersed repetitive DNA that might influence targeting efficiency. Those repetitive sequences can be sought using the Human Genome Browser Gateway (*http://genome.ucsc.edu/cgi-bin/hgGateway*). Merrihew *et al*. (*10*) proposed an idea that random integration into the

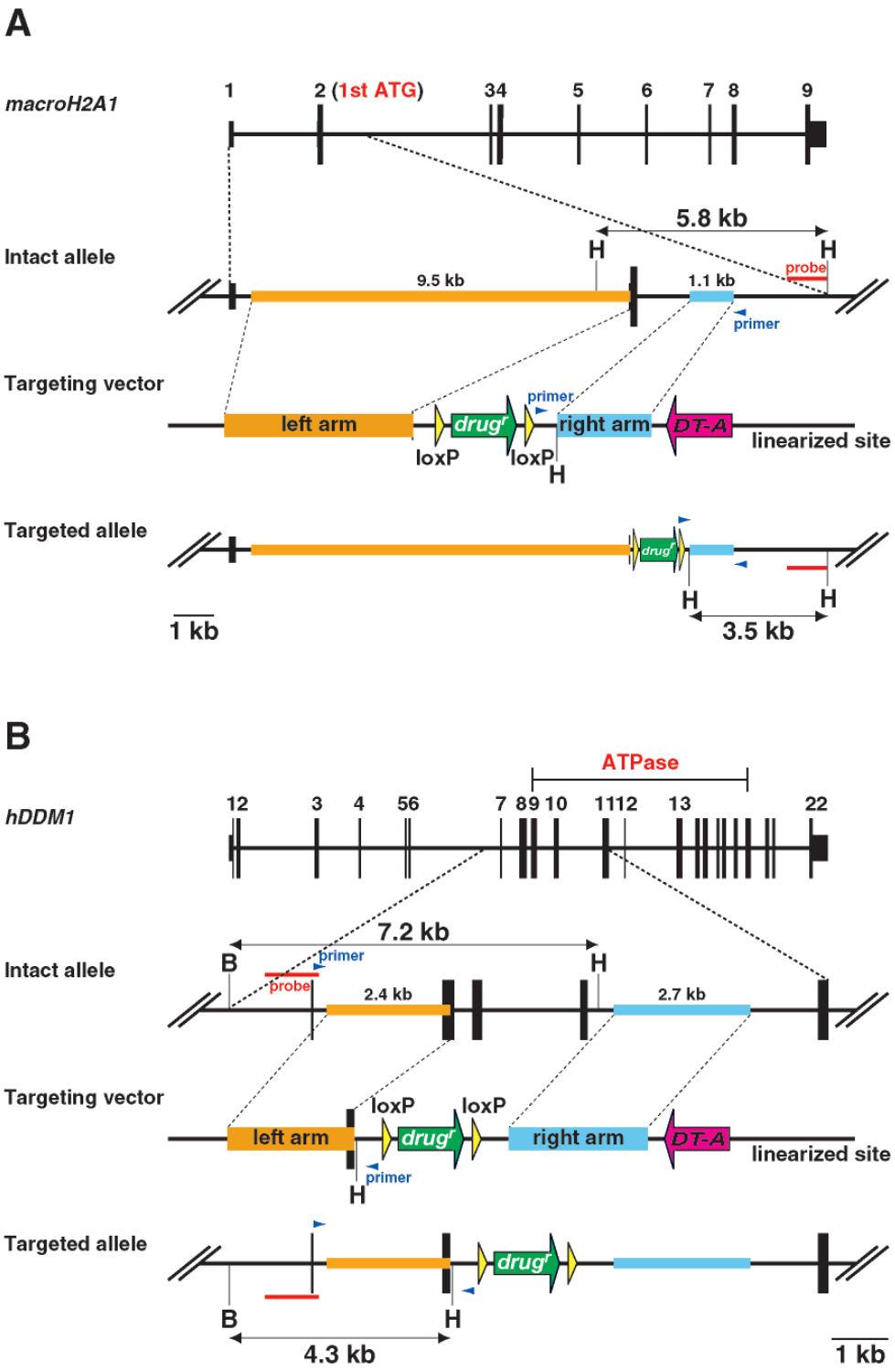

Figure 2. The strategy of knockout of the *GOI* in a Nalm-6 cell line. (A) An example of deletion of the exon containing the translation initiation codon. Diagrams to show the disruption of the *macroH2A1* locus. The *macroH2A1* gene consists of 9 exons with a histone fold (exon 2-4) and macro domain (exon 6-9). Codons coding methionine exist in exon 2 and exon 9. Therefore, gene-targeting vectors were constructed to delete the first ATG in exon 2. Black thin lines and closed boxes represent genomic DNA and exons, respectively. Orange and cyan box represent the left arm and the right arm of the targeting vector, respectively. The *drug*^{r} (green arrow box) and *DT-A* (magenta arrow box) cassettes are used for positive and negative selection, respectively. Through homologous recombination, exon 2 (including the 1st ATG) and introns are replaced with drug-resistant genes (*drug*^{r}): the histidinol resistance (*his*^{r}) gene for the first targeting, and the hygromycin resistance (*hyg*^{r}) gene for the second targeting. Yellow triangles represent *lox*P sequences, so *drug*^{r} could be removed from the targeted allele, if needed. In Southern blot analysis, the probe and expected bands are shown by a thick red line above the wild-type allele and double pointed arrows with their sizes and restriction sites: H for *Hin*dIII. In genomic PCR, the locations of the primers are indicated as blue arrowheads. (B) An example of the deletion of functional important coding exons. Diagrams to show the disruption of the human homolog of a decrease in DNA methylation 1 (*hDDM1*) locus. The *hDDM1* gene consists of 22 exons with a conserved ATPase domain extending from exon 9 to exon 19. Therefore, gene-targeting vectors were constructed to delete part of exon 8 and all of exons 9/10, in which the left arm harbored the stop codon in the frame and other frames (Figure 3); translation of *hDDM1* mRNA derived from targeted allele should be stopped. Through homologous recombination, a part of exon 8 and entire exons 9/10 including introns are replaced by *drug*^{r}. The symbols and terms used are the same as in A except where noted. The *puro*^{r} gene is used for the second targeting. The restriction sites B for *Bam*HI and H for *Hin*dIII are used for Southern blot analysis. These figures are drawn to scale.

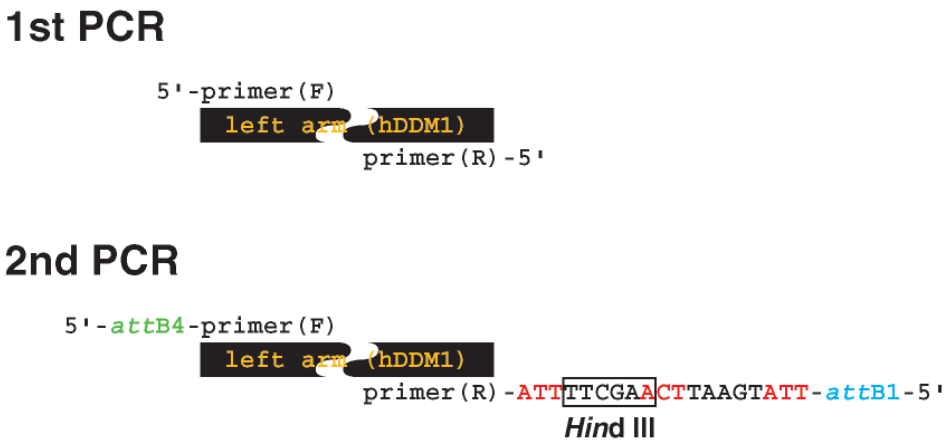

Figure 3. Primer design to amplify the DNA fragment with additional appended sequences using two-step PCR for the arm of the targeting vector. In the first step, the target genomic region is amplified using the primers simply corresponding to genome sequences. In the second step, the arm fragment is re-amplified using the primers with additional sequences: *att*B site for the MultiSite Gateway system (Figure 4), stop codon for the translation stop, and restriction enzyme site for Southern blot analysis. In this case, three frame stop codons (red characters) and a restriction enzyme site (*Hin*dIII, boxed AAGCTT) are appended at the 5'-end of the reverse primer for the left arm. The *att*B sequences differ from one another: *att*B4 sequence (green character) for the forward primer of the left arm, *att*B1 sequence (cyan character) for the reverse primer of the left arm, *att*B2 sequence for the forward primer of the right arm, and *att*B3 sequence for the reverse primer of the right arm.

genome was stimulated by short homological sequences. It is suggested that highly repetitive sequences should be excluded from the homologous arms as much as possible. However, the impact of repetitive sequences on gene targeting is a difficult issue, since it was reported that a flanking repetitive sequences enhanced the homologous recombination (*11*). Gene targeting of the *GOI* with pseudogenes or highly homologous genes often causes complications, Therefore, special care is needed in those cases. A BLAST search of the cDNA of the *GOI* or the arm sequences should always be run against the human genome assembly (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*) before finally deciding the arms. The sequence of the arms should be as identical as possible to the corresponding genomic regions, but for convenience the arms can, using the genomic DNA prepared from Nalm-6 cells as a template, be amplified by genomic PCR using proof-reading taq polymerases. Even with these proof-reading polymerases, amplification of the genomic regions often results in sequence errors, and thus the best clone with the sequence matching the corresponding genomic regions is chosen from the database.

3.3. *Positive selection cassette*

There are several positive selection marker genes available, including neomycin, hygromycin, puromycin, and histidinol resistance genes. The promoter for the marker expression used in the Nalm-6 system is mouse phosphoglycerate kinase (PGK) promoter or chicken β-actin promoter. Virus-derived promoters such as SV40 (Simian virus 40)-derived promoters should be avoided because some virus-derived promoters do not ensure stable expression in Nalm-6 cells (unpublished data). The cassette can be placed in either forward or reverse orientation relative to the direction of the *GOI*. The presence of a selectable marker cassette with its exogenous promoter may influence gene expression at the targeted locus or its surrounding genes. To avoid these unexpected and undesirable possibilities, the marker gene cassette should be flanked by loxP sequences or FRT sequences to provide the option for the subsequent removal of the marker gene cassette with Cre recombinase (*12*) or Flp recombinase (*13*). In addition, this option provides the opportunity to remove the selection marker cassette from the targeted locus for the subsequent targeting of an additional gene(s) to generate double or triple gene knockout cell lines. A point of note is that a "promoterless" drug resistance cassette is often used efficiently; the exons proximal to a promoter region are replaced and an endogenous promoter for the *GOI* is borrowed for the expression of the drug resistance gene (*4*).

3.4. *Negative selection cassette*

There are primarily two different ways of negative selection commonly used in gene targeting; these are used to preferentially kill the cells in which the targeting construct is incorporated in a random fashion. The first is a herpes simplex thymidine kinase gene expression cassette with gancyclovir or FIAU (1-2-deoxy-2-fluoro-1-β-D-arabinofuranosyl-5-iodouracil). An alternative strategy is to express the diphtheria toxin A-chain (DT-A) gene (*14*). Use of DT-A is preferred by the current authors because no drug need be added to the culture medium. The negative selection cassette can be placed in either forward or reverse orientation relative to the direction of *GOI* transcription. However, an important point is that even after positive/negative selection many of the resulting colonies are nontargeted clones (random integration or ectopic homologous recombinants; see 7. Troubleshooting).

3.5. *Linearization of the targeting vector*

The targeting vector must be linearized outside of the regions of homology before introduction into the Nalm-6 cells, and thus adequate restriction sites should be prepared in advance. The current authors usually place the restriction sites outside the negative selection cassette, as outlined in Figure 2 (see section 6.2.3).

3.6. *Southern blot analysis*

In the course of design of the targeting vector, genomic regions that can serve as Southern blot probes to recognize targeting events must also be determined. Southern analysis is performed with probes that are external to the regions of homologous arms in the targeting vector (Figure 2). Therefore, an important aspect to consider is restriction enzyme sites that can be used to discriminate between targeted and nontargeted alleles prior to construction of the targeting vector. Not all enzymes provide complete DNA restriction, and thus a pilot reaction with the enzyme of choice should be performed before screening is started. For convenience, ideal restriction enzyme sites are introduced in the downstream 3' primer to amplify the left arm or the upstream 5' primer to amplify the right arm, as shown in Figure 2. These restriction enzyme sites should be located outside the genomic region of the homologous arm and also at the genomic region to be disrupted (Figure 2). In this way, diagnostic digestion will yield a smaller DNA fragment for the mutant allele to serve as the recognized external probe. For the clear resolution of the DNA in agarose gels, the agreeable length of DNA fragment would be less than 15 kb. The length of the probe is also important for the efficiency of random oligo labeling (see section 6.2.4), and thus a 300~1,000 bp fragment is usually generated for the probe. Another step to take is avoiding the inclusion of highly-repetitive sequences in the probe and checking the probe sequence against the human genome assembly to exclude pseudogene loci and genetic regions with highly identical sequences. The probe is obtained by PCR amplification and its sequence confirmed before use.

3.7. *Primer design*

Genomic regions are amplified in two steps. In the first step, the genomic regions are simply amplified using the primers with 20-25 nucleotide sequences corresponding to the targeting locus. Sequences of primer with 55~68°C of the calculated Tm value and with G or C at the 3′ end are selected. Genetic analysis tools are also used to avoid primer sequences that tend to form a primer-primer dimer or hairpin-like structures as much as possible. The amplified DNA fragments are always confirmed by sequencing and/or restriction mapping analysis. In the second step, the confirmed DNA fragment is re-amplified with primers harboring additional sequences required for subsequent analysis. For instance, an in-frame stop codon and restriction sites for Southern blot analysis are added at the 5′ ends of the reverse primer for the left arm and the forward primer for the right arm (Figure 3). In addition, *attB* sequences are added to all of the primers at their 5′ ends when the MultiSite Gateway System is used to construct the targeting vector (see section 6.2.3).

4. Conditional gene targeting

Conditional gene targeting, in which gene disruption or gene inactivation occurs in a conditional manner, is especially valuable when null mutations are lethal or compensation experiments are required. The two main strategies for conditional targeting are using an inducible gene expression system such as a tetracycline-inducible gene expression system (Tet-Off system) or an inducible recombination such as *loxP* sequences and Cre site-specific recombinase (Cre/*loxP* system), CreMer, which carries a mutated hormone-binding domain of estrogen receptor that binds to the antagonist 4-hydroxytamoxifen (*15*). Both systems have advantages and disadvantages. For instance, the Tet-Off system is more tractable and suppression of gene expression can be achieved in a more synchronous manner. In addition, depleted gene product can be rapidly recovered by removing tetracycline. On the other hand, the Cre/*loxP* system can generate null alleles in principle and allows the use of endogenous promoters. However, Cre-mediated deletion does not occur synchronously and thus may not occur in all of the tamoxifen-treated cells (*16*). The Tet-Off system can be used efficiently in the Nalm-6 cell line with technical modifications (Ono *et al*., submitted). Use of the Tet-Off system is therefore recommended in Nalm-6 cell lines as the first choice to render cells conditionally null for the *GOI* because depletion is more synchronous and the system is more tractable.

5. Materials

5.1. *Web resources*

1. Human Genome Browser Gateway: *http://genome.ucsc.edu/cgi-bin/hgGateway*
2. NCBI BLAST Home: *http://blast.ncbi.nlm.nih.gov/Blast.cgi*

5.2. *Cell cultures*

1. Medium: 500 mL ES medium (Nissui, Tokyo, Japan) supplemented with 50 mL (10%) fetal bovine serum (FBS; Kohjin-Bio, Saitama, Saitama, Japan; *Note*: tetracycline-negative!), 5 mL (1%) penicillin-streptomycin solution (Sigma-Aldrich,

St Louis, MO, USA), 5 mL (2 mM) glutamine (Sigma-Aldrich), and 50 μM 2-mercaptoethanol (2-ME). *Note*: Adding too much 2-ME causes poor growth of cells!
2. Petri dish for suspension culture (10 cm × 2 cm; Greiner Japan, Tokyo, Japan).
3. Cell banker for frozen stock of cells (Nihon Zenyaku, Fukushima, Japan).

5.3. *Construction of the targeting vector*

1. GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich)
2. Proteinase K solution (Sigma-Aldrich)
3. RNase A solution (Sigma-Aldrich)
4. GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA)
5. PrimeSTAR HS DNA polymerase (Takara Bio, Ohtsu, Japan)
6. Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA)
7. PCR-Script Amp Cloning Kit (Stratagene, La Jolla, CA, USA)
8. Chemically competent DH5α or XL10-Gold cells
9. TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA
10. QIAprep Spin Miniprep Kit (Qiagen, Tokyo, Japan)
11. MultiSite Gateway System (Invitrogen, Carlsbad, CA, USA)

5.4. *Gene targeting screening*

1. Transfection: Nucleofector I (Amaxa Inc., Gaithersburg, MD, USA), Nucleofector Kit T (Amaxa Inc.).
2. Drug screening: 1.0 mg/mL of L-histidinol (Sigma-Aldrich), 0.2 μg/mL of puromycin (BD Biosciences, San Jose, CA, USA), 1.0 mg/mL of G418 (GIBCO, Grand Island, NY, USA), or 0.35 μg/mL of hygromycin B (Clontech, Mountain View, CA, USA). *Note*: Optimal concentrations for drug screening should be checked beforehand.
3. 96-well, 24-well, and 6-well flat-bottom plates for suspension culture (Sumitomo Bakelite Co. LTD, Tokyo, Japan).
4. 96-well V-bottom plate (Nunc, Roskilde, Denmark)
5. 8-channel and 12-channel pipettes (Thermo Scientific Inc., Rockford, IL, USA)
6. Lysis buffer for genomic DNA preparation: 100 mM Tris-HCl (pH 8.5), 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 100 μg/mL RNase A, and 100 μg/mL Proteinase K.
7. Plate seal 1 (Nunc)
8. Aluminum plate seal 2 (Simport, Beloeil, QC, Canada)
9. Optical 96-well reaction plate and MicroAmp 8-cap strip (ABI)
10. Takara Ex Taq (Takara Bio)
11. Takara LA Taq (Takara Bio)
12. Gel-loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 40% (w/v) sucrose in water
13. Biodyne B membrane (Pall, Pensacola, FL, USA)
14. Denaturation solution (for Southern): 1.5 M NaCl, 0.5 M NaOH
15. Neutralization buffer (for Southern): 1.5 M Tris-HCl (pH7.4), 1.5 M NaCl
16. Megaprime DNA labelling systems (GE Healthcare, Piscataway, NJ, USA)
17. Illustra ProbeQuant G-50 micro columns (GE Healthcare)
18. Hybridization oven HI-80M (Kurabo, Osaka, Japan)
19. Hybridization solution: 6× SSC, 5× Denhardt's reagent, 0.5% SDS, 50% formamide, 100 μg/mL salmon sperm DNA
20. [α-^{32}P] dCTP

6. Protocols

6.1. *Culture of Nalm-6 cells*

The Nalm-6 cell line is maintained in the medium described in section 5.2. The doubling time of the cells is around ~20 h and thus the cells 1:10 were diluted in three days. The cells grow to a maximum of 1.5 × 10^6 cells/mL in a logarithmic manner, but cells were kept under a concentration of 0.1~0.8 × 10^6 cells/mL. Cloning efficiency of the cells is quite good and the cells can be diluted when needed.

6.2. *Construction of the targeting vector and preparation of Southern probe*

6.2.1. *Preparation of genomic DNA*

The genomic DNA was prepared manually or by using the GenElute Mammalian Genomic DNA Miniprep kit. Starting cells are usually 5 × 10^6~1 × 10^7. Other kits or any manual protocols can also be used to prepare genomic DNA.

6.2.2. *Amplification of arms*

For the amplification of the genomic fragments, proofreading polymerase (with a 3′-5′ exonuclease activity) should be used. Here, PrimeSTAR HS DNA polymerase was used. A typical reaction is as follows:

1. Prepare a 50 μL reaction mixture as follows:

5× Polymerase buffer	10.0 μL
Genomic DNA (section 6.2.1)	2.0 μL (50 ng)
Forward primer (fin. 0.2 μM)	0.5 μL
Reverse primer (fin. 0.2 μM)	0.5 μL
dNTP (fin. 0.2 mM each)	4.0 μL
PrimeSTAR HS DNA polymerase	0.5 μL (1.25 U)
MilliQ water	32.5 μL

2. PCR program:

98°C	3 min	
98°C	10 sec	] 30 cycles
55°C	5 sec	
72°C	1 min/kb	
4°C	∞	

3. After checking and confirming the amplification of the DNA fragment through agarose gel electrophoresis, an amplified DNA band with an expected size is cut and purified by Wizard SV Gel and PCR Clean-Up System. *Note*: A recommended final elution volume is 50 μL according to the manufacturer's protocol, and ethanol precipitation is often performed here to concentrate DNA for subsequent cloning.
4. The purified DNA fragment is cloned into pPCR-Script Amp SK(+) cloning vector, as described in the manufacturer's protocol. For transformation, chemically competent DH5α or XL10-Gold cells were used. *Note*: Confirm the sequence of the cloned DNA because sequence matches influence targeting efficiency.

6.2.3. *Construction of the targeting vectors with the MultiSite Gateway System*

The MultiSite Gateway System, as mentioned previously (Figure 4; *ref. 17*), is quite useful at constructing targeting vectors, and the following is an example of constructing targeting vectors with this system. In this system, four DNA fragments of the left arm, the drug resistance expression cassette, the right arm, and the negative selection cassette/the plasmid backbone were conjugated efficiently in a one-tube reaction (Figure 4). In addition, two targeting vectors each containing different drug resistance gene cassettes are usually required to target the 1st and 2nd allele. Actually, three different targeting vectors can be generated simultaneously with the same arms but different drug resistant cassette vectors, as shown in Figure 4.

Step I:

1. Re-amplify the arm fragment with the primers containing attB sequences to fit the Gateway recombination system (Figure 4).
2. Prepare 10 μL reaction mixtures as follows:

	Left Arm	Right Arm
pDONR vector	50 fmoles (P4-P1R)	50 fmoles (P2R-P3)
Re-amplified PCR product	50 fmoles	50 fmoles
TE buffer	to 8.0 μL	to 8.0 μL

3. Add 2.0 μL BP Clonase II enzyme to the reaction mixture and mix well.
4. Incubate at 25°C for 1 h.
5. Add 1.0 μL Proteinase K and incubate at 37°C for 10 min to stop reaction.
6. Transformation of DH5α competent cells with 1 μL reaction mixture. *Note*: Transformants should be spread on pre-warmed LB plates containing 50 μg/mL kanamycin.
7. Prepare the plasmid DNA using the QIAprep Spin Miniprep Kit and select appropriate recombinant plasmid DNA by restriction enzyme digestion.
8. Confirm the sequence of the prepared recombinant plasmid DNA in this step and the resulting plasmids are termed as 5'-Entry clone and 3'-Entry clone.

Step II:

1. Prepare 20 μL reaction mixtures as follows:

Destination vector (pDEST DTA-MLS)	25 fmoles
Entry vector (pENTR lox-hisD)	25 fmoles
or (pENTR lox-puro)	25 fmoles
5'-Entry clone (L4-left arm-R1)	25 fmoles
3'-Entry clone (R2-right arm-L3)	25 fmoles
5× LR Clonase Plus Reaction Buffer	4.0 μL
TE buffer	to 16.0 μL

2. Add 4 μL LR Clonase Plus enzyme mix to the reaction mixture and mix well.
3. Incubate at 25°C for 16 h.
4. Add 2 μL Proteinase K solution and incubate for 10 min at 37°C to stop reaction.
5. Transformation of XL10-Gold competent cells with 1 μL reaction mixture. *Note*: Transformants should be spread on pre-warmed LB plates containing 100 μg/mL ampicillin.
6. Prepare the plasmid DNA using the QIAprep Spin Miniprep Kit and select appropriate recombinant plasmid DNA by restriction enzyme digestion.

6.2.4. *Preparation of southern probe*

For the amplification of the Southern probe, PrimeSTAR HS DNA polymerase should be also used. Basically subclone the amplified DNA fragment into the pPCR-Script Amp SK(+) cloning vector and confirm the sequence before use. The subcloned DNA fragment derived from pPCR-Script using the appropriate restriction enzyme was purified through agarose gel electrophoresis and stored.

6.3. *Targeting of the 1st allele of the GOI*

6.3.1 *Transfection of Nalm-6 cells and screening for drug-resistant colonies*

Day 0:

1. Linearize the targeting vector prepared in section

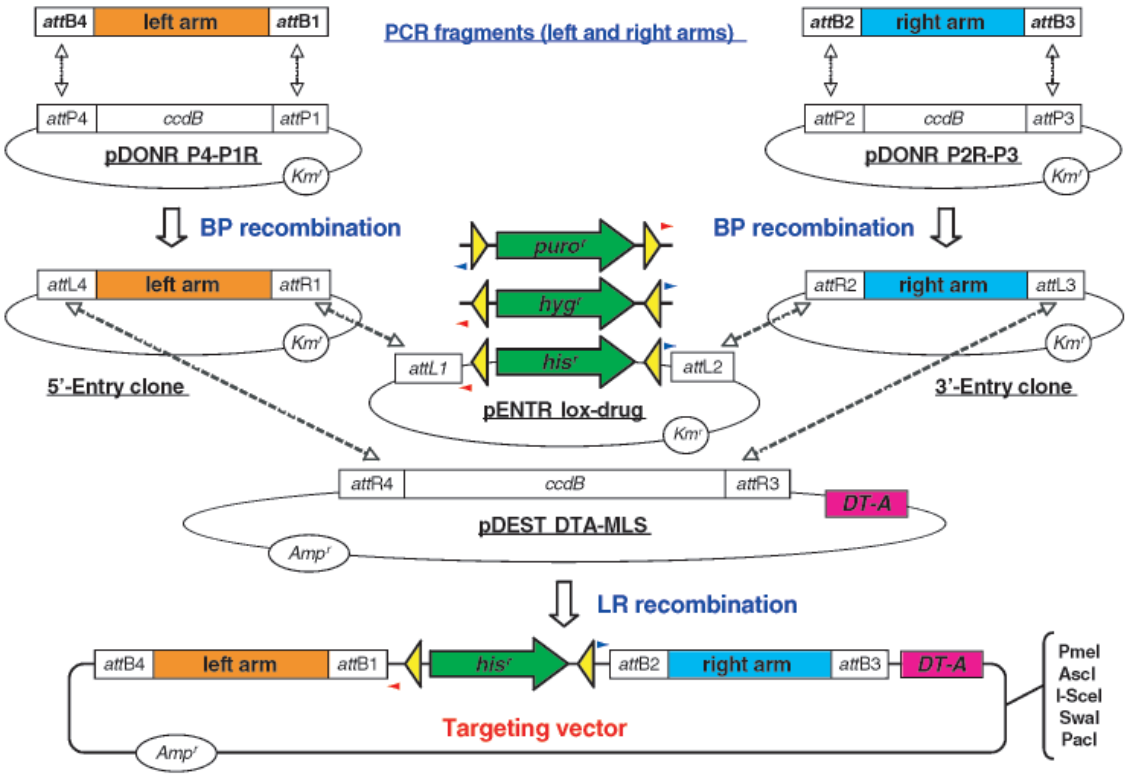

Figure 4. Diagram of the method of constructing a targeting vector based on the MultiSite Gateway system. This system consists of three steps: (1) PCR amplification with attB-containing primers, (2) BP recombination between each arm fragment and donor vector, and (3) LR recombination to construct the targeting vector using four DNA fragments. BP recombination is carried out between the left arm and pDONR P4-P1R donor vector and the right arm and pDONR P2R-P3 donor vectors, resulting in a 5'-Entry clone and 3'-Entry clone, respectively. In LR recombination, two targeting vectors for two rounds of targeting are generated containing different drug resistance gene cassettes; this was done using the same Entry clone but different drug resistant cassette vectors. Arrowheads indicate the two universal primers outside the *lox*P sequence. Yellow triangles represent *lox*P sequences for removal of the selection marker cassette from the targeted locus. *his*^{r}, a histidinol resistance gene; *hyg*^{r}, a hygromycin resistance gene; *puro*^{r}, a puromycin resistance gene; *DT-A*, a gene that codes for a diphtheria toxin A fragment; *Km*^{r}, a kanamycin resistance gene; *Amp*^{r}, an ampicillin resistance gene.

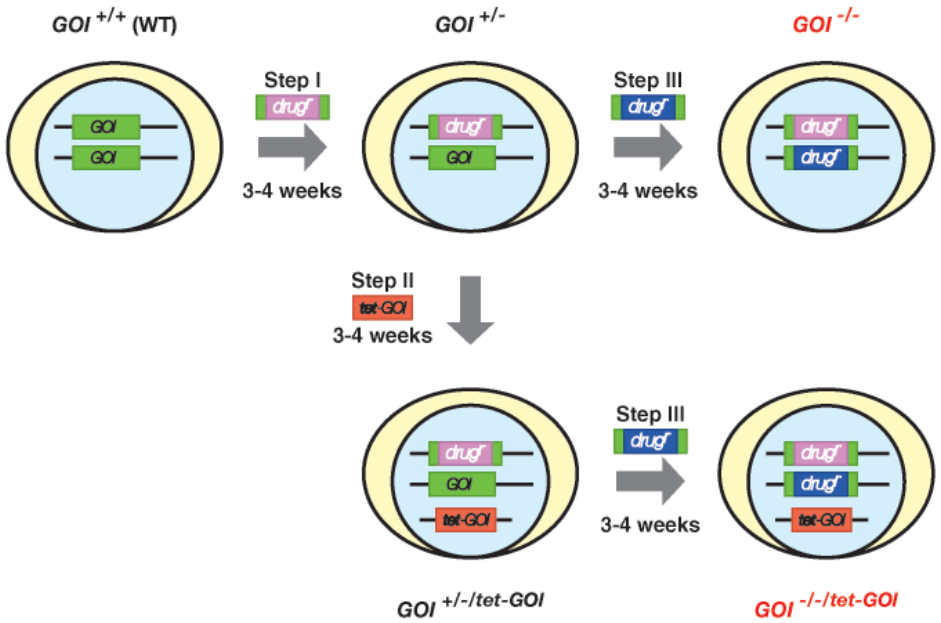

Figure 5. A flow chart for the generation of gene knockout human Nalm-6 cell clones. If the *GOI* is on the autosome, the cell harbors two alleles of the *GOI* (green box). Step I represents the first targeting of one *GOI* using the targeting vectors containing one drug resistant cassette (magenta box) to produce heterozygous *GOI*$^{+/-}$ cell clones, which takes 3-4 weeks. Step II is a process to obtain *GOI*$^{+/-/tet\text{-}GOI}$ cell clones expressing the exogenous *GOI* (orange box) in a tetracycline-regulated manner, which takes 3-4 weeks. This step is required only when tetracycline-inducible conditional gene-targeted cell clones are generated. Step III represents the second targeting of the other *GOI*, using the targeting vectors containing another drug resistant cassette (blue box), to generate homozygous *GOI*$^{-/-}$ or *GOI*$^{-/-/tet\text{-}GOI}$ cell clones, which also takes 3-4 weeks.

6.2, using a single cutter.
2. Digest overnight.

Day 1:

3. Perform phenol-chloroform extraction and ethanol precipitation and then rinse with ice-cold 70% ethanol.
4. Dry the DNA on a clean bench and suspend the DNA pellet in sterile MilliQ water.
5. Check the concentration of the DNA before transfection.
6. Pre-warm the complete culture medium (0.5 mL per one sample) in a 50 mL tube at 37°C.
7. Pre-warm the Nucleofector Solution T at room temperature (RT).
8. Perform a cell viability check for Nalm-6 cells. *Note*: Cells for transfection should be healthy and 2 × 10^6 cells per each transfection are required.
9. Harvest the cells by centrifugation at 90 g for 10 min at 22°C and completely aspirate the supernatant (Do not lose cells because cell pellets are less-tightly packed in this step!).
10. Suspend the cells with 100 μL of Nucleofector Solution T.
11. Add the linearized plasmid DNA (up to 2 μg) to the suspended cells and transfer the cell/DNA mixture into an Amaxa certified cuvette.
12. Perform transfection with Nucleofector I using the C-05 program of the machine.
13. Quickly add 500 μL pre-warmed culture medium into the cuvette and mix it well but slowly.
14. Transfer the cell/DNA mixture to a 6-well plate with a pipet.
15. Culture the cells in a humidified 5% CO_2 incubator at 37°C for 24 h.

Day 2:

16. Prepare the screening medium containing a selection drug at the appropriate concentration. *Note*: The optimal concentration of the drug should be determined whenever new lots of the drug are used!
17. Count the cells to check the viability of the transfected Nalm-6 cells. *Note*: Around 80% cell viability is preferred.
18. Seed the cells at 1,000-2,000 viable cells per well in the screening medium on 96-well flat-bottom plate.

Days 14-21:

19. After 2-3 weeks of culturing, drug-resistant colonies were selected and transferred to new 96-well flat-bottom plates.
20. These cell colonies were grown for the preparation of genomic DNA.

6.3.2. *Preparation of genomic DNA*

1. Transfer 150 μL cell (around 5 × 10^4 cells) suspensions from the 96-well flat-bottom plate to a 96-well V-bottom plate. *Note*: Wells on a flat-bottom plate should be filled with new medium and cells should then be cultured.
2. Centrifuge at 1,000 rpm (190 g) for 5 min at 4°C, and discard the supernatant by turning the plate over on a paper towel.
3. Add 50 μL lysis buffer per well, and seal the plate with a plate seal 1.
4. Mix the cells completely but gently using a plate mixer.
5. Incubate the mixtures at 55°C for more than 60 min.
6. After incubation, add 16 μL of 5 M NaCl per well, and mix them completely but gently using the plate mixer (Low speed, 1/3 max, 5 sec).
7. Add 70 μL 2-isopropanol per well, seal the plate with an aluminum plate seal 2, and mix vigorously using the plate mixer.
8. Centrifuge at 3,500 rpm (2,380 g) for 15 min at 4°C and discard supernatants by turning the plate over on a paper towel.
9. Resuspend the DNA pellet with 100 μL of ice-cold 70% ethanol per well, centrifuge at 3,500 rpm (2,380 g) for 5 min at 4°C, and discard supernatants by turning the plate over on a paper towel.
10. Dry for 10 min at RT.
11. Add 100 μL of TE buffer per well, seal the plate with a plate seal 1, and mix for more than 30 min using the plate mixer to completely dissolve the DNA pellet.
12. Store at 4°C for subsequent analysis.

6.3.3.*Target screening (Genomic PCR)*

1. Prepare a reaction pre-mixture as follows:

10× Ex Taq buffer	2.0 μL (×sample number)
Forward primer (100 μM)	0.1 μL (×sample number)
Reverse primer (100 μM)	0.1 μL (×sample number)
dNTP mixture (2.5 mM each)	1.6 μL (×sample number)
Ex Taq polymerase (5 units/μL)	0.1 μL (×sample number)
MilliQ water	15.1 μL (×sample number)
	19.0 μL (×sample number)

2. Add 1 μL of the genomic DNA prepared in section 6.3.2. to a MicroAmp Optical 96-well reaction plate.
3. Add 19 μL of well-mixed reaction mixture to the 96-well plate and mix it gently by pipetting, using the 8-channel multipipette.
4. Seal the plate tightly with MicroAmp 8-cap strip.
5. Perform PCR with the GeneAmp PCR System 9700 as follows:

98°C	5 min	
98°C	5 sec	] 40 cycles
65°C	30 sec	
68°C	1 min/kb	
72°C	5 min	
4°C	∞	

6. Check the PCR screen through agarose gel electrophoresis.
7. Select appropriate clones and transfer to a 6-well flat-bottom plate and then to a 10-cm plate for subsequent preparation of genomic DNA for Southern blotting. *Note*: If the target DNA fragment was not amplified, a different DNA polymerase could be used, such as LA taq, or parameters for PCR could be changed. If possible, several primers should be prepared.

6.3.4. *Target screening (Genomic Southern blotting)*

Day 0: Preparation of genomic DNA

The genomic DNA for Southern blotting is prepared from cell clones grown in a 24-well flat-bottom plate ~ 10-cm plate, as described in section 6.2.1.

1. Digest 10-20 μg genomic DNA with one or two restriction enzymes.

Day 1: Blotting

2. If necessary, concentrate the DNA fragments by ethanol precipitation and dissolve in ~15 μL of TE.
3. Add 0.15 vol of gel-loading buffer and separate the fragments of DNA by electrophoresis through an agarose gel (for most genomic DNAs, a 0.7% gel cast in 1× TBE may be used). Maintain a low voltage through the gel (about < 1 V/cm) to allow the DNA to migrate DNA slowly. *Note*: If the digested DNA has been stored at 4°C, it should be pre-heated to 56°C for 2-3 min! This heating disrupts any base pairing.
4. After electrophoresis is complete, stain the gel with ethidium bromide and photograph the gels. *Note*: Place a ruler alongside the gel in order to estimate the length of migrated DNA fragments.
5. Then soak the gel for 45 min at RT in a sufficient volume of denaturation solution with constant gentle agitation on a horizontal shaker.
6. Rinse the gel briefly in MilliQ water and then neutralize it by soaking for 30 min at RT in a sufficient volume of neutralization buffer with constant gentle agitation. Change the neutralization buffer and continue soaking for 15 min.
7. Use of the Biodyne B nylon membrane is suggested. Prepare the membrane as described (Molecular Cloning, 3rd edition).
8. Assemble the transfer apparatus and transfer the DNA to the prepared membrane with 0.4 M NaOH overnight, as described (Molecular Cloning, 3rd edition).

Day 2: Probe labeling

9. Labeling reaction: Prepare the following 50.0 μL reaction mixture.

probe	25 ng
random primers	5.0 μL
MilliQ water	to 33.0 μL

10. Incubate at 100°C for 5 min and transfer it to RT.
11. Add the following solutions to the mixture,

labeling buffer	10.0 μL
[α-^{32}P] dCTP	5.0 μL
Enzyme (Klenow fragment)	2.0 μL

12. Incubate at 37°C for 10 min.
13. Purify the labeled probe using the G-50 micro column (see Section 5.4).
14. Incubate the eluted DNA at 100°C for 5 min and rapidly cool it on ice for at least 2 min.

Day 2: Hybridization

15. Soak the membrane by floating it with a sufficient volume of neutralizing buffer in a tray for 15 min at RT.
16. Soak the membrane with a sufficient volume of 6× SSC in a tray for 5 min at RT.
17. Pre-hybridization: Place the membrane in a hybridization bottle containing 5 mL of hybridization solution, and incubate in a hybridization oven at 42°C for 2 h.
18. Pour off the hybridization solution from the hybridization bottle.
19. Hybridization: Add a 5 mL fresh hybridization solution containing a labeled probe, and incubate in a hybridization oven at 42°C over night.
20. Washing: wash the membrane as follows. *Note*: During washing, monitor the amount of radioactivity on the membrane using a hand-held Geiger counter!

In 2× SSC, 0.5% SDS	at RT for 5 min
In 2× SSC, 0.1% SDS	at RT for 5 min
In 0.1× SSC, 0.1% SDS	at 65°C for 30 min
In 0.1× SSC, 0.1% SDS	at 65°C for 1 h

21. Place the damp membrane on a sheet of plastic wrap, and expose the membrane to X-ray film for 36 h at –80°C with an intensifying screen.

6.4. *Isolation of stable cell clones expressing a tetracycline-inducible GOI*

As described in the section above, the most critical point here is to determine whether a full-length protein

or a tag-conjugated full-length protein is expressed to rescue the knockout phenotype. When a tagged-fusion protein is expressed, tetracycline-regulated expression of the transgenes is much easier to confirm with Western blot analysis, although tag-fusion may sometimes cause functional disability. cDNA is amplified by reverse-transcription PCR (RT-PCR) or can be purchased from Invitrogen (full-length cDNA of human genes is commercially available as Image clones). Detailed protocols for the applications of the improved Tet-Off system to the Nalm-6 cell line will be discussed in another opportunity (Nishijima *et al.*, in preparation).

6.5. *Targeting of the 2nd allele of the GOI*

To generate the homozygous null ($GOI^{-/-}$) or tetracycline-inducible conditional homozygous null ($GOI^{-/-/tet\text{-}GOI}$), the heterozygote for the *GOI* ($GOI^{+/-}$ or $GOI^{+/-/tet\text{-}GOI}$) is transfected with the 2nd targeting vector, as described in section 6.3.1. To screen for the $GOI^{-/-}$ or $GOI^{-/-/tet\text{-}GOI}$, genomic PCR (see sections 6.3.2 and 6.3.3), genomic Southern blot (see sections 6.2.1 and 6.3.4), or Western blot analysis can be used. Here, genomic PCR is usually performed for the first screening. For PCR screening, a specific primer outside of a homologous arm region is used with either of the two universal primers outside of the *loxP* sequences (Figure 4). If targeting vectors harboring a histidinol resistance cassette for the 1st targeting and a puromycin resistance cassette for the 2nd targeting are used, a universal primer with corresponding red arrowhead for the 1st screening and blue arrowhead for the 2nd screening can be used with a specific primer upstream of the left arm as was previously prepared. Additionally, PCR is performed using a primer set that corresponds to the region to be replaced by homologous recombination.

7. Troubleshooting

In addition to random integration, undesirable 'ectopic gene targeting' has often been observed in the Nalm-6 cell line as well as in mouse ES cells and a chicken DT40 cell line (unpublished data and personal communications). It is believed that ectopic gene targeting is an aborted event of homologous recombination in which a recombinant DNA molecule produced accidentally through homologous recombination between the introduced targeting vector and the target locus is randomly integrated into another genomic locus without completing targeting. Differentiating between this 'ectopic gene targeting' and true homologous recombination during screening for the heterozygotic allele is critical; otherwise, homozygotic alleles ($GOI^{-/-}$ or $GOI^{-/-/tet\text{-}GOI}$) can never be isolated. Thus, the signal strength of the bands derived from a wild-type allele and a targeted allele is always carefully compared in Southern blot analysis, as shown in Figure 6. The band intensity of the target allele is weaker than that of a wild-type allele in the cell clones in which 'ectopic gene targeting' takes place. In addition, a Southern probe is usually prepared at both the 5′ and 3′ ends to make sure.

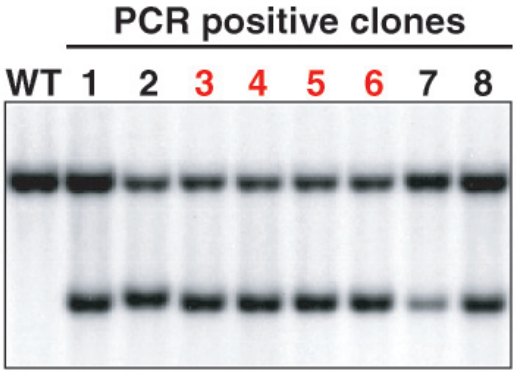

Figure 6. Southern blot analysis for 1st targeting. Genomic DNA was digested by restriction enzyme and hybridized with the [α-^{32}P] dCTP-labeled probe. Ten micrograms of DNA was loaded on each lane. The upper and lower bands are derived from a wild-type allele and a targeted allele, respectively. The signal strength of upper bands is no more than that of lower bands in clones 3, 4, 5 and 6, in which target gene disruption is performed through homologous recombination in all likelihood. The signal strength of upper bands in these clones is obviously weaker than that in the wild-type (WT). This does not apply, however, to clones 1, 7, and 8, in which 'ectopic gene targeting' takes place. These cell lines were not selected as a heterozygotic clone as they would never yield a knockout cell. Clone 2, in which the lower band has a different than expected size, was also not selected.

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Brief Report

The role of village doctors on tuberculosis control and the DOTS strategy in Shandong Province, China

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Summary **This study aims to assess the knowledge, attitudes, and practices of village doctors based on current national policies and to explore factors determining their roles in the directly observed therapy, short course (DOTS) strategy and tuberculosis (TB) control in rural China. In this cross-sectional study conducted in Shandong Province, we interviewed a total of 1,824 village doctors from 36 villages using a semi-structured questionnaire that included demographic characteristics, knowledge of TB treatment and current policies related to TB prevention and control, attitudes towards TB patients, and practices of educating rural residents, detection of TB cases, and supervision of patients. Results indicated that most village doctors underwent a training program and were willing to receive additional training while almost half recognized that their current knowledge was insufficient to meet the demands of their work. Village doctors implemented health education regarding TB control in a variety of forms for rural residents. Some practices of village doctors were inappropriate and remain so. In conclusion, this study revealed the need to provide sufficient, practical, and feasible training programs in order to administratively regulate the practices of village doctors and the need to enrich the content of incentives with increased financial subsidies.**

Keywords: Tuberculosis, DOTS, Village doctors, Management, Health education, China

1. Introduction

China has the second highest incidence of tuberculosis worldwide. Approximately one-third of the total population has been infected with Mycobacterium Tuberculosis. Among the infected population, 10% will develop the disease. The prevalence of smear-positive and infective tuberculosis (TB) is reported to be 121.6/100,000 and 157.8/100,000, respectively. According to a news release from the Ministry of Health of China, active TB patients number 450 million, of which 150 million are infective cases, and about 13 million annually die from the disease (*1*). The drug resistance rate is as high as 28%. Moreover, in terms of geography and socioeconomics, 85% of TB patients live in rural areas, where the prevalence of smear-positive and infective TB is much higher than in urban areas. TB is the first attributor to the vicious circle of illness and poverty among rural residents. Therefore, TB control in rural areas is an urgent priority for China.

Since the 1990s, the Chinese government has launched a series of projects directed at TB involving the use of directly observed therapy, short course (DOTS). The government provides free treatment for patients. DOTS coverage has reached 100%, and the case detection and treatment success rate has surpassed the goal of 70% and 85% at the global level (*2,3*). On the other hand, however, there is still a gap between national policies and actual practices. Because of the long duration of treatment, patients tend to discontinue that treatment, resulting in failure to cure the disease.

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In the DOTS strategy, village doctors are responsible for implementing health education in the community in order to promptly detect symptomatic TB patients and provide direct referral to an appropriate facility and in order to supervise and manage the drug use and treatment of patients in rural areas. They have to promptly report inappropriate drug use, discontinuation, and side effects occurring in patients to attending physicians at higher level health care facilities. In order to improve patients' adherence to treatment and improve the attitudes of village doctors, economic incentives such as bonuses for case detection and patient supervision have been introduced as part of the current national policies. The management by village doctors and effects of incentives in their work need to be systematically investigated in order to assist policymakers with their decision-making. The objectives of this study are to assess the knowledge, attitudes, and practices of village doctors based on current national policies and to explore factors determining their roles in the DOTS strategy and TB control.

2. Methods

A cross-sectional study was conducted from October to November 2007 in Shandong Province. A stratified-cluster random sampling was used to recruit participants, *i.e.* village doctors. First, based on GDP per capita, 17 districts in Shandong Province were divided into three categories, well-developed, middle developed, and less developed, and one district was selected from each category. Then, two counties from each district were selected for a total of six villages from each county. All registered village doctors in the 36 villages that were ultimately selected were interviewed with a structured questionnaire. The sample size was 1,824 in total. The content of the questionnaire included demographic characteristics, knowledge of TB treatment and current

Table 1. Demographic characteristic of the surveyed village doctors (n = 1,824)

		Frequency	%
Age	20 ~ 35	601	32.9
	35 ~ 50	723	39.6
	50 ~ 65	482	26.4
	65 ~ 80	18	1.0
Gender	Male	1,483	81.3
	Female	341	18.7
Education	Middle school and below	259	14.2
	High school or technical school	267	14.6
	Secondary medical school	1,229	67.4
	Junior medical college and above	69	3.8
Working years	0 ~ 10	640	35.1
	10 ~ 20	588	32.2
	20 ~ 30	311	17.1
	30 ~ 40	244	13.4
	40 ~ 50	41	2.2
Position level	Village doctor	235	12.9
	Assistant doctor	846	46.4
	Doctor and above	743	40.7
Major	Clinical medicine	1,655	90.7
	Public health	59	3.2
	Nursing	32	1.8
	Others	78	4.3
Income per month	Less than 1,000 RMB	1,465	80.3
	1,000 ~ 2,000 RMB	340	18.6
	2,000 ~ 3,000 RMB	19	1.0
Training experience	Yes	1,479	81.1
	No	345	18.9
Knowledge meeting the needs in the work	Yes	976	53.5
	No	848	46.5
Willingness for further training	Yes	1,463	80.2
	No	361	19.8
Free treatment for TB patients	Yes, I know	1,551	85.0
	No, I don't know	273	15.0
Incentive policy	Yes, I know	1,366	74.9
	No, I don't know	458	25.1

policies related to TB prevention and control, attitudes towards TB patients, and practices of educating villagers, TB case detection, and supervision of patients.

Interviewers were trained, a pilot study was conducted, and a guideline for field work was drafted ahead of time in order to coordinate experts from the Shandong Province Tuberculosis Prevention Center in the field work. Questionnaire development and data entry checking were also done in order to ensure the quality of the study.

Descriptive analysis, univariate tests including a Chi-square test and Kruskal-Wallis H test, was performed and a logistic regression model was constructed with SPSS 13.0 for Windows in order to determine factors influencing the village doctors' practices related to TB prevention and treatment in the rural community, including health education of rural residents, TB case detection, and supervision & management of TB patients. $p < 0.05$ was considered to indicate statistical significance.

Shandong University approved this study and ethical permission was obtained. All participants were informed about the study procedures and the study was conducted after their informed consent was obtained

3. Results and Discussion

3.1. *Demographic characteristic, training, and knowledge of related policies*

Table 1 is a summary of demographic characteristics, training and knowledge of related policies of the village doctors surveyed. The average age of village doctors was 42.14 years. Most participants (71.2%) had an educational background of secondary medical school or higher. Average work experience was 17.88 years, and 87.1% of village doctors were an assistant doctor or above. Of all of the village doctors, 90.9%

Table 2. Factors associated with health education provision (n = 1,824)

		Yes	No	Proportion of health education provision (%)	Univariate analysis[a]	Multivariate analysis[b]
Age	20 ~ 35	526	75	87.5	< 0.001	0.003
	35 ~ 50	567	156	78.4		
	50 ~ 65	407	75	84.4		
	65 ~ 80	14	4	77.8		
Education	Middle school and below	190	69	73.4	< 0.001	NS
	High school or technical school	230	37	86.1		
	Secondary medical school	1,031	198	83.9		
	Junior medical college and above	63	6	91.3		
Working years	0 ~ 10	545	95	85.2	0.001	0.002
	10 ~ 20	458	130	77.9		
	20 ~ 30	260	51	83.6		
	30 ~ 40	216	28	88.5		
	40 ~ 50	35	6	85.4		
Position level	Village doctor	219	16	93.2	< 0.001	NS
	Assistant doctor	703	143	83.1		
	Doctor and above	592	151	79.7		
Major	Clinical medicine	1,389	266	83.9	< 0.001	NS
	Public health	32	27	54.2		
	Nursing	21	11	65.6		
	Others	72	6	92.3		
Income per month	Less than 1,000 RMB	1,217	248	83.1	NS	
	1,000 ~ 2,000 RMB	284	56	83.5		
	2,000 ~ 3,000 RMB	13	6	68.4		
Training experience	Yes	1,372	107	92.8	< 0.001	< 0.001
	No	142	203	41.2		
Knowledge meeting the needs in the work	Yes	837	139	85.8	0.001	NS
	No	677	171	79.8		
Willingness for further training	Yes	1,285	178	87.8	< 0.001	< 0.001
	No	229	132	63.4		
Free treatment for TB patients	Yes, I know	1,326	225	85.5	< 0.001	0.013
	No, I don't know	188	85	68.9		
Incentive policy	Yes, I know	1,246	120	91.2	< 0.001	< 0.001
	No, I don't know	268	190	58.5		

[a] Chi-square test; [b] Multiple logistic regression test; NS = no significant.

specialized in clinical medicine. Average monthly income was 856.66 RMB (approximately 128 US dollars). Regarding occupational training in TB control and treatment, 81.8% of village doctors underwent a training program while nearly half recognized that their current knowledge was insufficient to meet the demands of their work. Most village doctors were familiar with national policies such as free treatment for TB patients and economic incentives for case detection, management, and supervision. Nonetheless, most participants were still willing to receive additional training in the future. The fact that almost half considered their current knowledge to be insufficient for their work represents an obstacle to providing adequate health care services to patients. According to the interviews, most participants were willing to improve their knowledge of prevention, early detection, and management of TB cases. Improving their knowledge of TB is crucial to increasing direct referrals to TB specialists and promptly detecting symptomatic TB patients (*4*).

3.2. *Role of village doctors in health education, case detection, and supervision*

Of village doctors surveyed, 83.0% provided an educational program on TB prevention for patients and rural residents. The main methods of education included distribution of pamphlets, verbal announcements, village broadcasts, and bulletins. Among those 1,514 village doctors, 86.5% informed rural residents of the typical symptoms of TB, 82.3% informed them of national policies such as free diagnosis and treatment, 70.1% informed them of correct practices for sterilization, 69.4% informed them about quarantines, 56.6% informed them of clinical practices for treatment, and 42.5% informed them of methods and times for sputum sample collection.

Table 3. Factors associated with TB case finding (*n* = 1,824)

		Yes	No	Proportion of TB case finding (%)	Univariate analysis[a]	Multivariate analysis[b]
Age	20 ~ 35	157	444	26.1	< 0.001	< 0.001
	35 ~ 50	343	380	47.4		
	50 ~ 65	177	305	36.7		
	65 ~ 80	8	10	44.4		
Education	Middle school and below	119	140	45.9	0.007	NS
	High school or technical school	108	159	40.4		
	Secondary medical school	431	798	35.1		
	Junior medical college and above	27	42	39.1		
Working years	0 ~ 10	204	436	31.9	< 0.001	< 0.001
	10 ~ 20	267	321	45.4		
	20 ~ 30	117	194	37.6		
	30 ~ 40	81	163	33.2		
	40 ~ 50	16	25	39.0		
Position level	Village doctor	53	182	22.6	< 0.001	< 0.001
	Assistant doctor	328	518	38.8		
	Doctor and above	304	439	40.9		
Major	Clinical medicine	587	1,068	35.5	< 0.001	NS
	Public health	42	17	71.2		
	Nursing	15	17	46.9		
	Others	41	37	52.6		
Income per month	Less than 1,000 RMB	549	916	37.5	0.03	NS
	1,000 ~ 2,000 RMB	129	211	37.9		
	2,000 ~ 3,000 RMB	7	12	36.8		
Training experience	Yes	528	951	35.7	0.001	NS
	No	157	188	45.5		
Knowledge meeting the needs in the work	Yes	373	603	38.2	NS	
	No	312	536	36.8		
Willingness for further training	Yes	573	890	39.2	0.004	0.001
	No	112	249	31.0		
Free treatment for TB patients	Yes, I know	625	926	40.3	< 0.001	< 0.001
	No, I don't know	60	213	22.0		
Incentive policy	Yes, I know	502	864	36.7	NS	
	No, I don't know	183	275	40.0		

[a] Chi-square test; [b] Multiple logistic regression test; NS = no significant.

Of 1,824 village doctors, 685 had detected TB cases or quasi-cases since 2006. They identified a total of 1,331 cases, of which 1,212 were referred to a higher health care facility and 834 cases were definitively diagnosed. Among these 685 village doctors, 90.9% referred cases or quasi-cases to a specialized TB dispensary, 6.7% referred them to a hospital, 0.6% treated the case themselves, and 1.8% took other measures.

Of all of the village doctors surveyed, 660 had supervised patients since 2006. The average duration of that supervision was 11 days per case. Regarding the site of supervision, 25.3% supervised patients at a village health care center, 74.1% at the patient's home, and 0.6% at another location. Only 31.7% of these village doctors had charged for drugs or inappropriately ordered the family or other individuals to keep the drugs. A total of 1,012 patients had been supervised since 2006, of which 19% failed to continue to adhere to the course of the treatment ordered.

Factors associated with provision of educational programs, case detection, and supervision by village doctors were input into a logistic regression model. Independent factors contributing to practices were identified as age, work experience (in years), training, willingness to receive further training, and knowledge of free treatment for TB patients, suggesting that adequate training programs will help these village doctors to provide adequate services in a rural community (Tables 2-4).

Since DOTS coverage has quantitatively reached 100%, the new issue for policymakers is the provision of quality TB care. The role of village doctors is crucial to the improvement of DOTS quality and TB control in rural areas. Most participants provided health education in various styles in the community. However, this study also found inappropriate practices in terms of patient referrals after case detection and supervision of patient

Table 4. Factors associated with supervision and management (n = 1,824)

		Yes	No	Proportion of supervision & management (%)	Univariate analysis[a]	Multivariate analysis[b]
Age	20 ~ 35	151	450	25.1	< 0.001	< 0.001
	35 ~ 50	329	394	45.5		
	50 ~ 65	172	310	35.7		
	65 ~ 80	8	10	44.4		
Education	Middle school and below	118	141	45.6	0.002	NS
	High school or technical school	106	161	39.7		
	Secondary medical school	412	817	33.5		
	Junior medical college and above	24	45	34.8		
Working years	0 ~ 10	197	443	30.8	< 0.001	< 0.001
	10 ~ 20	254	334	43.2		
	20 ~ 30	121	190	38.9		
	30 ~ 40	71	173	29.1		
	40 ~ 50	17	24	41.5		
Position level	Village doctor	45	190	19.1	< 0.001	NS
	Assistant doctor	319	527	37.7		
	Doctor and above	296	447	39.8		
Major	Clinical medicine	561	1094	33.9	< 0.001	NS
	Public health	40	19	67.8		
	Nursing	18	14	56.2		
	Others	41	37	52.6		
Income per month	Less than 1,000 RMB	524	941	35.8	NS	
	1,000 ~ 2,000 RMB	129	211	37.9		
	2,000 ~ 3,000 RMB	7	12	36.8		
Training experience	Yes	514	965	34.8	0.008	0.003
	No	146	199	42.3		
Knowledge meeting the needs in the work	Yes	367	609	37.6	NS	
	No	293	555	34.6		
Willingness for further training	Yes	560	903	38.3	< 0.001	< 0.001
	No	100	261	27.7		
Free treatment for TB patients	Yes, I know	602	949	38.8	< 0.001	< 0.001
	No, I don't know	58	215	21.2		
Incentive policy	Yes, I know	482	884	35.3	NS	
	No, I don't know	178	280	38.9		

[a] Chi-square test; [b] Multiple logistic regression test; NS = no significant.

drug use that negatively affected the quality of DOTS. Those practices should be corrected and improved by effective training programs and administrative directives.

3.3. *Incentives*

The purpose of introducing economic incentives as part of the current policy is to enhance the role of village doctors in rural TB control and detection and management of patients. However, this study failed to confirm that knowledge of economic incentives had a significant impact on case detection and supervision (Tables 3 and 4), suggesting that economic incentives did not affect village doctors as expected. In the interviews, most participants mentioned that while the current national policy on economic incentives was welcome the actual implementation in some counties was worrisome, which may hinder their attitude towards their work. Some participants felt that economic incentives should be increased with financial investment from the government and they also cited the importance of non-economic incentives as well. In that regard, non-economic incentives should be diverse, including individual awards, team or facility awards, promotions, more training opportunities, and commendations from the county or higher authorities. Based on the findings of this study, the government must seek to develop appropriate financial measures to enhance the role of village doctors since a shortage of financial subsidies accompanying the decentralization of health financing and lack of skill, equipment, and incentives causes a delay in case detection and prompt referral (*5*).

In conclusion, this study revealed the need to provide adequate and practical training programs to village doctors in order to administratively regulate their practices and the need to enrich the content of incentives for those doctors with increased financial subsidies and feasible models.

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Original Article

Correlates of life satisfaction among older Nepalese adults living with a son

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Summary **The objective of this study was to identify the correlates of life satisfaction in elderly Nepalese adults living with a son. A convenience sample of 489 urban elderly was recruited from Kathmandu, Nepal. Socio-demographic, economic, health, and social support variables were used as correlates of life satisfaction. Life satisfaction was measured using 9 of the 11 items of the Life Satisfaction Index by Liang. Results showed that the strongest correlate of life satisfaction was perceived financial satisfaction ($p < 0.0001$). High life satisfaction was also more likely reported by elderly who were educated ($p < 0.05$), had fewer functional limitations ($p < 0.005$), and better perceived health ($p < 0.0001$). Other correlates with high life satisfaction were instrumental support ($p < 0.05$) but not emotional support from the son. Further research on correlates of life satisfaction in developing countries will assist health care practitioners and policy makers involved in the well-being of aged populations.**

Keywords: Life satisfaction, Older adults, Developing countries, Social support, Nepal

1. Introduction

Gerontologists have indicated that quality of life should be considered not only in terms of the length of life but equally in the degree of life satisfaction. For the elderly, life satisfaction is defined as an overall assessment of one's life (*1*). Factors affecting life satisfaction in the elderly include socio-demographic characteristics, general health status, personal characteristics, and family relationships. Results of research into the determinants of life satisfaction in Western countries have been mixed and inconsistent. Major differences are consistently found in the demographic context of aging for men and women (*2*). A meta-analysis of 300 empirical studies on gender differences in life satisfaction revealed that socio-economic status more strongly correlated with life satisfaction in elderly men than in elderly women (*3*).

Various correlates of life satisfaction of the elderly have been reported in the literature. Findings on whether life satisfaction increases, decreases, or remains stable as people age are inconsistent. Some studies showed no empirical relationship between age and life satisfaction of the elderly, while others reported that life satisfaction decreased with age (*4*).

Still other studies have reported that life satisfaction is related to the gender and marital status of the elderly (*5*). A study among community-dwelling elderly in a rural part of Japan found life satisfaction was related to mental health and age in women but to mental health status and social support from others in men (*6*). Marital status was positively associated with life satisfaction among elderly Canadians (*7*). The effect of marital status on life satisfaction appears to be gender-specific. One study found that being married had a significantly higher association with life satisfaction for men than for women (*8*). Financial strain has been negatively associated with life satisfaction (*9*). One study found no significant relationship between income and life satisfaction (*10*), while another found a

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significant positive relationship between life satisfaction and education and income (*11*). Educational attainment has been positively associated with life satisfaction of the elderly (*12*).

Biologically, physical health deteriorates as people age. Health is an important variable influencing the life satisfaction of older adults. Associations between higher life satisfaction and better health, level of functioning, and self-perceived health have been consistently shown (*2*). There are cross-cultural variations in the correlates of life satisfaction of older adults. For example, emotional attributes may be a very important correlate of life satisfaction in individualist countries while they may be less important in collectivist countries where social factors are more significant (*13*).

The quality and quantity of reciprocal assistance plays a crucial role in the life satisfaction of the elderly. The literature shows that the elderly who receive a high level of social support report higher life satisfaction (*9*). Instrumental and emotional support are among the major types of social support provided to the elderly. A previous study in Nepal found that social support from children living with the elderly and social support for a spouse, children living together, and friends and neighbors reduced the loneliness of the elderly while support for children living apart increased subjective well-being life-satisfaction (cognitive long-term well-being) and support from children living together and support for children living together and apart increased subjective well-being life-stability (emotional short-term well-being). The study was, however, limited to one caste group, Brahman, in Nepal (*14*). The present study focuses on the general population of the elderly in Nepal.

Life satisfaction of the individual is a complex, subjective, and culturally determined phenomenon. Although much research has been conducted regarding the correlates of life satisfaction of the elderly in developed countries, few studies have been reported from developing countries (*15*). Population aging is a global phenomenon and is proceeding rapidly in developing countries. The population of the elderly has been increasing faster than the total population in Nepal over the last three decades, and the aged population is expected to double in 20 years (*16*). Life expectancy at birth increased in Nepal by almost 20 years, from 41 years in the 1970s to 60 years in 2001. In addition the average family size declined from 6 children (for every parent) in the 1970s to 4.1 in 2001 to 3.1 in 2006 (*16*). This increase in the older adult population in Nepal highlights the need to study those factors that impact well-being and life satisfaction. Norms of filial responsibility govern intergenerational relationships in Nepal as in other Asian countries. This is evident by the fact that as many as 80.0% of older Nepalese live with an adult child. The present study explores the correlates (socio-demographic, economic, health, and social support variables) of life satisfaction among Nepalese elderly living with a son.

2. Materials and Methods

2.1. *Subjects*

The study sample was derived from a survey that sought to examine the relationship between the elderly and a married son. A convenience sample of people aged 60 and above was recruited from a municipal area within Kathmandu, Nepal (Ward number 10). Elderly constitute 6.5% of the total population of Nepal (CBS, 2003). The 2004 voter list was used to identify adults aged 60 years or older. A total of 1,539 older adults aged 60 and above were identified in ward number 10. Only those elderly able to communicate well in Nepali were included. If there was more than one elderly adult in a household, only one elderly adult was selected for an interview based on his or her willingness to participate. A face-to-face structured interview was conducted by 1 of 10 researchers who had undergone an intensive one-day training course on the process of data collection. A total of 489 respondents (247 men and 242 women) were interviewed. Sampling procedures are detailed elsewhere (*17*).

Verbal informed consent was obtained from all respondents prior to the study. Due to the high rate of illiteracy among older adults, written informed consent was not possible (*14*). The study protocol was approved by the Institutional Review Board of the Graduate School of Medicine, The University of Tokyo.

2.2. *Dependent variable*

Life satisfaction was measured using the Life Satisfaction Index developed and validated by Liang (*18*) (LSI-L) from the original Life Satisfaction Index A developed by Neugarten and colleagues (*1*). The reliability and validity of this scale has been tested internationally (*19*). LSI-L is an 11-item scale measuring three dimensions of life satisfaction: mood, zest, and congruence. In the current data, two questions based on assumptions ("my life could be happier than it is now", and "I would not change my past life, even if I could") were removed because of lower or negative correlations with other items. Confirmatory factor analysis of the remaining nine items in the data of this study showed a good fit of the model with the second-order factor of general life satisfaction and the three first-order factors of mood, zest, and congruence (chi-square = 87.6, DF = 26, GFI = 0.961, AGFI = 0.933, RMSEA = 0.070). The total score of the nine-item version of the LSI-L was used in this study. Each item had response options of "agree" and "disagree," resulting in a score ranging from 0 to 9. The Cronbach reliability coefficient for the present study was 0.838.

Initially, the English questionnaire was translated into the Nepali language by the first author. The draft questionnaire was then reviewed and revised by three Nepali students at the University of Tokyo proficient at both the English and Nepali languages. The Nepali version of the questionnaire was re-translated into English by a professional Nepali-English translator who had not previously seen the questionnaire. After checking the original English version and the back-translated versions of the questionnaire, all differences were corrected by the first author and the final version of the questionnaire was developed.

2.3. *Independent variables*

Socio-demographic variables measured were age, gender, marital status, education, and economic status. Education was coded into four categories: illiterate (= 1), literate but no formal schooling (= 2), schooling through high school (= 3), and college & above (= 4). Economic variables were measured by self-perceived financial satisfaction, currently in paid employment, and possession of inherited property. Self-perceived financial satisfaction was measured by asking, "Compared to other people of your age in your neighborhood, are you financially satisfied?" Response options were categorized as dissatisfied (= 1), fairly satisfied (= 2), and satisfied (= 3). Respondents were asked if they were currently in paid employment and whether or not they possessed inherited property (yes = 1; no = 0). In Nepal, inherited property is transferred from parents to sons. Some older adults transfer all of their inherited property to their sons while still alive, some make provisions for transfer after death, and others keep a share of the property and transfer the rest to their sons. The importance of maintaining inherited property to the well-being of older Nepalese adults in later life has been confirmed in a qualitative study (*20*).

The health measures included Instrumental Activities of Daily Living (IADL), self-perceived health, and number of chronic illnesses. IADL was measured by five items (travel by public transportation, shopping for groceries, preparation of meals, light housework, taking the correct dose of medicine) taken from the seven-item Lawton IADL scale (*21*), which has been used previously among older adults in Nepal (*14*). Responses to the IADL are measured on a four-point Likert scale ranging from "unable to do so at all" to "no difficulty," and scores ranged from 0 to 15, with higher scores indicating better functional status. The Cronbach reliability coefficient of the IADL in the present study was 0.908. Self-reported general health (SRH) was measured by asking "In general, how do you describe your health?" with responses categorized as: poor health (= 1), fair health (= 2), and good health (= 3). The number of chronic illnesses was self-reported by referring to a list of nine common illnesses (high blood pressure, diabetes, heart diseases, stroke, cancer, arthritis, back pain, liver or gallbladder problems, or respiratory problems), with the responses categorized as no chronic illness (= 1), one chronic illness (= 2), and two or more chronic illnesses (= 3).

Social support was defined as support from a son living with the elderly for the preceding year and measured by six items selected from previous studies (*14,22*). Factor analysis (varimax rotation) showed a two-factor model: emotional support (listening to the parent when he/she needs to talk, sharing one's most private worries with the parent, and providing good advice to the parent in times of crisis) and instrumental support (financial assistance, home repairs and household work, and transportation support from the son). The Cronbach reliability alpha coefficient was 0.825 for emotional support and 0.709 for instrumental support. Respondents chose either "yes (= 1)" or "no (= 0)" for each of the three items of instrumental and emotional support, resulting in a possible score ranging from 0 to 3, with a higher score indicating more support.

2.4. *Statistical analysis*

Subject characteristics were implied using descriptive statistics. Variables that were previously reported in the literature as correlates of life satisfaction were selected as independent variables in the present study. The correlates of the life satisfaction of older adults were determined by multiple regression analysis using SPSS v. 15.0 for Windows (Chicago, IL, USA). Multicollinearity was checked by tolerance and variance-inflation-factor (VIF). The norms of multicollinearity were not violated in the present study. The value of beta (β) was considered to analyze the strength of the correlating variables. $P < 0.05$ was considered to be statistically significant in this study.

3. Results

3.1. *Subject characteristics*

Socio-demographic characteristics of the subjects are presented in Table 1. The sample consisted of 247 (50.5%) men and 242 women (49.5%) with a mean age of 69.9 years (range: 60 to 100 years). Fifty-four percent were currently married while 46.0% were widowed. A higher number of women were widowed (64.0%) than men (28.3%). Among respondents, 56.6% were illiterate, 13.7% had no formal education but were literate, 22.3% had a high school education, and 7.4% a college-level or higher education. Illiteracy was higher among women (81.8%) than men (32.0%). About 49.3% of respondents reported their financial satisfaction as fair, 33.5% as satisfied, and 17.2% as dissatisfied. Respondents who were in paid employment accounted

for 16.2%, and 81.0% possessed some type of private property. A total of 50.7% described their general health as poor, 40.1% as fair, and 9.2% as good. Twenty percent reported no chronic illness, 54.4% one chronic illness, and 25.2% two or more chronic illnesses.

Table 1. Subject characteristics

		Total (n = 489) n (%)
Age in years (Mean ± SD)		69.9 ± 8.1
Gender		
	Male	247 (50.5)
	Female	242 (49.5)
Marital status		
	Married	264 (54.0)
	Widowed	225 (46.0)
Education		
	Illiterate	277 (56.6)
	No schooling but literate	67 (13.7)
	High school	109 (22.3)
	College & above	36 (7.4)
Financial satisfaction		
	Dissatisfied	84 (17.2)
	Fair	241 (49.3)
	Satisfied	164 (33.5)
Paid employment		
	Yes	79 (16.2)
	No	410 (83.8)
Possess inherited property		
	Yes	396 (81.0)
	No	93 (19.0)
IADL (Mean ± SD)		11.9 ± 4.3
Perceived health		
	Poor	248 (50.7)
	Fair	196 (40.1)
	Good	45 (9.2)
Number of chronic illness		
	No	100 (20.4)
	One	266 (54.4)
	Two or more	123 (25.2)
Instrumental support (Mean ± SD)		2.0 ± 1.1
Emotional support (Mean ± SD)		2.1 ± 1.2
Dependent variable		
	LSI (Mean ± SD)	4.9 ± 2.8

SD: Standard deviation.
LADL: Instrumental activities of daily living; score ranges from 0-15.
LSI: Life satisfaction index; score ranges from 0-9.
Instrumental and emotional support received score ranges from 0-3.

3.2. *Correlates of life satisfaction*

In a regression model developed to explore the correlates of life satisfaction in older adults (Table 2), 28.0% of variance was explained by the predictor variables in the model. Financial satisfaction (β = 0.30; $p < 0.0001$) and perceived health (β = 0.17; $p < 0.0001$) were the strongest predictors of life satisfaction. Education (β = 0.14; $p < 0.05$), higher functional ability (IADL) (β = 0.16; $p < 0.005$), and higher instrumental support (β = 0.13; $p < 0.05$) were significantly correlated with higher life satisfaction.

4. Discussion

Life satisfaction is an important component of successful aging. The current study found that financial satisfaction was the strongest correlate of life satisfaction among urban elderly in Nepal. Additional correlates included education, functional status, self-perceived health, and instrumental support from a son.

Deteriorating health and financial needs affect not only the elderly themselves, but also have a wider impact on care-giving families as well as on society itself if the individual becomes the responsibility of the state. In this study, financial satisfaction was the strongest correlate of life satisfaction. Previous studies support the current finding that perceived financial satisfaction and health status are stronger correlates of life satisfaction than a social network and social support (*23*). A study in Hong Kong showed significant positive associations between life satisfaction and other variables including less financial strain, better social support, fewer somatic complaints, and higher education (*24*).

In the current subjects, better functional status,

Table 2. Correlates of life satisfaction (multiple regression)

Variable		B	SE	ß	p
Socio-demographic					
	Age	0.01	0.02	0.02	0.758
	Gender (male)	-0.13	0.26	-0.02	0.631
	Marital status (married)	0.35	0.25	0.06	0.163
	Education	0.38	0.13	0.14	0.003
Economic					
	Financial satisfaction	1.21	0.17	0.30	0.000
	Paid employment (yes)	-0.09	0.32	-0.01	0.781
	Possess property (yes)	-0.29	0.29	-0.04	0.316
Health					
	Instrumental Activities of Daily Living	0.10	0.03	0.16	0.002
	Perceived health	0.73	0.21	0.17	0.000
	Number of chronic illnesses	-0.20	0.19	-0.05	0.283
Social support received					
	Instrumental	0.33	0.12	0.13	0.006
	Emotional	0.04	0.11	0.02	0.700
Adjusted R^2		0.279			

B: Unstandardized coefficient; SE: Standard error; ß: Standardized coefficient; Variables in the parentheses indicate the reference categories. p value significant at < 0.05.

as indicated by a higher IADL score, also correlated strongly with life satisfaction. Previous studies have also found that life satisfaction is negatively associated with recent health problems, chronic illness (*25*) and functional impairment (*26*), and that health is a stronger correlate of life satisfaction than age (*27*). Longitudinal studies provide further evidence that age is not what causes a decline in life satisfaction but rather that health constraints play a major role (*28*). In the present sample, better perceived health also correlated strongly with life satisfaction. Previous studies have also found that perceived health is the strongest correlate of life satisfaction (*2*). However, the possibility of a converse causal association has also been noted, namely that poor life satisfaction may lead to poor health (*11*). Future longitudinal studies will help explain the causal relationship of health and life satisfaction among the elderly of Nepal.

Literacy is low in Nepal and it is even lower for the elderly. Education provides individuals the ability to view life from different perspectives. Education was a correlate of life satisfaction among elderly Nepalese. Previous studies have also found that life satisfaction is likely to be higher in educated rather than uneducated individuals (*12*). Because the sample of this study was from an urban area, older adults who were educated might have better adapted to rapid social and economic changes, providing them with higher life satisfaction than those who were not educated.

In the present study, instrumental support positively correlated with life satisfaction while emotional support did not. A previous study in Nepal also reported social support from children living together increased the subjective well-being life stability. The study did not differentiate social support into emotional and instrumental support (*14*). Previous studies in other countries, however, have consistently reported a positive association between social support from social networks and life satisfaction among the elderly (*29*), as well as the benefit of social support, particularly emotional support, to their well-being (*30*). Nevertheless, a study among the elderly in Hong Kong found that material and instrumental support were more influential than emotional support in preventing depression (*31*). The reason for the greater importance of instrumental than emotional support in the present sample may be due to the absence of formal support systems for the elderly in Nepal.

Several limitations of this study should be mentioned. The sample consisted of the elderly from an urban area who were living with a son or daughter-in-law, so the findings may not be generalizable to elderly with other living arrangements. Economic variables were measured subjectively as financial satisfaction and real income or objective economic status was not measured in the present study. Further, the cross-sectional nature of the study prevents the description of causal relationships. Nonetheless, this study is one of the first to explore the correlates of life satisfaction among older Nepalese adults. Further investigation of life satisfaction among older adults in Nepal is warranted.

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Original Article

Prevalence and potential risk factors of rhinitis and atopic eczema among schoolchildren in Vientiane capital, Lao PDR: ISAAC questionnaire

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Summary **In 1998, an epidemiological study on asthma and allergic diseases using ISAAC questionnaire in Laos was first conducted in the recommended schools located in Vientiane capital showing that the prevalence of rhinoconjunctivitis and atopic eczema were 23.7% and 7.1% among children aged 13-14 year-old, respectively. This study aimed to reassess the prevalence of rhinoconjunctivitis and atopic eczema using the same ISAAC questionnaire by employing random sampling method and to identify the potential risk factors for these rhinitis and atopic eczema. This school-based cross-sectional study was conducted in Vientiane capital from December 2006 to February 2007. Of 536 children, prevalence of rhinoconjunctivitis and atopic eczema among schoolchildren were 9.3% and 11.8%, respectively. Children with early respiratory infection (AOR = 4.06; p = 0.001), parasitic infestation especially by *Opisthorchis viverrini* (AOR = 3.41; $p < 0.05$) were more likely to have rhinitis. While history of measles (OR = 2.24; $p < 0.01$) and respiratory infection (OR = 1.96; $p < 0.05$), eating vegetables everyday (AOR = 2.96; $p < 0.01$) were associated with atopic eczema. The similarity of prevalence of rhinitis and rhinoconjunctivitis were also revealed between children aged 13-14 year-old in this study and 6-7 in the previous study in 1998. The validation study on ISAAC questionnaire in Lao language is needed in order to generalize this questionnaire in Lao.**

Keywords: Allergic diseases, Risk factors, Schoolchildren, ISAAC questionnaire, Laos

1. Introduction

High prevalence of allergies among children was observed in many developed countries. In 1990, the international study on asthma and allergies in childhood (ISAAC) questionnaire was developed providing the comparability of prevalence of asthma and allergies among schoolchildren aged 6-7 year-old and 13-14 in different areas around the world (*1*). Based on this standard questionnaire, the international study was conducted in 155 collaborating centers in 56 countries worldwide, and questionnaire was translated into 39 local language (*2*,*3*). In 2000, the worldwide variation of the prevalence of rhinoconjunctivitis and atopic eczema among children aged 13-14 year-old was demonstrated that the highest was reported in Nigeria (39.8% and 17.7%, respectively) and the lowest in Albania (4.0% and 0.8%) (*3*). These prevalence were consequently observed in many developing countries such as Brazil (*4*), Costa Rica (*5*), Turkey (*6*), Thailand (*7*,*8*) and Viet Nam (*9*) using this standard questionnaire.

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In 1998, an epidemiological study on asthma and allergic diseases using ISAAC questionnaire in Laos was first conducted in Vientiane capital, showing that the prevalence of rhinitis, rhinoconjunctivitis and atopic eczema among children aged 13-14 year-old were 49.1%, 24.4% and 7.1%, respectively (*10*). The prevalence of rhinoconjunctivitis in this study was higher than some western countries such as New Zealand (18.9%), Australia (19.6%), UK (18.5%), USA (18.9%), as well as Asian countries such as South Korea (10.2%), Japan (14.8%), Indonesia (5.3%), Philippines (15.3%), Thailand (15.5%), Malaysia (13.7%) and Singapore (15.1%) (*3*).

This high prevalence of rhinoconjunctivitis in Laos would have been due to the schools (3 elementary schools and a high school) were non-randomly recruited. Therefore, in this study, the random sampling technique was employed to select schools which were located in the highest densely populated area and to recruit the schoolchildren, aiming at ensuring the representation of schoolchildren in Vientiane capital. The objectives of this study were to reassess the prevalence of rhinitis, rhinoconjunctivitis and atopic eczema and to identify the risk factors for rhinitis and atopic eczema among schoolchildren residing in Vientiane capital, Lao PDR.

2. Methods

2.1. *Study design*

The school based, cross-sectional survey was conducted in the highest densely populated area in Vientiane capital from December 2005 to February 2006. Four elementary and 4 high schools located in this area, and the students aged 13-14 year-old and the parents of students aged 6-7, were recruited by using multistage random sampling: schools were selected using simple random sampling method; while students were selected using systematic random sampling method. The ethical consideration of this study was approved by the University of Tokyo in Japan and the University of Health Sciences, Ministry of Health in Laos. The participants were requested to give the written informed consent before the study was conducted.

2.2. *Data collection*

The original English version of standard ISAAC questionnaire was translated into Lao language by a Laotian researcher in Japan. Additional questions were included into the core questionnaire to investigate the socio-demographics and potential factors which might influence the development of allergies among these children. Two day training course for research assistants and the pilot study was conducted in Vientiane. The questionnaire was revised and back translated by Laotian research assistants in Laos. The face to face interview using ISAAC questionnaire was conducted to students aged 13-14 year-old, and to parents of students aged 6-7 by the trained assistants. The stool examination was performed to investigate the association between allergies and parasitic infestation, so the stool containers were distributed to all participated students and they were requested to bring their stool to the school on the next day.

2.3. *Operational Definition*

The questionnaire was developed mainly based on the English version of ISAAC questionnaire; however, the additional questions exploring the socio-demographic, hygiene behavior, history of infection and eating habit were also included in the main questionnaire for this study.

According to the ISAAC questionnaire, the study therefore defined rhinitis, rhinoconjunctivitis and atopic eczema as follows:

Rhinitis ever:	*Ever had a problem with sneezing, runny, or blocked nose when did not have cold or the flu*
Rhinitis:	*Had a problem with sneezing, runny, or blocked nose when did not have cold or the flu in the past 12 months*
Rhinoconjunctivitis:	*Had a problem with sneezing, runny, or blocked nose when did not have cold or the flu eyes in the past 12 months had been companied by itchy-watery*
Atopic eczema ever:	*Ever had an itchy rash which was coming and going for at least six months*
Atopic eczema:	*Had an itchy rash at any time in the past 12 months*

2.4. *Data analysis*

All data were entered and cleaned in SPSS version 12.01 for window (SPSS Inc., Chicago, IL, USA). The descriptive analysis, Chi-square test and binary logistic regression were employed. Because a few statistically significant differences between rhinitis and atopic eczema, and its potential risk factors were observed, the variables such as socio-demographic, environment, family history of allergies, history of infection and eating habit were input into logistic regression model (Table 3 and 4), and the binary logistic regression was performed backwardly. The *p*-value of less than 0.05 was considered to be statistically significant difference.

3. Results

3.1. *Socio-demographics*

Five hundred thirty six children were recruited in this study. Of these, 186 (34.7%) were children aged 6-7 year-old and 350 (65.3%) were 13-14. Three hundred nine (57.6%) were female, and 535 (97.9%) children were lowland Lao. Of the 536 children, 299 (58.1%) of stool samples were collected from the children.

3.2. *Prevalence of rhinitis*

Of the 536 children, 32.5% children reported on having rhinitis ever, of which 30.1% of 186 children aged 6-7 year-old and 33.7% of 350 children aged 13-14 (Table 1). The prevalence of rhinitis was 21.8% (21.0% of younger children and 22.3% of older). Fifty (9.3%) were rhinitis accompanied with conjunctivitis (rhinoconjunctivitis), in which 7.5% of younger children and 10.3% of older. It was also inline with the prevalence of hay fever of 24.1%, with 23.7% for younger children and 24.3% for older.

The prevalence of rhinitis by month in 2005 is shown in Figure 1. The seasonal pattern of the prevalence was peaked in November and December which were 28.4% and 31.4%, respectively. On the other hand, April and March had the lowest prevalence of only 1.5%.

3.3. *Prevalence of atopic eczema*

Of the 536 children, 15.9% children had experience with atopic eczema ever; 16.7% for children aged 6-7 year-old and 15.4% for children aged 13-14 (Table 1). The prevalence of atopic eczema was 11.8%, in which 13.4% of younger children 10.9% of older. Forty two (7.8%) children reported on having atopic dermatitis ever, in which 16 (8.6%) of younger children and 26 (7.4%) of older.

3.4. *Risk factors for rhinitis*

The bivariate analyses were performed to identify factors associated with having rhinitis; however, only a few factors showed the statistically significant association. In Table 2, children who were from low income family were less likely to have rhinitis in the past 12 months (OR = 0.57; $p = 0.01$); while, children who had history of respiratory infection (OR = 1.64; $p < 0.05$) and those who were infested by *Opisthorchis viverrini* (OR = 1.88; $p < 0.05$), were more likely to have rhinitis.

Because a few variables showed the statistical association with rhinitis, the variables such as socio-demographic, environment, family history of allergies, history of infection and eating habit were input into logistic regression model (Table 3); and the results have

Table 1. The prevalence of rhinitis, atopic eczema, and severity among schoolchildren: Result from ISAAC questionnaire

		Total (*n* = 536)		**6-7 ys (*n* = 186)**		**13-14 ys (*n* = 350)**	
		n	**%**	***n***	**%**	***n***	**%**
Rhinitis ever	Yes	174	32.5	56	30.1	118	33.7
	No	362	67.5	130	69.9	232	66.3
If "yes"							
Rhinitis	Yes	117	21.8	39	21.0	78	22.3
If "yes"							
Rhinoconjunctivitis	Yes	50	9.3	14	7.5	36	10.3
	No	67	12.5	25	13.4	42	12.0
Interfere with your daily activities	Not at all	45	8.4	24	12.9	21	6.0
	A little	51	9.5	9	4.8	42	12.0
	A moderate	14	2.6	4	2.2	10	2.9
	A lot	2	0.3	0	0	2	0.6
	Missing	5	0.9	2	1.1	3	0.9
Hay fever	Yes	129	24.1	44	23.7	85	24.3
	No	403	75.2	141	75.8	262	74.9
	Missing	4	0.7	1	0.5	3	0.8
Atopic eczema ever	Yes	85	15.9	31	16.7	54	15.4
	No	451	84.1	155	83.3	296	84.6
If "yes"							
Atopic eczema	Yes	63	11.8	25	13.4	38	10.9
If "yes"							
Body rash	Yes	52	9.7	20	10.8	32	9.1
	No	8	1.5	3	1.6	5	1.4
	Missing	3	0.6	2	1.1	1	0.3
Precise skin rash	Yes	53	9.9	19	10.2	34	9.7
	No	7	1.3	4	2.2	3	0.9
	Missing	3	0.6	2	1.1	1	0.3
Frequency of skin rash	0	22	41.0	10	5.4	12	3.4
(night/week)	< 1	28	4.2	8	4.3	20	5.7
	≥ 1	13	2.4	7	3.8	6	1.7
Atopic dermatitis ever	Yes	42	7.8	16	8.6	26	7.4
	No	485	90.5	168	90.3	317	90.6
	Missing	9	1.7	2	1.1	7	2.0

shown that children who were from high income family (AOR = 2.23; $p < 0.05$), eating eggs more than once a week (AOR = 3.47; $p < 0.05$), history of respiratory infection (AOR = 4.06; $p = 0.001$), parasitic infestation by *Opisthorchis viverrini* (AOR = 3.41; $p < 0.05$) were more likely to have rhinitis compared to other.

3.5. *Risk factors for atopic eczema*

The bivariate analyses were also performed to identify risk factors for atopic eczema; once again, however, only a few risk factors were identified. As the Table 2 shown, children with history of measles infection (OR = 2.24; $p < 0.01$) and respiratory infection (OR = 1.96; $p < 0.05$) were more likely to have atopic eczema. Moreover, children who spend more than 30 minutes on the road per day, were more likely to have atopic eczema (OR = 1.95; $p < 0.05$).

Similar to rhinitis, all variables were input into logistic regression model (Table 4); and the results have shown that children who eat vegetables everyday were more likely to have atopic eczema (AOR = 2.96; $p <$

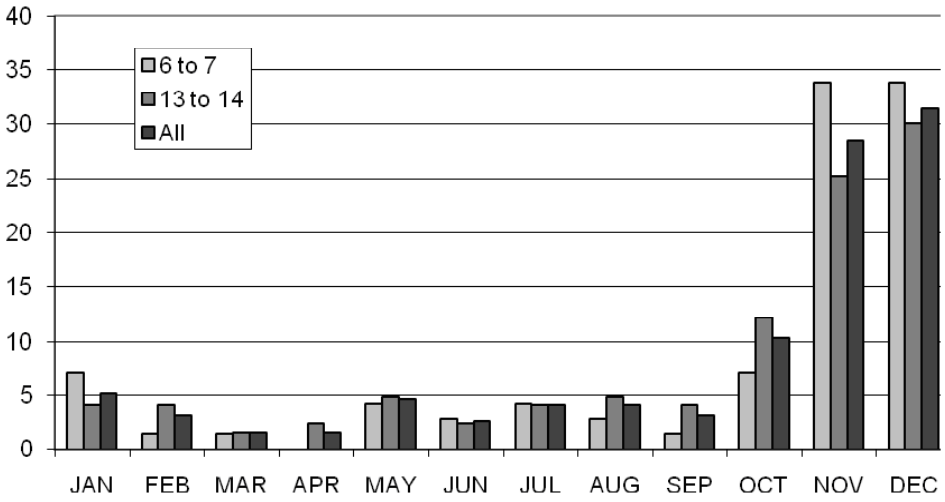

Figure 1. The prevalence of rhinitis by month in year 2005 among schoolchildren residing in Vientiane capital city, Lao PDR.

Table 2. Factors affecting rhinitis and atopic eczema in the past 12 months

	Rhinitis (*n* = 117)		Atopic eczema (*n* = 63)	
	n	**OR (95% CI)**	***n***	**OR (95% CI)**
Demographic status				
Boy	50	1.01 (0.67 - 1.53)	28	1.11 (0.65 - 1.88)
Younger children (6-7 ys)	39	0.92 (0.60 - 1.42)	25	1.27 (0.74 - 2.18)
High income family	48	1.74 (1.14 - 2.67) *	17	0.78 (0.43 - 1.40)
No sibling	6	0.65 (0.27 - 1.60)	5	1.19 (0.44 - 3.16) †
First child	44	0.93 (0.61 - 1.43)	25	1.04 (0.60 - 1.79)
Environmental factors				
Dog ownership	46	0.97 (0.64 - 1.48)	22	0.80 (0.46 - 1.38)
Cat ownership	26	1.30 (0.78 - 2.14)	10	0.78 (0.38 - 1.60)
Feeding chicken/duck	38	0.94 (0.61 - 1.46)	21	1.02 (0.58 - 1.78)
Contact pet > 1 per week	43	0.85 (0.55 - 1.29)	25	1.03 (0.60 - 1.78)
Time on road (> 30 minutes)	49	0.89 (0.59 - 1.34)	37	1.95 (1.15 - 3.33) *
Sharing bed	77	0.69 (0.44 - 1.07)	50	1.65 (0.85 - 3.20)
Air condition used	23	1.12 (0.66 - 1.88)	11	0.91 (0.45 - 1.81)
Parent smoke	46	1.16 (0.76 - 1.76)	24	1.10 (0.64 - 1.90)
Family history of asthma				
Parental asthma and allergy	6	2.23 (0.79 - 6.28) †	3	1.78 (0.49 - 6.44) †
Sibling asthma and allergy	5	1.63 (0.55 - 4.78) †	3	1.83 (0.51 - 6.62) †
History of infection in the past				
Measles infection	12	0.57 (0.30 - 1.09)	17	2.24 (1.21 - 4.14) **
Respiratory infection	37	1.64 (1.04 - 2.59) *	23	1.96 (1.11 - 3.45) *
Food				
Fish ≤ 1 day per week	27	1.04 (0.64 - 1.70)	17	1.34 (0.74 - 2.43)
Milk >1 day per week	59	1.25 (0.83 - 1.88)	28	0.93 (0.55 - 1.58)
Vegetable sometimes	43	0.72 (0.47 - 1.10)	25	0.86 (0.50 - 1.47)
Egg > 1 day per week	95	1.18 (0.70 - 1.99)	52	1.30 (0.65 - 2.58)
Intestinal parasitic infestation (*n* = 299)				
Ascaris lumbricoides	10	0.76 (0.36 - 1.60)	9	1.06 (0.48 - 2.35)
Opisthorchis viverrini	19	1.88 (0.99 - 3.54) *	3	0.36 (0.11 - 1.22) †

Pearson Chi-Square Test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; † Fisher's Exact Test

Table 3. Multivariate: Binary logistic regression analysis of rhinitis

		Rhinitis	
		AOR (95% CI)	***p* value**
Family income	Low	1	0.04
	High	2.23 (1.04 - 4.81)	
Eating eggs (day per week)	≤ 1	1	0.036
	> 1	3.47 (1.09 - 11.08)	
History of respiratory infection	No	1	0.001
	Yes	4.06 (1.83 - 9.01)	
Opisthorchis viverrini infestation	No	1	0.045
	Yes	3.41 (1.03 - 11.29)	

Model: *-2 Log likelihood = 202.102*; *Cox & Snell R Square = 0.088*; *Nagelkerke R Square = 0.143*. Variables entered on Step 1: *Sex, Age, Number of sibling, Parity, Parents education, Family income, Cat ownership, Dog ownership, Feeding chicken, Pets ownership, Sharing bed, Air condition use, Measles infection, Respiratory infection, Time on road, Frequency of meat consumption, Frequency of fish consumption, Frequency of vegetable consumption, Frequency of cow milk consumption, Frequency of fast food consumption, Frequency of eggs consumption, Intestinal parasitic infestation, Ascaris lumbricoides infestation, Opisthorchis viverrini infestation.*

Table 4. Multivariate: Binary logistic regression analysis of atopic eczema

		Atopic eczema	
		AOR (95% CI)	***p* value**
Feeding chicken/duck	Yes	1	0.092
	No	1.92 (0.90 - 4.07)	
Frequency of contact with pets* (day per week)	≤ 1	1	0.095
	> 1	1.79 (0.90 - 3.53)	
Eating vegetable**	Sometimes	1	0.002
	Everyday	2.96 (1.49 - 5.88)	
Time on the road (minute per day)	< 30	1	0.059
	> 30	1.85 (0.98 - 3.50)	

Model: *-2 Log likelihood = 239.176*; *Cox & Snell R Square = 0.074*; *Nagelkerke R Square* = 0.110; *Pets = dogs or cats; **all types of vegetable. Variables entered on Step 1: *Sex, Age, Number of sibling, Parity, Parents education, Family income, Cat ownership, Dog ownership, Feeding chicken, Pets ownership, Sharing bed, Air condition use, Measles infection, Respiratory infection, Time on road, Frequency of meat consumption, Frequency of fish consumption, Frequency of vegetable consumption, Frequency of cow milk consumption, Frequency of fast food consumption, Frequency of eggs consumption, Intestinal parasitic infestation, Ascaris lumbricoides infestation, Opisthorchis viverrini infestation.*

0.01). While spending time more than 30 minutes on the road seems to be risk for atopic eczema (AOR = 1.85; $p = 0.059$).

4. Discussion

This study revealed much lower prevalence of rhinitis and rhinoconjunctivitis among 13-14 year-old than in the previous study which was also conducted in Vientiane capital, while small difference was observed among children aged 6-7 year-old: among younger children, rhinitis (21.0% *vs*. 26.7%) and rhinoconjunctivitis (7.5% *vs*. 14.2%), and among older children, rhinitis (22.3% *vs*. 49.1%) rhinoconjunctivitis (10.3% *vs*. 22.3%). The previous study was conducted eight years ago, when the older children in the present study were 7-8 year-old. Interestingly, the similar prevalence of rhinitis and rhinoconjunctivitis among the older children in the present study and the younger children in the previous study was observed. High prevalence of rhinitis and rhinoconjunctivitis among the older children in the previous study probably due to unrepresentative schoolchildren of the Vientiane capital: only Vientiane high school, where students were mainly from high society and rich families, was included (*10*). There was also a possibility that the prevalence of rhinitis and rhinoconjunctivitis among schoolchildren in Vientiane capital has been changed over 8 years. A study in Brazil, which compared between ISAAC phases one in 1994 and three in 2001 in 4 cities, suggested changing either decrease or increase in prevalence of asthma and allergies (*4*).

The prevalence of rhinitis was as low as around five percent from January to September, and then sharply increased to 10% in October and peaked as high as 30% in December. This epidemiology was observed regardless of younger or older age group. The fluctuation of its prevalence in Laos was likely to be influenced by characteristics of season. When October and November comes, rainy season shifts to dry season and harvest season begins in Laos; this seasonal change might influence its prevalence of rhinitis. The similar pattern was found from a study among children aged 6-7 year-old in Beijing and Urumqi, China (*11*). Whereas two studies in Bangkok, Thailand, using ISAAC questionnaire showed the perennial fluctuation of its prevalence (*7,8*), notwithstanding that Thailand is more similar to Laos than China in terms of culture, life style and eating habit.

The ISAAC questionnaire has been developed basically for compare the worldwide prevalence. The prevalence of rhinoconjunctivitis (10.3%) among children aged 13-14 year-old in our study was lower than in Hong Kong (24.0%), Thailand (15.5%), Philippines (15.3%), Singapore (15.1%), Malaysia (13.7%), and Japan (13.4%); similar to Taiwan (11.3%), Viet Nam (10.7%), South Korea (10.2%); and higher than China (7.2%), Indonesia (5.3%), and India (5.6%) (*2,3,9*); indicating that the prevalence of rhinoconjunctivitis in Southeast Asia countries, including Laos but excluding Indonesia, ranked in the middle of worldwide prevalence. Unlike rhinoconjunctivitis, the prevalence of atopic eczema (10.9%) among these older children in our study was higher than Thailand (8.2%), Malaysia (8.0%), Taiwan (1.4%), Hong Kong (2.7%), Singapore (7.4%), South Korea (3.8%) and Japan (10.2%), and lower than UK (15.8%), Finland (15.6%), Ireland (13.6%), and New Zealand (12.7%) (*3*). The same standard questions were used in the ISAAC questionnaire; however, the original was written in English, and translated into local languages, which would affect the reliability and validity of the questionnaire. In addition, local languages of Laos and Thai (*8*), there were no specific terms to describe the technical terms used in ISAAC

questionnaire English version such as wheeze, hay fever and atopic eczema. This suggests the necessity of the validation study on ISAAC questionnaire in local languages.

In 1989, the hygiene hypothesis was first introduced by David Strachan that poor hygiene during childhood might prevent the development of allergy in adolescence and adulthood (*12*); The consistent finding was found in our study when children from high income family, where the living condition and hygiene behaviors were better than children from low income family, were more likely to have rhinitis.

Ramsey and Celedon in 2005 reviewed the contradictory findings of the history of infection as a risk for allergy in several studies (*13*). The result in our study shown that the history of respiratory infection was significantly, directly associated with development of rhinitis among targeted children.

Eating behavior was also investigated in our study, and the result showed that eating egg regularly was associated with the development of rhinitis. The consistent finding was found in a many studies: a study, for example, in Italy shown the higher prevalence of sensitization to egg among children with than without rhinitis (*14*).

The helminthic and parasitic infestation has taken to consider as one of factors influencing the development of allergies due to its competitive human immune response. In our study, we found that the *Opisthorchis viverrini* infestation was a risk of rhinitis; whereas there was no association between *Ascaris lumbricoides* infestation and rhinitis (several studies contradictorily demonstrated its association between *Ascaris spp*. infestation and allergic diseases). The study in former East Germany, for example, demonstrated the direct association between *Ascaris*-IgE sero-positive and high prevalence of allergic rhinitis (*15*). Another study in rural China also showed an increasing risk of asthma and skin picked test positive among subjects who were infested by *Ascaris lumbricoides* (*16*). The contradictory finding was found in other studies: a study by Cooper *et al*. reported the protective effect of geohelminth and *Ascaris lumbricoides* infestation against allergen skin test reaction among children aged 5-19 year-old in Ecuador (*17*); and van den Biggelaar *et al*. in Gabon, which reported the anhelminthic treatment of chronically infected children results in increased atopic reaction (*18*). No association between *Ascaris spp*. Infestation and rhinitis was observed in this study probably due to small sample.

Children who spent more than 30 minutes on the road per day were more likely to have atopic eczema; this association was also found when adjudged by other confounding despite being not statistically significant. The air pollution was not measured in our study. Therefore, further study employing the measurement for ambient air pollution is needed to explore its association in Laos.

Eating vegetables everyday had strong association with high prevalence of atopic eczema. Possible explanation is that chemical insecticides and fertilizers have been commonly used for plantation in Laos. A study in China showed the association between regularly contact with chemical insecticide, fertilizers and emissions from domestic fuel and wheezing, asthma attack and asthma medication among general population (*19*), although the study did not include other allergic diseases. The avoidance of using chemical insecticide for plantation in Lao is beneficial not only for reducing risk for atopic eczema, but for Lao people's health due to consuming free chemical vegetable which would not harm their body. Further study is needed to reveal more precise role of vegetables on the development of allergic diseases such as types and amount of vegetable intake and the level of chemical residue on vegetable.

This study revealed the similarity in the prevalence of rhinitis in 13-14 year-old of the present study and 6-7 year-old in the previous study which was also conducted in Vientiane capital eight years ago, and these prevalence were higher than that in some Southeast Asia countries. The validation study on Lao language version of ISAAC questionnaire is needed in order to generalize this questionnaire in Lao. History of respiratory infection and parasitic infestation, egg eating, and children from high family income promoted the development of rhinitis; while increasing in eating vegetable and spending more time on the road promoted atopic eczema. Our study also recommends the avoidance of chemical agents used in plantation is not only for reducing risk for atopic eczema but also for reducing chemical toxicity by consuming free chemical vegetables.

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Original Article

Comparison of body fat mass changes during the third trimester and at one month postpartum between lactating and nonlactating Japanese women

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Summary **The purpose of the present study was to compare which parts of the body fat mass tended to increase during the third trimester and at 1 month postpartum between lactating and nonlactating Japanese women. This prospective study examined 49 healthy pregnant women in the third trimester, and at 1 month postpartum. Demographic data, including lactation status, were obtained from a self-administered questionnaire. Newborn information was obtained from hospital charts. Anthropometric data, including body fat mass, were measured by the bioelectrical impedance analysis (BIA) method in the third trimester, and at 1 month postpartum. At 1 month postpartum, 16 mothers (32.7%) were lactating and 33 (67.3%) were mainly feeding formula. There were no significant differences between the lactating and nonlactating women regarding their demographic data, energy intakes and body fat mass changes during the third trimester of pregnancy. The trunk fat mass change showed a significant difference (p = 0.008) between the third trimester and 1 month postpartum, after adjustment by age and parity using repeated measurement ANCOVA, in the lactating and nonlactating women. In conclusion, the total body fat mass and body fat mass in the trunk at 1 month postpartum was significantly greater in lactating women than in nonlactating women.**

Keywords: Bioelectrical impedance analysis, Fat mass, Lactating, Postpartum, Pregnancy

1. Introduction

Lactation has many beneficial health effects (*1*), and the World Health Organization recommends exclusive lactation for 6 months after birth. In Japan, however, only 44.8% of mothers are still lactating at 1 month postpartum (*2*). The nutritional situation during reproduction is considered to be important for the health of the mother and their child.

Among previous reports regarding the relationships between postpartum weight or body fat retention and lactation, six studies found no relationship between postpartum weight or body fat retention and lactation (*3-8*) while another three studies showed very weak negative associations between lactation and weight retention (*9-11*). These findings were controversial. One of the reason for having no relation between postpartum weight retention and lactation may be higher energy intakes and expenditure in lactating women than in nonlactating women (*12*), because of a physiologic

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consequence of elevated concentrations of prolactin, an appetite stimulant in lactational period (*13*). Most previous studies have focused on obesity after delivery. Compared with Caucasian, White, Black or Brazilian women (*4-11*), Asian women have different energy intakes and metabolic energies that are reflected in body fat mass measurements.

In Japan, only one study examined waist/hip ratio during first trimester to 1 month postpartum in well nourished Japanese women (*14*) but there are no study examined the postpartum fat mass change in region with energy intakes information or lactating status among Japanese.

Body fat mass changes may different between populations or experimental design or methods. It remains unknown which parts of the body tend to store body fat mass after birth in lactating and nonlactating women especially in Japanese women.

In the present study, we carried out a longitudinal examination with the following objective to assess their energy intakes and which parts of the body fat mass tend to increase during the third trimester and at 1 month postpartum in healthy lactating and nonlactating Japanese women.

2. Methods

2.1. *Study design and sample selection*

This prospective study was conducted between July 2004 and November 2005 and involved women attending an antenatal clinic in Saitama. The clinic was located in a suburb of the Tokyo metropolitan area in Japan.

Criteria for inclusion were a singleton birth with no obstetric complications, age over 18 years, and intent to deliver at the clinic. Women with intrauterine growth retardation, polyhydramnios or oligohydramnios, spontaneous abortion or preterm delivery were excluded from the study.

During the recruitment period, 100 women attended pregnancy check-ups during their first trimester of pregnancy. Of these 100 women, 25 were excluded from the study for spontaneous abortion (n = 1), intention to deliver at a different clinic (n = 13) or changing the time of their check-up to the afternoon (n = 11). After receiving detailed information, 62 of the remaining 75 pregnant women (83%) agreed to participate in the study. One woman with intrauterine growth retardation during the third trimester and 7 women with incomplete data were excluded. Finally, a total of 54 pregnant women (72%) completed the measurements at the third trimester. Data at 1 month postpartum were available for 49 women. Five women dropped out of the study in the postpartum period, comprising 4 women who did not attend the 1-month check-up and one woman who refused to continue participating in the study because she did not have time.

2.2. *Ethical considerations*

Approval for the study was obtained from the Institutional Review Board of The University of Tokyo. Subjects were provided with information about the purpose and methods of the study and informed that participation was voluntary, that they were free to withdraw at any time and that full confidentiality was guaranteed. Written informed consent was obtained from all participants.

2.3. *Data collection*

The data were measured in each woman during pregnancy at 32 weeks of gestation and at 1 month postpartum when they attended the clinic for check-ups.

A self-administered questionnaire was used to obtain data regarding age, height, smoking and drinking habits, self-reported diseases, use of medications and lactation status. Lactation was defined as more than 8 lactation events/day and adding < 200 mL/day of formula. Women who gave > 200 mL/day of formula were classified as nonlactating. We selected the cutoff value of 200 mL because adding formula only once or twice per day can sustain 8 lactation events/day.

Dietary habits over the previous month were assessed with a brief self-administered diet history questionnaire (BDHQ) (*15*). The BDHQ used was a short version of a previously developed and validated comprehensive self-administered diet history questionnaire (DHQ) (*16*). It consisted of 74 questions for frequency and a part for portion sizes of 62 selected food items. Energy and nutrient intakes were calculated using an ad hoc computer algorithm based on the Standard Tables of Food Composition in Japan (5th edition) (*17*).

Data for self-reported prepregnancy weight, parity, past history, pregnancy complications, estimated fetal weight from ultrasonic echography, estimated amniotic fluid during pregnancy and at delivery, birth weight, infant sex and delivery time in weeks were taken from the hospital chart.

Weight and body fat mass were measured by the 8-electrode bioelectrical impedance analysis (BIA) method using a multi-frequency body composition analyzer (MC-190EM; Tanita Corp., Tokyo, Japan) after micturition. Ueda (*18*) developed a new system for measuring maternal total fat mass using the BIA method by compensating for the intrauterine component weight, as estimated by direct measurement or ultrasound examination, and assessed its validity for pregnant women. In the present study, BIA measurements were performed using a body fat analyzer (Model TBF-410; Tanita Corp.), which estimates body composition on the basis of maternal body density, and calculated by prediction equations from the impedances (50 kHz, single frequency) obtained at four electrodes placed

in contact with the soles of the subjects' bare feet in a standing position. To compensate for intrauterine weight, fetal weight was estimated by ultrasound examination, placental weight was calculated as described previously (*19*) and amniotic fluid weight was estimated by the Brace method (*20*). Body fat mass was measured in a total of five body parts, namely each arm, each leg and the trunk plus head (Figure 1). This BIA method provided high correlations with mean regional lean soft tissue and whole-body skeletal muscle mass estimates using the reference method of dual-energy x-ray absorptiometry (DXA) (*21*) for non pregnant women. Overall, the total body fat mass measurements showed high validity with DXA analysis during pregnancy, and the regional fat mass measurements showed high validity with DXA analysis after delivery.

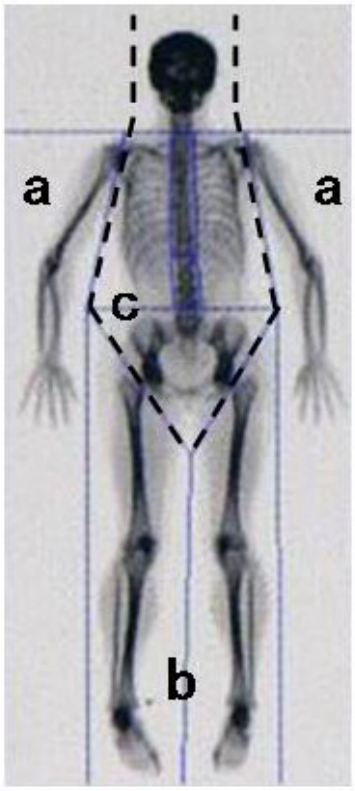

Figure 1. Parts of the body measured for body fat mass. (Modified from Lunar Rad. Co., USA). (a) Arms; (b) legs; (c) trunk.

2.4. *Statistical analysis*

The student's *t*-test was used to compare the mean characteristics of the participants and group differences for changes in body fat mass between lactating and nonlactating women. Repeated measurement analysis of covariance (ANCOVA) was used to test each independent variable which was total body fat mass, trunk fat mass, arms fat mass and legs fat mass among the third trimester and 1 month postpartum, dependent variables were lactating status adjusted by age and parity. All statistical analyses were carried out using SPSS for Windows version 16 for Japan.

3. Results

3.1. *Demographic data*

The demographic data are shown in Table 1. Forty-nine women continued to participate until 1 month postpartum. All participants delivered between 37 and 42 weeks of gestation. At 1 month postpartum, 16 of 49 mothers (32.7%) were lactating and 33 mothers (67.3%) were mainly feeding formula (nonlactating). The lactating and nonlactating women showed no significant differences in their demographic data and energy intakes at the third trimester and at 1 month postpartum.

The mean (SD) weight gain from prepregnancy until the last perinatal check-up was 9.5 (3.5) kg. The mean (SD) weight reduction from delivery to 1 month postpartum was 7.6 (2.0) kg.

Table 1. Characteristics of the participants

		Lactating (n = 16)	Non lactating (n = 33)	p-value [a)]
Maternal	Age (years)	31.4 ± 2.9	30.9 ± 3.8	n.s.
	Height (cm)	158.1 ± 4.6	157.2 ± 5.7	n.s.
	Prepregnancy body mass index (BMI:kg/m^2)	21.2 ± 2.5	21.7 ± 4.3	n.s.
	Gestational weight gain (kg)	10.4 ± 4.1	9.1 ± 3.4	n.s.
	Parity: primipara	5 (31)	13 (39)	n.s.
	Total energy intake (kcal/day)			
	Third trimester	1,428 ± 298	1,423 ± 398	n.s.
	One month postpartum	1,676 ± 374	1,608 ± 541	n.s.
	Weight (kg)			
	Prepregnancy	53.3 ± 8.2	53.2 ± 8.5	n.s.
	Third trimester	61.2 ± 7.6	60.3 ± 7.9	n.s.
	One month postpartum	56.2 ± 7.6	54.6 ± 7.6	n.s.
	Third trimester to one month postpartum	-5.1 ± 1.8	-5.7 ± 2.2	n.s.
	Week of delivery (week)	38.9 ± 0.9	39.1 ± 1.1	n.s.
	Placenta weight (g)	560 ± 108	589 ± 107	n.s.
Neonatal	Birth weight (g)	2,978 ± 399	3,214 ± 429	n.s.
	Birth height (cm)	48.4 ± 2.0	49.3 ± 2.0	n.s.
	Infant sex: boy	9 (56)	22 (67)	n.s.

Mean ± SD or n (%); n.s.: nonsignificant; [a)] Student's t-test.

The mean (SD) fat mass reductions from the third trimester to 1 month postpartum were: total body, 0.9 (2.5) kg; arms, 0.1 (0.9) kg; legs, 0.6 (0.8) kg; and trunk, 0.1 (1.9) kg. The trunk fat mass showed the largest increase among the different parts of the body examined.

As shown in Figure 2, at 1 month postpartum, nonlactating women exhibited a decrease in their total body fat mass and there was a significant difference (p = 0.026) between the lactating and nonlactating women. As shown in Figure 3, the lactating women showed an increase in their trunk fat mass while the nonlactating women exhibited a decrease, and there was a significant difference (p = 0.008) from the third trimester to 1 month postpartum between the lactating and nonlactating women.

The fat masses in the arms (Figure 4) and legs (Figure 5) exhibited similar changes at the third trimester and at 1 month postpartum in the lactating and nonlactating women.

4. Discussion

We measured the amounts of body fat mass using the BIA method in pregnant women followed up longitudinally during the third trimester and at 1 month postpartum and analyzed the differences between lactating and nonlactating women.

A main finding of our study is the trunk fat mass change showed a significant difference between the third trimester and 1 month postpartum in the lactating and nonlactating women.

Our study further found that there were no differences between energy intakes during the third trimester to one month postpartum between lactating and nonlactating women. We used the BDHQ to assess

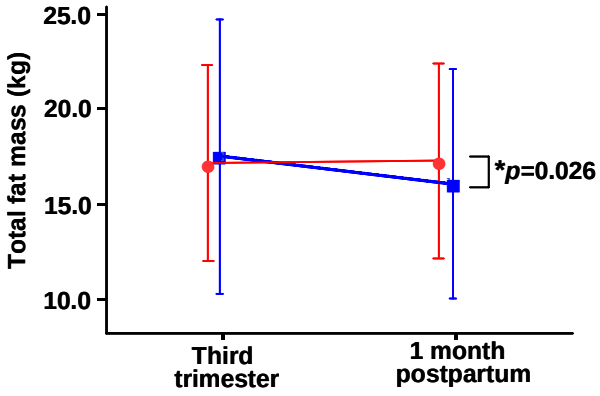

Figure 2. Changes in total fat mass in lactating (–●–; *red*) and nonlactating (–■–; *blue*) women. Data represent the crude mean ± SD (kg). * p = 0.026, by ANCOVA[1)] (n = 49). [1)] Adjusted by age and parity.

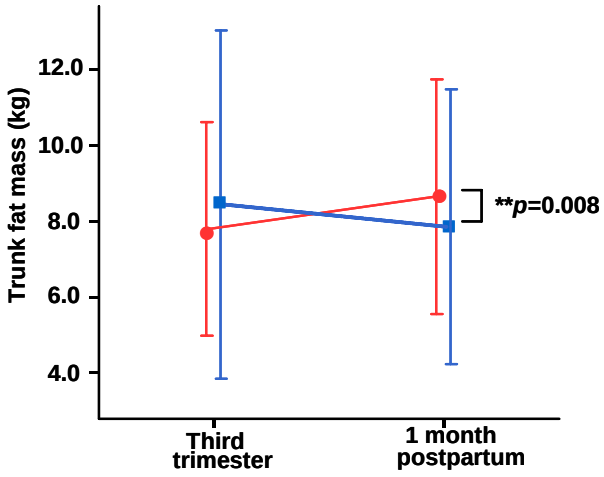

Figure 3. Changes in trunk fat mass in lactating ((–●–; *red*) and nonlactating (–■–; *blue*) women. Data represent the crude mean ± SD (kg). ** p = 0.026, by ANCOVA[1)] (n = 49). [1)] Adjusted by age and parity.

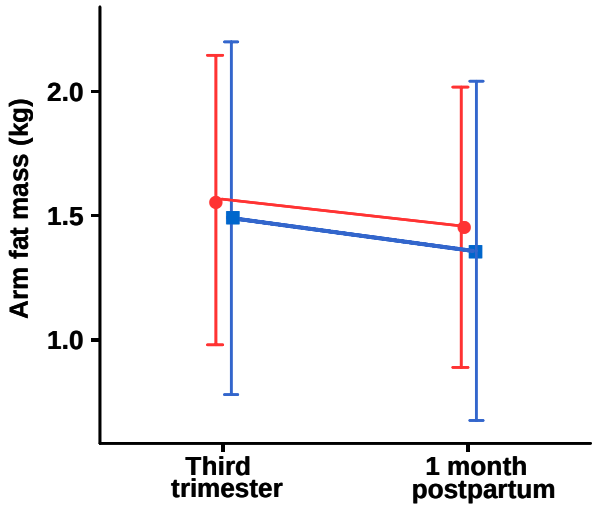

Figure 4. Changes in arm fat mass in lactating (–●–; *red*) and nonlactating (–■–; *blue*) women. Data represent the crude mean ± SD (kg). No significant differences were detected by ANCOVA[1)] (n = 49). [1)] Adjusted by age and parity.

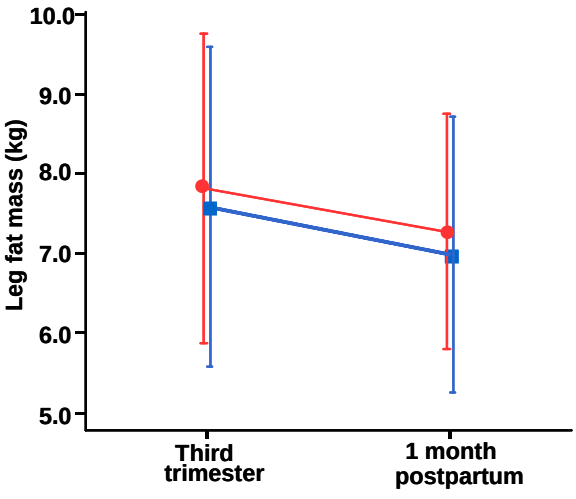

Figure 5. Changes in leg fat mass in lactating (–●–; *red*) and nonlactating (–■–; *blue*) women. Data represent the crude mean ± SD (kg). No significant differences were detected by ANCOVA[1)] (n = 49). [1)] Adjusted by age and parity.

total energy intake because of its feasibility and validity. However, because the BDHQ cannot directly observe the participants' dietary intake, the results should be interpreted cautiously. The total energy intake assessed with the BDHQ was seriously underreported compared with the estimated energy requirements (*22*), consistent with another study (*23*). However, it was unlikely to introduce a serious bias for comparisons of the intakes between the lactating and nonlactating women.

It is very difficult to measure body fat mass during pregnancy according to body parts or regions by DXA because of the perceived ionizing radiation hazard during pregnancy. The BIA method has high validity compared with DXA ($r = 0.87$, $p < 0.001$) for the total body fat mass and different parts of the body (*24*). However, it has a limitation in being unable to identify the specific body parts involved in the trunk fat mass gain, such as breast fat mass or abdominal fat mass. This is the first study to compare body fat mass in different regions between lactating and nonlactating women during pregnancy using the BIA method.

There is evidence of differences in fat mass storage in regions with high lipoprotein lipase (LPL) activation (*25*). Specifically, it was reported that both nonpregnant and pregnant women favored lipid assimilation in the femoral depot (*25*). However, the metabolic pattern changes during lactation because LPL activity decreases and lipid mobilization increases in the femoral depot. These changes are much less pronounced in the abdominal region. The fat cells from these different regions show different responses during pregnancy and lactation (*25*). In addition, abdominal adipose tissue has a higher adenosine content than femoral adipose tissue in lactating women (*26*). High estrogen levels during pregnancy promote a gynoid type of fat distribution (gluteofemoral), and low levels of estrogen during lactation may favor partitioning of the body fat to the upper body (*27*).

The present data are consistent with those in a previous longitudinal study using magnetic resonance imaging before pregnancy and at 5-10 days ($n = 15$), 2 months ($n = 15$), 6 months ($n = 13$) and 12 months ($n = 10$) postpartum, which showed that body fat mass exhibited greater decreases in the legs than in the trunk (*34*). Another previous study compared the waist/hip ratios in well-nourished Japanese women ($n = 41$) between the first trimester and 1 month postpartum and found significant retention of fat in the upper body (*14*).

Previous studies have compared nonpregnant women or lactating women longitudinally. It is therefore unclear whether the trunk fat mass gain after delivery is stimulated by lactation or in all women after birth regardless of lactation. Most of the previous studies comparing lactating and nonlactating women were longitudinal and aimed to solve obesity by identifying which groups had returned to their prepregnancy weights at 6 months or 1 year after birth (*6-8*). From our findings, trunk fat mass changes only occur in lactating women, and the lack of differences in the energy intakes during the third trimester and at 1 month postpartum and other factors between the lactating and nonlactating women suggests that lactation itself may stimulate these metabolic pattern changes.

In addition, limitation of this study is that we focused on only healthy pregnant women and one setting and small sample size, it is difficult to extrapolate the results of our study. Further studies are required to measure fat retention for a longer time and more sample size including underweight women who gain insufficient fat mass during pregnancy.

In conclusion, the total body fat mass and body fat mass in the trunk at 1 month postpartum was significantly greater in lactating women than in nonlactating women.

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Original Article

Overexpression of TIMP-2 mediated by recombinant adenovirus in rat abdominal aorta inhibits extracellular matrix degradation

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Summary **To investigate the effects of a tissue inhibitor of the matrix metalloproteinase-2 (TIMP-2) gene on the extracellular matrix of the abdominal aorta, models of rat abdominal aortic aneurysm were utilized and a solution of AdTIMP-2 was perfused into the abdominal aorta. The models of rat abdominal aortic aneurysm were constructed with intraluminal perfusion of the abdominal aorta with porcine pancreatic elastase, and an adenovirus solution carrying the TIMP-2 gene was transferred into the aorta by local perfusion. After 14 days, aortic wall elastin and collagen concentrations were measured with an image analysis system and fixed aortic tissues were examined by light microscopy for inflammation. No abdominal aortic aneurysms developed in TIMP-2 gene-transferred rat models, representing a marked decreased from control rats ($p < 0.01$). The elastic fibers and collagenous fibers were preserved with greater integrity in the AdTIMP-2 group than in the control group and inflammatory cells were seen in adventitial areas. The TIMP-2 gene mediated by adenovirus can renew the balance of degradation of the extracellular matrix caused by elastase and inhibit the formation of an abdominal aortic aneurysm. This finding provides a new strategy for treatment of abdominal aortic aneurysms.**

Keywords: Tissue inhibitor of matrix metalloproteinase, Abdominal aorta, Extracellular matrix, Gene therapy

1. Introduction

The formation of an abdominal aortic aneurysm (AAA) involves several pathological factors, the most important of which is the degradation of the extracellular matrix of the aorta wall resulting in the reformation of the vascular wall. A recombinant adenovirus vector-carrying tissue inhibitor of the matrix metalloproteinase-2 (TIMP-2) gene (*1*) was successfully constructed, and studies have proven that the TIMP-2 gene can inhibit vascular smooth muscle cell migration and proliferation *in vitro* (*2*). In this study, a rat model of AAA with local perfusion of a solution of the adenovirus vector was utilized to investigate the effects of the TIMP-2 gene on degradation of the aortic extracellular matrix with morphological and pathological techniques.

2. Materials and Methods

2.1. *Construction of rat AAA models and group assignments*

Twenty four grown male SD rats, weighing 350-400 g, were provided by the experimental animal center of the Second Military Medical University, Shanghai,

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China. The rat models were constructed by perfusing elastase into the abdominal aorta with reference to the literature (*3*). Porcine pancreatic elastase was purchased from EMD Chemicals Inc., San Diego, CA, USA. The rats were randomly divided into three groups: an AdTIMP-2 group, an AdCMV group, and a blank control group.

2.2. *Perfusion of the adenovirus solution*

Following perfusion of the adenovirus solution, an elastase solution was subsequently perfused into the rat abdominal aorta with a small-diameter syringe. The adenovirus solution was diluted to 10^9 pfu/mL and perfused at 1 mL into the rat aorta continuously for 30 min. After the blood flow through aorta was reestablished and the incision was closed, each rat was fed separately. The diameter of the rat abdominal aorta was measured before perfusion and immediately afterwards. Two weeks later, all of the rats were euthanized and surgery was performed to dissect the suprarenal segment of the abdominal aorta to evaluate the efficacy of systemic gene therapy with TIMP-2. The rats were killed after measuring the diameter of the aorta with vernier calipers. The dissected aorta segment was fixed in formalin and embedded in paraffin for morphological and pathological analyses.

2.3. *Pathological analyses*

Paraffin sections of vessel segments 4-5 μm thick were stained with hematoxylin-eosin and elastin-van Gieson. Morphometric analyses were performed on 3 to 4 cross sections for each vessel to microscopically determine the inflammation of the vessel wall. Computerized quantification of elastin degradation in the media was done using image analysis software.

2.4. *Statistical analysis*

The data were calculated as mean ± standard deviation. Comparisons between two groups were done using a Student's *t* test with $p < 0.05$ considered significant.

3. Results

3.1. *Common conditions after surgery*

A second surgery was performed on a total of 24 rats. The rats failed to move their lower limbs and activity decreased in the early stages after perfusion, usually recovering in 3 to 5 days.

3.2. *Measurement of the rat abdominal aorta diameter*

Table 1 shows the results of ADpre and ADpost separately for three groups of rats. As is apparent, there are no significant differences between the diameters of each group before and just after perfusion ($p > 0.05$). The diameter of the aorta on day 14 (AD14d) and the percent enlargement of the abdominal aorta (ADpet) were much lower for the AdTIMP-2 group than for the AdCMV and control groups. Similarly, the rate of AAA formation was also significantly lower for the AdTIMP-2 than for the other two groups ($p < 0.05$). AAA formation was completely prevented in the AdTIMP-2 treated group ($n = 8$), while all mice in the AdCMV and PBS control groups developed an AAA. These results demonstrate that overexpression of TIMP-2 in the vascular wall prevents AAA development in a rat model.

3.3. *Pathological analyses*

In the AdTIMP-2 group, the elastin fibers of media were preserved with integrity and degradation of collagen fibers was inhibited. Inflammatory cell infiltration was observed in the vessel wall (Figure 1A). The lumen of the abdominal aorta expanded and neointima formed in the AdCMV and control groups. In these two groups, the intima was split and elastin of the media was degraded. Collagen fibers were more badly degraded. Inflammatory

Table 1. Diameters of abdominal aortas in three groups of rats

Group	*n*	AD_{pre} (mm)	AD_{post} (mm)	AD_{14d} (mm)	AD_{pet} (%)	AAA (%)
AdTIMP-2 group	8	1.58 ± 0.04	2.03 ± 0.09	2.33 ± 0.06*	48 ± 4*	0**
AdCMV group	8	1.60 ± 0.04	2.09 ± 0.07	$3.52 \pm 0.11^{\Delta}$	120 ± 6	100
PBS group	8	1.57 ± 0.05	2.07 ± 0.08	$3.43 \pm 0.09^{\Delta}$	118 ± 5	100

Compared to the AdCMV and control test groups, * $p < 0.05$, ** $p < 0.01$; Compared to AD_{pre} and AD_{post}, $^{\Delta}p < 0.05$.

Table 2. Degrees of inflammation and degradation of rat abdominal aortic elastase-collagen in the three groups

Group	*n*	Inflammation of aorta			Degradation of elastin and collagen			
		(+)	(++)	(+++)	I	II	III	IV
AdTIMP-2 group	8	5	3	0	3	5	0	0*
AdCMV group	8	3	3	2	0	0	2	6
PBS group	8	3	2	3	0	1	1	6

Compared to the AdCMV and control test groups, * $p < 0.05$.

cells infiltrated through the vessel wall, especially in the outer layer (Figure 1B and C). Table 2 shows the degrees of inflammation and degradation of rat abdominal aortic elastin-collagen for the three groups of rats. The AdTIMP2 group displayed better preservation of elastin fibers and collagen, while the AdCMV and control groups displayed significant destruction of elastin fibers and the medial layer (Figure 2A-C).

4. Discussion

Previous studies by the current authors have shown that

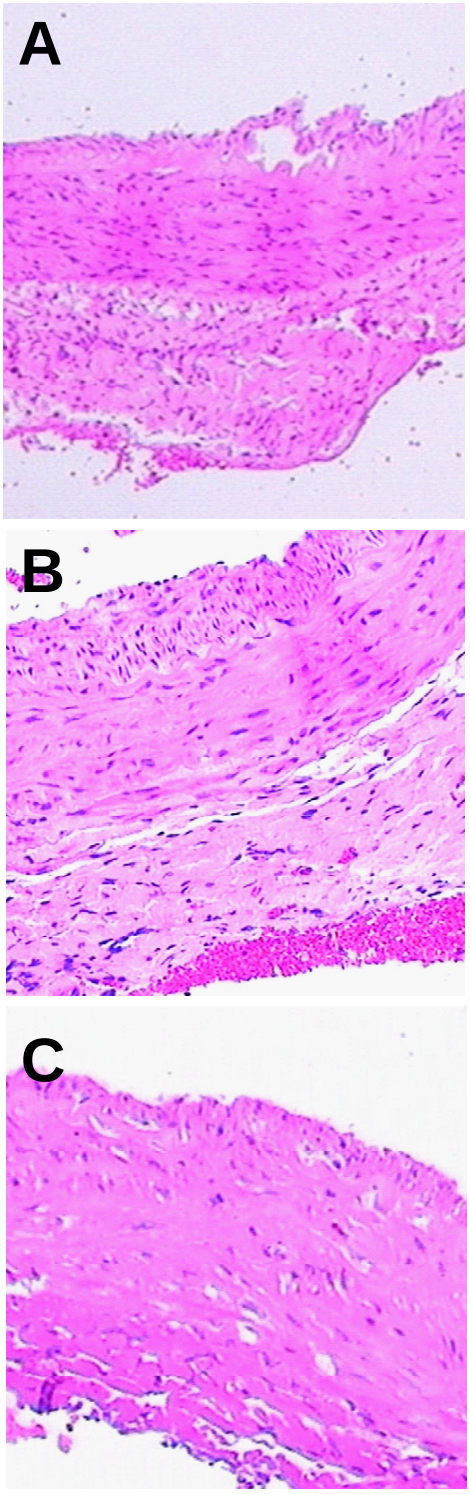

Figure 1. Histopathological changes in three groups of rats (magnification, × 200). (A) The AdTIMP2 group displayed better preservation of the medial layer; (B) The AdCMV group displayed hyperplasia of the intimal layer, destruction of the medial layer, and infiltration of inflammatory cells; (C) The PBS group displayed significant destruction of the medial layer and elastin fibers.

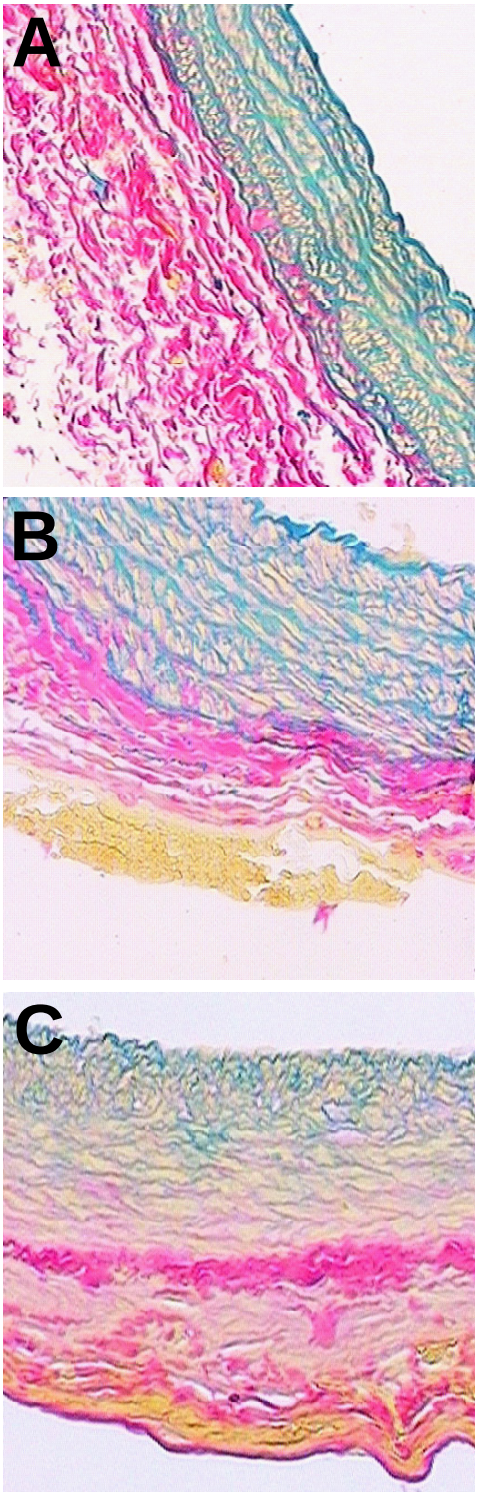

Figure 2. Verhoeff-van Gieson stain for elastin in three groups of rats (magnification, × 200). (A) The AdTIMP2 group displayed better preservation of elastin fibers and the medial layer; (B) The AdCMV group displayed significant destruction of elastin fibers and the medial layer; (C) The PBS group displayed disappearance of the medial layer and elastin fibers.

matrix metalloproteinases (MMPs) increased in the tissue of aneurysms and that their expression correlated with the inflammation of vessel walls, which can also be inhibited by tetracycline, thus inhibiting the formation of aneurysms (*4*). These results indicate that MMPs play an important role in the formation and progression of AAA in this rat model and provide valuable clues to the treatment of aneurysms. Presumably we can selectivity inhibit the MMPs activities in the vascular wall, the aneurysm can be cured fortunately.

Anidjar first reported creation of a rat AAA model by digesting the vessel wall with elastase (*3*). This model was similar to a human AAA in terms of histology and biology. Here, the TIMP-2 gene was delivered to the local area of vessel wall by gene transfer, consequently sustaining protein expression for a period of time. This can have a therapeutic effect and eliminate side effects in non-target organs.

Most adenovirus-mediated genes transferred to the vessel wall have delivery times of 20 min or longer or include 30 min incubation post-delivery from an infusion device (*5,6*). Regardless of the device used, most studies have delivered viral doses ranging from 1×10^9 to 1×10^{10} pfu/mL in delivery volumes of approximately 1 mL (*7*). Transduction efficacy can be improved by incubation of the vessel with a viral solution over an extended period of time. Although these conditions have been widely used, a lack of systematic study of delivery parameters suggests that delivery conditions were not necessarily optimal and that the clinical significance of such delivery is questionable. Here, a 10^9 pfu/mL viral solution was used and this concentration was found to have a therapeutic effect.

Impairment of ECM biosynthesis is thought to play a role in the pathogenesis of AAA because AAA is accompanied by a progressive decrease in the number of vascular smooth muscle cells that normally synthesize ECM (*8,9*). Disruption of elastin is sufficient for aneurysmal dilation of the aorta, and degradation of collagen is responsible for its rupture (*10,11*). Abundance studies have shown that MMPs were the main enzyme to digest elastin and collagen fibers of the vessel wall (*12,13*). Here, an adenovirus-mediated gene transfer of TIMP-2 to a local segment of rat aorta was utilized to block MMPs. The main components of ECM, elastin and collagen, were found to be preserved with greater integrity. Final results for TIMP-2 gene-transferred rats indicated a lower rate of AAA formation.

The current study also found that the inflammatory reaction was severe in the wall of artery although ECM degradation was inhibited. There was a great deal of inflammatory cell infiltration in the vessel wall, which suggests that other important factors may play roles in the formation of AAA. There may be two reasons for this: first, the adenovirus itself may be an immunogenic factor and stimulate an inflammatory reaction; second, vessels may be injured during model construction.

Other studies have found that mice deficient in MMP-9 and/or MMP-2 are protected from the development of AAA by infusion of elastase or $CaCl_2$ treatment of the aorta but are not protected from an inflammatory response (*12,13*). Doxycycline treatment inhibits the development of AAA experimentally, but it does not eliminate the inflammatory response (*14*), suggesting that the inflammatory response is maintained independently of MMP activity.

At present, the treatment of AAA relies on conventional surgery and there is no agent to inhibit the formation of AAA. This study has shown that adenovirus-mediated gene transfer of the TIMP-2 gene blocks degradation of ECM caused by proteinase, rebalanced the protein dissolution process and therefore preventing the formation of AAA, providing a new therapeutic strategy for the treatment of this condition.

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Original Article

Audiological characteristics of infants with abnormal transient evoked otoacoustic emission and normal auditory brainstem response

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Summary **Audiological characteristics were investigated in 81 ears of 53 infants with abnormal transient evoked otoacoustic emission (TEOAE) and normal auditory brainstem response (ABR). The relationship between ABR and other hearing testing methods, including 40Hz auditory event-related potential (40Hz-AERP), auditory steady state response (ASSR), distortion product otoacoustic emission (DPOAE), tympanometry, and acoustic reflex, was analyzed. Of the 81 ears, 18 ears (22.2%) were normal, while 63 ears (77.8%) were abnormal according to the tests. Testing of the 40 Hz AERP (36 ears) and ASSR (45 ears) revealed that 14 ears (38.9%) and 27 ears (60.0%) were abnormal, respectively. Testing of DPOAE in 68 ears revealed that 50 ears (73.5%) were abnormal. Testing of tympanometry in 50 ears and acoustic reflex in 47 ears revealed that 9 ears (18%) and 27 ears (57.4%) were abnormal, respectively. The present data suggests that the hearing of infants cannot be sufficiently evaluated with ABR only and that it must be evaluated with integrative audiological testing methods.**

Keywords: Neonatal screening, Audiology, Evoked potentials, Auditory, Otoacoustic emissions

1. Introduction

Congenital hearing loss, a frequent birth defect, can directly slow the development of language, emotion, mentality, and social communication in infants and significantly affect school attendance and employment. These impacts of congenital hearing loss on the family and society can be huge. There is evidence that the age of discovery and identification of hearing loss and the age at which intervention is performed positively and significantly affect language development in infants (*1,2*). However, there is no unified standard for diagnosis and evaluation of congenital hearing loss in China. With the increasing number of newborns and infants who fail hearing screening, correct diagnosis in the early stage of hearing loss becomes crucial. The present study analyzed hearing testing results for newborns and infants with abnormal transient evoked otoacoustic emission (TEOAE) and normal auditory brainstem response (ABR). This was done was to study the relationship between ABR and other hearing testing methods, including 40 Hz auditory event related potential (40 Hz AERP), auditory steady state response (ASSR), distortion product otoacoustic emission (DPOAE), tympanometry, and acoustic reflex, and the relationship between hearing screening and hearing diagnosis.

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2. Methods

2.1. *Subjects*

Data were collected for all newborns and infants who underwent audiological diagnosis at the children's

hearing diagnosis center of this hospital from December 2003 to November 2005. During this period, all subjects were screened with a TEOAE hearing test at a local newborn hearing screening center or maternal and child health care hospital. Subjects with an abnormal ear or ears in preliminary screening at the age of 2 to 7 days and in duplicate screening at the age of 30 or 42 days then underwent the ABR test at this hospital. Of 53 subjects (81 ears: 44 left ears and 37 right ears) with normal ABR, 34 were male (53 ears) and 19 were female (28 ears). The mean age was 4.1 months and ranged from 1.5 to 38 months.

2.2. *ABR test*

An evoked potential tester (Nicolet Spirit, Nicolet Inc., Madison, WI, USA) was used with an alternatively inverted click stimulus, pulse width of 0.1 ms, initial intensity of sound stimulus of 80 dB nHL, stimulus repetition rate of 11.9 times/s, analysis time of 10 ms, band-pass filtering of 10~3,000 Hz, and 2 or 3 replications of 1,000 sweeps. The box of electrodes consisted of four electrodes: a forehead electrode as the recording electrode, acoustic stimulation of the bilateral mastoids as the reference electrodes, and a glabella electrode as the ground electrode. The impedance values of the electrodes were below 5 Kohms. A V response threshold for the ABR wave equal to or less than 30 dB nHL served as an index of normal hearing in the range of 2 kHz to 4 kHz (*3*).

2.3. *40 Hz AER test*

The instrument and electrode placement in this test was the same as in the ABR test. Acoustic stimulation (2 ms-2 ms-2 ms) was presented as a tone burst at 500 Hz with an initial intensity of 80 dB nHL, a rhythm of 40 stimulations per second, an analysis time of 100 ms, band-pass filtering of 10 Hz-100 Hz, and 2 or 3 replications of 500 sweeps. The acoustic stimulus intensity sufficient to cause a reaction was the 40 Hz AERP response threshold. A value equal to or less than 40 dB nHL served as an index of normal hearing (*4*).

2.4. *ASSR test*

ASSRs were recorded using the Intelligent Hearing Smart ASSR evoked potential system (Intelligent Hearing Inc., Miami, FL, USA). The carrier frequencies of the acoustic stimulation signals were 0.5 kHz, 1 kHz, 2 kHz, and 4 kHz. The sine wave frequencies of amplitude modulation were 77 Hz, 85 Hz, 93 Hz, and 101 Hz for the left ear, and 79 Hz, 87 Hz, 95 Hz, and 103 Hz for the right ear, with modulation depth of 100%. Many brief sound signals were sent to both ears at the same time using ER-3A standard plug-in headphones to perform calibration. The recording electrode was placed on the forehead. Electrodes at the bilateral mastoids functioned as reference electrodes and a glabella electrode served as the ground electrode. The impedance values of the electrodes were below 3 Kohms. Band-pass filtering was 30~300 Hz and the amplifier gain was fixed at 10^5. According to the ASSR testing results, response thresholds of 0.5 kHz, 1 kHz, 2 kHz, and 4 kHz were determined. The mean value for 4 frequencies that was equal to or less than 35 dB nHL served as an index of normal hearing of ASSR (this laboratory's standard).

2.5. *DPOAE test*

An ILO-96 otoacoustic emission instrument (Otodynamics Ltd., Herts, UK) was used. Test conditions were as follows: using two stimulation pure tones, f1 and f2, at the same time, and f1 = f2 = 70 dB SPL, f2: f1 = 1.22. The f2 frequencies were 696 Hz, 1,001 Hz, 1,501 Hz, 2,002 Hz, 3,003 Hz, 4,004 Hz, 5,005 Hz, and 6,006 Hz. The normal criteria for DPOAE were that the distortion product (DP) value (2f1–f2) of each analysis frequency was in the normal range while the DP value of the frequency was at least as large as the noise value of 3 dB SPL and that passing scores were obtained for at least 4 of the 8 observed frequencies.

2.6. *Tympanometry test*

A GSI TympStar middle ear analyzer (Grason Stadler Inc., Madison, WI, USA) was used for testing. Sound was deemed to be detected at 226 Hz. Normal standards for the tympanometry test were that the tympanogram was an A type, tympanic cavity pressure was in the range of +100 mmH_2O, the extent of acoustic compliance was 0.3~1.6 mL, and the volume of the tympanic cavity was 0.5~1.0 mL.

2.7. *Acoustic reflex test*

The same instrument as used in tympanometry was used to perform acoustic reflex testing. Sound was deemed to be detected at 226 Hz. Normal standards for an acoustic reflex were as follows: a homolateral acoustic reflex was detectable and the reflex threshold was equal to or less than 90 dB SPL. Newborns and infants were tested while sleeping after they had taken 10% chloral hydrate. All testing was done in a noise-shielded room with indoor environmental noise of 18 dB (A).

3. Results

Of the 81 ears, 18 ears (22.2%) were normal in 12 subjects according to tests. Of these 12 subjects, 10 infants were male (16 ears, 30.2% of all male infant ears) and 2 were female (2 ears, 7.1% of all female infant ears). Of these normal ears, 9 were left ears

(20.5% of all left ears) and 9 were right ears (24.3% of all right ears). In contrast, 63 ears (77.8% of all ears) in 41 subjects with normal ABR were abnormal in one or more other hearing tests. Of these 41 subjects, 24 were male (37 ears, 69.8% of all male ears) and 17 were female (26 ears, 92.9% of all female ears). Of these 63 abnormal ears, 35 were left ears (79.5% of all left ears) and 28 were right ears (75.7% of all right ears). The abnormal rate in DPOAE testing was the highest among all of tests, followed by ASSR and acoustic reflex, while it was lowest in tympanometry (Table 1). Abnormal rates for female infants were significantly higher than for male infants in all of the tests (Table 2). Abnormal rates for left ears were significantly higher than for right ears in all tests except for the acoustic reflex (Table 3).

4. Discussion

Otoacoustic emission (OAE) is a type of audio energy that is generated in the cochlea, conducted through the ossicular chain and the tympanic membrane, and released into the external acoustic meatus (*5,6*). OAE differs from ABR and is only sensitive to the integrity of the peripheral auditory system rather than the level of hearing (*7*). The normal peripheral auditory system can be confirmed by OAE signals. OAE can be detected quickly and objectively in newborns and infants with a normally functioning outer, middle and inner ear (*8,9*), so it is thus often used to evaluate the hearing of children and screen the hearing of newborns. When the amplitude value of OAE is reduced or missing, further audiological evaluation is required. ABR is an essential objective method to evaluate hearing especially for those infants who cannot be measured with audiometry (*10*). The response threshold of ABR often serves as an objective clinical standard for high-frequency hearing in infants (*11*). However, ABR does not fully satisfy clinical requirements. The present study analyzed all hearing tests of infants with abnormal TEOAE and normal ABR.

Of the 81 ears with normal ABR, only 18 ears (22.2%) were normal in the 5 other hearing tests. ABR energy is mainly in the high frequency range (2 ~4 kHz) because of the induction of clicks in ABR. Although a click-induced ABR is closely correlated to a behavior auditory threshold of 2~4 kHz, ABR lacks frequency specificity and cannot reflect the levels of hearing at low and medium frequencies. A 40 Hz AERP is induced by a tone burst and has some advantages, such as stable waveform, large amplitude, good reproducibility, easy identification, determined threshold, and frequency specificity. This test mainly evaluates low-frequency (0.5~1 kHz) hearing and can compensate for the insufficiency of ABR testing to measure low-frequency hearing. Of the 36 ears with normal ABR, 38.9% were abnormal in the 40 Hz AERP test. This result suggests that low-frequency hearing loss could not be detected effectively with the ABR.

Table 1. Comparison of the abnormal rate for different tests

Testing method	Total tested subjects (number of ears)	Abnormal subjects (number of ears)	Abnormal rate (%)
40 Hz AERP	22 (36)	11 (14)	38.9
ASSR	31 (45)	20 (27)	60.0
DPOAE	45 (68)	34 (50)	73.5
Tympanogram	32 (50)	8 (9)	18.0
acoustic reflex	31 (47)	18 (27)	57.4

Table 2. Comparison of abnormal rates for male and female infants according to different testing methods

Testing method	Male			Female		
	Tested subjects (number of ears)	Abnormal subjects (number of ears)	Abnormal rate (%)	Tested subjects (number of ears)	Abnormal subjects (number of ears)	Abnormal rate (%)
40 Hz AERP	13 (23)	6 (8)	34.8	9 (13)	5 (6)	46.2
ASSR	20 (29)	12 (17)	58.6	11 (16)	8 (10)	62.5
DPOAE	28 (42)	19 (27)	64.3	17 (26)	15 (23)	88.5
Tympanogram	21 (32)	4 (4)	12.5	11 (18)	4 (5)	27.8
acoustic reflex	20 (29)	12 (16)	55.2	11 (18)	6 (11)	61.1

Table 3. Comparison of abnormal rates for left and right ears according to different testing methods

Testing method	Left ear		Right ear	
	Ears tested	Abnormal ears (%)	Ears tested	Abnormal ears (%)
40 Hz AERP	20	10 (50.0)	16	4 (25.0)
ASSR	23	15 (65.2)	22	12 (54.5)
DPOAE	38	30 (78.9)	30	20 (66.7)
Tympanogram	26	5 (19.2)	24	4 (16.7)
acoustic reflex	24	13 (54.2)	23	14 (60.9)

Nevertheless, a 40 Hz AERP is affected by the depth of sleep and sedatives and its waveform differentiation and response threshold differ in some cases. In recent years, ear, nose, and throat (ENT) doctors have assigned importance to the ASSR to evaluate hearing in infants. When many amplitude modulation acoustic signals are sent simultaneously through the ears, the ASSR can record the response to the sound stimulus and objectively judge the values for the hearing response threshold at frequencies of 0.5 kHz, 1 kHz, 2 kHz, and 4 kHz at the same time in both ears. ASSR possesses better frequency specificity and its high frequency is closely correlated with the behavioral hearing threshold (*12*). In addition, it is not affected by tranquilizers and sleep (*13*). An abnormal ASSR mainly reflects a rise in the hearing response threshold for low frequencies (500 Hz) (*14*). In the present study, there were 27 abnormal ears among the 45 ears tested by ASSR, with an abnormal rate of 60.0%. The abnormal rate for ASSR testing was significantly higher than for the 40 Hz AERP. This result demonstrates that the ASSR tends to underestimate low-frequency hearing. Many reports have mentioned that there is no positive correlation between a hearing response threshold of 500 Hz and the behavioral threshold when the ASSR was used to assess the hearing of normal infants (*15-17*). Accordingly, ASSR results must be compared with other hearing test results when assessing the low-frequency hearing of infants.

OAE is a sensitive index used to evaluate the integrity of the peripheral auditory system and can be detected only when the external ear, middle ear, and inner ear are functioning normal or close to normal (*18*). Studies have shown that different sound intensity levels of sound stimuli produce different levels of sensitivity in damaged cochlea (*5*). The sound intensity level of the sound stimulus in TEOAE is 80 dB SPL and the test can identify hearing loss of 20~30 dB HL (*19*), while the sound intensity level of the sound stimulus in DPOAE is 70 dB SPL and it can identify loss of up to 35~45 dB HL (*20*). There is no continuity between TEOAE and DPOAE test results; that is, a normal TEOAE does not mean a normal DPOAE, and vice versa (*21*). Frequency specificity in DPOAE is better than in TEOAE. In this study, 50 ears (73.5% of 68 ears with abnormal TEOAE) were abnormal according to the DPOAE test.

Tympanometry testing is mainly used to measure the function of the middle ear in children (*22*,*23*). A type B or C tympanogram shows that there is fluidity in the tympanic cavity while failure to induce an acoustic reflex indicates a problem with conduction in the middle ear (*24*). In conductive hearing loss, such as the congenital development malformation of the middle ear, the tympanogram is type A or type As and thus normal but acoustic reflex cannot usually be induced or has a higher threshold (*25*). In testing with tympanometry and the acoustic reflex, the overall abnormal rate was 75.4%. The abnormal rate in tympanometry (18.0%) was significantly lower than that in acoustic reflex (57.4%) testing. This suggests a higher proportion of middle ear disease in infants with abnormal TEOAE and normal ABR; 226 Hz tympanometry was less sensitive than acoustic reflex in testing of the middle ear disease in infants.

Analysis by gender demonstrated that the abnormal rate was significantly higher among females (92.9%) than males (69.8%). This was more prominent in results of 40 Hz AERP (male 34.8%, female 46.2%), DPOAE (male 64.3%, female 88.5%), and tympanometry (male 12.5%, female 27.8%). These results suggest a high false positive rate for screening of male infants. This finding agreed with a previous study by the authors (*26*). Total abnormal rates for left and right ears were 79.5% and 75.7%, respectively, and there was no significant difference between the two ears. Except for acoustic reflex test, other tests indicated higher abnormal rates for left ears than for right ears. In the 40 Hz AERP test, the abnormal rate for left ears was significantly higher than that for right ears (left 50.0%, right 25.0%).

In conclusion, these different hearing diagnostic methods possess their own characteristics and can supplement each other. However, hearing loss cannot be sufficiently determined with ABR alone in newborns and infants who fail hearing screening. ABR must be combined with other audiological tests and all testing results must be analyzed.

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