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Low Memorial Library, Columbia University

Columbia University is a member of the Ivy League of USA and has produced a lot of leaders in various fields. Low Memorial Library (*photo*) is a prominent building on the university's campus. The library was built in 1897 by University President Seth Low in memory of his father, Abiel Abbot Low and served as the main library until 1934. The building designed with a lot of elements of Roman and Greek style is designated as a National Historic Landmark.

(*Photo by Hao Qian*)

Review

Improved applications of the tetracycline-regulated gene depletion system

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Summary **Tightly controlled expression of transgenes in mammalian cells is an important tool for biological research, drug discovery, and future genetic therapies. The tetracycline-regulated gene depletion (Tet-Off) system has been widely used to control gene activities in mammalian cells, because it allows strict regulation of transgenes but no pleiotropic effects of prokaryotic regulatory proteins. However, the Tet-Off system is not compatible with every cell type and this is the main remaining obstacle left for this system. Recently, we overcame this problem by inserting an internal ribosome entry site (IRES) to drive a selectable marker from the same tetracycline-responsive promoter for the transgene. We also employed a CMV immediate early enhancer/β-actin (CAG) promoter to express a Tet-controlled transactivator. Indeed, the Tet-Off system with these technical modifications was applied successfully to the human pre-B Nalm-6 cell line in which conventional Tet-Off systems had not worked efficiently. These methodological improvements should be applicable for many other mammalian proliferating cells. In this review we give an overview and introduce a new method for the improved application of the Tet-Off system.**

Keywords: Tetracycline-regulated gene depletion system, Tet-Off system, gene targeting

1. Introduction

The tetracycline (Tet)-regulated gene depletion (Tet-Off) system, originally proposed by Gossen and Bujard (*1*), has been widely applied to control gene activity in eukaryotes. The system functions in cultured cells from mammals, plants, amphibians, and insects, as well as, in organisms including: yeast, *Drosophila*, plants, mice, and rats (*http://www.zmbh.uni-heidelberg.de/Bujard/homepage.html*). In order to set up a functional Tet-Off system, a stable host cell line containing two different expression units needs to be isolated, as shown in Figure 1. The first unit is a plasmid to express a regulatory protein termed Tet-controlled transactivator (tTA), which is a fusion protein of an *Escherichia coli* (*E. coli*) Tet repressor (TetR) and the transcriptional transactivation domain of herpes simplex virus protein 16 (VP16AD). The second unit is a response plasmid that expresses a gene of interest (GOI) under the control of a Tet-response element (TRE), which is a minimal RNA polymerase II promoter sequence fused downstream from the multiple Tet resistance operator in *Tn10* of *E. coli*. In the doubly stable cell harboring the tTA expression vector and the response vector, the tTA can bind to the TRE to stimulate the onset of transcription of *GOI* if the cells are cultured in the

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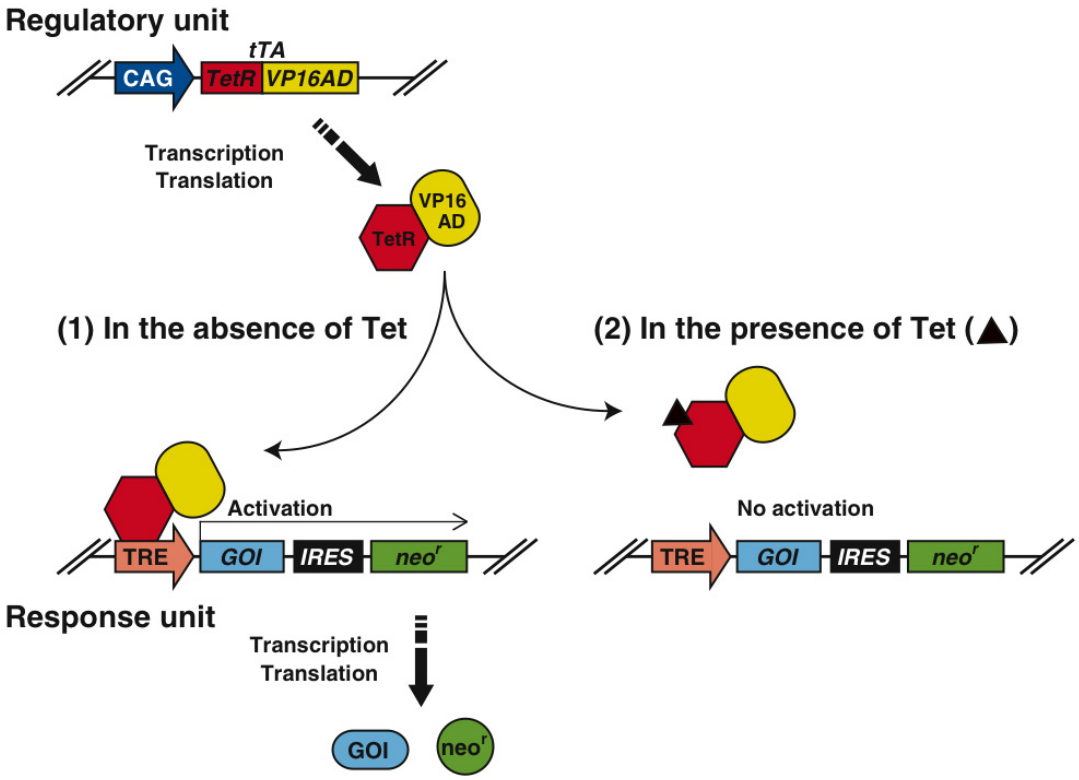

Figure 1. The principles and the strategy of the improved Tet-Off System. To set up a Tet-Off system, two expression units are required to be integrated into the genome of the host cells. The first is a regulatory unit to express tTA, a fusion protein of TetR and VP16AD). The second is a response unit in which *GOI* is placed downstream of TRE to express *GOI* in a tTA/TRE-dependent manner. **(1)** In the absence of Tet, tTA binds to TRE and activates TRE-derived transcription of *GOI*. **(2)** In the presence of Tet (closed triangle), TRE-derived expression of *GOI* is shut off promptly since Tet-bound tTA cannot access TRE. In our improved Tet-Off system, CAG promoter is used to express tTA in place of CMV promoter. In addition, IRES was inserted between *GOI* and drug-resistant gene (*neo*^{r}) in the response unit. The cell clones that express optimal amounts of TRE-derived transgenes can be isolated efficiently as drug-resistant clones.

absence of Tet. In contrast, the tTA is prevented from binding to the TRE and consequently transcription of *GOI* is abolished if the cells are cultured in the presence of Tet, even at concentrations far below cytotoxic levels for living cells.

Among a number of inducible gene expression systems so far developed (*1-5*), the Tet-Off system has been widely used, because it has been improved to overcome the initial technical problems, including leaky expression, loss of regulation, and slow suppression (see reviews in *6,7*). The main problem left for the Tet-Off system was that it does not work effectively in every cell type, as described (a protocol issued by Clontech 13 September 2005; *8-10*). However, this problem was overcome using the following technical modifications: switching of the promoter to express tTA from the human cytomegalovirus IE (CMV) to CMV immediate early enhancer/β-actin (CAG) (*11*) in the tTA expression vector; insertion of an internal ribosome entry site (IRES) followed by a drug-resistant gene in the response vector. These improvements enabled us to obtain rare clones that express robust amounts of tetracycline-regulated transgenes, in the human Nalm-6 pre-B cell line. This is a cell line that is not always compatible with the conventional Tet-Off system. As the Nalm-6 is a rare cell line that is efficient for gene targeting by homologous recombination, the successful application of the Tet-Off system allowed us to generate a 'tetracycline-inducible conditional gene deficient' Nalm-6 cell clone in which the expression of *GOI* can be depleted with the addition of Tet on a knockout background (*12,13*).

As the CAG promoter and the IRES sequence work effectively in most vertebrate cells, we expect that the improved Tet-Off system will be applicable to many other proliferating cells in which the conventional Tet-Off system would not work. In this review, the methodological improvements introduced in the Tet-Off system is summarized and a protocol for its application to the Nalm-6 cell line is introduced as a compelling example.

2. The choice of promoter to express tTA

The promoter to express tTA should be selected dependent on what types of the cells are used. For example, the CAG promoter (*11*) is a better choice than the CMV promoter for the human Nalm-6 cell line. Figure 2 is a transient Luc assay in which a Luc reporter vector was introduced into the Nalm-6 cell

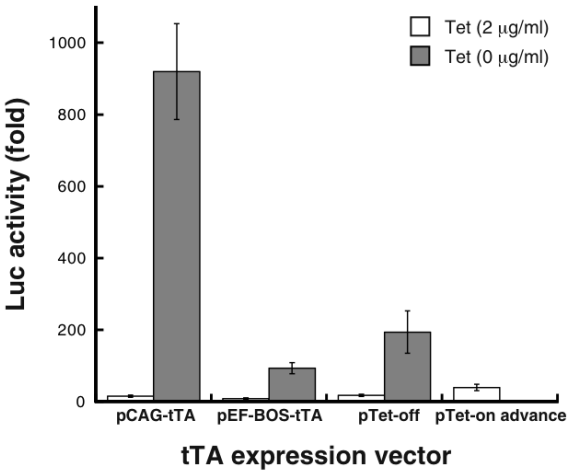

Figure 2. The efficacy of the promoter to express tTA. The histogram represents the fold increase in the induction of luciferase (Luc) expression in the transient Luc assay, in which pUHC13-3 (pTRE-Luc) was introduced alone or with pCAG-tTA (CMV enhancer and chicken β-actin promoter), pEF-BOS-tTA (SV40 enhancer and human EF-1α promoter), pTet-off or pTet-On advance (CMV promoter IE) into Nalm-6 cells. Twelve hours after transfection, the cells were separated into two aliquots, one was used for the depletion by Tet and the other for no depletion. After incubation for 36 h, cell extracts were prepared to measure the Luc activities as relative light units (RLUs). The Luc activity was normalized by the RLUs of the extract from the cells transfected with pTRE-Luc alone, and used to calculate the relative Luc activity (y-axis). Each independent experiment was performed in triplicate, and the results are expressed as the mean fold induction compared with the negative control (pTRE-Luc alone) ± standard error of mean (SEM).

line to monitor TRE-derived Luc expression. It was demonstrated that the Nalm-6 cell lines in which tTA was expressed under the control of CAG promoter (pCAG-tTA) displayed higher levels of Luc activities (on average about four-fold higher) than the Nalm-6 cell lines in which tTA was expressed under the control of CMV promoter (pTet-Off; BD Biosciences, San Jose, CA, USA). As the CAG promoter insures a stable expression of transgene in a wide rage of mammalian cells, it may be the first choice for general applications. The map of the pCAG-tTA we constructed is illustrated in Figure 3A.

3. Insertion of the IRES-drug resistance gene in the response vector

The IRES is a virus-derived nucleotide sequence in which a cap-independent translation is allowed to commence even in the middle of mRNA. Therefore, when the IRES sequence is located between the first *GOI* and the second drug resistance gene in the response vector, it permits the expression of the second gene used to monitor the expression of the first gene (*14,15*). Taking advantage of this property of the IRES, a few cell clones that express a TRE-derived transgene can be isolated efficiently by seeking drug resistance clones, instead of examining the expression of the transgene in each clone by Western blotting.

The conventional protocol for the Tet-Off system recommends a sequential transfection of the tTA expression vector and then the response vector. However, the screening system using the IRES-drug-resistant gene allowed us to transfect these two vectors simultaneously to obtain cell clones with optimal levels of TRE-derived transgene expressions, saving time by approximately a month or two. Importantly, the system worked effectively in other cell lines than the Nalm-6. The map of the typical response vector containing IRES-drug-resistance gene is shown in Figures 3B-D.

4. Materials

4.1. *Web resources*

1. Human genome database: *http://genome.ucsc.edu/cgi-bin/hgGateway*
2. NCBI BLAST home: *http://blast.ncbi.nlm.nih.gov/Blast.cgi*
3. Kazusa DNA Research Institute for full-length cDNAs of KIAA clones: *http://www.kazusa.or.jp/j/resources/clone.html*
4. Invitrogen for full-length cDNAs of IMAGE clones: *http://clones.invitrogen.com/index.php*

4.2. *Cell cultures*

1. Medium: Dulbecco's Modified Eagles Medium and Roswell Park Memorial Institute-1640 medium were used for adherent and suspension cells, respectively. The medium was supplemented with 50 mL (10%) fetal bovine serum (Kohjin-Bio, 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. Petri dish for adherent culture (9.4 cm × 1.6 cm; Greiner Japan, Tokyo, Japan).
3. Petri dish for suspension culture (9.4 cm × 2 cm; Greiner Japan).
4. Cell banker for frozen stocks of cells (Nihon Zenyaku, Fukushima, Japan).

4.3. *Preparation of cDNA*

1. TRIzol Reagent (Invitrogen, Carlsbad, CA, USA).
2. SuperScript™ First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen).
3. PrimeSTAR™ HS DNA polymerase (Takara Bio, Otsu, Japan).
4. Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA).
5. PCR-Script™ Amp Cloning Kit (Stratagene, La Jolla, CA, USA).

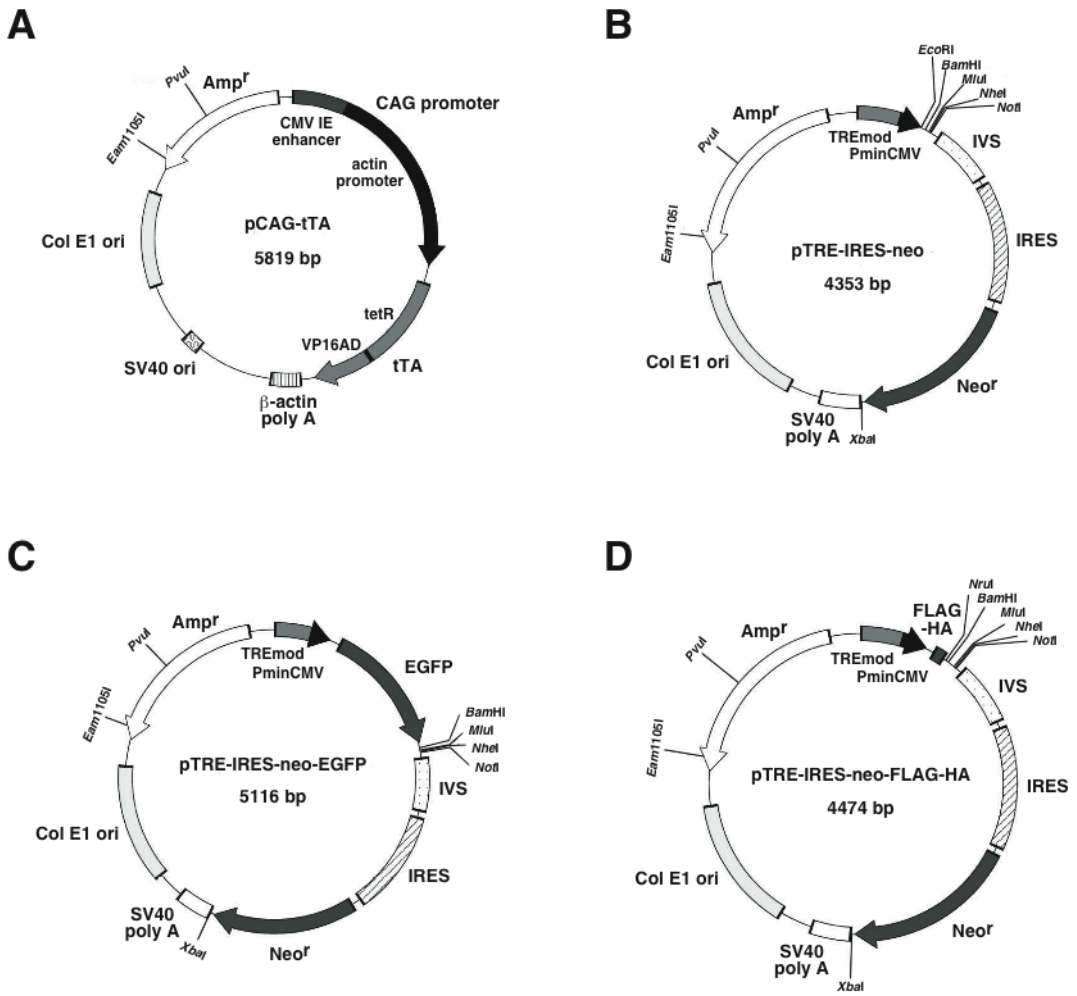

Figure 3. Schematic diagrams of tTA expression vector and response vectors. (A) Expression of tTA is driven by the CAG promoter in the pCAG-tTA. The tTA expression vector harboring neomycin resistant gene (pCAG-tTA-neo) for the sequential transfection procedure is also available. **(B-D)** A series of response plasmids were constructed from pTRE-tight vector. The response vector containing the hygromycin or puromycin resistant gene is available. Detailed information of these vectors is available upon request.

6. GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA).
7. Chemically competent DH5α or XL10-Gold cells.

4.4. *Transfection and Drug Screening*

1. Transfection: Nucleofector I (Amaxa Inc., Gaithersburg, MD, USA), Nucleofector Kit T (Amaxa Inc.).
2. Drug screening: For the case of Nalm-6 cell lines, 1.0 mg/mL of L-histidinol (Sigma-Aldrich), 0.2 μg/mL of puromycin (BD Biosciences), 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 for each cell line!
3. 96-well, 24-well, and 6-well flat-bottom plates for suspension culture (Sumitomo Bakelite Co. LTD., Tokyo, Japan).
4. 8-channel and 12-channel pipettes (Thermo Scientific Inc., Rockford, IL, USA).
5. Gel-loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 40% (w/v) sucrose in water.

4.5. *Luciferase (Luc) assay*

1. Luciferase Assay System (Promega). *Note*: Reconstituted Luc assay reagent should not be thawed above 25°C and also avoid multiple freeze-thaw cycles.
2. Luminat LB9507 (Berthold, Wildbad, Germany).

4.6. *Western blot analysis*

1. 2× Sodium Dodecyl Sulfate (SDS) sample buffer: 100 mM Tris-HCl (pH 6.8), 4.0% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol.
2. Phosphate-buffered saline: 2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4.
3. SuperSignal WestFemto Maximum Sensitivity Substrate (Thermo Scientific Inc.).

5. Protocols

5.1. *Preparation of cDNA*

A full-length cDNA is prepared by RT-PCR with the total RNA obtained from the cells or the organisms of interest, but some full-length cDNAs or ESTs can be obtained (IMAGE clones, KIAA clones, and so on: see *4.1*. Materials). The sequence of the obtained cDNA should be confirmed carefully to discriminate among alternative splicing products, polymorphisms, and amplification errors.

5.1.1. *Preparation of total RNA from cultured cells*

Total RNA is prepared using TRIzol Reagent. Roughly, 1×10^7 cells are required to prepare > 10 μg total RNA which is sufficient for the subsequent RT-PCR reactions.

5.1.2. *Preparation of cDNA*

1st strand synthesis – A typical reaction of RT-PCR with oligo (dT) primer is shown as follows, although the RT-PCR with specific primers is acceptable.

1. Prepare a 10 μL reaction mixture (I) as follows:

Prepared total RNA	to make 5.0 μg
dNTP (10 mM)	1.0 μL
Oligo(dT) primer (50 μM)	1.0 μL
DEPC-treated MilliQ water	to make 10 μL

2. Incubate at 65°C for 5 min and then put it on ice for 1 min.
3. Prepare a 9 μL reaction mixture (II) as follows:

10× RT buffer	2.0 μL
25 mM $MgCl_2$	4.0 μL
0.1 M DTT	2.0 μL
RNase inhibitor	1.0 μL

4. Mix 10.0 μL of the reaction Mixture (I) and 9.0 μL of the reaction Mixture (II).
5. Add 1.0 μL of SuperScript II reverse transcriptase, incubate at 50°C for 50 min, incubate at 85°C for 5 min, and then put it on ice.
6. Add 1.0 μL of RNase H, and incubate at 37°C for 20 min.
7. Resulting 1st strand cDNA was kept on ice or stored at -20°C until the next procedures.

PCR amplification – Primers used for PCR are designed for the subsequent construction of the TRE-responsive expression vectors, especially when an epitope-tag fused protein is expressed. For the amplification of full-length cDNA, proofreading polymerase (with a 3′-5′ exonuclease activity) should be used. A typical PCR reaction with PrimeSTAR HS DNA polymerase is shown below.

1. Prepare a 50 μL reaction mixture (III) as follows:

5× PCR buffer	10.0 μL
cDNA	1.0~2.0 μL
5′ primer (10 μM)	1.25 μL (final 250 nM)
3′ primer (10 μM)	1.25 μL (final 250 nM)
PrimeSTAR HS DNA polymerase	0.5 μL
dNTP (2.5 mM)	4.0 μL (final 200 μM each)
Sterile MilliQ water	to make 50 μL

2. PCR program:

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

3. Purify the PCR product using Wizard® SV Gel and PCR Clean-Up System.
4. Subclone the PCR product into pPCR-Script using PCR-ScriptTM Amp Cloning Kit.
5. Confirm the sequence.

5.1.3. *Construction of the Tet-responsive expression vector*

To obtain a Tet-responsive expression vector, the cDNA prepared to express a full-length or a partial, a wild-type or a mutated, or an epitope-tagged or a non-tagged protein, is integrated at the given multiple cloning sites of the pTRE-IRES-neo vector (Figures 3B-D). Expression of a tagged-fusion protein is advantageous because it enables easy confirmation of the Tet-responsive expression of the transgenes by Western blot analysis using anti-tag antibody. Expression of green fluorescence proteins (GFP)-fusion also makes it possible to analyze localization and dynamics of the expressed proteins by microscopic observation even when the specific antibodies against the expressed proteins are not available. The pTRE-IRES-series vectors for FLAG-HA- or GFP-fusion can be obtained upon request (Figures 3C and 3D).

5.2. *Transfection and screening of drug-resistant colonies*

In the following protocol, the tTA expression vector and the TRE-responsive vector are simultaneously introduced into the cells to save time, although the protocol issued by Clontech recommends a sequential transfection of the two vectors (*www.clontech.com*; Cat. No. 630921, published in September 13, 2005). The method to deliver the vector into the cells is dependent on the cell type. Lipofection is effective for adherent cells, while electroporation works better for suspension cells. Nucleofection is a transfection method that enables efficient transfer of nucleic acids

into cells considered difficult or even impossible to transfect. Nucleofection uses a combination of optimized electrical parameters, generated by a special device called Nucleofector (Amaxa Inc.), with cell-type specific reagents (Amaxa Inc.). As standard electroporation is not efficient in some suspension cells, nucleofection is recommended for those cells. In this section, a protocol to introduce DNA into human pre-B Nalm-6 cells using Nucleofector is shown as an example.

Day 0:

1. Prepare healthy growing Nalm-6 cells.
2. Linearize the pCAG-tTA vector (Figure 3A) and pTRE-IRES-neo vector prepared in section *5.1.3.*, using a single cutting enzyme.

Day 1:

1. Perform phenol-chloroform extraction, ethanol precipitation and then rinse with ice-cold 70% ethanol.
2. Dry the DNA on a clean bench and suspend the DNA pellet in sterile MilliQ water.
3. Check the concentration of the DNA before transfection.
4. Pre-warm the complete culture medium (0.5 mL per sample) at 37°C.
5. Pre-warm Nucleofector Solution T at room temperature (RT).
6. Perform a cell viability check for Nalm-6 cells. *Note*: Cells for transfection should be healthy and 2 × 10^6 cells for each transfection are required.
7. Harvest the cells by centrifugation at 90 g for 5 min at 22°C and completely aspirate the supernatant (Be careful because cell pellets are less-tightly packed in this step!).
8. Suspend the cells with 100 µL of Nucleofector Solution T.
9. Add < 5 µg digested plasmid DNAs to the suspended cells (The molar ratio of the pCAG-tTA and pTRE-IRES-neo expression vector is roughly 5 to 1, and the total amount of DNA is limited to less than 5 µg for the Amaxa system).
10. Transfer the cell/DNA mixture into an Amaxa certified cuvette.
11. Perform transfection with Nucleofector I using the C-05 program of the machine.
12. Quickly add 500 µL pre-warmed culture medium into the cuvette and mix it well but slowly.
13. Transfer the cell/DNA mixture to a 6-well plate with a pipet.
14. Culture the cells in a humidified 5% CO_2 incubator at 37°C for 24 h.

Day 2:

1. 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!
2. Count the cell number to check the viability of the transfected Nalm-6 cells. *Note*: Around 80% cell viability is preferred.
3. Seed the cells at around 2,000~5,000 viable cells per well in the screening medium on 96-well flat-bottom plate.

Day 14-21

1. After 2~3 weeks of culturing, drug-resistant colonies were selected and transferred to new 96-well flat-bottom plates.
2. These cell colonies were grown for the preparation of SDS-PAGE samples.

5.3. *Confirmation of the TRE-responsive expression*

1. When there are colonies in the 96-well flat-bottom plate, the cell clones are transferred to a 6-well plate or 10-cm dish to allow cells to grow until there are more than 1 × 10^6 cells.
2. Count the number of the cells, harvest more than 1 × 10^6 cells in a 15-mL tube by centrifugation at 190 g for 3 min at 4°C, and carefully discard the supernatant.
3. Suspend the cell pellet in 1 mL PBS (-), transfer it to a 1.5-mL tube, centrifuge at 1,000 rpm (190 g) for 3 min at 4°C, and carefully discard the supernatant.
4. Suspend the cell pellet in 50 µL of 2× SDS sample buffer per 1 × 10^6 cells.
5. Incubate the cells at 95°C for 3 min. The samples are not very viscous at this concentration, but they can be sonicated in a water bath sonicator, if necessary.
6. Load 10 µL of heat-denatured sample into each lane (*Note*: Whole cell extracts derived from around 1~2 × 10^5 cells are appropriate amounts for 1 mm-thick SDS-PAGE and western blot analysis).
7. SDS-PAGE analysis and western blot analysis are performed as described (Molecular Cloning, 3rd edition).

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Review

Two evolutionarily conserved essential β-barrel proteins in the chloroplast outer envelope membrane

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Summary **Chloroplasts are organelles specific to photosynthetic eukaryotes that support the lives of most organisms on earth. Chloroplasts were derived from an ancient cyanobacterium by endosymbiosis, and one characteristic shared between them and extant cyanobacteria is the presence of β-barrel proteins in the outer membrane. These integral membrane proteins are also found in the outer membranes of proteobacteria and mitochondria. In particular, a group of homologous β-barrel proteins called BamA homologs are present in all Gram-negative bacteria and the endosymbiotic organelles, *i.e.*, chloroplasts and mitochondria. It was recently revealed that, in both proteobacteria and mitochondria, there is a single essential BamA homolog that mediates β-barrel protein assembly. In a chloroplast, there are two distinct BamA homologs, Toc75 and OEP80, which diverged early in the evolution of chloroplasts from their common ancestor with extant cyanobacteria. Recent genetic studies demonstrated that each of these proteins is indispensable for viability of plants although neither has been shown to be involved in β-barrel protein assembly. Toc75 catalyzes import of nuclear-encoded precursor proteins, a process that is not required for bacteria, whereas the molecular function of OEP80 remains elusive. Establishment of a protein import apparatus was required to facilitate the transfer of genes from the endosymbiont to the host cell nucleus. Hence, we propose that the gene duplication giving rise to the two essential BamA homologs was a prerequisite for the successful conversion of the cyanobacterial endosymbiont into the chloroplast. Consequently, continued study of these two chloroplast proteins should advance our understanding of endosymbiosis and evolutionarily conserved proteins in general.**

Keywords: β-barrel membrane proteins, chloroplast outer envelope membrane, endosymbiosis, OEP80, Toc75

1. Introduction

Oxidative photosynthesis supports the lives of virtually all organisms on earth. In eukaryotes, this reaction takes place in the chloroplast, the organelle specific to photosynthetic protists (eukaryotic algae) and plants. In higher plants, chloroplasts are further integrated into the development of organisms by differentiating into various inter-convertible non-photosynthetic plastid types, such as chromoplasts in red and orange fruit and floral petals, and amyloplasts in root tips (*1*). In addition to photosynthesis, plastids perform many functions essential for normal growth and development of plants. These include assimilation of nitrogen and sulfur, biosynthesis of amino acids, fatty acids, carotenoids, tocopherols, and precursors of plant growth regulators such as abscisic acid and gibberellins (*2-5*), and gravity sensing (*6*). A number of genes encoding enzymes responsible for chloroplast metabolism have been

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identified and characterized. These achievements have made possible genetic engineering of crop plants that produce high amounts of compounds beneficial for human nutrition (*2*). In addition, the knowledge of biochemical processes in chloroplasts has been used to develop and test a computational model for a metabolic network (*7*).

Chloroplasts originated from an ancestral cyanobacterium, which was engulfed by a primitive mitochondriate eukaryotic cell about a billion years ago (*8*). This event, called primary endosymbiosis, gave rise to three major autotrophic lineages, Glaucophyta, Rhodophyta (red algae) and Viridiplantae (green algae and land plants), all of which contain chloroplasts surrounded by an envelope consisting of two distinct membranes (*9-11*). The outer membrane of the chloroplast envelope used to be considered as a remnant of the phagosomal membrane of the eukaryotic host (*12*). However, the presence of galactolipids and β-barrel proteins, a feature shared with membranes of extant cyanobacteria but not with the eukaryotic endomembrane systems, supports the prokaryotic origin of the chloroplast outer membrane (*13,14*). Chloroplasts spread into other protist lineages, too, such as diatoms, which play a major role in net primary oxygen production in the ocean, through multiple eukaryote-eukaryote (secondary and tertiary) endosymbioses (*15*). A secondary endosymbiotic event also gave rise to apicoplasts, organelles that lost photosynthetic capacity but still play important roles in viability of protozoan parasites (*16*). Hence, the chloroplast provides footprints of evolutionary events relevant to global energy production and human disease.

The presence of two distinct membranes, the outer and inner membranes, is a feature shared not only by cyanobacteria and chloroplasts, but also by all the other Gram-negative bacteria and mitochondria of eukaryotic cells. Furthermore, β-barrel membrane proteins are found exclusively in the outer membranes of these bacteria and the endosymbiotically derived organelles. Consequently, addressing questions about the chloroplast outer envelope should provide insights into the functions and evolution of various membrane systems. In this review, we intend to highlight a limited but interesting feature of the outer envelope of higher plant chloroplasts, which has generally been under-examined compared to its counterparts in bacteria and mitochondria. We propose that two chloroplast β-barrel membrane proteins, which belong to an evolutionarily conserved group of proteins in Gram-negative bacteria and the endosymbiotic organelles, played key roles in the successful conversion of the free-living prokaryote into the organelle. This review aims to emphasize the relevance of the study of these proteins for understanding endosymbiosis and the evolutionary event in general.

2. The outer membranes of Gram-negative bacteria, mitochondria, and chloroplasts

2.1. *Conserved and divergent properties of the outer membranes*

The outer and inner membranes of Gram-negative bacteria and the endosymbiotic organelles function as a physical barrier that separates two aqueous compartments, *i.e.*, the inside (the cytoplasm for the bacteria, the matrix for mitochondria, and the stroma for chloroplasts) and the outside (extracellular space for the bacteria, and the cytoplasm for mitochondria and chloroplasts), and catalyze communications between them. These two membranes also facilitate metabolic compartmentalization by providing an aqueous space in between, *i.e.*, the periplasm in the bacteria and the intermembrane space in mitochondria and chloroplasts. A number of features are conserved among the outer membranes of Gram-negative bacteria and the endosymbiotic organelles. However, these membranes have also evolved many distinct properties (Table 1).

It has been postulated that immediately after the engulfment by the host cell, the eubacterial endosymbionts were surrounded by a phagosomal membrane in addition to their own outer and inner membranes. This host-derived outermost membrane, however, quickly disappeared (*9*), and the remaining two lipid bilayers started evolving to adapt to the new environment (*17*). One of the significant changes in these two membranes was the establishment of protein import apparatus. This was required by the endosymbiont to complete gene transfer to the host nucleus, which is essential for its successful conversion to an organelle. Endosymbiotic gene transfer consists of i) duplication and transfer of genes in the endosymbiont to the host nucleus, ii) expression of the transferred genes in the nucleus, iii) targeting of the nuclear-encoded proteins back to its original location in the endosymbiont, and iv) loss of the genes from the endosymbiont. Consequently, most proteins currently found in chloroplasts and mitochondria are encoded in the nuclear genome, although each of these organelles still carries its own genome (*18*). In addition to protein import apparatus, the endosymbiont had to develop various machineries at the surrounding membranes to exchange numerous metabolites and solutes in order to integrate their metabolic processes into that of the host cell. While bacterial outer membranes play roles in symbiotic and/or pathogenic interactions with host cells (*19,20)*, their counterparts in the endosymbiotic organelles play significant roles in other processes, such as organelle division, movement, and the biosynthesis of membrane lipids (*14,21,22*). Recent studies have also demonstrated the role of the mitochondrial outer membrane in apoptosis

Table 1. Comparisons of the outer membranes of Gram-negative bacteria and the endosymbiotic organelles*

	Gram-negative bacteria		Endosymbiotic organelles	
	Proteobacteria**	Cyanobacteria	Mitochondria	Chloroplasts
Peptidoglycan layer	~6 nm[a]	12 nm[b]	None	None (except cyanelles)
Exclusion limit	~0.6 kD[c], ~6 kD[d]	~2 kD[e]	~3.4-6.8 kD[f]	~10 kD[g]
Protein content (wt%)	50[h]	30[i]	60[j]	25-30[k]
Number of integral membrane proteins identified/predicted***	42 (β-barrel) + 1 (α-helical)[l]	17 (β-barrel)[m]	4 (β-barrel) + 28 (α-helical)[n]	8 (β-barrel) + 26 (α-helical)[o]
Major non-protein components****	Lipopolysaccharides Phospholipids	Lipopolysaccarides Phospholipids	Phospholipids	Phospholipids Galactolipids
Functions*****	• Permeability barrier • Passive and active solute transport • Defense • Symbiotic and/or pathogenic interaction with host cells	• Permeability barrier • Passive and active solute transport • Symbiotic interaction with host cells	• Permeability barrier • Protein import • Solute transport (passive) • Biosynthesis of lipids, nicotinic acid, cysteine, erythroascorbic acid • Regulation of organelle morphology (shape, fission, and fusion) • Apoptosis	• Permeability barrier • Protein import • Biosynthesis of membrane lipids • Organelle division • Anchorage to the plasma membrane • Organelle movement • Sugar signaling

*The listed data are from the following species: [a], *Escherichia coli* (*102*); [b], *Synechocystis sp*. PCC 6714 (*103*); [c], *E. coli* (*104*); [d], *Pseudomonas aeruginosa* (*105*); [e], *Anabaena variabilis* (*106*); [f], *Neurospora crassa* (*107*); [g], *Spinacia oleracea* (spinach) (*108*); [h], *Salmonella typhimurium* (*109*); [i], *Synechocystis sp*. PCC6714 (*110*); [j], *Saccharomyces cerevisiae* (*111*); [k], *S. oleracea* (*112*); [l], *E. coli* (*113,114*, the White lab website (*http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html*)]; [m], *Anabaena sp*. strain PCC 7120 (*115*); [n], *N. crassa* (*22*); [o], *A. thaliana* (*14*); the Inoue lab website (*http://www.plantsciences.ucdavis.edu/kinoue/OM.htm*)]; **This phylum includes *Alphaproteobacteria*, the closest prokaryotic homologs of mitochondria (*116*); ***These should be underestimated numbers; ****These are in *E. coli* (*109,117*) for proteobacteria; *Synechocystis sp*. PCC6714 (*110*) for cyanobaceria; *N. crassa*, rat liver (*118*), and *S. Cerevisiae* (*111*) for mitochondria; *S. oleracea* (*21*) for chloroplasts. Galactolipids have been found in the inner but not in the outer membranes of extant cyanobacteria (*110,119*), whereas they are present in both membranes of chloroplasts (*112*). Hence, galactolipids in the chloroplast outer membrane may have been recruited from the inner membrane of the cyanobacterial endosymbiont; *****See text for appropriate references.

(*23*), and that of the chloroplast outer membrane in sugar-sensing (*24*). Indeed, the composition of integral membrane proteins has diverged significantly between the outer membranes of the extant bacteria and those of the endosymbiotic organelles (Table 1). These proteins in the organelles must have evolved either from those already present in the endosymbiont, or from gene products of the host cell (see section 3.1. for protein import components as examples).

2.2. *Beta-Barrel proteins – the common constituents of the outer membranes of Gram-negative bacteria and the endosymbiotic organelles*

One of the common features shared by Gram-negative bacteria, mitochondria, and chloroplasts is the presence of β-barrel proteins in the outer membrane (Table 1). These integral membrane proteins are postulated to form pores consisting of multiple transmembrane β-strands, which are laterally hydrogen-bonded in a circular pattern (*25-27*). A number of bacterial β-barrel proteins have been shown to use these hydrophilic pores directly to transport various solutes, metabolites and proteins (*20*). Furthermore, in some cases, these transmembrane structures appear to function as a membrane anchorage to support the soluble catalytic sites located in *cis*, as seen in OmpLA (*28*) and OmpT (*29*), and may also provide physical integrity of the lipid bilayer, as seen in OmpA (*20*). Three-dimensional structures of a number of bacterial β-barrel proteins, which consists of even numbers (8 to 24) of transmembrane β-strands, have been demonstrated by X-ray crystal analyses (*30,31*). In addition, electron microscopic studies have shown pores formed by putative β-barrel membrane proteins including those from mitochondria (*32,33*). Using these data, a number of hidden Markov model prediction programs have been developed to examine the presence of transmembrane β-strands in a given protein (*34-36*). Recently, three independent groups reported the first three-dimensional structures of non-bacterial β-barrel membrane proteins, VDACs (Voltage-Dependent Anion Channels), from human and mouse mitochondria (*37-39*). Unlike bacterial proteins, these mammalian proteins consist of an odd number of transmembrane β-strands. Interestingly, however, their *N*-terminal α-helical domain was found to bind to the β-barrel, orienting both the *N*- and *C*-termini towards the space between the outer and inner membranes, similar to the topology of the bacterial β-barrel proteins (*25*). Finally, there is no report on the crystal structure of any chloroplast β-barrel membrane proteins yet, although some of them can be predicted to contain an even number (8 to 16) of transmembrane β-strands by the programs such as PRED-TMBB (*34*) and PROFtmb (*35*), which are designed for

bacterial β-barrel membrane proteins (Inoue and Hsu, unpublished).

In *Escherichia coli*, 42 proteins have been found and/or predicted to integrate into lipid bilayers with multiple β-strands, whereas only a handful of β-barrel proteins have been identified in mitochondria and chloroplasts (Table 1) (*14,40*). Quite interestingly, although the proteins in the endosymbiotic organelles may have evolved from bacterial ancestors, only BamA homologs (see below) show apparent sequence similarities to prokaryotic proteins (*41*). Furthermore, the outer membranes of both mitochondria and chloroplasts are enriched with proteins with α-helical transmembrane domains, which seem to be very rare in the bacterial outer membranes (Table 1). Nonetheless, the major integral constituents of the outer membranes of mitochondria and chloroplasts are represented by β-barrel proteins, VDAC (*42*) and Toc75 (*43*), respectively. Hence, the importance of β-barrel proteins in the outer membranes appears to be conserved between the bacteria and the organelles.

3. Two essential β-barrel proteins in the chloroplast outer envelope – Toc75 and OEP80

3.1. *Chloroplast protein import*

Currently, most nuclear-encoded proteins targeted to the interior of chloroplasts are synthesized by cytoplasmic ribosomes with an *N*-terminal extension called a transit peptide. Extensive biochemical and genetic studies have identified multiple proteinaceous components involved in the transit peptide-dependent import of these precursor proteins at the chloroplast envelope membranes, and they are designated as Toc and Tic (Translocon at the outer- and the inner-envelope-membranes of chloroplasts) proteins (*44-51*). How did the pre-organelle establish these protein import machineries? The presence of apparent homologs in extant cyanobacteria suggests that some components evolved from proteins in the eubacterial endosymbiont, whereas other proteins may have been recruited from the host eukaryote (*52*). Some non-essential components may have adopted multi-functionality during evolution (*17*). Overall, however, the mechanism by which the protein import machinery was established remains largely unexplored, mainly due to the lack of appropriate tools.

3.2. *The protein translocation channel in the chloroplast outer envelope, Toc75*

As described in section 3.4., one of the chloroplast protein import components that was derived from prokaryotic proteins is the major β-barrel outer membrane protein Toc75, originally identified in seedlings of pea (*Pisum sativum*). Chemical cross-linking assays (*53,54*) and reconstitution into liposomes (*55,56*) have established that Toc75 is a major protein translocation pore. It forms a hetero-oligomeric complex in the outer membrane with two homologous GTPases, Toc159 and Toc34, which expose their large *N*-terminal portions to the cytoplasmic surface (*57*). Toc34 has been shown to be anchored to the membrane with a transmembrane α-helical domain, whereas the exact conformation of the transmembrane domain in Toc159 is not completely understood (*46*). Toc75 also plays a role in the insertion of a signal-anchored α-helical outer membrane protein, OEP14, which does not carry a transit peptide (*58*). Because Toc75 itself is encoded in the nuclear genome, it also has to be targeted to the organelle posttranslationally and inserted into the membrane. It is intriguing that unlike other outer membrane proteins in chloroplasts and mitochondria that do not need cleavable targeting sequences, Toc75 requires a transit peptide, which consists of two parts and is removed by two steps, for its correct targeting (*59-63*). In particular, Toc75 depends for its complete maturation on a membrane-bound protein called Plsp1 (plastidic type I signal peptidase 1) (*64,65*). Interestingly, similar to Toc75, Plsp1 appears to have derived from a protein in a cyanobacterial ancestor (*66*).

3.3. *A distinct paralog of Toc75 in chloroplasts, OEP80*

There are four genes in the genome of the model plant *Arabidopsis thaliana* that encodes apparent Toc75 homologs (*67,68*). Among them, the one on chromosome I was shown to be a pseudo-gene (*69*). The protein encoded on chromosome III is the sole functional Toc75, which contains the unique bipartite transit peptide (*61*), and is essential for plant viability as its gene knockout disrupted embryo development as early as at the two-cell stage (*69,70*). By contrast, functions of the other two Toc75 paralogs remain largely elusive. The protein encoded on chromosome IV is a truncated form of Toc75 without the N-terminus. Its gene knockout caused slight abnormalities in the structure of non-photosynthetic plastids, but did not significantly disrupt normal plant growth (*69*). The paralog encoded on chromosome V of *A. thaliana* was annotated to encode an 80 kDa protein, which shows only 22% sequence identity to Toc75. A pea ortholog of this protein, which was named Toc75-V, appears to be 66 kDa and was not found in Toc complexes prepared by sucrose-gradient centrifugation (*67*). Later immunoblotting results using an antibody against part of the deduced sequence and *in vitro* import data suggested that the size of this Toc75 paralog may be similar to that of the precursor, 80 kDa (*71*). This protein from *A. thaliana*, which was named OEP80 for outer envelope protein 80, does not require an *N*-terminal cleavable bipartite transit peptide as does

Toc75 for targeting to chloroplasts (*71*). More recently, disruption of the OEP80 gene was shown to cause embryo abortion in *A. thaliana* at a stage later than that affected by *TOC75* knockout (*72*). This indicates that, while both Toc75 and OEP80 are essential for viability of plants from very early stages of development, they probably have distinct functions (*73*).

3.4. *The evolutionary origin of Toc75*

The prokaryotic origin of Toc75 was first suggested in the late 1990s, when a gene encoding an apparent Toc75 homolog was found in the cyanobacterium *Synechocystis* sp. PCC6803 (*74,75*). The encoded protein Slr1227 was localized in the bacterial outer membrane and could be reconstituted as a voltage-gated channel in artificial liposomes (*74*). A genetic study demonstrated that Slr1227 is essential for cell viability (*75*) although its exact function remains unknown.

Interestingly, proteins homologous to Toc75 are found not only in cyanobacteria, but also in a wide range of Gram-negative bacteria and mitochondria. These homologs include surface antigens named D15 and TpsB transporters of two partner secretion systems (*52*). The conserved features of these proteins include an *N*-terminal soluble portion and a *C*-terminal transmembrane region consisting of 10 to 16 β-strands in the predicted structure (Figure 1) (*76*). The soluble portion contains one to five polypeptide translocation associated (POTRA) domains, which were also found in the *N*-terminal regions of FtsQ/DivIB bacterial division protein family (*77*). The initial prediction suggested that a typical POTRA domain consists of 70 to 90 amino acids, containing three β-strands and two α-helices (*77*). Recent structural studies have revealed the core βααββ motif of a POTRA domain, in which two α-helices are packed against a three-strand mixed β-sheet (*78-81*). The number of POTRA domains varies: three each for homologs from chloroplasts and cyanobacteria, one for those from mitochondria, and one to five for the Omp85 homologs from proteobacteria including *Alphaproteobacteria*, which are believed to share the common ancestor with mitochondria (Figure 1) (*82*). In the case of a subset of proteobacterial and mitochondrial proteins, the *N*-terminal portion containing the POTRA domains is postulated to exist in the space between the outer and inner membranes. This model is supported by extensive sequence comparisons between various putative homologs (*83*), proteolytic fragmentations of the proteins in the intact cells or organelles (*32,79,84*), reconstitution assays (*85*), and *in vitro* association of the POTRA domains with lipoproteins located in

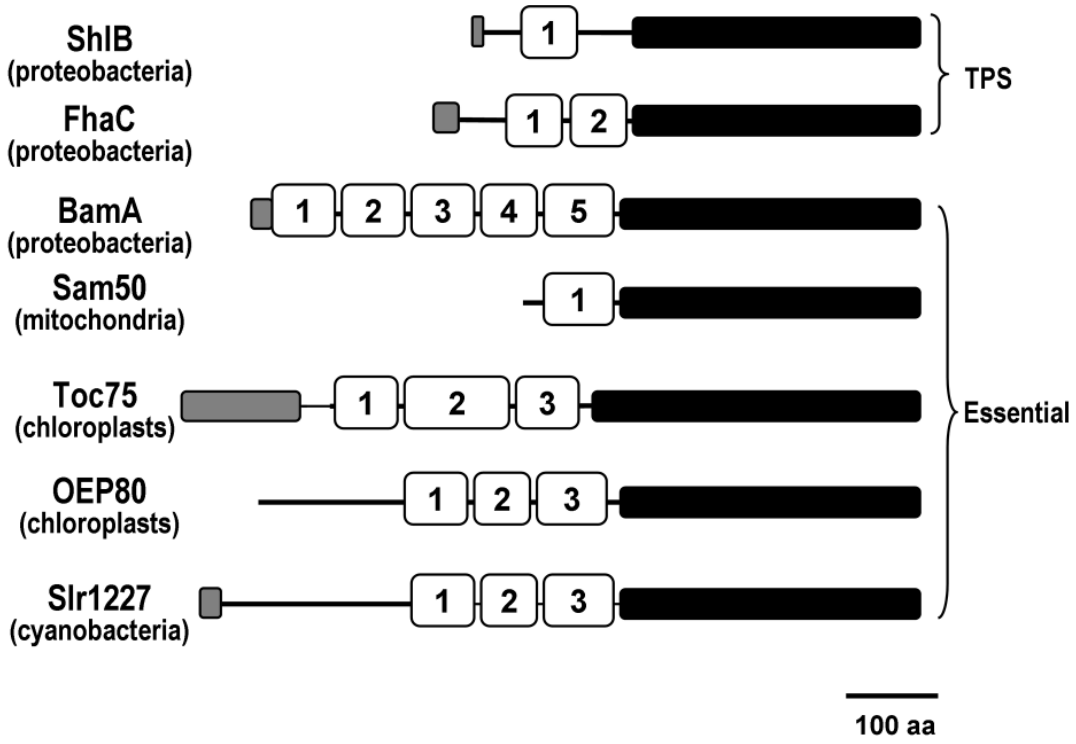

Figure 1. Schematic representation of the domain architectures of representative BamA homologs. The proteins are drawn approximately to scale. The signal peptides of four bacterial proteins (ShlB, FhaC, BamA and Slr1227) and the bipartite transit peptide of Toc75 are indicated with gray bars. POTRA and β-barrel transmembrane domains are indicated with white (with numberings) and black bars, respectively. ShlB and FhaC play a role in two-partner secretion (TPS) in proteobacteria, *Serratia marcescens* and *Bordetella bronchiseptica*, respectively (*100*), and the latter protein was shown to be dispensable for viability of the bacteria (*101*). The structures of FhaC (full sequence) and BamA (the first four POTRA domains) were confirmed by crystallography (*78,80*). Models for other structures are based on publications (*40,77*).

the periplasmic side of the bacterial outer membrane (*80*). By contrast, topologies of the homologs in chloroplasts and cyanobacteria have been less defined. Indeed, an initial model of Toc75 included the putative POTRA domains in the membrane-anchored region consisting of 16 β-strands, based on secondary structure prediction and proteolytic fragmentation patterns of proteins reconstituted into liposomes (*86*). Recently, however, a distal *C*-terminal part of Toc75 by itself was shown to form a pore *in vitro*, and a new model was presented, in which the predicted POTRA domains were included in the "*N*-terminal soluble domain" apart from newly-assigned 16 transmembrane β-strands (*87,88*).

Because protein import is not an essential process for free-living prokaryotes, it is postulated that Toc75 evolved from an ancestral cyanobacterial protein whose function was modified during endosymbiosis (*52*). A detailed phylogenetic analysis indicated that Toc75 and OEP80 diverged early in the evolution of chloroplasts from their common ancestor with extant cyanobacteria (*71*). Thus, it may be possible that both Toc75 and OEP80 evolved to take on distinct functions, which are essential for organelle development but are distinct from that of the bacterial protein. Another possibility is that OEP80 has retained the function of the ancestral protein which is essential for viability of both bacteria and chloroplasts, whereas Toc75 acquired a new role in order to facilitate the conversion of the endosymbiont to the organelle (*73*).

4. BamA and its homologs – evolutionarily conserved essential proteins in Gram-negative bacteria, mitochondria and chloroplasts

4.1. *Mechanism of β-barrel protein sorting in proteobacteria and mitochondria*

In Gram-negative bacteria, all of the β-barrel membrane proteins are synthesized in the cytoplasm, targeted first to the plasma membrane, then to the periplasm before getting sorted into the outer membrane. Although they can spontaneously be inserted and assembled in artificial liposomes (*89*), involvement of proteinaceous machinery in their targeting *in vivo* had been predicted because their insertion is unidirectional and specific to the outer membrane. Nonetheless, the mechanism of β-barrel protein insertion remained unknown until a breakthrough was made in 2003, when one of the D15-related proteins called Omp85 (Outer membrane protein 85) that has five POTRA domains was found to be responsible for accumulation of β-barrel proteins in the outer membrane of a Gram-negative bacterium, *Neisseria meningitidis* (*83*). Omp85 was shown to be part of a high molecular weight oligomeric complex of unknown composition. Later, an Omp85 homolog in *Escherichia coli* called YaeT was also shown to play an essential role in accumulation of outer membrane proteins, and to form a hetero-oligomeric complex with lipoproteins, YfgL, YfiO, NlpB, and SmpA (*90,91*). The Omp85 homolog and other four complex partners in *E. coli* were later renamed as BamA and BamB-E, respectively, for β-barrel assembly machine proteins A-E (*92*).

In contrast to their bacterial counterparts, all the β-barrel proteins in mitochondria are synthesized outside the organelle, and first traverse the outer membrane. However, they are eventually inserted into the lipid bilayers from the space between the outer and inner membranes (*93*). Following the discovery of the function of Omp85 in *N. meningitidis*, the evolutionarily conserved β-barrel protein sorting to the mitochondrial outer membrane was found to be catalyzed by novel eukaryotic BamA homologs, Tob55 (Topogenesis of mitochondrial outer membrane β-barrel proteins 55) from *Neurospora crassa* and Sam50 (Sorting and assembly machinery 50) from *Saccharomyces cerevisiae* (*32,94,95*). Similar to the bacterial homologs, both Tob55 and Sam50 are also part of a multi-subunit protein complex (*41*).

Recent studies have also revealed species-specific features of β-barrel protein sorting. First, all the essential proteobacterial BamA homologs have five, whereas mitochondrial counterparts have only one POTRA domain (Figure 1). Second, some of the subunits that form a complex with the BamA homologs appear to be unique to an individual species (*96*). Third, bacterial β-barrel precursor proteins contain species-specific *C*-terminal signal sequences recognized by bacterial BamA homologs (*97*), whereas each mitochondrial substrate contains a specific sorting signal that is recognized by one of the Sam50-interacting partners, Sam35, which is unique to this organelle (*40*). Finally, the complex containing Sam50 mediates integration of not only β-barrel, but also α-helical proteins in the outer membrane of yeast mitochondria (*98*), whereas there has been no report on such an activity in bacterial BamA homologs.

In summary, bacteria and mitochondria share a homologous mechanism of β-barrel protein assembly, but the similarity is limited to the core subunits (BamA homologs), while the sorting signal and partner proteins are distinct.

4.2. *Functions of POTRA domains in BamA homologs*

Recent genetic, biochemical and structural studies have also revealed the possible species-specific functions of the POTRA domains of BamA homologs. For the bacterial proteins, Kim *et al*. showed *in vivo* that the three *C*-terminal domains (POTRA3, 4, and 5) are indispensable for the function of BamA, and also that all but POTRA1 are necessary for

interaction of BamA with one of oligomeric partners, BamB (YfgL) (*80*). Furthermore, three-dimensional structures resolved by X-ray crystallography and NMR indicated that the POTRA functions as a scaffold for either substrate proteins or interacting partners by β-augmentation (*79-81*). Interestingly, in contrast to the case with the *E. coli* homolog, only the most *C*-terminal domain (POTRA5) was found to be indispensable for the function of the *N. meningitidis* Omp85 *in vivo* (*99*). As for the mitochondrial homologs that contain only one POTRA domain, a genetic study showed that this domain is not required for the proper function of the protein *in vivo* (*40*). Interestingly, however, deletion of part of the *N*-terminus of the POTRA domain, which left its *C*-terminal 18 residues containing the last β-strand intact, disrupted the function of Tob55/Sam50, but not its complex formation, causing a growth defect in yeast (*40,84*). In addition, Habib *et al*. showed *in vitro* that the sequence containing the entire POTRA domain could specifically bind to the mitochondrial β-barrel protein VDAC and inhibit its import into the organelle *in vitro* (*84*). This result indicates that the POTRA domain of the mitochondrial BamA homolog may also have a chaperone-like activity.

4.3. *BamA homologs in chloroplasts*

Among the two BamA homologs, Toc75 functions as a conducting channel for various cytoplasmically synthesized precursor proteins. Hence, it is tempting to speculate that another homolog, OEP80, plays an evolutionarily conserved role, which is to sort β-barrel proteins into the chloroplast outer membrane. Currently, however, there is no experimental evidence to support this idea. Indeed, we know almost nothing about the mechanism of β-barrel protein insertion into the chloroplast outer membrane. Nonetheless, several biochemical studies suggested the conserved chaperone-like functions of the POTRA domains of Toc75 and its cyanobacterial homolog (*87,88*). It is also worth mentioning that no gene product has been identified in *A. thaliana* chloroplasts that are similar to any proteins forming the complexes with BamA homologs in proteobacteria and mitochondria (Hsu and Inoue, unpublished).

5. Conclusions

Chloroplasts of higher plants and some algae play vital roles in survival of most organisms on earth. These organelles have also served as systems that allow us to address various biochemical and evolutionary questions. The presence of BamA homologs in the outer membranes of proteobacteria, cyanobacteria, mitochondria, and chloroplasts reflects the common evolutionary origin of these membranes. This idea is further supported by recent findings of the conserved β-barrel protein-sorting pathway mediated by BamA homologs in proteobacteria and mitochondria, although this has not been demonstrated in cyanobacteria and chloroplasts yet. There appears to be one single essential BamA homolog each in Gram-negative bacteria and mitochondria, whereas two distinct homologs are present in chloroplasts. One of them, Toc75, was identified as the main component of the protein import machinery in the 1990s, which is long before the proliferation of studies of the bacterial and mitochondrial homologs began in 2003. Another chloroplast BamA homolog, OEP80, which was first reported in 2002, is essential for viability of organisms and is targeted from the cytoplasm to chloroplasts by a mechanism distinct from that used by Toc75. Although the molecular function of OEP80 remains unknown, the presence of two essential BamA homologs is a feature unique to the chloroplast outer envelope among the evolutionarily conserved biological membrane systems. The obvious question is if Toc75 and/or OEP80 play a role in β-barrel protein sorting, similar to their homologs in proteobacteria and mitochondria. Another question is if Toc75 and OEP80, as they are β-barrel proteins themselves, share the same mechanism of insertion into the outer membrane. Addressing these questions should not only define the properties of evolutionarily conserved membrane systems, but also advance our understanding of mechanisms underlying the endosymbiotic event.

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Review

Intrinsic regenerative mechanisms of central nervous system neurons

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Summary **Injuries to the adult central nervous system (CNS), such as spinal cord injury and brain contusion, can cause permanent functional deficits if axonal connections are broken. Spontaneous functional recovery rarely occurs. It has been widely accepted that the extracellular environment of the CNS inhibits neuronal regeneration. However, it should be noted that another reason for injured neurons failing to regenerate is their weak intrinsic ability to do so. The regeneration of injured neurons is a process involving many intracellular phenomena, including cytoskeletal changes, gene and protein expression, and changes in the responsiveness to extracellular cues. The capacity of injured neurons to regenerate is modulated to some extent by changes in the expression of intracellular signaling molecules such as glycogen synthase kinase-3β and cyclic adenosine 3′,5′-monophosphate. Knowledge of these effects has guided the development of animal models for regenerative therapies of CNS injury. Enhancing the intrinsic regenerative machinery of injured axons in the adult CNS is a potentially powerful strategy for treating patients with a CNS injury.**

Keywords: Axon, CNS, spinal cord injury, GSK-3β, cAMP

1. Introduction

Lesions of the adult central nervous system (CNS), such as brain contusion and spinal cord injury lead to functional deficits due to neuronal cell death and a loss of axonal connections. Many strategies, including cell transplantation, activation of neurogenesis of neural stem/progenitor cells, and use of undamaged neurons after injury, have been attempted to develop new therapies for recovering the neuronal dysfunction. Importantly, in each approach, axonal regrowth is essential to restore damaged neuronal connections. Unlike embryonic or peripheral nervous system neurons, however, neurons in the adult mammalian brain and spinal cord do not spontaneously regenerate. This prevents neurological symptoms from improving after an injury.

The main reason for injured adult neurons failing to regrow is the inhibitory nature of their extracellular environment. Indeed, providing CNS neurons with a permissive environment, such as peripheral nerve environment, can promote regrowth. For example, the transected axons grow into a transplanted segment of sciatic nerve in the injured spinal cord (*1*,*2*). In dorsal root ganglia (DRG) neuron that sends one axon into peripheral target tissue and the other axon into CNS, the axon can regenerate into peripheral target, but not into CNS after injury (*3*). These findings can be considered as evidence of extracellular mechanisms that inhibit axon growth in the adult CNS. Several types of molecules are implicated in these inhibitory mechanisms, including proteins that are derived from either myelin, reactive astrocytes or those that are

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known to repulse axonal extension during ontogenesis (*4-6*).

Despite the inhibitory environment of the adult CNS, spontaneous regeneration and reorganization of axons are also found in animal models of CNS injury. For example, spontaneous axon sprouting of the corticospinal tract, one of the most important descending motor pathways for skilled movements in all mammalian species, was observed at the proximal sites of injuries in the spinal cord injury (SCI) (*5*). It was also observed that a few damaged corticospinal axons regrew beyond the lesion site in SCI mice (*7*). Importantly, these reorganization and regeneration of axonal network have been considered to contribute to a partial recovery of locomotor function in animal models of SCI. In addition, it suggests that adult CNS neurons may have an intrinsic capacity to evoke mechanisms that promote spontaneous axonal regeneration after CNS injury. This has led us to focus on the intrinsic regenerative capacity of adult CNS neurons.

Neutralizing the extracellular factors that inhibit regrowth promotes axonal regeneration to some extent *in vivo*. However, it is expected that the regrowth of axons in the adult CNS can be further improved by enhancing their intrinsic capacity to regenerate. The capacity of adult CNS neurons to regenerate is low compared to that of neurons during earlier stages of development. The growth of axons during development is sustained primarily by (i) cytoskeletal changes, (ii) gene expression and protein synthesis, and (iii) changes in the response to extracellular cues. Thus, regulation of these phenomena in adult CNS neurons may be a powerful strategy for re-establishing neuronal networks after injuries. In consideration of this, our review focuses on the intrinsic molecular mechanisms that promote axon regeneration in the CNS.

2. Molecular mechanisms of modulation in cytoskeletal assembly

Axon elongation is the product of intracellular machinery that assembles cytoskeletal elements when activated by intrinsic signals or extracellular factors. Of particular relevance to the growth of an axon is the assembly of microtubules and actin filaments.

Glycogen synthase kinase-3β (GSK-3β) is an inhibitory molecule to the processes involved in microtubule assembly, and thus prevents axon growth. Treatment with a GSK-3β inhibitor to SCI rats promotes the growth of corticospinal and serotonergic axons in the spinal cord, and thereby promotes functional recovery (*8*). GSK-3β phosphorylates and inactivates collapsin response mediator protein 2 (CRMP-2). It is known that CRMP-2 binds to tubulin heterodimers and actin filaments, thus promoting neurite elongation. During normal neural development, the active form of CRMP-2 has been found to counteract the inhibition of axon elongation produced by constitutively active GSK-3β (*9*). In adult CNS, increased expression of a non-active form of CRMP-2 was observed in spinal cord neurons that failed to regenerate after SCI (*10*). These findings suggest that, because of its effects on CRMP-2 activity, inhibition of GSK-3β may be an appropriate target in the treatment of CNS injuries.

Besides CRMP-2, GSK-3β has other physiological substrates in growing axons; adenomatous polyposis coli (APC) and microtubule binding protein 1B (MAP1B). APC appears to have roles in cytoskeletal integrity of actin and microtubule dynamics, and in promoting neurite elongation. As in the case of CRMP-2, phosphorylation of APC by GSK-3β inhibits neurite elongation by blocking its ability to bind to microtubules (*11*). Similarly, phosphorylation of MAP1B by GSK-3β increases the population of unstable microtubules, thus negatively regulates axon growth (*12*). Targeting the APC, MAP1B, or CRMP may be effective strategies to treat patients with a CNS injury.

In contrast to the beneficial effects of CRMP activation on neurite outgrowth, injury-induced proteolytic cleavage of CRMP seems to have negative effects on it. Calpain, which is a calcium-dependent protease, cleaves a large number of substrates including CRMPs. Interestingly, calpain activity is increased in the focal cerebral ischemic brain, and cleavage of CRMP also occurs following cerebral ischemia (*13*). The expression of cleaved product of CRMP is correlated with neuronal death during cerebral ischemia. Indeed, overexpression of cleaved products of CRMP into cultured cerebellar granule neurons causes neuronal death (*14*). These researches indicate that truncated CRMP plays a key role in neuronal survival. In parallel, the truncated CRMP also seems to regulate neurite outgrowth and degeneration. Short isoform of CRMP-2, identified as a C-terminal processed form by calpain, inhibits neurite outgrowth in cultured cortical neurons (*15*). This finding suggests that truncated CRMPs may negatively regulate the axon elongation. The role of CRMP cleavage on neurite degeneration have also become clear in the researches using *Wld*^{S} (Wallerian degeneration slow) mice, in which neurite degeneration is delayed. The neurites of cultured suprior cervical ganglias (SCGs) are degenerated by NGF deprivation. CRMP proteins are cleaved during neurite degeneration in NGF-deprived neurite of wild-type but not *Wld*^{S} mutant mice. Increase in truncated form of CRMP parallels the beading formation in degenerating neurites in SCGs (*16*). Thus, it is possible that CRMP cleavage is also involved in the process of neurite degeneration, and in turn, inhibits regeneration due to the loss of active form of CRMP. These findings suggest that injury-induced proteolytic cleavage of CRMP may act negatively on axonal regeneration. Hence, in order to explain the regenerative effects of CRMP, we must

focus on both activation (positive effect) and protein cleavage (negative effect) of CRMP.

The second messenger cyclic adenosine 3′,5′-monophosphate (cAMP) is another molecule involved in cytoskeletal rearrangement, and contributes to axon regeneration. Rolipram is a selective cAMP phosphodiesterase type IV inhibitor, and elevates the intracellular cAMP levels. It was shown that treatment with rolipram promotes both the regeneration of raphespinal tract neurons and functional recovery in SCI rats (*17*). In the downstream cascades, cAMP activates protein kinase A (PKA), which, in turn, restricts the inhibition of axonal growth by inactivating members of the Rho family of small GTPase proteins. RhoA and Rho kinase have emerged as negative regulators of the actin and tubulin polymerizations by a mechanism dependent on CRMP-2 inactivation (*6*,*10*). Treatment with C3 transferase, a Rho inhibitor, or the Rho kinase inhibitor, Y27632, causes axon regeneration in the injured spinal cord and promotes recovery of motor function (*18*). Thus, inhibition of Rho-Rho kinase pathway by the activation of cAMP-PKA signaling may also be useful to induce cytoskeletal changes that facilitate axon regeneration.

3. Molecular mechanism of gene expression and protein synthesis

A lack of gene expression and protein synthesis is thought to be responsible for the poor regeneration of neurons in the adult CNS. It is thus likely that transcriptional activation can be targeted so as to beneficially affect axon growth and functional recovery.

The cAMP-CREB-arginase 1 (Arg1) system is the most promising candidate target for approaches to promote axon regeneration by gene expression. A decrease in endogenous cAMP level has been found to coincide with a loss of the capacity of neurons to regenerate (*19*). This suggests that cAMP signaling may enhance the intrinsic regenerative capacity of adult CNS neurons. One of the downstream molecules of cAMP is the cAMP response element binding protein (CREB). It has been shown in cultured DRG neurons that the expression of constitutively active CREB overcomes the inhibitory effect of myelin-associated glycoprotein (MAG) on neurite growth (*20*). Moreover, in SCI rats, CREB activation is reported to promote the growth of dorsal column axons through the lesion site (*20*). It thus seems that cAMP-CREB signaling makes an important contribution to axon regeneration of the adult CNS. Activated CREB further regulates the transcription of many growth factor genes and facilitates an increased expression of the enzyme Arg1. Overexpression of Arg1 in cerebellar neurons can overcome the inhibition of axon growth by MAG and myelin, without elevation of cAMP (*21*). An increase in Arg1 expression (as induced by CREB) results in the synthesis of the polyamine putrescine, which is converted to spermidine. Administration of spermidine also promotes nerve regeneration after optic nerve crush (*22*). Thus, promotion of polyamine synthesis may be able to contribute to the axon regeneration. In addition, Arg1 levels in developing DRG neurons gradually decrease, that correlates with a decrease in endogenous cAMP level and the capacity for regeneration (*21*). From these findings, it appears that cAMP-CREB-Arg1 signal pathway and resulting polyamine synthesis are attractive targets to overcome factors that inhibit axon elongation.

It is thought that the GSK-3β pathway is also closely related to transcriptional system that enhances axonal growth. GSK-3β is inactivated by phosphatydilinositol 3-kinase (PI3 kinase) and extracellular signal-regulated kinase (ERK), both of which can increase protein systhesis to promote axonal growth. The deficiency of phosphatase and tensin homolog (PTEN), a positive regulator of PI3 kinase/mTOR (the mammalian target of rapamycin) pathway, enhances protein translation and promotes the regeneration of retinal ganglion cell (RGC) axons (*23*). Activation of ERK, which is involved in protein synthesis, induces the intrinsic growth state of adult corticospinal tract, thus promotes axon regeneration into subcortical lesion site (*24*). Although there seems to be few evidences, it might be possible that PI3kinase and ERK activations promote axon regeneration through inactivation of GSK-3β followed by protein synthesis. Indeed, it is reported that activated GSK-3β inhibits the phosphorylation and DNA-binding ability of CREB, a major promoter of protein synthesis (*25*). Thus, it is possible that activated GSK-3β limits the ability of CNS axons to regenerate by suppressing gene expression and protein synthesis.

4. Changes in the responsiveness to extracellular cues

Another aspect that should be investigated is the changes in the responses to the inhibitory environment, which is influenced by intracellular condition. The effects of environmental cues involved in axonal guidance are thought to be influenced by the intracellular levels of second messengers like cAMP and cyclic guanosine monophosphate (cGMP). Along these lines, several studies, which mainly focus on axonal growth in development, have investigated relationships between the responsiveness and cyclic nucleotide levels.

It has been shown that the repulsive response to recombinant proteins consisting of the extracellular domain of MAG can be elevated by cAMP level in *Xenopus* spinal axons (*26*), suggesting that cAMP elevations may modulate the response of neurons to environmental cues. Some of the most defined studies have focused on netrin-1, an axon guidance factor during development that promotes neurite elongation *via* deleted in colorectal cancer (DCC) receptor. Activation

of protein kinase A (PKA), a major downstream signal molecule of cAMP, enhances the DCC mobilization to the plasma membrane and increases axon extension in response to netrin-1 in cultured commissural neurons (*27*). Inhibition of RhoA that is inactivated by cAMP elevation also leads to the recruitment of additional DCC to the plasma membrane on the neurites of cultured explants of embryonic spinal cord (*28*). These findings implicate that elevation of cAMP level enhances the attractive responsiveness to extracellular cue, such as netrin-1, thus promotes neurite elongation. Hence, targeting of molecular mechanism that modulates the expression of guidance receptors may be an important approach for promoting the spontaneous regeneration of neurons.

cGMP level also affects the responses to extracellular cues. Sema3A, a guidance molecule, triggers the repulsive effect to cultured sensory neurons, and the repulsion can be converted to attraction by pharmacological activation of cGMP pathway (*26*). One major downstream targets of cGMP is cGMP-dependent protein kinase 1 (cGK1), which is activated by elevation of cGMP level. It was found in DRG explants of cGK1-deficient mice that raising cytosolic cGMP levels did not prevent the inhibitory effect of Sema3 (*29*), consistent with cGMP-cGK1 signaling being a main pathway that contribute to the change of responsiveness of neurons to extracellular cues. Although the role of cGMP in functional recovery after CNS injury in mammals has not yet been investigated, it has also been proposed that cGMP signaling is thought to play a key role in the regeneration of optic nerves in the goldfish (*30*). Further research on the role of cGMP in the response to inhibitory molecule would clarify the possible involvement of axon regeneration.

The results of several studies suggest that it is not simply their individual levels, but the ratio of cAMP to cGMP is also important for determining the response of extending axons to guidance molecules (*31*). This supports the likelihood of a variety of second messengers having crucial roles in the regulation the responses of the neuronal growth cones to extracellular cues. Although little is known about the molecular mechanisms that modulate the intrinsic response to extracellular cues by second messengers, research concerning the molecular mechanisms of intrinsic responsiveness of adult CNS neurons to extracellular cues may lead to the development of new therapies for CNS injuries.

5. Conclusions

While the extracellular environment of the adult CNS does not support axonal regeneration, a spontaneous partial regeneration of axons after CNS injury has been observed. This suggests that adult CNS neurons may have an intrinsic capacity to evoke mechanisms that promote spontaneous axonal regeneration after CNS injury. Adult neurons need appropriate regulation of cytoskeletal reorganization and protein synthesis to regenerate. In addition, changing the responsiveness of injured axon to chemoattractants may facilitate their regrowth. Extensive researches have revealed the intrinsic molecular mechanisms for axonal outgrowth. An understanding of how these powers are enhanced will contribute to the development of effective therapies for CNS injuries.

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Original Article

A decision analysis of the effectiveness of the pediatric telephone triage program in Japan

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Summary **Pediatric telephone triage programs have been initiated to reduce overcrowding of pediatric medical facilities, yet it is unclear what impact these programs have on reducing after hours room visits. This study used a decision analysis model and data from reference literature to investigate the effectiveness of this program and determine the factors that influence the results. The decision analysis model focused on a hypothetical group of children who were not undergoing ongoing treatment but developed symptoms or sustained injuries thought to require a nighttime visit to a medical facility. The model differentiated between urgent, semi-urgent, and non-urgent cases. Model parameters were estimated from the literature whenever possible. We estimated the difference in the number of children who received emergency medical attention, the number of semi-urgent cases in which attention was delayed, and the cases that did not receive medical care, between the telephone triage group and the control group. Telephone triage reduced the number of after hours visits by approximately 4%. There was no change in the number of semi-urgent cases for which medical care was delayed, and the number of semi-urgent cases that did not receive medical attention increased by three. The sensitivity analysis showed that increasing telephone triage use from 10% to 20% would reduce after hours visits by approximately 8%. We conclude that the benefits of this program to the region as a whole would outweigh the harmful effects. However, the effectiveness of telephone triage programs will be limited unless its usage is expanded.**

Keywords: Telephone triage, decision analysis, after-hours care

1. Introduction

In recent years Japan has experienced extreme overcrowding at pediatric medical facilities. There has been a shortage of pediatricians and a decrease in the number of pediatric medical institutions and parents' or guardians' child-care and health anxieties (*1*). The Japan Pediatric Society formulated the Pediatric Care Reform and Emergency Project (*2*) and established a website for parents about emergency pediatric care (*3*) in an attempt to eliminate the disorder of emergency pediatric care and compensate for the lack of manpower. The Ministry of Health, Labor and Welfare, acting in concert with the Japan Pediatric Society, carried out a preliminary survey beginning in 2002, and in 2004 initiated a pediatric emergency telephone triage program (*4*). Under this program, parents whose children are in need of emergency medical attention are instructed to dial the hotline number (#8000), and undergo telephone triage, during which pediatricians and nurses evaluate the need for emergency medical attention (*5*). As of June 2009, almost all prefectures in Japan (45/47 prefectures) had implemented this program.

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Based on reports that callers usually follow the triage pediatrician's instructions, and given the high satisfaction level of those using the program, it seems the pediatric emergency telephone triage program is effective (*6*). It is our hope that Japan will make greater use of this program in the future to further alleviate the overcrowding of pediatric emergency services. It remains unclear, however, to what degree this program contributes to a reduction in after hours visits including trips to emergency rooms. Therefore, to create a system that effectively utilizes the limited number of pediatricians and provides the necessary medical services to the appropriate people, we conducted this study with the following objectives in mind, conceived from the societal perspective of a regional health policy maker (*7*).

(i) In terms of the benefits of the pediatric emergency telephone triage program, we estimated the degree to which this program reduces the number of children visiting the after hours medical facility.

(ii) In terms of the harmful effects of the pediatric emergency telephone triage system, we estimated the degree to which medical attention was delayed as a result of the program.

(iii) In terms of the effectiveness of the program, we assessed the factors related to the pediatric emergency telephone triage system that most influence on reduction in the number of children visiting after hours medical facilities.

2. Methods

We performed decision analysis8 using data from the literature on this subject. The decision analysis model was determined through discussion with two pediatricians and two clinical epidemiologists. The statistical package DATA 3.5 (TreeAge Software, Inc., Williamstown, Massachusetts) was used for the analysis.

2.1. *Target population*

The target population of this study was a hypothetical group of children (age 0-15) who were not currently undergoing treatment and who developed symptoms or sustained injury thought to require emergency medical attention between 7:00-11:00 p.m. Sunday through Thursday, excluding holidays. At the time this study was initiated, these were the operating hours of most telephone consultation programs run at the prefectural level. Fridays and Saturdays were excluded because the time when regular medical attention is available differs on these days.

2.2. *Model parameters*

The model was developed based on the following 6 parameters:

(i) *Degree of urgency of child's symptoms*

The degree of urgency of a child's symptoms was classified as "urgent", "semi-urgent", and "non-urgent" based on the following definitions:

Urgent cases: Those requiring immediate examination and treatment (*i.e.* immediate attention is appropriate).

Semi-urgent cases: Those requiring examination and treatment as swiftly as possible (*i.e.* cases which should get medical attention as soon as possible).

Non-urgent cases: Those for which postponed examination and treatment is appropriate or for which further monitoring is adequate (*i.e.* cases which can be postponed until the following morning).

(ii) *After hours visits based on parents' discretion*

-In urgent cases, all parents will seek medical attention at emergency rooms.

-In semi-urgent and non-urgent cases, some parents will seek after hours medical care for their children, while the remainder wait and observe further.

-In cases that are being monitored and the symptoms worsen, emergency medical attention will be sought immediately.

(iii) *Changes in urgency during monitoring*

-When semi-urgent cases are monitored further, some become urgent or non-urgent.

-When non-urgent cases are monitored further, some become urgent or semi-urgent.

(iv) *Use of the pediatric emergency telephone triage program*

-In urgent cases, all parents will seek medical attention at emergency rooms rather than use the pediatric emergency telephone triage service.

-In semi-urgent and non-urgent cases, some parents will use the pediatric emergency telephone triage service while some will rely on their own judgment.

(v) *Classifying the urgency of symptoms during telephone triage*

-A pediatrician will handle the telephone consultations, and determine the degree of urgency.

-The telephone triage pediatrician will classify some semi-urgent cases as non-urgent, and some as urgent.

-The telephone triage pediatrician will classify some non-urgent cases as semi-urgent, and some as urgent.

(vi) *Compliance with telephone triage pediatrician's recommendation concerning medical care*

-The telephone triage pediatrician will recommend that all cases classified as semi-urgent seek emergency medical attention.

-The telephone triage pediatrician will encourage

all cases classified as non-urgent to follow up by monitoring progress until the next morning (including seeking regular medical attention the next morning).

-Some parents will follow the telephone triage pediatrician's recommendation to seek emergency medical attention while some will choose instead to monitor progress further.

-Some parents will follow the telephone triage pediatrician's recommendation to monitor the child's progress further while some will instead seek emergency medical attention.

2.3. *Outcome*

We used the model to evaluate the following outcomes:

(I): Number of children visiting the after hours medical facility: the number of children who were taken to the after hours medical facility, regardless of the urgency of their symptoms.

(II): Number of children for whom medical treatment was delayed: the number of children who should have received medical attention, but were monitored and worsened before receiving attention.

(III): Lost opportunities for appropriate medical attention: Number of children who should have received medical attention but did not, including semi-urgent cases that were monitored but did not receive treatment.

To determine the potential benefit of the telephone triage program, we compared the difference in (I) between the group using the telephone triage program and the control group (not using the program). To measure the potential harm, we found the difference in (II) and (III) between these two groups.

2.4. *Literature search*

To estimate the parameters in our model, we conducted a search of literature databases, including MEDLINE, the Japan Central Review of Medicine, and the Ministry of Health, Labor and Welfare. We searched each database for the terms "pediatrics," "emergency," "after hours," "out of hours," "nighttime," "telephone," and "triage," occurring together. In addition, for parameters for which it was not possible to find appropriate reference literature, we conducted a search of websites using Google (*http://www.google.co.jp*).

2.5. *Sensitivity analysis*

Each parameter used in the model was subject to sensitivity analysis by multiplying the value used in the basic analysis by 0.5 and 2.0 (for lower and upper limits) or by varying the parameter value within the appropriate range determined from the literature or author discussions

3. Results

3.1. *Decision tree* (Figure 1)

The figure displays a decision tree based on the results of discussion by the authors, taking into consideration the consistency of actions to seek medical attention in actual pediatric emergencies.

3.2. *Parameter estimation* (Table 1)

(i) *Proportion of degree of urgency of child's symptoms*

We could not find any studies directly measuring the proportions of urgent, semi-urgent, and non-urgent emergencies occurring within the region. We therefore made the following estimates based on surveys of residents regarding pediatric emergency treatment (*9*) and a study of the level of urgency of pediatric patients at sites of pediatric emergency care (*10*), which we took as relevant reference literature.

First, based on the survey of area residents (*9*), we divided cases based on the response to the onset of symptoms (including external injury) which were thought to need emergency medical attention at a medical facility into those that received medical attention (62.0%) and those that did not (38.0%). Then, based on the study of the degree of urgency of pediatric patients at emergency treatment facilities (*10*), we divided cases into three categories: urgent cases (62.0% × 4.3% = 2.7%), semi-urgent cases (62.0% × 23.9% = 14.8%), and non-urgent cases (62.0% × 1.8% = 44.5%). Finally, we defined non-urgent cases as the sum of cases that did not receive medical attention and those cases that did receive attention that were classified as non-urgent (38.0% + 44.5% = 82.5%).

The two studies were not limited to cases occurring during night, and the each urgency category described above were assumed to be consistent throughout the whole day. We found no data suggesting that the proportion of each urgency category varied significantly between day and night, so this hypothetical proportion was set.

(ii) *Rate of after hours visits based on parents' discretion*

We were unable to identify any studies reporting this figure. The authors discussed this and assumed the percentage was 90% for semi-urgent cases and 60% for non-urgent cases.

(iii) *Chance of change in urgency during monitoring*

We were unable to identify any studies reporting this figure. The authors discussed and hypothesized that there was a 10% chance of a case changing from one category to the next category, in the order of urgent ↔ semi-urgent ↔ non-urgent. Furthermore, there was a 1% chance of a case changing by two categories.

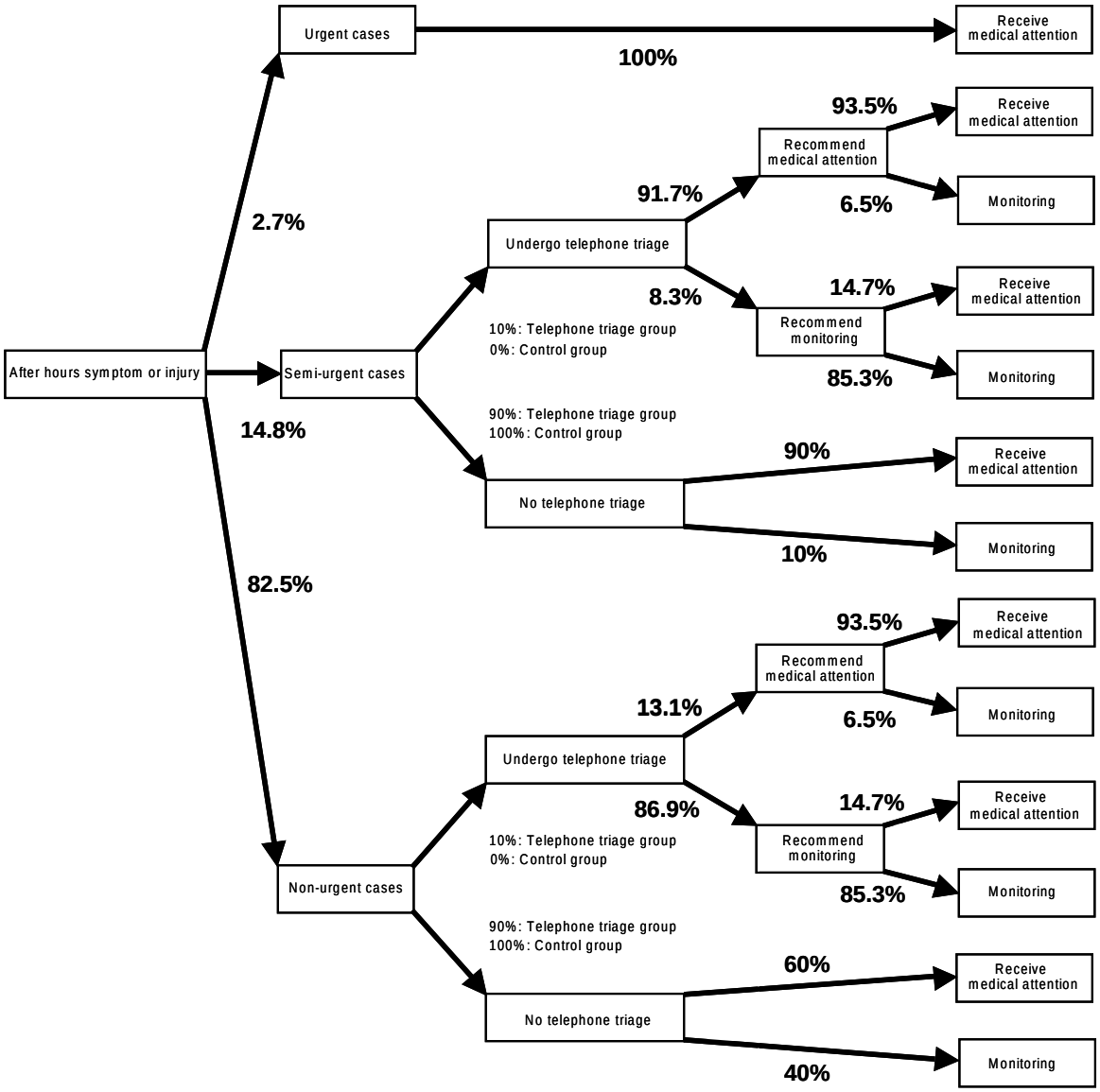

Figure 1. Decision tree.

(iv) *Rate of use of the pediatric emergency telephone triage program*

We were unable to identify any studies reporting this figure. Following discussion among the authors, we set the value at 10% in both semi-urgent and non-urgent cases, as parents who would use it would want to know after hours visit was not only necessary but also unnecessary.

(v) *Accuracy of classification of symptom urgency by telephone triage pediatricians*

We were unable to identify any studies reporting this figure. The authors discussed and hypothesized that 1 out of 10 cases might be misclassified into the next category, in the order of urgent ↔ semi-urgent ↔ non-urgent. Furthermore, 1 case out of 20 may have been misclassified by two categories (*e.g.* an non-urgent case misclassified as urgent).

(vi) *Rate of compliance with telephone triage pediatrician's recommendation concerning medical care*

There were two studies (*11*,*12*) reporting the rate at which parents follow the recommendations given by physicians during telephone triage. One study (*11*) was a randomized clinical trial that compared the results of telephone triage by physicians and nurses, but because over half of the participants dropped out of the study at the stage at which consent was to be obtained, we used the results of the remaining cohort study (*12*). In this study, the advice regarding medical care was divided into three categories: emergency medical attention, examination the following day, and telephone advice alone. The rate at which participants in this study followed recommendations to seek emergency medical treatment was used as the rate of compliance with the recommendation to visit the medical facility in our simulation, and the rates at which participants

followed advice to get an examination the following day or only receive advice on the telephone were collectively taken as the rate at which parents complied with recommendations for further monitoring and follow up.

3.3. *Basic analysis* (Table 2)

Of 10,000 cases arising outside regular hours, we estimated that 8,251 were non-urgent. In the control group, there were 6,757 after hours visits. In the telephone triage group, 973 cases underwent telephone triage, and the number of after hours visits was reduced by 273 compared to the control group, which corresponds to 4% of the control group's after hours visits. There was no increase or decrease in the number of semi-urgent cases for which medical attention was delayed, and the number of semi-urgent cases that did not receive attention increased by three.

3.4. *Sensitivity analysis* (Table 3)

If the rate of use of the telephone triage program varied

Table 1. Basic variable data

Variable	Value	Reference
Urgency of child's symptoms		
Urgent cases	2.7%	References *9*,*10* (See main text)
Semi-urgent cases	14.8%	
Non-urgent cases	82.5%	
Rate of after hours visits based on parents' discretion		
Urgent cases	100.0%	Definition
Semi-urgent cases	90.0%	Hypothesis
Non-urgent cases	60.0%	Hypothesis
Changes in urgency		
Semi-urgent cases		
Change to urgent	5.0%	Hypothesis (See main text)
Remain semi-urgent	90.0%	
Change to non-urgent	5.0%	
Non-urgent cases		
Change to urgent	1.0%	Hypothesis (See main text)
Change to semi-urgent	5.0%	
Remain non-urgent	94.0%	
Usage rate of pediatric emergency telephone triage program	10.0%	Hypothesis
Accuracy of triage pediatrician' classification of symptom urgency		
Semi-urgent cases		
Classified as urgent	8.3%	Hypothesis (See main text)
Classified as semi-urgent	83.4%	
Classified as non-urgent	8.3%	
Non-urgent cases		
Classified as urgent	4.4%	Hypothesis (See main text)
Classified as semi-urgent	8.7%	
Classified as non-urgent	86.9%	
Parents' compliance vis-à-vis recommendation about medical attention		
Recommend medical attention		
Compliance	93.5%	Reference *12*
Non-compliance	6.5%	
Recommend monitoring		
Compliance	85.3%	Reference *12*
Non-compliance	14.7%	

Table 2. Basic analysis (10,000 after hours cases)

Outcome	Telephone triage		Difference
	Yes	No	
No. of cases by urgency			
Urgent cases	267	Same	–
Semi-urgent cases	1,482	Same	–
Non-urgent cases	8,251	Same	–
Telephone triage cases	973	0	+973
After hours visits	6,484	6,757	-273
Urgent cases (reshown)	310	307	+3
Semi-urgent cases (reshown)	1,510	1,499	+11
Non-urgent cases (reshown)	4,664	4,951	-287
No. of semi-urgent cases that became urgent and received medical attention	7	7	+/-0
No. of semi-urgent cases that did not get attention	137	134	+3

Table 3. Sensitivity analysis (10,000 after hours cases)

Parameter	Parameter range of values (Lower limit-basic analysis-upper limit)	Outcome	Lower limit	Basic analysis	Upper limit
Rate of telephone triage usage (%)	5-10-20	# of after hours visits	-137	-273	-546
Rate of medical attention received based on guardian's discretion, non-urgent cases (%)	30-60-90	# of after hours visits	-40	-273	-505
Rate of medical attention received based on guardian's discretion, semi-urgent cases (%)	45-90-100	# of after hours visits	-209	-273	-287
Accuracy of classification of symptom urgency, semi-urgent cases classifi ed as non-urgent (%)	4.5-8.3-14.3	# of after hours visits	-298	-273	-238
Proportion of urgency level, semi-urgent cases (%)	7.4-14.8-29.6	# of after hours visits	-297	-273	-253
Rate of telephone triage usage (%)	5-10-20	# of semi-urgent cases that became urgent and got medical attention	+/-0	+/-0	+/0
Rate of telephone triage usage (%)	5-10-20	# of semi-urgent cases that did not receive medical attention	+/-0	+3	+6

from 5% to 20%, then the reduction in after hours cases would vary widely from approximately 2% (137/6,757 cases) to 8% (546/6,757 cases).

When the percentage of after hours visits for non-urgent cases based on parent discretion is changed from 30% to 90%, the reduction in after hours visits varies from approximately 0.6% (40/6,757 cases) to approximately 7% (505/6,757 cases).

Changing the percentage of after hours visits for semi-urgent cases based on parent discretion changed the number of after hours visits, but that variation was more limited than of the variation for non-urgent cases. On the other hand, the number of semi-urgent cases that did not receive medical attention changed greatly, and the rate of medical visits for semi-urgent cases based on parents' discretion was below 87.0%, which was lower for the telephone triage group than the control group (data not shown).

4. Discussion

By carrying out telephone triage in roughly 1,000 cases, we found that approximately 30% (273 cases) avoided after hours visits. Therefore, the effectiveness of telephone triage in reducing after hours visits was not extremely high. Most of this reduction in after hours visits is attributable to the fact that, as a result of telephone triage, non-urgent cases are directed to monitor developments further, thus avoiding the need for immediate medical attention. The accuracy of urgency classification during telephone triage, compliance with recommendations to continue monitoring the situation, and changes in the degree of urgency during monitoring all had a cumulative impact on the effectiveness of the program. Additionally, some of the monitoring cases received postponed medical attention the following morning, so the overall reduction in medical facility visits, during and outside of regular hours, may be smaller than that shown in the results.

Based on the hypothesis that the rate of use of telephone triage is 10%, only about 4% (273/6,757 cases) of after hours visits were avoided. This result suggests that the impact of this program as a means of easing the overcrowding of emergency medical facilities is slight. If the rate of use is increased, the effect will be greater; for instance, if all after hours cases other than urgent ones first undergo telephone triage, approximately 40% of those currently coming in for emergency attention would not do so, and we could expect emergency medical facility overcrowding to be decreased considerably.

A wide variation in assessment of emergency levels by each triage pediatrician participating in the running program may exist due to a lack of standard telephone triage training required for its participation. Extreme variation in emergency level assessment would therefore lead to a diminution of the effect of reducing after hours visits. However, because there were no figures for accuracy of the assessment, our decision model utilized an estimate of the average figures.

The increase in semi-urgent cases that do not receive attention, which is a negative outcome of telephone triage, was only three cases out of the 1,000 that underwent telephone triage. This is a comparatively small number. In addition, this increase in semi-urgent cases that did not receive medical attention disappeared when the rate of medical visits based on the discretion of parents declined slightly (from 90.0% to 87.5%). Therefore, although it depends on one's perspective in comparing the benefits of the telephone triage program (reduction in after hours visits) with its potential harm (delay in medical attention), we think that the harm remains within an acceptable range and the benefits are greater.

This study is only an estimate based on a hypothetical model, and it is important to do an actual outcome survey. It is important to confirm that life-threatening outcomes will not occur for semi-urgent cases left without medical care until the start of regular hours of operation the following day (at most, 14 hours from 7 p.m. to 9 a.m. the following morning). It is necessary to consider the possibility that such things would cancel telephone triage's potential benefit of reducing the use of

emergency services.

Among the potential benefits of the telephone triage program that we were unable to consider in this study are the enhanced peace of mind that offering the telephone triage program gives to parents and guardians (*6*), and the fact that reducing the number of emergency medical facilities patients makes it possible for pediatricians to give medical care to children with more serious ailments. The peace of mind of parents and guardians is difficult to measure, as are the benefits to the group, but these are important aspects that cannot be ignored.

There are several hurdles that must be cleared before telephone triage can be used more widely. First, there is the issue of finding pediatricians to participate in telephone triage. The results of this study show that to increase the effect of telephone triage on the reduction of emergency medical service use, it is necessary to carry out multiple triages. If pediatricians handle all of these telephone triages, then it will be necessary to hire a very large number of pediatricians, and realistically this is an extremely difficult proposition. In a survey of pediatricians in Hiroshima who took part in telephone triage, some reported that "taking part in telephone triage in addition to our normal work feels like a burden" (*13*). Therefore, rather than having pediatricians handle all telephone triage, there are programs being developed in Japan (*14*) to cultivate trained triage nurses like those in the U.S.(*15*). We think that the use of such triage nurses for telephone triage should be explored.

In addition, current telephone triage programs are run by individual prefectures. However, there is no particular reason why these programs should be provided at the prefectural level, and we think it may be more efficient, in terms of the allocation of triage personnel, to have one or several triage centers nationally.

On the other hand, if the telephone triage program is to be made more widely available, the issue of cost cannot be ignored. It would be best if a study of the telephone triage program's cost effectiveness was carried out swiftly, and the health service provider (the national government, prefecture, *etc.*) used these results as a reference in prioritizing it along with other health services (*12*).

The present study has several limitations. First, there is the great shortage of data estimating the variables used in the model. Therefore, there is the possibility that the absolute values of the reduction of after hours visits through telephone triage will vary widely, as shown in sensitivity analysis. In addition, there is the fact that we were unable to integrate both the beneficial and harmful effects of telephone triage.

In conclusion, we think that the pediatric emergency telephone triage service is a valuable health service with benefits that outweigh the possible harmful effects, but unless the use of the program is expanded, it is likely that the effectiveness in reducing after hours medical visits will be limited.

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Original Article

Smoking intensity, oxidative stress and chemotherapy in non-small cell lung cancer: A correlated prognostic study

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Summary **Cigarette smoking is a well known environmental risk factor for lung cancer; furthermore it can also enhance lung carcinogenesis by free radical mediated reactions. In addition smoking affects the rates of metabolism of several drugs and may contribute to poor cancer survival. The purpose of the present work, therefore, was to see the relationship of different smoking intensities with oxidative stress and survival after platinum based chemotherapy in non-small cell lung cancer (NSCLC). The oxidative stress levels (LPO, NO, SOD, and GSH) of 144 control subjects and 203 advanced stage NSCLC patients were assessed at day '0', after the 3rd and 6th cycle of chemotherapy. Pack year (PY) was stratified in groups (1-20, 21-50, > 50) for further analysis. Groups were compared using repeated measured ANOVA, while survival curves were compared by Kaplan-Meier methods. Oxidative stress levels of smokers were significantly high ($p < 0.01$ or $p < 0.05$) as compared to non-smoker at pretreatment, after the 3rd cycle and 6th cycle of chemotherapy but not well correlated with the PY exposures. Overall mean survival of smoker patients were significantly low when compared to non-smokers. The survival of > 50 PY group was significantly lowered ($p < 0.01$) as compared to others PY groups, indicating that survival after chemotherapy in smoker NSCLC patients may be dependent on their PY exposures. In conclusion, smoking is a bad prognostic factor in lung cancer therapy, besides its role in oxidative stress, and poor survival. Therefore, this factor can be used in patient selection for chemoprevention.**

Keywords: Lung cancer, smoking intensity, pack year, oxidative stress, prognostic factor

1. Introduction

Lung cancer is strongly associated with exposure to environmental carcinogens, with the highest risk being from cigarette smoking (*1*). Smoking is the most important risk factor for lung cancer, as supported by epidemiologic evidence since the 1950s (*2*,*3*). Thirty percent of all cancer is caused by smoking and approximately 85-90% of lung cancer cases are attributed to it (*4*,*5*).

Cigarette smoke (CS) can be divided into two phases; the gaseous phase and particulate matter (tar). Both phases are harmful, containing high concentration of toxic and carcinogenic compounds (*6*) and are both associated with diverse pulmonary disorders, including cancers. Although it is well established that tar contains a large number of carcinogens, previous studies suggest that chemicals in the gaseous phase of tobacco smoke are

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of major importance in the cytotoxic and carcinogenic effects of tobacco on bronchopulmonary epithelial cells (*7,8*). At present it is well known that for these lesions to occur both phases of tobacco smoke are required (*9*).

Furthermore, cigarette smoke is a major public health hazard which exposes the respiratory tract to substantial oxidative stress. Benzo(a)pyrene, one of the most representative carcinogens of tobacco smoke, may be the origin of free radical derivatives (*10*). One puff from a cigarette contains approximately 10^{14} oxidant radicals in the combined gaseous and particulate states. Cigarette smoke contains over 4,700 chemical compounds, a high concentration of oxidants (10^{14} molecules/puff), and 3,000 ppm NO/puff (*11*). The nature of oxidant species found within CS varies from short lived oxidants, such as super oxide radical (O_2^-) and the nitric oxide molecule (NO), to long lived organic radicals, such as, semiquinones that can undergo redox cycling within the epithelial lining fluid of smokers for an extended amount of time (*12,13*).

It was also seen that formation of free radicals and consequent lipid peroxidation (LPO) has been associated with lung cancer (*14*). The principal radical in the tar phase, a quinine/hydroquinone complex, is capable of reducing molecular oxygen to superoxide radicals (*15*). The gas phase of CS contains small oxygen and carbon centered radicals that are much more reactive than are all tar phase radicals (*11*). The major antioxidant in lung lining fluid is reduced glutathione (GSH) (*16*). It can also be found intracellularly and at lower concentrations in plasma. It is a powerful scavenger of both reactive oxygen and nitrogen species and can protect proteins against nitration, particularly from the nitrogen dioxide radical, nitrous oxide found in cigarette smoke (*17*).

In addition, smoking is associated with factors that may contribute to poor cancer survival (*18*). There is now strong evidence that lung cancer in non-smokers shows different patterns than those observed in smokers (*19,20*). Several studies have also shown a positive association between smoking status and shortened survival after treatment (*21-24*).

Previous studies indicated that lower smoking intensity is associated with favorable overall survival of non-small cell cancer (*25,26*). This is because smoking affects the rates of metabolism for several drugs. It was also seen that lung functions in smokers may be more chemo resistant (*4*). The resistance of cancer cells to anticancer drugs is a serious clinical problem encountered in the chemotherapy (CT) of lung cancer patients.

We hypothesized that because smoking is a bad prognostic factor in lung cancer therapy, besides its role in oxidative stress and lung cancer genesis, that this factor can be used in patient selection for chemoprevention. The aim of the present work was to study the relationship of different smoking intensity with oxidative stress and survival after platinum based chemotherapy in non-small cell lung cancer patients.

2. Materials and Methods

2.1. *Patient characteristics*

Two hundred and three non-small cell lung cancer (NSCLC) patients (age, range 30-88 years) (155 males, 48 females) previously untreated, histologically or cytologically confirmed, admitted to the Department of Pulmonary Medicine, Chhatrapati Shahuji Maharaj Medical University, (C.S.M.M.U) Lucknow, India, and 144 age and sex matched healthy subjects (Control) were recruited for the study from October 2006 to December 2008. Eligible patients had an eastern cooperative oncology group (ECOG) performance status (PS) of 0, 1, 2, and 3. Chest radiographs and computed tomography for staging, sputum cytology, lavage examination, bronchoscopic biopsy, fine needle aspiration biopsy and cytology (if required) were performed in all lung cancer patients for histological diagnosis (adenocarcinoma, squamous cell carcinoma, large cell, and others), stage (IIIA, IIIB, and IV), and site of lesion.

Smoking data were recorded for all cases. All subjects were systematically interviewed through a standard questionnaire about their current and life time smoking status; detailed data were obtained about tobacco consumption, including: smoking start age, current smoking status, duration, intensity, amount of pack year of smoking, and time since quitting were noted. The patients were classified into two categories based on cigarette smoking status: non-smoker, *i.e*. patients who had never smoked or smoked less than 100 cigarettes in their life time; and smokers, *i.e*. patients who smoked and who continued smoking. The patients who had stopped smoking recently were considered as current smokers. Patients who had quit smoking for more than 1 year were considered former smokers. Smoking intensity of the smokers was calculated as pack year (PY) smoked. The total number of pack year was calculated by multiplying the number of packs smoked a day by the number of years of regular cigarette smoking. To explore whether smoking intensity at diagnosis is an independent prognostic factor, the smallest significant cutoff smoking intensity was identified by patients' stratification. Patients were stratified into 3 groups (1-20, 21-50, and > 50) by different smoking intensity cutoff values in the analysis. In our study the use of a cutoff point of PY was based on a previous study (*26*).

Patients received cisplatin (50-75 mg/m^2 of body surface area) divided into 3 doses on day 1, 2, and 3 and etoposide (70-100 mg/m^2 of body surface area) on day 1, 2 and 3 repeated every 3 weeks for a maximum of six cycles. Date of therapy initiation, date of therapy discontinuation, date of death, date of last follow-up, and status at last follow-up were recorded. The survival time was defined as the interval between the date of initial treatment and the date of last follow-

up examination. Patients who were deceased were calculated from the last date they were known to be alive based on the date of last contact. This date was verified by inpatient and outpatient medical records, and/or confirmation with the patient's primary care physician and/or family. Details of demographic characteristics of patients are given in Table 1.

The study protocol was approved by the ethical committee of Chhatrapati Shahuji Maharaj Medical University Lucknow, Uttar Pradesh, India (vide communication, *ref.* code- XXII ECM/P9). Before enrolment, written informed consent from each subject was obtained.

2.2. *Biochemical assay*

Blood specimens (5 mL) were aseptically drawn in EDTA prior to initiation of each chemotherapy course (first day). Blood samples were again collected after the 3rd and 6th cycle of chemotherapy for oxidative stress measurement. Levels of LPO, NO, GSH, and SOD were assessed at pretreatment (day '0') and post treatment (after 3rd and 6th cycle). Hemolysate was prepared using the method of Beutler *et al*. (*27*). The baseline (day '0') values of all these biochemical parameters were also assessed in 144 control subjects.

Total amount of lipid peroxidation products was estimated using the thiobarbituric acid (TBA) method, which measured the malondialdehyde (MDA)-TBA complex (*28*). The intensity of pink color of the product was read at 532 nm. Results were expressed as nmol MDA/mL. Activity of superoxide dismutase was determined by the method of McCord and Fridovich (*29*). The xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion reduces nitroblue tetrazolium (NBT) to formazan, which was monitored at 560 nm. The level of this reduction was used as a measure of SOD activity. The reduced glutathione (GSH) level was determined using the method of Ellman *et al*. (*30*). The technique involved protein precipitation by metaphosphoric acid, and a spectrophotometric assay at 412 nm of the yellow derivative obtained from the reaction of supernatant with 5,5'-dithio-bis-2-nitrobenzoic acid. Plasma nitrite levels were measured with the method of Green *et al*. (*31*), using Griess reagent (sulfanilamide and *N*-(1-naphthyle)ethylenediamine). The method is based on a two step process. The first step is the conversion of nitrate to nitrite using nitrate reductase. The second step is the addition of Griess reagent that converts nitrite into a deep-purple azo compound. Photometric measurement of the absorbance at 540 nm of this azochromophore accurately determines the nitrite concentration (sodium nitrate is used as a standard) using a Microlab 300 semi-automated clinical chemistry analyzer (vital scientific) Merck, The Netherlands.

2.3. *Statistical analysis*

Independent groups were compared by using one way analysis of variance (ANOVA) while independent and dependent groups were compared with two factor repeated measures ANOVA followed by Newman-Keuls post hoc test. The Kaplan-Meier methods (Log rank test and Cox proportional hazard ratio) were used to compare survival between groups. A two-tailed ($\alpha = 2$), probability (p) value $p < 0.05$ was considered to be statistically significant. Graph Pad Prism (version 5) and STATISTICA (version 7) were used for the analysis.

For easy interpretation of the data, the percent mean change (from baseline to final evaluation) of one group over another was also evaluated as

$$\text{Mean change (\%)} = \frac{\text{Mean}_1 - \text{Mean}_2}{\text{Mean}_1} \times 100$$

where Mean_1 and Mean_2 denote means of 1st and 2nd groups, respectively.

Table 1. Demographic characteristics of patients

Characteristics	Number (%)
No. of patients	203 (100 %)
Sex - Male : Female	155 (76.4%): 48 (23.6%)
Smoker : Non-smoker	141 (69.5%): 62 (30.5%)
Pack year (PY) smoked , Mean ± SD, Median, Range	36 ± 26.1, 30, 3-162
PY-1-20 : PY-21-50 : PY- > 50 (n = 141)	36 (25.5%): 80 (56.8%): 25 (17.7%)
Age (Years)- Median, Range	55, 30-88
ECOG performance status- 0 : 1: 2	40 (19.7%): 107 (52.7%): 56 (27.6%)
Disease stage- IIIA : III B : IV	15 (7.4%): 142 (70.0%): 46 (22.7%)
Histological type- SCC: AC: LCC: O	82 (40.4%): 57 (28.1%): 27 (13.3%): 37 (18.2%)
Follow-up time (weeks)	88

Abbreviations: ECOG, Eastern co-operative oncology group performance status; SCC, squamous cell carcinoma; AC, Adenocarcinoma; LCC, large cell carcinoma; O, others (mixed).

3. Results

3.1. Oxidative stress levels of normal healthy subjects and non-smoker and smoker NSCLC patients at baseline

The baseline (pretreatment) oxidative stress levels of LPO, NO, GSH and SOD of normal healthy subjects (control) and non-smoker and smoker NSCLC patients (patients) are summarized in Table 2. Table 2 showed that pretreatment mean levels of LPO and NO in patients were comparatively high while the levels of GSH and SOD were comparatively low as compared to respective levels of control subjects and the increase and decrease were high in smokers as compared to non-smokers.

On comparing the mean level of LPO and NO in both non-smoker and smoker NSCLC patients were found to be significantly different and higher ($p < 0.01$) while the level of GSH and SOD were significantly lower ($p < 0.01$) as compared to control (Table 2). Similarly, the mean level of LPO and NO in NSCLC smoker patients were also found to be significantly higher ($p < 0.05$) while the level of GSH and SOD were significantly lower ($p < 0.05$) as compared to NSCLC non-smoker patients (Table 2).

Table 2. Baseline (pretreatment) biochemical parameters of normal healthy subjects (control) and NSCLC patients

Parameters	Control (n = 144)	NSCLC patients	
		Non-smoker (n = 62)	Smoker (n = 141)
LPO	2.24 ± 0.47	6.24 ± 0.92[a]	6.45 ± 0.70[a,b]
NO	11.88 ± 3.48	25.84 ± 4.58[a]	26.09 ± 5.58[a,b]
GSH	11.77 ± 1.30	3.20 ± 1.02[a]	3.01 ± 1.00[a,b]
SOD	2.89 ± 0.48	1.49 ± 0.61[a]	1.38 ± 0.56[a,b]

Data are shown as mean ± SD; [a] $p < 0.01$ (control *vs*. non-smoker or smoker); [b] $p < 0.05$ (non-smoker *vs*. smoker).

3.2. Oxidative stress levels of non-smokers and smokers NSCLC patients before and after chemotherapy

The oxidative stress levels of LPO, NO, GSH, and SOD in non-smoker and smoker NSCLC patients before (pretreatment or 0 cycles) and after 3rd and 6th cycles of chemotherapy are summarized in Table 3.

Comparing the levels within the groups (between cycles) (Table 3), the mean level of LPO and NO in both the non-smoker and smoker NSCLC patients at 3rd and 6th cycles were found to be significantly higher ($p < 0.01$) as compared to their respective pretreatment (0 cycle) levels. The respective levels of these in both the non-smoker and smoker NSCLC patients at 6th cycles were also found to be significantly higher ($p < 0.01$) as compared to their respective levels at 3rd cycle. However, the mean levels of GSH and SOD in both the non-smoker and smoker NSCLC patients at 3rd and 6th cycles were found to be significantly lower ($p < 0.01$) as compared to their respective pretreatment levels. The respective levels of these in both the non-smoker and smoker NSCLC patients at 6th cycles were also found to be significantly lower as compared to their respective levels at 3rd cycle.

Similarly, comparing the levels between the groups (non-smoker *vs*. smoker), the mean level of all oxidative stress parameters between the two groups at pretreatment were found to be the same, that is, levels did not differ significantly ($p > 0.05$) while at 3rd and 6th cycles these differed significantly (either $p < 0.05$, $p < 0.01$).

The percent mean change (0 cycles-6th cycles) (Table 3, last column) also showed more aggragation in smoker NSCLC patients than the non-smoker NSCLC patients. The LPO, NO, GSH, and SOD in NSCLC smoker patients aggragated (*i.e*. ratio of percent mean change of non-smoker and smoker) by a factor of 1.2, 1.2, 1.2, and 1.3 times more respectively than the non

Table 3. Biochemical parameters of non-smoker and smoker NSCLC patients before and after 3rd and 6th cycles of chemotherapy

Variables	Patients	At '0' cycle	After 3rd cycle	After 6th cycle	Mean change (%)
LPO	Non-smokers	6.24 ± 0.92 (62)	6.70 ± 0.92[a] (61)	7.22 ± 0.88[a,b] (53)	7.2
	Smokers	6.45 ± 0.70 (141)	7.04 ± 0.69[a] (131)	7.68 ± 0.60[a,b] (92)	8.3
NO	Non-smokers	25.84 ± 4.58 (62)	30.24 ± 4.25[a] (61)	31.83 ± 3.97[a,b] (53)	5.0
	Smokers	26.09 ± 5.58 (141)	32.01 ± 5.86[a] (131)	33.75 ± 4.53[a,b] (92)	5.2
GSH	Non-smokers	3.20 ± 1.02 (62)	2.53 ± 0.99[a] (61)	2.04 ± 0.92[a,b] (53)	7.2
	Smokers	3.01 ± 1.00 (141)	2.23 ± 0.86[a] (131)	1.70 ± 0.67[a,b] (92)	8.3
SOD	Non-smokers	1.49 ± 0.61 (62)	1.10 ± 0.55[a] (61)	0.98 ± 0.56[a,b] (53)	12.1
	Smokers	1.38 ± 0.56 (141)	0.95 ± 0.29[a] (131)	0.78 ± 0.20[a,b] (92)	23.0

Data are shown as mean ± SD. Values in parentheses are number of patients; [a] $p < 0.01$ (0 cycle *vs*. 3rd cycle or 6th cycle); [b] $p < 0.01$ (3rd cycle *vs*. 6th cycle).

smoker NSCLC patients. In other words, oxidative stress was well correlated with the patient exposures (non smoking and smoking).

3.3. *Pack year wise oxidative stress levels of smokers NSCLC patients before and after chemotherapy*

The pack year wise oxidative stress levels of LPO, NO, GSH, and SOD in smoker NSCLC patients before and after 3rd and 6th cycles of chemotherapy are summarized in Table 4.

Comparing levels within the groups (between cycles) (Table 4), the mean level of LPO and NO in all pack year groups of smoker NSCLC patients increased significantly ($p < 0.01$) after 3rd and 6th cycles of chemotherapy as compared to their respective pretreatment (0 cycle) levels. The respective levels of these were also significantly higher ($p < 0.001$) at 6th cycle as compared to their respective levels at 3rd cycle. Similarly, the mean levels of GSH and SOD in all pack year groups of smoker NSCLC patients decreased significantly ($p < 0.001$) after 3rd and 6th cycles of chemotherapy as compared to their respective pretreatment levels. The respective levels of these were also significantly lower ($p < 0.05$ or $p < 0.001$) at 6th cycle as compared to their respective levels at 3rd cycle.

Similarly, comparing the levels between the groups, except SOD, the mean level of all oxidative stress parameters in all three periods were found to be the same, that is, levels did not differ significantly ($p > 0.05$). The mean SOD of pack year > 50 at 0 cycle and 3rd cycle was found to be significantly higher ($p < 0.05$) than the pack year 1-20 and 21-50 groups.

The percent mean change (0 cycles-6th cycles) (Table 4, last column) of all oxidative stress parameters did not show any trend with pack year groups. In other words, oxidative stress did not correlate significantly with pack year exposures.

3.4. *Survivals*

The two years (or 88 wks) overall survival of all NSCLC patients (smoker + non-smoker), between non smoker and smoker NSCLC patients and among smoker NSCLC patients (pack year wise) has been summarized graphically in Figure 1.

The overall median survival of all NSCLC patients was found to be 28.50 wks (Figure 1a). The overall median survival of smoker NSCLC patients was significantly lower (Log rank test: $\chi^2 = 12.86$; $p < 0.01$) as compared to non-smoker NSCLC patients and the death rates in smoker NSCLC patients were also 0.22 times higher (Hazard ratio: ratio = 0.22; 95% CI of ratio = 0.19 to 0.61) than the death rate of non-smoker NSCLC patients (Figure 1b). Similarly, the overall median survival among different pack year groups of smoker NSCLC patients differed significantly (Log rank test: $\chi^2 = 27.53$; $p < 0.01$) with each other (Figure 1c). The survival of pack year > 50 smoker NSCLC patients was significantly lower as compared to the other pack year groups. Though the survival of pack year 21-50

Table 4. Pack year wise biochemical parameters of smoker NSCLC patients before and after 3rd and 6th cycles of chemotherapy

Parameters	Pack year	At '0' cycle	After 3rd cycle	After 6th cycle	Mean change (%)
LPO	1-20	6.44 ± 0.75 (36)	6.99 ± 0.75[a] (36)	7.82 ± 0.65[a,c] (27)	17.6
	21-50	6.46 ± 0.69 (80)	7.09 ± 0.64[a] (74)	7.67 ± 0.51[a,c] (58)	15.8
	> 50	6.46 ± 0.68 (25)	6.94 ± 0.79[a] (21)	7.25 ± 0.89[a,d] (7)	11.0
NO	1-20	25.88 ± 6.31 (36)	31.61 ± 6.35[a] (36)	34.39 ± 5.42[a,c] (27)	24.8
	21-50	26.28 ± 5.57 (80)	31.63 ± 5.80[a] (74)	33.25 ± 4.21[a,c] (58)	21.0
	> 50	25.76 ± 4.57 (25)	34.04 ± 4.96[a] (21)	35.49 ± 2.82[a,d] (7)	27.4
GSH	1-20	3.11 ± 1.07 (36)	2.20 ± 0.90[a] (36)	1.60 ± 0.65[a,c] (27)	48.4
	21-50	2.96 ± 0.97 (80)	2.31 ± 0.85[a] (74)	1.73 ± 0.68[a,c] (58)	41.5
	> 50	3.03 ± 1.04 (25)	1.99 ± 0.80[a] (21)	1.84 ± 0.68[b,d] (7)	39.1
SOD	1-20	1.32 ± 0.47 (36)	0.93 ± 0.24[a] (36)	0.81 ± 0.21[a,c] (27)	39.1
	21-50	1.30 ± 0.53 (80)	0.92 ± 0.24[a] (74)	0.76 ± 0.18[a,c] (58)	41.5
	> 50	1.73 ± 0.63 (25)	1.13 ± 0.43[a] (21)	0.79 ± 0.37[a,c] (7)	54.0

Data are shown as mean ± SD. Values in parentheses are number of patients; [a] $p < 0.01$ (0 cycle *vs*. 3rd cycle or 6th cycle); [b] $p < 0.05$ (0 cycle *vs*. 6th cycle); [c] $p < 0.01$ (3rd cycle *vs*. 6th cycle); [d] $p < 0.05$ (3rd cycle *vs*. 6th cycle).

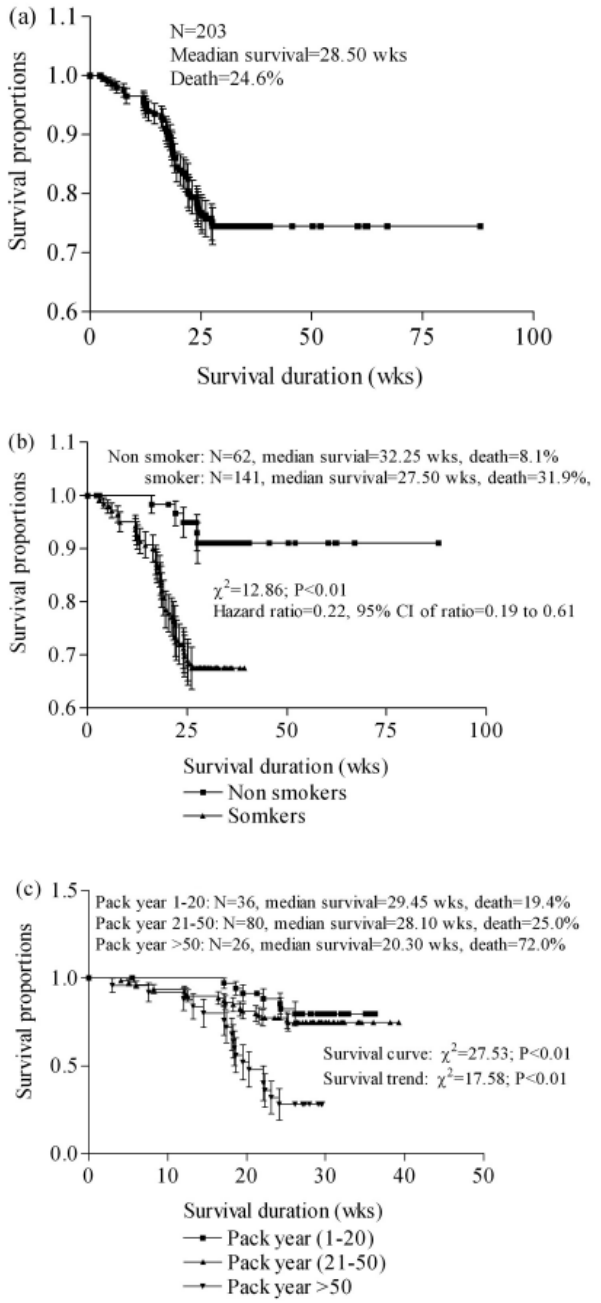

Figure 1. The two years overall survival. (a), All NSCLC patients; **(b)**, Non-smoker and smoker NSCLC patients; **(c)**, Different pack year groups of smoker NSCLC patients.

smoker NSCLC patients was also lower as compared to pack year 1-20 but seems statistically insignificant ($p > 0.05$) (Figure 1c). The survival trend among the three pack year exposure groups was also found to be significant (Log rank test for trend: $\chi^2 = 17.58$; $p < 0.01$) indicating that survival after chemotherapy in smoker NSCLC patients may be dependent on their pack year exposure.

4. Discussion

Cigarette smoking is a well known environmental risk factor for lung carcinogenesis. Tobacco smoke contains many mutagenic and carcinogenic chemicals (*32*,*33*) that might be associated with mutation in genes (*34*,*35*). Cigarette smoke is a composite of numerous pollutants in rather high concentrations. Well over one thousand constituents of smoke, including many oxidants, pro-oxidants, free radicals and reducing agents, have been identified (*36*). Also, Pryor and associates (*11*,*15*) have identified two different populations of free radicals , one in tar and the other in gas phase of CS. The principal radical in the tar phase, a quinone/hydroquinone complex is capable of reducing molecular oxygen to superoxide radicals. The gas phase of cigarette smoke contains small oxygen and carbon centered radicals that are much more reactive than are all tar phase radicals. Thus cigarette smoke contains many oxidants, free radicals and metastable products derived from free radical reactions that are capable of reacting with or inactivating essential cellular constituents.

An increased oxidant burden in smokers derives from the fact that cigarette smoke contains an estimated 10^{14} oxidants and 3,000 ppm NO/puff, and many of these are relatively long lived including tar-semiquinone, which can generate OH^- and hydrogen peroxide in the presence of free iron through the Fenton reaction (*11*). The increased oxidative metabolism of phagocytes is accompanied by increased generation of reactive oxygen species (ROS); such as, hydrogen peroxide, hydroxyl radicals and superoxide radicals. These ROS can attack DNA directly or cause membrane damage, and they can activate oxygen, a process that has been associated with tumor promotion. Earlier studies found that smokers have higher plasma levels of lipid peroxidation products, measured through the thiobarbituric acid (TBA)-malondialdehyde method (*37*). We therefore studied lipid peroxidation in smoker and non-smoker lung cancer patients. In addition there is a decrease in antioxidants in the distal airways of smokers, as well as a decrease in vitamin E in the bronchoalveolar lavage fluid, when compared to non smokers (*38-40*).

Some studies also discussed that CS increases the formation of reactive nitrogen species (RNS) and results in nitration and oxidation of plasma proteins. The levels of nitrated proteins (fibrinogen, transferrin, plasminogen, and ceruloplasmin) were higher in smokers compared to non-smokers (*41*). Evidence of increased $NO/ONOO^-$ activity in plasma and epithelial lining fluid has been shown in chronic smokers resulting in elevated formation of 3-nitrotyrosine (*42*).

The major antioxidants in lung lining fluid are GSH (*16*). Cofgreave *et al*. have shown that acute cigarette smoke inhalation for one hour caused significant depletion of GSH in the lungs, lavaged cells and lavaged fluid of rats (*43*). GSH is a powerful scavenger of both ROS and RNS and can protect proteins against nitration, particularly from the nitrogen dioxide radical, and nitrous oxide found in cigarette smoke (*17*).

The actual mechanisms of the carcinogens have

not been identified, even though polycyclic aromatic hydrocarbon and tobacco specific nitrate compounds have been indicated (*44*). Cigarette smoke alters the lung metabolism of many endogenous compounds as well as the activities of many biotransforming enzymes in lung tissue (*45*). It has been also well established that smoking is associated with many mortality-related pathological conditions and causes irreversible damage to lung parenchyma (*46*).

Smoking also affects the rates of metabolism for several drugs (*4*). In addition, it is possible that lung tumors in smokers may be more chemo resistant. The actual mechanism of this drug resistance is under investigation (*4*). The resistance of cancer cells to anticancer drugs is a serious clinical problem encountered in the chemotherapy of lung cancer patients.

In addition, smoking is associated with factors that may contribute to poor cancer survival; an increased mutation burden that could lead to accelerated carcinogenesis and progression (*47*). This is one of the most important determinants because smoking is associated with numerous diseases (*18,48*). Thus patients with lung cancer with a smoking history are at risk of dying from a spectrum of smoking associated diseases.

Despite the indisputable link between smoking status and increased risk of lung cancer, the data on the inclusion of this predictor in prognostic survival analysis has been scarce. Although a large number of papers have been published evaluating prognostic factors in lung cancer, tumor node metastasis (TNM)-staging is the most important tool to estimate the prognosis of lung cancer patients and to define the best treatment modality (*49*). TNM-staging gives an accurate estimate of localization and disease progression at the time of diagnosis, but it does not account for survival differences within the same stage. Pretreatment variables on survival are not necessarily identical to predicting response to chemotherapy (CT). Therefore, identification of these factors would be useful in analysis of the response rate to CT in lung cancer patients. Recently, a study indicated a beneficial effect on ECOG performance status when patients stopped smoking after diagnosis of NSCLC (*50*). Previous studies (*51,52*) suggest that the benefit consistently increases with the number of years since smoking abstinence. This is important since the prognosis of lung cancer patients might significantly be improved by smoking cessation (*53*). Current smoking at the time of diagnosis was an independent predictor of poor prognosis in lung cancer patients (*54*). Furthermore, a worse survival rate was seen in 215 SCLC patients who smoked throughout chemotherapy (*55*). However, a study with 154 SCLC patients showed that continued smoking during chemotherapy did not affect the outcome of this treatment (*24*). Our study examined whether smoking intensity by inducing oxidative stress affected the survival of patients with lung cancer treated with chemotherapy.

We observed a poorer response in patients with PY > 50. Patients with PY > 50 had a worse response than those with PY < 50. Factors related to tobacco smoking have been implicated in poorer response for current and former smokers. At least 55 of the > 2,000 chemical compounds identified in the tobacco leaf are proven carcinogens (*56*) and many of them could be responsible for the poor response. In a study of 369 patients with NSCLC, smoking pack years before surgery had a prognostic value (*46*). Also patients were classified into two group in a previous study with > 30 and < 30 smoking pack year. Smoking with 30 or more pack year was associated with poor prognosis and non-smokers have better prognosis compared with smokers (*25*). In a Japanese population based study stage I lung adenocarcinoma patients with a smoking intensity of less than 20 pack year showed a more favorable prognosis than those with a PY of 20 or more (*26*). It was seen that in our study that smokers show more oxidative stress than non-smokers before and after chemotherapy. Survival of the smoker NSCLC patients was also low as compared to non-smokers. Also the patients with > 50 PY showed worse survival than the other PY groups. We can conclude that smoking is a bad prognostic factor in lung cancer therapy, besides its role in oxidative stress, lung cancer genesis and poor survival. Therefore, this factor can be used in patient selection for chemoprevention.

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Case Report

Case report: Occurrence of fallopian tube cancer in a patient with previous history of estrogen receptor positive breast cancer

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Summary **Fallopian tube cancer is very rare, it accounts less than 1% of all genital tract cancer in women. The annual incidence is 3.6 per million women per year. The vast majority of fallopian tube cancers are papillary serous adenocarcinomas. Most women with fallopian tube cancer are usually diagnosed at age 50s to 60s. Vaginal bleeding or discharge is the most common presentation. We would like to present a case in which fallopian tube cancer was diagnosed in a patient with post menopausal bleeding, abnormal ultrasound finding and history of estrogen receptor (ER) positive breast cancer.**

Keywords: Fallopian tube cancer, estrogen receptor (ER) positive breast cancer

1. Introduction

Fallopian tube cancer is very rare and it accounts less than 1% of all genital tract cancer in women. Metastatic cancer to fallopian tube from ovary, uterus, breast and gastrointestinal tract is more common. Most women with fallopian tube cancer are usually diagnosed at age 50s to 60s. Vaginal bleeding or discharge is the most common presentation, other symptoms include pelvic pain and mass. Inherited BRCA mutation is an important risk factor for fallopian tube cancer. We would like to present a case in which fallopian tube cancer was diagnosed in a patient with post menopausal bleeding, abnormal ultrasound finding and history of estrogen receptor (ER) positive breast cancer.

2. Case report

The patient is a 56 year-old Caucasian women who had history of ER positive breast cancer, status post bilateral mastectomy and chemotherapy 2 years ago. Menopause was at age 51. She presented to gynecology clinic with a few weeks of post menopausal spotting. Endometrial biopsy was performed and the pathology report showed benign endometrium. Pelvic ultra sound revealed 5 cm complex right adnexal mass. The patient was then referred to gynecologic oncology service and underwent surgery. Frozen section of the right tube and ovary revealed fallopian tube cancer. Total abdominal hysterectomy, bilateral salpingo-oophorectomy and surgical staging were then performed. The final pathology showed a high grade primary right fallopian tube serous adenocarcinoma (Figure 1) with no evidence of metastasis. Patient received no further treatment but follow up.

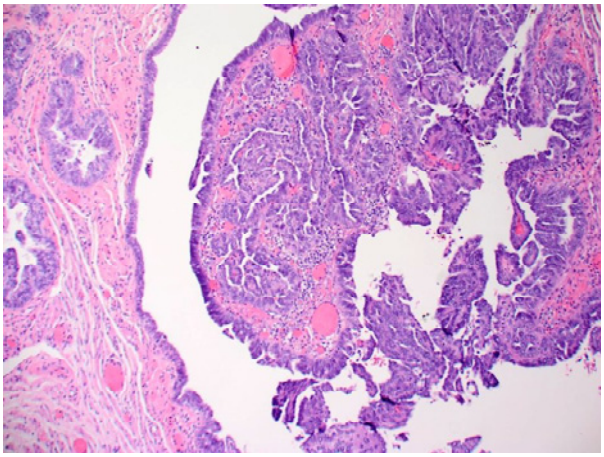

Figure 1. Serous adenocarcinoma with typical moderate to poorly differentiated solid nests of cells. The cells demonstrated proliferative, nuclear atypia, and mitotic figures. ×100.

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3. Discussion

Fallpian tube cancer is very rare, it accounts less than 1% of all genital tract cancer in women. The classical triad of symptoms are watery vaginal discharge, pelvic mass and pelvic pain. However, most patients do not present with these symptoms. In our patient, she presented with postmenopausal bleeding, and pelvic ultrasound revealed complex right adnexal mass. The interesting part of our patient was that she had history of ER positive breast cancer.

Even though the causes of fallopian tube cancer have not been extensively studied, it has been reported that this cancer is associated with chronic tubal inflammation, infertility, tuberculous salpingitis, and tubal endometriosis (*1*,*2*). Cytogenetic studies have also shown that this disease is associated with the overexpression or alternation of p53 (81%), HER2/neu (89%), c-myc (61%), and serum CA 125 expression profiles (*2*).

Inherited BRCA mutation is an important risk factor for fallopian tube cancer. The incidence of fallopian tube cancer increases 120-fold in BRCA1 mutation carriers when compared with Surveillance, Epidemiology, and End Results (SEER) (*1*). In addition, a study in Ontario, Canada showed a modest increase in the risk of ovarian cancer (relative risk (RR) = 2.2) and of early-onset breast cancer (RR = 2.4) was observed in the first-degree relatives of the fallopian cancer cases (*3*). 11% were positive for a mutation in BRCA1 and 5% were positive for a BRCA2 mutation (*3*). In our case, although we do not have the BRCA status of our patient, she did present with ER positive breast cancer followed by the diagnosis of fallopian tube cancer a few years later. We hope, through this case report, to raise the awareness of the possible association of breast cancer and other genital tract cancer such as fallopian tube cancer. Any patient with history of breast cancer and abnormal vaginal bleeding needs further evaluation of possible genital tract cancers. Similarly, the diagnosis of fallopian tube cancer should warrant further evaluation of possible clinical component of the hereditary breast-ovarian cancer syndrome which may be associated with BRCA1 and BRCA2 mutations.

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