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Review

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Using silkworms to search for lactic acid bacteria that contribute to infection prevention and improvement of hyperglycemia

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SUMMARY *Bombyx mori*, the silkworm, has biological functions in common with mammals, including humans. Since the molecular design of silkworm's innate immune system is analogous to that of mammals, understanding the silkworm's innate immunity is expected to contribute to the control of infection in humans. It is also possible to use silkworms to explore foodstuffs that activate innate immunity. Lactic acid bacteria have long been used in the production of fermented foods, and in recent years, their use as supplements has been attracting attention. Using silkworms, which are laboratory animals, functional lactic acid bacteria can be explored and isolated at low cost. Fermented foods produced by this method are expected to contribute to the maintenance of human health. In addition to the immune system, humans and silkworms share a common mechanism for maintaining blood glucose homeostasis, and it is possible to construct a pathological model of diabetes and search for therapeutic substances using silkworms. Taken together, we propose that the silkworm is useful for assessing the functions of lactic acid bacterial for health purposes.

Keywords silkworm, functional foods, immune priming, diabetes

1. Introduction

With the outbreak of the new coronavirus infection, functional foods that enhance immunity have been attracting attention. In addition, in Japan's super-aging society, there is growing interest in the prevention of lifestyle-related diseases such as diabetes (*1*). Lactic acid bacteria is one of the best food materials to meet these needs and have been used in the production of fermented foods such as pickles, yogurt, and breads since ancient times (*2,3*). Recently, the usefulness of the lactic acid bacteria for health maintenance has been emphasized. Maintaining people's health by consuming natural and functional foods that utilize lactic acid bacteria will provide a new approach to combating these diseases, which until now have relied on antibiotics and diabetic drugs. A variety of lactic acid bacteria are sold as functional foods for health promotion. However, there are not many of these products that have solid evidence (*4-6*). In general, foods are often evaluated for their health using mammals such as mice. However, it has been pointed out that using mice is not only costly but also ethically problematic in terms of animal welfare (*7*). Therefore, it is becoming increasingly difficult to obtain evidence from animal experiments using mice. Furthermore, in order to obtain evidence for health foods, it is ultimately necessary to conduct tests using humans. However, this requires huge costs ($3000-5000 per human subject (*8*)), and for this reason, health foods are currently being sold with little scientific evidence.

In an attempt to solve this problem, we have made use of silkworms. The silkworms have been used for sericulture since ancient times and have produced silk as a domesticated insect species. Japan has been at the forefront of the world in terms of silkworm breeding methods and strain maintenance. Recently, the production of genetically modified silkworms has become possible, and the silkworm has acquired the status of an experimental material for cutting-edge biology (*9*). However, since the silkworm 'looks' very different from humans, many people wonder whether the results of experiments on silkworms can be applied to humans. Let us consider what functions humans have that silkworms do not. The silkworm feeds on mulberry leaves. The silkworm recognizes mulberry leaves as food, and this is due to the function of the silkworm's brain. The mulberry leaves are digested in the digestive tract, and the nutrients are absorbed into the bloodstream. The silkworm has the muscles and nervous system necessary for feeding behavior. Silkworms also have blood, but not red-colored because it lacks the red blood

cells. The silkworm's blood contains immune cells that correspond to human white blood cells. Invertebrates, including the silkworm, have no genes encoding antibodies and they protect themselves from pathogens using an antibody-independent immune mechanism called innate immunity (*10-13*). Although it is true that the silkworm does not have some aspects of human body functions (*e.g.*, it lacks bones so that it may not be the best animal to create an osteoporosis model), most of the basic biological functions are shared by both silkworms and humans. Therefore, in principle, it is possible to create models of many diseases that humans suffer from using silkworms. We have mainly studied infectious disease models in silkworms, where we have found that most of the bacteria and fungi that infect mammals can infect and kill silkworms (*14,15*). We have also reported that antibiotics used as therapeutic agents in human clinical practice were effective in the silkworm infection model (*16*), and that the ED_{50} value, an index of therapeutic efficacy, was consistent between silkworms and mammals (*15*). We conducted a search for antibiotics against *Staphylococcus aureus* using this silkworm infection model as a screening tool for therapeutic efficacy, and succeeded in discovering Lysocin E, an anti-MRSA infection treatment, from extracts of soil bacteria (*17*). Animal studies on Lysosin E were conducted (unpublished), and it is now at the stage of planning human trials.

2. Innate immunity activation test using the silkworm muscle contraction system

As mentioned above, invertebrates rely on innate immunity for pathogen defense. This make a merit for the silkworm to be an innate immunity model, because, in mammals, it is not always easy to quantitatively measure innate immune activation at the individual level due the intricated cross-talks and interactions between the innate immune system and the adaptive immune system. Although *Drosophila* (fruit flies) and *Caenorhabditis elegans* (nematodes) have been used in biological research, the body size of the silkworm (they are much larger than fruit flies and nematodes) makes it easier to collect a good amount of blood sample by cutting its legs with scissors and to inject a certain volume of sample solution into the blood stream using a tuberculin syringe with needle. In addition, organs such as the intestinal tract can be removed to conduct experiments such as drug permeability test (*15*).

We reported that the silkworm's immune system is activated by the cell wall components of bacteria and fungi, resulting in contraction of silkworm muscles (*18*). This is a phenomenon in which immunocompetent cells in the silkworm's blood (often called hemolymph) recognize pattern molecules *via* innate immune receptors and release reactive oxygen species that lead to activation of paralytic peptides (BmPP; an insect cytokine), and then muscle contraction as a pharmacological effect of BmPP. Using this unique function, it is possible to quantitatively evaluate innate immunity activators in foods using the silkworm muscle contraction assay system.

The advantage of the silkworm muscle contraction as an indicator of innate immune activation is that it does not require costly facilities such as fancy fluorescent microscopes or mass spectrograms. It is worth noting here that the muscle contraction of the silkworm is not affected by lipopolysaccharides (LPS), which is always a problem in cell culture-based *in vitro* immune activation tests. LPS is a component of the outer wall of Gram-negative bacteria such as *Escherichia coli*, but since it acts on mammalian immune cells at extremely low concentrations, it is known to be easily contaminated by environmental contaminants such as tap water, which frequently affect the quantification of immunoreactive substances. It is a huge technical merit that the muscle contraction system can evaluate the activation of innate immunity without being affected by LPS. Using the silkworm muscle contraction system, we have found that extracts of green tea and broccoli have high immunoreactivity. Also, some lactic acid bacteria show very high immunoreactivity in this system. When various lactic acid bacteria were collected and tested, they show a wide range of activity from less than 1 units/mg to 105 units/mg (Table 1). It is also possible to identify subspecies of lactic acid bacteria that have outstanding immunoreactivities. We have identified a subspecies of *Lactococcus lactis*, 11/19-B1, using this system (*19*).

3. Primed immune responses found in the silkworm

When *Pseudomonas aeruginosa* is injected into the silkworm after pre-administration of an immune

Table 1. Immunoreactivity (silkworm muscle contraction activity) of different lactic acid bacteria.

No.	Phylogenetic Identity	Activity (U/mg)
1	*Lactococcus lactis*	105
2	ND (Not Determined)	77
3	*Lactococcus lactis*	45
4	*Streptococcus thermophilus*	43
5	*Lactococcus lactis*	33
6	*Lactobacillus bulgalicus*	29
7	ND	28
8	*Lactobacillus bulgalicus*	24
9	ND	23
10	*Lactobacillus casei*	18
11	*Lactococcus lactis*	17
12	ND	7.1
13	ND	2.2
14	ND	2
15	*Enterococcus casseliflavus*	0.83
16	ND	0.45
17	ND	< 0.30

A list of lactic acid bacteria is shown in the table with corresponding muscle contraction activity. Data from our previous work (*19*).

activator (immune primer), the silkworm may become resistant to *P. aeruginosa* infection. This function is called the primed immune responses in silkworms (*12,20*). *P. aeruginosa*, a Gram-negative bacterium, is a causative agent of opportunistic infections in humans and is naturally resistant to many antibiotics. More recently, nosocomial infections caused by multidrug-resistant *P. aeruginosa* (MDRP) have become a problem. We have found that several lactic acid bacteria are effective in this system. It is noteworthy that lactic acid bacteria can be added in the diet of silkworm, *i.e.*, oral administration shows immune-priming effects and protects silkworms from infection. In our past experience of searching for antibiotics, the probability of finding a drug that works against Gram-negative bacteria is extremely low. Therefore, it was a great surprise when we discovered this orally effective measure against *P. aeruginosa* infection. We are currently working on purification of the active substance, identification of its chemical structure, and the molecular mechanism of its action.

4. Evaluation of hypoglycemic substances using silkworms

For the uninitiated, it may come as a surprise that silkworms have blood and blood glucose levels. In the long history of sericulture, silkworms have probably never been fed sweets. Yet, in fact, when the silkworm is fed sugar, the blood glucose level rises and the weight gain stops, where glycation of blood proteins similar to that in human clinical practice is found. Insulin is a drug prescribed for diabetic patients, but even in silkworms whose blood glucose levels are elevated by the administration of sucrose, injecting human recombinant insulin lowers the blood glucose levels (*21*). Insulin also cancels the growth inhibition caused by the elevated blood glucose levels (*21*). It is also known that prolonged administration of glucose to mammals for more than a year can lead to type 2 diabetes, a condition in which insulin is ineffective. In the case of silkworms, continuous administration of glucose for only one day results in insulin resistance. Moreover, type 2 diabetes drugs such as metformin are effective in silkworms. These results suggest that the mechanism of blood glucose maintenance in the silkworm is analogous to that in mammals. In fact, the phosphorylation of Akt protein in silkworm fat body cells (a counterpart of human adipocytes) is increased by insulin and decreased by the inhibitor wortmannin, showing the similarity between silkworm and mammals at the molecular level (*21*).

We used this silkworm model of diabetes to search for ingredients in foodstuffs that exhibit hypoglycemic effects. *Enterococcus faecalis* is a lactic acid bacterium that causes human diseases as enterococci, but non-pathogenic variants are known and used in the production of various fermented foods. We found a sub-strain named YM0831 that was capable of lowering blood glucose levels in silkworms (*22*). We also confirmed that this bacterium has no pathogenic gene and is safe as food. The bacterium was found to suppress glucose uptake *in vitro* by Caco-2 cells derived from the human intestinal tract (*22*). This may be the mechanism of the hypoglycemic effect of this bacterium in the silkworm. We also conducted a blood glucose lowering test using healthy human volunteers. The results showed that the sucrose-induced increase in blood glucose level was suppressed by prior oral intake of the live bacteria (*22*). This finding endorses the usefulness of silkworm model for the search of human hyperglycemia drugs.

5. Future development

Lactic acid bacteria have been used in various fermented foods as non-pathogenic bacteria. We would propose a novel approach to evaluate the immune-activating and blood glucose-lowering effects of lactic acid bacteria using silkworm models, providing promising paths towards human trials on lactic acid bacteria if showing outstanding effects. This new approach will avoid the huge cost of conducting unpromising human trials and the ethical problem of mammalian sacrifice. The approach can be applied to: development of pet foods to which functional lactic acid bacteria are added, feed for farmed fish and livestock, and supplements for humans. Food products fermented with functional lactic acid bacteria can be expected to have a variety of health benefits and are the potent target of commercialization.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Original Article

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Ezh1 regulates expression of *Cpg15/Neuritin* in mouse cortical neurons

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SUMMARY Immature neurons undergo morphological and physiological maturation in order to establish neuronal networks. During neuronal maturation, a large number of genes change their transcriptional levels, and these changes may be mediated by chromatin modifiers. In this study, we found that the level of Ezh1, a component of Polycomb repressive complex 2 (PRC2), increases during neuronal maturation in mouse neocortical culture. In addition, conditional knockout of Ezh1 in post-mitotic excitatory neurons leads to downregulation of a set of genes related to neuronal maturation. Moreover, the locus encoding Cpg15/Neuritin (Nrn1), which is regulated by neuronal activity and implicated in stabilization and maturation of excitatory synapses, is a direct target of Ezh1 in cortical neurons. Together, these results suggest that elevated expression of Ezh1 contributes to maturation of cortical neurons.

Keywords epigenetics, Polycomb group proteins, neuronal maturation, depression, Alzheimer's disease, Nrn1

1. Introduction

The mammalian neocortex processes various kinds of information and governs higher-order functions such as behavior and cognition. Neurons are the most fundamental elements of the brain, and in order for the brain to perform its higher-order brain functions, they must form intricate networks. To this end, neurons must undergo dramatic maturation processes. During neocortical development, neurons are generated from neural precursor cells (NPCs) and migrate toward the pial surface (*1*). After reaching the pia, they stop migration and undergo dramatic morphological and functional maturation, including outgrowth of axons and dendrites, synapse formation, and establishment of membrane potential (*2-4*). The processes of neuronal fate decision and maturation into elaborate neurons involves the products of many genes. Indeed, global patterns of gene expression change during neuronal maturation (*5*): microarray analysis revealed that 49% of 14,213 mRNAs in mouse neocortex change their expression levels during this process. It remains unclear, however, how genome-wide transcription pattern is coordinately regulated during this maturation process.

Chromatin-level mechanisms such as DNA methylation and histone modifications play important roles in transcriptional regulation (*6-8*). Chemical modifications at histone tails change the chromatin state, leading to transcriptional activation or repression. For example, tri-methylation of histone H3 at lysine-4 (H3K4me3) is associated with transcriptional activation, whereas H3K9me3 and H3K27me3 are associated with transcriptional repression. Polycomb group (PcG) proteins are chromatin modifiers that regulate gene expression patterns, primarily through transcriptional repression. PcG assemble to form two protein complexes, polycomb repressive complex 1 (PRC1) and PRC2. These complexes catalyze ubiquitylation of histone 2A at lysine-119 (H2AK119ub) and trimethylation of histone 3 at lysine-27 (H3K27me3), respectively (*9,10*). PRC2-mediated H3K27me3 plays critical roles in the stage-specific repression of developmental regulator genes, and frequently exhibits bivalency along with an active histone modification, H3K4me3, during cellular differentiation (*11,12*).

The core components of PRC2 are Eed, Suz12, and the H3K27me3 transferase enhancer of zeste homolog

(Ezh) 1 or 2. Although the catalytic SET domains of Ezh1 and Ezh2 are highly homologous to each other, the H3K27me3 transferase activity of Ezh1 is much weaker than that of Ezh2 in NIH3T3 cells (*13*). To function, Ezh proteins must form complexes with other PRC2 components (*13-15*). Ezh1 and Ezh2 compete for other PRC2 components, and are therefore mutually exclusive in complex formation (*13-15*). Moreover, the expression patterns of Ezh1 and Ezh2 tend to be mutually exclusive. Expression of Ezh2 is frequently associated with a proliferative state (*16,17*), whereas Ezh1 is primarily expressed in post-mitotic or quiescent cells including hippocampal neurons, medium spiny neurons (MSNs) in the striatum, myotubes, aging kidney, and hematopoietic stem cells (*13,15,18-22*). Although Ezh proteins are primarily associated with gene repression through PRC2, several lines of evidence suggest that they also play non-canonical roles in gene activation in a context-dependent manner (*15,18,20,21*). However, in cortical neurons, whether expression levels of PRC2 components including Ezh proteins are changed, and whether Ezh proteins repress or activate their target genes are not investigated.

In NPCs, PRC2 plays various roles in maintaining the undifferentiated state of NPCs and temporal regulation of their fate restriction (*23-25*). The functions of PRC2 during neuronal maturation have also been explored. For example, in a rat hippocampal neuronal culture, knockdown of Ezh2 derepresses transcription of PSD95, a postsynaptic marker in excitatory neurons, and decreases secondary and tertiary branching of dendrites, whereas knockdown of Ezh1 decreases transcription of PSD95 (*18*). In addition, knockdown of Ezh2 increases neuronal firing frequency and febrile seizure susceptibility *in vivo* (*26*). Conditional knockout of *Ezh2* in neural progenitors results in derepression of HoxPG5 and Netrin-1 and aberrant pontine neuronal migration (*27*). In mature MSNs, PRC2 contributes to suppression of a transcriptional program that is detrimental to adult neuron function and survival (*22*). Knockdown of Ezh1 in the adult mouse prefrontal cortex attenuates sociability and promotes motivational behaviors (*28*). Therefore, it is of interest to determine which targets can be regulated by Ezh1 in postmitotic neurons.

In this study, we investigated the role of Ezh1 in the maturation of mouse neocortical neurons in an *in vitro* culture system. To this end, we generated a mouse line in which *Ezh1* could be conditionally deleted. We found that expression of Ezh1 increased during neuron maturation in primary neocortical culture. Moreover, neuron-specific deletion of *Ezh1* resulted in downregulation of a set of genes related to neuronal maturation. Among these genes, Ezh1 directly associated with the locus encoding Cpg15/Neuritin (Nrn1), which is regulated by neuronal activity and mediates the neuronal maturation process. Our results suggest that Ezh1 contributes to neuronal maturation by regulating expression of Nrn1.

2. Materials and Methods

2.1. Animals

All animals were maintained and studied according to protocols approved by the Animal Care and Use Committee of The University of Tokyo (approval numbers: P25-8 and P30-4). *Ezh1*$^{flox/flox}$ mice, described below, were crossed with *NEX-Cre* transgenic mice (*29*). JCL: ICR (CLEA Japan, Tokyo, Japan) or Slc: ICR (SLC Japan, Shizuoka, Japan) mice were used as wild-type animals. All mice were maintained in a temperature- and relative humidity-controlled environment (23 ± 3°C and 50 ± 15%, respectively) with a normal 12-h-light, 12-h-dark cycle. Two to six animals were housed per sterile cage (Innocage, Innovive, Sandiego, CA, USA) with chips (PALSOFT, Oriental Yeast, Tokyo, Japan), and with irradiated food (CE-2, CLEA Japan) and filtered water were available ad libitum.

2.2. Production of *Ezh1*$^{flox/flox}$ mice

All experiments were performed according to protocols approved by the Animal Care and Use Committee of The University of Tokyo and Niigata University. The *Ezh1*$^{flox/flox}$ mouse line was produced by homologous recombination using the ES cell line RENKA, which was developed from the C57BL/6N strain (*30*). The targeting vector was constructed in accordance with the mouse genomic DNA databases contained from exon 2 to exon 7 of *Ezh1* gene with the 6.76 kb upstream and 6.26 kb downstream (Figure 2a; exons are indicated by boxes with the exon number above). The neomycin phosphotransferase gene (*Neo*) and the gene encoding fragment A of diphtheria toxin (*DT-A*) were included for positive and negative selection, respectively. A DNA fragment containing a 34-bp loxP sequence and *Pgk1* promoter-driven *Neo* flanked by a pair of flippase recombinase target (FRT) sequences was inserted 1523 bp upstream of exon 5. The other loxP site was introduced 1,063 bp downstream of exon 2. Thus, Cre-loxP deletion would delete exons 3 and 4, resulting in a nonsense mutation in the *Ezh1* gene. Introduction of the targeting vector into mouse ES cells, screening for homologous recombinants with southern blot analysis, production of chimeric mice, were carried out as described previously (*30*). A diagnostic external probe for southern blot analysis is shown in a thick line. The resultant chimeric mice were mated with C57BL/6N mice, and offspring [*Ezh1*$^{+/lox(neo)}$] were further crossed with *Actb-Flp* mice to remove the neo cassette. The *flp* gene was bred out in the next generation. After confirming deletion of the neo cassette, heterozygous (*Ezh1*$^{flox/+}$) mice were mated to generate homozygous (*Ezh1*$^{flox/flox}$) mice. PCR genotyping was performed using primers 5'- AGATTGCAGGCATTCTCTGT -3' (forward) and 5'- TGTCGAAGCCGCATATACTC -3' (reverse),

which yielded PCR products of 530 bp for the floxed allele and 430 bp for the wild-type allele.

2.3. Primary neuron culture

The cortex was isolated from ICR mice at E14.5 or *NEX-Cre*$^{-/-\text{ or }+/-}$; *Ezh1*$^{flox/flox}$ mice at E15.5, with the appearance of the vaginal plug considered to be E0.5. Dissected cortices were subjected to enzymatic digestion with a papain-based solution (FUJIFILM Wako chemicals, Tokyo, Japan), and the dissociated cells were plated directly and cultured on dishes coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) and were maintained in differentiation-inducing medium, which contains Neurobasal (Thermo Fisher Scientific, Waltham, MA, USA) and Neuron Culture Medium (FUJIFILM Wako chemicals), supplemented with B27 and GlutaMAX (Thermo Fisher Scientific), for several days. At 5 or 6 days in vitro (DIV), half of the medium was replaced, and cytosine arabinoside (Sigma-Aldrich) were added to the culture medium at a concentration of 5 μM to prevent expansion of glial cells.

Genotypes of *NEX-Cre*$^{-/-\text{ or }+/-}$; *Ezh1*$^{flox/flox}$ mice were evaluated after plating. Tissue from each embryo was collected and lysed with 50 mM NaOH, and then incubated at 98°C for 10 min. After mixing with 1 M Tris-HCl (pH 8.0), each lysate was subjected to PCR using KOD-FX polymerase (Toyobo, Osaka, Japan). PCR primers for *Nex-Cre* are described in (*29*).

2.4. Reverse transcription-quantitative PCR analysis

Total RNA was isolated from cells using Trizol (Thermo Fisher Scientific) or RNA IsoPlus (Toyobo), and up to 0.5 μg of the RNA was subjected to RT using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo). The resultant cDNA was subjected to real-time PCR analysis in a LightCycler 480 instrument (Roche, Basel, Switzerland) with Thunderbird SYBR qPCR mix (Toyobo). The level of each target mRNA was normalized against the corresponding level of *Actb* mRNA. Primer sequences are provided in Table S1 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=69*).

2.5. Immunoblot analysis

Cells were lysed with RIPA buffer containing 50 mM Tris- HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium-deoxycholate, 0.1% SDS, and protease-inhibitor (1 mg/mL). The lysates were subjected to immunoblot analysis with antibodies against Cre (1:500 dilution; 69050, Novagen, Darmstadt, Germany), Ezh1 (1:500; HPA005478, Sigma-Aldrich or 1:500; 20852-1-AP, Protein-tech, Tokyo, Japan), H3 (1:2,000; ab1791, Abcam, Cambridge, UK), H3K27me3 (1:1,000; 07-449, Millipore, Darmstadt, Germany or 1:1,000; 9733, CST, Danvers, MA, USA), and ßIII-tubulin (1:1,000; MMS-435P, Covance, Princeton, NJ). Immune complexes were detected with horseradish peroxidase conjugated secondary antibodies (GE Healthcare, Chicago, IL, USA) and Luminata Forte Western HRP substrate or Immobilon ECL Ultra Western HRP Substrate (Millipore) on an Image Quant LAS4000 instrument (GE Healthcare). Band intensities were measured using the ImageJ Software.

2.6. Chromatin immunoprecipitation-quantitative PCR analysis

Chromatin immunoprecipitation (ChIP) for Ezh1 and H3K27me3 was carried out as described in (*31*). Cells were fixed with 1% formaldehyde and stored at –80°C until analysis. The cells were thawed, suspended in RIPA buffer for sonication (10 mM Tris-HCl at pH 8.0, 1 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate) and subjected to ultrasonic treatment on a Picoruptor (15 cycles of 30 s ON and 30 s OFF) (Diagenode, Seraing, Belgium). Cell lysates were then diluted with RIPA buffer for immunoprecipitation (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 0.5% sodium deoxycholate) and incubated for 1 h at 4°C with Protein A/G Magnetic Beads (Pierce, Waltham, MA, USA) to clear non-specific reactivity. They were then incubated overnight at 4°C with Protein A/G Magnetic Beads that had previously been incubated for overnight at 4°C with antibodies. The beads were isolated and washed first three times with wash buffer (2 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 20 mM Tris-HCl at pH 8.0) and then once with wash buffer containing 500 mM NaCl. Immune complexes were eluted from the beads for 15 min at 65°C with a solution containing 10 mM Tris-HCl at pH 8.0, 5 mM EDTA, 300 mM NaCl, and 0.5% SDS, and they were then subjected to digestion with proteinase K (Nacalai Tesque, Kyoto, Japan) for > 6 h at 37°C, removal of cross links by incubation for > 6 h at 65°C, and extraction of the remaining DNA with phenol-chloroform-isoamyl alcohol and ethanol. The DNA was washed with 70% ethanol, suspended in water, and subjected to real-time PCR analysis in a LightCycler 480 (Roche) with Thunderbird SYBR qPCR Mix (Toyobo). Primer sequences are provided in Table S2 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=69*).

2.7. RNA-seq analysis

Libraries for RNA-seq analysis were constructed from total RNA isolated as described above for RT-qPCR analysis. The SMART-seq stranded kit (Takara, Shiga, Japan) was used for template preparation, followed by deep sequencing on the Illumina HiSeqX platform to obtain 151-base paired-end reads. Approximately 10-

50 million sequences were obtained from each sample. Sequences were mapped to the reference mouse genome (mm10) with Hisat2 (*32*). Only uniquely mapped tags with no base mismatches were used for the analysis. Reads in each gene locus were counted using the featureCounts software (*33*) and gene expression levels were quantitated as reads per kilobase of mRNA model per million total reads (RPKM). Differentially expressed genes (DEGs) were identified using *edgeR* from the *R* package (*34,35*) as genes whose *q* values were < 0.25. GO analysis was carried out using DAVID Bioinformatic Resources (*36,37*), and GO terms whose Benjamini score was < 0.25 were considered as significant.

2.8. Statistical analysis

Data are presented as means ± SEM and were compared with the two-tailed Student's unpaired *t* test or by analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test.

2.9. Data availability statement

The sequence data have been deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive under the following ID: DRA011526.

3. Results

3.1. Expression of PRC2 components changes during maturation of cortical neurons *in vitro*

Expression levels of PRC2 components have been investigated in MSNs in the mouse striatum, as well as in rat hippocampal neurons (*18,22*), but not in the cortical neurons. Hence, we investigated whether expression of PRC2 components changes during maturation of mouse neocortical neurons. To this end, we first examined mRNA levels of PRC2 core components by reverse transcription-quantitative PCR (RT-qPCR) (Figure 1a). We prepared primary neuronal cultures isolated from E14.5 neocortex and cultured them for several days *in vitro* (DIV) in a differentiation-inducing medium. Expression of *Tubb3*, a neuronal marker, was higher at 7 DIV *vs.* 1 DIV, suggesting that neuronal maturation had progressed (Figure 1a). By contrast, the levels of *Eed* and *Suz12* mRNA did not significantly change between at 1 and 7 DIV (Figure 1a). The level of *Ezh1* mRNA increased at 7 DIV *vs*. 1 DIV, whereas the level of *Ezh2* decreased (Figure 1a). Next, we examined protein level of *Ezh1* (Figures 1b and 1c). Immunoblot analysis revealed that expression of βIII-tubulin increased at 7 DIV *vs*. 0 DIV (Figures 1b and 1c). Also, the level of Ezh1 proteins increased at 7 DIV *vs*. 0 DIV along with the level of *Ezh1* mRNA (Figures 1b and 1c). On the other hand, the level of H3K27me3 did not significantly change at 7 DIV *vs*. 0 DIV (Figures 1b and 1c). Together, these results suggest that expression of Ezh1 increases during the maturation of neocortical neuron.

3.2. Establishment of an excitatory neuron specific knockout of Ezh1

Given that the expression level of Ezh1 increases during neuronal maturation, we investigated the possible roles of Ezh1 during this process. For this purpose, we generated a mouse strain (*Ezh1*$^{flox/flox}$) in which loxP sequences were inserted downstream of exon 2 and upstream of exon 5 (Figure 2a and 2b). We then induced conditional knockout of the *Ezh1* gene specifically in post-mitotic excitatory neurons by crossing *Ezh1*$^{flox/flox}$ mice with *NEX-Cre* transgenic mice, which express Cre recombinase in differentiating excitatory neurons under the control of the *Math2* (*Neurod6*) promoter (*29,38*). Before conducting experiments, we first confirmed the deletion of *Ezh1* by this conditional KO (cKO). Immunoblotting of extracts from neocortical culture isolated from E15.5 cortex and cultured for 10 DIV revealed that the level of Ezh1 protein significantly decreased following Ezh1 cKO (Figure 2c), indicating that *Ezh1* was successfully deleted. Interestingly, the global level of H3K27me3 was not significantly reduced by Ezh1 cKO (Figures 2c and 2d), implying the existence of compensatory mechanisms or slow turnover of H3K27me3 in these cells.

3.3. Transcriptional changes caused by Ezh1 cKO in cortical neurons *in vitro*

Next, using the newly-established Ezh1 cKO mice, we examined the effects of *Ezh1* deletion on gene expression patterns. Specifically, we conducted RNA sequencing (RNA-seq) analysis and compared the gene expression patterns of control and Ezh1 cKO cortical neurons. For these experiments, we prepared E15.5 neocortices from either control or Ezh1 cKO mice and cultured them for 10 DIV under differentiation-inducing conditions. We analyzed differentially expressed genes (DEGs) determined using *edgeR* of the *R* package (*34,35*). We identified upregulated DEGs (3059) and downregulated DEGs (1626) in neocortical neuronal cultures from Ezh1 cKO mice relative to those from control mice (Figures 3a and 3b). In contrast to previous reports on rat hippocampal neurons (*18*), expression of *Dlg4/PSD95* did not significantly change following Ezh1 cKO in our cortical cultures (Table 1). To explore the possibility that Ezh1 contributes to neuronal maturation, we performed Gene Ontology (GO) analysis for both upregulated and downregulated gene sets using the DAVID Bioinformatic Resources (*36,37*) (Figures 3c and 3d). Genes upregulated by Ezh1 cKO were enriched for many non-neuronal GO terms, including defense response, system process, cytokine production, cell activation, and response to external stimulus (Figure 3c). *Ezh1* and *Ezh2* double knockout in MSNs leads to derepression of non-

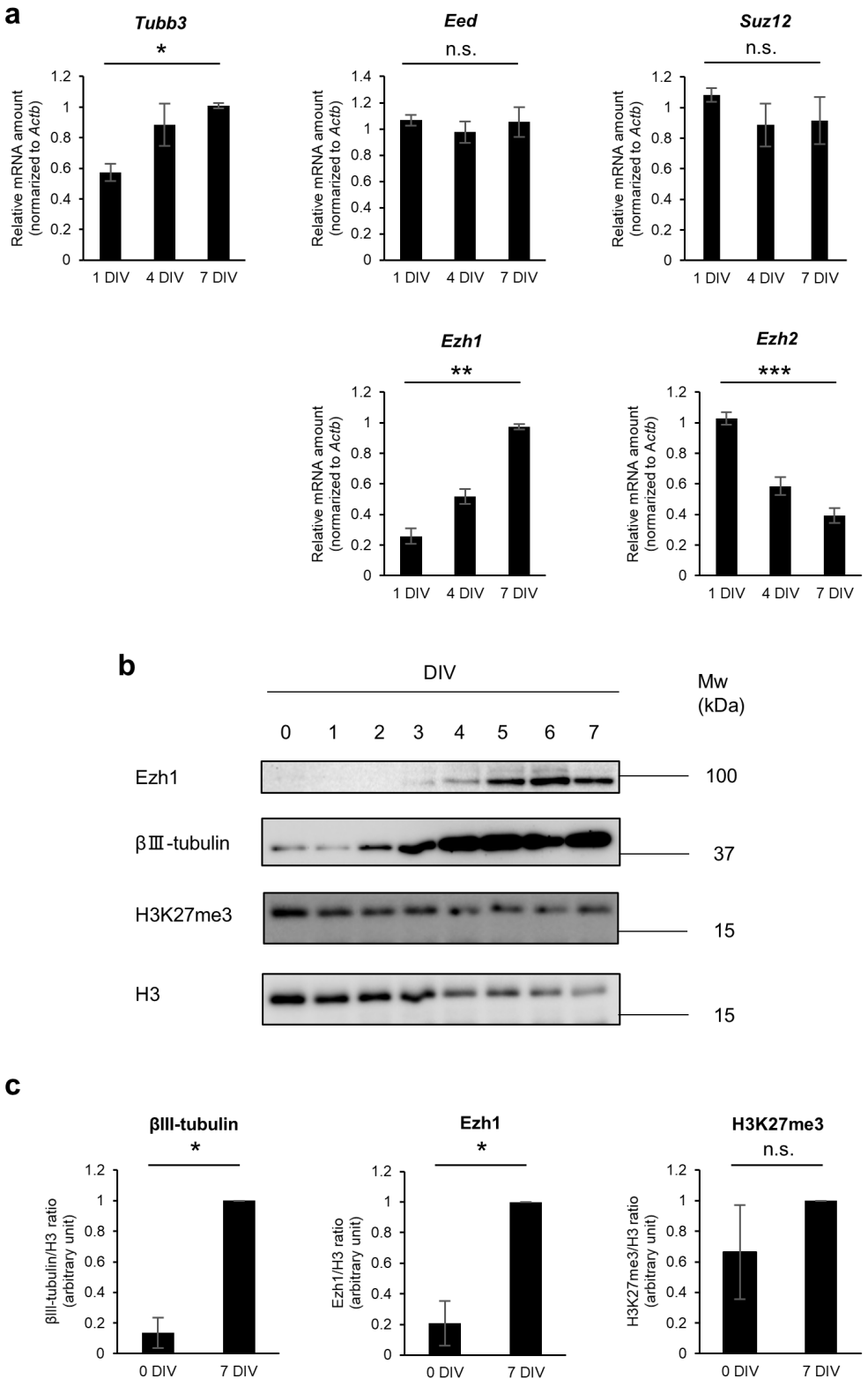


Figure 1. Changes in expression levels of PRC2 components during neuronal maturation *in vitro*. (a) RT-qPCR analysis of *Tubb3* (neuronal marker) and PRC2 components, *Ezh1*, *Ezh2*, *Eed*, and *Suz12* in cortical neurons isolated from E14.5 mouse cortex and cultured for 1 to 7 DIV under differentiation-inducing conditions. RT-qPCR data for each mRNA were normalized against the corresponding levels of *Actb* mRNA; means ± SEM from three independent experiments are shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (two-tailed unpaired Student's *t*-test). **(b)**, **(c)** Immunoblot analysis with antibodies against the indicated proteins in cortical neurons isolated from E14.5 mouse cortex and cultured for 0 to 7 DIV under differentiation-inducing conditions. The representative images of three independent experiments are shown in **(b)**. Quantifications of each amount of βIII-tubulin, Ezh1, and H3K27me3 normalized against the corresponding levels of H3 at 0 and 7 DIV are shown in **(c)**; means ± SEM from three independent experiments are shown. *$p < 0.05$ (two-tailed unpaired Student's *t*-test).

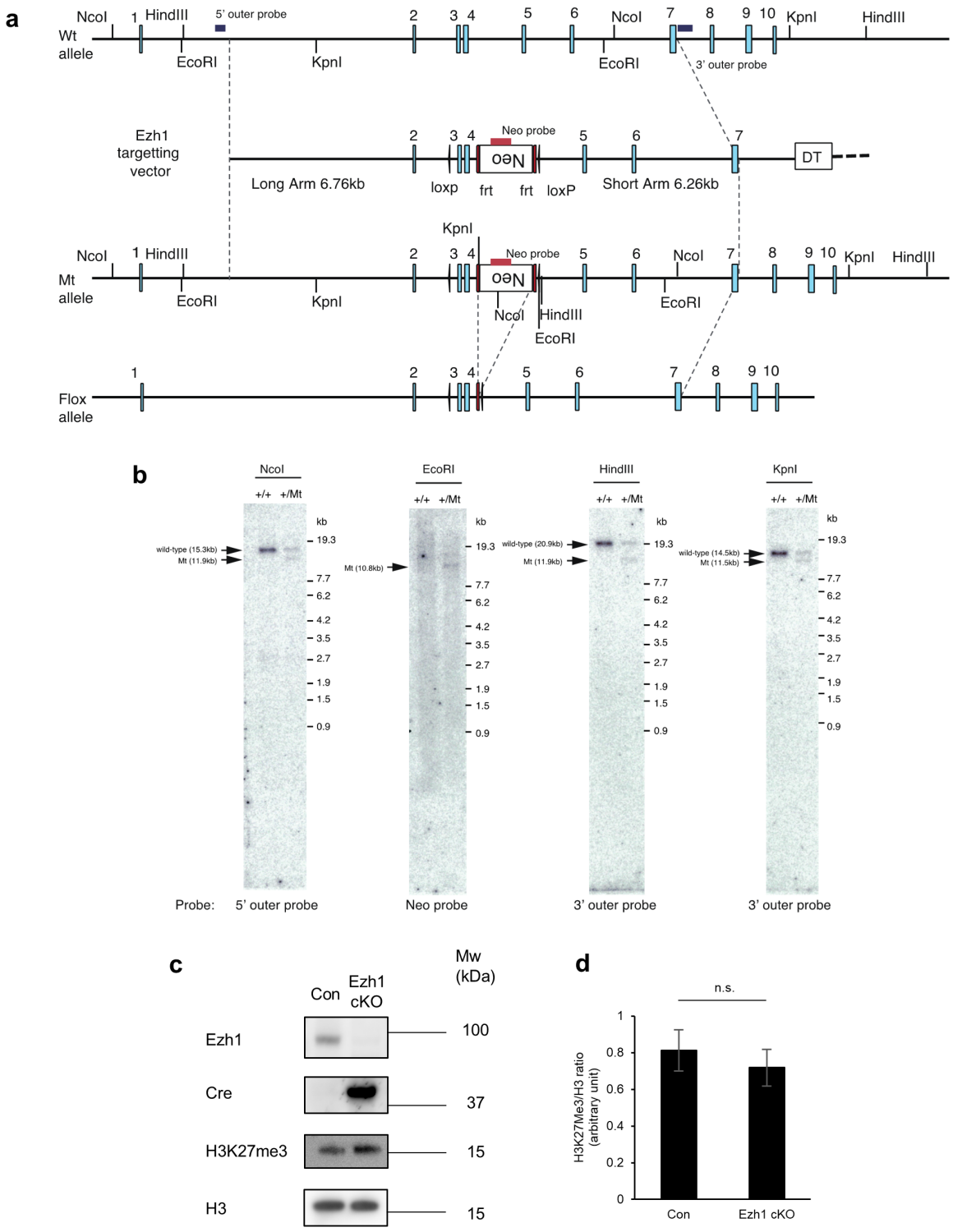
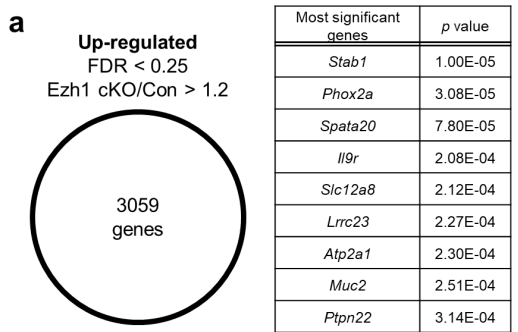
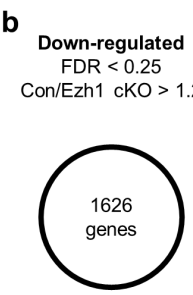


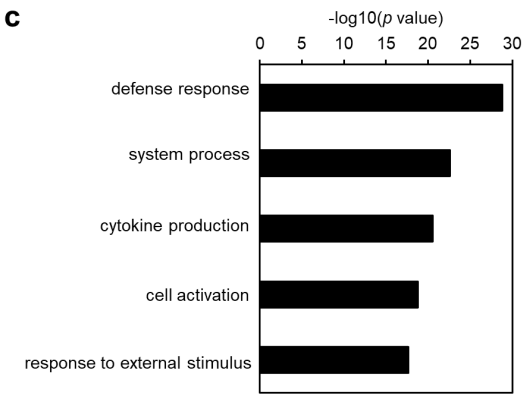
Figure 2. Establishment of excitatory neuron specific knockout of *Ezh1*. (a) Conditional disruption of the mouse *Ezh1* gene locus. Schematic representations show the wild-type allele, the targeting vector, and the resultant mutant and floxed alleles. **(b)** Southern blot analysis of ES cells of the indicated genotypes, using the indicated probes and restriction enzymes. **(c)** Immunoblot analysis with antibodies against the indicated proteins in cortical neurons isolated from *NEX-Cre*$^{-/-\text{ or }+/-}$; *Ezh1*$^{flox/flox}$ E15.5 mouse cortex and cultured for 10 DIV under differentiation-inducing conditions. **(d)** Quantification of H3K27me3/H3 ratio. Data are means ± SEM from three independent samples (two-tailed unpaired Student's *t*-test).

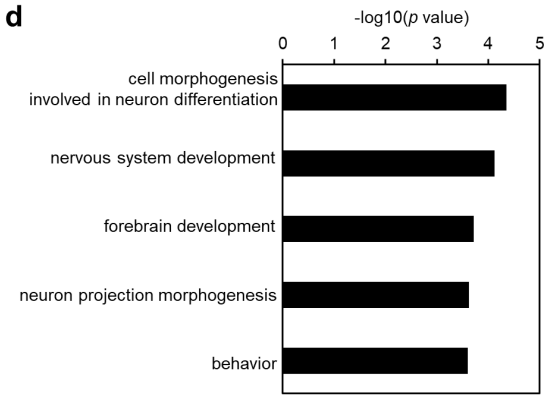


Most significant genes	*p* value
Stab1	1.00E-05
Phox2a	3.08E-05
Spata20	7.80E-05
Il9r	2.08E-04
Slc12a8	2.12E-04
Lrrc23	2.27E-04
Atp2a1	2.30E-04
Muc2	2.51E-04
Ptpn22	3.14E-04
Syne3	3.23E-04



Most significant genes	*p* value
Rps6	5.86E-08
Zfp955a	8.11E-05
Erbb4	1.24E-04
Bcap29	1.94E-04
Cmc1	2.26E-04
Luzp2	2.62E-04
Vstm2b	2.92E-04
Zfp946	3.01E-04
Wwp1	3.12E-04
Adcy2	3.71E-04





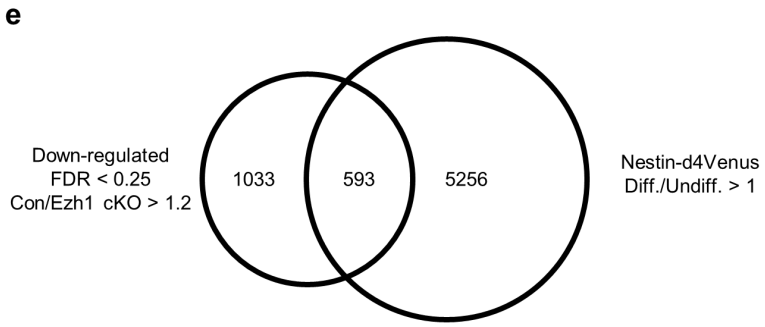


Figure 3. Genome-wide gene expression analysis of Ezh1 cKO cortical neurons. (a and **b)** Cortical neurons were isolated from the *NEX-Cre*$^{-/-}$; *Ezh1*$^{flox/flox}$ (Control) or *NEX-Cre*$^{+/-}$; *Ezh1*$^{flox/flox}$ (Ezh1 cKO) E15.5 mouse cortex and cultured for 10 DIV under differentiation-inducing conditions. Genes whose expression was upregulated **(a)** or downregulated **(b)** by Ezh1 cKO were defined as those whose Ezh1 cKO/control or control/Ezh1 cKO fold change was > 1.2 on average, with a false discovery rate (FDR) of 0.25. The genes with the 10 lowest *p* values (determined with *edgeR*) in each category are also listed (right panels). **(c** and **d)** Enriched GO terms and their *p* values, determined by functional annotation of all upregulated DEGs **(c)** and all downregulated DEGs **(d)** using the DAVID software. **(e)** Venn diagram representing the overlap between DEGs downregulated by Ezh1 cKO and genes expressed more strongly in d4Venus^{-} cells (differentiated neurons) than in d4Venus^{high} cells (undifferentiated NPCs).

MSN and non-neuronal lineage-specific transcription factors (*22*). Similarly, our gene expression data suggest that non-neuronal genes were upregulated by Ezh1 cKO.

Because Ezh1 can positively regulate the transcription of some target genes in addition to playing its conventional role as a transcription repressor (*15,18,20,21*), we next focused on genes that were downregulated gene by Ezh1 cKO. Downregulated DEGs were enriched for neuron-related terms like cell morphogenesis involved in neuron differentiation, nervous system development, forebrain development, neuron projection morphogenesis, and behavior (Figure 3d), indicating that Ezh1 cKO decreases expression of genes related to neuronal maturation. To find genes related to neuronal maturation that were regulated by Ezh1, we combined our RNA-seq data with a dataset obtained by comparing FACS-

Table 1. The list of representative genes downregulated by Ezh1 cKO

Gene Symbol	Con [rpkm]	Ezh1 cKO [rpkm]	Ezh1 cKO/Con	*p* value	Excitatory or Inhibitory
Erbb4	7.80	4.49	0.576	1.24E-04	Inhibitory
Kcnt2	11.46	7.04	0.614	4.55E-04	Excitatory
Epha3	11.22	7.46	0.665	5.16E-04	Both
Nptx2	8.96	5.48	0.612	6.21E-04	Excitatory
Cntnap2	9.63	6.57	0.682	6.23E-04	Both
Zfp804a	18.28	10.17	0.557	6.58E-04	Inhibitory
Gabra1	25.59	17.08	0.667	9.90E-04	Both
Ube3a	16.38	10.58	0.646	1.36E-03	Both
Lin7a	8.71	4.58	0.525	1.41E-03	Both
Grin2b	31.69	19.15	0.604	1.58E-03	Both
Neurod6(NEX)	57.29	41.51	0.725	1.80E-03	Excitatory (Control)
Grik3	9.88	7.72	0.782	3.58E-03	Both
Nrn1	13.65	10.54	0.772	4.31E-03	Excitatory
Nptx1	7.43	5.68	0.765	5.43E-03	Excitatory
Maf	5.27	4.14	0.786	7.36E-03	Inhibitory
Gad2	21.54	15.72	0.730	1.90E-02	Inhibitory
Dlg4(PSD95)	27.55	30.96	1.124	5.54E-01	Both (*18*)

List of representative genes that were downregulated by Ezh1 cKO and expressed more strongly in d4Venus^{-} cells (differentiated neurons) than in d4Venus^{high} cells (undifferentiated NPCs) and are related to neuronal maturation. The table shows average RPKM (reads per kilobase of mRNA per million total reads) of control or Ezh1 cKO three samples and *p* values (determined with *edgeR*). Judgement for genes expressed mainly in excitatory neurons, expressed mainly in inhibitory neurons, or expressed in both excitatory and inhibitory neurons is based on (*48*).

separated undifferentiated cells and differentiated cells from *Nestin-d4Venus* transgenic mice at E14.5 (*39*). A total of 593 genes that were downregulated by Ezh1 cKO were also more highly expressed in differentiated cells than in undifferentiated cells (Figure 3e). The set of DEGs that were downregulated by Ezh1 cKO included genes expressed mainly in excitatory neurons such as *Kcnt2*, *Nptx1*, *Nptx2*, and *Nrn1*, genes expressed mainly in inhibitory neurons such as *Erbb4*, *Gad2*, *Maf*, and *Zfp804a*, and genes expressed in both excitatory and inhibitory neurons such as *Cntnap2*, *Epha3*, *Gabra1*, *Grik3*, *Grin2b*, *Lin7a*, and *Ube3a*, all of which have been implicated in regulation of neuronal maturation (Table 1). Because we constructed the cKO mice with *NEX-Cre* mice, in which Cre is exclusively expressed in excitatory neurons (*29*), we expected *Ezh1* to be knocked out specifically in excitatory neurons and guessed that Ezh1 directly regulates gene expression in excitatory neurons. Therefore, downregulation of a set of genes mainly expressed in inhibitory neurons might not be a direct effect by Ezh1 cKO, but might be a secondary effect by changing transcriptional status of excitatory neurons by Ezh1 cKO. These results suggest that *Ezh1* deletion decreases the expression levels of a set of genes related to neuronal maturation.

3.4. Ezh1 binds directly to the promoter of *Nrn1*

Given the dysregulation of neuronal maturation-related gene expression induced by *Ezh1* deletion, we next investigated whether these genes, especially excitatory neuron-related genes, were direct targets of PcG proteins. To this end, we performed ChIP-qPCR assays for H3K27me3, a histone modification catalyzed by PRC2, as well as for Ezh1, on neocortical cultures prepared from wild-type mice at E14.5 and cultured for 10 DIV under differentiation-inducing conditions. Among the genes related to neuronal maturation mentioned above, *Cpg15/Nrn1* is a direct target of several PRC1 components in human fibroblasts (*40*), but it remains unknown whether this gene is a target of PcG components in neurons. *Cpg15/Nrn1* is an activity-regulated gene whose expression in the mammalian cortex is experience-dependent (*41,42*), and it is necessary for experience-dependent spine and synapse stabilization (*43*). Knockout mice exhibit developmental delays in synapse formation and poor learning (*44*) and aberrant plasticity in visual cortical networks (*45*). We detected significant deposition of H3K27me3 at the promoter of *Nrn1* at levels similar to those apparent at the promoter of *Hoxd3* (a positive control) (Figure 4a). Also, we detected Ezh1 at the promoter of *Nrn1* (Figure 4b). These results suggest that Ezh1 directly upregulates the expression of *Nrn1* in neocortical neurons.

4. Discussion

In this study, we found that expression of Ezh1 increases during neuronal maturation in the mouse neocortical culture, as in hippocampal neurons and medium spiny neurons (*18,22*). In addition, we found that excitatory neuron-specific knockout of Ezh1 leads to downregulation of a set of genes related to neuronal maturation. Finally, we identified the *Nrn1* promoter as a novel target of Ezh1 in neurons.

Previous studies in rat hippocampal neurons and mouse MSNs show that the level of Ezh2 decreases during neuronal maturation, whereas that of Ezh1 does not (*18,22*). Our results are consistent with these reports. In addition, we showed that Ezh1 regulates expression

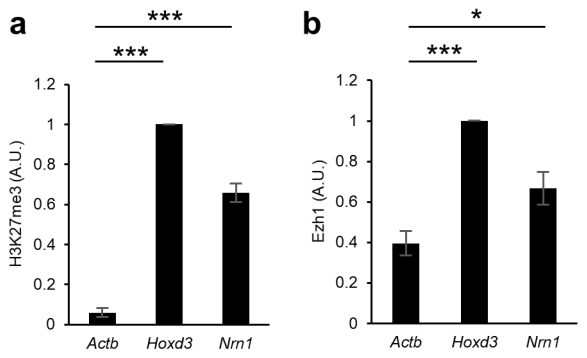


Figure 4. H3K27me3 deposition and Ezh1 binding at *Nrn1* locus in cortical neurons. Cortical neurons isolated from E14.5 mouse cortex and cultured for 10 DIV under differentiation-inducing conditions were subjected to ChIP-qPCR analysis with antibodies to H3K27me3 and Ezh1. ChIP-qPCR analysis of H3K27me3 deposition **(a)** and Ezh1 binding **(b)** at the indicated promoters. *Hoxd3* was examined as a positive control, and *Actb* as a negative control. Data are expressed as arbitrary units normalized against percent input of *Hoxd3* of each experiment. Data are means ± SEM from four independent experiments. Differences were evaluated by one-way ANOVA followed by Dunnett's multiple-comparison test. $*p < 0.05$, $***p < 0.001$.

of *Nrn1* in cortical neurons. Although *Ezh1* regulates the expression of PSD95 and binds to the *Psd95* promoter in the rat hippocampus (*18*), no other reports on target of Ezh1 have been reported in the nervous system. Ezh1 is enriched in the adult mouse brain, and knockdown of Ezh1 in the mouse prefrontal cortex attenuates sociability and promotes motivational behaviors (*28*). However, the molecular mechanisms responsible for these behavior phenotypes are not fully understood. Although *Nrn1* is a direct target of several PRC1 components in Hs68 cells (*40*), *Nrn1* has not been reported to be a direct target of PcG components in post-mitotic cells such as neurons. Our results demonstrate that *Nrn1* is a direct target of PcG components in post-mitotic neurons. It will be of interest to determine whether Ezh1 mediates neuronal maturation of cortical neurons, and if so, whether it does so by regulating Nrn1 expression.

Although Ezh1 and Ezh2 are primarily associated with gene repression, several lines of evidence suggest that they both play non-canonical roles in gene activation (*15,18,20,21*). In myotubes, Ezh1 shows genome-wide association with H3K4me3 and RNA polymerase, and with reduced levels of H3K27me3 (*20*). In rat hippocampal neurons, during neuronal maturation, binding of Ezh1 at the *Psd95* promoter increases concomitantly with acetylation of histone H3 at lysine-27 (H3K27ac) and phosphorylation of histone H3 at serine-28 (H3S28ph), both of which are active histone modifications, although their relationships with Ezh1 are unknown (*18*). Our results show that Ezh1 and H3K27me3 are associated with the *Nrn1* promoter (Figure 4), even though *Nrn1* is expressed in neurons. It is possible that the balance between H3K27me3 and other active histone modification determines the extent of *Nrn1* expression. In future studies, we will seek to determine whether another histone modification such as H3K4me3, H3K27ac, or H3S28ph is deposited on the *Nrn1* promoter, and if so, whether the amounts of such modifications change during neuronal maturation.

Our RNA-seq data indicate that 3,059 genes were upregulated by Ezh1 cKO, but 86.4% of those were < 3 RPKM on average in cKO samples, suggesting expression of these genes is not high even in derepressed status (Figure 3). Additionally, the level of H3K27me3 did not significantly differ between Ezh1 cKO and control (Figures 2c and 2d). Two studies suggested that Ezh1 depletion alone does not affect the global level of H3K27me3 (*13,22*), suggesting a compensatory role of Ezh2 upon *Ezh1* deletion. In MSNs, *Ezh1* and *Ezh2* double deletion leads to a dramatic decrease in the level of H3K27me3 and upregulation of death-promoting genes and associated neurodegenerative changes (*22*). *Ezh1* and *Ezh2* double deletion may also lead to a global decrease in the level of H3K27me3 in cortical neurons and more robust derepression of their common target genes. Moreover, other genes related to neuronal maturation were downregulated by Ezh1 cKO (Figure 3), although Ezh1 did not bind directly to their promoters (data not shown). It is possible that these genes were affected by downregulation of Nrn1. In future work, we will investigate whether these genes contribute to the defects in neuronal maturation caused by Ezh1 cKO.

Nrn1 has been reported to be down-regulated by chronic stress which worsen depression, and in the cerebral cortex and hippocampus of patients with Alzheimer's disease (*46,47*). Moreover, viral-mediated expression of Nrn1 in the hippocampus reduced symptoms of these diseases (*46,47*). Therefore, given that Ezh1 regulates Nrn1 expression, up-regulation of Ezh1 expression may treat these disorders.

In conclusion, we showed that expression of Ezh1 increases during neuronal maturation and that Ezh1 positively regulates transcription of *Nrn1*. Future studies will elucidate the role of Ezh1-mediated regulation of Nrn1 in the context of neuronal maturation.

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Original Article

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c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase-associated protein 1 (JSAP1) attenuates curcumin-induced cell death differently from its family member, JNK-associated leucine zipper protein (JLP)

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SUMMARY Curcumin, a major component of turmeric, is known to exhibit multiple biological functions including antitumor activity. We previously reported that the mitogen-activated protein kinase (MAPK) scaffold protein c-Jun NH_2-terminal kinase (JNK)-associated leucine zipper protein (JLP) reduces curcumin-induced cell death by modulating p38 MAPK and autophagy through the regulation of lysosome positioning. In this study, we investigated the role of JNK/stress-activated protein kinase-associated protein 1 (JSAP1), a JLP family member, in curcumin-induced stress, and found that JSAP1 also attenuates curcumin-induced cell death. However, *JSAP1* knockout showed no or little effect on the activation of JNK and p38 MAPKs in response to curcumin. In addition, small molecule inhibitors of JNK and p38 MAPKs did not increase curcumin-induced cell death. Furthermore, JSAP1 depletion did not impair lysosome positioning and autophagosome-lysosome fusion. Instead, we noticed substantial autolysosome accumulation accompanied by an inefficient autophagic flux in *JSAP1* knockout cells. Taken together, these results indicate that JSAP1 is involved in curcumin-induced cell death differently from JLP, and may suggest that JSAP1 plays a role in autophagosome degradation and its dysfunction results in enhanced cell death. The findings of this study may contribute to the development of novel therapeutic approaches using curcumin for cancer.

Keywords autophagy, lysosome, MAP kinase

1. Introduction

Curcumin, a polyphenolic compound extracted from turmeric (*Curcuma longa*), has been reported to have antitumor activity in various types of cancer (*1*). Curcumin is known to have a hormetic character, in which at lower concentration it possesses antioxidant activity but induces reactive oxygen species (ROS) at higher concentration (*2*). The antitumor activity of curcumin seems to be attributed to its ability to induce ROS, mitogen-activated protein kinase (MAPK) activity, and/or apoptosis (*3-5*). Autophagy, a lysosome-dependent intracellular degradation system, is regarded as one protective mechanism against a curcumin-induced stress (*3,6*). Cargo transport in autophagy is often mediated by c-Jun NH_2-terminal kinase (JNK)-interacting proteins (JIPs), a family of MAPK scaffold proteins, which can also function as motor adaptors. These include JNK/stress-activated protein kinase-associated protein 1 (JSAP1, also known as JIP3) (*7,8*) and the structurally related JNK-associated leucine zipper protein (JLP, also known as JIP4 or SPAG9) (*9,10*).

JSAP1 has been identified as a scaffold protein for the JNK MAPK signaling pathway (*7,8*), as well as an adaptor for kinesin-1 and dynein motor proteins (*11-13*). Hill *et al.* (*14*) reported that *Unc-16* (an ortholog of JSAP1 and JLP in *Caenorhabditis elegans*) is involved in the transport and clearance of autophagosomes. In mammalian neurons, Gowrishankar *et al.* (*15*) demonstrated that JSAP1 plays a role in axonal transport of lysosomes and that its dysfunction causes axonal accumulation of organelles, including

lysosomes. In addition, recent human genetic studies strongly suggested that JSAP1 mutation is associated with several neurological disorders, such as spastic diplegia and intellectual disability (*16,17*). Despite mainly expressed in neurons, several studies have reported that JSAP1 plays an important role in cancer cells (*18,19*).

We recently showed that JLP modulates p38 MAPK and autophagy in response to curcumin, leading to an overall protective effect from curcumin-induced cell death (*4*). To date, however, whether JSAP1 is involved in curcumin-induced stress remains unknown. In this study, we explored the role of JSAP1 in curcumin-induced cell death, focusing on MAPK signaling, lysosome transport, and autophagy.

2. Materials and Methods

2.1. Cell culture and reagents

Human colon carcinoma HCT116 and DLD-1 cells were cultured in Dulbecco's Modified Eagle Medium (Wako, Tokyo, Japan) and Roswell Park Memorial Institute 1640 (Wako), respectively, as described previously (*20*). Curcumin, puromycin, 4',6-diamidino-2-phenylindole (DAPI), and chloroquine were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plasmids and viral vector preparation

The following annealed oligonucleotides were inserted into pLVTH lentivirus plasmid vector (*4*) to express short hairpin RNAs (shRNAs) against human JSAP1: shJSAP1#1, forward, 5′- GATCCCCGTTTGAAGATGCTCTGGAATTCAAGAGATTCCAGAGCATCTTCAAACTTTTTGGAAA-3′; reverse, 5′- AGCTTTTCCAAAAAGTTTGAAGATGCTCTGGAATCTCTTGAATTCCAGAGCATCTTCAAACGGG-3′; shJSAP1#2, forward, 5'-GATCCCCGAACAAAGCTTTCGGCATCTTCAAGAGAGATGCCGAAAGCTTTGTTCTTTTTGGAAA-3′; reverse, 5′-AGCTTTTCCAAAAAGAACAAAGCTTTCGGCATCTCTCTTGAAGATGCCGAAAGCTTTGTTCGGG-3'. The pLVTH expression vectors for scramble shRNA (pLVTH-shScr) and JLP shRNA (pLVTH-shJLP), and the pCL20c expression vectors for tandem monomeric red fluorescent protein (mRFP) and green fluorescent protein (GFP)-tagged microtubule-associated protein 1 light chain 3 (LC3) (pCL20c-CMV-mRFP-GFP-LC3) and hemagglutinin-tagged wild-type human JLP (pCL20c-CMVΔ4-HA-JLP) were described previously (*4*). A human JSAP1 cDNA (RefSeq accession number NM_001040439) encoding the full-length JSAP1 protein was prepared as for the human JLP cDNA (*20*), and inserted into pCL20c-HA (*21*) to generate pCL20c-CMV-HA-JSAP1. To express the HA-JSAP1 at suboptimal levels in cells, the CMV promoter/enhancer region was deleted and the resulting plasmid, named pCL20c-ΔCMV-HA-JSAP1, was used for the analysis in Figure 2C. The CRISPR/Cas9-based vector for targeting *autophagy-related protein 5* (*ATG5*), PX459-sgATG5, was previously described (*4*). The following annealed oligonucleotides were inserted into *Bbs*I-digested PX459 V2.0 (Addgene; Plasmid #62988, Cambridge, MA, USA), and the resulting plasmid PX459-sgJSAP1 was used to knockout *JSAP1*: forward, 5′-CACC GCGGCGGCGTGGTGGTGTACC-3'; reverse, 5′- AAACGGTACACCACCACGCCGCCGC-3'. Lentiviral vectors were produced as previously described (*21*).

2.3. Lactate dehydrogenase (LDH) assay and immunoblotting

LDH assay using the LDH-Cytotoxic Test (Wako) was performed as described previously (*4*). Total cell lysates were prepared and analyzed by immunoblotting as previously described (*22*), using anti-ATG5 (12994), anti-Phospho-JNK (9251), anti-Phospho-p38 (4631) (each diluted 1:1,000, from Cell Signaling, Boston, MA, USA), anti-HA (1:1,000; 11867423001), anti-α-tubulin (1:3,000; T5168) (two from Sigma-Aldrich) antibodies. Anti-JLP and anti-JSAP1 antibodies were affinity purified as described previously (*20*), and used for immunoblotting at 0.25 μg/mL and 0.33 μg/mL, respectively.

2.4. Immunocytochemistry, fluorescence, and quantification

Immunocytochemistry was carried out following standard protocols as described previously (*22*), using anti-HA (1:100; 11867423001, Sigma-Aldrich) and anti-LAMP-1 (1:300; H4A3, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) antibodies. The secondary antibodies were goat Alexa Fluor 568- and 647-conjugated anti-mouse IgG antibody (both 1:500; Thermo Fisher Scientific, Waltham, MA, USA). For the mRFP-LC3 analysis in Figure 5E, the cells were fixed in 4% paraformaldehyde (Wako) and washed with 1X phosphate-buffered saline without detergent. Fluorescent images were captured using a confocal laser scanning microscope (TCS SP8; Leica, Wetzlar, Germany). Quantitative analyses of lysosome distribution (Figure 4B), and colocalization of mRFP and GFP signals with LAMP-1 (Figures 5C and 5D) were performed as described previously (*22,23*). For quantification of mRFP-LC3 puncta, the area of mRFP-LC3 in each cell was measured using ImageJ (NIH).

2.5. Statistical analysis

Significance was determined using a two-tailed unpaired Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. JSAP1 knockdown enhances curcumin-induced cell death

We first examined whether JSAP1 is involved in curcumin-induced cancer cell death. To this end, we infected cells with lentiviral vectors encoding control scramble shRNA (shScr) or two distinct JSAP1 shRNAs, shJSAP1#1 and shJSAP1#2. As shown in Figure 1A, the protein levels of JSAP1 were substantially reduced in HCT116 and DLD-1 cells expressing shJSAP1s, as compared to their respective parent cells and control cells expressing shScr. We then evaluated the effect of JSAP1 depletion on curcumin-induced cell death. Significantly increased cell death was observed in *JSAP1* knockdown cells (Figure 1B), indicating that JSAP1 plays a protective role in curcumin-induced cell death.

3.2. JSAP1 is involved in curcumin-induced cell death differently from JLP

We previously showed that JLP, a family member of JSAP1, suppresses curcumin-induced cell death (*4*). We therefore investigated whether JSAP1 acts in a similar manner to JLP or *via* a different mechanism. To this end, we first generated *JSAP1* knockout HCT116 cells using CRISPR/Cas9 system (Figure 2A and Figure S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=70*), and confirmed that *JSAP1* knockout enhanced curcumin-induced cell death (Figure 2B), as observed in *JSAP1* knockdown cells (Figure 1B). Next, we performed rescue experiments by lentivirally expressing hemagglutinin-tagged JSAP1 (HA-JSAP1) or JLP (HA-JLP), and found that HA-JSAP1, but not HA-JLP, reversed the increased cell death (Figures 2C and 2D). The expression levels of HA-JSAP1 and HA-JLP were comparable in the HCT116 cells (Figure 2C), in which the percentage of hemagglutinin-positive cells were almost 100% (Figure S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=70*). In addition, a further increase of cell death was observed by *JLP* knockdown in *JSAP1* knockout cells (Figure S3, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=70*). Taken together, these results indicate that JSAP1 inhibits curcumin-induced cell death in a different manner to JLP.

3.3. JSAP1 protects cell death induced by curcumin independently from JNK and p38 MAPK signaling pathways

As curcumin is known to activate JNK and p38 MAPK

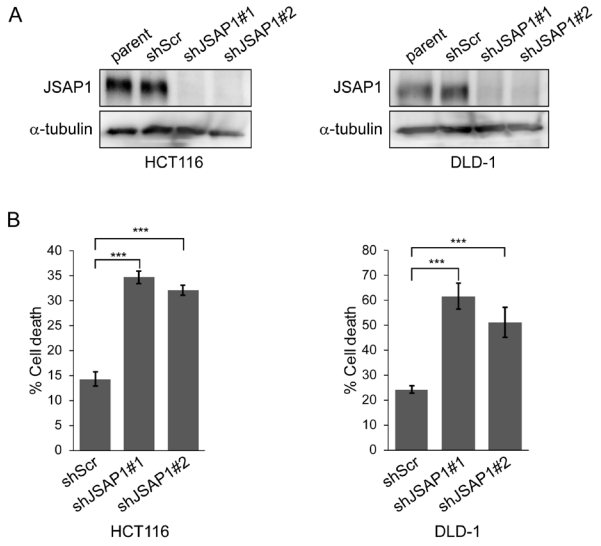

Figure 1. *JSAP1* knockdown enhances curcumin-induced cell death. **(A)** HCT116 and DLD-1 cells were transduced or not (parent cells) with lentiviral vectors for shScr, shJSAP1#1, or shJSAP1#2, as indicated, and the expression levels of JSAP1 were analyzed by immunoblotting with an anti-JSAP1 antibody. α-tubulin was used as a loading control. **(B)** HCT116 and DLD-1 cells expressing shScr, shJSAP1#1, or shJSAP1#2 were treated with or without 40 μM curcumin for 24 hours and subjected to LDH cell death assay as described in Materials and Methods. Quantitative data are expressed as means ± S.E.M of three independent experiments. $^{***}p < 0.001$.

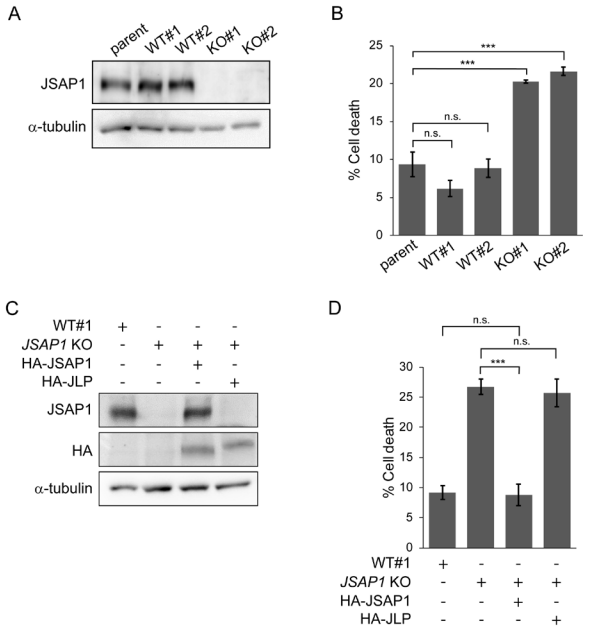

Figure 2. JSAP1 is involved in curcumin-induced cell death independently from JLP. (A) Expression of JSAP1 in HCT116 parental cells (parent), *JSAP1* wild-type clones (WT#1 and WT#2), and knockout (KO) clones (KO#1 and KO#2) was examined by immunoblotting using anti-JSAP1 antibody. **(B)** Parent, WT#1, WT#2, KO#1, and KO#2 cells were treated with 40 μM curcumin for 24 hours and subjected to a cell death assay, as in Figure 1B. **(C)** WT#1 and KO#1 (*JSAP1* KO) cells were transduced or not with lentiviral vectors for HA-JSAP1 or HA-JLP, as indicated, and the expression levels of JSAP1 and HA-JSAP1 or HA-JLP were analyzed by immunoblotting with antibodies against JSAP1 and hemagglutinin (HA). **(D)** The cells in (C) were treated with 40 μM curcumin for 24 hours and subjected to a cell death assay, as in Figure 1B. α-tubulin was used as a loading control. Quantitative data are expressed as means ± S.E.M of three independent experiments. $^{***}p < 0.001$; n.s., not significant.

pathways (*24*), we asked whether JSAP1-mediated activation of JNK and/or p38 contributes to curcumin-induced cell death. We first performed immunoblotting with anti-phospho-JNK and -p38 antibodies. The activation of both JNK and p38 was observed in control HCT116 cells after curcumin challenge as expected, however, which was not attenuated by *JSAP1* knockout (Figure 3A). Furthermore, we examined the effects of JNK and p38 inhibitors, SP600125 and SB203580, on cell death induced by curcumin. The JNK inhibitor SP600125 reduced cell death in control and *JSAP1* knockout cells (Figure 3B), suggesting a pro-cell death role of JNK pathway. The p38 inhibitor SB203580 showed no significant difference of cell death in control and *JSAP1* knockout cells (Figure 3C). Together, these results strongly suggest that JSAP1 protects curcumin-induced cell death independently from JNK and p38 MAPK signaling pathways.

3.4. JSAP1 may play a role in autophagosome degradation

It is known that curcumin alters lysosomal positioning (*4,24*) and activates autophagy to counteract curcumin-induced cell death (*4*). Indeed, the gene inactivation of *ATG5*, an essential gene for autophagy, enhanced curcumin-induced death of HCT116 cells (Figure S4, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=70*).

We first assessed the distribution of lysosomes in curcumin-treated control and *JSAP1* knockout cells by immunostaining with an antibody against LAMP-1, a lysosomal marker. As shown in Figure 4, there was no significant difference in the distribution between control and *JSAP1* knockout cells, suggesting that JSAP1 is not involved in the regulation of lysosomal positioning. Next, we asked whether autophagosome-lysosome fusion is impaired in *JSAP1* knockout cells. To examine this possibility, we lentivirally expressed mRFP-GFP-LC3 (*25*), a tandem fluorescent-tagged LC3 protein useful for dissecting the autophagosome maturation process, in control and *JSAP1* knockout cells, treated cells with curcumin, and analyzed by immunostaining with an anti-LAMP-1 antibody. As shown in Figures 5A-5D, the percentage of colocalization of mRFP with LAMP-1 was much higher, compared to that of GFP with LAMP-1 in both control and *JSAP1* knockout cells. Besides, most of GFP-positive punctum were mRFP-postive in control cells, which was consistent in *JSAP1* knockout cells (Figures 5A-5D). These results suggest that JSAP1 is dispensable for the autophagosome-lysosome fusion.

During analysis with mRFP-GFP-LC3, we noticed that the number of mRFP puncta in curcumin-treated *JSAP1* knockout cells was higher than that in curcumin-treated control cells. Indeed, quantitative analysis showed a significant increase of the number of mRFP puncta in *JSAP1* knockout cells (Figures 5E and 5F). However, when we blocked the autophagic flux by impairing autophagosome-lysosome fusion with chloroquine, no significant difference in number of mRFP puncta was observed between control and *JSAP1* knockout cells (Figures 5E and 5F). Taken together, these results may indicate that JSAP1 plays a role in autophagosome degradation and its dysfunction results in enhanced cell death.

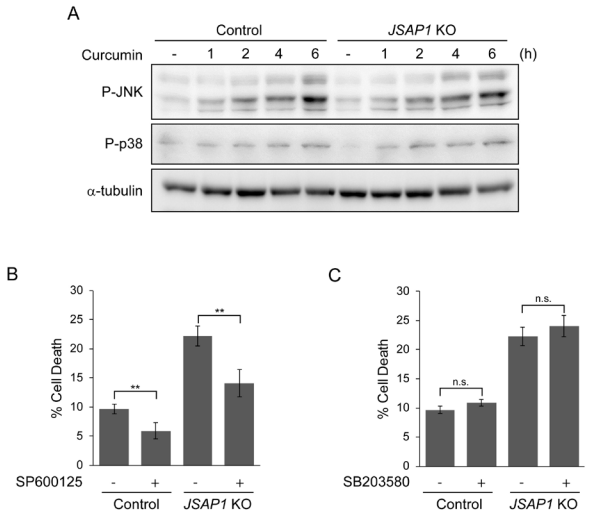

Figure 3. JSAP1 protects cell death induced by curcumin independently from JNK and p38 MAPK signaling pathways. (A) *JSAP1* WT#1 (control) and KO#1 (*JSAP1* KO) HCT116 cells were treated with or without 40 μM curcumin for the indicated time points and subjected to immunoblotting using anti-Phospho-JNK (P-JNK) and -Phospho-p38 (P-p38) antibodies. **(B, C)** Control and *JSAP1* KO cells in (A) were treated with 40 μM curcumin in the presence or absence of 40 μM JNK inhibitor SP600125 (B) or SB203580 (C), as indicated, and subjected to a cell death assay, as in Figure 1B. Quantitative data are expressed as means ± S.E.M of three independent experiments. $^{**}p < 0.01$; n.s., not significant.

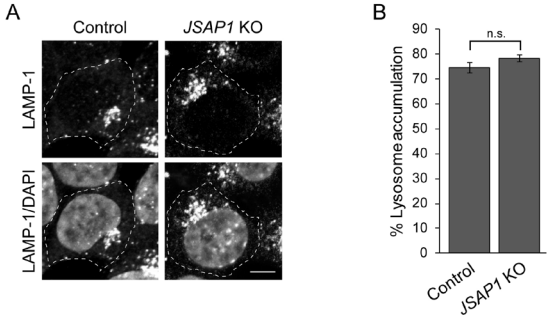

Figure 4. *JSAP1* knockout does not impair lysosome positioning in response to curcumin. (A) *JSAP1* WT#1 (control) and KO#1 (*JSAP1* KO) HCT116 cells were treated with 40 μM curcumin for12 hours, fixed and immunostained using an anti-LAMP-1 antibody. Nuclei were stained with DAPI. Z-stack images were obtained by confocal microscopy. Scale bar, 10 μm. **(B)** Quantification of the lysosome distribution results shown in (A). Quantitative data are expressed as means ± S.E.M of three independent experiments. The perinuclear region was defined as 0-5 μm from nuclear rim. At least 20 cells per experiment were analyzed. n.s., not significant.

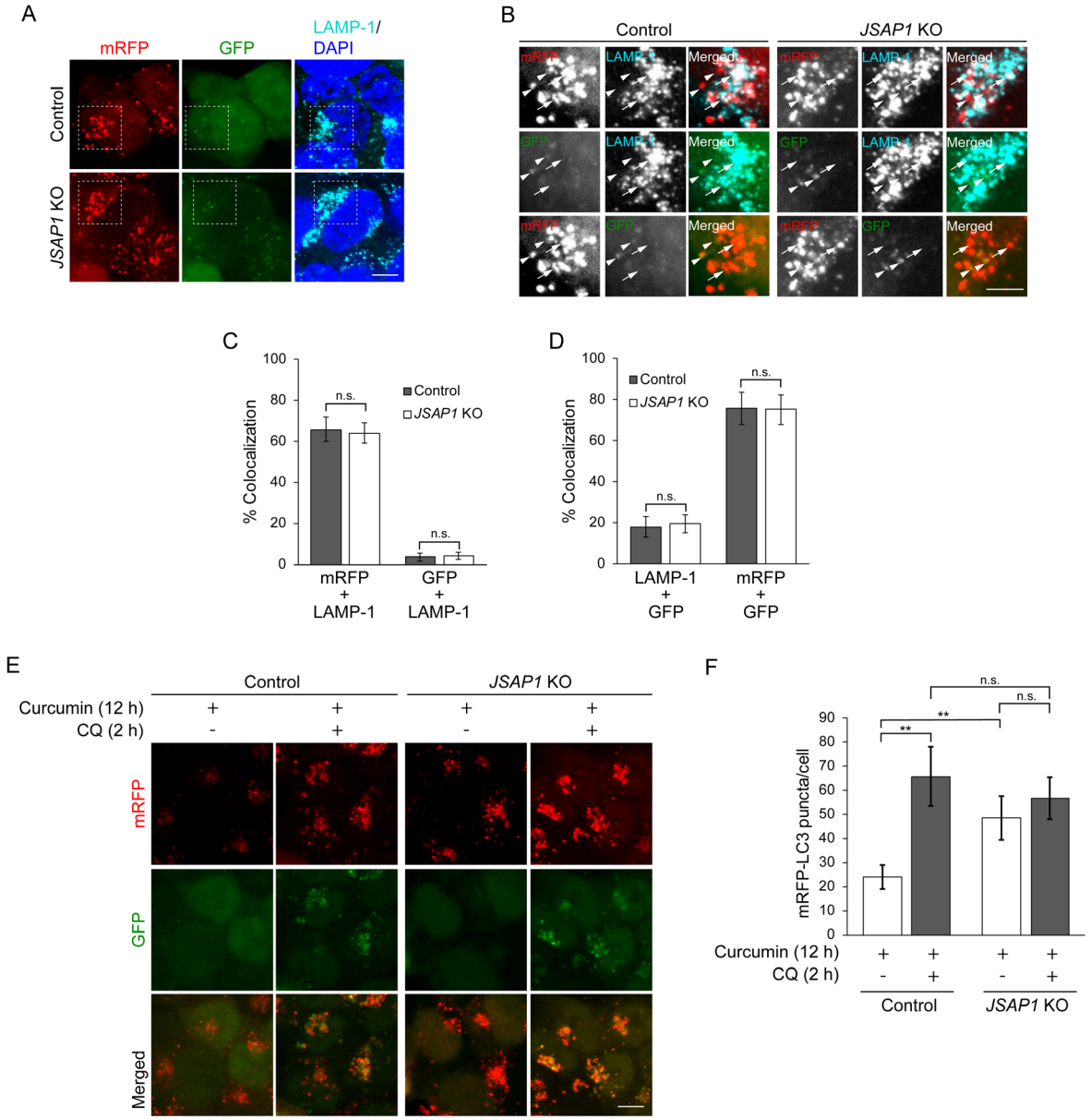

Figure 5. JSAP1 may play a role in autophagosome degradation. (A) *JSAP1* WT#1 (control) and KO#1 (*JSAP1* KO) HCT116 cells were infected with a lentiviral vector for mRFP-GFP-LC3, treated with 40 μM curcumin for 12 hours, immunostained using an anti-LAMP-1 antibody, fixed, and analyzed by confocal microscopy. Nuclei were stained with DAPI. Areas in the boxes indicated by dotted-lines are shown at a higher magnification in (B). Scale bar, 10 μm. **(B)** Confocal images of the boxed areas in (A) were merged to examine the colocalization of mRFP and LAMP-1, GFP and LAMP-1, and GFP and mRFP. Arrows indicate typical examples of colocalization of mRFP and LAMP-1. Arrowheads indicate typical examples of colocalization of mRFP and GFP. Scale bar, 2 μm. **(C, D)** Quantification of the results of colocalization analyses in (A) and (B). Colocalization of mRFP and GFP signals with LAMP-1 (C) and LAMP-1 and mRFP signals with GFP (D). **(E)** The control and *JSAP1* KO cells expressing mRFP-GFP-LC3, as in (A), were treated with or without 50 μM chloroquine (CQ) in the presence of 40 μM curcumin for the indicated times, fixed, and analyzed by confocal microscopy. Scale bar, 10 μm. **(F)** Quantification of mRFP-LC3 puncta per cell in (E). Quantitative data are expressed as means ± S.E.M of three independent experiments. At least 20 cells per experiment were analyzed. $^{**}p < 0.01$; n.s., not significant.

4. Discussion

In the present study, we explored the role of JSAP1 in curcumin-treated cells and found that JSAP1 depletion enhances curcumin-induced cell death, indicating a protective role of JSAP1 in response to cell death stimulation. We recently reported that JLP, a JSAP1 family member, also possesses a similar function for curcumin (*4*). In addition, several studies have shown functional redundancy between JSAP1 and JLP in some cellular processes (*26-28*). However, due to the following observations, it is unlikely that JSAP1 attenuates curcumin-induced cell death in a similar way with JLP: (1) *JLP* knockdown in *JSAP1* knockout cells further increased curcumin-induced cell death (Figure S3), and (2) exogenously expressed JLP, but not JSAP1, was unable to cancel the increase of cell death seen in curcumin-treated *JSAP1* knockout cells, although the

expression levels of both the exogenous JSAP1 and JLP proteins were comparable (Figures 2C and 2D).

It is known that JIP family proteins, including JSAP1, can function as scaffolding proteins for JNK signaling cascades (*29,30*). However, upon curcumin challenge, no or very little effect of downregulation of JNK activation was observed in *JSAP1* knockout cells (Figure 3A). Furthermore, the results of experiments using SP600125, a JNK inhibitor, did not support the involvement of JSAP1-JNK pathways in curcumin-induced cell death, but rather suggest a pro-cell death role of JNK pathways (Figure 3B). Other JIP family proteins, such as JIP1 and JIP2, might mediate JNK signaling pathways. To date, it is an open question how appropriate MAPK scaffold proteins would be selected for proper MAPK activation in response to extracellular and intracellular stimuli.

Autophagy consists of several sequential steps, including autolysosome formation. We previously demonstrated that JLP plays a role in lysosome positioning through retrograde transport along microtubules and its dysfunction leads to the inhibition of autophagosome-lysosome fusion (*4*). However, our results in this study showed that JSAP1 depletion did not impair lysosome positioning and autophagosome-lysosome fusion in curcumin-treated cells (Figures 4 and 5A-5D). Instead, we noticed substantial autolysosome accumulation accompanied by an inefficient autophagic flux in *JSAP1* knockout cells (Figures 5E and 5F). During the preparation of this manuscript, another study by Cason *et al.* (*31*) reported the accumulation of stationary autolysosomes in the proximal axon of hippocampal neurons after JIP3/JSAP1 depletion. Together, it is likely that JSAP1 is involved in the later step of autophagy, *i.e.*, the steps after autolysosome formation. Further study is needed to clarify the molecular mechanism of how JSAP1 depletion leads to the accumulation of autolysosomes.

The precise mechanism of how JSAP1 protects cells from curcumin-induced cell death is unclear at present. Autolysosome accumulation has been associated with inefficient autophagic lysosome reformation, a lysosome recycling process at the terminal step of autophagy (*32,33*). As a result, the number of free lysosomes is reduced and eventually hinders autophagic clearance, leading to cell death during the peak of autophagy (*34-36*). Thus, the autolysosome accumulation with attenuated autophagic flux in *JSAP1* knockout cells may increase sensitivity toward curcumin-induced cell death. However, we cannot rule out the possibility that other unknown functions of JSAP1 may be involved in curcumin-induced cell death.

Curcumin has been viewed as a potential chemotherapeutic agent for cancer. The findings of this study would contribute to the development of novel therapeutic approaches for cancer.

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Original Article

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Evaluation of pathogenicity and therapeutic effectiveness of antibiotics using silkworm *Nocardia* infection model

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SUMMARY *Nocardia* is a ubiquitous environmental microbe that causes nocardiosis against immunosuppressed and immunocompromised hosts. The assay system for the quantitative evaluation of virulence of *Nocardia* sp. or therapeutic effectiveness of antimicrobials for treatment of nocardiosis is not established so far. In this study, we established an infection model of *Nocardia* sp. using silkworm as an alternative animal model. We found that all tested *Nocardia* sp. such as *Nocardia asiatica*, *Nocardia elegans*, *Nocardia exalbida*, *Nocardia farcinica*, and *Nocardia nova* killed silkworm and their killing ability were different by species. *N. farcinica* showed higher pathogenicity among tested strain, similar to the mouse model as previously reported. In addition, we found that antimicrobials such as amikacin and minocycline showed therapeutic effectiveness in silkworms infected with N. farcinica, and we could determine effective doses 50 (ED_{50}) values. These results suggest that silkworm is a useful alternative animal to evaluate the pathogenicity of *Nocardia* pathogen and the therapeutic effects of antimicrobials against *Nocardia* sp. in a quantitative manner.

Keywords *Nocardia*, virulence, silkworm infection model, nocardiosis, *N. farcinica*

1. Introduction

Nocardia species are Gram-positive slow-growing bacteria and an acid-fast aerobic actinomycete, ubiquitous in the environment such as soil organic material and water. *Nocardia* sp. rare, although, sometimes causes localized or systemic disease in humans and animals. Manifestations of the disease range from cutaneous infection caused by traumatic inoculation of the organism in a normal host to severe pulmonary or central nervous system (CNS) disease in an immunosuppressed host (*1*). The severe bacteremia by *Nocardia* shows high mortality. The systemic review suggested that overall all-cause mortality was 40% (*2*). Recently, the genus *Nocardia* expanded and currently contains more than one hundred species (*3*), and *N. asteroids*, *N. brasiliensis*, *N. cyriacigeorgica*, *N. farcinica*, and *N. nova* have been reported as the main bacterial species that cause nocardiosis in the world (*4,5*). However, there is little literature regarding elucidating pathogenic properties of *Nocardia* sp. (*6*). Mice infection models have been established (*7*) and therapeutic efficacy of antimicrobials was reported (*8,9*), however, quantitative evaluation of the effectiveness of those antimicrobials were not reported to the best of our knowledge.

Nowadays, conducting experiments using mammals has been limited due to ethical problems and high breeding costs (*10*). Recently, silkworm, *Bombyx mori*, has paid attention as an alternative animal model since it causes less ethical issues and required small cost and space (*11*). We further established several pathogens infection model to evaluate antimicrobials using silkworm, such as *Staphylococcus aureus* (*12*), *Candida albicans* (*12*), *Cryptococcus neoformans* (*13*), and even for acid-fast aerobic *Mycobacterium* species (*14-16*). In this study, we demonstrated that a silkworm model is a useful model to evaluate the pathogenicity of *Nocardia* sp. and therapeutic efficacy of antimicrobials against *N. farcinica*, the most frequently isolated from the clinical.

2. Materials and Methods

2.1. Bacterial strains, identification and culture

Five pathogenic *Nocardia* strains were isolated from the different patients with the lower respiratory tract infection at Nippon Medical School Hospital, Japan, in 2014. Identification of genus of five strains from clinical specimens was based on positive Gram stain (Gram-positive filamentous bacilli) and positive modified acid-fast stain, colonial morphotypes and conventional biochemical reactions.

The identification of the bacterial species was performed by analyzing the 16S rRNA gene sequence. A portion of the colonies on BHI agar was picked up by toothpick and added to the PCR reaction solution. The primers were E9F (5'-GAGTTTGATCCTGGCTCAG-3'), E1541R (5'-AAGGAGGTGATCCAGCC-3') (*17*) were used. Ten cycles of 98°C for 10 sec, 55°C for 30 sec, and 68°C for 1 min 30 sec were performed and followed by 15 cycles of 98°C for 10 sec, 45°C for 30 sec, and 68°C for 1 min 30 sec were performed. The amplified DNA fragments were separated by agarose gel electrophoresis (1%, 100 V, 30 min). The target DNA fragments were cut from the gel and extracted using the QIAEX II Gel Extraction Kit. 16S rRNA sequences were obtained by cycle sequencing reactions using the Sanger method with the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Tokyo, Japan) and analyzed by ABI PRISM 3130xl genetic Analyzer (Thermo Fisher Scientific, Tokyo, Japan). The obtained sequences were used to perform a blast search against the Pubmed 16S rRNA database (BLAST: Basic Local Alignment Search Tool (nih.gov)). The bacterial species with the highest nucleotide sequence homology was determined as the species of the sample. These strains were stored at - 80°C using a microbank tube (Iwaki & Co., Ltd., Tokyo, Japan). These stocked strains were spread onto brain heart infusion agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) or 5% sheep blood agar (Eiken Chemical Co., Ltd., Tokyo, Japan), and cultured at 37°C in an atmosphere of 5 % carbon dioxide (CO_2) for three days.

2.2. Silkworm rearing

Silkworm eggs (Hu·Yo × Tukuba·Ne) were purchased from Ehime Sansyu (Ehime, Japan) and fed Silkmate 2S (Katakura Industries Co., Ltd. Tokyo, Japan) until they developed to the fourth molted larva. On the first day of fifth-instar larvae, silkworms were fed for one day with an antibiotic-free artificial food, Silkmate (Katakura Industries Co., Ltd., Tokyo, Japan).

2.3. Comparison of silkworm killing ability of *Nocardia* sp. strain

The colonies cultured on BHI agar medium (Eiken Chemical, Tokyo) were picked up using a loop, suspended in sterile saline and adjusted to OD_{600} = 0.175. A two-step dilution series of this bacterial solution was prepared using sterile saline and injected 50 µL into the hemolymph of silkworms. Silkworms were kept in an incubator at 27°C without feeding, and the number of surviving silkworms on 4 days after injection was counted (n = 3). To calculate the number of bacterial cells injected into the silkworm, the solution used for injection was diluted with sterile saline solution, and 100 µL was spread on BHI agar medium. After incubated under aerobic conditions at 37°C for three days and appeared colonies were counted. The LD_{50} value was defined as the activity that killed half of the silkworms (50% lethality).

2.4. Antimicrobial susceptibility test

Antimicrobial activity was determined by the microdilution method according to CLSI (*18*). After 72 hours of incubation at 37°C under aerobic conditions, bacterial growth was visually confirmed. The minimum concentration of antimicrobial agent that completely inhibited bacterial growth was determined as the minimum inhibitory concentration (MIC). The antimicrobial agents used were amikacin, minocycline, imipenem, sulfamethoxazole, trimethoprim, linezolid, erythromycin, oxacillin (FUJIFILM Wako Pure Chemical Corporation Tokyo, Japan), levofloxacin (Tokyo Pure Chemical Corporation Tokyo, Japan).

2.5. Therapeutic trial for *Nocardia* infected silkworms

Determination of the 50% effective dose (ED_{50}, µg/larva) of antimicrobial agents against *N. farcinica* TUTN006 strain was performed as described previously by Hamamoto H *et al.* (*12*).

Fifty microliters of *N. farcinica* TUTN006 (3.3×10^7 CFU/mL) suspended in 0.9% sterile saline were injected into hemolymph of silkworm on the day 2 of 5^{th} molted. Then 50 µL of antimicrobial agent diluted in saline was injected into the hemolymph (n = 3) by 27G needle with a syringe (Terumo, Tokyo Japan). After injection, the silkworms were kept without feeding in an incubator at 27°C. The survival number of silkworms were counted on day 3. The ED_{50} values were determined as the amount of drug of silkworm required for 50% survival.

3. Results

3.1. Establishment of a *Nocardia*-infected silkworm model

First, we performed the identification of bacterial species, the clinical isolates of *Nocardia* strain used in this study named TUTN001 to 007, by 16S rRNA sequencing. These strains were identified as *N. asiatica*, *N. elegans*, *N. exalbida*, *N. farcinica*, and *N.*

Table 1. Identification of bacterial species

Strain Name	Identification	Identities (%)	Top hit of Accession no.	Origin
TUTN001	*Nocardia asiatica*	1425/1432 (99)	NR_117244.1	Clinical
TUTN002	*Nocardia elegans*	1443/1443 (100)	NR_042353.1	Clinical
TUTN003	*Nocardia elegans*	1443/1443 (100)	NR_042353.1	Clinical
TUTN004	*Nocardia elegans*	1443/1443 (100)	NR_042353.1	Clinical
TUTN005	*Nocardia exalbida*	1432/1432 (100)	NR_117321.1	Clinical
TUTN006	*Nocardia farcinica*	1446/1446 (100)	MN100049.1	Clinical
TUTN007	*Nocardia nova*	1442/1444 (99)	AB630968.1	Clinical

Table 2. LD_{50} of *Nocardia* in silkworm

Strain Name	Species	LD_{50} at 4days (CFU/larva)
TUTN001	*N. asiatica*	2.0×10^6
TUTN002	*N. elegans*	7.0×10^5
TUTN003	*N. elegans*	6.3×10^5
TUTN004	*N. elegans*	1.4×10^5
TUTN005	*N. exalbida*	1.4×10^4
TUTN006	*N. farcinica*	1.4×10^4
TUTN007	*N. nova*	4.6×10^5

nova, respectively (Table 1). We injected suspension of these cells into silkworm hemolymph, and these strains killed silkworms. Next, we compared the killing ability of these species was examined by determining the LD_{50} value. The results showed that the killing ability of silkworms differed among the species, with *N. farcinica* and *N. exalbida* having the lowest LD_{50} values compared to the other *Nocardia* species (Table 2). These results suggested that *N. farcinica* and *N. exalbida* showed high virulence in the silkworm model. This result was consistent with the previous report (*7*) that *N. farcinica* showed higher virulence than *N. nova* in a mouse model. Therefore, we considered that the *Nocardia*-infected silkworm model was established.

3.2. ED_{50} of antibiotics against silkworm *N. farcinica* infection model

Next, the drug susceptibility test of the bacteria used in this study was conducted. As a result, all strains used in the experiment showed high susceptibility to amikacin, minocycline, linezolid and erythromycin. On the other hand, several strains showed low susceptibility to oxacillin, levofloxacin and ST fixed-dose combination (Table 3). These results are consistent with reports that a large proportion of *Nocardia* spp. are highly susceptible to amikacin, linezolid and minocycline (*19-21*). Next, we investigated whether the therapeutic efficacy of antimicrobial agents could be assessed using a *Nocardia*-infected silkworm model. We examined amikacin and minocycline, and found that these antimicrobials showed therapeutic effects on *Nocardia*-infected silkworms in a dose-dependent manner (Table 4). The ED_{50} values were determined as 5.2 μg/larva for amikacin, and 60 μg/larva for minocycline. The ED_{50} values for minocycline was higher than expected from its MIC, although, these results were consistent with the previous report in the mice model (*22*). In that mice model, amikacin treatment reduced the number of bacterial cells in the brain of *Nocardia*-infected mice. In contrast, minocycline treatment did not reduce cells in the brain, although amikacin and minocycline showed high antibacterial activity against the strain used for infection assay *in vitro*. Therefore, we concluded that a quantitative evaluation system for the therapeutic effects of antimicrobial agents using the *Nocardia*-infected silkworm model was established.

4. Discussion

In this study, we aimed to establish a model of *Nocardia* infection using the silkworm. The silkworm is an alternative animal, with the advantages of low cost, fewer ethical issues, and the ability to use large numbers of individuals for experiments. We found that silkworms were killed by all tested strains. Furthermore, killing ability of *Nocardia* sp. against silkworm is different among tested strain. The results were consistent with reports from mouse models (*7*). Therefore, we conclude that we have established a *Nocardia* infection model using the silkworm. Recently, novel virulence factors of *S. aureus*, *Serratia marcescens*, *C. neoformans* and enterohemorrhagic *Escherichia coli* have been identified using the silkworm model (*23-28*). These virulence factors also required for exerting virulence of pathogens against the mouse. So far, there has been little evaluation of virulence factors in *Nocardia* using animal models (*7,29*). Thus, this silkworm model would facilitate the research regarding the virulence of *Nocardia* sp.

Furthermore, we found that clinically used antimicrobial agents showed therapeutic effectiveness on *Nocardia*-infected silkworms. There are very few studies that have evaluated the therapeutic effects of antimicrobials in mice (*9,30,31*), and these studies were not quantitative. Thus, this study is the first report to quantitative evaluation of the therapeutic effect of antimicrobial agents in the *Nocardia* infection model. In addition, we found a discrepancy in the therapeutic efficacy of amikacin and minocycline in the silkworm model, compared with the susceptibility of these antimicrobials against *N. farcinica in vitro*.

Table 3. Antimicrobial susceptibility to *Nocardia*

Strain Name	*Species*	MIC(μg/mL)							
		AMK	MINO	LZD	MPIPC	LVFX	TMP/SMX	IMP	EM
TUTN001	*N. asiatica*	< 0.8	< 0.8	< 0.8	13	50	< 0.8	3.1	< 0.8
TUTN002	*N. elegans*	< 0.8	< 0.8	< 0.8	6.3	3.1	13	< 0.8	< 0.8
TUTN003	*N. elegans*	< 0.8	< 0.8	< 0.8	25	6.3	50	< 0.8	< 0.8
TUTN004	*N. elegans*	< 0.8	< 0.8	< 0.8	6.3	3.1	< 0.8	< 0.8	< 0.8
TUTN005	*N. exalbida*	< 0.8	< 0.8	< 0.8	6.3	3.1	50	< 0.8	< 0.8
TUTN006	*N. farcinica*	0.8	< 0.8	< 0.8	> 100	6.3	< 0.8	< 0.8	< 0.8
TUTN007	*N. nova*	< 0.8	< 0.8	< 0.8	50	13	25	< 0.8	< 0.8

Table 4. ED_{50} of antifungal agents in a silkworm model with *N. farcinica*

Antimicrobial	ED_{50} (μg/larva)
amikacin	5.2
minocycline	60

This trend was consistent with that reported in the mouse model (*9*). Therefore, the silkworm model is useful that to evaluate the therapeutic effectiveness of antimicrobial agents against *Nocardia* infections. We further speculated that this model is applicable to the development of novel antimicrobials that are effective against *Nocardia* infection.

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Original Article

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Factors related to the composition and diversity of wound microbiota investigated using culture-independent molecular methods: a scoping review

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SUMMARY All open wounds are often colonized by commensal microbes as a loss of skin can provide a ready portal of entry for microorganisms. Although the wound microbiota is known to be associated with wound infection and with delayed healing, the factors related to the formations of wound microbiota contributing to such poor clinical outcomes are not clear and have not led to effective infection prevention interventions. This review aimed to scope the factors related to the composition and diversity of wound microbiota that have been investigated using culture-independent molecular methods. Original articles on wound microbiota published from January 1986 to February 2020 were included in this review. Thirty-one articles met the inclusion criteria and were grouped according to wound types: chronic, acute, and animal model wounds. The factors identified were categorized according to patient characteristics, wound characteristics, treatment, and sampling. Although some studies reported the effect size of the factors, the values were small. No studies elucidated the mechanism of wound microbiota formation. The results of this scoping review highlight that the factors associated with the diversity of wound microbiota are poorly understood and that further studies are needed.

Keywords microbiota, biodiversity, sequence analysis, whole genome sequencing, wound infection

1. Introduction

A wound involves an interruption to the structure and function of fundamental skin tissue (*1*). Wounds result from a variety of mechanisms, such as surgical intervention, injury, extrinsic factors, and underlying conditions, and they are often classified as a result of their underlying cause into acute wounds, such as surgical wounds and burns, and chronic wounds, such as leg ulcers, diabetic foot ulcers (DFUs), and pressure ulcers (*2*). Wounds are exposed to external bacteria from the skin defect, and bacteria colonized on the wound bed assemble into a microbiota. Wound microbiota causes wound infection, which increases financial burdens on patients and the healthcare system and consequently increases mortality (*3-5*). For the development of infection, three elements are necessary: infectious host, source of infection, and route of transmission. Thus, interventions to improve host immunity (*e.g.* nutritional management and treatment for the underlying disease), to reduce the bioburden on the wound bed (*e.g.* wound cleansing and debridement), and to break the route of transmission (*e.g.* using wound dressing and disinfection of peri-wound skin) have been implemented for patients with wounds (*6-9*). However, despite these preventative measures taken, wound infections continue, and new approaches to wound infection prevention are needed.

More than 100 trillion symbiotic microorganisms, including bacteria, archaea, viruses, and eukaryotic microbes, live on and within the human body (*10*), and ensuring wound sterility is not possible. In infection control, the culture method has been used to assess the bacterial bioburden; however, this method underestimates the bacterial bioburden in a wound because most microorganisms circulating in the environment are not easily cultured (*11*). Given this, a growing number of studies have investigated wound

microbiota using culture-independent methods. Those previous studies have reported that wound microbiota is associated both with wound healing and wound infection (*12,13*). However, the factors related to the formations of wound microbiota contributing to such poor clinical outcomes are not clear. Thus, effective interventions targeting wound microbiota have also not been established. This scoping review aimed to identify which factors are related to the composition and diversity of wound microbiota in studies that used culture-independent molecular methods. A better understanding of the factors related to the diversity and composition of wound microbiota may lead to innovative preventive wound infection strategies, such as intervening in those factors to inhibit an adverse wound microbiota formation or alter it in a more positive direction. Furthermore, the results of this review are likely to be useful for researchers who study wound microbiota in helping to determine the direction of future research.

2. Materials and Methods

2.1. Protocol and registration

A review protocol has not been published. We used the Preferred Reporting Items for Systematic reviews and Meta-Analyses extension for Scoping Reviews checklist to guide this review (*14*).

2.2. Eligibility criteria

For this study, culture-independent molecular methods were defined as methods for identifying microbiota based on direct analysis of DNA without any culturing step. We limited our search to research articles published from January 1, 1986, to February 17, 2020 because the earliest device using a culture-independent molecular method was developed in 1986 (*15*). The languages of publications were restricted to English and Japanese. We included studies that involved participants of any age who had been described as having wounds in any setting, including acute care, aged care, and at home. Studies using animal wound models were also included because such experiments are required to identify the function of the researched microbiota. We included original research in this scoping review. Literature reviews, meta-analysis, practice guidelines, editorials, case studies, letters, conference notes/abstracts, posters, and oral presentations were excluded.

2.3. Information sources

To identify potentially relevant documents, the following bibliographic databases were searched: PubMed and the Cumulative Index to Nursing & Allied Health Literature. Additionally, we searched the Japan Medical Abstracts Society (JAMAS) database to collect articles in Japanese. The search strategies were developed through team discussion. The final search results were exported to Mendeley and duplicates were removed prior to screening by two researchers.

2.4. Search

The search was performed using a combination of search terms, including "burns" OR "open fractures " OR "lacerations" OR "surgical wound" OR "penetrating wounds" OR "abrasion" OR "pressure ulcer" OR "pressure injury" OR "leg ulcer" OR "diabetic foot" OR "varicose ulcer" OR "traumatic wound" OR "acute wound" OR "chronic wound" AND "microbiota" OR "microbiome". In the JAMAS database, we used the same combination of keywords in Japanese.

2.5. Selection of sources of evidence

Potentially relevant literature was imported into Rayyan for screening (*16*). Titles and abstracts were screened by two researchers (MK and YK) independently, and those that clearly did not fit the inclusion criteria were excluded. Potentially eligible full-text articles were screened for inclusion by two independent reviewers (MK and YK) according to the inclusion criteria. Disagreements on study selection were resolved through discussion.

2.6. Data charting process

Table S1 (online data: *http://www.ddtjournal.com/action/getSupplementalData.php?ID=73*) provides an overview of all included manuscripts. A data-charting form was developed by one author (MK) to determine which variables to extract. The form captured the relevant information concerning a study's characteristics and the specific factors found to be related to the composition and diversity of wound microbiota. To assess the factors found to be related, the composition and diversity of wound microbiota were evaluated based on the relative abundance of bacteria and according to the index of alpha and beta diversity, respectively. Alpha diversity is species richness within a single microbiota and Beta diversity shows the differences in the microbiota between different environments (*17*). Data were extracted by a single author (MK) and verified by co-authors (YK and GN). Discrepancies in the extracted data were resolved through discussion between the three authors.

2.7. Data items

We extracted the following data from eligible literature identified in our search: the study attributes: author (s), publication year, country, and title; study objectives; study design; study population: sample size, human

or animal, wound type, and sample type; the method used to obtain the microbiota data: analysis techniques, sequencer, and the region of bacterial DNA; outcome measures: alpha and beta diversity indices; factors related to the composition and diversity of wound microbiota.

2.8. Synthesis of results

We grouped the studies according to wound types and presented them in three tables: chronic wounds (Table S2) (online data: *http://www.ddtjournal.com/action/getSupplementalData.php?ID=73*), acute wounds (Table 1), and animal models (Table 2). The factors related to the composition and diversity of wound microbiota were summarized for each study along with the study attributes, objective, study design, populations, and analysis techniques. Where we identified a study that had investigated the effect on wound microbiota diversity in relation to certain factors, we summarized the indicators of the effect size.

3. Results

3.1. Selection of the sources of evidence

Figure 1 shows a flow chart of the study selection process. We identified 792 records through the database searches. Following de-duplication, 743 titles and abstracts were screened for eligibility. Of these, 53 studies were retained for full-text screening and 22 failed to meet the inclusion criteria. Of the excluded full texts, 12 studies (54.5%) did not investigate factors related to the composition and diversity of wound microbiota. Five studies (22.7%) did not meet the inclusion criteria in relation to publication type. Three studies (13.6%) investigated microbiome samples collected from non-wound sites. One study (4.5%) did not use culture-independent molecular methods. One study (4.5%) investigated certain factors but found no significant differences between the groups. The remaining 31 studies met our eligibility criteria.

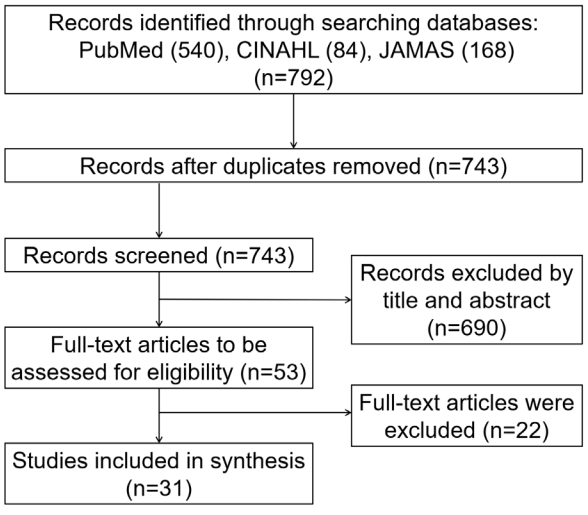

Figure 1. Flow diagram of the study selection process. CINAHL, the Cumulative Index to Nursing & Allied Health Literature; JAMAS, the Japan Medical Abstracts Society database.

3.2. Characteristics of the sources of evidence

The characteristics of the included studies are presented in Tables (Table S2 (online data: *http://www.ddtjournal.com/action/getSupplementalData.php?ID=73*), Table 1, and Table 2), along with data relevant to the scoping review question. All the studies were written in English and published between 2008 and 2020, and no Japanese papers were included.

3.3. Synthesis of the results of studies on chronic wounds

We included 25 studies (Table S2, online data: *http://www.ddtjournal.com/action/getSupplementalData.php?ID=73*): 13 from the United States; three each from the United Kingdom and Australia; two studies from India, and one each from Korea, Denmark, China, and Canada. All the included studies except one were prospective cohort studies (58%) or cross-sectional studies (38%).

In the 10 studies investigating patient characteristics, diabetes mellitus was investigated as a factor related to microbial diversity in wounds in seven studies. Indicators used included a diagnosis of diabetes mellitus (*18,19*), hemoglobin A1c (HbA1c) levels (*11,20-23*), and the duration of diabetes mellitus (*20*). Among them, both HbA1c levels and the duration of a patient's diabetes mellitus correlated with the alpha diversity index (dominance, $p = 0.0174$; diversity, $p = 0.0168$, the correlation coefficient was not shown) (*20*). Bacteria shown to be associated with indicators of diabetes mellitus included *Streptococcaceae* (*18*), *Curvibacter* sp. (*19*), *Bacteroidetes*, *Peptoniphilus*, *Streptococcus* (*22*), and *Streptococcus* species (*21*). Followed diabetes mellitus, sex was the second most commonly reported factor, which was considered in three studies. The dominant bacteria in the female samples included *Clostridiales* (*24*), *Burkholderia*, and *Proteus* (*25*). *Actinomycetale*s was dominant in the males' wound samples (*24*). Moreover, analysis using the beta diversity index showed that different wound microbiota formed in males and females (*23*). Age was considered in two studies. The wounds of patients aged < 65 years contained more bacteria types than patients aged > 65 years and the dominant bacterium was also different (< 65 years, *Clostridiales*; > 65 years, *Actinomycetales*) (*24*). Analysis using permutational multivariate analysis of variance showed statistical significance for age ($R^2 = 0.0454$, $p = 0.0001$) (*23*). Other factors identified

Table 1. Details on studies of acute wounds included in the review

Study	Design	Sample size	Sample type	Wound type	Analysis techniques	Region of DNA or primer	Alpha diversity index	Beta diversity index	Factors related to microbiota (diversity result)
Hannigan *et al*., (2014) (*43*)	Prospective	74 samples (30 patients)	Swab	Open fractures	16S rRNA sequencing by MiSeq	V4	PD, Observed OTUs	Bray-Curtis	Injury mechanism, location, severity, complication
Romano-Bertrand *et al*., (2015) (*45*)	Prospective	25 patients	Swab	Incision sites	TTGE	27F, 1492R	N/A	N/A	Antisepsis
Bartow-McKenney *et al*., (2018) (*44*)	Prospective	208 samples (52 patients)	Swab	Open fractures	16S rRNA sequencing	V1-V3	PD	Weighted UniFrac	Injury mechanism, sampling time-points

TTGE, temporal temperature gel electrophoresis; PD, Faith's phylogenetic diversity index; OTU, operational taxonomic unit; rRNA, ribosomal RNA.

Table 2. Details on animal studies included in the review

Study	Design	Sample size	Sample type	Wound type	Analysis techniques	Region of DNA or primer	Alpha diversity index	Beta diversity index	Factors related to microbiota (diversity result)
Grice *et al*., (2010) (*46*)	Animal experiments	20 mice	Swab	6-mm full thickness excisional wound	16S rRNA sequencing by ABI 3730xl sequencer	27F, 1492R	Shannon	N/A	Sampling time-points, DM
Kim *et al*., (2019) (*47*)	Animal experiments	37 samples (37 mice)	Swab	7-mm full thickness skin excision wound	16S rRNA sequencing by MiSeq	ITS-1507F, ITS-23SR, 129F, 1492R	Shannon	N/A	Level of oxidative stress
Sanjar *et al*., (2019) (*48*)	Animal experiments	24 samples (24 rats)	Biopsy	Deep partial-thickness burn	16S rRNA sequencing by MiSeq	V3-V4	Chao1, Shannon, PD, Simpson, Observed OTUs	Bray–Curtis, Unweighted UniFrac	Sampling time-points

DM, diabetes mellitus; OTU, operational taxonomic unit; PD, Faith's phylogenetic diversity index; rRNA, ribosomal RNA.

included serum C-reactive protein levels (*21*), white blood cell counts (*21*), autoimmune disease (*26*), disease course (*24*), and end-stage renal disease (*22*), all of which were reported in a single study. However, in one large study of 2,963 chronic wounds, no differences were found in microbial diversity for sex, age, ethnicity, and the presence of diabetes mellitus (*27*).

In total, 17 studies compared wound characteristics. The factor reported in most studies was the healing outcome (9/17). Wounds were classified based on whether a patient's wound had healed at 6 weeks (*28*), 7 weeks (*29*), 8 weeks (*30*), 12 weeks (*21,31,32*), and 6 months (*33*); whether the wound area had been found to have enlarged at the next visit (*26*), and whether there was a 50% wound size reduction after 4 weeks (*23*). In non-healing wounds, *Anaerococcus* (*29*), *Actinomycetales* (*28*), *Bacteroidales* (*28,32*), *Pseudomonas* (*26*), and *Ascomycota* (*30*) were reported as the dominant bacteria. Furthermore, *Staphylococcus aureus* 10757 was found only in non-healing wounds (*31*), whereas *Staphylococcus* (*29*), *Gammaproteobacteria*, and *Pseudomonadacea* (*28*) were reported as the dominant bacteria in healing wounds. Regarding the index of diversity, beta biodiversity was significantly lower within the healing wounds than within the non-healing wounds (*32*). Non-healing wounds were less likely to transition away to other clusters of microbiota compared with healing wounds (*21,33*). Additionally, a traditional linear model showed a negative association between healing time and changes in microbiota between baseline and the next follow-up visit (2 weeks' time) ($R^2 = 0.16$, $p < 0.0001$) (*21*). Wound duration was the second most frequently reported factor in relation to wound characteristics, which was reported in four studies. Beta diversity was shown to significantly differ in terms of wound duration (*23*). Longer duration wounds were associated with a greater relative abundance of *Proteobacteria* (*34,35*), and shorter duration wounds were associated with a greater relative abundance of *Firmicutes* (*34*). Furthermore, wound duration was negatively correlated with a relative abundance of *Staphylococcus* ($\rho = –0.30$; $p = 0.0333$), and positively correlated with the number of operational taxonomic units (OTUs) ($\rho = 0.41$; $p = 0.0022$), the Shannon diversity index ($\rho = 0.32$; $p = 0.020$), and a relative abundance of *Proteobacteria* ($\rho = 0.38$; $p = 0.0059$) (*11*). Additionally, higher frequencies of DFUs containing obligate anaerobes were correlated with a longer duration ($p = 0.03$), but the correlation coefficient was not shown (*34*). Ulcer depth and wound infection were reported in three studies, respectively. Ulcer depth was negatively associated with a relative abundance of *Staphylococcus* ($\rho = –0.47$; $p = 0.0005$) and positively associated with a relative abundance of anaerobic bacteria ($\rho = 0.33$; $p = 0.0182$) (*11*), as well as anaerobe levels (*21*). In addition, a study using whole shotgun metagenome sequencing shows differences in microbiota function by ulcer depth (*31*). Regarding wound infection, samples from infected wounds exhibited lower microbial diversity than those from uninfected wounds (*33*), and diversity depended on the severity of the infection (*34*). However, the mycobiome diversity in specimens of an infection-related complication was significantly higher (*30*). Surface area, tissue oxygenation, wound location, and ulcer severity was reported in two studies, respectively. In terms of DFU surface areas, a weak but significant positive correlation was found with OTU richness ($\rho = 0.27$; $p = 0.051$) (*11*). For tissue oxygenation, it was correlated with alpha diversity (the number of observed OTUs, $\rho = –0.258$, $p = 0.046$; phylogenetic distance, $\rho = –0.295$, $p = 0.022$, respectively) (*30*) and microbial functional profiles (*31*). In terms of ulcer location, differences in alpha diversity were reported between DFUs on the forefoot and the hindfoot (*30*) and between non-healing wounds located on a foot or leg and others (*19*), respectively. The severity of ulcers was assessed using the Wagner classification in both studies. *Firmicutes* in tissue samples were more abundant in the grade 0-2 group whereas *Bacteroidetes*, *Prevotella*, *Peptoniphilus*, *Porphyromonas*, and *Dialister* were more abundant in the grade 3-5 group (*22*). The DFUs in the grade 5 group were relatively more diverse than in the other wound grades (*25*). Other factors included recurrent ulcers (*20*), metabolite concentrations in wounds (*36*), and slough (*34*), all of which were reported in a single study. In particular, metabolite concentrations strongly correlated with a relative abundance of bacteria ($\rho > 0.700$, $p < 0.05$) (*36*).

Treatment interventions altered chronic wound microbiota. Four studies reported on antibiotic therapy. Antibiotic-treated and untreated wound microbiota had significantly different composition (*18,19*) and alpha diversity (*30*). Multiple logistic regression showed that antibiotic use was associated with a 41% reduction in risk of *Streptococcus* colonization ($p = 0.009$, the odds ratio was not shown) (*19*). Furthermore, both complications and antibiotics use contributed to bacterial community disruption, although the larger effect was noted for antibiotics use (*21*). Additionally, the composition of the microbiota altered in treatments other than antibiotic therapy. A modification of the wound microbiota was observed in samples following an angioplasty procedure (*37*), debridement (*31,37*), and in the use of cadexomer iodine (*i.e.* antiseptics) (*38*) and traditional Chinese medicine (*24*).

The sampling region and sampling time-points were reported as factors associated with sampling in three studies (*36,39,40*) and one study (*41*), respectively. Although the microbiota in separate samples collected from the same wound differed in diversity (*40*), the microbiota in samples collected from different sites within the same wound (*39*), from the superior and inferior sections of the wound (*36*), and at different time points (*41*) were similar. Most variations between

the samples could be explained in terms of the individuals involved (*23*), although one study reported a high level of dissimilarity within individuals (*30*). Additionally, one study reported that different wound types demonstrated different microbial composition and diversity (*42*), whereas another study found no differences in terms of wound types and patient characteristics (*27*).

3.4. Synthesis of the results of studies on acute wounds

Three prospective cohort studies investigated acute wounds (Table 1); two studies from the United States (*43,44*) and one from France (*45*). Factors regarding the wound characteristics, treatment, and sampling were reported.

Two studies (*43,44*) investigated open fracture wounds. The change in the relative abundance of *Corynebacterium* and unclassified *Enterobacteriaceae* between the first and second visit time points was significantly different for penetrating and blunt wounds ($p = 0.006$ and $p = 0.038$, respectively). Besides, location, severity, and complications have been reported as factors associated with diversity (*43*). Moreover, significant differences in microbial communities were found according to the mechanism of injury ($p < 0.05$), and the wound microbiota in penetrating wounds was more similar to the adjacent skin microbiota over time. (*44*). The third study investigated surgical wounds. Antisepsis was considered as a factor and the trend of microbiota dynamics in wounds observed after antisepsis showed a decrease of *Firmicutes* and *Actinobacteria* and an increase of *Proteobacteria* (*45*).

3.5. Synthesis of the results of studies on acute wounds

Three studies used animals for investigating wound microbiota (Table 2) and all three studies had been performed by groups of researchers in the United States (*46-48*). Factors regarding patient characteristics (*i.e.*, animal characteristics), wound characteristics, and sampling were reported.

In one study, a 6-mm full-thickness excisional wound was created in mice with Leprdb mutations (db/db) and age-matched nondiabetic heterozygous littermates (db/+) (*46*). After wounding on day 3, both db/db and db/+ wounds showed a significant decrease in diversity as compared with day 1 ($p = 1.1 \times 10^{-4}$ and 0.024, respectively). The OTU diversity of the db/+ wounds was significantly greater than the db/db on day 7 ($p = 0.026$). In another study, a 7-mm full-thickness skin excision wound was created in db/db$^{-/-}$ mice, and higher doses of antioxidant inhibitors were applied to create increasing levels of oxidative stress (*47*). The level of oxidative stress significantly contributed to a difference in the Shannon diversity index ($p < 0.0001$). Diversity across time was also significantly related ($p = 0.0198$). The third study used a mouse burn model (*48*). A deep partial-thickness burn was created in mice comprising a 10% burn of the total body surface area. In the burn wound phyla profile, the abundance of *Actinobacteria* increased from 4.86% on post-wounding day 1 to 22.9% on post-wounding day 11, whereas the abundance of *Proteobacteria* declined from 39.8% on post-wounding day 1 to 16.8% on post-wounding day 11.

4. Discussion

We conducted a scoping review to investigate wound microbiota in studies using culture-independent molecular methods. We included 31 papers, and consequently the factors obtained through the review were categorized in terms of patient characteristics, wound characteristics, treatment, and sampling.

To our knowledge, this scoping review is the first to summarize the factors related to microbiota across chronic and chronic wounds. Previous reviews focusing on wound microbiota have mainly targeted chronic wounds (*12,13*). In contrast, this review was not limited to chronic wounds, but also included studies on acute wounds and animal experimental studies. This approach allowed for a clearer picture of the current state of microbiota research on wounds to emerge. First, there is a paucity of studies investigating factors related to acute wound microbiota, and only three were included in this review. Future studies are needed to investigate the microbiota of acute wounds for more effective treatment. Second, it was found that there were factors related to the diversity of wound microbiota that were common in chronic wounds, acute wounds, and wounds created in animals. Sampling time-points were the factor obtained in all the wound group types and wound microbiota changes during the healing process. Furthermore, a difference in microbiota diversity was observed between animals with diabetes mellitus and controls, similar to the results of the clinical studies. Based on the results revealed in the clinical data, further research is required to elucidate phenomena occurring within wound microbiota (*e.g.*, mechanisms of microbiota formation and host interactions) using animal models.

This scoping review showed that factors related to the composition and diversity of wound microbiota could be categorized into the patient and wound characteristics, treatment, and sampling. Diabetes mellitus, autoimmune disease, and end-stage renal disease are known as factors associated with a patient's immunity (*49,50*). Factors of wound characteristics, such as wound area and depth, are related to the bacterial bioburden and the treatment factor included the removal of bacteria in the wound, such as through antimicrobial treatment and debridement. These results suggest that current research on wound microbiota is largely focused on the infectious host or the source of infection, with little focus on the route of infection.

Therefore, future investigations of wound microbiota should investigate the relevant factors according to the route of infection.

Several studies reported the impact of factors on wound microbiota diversity. However, these studies did not report indicators in relation to the effect size (*e.g.*, correlation coefficients and odds ratios) or showed limited values, and a strong correlation was only found concerning metabolite concentrations. Given this situation, it is likely that the relevant factors associated with the diversity of the wound microbiota have not been fully investigated. Also, many of the studies included in this review were cross-sectional, and the causal relationship between factors and the diversity of microbiota could not be determined. For example, it is unclear whether metabolite concentrations increased due to the production of bacteria or whether the presence of metabolites facilitated the growth of specific bacteria. Thus, further research is needed to investigate the causal relationship between the identified factors and microbiota diversity, as well as the varying specific effects of these factors.

For this scoping review, we used a database containing articles in Japanese. However, no documents in Japanese were included for full-text screening. Healthcare systems differ globally, and it is also possible that relevant factors in relation to patients' backgrounds and the causes of wound development may differ between countries or ethnicities. Thus, more studies are needed in Japan to investigate the microbiota of wounds as well as comparative studies across countries.

Our scoping review had some limitations. The quality of the studies identified was not assessed systematically in this scoping literature review. Furthermore, the studies frequently had small sample sizes or did not describe the wound type in detail. However, the healing process differs depending on the depth of the wound, that is, whether there has been partial or full thickness loss of dermal tissue. However, no studies specified wound depth, and the influence of different factors within the same or differing wound depths was not clear. This review also included studies that used different methods and devices to identify microbiota. Additionally, even if the same next-generation sequencer had been used across some studies, the target region may have differed. Caution should be exercised in comparing the results obtained using these different methods.

In conclusion, our scoping review that factors related to the diversity and composition of wound microbiota included patient characteristics, wound characteristics, treatment, and sampling. Further research is needed to implement these results in wound infection prevention.

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Original Article

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Generic selection criteria for safety and patient benefit [X]: Water-vapor permeability and peel force properties of brand-name and generic ketoprofen tapes

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SUMMARY Tape products containing ketoprofen have transdermal analgesic and anti-inflammatory effects. We compared the physicochemical properties (water-vapor permeability, peel force, peel force-time curve) between one brand-name product and eight generic products. Regarding the measurement of water-vapor permeability, the formulations using methacrylic acid n-butyl acrylate copolymer (MBA) adhesives showed higher water-vapor permeability than those using styrene isopropyl styrene block copolymer (SIS) adhesives. In the case of the formulation using SIS adhesive, the central part of the formulation had higher water-vapor permeability than both ends. In the 90-degree peel test using the methods of adhesion testing, significant differences were observed between the products, especially as the various application times (5 min, 30 min, 9 h and 24 h) increased. This may be because the longer the time of attachment to the adherend, the more the adhesive force with the adherend increased due to the "anchoring effect" of the adhesive. The measurement of the peel force-time curve showed different curves among the products, especially in the peel force curve of Teikoku after 24 h, which showed two characteristic peak curves. Furthermore, when the peel forces at 25°C and 40°C were compared, Mohrus and Toko showed significantly higher values at 40°C compared to 25°C. This study showed that there are many generic drugs with formulation characteristics different from those of brand-name drugs, and that there is a large difference among the products in terms of adhesion and detachment.

Keywords Water-vapor permeability, peel adhesion, tape containing ketoprofen, brand-name drug, generic drug

1. Introduction

In recent years, transdermal analgesic and anti-inflammatory tape formulations of nonsteroidal anti-inflammatory drugs (NSAIDs), which are indicated for the treatment of lumbago, osteoarthritis, tendinitis and rheumatoid arthritis, have been widely used in clinical practice in the field of orthopedics in Japan (*1*). In particular, the tape formulation is very thin and soft, and its strong adhesiveness allows it to be used on movable parts of joints, such as elbows, knees, hips and shoulders, which is why it is often used by the elderly. Since many generic drugs are now available on the market, the Japanese government is actively promoting the use of generic drugs in order to reduce medical costs (*2*). However, the switch from brand-name to generic drugs has been slow. This is because patients placed highest importance on the "efficacy" (analgesic and anti-inflammatory effects) of the formulation, and they were highly satisfied with the current tape formulation and tended to continue using the same product (*3*). In addition, even if pharmacists recommend patients to change from brand-name to generic drugs for the purpose of reducing medical costs, such change has not actually progressed much. The reason is that the patient's burden is extremely small due to the application of medical insurance (the patient's burden is 10 to 30% of the total amount), so the low drug price, which is an advantage of generic drugs, is actually not advantageous. In addition, even after switching from brand-name to generic drugs, some patients reportedly return to brand-name drugs due to reasons related to efficacy, such as "low analgesic and anti-inflammatory efficacy", as well as the feeling of use, such as "ease of peeling off during movement", "uncomfortable application" and "difficulty in application" (*3*).

Ketoprofen-containing tape has always been the top-selling prescription drug in Japan since the brand-

Table 1. Tape products used in this experiment

Product name	Abbreviated name	Class	Company name	Lot number
Mohrus® Tape 20mg	Mohrus	BN	Hisamitsu Pharm. Co., Inc.	Y428
Ketoprofen tape 20mg "Teikoku"	Teikoku	GE	Teikoku Pharm. Co., Inc.	A183S
Ketoprofen tape 20mg "Toko"	Toko	GE	Toko Pharm. Co., Ltd.	SC06T
Ketoprofen tape 20mg "BMD"	BMD	GE	Biomedics Co., Ltd.	5H17
Ketoprofen tape 20mg "SN"	SN	GE	Shiono Chemical Co., Ltd.	EZ02
Ketoprofen tape 20mg "Kyorin"	Kyorin	GE	Kyorin Rimedio Co., Ltd.	04AL
*Touchron® Tape 20	Touchron	GE	Kyukyu Pharm. Co., Ltd.	6TAL
**Frestol® Tape 20mg	Frestol	GE	Towa Pharm. Co., Ltd.	A056
Ketoprofen tape 20mg "Nichi-Iko"	Nichi-Iko	GE	Nichi-Iko Pharm. Co., Ltd.	5A17

BN: brand-name drug, GE: generic drug. Currently, *Touchron® Tape 20 has its name changed to Ketoprofen tape 20mg "Sanwa", **Frestol® Tape 20mg has its name changed to Ketoprofen tape 20mg "Towa".

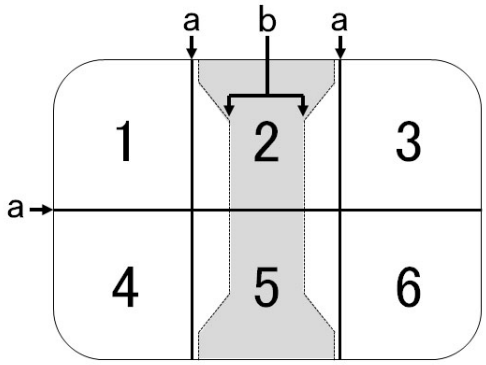

Figure 1. Tape cut area (1-6) and liner film cut position of Ketoprofen Tape "Teikoku". Solid line **(a)** was cut to obtain six fractions, and the dotted line **(b)** shows the cut position in the liner film. The gray area indicates the center flap of the liner film.

name drug, Mohrus® tape, was launched in December 1995 (*4*). It is a drug that is used frequently, especially by the elderly. We previously reported the formulation characteristics (peel force, water-vapor permeability, stretchability, rigidity and softness, *etc.*) of Mohrus® tape (*5,6*). In the current study, we report new findings on the peel power due to the difference in water-vapor permeability and temperature, which is directly related to the feeling of use in the formulation characteristics of Mohrus® tape.

2. Materials and Methods

2.1. Materials

Mohrus® Tape 20 mg (Hisamitsu Pharmaceutical Co., Inc.: Tokyo, Japan), Ketoprofen tape 20 mg "Teikoku" (Teikoku Pharmaceutical Co., Inc.: Kagawa, Japan), Ketoprofen tape 20 mg "Toko" (Toko Pharmaceutical Co., Ltd.: Tokyo, Japan), Ketoprofen tape 20 mg "BMD" (Biomedics Co., Ltd.: Tokyo, Japan), Ketoprofen tape "SN" (Shiono Chemical Co., Ltd.: Tokyo, Japan), Ketoprofen tape 20 mg "Kyorin" (Kyorin Rimedio Co., Ltd.: Tokyo, Japan), Touchron® tape 20 (Kyukyu Pharmaceutical Co., Ltd.: Toyama, Japan), Frestol® tape 20 mg (Towa Pharmaceutical Co., Ltd.: Osaka, Japan) and Ketoprofen tape 20 mg "Nichi-Iko" (Nichi-Iko Pharmaceutical Co., Ltd.: Toyama, Japan) was purchased. Table 1 shows the product name, manufacturer and serial number of each formulation used in this study. All other reagents used were special grade products.

2.2. Measurement of the water-vapor permeability

Water-vapor permeability was measured with reference to the method of Sawai *et al.* (*7*). After cutting the tape into 6 equal parts (Figure 1), the opening of the sample tube containing purified water was covered in advance, and the weight (W_1) was measured. Then, the tube was allowed to stand in a constant temperature and humidity tester KCL-2000W type (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) with a temperature of 40 ± 2°C and humidity of 50 ± 5% relative humidity (RH). After 24 h, the weight (W_1) of the sample tube was measured, and the water-vapor permeability W (g/m^2/24 h) was calculated and evaluated by the following formula. The average of various tapes was calculated after 6 times.

$$W\ (g/m^2/24\ h) = (W_0 - W_1)/A$$

W_0: Weight before test (g), W_1: Weight after test (g), A: Opening area of sample tube (m^2)

2.3. Measurement of the peel force

According to the 90-degree peel test (*8*) of the Japanese pharmacopoeia 17th edition, various tapes (45 mm × 77 mm) cut into a test plate (stainless steel) were attached using a crimping roller (2 kg) (Japanese Industrial Standards: JIS, Z0237, 2009). After crimping, the mixture was allowed to stand for 5 min, 30 min, 9 h, and 24 h under temperature of 25 ± 2°C or 40 ± 2°C and humidity 50 ± 5% RH. After that, the short side of the sample was peeled off completely with a digital force gauge ZTS-20N (Imada Corp. Ltd., Aichi, Japan) at a constant speed at 90 degrees to the adhesive surface. The peeling speed was moved at 5.0 mm (300 mm/min) per second, and the pressure

over time was measured 6 times with a recorder. The peel force of 50% length peeled from the test plate was averaged. In addition, the adhesive force-time curve from the start of peeling to complete peeling was measured using the graph drawing software Force Recorder Standerd Ver. 1.03 (Imada Co., Ltd., Aichi, Japan). Furthermore, the peel force of each product at 25℃ or 40℃ 30 minutes after application was measured.

2.4. Statistical analysis

For each experimental result, significant differences were analyzed using the paired *t*-test and the *Bonferroni/Dunn*-test of multiple comparison test (*9*). When $p \leq 0.05$ and $p \leq 0.01$, there was a difference at a significance level of 0.05 and 0.01, respectively.

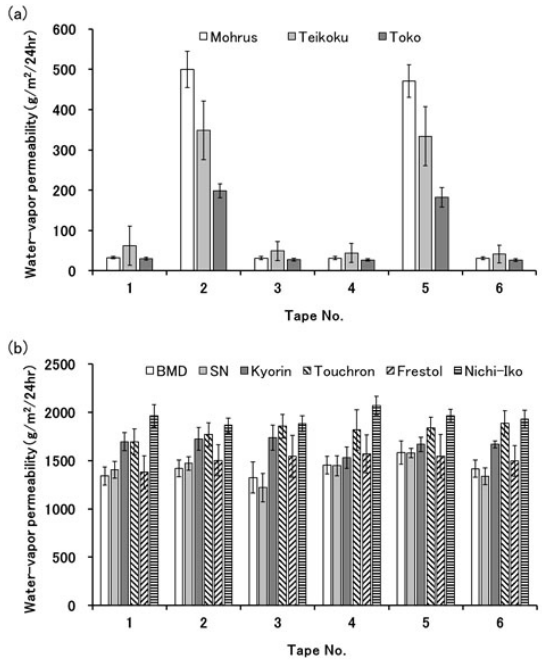

Figure 2. Water-vapor permeability of various tapes (n = 6). **(a)** Tapes containing SIS adhesives, **(b)** Tapes containing MBA adhesives.

3. Results

3.1. Measurement of the water-vapor permeability

Figure 2 shows the results of water-vapor permeability measurement for each product. In Figure 2, these nine preparations used in this study were divided into two groups, that is, Mohrus, Teikoku and Toko (Figure 2a) showed predominantly lower water-vapor permeability than six generic drugs group (BMD, SN, Kyorin, Touchron, Frestol and Nichi-Iko) (Figure 2b). In addition, preparations using styrene isopropyl styrene block copolymer (SIS) adhesives (Mohrus, Teikoku and Toko) show water-vapor permeability of 82 to 183 g/m^2/24 hr, whereas, preparations using methacrylic acid n-butyl acrylate copolymer (MBA) adhesives (BMD, SN, Kyorin, Touchron, Frestol and Nichi-Iko), showed water-vapor permeability of 1,410 to 1,810 g/m^2/24 h, which is about 10-fold higher (Table 2). Furthermore, Mohrus, Teikoku, and Toko had significantly higher water-vapor permeability in the central part (Tape No. 2 and 5) of the preparation than in the other parts (Tape No. 1, 3, 4 and 6). (Figure 2a).

3.2. Measurement of the peel force

3.2.1. 90-degree peel force test

Figure 3 shows the results of 90-degree peel adhesive

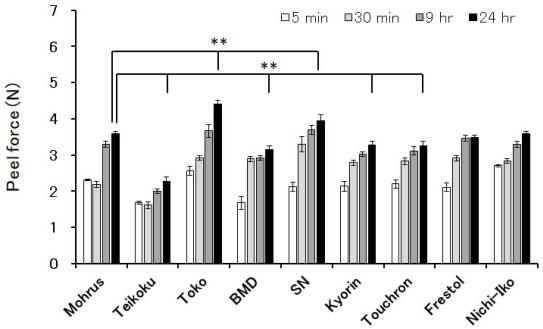

Figure 3. Comparison of 5 min, 30 min, 9 h and 24 h peeling force in various tapes. (n = 6, $^{**}p < 0.01$, Bonferroni/Dunn-test).

Table 2. Measurement of water-vapor permeability (n = 6)

Product name	Class	Water-vapor permeability (g/m^2/24h)	Types of Adhesives
Mohrus	BN	183.0 ± 234.6	SIS
Teikoku	GE	146.6 ± 151.1	SIS
Toko	GE	82.1 ± 84.3	SIS
BMD	GE	1423.6 ± 92.9	MBA
SN	GE	1410.3 ± 122.0	MBA
Kyorin	GE	1671.6 ± 74.4	MBA
Touchron	GE	1810.3 ± 69.3	MBA
Frestol	GE	1506.8 ± 67.6	MBA
Nichi-Iko	GE	1945.5 ± 72.7	MBA

BN: brand-name drug, GE: generic drug, ± SD: standard deviation, SIS: Styrene isopropyl styrene block copolymer, MBA: Methacrylic acid n-butyl acrylate copolymer.

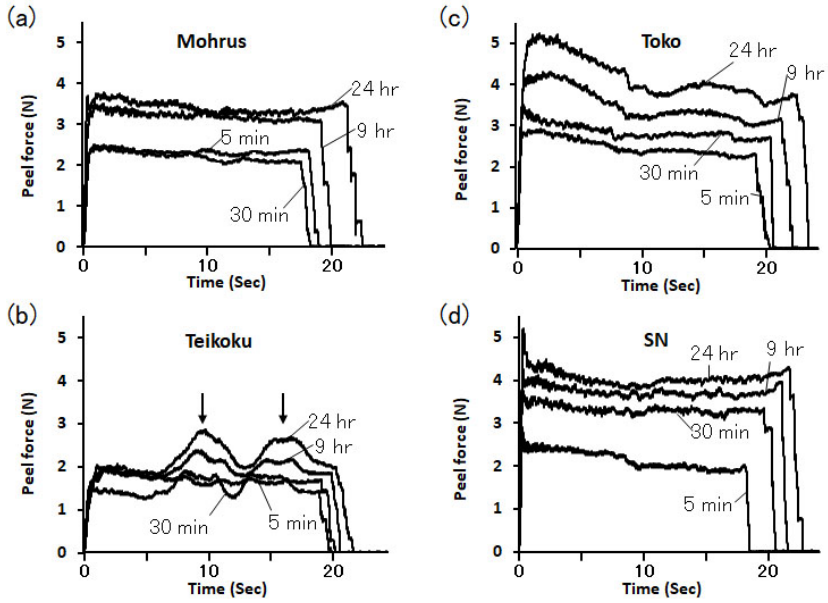

Figure 4. Comparison of 5 min, 30 min, 9 h, and 24 h peel force-time curve in various tapes (n = 6). (a) Mohrus, **(b)** Teikoku, **(c)** Toko, **(d)** SN.

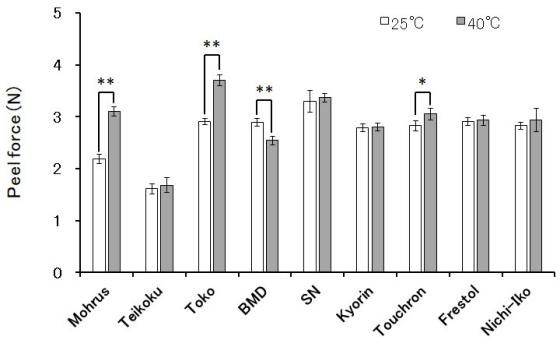

Figure 5. Comparison of peel force due to difference in temperature (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ (25℃ *vs.* 40℃, Paired *t*-test)

force measurement for each product. In Figure 3, the peel force when each product was separated after 5 min, 30 min, 9 h and 24 h from the start of application was measured, and the peel force tended to increase as the application time increased. In addition, when the peel force of each product after 24 h was compared, the generic drugs Toko (4.4 N) and SN (4.0 N) showed significantly higher values than the brand-name drug Mohrus (3.6 N). On the other hand, Teikoku (2.3 N), BMD (3.2 N), Kyorin (3.3 N) and Touchron (3.3 N) showed significantly lower values.

3.2.2. Comparison of the peel force-time curve

Figure 4 shows the measurement results of the peel force-time curve. When comparing the peel force-time curves of Mohrus (Figure 4a), Teikoku (Figure 4b) and Toko (Figure 4c) using SIS adhesives, Mohrus (Figure 4a) and Toko (Figure 4c) showed similar curves, although there were differences in the peel force. However, Teikoku showed a peculiar curve with two peaks at 9 and 24 h. On the other hand, the MBA adhesives (BMD, Kyorin, Touchron, Frestol and Nichi-Iko) showed similar curves to SN (Figure 4d), although there was a difference in peel force.

3.2.3. Comparison of the peel force due to difference in temperature

Figure 5 shows the measurement results of the peel force due to the difference in temperature. In Figure 5, when the peel force was measured 30 min after the start of application under the conditions of 25 or 40℃, Mohrus showed significantly higher values at 40℃ (3.1 N) than at 25℃ (2.2 N) (difference of 0.9 N). Similarly, the generic drug Toko showed significantly higher values at 40℃ (3.7 N) than at 25℃ (2.9 N) (difference of 0.8 N). On the other hand, BMD, showed significantly lower values at 40℃ (2.5 N) than at 25℃ (2.9 N) (difference of 0.4 N).

4. Discussion

The tape has a strong adhesive force, is difficult to peel off, has good followability to the skin even on moving parts (such as elbows and knees) and can be applied for a relatively long time (*10*).

Since an excellent therapeutic effect can be obtained by adhering to the skin and absorbing the drug through the skin, the component of the adhesive used in the plaster is an important factor affecting the transdermal absorption of the drug. However, it is known that strong adhesive strength and low water-vapor permeability cause physical irritation such as exfoliation of keratin and stuffiness.

The types of adhesives used in tapes are mainly classified into three types (rubber, acrylic, and silicon), and many products use the synthetic rubber type (SIS adhesives) or acrylic type (MBA adhesives). Mohrus, Teikoku and Toho are SIS adhesives listed in the package insert of each formulation, whereas BMD, SN, Kyorin, Touchron, Frestol and Nichi-Iko are MBA adhesives. According to the results of water-vapor permeability measurement, the SIS adhesives was about 10-fold less breathable than the MBA adhesives and tended to get stuffy when applied as a tape (Table 2). In addition, for Mohrus, Teikoku and Toho, which use SIS adhesives, the standard deviation value of water-vapor permeability measurement fluctuated greatly, suggesting that the water-vapor permeability may differ depending on the part of the tape. Therefore, when the tape agent was cut into 6 parts (No. 1 to 6) (Figure 1), and the water-vapor permeability of each piece was measured, Mohrus, Teikoku and Toho showed significantly higher water-vapor permeability in part No. 2 and 5 than No. 1, 3, 4 and 6 (Figure 2a). On the other hand, no such tendency was observed for BMD, SN, Kyorin, Touchron, Frestol and Nichi-Iko (Figure 2b). Such differences may be attributed to the manufacturing process of the tape preparation. The manufacturing process of tapes mainly involves: (I) Mixing (adhesive + drug), (II) Coating/Drying/Support and liner lamination, (III) Cutting/Back cutting, and (IV) Cutting to tape size/Packaging/Manufacturing with four steps of quality inspection. In (III), after the liner is attached to the tape agent, "back cutting" is performed to make a cut for the center flap in the liner to make it easier to attach when using the tape agent (Figure 1b). At the time of this back cutting, not only the liner but also the plaster was lightly cut; thus, it was considered that No. 2 and 5 showed significantly higher water-vapor permeability (Figure 2a). On the other hand, it is possible that BMD, SN, Kyorin, Touchron, Frestol and Nichi-Iko also have light cuts in the plaster, but since the MBA adhesive itself has high water-vapor permeability, a remarkable effect was not observed. From the above results, by confirming the type of adhesive in the package insert, since the SIS adhesives is a product that is easier to get stuffy than the MBA adhesives, such information would be informative to patients.

Next, when the 90-degree peel force was measured by the methods of adhesion testing, differences were observed among the products, and the peel force tended to increase with the extension of the application time (5 min, 30 min, 9 h and 24 h) (Figure 3). This is because when the adhesion time is short (5 min and 30 min), air bubbles are not released between the skin and the plaster containing the adhesive, and the adhesion area is expected to be small. On the other hand, as air bubbles gradually escape with the passage of time (9 and 24 h), a mechanical bonding that increases the contact area, the so-called "anchor effect (also called fastener effect)", occurs (*11,12*). As a result, the peel force was considered to have increased.

When the peel force-time curve was measured, different curves were shown for each product (Figure 4). For the brand-name drug Mohrus using SIS adhesives, the 5 min and 30 min curves were almost the same, and the 9- and 24-h curves were considered to show high values due to the anchoring effect (Figure 4a). The generic drug Teikoku using SIS adhesives showed a lower peel force when compared with Mohrus, and also showed two peaks on the 9 h and 24 h curves (Figure 4b). These peaks were consistent with the position of the cut for the center flap (Figure 1b), suggesting that the anchoring effect was enhanced at the gap. In the case of Toko, the peel force gradually increased with time, 5 min, 30 min, 9 h and 24 h, and the value at 24 h was the highest among other preparations (Figure 4c). The SN using MBA adhesives showed almost the same curve as Toko, and the similar peel force-time curve was shown on other pharmaceuticals (BMD, SN, Kyorin, Touchron, Frestol and Nichi-Iko) (Figure 4d).

In addition, the peel force after 30 min from the application start under the condition of 25℃ or 40℃ was measured, and Mohrus (brand-name drug) and Toko (generic drug) showed significantly higher values at 40℃. On the other hand, BMD of generic drugs was significantly higher at 25℃. As additives other than SIS or MBA adhesives, hydrogenated rosin glycerin ester (adhesion promoter, adhesive, *etc.*) and polyisobutylene (adhesive) are contained in Mohrus, terpene resin (tackifying resin) in Toko and polybutene (adhesive) in BMD (Table 3). Mohrus and Toko were each added with different adhesives, but both were more difficult to peel off at 40℃. It was also shown that BMD was difficult

Table 3. Main additives described in the package insert of each product

Abbreviated name/Adhesive agent	Mohrus /SIS	Teikoku /SIS	Toko /SIS	BMD /MBA	SN /MBA	Kyorin /MBA	Touchron /MBA	Frestol /MBA	Nichi-Iko /MBA
Adhesive resin									
Hydrogenated rosin glycerol ester (HRG)	●								
Alicyclic saturated hydrocarbon resin		●							
Terpene resin			●						
Polyisobutylene	●								
Polybutene		●		●	●	●	●	●	●

SIS: Styrene isopropyl styrene block copolymer, MBA: Methacrylic acid n-butyl acrylate copolymer.

to peel off at 25℃, but other using MBA adhesives products also contained polybutene, suggesting a difference in the amount of polybutene added.

In this study, the difference in partial water-vapor permeability of each pharmaceutical as well as peel force by the difference in pasting time and temperature condition were clarified. Grasping these detailed pharmaceutical characteristics may be helpful for pharmacists in selecting the appropriate pharmaceutical for the patient.

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Original Article

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Effect of interprofessional collaboration among nursing home professionals on end-of-life care in nursing homes

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SUMMARY As end-of-life (EOL) care in nursing homes is gradually increasing, interprofessional collaboration in EOL care in nursing homes is becoming important. However, a method for measuring interprofessional collaboration has not been established. Therefore, this study aimed to clarify the effect of interprofessional collaboration on EOL care in nursing homes. Questionnaires were mailed to the facility directors of 378 nursing homes in Kanagawa Prefecture, Japan, and distributed to nurses, care managers, and professional caregivers. Three professionals from each nursing home completed the same questionnaire, which included 9 items on EOL care: shared facility policy, residents' wishes, each professional's roles, person in charge of the facility, residents' conditions, mental status of residents' families, emergency codes, residents' key people, and sufficient discussion among professionals. Based on the professionals' responses, interprofessional collaboration was assessed. We used multivariable analysis, with interprofessional collaboration as an independent factor. The outcome was the amount of EOL care in the nursing home. A total of 180 (47.6%) nursing homes participated. Multivariable analysis showed that interprofessional collaboration (beta [β] coefficient 2.5, 95% confidence interval [CI] 0.45-4.48; $p = 0.017$), availability of EOL care bonuses (β coefficient 4.4, 95% CI 1.41-7.38; $p = 0.004$), physician support for emergency care during off time (β coefficient 5.4, 95% CI 1.86-8.94; $p = 0.003$), and EOL care conferences (β coefficient 4.1, 95% CI 1.19-6.99; $p = 0.006$) were significant factors associated with the amount of EOL care in the nursing homes. We found evidence in the adjusted model that interprofessional collaboration among facility professionals is effective for EOL care in nursing homes.

Keywords nurse, interprofessional working, care manager, professional care giver, perception, differences in perception

1. Introduction

End-of-life (EOL) care is of interest in ageing societies, especially in the super-aged society of Japan (*1*), and it requires interprofessional collaboration among multiple professionals (*2,3*). However, in Japan, most professionals do not receive sufficient interprofessional education before university graduation (*4*). Therefore, the provision of EOL care based on interprofessional collaboration is an important issue.

There are many issues involved in the implementation of EOL care in nursing homes, including interprofessional collaboration and legal situations. Concerning the location where people die, EOL care in nursing homes is less common (7%) than hospital death (73%) in Japan (*5*). The rate of hospital deaths in other countries is lower than that in Japan. EOL care in nursing homes is important for preventing the undesirable transfer of patients to hospitals and is a key issue in Japan, as the country with the largest superaged population (*1*). The Japanese government initiated public long-term care insurance (LTCI) in 2010, making people aged 65 and over eligible for elderly facility service benefits, based strictly on physical and mental disability. Elderly facilities with LTCI coverage are mainly geriatric health service facilities (GHSFs) and nursing homes. GHSFs provide physical therapy to older persons to support their daily living functions so they can resume independent living at home; thus, they act as 'intermediate' facilities. A previous study reported

that 26.8% of residents died in GHSFs (*6*). On the other hand, nursing homes provide only chronic care, with an average length of stay of approximately 4 years (*7*), which is longer than that in other countries. However, it is difficult to provide EOL care in nursing homes, and palliative care services in nursing homes cannot be extended because such services are generally available only in hospitals. Moreover, euthanasia is not legal even at the terminal stage in Japan, which is also a different situation than that in other countries. To implement EOL care in nursing homes, consistent communication between facility professionals and residents' families from admission to the EOL care period is necessary. Therefore, interprofessional collaboration that includes families is essential for EOL care in nursing homes.

Interprofessional collaboration among nurses, care managers, and professional caregivers is required for EOL care in Japanese nursing homes. A nurse usually assesses residents' medical conditions and requires consultation with a physician during the EOL care period. A care manager is a professional who is responsible for assessing residents' wishes for EOL care, creating care plans, and organizing services during the EOL care period (*8*). The care manager role has been introduced and covered in long-term care facilities, including nursing homes and social services, by the LTCI system in Japan (*9*). A professional caregiver provides daily care for residents from admission to EOL care. Since professional caregivers are the most common staff in nursing homes, professional caregivers play an important role in interprofessional collaboration for EOL care in nursing homes.

Although EOL care bonuses, physician support for emergency care, proximity to affiliated hospitals, and physician EOL care conferences are important factors of EOL care in nursing homes (*7,10-12*), interprofessional collaboration has not been reported to affect EOL care in nursing homes. The EOL care bonus system was initiated in 2006 and provides financial support for EOL care in nursing homes from the Japanese government. In addition, bonuses result in higher-quality EOL care, such as advance care planning (*10*). Because nurses in almost all nursing homes work on call at night, professional caregivers are often needed to provide EOL care in nursing homes at that time. In general, many nurses have prior work experience in hospitals before working at nursing homes (*13*). However, almost all professional caregivers have not previously had careers in which they might witness the death of a person before working at nursing homes (*14*). Professional caregivers' provision of EOL care in nursing homes at night without the presence of a nurse has raised concerns about the quality of EOL care in nursing homes. Therefore, interprofessional collaboration is important to ease caregivers' anxiety; in particular, EOL care conferences are required to effectively provide EOL care (*15,16*). However, to the best of our knowledge, there have been few reports investigating interprofessional collaboration in EOL care in nursing homes.

In the present study, we focused on interprofessional collaboration in EOL care in nursing homes, but quantifying interprofessional collaboration is difficult (*17,18*). An assessment of interprofessional collaboration among nursing home professionals providing EOL care in nursing homes will offer necessary insight. However, previous research has not reported the contribution of interprofessional collaboration to EOL care in nursing homes. Therefore, the objective of the present study was to investigate the effect of interprofessional collaboration on EOL care in nursing homes.

2. Materials and Methods

2.1. Study design and participants

This was a cross-sectional study of nursing homes in Kanagawa Prefecture. Data were collected via a longitudinal questionnaire survey from November 2015 to January 2016 (*10*). The survey was sent to the facility directors of all 378 nursing homes in Kanagawa Prefecture that were registered in the LTCI Services Informational Publication System in November 2015 (*19*). The facility directors distributed the survey forms to 3 representative professionals working in each facility: a nurse, a care manager, and a professional caregiver. Nursing homes that returned completed questionnaires were included in the study. Facilities that returned questionnaires with missing data were excluded. Gift cards with a value of 500 Japanese yen were used as an incentive to encourage study participation. We requested participation repeatedly by calling and faxing nonresponding nursing homes. The directors of the Health and Welfare Departments in Yokohama city, Kawasaki city, Yokosuka city, Sagamihara city, and Kanagawa Prefecture cooperated in the implementation of the present survey.

2.2. Setting

The setting of the present study included all nursing homes in Kanagawa Prefecture, Japan. This prefecture is near the national capital of Tokyo, which had a population of approximately nine million people as of 2014, 22.5% of whom were older persons (*20,21*). Kanagawa Prefecture is facing many issues related to the rapid increase in the population of older persons that will occur in Japan over the next 20 years (*22*).

2.3. Questionnaire

Identical questionnaires were distributed to all three professionals (nurses, care managers, and caregivers); the questionnaires included questions about perceptions

of interprofessional collaboration in EOL care in each nursing home. The three professionals were asked to respond 'Yes' or 'No' to the 9 items on interprofessional collaboration in EOL care (Supplemental data). For the study feasibility assessment, two nursing home visits were conducted to interview all three professionals (nurses, care manager, and caregivers), and a pilot survey was performed with the same survey questionnaire at 14 nursing homes in August 2014. The questionnaire included nine items designed to reveal each professional's perceptions of interprofessional collaboration in EOL care in each nursing home, and questionnaire content was developed based on the interviews conducted at the two nursing homes (Supplemental data).

2.4. Ethical considerations

This study was approved by the medical study institutional review boards of Yokohama City University (No. A140522015, approved on 24 July 2014) and performed in accordance with the Declaration of Helsinki. We explained the research content and provided a written description. We asked only the nursing homes that agreed to participate after being informed of the above information to complete the set of questionnaires. Therefore, consent was implied by the return of the questionnaires by the nursing home facilities.

2.5. Statistical analysis

Univariable analysis was performed using a simple regression model of the amount of EOL care in nursing homes. The factors were the eight identified response patterns (① to ⑧) for the 9 items (question 1 [Q1] to Q9). Multivariable analysis was conducted using a linear regression model. The primary outcome was the number of EOL care residents in the nursing home with adjustment per 100 beds as of 2014. According to our hypothesis, interprofessional collaboration was included as a variable in the multivariable linear regression model. Availability of EOL care bonuses, physician support for emergency care during off time, proximity to affiliated hospitals, and EOL care conferences were also included as variables (10,12). Interprofessional collaboration was quantified based on the perceptions of interprofessional collaboration in EOL care of the three professionals from each nursing home. The quantified value was stratified into three levels. For the higher level, the three professionals agreed with all 9 interprofessional collaboration questions. The middle level was between the higher level and lower level. For the lower level, any of the three professionals disagreed with any of the questions. All *p*-values were two-tailed, and all analyses in this study were performed using SPSS version J21 (IBM, Tokyo, Japan). A p-value < 0.05 was considered statistically significant in all analyses.

3. Results

3.1. Study participants

Of the 378 nursing homes that were sent questionnaires, 237 returned them (62.7% response rate) (Figure 1). The remaining 141 nursing homes (37.3%) did not return responses. Among the 237 responding nursing homes, one of the three professionals in 21 facilities (8.9%) did not answer any of the nine questions. Thirty-six facilities (15.2%) returned partially completed questionnaires. Ultimately, 180 nursing homes (47.6%) were included in the study.

3.2. Characteristics of the participating facilities

A total of 176 of the facilities (97.8%) were subsidized by the national government, and four facilities (2.2%) were subsidized by the local government. The mean number of beds was 60.0 (standard deviation [SD]: 39.8). A total of 160 (88.9%) nursing homes had individual rooms, and the median number of rooms was 21 (interquartile range [IQR]: 5.5-85). Among the 3,739 discharged residents from 180 nursing homes (mean ± SD: 21.5 ± 10.4 resident/facility), 2,804 residents (mean±SD: 15.8 ± 8.9 resident/facility) were discharged due to death during the year. Of those who died, 1,698 residents died in the nursing home (60.6%; mean ± SD: 9.4 ± 8.4 resident/facility). With adjustment for the number of deaths per 100 beds, the mean number of residents who died in the nursing home was 11.3 per year (SD: 9.7). Of the 180 nursing homes included in the study, 108 (65.6%) adopted the EOL care bonus system.

3.3. Characteristics of the three types of professionals in the responding facilities

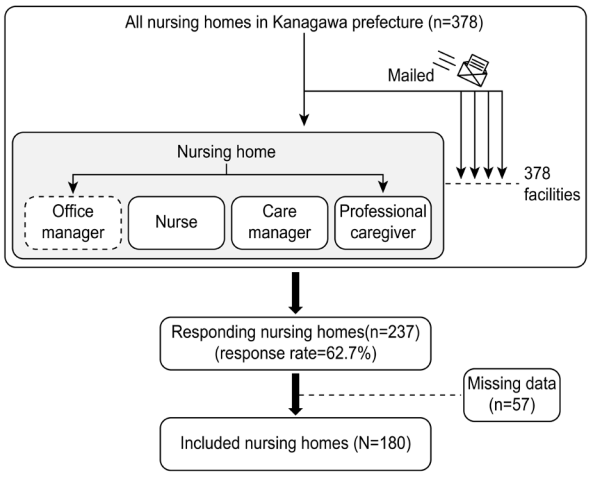

Figure 1. Flow charts of the inclusion criteria for the study. Among the responding nursing homes (n = 237), those with complete responses from the three types of professionals were included.

Table 1. Characteristics of the three types of participating professionals from each of the nursing homes (*n* = 180)

Variable	Nurse	Care manager	Professional caregiver
Age			
< 40	23 (12.8%)	59 (32.8%)	95 (52.8%)
40-49	42 (23.3%)	68 (37.8%)	58 (32.2%)
50-59	66 (36.7%)	41 (22.8%)	24 (13.3%)
60-69	47 (26.1%)	9 (5.0%)	2 (1.1%)
≥ 70	1 (0.6%)	3 (1.7%)	0 (0%)
Female gender	162 (90.0%)	98 (54.4%)	80 (44.4%)
Experience in their nursing homes [mean years ± SD]	7.3 ± 6.8	8.8 ± 5.9	9.2 ± 5.3
Experience in their areas of expertise [mean years ± SD]	26.1 ± 11.0	9.2 ± 5.3	9.7 ± 5.3
Experience of EOL care in their nursing homes (0, 1-4, ≥ 5 times)	28, 19, 129	33, 36, 109	25, 50, 103

SD: standard deviation; EOL: end of life.

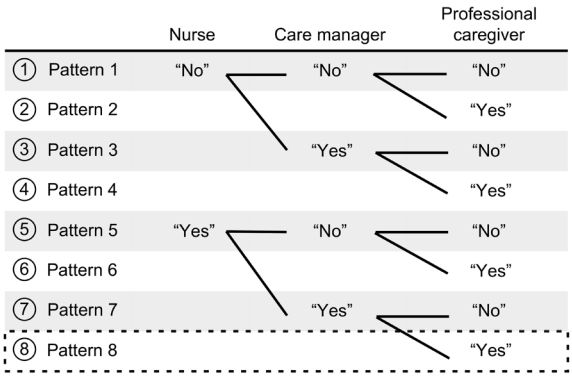

Figure 2. Eight patterns of 'Yes' or 'No' responses to the survey questions. Theoretically, Pattern 1 indicates the most negative response and Pattern 8 indicates the most positive response regarding interprofessional collaboration in EOL care in nursing homes.

Table 1 shows that almost all nurses were female. However, there were equal numbers of females and males among the care managers and professional caregivers. The findings also revealed that the participating care managers and professional caregivers were younger and had less expert experience than the nurses. Moreover, the care managers and professional caregivers had worked the majority of their careers at the nursing homes.

3.4. Differences in perception among the three types of professionals

The 'Yes' or 'No' responses to the questions in the present survey from the three types of professionals were categorized into eight patterns (①-⑧), as shown in Figure 2. Table 2 shows the results regarding the number of types of professionals who indicated the presence of interprofessional collaboration in EOL care in relation to each item. The following response patterns were observed: three professionals (⑧) > two professionals (④, ⑥, ⑦) > one professional (②, ③, ⑤) > none of the professionals (①). The univariable analysis showed that Q1, Q2, Q3, and Q7 received 'Yes' responses from none of the professionals (①); Q1, Q2, Q5, Q8, and Q9 received 'Yes' responses only from the nurse (⑤); and Q1, Q2, Q3, Q5, Q6, Q7, and Q8 received 'Yes' responses from all three professionals (⑧). These items were significantly associated with the amount of EOL care in nursing homes (Table 2).

3.5. Multivariable analysis results

In the multivariable analysis, the extent of interprofessional collaboration (adjusted coefficient 2.5, 95% confidence interval [CI] 0.45-4.48; $p = 0.017$), availability of EOL care bonuses (adjusted coefficient 4.4, 95% CI 1.41-7.38; $p = 0.004$), physician support for emergency care during off time (adjusted coefficient 5.4, 95% CI 1.86-8.94; $p = 0.003$), and EOL care conferences (adjusted coefficient 4.1, 95% CI 1.19-6.99; $p = 0.006$) were significant factors associated with the amount of EOL care provided in nursing homes (Table 3).

4. Discussion

4.1. Summary of the findings

To our knowledge, this is the first survey to reveal that interprofessional collaboration is associated with the amount of EOL care in nursing homes. Additionally, in the univariable analysis, the perception of the presence of interprofessional collaboration in EOL care by all three professionals (the nurse, care manager, and professional caregiver) was related to an increase in EOL care in nursing homes, whereas the perception of the presence of interprofessional collaboration in EOL by none of the professionals or exclusively by the nurse was related to a decrease in EOL care in nursing homes.

4.2. Interpretation and explanation of the results

The findings presented in Table 2 revealed that the perceptions of interprofessional collaboration differed across items and professionals. The perception of interprofessional collaboration was most frequently reported by all three professionals, followed by the

Table 2. Positive responses regarding interprofessional collaboration in end-of-life care in the nursing homes (n = 180)

Variable	1. None		2. Only PCG		3. Only CM		4. Only 2 professionals (CM+PCG)		5. Only NS		6. Only 2 professionals (NS+PCG)		7. Only 2 professionals (NS+CM)		8. 3 professionals	
	Coefficient (95%CI)	p-value	Coefficient (95%CI)	p-value	Coefficient (95%CI)	p-value	Coefficient (95%CI)	p-value	Coefficient (95%CI)	p-value	Coefficient (95%CI)	p-value	Coefficient (95%CI)	p-value	Coefficient (95%CI)	p-value
Q1. Facility policy on EOL care, number (ratio)	13 (7.2%) -9.8 (-15.2-4.5)	< 0.001**	8 (4.4%) -3.0 (-9.9-3.9)	0.394	4 (2.2%) -3.0 (-9.9-3.9)	0.394	9 (5.0%) 6.4 (-0.1-12.9)	0.052	4 (2.2%) -11.6 (-21.1-2.1)	0.020*	10 (5.6%) 0.6 (-5.7-6.8)	0.857	16 (8.9%) -1.1 (-6.1-3.9)	0.659	116 (64.4%) 3.9 (0.9-6.8)	0.001*
Q2. Residents' wishes for EOL care, number (ratio)	9 (5.0%) -9.5 (-15.9-3.1)	0.004*	2 (1.1%) -5.2 (-18.8-8.4)	0.454	3 (1.7%) -5.2 (-18.8-8.4)	0.454	12 (6.7%) 4.7 (-1.0-10.4)	0.106	4 (2.2%) -11.3 (-20.9-1.8)	0.020*	10 (5.6%) -0.5 (-6.7-5.7)	0.876	11 (6.1%) -0.6 (-6.6-5.4)	0.842	129 (71.7%) 3.4 (0.3-6.6)	0.030*
Q3. Each professional's role in EOL care, number (ratio)	14 (7.8%) -9.5 (-14.6-4.3)	< 0.001**	5 (2.8%) -6.2 (-14.9-2.4)	0.156	3 (1.7%) -6.2 (-14.9-2.4)	0.156	13 (7.2%) 4.4 (-1.1-9.9)	0.116	4 (2.2%) -8.4 (-18.0-1.2)	0.086	15 (8.3%) 0.1 (-5.1-5.2)	0.979	18 (10.0%) -1.9 (-6.6-2.9)	0.436	108 (60.0%) 4.0 (1.1-6.8)	0.007*
Q4. Key worker to contact for each resident, number (ratio)	6 (3.3%) -6.6 (-14.5-1.3)	0.103	9 (5.0%) -0.9 (-7.5-5.6)	0.784	6 (3.3%) -0.9 (-7.5-5.6)	0.784	9 (5.0%) -2.8 (-9.3-3.8)	0.405	2 (1.1%) 2.3 (-11.4-15.8)	0.748	18 (10.0%) 1.7 (-3.0-6.5)	0.474	14 (7.8%) -0.3 (-5.6-5.0)	0.907	116 (64.0%) 1.4 (-1.6-4.4)	0.346
Q5. Residents' conditions during EOL care, number (ratio)	2 (1.1%) -6.4 (-20.0-7.2)	0.353	2 (1.1%) -9.4 (-23.0-4.1)	0.172	1 (0.6%) -9.4 (-23.0-4.1)	0.172	16 (8.9%) 0.5 (-4.5-5.5)	0.846	5 (2.8%) -11.0 (-19.5-2.4)	0.012*	10 (5.6%) -4.7 (-10.9-1.5)	0.139	10 (5.6%) -0.4 (-6.6-5.8)	0.898	134 (74.4%) 4.0 (0.8-7.2)	0.015*
Q6. Sufficient discussion among professionals, number (ratio)	7 (3.9%) -6.0 (-13.4-1.3)	0.942	4 (2.2%) -6.9 (-16.5-2.8)	0.161	7 (3.9%) -6.9 (-16.5-2.8)	0.161	9 (5.0%) 1.7 (-4.9-8.2)	0.611	7 (3.9%) -6.1 (-14.0-1.8)	0.130	9 (5.0%) -3.0 (-8.5-2.5)	0.289	17 (9.4%) -0.2 (-5.1-4.7)	0.942	117 (65.0%) 3.1 (0.1-6.0)	0.043*
Q7. Mental status of residents' families, number (ratio)	9 (5.0%) -9.7 (-16.0-3.3)	0.003*	7 (3.9%) -2.1 (-9.5-5.3)	0.573	9 (5.0%) -2.1 (-9.5-5.3)	0.573	14 (7.8%) 2.7 (-2.6-8.1)	0.310	42 (23.3%) -2.3 (-7.8-3.2)	0.415	18 (10.0%) 2.6 (-2.1-7.3)	0.280	7 (3.9%) -2.1 (-6.2-2.0)	0.314	85 (47.2%) 3.0 (0.1-5.8)	0.040*
Q8. Emergency codes during on-call hours, number (ratio)	2 (1.1%) -6.4 (-20.0-7.2)	0.353	1 (0.6%) -0.2 (-19.4-19.0)	0.981	2 (1.1%) -0.2 (-19.4-19.0)	0.981	5 (2.8%) 2.8 (-5.9-11.4)	0.532	6 (3.3%) -11.5 (-20.0-2.9)	0.009*	11 (6.1%) -2.1 (-8.1-3.8)	0.479	9 (5.0%) -3.0 (-9.5-3.6)	0.370	144 (80.0%) 4.7 (1.2-8.2)	0.009*
Q9. Residents' key people, number (ratio)	2 (1.1%) 3.2 (-10.4-16.8)	0.643	4 (2.2%) 2.2 (-7.5-11.9)	0.658	3 (1.7%) 2.2 (-7.5-11.9)	0.658	7 (3.9%) -2.3 (-9.7-5.0)	0.532	2 (1.1%) -11.1 (-22.1-0.0)	0.049*	12 (6.7%) -3.7 (-9.4-2.0)	0.208	12 (6.7%) -2.8 (-8.5-2.9)	0.333	137 (76.1%) 3.7 (0.4-7.0)	0.027

NS: nurse; CM: care manager; PCG: professional caregiver; CI: confidence interval; Q: question; EOL: end of life. Statistical analysis was conducted based on a simple regression model for the amount of EOL care in nursing homes; *$p < 0.05$; **$p < 0.001$.

Table 3. Multivariable analysis of factors associated with end-of-life care in the nursing homes (n = 180)

Variable	Coefficient	(95%CI)	p-value
Interprofessional collaboration	2.5	(0.45-4.48)	0.017*
Availability of EOL care bonuses	4.4	(1.41-7.38)	0.004*
Physician support for emergency care during off time	5.4	(1.86-8.94)	0.003*
Proximity to affiliated hospital	2.0	(-3.82-7.89)	0.494
EOL care conferences	4.1	(1.19-6.99)	0.006*

Based on multiple linear regression analysis; EOL: end of life; CI: confidence interval; *$p < 0.05$.

perception of interprofessional collaboration by two professionals, one professional, and no professionals. These results were expected to some extent. In the univariable analysis, the perception of interprofessional collaboration by all three professionals indicated increased EOL care in nursing homes. On the other hand, the perception of interprofessional collaboration exclusively by only the nurse or by none of the professionals indicated decreased EOL care in nursing homes. The results suggest that nurse leaders in EOL care need to attend to other professionals' perceptions of interprofessional collaboration in EOL care.

The results of the multivariable analysis revealed that interprofessional collaboration was associated with the amount of EOL care in nursing homes. The results are novel in revealing the importance of interprofessional collaboration.

In the other results of the multivariable analysis, availability of EOL care bonuses, physician support for emergency care during off time, and EOL care conferences were shown to be independent factors associated with EOL care in nursing homes, which is consistent with previous research (10). The univariable analysis indicated that the perception of interprofessional collaboration by all three types of professionals increased the amount of EOL care in nursing homes, while the perception of interprofessional collaboration exclusively by the nurse or by none of the professionals decreased the amount of EOL care in nursing homes. The results on the perception of interprofessional collaboration by three professionals and no professionals were readily understood. The results strongly suggested that the perception of interprofessional collaboration in EOL care in nursing homes by only nurses may be an interfering factor. The results indicated that nurses in nursing homes need interprofessional collaboration with other professionals, not only subjective perceptions of EOL care. For the implementation of EOL care in nursing homes, communication, interviews and surveys with many professionals can be helpful.

4.3. Study limitations

Our study has several limitations. First, causal relationships could not be established due to the retrospective design. Second, there was selection bias because the setting was one prefecture in Japan, and the sample size was small because facilities without subjective responses from all three professionals were excluded. Future surveys should have a sufficient sample size to accommodate the eight patterns shown in Figure 2. Third, the present study was a questionnaire survey and may have been affected by response bias due to the possibility that respondents provided socially desirable responses to the questionnaire (*23*). Fourth, the study was an exploratory assessment of interprofessional collaboration in EOL care in nursing homes, and interprofessional collaboration was measured based on professionals' perceptions. Future studies are needed to determine the validity of this measure.

4.4. Notable characteristics of this study compared to other studies

In the field of interprofessional collaboration, few studies have undertaken quantitative evaluations due to the difficulty of measuring collaboration (*24,25*). Moreover, studies of interprofessional collaboration in EOL care in nursing homes are limited (*16,26-28*). The survey method used in this study was unique in that the same questions were asked of three types of professionals who ostensibly worked together in the same facility. To the best of our knowledge, this is the first such study of EOL care in nursing homes in the global literature. In research on perceptions of interprofessional collaboration, team training has been shown to be effective in the perception of collaboration (*29*). However, previous literature has not examined the interprofessional collaboration among nursing home professionals. Therefore, our approach will undoubtedly contribute to awareness of the reality of EOL care in nursing homes.

Given the cooperation of the directors of the health and welfare departments, the response rate (62.7%) in the present study was higher than that of a general survey. Therefore, the results of our study have high external validity.

4.5. Practical considerations and future work

According to the results of the present study, interprofessional collaboration among nurses, care managers, and professional caregivers is important to promote EOL care in nursing homes. Therefore,

it will be useful to regularly assess the perception of interprofessional collaboration in EOL care based on each type of professional in nursing homes. A questionnaire survey of multiple professionals, such as that used in the present study, can be useful for the assessment of interprofessional collaboration in EOL care research given that the present study revealed differences in the perceptions of interprofessional collaboration among professionals and was successful in quantitatively assessing interprofessional collaboration.

In conclusion, interprofessional collaboration in EOL care is associated with the amount of EOL care in nursing homes. The perception of interprofessional collaboration exclusively by nurses or by no professionals may tend to decrease the amount of EOL care in nursing homes. Interprofessional collaboration among nursing home professionals is effective for EOL care in nursing homes.

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Supplemental Data

Questionnaire (Survey of End-of-Life Care)

Q1. Do you think that each professional shares a basic policy for end-of-life care in your nursing home? 1) Yes 2) No

Q2. Do you think that each professional shares residents' or residents' families' wishes regarding end-of-life care in your nursing home? 1) Yes 2) No

Q3. Do you think that the role of each professional in end-of-life care is clear in your nursing home? 1) Yes 2) No

Q4. Do you think that a key worker is chosen for each resident during end-of-life care in your nursing home? 1) Yes 2) No

Q5. Do you think that each professional shares information on residents' conditions during end-of-life care in your nursing home? 1) Yes 2) No

Q6. Do you think that there is sufficient discussion by each professional of residents during end-of-life care in your nursing home? 1) Yes 2) No

Q7. Do you think that each professional shares information on the mental statuses of residents' families during end-of-life care in your nursing home? 1) Yes 2) No

Q8. Do you think that each professional shares the emergency codes for a resident in your nursing home? 1) Yes 2) No

Q9. Do you think that each professional knows who the key person for each resident is in your nursing home? 1) Yes 2) No

Original Article

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Evaluation of rapid drug safety communication materials for patients in Japan

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SUMMARY Since 2011, pharmaceutical companies in Japan have been required to issue two types of documents regarding severe adverse drug reactions reported post-marketing, namely the Rapid Safety Communication Materials for Patients and the Related Materials. However, the adequacy of these documents has not yet been systematically assessed. The aim of this study was to evaluate the adequacy of these two types of materials. The Rapid Safety Communications for Patients were obtained from the Pharmaceuticals and Medical Devices Agency (PMDA) website. The Related Materials were obtained from pharmaceutical companies or the PMDA website. Three assessors independently scored the Rapid Safety Communication for Patients and the Related Materials using the Centers for Disease Control and Prevention Clear Communication Index (CCI). In addition, the contents and descriptions of the materials were analyzed. In total, 13 materials for seven drugs were assessed. Almost all materials contained the "main message" and "call to action". However, the average CCI scores for the Rapid Safety Communication for Patients and Related Materials for Patients were 68.8 and 74.3 (out of 100), respectively. Further, none of the evaluated materials were scored above the CCI threshold score (*i.e.*, ≥ 90%). Descriptions regarding "language", "state of science", and "risk" were not adequate. In particular, the terminology used in materials seemed difficult for patients to understand. In conclusion, the Japanese Rapid Communication Materials for Patients require improvement. Furthermore, a system for evaluating these materials prior to publication should be established.

Keywords Risk communication, Clear Communication Index, drug safety

1. Introduction

Japan has experienced several pharmaceutical drug-related disasters ("*Yakugai*" in Japanese), and a possible reason for these is the lack of prompt risk communication to the public, including patients. Risk communication refers to "communication intended to supply lay people with information they need to make informed, independent judgment about risks to health, safety, and the environment" (*1*). For effective risk communication, adequate drug safety information must be provided to patients, which can prevent severe adverse drug reactions.

In 2011, the Ministry of Health, Labour, and Welfare (MHLW) set up a risk communication framework for patients based on the post-marketing phase of drug evaluation, in addition to the framework already available for healthcare professionals. The MHLW requires pharmaceutical companies to immediately issue two types of special warning communication letter, for healthcare professionals and patients, when severe or fatal adverse drug reactions occur (*2*): the Rapid Safety Communication (Blue Letter) and the Emergent Safety Communication (Yellow Letter) (*3*). The latter is issued in more serious cases, especially when urgent safety measures are necessary. These communication

letters for patients are A4-sized, single-page documents, based on a template standardized by the MHLW. As of January 2020, seven Blue Letters for patients and no Yellow Letters have been issued. In addition, the MHLW requests that pharmaceutical companies prepare similar, easily understood Related Materials documents using their own format (*4*). Generally, these documents are handed directly to the patients at the medical institution and also made available on the website of the Pharmaceuticals and Medical Devices Agency (PMDA), which is the Japanese national regulatory body, or on that of the pharmaceutical companies. However, the quality of these communication letters has not yet been systemically evaluated.

Clarity is critical in order for written communication materials to be comprehensible to a general audience with varying literacy levels. Recently, governmental organizations in developed countries have introduced standards (criteria) for providing clear health information to patients. In the USA, "Clear & Simple" (*5*) from the National Institutes of Health and the "Toolkit for Making Written Material Clear and Effective" (*6*) are issued by the Center for Medicare and Medicaid Services. Furthermore, the Centers for Disease Control and Prevention (CDC) published the "CDC Clear Communication Index (CCI)" (*7*) in 2014. The CCI has been used to identify important communication characteristics that enhance clarity and help readers to understand public messages and materials (*8*). This index is the most comprehensive and widely used tool for assessing and developing such materials (*9-12*). However, to the best of our knowledge, no study has applied the CCI for the assessment or during the development of rapid safety communication materials intended for patients. In this study, we aimed to evaluate the adequacy of all Blue Letter and Related Materials documents in Japan using the CCI.

2. Materials and Methods

2.1. Target materials

We searched the PMDA website for Blue Letters (including the Blue Letters for Healthcare Professionals and Blue Letters for Patients) issued as of April 2017 for the following six drugs: RANMARK® Subcutaneous Injection, Careram® Tablets, YAZ®, XEPLION® Aqueous Suspension, SOVRIAD® Capsules, and Lamictal® Tablets. Because the Related Materials for Patients were not available on the websites of PMDA or the pharmaceutical companies, we obtained the respective Related Materials for Patients directly from the companies. In November 2019, we obtained the Blue Letter for Healthcare Professionals and Patients as well as the Related Material for Patients for another drug, Verzenio®, from the PMDA website.

2.2. Blue Letter basic information

The international nonproprietary names (INNs) and indications were extracted from the package insert for each drug by T.S. (a pharmacist). Thereafter, information regarding severe adverse reactions was extracted from the Blue Letters by T.S. in January 2020.

2.3. Format and contents of the Blue Letters for Patients and Related Materials for Patients

In January 2020, A.Y. (Master of Public Health, MPH) and T.S. independently counted the pages of the Related Materials for Patients and extracted the date of development as well as contents from each Related Materials for Patients document. Thereafter, the materials were assessed. To count the characters in the materials, the PDF documents were converted to Microsoft Word® format, and the software's character-counting tool was used.

2.4. Assessment of materials based on the CCI

The CCI was downloaded from the CDC website (*13*) along with the user's guide (*8*) and full index (*14*). The author and co-authors confirmed the criteria in the CDC CCI user's guide for the assessment of materials.

The CCI is divided into the following seven categories: main message and call to action, language, information design, state of science, behavioral recommendations, numbers, and risk. Each of these contains 20 items, with a rating of 0 or 1. The individual scores were converted to an overall score of 100. A CCI percentage score of ≥ 90 would indicate that the evaluated material is clear and understandable.

The materials were assessed between January 20 and February 26, 2020. A.Y. (MPH), M.Y. (Pharmacist), K.Y. (Pharmacist), and T.N. (MD) discussed and defined "the main message" and "call to action" in order to understand the details and factors that led to the issue of the document (*i.e.*, the Blue Letter), with a focus on severe adverse reactions as well as signs and symptoms. Furthermore, three assessors (A.Y., M.Y., and K.Y.) confirmed the "behavioral recommendations" and "risk" in these materials. Based on "the main message" and "call to action", the three assessors independently scored the Blue Letter for Patients and Related Materials for Patients, with one point for "yes" and zero for "no" per CCI item. After the first round of scoring, the first author (A.Y.) collected the scores from the other assessors and compared them. For items with different scores, each author described their reason for scoring, and the assessors once again scored the items independently (the second round of scoring). After discussing differences in the third round of scoring, the scores were revised to obtain the final scores. Finally, A.Y. compiled all the scores.

2.5. Statistics

The internal agreement of assessors for CCI scoring was computed using the Fleiss's κ-value (*15*). Other statistical analyses (the mean score and standard error of mean, *t*-test to assess the difference in the average scores of two materials) were performed using R® software version 3.4.3. and Microsoft Excel®.

3. Results

3.1. Materials obtained

Seven Blue Letters, one for each of the seven drugs, were issued before January 10, 2020. For each drug, the INN, indications, and severe adverse reactions list, as well as the issue date and the number of characters in the relevant Blue Letter for Patients, are summarized in Table 1. The number of characters varied between 636 and 1005.

In addition, we obtained seven Related Materials for Patients from pharmaceutical companies or the PMDA website. The material for XEPLION® Aqueous Suspension was not available, and we obtained two separate documents for Lamictal® Tablets.

3.2. Format and contents of the Related Materials for Patients

Table 2 presents a summary of the format and contents of the obtained Related Materials for Patients. Some patient materials did not contain an issue date. They varied in the number of pages (between 1 and 6) and characters (between 447 and 2832). All materials were presented in color, with illustrations lacking only in one. Three materials contained the indications of the relevant drugs. Two of the materials included photographs of the drug. All materials, excluding that for SOVRIAD® Capsules, contained information on the signs and symptoms of severe adverse reactions, and all materials contained the recommendation to consult a physician or pharmacist. We then searched materials for the contact information for the pharmaceutical companies and medical institutes. One material contained only the former, another included only the latter, three contained both, and two included no contact information at all.

3.3 Evaluation of materials in accordance with the CCI

The "main message" and "call to action" were defined as follows:

Main message: Because severe adverse reactions have occurred, be aware of the relevant signs and symptoms.

Call to action: If these signs and symptoms appear (or in the case of contraindication), consult your physician or pharmacist.

Table 1. Characteristics of Blue Letters[1]

Blue Letters for healthcare professionals							
Product name	RANMARK® Subcutaneous Injection	Careram® Tablets	YAZ®	XEPLION® Aqueous Suspension	SOVRIAD® Capsules	Lamictal® Tablets	Verzenio®
International Nonproprietary Name (INN)	Denosumab	Iguratimod	Drospirenone, Ethinyl estradiol	Paliperidone	Simeprevir	Lamotrigine	Abemaciclib
Indication[2]	Bone lesion associated with multiple myeloma or bone metastases from solid tumors	Rheumatoid arthritis	Dysmenorrhea	Schizophrenia	Chronic hepatitis C	Bipolar disorder, epilepsy	Breast cancer
Severe adverse reactions	Serious hypocalcemia	Serious bleeding	Thrombosis	Fatal cases	Hyperbilirubinemia	Serious skin disorders	Interstitial pneumonia
Issue date for professionals	2012.9.11	2013.5.17	2014.1.17	2014.4.17	2014.10.24	2015.2.4	2019.5.17
Blue Letters for Patients							
Material No.	1-1	2-1	3-1	4-1	5-1	6-1	7-1
Number of characters	1005	1002	657	636	639	696	646

[1]Blue Letters: Rapid Safety Communications. [2]Excerpt from the drug package inserts.

Table 2. Characteristics of the Related Materials for Patients

Product name	RANMARK® Subcutaneous Injection	Careram® Tablets	YAZ®	XEPLION® Aqueous Suspension	SOVRIAD® Capsules	Lamictal® Tablets	Verzenio®
Material No.	1-2	2-2	3-2	No material	5-2	6-2	6-3	7-2
Issue date for patients	Not found	2016.5	Not found		2016.5	2015.3	2015.6	2019.6
Number of pages	6	2	4		2	3	6	1
Number of characters	1732	855	764		1164	447	2832	513
Contents[1]								
Indication		○	○				○	
Contraindication		○			○			
Risk of severe adverse reactions	○	○	○			○	○#	○
Subjective symptoms of severe adverse reactions	○	○	○			○	○#	○
How to take the drug		○			○		○#	
Drug photo	–	○			○			
Recommendation to consult a doctor	○	○	○		○	○	○#	○
Recommended preventive behavior	○	○	○			○	○#	
Taking the drug as directed		○				○	○	
Contact information; pharmaceutical company	○	○	○					○
Contact information; medical institute	○	○				○		○
Others	● High-risk patients ● Mechanism of serious adverse reaction		● Be cautious of the adverse reactions in carriers of the card. ● Instruction to follow doctor's directions. ● Space for medical center use.		● How to store the medicine. ● Symptoms of main adverse reactions. ● Caution on pregnancy and breastfeeding during treatment.	Main adverse effects# #Mention in FAQ.		Supervisor

[1]Two reviewers extracted data from each material and summarized them.

Based on the "main message" and "call to action," three assessors graded the seven Blue Letters and six Related Materials for Patients with a score of 0 or 1 per CCI item. The assessment results are presented in Table 3.

The material for SOVRIAD® Capsules was excluded from CCI base assessment because it did not mention severe adverse events and only provided information on how to take the medicine. In total, 13 materials were assessed. Reliability analysis showed substantial agreement among the assessors ($\kappa = 0.64$). The average scores and standard errors of the means for the three assessors were 68.8 ± 2.8 and 74.3 ± 2.8 for the Blue Letters for Patients and Related Materials for Patients, respectively. None of the materials reached a CCI score of 90%, which is the threshold for patient materials to be considered comprehensible.

3.3.1. Core

The "main message" (item #1) and "call to action" (#5) were found in almost all the Blue Letters for Patients. All Blue Letters for Patients were evaluated with "No" for #4 (visual support). However, some of the illustrations supported the main message in four of the Related Materials for Patients. All text in the Related Materials for Patients was in the active voice (#6). In a few of the Blue Letters for Patients, the main message was written in the passive voice. Approximately half of the materials included words that are not commonly used by the primary audience (#7). Bulleted or numbered lists were included (#8), and the most important information (main message) was presented at the top of the materials (#10) in all documents evaluated. "What's known and what's not" (#11, state of science,) was explained in only one material (4-1).

3.3.2. Behavioral recommendation

"Call to action" was regarded as a "behavioral recommendation," and #12-14 were scored with "Yes" for all materials, except one (2-2).

3.3.3. Numbers and risk

We evaluated only the numbers related to severe adverse reactions. None of the materials were scored on this part. Thus, part C was excluded from the calculation of total score percentage. The nature of the risk (#18) was not explained in three of the Blue Letters for Patients and four of the Related Materials, while the risks and benefits of the recommended behaviors (#19) were not addressed in any of the materials.

4. Discussion

In this study, we evaluated the adequacy of two types of rapid safety communication materials regarding

Table 3. Clear Communication Index Scores for Blue Letters for patients and the Related Materials

Part	No.	Questions	Blue Letter for Patients[1]								Related Materials for Patients[2]							
			1-1	2-1	3-1	4-1	5-1	6-1	7-1	Mean score of 3 reviewers for 7 materials	1-2	2-2	3-2	5-2	6-2	6-3	7-2	Mean score of 3 reviewers for 6 materials
			No. of reviewers who answered 'Yes'								No. of reviewers who answered 'Yes'							
Part A: Core																		
Main Message and Call to Action	1	Does the material contain one main message statement?	3	3	3	3	3	3	3	3.0	3	2	3	-	3	3	3	2.8
	2	Is the main message at the top, beginning, or front of the material?	3	3	3	0	3	3	3	2.6	3	2	3	-	3	3	3	2.8
	3	Is the main message emphasized with visual cues?	3	3	3	0	3	2	3	2.4	3	2	2	-	3	3	3	2.7
	4	Does the material contain at least one visual that conveys or supports the main message?	0	0	0	0	0	0	0	0.0	3	0	0	-	3	3	3	2.0
	5	Does the material include one or more calls to action for the primary audience?	3	3	3	3	3	3	3	3.0	3	3	3	-	3	3	3	3.0
Language	6	Do both the main message and the call to action use the active voice?	1	1	3	1	3	3	3	2.1	3	3	3	-	3	3	3	3.0
	7	Does the material always use words the primary audience uses?	2	0	3	3	0	3	0	1.6	0	3	3	-	3	0	0	1.5
Information Design	8	Does the material use bulleted or numbered lists?	3	3	3	3	3	3	3	3.0	3	3	3	-	3	3	3	3.0
	9	Is the material organized in chunks with headings?	0	0	3	0	3	3	3	1.7	3	3	3	-	0	3	3	2.5
	10	Is the most important information the primary audience needs summarized in the first paragraph or section?	3	3	3	3	3	3	3	3.0	3	3	3	-	3	2	3	2.8
State of Science	11	Does the material explain what authoritative sources, such as subject matter experts and agency spokespersons, know and don't know about the topic?	0	0	0	2	0	0	0	0.3	0	0	0	-	0	0	0	0.0
Part B: Behavioral Recommendations	12	Does the material include one or more behavioral recommendations for the primary audience?	3	3	3	3	3	3	3	3.0	3	3	3	-	3	3	3	3.0
	13	Does the material explain why the behavioral recommendation(s) is important to the primary audience?	3	3	3	3	3	3	3	3.0	3	0	3	-	3	3	3	2.5
	14	Does the behavioral recommendation(s) include specific directions about how to perform the behavior?	3	3	3	3	3	3	3	3.0	3	3	3	-	3	3	3	3.0
Part C: Numbers	15	Does the material always present numbers the primary audience uses?																
	16	Does the material always explain what the numbers mean?																
	17	Does the audience have to conduct mathematical calculations?																
Part D: Risk	18	Does the material explain the nature of the risk?	3	0	3	3	0	0	0	1.3	3	0	0	-	0	0	3	1.0
	19	Does the material address both the risks and benefits of the recommended behaviors?	0	0	0	0	0	0	0	0.0	0	0	0	-	0	0	0	0.0
	20	If the material uses numeric probability to describe risk, is the probability also explained with words or a visual?																
		Mean total score % for 3 reviewers	68.8	58.3	81.3	62.5	68.8	72.9	68.8	68.8	81.3	62.5	72.9	-	75	72.9	81.3	74.3
		Standard error of mean	3.6	2.1	0.0	0.0	0.0	2.1	0.0	2.8	0.0	6.3	2.1	-	0.0	2.1	0.0	2.8
																	Overall κ-value	0.64

[1]Blue Letter for Patients; 1-1 RANMARK® Subcutaneous Injection, 2-1 Careram® Tablets, 3-1 YAZ®, 4-1 XEPLION® Aqueous Suspension, 5-1 SOVRIAD® Capsules, 6-1 Lamictal®. [2]Related Materials for Patients; 1-2 RANMARK® Subcutaneous Injection, 2-2 Careram® Tablets, 3-2 YAZ®, 5-2 SOVRIAD® Capsules, 6-2 Lamictal® Tablets, 6-3 Lamictal® Tablets

severe adverse reactions for patients in Japan and found that these materials have several areas that require improvement. In particular, none of the assessed materials achieved the threshold (≥ 90%) CCI score. Furthermore, the descriptions regarding "language," "state of science," and "risk" were not adequate. The effectiveness of risk communication is often judged based on whether the intended audience took effective action (*16*). Fischhoff (*16*) lists the following requirements for adequate risk communication: (a) the information needed for effective decision making, (b) accessibility of the information, and (c) comprehensibility. The results of our study suggest that, overall, Japanese rapid safety communication materials for patients have a few issues, particularly related to the comprehensibility of risks.

Most of the materials in the Blue Letters for Patients and Related Materials for Patients contained a "main message" (#1, *i.e.*, "the occurrence of severe adverse reactions and warning of the symptoms") and a "call to action" (#5, *i.e.*, "consult a physician or pharmacist"). These items are considered sufficient minimum information with which to make a decision (*16*). However, there was a lack of information on "what's known and what's not" provided by authorities (state of science, #11), such as the details and number of severe adverse reaction cases or the uncertainty of association between adverse reactions and the drug concerned, as well as on the "risks and benefits of the recommended behaviors" (#19). Davis reported that patients preferred specific, detailed information about adverse effects (*17*). According to Suka *et al.*, several Japanese lay people indicated that they believe all information should be disclosed (*18*). These patients' information needs should be considered.

In the current study, we did not find detailed information on adverse reaction frequency in any of the materials (Part C). The quality of information on severity and frequency affects risk perception in relation to adverse reactions (*19*). Further research is required to establish effective adverse reaction risk perception in Japanese patients.

Accessibility refers to the following two aspects: access to information sources (*i.e.*, drug safety materials) and access to message content (*i.e.*, main message and call to action) (*16*). The CCI user's guide highlights how to disseminate rapid safety communication materials, and Japanese authorities have requested that materials be widely disseminated to the public as well as patients. To the best of our knowledge, there are no studies assessing ease of access to rapid safety communication materials. Accessibility, defined as access to message content, can be evaluated based on CCI items #2-4 (*7,10*). The Blue Letters for Patients lacked visual components (#4), such as illustrations, due to the requirement for their rapid publication. Nevertheless, the materials were concise, and the main messages were accessible because they were presented in the front (#2) and emphasized with visual cues (#3), except for one material. In contrast, most Related Materials for Patients varied in format and content, were colorful, and had a user-friendly appearance. However, their main messages may be unclear to patients, due to the substantial amount of information distributed across several pages, including extensive information on dosage.

In the current study, we identified some key issues regarding the comprehensibility of Japanese rapid drug safety information materials for patients. First, the risks of severe adverse reactions were not explicitly mentioned in some of the materials. To prevent severe adverse reactions, it is of utmost importance that patients are able to perceive the signs and symptoms and take appropriate actions (*20*). However, some materials did not clearly explain the severity of health outcomes (for example, "interstitial pneumonia"). Second, some materials used only medical terms (for example, "jaundice"), while it is necessary to explain risks and symptoms in a manner that lay people can understand. Illustrations may help promote a better understanding of medical terms. Third, with regard to voice (#6 in the CCI), the main messages of some materials were presented in the passive voice, as in "the case was reported." Japanese people tend to use the passive voice more often than Westerners, due to the characteristics of the language. However, in the Japanese language, the passive voice is also used to imply possibility, spontaneity, and respect. For this reason, drug safety materials should be written in the active voice as much as possible, in order to improve comprehensibility among the broader readership.

The current study had some limitations. First, although the Related Materials were obtained from the pharmaceutical companies, some materials might have been developed with intentions other than warning patients about serious adverse reactions. Further, as pharmacists and/or MPH professionals, the assessors might have been generous in scoring item #7 "use words that the primary audience use."

In conclusion, several concerns regarding the comprehensibility of Japanese rapid safety communication materials on drugs for patients were identified in this study. In particular, the language used and the explanation of the nature of risks should be improved. In addition, Japanese authorities should build a system for evaluating materials for patients based on indices, such as CCI, for use prior to material publication.

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Supplementary Material

The Blue Letters for patients
1-1 https://www.pmda.go.jp/files/000148240.pdf
2-1 https://www.pmda.go.jp/files/000148446.pdf
3-1 https://www.pmda.go.jp/files/000148622.pdf
4-1 https://www.pmda.go.jp/files/000148732.pdf
5-1 https://www.pmda.go.jp/files/000147341.pdf
6-1 https://www.pmda.go.jp/files/000198340.pdf
7-1 https://www.pmda.go.jp/files/000229599.pdf

Brief Report

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Clinical features of Barré-Lièou syndrome and efficacy of trazodone for its treatment: A retrospective single center study

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SUMMARY Barré-Lièou syndrome (BLS) is a manifestation of various autonomic and secondary symptoms including muscle stiffness, tinnitus, dizziness, and pain in various body parts. Although considered to be caused by hyperactivation of the autonomic nervous system due to trauma, there is currently no firmly established etiology or evidence on the treatment and clinical features of BLS. We retrospectively examined the clinical features of BLS and evaluated the efficacy of trazodone (TZD) for its treatment. We conducted a retrospective analysis of the data of 20 consecutive cases with suspected BLS who were treated in our hospital between 2016 and 2019. BLS symptoms were rated on a 10-point scale, and two groups were defined, that is, a mild-BLS group (BLS scores, 1-5) and a severe-BLS group (BLS scores, 6-10). Univariate analysis of patient factors was performed. The BLS score was 6.0 ± 1.7, and the maximum TZD dose was 80 ± 34 mg/day; nine patients (45%) were TZD free, and no TZD side effects were observed, while all patients had a good clinical outcome. There were significant differences between the mild-BLS and severe-BLS groups in the period from injury to diagnosis ($p = 0.015$), chest/back pain ($p < 0.001$), constipation ($p = 0.001$), and maximum TZD dose ($p = 0.008$). BLS involves posttraumatic autonomic symptoms accompanied by depression and insomnia. The sympathetic hypersensitivity theory could explain its etiology. TZD could effectively and safely treat BLS, and early diagnosis and treatment can contribute toward good clinical outcomes. Enhanced recognition and understanding of this disease are warranted.

Keywords Nonspecific symptoms, posttraumatic autonomic symptoms, hyperactivation

1. Introduction

Barré-Lièou syndrome (BLS) (*1-3*) is a manifestation of various autonomic and secondary symptoms, such as muscle stiffness, tinnitus, dizziness, and pain in the head, neck, eyes, throat, ears, chest, and back. The symptoms are as follows: 1) inner ear symptoms: dizziness, tinnitus, and feelings of obstruction of the ear; 2) eye symptoms: blurred vision, fatigue, discomfort, and sight loss (asthenopia); 3) chest and back symptoms: chest pain, back pain, arrhythmia, and respiratory distress; 4) pharyngeal and laryngeal symptoms: hoarseness, discomfort in the throat, and swallowing difficulty; 5) head and neck symptoms: headache, sense of head weight, and back of neck pain; 6) others: upper limb and whole body fatigue, upper limb numbness, distraction; 7) secondary psychiatric symptoms (anxiety, depression, and others), and 8) insomnia (*3*).

Although considered to be caused by hyperactivation of the autonomic nervous system due to trauma, there is currently no firmly established etiology. This and the nonspecific nature of many of its symptoms present a challenge both for clinicians, who must provide a correct diagnosis, and for patients, who are often misdiagnosed or face undue scrutiny from insurance companies. To date, there is no established evidence on the treatment and clinical features of BLS (*1-3*). We retrospectively investigated the clinical features of BLS and evaluated the efficacy of trazodone (TZD) for its treatment aiming to contribute novel evidence toward uncovering the nature of this obscure syndrome and provide viable treatment solutions.

2. Materials and Methods

This was a single-center retrospective cohort study,

enrolling 20 consecutive patients treated in our hospital between 2016 and 2019, with no positive traumatic changes on head and neck computed tomography or magnetic resonance imaging. They were suspected to have BLS based on the presence of posttraumatic autonomic symptoms. Although there are no specific diagnostic criteria for BLS, the following were adopted (*1-3*): autonomic symptoms observed after trauma, no significant traumatic changes observed on imaging, and improved symptoms through symptomatic blockade treatment. TZD was started at 20 mg/day and increased as needed in all cases where BLS was suspected. Combined use of stellate ganglion block was also considered.

To evaluate the severity of BLS, we calculated a "BLS score" as follows. Autonomic symptoms by organ (head, neck, eyes, ears, throat, chest and back, constipation, others), depression, and insomnia were assigned 1 point each, thus constituting the BLS score (range, 0-10). "Others" included upper limb numbness. TZD was discontinued in patients with a BLS score of 0 (TZD-free). The BLS score (after TZD) was also assessed at the last follow-up. When the BLS score improved by 2 points or more and the condition did not interfere with daily life, we considered that the patient had a good clinical outcome; otherwise, the outcome was considered poor.

Study parameters included age, sex, type of injury (whether or not the patient had been involved in a traffic accident), period from injury to diagnosis, follow-up duration, duration of TZD treatment, history of visiting other departments, presence or absence of autonomic symptoms and depression or insomnia, maximum TZD dose, whether or not TZD could be discontinued (TZD-free), presence or absence of TZD side effects, presence or absence of concomitant stellate ganglion block, and clinical outcome. The effective factors for TZD treatment were also investigated between initial mild (BLS score: 1-5) and severe groups (BLS score: 6-10).

The variables are expressed as percentage values or mean ± standard deviation. Fisher's exact test was used for categorical variables, and the Mann-Whitney U test was used for continuous variables. A p value < 0.05 was considered statistically significant. This study was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from the patients for participation in the study and publication of these cases and the accompanying images. The study design was approved by the appropriate ethics review board of our hospital.

3. Results and Discussion

Table 1 shows the clinical features of all 20 cases. The mean (± SD) age was 61.5 ± 13.6 years; there were 8 men and 12 women, and 16 patients (80%) had been injured in car accidents. The period from injury to diagnosis was 25.7 ± 62.8 months, the follow-up duration was 16.6 ± 8.4 months, the duration of TZD treatment was 12.0 ± 3.8 months, and other departments had been visited by 16 patients (80%). The initial BLS score was 6.0 ± 1.7, BLS score (after TZD) was 0.9 ± 1.0, the maximum dose of TZD was 80 ± 34 mg/day, 9 patients (45%) were TZD free, and no TZD side effects (especially

Table 1. The characteristics of the 20 consecutive cases of suspected Barré-Lièou Syndrome

Case No.	Age (years)	Sex	Type of injury (car accident)	Period from injury to diagnosis (days)	Observation period (months)	Duration of TZD treatment (months)	History of visiting other departments	BLS score	BLS score (after TZD)	TZD (max dose, mg)	TZD free	Stellate ganglion block
1	68	Female	+	270	37	12	+	9	0	100	+	-
2	44	Male	+	120	28	11	+	5	0	100	+	-
3	70	Female	+	4	27	16	+	5	0	50	+	-
4	40	Female	+	1	26	17	+	7	0	100	+	-
5	66	Female	+	6	23	17	+	5	0	100	+	-
6	46	Male	+	31	21	21	+	9	3	150	-	+
7	53	Male	+	1	20	12	+	4	0	50	+	-
8	64	Female	+	18	18	10	+	7	0	50	+	-
9	72	Female	+	9	17	11	-	6	0	50	+	-
10	45	Female	+	36	17	16	+	6	0	100	+	-
11	69	Male	+	21	15	15	-	8	2	100	-	-
12	56	Female	-	25	12	12	+	7	1	100	-	-
13	53	Male	+	5	10	10	+	8	3	150	-	+
14	73	Female	+	3	10	10	+	5	1	50	-	-
15	85	Male	+	2	9	9	+	4	1	50	-	-
16	72	Male	+	1	9	9	-	4	1	50	-	-
17	80	Female	-	12	9	9	+	7	2	100	-	-
18	72	Female	+	4	9	9	-	4	1	50	-	-
19	63	Male	-	3	8	8	+	4	1	50	-	-
20	39	Female	-	1	6	6	+	5	1	50	-	-

Abbreviations. BLS: Barré-Lièou syndrome, n: number of patients, SD: standard deviation, TZD: trazodone.

Table 2. Differences in patient characteristics and clinical outcomes between the mild and severe groups before trazodone administration

Patient factors	BLS score: 1-5 (n = 10)	BLS score: 6-10 (n = 10)	p value
Age, mean ± SD (years)	63.7 ± 14.3	59.3 ± 13.3	$p > 0.1$*
Sex (female), *n* (%)	5 (50)	7 (70)	$p > 0.5$†
Type of injury (car accident), *n* (%)	8 (80)	8 (80)	$p > 0.5$†
Period from injury to diagnosis, mean ± SD (days)	14.5 ± 37.1	42.8 ± 80.6	$p = 0.015$*
Follow-up duration (months)	14.9 ± 8.6	18.2 ± 8.3	$p > 0.1$*
Duration of TZD treatment, mean ± SD (months)	10.7 ± 3.5	13.3 ± 3.8	$p > 0.1$*
TZD free, *n* (%)	4 (40)	5 (50)	$p > 0.5$†
History of visiting other departments, *n* (%)	8 (80)	8 (80)	$p > 0.5$†
Autonomic symptoms Head, *n* (%)	9 (90)	10 (100)	$p > 0.5$†
Neck, *n* (%)	10 (100)	10 (100)	NA
Eyes, *n* (%)	8 (80)	7 (70)	$p > 0.5$†
Ears, *n* (%)	4 (40)	7 (70)	$p > 0.1$†
Throat, *n* (%)	9 (90)	10 (100)	$p > 0.5$†
Chest/Back, *n* (%)	0 (0)	9 (90)	$p = 0.00001$†
Constipation, *n* (%)	1 (10)	9 (90)	$p = 0.001$†
Others	0 (0)	1 (10)	$p > 0.5$†
Depression, *n* (%)	0 (0)	3 (30)	$p > 0.1$†
insomnia, *n* (%)	4 (40)	8 (80)	$p > 0.1$†
TZD max dose, mean ± SD (mg)	60 ± 21	100 ± 33	$p = 0.008$*
Stellate ganglion block, *n* (%)	0 (0)	2 (20)	$p > 0.1$†
Good clinical outcome, *n* (%)	10 (100)	10 (100)	NA

Abbreviations. BLS: Barré-Lièou syndrome, n: number of patients, NA: not applicable, SD: standard deviation, TZD: trazodone. *Note*: †Fisher exact test, *Mann-Whitney *U*-test

cardiac overload as an anticholinergic effect, which is considered a serious side effect; not shown in Table 1) were observed. There was concomitant stellate ganglion block in 2 cases (10%), and all patients had good clinical outcome.

Table 2 shows the comparative results of the univariate analysis for all examined patient factors between the initial mild (BLS score: 1-5, n = 10) and severe (BLS score: 6-10, n = 10) groups. There were significant differences between the mild group (BLS scores, 1-5) and severe group (BLS scores, 6-10) for the period from injury to diagnosis (14.5 ± 37.1 *vs*. 42.8 ± 80.6, $p = 0.015$), chest/back pain (0 [0%] *vs*. 9 [90%], $p < 0.001$), constipation (1 [10%] *vs*. 9 [90%], $p = 0.001$), and maximum TZD dose (60 ± 21 *vs*. 100 ± 33, $p = 0.008$). There were no significant differences in other patient factors.

This study showed the efficacy and safety of TZD for the treatment of BLS. Furthermore, the fact that TZD, an oral sympathetic nervous system blocker, was effective, supports the cervical sympathetic hyperactivity theory for the etiology of BLS (*1-6*), that is, posttraumatic cervical sympathetic hypersensitivity could cause BLS. In addition, it provides evidence for the validity of our BLS diagnostic criteria.

Treatment for BLS is supposed to follow the treatment protocol for cervical sprains, but a stellate ganglion block (one of the cervical sympathetic ganglia) or an α-blocker (a sympatholytic drug) is recommended. In addition, various treatments such as neck epidural block, intravenous drip infusion of prostaglandin E1, and acupuncture are performed, but a clear treatment effect has not been obtained and there have been numerous reported refractory cases (*2,3*).

TZD (*7-10*) is an antidepressant used worldwide as a 5-hydroxytryptamine and alpha 1-adrenergic receptor antagonist and serotonin reuptake inhibitor (7). The alpha-blocking effect of TZD is thought to be responsible for symptomatic improvement in patients with BLS (*3*) and paroxysmal sympathetic hyperactivity (*9,10*), which is considered to be a condition similar to sympathetic hyperactivity. TZD, which is commonly used to treat depression because of the mechanism associated with alpha-adrenergic receptor inhibition (*8*), was additionally effective for symptomatic amelioration in BLS (7-*10*).

TZD is relatively safe for use in the elderly, with fewer reported cases of its side effects (*8*). In our study, no patients experienced serious side effects of cardiac overload, and some of the minor side effects of TZD, such as constipation and dizziness showed early improvement.

The results presented in Table 2 suggest that delay in diagnosis could worsen BLS severity. Interestingly, symptoms of chest/back pain and constipation were significantly more frequent in the severe-BLS group than in the mild-BLS group, and the severe-BLS group received, on average, a significantly higher maximum dose of TZD compared to the mild-BLS group. However, when administered at appropriate doses, TZD could eventually be discontinued, and the clinical outcomes were good in both groups.

Because this syndrome presents with autonomic and psychiatric symptoms, insomnia, and others, it is treated as an undetermined complaint, and a physician unfamiliar with the disease cannot establish

a definitive diagnosis (*3-6*). As mentioned above, BLS' pathophysiology has not been established, and thus has not been internationally recognized as an independent syndrome. In the clinical setting, BLS can be diagnosed as a tension-type headache, cervical arm syndrome, traumatic cervical syndrome, or autonomic imbalance (*3,4*). As the mechanisms of these conditions remain undetermined, BLS could be considered when they manifest. In Japan, BLS is not widely recognized by either neurosurgeons or physicians involved in trauma care. In our study, the high percentage (80%) of patients with history of visiting other departments suggested that low awareness of this disease might be a contributing factor to the delay in diagnosis. Clinicians involved in trauma care should understand the pathology and clinical features of the disease and encourage the active use of TZD when BLS is suspected. Based on our findings, early BLS diagnosis and treatment with TZD, based on our diagnostic criteria and BLS score, could contribute toward a good clinical outcome for patients.

Our study has some limitations. This study involved selection bias due to its retrospective nature. As this retrospective study included a relatively small sample size, future prospective studies with a larger number of cases are suggested to confirm our findings.

In conclusion, BLS involves posttraumatic autonomic symptoms and may be accompanied by depression and insomnia. This study provided evidence supporting the sympathetic hypersensitivity theory to explain the etiology of BLS. TZD was shown to be safe and effective for BLS treatment. Severe BLS is often accompanied by chest/back pain and constipation, but early diagnosis and treatment can contribute toward good clinical outcomes. Enhanced recognition and understanding of this disease by physicians and society are warranted.

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Case Report

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Focal nodular hyperplasia mimicking hepatocellular adenoma and carcinoma in two cases

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SUMMARY Focal nodular hyperplasia (FNH) is a solid benign tumor of the liver, predominantly in young women. A correct diagnosis of FNH is essential for making appropriate clinical decisions and avoiding unnecessary liver resection. Herein, we reported that two male cases with FNH, who initially presented with persistent abdominal discomfort, were misdiagnosed with hepatocellular adenoma (HCA) and hepatocellular carcinoma (HCC) on contrast-enhanced magnetic resonance imaging and computed tomography scans, respectively. After surgery, a histological diagnosis of FNH was finally established. In this paper, we also reviewed the knowledge regarding diagnosis and differential diagnosis of FNH on imaging examinations, which are helpful for avoiding misdiagnoses and guiding clinical interventions.

Keywords Focal nodular hyperplasia, hepatobiliary contrast agents, contrast-enhanced ultrasound, hepatocellular adenoma, hepatocellular carcinoma

1. Introduction

Focal nodular hyperplasia (FNH) is a benign tumor of the liver with a prevalence of 0.3-3% in the general population (*1,2*). It is predominant in women aged 35-50 years old (*3*). In pathophysiology, arterial malformation leads to abnormal blood perfusion and secondary hyperplasia in the liver parenchyma (*4*). In histology, FNH is composed of hyperplastic hepatocytes separated by fibrous septum which contains hyperplastic bile ducts, tiny arterial branches, and infiltrating inflammatory cells (*4*). Most FNH patients are asymptomatic (*4*). Imaging examinations can usually achieve a definite diagnosis; if obscure, liver biopsy is recommended (*3,4*) with a great diagnostic accuracy of 95% (*5*). FNH patients mostly need conservative treatment alone (*3,4,6*), and undergo interventions when the symptoms are persistent and/or the diagnosis is unclear (*3,7-9*).

Hepatocellular adenoma (HCA) is another benign liver tumor with a prevalence estimated to be 0.001-0.004% (*4*). Unlike FNH, HCA has a risk of haemorrhage and malignant transformation. Lifestyle change, close imaging follow-up, and surgical resection are major treatment options for HCA (*10*). Hepatocellular carcinoma (HCC), the most common primary liver malignancy, mainly develops in patients with liver cirrhosis secondary to viral hepatitis and alcohol abuse (*3*). Current treatments of HCC include liver transplantation, liver resection, transarterial chemoembolization, radiofrequency ablation, and molecular targeted therapy (*11,12*).

The use of modern imaging techniques, including contrast-enhanced magnetic resonance imaging (CE-MRI), computed tomography (CECT), and ultrasound (CEUS), is valuable for the diagnosis of liver tumors, further guiding the treatment selection. However, atypical FNHs can mimic HCA or HCC on imaging, because all of them are hypervascular. Therefore, a differential diagnosis of FNH with HCA and HCC is of particular significance.

2. Case presentations

2.1. Case 1

On June 1, 2020, a 55-year-old male complained of persistent abdominal pain for half a year at our department. He had been treated with continuous oral

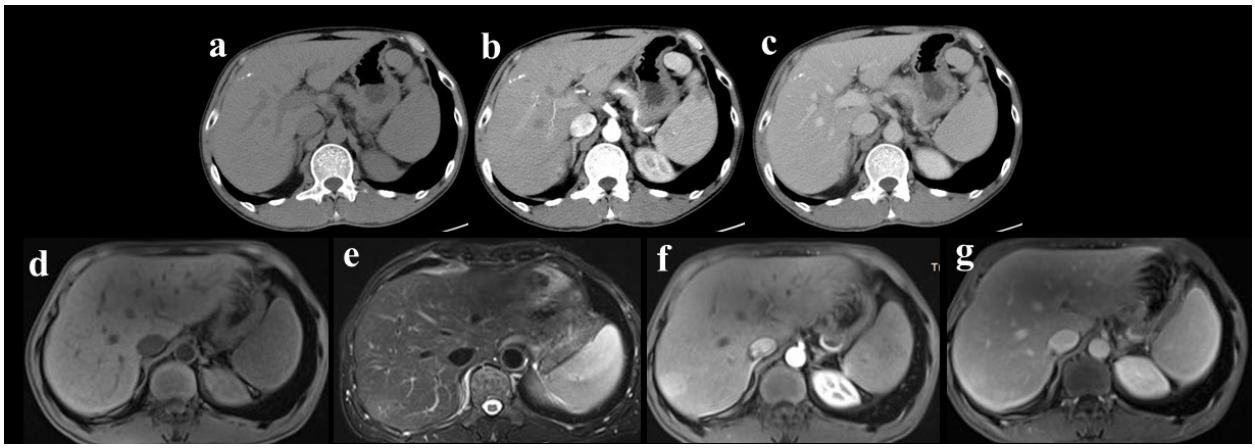

Figure 1. Imaging features of FNH in the case 1. (a-c) On multiphase CECT, no abnormal liver lesions were found, except for intrahepatic bile duct stones or calcification. **(d-g)** On conventional CE-MRI, a mass was not well-circumscribed in the 7th segment of the liver, which showed isointensity on T1-weighted images **(d)**, slight hyperintensity on T2-weighted images **(e)**, homogeneous and strong hyperintensity on arterial phase **(f)**, and slight hyperintensity on portal phase **(g)**.

clopidogrel bisulfate and hydroxyurea for essential thrombocythemia for 5 years. He also had histories of tuberculosis, appendectomy, and smoking and alcohol cessation as well as a family history of liver cirrhosis. No positive abdominal signs were found on physical examinations. On laboratory tests, the platelet count was 549,000/mm^3 (reference range: 125,000-350,000/mm^3); fecal occult blood was negative; serum lipase and amylase, liver function parameters, and serum albumin were within the reference range; tumor markers, including alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), β2-microglobulin (β2-MG), carbohydrate antigen-50 (CA-50), CA19-9, and CA24-2, were negative; hepatitis B virus antigen and hepatitis C virus antibody were negative. No positive lesions were found on esophagogastroduodenoscopy and X-ray gastrointestinal fluoroscopy. Neither superior mesenteric artery occlusion/stenosis nor left renal vein compression was found on abdominal color Doppler ultrasonography. Abdominal CECT showed intrahepatic bile duct stones or calcification, splenomegaly, and splenic infarction (Figure 1). Abdominal CE-MRI further found a mass, which was not well-circumscribed, in the 7th segment of the liver, with isointensity on T1-weighted images, slight hyperintensity on T2-weighted images, homogeneous and strong hyperintensity on arterial phase, and slight hyperintensity on portal phase (Figure 1). A possible diagnosis of HCA was considered.

On June 17, 2020, this patient underwent surgery at the Department of Hepatobiliary Surgery after obtaining his and his relatives' written informed consents. A mass with a diameter of about 3 cm was detected in the 7th segment of the liver under ultrasonic guidance, and then the 7th segment of the liver was completely resected. Histology confirmed a diagnosis of FNH (Figure 2). After surgery, abdominal pain disappeared, but liver dysfunction developed with increased serum alanine aminotransferase (ALT) level of 310.32 U/L (reference range: 9-50 U/L), serum aspartate aminotransferase (AST) level of 514.61 U/L (reference range: 15-40 U/L), serum total bilirubin (TBIL) level of 152.0 μmol/L (reference range: 5.1-22.2 μmol/L), direct bilirubin (DBIL) level of 97.5 μmol/L (reference range: 0-8.6 μmol/L), alkaline phosphatase (AKP) level of 169.39 U/L (reference range: 45-125 U/L), and gamma-glutamytransferase (GGT) level of 60.93 U/L (reference range: 10-60 U/L), and a decreased serum albumin (ALB) level of 29.4 g/L (reference range: 40-55 g/L). Conservative treatment was given for liver dysfunction.

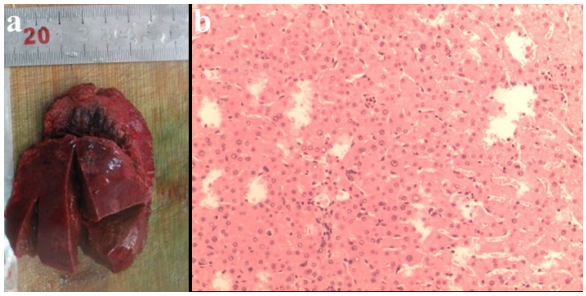

Figure 2. Surgical specimen and microscopic image of FNH in the case 1. (a) Surgical specimen had a diameter of about 3 cm. **(b)** Histology confirmed the diagnosis of FNH combined with focal steatosis (hematoxylin and eosin, ×100).

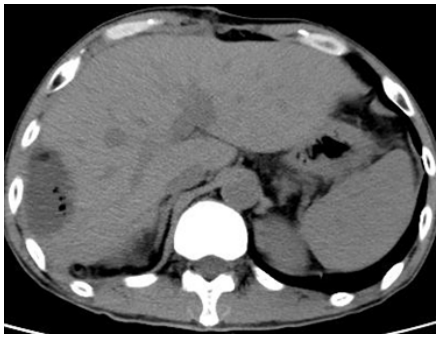

Figure 3. Postoperative abdominal CT scan showing that the 7th segment of the liver was absent in the case 1.

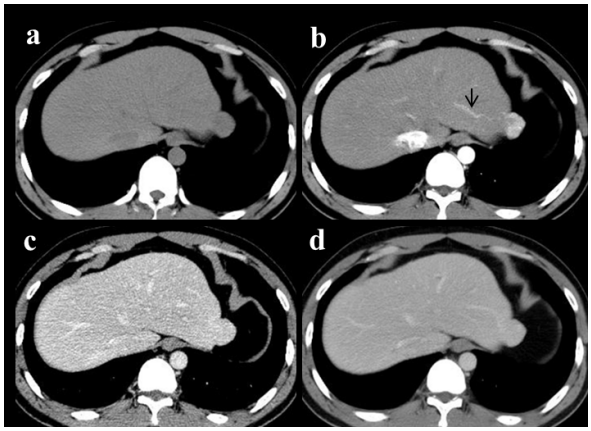

Figure 4. Abdominal multiphase CECT imaging features of FNH in the case 2. There was an irregularly exogenous mass in the left lateral segment of the liver on unenhanced phase **(a)**, hyperintensity on arterial phase **(b)**, and isointensity on portal **(c)** and delayed **(d)** phases. Notably, there were a thickened vessel (arrow) and its tiny branches into the lesion on arterial phase **(b)**.

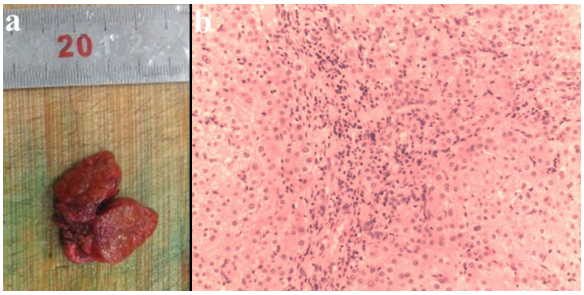

Figure 5. Surgical specimen and microscopic image of FNH in the case 2. (a) The lesion resected was lobulated in appearance with about 2.6 × 2.3 cm in size. **(b)** Histology showed nodular hyperplasia of hepatocytes with atypical hyperplasia and lymphocytes infiltration in the portal regions (hematoxylin and eosin, ×100).

Abdominal CT was re-examined and showed that the 7th segment of the liver was absent (Figure 3). He was discharged on June 28. On a recent telephone follow-up visit, he was diagnosed with a hepatic vein stenosis, and at a liver transplantation waiting list.

2.2. Case 2

On May 10, 2020, a 26-year-old male complained of intermittent diarrhea for more than one year and abdominal pain for 20 days at our department. He had histories of upper limb fracture and smoking. He denied the history of alcohol or drug abuse and family history of liver diseases. No positive signs were found on physical examinations. On laboratory tests, fecal occult blood and fecal bacterium cultures were negative; complete blood cell count, serum lipase and amylase, liver function parameters, and serum albumin were within the reference range; tumor markers, including AFP, CEA, β2-MG, CA-50, CA19-9, and CA24-2, were negative; hepatitis B virus antigen and hepatitis C virus antibody were negative. Esophagogastroduodenoscopy and colonoscopy revealed verrucous gastritis and proliferative changes of terminal ileum lymphatic follicles, respectively. Abdominal CECT revealed an irregularly exogenous mass with a size of about 2.6 × 2.3 cm in the left lateral segment of the liver, with hyperintensity on arterial phase and isointensity on portal and delayed phases (Figure 4). Notably, there was a thickened vessel and its branches into the lesion on arterial phase. Thus, a possible diagnosis of HCC was considered.

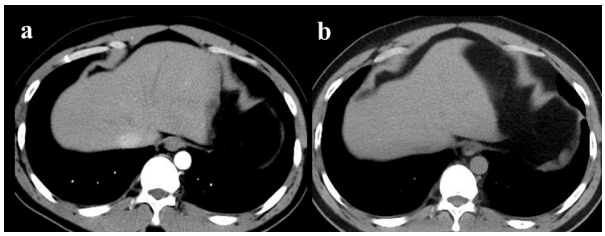

Figure 6. Postoperative abdominal CT scans showing that the left lateral segment of the liver was absent in the case 2. (a) Abdominal CECT in the first postoperative month. **(b)** Abdominal unenhanced CT scan in the 6th postoperative month.

On May 18, 2020, this patient underwent surgery at the Department of Hepatobiliary Surgery after obtaining his and his relatives' written informed consents. Intraoperatively, an exogenous mass with a diameter of about 3 cm was seen in the left lateral segment of the liver, and then the left lateral segment of the liver was completely resected. Histology confirmed a diagnosis of FNH with atypical hyperplasia (Figure 5) and the immunohistochemical staining revealed the absence of focal malignancy. He was complicated with mild liver dysfunction after surgery, with increased ALT level of 76.14 U/L (reference range: 9-50 U/L) and AST level of 48.98 U/L (reference range: 15-40 U/L). He was discharged on May 28. Abdominal CT at one month and six months after surgery showed that the left lateral segment of the liver was absent (Figure 6).

3. Discussion

A minority of FNH cases may present with abdominal pain or discomfort, which is secondary to the compression of large FNHs on adjacent organs (*1*). Retraction of FNHs after therapy can relieve abdominal pain, which may explain a potential correlation of abdominal pain with FNHs (*9*). In the case 1, abdominal pain alleviated after resection of this lesion, indicating that his abdominal symptoms might originate from FNH. Certainly, abdominal pain could also be attributed to other comorbidities (*13*), such as dyspepsia (*14*). In the case 2, abdominal pain remained after surgery.

Based on the CECT findings, the diagnosis was inaccurate in the case 2, and even hepatic lesion was not visualized in the case 1. CE-MRI has a higher diagnostic performance of focal liver lesions than

Table 1. The main imaging characteristics of FNH, HCA, and HCC

Items	Hepatocyte-targeted CE-MRI	Contrast-enhanced computed tomography
FNH	**Lesion parenchyma** T1WI: iso-/slight hypointensity; T2WI: iso-/slight hyperintensity; Arterial phase: hyperintensity; Portal phase: slight hyper-/isointensity; Delayed phase: iso-/slight hyperintensity; Hepatobiliary phase: iso-/hyperintensity. **Central scar** T1WI: hypointensity; T2WI: hyperintensity; Arterial phase: hypointensity; Portal phase: hypointensity; Delayed phase: hyperintensity; Hepatobiliary phase: hypointensity (*4,5,17,18,21*).	Arterial phase: ***typical*** centrifugal enhancement pattern, sometimes with a spoke-wheel morphology (mainly in lesions less than 3.1 cm); ***atypical*** centripetal and diffuse enhancement patterns (mainly in lesions larger than 3.1 cm); Portal and/or delayed phases: sustained enhancement (*23*).
HCA#	T1WI: iso-/hypointensity; T2WI: mostly hyperintensity; Arterial phase: hyperintensity; Portal phase: iso-/hyper-/hypointensity; Delayed phase: iso-/hyper-/hypointensity; Hepatobiliary phase: hypointensity (*4,18,21*).	Arterial phase: ***typical*** centripetal enhancement pattern; ***atypical*** diffuse enhancement pattern, without a spoke-wheel morphology, and unaffected by the lesion size; Portal and/or delayed phases: wash-out appearance (*25*).
HCC#	T1WI: variable; T2WI: variable/hyperintensity; Arterial phase: hyperintensity; Portal phase: iso-/hypointensity; Delayed phase: hypointensity; Hepatobiliary phase: hypointensity (*3,26*).	Arterial phase: hyperenhancement; Portal and/or delayed phases: wash-out appearance (*28*).

Notes: The signal intensity refers to the signal intensity relative to the normal liver parenchyma surrounding the lesion. # the signal intensity of the lesion on MRI is sometimes inhomogenous. *Abbreviations*: CE-MRI, contrast-enhanced magnetic resonance imaging; FNH, focal nodular hyperplasia; T1WI, T1-weighted images; T2WI, T2-weighted images; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma.

CECT (*15*). Notably, CE-MRI is considered as the preferred diagnostic approach in the case where the diagnosis is obscure (*4*). In a retrospective study involving a total of 124 focal liver lesions undetermined by CECT, the diagnostic accuracy of CE-MRI could be 58% (*15*). Among the small focal liver lesions (< 2 cm) that cannot be diagnosed by CECT, the diagnostic accuracy of CE-MRI could be 87.7% (*16*). The main imaging features of FNH on multiphase CE-MRI are summarized in Table 1 (*4,5,17*).

Several points should be helpful to differentiate between FNH and HCA. First, central scar is of great significance for the diagnosis of FNH, but it is only observed in approximately 50% of FNH cases and is usually present in FNH lesions larger than 3 cm. If such a typical sign is missing, it is difficult to obtain a confident diagnosis of FNH on conventional MRI (*18,19*). The case 1 with a small hepatic lesion did not have any signal intensity symbolizing the central scar on CE-MRI. Second, in the case 1, gadoterate meglumine, which is an extracellular space contrast agent, was used for multiphase CE-MRI examination. However, the imaging features on CE-MRI using gadoterate meglumine are not significantly different between FNH and HCA (*20*). By comparison, on hepatobiliary phase of CE-MRI with novel hepatocyte-selective contrast agents, such as gadoxetate disodium and gadobenate dimeglumine, there is a difference in signal intensity between FNH and HCA. The former often presents as iso-/hyperintensity, but the latter as hypointensity (*4,18,21*). Of course, some contrasting situations should not be neglected (*21-23*), which may be related to different expression levels of organic anion transporting polypeptide (OATP) on hepatocyte membrane (*24*). Third, serious complications, such as spontaneous rupture and haemorrhage, are extremely rare in FNHs (*4*), but they can develop in HCAs larger than 5 cm (*24*). Notably, no complication was observed in the case 1. Fourth, the sensitivity of MRI is low for the diagnosis of FNHs less than 3 cm where central scar is often missing (*4*). In this setting, we could consider CEUS as an alternative diagnostic approach to evaluate small FNHs (*23*). On CEUS, FNH can present as a typical centrifugal filling pattern with or without spoke-wheel morphology; by contrast, HCA can present as a typical centripetal filling pattern (*25*). Unfortunately, the case 1 did not undergo CEUS. Fifth, a diagnosis can be further established according to the texture analysis on hepatocyte-targeted CE-MRI (*21*) as well as a combination of risk factors and imaging features for suspected liver lesions (*22*).

On CECT or CE-MRI, a "fast-forward and fast-out" enhancement pattern in a focal liver lesion, which shows strong enhancement on arterial phase

and fast wash-out on portal or delayed phases, is one of the most important diagnostic criteria of HCC (*3*). Such an imaging feature is observed in the case 2. However, he was finally diagnosed with FNH by histology. This may be related to abundant backflow veins inside his hepatic lesion. There are several points to be concerned for a differential diagnosis of FNH with HCC. First, hepatocyte-targeted CE-MRI can be considered for differentiating FNH with HCC. FNHs show iso-/hyperintensity on hepatobiliary phase, but HCCs show hypointensity (*26*). But it's important to note that a genetic subtype of HCC can also show iso-/hyperintensity on hepatobiliary phase due to its overexpression of OATP 1B3 (*27*). Second, central scar on imaging usually favors the diagnosis of FNH, rather than HCC. But the scar-like feature can also be observed in fibrolamellar HCC or scalloped HCC (27). The case 2 was lacking of central scar. Third, on CEUS, HCC shows hyperenhancement on arterial phase and wash-out appearance on portal and delayed phases, but FNH often shows continuous enhancement (*28*). No CEUS examination was further performed in the case 2. Fourth, the natural disease course is often different between FNH and HCC. FNH shows a varied change in size of lesions (slowly increased, stable, or decreased), while HCC often shows a progressively increased size of lesions (*27*). Fifth, the risk factors and laboratory tests of suspicious liver diseases are often valuable (*3,11*). The case 2 was a young male without any underlying liver disease, and his AFP level was within the reference range, which were not consistent with the diagnosis of HCC.

In conclusion, FNH larger than 3 cm, rather than HCA and HCC, usually shows the presence of central scar. It is often difficult to distinguish FNH from HCA and HCC on CECT and conventional CE-MRI. By comparison, CE-MRI with hepatocyte-targeted contrast agents can provide more diagnostic clues, where FNH usually shows iso-/hyperintensity on hepatobiliary phase, but HCA and HCC often show hypointensity. Additionally, various CEUS findings and risk factors should be helpful for a differential diagnosis.

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Retracted: The safety of ritodrine hydrochloride: Adverse effects on fetuses and newborns

This article entitled "The safety of ritodrine hydrochloride: Adverse effects on fetuses and newborns" (*1*) has been retracted at the request of the authors due to wrong data extraction and processing.

Reference

1. Yonaga Y, Ito A. The safety of ritodrine hydrochloride: Adverse effects on fetuses and newborns. *Drug Discov Ther. 2021; 15(1):14-19. DOI: 10.5582/ddt.2021.01016.*

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