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Tel.: +81-3-5840-9697
Fax: +81-3-5840-9698
E-mail: office@ddtjournal.com

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Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
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Sulfonyl phosphonic 1,4-dithia-7-azaspiro[4,4]nonane derivatives as matrix metalloproteinase inhibitors: Synthesis, a docking study, and biological evaluation

Hao Zhang[1], Xuan Li[2], Xuejian Wang[1], Wenfang Xu[3], Jian Zhang[1,*]

[1] *Department of Medical Chemistry, School of Pharmacy, Weifang Medical University, Weifang, Shandong, China;*
[2] *Department of Cardiology, Weifang Chinese Medicine Hospital, Weifang, Shandong, China;*
[3] *Department of Medicinal Chemistry, School of Pharmacy, Shandong University, Ji'nan, Shandong, China.*

Summary **A series of novel sulfonyl phosphonic 1,4-dithia-7-azaspiro[4,4]nonane derivatives were designed, synthesized, and assayed for their activity against matrix metalloproteinase-2 (MMP-2). Results indicated that all of the compounds exhibited moderate inhibitory activity against MMP-2 compared to LY52 (the control) (IC_{50} = 0.95 ± 0.09 μM). Several selected compounds were also examined for their antiproliferative activity against SKOV3, HL60, and A549 cells. Notably, all of the tested compounds had slightly lower antiproliferative activity against SKOV3 cells than that of LY52. Compound 6d displayed the greatest inhibitory activity in an enzymatic assay and a cell-based assay, which means that this compound is a good candidate for further development of phosphonate-based MMP inhibitors.**

Keywords: Matrix metalloproteinase-2, 1,4-dithia-7-azaspiro[4,4]nonane derivatives, inhibitors, synthesis

1. Introduction

The integrity of the extracellular matrix (ECM), a complex network of proteins and polysaccharides surrounding each cell, is a prerequisite for the normal functioning and survival of an organism. Alterations of the ECM are performed by a family of structurally and functionally related zinc-dependent endopeptidases called matrix metalloproteinases (MMPs) that play important roles in physiological and pathological processes such as development, ovulation, wound healing, and angiogenesis (*1,2*). To date, at least 26 members of the MMP family have been identified in humans, and MMPs can be mainly grouped into five classes: collagenases, gelatinases, stromelysins, membrane-type MMPs (MT-MMPs), and matrilysin (*3*). MMPs are minimally expressed and strictly regulated at multiple levels to ensure proper functioning in physiological processes, whereas their overexpression and excessive activity have been implicated in a variety of pathological disorders ranging from cardiovascular disease to cancer (*4-7*). Of all of the identified MMP subtypes, MMP-2, also known as gelatinase A due to its close correlation with tumor progression (*8-10*), has been considered an attractive target for structure-based drug design, and research on MMP-2 inhibitors is a very promising strategy for cancer therapy and development of anticancer drugs (*11*).

The rapid increase in research on the solution and crystal structures of MMP-inhibitor complexes has led to a detailed depiction of the structure of MMPs. Briefly, except for Zn^{2+} in the conserved catalytic center of the MMP-2 enzyme, MMPs have two hydrophobic domains (S_1' and S_2' pockets, respectively) that are located in proximity to the catalytic zinc center. The S_1' pocket, a deep and narrow channel, is the most prominent domain with which to distinguish the selectivity of various MMPs, and this pocket is responsible for most of the observed substrate specificity of a given MMP, while the S_2' pocket is a solvent-exposed cleft (*12,13*). Effective MMP inhibitors

**Address correspondence to:*
Dr. Jian Zhang, Department of Medical Chemistry, School of Pharmacy, Weifang Medical University, 7166, West Baotong Road, Weifang, Shandong, 261053, China.
E-mail: zhangjian_3323@163.com

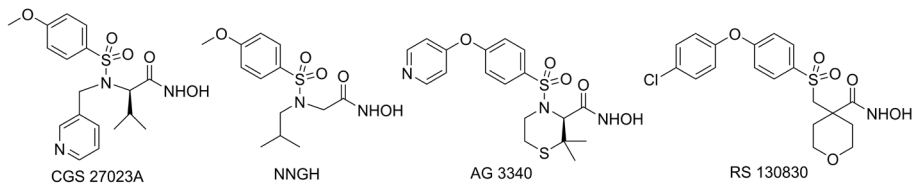


Figure 1. The Structures of CGS27023A, NNGH, AG3340, and RS 130830.

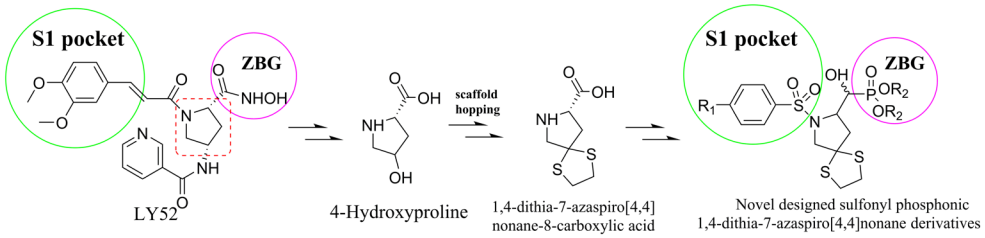


Figure 2. Construction of sulfonyl phosphonic 1,4-dithia-7-azaspiro [4,4] nonane derivatives.

are characterized by: *i*) a "warhead" for chelating with Zn^{2+}, also known as a zinc-binding group (ZBG); *ii*) one or more side chains effectively interacting with active subsites, the primary of which is the S_1'pocket; and *iii*) functional groups providing hydrogen bond interactions with the enzyme backbone (*14,15*).

The discovery of CGS 27023A (Figure 1) opened up a new avenue in the design and development of novel *N*-arylsulfonyl MMPs inhibitors (*16*). Other sulfonamide-based derivatives, including NNGH, AG 3340, and RS 130830, have also been shown in Figure 1 (*16-18*). The vast body of relevant literature indicates that the sulfonamide group was incorporated into MMP inhibitors for the following reasons: *i*) the sulfonyl group can improve enzyme-inhibitor binding by forming effective hydrogen bonds; *ii*) the sulfonyl group can properly anchor and orient the hydrophobic substituent to the S_1' groove *via* a gauche conformation, enabling it to plunge deep into the enzyme-binding domain (*18*).

The current authors' and their colleagues have recently endeavored to identify pyrrolidine derivatives as effective MMP inhibitors, exemplified by LY52 (Figure 2) (*19-22*). Moreover, there are more than 60% hydroxyproline (Hyp) and glycine (Gly) residues among the amino acids in the primary structure of collagen (*23*), which is the specific substrate of gelatinases. Buoyed by these findings, a new class of heterocyclic skeleton, 1,4-dithia-7-azaspiro[4,4]nonane-8-carboxylic acid (Figure 2), was chosen since derivatives or analogues of 4-hydroxyproline hold the promise of recognizing its substrate and subsequently interacting with the active sites of MMPs in a competitive manner. In particular, a 1,3-dithiane ring was reported to have an enormous impact on the *in vivo* efficacy of some antitumor molecules (*24*). Based on the "molecular hybridization principle," a reasonable conjecture was made that such attributes might potentially result in a synergistic effect on MMP-2 inhibition. Pursuant to this hypothesis and in light of the role of the sulfonyl group in MMP inhibitors, the current authors therefore designed sulfonyl phosphonate 1,4-dithia-7-azaspiro[4,4] nonane derivatives, wherein the arylsulfonyl group is incorporated at the 1-N position and the phosphonate group or phosphoric acid is incorporated as a zinc-binding group (ZBG).

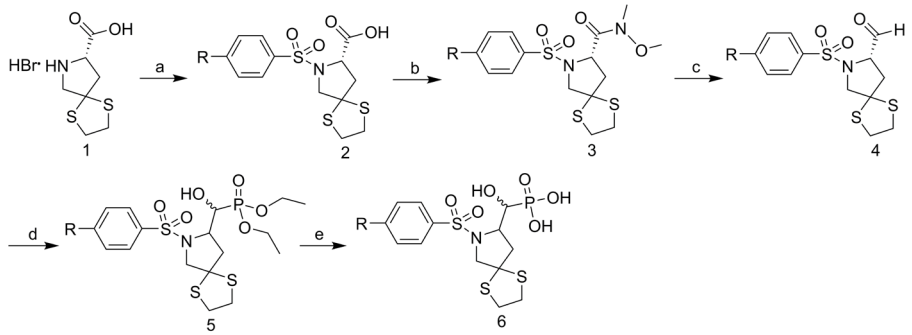
The current study describes the synthesis and biological activity of all of these sulfonyl phosphonate 1,4-dithia-7-azaspiro[4,4]nonane derivatives as well as docking studies of their interactions. Their structure-activity relationships have also been discussed.

2. Materials and Methods

2.1. *Chemicals and general procedures*

Unless otherwise noted, all of the materials, including reagents and solvents, were commercially available and used without further purification. All reactions were monitored by TLC with 0.25-mm silica gel plates (60GF-254) and were visualized with UV light or iodine vapor. Flash column chromatography was performed using 200-300-mesh silica gel. Melting points were determined on an electrothermal melting point apparatus (uncorrected). Proton NMR spectra were determined on a Brucker DRX spectrometer (300 MHz), with δ in parts per million and *J* in Hertz, using TMS as an internal standard. Measurements were made in DMSO-d_6 solutions. ESI-MS spectra were determined on an API 4000 spectrometer. HR-MS spectra were determined on an Agilent Q-TOF-6250 spectrometer at the Shandong Analysis and Test Center in Ji'nan, China. Anhydrous reactions were carried out in over-dried glassware in a nitrogen atmosphere.

The target compounds were efficiently synthesized following the procedures as illustrated in Scheme 1. The chemical structures of the target compounds were



Scheme 1. Reagents and conditions: (a) $RC_6H_4SO_2Cl$, DMAP, Et_3N; (b) TBTU, Et_3N, DCM; (c) $LiAlH_4$, THF; (d) Diethyl phosphite, Al_2O_3; (e) TMSBr, $CHCl_3$.

analytically confirmed with ^{1}H-NMR, ^{1}P-NMR, and HR-MS (see the Experimental Section).

Starting with a commercially available compound (**1**) as a chiral hydrobromide salt, sulfonamide intermediates (**2a-e**) were prepared *via* sulfonation with various sulfonyl chlorides and 4-*N*,*N*-dimethylaminopyridine (DMAP) as a catalyst and triethylamine (TEA) as a base. Condensation of **2a-e** with *N*-methoxymethanamine in dichloromethane (DCM) yielded the intermediates **3a-e**, which were then reduced with lithium tetrahydridoaluminate ($LiAlH_4$) to their aldehyde derivatives **4a-e** in anhydrous tetrahydrofuran (THF). Solvent-free nucleophilic addition of **4a-e** with diethyl phosphite and Al_2O_3 as a catalyst and medium produced α-hydroxyphosphonates **5a-e** (*25*), each of which was a mixture of two isomers that produced NMR spectra. The ethyl group of compounds **5a-d** was removed to obtain compounds **6a-d**, each of which was also a mixture of two isomers.

2.2. *In vitro MMP-2 inhibition assay*

IC_{50} values against MMP-2 were determined using succinylated gelatin as a substrate and MMP-2 (Gelatinase A, Sigma) as an enzyme or the supernatant of SKOV-3 cells in PBS (1×10^5/well). The enzyme and inhibitors were dissolved in sodium borate (pH 8.5, 50 mmol/L) and incubated in 96-well microtiter plates for 10 min at 37°C. The substrate was added and the mixture was incubated for another 30 min at 37°C. Then 0.03% TNBS was added and the mixture was incubated for an additional 20 min. The OD450 values of the resulting solution were determined at a wavelength of 450 nm with a plate reader (Varioskan, Thermo). Data were analyzed using OriginPro 7.5 software and IC_{50} values were determined.

2.3. *In vitro MMP-9 inhibition assay*

Active human MMP-9 full length protein was purchased from Abcam and the fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH_2 was purchased from AnaSpec. The inhibition of MMP-9 by the test compounds (**6a-d**) was fluorometrically assayed at excitation and emission wavelengths of 328 and 393 nm using 384-well plates and a plate reader (Varioskan, Thermo). Substrate hydrolysis was monitored for 15 min in a buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Brij-35, and 1% DMSO) containing 10 μM substrate. For those compounds displaying > 50% inhibitory activity at a concn of 10 μM, their IC_{50} values were determined based on dose-response measurements using an inhibitor range of concentrations (1 nM-10 μM) and an enzyme concentration equal to 3 nM. The enzyme was preincubated with the inhibitor 2 h before assessment of activity. Data were analyzed using the software OriginPro 7.5.

2.4. *MTT assay*

Cell lines were grown in RPMI1640 medium containing 10% FBS at 37°C in a humidified incubator containing 5% CO_2. Cell proliferation was determined using a 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2*H*-tertazolium bromide (MTT) assay. Briefly, cells were plated on 96-well plates (10,000/well) and cultured for 4 h in complete growth medium and then treated with various concentrations of the test compounds. The plates were incubated for an additional 48 h, and then 0.5% MTT was added to each well. Four hours later, formazan formed from MTT was dissolved with DMSO for 15 min. Finally, the optical density values were determined at 570 nm using an ELISA reader.

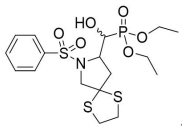
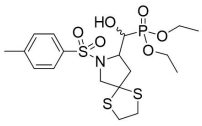
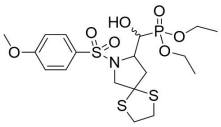
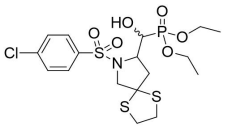
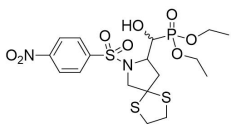
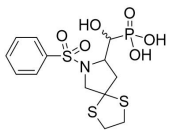
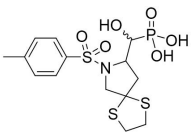
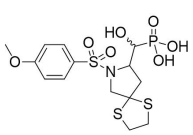
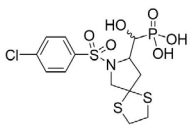
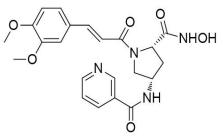
2.5. *Computational docking assay*

A docking study was conducted as follows: the selected compound was constructed with the Sybyl/Sketch module and its geometry was optimized with the Tripos force field and the Powell conjugate gradient algorithm with the convergence criterion set at 0.05 kcal/mol Å, and charges were assigned using the Gasteiger-Hückel method. The docking study of the selected compound with the active site of MMP-2 was performed using the Sybyl/ FlexX module. The active site was defined as a circle with a radius of 10.0 Å around Zn^{2+} (PDB: 1HOV).

3. Results

The newly synthesized sulfonyl phosphonic 1,4-dithia-7-azaspiro[4,4]nonane derivatives were assayed for their inhibitory activity against MMP-2, and **LY52** served as the positive control. Compounds **5a-d** and **6a-d** had IC_{50} values in the micromole range and displayed moderate inhibitory activity compared to **LY52** (the control) (IC_{50} = 0.95 ± 0.09 μM).

Table 1. The structures of the target compounds and their inhibitory activity against MMP-2

Compd	Structure	IC_{50} [a](μM)	
		MMP-2	MMP-9
5a		80.39 ± 2.52	ND
5b		63.16 ± 2.24	ND
5c		56.81 ± 1.79	ND
5d		38.24 ± 1.15	ND
5e		45.73 ± 1.28	ND
6a		14.58 ± 0.23	26.32 ± 0.20
6b		13.87 ± 0.21	25.75 ± 0.18
6c		10.25 ± 0.18	22.47 ± 0.23
6d		8.46 ± 0.14	15.26 ± 0.16
LY52		0.95 ± 0.09	1.72 ± 0.12

ND: not determined. [a]IC_{50} values are the mean of three experiments and the standard deviation is shown.

Compounds **6a-d** displayed greater inhibitory activity against MMP-2 and were thus assayed for their activity against MMP-9. Those compounds displayed moderate inhibitory activity against MMP-9 compared to **LY52** (the control) (IC_{50} = 1.72 ± 0.12 μM). Inhibition results are summarized in Table 1.

Furthermore, compounds **6a-d** were assayed for their inhibitory activity against human MMP-2 derived from cultured SKOV3 human ovarian carcinoma cells expressing a high level of MMP-2. As is apparent in Figure 3, all of the tested compounds exhibited moderate inhibitory activity against MMP-2 from SKOV3 cells compared to **LY52** (IC_{50} = 43.75 ± 1.12 μM).

Additionally, the MTT assay was used to evaluate compounds **6a-d** for their *in vitro* antiproliferative activity against a human ovarian tumor cell line (SKOV3), a leukemia cell line (HL60), and a lung cancer cell line (A549). HL60 and A549 cells over-expressed APN while SKOV3 cells over-expressed MMP-2. The results are shown in Table 2. Compounds **6a-d** had greater antiproliferative activity against SKOV3 cells than against HL60 and A549 cells, which may be due to the higher level of MMP-2 expression by SKOV3 cells than by the other two types of cells. However, a noteworthy finding was that compounds **6a-d** had slightly lower antiproliferative activity against SKOV3 cells than that of **LY52** (with respective IC_{50} values of 415.76, 346.82, 281.39, 173.58, and 697.14 μM), which was not consistent with the previous results of enzyme inhibition. This result could have been caused by several

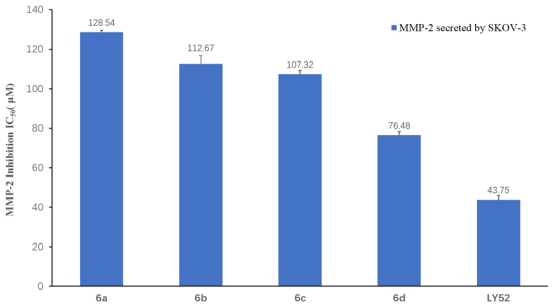


Figure 3. Inhibitory activity of compounds 6a, 6b, 6c, 6d, and LY52 against MMP-2 in a supernatant of SKOV-3 cells. Data are expressed as the mean values of three experiments.

Table 2. Anti-proliferative activity of compounds 6a, 6b, 6c, 6d, and LY52 against SKOV3, HL60, and A549 cells

Compd	IC_{50} [a](μM)		
	SKOV3	HL60	A549
6a	415.76	>1000	>1000
6b	346.82	>1000	>1000
6c	281.39	>1000	>1000
6d	173.58	>1000	>1000
LY52	697.14	>1000	>1000

[a]Mean values and the standard deviation of three experiments are shown.

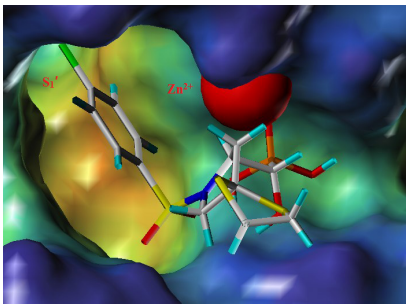


Figure 4. The FlexX docking of Compound 6d with MMP-2.

factors, such as cell membrane permeability, metabolic stability, subcellular localization, and cell mechanisms of exporting xenobiotics.

A docking analysis of the most potent compound, **6d**, was performed using Sybyl 8.0 from Tripos. The interaction of the compound with MMP-2 (PDB: 1HOV) is depicted in Figure 4 and results of the analysis suggested that the phosphinate group chelates Zn^{2+}, which is a crucial catalytic active site, while the arylsulfonyl group is incorporated into the S_1' pocket. Although the computational results partially supported this contention, the exact mode by which compound **6d** binds with MMP-2 needs to be determined in further X-ray crystal studies.

4. Discussion

All of the tested compounds displayed moderate inhibitory activity against MMP-2 and MMP-9 compared to **LY52** (the control). There was no obvious subtype selectivity between MMP-2 and MMP-9 for these sulfonyl phosphonic 1,4-dithia-7-azaspiro[4,4]nonane derivatives.

Compounds **6a-d** were more potent than compounds **5a-e**, which might be attributed to the ZBG. Phosphoric acid and phosphonate are the respective ZBGs for **6a-d** and **5a-e**, and both can chelate the zinc ion in the catalytic center of the enzyme. However, the phosphoric acid group was a more potent ZBG than the phosphonate group.

Among compounds **5a-e**, compounds **5b-e** contained a substituted arylsulfonyl group and displayed more potent inhibitory activity compared to benzenesulfonyl derivative **5a**. In particular, the chloro-substituted compound **5d** had greater inhibitory activity than the other compounds. Moreover, methyl substitution or methoxy substitution of the arylsulfonyl group at the C-4 position did not markedly affect inhibitory activity, but a compound with methoxy substitution displayed slightly greater inhibitory activity. A similar finding was noted for compounds **6a-d**.

In summary, this study has described the synthesis and biological evaluation of a series of sulfonyl phosphonic 1,4-dithia-7-azaspiro[4,4]nonane derivatives as MMP-2 inhibitors. All of the target compounds displayed moderate inhibitory activity against MMP-2 compared to **LY52** (the control). Several selected compounds were also assayed for their antiproliferative activity against SKOV3, HL60, and A549 cells. Compound **6d**, which displayed the greatest inhibitory activity in both an enzymatic assay and a cell-based assay, could be used as a candidate for further structural optimization to develop MMPIs in the future.

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References

1. Massova I, Kotra LP, Fridman R, Mobashery S. Matrix metalloproteases: Structures, evolution and diversification. FASEB J. 1998; 12:1075-1095.
2. Forget MA, Desrosier RR, Béliveau R. Physiological roles of matrix metalloproteinases: Implications for tumor growth and metastasis. Can J Physiol Pharmacol. 1999; 77:465-480.
3. Tu GG, Xu WF, Huang HM, Li SH. Progress in the development of matrix metalloproteinase inhibitors. Curr Med Chem. 2008; 15:1388-1395.
4. Hu J, Van den Steen PE, Sang QX, Opdenakker G. Matrix metalloproteinase inhibitors as therapy for inflammatory matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. Nat Rev Drug Discov. 2007; 6:480-498.
5. Macfadyen RJ. Can matrix metalloproteinase inhibitors provide a realistic therapy in cardiovascular medicine? Cur Opin Pharmacol. 2007; 7:171-178.
6. Lin J, Kakkar V, Lu X. Impact of matrix metalloproteinases on atherosclerosis. Curr Drug Targets. 2014; 15:442-453.
7. Kessenbrock K, Wang CY, Werb Z. Matrix metalloproteinases in stem cell regulation and cancer. Matrix Biol. 2015; 44:184-190.
8. Björklund M, Koivunen E. Gelatinase-mediated migration and invasion of cancer cells. Biochim Biophys Acta. 2005; 1755:37-69.
9. Zou Y, Chen Y, Jiang Y, Gao J, Gu J. Targeting matrix metalloproteinases and endothelial cells with a fusion peptide against tumor. Cancer Res. 2007; 67:7295-7300.
10. Deryugina EI, Quigley JP. Pleiotropic roles of matrix metalloproteinases in tumor angiogenesis: Contrasting, overlapping and compensatory functions. Biochim Biophys Acta. 2010; 1803:103-120.
11. Dufour A, Overall CM. Missing the target: Matrix metalloproteinase antitargets in inflammation and cancer. Trends Pharmacol Sci. 2013; 34:233-242.
12. Verma RP, Hansch C. Matrix metalloproteinases (MMPs): Chemical-biological functions and (Q)SARs. Bioorg Med Chem. 2007; 15:2223-2268.
13. Aureli L, Gioia M, Cerbara I, Monaco S, Fasciglione GF, Marini S, Ascenzi P, Topai A, Coletta M. Structural bases for substrate and inhibitor recognition by matrix metalloproteinases. Curr Med Chem. 2008; 15:2192-2222.

14. Gupta SP, Patil VM. Specificity of binding with matrix metalloproteinases. In: Matrix Metalloproteinase Inhibitors. Springer Basel, 2012; pp. 35-56.
15. Kontogiorgis CA, Papaioannou P, Hadjipaviou-Litina DJ. Matrix metalloproteinase inhibitors: A review on pharmacophore mapping and (Q)Sars results. Curr Med Chem. 2005; 12:339-355.
16. MacPherson LJ, Bayburt EK, Capparelli MP, *et al*. Discovery of CGS 27023A, a non-peptidic, potent, and orally active stromelysin inhibitor that blocks cartilage degradation in rabbits. J Med Chem. 1997; 40:2525-2532.
17. Jeng AY, Chou M, Parker DT. Sulfonamide-based hydroxamic acids as potent inhibitors of mouse macrophage metalloelastase. Bioorg Med Chem Lett. 1998; 8:897-902.
18. Cheng XC, Wang Q, Fang H, Xu WF. Roles of sulfonamide group in matrix metalloproteinase inhibitors. Curr Med Chem. 2008; 15:368-373.
19. Zhang J, Li XY, Jiang YQ, Feng JH, Li XG, Zhang YJ, Xu WF. Design, synthesis and preliminary evaluation of α-sulfonyl γ-(glycinyl-amino) proline peptidomimetics as matrix metalloproteinase inhibitors. Bioorg Med Chem. 2014; 22:3055-3064.
20. Zhang J, Li X, Zhu HW, Wang Q, Feng JH, Mou JJ, Li YG, Fang H, Xu WF. Design, synthesis and primary activity evaluation of pyrrolidine derivatives as matrix metalloproteinase inhibitors. Drug Discov Ther. 2010; 4:5-12.
21. Cheng XC, Wang Q, Fang H, Tang W, Xu WF. Design, synthesis and preliminary evaluation of novel pyrrolidine derivatives as matrix metalloproteinase inhibitors. Eur J Me Chem. 2008; 43:2130-2139.
22. Qu XJ, Yuan YX, Xu W.F, Chen MH, Cui SX, Li YL, Makuuchi M, Nakata M, Tang W. Caffeoyl pyrrolidine derivative LY52 inhibits tumor invasion and metastasis *via* suppression of matrix metalloproteinase activity. Anticancer Res. 2006; 26:3573-3578.
23. Kramer RZ, Bella J, Mayville P, Brodsky B, Berman HM. Sequence dependent conformational variations of collagen triple-helical structure. Nat Struct Biol. 1999; 6:454-457.
24. Huang F, Zhao M, Zhang X, Wang C, Qian K, Kuo RY, Morris-Natschke S, Lee KH, Peng S. Synthesis, DNA intercalation and 3D QSAR analysis of cis-2,4,5-trisubstituted-1,3-dithiolanes as a novel class of antitumor agents. Bioorg Med Chem. 2009; 17:6085-6095.
25. Françoise TB, André F. Synthesis of 1-hydroxyalkane phosphonic esters on alumina. Synthesis. 1982; 1982:916.

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Appendix

1.

7-(Phenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-carboxylic acid (2a)

1,4-Dithia-7-azaspiro[4,4]nonane-8-carboxylic acid (14.3 g, 50 mmol) was dissolved in a solution of water/dioxane (1:1, 200 mL), and then triethylamine (Et_3N, 17.5 mL, 125 mmol) and 4-(dimethylamino)pyridine (DMAP, 0.61 g, 5 mmol) were successively added. After the addition of benzenesulfonyl chloride (9.73 g, 55 mmol) in several portions below 0°C in an ice-salt bath, the mixture was allowed to warm to room temperature and stirred overnight. The solvent was removed under a vacuum and the resulting residue was partitioned between EtOAc and 1 N aqueous HCl. The organic layer was separated and washed with 1 N HCl (3 × 50 mL) and then washed with brine (2 × 50 mL), and the organic layer was then dried over anhydrous Na_2SO_4, filtered, and concentrated in a vacuum to yield target compound **2a**. The crude product was purified *via* recrystallization in 75% ethanol/H_2O to yield 12.80 g of **2a** as white powder (74.1%). *m.p*. 178-180°C, ESI-MS *m/z*: 344.7 $[M-H]^+$.

Compounds **2b-e** were synthesized following the general procedure described above.

7-Tosyl-1,4-dithia-7-azaspiro[4,4]nonane-8-carboxylic acid (2b):

White powder, yield 68.4%, *m.p*. 147-149°C. ESI-MS *m/z*: 359.3 $[M-H]^+$.

7-*p*-Methoxyphenylsulfonyl-1,4-dithia-7-azaspiro[4,4]nonane-8-carboxylic acid (2c)

White powder, yield 78.2%, *m.p*. 143-145°C. ESI-MS *m/z*: 375.2 $[M-H]^+$.

7-*p*-Chlorophenylsulfonyl-1,4-dithia-7-azaspiro[4,4]nonane-8-carboxylic acid (2d)

White powder, yield 75.6%, *m.p*. 149-151°C. ESI-MS *m/z*: 378.9 $[M-H]^+$.

7-p-Nitrophenylsulfonyl-1,4-dithia-7-azaspiro[4,4]nonane-8-carboxylic acid (2e)

White powder, yield 71.5%, *m.p*. 170-172°C. ESI-MS *m/z*: 389.5 $[M-H]^+$.

2.

***N*-methoxy-*N*-methyl-7-(phenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-carboamide (3a)**

Compound **2a** (3.45 g, 10 mmol) was dissolved in 100 mL anhydrous DCM with Et_3N (3.5 mL, 11 mmol) and then treated with 3.53 g (11 mmol) of O-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) at 0°C. After 30 minutes, *N*-methoxymethanamine was added and the mixture was stirred at room temperature for 12 h. The mixture was washed with 1 M HCl (3 × 50 mL), saturated $NaHCO_3$ solution (3 × 50 mL), and brine (2 × 50 mL) and then dried over Na_2SO_4. Evaporation of DCM yielded a pale yellow solid (57.3%). *m.p*.: 91~93°C, ESI-MS *m/z*: 389.4 $[M+H]^+$.

Compounds **3b-e** were synthesized following the

general procedure described above.

***N*-methoxy-*N*-methyl-7-tosyl-1,4-dithia-7-azaspiro [4,4]nonane-8-carboamid (3b)**

Pale yellow solid, yield 67.8%, *m.p*. 102-104°C. ESI-MS *m/z*: 403.5 $[M+H]^+$.

***N*-methoxy-N-methyl-7-(*p*-methoxyphenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-carboamide (3c)**

Pale yellow solid, yield 72.6%, *m.p*. 121-123 °C. ESI-MS *m/z*: 419.4 $[M+H]^+$.

***N*-methoxy-*N*-methyl-7-(*p*-chlorophenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-carboamide (3d)**

Pale yellow solid, yield 74.1%, *m.p*. 125-127 °C. ESI-MS *m/z*: 423.3 $[M+H]^+$.

***N*-methoxy-*N*-methyl-7-(*p*-nitrophenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-carboamide (3e)**

Yellow solid, yield 54.2%, *m.p*. 140-142 °C. ESI-MS *m/z*: 434.5 $[M+H]^+$.

3.

7-(Phenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-carbaldehyde (4a)

Compound **3a** (3.88 g, 10 mmol) was dissolved in anhydrous THF below 0°C in an ice-salt bath and treated with LiAlH4 (3.5 mL, 10 mmol) in several portions. After 30 minutes, the ice bath was removed and the resulting mixture was stirred at room temperature for 6 h. The reaction was quenched with 1 M NaOH and filtered through a thin layer of Celite. The resulting mixture was diluted with EtOAc (100 mL) and separated. The organic phase was washed successively with H_2O (2 × 50 mL), 1 M citric acid (2 × 50 mL), saturated $NaHCO_3$ (2 × 50 mL), and brine (50 mL), and the organic phase was dried over anhydrous Na2SO4. Evaporation of EtOAc yielded a pale yellow oil (**4a**). ESI-MS *m/z*: 330.3 $[M+H]^+$.

Compounds **4b-e** were synthesized following the general procedure described above.

7-Tosyl-1,4-dithia-7-azaspiro[4,4]nonane-8-carbaldehyde (4b)

Pale yellow oil. ESI-MS *m/z*: 344.3 $[M+H]^+$.

7-(*p*-Methoxyphenylsulfonyl)-1,4-dithia-7-azaspiro [4,4]nonane-8-carbaldehyde (4c)

Pale yellow oil. ESI-MS *m/z*: 360.4 $[M+H]^+$.

7-(*p*-Chlorophenylsulfonyl)-1,4-dithia-7-azaspiro[4,4] nonane-8-carbaldehyde (4d)

Yellow oil. ESI-MS *m/z*: 364.9 $[M+H]^+$.

7-(*p*-Nitrophenylsulfonyl)-1,4-dithia-7-azaspiro[4,4] nonane-8-carbaldehyde (4e)

Yellow oil. ESI-MS *m/z*: 375.4 $[M+H]^+$.

4.

Diethyl(hydroxyl(7-(phenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-yl) methyl)phosphonate (5a)

The crude oil **4a** (about 5 mmol), diethyl phosphate (0.64 mL, 5 mmol), and Al_2O_3 (1.5 g) were stirred at room temperature for 2 h. The mixture was extracted with DCM (30 mL × 3). The organic phase was dried over Na_2SO_4 and filtered through a thin layer of Celite to remove the solid. The filtered solution was concentrated under a vacuum to yield the crude product, which was purified using flash chromatography on silica gel (PE:EA = 2:1 to 1:2) to yield a pale yellow solid. Yield 47.3%, *m.p*.: 94-97°C; HRMS *m/z*: calcd. for C17H26NO6PS3 $[M+H]^+$ 468.0738, found 468.0734; ^{1}H NMR: (DMSO-d_6, ppm) δ: 1.253 (t, *J* = 3.6 Hz, 3H, CH_3), 1.288 (t, *J* = 3.6 Hz, 3H, CH_3), 2.131-2.293 (m, 1H, CH), 2.665-2.743 (m, 1H, CH), 3.014-3.090 (m, 2H, SCH_2), 3.193-3.229 (m, 2H, SCH_2), 3.608 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.752 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.847-3.902 (m, 1H, CH), 3.965-4.020 (m, 2H, OCH_2), 4.031-4.127 (m, 2H, OCH_2), 4.470-4.665 (m, 1H, CH, CH-$PO(OEt)_2$), 6.194-6.252 (m, 1H, OH), 7.628 (t, *J* = 7.2 Hz, 2H, ArH), 7.711 (t, *J* = 7.2 Hz, 1H, ArH), 7.828-7.875 (m, 2H, ArH). ^{31}P NMR: (DMSO-d_6, ppm) δ: 21.784, 22.967.

Compounds **5b-e** were synthesized following the general procedure described above.

Diethyl(hydroxyl(7-tosy -1,4-dithia-7-azaspiro[4,4] nonane-8-yl) methyl)phosphonate (5b)

Pale yellow solid, yield 51.6%, *m.p*. 100-103°C; HRMS *m/z*: calcd for C18H28NO6PS3 $[M+H]^+$ 482.0895, found 482.0892; ^{1}H NMR: (DMSO-d_6, ppm) δ: 1.255 (t, *J* = 3.3 Hz, 3H, CH_3), 1.286 (t, *J* = 3.3 Hz, 3H, CH_3), 2.131-2.293 (m, 1H, CH), 2.402 (s, 3H , $ArCH_3$), 2.656-2.733 (m, 1H, CH), 3.090-3.161 (m, 2H, SCH_2), 3.228-3.285 (m, 2H, SCH_2), 3.602 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.702 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.820-3.874 (m, 1H, CH), 4.002-4.048 (m, 2H, OCH_2), 4.063-4.120 (m, 2H, OCH_2), 4.434-4.656 (m, 1H, CH, CH-$PO(OEt)_2$), 5.895-6.224 (m, 1H, OH), 7. 406 (d, *J* = 6.6 Hz, 1H, ArH), 7.431 (d, *J* = 6.6 Hz, 1H, ArH), 7.689 (d, *J* = 8.1 Hz, 1H, ArH), 7.735 (d, *J* = 8.1 Hz, 1H, ArH). ^{31}P NMR: (DMSO-d_6, ppm) δ: 21.865, 22.979.

Diethyl(hydroxyl(7-(p-methoxyphenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-yl) methyl) phosphonate (5c)

Pale yellow solid, yield 45.8%, *m.p*. 81-84°C; HRMS *m/z*: calcd. for C18H28NO7PS3 $[M+H]^+$ 498.0844, found 498.0837; ^{1}H NMR: (DMSO-d_6, ppm) δ: 1.243 (t, *J* = 3.6 Hz, 3H, CH_3), 1.290 (t, *J* = 3.6 Hz, 3H, CH_3), 2.138-2.297 (m, 1H, CH), 2.513-2.735 (m, 1H, CH),

3.156-3.215 (m, 2H, SCH_2), 3.231-3.288 (m, 2H, SCH_2), 3.599 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.719 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.816-3.842 (m, 1H, CH), 3.850 (s, 3H, OCH_3), 4.031-4.054 (m, 2H, OCH_2), 4.066-4.127 (m, 2H, OCH_2), 4.475-4.662 (m, 1H, CH, CH-PO(OEt)$_2$), 5.895-6.220 (m, 1H, OH), 7. 113 (d, *J* = 6 Hz, 1H, ArH), 7.142 (d, *J* = 6 Hz, 1H, ArH), 7.742 (d, *J* = 9 Hz, 1H, ArH), 7.790 (d, *J* = 9 Hz, 1H, ArH). ^{31}P NMR: (DMSO-d_6, ppm) δ: 21.926, 23.060.

Diethyl(hydroxyl(7-(p-chlorophenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-yl) methyl) phosphonate (5d)

Pale yellow solid, yield 54.2%, *m.p*. 86-88°C; HRMS *m/z*: calcd. for C17H25NO6PS3Cl $[M+H]^+$ 502.0348, found 502.0342; ^{1}H NMR: (DMSO-d_6, ppm) δ: 1.252(t, *J* = 3.6 Hz, 3H, CH_3), 1.286 (t, *J* = 3.6 Hz, 3H, CH_3), 2.151-2.316 (m, 1H, CH), 2.679-2.757 (m, 1H, CH), 3.139-3.173 (m, 2H, SCH_2), 3.235-3.261 (m, 2H, SCH_2), 3.601 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.713 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.834-3.890 (m, 1H, CH), 4.022-4.054 (m, 2H, OCH_2), 4.078-4.127 (m, 2H, OCH_2), 4.416-4.618 (m, 1H, CH, CH-PO(OEt)$_2$), 5.952-6.267 (m, 1H, OH), 7. 681 (d, *J* = 6.9 Hz, 1H, ArH), 7.709 (d, *J* = 6.9 Hz, 1H, ArH), 7.824 (d, *J* = 9.0 Hz, 1H, ArH), 7.880 (d, *J* =9.0 Hz, 1H, ArH). ^{31}P NMR: (DMSO-d_6, ppm) δ: 21.644, 22.880.

Diethyl(hydroxyl(7-(p-nitrophenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-yl) methyl) phosphonate (5e)

Yellow solid, yield 39.4%, *m.p*. 97-99°C; HRMS *m/z*: calcd. for C17H25N2O8PS3 $[M+H]^+$ 513.0589, found 513.0586; ^{1}H NMR: (DMSO-d_6, ppm) δ: 1.258(t, *J* = 3.6 Hz, 3H, CH_3), 1.292 (t, *J* = 3.6 Hz, 3H, CH_3), 2.147-2.215 (m, 1H, CH), 2.693-2.776 (m, 1H, CH), 3.130-3.187 (m, 2H, SCH_2), 3.193-3.249 (m, 2H, SCH_2), 3.601 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.732 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.846-3.947 (m, 1H, CH), 4.029-4.059 (m, 2H, OCH_2), 4.078-4.133 (m, 2H, OCH_2), 4.392-4.571 (m, 1H, CH, CH-PO(OEt)$_2$), 5.952-6.301 (m, 1H, OH), 8. 101 (d, *J* = 6.9 Hz, 1H, ArH), 8.156 (d, *J* = 6.9 Hz, 1H, ArH), 8.397 (d, *J* = 9.0 Hz, 1H, ArH), 8.460 (d, *J* =9.0 Hz, 1H, ArH). ^{31}P NMR: (DMSO-d_6, ppm) δ: 21.389, 22.721.

5.

(Hydroxyl(7-(phenylsulfonyl)-1,4-dithia-7-azaspiro [4,4]nonane-8-yl)methyl)phosphonic acid (6a)

Compound **5a** (0.47g, 1 mmol) in 10 mL anhydrous DCM was dealkylated in the presence of bromotrimethylsilane for 2 h at room temperature. The solvent was removed under a vacuum to yield the crude product, which was purified using reversed phase column chromatography to yield compound 6a (H_2O:MeOH = 100% to 65:35). Pale yellow semisolid: yield 37.6%. HRMS *m/z*: calcd. for C13H18NO6PS3 $[M+H]^+$ 412.0112, found 412.0105; ^{1}H NMR: (DMSO-d_6, ppm) δ: 2.153-2.265 (m, 1H, CH), 2.666-2.765 (m, 1H, CH), 3.090-3.147 (m, 2H, SCH_2), 3.187-3.223 (m, 2H, SCH_2), 3.584 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.720 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.975-4.046 (m, 1H, CH), 4.203-4.535 (m, 1H, CH, CH-PO(OEt)$_2$), 7.592 (t, *J* = 7.5 Hz, 2H, ArH), 7.625-7.718 (m, 1H, ArH), 7.815-7.865 (m, 2H, ArH). ^{31}P NMR: (DMSO-d_6, ppm) δ: 18.303, 18.678.

Compounds **6b-d** were synthesized following the general procedure described above.

(Hydroxyl(7-tosyl-1,4-dithia-7-azaspiro[4,4]nonane-8-yl)methyl) phosphonic acid (6b)

Pale yellow semisolid: yield 41.8%. HRMS *m/z*: calcd for C14H20NO6PS3 $[M+H]^+$ 426.0269, found 426.0261; ^{1}H NMR: (DMSO-d_6, ppm) δ: 2.164-2.263 (m, 1H, CH), 2.408 (s, 3H, $ArCH_3$), 2.654-2.767 (m, 1H, CH), 3.096-3.155 (m, 2H, SCH_2), 3.193-3.239 (m, 2H, SCH_2), 3.576 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.712 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.933-3.975 (m, 1H, CH), 4.170-4.456 (m, 1H, CH, CH-PO(OEt)$_2$), 7.412 (d, *J* = 7.2 Hz, 2H, ArH), 7.785 (d, *J* = 7.2 Hz, 2H, ArH). ^{31}P NMR: (DMSO-d_6, ppm) δ: 18.394, 18.705.

(Hydroxyl(7-(p-methoxyphenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-yl) methyl)phosphonic acid (6c)

Yellow semisolid: yield 35.2%. HRMS *m/z*: calcd. for C14H20NO7PS3 $[M+H]^+$ 442.0218, found 442.0214; ^{1}H NMR: (DMSO-d_6, ppm) δ: 2.176-2.261 (m, 1H, CH), 2.648-2.727 (m, 1H, CH), 3.077-3.137 (m, 2H, SCH_2), 3.192-3.236 (m, 2H, SCH_2), 3.570 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.706 (d, *J* = 12 Hz, 1H, CH_2-N-), 3.838 (s, 3H, $ArOCH_3$), 3.914-3.971 (m, 1H, CH), 4.163-4.451 (m, 1H, CH, CH-PO(OEt)$_2$), 7.107 (d, *J* = 9.0 Hz, 2H, ArH), 7.750 (d, *J* = 9.0 Hz, 2H, ArH). ^{31}P NMR: (DMSO-d_6, ppm) δ: 18.447, 18.738.

(Hydroxyl(7-(p-chlorophenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-yl) methyl)phosphonic acid (6d)

Yellow semisolid: yield 43.7%. HRMS *m/z*: calcd. for C13H17ClNO6PS3 $[M+H]^+$ 445.9722, found 445.9716; ^{1}H NMR: (DMSO-d_6, ppm) δ: 2.312-2.377 (m, 1H, CH), 2.801-2.879 (m, 1H, CH), 3.080-3.142 (m, 2H, SCH_2), 3.199-3.268 (m, 2H, SCH_2), 3.706 (d, *J* = 12.3 Hz, 1H, CH_2-N-), 3.799 (d, *J* = 12.3 Hz, 1H, CH_2-N-), 4.074-4.130 (m, 1H, CH), 4.727-4.770 (m, 1H, CH, CH-PO(OEt)$_2$), 7.616 (d, *J* = 6.6 Hz, 2H, ArH), 7.908 (d, *J* = 6.6 Hz, 2H, ArH). ^{31}P NMR: (DMSO-d_6, ppm) δ: 20.202.

Original Article

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Validation of a sheet-shaped body vibrometer for screening of obstructive sleep apnea

Takamasa Kogure[1,2], Mina Kobayashi[3,4], Takashi Okawa[5], Tsuneya Nakajima[6], Yuichi Inoue[1,3,4,*]

[1] *Department of Somnology, Tokyo Medical University, Tokyo, Japan;*
[2] *Paramount Bed Sleep Research Laboratory, PARAMOUNT BED CO., LTD., Tokyo, Japan;*
[3] *Neuropsychiatric Research Institute, Japan Somnology Center, Tokyo, Japan;*
[4] *Foundation of Sleep and Health Science, Tokyo, Japan;*
[5] *Department of clinical inspection, Tokyo Dental College Ichikawa General Hospital, Chiba, Japan;*
[6] *Department of Otorhinolaryngology, Tokyo Dental College Ichikawa General Hospital, Chiba, Japan.*

Summary **We assessed the validity of using a sheet-shaped body vibrometer (SBV) as a portable monitoring device for obstructive sleep apnea (OSA) screening. Seventy consecutive patients with suspected OSA underwent simultaneous in-laboratory polysomnography (PSG) and SBV. We evaluated the screening accuracy of the respiratory event index (REI) obtained with the SBV, using the REI based on either the estimated total sleep time (REI_eTST) or time in bed (REI_TIB); these were compared to the apnea-hypopnea index (AHI) obtained *via* PSG. Bland-Altman plots indicated that the mean difference between REI_eTST and AHI was lower than that between REI_TIB and AHI (1.2 ± 19.8 *vs.* 6.5 ± 16.8). For AHI ≥ 15, the sensitivity and specificity at an optimal REI_eTST of 17.0 were 90.9% and 76.9%, whereas those at an optimal REI_TIB of 15.9 were 86.4% and 80.8%, respectively; moreover, for AHI ≥ 30, these values at an optimal REI_eTST of 26.0 were 89.5% and 88.2%, whereas those at an optimal REI_TIB of 23.8 were 84.2% and 92.2%, respectively. The optimal cutoff values of REIs for AHI of ≥ 5 were markedly different from those for AHI obtained *via* PSG (REI_eTST, 14.9; REI_TIB, 15.0), but close to those for AHI of ≥ 15; both had good sensitivities and specificities. REIs obtained *via* SBV performed well in moderate-to-severe, but not mild, OSA screening; REI_eTST showed a slightly higher sensitivity and a relatively closer value to the AHI obtained *via* PSG when compared to REI_TIB. We consider the SBV less acceptable for screening mild cases than more severe cases.**

Keywords: Obstructive sleep apnea, sheet-shaped body vibrometer, portable monitor, validation, estimated total sleep time

1. Introduction

Obstructive sleep apnea (OSA) is a known risk factor for cardiovascular morbidities, and is associated with mortality, cognitive dysfunction, deteriorated health-related quality of life, and sleepiness-related motor vehicular or occupational accidents (*1*). The prevalence of moderate-to-severe OSA in cases with an apnea-hypopnea index (AHI) of ≥ 15 events/h during overnight full polysomnography (PSG) was estimated as 7-14% in men and 2-7% in women in Western countries (*2-4*). In Asia, however, the prevalence of the disorder is estimated as 10.1% in men and 4.7% in women in the Korean population aged 40-69 years (*5*), and 5.3% in men (*6*) and 1.2% in women (*7*) in the Chinese population aged 30-60 years.

Attended in-laboratory PSG with subsequent manual scoring of the data is the gold standard for OSA diagnosis. However, PSG cannot be performed in all patients suspected to have OSA, as this examination requires a specialized laboratory for recording and is both labor and time consuming. Hence, it is believed 82% of men and 93% of women with moderate-to-severe OSA remain undiagnosed (*8*). Therefore, there

**Address correspondence to:*
Dr. Yuichi Inoue, Department of Somnology, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan.
E-mail: inoue@somnology.com

is a need for a convenient and ambulatory portable monitor (PM) with a high screening accuracy for OSA that facilitates a reduced time to diagnosis (*9,10*).

Data loss due to detached sensors during PM recording (*11*), and discomfort from sensor attachment (*12*), have been recognized as important issues of PM. The longer duration required for attaching the PM sensors may also increase patient discomfort (*11*). These issues can be resolved by PM recording without the need for attaching sensors, *i.e.*, non-wear PM devices. Previous studies on the validity of OSA screening with non-wear PM devices, such as a static charge sensitive bed (SCSB) (*13,14*) and a sheet-shaped device placed on a mattress (*15-17*), have been conducted. However, due to the insufficient number of validation studies, these devices have been classified as type 4 PM for the screening of OSA, and have hence not been generally accepted (*9,11*).

In fact, both type 3 and type 4 PM devices do not record variables required for sleep stage scoring (*i.e.*, electro-encephalography, electro-oculography, and electro-myography). Hence, the respiratory event index (REI) recorded with these types of PM has not been calculated as respiratory events per hour of total sleep time (TST), but as the events per hour of time in bed (TIB), which is longer than the TST (*18*). Therefore, the REI value is likely to be lower than the AHI, even though the respiratory events may be accurately measured with these devices. This limitation can be partially overcome through the combined use of wrist actigraphic recording, which allows TST estimation (*18*). However, the combined use of these devices increases the difficulty and complexity of the procedure.

NEMURI SCAN (NN-1100; PARAMOUNT BED CO., LTD., Tokyo, Japan) is a sheet-shaped body vibrometer (SBV), equipped with a highly sensitive pressure sensor, which detects body vibration through a mattress. This system has been shown to score sleep/wake states and calculate the estimated total sleep time (eTST) with almost the same accuracy as wrist actigraphy (*19*). Moreover, a SBV set under a mattress can detect small respiration- or heartbeat-related movements. Thus, by analyzing respiratory movements, the SBV can identify and score respiratory disturbances (*i.e.*, apneas or hypopneas), and accordingly calculate both eTST and respiratory events simultaneously. In our preliminary study on 20 patients with OSA, REI based on eTST (REI_eTST) was more similar to the AHI obtained *via* PSG in moderate ($15 \leq AHI < 30$) to severe ($AHI \geq 30$) OSA patients relative to REI based on TIB (REI_TIB) (*20*). However, we could not evaluate the screening accuracy of all OSA cases, including the mild OSA cases ($AHI \geq 5$) in that study, because most of the subjects had $AHI \geq 15$. Hence, in the present study, we aimed to assess the validity of SBV for OSA screening in a larger sample of not only moderate-to-severe OSA cases, but also mild cases and normal subjects.

2. Materials and Methods

2.1. *Subjects*

The study protocol was approved by the institutional review boards of both the Neuropsychiatric Research Institute and Tokyo Dental College Ichikawa General Hospital. We enrolled 70 consecutive patients (men, 58; women, 12; mean age, 48.5 ± 13.1 years; mean BMI, 26.1 ± 5.2 kg/m^2) who visited the outpatient clinic of the Yoyogi Sleep Disorder Center from January 2013 to November 2013 or Tokyo Dental College Ichikawa General Hospital from June 2011 to July 2011, with suspected OSA, based on findings of excessive daytime sleepiness, habitual snoring, or apnea events reported by their family members. They provided written informed consent for study participation, and consented to the simultaneous recordings of in-laboratory PSG and SBV. Among these patients, 20 from Tokyo Dental College Ichikawa General Hospital were already examined in our preliminary study (*20*).

2.2. *Polysomnography*

Diagnostic nocturnal PSG was performed using Alice 5 (Philips Respironics, Murrysville, PA, USA) or Embla N7000 (Natus Medical Inc., San Carlos, USA). The PSG montage included electroencephalogram (EEG; C3-A2, C4-A1, O1-A2, O2-A1), bilateral electro-oculogram, submental electromyogram, electrocardiogram, respiratory airflow (nasal pressure and thermistor), respiratory movements of the thorax and abdomen (inductance plethysmography), percutaneous oxyhemoglobin saturation (SpO2), snoring sound, and body position. The sleep stages were scored every 30 seconds according to the criteria of Rechtschaffen and Kales (*21*), whereas arousals were scored according to the American Sleep Disorders Association (ASDA) arousal criteria (*22*). The episodes of apnea/hypopnea were determined based on the American Academy of Sleep Medicine (AASM) criteria (*23*); accordingly, apnea was defined as the complete cessation of airflow for ≥ 10 s, whereas hypopnea was defined as a ≥ 50% reduction in airflow amplitude for ≥ 10 s or a discernible reduction for ≥ 10 s related to either arousal or oxygen desaturation of at least 3%.

2.3. *Sheet-shaped body vibrometer*

The SBV is equipped with a highly sensitive pressure sensor that detects body vibration generated by an examinee lying on a mattress. The pressure detected by the SBV changes in synchrony with expiration and inspiration; thus, the SBV measures respiratory-induced pressure changes, which are automatically adjusted for, to generate a respiratory waveform. The measured SBV value reaches the ceiling of the measurement range when

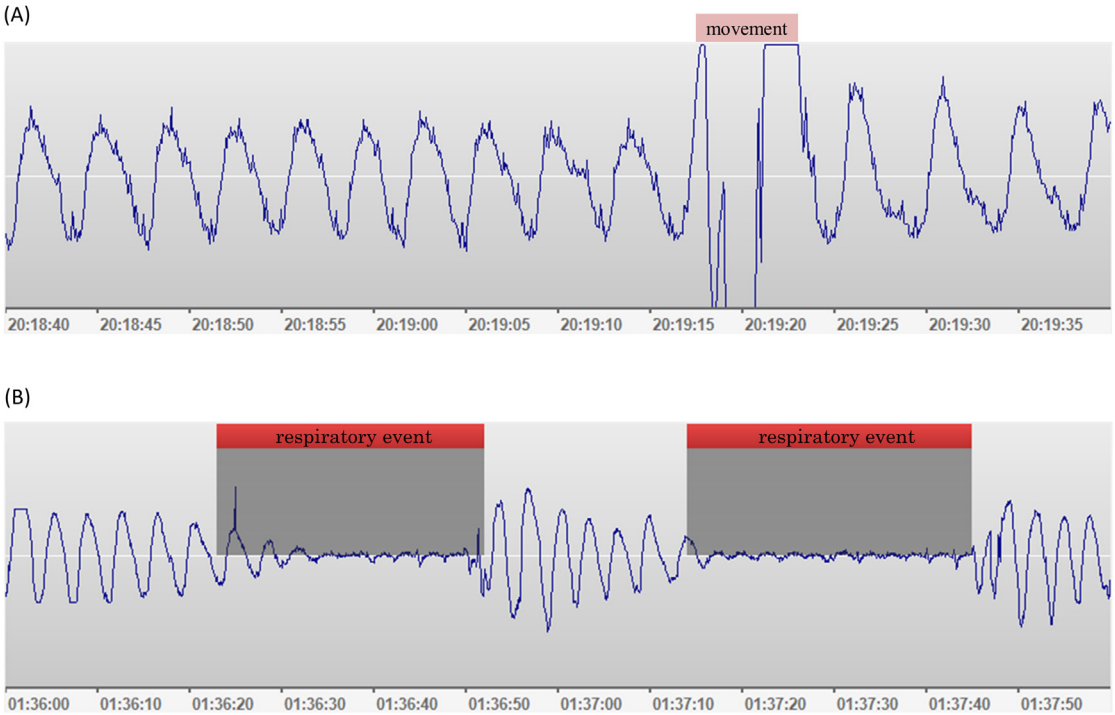

Figure 1. Respiratory waveform measured with the sheet-shaped body vibrometer. (A) movement of the examinee; **(B)** serial appearance of apnea-hypopnea events.

the examinee moves (Figure 1a). Thus, the respiratory waveform amplitudes represent the level of respiratory effort, and change (increase/decrease) based on the occurrence of apnea or hypopnea events (Figure 1b).

In the present study, the SBV was placed under a mattress, approximately 40 cm apart from its upper edge (Yoyogi Sleep Disorder Center: width, 120 cm; thickness, 15.5 cm; length, 195 cm; Tokyo Dental College Ichikawa General Hospital: width, 91 cm; thickness, 8.5 cm; length, 191 cm). The length, width, and thickness of the SBV itself was approximately 28.6 cm, 77 cm, and 1.1 cm, respectively. Using the SBV, the patients' body vibrations, including respiratory movements, were recorded simultaneously along with the PSG recordings.

The respiratory events obtained with the SBV were automatically scored, based on the findings of our preliminary study (*20*). Accordingly, respiratory events (apnea or hypopnea) were defined as follows: 1) a ≥ 30% reduction in the amplitude of the respiratory waveform from the mean amplitude of the previous 2 breaths, which lasted for at least 10 s, followed by body movement or amplitude recovery to a level greater than the mean amplitude; or 2) a consecutive increase in the amplitude of respiratory effort, more than 5 times. We also calculated the eTST based on the SBV sleep/wake data, scored according to our already published algorithm (*19*). The 2 REIs obtained *via* SBV included the respiratory events per hour of eTST (REI_eTST) and the value per hour of the total time from light-off to light-on (REI_TIB).

2.4. *Statistical analysis*

For comparisons between eTST obtained *via* SBV and TST obtained *via* PSG, between REI_TIB (/h) and AHI (/h) obtained *via* PSG, and between REI_eTST (/h) and AHI, the Wilcoxon signed rank test was performed. Pearson's correlation coefficient was used to analyze the correlations between eTST and TST, between REI_eTST and AHI, and between REI_TIB and AHI. Bland-Altman plots (*24*) were used to assess the agreement between the REIs and AHI. In the present study, we conducted receiver operating characteristic (ROC) curve analysis to determine the optimal cutoff value for predicting AHI of 5 events/h, 15 events/h, and 30 events/h by calculating the area under the ROC curves (AUC). Therefore, we calculated the sensitivities, specificities, positive and negative predictive values, positive and negative likelihood ratios, and the kappa coefficient at the respective optimal REI values for AHI of 5 events/h, 15 events/h, and 30 events/h. Statistical analyses were performed using EZR (*25*) (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). p values < 0.05 were considered statistically significant.

3. Results

All the patients successfully underwent simultaneous recordings with PSG and SBV without any data loss. The demographic variables and sleep variables for both

PSG and SBV recordings are presented in Table 1.

The Wilcoxon signed rank test indicated that the eTST (401 ± 75.0 min) was significantly longer than the TST (380 ± 66.2 min; $p < 0.001$). Moreover, the eTST was significantly correlated with the TST ($r = 0.431$, $p < 0.001$).

The Wilcoxon signed rank test also showed that the REI_TIB (19.5 ± 9.1) was significantly lower than the AHI (26.1 ± 22.7; $p = 0.040$). However, the REI_eTST did not significantly differ from the AHI ($p = 0.84$). Fair correlations between the REI_TIB and AHI ($r = 0.764$, $p < 0.001$; Figure 2a) and between the REI_eTST and AHI ($r = 0.625$, $p < 0.001$; Figure 2b) were noted.

Bland-Altman plots revealed that both the REI_TIB and REI_eTST tended to overestimate the REI, relative to the AHI, in cases with low AHI, and also tended to underestimate the REI as the AHI value increased (Figure 3). The mean difference between the REI_eTST and AHI was lower than that between the REI_TIB and AHI (1.2 ± 19.8 *vs*. 6.5 ± 16.8; Figure 3).

The results of ROC curve analysis are presented in Figure 4. The optimal cutoff values for predicting AHI ≥ 5 were 14.9 for REI_eTST and 15.1 for REI_TIB, those for predicting AHI ≥ 15 were 17.0 for REI_eTST and 15.9 for REI_TIB, and those for predicting AHI ≥ 30 were 26.0 for REI_eTST and 23.8 for REI_TIB.

The sensitivity, specificity, and kappa coefficient for a REI_eTST of 14.9 as a cutoff value for predicting

Table 1. Demographic and polysomnographic parameters of the participants ($n = 70$)

Variable	Value	Range
Gender (male/female)	58:12	
Age (years)	48.5 ± 13.1	20 - 80
Body mass index (kg/m^2)	26.1 ± 5.2	18.7 - 46.3
Height (cm)	168 ± 8.8	142 - 184
Weight (kg)	74.1 ± 18.4	46.0 - 137
PLMI (episodes/h)	6.4 ± 14.2	0 - 65.5
AHI (episodes/h)	26.1 ± 22.7	0.8 - 90.8
REI_TIB (episodes/h)	19.5 ± 9.1	4.7 - 47.5
REI_eTST (episodes/h)	24.9 ± 23.0	5.0 - 184.9
Time in bed (min)	461 ± 41.7	381 - 578
Total sleep time (min). measured by PSG	380 ± 66.2	242 - 510
Total sleep time (min), estimated by SBV	401 ± 75.0	111 - 562
Sleep efficiency (%), measured by PSG	82.3 ± 11.8	49.8 - 97.7
Sleep efficiency (%), estimated by SBV	87.2 ± 13.7	21.0 - 99.5

PLMI: periodic leg movement index; AHI: apnea hypopnea index; REI: respiratory event index; REI_TIB: REI per hour of time in bed; REI_eTST: REI per hour of estimated total sleep time; PSG: polysomnography; SBV: sheet-shaped body vibrometer.

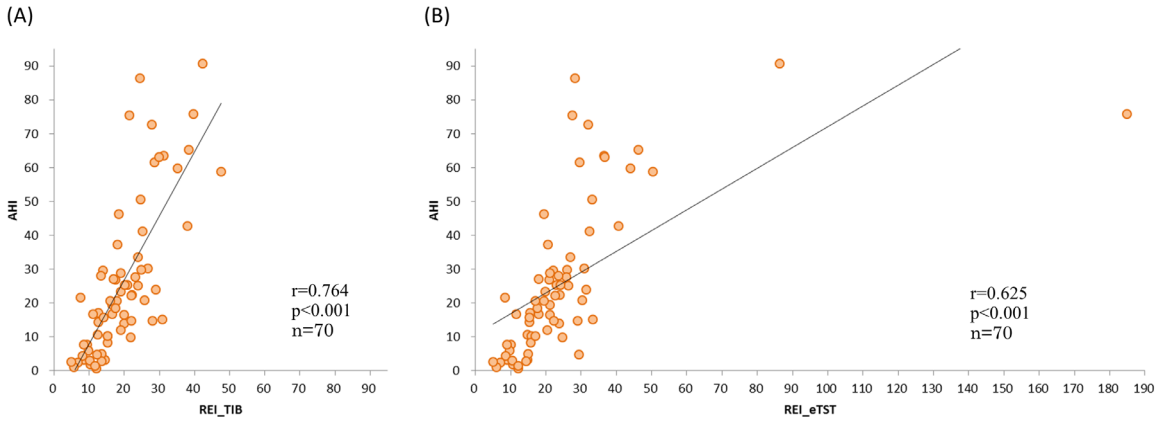

Figure 2. Pearson's correlation coefficient between the apnea-hypopnea index (AHI) and respiratory event index (REI). (A) REI per hour of time in bed (REI_TIB) and AHI; **(B)** REI per hour of estimated total sleep time (REI_eTST) and AHI.

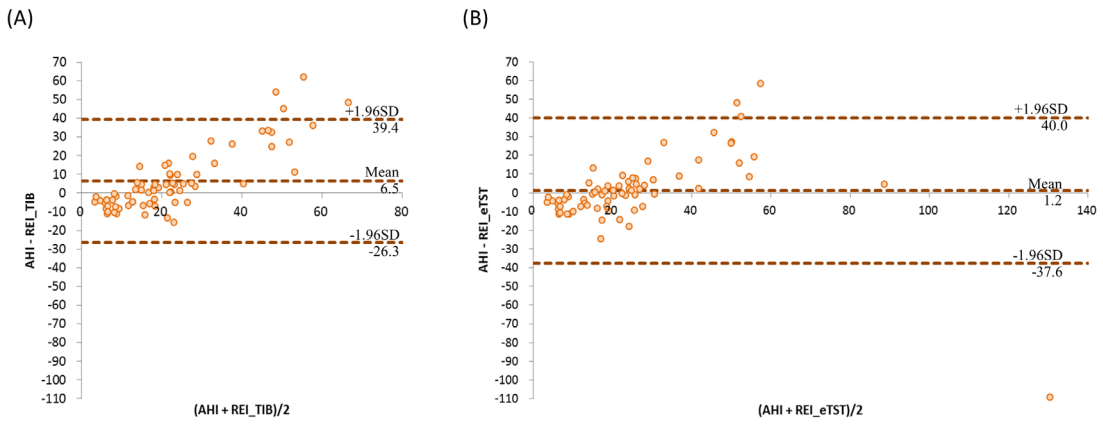

Figure 3. Bland-Altman plot for the apnea-hypopnea index (AHI) and respiratory event index (REI). (A) REI per hour of time in bed (REI_TIB) *vs*. AHI; **(B)** REI per hour of estimated total sleep time (REI_eTST) *vs*. AHI. Dotted lines represent the mean difference and the mean difference ± 1.96 standard deviation.

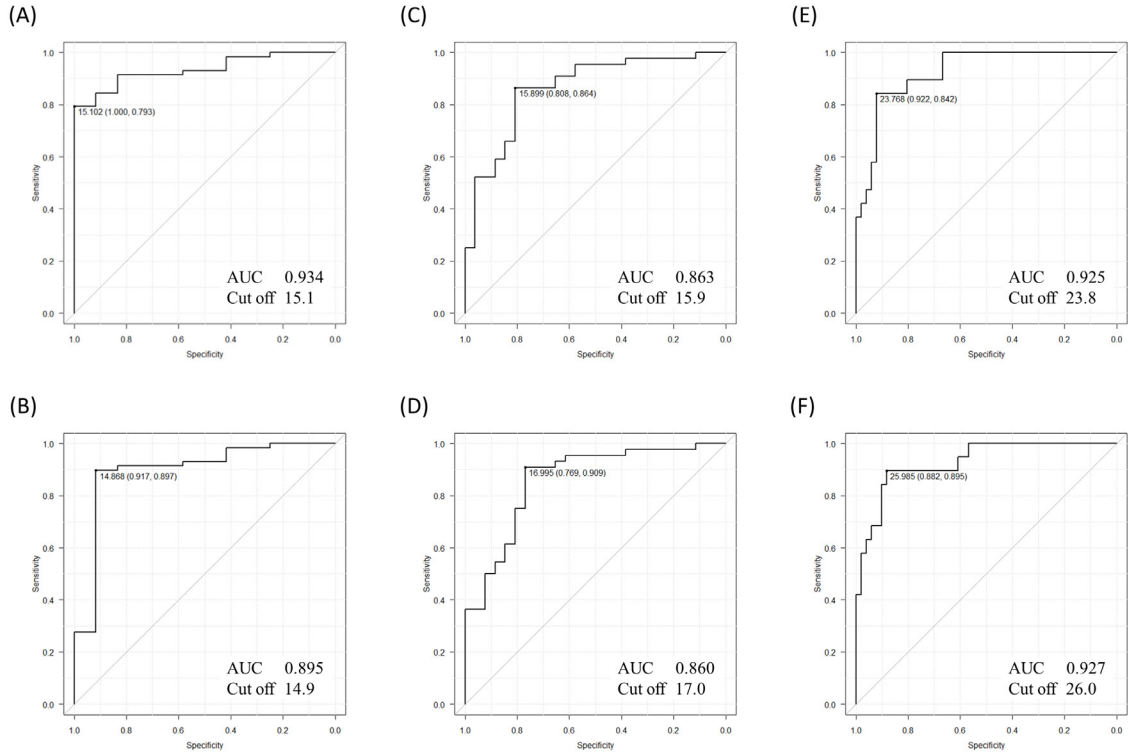

Figure 4. Receiver operating characteristic (ROC) curves of the respiratory event index (REI) for different apnea-hypopnea index (AHI) cut-off levels. (A) REI per hour of time in bed (REI_TIB) for AHI cutoff of 5; **(B)** REI per hour of estimated total sleep time (REI_eTST) for AHI cutoff of 5; **(C)** REI_TIB for AHI cutoff of 15; **(D)** REI_eTST for AHI cutoff of 15; **(E)** REI_TIB for AHI cutoff of 30; **(F)** REI_eTST for AHI cutoff of 30.

Table 2. Concurrent validity of the vibrometer-acquired respiratory event index for polysomnographically acquired apnea-hypopnea indexes of ≥ 5, ≥ 15, and ≥ 30

Variable	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	+LR	–LR	kappa
AHI≥5, REI_TIB≥15.1	79.3	100	100	50.0	–	0.21	0.57
AHI≥15, REI_TIB≥15.9	86.4	80.8	88.4	77.8	4.5	0.17	0.67
AHI≥30, REI_TIB≥23.8	84.2	92.2	80.0	94.0	10.7	0.17	0.75
AHI≥5, REI_eTST≥14.9	89.7	91.7	98.1	64.7	10.8	0.11	0.70
AHI≥15, REI_eTST≥17.0	90.9	76.9	87.0	83.3	3.9	0.12	0.69
AHI≥30, REI_eTST≥26.0	89.5	88.2	73.9	95.7	7.6	0.12	0.73

PPV: positive predictive value; NPV: negative predictive value; +LR: positive likelihood ratio; –LR: negative predictive likelihood ratio; Kappa: the kappa coefficient; AHI: apnea hypopnea index; REI: respiratory event index; REI_TIB: REI per hour of time in bed; REI_eTST: REI per hour of estimated total sleep time.

AHI ≥ 5 were 89.7%, 91.7%, and 0.70, respectively, whereas those for a REI_TIB of 15.1 as the cutoff value for predicting AHI ≥ 5 were 79.3%, 100%, and 0.57, respectively (Table 2). When the cutoff values of both REI_eTST and REI_TIB were set at 5 for AHI ≥ 5, the sensitivities, specificities, and kappa coefficients were found to be 100%, 8.3%, and 0.131, respectively. With regard to the prediction of AHI ≥ 15, the screening sensitivity, specificity, and kappa coefficient for REI_eTST of 17.0 as the optimal cutoff value were 90.9%, 76.9%, and 0.69, whereas those for REI_TIB of 15.9 as the optimal cutoff value were 86.4%, 80.8%, and 0.67, respectively. Moreover, with regard to the prediction of AHI ≥ 30, the sensitivity, specificity, and kappa coefficient for REI_eTST of 26.0 were 89.5%, 88.2%, and 0.73, whereas those for REI_TIB of 23.8 were 84.2%, 92.2%, and 0.75, respectively.

4. Discussion

In the present study, we aimed to evaluate the validity of SBV for OSA screening, while focusing on whether eTST could improve the consistency between AHI and REI. Therefore, we compared the screening accuracy of REI_eTST with that of REI_TIB according to the OSA severity cut-off levels. In particular, the sensitivity and specificity of non-wear PM devices for predicting severe OSA (AHI ≥ 30 events/h) have not been reported previously (*13-17*). However, in the present study, both REI_eTST and REI_TIB measured with the SBV

showed relatively high sensitivity and specificity at optimal cutoff values for predicting OSA with all three criteria (AHI ≥ 30, AHI ≥ 15, AHI ≥ 5), Moreover, data loss in PM recording using wearable sensors such as oronasal and respiratory effort sensors is considered an important problem (*11*). The fact that no data loss occurred during non-invasive SBV recording in the present study may be a valuable salient feature.

As reported previously, the REI_TIB is likely to be lower than the AHI in both type 3 and type 4 PM devices, which do not record the variables required for sleep stage scoring. In the present study, we also noted that the REI_TIB was significantly lower than the AHI. In contrast, there was no significant difference between the REI_eTST and AHI. In fact, the mean difference between the REI_eTST and AHI on Bland-Altman plots was also smaller than that between the REI_TIB and AHI. These findings suggest a somewhat beneficial feature of using REI calculation with eTST to reduce the difference between REI and AHI. However, this benefit may be limited by the accuracy of eTST, *i.e*., movement-based eTST, which can lead to TST overestimation when examinees do not move even when awake (*26*). In the present study, the underestimation of the event rate with REI_eTST with an increase in the AHI value appeared to reflect this phenomenon, as most of the patients with severe OSA exhibited TST overestimation (*18,20*).

In the present study, the optimal cutoff values for AHI ≥ 5 were approximately 15 episodes/h (14.9 for REI_eTST and 15.1 for REI_TIB) and were very close to those for AHI ≥ 15 (17.0 for REI_eTST and 15.9 for REI_TIB) despite relatively high sensitivity and specificity. Moreover, if the cutoff value was set at 5/h for both REI_eTST and REI_TIB, the specificities and kappa coefficients for predicting AHI ≥ 5 were clearly low with the 2 REIs. These results suggest that screening of AHI ≥ 5 with the SBV may be difficult, a problem that has been noted with wearable PMs (*12,27,28*). In contrast, when the REI value was set to 17.0 for REI_eTST or 15.9 for REI_TIB, the sensitivities and specificities for AHI ≥ 15 were good. Similarly, the 2 REI values for predicting AHI ≥ 30 had sufficient sensitivity and specificity. Thus, SBV was thought to be suitable for screening moderate-to-severe OSA, but was less acceptable for the screening of overall cases, including those with mild OSA (AHI ≥ 5).

The present study had certain limitations. First, the present study was conducted in a laboratory. In a study in which PSGs were conducted on different nights, 25% of individuals showed night-to-night variability of AHI greater than 20 events/hour (*29*). Considering this, we aimed to accurately evaluate the validity of SBV for OSA screening, using PSG-derived AHI on the same night in our laboratory as a reference. The 0% data loss and the screening ability could be partially attributable to this well-controlled environment. The data loss due to inaccurate device installation or forgetting to start the recording would possibly be greater during home recordings. Second, we scored apnea-hypopnea events using the AASM Chicago criteria, but did not use the AASM 2007 criteria (*30*) for PSG data. Ruehland *et al*. indicated that AHIs determined using the AASM Chicago criteria are significantly greater than those based on the AASM 2007 criteria (*31*). Thus, the screening ability of SBV could change if the AASM 2007 criteria are used. Future studies would be necessary to confirm the screening ability of SBV using AASM 2007 criteria.

In conclusion, SBV may be a clinically advantageous PM device due to the ability of REI to screen for moderate-to-severe OSA. REI_eTST showed a small but higher sensitivity and a relatively closer value to the AHI obtained *via* PSG as compared to REI_TIB. However, SBV appeared to be less acceptable for OSA screening in mild cases relative to moderate or severe cases. These characteristics should be confirmed in future home studies.

Acknowledgement

Part of this study was funded by PARAMOUNT BED CO., LTD.

Conflict of Interest

Takamasa Kogure is an employee of the company (PARAMOUNT BED CO., LTD.) that produces and distributes the sheet-shaped body vibrometer (NEMURI SCAN) used in this study.

References

1. Young T, Peppard PE, Gottlieb DJ. Epidemiology of obstructive sleep apnea: A population health perspective. Am J Respir Crit Care Med. 2002; 165:1217-1239.
2. Young T, Palta M, Dempsey J, Skatrud J, Weber S, Badr S. The occurrence of sleep-disordered breathing among middle-aged adults. N Engl J Med. 1993; 328:1230-1235.
3. Bixler EO, Vgontzas AN, Lin HM, Ten Have T, Rein J, Vela-Bueno A, Kales A. Prevalence of sleep-disordered breathing in women: Effects of gender. Am J Respir Crit Care Med. 2001; 163:608-613.
4. Durán J, Esnaola S, Rubio R, Iztueta A. Obstructive sleep apnea-hypopnea and related clinical features in a population-based sample of subjects aged 30 to 70 yr. Am J Respir Crit Care Med. 2001; 163:685-689.
5. Kim J, In K, Kim J, You S, Kang K, Shim J, Lee S, Lee J, Lee S, Park C, Shin C. Prevalence of sleep-disordered breathing in middle-aged Korean men and women. Am J Respir Crit Care Med. 2004; 170:1108-1113.
6. Ip MS, Lam B, Lauder IJ, Tsang KW, Chung KF, Mok YW, Lam WK. A community study of sleep-disordered breathing in middle-aged Chinese men in Hong Kong. Chest. 2001; 119:62-69.
7. Ip MS, Lam B, Tang LC, Lauder IJ, Ip TY, Lam WK. A

community study of sleep-disordered breathing in middle-aged Chinese women in Hong Kong: Prevalence and gender differences. Chest. 2004; 125:127-134.
8. Young T, Evans L, Finn L, Palta M. Estimation of the clinically diagnosed proportion of sleep apnea syndrome in middle-aged men and women. Sleep. 1997; 20:705-706.
9. Oliveira MG, Garbuio S, Treptow EC, Polese JF, Tufik S, Nery LE, Bittencourt L. The use of portable monitoring for sleep apnea diagnosis in adults. Expert Rev Respir Med. 2014; 8:123-132.
10. Chiner E, Andreu AL, Sancho-Chust JN, Sánchez-de-la-Torre A, Barbé F. The use of ambulatory strategies for the diagnosis and treatment of obstructive sleep apnea in adults. Expert Rev Respir Med. 2013; 7:259-273.
11. Portable Monitoring Task Force of the American Academy of Sleep Medicine. Clinical guidelines for the use of unattended portable monitors in the diagnosis of obstructive sleep apnea in adult patients. Portable Monitoring Task Force of the American Academy of Sleep Medicine. J Clin Sleep Med. 2007; 3:737-747.
12. Yin M, Miyazaki S, Itasaka Y, Shibata Y, Abe T, Miyoshi A, Ishikawa K, Togawa K. A preliminary study on application of portable monitoring for diagnosis of obstructive sleep apnea. Auris Nasus Larynx. 2005; 32:151-156.
13. Polo O, Brissaud L, Sales B, Besset A, Billiard M. The validity of the static charge sensitive bed in detecting obstructive sleep apneas. Eur Respir J. 1998; 1:330-336.
14. Svanborg E, Larsson H, Carlsson-Nordlander B, Pirskanen R. A limited diagnostic investigation for obstructive sleep apnea syndrome. Oximetry and static charge sensitive bed. Chest. 1990; 98:1341-1345.
15. Agatsuma T, Fujimoto K, Komatsu Y, Urushihata K, Honda T, Tsukahara T, Nomiyama T. A novel device (SD-101) with high accuracy for screening sleep apnoea-hypopnoea syndrome. Respirology. 2009; 14:1143-1150.
16. Kobayashi M, Namba K, Tsuiki S, Nakamura M, Hayashi M, Mieno Y, Imizu H, Fujita S, Yoshikawa A, Sakakibara H, Inoue Y. Validity of sheet-type portable monitoring device for screening obstructive sleep apnea syndrome. Sleep Breath. 2013; 17:589-595.
17. Tsukahara M, Sakao S, Jujo T, Sakurai T, Terada J, Kunii R, Tanabe N, Tatsumi K. The accuracy and uncertainty of a sheet-type portable monitor as a screening device to identify obstructive sleep apnea-hypopnea syndrome. Intern Med. 2014; 53:1307-1313.
18. Standards of Practice Committee of the American Academy of Sleep Medicine. Practice parameters for the use of actigraphy in the assessment of sleep and sleep disorders: An update for 2007. Sleep. 2007; 30:519-529.
19. Kogure T, Shirakawa S, Shimokawa M, Hosokawa Y. Automatic sleep/wake scoring from body motion in bed: Validation of a newly developed sensor placed under a mattress. J Physiol Anthropol. 2011; 30:103-109.
20. Kogure T, Okawa T, Nakajima T, Kobayashi M, Inoue Y. Preliminary study of sheet-shaped body vibrometer for screening obstructive sleep apnea syndrome. Japanese Journal of Sleep Medicine. 2015; 9:561-571. (In Japanese)
21. Rechtchaffen A, Kales A. A manual of standardized terminology, techniques and scoring system for sleep stages of human subjects. USPHS Publication No. 204. US Government Printing Office, Washington, DC, USA, 1968.
22. Atlas Task Force of the American Sleep Disorders Association. EEG arousal: Scoring rules and examples: A preliminary report from the Sleep Disorders. Sleep. 1992; 15:173-184.
23. The Report of the American Academy of Sleep Medicine Task Force. Sleep-related breathing disorders in adults: Recommendations for syndrome definition and measurement techniques in clinical research. Sleep. 1999; 22:667-689.
24. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet. 1986; 1:307-310.
25. Kanda Y. Investigation of the freely-available easy-to-use software "EZR" (Easy R) for medical statistics. Bone Marrow Transplant. 2013; 48:452-458.
26. Paquet J, Kawinska A, Carrier J. Wake detection capacity of actigraphy during sleep. Sleep. 2007; 30:1362-1369.
27. Bianchi MT, Lipoma T, Darling C, Alameddine Y, Westover MB. Automated sleep apnea quantification based on respiratory movement. Int J Med Sci. 2014; 11:796-802.
28. BaHammam AS, Sharif M, Gacuan DE, George S. Evaluation of the accuracy of manual and automatic scoring of a single airflow channel in patients with a high probability of obstructive sleep apnea. Med Sci Monit. 2011; 17:MT13-19.
29. Levendowski DJ, Zack N, Rao S, Wong K, Gendreau M, Kranzler J, Zavora T, Westbrook PR. Assessment of the test-retest reliability of laboratory polysomnography. Sleep Breath. 2009; 13:163-167.
30. Iber C, Ancoli-Israel S, Chesson A, Quan SF. The AASM Manual for the Scoring of Sleep and Associated Events: Rules, Terminology and Technical Specifications. 1st ed., American Academy of Sleep Medicine, Westchester, IL, USA, 2007.
31. Ruehland WR, Rochford PD, O'Donoghue FJ, Pierce RJ, Singh P, Thornton AT. The new AASM criteria for scoring hypopneas: Impact on the apnea hypopnea index. Sleep. 2009; 32:150-157.

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Original Article

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Experience with long-term administration of tolvaptan to patients with acute decompensated heart failure

Shunsuke Kiuchi*, Takahiro Fujii, Shinji Hisatake, Takayuki Kabuki, Oka Takashi, Shintaro Dobashi, Takanori Ikeda

Department of Cardiovascular Medicine, Toho University Graduate School of Medicine, Tokyo, Japan.

Summary **Tolvaptan (TLV) is an oral selective vasopressin type 2 receptor antagonist. Long-term use of TLV is not recommended in patients with heart failure (HF) if fluid retention disappears and/or body weight is within the target range. However, some patients require long-term use of TLV. The current study investigated the efficacy and safety of long-term use of TLV. Subjects were 258 consecutive patients with HF who received TLV during hospitalization from January 2011 to March 2015. The rate of continuing administration of TLV was evaluated. Moreover, the one-year mortality rate and rate of re-hospitalization either with or without TLV were investigated. Results at discharge and one year later were compared for patients who continued to receive TLV one year after discharge. Oral concomitant medications, blood pressures, heart rate, blood tests, chest X-ray and transthoracic echocardiography were investigated. In-hospital and one-year mortality rates were 15.9% and 27.8%, respectively. Moreover, the mortality rate and/or rate of re-hospitalization within one year was 54.4%. The rate of re-hospitalization for HF was significantly higher in patients who continued to receive TLV after discharge compared to patients who ceased receiving TLV after discharge ($p < 0.001$). However, the subjects who continued to receive TLV for up to one year after discharge tended to have a longer duration until re-hospitalization for HF and significantly decreased brain natriuretic peptide levels (577.6 ± 528.5 pg/mL to 397.3 ± 365.8 pg/mL, $p = 0.015$). Long-term use of TLV might delay re-hospitalization for HF in patients with severe HF. Large-scale clinical studies are necessary to verify these results.**

Keywords: Tolvaptan, long-term use, in-hospital death, re-hospitalization, heart failure

1. Introduction

Tolvaptan (TLV), an oral selective vasopressin type 2 receptor antagonist, was approved in Japan on October 27, 2010 and came on the market on December 14th of that same year. Patients with acute decompensated heart failure (ADHF) refractory to diuretics and fluid retention have been treated with TLV in Japan (*1*), but TLV has only been used to treat hyponatremia in other countries. Previous studies revealed that TLV alleviated worsening renal function (*2*) and decreased blood pressure (BP) as a result of taking diuretics (*3*). The duration of use of TLV tended to increase in accordance with its effectiveness.

Initiating TLV during hospitalization while monitoring the patient's serum sodium level has been recommended. Moreover, the package insert also recommends not administering TLV over a prolonged period if fluid retention disappears and/or body weight is within the target range. However, a study has revealed that some patients require TLV over a prolonged period (*4*). In addition, the long-term use of TLV is reported to reduce the dosage of loop diuretics (*5*).

TLV became available at this Hospital in November 2011, and some patients have received TLV for a prolonged period (more than one year). The aim of the current study was to examine the actual consequences of use of TLV and the effects of the long-term use of TLV at this Hospital.

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**Address correspondence to:*
Dr. Shunsuke Kiuchi, Department of Cardiovascular Medicine, Toho University Graduate School of Medicine, 6-11-1 Omori-nishi, Ota-ku, Tokyo 143-8541, Japan.
E-mail: syunnsuke@med.toho-u.ac.jp

2. Materials and Methods

2.1. *Statement of ethics*

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Omori Medical Center Ethical Committee of Toho University (24-123). The current study was a single-center, open retrospective study. Formal consent was not required for this type of study.

2.2. *Study subjects*

Subjects were 258 consecutive patients with ADHF who received TLV during hospitalization from January 2011 to March 2015. ADHF was diagnosed according to the Framingham criteria (*6*). The in-hospital mortality rate and the rate of continued administration of TLV were investigated after discharge and one year later. In addition, subjects were divided into three groups (Group A ceased receiving TLV after discharge, Group B continued receiving TLV upon discharge and ceased receiving TLV within one year of discharge, and Group C continued receiving TLV one year after discharge), and the rate of re-hospitalization for HF within one year was examined in these groups. Findings at discharge were compared among these groups. Differences in findings at discharge and one year later were investigated for patients who continued to receive TLV one year after discharge.

The need to continue receiving TLV was determined at discharge using an on-off test. An on-off test was performed after improvement of ADHF. In the on-off test, TLV was deemed unnecessary when ADHF did not worsen two days after TLV was discontinued. The need for outpatients to continue receiving TLV was determined by the attending physician given the patient's living conditions and diet.

2.3. *Concomitant oral medications*

Changes in the type and dosage of TLV and other concomitant medications were examined. The rate of administration of loop diuretics, a renin-angiotensin-aldosterone system inhibitor (RAAS-I), and a beta blocker (BB) were investigated. An RAAS-I was defined as an angiotensin-converting enzyme inhibitor, an angiotensin II type 1a receptor blocker, or a mineral corticoid receptor antagonist. The dose of a loop diuretic, converted to the furosemide dose (20 mg of furosemide is equivalent to 30 mg of azosemide), was evaluated.

2.4. *Clinical profile*

The New York Heart Association Classification (NYHA) was used to evaluate the severity of HF. BP was measured twice with an aneroid sphygmomanometer after the subject had been seated comfortably for at least five minutes, and the average was calculated (*7*). Systolic BP and diastolic BP were evaluated. Heart rate (HR) was evaluated using standard 12-lead electrocardiography (ECG). ECG was performed after the patient remained in a resting position.

2.5. *Laboratory analysis*

Changes in electrolytes (sodium, potassium, and chloride), liver function (aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase), renal function (blood urea nitrogen, and creatinine), and uric acid (UA), hemoglobin, and brain natriuretic peptide (BNP) levels were measured. All serum samples were obtained after fasting and resting in a supine position for at least five minutes.

2.6. *Chest X-ray and transthoracic echocardiography*

The cardiothoracic ratio (CTR) was determined from a chest X-ray obtained in the standing position on admission and was assessed by two physicians blinded to the examination. CTR was calculated utilizing the maximal cardiac diameter and the intrathoracic diameter. Transthoracic echocardiography (TTE) was performed to evaluate cardiac size (left atrial dimension and left ventricular end-diastolic/end-systolic dimensions), systolic function (ejection fraction (EF)), wall thickness (interventricular septal wall thickness and posterior wall thickness at end-diastole) were evaluated. EF was calculated with the Teichholz method (*8*) using a parasternal long-axis view or with a modified form of Simpson's method (*9*) using an apical two or four-chamber view.

2.7. *Statistical analysis*

Continuous variables were expressed as the mean ± standard deviation. Differences in findings after discharge were examined among the three groups using an unpaired Student's *t*-test. Values at discharge and one year later were compared using a paired *t*-test. Analyses were performed with Microsoft Excel and the statistical package Stat View (Stat View 4.0, SAS Institute Inc.). A probability (*p*) value of less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. *Prognosis for subjects*

Of the current subjects, 41 (15.9%) died in hospital during follow-up. Thirty-four of those patients (13.2%) died due to cardiovascular disease. One hundred and seven patients (49.3%) continued to receive TLV after discharge, excluding patients who died in hospital.

Table 1. Characteristics of Groups A, B, and C at discharge (no significant differences were apparent)

Items	Group A, *n* = 104	Group B, *n* = 57	Group C, *n* = 48
Age	71.6 ± 14.2	73/2 ± 15.1	73.8 ± 12.4
Gender male/female	55/49	31/26	23/25
NHYA (I/II/III/IV)	0/4/86/14	0/7/35/15	0/4/32/12
Rate of administration of loop diuretics (*n*, %)	72, 69.2%	45, 78.9%	43, 89.6%
Loop diuretics (mg)	25.8 ± 20.9	36.1 ± 25.4	29.3 ± 20.9
Rate of administration of ACE-I / ARB/ MRA (*n*, %)	82, 78.8%	44, 77.2%	39, 81.3%
Rate of administration of beta blocker (*n*, %)	82, 78.8%.	45, 78.9%	40, 85.4%
Systolic BP (mmHg)	108.9 ± 15.2	108.1 ± 24.1	105.5 ± 15.6
Diastolic BP (mmHg)	58.4 ± 8.4	60.1 ± 11.4	58.1 ± 8.9
Heart rate (bpm)	71.6 ± 11.4	74.7 ± 12.5	72.6 ± 12.7
Sodium (mEq/L)	137.1 ± 4.5	138.3 ± 4.3	139.5 ± 4.1
Potassium (mEq/L)	4.2 ± 0.5	4.2 ± 0.5	4.3 ± 0.5
Chloride (mEq/L)	103.0 ± 4.7	103.9 ± 4.8	105.2 ± 4.4
AST (IU/L)	24.9 ± 13.3	23.2 ± 11.3	24.6 ± 11.2
ALT (IU/L)	20.3 ± 16.4	16.7 ± 14.0	19.0 ± 12.6
LDH (IU/L)	224.2 ± 66.6	234.0 ± 69.3	235.4 ± 81.2
BUN (mg/dL)	29.4 ± 14.2	35.5 ± 21.9	29.0 ± 12.3
Creatinine (mg/dL)	1.32 ± 0.74	1.60 ± 0.87	1.50 ± 0.87
Uric acid (mg/dL)	6.7 ± 1.7	6.9 ± 2.2	7.5 ± 2.0
Hemoglobin (mg/dL)	11.7 ± 1.8	11.2 ± 1.9	11.6 ± 1.9
BNP (pg/mL)	460.4 ± 592.0	738.5 ± 731.6	572.0 ± 520.2
Cardiothoracic ratio (%)	58.0 ± 7.8	59.8 ± 7.3	58.3 ± 7.9
Left atrial dimension (mm)	42.6 ± 10.4	44.5 ± 8.8	46.8 ± 10.0
LVDd (mm)	54.9 ± 9.8	56.0 ± 12.6	55.6 ± 12.0
LVDs (mm)	40.7 ± 11.8	42.3 ± 14.3	43.0 ± 14.0
IVSTd (mm)	0.89 ± 0.20	0.91 ± 0.28	0.89 ± 0.21
PWTd (mm)	0.93 ± 0.20	0.97 ± 0.25	0.94 ± 0.24
Ejection fraction (%)	49.5 ± 17.3	48.0 ± 19.4	45.8 ± 18.1

NHYA: New York Heart Association classification, ACE-I: angiotensin-converting enzyme inhibitor, ARB: angiotensin II type 1a receptor blocker, MRA: mineralocorticoid receptor antagonist, BP: blood pressure, AST: aspartate aminotransferase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase, BUN: blood urea uitrogen. BNP: brain natriuretic peptide, LVDd: left ventricular end-diastolic dimension, LVDs: left ventricular end-systolic dimension, IVSTd: interventricular septal wall thickness at end-diastole, PWTd: posterior wall thickness at end-diastole. Continuous data are expressed as the mean ± standard deviation. We analyzed with the Student's *t*-test.

Eight patients were missing during follow-up. Thus, 209 patients (104 patients in Group A who ceased receiving TLV after discharge and 105 patients who continued to receive TLV after discharge (Groups B and C)) were evaluated after discharge. Group B consisted of 57 patients (54.3%) and Group C consisted of 48 patients (45.7%). Of the 209 patients, 58 (27.8%) died within one year of discharge.

3.2. *Comparison of findings at discharge among the three groups*

Differences in findings among the three groups are shown in Table 1. There were no significant differences in any findings among the three groups. This indicated that the severity of HF at discharge did not differ significantly among the three groups. In addition, the rate of administration of cardio-protective medications such as RAAS-I and BB did not differ significantly among the three groups. There were no significant differences in the dosage of loop diuretics among the three groups.

3.3. *One-year mortality rate and/or rate of re-hospitalization and TLV*

Twenty-six patients (25.0%) died in Group A. Similarly, 32 (30.5%) of 105 patients who continued to receive TLV after discharge died within one year of discharge. The mortality rate did not differ significantly ($p = 0.811$). Within one year of discharge, 47 patients (45.2%) in Group A and 67 (63.8%) out of 105 patients who continued to receive TLV after discharge died from any cause or were re-hospitalized for HF. Twenty-six patients (25.0%) in Group A were re-hospitalized for HF within one year of discharge. Twenty-nine patients (50.9%) in Group B were re-hospitalized for HF within one year of discharge. Similarly, 23 patients (47.9%) in Group C were re-hospitalized for HF within one year of discharge. The rate of re-hospitalization for HF was significantly higher in Groups C and B compared to that in Group A (Figure 1, $p < 0.001$). Continuous administration of TLV for one year after discharge tended to delay re-hospitalization for HF (Figure 1), but the duration until re-hospitalization did not differ significantly between Groups C and B.

3.4. *Changes in oral concomitant medication*

Forty-five patients who received TLV after discharge and who continued to receive TLV for one year were evaluated, excluding three patients who were

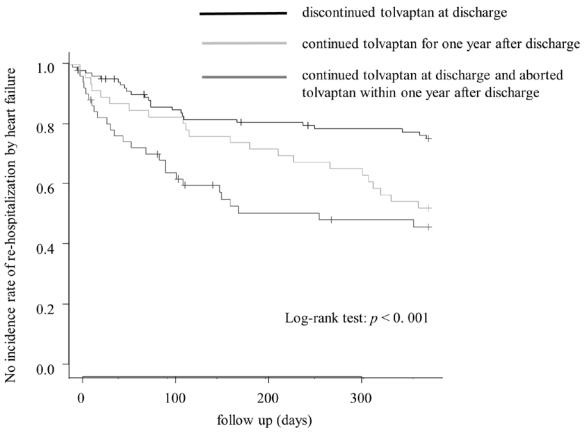

Figure 1. Re-hospitalization for heart failure within one year of discharge. Patients who continued to receive tolvaptan after discharge had a significantly higher rate of re-hospitalization for heart failure within one year of discharge compared to patients who ceased receiving tolvaptan after discharge. Continued administration of tolvaptan after discharge tended to extend the duration until re-hospitalization for heart failure. *p*-values were determined using the log rank test.

unavailable for follow-up. These patients consisted of 23 males (51.1%) with an average age of 73.8 ± 12.4 years (range: 45 to 91 years).

Forty-two patients (93.3%) received a loop diuretic one year after discharge, 37 (82.2%) received an RAAS-I, 39 (86.7%) received a BB, 9 (20.0%) received pimobendan, and 8 (17.8%) received amiodarone. The changes in concomitant medications, including TLV, did not differ significantly (Table 2).

3.5. *Changes in laboratory results and findings from chest X-rays and transthoracic echocardiography*

There were no significant changes in BP and HR (Table 2). Laboratory results indicated that sodium and UA levels decreased significantly (sodium: 139.8 ± 3.9 mg/dL to 138.6 ± 3.5 mg/dL, $p = 0.043$, UA: 7.9 ± 1.9 mg/dL to 6.8 ± 1.9 mg/dL, $p = 0.023$, Table3). In addition, BNP levels also decreased significantly (577.6 ± 528.5 pg/mL to 397.3 ± 365.8 pg/mL, $p = 0.015$, Table 3).

Table 2. Changes in concomitant medication and blood pressure at discharge and one year later in patients who continued to receive tolvaptan for one year

Items	At discharge	One year later	*p* value
Tolvaptan (mg)	9.21 ± 6.37	9.50 ± 5.57	0.619
Rate of administration of loop diuretics (*n*, %)	39, 86.7%	42, 93.3%	0.851
Loop diuretics (mg)	28.10 ± 21.89	27.14 ± 26.90	0.395
Rate of administration of ACE-I / ARB/ MRA (*n*, %)	37, 82.2%	35, 77.8%	0.210
Rate of administration of beta blocker (*n*, %)	39, 86.7%	42, 93.3%	0.958
Systolic BP (mmHg)	106.5 ± 15.5	115.0 ± 13.8	0.998
Diastolic BP (mmHg)	58.7 ± 8.6	63.9 ± 10.8	0.991
Heart rate (bpm)	69.1 ± 8.2	73.6 ± 10.8	0.980

ACE-I: angiotensin-converting enzyme inhibitor, ARB: angiotensin II receptor blocker, MRA: mineralocorticoid receptor antagonist, BP: blood pressure. Continuous data are expressed as the mean ± standard deviation. *p*-values were determined using the paired *t*-test.

Table 3. Changes in laboratory results and findings from chest X-rays and transthoracic echocardiography at discharge and one year later in patients who continued to receive tolvaptan for one year

Items	At discharge	One year later	*p* value
Sodium (mEq/L)	139.8 ± 3.9	138.6 ± 3.5	0.043
Potassium (mEq/L)	4.3 ± 0.5	4.5 ± 0.6	0.959
Chloride (mEq/L)	105.2 ± 4.4	104.4 ± 4.4	0.102
AST (IU/L)	24.3 ± 11.4	23.8 ± 6.4	0.356
ALT (IU/L)	18.4 ± 12.7	16.4 ± 7.4	0.135
LDH (IU/L)	238.5 ± 83.9	236.9 ± 48.2	0.456
BUN (mg/dL)	28.4 ± 12.0	33.4 ± 18.3	0.974
Creatinine (mg/dL)	1.51 ± 0.89	1.59 ± 0.86	0.951
Uric acid (mg/dL)	7.9 ± 1.9	6.8 ± 1.9	0.023
Hemoglobin (mg/dL)	11.6 ± 1.9	11.8 ± 1.9	0.780
BNP (pg/mL)	577.6 ± 528.5	397.3 ± 365.8	0.015
Cardiothoracic ratio (%)	59.4 ± 8.0	59.4 ± 9.2	0.508
Left atrial dimension (mm)	46.9 ± 9.5	45.4 ± 10.6	0.057
LVDd (mm)	55.4 ± 12.3	54.9 ± 12.8	0.345
LVDs (mm)	43.2 ± 14.3	45.8 ± 33.9	0.706
IVSTd (mm)	0.90 ± 0.21	0.91 ± 0.23	0.594
PWTd (mm)	0.95 ± 0.23	0.91 ± 0.20	0.179
Ejection fraction (%)	45.2 ± 18.4	50.0 ± 18.7	0.955

AST: aspartate aminotransferase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase, BUN: blood urea uitrogen. BNP: brain natriuretic peptide, LVDd: left ventricular end-diastolic dimension, LVDs: left ventricular end-systolic dimension, IVSTd: interventricular septal wall thickness at end-diastole, PWTd: posterior wall thickness at end-diastole. Continuous data are expressed as the mean ± standard deviation. *p*-values were determined using the paired *t*-test.

CTR and TTE findings did not change significantly (Table 3).

4. Discussion

4.1. *The severity of heart failure in subjects*

In the current study, the in-hospital mortality rate was 15.9% and the one-year mortality rate was 27.8%. Moreover, the mortality rate and/or rate of re-hospitalization within one year was 54.4%. Previous studies in Japan revealed that the in-hospital mortality rate was about 6% and the mortality rate and/or rate of re-hospitalization within one year of ADHF was about 40% (*10-12*). Therefore, the current study may have included patients who had more severe ADHF. Patients with worsening renal function are often encountered during the treatment of ADHF. Previous studies also reported that TLV alleviated renal dysfunction while treating ADHF, so TLV is a useful medication for treatment of patients with ADHF and renal dysfunction (*2,13*). In addition, serum sodium levels must be cautiously monitored because TLV was developed as a medication to treat hyponatremia (*14*). That said, hyponatremia has been reported to worsen the prognosis for ADHF (*15,16*). The use of TLV might have a renal protective effect and elevate serum sodium levels, but long-term use of TLV did not alleviate renal dysfunction or reduce serum sodium levels in the current study. These results were presumably due to the severity of HF in the current sample. Two patients ceased to receive RAAS-I one year after discharge due to renal dysfunction. This result could be due to the fact that BP tended to be high one year after discharge. However, BP was controlled to within the optimal range recommended by the Guidelines of the Japanese Society of Hypertension (*7*).

4.2. *TLV and the long-term prognosis for heart failure*

In the current study, the long-term use of TLV did not reduce the dosage of loop diuretics or the mortality rate. A study has reported that continuous administration of TLV does not improve the prognosis for HF (*17*). In contrast, another study reported that continuous administration of TLV reduced the rate of re-hospitalization (*18*). In the current study, however, the long-term use of TLV did not reduce the rate of re-hospitalization. These results were presumably due to the severity of HF in the current sample. However, the long-term use of TLV tended to delay re-hospitalization for HF and significantly reduced BNP levels. Long-term use of TLV is possibly a useful treatment for patients with severe ADHF. However, the effects of the long-term use of TLV are still unclear, and the current study was an open-label retrospective study of 45 patients. Therefore, large-scale clinical studies need to be conducted to verify these results.

4.3. *The necessity for the long-term use of TLV*

The need for continuous administration of TLV after discharge was evaluated with an on-off test. Since many studies have reported that the diuretic effect of TLV lasts several days, the on-off test was conducted two days after the discontinuation of TLV (*19,20*). An attending physician evaluated the patient's general condition based on urine volume, a chest X-ray, and symptoms such as dyspnea. The on-off test includes subjective evaluations, but assessment of the necessity for the long-term use of TLV in the on-off test helped to evaluate re-hospitalization for HF.

4.4. *Study limitations*

The current study was a small-scale, single-center, open retrospective study. The sample included only 45 patients who continued to receive TLV for one year after discharge. Thus, the classification of HF-reduced EF (HFrEF) and HF-preserved EF (HFpEF) was difficult. Therapeutic medications for HFrEF and HFpEF differ, but TLV has been reported to be useful and effective in treating both (*21*). Medications with a cardio-protective effect such as RAAS-I and BB are a class I recommendation for patients with HFrEF, but the only class I medication for patients with HFpEF is a diuretic. These cardio-protective medications also have antihypertensive action. BP also affects the prognosis for HF. Therefore, the different uses of these medications might have affected the current results. A second limitation of this study was that the duration of use of TLV has changed from year to year. When TLV originally became available, its use was only recommended for patients with more severe HF in comparison recent recommendations for it use. Thus, changes in the usage of TLV might have affected the current results. Further evaluation was difficult in the current study because the sample was small.

5. Conclusions

The current study has described experience with long-term administration of TLV at this hospital. Patients with ADHF treated with TLV after discharge had a higher rate of re-hospitalization at one year compared to patients with ADHF who ceased receiving TLV after discharge. However, long-term use of TLV decreased BNP levels in patients with ADHF. Patients who required continued administration of TLV had more severe HF, and the long-term use of TLV might delay re-hospitalization in patients with ADHF. Large-scale clinical studies are necessary to verify these results since the current study was a small-scale, single-center, retrospective study.

Conflict of Interest

T.I. has received grant support through his institution from Daiichi Sankyo, Bristol-Myers Squibb, and Boehringer Ingelheim and honoraria for lectures from Bayer Healthcare, Daiichi Sankyo, Bristol-Myers Squibb, Pfizer, Tanabe-Mitsubishi, and Ono Pharmaceutical. The author declares that he has no potential conflicts of interest with regard to current study. The co-authors report that they also have no conflicts of interest with regard to current study.

References

1. Matsuzaki M, Hori M, Izumi T, Fukunami M; Tolvaptan Investigators. Efficacy and safety of tolvaptan in heart failure patients with volume overload despite the standard treatment with conventional diuretics: A phase III, randomized, double-blind, placebo-controlled study (QUEST study). Cardiovasc Drugs Ther. 2011; 25 (Suppl 1):S33-S45.
2. Matsue Y, Suzuki M, Seya M, Iwatsuka R, Mizukami A, Nagahori W, Ohno M, Matsumura A, Hashimoto Y. Tolvaptan reduces the risk of worsening renal function in patients with acute decompensated heart failure in high-risk population. J Cardiol. 2013; 61:169-174.
3. Udelson JE, Orlandi C, Ouyang J, Krasa H, Zimmer CA, Frivold G, Haught WH, Meymandi S, Macarie C, Raef D, Wedge P, Konstam MA, Gheorghiade M. Acute hemodynamic effects of tolvaptan, a vasopressin V2 receptor blocker, in patients with symptomatic heart failure and systolic dysfunction: An international, multicenter, randomized, placebo-controlled trial. J Am Coll Cardiol. 2008; 52:1540-1545.
4. Xiong B, Huang Y, Tan J, Yao Y, Wang C, Qian J, Rong S, Deng S, Cao Y, Zou Y, Huang J. The short-term and long-term effects of tolvaptan in patients with heart failure: A meta-analysis of randomized controlled trials. Heart Fail Rev. 2015; 20:633-642.
5. Ogawa H, Ajioka M, Ishii H, Okumura T, Murase Y, Osanai H, Nakasima Y, Asano H, Sakai K. Long-term effects of tolvaptan in patients requiring recurrent hospitalization for heart failure. Nagoya J Med Sci. 2015; 77:355-362.
6. McKee PA, Castelli WP, McNakamura PM, Kannel WB. The natural history of congestive heart failure: The Framingham study. N Engl J Med. 1971; 285:1441-1446.
7. Shimamoto K, Ando K, Fujita T, *et al*. The Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH 2014). Hypertens Res. 2014; 37:253-390.
8. Teichholz LE, Kreulen T, Herman MV, Gorlin R. Problems in echocardiographic volume determinations: Echocardiographic-angiographic correlations in the presence or absence of asynergy. Am J Cardiol. 1976; 37:7-11.
9. Schiller NB, Shah PN, Crawford M, *et al*. Recommendations for quantitation of the left ventricle by two-dimensional echocardiography. American Society of Echocardiography Committee on Standards, Subcommittee on Quantitation of Two-Dimensional Echocardiograms. J Am Soc Echocardiogr. 1989; 2:258-267.
10. Wakabayashi K, Sato N, Kajimoto K, Minami Y, Mizuno M, Keida T, Asai K, Munakata R, Murai K, Sakata Y, Suzuki H, Takano T; ATTEND investigators. Incidence and predictors of in-hospital non-cardiac death in patients with acute heart failure. Eur Heart J Acute Cardiovasc Care. 2015. doi 10.1177/2048872615593388
11. Mizuno M, Kajimoto K, Sato N, Yumino D, Minami Y, Murai K, Munakata R, Asai K, Keida T, Sakata Y, Hagiwara N, Takano T; ATTEND Investigators. Clinical profile, management, and mortality in very-elderly patients hospitalized with acute decompensated heart failure: An analysis from the ATTEND registry. Eur J Intern Med. 2016; 27:80-85.
12. Kajimoto K, Minami Y, Sato N, Kasanuki H; Investigators of the Acute Decompensated Heart Failure Syndromes (ATTEND) Registry. Etiology of heart failure and outcomes in patients hospitalized for acute decompensated heart failure with preserved or reduced ejection fraction. Am J Cardiol. 2016; 118:1881-1887.
13. Matsue Y, Suzuki M, Torii S, *et al*. Clinical effectiveness of tolvaptan in patients with acute heart failure and renal dysfunction. J Card Fail. 2016; 22:423-432.
14. Schrier RW, Gross P, Gheorghiade M, Berl T, Verbalis JG, Czerwiec FS, Orlandi C; SALT Investigators. Tolvaptan, a selective oral vasopressin V2-receptor antagonist, for hypotension. N Engl J Med. 2006; 355:2099-2122.
15. Sato N, Gheorghiade M, Kajimoto K, Munakata R, Minami Y, Mizuno M, Aokage T, Asai K, Sakata Y, Yumino D, Mizuno K, Takano T; ATTEND Investigators. Hyponatremia and in-hospital mortality in patients admitted for heart failure (from the ATTEND registry). Am J Cardiol. 2013; 111:1019-1025.
16. Gheorghiade M, Abraham WT, Albert NM, Gattis Stough W, Greenberg BH, O'Connor CM, She L, Yancy CW, Young J, Fonarow GC; OPTIMIZE-HF Investigators and Coordinators. Relationship between admission serum sodium concentration and clinical outcomes in patients hospitalized for heart failure: An analysis from the OPTIMIZE-HF registry. Eur Heart J. 2007; 28:980-988.
17. Konstam MA, Gheorghiade M, Burnett JC Jr, Grinfeld L, Maggioni AP, Swedberg K, Udelson JE, Zannad F, Cook T, Ouyang J, Zimmer C, Orlandi C; Efficacy of Vasopressin Antagonism in Heart Failure Outcome Study With Tolvaptan (EVEREST) Investigators. Effects of oral tolvaptan in patients hospitalized for worsening heart failure. The EVEREST Outcome Trial. JAMA. 2007; 297:1319-1331.
18. Blair JE, Khan S, Konstam MA, Swedberg K, Zannad F, Burnett JC Jr, Grinfeld L, Maggioni AP, Udelson JE, Zimmer CA, Ouyang J, Chen CF, Gheorghiade M; EVEREST Investigators. Weight changes after hospitalization for worsening heart failure and subsequent re-hospitalization and mortality in the EVEREST trial. Eur Heart J. 2009; 30:1666-1673.
19. Kinugawa K, Sato N, Inomata T, Shimakawa T, Iwatake N, Mizuguchi K. Efficacy and safety of tolvaptan in heart failure patients with volume overload. Circ J. 2014; 78:844-852.
20. Udelson JE, Bilsker M, Hauptman PJ, Sequeira R, Thomas I, O'Brien T, Zimmer C, Orlandi C, Konstam MA. A multicenter, randomized, double-blind, placebo-controlled study of tolvaptan monotherapy compared

to furosemide and the combination of tolvaptan and furosemide in patients with heart failure and systolic dysfunction. J Card Fail. 2011; 17:973-981.
21. Imamura T, Kinugawa K. Tolvaptan improves the long-term prognosis in patients with congestive heart failure with preserved ejection fraction as well as in those with reduced ejection fraction. Int Heart J. 2016; 57:600-606.

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Original Article

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N-acetyl glucosamine and proteoglycan containing supplement improves the locomotor functions of subjects with knee pain

Yuna Naraoka[1,*], Hiroshi Harada[1,2], Mikiyuki Katagiri[1], Haruo Yamamura[3], Takuji Shirasawa[1]

[1] *Shirasawa Anti-Aging Medical Institute, Tokyo, Japan;*
[2] *Department of Neurosurgery, Juntendo University, Tokyo, Japan;*
[3] *CHARLE CO., LTD., Kobe, Japan.*

Summary **The aim of this study was to investigate the effect of *N*-acetyl glucosamine and proteoglycan-containing supplement (NGPS) on knee pain and locomotor functions in middle-aged and elderly persons with knee pain. An open trial was conducted on 19 subjects suffering from knee pain. The subjects, aged (55.6 ± 6.9) years, were given the NGPS tablets, which they must take 3 times per day, that contain 526.5 mg of *N*-acetyl glucosamine (GlcNAc) and 33.6 mg of proteoglycan for 12 weeks. Subjective pain was evaluated using the Visual Analog Scale (VAS), while the function of the knee with regard to daily operation was evaluated using the Japanese Knee Osteoarthritis Score (JKOM). Walking, stair-climbing and swelling were evaluated using the Japanese Orthopedic Association Score (JOA). These items were evaluated at a baseline, and after 4, 8, and 12 weeks of NGPS treatment. The VAS scores at 8 ($p = 0.004$) and 12 ($p < 0.001$) weeks were significantly lower than that at the baseline. The JKOM total score was significantly lower at 8 and 12 weeks ($p = 0.001$) than that at the baseline. The JOA score in the more painful side of the leg was significantly higher at 12 weeks ($p = 0.002$) than that at the baseline. The present study reveals that intake of NGPS is effective for relieving knee pain and improving knee function when walking or climbing stairs, swelling and bending or stretching.**

Keywords: Oligosaccharide, gonalgia, osteoarthritis, alternative medicine

1. Introduction

Osteoarthritis (OA) is the most common form of arthritis and is a leading cause of disability (*1*). Current treatment of OA includes both non-pharmacologic and pharmacological therapies (*2*). Among pharmacological treatment, analgesic and non-steroidal anti-inflammatory drugs (NSAIDs) are the primary treatment methods. However, the use of NSAIDs is limited by their serious side effects on the gastrointestinal tract and cartilage metabolism (*3,4*). Therefore, attention has been focused on safe and causal treatment, but not supportive treatment, in response to clinical symptoms of OA. The causal treatment has been performed with glucosamine. Kongtharvonskul and colleagues have shown that NSAIDs and glucosamine are equally efficacious for symptom relief in knee OA but NSAIDs have more side effects as observed on meta-analysis (*5*).

Tsuji *et al*. reported that supplementation with *N*-acetyl glucosamine (GlcNAc), one of the components of cartilage, improved knee function (*6*). Since, with age, the body starts to decrease the production of glycosaminoglycans from glucose (*7*), oral intake of GlcNac may increase synthesis of cartilage glycosaminoglycans and improve the symptoms of the knee joint (*8*). Because glucosamine consists of glycosaminoglycan after being converted into GlcNac in the cells of the target tissue, GlcNac is considered to be more effective in small amounts than glucosamine for improving knee functions. However, to our knowledge, no study on the effect of small amounts of GlcNac on OA has been conducted so far. In the present study, we investigated the effects of 12 weeks of treatment with a supplement containing 526.5 mg of GlcNAc and 33.6 mg of proteoglycan on knee functions in subjects with knee pain but who were not diagnosed

**Address correspondence to:*
Dr. Yuna Naraoka, Shirasawa Anti-Aging Medical Institute, 3-5-4 Hongo, Bunkyoku, Tokyo 113-0033, Japan.
E-mail: ynaraoka@juntendo.ac.jp

with OA.

The purpose of this study was to investigate the effectiveness of *N*-acetyl glucosamine and proteoglycan-containing supplement (NGPS) for 12 weeks of oral supplementation in middle aged subjects with chronic knee pain. Because the aim of this NGPS dosing is for primary prophylaxis, we recruited participants who have chronic knee pain but not diagnosed. In addition, we confirm the safety of NGPS.

2. Materials and Methods

2.1. *Subjects*

A total of 21 subjects, recruited from the Japanese Red Cross Society Iyama Hospital (Iyama hospital), expressed interest in participating in this study. Participants' eligibility was tested using a screening questionnaire. The criteria were the participants' age (40-69 years old) and conscious awareness of knee pain but not diagnosed. The exclusion criteria were knee pain due to an injury, accident or ligament damage within a year, progressive or possibility of rheumatoid arthritis, gout and calcium pyrophosphate dehydrate deposition disease, artificial joint, recent (within a month period) injected hyaluronan-modified in knee, routine use of supplements for knee pain (*e.g*., glucosamine, chondroitin, hyaluronan), cardiovascular disturbance, dyslipidemia, hepatic disease, kidney disease, circulatory condition, endocrine disease, alimentary disease, mental disorder, food allergy, improper lifestyle (*e.g*., dietary abnormality, alcohol dependence, night shift workers, takers of irregular holidays), participation in other clinical investigations within a month, pregnancy or lactation, or deemed unsuitable as per doctor's discretion.

After the pre-study screening, 4 eligible men and 15 eligible women volunteered to participate in this study. The participants' average [mean ± standard error (SE)] age, body weight, height, and heart rate were 55.6 ± 6.9 years, 64.6 ± 10.1 kg, 160.8 ± 5.9 cm, and 75.1 ± 10.0/min, respectively. They were informed about the possible risks and discomforts involved in the experiment prior to giving their written consent to participate in the study. Written consent forms were collected from all participants. The study design was approved by the Kenshokai Ethical Review Board and conducted in accordance with the principles of the amended Declaration of Helsinki.

2.2. *Test supplement*

NGPS containing N-acetyl glucosamine (526.5 mg) and proteoglycan (33.6 mg) as its main active ingredients in a tablet form (3 tablets a day) were supplied by CHARLE (CHARLE CO., LTD., Hyogo, Japan). The tablets also included Maltitose, Shark Fin Cartilage Extract (Type II Collagen and Chondroitin), Bosvvellia Serrata Extract, Ajuga Extract, crystalline cellulose, aroma chemical, Silicone dioxide (fine), calcium stearate and hyaluronan-modified.

2.3. *Procedures*

After the pre-study screening, the eligible subjects were assigned to a 12 week dietary intervention. During the intervention, the subjects ingested 3 tablets of NGPS per day. Every day, they recorded the time of ingestion and their physical condition in case they had a general feeling of unwellness. They underwent anthropometric tests, blood pressure tests, blood exam, analysis of urine and pain assessment before intake and every 4, 8, and 12 weeks after intake at the Japanese Red Cross Society Iyama Hospital. Moreover, as a posteriori survey, they underwent the same medical checks after 4 weeks of dietary intervention (at week 16).

2.4. *Anthropometric assessment and blood pressure*

The subjects' height, weight, and blood pressure were recorded at baseline and at the 4^{th}, 8^{th}, and 12^{th} week. Blood pressure was determined after 5 minutes of complete rest in a seated position. The Body Mass Index (BMI) was calculated based on the measurements of height and weight. All measurements were recorded by nurses at the Iyama Hospital.

2.5. *Hematological assessment and urinalysis*

Hematological assessment and urinalysis were performed to confirm the safety of NGPS. The following blood indices were analyzed: white blood cells, red blood cells, blood pigment, hemoglobin, hematocrit, leukocyte count, blood platelets, whole protein, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (γ-GTP), urea nitrogen, creatinine, uric acid, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglyceride, natrium, kalium, calcium, and hemoglobin A1c. Food intake and beverages other than water were not allowed 8 hours prior to blood sampling. Blood samples were drawn from the antecubital vein. The samples were immediately stored in a cooler box, which was maintained at 4°C until centrifugation was done in a refrigerated centrifuge at 4°C. Samples were analyzed at a clinical laboratory in the Iyama Hospital.

Samples for urinalysis were collected from each participant and uric acid, creatinine, qualitative protein, qualitative sugar and urobilinogenuria were measured.

2.6. *Assessment of efficacy*

Subjects answered the following three questionnaires for rating knee pain during rest, while walking and while doing stepping exercises: (1) Visual Analog

Table 1. The mean characteristics of the subjects

Items	0 w, Mean ± SD	4 w, Mean ± SD	*p* (*vs*. 0 w)	8 w, Mean ± SD	*p* (*vs*. 0 w)	12 w, Mean ± SD	*p* (*vs*. 0 w)
Age (years)	55.6 ± 6.9	-		-		-	
Height (cm)	160.8 ± 5.9	-		-		-	
Weight (kg)	64.6 ± 10.1	64.7 ± 10.0	0.267	64.7 ± 10.1	0.370	64.9 ± 10.1	0.029*
BMI (kg/m^2)	25.0 ± 3.9	25.1 ± 3.9	0.258	25.1 ± 4.0	0.367	25.1 ± 4.0	0.027*
Systolic blood pressure (mmHg)	127.3 ± 10.7	127.2 ± 10.1	0.958	127.1 ± 14.1	0.940	125.2 ± 11.8	0.435
Diastolic blood pressure (mmHg)	78.3 ± 6.9	77.2 ± 5.7	0.508	78.3 ± 6.8	0.976	76.5 ± 6.4	0.249
Heart race (beats/min)	75.1 ± 10.0	72.5 ± 11.0	0.249	74.0 ±8.6	0.564	74.9 ± 8.4	0.934

Note: *n* = 19 (4 male and 15 female).

Table 2. Assessment of efficacy

Items	0 w Mean ± SD	4 w Mean ± SD	*p* (*vs*. 0 w)	8 w Mean ± SD	*p* (*vs*. 0 w)	12 w Mean ± SD	*p* (*vs*. 0 w)	16 w Mean ± SD	*p* (*vs*. 12 w)
VAS (mm)	50.5 ± 24.7	33.6 ± 22.3	0.003**	30.6 ± 26.7	0.003**	18.8 ± 18.4	0.000**	28.9 ± 27.1	0.205
JKOM	43.6 ± 7.9	38.6 ± 5.9	0.156	35.9 ± 6.0	0.001**	35.8 ± 6.5	0.000**	36.5 ± 9.0	0.717
JOA*[1]	83.9 ± 8.6	90.5 ± 0.0	0.035*	88.9 ± 8.4	0.360	91.8 ± 6.3	0.001**	92.9 ± 7.7	0.508
Each evaluated items in JKOM									
Pain and stiffness in the knee	17.3 ± 4.4	13.9 ± 3.6	0.001**	13.1 ± 3.4	< 0.001**	13.2 ± 3.6	0.001**	13.5 ± 4.9	0.925
State of daily life	14.6 ± 4.0	13.3 ± 3.4	0.030*	13.1 ± 3.0	0.029*	12.9 ± 3.2	0.009**	12.9 ± 3.9	0.751
Daily activities	7.2 ± 1.9	7.6 ± 1.9	0.291	6.3 ± 1.0	0.106	6.2 ± 1.4	0.030*	6.6 ± 1.3	0.101
Condition of health	4.5 ± 1.3	3.8 ± 1.4	0.103	3.5 ± 1.2	0.003**	3.5 ± 1.1	0.009**	3.4 ± 1.3	0.603

*[1]: JOA score evaluated more painful side of the legs. *$p < 0.05$ and **$p < 0.01$.

Scale (VAS): respondents specified their pain level on a continuous scale from 0 to 10, (2) Japanese Knee Osteoarthritis Measure (JKOM) respondents evaluated the pain and stiffness suffered, the state of their daily life, daily activities, and the condition of their health with a total score of 125 (*9*), and (3) Japanese Orthopedic Association score (JOA score) respondents evaluated their ability to walk (30 points), ability to climb up and down stairs (25 points), range of motion (ROM; 35 points), and joint swelling (10 points) (*10*).

2.7. *Statistical analysis*

Statistical tests were carried out using SPSS ver. 20.0 (SPSS, IBM). A significance level of $p < 0.05$ was used.

For the assessment of efficacy, a non-parametric multiple comparison test was performed using the significant findings (Friedman test) of each pain assessment (JKOM, JOA, and VAS) questionnaire at each measurement point (0, 4, 8, and 12 weeks). For safety examination, hematological assessment, biochemical tests, urinalysis, and physical measurement other than qualitative tests, paired *t*-test using Excel 2013 (Microsoft) were used. All data are expressed as mean ± standard error, unless otherwise specified.

3. Results

The mean characteristics of the subjects are shown in Table 1. Body weight and BMI were significantly higher at 12 weeks ($p = 0.029$ and $p = 0.027$) than at the baseline. However, such a change is not significant to the study that it does not figure at all in the clinical data.

The knee pain assessment showed improvement in knee function for all evaluated items at the 12th week in Table 2. VAS scores at the 8th ($p = 0.004$) and 12th week ($p < 0.001$) were significantly lower than at the baseline in Figure 1A.

JKOM total scores at the 8th week ($p = 0.003$) and 12th week ($p < 0.001$) were significantly lower than at the baseline in Figure 1B. Pain-and-stiffness-score at the 4th week ($p < 0.001$), 8th week ($p < 0.001$), and 12th week ($p < 0.001$) were significantly lower than at the baseline in Figure 2A. The state-of-daily-life scores at the 4th week ($p = 0.024$), 8th week ($p = 0.033$), and 12th week ($p = 0.007$) were significantly lower than at the baseline in Figure 2B. Soreness due to daily activities score at the 12th week ($p = 0.049$) was significantly lower than at the baseline in Figure 2C. The Condition-of-health-scores at the 8th week ($p < 0.001$) and 12th week ($p = 0.009$) were significantly lower than at the baseline in Figure 2D. The JOA score at the 12th week ($p = 0.002$) was significantly higher than at the baseline for the more painful side of the leg in Figure 1C. However, these scores returned to the baseline level after 4 weeks of washout period (16 weeks); no significant difference was observed compared to the one on the 12th week.

On the safety testing, no variation of the value on clinical importance for safety was observed in hematological assessment and urinalysis.

4. Discussion

This investigation showed that 12 weeks of NGPS supplementation was effective for pain relief and improvement of the function of the knee.

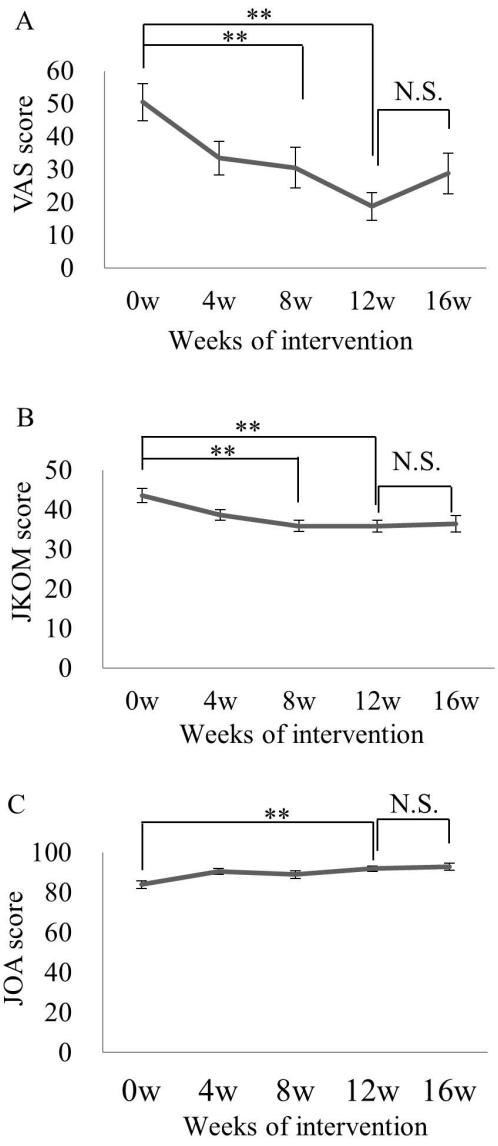

Figure 1. Effects of oral administration of NGPS on knee pain and function on time dependent change. A; the score of visual analog scale, **B**; Total scores of Japanese Knee Osteoarthritis, **C**; Total scores of Japan Orthopedic Association. Values represent mean ± SD.

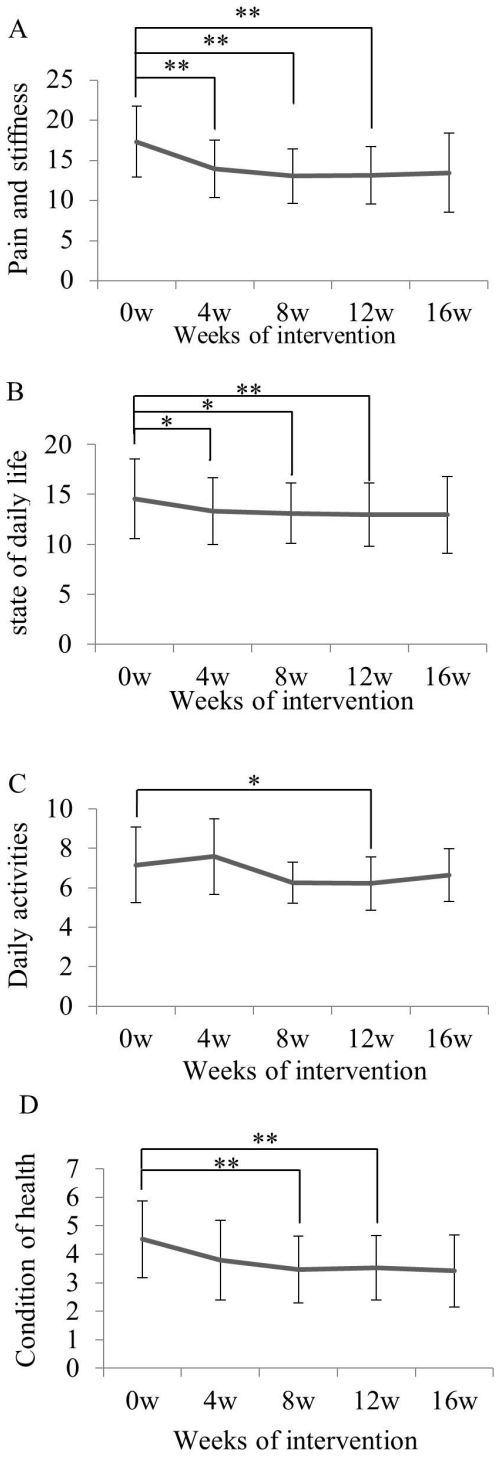

Figure 2. Effects of oral administration of NGPS on time dependent change of the each evaluated item in JKOM. Evaluated scores shows **A**; pain and stiffness, **B**; state of daily life, **C**; daily activities, **D**; condition of health in JKOM. Values represent mean ± SD.

The efficacy assessment revealed that NGPS supplementation decreased in VAS point and JKOM total scores and improved JOA total score after 12 weeks. However, 4 weeks after discontinuing supplementation, both VAS point and JKOM total score improved compared to the scores at 12 weeks. Similarly, at 16 weeks, JOA score decreased from that at 12 weeks. Giordano and colleagues showed 12 weeks of carry over effect of glucosamine sulfate in a randomized, double blind, placebo-controlled trial (*11*). They revealed that VAS scores tended to increase even after 4 weeks between the placebo group at 8 weeks and from the baseline. Since the results of this study are consistent with their report, we suggest that supplementation of NGPS is effective for improving knee-joint function.

A previous study has shown the effects of glucosamine hydrochloride supplementation on knee pain (*11*). The relationship of glucosamine and knee pain may be explained by the anti-inflammatory and chondroprotective activities of glucosamine hydrochloride (*13,14*), GlcNAc (*15*), chondroitin sulfate (*16*) and quercetin (*17,18*). Chan and colleagues

reported that glucosamine and chondroitin showed complementary anti-inflammatory effects when compared with glucosamine or chondroitin alone (*19,20*). Since in the result of JKOM, pain-and-stiffness-score showed the highest change ratio among the other evaluated items, the structure of the knee joint may have changed. The main components of this test supplement are a compound of GlcNAc and proteoglycan; the results that structural modification in the knee joint can be considered to be the composite effect of such anti-inflammatory reactions and chondroprotective activities. Ozkan and colleagues demonstrated that intraarticular *N*-acetyl glucosamine and intraarticular hyaluronate play a role in slowing the degenerative process and protecting the cartilage surface during the early stages of osteoarthritis in rabbits (*21*). Because test supplements also included hyaluronic acid as proteoglycan in addition to the GlcNAc, it was suggested to be effective for the improvement of the knee joint by chondroprotective and anti-inflammatory effects. In addition, because all participants in this study were not diagnosed with OA despite having knee pain, they might have only mild symptoms of knee pain, which is considered to be a factor influencing the improvement of knee function. These results suggest that glucosamine and proteoglycan intake in the early phase of knee pain inhibit the deformation of the knee cartilage, thus preventing OA.

In the safety assessment, all hematological evaluation items showed do not figure at all in the clinical data and no treatment-related adverse effects were experienced during the intervention periods. Moreover, there was no controversial weight gain likely to progress knee OA. These results demonstrated that NGPS can be taken safely. However, Hathcock and colleagues revealed that the safety only applies to intakes of up to 2,000 mg/day for glucosamine, and 1,200 mg/day for chondroitin sulfate (*22*); therefore intake beyond that which is stated above should be avoided.

There are some limitations to the present study. First, because no comparison control group was used, it is impossible to evaluate the placebo effect. Therefore, setting a placebo control group may be needed to clarify the actual effect of NGPS supplementation on knee function. Second, since there was no physiological endpoint, it is impossible to assess the state of the knee cartilage. To assess the structural conditions of the knee, metabolism markers, such as collagen in blood samples should have been evaluated. Third, NGPS had several components, further studies should be conducted to clarify the role of each component of NGPS on knee function.

The present results revealed that GlcNAc and proteoglycan containing supplement is effective for relieving knee pain and the improvement of knee function when walking or climbing stairs, swelling and bending or stretching. Moreover, the safety of this supplement was confirmed.

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Conflict of Interest

This study was funded by the CHARLE CO., LTD., Kobe, Japan. Haruo Yamamura is an employee of the Charle CO., LTD. No other authors declare any potential conflict of interest.

References

1. Arden N, Nevitt MC. Osteoarthritis: Epidemiology. Best Pract Res Clin Rheumatol. 2006; 20:3-25.
2. Jordan KM, Arden NK, Doherty M, *et al*. Standing Committee for International Clinical Studies Including Therapeutic Trials ESCISIT. EULAR Recommendations 2003: An evidence based approach to the management of knee osteoarthritis: Report of a Task Force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCISIT). Ann Rheum Dis. 2003; 62:1145-1155.
3. Ofman JJ, MacLean CH, Straus WL, Morton SC, Berger ML, Roth EA, Shekelle P. A metaanalysis of severe upper gastrointestinal complications of nonsteroidal antiinflammatory drugs. J Rheumatol. 2002; 29:804-812.
4. Huskisson EC, Berry H, Gishen P, Jubb RW, Whitehead J. Effects of antiinflammatory drugs on the progression of osteoarthritis of the knee. LINK Study Group. Longitudinal Investigation of Nonsteroidal Antiinflammatory Drugs in Knee Osteoarthritis. J Rheumatol. 1995; 22:1941-1946.
5. Kongtharvonskul J, Anothaisintawee T, McEvoy M, Attia J, Woratanarat P, Thakkinstian A. Efficacy and safety of glucosamine, diacerein, and NSAIDs in osteoarthritis knee: A systematic review and network meta-analysis. Eur J Med Res. 2015; 13:20-24.
6. Tsuji T, Yoon J, Kitano N, Okura T, Tanaka K. Effects of *N*-acetyl glucosamine and chondroitin sulfate supplementation on knee pain and self-reported knee function in middle-aged and older Japanese adults: A randomized, double-blind, placebo-controlled trial. Aging Clin Exp Res. 2016; 28:197-205.
7. Shuster S, Black MM, McVitie E. The influence of age and sex on skin thickness, skin collagen and density. Br J Dermatol. 1975; 93:639-643.
8. Altman RD, Manjoo A, Fierlinger A, Niazi F, Nicholls M. The mechanism of action for hyaluronic acid treatment in the osteoarthritic knee: A systematic review. BMC Musculoskelet Disord. 2015; 16:321.
9. Akai M, Doi T, Fujino K, Iwaya T, Kurosawa H, Nasu T. An outcome measure for Japanese people with knee osteoarthritis. J Rheumatol. 2005; 32:1524-1532.

10. Okuda M, Omokawa S, Okahashi K, Akahane M, Tanaka Y. Validity and reliability of the Japanese Orthopaedic Association score for osteoarthritic knees. J Orthop Sci. 2012; 17:750-756.
11. Giordano N, Fioravanti A, Papakostas P, Montella A, Giorgi G, Nuti R. The efficacy and tolerability of glucosamine sulfate in the treatment of knee osteoarthritis: A randomized, double-blind, placebo-controlled trial. Curr Th Res Clin Exp. 2009; 70:185-196.
12. Kanzaki N, Ono Y, Shibata H, Moritani T. Glucosamine-containing supplement improves locomotor functions in subjects with knee pain: A randomized, double-blind, placebo-controlled study. Clin Interv Aging. 2015; 10:1743-1753.
13. Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. Glucosamine HCl reduces equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage. 2000; 8:258-265.
14. Gouze JN, Bordji K, Gulberti S, Terlain B, Netter P, Magdalou J, Fournel-Gigleux S, Ouzzine M. Interleukin-1β down-regulates the expression of glucuronosyltransferase I, a key enzyme priming glycosaminoglycan biosynthesis: Influence of glucosamine on interleukin-1beta-mediated effects in rat chondrocytes. Arthritis Rheum. 2001; 44:351-360.
15. Shikhman AR, Kuhn K, Alaaeddine N, Lotz M. *N*-acetylglucosamine prevents IL-1 beta-mediated activation of human chondrocytes. J Immunol. 2001; 15; 166:5155-5160.
16. Iovu M, Dumais G, du Souich P. Anti-inflammatory activity of chondroitin sulfate. Osteoarthritis Cartilage. 2008; 16 (Suppl 3):S14-18.
17. Jackson JK, Higo T, Hunter WL, Burt HM. The antioxidants curcumin and quercetin inhibit inflammatory processes associated with arthritis. Inflamm Res. 2006; 55:168-175.
18. Mamani-Matsuda M, Kauss T, Al-Kharrat A, Rambert J, Fawaz F, Thiolat D, Moynet D, Coves S, Malvy D, Mossalayi MD. Therapeutic and preventive properties of quercetin in experimental arthritis correlate with decreased macrophage inflammatory mediators. Biochem Pharmacol. 2006; 72:1304-1310.
19. Chan PS, Caron JP, Orth MW. Effect of glucosamine and chondroitin sulfate on regulation of gene expression of proteolytic enzymes and their inhibitors in interleukin-1-challenged bovine articular cartilage explants. Am J Vet Res. 2005; 66:1870-1876.
20. Chan PS, Caron JP, Rosa GJ, Orth MW. Glucosamine and chondroitin sulfate regulate gene expression and synthesis of nitric oxide and prostaglandin E_2 in articular cartilage explants. Osteoarthritis Cartilage. 2005; 13:387-394.
21. Ozkan FU, Ozkan K, Ramadan S, Guven Z. Chondroprotective effect of *N*-acetylglucosamine and hyaluronate in early stages of osteoarthritis – an experimental study in rabbits. Bull NYU Hosp Jt Dis. 2009; 67:352-357.
22. Hathcock JN, Shao A. Risk assessment for glucosamine and chondroitin sulfate. Regul Toxicol Pharmacol. 2007; 47:78-83.

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Original Article

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Association between type 1 diabetes mellitus and risk of epilepsy: A meta-analysis of observational studies

Dandan Yan[1], Enfa Zhao[1], Hong Zhang[2], Xiaohui Luo[1], Yajuan Du[1,*]

[1] *Department of Structural Heart Disease, the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, China;*
[2] *Department of Neurology, the Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, China.*

Summary **A potential association between type 1 diabetes mellitus and subsequent epilepsy emerged in recent studies. This study aimed to evaluate the possible relationship between type 1 diabetes mellitus and epilepsy using meta-analysis. Pubmed, ISI Web of Knowledge, Embase and Cochrane Library were searched for potential studies of the association between type 1 diabetes mellitus and epilepsy from inception to February 1, 2017. Two investigators independently screened studies for inclusion and extracted related data; discrepancies were solved by consensus. Random effects model of Hazard Ratio (HR) was used to estimate the strength of association. We identified 13 papers from potentially relevant articles of which 3 cohort studies met the inclusion criteria. Random effects meta-analysis showed that type 1 diabetes mellitus was associated with an increased risk of epilepsy with HR = 3.29 (95% CI: 2.61-4.14; I^2 = 0, p = 0.689). Similar results were observed in type 1 diabetes mellitus patents younger than 18-years-old with HR = 2.96 (95% CI: 2.28-3.84; I^2 = 0, p = 0.571). Meta-analysis of 2 studies that adjusted for potential confounders yielded an increased risk of epilepsy with HR = 2.89 (95% CI: 2.26-3.70; I^2 = 0, p = 0.831). The meta-analysis indicates that type 1 diabetes mellitus is associated with a statistically significant increased risk for epilepsy compared to those without type 1 diabetes mellitus.**

Keywords: Type 1 diabetes mellitus, epilepsy, meta-analysis

1. Introduction

Type1 diabetes mellitus (T1DM) was characterized by destruction of pancreatic beta cells in the pancreatic islets and required lifelong dependence on exogenous insulin (*1*). In recent years, the incidence of T1DM has increased in children younger than 5 years and adolescents (*2*). Patients with T1DM were at an increased risk of suffering several severe health issues and mortality (*3*). Epilepsy was the most frequent serious neurological disorder. It was reported that the estimated prevalence of active epilepsy ranged from 0.2% to 4.1% (*4*). Varied causes of epilepsy were commonly reported, such as metabolic disturbances, structural, autoimmunity or genetic causes; however, the potential causes of epilepsies were still unclear (*5*). There was increased interest in a potential possible association between epilepsy and T1DM. However, the reported results were still inconsistent. Chou *et al*. found a positive association between type 1 diabetes mellitus and epilepsy with an HR of 2.84 (*6*), Dafoulas *et al*. *established* a positive association with an HR of 3.01 (*1*). Ramakrishnan *et al*. even found an almost six-fold increase in epilepsy in UK children with T1DM (*7*), whereas, some authors failed to confirm these associations (*8,9*). Thus, we conducted a meta-analysis to accurately evaluate the relationship between type 1 diabetes mellitus and risk for epilepsy.

2. Materials and Methods

2.1. *Search strategy*

We performed a systematic literature search of Pubmed, Embase, ISI Web of Knowledge and Cochrane Library from inception to February 1, 2017, for human studies of type 1 diabetes mellitus and epilepsy without a language restriction. The overall search strategy

**Address correspondence to:*
Dr. Yajuan Du, Department of Structural Heart Disease, the First Affiliated Hospital of Xi'an Jiaotong University, 277 Yanta West Road, Xi'an 710061, Shaanxi 710004, China.
E-mail: xmy505@163.com

referred to medical subject heading terms and/or text words: seizures or epilepsy and type 1 diabetes mellitus or T1DM. The references list of all included studies was also manually reviewed for potential studies. Abstracts and citations were screened independently by two investigators, and all included articles had a further screen for full-text reports.

2.2. *Inclusion and exclusion criteria*

A study was included in the meta-analysis if it met the following criteria: *i*) studies for type 1 diabetes mellitus; *ii*) the study evaluated the association between type 1 diabetes mellitus and subsequent epilepsy risk; *iii*) one of the outcomes contains epilepsy; and *iv*) study must contain a reference group. Editorials, letters, systematic reviews, comments or reports lacking sufficient data were excluded. If the works were shared or duplicated in more than one study, the most recent publication was included. All identified papers were independently reviewed by two authors.

2.3. *Data extraction*

Two investigators independently extracted the following data from each study: First author, year of publication, country, study type, matching conditions, crude HR, adjusted HR, Incidence rate, follow-up period and T1DM age. Disagreements were resolved by detailed discussion, consensus and arbitration by the third author.

2.4. *Statistical analysis*

All statistical analyses were carried out with Stata version 11.0 software (StataCorp, College Station,TX). Hazard ratio (HR) with 95% confidence interval (CIs) was used to estimate the effect sizes. I^2 was used to describe the statistical heterogeneity among studies. $I^2 > 50\%$ was considered to show severe heterogeneity. A random-effect model was used if $p > 0.05$ and $I^2 < 50\%$, otherwise, a fixed-effect model was selected. We used Begg's test (rank correlation method) (*10*) to evaluate possible publication bias and a p value of < 0.1 was considered as significant statistical publication bias.

3. Results

3.1. *Characteristics of the subjects in the included studies*

Detailed studies retrieval procedures are summarized in Figure 1. A total of 487 references were preliminarily identified according to the search strategy. 284 records remained after excluding 203 duplicate articles. We screened titles and abstracts of all identified papers and 171 clearly irrelevant records were excluded. After reviewing the remaining articles in more detail, 10 of the full-text articles were excluded for 3 reviews, 3 for insufficient data, 2 without control group and 2 letters. Finally, 3 cohort studies were eventually included in the study. Characteristics of 3 eligible studies are shown in Table 1.

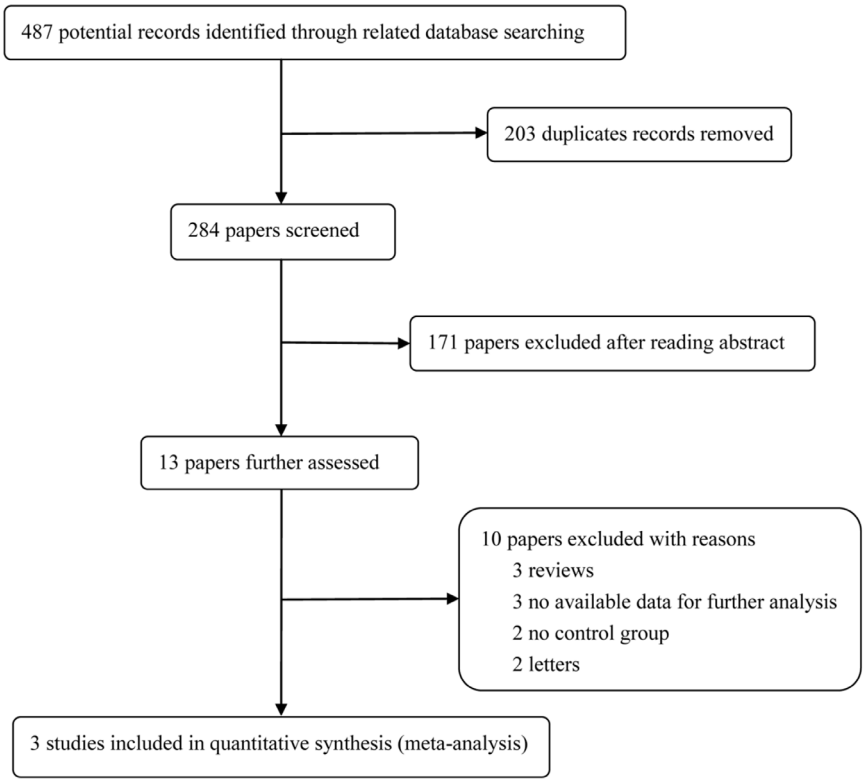

Figure 1. Flow chart of study selection process in the meta-analysis.

Table 1. Characteristics of 3 eligible studies in the meta-analysis

Author	Year	Country	Matched	Adjustment	Crude	Incidence rate (per 100,000 person-years), *n*		Follow-up	T1DM age
						Comparision cohort	T1DM cohort		
Chou C	2016	China	sex, urbanisation of residence area and index year with ten patients without type 1 diabetes	HR 2.84 [95% CI 2.11-3.83]	HR 3.26[95% CI]:(2.43, 4.37)	1.04	3.37	12 years	aged ≤ 18 years
George E	2016	UK	matched with up to four individuals without type 1 diabetes mellitus, based on age, sex and participating general practice	HR 3.01[95% CI1.93-4.68])/ HR 3.40[95% CI]: (1.97, 5.88) (≤ 18 years)	HR 3.02[95% CI]:(1.95, 4.69)/ HR 3.44[95% CI]:1.99-5.96 (≤ 18 years)	44/45 (≤ 18 years)	132/150 (≤ 18 years)	mean of 5.4 years	≤ 40 years (≤ 18 years)
McCorry D	2006	UK	age-matched patients with idiopathic generalized epilepsies (IGEs)		HR 4.4[95% CI]:(2.1-9.2)	150,000 subjects 15- to 30-year-olds	518 15- to 30-year-olds13with IGE	13 years	15-30 years

3.2. *Meta-analysis results*

As shown in Figure 2, the pooled results indicated that type 1 diabetes mellitus was associated with a statistically significant increased risk for epilepsy compared to those without type 1 diabetes mellitus. Random effects meta-analysis showed that T1DM was associated with an increased risk of epilepsy without noticeable heterogeneity with HR = 3.29 (95% CI: 2.61-4.14; $I^2 = 0$, $p = 0.689$). Similar results were observed in type 1 diabetes mellitus patients younger than 18-year-old age with HR = 2.96 (95% CI: 2.28-3.84; $I^2 = 0$, $p = 0.571$) (Figure 3). Meta-analysis of 2 studies that adjusted for potential confounders yielded an increased risk of epilepsy with HR = 2.89 (95% CI: 2.26-3.70; $I^2 = 0$, $p = 0.831$) (Figure 4).

3.3. *Publication bias*

To evaluate potential bias across studies, Begg's test with funnel plot asymmetry was used to identify small study effects of the association between T1DM and the risk of epilepsy. The funnel plot shown in Figure 5 was symmetrical, which indicated a low potential publication bias ($p = 0.526$).

4. Discussion

To our knowledge, this is the first meta-analysis that evaluated the possible effect of type 1 diabetes mellitus on subsequent epilepsy using the results of previous published studies. In this study, we found that type 1 diabetes mellitus was significantly associated with an increased risk for epilepsy compared to those without type 1 diabetes mellitus.

In recent years, there has been increasing support for the potential association between T1DM and the risk of epilepsy, although the exact mechanisms of the association remain unclear. This comorbid association was not isolated (*11*). Several hypotheses concerning the potential possible pathophysiology of the comorbidity, including genetic factors, immune abnormalities, brain lesions and metabolic abnormalities have been proposed by some researchers (*5,12*). Previously study found that glutamic acid decarboxylase antibodies (GAD-Abs) were a significant marker in TIDM patients. It was reported that GAD-Abs have been associated with T1DM and epilepsy (*13*). GAD-Abs were observed in about 60% to 70% of diabetes mellitus patients at the time of disease onset. Caietta *et al*. studied 10 T1DM children complicated with epilepsy and GAD-Abs was detected in most cases (*14*). The inactivation of gamma-amino butyric acid (GABA) receptors can also result in epilepsy (*15*). Owing to the central GABA increase in metabolism induced by hyperglycemia, GABA expression and epilepsy threshold were suppressed, thus facilitating the occurrence of seizures. Previous

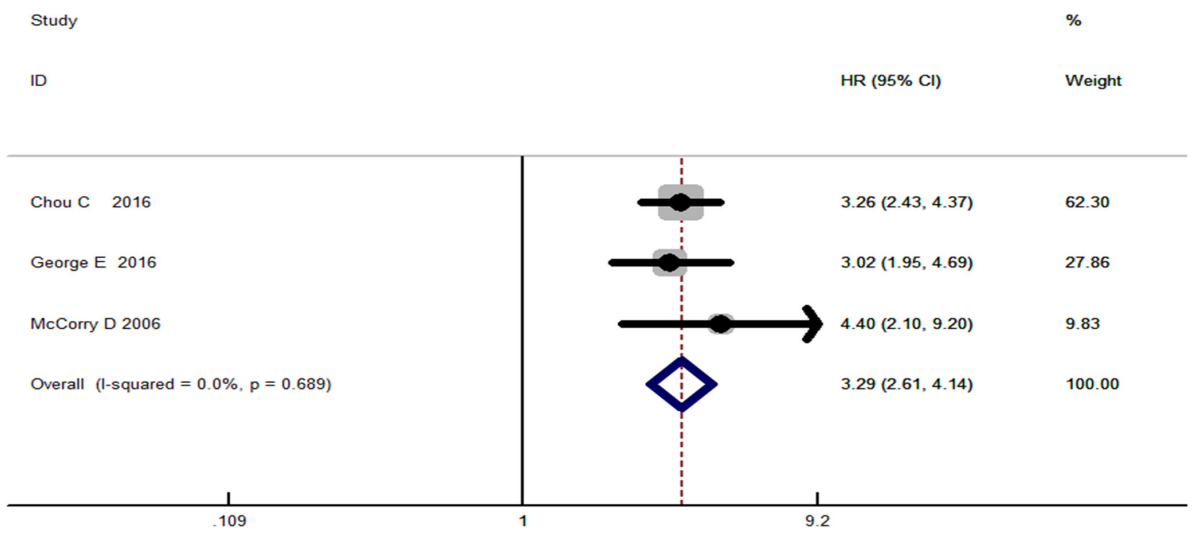

Figure 2. Pooled Hazard Ratio (HR) for the association between T1DM and epilepsy in 3 cohort studies.

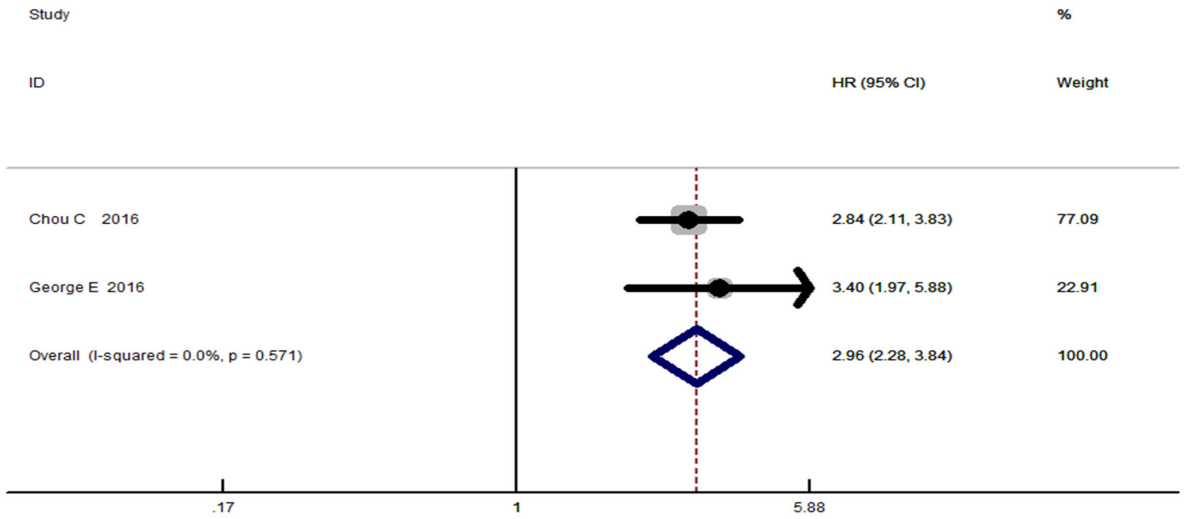

Figure 3. Pooled Hazard Ratio (HR) for the association between T1DM and epilepsy in patients younger than 18-years-old.

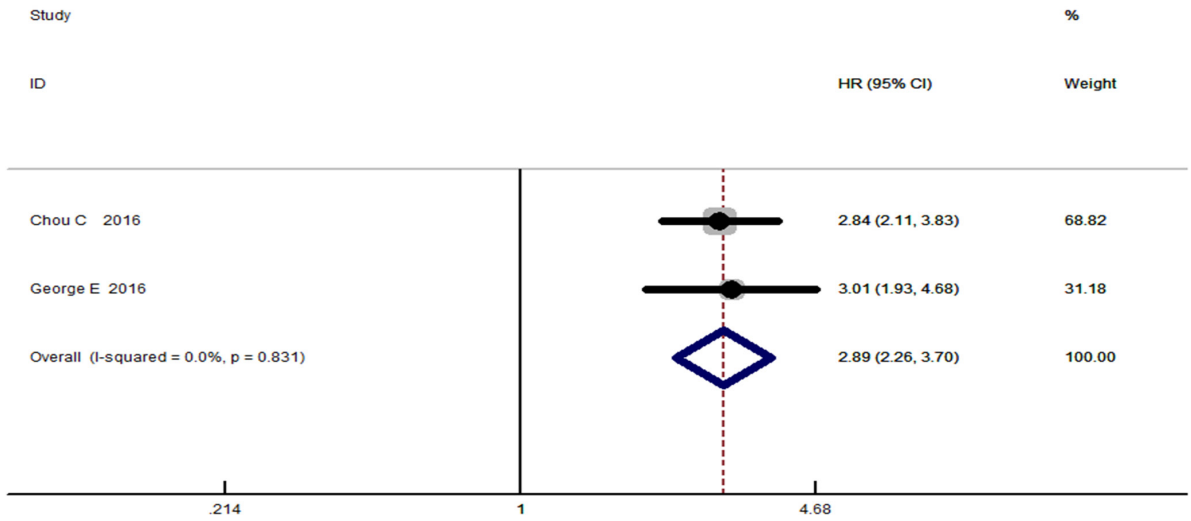

Figure 4. Pooled Hazard Ratio (HR) for the association between T1DM and epilepsy in patents adjusted for potential confounders.

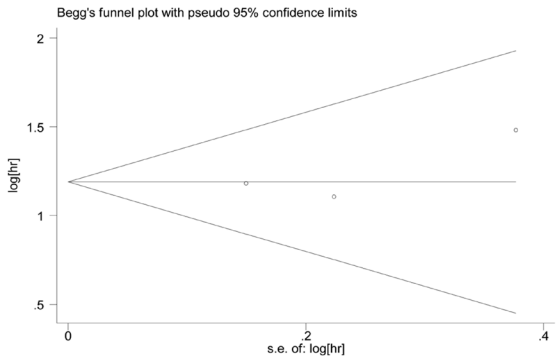

Figure 5. Funnel plot of studies evaluating the association between T1DM and epilepsy. Begg's regression asymmetry test (p = 0.526).

reports have found that approximately 15% of the diabetic patients complicated with seizures also suffered local brain damage under head computed tomography scanning. Seizures may be the result of T1DM patients' cerebrovascular complications that were commonly associated with varied types of brain damage (*6*). A possible relationship between cerebral infarction caused by diabetes mellitus and partial epilepsy has been also noted by Schomer *et al*. (*16*). Diabetes mellitus can result in pathological capillary changes, leading to neurological complications, such as epilepsy (*5*). The frequency that hypoglycemia occurred in diabetes mellitus was closely connected with the frequency of seizures (*17*). Seizures would gradually disappear when metabolic factors, such as hypoglycemia were removed. A case report found that transient hypoglycemia was caused by insulin administration and later presented with a focal seizure (*18*). The above indicated that metabolic disorders result from diabetes mellitus were closely related to epilepsy risk. Most diabetic mellitus patients complicated with seizures presented neither evident metabolic abnormalities nor serious brain damage, according to CT and MRI scanning, which indicated that there were perhaps some potential unknown pathogenesis that resulted in seizures. Previous study found that IER3IP1 mutations were the key factors, which account for the pathogenesis of early onset diabetes mellitus and infantile epilepsy. Gene mutation might act as a vital role in the pathogenesis of diabetic infantile epilepsy.

In addition, in this study, we found that T1DM patients younger than 18-years-old were also associated with an increased risk of developing epilepsy. Previous studies have demonstrated that young age, early onset and hypoglycemia can result in electroencephalographic abnormalities (*19*). The study revealed that patients who suffered electroencephalographic abnormalities were younger and had an earlier onset of diabetes (*6*). However, our meta-analysis also has limitations. However, even though we performed a systematic literature search only three studies were included. Besides, some negative studies failed to provide specific data for further analysis, which perhaps resulted in potential publication bias. Second, because of only three studies included, study quality assessment was not performed.

In conclusion, our meta-analysis indicated that patients with type 1 diabetes mellitus were associated with a higher incidence of increased risk for epilepsy compared to those without type 1 diabetes mellitus. The associations remained unchanged even when adjusted for potential confounders and in T1DM patients younger than 18-years-old. The specific mechanisms of the link between type 1 diabetes mellitus and epilepsy remained unclear. The causative factors require further investigation in future studies with a larger sample size.

References

1. Dafoulas GE, Toulis KA, McCorry D, Kumarendran B, Thomas GN, Willis BH, Gokhale K, Gkoutos G, Narendran P, Nirantharakumar K. Type 1 diabetes mellitus and risk of incident epilepsy: A population-based, open-cohort study. Diabetologia. 2017; 60:258-261.
2. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. Lancet. 2014; 383:69-82.
3. Zhao Y, Ye W, Boye KS, Holcombe JH, Swindle R. Healthcare charges and utilization associated with diabetic neuropathy: Impact of Type 1 diabetes and presence of other diabetes-related complications and comorbidities. Diabet Med. 2009; 26:61-69.
4. Ottman R, Lipton RB, Ettinger AB, Cramer JA, Reed ML, Morrison A, Wan GJ. Comorbidities of epilepsy: Results from the Epilepsy Comorbidities and Health (EPIC) survey. Epilepsia. 2011; 52:308-315.
5. Yun C, Xuefeng W. Association between seizures and diabetes mellitus: A comprehensive review of literature. Curr Diabetes Rev. 2013; 9:350-354.
6. Chou IC, Wang CH, Lin WD, Tsai FJ, Lin CC, Kao CH. Risk of epilepsy in type 1 diabetes mellitus: A population-based cohort study. Diabetologia. 2016; 59:1196-1203.
7. Ramakrishnan R, Appleton R. Study of prevalence of epilepsy in children with type 1 diabetes mellitus. Seizure. 2012; 21:292-294.
8. O'Connell MA, Harvey AS, Mackay MT, Cameron FJ. Does epilepsy occur more frequently in children with Type 1 diabetes? J Paediatr Child Health. 2008; 44:586-589.
9. Mancardi MM, Striano P, Giannattasio A, Baglietto MG, Errichiello L, Zara F, Prato G, Minuto N, Veneselli E, Lorini R, D'Annunzio G. Type 1 diabetes and epilepsy: More than a casual association? Epilepsia. 2010; 51:320-321.
10. Begg CB, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. Biometrics. 1994; 50:1088-1101.
11. Sander JW, Novy J, Keezer MR. The intriguing relationship between epilepsy and type 1 diabetes mellitus. Diabetologia. 2016; 59:1569-1570.
12. Keezer MR, Novy J, Sander JW. Type 1 diabetes mellitus in people with pharmacoresistant epilepsy: Prevalence and clinical characteristics. Epilepsy Res. 2015; 115:55-57.
13. Striano P, Errichiello L, Striano S. Autoantibodies to

glutamic acid decarboxylase in patients with epilepsy: What is their clinical relevance? Epilepsy Behav. 2011; 20:145.
14. Caietta E, Halbert C, Lepine A, Khammar A, Cano A, Gavaret M, Mancini J, Villeneuve N, Chabrol B, Simonin G, Reynaud R, Milh M. Association of type 1 diabetes mellitus and epilepsy in children. A cohort of 10 cases. Arch Pediatr. 2012; 19:9-16.
15. Cossette P, Liu L, Brisebois K, Dong H, Lortie A, Vanasse M, Saint-Hilaire JM, Carmant L, Verner A, Lu WY, Wang YT, Rouleau GA. Mutation of GABRA1 in an autosomal dominant form of juvenile myoclonic epilepsy. Nat Genet. 2002; 31:184-189.
16. Schomer DL. Focal status epilepticus and epilepsia partialis continua in adults and children. Epilepsia. 1993; 34 (Suppl 1):S29-36.
17. Lahat E, Barr J, Bistritzer T. Focal epileptic episodes associated with hypoglycemia in children with diabetes. Clin Neurol Neurosurg. 1995; 97:314-316.
18. Lapenta L, Di Bonaventura C, Fattouch J, Bonini F, Petrucci S, Gagliardi S, Casciato S, Manfredi M, Prencipe M, Giallonardo AT. Focal epileptic seizure induced by transient hypoglycaemia in insulin-treated diabetes. Epileptic Disord. 2010; 12:84-87.
19. Soltesz G, Acsadi G. Association between diabetes, severe hypoglycaemia, and electroencephalographic abnormalities. Arch Dis Child. 1989; 64:992-996.

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Brief Report

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Intratumor dihydropyrimidine dehydrogenase mRNA expression levels are decreased in extramammary Paget's disease

Yuki Nakamura, Ikko Kajihara*, Saori Yamada-Kanazawa, Saki Maeda-Otsuka, Takamitsu Johno, Jun Aoi, Toshikatsu Igata, Shinichi Masuguchi, Satoshi Fukushima, Masatoshi Jinnin, Hironobu Ihn

Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Honjo, Kumamoto, Japan.

Summary **S-1, a 5-fluorouracil (5-FU)-based anti-cancer agent, is an important drug for treating metastatic extramammary Paget's disease (EMPD). Although intratumor expression levels of 5-FU metabolism enzymes have been studied widely in many solid tumors, no studies have examined on the expression levels of thymidylate synthase (TS), orotate phosphoribosyl-transferase (OPRT) or dihydropyrimidine dehydrogenase (DPD) in skin cancers. The aim of this study was to estimate the intratumoral mRNA expression levels of these genes in EMPD by real time PCR. Intratumoral DPD mRNA levels were decreased in EMPD compared to those in normal skin, but its intratumoral DPD mRNA expression levels were not correlated with clinical manifestations. Intratumoral DPD mRNA levels were positively correlated with OPRT mRNA levels in EMPD. Based on these results, low expression of intratumoral DPD mRNA in EMPD may contribute to the pathogenesis of this disease.**

Keywords: S-1, extramammary Paget's disease, mRNA, real time PCR, thymidylate synthase, orotate phosphoribosyl-transferase, dihydropyrimidine dehydrogenase

1. Introduction

Extramammary Paget's disease (EMPD) is a rare skin cancer that shows erosive erythema and nodules in pubic or axillary lesions. Although the prognosis of EMPD with distant metastasis is poor, S-1 monotherapy (*1*) or S-1/docetaxel therapy (*2,3*) is an effective treatment. In cutaneous malignancy except for EMPD, S-1 based chemotherapy has also been reported to be a promising treatment for advanced squamous cell carcinoma (*4*) and angiosarcoma (*5*).

S-1 is an oral anti-tumor drug containing tegafur, potassium oxonate and 5-chloro-2,4-dihydroxypyrimidine (CDHP) and tegafur is a prodrug of 5-fluorouracil (5-FU) (*6*). The main enzymes responsible for the effect of S-1 are thymidylate synthase (TS), orotate phosphoribosyl-transferase (OPRT) and dihydropyrimidine dehydrogenase (DPD) (*7*). Fluorodeoxyuridine monophosphate, a 5-FU metabolite, inhibits TS which is responsible the DNA synthesis (*8*). OPRT converts 5-FU into 5-fluorouridine monophosphate, leading to inhibition of RNA synthesis (*9*). CDHP inhibits DPD which plays an important role in the inactivation of 5-FU (*6*).

The intratumor expression levels of these enzymes have been investigated in many solid tumors. TS and DPD activity in gastric and non-small lung cancer tissues are higher than those in normal tissues (*10*). In metastatic colon-rectal cancer, high TS and DPD mRNA expression levels in cancer tissues are negatively correlated with survival time (*11,12*). In metastatic gastric cancer patients, low TS and DPD mRNA expression levels were found associated with good response to S-1 (*13*).

However, to our knowledge, there have been no studies on the TS, DPD and OPRT expression levels in skin cancer. The aim of this study was to evaluate the mRNA expression levels of TS, DPD and OPRT in EMPD.

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**Address correspondence to:*
Dr. Ikko Kajihara, Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, 860-8556, Japan.
E-mail: kajiderma@gmail.com

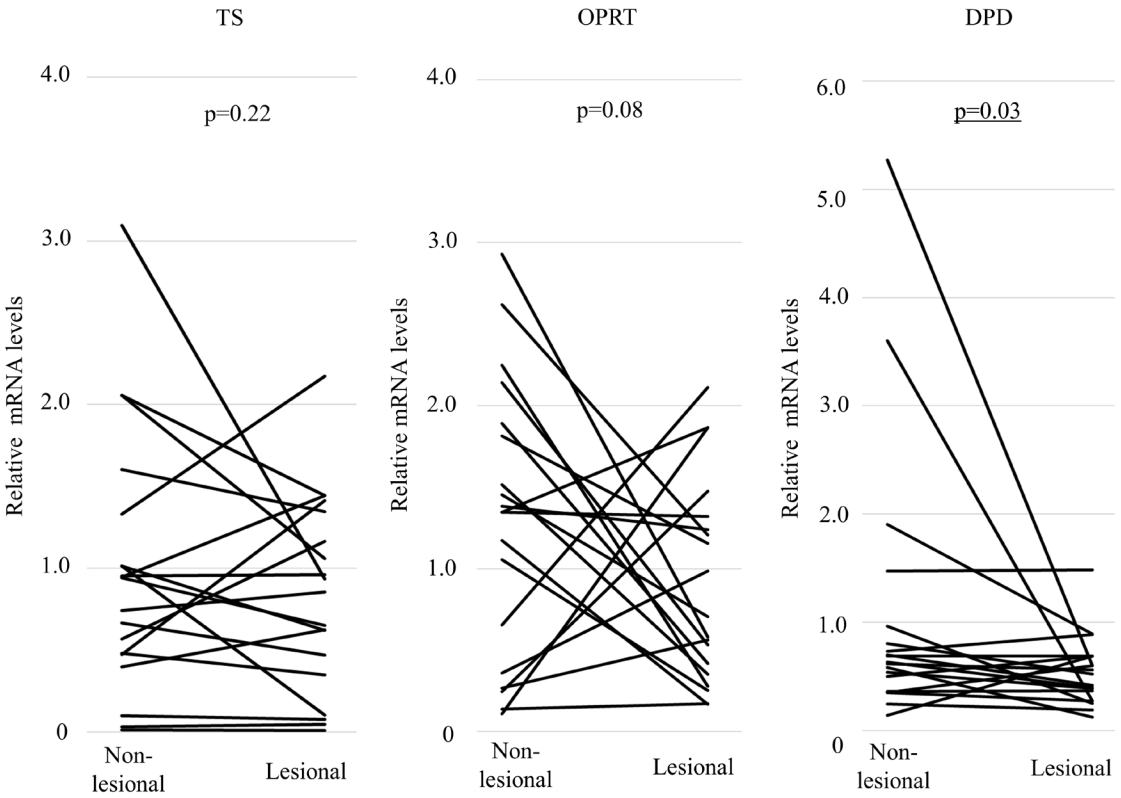

Figure 1. Relative intratumoral TS, OPRT and DPD mRNA expression levels in EMPD compared to those in normal tissues. TS: thymidylate synthase, OPRT: orotate phosphoribosyl-transferase, DPD: dihydropyrimidine dehydrogenase (DPD), EMPD: extramammary Paget's disease.

2. Materials and Methods

2.1. *Patients*

All patients were diagnosed with EMPD at Kumamoto University Hospital between November 2008 and August 2014. Eligible patients fulfilled the following criteria: histological diagnosis of extramammary Paget's disease, enforcement of mapping biopsy using punch biopsy before operation and sufficient tissue available in paraffin blocks for the assessments by real time polymerase chain reaction (PCR). Lesional or non-lesional skin was assessed by hematoxylin and eosin staining. Institutional review board approval and written informed consent for this study were obtained according to the Declaration of Helsinki.

2.2. *RNA isolation from tissue, cDNA synthesis and real time PCR analysis*

RNA was isolated from paraffin sections of skin samples using the RNeasy FFPE kit (Qiagen, Hilden, Germany). cDNA was synthesized using the first-strand cDNA using the RT^2 First Strand Kit (SABiosciences, Frederick, MD, USA). Quantitative real-time PCR was performed as previously described (*14*). Primer sets for TS, OPRT, DPD and GAPDH were obtained from SABiosciences. DNA was amplified for 50 cycles of denaturation for 15 seconds at 95°C and 35 seconds at 55°C, and annealing for 30 seconds at 72°C. Each transcript level was normalized to that of GAPDH.

2.3. *Statistical analysis*

Statistical analyses were performed using the Wilcoxon signed-rank test to compare matched mRNA expression levels in lesional and non-lesional skin. Correlations were assessed according to Fisher's correlation coefficient. A *p*-value of < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. *DPD mRNA levels in tumor tissue were decreased compared to in normal skin*

We evaluated the mRNA expression levels of 5-FU metabolism in EMPD using real time PCR. Although there were no differences in the TS and OPRT mRNA levels between paired tumor and normal sections, DPD mRNA levels in tumors were significantly lower than (less than 50%) those in non-lesional skin (Figure 1). Next, we examined the correlations between up/down-regulated DPD mRNA levels in tumor tissues and clinical manifestations (sex, age, degree of invasiveness and dying of EMPD). Intratumoral DPD mRNA expression levels in EMPD were not correlated with

Table 1. Correlation of DPD mRNA expression levels and clinical features

DPD mRNA	< 1 (n = 12)	≥ 1 (n = 7)	p-values
Age	79.7 ± 8.9	69.4 ± 6.2	0.06
Sex			
Male	9	4	0.62
Female	3	3	
Degree of invasiveness			
in situ	11	5	0.52
microinvasion/carcinoma	1	2	
Death from cancer	1	1	1.00

Relative DPD mRNA levels in EMPD tissues were lower (< 1) or higher (≥ 1) than those in normal tissues.

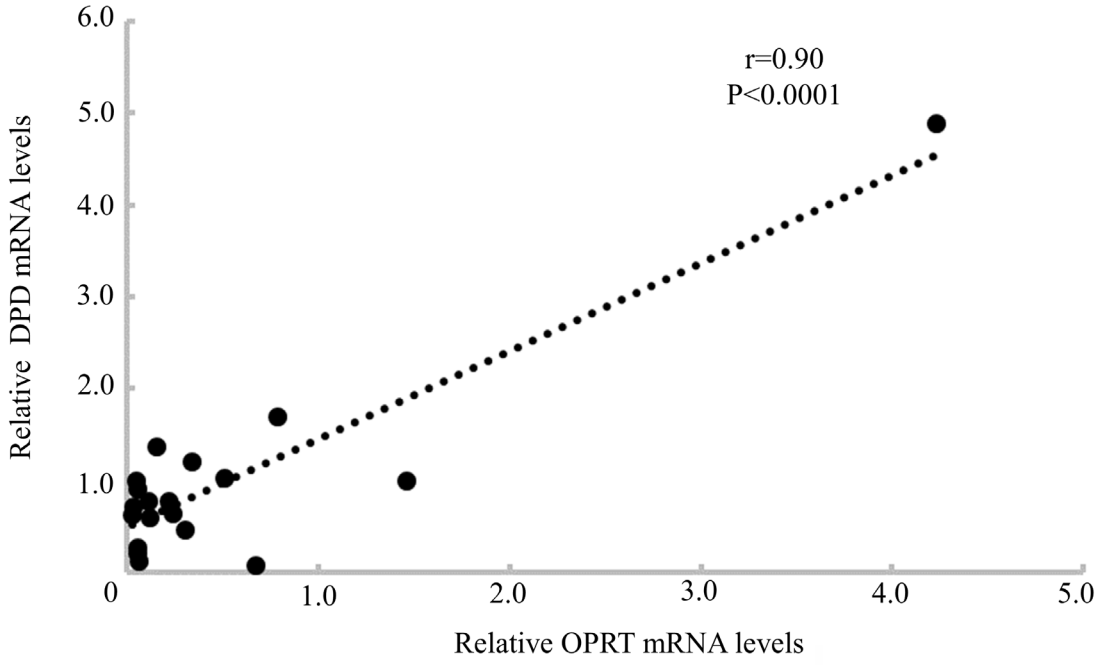

Figure 2. Correlation between intratumoral OPRT and DPD mRNA expression levels in EMPD.

any clinical information (Table 1).

3.2. *Intratumoral DPD mRNA levels positively correlated with OPRT mRNA levels*

We analyzed the relationship among intratumoral TS/OPRT/DPD mRNA expression levels compared to those in non-lesional tissues in EMPD. Although there were no differences in the correlation between TS/DPD (r < 0.01) or TS/OPRT (r = 0.05) mRNA levels, there was statistical correlation between OPRT and DPD mRNA expression levels (r = 0.90, p < 0.0001) (Figure 2).

We have investigated the intratumoral mRNA expression levels of 5-FU metabolism in EMPD and revealed three major findings. First, intratumoral DPD mRNA expression levels in EMPD were significantly lower compared to those in normal skin. Although the correlation between expression levels of 5-FU metabolism and the clinical efficacy of S-1 based therapy has been investigated, few studies have compared these expression levels in paired lesional and non-lesional tissues. Intratumoral DPD mRNA levels in colorectal cancers are higher compared with normal tissues, while those in gastric cancers are lower (*15*). This suggests that the mRNA expression levels of 5-FU metabolism differ in different for various types of malignant tumors.

Second, intratumoral DPD mRNA expression levels were not correlated with clinical features including prognosis. This result may be because of the small sample size. Using immunohistochemistry, it was determined that patients with DPD-positive tumors have significantly poorer prognosis than those with DPD-negative tumors in breast cancer (*16*). In several studies, lower intratumoral DPD mRNA levels were found to be correlated with good response to 5-FU (*17,18*). Additionally, in non-small cell lung cancer, low DPD protein expression level was correlated with longer survival and positive response to S-1/carboplatin therapy (*19*). There is no report about the connection between intratumoral DPD mRNA expression levels

and therapeutic response to S-1 in EMPD. To clarify this important subject, the accumulation of clinical case study is necessary. Taken together, a large sample size may confirm that EMPD is associated with good prognosis and efficacy following 5-FU-based chemotherapy because intratumoral DPD mRNA levels are decreased in EMPD.

Finally, DPD mRNA levels were positively correlated with OPRT mRNA levels although intratumoral OPRT mRNA expression levels were not increased. This result may be an outlier, with remarkably increased DPD and OPRT levels. In large-scale population analysis, the DPD/OPRT ratio varies in several cancers (*15*). Further investigations are needed to be clarify this.

In conclusion, we found that intratumoral DPD mRNA levels are overexpressed in EMPD. However, our results are limited because of this retrospective study evaluated a small sample size.

Acknowledgements

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References

1. Mikoshiba Y, Uhara H, Kubo H, Okuyama R. S-1 induced a durable response in metastatic extramammary Paget's disease. J Dermatol. 2013; 40:664-665.
2. Matsushita S, Yonekura K, Mera K, Kawai K, Kanekura T. Successful treatment of metastatic extramammary Paget's disease with S-1 and docetaxel combination chemotherapy. J Dermatol. 2011; 38:996-998.
3. Egashira S, Kajihara I, Kanemaru H, Uemura-Kiyohara M, Yamada-Kanazawa S, Nakahara S, Nagamoto E, Fukushima S, Jinnin M, Inoue Y, Ihn H. Achieved good response of S-1 and docetaxel combination chemotherapy in two patients with metastatic extramammary Paget's disease. J Dermatol. 2017; 44:e103-e104.
4. Teramoto Y, Nakamura Y, Yamada K, Yamamoto A. Oral S-1 in advanced cutaneous squamous cell carcinoma. J Dermatol. 2014; 41:494-497.
5. Kajihara I, Kanemaru H, Miyake T, Aoi J, Masuguchi S, Fukushima S, Jinnin M, Ihn H. Combination chemotherapy with S-1 and docetaxel for cutaneous angiosarcoma resistant to paclitaxel. Drug Discov Ther. 2015; 9:75-77.
6. Chhetri P, Giri A, Shakya S, Sapkota B, Pramod KC. Current Development of Anti-Cancer Drug S-1. J Clin Diagn Res. 2016; 10:XE01-XE05.
7. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: Mechanisms of action and clinical strategies. Nat Rev Cancer. 2003; 3:330-338.
8. Peters GJ, Backus HH, Freemantle S, *et al*. Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism. Biochim Biophys Acta. 2002; 1587:194-205.
9. West CM, Jones T, Price P. The potential of positron-emission tomography to study anticancer-drug resistance. Nat Rev Cancer. 2004; 4:457-469.
10. Fukushima M, Morita M, Ikeda K, Nagayama S. Population study of expression of thymidylate synthase and dihydropyrimidine dehydrogenase in patients with solid tumors. Int J Mol Med. 2003; 12:839-844.
11. Salonga D, Danenberg KD, Johnson M, Metzger R, Groshen S, Tsao-Wei DD, Lenz HJ, Leichman CG, Leichman L, Diasio RB, Danenberg PV. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. Clin Cancer Res. 2000; 6:1322-1327.
12. Ichikawa W, Uetake H, Shirota Y, Yamada H, Nishi N, Nihei Z, Sugihara K, Hirayama R. Combination of dihydropyrimidine dehydrogenase and thymidylate synthase gene expressions in primary tumors as predictive parameters for the efficacy of fluoropyrimidine-based chemotherapy for metastatic colorectal cancer. Clin Cancer Res. 2003; 9:786-791.
13. Ichikawa W, Takahashi T, Suto K, Shirota Y, Nihei Z, Shimizu M, Sasaki Y, Hirayama R. Simple combinations of 5-FU pathway genes predict the outcome of metastatic gastric cancer patients treated by S-1. Int J Cancer. 2006; 119:1927-1933.
14. Kusumoto S, Kajihara I, Nagamoto E, Makino K, Ichihara A, Aoi J, Johno T, Makino T, Fukushima S, Jinnin M, Ihn H. Increased CCL22 expression in psoriatic skin predicts a good response to infliximab therapy. Br J Dermatol. 2014; 171:1259-1261.
15. Fukui Y, Oka T, Nagayama S, Danenberg PV, Danenberg KD, Fukushima M. Thymidylate synthase, dihydropyrimidine dehydrogenase, orotate phosphoribosyltransferase mRNA and protein expression levels in solid tumors in large scale population analysis. Int J Mol Med. 2008; 22:709-716.
16. Horiguchi J, Takei H, Koibuchi Y, Iijima K, Ninomiya J, Uchida K, Ochiai R, Yoshida M, Yokoe T, Iino Y, Morishita Y. Prognostic significance of dihydropyrimidine dehydrogenase expression in breast cancer. Br J Cancer. 2002; 86:222-225.
17. Beck A, Etienne MC, Cheradame S, Fischel JL, Formento P, Renee N, Milano G. A role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumour sensitivity to fluorouracil. Eur J Cancer. 1994; 30A:1517-1522.
18. Ishikawa Y, Kubota T, Otani Y, Watanabe M, Teramoto T, Kumai K, Kitajima M, Takechi T, Okabe H, Fukushima M. Dihydropyrimidine dehydrogenase activity and messenger RNA level may be related to the antitumor effect of 5-fluorouracil on human tumor xenografts in nude mice. Clin Cancer Res. 1999; 5:883-889.
19. Takeda M, Okamoto I, Hirabayashi N, Kitano M, Nakagawa K. Thymidylate synthase and dihydropyrimidine dehydrogenase expression levels are associated with response to S-1 plus carboplatin in advanced non-small cell lung cancer. Lung Cancer. 2011; 73:103-109.

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Brief Report

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Properties of induced antimicrobial activity in *Musca domestica* larvae

Kiyoshi Kawasaki*, Minako Andoh

Faculty of Pharmaceutical Sciences, Doshisha Women's College, Kyoto, Japan.

Summary **Insects produce antimicrobial molecules that contribute to their innate immune responses to eliminate invading microorganisms. To explore the potential utility of these antimicrobial molecules, we focused on larvae of the house fly *Musca domestica*, which is an efficient processor of organic waste and a good resource of protein and oil for animal feeding. The induction of hemagglutinating activity, which is usually accompanied by activation of innate immune responses in fly larvae, was observed in the hemolymph following needle injury. Hemolymph collected from injured larvae demonstrated potent antimicrobial activities against both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Furthermore, the antimicrobial activity was significantly retained in hemolymph after heat-treatments, suggesting that pasteurization of animal feed prepared from fly larvae would be a useful sterilization method. These observations indicate that injured *Musca domestica* larvae are a source of antimicrobial agents, and highlight the utility of preparing animal feed from these larvae.**

Keywords: House fly, hemolymph, antimicrobial agents, innate immunity

1. Introduction

Insects respond to bacterial infection using innate immunity, consisting of germline-encoded sensing and effector molecules, among which antimicrobial peptides are prominent (*1*). Recognition of microorganisms induces synthesis of potent antimicrobial peptides in the fat body. Secretion of antimicrobial peptides into the hemolymph plays a role in inhibiting the growth of invading microorganisms. Over 150 insect antimicrobial peptides have been purified or identified, and they have potential applications in medicine and agriculture (*2*).

Antimicrobial peptides contain a region of positively-charged amino acids that specifically bind to negatively-charged surface molecules, such as bacterial lipopolysaccharide (*3-5*). This interaction disrupts the bacterial membrane and leads to cell lysis and/or cell death (*6-8*). Antimicrobial peptides were biochemically identified in the hemolymph of insects such as *Sarcophaga peregrina* (flesh fly) larvae and silkworm larvae (*9-13*). Furthermore, the excretions or secretions of medicinal maggots of the blowfly *Lucilia sericata* contain antimicrobial peptides in the absence of injury or invading bacteria (*14*). These findings indicate that insects express inducible and/or constitutive antimicrobial peptides.

Antimicrobial peptides are considered to be a novel class of antibiotics because they exhibit broad-spectrum antimicrobial activities, and they are not likely to induce resistance (*15*). Chemically synthesized or modified antimicrobial peptides were developed based on amino acid sequences of insect antimicrobial peptides. For example, the undecapeptide KLKLLLLLKLK-NH_2 was developed by modifying the primary structure of an antimicrobial peptide of *Sarcophaga peregrina*, Sapecin B (*16,17*). KLKLLLLLKLK-NH_2 exhibits broad-spectrum antimicrobial activities against Gram-positive bacteria, Gram-negative bacteria, and fungi (*17*). It also enhances mammalian immune responses, and its potential usefulness as an adjuvant has been previously demonstrated (*18-20*). Furthermore, KLKLLLLLKLK-NH_2 synthesized using D-amino acids displays higher antimicrobial activity than its L-form (*21,22*). These observations highlight the importance of native

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**Address correspondence to:*
Dr. Kiyoshi Kawasaki, Faculty of Pharmaceutical Sciences, Doshisha Women's College, Kodo, Kyotanabe, Kyoto 610-0395, Japan.
E-mail: kkawasak@dwc.doshisha.ac.jp

antimicrobial peptides as a resource for antimicrobial agents as well as immune modulators.

The house fly (*Musca domestica*) is a well-known carrier of pathogens that affect human and animal health. However, *Musca domestica* larvae are efficient processors of organic waste, and are a good source of protein and oil for animal feed (*23,24*). Animal feed prepared from *Musca domestica* larvae, in which antimicrobial peptide were induced, may be beneficial to overall animal health because it is well established that the addition of antimicrobial supplements to animal feed increases animal weight (*25*). Therefore, we sought to examine whether injury can induce antimicrobial activities in *Musca domestica* larvae. Induced antimicrobial molecules are useful sources of antimicrobial agents and valuable supplements in animal feeding.

2. Materials and Methods

2.1. *Fly larvae, bacteria, and reagents*

Musca domestica larvae were provided by E's Inc. (Tokyo, Japan). *Staphylococcus aureus* (NBRC100910), *Staphylococcus epidermidis* (NBRC100911), and *Pseudomonas aeruginosa* (NBRC12689) were purchased from National Institute of Technology and Evaluation (Kisarazu, Chiba, Japan). *Escherichia coli* XL1-blue was purchased from Stratagene (Agilent Technologies, Santa Clara, California, USA). Mannitol salt agar and cetrimide agar were purchased from Nissui Pharmaceutical (Tokyo, Japan). Mueller-Hinton II broth was purchased from Becton Dickinson (Franklin Lakes, New Jersey, USA). LB broth was purchased from Nacalai tesque (Kyoto, Japan). Agar for bacterial culture was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. *Injury of larvae and collection of hemolymph*

Musca domestica larvae were anesthetized by incubation on ice prior to being pricked once with a 0.30 × 12 mm needle (Dentronics, Tokyo, Japan). The injured larvae were incubated in an insect saline solution (130 mM NaCl, 5 mM KCl, 1mM $CaCl_2$) at 30°C. After 24 h, the anterior tip of the larva was cut off using fine scissors and the hemolymph was collected in a tube on ice. Approximately 500 µL of hemolymph was collected from 500 larvae. The hemolymph was centrifuged for 10 min at 100× g to remove hemocytes, and the supernatant was stored at –30°C.

2.3. *Assay for antimicrobial activity*

Bacteria were grown in LB broth and log-phase cells (OD_{600} = 0.15-0.3) were used for the analysis. The bacteria in growth medium (2 µL) was mixed with 18 µL of hemolymph sample or Mueller-Hinton II broth. To evaluate the effect of pH, 16 µL of the hemolymph sample or Mueller-Hinton II broth were mixed with 2 µL of 0.2 M phosphate buffer or 0.2 M phosphate/0.1 M citrate buffer for pH adjustment, and the pH adjusted samples were mixed with 2 µL of the bacterial suspension. The bacteria/hemolymph assay mixtures were incubated at 30°C for 1 h, and then serially diluted with Mueller-Hinton II broth. The diluted assay mixtures (100 µL) were plated onto agar plates and incubated at the appropriate temperature (30 or 37°C) for 1 or 2 days. The appropriate selective medium was used for each bacterial strain: mannitol salt agar (for *S. aureus* and *S. epidermidis*), LB agar containing 50 µg/mL tetracycline (for *E. coli* XL1-blue), and cetrimide agar (for *P. aeruginosa*). After cultivation, the colony forming units (CFU) in the bacteria/hemolymph assay mixtures were determined. Means of CFU were determined from triplicate or duplicate agar plates, and standard deviations (SD) were determined from triplicate plates.

2.4. *Assay for hemagglutinating activity*

Commercially available rabbit red blood cells were washed twice with 5 volumes of buffered insect saline (10 mM Tris/HCl (pH 7.9) containing 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$), and suspended in 10 volumes of phosphate buffered saline. Hemagglutinating activity was measured using serial two-fold dilutions of hemolymph in microtiter V-plates, and activity unit was determined as $titer^{-1}$. Each well contained a 50 µL suspension of red blood cells and 50 µL of hemolymph diluted with buffered insect saline. Agglutination was determined as previously described (*26*).

3. Results and Discussion

3.1. *Induction of antimicrobial activity in the hemolymph of Musca domestica larvae by injury*

We collected hemolymph from *Musca domestica* larvae injured with a needle and from larvae which were not injured as an experimental control. Initially, we examined the induction of hemagglutinating activity using rabbit red blood cells because previous reports indicated that hemagglutinating activity occurred concomitantly to the induction of innate immune responses in *Sarcophaga peregrina* larvae (*9,26*). Hemolymph collected from injured larvae usually exhibited 4- to 8-fold higher hemagglutinating activity than those from uninjured larvae, suggesting that needle injury induced innate immune responses in *Musca domestica* larvae.

Antimicrobial activities of the hemolymph were examined against several bacterial species including *S. aureus*, *S. epidermidis*, and *P. aeruginosa*. They were selected based on the availability of selective growth medium, which supports the growth of the desired bacteria while repressing the growth of environmental

bacteria. As shown in Figure 1, CFUs of *S. aureus*, *S. epidermidis*, and *P. aeruginosa* were reduced by treatment with hemolymph collected from injured larvae. However, CFUs were not reduced by treatment with hemolymph collected from larvae without injury. These observations indicate that antimicrobial activities against *S. aureus*, *S. epidermidis*, and *P. aeruginosa were* induced in the hemolymph of injured *Musca domestica* larvae.

3.2. *Characterization of the induced antimicrobial activity of hemolymph*

Charge-based interactions between bacterial membranes and antimicrobial peptides are essential for their antimicrobial activity, and it is likely that pH plays a critical role. Therefore, antimicrobial activity of hemolymph from injured larvae was examined across various pH conditions. As shown in Figure 2A, antimicrobial activity against *S. epidermidis* was increased as pH decreased, indicating that acidic environments are desirable for optimal antimicrobial effects. Moreover, antimicrobial activity against *E. coli* was observed at pH 5.8 but was not apparent at pH 7.8 (Figure 2B). These observations indicate that pH is an important factor for induced antimicrobial activity in *Musca domestica* larvae.

Stability of antimicrobial activity following heat-treatment was examined. Hemolymph collected from injured larvae was incubated at 65 or 75°C for 10 min. After heat-treatment, antimicrobial activity against *P. aeruginosa* was examined. Antimicrobial activity was retained in hemolymph samples subjected to heat-

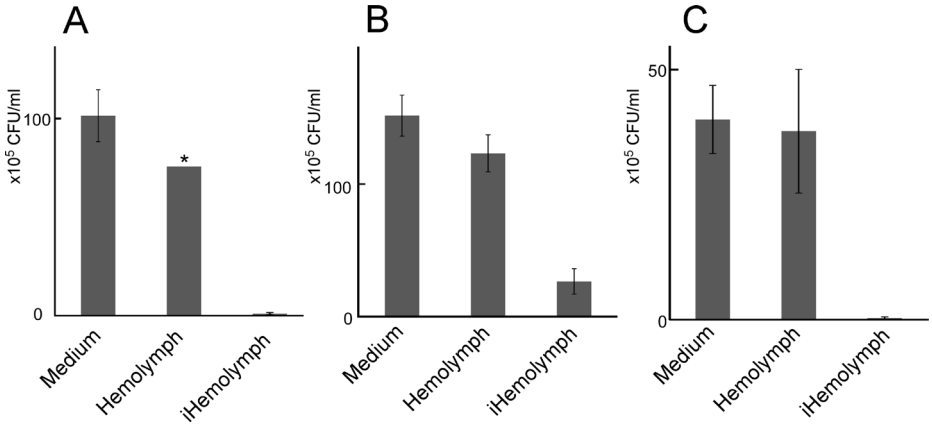

Figure 1. Antimicrobial activities were induced in hemolymph of Musca domestica larvae by needle injury. *S. aureus* **(A),** *S. epidermidis* **(B)**, and *P. aeruginosa* **(C)** were used to detect antimicrobial activities. The bacteria were combined with Mueller-Hinton II medium (Medium), hemolymph collected from larvae without injury (Hemolymph) or hemolymph collected from injured larvae (iHemolymph). The CFUs of the assay mixtures were determined, and the bars indicate the mean from duplicate plates (*) or the means ± SD from triplicate plates.

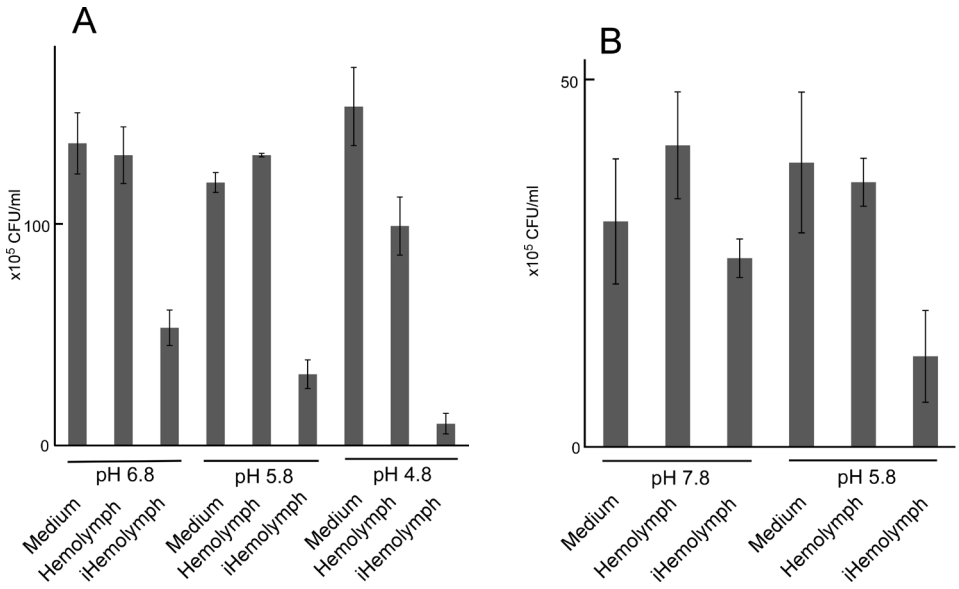

Figure 2. Effect of pH on the antimicrobial activity of hemolymph from injured larvae. *S. aureus* **(A)** and *E. coli* **(B)** were used for the analysis of antimicrobial activities. Values of pH in the assay mixtures were adjusted with phosphate/citrate buffer **(A)** and phosphate buffer **(B)**. CFUs in the assay mixtures (means ± SD) were presented.

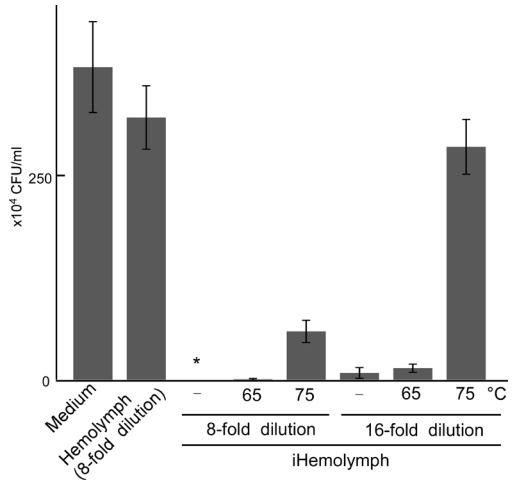

Figure 3. Heat-stability of antimicrobial activity of hemolymph from injured larvae. Diluted hemolymph in Mueller-Hinton II medium (18 μL of 8-fold and 16-fold) or Mueller-Hinton II medium alone were incubated at 65°C, 75°C, or on ice (–) for 10 min prior to combining with *P. aeruginosa*. CFUs in the assay mixtures (means ± SD) were presented. Asterisk (*) indicates that no colonies were detected (less than 8.3×10^3 CFU/mL).

treatment at 65°C (Figure 3). However, antimicrobial activity following heat-treatment at 75°C was only observed in 8-fold dilutions of hemolymph (Figure 3). Although these findings suggest that animal feed prepared from *Musca domestica* larvae should retain antimicrobial activity following pasteurization, future studies are needed to elucidate the optimal sterilization strategy.

In this study, we observed the induction of antimicrobial activity in *Musca domestica* larvae against several bacterial species following injury using a needle. Our current findings are consistent with previous reports on the upregulation of genes involved in innate immunity against invading bacteria in *Musca domestica* larvae (*27*). We speculate that the induction of antimicrobial activity was mediated by *imd* as well as *Toll* pathway, which were essential innate immune responses in *Drosophila* (*1*). The hemolymph collected from injured larvae contains various antimicrobial materials, including antimicrobial peptides, which may be ideal sources of antimicrobial agents. Furthermore, the antimicrobial properties of *Musca domestica* larvae make them a beneficial animal feed, because the addition of antimicrobial supplements to animal feed increases animal weight (*25*).

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Conflict of interest

K.K. is an advisor for E's Inc.

References

1. Hoffmann JA. The immune response of *Drosophila*. Nature. 2003; 426:33-38.
2. Yi HY, Chowdhury M, Huang YD, Yu XQ. Insect antimicrobial peptides and their applications. Appl Microbiol Biotechnol. 2014; 98:5807-5822.
3. Vaara M, Viljanen P. Binding of polymyxin B nonapeptide to gram-negative bacteria. Antimicrob Agents Chemother. 1985; 27:548-554.
4. Gunn JS, Lim KB, Krueger J, Kim K, Guo L, Hackett M, Miller SI. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol Microbiol. 1998; 27:1171-1182.
5. Kawasaki K, China K, Nishijima M. Release of the lipopolysaccharide deacylase PagL from latency compensates for a lack of lipopolysaccharide aminoarabinose modification-dependent resistance to the antimicrobial peptide polymyxin B in *Salmonella enterica*. J Bacteriol. 2007; 189:4911-4919.
6. Shai Y. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim Biophys Acta. 1999; 1462:55-70.
7. Matsuzaki K. Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. Biochim Biophys Acta. 1999; 1462:1-10.
8. Huang HW. Molecular mechanism of antimicrobial peptides: The origin of cooperativity. Biochim Biophys Acta. 2006; 1758:1292-1302.
9. Okada M, Natori S. Purification and characterization of an antibacterial protein from haemolymph of *Sarcophaga peregrina* (flesh-fly) larvae. Biochem J. 1983; 211:727-734.
10. Ando K, Okada M, Natori S. Purification of sarcotoxin II, antibacterial proteins of *Sarcophaga peregrina* (flesh fly) larvae. Biochemistry. 1987; 26:226-230.
11. Baba K, Okada M, Kawano T, Komano H, Natori S. Purification of sarcotoxin III, a new antibacterial protein of *Sarcophaga peregrina*. J Biochem. 1987; 102:69-74.
12. Faye I, Pye A, Rasmuson T, Boman HG, Boman IA. Insect immunity. 11. Simultaneous induction of antibacterial activity and selection synthesis of some hemolymph proteins in diapausing pupae of *Hyalophora cecropia* and *Samia cynthia*. Infect Immun. 1975; 12:1426-1438.
13. Miyashita A, Kizaki H, Kawasaki K, Sekimizu K, Kaito C. Primed immune responses to gram-negative peptidoglycans confer infection resistance in silkworms. J Biol Chem. 2014; 289:14412-14421.
14. Cerovsky V, Zdarek J, Fucik V, Monincova L, Voburka Z, Bem R. Lucifensin, the long-sought antimicrobial factor of medicinal maggots of the blowfly *Lucilia sericata*. Cell Mol Life Sci. 2010; 67:455-466.
15. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature. 2002; 415:389-395.
16. Matsuyama K, Natori S. Purification of three antibacterial proteins from the culture medium of NIH-Sape-4, an embryonic cell line of *Sarcophaga peregrina*. J Biol Chem. 1988; 263:17112-17116.
17. Yamada K, Natori S. Characterization of the antimicrobial peptide derived from sapecin B, an antibacterial protein of *Sarcophaga peregrina* (flesh fly). Biochem J. 1994; 298 Pt 3:623-628.
18. Okuyama-Nishida Y, Akiyama N, Sugimori G, Nomura K,

Ogawa K, Homma KJ, Sekimizu K, Tsujimoto M, Natori S. Prevention of death in bacterium-infected mice by a synthetic antimicrobial peptide, L5, through activation of host immunity. Antimicrob Agents Chemother. 2009; 53:2510-2516.
19. Fritz JH, Brunner S, Birnstiel ML, Buschle M, Gabain A, Mattner F, Zauner W. The artificial antimicrobial peptide KLKLLLLLKLK induces predominantly a TH2-type immune response to co-injected antigens. Vaccine. 2004; 22:3274-3284.
20. Schellack C, Prinz K, Egyed A, Fritz JH, Wittmann B, Ginzler M, Swatosch G, Zauner W, Kast C, Akira S, von Gabain A, Buschle M, Lingnau K. IC31, a novel adjuvant signaling via TLR9, induces potent cellular and humoral immune responses. Vaccine. 2006; 24:5461-5472.
21. Alvarez-Bravo J, Kurata S, Natori S. Novel synthetic antimicrobial peptides effective against methicillin-resistant Staphylococcus aureus. Biochem J. 1994; 302(Pt 2):535-538.
22. Manabe T, Kawasaki K. D-form KLKLLLLLKLK-NH_2 peptide exerts higher antimicrobial properties than its L-form counterpart via an association with bacterial cell wall components. Sci Rep. 2017; 7:43384.
23. Hussein M, Pillai VV, Goddard JM, Park HG, Kothapalli KS, Ross DA, Ketterings QM, Brenna JT, Milstein MB, Marquis H, Johnson PA, Nyrop JP, Selvaraj V. Sustainable production of housefly (*Musca domestica*) larvae as a protein-rich feed ingredient by utilizing cattle manure. PLoS One. 2017; 12:e0171708.
24. Niu Y, Zheng D, Yao B, Cai Z, Zhao Z, Wu S, Cong P, Yang D. A novel bioconversion for value-added products from food waste using *Musca domestica*. Waste Manag. 2017; 61:455-460.
25. Butaye P, Devriese LA, Haesebrouck F. Antimicrobial growth promoters used in animal feed: Effects of less well known antibiotics on gram-positive bacteria. Clin Microbiol Rev. 2003; 16:175-188.
26. Komano H, Mizuno D, Natori S. Purification of lectin induced in the hemolymph of *Sarcophaga peregrina* larvae on injury. J Biol Chem. 1980; 255:2919-2924.
27. Tang T, Li X, Yang X, Yu X, Wang J, Liu F, Huang D. Transcriptional response of *Musca domestica* larvae to bacterial infection. PLoS One. 2014; 9:e104867.

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Case Report

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Beta blocker and steroid therapy in the treatment of infantile hepatic hemangioendothelioma

Zeynep Canan Özdemir[1,*], Yeter Düzenli Kar[1], Nurullah Cihan Şöhret[2], Mahmut Kebapçı[3], Özcan Bör[1]

[1] *Division of Pediatric Hematology/Oncology, Department of Pediatrics, Eskişehir Osmangazi University Faculty of Medicine, Eskişehir, Turkey;*
[2] *Department of Pediatrics, Eskişehir Osmangazi University Faculty of Medicine, Eskişehir, Turkey;*
[3] *Department of Radiology, Eskişehir Osmangazi University Faculty of Medicine, Eskişehir, Turkey.*

Summary **Infantile hepatic hemangioendothelioma (IHHE) is the most common benign vascular liver tumor and typically occurs during the first 6 months of life. A 4-month-old male patient presented with abdominal distention. A physical examination revealed massive hepatomegaly. Liver enzyme levels were normal. The alpha fetoprotein level was 1,323 mg/dL (6-1,000). Abdominal magnetic resonance imaging (MRI) showed multiple, well-defined and hyperintense nodular lesions in the liver. MRI findings suggested IHHE. The thyroid stimulating hormone (TSH) level was high (177.2 µU/mL). He was started on sodium levothyroxine 50 µg daily. The patient has hypoxemia due to abdominal distention during the follow-up period. Oral methylprednisolone therapy was started at a dose of 2.5 mg/kg/dose, and propranolol at a dose of 1 mg/kg/dose, bid. Fifteen days later his TSH level remained elevated at 212.3 µU/mL despite repeatedly increasing the dose of levothyroxine up to 200 µg/daily. One month after the initial presentation, his TSH level was reduced to 11.28 µU/mL. We observed a marked improvement in abdominal distention and respiratory distress within 15 days and an average reduction of 50% in the lesion diameters after a month. Despite its benign nature, IHHE may lead to development of complications. Steroid and propranolol treatment may be useful in in the management of emergency complications.**

Keywords: Infantile hepatic hemangioendothelioma, methylprednisolone, propranolol

1. Introduction

Infantile hepatic hemangioendothelioma (IHHE) represents 12% of the childhood hepatic tumors. In addition, it is the most common tumor of the liver (*1-3*). Hepatic hemangiomas are thought to result from placental angioblasts (*4*). Approximately 85% of all cases are diagnosed within the first 6 months of life and they most commonly become symptomatic during this period (*5,6*). The most commonly finding is abdominal distention. Other findings include cardiac failure, skin hemangioma, respiratory failure, fulminant hepatic failure, consumption coagulopathy and abdominal compartment syndrome (*6-8*). Recent studies report that IHHE could be associated with hypothyroidism (*9-11*). While asymptomatic lesions spontaneously regress in years, symptomatic lesions require aggressive treatment since they have fatal risk (*1*). Therefore, accurate diagnosis of the disease and appropriate treatment is important. There is no standard therapeutic approach. Steroids and propranolol are the primary treatment choices. Surgery may be required in patients with complications and no response to medical treatment (*12-14*). This case is presented to emphasize that propranolol and methylprednisolone combination therapy may be effective in combating emergency complications.

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**Address correspondence to:*
Dr. Zeynep Canan Özdemir, Division of Pediatric Hematology/Oncology, Department of Pediatrics, Eskişehir Osmangazi University Faculty of Medicine, 26480, Eskişehir, Turkey.
E-mail: efecanan@yahoo.com

2. Case Report

A 4-month-old male patient was brought in with

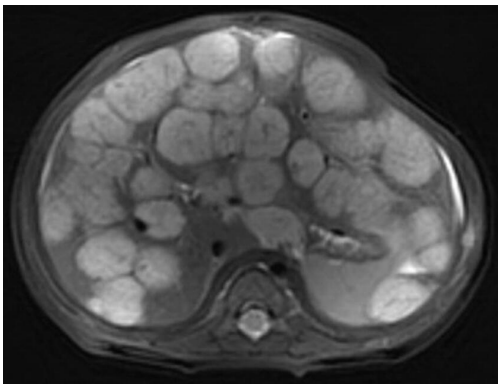

Figure 1. Before treatment T2-weighted axial MR image shows multiple hyperintense nodular lesions in the liver.

the complaint of abdominal distention. A physical examination revealed massive hepatomegaly. The liver was palpable 9 cm below the right costal margin. The liver extended towards the left of the midline and filled the splenic lodge and was palpable 5 cm below the left costal margin. The patient had a heart rate of 140 beats per minute, a blood pressure of 80/50 mmHg, and a respiratory rate of 32 breaths per minute. He had millimetric-sized hemangiomas on the trunk. Biochemistry investigation showed the following: ALT: 15 U/L, AST: 49 U/L, GGT: 495 U/L (8-61), ALP: 230 U/L (122-469), total bilirubin: 0.61 mg/dL, direct bilirubin: 0.08 mg/dL. Alpha-fetoprotein (AFP): 1,323 mg/dL (6-1,000), thyroid stimulant hormone (TSH): 177.2 μU/mL (0.73-8.35), free thyroxin (sT4): 1.29 ng/dL (0.92-1.99). Sodium levothyroxine treatment was initiated at a dose of 50 μg daily. The abdominal ultrasonography (USG) revealed an increased liver size (135 mm) and multiple hypoechoic lesions. Multiple nodular lesions covering the whole hepatic parenchyma were observed on abdominal dynamic magnetic resonance imaging (MRI) (Figure 1). The largest of these had a size of 35 × 20 mm with well defined and marked increased hyperintense vascularization on T2-weighted imaging. Doppler USG showed increased hepatic vascularization and hepatic arterial diameter. Diagnosis of infantile hepatic hemangioendothelioma (IHEE) was established based on the imaging findings. Since there was a high risk of bleeding because the tumor was of vascular origin, and USG showed increased vascularization, biopsy could not be performed. Respiratory distress developed during the follow-up period. Oxygen saturation decreased to 86% and oxygen therapy was required. Since the patient was symptomatic, oral methylprednisolone therapy was started at a dose of 2.5 mg/kg/dose, and propranolol at a dose of 1 mg/kg/dose, bid. TSH levels were 278.2 μU/mL and 212.3 μU/mL at 1 and 2 weeks after levothyroxine treatment, respectively. For this reason sodium levothyroxine dose gradually increased to 200 μg/day. On day 15 of treatment, a decrease in liver size

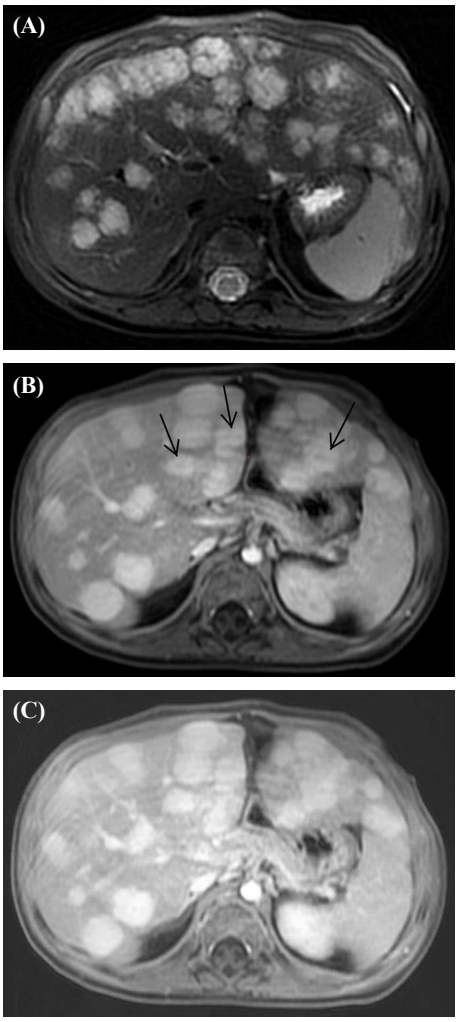

Figure 2. Follow-up T2-weighted axial MR image obtained after 9 months of therapy shows marked reduction number and size of lesions (A); after gadolinium contrast injection, there is peripheral enhancement in the arterial phase (B); and homogenous enhancement in the late phase (C).

began and the patient's additional oxygen requirement disappeared. The abdominal USG showed a decrease in the size of the largest lesion (22 × 17 mm), and the vertical length of the liver had decreased from 135 to 95 mm over the 1-month period. At that time, the TSH level was reduced to 11.28 μU/mL. The levothyroxine dose was then decreased to 75 μg daily and continued. At the third month of treatment, steroids were gradually reduced and discontinued, but propranolol therapy continued. A subsequent abdominal USG showed an increase in the size of the largest lesion (30 × 22 mm) and the vertical length of the liver increased from 95 to 124 mm in the fourth month; steroid treatment was then restarted at a dose of 0.5 mg/kg/dose, bid. The patient remained under steroid, propranolol and sodium levothyroxine treatment for nine months. At follow-up dynamic abdomen MRI showed a marked reduction number and size of all of the liver nodules (Figure 2A) which enhanced periphery post-contrast during early arterial phase (Figure 2B) with homogenous enhancement during the late phase (Figure 2C).

3. Discussion

The similarity of age between IHHE and hepatoblastomas at the time of diagnosis is interesting. Of all IHHE patients, 86% are diagnosed within the first 6 months and approximately 30-50% of patients with hepatoblastomas are diagnosed within the first year of life. Hepatoblastomas are the most common malignant liver tumor, representing 40-60% of all pediatric liver tumors (*2,15,16*). AFP is used for the differential diagnosis of pediatric liver tumors. AFP may increase up to 40,000 ng/mL at birth. After birth, it rapidly decreases; however, even at 6 months of age, it remains above adult values (*17*). In IHHE cases, high AFP values are not expected, but recent studies have shown that hepatocytes located nearby or trapped inside the tumor cells could be the source of the increased AFP levels (*15*). In our case, we considered a hepatoblastoma for a differential diagnosis; however, the AFP level was only slightly above the upper level. Ninety percent of hepatoblastoma tumors secrete a large amount of AFP due to the high level of hepatoblast cells they contain (*2,14*). Therefore, AFP levels are expected to be much higher.

Imaging methods are important in the diagnosis of liver tumors. Commonly practiced methods include tomography, MRI and ultrasound. Ultrasonographic characteristics of IHHE show variability. Well-defined hypoechoic lesions and abnormally large vascular structures are detected in the liver while color Doppler USG can define flow patterns of arteriovenous shunts in large abnormal vascular structures (*15*). The number of lesions cannot be used as a marker in differentiating between IHHE and hepatoblastomas because, in both diseases, lesions can be single or multiple (*15*). A criterion that can be used for the differential diagnosis is a venous thrombus. While this is a common finding with hepatoblastomas, it is not observed in IHHE (*3*). Contrast dynamic MRI and tomography are specific and diagnostic for IHHE. In MRI scans, IHHE lesions are observed as hypointense on T1 images and hyperintense on T2 images (*16*). In our case, there were many hypoechoic solid lesions; the hepatic artery diameter and liver vascularization increased and there was no hepatic venous thrombus. An abdominal dynamic MRI showed homogenous contrast uptake in the lesions. The patient was diagnosed with IHHE based on these findings.

In IHHE, the most common symptom is abdominal distention. In case of rapidly progressing IHHE, cardiac failure may lead to life-threatening complications such as respiratory distress and consumption coagulopathy (*6-8*). Our patient presented with the complaint of abdominal distention. During the follow up, he developed hypoxemia and needed oxygen support.

Hypothyroidism may also occur as a complication of IHHE (*11,12*). Hypothyroidism has been shown to be associated with type 3 iodothyronine deiodinase activity inside the tumor (*20,21*). This enzyme deiodinates thyroxin, thereby converting it into the biologically inactive form, triiodothyronine. A clinical manifestation, termed consumption hypothyroidism, occurs as a result of thyroid hormone inactivation exceeding the capacity of the thyroid hormone, which is synthesized by the thyroid gland (*18,19*). The biochemical features of this manifestation are very similar to the manifestations observed in primary hypothyroidism; however, the function of the thyroid hormone is normal and thyroid hormone replacement is needed to correct hypothyroidism. In our patient, we detected no structural or functional abnormalities in the thyroid gland by ultrasound or scintigraphy. Therefore, we attributed hypothyroidism to the activity of the type 3 iodothyronine deiodinase, which was secreted from the tumor tissue. The suppression of TSH along with a reduction in the tumor supported this idea.

The parameters affecting the treatment decision include the severity of the symptoms and the tumor size. There is no consensus on the mode of treatment. However, the use of systemic corticosteroids has become the mainstay of treatment (*12*). The mechanism of action for this treatment remains unclear. However, it is thought that the proliferation of endothelial cells and smooth muscle cells is inhibited and thus, reduction is achieved. Generally, prednisolone (2-3 mg/kg/day) is administered. After 2-3 weeks of medication, the drug is gradually reduced and used for 2-3 months. Recent studies have reported that complete or partial resolution is achieved in lesions with propranolol use (*13,14*). Some of the proposed hypotheses associated with propranolol's effects on hemangiomas include vasoconstriction, decreased renin production, inhibition of angiogenesis, and stimulation of apoptosis (*20,21*). Hepatic artery ligation or embolization may be performed in patients with severe symptoms and are refractory to medical treatment (*12*). Our patient had partial response to combined therapy with steroid and propranolol.

In conclusion, IHHE may manifest with massive hepatomegaly and respiratory distress during infancy and lead to life-threatening complications. Radiologic imaging is generally sufficient in making a diagnosis. Therapeutic decisions should be based on the severity of the symptoms and a propranolol and methylprednisolone combination may be useful in patients with severe symptoms.

References

1. Kim EH, Koh KN, Park M, Kim BE, Im HJ, Seo JJ. Clinical features of infantile hepatic hemangioendothelioma. Korean J Pediatr. 2011; 54:260-266.
2. Isaacs H Jr. Fetal and neonatal hepatic tumors. J Pediatr Surg. 2007; 42:1797-1803.

3. Pan FS, Xu M, Wang W, Zhou LY, Xie XY. Infantile hepatic hemangioendothelioma in comparison with hepatoblastoma in children: Clinical and ultrasound features. Hepat Mon. 2013; 13:e11103.
4. Barnes CM, Huang S, Kaipainen A, Sanoudou D, Chen EJ, Eichler GS, Guo Y, Yu Y, Ingber DE, Mulliken JB, Beggs AH, Folkman J, Fishman SJ. Evidence by molecular profiling for a placental origin of infantile hemangioma. Proc Natl Acad Sci U S A. 2005; 102:19097-19102.
5. Emre S, McKenna GJ. Liver tumors in children. Pediatr Transplant. 2004; 8:632-638.
6. Ganguly R, Mukherjee A. Infantile hemangioendothelioma: A case report and discussion. Pathol Res Pract. 2010; 206:53-58.
7. Sevinir B, Özkan TB. Infantile hepatic hemangioendothelioma: Clinical presentation and treatment. Turk J Gastroenterol. 2007; 18:182-187.
8. Moon SB, Kwon HJ, Park KW, Yun WJ, Jung SE. Clinical experience with infantile hepatic hemangioendothelioma. World J Surg. 2009; 33:597-602.
9. Ho J, Kendrick V, Dewey D, Pacaud D. New insight into the pathophysiology of severe hypothyroidism in an infant with multiple hepatic hemangiomas. J Pediatr Endocrinol Metab. 2005; 18:511-514.
10. Huang SA, Tu HM, Harney JW, Venihaki M, Butte AJ, Kozakewich HP, Fishman SJ, Larsen PR. Severe hypothyroidism caused by type 3 iodothyronine deiodinase in infantile hemangiomas. N Eng J Med. 2000; 343:185-189.
11. Çetinkaya S, Kendirci HN, Ağladioğlu SY, Baş VN, Özdemir S, Bozkurt C, Aycan Z. Hypothyroidism due to hepatic hemangioendothelioma: A case report. J Clin Res Pediatr Endocrinol. 2010; 2:126-130.
12. Dasgupta MK, Das S, Patra C, Sarkar S. Symptomatic Infantile Hepatic Hemangioendothelioma successfully treated with steroid. J Clin Neonatol. 2013; 2:187-189.
13. Emir S, Ekici S, İkiz MA, Vidinlisan S. The association of consumptive hypothyroidism secondary to hepatic hemangioma and severe heart failure in infancy. Turk Pediatri Ars. 2016; 51:52-56.
14. Mazereeuw-Hautier J, Hoeger PH, Benlahrech S, Ammour A, Broue P, Vial J, Ohanessian G, Léauté-Labrèze C, Labenne M, Vabres P, Rössler J, Bodemer C. Efficacy of propranolol in hepatic infantile hemangiomas with diffuse neonatal hemangiomatosis. J Pediatr. 2010; 157:340-342.
15. Kim TJ, Lee YS, Song YS, Park CK, Shim SI, Kang CS, Lee KY. Infantile hemangioendothelioma with elevated serum alpha fetoprotein: Report of 2 cases with immunohistochemical analysis. Hum Pathol. 2010; 41:763-767.
16. Mortele KJ, Vanzieleghem B, Mortele B, Benoit Y, Ros PR. Solitary hepatic infantile hemangioendothelioma: Dynamic gadolinium-enhanced MR imaging findings. Eur Radiol. 2002; 12:862-865.
17. Blohm ME, Vesterling-Horner D, Calaminus G, Gobel U. Alpha 1-fe¬toprotein (AFP) reference values in infants up to 2 years of age. Pediatr Hematol Oncol. 1998; 15:135-42.
18. Bessho K, Etani Y, Ichimori H, Miyoshi Y, Namba N, Yoneda A, Ooue T, Chihara T, Morii E, Aoki T, Murakami M, Mushiake S, Ozono K. Increased type 3 iodothyronine deiodinase activity in a regrown hepatic hemangioma with consumptive hypothyroidism. Eur J Pediatr. 2010; 169:215-221.
19. Huang SA, Fish SA, Dorfman DM, Salvatore D, Kozakewich HP, Mandel SJ, Larsen PR. A 21-year-old woman with consumptive hypothyroidism due to a vascular tumor expressing type 3 iodothyronine deiodinase. J Clin Endocrinol Metab. 2002; 87:4457-4461.
20. Schiestl C, Neuhaus K, Zoller S, Subotic U, Forster-Kuebler I, Michels R, Balmer C, Weibel L. Efficacy and safety of propranolol as first-line treatment for infantile hemangiomas. Eur J Pediatr. 2011; 170:493-501.
21. Chim H, Armijo BS, Miller E, Gliniak C, Serret MA, Gosain AK. Propranolol induces regression of hemangioma cells through HIF-1a-mediated inhibition of VEGF-A. Ann Surg. 2012; 256:146-156.

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Case Report

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Chryseobacterium indolegenes infection in a patient with chronic obstructive pulmonary disease

Sevda Soydan[1], Seyda Ignak[2,*], Ozlem Unay Demirel[3], Gülkan Karadağ[1], Ayşen Aykent[4], Sinan Aslan[5]

[1] *Derince Training and Research Hospital, Microbiology Clinic, Kocaeli, Turkey;*
[2] *Bahcesehir University School of Medicine, Department of Medical Biology, Istanbul, Turkey;*
[3] *Bahcesehir University School of Medicine, Department of Biochemistry, Istanbul, Turkey;*
[4] *Derince Training and Research Hospital Biochemistry Clinic, Kocaeli, Turkey;*
[5] *Derince Training and Research Hospital, Chest Diseases Clinic, Kocaeli, Turkey.*

Summary ***Chryseobacterium indolegenes* is a rare pathogen that causes a variety of infections in inviduals who are mostly hospitalized with severe underlying diseases. Here we present a case of *C. indolegenes* in a 69-year-old male with chronic obstructive pulonary disease (COPD) who was admitted to the chest disease outpatient clinic with symptoms like cough, fever and sputum production and followed up on a suspicion of pneumonia. Despite the fact that our patient did not have any history of hospitalization for at least one year, pneumonia cause was due to *C. indolegenes*. Clinicians should pay attention to the rare pathogens such as *C. indologenes* while managing COPD patients without prior hospitalization history.**

Keywords: *Chryseobacterium indologenes*, chronic obstructive pulmonary disease, penumonia, multidrug resistant

1. Introduction

Chryseobacterium genus belong to Flavobacteriaceae family and it is firstly described in 1994 (*1*). *Chryseobacterium* spp. is a catalase positive, indole positive, oxidase positive, non-glucose fermenting, aerobic Gram negative bacilli. *C. indologenes* is not a part of human microflora (*2*). It is widely distributed in nature primarily in soil and water sources. It was reported that it can survive even in chlorine-treated water, so can be a good source for healthcare associated infection (*3*). The infections due to *C. indologenes* are mostly associated with long term hospitalization, especially in patients who are immunocompromised, using medical devices (respirators, humidifiers, intravascular catheters, intubation tubes, *etc*.) and subject to prolonged exposure to broad spectrum antibiotics (*4,5*). In this case report, we report a *C. indolegenes* which was isolated from a 69 year old male with chronic obstructive pulmonary disease (COPD) admitted to the hospital with cough, fever, and sputum production. Authors emphasize that *C. indologenes* must be kept in mind as a cause of infection in chronic diseases like COPD.

2. Case Report

In this study, a 69-years-old male with COPD was admitted to the chest disease outpatient clinic with symptoms such as cough, fever, and sputum production and followed up on suspicion of pneumonia. The patient had no history of hospitalization at least for one year. First of all laboratory tests indicated a C-reactive protein (CRP) level of 17,71 mg/L (reference range, 0-5 mg/L) and a white blood count (WBC) of 17,600/mm^3 with 77.8% neutrophils. After samples were taken for blood and sputum cultures, emprical treatment was started with imipenem and levofloxacin. Yellow-pigmented Gram negative bacilli colonies were isolated

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**Address correspondence to:*
Dr. Seyda Ignak, Bahcesehir University, School of Medicine, Medical Biology Department, BAU TIP Temel Bilimler Binası, Sahrayıcedid Mah. Batman Sok. No:66-68, Yenisahra, E-5 Uzeri Kadikoy, Istanbul, Turkey.
E-mail: seyda_ignak@hotmail.com

from a sputum sample after 24 hours incubation in a 5% sheep blood agar. The microorganism was found as oxidase positive and non-lactose fermenting. The isolated bacterium was identified as *C. indologenes* by VITEK2 identification and antibiogram system (bioMerieux, Nürtingen, Germany). The strain was found to have intermediate resistance to levofloxacin 4 µg/mL, cefoperazone-sulbactam 32 µg/mL and resistant to ampicillin ≥ 32 µg/mL, trimethoprim-sulfamethoxazole ≥ 320 µg/mL, cefuroxime ≥ 64 µg/mL, cefoxitin ≥ 64 µg/mL, tobramycin ≥ 16 µg/mL, ampicillin/sulbactam ≥ 32 µg/mL, piperacillin ≥ 128 µg/mL, piperacillin-tazobactam ≥ 128 µg/mL, ceftazidime ≥ 64 µg/mL, cefepime ≥ 64 µg/mL, imipenem ≥ 16 µg/mL, meropenem ≥ 16 µg/mL, amikacin ≥ 64 µg/mL, ciprofloxacin ≥ 4 µg/mL, tetracyline ≥ 16 µg/mL, tigecycline ≥ 8 µg/mL, colistin ≥ 16 µg/mL, amoxicillin/clavulanic acid ≥ 32 µg/mL.

The clinical findings, growing of *C. indologenes* in sputum culture, high serum CRP level and increased WBC and neutrophil count lead the clinician to the diagnosis of pneumonia. Imipenem treatment was stopped and treatment was continued with levofloxacin (500 mg/IV). The clinical and laboratory findings of patient improved and there was no growth in control cultures after 14 days of treatment.

3. Discussion

COPD is a progressive lung disease which is characterized by airflow obstruction that is progressive and partly reversible. It is associated with abnormal inflamatory responses which are triggered by noxious particles or gases. A rapid decline in clinical status of COPD occur by exacerbations which are associated with microbial and airway inflammation (*6*). According to the Guidelines for management of COPD the impact of exacerbations could be minimised by using appropriate treatment with oral steroids and/or antibiotics. Up to now no sufficient evidence is found to begin prophylactic antibiotic therapy for managing stable COPD (*7*). In the literature *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Haemophilus parainfluenzae*, *Serratia marcessens*, *Acinetobacter* spp. are bacterial pathogens isolated from patients experiencing exacerbation of COPD (*8,9*). In our case we identified an uncommon pathogen, *C. indologenes*, in our COPD patient.

The natural habitat of *Chryseobacterium* spp. is water, soil, foodstuffs and plants. They are not a part of normal human flora (*4*). It was reported that *C. indologenes* are responsible from various clinical conditions, such as bacteremia, sepsis, pneumonia, shunt infection, urinary tract infection, infection of the central nervous system (*10-15*). It was reported that some underlying conditions such as indwelling devices, malignancies, hypertension diabetes mellitus lead to severe infections in hospitalized patients (*10*). Although it is rising importance in healthcare associated infections, there is no guideline for management of *C. indologenes* infections (*3,4*).

Although being low-virulent, they may cause serious infections in patients with underlying conditions such as long term hospitalization, being immunocompromised, use of medical devices (respirators, humidifiers, intravascular catheters, incubation tubes, *etc*.) and prolonged exposure to broad spectrum antibiotics (*5,10*). Despite the fact that our patient had no history of hospitalization for at least one year, pneumonia cause was found to be *C. indolegenes*.

Chryseobacterium is intrinsically resistant to carbapenems and cephalosporins *via* class A beta lactamase and class B carbapenem hydrolyzing beta lactamase activity. According to literature *C. indologenes* is frequently resistant to aminoglycosides, chloramphenicol, linezolid, and glycopeptides and susceptible to levofloxacin, ciprofloxacin, trimethoprim-sulfamethoxazole and piperacillin-tazobactam (*5,10*). In our case the strain was found to have intermediate resistance to levofloxacin and sefaperazon-sulbactam whereas it was resistant to other tested antibiotics.

In conclusion, surveillance programs are needed to delineate the suitable antimicrobial therapy for rarely isolated pathogens like *C. indologenes* and clinicians should keep in mind the rare pathogens while managing COPD patients without prior hospitalization history.

References

1. Vandamme P, Bernardet JF, Segers P, Kersters K, Holmes B. New perspectives in the classification of the Flavobacteria: Description of *Chryseobacterium* gen. nov., *Bergeyella* gen. nov., and *Empedobacter* norm. rev. Int J Syst Bacteriol. 1994; 44:827-831.
2. Schreckenberger PC, Daneshvar MI, Hollis DG. Acinetobacter, Achromobacter, Chryseobacterium, Moraxella, and other nonfermentative Gram-negative rods. In: Manual of Clinical Microbiology (Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, eds.). Washington, DC, USA, 2007; pp. 770-802.
3. Kirby JT, Sader HS, Walsh TR, Jones RN. Antimicrobial susceptibility and epidemiology of a worldwide collection of *Chryseobacterium* spp: Report from the SENTRY Antimicrobial Surveillance Program (1997-2001). J Clin Microbiol. 2004; 42:445-448.
4. Chen FL, Wang GC, Teng SO, Ou TY, Yu FL, Lee WS. Clinical and epidemiological features of *Chryseobacterium indologenes* infections: Analysis of 215 cases. J Microbiol Immunol Infect. 2013; 46:425-432.
5. Atıcı S, Unkar ZA, Erdem K, Kadayifci EK, Karaaslan A, Memisoglu AC, Soysal A, Toprak NU, Söyletir G, Ozek E, Bakır M. Ventilator-associated pneumonia caused by *Chryseobacterium indologenes*: A rare infant case and review of the literature. Springerplus. 2016; 5:1741.
6. Bhowmik A, Seemungal T, Sapsford R, Wedzicha J.

Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. Thorax. 2000; 55:114-120.
7. Chronic Obstructive Pulmonary Disease: Management of Chronic Obstructive Pulmonary Disease in Adults in Primary and Secondary Care. *https://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0033903/pdf/PubMedHealth_PMH0033903.pdf* (Accessed May 12, 2017).
8. Sethi S. Bacteria in exacerbations of chronic obstructive pulmonary disease: Phenomenon or epiphenomenon? Proc Am Thorac Soc. 2004; 1:109-114.
9. Erkan L, Uzun O, Findik S, Katar D, Sanic A, Atici AG. Role of bacteria in acute exacerbations of chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis. 2008; 3:463-467.
10. Lin YT, Jeng YY, Lin ML, Yu KW, Wang FD, Liu CY. Clinical and microbiological characteristics of *Chryseobacterium indologenes* bacteremia. J Microbiol Immunol Infect. 2010; 43:498-505.
11. Ceylan A, Guducuoglu H, Akbayram S, Bektas A, Berktas M. Sepsis caused by *Chryseobacterium indologenes* in a patient with hydrocephalus. Mikrobiyoloji Bulteni. 2011; 45:735-740.
12. Monteen MR, Ponnapula S, Wood GC, Croce MA, Swanson JM, Boucher BA, Fabian TC. Treatment of *Chryseobacterium indologenes* ventilator-associated pneumonia in a critically ill trauma patient. Ann Pharmacother. 2013; 47:1736-1739.
13. Al-Tatari H, Asmar BI, Ang JY. Lumboperitonial shunt infection due to *Chryseobacterium indologenes*. Pediatr Infect Dis J. 2007; 26:657-659.
14. Bhuyar G, Jain S, Shah H, Mehta VK. Urinary tract infection by *Chryseobacterium indologenes*. Indian J Med Microbiol. 2012; 30:370-372.
15. Olbrich P, Rivero-Garvía M, Falcón-Neyra MD, Lepe JA, Cisneros JM, Marquez-Rivas J, Neth O. *Chryseobacterium indologenes* central nervous system infection in infancy: An emergent pathogen? Infection. 2014; 42:179-183.

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Letter

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Combination of triple biomarkers AFP, AFP-L3, and PIVAKII for early detection of hepatocellular carcinoma in China: Expectation

Jianjun Gao[1,*], Peipei Song[2]

[1] *Department of Pharmacology, School of Pharmacy, Qingdao University, Qingdao, Shandong, China;*
[2] *Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa-shi, Chiba, Japan.*

Summary **Hepatocellular carcinoma (HCC) remains a severe health threat in China. Early tumor detection is crucial for improving the prognosis of patients. Currently, ultrasound plus biomarker alpha fetoprotein (AFP) is recommended by Chinese Liver Cancer Diagnosis and Treatment Guidelines in China. However, most HCC continues to be diagnosed beyond an early stage due to insufficient sensitivity and specificity of current surveillance tools, highlighting the need for more accurate biomarkers to improve early HCC detection. In Japan, ultrasound plus triple biomarkers AFP, *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3), and prothrombin induced by vitamin K absence II (PIVKA II) has been routinely used for HCC surveillance and achieved increased early HCC detection rate. Very recently, the assay of triple biomarkers AFP, AFP-L3, and PIVKA II using μTASWako i30 immuno-analyzer was brought into China. The prospect of the modality of ultrasound plus triple biomarkers for early HCC detection in China is expected in the future.**

Keywords: AFP, AFP-L3, PIVKA II, DCP, HCC

Liver cancer is the fourth most common cancer in China and the third most common cause of death from cancer in China in 2015 (*1*). It is estimated that over 50% of new cases of liver cancer in the world occurred in China each year (*2*). As the most common type of liver cancer, hepatocellular carcinoma (HCC) has a dismal prognosis because most patients (about two thirds) had lost the opportunity of surgical therapy when HCC was detected at advanced stage (*3*). Surveillance at regular intervals and early diagnosis of HCC is crucial for improving the patients' survival.

Currently, early detection of HCC is primarily based on noninvasive imaging methods, such as ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI) and expression patterns of serologic tumor markers such as alpha-fetoprotein (AFP) (*4*). Guidelines from the American Association for the Study of Liver Diseases (AASLD) and European Association for the Study of the Liver (EASL) recommend surveillance using ultrasound alone. Chinese Liver Cancer Diagnosis and Treatment Guidelines recommend ultrasound and serum AFP level as monitoring methods for surveilling high-risk groups. However, most HCC continues to be diagnosed beyond an early stage due to insufficient sensitivity and specificity of current surveillance tools, highlighting the need for more accurate biomarkers to improve early HCC detection.

Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), the glycosylated isoform of AFP, has been suggested as a biomarker for HCC early detection given its higher specificity than AFP. Clinical studies revealed a specificity of 92% and a sensitivity of 37-49% of AFP-L3 for early stage HCC detection when used alone (*5-7*). Prothrombin induced by vitamin K absence II (PIVKA II), also known as des-γ-carboxy prothrombin (DCP), is an abnormal prothrombin protein that is generated as a result of an acquired defect in posttranslational carboxylation (*8*). Several studies suggested that DCP had a high specificity of approx. 90% and a sensitivity of 56% for early stage HCC detection when used alone (*6,9*). Although AFP-L3 or PIVKA II appears to have insufficient sensitivity when used alone, it may have potential additive benefit to AFP which may cause false

*Address correspondence to:
Dr. Jianjun Gao, Department of Pharmacology, School of Pharmacy, Qingdao University, 38 Dengzhou Road, Qingdao 266021, Shandong, China.
E-mail: gaojj@qdu.edu.cn

positives with non-HCC malignancies. A recent clinical study showed that the combination of AFP, AFP-L3 and PIVKA II is superior to a single biomarker in HCC detection (*10*). The GALAD scoring algorithm based on AFP, AFP-L3, and PIVKA II significantly improves detection of BCLC early stage HCC, with a specificity of 93.3% and sensitivity of 85.6% (*11*). Thus far, ultrasound examination plus simultaneous measurement of triple tumor biomarkers AFP, AFP-L3, and PIVKA II has been recommended for surveillance of high-risk populations by Evidence-based Clinical Practice Guidelines for Hepatocellular Carcinoma: The Japan Society of Hepatology (JSH-HCC Guidelines). Studies showed that early HCC detection rate and patients' 5-year survival in Japan was 68% and 45.2% (*12,13*), which are obviously higher than those in the US and China, suggesting the promising value of combination of triple biomarkers in early HCC detection.

On June 28, 2017, Wako Pure Chemical Industries, Ltd. (Japan), the developer of μTASWako i30 immuno-analyzer for detection of AFP, AFP-L3, and PIVKA II, and Techpool Bio-pharma Co., Ltd. (China) announced in Shanghai, China that the two companies would cooperate to promote the application of the triple biomarkers detection assay in China. The prospect of improved HCC early detection in China is expected in the future.

References

1. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. CA Cancer J Clin. 2016; 66:115-132.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015; 65:87-108.
3. Ding XX, Zhu QG, Zhang SM, *et al*. Precision medicine for hepatocellular carcinoma: driver mutations and targeted therapy. Oncotarget. 2017.
4. Song P, Feng X, Inagaki Y, *et al*. Clinical utility of simultaneous measurement of alpha-fetoprotein and des-gamma-carboxy prothrombin for diagnosis of patients with hepatocellular carcinoma in China: A multi-center case-controlled study of 1,153 subjects. Biosci trends. 2014; 8:266-273.
5. Sterling RK, Jeffers L, Gordon F, Sherman M, Venook AP, Reddy KR, Satomura S, Schwartz ME. Clinical utility of AFP-L3% measurement in North American patients with HCV-related cirrhosis. Am J Gastroenterol. 2007; 102:2196-2205.
6. Marrero JA, Feng Z, Wang Y, *et al*. Alpha-fetoprotein, des-gamma carboxyprothrombin, and lectin-bound alpha-fetoprotein in early hepatocellular carcinoma. Gastroenterology. 2009; 137:110-118.
7. Oda K, Ido A, Tamai T, *et al*. Highly sensitive lens culinaris agglutinin-reactive alpha-fetoprotein is useful for early detection of hepatocellular carcinoma in patients with chronic liver disease. Oncol Rep. 2011; 26:1227-1233.
8. Xia J, Song P, Sun Z, Sawakami T, Jia M, Wang Z. Advances of diagnostic and mechanistic studies of gamma-glutamyl transpeptidase in hepatocellular carcinoma. Drug Discov Ther. 2016; 10:181-187.
9. Marrero JA, Su GL, Wei W, Emick D, Conjeevaram HS, Fontana RJ, Lok AS. Des-gamma carboxyprothrombin can differentiate hepatocellular carcinoma from nonmalignant chronic liver disease in american patients. Hepatology. 2003; 37:1114-1121.
10. Caviglia GP, Abate ML, Petrini E, Gaia S, Rizzetto M, Smedile A. Highly sensitive alpha-fetoprotein, Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein and des-gamma-carboxyprothrombin for hepatocellular carcinoma detection. Hepatol Res. 2016; 46:E130-135.
11. Best J, Bilgi H, Heider D, Schotten C, Manka P, Bedreli S, Gorray M, Ertle J, van Grunsven LA, Dechene A. The GALAD scoring algorithm based on AFP, AFP-L3, and DCP significantly improves detection of BCLC early stage hepatocellular carcinoma. Z Gastroenterol. 2016; 54:1296-1305.
12. Oeda S, Iwane S, Takasaki M, Furukawa NE, Otsuka T, Eguchi Y, Anzai K. Optimal follow-up of patients with viral hepatitis improves the detection of early-stage hepatocellular carcinoma and the prognosis of survival. Intern Med. 2016; 55:2749-2758.
13. Berhane S, Toyoda H, Tada T, *et al*. Role of the GALAD and BALAD-2 serologic models in diagnosis of hepatocellular carcinoma and prediction of survival in patients. Clin Gastroenterol Hepatol. 2016; 14:875-886.e6.

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