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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 Zhang¹, Xuan Li², Xuejian Wang¹, Wenfang Xu¹, Jian Zhang¹,*

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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) (IC₅₀ = 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 Zn²⁺ in the conserved catalytic center of the MMP-2 enzyme, MMPs have two hydrophobic domains (S₁’ and S₂’ pockets, respectively) that are located in proximity to the catalytic zinc center. The S₁’ 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 S₂’ 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
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 \(\delta\) 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
analytically confirmed with 1H-NMR, 13P-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 (LiAlH4) to their aldehyde derivatives 4a-e in anhydrous tetrahydrofuran (THF). Solvent-free nucleophilic addition of 4a-e with diethyl phosphate and Al2O3 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

IC50 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 x 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 IC50 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-NH2 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 CaCl2, 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 IC50 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% CO2. Cell proliferation was determined using a 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-terazolium 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 Zn2+ (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 IC50 values in the micromole range and displayed moderate inhibitory activity compared to LY52 (the control) (IC50 = 0.95 ± 0.09 µM).

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

<table>
<thead>
<tr>
<th>Compd</th>
<th>Structure</th>
<th>IC50 (µM)</th>
<th>MMP-2</th>
<th>MMP-9</th>
</tr>
</thead>
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<tr>
<td>5a</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>80.39 ± 2.52</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5b</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>63.16 ± 2.24</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5c</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>56.81 ± 1.79</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5d</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>38.24 ± 1.15</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5e</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>45.73 ± 1.28</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>6a</td>
<td><img src="image6.png" alt="Structure" /></td>
<td>14.58 ± 0.23</td>
<td>26.32 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>6b</td>
<td><img src="image7.png" alt="Structure" /></td>
<td>13.87 ± 0.21</td>
<td>25.75 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>6c</td>
<td><img src="image8.png" alt="Structure" /></td>
<td>10.25 ± 0.18</td>
<td>22.47 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>6d</td>
<td><img src="image9.png" alt="Structure" /></td>
<td>8.46 ± 0.14</td>
<td>15.26 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>LY52</td>
<td><img src="image10.png" alt="Structure" /></td>
<td>0.95 ± 0.09</td>
<td>1.72 ± 0.12</td>
<td></td>
</tr>
</tbody>
</table>

ND: not determined. *IC50 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) (IC50 = 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 (IC50 = 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 IC50 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.](image11.png)

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

<table>
<thead>
<tr>
<th>Compd</th>
<th>SKOV3 (µM)</th>
<th>HL60 (µM)</th>
<th>A549 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6a</td>
<td>415.76</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>6b</td>
<td>346.82</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>6c</td>
<td>281.39</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>6d</td>
<td>173.58</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>LY52</td>
<td>697.14</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

*Mean values and the standard deviation of three experiments are shown.
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 phosphate 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.

Acknowledgements

This work was supported by the Natural Science Foundation of Shandong Province (BS2015YY016) and the Doctoral Foundation of Weifang Medical University.

References

Evaporation of DCM yielded a pale SO₂, filtered, 24O to yield 12.80 g of 3. 

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 (Et₃N, 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 Na₂SO₄, filtered, and concentrated in a vacuum to yield target compound 2a. The crude product was purified via recrystallization in 75% ethanol/H₂O 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-carboxamide (3a)

Compound 2a (3.45 g, 10 mmol) was dissolved in 100 mL anhydrous DCM with Et₃N (3.5 mL, 11 mmol) and then treated with 3.53 g (11 mmol) of O-(Benzotriazol-1-yl)-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 NaHCO₃ solution (3 × 50 mL), and brine (2 × 50 mL) and then dried over Na₂SO₄. 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.

\[ N\text{-methoxy-N-methyl-7-tosyl-1,4-dithia-7-azaspiro}[4,4]nonane-8-carboamide (3b) \]
Pale yellow solid, yield 67.8%, m.p. 102-104°C. ESI-MS \( m/z \): 403.5 \([\text{M}+\text{H}]^+\).

\[ N\text{-methoxy-N-methyl-7-(p-methoxyphenylsulfonyl)-1,4-dithia-7-azaspiro}[4,4]nonane-8-carboamide (3c) \]
Pale yellow solid, yield 72.6%, m.p. 121-123 °C. ESI-MS \( m/z \): 419.4 \([\text{M}+\text{H}]^+\).

\[ N\text{-methoxy-N-methyl-7-(p-chlorophenylsulfonyl)-1,4-dithia-7-azaspiro}[4,4]nonane-8-carboamide (3d) \]
Pale yellow solid, yield 74.1%, m.p. 125-127 °C. ESI-MS \( m/z \): 423.3 \([\text{M}+\text{H}]^+\).

\[ N\text{-methoxy-N-methyl-7-(p-nitrophenylsulfonyl)-1,4-dithia-7-azaspiro}[4,4]nonane-8-carboamide (3e) \]
Yellow solid, yield 54.2%, m.p. 140-142 °C. ESI-MS \( m/z \): 434.5 \([\text{M}+\text{H}]^+\).

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 H2O (2 × 50 mL), 1 M citric acid (2 × 50 mL), saturated NaHCO3 (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 \([\text{M}+\text{H}]^+\).

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 \([\text{M}+\text{H}]^+\).

7-(p-Methoxyphenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-carbaldehyde (4c)
Pale yellow oil. ESI-MS \( m/z \): 360.4 \([\text{M}+\text{H}]^+\).

7-(p-Chlorophenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-carbaldehyde (4d)
Yellow oil. ESI-MS \( m/z \): 364.9 \([\text{M}+\text{H}]^+\).

7-(p-Nitrophenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-carbaldehyde (4e)
Yellow oil. ESI-MS \( m/z \): 375.4 \([\text{M}+\text{H}]^+\).

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 Al2O3 (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 Na2SO4 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 \( m/z \): calcld. for C17H26NO6PS3 \([\text{M}+\text{H}]^+\) 468.0738, found 468.0734; \(^1\)H NMR: (DMSO-\(d_6\), ppm) \( \delta \): 1.255 (\( t, J = 6.6 \text{ Hz}, 3 \text{H}, \text{CH} \)), 1.286 (\( t, J = 3.6 \text{ Hz}, 3 \text{H}, \text{CH} \)), 2.656-2.735 (m, 1H, CH), 3.090-3.161 (m, 2H, SCH), 3.193-3.292 (m, 2H, SCH), 3.608 (d, \( J = 12 \) Hz, 1H, CH2-N=), 3.752 (d, \( J = 12 \) Hz, 1H, CH2-N=), 3.847-3.902 (m, 1H, CH), 7.682 (d, \( J = 7.2 \) Hz, 2H, ArH), 7.711 (d, \( J = 7.2 \) Hz, 1H, ArH), 7.828-7.875 (m, 2H, ArH). \(^3\)P NMR: (DMSO-\(d_6\), ppm) \( \delta \): 21.784, 22.967.

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

Diethyl(hydroxyl-(7-tosyl-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 \( m/z \): calcld. for C18H28NO6PS3 \([\text{M}+\text{H}]^+\) 482.0895, found 482.0892; \(^1\)H NMR: (DMSO-\(d_6\), ppm) \( \delta \): 1.255 (\( t, J = 3.3 \text{ Hz}, 3 \text{H}, \text{CH} \)), 1.286 (\( t, J = 3.3 \text{ Hz}, 3 \text{H}, \text{CH} \)), 2.131-2.293 (m, 1H, CH), 2.402 (s, 3H, ArCH3), 2.656-2.733 (m, 1H, CH), 3.090-3.161 (m, 2H, SCH), 3.228-3.285 (m, 2H, SCH), 3.602 (d, \( J = 12 \) Hz, 1H, CH2-N=), 3.702 (d, \( J = 12 \) Hz, 1H, CH2-N=), 3.820-3.874 (m, 1H, CH), 4.002-4.048 (m, 2H, OCH3), 4.063-4.120 (m, 2H, OCH3), 4.434-4.656 (m, 1H, CH), 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). \(^3\)P NMR: (DMSO-\(d_6\), ppm) \( \delta \): 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 \( m/z \): calcld. for C19H28NO6PS3 \([\text{M}+\text{H}]^+\) 498.0844, found 498.0837; \(^1\)H NMR: (DMSO-\(d_6\), ppm) \( \delta \): 1.243 (\( t, J = 3.6 \text{ Hz}, 3 \text{H}, \text{CH} \)), 1.290 (\( t, J = 3.6 \text{ Hz}, 3 \text{H}, \text{CH} \)), 2.138-2.297 (m, 1H, CH), 2.513-2.735 (m, 1H, CH), 3.965-4.063 (m, 1H, CH), 4.031-4.127 (m, 2H, OCH3), 4.470-4.665 (m, 1H, CH), 6.914-6.252 (m, 1H, OH), 7.428 (d, \( J = 7.2 \) Hz, 2H, ArH), 7.711 (d, \( J = 7.2 \) Hz, 1H, ArH), 7.828-7.875 (m, 2H, ArH). \(^3\)P NMR: (DMSO-\(d_6\), ppm) \( \delta \): 21.865, 22.979.
Diethyl(hydroxyl(7-(p-chlorophenylsulfonyl)-1,4-dithia-7-azaspiro[4,4]nonane-8-yl) methyl) phosphonate (6d)

Pale yellow solid, yield 54.2%, m.p. 86-88°C; HRMS m/z: calcd. for C17H25N2O8PS3 [M+H]+ 502.0348, found 502.0342; 1H NMR: (DMSO-d6, ppm) δ: 1.252 (t, J = 3.6 Hz, 3H, CH3), 1.286 (t, J = 3.6 Hz, 3H, CH3), 2.151-2.316 (m, 1H, CH), 2.679-2.757 (m, 1H, CH), 3.139-3.173 (m, 2H, SCH2), 3.235-3.261 (m, 2H, SCH2), 3.601 (d, J = 12 Hz, 1H, CH2-N), 3.713 (d, J = 12 Hz, 1H, CH2-N), 3.834-3.890 (m, 1H, CH), 4.022-4.054 (m, 2H, OCH2), 4.078-4.127 (m, 2H, OCH), 4.164-4.168 (m, 1H, CH, CH-P(O)(OEi)), 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). 31P NMR: (DMSO-d6, ppm) δ: 21.926, 22.060.

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

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]+ 513.0586, found 513.0586; 1H NMR: (DMSO-d6, ppm) δ: 1.258 (t, J = 3.6 Hz, 3H, CH3), 1.292 (t, J = 3.6 Hz, 3H, CH3), 2.147-2.215 (m, 1H, CH), 2.693-2.776 (m, 1H, CH), 3.130-3.187 (m, 2H, SCH2), 3.193-3.249 (m, 2H, SCH2), 3.601 (d, J = 12 Hz, 1H, CH2-N), 3.732 (d, J = 12 Hz, 1H, CH2-N), 3.846-3.947 (m, 1H, CH), 4.029-4.059 (m, 2H, OCH2), 4.078-4.133 (m, 2H, OCH2), 4.392-4.571 (m, 1H, CH, CH-P(O)(OEi)), 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). 31P NMR: (DMSO-d6, ppm) δ: 21.389, 22.721.

5.

(2-Hydroxyl(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 (H2O:MeOH = 100% to 65:35). Pale yellow semisolid: yield 37.6%. HRMS m/z: calcd. for C13H18NO6PS3 [M+H]+ 412.0112, found 412.0105; 1H NMR: (DMSO-d6, ppm) δ: 2.153-2.265 (m, 1H, CH), 2.666-2.765 (m, 1H, CH), 3.090-3.147 (m, 2H, SCH2), 3.187-3.223 (m, 2H, SCH2), 3.584 (d, J = 12 Hz, 1H, CH2-N), 3.720 (d, J = 12 Hz, 1H, CH2-N), 3.975-4.046 (m, 1H, CH), 4.203-4.535 (m, 1H, CH, CH-P(O)(OEi)), 7.592 (t, J = 7.5 Hz, 2H, ArH), 7.625-7.718 (m, 1H, ArH), 7.815-7.865 (m, 2H, ArH). 31P NMR: (DMSO-d6, ppm) δ: 18.303, 18.678.

(2-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 C14H12NO6PS3 [M+H]+ 426.0269, found 426.0261; 1H NMR: (DMSO-d6, ppm) δ: 2.164-2.236 (m, 1H, CH), 2.408 (s, 3H, ArCH3), 2.654-2.767 (m, 1H, CH), 3.096-3.155 (m, 2H, SCH2), 3.193-3.239 (m, 2H, SCH2), 3.576 (d, J = 12 Hz, 1H, CH2-N), 3.712 (d, J = 12 Hz, 1H, CH2-N), 3.933-3.975 (m, 1H, CH), 4.170-4.456 (m, 1H, CH, CH-P(O)(OEi)), 7.412 (d, J = 7.2 Hz, 2H, ArH), 7.785 (d, J = 7.2 Hz, 2H, ArH). 31P NMR: (DMSO-d6, ppm) δ: 18.394, 18.705.

(2-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 C14H12NO6PS3 [M+H]+ 442.0218, found 442.0214; 1H NMR: (DMSO-d6, ppm) δ: 2.176-2.261 (m, 1H, CH), 2.648-2.727 (m, 1H, CH), 3.077-3.137 (m, 2H, SCH2), 3.192-3.236 (m, 2H, SCH2), 3.570 (d, J = 12 Hz, 1H, CH2-N), 3.706 (d, J = 12 Hz, 1H, CH2-N), 3.838 (s, 3H, ArOCH3), 3.914-3.971 (m, 1H, CH), 4.163-4.451 (m, 1H, CH, CH-P(O)(OEi)), 7.107 (d, J = 9.0 Hz, 2H, ArH), 7.750 (d, J = 9.0 Hz, 2H, ArH). 31P NMR: (DMSO-d6, ppm) δ: 18.447, 18.738.

(2-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 C13H17ClO6PS3 [M+H]+ 445.9722, found 445.9716; 1H NMR: (DMSO-d6, ppm) δ: 2.312-2.377 (m, 1H, CH), 2.801-2.879 (m, 1H, CH), 3.080-3.142 (m, 2H, SCH2), 3.199-3.268 (m, 2H, SCH2), 3.706 (d, J = 12.3 Hz, 1H, CH2-N), 3.799 (d, J = 12.3 Hz, 1H, CH2-N), 4.074-4.130 (m, 1H, CH), 4.727-4.770 (m, 1H, CH, CH-P(O)(OEi)), 7.616 (d, J = 6.6 Hz, 2H, ArH), 7.908 (d, J = 6.6 Hz, 2H, ArH). 31P NMR: (DMSO-d6, ppm) δ: 20.202.
Validation of a sheet-shaped body vibrometer for screening of obstructive sleep apnea

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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

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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 ≤ AHI < 30) to severe (AHI ≥ 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 ≥ 5) in that study, because most of the subjects had AHI ≥ 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/m²) 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
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 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 (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

(A)

(B)

Figure 1. Respiratory waveform measured with the sheet-shaped body vibrometer. (A) movement of the examinee; (B) serial appearance of apnea-hypopnea events.
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 \( \geq 5 \) were 14.9 for REI_eTST and 15.1 for REI_TIB, those for predicting AHI \( \geq 15 \) were 17.0 for REI_eTST and 15.9 for REI_TIB, and those for predicting AHI \( \geq 30 \) 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 (\( n = 70 \))

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

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.
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 AH1 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

7. Ip MS, Lam B, Tang LC, Launder IJ, Ip TY, Lam WK. A

(Received February 27, 2017; Revised May 17, 2017; Accepted June 11, 2017)
Experience with long-term administration of tolvaptan to patients with acute decompensated heart failure

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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.
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. 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. 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.
Comparison of findings at discharge among the three groups. There were no significant differences in the rate of administration of cardio-protective medications such as RAAS-I and BB did not differ significantly among the three groups. In addition, the rate of administration of loop diuretics among the three groups. There were no significant differences in the dosage of loop diuretics among the three groups.

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. Twenty-nine patients (50.9%) in Group A were re-hospitalized for HF within one year of discharge. Twenty-six patients (25.0%) in Group A 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.

### 3.2. 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...
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, Table 3). 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

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

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

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

AST: aspartate aminotransferase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase, BUN: blood urea nitrogen. BNP: brain natriuretic peptide, LVDD: left ventricular end-diastolic dimension, LVDSs: 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.
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


(Received March 21, 2017; Revised May 27, 2017; Accepted June 6, 2017)
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. Kongthavonskul 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...
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), Bosvellia 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 4th, 8th, and 12th 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
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 12\textsuperscript{th} week in Table 2. VAS scores at the 8\textsuperscript{th} week \( (p = 0.004) \) and 12\textsuperscript{th} week \( (p < 0.001) \) were significantly lower than at the baseline in Figure 1A.

JKOM total scores at the 8\textsuperscript{th} week \( (p = 0.003) \) and 12\textsuperscript{th} week \( (p < 0.001) \) were significantly lower than at the baseline in Figure 1B. Pain-and-stiffness-score at the 4\textsuperscript{th} week \( (p < 0.001) \), 8\textsuperscript{th} week \( (p < 0.001) \), and 12\textsuperscript{th} week \( (p < 0.001) \) were significantly lower than at the baseline in Figure 2A. The state-of-daily-life scores at the 4\textsuperscript{th} week \( (p = 0.024) \), 8\textsuperscript{th} week \( (p = 0.033) \), and 12\textsuperscript{th} week \( (p = 0.007) \) were significantly lower than at the baseline in Figure 2B. Soreness due to daily activities score at the 8\textsuperscript{th} week \( (p = 0.009) \) and 12\textsuperscript{th} week \( (p < 0.001) \) were significantly lower than at the baseline in Figure 2C. The Condition-of-health-scores at the 8\textsuperscript{th} week \( (p < 0.001) \) and 12\textsuperscript{th} week \( (p < 0.001) \) were significantly lower than at the baseline in Figure 2D. The JOA score at the 12\textsuperscript{th} 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 12\textsuperscript{th} 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.

Table 1. The mean characteristics of the subjects

<table>
<thead>
<tr>
<th>Items</th>
<th>0 w Mean ± SD</th>
<th>4 w Mean ± SD</th>
<th>8 w Mean ± SD</th>
<th>12 w Mean ± SD</th>
<th>p (vs. 0 w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55.6 ± 6.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>160.8 ± 5.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>64.6 ± 10.1</td>
<td>64.7 ± 10.0</td>
<td>0.267</td>
<td>64.7 ± 10.1</td>
<td>0.370</td>
</tr>
<tr>
<td>BMI (kg/m\textsuperscript{2})</td>
<td>25.0 ± 3.9</td>
<td>25.1 ± 3.9</td>
<td>0.258</td>
<td>25.1 ± 4.0</td>
<td>0.367</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>127.3 ± 10.7</td>
<td>127.2 ± 10.1</td>
<td>0.958</td>
<td>127.1 ± 14.1</td>
<td>0.940</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>78.3 ± 6.9</td>
<td>77.2 ± 5.7</td>
<td>0.508</td>
<td>78.3 ± 6.8</td>
<td>0.976</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>75.1 ± 10.0</td>
<td>72.5 ± 11.0</td>
<td>0.249</td>
<td>74.0 ±8.6</td>
<td>0.564</td>
</tr>
</tbody>
</table>

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

Table 2. Assessment of efficacy

<table>
<thead>
<tr>
<th>Items</th>
<th>0 w Mean ± SD</th>
<th>4 w Mean ± SD</th>
<th>8 w Mean ± SD</th>
<th>12 w Mean ± SD</th>
<th>p (vs. 0 w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAS (mm)</td>
<td>50.5 ± 24.7</td>
<td>33.6 ± 22.3</td>
<td>0.003**</td>
<td>30.6 ± 26.7</td>
<td>0.003**</td>
</tr>
<tr>
<td>JKOM</td>
<td>43.6 ± 7.9</td>
<td>38.6 ± 5.9</td>
<td>0.156</td>
<td>35.9 ± 6.0</td>
<td>0.001**</td>
</tr>
<tr>
<td>JOA*</td>
<td>83.9 ± 8.6</td>
<td>90.5 ± 0.0</td>
<td>0.035*</td>
<td>88.9 ± 8.4</td>
<td>0.360</td>
</tr>
</tbody>
</table>

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 \( 4.5 ± 1.3 \) \( 3.8 ± 1.4 \) \( 0.103 \) \( 3.5 ± 1.2 \) \( 0.003** \) \( 3.5 ± 1.1 \) \( 0.009** \) \( 3.4 ± 1.3 \) \( 0.603 \)

*: JOA score evaluated more painful side of the legs. *\( p < 0.05 \) and **\( p < 0.01 \).
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. Ozkakan 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, Hathcoek 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.

Acknowledgements

We thank all the participants and the professional staff, Takafumi Koide, Miyuuki Seki, at the Japanese Red Cross Society Iyama Hospital. 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 interests.

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


(Received March 23, 2017; Revised June 9, 2017; Revised June 12, 2017; Accepted June 13, 2017)
Association between type 1 diabetes mellitus and risk of epilepsy: A meta-analysis of observational studies

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1. Introduction

Type 1 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...
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.](www.ddtjournal.com)
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 patients adjusted for potential confounders.
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

13. Striano P, Errichelli L, Striano S. Autoantibodies to
glutamic acid decarboxylase in patients with epilepsy: What is their clinical relevance? Epilepsy Behav. 2011; 20:145.


(Received March 24, 2017; Revised May 29, 2017; Accepted June 18, 2017)
Intratumor dihydropyrimidine dehydrogenase mRNA expression levels are decreased in extramammary Paget's disease


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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.

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

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www.ddtjournal.com
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<sup>2</sup> 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 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...
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 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.

![Figure 2. Correlation between intratumoral OPRT and DPD mRNA expression levels in EMPD.](image)

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

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

We thank Ms. Ayaka Hirano and Ms. Chiemi Shiotsu for providing valuable technical assistance. This study was supported in part by a grant for scientific research from the Japanese Ministry of Education, Science, Sports and Culture from the Japanese Ministry of Health, Labour and Welfare.

References


(Received April 8, 2017; Revised May 21, 2017; Accepted June 3, 2017)
Properties of induced antimicrobial activity in *Musca domestica* larvae

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**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-NH₂ was developed by modifying the primary structure of an antimicrobial peptide of *Sarcophaga peregrina*, Sapecin B (16,17). KLKLLLLLKLK-NH₂ 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-NH₂ synthesized using D-amino acids displays higher antimicrobial activity than its L-form (21,22). These observations highlight the importance of native
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 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, 1 mM CaCl₂) 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 (OD₆₀₀ = 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 CaCl₂), 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 titer¹. 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](image1.png)

**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](image2.png)

**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.
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).

Acknowledgements

This work was supported in part by a grant from E’s Inc. and by Adaptable and Seamless Technology transfer Program through Target-driven R&D (A-STEP) from Japan Science and Technology Agency (JST).

Conflict of interest

K.K. is an advisor for E’s Inc.

References


(Received May 23, 2017; Revised June 1, 2017; Accepted June 17, 2017)
Beta blocker and steroid therapy in the treatment of infantile hepatic hemangioendothelioma

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1. Introduction

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

2. Case Report

A 4-month-old male patient was brought in with...
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 ng/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 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


(Received April 19, 2017; Revised June 5, 2017; Accepted June 17, 2017)
Chryseobacterium indolegenes infection in a patient with chronic obstructive pulmonary disease

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Summary

Chryseobacterium indolegenes is a rare pathogen that causes a variety of infections in individuals 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 pulmonary 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, pneumonia, 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. indologenes 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/ mm³ with 77.8% neutrophils. After samples were taken for blood and sputum cultures, empirical treatment was started with imipenem and levofloxacin. Yellow-pigmented Gram negative bacilli colonies were isolated.
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, Nüritingen, 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, cefoxime ≥ 64 µg/mL, ceftazidime ≥ 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, tetracycline ≥ 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 inflammatory 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. indologenes*.

*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


(Received May 26, 2017; Revised May 31, 2017; Accepted June 18, 2017)
Combination of triple biomarkers AFP, AFP-L3, and PIVAKII for early detection of hepatocellular carcinoma in China: Expectation

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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
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 immunoanalyzer 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

(Received June 29, 2017; Accepted June 30, 2017)
Guide for Authors

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(Revised February 2013)

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