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CONTENTS

Volume 18, Number 1, 2024

Review

1-9 **A systematic review of the mechanistic actions of microRNAs within integrated traditional Chinese medicine and western medical treatment for endometriosis.**
Lisha Li, Yiqin Zhang, Jing Zhou, Jing Wang, Ling Wang

Original Article

10-15 **Usefulness of a new immunochromatographic assay using fluorescent silica nanoparticles for serodiagnosis of Thai patients with amebiasis.**
Azumi Kakino, Urassaya Pattanawong, Napaporn Kuamsab, Tatsuya Imai, Chaturong Putaporntip, Satomi Asai, Xunjia Cheng, Somchai Jongwutiwes, Hiroshi Tachibana

16-23 **Inhibitory effects of kaempferol, quercetin and luteolin on the replication of human parainfluenza virus type 2 *in vitro*.**
Kae Sakai-Sugino, Jun Uematsu, Hidetaka Yamamoto, Sahoko Kihira, Mitsuo Kawano, Miwako Nishio, Masato Tsurudome, Hidehisa Sekijima, Myles O'Brien, Hiroshi Komada

24-33 **Astragalus root increases Treg and Th17 involvement in embryo implantation and pregnancy maintenance by decreasing CTLA-4^{+} Tregs.**
Kyoko Kobayashi, Kenroh Sasaki

34-43 **PD-1/PD-L1 inhibitors associated hypophysitis: An analysis from the FAERS database and case reports.**
Shanshan Chen, Linqi Ouyang, Lian Li, Yuyang Xiao, Shengfeng Wang

44-53 **Quantitative parameters of contrast-enhanced ultrasound effectively promote the prediction of cervical lymph node metastasis in papillary thyroid carcinoma.**
Biao Su, Lisha Li, Yingchun Liu, Hui Liu, Jia Zhan, Qiliang Chai, Liang Fang, Ling Wang, Lin Chen

Brief Report

54-59 **Medication incidents associated with the provision of medication assistance by non-medical care staff in residential care facilities.**
Hayato Kizaki, Daisuke Yamamoto, Hideyuki Maki, Kotaro Masuko, Yukari Konishi, Hiroki Satoh, Satoko Hori, Yasufumi Sawada

60-66 **Association between the experience of exertional heat illness (EHI) and living conditions of collegiate student athletes.**
Yoko Iio, Mamoru Tanaka, Hana Kozai, Yuka Aoyama, Yukihiro Mori, Manato Seguchi, Morihiro Ito

Correspondence

67-70 **Evolving immune evasion and transmissibility of SARS-CoV-2: The emergence of JN.1 variant and its global impact.**
Guanyong Ou, Yang Yang, Shengjie Zhang, Shiyu Niu, Qingxian Cai, Yingxia Liu, Hongzhou Lu

71-74 **Comparison of the physicochemical properties of branded and generic glucose-added maintenance hypotonic infusion fluids to assess the potential for phlebitis and incompatibility with other drugs.**
Sawako Takei, Soh Katsuyama, Yusuke Hori

Review

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A systematic review of the mechanistic actions of microRNAs within integrated traditional Chinese medicine and western medical treatment for endometriosis

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SUMMARY Endometriosis (EM), also known as Zhengjia in traditional Chinese medicine, is a common disease that significantly impacts women's health. An integrated treatment approach combining traditional Chinese medicine (TCM) and western medicine has demonstrated significant clinical efficacy in the management of this condition. Specifically, it has been effective in addressing blood circulation and other diseases. MicroRNAs (miRNAs), which are molecules important in gene regulation, have been implicated in various physiologic and pathologic processes. In this review, we systematically summarized the potential mechanisms underlying the integrated EM treatment, with a focus on the role of microRNAs (miRNAs). Current research suggests that integrated TCM and western medicine treatment may exert their therapeutic effects on EM by influencing the expression of miRNAs. Through miRNA modulation, such a treatment approach may inhibit the growth of ectopic lesions and alleviate clinical symptoms. This review will shed light on the specific miRNAs that have been implicated in the integrated treatment of EM, as well as their potential mechanisms of action. By consolidating the existing evidence, we aim to provide clinicians and researchers with a clearer understanding of the therapeutic benefits of the integrated approach and potentially identify new avenues for improving clinical treatment outcomes. Ultimately, this review will contribute to the growing body of knowledge in this field, providing a basis for further research and the development of more targeted and efficient treatment strategies for EM.

Keywords endometriosis, microRNA, traditional Chinese medicine, herb

1. Introduction

Endometriosis (EM) is a chronic benign disease with malignant biological features (*1*). It is characterized by the presence of endometrioid tissue outside the uterine cavity, leading to chronic painful symptoms and various comorbidities, including infertility (*2*). The prevalence of EM ranges from 5% to 21% among women enduring pelvic pain and 5% to 50% among infertile women, affecting approximately 10% of reproductive-aged women (*3*). The impact of EM on patients is substantial and encompasses both physical and psychological aspects (*4,5*). The economic burden of EM is also noteworthy, with patients facing high medical expenses, work loss, and healthcare costs (*6,7*). Therefore, there is an urgent need to explore more efficient and cost-effective treatment options to alleviate the suffering and overall burden experienced by patients. Gynecology in relation to traditional Chinese medicine (TCM) has a 3,000-year history (*8*). EM is recorded as Zhengjia in TCM books, and first mentioned in Huangdi Neijing (*9*). TCM is widely used as Chinese medicinal compounds, patented Chinese patent medicines and acupuncture (*10*); and is a popular treatment for EM in China due to its significant therapeutic function and few side effects. Both Asian and international scholars are now experimenting with the combined use of TCM and western medicine for improved treatment of EM.

MicroRNAs (miRNAs) have emerged as critical regulators of gene expression (*11*). These small noncoding RNA molecules work by binding to the 3'untranslated region of target messenger RNA (mRNA),

thus modulating its stability (*12*). The regulation of miRNAs has been recognized in recent years as a therapeutic strategy in numerous diseases, with several drugs targeting specific miRNAs for therapeutic purposes (*13,14*). The role of miRNAs in the treatment of EM with TCM is currently an active area of research, and understanding the involvement of miRNAs in the mechanisms subserving TCM treatment for EM could provide valuable insights into their therapeutic effects and facilitate the identification of miRNA targets for possible intervention.

By summarizing the findings from previous clinical and experimental studies, we can gain a better understanding of the role of miRNAs in treatment approaches, and provide valuable insights into the superiority and effectiveness of this integrated treatment strategy. This review will shed light on the specific miRNAs that have been implicated in the integrated treatment of EM, as well as their potential mechanisms of action. We aim to provide clinicians and researchers with a clearer understanding of the therapeutic benefits of such an integrated approach and potentially identify novel avenues for improving clinical-treatment outcomes.

2. Research status of endometriosis

EM is a complex and heterogeneous disease that presents with various phenotypes, including ovarian endometriosis (OMA), superficial peritoneal lesions (SUP), and deep infiltrating endometriosis (DIE) (Figure 1) (*1*). It is worth noting that DIE lesions are typically multifocal rather than isolated (*15*).

The theory of retrograde menstruation is the most widely accepted pathophysiologic hypothesis for EM. However, other mechanisms, such as inflammation, immune dysregulation, hormones, genetics, epigenetics, and environmental factors may also contribute to the development of EM (*16*). However, how these mechanisms interact to give rise to different phenotypes of EM remains unclear. Further research is therefore required to elucidate the intricate interplay between these mechanisms and their roles in determining the phenotype of EM.

As EM is a disease with a significant global patient population and a long-standing history, it has become a major area of interest to both clinical and scientific researchers. The management of EM typically involves pharmacologic, surgical, or combination treatments (*17*); and numerous clinical studies have entailed the efficacy and outcomes of different treatment modalities on EM (*18-22*).

In this context, this article will primarily focus on the pharmacologic and nonsurgical methods for the treatment of EM; and we will review the advantages and disadvantages associated with these various approaches based on the existing literature. By synthesizing the available evidence, we expect to provide a comprehensive overview of the current treatment options for EM, facilitating informed decision-making for clinicians and patients alike.

3. Western medical treatment in endometriosis

There are currently several common clinical medical therapies available for the management of EM; these therapies can be classified into non-hormonal and hormonal treatments. Non-hormonal treatments for EM often involve the application of nonsteroidal anti-inflammatory drugs (NSAIDs), certain antidepressants and anticonvulsant medications that can be used as first-line treatments to alleviate the symptoms of

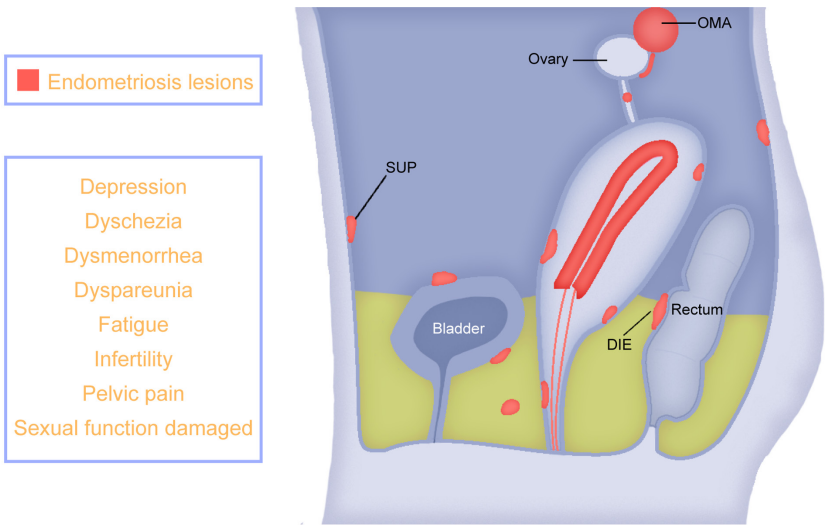

Figure 1. Clinical and disease characteristics of endometriosis. The endometrium can be ectopic to multiple sites. Three different phenotypes of endometriosis are common: superficial peritoneal endometriosis (SUP), ovarian endometriomas (OMA), and deeply infiltrating endometriosis (DIE). Endometrial tissue can also invade the myometrium leading to adenomyosis. Patients often experience physical and psychological discomforts.

dysmenorrhea (*23,24*). These medications can also assist in alleviating pain symptoms, but may not directly target the underlying mechanisms of EM.

Hormonal treatments, however, are commonly used in the management of EM. Combined oral contraceptives (COCs), which contain both estrogen and progestin, are often prescribed for the treatment of EM-related dysmenorrhea (*25*), and the continuous use of COCs has been shown to be superior in managing the dysmenorrheic symptoms of EM (*2*). Progestin use (another type of hormonal treatment) can be used in a cyclic or continuous manner to manage EM-associated pain; and continuous administration of progestogens (including medroxyprogesterone acetate) can help suppress the growth of endometrial lesions and alleviate pain symptoms (*26*). However, it is worth noting that the routine medical use required with these hormonal treatments can be tedious, leading to poor patient compliance (*2*).

In conclusion, non-hormonal treatments such as NSAIDs and certain medications may provide relief for the symptoms of EM, primarily by targeting pain management. Hormonal treatments, such as COCs, progestins, and gestrinone, offer additional options for managing dysmenorrhea and EM-associated pain. However, it is essential to consider individual patient preferences and factors such as patient compliance when selecting the appropriate treatment approach for EM.

Gonadotropin-releasing hormone analogues (GnRHa) comprise a popular treatment option, primarily due to their ease of use and high patient compliance (*27*). Emerging evidence from randomized controlled trials (RCTs) and meta-analyses suggests that GnRHa are effective in relieving symptoms of EM-associated pain (*9,28,29*). To mitigate the menopause-like symptoms of GnRHa treatment and to prevent bone loss, the addition of hormone replacement therapy (HRT) can be considered (*30*). While there is limited evidence on the optimal dosage, duration, and need for add-back HRT, the results of clinical trials combining GnRHa with HRT have shown promise (*31-33*). Individualized treatment plans, taking into account patient preferences and desired fertility outcomes, are crucial in the management of EM.

4. Traditional Chinese medicine in endometriosis

TCM has a long history of use in treating various gynecologic diseases, and new research on TCM for the treatment of EM is reported annually (Figure 2) (*34*). One study showed that combining dienogest, a conventional western medical treatment for EM, with TCM resulted in better outcomes in patients who did not respond well to standard western medical treatments (*9*). Moreover, RCTs and meta-analyses have revealed that TCM management of female infertility can lead to improved pregnancy rates compared to western medical drug therapy (*35*). These findings suggest that integrating TCM into the treatment of EM and female infertility may offer potential benefits and improved outcomes. However, it is important to note that additional research, including well-designed clinical trials, is needed to confirm the effectiveness and safety of combining TCM and western medicine in these treatments. Nonetheless, the increasing interest and research in this area indicate the potential value of an integrated approach. So we analyze the potential mechanisms of traditional Chinese medicine treatment through the following classic formulas for treating EM.

4.1. Guizhi Fuling pills (GZFLP)

GZFLP is a classical prescription in TCM that is commonly used for the treatment of EM. Its primary efficacy lies in invigorating the blood circulation and eliminating abnormal blood circulation (*33,36*). GZFLP is typically composed of five traditional Chinese herbs: *Cassia twig*, *Poria cocos*, *peach kernel*, *red peony root*, and *cortex moutan* (Paeonia suffruticosa *Andr.*) (*8*). When used together, GZFLP and western medicine have been found to significantly reduce the levels of leptin, vascular endothelial growth factor (VEGF), and interleukin-8 (IL-8) in the serum of EM patients. Moreover, this combination treatment has shown considerable effects in inhibiting the growth of ectopic lesions and relieving dysmenorrheic symptoms (*8*). It is intriguing that the activity and proportions of natural killer cells (NK cells) and $CD4^+$ T lymphocytes were significantly enhanced in the GZFLP-treatment group in a rat model of EM. This suggests that GZFLP may exert immune-modulating effects that lead to the regression of EM implants (*37*).

In addition to GZFLP, derivative drugs based on this

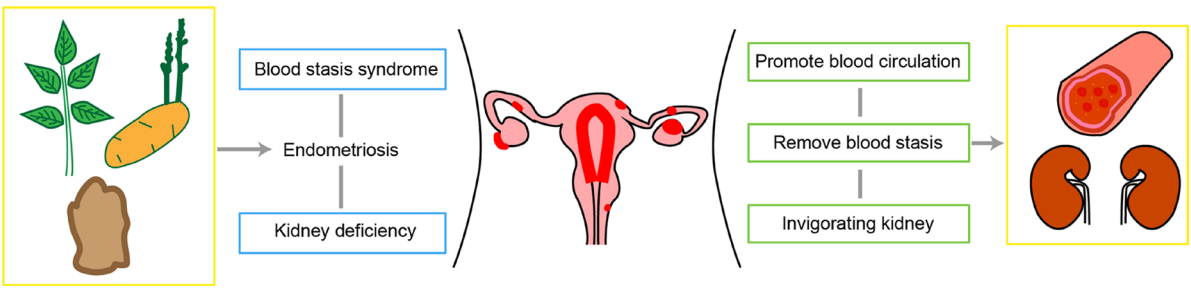

Figure 2. Endometriosis in Chinese traditional medicine treatment and theory. There is a correspondence between the clinical symptoms of endometriosis and the theories of Chinese medicine, which can be treated with Chinese herbs.

formulation have shown efficacy in treating EM (*33,37*). These findings suggest that TCM formulations may exert their therapeutic effects on EM through various mechanisms, including immune modulation and anti-inflammatory actions. However, further research is necessary to elucidate the exact mechanisms of action and to evaluate the safety and efficacy of these TCM formulations in human subjects.

4.2. Bushen Huoxue prescription (BSHXP)

BSHXP is commonly used in TCM to treat abnormal blood circulation in women, including EM. BSHXP is composed of *Rehmanniae Radix*, *Salviae miltiorrhizae Radix et Rhizoma*, *Puerariae lobatae Radix*, and *Ginseng Radix et Rhizoma*; and has been suggested to be effective and safe for EM patients (*38*). Combining BSHXP with laparoscopic surgery has shown promising results in improving clinical outcomes, as this combination treatment can significantly enhanced the clinical curative effect (*38*). However, more research is needed to further investigate the effectiveness and safety of this particular approach.

Additionally, Shaofu Zhuyu decoction, a TCM prescription known for activating the blood circulation, has demonstrated positive clinical effects when combined with western medicine in treating EM. This combination therapy has been found to improve endometrial receptivity scores, optimize oxidative stress levels, and reduce the recurrence rate for EM (*39*). While these TCM prescriptions show overall promise in treating EM, further high-quality analyses are necessary to provide more conclusive evidence regarding their effectiveness and safety.

4.3. Kuntai Capsule (KTC)

KTC is formulated with six herbs, *Rehmanniae Radix Praeparata*, *Coptidis Rhizoma*, *Paeoniae Radix Alba*, *Scutellariae Radix*, *Asini corii Colla*, and *Poria*. Authors have suggested that KTC inhibits the growth of ectopic lesions by regulating the level of tumor necrosis factor-α (TNF-α) and its downstream signaling molecules such as caspases and cytochrome c (*40*). These results provide some insights into the mechanisms by which KTC exerts its therapeutic effects on EM. KTC has also shown effectiveness in managing the peri-menopausal symptoms induced by postoperative GnRHa administration (*41*). This indicates the potential clinical value of KTC as a postoperative medication and in combined treatments with TCM and western medicine. However, it is important to note that clinical trials are necessary to further validate the efficacy and safety of KTC as a treatment for EM and peri-menopausal symptoms.

4.4. Fuke Qianjin capsule (FKQJC)

FKQJC is composed of eight TCMs: *Moghaniae Radix*, *Mahoniae Caulis*, *Andrographis Herba*, *Zanthoxylum dissitum Hemsl*, *Spatholobi Caulis*, *Angelicae sinensis Radix*, *Codonopsis Radix*, and *Rosae laevigata Radix.* FKQJC, when used in combination with dydrogestrel, inhibited the levels of VEGF, reduced inflammation, and diminished the incidence of adverse reactions in the treatment of EM (*42*). This suggests that FKQJC, along with dydrogestrel, may have potential benefits in managing EM symptoms. Additionally, it was observed that combining goserelin (a medication used to suppress hormone production) with FKQJC was both safe and effective in treating postoperative EM patients (*43*). This combination treatment also revealed improvements in hemodynamics and estrogen concentrations. Furthermore, when FKQJC was used in conjunction with gossypol acetate tablets, it improved ovarian function and attenuated the levels of carbohydrate antigen 125 (CA125, a biomarker for EM), retinol binding protein 4 (RBP4), and high mobility group box 1 (HMGB1) protein (*44*). These findings suggest that the combination of FKQJC and gossypol acetate exert potent therapeutic effects on ovarian function and biomarker levels in EM patients. However, more research, including clinical trials with larger sample sizes, is needed to further validate the safety, efficacy, and mechanisms of action of FKQJC in combination with these medications in the treatment of EM.

4.5. Sanjie Zhentong capsule (SJZTC)

SJZTC consists of the original powders of four natural plants products: *resina draconis*, *Panax notoginseng*, *Fritillariae thunbergii Bulbus*, and *Coicis semen*. In addition, a clinical study also revealed significant findings regarding acupuncture's effectiveness in reducing pain and serum CA125 levels in patients with EM, irrespective of the control intervention used (*39*). The use of SJZTC has shown promising results in effectively relieving dysmenorrheic symptoms (*41*), and both SJZTC itself and its two main components have demonstrated usefulness in reducing the development of the disease in a rat model (*41*). These findings indicate the potential benefits of SJZTC in managing EM symptoms and potentially slowing down the progression of the disease. However, further evaluation is needed of the efficacy and safety of SJZTC and its components in human subjects. Overall, these studies highlight the potential of acupuncture and SJZTC as alternative treatment options for EM, but additional study is needed to validate these findings and establish their clinical value.

4.6. Danefukang (DEFK)

The principal ingredients of DEFK are *Panax pseudoginseng* and *Salviae miltiorrhizae Radix et*

Rhizoma. DEFK has been shown to improve symptoms associated with EM, alleviate depression and anxiety, and reduce the levels of pro-inflammatory cytokines and CA125 (*42*). Investigators demonstrated that TCM treatment resulted in a significant drop in plasma levels of Prostaglandin F2α (PGF2α) and prostaglandin E2 (PGE2), while levels of 6-keto-PGF1 alpha, plasma beta-endorphin (beta-EP), and hydroxyproline (HYP) rose significantly. This suggests that TCM treatment not only provided symptom relief but also modulated the prostaglandin levels associated with EM (*43*). These findings demonstrate the potential effectiveness of DEFK and TCM in managing EM symptoms, and in modulating inflammatory and hormonal pathways. However, the findings necessitate elaboration to fully understand the underlying mechanisms of action and to validate them findings in larger clinical trials.

5. Roles of miRNAs in the integrated TCM and western medical treatment of EM

As research progresses, the roles of miRNAs in the treatment of EM are being explored. In one study, the authors investigated the effects of leuprolide acetate (a commonly used hormonal treatment) on the expression of miRNAs associated with EM (*45*). And their results showed that treatment with leuprolide acetate modulated the expression of seven miRNAs, and that expression correlated with the disease state. This suggests that miRNAs play a role in the pathogenesis of EM and could be potential targets for treatment. Another study focused on the use of aromatase inhibitors, specifically letrozole, in EM treatment (*44*). These authors ascertained that letrozole treatment significantly elevated the expression of a specific miRNA called miR-let-7f in Ishikawa cells and endometrial stromal cells (ESCs), and that it effectively arrested the migratory capability of endometrial cells. These studies highlight the role of miRNAs in the development and treatment of EM, and understanding their roles and deciphering their mechanisms of action should provide valuable insights for developing targeted therapies in the future.

Although miRNAs constitute a key factor in the treatment of many diseases, the role of miRNAs in the integrated treatment of TCM and western medicine remains arcane. By summarizing the roles and functions of miRNAs in TCM treatment, we can demonstrate the superiority of their integrated treatment in EM (Figure 3).

5.1. Role of miRNAs in GZFLP treatment

In a study that systematically summarized the active ingredients and pharmacologic mechanism of GZFLP, Kyoto Encyclopedia of Genes and Genomes (KEGG) results of the potential pathway of GZFLP included "miRNAs in cancer", which hinted at the critical importance miRNAs (*8*). A study on the molecular mechanisms and potential drug targets of Qu's formula (QUF) on EM indicated that "miRNAs in cancer" were also closely related to EM (*33*). Cais' Neiyi II prescription, of which GZFLP is the primary ingredient,

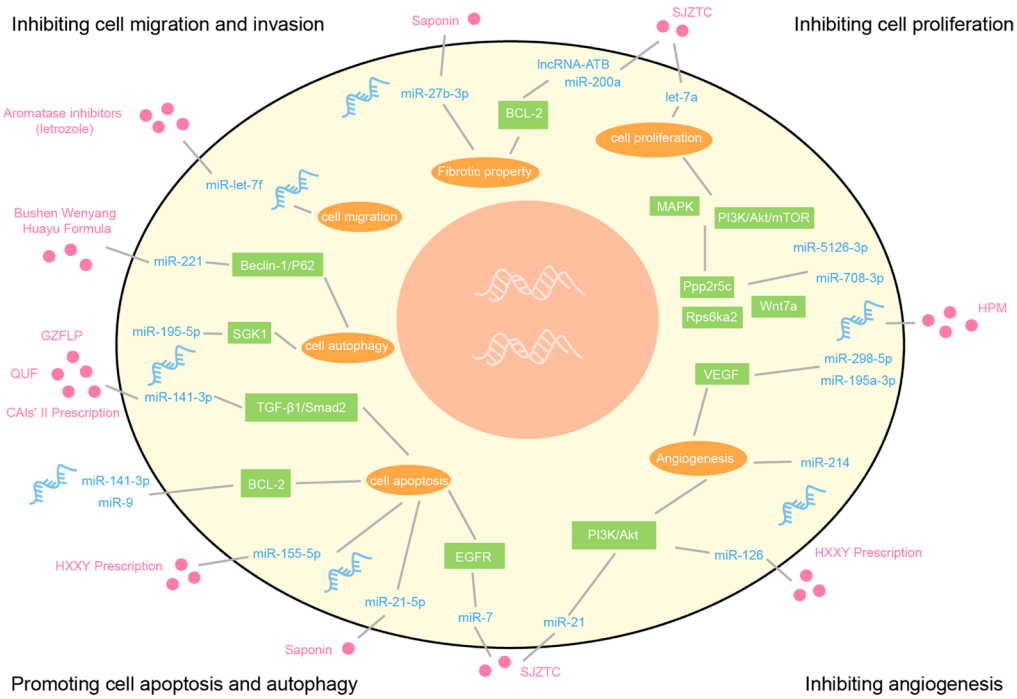

Figure 3. An overview of the role of miRNAs in the treatment of endometriosis by integrated Chinese and Western medicine. The combination of Western and Chinese traditional medicine regulates downstream genes and pathways by affecting the expression of miRNAs, through influencing cell proliferation, cell apoptosis and other functions to treat endometriosis.

was found to exert a therapeutic effect by elevating the expression levels of miR-141-3p in ESCs, inhibiting the activity of the downstream TGF-β1/Smad2 signaling pathway components, and promoting cellular apoptosis. GZFLP also affected the expression profile of miRNAs in a rat model of gynecologic diseases, suggesting that GZFLP was involved in a variety of biologic processes, including signal transduction and gene regulation. After treatment with high-dose of GZFLP, the expression of miR-187-3p and miR-330-5p was upregulated in the uterine tissue of EM rat models. According to another study on uterine fibroid patients, the expression of miR-214 increased and the miR-214-PI3K-AKT pathway was suggested to be one of the mechanisms with which to treat uterine fibroids as a women's abnormal blood-circulation disease. Although EM is usually known as a benign disorder, EM is also recognized as a precursor lesion for several malignant tumors and EM-related cancers in clinical practice (*46*). GZFLP possesses an anti-endometrial cancer activity, possibly *via* the inhibition of the lncRNA H19-mediated miR-195-5 pathway, suppressing tumor cell viability and promoting autophagy in endometrial cancer cells.

5.2. Role of miRNAs in activating blood circulation

Huoxue Xiaoyi granule (HXXY) is a traditional Chinese medicine that promotes blood circulation and improves abnormal circulatory function. When used to treat EM patients, HXXY was found to induce the differential expression of serum miRNAs. Specifically, certain miRNAs, such as miR-9-5p, miR-155-5p, and miR-202-3p, were up-regulated, while miR-216a-5p and miR-518a-5p were down-regulated. Among these, miR-155-5p was found to be associated with apoptosis. Treatment with HXXY resulted in smaller epithelial cells and reduced glandular cells in ectopic lesions, indicating that HXXY may restrained the growth of EM lesions by modulating the expression of miRNAs. This highlights the potential of miRNAs as novel targets in the treatment of EM.

Another effect of TCM treatments that promotes blood circulation and improves abnormal circulation is the inhibition of angiogenesis. This is achieved *via* the modulation of miR-126, which is highly expressed in vascular endothelial cells and regulates angiogenesis by affecting various transcription factors (*47*). Additionally, the TCM formula of Bushen Wenyang Huayu, which also promotes blood circulation, has been shown to inhibit autophagy by regulating the activities of the autophagy genes *Beclin-1* and *P62*; and this mechanism may be associated with a reduction in the expression levels of miR-221. These findings highlight the potential role of miRNAs in the therapeutic effects of TCM treatments in EM. Further research is required to sufficiently understand the mechanisms of action and to validate these findings in the clinical setting.

5.3. Role of miRNAs in herb-partitioned moxibustion (HPM)

HPM is a popular TCM treatment in the Chinese clinical field, particularly when combined with medical treatment (*48*). HPM is a combination of herbs and moxibustion and reflects the multiple functions of acupoint stimulation, moxibustion, and herb formulas, showing remarkable therapeutic effects in clinical use (*49*). Several studies have revealed that HPM reduced menstrual pain and improved quality-of-life for up to three months after a single treatment, or treatment combined with surgery or other medications (*50*). According to an investigation on the effect of HPM on miRNA expression profiles in nude rats using an EM model, five upregulated miRNAs and nine downregulated miRNAs were differentially expressed in the HPM group, but not in the model group. The Gene Ontology (GO) and KEGG results of the target genes of differentially expressed miRNAs were related to a combination of proteins, hormone metabolism, and downstream signaling pathways invloving cytochrome P450 drug metabolism. These differentially expressed miRNAs (miR-195a-3p and miR-298-5p) were found to be related to the *VEGF* and MAPK signaling pathways. After being verified by quantitative real-time PCR (qRT-PCR), miR-708-3p and miR-5126-3p were revealed to regulate the target gene expression of VEGF, protein phosphatase 2 regulatory subunit B gamma (*PPP2R5C*), Wnt-family member (*Wnt7a*) and ribosomal protein S6 kinase A2 (*RPS6KA2*). These studies preliminarily depicted the therapeutic mechanisms underlying HPM action, and indicated the possible functions of differentially expressed miRNAs and corresponding target genes that may regulate the mitogen-activated protein kinase (MAPK) signaling pathway and phosphoinositide-3-kinase/AKT serine threonine kinase/ mechanistic target of rapamycin kinase (PI3K/Akt/mTOR) signaling pathways. As a traditional treatment method, the role of HPM in reducing celluar adhesion, proliferation and angiogenesis deserves deeper exploration so as to elucidate the mechanisms underlying miRNA action.

5.4. Role of miRNAs in saponin action

As a well-known TCM herb, saponin is widely implemented in prescriptions to treat EM. A study conducted by Park *et al*. investigated the effects of saponin extracts from red ginseng on endometrial stromal cells (ESCs) in EM (*51*), and observed that treatment with saponin extracts led to a significant decline in the expression of miR-21-5p in ectopic ESCs; and that inhibition of miR-21-5p resulted in an increased apoptotic potential of ESCs. This suggests that saponins may exert their therapeutic potential on EM by modulating the expression of specific miRNAs,

such as miR-21-5p. Furthermore, another component of red ginseng called Rg3 was found to effectively alter the fibrotic properties of ESCs taken from EM patients (*52*). We posit that this effect may be attributed to the modulation of miR-27b-3p. These findings indicate that miRNAs, such as miR-21-5p and miR-27b-3p, play roles in the therapeutic effects of saponins extracted from red ginseng in EM. However, further research is needed to fully understand the mechanisms of action and to validate these findings in clinical settings. Nonetheless, these studies provide valuable insights into the potential of saponins from red ginseng to modulate specific miRNAs in the treatment of EM.

5.5. Role of miRNAs in analgesic Chinese medicine

Since SJZTC is frequently used in the treatment of diseases such as EM and several cancers, its work mechanism of action is of interest to scholars. Adenomyosis is characterized by the presence of endometrial stroma and glands within the myometrium, also constituting a disease caused by ectopic endometrium (*53*). SJZTC shows substantial clinical effects on treating adenomyosis, and several studies have suggested that its therapeutic mechanism is related to differentially expressed miRNAs. While these investigators ascertained that SJZTC treatment modulated the levels of miR-22-3p, miR-101-3p, miR-143-3p and miR-103-3p in the serum of EM patients, their exact mechanism of action needs further examination. These studies indicate that the activities of SJZTC are related to miRNAs in the treatment of tumors and adenomyosis and have provided avenues for exploring the possible mechanisms of miRNA action in the integrated TCM and western medical treatment of EM.

6. Discussion and Prospect

EM is a complex gynecological condition that causes various challenges and complications for women of reproductive age, including ovarian cysts, dysmenorrhea, and infertility. While western medical treatments are commonly used, they often possess disadvantages such as notable side effects and elevated recurrence rates. TCM treatments present a valuable alternative, as they offer potential benefits with fewer adverse effects. Many Chinese herbal medicines have shown promise in the treatment of EM, and understanding their mechanisms of action-particularly with regard to miRNAs-is of utmost importance. We herein analyzed articles that encompass pharmacology, animal experiments, and clinical trials so as to provide a comprehensive understanding of the role of miRNAs in the integrated treatment of EM using TCM and western medicine.

As EM belongs to a syndrome of abnormal blood circulation in classic TCM records, activating blood circulation and removing stagnation constitute its main treatment concept. miRNAs have been proven to display a wide range of effects in combined treatment, including affecting several key physiologic components and mechanisms such as cellular autophagy, proliferation, and migration; angiogenesis; and the release of prostaglandins (*54*). EM is now gradually being recognized as a fibrosis-related disease, and infertility in women with EM may be due to anatomical abnormalities caused by adhesions and fibrosis (*55*). In therapeutic fibrosis-related aspects, miRNAs are important in the therapeutic effects of TCM (*56*), as several studies on liver fibrosis indicated that miRNAs are related to regulatory mechanisms such as cellular growth, proliferation, apoptosis, differentiation, and angiogenesis in TCM treatments (*57*). Core herbs in the treatment of liver fibrosis are also the primary components in EM treatments, including *Salviae Miltiorrhizae* and *Astragali Radix* (*56*). These core prescriptions may regulate miRNA signaling and target the expression of *STAT3*, *PTGS2*, and *EGFR* (*56*). Researchers on heart fibrosis have demonstrated that cross-talk among miRNAs is involved in the underlying mechanism of TCM treatment (*54*). These anti-fibrotic effects of TCM are based on countering metabolic dysregulation, maintaining autophagic balance, and targeting miRNAs (*54*).

The exact mechanism of miRNAs in TCM prescription for EM treatment remains to be explored. The understanding of miRNAs as biomarkers on the degree of disease remission is insufficient. The exact mechanism of miRNAs affecting which gene's expression and which downstream pathway deserves to be further explored. The related researches on the principle of leading to a favorable reproductive outcome for the patients are few and lack experiments. The exploration of mechanism is conducive to the better research and development of related drugs and the combination of clinical treatments, so as to make contributions to relieving the symptoms of patients and improving the prognosis of patients.

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Original Article

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Usefulness of a new immunochromatographic assay using fluorescent silica nanoparticles for serodiagnosis of Thai patients with amebiasis

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SUMMARY A fluorescence immunochromatography (FIC) kit was developed recently using fluorescent silica nanoparticles coated with a recombinant C-terminal fragment of the surface lectin intermediate subunit (C-Igl) of *Entamoeba histolytica* to establish rapid serodiagnosis of amebiasis. We further evaluated the system using serum samples from 52 Thai patients with amebiasis. Of the patients, 50 (96%) tested positive using FIC. The samples were also tested using enzyme-linked immunosorbent assay (ELISA) with C-Igl as the antigen. Two samples were negative on ELISA but positive on FIC. The correlation coefficient between the fluorescence intensity using FIC and the optical density value using ELISA was 0.5390, indicating a moderate correlation between the two tests. Serum samples from 20 patients with malaria and 22 patients with *Clostridioides difficile* infection were also tested using FIC. The false-positive rates were 4/20 (20%) and 1/22 (4%) in patients with malaria and *C. difficile* infection, respectively. Combining the data from the present study with our previous study, the sensitivity and specificity of FIC were determined to be 98.5% and 95.2%, respectively. The results of the 50 samples were studied using a fluorescence scope and a fluorescence intensity reader, and the findings were compared. Disagreements were found in only two samples showing near-borderline fluorescence intensity, indicating that the use of scope was adequate for judging the results. These results demonstrate that FIC is a simple and rapid test for the serodiagnosis of amebiasis.

Keywords laboratory diagnosis, *Entamoeba histolytica*, lectin intermediate subunit, fluorescence immunochromatography

1. Introduction

Amebiasis, caused by the protozoan parasite *Entamoeba histolytica*, is a major disease in both developing and developed countries. Annually, an estimated 50 million cases of colitis and liver abscesses result in 55,000 deaths worldwide (*1*). Laboratory diagnosis of amebiasis by the detection of organisms, antigens, or DNA of *E. histolytica* is crucial for the early initiation of treatment (*2-4*). Serological tests to detect antibodies against *E. histolytica* are also useful, particularly in cases of extra-intestinal amebiasis. Several recombinant proteins of *E. histolytica* have been identified as candidates for the serodiagnosis of amebiasis (*5-9*).

We have previously demonstrated that the recombinant C-terminal fragment of the surface lectin intermediate subunit (C-Igl) of *E. histolytica* is useful as an antigen for enzyme-linked immunosorbent assays (ELISA) (*10-12*) as well as for multiple infectious disease detection systems (*13*). We recently developed a sensitive immunochromatographic kit using fluorescent silica nanoparticles coated with C-Igl for rapid serodiagnosis of amebiasis (*14*). However, all serum samples from the patients with amebiasis in the study were obtained from Japan.

Therefore, in the present study, we examined serum

samples from Thai patients with amebiasis to determine whether this kit is suitable for a wider application in Asia. Because several false-positive results were found in serum samples from patients with malaria and *Clostridioides difficile* infection in a previous study (*14*), further evaluation was performed using additional serum samples from patients with these infectious diseases. We also evaluated the use of a handheld fluorescence scope to facilitate the reading of the results.

2. Materials and Methods

2.1. Serum samples

Serum samples from a total of 52 patients with amebiasis in Thailand were obtained from the King Chulalongkorn Memorial Hospital. Of the patients, 30 samples were from patients with liver abscesses and 4 were from amebic dysentery. The patients were confirmed to respond to metronidazole treatment. The remaining 18 suspected cases were determined based on positive serology. All of the samples tested positive using an indirect hemagglutination (IHA) test (IHA Cellognost® Amoebiasis, Behring Diagnostics, Marburg, Germany) with various titers ranging from 1:128 (lowest positive) to ≥ 1:4,096 (highest positive). These samples were also confirmed to be positive using an indirect fluorescent antibody (IFA) test at a titer of 1:64. IFA was performed as described previously (*15,16*). Serum samples from 20 patients with malaria diagnosed in Thailand and serum samples from 22 patients with *C. difficile* infection obtained from Tokai University Hospital in Japan were used for the evaluation. Serum samples from 60 healthy Japanese individuals with negative serology in the IFA test were used as negative controls for ELISA to determine the cut-off value. The serum samples were stored at -80ºC, -30ºC or -20ºC before use. The study conformed to the provisions of the Declaration of Helsinki (as revised in 2013, *https://wma.net/what-we-do/medical-ethics/declaration-of-helsinki*). This study was approved by the review boards of Tokai University (17R017) and Chulalongkorn University (246/61).

2.2. Fluorescence immunochromatography

The immunochromatographic kit used in this study was prepared as described previously (*14*). Twenty µL of serum sample and 60 µL of 50 mM borate buffer (pH 8.0) were added to a tube containing freeze-dried silica nanoparticles. After rehydrating the particles, the solution was added to the well of an immunochromatographic cassette. After 30 min, the cassette was scanned using an immunochromatographic reader (DiaScan; Otsuka Electronics, Osaka, Japan), and the optimized value in the reader window was recorded. The measurement was repeated twice and the mean value was used. Based on a previous study, the cut-off fluorescence intensity value was set to 2,181 (*14*). Cassettes showing various fluorescence intensities were selected using an immunochromatographic reader and the fluorescence intensity of each sample was measured using a handheld fluorescence microscope. Each cassette was set in a QD Scope (Furukawa Electric Advanced Engineering, Ichihara, Japan) and then judged as positive or negative by three raters independently, based on the recognition of an obvious fluorescent band with the naked eye. The fluorescence intensity was measured again using a DiaScan α reader, and the value was used for the analysis of correlation with judgment based on the fluorescence scope.

2.3. Enzyme-linked immunosorbent assay

ELISA using recombinant C-Igl as an antigen was performed as previously described (*11*). The wells of 96-well flat-bottom Costar EIA/RIA plates (Corning Incorporated, Kennebunk, ME) were incubated with 100 ng of C-Igl in 50 mM sodium bicarbonate buffer (pH 9.6) overnight at 4°C. The wells were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween) and then treated with PBS containing 1% skim-milk for 1 h. A total of 100 µL of serum diluted 1:400 with PBS was added to each well and incubated for 1 h at 23°C. After washing with PBS-Tween, 100 µL of horseradish peroxidase-conjugated goat immunoglobulin G (IgG) to human IgG (whole molecule; MP Biomedicals, Solon, Ohio) diluted 1:2,000 with PBS containing 1% skim-milk was added to each well and the wells were incubated for 1 h at 23°C. After being washed with PBS-Tween, the wells were incubated with 200 µL of substrate solution (0.4 mg/mL of *o*-phenylenediamine in citric acid-phosphate buffer [pH 5.0] containing 0.001% hydrogen peroxide). After 30 min, the reaction was stopped by the addition of 50 µL of 2 M H_2SO_4 and the optical density (OD) value at 490 nm was measured using a SpectraMax i3 plate reader (Molecular Devices Japan, Tokyo, Japan). The cut-off value for a positive result was defined as an OD value > 3 standard deviations above the mean of 60 healthy negative controls.

2.4. Statistical analysis

Descriptive statistics including frequency and percentage were calculated for characteristics of the samples. Mean and Standard divisions were calculated for the continuous variables. Sensitivity and Specificity for the tests were calculated using the data from the present study as well as the data from the previous study. The level of significance was fixed at $P = 0.05$ and any value less than or equal to 0.05 was considered to be statistically significant. Prism 6 (GraphPad Software, San Diego, Calif., USA) was used for plotting the values and for analysis.

3. Results

3.1. Evaluation of fluorescence immunochromatography using serum samples from Thai patients with amebiasis

The 52 serum samples from Thai patients with amebiasis were tested using FIC. The correlation between fluorescence intensity and the IHA titer is shown in Figure 1. Of the samples, 50 tested positive for FIC. One of the four samples with the lowest IHA-positive titer (1:128) and one of the five samples with an IHA titer of 1:256 were scored as negative on FIC. However, all of samples with IHA titers ≥ 1:512 were positive on FIC indicating that the sensitivity of FIC in Thai patients was 96.2% (95% CI, 86.8-99.5).

3.2. Comparison of fluorescence immunochromatography and the enzyme-linked immunosorbent assay

The 52 serum samples from Thai patients with amebiasis were also examined using ELISA with recombinant C-Igl as the antigen. The correlation between the fluorescence intensity on FIC and the OD value on ELISA is shown in Figure 2. Using an OD cut-off value of 0.201 based on the mean plus 3 standard deviations of samples from healthy Japanese population, two samples were scored as negative, demonstrating a sensitivity of 96.2% for ELISA. One of the two serum samples with negative results had the lowest positive IHA titer, and the other had an IHA titer of 1:1024; these were positive for FIC. The correlation coefficient between the fluorescence intensity on FIC and the OD value on ELISA was 0.5390, indicating a moderately positive correlation between the two tests.

3.3. Specificity of fluorescence immunochromatography

Because several false-positive reactions were recorded in patients with malaria and *C. difficile* infection in a previous study, further evaluation of serum samples from patients with these infectious diseases was performed in the present study. Four of 20 serum samples (20%) from the malaria patients and one of the 22 serum samples (4.5%) from patients with *C. difficile* infection were scored as positive for FIC (Figure 3). When the five samples were tested by ELISA and IFA, none were positive, indicating that the positive reaction to FIC was a false positive. In the evaluation of FIC, by the addition of data from the present study to our previous study, the sensitivity and specificity were determined to be 98.5% ([80 + 50]/[80 + 52]; 95% CI, 94.6-99.8) and 95.2% ([122 + 37]/[125 + 42]; 95% CI, 90.8-97.9), respectively.

3.4. Evaluation of reading of the fluorescence immunochromatography result using a hand-held viewer

Fifty cassettes showing a range of fluorescence intensity values were read using a QD Scope fluorescence viewer (Figures 4A and 4B). The correlation between the positive rates by the three raters using a QD Scope and the fluorescence intensity read by DiaScan is shown in Figure 4C. Among the serum samples showing a fluorescence intensity of less than 2,181, two samples with values of 2,072 and 2,071 were scored as positive by all three and two of the three raters, respectively. The

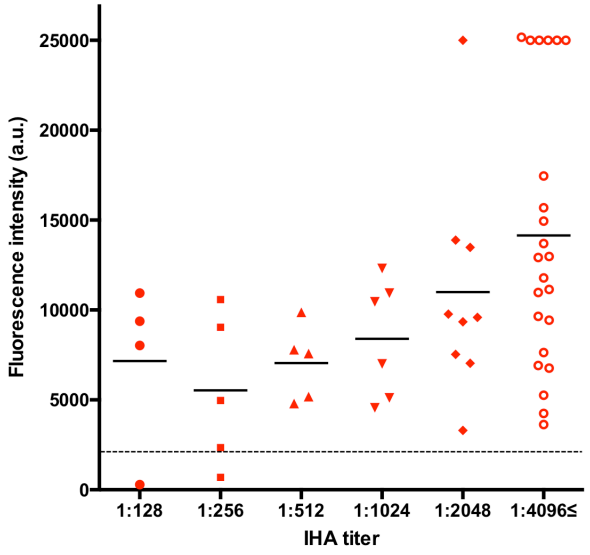

Figure 1. Correlation between fluorescence intensity on fluorescence immunochromatography and the indirect hemagglutination titer of 52 serum samples from Thai patients with amebiasis. The horizontal bars show the mean intensity. The broken line shows the cut-off value. For samples with fluorescence intensity above the measurement range using a DiaScan α reader, the value was recorded as 25,000.

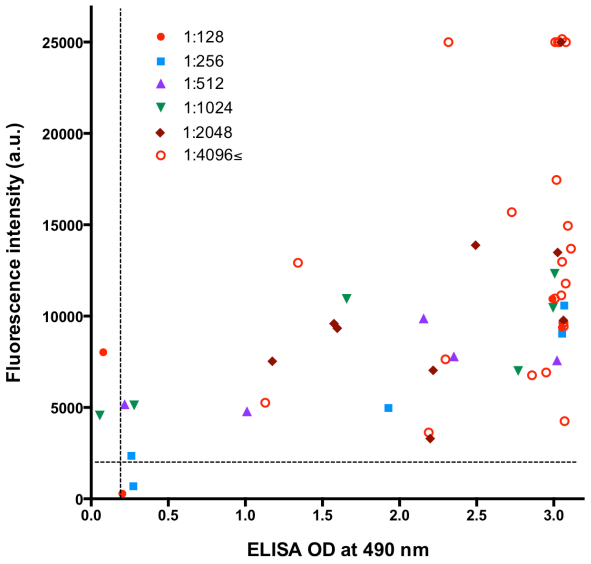

Figure 2. Correlation between fluorescence intensity on fluorescence immunochromatography and the enzyme-linked immunosorbent assay optical density value of 52 serum samples from Thai patients with amebiasis. The broken lines show the cut-off value. The indirect hemagglutination titers are shown by various types of dots.

results of the other samples were the same by the two reading methods.

4. Discussion

The present study demonstrated the high sensitivity of the FIC kit for testing serum samples from Thai patients with amebiasis. The use of C-Igl for the serodiagnosis of amebiasis by ELISA and FIC was originally studied using serum samples from patients with amebiasis collected in Japan (*11,14*). ELISA using C-IgL was useful for the epidemiological surveys of *E. histolytica*-infection in China (*10,12*). It has also been demonstrated that C-Igl is a useful antigen for studying microfluidic devices in China (*17*). In an evaluation of a microsphere-based multiplex assay using C-Igl, the sensitivity and specificity were 100% using serum samples from patients with amebic liver abscesses from Bangladesh with serum samples from Japan as negative controls (*13*). These results suggest that C-Igl is a useful antigen for the serodiagnosis of amebiasis in all over Asia using several methods, including FIC. This may be because the primary structure of C-Igl is well-conserved among *E. histolytica* strains derived from various countries (Tachibana *et al*., unpublished data).

In addition, this study demonstrated that the sensitivity of the FIC was comparable to that of ELISA using same samples. Recombinant C-Igl was used as the antigen in both tests, which confirmed the suitability of C-Igl despite the different detection systems used. ELISA is useful for testing large numbers of samples, whereas the FIC may be useful for testing small numbers of samples. It would be useful to have several different assays with different characteristics for the serodiagnosis of amebiasis. As false-positive reactions were recorded in two of nine samples from patients with malaria and in one of ten samples from patients with *C. difficile* infection in a previous study (*14*), further evaluation of serum samples from patients with these infectious diseases was performed in the present study. In this study, there was a relatively high rate of false-positive results in serum samples from patients with malaria on testing using FIC, whereas only one of the 22 serum samples from patients with *C. difficile* infection was false-positive. As these serum samples tested negative using ELISA with the same antigen, this rules out the possibility that the false-positivity rate on FIC was due to the existence of common epitopes

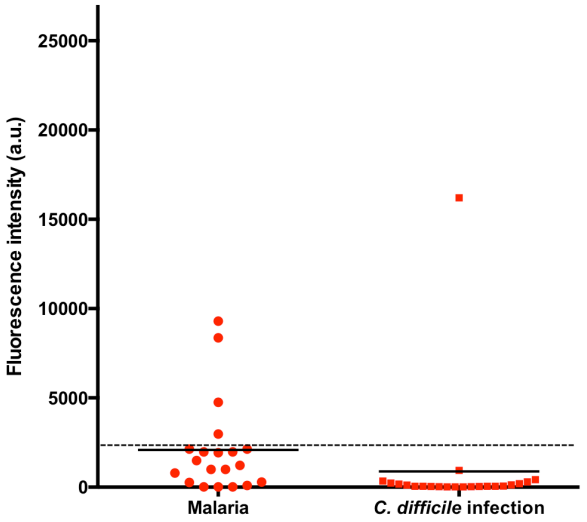

Figure 3. Fluorescence intensity on fluorescence immunochromatography of serum samples from patients with malaria and *C. difficile* infection. The horizontal bars show the mean intensity. The broken line shows the cut-off value.

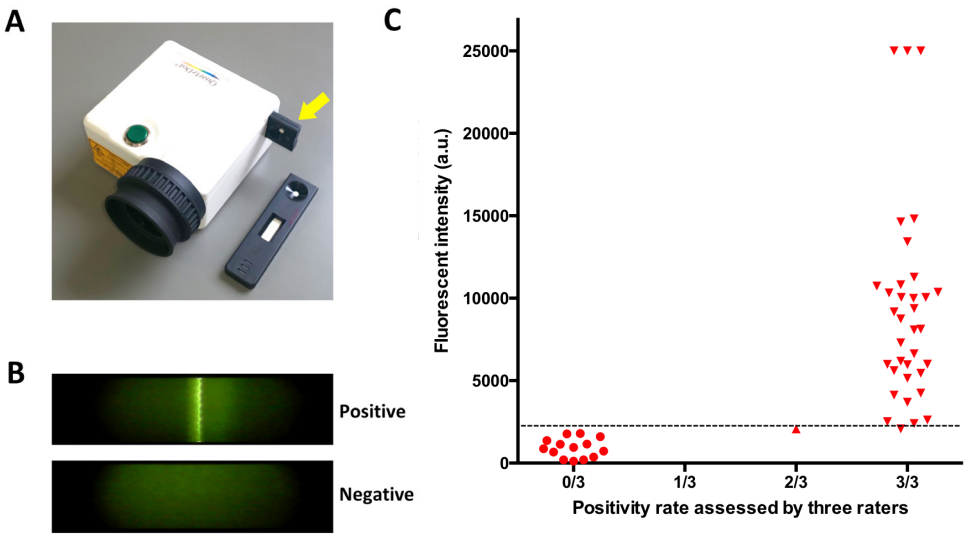

Figure 4. Application of a QD Scope for assessment of positive serology on fluorescence immunochromatography (FIC). (A) Photograph of the QD Scope and a cassette used in this study. The arrow indicates the inserted cassette. **(B)** Photograph of a fluorescent band in the window of the cassette. Representative pattern of positive and negative cases on FIC. **(C)** Correlation between the fluorescence intensity on immunochromatography and the positivity rate assessed by three raters using a QD Scope. The broken line shows the cut-off value on FIC.

between C-Igl and *Plasmodium* antigens. There is a chance that the sera of some malaria patients contain an unknown factor that affects the reaction to FIC. If such a factor exists, the factor can be removed during the washing step after the incubation of serum samples with C-Igl, using an ELISA system. The principle of this FIC system is that C-Igl on the surface of fluorescent silica nanoparticles and another C-Igl on the membrane are linked by bivalent IgG and/or divalent IgM molecules specific for C-Igl (*14*). Another possible system for capturing specific antibodies bound to C-Igl on the particles is the use of anti-human IgG and IgM antibodies on the membrane. It will be valuable to test the system in future studies because of the possibility that false positives will be reduced in addition to the detection of IgG and IgM separately (*18*).

In our previous study, the sensitivity and specificity of the FIC were 100 % and 97.6%, respectively (*14*); and by combining the present data, the values decreased to 98.5% and 95.2%, respectively. However, these values are comparable to the sensitivity and specificity of commercial ELISA and IHA kits of 69.0-100% and 87.5-99.8%, respectively (*2,19-22*).

Fluorescence assays have the advantage of higher intensity compared with visible wavelengths; however, specialized equipment is required to detect fluorescence (*18,23*). A handheld reader of fluorescence intensity, such as DiaScan, α is compact and useful for quantitative evaluation, but relatively expensive, and its program must be customized for the kit (*14*). In contrast, fluorescence viewers are economical and versatile without customization (*24*). This study showed that the results obtained using the two reading methods were consistent, except for samples with borderline values. Additionally, the fluorescence viewer exhibited excellent interrater reliability. This study demonstrated that the use of a handheld fluorescence scope was adequate for reading the results of the FIC assay.

In conclusion, the FIC kit using fluorescent silica nanoparticles and C-Igl appear to be potentially useful for the simple and rapid serodiagnosis of amebiasis in Asia. This study confirmed that the assay has high sensitivity and specificity except for a 20% false positive rate in patients with malaria. Additionally, the use of a fluorescence scope was effective in distinguishing between positive and negative results.

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Original Article

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Inhibitory effects of kaempferol, quercetin and luteolin on the replication of human parainfluenza virus type 2 *in vitro*

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SUMMARY The eight flavonoids, apigenin, chrysin, hesperidin, kaempferol, myricetin, quercetin, rutin and luteolin were tested for the inhibition of human parainfluenza virus type 2 (hPIV-2) replication. Three flavonoids out of the eight, kaempferol, quercetin and luteolin inhibited hPIV-2 replication. Kaempferol reduced the virus release (below 1/10,000), partly inhibited genome and mRNA syntheses, but protein synthesis was observed. It partly inhibited virus entry into the cells and virus spreading, and also partly disrupted microtubules and actin microfilaments, indicating that the virus release inhibition was partly caused by the disruption of cytoskeleton. Quercetine reduced the virus release (below 1/10,000), partly inhibited genome, mRNA and protein syntheses. It partly inhibited virus entry and spreading, and also partly destroyed microtubules and microfilaments. Luteolin reduced the virus release (below 1/100,000), largely inhibited genome, mRNA and protein syntheses. It inhibited virus entry and spreading. It disrupted microtubules and microfilaments. These results indicated that luteolin has the most inhibitory effect on hPIV-2 relication. In conclusion, the three flavonoids inhibited virus replication by the inhibition of genome, mRNA and protein syntheses, and in addition to those, by the disruption of cytoskeleton *in vitro*.

Keywords Virus replication, flavonoid, recombinant green fluorescence protein-expressing hPIV-2 without matrix protein

1. Introduction

Human parainfluenza virus type 2 (hPIV-2) is one of the major human respiratory tract pathogens of infants and children. hPIV-2 is a member of the genus *Rubulavirus* in the family *Paramyxoviridae*, and it possesses a single-stranded, non-segmented, negative stranded RNA genome of 15,654 nucleotides (*1*). hPIV-2 has 7 structural proteins, NP, V, phospho (P), matrix (M), F, HN and large (L) proteins. The gene order of hPIV-2 is 3'-(leader)-NP-V/P-M-F-HN-L-(trailer)-5'. All genes of hPIV-2 were sequenced by our group (*2-7*). Monoclonal antibodies (mAbs) were made, and antigenic diversity of clinical isolates was investigated by Tsurudome (*8*). The infectious hPIV-2 from cDNA clone was constructed by Kawano, and it was shown that its growth property was the same as that of control natural hPIV-2 (*9*).

In the present investigation, eight flavonoids which have inhibitory effect on major viruses (*10*) were tested for hPIV-2 growth, and it was found that three flavonoids, kaempferol, quercetin and luteolin, out of the eight had dose-dependent inhibitory effect on hPIV-2. The three had no or sufficiently low cytotoxicity at the concentration used in the present investigation (*11-15*). To investigate the effects of the flavonoids on viral genome synthesis, virus RNA was prepared and analyzed by PCR and real-time PCR. To elucidate the effects of the three flavonoids on mRNA synthesis, cDNA was synthesized using oligo(dT) primer and PCR was carried out. Virus protein expression was observed by indirect immunofluorescence study using mAbs against NP, F and HN proteins of hPIV-2 (*8*). The inhibitory effects of

the three flavonoids on cell-to-cell spreading of hPIV-2 were analyzed using a recombinant green fluorescence protein-expressing hPIV-2 without matrix protein (rhPIV-2ΔMGFP) (*9,16,17*). The number of viruses released from infected cells was determined. Cytoskeleton was reported to have an important role in paramyxovirus replication. Actin microfilaments are important in the hPIV-3 life cycle, specifically at the level of viral transport and replication (*18*). Tubulin also acts as a positive transcription factor for *in vitro* RNA synthesis by Sendai virus (*19*). The effects of the three flavonoids on actin microfilaments and microtubules were analyzed using rhodamine phalloidin and anti-tubulin α mAb, respectively.

2. Materials and Methods

2.1. Flavonoids

Eight flavonoids, apigenin ($C_{15}H_{10}O_5$: molecular weight (MW) 270.24), chrysin ($C_{15}H_{10}O_4$: MW 254.24), hesperidin ($C_{28}H_{34}O_{15}$: MW 610.56), kaempferol ($C_{15}H_{10}O_6$: MW 286.24), myricetin ($C_{15}H_{10}O_8$: MW 318.24), quercetin dihydrate ($C_{15}H_{10}O_7 \cdot 2H_2O$: formula weight 338.27), rutin ($C_{27}H_{30}O_{16}$: MW 610.52) and luteolin ($C_{15}H_{10}O_6$: MW 286.24) were purchased from Fuji Film Wako Pure Chemical (Osaka, Japan).

Apigenin was extracted from the flowers or leaves of various plants, for example parsley and chamomile. Chrysin is a naturally occurring flavone chemically extracted from the blue passion flower (*Passiflora caerulea*). Hesperidin is contained in the envelope of citrus fruits. Kaempferol mainly exists in raspberries, capers, brussels sprouts, black beans and grapes. Myricetin is a naturally-occurring flavonoid found in many grapes, berries, fruits, vegetables, herbs, as well as other plants. Quercetin is in red grape wine, leaves of radish and fennel. Luteolin is in leaves of basil, parsley and spinach.

They were dissolved in an appropriate solvent or vehicle at a concentration of 10 mg/mL, and added to the cell culture. Apigenin was dissolved in methanol, chrysin in dimethyl sulfoxide (DMSO), hesperidin in phosphate buffered saline (PBS) with 1/10 volume of 1 mol/L NaOH added. Kaempferol, myricetin, quercetin dihydrate, rutin and luteolin were dissolved in ethanol. The flavonoid solutions were stored in aliquots of 50 μL at −80°C until use, and not reused. 5 μL/mL methanol, DMSO or ethanol in culture medium was not toxic to the cells examined by cell culture microscope.

2.2. Virus and recombinant virus

The virus and the recombinant virus were approved by the relevant biosafety committees of Suzuka University of Medical Science and Mie University. hPIV-2 (Toshiba strain) was used. rhPIV-2ΔMGFP was constructed according to the method described previously (*9,16,17*), and it was shown that it did not produce infectious virus particles without addition of M protein gene *in trans* (data not shown). The virus titer was determined using Vero cells and the titer was about 1×10^5 $TCID_{50}$/mL.

2.3. Cell line and cultivation of cells

$LLCMK_2$ cells (rhesus monkey kidney cell line) were cultured in a flat-bottomed 24-well plate in 1 mL culture medium. Minimum essential medium α (MEMα: Fuji Film Wako Pure Chemical), supplemented with 2% fetal calf serum (FCS) and 0.1 mg/mL kanamycin, was used. The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. After three days, when the cells became confluent (5×10^5 cells), the medium was changed to MEMα with 0.5% FCS and 0.1 mg/mL kanamycin. The flavonoid solution was added to the cells, and the cells were infected with hPIV-2 (3×10^2 $TCID_{50}$).

2.4. Cytopathogenic assay

Cell fusion was observed at four days post infection under a cell culture microscope.

2.5. RNA preparation, cDNA synthesis, real-time PCR and PCR

RNA was extracted from the cells (2×10^6 cells) cultured in a flat-bottomed 6-well plate using TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's method. cDNA was synthesized with 1 μg RNA using Reverse Tra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) and NP gene specific primer (nucleotide number 1661-1679: 5'-CAACATTCAATGAATCAGT-3'). Real-time PCR was performed on the ABI PRISM 7700 Sequence Detection System (Life Technologies, Tokyo, Japan) using TaqMan Probe (1932-1956: 5'-FAM-AAGCACCGGATTTCTAACCCGTCCG-TAMRA-3'), forward primer (1851-1875: 5'-ACACACTCATCCAG ACAAATCAAAC-3'), and reverse primer (1958-1980: 5'-TGTGGAGGTTATCTGATCACGAA-3').

cDNA was synthesized with 1 μg RNA using forward primers for NP (nucleotide number 1,081-1,100: 5'-CATGGCCAAGTACATGGCTC-3'), F (5,821-5,840: 5'-CCCTATCCCTGAATCACAAT-3') and HN (7,741-7,760: 5'-ATTTCCTGTATATGGTGGTC-3') and superscript II reverse transcriptase (Invitrogen), and PCR was carried out with forward primers for NP (nucleotide number 1,081-1,100), F (5,821-5,840) and HN (7,741-7,760), and reverse primers for NP (1,466-1,489: 5'-CC TCCGAGTATCGATTGGATTGAA-3'), F (6,661-6,681: 5'-TGTCACGAGACGTTACGGACA-3') and HN (8,481-8,500: 5'-GAACTCCCCTAAAAGAGATG-3') genes and Ex Taq (Takara Bio, Kusatsu, Japan).

2.6. Detection of messenger RNA (mRNA)

cDNA was synthesized with 1 μg RNA using oligo(dT) primer and superscript II reverse transcriptase (Invitrogen), and PCR was carried out with forward primers for NP (nucleotide number 1,081-1,100: 5'-CATGGCCAAGTACATGGCTC-3'), F (5,821-5,840: 5'-CCCTATCCCTGAATCACAAT-3') and HN (7,741-7,760: 5'-ATTTCCTGTATATGGTGGTC-3') genes of hPIV-2, and reverse primers for NP (1,466-1,489: 5'-CC TCCGAGTATCGATTGGATTGAA-3'), F (6,661-6,681): 5'-TGTCACGAGACGTTACGGACA-3') and HN (8,481-8,500: 5'-GAACTCCCCTAAAAGAGATG-3') genes and Ex Taq (Takara Bio).

2.7. Immunofluorescence study

To detect virus proteins in the infected cells, the cells were fixed with 3.7% formaldehyde solution in PBS at room temperature for 15 min. The cells were further incubated with 0.05% Tween-20 in PBS at room temperature for 15 min to detect NP protein that exists mainly in the cytoplasm, or 3 min to detect F and HN proteins that are both in the cytoplasm and in the cell membrane, washed with PBS, and incubated with a mouse mAb against NP, F or HN protein of hPIV-2 at room temperature for 30 min. After washing with PBS, the cells were incubated with Alexa 488 conjugated secondary antibody anti-mouse IgGs (Invitrogen) at room temperature for 30 min, and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Actin was detected using rhodamine phalloidin (Invitrogen) and microtubules were observed using anti-tubulin α mAb against sea urchin tubulin α (clone B-5-1-2, Sigma-Aldrich, St Louis, MO, USA) at four days of cultivation. The cells were fixed with 3.7% formaldehyde solution in PBS at 37°C for 15 min, washed with PBS, and further incubated with 0.05% tween 20 in PBS at 37°C for 3 min to detect actin and for 15 min to detect microtubules.

2.8. Cell-to-cell spreading of hPIV-2

The flavonoid was added to the cells, and immediately after the addition, the cells were infected with rhPIV-2ΔM-GFP (1×10^4 $TCID_{50}$), and cultured for four days. They were then fixed with 1.2% formaldehyde solution in PBS at room temperature for 15 min and observed under a fluorescence microscope.

3. Results

3.1. Inhibitory effects of the three flavonoids

Different doses of the flavonoids (1 μg to 50 μg) were added to the 1 mL cell culture medium, and immediately after the addition the cells were infected with hPIV-2 (3×10^2 $TCID_{50}$), and cultured for four days. The cell fusion was observed under cell culture microscope at four days post infection. The three exhibited dose-dependent inhibitory effects. Kaempferol, quercetin dihydrate and luteolin almost completely inhibited hPIV-2 induced cell fusion, at 25 μg/mL, 20 μg /mL (17.9 μg /mL as quercetin) and 20 μg/mL, respectively.

3.2. Effect of the flavonoids on the release of hPIV-2

The supernatants of the cells were harvested at four days post the flavonoid addition and virus infection. The harvested supernatants were diluted, infected to the cells, and the virus titer was determined by the observation of cell fusion at four days post addition of the supernatants. Figure 1 shows the titer of the virus of the supernatants, indicating that the three flavonoids inhibited the virus release dose-dependently. Both kaempferol (25 μg/mL) and quercetin dihydrate (20 μg/mL) inhibited the release of the virus into the medium (below 1/10,000). Luteolin (20 μg/mL) almost completely inhibited the virus release (below 1/100,000). The three flavonoids of the concentration mentioned above were used in the following experiments.

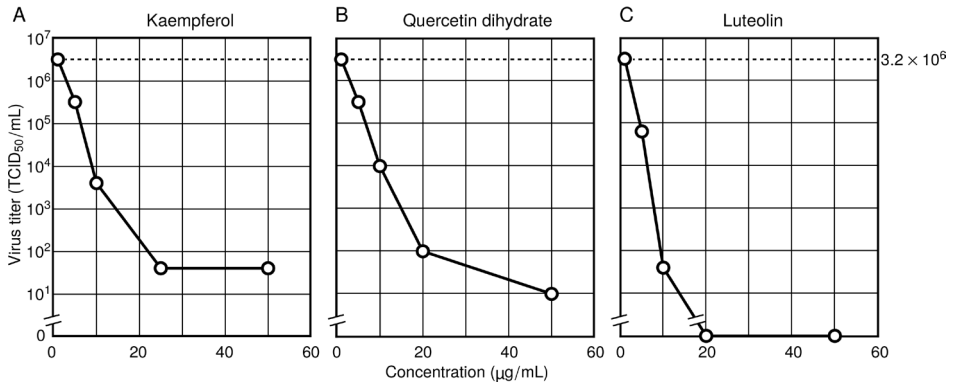

Figure 1. Dose-dependent virus release inhibition into culture medium by the three flavonoids. Both kaempferol (25 μg/mL) (**A**) and quercetin dihydrate (20 μg/mL) (**B**) inhibited the virus release into the medium (below 1/10,000). Luteolin (20 μg/mL) (**C**) almost completely inhibited the virus release (below 1/100,000).

Table 1. Effect of the three flavonoids on viral genome RNA synthesis analyzed by quantitative real-time PCR

	No drug	Kaempferol	Quercetin	Luteolin
Number of virus genome copies	1,219,753	79,435	47,291	3,757
Relative amount of virus genome RNA	1	0.065	0.039	0.003

The flavonoids were added to the cell culture, which was then infected with hPIV-2 and cultured for four days. RNA was extracted and viral genome RNA was analyzed by real-time PCR. The value of the virus infected cells was shown as 1. Kaempferol: 0.065, quercetin: 0.039 and luteolin: 0.003. Luteolin has a good effective inhibition ability to hPIV-2.

3.3. Effects of the flavonoids on viral genome RNA and mRNA syntheses

RNA was prepared from the flavonoid-treated infected cells using TRIZOL reagent according to the manufacturer's method at four days post infection, and viral genome RNA was analyzed by both real-time PCR and PCR. Viral mRNA was also analyzed by PCR.

Real-time PCR shows that kaempferol, quercetin and luteolin almost completely inhibited viral genome RNA syntheses. The inhibitory effect of luteolin was the most outstanding (Table 1).

Quite similar results were obtained by PCR (Figure 2). In control cells, no bands were seen. In virus-infected cells NP, F and HN bands were clearly detected. HN has two bands, because the primers might bind to similar nucleotide sequences. In kaempferol-treated infected cells, both NP gene and F gene bands were slightly detected, but the HN gene band was very faint. In quercetin-treated infected cells, NP and F genes were visible. In luteolin-treated infected cells, NP, F and HN gene syntheses were almost completely inhibited. These results are similar to those of real-time PCR.

Figure 3 shows the three flavonoids also inhibited viral mRNA syntheses. NP, HN or F mRNA was not detected in control cells, but all three mRNAs were clearly seen in virus infected cells. In kaempferol or quercetin-treated infected cells, NP, F and HN mRNA were slightly detected. However, in luteolin treated-infected cells, no visible band of NP, F or HN of mRNA was detected. The result of gene syntheses and mRNA syntheses were in good accordance. These results indicated that the three flavonoids had inhibitory effects on both viral genome and mRNA syntheses.

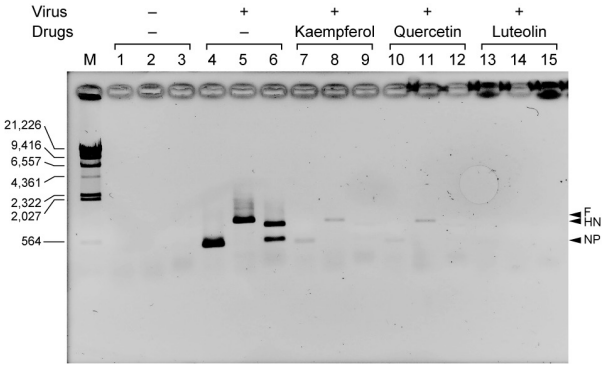

Figure 2. Effect of the flavonoids on viral genome RNA synthesis analyzed by PCR. NP, F and HN genes were detected using specific primers. In virus infected cells, NP, F and HN genes were clearly detected. In kaempferol-treated cells, NP and F genes were faintly detected. Quercetin inhibited HN gene synthesis. Luteolin almost completely inhibited the three gene syntheses.

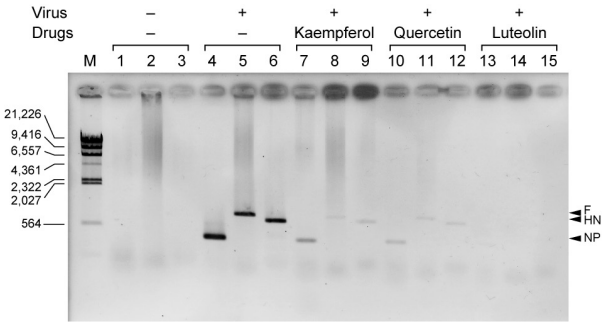

Figure 3. Effect of the flavonoids on viral mRNA synthesis analyzed by PCR. RNA was extracted from the cells and cDNA was synthesized by oligo(dT) primer. NP, F and HN mRNA were detected using specific primers. Both kaempferol and quercetin slightly inhibited NP, F and HN mRNA syntheses. Luteolin almost completely inhibited NP, F and HN mRNA syntheses.

3.4. Effects on protein syntheses

Indirect immunofluorescence study was carried out using mAbs against NP, HN and F proteins to examine the effects of the three flavonoids on viral protein syntheses at four days post infection (Figure 4). In non-infected cells, NP, HN or F protein was not detected (data not shown). Figures 4A, 4B and 4C show the NP, F and HN protein expression in hPIV-2 infected cells, respectively. In hPIV-2 infected cells, NP, F and HN proteins were observed in almost all the cells: NP protein was observed in many big strong fluorescent dots mainly in the cytoplasm, while F and HN proteins were seen in small dots in the cytoplasm and on the cell surface. Kaempferol itself has auto-fluorescence (Figures 4D, 4E and 4F). It only slightly inhibited the protein syntheses: there were many big fluorescence dots of NP protein. A large number of cells had many small fluorescent dots of F and HN proteins of infected cells cultured with kaempferol (Figures 4D, 4E and 4F, respectively). Quercetin largely inhibited NP, F and HN protein syntheses. (Figures 4G, 4H and 4I). In luteolin-treated cells, only a few positive cells were found (Figures 4J, 4K and 4L; NP, F and HN, respectively), indicating that luteolin inhibited almost completely the synthesis of viral proteins.

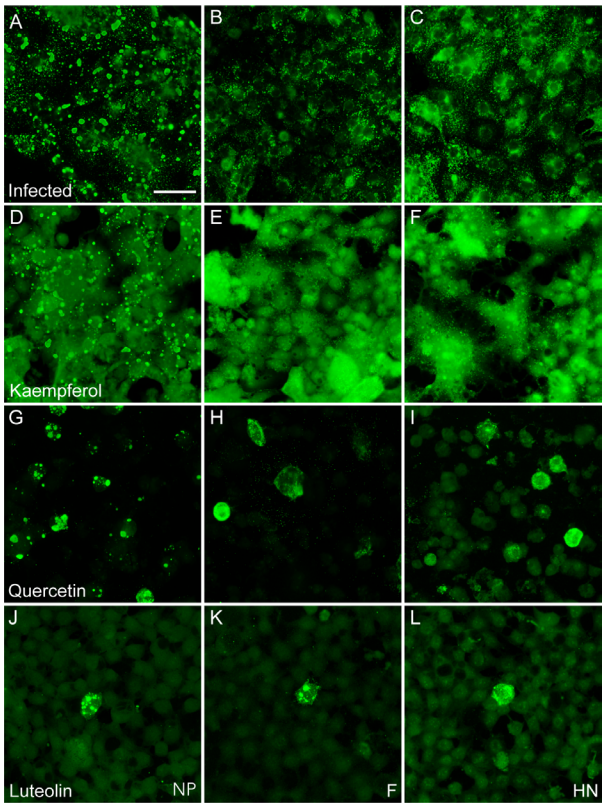

Figure 4. Effect of the three flavonoids on viral protein synthesis. NP (**A**), F (**B**) and HN (**C**) are virus infected positive controls. Kaempferol only partly inhibited NP (**D**), F (**E**) and HN (**F**) protein syntheses: there are many positive spots. Kaempferol itself has auto green fluorescence, indicating that kaempferol penetrated into the cells and stayed there. Quercetin largely inhibited NP (**G**), F (**H**) and HN (**I**) protein syntheses. Luteolin inhibited NP (**J**), F (**K**) and HN (**L**) protein syntheses: there are few positive cells. Bar: 50 µm.

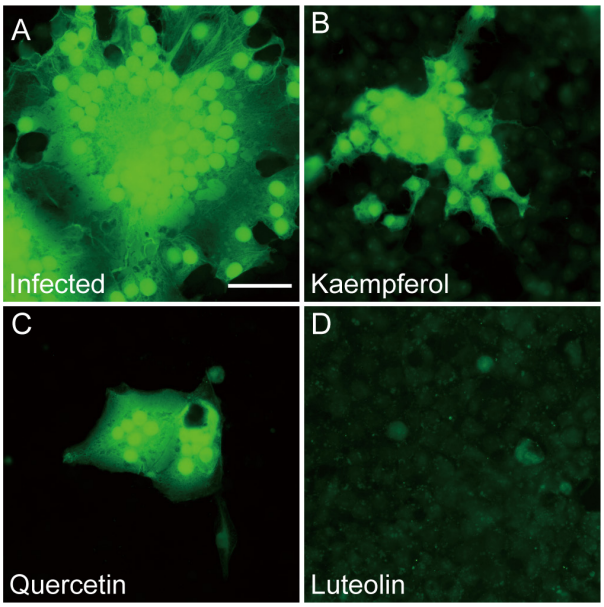

Figure 5. Effect of the flavonoids on multinucleated giant cell formation. The cells were added with the flavonoids and infected with rhPIV-2ΔM-GFP. Immunofluorescence study was carried out at four days of post infection. In positive control cells, many multinucleated giant cells with strong fluorescence were observed (**A**). Kaempferol (**B**), quercetin (**C**) largely inhibited giant cell formation: the fused cell size is small. Luteolin inhibit cell fusion. There were no fused fluorescent cells. Bar: 50 µm.

3.5. Effects on the multinucleated giant cell formation

The flavonoids were added to the cells, and immediately after that they were infected with rhPIV-2ΔM-GFP (1×10^4 $TCID_{50}$) and cultured for four days. The cells were fixed with 1% paraformaldehyde and observed under the fluorescence microscope. Figure 5A is a positive control. There are many multinucleated giant cells with strong fluorescence. Kaempferol (Figure 5B) and Quercetin (Figure 5C) largely inhibited the giant cell formation: there were a small number of fused cells, but the size is smaller than that of the positive control. In luteolin-treated cells (Figure 5D), no fluorescent cells were found, indicating that luteolin almost completely inhibited the infection of hPIV-2 to the neighboring cells, and as a result multinucleated giant cell formation was not observed. The multinucleated giant cell formation, the number of released virus from the cells, genome RNA syntheses and protein syntheses were in good accordance.

3.6. Effects on actin microfilaments

The three flavonoids were added to the cell culture without virus infection and cultured for four days. F-actin was stained with rhodamine phalloidin. Figure 6A is the positive control: bundles of actin microfilaments were clearly seen. Kaempferol partly disrupted actin microfilaments (Figure 6B), but quercetin (Figure 6C) and luteolin (Figure 6D) caused severe damage in actin microfilaments. These results showed the damage to actin microfilaments caused some inhibitory effects on the release of virus from the cells to culture medium.

3.7. Effects on microtubules

The cells were added with the flavonoids without virus infection, and cultured for four days. Microtubules were stained with anti-tubulin α mAb. Figure 7A is the positive control: microtubule networks were seen in the cytoplasm. Kaempferol (Figure 7B), quercetin (Figure 7C), and luteolin (Figure 7D) partly disrupted microtubules. Microtubules are also important for virus replication, so one of the causes of virus replication inhibition had some relation with the disruption of microtubules.

4. Discussion

Three flavonoids, kaempferol, quercetin and luteolin were tested for hPIV-2 replication *in vitro*. In the present investigation, the effects of the three flavonoids on genome RNA synthesis, viral mRNA synthesis, protein expression, multi-nucleated giant cell formation and

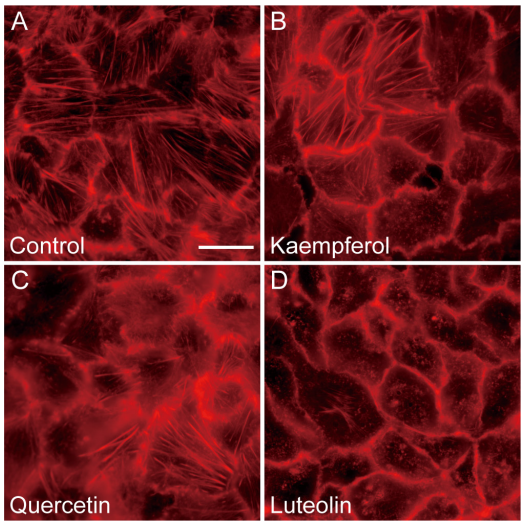

Figure 6. Effect of the flavonoids on actin microfilaments. The flavonoids were added to the cell culture without virus infection, and cultured for four days. The cells were stained with rhodamine phalloidin. In non-treated cells (**A**), actin microfilaments were clearly seen. Kaempferol (**B**) partly destroyed the filaments. Quercetin (**C**) and luteolin (**D**) caused damage the filaments. Bar: 50 μm.

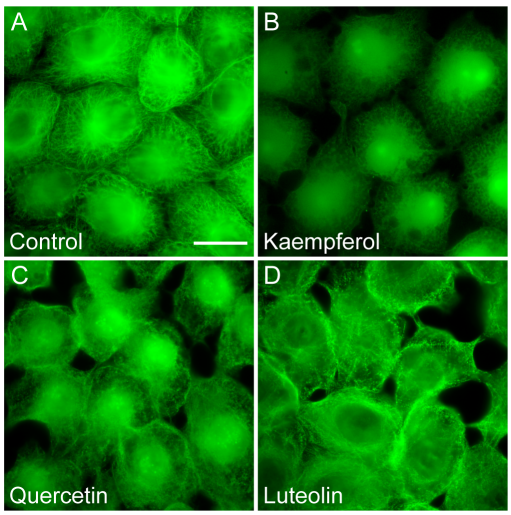

Figure 7. Effect of the flavonoids on microtubules. The flavonoids were added to the cell culture without virus infection, and cultured for four days. The cells were stained with anti-tubulin α mAb against sea urchin tubulin α. In non-treated cells (**A**), microtubules were clearly seen in the cytoplasm. Kaempferol (**B**) and quercetin (**C**) slightly destroyed microtubules. Luteolin (**D**) destroyed microtubules. Bar: 50 μm.

cytoskeleton (actin microfilaments and microtubules) were analyzed.

The three had inhibitory effects on hPIV-2 replication. Kaempferol, quercetin and luteolin reduced the release of the virus from the cells, and they had inhibitory effects on viral genome and mRNA syntheses. They inhibited largely the protein syntheses and multi-nucleated giant cell formation. They caused slight damage to actin microfilaments and microtubules, indicating that the inhibition of virus release was in part caused by the cytoskeletal damage.

Kaempferol has inhibitory activity against human cytomegalovirus (*20*). Kaempferol acts on the influenza virus neuraminidase and inhibits H1N1 and H9N2 virus (*21*). hPIV-2 also has hemagglutinin-neuraminidase (HN) protein, so kaempferol might act on HN protein, and inhibit release of hPIV-2 from the cells. It was also shown that kaempferol and kaempferol-7-*O*-glucoside (100 μg/mL) have strong inhibitory effect on human immunodeficiency virus 1 (HIV-1) reverse transcriptase (*22*). Kaempferol-3-*O*-glucoside binds to HIV-1 reverse transcriptase (*23*). Kaempferol exhibited potent inhibitory activity against feline calicivirus (*24*).

Anti-viral effect of quercetin on viruses were extensively investigated by many researchers. It has dose-dependent inhibitory effects on herpes simplex virus 1 (HSV-1) and HSV-2 in cell culture (*25,26*). Quercetin blocks binding and penetration of HSV-1 and -2 into host cells (*25*). It also inhibits H1N1, H5N2, H7N3 and H9N2 influenza virus *in vivo* (*27*). It may be an inhibitor of neuraminidase of type H1N1 and H7N9 influenza virus (*28-30*), and interacts with hemagglutinin of influenza virus (*31*), resulting in cell fusion between virus and host cells. Quercetin has anti-rhinovirus activity by inhibiting endocytosis, genome transcription and protein synthesis (*32*). In addition, it has inhibitory activity for many other viruses, such as cytomegalovirus (*12*), canine distemper virus (*33,34*), porcine diarrhea virus (*35,36*), dengue virus serotype 2 (*37*), *etc*.

Luteolin was also reported to have antiviral activity *in vitro*. For example, it has antiviral activity against HIV-1 (*38*), Epstein-Barr virus (*39*), severe acute respiratory syndrome-related coronavirus (SARS-CoV) (*40,41*), and Japanese encephalitis virus (*13*). It also has inhibitory activity for influenza A virus by interfering with the coat protein I complex expression (*14*).

Many investigators have shown the inhibitory effects of flavonoids on a wide range of viruses, for example, genistin inhibits adenovirus, arenavirus, HSV-1, HSV-2, human herpesvirus-8, rotavirus and respiratory syncytial virus (*43*), quercetin inhibits adenovirus (*10*), arenavirus (*43*) and coronavirus (*44*), luteolin inhibits coronavirus (*45*), kaempferol inhibits HSV-1 (*10*), myricetin inhibits Moloney murine leukemia virus and SARS-CoV (*10*), chrycin inhibits HSV-1 and coxsackie B virus (*10*), morin inhibits canine distemper virus and Moloney murine leukemia virus (*10*), *etc*.

The antiviral mechanisms of flavonoids are viral binding inhibition, inhibition of viral genome, mRNA and protein syntheses. In the present investigation, the inhibitory mechanisms are similar among the three flavonoids. The three flavonoids had inhibitory effects on viral genome RNA, mRNA and protein syntheses. They also inhibited multinucleated giant cell formation in size and number, indicating that they might inhibit virus entry and/or cell-to-cell spreading. In addition,

they caused damage in actin microfilaments and microtubules, resulting in the inhibition of virus release from the infected cells. These results are based on the *in vitro* study. Some researchers reported *in vivo* effects of quercetin (*27,32,36*). The next aim is to elucidate *in vivo* effects of the three flavonoids.

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Original Article

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Astragalus root increases Treg and Th17 involvement in embryo implantation and pregnancy maintenance by decreasing CTLA-4$^+$ Tregs

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SUMMARY Maintenance of pregnancy is highly dependent on the maternal immune system. High levels of regulatory T cells (Tregs) accumulate in the maternal placenta to suppress immunoreactivity against fetal antigens. We assessed whether Astragalus root (AsR) and AsR-containing Kampo medicines modulate immunoreactivity and thereby increase mouse litter size. AsR-exposed murine splenocytes exhibited significantly increased IL-2 secretion. In AsR-exposed mice, total Tregs were significantly increased, whereas cytotoxic T lymphocyte antigen 4 (CTLA-4)-positive Tregs were decreased in AsR-exposed mice. Tregs express IL-2 receptor subunit alpha and are activated by IL-2. CTLA-4 interacts with B7 expressed in antigen-presenting cells (APCs) with high affinity, and CTLA-4/B7 signaling plays a critical role in inhibiting APC activity, thereby suppressing CD4$^+$ T cell proliferation and activation. The decrease in CTLA-4$^+$ Tregs in AsR-exposed mice is thought to induce an increase in CD4$^+$ T cells, leading to increased IL-2 secretion from CD4$^+$ T cells followed by Treg activation. Th17 cells prevent trophoblast apoptosis, resulting in trophoblast invasion into the decidua. AsR increases Th17 cells, thereby inducing dose-dependent increases in litter size. Although Keishikaogito (KO)- and Ogikenchuto (OK)-exposed mice exhibited increased IL-2 secretion and splenic Tregs, KO also increased CTLA-4$^+$ Tregs. Therefore, KO promoted immunosuppression by increasing CTLA-4$^+$ Tregs, which induced a decrease in Th17 and exerted little effect on litter size. Therefore, an increase in both Tregs and Th17 cells can be considered necessary for embryo implantation and pregnancy maintenance.

Keywords *Astragalus membranaceus*, Kampo medicine, IL-2, Treg/Th17, litter size

1. Introduction

Excessive immune responses to harmless environmental substances and self-antigens are known to cause autoimmune diseases, such as pollen allergy and inflammatory bowel disease. The proliferation and activation of regulatory T cells (Tregs) contribute to the suppression of immune responses against self-antigens, allergens, and allografts. Kakkonto, which has been used for the initial symptoms of colds such as chills, fever, and inflammatory congestive symptoms in the back and shoulder, was recently reported to induce immune tolerance (immunosuppression) against food antigens through the increase in Tregs in the intestinal mucosa (*1*). CD4$^+$ T cells differentiate into Th1, Th2, Th17, and Treg cells in response to T-cell receptor (TCR) stimulation by antigens presented on antigen-presenting cells (APCs). Tregs differentiated from CD4$^+$ T cells express abundant CD25 as the IL-2 receptor alpha chain and forkhead Box P3 (Foxp3) as the master transcription factor. CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs are activated by IL-2 released from CD4$^+$ T cells and can generate an immunosuppressive microenvironment by secreting suppressive cytokines such as TGF-α and IL-10. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) expressed on CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs has the ability to suppress the maturation of APCs, by which Tregs bind to B7 (CD80/86) expressed on APCs. Tregs are classified into thymic-derived Tregs (tTregs) and peripheral-induced Tregs (pTregs) based on their origin. Leaving the thymus, tTregs migrate toward peripheral lymphoid tissues, *e.g.*, lymph nodes and spleen, and peripheral nonlymphoid tissues, such as intestinal mucosa, lung, liver, adipose tissue, infected tissues, grafts, placenta, and tumors (*2*). pTregs in the placenta are required for the maintenance of pregnancy stability. The maintenance of pregnancy is highly dependent on the maternal immune system against a semialleogenic fetus. In fact, pTreg-deficient mice have

been reported to raise the possibility of fetal resorption to the uterus (*3*), and it has also been reported that pTregs highly accumulate in the placenta of a maternal mouse, concomitant with an absolute decrease in circulating Tregs (*4*).

Weight loss in female athletes and excessive dieting lead to irregular menstruation, amenorrhea, and pregnancy failure because of homeostasis breakdown. Kampo medicines effectively improve immune system disorders, frailty, and irregular menstruation, which are difficult to treat with chemical drugs, by restoring and maintaining homeostasis. Astragalus root, the root of *Astragalus membranaceus*, has been used as a tonic mixed in Kampo medicines to modulate the immune system. We recently reported that the hot water extract of Astragalus root (AsR) increased blood estrogen levels in female mice and enhanced murine ovarian β-oxidation through the activation of PPARα (*5*). Furthermore, AsR increased the expression of Wnt/β-catenin signaling factors contributing to endometrial proliferation and decidual formation (*6*). Based on this background, we verified the possibility that AsR and AsR-containing Kampo medicines improve embryo implantation and fertility by enhancing maternal immune tolerance against the fetus.

2. Materials and Methods

2.1. Animals

Five-week-old SPF/BALB/c female mice (18-19 g, n = 96) and 7-week-old SPF/BALB/c male mice (22-23 g, n = 10) wern e purchased from Japan SLC Inc. (Shizuoka, Japan) and housed and maintained under standardized conditions of temperature (25 ± 1°C) and humidity (55 ± 5%) in a light cycle room (light from 07:00 a.m. to 07:00 p.m.; dark from 07:00 p.m. to 07:00 a.m.). The mice were fed standard chow (CE-2; CLEA Japan, Inc., Tokyo, Japan) and used for the experiment after being acclimatized to the room for one week. All experiments were approved by the Animal Experimental Committee of Tohoku Medical and Pharmaceutical University (approval No. 22044-cn), and the experimental procedures were conducted in accordance with the ethical guidelines of the university.

2.2. Extractions of samples

The dried, chopped crude drugs were purchased from Tochimoto Tenkaidou Co. Ltd. (Osaka, Japan). Five grams of Astragalus root, the root of *Astragalus mongholicus* or *A. membranaceus*, was boiled in 600 mL of distilled water to reduce the final volume by half. The boiled solution was filtered and concentrated in a rotary evaporator under reduced pressure at 50°C, and then the filtrate was freeze-dried to obtain AsR. The formulations of Japanese herbal medicines were prescribed as follows: Boiogito (Astragalus root, 5.0 g; Atractylodes rhizome, 3.0 g; Ginger, 1.0 g; Glycyrrhiza, 2.0 g, Jujube, 4.0 g; Sinomenium stem and rhizome, 4.0 g), Keishikaogito (Astragalus root, 3.0 g; Cinnamon bark, 3.0 g; Ginger, 1.0 g; Glycyrrhiza, 2.0 g; Jujube, 4.0 g; Peony root, 3.0 g), Kigikenchuto (Astragalus root, 2.0 g; Cinnamon bark, 4.0 g; Ginger, 1.0 g; Glycyrrhiza, 2.0 g; Japanese Angelica root, 4.0 g; Jujube, 4.0 g; Peony root, 5.0 g), Ogikenchuto (Astragalus root, 1.5 g; Cinnamon bark, 3.0 g; Ginger, 1.0 g; Glycyrrhiza, 3.0 g; Jujube, 3.0 g; Peony root, 6.0 g). Each formulation was boiled in 600 mL of distilled water to reduce the final volume by half. The decoctions were filtered and concentrated by a rotary evaporator under reduced pressure at 50°C and then freeze-dried to obtain the extracts of Boiogito (BO), Keishikaogito (KO), Kigikenchuto (KK) and Ogikenchuto (OK). The yields of extracts were measured to determine the dosages to mice.

2.3. WST-8 assay

Splenocyte proliferation was determined by using a WST-8 assay. BALB/c female mouse (n = 6) spleen was removed under sterile conditions after euthanasia by isoflurane anesthesia, minced in RPMI medium (RPMI-1640, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), filtered through 40 μm mesh, and hemolyzed with red blood cell lysis buffer (pluriSelect Life Science, Leipzig, Germany). Splenocytes were suspended in RPMI medium containing 0.05 mM 2-mercaptoethanol (FUJIFILM Wako Pure Chemical Corporation), 10% heat-inactivated FBS (Gibco Fetal Bovine Serum, Qualified, ThermoFisher Scientific Inc, Tokyo Japan), antibiotic, and antimycotic (penicillin-streptomycin mixed stock solution, Nacalai Tesque Inc., Kyoto, Japan) and were adjusted to 5×10^4 viable cells per milliliter. Splenocyte suspension (100 μL) was seeded in each well of a 96-well plate and incubated with 10 μL of 0.4 mg/mL concanavalin A (concanavalin A from *Canavalia ensiformis*, Jack bean, Merck KGaA, Darmstadt, Germany) at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Culture medium-dissolved test samples were added to each well. After 24 h of incubation, the cell count reagent (Cell Count Reagent SF, Nacalai Tesque Inc.) was added to each well and incubated for 4 h. Absorbance was measured at 450 nm with a reference wavelength of 600 nm using a microplate reader (SH-1300 microplate reader, Corona Electric Co., Ltd., Ibaraki, Japan).

2.4. IL-2 secretion test

Six-week-old female BALB/c mice (19-20 g, n = 4 in each group) were allowed free access to water solutions for 6 groups for 14 days: 0.8 (w/v) % AsR, 2.25 (w/v) % KO, 3.1 (w/v) % BO, 12.9 (w/v) % OK, and 3.0 (w/v) % KK, and control groups. After a 4-day

withdrawal of these solutions, 50 μg of concanavalin A (Concanavalin A from *Canavalia ensiformis*, Jack bean, Merck KGaA) suspended in 50 μL of complete Freund's adjuvant (Merck KGaA, Darmstadt, Germany) was injected subcutaneously through the tail to stimulate T cells. Seven days later, *i.e.*, 25 days after the start of sample administration, all mice (n = 24) were euthanasia by excessive inhalation of isoflurane. The spleen was extirpated under sterile conditions, minced in RPMI medium (RPMI-1640, FUJIFILM Wako Pure Chemical Corporation), filtered through 40 μm mesh and hemolyzed with red blood cell lysis buffer (pluriSelect Life Science). Splenocytes were suspended in RPMI medium containing 0.05 mM 2-mercaptoethanol (FUJIFILM Wako Pure Chemical Corporation), 10% heat-inactivated FBS (Gibco Fetal Bovine Serum, Qualified, ThermoFisher Scientific Inc), antibiotic and antimycotic (Penicillin-Streptomycin mixed stock solution, Nacalai Tesque Inc.) and were adjusted to 5 × 10^6 viable cells per milliliter. One milliliter of splenocyte suspension was seeded in each well of 24-well plates and incubated with 10 μL of 0.4 mg/mL concanavalin A (Concanavalin A from *Canavalia ensiformis*, Jack bean, Merck KGaA) at 37°C in a humidified atmosphere containing 5% CO_2. The medium was collected 18 h and 27 h after culture initiation, and the IL-2 concentrations in the medium were measured by ELISA (Mouse IL-2 Quantikine ELISA Kit, R&D Systems Inc., Minnesota, USA).

2.5. Flow cytometry

2.5.1. Sample preparation

Six-week-old female BALB/c mice (19-20 g, n = 6 in each group) were allowed free access to water solutions for 6 groups for 14 days: 0.8 (w/v) % AsR, 2.25 (w/v) % KO, 3.1 (w/v) % BO, 12.9 (w/v) % OK, and 3.0 (w/v) % KK, and control groups. Astragaloside IV (Biosynth Ltd., Staad, Switzerland), and formononetin (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) were subcutaneously injected once a day at 10 mL/kg for 14 days. After a 4-day withdrawal of these solutions, 50 μg concanavalin A suspended in 50 μL complete Freund's adjuvant (Merck KGaA) was injected subcutaneously through the tail to stimulate T cells. Seven days later, *i.e.*, 25 days after the start of sample administration, all mice (n = 36) were euthanasia by excessive inhalation of isoflurane. The spleen was extirpated under sterile conditions, minced in RPMI medium (RPMI-1640, FUJIFILM Wako Pure Chemical Corporation), filtered through 40 μm mesh and hemolyzed with red blood cell lysis buffer (pluriSelect Life Science) to prepare splenocytes.

2.5.2. Cell staining and flow cytometric analysis

Before cell staining, splenocytes were fixed and permeabilized by Fixation/Permeabilization solution (Fixation/Permeabilization Concentrate and Diluent, ThermoFisher Scientific Inc.). Then, splenocytes suspended in 400 μL buffer were divided 100 μL into 4 tubes (A~D). For analysis of $CD4^+$ T cells, splenocytes in tube B were stained with Super Bright 600-labeled CD4 antibody. For analysis of Treg, IL-10^+ Treg, and CTLA-4^+ Treg cells in tube C were stained with Super Bright 600-labeled CD4 antibody, PE-Cyanine 5-labeled CD25 antibody, PE-labeled CD152 antibody, Alexa Fluor 700-labeled IL-10 antibody and Alexa Fluor 488-labeled Foxp3 antibody. For analysis of Th17 cells, splenocytes in tube D were stained with Super Bright 600-labeled CD4 antibody and eFluor 450-labeled IL-17A antibody. All antibodies for cell-staining were purchased from ThermoFisher Scientific Inc. Splenocytes in tube A were not stained with antibodies. After adding the antibodies, splenocytes in tubes B, C, and D were incubated on ice for 1 hour and then washed once with PBS. Measurements by flow cytometry of splenocytes in tubes A, B, C, and D were performed on an Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific Inc.), and these data were collected and analyzed by Attune NxT Software (Thermo Fisher Scientific Inc.).

2.6. Mating test

Six-week-old female BALB/c mice (18-19 g, n = 6 per group) were free-accessed to water solutions for 5 groups for 14 days: 0.4 and 0.8 (w/v) % AsR and 1.125 and 2.25 (w/v) % KO, and control groups. Then, 3 female mice per 10-week-old BALB/c male mouse were mated in the same cage. Pregnancy was assessed by their appearance and body weight gain from the mating day. Approximately 3 days before parturition, pregnant mice were housed singly. Three days after parturition, the number of neonatal mice per maternal mouse was counted as the litter size.

2.7. Statistical analysis

All data are expressed as the mean values ± standard deviations (SD). Comparisons among the means were performed using Sigma Stat statistical software ver. 2.03 (SPSS, Inc., CA, USA): Student's unpaired t test or one-way ANOVA followed by Dunnett's multiple comparison test was performed for comparisons against a control group. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Splenocyte proliferation evaluation and IL-2 secretion

To determine whether splenocyte proliferation was induced or inhibited, splenocytes harvested from female BALB/c mice were exposed to AsR, KO, BO,

OK, or KK. Compared with the control (100% of cell proliferation), AsR-treated splenocytes exhibited a substantial increase, and did not inhibit over the measured reaction concentration range (Figure 1A). KO-exposed splenocytes tended to exhibit increased cell proliferation, and the highest reaction concentrations of KO showed no increase in cell proliferation from the initial cell number. As shown in Figure 1B, BO, OK, and KK showed no increase in cell proliferation, but decreased cell proliferation at higher concentrations.

To determine whether IL-2, which is essential for Treg activation, was increased by AsR, KO, BO, OK, and KK extract, splenocytes harvested from female BALB/c mice were cultured. AsR-exposed mice showed significantly increased IL-2 secretion at 18 and 27 h after culture initiation, whereas KO- and OK-exposed mice showed significantly increased IL-2 secretion at 27 h (Figure 1C).

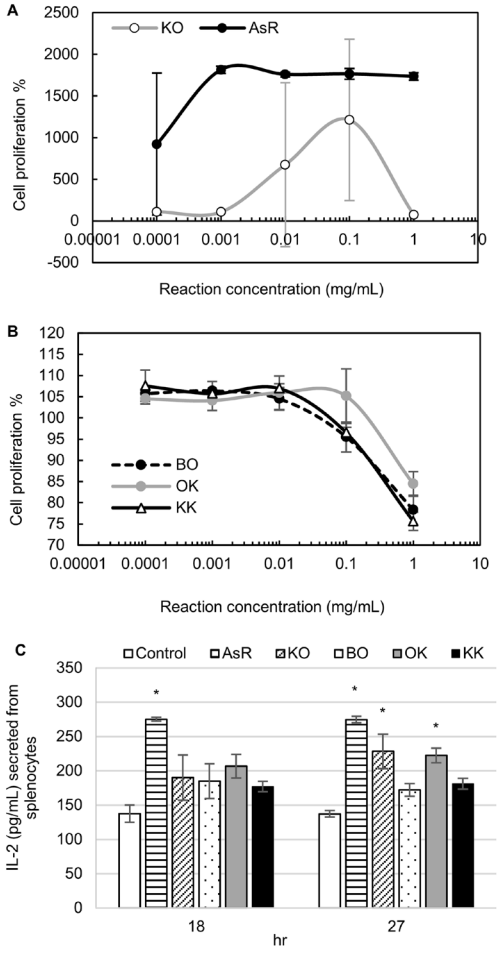

Figure 1. Effects of Astragalus root (AsR) and AsR-containing Kampo medicine exposure on splenocyte proliferation and IL-2 secretion from cultivated splenocytes. Splenocyte proliferation and IL-2 secretion levels from the splenocytes of AsR-, KO-, BO-, OK-, and KK-exposed female mice were compared with those in control mice. AsR-exposed splenocytes demonstrated dramatic increases in cell proliferation at 0.001-1.0 mg/mL, while KO-exposed splenocytes exhibited increased cell proliferation at 0.01-0.1 mg/mL (**A**). BO-, OK-, and KK-exposed splenocytes decreased cell proliferation at 1.0 mg/mL (**B**). AsR-exposed mice showed significantly increased IL-2 secretion levels from cultured splenocytes at 18 and 27 h of incubation, and KO and OK-exposed mice showed significantly increased IL-2 secretion levels at 27 h (**C**). AsR, Astragalus root; KO, Keishikaogito; BO, Boiogito; OK, Ogikenchuto; KK, Kigikenchuto. One-way ANOVA was used for data analysis, followed by Dunnett's multiple comparison test; $*P < 0.05$. The bar graphs and error bars represent the means ± SDs ($n = 4$).

3.2. Evaluation of splenic Treg and Th17 on AsR-exposed mice

To determine whether Tregs essential for accepting an allogeneic fetus to the decidua were induced by AsR, splenic Tregs were measured *via* flow cytometry analysis. After measuring the percentage of $CD4^+$ T cells in the splenic lymphocyte population, the percentage of $CD25^+$ $Foxp3^+$ T cells in the $CD4^+$ T-cell population was measured. Tregs were defined as $CD4^+$ $CD25^+$ $Foxp3^+$ T cells. Representative flow cytometry dot plots show gating (right upper quadrant) for $CD25^+$ $Foxp3^+$ T cells in $CD4^+$ T cells (Figure 2A). Compared with the control group, the AsR-administered mice had a significantly increased percentage of Tregs among $CD4^+$ T cells (Figure 2B).

To determine whether AsR generates an immunosuppressive environment by increasing the suppressive cytokine IL-10, splenic $IL\text{-}10^+$ Tregs were measured *via* flow cytometry analysis. After measuring the percentage of $CD4^+$ T cells in the splenic lymphocyte population, the percentage of $IL\text{-}10^+$ $Foxp3^+$ T cells was measured. $IL\text{-}10^+$ Tregs were defined as $CD4^+$ $IL\text{-}10^+$ $Foxp3^+$ T cells. Representative flow cytometry dot plots show gating (right upper quadrant) for $IL\text{-}10^+$ $Foxp3^+$ T cells in $CD4^+$ T cells (Figure 2A). Compared with the control group, $IL\text{-}10^+$ Tregs were significantly increased in AsR-administered mice (Figure 2C). To determine whether Th17 cells essential for promoting trophoblast invasion into decidua were induced by AsR, splenic Th17 cells were measured *via* flow cytometry analysis. After measuring the percentage of $CD4^+$ T cells in the splenic lymphocyte population, the percentage of $CD4^+$ $IL\text{-}17A^+$ T cells in $CD4^+$ T cells was measured. Th17 cells were defined as $CD4^+$ $IL\text{-}17A^+$ T cells. Representative flow cytometry dot plots show gating (right upper quadrant) for $CD4^+$ $IL\text{-}17A^+$ T cells in $CD4^+$ T cells (Figure 2D). Compared with the control group, AsR-exposed mice had a significantly increased percentage of Th17 cells among $CD4^+$ T cells (Figure 2E). An imbalance in the Treg/Th17 ratio has been observed in patients with recurrent spontaneous abortion. AsR-exposed mice showed no difference in the ratio compared with that of control mice (Figure 2F).

CTLA-4, known as CD152 expressed on Tregs, has the ability to strip off B7 (CD80/CD86) from APCs and impair the antigen-presenting activity of APCs, which leads to immunosuppression. To determine whether the expression of CTLA-4 on Tregs was increased or decreased by AsR, splenic $CTLA\text{-}4^+$ Tregs were

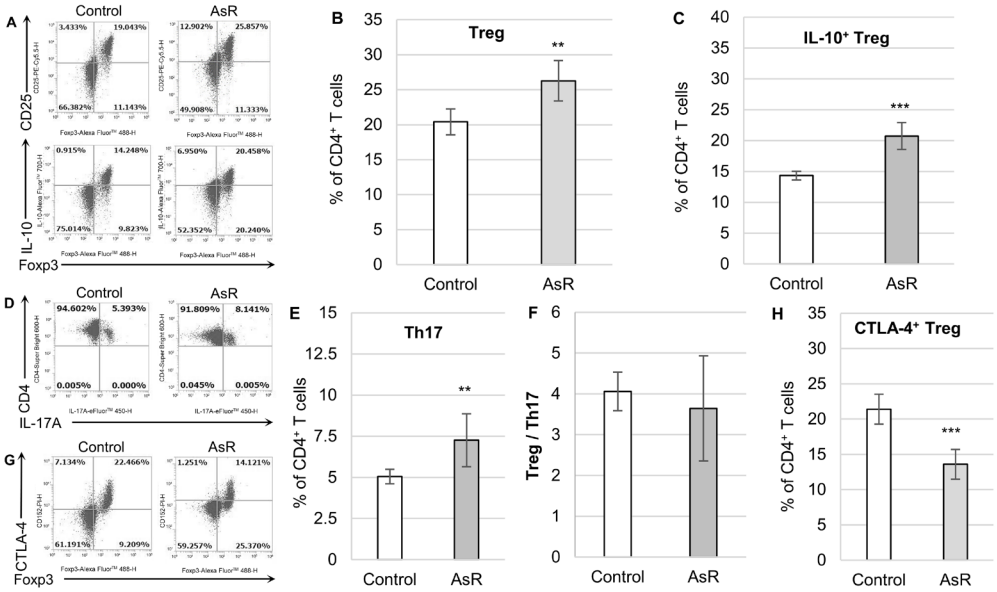

Figure 2. Effect of Astragalus root (AsR) exposure on splenic Tregs, IL-10$^+$ Tregs, Th17, Treg/Th17 ratio, and CTLA-4$^+$ Tregs. Tregs, IL-10$^+$ Tregs, Th17 cells, Treg/Th17 ratio, and CTLA-4$^+$ Tregs in CD4$^+$ lymphocytes of splenocytes prepared from AsR-exposed BALB/c female mice. Representative flow cytometric analysis of Tregs and IL-10$^+$ Tregs (**A**, CD25$^+$ Foxp3$^+$ T cells, or IL-10$^+$ Foxp3$^+$ T cells in CD4$^+$ T cells, right upper quadrant) in AsR-exposed and control mice. AsR-treated mice showed significantly increased Treg (**B**) and IL-10$^+$ Treg levels (**C**). Representative flow cytometric analysis of Th17 (**D**, IL-17$^+$ T cells in CD4$^+$ T cells, right upper quadrant) in AsR-exposed and control mice. AsR-exposed mice show an increased number of Th17 cells (**E**). The Treg/Th17 ratio was not significantly different (**F**). Representative flow cytometric analysis of CTLA-4$^+$ Tregs (**G**, Foxp3$^+$ CTLA-4$^+$ T cells in CD4$^+$ T cells, right upper quadrant) of AsR-treated and control mice. AsR-treated mice showed significantly decreased levels of CTLA-4$^+$ Tregs (**H**). AsR, Astragalus root. Student's *t* test was used for data analysis; $^{**}P < 0.01$, and $^{***}P < 0.001$. The bar graphs and error bars represent the means ± SDs ($n = 6$).

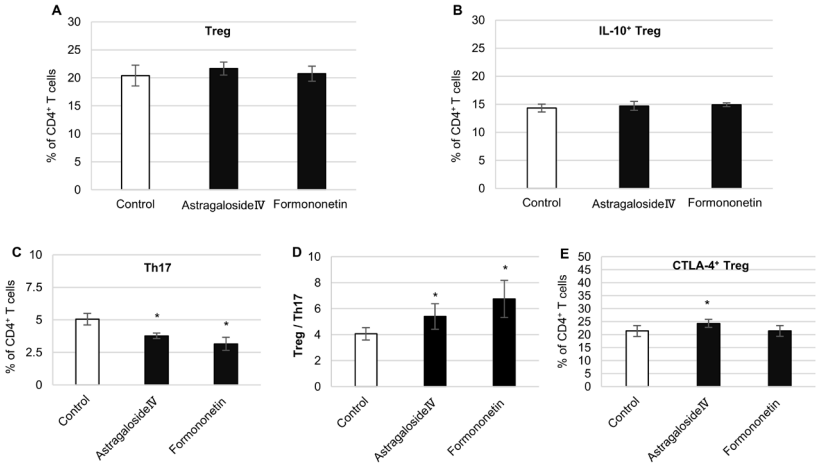

Figure 3. Effects of astragaloside IV and formononetin on splenic Tregs, IL-10$^+$ Tregs, Th17 cells, Treg/Th17 ratio, and CTLA-4$^+$ Tregs in mice. Treg (**A**), IL-10$^+$ Treg (**B**), and Th17 (**C**) in CD4$^+$ lymphocytes in splenocytes prepared from astragaloside IV- and formononetin-exposed BALB/c female mice. Astragaloside IV- and formononetin-exposed mice had a significantly decreased Th17 compared with control mice, thereby increasing the Treg/Th17 ratio (**D**). Astragaloside IV-exposed mice had a significantly increased CTLA-4$^+$ Treg (**E**). One-way ANOVA was used for data analysis, followed by Dunnett's multiple comparison test; $^{*}P < 0.05$. The bar graphs and error bars represent the means ± SDs ($n = 6$).

measured *via* flow cytometry analysis. After measuring the percentage of CD4$^+$ T cells in the splenic lymphocyte population, the percentage of CTLA-4$^+$ Foxp3$^+$ T cells in the CD4$^+$ T-cell population was measured. CTLA-4$^+$ Tregs were defined as CD4$^+$ Foxp3$^+$ CTLA-4$^+$ T cells. Representative flow cytometry dot plots show gating (right upper quadrant) for CTLA-4$^+$ Tregs (Figure 2G). AsR-exposed mice had significantly decreased CTLA-4$^+$ Tregs (Figure 2H).

3.3. Evaluation of splenic Treg and Th17 on formononetin and astragaloside IV-exposed mice

Formononetin (an isoflavone molecule) and astragaloside IV (a triterpene glucoside) contained in Astragalus roots were the main compounds that had no effect on the percentage of Tregs (Figure 3A) and IL-10$^+$ Tregs in CD4$^+$ T cells (Figure 3B). On the other hand, formononetin and astragaloside IV significantly

decreased the percentage of Th17 cells among $CD4^+$ T cells (Figure 3C), and thus significantly increased the Treg/Th17 ratio (Figure 3D) relative to that of the control. Furthermore, astragaloside IV-exposed mice showed a significant increase in CTLA-4^+ Tregs (Figure 3E).

3.4. Evaluation of splenic Treg and Th17 on AsR-containing Kampo medicines-exposed mice

To determine whether Kampo medicines containing Astragalus root used for the treatment of frail conditions increase splenic Treg, KO-, BO-, OK-, and KK-exposed mice were measured for the percentage of Treg in $CD4^+$ T cells *via* flow cytometry analysis. Representative flow cytometry dot plots show gating (right upper quadrant) for $CD25^+$ $Foxp3^+$ Tregs in $CD4^+$ T cells (Figure 4A). KO-, OK-, and KK-exposed mice had significantly more Tregs (Figure 4B) than control mice. KO-, BO-, OK-, and KK-exposed mice were measured for IL-10^+ Tregs in $CD4^+$ T cells *via* flow cytometry analysis. Representative flow cytometry dot plots show gating (right upper quadrant) for IL-10^+ Tregs in $CD4^+$ T cells (Figure 4A). KO-, BO-, and OK-exposed mice had significantly increased IL-10^+ Tregs (Figure 4C) relative to control mice. KO-, BO-, OK-, and KK-exposed mice were measured for the percentage of Th17 cells among $CD4^+$ T cells *via* flow cytometry analysis. Representative flow cytometry dot plots show gating (right upper quadrant) for $CD4^+$ IL-$17A^+$ T cells in $CD4^+$ T cells (Figure 4D). KO-exposed mice had a significantly decreased percentage of Th17 cells among $CD4^+$ T cells (Figure 4E) relative to control mice. KO-, and OK-exposed mice significantly increased the Treg/Th17 ratio (Figure 4F).

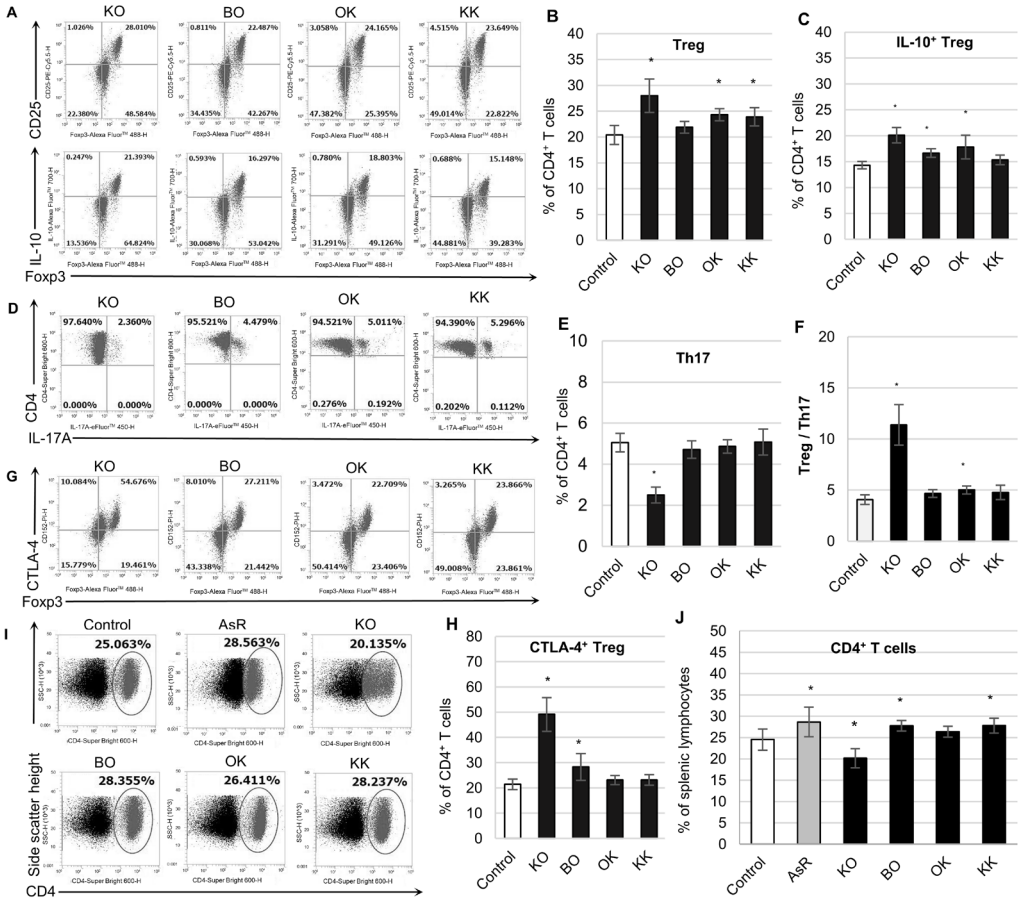

Figure 4. Effects of AsR-containing Kampo medicines exposure on splenic Tregs, IL-10^+ Tregs, Th17, Treg/Th17 ratio, CTLA-4^+ Tregs, and $CD4^+$ T cells. The Treg, IL-10^+ Treg, Th17, Treg/Th17 ratio, and CTLA-4^+ Treg in $CD4^+$ lymphocytes of splenocytes prepared from KO-, BO-, OK-, and KK-exposed BALB/c female mice. Representative flow cytometric analysis of Tregs and IL-10^+ Tregs (**A**, $CD25^+$ $Foxp3^+$ T cells, or IL-10^+ $Foxp3^+$ T cells in $CD4^+$ T cells, right upper quadrant) in KO-, BO-, OK-, KK-exposed and control mice. KO-, OK-, and KK-treated mice showed significantly increased Treg levels (**B**). KO-, BO-, and OK- treated mice showed significantly increased IL-10^+ Tregs (**C**). Representative flow cytometric analysis of Th17 (**D**, IL-17^+ T cells in $CD4^+$ T cells, right upper quadrant) in KO, BO, OK, KK-exposed and control mice. KO-exposed mice showed significantly decreased Th17 levels (**E**). KO and OK-exposed mice showed a significantly increased Treg/Th17 ratio (**F**). Representative flow cytometric analysis in CTLA-4^+ Treg (**G**, CTLA-4^+ $Foxp3^+$ T cells in $CD4^+$ T cells, right upper quadrant) of KO, BO, OK, KK-exposed and control mice. KO- and BO-exposed mice showed significantly increased CTLA-4^+ Tregs (**H).** Representative flow cytometric analysis of $CD4^+$ T cells in lymphocytes (**I**, circle) of AsR, KO, BO, OK, KK-exposed and control mice. AsR-, BO-, and KK-exposed mice showed significantly increased $CD4^+$ T cells, and KO-exposed mice showed a significant decrease in $CD4^+$ T cells **(J)**. KO, Keishikaogito; BO, Boiogito; OK, Ogikenchuto; KK, Kigikenchuto. One-way ANOVA was used for data analysis, followed by Dunnett's multiple comparison test; $*P < 0.05$. The bar graphs and error bars represent the means ± SDs ($n = 6$).

KO-, BO-, OK-, and KK-exposed mice were measured for the percentage of CTLA-4$^+$ Tregs in CD4$^+$ T cells *via* flow cytometry analysis. Representative flow cytometry dot plots show gating (right upper quadrant) for CTLA-4$^+$ Tregs (Figure 4G). Compared with the control group, KO- and BO-exposed mice had an increased percentage of CTLA-4$^+$ Tregs (Figure 4H), particularly KO-exposed mice, which showed an approximately 2-fold increase relative to the control.

3.5. Evaluation of splenic CD4$^+$ T cells

To determine whether the increase in CTLA-4$^+$ Tregs leads to a decrease in CD4$^+$ T cells, CD4$^+$ T cells in the splenic lymphocyte population were measured *via* flow cytometry analysis. Representative flow cytometry dot plots show gating (circle) for CD4$^+$ T cells (Figure 4I). KO-exposed mice significantly decreased the percentage of CD4$^+$ T cells in the splenic lymphocyte population, and AsR-, BO-, and KK-exposed mice significantly increased the percentage of CD4$^+$ T cells (Figure 4J).

3.6. Assessment of pregnancy

To determine whether fertility depends on the Treg/Th17 rate, AsR- and KO-exposed BALB/c mice were assessed with the average number of litters. The number of litters per maternal mouse was measured 4 days postpartum. AsR-exposed mice, which had no difference in Treg/Th17 compared with control mice, showed a trend toward a dose-dependent increase in the average number of litters (Figure 5A). KO-exposed mice, which had a significantly increased Treg/Th17 rate, showed no increase in the average number of litters compared with the control group (Figure 5B).

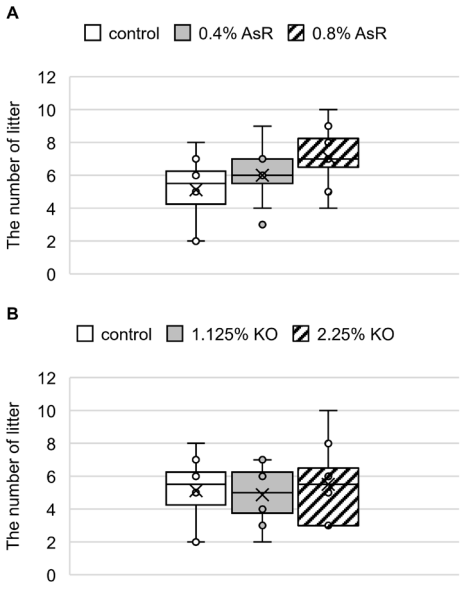

Figure 5. Effect of AsR and KO on the number of litters. A box-whisker plot was applied to indicate the number of litters. AsR-exposed mice showed a trend toward a dose-dependent increase in the average number of litters (**A**), whereas KO-exposed mice did not (**B**). The average number of litter was presented by "×" (n = 8). The number of litter in each maternal mouse was presented by "○" and "the ends of the whiskers". The lower and upper edges of the box signal the 25th percentile and 75th percentile, respectively. The parallel line across the box indicates the median of the distribution. AsR, Astragalus root; KO, Keishikaogito.

4. Discussion

The present study shows that AsR, which is used as a tonic contained in Kampo medicines, has the ability to promote embryo implantation and maintain pregnancy by improving the immune system. AsR further increased concanavalin A-enhanced splenocyte proliferation (Figure 1A). Kampo medicines, except KO, demonstrated no increase in cell proliferation (Figure 1B), suggesting that AsR and KO may promote T cell immunostimulation. IL-2 is secreted from antigen-activated CD4$^+$ helper T cells and consumed to activate Tregs and convert naïve CD8$^+$ T cells to effector CD8$^+$ T cells (*7*). Therefore, IL-2 is currently used to suppress immune responses in patients with autoimmune diseases and to enhance immune responses in patients with cancer and HIV-infected diseases. The administered dose of AsR (0.8% AsR-containing water solution, free access for 14 days) in this study was determined by the yield of the extract. In Figure 1C, cultured splenocytes of AsR-administered mice showed a significant increase in IL-2 secretion at 18 h and 27 h from incubation initiation. Therefore, AsR was thought to contribute to the activation of CD4$^+$ T cells.

In the secondary lymphoid tissue (spleen, lymph nodes), the CD4$^+$ T-cell subset comprises 10-15% of the Treg subset. CD4$^+$ Foxp3$^-$ T cells circulating in secondary lymphoid tissues are differentiated by IL-2 into CD4$^+$ Foxp3$^+$ Tregs, which accumulate in peripheral nonlymphoid tissue. Tregs are potent suppressors of inflammatory effector T cells, such as CD8$^+$ T cells, conventional CD4$^+$ T cells, and NK cells. It is well established that lymphocytes migrate into the maternal endometrium during the luteal phase and pregnancy (*8*), and Tregs particularly increase in the decidua during the first and second trimesters of pregnancy (*4*). Tregs in mice increase during the estrous cycle and early pregnancy (*9*). As shown in Figure 2B, Treg in splenic lymphocytes was significantly increased in AsR-administered mice, and thus AsR might increase the migrated number of Treg to peripheral nonlymphoid tissue, such as decidua, from the spleen.

Maternal immune tolerance induced by Tregs is essential for accepting fetal antigens to the decidua. CTLA-4, known as CD152, is a cell surface molecule constitutively expressed in Foxp3$^+$ Tregs but absent in naïve conventional T cells. CTLA-4 has a high affinity for B7 expressed in APCs, and CTLA-4/B7 signaling plays a critical role in inhibiting the activity of APCs, thereby suppressing the proliferation and activation of T

cells and the production of cytokines. It was reported that blocking the interaction of CTLA-4/B7 increases T-cell proliferation and IL-2 production (*10*). Furthermore, the blockade of CTLA-4 by ligands increases Tregs due to increased IL-2 production from $CD4^+$ T cells through enhanced CD28/B7 signaling (*11*). CTLA-4-Ig gene transfer-model mice have decreased embryonic absorption rates and improved recurrent pregnancy loss in association with increased levels of peripheral Treg (*12*). CTLA-4^+ Tregs were decreased in AsR-administered mice (Figure 2H), leading to an increase in the percentage of $CD4^+$ T cells in the splenic lymphocyte population (Figure 4J). These findings suggest a mechanism of AsR, as shown in Figure 6.

The trophoblast cells anchoring the placenta to the uterine wall in mice express indoleamine 2,3-dioxygenase (IDO), which mediates tryptophan catabolism into kynurenine, which drives Treg expansion. Lower IDO levels in mice have been reported to induce pregnancy loss associated with a lower number of splenic Tregs in maternal mice (*13-15*). Tregs recognize inflammatory effector cells, such as $CD8^+$ cytotoxic T cells (CTLs), $CD4^+$ T cells, and NK cells which attack fetal antigens in the murine endometrium. Th17 cells, which produce the proinflammatory cytokine IL-17, play a crucial role in the induction of autoimmune diseases such as rheumatoid arthritis, and an imbalance in Th17/Treg cells was observed in patients with recurrent spontaneous abortion. However, Th17 cells promote the proliferation and invasion of human trophoblasts into the maternal decidua (invaded trophoblasts named extravillous trophoblast cells: EVTs), and IL-17 excludes other cytokines and prevents apoptosis of EVTs (*8*). Murine trophoblasts (trophoblast giant cells and glycogen trophoblasts) are modestly more invasive than human trophoblasts. AsR increased peripheral Th17 (Figure 2E) and the number of litters in an AsR dose-dependent manner (Figure 5A), which suggests that AsR prevents trophoblast apoptosis.

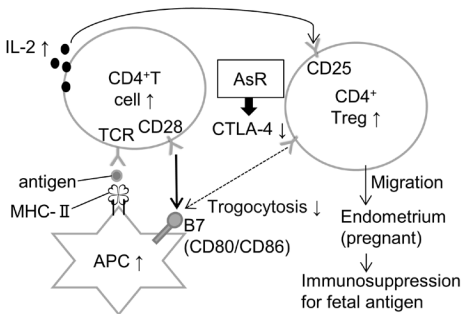

Figure 6. AsR-induced immunosuppressive milieu in splenic lymphocytes. AsR leads to APC activation by attenuated expression of CTLA-4 on Tregs. $CD4^+$ T cells increased by APC activation secrete IL-2, which binds to CD25 to activate Tregs, creating an immunosuppressive milieu. AsR, Astragalus root; APC, antigen-presenting cells; CTLA-4, cytotoxic T lymphocyte antigen 4; Treg, regulatory T cells; TCR, T-cell receptor; MHC-II, major histocompatibility complex II.

Formononetin, an isoflavone molecule contained in the Astragalus root, shows a wide range of pharmacological activities, such as anti-inflammatory (*16*), antidiabetic (*17*), neuroprotective (*18*), and cerebral anti-ischemia (*19*) activities. In this study, formononetin decreased splenic Th17 cells (Figure 3C) but had no effect on Tregs. Formononetin inhibits the activation of STAT3 (*20*), a Th17 transcription factor. Formononetin-administered BALB/c mice appeared to inhibit the activation of nuclear factor kappa β (NF-kB) related to the IL-17 signaling pathway (*21*). Astragaloside IV, a triterpene glucoside contained in AsR, plays important roles in the improvement of the immune system to suppress the migration of cervical cancer cells (*22*) and type 2 diabetes mellitus to prevent mitochondrial dysfunction (*23*). Astragaloside IV decreases IL-6 secretion from dendritic cells (DCs), which downregulates Th17 differentiation from $CD4^+$ T cells (*24*). In this study, astragaloside IV showed a significant increase in CTLA4^+ Tregs (Figure 3E) and a significant decrease in Th17 cells (Figure 3C). Astragalus polysaccharide (ASP) is an immunoreactive molecule, and ASP-treated DCs displayed mature morphology and augmented the cell surface expression of MHC-II (*25*). ASP decreases CTLA-4 mRNA expression and increases IL-2 mRNA expression (*26*). In our study, ASP was significantly decreased in murine splenic $CD4^+$ CTLA-4^+ T cells (data not shown, $P < 0.01$, *t* test) but did not decrease in CTLA-4^+ Tregs. Blockage of CTLA-4 on $CD4^+$ T cells promotes the proliferation of $CD4^+$ cells more than $CD8^+$ cells (*27*). We found no components in AsR that showed a mechanism in a manner similar to AsR for increasing Tregs.

To evaluate whether AsR-containing Kampo medicines (KO, BO, OK, KK) show a mechanism similar to that of AsR the percentages of Tregs, IL-10^+ Tregs, CTLA-4^+ Tregs, and Th17 cells in $CD4^+$ lymphocytes were measured. KO-administered mice exhibited the most strongly enhanced percentage of splenic Tregs (Figure 4B). KO-administered mice had increased IL-10^+ Tregs (Figure 4C) and decreased splenic Th17 cells (Figure 4E). The percentages of splenic CTLA-4^+ Tregs were dramatically increased in the spleens of KO-administered mice (Figure 4H). CTLA-4 expressed on Tregs has the ability to strip off MHC-II and the costimulatory molecule B7 from APCs and express B7 on the surface of CTLA-4^+ Tregs, resulting in impaired antigen-presenting activity of APCs (*28*). KO was thought to strongly induce this 'trogocytosis' by the overexpression of CTLA-4 on Tregs, resulting in a decrease in the percentage of $CD4^+$ T cells in the splenic lymphocyte population (Figure 4J).

Disruption of maternal-fetal immune balance, especially Treg/Th17 imbalance, is linked to recurrent spontaneous abortion (RSA). The immunosuppressant cyclosporine A, which is used for the treatment of RSA (*29*), improves the Treg/Th17 imbalance in RSA patients

(increased Th17 and decreased Treg compared with normal early pregnancy) and increases the live birth rate (*30*). To determine whether Treg/Th17 imbalance impacts pregnancy, the number of litters in AsR- and KO-exposed female mice was investigated. The number of litters showed a trend toward a dose-dependent increase in AsR-administered mice (Figure 5A) but did not increase in KO-administered mice (Figure 5B). AsR increased the percentage of both Treg and Th17 cells through a decrease in CTLA-4 expression on Treg cells and did not exhibit a Treg/Th17 imbalance, which could be one reason for the decreased litter size. AsR was thought to create an environment of immunosuppression and easy trophoblast proliferation in the endometrium to improve embryo implantation.

Multicomponent drugs, such as crude drugs and herbal medicines, have multiple targets in several signaling pathways, and it is difficult to identify their potential targets. The Network Pharmacology approach is effective in predicting all the main active ingredients of multicomponent drugs against the core targets of the disease. The seven ingredients, including formononetin, contained in the mixture of *Astragalus membranaceus* and *Angelica sinensis* were predicted to influence the core targets in the Toll-like receptor, IL-17, and TNF signaling pathways in atherosclerosis (*31*). Furthermore, the combination of *A. membranaceus* and *Ligusticum chuanxiong* treatment predicted that 14 components of *A. membranaceus* including formononetin, activated the core targets of three pathways in ischemic stroke: the Toll-like receptor, IL-17, and TNF signaling pathways (*32*). In addition, Buyang Huanwu's decoction containing *A. membranaceus* predicted that the quercetin content is linked to the core targets of the Il-17 signaling pathway in myocardial fibroblast, and *in vivo* assays revealed increased IL-6, IL-1β, and MMP expression in the IL-17 signaling pathway (*33*). *A. membranaceus* was significantly increased in splenic Th17. Conclusively, these findings suggest that the components of *A. membranaceus* may compositely activate the IL-17 pathway to improve embryo implantation.

The present research shows that AsR exposure improves embryo implantation and maintaining pregnancy and suggests a mechanism that involves enhanced maternal-fetal immune tolerance through increased Treg and Th17 numbers in splenic lymphocytes. Immune tolerance due to a decrease in T cells mediated by an increase in CTLA-4 on Tregs is thought to adversely affect maintaining pregnancy. Therefore, to create an immune tolerance milieu of peripheral nonlymphoid tissues such as the uterine mucosa, it would be worth considering ways to increase the number of immune cells in peripheral lymphoid tissue and promote Treg migration to peripheral nonlymphoid tissues. Specific microRNA expression is correlated with endometrium decidualization and trophoblast cell proliferation in the placenta during pregnancy (*34*). We would like to investigate the correlation between Treg, Th17, and the microRNA expression levels in decidua and further clarify the mechanism of maintaining pregnancy.

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Original Article

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PD-1/PD-L1 inhibitors associated hypophysitis: An analysis from the FAERS database and case reports

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SUMMARY To get a thorough understanding of PD-1/L1 inhibitor-related hypophysitis (PD-1/L1-irH), we utilized a combination of disproportionality analysis and case analysis to comprehensively characterize the clinical features of PD-1/L1-irH. Significant signals of hypophysitis were detected for all PD-1/PD-L1 inhibitors in the FAERS (FDA Adverse Event Reporting System). As revealed by both FAERS and the case analysis, PD-1/L1-irH occurred more commonly in males, PD-1 inhibitors users and patients older than 65 years. The median onset time was 101 days in FAERS and 8 cycles in the case analysis. In the case analysis, eight late-onset PD-1/L1-irHs occurred even after a discontinuation of several months (4-15 months). As revealed in FAERS, the outcome of PD-1/L1-irH tended to be poor, generally resulting in 64.66% hospitalization and 12.59% death. Fatigue was the most prominent symptom of PD-1/L1-irH, followed by anorexia, hyponatremia, and hypotension, as revealed by the analysis of 84 cases. Meanwhile isolated adrenocorticotropic (ACTH) deficiency was particularly prevalent for PD-1/L1-irH (85.71%), while gonadal hormones or posterior pituitary hormones deficiencies were rare. Glucocorticoids were administered to almost all cases (81/84), with a physiologic or stress dosage in 61.9% of cases, and a high-dose in 26.2% of cases. Most cases (58.3%) showed a favorable tumor response before diagnosis of PD-1/L1-irH. PD-1/L1-irH may occur throughout the whole therapy period even after discontinuation. Clinicians should pay more attention to PD-1 inhibitor users, males and older patients. Early diagnosis and prompt managements are crucial for PD-1/L1-irH as its potentially life-threatening nature.

Keywords PD-1/PD-L1 inhibitors, hypophysitis, disproportionality analysis, characteristic, managements

1. Introduction

Immunotherapy has revolutionized the field of cancer treatment over the past decade. Immune checkpoint inhibitors (ICIs), a novel class of medications in cancer therapy, have quickly gained traction in the treatment of various types of cancer (*1*). Currently, ICIs include antibodies that target certain immune checkpoints, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death 1 (PD-1) or its ligand (PD-L1), resulting in T-cell activation and antitumor activity. Despite the significant potential of ICIs, their success has been somewhat limited by a diverse spectrum of immune-related adverse events referred to as irAEs, which may affect every system (*2,3*).

Hypophysitis, characterized by inflammation of the pituitary gland, can result in the impairment of pituitary function and lead to irreversible hypopituitarism. If left untreated, it can also potentially lead to adrenal crisis, a life-threatening condition. Many reports have focused on immune-related hypophysitis induced by anti-CTLA-4 monoclonal antibodies (mAbs) like ipilimumab (*4*), while relatively fewer existed for anti-PD-1 mAbs, and even fewer for anti-PD-L1 mAbs. The underlying high rates of ipilimumab-mediated hypophysitis is thought to be related to expression of CTLA-4 in the pituitary (*5,6*). However PD-1/L1-irH may be a clinical entity distinct from CTLA-4 inhibitors related hypophysitis (*7*). Given the widespread use of PD-1/PD-L1 inhibitors in clinical practice and the potentially life-threatening nature of hypophysitis if not promptly recognized and treated, it is essential for clinicians to have a thorough understanding

of the clinical manifestations and management of PD-1/L1-irH.

In the study, we firstly performed a disproportionality analysis leveraging a large pharmacovigilance database (FAERS) to characterize and evaluate PD-1/L1-irH. As pharmacovigilance data may lack detailed clinical information, we subsequently conducted a systematic search of cases to gather additional information on clinical features, management and outcomes for PD-1/L1-irH.

2. Methods

2.1. Pharmacovigilance study procedures

We performed a retrospective pharmacovigilance study from Quarter 1 (Q1) in 2004 to Q3 in 2022 using the FAERS database (Figure 1A). Data downloaded from FAERS database were deduplicated following the strategy described by the database. Our study included six approved PD-1/PD-L1 inhibitors: nivolumab, pembrolizumab, camrelizumab, atezolizumab, avelumab, and durvalumab. Both generic and brand names from Drugbank were used as keywords for the database retrieval (Table S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=187*). Adverse events (AEs) reported in FAERS were coded by preferred terms (PTs) from the Medical Dictionary for Drug Regulatory Activities (MedDRA). All hypophysitis-relevant PTs (immune-mediated hypophysitis, hypophysitis, secondary adrenocortical insufficiency, secondary hypogonadism, secondary hypothyroidism, hypopituitarism) from MedDRA 24.0 were searched in REAC files of the database. Exposure assessment was considered when PD-1/PD-L1inhibitors were recorded as 'primary suspect'. We also collected information such as reporting source, gender, age, treatment regimen, start and end dates of therapy, onset time, and outcomes of adverse events. The anti-CTLA-4 mAbs are often used in combination with PD-1/PD-L1 inhibitors for the therapy of melanoma or hepatocellular carcinoma. To eliminate any potential interference from ipilimumab or tremelimumab, reports solely involving PD-1/PD-L1 inhibitors were selected for further analysis. Disproportionality analyses were conducted by reporting odds ratio (ROR) (*8*). The calculation formulas of ROR was listed in (Tables S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=187* and S3, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=187*). An AE signal was generated when both the ROR value was greater than 2 and the lower limit of the 95% CI of the ROR was greater than 1, and at least three cases were required to define a signal. Usually speaking, the higher the value, the stronger the association between the PD-1/PD-L1 inhibitors and PD-1/L1-irH.

2.2. Descriptive study

A systematic search regarding PD-1/L1-irH of multiple electronic databases was conducted up to March 30, 2023, including PubMed, Web of science, Wanfang, and China National Knowledge Infrastructure (CNKI) , with no language restrictions (Figure 1B). The search strategy and terms were listed in Table S4 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=187*). Case reports and case series were included, and reviews, mechanistic studies, animal studies, and articles without available full text were all excluded. To avoid potential interference, cases involving the concomitant use of ipilimumab or tremelimumab were also excluded from the analysis. Data including the baseline characteristics of patients (age, sex, tumor type), therapy(regime, start time and end time of the treatment, efficacy on tumor) and AEs (onset time, outcomes) were extracted. Two authors independently screened references for eligibility of data extraction and consulted a third author to resolve

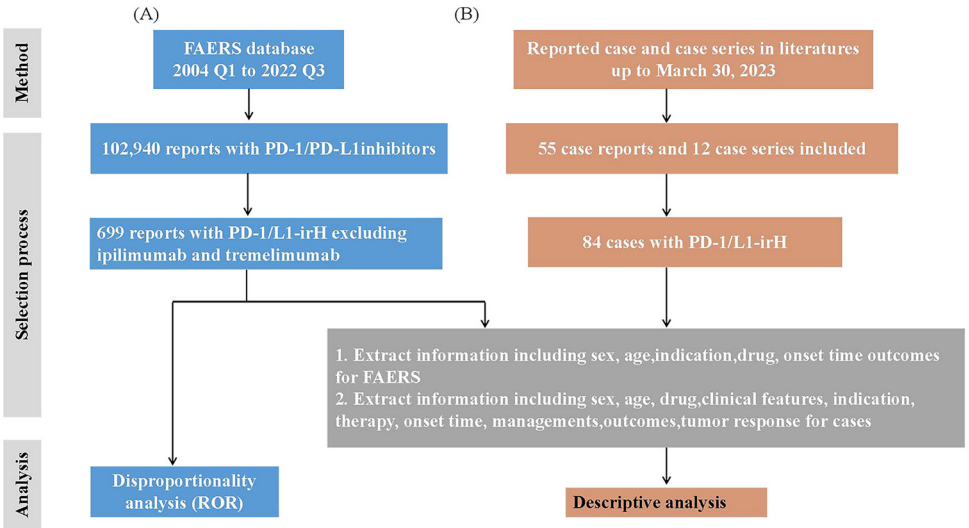

Figure 1. The flow diagram of (A) the pharmacovigilance study and (B) the descriptive study.

Table 1. Characteristics of patients with PD-1/L1-irH in FAERS

Drug	All (*n* = 699)	Nivolumab (*n* = 345)	Pembrolizumab (*n* = 262)	Atezolizumab (*n* = 70)	Avelumab (*n* = 6)	Durvalumab (*n* = 16)
Gender						
Male	421 (60.23%)	218 (63.19%)	143 (54.58%)	45 (64.29%)	3 (50.00%)	12 (75.00%)
Female	189 (27.04%)	92 (26.67%)	73 (27.86%)	18 (25.71%)	3 (50.00%)	3 (18.75%)
Unknown	89 (12.73%)	35 (10.14%)	46 (17.56%)	7 (10.00%)	0 (0.00%)	1 (6.25%)
Age						
Mean (SD)	66.6 ± 11.0	66.0 ± 11.4	66.7 ± 11.0	68.3 ± 8.6	61.7 ± 15.0	70.3 ± 7.5
Median (IQR)	68 (60-74)	68 (60-74)	68 (60-74)	68 (63-74)	66 (52-72)	67 (65-76)
≥ 65 y	371 (53.08%)	175 (50.72%)	145 (55.34%)	35 (50.00%)	4 (66.67%)	12 (75.00%)
< 65 y	222 (31.76%)	115 (33.33%)	74 (28.24%)	27 (38.57%)	2 (33.33%)	4 (25.00%)
Unknown	106 (15.16%)	55 (15.94%)	43 (16.41%)	8 (11.43%)	0 (0.00%)	0 (0.00%)
Indications						
Melanoma	147 (21.03%)	91 (26.38%)	54 (20.61%)	2 (2.86%)	/	/
Lung cancer	238 (34.05%)	90 (26.09%)	108 (41.22%)	26 (37.14%)	/	14 (87.50%)
Gastric cancer	43 (6.15%)	42 (12.17%)	/	/	1 (16.67%)	/
Renal cancer	56 (8.01%)	42 (12.17%)	12 (4.58%)	/	2 (33.33%)	/
Head and neck cancer	35 (5.01%)	24 (6.96%)	11 (4.20%)	/	/	/
Other & Unspecified cancer	180 (25.75%)	56 (16.23%)	77 (29.39%)	42[a] (60.00%)	3 (50.00%)	2 (12.50%)
Outcomes						
DE	88 (12.59%)	45 (13.04%)	29 (11.07%)	9 (12.86%)	/	5 (31.25%)
LT	87 (12.45%)	54 (15.65%)	27 (10.31%)	1 (1.43%)	/	5 (31.25%)
HO	452 (64.66%)	224 (64.93%)	172 (65.65%)	46 (65.71%)	5 (83.33%)	5 (31.25%)
DS	36 (5.15%)	17 (4.93%)	17 (6.49%)	2 (2.86%)	/	/
CA	2 (0.29%)	/	2 (0.76%)	/	/	/
OT	513 (73.39%)	276 (80.00%)	183 (69.85%)	37 (52.86%)	3 (50.00%)	14 (87.50%)
Unknown	59 (8.44%)	15 (4.35%)	38 (14.50%)	5 (7.14%)	/	1 (6.25%)

[a]:19 Hepatocellular cancer in 42 cases. DE: Death; LT: Life-Threatening; HO: Hospitalization - Initial or Prolonged; DS: Disability; CA: Congenital Anomaly; RI: Required Intervention to Prevent Permanent Impairment/Damage; OT: Other Serious (Important Medical Event).

disagreements.

2.3. Ethical considerations

The study was conformed to the provisions of the Declaration of Helsinki (as revised in 2013, https://wma.net/what-we-do/medical-ethics/declaration-of-helsinki)

2.4. Statistical analysis

Data was dealt with SPSS 23.0. Quantitative variables were expressed by the mean, standard deviation, median, and interquartile range (IQR), while qualitative variables were represented using numerical values and rates.

3. Results

3.1. Disproportionality analysis

During the study period, a total of 102,940 PD-1/PD-L1inhibitors associated AEs were documented in the FAERS database: 57,620 for nivolumab, 28,068 for pembrolizumab, 11,066 for atezolizumab, 1,513 for avelumab, 4,673 for durvalumab. After excluding ipilimumab and tremelimumab, 699 reports of PD-1/L1-irH were consisted of 345(49.36%) for nivolumab, 262 (37.48%) for pembrolizumab, 70 (10.01%) for atezolizumab, 6 (0.86%) for avelumab, 16 (2.29%) for durvalumab. Camrelizumab was not included in our analysis as only one relevant case was found.

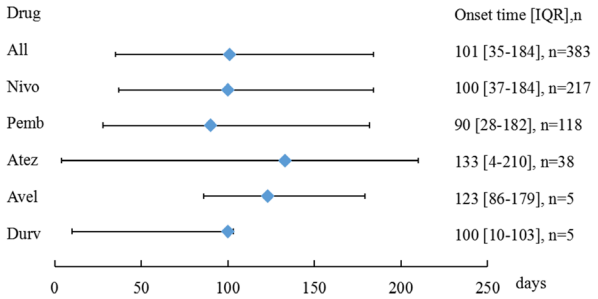

Figure 2. The median onset time of PD-1/L1-irH. Nivo: Nivolumab; Pemb: Pembrolizumab; Atez: Atezolizumab; Avel: Avelumab; Durv: Durvalumab.

The patients' characteristics were summarized in Table 1. Males were presented a larger proportion of PD-1/L1-irH than females, accounting for 60.23%. The average age at diagnosis of PD-1/L1-irH was 66.6 years, which did not differ significantly among each PD-1/PD-L1inhibitors. Lung cancer was the most common indication (34.05%), followed by melanoma (21.03%). While renal cancer was the most common indication for avelumab (33.33%). In our analysis, we found the outcome of PD-1/L1-irH tended to be poor, generally resulting in 64.66% hospitalization and 12.59% death. Among all PD-1/PD-L1inhibitors, the highest fatality proportion occurred in durvalumab (31.25%, 5 death out of 16 cases). As revealed in Figure 2, the overall median

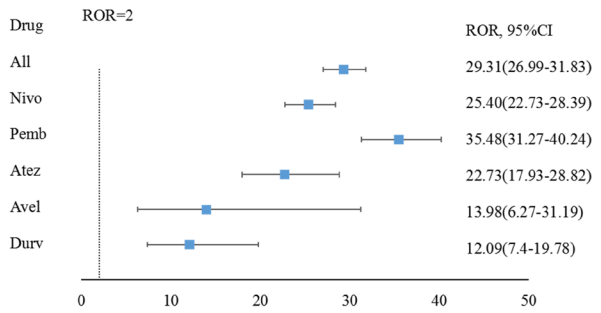

Figure 3. RORs of PD-1/L1-irH. Nivo: Nivolumab; Pemb: Pembrolizumab; Atez: Atezolizumab; Avel: Avelumab; Durv: Durvalumab.

onset time was 101 days (IQR: 35-184). There were also 28 reports with an onset time even longer than one year.

The signals of hypophysitis were detected significantly for each PD-1/PD-L1inhibitor, with an overall ROR 29.31 (95% CI, 26.99-31.83), 25.4 (95% CI, 22.73-28.39) for nivolumab, 35.48 (95% CI, 31.27-40.24) for pembrolizumab, 22.73 (95% CI, 17.93-28.82) for atezolizumab, 13.98 (95% CI, 6.27-31.19) for avelumab and 12.09 (95% CI, 7.4-19.78) for durvalumab, as shown in Figure 3.

3.2. Descriptive analysis

A total of 84 PD-1/L1-irH cases were extracted from 55 case reports (9-63) and 11 case series 64-74). The patients' information were summarized in Table 2. Males seemed to develop hypophysitis more probably than females (male:female, 65:19) as revealed in FAERS. The mean age for PD-1/L1-irH was 65 years old. Furthermore hypophysitis seemed to be induced by PD-1 inhibitors more likely than PD-L1inhibitors (92.86% *vs.* 7.14%). Nivolumab and pembrolizumab emerged as the predominant culprits in these cases, with prevalence rates of 61.9% and 22.62%, respectively. The primary indication of PD-1/PD-L1inhibitors was lung cancer (37 cases, 44.1%), followed by malignant melanoma (20 cases, 23.8%), renal cancer (10 cases, 11.9%). It is widely recognized that patients with PD-1/L1-irH may develop other irAEs concurrently. In our study, thyroiditis (9 cases) and type I diabetes (5 cases) were emerged as the most prevalent concomitant irAEs. Additionally, other irAEs including hepatitis, pancreatitis, pneumonia, pericarditis, as well as cerebritis and Guillain-Barré syndrome, were also found to occur concurrently with hypophysitis. The majority of patients (49 cases, 58.3%) exhibited a favorable tumor response including CR, PR or SD before diagnosis of PD-1/L1-irH.

The clinical features of PD-1/L1-irH were summarized in Table 3. The time between administration and symptom onset varied from two cycles to fifty cycles, with a median period of eight cycles. Interestingly, eight cases had developed hypophysitis even after discontinuing PD-1/PD-L1 inhibitors for several months (4-15 months) in our study. Fatigue (80.95%) emerged as the most prominent symptom, followed by anorexia (52.38%), hypotension (32.14%), and nausea/vomiting (30.95%). Weight loss (11.90%) and disorders of consciousness (11.90%) might also be presented in patients with PD-1/L1-irH. Headache (5.95%) and visual disturbances (1.19%) were rarely observed. As for endocrine, almost all patients exhibited central adrenal insufficiency (81/84, 96.4%), among which central hypothyroidism was coexisted in six cases, and central hypogonadism was coexisted in two cases. Furthermore, a simultaneous involvement of all three aforementioned pituitary axes occurred in one patient. Rarely, only one case was presented as central hypothyroidism, hypogonadism and central diabetes

Table 2. Demographics and baseline characteristics of 84 cases with PD-1/L1-irH

Parameter	*n*	Percentage (%)
Gender		
Male	65	77.38
Female	19	22.62
Age (years) at diagnosis, mean ± SD	65.42 ± 9.74	
Age (years) at diagnosis, medium (IQR)	65(59-73)	
Types of PD-1/PD-L1 inhibitors:		
PD-1 inhibitors	78	92.86
Nivolumab	52	61.90
Pembrolizumab	19	22.62
Camrelizumab	4	4.76
Sintilimab	1	1.19
Sintilimab+tislelizumab	1	1.19
HX008	1	1.19
PD-L1 inhibitors	6	7.14
Atezolizumab	3	3.57
Durvalumab	2	2.38
Avelumab	1	1.19
Cancer type		
Lung cancer	37	44.05
Melanoma	20	23.81
Renal cancer	10	11.90
Ureteral cancer	5	5.95
Liver and biliary tract cancer	2	2.38
Head and neck tumor	5	5.95
Breast cancer	2	2.38
Colon cancer	1	1.19
Neuroendocrine tumor	2	2.38
Additional irAEs		
Thyroiditis	9	10.71
Hepatitis	1	1.19
Pancreatitis	1	1.19
Pneumonia	2	2.38
Cerebritis	1	1.19
Type I diabetes	5	5.95
Pericarditis	1	1.19
Skin exfoliative dermatitis	1	1.19
Guillain-Barré syndrome	1	1.19
Tumor response		
PD	12	14.28
SD	20	23.81
PR	25	29.76
CR	4	4.76
Unknown	23	27.38

PD: progressive disease; SD: stable disease; PR: partial response; CR: complete response.

Table 3. The clinical features of PD-1/L1-irH (*n* = 84)

Parameter	*n*	(%)
Symptoms		
Fatigue	68	80.95
Anorexia	44	52.38
Hypotension	27	32.14
Nausea/vomiting	26	30.95
Weight loss	10	11.90
Disorders of consciousness	10	11.90
Headache	5	5.95
Visual disturbances	1	1.19
Median cycles to onset NO. (range)	8 (2-50)	
Pituitary hormone disturbance		
ACTH	72	85.71
ACTH+TSH	6	7.14
ACTH+FSH/LH	2	2.38
TSH+FSH/LH+DI	1	1.19
ACTH+TSH+FSH/LH	1	1.19
FSH/LH	1	1.19
DI	1	1.19
Other disturbance		
Hyponatremia	46	54.76
MRI findings (*n* = 74)		
Normal	56	75.68
Enhancement of the pituitary gland or Pituitary stalk	11	14.86
Heterogeneous enhancement	2	2.70
Atrophy	4	5.41
Abnormal of posterior pituitary	3	4.05

ACTH: isolated adrenocorticotropic deficiency; ACTH+TSH: adrenocorticotropic deficiency concurrent with central hypothyroidism; ACTH+FSH/LH: adrenocorticotropic deficiency concurrent with central hypogonadism; TSH+FSH/LH+DI: central hypothyroidism, hypogonadism, and diabetes insipidus; FSH/LH: isolated hypogonadism; DI: central diabetes insipidus; MRI: magnetic resonance imaging.

Table 4. Managements and outcomes of PD-1/L1-irH (*n* = 84)

Parameter	*n*	(%)
PD-1/L1 discontinuation		
NO	21	25.00
YES, temporarily	15	17.86
YES, permanently	27	32.14
Unknown	21	25.00
Hormonal replacement (HRT)		
Glucocorticoids	81	96.43
High-dose[a]	22	26.19
Physiologic or stress dose[b]	52	61.90
Unspecified dose	7	8.33
Other hormone		
Thyroid hormone[c]	5	5.95
Testosterone	1	1.19
Desmopressin	1	1.19
Unspecified treatments	1	1.19
Outcomes		
Improved after HRT	47	55.95
Unknown	37	44.05
Hormonal recovery (*n* = 24)		
Follow-up (months), median (range)	6 (3-12)	
ACTH (*n* = 24)	2	8.33
TSH (*n* = 3)	1	33.33

[a]A high-dose refers to 0.5-2 mg/kg/day methylprednisolone or equivalent. [b]A physiologic or stress dose steroid refers to a dose of hydrocortisone less than 100 mg or equivalent. [c]Meant that thyroid hormone was given concurrently with steroids.

insipidus while maintaining normal in pituitary-adrenal axis. Additionally, one case had isolated hypogonadism, and one had isolated central diabetes insipidus. Hyponatremia was observed in the majority of cases (54.8%). MRI was taken in 74 patients, with the majority of cases showing normal pituitary imaging (75.68%). Enhancement of the pituitary gland or stalk thickening was only presented in 11 cases (14.86%). Abnormal imaging of posterior pituitary occurred in only three cases. Meanwhile only one of three cases had a clinical symptom of central diabetes insipidus.

The managements and outcomes of PD-1/L1-irH were listed in Table 4. Among 84 cases, 21 cases did not mention information about PD-1/PD-L1inhibitors discontinuation in the reports. PD-1/PD-L1inhibitors were continued for 21 cases, temporarily discontinued for 15 cases, and permanently discontinued for 27 cases. Glucocorticoids was usually initiated promptly at diagnosis, including methylprednisolone, prednisone or hydrocortisone. Nearly all patients (81/84) received glucocorticoids, with five also receiving thyroid hormone concurrently. Apart from seven cases where the dose was not mentioned, the majority of patients (52 /84, 61.90%) initiated a physiologic or stress dose of steroids, while thirteen cases (22/84, 26.19%) received high-dose steroids. A physiologic or stress dose steroid refers to a dose of hydrocortisone less than 100 mg or equivalent other glucocorticoids while a high-dose refers to 0.5-2 mg/kg/day methylprednisolone or equivalent. Furthermore, it is worth noting that one case with isolated hypogonadotropic hypogonadism was treated by testosterone, and desmopressin was given to one with central diabetes insipidus. A total of 24 patients were followed up for a median duration of 6 months (range: 3-12 months). Significant improvements in symptoms were observed following hormone treatments. One of three cases with pituitary-thyroid deficiency experienced a full recovery. However, the restoration of the pituitary-adrenal axis was uncommon. Only two cases (2/24) demonstrated recovery after two and three months respectively.

4. Discussion

Undoubtedly, ICIs such as PD-1/PD-L1 inhibitors have become a game changer in cancer treatment following the unprecedented and satisfactory response rate in recent years. However, a series of unique irAEs accompanied by the increased usage of PD-1/PD-L1 inhibitors also bother the clinicians. Initially as a specific irAE of ipilimumab (*75*), hypophysitis has also been reported to be associated with PD-1/PD-L1 inhibitors though less frequently. To the best of our knowledge, our study is, as of today, the first and largest case-analysis and disproportionality analysis, specifically focused on PD-1/

L1-irH, rather than encompassing all immune checkpoint inhibitors-related hypophysitis.

Hypophysitis associated with PD-1 inhibitors seemed to be reported more frequently than PD-L1 inhibitors whether from the case analysis or FAERS. The trend was presented similarly in a meta-analysis conducted in 2019 (PD-1 inhibitors *vs.* PD-L1 inhibitors 1.2% *vs.* 0.8%) (*76*). The male preponderance of anti-CTLA-4 mAbs-induced hypophysitis was confirmed by a recent meta-analysis of de Filette *et al.* (*77*) which kept the same trend even after adjusting sex difference caused by primary tumor *i.e.* melanoma (*78*). It has not studied whether PD-1/L1-irH also had the same sex difference. Our study had firstly shown that males had a higher reporting rate of PD-1/L1-irH than females. Moreover the male preponderance was observed for each PD-1/PD-L1 inhibitor as evidenced by FAERS. The phenomenon may be explained by the indication of PD-1/PD-L1 inhibitors, in particular lung cancer, kidney cancer and melanoma which affect men more than women (*79-81*).

PD-1/L1-irH was found with a higher reporting rate in patients older than 65 years old, especially revealed in FAERS regardless the type of PD-1/PD-L1 inhibitors. A similar median age (66 years old) was revealed in a retrospective study including all ICIs from VigiBase (*82*). The onset time of PD-1/L1-irH had been varied from one month to over a year, as revealed in both database and case analysis. This might be explained by different exposure of PD-1/PD-L1 inhibitors which might be influenced by factors such as sex, baseline eGFR, age, race *etc.* (*83,84*). A notable finding in the case analysis was that eight patients (nivolumab: 5 cases; pembrolizumab: 3 cases) had the late-onset hypophysitis even after discontinuing the offender-drugs for several months, with the longest interval as fifteen months (*30,36,37,49,51,60,64*). The interesting phenomenon could be explained by the pharmacokinetics and pharmacodynamics of PD-1/PD-L1 inhibitors to some degree. A pharmacodynamics analysis of nivolumab demonstrated that even after a single infusion, a mean occupancy of > 70% for PD-1 molecules on circulating T cells was sustained for a period of 2 months, regardless of dose (*85*). This indicated that nivolumab could block PD-1-mediated signaling even when it is undetectable in serum. T-cell memory for tumor antigens may also be reactivated by ICIs, with the resulting antitumor effect being maintained for several months. Indeed, a long-term antitumor action was observed in patients with non-small cell lung cancer (NSCLC) or melanoma even after discontinuation (*86,87*), suggesting that irAEs might also occur even after nivolumab withdrawal.

The nonspecific symptoms presented by PD-1/L1-irH, such as fatigue, anorexia, nausea, vomiting, could be confused with chemotherapy or other irAEs or cancer itself. So it may pose a challenge for diagnosis of hypophysitis in these particular patients in the absence of abnormal pituitary MRI findings (*88*). Hypotension and hyponatremia might be another two common features of PD-1/L1-irH. Hyponatremia was even be reported as a powerful predictor of the acute development of isolated ACTH deficiency caused by anti-PD-1/PD-L1mab (*71*). Visual disturbances were uncommon, occurring in only 1% PD-1/L1-irH in our study. And the incidence of headaches was approximately 6% in patients with PD-1/L1-irH, which demonstrated a much lower prevalence compared to anti-CTLA-4 hypophysitis (13/15, 86.7%) (*89*). This could potentially be attributed to a heightened prevalence of pituitary enlargement in anti-CTLA-4 hypophysitis (12/15, 80.0%) (*89*), while lower for PD-1/L1-irH accounting for 21.4% and even lower in our study, accounting for 14.86%. Unlike the hypervascular state observed in the early stage, pituitary atrophy and empty sella might be present as the final outcome of PD-1/L1-irH (*90*).

As revealed in 84 cases, PD-1/L1-irH mainly involved ACTH deficiency (96.4%) especially isolated ACTH deficiency (85.7%), as opposed to whole hypophysitis caused by anti-CTLA4 (*91*). The multiple hormonal abnormalities were not common in these cases accounting for 12%. Other pituitary-endocrine axis might be affected alone without ACTH deficiency like one case concurrently presented as central hypothyroidism, hypogonadism and diabetes insipidus, one as central diabetes insipidus, and one as isolated hypogonadotropic hypogonadism (*25*). Mechanistically, the "ectopic" expression of PD-1 on corticotrophs cells could potentially elucidate the exquisite predilection for ACTH-deficiency in PD-1/L1-irH (*92*). Conversely, the "ectopic" expression of CTLA-4 on adenohypophyseal cells may partially account for the whole hypophysitis induced by anti-CTLA4 (*5*). The recovery of ACTH is typically challenging, often requiring lifelong hormone supplementation. In contrast, the restoration of gonadotropic hormone and thyroid-stimulating hormone is relatively straightforward. The predictors of immunotherapy related hypophysitis had also drawn researchers' interests. In one case-control study, anti-pituitary cell antibody was positive for most hypophysitis induced by ICIs including anti-CTLA-4 mAbs and anti-PD1/L1 mAbs. Furthermore, different human leukocyte antigen (HLA) types were found between isolated ACTH and ICI-induced hypophysitis (*93*). However only one of four cases tested positive for anti-pituitary cell antibodies in our study. Additionally, due to the limited sample size of six cases, we were unable to determine the specific type(s) of HLA that may be associated with PD-1/L1-irH.

The discontinuation of anti-PD-L1/PD-L1 mAbs did not come to agreement now which might depend on the severity of hypophysitis and the special condition of patients. In cases of mild or moderate hypophysitis, it is advisable to continue anti-PD-L1/PD-L1 mAbs , while the option to suspend or discontinue treatment may be warranted for severe hypophysitis (*94*). For example,

a NSCLC patient manifested moderate hypophysitis following 33 administrations of nivolumab, while subsequently managing to sustain an additional 50 courses concurrent with hormone replacement (*10*).

Glucocorticoids remain the primary therapy for PD-1/L1-irH until now. Generally, a physiologic regimen was recommended for mild hypophysitis whereas a high-dose protocol for severe cases (*95*). However the dosage of glucocorticoids for severe cases varied widely, ranging from 50 mg of hydrocortisone to 1 mg/kg/day of prednisolone in our study (*14,17,22*). While a high dose of glucocorticoids has been reported to be correlated with a reduced survival in ipilimumab-induced hypophysitis (*96,97*). Therefore, it's urgent to gather more evidence regarding the appropriate dosage of glucocorticoids for immunotherapy related hypophysitis. Isolated hypogonadotropic deficiency was uncommon for PD-1/L1-irH (*58*), which could be corrected by synthetic sex hormones in order to prevent bone loss and osteoporotic fractures in women, and muscular mass loss in men. Posterior hypophysitis, such as diabetes insipidus, was extremely rare and could be treated with oral desmopressin in mild cases (*59*).

A prospective study revealed that hypophysitis accompanied by ACTH deficiency was associated with improved overall survival in patients with NSCLC and melanoma who were treated with physiological doses of hydrocortisone (*98*). In our study, most patients with PD-1/L1-irH showed a positive tumor response before the diagnosis of hypophysitis. However, further information and evidence are required to fully understand the relationship between immunotherapy-related hypophysitis and the efficacy of immunotherapy.

In conclusion, clinicians should pay attention to patients treated by PD-1/L1 inhibitors especially for PD-1 inhibitor users, males, and older patients during the whole therapy period, even for several months after discontinuation. Early diagnosis and prompt managements are crucial for PD-1/L1-irH as its potentially life-threatening nature. Further studies should focus on the proper dosage of glucocorticoids and the predictors for PD-1/L1-irH.

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Original Article

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Quantitative parameters of contrast-enhanced ultrasound effectively promote the prediction of cervical lymph node metastasis in papillary thyroid carcinoma

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SUMMARY Papillary thyroid carcinoma (PTC), the most common endocrine tumor, often spreads to cervical lymph nodes metastasis (CLNM). Preoperative diagnosis of CLNM is important when selecting surgical strategies. Therefore, we aimed to explore the effectiveness of quantitative parameters of contrast-enhanced ultrasound (CEUS) in predicting CLNM in PTC. We retrospectively analyzed 193 patients with PTC undergoing conventional ultrasound (CUS) and CEUS. The CUS features and quantitative parameters of CEUS were evaluated according to PTC size ≤ 10 or > 10 mm, using pathology as the gold standard. For the PTC ≤ 10 mm, microcalcification and multifocality were significantly different between the CLNM (+) and CLNM (-) groups (both $P < 0.05$). For the PTC > 10 mm, statistical significance was noted between the two groups with respect to the margin, capsule contact, and multifocality (all $P < 0.05$). For PTC ≤ 10 mm, there was no significant difference between the CLNM (+) and CLNM (-) groups in all quantitative parameters of CEUS (all $P > 0.05$). However, for PTC > 10 mm, the peak intensity (PI), mean transit time, and slope were significantly associated with CLNM (all $P < 0.05$). Multivariate analysis showed that PI > 5.8 dB was an independent risk factor for predicting CLNM in patients with PTC > 10 mm ($P < 0.05$). The area under the curve of PI combined with CUS (0.831) was significantly higher than that of CUS (0.707) or PI (0.703) alone in the receiver operator characteristic curve analysis ($P < 0.05$). In conclusion, PI has significance in predicting CLNM for PTC > 10 mm; however, it is not helpful for PTC ≤ 10 mm.

Keywords contrast-enhanced ultrasound, quantitative parameter, papillary thyroid carcinoma, cervical lymph node metastasis

1. Introduction

Papillary thyroid carcinoma (PTC) accounts for approximately 80% of all malignant thyroid tumors, and its incidence has increased in recent decades (*1-3*). Although, as compared to other cancers, most PTC demonstrate benign behavior and have a better prognosis after surgery, the occurrence of cervical lymph node metastasis (CLNM) contributes to a poor prognosis, including local recurrence, distant metastasis, and even death (*4,5*). PTC has a high rate of CLNM, ranging from 20% to 90% (*6,7*). CLNM commonly occurs first in the central region of the neck (*8*). According to the revised American Thyroid Association guidelines, PTC patients should undergo preventive central lymph node dissection (CLND) (*9*). However, prophylactic CLND increases the extent of surgery, temporary laryngeal nerve injury, and other complications (*10-12*). The effectiveness versus risk of central lymph node dissection remains controversial when complications after surgery are considered.

Some studies have reported an association between tumor size and CLNM (*7,13,14*). However, the risk factors for CLNM correlating with the size of the PTC are not always consistent. Li *et al.* (*15*) reported that PTC > 10 mm correlated with CLNM, whereas in a multivariate analysis, Chen *et al.* (*16*) found that PTC size was not associated with CLNM. Conventional

ultrasound (CUS) is the first imaging method used to assess CLNM and preoperatively stage patients with PTC to determine the extent of surgery. However, CUS can detect only 20%–31% of PTC patients with central neck lymph node metastasis (*17*). Contrast-enhanced ultrasound (CEUS) with sensitive detection of tissue blood perfusion has been used to differentiate malignant from benign thyroid nodules (*18-20*). To our knowledge, some researchers have focused on the ability of CEUS to predict CLNM in patients with PTC using qualitative or quantitative analysis, and the value remains controversial owing to discrepant results or different methodologies (*21,22*).

Therefore, this study mainly focuses on the CEUS characteristics of PTC to identify appropriate quantitative parameters and the best cutoff value to predict CLNM according to the nodule size.

2. Materials and Methods

2.1. Patients

Between August 2019 and March 2021, 268 PTC in 268 patients underwent CEUS after a CUS examination. The inclusion criteria were as follows: (1) patients who had undergone total or near-total thyroidectomy combined with neck lymph node dissection; and (2) thyroid nodules confirmed as PTC by histopathological examination. The exclusion criteria were as follows: (1) patients who had not undergone neck lymph node dissection; and (2) insufficient CUS and CEUS images of the thyroid nodules for analysis. Of the 268 patients, 46 (17.2%) were excluded because of the absence of neck lymph node dissection, 11 (4.1%) because of incomplete CUS documents, and 18 (6.7%) because of incomplete CEUS images. Thus, 193 patients (101 women and 92 men) with confirmed PTC were included. Among the 193 patients with PTC, 79 had CLNM and 114 did not. The thyroid nodule size ranged from 2 to 58 mm (9.8 ± 6.7mm). The patient's age ranged from 22-74 years (45.6 ± 12.6 years). Figure 1 depicts the flowchart of the 193 patients selected.

This study was approved by the Institutional Review Board and Ethics Committee of Fudan University and conducted in accordance with the Declaration of Helsinki. Each patient signed an informed consent form prior to the ultrasound examination.

2.2. CUS and CEUS examinations

All thyroid lesions were examined by a sonographer (L.C.) with more than 10 years of experience in CUS and CEUS. An Aplio500 system (Toshiba Medical Systems Corp., Tokyo, Japan) with a 5-14 MHz linear transducer was used. Grayscale ultrasound and CDFI were performed for each thyroid nodule to assess nodule morphology and blood flow characteristics. The largest dimension of the nodule on CUS was measured and its size documented. CEUS examination was performed after CUS using the Aplio500 system at a low

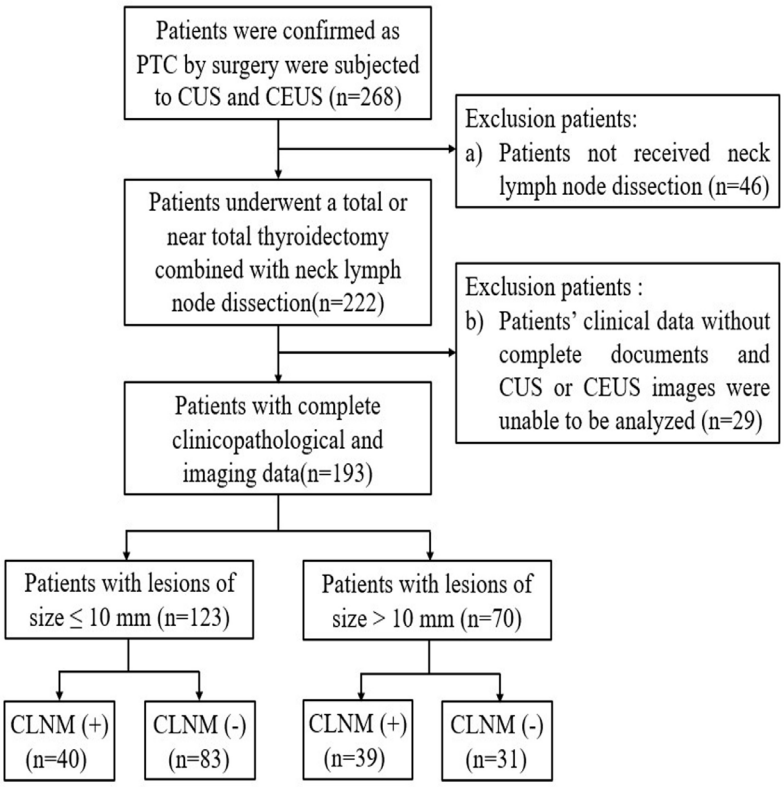

Figure 1. Flowchart of study participants included and excluded in the study.

mechanical index of 0.05-0.10. The contrast agent used in this study, SonoVue, is composed of microbubbles filled with sulfur hexafluoride (SF6) and phospholipids. For this, 5 mg of SonoVue was mixed with 5 mL 0.9% physiological saline and gently shaken to create a microbubble suspension. The SonoVue suspension (1.2 mL) was injected as a bolus, followed by an immediate rinse with 5 mL saline. At the same time, the timer and recording software were started, the imaging lasted at least 2 min, and the images were stored in digital form.

2.3. Image review and data evaluation

The CUS and CEUS characteristics of the thyroid nodules were reviewed by two radiologists (B.S. and Y.C.L.), who were blinded to the patient identities and pathological results. The CUS characteristics of thyroid nodules that were documented and described included location (lower, upper, middle, or isthmus), echogenicity (iso/hyperechoic, hypoechoic, or markedly hypoechoic), margin (regular or irregular), composition (mixed or solid), shape (wider than tall, taller than wide), microcalcification (present or absent), halo sign (present or absent), capsule contact (present or absent), diffuse lesion (present or absent), multifocality (present or absent), and blood flow signal on CDFI (avascularity, peripheral vascularity, central vascularity, or mixed vascularity). Multifocality was defined as the presence of multiple lesions discovered on ultrasonography and confirmed by pathology. Time-intensity curve (TIC) analysis software was used to obtain quantitative parameters. The following quantitative parameters were obtained from the TIC of CEUS: (1) peak intensity (PI, in dB): the intensity corresponding to the highest point on the curve; (2) time to peak (TTP, in seconds): the time from the origin to the point of PI; (3) mean transit time (MTT, in seconds): the time of intensity dropped from peak to 50% on the curve; (4) slope (SL, in dB/s): the slope coefficient of the ascent curve; (5) area under the curve (AUC, dB.s): the area under the entire curve; (6) area wash in (AWI, dB.s): the area under the ascending portion of the curve; and (7) area wash out (AWO, dB.s): the area under the decreasing portion of the curve.

Based on the images, the two radiologists made the final conclusion. If there were differences in the evaluation of specific thyroid images, a final consensus was reached through mutual consultation.

2.4. Statistical analysis

Continuous quantitative data was represented by the mean ± standard deviation and compared with the independent-sample *t* test. The χ^2 test and Fisher's exact tests were employed for discrete variables that were represented by numbers and percentages. Multivariate logistic regression analysis was used to identify the independent predictors of CLNM in patients with PTC. A receiver operating characteristic (ROC) curve was constructed to determine AUC, sensitivity, and specificity. Differences between the various methods were compared using the Z-test. Statistical Product and Service Solutions (SPSS) 25.0, and MedCalc Statistics version 19.6 were used for the statistical analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Baseline characteristics and pathological findings

Of the 193 PTC, 123 (63.7%) were ≤ 10 mm, 40 (20.7%) were CLNM (+), 83 (43.0%) were CLNM (-), 70 (36.3%) were > 10 mm, 39 (20.2%) were CLNM (+), and 31 (16.1%) were CLNM (-). For the PTC ≤ 10 mm, significant differences between the CLNM (+) and CLNM (-) groups were noted with respect to size and age (both $P < 0.05$). In contrast, for PTC > 10 mm, there was only a significant difference in size between the CLNM (+) and CLNM (-) groups ($P < 0.05$). As regards the sex and location for PTC ≤ 10 mm and the age, sex, and location for PTC > 10 mm, the proportion of the CLNM (-) groups was similar to the CLNM (+) groups ($P > 0.05$ for all) (Table 1).

3.2. CUS features

For the PTC ≤ 10 mm on CUS, the microcalcification

Table 1. Baseline characteristics and pathological findings

Characteristics	≤ 10 mm (*n* = 123)		*P*	> 10 mm (*n* = 70)		*P*
	CLNM (-) (*n* = 83)	CLNM (+) (*n* = 40)		CLNM (-) (*n* = 31)	CLNM (+) (*n* = 39)	
Size	5.9 ± 1.8	7.0 ± 1.9	0.002	12.9 ± 4.4	18.9 ± 8.9	0.000
Mean age	48.0 ± 11.6	41.9 ± 13.3	0.010	47.3 ± 12.2	42.2 ± 14.3	0.119
Sex			0.239			0.851
Male	34 (27.6)	12 (9.8)		20 (28.6)	26 (37.1)	
Female	49 (39.8)	28 (22.8)		11 (15.7)	13 (18.6)	
Multifocal			0.019			0.037
Absent	63 (51.2)	22 (17.9)		22 (31.4)	18 (25.7)	
Present	20 (16.3)	18 (14.6)		9 (12.9)	21 (30.0)	

CLNM, cervical lymph node metastasis; PTC, papillary thyroid carcinoma; Values are presented as the number (%).

and multifocality were significantly different between the CLNM (+) and CLNM (-) groups (P = 0.000 and 0.019, respectively). There was no statistical significance between the two groups in terms of composition, echogenicity, shape, margin, halo sign, capsule contact, diffuse lesion, and CDFI pattern (P > 0.05 for all). However, for PTC > 10 mm, statistically significant differences were noted between the CLNM (+) and CLNM (-) groups in terms of margin, capsule contact, and multifocality on CUS (P = 0.024, 0.030, and 0.037, respectively). No significant difference was noted between the groups in terms of the composition, echogenicity, shape, microcalcification, halo sign, diffuse lesion, multifocality, and CDFI patterns (P > 0.05 for all) (Figure 2). Table 2 details the CUS features of the CLNM (+) and CLNM (-) groups for PTC ≤ 10 mm and PTC > 10 mm.

3.3. Quantitative parameters of CEUS

For PTC ≤ 10 mm, there were no statistically significant differences between the groups CLNM (+) and CLNM (-) for all quantitative parameters of CEUS (P > 0.05). However, for PTC > 10 mm, the two groups differed significantly in terms of PI, MTT, and SL (P = 0.032, 0.031, and 0.007, respectively). There were no significant differences in TTP, AUC, AWI, or AWO (P > 0.05 for all) (Table 3, Figure 2).

3.4. Independent indicators correlated with CLNM

Multivariate analysis indicated that for PTC ≤ 10 mm, microcalcification (P = 0.001, OR = 4.165, 95% CI: 1.798 – 9.646) and multifocality (P = 0.031, OR = 2.540, 95% CI: 1.088 – 5.929) on CUS were independent indicators for predicting CLNM (Table 4). However, for PTC > 10 mm, capsule contact (P = 0.015, OR = 4.401, 95% CI: 1.335 – 14.516) and multifocality (P = 0.021, OR = 4.233, 95% CI: 1.240 – 14.445) on CUS and PI (P = 0.021, OR = 5.898, 95% CI: 1.312 – 26.508) on CEUS were independent predictors of CLNM (Tables 4 and 5, respectively).

3.5. Predicting values of CLNM for PTC

For PTC ≤ 10 mm, the AUC for CUS (microcalcification combined with multifocality) was 0.716 with 87.500% sensitivity and 48.193% specificity (Table 6). For PTC > 10 mm, the AUC of PI was 0.703 with 92.308% sensitivity and 48.387% specificity by the cut-off of 5.8 dB, and the AUC of PI combined with CUS (capsule contact combined with multifocality) was 0.831 with 84.615% sensitivity and 74.193%, was significantly higher than that of PI and CUS (both P < 0.05) (Table 6). Figure 3 shows the ROC curves of capsule contact, multifocality, and PI correlated with CLNM.

4. Discussion

As CLNM is associated with the prognosis and treatment of PTC, correctly predicting CLNM is essential for PTC patients (*23-25*). CUS is generally the preferred choice for predicting CLNM in PTC because it is conveniently available, non-invasive, inexpensive, and high resolution. However, CUS may not always be reliable because of its overlap with CUS features associated with PTC with or without CLNM. In various studies, the risk factors of CUS for predicting CLNM have differed, including shape, size, boundary, multifocality, capsule contact, calcification, and blood flow (*26-30*). In the current study, we analyzed the CUS characteristics of PTC based on tumor size and calculated the prediction efficiency

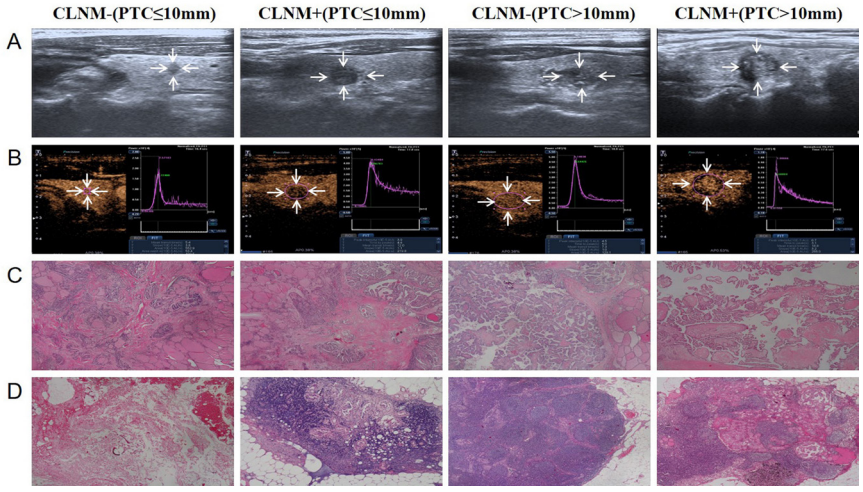

Figure 2. CUS, CEUS, pathology, and corresponding lymph node pathological images of PTC (≤ 10 mm and > 10 mm). **(A)** CUS feature of PTC (≤ 10 mm and > 10 mm) with or without CLNM. **(B)** The quantitative parameters of CEUS of PTC (≤ 10 mm and > 10mm) with or without CLNM. **(C)** Histopathology of PTC (≤ 10 mm and > 10 mm) with or without CLNM. **(D)** Histopathology of benign lymph nodes and metastatic lymph nodes in the neck (≤ 10 mm and > 10 mm). Above figures show that in PTC ≤ 10 mm, microcalcifications are more prone to cervical lymph node metastasis, while PTC > 10 mm, capsule contact and PI > 5.8 dB are more prone to lymph node metastasis.

Table 2. CUS features for PTC

Characteristics	≤ 10 mm (*n* = 123)		*P*	> 10 mm (*n* = 70)		*P*
	CLNM (-) (*n* = 83)	CLNM (+) (*n* = 40)		CLNM (-) (*n* = 31)	CLNM (+) (*n* = 39)	
Location			0.279			0.058
Lower	17 (13.8)	5 (4.1)		7 (10.0)	35 (50.0)	
Upper/middle	66 (53.6)	35 (28.5)		24 (34.3)	4 (5.7)	
Composition			0.148			1.000
Solid	83 (67.5)	39 (31.7)		30 (42.9)	38 (54.3)	
Mixed	0 (0)	1 (0.8)		1 (1.4)	1 (1.4)	
Echogenicity			0.377			0.287
hypoechoic	77 (62.6)	35 (28.5)		29 (41.4)	36 (51.4)	
Iso/hyperechoic	6 (4.9)	5 (4.1)		2 (2.9)	3 (4.3)	
Shape			0.085			0.095
Taller than wide	34 (27.6)	23 (18.7)		27 (38.6)	38 (54.3)	
Wider than tall	49 (39.8)	17 (13.8)		4 (5.7)	1 (1.4)	
Margin			0.514			0.024
Regular	32 (26.0)	13 (10.6)		17 (24.3)	11 (15.7)	
Irregular	51 (41.5)	27 (21.9)		14 (20.0)	28 (40.0)	
Microcalcification			0.000			0.250
Absent	51 (41.5)	11 (8.9)		17 (24.3)	16 (22.9)	
Present	32 (26.0)	29 (23.6)		14 (20.0)	23 (32.9)	
Halo sign			0.349			0.425
Absent	80 (65.0)	37 (30.1)		29 (41.4)	38 (54.3)	
Present	3 (2.4)	3 (2.4)		2 (2.9)	1 (1.4)	
Capsule contact			0.629			0.030
Absent	75 (61.0)	35 (28.5)		20 (28.6)	15 (21.4)	
Present	8 (6.5)	5 (4.1)		11 (15.7)	24 (34.3)	
Diffuse lesion			0.957			1.000
Absent	77 (62.6)	37 (30.1)		29 (41.4)	37 (52.9)	
Present	6 (4.9)	3 (2.4)		2 (2.9)	2 (2.9)	
Multifocal			0.019			0.037
Absent	63 (51.2)	22 (17.9)		22 (31.4)	18 (25.7)	
Present	20 (16.3)	18 (14.6)		9 (12.9)	21 (30.0)	
CDFI patterns			0.199			0.512
Avascularity	56 (45.5)	23 (18.7)		8 (11.4)	6 (8.6)	
Central	16 (13.0)	6 (4.9)		5 (7.1)	7 (10.0)	
Peripheral	5 (4.1)	3 (2.4)		6 (8.6)	5 (7.1)	
Mixed	6 (4.9)	8 (6.5)		12 (17.1)	21 (30.0)	

CDFI, color Doppler flow imaging; CUS, conventional ultrasound; CLNM, cervical lymph node metastasis; PTC, papillary thyroid carcinoma; Values are presented as the number (%).

Table 3. Quantitative parameters of CEUS for PTC

Characteristics	≤ 10 mm (*n* = 123)		*P*	> 10 mm (*n* = 70)		*P*
	CLNM (-) (*n* = 83)	CLNM (+) (*n* = 40)		CLNM (-) (*n* = 31)	CLNM (+) (*n* = 39)	
PI (dB)	14.8 ± 19.6	11.9 ± 9.7	0.389	12.5 ± 13.1	25.3 ± 33.2	0.032
TTP (s)	7.6 ± 6.9	12.5 ± 26.1	0.215	5.9 ± 3.6	4.6 ± 1.9	0.053
MTT (s)	40.8 ± 57.8	40.3 ± 60.3	0.965	53.9 ± 67.3	23.2 ± 41.4	0.031
SL (dB / s)	7.1 ± 26.1	4.1 ± 4.7	0.467	2.9 ± 2.9	8.2 ± 10.9	0.007
AUC (dB · s)	716.6 ± 875.9	638.2 ± 646.7	0.416	864.7 ± 1312.5	1037.2 ± 1224.4	0.573
AWI (dB · s)	42.5 ± 55.9	67.4 ± 176.4	0.244	50.7 ± 79.9	66.3 ± 84.9	0.435
AWO (dB · s)	676.5 ± 822.9	585.4 ± 612.6	0.535	814.1 ± 1234.5	970.7 ± 1141.6	0.484

AUC, area under the receiver operating characteristic curve; AWI, area wash in; AWO, area wash out; CEUS, contrast-enhanced ultrasound; CLNM, cervical lymph node metastasis; MTT, mean transit time; PTC, papillary thyroid carcinoma; PI, peak intensity; TTP, time to peak; SL, slope. Values are presented as the number (%).

of CLNM. We confirmed that microcalcification and multifocality on CUS are related to CLNM in nodules ≤ 10 mm. For nodules > 10 mm in size, capsule contact and multifocality on CUS were independent predictors of CLNM. The results of the present study show that CLNM is strongly related to multifocality, whether the nodule is ≤ 10 mm or > 10 mm. These findings are in line with previous reports, which documented

Table 4. Multivariate analysis of CUS characteristics associated with CLNM in PTC

Independent risk factor	β	S.E.	Wald	df	*P*	OR	95% CI
≤ 10 mm PTC							
Microcalcification	1.427	0.428	11.085	1	0.001	4.165	1.798 - 9.646
Multifocal	0.932	0.432	4.648	1	0.031	2.540	1.088 - 5.929
Constant	-1.846	0.379	23.780	1	0.000	0.158	/
> 10 mm PTC							
Margin	0.717	0.547	1.719	1	0.190	2.047	0.701 - 5.976
Capsule contact	1.482	0.609	5.924	1	0.015	4.401	1.335 - 14.516
Multifocal	1.443	0.626	5.309	1	0.021	4.233	1.240 - 14.445
Constant	-1.496	0.579	6.678	1	0.010	0.224	/

CI, confidence interval; CUS, conventional ultrasound; CLNM, cervical lymph node metastasis; df, degrees of freedom; OR, odds ratio; PTC, papillary thyroid carcinoma; S.E., standard error.

Table 5. Multivariate analysis of quantitative parameters of CEUS associated with CLNM in PTC (> 10 mm)

Independent risk factor	β	S.E.	Wald	df	*P*	OR	95% CI
PI	1.775	0.767	5.356	1	0.021	5.898	1.312 - 26.508
MTT	1.314	0.779	2.842	1	0.092	3.720	0.808 - 17.129
SL	0.727	0.652	1.243	1	0.265	2.069	0.576 - 7.429
Constant	-2.048	0.854	7.945	1	0.005	0.090	/

CEUS, contrast-enhanced ultrasound; CLNM, cervical lymph node metastasis; df, degrees of freedom; MTT, mean transit time; OR, odds ratio; PTC, papillary thyroid carcinoma; PI, peak intensity; S.E., standard error; SL, slope.

Table 6. ROC analysis for predicting CLNM in PTC

Independent risk factor	Cut-off value	Sensitivity	Specificity	AUC (95% CI)
≤ 10 mm PTC				
Multifocal[1]	Present	18 / 40 (45.0)	63 / 83 (75.9)	0.605 (0.512 - 0.961)
Microcalcification[2]	Present	29 / 40 (72.5)	51 / 83 (61.4)	0.670 (0.579 - 0.752)
1 + 2	1 + 2	35 / 40 (87.5)	40 / 83 (48.2)	0.716 (0.628 - 0.794)
> 10 mm PTC				
Capsule contact[3]	Present	24 / 39 (61.5)	20 / 31 (64.5)	0.630 (0.506 - 0.743)
Multifocal[4]	Present	21 / 39 (53.8)	22 / 31 (70.9)	0.624 (0.500 - 0.737)
3 + 4	3 + 4	36 / 39 (92.3)	13 / 31 (41.9)	0.707 (0.586 - 0.810)
PI[5]	5.8	36 / 39 (92.3)	15 / 31 (48.4)	0.703 (0.582 - 0.807)
3 + 4 + 5	3 + 4 + 5	33 / 39 (84.6)	23 / 31 (74.2)	0.831 (0.722 - 0.910)

AUC, area under the receiver operating characteristic curve; CLNM, cervical lymph node metastasis; PTC, papillary thyroid carcinoma; PI, peak intensity; ROC, receiver operating characteristic. Values are presented as the number (%).

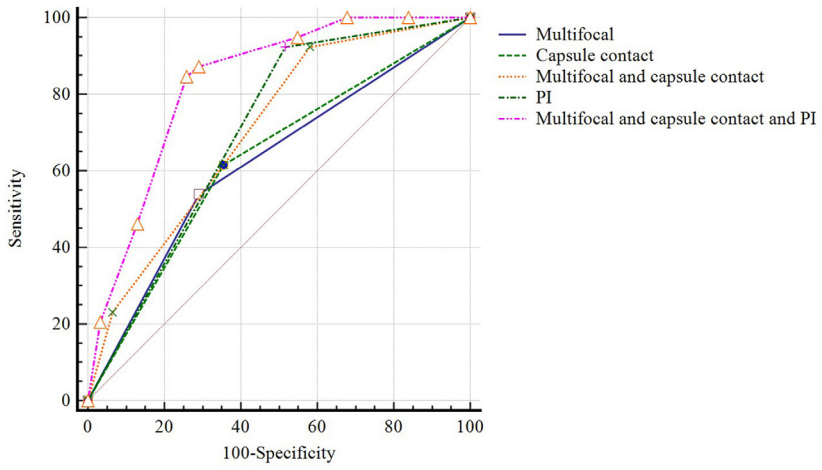

Figure 3. ROC analysis for the features of CUS and quantitative parameters of CEUS in predicting CLNM in patients with PTCs (> 10 mm). The ROC curves of capsule contact, multifocality and PI correlating with CLNM.

that multifocality in PTC is associated with metastatic spread in histopathology (*31,32*). The difference in the results between nodules ≤ 10 and > 10 mm may be due to tumor growth and metabolism. Microcalcification is a malignant feature of CUS. In histopathology, microcalcification in PTC is mainly caused by psammoma bodies with a diameter of 10-100 μm and may be related to the aggressive behavior of PTC (*33*). Owing to other pathological structures, such as focal fibrosis in nodules > 10 mm, distinguishing fibrosis from microcalcification is difficult (*34*). Capsule contact was identified as a risk factor for CLNM in nodules measuring > 10 mm, probably because cancer cells are easily destroyed by the capsule and spread to other parts, such as fat, muscle, and lymphoid tissue (*32*). Though CUS had a high sensitivity in the prediction of CLNM for PTC ≤ 10 mm and PTC > 10 mm (87.5%, 92.3%, respectively), it showed a low specificity for that (48.2%, 41.9%, respectively). Therefore, further imaging studies are required to predict cervical lymph node metastasis in patients with PTC.

Presently, CEUS is a topic of active research in ultrasound medicine. This modality can detect both large-diameter vessels and high-velocity blood flow, significantly improving the detection of small vessels within a lesion (*35-37*). Furthermore, CEUS offers advantages over other imaging techniques such as CT and MRI, including the ability for repeated examinations, the absence of radiation exposure, no hepatorenal toxicity, and a low risk of allergic reactions. Previous studies have identified the usefulness of CEUS for predicting CLNM in PTC (*38-40*). However, most of these studies focused on the qualitative analysis of CEUS features (*41,42*). The fact that qualitative analysis based on visual observation is subjective and may not provide an objective evaluation of the details of PTC perfusion is worth noting. In contrast, quantitative CEUS can provide objective perfusion characteristics with exceedingly good reproducibility, reduce sonographic dependency and subjective errors, and provide steady and reliable results.

Several studies have confirmed that microvascular density correlates with CLNM in PTC (*43,44*). A higher microvascular density in the tumor tissue is associated with a higher likelihood of CLNM (*45*). Microvascular density is considered the "gold standard" to quantitatively evaluate the formation of tumor neovascularization, but it requires invasive procedures such as surgery or puncture to obtain tissue specimens (*46*). Fortunately, time-intensity curve (TIC) parameters derived from CEUS reflect the process of intensity enhancement in the region of interest (ROI) over time following contrast agent injection. These quantitative parameters of CEUS provide an objective reflection of tissue microcirculation changes, making it a non-invasive alternative for assessing neovascularization and predicting CLNM in PTC. PI positively correlated with microvascular density and was defined as the intensity at which the contrast agent reached peak perfusion. The results of this study indicated that for >10 mm PTC, the difference in PI between the CLNM (+) and CLNM (-) groups was statistically significant (25.30 ± 33.17 *vs.* 12.50 ± 13.10 dB, $P = 0.032$). Further, the multifactor analysis suggested that PI was a predictive factor for CLNM, which is in agreement with earlier findings (*47,48*). The slope is defined as the rate at which the contrast agent reaches its peak intensity. Because of the larger new capillaries in the tumor, a larger amount of contrast agent centralizes in the PTC, resulting in a steeper slope. The slope of PTC > 10 mm with CLNM was higher than that of PTC > 10 mm without CLNM. The MTT reflects the retention time of the contrast agent in the ROI, which is closely related to the blood flow velocity. Wei *et al*. (*49*) reported that the internal blood vessels of malignant tumors are thicker than those of benign nodules and that arteriovenous fistulas are easy to form, which shortens the retention time of contrast agents in the blood vessels (*50*). The present study found that the MTT of PTC > 10 mm in the CLNM (+) group was shorter than the CLNM (-) group. However, the results of multivariate analysis suggested that SL and MTT were not associated with CLNM for PTC > 10 mm ($P > 0.05$). All quantitative parameters of CEUS in PTC ≤ 10 mm were not statistically different between the two groups ($P > 0.05$). The finding may be due to the fact that small tumor neovascularization beds in PTC ≤ 10 mm are not sufficiently developed; insufficient number and small diameter result in less perfusion of contrast agent. Further, owing to the inevitable breathing and tension movements of patients, the dynamic ROI image of nodules ≤ 10 mm is often unstable. Because the lesion is quite small, a slight movement will cause a deviation in the parameters from the ROI. Thus, accurate tracking and identification of small lesions are difficult. Consequently, the quantitative parameters obtained after analysis may have been inaccurate, which could explain why these parameters did not show any differences. Therefore, we consider that the use of quantitative CEUS in nodules ≤ 10 mm to predict CLNM is limited.

Furthermore, in our study, we calculated and compared the AUC for predicting CLNM in PTC using the quantitative parameters of CEUS, CUS, and a combination of both. For nodules ≤ 10 mm, the AUC for CUS features in predicting CLNM was 0.716, which was higher than the AUC of 0.687 reported by Luo *et al*. (*51*). The AUCs for CUS and CEUS in predicting CLNM in nodules > 10 mm were 0.706 and 0.703, respectively. Additionally, our study demonstrated that the AUC for the combination of CUS and CEUS was 0.831, which was significantly higher than that for CUS alone ($P < 0.05$). These findings suggest that the combination of CUS and CEUS can improve the predictive accuracy of CLNM, particularly for nodules larger than 10 mm.

Acknowledging the limitations of this study is important. First, this being a single-center study, a

selection bias may have been introduced. Therefore, large-scale, multicenter studies are necessary to validate these results. Second, although quantitative evaluation of CEUS is considered more objective than qualitative evaluation, it requires skilled operators and thus may be less flexible. Even a slight movement of the ROI in a target lesion can lead to inaccurate results. Standardization of the technique and further training of operators may be necessary to improve accuracy. Third, the study did not survey the factors related to long-term survival or locoregional recurrence rates. Future research should address these questions to provide a deeper understanding of the topic.

In conclusion, this study found that certain characteristics, such as microcalcification and multifocality, observed in CUS are helpful in predicting CLNM in nodules that are 10 mm or smaller. However, the use of quantitative CEUS to predict lymph node metastasis in smaller nodules is limited. For nodules larger than 10 mm, characteristics such as capsule contact, multifocality observed on CUS, and peak intensity observed on CEUS can be used to predict CLNM. Quantitative parameters obtained from CEUS are valuable for evaluating CLNM. The combination of CUS and CEUS can be an effective approach for predicting CLNM in nodules larger than 10 mm.

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Brief Report

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Medication incidents associated with the provision of medication assistance by non-medical care staff in residential care facilities

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SUMMARY The shift towards community-based care in Japan has led to increased medication assistance for older people by non-medical care staff. These staff members help take pre-packaged medications, apply patches, and administer eye drops. This study assessed the risks associated with such assistance by reviewing medication-related incidents across 106 residential care facilities between April 1, 2015, and March 31, 2016. An analysis of incident reports showed that all incidents were minor, with no serious outcomes. The incidents were categorized into four types: dropped drugs, misdelivery/misuse of medicines, forgetting to take medicines, and loss of medicines, with dropped drugs being the most frequent. Most incidents occurred in the morning and primarily involved residents with intermediate nursing care needs. These findings indicate a low risk of serious incidents because of medication assistance from non-medical staff. However, the frequency and nature of the incidents were influenced by the timing of medication administration and the care needs of the residents. These insights highlight the need for customized approaches to medication assistance, considering the residents' care levels and potentially optimizing medication administration times to improve safety in residential care settings.

Keywords risk management, medication-related incidents, home care services, residential facilities

1. Introduction

Recently, there has been an increase in the population of older people, leading to a shift from hospital-based to community-based care in Japan because of the inability of hospitals to accommodate long stays for elderly patients. The "community-based integrated care system," which offers comprehensive services, including health care provision, nursing care, prevention, housing, and livelihood support, has emerged in response (*1*). Non-medical care staff without a license as nurses or doctors are increasingly involved in providing medical care and daily lifestyle support to individuals in homes or residential care facilities. For instance, care staff in these facilities may assist older people with pre-packaged medications prepared by pharmacists. According to the interpretation of the Medical Practitioners' Act Article 17, the Dental Practitioners' Act Article 17, and the Public Health Nurse, Midwife, and Nurse Act Article 31 issued by the Ministry of Health, Labour and Welfare, while care staff is not authorized to perform "medical services," assisting with pre-packaged medications, applying patches, or administering eye drops is not considered a "medical service," thus, can be performed by care staff. This clarification allows non-medical staff to provide certain care services under specific conditions.

Under these conditions, the potential for incidents arising from medication assistance by non-medical staff raises concerns. A considerable number of elderly residents in such facilities receive pharmacotherapy for chronic conditions, with data indicating that approximately 40% of nursing home residents in the USA are prescribed nine or more medications (*2*). Despite the presumed lower risk of incidents in Japanese residential care settings compared to hospitals, evidence from our previous survey suggests that care staff find providing medication assistance challenging (*3*).

While past research has addressed medication incidents in hospitals (*4-7*) or primary care (*8,9*) and nursing homes (*10*), there has been little focus on incidents involving non-medical staff. Our previous

studies examined the factors contributing to certain types of incidents by non-medical staff in residential care facilities (*11*). However, a comprehensive investigation of all incident types by non-medical staff in such settings has not been conducted.

This study aimed to assess the risks associated with medication assistance by non-licensed care staff by analyzing medication-related incidents in residential care facilities where integrated care is provided.

2. Materials and Methods

2.1. Design

This multicenter retrospective study analyzed incident reports related to medication assistance by non-medical care staff in 106 long-term residential care facilities operated by a single company. These facilities shared characteristics, such as having at least one staff member present 24 hours a day, one caregiver or one nurse for every three persons requiring nursing care, or one caregiver or one nurse for every ten persons requiring support, and private living quarters for all residents. The residents of these facilities underwent routine physician visits. An incident was defined as any event leading to a resident's failure to take their prescribed medication accurately.

2.2. Data collection

This study included incidents from April 1, 2015, to March 31, 2016, focusing only on those involving non-medical care staff who were not medical professionals. Medical staff incidents were excluded. Most residents in the participating facilities received medication assistance, forming the study's basis.

Following any incident, a detailed incident report was filled out, covering the following points: conditions at the time of the incident, nature of the incident, time, place, and basic information about the resident who experienced the incident, such as age, sex, and nursing care requirement level. The responsibility for completing these reports was not confined to the care staff directly involved in the incident; facility managers or nurses were also authorized to document incidents. Policies at these facilities encourage the reporting of minor incidents.

An identifying code was assigned to the facility name, care staff name, and resident name in the incident report, anonymized to maintain confidentiality.

2.3. Data analysis

These incidents were classified into four distinct categories: dropped drugs, misdelivery/misuse of medicines, forgetting to take medicines and loss of medicines. The "taking medicines" scope included eye drops, patches, or ointments. These categories are defined as follows. Dropped drugs: Finding drugs in an unexpected location in an unwrapped state. Misdelivery/misuse of medicines: providing support in a manner different from the standard procedures for residents taking medicines. Forgetting to take medicines: failure to take medicines at the prescribed time. Loss of medicines: inability to find medicines.

The number of incidents was calculated for each category, and the correlation coefficient between the number of incidents per facility and the facility population size was calculated. The significance of this correlation was evaluated at the 5% level. Additionally, the impact of the timing of incidents and residents' care needs, represented by their nursing care requirement levels, on incident rates was analyzed. The nursing care requirement level measures the extent of residents' care needs, ranging from support level 1 to long-term care level 5, with increasing levels indicating a greater need for care. The grading system is shown in Table 1.

The statistical analysis was conducted using Microsoft Excel version 16.28.

2.4. Ethical approval

All the procedures were performed according to the principles of the Declaration of Helsinki. This study was approved by the Research Ethics Review Committee of the Faculty of Pharmaceutical Sciences, University of Tokyo (accession number 28-15, approved on November 1, 2016) and the Research Ethics Review Committee of the Faculty of Pharmacy, Keio University (accession number 180731-7, approved on July 31, 2018).

3. Results and Discussion

This study included 2,142 incidents from 106 facilities, with an average of 20.4 incidents per facility (median, 18). The correlation coefficient between the number of residents and the incidents in each facility was 0.33 ($P < 0.05$), indicating a weak but significant correlation.

The most common incident type was "dropped drugs," comprising 55.0% of all reported incidents (Table 2). Within this category, incidents were split between "discovery of dropped drugs" and "spitting up/falling while taking medicines." Additionally, the category of "mis-delivery/misuse of medicines" was divided into "wrong person," "wrong dose," "wrong time," "forgetting to remove medicines," and "other mis-delivery/misuse" (Table 2). Cases of forgetting to remove medications were related to forgetting to remove patches. The outcome of all incidents was either "there is no change about residents' conditions" or "we conduct a follow-up for residents' conditions," and none required residents to undergo additional procedures such as emergency medical examination. More than half the incidents

Table 1. Nursing care requirement levels

a. Requiring Support, Requiring Care, Independence	
Requiring Support	They can perform basic activities of daily living, such as walking and getting up on their own, but need some support with daily living instrumental activities.
Requiring Care	Unable to perform basic activities of daily living, such as walking and getting up on their own, they need some support.
Independence	They can perform basic activities of daily living, such as walking and getting up on their own, and daily living activities, such as taking medicine and using the telephone.
b. Status of persons requiring care at each nursing care requirement level	
Long-Term Care Level 1	The ability to perform instrumental activities of daily living is further impaired compared with persons whose status is "requiring help," and partial nursing care is required.
Long-Term Care Level 2	In addition to the condition with long-term care level 1, partial care is also needed for basic activities of daily living.
Long-Term Care Level 3	The ability to perform basic and instrumental activities of daily living is markedly lower compared with persons whose status is long-term care level 2, and almost total support is required.
Long-Term Care Level 4	In addition to long-term care level 3 conditions, the ability to move is further decreased, and it is difficult to perform daily living without care.
Long-Term Care Level 5	The ability to move is further decreased compared with persons whose status is long-term care level 4, and it is almost impossible to perform daily living without care.

This table is available at https://www.mhlw.go.jp/topics/kaigo/kentou/15kourei/sankou3.html (Japanese website).

Table 2. Types of incidents with medication support involved by care staff

Category of Incidents	Cases (%)
Dropped drugs	
Spitting up/falling while taking medicines	160 (7.5)
Discovery of dropped drugs	1017 (47.5)
Mis-delivery/misuse of medicines	
Wrong person	139 (6.5)
Wrong dose	23 (1.1)
Wrong timing	85 (4.0)
Forgetting to remove medicines	30 (1.4)
Other mis-delivery/misuse	31 (1.5)
Forgetting to take medicines	639 (29.8)
Loss of medicines	18 (0.8)

occurred in the communal setting of a restaurant facility, highlighting the complexity of managing medication assistance in shared spaces. This finding suggests that communal areas present challenges to medication safety, necessitating strategies that consider the unique dynamics of these environments.

Each day, the number of incidents per type was calculated (Table 3). Overall, the highest number of incidents occurred in the morning, followed by the evening. While incidents of "forgetting to take medicines" tended to occur more frequently right after waking, incidents of "dropped drugs" were more common during the morning hours. This pattern suggests a potential risk concentration at specific times of day, particularly when medication administration coincides with other care activities. In the case of "dropped drugs" and "loss of medicines," many of the incidents where the time of incident occurrence could not be determined were classified as unidentified.

The incidence rate was examined by nursing care requirement level which indicates residents' need for nursing care (Figure 1). One hundred and forty-two incidents were excluded from the analysis because the residents involved could not be identified. The total number of included incidents were 1,998. The number of incidents corrected for the number of residents in each care requirement level tended to be higher for residents at long-term care levels 2, 3, or 4 and lower for residents at support level 1 or long-term care level 5. There was little shifting in the number of incidents of "forgetting to take medicines" or "mis-delivery/misuse of medicines" according to the nursing care requirement level. However, the number of incidents of "dropped drugs" tended to be especially lower among residents at long-term care level 5. These distributions underscore the influence of resident care needs on the likelihood of medication-related incidents, with a particular vulnerability observed among those with intermediate care requirements.

Our results suggest that the medication support provided by non-medical care staff in residential care settings involves a low risk of serious incidents and that the outcomes of incidents were not serious. In the systematic review, Ferrah *et al.* reported that the serious effects of medication errors were low in nursing home settings (*12*), which is consistent with our research. However, most of the previous studies in their systematic reviews focused on medication errors involving medical professionals, and few focused on

Table 3. Timing of the incidents

Timing	Category of Incidents			
	Dropped drugs	Mis-delivery/misuse of medicines	Forgetting to take medicines	Loss of medicines
Right after waking	4 (0.3)	3 (1.0)	49 (7.7)	0 (0.0)
In the morning	346 (29.4)	85 (27.6)	182 (28.5)	1 (5.6)
In the afternoon	71 (6.0)	47 (15.3)	58 (9.1)	1 (5.6)
In the evening	165 (14.0)	73 (23.7)	155 (24.3)	1 (5.6)
At bedtime	88 (7.5)	25 (8.1)	70 (11.0)	3 (16.7)
Other time	6 (0.5)	74 (24.0)	118 (18.5)	1 (5.6)
Unidentified	497 (42.2)	1 (0.3)	7 (1.1)	11 (61.1)
total	1177	308	639	18

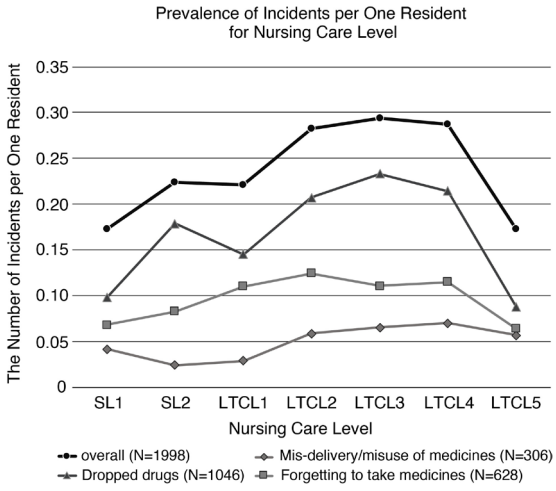

Figure 1. Prevalence of incidents per one resident according to nursing care requirement level. The number of incidents at each nursing care requirement level was divided by the number of persons in all facilities to obtain the number of incidents experienced per person. Plots show the total number of incidents and each incident type. The loss of medicines is not shown because the number was too small. LTCL: Long-term care level. SL: Support level.

incidents involving non-medical staff assisting patients with their medications. Our study is the first to focus on care staff who are not medical professionals and to investigate the prevalence of medication incidents involving non-medical care staff in a residential care setting.

This study identified four primary medication-related factors in residential care facilities. Incidents of "forgetting to take medicines" and "mis-delivery/misuse of medicines" reflect patterns that are also observed in hospital settings (*4,13*). The most common type of incident was "dropped drugs" in our study. Notably, this study quantitatively showed that "dropped drugs" are a common incident in residential care facilities in Japan, highlighting operational challenges in residential care facilities.

None of the 2,142 medication-related incidents documented yearly required emergency medical care. This finding indicates a minimal risk of severe incidents from medication assistance by non-medical staff in residential care, likely because of the limited scope of such assistance. In addition, incidents like "wrong person" or "wrong dose" seen in this study have also been reported as medication errors in hospital settings (*4-6,13,14*) or in care home settings (*11,15,16*), and some of these incidents have been harmful reportedly (*13,16*) . Although no harmful incidents were observed in our study, efforts to prevent even minor incidents are important for risk management in residential care settings.

Some incidents occurred at unknown times of the day, and most were cases in which dropped drugs, which accounted for the largest number of incidents, were discovered later. This category may include cases of "refusal," in which the patient pretended to take medicines on the spot but intentionally did not ingest them, or "leftover," in which the medicine was not completely swallowed and remained in the mouth, even if medication assistance was provided properly at the time. The highest number of incidents occurred in the morning, possibly because many residents take their medications in the morning, thus concentrating the staff's workload. A study conducted in residential facilities in Japan showed that residents consume more medicines in the morning (*17*). Therefore, the dispersion of drug administration times could be a method for preventing such incidents.

In this study, residents' conditions were evaluated based on the nursing care requirement level, as this could be a factor in the occurrence of incidents. Notably, the frequency of "dropped drugs" incidents varied significantly with the nursing care requirement level. Incidents were less common among residents classified as requiring long-term care level 5. Since it is impossible for residents requiring long-term care level 5 to lead their daily lives without nursing care, it is possible that the care staff more strongly recognized the need for care, including assistance in taking medicines. However, the number of incidents tended to be higher among residents with requirements for long-term care levels 2, 3, or 4 (intermediate care level). These levels represent a gradual decline in the ability to conduct activities of daily living, and the care staff did not adequately recognize this decline when providing medicines. It may be necessary to appropriately assess

the status of residents' activities of daily living and provide additional support for taking medication.

There may be potential for pharmacists to improve these residential facility conditions. Community pharmacists generally dispense and deliver pre-packaged medicines to residents. Our previous research highlighted the need for pharmacists to be involved in medication support by care staff (*3*) . Thus, there may be room for pharmacists to improve medical safety in residential care facilities in Japan through comprehensive medical reviews.

Most of the facilities examined in this study were situated around urban areas in Japan, thereby indicating a high degree of generalizability for urban facilities. However, basic information regarding the care staff involved in the incidents, such as sex, age, and work experience, could not be analyzed because they were not identified in the incident reports used in this analysis. As our previous study showed that the mental burden of caregivers contributes to the occurrence of incidents (*11*), further analysis of incident occurrence situations, considering staff background information, should be conducted. Furthermore, the period of the incidents covered in this study was one year, from April 2015 to March 2016, and the possibility that the situation changed cannot be ruled out. However, since there are no major updates to the section on medication assistance not related to medical services in the notice issued by the Ministry of Health, Labour and Welfare in 2022, and there seems to be no major changes in medication-related tasks that can be performed by non-medical staff. Thus, it can be assumed that the trend of incidents will not change significantly.

In summary, many minor medication-related incidents were observed, and the outcomes were not serious, indicating that the risk of serious incidents occurring with medication support by non-medical staff, who are not doctors or nurses, should be low. Among them, dropped drugs occurred most frequently, and because their occurrence was related to the residents' nursing care requirement level, it is important to develop countermeasures that consider this factor. In addition, most of the incidents occurred in the morning. Thus, from the pharmacological management perspective, improving medical safety in residential care facilities may be effective by dispersing the administration time of medicines.

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Brief Report

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Association between the experience of exertional heat illness (EHI) and living conditions of collegiate student athletes

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SUMMARY Exertional heatstroke (EHS), a severe form of exertional heat illness (EHI), is the third leading cause of death in athletes; thus, early detection and prevention of EHI can help prevent EHS, which is a life-threatening condition. This study aimed to clarify the association between the cognizance of experiencing EHI and living conditions and specific EHI symptoms among collegiate athletes. This study was conducted in October 2022 by administering a questionnaire to 237 male collegiate athletes. Of the 215 (90.7%) respondents, 197 (91.6%) provided valid responses; among them, 88 (44.7%) responded they had experienced EHI, while 109 (55.3%) had not. A history of medical examinations due to EHI, having experienced headaches during summer activities, and having read the EHI manual were factors indicating cognizance of EHI. The number of times meals containing a staple food, main dish, and side dish were eaten in a day was a factor in preventing EHI. Early detection of EHI is important for its prevention, and it is important that athletes themselves have knowledge of symptoms and can correctly self-diagnose EHI. Emphasizing the potential of a well-balanced dietary intake has the potential to prevent EHI is crucial.

Keywords EHI, EHS, male collegiate athletes, cognizance of EHI, self-diagnose EHI, prevention

1. Introduction

As global temperatures increase due to climate change, health hazards caused by heat are increasing. In particular, physical exercise in a hot and humid environment inhibits the heat dissipation system by sweating and increases the core body temperature (*1*). As a result of an increase in core body temperature, exertional heat illnesses (EHI) can occur in various ways, ranging from mild, non-life-threatening conditions, to life-threatening conditions, such as common and exertional heatstroke (*2*). Common heat stroke refers to a sudden increase in core body temperature to 40°C or higher, causing central nervous system dysfunction (*3*). Exertional heatstroke (EHS) is usually caused by strenuous physical activities performed in a hot and humid environment and is characterized by central nervous system dysfunction (such as collapse, convulsions, and coma), followed by organ and tissue damage, and even death in patients with hyperthermia (*4*). The incidence of EHI is estimated to be 1 in 1000 athletes, and it is the third leading cause of death among athletes during physical activities, with epidemiological data indicating a mortality rate of approximately 27% (*5*).

In a study of sports-related deaths in Japanese high schools between 2009-2018, the leading causes were heart-related (47.6%), followed by head and neck injuries (23.8%) and EHS (22.2%) (*6*). Moreover, from 1998-2018, the causes of nontraumatic fatalities among U.S. high school and college football players were psychogenic (57.7%) and EHS (23.6%) (*7*). Other studies found that since 2000, more than 40 American high school football players and 10 college football players have died due to EHI in the United States (*8*). As shown in previous literature, EHI is a leading cause of death during physical activity. Importantly, it is also preventable. Enforcing the correct preventive measures and administering appropriate treatment is a necessity to avoid EHS-related complications. Above all, detection during the stage of mild symptoms of EHI is crucial to prevent its progression to EHS.

Early detection of EHI and rapid cooling (body

cooling by immersion up to the neck in a tub of cold/ice water) were shown to reduce both morbidity and mortality associated with EHI (*8, 9*). Diagnostic signs for early detection of EHI include collapse, confusion, and convulsions; however, these signs and symptoms are likely to be missed if not carefully observed by instructors and staff (*9*). Therefore, athletes themselves should be able to promptly detect and report subjective symptoms.

Prevention is more important than any existing treatment for EHI (*5*); thus, it is necessary to know the risk factors of EHI to implement adequate preventive measures. The current risk factors for EHI include low physical fitness, being overweight, exercising during hot weather, sleep deprivation, improper inadequate acclimatization, high heat index in accordance with Wet Bulb Globe Temperature (WBGT), solar radiation, physical exertion beyond one's physical fitness capacity, inadequate physical activity/rest cycle, lack of proper medical triage, and inadequate treatment (*10*). On the other hand, little attention has been paid to dietary intake as a strategy to prevent EHI.

The relationship between individual nutrients such as moderate fluid intake with glucose and sodium (*11*), glutamine supplementation (*12*), and protein intake after exercise (*13*) has been reported on the possibility of preventing or reducing the severity of EHI through nutrition and dietary strategies. However, little has been reported on the relationship between EHI prevention and daily dietary intake.

In this study, we attempted to clarify the relationship between the cognizance of experiencing EHI among college students involved in athletic clubs and their living conditions, including daily eating habits and specific EHI symptoms. We focused on EHI rather than EHS because EHI is recognized as a more common and comprehensive heat-related symptom. In addition, we believe that early detection and response to EHI are important to prevent EHS, which is a serious condition of EHI. We hypothesized that the occurrence of EHI during athletic club activities is associated with various living conditions and daily eating habits. We also aimed to identify factors influencing athletes' cognizance and new risk factors for EHI. We believe that by investigating their living conditions, we can clarify the factors that cause EHI and establish new preventive measures.

2. Materials and Methods

2.1. Study design and data collection

In October 2022, a cross-sectional study was conducted to identify the occurrence of EHI during club activities in the summer and its associated factors by administering a questionnaire to 237 male students involved in athletic clubs at University A. Of the 215 (90.7%) respondents, 197 (91.6%) provided valid responses. Only male students were included in this study, as few female students were involved in athletic club activities at the university.

A statistical power analysis was conducted using IBM SPSS version 28 (Statistical Package for Social Science, Chicago, IL, USA) to ascertain the sample size required for this study. A sample size of 180 was required for 27 explanatory variables with a power of 0.8 and a significance level of 0.05; the sample size for this study met this requirement. Additionally, this study was approved by the Ethics Review Committee of Chubu University (Approval No.: 20220055).

2.2. Survey items

Data collection was performed using a web-based questionnaire designed with an anonymous Google form. A total of 238 items were surveyed in the questionnaire. Paper-based recruitment forms were distributed to members of university athletic clubs to recruit eligible participants. Students who agreed to participate accessed the web survey by scanning the QR code of the Google form URL using their mobile phones. Information on basic attributes, such as gender, faculty affiliation, and club activities, was collected. Questions were also asked regarding the students' previous EHI experiences, lifestyle habits (such as food intake and sleep), and subjective symptoms of EHI experienced during summer club activities. For questions regarding subjective EHI symptoms, the 13 subjective symptoms were defined based on the classification recommended by the Committee on Heat Stroke and Hypothermia of the Japanese Association for Acute Medicine and previous studies (*14*).

To prevent subjects from associating these subjective symptoms with EHI, they were simply asked if they experienced these symptoms during club activities in the summer. This was done to enable clarification of the association between the subject's cognizance of "having EHI" and specific, subjective symptoms.

Daily physical activity was assessed using the International Physical Activity Questionnaire-Short Version (IPAQ-SV), which comprises seven questions regarding how many hours per day and days per week subjects spent walking or engaging in moderate- or high-intensity physical activity during an average week. Activity was categorized into three levels, "high," "moderate," and "low", depending on the intensity, duration, and number of days of physical activity per week (*15*).

2.3. Statistical analysis

The collected data was organized using simple tabulation. To test our hypothesis, Pearson's Chi-squared test was used to analyze the association between the occurrence of EHI as cognized by the subjects and their lifestyle

habits, food intake statuses, and symptoms attributed to EHI experienced during club activities in summer (Tables 2 and 3). Binomial logistic regression analysis (stepwise variable selection method) was performed using the items that were found to be significant, and factors contributing to the occurrence of EHI were extracted.

The dependent variable was the presence or absence of EHI experience, and the explanatory variables were lifestyle habits, physical activity habits, physical symptoms during club activities in the summer, and nutrient and food intake. A dummy variable with settings of "3 times" = 0 or "less than 2 times" = 1 was used as the explanatory variable for questionnaire item E in Table 2; for K and L in Table 2 and items in Table 3, dummy variables with settings of "Yes" = 0 or "No" = 1 were used. Odds ratios and 95% confidence intervals (95% CI) were calculated for each explanatory variable. In addition, the Kruskal Wallis test was utilized to confirm the difference in the amount of exercise (IPAQ) depending on each club activities. The significance level was set at $P < 0.05$ for all items. The statistical analysis software IBM SPSS version 28 was used for this analysis.

3. Results and Discussion

Participant characteristics are shown in Table 1. All 197 subjects were male, with a mean age of 19.8 years (SD: ± 1.1). 64 participants (32.5%) were in their first year of university, 56 (28.4%) in their second year, 57 (28.9%) in their third year, and 20 (10.2%) in their fourth year; 55 participants (27.9%) played soccer, 31 (15.7%) played handball, 100 (50.8%) played baseball, and 11 (5.6%) played rugby. Eighty-Eight participants (44.7%) responded that they had previously experienced EHI, and 109 (55.3%) responded that they had never experienced EHI. As shown in Table 2, year and club affiliation were not associated with cognizance of experiencing EHI.

Table 2 shows the association between the cognizance of experiencing EHI and attributes, living conditions (such as sleep, physical activities, and diet), and EHI-related questions. Athletes who reported having meals containing a staple food, main dish, and side dish three times a day were less likely to have experienced EHI than those who had similar meals two or fewer times a day. Most of the athletes who responded as having a history of medical examinations due to EHI also indicated that they had previously experienced EHI. Additionally, athletes who indicated that they had read the EHI Prevention Manual were more likely to report that they had previously experienced EHI than those who indicated that they had not read the manual. Other living conditions, including IPAQ, were not related to EHI experiences (Table 2). Also, there was no difference in the amount of physical activity (IPAQ) by club activity (Data not shown).

Table 1. Basic attributes

Items	*n*	%
Total	197	100.0
Age: 19.8 (SD1.12)		
Year in college		
First	64	32.5
Second	56	28.4
Third	57	28.9
Fourth	20	10.2
Club activities		
Soccer	55	27.9
Hand-ball	31	15.7
Baseball	100	50.8
Rugby	11	5.6
Experience of EHI		
No	109	55.3
Yes	88	44.7

Table 3 shows the association between the cognizance of experiencing EHI and subjective symptoms during summer activities. Athletes who reported experiencing "headache," "dizziness," "weakness or fatigue," "abnormally fast breathing," and "fast or weak pulse" during summer activities were more likely to report having had EHI than those who did not experience any of these symptoms. Additionally, 159 athletes (80.7%) reported experiencing some EHI-related symptoms during summer activities, while 38 (19.3%) did not.

Table 4 presents the results of a binomial logistic regression analysis of the factors contributing to the cognizance of experiencing EHI. A history of medical examination due to EHI (odds ratio [OR], 18.607; 95% confidence interval [CI], 4.040-85.693; $P < 0.001$), experiencing headaches during club activities in summer (OR, 3.061; CI, 1.597-5.867; $P < 0.001$), and reading the EHI manual (OR, 0.430; CI, 0.190-0.972; $P = 0.043$) were factors for the cognizance of EHI. Conversely, the number of times meals containing a staple food, main dish, and side were dishes eaten in a day (OR, 2.217; CI, 1.066-4.611; $P = 0.033$) was a factor in the prevention of EHI.

In this cross-sectional study, students in college athletic clubs were surveyed to determine their experience with EHI and its predictors. Medical examination for EHI, headaches during summer club activities, knowledge of EHI and daily eating habits were considered likely to affect cognizance of EHI.

Regarding the experience of EHI among college student athletes, nearly half of the athletes responded that they had previously experienced EHI. However, this question was asked such that the response was based on the athletes' own judgment of whether they had experienced EHI, which may differ from the actual occurrence of EHI. Although all 13 symptoms suspected to be associated with EHI should correspond with whether the athlete was cognizant of EHI or not, only five symptoms of EHI ("headache," "dizziness," "weakness and fatigue," "abnormal fast breathing," and "weak or rapid pulse") corresponded. This likely

Table 2. Association between cognizance of experiencing EHI and basic attributes, living conditions, *etc.*

Items	Experience of EHI		Total	*P*-Value
	No	Yes		
	n (%)	*n* (%)	*n* (%)	
	109 (55.3)	88 (44.7)	197 (100.0)	
Basic attributes				
Year in college				0.287
First	37 (57.8)	27 (42.2)	64 (32.5)	
Second	25 (44.6)	31 (55.4)	56 (28.4)	
Third	35 (61.4)	22 (38.6)	57 (28.9)	
Fourth	12 (60.0)	8 (40.0)	20 (10.2)	
Club activities				0.324
Soccer	36 (65.5)	19 (34.5)	55 (27.9)	
Hand-ball	17 (54.8)	14 (45.2)	31 (15.7)	
Baseball	51 (51.0)	49 (49.0)	100 (50.8)	
Rugby	5 (45.5)	6 (54.5)	11 (5.6)	
Lifestyle, etc.				
A. Average amount of sleep				0.798
More than 7 hours	29 (56.9)	22 (43.1)	51 (25.9)	
Less than 7 hours	80 (54.8)	66 (45.2)	146 (74.1)	
B. Training Environment				0.650
Outdoor	93 (56.0)	73 (44.0)	166 (84.3)	
Indoor	16 (51.6)	15 (48.4)	31 (15.7)	
C. IPAQ				0.706
Low	10 (62.5)	6 (37.5)	16 (8.1)	
Moderate	11 (61.1)	7 (38.9)	18 (9.1)	
High	88 (54.0)	75 (46.0)	163 (82.7)	
D. Frequency of breakfast intake				0.565
Every day	70 (56.9)	53 (43.1)	123 (62.4)	
Sometimes skipped	39 (52.7)	35 (47.3)	74 (37.6)	
E. Number of times meals containing a staple food, main dish, and side dish were eaten per day				0.049*
Three times	36 (66.7)	18 (33.3)	54 (27.4)	
Less than 2 times	73 (51.0)	70 (49.0)	143 (72.6)	
F. Daily food intake [breakfast]				0.773
Skip	5 (62.5)	3 (37.5)	8 (4.1)	
Small serving	35 (50.7)	34 (49.3)	69 (35.0)	
Just right serving	55 (56.7)	42 (43.3)	97 (49.2)	
Large serving	14 (60.9)	9 (39.1)	23 (11.7)	
G. Daily food intake [lunch]				0.347
Skip	0 (0.0)	1 (100.0)	1 (0.5)	
Small serving	8 (47.1)	9 (52.9)	17 (8.6)	
Just right serving	71 (59.7)	48 (40.3)	119 (60.4)	
Large serving	30 (50.0)	30 (50.0)	60 (30.5)	
H. Daily food intake [dinner]				0.845
Skip	1 (50.0)	1 (50.0)	2 (1.0)	
Small serving	2 (40.0)	3 (60.0)	5 (2.5)	
Just right serving	40 (53.3)	35 (46.7)	75 (38.1)	
Large serving	66 (57.4)	49 (42.6)	115 (58.4)	
I. Frequency of intake [sports drinks]				0.633
Often	9 (50.0)	9 (50.0)	18 (9.1)	
Never	100 (55.9)	79 (44.1)	179 (90.9)	
J. Frequency of intake [energy drinks]				0.571
Often	4 (66.7)	2 (33.3)	6 (3.0)	
Never	105 (55.0)	86 (45.0)	191 (97.0)	
EHI Related				
K. History of medical examination for EHI				< 0.01**
No	107 (61.5)	67 (38.5)	174 (88.3)	
Yes	2 (8.7)	21 (91.3)	23 (11.7)	
L. Read the EHI countermeasures manual				0.029*
No	94 (59.1)	65 (40.9)	159 (80.7)	
Yes	15 (39.5)	23 (60.5)	38 (19.3)	

indicates that the subjects did not correctly understand the symptoms of EHI.

Of these subjects, those who reported having headaches during summer activities were three times more likely to have experienced EHI than those who did not. While headache is a moderate symptom of EHI, it is important to be able to recognize even minor symptoms, such as "dizziness" and "muscle cramps," as EHI-related (*16*). As these symptoms suspected to be EHI-related can also be caused by non-EHI factors, it is likely that they are less related to whether the student athlete has experienced EHI. However, any suspicious symptoms should be regarded as a sign of EHI, especially if they occur during physical activities in the summer, and it is necessary to update student athletes' cognizance of EHI so they can better judge the aforementioned symptoms. A

Table 3. Association between cognizance of experiencing EHI and symptoms during club activities in summer

Symptoms Response	Experience of EHI		Total	*P*-Value
	No	Yes		
	n (%)	*n* (%)	*n* (%)	
	109 (55.3)	88 (44.7)	197 (100.0)	
Headache				0.001**
No	61 (67.8)	29 (32.2)	90 (45.7)	
Yes	48 (44.9)	59 (55.1)	107 (54.3)	
Dry mouth				0.437
No	28 (50.9)	27 (49.1)	55 (27.9)	
Yes	81 (57.0)	61 (43.0)	142 (72.1)	
Dizziness				0.038*
No	62 (62.6)	37 (37.4)	99 (50.3)	
Yes	47 (48.0)	51 (52.0)	98 (49.7)	
Weakness and fatigue				0.048*
No	60 (62.5)	36 (37.5)	96 (48.7)	
Yes	49 (48.5)	52 (51.5)	101 (51.3)	
Difficulty concentrating or thinking clearly				0.227
No	54 (60.0)	36 (40.0)	90 (45.7)	
Yes	55 (51.4)	52 (48.6)	107 (54.3)	
Nausea				0.058
No	75 (60.5)	49 (39.5)	124 (62.9)	
Yes	34 (46.6)	39 (53.4)	73 (37.1)	
Muscle cramp				0.250
No	61 (59.2)	42 (40.8)	103 (52.3)	
Yes	48 (51.1)	46 (48.9)	94 (47.7)	
Abnormally fast breathing				0.042*
No	76 (60.8)	49 (39.2)	125 (63.5)	
Yes	33 (45.8)	39 (54.2)	72 (36.5)	
Weak or rapid pulse				0.031*
No	78 (60.9)	50 (39.1)	128 (65.0)	
Yes	31 (44.9)	38 (55.1)	69 (35.0)	
Numbness of the lips				0.333
No	88 (57.1)	66 (42.9)	154 (78.2)	
Yes	21 (48.8)	22 (51.2)	43 (21.8)	
Strange behavior				0.552
No	88 (56.4)	68 (43.6)	156 (79.2)	
Yes	21 (51.2)	20 (48.8)	41 (20.8)	
Fainting				0.609
No	91 (56.2)	71 (43.8)	162 (82.2)	
Yes	18 (51.4)	17 (48.6)	35 (17.8)	
Hallucinating				0.921
No	91 (55.5)	73 (44.5)	164 (83.2)	
Yes	18 (54.5)	15 (45.5)	33 (16.8)	
Exhibiting any symptom				0.473
No	23 (60.5)	15 (39.5)	38 (19.3)	
Yes	86 (54.1)	73 (45.9)	159 (80.7)	

**$P < 0.01$; *$P < 0.05$.

Table 4. Odds ratios for cognizance of experiencing EHI with respect to lifestyle habits, subjective symptoms, *etc.*

Items	Odds ratio	Confidence Interval		*P*-Value
		Lower Limit	Upper Limit	
• History of medical examination for EHI [a]	18.607	4.040	85.693	< 0.001**
• Number of times meals containing a staple food, main dish, and side dish were eaten per day[b]	2.217	1.066	4.611	0.033*
• Read the EHI manual[c]	0.430	0.190	0.972	0.043*
• Experienced headaches during club activities in summer[a]	3.061	1.597	5.867	< 0.001**

**$P < 0.01$; *$P < 0.05$; [a]No = 0, Yes = 1; [b]3 times = 0, Less than 2 times = 1; [c]Yes = 0, No = 1.

relationship emerged between participants who indicated having read the EHI manual and those who indicated they had experienced EHI in the past, which suggests that these students already had a sound knowledge of EHI, which in turn enabled them to recognize their own symptoms.

It is not surprising that most athletes who reported a history of medical examination due to EHI cognized their experience of EHI; however, it can also be interpreted that they did not cognize EHI until they were examined and diagnosed with EHI at a hospital. While early detection and prompt response (rehydration, cold water immersion) are the best ways to prevent serious illness and death due to EHI (*9,17*), it is also likely that the patients were already seriously ill by the time they were transported to the hospital. A history of EHI has also been noted as a risk factor for EHI, and it is vital to detect and respond to early EHI symptoms before they become more serious (*18*). Moreover, clinical changes in EHI are subtle, and early detection by athletic instructors and others, such as team staff, teammates, and bystanders, is difficult as signs may be overlooked (*9*). Therefore, it is of utmost importance that athletes themselves become highly cognizant and able to self-diagnose and self-report EHI at an early stage; to achieve this, athletes need to be taught how to correctly recognize the various symptoms indicative of EHI.

This study clarified that daily eating habits is important for preventing EHI. The number of times meals containing a staple food, main dish, and side dish were eaten in a day was associated with whether the athlete experienced EHI or not. Athletes who reported having meals containing a staple food, main dish, and side dish less than twice a day had more than twice the risk of experiencing EHI compared with those who reported having these meals three times a day. This highlights the importance of a balanced intake of all three meals in preventing EHI. A systematic review of previous studies reported that the more frequent the number of meals consisting of a staple food, main dish, and side dish, the higher the intake of energy, protein, and various vitamins and minerals, which is consistent with the Dietary Reference Intakes for Japanese individuals (*19,20*). The results of this survey suggest that the athletes who answered that they consumed a meal consisting of a staple food, main dish, and side dish "three times" a day were likely to have had a well-balanced daily diet.

In general, the amount of physical activity is thought to vary depending on the sports activities, and the incidence of sports-related deaths, including EHI, has also been reported to vary by activity (*6,21*). Therefore, the experiences of EHI were examined by club activities in four categories, but no relationship was found.

Early detection is key to preventing EHI; this requires the individual to be cognizant of EHI and promptly informing those around them for swift response. Therefore, having knowledge of the EHI symptoms and the ability to self-diagnose are vital. Additionally, guidance on a well-balanced dietary intake by having three balanced meals (each containing a staple food, main dish, and side dish) per day should be emphasized, especially considering the likelihood of preventing EHI. Both athletic training instructors/leaders and athletes should be educated about these factors from an early stage. In addition to the existing EHI countermeasure manuals, posters and pamphlets should be prepared and distributed, and workshops should be held to inform people about the symptoms of EHI, enable them to correctly detect and report EHI at an early stage, and promote the recommendation of a well-balanced dietary intake.

A limitation of this study is that no gender comparisons were made, as the data was only gathered from male athletes. Only male athletes were surveyed in this study, as the university where the study was conducted had very few female students who were involved in athletic club activities. Additionally, the sample size of this study is limited, and further research is needed to generalize the results. Moreover, the results may not be representative of the general public, as climates, lifestyles, and social conditions vary depending on the country and region.

In conclusion, a well-balanced intake of all three meals may reduce the incidence of EHI. It was also noted that athletes need to accurately understand the symptoms of EHI. Detecting and preventing EHI at an early stage necessitates urgent education for athletes on well-balanced food intake and accurately identifying EHI symptoms through workshops, as well as the creation and distribution of materials relevant to these topics.

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Evolving immune evasion and transmissibility of SARS-CoV-2: The emergence of JN.1 variant and its global impact

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SUMMARY The continuous evolution of SARS-CoV-2 variants constitutes a significant impediment to the public health. The World Health Organization (WHO) has designated the SARS-CoV-2 variant JN.1, which has evolved from its progenitor BA.2.86, as a Variant of Interest (VOI) in light of its enhanced immune evasion and transmissibility. The proliferating dissemination of JN.1 globally accentuates its competitive superiority and the potential to instigate fresh surges of infection, notably among cohorts previously infected by antecedent variants. Notably, prevailing evidence does not corroborate an increase in pathogenicity associated with JN.1, and antiviral agents retain their antiviral activity against both BA.2.86 and JN.1. The sustained effectiveness of antiviral agents offers a beacon of hope. Nonetheless, the variant's adeptness at eluding the immunoprotective effects conferred by extant vaccines highlights the imperative for the development of more effective vaccines and therapeutic approaches. Overall, the distinct evolutionary trajectories of BA.2.86 and JN.1 underscore the necessity for ongoing surveillance and scholarly inquiry to elucidate their implications for the pandemic's evolution, which requires the international communities to foster collaboration through the sharing of data, exchange of insights, and collective scientific endeavors.

Keywords SARS-CoV-2 variants, JN.1, immune evasion, vaccine refinement, global public health

Since the emergence in late 2021, SARS-CoV-2 Omicron variant (Pango lineage designation B.1.1.529) has perpetuated successive waves of incidence on a global scale, attributable to their evolving immune evasion (*1*). Of particular concern, BA.2.86 subvariant of Omicron emerged in 2023, characterized by a markedly increased number of mutations in the spike protein when compared with its BA.2 predecessor (*2*). Moreover, JN.1 subvariant (also referred to as BA.2.86.1.1) evolving from BA.2.86 has escalated internationally since its emergence in late 2023 with notable increases in Europe and the United States (*3*).

The BA.2.86 variant, delineated as the "second generation of BA.2", is characterized by the possession of another 34 mutations in the spike protein, distinguishing significantly from both BA.2 and XBB.1.5 variants (Figure 1). These mutations are predominantly located within the N-terminal domain (NTD) and the receptor-binding domain (RBD), impacting the variant's receptor binding and immune evasion capabilities (*4*). Emerging from BA.2.86, JN.1 is characterized by an additional mutation, L455S, within the RBD of the spike protein (*5*). This mutation endows JN.1 with enhanced transmissibility and immune evasion, setting it apart from its antecedent, BA.2.86.1, as well as other variants within the XBB lineage (*6*). The *in vitro* analyses reveal that JN.1 exhibits diminished binding affinity for the human angiotensin-converting enzyme 2 (ACE2) receptor, a pivotal mechanism facilitating viral ingress into host cells (*6*). Nevertheless, JN.1 is associated with elevated infectivity relative to the BA.2.86 variant (*5*), and epidemiological modeling has elucidated that the JN.1 variant is characterized by advantageous growth and spread dynamics with a growth rate approximately 2.3 times superior to that of the EG.5.1.1 variant (*5*). Furthermore, JN.1 displayed significantly enhanced immune evasion against previous infection induced neutralization and extensive resistance against class 1, and 3 monoclonal antibodies targeting the RBD (*5*). This phenomenon underscores a sophisticated interplay between the variant's immune evasion strategies and its efficiency in receptor binding.

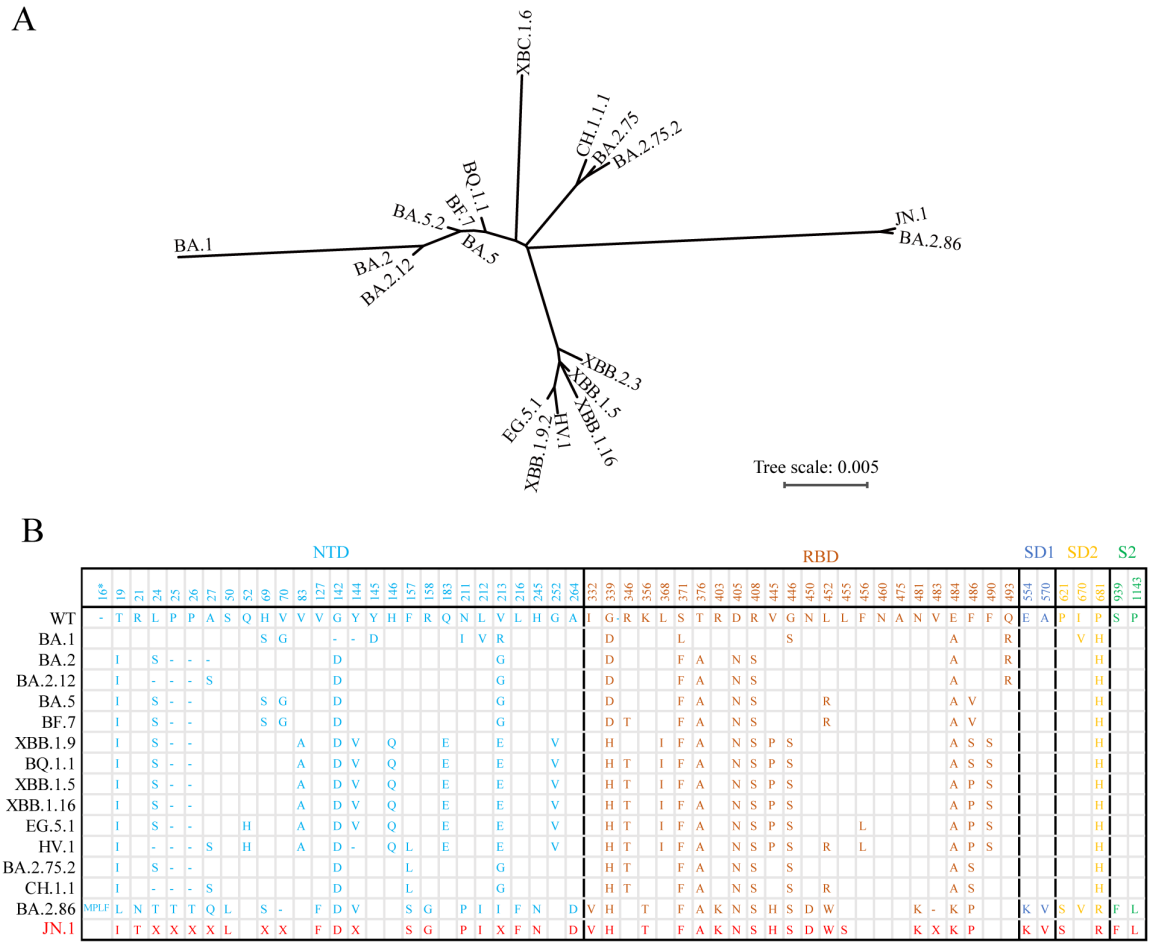

Figure 1. The phylogenetic evolution and key amino acids mutations of Omicron sub-variants. A: Phylogenetic tree of Omicron sub-variants based on spike sequences. **B:** Key amino acid mutations in the spike protein of the Omicron sub-variants.

The World Health Organization (WHO) has subsequently classified JN.1 as a Variant of Interest (VOI), positioning it within the second-tier category of its variant monitoring framework (*7*). The ascendancy of JN.1 correlates with increased detections in wastewater surveillance and a surge in hospital admissions within regions such as the Netherlands and Singapore (*8*). Numerous studies have been conducted to track the proliferation of SARS-CoV-2 through the analysis of regional wastewater samples (*9-11*). A report published by the Centers for Disease Control and Prevention (CDC) on January 5 highlighted a notable intensification in viral activity, particularly evidenced by a 27% augmentation in wastewater viral loads compared to the preceding year (*10*). Furthermore, subsequent to its initial identification in wastewater samples in Germany, JN.1 has rapidly ascended to a position of major endemic prevalence (*11*). As of January 20, 2024, a total of 78,178 JN.1 and 95,183 BA.2.86 related sequences were detected globally (Figure 2A and 2B), according to the data sourced from the cov-spectrum database (*https://cov-spectrum.org*) (*12*). Notably, JN.1 accounted for 88.6% of these sequences, and demonstrated a persistent upward trajectory with rapid spread to multiple regions around the world (Figure 2C). To date, there is no empirical evidence to suggest that the JN.1 variant is implicated in exacerbating clinical symptoms or augmenting pathogenicity (*13*). The clinical manifestations associated with JN.1 infection align closely with those observed in preceding Omicron variants, predominantly presenting as fever, cough, headache, and in many instances, asymptomatic or mild conditions (*14*). Moreover, one recent study has elucidated that antiviral agents, including paxlovid, remdesivir, and molnupiravir, retain their antiviral activity against both BA.2.86 and JN.1 (*15*).

In light of this waning SARS-CoV-2 specific immunity from infection and vaccination, particularly pronounced among the elderly demographic, the evaluation of booster vaccination strategies has been undertaken (*2,8*). Bivalent booster vaccinations have been shown to transiently augment neutralization titers, while their immunogenic potency also diminishes within a six-month timeframe (*8*). Moreover, with the rapid evolution of SARS-CoV-2 during the continuous spread, researches highlight the urgent need to update vaccine formulations in response to emerging SARS-

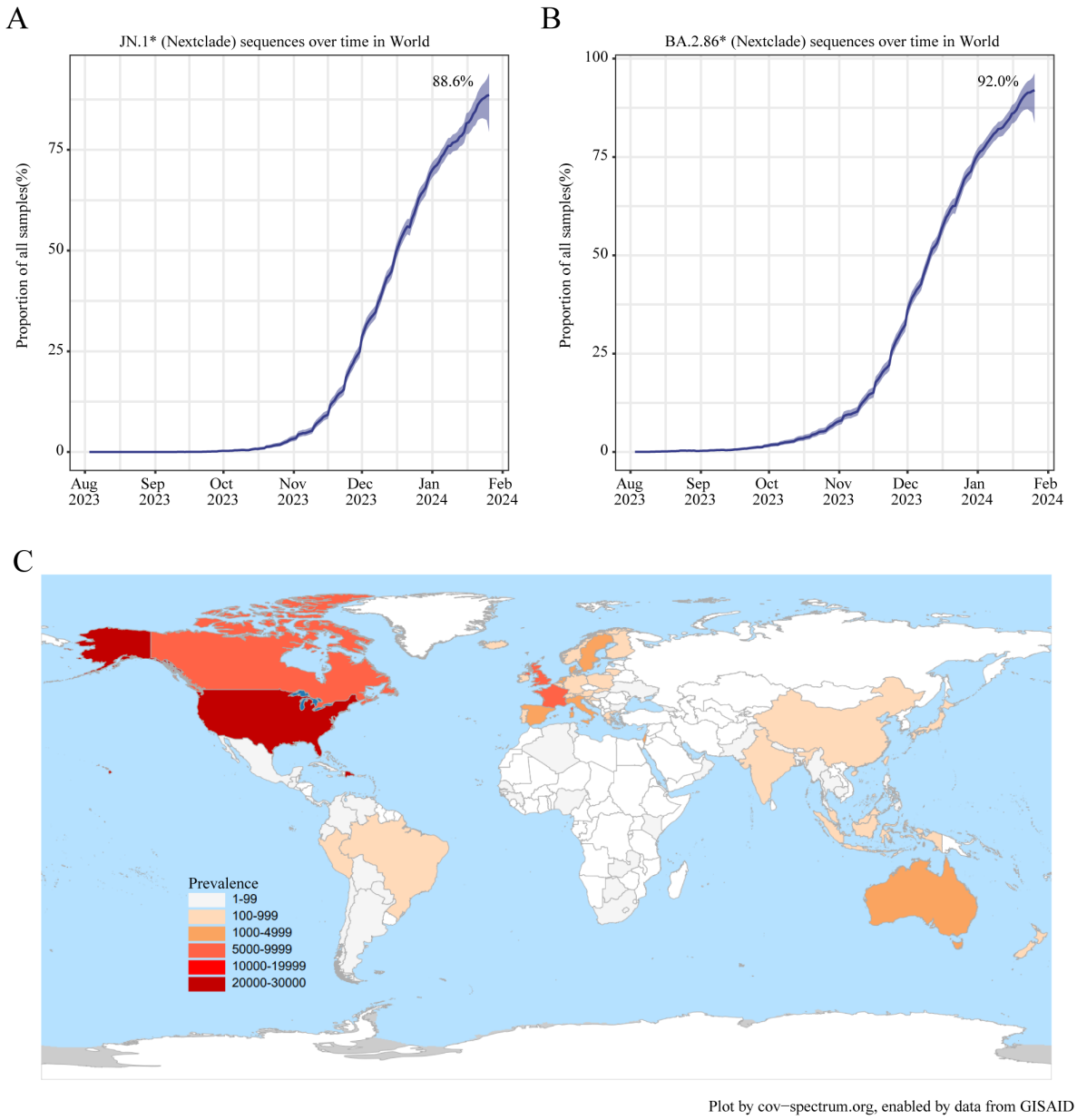

Figure 2. The global spread and prevalence of JN.1 or BA.2.86 related variants. The proportion of emerging SARS-CoV-2 variants JN.1* **(A)** and BA.2.86* **(B)**. The global geographical distribution of JN.1* prevalence **(C)**. The figure was generated by *https://cov-spectrum.org/*. *, including all the JN.1 or BA.2.86 related variants.

CoV-2 variants (*16*). The immune responses initially shaped by vaccines based on the ancestral strain are less effective against Omicron-based boosters due to immune imprinting (*17,18*). Studies in both mice and humans have shown that the efficacy of a single Omicron booster is compromised by immune imprinting, a challenge that can be addressed with a second Omicron booster (*16*). This approach not only mitigates the imprinting effect but also induces a broad neutralizing response. Further analysis revealed that individuals with repeated Omicron exposures develop mature, Omicron-specific antibodies, markedly different from those triggered by the ancestral strain, effectively reducing immune imprinting (*16*). Thus, removal of the ancestral strain component from future vaccine updates should be advocated, and it is recommended that individuals lacking prior Omicron exposure should receive two updated booster doses to enhance protection. Vaccines recently updated to target variants such as XBB.1.5 have demonstrated cross-reactivity with JN.1, intimating their prospective efficacy against emerging variants (*8*).

The distinct evolutionary trajectories of BA.2.86 and JN.1 underscore the necessity for ongoing surveillance and scholarly inquiry to elucidate their implications for the pandemic's evolution. The propensity of JN.1 to evade immune responses presents formidable challenges to extant vaccines and therapeutic antibodies, highlighting the imperative for the development of more

effective vaccines and therapeutic approaches. Moreover, the escalating prevalence of JN.1 may be attributable to factors beyond mere escape from neutralization, which merits comprehensive investigation. Therefore, in the face of persistent mutations and the proliferation of SARS-CoV-2, it is imperative for international communities to foster collaboration through the sharing of data, exchange of insights, and collective scientific endeavors.

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Comparison of the physicochemical properties of branded and generic glucose-added maintenance hypotonic infusion fluids to assess the potential for phlebitis and incompatibility with other drugs

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SUMMARY In Japan, the switch from branded to generic infusion fluids has been promoted as a national policy. Recently, as generic products have been in short supply, the switch from generic to branded infusion fluids has increased. However, certain additives for injectable infusion fluids, such as nonvolatile acids like acetic acid and hydrochloric acid, are not required to be listed in the package insert. We hypothesized that the addition of nonvolatile acids may be one of the reasons for the differences in physicochemical properties between the branded and generic infusion fluids. We have previously reported that in other types of electrolyte infusion fluids, a variation in pH can cause incompatibility with other drugs, and variation in titratable acidity and osmolality can lead to phlebitis. Glucose-added maintenance hypotonic infusion fluid (listed as type-3G) is commonly used as a maintenance solution when energy support is needed. However, nonvolatile acid is added to prevent the caramelization of glucose, resulting in higher osmolality and titratable acidity and lower pH. Therefore, we hypothesized that both phlebitis and incompatibility with other drugs are likely to occur; hence, we measured and evaluated the physicochemical properties of branded and generic type-3G infusion fluids. We show that the osmolality, pH, and titratable acidity of all evaluated branded and generic products differed significantly and that these properties should be evaluated together to avoid phlebitis and incompatibility with other drugs when switching between branded and generic type-3G infusion fluids.

Keywords infusion fluids, titratable acidity, pH, osmolality, phlebitis, incompatibility

In Japan, four types of hypotonic infusion fluids with different compositions are used according to their specific purposes. Some additives for injectable infusion fluids that are intended to adjust pH or make the infusion fluid isotonic are not required to be listed on the package insert (*1*). Of these, weakly acidic nonvolatile acids, such as acetic, do not fully dissociate in the infusion fluids due to their weakly acidic pH. However, they almost completely dissociate in blood, which is weakly basic. Consequently, the concentration of hydrogen ions in these weakly acidic nonvolatile acids is not indicated by the pH of the infusion but is represented by the titratable acidity, which measures the hydrogen-ion concentration in the infused solution within the bloodstream. Titratable acidity is defined as the amount of base (NaOH) required to neutralize the pH of 100 mL of an infusion fluid to physiological pH (7.4) (*2*). Based on these facts, we hypothesized that differences in additives may contribute to differences in the physicochemical properties, such as pH, titratable acidity, and osmolarity, between branded and generic infusion fluids. Variations in pH lead to possible incompatibility with other drugs (*3*), while changes in pH (*4-7*), titratable acidity (*6,7*), and osmolarity (*5,8-10*) can lead to phlebitis. In our previous studies, we measured the pH, titratable acidity, and osmolality of isotonic electrolyte infusion fluids and three types of hypotonic electrolyte infusion fluids to determine any differences, and reported properties that should be evaluated when switching between branded and generic infusion fluids (*11-14*). In this study, we measured the pH, titratable acidity, and osmolarity of branded and generic glucose-added maintenance hypotonic infusion fluids (listed as type-3G), which are commonly used as

maintenance solutions when energy support is needed, and we considered which physicochemical properties should be evaluated when switching between branded and generic type-3G infusion fluids.

Experiments were performed using four branded infusion fluids (labeled "Brand 1", "Brand 2", "Brand 3", and "Brand 4"), and the generic version of each (labeled "Generic 1", "Generic 2", "Generic 3", and "Generic 4"). Table 1 lists the constituents of each infusion fluid. All measurements were performed on five preparations each of formulations with the same lot numbers. Normality was confirmed using Shapiro-Wilk's W test. Two-group comparisons were performed using the two-sided Student *t*-test or Wilcoxon rank-sum test. All statistical analyses were performed using JMP® 15 (SAS Institute Inc., Cary, NC, USA), and results with $P < 0.05$ were considered statistically significant.

The results are shown in Table 2. There were significant differences in osmolality between the branded and generic versions in each group. The osmolality of Generic 1 was ~1.02-fold higher than that of Brand 1, that of Brand 2 was ~1.01-fold higher than that of Generic 2, that of Generic 3 was ~1.01-fold higher than that of Brand 3, and that of Brand 4 was ~1.01-fold higher than that of Generic 4. There were significant differences in pH between the branded and generic versions in each group. The pH of Generic 1 was ~0.4 higher than that of Brand 1, that of Generic 2 was ~0.44 higher than that of Brand 2, that of Generic 3 was ~0.04 higher than that of Brand 3, and that of Brand 4 was ~0.12 higher than that of Generic 4. There were significant differences in the titratable acidity between the branded and generic versions in each group. The titratable acidity of Brand 1 was ~2.35-fold higher than that of Generic 1, that of Brand 2 was ~1.29-fold higher than that of Generic 2, that of Brand 3 was ~1.04-fold higher than that of Generic 3, and that of Generic 4 was ~1.44-fold higher than that of Brand 4.

Although there is insufficient evidence for tolerable osmolality in phlebitis, there have been scattered reports recommending a threshold of 600 mOsm/kg (5,8,10).

Table 1. Composition of type-3G infusion fluids

Classification	Branded	Generic	Branded	Generic	Branded	Generic	Branded	Branded
Labeled name	Brand 1	Generic 1	Brand 2	Generic 2	Brand 3	Generic 3	Brand 4	Brand 4
Components*								
Na^+ (mEq/L)	35	35	40	40	35	35	35	35
K^+ (mEq/L)	20	20	35	35	20	20	20	20
Ca^{2+} (mEq/L)	–	–	–	–	5	5	–	–
Mg^{2+} (mEq/L)	–	–	–	–	3	3	3	3
Cl^- (mEq/L)	35	35	40	40	28	28	38	38
$Lactate^-$ (mEq/L)	20	20	20	20	–	–	20	20
$Acetate^-$ (mEq/L)	–	–	–	–	20	20	–	–
$Gluconate^-$ (mEq/L)	–	–	–	–	5	5	–	–
$Citrate^{3-}$ (mEq/L)	–	–	–	–	–	–	–	–
P (mmol/L)	–	–	8	8	10	10	–	–
Glu (%)	7.5	7.5	10	10	10	10	10	10

* Data on constituents were obtained from the infusion fluid package insert of each preparation.

Table 2. Comparison of the osmolality, pH, and titratable acidity of branded and generic infusion fluids

Classification	osmolality		pH		titratable acidity	
Labeled name	mean ± SD* (mOsm/kg)	*P* value	mean ± SD* or median [IQR]**	*P* value	mean ± SD* or median [IQR]** (mEq/L)	*P* value
Group 1		< 0.0001		0.0109		0.0117
Brand 1	543 ± 2*		5.35 [5.35-5.37]**		0.532 [0.531-0.534]**	
Generic 1	552 ± 1*		5.75 [5.72-5.75]**		0.226 [0.226-0.236]**	
Group 2		0.0005		< 0.0001		0.0122
Brand 2	762 ± 3*		5.18 ± 0.002*		9.050 [9.033-9.082]**	
Generic 2	753 ± 1*		5.62 ± 0.005*		7.013 [6.820-7.018]**	
Group 3		0.0017		< 0.0001		< 0.0001
Brand 3	728 ± 1*		5.02 ± 0.004*		15.156 ± 0.150*	
Generic 3	738 ± 3*		5.06 ± 0.006*		14.536 ± 0.054*	
Group 4		< 0.0001		< 0.0001		< 0.0001
Brand 4	732 ± 1*		4.74 ± 0.005*		1.879 ± 0.006*	
Generic 4	723 ± 2*		4.62 ± 0.008*		2.715 ± 0.019*	

* Continuous variables are presented as mean ± standard deviation (SD). ** Continuous variables are presented as median [interquartile range].

Furthermore, regarding the relationship between tolerable osmolality of peripheral veins and the duration of administration, it has been reported that the upper limit of osmolality that can be administered through a peripheral vein is approximately 820, 690, and 550 mOsm/kg for 8, 12, and 24 h, respectively. Furthermore, the longer the duration of administration, the lower the tolerable osmolality in the peripheral veins (*9*). In the current study, there were clear differences in osmolality between the branded and generic infusion fluids in all groups; however, except for Group 1, all groups had an osmolality greater than 600 mOsm/kg. Therefore, the possibility of phlebitis is high, and avoidance measures are necessary regardless of switching in Groups 2-4. In contrast, type-3G infusion fluids contain potassium ions and glucose, each with a dosage rate limitation (*15,16*). The time required to administer 2,000 mL to a 60-kg patient, the average weight of a Japanese patient (*17*), within this dosing rate limit is more than 5 h for Group 1 and more than 7 h for Groups 2-4. Compared to the data on the duration of administration and peripheral venous tolerable osmolality reported in previous studies (*18*), our estimate suggests that Group 1 can be safely administered for up to 24 h, whereas Groups 2-4 are safe for administration within an approximate 8-h window. These estimates indicate that switching between branded and generic infusion fluids should not be a problem within these timeframes. Based on these findings, we determined that osmolality should be evaluated along with the duration of administration when switching between branded and generic type-3G infusion fluids to avoid phlebitis.

Differences in pH can cause incompatibility with other drugs (*3*) and phlebitis (*4,6,7,9*). The present study revealed significant differences in pH between branded and generic type-3G infusion fluids in each group investigated. If 4 mg of bromhexine hydrochloride (Bisolvon® Injection 4 mg) (*19*) is administered through the side tube for Brand 4 (pH 4.74) and Generic 4 (pH 4.62), no change is expected for Generic 4, whereas the solution is expected to become cloudy for Brand 4. Bisolvon® Injection is formed by adding hydrochloric acid to bromhexine, a weakly basic substance, to produce the hydrochloride salt. Based on the results of experiments conducted by the manufacturer (*19*), it is anticipated that the proportion of insoluble molecular forms will increase and the solution may become turbid if the pH of the solution is higher than 4.71.

Based on previous studies that showed that the upper limit of pH tolerated for phlebitis is approximately 6.5 (*4*), all products evaluated in the current study are likely to cause phlebitis. Okamura *et al*. (*6*) examined the effects of pH and titratable acidity on phlebitis in hospitalized patients using two parenteral nutritional infusion fluids with nearly identical osmolality but different pH and titratable acidity (pH 5.1 and titratable acidity 17.5 *vs.* pH 6.7 and titratable acidity 7) and found that products with lower pH and higher titratable acidity had a significantly higher incidence of phlebitis. Kuwahara *et al*. (*7*) evaluated the same formulations pathologically in animal studies and reported results similar to those of Okamura *et al*. (*6*), and further reported that for equally low pH, the higher the titratable acidity, the higher the likelihood of phlebitis.

The titratable acidity of branded and generic infusion fluids may differ due in part to non-volatile acids. Moreover, titratable acidity is equivalent to a value that indicates the total amount of dissociated and undissociated acid in the infusion fluid and is not predictable from the pH. When infusion fluids with high titratable acidity are administered to blood, hydrogen ions are supplied by both dissociated and undissociated acids in the infusion fluid and are more difficult to neutralize. Therefore, infusion fluids with high titratable acidity are more likely to irritate venous endothelial cells with hydrogen ions for a longer period and cause phlebitis than infusion fluids with low titratable acidity (*20*). In the present study, the pH of all products was below 6.5 (*4*), the upper tolerable pH limit for phlebitis, and there was a significant difference in titratable acidity between the branded and generic infusion fluids in each group. Considering the above-mentioned studies by Okamura *et al*. (*6*) and Kawahara *et al*. (*7,20*) on infusion pH and titratable acidity, the potential for phlebitis is more likely in Brands 1–3 and Generic 4. Based on these findings, we determined that titratable acidity and pH are important when switching between branded and generic type-3G infusion fluids because they allow for more accurate avoidance of phlebitis.

In conclusion, the present showed that pH, titratable acidity, and osmotic pressure are the physicochemical properties that should be evaluated when switching between branded and generic type-3G infusion fluids. Our results from the present and previous studies (*11-14*) highlighted physicochemical properties that should be evaluated when switching between branded and generic electrolyte infusion fluids for all types. We believe that it is our urgent task to integrate the results of this study with our previous studies (*11-14*) to provide practical information that can be used in clinical practice.

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