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Rapid evolution of the COVID-19 pandemic calls for a unified public health response

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SUMMARY The globe has witnessed the rapid evolution of SARS-CoV-2 mutations and emerging variants of concern (VOCs) and variants of interest (VOIs) that have broadly impacted the transmissibility, antigenicity, morbidity, and mortality of the virus. Although around 2.5 billion vaccine doses have been administered worldwide, vaccine coverage remains far behind the minimum threshold needed to achieve herd immunity overall and it varies substantially by country. Many countries, and especially low- and middle-income countries (LMICs), are struggling with access to COVID-19 vaccines and a lack of personnel to perform mass vaccination. Effective nonpharmaceutical interventions (NPIs) are also not unanimously accepted and strictly complied with by the public and local communities. Moreover, the global fight against COVID-19 is and continues to face geopolitical, social, economic, and human rights concerns. Taken together, these circumstances call for a unified public health response with well-organized individual, local, national, and global efforts and actions to achieve success in controlling the COVID-19 pandemic and achieving sustainable health and development goals.

Keywords SARS-CoV-2; evolution; vaccine; NPIs; unified public health response

As of 5:44 PM CEST on June 22, 2021, there have been 178,503,429 confirmed cases of COVID-19 globally, including 3,872,457 deaths, reported to the WHO (*1*). The global number of weekly confirmed cases has been decreasing since April 26 (Figure 1A, 1B), but such declines have not been observed universally (*2*). In the week of June 14-20, a marked increase in the number of weekly cases compared to the previous week was recorded in the African Region and some countries such as Brazil (505,344 new cases, representing an 11% increase), Colombia (193,907 new cases, representing a 10% increase), and the Russian Federation (108,139 new cases, representing a 31% increase) (*2*). The number of new deaths weekly has also been decreasing across all regions except for the African, the South-East Asia, and the Eastern Mediterranean Regions. Nevertheless, the global mortality remains high, with around 70,000 deaths reported each week (Figure 1C, 1D).

Rapid evolution of SARS-CoV-2

Over two million SARS- CoV-2 sequences have been submitted and shared *via* the Global Initiative on Sharing All Influenza Data (GISAID) (*https://www.gisaid.org*). Since late 2020, after 11 months of relative evolutionary stasis following the emergence of SARS-CoV-2 in late 2019, the virus has been identified as having mutations, in the context of 'variants of concern (VOCs)', that impact its characteristics, including transmissibility and antigenicity (*3*). As of June 22, 2021, the WHO has designated four SARS-CoV-2 VOCs: Alpha (B.1.1.7, first documented in the UK), Beta (B.1.351, first documented in South Africa), Gamma (P.1, first documented in Brazil), and Delta (B.1.617.2, first documented in India) (*2*). The WHO has also designated seven variants of interest (VOIs): Epsilon (B.1.427/B.1.429, first documented in the US), Zeta (P.2, first documented in Brazil), Eta (B.1.525, first documented in multiple countries), Theta (P.3, first documented in the Philippines), Iota (B.1.526, first documented in the US), Kappa (B.1.617.1, first documented in India) and Lambda (C.37, first documented in Peru).

Figure 2 shows the timeline for a total of eleven VOCs or VOIs from earliest documentation to designation as VOCs or VOIs. The Delta variant, first documented in October 2020 in India, was designated as a VOI on April 4 and as a VOC on May 11 (Figure 2). Globally, the Alpha variant has been reported in 170 countries (seven new countries in the week of June 14-

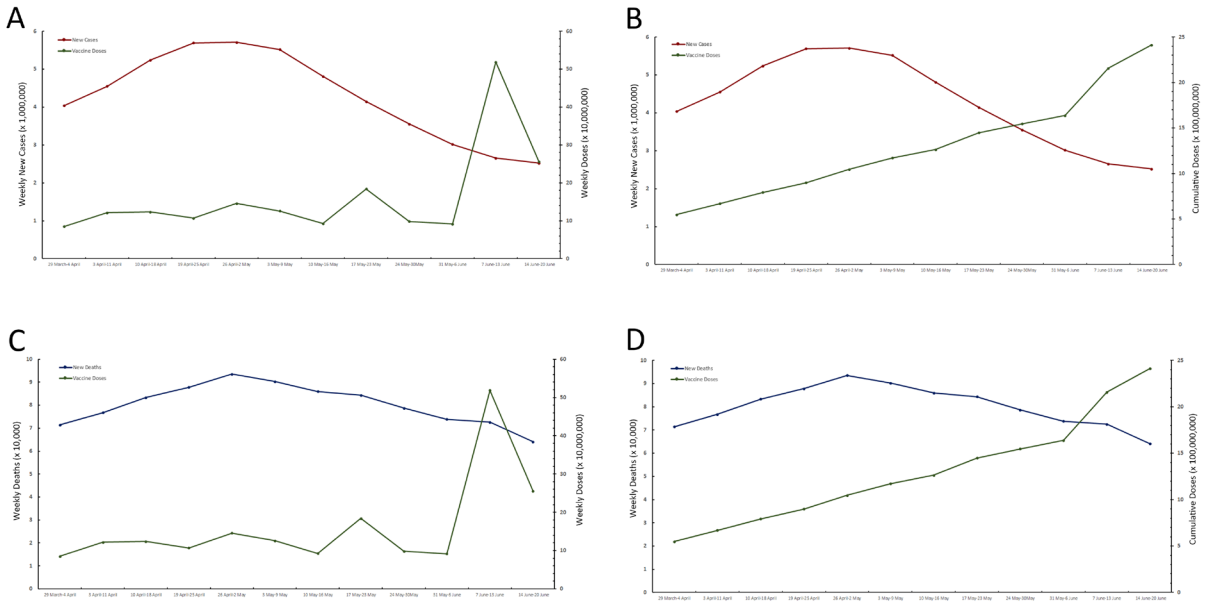

Figure 1. Weekly new confirmed cases or deaths versus vaccine doses administered weekly or cumulatively since April 2021.

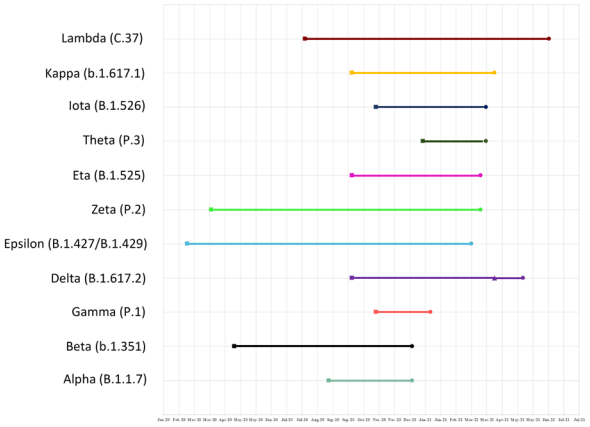

Figure 2. Timelines of SARS-CoV-2 variants of concern (VOCs) and variants of interest (VOIs) from the date of the earliest documentation in samples to the date of designation as VOCs or VOIs, as of June 22, 2021.

20), the Beta variant has been reported in 119 (four new countries), the Gamma variant has been reported in 71 (three new countries), and the Delta variant has been reported in 85 countries (six new countries) (*2*). The Delta variant has a much higher transmissibility and shorter incubation period than other variants (*4*).

Mass vaccination campaigns

Successful control of the current COVID-19 pandemic relies to a large extent on herd immunity achieved as early as possible. By 22 June 2021, a total of 2,414,347,324 vaccine doses have been administered globally (*1*). The vaccine doses administered weekly have not risen over the past two and a half months (Figure 1A, 1C), and vaccine coverage remains far behind the minimum threshold needed to achieve herd immunity. Vaccination coverage of 60-75% would be necessary to achieve this goal (*5,6*). However, the size of populations for COVID-19 vaccination varies markedly by geographical region (*7*). Moreover, many countries are still struggling to provide access to vaccines.

Addressing the scope of COVID-19 vaccine hesitancy in various countries is recommended as an initial step to building trust in COVID-19 vaccination efforts (*8-10*). Various individual and organizational factors, social networks, and media shape public attitudes towards COVID-19 vaccines (*10*). There are increasing global concerns about vaccine effectiveness and how it may be affected by the rapidly emerging VOCs and VOIs. Limited clinical trials have indicated that some vaccines protect against certain variants (*11,12*), but emerging evidence indicates that vaccinated serum is less capable of neutralizing some SARS-CoV-2 variants and that vaccines are less effectiveness against emerging variants (*13-15*). As shown in Figure 1B and 1D, the increase in the cumulative number of vaccine doses crisscrosses the decreases in the weekly reported numbers of new cases and new deaths, and the decrease in the number of new deaths is marked.

Non-pharmaceutical interventions

Stringent non-pharmaceutical interventions (NPIs) including facemasks, individual hygiene, restrictions on public and community activities, regional lockdowns, and international quarantines have played a critical role in curbing the pandemic and will continue to be crucial aspects of the discussion and debate among the general public, governments, and the international community

regarding mandatory self-isolation, limiting sizes of gathering, and business closures (*16-18*), which should all be addressed (*17,19*).

Discussion

There is no doubt that the evolution of SARS-CoV-2 is inevitable and will continue as the pandemic continues to rage worldwide. The more the virus circulates, the greater the risk that the virus evolves into variants that have higher infectivity, transmissibility, and virulence. Established and proven public health measures including mass vaccination and various NPIs such as individual hygiene, social distancing, travel restrictions, and international quarantine as well as public health surveillance of and screening for COVID-19 are all crucial aspects of the global strategy to reduce viral transmission and the occurrence of mutations that negatively impact disease control (*20,21*). Nevertheless, the key to successful global control of the COVID-19 pandemic is the planning and implementation of a unified public health response among all countries, territories, areas, communities, families, and citizens; in this era of globalization, no person or entity can avoid the pandemic (*2,22,23*).

A *Unified Public Health Response* to rapidly evolving SARS-CoV-2 involves the integration of efforts at four levels linked by trust, respect, rights, and obligations: personal or individual, community or local, national, and global. At the personal or individual level, keeping individual hygienic behaviors, complying with preventive measures and interventions continue to be essential for halting viral transmission (*24*). Vaccine hesitancy should be closely examined and addressed as COVID-19 vaccine acceptance depends on personal beliefs and attitudes, as well as the characteristics of new vaccines, vaccination strategy and various other factors.

At the community or local level, understanding various personal and population needs and the factors shaping public attitudes towards access to, acceptance of, and the effectiveness of vaccines would facilitate the planning of multiple evidence-based interventions in order to promote vaccine acceptance. Public concerns about public activities and social interaction as well as the need for healthcare unrelated to COVID-19 also need to be carefully addressed. A supportive and trusting community is critical and conducive in terms of promoting the public response to COVID-19.

At the national level, the government should foster public trust, create multi-sectorial consortia, and devise strategic response plans including greater political commitment, collection and allocation of various resources, more precise and timely public health surveillance, rapid large-scale contact tracing and screening, public education to promote NPIs, and the roll out of mass vaccination (*22,25*). Governments should evaluate different strategies and allocation schemes based on local epidemiological characteristics, the general health of the population, projections of the number of available vaccine doses, and vaccination strategies that offer direct or indirect benefits (*7*).

At the global level, the UN and WHO should continue to work closely with national governments and authorities, regional organizations, non-government organization (NGOs), donors, and vaccine manufacturers to improve access to and distribution of vaccines, and especially in low- and middle-income countries (LMICs) (*25*). More importantly, international organizations under the UN should play a constructive role as platforms in coordinating COVID-19 control efforts among countries, territories, and areas, *e.g.*, creating mechanisms for more effective global allocation of vaccines and mutual acceptance of vaccination certificates between countries using different vaccines, as well as instituting quarantines for international travel and trade (*23,26*).

One hundred years ago, C.E.A. Winslow, the first chairman of the Department of Public Health at the Yale University School of Medicine, set forth a new definition of public health that has widely served as a guide for public health efforts over the past century: *Public health is the science and the art of preventing disease, prolonging life, and promoting physical health and efficiency through organized community efforts for the sanitation of the environment, the control of community infections, the education of the individual in principles of personal hygiene, the organization of medical and nursing services for the early diagnosis and preventive treatment of disease, and the development of the social machinery which will ensure to every individual a standard of living adequate for the maintenance of health; organizing these benefits in such a fashion as to enable every citizen to realize his birthright of health and longevity* (*27*). According to this concept, the core elements of a successful public health response are organized and unified individual, local, national, and global efforts and actions to achieve sustainable health goals and longevity. This has been proven in the global fight against the COVID-19 pandemic (*2,16,28,29*) and is particularly relevant at this moment in time. Given the accelerated emergence of viral mutations and variants, vaccination programs need to be rapidly rolled out and NPIs need to continue to be implemented under the rubric of a *Unified Public Health Response* (*3,30-32*).

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Editorial

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Traditional Chinese medicine for treatment of novel infectious diseases: Current status and dilemma

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SUMMARY Traditional Chinese medicine (TCM) is a valuable form of medicine with a long history in China. It has played a significant role in the control and prevention of infectious diseases including SARS and H7N9 flu. After the outbreak of COVID-19, China's National Health Commission included TCM in the Diagnosis and Treatment Protocol for COVID-19. During the COVID-19 pandemic, three traditional Chinese medicines (Jinhua Qinggan granules, Lianhua Qingwen medicine, and a Xuebijing Injection) and three TCM preparations (a Qingfei Paidu decoction, a Huashi Baidu decoction, and a Xuanfei Baidu decoction) have been screened for their efficacy against COVID-19. More than 150 trials involving TCMs are registered in the Chinese Clinical Trial Registry (ChiCTR), and those trials cover prevention, treatment, recovery, and illnesses diagnosed in accordance with TCM principles. TCM can effectively alleviate the symptoms of patients with COVID-19, delay the disease's progression from mild to severe or critical, and reduce severe and critical all-cause mortality. The underlying mechanisms of TCM mainly involve action against SARS-CoV-2, anti-inflammatory and immunomodulatory action, and organ protection. The current work provides a brief description of the current status of and issues with TCM to treat this novel infectious disease. The hope is that TCM can help considerably to control this global epidemic.

Keywords Traditional Chinese Medicine, COVID-19, Chinese protocol, clinical trials, underlying mechanisms

In contrast to other human diseases, infectious diseases may be significant causes of human and animal morbidity and mortality, leading to extensive outbreaks and epidemics. Over the past few decades, several epidemics of new viral respiratory tract infections, such as severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome coronavirus infection, have emerged and threatened global health security (*1*). Currently, coronavirus disease 2019 (COVID-19) has been identified as an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and was first reported in 2019 in Wuhan; COVID-19 was declared a pandemic by the World Health Organization (WHO) on March 11, 2020 (*2*).

Soon after COVID-19 emerged in China at the beginning of 2020, the Chinese Government immediately implemented strong measures to contain the outbreak. Thanks to considerable effort, the number of COVID-19 cases has stabilized in China as a whole, although a small number of imported cases emerge intermittently. A rapid epidemic began to spread around the world starting in April 2020. On June 11, 2021, the total number of confirmed cases reached 175,902,115, with 3,794,612 deaths reported in 215 countries and territories (*3*). However, the current management of COVID-19 is based generally on supportive therapy and treatment to prevent respiratory failure. Effective options in the form of antiviral therapy and vaccination are currently being developed and evaluated.

Traditional Chinese medicine (TCM) is a valuable form of medicine with a long history in China. It showcases the wisdom of Chinese people and has played a significant role in the control and prevention of infectious diseases. Based on past experiences with the treatment of infectious diseases in China, TCM has proven effective in treating contagious diseases including SARS and H7N9 flu (*4*). Therefore, TCM was often used to treat COVID-19 in China from the start. As is well known, TCM has played a positive role in controlling the COVID-19 pandemic. The current work provides a brief description of the current status of and issues with TCM to treat this novel infectious disease.

Chinese protocol for the treatment of COVID-19

Following the outbreak of COVID-19, China's National

Health Commission successively issued eight versions of its Diagnosis and Treatment Protocol for COVID-19, and TCM has been included ever since the third version of the integrative treatment protocol. A body of evidence from clinical practice and research has shown that integrated traditional Chinese and Western medicine played an important role for China's successful battle with COVID-19 (*5*). The National Health Commission China declared that 92% of the confirmed COVID-19 cases were treated with TCM in combination with Western medicine, and patients responded well to the treatment by improving substantially or fully recovering in over 90% of cases (*6*).

As demonstrated by clinical data, three traditional Chinese medicines (Jinhua Qinggan granules, Lianhua Qingwen medicine, and a Xuebijing Injection) and three TCM preparations (a Qingfei Paidu decoction, a Huashi Baidu decoction, and a Xuanfei Baidu decoction) performed well in treating COVID-19 and are recommended in the Diagnosis and Treatment Protocol for COVID-19 (*7*). Moreover, the three traditional Chinese medicines are now approved for treatment of COVID-19 according to the National Medical Products Administration, and two of the three TCM preparations (Qingfei Paidu Fang and Huashi Baidu Fang) have been approved by the National Medical Products Administration to undergo clinical trials as treatments for COVID-19.

Clinical trials on COVID-19

At the frontline of the fight against the epidemic, TCM personnel have also worked extensively to identify TCM therapies that are effective against the virus. Numerous clinical trials on COVID-19 have been conducted to confirm the efficacy and safety of several traditional Chinese medicines and preparations. As of May 30, 2021, 840 clinical trials addressing various aspects of COVID-19 have been registered in the Chinese Clinical Trial Registry (ChiCTR), including more than 150 studies on TCM (*8*). Nearly 20% of all trials registered in ChiCTR involved TCM, and those trials covered prevention, treatment, recovery, and illnesses diagnosed in accordance with TCM principles, indicating that the use of TCM has been investigated in the management of COVID-19 in China.

To date, randomized controlled clinical trials on several traditional Chinese medicines and preparations to treat COVID-19 have been published. These TCMs include Lianhua Qingwen capsules, Jinhua Qinggan capsules, and Shufeng Jiedu capsules. Three trials including 245 patients with COVID-19 indicated that Lianhua Qingwen capsules had significant efficacy in improving clinical symptoms such as fever, cough, and fatigue and in curbing progression to severe or critical disease (*9*). Shufeng Jiedu capsules have also been recommended for treatment of COVID-19 since the fifth version of the Diagnosis and Treatment Protocol; some clinical cases have been reported and some clinical trials have been registered and are underway (*10,11*). A retrospective cohort study including 200 patients with COVID-19 at Wuhan Hospital indicated that Shufeng Jiedu capsules combined with Arbidol reduces the duration of symptoms and increases the likelihood of clinical efficacy without causing significant adverse reactions (*12*).

Although some randomized and controlled clinical trials on TCM for the treatment of COVID-19 have been published, most might not be able to continue or may have little significance to the use of TCM in the global management of COVID-19 based on an analysis of the details of the registered trials and the current status of the epidemic in China. This situation might be explained by the following three factors: (*i*) the generally low methodological quality and limited sample size mean that many trials are unlikely to generate strong evidence regarding the efficacy of TCM in China; (*ii*) the COVID-19 epidemic has been controlled in China, so many studies may not be completed due to a lack of patients and will have to be terminated; (*iii*) interventions in these trials lacked sufficient evidence in previous clinical practice or were not available outside of China, such as personalized treatment based on identification of an illness according to TCM principles (*13,14*). Hence, due to the limited quantity and quality of the current studies, better quality, rigorously designed, and multi-center randomized controlled trials with large samples need to be conducted in order to provide more clinical evidence of the clinical efficacy of TCM.

Underlying mechanisms of TCM in treating COVID-19

To the extent know, COVID-19 can cause fatal systemic complications due to a strong immune response or cytokine storm and also cause multiple organ dysfunction syndrome (MODS), which is the main cause for a transition from mild to severe disease or even death in patients with COVID-19 (*15*). Mounting evidence supports the therapeutic efficacy of TCM in alleviating the clinical symptoms of COVID-19; the underlying mechanisms of TCM mainly involve antiviral, anti-inflammatory, and immunomodulatory actions and organ protection (*16*). TCM can inhibit the replication and transcription of SARS-CoV-2, prevent the entry of SARS-CoV-2 into host cells, and attenuate the cytokine storm, immune deficiency, and coagulation abnormalities caused by the virus infecting the human body.

One network pharmacology analysis indicated that Lianhua Qingwen capsules modulated the inflammatory process, had antiviral action, and repaired lung injury caused by COVID-19 (*17*). Moreover, they were also

able to alleviate the cytokine storm and symptoms caused by abnormal ACE2 expression. Another network pharmacology analysis indicated that a Qingfei Paidu decoction had immunoregulatory, anti-infection, and anti-inflammatory action and provided protection from SARS-CoV-2 in multiple organs (*18*). Four compounds (baicalin, glycyrrhizic acid, hesperidin, and hyperoside) and 7 targets (AKT1, TNF-α, IL-6, PTGS2, HMOX1, IL-10, and TP53) were identified as key molecules involved in the effects of a Qingfei Paidu decoction.

Although the underlying mechanisms of TCM have been briefly evaluated using network pharmacology analysis, the in-depth mechanisms remain unclear, such as antiviral action in a model of viral infection, the immune regulatory response, and the cytokine storm induced in host cells (*19*). Therefore, the exact mechanism and efficacy of TCM still need to be studied using molecular biological techniques, including genomics, proteomics, and metabolomics, and in vitro and in vivo models.

Conclusion

Efforts to control COVID-19 outbreaks in China have demonstrated the superiority of TCM. TCM can effectively alleviate the symptoms of patients with COVID-19, delay the disease's progression from mild to severe and critical, and reduce severe and critical all-cause mortality. The underlying mechanisms of TCM mainly involve action against SARS-CoV-2, anti-inflammatory and immunomodulatory action, and organ protection. Given that the global epidemic is still raging, better quality, rigorously designed, and multi-center randomized controlled trials with large samples need to be conducted in order for TCM to serve patients around the world. The myriad components, targets, and pathways of TCMs also need to be studied using molecular biological techniques in order to determine their exact mechanism and efficacy. In short, the hope is that TCM can help to control this global epidemic.

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Mini-Review

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Opportunities and challenges to the use of neutralizing monoclonal antibody therapies for COVID-19

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SUMMARY The coronavirus disease 2019 (COVID-19) pandemic has resulted in a substantial global public healthcare crisis, leading to the urgent need for effective therapeutic strategies. Neutralizing antibodies (nAbs) are a potential treatment for COVID-19. This article provides a brief overview of the targets and development of nAbs against COVID-19, and it examines the efficacy of nAbs as part of both outpatient and inpatient treatments based on emerging clinical trial data. Assessment of several promising candidates in clinical trials highlights the potential of nAbs to be an effective therapeutic to treat COVID-19 in outpatient settings. Nevertheless, the efficacy of nAbs treatment for hospitalized patients varies. In addition, this review identifies challenges to ending the COVID-19 pandemic, including concerns over nAbs development and clinical use. Resistant variants significantly threaten the availability of nAb-based therapeutics. This review also discusses other approaches that may improve the clinical benefit of neutralizing mAbs.

Keywords SARS-CoV-2, COVID-19, neutralizing antibody, monoclonal antibody, clinical therapy

1. Introduction

A novel coronavirus, SARS-CoV-2, caused the global coronavirus disease 2019 (COVID-19) pandemic. COVID-19 results in substantial levels of morbidity and mortality, though a considerable proportion of the infected have only mild to moderate symptoms (*1-3*). The COVID-19 pandemic poses a massive threat to worldwide health along with widespread economic disruption, necessitating the urgent development of novel antivirals and effective therapeutic options to alleviate the disease's adverse outcomes.

Given these circumstances, broad-spectrum antivirals (remdesivir, lopinavir/ritonavir, *etc*.) and immune-modulators (tocilizumab and dexamethasone, *etc*.) were initially investigated and found to have varying degrees of efficacy (*4-8*). Hopes were raised by convalescent plasma therapy, *i.e*. use of blood from recovered patients, but its efficacy had been generally proved disappointing due to the lack of standardized doses and a consistent titer of active neutralizing antibodies (nAbs) (*9,10*). That said, the use of monoclonal antibodies (mAbs) offers a new avenue for the treatment of infectious diseases. nAbs are created to exclusively bind to the special epitope regions of a virus that are indispensable to its cellular entry, infectivity, and replication to decrease these events (*11,12*). Neutralizing mAbs serve as potent alternative to most of the current treatments for viral infections. The outstanding efficacy of nAbs against aggressive fatal viruses, like Ebola virus and respiratory syncytial virus (RSV) (*13,14*), substantiate the great potential of nAbs to serve as COVID-19 therapies.

2. Targets of SARS-CoV-2 neutralizing mAbs

The surface spike glycoprotein (S protein) on SARS-CoV-2 is a rational target for nAb-based therapies, as it facilitates virus entry into host cells *via* interaction with the cellular angiotensin-converting enzyme 2 (ACE2) receptor (*15,16*). The S protein contains two subunits. Its S1 subunit has an N terminal domain (NTD) and receptor-binding domain (RBD) (*15*). Components of the S2 subunit promote viral fusion (*17*). Due to its crucial role in facilitating direct viral contact with the ACE2 receptor, the RBD is the major target for nAbs to block SARS-CoV-2 from entering human cells (*15*). The NTD in the S1 subunit or S2 subunit of SARS-CoV-2 could likely serve as a potential target for nAb as well, but the mechanisms are unclear (*18-21*).

A point worth noting, however, is that the structure of the S protein fluctuates dynamically in that it has

two conformations: a closed state and an open state. In the closed ("down") conformation, the three RBDs are inaccessible, which sterically hinders binding (*22,23*). In contrast, an RBD that is necessary for SARS-CoV-2 fusion is exposed in the open ("up") state. (*22,24*). This character of the S protein poses a challenge to the development of mAbs that may bind to an RBD but fail to neutralize SARS-CoV-2 *in vitro*. The dynamic conformation of the S protein might also directly give rise to generation of infectivity-enhancing antibodies in patients with severe COVID-19. Most recently, researchers found that some anti-NTD mAbs from patients with COVID-19 were able to induce the RBD to transition into the "up" conformation to enhance the binding affinity of the S protein to ACE2 and increase the infectivity of SARS-CoV-2. Structural results indicated that almost all of the infectivity-enhancing mAbs bound to NTD in a similar manner (*25*), implying the imperative need to elucidate the complicated etiology of COVID-19.

3. Clinical development of and concerns regarding SARS-CoV-2 neutralizing mAbs

To date, a range of technologies has been adopted to elicit anti-SARS-CoV-2 nAbs. Most of the promising nAb candidates for COVID-19 therapy are generated by screening enriched B cells from the peripheral blood of convalescent patients (*20,26,27*). Similarly, phagedisplay mediated bio-panning or genetically humanized mice immunized with SARS-CoV-2 to produce fully human nAbs have been used to identify the best candidates (*27-29*).Some approaches to improve availability and pharmacological properties have been used during the development of nAbs against SARS-CoV-2. VIR-7831, an anti-SARS-CoV-2 nAbs from a convalescent patient who recovered from SARS, was engineered with mutations and modification of the Fc region of immunoglobulin G (IgG) as well as the neonatal Fc receptor (FcRn), to increase its affinity, extend the antibody half-life, and enhance lung bio-availability (*30*).

There are concerns about immune enhancement of nAbs against COVID-19. Some viral infections, including SARS and MERS, exhibit antibody-dependent enhancement (ADE) (*31,32*). ADE can activate or enhance various categories of processes, such as antibody-mediated boosting of viral entry and replication, complement activation, and cytokine release (*33-36*). The Fc domain could be modulated to attenuate interactions between nAbs and cellular Fc receptors, and thus, to minimize ADE-related events. A typical example is the evolution of AZD7442, a cocktail of two nAbs for treatment of COVID-19 (*37*). Similarly, point mutations (at positions 234 and 235) were introduced into the Fc regions of etesevimab (JS016) to reduce the risk of ADE phenomenon (*38,39*).

4. The clinical utility of SARS-CoV-2 neutralizing mAbs

The excellent pre-clinical evidence has given rise to accumulated clinical trials of anti-SARS-CoV-2 mAbs so far, but a limited number of nAbs have progressed to phase 3 trials for COVID-19 therapies (Table 1).

There are detailed data on the efficacy of bamlanivimab and bamlanivimab/etesevimab and casirivimab/imdevimab cocktails as therapies for ambulatory patients with COVID-19 from Phase 3 trials. The single nAb bamlanivimab (also known as LY-CoV555 or LY3819253) and a bamlanivimab/etesevimab cocktail (designated as LY-CoV016 or LY3832479), derived from convalescent patients by targeting the RBD, were developed by Eli Lilly and AbCellera (*40,41*). Bamlanivimab was well tolerated at a wide-range of doses without serious severe adverse events (AEs) (*41*). Administration of bamlanivimab resulted in fewer patients requiring hospitalization and a significant decrease in the viral load in patients receiving the 2800-mg dose (medium dose) in comparison to a placebo, but, surprisingly, did not have that effect at 7000 mg (a higher dose) (*42*). This might involve the "prozone effect". Thus, the US Food and Drug Administration (FDA) issued emergency use authorization (EUA) for bamlanivimab to treat patients with mild to moderate COVID-19, including those hospitalized (*43*). Further viral load and pharmacodynamic/pharmacokinetic data revealed a marked decrease in the log10 viral load on d 11 in the group receiving a bamlanivimab/etesevimab cocktail (bamlanivimab 700 mg and etesevimab 1400 mg), and this decrease was more obvious than that in the group receiving bamlanivimab mono-therapy (*41*). In a Phase 3 trial, the cocktail decreased the risk of hospitalization (by 70%) and death (0 *vs*. 10) in patients with COVID-19 (*44*). Based on the clinic trial data, the FDA granted an EUA temporarily authorizing administration of the cocktail to treat patients with mild to moderate COVID-19 who were at risk of developing severe COVID-19; the cocktail's safety and efficacy continue to be investigated in hospitalized patients (*45*).

Regeneron collaborated with F. Hoffmann-La Roche to develop a novel nAbs- casirivimab and imdevimab cocktail (REGN10987 and REGN10933) to treat COVID-19 (*46*). This cocktail for ambulatory patients reduced the viral load in patients (a 10-fold reduction, on average) in different countries compared to that in patients receiving a placebo. It also markedly reduced the risk of hospitalization by 70% (1200 mg) and 71% (2400 mg) (*47*). Both doses were well tolerated without severe SAEs (*48*). The cocktail has been issued an EUA by the FDA for ambulatory patients (*49*). A similar authorization was issued by the European Medicines Agency, which recommended it for patients who are at risk of developing severe COVID-19 (*50,51*).

Hospitalized patients with COVID-19 are a

Table 1. Neutralizing mAb-based therapeutics for COVID-19 in clinical trials[a]

Neutralizing antibody	Monotherapy or Cocktail	Sponsor	nAb Source	Phase
LY-CoV555	Monotherapy	AbCellera/Eli Lilly	Convalescent plasma	Phase 2/3
LY-CoV016 (JS016)	Monotherapy	Junshi Biosciences/Institute of Microbiology/Eli Lilly	Recombinant	Phase 2
LY-CoV555 + LY-CoV016	Cocktail	AbCellera/Eli, Lilly/Junshi Biosciences	Convalescent plasma/Recombinant	Phase 3
REGN10933 + REGN10987	Cocktail	Regeneron/F. Hoffmann-La Roche Ltd.	Convalescent plasma/humanized mice	Phase 1/2/3
BGB DXP593	Monotherapy	BeiGene/Singlomics Biopharmaceuticals	Convalescent plasma	Phase 2
CT-P59	Monotherapy	Celltrion	Convalescent plasma	Phase 2/3
TY027	Monotherapy	Tychan Pte. Ltd.	Engineered	Phase 3
BRII-196+ BRII-198	Cocktail	Brii Bio/TSB Therapeutics	Convalescent plasma	Phase 3
VIR-7831	Monotherapy	Vir Biotechnology, Inc. GlaxoSmithKline	Convalescent plasma	Phase 3
SCTA01	Monotherapy	Sinocelltech Ltd.	Recombinant	Phase 2/3
HLX70	Monotherapy	Hengenix Biotech, Inc.	Convalescent plasma	Phase 1
STI-1499	Monotherapy	Sorrento/Mount Sinai Health System	Convalescent plasma	Phase 1
MW33	Monotherapy	Mabwell (Shanghai), Bioscience Co., Ltd.	Convalescent plasma	Phase 2
SI-F019	Monotherapy	Sichuan Baili Pharmaceutical Co., Ltd.	Recombinant	Phase 1
HFB30132A	Monotherapy	HiFiBiO Therapeutics	Recombinant	Phase 1
ADM03820	Cocktail	Ology Bioservices	Convalescent plasma/Recombinant	Phase 1
APN-01	Monotherapy	Aperion Biologics	Recombinant	Phase 2

[a]Data in Table 1 are from the World Health Organization (International Clinical Trials Registry Platform (ICTRP) (who.int)) and National Institutes of Health (*https://clinicaltrials.gov/*) databases and drug company webpages to cite clinical trials investigating of antiviral mAbs as treatments for COVID-19.

difficult-to-treat population since they have extremely poor outcomes and significant critical care needs (*52,53*). In Phase 2-3 trials, bamlanivimab failed to provide a clinical benefit in hospitalized patients (*54*). Like bamlanivimab, a casirivimab/imdevimab cocktail did not significantly reduce the risk of death, but the RECOVERY trial is ongoing. Several other neutralizing nAbs, including VIR-7831, a BRII-196/BRII-198 cocktail, and SCTA01 (a humanized recombinant anti-SARS-CoV-2 mAb), are going to be assessed in hospitalized patients with COVID-19 (*55*).

5. The challenges of SARS-CoV-2 neutralizing mAbs in clinical settings

Substantial challenges have hampered clinical trials on and use of nAbs to treat SARS-CoV-2. Cost/access is one hurdle, as is large-scale manufacturing and storage. Since most people with an early infection recover, specifying a clinical endpoint with which to gauge the benefit relative to a placebo is difficult. Likewise, inflammation and coagulopathy may pose a more serious threat than viral replication in patients with severe disease, so determining the benefit of nAbs in that cohort is difficult.

There are also concerns about the route of administration in clinical settings. Administration *via* IV infusion (*e.g*., bamlanivimab and the bamlanivimab/etesevimab and casirivimab/imdevimab cocktails) is difficult in a community setting while far easier in a hospital. Clearly, oral administration would have an edge in an outpatient setting and limit damage to respiratory epithelial cells, thus prompting efforts to optimize routes of administration (*56*). Another aspect is the timing of nAb administration. Some deaths due to COVID-19 in the later stages are reported to be driven by infection-related inflammation stimulated by innate mediators, *e.g*. IL-6 (*57,58*). Thus, early intervention with nAbs seems to be necessary when considering the delayed initiation of mAbs dose before the effective inhibitory concentration is reached in the lung.

An underlying limitation of nAbs for treatment of COVID-19 is the unknown bio-availability of passively infused IgG in tissues affected by the disease, and especially the lungs. Some patients are likely to experience either a nonallergic infusion-related reaction or an allergic infusion-related reaction. Infusion-related reactions could facilitate effector functions, including complement-dependent cytotoxicity (CDC), opsonization, the classical complement cascade, and antibody-dependent cellular cytotoxicity (ADCC), to cause a series of symptoms such as itching and hypotension (*59-61*).

Another priority consideration is the effect of variants that directly make available therapeutics substantially reduced. The general approach is to use nAb cocktails instead of monotherapy, since the nAbs

in a cocktail bind to distinct epitopes corresponding to the diversity of the S protein, thus decreasing treatment-emergent resistant variants (*46*). Another method is to select nAbs that target conserved epitopes indispensable for viral function, *e.g*., VIR-7831 (*62*). Thus, comprehensive and continued monitoring of SARS-CoV-2 variants should remain a priority.

6. Conclusion

Hundreds of neutralizing mAbs in pre-clinical studies as COVID-19 therapies have emerged. Evaluation of several promising candidates in clinical trials suggests that nAbs could serve as an effective therapeutic intervention for SARS-CoV-2 in ambulatory patients. Nevertheless, there are substantial challenges. The efficacy of nAb therapies for hospitalized patients with COVID-19 varies, highlighting the concern about anti-SARS-CoV-2 nAbs treatment in patients who already have severe symptoms. In addition, resistant variants threat the availability of nAb-based therapeutics. Therefore, the importance should be attached on the development of nAbs with improved availability and increased efficacy. Moreover, anti-SARS-CoV-2 nAbs therapies are likely to shed light on development of alternative interventions to treat other acute respiratory infections.

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Review

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New progress in elucidating the relationship between cancer therapy and cardiovascular toxicity

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SUMMARY Onco-cardiology is an emerging field linking cancer with cardiovascular injury. Understanding the mechanism of cardiac injury helps improve the quality of life of cancer survivors. A series of studies on adverse reactions to cancer or oncological treatments has indicated that adverse cardiovascular events related to cancer treatments may occur over a longer period of survival, and even years after therapy has concluded. Current cancer therapies, including chemotherapy, radiotherapy, targeted therapy, and immunotherapy, have been found to have latent cardiovascular toxicity. These toxic effects are often progressive and irreversible and ultimately lead to cardiovascular events such as heart failure, hypertension, coronary heart diseases, arrhythmia, and thromboembolism. Therefore, more emphasis should be placed on revealing the mechanism of cancer treatment-related cardiovascular toxicity. This would help to guide prevention, diagnosis, and treatment of CVDs in cancer survivors.

Keywords onco-cardiology, cardiovascular toxicity, cardiovascular diseases, cancer

1. Introduction

Cardiovascular diseases (CVDs) and cancer are the two leading causes of death worldwide. Mortality from cancer has been steadily declining over the past few decades, largely because of early detection strategies, improved surgical approaches, and advances in cancer therapeutics. However, recent data have indicated that CVDs are becoming the second leading cause of long-term morbidity and mortality among cancer survivors. This is largely due to the following reasons: *i*) Cancer and CVDs involve many common risks and pathologic factors, like smoking, diabetes, and aging (*1-3*); *ii*) Various cancer treatments cause direct or indirect damage to the cardiovascular system. According to previous investigations, over 60% of patients were prescribed cardiovascular risk drugs during cancer treatments (*4,5*). Conventional chemotherapy and targeted therapies are associated with an increased risk of cardiac damage including heart failure (HF), hypertension, arrhythmia, thromboembolism, and ischemic cardiomyopathy (CM), and may be life-threatening in some instances.

Oncologists puzzled by the necessity of cardiovascular assessment and monitoring when dealing with patients with tumor. The field of onco-cardiology has developed in response to a fusion of relevant theories in cardiology and oncology to optimize care for patients with cancer. Thus, further studies on the epidemiology and pathophysiology of cardiovascular damage & disfunction in cancer survivors need to be promptly conducted to identify screening, preventive, and therapeutic approaches.

2. Epidemiology: Cancer treatments and CVDs

The overall incidence of cancer increases steadily with age for over 80% of patients between the ages of 60 and 80. The International Agency for Research on Cancer (IRCA) has reported that there were approximately 19.3 million new patients with cancer worldwide and nearly 10 million deaths in 2020 (*6*). In China, there were close to 4.57 million new cancer cases in 2020, accounting for about a quarter of new patients with cancer worldwide. The number of patients found to have cancer is estimated to increase by 60% in 2030.

As a result of continued advances in cancer treatments, the survival rate has increased significantly. About 75% of cancer survivors suffer from one or more chronic diseases, and the leading cause of death for these survivors is CVDs (*7-9*). The largest cohort analysis of

cancer and CVDs in China reported that 18% of patients with cancer have at least one cardiovascular risk factor and that 5% of patients with cancer have CVDs (*4*). Patients with cancer and CVDs have a higher mortality risk than patients without CVDs. In addition, a 2008 study by Menna, Salvatorelli, & Minotti (in *Chemical Research in Toxicology*) found that many decades after diagnosis and treatment, patients with cancer had a 15-fold higher rate of HF, a 9-fold higher rate of stroke, and a 10-fold higher incidence of coronary atherosclerosis (*10,11*). Within 5 to 10 years after cancer treatments, more than 50% of survivors have subclinical signs of heart and blood vessel damage (*12,13*).

3. Mechanism of cancer treatment-related cardiovascular toxicity

Direct compression or infiltration of the heart by tumor, cytokines and growth factors secreted by tumor cells, and endocrine factors released by tissues surrounding tumors degrade the normal structure and function of the heart. The control of primary disease is the best way to alleviate these effects. However, mounting evidence has suggested that cancer treatments, such as drugs, surgery, and radiation (Table 1), induce cardiovascular impairment that may be related to cardiomyocyte apoptosis, mitochondrial damage, oxidative stress, proinflammatory response, and endothelial cell damage (Figure 1).

3.1. Cardiac toxicity

The structure and electrical behavior of the myocardium are the two leading components that maintain the normal physiological function of the heart. Various cancer treatments disrupt normal cell, tissue, and organ function, degrading cardiac function and ultimately leading to CVDs.

3.1.1. Structural dysfunction of the myocardium

Cardiomyocyte apoptosis has been reported to be a key mechanism of cancer treatment-induced cardiotoxicity, and it is frequently accompanied by oxidative stress and mitochondrial injury. Some evidence suggests that anthracyclines, one of the most widely used chemotherapeutic agents, induce apoptosis of cardiomyocytes. Anthracycline exposure reduces the number of mesenchymal progenitor and circulating cells, and thus reduce cardiorepartive capacity when the heart is exposed to stress (*14*). In addition, anthracyclines act on topoisomerase-II, which has been found to be one of the direct target molecules of cardiotoxic drugs. Dysfunction of topoisomerase II exacerbates the breakage of DNA, mitochondrial DNA damage, and oxidative stress, it prevents ligase repair, and it up-regulates the expression of MAPK, JNK, p38, and protein kinase B. Thus affecting Ca^{2+}-regulating proteins or Ca^{2+} channels and leading to events such as Ca^{2+} overload and cardiac remodeling (*15-17*). Another anti-cancer agent involved in apoptosis is imatinib, which produces a dose-dependent collapse of the mitochondrial membrane potential that damages cardiomyocyte mitochondria. At the subcellular level, a lack of functional mitochondria causes a decrease in intracellular ATP content, an endoplasmic reticulum stress response, and increased release of cytochrome C into the cytoplasm, thus accelerating cardiomyocyte apoptosis (*18*).

Another important factor causing abnormalities in the structure of the heart is due to cancer treatment-related cardiomyocyte and interstitial fibrosis. Human epidermal growth factor receptor 2 (HER2) inhibitor is a targeted drug to treat breast cancer that interferes with the heterodimerization of ErB2 and ErB4 in cardiomyocytes, thus eliminating the protective effect of neuregulin 1, which helps to repair cardiomyocytes

Table 1. Mechanism of cardiovascular toxicity induced by cancer treatments

Mechanism	Cancer treatments	Related CVDs
Cardiac apoptosis	• Anthracyclines • Antimetabolites • PKIs • Radiation • Topoisomerase II inhibitors	HF Arrythmia Heart valve disease
Endothelial damage	• Alkylating agents • Anthracyclines • PKIs • Taxanes • Proteasome inhibitors • Radiation • Platinum • VEGF inhibitors	CAD Hypertension Thromboembolism
Mitochondrial injury	• Anthracyclines • TKIs	HF Arrythmia CAD
Oxidative stress	• Alkylating agents • Anthracyclines • Antimetabolites • Mitomycin C	HF Pericarditis CAD
Proinflammatory response	• Anthracyclines • Antimetabolites • Interferon-α	HF Pericarditis Arrythmia CAD
Cytokine release syndrome	• Alkylating agents • Anthracyclines • Antimetabolites • Monoclonal antibodies • Amsacrine • Platinum • Melphalan	Thromboembolism Arrythmia HF CAD
Abnormal Ca^{2+} homeostasis	• Anthracyclines • Arsenic trioxide • Taxanes	Arrythmia

Abbreviation: PKIs, protein kinase inhibitors; TKIs, Tyrosine kinase inhibitors; HF, heart failure; CAD, coronary atherosclerosis; VEGF inhibitors, vascular endothelial growth factor inhibitors.

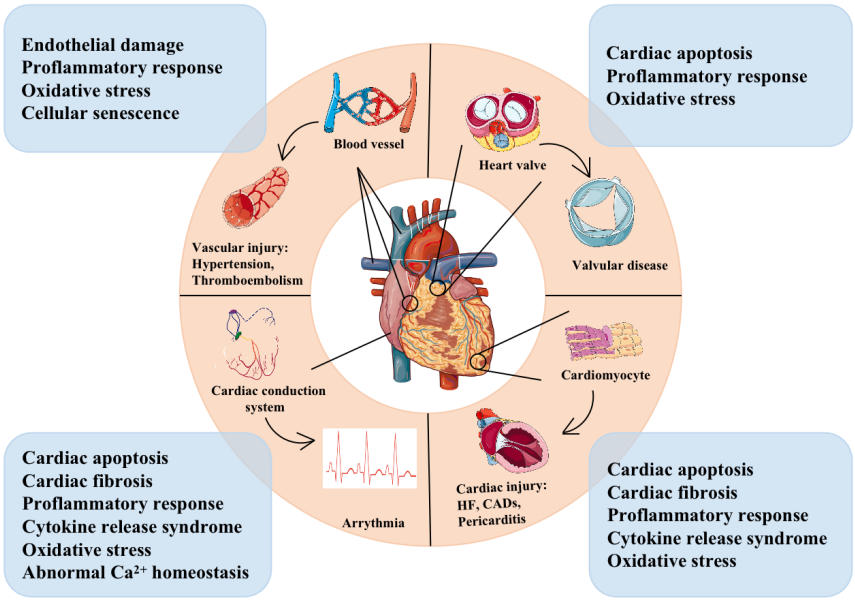

Figure 1. The mechanism of cardiovascular toxicity induced by cancer treatments.

and maintain homeostasis (*19,20*). In addition to cardiac fibrosis directly caused by drugs, physical therapy also significantly accelerates this process. Radiation therapy continues to advance, and it has become the primary modality for clinical treatment of malignancies. Radiation therapy kills cancer cells and prevents them from returning via a variety of mechanisms. However, it simultaneously kills neighboring normal cells, so the radiation dose and volume exposed must be tightly controlled. Prolonged radiation exposure affects cardiac capillary endothelial cells, leading to their proliferation, damage, swelling, and degeneration, and it significantly reduces the number of capillaries (*21*). Capillary network damage is associated with decrease in the myocardial blood supply, eventually contributing to the development of myocardial fibrosis. Radiotherapy also induces fibrosis of the heart valves and cusps (*22*).

In addition to mechanisms mentioned thus far, proinflammatory response and cytokine release syndrome (CRS) often occurs together during immunotherapy and biological treatment. Immune effector cells cross-link with tumor cells, contributing to the release of a large number of cytokines and inflammatory factors. Cytokines are mainly derived from the target cells themselves and immune cells recruited to the tumor. Aggregation of cytokines can lead to excessive activation of immune cells such as macrophages, which create a positive feedback loop that promotes the activation of cytokines such as IL-1, IL-6, IL-8, IL-10, and MCP-1, eventually leading to a cytokine storm and excessive inflammation (*23,24*).

3.1.2. Myocardial electrical dysfunction

Studies have indicated that cancer treatments regulate cardiomyocyte ion channels. HER2 inhibitors regulate cardiac sympathetic nerve tension and affect a variety of ion channels, such as I_{to}, I_{K1}, and $I_{K,Ach}$ (*3*). Arsenic trioxide, which is often used to treat hematologic cancers, blocks I_{Kr} and I_{Ks} and activates $I_{K,Ach}$ (*25*). An increase in myocyte automaticity and altered expression of ion channels induces prolongation of the QT interval and early post-depolarization and it triggers abnormal cardiac electrical activity.

In addition, calcium homeostasis plays a key role in myocardial excitation-contraction coupling, which is involved in the changes in cardiac electrical activity. Studies have indicated that paclitaxel increases the expression of neuronal calcium sensor 1 (NCS-1), it modulates the release of calcium from cells depending on the inositol-1,4,5-triphosphate receptor (InsP3R), and it affects calcium signaling, calcium oscillation, and calcium homeostasis in cardiomyocytes. Thus disrupting the normal electrophysiology of the myocardium (*26*).

3.2. Vascular toxicity

Cardiotoxicity has received extensive scholarly attention, but vascular toxicity cannot be ignored. Endothelial injury is a key feature of vascular toxicity and it is caused by cell aging, oxidative stress, and pro-inflammatory response.

Cisplatin and carboplatin are platinum-based agents that have been in widespread use for many years to treat several forms of cancer, and platinum-based agents are considered to be vasotoxic. Cisplatin can still be detected in serum several years after treatment, and it continues to affect vascular endothelium (*27*). Cisplatin exposure activates endothelial cells and then up-regulates intracellular adhesion molecule 1, tissue-

type plasminogen activator, and plasminogen activator inhibitor type 1 in endothelial cells, suggesting an acceleration of atherosclerosis (*11,27*). After many years of cisplatin therapy, patients have an increased carotid intima media thickness (C-IMT) and aortic IMT (A-IMT). Many other types of anticancer drugs appear to be vasotoxic. 5-Fu and capecitabine are commonly used antimetabolites that promote oxidative stress, causing cellular damage and readily inducing endothelial injury followed by thrombosis (*28*).

Radiotherapy is known to cause the shortening and dysfunction of telomeres, thus accelerating cellular senescence. Senescence of vascular endothelial cells increases the production of reactive oxide species (ROS) and decreases the vascular protective effect of nitric oxide, contributing to the senescence-induced apoptosis of endothelial cells. Cellular senescence is thought to be a gradual decline in the ability of cells to cope with various stresses that may trigger chronic pathological conditions, including CVDs (*29*).

4. CVDs associated with cancer treatments

Therapy of a longer duration is considered to involve an increased risk of cardiovascular events such as HF, hypertension, CAD, arrhythmia, thromboembolism, heart valve disease, and pericarditis. These events are common, and they involve a high rate of morbidity and disability. Therefore, the cardiovascular system should be monitored, and cardiovascular events prevented in all patients with cancer.

4.1. HF

HF is one of the most common complications in patients with cancer, and it influences the overall outcomes for those patients, particularly the elderly. Conventional chemotherapeutics, such as anthracyclines, antimetabolites, and cyclophosphamide, can induce permanent myocardial cell injury, leading to left ventricular (LV) dysfunction (LVD) and diastolic and systolic dysfunction and eventually progressing to acute or chronic HF (*30*). Anthracyclines, especially doxorubicin, are the greatest concern, since they can lead to HF and significantly shortener lifespan. Anthracycline-related LVD has historically been considered to be dose-dependent, cumulative, and progressive; over 50% of patients receiving anthracycline-based chemotherapy have a certain degree of cardiac dysfunction 10-20 years after chemotherapy, 5% have obvious HF, and the mortality rate is close to 60% (*31*). A study has indicated that myocyte injury may also be reversible if LVD, diastolic as well as systolic dysfunction are detected early, and appropriate HF-based treatment is instituted (*32*).

Surveillance strategies are currently based on expert consensuses. An integrated approach combining cardiac biomarkers as well as imaging data plays a major role in identification, assessment, and monitoring of antitumor drug-induced cardiotoxicity. Troponins may facilitate the detection of cardiotoxicity in the preclinical phase and help to predict the severity of future LVD in patients treated with antitumor drugs (*33,34*).

The left ventricular ejection fraction (LVEF) is the most commonly used parameter of cardiac function that independently predicts short-term and long-term mortality from cardiovascular events. The Clinical Practice Guidelines of the American Society of Clinical Oncology (ASCO) recommend assessment of LVEF prior to and during exposure to known cardiotoxic agents in high-risk patients (*35*). Recent improvements in imaging technology have led to more sensitive and precise assessment of cardiac function. New techniques, including spot tracking and measurement of strain, make up for the technical shortcomings of conventional ultrasound (*36*). These imaging technologies have enabled early detection of structural injury to the heart and cardiac injury as a result of cancer therapy.

The primary goal of treating cancer treatment-related HF is maintaining the LVEF and limiting myocardial remodeling. There is growing evidence suggesting that several agents, such as β-blockers, angiotensin antagonists, statins, angiotensin-converting enzyme inhibitors (ACEIs), and angiotensin receptor blockers (ARBs), play a cardioprotective role in preventing cancer treatment-induced cardiotoxicity. However, patients receiving anthracycline-based chemotherapy have a significant increase in adverse reactions to ACEIs/ARBs, such as a dry cough, hypotension, hyperkalemia, and angioedema (*37*). For these patients, carvedilol is a better choice to reduce HF.

4.2. Hypertension

Hypertension is a common comorbidity in patients with cancer. Nearly 70% of patients with prostate or bladder cancer develop hypertension. Moreover, the toxicity of anticancer drugs accelerates the development of hypertension. Monoclonal antibodies (such as bevacizumab), TKIs, and vascular endothelial growth factor (VEGF) signaling pathway (VSP) inhibitors disrupt endothelial cell receptor signaling, which affects the activity of nitric oxide synthase, nitric oxide levels, and endothelin synthesis. Increased vasoconstriction capacity and decreased peripheral angiogenesis induce hypertension. In addition, alkylating agents, taxanes, platinum-based agents, and neuroendocrine drugs can also cause hypertension.

The primary goal of treating patients with hypertension is to minimize damage to target organs including the brain, kidneys, and heart by standard management of blood pressure (BP). Effectively lowering BP reduces morbidity and mortality. The European Society of Cardiology recommends a target systolic BP (SBP) < 140 mmHg and a diastolic BP (DBP) < 90

mmHg for patients taking a VSP inhibitor (*7*). BP should be actively monitored throughout treatment, with weekly assessments during the first cycle of treatment and assessments at least every 2 to 3 weeks during the remaining cycles. In addition, the target BP should be adjusted to < 130/80 mmHg for patients with multiple pre-existing risk factors for adverse consequences of high BP, such as chronic kidney disease (*38*).

Specific classes of antihypertensives have not been recommended for patients with cancer treatment-related hypertension. ACEIs or ARBs, calcium channel blockers (CCBs), and β-blockers are the treatment of choice. However, non-dihydropyridine calcium channel blockers (non-DHP CCBs), such as verapamil and diltiazem, should be avoided in patients receiving sunitinib or sorafenib due to underlying drug interactions (*7*). Multi-agent antihypertensive therapy is the preferred treatment for patients with cancer and poorly controlled BP. However, clinical guidelines for the management of hypertension recommend the temporary withdrawal of anticancer drugs if BP cannot be controlled and hypertension-related adverse events occur (*39*).

4.3. Coronary artery diseases (CADs)

The coexistence of CAD and cancer is largely driven by common risk factors (such as aging, smoking, obesity, and diabetes) and the increasing use of cancer treatments. CAD is triggered by anti-cancer reagents in cancer patients due to increased low-density lipoprotein levels, platelet activation, endothelial damage, and vasospasms (*40*). The combination of bevacizumab, bleomycin, and vinblastine increases the long-term risk of CAD and myocardial infarction by 1.5- to 7-fold (*41*). Moreover, radiotherapy has also been found to have negative effects: coronary events occur within 5 years after initial exposure to radiation, lasts up to 30 years post-exposure, and they increase linearly with an increase in the radiation dose (*42*).

As is true for all patients with a heart condition, non-invasive stress testing, ECG, CTA, and coronary angiography represent important modalities for the assessment and management of cancer survivors with CAD (*43,44*). ECG and cardiac biomarkers are recommended for initial diagnosis of symptoms suggestive of an acute coronary syndrome (ACS). Clinical practice guidelines recommend the temporary withdrawal of chemotherapy if ECG from patients receiving pyrimidine analogue indicates acute myocardial ischemia, such as ST segment changes or T wave inversion.

In patients with angina that fails to respond to optimal medical therapy or in patients with a high TIMI score, invasive evaluation and treatment should be considered. Percutaneous coronary intervention (PCI) is the gold standard for ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI)/unstable angina (*40*). After surgery, patients receive routine anticoagulation therapy for at least 12 months. For patients with cancer and a high risk of bleeding, the European Society of Cardiology (ESC) recommends triple therapy [dual anti-platelet therapy (DAPT) and an oral anticoagulant (OAC)] for one month. Patients should subsequently receive antiplatelet drug and OAC for 11 months. If the risk of bleeding is extremely high, administration of an antiplatelet drug and an OAC for 12 months should be considered, and then OAC can be taken alone (*45*).

Other important recommendations for patients with cancer and CAD are control of symptoms, prevention of the progression of atherosclerosis, and prevention of the development of acute coronary syndrome via active medical treatment, and particularly with statins and β-blockers.

4.4. Arrhythmia

Kravchenko et al. reported that 28.6% of patients with lung cancer had arrythmia, including those who received chemotherapy or radiotherapy (*46*). In general, cancer therapy-related arrhythmias can be differentiated into bradycardia and tachycardia such as atrial fibrillation (AF), ventricular tachycardia (VT), long Q-T syndrome, and heart block.

Cardiac arrhythmias were first found to occur with paclitaxel, which mainly induces episodes of asymptomatic bradycardia. All other rare paclitaxel-induced arrythmias, including supraventricular tachycardia and premature ventricular, are self-limiting and resolve 48h after treatment is stopped. Radiation therapy is another classic cancer treatment that produce different forms of bradycardia, up to a grade III atrioventricular block. Exposure to radiation causes fibrosis in the conduction system, including the sinus node, atrioventricular and His bundle, and bundle branches.

QTc prolongation and related VT has been noted with arsenic trioxide and various TKIs. These effects are attributed to an increase in the inward current and a decrease in the outward current in the ventricle.

AF is the most frequent sustained arrhythmia in clinical practice, and its prevalence increases with age. Initially, reported risk factors for AF were neoplastic infiltration, mechanical pressure on the heart, or a complication of oncological thoracic surgery. The association between cancer therapy and AF was subsequently identified. During prolonged treatment, the release of proinflammatory cytokines, abnormalities in calcium homeostasis, direct myocardial damage, and an increase in vagal and adrenergic tones can interfere with the electrical conduction system of the heart and serve as major risk factors for AF.

ECG is gold standard for diagnosis of arrhythmia. Clinicians should investigate the QT interval with a

routine 12-lead ECG and calculate the QTc (heart rate-corrected QT interval) both before and after cancer treatments (*47*). During treatment, discontinuation or adjustment of the anti-cancer regimen should be considered for patients with a QTc> 500ms, QTc prolonged more than 60ms, or new arrhythmia. In addition, 24-hour continuous Holter recording is helpful in diagnosing suspected arrhythmias, and establishing their frequency, relating them to symptoms, and assessing the response to therapy (*48*).

Management and prevention of arrhythmia in oncology patients have the same goals as in the general population. Amiodarone and β-blockers are used as first-line treatments. Ca^{2+} channel blockers (CCBs) are recommended to reduce the heart rate, but recent studies have indicated that ibrutinib increases the plasma levels of CCBs, amiodarone, and digoxin (*47,48*). Therefore, caution is recommended when these drugs are combined to manage tachyarrhythmia.

Adequate anticoagulation therapy is the cornerstone of AF treatment, but it must be carefully administered to patients receiving cancer therapies. CHADS2 (CHA2DS2-VASc) and HAS-BLED scoring standards are currently the general methods for assessing AF. However, neither consider cancer and cancer treatment-induced hypercoagulability. Thus, both the score and the patient's condition need to be considered when determining whether to administer anticoagulant therapy.

4.5. Thromboembolism

Hypercoagulability associated with cancer seems to be a major problem leading to thrombosis. Thromboembolism can be divided into venous thrombosis and arterial thrombosis. Venous thrombosis often occurs in patients with advanced cancer; it poses a huge burden and has a poor prognosis (*49*). Arterial embolism is mainly evident as acute coronary syndrome, acute ischemic stroke, or peripheral vascular disease. Coronary thrombosis is the most common form of acute coronary syndrome. Cancer treatments accelerate thrombosis due to injury to the vascular endothelium and disruption of normal coagulation (*50*). Anti-angiogenic drugs or TKIs lead to arterial thrombosis such as thrombosis or embolism; thalidomide or lenalidomide are major causes of venous thrombosis, which manifests as deep vein or pulmonary embolism.

Excellent tumor control is associated with a decreased risk of thromboembolism. Therefore, anti-cancer drugs are not considered to be absolutely contraindicated. Given a high risk of arterial embolism, VEGF should be avoided and aspirin or clopidogrel should be used to prevent thromboembolism. Warfarin and low molecular weight heparin (LMWH) are recommended to treat patients with an increased risk of venous thrombosis (*51*). A point worth mentioning is that deep vein thrombosis is a serious complication after surgery for cancer, regardless of the type of cancer or the patient's age or sex. Khorana scoring can be used to predict the risk of chemotherapy-associated VET. In high-risk patients undergoing surgery, bleeding and the risk of an embolism should be assessed preoperative and anticoagulants should be administered to prevent embolism.

4.6. Other CVDs

Pericarditis, myocarditis, and heart valve disease are seen in patients with cancer, and especially those undergoing radiotherapy (*21*). Acute radiation pericarditis typically occurs within days to a few weeks after treatment and gradually develops into chronic pericarditis, which manifests as pericardial effusion or constrictive pericarditis. Mitral valve dysfunction is the main feature of radiation-related heart valve disease, and the degree of damage is correlated with the radiation dose.

The main methods of monitoring pericarditis, myocarditis, and heart valve disease include ECG and echocardiography. Cardiac ultrasound is generally the method of choice for monitoring pericarditis and heart valve disease, and it can be used to evaluate the structure and function of the heart valves and pericardium (*34*). A conventional 12-lead ECG is recommended as an initial diagnostic test for patients suspected of having myocarditis.

Clinical data on the prevention and treatment of cancer treatment-related pericardial and valvular disease are limited. As in patients who do not have cancer, conventional surgical resection and interventional valve replacement are indicated for patients with cancer treatment-related pericardial or valvular disease. Due to the potential adverse effects of radiotherapy, a lower radiation dose or new techniques to precisely locate and expose less of the heart are crucial to reducing valvular and pericardial injury.

5. Conclusion

Improvements in cancer diagnosis and treatment have led to a significantly increased survival rate for patients with cancer. Long-term quality of life after therapy has become a topic of increasing interest for both cancer survivors and experts. Cancer therapies cause short-term and long-term adverse reactions involving the heart and circulation and they exacerbate existing CVDs. Therefore, large-scale prospective studies need to be conducted and the mechanism of cancer treatment-related cardiovascular toxicity needs to be understood further to improve guidelines on appropriate monitoring, prevention, and treatment of cancer treatment-related cardiovascular events.

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Original Article

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The impact of COVID-19 pandemic on the utilization of ambulatory care for patients with chronic neurological diseases in Japan: Evaluation of an administrative claims database

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SUMMARY The COVID-19 pandemic has affected not only the emergency medical system, but also patients' regular ambulatory care, as such decrease in the number of patients visiting outpatient clinics decreased in 2020 than in 2019, or the ban lifting of subsequent visits by telephone for outpatient clinics since March 2020 in lieu of ambulatory care for chronic diseases. In this context, we investigate the impact of the COVID-19 pandemic on ambulatory care at Japanese outpatient clinics for patients with chronic neurological diseases during 2020. We collected data from the administrative claims database (DeSC database) covering more than 1 million individuals. Serial changes in the frequency of subsequent outpatient visits to clinics or hospitals (excluding large hospitals) for chronic ambulatory care of epilepsy, migraine, Parkinson's disease (PD), and Alzheimer's disease (AD) in 2020 were measured. As a result, since April 2020, the monthly outpatient visits for epilepsy, PD, and AD decreased slightly but significantly (approximately 0.90 in relative risk [RR]) but visits for migraine increased (RR = 1.15). Telephone visit was most frequently used in April-May, in less than 5% of monthly outpatient clinic visits for the examined neurological diseases. Outpatient visits for migraine treatment were more likely to be done by telephone than in case of other diseases (adjusted Odds ratio = 2.08). These results suggest that the impact of COVID-19 pandemic on regular ambulatory care for several chronic neurological diseases yielded different effect depending on the disease, in terms of the frequency or type of outpatient visits.

Keywords COVID-19, ambulatory care, chronic neurological disease, administrative claims data, telemedicine

1. Introduction

The global coronavirus disease (COVID-19) pandemic, since early 2020, has severely affected not only the emergency medical system in Japan (*1*), but also patient care at the outpatient clinics. To reduce the risk of COVID-19 infection, people were requested to refrain from unnecessary and nonurgent outings or from visiting crowded places, with a call to "avoid the three Cs" (closed spaces, crowded places, and close-contact settings) (*2*) based on the Act on Special Measures for Pandemic Influenza and New Infectious Diseases Preparedness and Response (*https://elaws.e-gov.go.jp/document?lawid=424AC0000000031*). People also refrained from visiting outpatient clinics (*3*), leading to a significant decrease in the number of ambulatory visits to internal medicine outpatient clinics in Japan by more than 10% in April-May 2020 compared to April-May 2019 (*4*). In addition, the ban on subsequent visits by telephone (or telephone re-examination) at outpatient clinics as an alternative to ambulatory care for chronic diseases was removed by the Ministry of Health, Labor and Welfare (MHLW) since March 2020 as an exceptional measure against the COVID-19 pandemic (*5*): in terms of reimbursement, it became newly available to claim a "subsequent visit fee" along with the "prescription fee", even in case of telephone visits.

What is concerned in these measures against COVID-19 pandemic is that they are not always feasible for some patients with chronic neurological diseases (*e.g.*, dementia, epilepsy, or Parkinson's disease), who are one of those considered as vulnerable to COVID-19 infection due to their old age or comorbid status (*6*). Since patients with chronic neurological diseases need

continuous medication and regular ambulatory care at the outpatient clinic, it is unfavorable to interrupt ambulatory care completely, even during a state of emergency (*7*). In accordance with the perceived risk of visiting outpatient clinics (*8*), some patients with neurological disease may have cancelled their routine visits, and others might have adapted by increasing the number of prescription days or by receiving ambulatory care using telephone (*9,10*), thereby attempting to decrease the frequency of direct visits to the outpatient clinic (*11*). However, because in-person visit is deemed essential especially for the ambulatory care of patients with neurological diseases (*12*), there may remain some medium- or long-term safety concerns about these measures against COVID-19.

Before attempting to evaluate the safety and efficacy of these measures to prevent COVID-19 infection for vulnerable individuals, we need to have basic data about the impact of the COVID-19 pandemic on the care for patients with chronic neurological diseases (*13*), of which evidence is limited in Japan. In a recent study using claims data from the United States, it is reported that in-person outpatient visits decreased to a degree that could not be complemented by the increased use of telemedicine (*14*). From Japan, the decrease in the number of ambulatory visits to outpatient clinics and hospitals, or the utilization rate of telephone visits, have only been reported in general remarks (*4*) or with a very limited samples (*15*), and have not been reported in detail by the area of neurology. Therefore, we herein aimed to assess the basic features of the change in care for patients with chronic neurological disease at the outpatient clinic during 2020. We used the DeSC claims database that is based on the Japanese public health insurance and comprises data on more than one million individuals in Japan. The database has a great advantage in terms of its high accessibility and analyzability compared to much larger Japanese claims databases (*e.g.*, NDB) (*16*), and thus, the use of the DeSC database in the current study would provide a starting point and an important foundation for further research.

2. Materials and Methods

2.1. Study design

This was a retrospective observational study using administrative claims data and was approved by the University of Tokyo Graduate School of Medicine Institutional Ethics Committee (ID: 11628-(3)). Informed consent is not required because this study only uses already-prepared anonymized information as required by the Act on the Protection of Personal Information in Japan (*https://elaws.e-gov.go.jp/document?lawid=415AC0000000057*). We applied for access to the DeSC database (*https://desc-hc.co.jp/archives/2188*) in March 2021, which was approved by DeSC Healthcare, Inc. (*https://desc-hc.co.jp/en*), permitting us to obtain the data in April and June 2021.

2.2. About the DeSC database

The DeSC database was built by anonymizing and processing data from the health insurance claims database provided by several Japanese public health insurers covering more than one million individuals. Three types of insurers are included: Society-managed, employment-based health insurance association (SHI), National Health Insurance (NHI), and Latter-Stage Elderly Heathcare System (we abbreviated as 'LSEHS' here) (*17*). In Japanese public health insurance system, there is no difference depending on the type of insurers regarding which clinics/hospitals to visit or regarding the range of medical treatments/practices covered by each insurer, and which type of insurer types for each individual is determined by their employment status (*17*): the SHI is provided for individuals working at large companies and their family members. NHI covers broader range of people younger than 75 years and not covered by other public health system, such as self-employed persons, freelancers, farmers, retirees, or college students. People who are 75 years or older are in principle covered by LSEHS specifically arranged for covering elderly individuals. So that the DeSC database contains patients with broad range of age and social background.

The details of individual health insurers who provided their data to the DeSC database, including their names or addresses, are completely confidential. The degree of overlap in the geographical medical areas of NHI, SHI, and LSEHS insurers is also undisclosed. The eligibility criteria for the DeSC database is as follows: insured individuals and their dependents who are in the age group of 19-74 years (in SHI), 0-74 years (in NHI), or 65 years or older (in LSEHS) as on November 30, 2021. The database includes all eligible patients' receipts claimed during the period between April 2015 and November 2021 (for SHI and NHI) or during the period between June 2018 and November 2021 for LSEHS.

As for database specifications, disease name, drugs, or medical procedures related to the following specific diseases are masked for anonymity: "designated infectious disease" such as COVID-19 or "type 1-3 infectious diseases" (*e.g.*, Ebola hemorrhagic fever, tuberculosis, SARS, or cholera) as specified in the Act on the Prevention of Infectious Diseases and Medical Care for Patients with Infectious Diseases in Japan (*https://elaws.e-gov.go.jp/document?lawid=410AC0000000114*), or "designated intractable diseases" (*18*) with a small prevalence rate.

2.3. Data preprocessing

The process of data handling and analyses were conducted using R software (version 3.5.1) in MacOS

Catalina. The preprocessing workflow is shown in Figure 1. First, the total number of insured individuals and their dependents registered by the insurers during the study period was 665,398 in SHI, 859,983 in NHI, and 300,070 in LSEHS after excluding those who had withdrawn from the insurers' list during the study period.

Among them, claims data of those who had ever visited outpatient clinic during the study period were extracted (Figure 1A). Receipts were claimed for each patient in each month of visit by each clinic or hospital, and for each type of medical setting (*e.g.*, ambulatory care, in-hospital care, or pharmacy); for our analysis, we only included the receipts claimed for ambulatory care at the facilities with less than 200 beds (Figure 1B). We limited the size of clinics or hospitals of each receipt because subsequent visit at facilities with 200 or more beds are claimed under "outpatient clinic fee" (medical practice code: 112011310) regardless of the use of telephone or any online devices, and consequently, the use of telephones for subsequent ambulatory care cannot be identified from the coding. In case of facilities with less than 200 beds, ambulatory care *via* the telephone or any electronic device can be identified, since it would be claimed as a "subsequent visit fee by telephone" (medical practice code [version in April 2020]: 112007950, 112008850, and 112023350) instead of simply "subsequent visit fee" (medical practice code: 112007410, 112015810, and 112008350). We have not considered the claims under "first visit fee" (medical practice code: 111000110) or "online medicine fee" (medical practice code: 112023210), because in case of the former, one cannot distinguish whether the examination was done by in person or *via* telephonic examination, and in the case of the latter claims, facilities are required to notify authorities in advance to start "online medicine", which have actually been hardly used by patients in this database even during the COVID-19 pandemic.

Subsequently, among the receipts claimed for ambulatory care at the outpatient clinic (Figure 1C), we considered only those that included the prescription of oral or external medications as well as the claims of "subsequent visit fee" or "subsequent visit fee by telephone" for the analysis (Figure 1D). Next, since different outpatient visits within the same month cannot be distinguished solely by each receipt, we additionally referred to the date of visits to differentiate between each visit. We use the term "date-based visit" to refer to the distinguished minimum unit of visits in order to conduct visit-based analyses.

2.4. Outcome definition

For the above-mentioned date-based visits, we determined the diseases for which each patient visited the

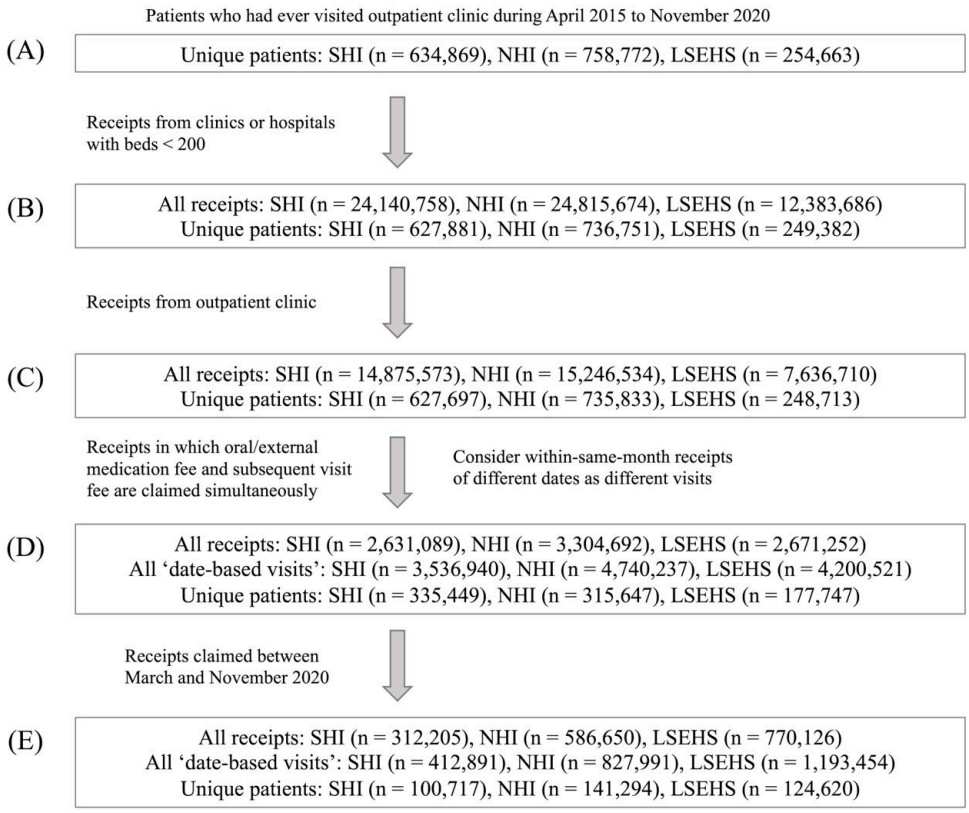

Figure 1. Data preprocessing workflow. Among approximately 1.5 million individuals who are insured by the SHI, NHI, and LSEHS insurers in the period between April 2015 and November 2020, those who had subsequent visit to outpatient clinic for their chronic neurological diseases were reviewed. Abbreviations: LSEHS, Latter-stage elderly healthcare system; NHI, National Health Insurance; SHI, society-managed, employment-based health insurance association.

outpatient clinic. Here, we specifically focus on several common neurological diseases, *i.e.*, Alzheimer's disease (AD) dementia, epilepsy, Parkinson's disease (PD), and migraine. This choice of disease is mainly due to the relatively limited sample size of the database (*i.e.*, 1-1.5 million individuals) and the low prevalence rate of many neurological diseases: extremely rare diseases by which recipients of 'intractable disease medical care ticket' (18) are less than 500 had been anonymized during the database creation process. Even in case of "uncommon" neurological diseases such as spinocerebellar ataxia, myasthenia gravis, or multiple sclerosis, they cannot always be sufficiently identified in the database: for example, disease prevalence rate of 10 per 100,000 persons in a database covering 1 million individuals means the number of included patients with the disease can become much fewer than 100 throughout the study period, because of the potential risk of under-estimation of patients based on the above-defined disease definition. In addition, although very common, we did not include chronic ischemic stroke or chronic hemorrhagic stroke because they cannot always be specifically identified by the name of the medication.

We determined whether each date-based outpatient visit was related to the care of each disease, defined by fulfilling both of the following criteria: (A) medications specific to the disease of interest are prescribed (Supplementary Table S1, A, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=77*), and (B) receipt claim (monthly) includes disease names related to the disease of interest (Supplementary Table S1, B, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=77*). This is in reference to earlier studies (*e.g.*, in PD (*19,20*) or epilepsy (*21,22*)). We referred to the Anatomical Therapeutic Chemical classification system (ATC code) for identifying medications (A) and the 10th version of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) for identifying diseases (B).

2.5. Statistical analyses

In Japan, COVID-19 positive case was first confirmed on January 16, 2020, and daily positivity gradually increased. Then the daily number of COVID-19 positivity started to increase exponentially since late March 2020, leading to the first-time declaration of State of Emergency (*6*). The first wave peaked out in the mid of April 2020. The next State of Emergency has not been declared until January 8, 2021 in the mid of third wave of COVID-19 positivity. Based on such serial events, in this study we investigated the serial changes in the monthly outpatient visits especially in terms of the first State of Emergency.

As described above, the DeSC database comprises three sub-datasets (*i.e.*, SHI, NHI, and LSEHS datasets), corresponding to data obtained from different types of insurers. The population covered by these insurers have different disease frequencies partly due to the differences in age distribution (as mentioned in the Results section) and social background. Therefore, we conducted the same analysis on each of the three datasets independently, and then comparatively described the results. In this study, we performed the following analyses.

i) Serial monthly changes in the date-based visit of neurological diseases of interest, in terms of before and after the nationwide declaration of a state of emergency (*6*) in 2020.
ii) Utilization rate of telephone visits among date-based subsequent visits since March 2020.
iii) Facility-dependent variance in the utilization (frequency and rate) of telephone visits.
iv) Features which promoted or discouraged the use of telephone in subsequent ambulatory care.

First, we evaluated the overlap in SHI, NHI, and LSEHS insurers among all visits in each facility, because the degree of overlap in the geographical medical areas of these insurers is uncertain. In Japan there is no restriction on patients regarding which hospitals to visit regardless of the type of health insurance ("free access"); therefore, if many facilities had received only the patients covered by one type of insurer, the locations of that type of insurer should be interpreted as geographically located away from others. Such insurer-deviation in each facility F is evaluated by the asymmetry index (AI), calculated by the following formula:

$$AI_F = \left| \frac{max\{N_{F,SHI}, N_{F,NHI}, N_{F,LSEHS}\} - min\{N_{F,SHI}, N_{F,NHI}, N_{F,LSEHS}\}}{max\{N_{F,SHI}, N_{F,NHI}, N_{F,LSEHS}\} + min\{N_{F,SHI}, N_{F,NHI}, N_{F,LSEHS}\}} \right|$$

where $N_{F,insurance}$ is the number of patients by each insurance at a certain facility F, and $0 \leq AI_F \leq 1$. If AI is not 1 in many of the facilities, the residing locations of patients insured by SHI, NHI, and LSEHS can then be interpreted as geographically overlapping with each other.

For Analysis [1], serial change at each month since the nationwide declaration of a state of emergency (first-time on April 7, 2020) in comparison with the previous year was measured by relative risk (RR), calculated by the following formula:

$$RR_m = \left(\frac{N_{m,2020}}{N_{m,2020} + N_{Jan,2020}} \right) \Big/ \left(\frac{N_{m,2019}}{N_{m,2019} + N_{Jan,2019}} \right)$$

where $N_{m,2020}$ denotes the total number of date-based visits of a certain disease of interest in month m of 2020. As a reference month, we used the total number of date-based visits in January 2019 and January 2020. The RR values from RR_{Mar} through RR_{Nov} were

synthesized by conducting generic inverse variance meta-analysis, to obtain synthesized RR (RR_{syn}) in a random effect model. When the upper 95% of the synthesized RR was less than 1, or when the lower 95% of the synthesized RR was larger than 1, the number of date-based visits was considered to have significantly changed compared to the previous year (March-November 2019). To calculate RR and its 95% CI, we used the R packages {*epitools*} (*23*) and {*meta*} (*24*).

In addition, we also applied interrupted time-series analysis (ITSA) (*25*) for Analysis [1], based on the impact model where there was a significant decline in the level along with the declaration of a state of emergency (in April 2020), while serial trend was maintained. The Poisson regression formula for the ITSA is as follows:

$$log(total\ count\ N\ of\ outpatient\ visit\ /month) = intercept + \beta_1 \cdot (month\ number\ since\ December\ 2018) + \beta_2 \cdot (being\ period: April{\sim}November\ 2020) + \beta_3 \cdot (dummy\ variable\ of\ month) + offset(total\ number\ of\ outpatient\ visits\ (or\ insured\ people)\ in\ each\ month)$$

where the term "*month number since December* 2018" denotes long-term trend (slope) of the month before impact, and the term "*being period: April~November* 2020" corresponds to the temporal change in the intercept as a consequence of the declaration of state of emergency. Seasonality in the serial change of monthly outpatient clinic visits is taken into account by the term "*dummy variable of month*". The visit count is also influenced by the total number of monthly insured individuals and is thus considered as the offset term. As in RR, when the upper 95% confidence interval (CI) of the adjusted odds ratio (OR) was less than 1, or when the lower 95% CI of the adjusted OR was larger than 1, the factor was considered to be significantly associated with the utilization of telephone visits in the regular outpatient visit (excluding the first-time one).

Whether telephone visit is used during regular ambulatory care depends not only on the patients' intention but also on the status of each facility (clinic/hospital) to enable the use of telephone visits, as well as any compelling need to use telephone visits, such as forced temporary closure of outpatient clinics due to the nosocomial outbreak of COVID-19 (*26*). This means that the utilization rate would vary depending on each facility. In Analysis [3], we calculated the coefficient of variation (CV_D) of each disease D to measure the facility-dependent variance in the utilization rate of telephone visits between March and November 2020, using the following formula:

$$T_k = total\ number\ of\ subsequent\ visits\ for\ D\ by\ telephone\ in\ facility\ k\ during\ the\ period$$

$$V_k = total\ number\ of\ subsequent\ visits\ for\ D\ in\ facility\ k\ during\ the\ period$$

$$CV_D = standard\ deviation\left(\frac{T_1}{V_1}, \frac{T_2}{V_2}, \ldots \frac{T_F}{V_F}\right) \Big/ mean\left(\frac{T_1}{V_1}, \frac{T_2}{V_2}, \ldots \frac{T_F}{V_F}\right)$$

where F ($1 \leq k \leq F$) is the total number of facilities, $T_k \geq 0$, and $V_k \geq 3$. We regarded the use of telephone visits for disease D as variable if $CV_D > 1$.

In Analysis [4], we merged SHI, NHI, and LSEHS datasets together, and applied a generalized linear mixed model to examine which features are associated with the use of telephone visits among all the date-based subsequent outpatient visits. Since there is a variance in the number of telephone visits used depending on the clinic or hospital (as observed in Analysis [3]), as well as differences between the two datasets in terms of age distribution and disease frequency, we incorporated the factor of patient care facility and the insurer of each patient into the mixed effects model as random effect terms. The model is described by the following formula (*27*):

$$Odds = \frac{P(Y_i = 1 \mid x_i, z_i)}{P(Y_i = 0 \mid x_i, z_i)} = exp(x_i^T \beta + z_i^T u)$$

where Y_i represents the binomial status of the use or non-use of telephone visits in the subsequent outpatient visits i for any diseases, β is the vector of fixed effect parameters, x_i is the covariate matrix for fixed effects, u is the vector of random effect parameters, and z_i is the covariate matrix of random effects. Covariates to measure fixed effects include patient's age at the time of outpatient clinic visit, sex of the patient, and binomial status whether the visit is for seeking care for a certain disease of interest. Covariates used to measure random effects (as the random intercept) denote the factor of the clinic or hospital where the receipt was claimed, and the type of insurance (*i.e.*, SHI, NHI, and LSEHS). For the multilevel analysis, we used the R package {*lme4*} (*28*). We have not conducted the Analysis [4] for the patient-based count table because of the difficulty in adjusting the factors influencing facility visits (*e.g.*, some patients regularly visit several different facilities in the same month).

3. Results

3.1. Basic demographic characteristics

During the period between March and November 2020, we included 412,891 date-based outpatient clinic visits claimed for 100,717 unique patients in SHI, 827,991 date-based visits claimed for 141,294 unique patients in NHI, and 1,193,454 date-based visits claimed for 124,620 unique patients in LSEHS (Figure 1E). As expected, the AI value was mostly 1 in many facilities (mean = 0.999, 95%-tile = 1.000, and 99.7%-tile = 1.000).

The summary demographic characteristics of eligible date-based visits and unique patients during the period between March and November 2020 are described in Table 1 (data during all eligible period

Table 1. Basic demographic characteristics of all eligible subsequent outpatient visits/patients from SHI, NHI, and LSEHS

(A) Summary by outpatient visits	SHI visits Mar.-Nov. 2020	NHI visits Mar.-Nov. 2020	LSEHS visits Mar-Nov 2020
N	412,891	827,991	1,193,454
Age (in Dec 2020)	54.5 (44.9-63.1)	69.2 (62.8-72.3)	82.5 (78.6-87.0)
Sex (female)	192,132 (46.5 %)	447,245 (54.0 %)	704,240 (59.0 %)
Telephone re-examination used	1,116 (0.3 %)	4,613 (0.6 %)	4,279 (0.4 %)
neurological diseases			
Alzheimer's disease	119 (0.0 %)	1,278 (0.2 %)	24,475 (2.1 %)
epilepsy	1,337 (0.3 %)	8,952 (1.1 %)	4,687 (0.4 %)
Parkinson's disease	348 (0.1 %)	5,032 (0.6 %)	3,673 (0.3 %)
migraine	837 (0.2 %)	741 (0.1 %)	206 (0.0 %)
(B) Summary by unique patients	SHI patients who have vis-ited during Mar-Nov 2020	NHI patients who have visited during Mar-Nov 2020	LSEHS patients who have visited during Mar-Nov 2020
N	100,717	141,294	124,620
Age (in Dec 2020)	51.0 (40.2-59.8)	68.8 (60.6-72.1)	82.5 (78.6- 87.1)
Sex (female)	49,076 (48.7 %)	80,002 (56.6 %)	76,616 (61.5 %)
Telephone re-examination used	861 (0.9 %)	1,783 (1.3 %)	2,295 (1.8 %)
neurological diseases			
Alzheimer's disease	27 (0.0 %)	239 (0.2 %)	3,778 (3.0 %)
epilepsy	222 (0.2 %)	1,273 (0.9 %)	724 (0.6 %)
Parkinson's disease	54 (0.1 %)	589 (0.4 %)	546 (0.4 %)
migraine	275 (0.3 %)	194 (0.1 %)	49 (0.0 %)

LSEHS, Latter-stage elderly healthcare system; NHI, National Health Insurance; SHI, society-managed, employment-based health insurance association.

is provided in Supplementary Table S2, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=77*). When categorized by visits (Table 1A), the median age of patients covered by each insurer clearly differed: median 54.5 years in SHI, 69.2 years in NHI, and 82.5 years in LSEHS. The proportion of female patients also differed: 46.5% in SHI, 54.0% in NHI, and 59.0% in LSEHS.

Before the deregulation of telephone visits on March 2020, among subsequent outpatient visits, telephone was used in 0.07% (2,194/3,124,049) in SHI, 0.02% (768/3,911,478) in NHI, and 0.04% (1,228/3,007,067) in LSEHS throughout the reviewed period; the utilization rate of telephone re-examination increased to 0.30% (1,116/412,891) in SHI, 0.58% (4,613/827,991) in NHI, and 0.36% (4,279/1,193,454) in LSEHS during the period between March and November 2020.

3.2. Serial changes in outpatient clinic visits

First, we reviewed serial changes in the date-based outpatient clinic visits within the last two years (December 2018-November 2020), as shown in Figure 2. Visual inspection revealed that the number of monthly visits for any disease in all insurers (Figure 2A, 2E), for epilepsy or PD in NHI (Figure 2C, 2G), or for AD in LSEHS (Figure 2D, 2H) appeared to be decreasing since the timing of April 2020.

This was statistically validated by RR and ITSA (without offset term), showing similar results (Table 2): regular outpatient visits for any diseases decreased both in synthesized RR (Table 2A) and in ITSA intercept term (column '*impact in April 2020*') regardless of the type of insurers (Table 2B). In addition, in terms of neurological diseases, in SHI, visits for migraine showed slight but significant increase in both RR (Table 2A) and ITSA (Table 2B), and other neurological diseases showed no significant change. In NHI and LSEHS, the number of monthly visits for all neurological diseases except for migraine significantly decreased in both RR (Table 2A) and ITSA (Table 2B).

In ITSA models including offset term (Supplementary Table S3, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=77*), there was a significant increase in the number of visits for epilepsy, PD, and migraine in proportion to all ambulatory visits (excluding the first-time ones).

3.3. Facility-dependent variability in telephone re-examination use

We calculated the *CV* of the telephone re-examination rate, revealing high facility-dependent variance (*CV* > 1) in the rate of telephone re-examination used in outpatient visits for each disease: CV_{AD} = 4.69, $CV_{epilepsy}$ = 4.63, CV_{PD} = 4.64, $CV_{migraine}$ = 3.37, while CV_{any} = 9.64 (Figure 3A).

Figure 3B and Figure 3C are bar plots showing the number of outpatient clinic visits by facility (clinics or hospitals) where telephone re-examination "subsequent visit fee" had been claimed from March to November 2020 for 10 or more visits (SHI, NHI, and LSEHS

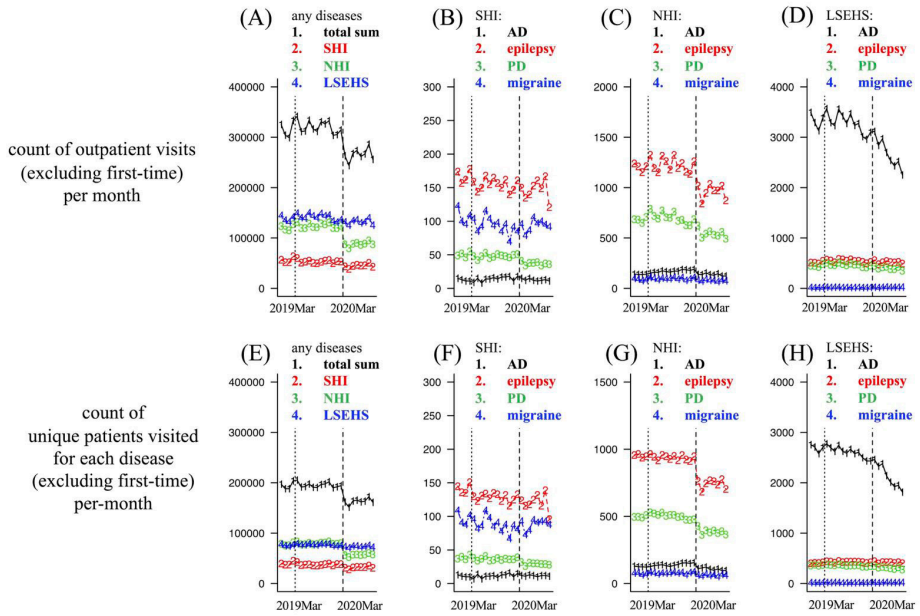

Figure 2. Serial change in the monthly count of visits / patients where subsequent visit fee were claimed, since December 2018 to November 2020. Monthly count by visits **(A) - (D)** and count by unique patients **(E) - (H)**. We calculated the count of unique patients of each month by enumerating those who have ever visited for the disease of interest and have any visits within the month. The vertical dashed line shows the timing of Declare of State of Emergency in early April 2020, and the vertical dotted line indicates the same day in 2019. Abbreviations: AD, Alzheimer's disease; LSEHS, Latter-stage elderly healthcare system; NHI, National Health Insurance; PD, Parkinson's disease; SHI, society-managed, employment-based health insurance association.

Table 2. Change in the number of monthly outpatient visits: RR and ITSA

(A) Synthesized RR results Visits for	RR in SHI	RR in NHI	RR in LSEHS
AD dementia	0.841 (0.694-1.018)	0.806 (0.753-0.863)*	0.933 (0.899- 0.968)*
Epilepsy	1.025 (0.973-1.08)	0.911 (0.875-0.947)*	0.95 (0.924-0.977)*
PD	0.945 (0.853-1.047)	0.914 (0.884-0.945)*	0.948 (0.919-0.978)*
Migraine	1.148 (1.077-1.225)*	0.987 (0.921-1.057)	1.032 (0.905-1.175)
any diseases	0.932 (0.904-0.961)*	0.834 (0.797-0.873)*	0.964 (0.953-0.976)*

*Upper 95% CI < 1 or lower 95% CI > 1.

(B) ITSA results (without offset term)	coefficients (95% CI)	
Visits for	Serial trend (slope)	Impact in April 2020
SHI		
AD dementia	1.021 (0.99-1.053)	0.737 (0.465-1.166)
Epilepsy	0.992 (0.983-1.001)	1.037 (0.905-1.187)
PD	0.997 (0.981-1.014)	0.803 (0.627-1.030)
Migraine	0.982 (0.97-0.993)*	1.205 (1.013-1.434)*
any diseases	0.996 (0.995-0.996)*	0.881 (0.875-0.888)*
NHI		
AD dementia	1.024 (1.014-1.033)*	0.626 (0.546-0.717)*
Epilepsy	1 (0.996-1.003)	0.775 (0.738-0.815)*
PD	0.997 (0.992-1.001)	0.777 (0.727-0.831)*
Migraine	1.008 (0.996-1.02)	0.722 (0.605-0.862)*
any diseases	1.002 (1.002-1.002)*	0.687 (0.684-0.690)*
LSEHS		
AD dementia	0.994 (0.992-0.996)*	0.851 (0.826-0.878)*
Epilepsy	1.001 (0.996-1.007)	0.914 (0.849-0.984)*
PD	0.998 (0.993-1.004)	0.856 (0.789-0.928)*
Migraine	1.007 (0.98-1.036)	1.059 (0.715-1.569)
any diseases	0.999 (0.999-0.999)*	0.924 (0.92-0.929)*

*Upper 95% CI < 1 or lower 95% CI > 1. CI, confidence interval; ITSA, interrupted time-series analysis; LSEHS, Latter-stage elderly healthcare system; NHI, National Health Insurance; RR, relative risk; SHI, society-managed, employment-based health insurance association.

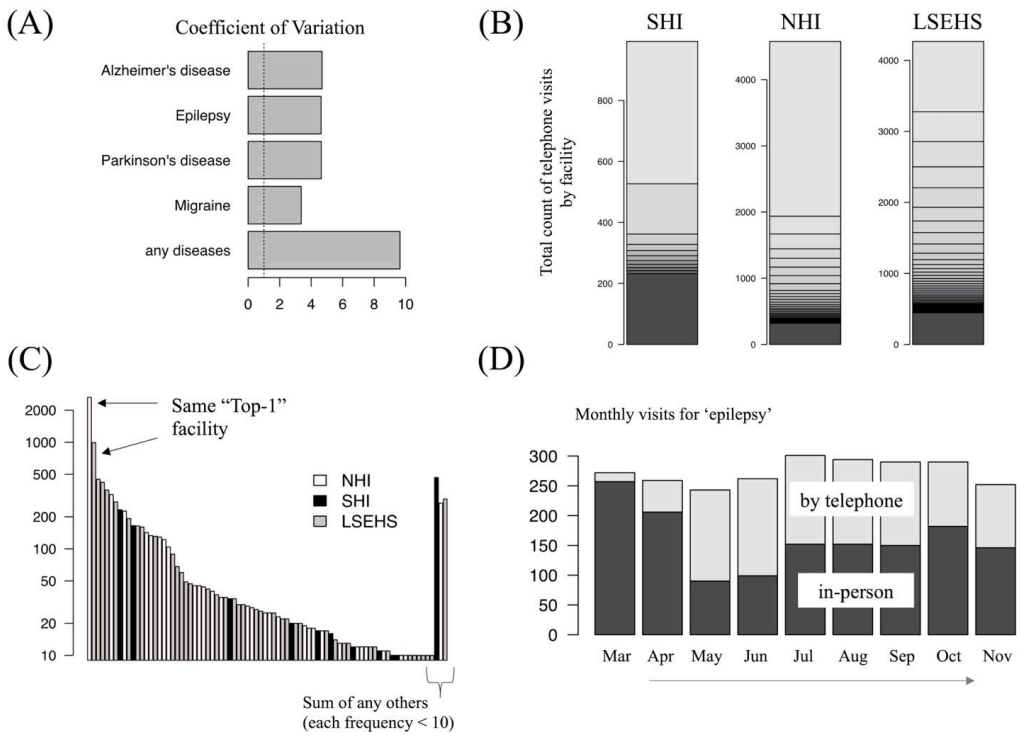

Figure 3. Facility-dependent variance in the number/rate of telephone re-examination used in subsequent outpatient visits between March and November 2020. (A) Degree of facility-dependent variation in the rate of telephone re-examination used for each disease. **(B)** Stacked bar plot of actual number of telephone re-examination used in each facility, summarized by the type of insurer and its parallel bar chart **(C)**. **(D)** Serial changes in the number of visits for ambulatory care for epilepsy at the 'Top-1' outpatient clinic. Abbreviations: SHI, society-managed, employment-based health insurance association; NHI, National Health Insurance; LSEHS, Latter-stage elderly healthcare system.

visits were separately counted in each facility). A large variability in the count was observed, and notably, the one facility which provided the largest number of telephone re-examination in NHI and LSEHS ('top-1' facility) accounted for 36.3% of all telephone re-examination visits in this database since March 2020 (Figure 3C). When we further focused on the "top-1" facility in terms of its care for epilepsy (Figure 3D), the proportion of telephone re-examination use clearly increased in May 2020.

3.4. Telephone re-examination utilization

Overall, visits for any diseases had an approximately 1% of utilization rate of telephone visits at best in May 2020, regardless of the type of insurers (Supplementary Figure S1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=77* A). Serial changes in the utilization rate of telephone visits in subsequent ambulatory care for neurological diseases in 2020 are plotted in Figure 4. In SHI (Figure 4A, D), among the neurological diseases of interest, telephone was most frequently used in visits for AD dementia in April, followed by migraine in May (Figure 4A). On the other hand, in NHI and LSEHS, telephone re-examination was most frequently used in visits for epilepsy among the neurological diseases of interest (Figure 4B-C, E-F), followed by AD dementia.

3.5. Factors associated with the use of telephone re-examination

We shall now examine which features of each visit may have contributed (or not contributed) to the use of telephone re-examination in regular visits. The results are shown in Table 3, where each row shows the result of fixed effect coefficient and its 95% CI in each model (models 1-4, depending on the type of disease as covariate). After adjusting the type of insurers and the facility-specific factor, older or female patients were found to be slightly more likely to use telephone re-examination than younger or male patients consistently (adjusted Odds ratio [OR] in female (compared to male) = 1.31 (95% CI: 1.25-1.37), and adjusted OR = 1.01 (95% CI: 1.006-1.011) per 1-year older). Being regular visits for neurological diseases of interest except for migraine was not associated with the use of telephone re-examination (adjusted OR in migraine = 2.08 [95%CI: 1.52-2.84]).

4. Discussion

In this study, we analyzed an administrative claims database to investigate the influence of the COVID-19 pandemic on ambulatory care for chronic neurological diseases in Japan in 2020.

The key findings are as follows: [1] regular visits

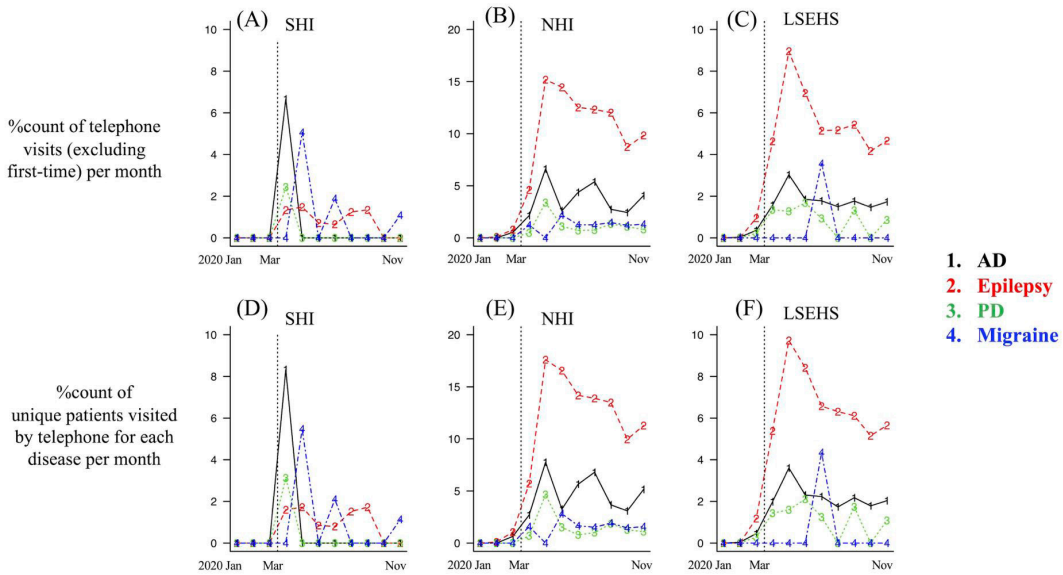

Figure 4. Serial increase in the monthly utilization rate (%) of telephone re-examination in re-examination visits for neurological diseases. Serial changes of %count by visit **(A-C)** or by unique patients **(D-F)** between January and November 2020. The vertical dotted line represents the timing of Declare of State of Emergency in April 2020. Abbreviations: AD, Alzheimer's disease; LSEHS, Latter-stage elderly healthcare system; NHI, National Health Insurance; PD, Parkinson's disease; SHI, society-managed, employment-based health insurance association.

Table 3. Results of the generalized linear mixed model for telephone visits among all (SHI + NHI + LSEHS) subsequent visits

Mixed models	Fixed-effect coefficients (in Odds ratio and 95%CI)		
	Age at each visit	Sex (female)	Being visits for the disease of interest
model 1	1.008 (1.006-1.011)*	1.308 (1.250-1.369)*	1.015 (0.901-1.144) [AD]
model 2	1.008 (1.006-1.011)*	1.309 (1.251-1.369)*	1.074 (0.984-1.173) [epilepsy]
model 3	1.008 (1.006-1.011)*	1.308 (1.251-1.367)*	0.850 (0.707-1.022) [PD]
model 4	1.008 (1.006-1.011)*	1.307 (1.249-1.367)*	2.077 (1.519-2.839)* [migraine]

*Upper 95% CI < 1 or lower 95% CI > 1. AD, Alzheimer's disease; CI, confidence interval; LSEHS, Latter-stage elderly healthcare system; NHI, National Health Insurance; PD, Parkinson's disease; SHI, society-managed, employment-based health insurance association.

for AD dementia, epilepsy, and PD mildly decreased since April 2020 in NHI and LSEHS; [2] the frequency and proportion of telephone re-examination use in ambulatory care visits for neurological diseases highly varied by each facility; [3] the telephone re-examination utilization rate generally reached its peak (approximately 5% of visits) in May 2020, after its lifting of the nationwide ban in March 2020; and [4] regular ambulatory visits for migraine were more likely to be done by telephone than visits for other diseases were. A major advantage of this study is that it used the DeSC database, which has higher accessibility and analyzability than the national database (NDB), to investigate recent claims data, despite its weakness of relatively smaller sample size. Our results thus provide basic insights about the trends in ambulatory care for chronic neurological disease patients at outpatient clinics during 2020, setting the stage for further research on the influence of COVID-19 on patients with neurological diseases.

In line with an earlier study from Japan (*4*), United States (*14*), or China (*29*) reporting reduced outpatient visits following the COVID-19 pandemic, we confirmed the decline in regular visits for AD dementia, epilepsy, Parkinson's disease, or for any diseases (Figure 2A, Table 3), presumably reflecting the social response to the COVID-19 pandemic – refraining from stepping out to reduce the risk of COVID-19 infection. For neurological disease patients who need regular visits to outpatient clinics for their requirement of continuous medications, some of them may have adapted by increasing the number of prescription days. Although the use of telephone re-examination may have led to an increase in the monthly visit count because according to the authors' observation many of the facilities available for regular visits by telephone re-examination self-regulated the prescription days to be shorter than those in case of in-person visits (*e.g.*, 30 days or so), the low utilization rate of telephone visits (Figure 4) could not have complemented the over decrease in the count of monthly visits, we suspect. It is uncertain about how many of patients with these neurological

diseases voluntarily ceased to visit hospitals and stop taking medications, and about its medium/long-term consequences.

Change in the monthly visits differed by the type of insurance. There was a decline in the monthly regular visits for AD, epilepsy, or PD in NHI or LSEHS but not in SHI (Table 3), while there was an elevation in the number of monthly visits for migraine in SHI (Table 3). This may be due to the difference in their median age distribution: older in the increasing order of SHI, NHI, and LSEHS. The perceived risk of COVID-19 infection may vary by age, as reported in a survey in the United State where more older people reported larger perception of fatality risk when infected with COVID-19 (*30*). Larger fear of death by COVID-19 infection will motivate social distancing more intensively. In addition, since mental stresses and lifestyle changes due to COVID-19 pandemic might lead to trigger migraine (*31,32*), which has in general more disease activity in younger age (*33*).

Until March 2020, the use of telephone re-examination in outpatient clinics in Japan had been largely limited, even for regular visits for chronic diseases. On February 28, 2020, the MHLW announced that they are lifting the nationwide ban on telephone re-examination for outpatient clinics as one of the exceptional measures against the COVID-19 pandemic (*5*). Aside from the initial plans to prevent COVID-19 infection risks, the consequences of lifting the ban on telephone re-examination, in terms of actual efficacy and safety, has hardly been validated in Japanese clinical settings. Although currently, there are no established guidelines, careful evaluation will be needed beforehand to determine the diseases or cases for which the use of telephone re-examination may be especially inappropriate (*12,34*). For example, patients with epilepsy and poor sleep quality were found to have an increased risk of worsening seizures during COVID-19 in Italy (*34*). Patients with neurological diseases, such as neurodegenerative diseases, often require in-person neurological examinations at outpatient clinics to be evaluated for disease progression or their current disease status, so that telephone re-examination in lieu of ambulatory care may be less appropriate for PD (*12*) compared to other neurological diseases such as epilepsy or migraine.

Overall, our study revealed that outpatient visits for ambulatory care for AD, PD, and epilepsy mildly but significantly decreased without any elevation in the utilization rate of telephone visits, while outpatient visits for ambulatory care for migraine mildly increased with higher utilization rate of telephone visits. These results showed that the impact of COVID-19 pandemic on the ambulatory care in 2020 yielded different effect depending on the disease, in terms of the frequency or type of outpatient visits. In addition, in case of AD dementia, epilepsy, and PD, potential medium/long-term risk of using telephone visits may be limited as a whole. Although the generalizability of these obtained results is limited given the relatively smaller size of claims database by nature, the degree of decline in patient visits or the utilization rate of telephone re-examination was similar to that of an earlier questionnaire survey (*4*), thereby supporting a certain level of validity in the results obtained in the current study.

Higher odds ratio of using telephone visits for migraine during COVID-19 pandemic also suggests the importance of paying attention to the treatment of migraine or other related headache patients, especially in terms of medication overuse (*35*). Although its direct evidence is limited, due to the psychological stresses following COVID-19 pandemic and the requirement of social distancing, as well as the higher convenience of telephone visits than in-person outpatient visits, might increase the frequency of migraine attack and the patients' demand for triptan drugs (*32*); such scenario is consistent with our results where outpatient visits for migraine in SHI increased after the timing of Declaration of State of Emergency (Figure 2B). In some migraine patients whose triptan demand increased, the risk of medication (or triptan) overuse elevates. In this context, we will need to evaluate the association between the incidence of medication overuse headache and the use of telephone visits (or telemedicine) under COVID-19 pandemic.

We observed very low utilization rate of telephone visits instead of conventional in-person visits for the ambulatory care of any diseases (*e.g.*, approximately 1% or less: Figure S1), and the high variability in facilities where telephone visits were used (Figure 3). As this measure was introduced as an exceptional measure against COVID-19 pandemic (*5*), the policy was insufficiently implemented throughout the overall clinics/hospitals, so that its direct effectiveness may have been insufficient by itself. Assessing facility-wise barriers/causes preventing to facilitate telemedicine may be also required as a policy consideration to promote telemedicine in the future.

Our study has several limitations. First, the disease definition of neurological diseases used in this study has not always been validated, and it is also impossible to return to the original electronic medical record for validation. The disease definitions used in this study are focused more on the content of prescriptions, which could lead to some underestimation of the actual diseases of interest: for example, patients with AD dementia do not always take anti-cholinesterase drugs because of their positive side effects or negative main effects. PD patients in their earliest stage would not be detected by our definition because they often do not receive any anti-PD medications. Furthermore, there is another concern about the overestimation of neurological diseases, especially in the case of epilepsy, since anti-epileptic drugs can sometimes be used for

other indications. For instance, valproic acid is often used for bipolar disorder or migraine prophylaxis, and carbamazepine can also be prescribed for bipolar disorder or trigeminal neuralgia.

Moreover, because the DeSC database used in this study does not disclose the details of the regions and localities of the insurers, we were unable to account for regional differences caused by differences in the timing and the extent to which the COVID-19 pandemic influenced the local medical systems across Japan, which may be one of the important confounders. These database specifications limit the generalizability of the current results; therefore, further validation studies using nationwide claims data (*e.g.*, NDB) will be necessary to obtain a more robust conclusion.

In conclusion, our study suggests that, in Japan, the impact of the COVID-19 pandemic on ambulatory care for certain chronic neurological diseases was relatively limited during the first half of 2020 in terms of the frequency or type of outpatient visits.

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Original Article

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Anti-neuroinflammatory activity of Shenqi Fuzheng Injection and its main active constituents

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SUMMARY Enhancement of alternative activation (M2) in microglia is a promising therapeutic target for microglia-mediated neuroinflammation. Shenqi Fuzheng Injection (SFI) is a clinical adjuvant treatment for cancer to reduce the side effects during cancer treatment, including boosting mood and improving appetite. However, the mechanism of SFI's effects on central symptoms is not clear. Therefore, using arginase 1 (*Arg1*) and transforming growth beta-1 (*Tgfb1*) as markers for M2 microglia activation, we found that compounds **1**, **5**, **12**, **14**, and **15** are the major M2-promoting constituents in SFI, which significantly upregulated *Arg1* or *Tgfb1* gene expression. Our results suggested that these compounds in SFI may promote M2 microglial activation and have potential uses in modulating microglial activation and alleviating neuroinflammation.

Keywords Neuroinflammation, Shenqi Fuzheng Injection (SFI), Arginase 1 (*Arg1*), Transforming growth factor beta-1 (*Tgfb1*), microglia

1. Introduction

Neuroinflammation contributes to most, if not all, neurodegenerative diseases, such as multiple sclerosis (*1-3*), Parkinson's disease (*4,5*), Alzheimer's disease (*6,7*), and Huntington's disease (*8,9*). As the resident innate immune cell in the central nervous system (CNS), microglia surveils brain parenchyma's homeostatic environment by phagocyting the cell and myelin debris and facilitating neuronal tissue repair. In recent studies, elevated microglial activity is also recognized in psychiatric disorders (*10,11*), such as major depression (*12,13*), bipolar disease (*14,15*), and schizophrenia (*16,17*). Under various physiopathological conditions, microglia respond to the stress signals and serves as a critical mediator in local neuroimmune regulation.

The rapid activation of microglia is heterogeneous and can be generalized into two major phenotypes: the classic pro-inflammatory M1 phenotype and the alternative protective M2 phenotype. M1 microglial cells release pro-inflammatory cytokines and reactive oxygen species, resulting in impaired mitochondrial function, disrupted bioenergetic homeostasis, and aggravated neuroinflammation. Elevated expression of inducible nitric oxide synthase (iNOS) and NADPH oxidase in M1 activation can further mediate local neurons and glia' cell death (*15,18*). Therefore, inhibiting M1 microglia activation is under investigation as a therapeutic target for neurodegenerative diseases and psychiatric disorders.

While chronic activation of M1 microglia is a hallmark of neuroinflammation, M2 polarization is considered to take the neuroprotective role by producing anti-inflammatory factors, such as arginase 1 (ARG1) and transforming growth factor beta-1 (TGFB1) (*19*). ARG1 is one of the canonical activation markers of M2 microglia. It blocks nitric oxide production by competing with iNOS for the same substrate L-arginine, thus reducing the cytotoxic effects during pathological events in the CNS. Alteration in arginine metabolism has been implicated in patients with schizophrenia and Alzheimer's disease in clinical studies (*20*). By converting L-arginine to the downstream neurotrophic L-ornithine and polyamines (*18*), ARG1 has been associated with neuroprotective functions, such as neurite growth, tissue repair, and secretion of anti-inflammatory cytokines. TGFB1 is another anti-inflammatory cytokine and an activation marker of M2 microglia. It has a dampening effect on activated pro-inflammatory immune cells to reduce

the cytotoxicity effects and protect neurons and glial cells (*21,22*). TGF-β is a highly conserved multipotent cytokine and has three isoforms: TGF-β1, TGF-β2, and TGF-β3. All belong to the TGF-β superfamily, which contributes to the CNS neurogenesis, homeostasis, and response to tissue injury (*23,24*). *In vitro* treatment with TGF-β1 reduces excitatory neuronal injury, and *in vivo* pretreatment with TGF-β1 before vascular occlusion reduced ischemic areas, suggesting that TGF-β1 plays a protective role against neuroinflammation (*25*). Besides, TGF-β also promotes neuronal survival and proliferation. For example, TGF-β2 perfusion can reduce the death of mature motor neurons caused by injury, which is more potent than glial cell-derived neurotrophic factor (GDNF) or brain-derived neurotrophic factor (BDNF) (*26*). Hence, promoting microglia to the M2 phenotype provides potential therapeutic benefits and is an attractive alternative strategy against neuroinflammation.

Shenqi Fuzheng injection (SFI) is an herbal extract of Dangshen (*Radix Codonopsis*), and Huangqi (*Radix Astragali Mongolici*) developed from Traditional Chinese Medicine (TCM) and has been widely used as adjuvant therapy for cancer patients in chemotherapy and radiation therapy. For example, SFI reduced radiation-induced brain injury by modulating inflammatory factors to achieve neuroprotective effects (*27,28*). The alleviation of cancer-related depression-like behaviors after SFI treatment was found in a murine model (*29*). Many clinical cases also support that SFI has significant effects on ameliorating some of the side effects of cancer treatment, including boosting mood, improving appetite, and strengthening immunity (*30-33*). These studies indicate that SFI may be associated directly or indirectly with neuroprotective roles against CNS-related inflammation during cancer treatment.

In the present study, we evaluated the effects of SFI using a murine microglial cell line (BV-2 cell) based on the interleukin-4 (IL-4) induced alternative activation of M2 microglia. By evaluating the gene expression levels of M2-activation markers *Arg-1* and *Tgfb1*, we explored the bioactive compounds in SFI that might have anti-neuroinflammatory effects *via* the M2 microglia activation.

2. Materials and Methods

2.1. Materials

SPE Bond Elut Plexa was from Agilent (Santa Clara, CA, USA). Fetal bovine serum (FBS) was purchased from Gibco Ltd. (Grand Island, NY, USA). Dulbecco's modified Eagle's medium (DMEM) was from Hyclone (Logan, UT, USA). Lipopolysaccharides (LPS) from Escherichia coli O55:B5 was from Sigma-Aldrich Co. (St. Louis, MO, USA). Recombinant Mouse IL-4 was from R & D system (Minneapolis, MN, USA). CCK-8 was from meilunebio (Dalian, Liaoning, China). RNAprep pure Cell/Bacteria Kit, FastKing gDNA Dispelling RT SuperMix, and SuperReal PreMix Plus (SYBR Green) were purchased from TIANGEN (Beijing, China). SFI was provided by Livzon Pharmaceutical Group Inc. (Zhuhai, Guangdong, China). Compounds **1**-**15** (purities ≥ 98%) were purchased from Nautre-Standard (Shanghai, China). 800TS Microplate Reader was from Bio-Tek (Winooski, VT, USA); Q5000 UV-Vis Spectrophotometer was from Quawell Technology, Inc. (San Jose, CA, USA); S1000 Thermocycler was from Bio-Rad (Hercules, CA, USA); Quantstudio 6 Flex Real-Time PCR System was from ThermoFischer Scientific (Waltham, MA, USA).

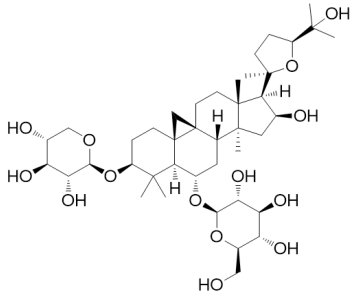
2.2. The preparation of AW and WW

The SPE column (filler: non-polar diethylene phenyl neutral polymer adsorbent, specification: 6 mL/200 mg, 45 μm) was conditioned with methanol and water, and 15 mL SFI was added to the column and eluted with 10 mL water. The elute at this step was collected as the WW component. The column was further eluted with 6 mL methanol. The methanol was evaporated under heat, and the residue was resuspended in water and set to 5 mL in a volumetric flask. The resuspended portion was filtered and used as the AW component. The final solution passed through a 0.22 μm filter before used in the cell culture. Compounds **1**-**15** are derived from AW components of SFI, which has been reported in previous literature (Table 1) (*34-38*). Table 1 was made using ChemOffice software (ver. 14.0).

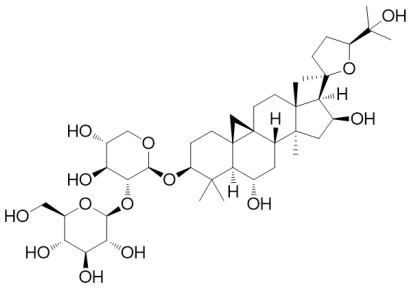
2.3. Cell culture and treatment

Murine microglial cell line BV-2 was a kind gift from Professor Linyin Feng at the Shanghai Institute of Materia Medica. Cells were cultured in DMEM with 10% FBS, 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When reaching 80-90% confluency, they were passaged at a culture ratio of 1:5 and used during their logarithmic growth phase in the following biological experiments. Only cells under 15 passages were used. BV-2 microglia were pretreated with 10% (v/v) SFI, SFI alcohol- or water-washing components 10% (v/v), or compounds (200 nM) for 1 h before polarization was induced. LPS (100 ng/mL) was applied to induce the M1-polarization of microglia, and IL-4 at 20 ng/mL was added to induce M2-type activation. For M1 microglia related experiment, aspirin (1.2 mM) was included as a positive control for its anti-inflammatory activity. Pretreatment with IL-4 (20 ng/mL) was included as a positive control for enhancement of M2-polarization. An equal volume of 1% BSA in phosphate buffer saline, normal saline, and DMEM was used as the vehicle control for SFI, IL-4, and SFI compounds, respectively.

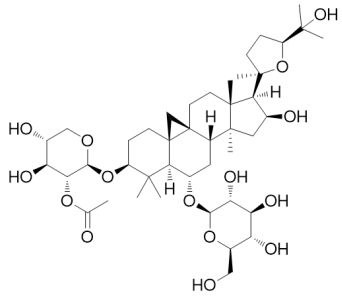
Table 1. The main components and their structures of SFI (Structure)



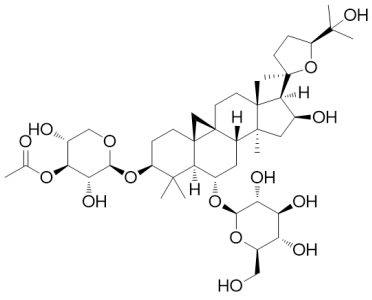
Astragaloside IV (**1**) (Ref. *34-38*)



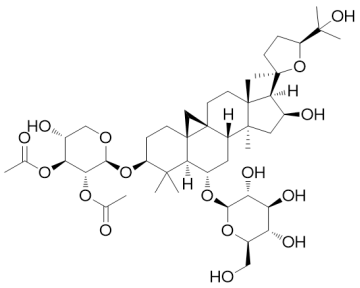
Astragaloside III (**2**) (Ref. *34-38*)



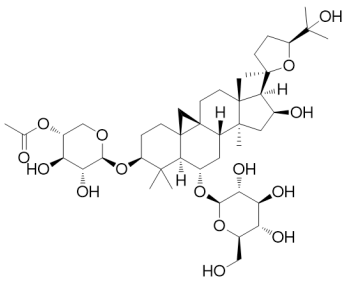
Astragaloside II (**3**) (Ref. *34-38*)



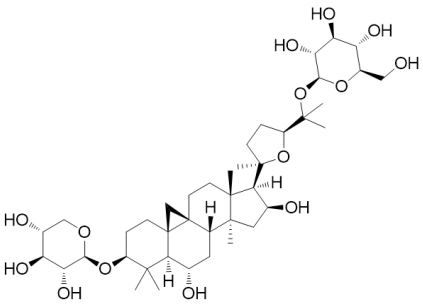
Isoastragaloside II (**4**) (Ref. *34-38*)



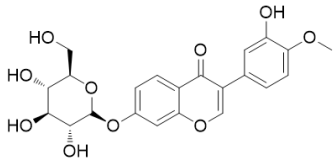
Astragaloside I (**5**) (Ref. *35,38*)



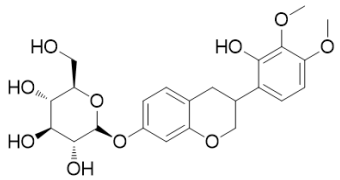
Cyclocephaloside II (**6**) (Ref. 35-37)



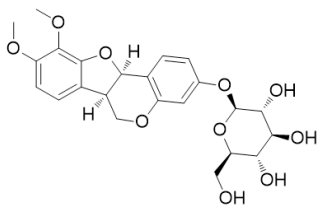
Isoastragaloside IV (**7**) (Ref. *34*)



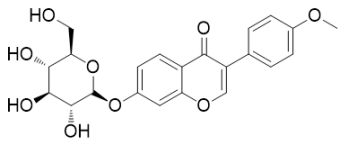
Calycosin-7-O-β-D-glucoside (**8**) (Ref. *34-38*)



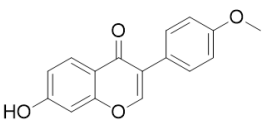
7,2-Dihydroxy-3,4-dimethoxyisoflavan 7-O-β-D-glucoside (**9**) (Ref. *35*)



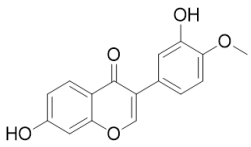
(6aR,11aR)-9,10-dimethoxy pterocarpan-3-O-β-D-glucopyanoside (**10**) (Ref. *36,38*)



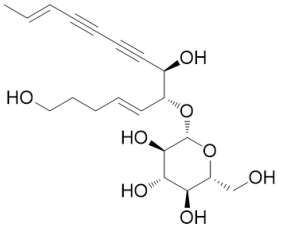
Formononetin glucoside (**11**) (Ref. *38*)



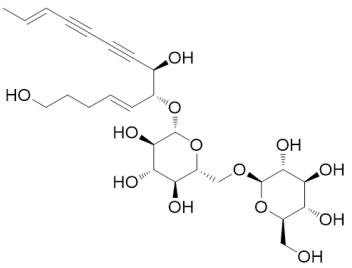
7,2-Dihydroxy-3,4-dimethoxyisoflavan 7-O-β-D-glucoside (**12**) (Ref. *38*)



Calycosin (**13**) (Ref. *35,38*)



Lobetyolin (**14**) (Ref. *34-38*)



Lobetyolinin (**15**) (Ref. *35,36*)

2.4. Cell viability assay

Cells were seeded in 96-well culture plates at a density of 8,000 cells/well. After overnight incubation, the culture medium was replaced with fresh medium containing 5% FBS per well. Cells were pretreated and stimulated as mentioned in Section 4.3. The cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 for 24 h. Before the viability assessment, the culture medium was replenished with 100 µL of serum-free culture medium, and 10 µL CCK-8 was added per well. The cells were further cultured at 37°C, and 5% CO_2 for 30 min and the absorbance (OD) was measured on a microplate reader at 450 nm. The cell viability of BV-2 cells was calculated using the following formula:

$$\text{Cell viability\%} = \frac{(OD_{sample} - OD_{blank})}{(OD_{control} - OD_{blank})} \times 100\%$$

2.5. Real-Time Quantitative PCR (qPCR)

In the gene expression studies, BV-2 microglia were pretreated with 10% (v/v) SFI or its components, or SFI compounds at indicated concentrations for 1 h, followed by 4 h stimulation of IL-4 (20 ng/mL). At the end of the treatment, the culture medium was removed, and cells were washed twice with cold PBS. Cells were collected, and total RNA extraction and RT-qPCR analysis were performed according to the manufacturer's instructions. Briefly, total RNA was isolated using RNAprep pure Cell/Bacteria Kit. Total RNA concentrations and purity was determined by a microvolume spectrophotometer. 1 µg of RNA was reverse transcribed to cDNA in a 20 µL mixture in the thermocycler, and cDNA was used as templates for qPCR on a Quantstudio 6 Flex Real-Time PCR System using a final reaction volume of 20 µL. Ct values were converted to ΔΔCt values using *Gapdh* as the reference gene and normalized to the vehicle control group for each probed gene. The primers were synthesized by Beyotime Biotechnology. Primers for qPCR sequences are as follows (from 5' to 3'):

Arg1:
forward 5′-CTCCAAGCCAAAGTCCTTAGAG-3′
and reverse 5′- GCATCCACCCAAATGACACAT-3′.
Tgfb1:
forward 5′-GGTCCTTGCCCTCTACAACC-3′
and reverse 5′-CCACGTAGTAGACGATGCGC-3′.
Gapdh:
forward 5′-CTCCACTCACGGCAAATTCAACG-3′
and reverse 5′-AGGGGCGGAGATGATGACCC-3′.

2.6. Statistical analysis

The results are presented as the means ± standard error (SD). Data were analyzed by one-way analysis of variance (ANOVA) of the differences among treatments, and Dunnett's multiple comparisons test was applied for comparison between the treatment groups with the mode group using GraphPad Prism 9.0 (San Diego, CA, USA). Differences with P values less than 0.05 was considered statistically significant.

3. Results

3.1. Chemistry

Since the SFI showed anti-neuroinflammatory activity, we analyzed its components. By solid phase extraction (SPE) column chromatography, the extracts were divided into alcohol-washing (AW) part and water-washing (WW) part. The activity test showed that the active ingredients were mainly in the alcohol washing part. Further composition study revealed that fifteen compounds were the main components of alcohol washing (Table 1). Among the fifteen compounds, compounds **1**-**13** were from *Radix Astragali Mongolici* and belong to saponins and flavonoids, while compounds **14** and **15** were from *Radix Codonopsis* and belong to alkynyl glycosides. These compounds are obtained through commercial purchase.

3.2. Biological evaluation

First, the effect of SFI on the viability of BV-2 microglia was measured by the Cell Counting Kit-8 (CCK-8) assay, and the results showed that SFI did not alter the viability of LPS-stimulated M1 or interleukin-4 (IL-4)-stimulated M2 microglia (Figure 1). These results demonstrated that SFI did not interfere with the cell proliferation or inhibition of BV-2 microglia.

Then, the effects of SFI on LPS-induced M1 microglia were investigated. The cells were pretreated with SFI (10% v/v) for 1 h before LPS (100 ng/mL) stimulation for 4 h. Aspirin was used as the positive control for its anti-inflammatory activity. The results showed that tumor necrosis factor-α (*Tnf*) and interleukin-6 (*Il6*) expression levels (Figure 2A-B) in BV-2 microglia were significantly upregulated after LPS-stimulation. SFI significantly upregulated

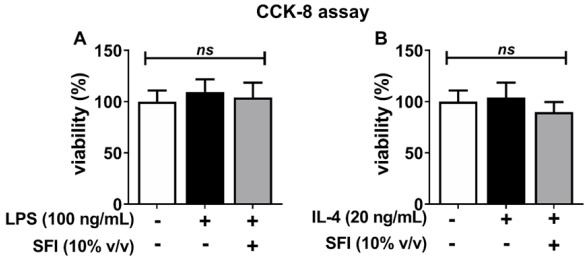


Figure 1. Effects of SFI (10% v/v) on cell viability of M1 and M2 microglia after 24 h treatment. All values were presented as mean ± SD.

Il6 expression level (Figure 2B) but did not alter *Tnf* expression at 4 h (Figure 2B). Then, to further evaluate the effect of SFI on the function of M2 microglia, we pretreated BV-2 cells with SFI (10% v/v) for 1 h, and stimulated the cells with IL-4 (20 ng/mL) for 4 h. We measured the transcription levels of *Arg1* and *Tgfb1*, and the results showed that IL-4 significantly enhanced *Arg1* ($P < 0.0001$) and *Tgfb1* ($P < 0.01$) expression in BV-2 cells (Figure 2C-D). The combination of SFI and IL-4 significantly increased the transcriptional expression of *Arg1* and *Tgfb1* in BV-2 cells compared with IL-4 alone ($P < 0.05$) (Figure 2C-D). These results indicated that the anti-neuroinflammatory and neuroprotective effects of SFI may be achieved *via* enhancing alternative activation (M2) rather than inhibiting the pro-inflammatory classic activation (M1).

To explore the material basis for the anti-neuroinflammatory compounds in SFI, we separated SFI into AW and WW components using an SPE column. The effects of both components on M2 microglia were evaluated, respectively. The results showed that the AW and WW components of SFI did not affect the cell viability of M2 microglia (Figure 3A). The AW components significantly increased the transcriptional expression of *Arg1* in BV-2 cells ($P < 0.05$), while the WW group did not affect the transcriptional expression of *Arg1* and *Tgfb1* (Figure 3B-C). These results demonstrated that for SFI, compounds with significant M2-promoting activity were in the AW component. The up-regulation of *Tgfb1* expression level was absent in the AW component (Figure 3B). It is possible that different compounds in the AW component have various regulatory effects on *Tgfb1* expression level in BV-2 cells, and the combined effects on *Tgfb1* expression level were not manifested. Therefore, the regulatory effect of the individual compound is worth further exploration.

As the AW component demonstrated M2-promoting activity, 15 compounds from the AW component were selected for further activity screening. None of the compounds showed inhibitory effects on M2 microglia cell viability at the concentration of 200 nM (Figure 4A). The 15 compounds demonstrated various capabilities in regulating transcription levels of *Arg1* and *Tgfb1*. Compounds **5** and **14** augmented the expression levels of both *Arg1* and *Tgfb1* ($P < 0.01$), while compounds **4**, **6**, and **7** posted an inhibitory effect on the expression of the M2 markers. Compounds **1**, **9**, **10**, and **14** only increased the transcription level of Arg1 ($P < 0.01$), while compounds **11**, **12**, and **15** only increased *Tgfb1* transcriptional expression ($P < 0.001$) (Figure 4B-C).

To measure the compounds' potency, compounds **1**, **5**, **12**, **14**, and **15** (Figure 5) were further examined for their regulatory effects on *Arg1* and *Tgfb1* expression level at doses of 12.5 nM, 50 nM, and 200 nM. Compounds **5** and **14** increased the expression

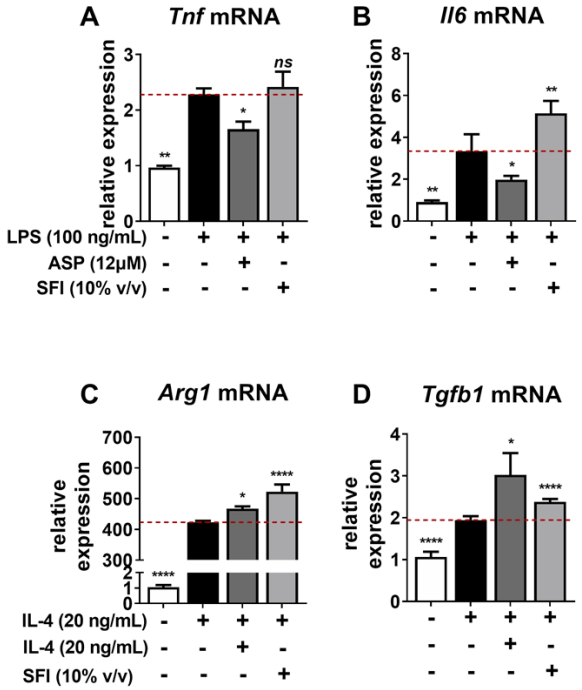


Figure 2. Effects of SFI on BV-2 microglia gene expression levels of M1- (A and B) and M2- (C and D) polarization markers. SFI showed significant promoting effects on the gene expression levels of BV-2 M2-microglia markers, arginase-1 (*Arg1*) **(C)** and transforming growth factor-β (*Tgfb1*) **(D)**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.001$ compared with IL-4 group. All values were presented as mean ± SD.

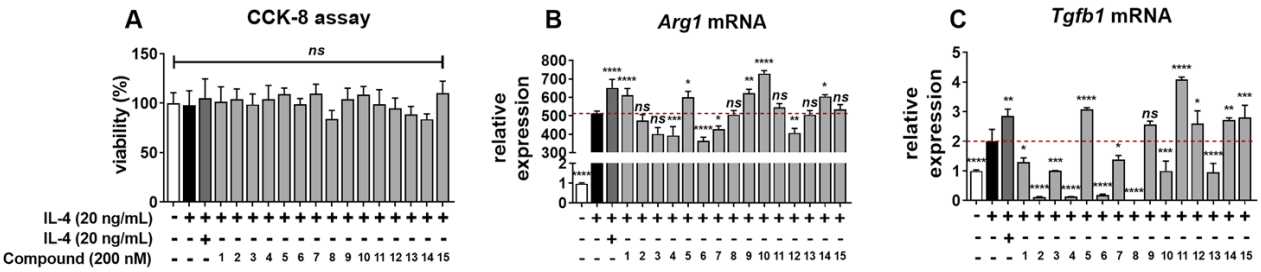


Figure 4. Effects of 15 compounds derived from AW component of SFI on BV-2 cell viability and gene expression levels of *Arg1* and *Tgfb1*. The 15 compounds did not alter cell viability at the concentration of 200 nM, and they demonstrated different regulatory effects on the expression level of *Arg1* and *Tgfb1*. $^{**}P < 0.01$, $^{****}P < 0.001$ compared with IL-4 group. All values were presented as mean ± SD.

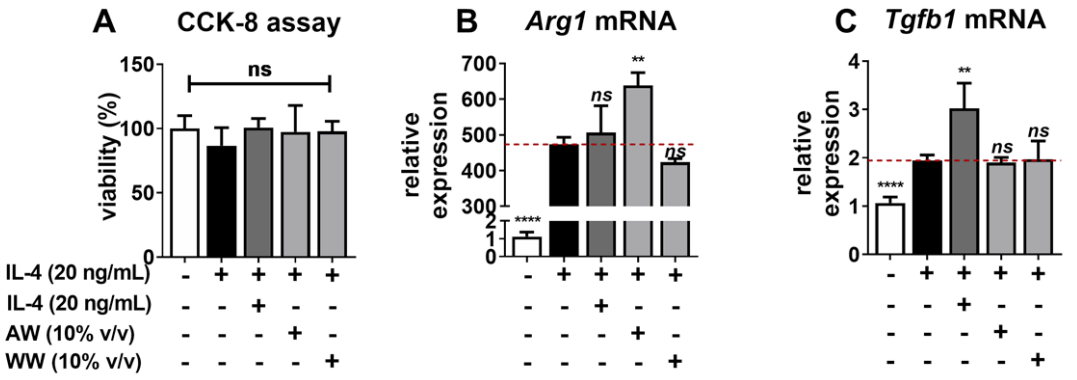


Figure 3. The regulatory effects of AW component and WW component derived from SFI. AW component of SFI significantly upregulated the gene expression level of Arg-1. $^{**}P < 0.01$, $^{****}P < 0.001$ compared with IL-4 group. All values were presented as mean ± SD.

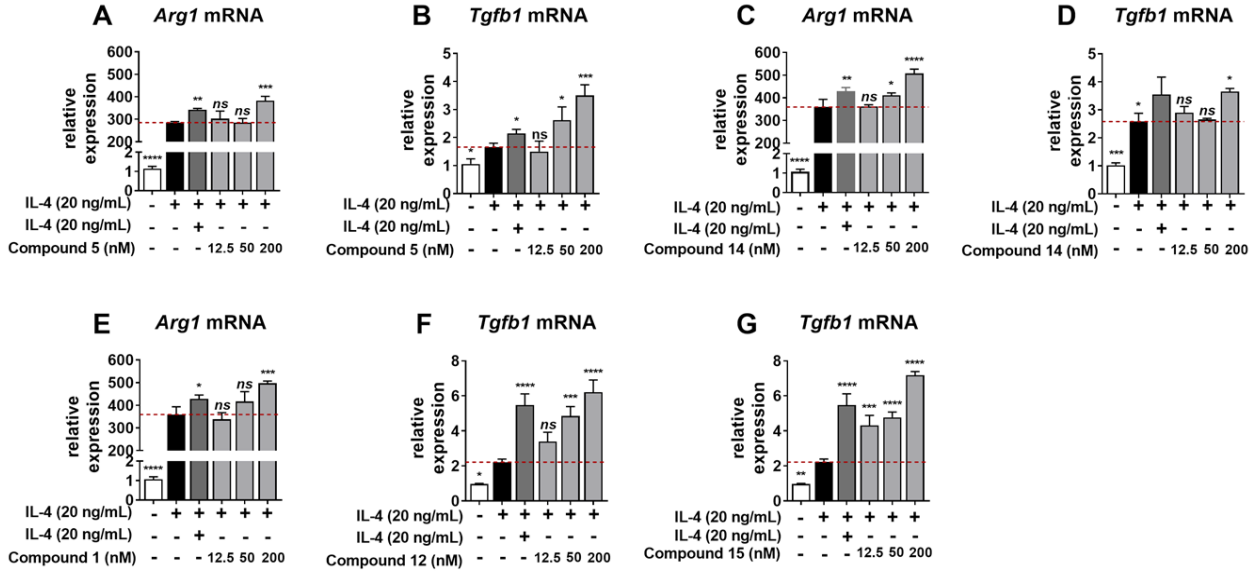


Figure 5. The regulatory effects of compounds 5 (A and B), 14 (C and D), 1 (E), 12 (F), and 15 (G) on the gene expression levels of *Arg1* (B) and *Tgfb1* (C) in IL-4-stimulated BV-2 cells. Cells were pretreated with compounds at indicated concentrations for 1 h before stimulation with 20 ng/mL IL-4 for 4 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with IL-4 group. All values were presented as mean ± SD.

levels of *Arg1* and *Tgfb1* in a dose-dependent manner in the range of 12.5 nM to 200 nM. Compounds **5** upregulated the expression of *Arg1* at the concentration of 200 nM (Figure 5A), while compound **14** showed a promoting effect on *Arg1* at a lower concentration of 50 nM (Figure 5C). The expression level of *Tgfb1* upregulated by compounds **5** and **14** at 50 nM and 200 nM (Figure 5B and D), respectively. Other compounds demonstrated limited enhancing activity on the chosen M2 markers. For example, compounds **1** only enhanced the expression of *Arg1* at 200 nM (Figure 5E), while compounds **12** and **15** upregulated *Tgfb1* at 50 nM (Figure 5F) and 12.5 nM (Figure 5G), respectively. These results indicated that different types of compounds in SFI showed good potency in regulating the expression level of *Arg1* and *Tgfb1*, but their effective concentrations were not the same.

4. Discussion

The aim of this study was to explore the bioactive constituents of SFI and their anti-neuroinflammatory effects. By using a murine microglial cell line (BV-2 cell) based on the IL-4 induced alternative activation of M2 microglia, the gene expression levels of M2-activation markers *Arg1* and *Tgfb1* were measured to indicate the anti-neuroinflammatory effects *via* the M2 microglia activation. We demonstrate that SFI augments the IL-4 mediated M2 microglia activation, characterized by upregulated gene expression of M2 markers *Arg1* and *Tgfb1*. Compounds **1**, **5**, **12**, **14**, and **15** in the AW components SFI are the major bioactive constituents that have regulatory effects on enhancing M2 microglia activation.

Our studies showed that the M2 microglia

promoting activity of SFI was found mainly in the alcohol washing components (Figure 3). It is in accordance with our findings that the characteristic compounds in Dangshen (*Radix Codonopsis*) and Huangqi (*Radix Astragali Mongolici*) (Table 1) were mainly in the alcohol washing component, and the water washing components are mainly composed of amino acids, nucleosides, and oligosaccharides (data not published), which may not be the major contributor to the microglia/macrophage alternative activation.

At the concentration of 200 nM, the regulatory effects of the 15 characteristic compounds on *Arg1* and *Tgfb1* were not identical (Figure 4). For example, Compounds **1** and **10** had promoting effects on *Arg1* but inhibitory effects on *Tgfb1*; Compounds **5** and **14** had the same up-regulatory effects on both genes as SFI (Figure 5A-D); Compounds **4**, **6**, and **7** had the opposite effect on both indexes. Studies have shown that the regulatory mechanism are quite different between *Arg1* and *Tgfb1*. As a significant anti-inflammatory cytokine, *Tgfb1* signaling plays a critical role in IL-4-induced M2 macrophage activation through co-signaling to Akt, as well as MAPK pathway, manifested by synergic upregulation with IL-4 on *Arg1* expression level in an autocrine and/or paracrine fashion (*39,40*). Another study showed that Compound **1** (astragaloside IV) were associated with promoting microglia polarization to M2 and improve tissue regeneration in peri-infarct regions in a rat cerebral ischemia/reperfusion injury model, which may be mediated by PPARγ pathway (*41*). The exact molecular mechanism of how Compound **1** and other compounds regulate microglia polarization is to be identified, but our study indicates that the two herbal ingredients Dangshen and Huangqi may contain compounds with immune-modulatory effects. Made from natural herbs, the complex constituents pose challenges in understanding the pharmacological action mechanisms of SFI. Therefore, more studies are required to determine the accurate concentration of each compound in SFI to further compare their contribution to the final efficacy. Also, compounds action mechanisms and their possible synergistic effects in regulating M2 microglial activation are worth further exploration.

In summary, the current study explores the bioactive constituents of SFI and their anti-neuroinflammatory effects. We demonstrate that SFI augments the IL-4 mediated M2 microglia activation, characterized by upregulated expression of M2 markers *Arg1* and *Tgfb1*. Compounds **1**, **5**, **12**, **14**, and **15** in the AW components SFI are the major bioactive constituents that have regulatory effects on enhancing M2 microglia activation. These results indicated that SFI might have protective and modulatory effects against neuroinflammation by polarizing microglia toward an anti-inflammatory M2 phenotype, and thus these bioactive compounds may provide beneficial effects in neuroinflammation-related CNS disorders.

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Original Article

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C-C chemokine receptor type 6 modulates the biological function of osteoblastogenesis by altering the expression levels of Osterix and OPG/RANKL

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SUMMARY Circulating inflammatory factors affect osteoblast and osteoclast formation and activity in osteoporosis. Estrogen affects the migration of Th17 cells *via* the C-C chemokine receptor type 6 (CCR6) and C-C chemokine ligand 20 (CCL20) signaling pathways to modulate bone metabolism; however, it is unclear whether and how CCR6 modulates bone homeostasis. In the present study, *CCR6* knockout (*CCR6*$^{-/-}$) mice were selected to investigate the effects of CCR6 in the regulation of homeostasis of osteoblasts and osteoclasts. Primary osteoblasts were isolated from the calvarium of newborn *CCR6*$^{-/-}$ or wild-type mice, followed by osteoblastic differentiation culture *in vitro*. *CCR6* deletion reduced osteoblast activity in terms of alkaline phosphatase (ALP) activity and inhibited osteoblast mineralization according to the results of Alizarin Red S staining, whereas it did not affect the proliferation of osteoblasts. *CCR6* deletion inhibited *Osterix* mRNA expression in osteoblasts during the late stage of mineralization *in vitro*, while it did not affect mRNA expression levels of runt-related transcription factor 2 (*Runx2*) and *Collagen-1*. The ratio of osteoprotegerin (*OPG*) / receptor activator of nuclear factor κ-B ligand (*RANKL*) mRNA level in osteoblasts was decreased by *CCR6* deficiency in the culture treated with 1,25(OH)2D3/PGE2, while there was no effect observed in the normal culture environment. The results provide novel insights, such as that *CCR6* deletion suppresses osteoblast differentiation by downregulating the expression levels of the transcription factor *Osterix*, and indirectly promotes osteoclast production by increasing transcription of RANKL. This may be one of the mechanisms *via* which *CCR6* deletion regulates bone metabolism.

Keywords osteoporosis, C-C chemokine receptor type 6, osteoprotegerin, receptor activator of nuclear factor κ-B ligand, osteoblast, osteoclast

1. Introduction

Bone metabolism balance is an important factor in maintaining the health of the body. Osteoporosis is a group of systemic skeletal diseases characterized by low bone mass, degeneration of the bone microstructure, increased bone fragility and fracture sensitivity (*1,2*). The main pathogenesis is the imbalance of bone remodeling. Bone remodeling mainly includes bone formation and bone resorption activities, both of which are initiated and modulated by a number of factors, including inflammation, hormone levels and mechanical stimulation (*3,4*). A decrease in estrogen level is the main cause of osteoporosis in postmenopausal women. Estrogen reduction affects the biological behavior of osteoblasts, osteoclasts and T cells by altering the levels of cytokines, such as TNF-α, IL-1, IL-6 and IL-17, which affects bone metabolism (*5-8*).

During the early stages of collagen-induced arthritis (CIA), estrogen treatment can increase the number of Th17 cells in lymph nodes and decrease the number of Th17 cells in joints of CIA mice (*9,10*). Studies have demonstrated that C-C chemokine receptor type 6 (CCR6) serves an important role in the differentiation of B cells driven by antigens, and can regulate the migration of dendritic cells and T cells in inflammatory and immune responses (*11-13*). In addition, estrogen can increase the expression levels of CCR6 and CCL20, which play a role as the ligand of CCR6 in Th17 cells of the lymph nodes. Furthermore, the increase in CCR6

and C-C chemokine ligand 20 (CCL20) expression in the lymph nodes impels Th17 cells to stay in the lymph nodes and hinders the migration of Th17 cells to the joints, thus reducing the recruitment of neutrophils into joints, and alleviating arthritis and erosion providing potential treatment targets (*14*). In the physiological state, osteoclasts are involved in bone resorption and form local bone resorption lacunae. Additionally, osteoclasts release cytokines and chemotaxins, recruit osteoblasts to local bone resorption lacunae, and participate in new bone formation, thus maintaining the balance of bone metabolism (*15-17*). Previous studies have revealed that global loss of CCR6 in mice markedly decreases trabecular bone mass coincident with reduced osteoblast numbers. CCL20 and CCR6 are co-expressed in osteoblast progenitors and the levels increase during osteoblast differentiation, indicating the potential for CCL20/CCR6 signaling to influence osteoblasts *via* both autocrine and paracrine pathways. CCL20/CCR6 signaling may serve an important role in regulating bone mass accrual, potentially by modulating osteoblast maturation, survival and the recruitment of osteoblast-supporting cells (*18*). Further studies investigating the role of CCR6 in the pathogenesis of bone metabolism-related diseases could provide novel ideas and methods for the treatment of osteoporosis.

2. Materials and Methods

2.1. Mice

CCR6$^{-/-}$ and wild-type (WT) mice (C57BL/6 mice), aged 10-12 weeks and weighing 20-30 g, were purchased from Jackson Laboratory. Mice were raised in carbonate plastic cages in the animal room (clean grade) of the Institute of Gynecology and Obstetrics of the Hospital Affiliated to Fudan University, Shanghai, China. The genotype of the *CCR6*$^{-/-}$ mice was identified according to the standard protocol provided by Jackson Laboratory, and the identified primer sequences are listed in Table 1. The experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Fudan ethics committee. Throughout the study period, the mice were housed in a temperature-controlled (23 ± 0.5˚C) and humidity-controlled (43 ± 8%) environment with a 12-h light-dark cycle and *ad libitum* access to food and water.

2.2. Genomic DNA isolation and genotyping

Genotypes of *CCR6*$^{-/-}$ mice were confirmed by PCR analyses of genomic DNA. Generally, tissue samples were collected and used for further PCR analyses with 2X GC rich PCR MasterMix (Tiangen Biotech Co., Ltd.). The PCR reaction was performed according to the protocol provided by Jackson Laboratory: 94˚C for 3 min, followed by 10 cycles of 94˚C for 30 sec, 65˚C for 15 sec and 68˚C for 10 sec, followed by another 28 cycles of 94˚C for 15 sec, 60˚C for 15 sec and 72˚C for 10 sec, and finally an additional step of 72˚C for 2 min prior to the end of the program.

Table 1. Summary of oligonucleotide primers for *CCR6*$^{-/-}$ mice genotyping

Oligonucleotide	Sequence* (5'-3')
Common forward primer	AAAACCCAAGTGTTGGTGGCATGAG
Wild-type reverse primer	CCCTAGAAGAGGTCAGAAACTTCAC
Mutant reverse primer	GGGTGGGATTAGATAAATGCCTGCTCT

*The oligonucleotide sequences for genotyping were obtained from the Jackson Laboratory website: *https://www.jax.org/search?q=+005793*

2.3. Primary osteoblast isolation and induced differentiation culture

Osteoblasts were collected from the calvarium of newborn mice after 2 days as previously described (*19*). Skull bones were extracted and digested (five times; 10 min each time) in α-minimum essential medium (α-MEM) containing 0.1% collagenase and 0.2% dispase. The supernatant from the first 10-min digestion was discarded. Cells obtained from the remainder of the digestions were pooled, and 5×10^5 cells were seeded onto 10% FBS (Gibco) and phenol red-free α-MEM supplemented with 10 U/mL penicillin and 10 μg/mL streptomycin in 6-well culture plates until they reached 80% confluence. The osteogenic differentiation medium consisted of 10% serum and phenol red-free α-MEM, 20 mM ascorbic acid, 1 M β-glycerophosphate disodium salt hydrate and 1 mM dexamethasone.

2.4. Cell culture

MC3T3-E1 cells were acquired commercially from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA). Cell growth medium (GM) was supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% antibiotic (Gibco, Rockville, MD, USA) in a humidified atmosphere of 5% CO_2 at 37˚C. After culturing MC3T3-E1 cells until sub-confluence, osteogenic differentiation was ensured by changing medium to osteogenic differentiation medium.

2.5. Cell transfection

The *CCR6*-specific small interfering RNA (siRNA) and negative control (si-NC) were purchased from

GenePharma (Shanghai, China). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, CA, USA). Cell transfection was performed independently at least three times.

2.6. Western blot assay

Cell protein samples were isolated, quantified and diluted in loading buffer to the same concentration. After denaturation, proteins were separated by electrophoresis on 10% sodium dodecyl sulphate (SDS) polyacrylamide gels, followed by transfer to Hybond membrane. Membranes were blocked in 5% skimmed milk at room temperature for 2 h and exposed to primary antibodies against Actin (ab8226, Abcam, USA), CCR6 (PA1-21615, Invitrogen, USA), runt-related transcription factor 2 (Runx2) (ab92336, Abcam,USA), Osterix (ab209484, Abcam, USA), Collagen-1(ab270993, Abcam, USA) overnight at 4°C. Following rinsing three times in Tris-buffered saline (TBST), membranes were incubated with secondary antibodies for 2 h. Proteins in the membranes were visualized using a chemiluminescence (ECL) system (Amersham Biosciences, USA). Actin (ab197345, Abcam, USA) was used as an internal reference. The experiment was independently repeated 3 times.

2.7. Alkaline phosphatase (ALP) staining and quantitative detection of ALP activity

For ALP detection, 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) was the preferred staining substrate. After 7 days of osteogenic induction medium treatment in 24-well plates, cells were washed with 500 μL PBS and fixed with 4% paraformaldehyde for 20 min at room temperature, followed by three washes with 500 μL PBS. The fixed cells were incubated in BCIP/NBT buffer, which was prepared according to the kit's protocol (3 ml ALP staining buffer, 10 μL 300X BCIP buffer and 20 μL 150X NBT buffer) for > 30 min at room temperature avoiding light until ALP-positive differentiated osteoblasts appeared blue-violet. The reaction was stopped by adding excess deionized water. The results were visualized using an HP scanner and recorded. A total of 5×10^5 primary osteoblasts were seeded into 6-well culture plates and cultured until they reached 90% confluence. The cells were digested with pancreatin and collected into a 1.5 mL Eppendorf tube. ALP activity in cell lysate was quantitated by using an Alkaline Phosphatase Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol.

2.8. Alizarin Red S staining

The primary osteoblasts were isolated and the cell density was adjusted to 1×10^5 cells/mL. After 21 days of osteogenic induction medium treatment in 24-well plates, Alizarin Red S stain was added to each well and the plate was incubated in the dark for 10 min at room temperature. The staining buffer was removed carefully when mineralized osteoblasts appeared bright orange-red and undifferentiated cells were slightly red or colorless.

2.9. RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

For PCR analysis, total RNA was isolated using an RNA extraction kit (Axygen; Corning Inc.) according to the manufacturer's protocol. The concentration of total RNA was measured using a NanoDrop 2000c (Thermo Fisher Scientific, Inc.). RNA (1 μg) was reverse transcribed into cDNA using a reverse transcriptase kit (Promega Corporation). qPCR was performed using SYBR Premix Ex Taq (Takara Bio, Inc.). The cDNA levels were quantified using the housekeeping gene GAPDH. Gene expression was normalized to the level of housekeeping gene GAPDH and analyzed using the standard $2^{-\Delta\Delta CT}$ method. Primer sequences are listed in Table 2.

2.10. Cell viability assay

Primary osteoblasts were isolated according to the method "*Primary osteoblast isolation and induced differentiation culture*", and the cell density was adjusted to 2×10^5 cells/mL. Subsequently, 100 μL medium containing 2,000 cells was added to each well of a 96-well cell culture plate. After 24 h, when the cells had adhered completely, the medium was exchanged with medium containing 25 μg/mL vitamin C, 10 mM

Table 2. The primers used in the study

Gene	Forward Primer	Reverse Primer
Osterix	GCTCGTAGATTTCTATCCTC	CTTAGTGACTGCCTAACAGA
Collagen-1	TGACTGGAAGAGCGGAGAGTA	GACGGCTGAGTAGGGAACAC
Runx2	GACAGTCCCAACTTCCTGTG	GCGGAGTAGTTCTCATCATTC
OPG	CCTTGCCCTGACCACTCTTAT	CGCCCTTCCTCACACTCAC
RANKL	CAAGATGGCTTCTATTACCTGT	TTGATGCTGGTTTTAACGAC
GAPDH	GTTGTCTCCTGCGACTTCA	GGTGGTCCAGGGTTTCTTA

Runx2: *runt-related transcription factor 2*; *OPG*: *osteoprotegerin*; *RANKL*: nuclear factor-κ B ligand.

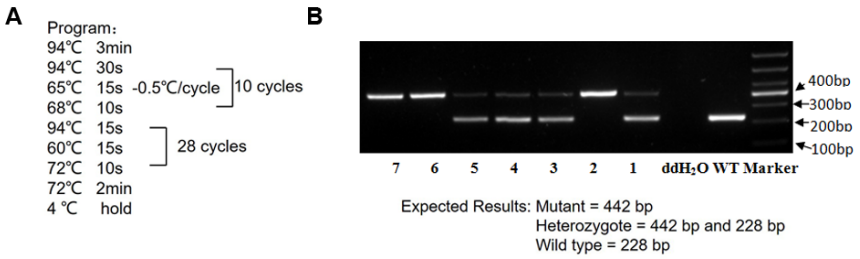

Figure 1. Gene identification of *CCR6*$^{-/-}$ mice. (A) Master protocol details provided by Jackson Laboratory. **(B)** Gel image of PCR products and the expected results of mice. Mutant, 442 bp; heterozygote, 442 and 228 bp; wild-type, 228 bp. *CCR6*, *C-C chemokine receptor type 6*.

β-glycerol phosphate and 100 nM dexamethasone (differentiation medium). Each well contained 100 μL medium, and six replicate wells were analyzed for each group. After 12, 24, 48 and 96 h, 10 μL MTT solution (5 mg/mL; 0.5% MTT) was added to each well, followed by incubation in a cell incubator for 4 h. Subsequently, 100 μL formazan solution was added to each well, followed by incubation in a cell culture box until the formazan was completely dissolved as observed under an ordinary optical microscope. The absorbance of each well was measured at a wavelength of 570 nm using an ELISA plate reader. The activity of OB after 12 h in WT mice was calculated and expressed as a percentage of that in the control group.

2.11. Treated primary osteoblasts with 1,25(OH)2D3/PGE2 *in vitro*

The primary osteoblasts were isolated according to the method "*Primary osteoblast isolation and induced differentiation culture*" and the cell density was adjusted to 1×10^5 cells/mL. A 24-well plate was used to inoculate 500 μL/well or a 6-well plate was used to inoculate 2 ml/well. The cells were cultured in an incubator at 37˚C with 5% CO_2. After 48 h, the medium containing 1×10^{-8} M 1,25-dihydroxyvitamin D3 and 1×10^{-6} M prostacyclin E2 (co-culture medium) was used to replace the medium, which simulated a microenvironment of co-culture of osteoblasts and osteoclasts (*20*). Subsequently, medium containing 1,25(OH)2D3/PGE2 was changed once every 48 h.

2.12. Statistical analysis

All data are presented as the mean ± SEM. Differences were assessed by Student's *t*-test using SPSS software (IBM Corp.). All experiments were repeated more than three times. $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Identification of *CCR6*$^{-/-}$ mice

The transgene identification of *CCR6*$^{-/-}$ mice was confirmed according to the standard protocol provided by Jackson Laboratory (stock number, 005793; strain name, B6.129P2-Ccr6tm1Dgen/J). The master protocol details are presented in Figure 1A. The expected product size of mutant (*CCR6*$^{-/-}$) was 442 bp, the sizes of heterozygote (*CCR6*$^{+/-}$) were 228 and 442 bp, and the size of WT was 228 bp (Figure 1B).

3.2. Deletion of *CCR6* inhibits the differentiation of osteoblasts *in vitro*

The mRNA expression of *CCR6* in primary osteoblasts from *CCR6*$^{-/-}$ mice was decreased obviously compared to WT controls (Figure 2A). The present study first examined the effect of *CCR6* deficiency on ALP activity of differentiated primary osteoblasts 7 days after osteogenic induction medium treatment. ALP staining revealed reduced osteoblastic differentiation in *CCR6*$^{-/-}$ mice compared with WT controls (Figure 2B). Additionally, ALP activity in the cell lysate was markedly decreased in *CCR6*$^{-/-}$ osteoblasts compared with WT controls (Figure 2C).

3.3. *CCR6* deficiency inhibits mineralization of differentiated primary osteoblasts while it has no effect on cell viability

Osteoblasts of WT and *CCR6*$^{-/-}$ mice were treated with induction differentiation medium for 21 days, and the number of calcium nodules was compared using Alizarin Red staining. Calcium nodules appeared orange-red following Alizarin Red staining. The results of Alizarin Red staining demonstrated that the number of osteoblastic calcium nodules in *CCR6*$^{-/-}$ osteoblasts cultured *in vitro* was lower than that in osteoblasts from WT mice (Figure 3A). Quantitative comparison of the number of calcium nodules per pore revealed that the number of osteoblastic calcium nodules cultured in *CCR6*$^{-/-}$ osteoblasts was markedly lower than that in WT osteoblasts (Figure 3B). Therefore, the present study suggests that *CCR6* deletion may weaken osteoblast activity and inhibit osteoblast mineralization *in vitro*. Furthermore, the proliferation activity of primary osteoblasts from WT and *CCR6*$^{-/-}$ mice was assessed. The results demonstrated that there was no significant

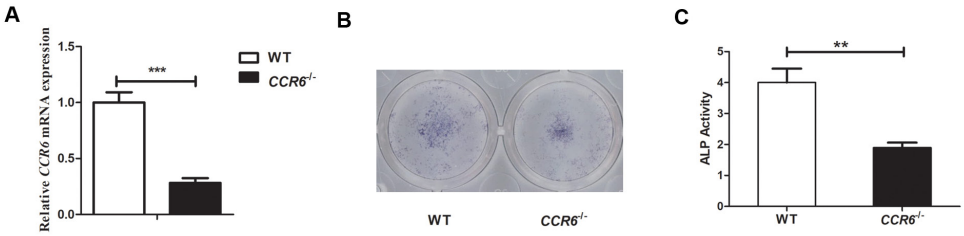

Figure 2. *CCR6* deficiency inhibits ALP activity in differentiated primary osteoblasts. (A) The mRNA expression of *CCR6* was detected in primary osteoblasts of WT and *CCR6*$^{-/-}$ mice. **(B)** Primary osteoblasts from *CCR6*$^{-/-}$ or WT mice were treated with osteogenic induction medium for 7 days, and ALP levels and activity were examined. ALP staining revealed reduced osteoblastic differentiation in *CCR6*$^{-/-}$ mice compared with WT controls. **(C)** ALP activity was decreased in *CCR6*$^{-/-}$ osteoblasts compared with WT controls. $^{*}p < 0.05$. All data are presented as the mean ± SEM and representative of at least three experiments.

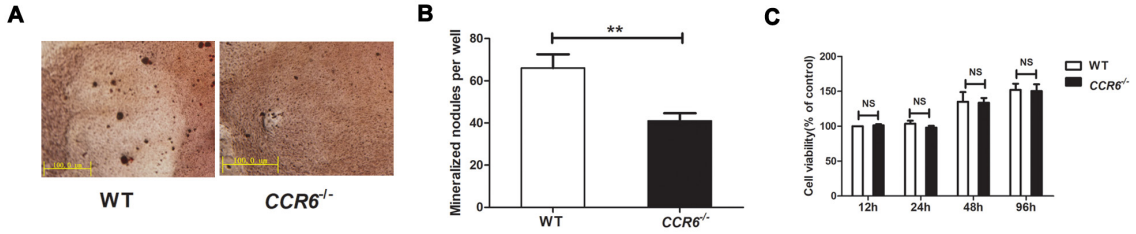

Figure 3. Mineralization of differentiated primary osteoblasts is inhibited in *CCR6*$^{-/-}$ mice. Following Alizarin Red S staining of primary osteoblasts isolated from *CCR6*$^{-/-}$ or WT mice, the number of calcium nodules was compared. **(A)** The numbers of osteoblastic calcium nodules in *CCR6*$^{-/-}$ osteoblasts were markedly lower than those in osteoblasts from WT mice *in vitro*. **(B)** The number of mineralized nodules per well was calculated for *CCR6*$^{-/-}$ and WT control osteoblasts. **(C)** *CCR6* deficiency did not affect the viability activity of primary osteoblasts. $^{*}p < 0.05$. All data are presented as the mean ± SEM and representative of at least three experiments.

difference in cell proliferation activity between WT and *CCR6*$^{-/-}$ osteoblasts at 12, 24, 48 and 96 h (Figure 3C). This indicated that *CCR6* deletion had no effect on the proliferation of osteoblasts in mice.

3.4. *CCR6* deficiency decreases *Osterix* expression in differentiated primary osteoblasts

Runx2 and Osterix are important transcription factors in osteoblast growth and differentiation. Runx2 is expressed in the early stage of osteoblast differentiation, whereas Osterix is only expressed in the late stage of osteoblast differentiation. The primary osteoblasts of WT and *CCR6*$^{-/-}$ mice were induced to differentiate and cultured *in vitro*. RT-qPCR was used to analyze the expression levels of *Runx2* and *Osterix* in the two groups. The results revealed that with the prolongation of cell culture time *in vitro* there was no significant difference in the mRNA expression levels of *Runx2* (Figure 4A). The mRNA expression levels of *Osterix* in *CCR6*$^{-/-}$ osteoblasts were markedly lower than those in WT osteoblasts after 21 days of culture (Figure 4B). Additionally, the present study analyzed the mRNA expression levels of the functional factor *Collagen-1* during osteoblastic differentiation *in vitro*. There was no significant difference in the mRNA expression levels of collagen-1 between *CCR6*$^{-/-}$ and WT osteoblasts (Figure 4C). We also transfected MC3T3-E1 cells with siRNA-*CCR6* to detect the expression of related proteins. The MC3T3-E1 cells were induced to differentiate, after 21 days of differentiation culture, we transfected the MC3T3-E1 with siRNA-*CCR6* and si-NC, then detected the protein expression of Runx2, Osterix and Collagen-1 in cells. There was no significant difference in the expression levels of Runx2 and Collagen-1 between si-NC and si-*CCR6* groups, while the expression level of Osterix in si-*CCR6* group was markedly lower than those in NC group (Figure 4D).

3.5. Osteoprotegerin (*OPG*)/receptor activator of nuclear factor κ-B ligand (RANKL) levels decreased in *CCR6*$^{-/-}$ osteoblasts treated with 1,25(OH)2D3/PGE2

The OPG/RANKL/receptor activator of nuclear factor κB (RANK) system is an important signaling pathway in osteoclast differentiation. Osteoblasts can regulate osteoclastogenesis by expressing OPG and RANKL. The primary osteoblasts of WT and *CCR6*$^{-/-}$ mice were divided into a common culture group and a treated group. The common culture group was cultured with common culture medium, whereas treated group was supplemented with 1,25(OH)2D3/PGE2 to common culture medium, which was used to simulate the co-culture environment. Total RNA was collected after 0, 1 and 2 days of culture, and the mRNA expression levels of OPG and RANKL in osteoblasts were analyzed by RT-qPCR. The results demonstrated that the mRNA expression levels of OPG in *CCR6*$^{-/-}$ osteoblasts were

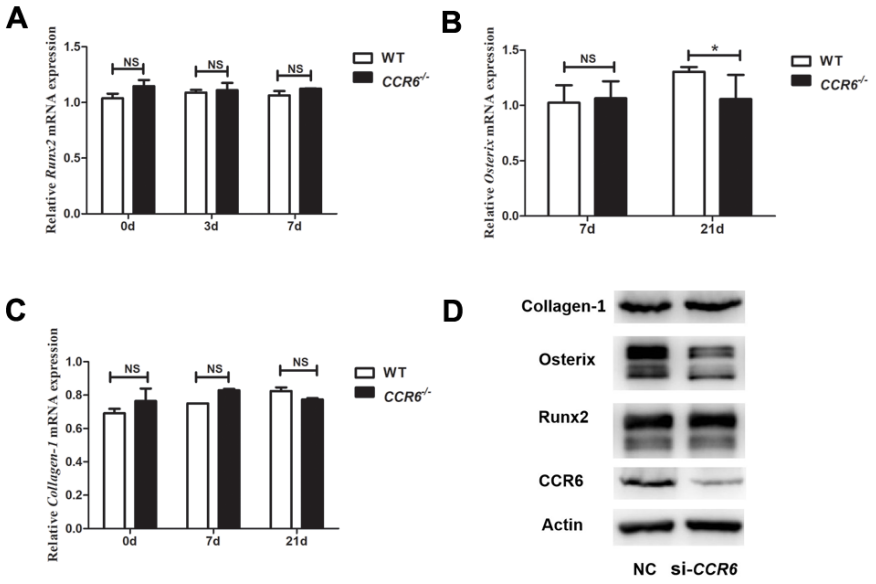

Figure 4. CCR6 affects mRNA expression levels of osteoblastogenesis-related genes. Primary osteoblasts from *CCR6*$^{-/-}$ or WT mice were treated with osteogenic induction medium for 0, 3, 7 and 21 days. The mRNA expression levels of **(A)** *Runx2*, **(B)** *Osterix* and **(C)** *Collagen-1* were detected. **(D)** The protein expression levels of Runx2, Osterix and Collagen-1 were detected by transfection with si-NC or si-*CCR6* in MC3T3-E1 cells. $^{*}p < 0.05$. All data are presented as the mean ± SEM and representative of at least three experiments.

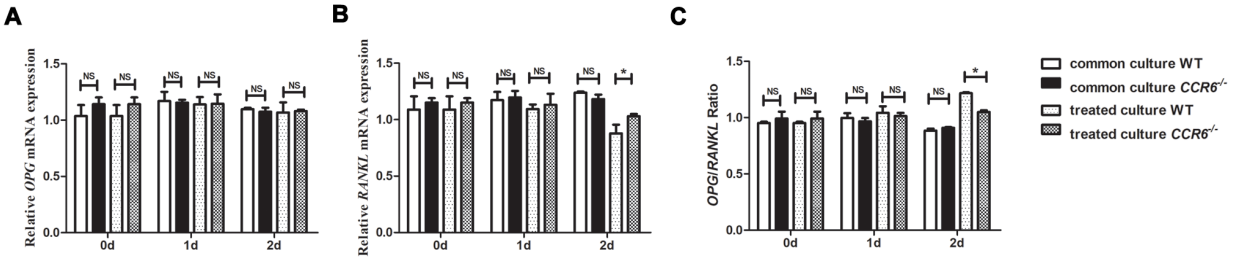

Figure 5. *CCR6* deficiency decreases the *OPG/RANKL* level in osteoblasts treated with 1,25(OH)2D3/PGE2. Primary osteoblasts from *CCR6*$^{-/-}$ or WT mice were cultured in growth medium or treated medium. **(A)** *CCR6* deficiency did not affect the mRNA expression levels of *OPG* in osteoblasts compared with WT controls in both the normal culture environment and the treated group. **(B)** *CCR6* deletion increased the mRNA expression levels of *RANKL* in osteoblasts compared with WT controls in the treated group. **(C)** The *OPG/RANKL* ratio was decreased in osteoblasts with *CCR6* deletion compared with WT control in the treated group, while no change was observed in the normal culture environment. $^{*}p < 0.05$. All data are presented as the mean ± SEM and representative of at least three experiments.

not significantly different from those in WT osteoblasts in both the normal culture environment and the treated environment (Figure 5A). Furthermore, *CCR6* deletion did not affect the mRNA expression levels of RANKL in osteoblasts in the normal culture environment, whereas it increased the mRNA expression levels of RANKL in osteoblasts in the treated group (Figure 5B). The ratio of OPG/RANKL was decreased in osteoblasts with CCR6 deletion compared with WT control cells in the treated group (Figure 5C).

4. Discussion

Previous studies have demonstrated that osteoblasts and osteoclasts can secrete CCR6 and CCL20, and the CCL20/CCR6 signaling pathway is closely associated with bone metabolism (*18*). When the overall level of CCR6 in mice decreases, trabecular bone mass decreases, which is consistent with the reduced number of osteoblasts. CCR6 and CCL20 are co-expressed in osteoblast progenitor cells. The expression levels of CCR6 and CCL20 are increased during the differentiation of osteoblasts, suggesting that the CCL20/CCR6 signaling pathway affects osteoblasts *via* autocrine and paracrine pathways (*21,22*). Whether CCR6 is involved in osteoblastogenesis and what role it serves remains to be elucidated. Therefore, the present study proposed the hypothesis that the decrease in CCR6 expression in osteoblasts suppresses osteoblast differentiation and regulates bone metabolism, which leads to osteoporosis.

The main cause of osteoporosis is the imbalance of bone formation induced by osteoblasts and bone resorption induced by osteoclasts. Osteoblasts are derived from mesenchymal stem cells (MSCs) and have the potential to differentiate into a variety of cells, such as chondrocytes, myoblasts or adipocytes. Osteoblast differentiation is divided into three stages:

cell proliferation, extracellular matrix formation and maturation, and mineralization. Each stage has a characteristic gene expression profile (*23-25*). Under suitable culture conditions, osteoblasts can secrete certain unique extracellular matrix proteins, including osteocalcin (OCN), ALP and a large amount of *Collagen-1* (*26,27*). The extracellular matrix does not mineralize at the beginning of deposition and is rich in *Collagen-1* (*28*). With the accumulation of calcium phosphate in the form of hydroxyapatite, the matrix mineralizes to form hard but light-weight sediments (both organic and inorganic), which are the main components of bone tissue (*29,30*). These osteoid calcium nodule deposits represent the end products of proliferation and differentiation of osteoblasts (*31*). In the present study, we isolated pre-osteoblasts from the calvarium of newborn *CCR6*$^{-/-}$ mice and detected ALP activity, calcium deposit formation, and osteoblastogenesis related factor expression 0, 3, and 7 days after differentiation. The activity of ALP decreased in *CCR6*$^{-/-}$ osteoblasts 7 days after differentiation compared to wild-type controls, combined with impaired calcium deposit formation that indicated inhibited mineralization of differentiated osteoblasts.

During different stages of osteoblast growth and development, sequential expression of different osteoblast-related genes has different effects on differentiation. Runx2 and Osterix are important transcription factors in osteoblast growth and differentiation. Runx2 is expressed in the early stage of differentiation, whereas Osterix is only expressed in the late stage of differentiation (*32-34*). Runx2 is essential for osteoblast differentiation in the process of chondrogenesis and intramembrane osteogenesis. Runx2 can directly stimulate the transcription of OCN, Collagen-1, osteopontin, collagenase 3 and suppression of Tumorigenicity 2 during the differentiation of bone marrow MSCs (BMSCs) into osteoblasts (*35-37*). Osterix is a downstream transcription factor of Runx2 in osteoblasts and is required for osteoblast differentiation (*38*). If Runx2 and Osterix expression is inhibited, this will affect the growth and differentiation of osteoblasts, which will lead to a differentiation disorder. Therefore, the present study isolated RNA from osteoblasts, which had been treated in different stages of differentiation and analyzed the expression levels of osteoblast differentiation-related genes using RT-qPCR. The results demonstrated that *CCR6* deletion did not affect the transcription levels of *Collagen-1* and *Runx2* in osteoblasts, whereas the transcription levels of *Osterix* were markedly lower in *CCR6*$^{-/-}$ osteoblasts than in WT osteoblasts, indicating that *CCR6* deletion inhibited *Osterix* expression in the late stage of mineralization of osteoblasts *in vitro*.

CCR6 deletion can weaken the activity of osteoblasts and inhibit the mineralization of osteoblasts *in vitro*. The present study assessed the osteoblasts of the two groups using an MTT assay, and the effect of CCR6 deletion on osteoblast proliferation was observed. The results revealed that there was no significant difference in the proliferation activity between the two groups at the four time points (12, 24, 48 and 96 h). The results demonstrated that CCR6 deletion did not affect the proliferation of osteoblasts. Therefore, it was concluded that CCR6 deletion may directly affect the differentiation of osteoblasts but not proliferation.

Postmenopausal estrogen deficiency is associated with increased bone resorption and increased production of pro-inflammatory factors, such as RANKL. A mature osteoclast is a multinucleated giant cell, which is induced and differentiated by bone marrow hematopoietic stem cells stimulated by macrophage colony-stimulating factor and RANKL. RANKL serves an important role in osteoclast generation. OPG, also known as osteoclast inhibitory factor, is a growth factor receptor belonging to the tumor necrosis factor receptor family (*39,40*). RANKL expression in osteoblasts and BMSCs can promote the differentiation and activation of osteoclasts and inhibits the apoptosis of osteoclasts. In addition, osteoblasts and BMSCs secrete and express OPG, which competitively binds with RANKL, preventing binding between RANKL and RANK (*41,42*). The OPG/RANKL/RANK system is an important signaling pathway in osteoclast differentiation. Numerous hormones and immune factors affect bone metabolism *in vivo* by affecting the expression levels of OPG or RANKL (*43,44*). The present results demonstrated that *CCR6* deletion did not affect the transcription levels of OPG in osteoblasts in the simulated co-culture environment or in the common culture environment, but increased the transcription of *RANKL* in mice osteoblasts in the treated culture environment only. Therefore, it was speculated that the deletion of the *CCR6* could indirectly promote osteoclast formation by increasing the transcription levels of *RANKL*, while the bone mass and bone microarchitecture of *CCR6*$^{-/-}$ and wild-type mice *in vivo* should be analyzed in further studies.

In conclusion, *CCR6* deletion weakened osteoblast activity and inhibited osteoblast mineralization *in vitro*, whereas it did not affect the proliferation of osteoblasts. This suggests that *CCR6* deletion may directly inhibit osteoblast differentiation by downregulating the expression levels of *Osterix*, a key transcription factor in osteoblast differentiation, and indirectly promote osteoclast production by increasing the transcription levels of *RANKL* in osteoblasts without affecting the transcription levels of *OPG*. However, the transcription levels of *Collagen-1* and *Runx2* were not significantly altered in *CCR6*$^{-/-}$ osteoblasts. Therefore, it was speculated that *CCR6* deletion can alter the biological function of osteoblasts in osteogenesis in mice. The present study provides novel evidence to explain the mechanisms *via* which *CCR6* deletion regulates bone metabolism.

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Original Article

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Replication Factor C4 in human hepatocellular carcinoma: A potent prognostic factor associated with cell proliferation

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SUMMARY Replication Factor c4 (RFC4) has been found to play important roles in many carcinomas and is correlated with poor prognosis. The present study was performed to investigate the specific role of RFC4 in hepatocellular carcinoma (HCC) and the underlying molecular mechanism. Public datasets including TCGA and GTEx were applied to explore the expression of RFC4 in HCC and its association with HCC prognosis. The results of bioinformatics analysis showed that RFC4 was overexpressed in HCC tissues compared with noncancerous tissues and significantly correlated with poor prognosis for HCC. Through immunohistochemistry, the association between RFC4 expression and clinical-pathological features of HCC patients was evaluated. Western blots were applied to investigate relative protein expression. Then *in vivo* and *in vitro* experiments were performed to explore the function of RFC4 in HCC tumor cells. The present results suggest that high level expression of RFC4 is associated with tumor size. In addition, RFC4 knockdown suppressed the cell proliferation and sphere formation of hepatoma cells *in vitro*. Moreover, silencing of RFC4 significantly decreased the growth of tumors in a xenograft tumor model. In conclusion, our study indicates that RFC4 is a potential prognostic predictor associated with poor outcomes for HCC patients. Furthermore, knocking down RFC4 could significantly inhibit tumor progression both *in vitro* and *in vivo*. Therefore, the present study can shed new light on the understanding of molecular mechanisms of HCC and may provide molecular targets and diagnostic biomarkers for the treatment of HCC.

Keywords RFC4, hepatocellular carcinoma, prognostic marker

1. Introduction

As the eukaryotic clamp loader, replication factor C (RFC) loads the DNA polymerase δ processivity factor proliferating cell nuclear antigen (PCNA) at primer-template junctions (*1-4*). RFC is a heteropentameric complex consisting of one large subunit (Rfc1) and four small subunits (Rfc2, -3, -4, and -5), all of which are essential for viability in yeast (*5-7*). Consistent with their role in DNA replication, mutant strains of the subunits display defects in the DNA replication checkpoint pathway (*7-9*). By interacting with RPA1, RFC4 is required for both DNA replication and DNA damage checkpoints in Saccharomyces cerevisiae (*7*). Therefore, the deregulation of the RFC4 can contribute to cell proliferation and tumorigenesis and its aberrant expression may indicate it is a promising prognostic marker in serval malignancies (*10-14*). However, the role of RFC4 in cancer initiation and progression and its correlation with HCC remains unclear.

In this study, we systematically explored the roles of RFC4 in HCC. The correlation between RFC4 expression and HCC prognosis was investigated. Furthermore, the function of RFC4 in tumor proliferation was also explored. Consequently, our findings in the study may provide further understanding of HCC development and lead to an improved diagnosis.

2. Materials and methods

2.1. Bioinformatics analysis

Data from GTEx database and TCGA database were applied for differential genetic analysis. Differential gene expression and survival analysis were measured using the GEPIA website (*http://gepia.cancer-pku.cn*). One-way ANOVA was applied for gene expression analysis between cancer and non-cancerous liver tissues. The disease-free survival time and overall survival time were obtained from the TCGA public

database. The results are shown below.

2.2. Patients and samples

Approved by our institutional research ethics committee, 142 patients with hepatocellular carcinoma who have been proved by pathology were incorporated into the study. Fresh samples were collected just after surgery and fixed in 10% formalin before embedding in paraffin wax. Patients' clinical and pathological data including age, gender, number of tumor nodes, tumor sizes and AFP level were identified. The histopathology of each specimen was reviewed by board-certified pathologists in our institution. Written informed consent was obtained from patients before the study. We have also complied with the World Medical Association Declaration of Helsinki involving the ethical conduct of research involving human subjects.

2.3. Immunohistochemistry

The paraffin-embedded tissues were sliced into 4um sections and baked at 75°C for 45 minutes. The sections were de-waxed in xylene and rehydrated in graded ethanol. Then, EDTA (PH = 8.0) and 3% H_2O_2 in methanol treated the slices for 10 minutes. The tissue sections were cultured with anti-RFC4 antibodies (rabbit, ab156780, Abcam) at a 1:250 dilution overnight at 4°C. Then second antibody was added and incubated at room temperature for 30 minutes. After DAB staining, the sections were counterstained using haematoxylin, dehydrated, and cleared with xylene. For the results analysis, a semi-quantitative H-score was computed for each sample by multiplying the staining intensities (0: negative, 1: weak staining, 2: moderate staining, 3: strong staining) and distribution areas (0-100%). All samples were classified as high expression and low expression group respectively according to the distribution of H-score.

2.4. Cell culture and transfection

Both human hepatocellular carcinoma cell lines (Hep3B and SNU-475) used in this study were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SNU-475 was cultured in RPMI-1640 medium while Hep3B was cultured in Dulbecco's modified Eagle's medium (DMEM), both contained 1% penicillin streptomycin and 10% fetal bovine serum. Cells were maintained at 37°C with 5% CO_2. Both cell lines were maintained in our institution. For *in vitro* study, both cell lines were transfected with shRNA to silence RFC4 according to the manufacture instructions. Short hairpin RNA (Target sequence: AAGGATCGAGGAGTAGCTGCCAG and GGACCACCTGGAACTGGAAAAAC) was obtained from the National Core Facility for Manipulation of Gene Function by RNAi, miRNA, miRNA sponges, and CRISPR/Genomic Research Center, Academia Sinica, Taipei, Taiwan. The stable cell lines were verified by RT-qPCR and Western blot before proceeding to the next experiment.

2.5. RT-PCR

All RNA extractions were performed from cells by using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific) according to protocols. After measuring content by ultraviolet analysis, RNA was used to synthesize cDNA for qPCR analysis. Quantitative PCR was performed on a Smart Cycler using SGExcel FastSYBR Mixture (With Low ROX) Plus (Sango Biotech, China). To further analyze the real-time PCR data, we applied a comparative threshold cycle (Ct) method, which compares differences in CT values between target RNA and common control (*15*). Forward and reverse primers are shown below (RFC4, F, 5′-GCGGAAACCTGAGGAACGAGCC-3 and R, 5′-TGGCAGCTACTCCTCGATCCTTG-3'; GAPDH, F, 5′-GAGTCAACGGATTTGGTCGT-3′ and R, 5′-TTGATTTTGGAGGGATCTCG-3′).

2.6. Western blot

Cell samples were lysed in RIPA lysis buffer, and protein concentrations were determined using the bicinchoninic acid (BCA) method. Then, an equal amount of denatured protein sample was loaded per lane, separated in SDS-PAGE gels and transferred to PVDF membranes. After blocking with 5% dry milk for 1h at room temperature, primary antibodies including RFC4 (1:1,500 dilution, ab156780, Abcam), mouse anti-β-actin (1:1,000 dilution, ab8226, Abcam plc, Cambridge, UK) were used. After incubation and washing, membranes were further incubated with secondary antibodies (polyclonal goat anti-rabbit/mouse, 1:10,000 dilutions, Rockland Immunochemicals Inc, PA) for 30 min at 37°C and detected by chemiluminescence. Protein bands were visualized by applying the SuperSignal™ West Femto Chemiluminescent Substrate (Pierce Biotechnology, USA).

2.7. Colony formation array

Hep3B and SNU-475 cells were seeded and cultured on 60 mm^2 plates at an initial density of 800/well, each group was measured in 3 parallel wells. After 2 weeks, cells were washed and then fixed with 10% formaldehyde for 15 min at room temperature. Cells were then stained with Giemsa for 15 min. Colony numbers were counted using an optical microscope.

2.8. MTT assay

Both Hep3B and SNU-475 cells were seeded and

cultured on 96-well plates at a density of 4,000 cells/well. The cell's proliferation capacity was measured using MTT (methyl thiazolyl tetrazolium) assay. 0.02 mL of 5 mg/mL MTT reagent were added into each well for 24 hours at 37°C. Then the medium was replaced by 0.15 mL of dimethyl sulfoxide (DMSO, Sigma) for 10 min incubation. Microplate spectrophotometer (Thermo Scientific, Franklin, MA) was applied to measure the optical density at 570 nm. All experiments were performed in triplicate.

2.9. Tumorigenicity assay

For *in vivo* xenograft studies, 5×10^6 Hep3B cells transfected with RFC4 shRNA and controls were subcutaneously injected into the left flank of 8-week-old BALB/c nude mice (Slac Laboratory Animal Co. Ltd, Shanghai, China). Mice were euthanized at 4 weeks post injection, and the tumors were excised and fixed for subsequent histopathological examination and analysis. Meanwhile, tumor volumes were measured twice a week after two weeks, tumor volume = 1/2 (length × width^2). All animal experiments were approved by the Animal Care and Use Committee of our institution.

2.10. Flow cytometry

Cell cycle progression was analyzed by flow cytometry. HCC cells were trypsinized, counted, washed, fixed by dropwise addition of 70% ethanol and stored at 4°C until analysis. Then cells were washed with PBS, resuspended in 50 ug/mL propidium iodide (PI) solution and analyzed by flow cytometry.

2.11. Statistical analyses

All data in our study were analyzed by SPSS.22.0. Data are presented as mean ± SEM. Student's *t*-test were used for continuous variables, χ^2 tests were applied to analyze categorical variables. Survival of patients was plotted using Kaplan-Meier method. In this study, a p value of < 0.05 was considered to indicate statistical significance.

3. Results

3.1. RFC4 was overexpressed in hepatocellular carcinoma

To explore the potential roles of RFC4 in hepatocellular carcinoma, we first analyzed the mRNA expression of RFC4 in hepatocellular carcinoma and normal liver tissues by Bioinformatics analysis. Differential gene expression analysis was conducted from GTEx and TCGA database and 369 primary HCC samples and 160 normal liver tissues were analyzed. As Figure 1A shows, RFC4 was significantly overexpressed in HCC. Moreover, we further explored the expression of RFC4 in several HCC cell lines through public dataset (CCLE) and further verified *via* RT-PCR. As Figure 1B-C shows, RFC4 represented a significantly higher expression level in HCC cell lines compared with normal liver cell lines. Next, we investigated the expression of

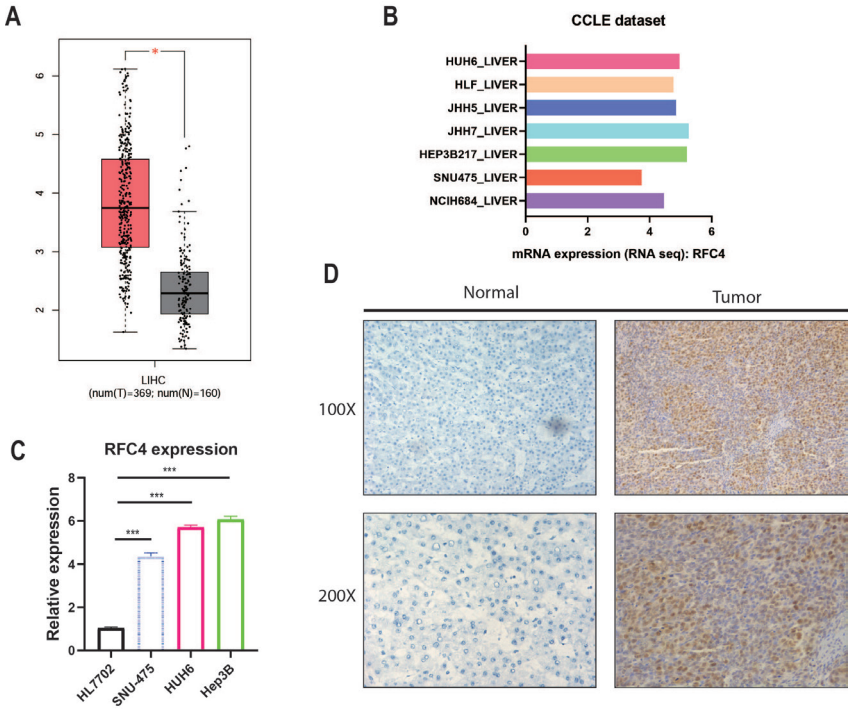

Figure 1. The expression of RFC4 in human HCC cell lines and tissues. (A) The expression of RFC4 mRNA level in HCC and normal liver tissues. **(B)** RFC4 mRNA expression in the CCLE dataset. **(C)** RFC4 mRNA expression in normal liver cell and HCC cell lines were explored by RT-PCR. **(D)** Immunostaining showed high and low expression of RFC4 in HCC and normal liver tissues.

RFC4 protein in HCC and normal liver tissues from 142 patients *via* IHC. Similar to mRNA expression, the expression of RFC4 protein was significantly overexpressed in HCC (Figure 1D). Moreover, the positive signal of RFC4 was mainly expressed in the nuclei of tumor cells and showed typical strong staining (Figure 1D). In contrast, normal liver tissues barely expressed RFC4 (Figure 1D).

3.2. High expression of RFC4 correlated with clinicopathological variables and prognosis of HCC

In order to investigate the correlation between RFC4 protein expression status and clinical-pathological characters of HCC patients, we divided HCC patients into two groups according to the expression of RFC4 protein. The associations between RFC4 protein expression and clinicopathological features are shown in Table 1. High expression of RFC4 protein was significantly correlated with bigger tumor size ($p = 0.029$). As for other clinicopathological features including age, gender, number of tumor nodes and AFP level, no associations were found (all $p > 0.05$). On the other hand, we assessed the association between RFC4 expression and prognosis to identify the prognostic value of RFC4 for HCC. Overall survival (OS) and disease-free survival (DFS) information were obtained from TCGA database. A significant correlation was found between RFC4 expression and adverse clinical outcome including short OS and DFS of HCC patients (Figure 2A-B).

3.3. RFC4 promotes the proliferation of HCC cancer cells

To understand the potential roles of RFC4 in malignant behavior of HCC, we inhibited the expression of RFC4 in HCC cancer cells. RFC4 expression was knocked

Table 1. Relationships of RFC4 and clinicopathological characteristics in 142 patients with hepatocellular carcinoma

Feature	All, $n = 142$	RFC4 expression		χ^2	p
		Low, $n = 60$	High, $n = 82$		
Age (year)				3.169	0.075
< 60	80	39	41		
≥ 60	62	21	41		
Gender				0.021	0.886
Male	72	30	42		
Female	70	30	40		
Number of tumor nodes				3.388	0.066
Single	60	20	40		
Multiple ≥ 2	82	40	42		
Tumor size				4.760	0.029
< 5 cm	63	33	30		
≥ 5 cm	79	27	52		
AFP (ng/mL)				0.483	0.487
< 50	52	20	32		
≥ 50	90	40	50		

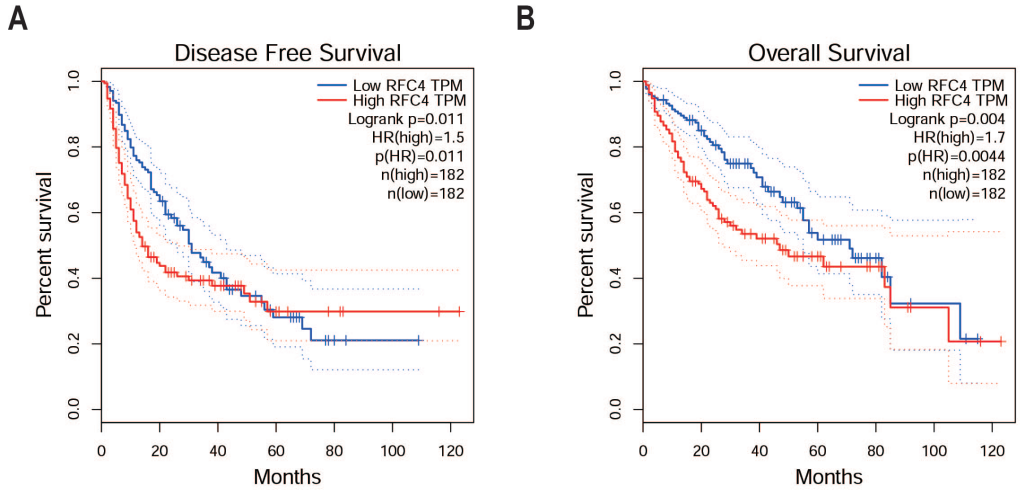

Figure 2. High expression of RFC4 as a prognostic factor of human HCC. (A) Kaplan-Meier survival analysis of disease-free survival for RFC4 expression in HCC. **(B)** Kaplan-Meier survival analysis of overall survival for RFC4 expression in HCC.

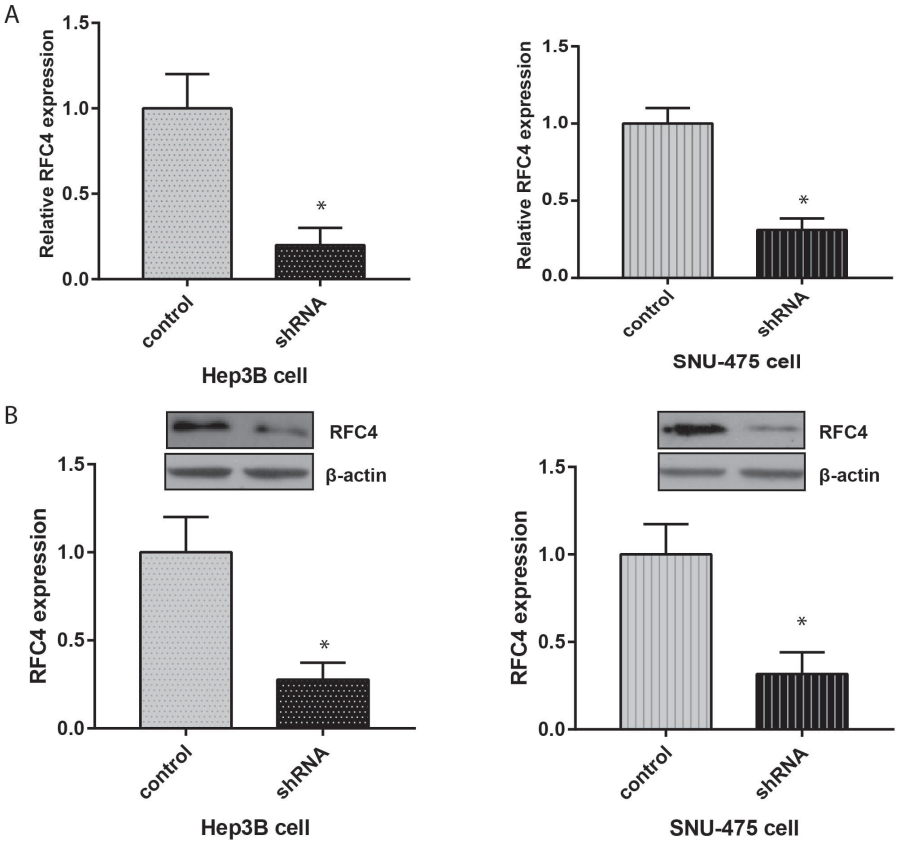

Figure 3. Stably knocking down of RFC4 by shRNA in both Hep3B and SNU-475 cells. (A, B) RT-PCR and Western blot showed lower expression level of RFC4 in shRNA group ($p < 0.05$), compared to the control group, just as expected.

down in both Hep3B and SNU-475 cells using shRNA. Both RT-PCR and Western blot results showed that shRNA worked as we expected, the expression of RFC4 was dramatically decreased in the shRNA group (Figure 3A-B).

Next, we tested whether loss-of-function of RFC4 is correlated with the proliferation of HCC cells by MTT assay and colony formation arrays. We found that down-regulation of the expression of RFC4 significantly inhibited the proliferation of Hep3B and SNU-475 cells compared to controls ($p < 0.05$), manifested as decreased cell proliferation rate and colony formation (Figure 4A-B). Flow cytometry further indicated that cell cycle arrest was induced after knocking down RFC4 (Figure 4C). Cells remaining in G2 phase significantly increased after RFC4 ablation.

3.4. Down-regulation of the expression of RFC4 suppressed the tumorigenicity of HCC cells *in vivo*

To further explore whether the role of RFC4 is associated with abnormal *in vitro* behavior and can be translated into abnormal tumorigenesis *in vivo*, cells from RFC4 shRNA group and control group were injected subcutaneously into athymic mice respectively. The tumor volumes were measured twice a week after two weeks. As the growth curve shows in Figure 5A, tumor growth of shRNA group was significantly slower than that of control group ($p < 0.05$). Meanwhile, we also performed IHC to detect the expression of RFC4 in subcutaneous tumors. Consistently, RFC4 expression was dramatically decreased in shRNA group, which indicated that an effective and stable knocking-down of RFC4 was built into mice tumors (Figure 5B).

4. Discussion

In this study, we explored the potential role of RFC4 in HCC. We demonstrated that RFC4 was overexpressed in HCC tissues and high RFC4 expression represents a poor clinical outcome. Furthermore, we investigated the function of RFC4 in HCC aggression. Consistent with previous reports, RFC4 may be an effective marker and probably even a potential therapeutic target for HCC.

RFC family members play important roles in eukaryotic DNA replication and DNA repair activities following DNA damage. Among them, RFC4, which encodes the fourth largest subunit of the RFC complex is also involved in several other biological processes (*7,16*). Deregulation of PFC4 may contribute to cell proliferation and tumorigenesis. Moreover, it has been reported to be deregulated in diverse malignancies, including cervical cancer, colorectal cancer, and prostate cancer (*10-13*). In our study, we indicated that RFC4 plays an important role in tumor cell proliferation *via* MTT assay and colony formation. The knockdown of RFC4 expression in HCC lines by siRNA resulted in

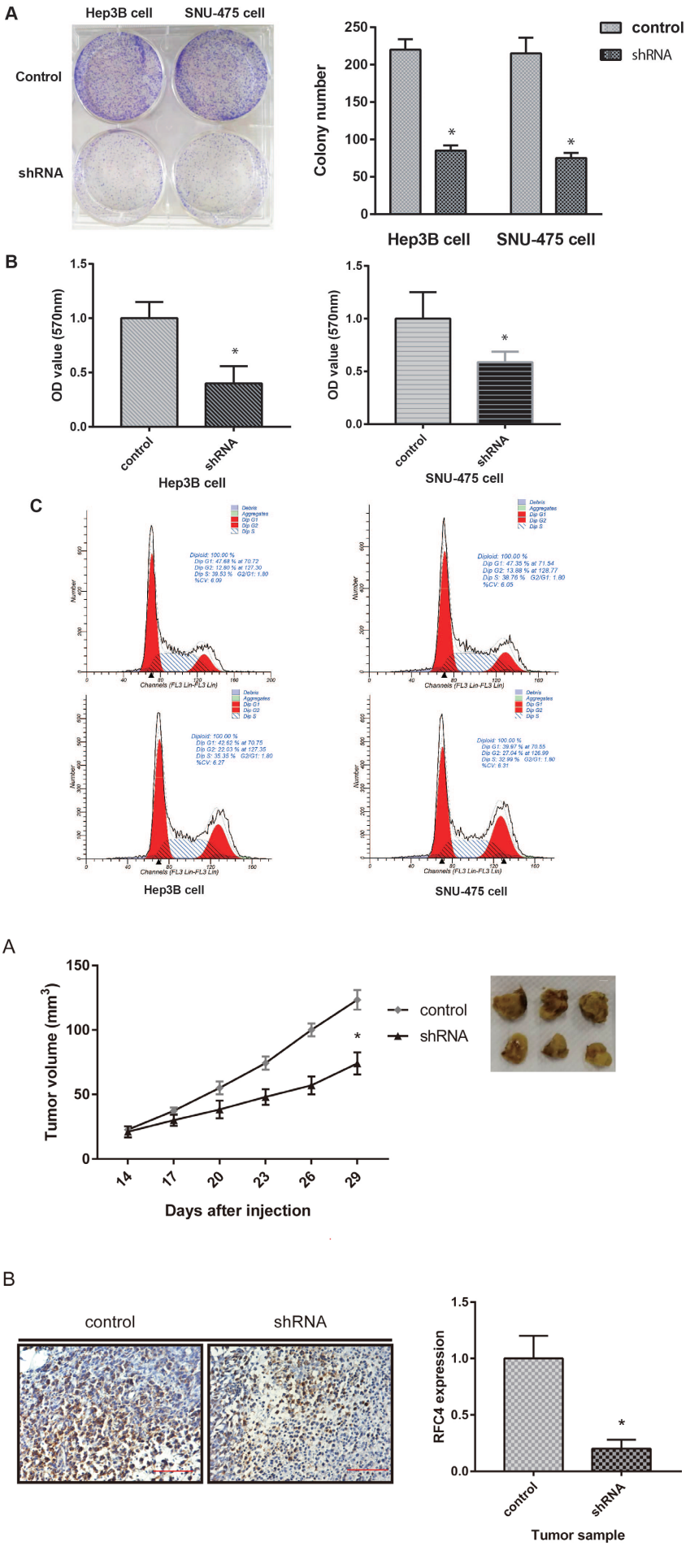

Figure 4. Knocking down RFC4 in HCC cancer cells inhibited tumor cell proliferation. (A) Typical images of colony-forming assay and its quantification demonstrated that colony rate of shRNA group was significantly lower than control group in both cancer cell lines ($p < 0.05$). **(B)** OD value of MTT assay suggested that cell proliferation rate of shRNA group was lower than control group in both cancer cell lines ($p < 0.05$). **(C)** Cell cycle arrest of HCC cells induced by RFC4 ablation. Cell cycle was analyzed by flow cytometry.

Figure 5. The influence of RFC4 on tumor growth of HCC in mice. (A) Representative images of tumors and tumor growth curves from both groups are shown. Tumor volumes of shRNA group were smaller than that of the control group ($p < 0.05$). Scale bar 2 mm. **(B)** Immunohistochemistry demonstrated that the expression of RFC4 was dramatically decreased in mice tumors ($p < 0.05$), which suggested a successful construction of RFC4 knocked down model in mice. Scale bar 100 um.

a significant decrease in cell proliferation. This result may suggest that RFC4 is involved in DNA replication in cancer cells. Consistent with our results, Arai *et al.* found that knock down of RFC4 in HepG2 cells arrested the growth of HepG2 cells in S phase and increased cell apoptosis.

The current status of HCC diagnosis and treatment urgently requires us to search for potent molecular targets for therapies directed against hepatocellular carcinoma. Several studies have reported that aberrant expressions of RFC4 can be a promising prognostic marker and even a therapeutic target in a number of malignancies (*14,15,17*). In our present analyses, data from public databases suggested that high expression of RFC4 was correlated with clinical-pathological characteristics and poor OS and DFS of HCC patients. Meanwhile, we also found that high expression of RFC4 protein was significantly correlated with larger tumor size ($p = 0.029$). All the above results suggested that RFC4 is a potential molecular target for modulating the growth of hepatocellular cancer cells. Similar to our findings, Wang *et al.* identified RFC4 as a radioresistant factor that promotes NHEJ-mediated DNA repair in colorectal cancer cells *via* genome-wide RNAi screen and the expression level of RFC4 predicted radiotherapy responsiveness (*10*). By using public databases, Kong *et al.* demonstrated that five core genes including RFC4 and relevant cell cycle-related pathways play significant roles in HCC progression and prognosis and could greatly improve knowledge about HCC progression (*11*). Niu *et al.* found that RFC4 was not only changed in high-grade squamous intraepithelial lesions, but also significantly changed in squamous cell carcinomas, indicating that its dysregulation may contribute to cervical cancer development (*12*). In addition, using immunohistochemistry analysis in a tissue microarray comprising 331 surgically resected colorectal cancer sections, Xiang *et al.* reported that RFC4 is frequently overexpressed in colorectal cancer, and is associated with tumor progression and a worse survival outcome (*13*).

In conclusion, we elaborately explored the roles of RFC4 in HCC. Though more research is still needed to explore and verify the exact underlying mechanisms of HCC development and progression, we speculate that RFC4 can be a prognostic marker and even a therapeutic target for HCC.

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

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Commentary

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Infectious disease activity during the COVID-19 epidemic in Japan: Lessons learned from prevention and control measures

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SUMMARY In Japan, the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients with Infectious Diseases (the "Infectious Diseases Control Law") classifies infectious diseases as category I-V infectious diseases, pandemic influenza, and designated infectious diseases based on their infectivity, severity, and impact on public health. COVID-19 was designated as a designated infectious disease as of February 1, 2020 and then classified under pandemic influenza as of February 13, 2021. According to national reports from sentinel surveillance, some infectious diseases transmitted by droplets, contact, or orally declined during the COVID-19 epidemic in Japan. As of week 22 (June 6, 2021), there were 704 cumulative cases of seasonal influenza, 8,144 cumulative cases of chickenpox, 356 cumulative cases of mycoplasma pneumonia, and 45 cumulative cases of rotavirus gastroenteritis; these numbers were significantly lower than those last year, with 563,487 cumulative cases of seasonal influenza, 31,785 cumulative cases of chickenpox, 3,518 cumulative cases of mycoplasma pneumonia, and 250 cumulative cases of rotavirus gastroenteritis. Similarly, many infectious diseases transmitted by droplets or contact declined in other countries and areas during the COVID-19 pandemic. One can reasonably assume that various measures adopted to control the transmission of COVID-19 have played a role in reducing the spread of other infectious diseases, and especially those transmitted by droplets or contact. Extensive and thorough implementation of personal protective measures and behavioral changes may serve as a valuable reference when identifying ways to reduce the spread of infectious diseases transmitted by droplets or contact in the future.

Keywords COVID-19, infectious diseases, influenza, chickenpox, mycoplasma pneumonia, rotavirus gastroenteritis

1. Introduction

As of June 30, 2021, the pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in 181,521,067 confirmed cases of coronavirus disease COVID-19 (COVID-19), including 3,937,437 deaths, worldwide according to a report from the World Health Organization (WHO) (*1*). Transmission of SARS-CoV-2 is mainly through direct, indirect, or close contact with an infected person by infectious secretions such as saliva, respiratory secretions, or droplets from an infected person, and the global population has generally been susceptible to COVID-19 (*2-4*).

Various prevention and control measures are being implemented globally to control the spread of SARS-CoV-2 (*5-7*). Studies indicated that COVID-19 prevention and control measures, including extensive and thorough implementation of personal protective measures, limiting of public gatherings, and social distancing, can play a role in reducing the spread of other infectious diseases, such as influenza (*8-10*). The current work has reviewed infectious disease activity during the COVID-19 epidemic in Japan and discussed the lessons learned from COVID-19 prevention and control measures as a way to reduce the spread of other infectious diseases, and especially those transmitted by droplets or contact.

2. Current status of infectious diseases in Japan during the COVID-19 epidemic

The first case of COVID-19 was reported on January 16, 2020 in Japan (*11*). COVID-19 was designated as a designated infectious disease as of February 1, 2020 (*12*) and then classified under pandemic influenza as of

February 13, 2021 (*13*).

In Japan, the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients with Infectious Diseases (the "Infectious Diseases Control Law") classifies infectious diseases into the following categories: *i*) Category I Infectious Diseases (includes 7 diseases), *ii*) Category II Infectious Diseases (includes 7 diseases), *iii*) Category III Infectious Diseases (includes 5 diseases), *iv*) Category IV Infectious Diseases (includes 44 diseases), *v*) Category V Infectious Diseases (includes 24 diseases), *vi*) Pandemic Influenza (includes 4 diseases), and *vii*) Designated Infectious Diseases. The specific infectious diseases in each category are listed in Table 1.

The categories of infectious diseases according to the Infectious Diseases Control Law are determined based on their infectivity, severity, and impact on public health. These aspects are the basis for preventing and controlling

Table 1. Current categories of infectious diseases in Japan

Category	Disease
Category I Infectious Diseases (Surveillance of nationally notifiable diseases)	(1) Ebola hemorrhagic fever, (2) Crimean-Congo hemorrhagic fever, (3) Smallpox, (4) South American hemorrhagic fever, (5) Plague, (6) Marburg disease, (7) Lassa fever.
Category II Infectious Diseases (Surveillance of nationally notifiable diseases)	(8) Poliomyelitis, (9) Tuberculosis, (10) Diphtheria, (11) Severe acute respiratory syndrome (SARS), (12) Middle East respiratory syndrome (MERS), (13) Avian influenza (H5N1), (14) Avian influenza (H7N9).
Category III Infectious Diseases (Surveillance of nationally notifiable diseases)	(15) Cholera, (16) Shigellosis, (17) Enterohemorrhagic E. coli infection, (18) Typhoid fever, (19) Salmonella serovar paratyphi A.
Category IV Infectious Diseases (Surveillance of nationally notifiable diseases)	(20) Hepatitis E, (21) West Nile fever (including West Nile encephalitis), (22) Hepatitis A, (23) Echinococcosis, (24) Yellow fever, (25) Psittacosis, (26) Omsk hemorrhagic fever, (27) Relapsing fever, (28) Kyasanur Forest disease, (29) Q fever (Coxiella burnetii), (30) Rabies, (31) Coccidioidomycosis, (32) Monkeypox, (33) Zika virus infection, (34) Severe fever with thrombocytopenia syndrome (Phlebovirus), (35) Hemorrhagic fever with renal syndrome, (36) Western equine encephalitis, (37) Tick-borne encephalitis, (38) Anthrax, (39) Chikungunya fever, (40) Scrub typhus, (41) Dengue fever, (42) Eastern equine encephalitis, (43) Avian influenza (excluding H5N1 and H7N9), (44) Nipah virus infection, (45) Japanese spotted fever, (46) Japanese encephalitis, (47) Hantavirus pulmonary syndrome, (48) B virus disease, (49) Glanders, (50) Brucellosis, (51) Venezuelan equine encephalitis, (52) Hendra virus infection, (53) Typhoid fever, (54) Botulism, (55) Malaria, (56) Tularemia, (57) Lyme disease, (58) Lyssavirus infection, (59) Rift Valley fever, (60) Melioidosis, (61) Legionnaires' disease, (62) Leptospirosis, (63) Rocky Mountain spotted fever.
Category V Infectious Diseases (Surveillance of nationally notifiable diseases)	(64) Amoebiasis, (65) Viral hepatitis (excluding hepatitis E and A), (66) Carbapenem-resistant enterobacteriaceae infections, (67) Acute flaccid paralysis (excluding acute poliomyelitis), (68) Acute encephalitis (excluding West Nile encephalitis, Western equine encephalitis, tick-borne encephalitis, Eastern equine encephalitis, Japanese encephalitis, Venezuelan equine encephalitis, and Rift Valley fever), (69) Cryptosporidiosis, (70) Creutzfeldt-Jakob disease, (71) Invasive group A streptococcal infection, (72) Acquired immune deficiency syndrome (AIDS), (73) Giardiasis, (74) Invasive Haemophilus influenzae disease, (75) Invasive meningococcal disease, (76) Invasive pneumococcal disease, (77) Varicella (the patient requires hospitalization), (78) Congenital rubella syndrome, (79) Syphilis, (80) Disseminated cryptococcosis, (81) Tetanus, (82) Vancomycin-resistant Staphylococcus aureus infection, (83) Vancomycin-resistant Enterococcus infection, (84) Pertussis, (85) Rubella, (86) Measles, (87) Multi-drug-resistant Acinetobacter infection.
Category V Infectious Diseases (Sentinel surveillance)	*Weekly Report*: (88) Respiratory syncytial virus infection, (89) Pharyngoconjunctival fever, (90) Group A streptococcus pharyngitis, (91) Infectious gastroenteritis, (92) Varicella, (93) Hand, foot, and mouth disease, (94) Erythema infectiosum, (95) Exanthema subitum, (96) Herpangina, (97) Mumps, (98) Influenza (excluding avian influenza, novel influenza, etc.), (99) Acute hemorrhagic conjunctivitis, (100) Epidemic keratoconjunctivitis, (105) Chlamydia pneumonia (excluding psittacosis), (106) Bacterial pneumonia (excluding pathogens causing Haemophilus influenzae, Meningococcus meningitidis, or Streptococcus pneumoniae), (107) Mycobacterial meningitis, (108) Mycoplasma pneumoniae, (109) Aseptic meningitis. *Monthly Report*: (101) Genital chlamydia infection, (102) Genital herpes virus infection, (103) Condyloma acuminatum, (104) Gonococcal infection, (107) Penicillin-resistant streptococcus pneumoniae infection, (110) Methicillin-resistant staphylococcus aureus infection, (111) Drug-resistant pseudomonas aeruginosa infection.
Pandemic Influenza (Novel Influenza or Re-emerging Influenza) (Surveillance of nationally notifiable diseases)	(112) Novel influenza, (113) Re-emerging influenza, (114) Novel coronavirus infection (COVID-19)*, (115) Re-emerging coronavirus infection*.
Designated infectious diseases (Surveillance of nationally notifiable diseases)	--

*Novel coronavirus infection (COVID-19) and re-emerging coronavirus infection have been changed from a designated infection to pandemic influenza since February 13, 2021. Data source: *http://idsc.tokyo-eiken.go.jp/survey/sikkan*

Table 2. Main measures based on the Infectious Diseases Control Law

Items	Hospitalization advised	Self-quarantine required	Building access and traffic restrictions	Asymptomatic patients	Work restrictions
Category I Infectious Diseases	Possible	Not possible	Possible	Possible	Possible
Category II Infectious Diseases	Possible	Not possible	Not possible	Not possible	Possible
Category III Infectious Diseases	Not possible	Not possible	Not possible	Not possible	Possible
Category IV Infectious Diseases	Not possible	Not possible	Not possible	Not possible	Not possible
Category V Infectious Diseases	Not possible	Not possible	Not possible	Not possible	Not possible
Pandemic Influenza (Novel Influenza or Re-emerging Influenza)	Possible	Possible	*Possible under certain conditions	Possible	Not possible
Designated infectious diseases	Possible	Possible	Possible	Possible	Possible

*Only the provisions of Article 44-4 of the Infectious Diseases Control Law apply. Data Source: *https://corona.go.jp/news/pdf/shiteikansensho_20200831.pdf*

the transmission and spread of infectious diseases. Restrictions differ for each category of infectious disease (Table 2).

Sentinel surveillance and surveillance of nationally notifiable diseases are used collect information on infectious diseases pursuant to the Infectious Diseases Control Law. To ascertain the epidemiology of an infectious disease, sentinel surveillance is performed for specific Category V infectious diseases, and designated hospitals or clinics are required to report the number of cases and clinical data to a public health center on a weekly or monthly basis (Table 1). Public health centers report to the prefectural government and the Ministry of Health, Labor, and Welfare *via* an online reporting platform. Since 1999, the National Institute of Infectious Diseases has collected and analyzed clinical data from the platform and published the Infectious Disease Weekly Report (IDWR) (*14,15*).

According to the IDWR (*16*), some infectious diseases transmitted by droplets, contact, or orally declined during the COVID-19 epidemic. An example is the following 4 Category V Infectious Diseases:

i) The incidence of seasonal influenza is based on 5,000 sentinel surveillance sites (3,000 sites in pediatrics and 2,000 sites in internal or general medicine at hospitals and clinics). As of week 22 (June 6, 2021), there were 704 cumulative cases, compared to a total of 563,487 cases last year.

ii) The incidence of chickenpox is based on 3,000 sentinel sites in pediatrics. As of week 22 (June 6, 2021), there were 8,144 cumulative cases, compared to a total of 31,785 cases last year.

iii) The incidence of mycoplasma pneumonia is based on 500 sentinel sites in general medicine at hospital and clinics (with more than 300 beds). As of week 22 (June 6, 2021), there were 356 cumulative cases, compared to a total of 3,518 cases last year.

iv) The incidence of rotavirus gastroenteritis is based on 500 sentinel sites in general medicine at hospitals and clinics (with more than 300 beds). As of week 22 (June 6, 2021), there were 45 cumulative cases, compared to a total of 250 cases last year.

Moreover, the incidence of the above four infectious diseases is significantly lower during the COVID-19 epidemic in Japan (2020-2021) compared with data from the past decade (2011-2020) (Figure 1).

3. Current status of infectious diseases around the world during the COVID-19 pandemic

Many infectious diseases transmitted by droplets or contact have declined in other countries and areas during the COVID-19 pandemic. The WHO reported significant declines in influenza virus infection in many countries such as the United States, Australia, and South Korea in 2020-2021. According to reports of visits for influenza-like illness (ILI) from the US Outpatient Influenza-like Illness Surveillance Network (ILINet), during week 22 (June 6, 2021), 1.2% of patient visits was due to ILI, which is below the national baseline of 2.6% (*17*). In Australia prior to week 22 (June 6, 2021), the National Notifiable Disease Surveillance System (NNDSS) reported 326 notifications of laboratory-confirmed influenza, which is significantly lower than the 5-year average for influenza. South Korea reported an overall weekly ILI rate of 1.9 ILI cases per 1,000 outpatient visits in week 23 (June 23) of 2021, and the ILI rate has been below the national epidemic threshold (5.8 ILI cases per 1,000 outpatient visits) since week 10 (March 8) of 2020 (*18*).

Moreover, the number of chickenpox cases has decreased in some areas of the United States during the COVID-19 pandemic. From January-April 2021, a total of 93 cases of chickenpox were reported in Florida, and 30 cases were reported in April 2021; this number is lower than the 5-year average (*19*). Minnesota reported 122 cases of chickenpox in 2020, which is the lowest incidence in 8 years compared to statistics from 2013 (*20*).

In China, a study noted a decrease in mycoplasma pneumonia among children after the outbreak of COVID-19 (*21*). Based on clinical data from 2017 to 2020, 34,977 patients with mycoplasma pneumonia were analyzed by year, season, sex, and age. The data revealed two outbreaks of mycoplasma pneumonia, the first occurring between October and December 2017 and the

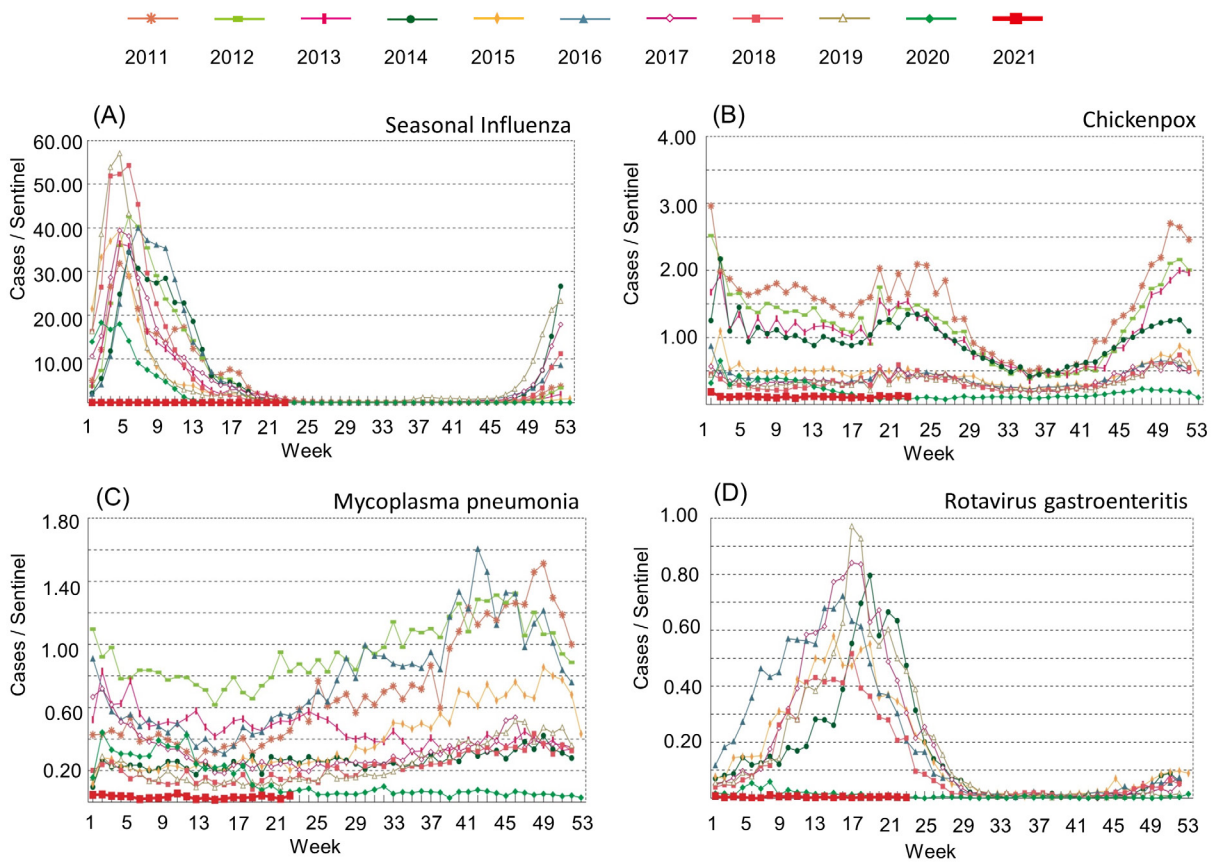

Figure 1. The number of cases reported from sentinel sites during 2011-2021 in Japan. (A) Seasonal influenza, **(B)** Chickenpox, **(C)** Mycoplasma pneumonia, and **(D)** Rotavirus gastroenteritis. Data source: *https://www.niid.go.jp/niid/images/idsc/idwr/IDWR2021/idwr2021-22.pdf*

second occurring between April 2019 and January 2020. However, mycoplasma pneumonia decreased due to restrictive measures and strict self-isolation implemented after the outbreak of COVID-19.

4. Have COVID-19 prevention and control measures reduced the incidence of infectious diseases transmitted by droplets or contact?

Infectious diseases are transmitted through multiple channels, such as droplets, contact, and orally. Seasonal influenza, chickenpox, and mycoplasma pneumonia are transmitted mainly through droplets and contact (*22-24*). Rotavirus gastroenteritis is mainly transmitted orally and by contact (*25*).

Various prevention and control measures have been and are being implemented during the COVID-19 epidemic in Japan, and especially during the three declared states of emergency (the first state of emergency was declared from April 7 to May 25, 2020, the second was declared from January 8 to March 21, 2021, and the third was declared from April 25 to June 20, 2021). Personal protective measures were thoroughly implemented, including wearing masks, handwashing, and avoiding confined spaces, crowded places, and close-contact settings. More importantly, behavioral changes adopted to constrain COVID-19 during the three declared states of emergency reduced population density and contact with people, including closing schools, asking restaurants to reduce their business hours, teleworking, curbing the flow of people during vacation week (*26*).

During the COVID-19 epidemic, other infectious diseases transmitted by droplets, contact, or orally, such as seasonal influenza, chickenpox, mycoplasma pneumonia, and rotavirus gastroenteritis, have declined in Japan. One can reasonably assume that various measures adopted to control the transmission of COVID-19 have played a role in reducing the spread of other infectious diseases, and especially those transmitted by droplets or contact. Extensive and thorough implementation of personal protective measures and behavioral changes may serve as a valuable reference when identifying ways to reduce the spread of infectious diseases transmitted by droplets or contact in the future.

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Commentary

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China should emphasize key issues inherent in rational medication management for the elderly

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SUMMARY According to China's Seventh National Census, 18.70% of a total of 1.41 billion people were 60 or older and 13.50% were 65 or older, so China's population is increasingly aging. In conjunction with China's socioeconomic and scientific and technological development and its promotion of medical insurance-related policies, rational medication management for the elderly is a concern in order to control the risk of polypharmacy. This paper summarizes and discusses the following five key issues inherent in rational medication management: *i*) an increase in serious polypharmacy and the potential risks of medication; *ii*) a lack of medication consultation service and medication withdraw without healthcare providers' supervision; iii) poor medication compliance among the elderly; *iv*) insufficient quantity and incompetence of pharmaceutical staffing; and *v*) limited awareness of pharmaceutical services and lack of trust in the ability of pharmacists. Based on a discussion of factors influencing these issues, suggestions have been put forward in the hopes that China emphasizes rational medication management in order to reduce the risk of polypharmacy and the disease burden of the elderly in China.

Keywords polypharmacy, aging of the population, clinical pharmacy, medication compliance

1. Introduction

On May 11, 2021, the State Council of China released the results of the country's Seventh National Census, which indicated that there are a total of 1.41 billion people in China (*1*). Of the total population, 18.70% were age 60 or older and 13.50% were age 65 or older. Compared to figures from 2010, this represented a 5.44% increase in people age 60 or older and a 4.63% increase in people age 65 or older. These figures indicate that the population is increasingly aging,and it is urgent to address challenges posed by aging.

One of the challenge is that the elderly with serious comorbidities are more prone to take multiple medications (*2*). In conjunction with China's socioeconomic and scientific and technological development and its promotion of medical insurance-related policies, the elderly have more accessibilities tomedical treatment and therapeutic drugs, resulting in an increased risk of irrational drug use (*3*). Due to the heavy concealment, high risk, and little attention paid to polypharmacy in the elderly, rational medication management for the elderly is a concern.

Rational medication management involves the whole process from prescription to taking a medication, including related policies and regulations, resource allocation, rational drug use-related training, medication taking behavior, physician care, provision of pharmaceutical services, and patient compliance with medication.

Based on the Donabedian model (structure, process, and outcomes) and Stakeholder theory, a theoretical framework for rational medication management was created (Figure 1), and issues inherent in rational medication management for the elderly were systematically identified in the literature and interviews and then compiled into a list. Through expert discussion, five key issues inherent in rational medication management for the elderly were ultimately identified (Figure 2), and those issues are discussed in this paper.

2. Key issues in terms of the elderly patients

A point worth noting is that three of the five key issues primarily involve patients, which implies that medication revolves around the elderly themselves.

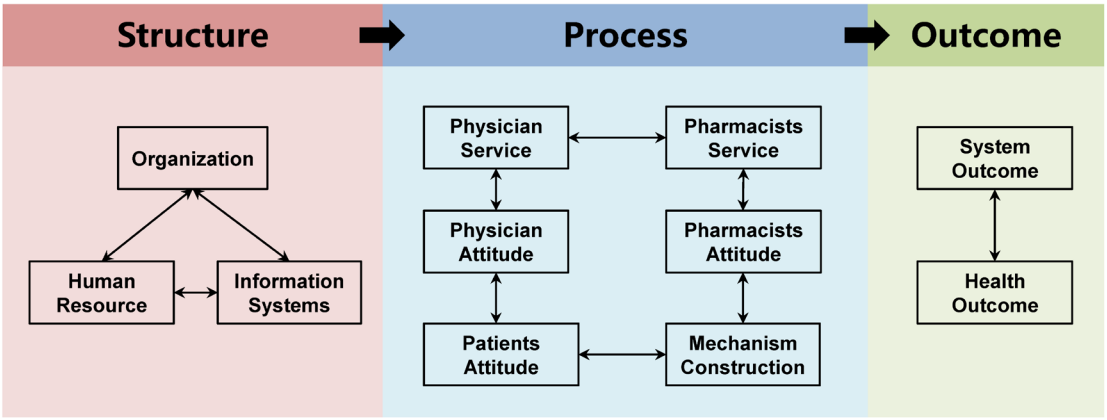

Figure 1. The theoretical framework for rational medication management. Rational medication management was defined, and an analytical framework for rational medication management was devised based on the Donabedian model (structure, process, and outcomes) and Stakeholder theory.

Issues Collection	***Literature Research Approach*** From 2010 to 2020 5454 papers were detected (removed duplicate and screened) **323 articles** were included	***Interview Research Approach*** Between August and September 2020 **5 interviews** were conducted A total of **50 people** (included family doctors, pharmacists, and health managers)
Issues List Formation	***Merge Collected Issues*** **Expert argumentation** by **82 experts** From 10 administrative regions of Shanghai (included family doctors, pharmacists, and health managers) A list of **35 issues** were identified and formed	
Key Issues Identification	***Identify Key Issues*** **Expert argumentation** by **82 experts** from expert base **5 key issues** through experts rated the severity, importance and solvability	

Figure 2. Flow chart for identification of key issues. Three hundred and twenty-three sources were reviewed by searching the relevant literature and excluding sources with little relevance to issues inherent in rational medication management. Fifty experts, including medical personnel and personnel rationally managing patient medication, in Shanghai were selected to identify issues inherent in rational medication management for the elderly. Thirty-five issues were identified and compiled into a list. Eighty-two experts rated the seriousness, significance, and solvability of the 35 issues and identified 5 key issues that need to be prioritized.

The elderly themselves are ultimately the ones taking medications so instructions in rational drug use, intervention by primary care physicians, and supervision by family members are particularly important.

2.1. An increase in serious polypharmacy and the potential risks of medication (*Key issue 1*)

First, a majority of the elderly need to take multiple medications for multiple comorbidities over a prolonged period time, resulting in a complex medication therapy and high risk of polypharmacy. Studies have found that elderly Chinese patients suffering from polypharmacy are taking an average of 10.3 ± 5.1 medications (*4*), and the incidence of polypharmacy in the elderly age 80 and over is as high as 82.4% (*5*).

Second, due to the high degree of specialization in medical care in China, elderly patients tend to visit physicians across specialties and medical institutions,

which results in dispersed treatment and medication information. Since information systems in medical facilities at all levels haven't not yet connected with one another, physicians in different medical facilities may prescribe the same or similar medications and primary care physicians may have difficulty determining contraindicated medications, increasing the risk of polypharmacy.

Third, misuse of supplements and traditional Chinese medicines without definitive indications is seriously widespread in the elderly. And due to the deeply rooted concept that "medicine and food homology" in China, most elderly believe that "the more medicines, the better" At the same time, improvement of the healthcare system means that the elderly are more accessible to medications and various healthcare products, increasing the risk of polypharmacy.

2.2. A lack of medication consultation service and medication withdraw without healthcare providers' supervision (*Key issue 2*)

First, some elderly perceive they have gained enough medical knowledge to treat themselves with long term sick experience. So they would prefer to choose medications based on their own experiences or information from people with similar symptoms, instead of consulting a physician or pharmacist at a regular medical facility.A study of 380 elderly outpatients found that 47.9% hadself-medication experiencesng with no diagnosis from a physician (*6*), and a survey in a community found that 88.43% of the elderly purchased and used drugs or other healthcare products without seeking advice from physicians or pharmacists (*7*). This is mainly due to a lack of trust in physicians and pharmacists among the elderly, as well as the limited effectiveness of instruction in rational drug use.

Second, the elderly tend to seekinformation from informal information sources because of a lack of communication with primary healthcare physicians. They often purchase drugs based on advertisements or recommendations from friends, which mainly stems from gimmicks and false claims, and it can result in adverse consequences for the elderly if unattended or unsupervised by family members.

2.3. Poor medication compliancen among the elderly (*Key issue 3*)

First, the elderly have difficulty taking medication on time and as instructed by a physician; they often take medication by mistake, forget to take it, or adjust the dosage according to the symptomsthemselves. A study of the elderly living at home found that only 41.9% of the patients were able to follow a physician's instructions (*8*). This is mainly due to poor memory, low awareness, and frequent change of medication therapy.

Second, psychological factors influence the medication taking behavior of the elderly a lot.For instance, some elderly choose to reduce the dosage with a fear of side effects, yet others choose to increase the dosage with an eagerness to get well. This is mainly due to the lack of direct communications between physicians and patients. In addition, unrecorded medication taking behavior at home results in huge information gap, and physicians cannot offer appropriate advice according to patients' actual medication taking scenarios.

3. Key issues in terms of care and service providers

The remaining two key issues are related to care and service providers. Specifically, they are related to the quantity and quality of care and service providers and the public's views of pharmaceutical personnel.

3.1 Insufficient quantityand incompetence of pharmaceutical staffing (*Key issue 4*)

In order to provide pharmaceutical services, medical facilities need to be staffed with sufficient pharmacy technicians. According to the Regulations on the Administration of Pharmaceutical Affairs in Medical Facilities, pharmacy personnel shall account for no less than 8% of all healthcare personnel in a medical facility (*9*). However, there is a serious dearth of pharmacists, and especially clinical pharmacists, which seriously restricts development of pharmaceutical services. As of 2018, there were only 460,000 pharmacists (practitioners) in China, which is far smaller than the number of licensed (assistant) physicians (3.607 million) and registered nurses (practitioners) (4.099 million) (*10*).

This lack of personnel is mainly due to 3 reasons. First, a relatively small number of pharmacists graduate in clinical pharmacy in China, and an even smaller number choose to work in clinical pharmacy. Second, there are no set criteria for promotion or forms of employment for pharmacists in China. Pharmacists are poorly paid, and many are leaving the profession. Third, the lack of a mechanism of assessing pharmacists providing pharmaceutical services seriously affects the quality of pharmaceutical personnel. In short, a pharmaceutical service fee needs to be determined and imposed.

3.2 Limited awareness of pharmacy services and lack of trust in the ability of pharmacists (*Key issue 5*)

A survey of patients and their families in 30 tertiary hospitals in Shanghai found that only 48.0% of patients and their families felt that they were familiar with the job of a clinical pharmacist, and 39.7% of patients knew little about clinical pharmaceutical services but had heard of them (*11*).

This is mainly due to three reasons. First,

pharmacy is still young profession in China, which is transitioning from traditional pharmacy to "patient-centered" clinical pharmacy. The public has little familiarity with pharmacists, so patients and physicians have limited awareness and trust in pharmacists and pharmaceutical services. Second, clinical pharmacists have difficulty doing their work properly due to the lack of corresponding standards for clinical pharmacists. Third, pharmacists and patients lack channels of communication. A few minutes when providing a patient with his or her medication is not enough time for pharmacists to fully understand the status of the patient's medications.

4. Suggestions

On March 29, 2017, the WHO proposed Medication Safety as the third Global Patient Safety Challenge (*12*). Governments need to take urgent measures to deal with polypharmacy by enhancing rational medication management for the elderly. As living conditions improve and medical technology advances, the demands of and consumption by the Chinese population, including the rational drug use by the elderly, will increase. A discussion identified several key issues with rational medication management for the elderly in China: *i*) a shortage of pharmaceutical personnel; *ii*) lack of a mechanism for compensation, such as a pharmaceutical service fee; and *iii*) inadequate an-home management of medication. Accordingly, China needs to: *i*) promote reforms in and the updating of pharmaceutical education; *ii*) establish a price system for pharmaceutical services; *iii*) energize primary pharmaceutical services using "Internet Plus", and *iv*) increase public awareness of rational medication management.

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BST
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Guide for Authors

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