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Editorial

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Trends in managing COVID-19 from an emerging infectious disease to a common respiratory infectious disease: What are the subsequent impacts on and new challenges for healthcare systems?

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SUMMARY Targeting the 9 countries with the highest cumulative number of newly confirmed cases in the past year, we analyzed the case fatality ratio (CFR) among newly confirmed cases and the vaccination rate (two or more doses of vaccine per 100 people) in the United States of America (USA), India, France, Germany, Brazil, the Republic of Korea, Japan, Italy, and the United Kingdom (UK) for the period of 2020-2022. Data reveal a decrease in the CFR among newly confirmed cases since the beginning of 2022, when transmission of the Omicron variant predominates, and an increase in vaccination rates. The Republic of Korea had the lowest CFR among newly confirmed cases (0.093%) in 2022 and the highest vaccination rate (86.27%). Japan had the second highest vaccination rate (83.12%) and a decrease in the CFR among newly confirmed cases of 1.478% in 2020, 1.000% in 2021, and 0.148% in 2022; while the average estimated fatality ratio for seasonal influenza from 2015-2020 was 0.020%. Currently, most countries are now easing COVID-19–related restrictions and are exploring a shift in management of COVID-19 from an emerging infectious disease to a common respiratory infectious disease that can be treated as the equivalent of seasonal or regional influenza. However, compared to influenza, infection with the Omicron variant still has a higher fatality ratio, is more transmissible, and the size of future outbreaks cannot be accurately predicted due to the uncertainty of viral mutation. More importantly, as countries shift their response strategies to COVID-19, there is an urgent need at this time to clarify what the subsequent impacts on healthcare systems and new challenges will be, including the clinical response, the dissemination of scientific information, vaccination campaigns, the creation of future surveillance and response systems, the cost of treatments and vaccinations, and the flexible use of big data in healthcare systems.

Keywords COVID-19, healthcare system, case fatality ratio (CFR), newly confirmed cases, vaccination, influenza

The COVID-19 global pandemic of the past three years has changed the way humans behave, the way governments respond to crises, and it has placed a heavy burden on the healthcare systems of the countries in question (*1*). In response to the global pandemic, countries have adopted different strategies based on the conditions in those countries, such as herd immunity, lockdowns, and "dynamic zero-COVID".

With the improved ability to detect the virus, accumulated experience with clinical treatment, advances in drug development, promotion of vaccination campaign, and especially the less virulent Omicron variant than earlier variants at the current stage (*2-5*), most countries are now easing COVID-19–related restrictions and are exploring a shift in the management of COVID-19 from an emerging infectious disease to a common respiratory infectious disease. For example, there is currently extensive discussion on whether COVID-19 can be treated as the equivalent of seasonal or regional influenza.

Targeting the 9 countries with the highest cumulative number of newly confirmed cases in the past year, we analyzed the case fatality ratio (CFR) among newly confirmed cases and the vaccination rate (two or more doses of vaccine per 100 people) in the United States of America (USA), India, France, Germany, Brazil, the Republic of Korea, Japan, Italy, and the United Kingdom (UK) for the period of 2020-2022.

Data reveal a decrease in the CFR among newly confirmed cases since the beginning of 2022, when transmission of the Omicron variant predominates, and an increase in vaccination rates. In 2022, the CFR among newly confirmed cases for the 9 countries, from lowest to highest, was 0.093% in the Republic of Korea, 0.115% in France, 0.130% in Germany, 0.136% in Japan, 0.226% in Italy, 0.350% in the UK, 0.480% in India, 0.507% in the USA, and 0.536% in Brazil (Figure 1). As of December 2022, the vaccination rates (two or more doses of vaccine per 100 people) for the 9 countries, from highest to lowest, are 86.27% in the Republic of Korea, 83.12% in Japan, 81.26% in Italy, 80.9% in Brazil, 78.33% in France, 76.18% in Germany, 75.19% in the UK, 68.86% in the USA, and 67.09% in India (Figure 2).

The country with the second highest vaccination rate is Japan. As of December 15, 2022, the total number of vaccine doses administered has reached 361,774,386. Nationwide, 80.4% of the total population has received the second dose of the vaccine and 67.4% has received the third dose; 92.4% of the population age 65 or older received the second dose of the vaccine and 90.9% of

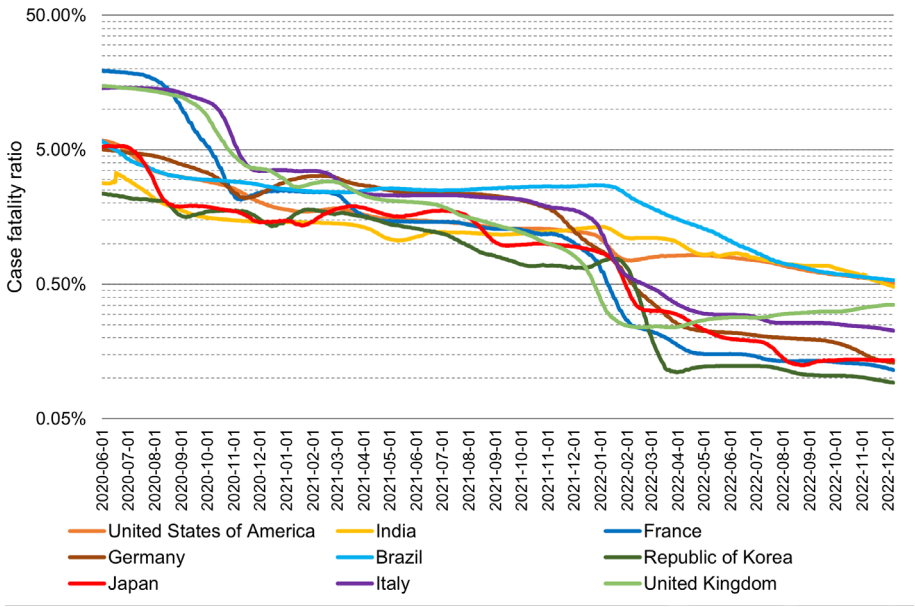

Figure 1. The case fatality ratio (CFR) among newly confirmed cases in 9 countries from 2020-2022*. Data reveal a decrease in the CFR among newly confirmed cases since the beginning of 2022, when transmission of the Omicron variant predominates. *Data are as of December 7, 2022. Data source: *https://covid19.who.int/WHO-COVID-19-global-data.csv*

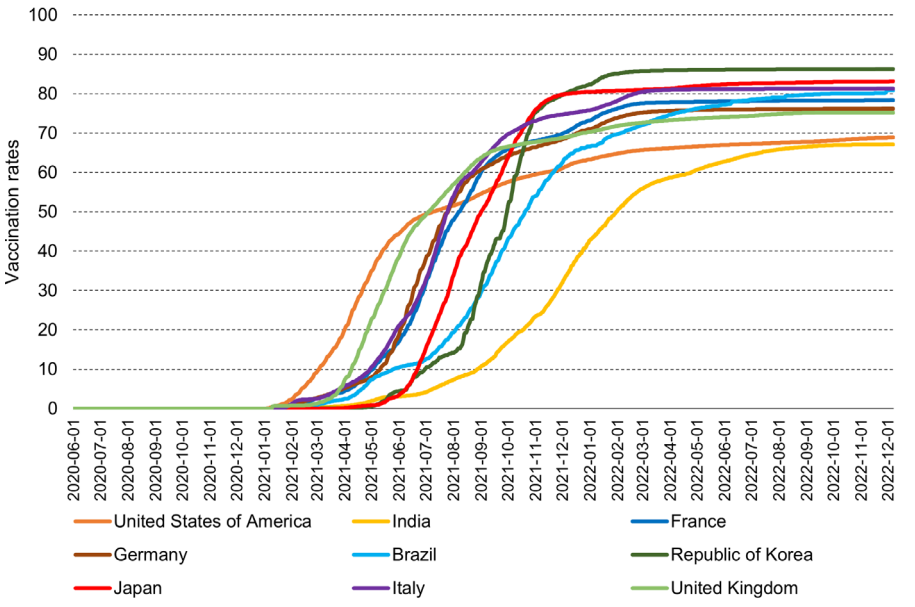

Figure 2. The number of people vaccinated in 9 countries from 2020-2022*. Data reveal an increase in vaccination rates (two or more doses of vaccine per 100 people) in 9 countries. *Data are as of December 7, 2022. Data source: *https://github.com/owid/covid-19-data/tree/master/public/data/vaccinations*

that population received the third dose (*6*).

Japan experienced its highest level of COVID-19 to date during a six-week period from July 20 to August 31, 2022, with an average of nearly 200,000 newly confirmed cases per day nationwide (*7*). This was followed by a decline to 13,053 new cases on October10, 2022 and the number has now risen to 168,494 new cases on December 15, 2022 (*8*). Although the number of new cases has increased more than before, mainly because of the enhanced transmissibility of the Omicron variant, few patients in critical condition needed specialized urgent care, such as intubation and extracorporeal membrane oxygenation (*9*).

To explore trends in COVID-19 among newly confirmed cases, we analyzed the CFR among new cases in Japan from 2020-2022 based on monthly, bimonthly, and annual data. Data reveal a decrease in the CFR among newly confirmed cases of 1.478% in 2020, 1.000% in 2021, and 0.148% in 2022 (Figure 3). For comparison, the average estimated fatality ratio for seasonal influenza in Japan for 2015-2020 was 0.020% (Figure 4).

In Japan, the Infectious Diseases Control Act classifies infectious diseases into five categories, Category I to V, based on how contagious the pathogens are and the severity of the disease they cause, and seasonal

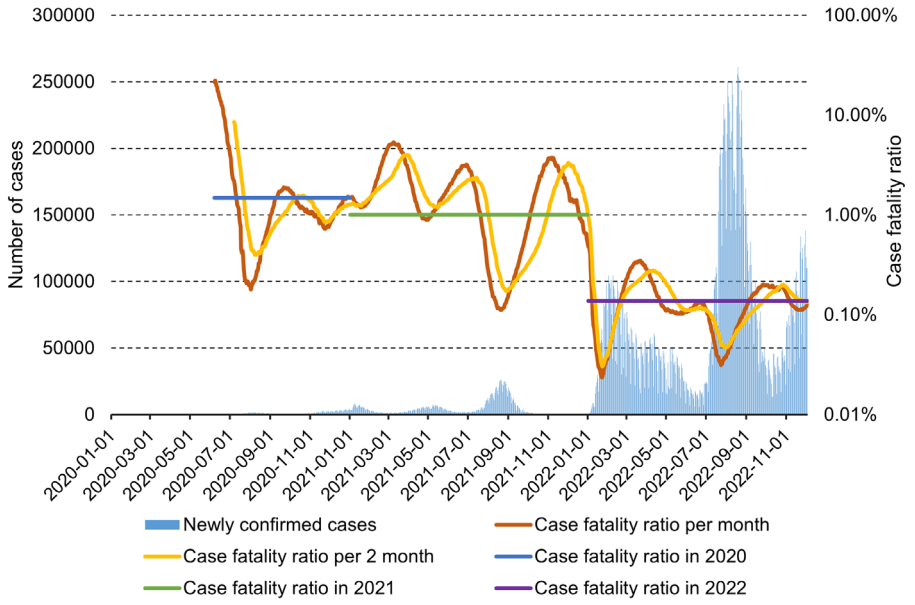

Figure 3. The case fatality ratio (CFR) among new infections in Japan from 2020-2022 based on monthly, bimonthly, and annual data*. Data reveal a decrease in the CFR among new infections of 1.478% in 2020, 1.000% in 2021, and 0.148% in 2022. *Data are as of December 3, 2022. Data source: *https://www.mhlw.go.jp/stf/covid-19/open-data.html*

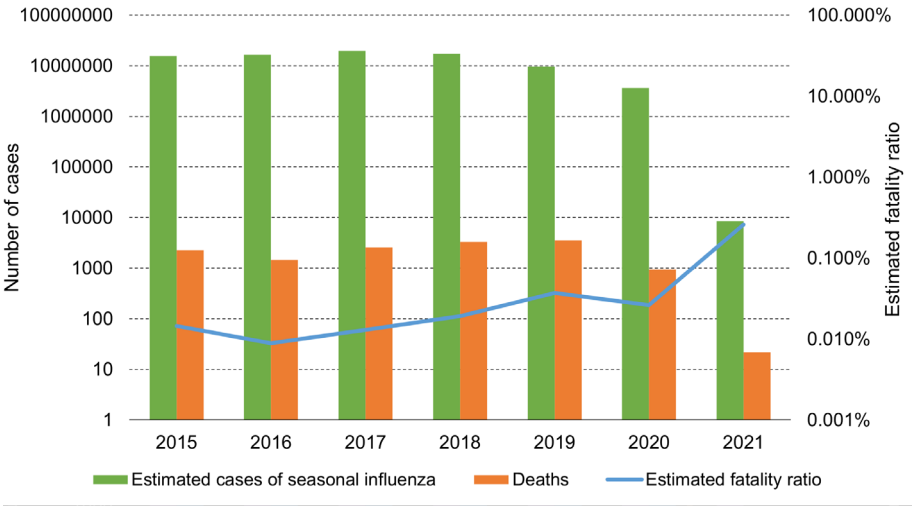

Figure 4. The estimated fatality ratio for seasonal influenza in Japan from 2020-2021. Data show that the estimated fatality ratio for seasonal influenza in Japan was 0.015% in 2015, 0.009% in 2016, 0.013% in 2017, 0.019% in 2018, 0.037% in 2019, 0.026% in 2020, and 0.259% in 2021. Data source: *https://www.mhlw.go.jp/toukei/list/81-1a.html*, *https://www.niid.go.jp/niid/ja/component/search/?searchword=iasr%20%E3%82%A4%E3%83%B3%E3%83%95%E3%83%AB%E3%82%A8%E3%83%B3%E3%82%B6%20%E3%82%B7%E3%83%BC%E3%82%BA%E3%83%B3*

influenza is classified as a Category V infectious disease. Since February 2020, COVID-19 was classified as a legally designated infectious disease (*10*), and medical expenses for hospitalization were basically covered by public funds. Currently, COVID-19 is classified under "novel influenza and other infectious diseases" that are equivalent to "Category II", and patients with COVID-19 are hospitalized in designated hospitals for specified infectious diseases. However, since November 30, 2022, the Japanese Government has started discussions on the classification of COVID-19 from Category II to Category V (*11*), which is equivalent to seasonal influenza. If COVID-19 is classified as a Category V infectious disease, patients with COVID-19 will be able to be treated in general medical facilities rather than in designated hospitals, which will help to alleviate the pressure on medical supplies during the peak of the epidemic. But on the other hand, the government will not be able to declare a state of emergency in relation to COVID-19 during the peak of the epidemic and take measures such as restricting travel and requiring the infected to isolate themselves.

Currently, there is an extensive discussion on whether COVID-19 can be treated as the equivalent of seasonal or regional influenza, not only in Japan but also in many countries such as China. Starting in December 2022, China's response strategy has shifted from "dynamic zero-COVID" to widespread easing of restrictions (*12*), such as the elimination of nationwide PCR testing, the elimination of the "Mobile Itinerary Card" (a nationwide mobile tracking application that collects data on users' travel activity). However, a point worth noting is that compared to influenza, infection with the Omicron variant still has a higher fatality ratio, is more transmissible, and the size of future outbreaks cannot be accurately predicted due to the uncertainty of viral mutation.

More importantly, as countries shift their response strategies to COVID-19, there is an urgent need at this time to clarify what the subsequent impacts on healthcare systems and new challenges will be. At least the following 6 important issues need to be carefully addressed: *i*) Developing response guidelines for current epidemics based on the coordination of national medical resources to ensure timely treatment of critically ill patients while avoiding hospital overcrowding; *ii*) Facilitating the dissemination of scientific information to avoid public panic and guiding the thorough implementation of basic infection control measures during an outbreak including wearing masks, handwashing, and avoiding confined spaces, crowded places, and close-contact settings; *iii*) Continuing to promote vaccination campaigns to reduce the overall amount of virus that can spread to the whole population, and especially to reduce the risk of infection among the elderly and vulnerable populations; *iv*) If COVID-19 is equated with seasonal or regional influenza, will it be incorporated into a national or even global influenza surveillance and response system? Will the COVID-19 vaccine be administered as regularly as the flu vaccine? *v*) Who will pay for future COVID-19 treatment and vaccination costs? In Japan, if COVID-19 is classified as a Category V infectious disease, treatment costs other than those covered by insurance and vaccinations other than those with special regulations will be paid for out-of-pocket; *vi*) Effective use of things that emerged during the COVID-19 response in the context of the rapid development of big data in healthcare, such as the joint development and use of point-of-care testing (POCT) and the Internet of Medical Things (IoMT) (*13*) to advance disease prevention and health maintenance.

In conclusion, after three years of the global COVID-19 pandemic, there appears to a trend from managing COVID-19 as an emerging infectious disease to managing it as a common respiratory infectious disease. As countries shift their response strategies to COVID-19, there is an urgent need at this time to clarify what the subsequent impacts on healthcare systems and new challenges will be, including the clinical response, the dissemination of scientific information, vaccination campaigns, the creation of future surveillance and response systems, the cost of treatments and vaccinations, and the flexible use of big data in healthcare systems.

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Editorial

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Focusing on development of novel sampling approaches and alternative therapies for COVID-19: Are they still useful in an era after the pandemic?

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SUMMARY The different viral characteristics of the Omicron variant of SARS-CoV-2 have fundamentally changed people's view concerning COVID-19. Many alternative sampling approaches and therapies have been developed that may be better suited to the Omicron variant, such as a saline gargle to detect SARS-CoV-2 and nasal irrigation with chlorine dioxide. The mechanisms of these methods of sampling and alternative therapies are briefly summarized here. Development of novel alternative sampling/therapeutic approaches for COVID-19 is crucial due to the uncertain future of emerging respiratory viruses, and their efficiency/safety needs to be verified in a post-pandemic era since viral infections of the respiratory tract have a similar route of transmission as SARS-CoV-2.

Keywords SARS-CoV-2, COVID-19, alternative therapy, sampling approach, viral infections of the respiratory tract

Since the COVID-19 pandemic started in 2019, several variants of SARS-CoV-2 have emerged from time to time. Undoubtedly, at present (the end of 2022) Omicron and its subvariants have become the predominant variant in terms of patients with COVID-19 due to its rapid spread and lower lethality. Unlike previous variants, the Omicron variant has peculiar clinical and epidemiologic characteristics: *i*) It tends to cause severe disease less often. The Omicron variant is less likely to induce pro-inflammatory cytokines and thereby cause a "cytokine storm". Hence, lung function is seldom diminished. *ii*) Upper respiratory symptoms such as a cough, fatigue, and a stuffy or runny nose are most commonly reported. *iii*) Most patients are asymptomatic or have mild symptoms (*1*). Although patients infected with Omicron are less likely to develop severe illness in comparison to previous variants, Omicron remains a danger in older people, and particularly in the unvaccinated (*2*). Moreover, the Omicron variant exhibits stronger immune evasion than previous strains, and easier to cause reinfection. Accordingly, the dangerousness of Omicron infection is far from being completely ignored and or underestimated. Taking measures to control the transmission of the Omicron variant remains an important public health task for global healthcare administrators.

Based on the nature of its potent infectivity and the existence of asymptomatic cases, conducting mass, frequent, and repeated sampling to identify asymptomatic patients is crucial to controlling viral transmission. The commonly reported methods of sampling are nasopharyngeal swabs (NPS), oropharyngeal swab (OPS), saliva and gargle (Figure 1A). The advantages and disadvantages of these sampling methods are listed in Table 1. Since the Omicron variant mainly affects the upper respiratory tract, saliva or gargle sampling might be a satisfactory solution compared to conventional methods. Qiao *et al*. conducted a pilot study to verify the efficiency of a saline gargle sampling at detection of the Omicron variant. Their sample was small, but they found that saline gargle sampling was no less efficient than an NPS. Interestingly, they found that saline gargle sampling might have better sensitivity at identifying asymptomatic patients. This was the first study to verify the efficiency of a saline gargle as a sampling method in the context of Omicron.

Another important issue is alternative therapy for the Omicron variant. Thus far, there is no specific treatment for COVID-19. The main treatments for COVID-19, such as corticosteroids and antivirals (remdesivir, lopinavir, ritonavir, *etc*.), vitamin supplements (vitamin D, vitamin C, vitamin E, and zinc), and antibiotics, are commonly used to treat patients infected with a previous variant, and particularly for those patients with severe disease (*3*). Patients who are asymptomatic or who have mild symptoms account for the majority of patients

infected with Omicron. Accordingly, few patients infected with the Omicron variant require treatments like corticosteroids and antibiotics. In contrast, many patients prefer convenient, simple, and low-cost alternative therapies. Mechanisms of these reported alternative therapies include: *i*) *Directly killing the virus*: Such methods usually use a disinfectant directly administered into the upper respiratory tract, such as nasal irrigation of one nostril with povidone-iodine (*4*), to achieve "physical killing". Nebulization of ozone (O_3) also falls under this type (*3*). *ii*) *Removal of the local virus*: Several studies have reported using non-disinfectants such as saline (*5*) and triamcinolone acetonide (*6*) for nasal irrigation.

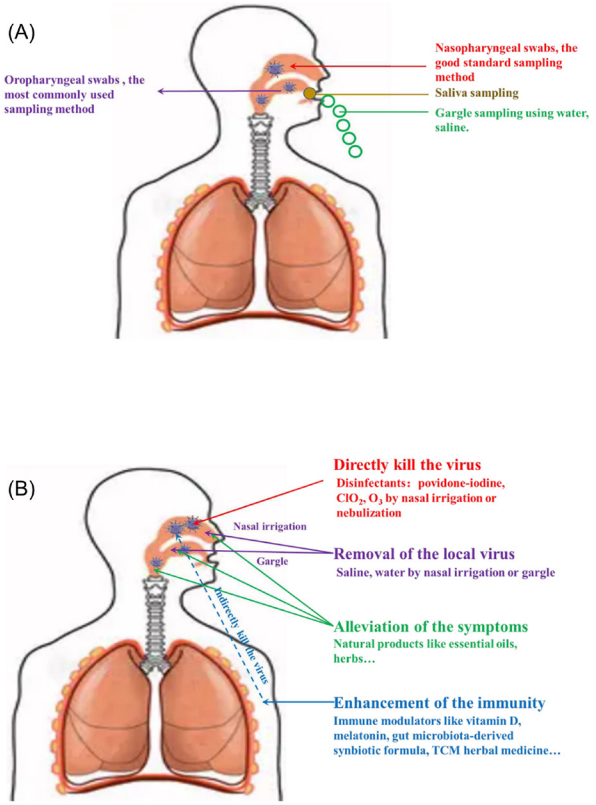

Figure 1. Mainstream sampling approaches and alternative therapies for SARS-CoV-2. (A), The reported sampling approaches to detect SARS-CoV-2. **(B)**, Mechanisms of the available alternative therapies to treat COVID-19.

They contend that such nasal irrigation may "wash out the nasal cavity", helping to reduce the viral load in the upper respiratory tract and thereby ameliorating COVID-19-related symptoms. Studies in Mexico even orally administered chlorine dioxide (ClO_2) in a low dose to prevent/treat COVID-19-related symptoms in relatives living with COVID-19 patients (*7,8*). *iii*) *Alleviation of symptoms using natural products*: This scenario includes a number of natural products such as Indonesian herbal compounds (*9*), Iran herbal medicine (*10*), propolis and honey (*11*), Indian herbs (*12*), essential oils (*13*), and traditional Chinese medicine (TCM) (*14-16*). Although all of these studies contend that these natural products "are efficacious and safe", these products are not amenable to becoming generally accepted therapeutics worldwide due to regional and cultural characteristics. Moreover, more rigorously designed randomized controlled trials need to be conducted to provide more compelling evidence regarding the safety and efficacy of these natural products. *iv*) *Enhancement of immunity by supportive treatment*: Naturopathic treatment (*17*), negative pressure therapy (*18*), and neurological music therapy (*19*) have also been cited as efficacious against COVID-19. Cao *et al*. discussed the possibility of using ClO_2, a safe and highly effective disinfectant, as a potential agent for nasal irrigation. In comparison to povidone-iodine that was previously used, ClO_2 seems to have better antiviral action and cause less irritation. Nasal irrigation with ClO_2 might be a more satisfactory solution to the Omicron variant of SARS-CoV-2. Hence, their final results are eagerly anticipated.

The Omicron variant has, in fact, markedly changed how people view SARS-CoV-2 as well as COVID-19. However, no one knows when the pandemic will finally abate. Moreover, no one knows what new variants of SARS-CoV-2 and what emerging respiratory viruses will emerge in the future. Coinfection with COVID-19 and the other respiratory viruses such as influenza might be a potential threat to public health (*20*). Viral infections of the respiratory tract have a similar route of transmission, so measures being taken against SARS-CoV-2 might also be potentially effective against other emerging viral infections that mainly affect the respiratory system. Thus,

Table 1. Advantages and disadvantages of the reported methods of sampling

Items	Advantages	Disadvantages
Nasopharyngeal swabs (NPS)	Gold standard with the best sensitivity	Uncomfortable, might be resisted by some individuals, not suitable for some patients, requires an experienced tester, might cause sneezing, which is risky for the tester.
Oropharyngeal swabs (OPS)	Acceptable sensitivity and acceptable comfort; the most commonly used method of sampling. Easier to perform than an NPS.	Lower sensitivity than OPS, is also risky because it might cause a cough.
Saliva	Convenient, easy to perform, allows self-sampling.	Not easy to standardize, lower sensitivity than NPS and OPS.
Gargle	Convenient, easy to perform, allows self-sampling. Easy to standardize.	Lower sensitivity than NPS and OPS for non-Omicron variants.

development of novel alternative sampling/therapeutic approaches for COVID-19, and verification of their efficiency/safety, is crucial in a post-pandemic era.

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Review

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How do RNA binding proteins trigger liquid-liquid phase separation in human health and diseases?

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SUMMARY RNA-binding proteins (RBPs) lie at the center of post-transcriptional regulation and protein synthesis, adding complexity to RNA life cycle. RBPs also participate in the formation of membrane-less organelles (MLOs) *via* undergoing liquid-liquid phase separation (LLPS), which underlies the formation of MLOs in eukaryotic cells. RBPs-triggered LLPS mainly relies on the interaction between their RNA recognition motifs (RRMs) and capped mRNA transcripts and the heterotypic multivalent interactions between their intrinsically disordered regions (IDRs) or prion-like domains (PLDs). In turn, the aggregations of RBPs are also dependent on the process of LLPS. RBPs-driven LLPS is involved in many intracellular processes (regulation of translation, mRNA storage and stabilization and cell signaling) and serves as the heart of cellular physiology and pathology. Thus, it is essential to comprehend the potential roles and investigate the internal mechanism of RPBs-triggered LLPS. In this review, we primarily expound on our current understanding of RBPs and they-triggered LLPS and summarize their physiological and pathological functions. Furthermore, we also summarize the potential roles of RBPs-triggered LLPS as novel therapeutic mechanism for human diseases. This review will help understand the mechanisms underlying LLPS and downstream regulation of RBPs and provide insights into the pathogenesis and therapy of complex diseases.

Keywords Biomacromolecule, phase transition, membrane-less organelles (MLOs), human diseases, therapeutic targets

1. Introduction

RNA binding proteins (RBPs) are a kind of protein family ubiquitously in eukaryotes and are considered key regulatory components and critical interaction partners for all cellular RNAs (*1*). In addition to the extensive physiological functions of RBPs, their defective expressions and intracellular mis-localization are contributed to a variety of human diseases, such as virus infection, cancer, aging-related diseases and neurodegenerative diseases (*2,3*). Generally, RBPs are characterized by the presence of RNA-binding domains (RBDs), through which they bind to target RNAs, thus regulating the fate or function of the bound RNAs. Moreover, some RBPs possess a high percentage of intrinsically disordered regions (IDRs) or prion-like domains (PLDs), which provokes great interest in deciphering the molecular mechanisms of RBPs in cellular compartmentalization and liquid-liquid phase separation (LLPS) (*4,5*). In other words, the unique structural features of RBPs allow them to assemble with RNAs/proteins to form dynamic liquid-like condensations *via* LLPS, thus controlling the RNA life cycle.

LLPS is a reversible and metastable de-mixing, where proteins and/or RNA spontaneously segregate to form two coexisting liquid phases (the condensed phase and the dilute phase) mediated by transient, multivalent interactions (*6*). LLPS is one of the important mechanisms by which organisms respond to external stimuli and protect themselves, regulating multiple physiological and pathological processes (*7*).

Moreover, LLPS acts as the foundation and driving force of the formation of membrane-less organelles (MLOs), which explains the self-assembly process of subcellular structures (*8*). MLOs, ubiquitous functional subunits of intracellular organization, are primarily responsible for localizing and regulating complex biochemical reactions intracellular, offering facile transport of substrates for cells. Additionally, numerous studies have shown that multiple aggregation-prone RBPs, such as Fused in sarcoma (FUS), Human antigen R (HUR), Ras-GAP SH3 domain binding protein 1 (G3BP1), TAR DNA-binding protein 43 (TDP-43) and T cell intracellular antigen-1 (TIA-1), spontaneously aggregate and develop MLOs by LLPS process (*9*). Notably, prominent MLOs condensed by RBPs, including stress granules (SGs), process bodies (P-bodies) and germ cells, are thought to orchestrate many important biological processes and in some cases drive diseases. However, there is still no systematic review of RBPs-triggered LLPS and their physiological and pathological functions. Therefore, in the present review, we will focus on the pioneering works of elucidating the molecular mechanism of RBPs-triggered LLPS and their physiological and pathological roles. This review will promote the current understanding of the molecular biology of RBPs-driven LLPS, provide new insights into the function of RBPs and offer future directions for RBPs and LLPS research.

2. Structural characteristics of RBPs involved in LLPS

RBPs are evolutionarily deeply conserved and generally ubiquitously expressed in eukaryotes, which just mirrors their central roles in the RNA life cycle. Structurally, RBPs are often characterized by the presence of RNA-binding domains (RBDs) and intrinsically disordered regions (IDRs) (*10*). Thereinto, RBDs are the functional units responsible for binding RNA in RBPs and primarily recognize their target sites through the sequences and shape of RNA. Moreover, RBDs of RBPs also serve important roles in the formation of membrane-less organelles (MLOs), thus participating in the compartmentalization, organization and stress response of the cells (*8*). On the other hand, IDRs in RBPs are repetitive and have a high content of glycine, arginine, lysine and tyrosine residues, which are commonly located in domains that interface with RNA. IDRs interaction is particularly important for assembly of RBPs and formation of multi-component MLOs (*11*). Interestingly, there is mounting evidence of RBPs containing RBDs and IDRs possess a particularly high LLPS propensity through complex interactions of multivalent protein-protein, protein-RNA and RNA-RNA (*5,12*). Moreover, RBPs also exert their regulatory roles through various post-translational modifications (PTMs), which are conducive to triggering LLPS to enable the cell to quickly and efficiently respond to stress stimuli (*3*). For example, multiple PTMs of FUS, such as serine and threonine phosphorylation as well as arginine methylation, can strongly influence the biophysical properties of FUS aggregation and LLPS (*13*). In a word, the unique structural composition and sequence characteristics of RBPs are conducive to their assembly and LLPS (Figure 1A).

3. LLPS underlies MLOs formation

As biological evolution, the emergence of diversification

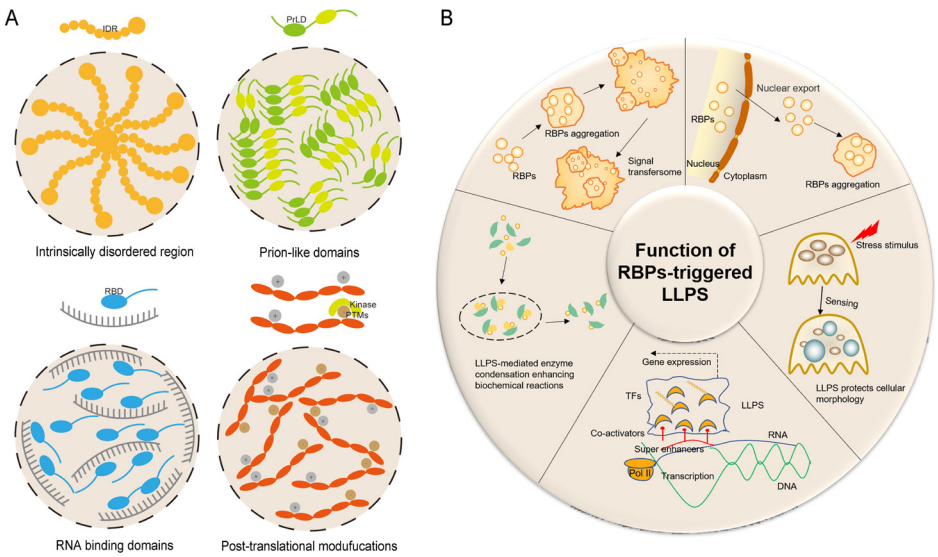

Figure 1. Structure characteristics of RBPs involved in LLPS and roles of RBPs-triggered LLPS in controlling RNA life cycle. A. The unique structures that drive the LLPS of RBPs. RBPs usually contain IDRs, PrLD, RBDs and PTMs which are vital for RBPs to undergo LLPS. **B**. The function of RBPs-triggered LLPS in RNA life cycle. RBPs and its LLPS are involved in regulating cellular mechanotransduction, intracellular biochemical reactions, cellular homeostasis, endocrine and gene transcription.

of organelles at the cellular level allows different biological reactions in specific organelles orderly. Similarly, MLOs are also involved in various cellular biological processes due to the concentrated nucleic acid and protein within them (*14*). LLPS is an important organizing principle and theoretical basis of MLOs, which explains the regulation mechanisms of MLOs in the assembly, composition and function.

Recently, numerous studies have indicated that these ubiquitously MLOs in eukaryotic cells modulate a diversity of physiological and pathological traits in multiple ways, which are closely related to the physical properties, types and intracellular localization of MLOs. Moreover, these MLOs formed by LLPS are distributed in the cytoplasm, nucleus, and on the membrane. Figure 2 displayed the various biomolecular condensates formed by LLPS and the assembly of stress granules (SGs). Cytoplasmic MLOs are dynamically assembled by the LLPS driven by the temporarily untranslated RNAs and proteins which coalesce into a concentrated state (condensed phase) in the cytoplasm. Prominent examples of cytoplasmic MLOs mainly include stress granules (SGs), processing bodies (P-bodies), RNA transport granules and germ granules. SGs are one of the predominant types of cytoplasmic MLOs formed by the crowded protein and RNA. Under stress, SGs immediately start to accumulate and regulate the mRNA utilization in eukaryotic cells, which is essential for maintaining cell integrity and intracellular homeostasis. Moreover, P-bodies are highly conserved cytoplasmic foci with properties of liquid droplets and have been observed in somatic cells originating from vertebrates and invertebrates, plants and yeast. P-bodies are formed by LLPS and are primarily composed of translation-arrested RNAs and RBPs related to mRNA decay, suggesting roles in post-transcriptional control. In addition, LLPS also appears to be important for driving the assembly of various nuclear-localized MLOs such as nucleoli, Cajal bodies and nuclear speckles, and underlie their biogenesis. Nucleolus, as the most prototypical and prominent MLO in nuclear, forms around regions of chromosomes containing stretches of tandem ribosomal DNA (rDNA) gene repeats, known as nucleolar organizer regions (NORs) (*15*). Nuclear speckles are another well-studied MLOs formed by LLPS in nuclear, exhibiting dynamic and irregular shapes. Nuclear speckles are subnuclear structures enriched in RBPs, particularly those involved in splicing, located in the interchromatin regions of the nucleoplasm of mammalian cells (*16*).

4. Well-studied RBPs involved in LLPS

Numerous RBPs, especially RBDs/IDRs-harboring RBPs, readily undergo concentration-dependent LLPS and mediate protein/RNA interactions to form MLOs (*17*). In eukaryotic cells, diverse stresses trigger coalescence and condensation of RBPs, which is an essential prerequisite for LLPS. Under stress stimuli, RBPs recruit the translation-stalled mRNA to form MLOs with liquid-like properties, which precisely support the fact that numerous dynamic MLOs (such as SGs) are rich in RBPs. The assemblies of condensates formed by phase transitions of RBPs are implicated in both health and disease, triggering interests in deciphering the molecular mechanisms of compartmentalization orchestrated by RBPs in both physiology and pathology. Until now, the LLPS of multiple RBPs, including TIA-1, FUS, G3BP1, HUR, poly (A)-binding protein (PABP),

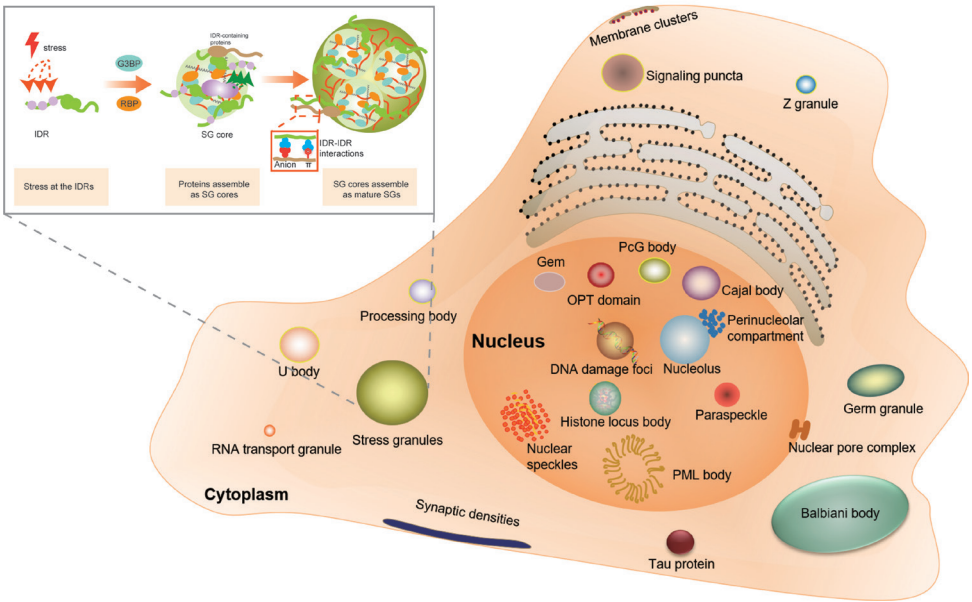

Figure 2. Types of membrane-less organelles (MLOs) in cells driven by LLPS. All these MLOs distribute in plasma membrane, cytoplasm and nucleus to perform their own duties. SGs are one of the most common MLOs in cytoplasm, whose formation process is driven through LLPS of RBPs.

Table 1. Overview of RBPs involved in the process LLPS.

RBPs	MLOs Association	Biological Function	Number of IDRs[a]	Overall percent disordered (%)[b]	Mechanisms of LLPS Occurrence
HUR	SGs (*34*)	RNA stability	8	19.33	The HNS domain makes it from the nucleus to the cytoplasm, accumulates aggregates, and colocalizes with G3BP1
TIA-1	SGs (*36*)	mRNA silencing	8	25.45	Self-association of the intrinsically disordered prion-like domain that facilitates LLPS
TDP-43	Nuclear gems (*90*); nuclear stress bodies (*91*); SGs (*24*); paraspeckles (*92*)	transcriptional and posttranscriptional regulation	8	35.78	The IDR, the unique structure of the C-terminal domain, and multiple RNA-binding sites for TDP-43 make it trigger LLPS
hnRNPA1	SGs (*93*); paraspeckles (*92*)	pre-mRNA splicing, mRNA stability	5	29.06	The 'prion-like' LC domain mediates hnRNPA1 self-association to trigger LLPS
FUS	Paraspeckles (*94*); Nuclear gems (*94*); SGs (20)	mRNA transport and translation, gene splicing	8	72.58	LC domain, C-terminal domain, and RRM, these self-assembling regions drive LLPS behavior, which is influenced by the various PTMs of FUS
G3BP1	SGs (*25*)	Ras signaling/marker of SGs	4	53.22	Three IDRs in G3BP1 interact according to the saturating concentration of RNA to change conformation and turn on LLPS
NCL	Nucleolus (*95*)	rDNA transcription, rRNA maturation, ribosome assembly	13	55.49	NLS enables it to enter the nucleus, where RBDs and RGG domains bind to other nucleolar components such as rRNA
PABP-1	SGs (*96*)	Translation stability	11	33.57	Unclear
EWSR1	DNA damage response foci (*97*); SGs; paraspeckles (*92*)	fuse with various partner genes	7	79.85	Interaction of the LC domain with the RGG domain contributes to self-association
TAF15	DNA damage response foci (*97*); SGs (*98*); paraspeckles (*92*)	Regulation of gene transcription	7	51.1	LC-RGG, LC-LC, and RGG-RGG interactions contribute to self-association
hnRNPA3	SGs (*99*)	pre-mRNA splicing, mRNA stability	7	38.76	LC region mainly drives self-association and RRM domain may function in the presence of RNA

[a], The number of IDRs within the proteins' sequences; [b], The proportion of disordered regions in the total protein sequence.

TDP-43, heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and nucleolin (NCL), have been well-studied. The detailed information of RBPs involved in LLPS was listed in Table 1.

4.1. Fused in sarcoma (FUS) drives LLPS

Fused in sarcoma (FUS) is a ubiquitously expressed and predominantly nuclear-localized RBP. Multiple well-established functions – mRNA transport and translation, gene splicing and gene expression – have been ascribed to FUS. Emerging evidence has shown that FUS undergoes a reversible dynamic phase transition between a dispersed state, liquid droplets, and hydrogels (*5,18*). Structurally, FUS maintains some self-assembly regions including prion-like low-complexity (LC) domains, C-terminal domains and RNA recognition motif (RRM), which are vital for driving LLPS behavior. Moreover, the various PTMs (*e.g.*, phosphorylation) of FUS also alter its localization, concentration and aggregation thus influencing the self-assembly or LLPS driven by FUS (Figure 3A). Monahan *et al*., showed that both phosphorylation and phosphomimetic variants reduce FUS propensity to aggregate and disrupt the LLPS and toxicity of FUS in the presence of RNA or salt (*19*). Collectively, the importance of FUS in the cellular life cycle, together with its facile self-assembled structures, raises the possibility that natural selection and evolution have preserved the LLPS propensity of FUS. The response of FUS to cellular stress also involves the formation of MLOs which are transient regulatory structures. For instance, under stress conditions like oxidative stress and hyperosmolar stress, FUS rapidly shuttles from the nucleus to the cytoplasm and assemble into SGs or P-bodies *via* LLPS (*20*). Upon removal of these cellular stress, liquid-like droplets of FUS disassemble in seconds. Noteworthy, excessive recruitment of FUS into phase-separated liquid-like droplets followed by aggregation has been proposed to drive disease. Taken together, proper assembly and aggregation of FUS through LLPS play essential roles in maintaining normal cellular physiological function. Inversely, aberrant LLPS driven by FUS mutants has been proposed as a cause of cellular dysfunction and

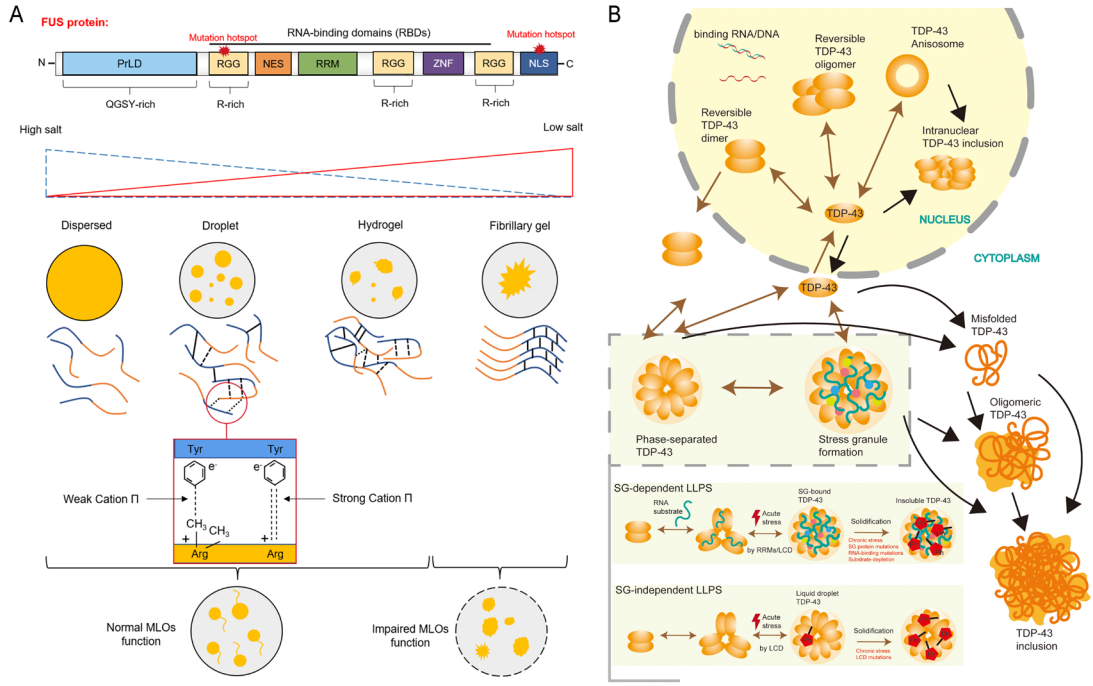

Figure 3. The molecular mechanisms of FUS- and TDP43-driven LLPS. A. FUS maintains self-assembly regions including prion-like low-complexity (LC) domains, C-terminal domains and RNA recognition motif (RRM), which are vital for driving LLPS behavior. **B.** TDP-43 can translocate from nucleus to cytoplasm under various stress conditions and form multiple MLOs through phase transition.

multiple severe diseases (be discussed in detail in the subsequent sections).

4.2. TAR DNA-binding protein 43 (TDP-43) drives LLPS

TAR DNA-binding protein 43 (TDP-43) is a highly conserved, ubiquitously expressed multi-domain RBP, belonging to the heterogeneous nuclear ribonucleoprotein (hnRNP) family (Figure 3B). TDP-43 is mainly implicated in the transcriptional and posttranscriptional regulation of mRNA transcripts that it binds (*21*). Under physiological conditions, TDP-43 is normally located in the nucleus of most cells. However, as a nucleocytoplasmic shuttling protein, TDP-43 also can translocate from nucleus to cytoplasm under various stress conditions, where it processes distinct cytoplasmic functions, including mRNA stabilization. Recently, several studies revealed that PrLD/IDR-containing TDP-43 undergoes LLPS and spontaneously develops membrane-less organelles. However, distinct from other RBPs, TDP-43 has no dominant LLPS motif in its intrinsically disordered C-terminal domain, how then can TDP-43 trigger LLPS? Structurally, there is a unique IDR with prion-like glutamine/asparagine (QN)-rich regions and central α-helical element at the C-terminus of TDP-43, which is critical for undergoing LLPS to form condensates. Specifically, the QN-rich domains in the IDR are predominately responsible for the aggregation of proteins. The α-helical element spans roughly 20 residues in the center of the domain and is involved in intermolecular interactions, that is just the reason why LLPS is controlled by fewer motifs in the C-terminal domain of TDP-43 (*22*). Moreover, this process of LLPS is primarily driven by three tryptophans in the α-helix, among which Trp-334 is the most important one and followed by Trp-385 and Trp-412. Notably, Trp-334 enables the α-helical element with a high intrinsic propensity for self-assembly, which enhances the intermolecular interaction and thus facilitates LLPS. On the other hand, several pieces of research showed that RNA binding also increases the liquid-like properties of TDP-43 condensates (*23*). The physiological interaction containing multiple binding sites of TDP-43 with RNAs significantly strongly nucleates TDP-43-driven multivalent LLPS and maintain its liquid properties. Collectively, the unique structures of IDR and C-terminal domain, as well as the multiple RNA binding sites of TDP-43, have prompted it more amenable to form various membrane-less organelles through LLPS.

Reportedly, TDP-43 forms different membrane-less organelles in various cell types, including Cajal bodies and paraspeckles in the nucleus as well as SGs in the cytoplasm (*24*). Given that TDP-43 is a nucleocytoplasmic shuttling protein, SGs are the most common type of membrane-less organelles formed by the accumulation of TDP-43 in the cytoplasm in response to stress. A variety of cellular stresses, such as heat shock, oxidative stress and osmotic, normally trigger TDP-43 to transiently localize to the cytoplasm and assemble into SGs. Moreover, localization of TDP-43 to SGs is generally mediated by both its RRM1 domain as well

as its C-terminal prion-like QN-rich domain. Notably, normal aggregation and assembly of TDP-43 into SGs are essential complexes that modulate RNA translation during stress. Nevertheless, sustained stress and ensuing TDP-43 misfolding or mislocalization are directly toxic and create aberrant SGs and pathogenic TDP-43 aggregates, which is a hallmark of a spectrum of neurodegenerative disorders (be discussed in next section).

4.3. G3BP1 drives LLPS

Ras-GAP SH3 domain binding protein 1 (G3BP1), one of the members of phosphorylation-dependent endoribonuclease that interacts with RasGAP, is a highly conserved multi-domain RNA binding protein involved in a variety of biological processes and diseases. Recently, G3BP1, acting as a molecular switch for the process of LLPS during the formation of MLOs (especially SGs), has drawn increasing attention among researchers (*25*). Several studies have revealed that G3BP1 is often involved in the initiation of SGs formation and is recruited to SGs in response to environmental stress (*26,27*). Therefore, G3BP1 is deemed as an essential determinant of the fate of mRNAs during cellular stress and a critical effector of SGs assembly. For example, Tourrière *et al*., showed that G3BP1 is rapidly recruited to SGs in cells exposed to sodium arsenite (SA), a well-recognized chemical stressor for inducing SGs (*28*). Moreover, Sun and colleagues observed that, upon newcastle disease virus (NDV) infections, endogenous G3BP1 was induced to present as punctate fluorescence and form stable SGs (*29*). Importantly, G3BP1 is often directly bonded by many viruses to specifically inhibit SGs formation thus evading the host's innate immune response (*27*). Another research also revealed that cells lacking both G3BP1 and G3BP2 cannot form SGs in response to p-eIF2α or eIF4A inhibition (*30*), indicating that G3BP is essential for the assembly of SGs initiated by p-eIF2α or eIF4A inhibition. Additionally, some other research holds the point that G3BP1 serves as the central node of the protein-RNA interaction network that triggers LLPS and subsequently assists SGs to assemble (*31*).

Generally, G3BP1 initiates and maintains the process of LLPS and SGs assembly *via* several well-recognized mechanisms. Specifically, one of the well-recognized mechanisms holds that the interplay between three IDRs in G3BP1 regulates its intrinsic propensity for LLPS and thus contributes to the SGs assembly (*25*). Interestingly, unlike the conventional IDRs, IDRs in G3BP1 have evolved to fine-tune the saturation concentration of RNA for LLPS. When RNA concentrations are low, the acidic IDR1 and the basic IDR3 favorably interact with each other to create a compact "closed" conformation. Above a threshold RNA concentration, RNA displaces IDR1 to bind IDR3, which permits the G3BP1 homodimer to adopt an expanded, "open" conformation, initiating LLPS. On the other hand, G3BP1 also facilitates and nucleates SG assembly by binding its RGG motif with 40S ribosomal subunits, which is also essential for G3BP1-mediated SGs formation. Moreover, the domain architecture of G3BP1 dimerization is another intrinsic property that influences the speed of LLPS and SGs assembly *in vitro*. Altogether, G3BP1 is a SGs-resident protein and acts as a tunable switch that triggers LLPS to nucleate SGs assembly through multiple interactions or peculiar structures.

4.4. Human antigen R (HuR) drives LLPS

Human antigen R (HuR), also known as HuA and ELAVL1 (embryonic lethal abnormal vision-like 1), belongs to the fourth member of the ELAVL family. As a well-established mRNA stabilizing RBP, HuR principally regulates the stability and translation of its target mRNAs to involve in multiple pathological processes. Although HuR appears predominantly localized in the nucleus, after exposure to specific stresses, it shuttles to the cytoplasm where HuR exerts its function in the stabilization of ARE-mRNA, which requires the HuR nucleocytoplasmic shuttling (HNS) domain (*32*). Recently, HuR has been repeatedly reported to assume essential roles in cellular stress responses, especially in the assembly of SGs. HuR aggregates and forms SGs in the cytoplasm under stresses, such as heat shock, oxidative stress and ultraviolet radiation (UV) (*33*). For example, Yoon *et al*., revealed that in human cervical carcinoma cells, sodium arsenite exposure enhances the accumulation of HuR in SGs and is accompanied by increased HuR binding to target transcripts SIRT1 and VHL mRNAs and by stabilization of these mRNAs (*34*). Moreover, heat shock treatment also promotes HuR to translocate from the nucleus into the cytoplasm, forming SGs, colocalizing with the SGs marker — G3BP1 protein, and regulating the translation of its binding mRNA (*35*). These studies indicated that HuR is an essential component of SGs and highlight that HuR presence in SGs associated with the fate of target mRNAs.

4.5. T cell intracellular antigen-1 (TIA-1) drives LLPS

T cell intracellular antigen-1 (TIA-1) is a prototypical prion-related RBP that consists of three N-terminal RRMs and a C-terminal intrinsically disordered low complexity domain (LCD), which play a central role in facilitating LLPS. TIA-1 is considered as one of the canonical scaffold proteins involved in nucleating LLPS, which regulates target mRNA translation in the cytosol under stresses *via* inducing a conformational change that favors LLPS (*36*). TIA-1 is a cellular stress response protein that shuttles into the cytoplasm promptly and facilitates the assembly of SGs upon stress. TIA-1 is a key component of SGs and makes cells

more sensitive to stress thus affecting SGs formation. As an RBP, TIA-1 often sequesters target RNAs into SGs that allow these RNAs to escape the unfavorable cellular stresses, such as heat shock, oxidative stress and hypertonic stress. Moreover, some evidence shows that TIA-1 overexpression is also sufficient to drive a spontaneous formation of SGs even in the absence of stress, presenting the importance of TIA-1 for SGs (*37*). Another study demonstrated that TIA-1 mutation slows the disassembly of SGs following heat shock in HeLa cells (*38*). How then does TIA-1 be involved in SGs assembly and disassembly? TIA-1 condenses and forms SGs *via* the self-association of its intrinsically disordered prion-like domain that facilitates LLPS. Besides, multiple RNA binding sites of TIA-1 are thought to induce aggregation of TIA-1 through LLPS, thus promoting the assembly and formation of SGs. The RRMs of TIA-1 are also essential for SGs formation (*37*). Specifically, interactions between folded RNA recognition motifs in granule proteins and RNA stimulate the formation of SGs.

5. RBPs-driven LLPS regulates physiological function

LLPS, a ubiquitous biophysical phenomenon of the cellular interior, governs multiple biochemical reactions therein. Notably, as key players in RNA metabolism and function, RBPs tend to aggregate and undergo LLPS to regulate various cell physiological activities and protect against extracellular stress perturbations. In this section, we will focus on the cellular physiologic function of RBPs-driven LLPS (Figure 1B).

5.1. Regulation of cellular mechanotransduction

Mechanical stimuli are essential for maintaining normal cell growth and development (*39*). Emerging evidence is revealing that LLPS plays essential roles in mechanosensing for regulating the mechanobiological signal coupling and mechanosensitivity of cells. For example, mechanical stretch regulates the SGs formation in smooth muscle cells thus affecting the protein translation and cell physiology (*40*). Another research revealed that biomacromolecule LLPS facilitates the assembly and organization of matured FAs, thus allowing for efficient mechano-transduction and cell migration. Moreover, the disturbance of LLPS can change the dynamics, mechanical sensitivity and durotaxis of cells (*41*). A new finding indicated that the mechanosensitive lncRNA Neat1 could undergo LLPS and form paraspeckle (an important nuclear MLO) under various mechanical stimuli, and subsequently enhances bone strength and promotes osteoblast function (*42*). These findings prompted that LLPS can respond to mechanical stimuli and thus regulate the mechanical sensitivity of cells, in turn, LLPS also requires mechano-transduction.

5.2. Regulation of intracellular biochemical reactions

LLPS is known to facilitate the subcellular compartmentalization (MLOs formation) and enrich proteins/RNAs locally, which allow the cells to carry out biochemical reactions smoothly and orderly. On the one hand, LLPS is in charge of concentrating biomacromolecules as MLOs thus promoting the biochemical reactions rate. On the other hand, LLPS can also inhibit some reactions by restricting molecular motion and sequestering the substrates from proteins (*43*). MLOs formed through LLPS, including SGs, nucleoli and P granules, are responsible for compartmentalizing the biochemical reactions in cells. Interestingly, RBPs concentration by LLPS also provide a highly cooperative mechanism to locally concentrate RNAs and promote cellular reactions (*44*). In addition, LLPS also regulate the cellular biochemical reactions through modulating the enzyme activities, which are important omnipresent catalysts of biochemical reactions. LLPS can selectively enrich or repel molecules, increase the molecular interactions, even modify the molecule conformations, and subsequently impart specificity to biochemical processes. For example, condensates formed by LLPS could condense an enzyme and its possible substrates to a specific subset, conferring specificity to the potentially promiscuous biochemical reaction (*8*). In summary, LLPS and the triggered RBPs condensation are crucial for cellular biochemical reactions.

5.3. Regulation of cellular homeostasis

Cells are continuously exposed to external stress such as salt concentration, pH, temperature and oxidative stress and are regulated by these external stressors. Accumulating evidence indicates that LLPS possesses both the ability to sense the external stress and the flexibility to respond to the changes, playing versatile roles in the maintenance of cellular homeostasis (*8*). Especially, RBPs condensation formed by LLPS can communicate with each other freely within the condensates and their surrounding solution, which enables cells to rapidly sense and respond to external stress signals. For instance, Riback *et al*., reported that the poly(A) binding protein Pab1, as an important RBP, synthesizes multiple thermal and pH signals into a unified quinary response through phase separation, thus enhancing cells to maintain cellular homeostasis and its adaptability to the environment (*45*). Moreover, LLPS of RBPs mediates multiple biological events related to redox maintenance by modifying the phase behavior of macromolecules, providing a membrane-less compartment that a cell can tap in response to oxidative stress. For example, Kato *et al*., reported that Pbp1 directly responds to redox imbalance, and the self-association of methionine-rich LC domain of Pab1 is readily oxidized upon oxidative stress induced by

dysfunctional mitochondria or H_2O_2 (*46*). Interestingly, the formation of LLPS is in turn modulated by these different external stress conditions. As the temperature is crucial for the cellular homeostasis and easily controlled *in vitro*, thermo-responsive biomolecular LLPS has been studied extensively. For example, both full-length FUS and its LC domain undergo LLPS in a temperature-dependent manner (*47*). LLPS of proteins such as folded egg-white lysozyme and the N-terminal intrinsically disordered regions (IDRs) of DEAD-Box helicase Ddx4 occurs only at temperatures below a critical temperature *Tc*, whereas LLPS of proteins like Pab1 and IDP α-elastin occurs at temperatures higher than *Tc* (*48*). Taken together, there is a reciprocal regulatory relationship existing between LLPS processes and the external stress the condensate environment in cells. LLPS is responsible for protecting against external stress perturbations in cells and maintaining cellular homeostasis during cellular stress.

5.4. Regulation of gene transcription

Transcription, as a vital contributor to functional cell states and intracellular gene expression, is strictly regulated by multiple factors including transcription factors (TFs), coactivators, enhancers as well as RBPs. RBPs-driven LLPS and the formed MLOs have emerged as a novel mechanism by which these factors regulate transcription (*49*). Transcription is driven by a type of protein termed RNA polymerases (Pol I, Pol II and Pol III), of which Pol II is responsible for producing messenger RNAs and non-coding RNAs and is considered the most important polymerase. Multiple RBPs prone to undergo LLPS are involved in the transcription regulation upon both normal and stress conditions. For example, representative RBP like FUS has been reported to be able to form phase-separated condensates thus recruiting the intrinsically disordered carboxy-terminal domain (ID-CTD) of Pol II to trigger the target gene transcription (*50*). Importantly, LLPS not only regulates transcription initiation but also modules the elongation phase of transcription. For instance, LLPS of the negative elongation factor (NELF) and positive transcription elongation factor b (P-TEFb) were found to modulate the transition from promoter-proximal pausing to transcription elongation (*51*). Specifically, P-TEFb, as the promotor of transcription elongation, translates from the paused Pol II into a condensate through phase separation to facilitate the phosphorylation of NELF and the CTD of Pol II, therefore forming elongation condensates and promoting transcription. In addition to RNA transcription regulation, LLPS also participates in DNA transcription regulation, particularly in the functions of CTD of Pol II and associated proteins, the disordered activation domains of transcription factors, and heterochromatin proteins (*52, 53*). The evidence reviewed here suggests that LLPS plays prominent roles in various stages of transcription regulation.

6. RBPs triggered-LLPS results in human diseases

In recent years, researchers have realized that the RBP-induced liquid-liquid separation process is the basis for the formation of MLOs, which is necessary to maintain normal cell functions. Nevertheless, there are emerging studies showing that abnormal assembly and LLPS of RBPs are also closely related to the pathogenesis of various human diseases, like neurodegenerative diseases, cancer and aging diseases. The following section introduces the roles of RBPs-triggered LLPS in human diseases (virus infection, cancer, neurodegenerative diseases and aging-related diseases) (Table 2 and Figure 4A).

6.1. Corona Virus Disease 2019 (COVID-19)

Recently, COVID-19 pandemic is becoming one of the largest global public health crises in modern history. As the etiologic agent of COVID-2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has also received widespread in society. Notably, SARS-CoV-2 nucleocapsid protein is an abundant RBP and is characterized by self-assembly, which is critical for viral genome packaging and transcription as well as viral replication (*54*). Lately, the mounting high-level studies have shown that SARS-CoV-2 nucleocapsid protein can undergo LLPS through its domain of dimerization *via* different ways and thus manage to outsmart host antiviral defense mechanisms. Factually, LLPS has been reported to serve as a scaffold for virus replication and accelerates viral assembly as well as virus production through proximity-dependent interactions (*55*). Here, we review and summarize the cooperative LLPS of the SARS-CoV-2 nucleocapsid protein and their roles during SARS-CoV-2 infection (Figure 4B).

For example, Chen *et al.*, have revealed that the interaction between SARS-CoV-2 nucleocapsid and single-stranded RNA (ssRNA) enables them to undergo LLPS in a Zn^{2+}-dependent manner, which further facilitated the viral assembly and transmission (*56*). Another study found that LLPS of SARS-CoV-2 nucleocapsid protein was mediated by the specific viral genomic RNA sequences and structures, which may be important for SARS-CoV-2 processes such as viral genome packaging and virus production (*57*). SARS-CoV-2 can assemble its nucleocapsid protein and genomic RNA through robust LLPS and then enters droplets formed by RBPs (FUS, TDP-43, hnRNPA2) associated with SGs formation, suggesting the essential roles of MLOs formed *via* LLPS in virus infection. Likewise, Wang and co-workers also found that the N-terminal IDRs are able to trigger the LLPS of SARS-CoV-2 nucleocapsid protein and enable them

Table 2. Pathological roles of RBPs Triggered-LLPS in human diseases

Type of disease/Specific type	Connection with LLPS	Substances involved	*Ref.*
Infectious diseases COVID-2019	LLPS manages to outsmart host antiviral defense mechanisms	SARS-Cov-2 nucleocapsid protein	(*56*)
Cancer leukemia	LLPS is essential for the development of leukemia with NUP98 fusion oncoprotein	NUP98 fusion oncoproteins (FOs)	(*60*)
Liver cancer	YAP and TAZ act as Hippo pathway effectors to inhibit liver cancer in mice through a competitive mechanism	YAP, TAZ	(*100*)
Neurodegenerative diseases Amyotrophic Lateral Sclerosis (ALS)	TDP-43 gene mutation induces ALS, Deposition of toxic protein aggregates containing RBPs (TDP-43, EWS, TAF15) can characterize related diseases	TDP-43, EWS, TAF15	(*101*)
Alzheimer disease (AD)	Abnormal deposition of Tau protein in the brain triggered by LLPS	Tau, amyloid-β	(*102*)
Parkinson	RBP induces LLPS to form pathological protein aggregates	FUS, α-synuclein	(*18*)
Aging-related diseases			
Diabetes	Islet amyloid polypeptide is gelled and aggregated by LLPS	Islet amyloid polypeptide	(*76*)

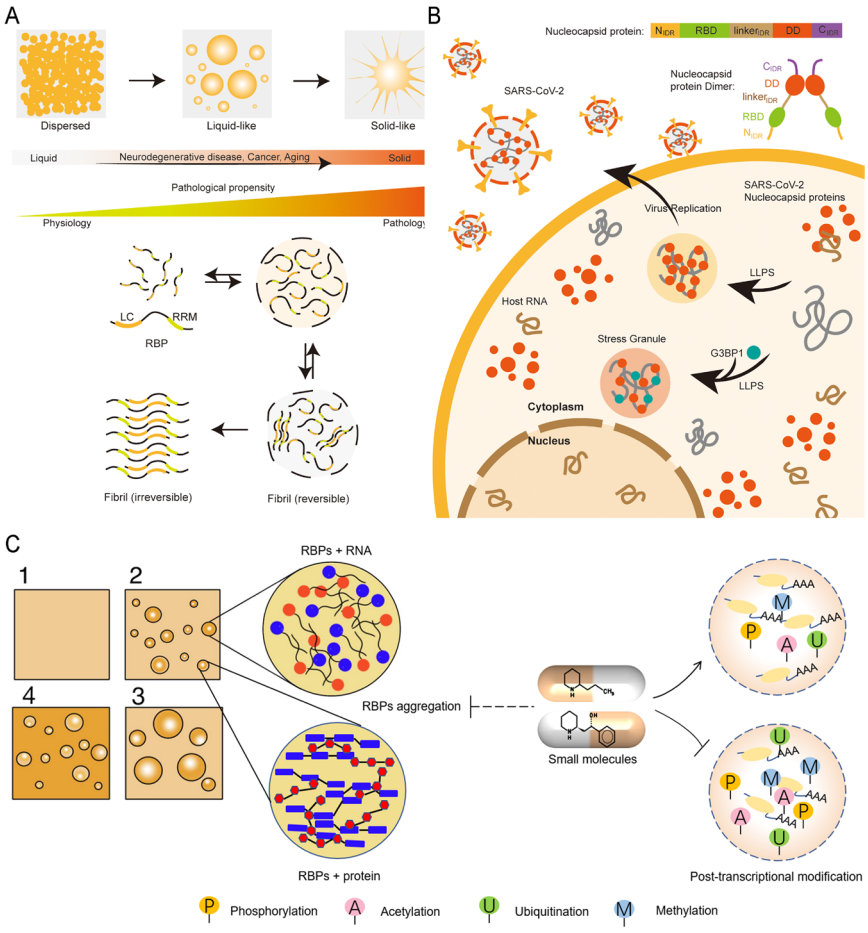

Figure 4. Pathological roles of RBPs-triggered LLPS in cells and human diseases. A. Abnormal assembly and LLPS of RBPs are also closely related to the pathogenesis of various human diseases, like neurodegenerative diseases, cancer and aging diseases. **B**. Roles of SARS-CoV-2-driven LLPS and SGs formation in virus infection of COVID-2019.

to assemble with G3BP1 into SGs to inhibit host cell innate immunity (*58*). However, differences of opinion lay behind the roles of SGs during virus infection. Some other studies showed that SARS-CoV-2 nucleocapsid protein enables to disrupt SGs assembly through interacting with G3BP1 and blocking the interaction between G3BP1 and other SG-related proteins to facilitate viral production. That is, well-assembled SGs are part of the antiviral responses during viral infection through sequestering host and viral mRNAs and proteins

(*59*).In general, LLPS of SARS-CoV-2 nucleocapsid protein may not only affect viral replication, genomic RNA as well as viral production, but also regulate host cell function to expedite viral transmission. More importantly, the LLPS activity of SARS-CoV-2 nucleocapsid protein may be a promising therapeutic target for pandemic COVID-2019 and new possibilities for the development of new antiviral drugs.

6.2. Cancer

Cancer is a pathological condition where cells uncontrollably divide and escape the regulatory bounds of normal homeostatic balance, which is maintained through precise spatiotemporal regulation. In fact, dysregulated LLPS and toxic aggregates of RBPs have been shown to regulate cancer cell pathology and are deemed as a hidden driver of oncogenic activity. In this part, we elaborate on how LLPS shapes the biochemical landscape of cancer cells.

Abnormal aggregation and assembly of RBPs lead to abnormal LLPS and drive carcinogenesis. For instance, IDRs-containing NUP98 (an important RBP) fusion oncoproteins (FOs) can undergo LLPS and thus result in aberrant transcriptional activity and transformation of hematopoietic stem and progenitor cells in pediatric leukemia (*60*). Similarly, NUP98-HOXA9, as the classical chimeric canceration protein, undergoes LLPS and forms droplet aggregates to further drives aberrant chromatin looping and cancer development (*61*). In addition, the virus-like domain of oncogenic EWS-FLI1 fusion protein enables it to undergo LLPS and assemble as condensates in Ewing sarcoma, which recruits unusual chromatin remodeling complex and promotes tumor gene expression (*62*). Besides, it has been verified that the intranuclear protein AKAP95 can undergo LLPS and regulate the splicing of cancer genes and tumor generation within the range of appropriate physical attributes. This discovery drives an unconventional idea for cancer treatment to inhibit cancer cells by controlling or interfering with biomolecule aggregates formed by LLPS.

In addition, LLPS triggered by RBPs have the function to regulate carcinogenic signals. A metabolic disorder is a typical feature of cancer cells, which is manifested in that cancer cells change the normal biosynthetic pathway to adapt to uncontrolled abnormal proliferation. LLPS serves as the main organizer of signal intervals to control carcinogenic signals. For example, PKA regulatory subunits undergo LLPS and form biomolecule aggregates rich in cAMP and PKA activity in liver cancer. Moreover, PKA fusion protein can block LLPS and induce abnormal cAMP signaling, which leads to abnormal cell activity (*63*). Besides, pathogenic SH2 mutant can lead to the alteration of the conformation of LLPS to trigger oncogene signal transduction and MAPK hyperactivation (*64*).

All in all, the close association between LLPS and cancer means that we are entering a new or exciting phase in cancer research. RBP-mediated LLPS are the hidden drivers of carcinogenesis, so it is of great research significance to regulate LLPS to directly control multiple processes in cancer. However, how the specific biochemical reactions of the aggregates formed by LLPS occur, and the exact functional relationship between the aggregates and cancer cell pathology need to be further expanded.

6.3. Neurodegenerative diseases

Neurodegenerative diseases are characterized by irreversibility and cause serious threats to human life. The toxic aggregate of RBPs and abnormal LLPS have been deemed as one of the pathological incentives and hallmarks for various neurodegenerative diseases (*65*). Therefore, abnormal RBPs and LLPS drive the exploration of new downstream mechanisms of neurodegenerative diseases.

Currently, multiple typical RBPs, such as FUS, TDP-43, TAU and they-driven LLPS are the key participants in neurodegenerative diseases (*66-68*). Specifically, genetic mutations in FUS and TDP-43 often lead to aberrant assembly condensates formation by LLPS (*68*). The prone-like LCDs of FUS enable its fibrillar amyloid assembly and thus result in amyotrophic lateral sclerosis (ALS) (*69*). The interaction of FUS with G4-RNA promotes its liquid-solid phase transition in ALS pathogenesis, which provides clues for the relationship between abnormal RBPs aggregation and ALS mechanism (*70*). Another study has revealed that ALS-FUS leads to decreased nucleocytoplasmic transport (NCT) and nucleoporin (Nup) density in the nuclear membrane of human neurons, which subsequently alters the phase separation characteristics and nucleocytoplasmic transport path in diseases (*71*). In addition, TDP-43 also participates in the pathogenic process of ALS. In 2006, mislocalization of TDP-43 was observed in the brains and spinal cord regions of ALS patients (*72*), which has been regarded as a symptom of most ALS. It was found that the excessive condensation of TDP-43 could affect its RNA networks in the context of disease (*73*). Notably, the pathological process of abnormal TDP-43 aggregation is associated with the formation of SGs. The accumulation of misfolded TDP-43 in the endoplasmic reticulum can activate PERK and phosphorylate elF2α to promote SGs formation (*74*). It has been speculated that a variety of neurodegenerative disease-related proteins are recruited to SGs when stress is applied, and are involved in the conformation of SGs. Besides, abnormal assembly of TAU and its solid phase accumulation are also involved in the development of neurodegenerative diseases (*67*). Phosphorylated free tau is mislocated and accumulates in dendrites and

somatic cells, which ultimately leads to the pathological features of neurodegenerative diseases (*75*). It has been demonstrated that aggregated TAU inhibits anterograde fast axonal transport, which supports the hypothesis that tau oligomers are toxic in neurodegenerative diseases and facilitates elucidation of the exact mechanism of tau-mediated neurotoxicity.

In summary, it is well established that aberrant RBP-mediated LLPS is one of the major causes of neurodegenerative diseases. The aberrant cellular functions are linked to the pathological liquid to solid phase transition of RBPs, which may pave the way for the potential treatment strategies of neurodegenerative diseases in the future.

6.4. Aging

Aging, an irreversible biological process, often manifests as a progressive loss of homeostasis in cells, which is characterized as RBPs aggregates. As the underlying mechanism of RBPs aggregates, LLPS is therefore believed to get involved in the pathological transitions during aging and drive the progression of age associated diseases. That is the changes of intracellular bioprocesses during aging can lead to the aberrant RBPs assembly and LLPS, in turn, these aberrant LLPS might promote aging and aging-associated diseases.

Generally, RBPs-driven LLPS can trigger the formation of mRNPs such as P-bodies and SGs to regulate the cellular functions in normal cells. As cells get aging, these mRNPs constantly aggregate and transformed into pathological accumulation that are harmful to cells. Therefore, some speculate that the abnormal phase transition of RBPs may be a vital pathogenesis for aging-related diseases. It is reported that islet amyloid polypeptide can undergo LLPS and induce hydrogelation and aggregation in amyloidogenic type II diabetes. During aging, the accumulation of misfolded polypeptides can lead to amyloidosis and affect LLPS-driven aggregation (*76*). RBP FUS converts into a gel-like state at a higher concentration level in cells and further transitions into solid-like fibrillar aggregates over time, which acts as the hallmarks of aging and related diseases (*77*). Moreover, the persistent SGs formed by RBPs irreversible aggregations and abnormal LLPS may also result in the pathogenesis of aging-related diseases. For example, PAB-1 and TIAR-2 excessively accumulate and form irreversible SGs and subsequently lead to the shorter lifespan of aged C. elegans (*78*). In addition, the irreversible amyloid-β oligomeric aggregates formed by TDP-43 disturb SG dynamics and thus cannot exchange materials with their surroundings and accelerate aging diseases (*79*). Another research showed that cellular senescence can lead to eIF2α hyperphosphorylation and disturb the formation of SGs in the stress response, indicating the interplay between aging and LLPS (*80*). Besides, SGs can recruit and assemble the pro-aging protein, plasminogen activator inhibitor-1 (PAI-1) thus performing their anti-aging effect (*81*). Therefore, irreversible RBPs aggregation by LLPS are believed to interfere with normal cellular functions during aging, which is worth investigating in the future.

Although the phase behavior existing in various diseases is not yet completely understood, it is still necessary to study phase separation in the future treatment of diseases. Some disease-related LLPS processes can be inhibited or promoted at the stage of separation, thereby changing the biological properties of related proteins. For example, treatment drugs taken for cancer affect the activity of medicine due to the formation of condensate (*82*). This situation can be improved by changing the mechanism of drug condensate formation to inhibit the formation of agglomerates. Therefore, this provides us with a new idea: controlling and regulating the LLPS procedure to change the drug activity for different diseases, this may be beneficial for disease treatment.

7. RBPs-driven LLPS as novel therapeutic mechanism

Nowadays, the broad physiological and pathological characteristics of RBPs-driven LLPS have raised interest of whether it can be a promising therapeutic mechanism and strategy for diseases. In this section, we draw attention to RBPs and they-induced LLPS as a new therapeutic area as well as introduce the therapeutic mechanisms of small molecules (including natural products) targeting RBPs (Figure 5).

At present, numerous small molecules have shown great potential to affect the process of RBPs aggregation and function to modulate LLPS-induced human diseases with different mechanisms. Especially, RBPs as the major components of SGs, small molecules targeting them can affect SG dynamics, including assembly, disassembly, maintenance and clearance. For example, a recent finding showed that targeting the liquid-like droplets formed by LLPS of SARS-CoV-2 nucleocapsid protein can restrain the virus replication and promote innate antiviral immunity (*83*). Troxerutin (also known as vitamin P4) has been shown to promote SG formation in a TIA-1-dependent manner (*84*). Another small molecule boric acid, promotes TIA-1 translocation from the nucleus to cytoplasmic SGs, thus exhibiting anticarcinogenic and bone-strengthening effects (*85*). As for mitoxantrone, it reduces the formation of TDP-43^{+} SGs and prevents the accumulation of mutant TDP-43 in SGs (*86*). Mitoxantrone also significantly suppresses the recruitment of FUS to SGs, and reduces the number and size of liquid FUS droplets formed *in vitro*, thus treating the neurodegenerative disease. Besides, compounds including lipoamide and lipoic acid also inhibit FUS accumulation and disrupt FUS-induced LLPS as well as

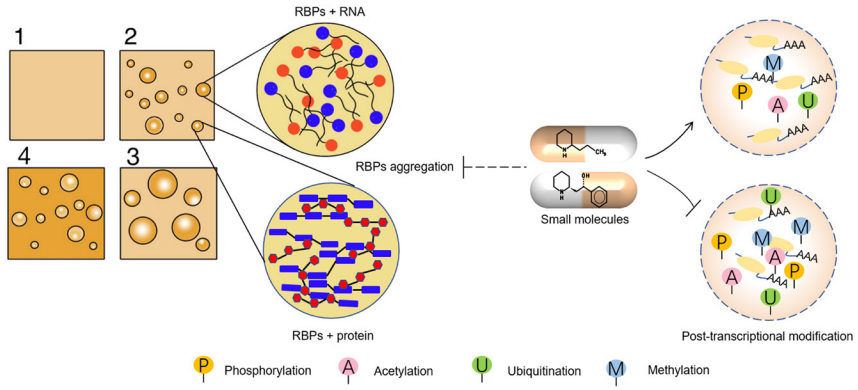

Figure 5. Small molecules targeting RBPs-triggered LLPS are the novel therapeutic strategy for human diseases.

SGs formation (*87*). It has been reported that the PARP inhibitor Olaparib delays the recruitment of TDP-43 and hnRNPA1 to SGs and delays SG assembly, which is beneficial in ALS and frontotemporal dementia (FTD) (*88*). Targeting the dysregulated PTMs which affect the function of RBPs is another therapeutic option for multiple diseases induced by abnormal LLPS. Some compounds target the dysregulated PTMs of RBPs to regulate the process of LLPS, thus alleviating diseases. For instance, multiple small compounds, such as silmitasertib, tetra bromo cinnamic acid and okadaic acid, could target G3BP1, the core component of SGs, to affect its dysregulated phosphorylation (*89*).

Altogether, targeting RBPs to inhibit their abnormal aggregations and restore the normal function of LLPS may be a novel therapeutic strategy in multiple diseases. RBPs-driven LLPS also open up a whole new field for the development of small molecule drugs. The huge drug discovery opportunities contained in the area LLPS have increasingly been recognized by researchers regardless of the exploration direction.

8. Concluding remarks and future perspectives

Ever since LLPS was first described, numerous studies have been dedicated to investigating its visible phase transitions, physicochemical properties, cellular functions and its possible involvement in human diseases. RNA binding proteins (RBPs) that contain IDRs, RBDs and LCDs are prone to aggregate, interact with other proteins/RNAs and undergo LLPS. In this review, we have tried to describe the whole development picture of LLPS and summarize the basic biological function of RBPs-driven LLPS and MLOs formation. As described in this review, aberrant RBPs aggregations and LLPS have become novel promising therapeutic targets for human diseases and opened up a whole new field for the development of small molecule drugs.

Despite such breakthroughs in the field of LLPS, our understanding of the LLPS is still in its infancy and a growing number of questions have also emerged. For example, what is the underlying mechanism in the regulation of RBPs condensates by their material properties during LLPS? How do disease-associated mutations of RBPs regulate the physical properties of their condensates? Whether RBPs which undergo LLPS can be used as therapeutic targets for diseases, and how to regulate the LLPS of RBPs to achieve the desired therapeutic effect still need to be further explored. Importantly, more quantitative tools or approaches need to be developed and applied to LLPS research.

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Review

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An overview: Management of patients with advanced hepatocellular carcinoma

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SUMMARY Hepatocellular carcinoma (HCC) has constituted a significant health burden worldwide, and patients with advanced HCC, which is stage C as defined by the Barcelona Clinic Liver Cancer staging system, have a poor overall survival of 6-8 months. Studies have indicated the significant survival benefit of treatment based on sorafenib, lenvatinib, or atezolizumab-bevacizumab with reliable safety. In addition, the combination of two or more molecularly targeted therapies (first- plus second-line) has become a hot topic recently and is now being extensively investigated in patients with advanced HCC. In addition, a few biomarkers have been investigated and found to predict drug susceptibility and prognosis, which provides an opportunity to evaluate the clinical benefits of current therapies. In addition, many therapies other than tyrosine kinase inhibitors that might have additional survival benefits when combined with other therapeutic modalities, including immunotherapy, transarterial chemoembolization, radiofrequency ablation, hepatectomy, and chemotherapy, have also been examined. This review provides an overview on the current understanding of disease management and summarizes current challenges with and future perspectives on advanced HCC.

Keywords Hepatocellular carcinoma, Advanced, Management, Molecularly targeted therapies, Portal vein tumor thrombosis

1. Introduction

Primary liver cancer is the sixth most common carcinoma and the third leading cause of cancer-related death (*1*), with 905,667 new cases and 830,180 deaths worldwide in 2020 (*2*). Hepatocellular carcinoma (HCC), accounting for about 90% of primary liver cancer, has constituted a significant health burden all around the world (*3*). The well-established risk factors for HCC include hepatitis virus infection (hepatitis B and C virus), alcohol consumption, obesity, diabetes, and aflatoxin exposure (*1*). The 5-year survival rate is less than 20% for patients with HCC and is determined by disease stage (*4*). From 2000 to 2015, the overall HCC death rate increased by 48.6% in males (95% CI: 43.9-53.4%; from 7.52 to 11.18 per 100,000 persons) and 34.7% in females (95% CI: 28.1-41.7%; from 2.82 to 3.80 per 100,000 persons) (*5*).

Advanced HCC, also referred to as Barcelona Clinic Liver Cancer (BCLC) stage C (BCLC-C) (*6,7*), is defined as patients with segmental or portal macrovascular invasion or extrahepatic spread who exhibit cancer-related symptoms (Eastern Cooperative Oncology Group Performance Status (ECOG PS) of 1-2) (*8*). Due to the tumor extent or cirrhosis, advanced HCC tends to be surgically unresectable, with a poor median survival of 6-8 months (*9,10*). Importantly, portal vein tumor thrombosis (PVTT) commonly occurs in patients with advanced HCC, which results in aggressive disease progression, impaired liver function reserve, an increased recurrence rate, and a reduced median survival time (2-4 months) (*11*). This review summarizes the current management of patients with advanced HCC (BCLC-C) (Figure 1) and it discusses recent developments as well as current challenges in this field.

2. Management of advanced HCC: Systemic therapies

Advanced HCC was historically incurable until the appearance of sorafenib (*12*), a tyrosine kinase inhibitor (TKI) (*13-15*). Currently, systemic therapy is the primary option for advanced HCC (*16*), and several updated molecularly targeted agents (*17-19*), together with combination therapy (immune checkpoint inhibitors

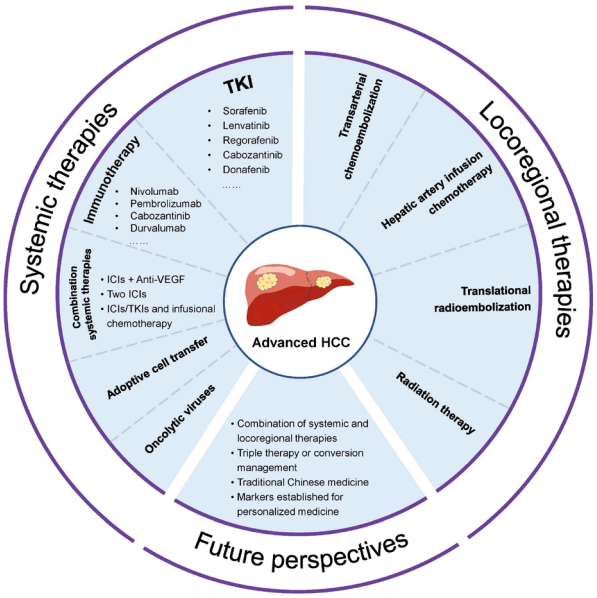

Figure 1. Current management of patients with advanced hepatocellular carcinoma. Systemic therapies include TKI, immunotherapy, combination systemic therapies, adoptive cell transfer, and oncolytic viruses. Locoregional therapies include transarterial chemoembolization, hepatic artery infusion chemotherapy, translational radioembolization, and radiation therapy. Potential management strategies are also listed, such as a combination of systemic and locoregional therapies, triple therapy or conversion management, traditional Chinese medicine, and markers for personalized medicine. HCC, hepatocellular carcinoma; TKI, tyrosine kinase inhibitor.

(ICIs) or other antibodies), have displayed remarkable efficacy (*20*).

2.1. Monotherapy with TKI

2.1.1. Sorafenib

Sorafenib is a TKI that inhibits the activity of kinases and pathways (platelet-derived growth factor receptor (*PDGFR*), *c-KIT*, vascular endothelial growth factor receptor (*VEGFR*), *RET*, *RAS*/*RAF*/mitogen-activated protein kinase (*MAPK*), *FLT-3* and Janus kinase (*JAK*)/signal transducer and activator of transcription protein (*STAT*)) to result in antiangiogenic, antiproliferative, and proapoptotic action (*21*). The survival benefit of sorafenib was first revealed in a phase 2 study involving 137 patients with advanced HCC (median overall survival (OS): 9.2 months) in 2006 (*22*). A multicenter randomized trial subsequently indicated that sorafenib (400 mg twice a day) resulted in a longer OS compared to a placebo (10.7 *vs.* 7.9 months; hazard ratio (HR): 0.69, 95% confidence interval (CI): 0.55-0.87) (*12*). Further subgroup analysis suggested that sorafenib could improve survival and disease control, regardless of etiology, baseline tumor burden, disease stage, or prior therapy (*23,24*).

Several studies reported the use of sorafenib in patients with advanced HCC and PVTT (Table 1) (*25-31*). In a study by Jeong *et al.* (*32*), 30 patients with advanced HCC and PVTT received sorafenib monotherapy. Among these individuals, 10% were reported to have a partial response to revascularization and 30% had stable disease (*32*). The median OS was 3.1 months (95% CI: 2.70-3.50), with a median progression-free survival (PFS) of 2.0 months (95% CI: 1.96-2.05). In addition, Ahn *et al.* (*28*) compared sorafenib to hepatic arterial infusion chemotherapy in patients with advanced HCC and PVTT. The group receiving sorafenib had a significantly shorter time to progression (TTP, 2.1 *vs.* 6.2 months) and a reduced disease control rate (37% *vs.* 76%) than the group receiving arterial infusion chemotherapy. Still, more solid evidence and further validation are required to support the administration of sorafenib in patients with advanced HCC and PVTT.

The combination of sorafenib with other drugs or treatments is another hot topic, and a growing number of studies have suggested the potential survival benefit of combination therapy to treat advanced HCC. Goyal *et al.* (*33*) combined sorafenib with FOLFOX (5-fluorouracil 1,200 mg/m^2/day continuous infusion for 46 hours, leucovorin 200 mg/m^2 and oxaliplatin 85 mg/m^2 twice a week) in patients with advanced HCC, who received sorafenib (400 mg twice daily) for 2 weeks followed by FOLFOX. The median TTP was 7.7 months (95% CI: 4.4-8.9), the overall response rate (ORR) was 18%, and the median OS was 15.1 months (95% CI: 7.9-16.9) (*33*). In a phase 2 trial comparing sorafenib (400 mg twice daily; n = 46) with sorafenib-GEMO (400 mg twice daily; 1000 mg/m^2 gemcitabine; 100 mg/m^2 oxaliplatin; n = 48), There were no significant differences in the median OS between the group receiving sorafenib alone and the group receiving sorafenib-GEMOX (14.8 months (90% CI, 12.2-22.2) *vs.* 13.5 months (90% CI: 7.5-16.2)). However, the median TTP improved in the group receiving sorafenib-GEMOX compared to the group receiving sorafenib alone (6.2 months (95% CI: 3.7-7.2) *vs.* 4.6 months (95% CI: 3.8-6.2)) (*34*). In addition, phase I trials have revealed the potential effect of the combination of sorafenib and trametinib (median PFS: 3.7 months; median OS: 7.8 months) (*35*) or enzalutamide (median PFS: 2.9 months; median OS: 6.7 months) (*36*) in patients with advanced HCC. In addition, a multicenter study compared sorafenib alone (n = 169) with the combination of sorafenib and transarterial chemoembolization (TACE) (n = 170) (*37*). There were no significant differences in the median OS (12.8 *vs.* 10.8 months, HR: 0.91, P = 0.290), while the median TTP (5.3 *vs.* 3.5 months, HR: 0.67, P = 0.003) and median PFS (5.2 *vs.* 3.6 months, HR: 0.73, P = 0.010) were significantly higher in the group receiving sorafenib and TACE. Similarly, in a retrospective study by Wu *et al. (38)*, the combination of sorafenib and TACE resulted in a significantly prolonged median OS (17.9 *vs.* 7.1 months) and median TTP (9.3 *vs.* 3.4 months) compared to the group receiving TACE alone.

Table 1. Recent studies on sorafenib-based therapy to treat advanced HCC with PVTT

Author	Year	Type	OS	PFS	TTP	ORR	Adverse events
Jeong *et al.*[32]	2013	Retrospective (n = 30)	3.1	2.0	NA	NA	Fatigue (43.3%) and hand-foot skin reaction (30.0%)
Nakazawa *et al.*[31]	2014	Retrospective (n = 97)	4.3	NA	NA	NA	Elevated AST/ALT (6%), anorexia/nausea (4%)
Song *et al.*[29]	2015	Prospective (n = 60)	5.5	NA	2.1	NA	NA
Kim *et al.*[30]	2015	Retrospective (n = 66)	3.2	NA	1.6	NA	NA
Choi *et al.*[25]	2018	Prospective (n = 29)	7.2	NA	2.7	3.40%	Hyperbilirubinemia (34.5%), hand-foot syndrome (31.0%), and elevated AST (27.6%)
Kodama *et al.*[27]	2018	Retrospective (n = 36)	5.3	2.1	NA	NA	Elevated AST/ALT (8.3%), elevated bilirubin (5.5%), diarrhea and general fatigue (13.9%)
Kaneko *et al.*[26]	2020	Retrospective (n = 291)	14.4	NA	NA	NA	NA
Ahn *et al.*[28]	2021	Retrospective (n = 35)	6.4	NA	2.1	NA	Anemia (20%), hand-foot skin reaction (28.6%), dyspepsia/anorexia (25.7%), and elevated AST (22.9%)

OS, overall survival; PFS, progression-free survival; TTP, time to progression; ORR, overall response rate; AST, aspartate transaminase; ALT, alanine transaminase; NA, not available.

The combination of selective internal radiation therapy and sorafenib was also studied in patients with advanced HCC, and the median OS was 12.1 months (sorafenib alone: 11.4 months, HR: 1.01, P = 0.953) (*39*). Although the efficacy of combination therapies seems encouraging, these therapies require adequate liver reserve given drug-related hepatotoxicity (such as elevated AST or ALT, diarrhea, or hyperbilirubinemia) (*34,35*).

Since sorafenib was the most widely used targeted drug in patients with advanced HCC, drug resistance posed a serious issue and significantly limited its efficacy (*40,41*). A poor prognosis (median TTP: 2.9 months) and liver function (Child-Pugh score, ≥ 7) were observed in patients with advanced HCC resistant to sorafenib (*42*). However, the specific rate of sorafenib resistance has not been reported, and few studies investigated the mechanisms underlying that drug resistance. Xu *et al.* (*43*) revealed that *circRNA-SORE* was significantly up-regulated in sorafenib-resistant HCC cells. When *circRNA-SORE* is silenced, the apoptosis of HCC cells induced by sorafenib increases, suggesting the pivotal role of circRNA-SORE in maintaining the resistance of HCC cells to sorafenib. In addition, circRNA-SORE was reported to induce sorafenib resistance by regulating β-catenin signaling and stabilizing Y-box binding protein 1 (*13*). Phosphoglycerate dehydrogenase is a critical molecule for sorafenib resistance in HCC (*44*). In addition, liver X receptor activation might also enhance sorafenib sensitivity in HCC (*45*).

Predicting the prognosis for patients with advanced HCC receiving sorafenib has recently been investigated. The acyl-CoA synthetase long-chain family member 4 protein was proposed as a biomarker of sorafenib sensitivity in HCC, given its negative association with IC_{50} values for sorafenib in HCC cell lines (R = -0.952, P < 0.001) (*46*). Colagrande *et al.* (*47*) performed contrast-enhanced CT on patients with advanced HCC receiving sorafenib before (T0) and 60-70 days (T1) after initiation of treatment, and the VED_{T0} and VED_{T1} values were calculated accordingly. Results revealed that patients with VED_{T0} > 70% had a higher median OS rate than those with lower VED_{T0} (451.5 *vs.* 209.5 days, P = 0.032), and the area under the curve was 0.716. In addition, magnetic resonance imaging can also be used to predict the prognosis for patients at BCLC-C receiving sorafenib (*48,49*).

In addition, the interaction between sorafenib and other drugs, which might influence efficacy, remains a major problem. A secondary analysis of phase 3 clinical trials (n = 542) revealed that administration of proton pump inhibitors did not induce adverse survival outcomes during sorafenib treatment (*50*). Combining pravastatin (*51*) (10.7 *vs.* 10.5 months, P = 0.975) with sorafenib had no significant influence on OS in patients with advanced HCC. Interestingly, patients receiving sorafenib and aspirin were reported to have a better prognosis than those receiving sorafenib alone (OS, 18.3 *vs.* 8.8 months, HR: 0.57, P < 0.001; PFS, 7.3 *vs.* 3.0 months, HR: 0.61, P < 0.001) (*52*).

2.1.2. Lenvatinib

Lenvatinib is an oral TKI recommended as a first-line therapy along with sorafenib (*53*). It inhibits *VEGFR*, *PDGFR*, *KIT*, *RET* and fibroblast growth factor receptor activity (*54*). To the extent known, the efficacy of lenvatinib was first indicated in a phase 2 study of lenvatinib in 46 patients with advanced HCC, who had a median OS of 18.7 months (95% CI: 12.7-25.1) and median TTP of 7.4 months (95% CI: 5.5-9.4) (*55*). A subsequent non-inferiority trial involved 954 patients and randomly assigned those patients to receive lenvatinib (n = 478) or sorafenib (n = 476). Results revealed that median OS was 13.6 months (95% CI: 12.1-14.9) in the group receiving lenvatinib, which was non-inferior to that in the group receiving sorafenib (12.3 months, 95% CI: 10.4-13.9; HR: 0.92, 95% CI: 0.79-1.06) (*15*). In addition, studies indicated that lenvatinib might be superior to sorafenib in maintaining liver function and improving the prognosis for patients with advanced HCC. A study by Terashima *et al.* (*56*) retrospectively examined 180 patients with advanced HCC and a Child-

Pugh score of 5-7, and a better Child-Pugh score was noted in patients receiving lenvatinib (n = 45) than those receiving sorafenib (n = 135) after 4 weeks (P = 0.048) and 12 weeks (P = 0.036). Similarly, Kim *et al.* (*57*) reported that lenvatinib treatment is significantly associated with a longer PFS, with an HR of 0.461, compared to sorafenib. When lenvatinib was combined with PD-1 blockades, patients with advanced HCC had a median PFS of 6.6 and OS of 11.4 months (*57*).

Despite the survival benefits, lenvatinib-related adverse events are frequent, and liver function and PVTT play an important role in the efficacy of lenvatinib. In a phase 2 study of lenvatinib administration (*55*), frequent adverse events including hypertension (76.1%), hand-foot syndrome (65.2%), decreased appetite (60.9%), and proteinuria (60.9%) were observed, which led to a dose reduction (34 patients, 74%) or discontinuation (10 patients, 22%) (*55*). Recently, two retrospective, real-world studies of lenvatinib in advanced HCC were conducted in South Korea and China. In South Korea, Cheon *et al.* (*58*) analyzed the survival outcomes of 67 patients with advanced HCC receiving lenvatinib as first-line therapy. In patients with Child-Pugh class A cirrhosis (n = 74), PFS was 4.6 months (95% CI: 3.1-6.1) and OS was10.7 months (95% CI: 4.8-16.5) while PFS was 2.6 months (95% CI: 0.6-4.6) and OS was 5.3 months (95% CI: 2.0-8.5) in patients with Child-Pugh class B cirrhosis (n = 18). Wang *et al.* (*59*) performed a real-world study involving 54 patients with HCC receiving lenvatinib in China, and an ORR of 22% was observed with a PFS of 168 days and an adverse event rate of 92.8%. That study noted that PVTT was significantly associated with a poor PFS as an independent risk factor (HR: 0.38, P = 0.037).

Interestingly, lenvatinib was studied in patients with advanced HCC and PVTT. Chuma *et al.* (*60*) indicated that patients with advanced HCC and tumor thrombus in the main portal vein trunk had a median PFS of 101 days and OS of 201 days after lenvatinib treatment. Similarly, in a retrospective study by Maruta *et al. (26)*, 54 patients with advanced HCC and PVTT who received lenvatinib treatment had an OS of 14.7 months. Patients with PVTT still had a significantly poorer survival than those without PVTT (6.5 *vs.* 14.2 months) (*61*). A point worth noting is that patients with advanced HCC and PVTT still had a poor prognosis despite systemic treatment.

Although lenvatinib may provide additional survival benefits over sorafenib in patients with advanced HCC, a lenvatinib-susceptible subgroup of patients with HCC needs to be selected. Myojin *et al.* (*62*) proposed a *ST6GAL1*-based stratification strategy for lenvatinib or sorafenib. They conducted genetic screening on a mouse model of HCC (C57BL/6J male mice) and evaluated the biomarker candidate (*ST6GAL1*) in human HCC cell lines (serum samples from 76 patients with advanced HCC receiving curative hepatectomy and 96 patients receiving TKI therapy). Results suggested that a high level of *ST6GAL1* expression was significantly associated with a better treatment response to lenvatinib than sorafenib. However, for patients with a low level of *ST6GAL1* expression, there were no significant differences in OS between lenvatinib and sorafenib treatment. The predictive factors for clinical outcomes of lenvatinib therapy have also been examined. A study by Shomura *et al.* (*63*) prospectively enrolled 46 patients with advanced HCC who received lenvatinib therapy and it followed them for about 2 years. Results revealed that grade 2/3 hypothyroidism occurred in patients with a shorter treatment duration than in those with grade 0/1 (HR: 4.28, P = 0.011). Patients with grade 2/3 hypothyroidism had a significantly longer OS than those with grade 0/1 (age-adjusted HR: 0.21, 95% CI: 0.05-0.94).

2.1.3. Regorafenib

Like sorafenib, regorafenib is an oral multi-kinase inhibitor that suppresses angiogenesis, oncogenesis, and the tumor microenvironment (*64*), and it has been recommended as a second-line therapy for advanced HCC by the European Association for the Study of the Liver (EASL) (*6*). The RESORCE trial involved 573 patients with HCC who tolerated sorafenib (≥ 400 mg/day for 28 days) and who had relatively good liver function (Child-Pugh class A) (*19*). When given regorafenib 160 mg, survival improved significantly compared to a placebo (median OS, 10.6 *vs.* 7.8 months, HR: 0.63, P < 0.001). However, several complications were reported, of which the most common grade 3 or 4 adverse events were hypertension (15% *vs.* 5%), a hand-foot skin reaction (13% *vs.* 1%), fatigue (9% *vs.* 5%), and diarrhea (3% *vs.* 0%). A subsequent real-world study indicated that regorafenib after sorafenib led to a prolonged OS in patients with advanced HCC compared to a placebo (9.7 *vs.* 6.0 months, P < 0.001) (*65*). In another real-world study, sequential therapy (regorafenib after sorafenib) was administered to 133 patients with HCC, who had a median OS of 10.0 months, a PFS of 2.7 months, and a TTP of 2.6 months (*66*). The survival outcomes were comparable to those in the RESORCE trial (n = 573, OS: 10.6 months) and a phase III study in Japan (n = 44, OS: 17.3 months) (*67*).

The safety and efficacy of regorafenib as a second-line agent to treat patients with advanced HCC and Child-Pugh class B cirrhosis have been indicated. Kim *et al.* (*68*) retrospectively examined 59 patients with advanced HCC and Child-Pugh class B cirrhosis who received regorafenib after sorafenib (37 receiving 2nd line systemic therapy and 22 receiving 3rd-4th line systemic therapy). The median OS was 4.6 months and PFS was 1.8 months, which were significantly worse than those in patients with Child-Pugh class A cirrhosis (P < 0.001 and P = 0.008, respectively). In addition, compared to patients with Child-Pugh class A cirrhosis, grade 3 or 4 adverse effects were more common in

patients with Child-Pugh class B cirrhosis (27.1% *vs.* 14.1%, $P = 0.017$), including increased blood bilirubin, a hand-foot skin reaction, and skin rash.

Recently, the combination of regorafenib and immunotherapy (*e.g.*, anti-PD-1 agents) was also examined in animal models. For example, Shigeta *et al.* (*69*) intraperitoneally injected regorafenib (at 10 mg/kg daily) or/and PD-1 antibodies (at 10 mg/kg thrice a week) in orthotopic HCC mice. Compared to regorafenib or anti-PD-1 alone, mice receiving regorafenib plus an anti-PD-1 antibody had a significant survival benefit (HR: 0.17, $P < 0.001$), which might be attributed to the promotion of cytotoxic T lymphocyte infiltration *via* the *CXCL10/CXCR3* axis.

In addition, several biomarkers with which to predict the OS of patients receiving regorafenib were examined. Teufel *et al.* (*70*) collected tumor tissues and baseline plasma samples from patients with advanced HCC in the RESORCE trial and reported that the decreased expression of 5 proteins in plasma was significantly associated with better OS after regorafenib treatment, including angiopoietin 1 (HR: 1.12, 95% CI: 1.05-1.19), the latency-associated peptide of transforming growth factor beta 1 (HR: 1.36, 95% CI: 1.12-1.65), cystatin B (HR: 1.46, 95% CI: 1.15-1.85), oxidized low-density lipoprotein receptor 1 (HR: 1.35, 95% CI: 1.16-1.57), and C-C motif chemokine ligand 3 (HR: 1.02, 95% CI: 1.01-1.04). In addition, Tong *et al.* (*71*) indicated that annexin A3 (*ANXA3*) is a potential biomarker with which to predict the effect of sorafenib and regorafenib treatment in a mouse model of HCC. A high level of *ANXA3* expression could increase the resistance of HCC cells to sorafenib and regorafenib. Interestingly, when *ANXA3* was inhibited in the immune-competent mouse model, a significantly decreased liver/body weight ratio was observed after both sorafenib and regorafenib treatment.

2.1.4. Cabozantinib

Cabozantinib is an inhibitor of multiple receptor tyrosine kinases (including *VEGFR*, *MET*, *RET*, *AXL* and *KIT*) that are associated with oncogenesis, angiogenesis, tumor growth, and metastasis (*72*). The CELESTIAL trial (a randomized, double-blind, phase 3 trial) (*17*) involved 707 patients with advanced HCC who had received sorafenib treatment, and it evaluated the effect of cabozantinib. Results revealed a significantly improved OS and PFS in the group receiving cabozantinib compared to the group receiving a placebo (OS: 10.2 *vs.* 8.0 months, $P = 0.005$; PFS: 5.2 *vs.* 1.9 months, $P < 0.001$). In addition, adverse events of grade 3 or 4 were 68% in patients receiving cabozantinib and 36% in patients receiving a placebo.

In a secondary analysis of the CELESTIAL trial, Shlomai *et al.* (*73*) evaluated the cost-effectiveness of cabozantinib according to the Markov model. Results revealed that the mean incremental cost of cabozantinib for patients with HCC was USD 76,406 and the incremental cost-effectiveness ratio compared to supportive care was USD 469,374/quality-adjusted life-year (QALY) (based on 60 mg cabozantinib daily), which is not cost-effective at conventional willingness-to-pay thresholds (USD 50,000-150,000 per QALY). Based on adjusted second-line populations in the RESORCE and CELESTIAL trials, Kelley *et al.* (*74*) compared patients receiving regorafenib ($n = 573$) with those receiving cabozantinib ($n = 266$). Results revealed no significant differences in median OS (10.6 *vs.* 11.4 months, $P = 0.347$), while the median PFS was longer in the cabozantinib group (5.6 *vs.* 3.1 months, $P < 0.001$). In a recent multicenter, real-life cohort study involving 88 patients with advanced HCC, a median OS of 7 months was reported after the start of cabozantinib treatment (*75*).

2.1.5. Donafenib

Donafenib is a novel small-molecule TKI developed by creatively substituting a trideuteriomethyl group for a methyl on sorafenib to inhibit *VEGFR*, *PDGFR*, and various *Raf* kinases (*76*). In a phase 2-3 trial (ZGDH3), donafenib was given 200 mg orally, twice daily. The PFS and ORR were similar, but donafenib displayed superiority over sorafenib in improving OS (12.1 *vs.* 10.3 months) (*77*). Moreover, improved safety and tolerability indicate a potential option for the first-line treatment of advanced HCC (*78*).

2.2. Immunotherapy

Antigen-presenting cells mediate T cell activation after recognizing a cancer cell antigen. However, immune tolerance by HCC can be induced by the increased differentiation of Treg cells, upregulated immunosuppressive cytokines, and elevated expression of co-inhibitory molecules (*e.g.*, PD-1 and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4)). The immunosuppressive microenvironment facilitates the growth and progression of HCC, which provides a therapeutic target for advanced HCC (*79*).

A meta-analysis of 2,402 patients with advanced HCC who received ICIs revealed that the mean OS was 15.8 months (*80*). Moreover, the overall ORR was 22.7% and the disease control rate was 60.7%. In the subgroup, the OS was 18.7 months for patients receiving nivolumab ($n = 846$) and 13.3 months for those receiving pembrolizumab ($n = 435$). The overall rate of treatment discontinuation due to adverse events was 14.9%.

A growing number of studies on ICIs have indicated that PD-1/PD-L1 blockade immunotherapy is a novel optional therapy with which to treat advanced HCC (*81-83*). In a real-world study based on 55 patients with advanced HCC receiving an anti-PD-1 agent, Cui *et al.* (*84*) noted a median OS of 15 months, a median PFS

of 10 months, and a disease control rate of 89%. In addition, a meta-analysis of 1,232 patients with advanced HCC receiving PD-1 or PD-L1 inhibitors revealed that the median PFS was 3.58 months (95% CI: 2.65-4.50), the median OS was 12.24 months (95% CI: 10.48-14.00), the overall ORR was 20% (95% CI: 0.16-0.24), the disease control rate was 60% (95% CI: 0.54-0.67), the rate of adverse events was 63% (95% CI: 0.45-0.78), and the rate of serious adverse events was 11% (95% CI: 0.06-0.22) (*85*).

2.2.1. Nivolumab (anti-PD1 antibody)

Nivolumab is a PD-1 ICI with a durable response and manageable safety, and it has been recommended for patients with advanced HCC by the American Society of Clinical Oncology (ASCO) owing to its additional survival benefits (*53*). A dose-escalation and expansion trial of nivolumab (CheckMate 040) was performed in 262 patients with advanced HCC (48 in the dose-escalation phase and 214 in the dose-expansion phase) (*18*). The ORR was 20% (95% CI: 15-26%) in patients receiving nivolumab 3 mg/kg in the dose-expansion phase and 15% (95% CI: 6-28%) in the dose-escalation phase. In dose escalation, 3 patients (6%) had serious treatment-related adverse events (including pemphigoid, adrenal insufficiency, and a liver disorder), and the incidence of adverse events was not significantly associated with the drug dose (*18*). Given the difference in incidence and pathogenesis in Asian and non-Asian populations (*86,87*), the safety and efficacy of nivolumab in Asians were further indicated in CheckMate 040 (*86*). ORR was 14% in the overall population and 15% in the Asian cohort. The median duration of response was longer in the overall population (19.4 months, 95% CI: 9.7-not evaluable) than in Asian patients (9.7 months, 95% CI: 5.6-not evaluable), while the median OS was similar between the overall population (15.1 months, 95% CI: 13.2-18.2) and Asian patients (14.9 months, 95% CI: 11.6-18.9).

Nivolumab was also studied in patients with advanced HCC and Child-Pugh class B cirrhosis. Kambhampati *et al.* (*88*) retrospectively studied the effect of nivolumab in 18 patients with advanced HCC and Child-Pugh class B cirrhosis from the Hepatobiliary Tissue Bank and Registry and CheckMate 040 trial, which reported an ORR value of 17% (3 of 18 patients, including 2 partial responses and 1 complete response). The median OS was 5.9 months (95% CI: 3.0 months-not evaluable), and the median PFS was 1.6 months (95% CI: 1.4-3.5 months). In addition, most patients (94%, 17 of 18 patients) experienced grade ≥ 3 adverse events (according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.03)), and treatment-related grade ≥ 3 adverse events were reported in 28% of patients (5 of 18 patients).

In addition, the safety and efficacy of nivolumab were recently studied in the real world. Fessas *et al.* (*89*) conducted an international, multicenter observational study (eight centers in North America, Europe, and Asia) involving 233 patients with advanced HCC receiving nivolumab alone. They reported that the ORR was 22.4% and the disease control rate was 52.1%, with a median OS of 12.2 months (95% CI: 8.4-16.0) and PFS of 10.1 months (95% CI: 6.1-14.2). Still, the OS was shorter in patients with Child-Pugh class B cirrhosis (n = 75) than in those with Child-Pugh class A cirrhosis (n = 158) (7.3 *vs.* 16.3 months, $P < 0.001$). Based on 203 patients with advanced HCC receiving nivolumab, Choi *et al.* (*90*) indicated that the median OS was significantly shorter in patients with Child-Pugh class B cirrhosis (n = 71) than Child-Pugh class A cirrhosis (n = 132) (11.3 *vs.* 42.9 weeks, HR: 2.10). In patients with Child-Pugh class B cirrhosis, those with a score of 8-9 had a worse OS than those with a score of 7 (7.4 *vs.* 15.3 weeks, HR: 1.93, $P < 0.020$). In another real-world study involving 34 patients with advanced HCC, Scheiner *et al.* (*91*) reported similar results with a PFS of 4.3 months (95% CI: 2.0-6.7) and an OS of 9.0 months (95% CI: 5.5-12.5). These studies indicated that the OS remained poor in patients with Child-Pugh class B cirrhosis despite nivolumab treatment, and more management therapies should be explored for patients with advanced HCC and Child-Pugh class B cirrhosis.

2.2.2. Pembrolizumab (anti-PD1 antibody)

Pembrolizumab (anti-PD1 antibody) is another ICI that was approved as second-line systemic therapy for the treatment of advanced HCC based on the results of a phase 2 trial (KEYNOTE-224) (*92*). In KEYNOTE-224, pembrolizumab performed well, resulting in a median PFS of 4.9 months (95% CI: 3.4-7.2), an OS of 12.9 months (95% CI: 9.7-15.5), and an ORR of 17% (95% CI: 11-26).

A subsequent phase III trial (KEYNOTE-240) compared pembrolizumab and a placebo, but results did not reach the prespecified boundaries of statistical significance in terms of OS and PFS (*93*). A presentation at the ASCO gastroenterology (GI) 2022 meeting indicated that pembrolizumab resulted in a better OS (14.6 *vs.* 13 months) and PFS (2.6 *vs.* 2.3 months) compared to a placebo.

2.2.3. Camrelizumab (anti-PD1 antibody)

Camrelizumab is a humanized monoclonal anti-PD1 antibody that has a different binding epitope than nivolumab and pembrolizumab (*94*). It was well tolerated in patients with an advanced solid tumor (*95,96*). A multicenter, phase 2 single-arm study (NCT02989922) indicated that the ORR to camrelizumab was 14.7% (95% CI: 10.3-20.2) and the OS probability at 6 months was 74.4% (95% CI: 68.0-79.7). Grade 3/4 treatment-related

adverse events (TRAEs) occurred in 22% patients and the rate of treatment-related death was 0.9% (*97*).

2.2.4. Durvalumab (anti-PD-L1)

Intravenous durvalumab is a human monoclonal antibody that has anti-tumor action by binding to the PD-L1 receptor on the surface of cancer cells (*98*). A phase 1/2 study (NCT01693562) evaluated the safety and efficacy of durvalumab in patients with HCC, and it noted an ORR of 10.3%, a median OS of 13.2 months (95% CI: 6.3-21.1), and a rate of Grade 3/4 TRAEs of 20%, as was reported at the ASCO GI 2017 meeting. However, most studies tend to favor anti-PD-1 over anti-PD-L1 therapy because of the poor pharmacokinetic properties of anti-PD-L1 antibodies and the additional blockade of PD-L2 interactions (*99-101*).

2.2.5. Tremelimumab (anti-CTLA-4)

Tremelimumab is a lgG2 monoclonal antibody specific for CTLA-4 that can promote T cell activation and proliferation by blocking the binding of CTLA-4 (*102*). A clinical trial (NCT01008358) of tremelimumab in patients with HCC indicated that the disease control rate was 76.4%, the median TTP was 6.48 months (95% CI: 3.95-9.14), and the median OS was 8.2 months (95% CI: 4.64-21.34) (*103*).

2.3. Combination systemic therapies

2.3.1. Combinations of ICIs and an anti-*VEGF* antibody

VEGF overexpression is a critical mechanism of tumor angiogenesis and is related to immunosuppressive action in HCC (*104*). Combinations of ICIs and an anti-*VEGF* antibody can lead to synergistic anti-tumor action against advanced HCC. Therefore, atezolizumab plus bevacizumab was recommended as first-line therapy according to guidelines (*53,105,106*). Atezolizumab is a PD-L1 blocker and bevacizumab is a *VEGF* inhibitor. Atezolizumab plus bevacizumab has superseded sorafenib as first-line treatment for unresectable HCC, and the former is now approved by the US FDA because of its superior performance (PFS: 6.8 *vs.* 4.3 months, OS: 19.2 *vs.* 13.4 months, ORR: 30 *vs.* 11%) (*16*).

Both lenvatinib and cabozantinib can inhibit *VEGF* receptors. Lenvatinib plus pembrolizumab displayed encouraging results with an ORR of 46.0% (95% CI: 36.0-56.3) and median PFS of 9.3 months (95% CI: 5.6-9.7) in a phase 1b trial (*107*). Atezolizumab plus cabozantinib was evaluated by the COSMIC-312 phase III trial, and results revealed that atezolizumab-cabozantinib was superior to sorafenib in terms of PFS (6.8 *vs.* 4.2 months).

2.3.2. Combinations of 2 ICIs

A combination of PD-1 and CTLA-4 antibodies has been used to treat numerous cancers including HCC. A previous trial (phase I/II) investigated the efficacy and safety of durvalumab-tremelimumab and found that T300 + D1500 (tremelimumab 300 mg plus durvalumab 1,500 mg (one dose each during the first cycle) followed by durvalumab 1,500 mg once every 4 weeks) displayed the most encouraging benefit-risk profile (*108*). T300 + D1500 (STRIDE) was further evaluated in a phase III trial (HIMALAYA), the results of which were reported at the ASCO GI 2022 meeting. T300 + D1500 led to a significantly better OS of 16.4 months compared to sorafenib alone at 13.8 months (HR: 0.78; 95% CI: 0.65-0.92; $P = 0.0035$). The ORR to STRIDE was 20.1%, which was higher than that for sorafenib alone (5.1%). T300 + D1500 is a promising treatment strategy for patients with HCC who are not eligible for atezolizumab plus bevacizumab (*109*).

Moreover, the US FDA recently approved the combined use of atezolizumab and bevacizumab in patients with advanced HCC who had not previously received systemic treatment (*110*). Atezolizumab is a monoclonal antibody that inhibits the interaction of PD-L1 with programmed cell death protein 1 (PD-1) and *CD80* receptors, whereas bevacizumab blocks vascular endothelial growth factor A. In a global open-label trial (IMbrave 150 trial), patients with advanced HCC without previous systemic treatment were randomly assigned to receive either atezolizumab plus bevacizumab ($n = 336$) or sorafenib ($n = 165$) in a 2:1 ratio (*16*). OS at 12 months was significantly longer in the patients receiving atezolizumab-bevacizumab (67.2% *vs.* 54.6%) than in those receiving sorafenib. The HR for all-cause death was 0.58 (95% CI: 0.42-0.79) in patients receiving atezolizumab-bevacizumab compared to patients receiving sorafenib ($P < 0.001$) (*16*). The survival rate at 12 months was 67.2% for patients receiving atezolizumab-bevacizumab and 54.6% for patients receiving sorafenib, and a longer PFS was observed in patients receiving atezolizumab-bevacizumab (6.8 *vs.* 4.3 months, HR: 0.59, $P < 0.001$). There were no significant differences in adverse events between patients receiving atezolizumab-bevacizumab (56.5%) and patients receiving sorafenib (55.1%), except for grade 3 or 4 hypertension (*16*). A network meta-analysis of 14 trials with 6,290 patients with advanced HCC further indicated a significantly prolonged OS in patients receiving atezolizumab-bevacizumab compared to patients receiving lenvatinib (HR: 0.63), sorafenib (HR: 0.58), or nivolumab (HR: 0.68, 95% CI) alone (*111*). Moreover, atezolizumab-bevacizumab ($n = 60$) resulted in better survival benefits than atezolizumab alone in patients with advanced HCC (PFS: 5.6 *vs.* 3.4 months, $P = 0.011$) (*16*). In a subsequent study, Chiang *et al.* (*112*) performed a cost-effectiveness analysis based on the IMbrave 150 trial. Atezolizumab-bevacizumab resulted in a gain of 0.44 QALYs with a cost of USD 79,074. The incremental

cost-effectiveness ratio of atezolizumab-bevacizumab was USD 179,729 per QALY compared to sorafenib. The decreased price of atezolizumab-bevacizumab by 20% was expected to lead to a cost-effectiveness ratio of USD 150,000/QALY and a decreased price by 29% was expected to lead to a cost-effectiveness ratio of USD 100,000/QALY. This would satisfy the willingness-to-pay threshold according to that study. Compared to sorafenib alone, atezolizumab-bevacizumab provided an additional 0.53 QALYs, thus resulting in an incremental cost-effectiveness ratio of USD 145,546.21 per QALY in China (the willing-to-pay threshold was USD 28,527.00/QALY) and USD 168,030.21 per QALY in the US (the willing-to-pay threshold was USD 150,000.00 / QALY) (*113*). Therefore, despite its marked efficacy, atezolizumab-bevacizumab might not be a cost-effective strategy for the first-line systemic treatment of patients with advanced HCC in China and the US.

On March 10, 2020, FDA approved nivolumab plus ipilimumab for the treatment of patients with HCC who had previously received sorafenib (*114*). The recommended regimen is nivolumab 1 mg/kg plus ipilimumab 3 mg/kg every three weeks for 4 cycles followed by nivolumab 240 mg biweekly. The FDA's approval was based on data from the CheckMate040 randomized clinical trial (*115*). This combination strategy is currently being studied as a first-line therapy in the phase III CHECKMATE-9DW trial.

2.3.3. Combinations of ICIs/TKIs and infusion chemotherapy

Previous studies revealed that chemotherapy can disrupt immune tolerance, facilitate an immune response, and induce immunogenic cell death (*116*). A phase 2 study investigated the combination of camrelizumab and oxaliplatin-based chemotherapy to treat advanced HCC (*117*). Results indicated that the ORR was 26.5%. A subsequent phase 3 study (NCT03605706) comparing the combination therapy to a placebo with chemotherapy is ongoing. Sorafenib plus chemotherapy was evaluated by the phase II randomized PRODIGE 10 trial (*34*). The additional clinical benefit from sorafenib plus chemotherapy seems limited, and no subsequent trial is planned.

To the extent known, there are few studies on intravenous chemotherapy treatment alone for advanced HCC since it is an alternative therapy with modest anti-tumoral responses and limited survival benefits. Abou-Alfa *et al.* (*118*) evaluated the efficacy of doxorubicin plus sorafenib compared to doxorubicin alone (n = 96). Patients receiving combination therapy had a significantly higher median OS (13.7 *vs.* 6.5 months, P = 0.006) and PFS (6.0 *vs.* 2.7 months, P=0.006).

2.3.4. Mechanisms of synergy between ICIs and other molecular therapies

All combinations of TKIs and ICIs that are efficacious against HCC are considered to be related to the inhibition of *VEGF* signaling. Although two treatments might lead to an additive effect, several experimental studies and clinical trials have provided evidence of a synergistic effect (*69,119-123*).

This synergy includes the effect of *VEGF* pathway inhibition on tumor vasculature and immune cells. Inhibition of *VEGF* causes vessel pruning (leading to hypoxia) and normalization (leading to improved drug delivery and enhancement of immune cell attachment and extravasation) (*124*). In addition, *VEGF* can affect the tumor microenvironment as a potent immunomodulatory molecule (*122*). Moreover, the effects of TKIs on HCC are not limited to *VEGF* signaling. TKIs can also block pathways that lead to immune cell exclusion, such as *MAPK*, *WNT-β-catenin*, *CDK4/6*, and *PI3K-PTEN* signaling (*125*).

2.4. Adoptive cell transfer

The mechanism of adoptive cell transfer (ACT) is the transfer of immune cells back to the body after they have been induced to possess more effective antitumor features (*126*). ACT therapies that displayed promising antitumor activity against HCC include chimeric antigen receptor T cells (CAR-T-cells), T cell receptor (TCR) engineered T cells, cytokine-induced killer cells (CIKs), and tumor-infiltrating lymphocytes (TILs) (*127*).

Autologous *GPC-3*-CAR-T cell therapy resulted in good clinical outcomes (an OS rate of 50.1% at 6 months, 42.0% at 1 year, and 10.5% at 3 years) in patients with *GPC-3*-positive advanced HCC in phase I studies (*128*). More than half of the patients with *CD133*-positive advanced HCC had a clinical benefit, with a median PFS of 6.8 months and OS of 12 months after reinfusion of *CD133*-CAR-T cells (*129*). In addition, several meta-analyses concluded that adjuvant CIK cell-based immunotherapy is a promising therapeutic approach for patients with BCLC stage B or lower HCC since it can improve OS and reduce recurrence (*130,131*). Further studies and clinical trials need to be conducted.

2.5. Oncolytic viruses

Oncolytic viruses (OVs) can preferentially infect tumor cells and cause lysis while sparing normal tissue, so they are promising treatment strategies that might be considered in multimodal therapy (*132*). In recent years, the oncolytic activity of LDO-GFP (a herpes simplex virus type 1-based oncolytic vector), Golgi protein 73-sphingosine kinase 1-short RNA-adenovirus serotype 5, and VV-IL-37 was noted *in vitro* and *in vivo* (*133-135*). OV dosing must be determined for clinical use, and thus more trials need to be conducted.

3. Management of advanced HCC: Locoregional therapies

3.1. TACE

Although TACE is not recommended for patients with advanced HCC according to the EASL (*6*) and the US Association for the Study of Liver Diseases (AASLD) (*136*), several studies suggested the survival benefits of TACE in patients with advanced HCC with or without PVTT (*137-139*). The BRIDGE study indicated that TACE was commonly used in many countries for patients with all stages of HCC (including North America, Europe, China, and South Korea) and TACE was used in about 50% of patients with BCLC-C HCC (*140*). To the extent known, Lo *et al.* (*141*) were the first to indicate that TACE could result in additional survival in patients with advanced HCC (relative risk of death: 0.49, 95% CI: 0.29-0.81, $P = 0.006$) compared to the best supportive care. Niu *et al.* (*137*) indicated the expanded OS in patients receiving TACE compared to patients receiving conservative treatment (8.67 *vs.* 1.4 months, $P < 0.001$). Recently, a randomized, multicenter prospective trial (TACTICS trial) (*142*) has revealed that TACE plus sorafenib significantly improved PFS compared to TACE alone in patients with advanced HCC, with a median PFS of 25.2 months and 13.5 months, respectively ($P = 0.006$). The 1-year survival rate was 96.2% and the 2-year survival rate was 82.7% in patients receiving combination therapy compared to rates of 77.2% and 64.6% in the TACE group.

TACE might be a treatment option for patients with advanced HCC and PVTT involving collateral vessels around the portal vein and relatively good liver function (*143*). In a large cohort of 164 patients with advanced HCC and PVTT, TACE significantly improved survival in patients with PVTT involving the segmental branches of the portal vein or above (*144*) compared to patients receiving conservative treatment. In a recent meta-analysis (*139*) of 1,933 patients with HCC and PVTT, TACE resulted in a median OS of 8 months (95% CI: 5-15) and a 1-year survival rate of 29% (95% CI: 20-40%), a 3-year survival rate of 4% (95% CI: 1-11%), and a 5-year survival rate of 1% (95% CI: 0-5%).

3.2. Hepatic artery infusion chemotherapy

As mentioned in the practice guidelines in Asian countries (*10,145*), hepatic artery infusion chemotherapy (HAIC) is widely used to treat unresectable HCC. HAIC injects a highly concentrated chemotherapeutic agent into the targeted lesion *via* the hepatic artery. A multicenter retrospective study conducted in South Korea indicated that HAIC led to favorable responses in patients with HCC and PVTT compared to sorafenib, with a longer median OS (7.1 *vs.* 5.5 months, $P = 0.011$) and TTP (3.3 *vs.* 2.1 months, $P = 0.034$) (*29*). A phase 3 trial (SILIUS, NCT01214343) conducted at 31 sites in Japan involved patients with unresectable advanced HCC to compare HAIC plus sorafenib and sorafenib monotherapy (*146*). There were no significant differences in the median OS of patients receiving HAIC-sorafenib and patients receiving sorafenib (11.8 months (95% CI: 9.1-14.5) *vs.* 11.5 months (95% CI: 8.2-14.8), $P = 0.955$). Grade 3-4 adverse events were more frequent in patients receiving HAIC-sorafenib. Nevertheless, HAIC resulted in a better response in patients with HCC and macroscopic vascular invasion. Therefore, HAIC cannot significantly provide an additional benefit for patients with advanced HCC who received sorafenib monotherapy, but it could be an additional treatment for patients with macroscopic vascular invasion (*147*).

3.3. Transarterial radioembolization

The 2018 AASLD guidelines (*148*) recommended transarterial radioembolization (TARE) as an alternative therapy to molecularly targeted agents for patients with BCLC stage C cirrhosis. In contrast to TACE, the therapeutic action of TARE is predominately radiation with yttrium 90, which is injected intra-arterially in the vessels feeding the HCC (*149*). A retrospective study conducted by Gramenzi *et al.* (*150*) noted the potential efficacy of TARE in patients with advanced HCC. Two clinical trials compared TARE and sorafenib in advanced HCC (*151,152*). The first, the SARAH (SorAfenib Versus Radioembolization in Advanced Hepatocellular Carcinoma) trial, found no significant differences in survival between patients receiving TARE or sorafenib (8.0 *vs.* 9.9 months, $P = 0.18$). The second randomized trial, the SIRveNIB (selective internal radiation therapy *vs.* sorafenib) trial, also reported no significant differences in OS between TARE and sorafenib (8.8 *vs.* 10.0 months, $P = 0.36$). Both trials rated TARE highly because of its tumor response rate and tolerability.

3.4. Percutaneous ablation

Ethanol injection (EI), microwave ablation (MWA), radiofrequency ablation (RFA), and cryoablation (CRA) are predominant forms of image-guided percutaneous ablation therapies with minimally invasive characteristics. They are frequently suggested for patients with small HCC (≤ 3 cm) and Child-Pugh class A or B hepatic functional reserve.

There was a time when EI was regarded as the standard in ablation. The survival rate in patients with HCC treated with EI has been reported to be 38-60% at 5 years (*153-156*). Nowadays, EI is seldom recommended unless RFA cannot be safely performed. A SEER database analysis indicated that EI resulted in similar clinical outcomes compared to RFA in patients with a single HCC of no more than 5 cm (*157*). Many centers considered EI as an adjuvant therapy for combination

strategies (*158*). Studies have indicated that EI can enhance the efficacy of RFA in the treatment of HCC (*159,160*), the underlying mechanism of which is that EI can induce microthrombi formation to occlude blood vessels and then reduce heat dissipation to increase the efficacy of RFA. EI combined with TACE was found to be safe for the treatment of advanced HCC and PVTT, and it resulted in a significant survival advantage over TACE alone (*161*).

MWA, in which tumor tissue is ablated by dielectric heat caused by microwave energy, has progressed beyond its initial use for early-stage HCC thanks to the development of equipment and techniques in recent years (*3*). Two clinical trials revealed that MWA is more efficacious than RFA in terms of eradicating larger tumors (size 3-5 cm) and requires less time (*162,163*). Nowadays, whether improved MWA can be suggested for intermediate-stage patients remains unknown. A recent multicenter retrospective study indicated that MWA can result in better survival for patients with HCC (BCLC stages 0-B) over a 12-year follow-up period (*164*). Only 1 randomized study of advanced HCC compared the safety and efficacy of TACE plus MWA and TACE alone, and it found that combined treatment was more efficacious (*165*). Further studies of MWA to treat advanced HCC need to be conducted.

RFA releases an electrical current within the radiofrequency range through a needle electrode and thus leads to heat-based thermal cytotoxicity (*166*). Studies revealed that RFA alone or combined with other therapies could provide additional survival benefits for patients with advanced HCC (*166-171*). For example, Duffy *et al.* (*169*) combined tremelimumab (3.5 mg/kg or 10 mg/kg for 6 doses every 4weeks) and RFA (on day 36) to treat advanced HCC, and the median OS was 12.3 months (95% CI: 9.3-15.4 months) with no dose-limiting toxicities. The 6-month probability of tumor PFS was 57.1% and the 12-month probability was 33.1% (*169*). Peng *et al.* (*168*) reported that the median OS improved significantly in patients receiving combination therapy (RFA plus TACE and sorafenib) than in patients receiving sorafenib alone (14.0 *vs.* 9.0 months, $P < 0.001$). In a study by Lyu *et al.* (*172*), patients with advanced HCC who received a PD-1 inhibitor (nivolumab or pembrolizumab) but who did not respond for at least 12 months underwent subtotal thermal ablation. Interestingly, an increased response rate from 10% (5/50) to 24% (12/50) was observed after RFA. Moreover, the efficacy and safety of RFA were evaluated in that proof-of-concept study, with a median TTP of 6.1 months (95% CI: 2.6-11.2), PFS of 5 months (95% CI: 2.9-7.1), and OS of 6.9 months (95% CI: 7.7-26.1).

CRA has an advantage of causing less damage when treating HCC compared to MWA and RFA because of its specific mechanism: tumor tissue injury is based on the formation of an ice ball at the tip of a cryoprobe (*173*). CRA is equally effective for locoregional treatment of early-stage HCC compared to RFA and MWA (*174,175*). CRA and RFA had similar rates of local tumor progression and safety even in elderly patients with small HCC (*176*). Currently, CRA is regarded as a safe alternative to RFA or MWA (*177*). Reported experience in using CRA to treat unresectable HCC is limited. Several retrospective studies compared the efficacy and safety of TACE combined with ablation (MWA, RFA, or CRA) for intermediate or advanced HCC (*178-180*). Although the efficacy of these combination strategies was comparable, TACE-MWA had the lowest complication rate (especially with regard to thrombocytopenia).

3.5. Hepatectomy

Hepatectomy might also be an optional therapy for patients with advanced HCC. It was not recommended by the EASL or JIS guidelines (*6,10*), but its survival benefits have been examined in many studies. A study by Komatsu *et al.* (*181*) involved 314 patients (BCLC-B, $n = 149$; BCLC-C, $n = 165$) who underwent complete hepatectomy or reductive hepatectomy. The median OS was 19.5 months for patients with BCLC-C undergoing complete hepatectomy and 17.6 months for those undergoing reductive hepatectomy ($P = 0.014$) but 48.9 months and 20.1 months in those with BCLC-B ($P = 0.008$). The 3-year OS rate was 18.6% for BCLC-C patients undergoing complete hepatectomy and 0% for those undergoing reductive hepatectomy. The 3-year OS rate was 47.5% in patients with BCLC-B undergoing complete hepatectomy and 0% in those undergoing reductive hepatectomy. A study by Yamamoto *et al.* (*182*), retrospectively examined 372 patients with advanced HCC and PVTT who underwent hepatectomy. Results indicated that the cumulative 5-year OS was 58.3% and the 5-year disease-free survival rate was 31.3%. A meta-analysis (*183*) compared hepatectomy to TACE or sorafenib alone for patients with advanced HCC and PVTT. There were no significant differences in survival between hepatectomy and TACE (odds ratio (OR): 0.96, 95% CI: 0.44-2.11), but hepatectomy resulted in an improved OS compared to sorafenib (OR=0.12, 95% CI: 0.06-0.24). Importantly, hepatectomy was superior in patients without PVTT in the main trunk compared to those with main portal vein invasion (OR = 2.18, 95% CI: 1.76-2.70). Still, this conclusion should be interpreted cautiously given the limited sample, publication bias, type of retrospective study, and different follow-up times.

3.6. Radiation therapy

HCC is considered to be a radiosensitive tumor, and its location in a radiosensitive organ limited the use of radiotherapy in the past. Nevertheless, recent advances in three-dimensional conformal radiation therapy (3D-CRT) have allowed safer use of radiotherapy without severe toxicity in patients with unresectable HCC. Technological

developments (intensity-modulated radiotherapy (IMRT) and stereotactic body radiotherapy (SBRT)) can target HCC precisely, thus providing a clinical benefit. 3D-CRT is suggested for symptomatic bony metastases caused by advanced HCC by the Asian-Pacific clinical practice guidelines (*184*) but is not recommended in the AASLD and EASL guidelines for treating HCC. A multicenter controlled study conducted by Wei *et al.* (*185*) indicated that neoadjuvant 3D-CRT provided significantly better survival outcomes than resection alone in HCC and PVTT. Even through the evidence is insufficient, 3D-CRT may be one of the promising treatment options for advanced HCC.

4. Future perspectives

4.1. Combinations of systemic and locoregional therapies

In addition to systemic treatments, local therapies such as radiotherapy, RFA, and TACE were found to induce an immune response by promoting the death of immunogenic tumor cells or destroying the tumor microenvironment thus augmenting immune simulation and antitumor action (*186-189*).

Several clinical trials are evaluating the safety and efficacy of the combination of systemic therapies (TKIs or ICIs) with locoregional treatments. As reported at the ASCO GI 2022 meeting, the LAUNCH phase III clinical trial was conducted to compare levatinib plus TACE to levatinib alone in terms of safety and efficacy. Levatinib plus TACE performed better than levatinib monotherapy with a longer OS (17.8 *vs.* 11.5 months, $P < 0.001$) and PFS (10.6 *vs.* 6.4 months, $P < 0.001$) and higher ORR (54.1% *vs.* 25.0%, $P < 0.001$), indicating that levatinib plus TACE is likely to become a novel first-line therapy. Tremelimumab plus ablation was assessed by a phase II trial, and ablation was found to be related to an increased response rate in patients with HCC who had an atypical response to an ICI after resistance to sorafenib (*169*). TARE or TACE in combination with ICIs is also being investigated in phase II trials (*190,191*).

The combination of local and systemic therapy may result in higher efficacy and fewer adverse reactions to HCC treatment. However, many details such as the optimal dose, timing, and sequence of the combination treatment strategies need to be determined, and these require a deeper understanding of their underlying mechanisms.

4.2. Triple therapy or conversion management

Successful combinations of systemic therapies and combinations of systemic and locoregional therapies allow for triple therapy. A triple therapy is a general combination of 2 systemic therapies (1 TKI and 1 ICI or 2 antibodies) and 1 locoregional therapy.

A number of retrospective studies have investigated treatment with TACE plus a PD-1 inhibitor combined with lenvatinib (*192-197*), and they have noted encouraging efficiency and manageable safety in patients with unresectable HCC. A phase II single center study revealed the safe and encouraging antitumor activity of HAIC plus levatinib-toripalimab in high-risk advanced HCC (*198*). In that trial involving 36 subjects, the primary end-point was met with a PFS rate of 80.6% (95% CI: 64.0-91.8%) at six months. Median PFS was 10.4 months (95% CI: 5.8-15.0) and OS was 17.9 months (95% CI: 14.5-21.3).

Several articles have reported on the use of triple therapy and they have also described the potential conversion of resection (*199,200*). Successful conversion criteria were: (*i*) at least partial remission; (*ii*) a future liver remnant (FLR) > 40% or non-cirrhosis > 30%; (*iii*) reversion to a branch thrombus; (*iv*) Child-Pugh < 7; and (*v*) no new resectable liver lesions during treatment. Two studies focusing on levatinib plus PD-1 inhibitors plus TACE/HAIC for advanced unresectable HCC were presented at the ASCO GI 2022 meeting. A prospective multicenter trial (NCT04997850) noted a conversion rate of 50%. Successful conversion therapy plus surgery is a type of quadruple therapy.

4.3. Traditional Chinese medicine

As mentioned in the Chinese guidelines, traditional Chinese medicine (TCM) may improve the clinical outcomes and reduce the adverse effects of other therapies (*145*). Several types of TCM (such as Huai'er granules and cinobufacini) have been used for the treatment of liver cancer in China.

Two multicenter randomized clinical trials indicated the efficacy of TCM on recurrence after curative resection of HCC (*201,202*). A trial conducted by Chen *et al.* (NCT01770431) involved 39 centers and 1,044 patients. The mean RFS in patients taking Huaier (patients who took Huaier orally) was longer than that in the control group (75.5 *vs.* 68.5 weeks; HR 0.67; 95% CI: 0.55-0.81). Another trial compared the efficacy and safety of TACE and TCM (a cinobufacini injection and Jiedu granules) for patients with HCC who underwent surgery. A TCM regimen was related to a diminished risk of HCC recurrence compared to TACE. Currently, there is insufficient evidence for TCM in patients with advanced HCC. Results of two retrospective studies indicated that TCM as adjuvant therapy can also prolong median survival time for patients with advanced HCC (*203*). In the future, TCM can be tried as a supplementary treatment for patients with HCC who have received comprehensive treatment.

4.4. Treatment-related toxicity

Combination therapies with TKIs, ICIs, and conventional therapies have actually revolutionized the management of

advanced HCC because of their marked curative effect. However, combination therapies (and especially those including more than 1 systemic therapy) are accompanied by increased toxicities and each combination strategy may cause different adverse events (*109*).

The optimal sequence of treatments and the selection of patients should be fully considered. In clinical practice, patients who do not have comorbidities and who have sufficient liver reserve will be considered for combination treatments. Most adverse events are moderate and controllable with conservative treatment, but the occurrence of rare and life-threatening toxicities should not be ignored (*204*). Patients require a detailed physical examination depending on the treatment strategy and need to be informed of precursory symptoms of adverse events for better self-monitoring during stages of treatment. For instance, patients with advanced HCC should undergo esophagogastroduodenoscopy prior to atezolizumab-bevacizumab therapy because the combination treatment is accompanied by a higher risk of bleeding (*205,206*). In addition, an evaluation for the presence of varices is recommended within 6 months of initiation of atezolizumab-bevacizumab (*110*).

4.5. Consideration of the etiology of HCC

Current international practice guidelines do not consider the influence of the etiology of HCC in their treatment algorithms (*6,148,184*). While locoregional treatments seem equally effective regardless of the etiology of HCC, little is known about the impact of non-alcoholic fatty liver disease (NAFLD) as an etiology on the efficacy of systemic therapy (*207*). An international cohort study of 5,201 patients (Europe and North America) concluded that NAFLD-driven HCC received a similar clinical benefit from sorafenib compared to other etiologies (*208*). TKIs are probably equally effective, but several studies have found that ICIs may be less effective in NAFLD-driven HCC than in viral HCC.

A meta-analysis of three randomized phase III trials involving 1,656 patients with advanced HCC found that immunotherapy did not improve survival in patients with non-viral HCC (*209*). Moreover, *in vivo* studies found that anti-PD-1 treatment did not result in regression of NAFLD-driven HCC. A recent meta-analysis of 8 trials including 3,739 patients revealed that ICIs are less efficacious in patients with non-viral HCC, while there were no differences associated with etiology in patients with HCC receiving a TKI or anti-VEGF antibody (*210*). For patients with NAFLD-driven HCC, the combination strategies may require a change, such as elimination of immunotherapy. Further clinical trials should be designed with prespecified stratification.

4.6. Molecular biomarkers

There is a clear need for reliable molecular biomarkers in HCC risk stratification, prognosis, and treatment response. Biomarkers were extensively studied in terms of microsatellite instability, PD-L1 expression, and the tumor mutational burden (*211*). Alpha fetoprotein (AFP), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) have been found to be related to HCC treatment outcomes (*212-214*). A recent study indicated that preoperative prothrombin induced by vitamin K absence-II (PIVKA-II) has a higher positive rate than AFP in detecting resectable HCC and predicting early postoperative recurrence (*215*). The level of PIVKA-II can serve as an indicator of the presence of PVTT and an advanced tumor stage (*216*). Although the EASL guidelines state that PIVKA-II is suboptimal in terms of cost-effectiveness, it has recently been a routinely measured tumor marker similar to AFP (*6*). In addition to circulating markers, gene expression assays were also used to identify biomarkers with which to predict the response to immunotherapy. Haber *et al*. constructed a novel 11-gene signature that can predict response and survival in patients with advanced HCC who were initially treated with an anti-PD1 antibody (*217*). However, gene expression assays require invasive biopsies before treatment.

4.7. Noninvasive imaging biomarkers and artificial intelligence (AI)

HCC staging systems (*e.g.*, Barcelona Clinic Liver Cancer, Hong Kong Liver Cancer, Cancer of the Liver Italian Program, and TNM systems) occupy the central role in HCC prognosis and management (*218*). However, these systems are inadequate at accurately predicting risk and none of them provide quantitative measures. A few biomarkers have been examined and validated in their prediction of drug susceptibility and prognosis, offering an opportunity to evaluate the clinical benefits of current therapies (*33,219-221*). Besides novel serum biomarkers, various imaging modalities can also offer precious information. Image findings and data mining algorithms can be used to capitalize on imaging data. Xu *et al.* (*222*) studied radiomics, image findings, and serum indices to predict microvascular invasion using nomograms. Radiomics models established by Ji *et al.* (*223*) can accurately provide quantifiable risk measures of recurrence for early-stage HCC. In addition to tumor characteristics, stages of liver fibrosis might also affect the treatment plan and can be also predicted with radiomics and machine learning techniques (*224,225*). A multicenter study conducted in France is developing an AL algorithm based on clinical, biological, and ultrasound data to stratify the risk of HCC emergence in high- and low-risk patients (*226*). In instances where a biomarker has yet to be identified (such as therapeutic response and treatment toxicity), AI-based prediction could significantly contribute to improving clinical outcomes

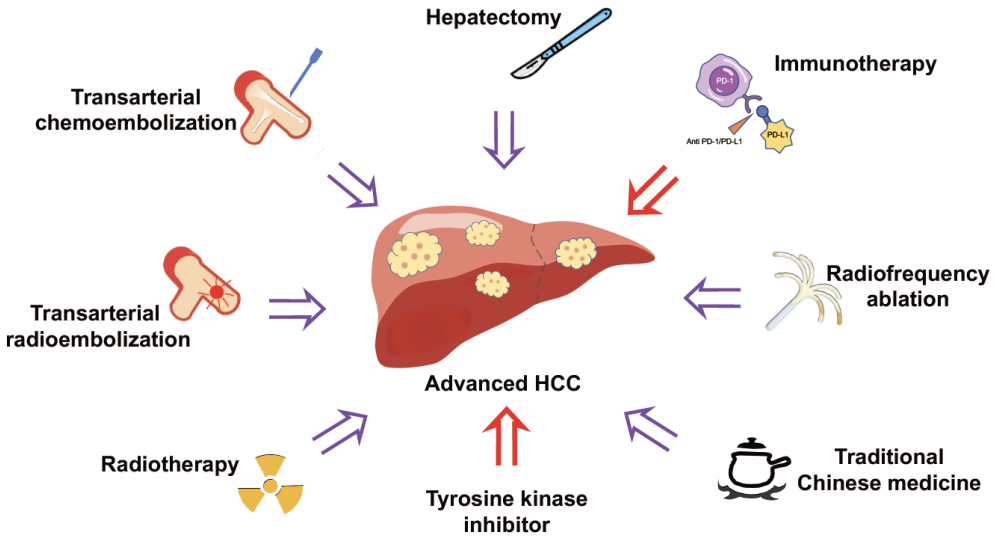

Figure 2. Management of patients with advanced hepatocellular carcinoma apart from molecularly targeted therapy. The red arrows indicate the first-line therapy to treat hepatocellular carcinoma. HCC, hepatocellular carcinoma.

and reducing healthcare expenditures. The potential of AI should be fully explored in prospective studies to improve the clinical management of patients with HCC. If tumor characteristics (such as high/low risk and resistance to chemotherapy or immunotherapy) can be determined noninvasively, more suitable treatment strategies can be formulated.

4.8. Challenges

Despite the promising developments, the optimal therapeutic strategy for advanced HCC remains vague. Several challenges regarding disease prognosis remain, and few therapies have provided additional survival benefits. Moreover, patients with advanced HCC might be further classified based on biomarkers or imaging parameters, which would help to devise proper therapies in various clinical settings. Furthermore, which targeted agents should be used once the first targeted agent (sorafenib or lenvatinib) fails remains unclear. Treatments for early or intermediate HCC (such as TACE, TARE, immunotherapy, ablation, hepatectomy, and intravenous chemotherapy) should be further examined for their potential therapeutic value in treating advanced HCC (Figure 2). Lastly, the resistance to both targeted agents and immunotherapy is another hot topic, and the underlying mechanisms should be examined to develop novel strategies to overcome drug resistance.

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Review

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Timing of parathyroidectomy for kidney transplant patients with secondary hyperparathyroidism: A practical overview

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SUMMARY Kidney transplantation remains the best treatment for patients with end-stage kidney disease, and it could partially mitigate systemic disorders of mineral and bone metabolism caused by secondary hyperparathyroidism. However, persistent hyperparathyroidism is still observed in 30-60% of patients 1 year after kidney transplantation, leading to impairment of allograft function and a disturbance of mineral metabolism. The timing of parathyroidectomy varies among transplant centers because the possible negative effects of parathyroidectomy on allograft outcomes are still unclear. This review provides a comprehensive and detailed overview of the natural course of hyperparathyroidism following kidney transplantation and the effects of the timing and extent of parathyroidectomy on allograft function. It aims to provide useful information for surgeons to propose an appropriate intervention strategy to break the vicious cycle of post-kidney transplantation hyperparathyroidism and deterioration of allograft function.

Keywords persistent hyperparathyroidism, allograft function, hemodynamic fluctuation, nephrocalcinosis

1. Introduction

As a common and formidable complication in end-stage renal disease (ESRD), chronic kidney disease-mineral and bone disorder (CKD-MBD) is initially stimulated by phosphorus retention, combined with a low calcium level, and abnormalities in 1.25-hydroxyvitamin D synthesis, together with a progressive increase in parathyroid hormone (PTH), which eventually results in secondary hyperparathyroidism (SHPT) (*1,2*). SHPT is likely to develop to an advanced stage that is resistant to medical treatment such as drug therapy, dialysis, and diet. Untreated or treatment-resistant SHPT results in serious complications such as kidney stones, osteoporosis, vascular calcification, and pathological fractures (*3-5*), affecting the quality of life in most patients with ESRD (*5,6*).

Kidney transplantation (KT) remains the best treatment for patients with ESRD (*7*). By restoring renal function, successful KT is thought to ameliorate the endocrinal and metabolic effects of SHPT and at least partially mitigate systemic disorders of mineral and bone metabolism, thus improving quality of life and increasing patient survival (*5,7-9*). However, in the early post-transplant period, persistence of preexisting hyperparathyroidism and related biochemical alterations are still observed in some patients. Hyperparathyroidism after KT may influence changes in allograft function and further disturb mineral metabolism (*4,7*), which leads to a vicious cycle involving the persistence of post-transplant hyperparathyroidism and impairment of allograft function. Two concerns regarding the management of hyperparathyroidism in kidney transplant recipients have been raised: (*i*) Which strategy is feasible and acceptable? To prevent potential persistent hyperparathyroidism (PHPT) in a KT candidate, or to treat a definite PHPT in a kidney transplant recipient? and (*ii*) If treatment of PHPT is ensured, what is the appropriate timing for surgery? Early or late?

The current review includes recent findings and it discusses the effects of the timing and extent of parathyroidectomy on allograft function.

2. Natural course of hyperparathyroidism following KT and prevalence of post-transplant PHPT

The preexisting mineral imbalances that lead to SHPT are usually normalized within several days to several weeks after KT (*7*), but changes in serum PTH levels usually take several months to manifest. Recent retrospective studies (*10,11*) have described the long-term natural history of SHPT after KT. The PTH level dropped significantly during the first 3 months post-transplant and decreased gradually throughout the

year, typically stabilizing thereafter. The development of hyperparathyroidism was determined based on the pre-transplant PTH level and the renal function at each checkpoint during follow-up. This indicated that remission of hyperparathyroidism following KT depended on the restoration of renal function. A decrease in the PTH level by 33% in the first 6 months of the first year after transplantation and by 57% in the second half indicated that the restoration of renal function might accelerate the remission of hyperparathyroidism (*10*). Studies have noted a significant correlation between PTH and the estimated glomerular filtration rate (eGFR) (*12,13*). With the restoration of a more normal eGFR, SHPT might resolve in mild cases or remain stable until the end of the first year post-transplant (*11,14*). Even with adequate allograft function, PHPT is still observed in 30-60% of kidney transplant recipients 1 year after KT and in 20% of patients even at 5 years (*4,14,15*). The direct and most prominent impact of PHPT is on the serum calcium level (*12,14*). Although the serum calcium level normalizes a few days after KT, hypercalcemia may develop as soon as 1 week after transplantation (*7,14*). Hypercalcemia is closely associated with PHPT in the majority of cases and is usually considered to be an indicator of PHPT, with a incidence between 10% and 30% (*9,14,15*). This variability in incidence may be attributable to various factors such as the use of different serum calcium levels for diagnosis, evaluation of the ionized or total calcium level, regardless of whether the calcium levels are corrected based on albumin, and the timing of diagnosis (*15*). A point worth noting is that the prevalence of hypercalcemia may decrease over time, but 5-10% of patients continue to have persistently high serum calcium levels over the long term (*7*).

3. Post-KT PHPT and its clinical significance

Like hypercalcemia, the divergence in the prevalence of post-KT PHPT also reflects the fact that the diagnostic cut-offs and evaluation time-points are inconsistent (*9,14,16-22*). In many studies, post-KT PHPT was referred to as tertiary hyperparathyroidism (THPT). There is a lack of a consensus regarding the exact definition for THPT. "THPT" refers to an advanced stage – and especially autonomous secretion of PTH – but does not necessarily define hyperparathyroidism as that occurring only after KT. Concomitant hypercalcemia is an indispensable criterion for THPT but not for PHPT. KT itself is just like a screening procedure, eliminating all of the hyperplastic parathyroid tissue that might return with re-established calcium-phosphate homeostasis, leaving only autonomous tissue that does not respond to feedback (*11*). Different percentages of THPT components remain in PHPT parathyroid glands at different time-points post-transplant (*22*). Even with small parathyroid glands, nodular hyperplasia might still be very severe (*20,22,23*). Therefore, THPT is considered a subset of PHPT post-KT (*22*), and the term PHPT has been used in a review of the relevant literature throughout this paper.

PHPT endangers both patient and allograft survival. First, PHPT is also associated with increased mortality, cardiovascular complications, fractures, and decreased quality of life (*11*). In a multivariate analysis comparing kidney transplant recipients with serum PTH>65 pg/mL to those with normal or low levels, an increase of 46% for all-cause death was noted (*24*). Second, with respect to specific concern about allograft survival, PHPT contributes to deterioration of allograft function. A longitudinal study of 911 kidney transplant recipients with a mean follow-up time of 47 months reported that the rate of allograft failure (defined as a return to dialysis) was 49/538 in the PHPT group *vs*. 10/343 in the non-PHPT group, and death-censored allograft survival was lower in the PHPT group (*19*). Another analysis of 1,609 kidney transplant recipients found that PHPT was independently associated with delayed allograft function and worse allograft survival (*5*). PHPT and hypercalcemia have a close causal relationship and both affect three aspects of allograft function: (*i*) PHPT and hypercalcemia promote nephrocalcinosis, which is characterized by tubular and interstitial deposits of calcium in the form of calcium oxalate or calcium phosphate. As the duration and severity of hypercalcemia increases, the extent of tubulointerstitial calcification increases, which may contribute to allograft dysfunction over the long term, and especially when the calcium level rises rapidly (*14,15*). (*ii*) Hypercalcemia promotes vascular calcification. PHPT-related hypercalcemia is associated with the development of vascular calcification, which then stiffens the vasculature and impairs arterial distensibility, thereby compromising perfusion of the allograft. Because vasculature supplying the allograft may also be involved, the possibility of allograft failure increases (*24,25*). Severe hypercalcemia can also cause acute injury of the allograft due to low perfusion by direct vasoconstriction (*14*). (*iii*) PHPT leads to hyperfiltration injury. The hemodynamic effect of PTH seems to significantly vasodilate preglomerular vessels while constricting efferent arterioles at the same time, resulting in hyperfiltration and consequent progressive deterioration of renal function.

4. To prevent PHPT in KT candidates, is pre-transplant parathyroidectomy an appropriate or excessive treatment?

There is a wide divergence of clinical practice and a lack of consensus in the area of surgical management of KT candidates with hyperparathyroidism (*4,26*). In a survey conducted in the United States in 2018, questions about the use of parathyroid surgical procedures to prepare patients for KT indicated that more than two-thirds of respondents did not consider a PTH level > 800 pg/mL as

an absolute or relative contraindication for transplantation (*26*). Approximately 66% of respondents answered that they occasionally recommended parathyroidectomy for SHPT prior to KT, and only 5% answered that they always recommended it in the context of SHPT. Sixty-three percent indicated that < 10% of their KT candidates underwent pre-transplant parathyroidectomy at their facilities. Sixty-six percent recommended post-transplant parathyroidectomy based on persistent, unsatisfactory PTH levels post-transplant.

4.1. Pre-transplant parathyroidectomy might be superior to post-transplant parathyroidectomy in terms of ensuring allograft function

Whether the parathyroidectomy is performed before or after KT, a sustained decrease in PTH and calcium levels was consistently achieved (*18,27,28,29-31*). However, the altered mineral metabolism and endocrine function associated with pre-KT parathyroidectomy might be more conducive to allograft survival compared to that associated with untreated SHPT. This could explain why pre-KT parathyroidectomy was considered to be superior to post-KT parathyroidectomy in terms of ensuring allograft function according to several comparative studies (*11,25,27,28,32,33*). First, the degree of metabolic and endocrine disturbances that an allograft is subjected to is more severe in patients not undergoing pre-KT parathyroidectomy than in those undergoing that procedure. Almost all of the studies noted that the pre-KT PTH level (the post-parathyroidectomy PTH level in the pre-transplant group roughly compared to the pre-parathyroidectomy level in the post-transplant group) and the prevalence of hypercalcemia, which were both risk factors contributing to worsening allograft function, were significantly higher in the post-transplant groups (*18,27,28*). This likely indicates that patients undergoing post-transplant parathyroidectomy usually have severe PHPT and impaired allograft function should be promptly remedied. Second, post-KT parathyroidectomy results in additional and drastic hemodynamic fluctuation in the allograft, related mainly to an abrupt reduction in PTH. Given that PTH has important preglomerular vasodilatory action as well as efferent vasoconstrictive action, a steep decline in PTH action on the renovascular system could lead to perivascular ischemia and cause irreversible interstitial tubule cell damage. In a study by Jeon *et al*., patients with impairment of > 25% 1 month after transplantation had a significantly lower baseline eGFR and significantly greater changes in the PTH level after parathyroidectomy (*27*). Third, the incidence of interstitial calcium deposition was significantly higher in the post-KT group than in the pre-KT group (*31*). This progressive nephrocalcinosis likely correlates with a high risk of reduced allograft function (*11,34*). The potential cause of further deterioration of allograft function after parathyroidectomy and whether parathyroidectomy halts the progress of interstitial calcification need to be examined further; information from a kidney biopsy might help in the differential diagnosis of allograft dysfunction after parathyroidectomy (*27*).

4.2. There are doubts about the need for pre-transplant parathyroidectomy to preclude the regression of mild or moderate SHPT in transplant candidates

In the literature, few KT candidates have undergone a parathyroidectomy (*22,25*). There are 3 doubts about the necessity for pre-transplant parathyroidectomy in transplant candidates:

1) *Spontaneous regression of SHPT after successful KT*: Clinical studies comparing the effect of pre- or post-transplant parathyroidectomy on allograft function have yielded inconsistent results. Should PHPT be prevented before KT or be treated after KT? Some authors believe that because SHPT can regress in up to 57% of patients with correction of mineral homeostasis after successful KT, spontaneous resolution may be expected in some patients (*5,16*), so parathyroidectomy can be postponed for some candidates (*30*).

2) *The alternative role of calcimimetics in the treatment of SHPT*: Since their approval for clinical use in 2004, calcimimetics have gained wide acceptance at both efficaciously and safely ameliorating SHPT, and the rate of prescription has increased over the years (*35*). Calcimimetics both contribute to normalization of PTH and calcium levels and also eliminate the complications of parathyroidectomy. The use of calcimimetics provides an opportunity for surgeons to decide whether to perform post-transplant parathyroidectomy based on the impact of successful KT on parathyroid function.

3) *Unpredictable changes in parathyroid function after pre-transplant parathyroidectomy*: The serum level of PTH is one of the most important indices with which to evaluate patients with hyperparathyroidism, but it is still the most difficult index to control by parathyroidectomy. The rate of hypoparathyroidism following surgical intervention in SHPT varied between 16.6% and 18.1% (*36*). Low levels of PTH before KT were associated with an increased risk of post-KT vascular events (*37*). Marked hypoparathyroidism and hypocalcemia related to pre-KT parathyroidectomy might also lead to slow allograft function, and suboptimal allograft function is significantly related to the diffuse hypoperfusion of the glomeruli (*38*). Conversely, the risk of hyperparathyroidism recurrence is always present if renal disease is not fully cured. A study reported that hyperparathyroidism recurred after subtotal parathyroidectomy in one-third of patients on chronic hemodialysis at the end of a 10-year follow-up (*39*). If hyperparathyroidism recurs and persists after KT, PHPT and hypercalcemia are confirmed risk factors for poor allograft outcomes (*15,40,41*). Thus, a trade-off between the possible benefit and this potential risk of

parathyroidectomy has to be evaluated in KT candidates.

Therefore, the relatively low proportion of patients who undergo pre-transplant parathyroidectomy could be partially explained by two reasons: (*i*) its necessity in light of the potential effect of KT on the regression of SHPT and (*ii*) its curative effect regarding unpredictable changes in mineral metabolism and parathyroid function.

4.3. The risk factors for development of PHPT should be evaluated before pre-transplant parathyroidectomy

Okada *et al*. (*31*) suggested the superiority of pre-transplant parathyroidectomy over post-transplant parathyroidectomy in terms of stabilizing post-KT PTH levels within the normal range and preserving renal allograft function. This means that the main significance of pre-transplant parathyroidectomy is to cure SHPT and to prevent post-KT PHPT, thereby allowing KT candidates to avoid post-transplant parathyroidectomy. Therefore, KT candidates with risk factors for PHPT are eligible for pre-transplant parathyroidectomy. The risk factors for development of PHPT that should be evaluated before surgery include a high pre-KT PTH level, a long history of dialysis, and treatment with calcimimetics. Patients with pre-KT PTH levels of 300-599 pg/mL are likely to develop PHPT, and the higher the preoperative level, the more possibility PHPT developing (*22*). In addition, a dialysis vintage > 6 years is a strong predictor of PHPT (*21,42*). The parathyroid mass grows gradually and progresses over time in patients undergoing longterm dialysis, indicating a high possibility of parathyroid gland autonomy (*42*). Tominaga *et al*. (*43*) reported that in SHPT patients on dialysis, an enlarged parathyroid gland weighing > 500 mg had a > 90% probability of containing nodular hyperplastic tissue, whereas a gland weighing < 150 mg mostly had diffuse hyperplastic tissue. Paradoxically, usage of calcimimetics was also cited as a risk factor. Some studies have reported a "rebound" effect on PTH because of the cessation of calcimimetics at the time of KT, leading to subsequent hyperparathyroidism and hypercalcemia (*8,21,22*). The deceptive action of calcimimetics blurred the true status of SHPT, thereby further hampering evaluation of the PHPT risk before KT. Once severe hyperparathyroidism is diagnosed, post-KT parathyroidectomy is the only option to treat that condition.

5. To cure PHPT in kidney transplant recipients, should post-transplant parathyroidectomy be performed early or late?

SHPT was not cured in all patients with KT, and PHPT was observed in 10-66% of patients 1 year after transplantation despite improvement in renal function (*16,19,44*). In a study by Lou *et al*. (*5*) with a more contemporary cohort with 1,609 cases, KT cured hyperparathyroidism in only 30.3% of cases at 1 year and 56.9% of cases at 2 years. PHPT has negative effects on allograft survival and can only be cured by surgical intervention. The current indications for post-KT parathyroidectomy include THPT, enlarged (> 500mg) parathyroids on imaging, persistent hypercalcemia, rapidly worsening vascular calcification, unexplained deterioration of allograft function, and progressive loss of bone mineral density (*27,44,45*). Although parathyroidectomy is intended to correct PHPT as well as hypercalcemia and to reverse the progression of allograft impairment, it is a double-edged sword in terms of its effect on allograft function. In most studies, parathyroidectomy preformed within 12 months after KT was classified as "early parathyroidectomy;" otherwise, it was classified as "late parathyroidectomy" (*18,28,30*). In addition, there is also debate over the impact of early versus late parathyroidectomy after KT on allograft function. The current guidelines and practice for PHPT recommend close observation, frequent monitoring, and waiting until 12 months after transplant prior to considering parathyroidectomy (*11*). How does post-transplant parathyroidectomy effect allograft function, and is late parathyroidectomy superior to early parathyroidectomy as current practices recommend?

5.1. The negative effect of post-transplant parathyroidectomy on allograft function is mainly by transient impairment and rarely by permanent deterioration

Several studies have reported that allograft function declined significantly 1-3 months after parathyroidectomy, it gradually recovered, and then it finally improved to the baseline level 12-15 months after parathyroidectomy (*3,16*). Long-term outcomes were comparable to those in patients who did not undergo parathyroidectomy, and permanent graft dysfunction was rare (*6,8,10,45*). Patecki *et al*.(*45*) reported that the median annual change in the eGFR between KT and parathyroidectomy was -0.5 mL/min, and then a significant drop of 25% in the eGFR was observed. In the interval between parathyroidectomy and 3 years later, a stable annual increase in the eGFR of 1.0 mL/min reflected improvement or recovery of renal function after the correction of hyperparathyroidism. A multicenter retrospective study including 185 patients compared the eGFR 5 years after transplantation in pre- and post-transplant parathyroidectomy groups, and it noted no differences in allograft function regardless of the timing of parathyroidectomy. Even though the post-transplant group had a > 25% decrease in the eGFR 3 months after parathyroidectomy, eGFR gradually recovered to the level in the pre-transplant group 1 year post-parathyroidectomy (*30*). In another multi-center study including 100 patients, acute deterioration of renal function was alleviated and stabilized 1 week after

parathyroidectomy in most patients; however, persistent impairment of renal function was noted in around 20% of patients. There were no significant differences in the eGFR in the pre- and post-transplant groups from 1 month to 5 years after transplantation (*27*). The decrease in the eGFR post-parathyroidectomy was associated with abnormalities of hemodynamics induced by parathyroidectomy-related hypoparathyroidism, and the decline might be transient. Therefore, post-transplant parathyroidectomy is necessary when PHPT impairs graft function, even though a transient decline in the eGFR may occur.

5.2. Late post-transplant parathyroidectomy provides a reasonable timeframe to allow spontaneous regression of hyperparathyroidism but it delays alleviation of allograft impairment due to PHPT

A marked decrease in the PTH level usually indicates the success of parathyroidectomy, but it is a significant predictor of allograft dysfunction (*40*). A decline in the PTH level of more than 80% is followed by a significant drop in creatinine clearance (*9,46*). In the initial phase after KT, PTH may be a driving force for glomerular filtration, simultaneously vasodilating preglomerular vessels and constricting efferent arterioles (*13*). A decline in PTH results in a reverse of glomerular hyperfiltration and reduced renal perfusion. This early hemodynamic injury induced by a low PTH after early post-transplant parathyroidectomy is certainly the main cause for early deterioration of renal function (*46*). Littbarski *et al*.(*28*) reported that parathyroidectomy within the first year post-transplant was more closely associated with compromised allograft function compared to pre-transplant parathyroidectomy, whereas late post-transplant parathyroidectomy was not. Late post-transplant parathyroidectomy allowed hyperparathyroidism to spontaneously regress in a considerable proportion of KT patients within a reasonable timeframe. A drastic hemodynamic fluctuation in the allograft due to post-transplant parathyroidectomy may be avoided in these patients.

However, in patients who required a post-transplant parathyroidectomy, PHPT is present at the time of transplantation and abnormal mineral metabolism may persist for several years. Chronic exposure to high PTH and calcium levels in patients with allograft calcification can facilitate further tubulointerstitial calcification and allograft impairment (*15,47*). Parathyroidectomy should be performed before PHPT progresses to an advanced stage (*20,45*). Research has indicated that achievement of a normal PTH level has a positive impact on overall allograft survival. Patients whose PTH levels normalized within 12 months displayed improved overall allograft survival compared to those whose levels normalized after 12 months (*5*). Normalization of calcium levels may contribute to regression of calcification in some patients (*15,41*). Therefore, the earlier an intervention is implemented, the better the prognosis with respect to the deteriorative role of PHPT and hypercalcemia (*47*).

5.3. Relatively limited parathyroidectomy (subtotal parathyroidectomy) is a procedure with proven effectiveness and safety in the early post-transplant period

Before considering a parathyroidectomy in the early period post-transplant, an important issue is to identify and treat patients who are prone to developing severe, non-resolving post-transplant hyperparathyroidism. A PTH level ≥ 150 pg/ml at 3 months post-transplant was found to be an independent predictor for allograft loss, decreased overall survival, and death with a functioning allograft during the period studied (*41*). In patients with severe hyperparathyroidism early after transplantation, the current guidelines consider severe hypercalcemia (serum calcium > 2.87 mmol/L) to be a strong indication for early parathyroidectomy (*48*). Limited parathyroidectomy might offer less possibility of a significant change in PTH (*7*) and lower the risk of hypoparathyroidism (*49*), thus having a positive effect on the rapid return of the eGFR to preoperative levels (*34,50*). Moreover, intraoperative PTH (ioPTH) monitoring provides a more precise profile of the PTH level (*51*). Von Beek *et al*.(*52*) found that there was a strong correlation between ioPTH and early postoperative PTH levels, indicating that ioPTH was a potentially useful index for predicting surgical effectiveness. In patients with functioning allografts, ioPTH levels 10 min and 20 min after resection of the parathyroid glands were equivalent to 2.6 times and 2.3 times the post-operative PTH levels, respectively, and were considered to possibly fall within the expected target range (*52*). With accurate monitoring, an acute decline in renal function after parathyroidectomy can be avoided and a stable renal function can be achieved throughout the 1-year follow-up (*51*).

The current issues on this topic are summarized in Figure 1. To prevent or to treat, that is the question. There is uncertainty about the necessity of surgery to prevent potential PHPT. When treating definite PHPT, there is a risk of endangering allograft function due to either a delayed surgical intervention or surgery with an abrupt drop in the PTH level.

The indications for surgical treatment of SHPT have been well-defined. Based on the current authors' own experience, parathyroidectomy should be performed, if indicated, to alleviate CKD-MBD-related symptoms, regardless of whether patients are KT candidates or not. A pre-KT parathyroidectomy would not affect the timetable for a planned KT but it would create relatively favorable conditions for a future allograft. The only additional consideration is the amount of residual parathyroid tissue. A subtotal parathyroidectomy or a

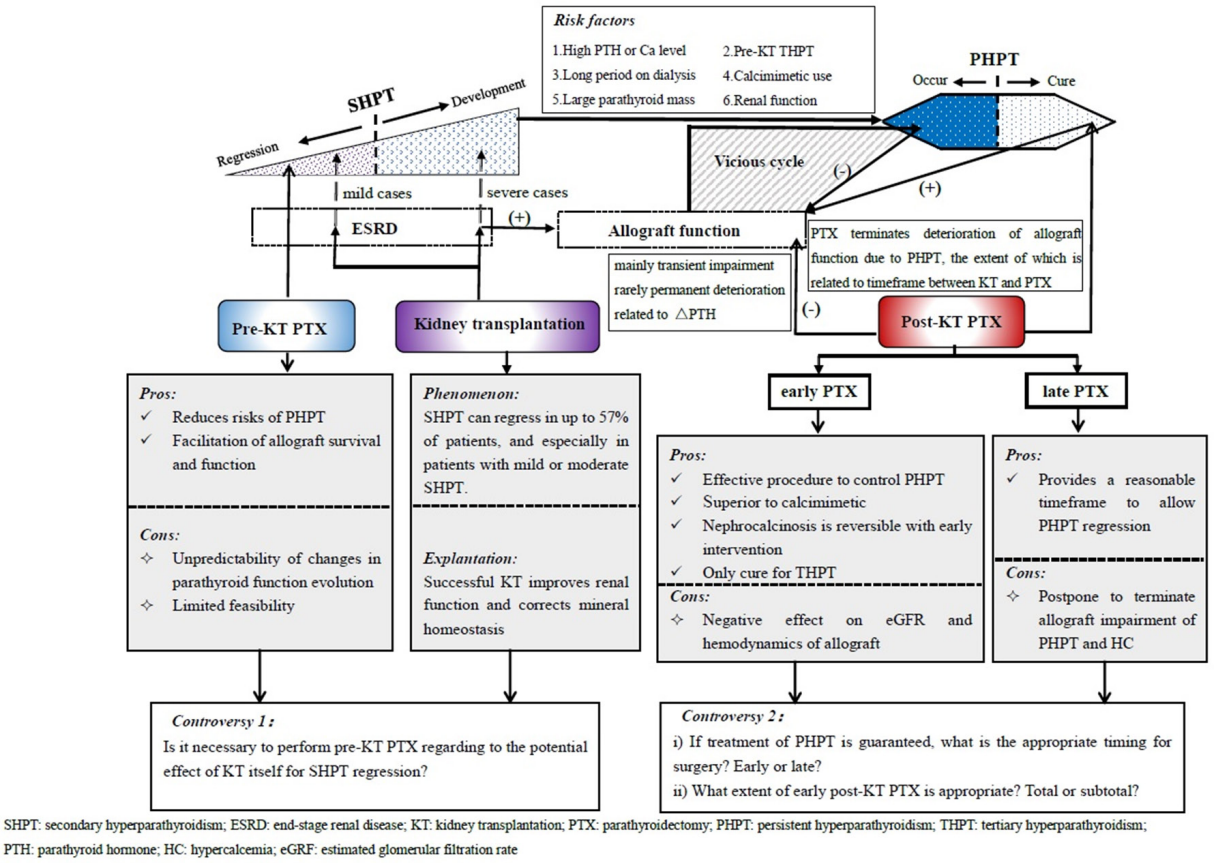

Figure 1. Flowchart showing current controversies pertaining to the timing of parathyroidectomy for kidney transplant patients with secondary hyperthyroidism.

total parathyroidectomy with auto-transplantation is a rational choice.

An "early" and "limited" surgical intervention is recommended for the treatment of PHPT. Several clinical indexes are feasible at predicting and confirming PHPT in the first six months post-KT; further observation has less value in confirming the diagnosis of PHPT and it has many adverse effects on allograft function. Thus, breaking this vicious cycle as soon as possible has clinical significance. A limited parathyroidectomy can reduce the negative impact of surgery on allograft function with a relatively mild change in the PTH level. Therefore, subtotal parathyroidectomy in the early period post-transplant may be a safe choice.

6. Conclusion

KT remains the best treatment for patients with ESRD but hyperparathyroidism might persist in a fairly large number of patients. To prevent or treat PHPT requires a consensus between doctors and patients. To determine the appropriate surgical intervention for PHPT, various factors for allograft impairment such as PHPT, hypercalcemia, the donor source, and parathyroidectomy itself should be evaluated. The rational timing of parathyroidectomy for KT patients with PHPT remains controversial with regard to the preservation of both allograft function and serum Ca levels. Additional studies with a large sample size and prospective trials should be conducted in the future.

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Longitudinal impact of compliance with routine CD4 monitoring on all cause deaths among treated people with HIV in China

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SUMMARY Keeping adherence to the continuous and standardized CD4 follow-up monitoring service is of great significance to the control of disease progression and the reduction of avoidable mortality for HIV-infected patients. As non-communicable diseases (NCDs) have become main causes of deaths for people with HIV (PWH) in the era of combination antiretroviral therapy (cART), how and to what extent does adherence to routine CD4 monitoring differentially impact on AIDS-related versus NCDs-related deaths in low- and middle-income countries (LMIC) remains elucidated. A CD4 test index was developed by dividing the actual number of received CD4 tests by the theoretical number of CD4 tests that should have been performed according to national treatment guidelines during the study period, with an index value of 0.8-1.2 reflecting compliance. From 1989 to 2020, 14,571 adults were diagnosed with HIV infection in Dehong Prefecture of Yunnan province in Southwestern China, 6,683 (45.9%) PWH had died with the all-cause mortality of 550.13 per 10,000 person-years, including 3,250 (48.6%) AIDS-related deaths (267.53 per 10,000 person-years). Among patients on cART, the median CD4 test index was 1.0 (IQR 0.6-1.3), and 35.2% had a CD4 test index less than 0.8. Cox proportional hazards regression analysis indicated that PWH with CD4 test index at 0.8-1.2 were at the lowest risk of both AIDS-related (aHR = 0.06; 95%*CI*: 0.05-0.07) and NCDs-related (aHR = 0.13; 95%*CI*: 0.11-0.16) deaths. Adherence to routine CD4 monitoring is critical for reducing both AIDS-related and NCDs-related mortality of PWH. An appropriate (once or twice a year) rather than an unnecessarily higher frequency of routine CD4 testing could be most cost-effective in reducing mortality in LMIC.

Keywords CD4 testing, adherence, mortality, non-communicable disease (NCD), HIV

1. Introduction

Combination antiretroviral therapy (cART) effectively reduces the morbidity and mortality of people with HIV (PWH) and extends their life expectancy to that of the general population (*1*). WHO guidelines proposed in 2015 recommend that HIV-infected patients should immediately initiate cART as soon as they are diagnosed (*2*), as it is related to decreasing morbidity and mortality, reducing HIV transmission (*i.e.*, treatment as prevention, TasP), and reducing loss to HIV healthcare (*3,4*).

Increasing lifespans of PWH and cART may both contribute to development of some non-communicable diseases (NCDs) among PWH. The prevalence of NCDs among PWH is much higher (29-44%) compared to that in the general population (15-25%) (*5*).The co-occurrence of HIV and NCDs remarkably challenges survival and quality of life as well as health care of PWH, especially in low- and middle-income countries (LMIC) (*6,7*). Despite a dramatic decrease in AIDS-related mortality among well-treated PWH, their life expectancy are compromised compared with persons without HIV (*8*). The excess mortality is primarily caused by NCDs, and NCDs had become the leading cause of the increased mortality of PWH globally (*9-11*). These suggest an importance of screening and managing NCDs within health care services provided for PWH.

CD4 testing plays an important role in settings with limited health resources where viral load testing cannot be carried out regularly (*12*). After initiating

cART, keeping adherence to the continuous and standardized CD4 follow-up monitoring service is of great significance to the control of disease progression and reduction of avoidable mortality for HIV-infected patients (*13*). Some recent studies suggest that in the early phase of cART, continuous CD4 monitoring helps identify immunosuppressed patients and detect the risk of progression to AIDS (*14,15*). However, it is noted that HIV-infected patients who are not immunocompromised and well adhere to cART may not benefit from regular CD4 testing (*16*).

Since the National Free Antiretroviral Treatment Program (NFATP) was established in 2003 in China (*17*), AIDS-related deaths among PWH have substantially decreased, particularly as cART has now been scaled up to over 95% of PWH and is available to all newly diagnosed HIV infections regardless of their CD4 counts or immunodeficiency status. However, consistent with other countries, there are emerging observations that PWH in China are at significantly higher risks of NCDs than the general population and are increasingly overtaken by NCDs in both morbidities and mortalities (*18*). Meanwhile, the national guideline for cART and health management of PWH in China has developed to the Fourth Edition, reflecting periodic changes and adjustments in standard clinical practices and treatment for PWH. Accordingly, the agenda of routine CD4 testing after initiating cART for regular care of PWH has also been changing. Before 2012, a CD4 test was performed in the 3rd, 6th, and 12th month in the first year after initiation of cART, and once per 6 months afterwards (1st Edition before 2008 and 2nd Edition during 2008-2012); from 2012 to 2016, a CD4 test was performed once per 6 months after initiation of cART (3rd Edition), and from 2016 to now, a CD4 test was performed at least once a year (4th Edition). Nonetheless, no longitudinal studies have been conducted to comprehensively evaluate the impact of compliance with routine CD4 testing on the health outcomes particularly AIDS-related and NCDs-related deaths among PWH in China.

The objective of this study was to characterize and determine the mortality of AIDS-related deaths and NCDs-related deaths among PWH in Dehong Prefecture of Yunnan province in Southwestern China, which has the longest history of HIV epidemic and most comprehensive anti-HIV campaigns (*19*), and to identify how and to what extent compliance with routine CD4 monitoring has impacted on AIDS-related versus NCDs-related deaths.

2. Materials and Methods

2.1. Study design

This is a retrospective cohort study conducted in Dehong Prefecture. Data were derived from China's HIV/AIDS Comprehensive Response Information Management System (CRIMS), a nationwide real-time reporting system composed of eight subsystems (*20*). Participants gave informed consent to participate in the study. This study was approved by the Institutional Review Board of Fudan University School of Public Health, Shanghai, China (IRB#2018-01-0655).

2.2. Setting and participants

2.2.1. Patients

Patients were eligible for the present study if they were older than 18 years at HIV diagnosis, were local residents of Dehong Prefecture and were not infected through mother-to-child transmission. For all eligible patients, the observational period was from the date of cART initiation (*i.e.*, baseline) through 31 December 2020 or death. Accordingly,14,571 adults diagnosed with HIV in Dehong Prefecture (the lost to follow-up rate was 0.02%) were subject to the analysis.

2.3. Definitions and variables

2.3.1. Definitions

CD4 test index: A CD4 test index reflecting compliance with CD4 testing was developed by dividing the actual number of received CD4 tests by the theoretical number of CD4 tests that should have been performed according to the national guidelines during the study period. The CD4 test index was then categorized into four groups as the following: ≤ 0.3 (severe incompliance), > 0.3 & ≤ 0.8 (medium incompliance), > 0.8 & ≤ 1.2 (compliance) and > 1.2 (over compliance).

AIDS-related deaths: since 2011, Dehong Prefecture Center for Disease Control and Prevention (CDC) has conducted three waves of confirmatory inspections on the causes of deaths among HIV-infected persons who had instantaneous registration of death in the CRIMS. For the present study, the detailed causes of deaths were extracted from the CRIMS and compared with the Coding of Death in HIV (CoDe) protocol to determine AIDS-related deaths. For patients whose causes of deaths were unknown, the deaths were considered to be AIDS-related if they had CD4 count < 200 cells/μL within 6 months before death.

NCDs-related deaths: if a patient died from any of cardiovascular diseases (CVDs), cancers, chronic respiratory diseases, diabetes, mental disorders recently added to the list of major NCDs, and other NCDs-related diseases defined in the International Classification of Diseases, 10th revision (ICD-10), the death was defined as NCDs-related.

2.3.2. Data collection

Information about socio-demographic variables, date of

HIV diagnosis, HIV transmission route, date of cART initiation, baseline and follow-up CD4 cell counts and HIV viral load (VL) of the participants was extracted from CRIMS.

2.4. Statistical analysis

For mortality rate estimation, the date of HIV diagnosis was used as time of entry, and the date of AIDS-related death or the date of NCDs-related death or the end of observation (December 31, 2020) was regarded as the end date. Those who died of non-AIDS-related and non-NCD-related deaths or were lost to follow-up for > 3 months were considered censored, and the date of death or the date of loss to follow-up was used as the end date. Pearson chi-square test was used to compare categorical variables.

For Kaplan-Meier survival curve of the HIV-infected patients who were receiving cART, the date of cART initiation was treated as time of entry, and the date of AIDS-related or NCDs-related death or the end of observation (December 31, 2020) was as the end date. Those who died of non-AIDS-related or non-NCD-related deaths or lost to follow-up for > 3 months were considered censored, and the date of death or the date of loss to follow-up was used as the end date. Tarone-Ware test was used to compare survivals between groups.

Cox proportional hazards regression model was employed to determine the association between CD4 test index and AIDS-related mortality or NCDs-related mortality. In multivariate analysis, sex, ethnicity, transmission route, age at cART initiation, baseline CD4 count, nadir CD4 count, and baseline HIV viral load were considered to be potential confounding variables that were then entered into the multiple regression model for adjustment, based on literature review and univariate analyses in the present study. Multiple imputation procedure was employed to complete the missing values for baseline viral load. All analyses were performed with SAS (version 9.4).

3. Results

3.1. Characteristics of study participants

From 1989 to 2020, 14,571 adults were diagnosed with HIV in Dehong Prefecture, China, contributing 121,479.9 person-years of follow up (Table 1). Among them, 10,700 (73.4%) were males, 6,848 (46.9%) were diagnosed before 31 years old, 5,122 (35.1%) were Dai ethnicity and 6,503 (44.6%) had acquired HIV through injection drug use (IDU). Noticeably, there was a striking increase of diagnosed HIV infections in 2004 due to a mass screening program, and cART was not available until 2004 (Figure 1A). Thus, only 9,606 (65.9%) participants had received cART.

3.2. CD4 test index

Among 9,606 HIV-infected patients with cART, 9,251 (96.3%) had been on cART for more than one year, had pretreatment baseline CD4 test, and had at least one posttreatment CD4 test. Of them, the median CD4 test index was 1.0 (IQR 0.6-1.3), and 35.2% had a CD4 test index less than 0.8, reflecting noncompliance to CD4 testing (Table 2). As shown in Table 2, the CD4 test index was significantly different by sex, age, ethnicity, HIV transmission route, baseline CD4 count, nadir CD4 count, and baseline HIV viral load.

3.3. AIDS-related *vs*. NCDs-related mortality

The annual number of all-cause deaths had been increasing since 1989, reached peak in 2007, and has then been decreasing (Figure 1B). By the end of 2020, 6,683 (45.9%) patients had died, including 3,250 (48.6%) AIDS-related deaths and 1,734 (26.0%) NCDs-related deaths (Table 1). The all-cause mortality was 550.13 per 10,000 person-years, the AIDS-related mortality was 267.53 per 10,000 person-years and the NCDs-related mortality was 142.74 per 10,000 person-years (Table 1). The all-cause mortality, the AIDS-related mortality, and the NCDs-related mortality were all lower in people with cART than those in people without cART, and were different by socio-demographic and HIV infection characteristics (Table 1).

3.4. Association between CD4 test index and mortality

3.4.1. Survival by CD4 test index

The Kaplan-Meier survival curve showed that treated patients with different CD4 test index had significantly different survival probabilities in terms of AIDS-related, NCDs-related as well as all-cause deaths (all *p-values* < 0.05) (Figure 2). Patients with CD4 test index lower than 0.3 had the lowest survival probability after cART, and patients with CD4 test index of 0.8-1.2 had the highest survival probability (Figure 2A for AIDS-related deaths, Figure 2B for NCDs-related deaths, and Figure 2C for all-cause deaths).

3.4.2. Associates of AIDS-related mortality

As shown in Table 3, univariate COX regression analysis indicated that the AIDS-related mortality was significantly associated with sex, age, ethnicity, HIV transmission route, baseline CD4 count, nadir CD4 count, baseline HIV viral load, and CD4 test index. Such associations remained significant in multivariate COX regression analysis adjusting for confounding variables. In particular, compared to patients with CD4 test index less than 0.3, patients with CD4 test index at 0.3-0.8 (aHR = 0.18, 95%*CI*: 0.15-0.22), 0.8-1.2

Table 1. Baseline characteristics of HIV-infected participants

Characteristics	*N* (%)	Person-years of follow up	All-cause Deaths		AIDS-related Deaths		NCDs-related Deaths		Other Deaths	
			N	Mortality (/10,000 person-years)	*N* (%)[a]	Mortality (/10,000 person-years)	*N* (%)[a]	Mortality (/10,000 person-years)	*N* (%)[a]	Mortality (/10,000 person-years)
Total	14,571	121,479.90	6,683	550.13	3,250 (48.6)	267.53	1,734 (26.0)	142.74	1,699 (25.4)	139.86
Gender										
Male	10,700 (73.4)	86,671.93	5,786	667.60	2,723 (47.0)	314.17	1,455 (25.2)	167.87	1,608 (27.8)	185.53
Female	3,871 (26.6)	34,807.92	897	257.70	527 (58.8)	151.40	279 (31.1)	80.15	91 (10.1)	26.14
Age at diagnosis, yr										
< 30	6,848 (46.9)	64,665.93	3,252	502.90	1,521 (46.8)	235.21	698 (21.4)	107.94	1,033 (31.8)	159.74
31-40	4,556 (31.3)	36,866.75	2,080	564.20	1,057 (50.8)	286.71	536 (25.8)	145.39	487 (23.4)	132.10
41 -50	1,977 (13.6)	13,403.74	806	601.32	424 (52.6)	316.33	259 (32.1)	193.23	123 (15.3)	91.77
≥ 51	1,190 (8.2)	6,543.44	545	832.90	248 (45.5)	379.01	241 (44.2)	368.31	56 (10.3)	85.58
Ethnicity										
Han	5,109 (35.1)	43,477.78	1,987	457.02	968 (48.7)	222.64	513 (25.8)	117.99	506 (25.5)	116.38
Dai	5,122 (35.1)	42,597.15	2,574	604.27	1,288 (50.0)	302.37	667 (25.9)	156.58	619 (24.1)	145.31
Jingpo	3,626 (24.9)	29,808.51	1,843	618.28	845 (45.8)	283.48	486 (26.4)	163.04	512 (27.8)	171.76
Others	714 (4.9)	5,596.41	279	498.53	149 (53.4)	266.24	68 (24.4)	121.51	62 (22.2)	110.79
HIV transmission route										
Sexual contacts	8,068 (55.4)	64,770.10	2,242	346.15	1,225 (54.6)	189.13	684 (30.5)	105.60	333 (14.9)	51.41
Injection drug use	6,503 (44.6)	56,709.75	4,441	783.11	2,025 (45.6)	357.08	1,050 (23.6)	185.15	1,366 (30.8)	240.88
Diagnostic Year										
1989 - 2004	4,773 (32.8)	45,961.66	3,516	764.99	1,717 (48.8)	373.57	776 (22.1)	168.84	1,023 (29.1)	222.58
2005 - 2012	7,214 (49.5)	65,166.81	2,807	430.74	1,420 (50.6)	217.90	796 (28.4)	122.15	591 (21.0)	90.69
2013 - 2020	2,584 (17.7)	10,351.38	360	347.78	113 (31.4)	109.16	162 (45.0)	156.50	85 (23.6)	82.11
Baseline CD4 count, cells/μL										
0 ~ 100	1,719 (11.8)	16,644.38	591	355.07	345 (58.4)	207.28	153 (25.9)	91.92	93 (15.8)	55.87
101 ~ 200	1,896 (13.0)	19,929.60	507	304.61	216 (42.6)	129.77	181 (35.7)	108.75	110 (21.7)	66.09
201 ~ 350	3,056 (21.0)	33,422.33	618	371.30	195 (31.5)	117.16	254 (41.1)	152.60	169 (27.4)	101.54
> 350	2,580 (17.7)	23,803.94	296	177.84	41 (13.9)	24.63	149 (50.3)	89.52	106 (35.8)	63.69
Missing	5,320 (36.5)	27,679.60	4,671	2,806.35	2,453 (52.5)	1,473.77	997 (21.3)	599.00	1,221 (26.1)	733.58
Baseline viral load, log10 copies/mL										
0	3,458 (23.7)	35,641.25	429	120.37	106 (24.7)	29.74	197 (45.9)	55.27	126 (29.4)	35.35
> 0 and ≤ 3	1,356 (9.3)	15,509.01	239	154.10	80 (33.5)	22.45	107 (44.7)	30.02	52 (21.8)	14.59
> 3	2,111 (14.5)	21,647.26	511	236.06	238 (46.6)	66.78	174 (34.0)	48.82	99 (19.4)	27.78
Missing	7,646 (52.5)	48,682.33	5,504	1,130.60	2,826 (51.3)	792.90	1,256 (22.8)	352.40	1,422 (25.8)	398.98
Combination ART										
No	4,965 (34.1)	23,438.78	4,622	1,971.95	2,432 (52.6)	1,037.60	977 (21.1)	416.83	1,213 (26.2)	517.52
Yes	9,606 (65.9)	98,041.07	2,061	210.22	818 (39.7)	83.43	757 (36.7)	77.21	486 (23.6)	49.57

[a] Proportion among all-cause death.

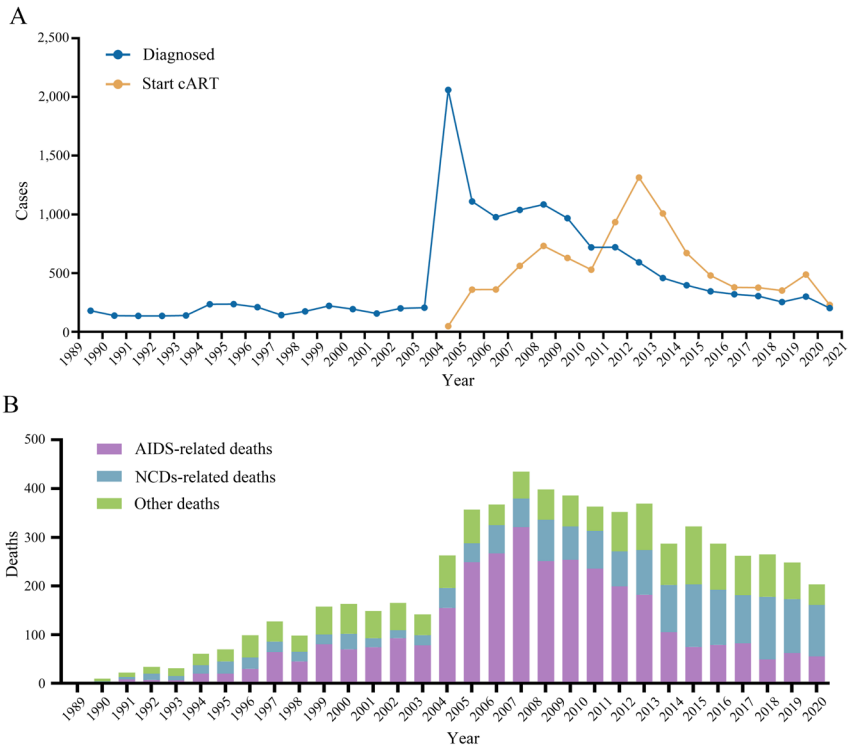

Figure 1. Annual number of HIV positive diagnoses, initiation of cART (A), and death (B) from 1989 to 2020 (deaths are classified by cause of death) in Dehong Prefecture, China.

Table 2. CD4 Test Index among HIV-infected patients in cART (*n* = 9,251)

Characteristics	Total (%)[a]	CD4 Test Index				χ^2	*p*
		≤0.3	> 0.3 and ≤ 0.8	> 0.8 and ≤ 1.2	> 1.2		
		N (%)[b]	*N* (%)[b]	*N* (%)[b]	*N* (%)[b]		
Total	9,251	1,342 (14.5)	1,914 (20.7)	4,571 (49.4)	1,424 (15.4)		
Gender						99.67	< 0.001
Male	6,044 (65.3)	937 (15.5)	1,399 (23.2)	2,793 (46.2)	915 (15.1)		
Female	3,207 (34.7)	405 (12.6)	515 (16.1)	1,778 (55.4)	509 (15.9)		
Age at ART initiation, yr						68.94	< 0.001
≤ 30	2,679 (29.0)	461 (17.2)	543 (20.3)	1,347 (50.3)	328 (12.2)		
31 ~ 40	3,593 (38.8)	447 (12.4)	746 (20.8)	1,843 (51.3)	557 (15.5)		
41 ~50	1,974 (21.3)	291 (14.7)	424 (21.5)	921 (46.7)	338 (17.1)		
≥ 51	1,005 (10.9)	143 (14.2)	201 (20.0)	460 (45.8)	201 (20.0)		
Ethnicity						117.02	< 0.001
Han	3,534 (38.2)	398 (11.3)	629 (17.8)	1,888 (53.4)	619 (17.5)		
Dai	3,042 (32.9)	471 (15.5)	693 (22.8)	1,452 (47.7)	426 (14.0)		
Jingpo	2,194 (23.7)	411 (18.7)	484 (22.1)	994 (45.3)	305 (13.9)		
Others	481 (5.2)	62 (12.9)	108 (22.5)	237 (49.3)	74 (15.4)		
HIV transmission route						106.46	< 0.001
Sexual contacts	6,462 (69.9)	877 (13.6)	1,194 (18.5)	3,396 (52.5)	995 (15.4)		
IDU	2,789 (30.1)	465 (16.7)	720 (25.8)	1,175 (42.1)	429 (15.4)		
Baseline CD4 count, cells/μL						253.10	< 0.001
0 ~ 100	1,719 (18.6)	248 (14.4)	240 (14.0)	950 (55.3)	281 (16.3)		
101 ~ 200	1,896 (20.5)	259 (13.7)	351 (18.5)	1,019 (53.7)	267 (14.1)		
201 ~ 350	3,056 (33.0)	373 (12.2)	617 (20.2)	1,641 (53.7)	425 (13.9)		
> 350	2,580 (27.9)	462 (17.9)	706 (27.4)	961 (37.2)	451 (17.5)		
Nadir CD4 count, cells/μL						175.32	< 0.001
0 ~ 100	1,420 (15.4)	261 (18.4)	264 (18.6)	684 (48.2)	211 (14.9)		
101 ~ 200	2,157 (23.3)	244 (11.3)	397 (18.4)	1,217 (56.4)	299 (13.9)		
201 ~ 350	2,924 (31.6)	351 (12.0)	568 (19.4)	1,552 (53.1)	453 (15.5)		
> 350	2,750 (29.7)	486 (17.7)	685 (24.9)	1,118 (40.7)	461 (16.7)		
Baseline viral load, log_{10} copies/mL						103.11	< 0.001
0	5,609 (60.6)	844 (15.0)	1,150 (20.5)	2,647 (47.2)	968 (17.3)		
> 0 and ≤ 3	1,545 (16.7)	198 (12.8)	244 (15.8)	872 (56.4)	231 (15.0)		
> 3	2,097 (22.7)	300 (14.3)	520 (24.8)	1,052 (50.2)	225 (10.7)		

[a] Frequency in the patients on cART; [b] Proportion among total.

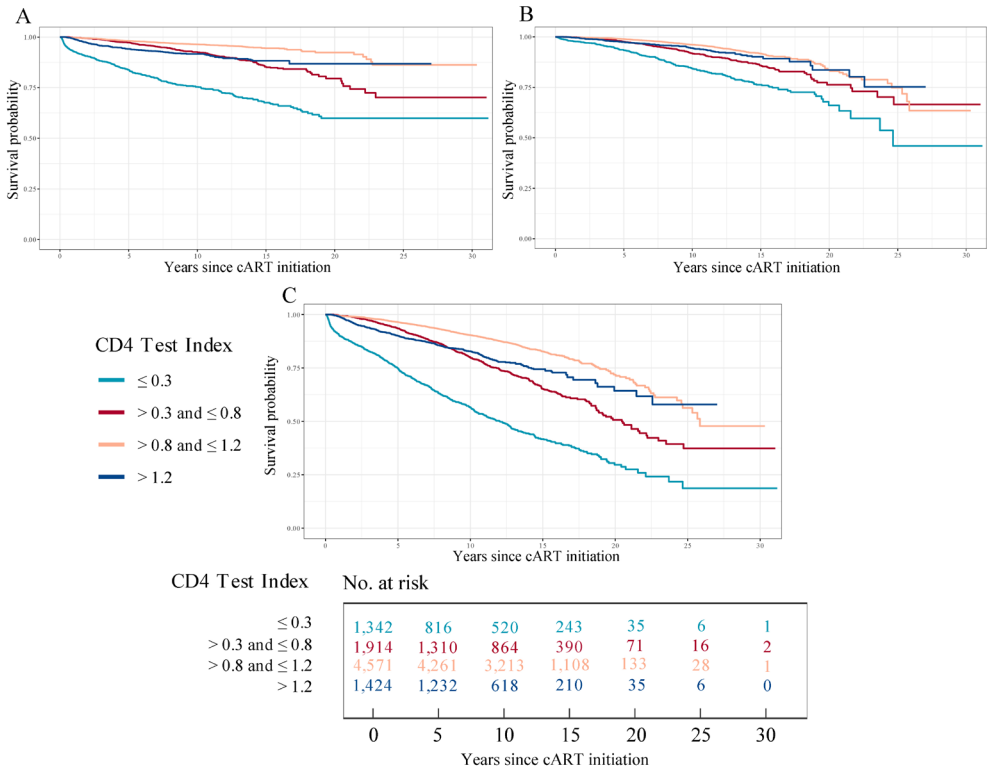

Figure 2. The Kaplan-Meier curve of AIDS-related cause (A), NCD-related causes (B), and all-cause (C) of death under CD4 test index.

Table 3. Univariate and multivariate COX analyses of factors associated with AIDS-related Deaths among HIV-infected patients with cART (*n* = 9,251)

Characteristics	Crude HR (95%*CI*)	*p*	Adjusted HR (95%*CI*)	*p*
Gender				
Male	1.00		1.00	
Female	0.45 (0.38, 0.53)	< 0.001	0.76 (0.63, 0.92)	0.005
Age at ART initiation, yr				
≤ 30	1.00		1.00	
31 ~ 40	1.33 (1.12, 1.59)	0.002	1.31 (1.09, 1.57)	0.004
41 ~50	1.47 (1.20, 1.79)	< 0.001	1.45 (1.18, 1.77)	< 0.001
≥ 51	1.53 (1.20, 1.95)	< 0.001	1.75 (1.36, 2.25)	< 0.001
Ethnicity				
Han	1.00		1.00	
Dai	1.17 (0.99, 1.38)	0.07	1.28 (1.08, 1.52)	0.004
Jingpo	1.31 (1.10, 1.56)	0.003	1.23 (1.02, 1.48)	0.028
Others	1.11 (0.80, 1.55)	0.54	1.13 (0.81, 1.57)	0.49
HIV transmission route				
Sexual relations	1.00		1.00	
IDU	2.16 (1.88, 2.48)	< 0.001	1.57 (1.34, 1.85)	< 0.001
Baseline CD4 count, cells/μL				
0 ~ 100	1.00		1.00	
101 ~ 200	0.54 (0.46, 0.64)	< 0.001	0.60 (0.50, 0.72)	< 0.001
201 ~ 350	0.29 (0.24, 0.35)	< 0.001	0.43 (0.35, 0.53)	< 0.001
> 350	0.10 (0.07, 0.13)	< 0.001	0.24 (0.17, 0.35)	< 0.001
Nadir CD4 count, cells/μL				
0 ~ 100	1.00		1.00	
101 ~ 200	0.36 (0.31, 0.43)	< 0.001	0.52 (0.43, 0.62)	< 0.001
201 ~ 350	0.20 (0.17, 0.24)	< 0.001	0.37 (0.30, 0.45)	< 0.001
> 350	0.06 (0.05, 0.08)	< 0.001	0.17 (0.12, 0.24)	< 0.001
CD4 Test Index				
≤ 0.3	1.00		1.00	
> 0.3 and ≤ 0.8	0.25 (0.21, 0.30)	< 0.001	0.18 (0.15, 0.22)	< 0.001
> 0.8 and ≤ 1.2	0.09 (0.08, 0.11)	< 0.001	0.06 (0.05, 0.07)	< 0.001
> 1.2	0.23 (0.19, 0.28)	< 0.001	0.17 (0.14, 0.21)	< 0.001
Baseline viral load, log_{10} copies/mL				
0	1.00		1.00	
> 0 and ≤ 3	5.00 (4.20, 5.96)	< 0.001	4.72 (3.94, 5.65)	< 0.001
> 3	3.83 (3.21, 4.56)	< 0.001	3.27 (2.73, 3.91)	< 0.001

Table 4. Univariate and multivariate COX analyses of factors associated with NCDs-related Deaths among HIV-infected patients with cART (*n* = 9,251)

Characteristics	Crude HR (95%*CI*)	*p*	Adjusted HR (95%*CI*)	*p*
Gender				
Male	1.00		1.00	
Female	0.42 (0.35, 0.50)	< 0.001	0.62 (0.51, 0.75)	< 0.001
Age at initiating, yr				
≤ 30	1.00		1.00	
31 ~ 40	1.92 (1.55, 2.40)	< 0.001	1.94 (1.55, 2.43)	< 0.001
41 ~50	2.85 (2.26, 3.60)	< 0.001	3.22 (2.54, 4.07)	< 0.001
≥ 51	5.38 (4.24, 6.82)	< 0.001	8.40 (6.56, 10.75)	< 0.001
Ethnicity				
Han	1.00		1.00	
Dai	1.37 (1.15, 1.64)	< 0.001	1.35 (1.13, 1.61)	0.001
Jingpo	1.62 (1.35, 1.96)	< 0.001	1.56 (1.28, 1.89)	< 0.001
Others	1.15 (0.79, 1.66)	0.47	1.24 (0.86, 1.80)	0.26
HIV transmission route				
Sexual relations	1.00		1.00	
IDU	2.39 (2.07, 2.77)	< 0.001	1.93 (1.62, 2.30)	< 0.001
Baseline CD4 count, cells/μL				
0 ~ 100	1.00		1.00	
101 ~ 200	1.04 (0.84, 1.29)	0.71	0.82 (0.65, 1.03)	0.090
201 ~ 350	0.88 (0.72, 1.08)	0.21	0.71 (0.56, 0.89)	0.003
> 350	0.83 (0.66, 1.05)	0.12	0.62 (0.46, 0.83)	0.001
Nadir CD4 count, cells/μL				
0 ~ 100	1.00		1.00	
101 ~ 200	0.89 (0.72, 1.10)	0.290	0.90 (0.72, 1.13)	0.36
201 ~ 350	0.77 (0.62, 0.95)	0.013	0.86 (0.68, 1.08)	0.20
> 350	0.66 (0.53, 0.83)	< 0.001	0.81 (0.61, 1.08)	0.15
CD4 Test Index				
≤ 0.3	1.00		1.00	
> 0.3 and ≤ 0.8	0.39 (0.32, 0.48)	< 0.001	0.30 (0.25, 0.38)	< 0.001
> 0.8 and ≤ 1.2	0.16 (0.13, 0.19)	< 0.001	0.13 (0.11, 0.16)	< 0.001
> 1.2	0.25 (0.19, 0.32)	< 0.001	0.18 (0.14, 0.24)	< 0.001
Baseline viral load, log_{10} copies/mL				
0	1.00		1.00	
> 0 and ≤ 3	0.73 (0.59, 0.90)	0.004	0.84 (0.68, 1.05)	0.12
> 3	0.96 (0.80, 1.14)	0.64	0.95 (0.79, 1.14)	0.56

(aHR = 0.06; 95%*CI*: 0.05-0.07), or > 1.2 (aHR = 0.17; 95%*CI*: 0.14-0.21) were at lower risk of AIDS-related death.

3.4.3. Associates of NCDs-related mortality

As shown in Table 4, univariate COX regression analysis indicated that the NCDs-related mortality was significantly associated with sex, age, ethnicity, HIV transmission route, nadir CD4 count, baseline HIV viral load, and CD4 test index. In multivariate COX regression analysis adjusting for confounding variables, NCDs-related mortality remained significantly associated with sex, age, ethnicity, HIV transmission route, and CD4 test index, but was no longer associated with nadir CD4 count and baseline HIV viral load. In particular, compared to patients with CD4 test index less than 0.3, patients with CD4 test index at 0.3-0.8 (aHR = 0.30, 95%*CI*: 0.25-0.38), 0.8-1.2 (aHR = 0.13; 95%*CI*: 0.11-0.16), or > 1.2 (aHR = 0.18; 95%*CI*: 0.14-0.24) were at lower risk of NCDs-related death.

4. Discussion

Our findings show that, as one of the earliest epicenters affected by HIV in China, although the annual number of deaths among HIV-infected people in Dehong Prefecture has been declining, the mortality here is still higher than in high-income countries (*21,22*). Among patients with cART, the median CD4 test index was 1.0 (IQR 0.6-1.3), and 35.2% had a CD4 test index less than 0.8 and the CD4 test index was significantly different by sex, age, ethnicity, HIV transmission route, baseline CD4 count, nadir CD4 count, and baseline HIV viral load. Patients who complied with routine CD4 testing had the lowest risk of both AIDS-related and NCDs-related deaths.

This study, for the first time, evaluated the impact of compliance with routine CD4 testing on all-cause mortality of PWH in China. A CD4 test index was developed and if over 0.8, indicates that the patients have well adhered to routine CD4 testing and have substantially reduced risks of deaths, whereas a lower CD4 test index was significantly associated with higher risk of death. However, it was the CD4 test index between 0.8 and 1.2 but not over 1.2 that was significantly linked to the lowest risk of both AIDS-related and NCDs-related deaths. This observation has two important implications. One is that routine CD4 monitoring is critical for survival of PWH, as many reports have demonstrated that routine CD4 testing helps healthcare providers monitor the immunodeficiency

status, understand the disease progression, and effectively improve the quality of life of HIV-infected patients (*23*). The other, however, is that an appropriate (once per 6 months or at least once a year) rather than an unnecessarily higher frequency of routine CD4 testing could be most cost-effective in reducing mortality of HIV patients. This is particularly relevant in resource-limited settings. In fact, there is literature showing that enriching the content and reducing the frequency of a single CD4 test could achieve a balance between decreasing the risk of death and saving health service resources, and reduces the economic burden of HIV disease in LMIC (*13,24*). According to the China national guidelines for cART, blood biochemical tests for aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), triglyceride (TG), creatinine (Cr), blood urea nitrogen (BUN), total bilirubin, and glucose (Glu) were preformed simultaneously with CD4 test, which would not only help monitoring side effects of cART but also assessing risks of certain NCDs.

cART was not available until 2003 in China when the government launched the national comprehensive anti-HIV/AIDS campaign including NFATP. In the pre-ART era, AIDS-related death was a major health threat for PWH (*25*). However, with the expansion of the combination ART and increasing life expectancy, the prevalence of NCDs among PWH in China has been increasing and AIDS-related deaths have been replaced by NCDs-related deaths as the main threat for PWH (*26*). In the present study, the main cause of deaths among PWH was also shifted from AIDS-related severe immunodeficiency during the early non-ART stage to non-AIDS-related diseases especially NCDs in the past decade. NCDs have become a great challenge for HIV-infected individuals and health care providers in China and other LMIC, which calls for integration of NCDs prevention, intervention and treatment into the existing anti-HIV/AIDS campaigns.

Nadir CD4 count and baseline viral load were significantly associated with AIDS-related deaths but not with NCDs-related deaths. The increasing lifespan of patients in Dehong Prefecture increased risks to NCDs and NCDs-related deaths, as patients were more likely to be exposed to unhealthy diets and harmful use of alcohol and tobacco (*27,28*). In addition, cART side effects predispose individuals further to NCDs and NCDs-related deaths (*29*).

Several published studies have shown correlation between gender and risk of death in HIV-infected patients (*30,31*). Women had lower risks of both AIDS-related and NCDs-related deaths than men in the present study. This is most likely because women have had better adherence to cART, lower prevalence of alcohol use and injection drug use (*32-35*). Other pathophysiological differences by sex are not ruled out and deserve further investigation.

The Dai ethnic patients have the highest AIDS-related mortality whereas the Jingpo ethnic patients have the highest NCDs-related mortality in this study. Previous studies in Dehong Prefecture found that smoking and heavy drinking were more prevalent among Jingpo patients (*35,36*). Whether there are genetic predisposing factors and gene-environment interactions for differential morbidities and mortalities between different ethnicities remains to be investigated in the near future.

This study is subject to several limitations. First, behavioral and environmental exposures to non-AIDS diseases especially NCDs were not systematically collected, refraining us from examining secular trends of such exposures and fully understanding the rising of NCDs-related deaths over the past decades. Second, although the causes of deaths were carefully investigated and verified by the researchers, misclassifications could still not be ruled out given that the study site is a remote rural area in Southwestern China where access to and utilization of health services for non-AIDS diseases were relatively limited, especially for those living in rural villages. Most likely, some HIV-infected patients died of NCDs might have never been diagnosed with NCDs and thus were not correctly attributed to NCDs-related deaths, underestimating the NCDs-related mortality. Third, the required frequency of routine CD4 testing for HIV-infected patients, although mostly twice per year, was not unanimous over the study period. However, according to the study results, an appropriate frequency of routine CD4 testing instead of too less or too more tests should be the best recommendation.

5. Conclusion

In conclusion, NCDs-related deaths have taken over as the main causes of death among treated PWH in rural China. Sex, age, ethnicity, HIV transmission route, baseline CD4 count, nadir CD4 count, and baseline HIV viral load were associated with the compliance level of CD4 test. PWH benefit from long-term compliance with appropriate routine CD4 monitoring in controlling all-cause mortality. Our study will provide new experience to routine HIV surveillance and testing in LMIC, that is, enriching the content and reducing the frequency (once per 6 months or at least once a year) of CD4 test could best reduce the burden of HIV disease. In addition, risk assessment and intervention, diagnosis and treatment of NCDs especially those common to HIV patients are urgently needed to be integrated into the existing comprehensive anti-HIV/AIDS programs.

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Thrombin cleaves recombinant soluble thrombomodulin into a lectin-like domain fragment and a fragment with protein C-activating cofactor activity

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SUMMARY Thrombomodulin (TM) is a transmembrane protein that plays an important role in regulating the coagulation system by acting as a cofactor for thrombin in protein C activation. Additionally, TM is involved in inflammation. Previous studies have shown that soluble fragments of TM of varying sizes, which are derived from membrane-bound TM, are present in plasma and urine. Soluble fragments of TM are speculated to exhibit biological activity. Among these, a lectin-like domain fragment (TMD1) is of particular importance. Recombinant TMD1 has previously been shown to attenuate lipopolysaccharide-induced inflammation. Here, we report that thrombin cleaves recombinant soluble TM, which is used for the treatment of disseminated intravascular coagulation associated with sepsis, into TMD1 and a fragment comprising the C-terminal portion of TM (TMD23), the latter of which retains the cofactor activity for activating protein C. Our findings suggest that thrombin not only activates protein C on membrane-bound TM but may also cleave TM to generate TMD1.

Keywords Thrombomodulin, thrombin, cleavage product, protein C-activating cofactor activity

Thrombomodulin (TM) is a transmembrane protein, expressed mainly on vascular endothelial cells, that has an anticoagulant role in the coagulation system (*1*). It is composed of a lectin-like domain (TMD1), a hydrophobic region, six tandem repeats of epidermal growth factor-like domains (TMD2), a Ser/Thr-rich domain (TMD3), a transmembrane segment (TMD4), and a cytoplasmic tail (TMD5). TM serves as a thrombin receptor and cofactor for thrombin. Thrombin and protein C interact with TMD2, resulting in activation of protein C by thrombin. Activated protein C in turn inactivates FVa and FVIIIa in the coagulation system. Studies with recombinant TMD1 have shown that TMD1 has several biological functions, including attenuation of lipopolysaccharide (LPS)-induced inflammation (*2*), suppression of high-mobility group box 1-mediated inflammation (*3,4*), suppression of vascular inflammation (*5*), and inhibition of angiogenesis (*6*). TM also has activating and inactivating functions in alternative pathways of the complement system (*7,8*) and can interfere with complement activation *via* the classical and lectin pathways (*9*). In addition, soluble fragments of TM of varying sizes are present in the plasma and urine (*10*). It has been speculated that proteases such as rhomboids and metalloproteases are involved in the release of TM from the cell membrane (*11,12*).

Soluble TM fragments containing TMD2 exhibit protein C-activating cofactor activity (*13*). Recombinant human soluble TM consisting of TMD1, TMD2, and TMD3 (rTM) has recently been used in the clinic to treat disseminated intravascular coagulation (DIC) associated with sepsis (Figure 1A) (*13-16*). Clinical trials have also assessed rTM in DIC with hematologic malignancy and hemolytic uremic syndrome. Here, we report that thrombin cleaves rTM into two fragments, TMD1 and TMD23, the latter of which retains the cofactor activity for protein C activation by thrombin.

Incubation of rTM with varying concentrations of human thrombin (2-20 μg/mL, *i.e.*, 50-500 nM) resulted in the appearance of a 59-kDa band below rTM (Figure 1B, left panel, arrow 1) and a faint 31 kDa band above the B chain of thrombin (Figure 1B, left panel, arrow 2) on SDS-PAGE in a concentration-dependent manner. An anti-TM antibody bound by immunoblotting to both the 59-kDa and 31 kDa proteins (Figure 1B, right panel, arrows 1 and 2). The sum of the molecular weights of the 59-kDa and 31-kDa fragments nearly match the molecular weight of rTM (89 kDa). These results indicate that thrombin cleaves rTM into these two fragments. The N-terminal amino acid sequence of the 59-kDa fragment was determined to be Gly-Ala-Asp-Phe-Gln-Ala-Leu-

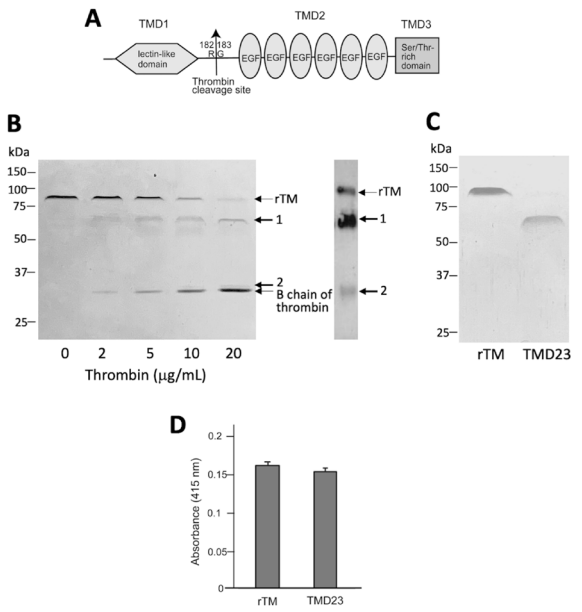

Figure 1. Cleavage of rTM into TMD1 and TMD23 by thrombin, and thrombin-cofactor activity of TMD23. (A) The domain structure of rTM and the identified thrombin cleavage site (arrow). **(B)** SDS-PAGE and immunoblot of thrombin-treated rTM. rTM (50 μg/mL) and thrombin (0-20 μg/mL) were incubated in 20 mM Tris, 150 mM NaCl, pH 8.0 at 37°C for 3 hrs, followed by SDS-PAGE under reducing condition (10% gel). The gel was stained with Coomassie Brilliant Blue R-250 (left panel). In the right panel, after incubation of rTM (50 μg/mL) and human thrombin (20 μg/mL), SDS-PAGE, and transfer to a PVDF membrane, the blots were probed with a rabbit polyclonal antibody against human TM. Horseradish peroxidase-conjugated anti-rabbit IgG was used as the secondary antibody and visualized using 3,3',5,5'-tetramethylbenzidine. In the both panels, arrow 1 and arrow 2 indicate a 59-kDa protein (TMD23) and a 31-kDa protein (TMD1), respectively. Positions of molecular weight markers are indicated on the left. **(C)** SDS-PAGE of purified TMD23. After rTM and thrombin were incubated, the reaction mixtures were subjected to anion-exchange chromatography on a Mono Q column, and TMD23 was purified. **(D)** Thrombin-cofactor activity of TMD23. TMD23 or rTM at concentrations of 1μM were incubated with thrombin and protein C at 37°C for 10 min. Then, hirudin was added, and the reaction mixture was incubated at 37°C for 10 min. After incubation, S-2366, a substrate for protein C, was added, followed by additional incubation at 37°C for 20 min. After adding acetic acid to terminate the reaction, absorbance was measured at 415 nm (n = 3).

Pro-Val-Gly, which is identical to the sequence spanning amino acids 183-192 of rTM. This region is located in the hydrophobic domain between TMD1 and TMD2, indicating that thrombin cleaves rTM between Arg^{182} and Gly^{183} to generate TMD1 and TMD23, respectively (Figure 1A).

Among the three domains of rTM, TMD2 is responsible for the thrombin cofactor activity. Therefore, we next examined whether TMD23 generated from rTM by thrombin retained its protein C-activating cofactor activity. We purified TMD23 from the reaction mixture of rTM and thrombin by anion-exchange chromatography using Mono Q. Purified TMD23 migrated as a single band on SDS-PAGE (Figure 1C). Using purified TMD23, we examined its protein C-activating cofactor activity and found that it exhibited activity similar to that of rTM (Figure 1D).

Thrombin acts on a variety of biological substrates. In the coagulation system, thrombin cleaves fibrinogen, FXIII, FVIII, FXI, protein C, and other coagulation factors. In addition, proteins such as C3 and C5 of the complement system are thrombin substrates, which shows its relatively broad specificity. Based on phage-display analysis, Gallwitz *et al*. proposed the following consensus recognition sequences of optimal substrates for thrombin: P2-Pro, P1-Arg, P1′-Ser/Ala/Gly/Thr, P2′-not acidic, and P3′-Arg (*17*). However, no natural substrates of thrombin display this consensus sequence, suggesting that exosite cooperativity is important for determining specificity and cleavage rate. The observed cleavage site of rTM has the sequences P2-Ala, P1-Arg, P1′-Gly, P2′-Ala, and P3′-Asp, which align to three positions in the consensus recognition sequence.

The physiological concentrations of free thrombin during coagulation reactions are estimated to range from 1 nM to approximately 500 nM (*18,19*). Thrombin at a relatively higher concentration (50 nM-500 nM) was used to examine rTM cleavage in this study. High plasma thrombin levels can be observed in certain pathophysiological conditions such as DIC (*20*).

The physiological relevance of thrombin cleavage of rTM into TMD1 and TMD23 is that thrombin may be involved in the generation of soluble TMD1 in plasma. It has been reported that an approximately 30-kDa protein that is regarded as possibly TMD1 is present in the plasma (*21*). Thrombin may cleave membrane-bound and/or soluble TM shed from the cells to generate TMD1. The involvement of metalloproteases in TMD1 shedding has also been previously reported (*22*). As described above, TMD1 has been shown to have several biological functions, including the attenuation of inflammation mediated by LPS. Soluble TMD1, but not membrane-bound TMD1, may play a crucial role in the attenuation of circulating LPS-induced inflammation.

We demonstrated that TMD23 generated from rTM by thrombin digestion has cofactor activity for protein C activation. It was previously reported that recombinant TMD23 has cofactor activity and exerts a 4.6-fold higher activity than rTM on a molar basis (*13*). However, TMD23 did not show a higher activity than rTM in this study (Figure 1D). A possible explanation for this observation is that TMD23 partially loses its cofactor activity during purification. The present results imply that TMD23 may retain its cofactor activity under conditions in which rTM is cleaved by thrombin in the blood.

In summary, thrombin cleaves rTM to generate TMD1 and TMD23, the latter of which retains its cofactor activity for protein C activation by thrombin. This finding suggests that thrombin not only activates protein C on membrane-bound TM but may also cleave TM to generate TMD1, which exerts several biological functions in the blood.

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Can nasal irrigation with chlorine dioxide be considered as a potential alternative therapy for respiratory infectious diseases? The example of COVID-19

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SUMMARY Chlorine dioxide (ClO_2) is a high-level disinfectant that is safe and widely used for sterilization. Due to the limitations on preparing a stable solution, direct use of ClO_2 in the human body is limited. Nasal irrigation is an alternative therapy used to treat respiratory infectious diseases. This study briefly summarizes the available evidence regarding the safety/efficacy of directly using ClO_2 on the human body as well as the approach of nasal irrigation to treat COVID-19. Based on the available information, as well as a preliminary experiment that comprehensively evaluated the efficacy and safety of ClO_2, 25-50 ppm was deemed to be an appropriate concentration of ClO_2 for nasal irrigation to treat COVID-19. This finding requires further verification. Nasal irrigation with ClO_2 can be considered as a potential alternative therapy to treat respiratory infectious diseases, and COVID-19 in particular.

Keywords chlorine dioxide (ClO_2), nasal irrigation, COVID-19, SARS-CoV-2, respiratory infectious diseases

Chlorine dioxide (ClO_2) is an oxidizing agent that is commonly used as a high-level disinfectant. It is effective at killing pathogenic microorganisms including bacteria, viruses, fungi, and spores, and it has almost no toxic effects on human or animal cells in daily use (*1*). ClO_2 has a molecular structure with 19 electrons in the outer layer, which contributes to its oxidizing action and penetration. It can adsorb to and penetrate the surface of microorganism without markedly destroying the integrity of the microbial shell (such as the cytoderm or protein capsid), and it markedly acts on enzymes containing sulfhydryl groups. The mechanism of disinfection by ClO_2 is *via*: i) Rapid damage to tyrosine on the capsid of the bacterium or virus, thereby suppressing their specific adsorption; ii) Suppression of protein synthesis; and iii) Killing these microorganisms, which account for its sterilizing action (*2*). In the context of SARS-CoV-2, ClO_2 directly affects the spike protein and RNA of the virus, ultimately killing the virus (*3*). Hence, ClO_2 has been long used for sterilization, both for sterilization of equipment and environments as well as for human disinfection, such as dental oral cleaning (*4-6*) and wound cleaning (*7*). Its disinfecting action in home environments, the water supply, environmental surfaces, and medical equipment have been well documented. However, there is a limitation to directly using ClO_2 on human body, namely the limited availability of a stable ClO_2 solution that can be stored for a prolonged period. A ClO_2 solution often needs to be prepared before using *via* a chemical reaction of precursors such as sodium chlorite ($NaClO_2$) or use of an effervescent tablet. Such "activation" procedures are inconvenient. Importantly, the concentration and stability of the obtained ClO_2 solution are not easily controlled, thereby limiting the use of ClO_2 to disinfect the human body. Fortunately, a stable ClO_2 solution (free of activation) has recently become available, and this offers hope for the direct use of ClO_2 in the human body.

1. Use of ClO_2 for human disinfection

Many previous animal studies have demonstrated the safety of ClO_2 as a sanitizer. Ma *et al*. verified the efficacy, toxicity, and safety of ClO_2 *in vitro* and *in vivo* (*8*). Their *in vitro* experiments found that ClO_2 at 5-20 ppm resulted in a 98.2% reduction in bacteria and fungi. ClO_2 at 200 ppm (37℃, 2 min) killed most strains of influenza A and B and enterovirus 71. In terms of

toxicity, cellular viability was 74.0% at 600 ppm, and 40.3% at 800 ppm. In *in vivo* experiments, inhalation of ClO_2 at 0-20 ppm (24 h) or oral administration of ClO_2 at 0-40 ppm (90 days) did not cause any pathological changes in the heart, lungs, liver, kidneys, or spleen of mice. Oral administration of ClO_2 at 0-40 ppm also did not cause any pathological changes in these organs. Use of 0.1 mL of ClO_2 at 50 ppm did not lead to ocular irritation in rabbits (*8*). These experiments verified the biosafety of ClO_2 in different animals.

However, evidence regarding the direct use of ClO_2 in humans is limited due to the aforementioned limitation. By far, the most common context is dental disinfection. Early in 2008, a Japanese team used 0.1% ClO_2 (1,000 ppm) mouthwash to treat healthy subjects with halitosis (*4*). They found that halitosis was alleviated, and no adverse events were reported (*5*). Later, the same team used ClO_2 at 1,000 ppm (7 days of mouthwash) in 15 subjects with halitosis. They found that accumulation of plaque, coating of the tongue, and the count of *Fusobacterium nucleatum* in saliva decreased. Only three subjects reported "dislike of the smell and taste of ClO_2". Recently, an Indian team also used ClO_2 at 1,000 ppm for disinfection in patients who underwent periodontal surgery (mouthwash bid for 14 days). They found that all of the patients were able to tolerate the ClO_2 mouthwash. No discomfort was reported (*6*). Noszticzius *et al*. used ClO_2 at 300 ppm as an antimicrobial agent for the wounds of patients with deep venous thrombosis or diabetic foot (*7*). They found that ClO_2 at 300 ppm displayed efficacy in killing all bacteria. It helped with wound healing without causing any toxic reactions. They contended that ClO_2 might be a good disinfectant for use in all living organisms (Table 1).

In terms of using ClO_2 in the context of COVID-19, most studies similarly concerning the environment. There are only limited studies in humans (Table 1). Aparicio-Alonso *et al*. orally administered ClO_2 at 3 ppm as a prophylactic agent to family members living with COVID-19 patients in Mexico (*9*). They found that ClO_2 was effective at preventing COVID-19, and no adverse events were reported. In another study, Aparicio-Alonso *et al*. orally administered a mean dose of 1.41 mg/kg to treat COVID-19 patients (*10*). They found that CIO2 helped to resolve COVID-19 symptoms and reduce the duration of treatment. Only 6.78% of patients reported mild and sporadic uncomfortable reactions such as headaches, dizziness, vomiting, diarrhea, and nausea. They hence concluded that ClO_2 might be considered as a safe alternative therapy with which to treat COVID-19.

There are only 2 studies reporting toxic reactions. Bathina *et al*. reported an unusual case of reversible acute kidney injury due to chlorine dioxide poisoning due to consumption of 250 mL of stable ClO_2 (*11*). Recently, Medina-Avitia *et al*. reported a 55-year male who developed acute kidney injury and disseminated intravascular coagulation due to the oral administration of CIO2 to prevent and treat COVID-19. After treatment with hemodialysis, the kidney injury was reversed (*12*). These cases imply that: i) Oral administration of CIO2 in a short period, in a large dose, or to patients with underlying illnesses might be risk factors for the development of acute kidney injury and ii) this ClO_2-related kidney injury is reversible.

2. Using nasal irrigation as an alternative therapy for COVID-19

Since there is no specific treatment for COVID-19, many alternative therapies have been considered. High titers

Table 1. The concentrations of ClO_2 in representative studies directly using ClO_2 in the human body

Studies/Country	Subjects	Intervention	Concentration of ClO_2 (ppm)	Safety	Efficacy
Shinada *et al*., 2008/Japan	15 healthy subjects	7 days of mouthwash	1,000	No adverse events reported	Relief of halitosis
Shinada *et al*., 2010/Japan	15 healthy subjects	7 days of mouthwash	1,000	Three subjects reported "dislike of the smell and taste"	Accumulation of plaque, coating of the tongue, and the count of Fusobacterium nucleatum in saliva decreased
Noszticzius *et al*., 2013/Hungary	One patient with thrombosis and two patients with diabetic foot	Direct administration of ClO_2 to the wound	300	No adverse events reported	Effective at wound disinfection and helped with wound healing
Kale *et al*., 2020/India	Patients who underwent periodontal surgery	14 days of mouthwash	1,000-2,000	No adverse events reported	ClO_2 contributed to the promotion of early wound healing after periodontal surgery
Aparicio-Alonso *et al*., 2021/ Mexico	Family members living with patients with COVID-19	14-day oral administration	3 ppm	No obvious adverse reactions reported	ClO_2 reduced COVID-19-related symptoms and contributed to prevention of the COVID-19
Aparicio-Alonso *et al*., 2021/ Mexico	Family members living with patients with COVID-19	14-day oral administration	3 ppm (1.41 mg/kg)	Only 6.78% of patients reported mild and sporadic uncomfortable reactions such as headaches, dizziness, vomiting, diarrhea, and nausea.	ClO_2 reduced COVID-19-related symptoms and contributed to prevention of the COVID-19

of SARS-CoV-2 can be detected in the upper airways of asymptomatic/symptomatic COVID-19 patients (*13*), with higher viral loads found in nasal swabs compared to pharyngeal swabs. Nasal irrigation has hence been considered as an alternative therapy to treat COVID-19. During the early days of the COVID-19 pandemic, Casale *et al*. (*14*), Ramalingam *et al*. (*15*), and Panta *et al*. (*16*) proposed that nasal irrigation may be a potential treatment for COVID-19. Later, Huijghebaert *et al*. reported that early nasal irrigation with saline may ameliorate COVID-19 symptoms (*17*). Yilmaz *et al*. found that nasal irrigation with hypertonic alkaline significantly reduced the viral load in patients with COVID-19 (*18*). Later, Yildiz *et al*. found that nasal saline irrigation with triamcinolone acetonide may relieve COVID-19-related hyposmia (*19*). Baxter *et al*. found that nasal irrigation with povidone-iodine or sodium bicarbonate helped to reduce disease severity and the duration of hospitalization in patients with COVID-19 (*20*). These studies seem to prove the efficacy of nasal irrigation to treat COVID-19. However, whether nasal irrigation can be used as a potential alternative therapy for COVID-19 requires further investigation because of the small samples in those studies. Moreover, those studies involved the early beta and delta strains; whether nasal irrigation is effective against the omicron strain warrants investigation.

Based on the aforementioned evidence, ClO_2, is a safe and efficient disinfectant, and it is particularly useful as an agent for nasal irrigation to treat respiratory infectious diseases, and COVID-19 in particular.

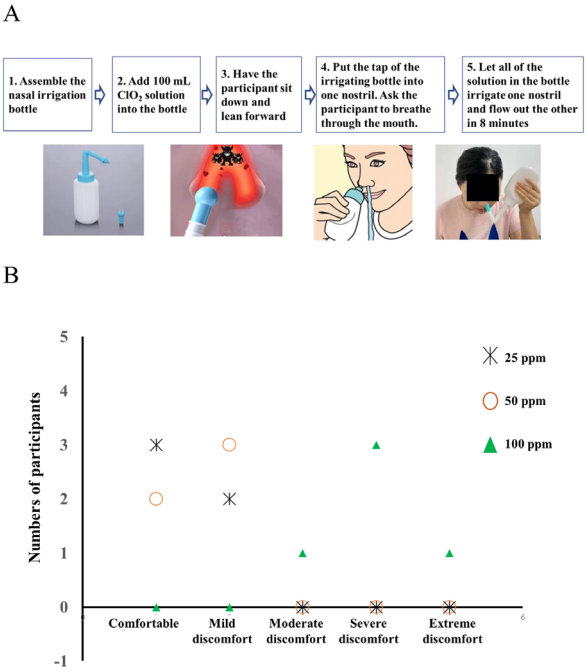

Figure 1. Results of a preliminary experiment exploring the doses of ClO_2 for nasal irrigation in 5 healthy participants. (A) Schematic diagram of nasal irrigation used in a preliminary experiment. **(B)** Results of the preliminary experiment with regard to discomfort. At a ClO_2 concentration of 25 ppm, 3 participants felt comfort and 2 participants felt mild discomfort. At a ClO_2 concentration of 50 ppm, two participants felt comfort and three participants felt mild discomfort. At a ClO_2 concentration of 100 ppm, one participant felt moderate discomfort, three participants felt severe discomfort, and one participant felt extreme discomfort. Hence, 25-50 ppm was considered to be an appropriate concentration range for nasal irrigation and was used in subsequent experiments.

3. Deduction of an appropriate dose of ClO_2 for nasal irrigation

The first consideration is safety. The dose, concentration, and method of administration are known to be the most crucial factors associated with the safety of ClO_2 in the context of COVID-19 (*3*). Hence, the dose/concentration of ClO_2 must be carefully and comprehensively determined by balancing efficacy, safety, and ease of solution preparation. Several aspects need to be considered to deduce the appropriate dose:

1). Hatanaka *et al*. reported that exposure to ClO_2 at 24 ppm for 10 s can kill 99.99% of SARS-CoV-2 (*1*). This suggests that SARS-CoV-2 is extremely sensitive to ClO_2.

2). Aparicio-Alonso *et al*. reported that oral administration of ClO_2 at 3 ppm in a dose of 0.3 mg/kg/day was safe (*9*). Assuming the body weight of an adult is 50 kg, oral consumption of 15 mg/day ClO_2 is safe. This is 20 times lower than the lowest observed adverse effect level (LOAEL) and 300 times lower than the LD_{50}.

3). Ma *et al*. found that ClO_2 at 50 ppm did not cause ocular irritation in rabbits, which proved that 50 ppm causes no mucosal irritation. Thus, it is safe for the nasal mucosa.

4). In a preliminary experiment performing nasal irrigation in 5 healthy subjects, 25 and 50 ppm did not cause any intolerable discomfort, whereas 100 ppm may cause discomfort due to the smell (Figure 1).

5). If using ClO_2 at 50 ppm for nasal irrigation (100 mL, bid), the nasal irrigation dose is 10 mg (for a 50 kg adult), about 2/3 of the dose in the study by Aparicio-Alonso *et al*. (3). This is 30 times lower than the LOAEL and 450 times lower than the LD_{50}

6). When preparing a ClO_2 solution, concentrations of 25 and 50 ppm are easily handled and stored.

Accordingly, 25-50 ppm was deemed to be an appropriate concentration range for nasal irrigation with ClO_2 in terms of safety and efficacy. Indeed, the current authors are now conducting a subsequent study to evaluate the safety and efficacy of nasal irrigation with ClO_2 at 25 or 50 ppm. The forthcoming results should help to provide evidence regarding whether nasal irrigation with ClO_2 can be used as an alternative therapy to treat COVID-19, as well as the other respiratory infectious diseases such as influenza.

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This experiment was approved and supervised by the Ethics Committee of The Third People's Hospital of Shenzhen (approval number 2022-182-03). The study protocol was explained to all of the participants who then provided written informed consent to participation in this study.

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Verification of the efficiency of saline gargle sampling for detection of the Omicron variant of SARS-CoV-2, a pilot study

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SUMMARY A saline gargle (SG) has proven to be an efficient method of sampling to detect SARS-CoV-2. The aim of this pilot study was to verify the efficiency of SG sampling in detecting the Omicron variant of SARS-CoV-2. Subjects were a total of 68 patients with COVID-19 (Omicron variant), and 167 pairs of samples were collected. A conventional oropharyngeal swab (OPS) was obtained and SG sampling was performed immediately afterward; both were subjected to RT-qPCR. A subgroup analysis of symptomatic and asymptomatic patients was performed. Results revealed no significant differences in the distribution of patients and cycle threshold (CT) values between the SG and OPS in overall data and data on days 1-3, 4-7, and 8-14. The subgroup analysis revealed no significant differences between the SG and OPS results in symptomatic patients. In asymptomatic patients, the CT values for the SG were significantly lower than those for the OPS, implying that SG sampling had better sensitivity in the context of the Omicron variant. These data indicate that the SG had satisfactory efficiency (*vs*. the OPS). An SG is a simple and less invasive method of sampling that is suited to mass, frequent, and repeated sampling to detect SARS-CoV-2.

Keywords saline gargle, SARS-CoV-2 detection, Omicron variant, oropharyngeal swab, COVID-19

By far, the gold standard for confirming COVID-19 remains SARS-CoV-2-specfic quantitative real-time polymerase chain reaction (RT-qPCR) sampling with a nasopharyngeal swab (NPS) (*1*). An NPS has the best sensitivity, but it can cause discomfort. Moreover, sampling with an NPS commonly requires well-trained and experienced medical staff. More convenient methods, such as an oropharyngeal swab (OPS), are also widely accepted for mass, frequent, and repeated sampling, but the latter sacrifices convenience and sensitivity. In China, an OPS is now the most widely used method of sampling to detect SARS-CoV-2. However, an OPS can also cause discomfort to the examinee. It can potentially enhance the exposure risk of medical staff since an OPS can sometimes cause coughing. Accordingly, alternative methods of sampling that are less invasive have been considered. Saliva testing is an important alternative method that is also accepted in many countries such as Japan. However, saliva sampling remains controversial because of its sensitivity (*vs*. an NPS and OPS). Moreover, a lack of standard methods of collection/processing has also been cited by some researchers (*2*), which consider saliva sampling inappropriate for the general population. Tan *et al*. pointed out that standardization of saliva sampling might be a solution to encourage it acceptance as an alternative method of detecting SARS-CoV-2 (*3*). However, developing a "replicable" standard method of saliva sampling is not easy. Accordingly, an alternative method of sampling should have the following characteristics: safe, convenient, sensitive, comfortable, simple, and replicable, so that it can easily be standardized. Thus, a saline gargle (SG) has been considered. Bennett *et al*. found that a gargle lavage sample is more sensitive than an OPS for respiratory pathogens (*4*). Early in 2020, Saito *et al*. first reported that testing a gargle sample for SARS-CoV-2 using 10 mL of normal saline can yield a positive result from a patient with COVID-19. They pointed out that an SG might be used as a safe and sensitive method with which to diagnose COVID-19 (*5*). Mittal *et al*. compared the efficiency of SARS-CoV-2 detection among an NPS, OPS, and SG (8-10 mL of normal saline) (*6*). They

found that an SG had satisfactory sensitivity at detecting SARS-CoV-2 but caused less discomfort. Later, Poukka *et al*. (*7*) and Lévesque *et al*. (*8*) also obtained similar results. Benoit *et al*. found that gargling with water for 5 s in the mouth and 5 s in the throat had a slightly lower sensitivity *vs*. an OPS and NPS (*9*). Gobeille Paré *et al*. found that gargling with natural spring water (5 mL of water for 20s) resulted in a lower sensitivity than an OPS or NPS (89.6% *vs*. 97.9%, $p = 0.005$) (*10*). Most of the aforementioned studies demonstrated the value of an SG as an alternative method of sampling to detect SARS-CoV-2, though it is less sensitive than conventional OPS and NPS. However, these studies are based on the old variants such as the beta and delta strains. Thus far, no study has verified the diagnostic value of an SG in detecting the Omicron variant, which mainly affects the upper respiratory tract. Moreover, most patients are asymptomatic. Given these facts, the current study was designed to evaluate the sensitivity of an SG in the context of the Omicron variant. A subgroup analysis was performed by dividing subjects into symptomatic and asymptomatic patients. This study attempted to determine the value of an SG in symptomatic and asymptomatic patients. The findings of this study may help to better understand the role of an SG in SARS-CoV-2 detection.

Subjects were a total of 68 patients who were confirmed to be infected with the Omicron variant of SARS-CoV-2 (2022 July-2022 August) based on an NPS. The inclusion and exclusion criteria are available in the supplementary materials (Table S1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=128*). Subjects were tested daily (9:00 AM) for SARS-CoV-2 with an OPS and an SG immediately afterwards after the initiation of this study. A total of 167 pairs of samples (including OPSs and SGs) were collected. The OPS was obtained per routine methods. SG samples were collected by asking subjects to rinse their mouth with 8 mL of saline water for 10 seconds. They then tilted their head back and gargled for another 10 seconds, finally spitting the water back into a 10-mL plastic tube. Samples were collected and stored in a refrigerator at -80℃. The time between sampling and refrigeration was limited to 4 hours. This study was strictly conducted per the guidelines of the Declaration of Helsinki of the World Medical Association (2000), and it was approved and supervised by the ethics committee of The Third People's Hospital of Shenzhen (approval number 2022-116-03). This study was registered with a Chinese clinical trial registry (ChiCTR2200063457). The study protocol was explained to all of the patients, who were asked to provide written informed consent to participation in this study. Samples were treated and tested for SARS-CoV-2 using a routine RT-qPCR assay. The software SPSS (ver23.00, IBM, US) was used for statistical analysis. Categorical variables were expressed as a percentage while continuous variables were expressed as a median with an interquartile range (IQR). The distribution of positive and negative patients was compared using a chi-squared test or Fisher's exact test. A paired *t*-test was used to compare the difference in the CT values for the SG and OPS (CT values were only available for positive patients). The detailed methodology is available in the supplementary materials.

This study involved a total of 68 subjects with COVID-19 (Omicron variant). Detailed information on the patients is listed in Table S2 (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=128*). Overall, a total of 126 (77.45%) SG samples tested positive, 12 (7.19%) of those had a single positive result, and 41 tested negative. Overall, a total of 126 (77.45%) OPS samples tested positive, and 11 (6.59%) of those had a single positive result. There were no significant differences in the distribution of

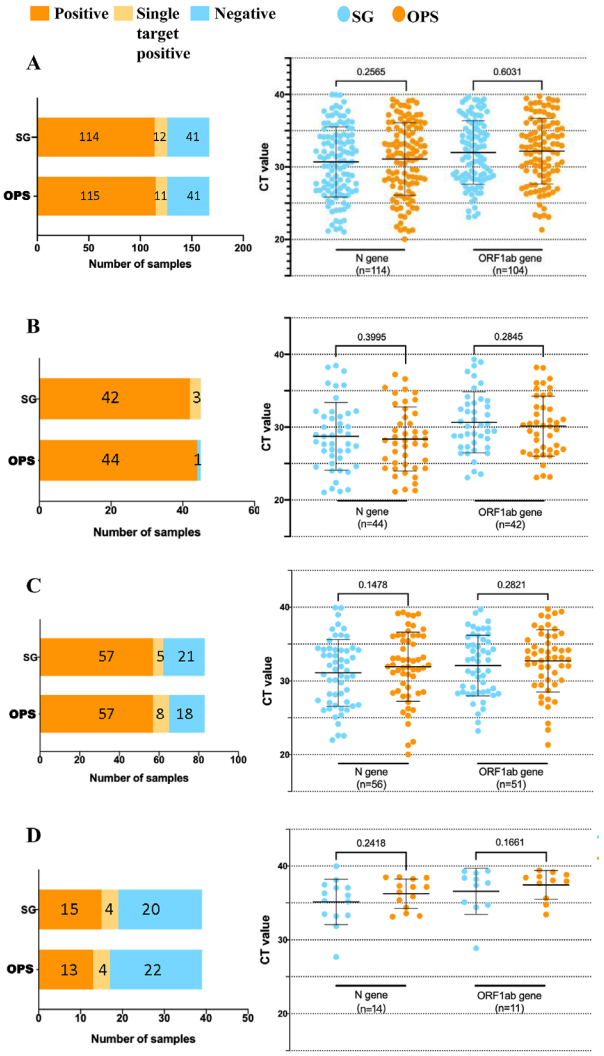

Figure 1. Comparison of SARS-CoV-2 detection in a saline gargle or oropharyngeal swab from subjects infected with the Omicron variant of SARS-CoV-2. (A), Overall data on SARS-CoV-2 detection in all patients. **(B)**, Data 1-3 days after the onset of COVID-19. **(C)**, Data 4-7 days after the onset of COVID-19. **(D)**, Data 8-14 days after the onset of COVID-19. The column on the left indicates the distribution of patients tested with RT-qPCR; the column on the right indicates the CT values determined with RT-qPCR. CT, cycle threshold; OPS, oropharyngeal swab; SG, saline gargle.

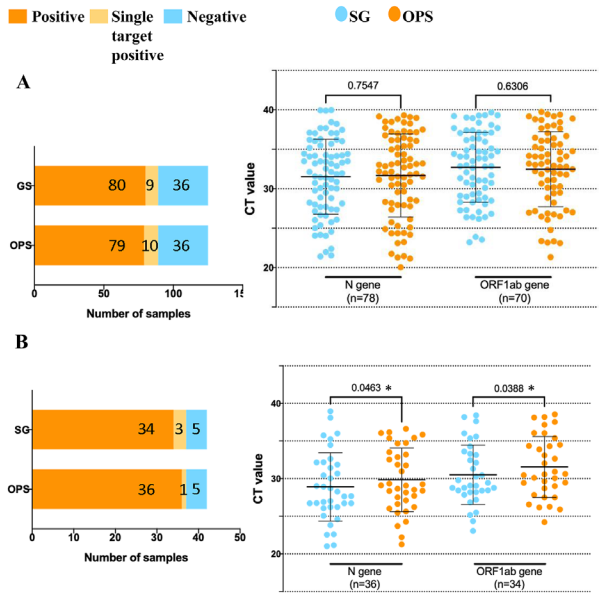

Figure 2. Comparison of SARS-CoV-2 detection (Omicron variant) in a saline gargle or oropharyngeal swab from symptomatic or asymptomatic subjects with COVID-19. (A), Data on symptomatic patients. **(B)**, Data on asymptomatic patients. The column on the left indicates the distribution of patients tested with RT-qPCR; the column on the right indicates the CT values determined with RT-qPCR. *indicates $p < 0.05$. CT, cycle threshold; OPS, oropharyngeal swab; SG, saline gargle.

patients (Figure 1A, column on the left). There were no significant differences in the CT values for the N gene or the Orf1/ab gene in SG and OPS samples (Figure 1A, column on the right). Nevertheless, the internal reference gene (RNase P) was significantly lower in SG samples than in OPS samples ($p < 0.0001$, Figure S1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=128*). Likewise, the data on days 1-3 (Figure 1B), 4-7 (Figure 1C), and 8-14 (Figure 1D) exhibited the same trend, namely, there were no significant differences in the distribution or patients or CT values (SG *vs*. OPS). The subgroup analysis found no significant differences in the distribution of patients or CT values in symptomatic patients (Figure 2A). In asymptomatic patients, there were no significant differences in the distribution of patients (Figure 2B, column on the left). The CT values for the N gene ($p = 0.0463$) and Orf1/ab ($p = 0.0388$) in patients sampled with the SG were significantly lower than those in patients sampled with the OPS (Figure 2B, column on the right).

The current study verified the use of an SG to detect SARS-CoV-2. Both overall data and data on days 1-3, 4-7, and 8-14 revealed that the SG had satisfactory sensitivity in comparison to the most commonly used OPS (Figure 1). In symptomatic patients, there were no significant differences between the SG and OPS (Figure 2A). Interestingly, in asymptomatic patients, the CT values for the N gene and Orf1/ab were significantly lower in SG samples than in OPS samples, implying that the SG had better sensitivity (Figure 2B). To the extent known, this is the first study to compare the efficiency of an SG and OPS in patients infected with the Omicron variant, either symptomatic or asymptomatic. Findings regarding the efficiency of an SG agree with the results of previous studies using saline (*5,6*) and water (*7-10*). However, all of those studies detected older variants in an insufficient number of asymptomatic patients. The current study tested patients for the Omicron variant, and there was a large enough sample of asymptomatic patients. Results revealed that there were no differences between the SG and OPS in symptomatic patients. A point worth noting is that the results for asymptomatic patients seem to indicate that the SG had better sensitivity (lower CT values). A potential explanation might be that an SG collects more infected tissues/cells than a conventional OPS. In comparison to older variants, the Omicron variant mainly affects the upper respiratory tract. Due to the small sample in this pilot study, this aspect requires further investigation. Nevertheless, the results of this study provide inspiring evidence that an SG has satisfactory efficiency in comparison to an OPS in detecting the Omicron variant of SARS-CoV-2 that is currently prevalent. Moreover, SG sampling is easy to standardize. Indeed, it is a simple, convenient, less invasive, and easily adapted to self-sampling, so it is suited to mass, frequent, and repeated sampling to detect SARS-CoV-2.

Ethical statements

This study was strictly conducted per the guidelines of the Declaration of Helsinki of the World Medical Association (2000), and it was approved and supervised by the ethics committee of the Third People's Hospital of Shenzhen (approval number 2022-116-03). This study was registered with a Chinese clinical trial registry (ChiCTR2200063457). The study protocol was explained to all of the patients, who were asked to provide written informed consent to participation in this study.

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Commentary

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Reflections on abortion rights: From policy to medicine

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SUMMARY On June 24, 2022, the US Supreme Court overturned Roe v. Wade, which marked a further restriction on women's abortion rights in the US. It has sparked a wide range of societal reactions around the world. Women in different countries enjoy diverse abortion rights due to conditions in their respective nations and cultures. Abortion protects women's rights to a certain extent, and especially in the event of unintended pregnancy. An inappropriate abortion ban will affect women's health and lives and all aspects of medicine, including the lives of doctors, patient access, and the development of medical technology. This review provides a gynecologist's perspective on the impact of abortion policies on women's health and the medical system. This review also attempts to determine the reason for the government's abortion ban.

Keywords abortion right, women, policy, risks and benefits, medical system

Abortion is a longstanding controversy regarding its moral, legal, medical, economic, and religious aspects. Between 2015 and 2019, there were approximately 121 million unintended pregnancies and 73 million abortions worldwide (*1*). A study conducted in 14 countries, including low-, middle-, and high-income countries, estimated that the most frequent reasons for an abortion were socioeconomic concerns or limiting childbearing (*2*). In order to call for the legalization of abortion, the United Nations (UN) has designated September 28 as International Safe Abortion Day to support women's fundamental rights to a safe abortion (*3*). However, as the fertility rate declines and the population rapidly ages, the debate over abortion has resurfaced. Whether abortion rights are a human right is worth considering. What will happen if those rights are restricted? The current review summarizes the abortion policies in the US, Europe, Japan, and China and it discusses the impact of these policies on individuals, doctors, and the medical system.

1. Abortion law and policies in different countries

▪ *US* Abortion rights have long been controversial in the US. About half a century ago, most states had strict restrictions on abortion rights until Roe v. Wade in 1973. A woman's right to an abortion began to be protected by the Constitution after Roe v. Wade. On June 24, 2022, the US Supreme Court overturned this case. Subsequently, several states immediately changed their laws on abortion. As of July 16, 44 states have banned abortions unless the mother's life is in danger or in other extreme circumstances (*4*) (Table 1). Healthcare providers are allowed to refuse to provide abortion services in 46 states (*5*).

▪ *Europe* Compared to the US, abortion policies in Europe are more liberal (*6*). Abortion is usually legal to protect the health of a pregnant woman. Some countries require women to undergo mandatory counseling. Abortion is not allowed only in 6 European countries. Andorra, Malta, and San Marino have a total abortion ban. Liechtenstein, Monaco, and Poland allow abortion when a woman's life or health is at risk or the pregnancy results from sexual assault. Moreover, abortion is permitted in Monaco and Poland when the pregnancy involves a severe fetal anomaly.

▪ *Japan* In Japan, an abortion is permitted within 22 weeks if a pregnancy is caused by violence, coercion, or rape, or if continuing pregnancy or childbirth will result in health risks or financial hardship under the current Maternal Protection Act (*7*). Methods of abortion, including emerging abortifacients, require spousal consent. Abortifacients were legalized in December 2021

Table 1. Restrictions on abortion in the US

Gestational limits
When the mother's life is in danger
Any time: Alabama, Arkansas, Missouri, Oklahoma, South Dakota, Texas
After 6 weeks: Ohio, Tennessee
After 15 weeks: –
After 22 weeks: Indiana, Iowa, Kansas, Kentucky, Nebraska, North Dakota, West Virginia, Wisconsin
After 24 weeks: Pennsylvania
Viability*: Michigan, Idaho, Montana, North Carolina, Wyoming
Third trimester: –
Physical and general health reasons
Any time: –
After 6 weeks: –
After 15 weeks: –
After 22 weeks: –
After 24 weeks: Massachusetts
Viability*: Arizona, California, Connecticut, Delaware, Hawaii, Illinois, Maine, Maryland, Minnesota, New York, Rhode Island, Washington
Third trimester: Virginia
Cases of rape, incest, or fetal abnormalities
Any time: Louisiana, Mississippi
After 6 weeks: South Carolina
After 15 weeks: Florida
After 22 weeks: Georgia
After 24 weeks: Massachusetts
Viability*: Delaware, Maryland, Utah
Third trimester: –

No restrictions:
Alaska, Colorado, Nevada, New Hampshire, New Jersey, New Mexico, Oregon, Vermont

*Viability: The point at which a fetus is capable of surviving outside the uterus, usually between 24 and 28 weeks.

and will be approved as early as December 2022 (*8*).

▪ *China* Abortion is legal in most regions of China but prohibited for gender selection. The Law of the People's Republic of China on the Protection of Women's Rights and Interests states that women have the freedom to choose to have a child or not (*9*). Most regions restrict abortion only after 28 weeks' gestation unless fetal malformations or the mother's safety are involved. To balance the sex ratio, some provinces have banned abortion after 14 weeks' gestation when a child's gender can be predicted *via* ultrasound (*10*).

2. The effects of abortion on patients

2.1. Methods of abortion and risks involved

The methods of abortion that are current available internationally include medical and surgical abortion. Misoprostol and mifepristone were used for medical abortion during the first trimester of pregnancy with an efficiency of 96.7%. The combination of these two drugs is recommended by the World Health Organization (WHO) for safe abortion care, and they are on the WHO's List of Essential Medicines (*11*). Medical abortion partly protects the patient's privacy and avoids invasive surgery. Surgical abortions include uterine aspiration and dilation and evacuation (D&E). Uterine aspiration is used primarily in the first 14 weeks of gestation, while D&E is used for gestational ages between 14 and 24 weeks. Compared to medical abortions, surgery takes less time and is less painful because anesthesia is used (*12*).

2.2. Effects on women's health

Abortion has harmful and beneficial effects on women's lives. Medical abortion can cause prolonged bleeding and cramping, and especially without a doctor's prescription; adverse drug reactions will occur, such as nausea, vomiting, diarrhea, headache, dizziness, fever, hot flashes, and chills (*13*). Surgical abortion can cause *i)* bleeding, *ii)* uterine perforation, *iii)* cervical trauma, *iv*) additional procedures due to insufficient aspiration, *v)* infertility, and *vi)* other symptoms including vasovagal syncope, asthma exacerbation, and disseminated intravascular coagulation. Either medical or surgical abortion may cause infection, retained products of conception, failed abortion, and continuing pregnancy (*14*). Women who underwent abortions are more likely to experience unsuccessful pregnancies or miscarriages than those who gave birth (*15*). A strict abortion policy may increase the cases of unsafe abortion, such as abortifacient abuse and risky intentional miscarriages, which increase the risk of gynecological diseases and harm women's health and lives. Besides physical health, women's mental health can also be significantly impacted by abortion. The risk of mental disorders is 81% higher for a woman who has undergone an abortion than one who has not, and nearly 10% of mental illnesses can be attributed to abortion (*16*). Abortion also increases the risk of a mental disorder recurring (*17*).

However, abortion protects women against a series of subsequent problems caused by unintended pregnancies. *i)* In the case of sexual assault or incest, abortion can reduce the harm to women, allowing them to continue their studies and lives. *ii)* Abortion procedures can be used to treat and prevent serious conditions, such as fetal death or incomplete abortion. *iii)* Abortion can prevent sexual dysfunction, a hormonal imbalance, or a distorted body shape after pregnancy (*18*). In addition, abortion can protect women and their families from the financial and mental stress of additional childbirth and childcare, improving their quality of life. Therefore, the right to a safe voluntary abortion is reasonable and essential for women.

2.3. The patients' decision

The enactment of abortion bans puts patients at risk. Due to severe legal restrictions and tight economic conditions, many women choose low-cost but unsafe abortions, such as self-induced abortions, clinics with poor hygiene, or even untrained personnel using dangerous methods. A study found that between 2010 and 2014, 25 million unsafe abortions were performed each year globally, accounting for 45% of all abortions (*19*). Most of those abortions occur in low- and middle-income countries due to more restrictive policies and

socioeconomic factors. Abortion restrictions further limit insurance coverage. Currently, 16 states cover all or most medically necessary abortions with Medicaid. In 33 states and the District of Columbia, however, state funds are unavailable unless a pregnancy is life-threatening or results from rape or incest (*20*). Patients' decisions will undoubtedly be influenced by financial concerns. Therefore, the WHO recommends that countries devise policies and financial commitments that support access to safe, legal abortions.

3. The impact of abortion bans on the medical system

3.1. The medical system

The imposition of abortion restrictions will affect the medical system in all aspects. In the aftermath of the overturning of Roe v. Wade, the number of abortion clinics and doctors has decreased in many US states, limiting patient access. To obtain an abortion or medical care, they must travel to other states where abortion is allowed. In addition, emergency abortions will not be possible in life-threatening circumstances due to a lack of doctors in some communities. Since the restriction on abortion, doctors have acted more cautiously given local legalization. For example, mifepristone, used to prevent miscarriages, is banned in abortion clinics in Alabama. This means that women experiencing incomplete abortions cannot receive timely treatment until their life is in danger. This ban also limits the development of medical technology because of its chilling effect. The live birth rate from in vitro fertilization is less than 30%, and abortion is the most common outcome (*21*).

3.2. The doctor's dilemma

Doctors have been significantly affected by abortion restrictions. Abortion providers experienced a considerable rise in stalking (600%), blockades (450%), hoax devices/suspicious packages (163%), attacks (129%), and assaults and battery (128%) in 2021, which may be exacerbated by the overturning of Roe v. Wade (*22*). The rate of resignation among abortion doctors has risen due to excessive legal burdens or declining patient numbers and salaries. Doctors who continue to perform abortions are forced to find a new approach, such as traveling across state lines to perform abortions or providing counseling or medication to patients *via* telemedicine. Most doctors who only perform abortions have relocated to states where abortion is legal (*23*).

4. From policy to action by the medical system: What's the impetus?

Medicine and policy have different origins and goals but they can affect each other to some degree. For a long time, harming life was considered illegal under the tenets of Christianity, even if a baby was involved. In the Middle Ages, English common law declared abortions a criminal offense after the "quickening," the moment that implied the presence of a human soul (*24*). Over time, the heartbeat theory and the conception theory have gradually emerged with advances in science and people's knowledge of the origin of life. Policies have increasingly rested primarily on modern scientific or technological evidence rather than moral authority or religion. Generally, medical policies are supposed to improve care by regulating and supporting medical technologies. Social issues, however, may require some adjustments for social stability or other reasons, even if they seem unreasonable. For example, during the COVID-19 pandemic, most countries in Europe recognized the shortcomings of current policies on abortion and care and made timely policy adjustments. Some countries, like France and England, utilized telemedicine instead of face-to-face visits. Northern Ireland introduced early medical abortion for the first time during the pandemic (*25*). Another example is China, the world's most populous country, which implemented a family planning policy in 1981 to control rapid population growth. As the population ages and birth rates decline, this restriction has been relaxed, along with an announcement to reduce "non-essential abortions" in 2021 (*26*). Thus, the right to abortion depends on public demand for a transition from policy to action by the medical system. The abortion ban may seem counterintuitive but reasonable based on the above factors, and we should remain objective.

5. Conclusion

In short, the issue of abortion is a longstanding topic that emerged with the emancipation of women. For women, abortion is a mixed blessing. Despite repeated calls from the United Nations and the WHO to legalize abortion, it differs across countries and regions today. Policymakers need to think holistically. A one-size-fits-all policy could lead to a severe blow to abortionists and related medical issues. Therefore, abortion policies should be carefully adjusted.

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Letter to the Editor

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Is targeting angiotensin-converting enzyme 2 (ACE2) a prophylactic strategy against COVID-19?

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SUMMARY Prophylaxis against COVID-19 is greatly needed for vulnerable populations who have a higher risk of developing severe disease. Vaccines and neutralizing antibodies against SARS-CoV-2 are currently the main approaches to preventing the virus infection. However, the constant mutation of SARS-CoV-2 poses a huge challenge to the effectiveness of these prophylactic strategies. A recent study suggested that downregulation of angiotensin-converting enzyme 2 (ACE2), the receptor of SARS-CoV-2 entry into human cells, can decrease susceptibility to viral infection *in vitro*, *in vivo*, and in human lungs and livers perfused *ex situ*. These findings indicate the potential to use agents to reduce ACE2 expression to prevent COVID-19, but the efficacy and safety should be verified in clinical trials. Considering ACE2 performs physiological functions, risks due to its downregulation and benefits from prophylaxis against SARS-CoV-2 infection should be carefully weighed. In the future, updating vaccines against variants of SARS-CoV-2 might still be an important strategy for prophylaxis against COVID-19. Soluble recombinant human ACE2 that acts as a decoy receptor might be an option to overcome the mutation of SARS-CoV-2.

Keywords ACE2, COVID-19, SARS-CoV-2, neutralizing antibodies, prophylaxis

To the Editor,

The rapid global spread of SARS-CoV-2 highlights the requirement for prophylaxis, and especially for vulnerable populations who have a higher risk of developing severe disease, such as older adults (over the age of 65) and patients with comorbidities including cardiovascular disease, chronic respiratory disease, and diabetes (*1,2*). A number of vaccines and neutralizing antibodies against SARS-CoV-2 have been developed thus far and they have played important roles in preventing or decreasing the rate of severe COVID-19 (*3,4*). However, the constantly emerging variants of the virus pose a huge challenge to the effectiveness of these prophylactic strategies (*5*).

Angiotensin-converting enzyme 2 (ACE2) is central to SARS-CoV-2 infection since it facilitates viral entry into human cells (*6*). ACE2 is ubiquitously expressed in the human body in respiratory epithelial cells, type II alveolar cells, small intestinal epithelial cells, vascular endothelial and artery smooth muscle cells, and the brush border of proximal tubular cells in the kidney (*7-9*). Increased ACE2 expression may confer increased susceptibility to host cell entry by SARS-CoV-2 (*7*), which suggests a possible strategy of preventing SARS-CoV-2 infection through modulation of ACE2 expression. A recent study elucidated a novel mechanism controlling ACE2 expression: farnesoid X receptor (FXR) was identified as a direct regulator of ACE2 transcription (*10*). A point worth noting is that ursodeoxycholic acid (UDCA), a drug widely prescribed for cholestatic disorders, was found to downregulate ACE2 expression by suppressing FXR signaling in gallbladder cholangiocytes, airway and intestinal organoids *in vitro*, in the respiratory, biliary, and intestinal epithelium in mice and hamsters, and in human lungs and livers perfused *ex situ*, leading to reduced susceptibility to SARS-CoV-2 infection (*10*). Moreover, treatment with UDCA reduces ACE2 expression in nasal epithelial cells of humans and correlates with lower serum ACE2 levels in patients with cholestatic liver disorders. This study indicated the potential to use FXR antagonists, such as UCDA, to prevent COVID-19, but the efficacy and safety should be verified in clinical trials.

ACE2 is a pivotal counter-regulatory enzyme to angiotensin-converting enzyme (ACE), a central enzyme of the renin-angiotensin system (RAS) (*11*). ACE catalyzes the conversion of angiotensin I (Ang I) to angiotensin II (Ang II), which activates AT_1R and induces vasoconstriction, renal sodium reabsorption and

potassium excretion, aldosterone synthesis, elevation of blood pressure, and activation of inflammatory and pro-fibrotic pathways (*12-14*). ACE2 cleaves Ang I and Ang II and ultimately yields angiotensin (1-7), which activates its receptor MasR and exerts vasodilatory, anti-inflammatory, anti-oxidative, and anti-fibrotic actions (*12,15*). Thus, the balance of ACE/ACE2 determines the availability of different angiotensin peptides, and ACE skewing may result in elevated concentrations of Ang II and contribute to increased oxidative stress, inflammation, and development of hypertension, metabolic syndrome, and diabetes (*14,16*). Knocking out ACE2 was found to lead to increased Angio II and a severe cardiac contractility defect in mice, suggesting that ACE2 is an essential regulator of cardiac function (*17*). ACE2 has also been found to be protective in severe acute lung injury and cardiovascular and metabolic diseases, including diabetes and its complications (*11,18,19*). In addition to its catalytic activity, ACE2 is required for the expression of B^0AT1, a neutral amino acid transporter on the luminal surface of intestinal epithelial cells (*20*). This RAS-independent function of ACE2 is associated with regulating intestinal amino acid homeostasis, expression of antimicrobial peptides, and the ecology of the gut microbiome (*20*). When challenged with factors inducing epithelial damage, mice deficient in ACE2 displayed increased susceptibility to intestinal inflammation (*20*). Given the physiological function of ACE2, risks due to its downregulation and benefits from prophylaxis against SARS-CoV-2 infection should be carefully weighed.

Updating vaccines against the variants of SARS-CoV-2 remains an important approach for prophylaxis against COVID-19. Considering SARS-CoV-2's affinity for binding to ACE2, recombinant human ACE2 (rhACE2) may act as a decoy receptor that blocks the virus to bind ACE2 located on the cell membrane. Alteration of enzyme activity but preservation of SARS-CoV-2 binding activity may be necessary for rhACE2 to avoid systemic cardiovascular reactions during systemic administration. Theoretically, this might be an alternative prophylactic or therapeutic strategy to overcome the mutation of SARS-CoV-2 to some extent.

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