ISSN 1881-7831 Online ISSN 1881-784X

DD&T

Drug Discoveries & Therapeutics

Volume 14, Number 4
August 2020

IRCA BSSA

IACMHR

www.ddtjournal.com

DD&T
Drug Discoveries & Therapeutics

ISSN: 1881-7831
Online ISSN: 1881-784X
CODEN: DDTRBX
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.

Drug Discoveries & Therapeutics is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group. It is published bimonthly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA.

Drug Discoveries & Therapeutics publishes contributions in all fields of pharmaceutical and therapeutic research such as medicinal chemistry, pharmacology, pharmaceutical analysis, pharmaceutics, pharmaceutical administration, and experimental and clinical studies of effects, mechanisms, or uses of various treatments. Studies in drug-related fields such as biology, biochemistry, physiology, microbiology, and immunology are also within the scope of this journal.

Drug Discoveries & Therapeutics publishes Original Articles, Brief Reports, Reviews, Policy Forum articles, Case Reports, News, and Letters on all aspects of the field of pharmaceutical research. All contributions should seek to promote international collaboration in pharmaceutical science.

Editorial Board

Editorial and Head Office:

Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan
E-mail: office@ddtjournal.com

Drug Discoveries & Therapeutics

Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

E-mail: office@ddtjournal.com
URL: www.ddtjournal.com

Editorial Board Members

CONTENTS

Volume 14, Number 4, 2020

Review

Original Article

Brief Report

Letter

Review

DOI: 10.5582/ddt.2020.03067

Laboratory diagnosis of COVID-19 in Africa: availability, challenges and implications

Elijah Kolawole Oladipo[1,2,*], Ayodeji Folorunsho Ajayi[3], Aduragbemi Noah Odeyemi[3], Olawumi Elizabeth Akindiya[4], Emmanuel Tayo Adebayo[3], Ayomide Samuel Oguntomi[2], Moyosoluwa Precious Oyewole[5], Esther Moradeyo Jimah[6,2], Abayomi Adio Oladipo[7], Olumuyiwa Elijah Ariyo[8], Bukola Bisola Oladipo[9], Julius Kola Oloke[10]

[1] Department of Microbiology, Laboratory of Molecular Biology, Bioinformatics and Immunology, Adeleke University, Ede, Osun State, Nigeria;
[2] Genomics Unit, Helix Biogen Consult, Ogbomosho, Oyo State, Nigeria;
[3] Department of Physiology, Ladoke Akintola University of Technology, Ogbomosho, Oyo State, Nigeria;
[4] Department of Biological Science, Ondo State University of Science and Technology, Okitipupa, Ondo State, Nigeria;
[5] Department of Biochemistry Ladoke Akintola University of Technology, Ogbomosho, Oyo State, Nigeria;
[6] Department of Medical Microbiology and Parasitology, University of Ilorin, Ilorin, Kwara State, Nigeria;
[7] Department of Haematology and Blood Grouping Serology, Obafemi Awolowo University Teaching Hospital Complex, Ile Ife Wesley Guild Hospital Wing, Osun State, Nigeria;
[8] Department of Medicine, Infectious Diseases and Tropical Medicine Unit, Federal Teaching Hospital, Ido-Ekiti, Ekiti State, Nigeria;
[9] Department of Clinical Nursing, Bowen University Teaching Hospital, Ogbomosho, Oyo State, Nigeria;
[10] Department of Natural Science, Precious Cornerstone University, Ibadan, Oyo State, Nigeria.

SUMMARY The COVID-19 infection has been a matter of urgency to tackle around the world today, there exist 200 countries around the world and 54 countries in Africa that the COVID-19 infection cases have been confirmed. This situation prompted us to look into the challenges African laboratories are facing in the diagnosis of novel COVID-19 infection. A limited supply of essential laboratory equipment and test kits are some of the challenges faced in combatting the novel virus in Africa. Also, there is inadequate skilled personnel, which might pose a significant danger in case there is a surge in COVID-19 infection cases. The choice of diagnostic method in Africa is limited as there are only two available diagnostic methods being used out of the six methods used globally, thereby reducing the opportunity of supplementary diagnosis, which will further lead to inappropriate diagnosis and affect the accuracy of diagnostic reports. Furthermore, challenges like inadequate power supply, the method used in sample collection, storage and transportation of specimens are also significant as they also pose their respective implication. From the observations, there is an urgent need for more investment into the laboratories for proper, timely, and accurate diagnosis of COVID-19.

Keywords Africa, challenges, COVID-19, implications, laboratory diagnosis

1. Introduction

The outbreak of the new coronavirus disease of 2019 (COVID-19) infection began on November 17, 2019, in the Hubei province of Wuhan, China (*1*). This infection was identified by real-time polymerase chain reaction (RT-PCR) assay on December 30, 2019, by the WHO office in China (*2*). The virus has spread across regions and territory of many countries since then (*3*). The causative agent of the disease is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is currently being diagnosed with the RT-PCR because of its reliability (*4*). SARS-CoV-2 is similar to the severe acute respiratory syndrome virus (SARS-CoV) by ~80%, also has ~50% similarity to the Middle East respiratory syndrome virus (MERS-CoV) and ~96% closeness to bat coronavirus (RaTG13) (*5*).

The COVID-19 has been classified as a pandemic by the World Health Organization (WHO) on March 11, 2020 (*6*). A total of 5,596,550 confirmed cases and 353,373 deaths have been reported globally. African Region has reported 124,733 cases, which are approximately 2.23% of the global confirmed cases as well as 3,700 deaths (1.05%) of the worldwide death toll. All record is given as of May 28, 2020 (*7*). The decreased number of confirmed cases and death rates in

Africa compared with the other continents may be due to the lower rate of testing, which undermines the incident rate and therefore not giving the real picture of the outbreak (*8*).

At the beginning of this pandemic, only two African countries were conducting COVID-19 diagnoses using the RT-PCR (*9*). Later on, many African countries introduced laboratory diagnostic tests. For example, Nigeria has four laboratories that can detect COVID-19 infection. These laboratories are facilitated by the African Center for Disease Control (ACDC) and Nigeria Center for disease control (*9,10*).

Generally, COVID-19 can be diagnosed using the following laboratory techniques: molecular methods (nucleic acid testing), serology (protein testing), rapid antigen test (point of care testing), cell culture, computed tomography. The molecular method using RT-PCR is the most specific and sensitive diagnostic method with as fast as three hours of reliable diagnosis (*10*). Africa as a case study, many laboratories diagnosing COVID-19 have proven to be a massive challenge due to various factors such as resource limitations, unavailability of sensitive diagnostic tests, and implementation of prevention strategies during diagnoses process. In contrast, in the developed nations of the world, multiple laboratory diagnostic methods are being employed. In the advent of a disease outbreak, developing an effective diagnostic protocol is always a clamour of the general masses and scientists. However, the scientific community is equally faced with the task of developing rapid diagnostic techniques and equipment, especially in severe cases of epidemic and pandemic where a need for mass diagnosis must be met (*11*).

The absence of extensive testing data has contributed to the inappropriate estimation of the outbreak and the response to it in Africa. Some deaths have been recorded. Therefore, swift responses are required in Africa to overcome the challenges of laboratory diagnosis to minimize the implications of COVID-19 infection. Also, there must be an appropriate protocol for sample collection, transportation, and precise diagnosis to help reduce the impact on the continent (*11*). Those, as mentioned above, will help us to unravel the real implication of the pandemic on African populace and take appropriate steps.

2. Laboratory techniques for COVID-19 diagnosis

2.1. Serology

In serology, antigen and antibody of viral proteins that elicit a response to SARS-CoV-2 infection are being used for COVID-19 diagnosis. Serology tests can be used to monitor the progress of the disease, past infection, and developed an immunity. Early-stage of infection is indicated by the presence of immunoglobulin M (IgM), while prior-infection and post-infection protection are indicated by immunoglobulin G (IgG) (*12*). The use of the test is also essential in epidemiological investigations and the development of vaccines (*12*). A recent study showed that both IgM and IgG antibodies were detected in all the 39 infected patients who were studied after 5 days of being infected (*11*).

The infection causes changes in viral load, which make viral proteins challenging to detect. Kelvin *et al*. (*13*) found high salivary viral loads in the first weeks, after the onsets of symptoms, which gradually declined with time, but on the other hand, antibodies produced in response to viral proteins provide a full-time frame for the indirect detection of SARS-CoV-2. This test targeting antibodies as markers of SARS-CoV-2 infection can be of use in COVID-19 diagnosis. The shortcoming of this method is the possibility of having cross-reactivity of SARS-CoV-2 antibodies with antibodies produced against other coronaviruses (*14*).

2.2. Rapid antigen test

Rapid antigen test is a lateral flow technology that uses a protein biomarker whereby the colourimetric signal is produced on the paper by the gold-coated antibodies, which confirm the presence of SARS-COV-2 (*15*). They are meant to provide a reasonable turnaround time, and they are of lower-cost in the diagnosis of COVID-19 (*11*). They are used for the determination of infection without sending samples to microbiological laboratories. Thus, rural communities without laboratories can conduct a diagnosis of suspected patients using rapid antigen tests (*14*). Though, rapid antigen test is limited by their reduced sensitivity. Lateral flow antigen detection for SARS-CoV-2 is an approach of quick antigen test, and this approach is undergoing development for diagnosis of COVID-19 (*14*).

2.3. Computed tomography (CT)

Usually, a chest radiograph is commonly employed as an imaging study for patients with respiratory illnesses. Chest CT scan was used for diagnosis of COVID-19 infection in the Hubei Province China due to the shortage of RT-PCR kits. In a chest CT scan, many X-ray measurements are taken at different angles across the chest of the patient; this is to produce cross-sectional images. The images are then subjected to analysis by radiologists to examine abnormal features that can raise a cause for further diagnosis (*14*).

There are controversies on the suitability of CT scan for diagnosis of COVID-19. Earlier studies have reported a high degree of sensitivity of CT scan for diagnosis of COVID-19 (*16,17*). But the reports have been faulted based on poor research design, incomplete methodology, and lack of gold standard. There are also many confounding variables, a short discussion, and pair review process query. Therefore, caution should be taken

in the use of this method (*18*). Also, typical CT scan results in COVID-19 patients at the early stage of disease infection (0-2 days) are not frequently precise (*19*). These shortcomings of CT scans make the diagnosis of COVID-19 challenging to be differentiated from other viral infections like pneumonia, and there is also the hysteresis of abnormal CT imaging in the process of diagnosis (*20*).

2.4. Cell culture

According to WHO interim guidance for COVID-19, virus isolation is not recommended as a routine diagnostic procedure. Isolation of human coronaviruses in cell culture is not a conventional diagnostic technique due to lack of permissive cell lines, labour, expertise requirements and lack of commercial antisera for culture confirmation (*11*). Also, there is a strict guideline for cell culture procedure for COVID-19. The biosafety practices in the laboratory concerning COVID-19 infection are classified under the biological safety level-3 (BCL-3), requiring personal protective device and respirators, scrub suits, and solid-front wraparound gown. Also, the biological safety cabinet (BSC) must be available, hand-free sink, eyewash, and sustained directional airflow towards a self-closing door (*21*). However, cell culture is essential for antiviral and pathogenesis research (*22*).

2.5. Nucleic-acid testing

2.5.1. Real-time polymerase-chain reaction

In Africa, only the molecular method of nucleic acid testing is being carried out in specialized laboratories. It will be essential to acknowledge that RT-PCR testing is the standard gold method for diagnosis of COVID-19 (*23*). This method is basically to genetically test for SARS-CoV-2 by using reverse transcription-polymerase chain reaction (RT-PCR) kit. Using RT-PCR kits, the SARS-CoV-2 RNA is reversely transcribed into complementary DNA strands, then subjected to amplification of specific regions of the cDNA (*14*). Real-time PCR is the technique of retrieving data throughout the process of PCR by joining amplification and detection into one single operation. The principle is using a variety of different fluorescent chemistries in correlation with the concentration of PCR product to florescent activity (*24*).

2.5.2. Real-time quantitative reverse transcription PCR

Real-time quantitative reverse transcription PCR is a reliable technique for product detection and measurement during each cycle of the PCR process. It is essential for effective counting of microbial load. Hence it is imperative for the diagnosis of viral infection. Depending on the kind of probe (SYBR Green or Taqman), the fluorescent dye binds to the amplified gene product, and the qPCR reads the CT value (*25*). QRT-PCR is done in a specific thermal cycler that can illuminate each sample with a light beam of a specific wavelength and afterward detect the fluorescence that the excited fluorophore is emitting (*26*). This technique is being used in diagnosing COVID-19 (SARS-COV-2) as earlier described by Chu *et al*. (*27*) and Tang *et al*. (*28*). The peculiarity of qRT-PCR lies in its ability to quantify the viral presence. The principle of qRT-PCR assays involves following the R.T. of RNA into cDNA. Therefore, useful detection chemistry is needed to report the presence of PCR products. An instrument that will monitor the amplification in real-time is also required, and appropriate software for quantitative analysis (*25*).

2.5.3. Droplet digital PCR

The ability of droplet digital PCR (ddPCR) to measure the levels of amplifiable targets precisely makes it an essential technique for measuring and determining copy number alterations (CNAs) in genomic biomarkers (*29*). Yu *et al*. (*30*) stated that COVID-19 was diagnosed in China using a digital PCR kit from one of their studies. The kit detects the ORFlab gene, N gene, and a positive reference gene. Ten copies/test was the detection limit (*30*). Studies have shown that digital droplet PCR (ddPCR) possess some advantages over RT-PCR in terms of absolute quantification and more sensitivity for virus detection (*31*). Yu *et al*. (*30*) reported that both RT-PCR and ddPCR showed reliable accuracy in samples with high viral load and negative examples, but digital droplet PCR (ddPCR) was better at detection in samples that possessed low viral load.

Out of all these laboratory diagnostic methods, Africa has concentrated firstly on qRT-PCR and the RT-PCR. There has not been much evidence of the laboratory techniques used in African from selected published reviewed papers. Likewise, there is a shortage of information on the use of ddPCR in Africa for the diagnosis of COVID-19 (*30*). This has a significant challenge on the determination of the virus by not combining other methods for proper diagnosis; this could affect the accurate transmission picture of the virus in each country from Africa. There will likely be records of false positivity and negativity by the quality of the diagnostic protocols. Though qRT-PCR has helped in detecting the virus in suspected COVID-19 patients for differential diagnosis of the unknown respiratory syndrome, screening of asymptomatic individuals and rescreening of confirmed COVID-19 patients that have receives treatment for the infection to be tested positive before being discharged. For the understanding of the pathogenesis of the circulating viral strains in Africa, cell culture should be encouraged, and facilities should be provided (*31*). This will help Africa to have a better understanding of the pathogenesis of the circulating

strain as much as enhancing antiviral research.

The better the understanding of the community-community-transmission rate, the more rural diagnosis should be carried out. Therefore, validation of the rapid antigen test is essential in this case because of their reduced sensitivity. But if the validation is appropriately carried out, it will, in turn, help the rural community in the detection of COVID-19 infection (*31*).

3. Challenges of laboratory diagnosis of COVID-19 in Africa

3.1. Africa health system

The health systems in Africa are strained and have minimal capacity to control the pandemic (*32*). Apart from this endemic infectious disease, the health systems in Africa are facing both communicable and non-communicable diseases, including injury, anaemia, malaria, HIV/AIDs, tuberculosis, and cancer. As a result, the health systems in Africa are stretched thin, to begin with, and there is very little access to the room to absorb the COVID-19 pandemic (*33*). Besides, Africa has the lowest capacity to provide critical care in the world. A recent analysis showed that countries with the highest numbers of intensive care beds per capital do not include any country from Africa (*33*). In Liberia, there are no intensive care units (ICU) with ventilators, for example. Uganda has 0.1 ICU bed/100,000 population. But, the United States has 34.7 beds/100,000 population (*24*). The potential to treat severe forms of COVID-19 depends on the availability of ventilators, electricity, and oxygen because this infectious disease could lead to respiratory failure requiring ventilation support (*33*). The challenges could be summarized under the headings, such as lack of sufficient human personnel, poor budget allocation to the health sector, and weak management and leadership policies (*32*).

3.2. Insufficient human resources

The problem of insufficient human resources comes first in Africa health sectors. There is the problem of a low number, uneven distribution, and immigration of African exports to European and Asian countries (*34*). Also, poor delivery of services due to incessant strikes, weak government policies for civil servants, poor work attitude are some of the problems facing African health sectors (*35*). The WHO organization have also identified that the sub-Sahara African countries are facing a shortage of health care providers, and proper attention has not to be given by the African governments. These problems may lead to poor treatment outcomes and an inability to fulfil health goals nationally and globally (*35*).

Also, most of the hospitals in Africa are faced with limited skilled healthcare personnel in which there have been mass inflows of both asymptomatic and symptomatic patients. With time, this will later overwhelm the inadequate health infrastructure available in Africa. Also, this will make it difficult to spare medical personnel to leave their health posts to conduct field tests. Limited health care personnel pose limitations to the distribution of test kits needed for the diagnosis can delay the service for laboratory testing of suspected patients (*36*).

3.3. Poor budget allocation to the health system

Poor budget allocation to the health system is another significant challenge for the African health care system, and this has become a chronic problem, and this is even more severe with the present economic issue as a result of the lockdown (*37*). Out-of-pocket payments (OOPs), a regressive mode of funding, have been responsible for 40% of total healthcare funding in 50% of African countries (*38*). Even after several declarations by African heads of states of improved financing of the healthcare system, the allocated funds remain inadequate in the majority of the countries (*37*).

The high cost of healthcare delivery, lack of financial sustainability, and absence of economic autonomy by the health care system are also contributing factors to the financial challenges of the health care system (*39*). The available budget to the healthcare system is a determinant factor in the success of the fight against the COVID-19 pandemic. Therefore, an improved budgetary allocation is vital.

3.4. Leadership and management problem

Poor leadership and management have had their toll on all sectors of governance, and the health sector had a significant share of it. It has been identified that the training, development, selection, and preparedness for a leadership position is one of the complex problems facing the future of the healthcare system in Africa countries (*41*). Unfamiliarity with the techniques of leadership, lack of political will, inefficient healthcare program integration, poor government policies, and corruption have been identified as the problems facing health care leadership and management structure (*40*). To effectively fight the menace of poor leadership, commitment to work, team spirit, job satisfaction, integrity, and dedication are expected of every potential leader.

3.5. Unavailability of the test kit

The swab and reagents that are needed to carry out the test for COVID-19 are in short supply for most of the African countries, and the kit cannot manufacture our own. A large amount of some of these supplies would have imported from China and some European countries. However, these countries are in small quantities, for they

too are affected by this virus. So that is a big challenge, and it can lead to an increase in the number of infected in the region (*33*).

3.6. Inadequate personal protective equipment (PPE)

Africa is exceptionally vulnerable because we are unable to manufacture enough PPE for this infection. The purchase of personal equipment such as masks, hand glove, and others are in short supply across the world. There is a competition on the protective purchase of this equipment with developed countries. That is the most unfortunate situation with this pandemic, and it is affecting every part of the world. For the outbreak of the Ebola virus in Africa, we counted on Europe or the U.S. to import supplies. But everybody is affected at the same time, and it becomes a big challenge (*33*).

3.7. Sample collection and analysis

Sample collection and analysis are critical steps in detecting the virus. The collection of samples for the laboratory must be done following the world health organization standard. The diagnostic test consists of a swab, a long stick with a piece of cotton or gauze at the end, taken from the throat and nose of the suspect case. The collection is the beginning of the process. Subsequent steps are essential, too. There are cases where samples are stated inadmissible. For example, it's only useable if it's accompanied by the right documentation (*42*).

Sample collection is challenging because of the nasal swab that is not made of cotton; it is long and flexible and can extend to the ear. Apart from the possibility of damaging the nose or ear, it could also inhibit sample collection and testing. Analysis in the lab requires expertise too, with accuracy and precision in terms of time and sample temperature (*42*).

3.8. Transportation

The samples must be controlled with the utmost caution. Unfortunately, we have come across cases where the person transporting the material mishandle it (*43*). It is also vital to respect the timeframe for carrying the sample, in particular, to make sure that it is stored and transported at a standard temperature of 2-8°C. If it is placed outside this standard temperature, it no longer conforming to the ideal. It must also be transported correctly and not put in a horizontal position or upside down. For this reason, it is essential to set the samples in a rack that shows the direction of storage with an arrow to keep them upright throughout the journey. Materials and specimens to be used in the laboratory should be placed in a secondary container to minimize breakage, spill, or contamination during transportation within and between laboratories (*33*).

3.9. A limited power supply

The already dilapidated power supply system contributes to the challenges facing laboratory diagnosis in Africa. Regular power cuts pose a further challenge to the national health system infrastructure. Limited energy access or reduction in supply hours would affect healthcare facilities such as testing, cooling systems, medical devices, and equipment (*44*).

4. The implication of the challenge

4.1. Test kit

Insufficiency of test kit can lead to a lot of casualties in Africa. As of May 28, 2020, the number of confirmed cases of COVID-19 in African region was 124,733, with a total death of 3,700. A total of 54 infected countries were reported in Africa with Egypt, Algeria, South Africa, and Cameroon leading the chart of most confirmed cases (*45*). The number of confirmed COVID-19 cases in Africa is not a true reflection of the outbreak due to insufficient kits. These issues may lead to misdiagnosis of the patient, inadequate treatment, and increased mortality rate. The laboratory personnel may tend to manage some of the malfunction test kits to avoid interruption in daily data to be compiled. This may lead to the inability to determine the true prevalence and spread o of the diseases. In addition, it results in a high mortality rate.

4.2. Personal protective equipment

World Health Organization has warned that severe and mounting disruption to the global supply of personal protective equipment (PPE) such as face shields, surgical masks, diagnostic swabs, ventilator components and reusable N95 respirators – caused by rising demand, panic buying, hoarding, and misuse – is putting lives at risk from the new coronavirus and other infectious diseases (*46*). According to findings, before the COVID-19 pandemic, the only country producing the PPE in Africa is South Africa. The shortage of PPE has given a lot of local industries, uprising companies, and small-scale firms to start the production of the PPE without proper precautions. It is well known that Africa did not have a trade of PPE before now, and the shortage has made many to start production. This is risky because the person producing it might also be an asymptomatic COVID-19 patient or might have come in touch with someone with it. This will increase the rate of spread of this SARS-COV-2 and also increased the mortality rate (*46*). These make African more vulnerable to this disease than any other continent. The shortage may lead to a reduction in the workforce of the laboratory personnel to avoid their lives being endangered. It may also lead to the management of PPE without proper care and making more lives vulnerable to these diseases.

4.3. Transportation

Africa has a high risk for the introduction of the novel coronavirus disease 2019 (COVID-19), because of the high volume of air traffic (*via* importing) and trade between China and Africa (*3*). Based on their analysis, the WHO International Health Regulations Monitoring and Evaluation Framework; Egypt, Algeria, and South Africa had the highest importation risk and a moderate to high capacity to respond to outbreaks. Also, Nigeria, Ethiopia, Sudan, Angola, Tanzania, Ghana, and Kenya had medium risk with variable size and high vulnerability (*3*). In the model, the risk mainly originates from Guangdong, Fujian, and Beijing. The study illuminates a valuable tool that can help countries in Africa prioritize and allocate resources as they prepare to respond to the potential exposure and spread of COVID-19 (*9*). This might also be a severe implication to laboratory personnel because most of the laboratory tools are imported from distant countries, and even most of the countries the goods will pass through are on lockdown. There might be a need for the approval of high governance body to allow the movement of the products, and there might be a delay which will elongate the work of laboratory personnel (*18*). If there is damage in the laboratory kits, it may not be easy to repair because of transportation issues, and this will reduce the workforce of the laboratory personnel, lengthening diagnosis, and increase the spread. Transportation of laboratory samples will be one of the utmost problems facing Africa. There is a timeframe for transporting specimens. If the samples are placed outside the ideal temperature, the example no longer conforms to the standard, which may cause a problem when analyzing or interpreting the sample results (*9*).

4.4. Skilled laboratory personnel

In African countries, there is inadequate laboratory personnel that understand the use of COVID-19 laboratory diagnostic tools. Some of these countries did not have this equipment before the start of COVID-19 pandemics, and nations that have the equipment have few-laboratory personnel. So, there will be a need to provide more laboratory diagnostic tools with sufficient and quality laboratory personnel. This will allow a rush-training to employ more people to work as laboratory personnel. The training might not be adequate, because the rate of assimilation of everyone is different. Besides, this may pose a threat to the lives of people, and misinterpretation of laboratory results might be frequent (*47*).

4.5. Power supply

In some countries in Africa, there is limited or no power supply. This allows the laboratory to make use of the power generator, and the power generator may heighten risk because of its carbon monoxide emission. This will also cripple telecommunication and ICT services. These would also delay minute to minutes monitoring and reporting (*48*).

5. Conclusion

This study has shown that tackling COVID-19 pandemic in Africa requires effective laboratory diagnosis. However, as noted from this review Africa as a continent has depended majorly on only two laboratory diagnostic methods (RT-PCT and qRT-PCR) for detecting COVID-19 infection. This showed us a limitation to the effective elaborate laboratory diagnosis of the vast African populace. Specifically, the insufficiency in test kits, personal protective equipment, limited skilled personnel, inadequate power supply, and bad transportation system have all impaired the accurate diagnosis of COVID 19 infection. These challenges could have led to misdiagnosis of patients, slow rate of diagnosis of suspected cases, increased number of infected persons, inadequate treatment, and ultimately increased mortality rate.

Having noted all these challenges, it is therefore imperative that Africa responds quickly to provide sufficient standard laboratory diagnostic tools. Also, more diagnostic techniques should be employed as alternatives and support to the existing methods. Moreover, laboratory personnel should be adequately trained to execute accurate diagnosis. There should be improved remuneration for health workers generally and better funding of the health sector. These will help to reduce the spread of SARS-CoV-2, and the mortality rate will be reduced consequently.

Acknowledgements

We thank the scientists around the world for their hard work and vital information on the diagnosis of the COVID-19 infection in Africa laboratories. We also thank the frontline health worker for their sacrifices in serving humanity. Also, from our heart, we say a big thank you to Helix Biogen Consult for their support and the sponsorship of this project.

References

1. First Covid-19 case happened in November, China government records show – report. *https://www.theguardian.com/world/2020/mar/13/first-covid-19-case-happened-in-november-china-government-records-show-report* (accessed March 21, 2020).
2. Report of the WHO-China Joint Mission on coronavirus disease 2019 (COVID-19). *https://www.who.int/docs/default-source/coronaviruse/who-china-joint-mission-on-covid-19-final-report.pdf* (accessed March 25, 2020).
3. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y,

Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneu¬monia in Wuhan, China: A descriptive study. Lancet. 2020; 395:507-513.
4. Hong KH, Lee SW, Kim TS, *et al*. Guidelines for laboratory diagnosis of coronavirus disease 2019 (COVID-19) in Korea. Ann Lab Med. 2020; 40:351-360.
5. Zhou P, Yang XL, Wang XG, *et al*. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020; 579:270-273.
6. World Health Organization. Statement on the second meeting of the International Health Regulations (2005) Emergency Committee regarding the outbreak of novel coronavirus (2019-nCoV). *https://www.who.int/news-room/detail/30-01-2020-statement-on-the-second-meeting-of-the-international-health-regulations-(2005)-emergency-committee-regarding-the-outbreak-of-novel-coronavirus-(2019-ncov* (accessed February 1, 2020).
7. World Health Organization. Infection prevention and control during health care when COVID-19 is suspected. *https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200417-sitrep-88-covid-191b6ccc d94f8b4f219377bff55719a6ed.pdf?sfvrsn=ebe78315_6* (accessed April 20, 2020).
8. Kobia F, Gitaka J. COVID-19: Are Africa's diagnostic challenges blunting response effectiveness? AAS Open Res. 2020.
9. Nkengasong JN, Mankoula W. Looming threat of COVID-19 infection in Africa: Act collectively, and fast. Lancet. 2020; 395:841-842.
10. Nigeria Centre for Disease Control. Frequently ask questions on coronavirus. *https://ncdc.gov.ng/news/232/frequently-asked-questions-on-coronavirus* (accessed March 2, 2020).
11. Loeffelholz MJ, Tang YW. Laboratory diagnosis of emerging human coronavirus infections - the state of the art. Emerg Microbes Infect. 2020; 9:747-756.
12. Carter LJ, Garner LV, Smoot JW, Li Y, Zhou Q, Saveson CJ, Sasso JM, Gregg AC, Soares DJ, Beskid TR, Jervey SR, Liu C. Assay techniques and test development for COVID-19 diagnosis. ACS Cent Sci. 2020; 6:591-605.
13. To kk, Tsang OT, Leung WS, *et al*. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: An observational cohort study. Lancet Infect Dis. 2020; 20:565-574.
14. Udugama B, Kadhiresan P, Kozlowski HN, Malekjahani A, Osborne M, Li V, Chen H, Mubareka S, Gubbay JB, and. Chan WCW. Diagnosing COVID-19: The disease and tools for detection. ACS Nano. 2020; 14:3822-3835.
15. Bosch I, de Puig H, Hiley M, *et al*. Rapid antigen tests for dengue virus serotypes and Zika virus in patient serum. Sci Transl Med. 2017; 9:eaan1589.
16. Fang Y, Zhang H, Xie J, Lin M, Ying L, Pang P, Ji W. Sensitivity of chest C.T. for COVID-19: Comparison to RT-PCR. Radiology. 2020; 296:E115-E117.
17. Ai T, Yang Z, Hou H, Zhan C, Chen C, Lv W, Tao Q, Sun Z, Xia L. Correlation of chest C.T. and RT-PCR testing in coronavirus disease 2019 (COVID-19) in China: A report of 1014 cases. Radiology. 2020; 296:E32-E40.
18. Hope MD, Raptis CA, Shah A, Hammer MM, Henry TS, six signatories. A role for CT in COVID-19? What data really tell us so far. Lancet. 2020; 395:1189-1190.
19. Bernheim A, Mei X, Huang M, Yang Y, Fayad ZA, Zhang N, Diao K, Lin B, Zhu X, Li K, Li S, Shan H, Jacobi A, Chung M. Chest CT findings in coronavirus disease-19 (COVID-19): Relationship to duration of infection. Radiology. 2020; 29:200463.
20. Li X, Geng M, Peng Y, Meng L, Lu S. Molecular immune pathogenesis and diagnosis of COVID-19. J Pharm Anal. 2020; 10:102-108.
21. Scott mechler biosafety level guidance for COVID-19 research. *https://consteril.com/biosafety-level-guidance-covid-19-research/* (accessed March 21, 2020).
22. Centre for Disease Control and Prevention. *http://www.cdc.gov/coronavirus/2019-ncov/php/grows-virus-cell-culture.html* (accessed April 1, 2020).
23. Centre for Disease Control and Prevention. *http://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html* (accessed July 9, 2020).
24. Wang W, Tang J, Wei F. Updated understanding of the outbreak of 2019 novel coronavirus (2019-nCoV) in Wuhan, China. J Med Virol. 2020; 92:441-447.
25. Bustin SA, Mueller R. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. Clin Sci (Lond). 2005; 109:365-379.
26. Chu DKW, Pan Y, Cheng SMS, Hui KPY, Krishnan P, Liu Y, Ng DYM, Wan CKC, Yang P, Wang Q, Peiris M, Poon LLM. Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. Clin Chem. 2020; 66:549-555.
27. Tang YW, Schmitz JE, Persing DH, Stratton CW. Laboratory diagnosis of COVID-19: Current issues and challenges. J Clin Microbiol. 2020; 58:e00512- e00520.
28. Hughesman CB, Lu XJD, Liu KYP, Zhu Y, Poh CF, Haynes C. A robust protocol for using multiplexed droplet digital PCR to quantify somatic copy number alterations in clinical tissue specimens. PLoS One. 2016; 11:e0161274.
29. Yu F, Yan L, Wang N, Yang S, Wang L, Tang Y, Gao G, Wang S, Ma C, Xie R, Wang F, Tan C, Zhu L, Guo Y, Zhang F. Quantitative detection and viral load analysis of SARS-CoV-2 in infected patients. Clin Infect Dis. 2020; 71:793-798.
30. Huang JT, Liu YJ, Wang J, Xu ZG, Yang Y, Shen F, Liu XH, Zhou X, Liu SM. Next generation digital PCR measurement of hepatitis B virus copy number in formalin-fixed paraffin-embedded hepatocellular carcinoma tissue. Clin Chem. 2015; 61:290-296.
31. Neema Kaseje. Surgical system research group. *https://www.weforum.org/agenda/2020/03/why-sub-saharan-africa-needs-a-unique-response-to-covid-19/* (accessed April 21, 2020).
32. World Health Organization. Laboratory biosafety guidance. *https://apps.who.int/iris/bitstream/handle/10665/331138/WHO-WPW-GIH-2020* (accessed March 30, 2020).
33. Omaswa F. Solutions for Africa's health workforce crisis through country based research. Hum Resour Health. 2014; 12:I1.
34. Bronwyn and Neil Africa source. Barriers to mass testing for COVID-19 in Africa. *https://atlanticcouncil.org/blogs/africasource/barriers-to-mass-testing-for-covid-19-in-africa/* (accessed April 30, 2020).
35. Djibouti M, Gotsadze G, Mataradze G, Menabde G. Human resources for health challenges of public health system reform in Georgia. Hum Resour Health. 2008; 6:1-7.
36. Musango L, Elovainio R, Nabyonga J, Toure B. The state of health financing in the African Region. Afr Health

Monit. 2013; 1:16.
37. Roncarolo F, Boivin A, Denis JL, Hébert R, Lehoux P. What do we know about the needs and challenges of health systems? A scoping review of the international literature. BMC Health Serv Res. 2013; 17:636.
38. Oleribe OO, Udofia D, Oladipo O, Ishola TA, Taylor-Robinson SD. Healthcare workers' industrial action in Nigeria: A cross-sectional survey of Nigerian physicians. Hum Resour Health. 2018; 16:54.
39. Dr Jonas Nsenda Nkongolo. Infectious diseases. *https://www.afro.who.int/news/algerias-main-lab-anchors-covid-19-response* (accessed April 3,2020).
40. Dr Derrar. Algeria's main lab anchors COVID-19 response. *https://www.afro.who.int/news/algerias-main-lab-anchors-covid-19-response* (accessed April 3,2020).
41. Samuel Ayokunle. *https://theconversation.com/covid-19-nigeria-should-prioritise-power-supply-to-health-care-facilities-134444* (accessed April 3,2020).
42. World Health Organization. Global surveillance for human infection with a novel coronavirus. *https://www.who.int/publications-detail/global-surveillance-for-human-infection-with-novel-coronavirus-(2019-ncov)* (accessed April 3, 2020).
43. Shortage of personal protective equipment endangering health workers worldwide. *https://www.who.int/news-room/detail/03-03-2020-shortage-of-personal-protective-equipment-endangering-health-workers-worldwide* (accessed March 30, 2020).
44. Gilbert M, Pullano G, Pinotti F, Valdano E, Poletto C, Boëlle P-Y, D'Ortenzio E, Yazdanpanah Y, Eholie SP, Altmann M, Gutierrez B, Kraemer MUG, Colizza V. Preparedness and vulnerability of African countries against importations of COVID-19: A modelling study. Lancet. 2020; 395:871-877.
45. Dolf GF, Boshell D, gan D. Bazilian N, Wagner RG. The role of renewable energy in the global energy transformation. Energy Strateg Rev. 2019; 24:38-50.

Received August 5, 2020; Revised August 17, 2020; Accepted August 26, 2020.

Address correspondence to:
E. K. Oladipo, Department of Microbiology, Laboratory of Molecular Biology, Bioinformatics and Immunology, Adeleke University, Ede, Osun State, Nigeria; Genomics Unit, Helix Biogen Consult, Ogbomosho, Oyo State, Nigeria.
E-mail: oladipo.elijah@adelekeuniversity.edu.ng; koladipo2k3@yahoo.co.uk

Review

DOI: 10.5582/ddt.2020.03064

Association of ACE2 receptor and ACEIs/ARBs with disease severity in COVID-19

Shweta Sinha[1], Alka Sehgal[2], Rakesh Sehgal[1,*]

[1] Department of Medical Parasitology, PGIMER, Chandigarh, India;
[2] Department of Obstetrics & Gynecology, Government Medical College & Hospital Sector 32, Chandigarh, India.

SUMMARY Coronavirus disease 2019 (COVID-19) is found to be associated with various comorbidities which include cardiovascular diseases, hypertension, and diabetes. The impaired regulation of renin-angiotensin-aldosterone system (RAAS) has been seen in COVID-19 patients, but whether RAAS inhibitors, such as angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II type 1 receptor blockers (ARBs), are responsible for worsening of clinical conditions remains unknown. Herein, we review the role of angiotensin-converting enzyme 2 (ACE2) expression in disease progression, its association with comorbidities and COVID-19, and summarize the clinical evidence for several potential directions for future research work on ACEIs/ARBs in COVID-19 patients.

Keywords COVID-19, ACE2, comorbidities, ACEIs, ARBs

1. Introduction

The recent outbreak of the coronavirus disease 2019 (COVID-19) is a serious threat to the human population all along the globe. The COVID-19 cases were first reported in December 2019, in the region of Wuhan, China, then it spread rapidly worldwide, and meanwhile, COVID-19 became a global public health emergency of utmost concern (*1*). The causative agent has been spotted as a novel enveloped RNA beta-coronavirus and has been named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (*2*). The clinical outcome of SARS-CoV-2 infection is mainly distinguished by respiratory tract symptoms, which include dry cough, fever, fatigue, pharyngodynia, and problems associated with pneumonia and acute respiratory distress syndrome (*3*).

Population with all age groups is vulnerable to SARS-CoV-2, but the older age population and those with other comorbidities are found to be more prone to critical outcomes. According to current knowledge, the death rate seems to be higher in patients with severe underlying diseases (*4*). This includes cardiovascular diseases, hypertension, diabetes mellitus, chronic obstructive pulmonary disease (COPD), malignancy, and chronic kidney disease (*5*). The causality rate in COVID-19 patients was examined to be larger in patients with underlying diseases, *i.e.* cardiovascular disease (10.5%) and hypertension (6.0%) when compared with the normal population (2.3%) (*6*). Apart from this, diabetes is another common disease that is linked to the worst outcomes in COVID-19 and is often linked to hypertension and prescribed with renin-angiotensin-aldosterone system (RAAS) inhibitors (*7*). However, the explanation behind this observation is mostly unknown.

RAAS inhibitors are commonly prescribed drugs for various indications such as hypertension, myocardial infarction, cardiac failure, kidney diseases, and complications of diabetes all over the globe (*7*). Now, the use of RAAS inhibitors is under the controversial discussion of assumptions of two hypotheses (deleterious *vs.* protective) (*8*). In the "deleterious effect" hypothesis, RAAS inhibition leads to the upregulation of angiotensin-converting enzyme 2 (ACE2) expression at the cell surface, which will promote SARS-CoV-2 entry. In the "protective effect" hypothesis, RAAS inhibition will decrease the formation of angiotensin II, which would otherwise, upon SARS-CoV-2 binding, activate angiotensin II type I receptor (AT_1R), driving inflammation and fibrosis in the lung. In response to this discussion, the Council on Hypertension of the European Society of Cardiology made the following statement, "The Council on Hypertension strongly recommends that physicians and patients should continue treatment with their usual anti-hypertensive therapy because there is no clinical or scientific evidence to suggest that treatment with angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II type 1 receptor blockers (ARBs) should be discontinued because of the COVID-19 infection"

(*9*). The preceding statement has been then supported by the various numbers of distinguished societies, suggesting a continuation of the current medication regimen to the hypertensive patient with COVID-19. On March 17, 2020, the American Heart Association, the Heart Failure Society of America, and the American College of Cardiology put out a joint statement commending to continue ACEIs/ARBs as prescribed and any changes in medications in the setting of COVID-19 should be done only after careful evaluation (*10*). Therefore, prompt attention is needed to answer the existing cause of disease severity in COVID-19 patients having underlying diseases that would support in making timely clinical decisions (*11,12*).

Herein, we review the role of ACE2 expression in disease progression, its association with comorbidities and COVID-19, and summarize the clinical evidence for several potential directions for future research work on ACEIs/ARBs in COVID-19 patients.

2. Expression of ACE2 receptors

ACE2, a transmembrane protein having extracellular N-terminus and an intracellular C-terminus, primarily distributed on endothelial cells. Its catalytic site, similar to that of ACE, is mainly faced to distinct vasoactive peptides that are in circulation (*13*). The action of ACE2 is regulated by its expression pattern on the cell surface and also *via* its cleavage from the cell membrane. *ACE2* genes are located on the X chromosome (*14*), and its receptors are ubiquitous. ACE2 receptor expression is broadly observed in distinct human tissues such as lung, endothelium, heart, intestine, and kidney, which indicates the entry point for viruses like SARS-CoV and SARS-CoV-2 for their infection and multiplication (*15*). However, ACE2 expression varies and depends upon various factors such as age, gender, ethnicity, and physiological state, for example, more expression of ACE2 is observed in Asian as compared to white and African-American population (*16*). This differential expression is often detrimental to the severity of the diseases (Figure 1).

The expression of ACE2 in the lungs is found to be declined with the progress of the age (*17*) which is more to be in men as compared to women (*17*). Reduced expression of ACE2 has been linked with clinical conditions like diabetes mellitus which may be under the effect of glycosylation (*18-20*). Also, various clinical and experimental studies show the relevance of ACE2 deficiency due to either inhibition or deletion as a causative outcome for hypertension (*21,22*). Additionally, its deficiency is linked to aggravation of hypertension as well as cardiac hypertrophy, which is stimulated by angiotensin II (*23*) which is also responsible for remodeling of defective left ventricular after myocardial infarction (*24*). Furthermore, ACE2 deficiency enables the patients more vulnerable to cardiac failure (*21*). Deletion of the heterozygote *ACE2* gene is found to be sufficient in increasing the vulnerability to cardiac diseases (*25*). Besides this, deficiency of ACE2 is found to be one of the critical factors in the pathogenesis of SARS-CoV-2 infection. The ACE2 down-regulation stimulated with the viral invasion assumed to be detrimental particularly in patients with threshold ACE2 deficiency for example in case of old age population, or having hypertension, diabetes or prior cardiovascular diseases.

Moreover, over-expression of ACE2 is found to be preventive or can reverse the heart failure conditions (*26*). Modulation of ACE2 expression is seen with some etiological factors, like 1) Environmental conditions: This mainly includes polluted air which should be considered. The ACE2 activity was increased massively about 100 fold, in experimental models after

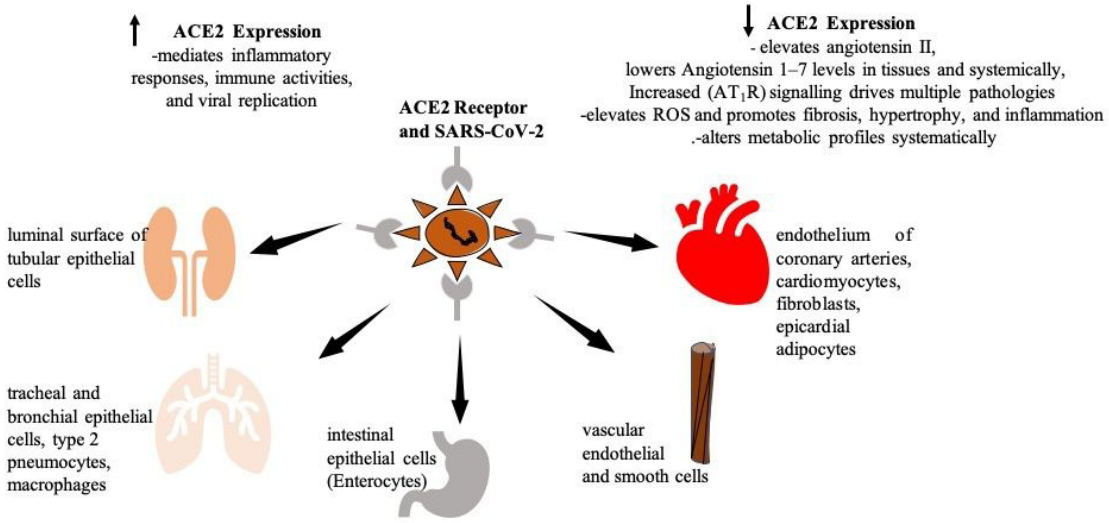

Figure 1. Expression of ACE2 receptors.

being exposed to NO_2, which concomitantly increases the binding of angiotensin II to its receptor (*27-29*). 2) Smokers: In the case of smokers, ACE2 is overexpressed on the epithelium of the lung airways, which has been observed previously in small animals, wherein exposure to smoke led to the upregulation of both the activity and the expression of ACE2 in the airways (*30,31*). Cai *et al.* recently elucidated over-expression of the *ACE2* gene in smoker groups juxtapose to non-smokers (*32*). Zhao *et al.* elicited clearly that the ACE2 is expressed in type-2 pneumocytes, wherein the genes controlling the viral multiplication and transmission are also highly expressed (*33*). Smoking has the ability to intensify the ACE2 receptor expression (*34*), active smoking and COPD leads to over-expression of ACE2 in lower airways, which to some extent may clarify the expanded threat of severity in COVID-19 patients. These observations feature the significance of withdrawal from smoking in these sub-populations, together with increased surveillance programs of these risk subgroups and rapid diagnosis would further prevent the infection from this virus (*35*). 3) Alcohol: Okuno *et al.* have detailed about the prolonged rise of the activity of serum ACE in alcoholics, and suggested that the levels of angiotensin II are increased because of incitement of ACE activity (*36,37*).

Nevertheless, during pregnancy, the ACE2 expression is markedly increased at the mRNA level (*38*). Li *et al.* showed profound expression of ACE2 in maternal-fetal interface cells. This includes perivascular cells of decidua and stromal cells, and cytotrophoblast and syncytiotrophoblast in placenta (*39*). The expression was also seen in particular types of cells in human fetal, *i.e.*, lung, liver, and heart but not seen in the kidney. This study elucidated about the widespread expression of ACE2 receptor in specific cell types of maternal-fetal interface and fetal organs which serve as an entry point for SARS-CoV-2 infection. The over-expression of ACE2 in these cells entails about the chances of placenta to be get infected by SARS-CoV-2 (*39*). Therefore, the illusion of any vertical transmission of the virus, pregnancy complication, and the placenta dysfunction or abortion due to SARS-CoV-2 must be clarified carefully with further investigation in clinical research and practice (*39*).

3. Association of ACE2 receptor in case of underlying disease or conditions in COVID-19

Various studies have given the factual basis of uses of ACE2 by the SARS-CoV and SARS-CoV-2 as a crucial cellular receptor to enable its infection. Both the viruses SARS-CoV-2 and SARS-CoV share 79% identity in nucleotide sequence (*40*). Studies have anticipated that the receptor-binding domain (RBD) of the spike glycoprotein present in both the viruses (SARS-CoV-2 and SARS-CoV) allocate almost similar conformation, and the association affinity between ACE2 and RBD was found to be much greater for SARS-CoV-2, on assessment with computer modeling and biophysical analysis (*41,42*).

Receptor binding is initiated *via* the viral spike protein, which is further processed by the TMPRSS2 protease (*43*). At this point, the spike protein associates with the extracellular domain of ACE2, which stimulates clathrin-dependent endocytosis of the complex (*43-45*). Curiously, this association is surprisingly improved in patients experiencing hypertension or coronary illness just as in diabetes or other comorbid conditions (*46-48*). After the entry of viruses, the viral RNA is discharged into the host-cell and further replicated by using the host cell's machinery to produce infectious virion which is released *via* exocytosis process. Moreover, apart from producing infectious virion, replication, and infection mechanism of SARS-CoV-2 also influences the ACE2 expression as well as its presentation. Receptor internalization leads to the decreased availability of these receptors for binding to the cell surface. The function of ACE2 is to degrade angiotensin I into angiotensin (1-7) peptides, which is responsible for lower blood pressure and vasodilation. The down-regulation of ACE2 disturbs the balance between ACE/angiotensin II and ACE2, however, the action of angiotensin II in the RAAS is increased due to the absence of antagonism, angiotensin (1-7) (*21*), shown in Figure 2. In order to know the significance of angiotensin II cleavage as a result of ACE2, there is need to understand the biological consequences of angiotensin II. Angiotensin II stimulates the release of hormone aldosterone and is a well-known vasoconstrictor. In various studies, angiotensin II has been observed for initiating a variety of adverse reactions consequences, like interstitial fibrosis, myocardial dysfunction and hypertrophy, endothelial malfunction, obesity-associated hypertension, enhanced inflammation, oxidative stress and blood coagulation (*15,21,44,49*). Moreover, angiotensin II upon binding to AT_1R exerts exacerbated pulmonary vascular permeability which results in increased hydrostatic pressure and finally pulmonary edema (*50*). In context to this, there are few experimental studies of lung injury that shows downregulation of ACE2 receptors stimulates inflammatory lesions in the tracheobronchial tree (thickening of alveolar walls, infiltrates of inflammatory cells, bleeding, edema) under the influence of angiotensin II (*51-54*). The significance of this RAAS activation as a causative factor of pulmonary edema in COVID-19 is still obscure (*29*). Nonetheless, a number of clinical observations are needed to decide whether the downregulated expression of ACE2 is a consequence of COVID-19 infection.

4. Role of ACEIs and ARBs in COVID-19

Obstruction of the RAAS is evinced to be favorable

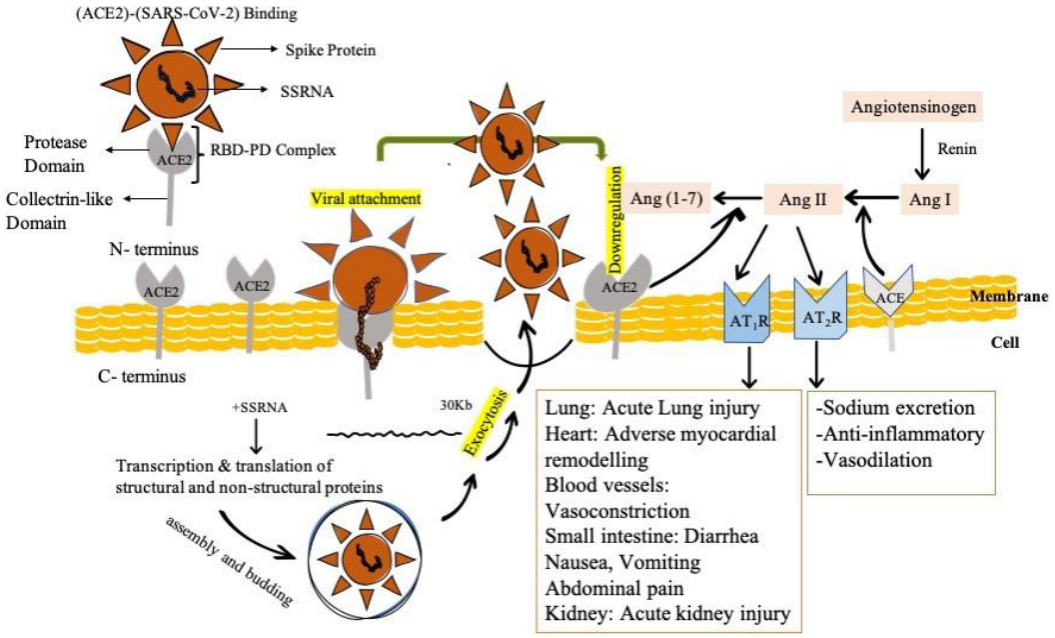

Figure 2. SARS-CoV-2 infection and its relation with renin angiotensin aldosterone system (RAAS). ACE2: angiotensin-converting enzyme 2; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; RBD: receptor binding domain; SSRNA: single stranded ribonucleic acid; Ang I: angiotensin I; Ang II: angiotensin II; Ang (1-7): angiotensin (1-7); AT_1R: angiotensin II type 1 receptor; AT_2R: angiotensin II type 2 receptor; ACE: angiotensin-converting enzyme.

in the restoration of kidney and cardiac function in the patients with clinical conditions such as kidney diseases, cardiac failure, and post-myocardial infarction (*55,56*). The two agents, ARBs and ACEIs are considered first-choice drugs in hypertension, heart failure, post-myocardial infarction, and chronic kidney disease and also increases the expression of ACE2 (*57*). ACEIs consist of a particular class of antihypertensive medication which is mainly marked for cardiac failure, post-myocardial infarction, asymptomatic left ventricular dysfunction, diabetic nephropathy, and proteinuria. ACEIs leads to vasodilation by reducing the production of angiotensin II (*58,59*). Furthermore, the activity of ACEIs is found to be high in the lungs due to the profound expression of ACE (*51*). So, during lung associated complications, administration of ACEIs leads in the augmented release of bradykinin in the lungs, which are mostly deteriorated by ACEIs. This may sensitize the airways and increases the cough reflex (*60,61*).

Moreover, ARBs act on the RAAS pathway through effective blocking of AT_1R and was initially developed for the treatment of hypertension. ARBs display pleiotropic defending effects as it is helpful in directly reducing the endothelial injury, organ fibrosis and inflammation, maintain mitochondrial activity, energy metabolism, and insulin sensitivity. It also protects the excessive metabolism of lipids and harmonize the coagulation processes (*62,63*). All these processes are the clinical features that are observed in a recovered patient with acute critical disorders (*62,63*). Because of these facts, ARBs are widely prescribed as first- line antihypertensives treatment options as well as for the treatment of other diseases like cardiovascular diseases, diabetes and kidney disease. Moreover, ARBs have the ability to decrease inflammation and debilitated epithelial and endothelium of various organs, which has been seen in various viral infections where ARBs protect the integrity of lung endothelial barrier directly that occurred due to acute injury (*64*). There is also considerable clinical evidence that shows straight consequences of ARB medication, which protects the lung from severe injury linked to sepsis, pneumonia sepsis, and influenza and reduces the mortality (*64*).

COVID-19 patients with hypertension, diabetes, and cardiovascular diseases (among other underlying disease conditions that are often treated with these agents) have been reported to have the highest case fatality rates (*6,65*). The ACEIs/ARBs treatment which has the ability to induce exacerbated ACE2 expression, a receptor for SARS-CoV-2 entry in COVID-19 patients, assumes to be having a controversial action because of reducing inflammatory responses in context to pathogen and at the same facilitating the virus entry (*66*). Moreover, the major concern is about ACEIs and ARBs treatment, which increases the severity as well as the mortality of COVID-19 patients by 2-fold. One assumption is that ACEIs could straight inhibit ACE2; however, ACE2 functions as a carboxypeptidase and is not hindered by clinically recommended ACEIs (*67*). Moreover, the topic is controversial and debatable, with a limited number of clinical evidence, shown in Table 1, which mostly suggest continuation of these medications till date.

Table 1. Studies showing use of ACEIs/ARBs in COVID-19

Authors	Hypothesis/Aim	Patient enrolled	Results/Outcome	Conclusion/ recommendation on use or not use of ACEIs or ARBs	Limitation
Bean *et al.*, 2020 (*68*)	"To evaluate the association between treatment with ACEI or ARB and disease severity in COVID-19 patients".	Multicentre retrospective study -cohort of 205 acute inpatients with COVID-19, 53 patients out of 205 patients reached the primary endpoint and out of this only 9 patients were taking ARBs.	Individuals diagnosed with hypertension had a similar likelihood of developing severe disease as those that were not diagnosed with hypertension.	Recommended for use of ARB and ACEIs	Relatively small sample taken from a single UK centre over a short follow-up.
Guo *et al.*, 2020 (*69*)	"To evaluate the association of underlying cardiovascular disease and myocardial injury with fatal outcomes in patients with COVID-19".	Single-centre, retrospective, observational study of 187 patients, 66 (35.3%) had underlying cardiovascular disease including hypertension, coronary heart disease, and cardiomyopathy, and 52 (27.8%) exhibited myocardial injury as indicated by elevated Troponin T levels.	The mortality rates of patients with and without use of angiotensin-converting enzyme inhibitors/angiotensin receptor blockers was 36.8% (7 of 19) and 25.6% (43 of 168).	Recommended for use of ARB and ACEIs in COVID-19 patients	Limited number of patients, the mortality of those treated with or without use of ACEIs/ARBs did not show a significant difference in outcome. -a larger cohort study is needed for further verification.
Li *et al.*, 2020 (*70*)	"To investigate the association between ACEIs/ARBs and severity of illness and mortality in patients with hypertension hospitalized for COVID-19 infection".	Retrospective, single-centre case series of the 1178 hospitalized patients with COVID-19 infections -362 patients with hypertension and of whom 115 were taking ACEIs/ARBs.	Data provide some reassurance that ACEIs/ ARBs are not associated with the progression or outcome of COVID-19 hospitalizations in patients with hypertension.	Data support current guidelines and societal recommendations for treating hypertension during the COVID-19 pandemic.	Enrolment of small number of patients with hypertension taking ACEIs/ARBs who were hospitalized with COVID-19. -biasness in maintenance of ACEIs/ ARBs treatment at baseline.
Liu *et al.*, 2020 (*71*)	"To investigate any difference in disease severity between COVID-19 patients with hypertension comorbidity".	Multicentre retrospective study, -clinical data of 511 COVID-19 patients -Patients were categorized into six sub-groups of hypertension comorbidity based on treatment using one of anti-hypertension drugs (ACEI, ARB, CCB, BB, thiazide), or none.	Elderly (age > 65) COVID-19 patients with hypertension comorbidity who were taking ARB anti-hypertension drugs may be less likely to develop severe lung disease compared to patients who were not taking anti-hypertension drugs.	Data support various society guidelines to continue current treatment of chronic disease conditions with either ACEIs/ARBs during the COVID-19 pandemic.	Limited number of sample. -lack of statistical difference between Contol and studied population
Mehta *et al.*, 2020 (*72*)	"To clarify the potential association of use of angiotensin-converting enzyme inhibitors (ACEI) and angiotensin II receptor blockers (ARB) with testing positive for coronavirus disease 2019 (COVID-19)".	Retrospective prospective, observational study, -Includes 18,472 patients who underwent COVID-19 testing, 2,285 (12.4%) were taking ACEIs or ARBs.	Taking either an ACEI or ARB was not associated with an increase in the likelihood of testing positive for SARS-CoV-2 infection (overlap propensity score-weighted odds ratio, 0.97; 95% CI, 0.81-1.15).	These data support various society guidelines to continue current treatment of chronic disease conditions with either ACEI or ARB during the COVID-19 pandemic.	The data did not include information on duration of ACEI or ARB use before or after testing. Limited to particular races/ ethnicities.

Table 1. Studies showing use of ACEIs/ARBs in COVID-19 (continued)

Authors	Hypothesis/Aim	Patient enrolled	Results/Outcome	Conclusion/ recommendation on use or not use of ACEIs or ARBs	Limitation
Meng *et al.*, 2020 (*73*)	"To evaluate the ability of RAAS inhibitors to protect against COVID-19 in patients with hypertension".	Single-centre, retrospective study. -42 patients receiving antihypertensive therapy were included and were divided: the ACEI/ARB group (17 patients) included patients treated with ACEI or ARB drugs, and the non-ACEI/ARB group (25 patients) included patients treated with other antihypertensive drugs, including calcium channel blockers (CCBs), β-blockers and diuretics.	First clinical evidence demonstrating that RAAS inhibitors improve the clinical outcomes of COVID-19 patients with hypertension.	Data support various society guidelines to continue current treatment of chronic disease conditions with either ACEIs/ ARBs during the COVID-19 pandemic.	Small sample size and selection bias.
Shi *et al.*, 2020 (*74*)	To explore the association between cardiac injury and mortality in patients with COVID-19.	The study population included 416 patients hospitalized with confirmed COVID-19: 82 patients (19.7%) with cardiac injury and 334 patients (80.3%) without cardiac injury.	ACEIs/ARBs was not included in the study.	Study demonstrates the statistically significant association between cardiac injury and mortality in patients with COVID-19. However, the mechanism of cardiac injury among these patients with COVID-19 was not revealed.	Larger populations and multiple centres are warranted to further confirm the outcomes of cardiac injury in COVID-19. Disease is not study to look into role of ARBS/ACEIS in COVID-19.
Wei *et al.*, 2020. (*75*)	"To explore the prevalence and immediate clinical implications of acute myocardial injury in a cohort of patients with COVID-19".	Single-centre, observational study -101 Covid-19 patients.	Explanations was hypothetical, and did not provide any clue to justify alteration of ACEIs/ARBs treatment in patients with COVID-19	Not specified	Limited with a small sample size, unable to draw definitive conclusions and acknowledge the existence of selection bias as complete documentation of exposure history and laboratory testing was not available for every patient, and echocardiography was only performed for clinical expediency.
Yang *et al.*, 2020 (*66*)	"To evaluate the correlation of ARBs/ACEIs usage with the pathogenesis of COVID-19 patients with pre-existing hypertension".	Retrospective, single-center study. -126 COVID-19 patients allocated to ARBs/ ACEIs group (n = 43) and non-ARBs/ ACEIs group (n = 83) according to their antihypertensive medication.	Within the hypertension group, patients on ARBs/ACEIs had a much lower proportion of critical patients and a lower death rate than those on non-ARBs/ACEIs medications, although these differences failed to reach statistical significance.	Finding of the study support the use of ARBs/ACEIs in COVID-19 patients with pre-existing hypertension.	Interpretation of findings was limited by the sample size and selection bias.

Table 1. Studies showing use of ACEIs/ARBs in COVID-19 (continued)

Authors	Hypothesis/Aim	Patient enrolled	Results/Outcome	Conclusion/ recommendation on use or not use of ACEIs or ARBs	Limitation
Zeng *et al.*, 2020 (*76*)	"To investigate and compare the demographic characteristics, coexisting diseases, severity of pneumonia, and the effect of antihypertensive drugs (ACEI/ ARB versus non-ACEI/ARB) in COVID-19 patients with coexisting hypertension".	Retrospective, Single-centre, observational study. -included data from all patients with clinically confirmed COVID-19. A total of 274 patients, 75 with hypertension and 199 without hypertension, were included in the analysis.	Patients with hypertension who had previously taken ACEI/ARB drugs for antihypertensive treatment have an increased tendency to develop severe pneumonia after infection with SARS-COV-2 ($p = 0.064$).	Not specified	The number of patients included was small.
Zhang *et al.*, 2020 (*77*)	"To determine the association between in-hospital use of ACEI/ ARB and all-cause mortality in COVID-19 patients with hypertension".	Retrospective, multi-centre study, included 1128 adult patients with hypertension diagnosed with COVID-19, including 188 taking ACEI/ARB (ACEI/ARB group; and 940 without using ACEI/ARB (non-ACEI/ ARB group.	The detected risk for all-cause mortality was lower in the ACEI/ARB group versus the non-ACEI/ARB group (adjusted HR, 0.42; 95% CI, 0.19-0.92; $p = 0.03$).	Recommended for use of ARB and ACEIs in COVID-19	Modest sample-size who received ACEI/ARB. -few parameters were not available in all patients, and in-hospital medications might be not fully recorded.

5. Conclusion

Patients taking common hypertensive medications were not found to be at higher risk for infection with SARS-CoV-2, or to be more likely to have severe complications from COVID-19. However, this could be one of the active areas of research and proper clinical trials. The published clinical evidence is limited by various confounding factors, like small sample size, selection bias, the inappropriate association of data, time period of ACEIs or ARBs administration prior to or after testing, *etc.* Moreover, result interpretation is also subjected to a different grade of variations between countries as a result of insufficient testing of COVID-19 patients and differences in disease prevalence (*78*). Moreover, few points that need to be considered from various studies in this context are:

(1) The *ACE2* gene is subjected to a larger degree of polymorphism and significantly associated with the phenomenon of arterial hypertension which has been observed in Han Chinese men and in women of diverse races and ethnicity (*29,79*). In addition to this, ACE2 polymorphism is also linked to susceptibility to diabetes mellitus, ventricular hypertrophy cerebral stroke, septal wall thickness, and coronary artery disease mainly in the Asian population (*29*). Nonetheless, any possible association of ACE2 polymorphism with regards to differences in vulnerability to COVID-19 infection is still needed to be answered.

(2) ACE2 binds more efficiently (about > 10-20 fold) to SARS-CoV-2 than to SARS-CoV. This data, along with the other interpretation, like people of diverse races and ethnicity, with different sexes and ages were all vulnerable to SARS-CoV-2 infection, which entails that expression of ACE2 inside the human body under normal physiological condition may be sufficient enough to contract SARS-CoV-2 infection, and additional upregulation would not be the reason for increased risk or severity (*66*).

(3) The RAAS inhibitors instead of inhibiting viral replication directly; there may be chances that they display their antiviral role through an indirect effect of inflammatory responses and immune regulation (*77*). Large prospective studies are needed to validate and explore this preliminary finding for further studies that will focus on the mechanism of action of ACEIs/ARBs to control the inflammatory response.

(4) A randomized controlled clinical trial evaluating the role of ACEIs/ARBs is the need of the situation, before reaching to any interpretation regarding the likely benefit of these drugs in COVID-19 patients. Moreover, the trial will also be beneficial in the futuristic approach in tackling such a pandemic in different geographical regions. Together, these efforts may lead to finding of some new therapeutic molecule that will target ACE2 in COVID-19 patients with other comorbidities.

References

1. Wang C, Horby PW, Hayden FG, Gao GF. A novel coronavirus outbreak of global health concern. Lancet. 2020; 395:470-473.
2. Lu R, Zhao X, Li J, *et al.* Genomic characterisation and epidemiology of 2019 novel coronavirus: Implications for virus origins and receptor binding. Lancet. 2020; 395:565-574.
3. Huang C, Wang Y, Li X, *et al.* Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497-506.
4. Sun K, Chen J, Viboud C. Early epidemiological analysis of the coronavirus disease 2019 outbreak based on crowdsourced data: A population-level observational study. Lancet Digit Health. 2020; 2:e201-e208.
5. Emami A, Javanmardi F, Pirbonyeh N, Akbari A. Prevalence of underlying diseases in hospitalized patients with COVID-19: A systematic review and meta-analysis. Arch Acad Emerg Med. 2020; 8:e35.
6. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: Summary of a report of 72,314 cases from the Chinese center for disease control and prevention. JAMA. 2020.
7. de Abajo FJ, Rodríguez-Martín S, Lerma V, *et al.* Use of renin-angiotensin-aldosterone system inhibitors and risk of COVID-19 requiring admission to hospital: A case-population study. Lancet. 2020; 395:1705-1714.
8. South AM, Tomlinson L, Edmonston D, Hiremath S, Sparks MA. Controversies of renin-angiotensin system inhibition during the COVID-19 pandemic. Nat Rev Nephrol. 2020; 16:305-307.
9. European Society of Cardiology. Position statement of the ESC Council on Hypertension on ACE-inhibitors and angiotensin receptor blockers. Published March 13, 2020. *https://www.escardio.org/Councils/Council-on-Hypertension-(CHT)/News/position-statement-of-the-esc-council-on-hypertension-on-ace-inhibitors-and-ang* (accessed May 15, 2020).
10. Angiotensin converting enzyme Inhibitors and angiotensin Receptor Blockers. What is the evidence? American Heart Association. HFSA/ACC/AHA statement addresses concerns re: using RAAS antagonists in COVID-19. *https://professional.heart.org/professional/ScienceNews/UCM_505836_HFSAACCAHA-statement-addresses-concerns-re-using-RAAS-antagonists-in-COVID-19.jsp* (accessed March 20, 2020).
11. Kwok KO, Li KK, Chan HHH, Yi YY, Tang A, Wei WI, Wong SYS. Community Responses during Early Phase of COVID-19 Epidemic, Hong Kong. Emerg Infect Dis. 2020; 26:1575-1579.
12. Groß S, Jahn C, Cushman S, Bär C, Thum T. SARS-CoV-2 receptor ACE2-dependent implications on the cardiovascular system: From basic science to clinical implications. J Mol Cell Cardiol. 2020; 144:47-53.
13. Warner FJ, Lew RA, Smith AI, Lambert DW, Hooper NM, Turner AJ. Angiotensin-converting enzyme 2 (ACE2), but not ACE, is preferentially localized to the apical surface of polarized kidney cells. J Biol Chem. 2005; 280:39353-39362.
14. Crackower MA, Sarao R, Oudit GY, *et al.* Angiotensin-converting enzyme 2 is an essential regulator of heart function. Nature. 2002; 417:822-828.
15. Zhang H, Penninger JM, Li Y, Zhong N, Slutsky AS. Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: molecular mechanisms and potential therapeutic target. Intensive Care Med. 2020; 46:586-590.
16. Batlle D, Wysocki J, Satchell K. Soluble angiotensin-converting enzyme 2: A potential approach for coronavirus infection therapy? Clin Sci (Lond). 2020; 134:543-545.
17. Xie X, Chen J, Wang X, Zhang F, Liu Y. Age- and gender-related difference of ACE2 expression in rat lung. Life Sci. 2006; 78:2166-2171.
18. Pal R, Bhansali A. COVID-19, diabetes mellitus and ACE2: The conundrum. Diabetes Res Clin Pract. 2020; 162:108132.
19. Tikellis C, Thomas MC. Angiotensin-converting enzyme 2 (ACE2) is a key modulator of the renin angiotensin system in health and disease. Int J Pept. 2012; 2012:256294.
20. Yamagata R, Nemoto W, Nakagawasai O, Takahashi K, Tan-No K. Downregulation of spinal angiotensin converting enzyme 2 is involved in neuropathic pain associated with type 2 diabetes mellitus in mice. Biochem Pharmacol. 2020; 174:113825.
21. Patel VB, Zhong JC, Grant MB, Oudit GY. Role of the ACE2/angiotensin 1-7 axis of the renin-angiotensin system in heart failure. Circ Res. 2016; 118:1313-1326.
22. Patel SK, Velkoska E, Freeman M, Wai B, Lancefield TF, Burrell LM. From gene to protein-experimental and clinical studies of ACE2 in blood pressure control and arterial hypertension. Front Physiol. 2014; 5:227.
23. Zhong J, Basu R, Guo D, Chow FL, Byrns S, Schuster M, Loibner H, Wang XH, Penninger JM, Kassiri Z, Oudit GY. Angiotensin-converting enzyme 2 suppresses pathological hypertrophy, myocardial fibrosis, and cardiac dysfunction. Circulation. 2010; 122:717-728.
24. Kassiri Z, Zhong J, Guo D, Basu R, Wang X, Liu PP, Scholey JW, Penninger JM, Oudit GY. Loss of angiotensin-converting enzyme 2 accelerates maladaptive left ventricular remodeling in response to myocardial infarction. Circ Heart Fail. 2009; 2:446-455.
25. Wang W, Patel VB, Parajuli N, Fan D, Basu R, Wang Z, Ramprasath T, Kassiri Z, Penninger JM, Oudit GY. Heterozygote loss of ACE2 is sufficient to increase the susceptibility to heart disease. J Mol Med (Berl). 2014; 92:847-858.
26. Guo J, Huang Z, Lin L, Lv J. Coronavirus disease 2019 (COVID-19) and cardiovascular disease: A viewpoint on the potential influence of angiotensin-converting enzyme inhibitors/angiotensin receptor blockers on onset and severity of severe acute respiratory syndrome coronavirus 2 infection. J Am Heart Assoc. 2020; 9:e016219.
27. Meulenbelt J, van Bree L, Dormans JA, Boink AB, Sangster B. Biochemical and histological alterations in rats after acute nitrogen dioxide intoxication. Hum Exp Toxicol. 1992; 11:189-200.
28. Patel JM, Sekharam KM, Block ER. Oxidant injury increases cell surface receptor binding of angiotensin II to pulmonary artery endothelial cells. J Biochem Toxicol. 1990; 5:253-258.
29. Alifano M, Alifano P, Forgez P, Iannelli A. Renin-angiotensin system at the heart of COVID-19 pandemic. Biochimie. 2020; 174:30-33.
30. Hung YH, Hsieh WY, Hsieh JS, Liu FC, Tsai CH, Lu LC, Huang CY, Wu CL, Lin CS. Alternative roles of STAT3 and MAPK signaling pathways in the MMPs activation and progression of lung injury induced by cigarette smoke exposure in ACE2 knockout mice. Int J Biol Sci. 2016;

12:454-465.
31. Yilin Z, Yandong N, Faguang J. Role of angiotensin-converting enzyme (ACE) and ACE2 in a rat model of smoke inhalation induced acute respiratory distress syndrome. Burns. 2015; 41:1468-1477.
32. Cai G. Tobacco-use disparity in gene expression of ACE2, the receptor of 2019-nCov. Preprint. 2020.
33. Zhao Y, Zhao Z, Wang Y, Zhou Y, Ma Y, Zuo W. Single-cell RNA expression profiling of ACE2, the putative receptor of Wuhan 2019-nCov. bioRxiv. 2020.
34. Brake SJ, Barnsley K, Lu W, McAlinden KD, Eapen MS, Sohal SS. Smoking upregulates angiotensin-converting enzyme-2 receptor: A potential adhesion site for novel coronavirus SARS-CoV-2 (Covid-19). J Clin Med. 2020; 9:841.
35. Leung JM, Yang CX, Tam A, Shaipanich T, Hackett TL, Singhera GK, Dorscheid DR, Sin DD. ACE-2 expression in the small airway epithelia of smokers and COPD patients: Implications for COVID-19. Eur Respir J. 2020; 55:2000688.
36. Okuno F, Arai M, Ishii H, Shigeta Y, Ebihara Y, Takagi S, Tsuchiya M. Mild but prolonged elevation of serum angiotensin converting enzyme (ACE) activity in alcoholics. Alcohol. 1986; 3:357-359.
37. Husain K, Ansari RA, Ferder L. Alcohol-induced hypertension: Mechanism and prevention. World J Cardiol. 2014; 6:245-252.
38. Brosnihan KB, Neves LA, Anton L, Joyner J, Valdes G, Merrill DC. Enhanced expression of Ang-(1-7) during pregnancy. Braz J Med Biol Res. 2004; 37:1255-1262.
39. Li M, Chen L, Zhang J, Xiong C, Li X. The SARS-CoV-2 receptor ACE2 expression of maternal-fetal interface and fetal organs by single-cell transcriptome study. PLoS One. 2020; 15:e0230295.
40. Wang H, Li X, Li T, Zhang S, Wang L, Wu X, Liu J. The genetic sequence, origin, and diagnosis of SARS-CoV-2. Eur J Clin Microbiol Infect Dis. 2020.
41. Li F. Structure, function, and evolution of coronavirus spike proteins. Annu Rev Virol. 2016; 3:237-261.
42. Gui M, Song W, Zhou H, Xu J, Chen S, Xiang Y. Cryo-electron microscopy structures of the SARS-CoV spike glycoprotein reveal a prerequisite conformational state for receptor binding. Cell Res. 2017; 27:119-129.
43. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell. 2020; 181:271-280.
44. Turner AJ, Hiscox JA, Hooper NM. ACE2: From vasopeptidase to SARS virus receptor. Trends Pharmacol Sci. 2004; 25:291-294.
45. Chen Y, Guo Y, Pan Y, Zhao ZJ. Structure analysis of the receptor binding of 2019-nCoV. Biochem Biophys Res Commun. 2020; 525:135-140.
46. Buhl KB, Oxlund CS, Friis UG, Svenningsen P, Bistrup C, Jacobsen IA, Jensen BL. Plasmin in urine from patients with type 2 diabetes and treatment-resistant hypertension activates ENaC in vitro. J Hypertens. 2014; 32:1672-1677.
47. Svenningsen P, Hinrichs GR, Zachar R, Ydegaard R, Jensen BL. Physiology and pathophysiology of the plasminogen system in the kidney. Pflugers Arch. 2017; 469:1415-1423.
48. Zheng H, Liu X, Sharma NM, Li Y, Pliquett RU, Patel KP. Urinary proteolytic activation of renal epithelial Na^+ channels in chronic heart failure. Hypertension. 2016; 67:197-205.
49. Kuba K, Imai Y, Penninger JM. Multiple functions of angiotensin-converting enzyme 2 and its relevance in cardiovascular diseases. Circ J. 2013; 77:301-308.
50. Cha SA, Park BM, Kim SH. Angiotensin-(1-9) ameliorates pulmonary arterial hypertension *via* angiotensin type II receptor. Korean J Physiol Pharmacol. 2018; 22:447-456.
51. Kuba K, Imai Y, Rao S, *et al.* A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. Nat Med. 2005; 11:875-879.
52. Imai Y, Kuba K, Rao S, *et al.* Angiotensin-converting enzyme 2 protects from severe acute lung failure. Nature. 2005; 436:112-116.
53. Hung YH, Hsieh WY, Hsieh JS, Liu FC, Tsai CH, Lu LC, Huang CY, Wu CL, Lin CS. Alternative roles of STAT3 and MAPK signaling pathways in the MMPs activation and progression of lung injury induced by cigarette smoke exposure in ACE2 knockout mice. Int J Biol Sci. 2016; 12:454-465.
54. Lin CI, Tsai CH, Sun YL, Hsieh WY, Lin YC, Chen CY, Lin CS. Instillation of particulate matter 2.5 induced acute lung injury and attenuated the injury recovery in ACE2 knockout mice. Int J Biol Sci. 2018; 14:253-265.
55. Lin SY, Lin CL, Lin CC, Hsu WH, Lin CD, Wang IK, Hsu CY, Kao CH. Association between angiotensin-converting enzyme inhibitors and lung cancer-a nationwide, population-based, propensity score-matched cohort study. Cancers (Basel). 2020; 12:747.
56. Makani H, Bangalore S, Desouza KA, Shah A, Messerli FH. Efficacy and safety of dual blockade of the renin-angiotensin system: Meta-analysis of randomised trials. BMJ. 2013; 346:f360.
57. Saleem TS, Bharani K, Gauthaman K. ACE inhibitors-angiotensin II receptor antagonists: A useful combination therapy for ischemic heart disease. Open Access Emerg Med. 2010; 2:51-59.
58. Amann B, Tinzmann R, Angelkort B. ACE inhibitors improve diabetic nephropathy through suppression of renal MCP-1. Diabetes Care. 2003; 26:2421-2425.
59. Herman LL, Padala SA, Annamaraju P, Bashir K. Angiotensin Converting Enzyme Inhibitors (ACEI). StatPearls. *https://www.ncbi.nlm.nih.gov/books/NBK431051/* (accessed August 15, 2020).
60. Fox AJ, Lalloo UG, Belvisi MG, Bernareggi M, Chung KF, Barnes PJ. Bradykinin-evoked sensitization of airway sensory nerves: A mechanism for ACE-inhibitor cough. Nat Med. 1996; 2:814-817.
61. Cassone A, Gucciardo D, Cauda R. A call to research: The relationship between SARS-2-CoV, ACE 2 and antihypertensives. Pathog Glob Health. 2020; 114:165-167.
62. Gurwitz D. Angiotensin receptor blockers as tentative SARS-CoV-2 therapeutics. Drug Dev Res. 2020; 81:537-540.
63. Fedson DS. Treating the host response to emerging virus diseases: Lessons learned from sepsis, pneumonia, influenza and ebola. Am Transl Med. 2016; 4:421.
64. Saavedra JM. Angiotensin receptor blockers and COVID-19. Pharmacol Res. 2020; 156:104832.
65. Li B, Yang J, Zhao F, Zhi L, Wang X, Liu L, Bi Z, Zhao Y. Prevalence and impact of cardiovascular metabolic diseases on COVID-19 in China. Clin Res Cardiol. 2020; 109:531-538.

66. Yang G, Tan Z, Zhou L, Yang M, Peng L, Liu J, Cai J, Yang R, Han J, Huang Y, He S. Effects of angiotensin II receptor blockers and ACE (angiotensin-converting enzyme) inhibitors on virus infection, inflammatory status, and clinical outcomes in patients with COVID-19 and hypertension: A single-center retrospective study. Hypertension. 2020; 76:51-58.
67. Rice GI, Thomas DA, Grant PJ, Turner AJ, Hooper NM. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. Biochem J. 2004; 383:45-51.
68. Bean DM, Kraljevic Z, Searle T, Bendayan R, Kevin O, Pickles A, Folarin A, Roguski L, Noor K, Shek A, Zakeri R, Shah AM, Teo JTH, Dobson RJB. Angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers are not associated with severe COVID-19 infection in a multi-site UK acute hospital trust. Eur J Heart Fail. 2020; 22:967-974.
69. Guo T, Fan Y, Chen M, Wu X, Zhang L, He T, Wang H, Wan J, Wang X, Lu Z. Cardiovascular Implications of Fatal Outcomes of Patients With Coronavirus Disease 2019 (COVID-19). JAMA Cardiol. 2020; 5:1-8.
70. Li J, Wang X, Chen J, Zhang H, Deng A. Association of renin-angiotensin system inhibitors with severity or risk of death in patients with hypertension hospitalized for coronavirus disease 2019 (COVID-19) infection in Wuhan, China. JAMA Cardiol. 2020; 5:1-6.
71. Liu Y, Huang F, Xu J, *et al.* Anti-hypertensive angiotensin II receptor blockers associated to mitigation of disease severity in elderly COVID-19 patients. medRxiv. 2020.
72. Mehta N, Kalra A, Nowacki AS, Anjewierden S, Han Z, Bhat P, Carmona-Rubio AE, Jacob M, Procop GW, Harrington S, Milinovich A, Svensson LG, Jehi L, Young JB, Chung MK. Association of use of angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers with testing positive for coronavirus disease 2019 (COVID-19). JAMA Cardiol. 2020.
73. Meng J, Xiao G, Zhang J, He X, Ou M, Bi J, Yang R, Di W, Wang Z, Li Z, Gao H, Liu L, Zhang G. Renin-angiotensin system inhibitors improve the clinical outcomes of COVID-19 patients with hypertension. Emerg Microbes Infect. 2020; 9:757-760.
74. Shi S, Qin M, Shen B, Cai Y, Liu T, Yang F, Gong W, Liu X, Liang J, Zhao Q, Huang H, Yang B, Huang C. Association of cardiac injury with mortality in hospitalized patients with COVID-19 in Wuhan, China. JAMA Cardiol. 2020; 5:802-810.
75. Wei JF, Huang FY, Xiong TY, *et al.* Acute myocardial injury is common in patients with covid-19 and impairs their prognosis. Heart. 2020; 106:1154-1159.
76. Zeng Z, Sha T, Zhang Y, Wu F, Hu H, Li H, Han J, Song W, Huang Q, Chen Z. Hypertension in patients hospitalized with COVID-19 in Wuhan, China: A single-center retrospective observational study. medRxiv. 2020.
77. Zhang P, Zhu L, Cai J, Han J, Song W, Huang Q, Chen Z. Association of inpatient use of angiotensin converting enzyme inhibitors and angiotensin II receptor blockers with mortality among patients with hypertension hospitalized with COVID-19. Circ Res. 2020.
78. Groß S, Jahn C, Cushman S, Bär C, Thum T. SARS-CoV-2 receptor ACE2-dependent implications on the cardiovascular system: From basic science to clinical implications. J Mol Cell Cardiol. 2020; 144:47-53.
79. Zhang Q, Cong M, Wang N, Li X, Zhang H, Zhang K, Jin M, Wu N, Qiu C, Li J. Association of angiotensin-converting enzyme 2 gene polymorphism and enzymatic activity with essential hypertension in different gender: A case-control study. Medicine (Baltimore). 2018; 97:e12917.

Received August 4, 2020; Revised August 17, 2020; Accepted August 26, 2020

Address correspondence to:
Rakesh Sehgal, Department of Medical Parasitology & Chairperson Group D Departments, Postgraduate Institute of Medical Education & Research, Chandigarh, India.
E-mail: sehgalpgi@gmail.com

Review

DOI: 10.5582/ddt.2020.03059

Review of current clinical management guidelines for COVID-19 with special reference to India

Rohit Kumar[1], Kutty Sharada Vinod[2], Ankit Mittal[1], Shreya Das Adhikari[3], Nitin Gupta[4,*]

[1] Department of Medicine, All India Institute of Medical Sciences, New Delhi, India;
[2] Department of Pulmonary Medicine, All India Institute of Medical Sciences, New Delhi, India;
[3] Departments of Oncoanaesthesia and Palliative Medicine, Dr Bhim Rao Ambedkar Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, New Delhi, India;
[4] Department of Infectious Diseases, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, Karnataka, India.

SUMMARY The healthcare sector has been overwhelmed by the global rise in the number of COVID-19 cases. The primary care physicians at the forefront of this pandemic are being provided with multiple guidelines (state, national, international). The aim of this review was to examine the existing guidelines for congruence and critically analyze them in light of current evidence. A discordance was noted between the national and state guidelines with respect to indication, duration and dosage of antivirals, steroids/immunomodulators, anticoagulation and convalescent plasma. The lack of concordance between various guidelines mandates the need for a unified national guideline that is regularly updated.

Keywords Coronavirus disease 19, hydroxychloroquine, remdesivir, dexamethasone, tocilizumab, convalescent plasma

1. Introduction

The current coronavirus disease 2019 (COVID-19) pandemic has put a challenging situation in front of an already overburdened health care system with an inadequate doctor to patient ratio. The primary care physicians are at the front line of this pandemic and are not only dealing with an increased number of patients but are also facing difficulties in the management of critically ill patients. To fill the current knowledge gap and guide the management plans of primary care physicians, several guidelines have been published all over the world. In a review by Kow *et al.*, inconsistencies and lack of consensus have been reported in recommendations given by several global bodies (*1*). The aim of this review was to compare and critically analyze the current existing guidelines for congruency.

2. Review strategy

The websites of Infectious Disease Society of America (*2*), World Health Organisation (*3*) and the Ministry of Health and Family Welfare of the central and state governments of India (*4*) were searched for the last available guidelines on COVID-19 as on 25.06.2020. The guidelines from the following states were available- Kerala (*5*), Karnataka, Maharashtra and West Bengal (*6*). These guidelines were reviewed independently by three reviewers. The data extracted from these guidelines are compiled according to severity (mild, moderate, severe) and are discussed under the following headings (Table 1).

3. Anticoagulation

Concerns on the possible role of coagulopathy in moderate-to-severe COVID-19 were raised in the initial reports from China. Raised D-Dimer was found as one of the predictors of mortality (*7*). A descriptive study by Carsana *et al.*, on post-mortem lung specimens of COVID-19 cases revealed the presence of microthrombi (*8*). Similar observations were reported in other histopathological studies as well (*9*). Based on these observations, a possible role of anticoagulation in prophylactic or therapeutic dose in moderate to severe illness has been proposed. However, the evidence suggesting the benefit of anticoagulant therapy is still weak. A retrospective observational study by Tang *et al.* revealed no overall benefit of heparin on 28-day-mortality. However, when adjusted for D-Dimer values, heparin prophylaxis was associated with a

Table 1. Comparison of national and state management guidelines (as on 25.06.2020)

National/ state (DOP)	Anti-coagulation	Antivirals	Steroids/ immunomodulators	Antibiotic at presentation	Convalescent plasma
			Mild- fever, cough, malaise, rhinorrhea, sore throat without shortness of breath		
WHO (27.05)	No recommendation	Not recommended outside clinical trial	Not recommended	Not recommended	Not recommended outside clinical trial
IDSA (25.06)	No recommendation	Not recommended outside clinical trial	Not recommended	No recommendation	Not recommended outside clinical trial
India (13.06)	Not recommended	HCQ recommended if high risk features*	Not recommended	Not recommended	Not recommended
Maharashtra (22.06)	If high risk features#- LMWH OD	If high risk#-HCQ or Favipiravir	No recommendation	If high risk#- Cefixime or Amoxicillin-clavulanate	No recommendation
Karnataka (15.05)	LMWH OD (If d–dimer > 1000ng/ml or ground glass opacities)	Oseltamivir plus Azithromycin plus HCQ	Not recommended	Not recommended	Not recommended
Kerala (24.03)	Not recommended	Not recommended	Not recommended	Not recommended	Not recommended
West Bengal	No recommendation	HCQ for high risk**	No recommendation	No recommendation	No recommendation
			Moderate- Pneumonia with Spo2 90-94% on room air		
WHO (27.05)	No recommendation	Not recommended outside clinical trial	Tocilizumab not recommended outside clinical trial. Steroids not recommended	Not recommended	Not recommended outside clinical trial
IDSA (25.06)	No recommendation	Not recommended outside clinical trial	Tocilizumab not recommended outside clinical trial. Steroids use recommended (Dexa 6 mg daily or equivalent)	No recommendation	Not recommended outside clinical trial
India (13.06)	LMWH OD	HCQ and/or remdesivir for those on oxygen	If oxygen requirement progressively increasing MPS 0.5 – 1 mg/Kg for 3 days If not improving on steroids-Tocilizumab	Older people, immunocompromised patients, and children < 5 years of age	If oxygen requirement progressively increasing despite use of steroids
Maharashtra (22.06)	LMWH OD	HCQ or favipiravir or remdesivir If HCQ contraindicated – Ivermectin plus doxycycline	MPS 0.5-1 mg/Kg for 3 days followed by oral pred tapered over 5 days If MPS not available, Dexa 6 mg OD for 10 days If oxygen requirement progressively increasing-Tocilizumab	Ceftriaxone	1.If P/F between 200-300 OR 2.Respiratory rate > 24/min, Sao2 < 93% on room air

Abbreviations: DOP, date of publication in date/month format; LMWH, low molecular weight heparin; HCQ, hydroxychloroquine; CQ, chloroquine; LPV/r, lopinavir/ritonavir; OD, once daily; BD, twice daily; Dexa, dexamethasone; Pred, prednisolone; MPS, methylprednisolone; 3GC, 3rd generation cephalosporins; AG, aminoglycosides; PT, piperacillin-tazobactam; NAC, N-acetyl cysteine; ULN, upper limit of normal; ARDS, acute respiratory distress syndrome. #High risk features, obesity, age > 60 yrs, DM, HTN, COPD/chronic lung disease, immunocompromised state, CKD. **Patients with age > 60 years; chronic lung diseases; chronic liver disease; chronic kidney disease; hypertension; cardiovascular disease; cerebrovascular disease; diabetes; HIV; cancers; on immunosuppressive drugs.

Table 1. Comparison of national and state management guidelines (as on 25.06.2020) (continued)

National/ state (DOP)	Anti-coagulation	Antivirals	Steroids/ immunomodulators	Antibiotic at presentation	Convalescent plasma
Karnataka (15.05)	LMWH OD	Oseltamivir plus Azithromycin plus HCQ	No recommendation	Recommended according to local antibiogram	Not recommended
Kerala (24.03)	No recommendation	HCQ plus Azithromycin plus Oseltamavir	Systemic steroids and Tocilizumab not recommended.	Not recommended	Not recommended
West Bengal	LMWH OD (if d-dimer > 6ULN)	HCQ plus Oseltamavir (when suspected)	Progressive deterioration of oxygenation, rapid worsening on imaging- MPS 1- 2mg/kg/day X 3-5 days. Tocilizumab - If IL-6 is more than 5 times of ULN	3GC / PT / Carbapenem+/- AG + Azithromycin	No recommendation on CP (Recommends therapeutic plasma exchange)
			Severe- pneumonia with Spo2 < 90% in room air AND Critical- ARDS, hypotension, worsening mental status, MODS		
WHO (27.05)	LMWH OD	Antivirals not recommended outside clinical trial	Tocilizumab not recommended outside clinical trial. Steroids not recommended	Not recommended	Not recommended outside clinical trial
IDSA (25.06)	No recommendation	Remdesivir recommended	Tocilizumab not recommended outside clinical trial. Steroids use recommended (Dexa 6 mg daily or MPS 40 mg or Pred 32 mg)	No recommendation	Not recommended outside clinical trial
India (13.06)	LMWH BD	HCQ use is to be avoided	MPS 1-2 mg/Kg for 5-7 days. No recommendation on tocilizumab	Older people, immunocompromised patients, and children < 5 years of age	No recommendation
Maharashtra (22.06)	LMWH OD If D-Dimer is raised 3-fold, then BD	Remdesivir may be considered	MPS 0.5-1 mg/Kg for 5-7 days and duration can be extended based upon D-dimer. If MPS not available, Dexamethasone 6 mg OD for 10 days. Tocilizumab can be considered	Meropenem or as per local antibiotic policy	May be considered
Karnataka (15.05)	LMWH BD days	Oseltamivir plus Azithromycin plus HCQs. Lopinavir/ritonavir on compassionate grounds. Remdesivir if patient progress to ARDS or septic shock.	Tocilizumab and/or steroids- if patient progresses to ARDS or septic shock Sepsivac recommended in septic shock	Ceftriaxone	Can be tried if patient progress to ARDS or septic shock
Kerala (24.03)	No recommendation	Oseltamivir plus Azithromycin plus HCQ. LPV/r can be added if HCQ contraindicated or progressive disease	Steroids for septic shock, macrophage activation syndrome. For grade 3 or 4 cytokine release syndrome- Tocilizumab (or steroids)	Not recommended	No recommendation
West Bengal			Same as moderate		

Abbreviations: DOP, date of publication in date/month format; LMWH, low molecular weight heparin; HCQ, hydroxychloroquine; CQ, chloroquine; LPV/r, lopinavir/ritonavir; OD, once daily; BD, twice daily; Dexa, dexamethasone; Pred, prednisolone; MPS, methylprednisolone; 3GC, 3rd generation cephalosporins; AG, aminoglycosides; PT, piperacillin-tazobactam; NAC, N-acetyl cysteine; ULN, upper limit of normal; ARDS, acute respiratory distress syndrome. #High risk features, obesity, age > 60 yrs, DM, HTN, COPD/chronic lung disease, immunocompromised state, CKD. **Patients with age > 60 years; chronic lung diseases; chronic liver disease; chronic kidney disease; hypertension; cardiovascular disease; cerebrovascular disease; diabetes; HIV; cancers; on immunosuppressive drugs.

statistically significant reduction in 28-day-mortality (*10*). There is a need to assess the safety and efficacy of anticoagulation in large scale prospective studies (*11*). Based on these observations, a possible role of anticoagulation has been proposed in patients requiring oxygen therapy. While most guidelines recommend the use of anticoagulation in patients with moderate/severe disease, there are some differences with respect to indication for a higher prophylactic dose (Table 1). Although the evidence to use anticoagulants in mild disease is lacking, some of the state guidelines have recommended thromboprophylaxis in this category as well (Table 1).

4. Antivirals

4.1. Hydroxychloroquine (HCQ)

HCQ was touted as the wonder drug at the beginning of the pandemic based on small observational studies. Although a recent study from Michigan showed decreased mortality in the HCQ arm, several confounders were not taken into account (*12*). Based on the preliminary results of the RECOVERY and SOLIDARITY trial, the HCQ arm was discontinued in both the trials. In a randomized controlled trial, HCQ did not substantially reduce the severity of symptoms in patients with mild COVID-19 (*13*). Studies have also highlighted that adverse events such as arrhythmia are higher in the HCQ arm when compared to standard of care. Based on some observational studies, it was initially recommended to combine azithromycin with HCQ for a potential synergistic effect; however, it did not stand the test of time (*14*). This combination may be potentially harmful because of the increased risk of QT prolongation. An observational study published from India showed that although the use of HCQ prophylaxis, on the whole, was not associated with decreased infection rate, > 4 doses were associated with a decreased chance of infection (*15*). A randomized controlled trial showed that receipt of post-exposure prophylaxis with HCQ within four days of exposure did not decrease infection rates (*16*). Despite the lack of evidence, the national and state guidelines have retained HCQ with/without azithromycin as the preferred antiviral for clinical management in mild with/without risk factors and moderate illness. While the national guideline mentions that HCQ should be avoided in severe cases, other states continue to recommend and enforce the use of HCQ (Table 1).

4.2. Favipiravir

Favipiravir is an RNA polymerase inhibitor that has been marketed in India for treatment of mild COVID-19. A very small study compared favipiravir versus lopinavir/ritonavir in patients with the non-severe disease and showed faster viral and radiographic clearance in patients on favipiravir (*17*). Although this study mandates need for larger studies, the evidence to use outside the purview of a clinical trial is not there.

4.3. Lopinavir/ritonavir

Lopinavir/ritonavir is a protease inhibitor that was initially tried for use in patients with SARS-COV-2. However, a randomized trial of 199 patients with severe COVID-19 showed that it did not help in improving the clinical outcomes and was also removed from the SOLIDARITY trial (*18*). Despite a lack of clear evidence, lopinavir/ritonavir has been recommended by some of the state guidelines.

4.4. Remdesivir

Remdesivir is a novel nucleotide analogue that is recommended for use in patients with COVID-19 requiring oxygen. In a multinational, randomized, placebo-controlled trial of around a thousand patients with confirmed COVID-19 pneumonia, remdesivir resulted in a faster time to recovery. There was a trend towards lower 14-day mortality in patients in the remdesivir arm (*19*). In another study with a smaller sample size from China, time to clinical improvement and mortality was not statistically different with remdesivir compared with placebo. However, in patients who received remdesivir within ten days of symptom onset, there was a trend to shorter time to improvement and lower mortality rates (*20*). While the national and some state guidelines have incorporated remdesivir into their management algorithms, some states are yet to update theirs.

4.5. Others

Despite very poor evidence that supports the use of agents like doxycycline and ivermectin, they have found a place in management algorithms and clinical practice. It is therefore important that management guidelines are updated frequently and are based on the most recent scientific evidence.

5. Steroids

The controversy around the use of steroids was somewhat mitigated with results of RECOVERY trial. Dexamethasone (6 milligrams once daily for ten days) was found to be useful as it decreased 28-day mortality in patients requiring oxygen or invasive ventilation. It did not show any benefit in patients not requiring oxygen (*21*). No increase in the adverse effects or secondary infections was noted with dexamethasone. While most guidelines have adopted steroids in clinical management, there is wide variability in the formulation

(dexamethasone, methylprednisolone, hydrocortisone), dose (as high as 2 milligrams per kilograms of methylprednisolone) and duration of steroids, creating a lot of confusion.

6. Immunomodulators

6.1. Tocilizumab

Tocilizumab is an interleukin-6 receptor inhibitor that is now being used for the management of hyper-inflammation in COVID. Pending randomized controlled trial, some observational studies have shown some benefit with tocilizumab. In a study of patients with severe COVID-19, tocilizumab was associated with a reduced risk of invasive mechanical ventilation or death (*22*). In another study of ventilated patients with COVID-19, tocilizumab was associated with lower mortality (*23*). Both the studies were associated with higher rates of superinfection. Although tocilizumab remains an investigational therapy, it has been recommended by some of the guidelines (Table 1).

6.2. Sepsivac

Sepsivac is a heat-killed *Mycobacterium W* that was developed as an immunomodulator for treating gram-negative sepsis. It has been proposed as a drug for COVID-19, but there are no published studies supporting this claim. However, it has been incorporated into the Karnataka state guidelines for patients of COVID-19 progressing into septic shock without much justification.

7. Antimicrobials

Although a possible coinfection in a patient with proven COVID-19 cannot be ruled out without doing microbiological tests, preliminary data indicates that coinfection with other pathogen is not very common in patients with COVID-19 (*24*). Blanket antibiotics in all patients with COVID-19 seems to be counterintuitive and goes against the principle of antimicrobial stewardship. Antibiotics should be used only in patients where bacterial infection is suspected (*e.g.* shock, new-onset consolidation after improvement, *etc.*). Empiric antibiotics, when initiated, should be promptly de-escalated based on microbiological cultures and/or negative procalcitonin reports. Despite India being a hub of antimicrobial resistance, several guidelines have recommended the use of prophylactic antibiotics in patients with moderate and severe disease (Table 1).

8. Convalescent plasma

Convalescent plasma has received much attention from the media and the public. It has been tried in the past for the treatment of various infectious diseases where no definitive therapy was available. It acts by acting as a source of neutralizing antibodies and inhibiting the replication of the virus (*25*). Since no single effective antiviral therapy is available, it may be a potentially effective therapeutic option for COVID-19. Use of convalescent plasma in the current pandemic is guided by experience from previous influenza and coronavirus epidemics (*26*). There has been limited published data for its use in COVID-19, mostly in the form of case reports or case series (*27-29*). In a Cochrane analysis, no conclusion could be drawn on the overall efficacy of convalescent plasma (*30*). Pending the results of few ongoing large-scale trials, most guidelines support the use of convalescent plasma only as an investigational therapy.

9. Conclusion

Due to the sheer numbers of people affected by the illness, the systems are moulding themselves and evolving in order to rise to the challenges thrown at them by the pandemic. In such a situation, having state, national and international guidelines that do not agree on recommendations can cause undue confusion. There is a need to have a unifying national guideline with ongoing appraisal and recommendations that are updated on the basis of evolving data. Similar incongruencies should be identified and addressed at the level of other countries as well to aid the clinical practice of physicians globally.

References

1. Kow CS, Capstick T, Zaidi STR, Hasan SS. Consistency of recommendations from clinical practice guidelines for the management of critically ill COVID-19 patients. Eur J Hosp Pharmt. 2020.
2. Bhimraj A, Morgan RL, Shumaker AH, *et al.* Infectious diseases society of America guidelines on the treatment and management of patients with COVID-19. Clin Infect Dis. 2020.
3. Clinical management of COVID-19. *https://www.who.int/publications-detail-redirect/clinical-management-of-covid-19* (accessed July 23, 2020).
4. RevisedNationalClinicalManagementGuidelineforCOVID1931032020.pdf. *https://www.mohfw.gov.in/pdf/RevisedNationalClinicalManagementGuidelineforCOVID1931032020.pdf* (accessed July 23, 2020).
5. interim_24032020.pdf. *http://dhs.kerala.gov.in/wp-content/uploads/2020/03/interim_24032020.pdf* (accessed July 23, 2020).
6. Management_Protocol_for_COVID-19_-_WB_2nd_Edition.pdf. *https://www.wbhealth.gov.in/uploaded_files/corona/Management_Protocol_for_COVID-19_-_WB_2nd_Edition.pdf* (accessed July 23, 2020).
7. Zhou F, Yu T, Du R, *et al.* Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: A retrospective cohort study. Lancet. 2020; 395:1054-1062.

8. Carsana L, Sonzogni A, Nasr A, *et al.* Pulmonary post-mortem findings in a series of COVID-19 cases from northern Italy: A two-centre descriptive study. Lancet Infect Dis. 2020.
9. Menter T, Haslbauer JD, Nienhold R, *et al.* Postmortem examination of COVID-19 patients reveals diffuse alveolar damage with severe capillary congestion and variegated findings of lungs and other organs suggesting vascular dysfunction. Histopathology. 2020.
10. Tang N, Bai H, Chen X, Gong J, Li D, Sun Z. Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. J Thromb Haemost. 2020; 18:1094-1099.
11. Marietta M, Vandelli P, Mighali P, Vicini R, Coluccio V, D'Amico R. Randomised controlled trial comparing efficacy and safety of high versus low Low-Molecular Weight Heparin dosages in hospitalized patients with severe COVID-19 pneumonia and coagulopathy not requiring invasive mechanical ventilation (COVID-19 HD): A structured summary of a study protocol. Trials. 2020; 21:574.
12. Arshad S, Kilgore P, Chaudhry ZS, Jacobsen G, Wang DD, Huitsing K, Brar I, Alangaden GJ, Ramesh MS, McKinnon JE, O'Neill W, Zervos M. Treatment with hydroxychloroquine, azithromycin, and combination in patients hospitalized with COVID-19. Int Infect Dis. 2020; 97:396-403.
13. Skipper CP, Pastick KA, Engen NW, *et al.* Hydroxychloroquine in nonhospitalized adults with early COVID-19: A randomized trial. Ann of Intern Med. 2020.
14. Gautret P, Lagier JC, Parola P, *et al.* Hydroxychloroquine and azithromycin as a treatment of COVID-19: Results of an open-label non-randomized clinical trial. Int J Antimicrob Agents. 2020; 56:105949.
15. Chatterjee P, Anand T, Singh KJ, Rasaily R, Singh R, Das S, Singh H, Praharaj I, Gangakhedkar RR, Bhargava B, Panda S. Healthcare workers & SARS-CoV-2 infection in India: A case-control investigation in the time of COVID-19. Indian J Med Res. 2020; 151:459-467.
16. Boulware DR, Pullen MF, Bangdiwala AS, *et al.* A randomized trial of hydroxychloroquine as postexposure prophylaxis for Covid-19. N Engl J Med. 2020; 383:517-525.
17. Cai Q, Yang M, Liu D, *et al.* Experimental treatment with favipiravir for COVID-19: An open-label control study. Engineering (Beijing). 2020.
18. Cao B, Wang Y, Wen D, *et al.* A trial of lopinavir-ritonavir in adults hospitalized with severe Covid-19. N Engl J Med. 2020; 382:1787-1799.
19. Beigel JH, Tomashek KM, Dodd LE, *et al.* Remdesivir for the treatment of Covid-19-preliminary report. N Engl J Med. 2020.
20. Wang Y, Zhang D, Du G, *et al.* Remdesivir in adults with severe COVID-19: A randomized, double-blind, placebo-controlled, multicentre trial. Lancet. 2020; 395:1569-1578.
21. Group RC, Horby P, Lim WS, *et al.* Dexamethasone in hospitalized patients with Covid-19-preliminary report. N Engl J Med. 2020.
22. Guaraldi G, Meschiari M, Cozzi-Lepri A, *et al.* Tocilizumab in patients with severe COVID-19: A retrospective cohort study. Lancet Rheumatol.2020; 2:e474-e484.
23. Somers EC, Eschenauer GA, Troost JP, *et al.* Tocilizumab for treatment of mechanically ventilated patients with COVID-19. medRxiv. 2020.
24. Rawson TM, Moore LS, Zhu N, Ranganathan N, Skolimowska K, Gilchrist M, Satta G, Cooke G, Holmes A. Bacterial and fungal co-infection in individuals with coronavirus: A rapid review to support COVID-19 antimicrobial prescribing. Clin Infect Dis. 2020.
25. Rojas M, Rodríguez Y, Monsalve DM, Acosta-Ampudia Y, Camacho B, Gallo JE, Rojas-Villarraga A, Ramírez-Santana C, Díaz-Coronado JC, Manrique R, Mantilla RD, Shoenfeld Y, Anaya JM. Convalescent plasma in Covid-19: Possible mechanisms of action. Autoimmun Rev. 2020; 19:102554.
26. Zhu T, Xu A, Bai X, He Y, Zhang H. Effect of convalescent plasma and immunoglobulin on patients with severe acute respiratory syndrome: A systematic review. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue. 2020; 32:435-438. (in Chinese).
27. Salazar E, Perez KK, Ashraf M, *et al.* Treatment of coronavirus disease 2019 (COVID-19) patients with convalescent plasma. Am J Pathol. 2020; 190:1680-1690.
28. Zeng QL, Yu ZJ, Gou JJ, Li GM, Ma SH, Zhang GF, Xu JH, Lin WB, Cui GL, Zhang MM, Li C, Wang ZS, Zhang ZH, Liu ZS. Effect of convalescent plasma therapy on viral shedding and survival in patients with coronavirus disease 2019. J Infect Dis. 2020; 222:38-43.
29. Duan K, Liu B, Li C, *et al.* Effectiveness of convalescent plasma therapy in severe COVID-19 patients. Proc Natl Acad Sci U S A. 2020; 117:9490-9496.
30. Valk SJ, Piechotta V, Chai KL, Doree C, Monsef I, Wood EM, Lamikanra A, Kimber C, McQuilten Z, So-Osman C, Estcourt LJ, Skoetz N. Convalescent plasma or hyperimmune immunoglobulin for people with COVID-19: A rapid review. Cochrane Database Syst Rev. 2020; 5:CD013600.

Received July 25, 2020; Revised August 12, 2020; Accepted August 26, 2020

**Address correspondence to:*
Nitin Gupta, Department of Infectious Diseases, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, Karnataka-576104, India.
E-mail: nityanitingupta@gmail.com

Original Article

DOI: 10.5582/ddt.2020.03061

A simple artificial diet available for research of silkworm disease models

Atmika Paudel[1], Suresh Panthee[1], Hiroshi Hamamoto[1], Kazuhisa Sekimizu[1,2,*]

[1] Teikyo University Institute of Medical Mycology, Tokyo, Japan;
[2] Genome Pharmaceuticals Institute Co., Ltd., Tokyo, Japan.

SUMMARY This study was performed with the aim of making a very simple recipe of silkworm diet for research purposes, especially screening of drug candidates. We prepared a diet containing mulberry leaves powder and soybean flour at different ratios, fed them to fifth instar silkworm larvae, and observed their growth. We selected the diet with 1:1 ratio of mulberry powder and soybean flour, named MS-11, and used for further experiments. MS-11 diet was available for oral administration of drugs in silkworm hyperglycemic model and infection model. The availability of a simple artificial diet for experiments that require feeding silkworms will enhance the use of silkworms for biological, biotechnological, and pharmacological researches.

Keywords Silkworm, artificial diet, mulberry, research

1. Introduction

Silkworm (*Bombyx mori*) is a lepidopteran invertebrate that has been domesticated for silk production with a long history of breeding (*1*). Apart from using them for silk, silkworm larvae have gained attention as animal model of various human diseases. The basic common pathways shared by silkworms with mammals, their body features and size and ease in handling, and less ethical concerns make silkworms appropriate for research purposes before moving on to higher animal models (*2*).

The fact that human pathogenic microorganisms kill silkworms and clinically used antibiotics cure those infections with comparable effective dose fifty (ED_{50}) values (*3*) to those of mammals make silkworm suitable for using as an infection model. The activation of innate immunity and response towards infection (*4*) makes them suitable for the study of host-pathogen interactions. Further, similarity in pharmacokinetics (*5,6*) and toxicities (*7*) of compounds make them more reliable and appropriate for using them to identify lead molecules that have therapeutic effect and a potential to further towards clinical applications.

Silkworms have been applied for the screening of novel antimicrobial agents with novel mechanism of actions from natural product (*8*) and chemical libraries (*9-11*), for screening of novel virulence factors of pathogenic microorganisms (*12,13*). The elevation of glucose in silkworm blood after ingestion of glucose and sucrose diet and lowering of this level by clinically used anti-diabetic drugs (*14*) makes them suitable for screening of compounds that may be applied as anti-diabetes agents. The fate of compounds after ingestion such as bioavailability, absorption, distribution, metabolism, and excretion can be studied as silkworms have basic metabolic pathway common to mammals with involvement of cytochrome P450 enzyme for metabolism (*5*). On the basis of pharmacokinetic parameters, therapeutic activities of compounds with similar *in vitro* activities could be differentiated by evaluation with silkworms (*11*). Therefore, silkworm larvae are appropriate for pharmacological researches.

Usually, silkworms are reared on mulberry leaves as they have a selective preference over it. As mulberry leaves are not available all year round, several artificial diets for silkworm rearing are commercially available. There have been several studies to establish artificial diets for silkworms focusing on improving the silk production (*15-17*). However, diets that can be easily prepared and suitable for establishing silkworms as disease models are lacking. In our laboratory, we have been routinely using artificial diet for research purposes. The comparison of the artificial diet with mulberry leaves on infection model of silkworm showed that the killing ability of a Gram-positive bacteria, *Staphylococcus aureus*, was same regardless of the diet fed (*18*).

Even when mulberry leaves are available for silkworm rearing, some disease models require the use of artificial diets, especially in conditions where silkworms must be fed with substances under study.

For instance, when screening for blood glucose level lowering agents, silkworms should be fed high glucose/sucrose diet, when determining oral toxicity of compounds, the compounds must be fed to silkworms. In such experiments, it is necessary that silkworms feed on the substances and this in only possible when artificial diet is available.

In this study, we established a simple artificial diet, MS-11, for pharmacological and biological experiments requiring feeding silkworms. The establishment of such diet will help researchers around the world to prepare their own artificial diet in the laboratories and perform desired researches. This will expand the use of silkworms in research which is limited to the laboratories where rearing silkworms in artificial diets is a common practice.

2. Materials and Methods

2.1. Preparation of artificial diet

Artificial diets were prepared by mixing five different proportions of mulberry leaves powder (Healthy Company, Japan) and soybean flour, and agar (Nacalai, Japan) in 100 mL water (Figure 1A). They were autoclaved at 121°C for 15 minutes, mixed and kept at 4°C for use. Ready to use artificial diet, Silkmate 2S diet, was purchased from Nosan Corporation, Japan.

2.2. Rearing of silkworms

Silkworms were reared according to previously described methods (*8,10*). Briefly, hatched larvae from silkworm eggs (Hu·Yo × Tukuba·Ne; Ehime Sanshu), were fed artificial diet, SilkMate 2S (Nihon Nosan Co., Ltd., Kanagawa, Japan) until 4th or 5th instar larvae as mentioned. Silkworms were maintained at 27°C all the time.

2.3. Measurement of glucose level in silkworm hemolymph

The 4th instar larvae were fed Silkmate diet or MS-11 diet until they become 5th instar larvae. The 5th instar larvae weighing between 0.9 to 1.1 g were starved overnight. Next day, the larvae were fed respective diets with or without 10% sucrose (w/w) (Fujifilm wako, Japan). Acarbose (80 mg/g, LKT laboratories Inc., USA), a known clinically used antidiabetic drug, was mixed with the 10% sucrose containing diets as a positive control. After 1 h of feeding, hemolymph was collected by cutting the abdominal prolegs, and glucose level was measured using a glucometer (Accu-Chek: Roche, Basel, Switzerland).

2.4. Determination of oral therapeutic activity of antibiotics

The 5th instar silkworm larvae were fed overnight with MS-11 diet. *Staphylococcus aureus* MSSA1 was grown in sheep blood agar plate at 37°C overnight, inoculated in tryptic soy broth, and grown at 37°C with shaking at 200 rpm for 18 hours. The full growth was 10-fold diluted with 0.9% saline, 50 µL of which was injected into the hemolymph of each silkworm larva. The infected larvae were fed with MS-11 diet containing various concentrations of chloramphenicol or tetracycline. The survival was observed and recorded for each dose when all the silkworms fed with no antibiotic diet died. ED_{50} values were calculated by logistic regression analysis using the logit link function.

3. Results

3.1. Growth of silkworms depends upon the component of artificial diet

We prepared five different artificial diets with varying proportions of mulberry leaves powder and soybean flour (Figure 1A). We fed 1 g of these diets to each of the 5th instar first day silkworm larvae and observed their growth. We found that the larvae preferred the diets containing mulberry leaves powder. Among the

A.

Diet	Ingredients per 100 mL water		
	Mulberry powder	Soybean flour	Agar
A	20 g	0 g	2.5 g
B	16 g	4 g	2.5 g
C	10 g	10 g	2.5 g
D	4 g	16 g	2.5 g
E	0 g	20 g	2.5 g
F	Silkmate diet		

B.

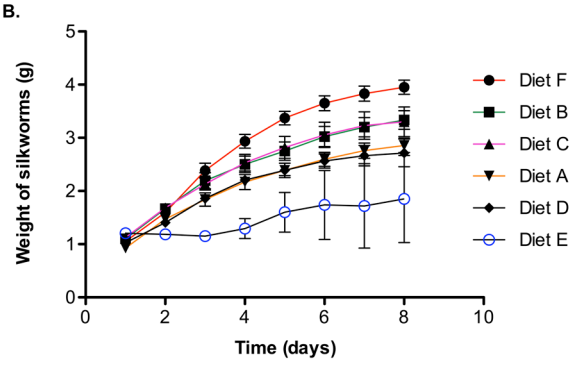

Figure 1. Silkworm artificial diet and growth of silkworm larvae. (A) Composition of the artificial diets used in this study. Diets were prepared as mentioned in Materials and Methods section. **(B)** Growth of the artificial diet fed silkworm larvae. One gram of each diet was fed per larva per day and remaining diets were removed each day. Growth of each larva was recorded each day for 8 days and data is shown as mean ± SEM.

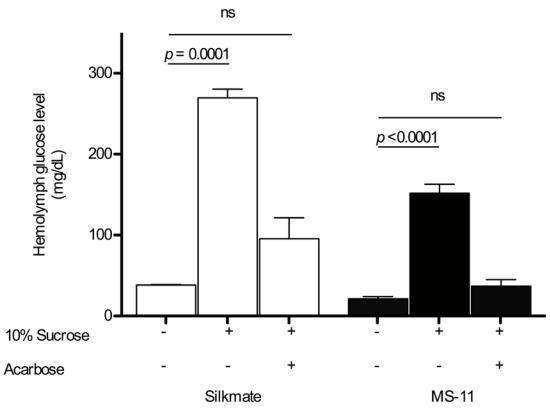

Figure 2. Hemolymph glucose level of silkworm larvae. Larvae were fed with 10% sucrose containing Silkmate or MS-11 diet with or without acarbose (80 mg/g). After one hour, glucose level in hemolymph was measured by a glucometer. Data is shown as mean ± SEM and analyzed by one-way analysis of variance (AVOVA) with Dunnett's multiple comparison test compared with no sucrose diet, ns: not significant.

five diets tested, the most weight gain was observed in silkworms fed with diet B and C, however, the growth of all the five diets fed silkworms was smaller than that of the commercially available diet F (Silkmate) fed silkworms (Figure 1B). Although the larvae grew normally until day 8, they could not form cocoon, suggesting that some essential components for cocoon formation are lacking on the diets.

3.2. Glucose lowering activity of an orally administered antidiabetic drug

We selected diet C (mulberry powder:soybean flour,1:1), named as MS-11, for further experiments. We used MS-11 to check the hemolymph glucose level in silkworm by mixing 10% sucrose in the diet and compared with the Silkmate diet. We found that like Silkmate diet, MS-11 diet containing 10% sucrose fed silkworm hemolymph showed higher glucose levels, which was lowered by a clinically used anti-diabetic drug acarbose (Figure 2). The overall high hemolymph glucose level in Silkmate diet-fed silkworms may be due to the sugar content in the diet itself. These results suggested that MS-11 diet containing 10% sucrose could be used for the glucose level lowering experiments and is suitable for screening for identification of blood-glucose-lowering agents.

3.3. Oral administration of antibiotics by using artificial diet in a silkworm infection model

To assess oral therapeutic effectiveness of antibiotics, we injected silkworms with a clinical isolate of methicillin-sensitive strain of *S. aureus*, MSSA1 into the hemolymph. We then mixed different concentrations of clinically used antibiotics chloramphenicol and tetracycline with MS-11 diet and fed the infected

Table 1. Therapeutic activity of antibiotics by oral administration

Antibiotic	ED_{50} value (μg/g larva)	
	MS-11 diet	Silkmate diet*
Chloramphenicol	41	40
Tetracycline	9	8

*ED_{50} values were previously reported (*3*).

silkworms. We counted the number of the survival of silkworms and calculated the ED_{50} values of the antibiotics. We found that the ED_{50} values of the antibiotics per oral route to be consistent with those previously obtained using the Silkmate diet (Table 1). These results suggest that MS-11 diet prepared in this study is available for determining therapeutic activities of orally effective antibiotics.

4. Discussion

Several studies have been performed to assess the components essential for the growth of silkworm and for optimum silk production by using artificial diet (*19*). Comparison between natural and artificial diets fed silkworms have also been done that analyzed gut microbiota (*20*) as well as proteomic (*21*) and metabolomic (*22*) profiles. Most of the studies on artificial diet have mainly focused on silk production, so far. In the recent scenario of growing interest of using silkworms as drug discovery models, artificial diet that is simple, can be widely used, and applicable for research purposes is desirable.

Silkworms sense the odor of mulberry and are attracted by β-γ-hexenol and α-β-hexenal components in the mulberry (*23*), which is sensed by *GR66* gene encoding a putative bitter gustatory receptor (*24*) that are responsible for the mulberry-specific feeding preference of the silkworms. As expected, we found that the larvae preferred diet that contained mulberry powder. Of note, the MS-11 diet is for research and not for silkworm rearing purpose. Silkworms can be reared in mulberry leaves and MS-11 diet can then be used for subsequent feeding experiments. MS-11 diet is simple with only two major ingredients and can be easily prepared in individual laboratories. Besides, this composition ensures that there is less interference of the diet components to the experiments. The diet is applicable to experiments that require feeding compounds/extracts to silkworms to assess various biological activities through oral route of administration. Here we showed two such examples; assessing blood glucose-lowering activity and oral antimicrobial activity of clinically used drugs. MS-11 diet can be utilized for other similar experiments including but not limited to assessing oral toxicity, bioavailability, absorption, digestion/metabolism of compounds, and effect of compounds including food

products, probiotics in immunity, and application in food/nutrition science.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number JP19K16653 (Grant-in-Aid for Early-Career Scientists) to AP, JSPS KAKENHI Grant Number JP19K07140 (Grant-in-Aid for scientific research) to HH and TBRF fellowship to SP.

References

1. Xia Q, Guo Y, Zhang Z, *et al.* Complete resequencing of 40 genomes reveals domestication events and genes in silkworm (*Bombyx*). Science. 2009; 326:433.
2. Panthee S, Paudel A, Hamamoto H, Sekimizu K. Advantages of the silkworm as an animal model for developing novel antimicrobial agents. Front Microbiol. 2017; 8:373.
3. Hamamoto H, Kurokawa K, Kaito C, Kamura K, Manitra Razanajatovo I, Kusuhara H, Santa T, Sekimizu K. Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms. Antimicrob Agents Chemother. 2004; 48:774-779.
4. Ishii K, Hamamoto H, Kamimura M, Sekimizu K. Activation of the silkworm cytokine by bacterial and fungal cell wall components *via* a reactive oxygen species-triggered mechanism. J Biol Chem. 2008; 283:2185-2191.
5. Hamamoto H, Tonoike A, Narushima K, Horie R, Sekimizu K. Silkworm as a model animal to evaluate drug candidate toxicity and metabolism. Comp Biochem Physiol C Toxicol Pharmacol. 2009; 149:334-339.
6. Hamamoto H, Horie R, Sekimizu K. Pharmacokinetics of anti-infectious reagents in silkworms. Sci Rep. 2019; 9:9451.
7. Usui K, Nishida S, Sugita T, Ueki T, Matsumoto Y, Okumura H, Sekimizu K. Acute oral toxicity test of chemical compounds in silkworms. Drug Discov Ther. 2016; 10:57-61.
8. Hamamoto H, Urai M, Ishii K, *et al.* Lysocin E is a new antibiotic that targets menaquinone in the bacterial membrane. Nat Chem Biol. 2015; 11:127-133.
9. Paudel A, Panthee S, Hamamoto H, Sekimizu K. GPI0363 inhibits the interaction of RNA polymerase with DNA in *Staphylococcus aureus*. RSC Adv. 2019; 9:37889-37894.
10. Paudel A, Hamamoto H, Panthee S, Kaneko K, Matsunaga S, Kanai M, Suzuki Y, Sekimizu K. A novel spiro-heterocyclic compound identified by the silkworm infection model inhibits transcription in *Staphylococcus aureus*. Front Microbiol. 2017; 8:712.
11. Paudel A, Panthee S, Urai M, Hamamoto H, Ohwada T, Sekimizu K. Pharmacokinetic parameters explain the therapeutic activity of antimicrobial agents in a silkworm infection model. Sci Rep. 2018; 8:1578.
12. Kaito C, Kurokawa K, Matsumoto Y, Terao Y, Kawabata S, Hamada S, Sekimizu K. Silkworm pathogenic bacteria infection model for identification of novel virulence genes. Mol Microbiol. 2005; 56:934-944.
13. Paudel A, Hamamoto H, Panthee S, Matsumoto Y, Sekimizu K. Large-scale screening and identification of novel pathogenic *Staphylococcus aureus* genes using a silkworm infection model. J Infect Dis. 2020; 221:1795-1804.
14. Matsumoto Y, Sumiya E, Sugita T, Sekimizu K. An invertebrate hyperglycemic model for the identification of anti-diabetic drugs. PLOS ONE. 2011; 6:e18292.
15. Cappellozza L, Cappellozza S, Saviane A, Sbrenna G. Artificial diet rearing system for the silkworm *Bombyx mori* (Lepidoptera: Bombycidae): effect of vitamin C deprivation on larval growth and cocoon production. Appl Entomol Zool. 2005; 40:405-412.
16. Ito T, Arai N. Artificial diets for the silkworm, *Bombyx mori*, with special references to a semi-synthetic diet for fifth-instar larvae, which includes a large amount of defatted soybean meal. Nippon Nōgeikagaku Kaishi. 1973; 47:397-401.
17. Etebari K, Matindoost L. Application of multi-vitamins as supplementary nutrients on biological and economical characteristics of silkworm *Bombyx mori* L. J Asia Pac Entomol. 2005; 8:107-112.
18. Anantaworasakul P, Hamamoto H, Sekimizu K, Okonogi S. Physiological comparison between mulberry ('Morus alba'L.) leaves diet and artificial diet on growth development as well as antibiotic therapeutic response of silkworms. Aust J Crop Sci. 2013; 7:2029.
19. Moise AR, Pop LL, Vezeteu TV, Domut Agoston B, Pasca C, Dezmirean DS. Artificial diet of silkworms (*Bombyx Mori*) Improved with bee pollen - biotechnological approach in global centre of excellence for advanced research in sericulture and promotion of silk production. Bull UASVM Anim Sci Biotechnol. 2020; 77:51-57.
20. Dong HL, Zhang SX, Chen ZH, Tao H, Li X, Qiu JF, Cui WZ, Sima YH, Cui WZ, Xu SQ. Differences in gut microbiota between silkworms (*Bombyx mori*) reared on fresh mulberry (*Morus alba* var. multicaulis) leaves or an artificial diet. RSC Adv. 2018; 8:26188-26200.
21. Zhou ZH, Yang HJ, Chen M, Lou CF, Zhang YZ, Chen KP, Wang Y, Yu ML, Yu F, Li JY, Zhong BX. Comparative proteomic analysis between the domesticated silkworm (*Bombyx mori*) reared on fresh mulberry leaves and on artificial diet. J Proteome Res 2008; 7:5103-5111.
22. Dong HL, Zhang SX, Tao H, Chen ZH, Li X, Qiu JF, Cui WZ, Sima YH, Cui WZ, Xu SQ. Metabolomics differences between silkworms (*Bombyx mori*) reared on fresh mulberry (Morus) leaves or artificial diets. Sci Rep. 2017; 7:10972.
23. Watanabe T. Substances in mulberry leaves which attract silkworm larvæ (*Bombyx mori*). Nature. 1958; 182:325-326.
24. Zhang ZJ, Zhang SS, Niu BL, Ji DF, Liu XJ, Li MW, Bai H, Palli SR, Wang CZ, Tan AJ. A determining factor for insect feeding preference in the silkworm, *Bombyx mori*. PLOS Biol. 2019; 17:e3000162.

Received July 29, 2020; Accepted August 8, 2020.

**Address correspondence to:*
Kazuhisa Sekimizu, Teikyo University Institute of Medical Mycology, 359 Otsuka, Hachioji, Tokyo, 192-0395, Japan.
E-mail: sekimizu@main.teikyo-u.ac.jp

Released online in J-STAGE as advance publication August 24, 2020.

Original Article

DOI: 10.5582/ddt.2020.01010

Identification of differentially expressed long non-coding RNAs associated with dilated cardiomyopathy using integrated bioinformatics approaches

Xiaohui Luo[1,§], Pengdan Luo[2,§], Yushun Zhang[1,*]

[1] Department of Structural Heart Disease, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China;
[2] Department of Obstetrics and Gynecology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China.

SUMMARY The aim of this study was to identify novel long non-coding RNA (lncRNA) biomarkers associated with dilated cardiomyopathy (DCM) and reveal the potential molecular mechanisms of DCM development using bioinformatics approaches. The array data of GSE5406, including 108 DCM samples and 16 non-failing control samples, were obtained from the Gene Expression Omnibus database. The differentially expressed lncRNAs were identified using limma package in R. Pearson's correlation analyses were performed between the differentially expressed lncRNAs and protein-coding genes based on their expression levels. Pathway enrichment of these lncRNAs was conducted based on the significantly co-expressed genes. From the receiver operating characteristic (ROC) curve, the area under the ROC curve (AUC) value was obtained and used for evaluating discriminatory ability. IDI2-AS1 and XIST were differentially expressed in DCM patients. A total of 510 co-expressed genes were identified. The enriched functions and pathways of the co-expressed genes mainly included NADH dehydrogenase activity, cardiac muscle contraction, and oxidative phosphorylation. The ROC curve analysis indicated that the two lncRNAs have favorable diagnostic values in DCM. The AUC values of XIST, IDI2-AS1, and the combination of XIST and IDI2-AS1 were 0.733 (95% CI: 0.646-0.809), 0.796 (95% CI: 0.715-0.863), and 0.823 (95% CI: 0.745-0.886), respectively. This study identified IDI2-AS1 and XIST lncRNAs and related pathways involved in the pathogenesis of DCM, thus providing potential diagnostic and therapeutic targets for DCM.

Keywords long Non-coding RNAs, biomarker, dilated cardiomyopathy, bioinformatics analysis

1. Introduction

Dilated cardiomyopathy (DCM) is the most common type of cardiomyopathy worldwide, characterized by the primary presence of left ventricular (LV) dilation and reduced systolic function in the absence of abnormal loading conditions or coronary artery disease (*1,2*). DCM may result in progressive end-stage heart failure and sudden cardiac arrest, which is closely associated with excessive morbidity and premature mortality (*3*). Despite the rapid advancement in therapeutic modalities, the 5-year mortality for DCM remains up to 20%, and most of them die from heart failure and ventricular arrhythmias (*4*). Moreover, patients with DCM are usually asymptomatic at the early stage, and the period of diagnosis and treatment may be considerably delayed (*5*). In the majority of cases of idiopathic DCM, the etiology remains still unknown.

Recently, an increasing number of studies have confirmed that lncRNAs play important roles in the heart, including DCM-induced chronic heart failure initiation and progression at the posttranscriptional level (*6*). LncRNAs represent a cluster of RNAs that are >200 nucleotides in length without protein coding potential (*7*). LncRNAs can perform multiple biological functions, such as RNA processing, regulation of chromatin remodeling, marker of cell fate, and as a competing endogenous RNA (*8-11*). Previous studies have reported that lncRNAs play critical roles in the progression as well as diagnostic and therapeutic targets of cardiovascular diseases (*12,13*). Nevertheless, further studies required to explore the potential functions of lncRNAs in the development of DCM. In this study, we aimed to explore more lncRNAs involved in DCM. We screened differentially expressed lncRNAs based on GSE5406, followed by a series of bioinformatics analyses to identify key lncRNAs involved in DCM.

2. Methods

2.1. Microarray gene expression

GSE5406 gene expression profile was obtained from Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*) database, based on the platform of the PL96 Affymetrix Human Genome U133A expression beadchip. The dataset was an mRNA expression profile chip, which contained 16 non-failing control samples and 108 samples from DCM patients. The platform contains both mRNAs and lncRNAs; therefore, we obtained the lncRNA expression profile by repurposing the probes in the mRNA expression profiles to lncRNAs according to the annotation in the GENCODE version GRCh38 (*http://www. gencodegenes.org*). The probe without a gene symbol was removed. For different multiple probe sets mapping to the same mRNA or lncRNA, the mean value of different probes was selected as the final expression value.

2.2. Differentially expressed lncRNA identification

The LIMMA package (Linear Models for Microarray Data) in R software (version 3.5.1) with multiple testing corrections based on the Benjamini & Hochberg method was used to screen out the differentially expressed lncRNAs between DCM and control groups (*14*). Adjusted false discovery rate p values < 0.05, along with fold change ≥ 1.5, were used as the thresholds for difference analysis. Then, cluster analysis was carried out using the Pheatmap package in R to further analyze the differentially expressed lncRNAs.

2.3. LncRNA-related protein-coding gene identification and enrichment analysis

The Pearson's correlation coefficients between the differentially expressed lncRNAs and the protein-coding genes were calculated to determine co-expression relationships (*15*). The protein-coding genes positively or negatively correlated with the differentially expressed lncRNAs were considered as lncRNA-related protein-coding genes (|Pearson's correlation coefficient| > 0.6 and p-value < 0.001). Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were also conducted using the biological tool. The clusterProfiler package was used to explore the biological meaning and pathways behind numerous genes (*16*).

2.4. Diagnostic value of differentially expressed lncRNAs in DCM

To test the predictive value of the differentially expressed lncRNAs, we generated a receiver operating characteristic (ROC) curve using the lncRNA expression data from 108 DCM patients and 16 controls. The area under the ROC curve (AUC) value was utilized to determine the diagnostic effectiveness in discriminating patients with DCM patients from normal individuals.

3. Results

3.1. Identification of differentially expressed lncRNAs

According to the selection criteria, a total of 107 lncRNAs were obtained, and two differentially expressed lncRNAs were identified between the DCM and control groups, including upregulated IDI2-AS1 and downregulated XIST. As shown in the heat map (Figure 1), the differentially expressed lncRNAs were predominantly separated into two clusters.

3.2. Functional enrichment analysis of pathways correlated with the identified lncRNAs in DCM

We examined the expression correlation between identified lncRNAs and genes by calculating the Pearson's correlation coefficient through expression profiles in 108 patients with DCM. Co-expression between 510 genes and the two lncRNAs (Pearson's correlation coefficient > 0.6 and p-value < 0.001) was found. The highest Pearson's correlation coefficient of determination between two lncRNAs and mRNA matrix is shown in Figure 2. We performed GO and KEGG enrichment analyses to uncover specific functional categories of the co-expressed genes. As a result, 510 co-expressed genes clustered most significantly in NADH dehydrogenase activity, cytochrome-c oxidase activity, cofactor binding, and oxidoreductase activity (Figure 3A). KEGG pathway enrichment showed that genes were mainly involved in oxidative phosphorylation and cardiac muscle contraction (Figure 3B).

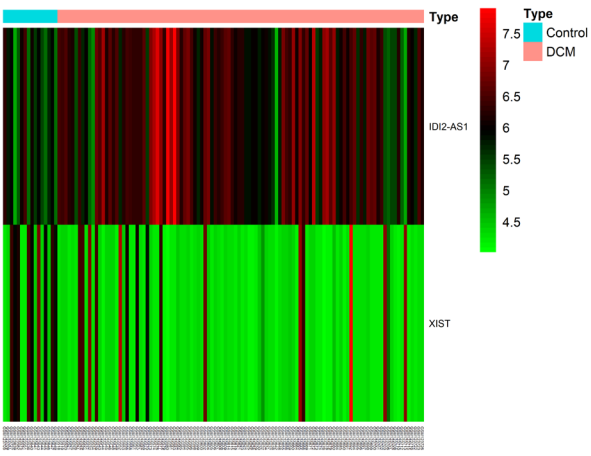

Figure 1. Heatmap results of differentially expressed long non-coding RNAs.

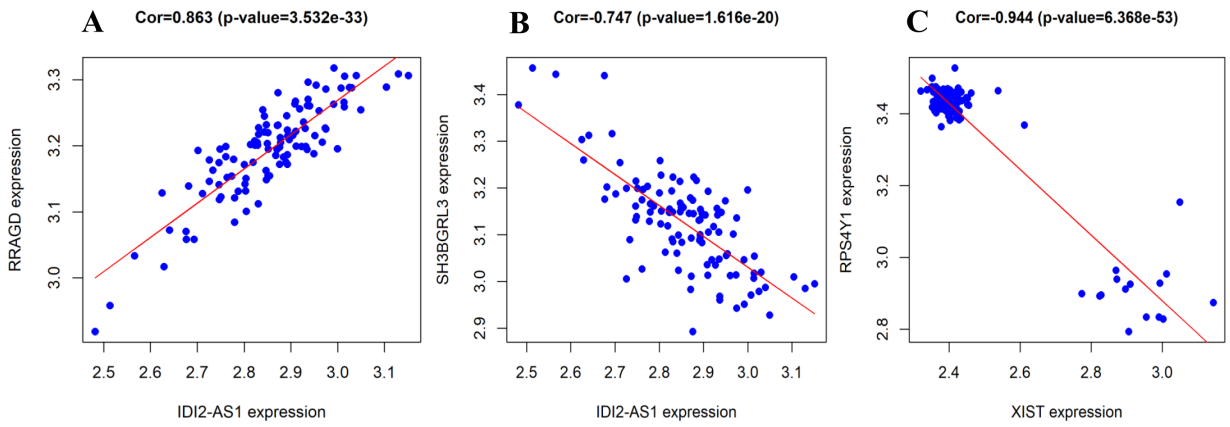

Figure 2. The highest Pearson's correlation coefficient of determination between two long non-coding RNAs and the mRNA matrix.

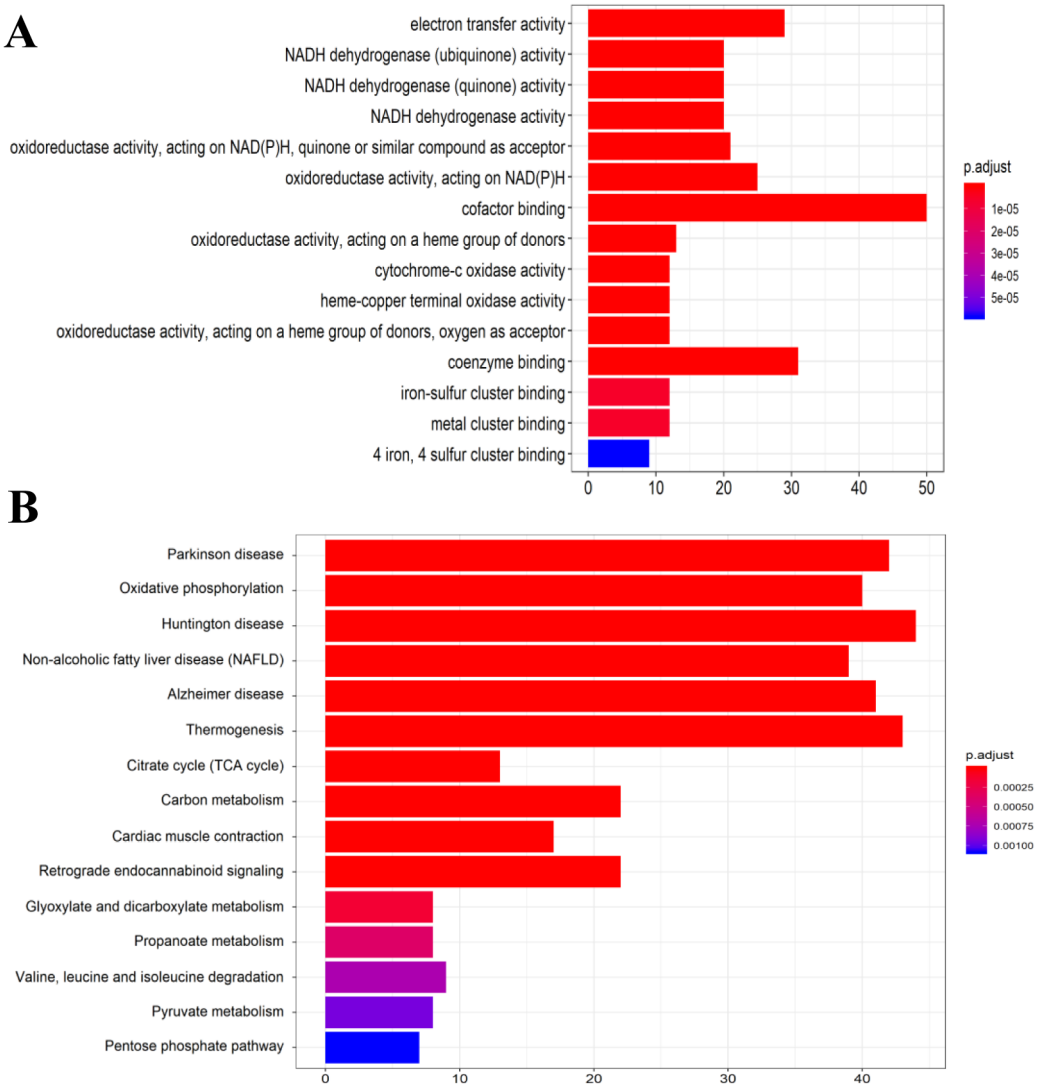

Figure 3. Enriched gene ontology (A) and Kyoto encyclopedia of genes and genomes (B) pathway analysis of co-expressed genes related to IDI2-AS1 and XIST.

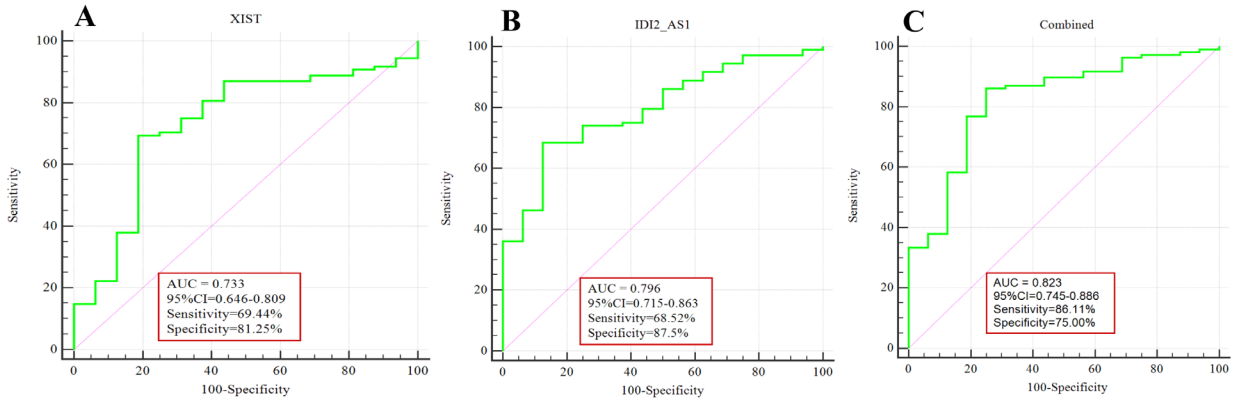

Figure 4. Diagnostic values of XIST (A), IDI2-AS1 (B), and IDI2-AS1 combined XIST (C) in discriminating dilated cardiomyopathy samples from non-failing control samples.

3.3. Diagnostic significance of differentially expressed lncRNAs

To explore the diagnostic significance of differentially expressed lncRNAs, ROC curves were generated. As shown in Figure 4, the diagnostic effectiveness of two lncRNAs in discriminating DCM from non-failing control ones revealed a favorable diagnostic value with an AUC of 0.733 (95% CI: 0.646-0.809) in XIST (Figure 4A) and AUC of 0.796 (95% CI:0.715-0.863) in IDI2-AS1 (Figure 4B). Moreover, when XIST and IDI2-AS1 were combined, the ROC curve yielded an AUC of 0.823 (95% CI: 0.745-0.886, Figure 4C).

4. Discussion

DCM is the third most common cause of congestive heart failure and sudden cardiac arrest (*17*). Previous studies have revealed that lncRNAs play important roles in cardiac development and participate in cardiac homeostasis and regeneration as epigenetic regulators of heart gene expression (*18,19*). Recently, increasing attention has been paid to improving the treatment of DCM. However, there remains a long road to better understand of the underlying mechanism. In this study, dysregulated lncRNAs between patients with DCM and non-failing controls were analyzed by RNA sequencing. In total, a differentially expressed lncRNA steadily upregulated and another steadily downregulated. In particular, based on the lncRNA-related protein-coding genes, the following functional enrichment analysis indicated that lncRNA-related co-expressed mRNAs were mainly enriched in NADH activity, cytochrome c oxidase activity, and oxidoreductase activity. KEGG pathway enrichment showed that these genes were mainly involved in oxidative phosphorylation and cardiac muscle contraction.

In recent years, several studies have explored the functions and clinical significance of lncRNAs in HCM (*20,21*). A recent study investigated 14 lncRNAs deregulated in patients with non-end-stage dilated hypokinetic ischemic cardiomyopathy, such as CDKN2B-AS1, EGOT, and H19 (*22*). A whole-transcriptome analysis of heart biopsy specimens from patients with DCM and healthy heart donors confirmed that myocardial infarction associated transcript was highly expressed in patients with DCM (*23*). To date, many lncRNAs have been revealed to be associated with myocardial pathophysiology, and research on the biological function and mechanism of lncRNAs has only begun and remained unclear (*24,25*). LncRNA X inactive-specific transcript (XIST) is indispensable for transcriptional silencing of the X chromosome in female mammals, which play vital role in the inactivation of the X chromosome (*26*). It has been widely used as an oncogene or a tumor suppressor in many malignancies (*27*). XIST has also been demonstrated to be remarkably upregulated in the hypertrophic hearts of mice induced by transaortic constriction and phenylephrine-treated rat cardiomyocytes (*28*). Furthermore, it was shown that XIST could inhibit myocardial cell proliferation and promote apoptosis. It was also reported that XIST contributed to tumor proliferation and suppressed tumor apoptosis in lung cancer and hepatocellular carcinoma (*29,30*). All these findings help to explain the potential contribution of XIST to DCM. As for isopentenyl-diphosphate delta isomerase 2 antisense RNA 1 (IDI2-AS1), its function in DCM has not been reported in the literature. Overexpression of IDI2-AS1 sensitized human cells to cell death in response to various stresses, such as ultraviolet irradiation, cycloheximide, hydrogen peroxide, and mercury II chloride (*31*). Therefore, its function should be explored in more studies.

Systolic dysfunction is the first hallmark pathophysiologic sign of DCM (*32*). In the normal heart, contraction is synchronous within the myocardium, with a normal symmetric distribution of negative strain across the wall. Conversely, in the heart with DCM, the contraction pattern is obviously dyssynchronous (*33*). Therefore, heart contraction may be a vital indicator of DCM. In heart failure, deterioration of cytosolic Ca^{2+} and Na^{+} handling hinders mitochondrial Ca^{2+} uptake

and the subsequent Krebs cycle-induced regeneration of the decreased forms of NADH and NADPH, leading to energetic deficit and oxidative stress (*34*). These results were consistent with the enrichment analysis that lncRNAs were mainly enriched in NADH and oxidoreductase activity and cardiac muscle contraction. This finding was confirmed by the fact that reduced sarcomere contractility can increase ventricular volumes to maintain cardiac output through the Frank-Starling mechanism, generating a thin-walled LV appearance that is discovered in DCM (*35*). DCM is usually diagnosed at ages between 30 and 40 years of age. However, owing to the vague symptoms and delay in diagnosis, the outcome for DCM is poor (*36*). DCM accounts for about one-third of heart failure patients. A recent study reported that lncRNAs ENST00000532365 and ENST00000507296 could serve as biomarkers for the diagnosis of DCM-related heart failure (*36*). In the present study, we found that XIST presented an AUC of 0.733, and IDI2-AS1 exhibited an AUC of 0.796. Moreover, when the two lncRNAs were combined, the ROC curve yielded an AUC of 0.823 with a sensitivity of 86.11% and specificity of 75.00%. Therefore, based on these results, XIST combined with IDI2-AS1 can be used as a novel diagnostic biomarker in DCM. However, the present study has some limitations. First, the sample size was relatively small. In addition, this study was performed based on bioinformatic methods, and the findings should be confirmed by experimental validation.

In summary, the present study identified IDI2-AS1 and XIST and related pathways that can help us understand the molecular mechanisms underlying the pathogenesis of DCM. XIST combined with IDI2-AS1 may be a novel promising novel biomarker for DCM diagnosis.

Data availability

The data that support the findings of this study are available from the Gene Expression Omnibus (GEO, *https://www.ncbi.nlm.nih.gov/geo/*), accession number GSE5406.

Acknowledgments

The authors thank the efforts of the National Cancer Institute in the creation of the Gene Expression Omnibus database

References

1. Elliott P. Cardiomyopathy. Diagnosis and management of dilated cardiomyopathy. Heart. 2000; 84:106-112.
2. Jefferies JL, Towbin JA. Dilated cardiomyopathy. Lancet. 2010; 375:752-762.
3. Hershberger RE, Hedges DJ, Morales A. Dilated cardiomyopathy: the complexity of a diverse genetic architecture. Nat Rev Cardiol. 2013; 10:531-547.
4. Felker GM, Thompson RE, Hare JM, Hruban RH, Clemetson DE, Howard DL, Baughman KL, Kasper EK. Underlying causes and long-term survival in patients with initially unexplained cardiomyopathy. N Engl J Med. 2000; 342:1077-1084.
5. Bozkurt B, Colvin M, Cook J, *et al*. Current Diagnostic and Treatment Strategies for Specific Dilated Cardiomyopathies: A Scientific Statement From the American Heart Association. Circulation. 2016; 134:e579-e646.
6. Thum T, Gross C, Fiedler J, *et al*. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature. 2008; 456:980-984.
7. Kung JT, Colognori D, Lee JT. Long noncoding RNAs: past, present, and future. Genetics. 2013; 193:651-669.
8. Gong C, Maquat LE. lncRNAs transactivate STAU1-mediated mRNA decay by duplexing with 3' UTRs *via* Alu elements. Nature. 2011; 470:284-288.
9. Fatica A, Bozzoni I. Long non-coding RNAs: new players in cell differentiation and development. Nat Rev Genet. 2014; 15:7-21.
10. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni I. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell. 2011; 147:358-369.
11. Ginger MR, Shore AN, Contreras A, Rijnkels M, Miller J, Gonzalez-Rimbau MF, Rosen JM. A noncoding RNA is a potential marker of cell fate during mammary gland development. Proc Natl Acad Sci U S A. 2006; 103:5781-5786.
12. Thum T, Condorelli G. Long noncoding RNAs and microRNAs in cardiovascular pathophysiology. Circ Res. 2015; 116:751-762.
13. Gomes CPC, Spencer H, Ford KL, Michel LYM, Baker AH, Emanueli C, Balligand JL, Devaux Y. The function and therapeutic potential of long non-coding RNAs in cardiovascular development and disease. Mol Ther Nucleic Acids. 2017; 8:494-507.
14. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015; 43:e47.
15. Bhardwaj N, Lu H. Correlation between gene expression profiles and protein-protein interactions within and across genomes. Bioinformatics. 2005; 21:2730-2738.
16. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS. 2012; 16:284-287.
17. Halliday BP, Cleland JGF, Goldberger JJ, Prasad SK. Personalizing risk stratification for sudden death in dilated cardiomyopathy: The past, present, and future. Circulation. 2017; 136:215-231.
18. Devaux Y, Zangrando J, Schroen B, Creemers EE, Pedrazzini T, Chang CP, Dorn GW, 2nd, Thum T, Heymans S. Long noncoding RNAs in cardiac development and ageing. Nat Rev Cardiol. 2015; 12:415-425.
19. Calore M, De Windt LJ, Rampazzo A. Genetics meets epigenetics: Genetic variants that modulate noncoding RNA in cardiovascular diseases. J Mol Cell Cardiol. 2015; 89:27-34.
20. Sun J, Wang C. Long non-coding RNAs in cardiac

hypertrophy. Heart Fail Rev. 2019.
21. Wang K, Long B, Zhou LY, Liu F, Zhou QY, Liu CY, Fan YY, Li PF. CARL lncRNA inhibits anoxia-induced mitochondrial fission and apoptosis in cardiomyocytes by impairing miR-539-dependent PHB2 downregulation. Nat Commun. 2014; 5:3596.
22. Greco S, Zaccagnini G, Perfetti A, Fuschi P, Valaperta R, Voellenkle C, Castelvecchio S, Gaetano C, Finato N, Beltrami AP, Menicanti L, Martelli F. Long noncoding RNA dysregulation in ischemic heart failure. J Transl Med. 2016; 14:183.
23. Frade AF, Laugier L, Ferreira LR, *et al*. Myocardial infarction-associated transcript, a long noncoding RNA, is overexpressed during dilated cardiomyopathy due to chronic chagas disease. J Infect Dis. 2016; 214:161-165.
24. Zhang J, Feng C, Song C, Ai B, Bai X, Liu Y, Li X, Zhao J, Shi S, Chen X, Su X, Li C. Identification and analysis of a key long non-coding RNAs (lncRNAs)-associated module reveal functional lncRNAs in cardiac hypertrophy. J Cell Mol Med. 2018; 22:892-903.
25. Chen C, Tang Y, Sun H, Lin X, Jiang B. The roles of long noncoding RNAs in myocardial pathophysiology. Biosci Rep. 2019; 39:BSR20190966.
26. Lo HC, Wu SC, Huang HC, Yeh CC, Huang JC, Hsieh CH. Laparoscopic simple closure alone is adequate for low risk patients with perforated peptic ulcer. World J Surg. 2011; 35:1873-1878.
27. Yang Z, Jiang X, Jiang X, Zhao H. X-inactive-specific transcript: A long noncoding RNA with complex roles in human cancers. Gene. 2018; 679:28-35.
28. Zhou T, Qin G, Yang L, Xiang D, Li S. LncRNA XIST regulates myocardial infarction by targeting miR-130a-3p. J Cell Physiol. 2019; 234:8659-8667.
29. Mo Y, Lu Y, Wang P, Huang S, He L, Li D, Li F, Huang J, Lin X, Li X, Che S, Chen Q. Long non-coding RNA XIST promotes cell growth by regulating miR-139-5p/PDK1/AKT axis in hepatocellular carcinoma. Tumour Biol. 2017; 39:1010428317690999.
30. Tang Y, He R, An J, Deng P, Huang L, Yang W. lncRNA XIST interacts with miR-140 to modulate lung cancer growth by targeting iASPP. Oncol Rep. 2017; 38:941-948.
31. Tani H, Torimura M. Development of cytotoxicity-sensitive human cells using overexpression of long non-coding RNAs. J Biosci Bioeng. 2015; 119:604-608.
32. Garfinkel AC, Seidman JG, Seidman CE. Genetic pathogenesis of hypertrophic and dilated cardiomyopathy. Heart Fail Clin. 2018; 14:139-146.
33. Curry CW, Nelson GS, Wyman BT, Declerck J, Talbot M, Berger RD, McVeigh ER, Kass DA. Mechanical dyssynchrony in dilated cardiomyopathy with intraventricular conduction delay as depicted by 3D tagged magnetic resonance imaging. Circulation. 2000; 101:E2.
34. Bertero E, Maack C. Calcium signaling and reactive oxygen species in mitochondria. Circ Res. 2018; 122:1460-1478.
35. Lakdawala NK, Thune JJ, Colan SD, Cirino AL, Farrohi F, Rivero J, McDonough B, Sparks E, Orav EJ, Seidman JG, Seidman CE, Ho CY. Subtle abnormalities in contractile function are an early manifestation of sarcomere mutations in dilated cardiomyopathy. Circ Cardiovasc Genet. 2012; 5:503-510.
36. Zhang X, Nie X, Yuan S, Li H, Fan J, Li C, Sun Y, Zhao Y, Hou H, Wang DW, Chen C. Circulating long non-coding RNA ENST00000507296 is a prognostic indicator in patients with dilated cardiomyopathy. Mol Ther Nucleic Acids. 2019; 16:82-90.

Received February 14, 2020; Revised July 4, 2020; Accepted July 23, 2020.

[§]These authors contributed equally to this work.

**Address correspondence to:*
Yushun Zhang, Department of Structural Heart Disease, The First Affiliated Hospital of Xi'an Jiaotong University, No.277, Yanta West Road, Xi'an 710061, China.
E-mail: zys2889@sina.com

Released online in J-STAGE as advance publication July 29, 2020.

Original Article

DOI: 10.5582/ddt.2020.03062

Diagnosis of papillary thyroid carcinoma by ^{1}H NMR spectroscopy-based metabolomic analysis of whole blood

Tiantian Wang[1,2,§], Zhigang Sun[2,3,§], Yong Wang[4], Feifei Li[1], Xiaoming Zhou[2], Xingsong Tian[2], Shuqi Wang[1,*]

[1] School of Pharmaceutical Sciences, Shandong University, Ji'nan, Shandong, China;
[2] Department of Thyroid and Breast Surgery, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Ji'nan, Shandong, China;
[3] Department of Colorectal Surgery and State Key Lab of Molecular Oncology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China;
[4] Department of Thyroid Surgery, The Second Affiliated Hospital of Zhejiang University College of Medicine, Hangzhou, Zhejiang, China.

SUMMARY The incidence rate of thyroid carcinoma, especially papillary thyroid carcinoma (PTC), has increased significantly over time. As a primary pathway for metastasis, the lymphatic system is an important prognostic factor for PTC patients. Although the metabolic changes in PTC patients have been investigated in extensive studies, few studies focused on the whole blood metabolic profiling of PTC patients. In this study, we investigated the ^{1}H NMR-based metabolic profiles of whole blood samples that were obtained from healthy individuals and PTC patients, with or without lymph node metastasis. The estimation of the predictive potential of metabolites was evaluated using multivariate statistical analyses, which revealed that the whole blood carries information that is sufficient for distinguishing between PTC patients and healthy individuals. However, PTC patients were not well classified as positive or negative according to the lymph nodes. We did not find a metabolite that could discriminate the presence of lymph node metastasis. Further studies with larger sample sizes are needed to elucidate significant metabolites to indicate the presence of lymph node metastasis in patients with PTC.

Keywords Thyroid, ^{1}H NMR spectroscopy, metabolomics, lymph node metastasis, papillary thyroid carcinoma

1. Introduction

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer, representing 75-85% of all thyroid cancer cases (*1*). Surgery is the elective treatment for papillary thyroid carcinoma. Preoperative distinction between benign and malignant conditions is crucial to avoid overtreatment of patients and morbidity linked with an inappropriate surgery (*2,3*). Clinical research in thyroid pathology is focusing on the development of new diagnostic tools to improve the stratification of nodules that have biological, practical, and economic consequences on the management of patients. Ultrasound plays an increasingly important role in the diagnosis of the thyroid nodule, and the ultrasound-guided fine-needle aspiration biopsy (FNAB) has become the gold standard for preoperative judgment in thyroid carcinoma patients (*4,5*).

As an invasive diagnostic method, FNAB presents a certain risk of bleeding and a low possibility of needle tract metastasis. Patients receiving FNAB often require surgery as soon as possible due to the risk of nodule enlargement or change in nodule nature. Therefore, the development of new diagnostic methods that could provide useful clinical information for the non-invasive diagnosis of the nature of thyroid nodules and lymph nodes is particularly important (*6*). We hope that this approach will decrease unnecessary repeated biopsies and surgical procedures.

Metabolomics is the systematic study of small molecular metabolites in cells, tissues, biofluids or cell culture medias, and provides tangible results regarding cellular processes or responses to environmental stresses (*7*). In general, cancer cells have an impaired energy metabolism, and metabolic intermediates typically accumulate in tumors (*8*). Metabolomics is now recognized as a powerful technique for identifying biomarkers and altering the metabolic pathways in cancer, including nucleotide synthesis, glycolysis, phospholipid and fatty acid metabolism, and amino acid metabolism (*9,10*). Metabolomics has been increasingly used to identify biomarkers for early diagnosis and

understanding the potential mechanisms of various cancers (*11*). In human studies, blood samples are used to capture a physiological average of the host's metabolic status and serum is routinely collected, which makes it a frequent and convenient sample for metabolomics studies.

However, the analysis of the serum metabolome does not consider the contribution of erythrocytes (*12,13*). Compelling evidence suggests a significant value of erythrocytes for metabolite profiling of whole blood in investigations of human health and diseases (*13-16*). The major advantage of whole blood analysis is that it provides access to both the plasma, serum, and red blood cell metabolome in one step and eliminates many of the pre-analytical processing problems with little additional effort compared to the traditionally used serum or plasma analysis (*12,17*). Thus, whole blood metabolomics offers an added opportunity to gain insights into additional metabolites and metabolic pathways.

In the present study, we investigated and compared the metabolomics characteristics of the whole blood from PTC patients and healthy controls using an untargeted metabolomics approach based on ^{1}H NMR, coupled with multivariate statistical analyses. The metabolic differences according to the presence or absence of lymph node metastasis (LN) in patients with PTC were also investigated in our study, in the search for a potential novel prognostic biomarker.

2. Materials and Methods

2.1. Chemical Regents

Deuterium water (D_2O, 99.8%) was purchased from Tenglong Weibo Technology Co., Ltd. (Qingdao, China). High-performance liquid chromatography (HPLC) grade methanol, and 3-(trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (DSS-d_6) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemical reagents were analytical grade.

2.2. Clinical samples and processing of whole blood

We collected whole blood samples from patients who underwent thyroid surgery (n = 80) from the department of breast and thyroid surgery between January 2017 and October 2018, with prior ethical approval by the Institutional Ethics Committee, Shandong Provincial Hospital. The whole blood samples were collected from age matched healthy volunteers (n = 47). All patients signed informed consent before participating in the study. The patients enrolled in this study did not receive any chemotherapy or radiation therapy before surgical treatment. The clinical details and pathological features of all study subjects are listed in Table 1. All samples were collected in EDTA-coated whole blood collection vials. Following collection, samples were placed in an ice-water bath. 1 ml aliquots of blood were placed in cryogenic tubes and immediately flash frozen in liquid nitrogen before being stored at 80ºC. Whole blood samples were stored at 80ºC until further experimentation.

2.3. Sample processing for NMR spectroscopy

The reagents were kept ice-cold before use. To extract the metabolites, 400 µL of whole blood was mixed with cold methanol in a 1:2 sample/methanol (v/v) ratio. All sample solutions were then vortexed for 30 s, and sonicated for 2 min at 4°C. The mixtures were centrifuged at 14,600 rpm for 20 min to pellet the proteins and cell debris (*12*). The clear aqueous solutions were transferred to fresh vials and evaporated to dryness at 45°C in a speedvac concentrator (SPD2010 Integrated SpeedVac, ThermoFisher Scientific, USA). The dried samples were mixed with 600 µL phosphate buffer containing 0.5 mM DSS-d_6, and the pH was adjusted to 7.0 with NaOH and/or HCl. Then, we spun the samples to sediment any residue, and the supernatants were transferred to 5 mm NMR tubes for analysis.

2.4. NMR experiments

All one-dimensional ^{1}H NMR spectra were acquired using a Bruker Avance III 600 MHz NMR spectrometer (Bruker GmbH, Rheinstetten, Germany) operating at

Table 1. The clinical information and pathological features of all patients

	All Patients	LN metastasis	No LN metastasis
Age (years, range)			
≥ 45	41	19	22
< 45	39	25	14
Sex			
Male	27	24	3
Female	53	20	33
TNM stage			
I	27	0	27
II	9	0	9
III	25	25	0
IV	19	19	0
Primary tumor numbers			
1	61	30	31
> 1	19	14	5
Primary tumor numbers (tumor size ≤ 10mm)			
1	35	13	22
≥ 1	13	8	5
Primary tumor size (mm)			
≤ 10	32	20	12
> 10	48	24	24
Primary tumor localization			
One side	69	35	34
Both side	11	9	2

a proton frequency of 600.1 MHz, equipped with a cryoprobe at 298 K. The ^{1}H NMR experiments were performed using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with water pre-saturation to remove broad signals of molecules. The CPMG spectra were recorded with 64,000 time domain data points, a 20.0 ppm spectral width, 32 scans, and a relaxation delay of 5 s. All data were manually phased, and baseline corrected. All resonances of the metabolites present in ^{1}H NMR were confirmed by referring to the Biological Magnetic Resonance Bank (BMRB), Human Metabolome Database (HMDB), published research articles, and by comparing with standard data.

2.5. Data processing and analysis

The data were preprocessed with MestReNova 12.0 (Mestrelab Research SL, Santiago de Compostela, Spain), and manual phasing adjustment of the raw NMR data (FID), and the post-processing consisted of Fourier transformation, phasing and baseline correction, cutting off the solvent peak (water peak, 4.75-4.91 ppm), and normalization. We then performed the piecewise binning; the spectral region from 1.1 to 9.00 ppm was segmented into 148 intervals with an equal width of 0.05 ppm. The data were exported, converted to Excel format, examined, and removed from the solvent peak section.

The normalized NMR data in Excel format were imported into statistical software SIMCA-P (Version 14.0; Umetrics AB, Umea, Sweden) for multivariate statistical analysis, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal projection to latent structure (OPLS) analysis. PCA was first performed to determine possible outliers. The model type selected was PCA-X. We used the scale selected Par data processing mode, completed automatic fitting, and examined the abnormal points. Generally, two times the red line value was used as the abnormal point standard. If there were abnormal points, we deleted the corresponding data in the comprehensive analysis and checked the fit again. After no outlier points remained, the model type selected PLS-DA to build the PLS-DA model, and the order of the samples in the dataset was randomized. The discriminant version of the partial least squares regression (PLS-DA) with a default k-fold cross validation procedure was used to determine the differences between the groups.

Perturbation testing of the model: The PLS-DA was cross-validated by a permutation analysis (200 times). All points should be at the right of the highest point, with the data separated between groups and clustered within groups. We calculated the R^2 and Q^2 values.

The OPLS model was built following the same procedure to obtain good separation between different groups. The R^2 and Q^2 obtained after fitting indicated the parameters of interpretability and the predictability of the model, respectively, which were used to evaluate the quality of the model. To identify the significant spectral peaks, variable importance in the projection (VIP) of > 1 was considered as a criterion for differences between different groupings, which was analyzed and taken as a coefficient from the OPLS model. The identified metabolites were chosen as discriminating ones, which met the conditions of VIP > 1. We generated an S-plots graph, and the variables of the two diagonals of the S-shape were considered as differential metabolites. According to the histogram of the VIP values and S-plots graph to preliminary screen differential metabolites, we used the MetaboAnalyst database to judge the types of metabolites and predict the metabolic pathways and intermediate metabolites in the metabolic pathways (*18-20*).

3. Results

3.1. ^{1}H NMR metabolic profiles of whole blood samples

For the ^{1}H NMR spectra of whole blood, the CPMG pulse sequence was used to suppress the resonances from macromolecules, such as proteins and lipoproteins. Typical ^{1}H NMR spectra of whole blood samples are shown in Figure 1. The ppm scale was expanded to focus on the range of 0.8-9.0 ppm. The resonances attributed to a series of endogenous compounds were identified according to the literature and some public databases (HMDB and BMRB). These peaks were further assigned as lactate, lysine, acetic acid, arginine, glutamic acid, methionine, proline, 3-hydroxybutyric acid, aspartate, tyrosine, 1-methylhistidine, creatine, D-glucose, acetoacetate, L-threonine, L-ornithine, isopropyl alcohol, L-histidine, L-phenylalanine, hypoxanthine, and formate.

3.2. Multivariate statistical analysis

To perform a comprehensive comparison of the metabolic profiles among the groups, we employed PCA and O2PLS-DA with the first two principal components (Figure 2). The PCA scores plot showed that the PTC groups (LN-negative and LN-positive) and the normal group samples were scattered into different regions, whereas overlaps between the LN-negative and LN-positive subjects were also observed in the PCA score plots. The majority of the samples were in the 95% confidence interval. Therefore, all the samples were used in the following analysis to ensure the maximum information.

To further identify the metabolic characteristics of the patients, pairwise OPLS-DA was performed. Obvious separations between the normal group and PTC group, the normal group and LN-negative group, the normal group and LN-positive group, and the

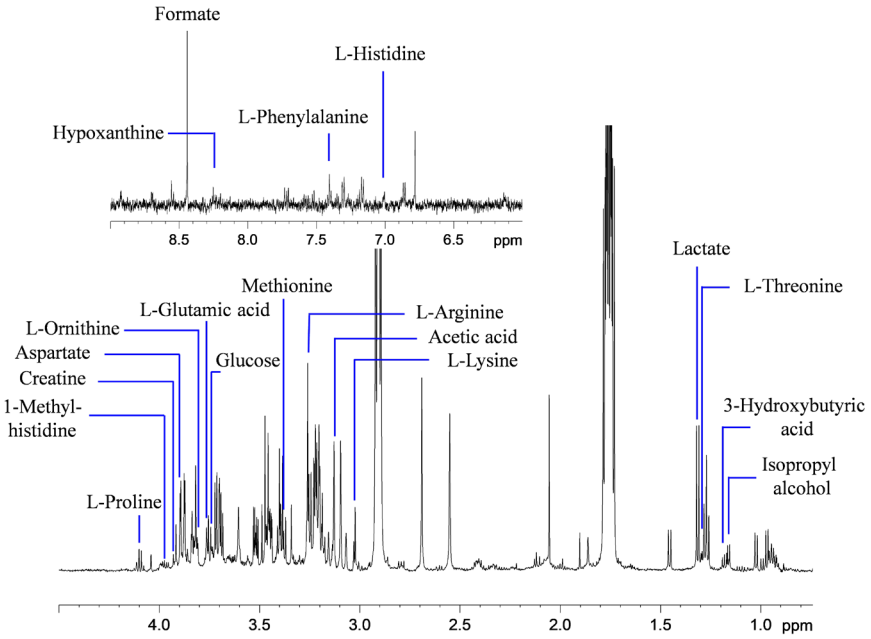

Figure 1. Representative whole blood 600 MHz ^{1}H NMR spectra.

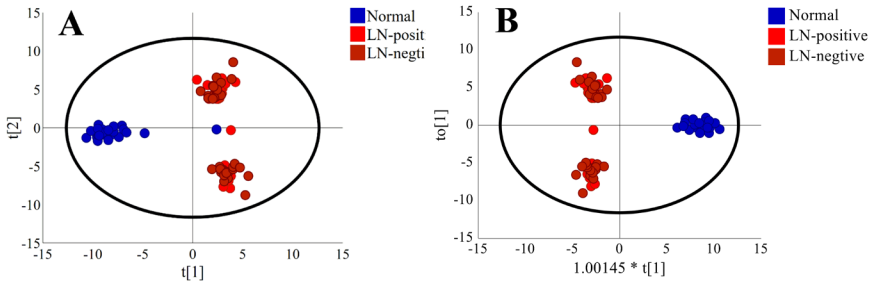

Figure 2. Metabolic profiling between the LN-negative PTC group, LN-positive PTC group and normal controls. A, principal component analysis (PCA) scores plot and **B**, O2PLS-DA scores plot.

LN-negative group and LN-positive (Figure 3) group were observed. A separation between the normal group and the PTC was shown in Figure 3A. The model parameters (R^2 = 0.954, Q^2 = 0.914) demonstrated a good quality of the obtained OPLS model. In the S-plot (Figure 3E), the variables far away from the center of the plot were assumed to have a greater contribution to the model separation. Whole blood metabolite expression patterns were observed in PTC patients and healthy controls, respectively. The pairwise PLS-DA between PTC patients and healthy controls revealed a clear separation with R^2Y (cum) = 0.77, Q^2 (cum) = 0.61 and R^2Y (cum) = 0.75, Q^2 (cum) = 0.61, respectively (Figure 3).

To identify the significantly distinguishing metabolites in discriminating between LN-negative and LN-positive PTC patients, further multivariate statistical analysis was performed. OPLS-DA was performed to separate the patients into two groups for each comparison. To the best of our knowledge, this is the first study to characterize the whole blood metabolic profile of PTC with or without LN, which is useful for identifying potential biomarkers and understanding the underlying molecular mechanism. However, the OPLS-DA score plots did not separate the two groups clearly for all three comparisons. When the patients were classified into LN-negative and LN-positive, the OPLS-DA score plot exhibited nonseparation between the two groups (Figure 3D).

We first identified 48 metabolites according to their corresponding chemical shift and multiplicity. The altered metabolite level in the whole blood of PTC patients and healthy controls was characterized. Based on the statistical analysis of the results (p < 0.05 and VIP > 1), 20 metabolites were identified as potential biomarkers for PTC and the color change for

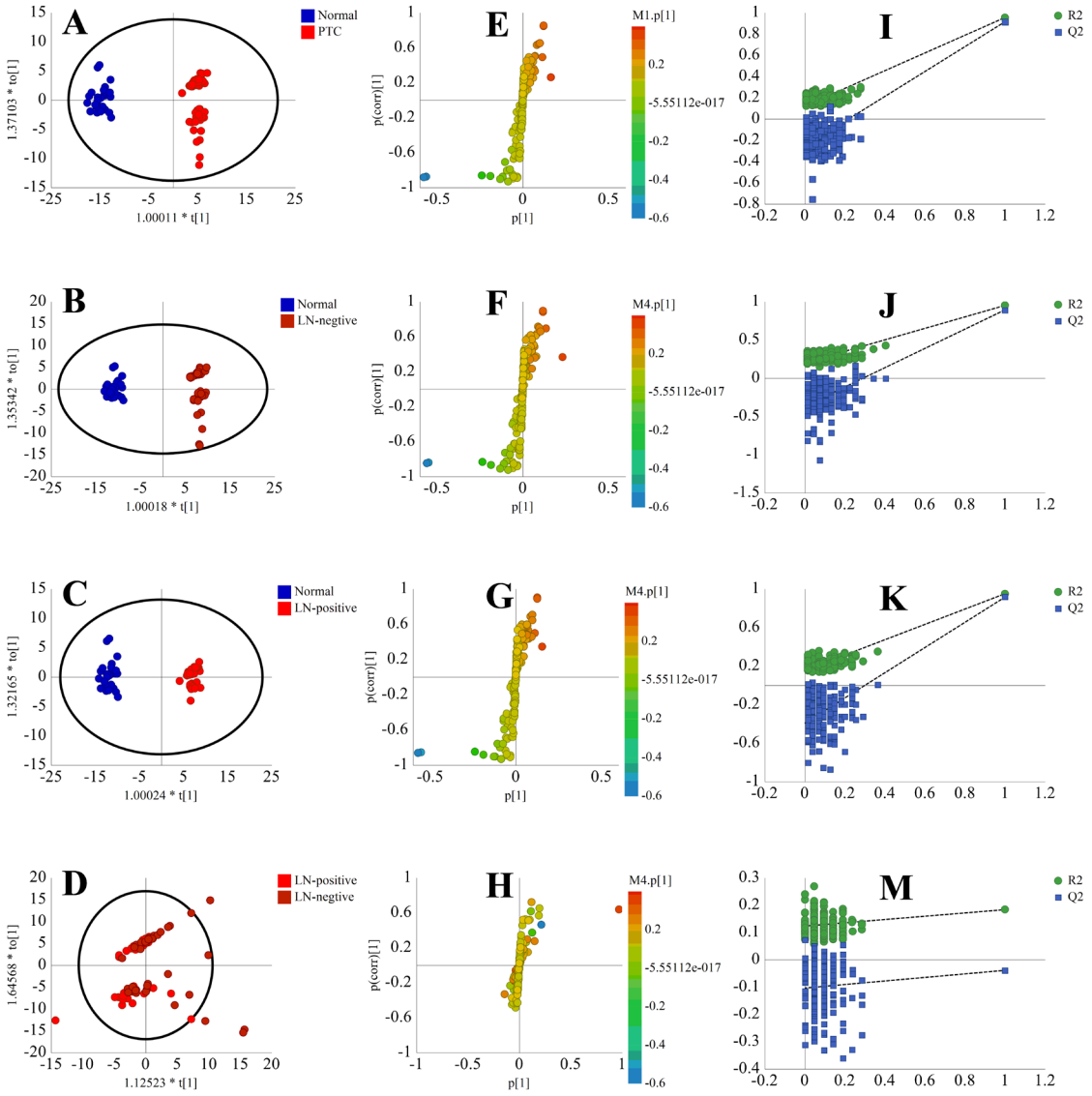

Figure 3. Metabolic profiling between the PTC and normal controls. A, B, C, and **D** are the orthogonal projection to latent structure discriminant analysis (OPLS-DA) scores plots between the PTC and normal controls, LN-negative and normal controls, LN-positive and normal controls, and LN-negative and LN-positive. **E, F, G**, and **H** are S-plots of the OPLS-DA model of the PTC and normal controls, LN-negative and normal controls, LN-positive and normal controls, and LN-negative and LN-positive, the variables that lie far away from the center of the plot were assumed to have a greater contribution to the model classification. **I, J, K**, and **M** are validation plots of the OPLS-DA model of the PTC and normal controls, LN-negative and normal controls, LN-positive and normal controls, and LN-negative and LN-positive using a permutation test that was randomly permuted 200 times. The explained variance (R2X) and predictivity (Q2Y) of the constructed model are indicated on the far right and remain higher than those of the 200 permuted models to the left.

the key metabolites according to the VIP-parameter is shown in Figure 3E and Table 2. We also evaluated the significance of the altered metabolites level in the LN-negative and LN-positive groups as compared to healthy subjects (Table 2). Compared to the normal controls, the concentrations of tyrosine, lactate, lysine, acetic acid, arginine, glutamic acid, methionine, hydroxybutyric acid, aspartate, tyrosine, acetoacetate, threonine, histidine, hypoxanthine, and formate decreased in PTC patients, whereas the concentrations of isobutyric acid, proline, 1-methylhistidine, creatine, glucose, ornithine, and isopropyl alcohol increased.

To evaluate the performance of each altered metabolite in distinguishing PTC from healthy subjects; we analyzed the diagnostic accuracy through receiver operating characteristic (ROC) curves analysis. The diagnostic accuracy in the form of the area under the ROC curve (AUC) was evaluated in the datasets of healthy *vs.* PTC, healthy *vs.* LN-negative, and healthy *vs.* LN-positive patients (Table 3). The metabolites were also used to construct an independent model, and we found that isobutyric acid, lactate, lysine, arginine, glutamic acid, methionine, proline, aspartate, tyrosine, creatine, glucose, threonine, ornithine, isopropyl alcohol, and formate performed with good diagnostic potential with AUC scores of more than 0.9 in all

Table 2. The differential metabolites among all papillary thyroid carcinoma (PTC) patients, LN-positive PTC patients, LN-negative PTC patients, and normal controls

Metabolites	Chemical shift[a] (ppm, multiplicity)	PTC *vs.* Normal			LN-positive *vs.* Normal			LN-negative *vs.* Normal		
		VIP[b]	P[c]	FC[d]	VIP[b]	P[c]	FC[d]	VIP[b]	P[c]	FC[d]
Lactate	1.32 (*d*)	1.141	9.45E-08	27.187	1.0958	6.54E-06	26.416	1.4698	6.48E-10	28.14
L-Lysine	3.03 (*t*)	2.0087	9.50E-04	0.011598	1.8146	5.45E-19	0.014733	1.7784	1.52E-16	0.0077247
Acetic acid	3.11 (*d*)	1.3528	3.29E-03	0.40239	1.1992	5.46E-07	0.43641	1.3331	5.40E-08	0.36032
L-Arginine	3.25 (*t*)	1.602	1.12E-02	0.25497	1.5119	1.81E-11	0.23638	1.4474	1.43E-09	0.27797
L-Glutamic acid	3.76 (*q*)	1.8954	2.62E-25	0.19049	1.7676	1.79E-17	0.2036	1.7881	7.82E-17	0.17427
Methionine	3.38 (*s*)	2.0955	3.07E-36	0.051506	1.9201	3.33E-23	0.061593	1.9079	3.37E-21	0.039035
L-Proline	4.10 (*m*)	1.0941	3.59E-07	9.7312	1.1348	2.67E-06	10.215	1.2316	8.19E-07	9.1315
3-Hydroxybutyric acid	1.22 (*d*)	1.2645	1.84E-09	0.2245	1.154	1.69E-06	0.22264	1.1268	9.15E-06	0.2268
Aspartate	3.90 (*q*)	2.0767	6.34E-35	0.091451	1.9829	1.67E-26	0.05952	1.8387	1.74E-18	0.13094
Tyrosine	4.42 (*m*)	2.0821	2.70E-35	0.0077964	1.8898	7.46E-22	0.0063454	1.837	1.99E-18	0.0095911
1-Methylhistidine	3.98 (*m*)	1.1721	3.72E-08	3.7893	1.1772	9.55E-07	3.5902	1.3416	4.21E-08	4.0355
Creatine	3.93 (*s*)	1.4811	2.98E-13	2.5773	1.5453	4.28E-12	2.5713	1.5156	1.16E-10	2.5848
D-Glucose	3.72 (*m*)	2.0161	3.56E-31	3.6886	1.9788	2.92E-26	3.8243	1.9263	4.92E-22	3.5207
L-Threonine	1.30 (*d*)	1.6894	3.08E-18	0.38636	1.7044	1.11E-15	0.38161	1.5688	1.30E-11	0.39223
L-Ornithine	3.81 (*t*)	1.5034	1.04E-13	2.8769	1.3843	2.25E-09	2.7964	1.3898	9.70E-09	2.9764
Isopropyl alcohol	1.17 (*d*)	1.1943	1.87E-08	3.2248	1.308	2.60E-08	2.5971	1.3666	1.99E-08	4.0013
L-Histidine	7.09 (*d*)	1.5729	2.99E-15	0.22942	1.5081	2.12E-11	0.2296	1.5272	7.33E-11	0.2292
L-Phenylalanine	7.44 (*d*)	1.1865	2.39E-08	0.30858	1.0246	2.96E-05	0.38102	1.5358	5.17E-11	0.219
Hypoxanthine	8.23 (*s*)	0.94239	1.59E-05	164.71	1.0839	8.51E-06	163.11	1.0141	8.43E-05	166.52
Formate	8.45 (*s*)	2.0866	1.32E-35	0.043805	1.9294	1.21E-23	0.032461	1.8646	1.96E-19	0.057839

[a]Multiplicity: *s* singlet, *d* doublet, *t* triplet, *q* quartet, *m* multiplet. [b]Variable importance in the projection was obtained from OPLS model. [c]*p*-value obtained from Student's *t*-test. [d]Fold change (FC) was calculated as a binary logarithm of the average mass response (normalized peak area) ratio between PTC versus normal controls, LN-positive versus normal controls or between LN-negative versus normal controls.

Table 3. The details of the ROC curves of all significantly altered metabolites in different groups

Metabolites	PTC *vs.* Normal		LN-negative *vs.* Normal		LN-positive *vs.* Normal	
	AUC	95% CI	AUC	95% CI	AUC	95% CI
Lactate	0.980	0.937-1.0	0.983	0.939-1.0	0.978	0.929-1.0
L-Lysine	0.982	0.953-0.999	0.985	0.958-1.0	0.979	0.945-0.999
Acetic acid	0.844	0.823-0.941	0.867	0.761-0.948	0.826	0.711-0.917
L-Arginine	0.954	0.893-0.991	0.951	0.887-0.99	0.957	0.901-0.995
L-Glutamic acid	0.964	0.919-0.996	0.969	0.923-1.0	0.960	0.908-1.0
Methionine	0.974	0.918-1.0	0.976	0.925-1.0	0.972	0.913-1.0
L-Proline	0.921	0.841-0.978	0.922	0.846-0.988	0.921	0.843-0.985
3-Hydroxybutyric acid	0.869	0.787-0.924	0.876	0.795-0.95	0.862	0.784-0.94
Aspartate	0.961	0.913-0.996	0.949	0.844-0.993	0.970	0.921-1.0
Tyrosine	0.973	0.93-1.0	0.970	0.923-1.0	0.975	0.937-1.0
1-Methylhistidine	0.875	0.798-0.942	0.890	0.794-0.959	0.862	0.772-0.944
Creatine	0.904	0.839-0.958	0.911	0.829-0.978	0.899	0.813-0.979
D-Glucose	0.986	0.966-1.0	0.989	0.958-1.0	0.984	0.955-1.0
L-Threonine	0.938	0.868-0.988	0.925	0.83-0.988	0.949	0.88-0.996
L-Ornithine	0.940	0.857-0.993	0.943	0.871-0.955	0.938	0.85-0.996
Isopropyl alcohol	0.935	0.853-0.984	0.945	0.872-0.997	0.926	0.838-0.989
L-Histidine	0.895	0.809-0.952	0.898	0.815-0.964	0.891	0.806-0.954
L-Phenylalanine	0.889	0.823-0.941	0.926	0.864-0.987	0.858	0.766-0.932
Hypoxanthine	0.680	0.581-0.78	0.767	0.649-0.871	0.609	0.488-0.733
Formate	0.980	0.942-1.0	0.975	0.926-1.0	0.984	0.956-1.0

PTC patients compared with the healthy subjects. To clearly demonstrate these differences, scatter plots were constructed to illustrate the relative concentration (Figure 4). In the LN-negative and LN-positive subjects, the significance of all these metabolites were similarly found in PTC patients.

3.3. Metabolic pathway analysis

A pathway analysis provides useful information about the biological roles of metabolites, which can further provide insights into the pathogenesis and mechanisms of a specific disease. To further explore the metabolic

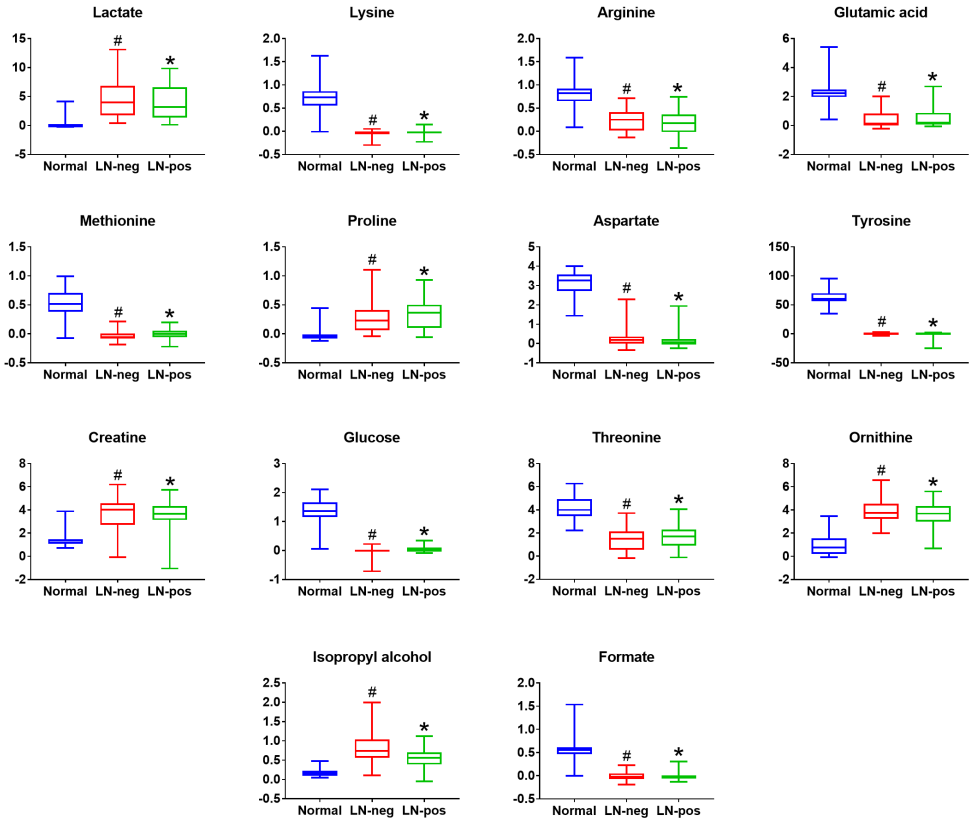

Figure 4. Scatter plots illustrating discrimination among normal controls, LN-negative and LN-positive PTC patients. The Y axis represents the relative abundance of NMR signals (normalized to the total peaks). $^{*}p < 0.01$ from the LN-positive PTC versus normal controls; $^{\#}p < 0.01$ from the LN-negative PTC versus normal controls.

Table 4. The results from pathway analysis using the software package KEGG graph

Pathway Name	Hits	*P*	-log (*p*)	Holm *p*	FDR	Impact
Histidine metabolism	4	3.3309E-5	10.31	0.0027313	9.3265E-4	0.22131
Arginine biosynthesis	4	1.8628E-5	10.891	0.0015461	7.8236E-4	0.2538
Phenylalanine, tyrosine and tryptophan biosynthesis	2	9.3496E-4	6.975	0.074797	0.015707	1.0
Arginine and proline metabolism	5	8.0243E-5	9.4304	0.0064997	0.0016851	0.34441
Phenylalanine metabolism	2	0.006693	5.0067	0.52875	0.086182	0.35714
Pyruvate metabolism	2	0.031315	3.4637	1.0	0.23913	0.14463
Alanine, aspartate and glutamate metabolism	2	0.048932	3.0173	1.0	0.31618	0.42068

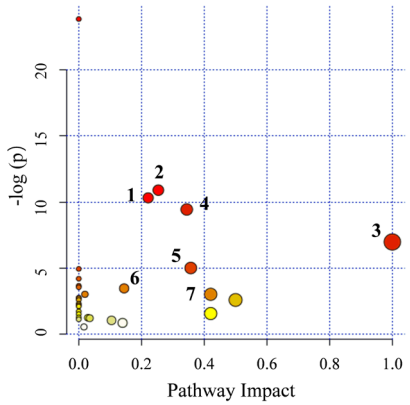

Figure 5. The impact of metabolic pathways in PTC. 1, Histidine metabolism; **2,** Arginine biosynthesis; **3,** Phenylalanine, tyrosine and tryptophan biosynthesis; **4,** Arginine and proline metabolism; **5,** Phenylalanine metabolism; **6,** Pyruvate metabolism; **7,** Alanine, aspartate and glutamate metabolism. The size and color of each circle is based on the pathway impact value and the *p*-value, respectively.

pathways that are involved in the regulation of PTC formation, the detected differential metabolites and their potential metabolic pathways were analyzed using KEGG graph software and the KEGG database. To explore metabolic pathway influences between the normal group and PTC group, pathway analysis performed by MetaboAnalyst 4.0, which combined results from powerful pathway enrichment analysis with the topology analysis.

Metabolic pathway analysis revealed that over 31 pathways were influenced (Table 4 and Figure 5). An impact value > 0.1 and a hit value > 2 were used as the threshold to identify the significantly altered metabolic pathways (*21*). Accordingly, seven potential target pathways were identified in whole blood samples, including arginine biosynthesis; histidine metabolism; arginine and proline metabolism; butanoate metabolism;

phenylalanine, tyrosine and tryptophan biosynthesis; the synthesis and degradation of ketone bodies, phenylalanine metabolism; alanine, aspartate and glutamate metabolism; and tyrosine metabolism. The pathways details are displayed in Table 4.

4. Discussion

Currently, fine-needle aspiration (FNA) cytology has been used on a growing number of patients to determine the benign or malignant nature of their thyroid nodules and enlarged lymph nodes before surgery. The natures of some nodules and lymph nodes are indeterminate with FNAB alone. Once nodules and lymph nodes are identified as uncertain in nature, this will affect the most appropriate management strategy (*22-24*). After surgery, only approximately 80% of these thyroid nodules are malignant, which indicates that about 20% of patients with benign nodules were undergoing unnecessary thyroidectomy (*25*). The prediction of PTC, including the risk prediction of lymph node metastasis, is not only significant for guiding the indication of surgery to improve prognosis but also more significant for avoiding overtreatment and improving the quality of life.

In the present study, we investigated and identified the metabolic characteristics of the whole blood samples from PTC patients and healthy controls using ^{1}H NMR-based metabolomics and multivariate pattern recognition analytical techniques for the first time. Our results revealed that the metabolic phenotype of PTC patients' blood was significantly different from that of healthy controls' blood. In detail, 20 metabolites were significantly altered in PTC patients compared with those in the healthy controls. The presence of LN in patients with PTC was also distinguished for the first time, where no clear associations was observed. Compared with the published reports of the metabolic profiling of PTC (*26-29*), we focused on the metabolite profiling of whole blood in investigations of human health and PTC patients. The identified metabolites may be the potential factors for the diagnosis and prognosis of PTC patients. They also were valuable in understanding the molecular mechanisms in the process of PTC.

The identification of specific metabolites displaying altered levels of their associative metabolic pathways can improve the understanding of the biological and pathological aspects involved in the process from normal to an eventually cancerous state. Altered pathways include changes in arginine biosynthesis; histidine metabolism; arginine and proline metabolism; butanoate metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; synthesis and degradation of ketone bodies, phenylalanine metabolism; alanine, aspartate and glutamate metabolism; and tyrosine metabolism.

Growth of cancer cells is rapid and divided into short cycles, requiring a higher energy supply to complete more biosynthesis, which requires reprograming of biochemical pathways to alter the metabolism of the cells. These alterations include increased glucose and glutamine uptake, lactate production, and abnormal biosynthesis of nucleic acids, proteins, and lipids (*30*). Deja *et al.* found that metabolic differences not only between thyroid cancer and normal tissues but also between different types of thyroid lesions, reflected the sensitivity of the metabolomic fluctuations. They concluded that the metabolic changes in thyroid carcinoma are mainly related to osmotic regulators, citrate, and amino acids that provide produce the TCA cycle (*31*).

In the present study, a decreased glucose level and increased lactate level in the LN-negative and LN-positive PTC groups compared to those in the healthy control group indicated that the energy supply pattern shifted from aerobic to anaerobic metabolism (Figure 4) (*26,29*). Creatine, phosphocreatine, and creatinine, through the creatine kinase reaction, played important roles in maintaining a constant ATP level (*32*). During arginine and proline metabolism, phosphocreatine and creatinine are the breakdown products of creatine, which reacts with ATP under the catalysis of creatine kinase (*33*).

In our results, the increased level of creatine in the PTC group suggested a disruption of their conversion to ATP, which may then cause an ATP depletion, and consequently may block the supply of energy in PTC patients. Aspartate, asparagine, glutamine, and glutamate are all involved in the alanine, aspartate, and glutamate metabolism. The decreased levels of glutamate and aspartate in PTC patients suggested that the generation from glutamine to glutamate was obstructed. The decrease in glutamate led to the distribution of the TCA cycle and the formation of glutathione.

The results of metabolomic studies are more contrasting regarding amino acids (*34*). Among the biomarkers identified, amino acids, such as glutamine, lysine, valine, and arginine were inversely associated with hepatocellular and prostate cancer risk in some prospective metabolomic studies (*35-38*). Li *et al.* analyzed normal thyroid and matched tumor tissues obtained from 16 patients and concluded that the metabolic components of PTC are characterized by increased glycolysis and inhibition of the tricarboxylic acid cycle, and an abnormal metabolism of carcinogenic amino acids, choline, and lipids (*39*).

In a similar study, Miccoli *et al.* studied 72 patients who underwent total thyroidectomy. They found that the thyroid tumor sample spectra had higher levels of lactate and acetylcholine, and lower levels of lipids and alanine than the normal sample (*40*). Our study showed that amino acid differences were mainly reflected in tyrosine, lysine, arginine, glutamic acid, methionine, aspartate, tyrosine, threonine, ornithine, and histidine. Future studies should focus on amino acid metabolisms, and on elucidating the mechanisms underlying the process of

LN of PTC before the clinical application of the FNA.

The prediction of PTC is not only significant for guiding the indication of surgery to improve prognosis but also for avoiding overtreatment and improving the quality of life. Metabolomics has the potential to change our existing understanding of the molecules involved in thyroid carcinoma, thus becoming a new diagnostic approach, providing metabonomic support for the preoperative evaluation of the nature of thyroid nodules, such as FNAB. Metabolomics can also improve our understanding of the process of cancer development and novel biomarkers. In the future, we hope to have more samples and more accurate metabonomic studies for the lymph node metastasis of papillary thyroid carcinoma, to realize the auxiliary judgment for the presence or absence of lymph node metastasis through the quantification of metabolites and metabolic pathways.

In conclusion, we were able to distinguish PTC patients from healthy controls with a high level of accuracy based on principal component analysis and linear discriminant analysis of the ^{1}H NMR metabolomic data obtained from whole blood samples. We first investigated the metabolic profiles between the LN-negative and LN-positive cancer subjects. No metabolite was found that could discriminate the presence of LN metastasis. The metabolic information obtained by ^{1}H NMR might play a significant role in screening biomarkers and in the early diagnosis of PTC cancer. Further studies with larger sample sizes are needed to elucidate significant metabolites to indicate the presence of LN metastasis in patients with PTC.

Acknowledgements

This work was supported by the Key Research, Development Program of Shandong Province (2017GSF218049) and Young Scholars Program of Shandong University (2018WLJH93), and Shandong medical and health science and technology development program (2017WS098).

References

1. Chen M, Shen M, Li Y, Liu C, Zhou K, Hu W, Xu B, Xia Y, Tang W. GC-MS-based metabolomic analysis of human papillary thyroid carcinoma tissue. Int J Mol Med. 2015; 36:1607-1614.
2. Haugen BR. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: What is new and what has changed? Cancer. 2017; 123:372-381.
3. Chan DS, Gong K, Roskies M, Forest V, Hier MP. Payne RJ. Re-visiting the ATA 2015 sonographic guidelines - who are we missing? A retrospective review. J Otolaryngol Neck Surg. 2018; 47:1-6.
4. Pagni F, Limperio V, Bono F, Garancini M, Roversi G, De Sio G, Galli M, Smith AJ, Chinello C, Magni F. Proteome analysis in thyroid pathology. Expert Rev Proteomics. 2015 12:375-390.
5. Cooper DS, Doherty GM, Haugen BR, Kloos RT, Lee SL, Mandel SJ, Mazzaferri EL, Mciver B, Pacini F, Schlumberger M. Revised American Thyroid Association Management Guidelines for Patients with Thyroid Nodules and Differentiated Thyroid Cancer. Thyroid. 2009; 19:1167-1214.
6. Wojtowicz W, Zabek A, Deja S, Dawiskiba T, Pawelka D, Glod M, Balcerzak W, Mlynarz P. Serum and urine ^{1}H NMR-based metabolomics in the diagnosis of selected thyroid diseases. Sci Rep. 2017; 7:9108.
7. German JB, Hammock BD and Watkins SM. Metabolomics: building on a century of biochemistry to guide human health. Metabolomics. 2005; 1:3-9.
8. Strand E, Tangen IL, Fasmer KE, Jacob H, Halle MK, Hoivik EA, Delvoux B, Trovik J, Haldorsen IS, Romano A. Blood Metabolites Associate with Prognosis in Endometrial Cancer. Metabolites. 2019; 9:302.
9. Bitencourt AGV, Goldberg J, Pinker K, Thakur SB. Clinical applications of breast cancer metabolomics using high-resolution magic angle spinning proton magnetic resonance spectroscopy (HRMAS ^{1}H MRS): systematic scoping review. Metabolomics. 2019; 15:148.
10. Beger RD. A review of applications of metabolomics in cancer. Metabolites. 2013; 3:552-574.
11. Wang H, Wang L, Zhang H, Deng P, Chen J, Zhou B, Hu J, Zou J, Lu W, Xiang P, Wu T, Shao X, Li Y, Zhou Z, Zhao YL. ^{1}H NMR-based metabolic profiling of human rectal cancer tissue. Mol Cancer. 2013; 12:121.
12. Nagana Gowda GA, Raftery D. Whole blood metabolomics by ^{1}H NMR spectroscopy provides a new opportunity to evaluate coenzymes and antioxidants. Anal Chem. 2017; 89:4620-4627.
13. Stringer KA, Younger JG, McHugh C, Yeomans L, Finkel MA, Puskarich MA, Jones AE, Trexel J, Karnovsky A. Whole blood reveals more metabolic detail of the human metabolome than serum as measured by ^{1}H-NMR spectroscopy: Implications for sepsis metabolomics. Shock. 2015; 44:200-208.
14. Catalan U, Rodriguez M, Ras M, Macia A, Mallol R, Vinaixa M, Fernandezcastillejo S, Valls R, Pedret A, Griffin JL. Biomarkers of food intake and metabolite differences between plasma and red blood cell matrices; a human metabolomic profile approach. Mol Biosyst. 2013; 9:1411-1422.
15. Chaleckis R, Ebe M, Pluskal T, Murakami I, Kondoh H, Yanagida M. Unexpected similarities between the Schizosaccharomyces and human blood metabolomes, and novel human metabolites. Mol Biosyst. 2014; 10:2538-2551.
16. Chaleckis R, Murakami I, Takada J, Kondoh H, Yanagida M. Individual variability in human blood metabolites identifies age-related differences. Proc Natl Acad Sci. 2016; 113:4252-4259.
17. McHugh CE, Flott TL, Schooff CR, Smiley Z, Puskarich MA, Myers DD, Younger JG, Jones AE, Stringer KA. Rapid, reproducible, quantifiable NMR metabolomics: Methanol and methanol: Chloroform precipitation for removal of macromolecules in serum and whole blood. Metabolites. 2018; 8:93.
18. Chong J, Wishart DS, Xia J. Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. Curr Protoc Bioinforma. 2019; 68:e86.
19. Chong J, Yamamoto M, Xia J. MetaboAnalystR 2.0: From raw spectra to biological insights. Metabolites. 2019; 9:57.
20. Chong J, Xia J. MetaboAnalystR: an R package for

flexible and reproducible analysis of metabolomics data. Bioinformatic. 2018; 34:4313-4314.
21. Jin H, Zhu B, Liu X, Jin J, Zou H. Metabolic characterization of diabetic retinopathy: An ^{1}H-NMR-based metabolomic approach using human aqueous humor. J Pharm Biomed Anal. 2019; 174:414-421.
22. Bailey V, Oltmann SC. Trends in radioactive iodine usage at a county hospital: How has practice changed in response to the 2015 American Thyroid Association Guidelines? J Am Coll Surg. 2019; 229:e105.
23. McGreevy A. The changing trend in thyroid surgery in a regional tertiary referral centre: a ten year review of practice. Br J Surg. 2011; 98:121-121.
24. Nikiforov YE, Ohori NP, Hodak SP, Carty SE, Lebeau SO, Ferris RL, Yip L, Seethala RR, Tublin ME, Stang MT. Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: A prospective analysis of 1056 FNA samples. J Clin Endocrinol Metab. 2011; 96:3390-3397.
25. Navascarrillo D, Rodriguez J, Montorogarcia S, Orenespinero E. High-resolution proteomics and metabolomics in thyroid cancer: Deciphering novel biomarkers. Crit Rev Clin Lab Sci. 2017; 54:446-457.
26. Seo JW, Han K, Lee J, Kim E, Moon HJ, Yoon JH, Park VY, Baek H, Kwak JY. Application of metabolomics in prediction of lymph node metastasis in papillary thyroid carcinoma. PLoS One. 2018; 13:e0193883.
27. Torregrossa L, Shintu L, Chandran JN, Tintaru A, Ugolini C, Magalhaes A, Basolo F, Miccoli P, Caldarelli S. Toward the reliable diagnosis of indeterminate thyroid lesions: A HRMAS NMR-based metabolomics case of study. J Proteome Res. 2012; 11:3317-3325.
28. Xu Y, Zheng X, Qiu Y, Jia W, Wang J, Yin S. Distinct metabolomic profiles of papillary thyroid carcinoma and benign thyroid adenoma. J Proteome Res. 2015; 14:3315-3321.
29. Zhou Q, Zhang L, Xie C, Zhang M, Wang Y, Liu G. Metabolomics as a potential method for predicting thyroid malignancy in children and adolescents. Pediatr Surg Int. 2020; 36:145-153.
30. Duarte IF, Gil AM. Metabolic signatures of cancer unveiled by NMR spectroscopy of human biofluids. Prog Nucl Magn Reson Spectrosc. 2012; 62:51-74.
31. Deja S, Dawiskiba T, Balcerzak W, Orczykpawilowicz M, Glod M, Pawelka D, Mlynarz P. Follicular adenomas exhibit a unique metabolic profile. ^{1}H NMR studies of thyroid lesions. PLoS One. 2013; 8:0084637.
32. Liu X, Zheng X, Du G, Li Z, Qin X. Brain metabonomics study of the antidepressant-like effect of Xiaoyaosan on the CUMS-depression rats by ^{1}H NMR analysis. J Ethnopharmacol. 2019; 235:141-154.
33. Liu Y, Jia H, Chang X, Ding G, Zhang H, Zou Z M. The metabolic disturbances of isoproterenol induced myocardial infarction in rats based on a tissue targeted metabonomics. Mol Biosyst. 2013; 9:2823-2834.
34. Lecuyer L, Bala AV, Deschasaux M, Bouchemal N, Triba MN, Vasson M, Rossary A, Demidem A, Galan P, Hercberg S. NMR metabolomic signatures reveal predictive plasma metabolites associated with long-term risk of developing breast cancer. Int J Epidemiol. 2018; 47:484-494.
35. Fages A, Duartesalles T, Stepien M, Ferrari P, Fedirko V, Pontoizeau C, Trichopoulou A, Aleksandrova K, Tjonneland A, Olsen A. Metabolomic profiles of hepatocellular carcinoma in a European prospective cohort. BMC Med. 2015; 13:242.
36. Huang J, Mondul AM, Weinstein SJ, Koutros S, Derkach A, Karoly ED, Sampson JN, Moore SC, Berndt SI, Albanes D. Serum metabolomic profiling of prostate cancer risk in the prostate, lung, colorectal, and ovarian cancer screening trial. Br J Cancer. 2016; 115:1087-1095.
37. Mondul AM, Moore SC, Weinstein SJ, Mannisto S, Sampson JN, Albanes D. 1-Stearoylglycerol is associated with risk of prostate cancer: results from a serum metabolomic profiling analysis. Metabolomics. 2014; 10:1036-1041.
38. Stepien M, Duartesalles T, Fedirko V, Floegel A, Barupal DK, Rinaldi S, Achaintre D, Assi N, Tjonneland A, Overvad K. Alteration of amino acid and biogenic amine metabolism in hepatobiliary cancers: Findings from a prospective cohort study. Int J Cancer. 2016; 138:348-360.
39. Li Y, Chen M, Liu C, Xia Y, Xu B, Hu Y, Chen T, Shen M, Tang W. Metabolic changes associated with papillary thyroid carcinoma: A nuclear magnetic resonance-based metabolomics study. Int J Mol Med. 2018; 41: 3006-3014.
40. Miccoli P, Torregrossa L, Shintu L, Magalhaes A, Chandran J, Tintaru A, Ugolini C, Minuto MN, Miccoli M, Basolo F, Caldarelli S. Metabolomics approach to thyroid nodules: A high-resolution magic-angle spinning nuclear magnetic resonance-based study. Surgery. 2012; 152:1118-1124.

Received July 31, 2020; Revised August 9, 2020; Accepted August 20, 2020

[§]These authors contributed equally to this work.
*Address correspondence to:
Shuqi Wang, School of Pharmaceutical Sciences, Shandong University, Jinan, 250012, China.
E-mail: wangsq@sdu.edu.cn

Released online in J-STAGE as advance publication August 27, 2020.

Brief Report

DOI: 10.5582/ddt.2020.03058

MicroRNA analysis of NCI-60 human cancer cells indicates that miR-720 and miR-887 are potential therapeutic biomarkers for breast cancer

Zhiyuan Lv[§], Shuo Wang[§], Wandong Zhao, Ningning He[*]

School of Basic Medicine, Qingdao University, Qingdao, China.

SUMMARY MicroRNAs (miRNAs) play a vital role in many biological processes, including cell growth, differentiation, apoptosis, development, differentiation, and carcinogenesis. Since miRNAs might play a part in cancer initiation and progression, they comprise an original class of promising diagnostic and prognostic molecular markers. In order to systematically understand the regulation of miRNA expression in cancers, the current study analyzed the miRNA expression profile in NCI-60 human cancer cell lines. Over 300 miRNAs exhibited unique expression profiles in cell lines derived from the same lineage. This study identified 9 lineage-specific miRNA expression patterns. Moreover, results indicated that miR-720 and miR-887 are expressed at relatively high levels in breast cancer cell lines compared to other types of cancer. Ultimately, matching NCI-60 drug response data to miR-720 and miR-887 expression profiles revealed that several FDA-approved drugs were inversely related to miR-720 and miR-887. Furthermore, the anti-cancer effect of perifosine was significantly enhanced by inhibiting miR-720 and decreased by miR-720 precursor treatment in breast cancer cell lines. 5-Fu treatment was enhanced by inhibiting miR-887 and decreased by miR-887 precursor treatment. The current results offer insight into the relationship between miRNA expression and their lineage types, and the approach used here represents a potential cancer therapy with the help of miRNAs.

Keywords miRNA, NCI-60, lineage-specific, miR-720, miR-887

1. Introduction

MicroRNAs (miRNAs) are small non-coding endogenous RNAs containing 20-25 nucleotides that regulate gene expression at the post-transcriptional level *via* sequence-specific interactions with 3'-untranslatedregions (UTRs) in miRNAs and also *via* inhibition of translation or degradation of miRNAs (*1-3*). miRNAs play a significant role in crucial biological processes, such as cell proliferation, apoptosis, development, differentiation, and metabolism, and miRNAs are especially associated with carcinogenesis (*4-8*). Several studies have indicated that aberrantly expressed miRNAs might serve as oncogenes or tumor suppressor genes in cancers (*9-12*).

NCI-60 from the Developmental Therapeutics Program (DTP) of NCI/NIH, which includes nine types of cancer lines (breast, central nervous system (CNS), colon, leukemia, melanoma, lung, ovarian, prostate, and renal) (*13,14*). Multiple high-throughput screening data is used in the NCI-60 cell line panel, including compound screening data (*15,16*), gene expression data (*17-19*), data on changes in the DNA copy number (*20*), protein analysis (*21*), DNA methylation (*22*), functional target analysis (*23*), and microRNA expression (*19,24*). This provides many opportunities at the molecular and genetic levels to identify specific pathways and mechanisms associated with cancer (*21,25*).

Because miRNAs are involved in the pathogenesis of cancer, they may be potential molecular biomarkers (*26-28*). miRNAs may help to classify cancers and predict their therapeutic response and also to identify novel targets (*29-31*). In *silico* models have provided a helpful tool for biomedical research (*32,33*). Here, data on miRNA expression by NCI-60 were used to determine the degree of miRNA enrichment in each pedigree, and 9 specific patterns of miRNA expression were identified. Moreover, matching NCI-60 drug response data to miR-720 and miR-887 expression profiles revealed several FDA-approved drugs that were closely inversely correlated with miR-720 and miR-887. Interestingly, the anti-cancer effect of perifosine treatment was improved by inhibiting miR-720 and decreased by miR-

720 precursor treatment in breast cancer cell lines. Inhibition of miR-887 can improve the anti-cancer effect of 5-Fu treatment, while inhibition of miR-887 precursor treatment can reduce the anti-cancer effect of 5-Fu treatment, especially in breast cancer cell lines. The current results provide insight into the relationship between miRNA expression and their lineages, and the approach used here can identify candidates with which to investigate drug resistance and mechanisms of sensitivity in the future.

2. Materials and Methods

2.1. Data acquisition and analysis

The GSE26375 dataset of NCI-60 miRNA expression data was obtained from Gene Expression Omnibus (GEO, *https://www.ncbi.nlm.nih.gov/geo/*). NCI-60 drug response data were obtained from the NCI/NIH DTP program (*www.dtp.nci.nih.gov*). The dataset, published in July 2012, provides the GI50 values describing the sensitivity of NCI-60 DTP human tumor cell lines to 50,839 compounds.

In order to compare the level of miRNA expression in different cancer lineages, the data on NCI-60 miRNA expression were subjected to cell line enrichment analysis (CLEA) (*34*). The priority of cell lines with specific lineages was analyzed on a receiver operating characteristic curve (ROC plot). The area under the ROC curve (AUC) is used to measure the "overexpression" of a given lineage. The *p*-value was used to calculate significance assessed through 1,000 permutations. An AUC value of 50 represents random enrichment. In the analysis of drug response data and miRNA expression data, Pearson's correlation coefficient (PCC) and its *p*-value were calculated.

2.2. The Kaplan-Meier plotter survival analysis

Kaplan Meier plotter (*http://kmplot.com/analysis/*) was used to determine the recurrence-free survival rate (RFs), and the prognostic value of a high level of miRNA expression specifically in Brest cancer samples was also evaluated. The Kaplan–Meier survival curve was plotted, and the log-rank *p* value and hazard ratio (HR) with 95% confidence intervals were calculated and plotted in R using the Bio-conductor package.

2.3. Cell line culture

All cells were purchased from ATCC, and their identity was confirmed and they were tested for contamination prior to shipment. Cells (A549, H460, H322M, MCF-7, T47-D and MDA-MB-231) were grown in RPMI medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptavidin (Gibco; Thermo Fisher Scientific, Inc.), and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.4. Quantitative real-time PCR (qPCR) analysis

In accordance with the manufacturer's instructions, RNA was isolated from tissues or cells using the mirVana miRNA Isolation Kit (Ambion; Thermo Fisher Scientific, Inc.). First-strand cDNA was synthesized using a PrimeScript 1st Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, Liaoning, China). The extraction concentration was determined with a NanoDrop spectrophotometer. The products were kept at –80˚C before further experiments. The cDNA was then amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the appropriate primers and an ABI 7500-fast thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). U6 served as the internal control. Relative expression was measured using the $2^{-\Delta\Delta CT}$ method (*35*).

2.5. MTT assay

Cell proliferation was determined using an MTT assay. 5-Fu and perifosine were purchased from Abmole (Abmole Bioscience Inc.; Houston, TX, USA). The miRNA precursor (has-miR-720 and has-miR-887), precursor-negative control, and miR-720 and miR-887 inhibitors were purchased from Biomics (Biomics Biotech; Jiangsu, Nanjing, China). The cells were seeded on a 96-well plate at a density of 2×10^3 cells per well in triplicate. After culturing for 24 h, the cells transfected with a miRNA precursor or miR-720 and miR-887 inhibitor and then treated with or without perifosine and 5-Fu. Cells were incubated for another 72 h and then their viability was measured using the MTT assay. Twenty µL of MTT (5 mg/mL) was added to the wells, and 4 h later the mixed medium was replaced with 150 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Subsequently, the 96-well plate was stirred at room temperature for 15 min. Then, the OD value of each well was determined using a fluorescence microplate reader (Sunrise Remote; Tecan Austria GmbH, Grödig, Austria) at a wavelength of 490 nm. All experiments were performed in triplicate.

2.6. Software support

Hierarchical clustering of miRNA expression in NCI-60 cancer cell lines or lineage types was performed using QCanvas for the best presentation (*36*). All images were formatted with Adobe Illustrator CS4 (Adobe Systems, Inc., San Jose, CA, USA). In order to determine significant differences between two groups, a Student's

t-test was performed to calculate the associated *p*-values. Statistical significance between multiple groups was evaluated using one-way analysis of variance (ANOVA) followed by a Newman-Keuls post hoc test using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results and Discussion

3.1. Clustering of NCI-60 cell lines based on miRNA expression

The NCI-60 miRNA expression profile consisted of > 1,000 miRNAs against 59 cancer cell lines. The unsupervised hierarchical clustering of the miRNA expression profile revealed that cell lines from the same lineage were generally located together, and this was especially true for CNS, renal, colon, ovarian, and breast cancer and melanoma (Figure 1A). To identify the expression patterns of lineage-specific miRNA, subsets of miRNAs were selected using CLEA analysis. The enrichment score (AUC value) in CLEA analysis was used to select lineage-specific miRNAs. An AUC value of 85 and a *p*-value of 0.01 were used as cutoff values to ensure that miRNAs had significant over-expression for a particular lineage type while an AUC value of 15 and a *p*-value of 0.01 were used as cutoff values for significant under-expression. As a result, a total of 332 miRNAs were found to satisfy the aforementioned criteria. These selected miRNAs were clustered hierarchically to classify the 9 pedigrees (Figure 1B).

3.2. Breast-specific miRNAs

A total of 13 miRNAs exhibited a breast cancer-

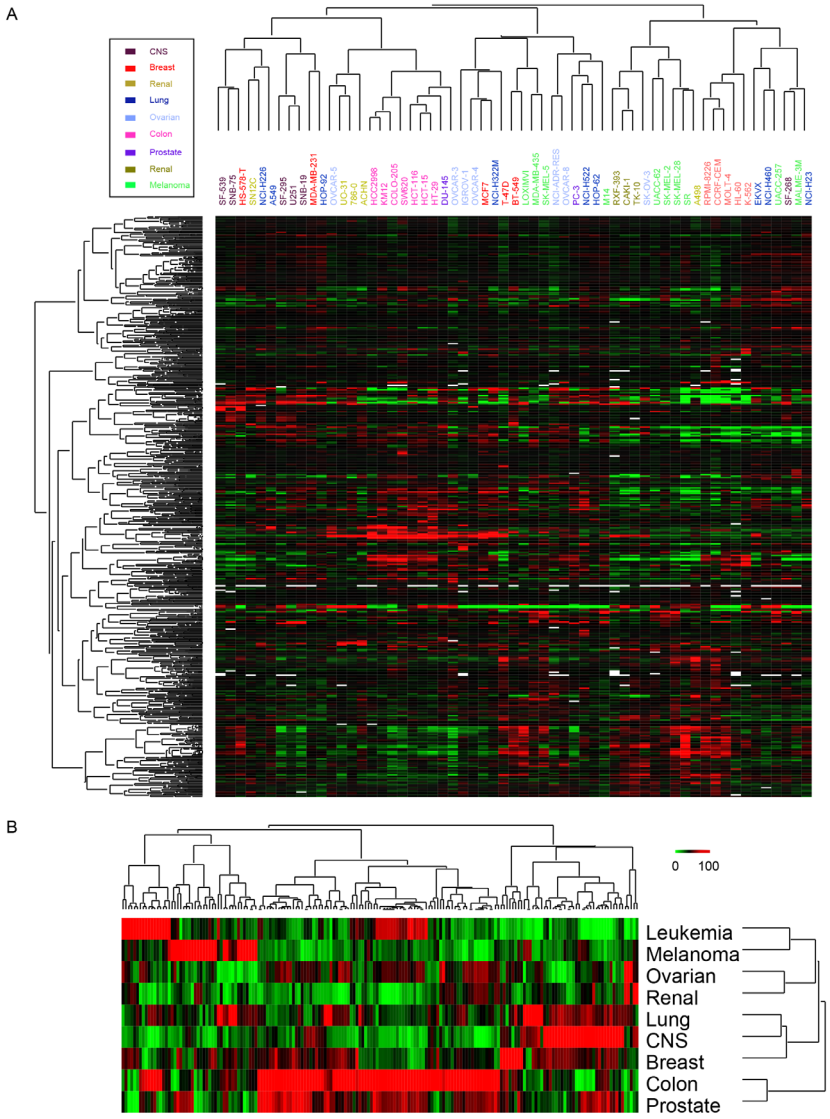

Figure 1. Hierarchical clustering of miRNA expression. (A) Expression profile of total miRNAs across NCI-60 cancer cell lines. **(B)** Expression profile of lineage-specific miRNAs across 9 lineage categories. Red represents over-expression, and green represents a down-expression.

Table 1. List of miRNAs specific to breast cancer

Gene ID	Gene Symbol	AUC	*p*-value
17583	hsa-miR-720	90	0.0013
17873	hsa-miR-887	90	0.0021
27672	hsa-miR-615-3p	91.4815	0.0013
21511	hsa-miRPlus-E1013	90	0.0008
17583	hsa-miR-720	90	0.0013
13148	hsa-miR-195	88.5185	0.0013
10941	hsa-miR-135a	88.5185	0.0016
28520	hsa-miR-454*	87.7778	0.0027
11121	hsa-miR-489	86.6667	0.0027
21704	hsa-miRPlus-E1233	85.9259	0.0025
27550	hsa-miR-572	85.9259	0.0041
17900	hsa-miR-1307	85.5556	0.0023
17943	hsa-miR-760	13.7037	0.0015

specific pattern of expression (Table 1). miR-720 and miR-887 were significantly over-expressed in breast cancer cell lines (Figures 2A and 2B). Several studies have found that miR-720 and miR-887 may be tumor suppressors in human cancers, such as renal cell carcinoma (*37*), cervical cancer (*37*), colon cancer (*38*), and breast cancer (*39*). To assess whether or not miR-720 and miR-887 are expressed at high levels in breast cancer, qPCR was used to detect miR-720 and miR-887 expression in 3 breast cancer cell lines and 3 lung cancer cell lines. miR-720 and miR-887 were significantly up-regulated in breast cancer cells (Figures 2C and 2D). Next, the prognostic value of miR-720 and miR-887 was assessed in breast cancer. High levels of miR-720 and miR-887 expression were correlated with a longer relapse-free survival (RFS) in all patients with breast cancer (HR = 1.3, $p = 0.031$ and HR = 1.29, $p = 0.02$) (Figures 2E and 2F).

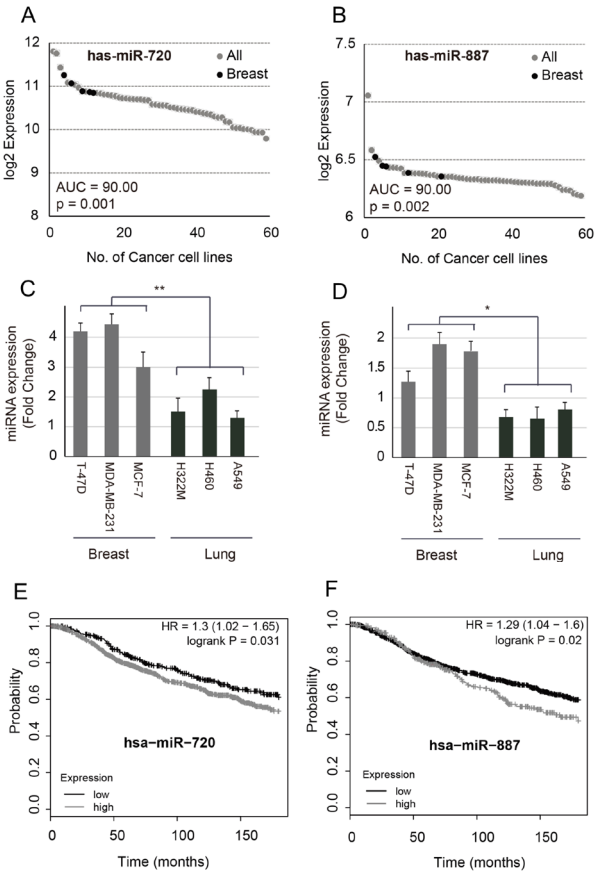

Figure 2. miR-720 and miR-887 are expressed at high levels in breast cancer cells. (A) miR-720 and **(B)** miR-887 are expressed at significantly high levels in breast cancer cell lines compared to other NCI-60 cancer cell lines. qPCR analysis of levels of **(C)** miR-720 and **(D)** miR-887 expression in lung cancer cells and breast cancer cells. The prognostic values of **(E)** miR-720 and **(F)** miR-887 in breast cancer. Data are expressed as the mean ± SEM ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ between the compared data.

3.3. miRNAs and drug response

The NCI-60 drug response dataset includes FDA-approved anticancer drugs. The expression profiles of miR-720 and miR-887 were compared to the drug response of the FDA-approved NCI-60 cancer cell line using PCC. The response to 6 drugs (chlorambucil, carmustine, perifosine, doxorubicin, plicamycin, and romidepsin) was inversely related to the expression of miR-720 (Figure 3A). The response to 4 drugs (chlorambucil, doxorubicin, 5-fluorouracil (5-Fu), and mitotane) was inversely related to the expression of miR-887 (Figure 3B). These findings may indicate that cell lines initially express high levels of miR-720 and miR-887 but that further treatment reduces those levels, indicating sensitivity to the drugs used. Perifosine is a third-generation oral alkyl phospholipid with antitumor activity (*40-42*). Perifosine has been found to be an effective and consistent Akt inhibitor in preclinical and clinical studies (*42-45*). In particular, perifosine has been found to be cytotoxic in mouse glioma, medulloblastoma, and neuroblastoma models (*45*). 5-Fu is one of the earliest and still most commonly used anticancer drugs (*46,47*). The current study verified that the anti-cancer effect of perifosine was significantly enhanced by inhibiting miR-720 (Figure 4A) and that the anti-cancer effect of 5-Fu was significantly enhanced by inhibiting miR-887 in breast cancer cells (Figure 4B). Therefore, miR-720 and miR-887, as suppressors of breast cancer, play an important role in the anti-tumor activity of chemotherapy drugs.

4. Conclusion

The biological characteristics of the NCI-60 cell lines are reflected in their miRNA landscape, as lineage-specific miRNA features are retained across all cell lines. The current study identified a number of miRNAs in each histology that displayed a lineage-specific pattern. This study determined the correlation between miR-720 and miR-887 expression and sensitivity to FDA-approved drugs. This approach can help identify new chemicals and miRNA-based biomarkers for personalized drugs. The current results indicated that miR-720 and miR-887 were over-expressed in breast

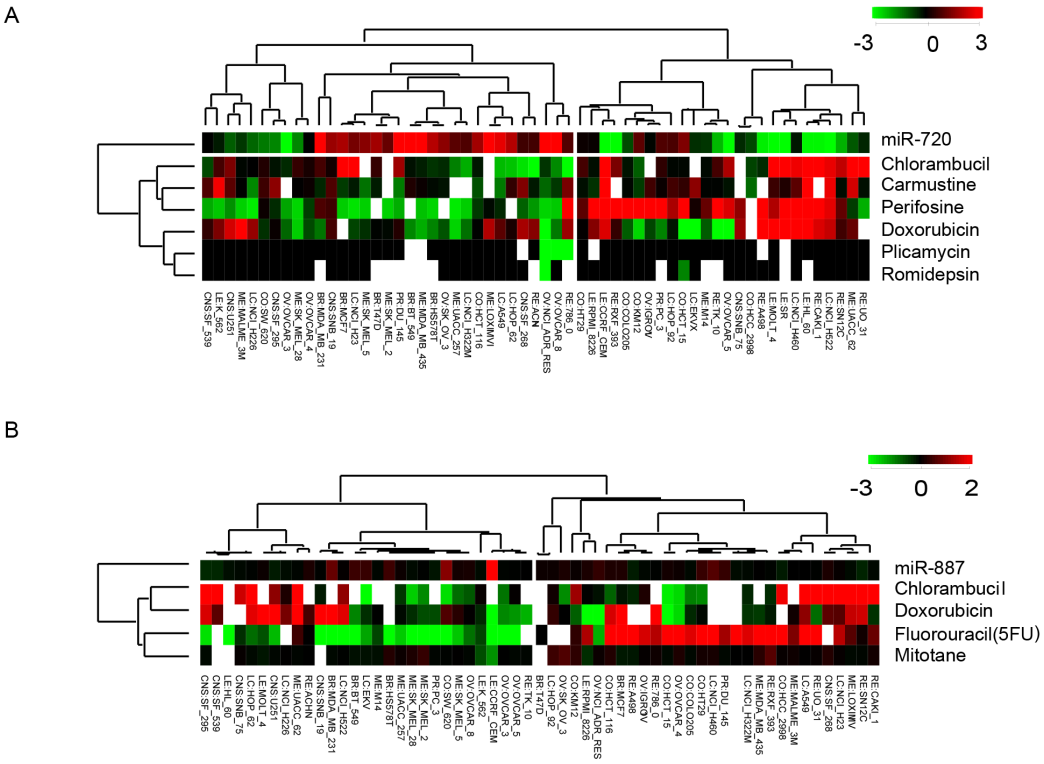

Figure 3. Correlation between miR-720 and miR-887 expression and drug sensitivity. (A) Profile of the drug response of NCI-60, which was negatively correlated with miR-720 expression. **(B)** Profile of the drug response of NCI-60, which was negatively correlated with miR-887 expression. For miRNA expression, red indicates a higher level of expression and green indicates a lower level of expression. For drug response, red indicates a sensitive response, green indicates a resistant response, and white indicates data are not available.

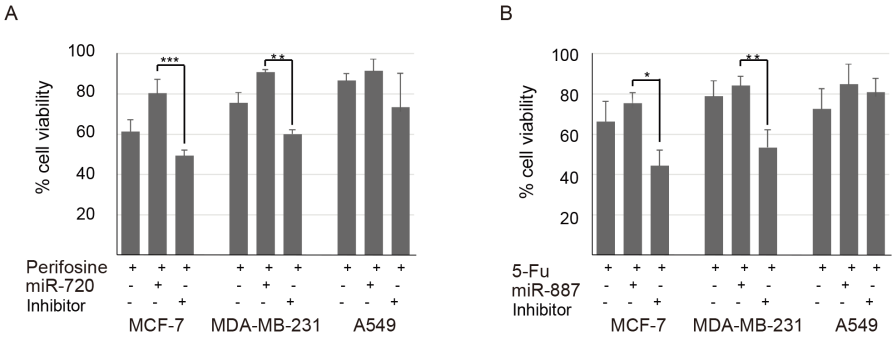

Figure 4. miR-720 and miR-887 as tumor suppressors in breast cancer. Investigation of the clinical potential of perifosine **(A)** and 5-Fu **(B)** combined with miR-720 and miR-887 inhibition for the treatment of breast cancer. Cancer cells were treated with perifosine in the presence of an miR-720 or miR-720 inhibitor and 5-Fu in the presence of miR-887 or miR-887 for 72 hours. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ between the compared groups.

cancers. Testing the combined effect of perifosine with miR-720 inhibition and 5-Fu with miR-887 inhibition revealed the enhancement of anti-cancer action. miR-720 and miR-887 are potent tumor suppressors and may become potential therapeutic tools for patients with breast cancer.

References

1. Ambros V. The functions of animal microRNAs. Nature. 2004; 431:350-355.
2. Kwak PB, Iwasaki S, Tomari Y. The microRNA pathway and cancer. Cancer Sci. 2010; 101:2309-2315.
3. Singh NK. microRNAs Databases: Developmental Methodologies, Structural and Functional Annotations. Interdiscip Sci. 2017; 9:357-377.
4. Hwang HW, Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer. 2007; 96 (Suppl):R40-44.
5. Lu J, Getz G, Miska EA, Alvarezsaavedra E, Lamb J, Peck D, Sweetcordero A, Ebert BL, Mak RH, Ferrando AA. MicroRNA expression profiles classify human cancers. Nature. 2005; 435:834-838.
6. Wang Y, Lee CG. MicroRNA and cancer--focus on apoptosis. J Cell Mol Med. 2010; 13:12-23.
7. Wu W, Sun M, Zou GM, Chen J. MicroRNA and cancer: Current status and prospective. Int J Cancer. 2010;

120:953-960.
8. Kohnehrouz BB, Bastami M, Nayeri S. *In silico* identification of novel microRNAs and targets using EST analysis in allium cepa L. Interdiscip Sci. 2018; 10:771-780.
9. Volinia S, Calin GA, Liu CG, *et al*. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006; 103:2257-2261.
10. Yang N, Kaur S, Volinia S, Greshock J, Lassus H, Hasegawa K, Liang S, Leminen A, Deng S, Smith L. MicroRNA Microarray Identifies Let-7i as a Novel Biomarker and Therapeutic Target in Human Epithelial Ovarian Cancer. Cancer Res. 2008; 68:10307.
11. Wei J, Gao W, Zhu CJ, Liu YQ, Mei Z, Cheng T, Shu YQ. Identification of plasma microRNA-21 as a biomarker for early detection and chemosensitivity of non-small cell lung cancer. Chin J Cancer. 2011; 30:407-414.
12. Liu R, Chen X, Du Y, Yao W, Shen L, Wang C, Hu Z, Zhuang R, Ning G, Zhang C. Serum microRNA expression profile as a biomarker in the diagnosis and prognosis of pancreatic cancer. Clin Chem. 2012; 58:610-618.
13. Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res. 1988; 48:4827-4833.
14. Lorenzi PL, Reinhold WC, Varma S, Hutchinson AA, Pommier Y, Chanock SJ, Weinstein JN. DNA fingerprinting of the NCI-60 cell line panel. Mol Cancer Ther. 2009; 8:713-724.
15. Weinstein JN. Spotlight on molecular profiling: "Integromic" analysis of the NCI-60 cancer cell lines. Mol Cancer Ther. 2006; 5:2601-2605.
16. Mondal SI, Khadka B, Akter A, Roy PK, Sultana R. Computer based screening for novel inhibitors against Vibrio cholerae using NCI diversity set-II: an alternative approach by targeting transcriptional activator ToxT. Interdiscip Sci. 2014; 6:108-117.
17. Blower PE, Verducci JS, Lin S, Zhou J, Chung JH, Dai Z, Liu CG, Reinhold W, Lorenzi PL, Kaldjian EP, Croce CM, Weinstein JN, Sadee W. MicroRNA expression profiles for the NCI-60 cancer cell panel. Mol Cancer Ther. 2007; 6:1483-1491.
18. Shankavaram UT, Reinhold WC, Nishizuka S, Major S, Morita D, Chary KK, Reimers MA, Scherf U, Kahn A, Dolginow D. Transcript and protein expression profiles of the NCI-60 cancer cell panel: an integromic microarray study. Mol Cancer Ther. 2007; 6:820-832.
19. Liu H, D'Andrade P, Fulmer-Smentek S, Lorenzi P, Kohn KW, Weinstein JN, Pommier Y, Reinhold WC. mRNA and microRNA expression profiles of the NCI-60 integrated with drug activities. Mol Cancer Ther. 2010; 9:1080-1091.
20. Ikediobi ON, Davies H, Bignell G, *et al.* Mutation analysis of 24 known cancer genes in the NCI-60 cell line set. Mol Cancer Ther. 2006; 5:2606-2612.
21. Nishizuka S, Charboneau L, Young L, Major S, Reinhold WC, Waltham M, Kouros-Mehr H, Bussey KJ, Lee JK, Espina V, Munson PJ, Petricoin E, 3rd, Liotta LA, Weinstein JN. Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays. Proc Natl Acad Sci U S A. 2003; 100:14229-14234.
22. Ehrich M, Turner J, Gibbs P, Lipton L, Giovanneti M, Cantor C, van den Boom D. Cytosine methylation profiling of cancer cell lines. Proc Natl Acad Sci U S A. 2008; 105:4844-4849.
23. Lee JS, Paull K, Alvarez M, Hose C, Monks A, Grever M, Fojo AT, Bates SE. Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute drug screen. Mol Pharmacol. 1994; 46:627-638.
24. Ma Q, Song J, Chen Z, Li L, Wang B, He N. Identification of potential gene and microRNA biomarkers for colon cancer by an integrated bioinformatical approach. Transl Cancer Res. 2018; 7:17-29.
25. Acharya PP, Khatri HR, Janda S, Zhu J. Synthesis and antitumor activities of aquayamycin and analogues of derhodinosylurdamycin A. Org Biomol Chem. 2019; 17:2691-2704.
26. Bhagirath D, Yang TL, Bucay N, Sekhon K, Majid S, Shahryari V, Dahiya R, Tanaka Y, Saini S. MicroRNA-1246 is an exosomal biomarker for aggressive prostate cancer. Cancer Res. 2018; 78:1833-1844.
27. Chand M, Keller DS, Mirnezami R, Bullock M, Bhangu A, Moran B, Tekkis PP, Brown G, Mirnezami A, Berho M. Novel biomarkers for patient stratification in colorectal cancer:A review of definitions,emerging concepts,and data. World J Gastrointest Oncol. 2018; 10:145-158.
28. Huang Y, Zhu J, Li W, Zhang Z, Xiong P, Wang H, Zhang J. Serum microRNA panel excavated by machine learning as a potential biomarker for the detection of gastric cancer. Oncol Rep. 2018; 39:1338-1346.
29. Gao X, Wu Y, Yu W, Li H. Identification of a seven-miRNA signature as prognostic biomarker for lung squamous cell carcinoma. Oncotarget. 2016; 7:81670-81679.
30. Li X, Shi Y, Yin Z, Xue X, Zhou B. An eight-miRNA signature as a potential biomarker for predicting survival in lung adenocarcinoma. J Transl Med. 2014; 12:159.
31. Gaur P, Chaturvedi A. Clustering and Candidate Motif Detection in Exosomal miRNAs by Application of Machine Learning Algorithms. Interdiscip Sci. 2017;1-9.
32. Lankapalli AR, Kannabiran K. Interaction of marine Streptomyces compounds with selected cancer drug target proteins by *in silico* molecular docking studies. Interdiscip Sci. 2013; 5:37-44.
33. Singh AK, Misra K. Human papilloma virus 16 E6 protein as a target for curcuminoids, curcumin conjugates and congeners for chemoprevention of oral and cervical cancers. Interdiscip Sci. 2013; 5:112-118.
34. Kim N, He N, Kim C, Zhang F, Lu Y, Yu Q, Stemke-Hale K, Greshock J, Wooster R, Yoon S, Mills GB. Systematic analysis of genotype-specific drug responses in cancer. Int J Cancer. 2012; 131:2456-2464.
35. Song J, Ma Q, Hu M, Qian D, Wang B, He N. The Inhibition of miR-144-3p on Cell Proliferation and Metastasis by Targeting TOP2A in HCMV-Positive Glioblastoma Cells. Molecules. 2018; 23:3259.
36. Kim N, Park H, He N, Lee HY, Yoon S. QCanvas: An Advanced Tool for Data Clustering and Visualization of Genomics Data. Genomics Inform. 2012; 10:263-265.
37. Bhat NS, Colden M, Dar AA, Saini S, Arora P, Shahryari V, Yamamura S, Tanaka Y, Kato T, Majid S, Dahiya R. MicroRNA-720 Regulates E-cadherin-alphaE-catenin Complex and Promotes Renal Cell Carcinoma. Mol Cancer Ther. 2017; 16:2840-2848.
38. Nonaka R, Miyake Y, Hata T, *et al.* Circulating miR-103

and miR-720 as novel serum biomarkers for patients with colorectal cancer. Int J Oncol. 2015; 47:1097-1102.
39. Fite K, Gomez-Cambronero J. Down-regulation of MicroRNAs (MiRs) 203, 887, 3619 and 182 Prevents Vimentin-triggered, Phospholipase D (PLD)-mediated Cancer Cell Invasion. J Biol Chem. 2016; 291:719-730.
40. Becher OJ, Millard NE, Modak S, *et al.* A phase I study of single-agent perifosine for recurrent or refractory pediatric CNS and solid tumors. PLoS One. 2017; 12:e0178593.
41. Kondapaka SB, Singh SS, Dasmahapatra GP, Sausville EA, Roy KK. Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. Mol Cancer Ther. 2003; 2:1093-1103.
42. Hideshima T, Catley L, Yasui H, Ishitsuka K, Raje N, Mitsiades C, Podar K, Munshi NC, Chauhan D, Richardson PG. Perifosine, an oral bioactive novel alkylphospholipid, inhibits Akt and induces *in vitro* and *in vivo* cytotoxicity in human multiple myeloma cells. Blood. 2006; 107:4053-4062.
43. Li Z, Tan F, DJ L, SM S, CJ T. *In vitro* and *in vivo* inhibition of neuroblastoma tumor cell growth by AKT inhibitor perifosine. J Natl Cancer Inst. 2010; 102:758-770.
44. Li Z, Oh DY, Nakamura K, Thiele CJ. Perifosine-induced inhibition of Akt attenuates BDNF/TrkB-induced chemoresistance in neuroblastoma *in vivo*. Cancer. 2011; 117:5412.
45. Momota H, Nerio E, Holland EC. Perifosine inhibits multiple signaling pathways in glial progenitors and cooperates with temozolomide to arrest cell proliferation in gliomas *in vivo*. Cancer Res. 2005; 65:7429-7435.
46. Pennathur A, Gibson MK, Jobe BA, Luketich JD. Oesophageal carcinoma. Lancet. 2013; 381:400-412.
47. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer. 2003; 3:330.

Received July 21, 2020; Revised August 16, 2020; Accepted August 25, 2020

[§]These authors contributed equally to this work.

**Address correspondence to:*
Ningning He, School of Basic Medicine, Qingdao University, #38 Dengzhou Road, Qingdao, China.
E-mail: heningning@qdu.edu.cn

Released online in J-STAGE as advance publication August 29, 2020.

Brief Report

DOI: 10.5582/ddt.2020.03060

Phenotypic analysis of human CYP2C9 polymorphisms using fluorine-substituted tolbutamide

Yuki Kitamura[1], Ken-ichi Saeki[1,2,*]

[1] College of Pharmacy, Kinjo Gakuin University, Nagoya, Aichi, Japan;
[2] Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Aichi, Japan.

SUMMARY To investigate the effect of fluorine substitution on tolbutamide (TB) hydroxylation catalyzed by CYP2C9, the hydroxylation of TB and its fluorinated derivative 3'-fluoro-tolbutamide (3'-F-TB) by recombinant human CYP2C9*1, CYP2C9*2, and CYP2C9*3 was analyzed. In general, fluorine substitution near the metabolic site may decrease enzymatic oxidation owing to its electron-withdrawing nature. Fluorine substitution reduced the Michaelis–Menten-derived K_m of 4'-hydroxylation of TB catalyzed by CYP2C9*1 from 115 (TB) to 77 (3'-F-TB) μM. In the case of TB hydroxylation catalyzed by CYP2C9*2, the K_m value of TB was also reduced by fluorine substitution from 129 to 88 μM. The greatest effect of fluorine substitution on the K_m in TB hydroxylation was observed in the catalysis by CYP2C9*3, in which the K_m value decreased from 287 to 117 μM. When a mixture containing TB and 3′-F-TB was hydroxylated by CYP2C9, the hydroxylated metabolite ratio in CYP2C9*3 was significantly increased compared with that in CYP2C9*1 and CYP2C9*2 ($p < 0.01$, Tukey–Kramer test). These results suggest that obtaining the metabolite profiles of fluorine-substituted analogs of the key substrate molecule may be useful as a new tool for phenotyping polymorphic CYP isoforms.

Keywords fluorine substitution, tolbutamide, CYP2C9 polymorphism, phenotyping

1. Introduction

CYP2C9 catalyzes the metabolism of several clinically used drugs, including S-warfarin, phenytoin, flurbiprofen, and tolbutamide (TB) (*1-4*). Human CYP2C9 has three major polymorphic isozymes: CYP2C9*1 (wild-type), CYP2C9*2 (Arg144Cys), and CYP2C9*3 (Ile359Leu) (5). The genetic variability of CYP2C9 is dominated by the *2 allele (11.7%) and *3 allele (7%) in Europeans, whereas the major allele in Asian and African populations is *3 (3.4% in East Asians and 11.3% in South Asians) (*6,7*). Generally, the difference in metabolic activity among the CYP2C9 isozymes may vary considerably. It is suggested that CYP2C9*2 shows moderate activity and CYP2C9*3 shows poor activity compared with that shown by CYP2C9*1 in the metabolism of most CYP2C9 substrates (*8*). Additionally, CYP2C9*3 has been reported to reduce the hydroxylation activity of TB and phenytoin (*9,10*).

Fluorine substitution of a hydrogen atom often exerts so-called mimic effects on interactions with biological molecules because the covalent and van der Waals' radii of the fluorine atom are similar to those of the hydrogen atom (*11*). However, the replacement of hydrogen with fluorine changes not only the distribution of electron density within the molecule but also the electric repulsive/attractive interactions with intra/intermolecular environments, which may significantly affect enzyme–substrate and enzyme–inhibitor interactions. In our previous study, the inhibitory effects of fluorinated benzo[h]quinolines (BhQ) on drug metabolism by recombinant human CYP2C9 were analyzed, and the position-specific substitution by a fluorine atom was shown to alter the ability of BhQ to inhibit CYP2C9 (*11*). The present study aimed to determine whether fluorine substitution improves the decrease in metabolic activity catalyzed by CYP2C9 polymorphic isozymes. In this study, we compared the changes in hydroxylation metabolism by CYP2C9*1, CYP2C9*2, and CYP2C9*3 using TB and 3'-fluoro-tolbutamide (3'-F-TB) as a fluorine-substituted TB analog (Figure 1).

2. Materials and Methods

2.1. Materials

Microsome preparations from baculovirus-infected insect cells coexpressing CYP2C9*1, *2, or *3 with

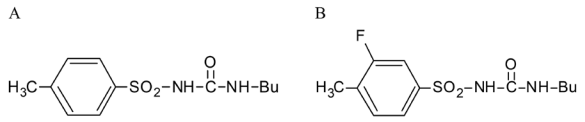

Figure 1. The chemical structures of (A) TB and (B) 3'-F-TB.

NADPH-CYP oxidoreductase were purchased from Gentest (Woburn, MA, USA); NADP, glucose-6-phosphate (G6P), and G6P dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan); and TB, 4'-hydroxytolbutamide, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, Mo, USA). The melting point was determined using a MP-500D (Yamato Scientific, Tokyo, Japan) micro melting point apparatus without correction. Mass spectra (electron impact ionization) were measured using an AX505HA spectrometer (JEOL, Tokyo, Japan). ^{1}H-NMR spectra were recorded using a JNM-A500 spectrometer (JEOL) in $CDCl_3$ using tetramethylsilane as an internal standard.

2.2. Synthesis of 3'-F-TB

4-[^{18}F] fluorotolbutamide, a fluorine-substituted TB analog, is used in positron analysis as an imaging reagent for pancreatic islets of Langerhans. 4-[^{18}F] fluorotolbutamide has previously been synthesized by a nucleophilic addition reaction of 4-[^{18}F] fluorobenzensulfonamide and butyl isocyanate (*12,13*). Similarly, 3'-F-TB was synthesized from 3-fluoro-4-metylbenzensulfonamide (3-F-4-Me-BSA) and butyl isocyanate. The melting point was 123-125°C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.91 (m, 3H, H-4'), 1.25-1.39 (m, 2H, H-3'), 1.43-1.52 (m, 2H, H-2'), 2.35 (d, 3H, CH_3), 3.13-3.22 (m, 2H, H-1'), 4.46 (br, 1H, 1-NH), 6.44 (br, 1H, 3-NH), 7.34 (t, 1H, H-5''), 7.55(dd, 1H, H-2''), 7.61 (dd, 1H, H-6''); $J_{F\text{-}CH3}$ = 2.1, $J_{2''\text{-}F}$ = $J_{5''\text{-}6''}$ = 8.7, $J_{2''\text{-}6''}$ = 2.0 Hz. HR-MS *m/z*: 288.0942, Calcd for $C_{12}H_{17}FN_2O_3S$: 288.0944.

2.3. Determination of CYP2C9 activity by TB hydroxylation

The determination of TB hydroxylase activity catalyzed by CYP2C9 was performed according to previous reports with slight modifications (*11,14*). Briefly, the incubation mixture (50 µL in a 1.5-mL microtube) contained 0.1 M potassium phosphate buffer (pH 7.4), 1.3 mM NADP, 3.3 mM G6P, 3.3 mM $MgCl_2$, 0.08 units of G6P dehydrogenase, TB, or 3'-F-TB, and 5.0 pmol CYP (approximately 8 µg protein). After incubation at 37°C for 40 min, the resulting metabolites were extracted with 2.5 volumes of ethyl acetate, and the organic solvent layer was evaporated. The residue was dissolved in 30 µL of a solution containing 0.01 M HCl and 20% CH_3CN and analyzed by HPLC. HPLC analysis was performed using a Shimadzu liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Model LC-10A solvent delivery system (Shimadzu), Model SPD-10AV UV-vis spectrophotometric detector (Shimadzu), and Wakosil II 5C18RS (ODS) column (2 mm × 150 mm) (Fujifilm Wako Pure Chemical, Osaka, Japan). The solvent system consisted of 40% CH_3CN-H_2O. The flow rate was 0.15 mL/min. Hydroxylated TB (OH-TB) was quantified from the peak areas measured by ultraviolet absorption at 236 nm with reference to 10 µM 4'-hydroxytolbutamide as the standard sample. Hydroxylated 3'-F-TB (3'-F-OH-TB) was semi-quantitatively determined from the integrated area measured at 236 nm, assuming that the ε value was the same as that of OH-TB. At least four independent experiments were performed.

2.4. Statistical analysis

The ratio of the hydroxylated metabolites of TB and 3'-F-TB was statistically analyzed using one-way analysis of variance with the Tukey-Kramer test (SPSS Statistics version 24; IBM, Armonk, NY, USA). A p value of < 0.05 was considered statistically significant.

3. Results and Discussion

Using TB and 3'-F-TB as substrates, the changes in hydroxylation metabolism by CYP2C9*1, CYP2C9*2, and CYP2C9*3 were compared. To determine the metabolic rate, the peak area of the hydroxylation reactions catalyzed by CYP2C9 was measured by comparison with that of 10 µM 4'-hydroxytolbutamide as the standard sample. The kinetics of metabolite formation by CYP2C9 was assessed in quadruplicate, and the substrate concentration ranged from 0.1 to 1.0 mM. V_{max} and K_m were then calculated using Eadie–Hofstee plots (Figure 2 and Table 1). Fluorine substitution reduced the Michaelis–Menten-derived K_m of TB 4'-hydroxylation catalyzed by CYP2C9*1 from 115 (TB) to 77 (3'-F-TB) µM. In the case of TB hydroxylation by CYP2C9*2, the K_m value was also reduced from 129 to 88 µM by fluorine substitution. The largest effect of fluorine substitution on the K_m value in TB hydroxylation was observed in the case of catalysis by CYP2C9*3, in which the K_m decreased from 287 to 117 µM.

The mixture containing equal amounts of TB and 3'-F-TB was quantified as the ratio of the hydroxylated metabolite by CYP2C9*1, CYP2C9*2, and CYP2C9*3 (Table 2). The ratio of OH-TB compared with that of 3'-F-OH-TB in the combination metabolism of 100 µM TB and 100 µM 3'-F-TB showed almost the same tendency as the ratio of K_m values, and the hydroxylated metabolite ratio (3'-F-OH-TB/OH-TB) obtained from the metabolism by CYP2C9*3 was significantly higher than that of CYP2C9*1 and CYP2C9*2 (p < 0.01,

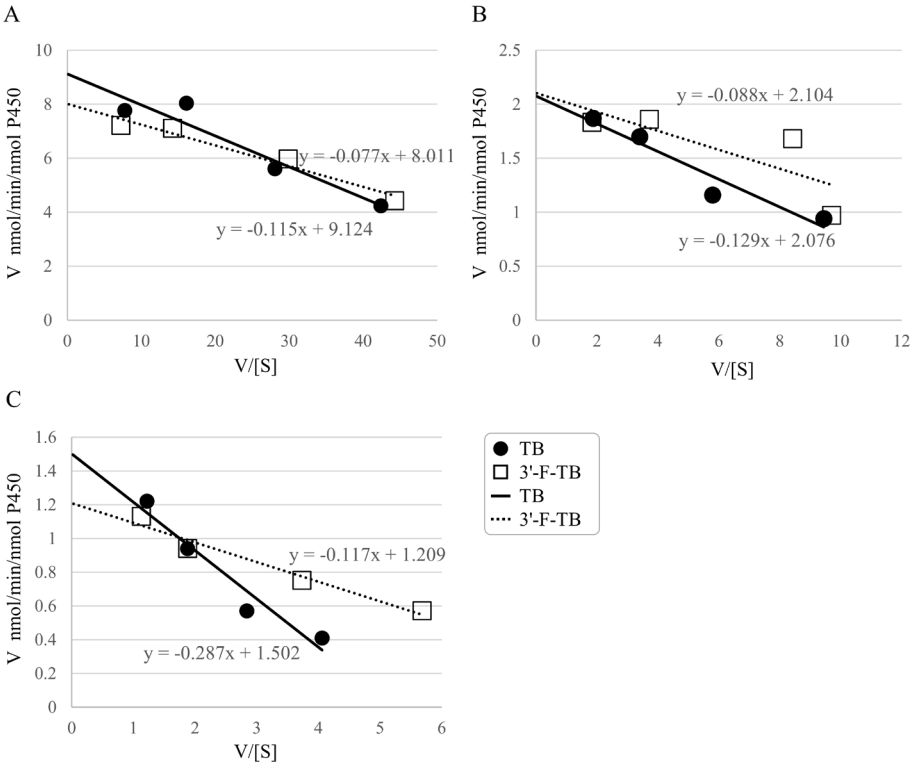

Figure 2. Eadie–Hofstee plots for the hydroxylation of TB and 3'-F-TB by (A) CYP2C9*1, (B) CYP2C9*2, and (C) CYP2C9*3. The hydroxylation catalyzed by CYP2C9 was quantified by comparison with 10 μM 4'-hydroxytolbutamide as the standard sample. Reactions were performed in quadruplicate, and the concentration of TB or 3'-F-TB ranged from 0.1 to 1.0 mM. Each point represents the mean of quadruplicate determinations. TB (black circles and solid lines) and 3'-F-TB (white squares and dotted lines).

Table 1. Hydroxylation metabolism parameters of TB and 3'-F-TB

Items	TB	3'-F-TB
CYP2C9*1		
V_{max} (nmol/min/nmol P450)	9.12	8.01
K_m (μM)	115	77
K_m ratio (TB/3'-F-TB)	1.49	
CYP2C9*2		
V_{max} (nmol/min/nmol P450)	2.08	2.10
K_m (μM)	129	88
K_m ratio (TB/3'-F-TB)	1.47	
CYP2C9*3		
V_{max} (nmol/min/nmol P450)	1.50	1.21
K_m (μM)	287	117
K_m ratio (TB/3'-F-TB)	2.45	

Table 2. Ratio of the hydroxylated metabolite (3'-F-OH-TB/OH-TB)

Items	3'-F-OH-TB/OH-TB
CYP2C9*1	1.73 ± 0.09
CYP2C9*2	1.67 ± 0.09
CYP2C9*3	2.28 ± 0.14$^{*,\#}$

Data are expressed as the mean ± S.D. (CYP2C9*1; $n = 7$, CYP2C9*2; $n = 8$, CYP2C9*3; $n = 8$). $^{*}p < 0.01$, *vs*. CYP2C9*1 by the Tukey–Kramer test. $^{\#}p < 0.01$, *vs*. CYP2C9*2 by the Tukey–Kramer test.

Tukey–Kramer test).

Many gene polymorphisms in CYP2C9 have been discovered, and it is well recognized that differences in genotypes cause differences in metabolic activity (*15*). It is important to determine the differences in metabolic activity among each isozyme to understand how they affect drug efficacy and safety, especially considering the possibility of serious side effects due to a reduction in the metabolic capacity of CYP2C9*3. It has been reported that the human plasma area under the curve of OH-TB and the ratio of OH-TB/TB were approximately twice as high in individuals with the wild-type CYP2C9 genotype than in heterozygous CYP2C9*3 individuals (*16*). In fact, we showed that the K_m value of TB in CYP2C9*1 was reduced compared with that of CYP2C9*3 (Table 1). In this study, we compared the changes in hydroxylation metabolism by CYP2C9 polymorphisms using a fluorine-substituted TB analog. 3'-F-TB reduced the K_m and increased the production of hydroxylated metabolites compared with TB (Table 1). These results indicate that fluorine substitution increases the affinity for CYP2C9 and is susceptible to hydroxylation by CYP2C9. The effect of fluorine substitution on TB suggests that TB hydroxylation in humans with CYP2C9*3 may be enhanced to the same extent as CYP2C9*1.

Phenotypic analysis directly reflects various external environments, intrinsic factors, and genotypes

that affect CYP activity, unlike genetic analysis (*17*). Therefore, phenotypic analysis is considered to provide more useful information than genetic analysis in clinical practice. However, very few simple and quick phenotype analysis methods have been established (*17*). When the mixture containing TB and 3'-F-TB was hydroxylated by CYP2C9, the hydroxylated metabolite ratio (3'-F-OH-TB/OH-TB) in CYP2C9*3 was significantly increased compared with that in CYP2C9*1 and CYP2C9*2 (Table 2). This result indicates that fluorine substitution in TB suppressed the decreased metabolic activity by CYP2C9*3 and/or the metabolism of TB was reduced by CYP2C9*3. It was reported that the inhibition of CYP2C9 by BhQ and its fluorinated derivatives varied with the position of the substituted fluorine (*11*). The effects of fluorine substitution on the inhibition of TB hydroxylation catalyzed by CYP2C9*1 were different compared with the effects on the hydroxylation catalyzed by CYP2C9*2 and CYP2C9*3 (*11*). Additionally, fluorine substitution in quinoline and 4-methylquinoline affected their mutagenicity and the formation of hydroxylated metabolites (*18,19*). Fluorine substitution could alter the metabolic profiles of other substrates, such as phenytoin, in addition to TB, which may enhance their efficacy and reduce their side effects.

In conclusion, we suggest that obtaining the metabolite profiles of fluorine-substituted analogs of the key substrate molecule may be useful as a new tool for phenotyping polymorphic CYP isoforms. Future studies should develop probes that can differentiate between the various metabolic enzymes in human liver microsomes to estimate the clinical relevance at *in vitro* levels. The development of compounds with a large difference in the K_m ratio between CYP2C9*1 and CYP2C9*3 may contribute to a new method for phenotypic analysis.

Acknowledgements

We are grateful to Hiroaki Amano and Noriko Matsuyama from the Graduate School of Pharmaceutical Sciences at Nagoya City University for their help with the experiments.

References

1. Rettie AE, Korzekwa KR, Kunze KL, Lawrence RF, Eddy AC, Aoyama T, Gelboin HV, Gonzalez FJ, Trager WF. Hydroxylation of warfarin by human cDNA-expressed cytochrome P-450: a role for P-4502C9 in the etiology of (S)-warfarin-drug interactions. Chem Res Toxicol. 1992; 5:54-59.
2. Veronese ME, Mackenzie PI, Doecke CJ, McManus ME, Miners JO, Birkett DJ. Tolbutamide and phenytoin hydroxylations by cDNA-expressed human liver cytochrome P4502C9. Biochem Biophys Res Commun. 1991; 175:1112-1118.
3. Srivastava PK, Yun CH, Beaune PH, Ged C, Guengerich FP. Separation of human liver microsomal tolbutamide hydroxylase and (S)-mephenytoin 4'-hydroxylase cytochrome P-450 enzymes. Mol Pharmacol. 1991; 40:69-79.
4. Veronese ME, Miners JO, Randles D, Gregov D, Birkett DJ. Validation of the tolbutamide metabolic ratio for population screening with use of sulfaphenazole to produce model phenotypic poor metabolizers. Clin Pharmacol Ther. 1990; 47:403-411.
5. Lee CR, Goldstein JA, Pieper JA. Cytochrome P450 2C9 polymorphisms: a comprehensive review of the in-vitro and human data. Pharmacogenetics. 2002; 12:251-263.
6. Kirchheiner J, Brockmoller J. Clinical consequences of cytochrome P450 2C9 polymorphisms. Clin Pharmacol Ther. 2005; 77:1-16.
7. Zhou Y, Ingelman-Sundberg M, Lauschke VM. Worldwide distribution of cytochrome P450 alleles: A meta-analysis of population-scale sequencing projects. Clin Pharmacol Ther. 2017; 102:688-700.
8. Yasar U, Eliasson E, Forslund-Bergengren C, Tybring G, Gadd M, Sjoqvist F, Dahl ML. The role of CYP2C9 genotype in the metabolism of diclofenac *in vivo* and *in vitro*. Eur J Clin Pharmacol. 2001; 57:729-735.
9. Goldstein JA, de Morais SM. Biochemistry and molecular biology of the human CYP2C subfamily. Pharmacogenetics. 1994; 4:285-299.
10. Veronese ME, Doecke CJ, Mackenzie PI, McManus ME, Miners JO, Rees DL, Gasser R, Meyer UA, Birkett DJ. Site-directed mutation studies of human liver cytochrome P-450 isoenzymes in the CYP2C subfamily. Biochem J. 1993; 289 (Pt 2):533-538.
11. Matsuyama N, Kato T, Kimura K, Mizutani T, Saeki K. Phenotype analysis of human cytochrome P450 2C9 polymorphism using a panel of fluorine substituted benzo[h]quinolines as inhibitor of tolbutamide hydroxylation. J Health Sci. 2006; 52:821-824.
12. Shiue GG, Schirrmacher R, Shiue CY, Alavi AA. Synthesis of fluorine-18 labeled sulfonureas as β-cell imaging agents. J Labelled Cpd Radiopharm. 2001; 44:127-139.
13. Schirrmacher R, Shiue GG, Shiue CY, Alavi AA. Synthesis of F-18 Labeled Tolbutamide as Beta-Cells Imaging Agent. *https://download.uni-mainz.de/fb09-kernchemie/jb1999/b6.pdf* (accessed Augest 10, 2020).
14. Relling MV, Aoyama T, Gonzalez FJ, Meyer UA. Tolbutamide and mephenytoin hydroxylation by human cytochrome P450s in the CYP2C subfamily. J Pharmacol Exp Ther. 1990; 252:442-447.
15. Daly AK, Rettie AE, Fowler DM, Miners JO. Pharmacogenomics of CYP2C9: Functional and Clinical Considerations. J Pers Med. 2017; 8:1.
16. Shon JH, Yoon YR, Kim KA, Lim YC, Lee KJ, Park JY, Cha IJ, Flockhart DA, Shin JG. Effects of CYP2C19 and CYP2C9 genetic polymorphisms on the disposition of and blood glucose lowering response to tolbutamide in humans. Pharmacogenetics. 2002; 12:111-119.
17. Streetman DS, Bertino JS Jr, Nafziger AN. Phenotyping of drug-metabolizing enzymes in adults: a review of *in-vivo* cytochrome P450 phenotyping probes. Pharmacogenetics. 2000; 10:187-216.
18. Kato T, Saeki K, Kawazoe Y, Hakura A. Effects of oligofluorine substitution on the mutagenicity of quinoline: a study with twelve fluoroquinoline derivatives. Mutat Res. 1999; 439:149-157.

19. Kato T, Hakura A, Mizutani T, Saeki K. Anti-mutagenic structural modification by fluorine-substitution in highly mutagenic 4-methylquinoline derivatives. Mutat Res. 2000; 465:173-182.

Received July 27, 2020; Revised August 21, 2020; Accepted August 25, 2020.

*Address correspondence to:
Ken-ichi Saeki, College of Pharmacy, Kinjo Gakuin University, 2-1723 Omori, Moriyama-ku, Nagoya 463-8521, Japan
E-mail: ksaeki@kinjo-u.ac.jp

Released online in J-STAGE as advance publication August 29, 2020.

Letter

DOI: 10.5582/ddt.2020.03065

A successful case of lupus myelitis treated with intravenous pulse methylprednisolone and pulse cyclophosphamide therapy

Kanako Sonoda-Shimada[1], Ikko Kajihara[1,*], Masatoshi Jinnin[1,2], Hironobu Ihn[1]

[1] Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan;
[2] Department of Dermatology, Wakayama Medical University Graduate School of Medicine, Wakayama, Japan.

SUMMARY Lupus myelitis is a rare but serious condition characterized by myelopathy in patients with systemic lupus erythematosus (SLE). Its presentation is usually acute or subacute, and it is often refractory to treatment. We reported a rare presentation of lupus myelitis in a 38-year-old Japanese woman with a 20-year history of SLE. She developed paraparesis and bladder/bowel dysfunction 6 months prior to presentation. Magnetic resonance imaging revealed atrophy of the entire thoracic spinal cord with high intensity on T1-weighted sequence. She was initially treated with intravenous pulse steroid therapy, and prednisolone (20 mg/day) was continued; mizoribine was changed to azathioprine (100 mg/day). In addition, she underwent a rehabilitation program to improve lower-extremity muscle weakness. Moreover, because of the refractory clinical condition, intravenous cyclophosphamide pulse therapy was added. Within 1 month, she could walk with a cane and had a desire to urinate and defecate. In conclusion, early and aggressive treatment improves the permanent damage of lupus myelitis.

Keywords systemic lupus erythematosus, central nervous system lupus, myelitis, methylprednisolone, cyclophosphamide

Lupus myelitis is a rare and serious complication in patients with systemic lupus erythematosus (SLE). Partial remission and complete recovery occur in 50.0-62.5% and 7.1-27.8% of these patients, respectively (*1*). Here, we report the improvement of a rare case of lupus myelitis using intravenous pulse methylprednisolone and pulse cyclophosphamide therapy.

A 38-year-old woman with SLE received oral prednisolone (20 mg/day) and mizoribine (100 mg/day) for 20 years at a nearby hospital. She was admitted to our hospital because she developed paraparesis and bladder/bowel dysfunction 6 months prior to presentation. She was bedridden because of the exacerbation of these symptoms. On admission, she presented with discrete maculopapular erythema on her face (Figure 1A), ulcers on the buttock and oral mucosal ulcers. Laboratory findings were as follows: antinuclear antibody at a titer of 1:1,280 in a speckled pattern, anti-double-stranded DNA antibody level > 200 IU/mL (< 12 IU/mL); total functional hemolytic complement (CH50) < 10 U (31.6-57.6 U/mL); C3, 22.8 mg/dL (73-138 mg/dL): C4, 1.3 mg/dL (11-31 mg/dL); anti-cardiolipin β-2-glycoprotein I, 1.3 U/mL (< 3.5 U/mL); anti-cardiolipin immunoglobulin G < 8 U/mL (< 10 U/mL); an anti-aquaporin 4 antibody < 1.3 U/mL (< 5.0 U/mL). Magnetic resonance imaging (MRI) revealed atrophy of the entire thoracic spinal cord with high intensity on T1-weighted sequence (Figure 1B). Cerebrospinal fluid (CSF) examination showed elevated protein levels of 47.6 mg/dL (8-43 mg/dL), and the immunoglobulin G index was 1.18. Oligoclonal bands in CSF were also positive. Pretherapeutic SLE disease activity index (SLEDAI) score was 16 (vasculitis, new rash, mucosal ulcers, low complement, increased DNA binding). Based on these results, she was diagnosed with myelitis associated with SLE (lupus myelitis). She was initially treated with intravenous pulse steroid therapy, and prednisolone (20 mg/day) was continued; mizoribine was changed to azathioprine (100 mg/day). In addition, she underwent a rehabilitation program to improve lower-extremity muscle weakness. One week after the initiation of this therapy, the SLEDAI score improved up to 4 (low complement, increased DNA binding). Because of refractory hypocomplementemia, high titer of anti-double-stranded DNA antibody and leukopenia, intravenous cyclophosphamide pulse therapy was added. Within 1 month, she could walk with a cane and had a desire to urinate and defecate.

Overall, 1-2% of patients with SLE have lupus myelitis (*2*). It occurs most commonly at the thoracic

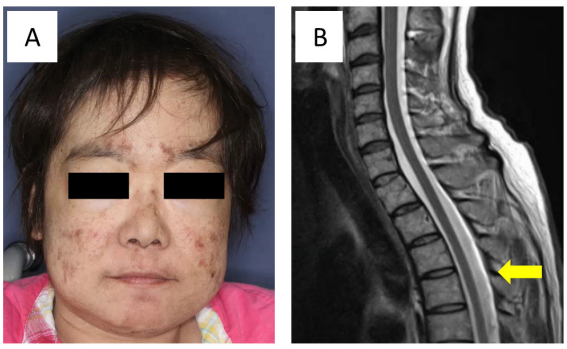

Figure 1. (A) Discrete maculopapular erythema on her face. (B) Magnetic resonance imaging (MRI) showed an atrophy of whole thoracic spinal cord.

levels, especially at the levels of T5-T8 (*2*). The factors associated with neurological outcomes of lupus myelitis include: extensive spinal cord lesions, severity of the symptoms at onset, antiphospholipid antibodies, and delay of treatment (*3*). Although the therapeutic strategy for lupus myelitis is based on SLE, initial treatment often involves intravenous pulse methylprednisolone and pulse cyclophosphamide (*1*). Rituximab, intravenous immunoglobulin, and plasma exchange therapy are sometimes added as combination therapies (*4*). Anticoagulation therapy has also been shown to be effective in patients who are antiphospholipid-positive (*1,5*). In conclusion, early and aggressive treatment improves the permanent damage of lupus myelitis.

References

1. Li XY, Xiao HB, Pai P. Myelitis in systemic lupus erythematosus. J Clin Neurosci. 2017; 44:18-22.
2. Kovacs B, Lafferty TL, Brent LH, DeHoratius RJ. Transverse myelopathy in systemic lupus erythematosus: an analysis of 14 cases and review of the literature. Ann Rheum Dis. 2000; 59:120-124.
3. Bertsias GK, Ioannidis JPA, Aringer M, *et al*. EULAR recommendations for the management of systemic lupus erythematosus with neuropsychiatric manifestations: report of a task force of the EULAR standing committee for clinical affairs. Ann Rheum Dis. 2010; 69:2074-2082.
4. Ye Y, Qian J, Gu Y, Chen X, Ye S. Rituximab in the treatment of severe lupus myelopathy. Clin Rheumatol. 2011; 30:981-986.
5. D'Cruz DP, Mellor-Pita S, Joven B, Sanna G, Allanson J, Taylor J, Khamashta MA, Hughes GR. Transverse myelitis as the first manifestation of systemic lupus erythematosus or lupus-like disease: good functional outcome and relevance of antiphospholipid antibodies. J Rheumatol. 2004; 31:280-285.

Received August 5, 2020; Revised August 17, 2020; Accepted August 25, 2020.

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

Released online in J-STAGE as advance publication August 29, 2020.

Letter

DOI: 10.5582/ddt.2020.03026

Choosing the therapy for neurological infection with rapidly growing mycobacteria

Atman Dash[1], Nitin Gupta[2], Yogiraj Ray[2], Parul Kodan[2], Binit Kumar Singh[2], Manish Soneja[2,*]

[1] Department of Neurosurgery, All India Institute of Medical Sciences, New Delhi, India;
[2] Department of Medicine, All India Institute of Medical Sciences, New Delhi, India.

SUMMARY The management of neurological infections due to non-tubercular mycobacteria is extremely challenging because of scarce literature, issues with penetration, lack of easily available susceptibility platforms and adverse effects associated with long term therapy. We report a case of a young girl with neurological infection due to rapidly growing mycobacteria to discuss the factors that should be considered while choosing the therapy for such rare and persistent infections.

Keywords non-tubercular mycobacteria, rapidly growing mycobacteria, carbapenems, aminoglycosides

Rapidly growing non-tubercular mycobacteria (NTM) are uncommonly associated with infections involving lungs, bones or soft tissues (*1-4*). They usually occur following trauma, contamination during surgery, hematogenous infection from a distant focus or contiguous spread from wounds or perforated gut (*6-7*). These organisms may very rarely involve the central nervous system (CNS) as well. The treatment of NTM is mostly based on small observational studies as there are no properly conducted clinical trials in this area. With the exception of a few case reports, there is hardly any literature on NTM involving the CNS. This, along with the issues of penetration, lack of easily available susceptibility platforms and adverse effects associated with long term therapy, makes the management extremely challenging. We discuss the factors that should be considered while choosing the therapy for such infections using a case of ventriculoperitoneal (VP) shunt infection with *Mycobacterium fortuitum* that we managed recently.

A 13-years old female patient, a known case of posterior fossa glioma with bilateral ventriculoperitoneal shunt *in-situ* (for four years) presented to an outside hospital with acute appendicitis. She was found to have an inflamed appendix which was removed laparoscopically. Fifteen days after the surgery, she presented to our hospital with high-grade fever and altered sensorium with convulsions. She was found to have a Glasgow Coma Score of 9 (Eye-3, Verbal-2, Motor-4). A large, firm and mobile swelling was noticed on the abdominal examination. Computerized tomography (CT) scan of the brain showed dilated ventricles, and CT abdomen showed a large uniloculated cyst (pseudocyst) enclosing the shunt tips. Cerebrospinal fluid examination (CSF) revealed a total leucocyte count of 280/μL (neutrophil 70%, lymphocyte 30%), a glucose of 80 mg/dl and a protein of 14 mg/dl. Ziehl Neelsen staining of the CSF showed multiple acid-fast bacilli (3+) and the growth in culture was identified as *Mycobacterium fortuitum* by line probe assay and sequencing (Figure 1). The likely source of shunt infection in our case was the laparoscope used in the appendectomy. We hypothesized that the infection in the CSF was a result of ascending infection from the peritoneal end of the VP shunt. She was started on imipenem (intravenous), amikacin (intravenous),

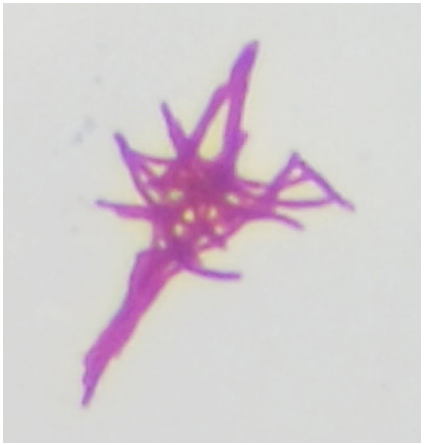

Figure 1. Ziehl Neelsen stain of cerebrospinal fluid showing acid-fast bacilli.

Table 1. Summary of drugs used for treatment of Mycobacterium fortuitum

Drug	Percentage susceptibility	Dose and formulation	AUCCSF/AUCS (uninflamed meninges)	Adverse effects
Amikacin	100	5 mg/kg/dose q8 h IV	0.2	Nephrotoxicity, Ototoxicity, neurotoxicity
Levofloxacin	100	500-750 mg q24h IV or oral	0.71	Gastrointestinal, headache, insomnia
Imipenem	100	500 mg q6 h IV	0.2	Anemia, thrombocytopenia, transaminitis
Linezolid	86	600 mg q12 h IV or oral	0.9	Diarrhea, leucopenia, thrombocytopenia
Doxycycline	50	100 mg q12 h IV or oral	0.2	Diarrhea, nasopharyngitis
Minocycline	50	200 mg loading followed by 100 mg q12 h	No data	Dizziness, fatigue, pruritus
Clarithromycin	50	500 mg q12 h oral	No data	Dysgeusia, diarrhoea

*AUCCSF/AUCS: Area under curve of drug in cerebrospinal fluid divided by area under curve in serum.

levofloxacin (oral) and linezolid (oral). All the shunt hard wares were removed, and a frontal Ommaya reservoir was inserted. The endoscopic third ventriculostomy was done after the CSF became sterile. She was discharged after two months with oral levofloxacin and linezolid. The therapy was discontinued after one and a half year of oral therapy.

Treatment in such cases requires appropriate antimicrobials effective against the species of NTM causing the infection. It is generally suggested to use at least one or two parenteral drugs for the initial part of therapy followed by a prolonged course of oral therapy (*5*). We chose a regimen containing four drugs for the initial part of the therapy considering the involvement of CNS, the severity of the condition and lack of susceptibility testing. The oral therapy with two drugs was prolonged beyond one year for the same reason. The choice was based on the percentage susceptibility, penetration in CSF and adverse effect profile (*8*) (Table 1). Shunt hardware is a source for colonization of the bacteria because of its inherent property to form biofilms. Removal of all such shunt material is necessary to achieve a good outcome, as noted in the literature. CNS infection with NTM is a rare but potentially fatal condition. Besides a high index of suspicion and early diagnosis, choosing the right therapy for the correct duration is paramount for a favourable outcome.

References

1. Wallace Jr RJ, Musser JM, Hull SI, Silcox VA, Steele LC, Forrester GD, Labidi A, Selander RK. Diversity and sources of rapidly growing mycobacteria associated with infections following cardiac surgery. J Infect Dis. 1989; 159:708-716.
2. Clegg HW, Foster MT, Sanders WE, Baine WB. Infection due to organisms of the *Mycobacterium fortuitum* complex after augmentation mammaplasty: clinical and epidemiologic features. J Infect Dis. 1983; 147:427-433.
3. Galil K, Miller LA, Yakrus MA, Wallace Jr RJ, Mosley DG, England B, Huitt G, McNeil MM, Perkins BA. Abscesses due to mycobacterium abscessus linked to injection of unapproved alternative medication. Emerg Infect Dis. 1999; 5:681.
4. Winthrop KL, Abrams M, Yakrus M, Schwartz I, Ely J, Gillies D, Vugia DJ. An outbreak of mycobacterial furunculosis associated with footbaths at a nail salon. N Engl J Med. 2002; 346:1366-1371.
5. Talati NJ, Rouphael N, Kuppalli K, Franco-Paredes C. Spectrum of CNS disease caused by rapidly growing mycobacteria. Lancet Infect Dis. 2008; 8:390-398.
6. Kanev PM, Sheehan JM. Reflections on shunt infection. Pediatr Neurosurg. 2003; 39:285-290.
7. Kulkarni AV, Drake JM, Lamberti-Pasculli M. Cerebrospinal fluid shunt infection: a prospective study of risk factors. J Neurosurg. 2001; 94:195-201.
8. Nau R, Sörgel F, Eiffert H. Penetration of drugs through the blood-cerebrospinal fluid/blood-brain barrier for treatment of central nervous system infections. Clin Microbiol Rev. 2010; 23:858-883.

Received May 4, 2020; Revised July 24, 2020; Accepted August 21, 2020.

*Address correspondence to:
Manish Soneja, Department of Medicine, Teaching Block, All India Institute of Medical Sciences, Ansari Nagar, New Delhi-110029, India.
E-mail: manishsoneja@gmail.com

Released online in J-STAGE as advance publication August 24, 2020.

DD&T
Drug Discoveries & Therapeutics

Guide for Authors

1. Scope of Articles

Drug Discoveries & Therapeutics (Print ISSN 1881-7831, Online ISSN 1881-784X) welcomes contributions in all fields of pharmaceutical and therapeutic research such as medicinal chemistry, pharmacology, pharmaceutical analysis, pharmaceutics, pharmaceutical administration, and experimental and clinical studies of effects, mechanisms, or uses of various treatments. Studies in drug-related fields such as biology, biochemistry, physiology, microbiology, and immunology are also within the scope of this journal.

2. Submission Types

Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables. Supplementary Data are permitted but should be limited to information that is not essential to the general understanding of the research presented in the main text, such as unaltered blots and source data as well as other file types.

Brief Reports definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Brief Reports are not intended for publication of incomplete or preliminary findings. Brief Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 4 figures and/or tables and 30 references. A Brief Report contains the same sections as an Original Article, but the Results and Discussion sections should be combined.

Reviews should present a full and up-to-date account of recent developments within an area of research. Normally, reviews should not exceed 8,000 words in length (excluding references) and should be limited to a maximum of 10 figures and/or tables and 100 references. Mini reviews are also accepted, which should not exceed 4,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 50 references.

Policy Forum articles discuss research and policy issues in areas related to life science such as public health, the medical care system, and social science and may address governmental issues at district, national, and international levels of discourse. Policy Forum articles should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 30 references.

Case Reports should be detailed reports of the symptoms, signs, diagnosis, treatment, and follow-up of an individual patient. Case reports may contain a demographic profile of the patient but usually describe an unusual or novel occurrence. Unreported or unusual side effects or adverse interactions involving medications will also be considered. Case Reports should not exceed 3,000 words in length (excluding references).

Communications are short, timely pieces that spotlight new research findings or policy issues of interest to the field of global health and medical practice that are of immediate importance. Depending on their content, Communications will be published as "Comments" or "Correspondence". Communications should not exceed 1,500 words in length (excluding references) and should be limited to a maximum of 2 figures and/or tables and 20 references.

Editorials are short, invited opinion pieces that discuss an issue of immediate importance to the fields of global health, medical practice, and basic science oriented for clinical application. Editorials should not exceed 1,000 words in length (excluding references) and should be limited to a maximum of 10 references. Editorials may contain one figure or table.

News articles should report the latest events in health sciences and medical research from around the world. News should not exceed 500 words in length.

Letters should present considered opinions in response to articles published in *Drug Discoveries & Therapeutics* in the last 6 months or issues of general interest. Letters should not exceed 800 words in length and may contain a maximum of 10 references. Letters may contain one figure or table.

3. Editorial Policies

For publishing and ethical standards, *Drug Discoveries & Therapeutics* follows the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (*http://www.icmje.org/recommendations*) issued by the International Committee of Medical Journal Editors (ICMJE), and the Principles of Transparency and Best Practice in Scholarly Publishing (*https://doaj.org/bestpractice*) jointly issued by the Committee on Publication Ethics (COPE), the Directory of Open Access Journals (DOAJ), the Open Access Scholarly Publishers Association (OASPA), and the World Association of Medical Editors (WAME).

Drug Discoveries & Therapeutics will perform an especially prompt review to encourage innovative work. All original research will be subjected to a rigorous standard of peer review and will be edited by experienced copy editors to the highest standards.

Ethics: *Drug Discoveries & Therapeutics* requires that authors of reports of investigations in humans or animals indicate that those studies were formally approved by a relevant ethics committee or review board. For research involving human experiments, a statement that the participants gave informed consent before taking part (or a statement that it was not required and why) should be indicated. Authors should also state that the study conformed to the provisions of the Declaration of Helsinki (as revised in 2013). When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed.

Conflict of Interest: All authors are required to disclose any actual or potential conflict of interest including financial

interests or relationships with other people or organizations that might raise questions of bias in the work reported. If no conflict of interest exists for each author, please state "There is no conflict of interest to disclose".

Submission Declaration: When a manuscript is considered for submission to *Drug Discoveries & Therapeutics*, the authors should confirm that 1) no part of this manuscript is currently under consideration for publication elsewhere; 2) this manuscript does not contain the same information in whole or in part as manuscripts that have been published, accepted, or are under review elsewhere, except in the form of an abstract, a letter to the editor, or part of a published lecture or academic thesis; 3) authorization for publication has been obtained from the authors' employer or institution; and 4) all contributing authors have agreed to submit this manuscript.

Cover Letter: The manuscript must be accompanied by a cover letter prepared by the corresponding author on behalf of all authors. The letter should indicate the basic findings of the work and their significance. The letter should also include a statement affirming that all authors concur with the submission and that the material submitted for publication has not been published previously or is not under consideration for publication elsewhere. The cover letter should be submitted in PDF format. For example of Cover Letter, please visit: Download Centre (*https://www.ddtjournal.com/downcentre)*.

Copyright: When a manuscript is accepted for publication in *Drug Discoveries & Therapeutics*, the transfer of copyright is necessary. A JOURNAL PUBLISHING AGREEMENT (JPA) form will be e-mailed to the authors by the Editorial Office and must be returned by the authors as a scan. Only forms with a hand-written signature are accepted. This copyright will ensure the widest possible dissemination of information. Please note that your manuscript will not proceed to the next step in publication until the JPA form is received. In addition, if excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article.

Peer Review: *Drug Discoveries & Therapeutics* uses single-blind peer review, which means that reviewers know the names of the authors, but the authors do not know who reviewed their manuscript. The external peer review is performed for research articles by at least two reviewers, and sometimes the opinions of more reviewers are sought. Manuscripts sent out for peer review are evaluated by independent reviewers. Peer reviewers are selected based on their expertise and ability to provide high quality, constructive, and fair reviews. For research manuscripts, the editors may, in addition, seek the opinion of a statistical reviewer. Consideration for publication is based on the article's originality, novelty, and scientific soundness, and the appropriateness of its analysis.

Suggested Reviewers: A list of up to 3 reviewers who are qualified to assess the scientific merit of the study is welcomed. Reviewer information including names, affiliations, addresses, and e-mail should be provided at the same time the manuscript is submitted online. Please do not suggest reviewers with known conflicts of interest, including participants or anyone with a stake in the proposed research; anyone from the same institution; former students, advisors, or research collaborators (within the last three years); or close personal contacts. Please note that the Editor-in-Chief may accept one or more of the proposed reviewers or may request a review by other qualified persons.

Language Editing: Manuscripts prepared by authors whose native language is not English should have their work proofread by a native English speaker before submission. If not, this might delay the publication of your manuscript in *Drug Discoveries & Therapeutics*.

The Editing Support Organization can provide English proofreading, Japanese-English translation, and Chinese-English translation services to authors who want to publish in *Drug Discoveries & Therapeutics* and need assistance before submitting a manuscript. Authors can visit this organization directly at *http://www.iacmhr.com/iac-eso/support.php?lang=en.* IAC-ESO was established to facilitate manuscript preparation by researchers whose native language is not English and to help edit works intended for international academic journals.

4. Manuscript Preparation

Manuscripts are suggested to be prepared in accordance with the "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals", as presented at *http://www.ICMJE.org*.

Manuscripts should be written in clear, grammatically correct English and submitted as a Microsoft Word file in a single-column format. Manuscripts must be paginated and typed in 12-point Times New Roman font with 24-point line spacing. Please do not embed figures in the text. Abbreviations should be used as little as possible and should be explained at first mention unless the term is a well-known abbreviation (*e.g.* DNA). Single words should not be abbreviated.

Title page: The title page must include 1) the title of the paper (Please note the title should be short, informative, and contain the major key words); 2) full name(s) and affiliation(s) of the author(s), 3) abbreviated names of the author(s), 4) full name, mailing address, telephone/fax numbers, and e-mail address of the corresponding author; and 5) conflicts of interest (if you have an actual or potential conflict of interest to disclose, it must be included as a footnote on the title page of the manuscript; if no conflict of interest exists for each author, please state "There is no conflict of interest to disclose"). Please visit Download Centre and refer to the title page of the manuscript sample.

Abstract: The abstract should briefly state the purpose of the study, methods, main findings, and conclusions. For article types including Original Article, Brief Report, Review, Policy Forum, and Case Report, a one-paragraph abstract consisting of no more than 250 words must be included in the manuscript. For Communications, Editorials, News, or Letters, a brief summary of main content in 150 words or fewer should be included in the manuscript. Abbreviations must be kept to a minimum and non-standard abbreviations explained in brackets at first mention. References should be avoided in the abstract. Three to six key words or phrases that do not occur in the title should be included in the Abstract page.

Introduction: The introduction should be a concise statement of the basis for the study and its scientific context.

Materials and Methods: The description should be brief but with sufficient detail to enable others to reproduce the experiments. Procedures that have been published previously should not be described in detail but appropriate references should simply be cited. Only new and significant modifications of previously published procedures require complete description. Names of products and manufacturers with their locations (city and state/country) should be given and sources of animals and cell lines should always be indicated. All clinical investigations must have been conducted in accordance with Declaration of Helsinki principles. All human and animal studies must have been approved by the appropriate institutional review board(s) and a specific declaration of approval must be made within this section.

Results: The description of the experimental results should be succinct but in sufficient detail to allow the experiments to be analyzed and interpreted by an independent reader. If necessary, subheadings may be used for an orderly presentation. All figures and tables must be referred to in the text.

Discussion: The data should be interpreted concisely without repeating material already presented in the Results section. Speculation is permissible, but it must be well-founded, and discussion of the wider implications of the findings is encouraged. Conclusions derived from the study should be included in this section.

Acknowledgments: All funding sources should be credited in the Acknowledgments section. In addition, people who contributed to the work but who do not meet the criteria for authors should be listed along with their contributions.

References: References should be numbered in the order in which they appear in the text. Citing of unpublished results, personal communications, conference abstracts, and theses in the reference list is not recommended but these sources may be mentioned in the text. In the reference list, cite the names of all authors when there are fifteen or fewer authors; if there are sixteen or more authors, list the first three followed by *et al*. Names of journals should be abbreviated in the style used in PubMed. Authors are responsible for the accuracy of the references. The EndNote Style of *Drug Discoveries & Therapeutics* could be downloaded at **EndNote** (*https://www.ddtjournal.com/examples/Drug_Discoveries_Therapeutics.ens*).

Examples are given below:

Example 1 (Sample journal reference):

Nakata M, Tang W. Japan-China Joint Medical Workshop on Drug Discoveries and Therapeutics 2008: The need of Asian pharmaceutical researchers' cooperation. Drug Discov Ther. 2008; 2:262-263.

Example 2 (Sample journal reference with more than 15 authors):

Darby S, Hill D, Auvinen A, *et al*. Radon in homes and risk of lung cancer: Collaborative analysis of individual data from 13 European case-control studies. BMJ. 2005; 330:223.

Example 3 (Sample book reference):

Shalev AY. Post-traumatic stress disorder: Diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

Example 4 (Sample web page reference):

World Health Organization. The World Health Report 2008 – primary health care: Now more than ever. *http://www.who.int/whr/2008/whr08_en.pdf* (accessed September 23, 2010).

Tables: All tables should be prepared in Microsoft Word or Excel and should be arranged at the end of the manuscript after the References section. Please note that tables should not in image format. All tables should have a concise title and should be numbered consecutively with Arabic numerals. If necessary, additional information should be given below the table.

Figure Legend: The figure legend should be typed on a separate page of the main manuscript and should include a short title and explanation. The legend should be concise but comprehensive and should be understood without referring to the text. Symbols used in figures must be explained. Any individually labeled figure parts or panels (A, B, *etc*.) should be specifically described by part name within the legend.

Figure Preparation: All figures should be clear and cited in numerical order in the text. Figures must fit a one- or two-column format on the journal page: 8.3 cm (3.3 in.) wide for a single column, 17.3 cm (6.8 in.) wide for a double column; maximum height: 24.0 cm (9.5 in.). Please make sure that artwork files are in an acceptable format (TIFF or JPEG) at minimum resolution (600 dpi for illustrations, graphs, and annotated artwork, and 300 dpi for micrographs and photographs). Please provide all figures as separate files. Please note that low-resolution images are one of the leading causes of article resubmission and schedule delays.

Units and Symbols: Units and symbols conforming to the International System of Units (SI) should be used for physicochemical quantities. Solidus notation (*e.g*. mg/kg, mg/mL, mol/mm^2/min) should be used. Please refer to the SI Guide *www.bipm.org/en/si/* for standard units.

Supplemental data: Supplemental data might be useful for supporting and enhancing your scientific research and *Drug Discoveries & Therapeutics* accepts the submission of these materials which will be only published online alongside the electronic version of your article. Supplemental files (figures, tables, and other text materials) should be prepared according to the above guidelines, numbered in Arabic numerals (*e.g*., Figure S1, Figure S2, and Table S1, Table S2) and referred to in the text. All figures and tables should have titles and legends. All figure legends, tables and supplemental text materials should be placed at the end of the paper. Please note all of these supplemental data should be provided at the time of initial submission and note that the editors reserve the right to limit the size and length of Supplemental Data.

5. Submission Checklist

The Submission Checklist will be useful during the final checking of a manuscript prior to sending it to *Drug Discoveries & Therapeutics* for review. Please visit Download Centre and download the Submission Checklist file.

6. Online Submission

Manuscripts should be submitted to *Drug Discoveries & Therapeutics* online at *https://www.ddtjournal.com*. The manuscript file should be smaller than 5 MB in size. If for any reason you are unable to submit a file online, please contact the Editorial Office by e-mail at *office@ddtjournal.com*.

7. Accepted Manuscripts

Proofs: Galley proofs in PDF format will be sent to the corresponding author *via* e-mail. Corrections must be returned to the editor (*proof-editing@ddtjournal.com*) within 3 working days.

Offprints: Authors will be provided with electronic offprints of their article. Paper offprints can be ordered at prices quoted on the order form that accompanies the proofs.

Page Charge: Page charges will be levied on all manuscripts accepted for publication in *Drug Discoveries & Therapeutics* ($140 per page for black white pages; $340 per page for color pages). Under exceptional circumstances, the author(s) may apply to the editorial office for a waiver of the publication charges at the time of submission.

Misconduct: *Drug Discoveries & Therapeutics* takes seriously all allegations of potential misconduct and adhere to the ICMJE Guideline (*http://www.icmje.org/recommendations*) and COPE Guideline (*http://publicationethics.org/files/Code_of_conduct_for_journal_editors.pdf*). In cases of suspected research or publication misconduct, it may be necessary for the Editor or Publisher to contact and share submission details with third parties including authors' institutions and ethics committees. The corrections, retractions, or editorial expressions of concern will be performed in line with above guidelines.

(As of June 2020)

Drug Discoveries & Therapeutics
Editorial and Head Office
Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan.
E-mail: office@ddtjournal.com