SUPPLEMENT 7

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Research Fund for the Control of Infectious Diseases

Research Dissemination Reports

控制傳染病研究基金

研究成果報告

Respiratory infectious diseases
呼吸道傳染病

Fungal diseases
真菌疾病

Other viral diseases
其他病毒性疾病

Supplement 7

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HONG KONG Volume 22 Number 6 **December 2016**

SUPPLEMENT 7

MEDICAL JOURNAL 香港醫學雜誌

Research Fund for the Control of Infectious Diseases

Research Dissemination Reports

RESPIRATORY INFECTIOUS DISEASES

FUNGAL DISEASES

OTHER VIRAL DISEASES

Editorial

Dissemination reports are concise informative reports of health-related research supported by funds administered by the Food and Health Bureau, for example, the *Research Fund for the Control of Infectious Diseases* (which was consolidated into the *Health and Medical Research Fund* in December 2011). In this edition, 13 dissemination reports of projects related to respiratory infectious diseases, fungal disease, and other viral diseases are presented. In particular, three projects are highlighted due to their potentially significant findings, impact on healthcare delivery and practice, and/or contribution to health policy formulation in Hong Kong.

In Hong Kong, seasonal influenza vaccination is recommended for all pregnant women, regardless of gestational age, to reduce the cardiopulmonary complications and hospitalisations associated with influenza infection. Tarrant et al[1] conducted a multi-centre, cross-sectional study to assess the prevalence of seasonal influenza vaccine uptake among 2846 pregnant women in Hong Kong. They found that only 49 (1.7%) reported receiving the seasonal influenza vaccine during pregnancy. The most common reasons for not being vaccinated were fear of side effects that could harm the foetus or themselves. Influenza vaccination uptake is extremely low among pregnant women in Hong Kong. The authors suggest that influenza vaccination uptake can be increased by providing more education to pregnant women about the importance of vaccination during pregnancy, informing them about current Centre for Health Protection recommendations about use of the vaccine during pregnancy, and having health care providers advise pregnant women to be vaccinated.

Yeasts belonging to the genus *Candida* cause infection (candidiasis) in susceptible patients. *Candida* infections have increased alongside advancements in medicine such as invasive procedures, the use of immunosuppressive drugs for organ transplants, and the frequent administration of broad-spectrum antibiotics. *C albicans* is the most common cause of candidiasis and is a major cause of nosocomial infection with mortality that can exceed 40%. To counter the development of resistance to current antifungal agents, Wong et al[2] conducted a search for potential new lead compounds from natural sources. They found that the aqueous extract of the fruiting bodies of the mushroom, *Russula nigricans*, elicited >20% inhibition of *C albicans*. Further characterisation of the inhibitory compound(s) and the mechanism of action were conducted. This study demonstrates the enormous potential of natural products as lead compounds in the development of effective antimicrobials.

In Hong Kong, the HIV prevalence was 4.31% in 2008 among men who have sex with men (MSM) and has subsequently increased to 5.85% in 2014. Lau et al[3] conducted a case-crossover study to identify event-specific variables that are predictive of episodes of unprotected anal intercourse among MSM in Hong Kong who were inconsistent condom users. They found that availability and possession of condoms, condom negotiation, and planning to use condoms were event-specific factors related to lower likelihoods of unprotected anal intercourse with both regular and non-regular male sex partners. The authors suggest that HIV prevention strategies should consider these factors and target specifically on inconsistent condom users.

We hope you will enjoy this selection of research dissemination reports. Electronic copies of these dissemination reports and the corresponding full reports can be downloaded individually from the Research Fund Secretariat website (http://www.fhb.gov.hk/grants). Researchers interested in the funds administered by the Food and Health Bureau also may visit the website for detailed information about application procedures.

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Prevalence of maternal immunisation with seasonal influenza vaccine in Hong Kong

MA Tarrant *, DYT Fong, ILY Lee, C Sing, CYS Yuen

KEY MESSAGES

1. Influenza vaccination uptake is extremely low among pregnant women in Hong Kong.
2. Influenza vaccination uptake can be increased by providing more education to pregnant women about the importance of vaccination during pregnancy, informing them about current Centre for Health Protection recommendations about use of the vaccine during pregnancy, and having health care providers advise pregnant women to be vaccinated.

Hong Kong Med J 2016;22(Suppl 7):S4-5
RFCID project number: 10090982

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In Hong Kong, seasonal influenza vaccination is recommended for all pregnant women, regardless of gestational age, to reduce the cardiopulmonary complications and hospitalisations associated with influenza infection.[1] The World Health Organization has identified pregnant women as the highest priority for seasonal influenza vaccination.[2] Concerns regarding vaccine safety during pregnancy, side effects, and possible birth defects are the major reasons pregnant women are reluctant to receive the vaccine during pregnancy.[3-5] It is important to further understand the barriers to vaccination uptake among this group so as to enable better pandemic planning and preparedness. This study aimed to assess the uptake rate of influenza vaccine among pregnant women and to determine the predictors of influenza vaccination.

In Hong Kong, 2846 postnatal women admitted to the obstetric unit of all eight public hospitals were recruited after giving birth during a 10-week period from April to June 2011. Data collected consisted of baseline sociodemographic data, maternal influenza status, knowledge and attitudes toward influenza vaccination during pregnancy, and maternal and infant data.

Of the 2846 women recruited, only 49 (1.7%) reported receiving the seasonal influenza vaccine during pregnancy. The most common reasons for not being vaccinated were fear of side effects that could harm the foetus or self. Vaccinated women were more likely to have a higher education level, to have more knowledge about the risks of influenza infection during pregnancy, to be aware of the vaccination recommendations, and to have received a vaccination recommendation from a health care provider. Participants in this study who were aware of the Centre for Health Protection recommendation were three times more likely to be vaccinated and participants who were advised by their health care provider to be vaccinated were almost seven times more likely to do so.

These findings show a very low seasonal influenza vaccination uptake rate among pregnant women in Hong Kong one year after the 2009 A/H1N1 influenza pandemic. In addition, the rate of influenza vaccine uptake in this study is one of the lowest reported in the recent literature. Despite their priority status for vaccination, there appears to be little actual promotion of influenza vaccine to pregnant women in Hong Kong. Encouraging and incentivising obstetric health care providers to recommend such vaccination and the on-site provision of influenza vaccine in antenatal clinics would also help to improve vaccination uptake. Interventions to increase influenza vaccine knowledge and uptake among both health care providers and pregnant women should be a priority for future pandemic planning and seasonal influenza vaccination campaigns.

Acknowledgements

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Results of this study have been published in:

Yuet Sheung Yuen C, Yee Tak Fong D, Lai Yin Lee I, Chu S, Sau-mei Siu E, Tarrant M. Prevalence and predictors of maternal seasonal influenza vaccination in Hong Kong. Vaccine 2013;31:5281-8.

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The role of balanced haemagglutinin-neuraminidase activity in the genesis of transmissible neuraminidase inhibitor-resistant variants in seasonal and novel pandemic influenza A H1N1 viruses

HL Yen *, JSM Peiris

KEY MESSAGES

1. The H275Y mutation reduced neuraminidase enzyme activity, increased neuraminidase K_M for 3'-sialyllactose or 6'-sialyllactose, decreased viral infectivity in mucin-secreting human airway epithelial cells, and attenuated pathogenicity in ferrets, when compared with its wild-type counterparts.
2. All H275Y variants of recombinant A(H1N1) pdm09 or seasonal H1N1 influenza viruses with different haemagglutinin-neuraminidase gene constellations were transmitted from inoculated ferrets to naïve direct contact or respiratory droplet contact ferrets, with the transmission efficiency minimally affected, when compared with their wild-type counterparts.

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RFCID project number: 10090142

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Introduction

Since 2009, the neuraminidase (NA) inhibitors have become the major weapon against seasonal H3N2 and the A(H1N1)pdm09 influenza viruses, as these viruses are largely resistant to the M2 ion channel blockers. Mutations that confer resistance to NA inhibitors at the NA catalytic or framework sites may impair enzyme function and compromise viral fitness. The fitness of the NA inhibitor-resistant variants can also be confounded by the haemagglutinin (HA) protein that possesses a counteracting activity to the NA protein. Resistance to NA inhibitors among circulating seasonal influenza viruses was low until 2007 when a H275Y variant emerged among seasonal H1N1 viruses and spread globally by the end of 2008. The A/Brisbane/59/07 (Brisbane)-like virus associated with the H275Y NA mutation had undergone a major antigenic drift from the previously circulating A/New Caledonia/20/99 (NewCal)-like virus.[1] The emergence of the epistatic mutations V234M and R222Q may increase surface NA expression and viral fitness while accommodating the H275Y NA mutation that emerged.[2]

The A(H1N1)pdm09 virus may acquire resistance to NA inhibitors through spontaneous NA mutation or via genetic reassortment with the seasonal H1N1 virus. To achieve a comprehensive understanding of the various degrees of NA functional loss that has been implicated by the H275Y NA mutation, we systematically generated three pairs of recombinant A(H1N1)pdm09 viruses with their NA genes derived from the CA04, NewCal, or Brisbane viruses. Viral fitness was evaluated using different in vitro and in vivo models. We also evaluated the effect of HA and NA derived from the Brisbane-like viruses, including the permissive V234M and R222Q NA mutations. This study provided an in vitro and in vivo comparison of the effect of the H275Y mutation across three antigenic strains exhibiting different epidemiological outcome.[3]

This study aimed to (1) evaluate the fitness and transmission potential of NA inhibitor-resistant pandemic H1N1 2009 virus with spontaneous NA mutations that confer resistance to NA inhibitors or with NA gene derived from the Brisbane-like oseltamivir-resistant viruses; (2) determine the effect of HA and NA of Brisbane-like oseltamivir-resistant seasonal H1N1 viruses on transmissibility; and (3) investigate the potential effect of the secondary permissive mutations (R222Q and V234M).

Methods

This study was conducted from January 2010 to June 2012.

Generation of recombinant viruses

The genome of A(H1N1)pdm09 or seasonal H1N1 influenza viruses were amplified by RT-PCR and cloned into pHW2000 plasmid. The H275Y NA mutation was introduced to the plasmids using site-directed mutagenesis.

Competitive growth between A(H1N1) pdm09 virus with or without the NA-H275Y mutation in cells

RG-CA04 and RG-CA04$^{NA\text{-}H275Y}$ were mixed in different ratios to infect pre-washed differentiated normal human bronchial epithelial (NHBE) cells or MDCK-SIAT1 cells (with or without 0.2 μM oseltamivir carboxylate). RNA was extracted at different times post-infection and the NA gene was amplified by RT-PCR and cloned into pCR4-TOPO vector to determine the ratio between the H275 and Y275 genotypes by Sanger sequencing.

NA kinetics

NA kinetics using 20-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA) and sialosides [3'sialyllactose (3'SL) and 6'sialyllactose (6'SL)] were determined with viruses standardised to 5000 or 1.74×10^6 PFU/mL, respectively. The data were fitted using nonlinear regression to determine the Michaelis constant (K_M) and maximum velocity (V_{max}) of substrate conversion.

Transmission experiments in ferrets

Transmissibility was evaluated in 4-6 month old male ferrets seronegative for influenza A NP protein by ELISA (ID.vet) and influenza B virus (B/Brisbane/60/08) by HA inhibition assay (≤20). All studies were conducted in compliance with applicable laws and with ethics approval. Donor ferrets were inoculated with 10^4 PFU of recombinant virus intra-nasally under isoflurane anaesthesia. At 1 day post-infection, naïve direct contact and respiratory droplet contact ferrets were introduced. Nasal washes were collected from all ferrets on alternate days for 14 days to monitor virus shedding.

Results

H275Y NA mutation led to differential NA functional loss in different NA backgrounds

Recombinant viruses carrying the H275Y mutation showed lower V_{max} and higher K_M values than their wild-type counterparts (Table). The K_M value of the RG-CA04×Brisbane^{NA} for MUNANA, 3'SL, or 6'SL was significantly lower than that of the RG-CA04×NewCal^{NA} or the RG-CA04 viruses (Table). Overall, the H275Y mutation led to decreased NA activity and increased K_M for NAs derived from seasonal or A(H1N1)pdm09 H1N1 influenza viruses.

Recombinant viruses carrying the H275Y mutation were compromised in establishing infection in mucin-secreting differentiated NHBE cells

We compared the ability of the recombinant viruses to infect mucin-secreting differentiated NHBE cells in the presence or absence of the mucin layer by washing the cells extensively or leaving them unwashed prior to infection. Among the pre-washed cells, all wild-type viruses (RG-CA04, RG-CA04×NewCal^{NA}, and RG-CA04×Brisbane^{NA}) were able to establish infection in 4/4 replicates; the H275Y variants, RG-CA04$^{NA\text{-}H275Y}$, RG-CA04×NewCal$^{NA\text{-}H275Y}$, RG-CA04×Brisbane$^{NA\text{-}H275Y}$, established infection in 3/4, 3/4, and 2/4 replicates, respectively. Comparable viral titres were observed between the replicates successfully infected by the wild-type and the H275Y counterparts. In cells that were left unwashed prior to infection, the wild-type viruses could establish infection in 4/5, 5/5, and 3/5 replicates of the differentiated NHBE respectively, whereas their H275Y variants could establish infection in only 2/5, 1/5, and 1/5 replicates respectively. Overall, recombinant viruses with the H275Y mutation, regardless of the origin of the NA gene, exhibited a reduced ability to establish infection in the differentiated NHBE cells.

Competitive growth between RG-CA04 and RG-CA04^{H275Y} viruses in vitro

RG-CA04 and RG-CA04$^{NA\text{-}H275Y}$ viruses were pre-mixed at different ratios to co-infect the washed differentiated NHBE cells or MDCK-SIAT1 cells. RG-CA04 genotype increased over time but was

TABLE. Neuraminidase enzyme kinetics using MUNANA, 3'-SL, and 6'-SL substrates (Permission from: Wong DD, Choy KT, Chan RW, et al. Comparable fitness and transmissibility between oseltamivir-resistant pandemic 2009 and seasonal H1N1 influenza viruses with the H275Y neuraminidase mutation. J Virol 2012;86:10558-70.)

Virus	MUNANA			3'SL			6'SL		
	K_M (μM)	V_{max}	V_{max} ratio	K_M (μM)	V_{max}	V_{max} ratio	K_M (μM)	V_{max}	V_{max} ratio
RG-CA04	26.5	4.0	1.0	622.7	101.0	1.0	3469	68.2	1.0
RG-CA04$^{NA\text{-}H275Y}$	61.9	3.5	0.9	905.3	105.8	1.1	4224	57.8	0.8
RG-CA04×NewCal^{NA}	42.3	4.3	1.0	741.5	111.3	1.0	2355	84.5	1.0
RG-CA04×NewCal$^{NA\text{-}H275Y}$	84.5	2.4*	0.6	1370.0	69.5*	0.6	3987	57.2	0.7
RG-CA04×Brisbane^{NA}	14.0†	6.8	1	454.2	152.0	1.0	2108	105.6	1.0
RG-CA04×Brisbane$^{NA\text{-}H275Y}$	26.4	4.3*	0.6	698.9	97.5*	0.6	2462	67.8*	0.6

* P<0.05 when compared to counterpart wild-type viruses

† P<0.05 when compared to RG-CA04×NewCal^{NA} virus

not able to completely dominate the oseltamivir-resistant Y275 genotype in the MDCK-SIAT1 cells. Overall, the RG-CA04 exhibited a slightly higher survival advantage than RG-CA04$^{NA\text{-}H275Y}$ virus when co-infected at different ratios in the absence of oseltamivir (Fig 1). In the absence of oseltamivir carboxylate, the proportion of RG-CA04 genotype decreased progressively over time.

Transmissibility of the recombinant A(H1N1)pdm09 viruses in ferrets

RG-CA04 caused greater levels of weight loss in ferrets compared with the RG-CA04$^{NA\text{-}H275Y}$ or RG-CA04×Brisbane$^{NA\text{-}H275Y}$ viruses (Fig 2). One of the two ferrets inoculated with RG-CA04 virus died on day 8 post-inoculation, whereas all ferrets survived in the RG-CA04$^{NA\text{-}H275Y}$ and RG-CA04×Brisbane$^{NA\text{-}H275Y}$ groups. Transmission from inoculated ferret to naïve direct contact and respiratory droplet contact ferrets was observed for all three recombinant CA04 viruses at day 4 post-inoculation (Fig 2). Overall, we observed that RG-CA04$^{NA\text{-}H275Y}$ or RG-CA04×Brisbane$^{NA\text{-}H275Y}$ viruses possessed attenuated pathogenicity but retained comparable transmission efficiency to the RG-CA04 virus.

Generation of recombinant seasonal H1N1 viruses with different HA and NA constellations

A series of recombinant viruses with identical internal genes but with different HA-NA gene constellation derived from NewCal and Brisbane were generated. Permissive mutations R222Q and V234M were introduced into the NA of the NewCal virus. All recombinant viruses replicated to comparable titres in MDCK cells. H275Y NA mutation consistently led to an increased K_M values for MUNANA, 3'SL, or 6'SL substrates. Interestingly, we observed that the permissive mutations R222Q and V234M did not significantly affect the K_M value of the wild-type NewCal NA, whereas the K_M values of RG-NewCal$^{HA,\ NA\text{-}R222Q,V234M,\ H275Y}$ were lower than that of the RG-NewCal$^{HA,NA\text{-}H275Y}$ virus.

Transmissibility of the recombinant seasonal H1N1 viruses in ferrets

The transmissibility of RG-Brisbane$^{HA,NA\text{-}H275Y}$, RG-NewCal^{HA,NA}, RG-NewCal$^{HA,NA\text{-}H275Y}$, or RG-NewCal^{HA}×Brisbane$^{NA\text{-}H275Y}$ recombinant seasonal H1N1 viruses was evaluated. All viruses could transmit to direct contact and respiratory droplet contact ferrets with minor differences in efficiency. By comparing the RG-NewCal^{HA,NA} and RG-NewCal$^{HA,NA\text{-}H275Y}$ viruses, we observed that the H275Y NA mutation slightly decreased the respiratory droplet transmissibility but not the direct contact transmissibility.

Discussion

The H275Y mutation led to reduced NA enzyme function, regardless of the origin of the NA gene segment. The NA of the Brisbane virus of which the H275Y variant spread globally exhibited a unique NA enzyme property compared with the NA derived from the NewCal or CA04 viruses. Specifically, the

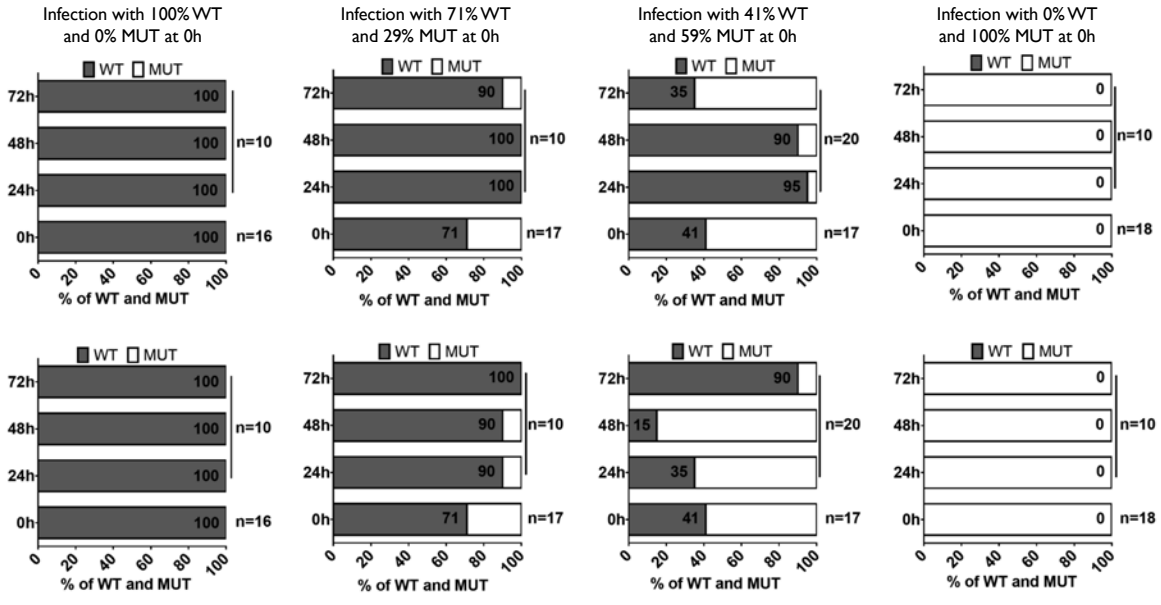

FIG 1. Competitive replication of RG-CA04 and RG-CA04$^{NA\text{-}H275Y}$ in differentiated NHBE cells (Permission from: Wong DD, Choy KT, Chan RW, et al. Comparable fitness and transmissibility between oseltamivir-resistant pandemic 2009 and seasonal H1N1 influenza viruses with the H275Y neuraminidase mutation. J Virol 2012;86:10558-70.)

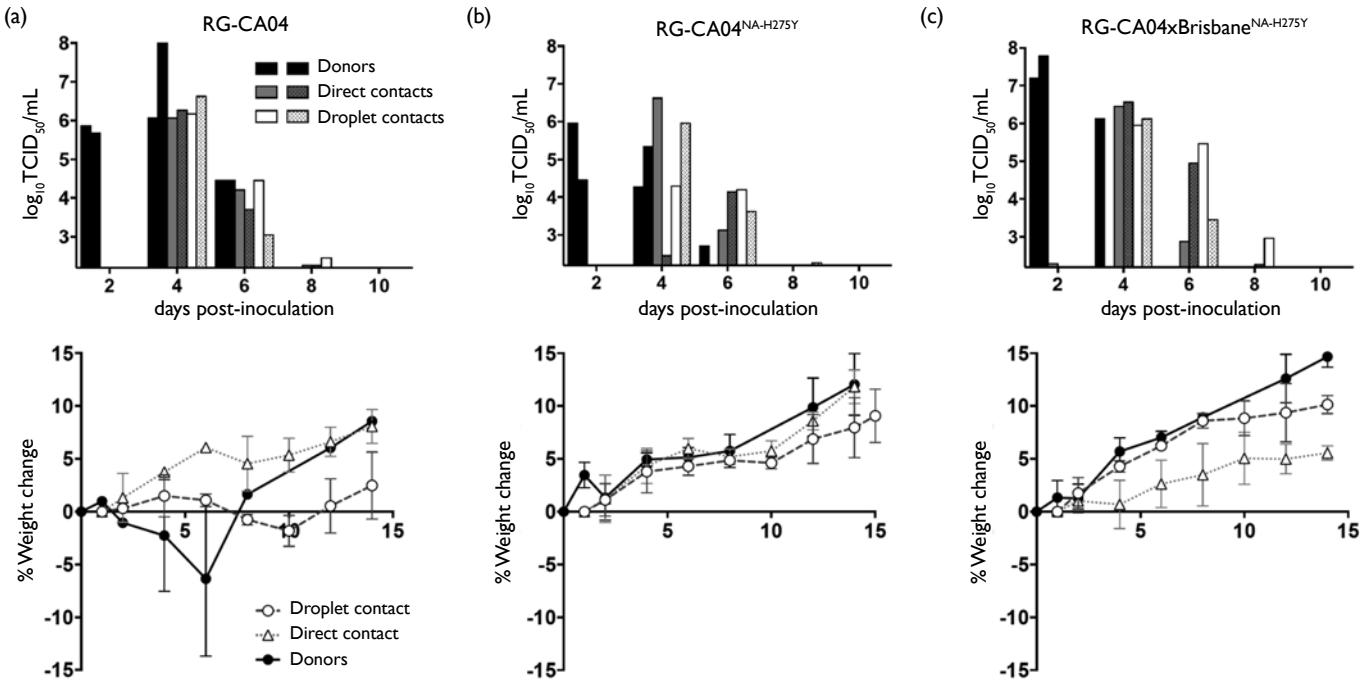

FIG 2. Transmission of (a) RG-CA04, (b) RG-CA04$^{NA-H275Y}$, and (c) RG-CA04x BrisbaneNA-H275Y viruses in ferrets: viral titres ($log_{10}TCID_{50}$/mL) and percentage of ferret weight changes for donor ferrets, direct contacts, and respiratory droplet contacts. (Permission from: Wong DD, Choy KT, Chan RW, et al. Comparable fitness and transmissibility between oseltamivir-resistant pandemic 2009 and seasonal H1N1 influenza viruses with the H275Y neuraminidase mutation. J Virol 2012;86:10558-70.)

K_M value of oseltamivir-sensitive Brisbane NA was consistently lower in catalysing MUNANA, 3'SL, or 6'SL when compared with the NewCal or CA04 NA, consistent with a previous study.[4] Introduction of the permissive R222Q and V234M mutations[2] into the NewCal NA did not significantly increase NA activity or the K_M value. The H275Y variants with reduced NA function showed decreased infectivity in the mucin-secreting differentiated NHBE cells. Direct competitive assay between the RG-CA04 and RG-CA04$^{NA-H275Y}$ variant suggested that the oseltamivir-sensitive RG-CA04 virus showed minor survival advantage over the H275Y variant in differentiated NHBE or in MDCK-SIAT1 cells.

Despite the detection of reduced viral competencies, both the oseltamivir-sensitive and the oseltamivir-resistant H275Y variants in recombinant A(H1N1)pdm09 viruses or the seasonal H1N1 viruses with different HA-NA constellations were able to transmit to naïve direct contact or respiratory droplet contact ferrets. Our results suggest that the H275Y mutation in H1N1 influenza leads to a minor reduction in viral fitness with its transmission potential being minimally affected in the naïve ferret model.

Conclusion

The oseltamivir-resistant H275Y variants have transmission potential; continued monitoring of this mutation among circulating A(H1N1)pdm09 strains is important.

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Results of this study have been published in: Wong DD, Choy KT, Chan RW, et al. Comparable fitness and transmissibility between oseltamivir-resistant pandemic 2009 and seasonal H1N1 influenza viruses with the H275Y neuraminidase mutation. J Virol 2012;86:10558-70.

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Prevention and treatment of swine-origin influenza virus with interferon: an in vivo and ex vivo study

JM Nicholls *, RWY Chan, E Fish

KEY MESSAGES

1. Prophylactic interferon reduces infection with influenza H1N1pdm in lung tissue. Therapeutic interferon is beneficial in lung tissue but not in bronchial tissue.
2. Exogenous interferon appears to be useful for pulmonary involvement of influenza viruses (H1N1 and H5N1) but may not be of significant benefit for bronchial infection. For influenza virus infections that are resistant to current antiviral agents, interferon therapy offers a potential benefit.

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Introduction

The influenza A/H1N1pdm virus that emerged in 2009 contained a unique mixture of genes that originated from Eurasian and North American swine. Even though this new virus was associated with a low mortality compared with seasonal influenza, there were concerns that the higher mortality in younger patients (contrasting with a bias towards elderly patients with seasonal influenza) and the potential for this virus to re-assort with avian influenza viruses would result in a high mortality.

This pandemic virus was a reassortant virus with the incorporation of a haemagglutinin gene from the classic swine lineage and a neuraminidase gene from Eurasian-like swine lineage. A younger population (<50 years of age) appeared to be immunologically naïve with respect to previous exposure, but the elderly were partially protected. A vaccine strategy was implemented early in the outbreak, and although effective, still had a lag of 3-4 months from the start of the outbreak. In the early stages of the outbreak, effective treatment relied on existing therapies. Unfortunately, H1N1pdm was resistant to amantadine and rimantidine and there were increased numbers of reports to suggest virus resistance to the neuraminidase inhibitor oseltamivir (Tamiflu).

We planned to investigate the role of interferon (IFN) in the prevention and treatment of H1N1pdm. When cells are infected with influenza virus, they begin to express IFN, which leads to an antiviral response through the activation of effector molecules. The type I IFNs (including IFNα) are produced early in the infection of the cell and function by inhibiting viral replication and protein synthesis. To counteract this host antiviral mechanism, viruses have developed mechanisms to evade the IFN response. For example, the NS1 protein of influenza virus targets either the post-transcriptional processing of IFN mRNA and/or the IFN-inducible antiviral proteins such as ISG15 and PKR. Evidence of a role for IFN in an anti-influenza virus response has been shown through the use of IFN α/ß receptor deficient mice.

IFNα has been shown to effectively control hepatitis B and C in humans, particularly active hepatitis C virus, and to have cured 98% of affected individuals. During the 2003 severe acute respiratory syndrome outbreak in Toronto, IFN alfacon-1, a novel synthetic consensus IFN, showed clinical benefit in patients treated with steroids and IFN compared with those treated with steroids alone. In guinea pigs and ferrets infected with H5N1 and seasonal influenza respectively, IFN treatment resulted in reduced viral titres and improved pulmonary pathology.[1,2] In the 1980s, intranasal administration of IFNα showed promise in preventing influenza infection, but side-effects prevented the widespread adoption of this route of administration. The Mx1 protein is a downstream protein induced by IFN and thus a suitable target for analysis.

Given that H1N1pdm emerged as a pandemic virus with a low but significant mortality in young patients, together with a potential for increased oseltamivir resistance, there is a need to explore the role of IFN in the management of lower respiratory tract infection. Clinically, there may be little benefit from the use of IFN in uncomplicated nasopharyngeal H1N1pdm infection. We thus explored the utility of IFN treatment in a human ex vivo bronchial/lung culture model.

Methods

This study was conducted from January 2010 to December 2011.

Ex-vivo culture

The ex vivo organ cultures of the lung and bronchial biopsies were obtained from lungs removed at surgery according to previously approved protocols. The bronchial tissues were placed on a sterile sponge to create minimal contact with enough growth medium (F12K + 1% PS) in an air-liquid interface situation and cultured for 24 and 48 hours with influenza viruses as listed below and also with pre-incubation with different concentrations of IFN-alfacon-1.

Viruses used

We used an influenza virus isolated from a Mexican patient with H1N1pdm disease in Hong Kong in 2009, (A/Hong Kong/415742/09), a virus from a patient with H5N1 disease in Vietnam (A/VN/3046/04) and Hong Kong (A/Hong Kong/483/97), a human seasonal influenza H1N1 virus (A/Hong Kong/54/98), and a seasonal influenza H3N2 virus (A/Oklahoma/1992/05).

Evaluation of cytokine profile by superarray

Total RNA was extracted from ex vivo tissues using the RNeasy mini Kit (Qiagen). The eluted total RNA (20 μL) was used for the first-strand cDNA synthesis with the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The gene expression of 84 key cytokine genes was then profiled by RT-PCR-based RT^2 Profiler Interferons and Receptors PCR Array (SABioscience, Frederick, MD, USA) in the ABI 7500 Real-Time PCR System (Applied Biosystems). Fold change of IFNs and IFN receptor expression in experimental samples relative to the control samples (eg mock-infected) was calculated using the ΔΔCt method. The ΔΔCt value of each sample was normalised by up to a total of five housekeeping genes (β-2-microglobulin, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, glyceraldehyde-3-phosphate dehydrogenase, and β-actin). All data were analysed by the RT^2 Profiler PCR Array Data Analysis Template v3.0 and all gene expression changes >2.5 fold were considered significant.

Detection of IFNAR receptors and Mx1 protein in bronchial and pulmonary tissues

IFNAR1 and IFNAR2 antibodies from Abcam and Lifespan biosciences gave no reaction to formalin fixed tissues. We then applied these to 10 fresh bronchial and lung biopsies that had been excised and embedded in OCT. After blocking with appropriate sera, antibodies were used at 1/50 (2 μg/mL) concentration for IFNAR1 and 1/50 (5 μg/mL) for IFNAR2 for 30 mins at room temperature. Secondary incubation with alkaline phosphatase conjugated streptavidin (Vectorlabs SA-5100) at 1/200 for 30 mins followed by development with Vector Red substrate kit (Vectorlabs SK-5100) at room temperature for 10 mins. The Mx1 protein antibody was obtained from Abcam and also used on frozen tissue.

Infection of mice by pandemic and non-pandemic H1N1

Wild type and knock-in Mx1$^{+/+}$ mice were bred in Toronto and infected with pandemic H1N1. Viral load and interferon gene expression was studied at 3 days post infection.

Results

Analysis of 10 pairs of bronchial and lung biopsies showed that five of these had weak apical expression of IFNAR1 and IFNAR2 in the bronchial epithelium and no significant staining in the pulmonary parenchyma (Fig 1). This staining was only partially membranous and also extracellular. This is consistent with a previous study that reported very low innate expression of IFNAR in the unstimulated epithelium of the respiratory tract. We attempted to determine the IFNAR1 and IFNAR2 expression on the surface of cells in ex vivo cultures after influenza virus infection. Nonetheless, the available IFNAR antibody did not permit staining of fixed tissues. The compulsory 10% formalin fixation of the tissue was due to the safety protocol required as a standard operation protocol. Mx1 protein was detected in the bronchial epithelium and in scanty alveolar macrophages (Fig 1).

To investigate the interaction of influenza with interferon, human lung explant tissues were pretreated with IFN-alfacon-1 for 16 hours prior to infection with H5N1 or H1N1 influenza A virus. At different time points post infection, RNA was extracted for cDNA synthesis. Analysis of influenza

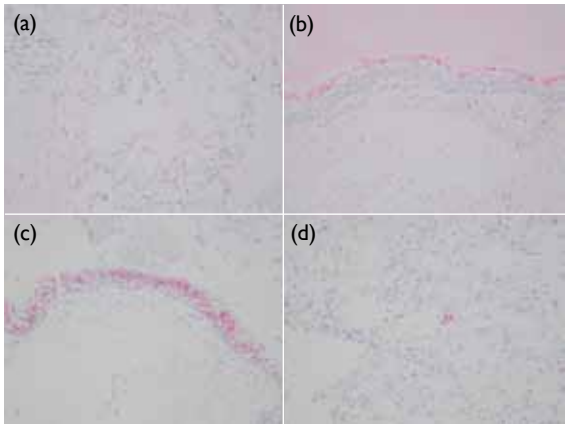

FIG 1. IFNAR1 staining of (a) lung and (b) bronchus from normal tissue (frozen section with Vector Red staining). Mx1 immunohistochemistry of (c) bronchus and (d) lung tissue.

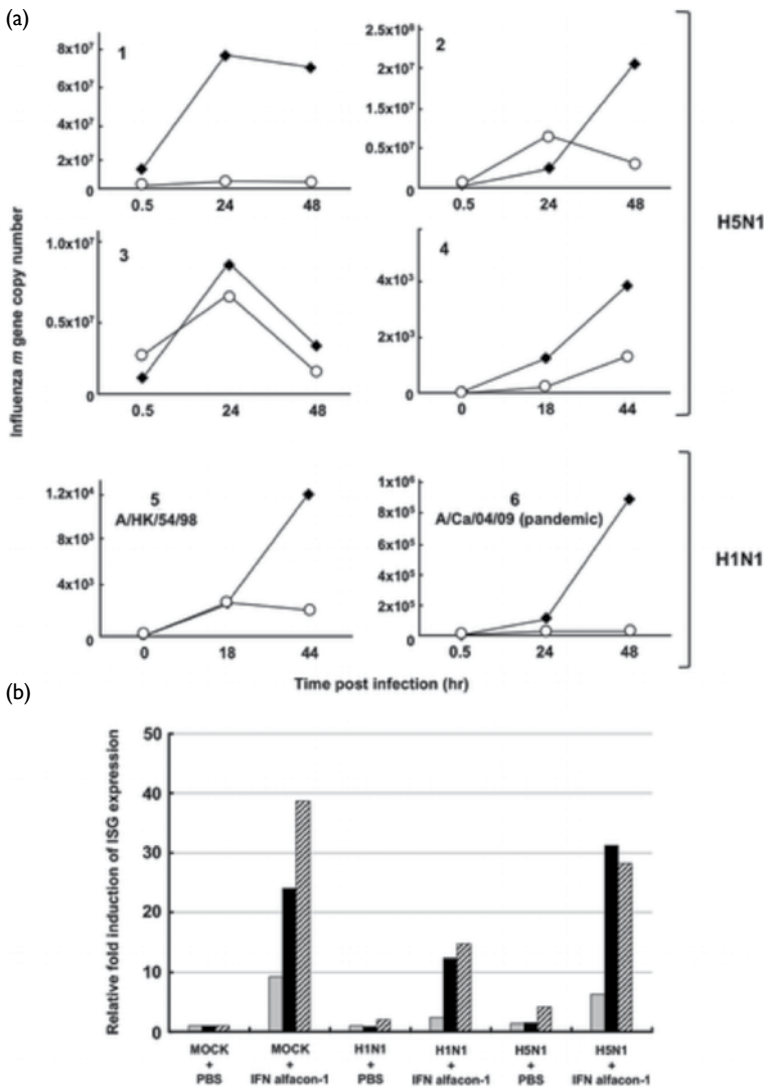

FIG 2. Treatment with IFN alfacon-1 inhibits H5N1 and H1N1 replication in primary human lung cells and upregulates ISG expression. Different human surgical lung tissue explants (1-6) were either left untreated (♦) or treated with IFN alfacon-1 (1.2x10^4 U/mL) (O) for 16 hours. Tissues were then infected with H5N1 (1-4) or H1N1 influenza A virus (5, 6), as indicated. At different time points post infection, RNA from cells was collected and cDNA synthesised. (a) Gene expression for influenza A M gene was measured by RT-PCR. (b) Gene expression for PKR (▩), ISG15 (▪), 2'5'-OAS (▨), and β-actin was measured by RT-PCR analysis at 18 hours post-infection with H5N1 or A/HK/54/98 H1N1. Data are representative of two independent experiments and normalised to mock infected controls.

A matrix (M) gene expression revealed that IFN alfacon-1 pretreatment effectively inhibited H5N1 and H1N1 influenza A replication (Fig 2). Gene expression analysis for 2'5'-OAS, PKR and ISG15, and IFN-stimulated genes (ISGs) associated with an IFN-inducible antiviral response revealed that the expression levels for these ISGs were not highly upregulated in H5N1 or H1N1 virus infected tissues. Notably, IFN alfacon-1 pretreatment of mock infected explants induced a high expression of ISGs. IFN-inducible ISG expression was observed in both H5N1 and H1N1 virus-infected human lung tissues pre-treated with IFN alfacon-1 (Fig 2).

We further examined the effects of IFN alfacon-1 on pandemic H1N1 influenza A infection when IFN was added post-challenge with virus. Three different human lung explants were infected with H1N1pdm virus, and then 24 hours post-infection treated with 1.2×10^4 U/mL IFN alfacon-1. At 24 and 48 hours post-treatment, the effects of IFN on viral replication were evaluated by measuring M gene expression and $TCID_{50}$ values. There was evidence for the protective effects of IFN treatment, even when added post-infection, as assessed by $TCID_{50}$ and M gene expression (Fig 3). These results were supported by evidence of a reduction in influenza A nucleoprotein expression, visualised in the IFN-treated lung explants (Fig 3).

In contrast to the lung data therapeutic treatment, the same IFN post-infection treatment regimen in bronchial tissues did not show a convincing protective effect against H1N1pdm influenza virus infection. In four bronchial biopsies from different patients, we found no protection (2 cases) or a nominal protective effect (2 cases) against H1N1pdm after the IFN post-infection treatment (Fig. 4). In two biopsies where IFN alfacon-1 was added 24 hours after infection, there was no inhibitory effect, whereas for two other biopsies there was an inhibitory effect (Fig 4). Overall, IFN post treatment in bronchial tissue showed no advantage when compared with control treatment during H1N1pdm infection. Similar experiments were not conducted using H5N1 virus as we found no H5N1 replication in human ex vivo bronchus culture (n=6).

In the initial experiments, either H1N1 or H5N1 influenza A virus was used to infect the intact human lung explant tissue. To determine if influenza A infection would inhibit either *ifnar1* or *ifnar2* expression, RNA was collected and analysed at 18 hours post-infection. Infection with both viruses led to a selective reduction in *ifnar1* gene expression when compared with mock-infected control tissues (Fig 5). Notably, infection with the H5N1 influenza A strain led to a greater reduction in *ifnar1* gene expression compared with infection with H1N1 virus. The inhibitory effects of H1N1 and H5N1 infection on *ifnar2* gene expression were not significant.

There was no significant change in weight loss, viral load, or interferon gene production in the wild-type or Mx1$^{+/+}$ mice identified.

Discussion

This study investigated how a newly emerging pandemic virus—H1N1pdm—affected IFN signalling and explored the potential therapeutic potential of exogenous IFN to override any virus-induced inhibitory effects associated with the virally-encoded NS1 protein of influenza virus. Lung biopsies indicated that H5N1 infection was able to inhibit IFNAR and

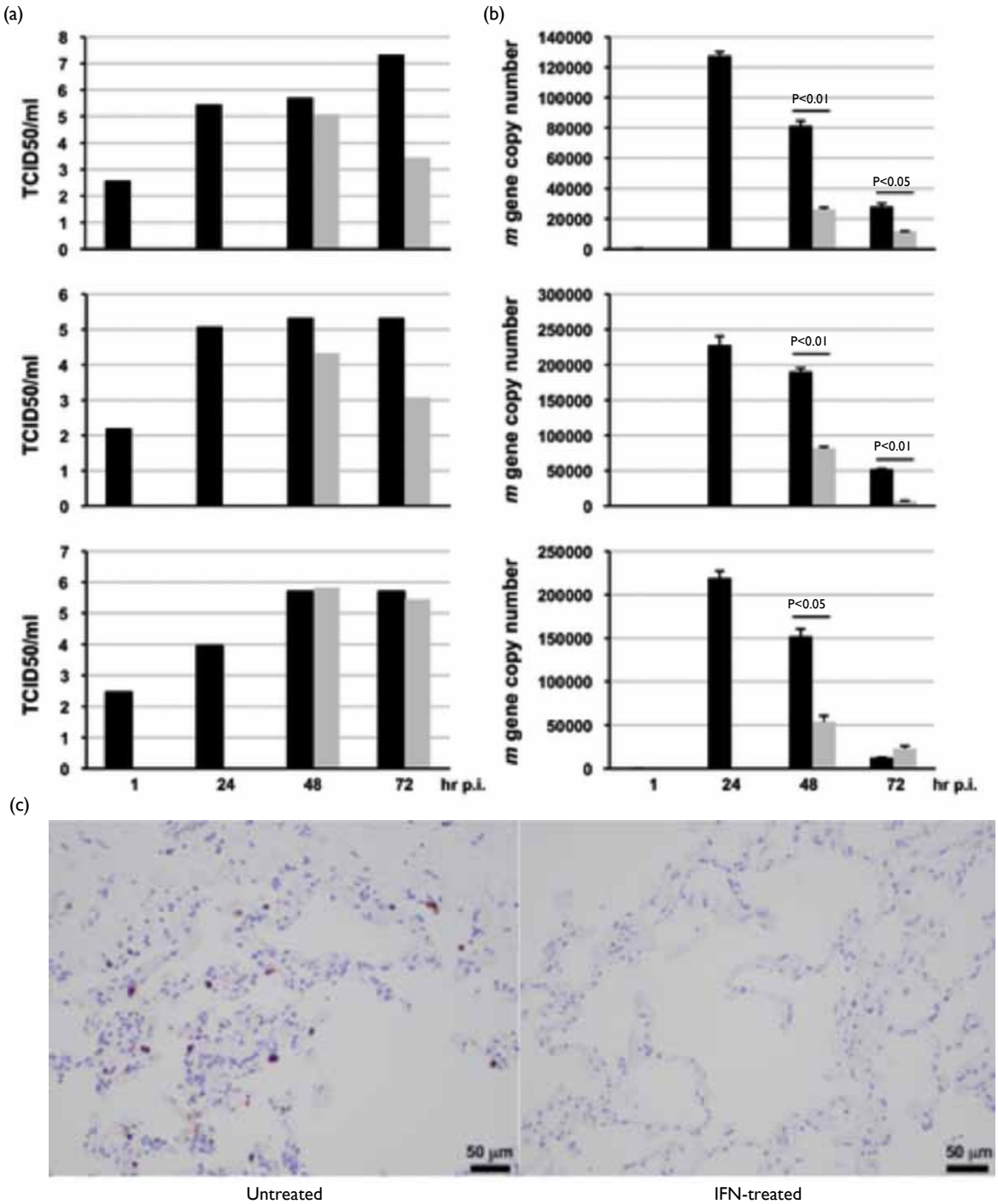

FIG 3. Three different human surgical lung tissue explants were infected with pandemic H1N1 2009 virus for 24 hours, and then left untreated (▪) or treated with IFN alfacon-1 ($1.2x10^4$ U/mL) (▩) for 48 hours. (a) Viral titres ($TCID_{50}$) and (b) influenza A M gene expression were measured. (c) Thin sections of untreated or IFN-treated infected human lung explants were stained for influenza A nucleoprotein (reddish brown).

SOCS protein expression and demonstrated that this may be due to inhibition of the IFN-inducible STAT pathway. Nevertheless, we have provided evidence that IFN treatment overrides the inhibitory effects of influenza virus infection. This IFN-inducible antiviral effect, however, was not reproduced in our ex vivo bronchial culture system. Our immunohistochemistry studies have shown that intrinsic IFNAR1 and IFNAR2 expression is very low in the bronchial epithelium. This anatomical site is not a successful therapeutic target for systemic IFN therapy.

Our ex vivo studies have shown that in the lung explant model, IFN pre-treatment was able to override the inhibitory effects of influenza virus infection. The differential effectiveness of IFN in suppressing both H5N1 and H1N1 subtypes may be

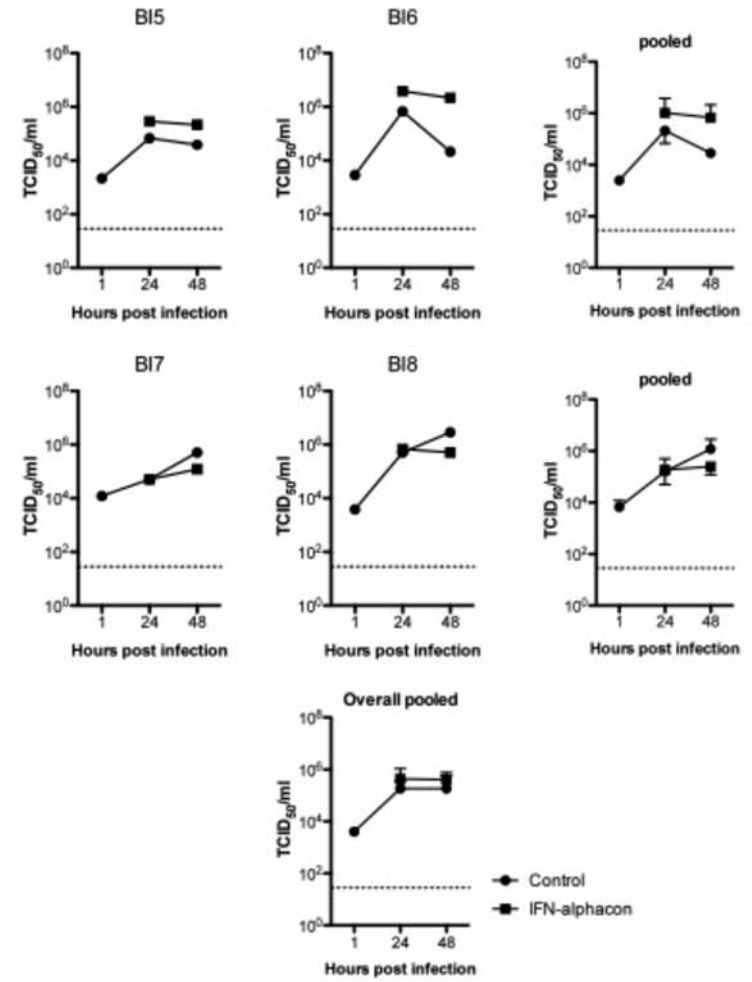

FIG 4. Therapeutic effect of IFN alfacon-1 on H1N1pdm replication in the bronchus. Replication was assessed by viral titration assay using tissue culture supernatant.

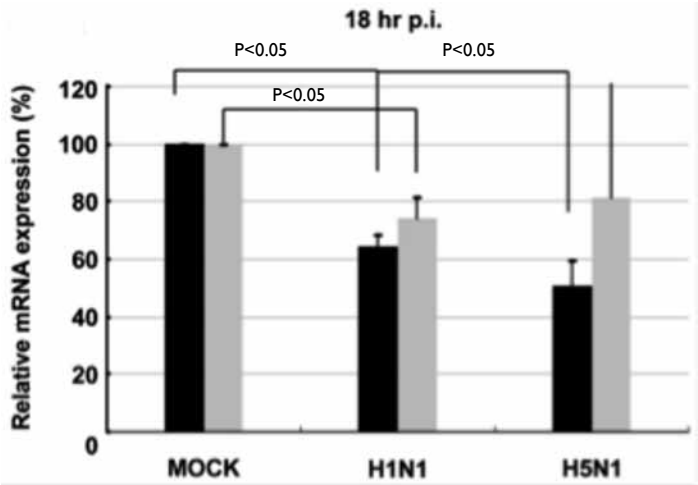

FIG 5. Human lung explant tissue was either mock-infected or infected with A/HK/483/97 H5N1 or A/HK/54/98 H1N1 influenza A virus. At 18 hours post-infection, tissue was processed to extract RNA. cDNA was synthesised and expression of *ifnar1*, *ifnar2*, and β-*actin* gene expression was measured by RT-PCR analysis. Gene expression was calculated relative to β-*actin* gene expression and normalised to mock infected tissues. Data are representative of two independent experiments.

due to the differential innate immune responses of the host to these different viruses immediately after infection. Specifically, H5N1 virus is well known to cause hyper-induction of cytokine and chemokines, whereas H1N1 and H1N1pdm do not. The differential regulation of the host sensing receptor by H5N1 and H1N1 virus was discussed in an earlier report in 2009. The differential regulation of the innate immune response between the two subtypes is shown in terms of quantity of the cytokines and chemokines produced and the intensity of the pathways triggered, instead of a qualitative difference. It appears that the innate immune response triggered by seasonal H1N1 and H1N1pdm is actually comparable, and H1N1pdm did not show hyper-induction of cytokines as for H5N1.[3,4]

Conclusion

We analysed IFN-induced signalling using a focused IFN pathway array. We found that exogenous IFN increased the induction/expression of antiviral genes, yet observed a decrease in the expression level of IFN receptors. This IFN-induction of antiviral proteins, eg Mx, OAS, likely contributed directly to the reduction in viral gene transcription and virus replication observed. Although our data indicated that effectiveness is limited, IFN treatment post-infection with H1N1pdm influenza virus did limit the viral replication in terms of viral titre, M gene, and influenza NP expression.

Acknowledgements

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Metagenomics of tuberculosis infection in Hong Kong

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KEY MESSAGES

1. Taxonomic assignment of the sequence reads using the Ribosomal Database Project Classifier showed that *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria*, and *Fusobacteria* were the major bacterial phyla recovered from sputum. They composed over 98% of the microbial community in both the tuberculosis (TB) and control samples.
2. Upon genera identification, 16 major bacterial genera were recovered in the sputum samples. The most abundant genera in the TB samples were *Neisseria* (28.0%), *Streptococcus* (27.8%), and *Prevotella* (16.8%), whereas those for the controls were *Streptococcus* (31.8%), *Neisseria* (22.0%), and *Prevotella* (14.4%). *Neisseria* and *Prevotella* were more dominant in the TB samples and *Streptococcus* was more dominant in the controls.
3. The microbial diversity was similar in both the TB and the control sputum samples. There was no relationship between microbial diversity and disease state. This may be because different diseases may involve different sets of microbiota.

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Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB) that kills about two million persons annually.[1] In addition, two billion people are estimated to be latently infected with this organism.[2] In Hong Kong as at November 2012, there were more than 4900 TB cases, representing more than 31% of the incidence of notifiable diseases.[3] The proximity of Hong Kong to other regions with a high incidence of multidrug-resistant (MDR)-TB or extensively drug-resistant (XDR)-TB strains undermines local TB control efforts.[4] This study aimed to determine the microbial communities in sputum during primary TB infection by comparing TB with non-TB sputum samples.

Methods

This study was conducted from November 2009 to July 2012 and was approved by the Joint CUHK-NTEC Clinical Research Ethics Committee of the Prince of Wales Hospital in Hong Kong.

Sputum samples were collected from the Tuberculosis Reference Laboratory of the Hong Kong Government. All samples were from Hong Kong Chinese patients free of HIV. No anti-TB or other antibiotic medication had been given to patients in the 4 weeks prior to sputum collection. Smear-positive and culture-positive TB sputum samples were collected from 13 males and 8 females aged 20 to 66 years. Non-TB sputum samples were collected from 6 males and 8 females aged 22 to 82 years. The sputum samples were treated with a 3% NaOH solution, neutralised with a phosphate buffer and then centrifuged. About 0.2 mL supernatant was subjected to genomic DNA extraction using the QIAamp DNA Mini Kit according to the manufacturer's protocol (Qiagen, Valencia [CA], USA).

Polymerase chain reaction (PCR) was performed by composite primers with adaptors and sample-specific multiplex identifiers, flanking the hypervariable V1-V2 region of the 16S rRNA gene using the Platinum PCR SuperMix High Fidelity Kit (Invitrogen, Carlsbad [CA], USA). The PCR profile consisted of an initial denaturation of 30 s at 94°C, followed by 28 cycles of 30 s at 94°C, 30 s at 55°C, and 40 s at 68°C. PCR products were purified using the MinElute PCR Purification Kit (Qiagen, Valencia [CA], USA) and the quality of the purified products was assessed using Agilent Bioanalyzer 2100. Pyrosequencing of the purified PCR products was performed using the Roche/454 GS FLX Titanium platform.

Raw sequencing reads were demultiplexed, quality-filtered, and analysed using QIIME 1.4.0. Quality-filtered reads were clustered into operational taxonomic units (OTUs) at 97% similarity. Taxonomic assignment of representative OTUs was performed using the Ribosomal Database Project (RDP) Classifier at a 0.8 confidence threshold against the Greengenes core set. DNA reads obtained from pyrosequencing against databases of known sequences using a comparison tool such as BLAST were also performed. The dataset was ratified before alpha diversity calculations. Principal coordinate analysis was performed using the weighted and unweighted UniFrac distances.

Results

Manipulation of samples and pyrosequencing results

For pyrosequencing, a total of 964 556 raw 16S rRNA reads were obtained. The filtering process removed about 14% of the raw sequencing reads. There were about 499 000 and 331 000 high-quality reads for the TB and control samples, respectively. The average read length was about 370 bp, after removal of the primer sequences. The average number of qualified reads was 22 660 for the TB group and 23 667 for the control group. The sequencing data were then submitted to the National Centre of Biotechnology Information short read archive with an accession number SRA058505.

Microbial diversity in the sputum samples microbiota

The qualified sequence reads were taxonomically assigned using the RDP Classifier. The major bacterial phyla identified were *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria*, and *Fusobacteria*. These bacterial phyla comprised over 98% of the microbial community in both the TB and control samples. Among the five major bacterial phyla identified, *Firmicutes, Proteobacteria*, and *Bacteroidetes* were the most abundant groups, comprising 37.6%, 31.2%, and 19.2% in the TB samples and 43.6%, 27.1%, and 17.0% in the controls, respectively. The relative abundance of *Actinobacteria* and *Fusobacteria* in the two groups was similar. *Proteobacteria* and *Bacteroidetes* were more dominant in the TB samples, whereas *Firmicutes* was more dominant in the controls. Further elaboration of the data revealed a large variation in the community structure among the tested individuals. This was supported by principal coordinate analysis, in which no obvious differential clustering was observed between members of the TB and control groups.

Upon genera identification, 16 major bacterial genera were recovered in the sputum samples. The most abundant genera in the TB samples were *Neisseria* (28.0%), *Streptococcus* (27.8%), and *Prevotella* (16.8%), whereas those for the controls were *Streptococcus* (31.8%), *Neisseria* (22.0%), and *Prevotella* (14.4%). *Neisseria* and *Prevotella* were more dominant in the TB samples, and *Streptococcus* was more dominant in the controls. *Lactococcus, Pseudomonas* and unclassified *Enterobacteriaceae* were less dominant and prevalent in the TB samples. Within the 16 major genera, only *Actinomyces, Fusobacterium, Leptotrichia, Prevotella, Streptococcus*, and *Veillonella* were recovered in all the TB samples, and all these genera, except *Leptotrichia* were found in all the 36 samples. Furthermore, comprehensive data analysis revealed that there were inter-member variations in the community structure at the genus level.

The three major genera: *Neisseria, Streptococcus*, and *Prevotella* identified were dominated by two to three major OTUs. Two OTUs comprised more than 90% of the dominant genus *Neisseria* in both the TB samples and controls. By BLAST search, OTU 6783 was found to be similar to an uncultured clone NSV3Q1b18 and OTU 7988 was similar to an uncultured clone 7H59. For *Streptococcus*, OTU 1734 was similar to a *S mitis* clone; OTU 6370 was similar to a *S parasanguinis* clone, and OTU 7204 was highly similar to the *S salivarius*. Interestingly, two other OTUs—OTU 2225 and OTU 7999—comprised more than one-third of the genus *Prevotella* in both TB samples and controls.

Genera *Moryella, Mogibacterium*, and *Oribacterium* were the less represented taxa. They were enriched in the TB samples ($P<0.05$). In addition, a genus belonging to the unclassified *Lactobacillales* was enriched in the control samples ($P<0.05$). Nonetheless, at the species level, eight OTUs, including those showing 99% identity to *Prevotella melaninogenica, Lactobacillus crispatus, Streptococcus anginosus*, and *Parvimonas micra*, were enriched in the TB samples ($P<0.05$). Two other OTUs, including one showing 99% similarity to the *Aggregatibacter aphrophilus* species, were enriched in the controls ($P<0.05$).

Discussion

Five major bacterial phyla were identified from the sputum samples: *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria*, and *Fusobacteria*. They are also found in other human body sites such as the oral cavity, skin, colon, the sputum of patients with cystic fibrosis and in the bronchial tract of patients with chronic obstructive pulmonary disease. This collectively suggests the prevalence of these major phyla in normal and diseased lung microbiota.[6]

Among the 16 major genera recovered,

only *Actinomyces, Fusobacterium, Leptotrichia, Prevotella, Streptococcus*, and *Veillonella* were found in all TB samples, and they may represent the core genera in the TB sputum microbiota. Similarly, with the exception of *Leptotrichia*, the remaining five genera were found in all 36 samples, and they may represent the core genera in the sputum microbiota in general.[6]

Streptococcus, Neisseria, and *Prevotella* were the three most dominant genera recovered in the sputum samples. They were also the major genera identified in the sputum of patients with chronic obstructive pulmonary disease, nosocomial pneumonia, and cystic fibrosis. This was in contrast to the normal lung microbiota, in which the genus *Pseudomonas* dominated. For *Streptococcus, Streptococcus pneumonia* is a well-known pathogen associated with pneumonia. Pathogenic *Neisseria* species such as *Neisseria meningitides* can cause pneumonia, and *Prevotella* can cause lower respiratory tract infection.[6]

Each of the three most dominant genera was dominated by two to three major OTUs. These OTUs comprised one-third to 90% of the corresponding groups. As the OTUs of genus showed high similarity to pathogenic strains causing a wide range of diseases, such as pneumonia, it is thus reasonable to speculate that the increased relative abundance of these opportunistic pathogens during TB infection could alter the microbial community in TB lung and affect disease progression.[6]

The less abundant taxa may also affect the dynamics of the microbial community and clinical outcomes. *Mogibacterium, Moryella*, and *Oribacterium* were the genera that were statistically abundant in the TB samples. For *Mogibacterium, M timidum* has been identified in cases of acute lung infection. *Moryella* and *Oribacterium* have been identified in recent decades although little information is available since their discovery.

Conclusion

The microbial diversity was similar in TB and control sputum samples. No special relationship between the microbial diversity and the disease state was observed. This may be because different diseases may involve different sets of microbiota.

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Gene regulatory function and cellular partners of SARS-associated coronavirus nucleocapsid protein

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KEY MESSAGES

1. SARS coronavirus (SARS-CoV) nucleocapsid (N) protein expressed in cultured human cells was predominantly found in the cytoplasm and was competent in repressing the transcriptional activity driven by interferon-stimulated response elements. Expression of N protein did not influence the transcription from FGL2 promoter. N protein did not modulate the expression of FGL2 mRNA or protein in transfected or SARS-CoV-infected cells.
2. SARS-CoV N and M proteins inhibit gene transcription of type I interferons through different mechanisms. M protein potently antagonises the activation of interferon-stimulated response element-dependent transcription by RIG-I, MDA5, TBK1, IKKε, and VISA, whereas N protein has no influence on these stimuli. The expression of M protein prevents the formation of TRAF3-TANK-TBK1/IKKε complex.

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Introduction

Severe acute respiratory syndrome (SARS) is a fatal infectious disease that spread in China and around the world in 2003. The primary aetiological agent was the SARS coronavirus (SARS-CoV), close relatives of which have been found in various bats.[1]

Coronavirus nucleocapsid (N) protein is a relatively conserved structural protein that binds genomic RNA and plays an important role in viral RNA synthesis, viral assembly, and formation of RNA replication complex.[2] Intracellularly, coronavirus N protein localises to the cytoplasm where it interacts with M protein to form an icosahedral core. In addition, N proteins from mouse hepatitis virus (MHV) and several other coronaviruses have also been shown to localise to the nucleolus to exert an impact on cell cycle progression.[3] In line with this, MHV N protein regulates the expression of cellular genes such as FGL2.[4] FGL2 encodes a prothrombinase termed fibrinogen-like protein 2 that causes vascular thrombosis and fibrin deposition.

Although nucleolar localisation has also been suggested, SARS-CoV N protein is more frequently found in the cytoplasm.[5] Interestingly, its gene regulatory function has also been documented in the context of AP-1-, NF-κB-, Smad-, and CCAAT/enhancer binding protein (C/EBP)-dependent transcription,[6,7] and interferon production.[8] Fibrosis and vascular thrombosis in the lung are also observed commonly in patients with SARS. In line with this, SARS-CoV N protein has been shown to regulate the expression of human FGL2 gene.[9] In addition to N protein, the inhibition of interferon production and signalling by SARS-CoV is thought to be mediated through other viral structural and non-structural proteins ORF3b, ORF6, nsp1, and PLpro.[10] In this way, SARS-CoV counteracts a major component of the host antiviral innate immunity at multiple levels.

This project aims to shed light on the gene regulatory function and cellular partners of SARS-CoV N protein. We found that N protein does not modulate FGL2 but inhibits transcription from interferon gene promoters through a mechanism distinct from M protein, and impeded the formation of TRAF3-TANK-TBK1/IKKε complex. Our findings point to a new model in which SARS-CoV circumvents the production of type I interferons.

Methods

N gene and other viral genes of SARS-CoV were subcloned and expressed in cultured mammalian cells. Properties of N and M protein were characterised in N or M gene-transfected and SARS-CoV- infected cells using Western blotting, luciferase reporter assay, confocal immunofluorescence microscopy, and immunoprecipitation.

Results

Expression and gene regulatory activity of SARS-CoV N protein

The distinct subcellular localisation patterns of MHV and SARS-CoV N proteins suggest that they might serve different functions inside the cell. Gene regulatory activity of SARS-CoV N protein has recently been demonstrated in different models. As a first step to characterise its possible roles in cellular pathogenesis, we expressed SARS-CoV N protein in HEK293 cells. Western blot analysis indicated that N protein was abundantly expressed in transfected cells.

SARS-CoV N protein did not influence transcription of FGL2 gene in transfected cells

The stimulation of FGL2 promoter by SARS-CoV N protein has implications not only in the pathogenesis of SARS, but also in the development of therapeutics. To shed light on whether and how SARS-CoV N protein might activate transcription of the FGL2 gene, we constructed reporter plasmid pFGL2-Luc, in which the expression of firefly luciferase is under the control of FGL2 promoter. To confirm the activity of this construct, we cotransfected it into HEK293 cells with an expression vector for Sp1, a known activator of FGL2 promoter. A more than two-fold stimulation of reporter expression by Sp1 demonstrated that pFGL2-Luc sensitively reflected intracellular activity of FGL2 promoter (Fig 1a). As a control for proper expression and activity of SARS-CoV N protein in HEK293 cells, we found that N protein was fully competent to repress ISRE transcriptional activity that controls interferon production.

Nonetheless, when we cotransfected pFGL2-Luc and an expression plasmid for SARS-CoV N protein into HEK293 cells, an induction of reporter activity was not observed (Fig 1b). To further characterise the influence of N protein on expression of the endogenous FGL2 gene, we analysed the steady-state amounts of FGL2 transcript in HEK293 cells overexpressing Sp1 or N protein. While the expression of Sp1 correlated with elevation of FGL2 transcript above the basal level, expression of N protein did not alter the relative amount of FGL2 mRNA in transfected cells. Consistently, the accumulation of Sp1 protein in the cell was associated with an abrupt increase in FGL2 protein level, whereas increased expression of N protein had no influence on the steady-state amount of FGL2. Thus, SARS-CoV N protein did not induce expression of FGL2 transcript or protein in transfected cells. We also showed that SARS-CoV N protein did not affect the expression of FGL2 in SARS-CoV-infected cells.

SARS-CoV N and M proteins counteract type I interferon production through different mechanisms

SARS-CoV N protein antagonises the production of type I interferons.[8] In our study to characterise the gene regulatory function of N protein, we included M protein as a control and found serendipitously that M protein was a more potent inhibitor of interferon β promoter when compared with N protein. To compare the mechanisms by which N and M proteins counteract interferon induction and signalling in cultured cells, we used RIG-I, a well-studied cytoplasmic sensor of dsRNA and activator of interferon production and signalling, to stimulate ISRE activity and then assessed the influence of N and M. Interestingly, coexpression of N did not modulate RIG-I-activated interferon production, whereas M was able to suppress RIG-I activity in a dose-dependent manner (Fig 2). Notably, this activity of M is similar to that of influenza A virus NS1, but

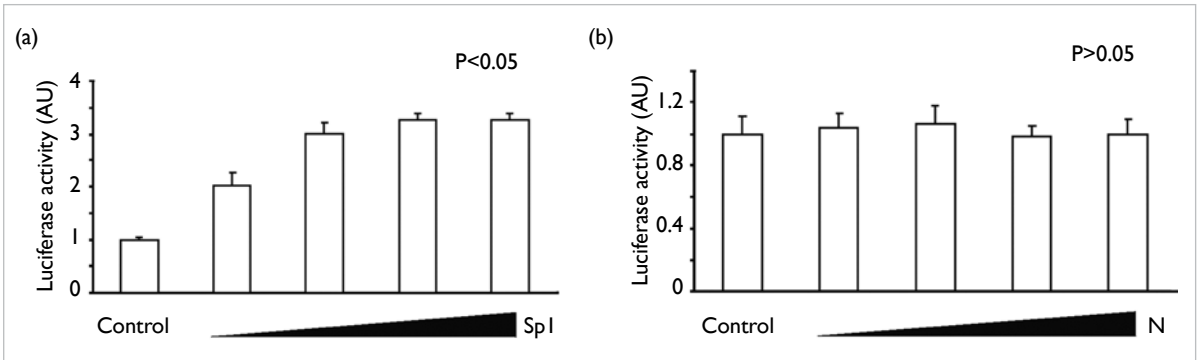

FIG 1. Expression of SARS-CoV N protein does not stimulate FGL2 promoter. HEK293 cells are transfected with 100 ng pFGL2-Luc plus progressively increasing amounts (100, 200, 400, and 600 ng) of (a) Sp1 expression plasmid and (b) SARS-CoV N protein expression plasmid, respectively. Control cells are transfected with pFGL2-Luc and empty vector.

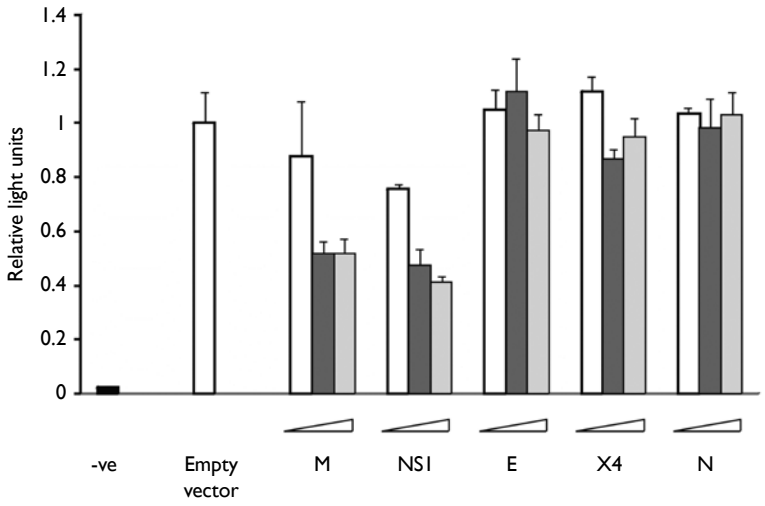

FIG 2. SARS-CoV M protein suppresses RIG-I-induced activation of ISRE. Assays are carried out as in Fig 1b, and RIG-I expression vector is transfected into all cells expect for the negative control group (-ve).

not SARS-CoV E or ORF7A (X4) proteins. We also found that while M protein inhibited the ability of MDA5, TBK1, IKKε and VISA to activate ISRE, N had no influence on any of these stimuli (data not shown). Hence, SARS-CoV N and M proteins suppress type I interferon production through different mechanisms.

SARS-CoV M protein counteracts type I interferon production by disrupting the formation of TRAF3-TANK-TBK1/IKKε complex

Since M protein displayed a unique pattern to inhibit RIG-I, we next investigated the mechanism by which it modulates interferon induction and signalling. Co-immunoprecipitation and confocal immunofluorescence microscopy revealed that M associated with RIG-I, TRAF3, TBK1, and IKKε. Because the formation of TRAF3-TANK-TBK1/IKKε complex is an essential step in stimulus-induced activation of IRF3 and IRF7, we explored whether expression of M might influence this step. Notably, the interactions of TRAF3 with TBK1, IKKε, and TANK were evident in the absence of M. In contrast, these three pairs of interaction were abolished in M-expressing cells. In other words, the expression of M prevented the formation of TRAF3-TANK-TBK1/IKKε complex, thereby inhibiting IRF3/7 phosphorylation and activation.

Discussion

We showed that SARS-CoV N protein did not modulate transcription of the human FGL2 gene. Our findings are opposed to those of a recent report on the induction of FGL2 promoter through activation of C/EBPα by SARS-CoV N protein.[9] It is noteworthy that the human FGL2 promoter used in our study contained all *cis* regulatory elements described by others including the C/EBP site.[9] In addition, the utility of our pFGL2-Luc construct was further supported experimentally by the significant activation induced by cellular Sp1 transcription factor (Fig 1a). Although we do not understand whether different experimental systems might explain different observations, our demonstration of the unaltered expression of FGL2 protein in infected cells might be more biologically relevant to SARS-CoV.

SARS-CoV N protein is structurally and functionally related to MHV N protein. As such, both proteins are capable of multimerisation and RNA binding.[11] In addition, both proteins have gene regulatory activity and can repress interferon production.[10] Nonetheless, SARS-CoV N protein also possesses properties that are not shared with its MHV counterpart. For example, SARS-CoV N protein is localised predominantly to the cytoplasm, whereas MHV N protein was found in the nucleolus.[5] In addition, SARS-CoV N protein did not activate FGL2 transcription. FGL2 is unlikely to be involved in the pathogenesis of SARS.

We compared the molecular mechanisms by which SARS-CoV N and M proteins antagonise type I interferon production and signalling. We found that M protein physically interacted with RIG-I, TBK1, and IKKε. In addition, M protein disrupted the formation of TRAF3- TANK-TBK1/IKKε complex to inhibit IRF3/7 phosphorylation and activation. Our work not only reveals a new interferon antagonist encoded by SARS-CoV, but also provides a new mechanism for SARS-CoV modulation of interferon production and signalling. Our findings are generally consistent with the concept that SARS-CoV encodes multiple structural and non-structural proteins to counteract the host antiviral response.[10] In addition, our results have implications in the rational design and use of anti-SARS CoV agents.

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Mechanistic study on the assembly and release of lentiviral particles pseudotyped with haemagglutinin of highly pathogenic avian influenza H5N1 viruses: implications for strain-specific pseudotype development

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KEY MESSAGES

1. We have developed and characterised lentiviral particles pseudotyped with avian influenza A virus H5 haemagglutinin (H5pp) from a Cambodian H5N1 isolate, which can be used as a safe tool for high-throughput serological studies without the requirement of biosafety level 3 facilities (BSL-3).
2. Not all H5 haemagglutinins (HA) give rise to efficient production of H5pp. The amino acid residue at position 134 of the influenza A virus HA protein receptor binding domain is a critical molecular switch to control the ability of H5 HA to pseudotype lentiviral vectors.

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The emergence and re-emergence of H5N1 virus in Hong Kong and China

There have been three influenza pandemics in the 20th century: the 1918 Spanish flu (H1N1), the 1957 Asian flu (H2N2), and the 1968 Hong Kong flu (H3N2). These influenza pandemics caused severe illness, especially the 1918 Spanish flu that claimed millions of lives worldwide. Avian influenza A virus (H5N1) is highly pathogenic in wild birds and poultry. Occasionally, it crosses the species barrier to infect humans. The first human outbreak of H5N1 influenza virus occurred in Hong Kong in 1997 following severe outbreaks at three chicken farms. By the end of 1997, 18 people had been confirmed to be infected with H5N1 of whom six died. Luckily, the highly pathogenic avian influenza H5N1 virus has caused only limited infection in humans since 1997. H5N1 virus has not gained efficient transmissibility from poultry to humans or between humans. Nonetheless, the deadly H5N1 viruses persist to re-emerge in the human population.

Since the first human outbreak in 1997, there have been more than 500 documented human cases of H5N1 infection with a mortality rate of about 60%.[1] After the traumatic experience of H5N1 outbreaks in poultry in 1997, the Hong Kong SAR government launched an intensive surveillance system in live poultry markets and conducted mass culling of poultry whenever a severe outbreak was identified. These measures were successful and no human cases of H5N1 infection were reported until 2003. In January 2003, three people in a family of five developed fever and severe respiratory illness after their visit to mainland China. One died before H5N1 infection was confirmed, and two were confirmed to have H5N1 infection based on virological evidence. Human infection with H5N1 virus in China has been detected almost every year since 2003. In light of the increasing human travel between Hong Kong and Mainland China, the threat of H5N1 is unlikely to disappear.

In 2009, a swine-origin pandemic H1N1 virus (S-OIV) emerged in Mexico and the USA. The virus quickly spread globally with high transmissibility between humans. It is known that pigs are the mixing vessels that can be co-infected by swine, human, and avian influenza viruses. The fear is that if the high pathogenicity of H5N1 influenza virus somehow combines with the high transmissibility of S-OIV, it would be devastating. There is an urgent need to speed up research on influenza viruses, including the highly pathogenic H5N1.

Virulence factors for highly pathogenic H5N1 viruses

H5N1 viruses have been characterised in various animal models in order to understand the virulence factors. In birds and poultry, the cleavability of the virus haemagglutinin (HA) surface protein plays a major role in virulence. In the mouse model, a polybasic amino acid cleavage site in HA and 627K of polymerase basic protein 2 are two important virulence factors. Viruses with these features replicate and spread systemically in mice and eventually lead to a lethal outcome. In the ferret model, HA and non-structural genes contribute to the high virulence of H5N1 in these animals.[2]

The HA of H5N1 contains a furin-dependent polybasic cleavage site that is characteristic of highly pathogenic H5N1 viruses.[3] Currently, the spread of H5N1 virus in the human population is limited. However, through mutation and reassortment, the virus may become more easily transmissible from birds to humans and/or between humans, posing a potential pandemic threat to public health worldwide. It is therefore important to fully understand the biology of H5N1 viruses and to develop sensitive and rapid diagnostic methods. Nonetheless, an obstacle to the study of H5N1 viruses is the stringent safety requirements. Thus, retroviral particles pseudotyped with H5-HA (H5pp) have been developed. Similar to the replication-competent virus, H5pp entry requires alpha-2,3 sialic acids, is pH-dependent, and can be neutralised by sera-containing anti-H5N1 antibodies,[4] thus making it a very useful and safe tool.

H5 haemagglutinin pseudotyped lentiviral particles are a safe tool for serological/epidemiological studies without the requirement for BSL-3 facilities

Previously, we reported the development of H5 HA pseudotyped lentiviral particles using HA of A/Cambodia/40808/2005 (H5Cam), a H5N1 isolate from a Cambodian patient who died from the infection. The lentiviral backbone is deficient in its original viral envelope protein and the entry of the particle is therefore dependent on the 'foreign' envelope protein that is pseudotyped on the surface of the particles. The H5pp is capable of conducting only a single round of transduction, thus making it a very useful and safe alternative for assays that normally involve the use of real H5N1 viruses. Assays using H5pp can be performed in BSL-2 facilities. This is especially useful when researchers do not have access to BSL-3 facilities. As a mimic of the real H5N1 virus, H5pp can be used in a wide range of applications, including sero-diagnosis, entry mechanism studies, and drug discovery.[5]

The H5 HA pseudotyped lentiviral particles can be engineered to contain only HA or both HA and neuraminidase (NA) [Fig 1]. The pseudotyped particles usually contain a reporter gene: luciferase or green fluorescent protein. Upon transduction of H5pp into permissive cells, H5pp enters the target cells in a sialic acid dependent manner. The reporter gene is subsequently expressed in the target cell and facilitates measurement of the activity of the pseudo-particles. Among the various applications of H5pp, the most widely used is probably sero-diagnosis and sero-surveillance (Fig 2). For this purpose, H5pp has certain advantages over H5N1-pp: it is specifically neutralised only by anti-HA antibodies, avoiding the confounding effect of antibodies against NA1 due to infection with influenza virus subtypes other than H5N1. H5Npp is easy to produce for all four H5N1 virus stains, but the efficiency to generate HA-only H5pp varies a great deal with HAs derived from different H5N1 virus strains. The underlying mechanism is not known.

Novel findings

From January 2009 to December 2010, a study was conducted to analyse the ability of HAs

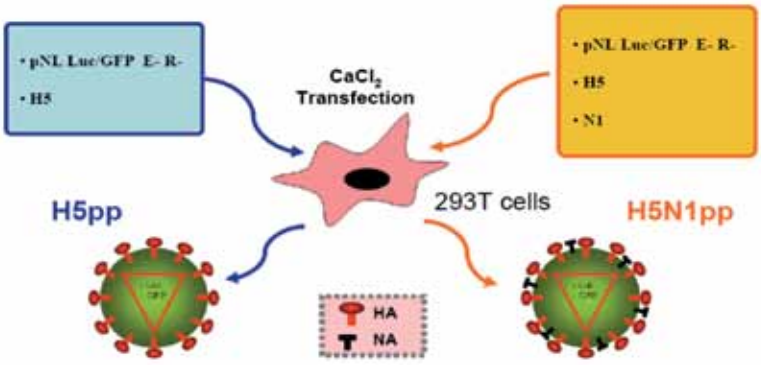

FIG 1. Production of H5pp and H5N1pp in 293T cells

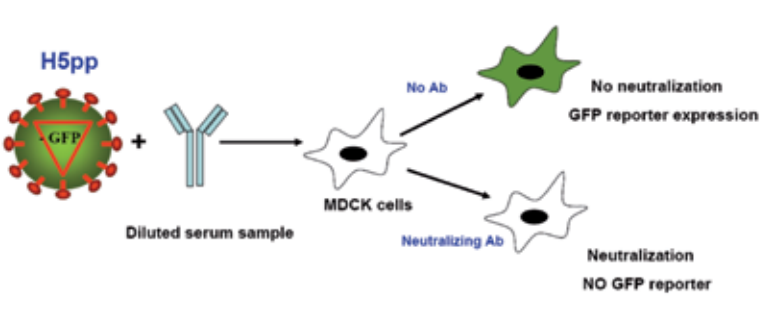

FIG 2. Neutralisation-based serodiagnostic assay using H5pp

from different clades of avian influenza virus to pseudotype lentiviral particles, which do not give rise to the same level of efficient H5pp production as H5Cam. In particular, the expression and cleavage of two H5 HAs (from A/Cambodia/2005/40808 and A/Anhui/2005/01) was compared, as was their ability to pseudotype lentiviral vector in HEK293T cells.

To ascertain the flexibility and adaptability of H5pp production in the event of novel emerging H5N1 virus strains, we developed strain-specific H5pp and compared the ability of three other strains belonging to different clades to pseudotype lentiviral particles. Unexpectedly, we observed significant differences in the efficiency of H5-HAs to generate H5pp in 293T cells. In particular, H5 HA from A/Anhui/2005/01 (H5Anh) was unable to produce H5pp, yielding low luminescence signals after particle transduction in Madin-Darby canine kidney (MDCK) cells, whereas H5-HA from A/Cambodia/40808/2005 (H5Cam) was the most efficient strain.

Through in depth mutagenesis studies, we revealed that when a single A134V mutation was introduced in the receptor-binding site, the ability of the usually inefficient H5Anh to generate H5pp was largely restored.[6] Differences in receptor binding ability and cell surface expression pattern, due to mutations in the receptor-binding domain of HA, may be the underlying mechanism. First, by multiple sequence alignment, we identified a small region around the 130-loop of receptor binding site of HA that seemed to be a 'hot spot' that harboured diverse sequence variations among different H5N1 strains. Then serial H5-HA mutants were generated, and eventually we found that one single A134V mutation was a critical switch to dictate the ability of H5 HA to produce H5pp. Using a cell-based ELISA binding approach, we found that H5Anh that contains 134A displayed strong binding to both MDCK and MDCK-SIAT cells (more alpha-2,6- and less alpha-2,3-sialic acid than parental MDCK); and A134V mutation reduced the binding to a dramatically lower level. It is very likely that strong binding of H5Anh to its cell surface receptors makes it difficult to release H5pp from the producer cells; and A134V mutation reduces the binding thus allowing the release of H5pp. We did not observe an increase in binding to MDCK-SIAT cells that contain more alpha-2,6-sialic acids on the cell surface, suggesting that A134V mutation probably leads to decreased binding to alpha-2,3-sialic acids rather than a switch to alpha-2,6-sialic acid binding.[6]

Implications

Our study has demonstrated the underlying molecular mechanism for the efficient production of H5pp. Through serial mutagenesis of two H5-HAs, we have revealed that differences in receptor binding ability, due to mutations in the receptor-binding domain of HA, may be the underlying mechanism. In addition, A134V mutation has been reported as a naturally occurring mutation in the human host. Our results may have implications for the understanding of human host adaptation of avian influenza H5N1 viruses.

Interestingly, alanine at position 134 is highly conserved in avian H5N1 viruses; to date A134V mutation has been found only in human isolates of H5N1 viruses. All avian H5N1 viruses possess 134A except for one that has 134S instead. Notably for human isolates of H5N1 viruses, more diversity is observed at this position: three H5N1 viruses isolated from human patients have 134T and eleven have 134V. In at least two cases (A/Cam/408008/2005 and A/Thailand/676/2005), viruses found in the original patient specimens were a mixture of both the wild type virus containing 134A in the HA and mutant virus containing 134V. It is possible that some human isolates of H5N1 viruses may actually contain an A134V mutation but were not detected in the process of virus isolation or sequencing of viral genomes. These observations suggest that 134V may be selected as the avian H5N1 viruses adapt for replication in human hosts. It may be of importance to monitor closely mutations in the receptor binding site of H5 HA; H5pp production together with soluble HA protein cell binding analysis may serve as convenient functional assays to monitor for mutations in H5N1 viruses with potential consequences for human host adaptation.

We have found that the A134V mutation not only exerts a critical influence in the determination of pseudotyping efficiency, but has an impact on H5N1 viruses. Both A/Cambodia/408008/2005 and A/Cambodia/V0401301/2011, two different H5N1 isolates carrying the same A134V mutation, could agglutinate human and guinea pig red blood cells (RBCs) but failed to agglutinate horse RBCs; two other strains of H5N1 viruses without the A134V mutation could also agglutinate horse RBCs. These observations indicate that an A134V mutation in H5-HA reduces virus binding to alpha-2,3-sialic acid. The differential RBC binding properties observed at the whole virus level, when both HA and NA are present, support the idea that A134V mutation in H5-HA can be biologically relevant.[6]

Through several independent lines of evidence, we have identified the molecular determinants in HA that enable efficient packaging into H5pp and have defined the underlying molecular mechanism. Our results have been discussed in the context of understanding human host adaptation of avian influenza H5N1 viruses.

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Screening of aqueous and organic extracts from a variety of fungi for their ability to antagonise the pathogenic yeast *Candida albicans*

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KEY MESSAGES

1. Aqueous extracts and ethyl acetate extracts from the fruiting bodies and mycelia of various fungi were tested for anti-*Candida albicans* activity.
2. The aqueous extracts of the fruiting bodies of two mushrooms, *Russula nigricans* and *Suillus placidus*, elicited some inhibition of *C albicans*.
3. The ethyl acetate extracts of the fruiting bodies of all mushrooms did not produce a conspicuous inhibition of *C albicans*.
4. Accumulation of the nuclear dye SYTOX green in *C albicans* cells did not occur after exposure to the *Russula nigricans* and *Suillus placidus* aqueous extracts, suggesting that inhibition of *C albicans* is not due to a membrane permeabilising effect.

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Introduction

Yeasts belonging to the genus *Candida* cause infection in susceptible patients. The increase in *Candida* infections accompanies advancements in medicine such as invasive procedures, the use of immunosuppressive drugs for organ transplants, and the frequent administration of broad-spectrum antibiotics. *C albicans* is the most common cause of candidiasis that represents the fourth most frequent nosocomial infection involving both mucosal and deep tissues, with mortality that can exceed 40%. Azoles, polyenes, and fluorinated pyrimidines are the currently available antifungal drugs. New antifungal agents are desirable. In view of the development of fungal resistance to antifungal agents,[1] a search for new ones is warranted.

Fungi produce antifungal molecules for defence against intruding fungi. The yeast *Saccharomyces boulardii* has been used to prevent and treat intestinal infections caused by bacterial pathogens. It demonstrates an antagonistic effect on filamentation, adhesion, and biofilm formation in *C albicans*. The fungi *Pichia angusta* and *Botrytis cinerea* interact with *Candida* species. Antifungal lipopeptides are produced by *Cryptosporiopsis* and *Mycogone* spp. *Aspergillus giganteus* and *A niger* also produce antifungal peptides. Antifungal cyclic peptides are produced by *Isaria felina* and *Clavariopsis aquatica*.

Defensins are defensive peptides. Plectasin, a fungal defensin, kills bacteria by binding to the bacterial cell-wall precursor Lipid II. Plant defensins can inhibit *C albicans*[2] and thus are potentially exploitable in the treatment of fungal diseases in humans.

Antifungal peptides and proteins against plant pathogenic fungi with different N-terminal sequences have been isolated in our laboratory from edible basidiomycete fungi (mushrooms). Most were prepared by aqueous extraction and inhibited mycelial growth with IC_{50} values at micromolar concentrations.

This study aimed to test the aqueous extracts and ethyl acetate extracts of a variety of fungal species for inhibitory activity against *C albicans*.

Study design and instruments

This study was conducted from December 2010 to December 2011.

Strains and growth conditions

Clinical strain 08-189073 and ATCC strain of *C albicans* were used. An isolated colony of fungi was inoculated into 5 mL of yeast nitrogen base (YNB, pH 5.5) broth (Difco Laboratories) and incubated overnight at 37°C. A 0.1 mL aliquot of this pre-culture was inoculated into 5 mL of YNB, incubated for 24 h at 37°C, and used for all experiments.

Sample preparation

Dried fruiting bodies of mushrooms were collected from local markets and Yunnan Province, China.

Mycelia of other fungi were obtained from the Department of Microbiology, China Agricultural University, Beijing, China. The collection was composed of fruiting bodies and mycelia of fungi belonging to different orders and families. To obtain aqueous extracts, the fruiting bodies or mycelia were homogenised in liquid nitrogen using a pestle. The homogenised powder was soaked in distilled water for 12 hours and centrifuged. The resulting supernatants were concentrated by freeze-drying. To obtain organic extracts of the fungi, the powder obtained above was extracted with ethyl acetate (1 g/5 mL) for 3 hours, with changes of ethyl acetate every half hour. The ethyl acetate was removed using a rotary evaporator. Organic extracts were dissolved in dimethyl sulfoxide to increase the solubility.

Assay for antifungal activity

C albicans was incubated in 10 mL nutrient broth for 12 hours at 37°C. The suspension (5 mL) was transferred to 50 mL nutrient broth and incubated for 6 h to shift growth to the mid-logarithmic phase. The yeast suspension was then centrifuged (2000 g, 10 min). The pellet was collected and resuspended in phosphate buffer saline. Each sample was prepared in triplicate; one aliquot of yeast was mixed with the test samples at 4, 2, 1, 0.5, and 0.25 mg/mL; one aliquot of yeast was mixed with culture medium as a negative control. The mixtures were incubated in a shaker and aliquots obtained at 0, 3, 6, and 12 h, then serially diluted with nutrient broth and spread on agar plates. After incubation at 37°C for 24 h, the colonies were counted. The average number of colonies for each condition and dilution was derived from the three plates. Amphotericin B was used as positive control.

Membrane permeabilisation

This assay is based on uptake of SYTOX Green. After incubation with the nuclear dye for 10 min, fungal cells were analysed with a fluorescence microscope for internalised dye. Controls were treated with amphotericin B.

Results

Antifungal activity

The results of the assay for the aqueous extracts and ethyl acetate extracts of various fungi for activity against *C albicans* (ATCC 90028) are shown in the Table. All aqueous extracts except those of *R nigricans* and *S placidus*, and all ethyl acetate extracts brought about 2% to 12% inhibition of *C albicans* reference strain ATCC 90028 (after treatment for 12 hours at 37°C) and thus were regarded as inactive for practical purposes. The aqueous extract of *R nigricans* showed 22-40% inhibition while the aqueous extract of *S placidus* showed 11-23% inhibition at the concentrations of 1-3 mg/mL. The aqueous extract of *R nigricans* (3 mg/mL) inhibited *C albicans* reference strain ATCC 90028 and clinical strain 08-189073 by about 60% after treatment for 12 hours at 37°C.

SYTOX green uptake

SYTOX green accumulation in *C albicans* cells was not observed after exposure to *R nigricans* aqueous extract.

Discussion

In this study, a number of fungal species belonging to different orders and families were examined for their ability to inhibit *C albicans*. The inhibition elicited by the vast majority of these fungal species was too low (<20%) to be considered significant. The aqueous extracts of two fungal species, *S placidus* and *R nigricans*, elicited >20% inhibition at a concentration of 4 mg/mL and 1 mg/mL, respectively. We focused on the aqueous extract of *R nigricans* in view of its higher anti-*C albicans* activity.

The lack of SYTOX green accumulation in *C albicans* cells following treatment with *R nigricans* aqueous extract suggests that the fungal extract does not adversely affect the permeability of *C albicans* membrane and that it inhibits *C albicans* by some other mechanism. This observation is in contrast to the findings that some antifungal peptides adversely affect permeability of the *C albicans* membrane.[3]

The presence of antifungal protein previously reported in some of the fungal species examined in the present investigation, which did not inhibit *C albicans*, suggests that the isolated antifungal proteins do not inhibit *C albicans* although they are active against other fungal species. Previously we have found that some antifungal proteins exhibit broad-spectrum activity against various fungal species but other antifungal proteins demonstrate activity against only one or two fungal species.[4]

Acknowledgement

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TABLE. Anti-*Candida* activity of fungal extracts toward reference strain ATCC 90028 (treatment for 12 hours at 37°C)

Scientific Name	Mean±SD % growth inhibition of *Candida albicans*					
	Aqueous extract (mg/mL)			Ethyl acetate extract (mg/mL)		
	1	2	3	1	2	3
Fruiting bodies of fungi						
Agaricus blazei	5±0.2	4±0.4	5±0.5	-	5±0.2	5±0.2
Agrocybe cylindracea	3±0.2	6±0.2	8±0.5	5±0.1	3±0.2	8±0.8
Amanita caesarea	4±0.4	5±0.7	11±0.7	-	-	-
Amanita manginiana	4±0.2	5±0.2	4±0.4	-	4±0.2	4±0.5
Boletus bicolour	5±0.3	7±0.7	9±0.5	4±0.3	5±0.3	8±0.5
Boletus edulis	6±0.4	8±1.2	8±0.5	-	6±0.7	8±0.3
Boletus sp.	2±0.1	-	-	2±0.1	3±0.1	5±0.5
Catathelasma ventricosum	-	5±0.4	8±0.5	-	-	5±0.4
Coprinus comatus	7±0.2	6±0.5	12±1.5	2±0.6	4±0.2	2±0.5
Cortinarius collinitus	4±0.8	-	3± 0.5	4±0.1	4±0.6	8±1.8
Cortinarius lilacinus	-	6±0.3	9±0.5	-	-	5±0.5
Dictyophora duplicata	7±0.6	7±0.5	11±0.7	7±0.6	7±0.6	11±2.5
Diehiomyces microsporus	5±0.4	6±0.6	10±0.8	-	5±0.4	10±1.5
Flammulina velutipes	3±0.3	8±0.7	9±0.5	-	3±0.3	9±0.5
Gloeostereum incarnatum	-	9±0.8	11±1.0	-	-	7±0.5
Gomphidius viscidus	6±0.6	7±0.2	7±0.5	6±0.6	6±0.6	7±0.4
Hericium erinaceum	7±0.3	6±0.5	9±0.8	7±0.3	7±0.3	9±0.2
Hypsizygus marmoreus	8±0.6	7±0.6	8±0.6	5±0.6	4±0.6	4±0.6
Lactarius volemus	9±0.4	8±0.3	8±0.5	-	3±0.4	8±0.5
Lentinula edodes	7±0.5	9±0.8	8±0.5	7±0.5	7±0.5	11±0.5
Marasmius oreades	-	5±0.3	7±0.5	-	-	9±0.5
Phellinus igniarius	5±0.4	6±0.2	6±0.5	5±0.4	5±0.4	8±0.4
Pholiota nameka	6±0.1	7±0.5	12±0.9	-	6±0.1	10±0.8
Pleurotus citrinopileatus	5±0.2	6±0.6	7±0.5	5±0.2	4±0.4	6±0.5
Pleurotus eryngii	6±0.3	6±0.7	5±0.5	-	6±0.6	5±0.2
Pleurotus ostreatus	7±0.6	6±0.3	6±0.5	-	7±0.6	8±0.5
Pycnoporus sanguineus	-	-	3±0.5	6±0.3	6±0.3	10±0.7
Russula lepida	8±0.7	8±1.1	11±0.5	8±0.7	8±0.7	11±0.8
Russula nigricans	**22±4.6**	**38±3.4**	**40±3.8**	**2±0.6**	**-**	**-**
Sarcodon aspratus	6±0.3	8±0.2	6±0.5	-	-	-
Suillus pictus	7±0.2	7±0.5	11±0.5	3±0.2	6±0.4	9±0.5
Suillus placidus	**11±2.0**	**16±3.4**	**23±2.5**	**-**	**1±0.2**	**3±0.3**
Thelephora ganbajun	5±0.4	6±0.2	9±0.5	5±0.3	5±0.2	6±0.2
Trametes versicolor	6±0.2	7±0.6	10±0.5	-	6±0.2	11±0.6
Tylopilus virens	6±0.4	7±0.4	9±0.5	6±0.3	6±0.4	6±0.8
Volvariella volvacea	6±0.1	8±0.6	9±0.5	-	-	2±0.5
Wolfiporia cocos	-	4±0.3	7±0.5	-	4±0.2	5±0.5
Mycelia of fungi						
Botrytis cinerea	7±0.2	8±0.9	10±0.5	-	-	9±0.5
Coprinus comatus	5±0.2	7±0.6	9±0.5	-	5±0.2	9±0.4
Fusarium oxysporum	-	6±0.8	8±0.5	-	-	8±0.5
Fusarium solani	5±0.2	6±0.8	8±0.5	2±0.2	3± 0.2	3±0.5
Helminthosporium maydis	5±0.4	7±0.7	8±0.5	5±0.4	5±0.4	10±1.2
Rhizoctonia solani	-	6±0.3	9±0.5	-	-	5±0.5
Tricholoma mongolicum	6±0.7	7±0.5	9±0.5	5±0.1	6±0.1	5±0.3
Valsa mali	6±0.3	6±0.6	10±0.5	2±0.2	3±0.3	3±0.5

'-' denotes no inhibition or even stimulation of proliferation; inhibition <20% is considered too low to be significant

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Functional analysis of apoptosis-inducing factor in the human fungal pathogen *Candida albicans*

WK Tsang *, WP Fong

KEY MESSAGES

1. We have cloned and expressed the three putative *Candida albicans* apoptosis-inducing factor (AIF) sequences (orf19.1438, orf19.2175, orf19.2671) in *Escherichia coli* BL21. The proteins are designated as CaAifp-orf19.1438, CaAifp-orf19.2175, CaAifp-orf19.2671, respectively.
2. The three CaAifp have been purified from *E coli* BL21 to homogeneity.
3. Among the three CaAifp, only CaAifp-orf19.2175 and CaAifp-orf19.2671 possess NADH oxidase activity. The former also exhibits DNase activity.
4. CaAifp-orf19.2175 can functionally complement *S cerevisiae Δaif1* mutant and sub-cellular translocation of this protein is evident upon apoptotic challenge.
5. CaAifp-orf19.2175 is a bona fide *C albicans* AIF.

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Introduction

Apoptosis is a highly organised cellular process that leads to cell death in multicellular organisms. Apoptotic features have been induced in *Candida albicans* following exposure to farnesol, acetic acid, H_2O_2, or a low dose of antifungal agent.[1] Mitochondria play a key role in energy production and cell death. Mitochondrial dysfunction has been regarded as the onset of apoptosis, exemplified by depolarisation of mitochondrial membrane potential, elevation of the level of reactive oxygen species, and release of apoptosis-inducing factor (AIF) and cytochrome *c*.[2] AIF is a highly conserved protein, and *C albicans* AIF has yet to be identified.

In our earlier studies of the antifungal activity of purpurin against *Candida* species, we hypothesised the existence of a mitochondrial-mediated cell death pathway in *C albicans*. In light of the phylogenetic relationship between *S cerevisiae* and *C albicans*, we performed a BLAST database search of the *C albicans* genome using the amino acid sequence of *S cerevisiae* AIF (YNR074c) as query and identified three putative AIF sequences (orf19.1438, orf19.2175, orf19.2671).

This project aimed to identify and characterise cell death mediators in *C albicans* by (1) cloning and expressing the three putative *C albicans* AIF sequences, and (2) determining the cellular localisation and functional role of AIF in energy production and cell death.

Methods

This study was conducted from December 2011 to December 2012.

Strains and cultivation

C albicans SC5314 was used for DNA isolation, and *C albicans* BWP17 (*ura3*, *his1*, *arg4*) was used for gene tagging experiments. They were cultivated on YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C. *Escherichia coli* DH5α (Novagen) was used for plasmid propagation, and *E coli* BL21 (Novagen) was used for protein expression. Bacterial strains were grown on LB medium at 37°C. *S cerevisiae Δaif1* mutant was obtained from EUROSCARF. *S cerevisiae* and its derivatives were cultivated on SC medium (0.67% yeast nitrogen base without amino acids (Difco), 0.077% CSM-ura supplements (BIO101), 2% dextrose) at 30°C. Unless otherwise specified, 2% bactoagar (Difco) was added to prepare the solid media.

Cloning, expression, and purification of *C albicans* apoptosis-inducing factor

The three putative *C albicans* AIF sequences were obtained by PCR using gene-specific primers (Table 1). The nucleotide sequences of the cloned genes were confirmed by automatic DNA sequencing (TechDragon). To circumvent the unusual codon usage in *C albicans*, the CUG codons in orf19.1438 and orf19.2175 were converted to UUG codon by site-directed mutagenesis using mutagenic primers (Table). The three AIF sequences were subcloned into pET28b (Novagen) and expressed in *E coli* BL21 as C-terminal 6×His-tagged fusion proteins (CaAifp-orf19.1438, CaAifp-orf19.2175, CaAifp-

orf19.2671). Protein expression was induced by IPTG (1 mM) at 18°C for 20 hours. After induction, the cells were harvested by centrifugation (2400 g, 15 min) at 4°C, washed once and resuspended in ice-cold Buffer A (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 8) supplemented with 1 mM phenylmethylsulphonyl fluoride and disrupted by sonication on ice (seven cycles, 30 s each, with 30 s intervals). Cell-free extract was obtained by centrifugation (13 000 G, 30 min) at 4°C and filtration (0.2 μm nylon membrane; Millipore). The filtrate was applied to a pre-equilibrated HisTrap HP column (GE Healthcare), and CaAifp were eluted with increasing concentrations of imidazole. The purity of the proteins was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 12% polyacrylamide gels. Protein concentration was determined by the Bradford method using bovine serum albumin as standard.

NADH oxidase activity

NADH oxidase activity of the purified CaAifp was determined in a 3-mL reaction mixture containing 50 mM KH_2PO_4, 0.1 mM FAD, and 0.07 mM NADH, pH 7 at 30°C. The reaction was followed by decreased absorbance at 340 nm.[3] One unit (U) of NADH oxidase activity was defined as the amount of protein that yielded 1 μmol of NAD^+ per min.

DNase activity

DNase activity of the purified CaAifp was determined in a 100-μL reaction mixture at 37°C containing 0.5 μg plasmid DNA or purified *C albicans* nuclei,[4] 20 μg purified CaAifp, 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.9. At the indicated time, DNA was precipitated by phenol/chloroform/isoamyl alcohol extraction and analysed using gel electrophoresis.

Sub-cellular localisation of CaAifp

The three putative AIF sequences were tagged with green fluorescent protein (GFP) at their C-terminal end using a PCR-based approach via homologous recombination.[5] Briefly, tag cassettes were synthesised by gene-specific primers (Table) and plasmid pGFP-URA3, and were transformed into *C albicans* BWP17. Correct integration of the tag-cassette into chromosomal AIF sequences was identified by PCR, using one primer that annealed within the tag-cassette and another that annealed the AIF sequence (Table). Sub-cellular localisation of the GFP-tagged *C albicans* AIF sequences was evaluated by confocal microscopy (excitation: 480 nm; emission: 530 nm). Mitochondria were stained with MitoTracker Red CMXRos (Invitrogen) (excitation: 579 nm; emission: 599 nm). To evaluate sub-cellular translocation of GFP-tagged CaAifp, fungal cells were challenged by H_2O_2 (5 mM) for 4 hours. Digital images were collected using a CCD camera and processed using Adobe Photoshop.

TABLE. Primers for cloning, site-directed mutagenesis, and gene tagging

Primer name	Sequence[a,b,c] (5' to 3')
Cloning*	
1438-F	**CATatg**acgccaaaagtggtaatcattg
1438-R	**GGATCC**ttaattgactgttgcaccaata
2175-F	**CATatg**tctaaagagaaaagtaaacaga
2175-R	**GGATCC**tcacgtaaaatgctccccagct
2671-F	**CATatg**acagatctcgccaaacaaccac
2671-R	**GGATCC**ttacatatctatagtcgctcta
Site-directed mutagenesis†	
1438-67	taaacacattaaaaAGCttaattaccaaaaa
1438-640	accagagccgatcAGCgatgctttcaaga
2175-718	gattattgaaacaAGCggccttttaccca
2175-847	agacgtggttgccAGCggtgcaaactcaa
Gene tagging‡	
1438-GFP-F	
1438-GFP-F	atcaatggttcaagagtatcaggattaccggttagggacatgtaagaagtttatt ggtgcaacagtcaatGGTGGTGGTTCTAAAGGTGAAGAATTATT
1438-GFP-R	atcgacgtgtaaaagtgagaaaaaaaaaaaaagttaatatttcagtgttgcaaac atatttcaatagttctTCTAGAAGGACCACCTTTGATTG
2175-GFP-F	accaagctttttaatttggtttgctaaggcaagagattttatgatttccaaagctg gggagcattttacgGGTGGTGGTTCTAAAGGTGAAGAATTATT
2175-GFP-R	cgaaattcacaaaaacgatttctcttaaacacatatatattttgctatctaggtat atggattattctatTCTAGAAGGACCACCTTTGATTG
2671-GFP-F	ggataatgagcattataagcaagagtatgaggattattgtataggtaaaattag agcgactatagatatgGGTGGTGGTTCTAAAGGTGAAGAATTATT
2671-GFP-R	cacaacaacactttgaataaccactaaacttctctgtttcctttattgcttacaca aacttccacaccacTCTAGAAGGACCACCTTTGATTG
Verification	
AIF-TagF	CCTATGAATCCACTATTGAACC
1438-TagR	ATCGACGTGTAAAAGTGAGAAA
2175-TagR	CGAAATTCACAAAAACGATTTC
2671-TagR	CACAACAACACTTTGAATAACC

* Lower case sequences are homology regions; restriction sites for cloning are in bold: *Nde*I (CATATG), *Bam*HI (GGATCC)

† Lower case sequences are homology regions; mutagenic sites are underlined

‡ Lower case sequences are homology regions; upper case sequences anneal tag cassettes

Functional characterisation of CaAifp

The three *C albicans* AIF sequences were subcloned into *S cerevisiae* episomal vector pRS426 (Novagen) and transformed into *S cerevisiae Δaif1* mutant. The transformants were challenged by H_2O_2 and cell survival was determined using a drop test assay. DNA fragmentation was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick

end labelling (TUNEL) assay using the *In Situ* Cell Death Detection Kit (Fluorescein) (Roche). The fluorescence was quantified using a FACSort flow cytometer (Becton Dickinson) at 530 nm. The data were analysed with the WinMDI 2.9 software.

Results

Preparation and characterisation of the purified CaAifp

The three *C albicans* AIF sequences were cloned. Protein expression was achieved in *E coli* BL21 and the CaAifp (CaAifp-orf19.1438, CaAifp-orf19.2175, CaAifp-orf19.2671) were obtained as C-terminal 6×His-tagged fusion proteins to homogeneity. NADH oxidase activity was only detected in CaAifp-orf19.2175 (4.2±0.5 U/mg) and CaAifp-orf19.2671 (3.9±0.4 U/mg). Only CaAifp-orf19.2175 was able to degrade purified *C albicans* nuclei and plasmid DNA in a time-dependent manner (Fig 1).

Sub-cellular localisation of CaAifp

The sub-cellular localisation of the three CaAifp could be visualised in *C albicans* using confocal microscopy. CaAifp-orf19.1438 and CaAifp-orf19.2671 were cytosolic proteins, whereas CaAifp-orf19.2175 was a mitochondrial protein. Upon apoptotic challenge by H_2O_2, CaAifp-orf19.2175 translocated from the mitochondria to the cytosol (Fig 2).

Complementation study

In the presence of an apoptotic level of H_2O_2, the *S cerevisiae* strain harbouring CaAifp-orf19.2175 exhibited normal apoptotic responses as demonstrated by the drop test assay. In addition, TUNEL assay showed that 54.87%±5.39% of the cell population was TUNEL-positive.

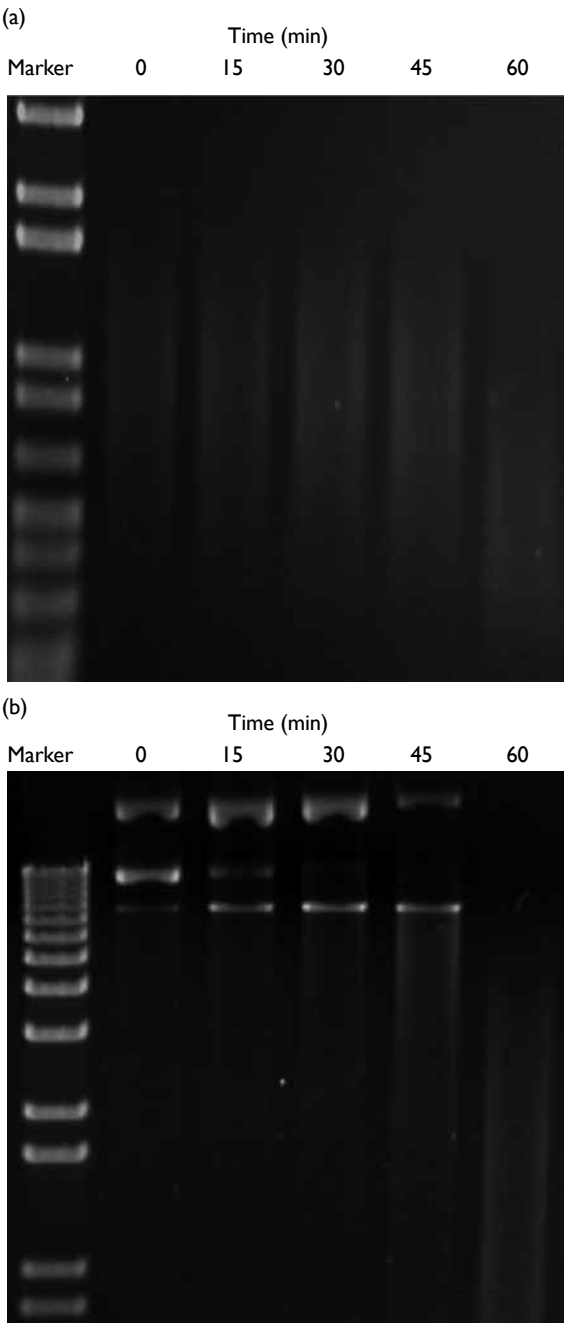

FIG 1. DNase activity of the purified CaAifp-orf19.2175: (a) purified *Candida albicans* nuclei, and (b) plasmid DNA.

Discussion

Three putative AIF sequences (orf19.1438, orf19.2175, orf19.2671) were identified in the *C albicans* genome. In *C albicans*, the CUG codon is translated into serine instead of leucine. Therefore, we performed site-directed mutagenesis to convert the CUG codon to UUG codon in orf19.1438 and orf19.2175 for proper (over)expression in *E coli*. At a lower temperature (18°C) and for a longer duration (20 hours) of IPTG induction to avoid the formation of inclusion bodies, we were able to express the three CaAifp in soluble form. In general, ~1 mg/mL protein was routinely obtained in the crude extract. Addition of a 6×His-tag allowed one-step purification using affinity chromatography.

AIF is a bifunctional enzyme that plays a pivotal role in energy production (NADH oxidase activity) and cell demise (DNase activity). CaAifp-orf19.2175 and CaAifp-orf19.2671 possess NADH oxidase activity, and the former also degrades DNA. More experiments are warranted to understand the nature of its NADH oxidase and DNase activities in terms of enzyme inhibition and co-factor requirements.

The sub-cellular localisation of the *C albicans* CaAifp was readily visualised by confocal microscopy using GFP tagging. Both CaAifp-orf19.1438 and CaAifp-orf19.2671 are cytosolic proteins. CaAifp-orf19.2175 is a mitochondrial protein, as the green and red fluorescence were shown superimposed. The apoptotic features of

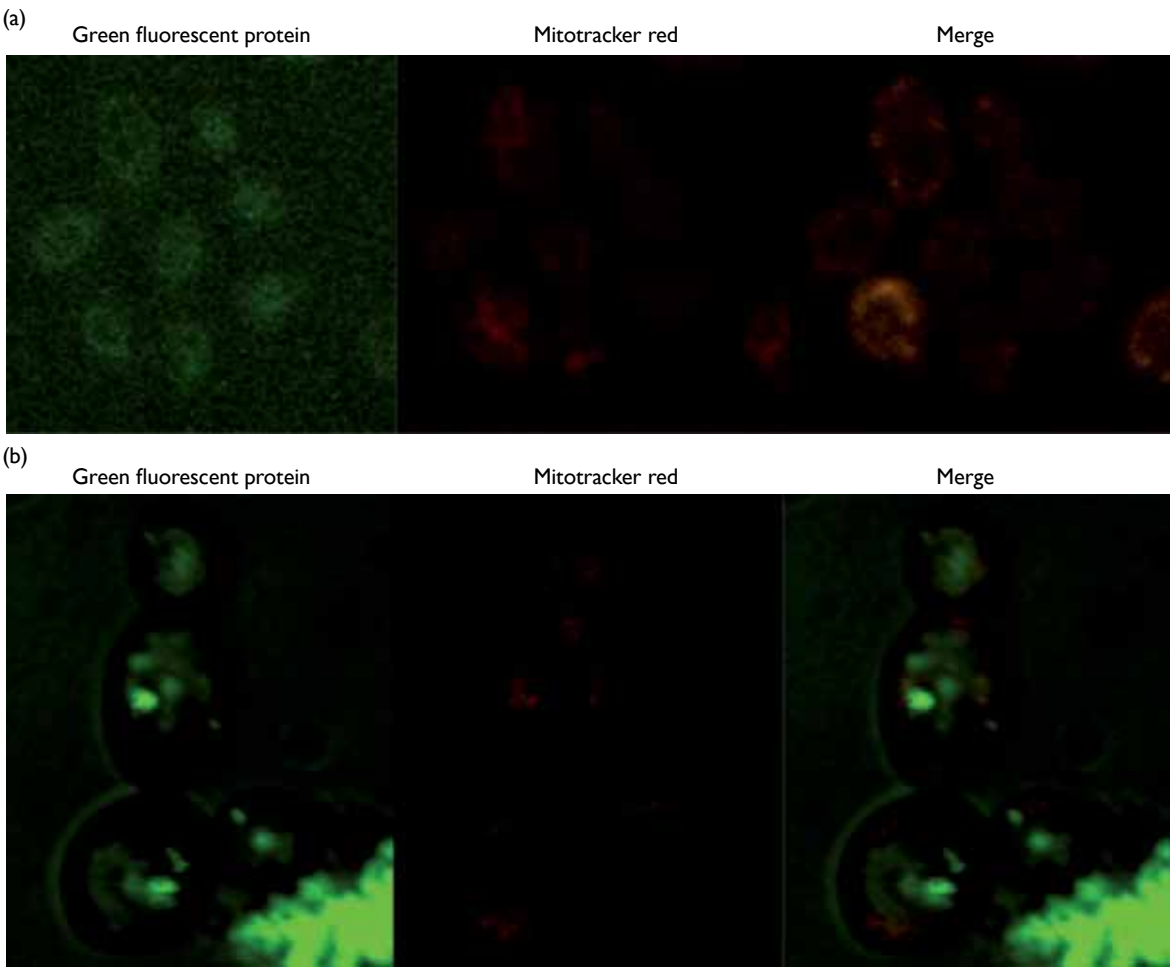

FIG 2. Sub-cellular translocation of CaAifp-orf19.2175 upon apoptotic challenge (a) before and (b) after H_2O_2 treatment

CaAifp-orf19.2175 were demonstrated by its sub-cellular translocation upon oxidative stress, the ability to functionally complement *S cerevisiae Δaif1* mutant, and the presence of TUNEL-positive cells in CaAifp-orf19.2175 complemented the *S cerevisiae* strain in the presence of an apoptotic level of H_2O_2. Our data also suggest phylogenetic conservation of a mitochondrial-mediated cell death mechanism in *S cerevisiae* and *C albicans*. Further studies are needed to establish linkage between the apoptotic functions of CaAifp, caspase, and other effectors in *C albicans* such as cyclophilin, Ras-cAMP-PKA pathway, and antifungal agents.

Conclusion

CaAifp-orf19.2175 is a bona fide *C albicans* AIF. It provides evidence of the phylogenetic conservation of the cell death mechanism in unicellular microorganisms, and opens up a new research area in *C albicans* that focuses on mitochondrial-mediated cell demise.

Acknowledgements

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Identification of antifungal molecules from novel probiotic *Lactobacillus* bacteria for control of *Candida* infection

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KEY MESSAGES

1. Probiotic lactic acid bacteria were identified to have antifungal properties against *Candida albicans*.
2. The cell-free supernatant of the lactic acid bacteria was successfully fractionated and purified using fast performance liquid chromatography.
3. Eight of the 41 fractions containing the antifungal components exhibited a growth inhibitory effect against *C albicans*.

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Introduction

Candida is a commensal fungus that inhabits various niches of the human body and can cause infection under certain circumstances, ranging from superficial mucous membrane infection to systemic disease with high mortality.[1] Rising drug resistance and undesirable side effects of currently available antifungal agents have prompted research of alternative therapeutic strategies.[2] Probiotics are live microorganisms that confer health benefits on the host when administered in adequate amounts in food or as a dietary supplement, according to the Food Agricultural Organization/World Health Organization. Probiotics, such as lactic acid bacteria (LAB), are well-recognised for their health benefits and have been shown to improve various medical conditions, including gastrointestinal disorders, cancer, and infectious disease.[3] Probiotic-containing products can reduce *Candida* infection such as oral candidiasis and vulvovagional candidiasis.[4] Probiotic products have preferable properties as antifungal agents for control of *Candida* infection, because they are natural, effective, and safe.

In a preliminary study, we found that LAB had antifungal properties. Subsequent experiments demonstrated that culture supernatant of LAB inhibited the hyphal formation of *C albicans* in a dose-dependent manner, and exhibited fungicidal acidity at slightly higher concentrations. LAB supernatant was effective in preventing *C albicans* infection in vitro. The present study aimed to identify the inhibitory antifungal molecules in the LAB supernatant using standard methodology.

Methods

This study was conducted from October 2012 to September 2013.

Preparation of cell-free supernatant

Lactobacillus species were cultured in Man Rogosa Sharpe broth (Difco Laboratories, USA) anaerobically at 37°C to the stationary phase. Cultures were centrifuged at 9500 × g for 15 min and filter-sterilised through a 0.45 μm pore filter (Advantec MFS, Japan). The cell-free culture filtrate was then used for downstream experiments.

Fractionation and purification of LAB supernatant

Proteins of the culture supernatant were purified using standard methodology. In brief, proteins in the cell-free supernatant were precipitated with ammonium sulfate, dissolved in sodium phosphate buffer and desalted by overnight dialysis in sodium phosphate buffer at 4°C. The mixture of proteins was then fractionated in a DEAE Sepharose Fast Flow column (HiPrep 16/10 DEAE-FF, GE Healthcare) mounted in a fast performance liquid chromatography (FPLC) system (Akta purifier, GE Healthcare). Activity was eluted at a flow rate of 1 mL/min using a running buffer of 0.05M Tris (pH 7.5) and an elution buffer of 0.05M Tris (pH 7.5) and 2M NaCl. A gradient of NaCl was applied linearly from 0 to 40% (elution buffer). A total of 41 fractions (5 mL each) were collected. The total protein concentration of each sample was measured using a 2D Quant kit (GE Healthcare).

Antifungal activity of the fractions

All fractions were measured for their antifungal activity against *C albicans* using a broth microdilution assay in a time-dependent manner. Inocula from

TABLE. Antifungal activity of the fractions of supernatants derived from lactic acid bacteria*

Fraction	Time-interval		
	24 h	48 h	72 h
A1	×	×	×
A2	×	×	×
A3	×	×	×
A4	×	×	×
A5	×	×	×
A6	×	×	×
A7	×	×	×
A8	100%	100%	50%
A9	100%	50%	×
A10	100%	100%	50%
A11	×	×	×
A12	100%	×	×
B1	×	×	×
B2	×	×	×
B3	×	×	×
B4	×	×	×
B5	×	×	×
B6	100%	50%	50%
B7	100%	50%	50%
B8	100%	100%	100%
B9	100%	100%	100%
B10	×	×	×
B11	×	×	×
B12	×	×	×
C1	×	×	×
C2	×	×	×
C3	×	×	×
C4	×	×	×
C5	×	×	×
C6	×	×	×
C7	×	×	×
C8	×	×	×
C9	×	×	×
C10	×	×	×
C11	×	×	×
C12	×	×	×
D8	×	×	×
D9	×	×	×
D10	×	×	×
D11	×	×	×
D12	×	×	×

* Complete (100%), partial (50%), or no (x) inhibition of growth was recorded for each fraction at each time point. Eight of the fractions displayed an inhibitory effect against *Candida albicans*. Four of them exhibited a complete inhibitory effect (100%) for 48 hours or more

24-hour *C albicans* cultures were harvested and suspended in RPMI 1640 medium with turbidity equivalent to McFarland standard 0.5 (1×10^6 cells/mL) and then diluted to approximately 0.5×10^3 to 2.5×10^3 cells/mL. The test was performed in pre-sterilised, flat-bottom 96-well polystyrene plates (Iwaki, Japan), with each well filled with 0.1 mL of *C albicans* suspension and 0.1 mL of the fraction. The plates were then incubated at 37°C. The inhibitory activity of the fractions was recorded by visual observations of growth inhibition after 24, 48, and 72 hours. To verify the results, at the end of the 72-hour incubation, aliquots of broth culture were plated on the Sabouraud Dextrose Agar and the number of colonies was recorded. Fractions with significant antifungal activity as shown in the aforementioned assay were used for downstream proteomic studies to identify the antifungal protein components.

Results

A total of 41 fractions were collected from the FPLC purification of the cell-free LAB supernatant. The antifungal activity of these fractions is shown in the Table. Interestingly, eight of the 41 fractions

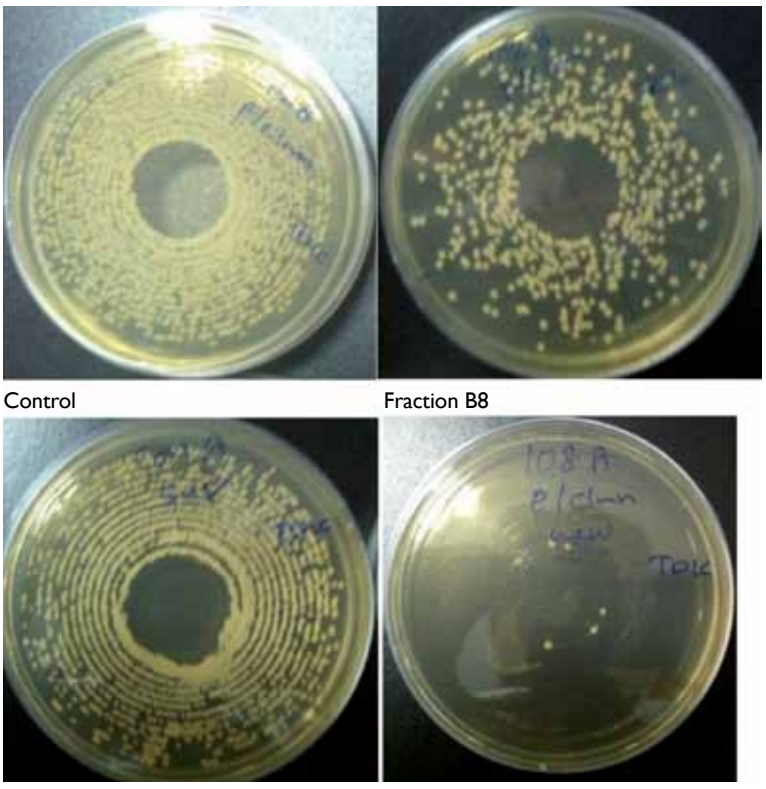

FIG. Growth inhibitory effect of each fraction was further evaluated by plating on Sabouraud Dextrose Agar and counting of the colony forming units (CFUs): the number of CFUs in Fractions B7 and B8 was significantly lower than that in controls, with 50% and 100% of the growth inhibitory effect against *Candida albicans*, respectively.

displayed a growth inhibitory effect against *C albicans*. Among these eight fractions, four (A8, A10, B8, and B9) exhibited a complete growth inhibitory effect (100%) in the broth microdilution assay when incubated with *C albicans* for 48 hours or more. This finding indicated that the antifungal components of the supernatant were successfully fractionated. The growth inhibitory effect of these fractions was further confirmed by plating on Sabouraud Dextrose Agar and counting of the colony forming units (Fig). These fractions were selected for further protein identification using liquid chromatography-mass spectrometry.

Discussion

Fungal infections, of which candidiasis is the most common form, have become a significant problem in the clinical setting worldwide, due to an increase in immunocompromised populations. In this study, we successfully identified LAB strains with antifungal activity against *C albicans*. The antifungal compounds of LAB are complex, and their isolation and purification from LAB are difficult.[5] Despite this, we successfully established a protocol for fractionation using FPLC, and isolated the working fractions from the probiotic supernatant that exhibited a growth inhibitory effect against *C albicans*. We are working on identification of the antifungal components in the selected fractions using a proteomics approach.

Conclusions

Candidiasis places a huge burden on public health, especially in immunocompromised populations. Prophylactic antifungal treatment is associated with undesirable side effects. Probiotic products, such as LAB, have been proven to be effective and safe to prevent and treat candidiasis. Therefore, it is important to identify appropriate probiotics and associated products. We successfully fractionated and purified the cell-free supernatant of LAB with antifungal activity using fast performance liquid chromatography, and eight of the 41 fractions exhibited antifungal effects in the broth microdilution assay against *C albicans*. Our findings facilitate further investigation of the molecular mechanisms of the antifungal activity of LAB.

Acknowledgement

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Antimicrobial activity of cathelicidin peptides and defensin against oral yeast and bacteria

JH Wong, TB Ng *, RCF Cheung, X Dan, YS Chan, M Hui

KEY MESSAGES

1. Human cathelicidin LL37 and its fragments LL13-37 and LL17-32 were equipotent in inhibiting growth of *Candida albicans*.
2. LL13-37 permeabilised the membrane of yeast and hyphal forms of *C albicans* and adversely affected mitochondria.
3. Reactive oxygen species was detectable in the yeast form after LL13-37 treatment but not in untreated cells suggesting that the increased membrane permeability caused by LL13-37 might also lead to uptake of the peptide, which might have some intracellular targets.
4. LL37 and its fragments also showed antifungal activity against *C krusei*, and *C tropicalis*.
5. A 5447-Da antifungal peptide with sequence homology to plant defensins was purified from king pole beans by chromatography on Q-Sepharose and FPLC-gel filtration on Superdex 75. It inhibited growth of fungi, including *Mycosphaerella arachidicola*, *Saccharomyces cerevisiae* and *C albicans* with an IC_{50} value of 3.9, 4.0, and 8.4 μM, respectively. The peptide increased fungal membrane permeability.
6. LL37 did not show obvious antibacterial activity below a concentration of 64 μM and its fragments did not show antibacterial activity below a concentration of 128 μM. Pole bean defensin exerted antibacterial activity on some bacterial species.

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Introduction

Fungal resistance to traditional drugs calls for new antimicrobial agents. In mammals, defensins and cathelicidins are two major families of antimicrobial peptides. Cathelicidins are cationic peptides with a conserved N-terminal cathelin-like domain and a variable C-terminal antimicrobial domain. Cathelicidins rapidly kill *Candida* and other yeast spp.[1] *Candida* infection with *C albicans* is the fourth most common cause of hospital-acquired bloodstream infection in the USA. Defensins are cationic cysteine-rich peptides found in mammals, fungi, insects, and plants.[2] Defensins interact with membrane phospholipids leading to compromised membrane permeability. Defensins gain entry into the cells to interact with intracellular targets and interfere with cellular activity.[3] This study aimed to (1) determine whether human cathelicidin LL37 and its fragments LL13-37 and LL17-32 can inhibit *C albicans*, and (2) purify an antifungal defensin from *Phaseolus vulgaris* cv. 'king pole bean' seeds to investigate its antimicrobial activity on the growth of pathogenic fungi and yeasts.

Methods

This study was conducted from November 2009 to January 2011. All peptides were synthesised using the solid-phase method of Fmoc chemistry. Cathelicidin and fragments were assayed for a cytotoxic effect on human peripheral blood monocytes and haemolytic effect on rabbit erythrocytes. Antifungal activity of LL37 and its fragments on *C albicans* and other *Candida* species was monitored using a XTT reduction assay.

Defensin was isolated from king pole beans by ammonium sulfate precipitation, chromatography on Q-Sepharose and gel filtration on Superdex 75. Molecular mass was determined by SDS-PAGE and mass spectrometry. Purified peptide was submitted to sequencing.

C albicans strains (SC5134 and ATCC 90028) were used. Yeast cells were cultured for 36 hours with or without LL13-37 before observation under a scanning electron microscopy (SEM).

To assay for permeabilisation of the fungal membrane, *C albicans* cultures were incubated with 5 μM LL13-37 at 37°C for 8 hours. SYTOX Green was then added up to 0.5 μM. After 10 minutes, cells were observed under a confocal microscope.[4] SYTOX Green, a high-affinity nuclear stain, penetrates cells with a compromised membrane.

To localise mitochondria in *C albicans* cells, the cells were cultured for 12 hours in RPMI 1640

with or without LL13-37, and washed with PBS. Mitotracker deep-red was added and incubated for 30 minutes. The medium was aspirated and cells were washed twice. Images were acquired using a confocal microscope.

C albicans cells were treated with bimane-labelled cathelicidin peptides and visualised under a confocal microscope (excitation: 330-385 nm).[5]

To measure reactive oxygen species production, *C albicans* cells were cultured for 24 hours with or without LL13-37, and washed. After incubation with 25 μM5-(and-6)-carboxy-2',7'-dihydrodichlorofluorescein diacetate (carboxy-H_2DCFDA) in PBS at 37°C for 30 minutes, the cells were washed and imaged using a confocal microscope.[6]

To assay induction of cytokine gene expression, cells were isolated from the spleen of BALB/c mice (20-25 g) by pressing the tissue through a sterilised stainless steel sieve and were resuspended in culture medium.[1] A 4-mL aliquot of cells was incubated with 10 mL medium containing 0.1 mL 2 μM isolated peptide solution and Con A, at 37°C in 5% CO_2 for 4 hours. Total RNA extraction was performed using TRIzol method followed by RT-PCR. Agarose gel electrophoresis was performed to visualise the PCR product.

To assay antibacterial activity, sterile Petri plates containing 10 mL of nutrient agar (8% agar) were used. A 3-mL aliquot of warm nutrient agar (0.7%) containing the bacterium was poured onto the plates. A sterile blank paper disk (0.625 cm in diameter) was placed on the agar. The test sample (10 μL) was added to the disk. The plate was incubated at 37°C for 24 hours. A transparent ring around the paper disk signified antibacterial activity.

Antibacterial activity against *Pseudomonas aeruginosa*, *Streptococcus sanguis*, *Staphylococcus aureus*, *Porphyromonas gingivalis*, and *Haemophilus influenzae* was assessed via broth macrodilution using Clinical and Laboratory Standards Institute methodology. Stock solutions of peptides were prepared to a concentration of 500 μg/mL in buffer. Organisms were subcultured and isolated on blood agar, suspended in 3 mL of Mueller-Hinton broth at a turbidity of a 0.5 M McFarland standard, and diluted to approximately 10^5 CFU/mL before introduction into a 96-well plate containing serially two-fold diluted peptides in Mueller-Hinton broth. The turbidity resulting from peptides solution in broth required the creation of a control well lacking microbes to serve as turbidity control. The plate was incubated overnight for 16-20 hours at 37°C.

Results

Characteristics of human cathelicidin and fragments

Treatment of human peripheral blood monocytes with 125 μM to 1 μM LL 37, LL13-37, and LL17-32 resulted in viability of 83-92%. There was no haemolytic effect on rabbit erythrocytes at 1 to 25 μM (91-99% viability), but LL37 and LL17-32 exerted a haemolytic effect at 125 μM.

Following exposure of cells to LL13-37, LL37, and LL17-32 for 24 hours at 37°C on a 96-well polystyrene plate, XTT assay revealed that *C albicans* cells were inhibited. The ranking of potencies was LL37 > LL13-37 > LL17-32.

On SEM images of *C albicans* (Fig 1), LL13-37, LL37, and LL13-37 had analogous potencies in inhibiting hyphal growth in *C albicans*. The hyphae were uniformly thick in the negative control. After treatment with LL13-37, the hyphae assumed a more slender appearance. Budding appeared less robust and death ensued. Following exposure to 50 μM LL 13-37, only the yeast form and no hyphal form was discernible (Fig 1).

LL13-37 at 5 μM permeabilised the membrane of the yeast and hyphal forms since the nuclear stain SYTOX Green was localised in both forms. There was no green fluorescence in the PBS-treated yeast or hyphal form.

Mycelia incubated with LL13-37 were stained by SYTOX green, but not by MitoTracker deep red, indicating mitochondria were not adversely affected by LL13-37 (Fig 2).

Bimane-labelled LL13-37 entered some but

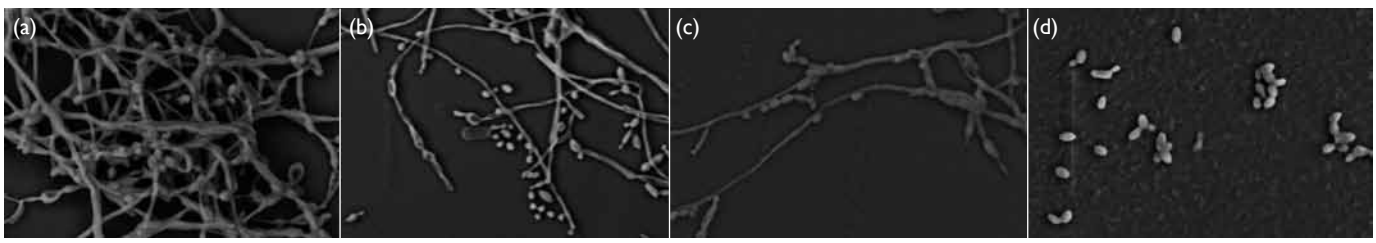

FIG 1. Scanning electron microscopic images of *C albicans* showing hypha formation in different LL13-37concentrations: LL 37 and LL13-37 have similar growth-inhibiting potencies (not shown). (a) In negative control (0 μM), hyphae were uniformly thick. In (b) 0.5 μM and (c) 5 μM LL13-37, hyphae became slender, budding became less healthy-looking and death resulted. (d) In 50 μM LL 13-37, only planktonic form and no hyphal form was discernible

not all of the *C albicans* hyphae.

Reactive oxygen species were demonstrable by confocal microscopy in the yeast form of *C albicans* cells after treatment with LL13-37, but not in the untreated cells. The difference due to LL13-37 treatment of the hyphal form was much less conspicuous and is not presented.

Respectively for *C albicans* (SC5134), *C albicans* (ATCC 90028), *C krusei* (ATCC 6258), and *C tropicalis* (ATCC 750), IC_{50} values of LL37 were 0.25±0.02, 0.56±0.08, 1.29±0.05, and 0.64±0.02 μM, whereas IC_{50} values of LL13-37 were 0.51±0.01, 0.67±0.06, 1.89±0.03, and 1.21±0.12 μM, and IC_{50} values of LL17-32 were 1.02±0.02, 1.51±0.12, 3.22±0.11, and 2.51±0.08 μM. LL37 did not show obvious antibacterial activity below a concentration of 64 μM and its fragments did not show antibacterial activity below a concentration of 128 μM.

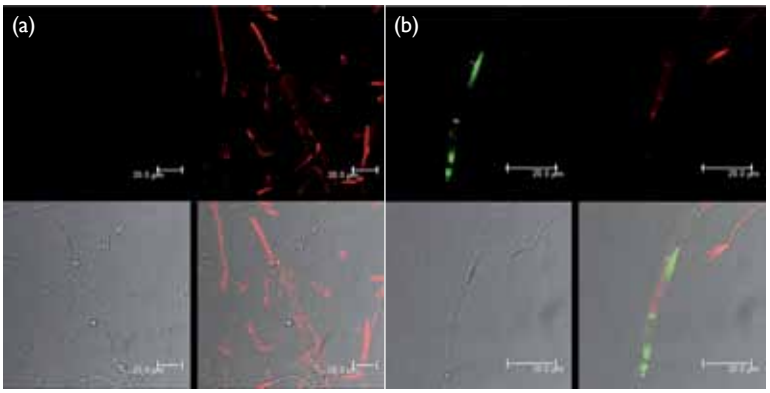

FIG 2. Confocal microscope pictures showing staining of *C albicans* mycelia with SYTOX green and MitoTracker deep red: (a) control (treated with PBS), and (b) treated with 5 μM LL13-37. Mycelia treated with LL13-37 stained with SYTOX green, but not with MitoTracker deep red. The upper left, upper right, lower left, and lower right quadrants refer to staining with SYTOX green, staining with MitoTracker deep red, bright field, and superimposed picture, respectively

Isolation and characterisation of bean defensin

Two peaks were demonstrated by Q-Sepharose chromatography. Anti-fungal activity in king pole bean seeds was recovered in the non-absorbed fraction, which was subsequently purified by gel filtration on Superdex 75. Three peaks were collected. Purified peptide (5 mg/50 g seeds) was located in fraction S3.

Molecular weight was determined by gel filtration on Superdex 75. The calibration curve was constructed using protein markers. Tricine gel electrophoresis of the peptide showed a single 5-kDa band (data not shown). Its accurate molecular mass was determined by mass spectrometry to be 5447.4 Da.

The purified peptide remained stable after exposure for 30 minutes from 0-80°C. However, its activity was lost after incubation for 30 minutes at or above 90°C. All activity remained at pH 4-10.

The N-terminal sequence of the antifungal peptide was KTCENLADTFRGPCFATSNC. It shared 75-89% identity with plant defensins.

The isolated peptide was active against *M arachidicola* and *S cerevisiae* with an IC_{50} value of 3.9 and 4.0 μM, respectively.

When the culture medium contained 5 mM $MgCl_2$, the IC_{50} value of the peptide to *M arachidicola* and *S cerevisiae* increased to 12 and 10.8 μM respectively.

Fluorescent microscopy revealed that the isolated peptide increased the membrane permeability in *S cerevisiae*, *M arachidicola*, and *C albicans* (Fig 3).

Only cytokine TNF-α was induced in murine splenocytes by the isolated peptide.

IC_{50} values of antibacterial activity of pole bean defensin toward *Mycobacterium phlei*, *Bacillus megaterium*, *Bacillus subtilis*, and *Proteus vulgaris* were 90±3, 100±7, 102±6, and 92±5 μM, respectively. For *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens*, no inhibition was observed at a dose of 150 μM pole bean defensin.

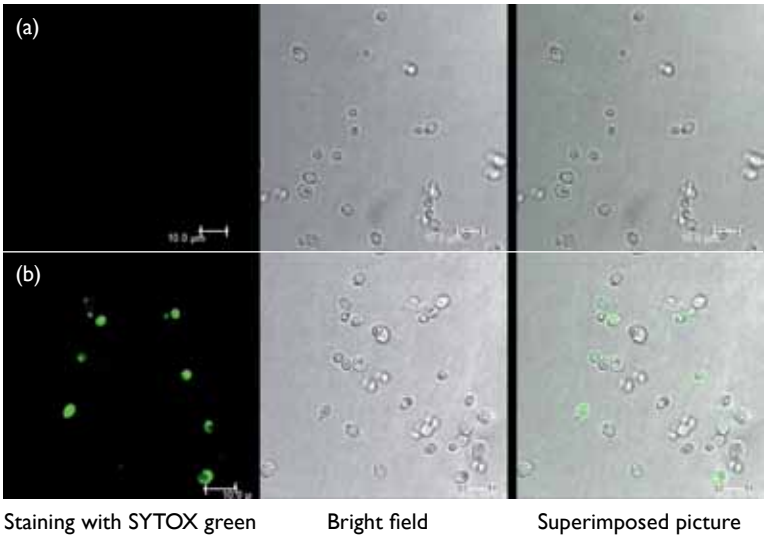

FIG 3. Membrane permeabilisation assay of different forms of *C albicans* cells treated with 20 μM pole bean antifungal peptide: (a) treated with PBS as controls, (b) treated with peptide. All fungal cells showed strong SYTOX green fluorescence in the presence of peptide, as compared with controls, in which fungi were grown without the peptide. All pictures were taken with a confocal microscope.

Discussion

LL13-37 was selected because of its lowest toxicity to human peripheral blood monocytes and rabbit

erythrocytes. Antimicrobial activity was most likely attributed to the cationic nature of LL37 and its fragments (LL13-37 and LL17-32) that carry +6, +4 and +5 net charges, respectively. Hence, they could interact with negatively charged components of the microbial surface and subsequently disrupt the membrane barrier via pore-formation or non-specific membrane permeabilisation. SYTOX Green, a high-affinity nucleic acid stain that does not traverse live cell membranes but penetrates cells with a compromised plasma membrane, is an indicator of membrane integrity. LL37 and fragments are positively charged at a neutral pH, α-helical, and contain many hydrophobic and basic residues, enabling them to bind and disrupt the negatively charged membrane of pathogens, culminating in cell death.

Findings on bimane-labelled LL13-37 revealed that LL13-37 enters fungal cells. The increased membrane permeability might not be the sole determinant of cell death but it might ensue in uptake of the peptides. Once inside cells, they may interfere with nucleic acid and/or protein synthesis. The peptides might have some intracellular targets.

Intracellular localisation of LL13-37 and its activity in *C albicans* cells was accompanied by reactive oxygen species accumulation, which can produce deleterious effects on nucleic acids, proteins and lipids. With this multiplicity of targets it is not easy to pinpoint the events that contribute to loss of cell viability following reactive oxygen species–induced damage.

After treatment with LL13-37, the mitochondria in *Candida* could not be labelled by Mitotracker deep red, a cell-permeant mitochondrion-selective dye. The results indicated that LL13-37 could enter *Candida* cells and adversely affect the mitochondrial membrane.

The isolated king pole bean defensin exerted potent antifungal activity against *S cerevisiae* and *C albicans* with an IC_{50} value of 4.0 and 8.4 μM, respectively. Its efficacy against *C albicans*, which can cause infection in immunocompromised patients, is noteworthy. The possibility of exploiting plant defensins as an antifungal therapy in humans has been proposed.[7] Its antifungal potency is similar to that reported for other plant defensins.

In the present study, fungi treated with the antifungal peptide were stained with SYTOX Green, a high-affinity nucleic acid stain that does not cross live cell membranes. Only cytokine TNF-α was induced in murine splenocytes by the isolated peptide suggesting that the peptide is a weak cytokine gene inducer. King pole bean defensin did not induce haemolysis in rabbit erythrocytes, indicating that it adversely affects fungal but not mammalian cell membranes. This enhances the therapeutic potential of the peptide.

Acknowledgements

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Event-specific risk factors predicting unprotected anal intercourse among Hong Kong men who have sex with men: a case-crossover study

JTF Lau *, J Gu, HY Tsui

KEY MESSAGES

1. Availability and possession of condoms, condom negotiation, and planning to use condoms were event-specific factors related to lower likelihoods of unprotected anal intercourse (UAI) with both regular and non-regular male sex partners (RP and NRP). Situational risk factors for UAI were anal sex taking place during the weekday and tiredness prior to anal sex. Other situational and environmental factors were display of reminders promoting condom use, home settings, travel, and nervous feelings.
2. HIV prevention should consider the aforementioned factors and target specifically on inconsistent condom users. It should therefore be based on research discriminating between episodes of UAI and protected anal intercourse (PAI) within inconsistent condom users, rather than those obtained from traditional studies discriminating between consistent and inconsistent condom users. This is especially important as our findings suggested that the factors derived by the two types of studies might differ.
3. HIV prevention should consider segmentation of RP and NRP, as predictors of UAI differ among the two partner types.
4. This study demonstrates the usefulness of a case-crossover design for investigating event-specific variations in behaviours.

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In 2008, the HIV prevalence was 4.3% among men who have sex with men (MSM) in Hong Kong.[1] The prevalence of unprotected anal intercourse (UAI) among MSM in Hong Kong ranged from 25% to 67%.[1,2] According to the socio-ecological model[3] advocated by HIV workers, factors associated with UAI exist at individual, interpersonal, and environment levels. Most associated factors are derived from cross-sectional surveys that discriminate between consistent and inconsistent condom users among MSM, but this cannot explain why an individual uses condoms inconsistently. According to the social learning theory,[4] event-specific factors are predictive of health-related behaviours.

The case-crossover design is developed to identify event-specific (episode-specific) variables that are predictive of health outcomes.[5] It has been increasingly used in different research areas. Nonetheless, only two studies applied the design to investigate factors associated with HIV-related behaviours among MSM, but these studies focused on a narrow range of situational risk factors. The present study applied this study design to investigate whether three types of event-specific factors (sex partners' attributes, situational factors, and environmental/settings factors) are predictive of episodes of UAI among MSM in Hong Kong who were inconsistent condom users.

Factors with regard to two contrasting events—an episode of UAI versus an episode of protected anal intercourse (PAI) of the same person—was compared.[5] A total of 428 MSM who had at least one episode each of UAI and PAI with a man in the previous 6 months were recruited; respectively 213 and 215 MSM involved regular and non-regular sex partners (RP and NRP) and completed the questionnaire.

A higher likelihood of UAI with RP was associated with alcohol use by the participants or the partner prior to sex, having the participant suggested UAI, the anal episode took place overseas, during a weekday, and not at home. Whereas a lower likelihood of UAI with RP was associated with planning and discussion about condom use, perceived partner's preference for condom use, suggestion to have PAI by the participant or the partner, condoms already placed at the venue, and the partner possessed a condom.

A higher likelihood of UAI with NRP was associated with feeling tired or nervous before having anal sex, perception that the partner was

unlikely use condoms, having the participant or partner suggested UAI, and the anal sex episode took place on a weekday. Whereas a lower likelihood of UAI with NRP was associated with partner of age ≤35 or unknown age, having had at least three times of anal sex with the NRP, perception that himself and the NRP had had asymmetrical sexual experience, perception that the NRP was feminine, and liking towards the NRP, as well as having had UAI with another men in the last week, discussion about condom use prior to anal sex, perceived partner's liking in condom use, the participants or partner's suggestion to have PAI, planning to use condoms, condoms already placed at the venue, display of condom use promotion materials, and the participant or partner possessed a condom.

Almost all factors related to condom negotiation and the perception that the partner liked to use condoms were protective factors against UAI with both RP and NRP. The protective effects of the availability of condoms are also evident. HIV prevention targeting inconsistent condom users should cultivate skills and rehearsals about speaking out the wish to use a condom and insisting on condom use. Venues such as gay saunas and hotels should ensure accessibility of condoms, and inconsistent condom users may put up reminders as their tailor-made cue to actions for PAI. Non-structural situational factors (eg sex taking place during a weekday, overseas, not at a home setting, tiredness and nervous feelings prior to anal sex) also trigger off UAI. MSM should be trained on how to relax and improve self-efficacy on condom use when such situations arise. Our findings provide important insights for designing new HIV prevention. A critical review of the appropriateness of relying only on the factors obtained from conventional studies that discriminate between consistent and inconsistent condom users is warranted.

Acknowledgements

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Molecular dissection of dengue virus egress: involvement of the class II ARF small GTPase

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KEY MESSAGE

The transmembrane domain 2 of prM is a possible binding domain by Arf4 protein

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Four serotypes of dengue viruses 1-4 are the pathogens of dengue fever, dengue haemorrhagic fever, and dengue shock syndrome. They are estimated to cause 50-100 million cases, including 25 000 deaths, every year.[1] Despite the increased health and economic impact of dengue virus infection, there is still no specific treatment. This is due, in part, to the incomplete understanding of specific host-pathogen interactions during the life cycle of the virus in infected cells. More research into the basic biology of dengue virus is needed.

Dengue virus particles can be viewed schematically as internal and external parts. The internal part is the core structure of the virus and comprises the genome and capsid protein, which binds to the genome so that the core structure can be well organised and packaged into the external part. The internal part carries viral genetic information, stored as a long RNA molecule, and can replicate itself to form nascent viruses. The external part consists of a lipid membrane and the viral structural envelope glycoproteins that are integrated in the lipid membrane. The lipid membrane is derived from the host cell when nascent viruses are formed. Dengue viruses have two structural envelope glycoproteins, prM and E.[2]

During viral maturation, prM is cleaved by furin, a cellular protease, to form pr and M, the former being released from the mature virion, and the latter remaining on it to form a heterodimer with E protein.[3] Glycoprotein prM and E play important roles in the dengue virus life cycle. The life cycle can be divided into three stages: entry, replication, and secretion. E protein is mainly responsible for binding with a cellular receptor and then triggering viral-host membrane fusion during viral entry, whereas prM protein is required for various stages of viral secretion. The secretion process describes the assembly and subsequent transportation of virus. First, prM protein functions as a chaperone in ER to help the proper folding of E protein. Second, prM protects E protein from conformational changes that may be triggered by the acidic environment along the secretion pathway, thus promoting fusion of the nascent virion with the membrane compartment and release inside, not outside, the cell. Third, immediately before dengue virus is released from host cells, prM protein is cleaved by furin, a major processing enzyme of the secretory pathway, to form M and soluble pr proteins. This step is a critical process for the infectivity of the nascent virus and is called maturation. Although the secretion of dengue virus is a complex process, compared with viral entry and replication, little attention has been paid to understand the various stages at a molecular level.

Assembly of dengue virus occurs in the endoplasmic reticulum (ER) and is driven by the interaction between E and prM proteins. Nascent virions in the ER thus need to be transported from the ER to the Golgi apparatus, and then transported to the plasma membrane before they are finally released. As described above, prM protein performs multiple functions during the secretion process. To achieve this, prM protein needs the help of many cellular factors. Utilisation of cellular factors is a common strategy by which viruses complete their life cycle. Although such cellular factors will not be assembled into newly formed viral particles, they are really indispensable to survival of the virus. Thus, if these crucial cellular factors are not utilised by the virus, viral replication will be inhibited. Identification of these host factors and the mechanisms that govern their interaction with a virus enables design of antiviral strategies.

To identify the cellular factors and their mechanism of action, we use dengue recombinant subviral particles (RSPs), which bear several similarities to dengue virus and are a safer and convenient tool in the laboratory. Dengue RSPs are generated by cells expressing glycoprotein prME in the absence of capsid protein and RNA genome. Thus, dengue RSPs consist of only the external structure

of dengue virus and cannot cause infection because of the lack of viral genome. In previous work, we have developed a stable dengue RSP-producing cell line (HeLa-prME) using a codon-optimised dengue prME gene that greatly increases the expression level of prME proteins in mammalian cells.[4] We have shown that dengue RSPs can mimic the secretion of dengue virus, and have validated this tool to analyse trafficking and secretion of dengue virus.[4]

Using the dengue RSP-producing cell line, we have identified two cellular factors: ADP-ribosylation factor 4 and 5 (Arf4 and Arf5), and demonstrated that they are indispensible for dengue virus secretion.[5] Simultaneous depletion of Arf4 and Arf5 blocks RSP secretion for all four dengue serotypes. Intriguingly, Arf4 and Arf5 are not required for the constitutive secretion of host cells, ie the mechanism by which cells export material into the extracellular space, indicating that they are specifically required by dengue virus. Arf4 and Arf5 belong to the ADP-ribosylation factor family, of which six members have been identified and all play an important role in intracellular transportation. A predominant function of Arf proteins is that they can bind to lipid membrane, recruit some other cellular proteins, and then curve the lipid membrane to a semi-spherical or spherical structure. Such curvature of the lipid membrane is an important step for the formation of trafficking vesicles, which are the universal form of intracellular transportation from one organelle to another. It is noteworthy that bending of lipid membranes also occurs during the formation of enveloped virus including dengue virus.

Based on amino acid sequence identity, six Arfs are grouped into three classes: class I (Arf1-3), class II (Arf4, 5), and class III (Arf6). Arf4 and Arf5 are mainly distributed in the peri-nuclear region. Arf4 participates in the transport of rhodopsin from the Golgi apparatus to the plasma membrane by binding to a VxPx motif at the C terminus of rhodopsin. Arf proteins are hijacked by various viruses for their life cycle. For example, Arf1 protein is utilised by viruses such as HIV and HCV for virus assembly or replication, whereas Arf6 protein is hijacked by viruses such as coxsackievirus and HIV for virus entry.

We designed experiments to investigate the molecular mechanism by which Arf4 and Arf5 affect dengue virus secretion. One important technique used is co-immunoprecipitation. Cells producing RSP are broken and the resulting lysate is incubated with beads covered with an antibody that specifically recognises prM protein. The prM protein and other proteins binding with prM attach to the bead through the bridge of anti-prM antibody and can be precipitated by centrifugation. This approach enables extraction of the cell molecules that can interact with the viral protein under study. Arf4 and Arf5 proteins and prM protein bind with each other in RSP-producing cells. This means that dengue prM protein may recruit Arf4 or Arf5, and that this interaction in turn facilitates virus secretion. Dengue prM protein has been reported to help the correct folding of E protein in ER and then protect it from fusing within the host cell before progeny viruses are released. Here, we demonstrated another important role of prM during the secretion process: recruiting Arf4 or Arf5, two crucial factors for intracellular trafficking, to the lipid membrane to facilitate virus secretion.

As mentioned above, Arf4 can recognise a VxPx (where X stands for any amino acid) motif that is found at the C terminus of four serotypes of dengue virus prM proteins. To study whether VxPx in prM is also recognised by Arf4 or Arf5, we have used molecular cloning techniques to generate mutant prME genes in which VxPx motif is either deleted (prME-DVxPx) or substituted (prME-AxAx) and then use transfection reagents to deliver these mutants as well as wild type prME to mammalian cells. We found that substitutions inside this motif (V161A and P163A) did not reduce the expression or secretion of RSP. Taken together, our results indicate that although VxPx at the C terminus of prM affects the expression level of the protein, it is not the sequence recognised by Arf4 or Arf5.

In further experiments, we used another technique called glutathione S-transferase (GST) pull-down assay to gain insight into the molecular basis of the interaction between prM and Arf proteins. The principle of GST pull-down is similar to that of co-immunoprecipitation. In this assay, instead of using an anti-prM antibody to co-immunoprecipitate interacting molecules, some parts of prM protein are fused to the GST gene and then immobilised at the surface of beads coated with glutathione, which interacts with high affinity with GST. Using the GST pull-down assay, we extended our previous observations and demonstrated that the four amino acid peptides VxPx or AxAx are not sufficient to pull down class II Arf protein. The efficient pull-down of Arf4 protein requires the additional presence of the second transmembrane domain of prM, suggesting that Arf4 might interact with this region of prM protein.

The different binding ability to Arf protein by rhodopsin VxPx and prM VxPx might be explained by considering their different topology. Whereas the VxPx motif of rhodopsin is located at the cytosolic side of the intracellular membrane compartment in which it is inserted and Arf protein is able to recognise it for interaction, the same sequence in prM is exposed to the luminal side of those compartments and theoretically inaccessible to Arf protein. The transmembrane domain of prM, which spans the lipid membrane, is therefore more likely

to interact with class II Arfs. Further experiments are needed to test the role of this transmembrane domain for prM interaction with Arf4 and Arf5.

In the second part, we focused on KDELRs. KDELRs (KDELR1-3) are seven-transmembrane proteins that regularly cycle between the ER and Golgi apparatus. Depletion of Arf4 and Arf5 blocks not only the secretion of RSP, but also the transportation of KDELR. The depletion of Arf4 and Arf5 results in a concentration of KDELR around the Golgi apparatus and a decrease of KDELR in the ER compartment, suggesting that in the absence of Arf4 and Arf5, KDELR cannot exit the Golgi apparatus and therefore accumulates in that organelle. Co-immunoprecipitation experiments found that prME proteins also interact with KDELR. Because KDELR shuttling between the ER and Golgi apparatus requires both Arf4 and Arf5, we postulate a novel mechanism of dengue virus secretion: dengue RSP are formed in the ER where they bind to KDELR as a means to be transported from the ER to the Golgi apparatus. RSPs and KDELRs dissociate in the Golgi apparatus by an unknown mechanism, and KDELR is retrieved back to the ER in a class II Arf dependent manner. In this model, the transportation of dengue virus from the ER to Golgi apparatus requires not only the presence of KDELR but also the normal cycle of KDELR, which is subject to Arf4/Arf5 availability. This model incorporates the experimental results in a testable model and we will design experiments to study this hypothesis in future.

Although the mechanism by which Arf4 and Arf5 are involved remains to be studied, our findings shed new light on a molecular mechanism used by dengue viruses during the late stages of the replication cycle and demonstrate a novel role for prM protein that could represent a novel therapeutic target. More basic knowledge of the life cycle of the dengue virus is needed in order to devise better strategies to treat this disease and reduce its burden worldwide.

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Inhibition of RIG-I-dependent innate immunity by herpes simplex virus type I Us11 protein

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KEY MESSAGES

1. Double-stranded RNA binding protein PACT activates RIG-I and thus optimally induces the production of type I interferon.
2. PACT associates with RIG-I in virus-infected cells. Activation of RIG-I by PACT triggers host anti-viral responses.
3. Herpes simplex virus 1 (HSV-1)-encoded Us11 protein inhibits the production of type I interferon in virus-infected cells. Mutant HSV-1 virus incapable of expressing Us11 protein induces higher amounts of type I interferon.
4. Us11 associates with PACT, and inhibits PACT-dependent type I interferon production.
5. Other viruses may apply the same strategy to shut down host antiviral responses by inhibiting PACT-dependent type I interferon production.

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Introduction

Infection with herpes simplex virus type 1 (HSV-1) is very common and causes oral and/or genital herpes. In addition, HSV-1 can infect the nervous system, resulting in encephalitis.[1] HSV-1 Us11 is a multifunctional protein required for full resistance to interferons, and inhibits protein kinase R (PKR) through an interaction with cellular dsRNA-binding protein PACT.[2] Nonetheless, exactly how Us11 antagonises innate immunity is not fully understood. In this project, we aimed to fully characterise this new mechanism by which Us11 counteracts PACT to inhibit RIG-I-dependent interferon production.

Methods

In vitro affinity binding and complex formation assays were performed with recombinant Us11, PACT and RIG-I expressed and purified from *Escherichia coli*. Co-fractionation and co-localisation experiments were carried out to verify the interaction between Us11 and PACT in transfected and HSV-1-infected cells. The roles of PACT and RIG-I in interferon-induced anti-HSV cellular response as well as the mechanisms by which Us11 inhibits PACT and RIG-I were investigated. In particular, the influence of Us11 on PACT-RIG-I complex formation was determined.

Results

PACT was a potent activator of RIG-I and resulted in optimal interferon production. PACT and RIG-I mediated interferon production was critical to combat viral infection. Experimentally, RNAi depletion of PACT led to inhibition of virus-induced and RIG-I-dependent activation of IFN production in three different cell lines, lung epithelial carcinoma A549, normal diploid fibroblast IMR-90, and primary mouse embryonic fibroblast MRFs. In addition, the stimulation of RIG-I by PACT did not require PKR or Dicer, but was mediated through a direct interaction with the C-terminal domain of RIG-I leading to activation of ATPase activity and plausibly a conformational change associated with the recruitment of downstream effectors.

We revealed a novel inhibitory role of HSV-1 Us11 protein in PACT-RIG-I mediated interferon production. Us11 protein associated with PACT, and thus inhibited the activation of RIG-I. This interaction required both dsRNA binding domains of Us11 and PACT, and this interaction was dsRNA-insensitive. Using HSV-1 wild type and mutant viruses, viruses that express Us11 demonstrated a stronger inhibition on interferon production. This inhibition was caused by the sequestration of PACT by Us11 protein.

Discussion

Our findings revealed PACT as a new target of a viral interferon-antagonising protein. We demonstrated the importance of the C-terminal dsRNA-binding domain of Us11 in its interaction with PACT. The same domain was also used in the interaction with RIG-I, melanoma differentiation-associated protein 5 (MDA5), Dicer, PKR and 2'-5'-oligoadenylate synthase. It would be of great interest to determine

whether Us11 also affects the normal function of these cellular proteins. Particularly, the interplay of Us11, PACT, RIG-I, and MDA5 in the activation of type I interferon production merits further investigation. Because other viral interferon-antagonising proteins such as influenza A virus NS1[3] and Ebola virus VP35[4] can also interact with PACT and perturb RIG-I function, suppression of PACT-augmented activation of RIG-I might represent a common viral countermeasure to combat the host antiviral response used by other viruses.

Conclusions

Our study revealed a novel mechanism by which HSV-1 circumvents innate antiviral immunity through Us11 inhibition of PACT and RIG-I. This finding provides a new opportunity in the development of novel antiviral agents against HSV-1 infection.

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